Supplementary Information

The Broad Spectrum Antibiotic Xanthocillin X Effectively Kills *Acinetobacter baumannii* via Dysregulation of Heme Biosynthesis

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Supplementary Discussion

Supplementary Discussion 1: Cloning of PbgS(wt) and PbgS(P241S)

In order to understand the impact of the mutation P241S on the enzyme activity, we first expressed the N-terminally His-tagged PbgS(wt) (pDest 17-\textit{abhemB}$_{\text{wt}}$) in \textit{E. coli} BL21 (DE3) and \textit{E. coli} Lemo21(DE3) expression strains. However, under all conditions tested (varying IPTG concentrations, duration and temperature of expression) we solely obtained aggregated protein as judged by SDS-PAGE after purification using Ni-NTA resin (Supplementary Figure 10). Only the expression as N-terminally His-tagged maltose-binding-protein (MBP) fusion constructs either with (pET MBP-1a-\textit{abhemB}$_{\text{wt}}$) or without (pET-41-K-\textit{abhemB}$_{\text{wt}}$) TEV-cleavage site in \textit{E. coli} Lemo21(DE3) resulted in partially soluble protein, which proved to be poorly soluble and unstable after cleavage of MBP using TEV protease. The N-terminally His-tagged MBP fusion constructs of mutant protein P241S showed comparable expression behaviour. After expression as partially soluble protein, it aggregated immediately after cleavage of MBP. Thus, we cloned PbgS(wt) and PbgS(P241S) tag free in plasmid pVRL2 and transformed resulting plasmids pVRL2-\textit{abhemB}$_{\text{wt}}$ and pVRL2-\textit{abhemB}$_{p241S}$ in \textit{A. baumannii} ATCC19606.

Supplementary Discussion 2: Determination of enzyme efficiency ($k_{\text{cat}}/K_m$)

PbgS(wt) and PbgS(P241S) were overexpressed tag-free in \textit{A. baumannii} (see Supplementary Discussion 1) and lysate was used for the activity assay to calculate the kinetic parameters ($K_m$(PbgS(wt)) = 153 $\mu$M, $v_{\text{max}}$(PbgS(wt)) = 0.027 min$^{-1}$, $K_m$(PbgS(P241S)) = 5670 $\mu$M, $v_{\text{max}}$(PbgS(P241S)) = 0.015 min$^{-1}$). For that, \textit{A. baumannii} (wt) lysate was included as control to subtract the basal activity of the native PbgS(wt), which was considerably lower compared to the activity measured in the overexpressed lysates. In order to compare enzyme efficiencies ($k_{\text{cat}}/K_m$ with $k_{\text{cat}} = v_{\text{max}}$/[Enzyme]) of PbgS(wt) and PbgS(P241S), we quantified the overexpressed enzymes in lysates via label-free LC-MS/MS analysis. LFQ intensities indicate comparable [Enzyme] quantities (Supplementary Figure 11B) and consequently, the ratio $v_{\text{max}}/K_m$ revealed a significant drop in enzyme efficiency for mutant PbgS(P241S) of about 60-fold.
Supplementary Figures

Supplementary Figure 1: Fluorescence titration of XanDME with Cu(II). The data represent average values ± s.d. of independent experiments (n = 3 per group).

Supplementary Figure 2: Synthesis of XP and XPP. A) Alkylation of Xan using propargyl bromide to yield XP. B) Alkylation of Xan using the minimal photocrosslinker 2 to yield XPP.
Supplementary Figure 3: Competitive ABPP experiments using XP in *E. coli* K12. A) The volcano plot shows enrichment of proteins after pre-treatment of intact *E. coli* cells with Xan (30 μM) on a log₂ scale. Green dots represent proteins that were significantly enriched by XP compared to DMSO control (Figure 2C). The vertical and horizontal threshold lines represent a log₂ enrichment ratio of $-0.9$ and a $-\log_{10} P$ value of 2 (two-sided two-sample t-test, n = 4 independent experiments per group), respectively. B) Table that allocates proteins above the set threshold from the ABPP experiment (Figure 2C).
Supplementary Figure 4: Target identification by chemical proteomic profiling in A. baumannii. A) ABPP experiment using XP in A. baumannii ATCC19606. The volcano plot shows enrichment of proteins after treatment of A. baumannii cells with XP (3 µM) compared with DMSO on a log2 scale. The vertical and horizontal threshold lines represent a log2 enrichment ratio of 2 and a −log10 P value of 1.3 (two-sided two-sample t-test, n = 3 independent experiments per group), respectively. B) Competitive ABPP experiment using XP in A. baumannii. The volcano plot shows enrichment of proteins after pre-treatment of intact A. baumannii cells with Xan (30 µM) on a log2 scale. Green dots represent proteins that were significantly enriched in the ABPP experiment (A). The vertical and horizontal threshold lines represent a log2 enrichment ratio of −0.9 and a −log10 P value of 1.3 (two-sided two-sample t-test, n = 3 independent experiments per group), respectively. C) Table that allocates proteins above the set threshold from the ABPP experiment (A). To determine essentiality, transposon mutant library generated by Gallagher et al. was used. For that, BLAST search was performed to assign homologous proteins in A. baumannii AB5075 (Program version BLASTP 2.10.1+, search limited to A. baumannii AB5075 (taxid:1116234), the resulting query coverage and percent identity are listed).
### Supplementary Figure 5: Gel-based labeling of recombinant AbCAT and EcCAT.

Recombinant protein (1 µM) was treated with indicated concentrations of XP or DMSO as control for 1 h. Heat inactivated protein samples were included as control for unspecific binding. After incubation, samples were clicked to rhodamine azide and analyzed by SDS-PAGE with subsequent in-gel fluorescence scanning and Coomassie-staining. The gel is representative for three independent experiments.

| Heat control | + | + | - | - | - | - | + | + | - | - | - | - |
|--------------|---|---|---|---|---|---|---|---|---|---|---|---|
| XP (eq.)     | 100| 10| 100| 10 | 5 | 1 | - | 100| 10| 100| 10 | 5 |

**Fluorescence**

**Loading control**

### Supplementary Figure 6: Influence of Xan on the catalases activity using catalase activity assay kit (BioVision, catalog number K773-100).

Hydroxylamine was used as positive control for CAT inhibition. CAT (final concentration: 25 nM) was pre-incubated with either Xan or Hydroxylamine for 30 min at RT and the reaction was started by the addition of H₂O₂ (final concentration: 16 µM). After 15 min at RT, the reaction was stopped by the addition of 10 µL of the stop solution and 50 µL of the develop mix (47.7 µL assay buffer, 0.3 µL OxiRed probe, 2 µL HRP). After incubation for 10 min, the fluorescence signal (λ<sub>ex</sub> = 535 nm, λ<sub>em</sub> = 587 nm) was measured. Values represent mean ± s.d. of averaged duplicates of independent experiments (n = 3) and are normalized to the DMSO-treated samples (positive control, 100%) and the negative control (without enzymes, 0%).
Supplementary Figure 7: Target identification by chemical proteomic profiling in *A. baumannii* ATCC19606 using XPP in soluble fraction. **A**) A/BPP experiment using XPP in *A. baumannii* ATCC19606 in soluble fraction. The volcano plot shows enrichment of proteins after treatment of *A. baumannii* cells with XPP (3 µM) compared with DMSO on a log2 scale. The vertical and horizontal threshold lines represent a log2 enrichment ratio of 2 and a −log10 *P* value of 2 (two-sided two-sample *t*-test, *n* = 4 independent experiments per group), respectively. **B**) Table gives detailed information on proteins above the set threshold from the A/BPP experiments. To determine essentiality, transposon mutant library generated by Gallagher et al. was used. For that, BLAST search was performed to assign homologous proteins in *A. baumannii* AB5075 (Program version BLASTP 2.10.1+, search limited to *A. baumannii* AB5075 (taxid:1116234), the resulting query coverage and percent identity are listed). Essential gene list published by Wang et al. was used to determine essentiality and BLAST search was limited to *A. baumannii* ATCC17978 (taxid: 400667, program version BLASTP 2.10.1+, query coverage and percent identity are listed). Proteins, for which BLAST search did not find any proteins significantly similar to proteins in *A. baumannii* AB5075 or *A. baumannii* ATCC17978 (no significant similarity found – n.s.s.f.), cannot be categorized according to essentiality.
Supplementary Figure 8: Target identification by chemical proteomic profiling in *A. baumannii* ATCC19606 using XPP in insoluble fraction. 

A) A/BPP experiment using XPP in *A. baumannii* ATCC19606 in insoluble fraction. The volcano plot shows enrichment of proteins after treatment of *A. baumannii* cells with XPP (3 µM) compared with DMSO on a log2 scale. The vertical and horizontal
Threshold lines represent a log₂ enrichment ratio of 2 and a −log₁₀ P value of 2 (two-sided two-sample t-test, n = 4 independent experiments per group), respectively. B) Table gives detailed information on proteins above the set threshold from the A/BPP experiments. To determine essentiality, transposon mutant library generated by Gallagher et al. was used.³ For that, BLAST search was performed to assign homologous proteins in A. baumannii AB5075 (Program version BLASTP 2.10.1+, search limited to A. baumannii AB5075 (taxid:1116234), the resulting query coverage and percent identity are listed).⁴ Proteins, for which BLAST search did not find any proteins significantly similar to proteins in A. baumannii AB5075 (no significant similarity found – n.s.s.f.), cannot be categorized according to essentiality.

**Table**

| Homo sapiens | E. coli | P. aeruginosa | A. baumannii |
|--------------|---------|---------------|--------------|
| --- | --- | --- | --- |
| sp| P| 157 | 157 | 157 |
| P0AC82 | E. coli | --- | --- |
| Q59643 | E. coli | --- | --- |
| loc | A. baumannii | --- | --- |

For that, BLAST search was performed to assign homologous proteins in A. baumannii AB5075 (Program version BLASTP 2.10.1+, search limited to A. baumannii AB5075 (taxid:1116234), the resulting query coverage and percent identity are listed).⁴ Proteins, for which BLAST search did not find any proteins significantly similar to proteins in A. baumannii AB5075 (no significant similarity found – n.s.s.f.), cannot be categorized according to essentiality.

**Supplementary Figure 9**: Multiple sequence alignment of orthologous protein sequences using Clustal O (Version 1.2.4).⁹ Important structural features are highlighted: Binding site for A-side 5-ALA and P-side 5-ALA (green), active site lid (blue) and amino acid mutation in Xan resistant isolates (yellow).⁹
**Supplementary Figure 10:** SDS-PAGE analysis of overexpression of PbgS(wt) and purification using Ni-NTA resin (Qiagen). *E. coli* Lemo21(DE3) and *E. coli* BL21(DE3) harboring plasmids pDest 17-ahemB<sub>wt</sub> or pET-41-K-ahemB<sub>wt</sub> were used for expression of fusions proteins with a molecular weight of 42.6 kDa (6×His-tag-PbgS) and 83.6 kDa (6×His-tag-MBP-PbgS), respectively. This gel is representative for all conditions tested (varying IPTG concentrations, temperature and duration of expression). Here, protein expression was induced with 400 µM IPTG, cells were incubated at indicated temperature for 20 h at 150 rpm. Afterwards, 5 mL of cell culture were purified using Ni-NTA matrix (Qiagen) following the manufacturer’s protocol. 50 µL of eluted sample was analyzed by SDS-PAGE and Coomassie-staining. Blue arrows indicate the expected position of the fusion proteins.

**Supplementary Figure 11:** Activity assay of PbgS(wt) in the presence of Xan and LFQ quantification of PbgS(wt) and PbgS(P241S) in lysate. A) Assay performed in lysates of *A. baumannii* that harbor pVRL2-ahemB<sub>wt</sub> for expression of PbgS(wt). Lysate of *A. baumannii* (wt) (without plasmid) was used as blank control in order to subtract the basal PbgS activity level. Xan (100 µM) was added to the lysate and incubated for 15 min at 37 °C before 5-ALA (100 µM) was added. Enzymatic reaction was stopped after 30 min by the addition of Ehrlich’s Reagent and absorbance was measured after 10 minutes. Data represent mean values ± s.d. of averaged duplicates of independent experiments (n = 2 per group). B) LFQ intensities of PbgS(wt) in lysate of *A. baumannii* (wt) and of *A. baumannii* harboring pVRL2-ahemB<sub>wt</sub> as well as PbgS(P241S) in lysate of *A. baumannii* harboring pVRL2-ahemB<sub>P241S</sub> used for the PbgS activity assays (**Figure 3B, Supplementary Figure 11A, Supplementary Discussion 2**).
Supplementary Figure 12: Investigation of the interaction of isonitrile compounds with heme. **A)** Structure of resveratrol (Res), which was used as negative control in the spectroscopic analysis of hemin (Figure 4A). **B)** UV-Vis spectra of hemin (20 µM, solid line) and protoporphyrin IX (PPIX, 20 µM, dashed lines) with either DMSO (black) or Xan (40 µM, green). C) UV-Vis spectra of hemin (20 µM) with DMSO (black) or XanDME (20 µM, olive green). D) UV-Vis spectra of hemin (20 µM) with DMSO (black) or XP (20 µM, light blue and 40 µM, dark blue) in 200 mM HEPES (pH 7.0). E) Inhibition of the GSH–mediated destruction of hemin by XanDME. Averaged technical quadruplicates were normalized to the respective DMSO-treated samples and values represent mean ± s.d. of independent experiments (n = 3). F) In vitro activity assay of reconstituted holoHRP in the presence of varying concentrations of Xan. Hemin (5 nM) was pre-incubated with either Xan (green bars) or apoHRP (light grey bars, 5 µM) followed by addition of apoHRP (5 µM) or Xan, respectively and activity of holoHRP was subsequently measured. Averaged technical duplicates were normalized to the respective DMSO-treated samples and values represent mean ± s.d. of independent experiments (n = 2). G) Reconstitution activity assay of holoHRP in *A. baumannii* cell lysate after treatment of intact cells with either 250 nM Xan or DMSO for 30 min. Cell lysates (2.5 µg protein) were pre-incubated with apoHRP (final concentration 10 µM) for 10 min at 4 °C and the activity of resulting holoHRP was measured. Lysates without apoHRP added were used as blank control. Averaged technical quadruplicates were normalized to the respective DMSO-treated samples and value represents mean ± s.d. of independent experiments (n = 3). Of note, due to the low solubility of PPIX and XanDME, the experiments described in B), C), and E) were recorded in DMSO / 200 mM HEPES (pH 7.0) (1:1, v/v).
**Supplementary Figure 13:** Accumulation of porphyrins. **A)** Fluorescence spectrum of extracted porphyrins. Intact *A. baumannii* ATCC19606 were incubated with *Xan* (1 µM) or DMSO for 1 h. After cell lysis, porphyrins were extracted and fluorescence spectra were recorded (λ_ex = 406 nm). Samples containing *Xan* without added bacteria (1 µM, violet) served as fluorescence control. Figure is representative for n = 6 independent extractions. **B)** Pellets of *A. baumannii* treated with either *Xan* (1 µM, left) or DMSO (right) for 4 h.

**Supplementary Figure 14:** Cytotoxicity of *Xan* against human cells (HeLa). Averaged technical sextuplicates were normalized to DMSO-treated samples and value represents mean ± s.d. of independent experiments (n = 3).
Supplementary Tables

**Supplementary Table 1** | Resistance development during serial passaging in the presence of sub-MIC concentrations of antimicrobials. Ciprofloxacin (Cip) served as positive control. For Xan and Cip, the highest concentrations tested were 25 µM (100-fold MIC) and 800 µM (~100-fold MIC), respectively. The table contains all individual data from n = 3 independent experiments.

| Day | Xan | | | | | | Cip | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|     | Replicate A | Replicate B | Replicate C | Replicate A | Replicate B | Replicate C |
|     | MIC [µM]    | MIC [µM]    | MIC [µM]    | MIC [µM]    | MIC [µM]    | MIC [µM]    |
| 1   | 0.25         | 0.25         | 0.25         | 16           | 8            | 8            |
| 2   | 0.50         | 0.50         | 0.5          | 64           | 32           | 16           |
| 3   | 1            | 0.5          | 0.5          | 128          | 32           | 64           |
| 4   | 1            | 1            | 1            | 500          | 128          | 64           |
| 5   | 2            | 2            | 2            | >800         | >500         | 256          |
| 6   | 2            | 2            | 2            | >800         | >800         | 500          |
| 7   | >8           | >8           | >8           | >800         | >800         | 800          |
| 8   | >25          | >25          | 25           | >800         | >800         | 800          |
| 9   | >25          | >25          | >25          | >800         | >800         | >800         |
| 10  | >25          | >25          | >25          | >800         | >800         | >800         |
| 11  | >25          | >25          | >25          | >800         | >800         | >800         |
**Supplementary Table 2** | Table containing detailed information on proteins upregulated in full proteome analysis of Xan-resistant mutants. For uncharacterized proteins, BLAST search was used to assign homologous proteins *A. baumannii* AB5075 (Program version BLASTP 2.10.1+, search limited to *A. baumannii* AB5075 (taxid:1116234), query coverage and percent identity are listed), which has a higher annotation rate than *A. baumannii* ATCC19606.4,5

| Function | Proteins | UniProt ID (ATCC19606) | BLAST Search | Query Coverage | Percent Identity | Gene name (AB5075) |
|----------|----------|-------------------------|--------------|----------------|------------------|--------------------|
| Acetoin metabolism | Acetoin:2,6-dichlorophenolindophenol oxidoreductase, alpha subunit | D0C7E8 | | | | |
| Dihydrolipoyl dehydrogenase | | D0C7E5 | | | | |
| TPP-dependent acetoin dehydrogenase complex, E1 component, beta subunit | | D0C7E7 | | | | |
| Redox enzyme | Uncharacterized protein | D0CAC1 | Heme oxygenase-like protein | 98% | 99% | ABUW_3351 |
| Catalase | D0C8B2 | | | | | |
| Uncharacterized protein | D0C8B3 | Heme oxygenase-like protein (Iron-containing redox enzyme family protein) | 99% | 99% | ABUW_2437 |
| RND transporter | Efflux transporter, RND family, MFP subunit | D0CF95 | | | | |
| Efflux transporter, HAE1/HME family, permease protein | D0CF96 | | | | | |
| Biofilm formation | Fimbrial usher protein | D0C5T3 | | | | |
| Protein CsuC | D0C5T2 | | | | | |
| Protein CsuE | D0C5T4 | | | | | |
| Spore Coat Protein U domain protein | D0C5T1 | | | | | |
| Poly-beta-1,6 N-acetyl-D-glucosamine export porin PgaA | D0C5Z9 | | | | | |
| Poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase PgaB | D0C600 | | | | | |
| Putative alpha,alpha-trehalose-phosphate synthase (UDP-forming) | D0C9J2 | | | | | |
| Trehalose 6-phosphate phosphatase | D0C9J3 | | | | | |
| Treptomycin resistance protein | D0C912 | | | | | |
| OmpA family protein | D0C913 | | | | | |
| DUF4142 domain-containing protein | D0CFS8 | | | | | |
| FMN-dependent oxidoreductase, nitroreductase family | D0C858 | | | | | |
| Oxidoreductase, short chain dehydrogenase/reductase family protein | D0C8B1 | | | | | |
| Reverse transcriptase domain-containing protein | D0CBF4 | | | | | |
| Rhs element Vgr protein | D0C9Y7 | | | | | |
| Transcriptional regulator, TetR family | D0C7Z9 | | | | | |
| Transcriptional regulator, TetR family | D0CAC2 | DUF2171 domain-containing protein | 100% | 100% | ABUW_1466 |
|--------------------------------------|--------|----------------------------------|------|------|-----------|
| Uncharacterized protein              | D0C5R3 | DUF2171 domain-containing protein | 100% | 100% | ABUW_1466 |
| Uncharacterized protein              | D0C5R4 | hypothetical protein             | 96%  | 99%  | not found |
| Uncharacterized protein              | D0C692 | DNA breaking-rejoining protein    | 100% | 98%  | ABUW_1659 |
| Uncharacterized protein              | D0C8B0 | Uncharacterized protein           | 100% | 98%  | ABUW_2434 |
| Uncharacterized protein              | D0C8B6 | hypothetical protein              | 100% | 99%  | ABUW_2440 |
| Uncharacterized protein              | D0C9C4 | no significant similarity found   | -    | -    | -         |
| Uncharacterized protein              | D0C9Y0 | Rhs element Vgr protein, putative | 11%  | 47%  | ABUW_2817 |
| Uncharacterized protein              | D0CAU6 | Chaperone modulatory protein CbpM (MerR HTH regulatory family protein) | 100% | 100% | ABUW_3516 |
| Uncharacterized protein              | D0CE49 | No significant similarity found   | -    | -    | -         |
| Uncharacterized protein              | D0CE50 | No significant similarity found   | -    | -    | -         |
| Uncharacterized protein              | D0CFS7 | 17 kDa surface antigen            | 100% | 96%  | ABUW_2678 |
**Supplementary Table 3** | Table containing detailed information on proteins downregulated in full proteome analysis of Xan-resistant colonies. For uncharacterized proteins, BLAST search was used to assign homologous proteins *A. baumannii* AB5075 (Program version BLASTP 2.10.1+, search limited to *A. baumannii* AB5075 (taxid:1116234), query coverage and percent identity are listed), which has a higher annotation rate than *A. baumannii* ATCC19606.4,5

| Proteins                                                                 | UniProt ID (ATCC19606) | BLAST Search        | Query Coverage | Percent Identity | Gene name (AB5075) |
|-------------------------------------------------------------------------|------------------------|---------------------|----------------|------------------|-------------------|
| Toxin-antitoxin system, toxin component, Bro domain protein             | D0C5H6                 |                     |                |                  |                   |
| Phosphomethylpyrimidine synthase                                        | D0CB46                 |                     |                |                  |                   |
| Uncharacterized protein                                                 | D0CC98                 | Uncharacterized protein | 100%           | 44%              | ABUW_0742         |
| Uncharacterized protein                                                 | D0CCB7                 | Uncharacterized protein | 82%            | 54%              | ABUW_1278         |
| Uncharacterized protein                                                 | D0CCD2                 | Uncharacterized protein | 83%            | 58%              | ABUW_0781         |
| Uncharacterized protein                                                 | D0CCD3                 | No significant similarity found |                |                  |                   |
| Sulfate ABC transporter, sulfate-binding protein                        | D0CCM4                 |                     |                |                  |                   |
| Protein PilG                                                             | D0CDH8                 |                     |                |                  |                   |
| Uncharacterized protein                                                 | D0CDU2                 | Putative tail fiber  | 59%            | 66%              | ABUW_0790         |
| Uncharacterized protein (Fragment)                                      | D0CDU3                 | Uncharacterized protein | 100%           | 89%              | ABUW_0791         |
| Uncharacterized protein                                                 | D0CED4                 | Uncharacterized protein | 31%            | 54%              | ABUW_2662         |
| Metallo-beta-lactamase domain protein                                   | D0CF44                 |                     |                |                  |                   |
| Cytochrome b562                                                         | D0CG11                 |                     |                |                  |                   |
### Supplementary Table 4: Table containing detailed information on proteins downregulated in full proteome analysis of Xan versus DMSO treated A. baumannii. For uncharacterized proteins, BLAST search was used to assign homologous proteins A. baumannii AB5075 (Program version BLASTP 2.10.1+, search limited to A. baumannii AB5075 (taxid:1116234), query coverage and percent identity are listed), that has a higher annotation rate than A. baumannii ATCC19606.\(^{4,5}\)

| #  | Proteins                                                                 | UniProt ID (ATCC19606) | BLAST Search               | Query Coverage | Percent Identity | Gene name (AB5075) |
|----|--------------------------------------------------------------------------|------------------------|----------------------------|----------------|------------------|--------------------|
| 1  | Uncharacterized protein                                                  | D0CC95                 | hypothetical protein       | 100%           | 99%              | ABUW_1256          |
| 2  | Uncharacterized protein                                                  | D0CC98                 | hypothetical protein       | 100%           | 44%              | ABUW_0742          |
| 3  | Toxin-antitoxin system, toxin component, Bro domain protein              | D0C5H6                 |                            |                |                  |                    |
| 4  | Uncharacterized protein                                                  | D0CDH3                 | YegP family protein        | 99%            | 100%             | ABUW_0673          |
| 5  | Transporter, branched chain amino acid:cation symporter (LIVCS) family protein | D0C8S3                 |                            |                |                  |                    |
| 6  | Aspartate racemase                                                       | D0C5W2                 |                            |                |                  |                    |
| 7  | Uncharacterized protein                                                  | D0CCB7                 | hypothetical protein       | 82%            | 54%              | ABUW_1278          |
| 8  | 5-oxoprolinase subunit A                                                 | D0C8S2                 |                            |                |                  |                    |
| 9  | Uncharacterized protein                                                  | D0C8S0                 | 5-oxoprolinase/urea amidolyase family protein | 100% | 97%              | ABUW_2604          |
| 10 | Metallo-beta-lactamase domain protein                                    | D0CF44                 |                            |                |                  |                    |
| 11 | Cytochrome d ubiquinol oxidase, subunit II                               | D0C6M3                 |                            |                |                  |                    |
| 12 | Type VI secretion system lysozyme-related protein                        | D0C8P2                 |                            |                |                  |                    |
| 13 | Diacylglycerol kinase catalytic domain protein                           | D0CBD5                 |                            |                |                  |                    |
| 14 | Uncharacterized protein (Fragment)                                       | D0C8P6                 | hypothetical protein       | 100%           | 100%             | ABUW_2581          |
| 15 | Uncharacterized protein                                                  | D0C773                 | hypothetical protein       | 100%           | 99%              | not found          |
| 16 | Molybdate ABC transporter, periplasmic molybdate-binding protein          | D0C793                 |                            |                |                  |                    |
| 17 | Fumarylacetoacetase                                                      | D0CFD2                 |                            |                |                  |                    |
| 18 | Uncharacterized protein                                                  | D0CD61                 | hypothetical protein       | 100%           | 98%              | ABUW_0514          |
| 19 | Conserved TM helix                                                       | D0C9F9                 |                            |                |                  |                    |
| 20 | Putative hydrolyase                                                      | D0C8S1                 |                            |                |                  |                    |
**Methods**

**Media**

Unless otherwise stated B medium (10 g/L casein peptone, 5 g/L NaCl, 5 g/L yeast extract, 1 g/L K$_2$HPO$_4$, pH 7.5) was used for cultivation of all *S. aureus* strains. LB medium (Lysogeny Broth; 10 g/L casein peptone, 5 g/L NaCl, 5 g/L yeast extract, pH 7.5) was used for cultivation of all *S. typhimurium* strains, all *A. baumannii* strains, both *E. coli* strains, and *P. aeruginosa* PAO1. BHB medium (Brain Heart Infusion, 7.5 g/L brain infusion, 10 g/L heart infusion, 10 g/L casein peptone, 5 g/L NaCl, 2.5 g/L Na$_2$HPO$_4$, 2 g/L glucose, pH 7.4) was used for cultivating *L. monocytogenes*, *K. pneumoniae*, *E. faecalis* and *E. faecium*.

For resistance development, *A. baumannii* was cultivated in cation-adjusted Mueller-Hinton broth (17.5 g/L acid hydrolysate of casein, 3 g/L beef extract, 1.5 g/L starch, pH 7.3).

**Stock solutions**

*Xan*, *XanDME*, *XP* and *XPP* were synthesized as described in section “Chemical Synthesis” and stock solutions were prepared with DMSO as solvent. 1 mM hemin working solution in 0.1 N aqueous NaOH was freshly prepared before use and stored in the dark.

Gentamicin (Gen) and Ciprofloxacin (Cip) stock solutions were prepared in sterile ddH$_2$O and 0.1 N aqueous HCl, respectively.

**Overnight cultures**

The appropriate medium (5 mL) for cultivation was inoculated (1:1,000) with the desired bacterial cryostock with a sterile pipette tip in a plastic culture tube. The culture was then incubated overnight (14 – 16 h, 37 °C, 200 rpm). A sterile control (medium containing no bacteria) was included each time.
Minimum inhibitory concentration (MIC)

Overnight cultures were diluted 1:10,000 into medium and directly used for the tests. Various dilutions of the compounds in DMSO were prepared and 1 µL thereof was pipetted in triplicates in 96 well-plates (transparent Nunc 96-well flat bottom, Thermo Fisher Scientific), including DMSO only, which served as growth control. Then, 99 µL of the diluted bacterial suspension were transferred to each well and a sterile control containing only medium was included. Bacteria were incubated for 24 h (37°C, 200 rpm) and the dilution series was analyzed for microbial growth, indicated by turbidity. The lowest concentration in the dilution series at which no growth of bacteria could be observed by eye was defined as the minimum inhibitory concentration (MIC) of the compound. MIC values were determined by three independent experiments.

Metal chelation assays

The assay was performed as described by Shapiro et al. with slight modifications. All fluorescence titrations were performed in MeOH on a Dual-FL™ (HORIBA Scientific) using a sub-micro fluorometer cell (open top, Starna, catalog no. 16.160F-Q-10/Z15). Briefly, a 12.5 µM stock solution of Xan (150 µL; 5% DMSO final concentration) was prepared and an emission spectrum was recorded (λexcitation = 366 nm, integration time = 1 s, accumulations = 10). The metal chloride stock solution (125 µM) was added in 3.75 µL portions (0.25 eq.), the solution was gently mixed, and an emission spectrum was recorded after each addition. All experiments were performed in triplicates. An emission spectrum of MeOH with 5% DMSO was used as a blank control and was subtracted from values. A fluorescence titration using MeOH served as a negative control for metal binding.

Gel-free activity-based protein profiling (ABPP) and affinity-based protein profiling (AfBPP)

Culture, labeling, lysis and click reaction for preparative ABPP in A. baumannii ATCC19606 and E. coli K12 with XP

Overnight cultures were diluted 1:100 in LB medium and incubated (37 °C, 200 rpm) until an OD_{600} of 2.0 was reached. Bacterial cells were harvested (6,000 ×g, 4 °C, 15 min), washed with PBS and resuspended in PBS to give a final OD_{600} of 40. This bacterial suspension was split into aliquots of 1 mL and either XP (3 µM, 1% DMSO final concentration) or DMSO (1%) was added, followed by incubation (37 °C, 200 rpm) for 2 h. For competition labeling, cells were treated with Xan (30 µM, 1% DMSO final concentration) for 2 h (37 °C, 200 rpm) before the addition of XP (3 µM, 1% DMSO final concentration). After compound treatment, bacteria were harvested.
(6,000 x g, 4 °C, 15 min) and washed with PBS. Cell pellets were resuspended in 0.4% (w/v) SDS in PBS (1 mL) and lysed by sonication (4 × 30 s, 75% intensity; Sonopuls HD 2070 ultrasonic rod, Bathelin electronic GmbH) with cooling breaks on ice. The cell lysate was clarified by centrifugation (20,000 x g, 30 min, 4 °C), protein concentration was measured using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Pierce Biotechnology) and sample concentrations were adjusted to equal protein amount. Next, 500 µL clear cell lysate (2 mg/mL protein concentration) were subjected to click-reaction (CuAAC) by adding 3 µL biotin azide (10 mM in DMSO), 10 µL tris(2-carboxyethyl) phosphine (TCEP; 52 mM in ddH2O), 30 µL TBTA ligand (1.667 mM in 80% tBuOH and 20% DMSO) and 10 µL CuSO4 (50 mM in ddH2O) and samples were incubated for 1 h at room temperature (RT). Following CuAAC, proteins were precipitated with ice-cold acetone (4 volumes) at –20 °C overnight, centrifuged (16,900 x g, 30 min, 4 °C), and washed twice with ice-cold methanol (1 mL). Pellets were resuspended in 0.4% (w/v) SDS in PBS (500 µL) at RT by sonication (10% intensity, 10 s; Sonopuls HD 2070 ultrasonic rod, Bathelin electronic GmbH). Affinity enrichment was performed with avidin agarose resin (product no. A9207, Sigma Aldrich, pre-washed three times with 0.4% (w/v) SDS in PBS (1 mL); 400 x g for 2 min was used to pellet beads; typically, 50 µL of bead slurry was used for enrichment). Samples were added to the resin in LoBind Eppendorf tubes and incubated with agitation for 1 h at RT. To remove unbound proteins, the beads were washed three times with 0.4% (w/v) SDS in PBS (1 mL), two times with 6 M urea (in ddH2O, 1 mL) and three times with PBS (1 mL). For quantitative mass spectrometric analyses, avidin agarose beads with bound proteins were resuspended in 200 µL denaturation buffer (7 M urea, 2 M thiourea in 20 mM pH 7.5 HEPES buffer). Upon on-bead reduction with TCEP (5 mM) at 37 °C for 1 h, proteins were alkylated using iodoacetamide (IAA, 10 mM) at 25 °C for 30 min and samples were quenched with dithiothreitol (DTT, 10 mM) at RT for 30 min. Samples were diluted with 600 µL triethylammonium bicarbonate (TEAB) buffer (50 mM in ddH2O) and digested with 1.5 µL trypsin (0.5 µg/µL; sequencing grade, modified, Promega) at 37 °C overnight under continuous shaking (450 rpm). On the next day, the digestion was stopped by adding formic acid (FA, 10 µL) and the suspension was centrifuged (17,000 x g, 3 min, RT) to pelletize the beads. The supernatant was loaded on 50 mg SepPak C18 columns (Waters) equilibrated with 0.1% trifluoroacetic acid (TFA). The peptides were washed three times with 1 mL 0.1% TFA and 500 µL 0.5% FA. Afterwards, the peptides were eluted three times with 250 µL elution buffer (80% acetonitrile (ACN), 0.5% FA), lyophilized and stored at –80 °C until further usage. The experiment was performed in n = 4 (E. coli K12) and in n = 3 (A. baumannii ATCC19606) independent experiments.
Culture, labeling, lysis and click reaction for preparative ABPP in *A. baumannii* ATCC19606 with XPP

Overnight cultures were diluted 1:100 in LB medium and incubated (37 °C, 200 rpm) until an OD₆₀₀ of 3.5 was reached. Bacterial cells were harvested (6,000 ×g, 4 °C, 15 min), washed with PBS and resuspended in PBS to give a final OD₆₀₀ of 40. This bacterial suspension was split into aliquots of 1 mL and either XPP (3 µM, 1% DMSO final concentration) or DMSO (1%) was added, followed by incubation (37 °C, 200 rpm) for 2 h. After compound treatment, samples were transferred to 6-well plates (flat bottom, VWR) and irradiated for 15 min with UV light (UV low-pressure mercury-vapour fluorescent lamp, Philips TL-D 18W BLB, 360 nm maximum). During irradiation a cool pack was placed under the plate. Subsequently, bacteria were harvested (6,000 ×g, 4 °C, 15 min) and washed with PBS. Cell pellets were resuspended in PBS (1 mL) and lysed (3 × 30 s at 6,500 rpm, 30 s cooling breaks on ice after each run; Precellys Glass/Ceramic Kit SK38 2.0 mL tubes; Precellys 24 Homogenizer, Bertin Technologies). The cell lysate was clarified by centrifugation (20,000 ×g, 30 min, 4 °C). The soluble fraction was transferred to a new Eppendorf tube. The insoluble fraction was washed twice with PBS and resuspended in PBS (600 µL). Protein concentration was measured using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Pierce Biotechnology) and sample concentrations were adjusted to equal protein amount (soluble fraction, 1 mg/mL; insoluble fraction, 2 mg/mL). Next, 10% (w/v) SDS in PBS was added to 500 µL of adjusted protein samples to get a final concentration of 0.4% (w/v) SDS in PBS. Afterwards, the experimental procedure was analogous to “preparative ABPP in *A. baumannii* ATCC19606 and *E. coli* K12 with XP” as described above. The experiment was performed in n = 4 independent experiments.

**LC-MS/MS analysis**

Before MS measurements, the lyophilized peptides were resolved in 25 µL 1% FA and filtered through 0.22 µm PVDF filters (Millipore), which were equilibrated with 300 µL 1% FA. The filtrates were transferred into MS-vials and stored at −20 °C until the measurements were performed.

Samples were analyzed with an UltiMate 3000 nano HPLC system (Dionex) using an Acclaim C18 PepMap100 (75 µm ID × 2 cm) trap column and an Acclaim PepMap RSLC C18 (75 µm ID × 50 cm) separation column coupled to a Q Exactive Plus (Thermo Fisher) in EASY-spray setting. Samples were loaded on the trap column and washed with 0.1% TFA, then transferred to the analytical column (buffer A: H₂O with 0.1% FA, buffer B: ACN with 0.1% FA, flow 300 nL/min, gradient 5 to 22% buffer B in 115 min, then to 32% buffer B in 10 min, then to 90% buffer B in 10 min and hold 90% buffer B for 10 min, then to 5% buffer B in 0.1 min and hold 5% buffer B for
9.9 min). Q Exactive Plus was operated in a TOP10 data dependent mode. Full scan acquisition was performed in the orbitrap at a resolution of 140,000 and an AGC target of 3e6 (maximum injection time of 80 ms) in a scan range of 300−1,500 m/z. Monoisotopic precursor selection as well as dynamic exclusion (exclusion duration: 60 s) was enabled. Precursors with charge states of >1 and intensities greater than 1e5 were selected for fragmentation. Isolation was performed in the quadrupole using a window of 1.6 m/z. Precursors were collected to an AGC target of 1e5 (maximum injection time of 100 ms) and acquisition was performed at a resolution of 17,500 in a scan range of 200−2,000 m/z. Fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 27%) and detected in the orbitrap.

**MS data analysis**

Raw files were analyzed using MaxQuant software (version 1.6.2.10) with the Andromeda search engine. The following settings were applied: fixed modification: carbamidomethylation (cysteine); variable modification: oxidation (methionine), acetylation (N-terminus); proteolytic enzyme: trypsin/P; missed cleavages: 2; main search tolerance: 4.5 ppm; MS/MS tolerance: 0.5 Da; false discovery rates: 0.01. The options “LFQ” and “match between runs” (0.7 min match and 20 min alignment time windows) were enabled; “second peptides” was disabled. Searches were performed against the UniProt database for *A. baumannii* ATCC19606 (taxid: 575584, 29.06.2020) or *E. coli* K12 (taxid: 83333, 01.07.2020). Statistical analysis of the data was performed using Perseus (version 1.6.13.0). Putative contaminants, reverse peptides and peptides only identified by site were deleted. LFQ intensities were log2-transformed and data was filtered for either three valid values in at least one group (ABPP with XP in *E. coli*, ABPP with XPP in *A. baumannii*) or two valid values in at least one group (ABPP with XP in *A. baumannii*) and missing value imputation was performed over the total matrix. For statistical evaluation, −log10 (*P* values) were obtained by a two-sided two sample Student’s *t*-test.

**Gel-based ABPP**

For labeling of recombinant protein, 100 µL of the protein in PBS (1 µM) were incubated with various concentrations of XP (1% DMSO final concentration) for 1 h at RT. Heat control (h.c.) protein samples were prepared in 1% (w/v) SDS in PBS and were boiled at 95 °C for 20 min prior to the addition of the probe. After compound treatment, the samples were subjected to CuAAC by adding 2 µL rhodamine azide (10 mM in DMSO), 2 µL TCEP (52 mM in ddH2O), 6 µL TBTA ligand (1.667 mM in 80% tBuOH and 20% DMSO) and 2 µL CuSO4 (50 mM in ddH2O) and incubated for 1 h at RT. One equivalent of 2× Laemmli buffer (125 mM Tris–HCl, 20% (v/v) glycerol, 4% (w/v)
SDS, 0.005% (w/v) bromphenol blue, 10% (v/v) 2-mercaptoethanol) was added and samples were subsequently analyzed by SDS-PAGE (12.5% polyacrylamide gels).

The BenchMark™ Fluorescent Protein Standard (Thermo Fisher Scientific) and the Roti®-Mark Standard (Carl Roth) were used as size markers. An ImageQuant LAS- 4000 image reader (GE Healthcare) equipped with a Fujinon VRF43LMD3 lens and a 575DF20 filter (Fujifilm) was used for visualization of labelled proteins. Gels were then Coomassie-stained by overnight incubation in staining solution (0.25% (w/v) Coomassie Brilliant Blue R-250, 9.2% AcOH, 45.4% EtOH), followed by destaining with 10% AcOH, 40% EtOH in ddH₂O.

**Cloning**

| Plasmid          | Description                                                                 | Cloning method     | Source       |
|------------------|-----------------------------------------------------------------------------|--------------------|--------------|
| pDONR 207        | Gateway donor vector; rrnB T2, rrnB T1, attP1, ccdB, CmR, attP2, GenR, pUC ori | -                  | Invitrogen   |
| pDONR 207- abhemB<sub>wt</sub> | Gateway donor vector; rrnB T2, rrnB T1, attL1, attL2, GenR, pUC ori, gene hemB cloned into attP1 and attP2 sites of pDONR207 | Gateway cloning    | This study   |
| pDONR 207- abkatG | Gateway donor vector; rrnB T2, rrnB T1, attL1, attL2, GenR, pUC ori, gene AbkatG cloned into attP1 and attP2 sites of pDONR207 | Gateway cloning    | This study   |
| pDONR 207- eckatG | Gateway donor vector; rrnB T2, rrnB T1, attL1, attL2, GenR, pUC ori, gene EckatG cloned into attP1 and attP2 sites of pDONR207 | Gateway cloning    | This study   |
| pDest 17         | Gateway destination vector; T7 promotor, N-6×His, attR1, CmR, ccdB, attR2, AmpR, pBR322 ori | -                  | Invitrogen   |
| Vector Name          | Description                                                                 | Gateway or Restriction Enzyme-Based Cloning | Source          |
|---------------------|-----------------------------------------------------------------------------|---------------------------------------------|-----------------|
| pET-41-K            | Gateway destination vector; T7 promoter, *lacI*, N-6×His, MBP, *attR1*, Cm<sup>R</sup>, *ccdB*, *attR2*, Kan<sup>R</sup>, pBR322 ori | -                                           | EMBL            |
| pET 300             | Gateway destination vector; T7 promoter, *lacI*, N-6×His, *attR1*, Cm<sup>R</sup>, *ccdB*, *attR2*, Amp<sup>R</sup>, pBR322 ori | -                                           | Invitrogen      |
| pDest 17-*abhemB<sub>wt</sub>* | Expression vector; *attB1*, *attB2*, Amp<sup>R</sup>, T7 promoter, pBR322 ori, N-6×His, gene *hemB* cloned into *attR1* and *attR2* sites of pDest17 | Gateway cloning                          | This study      |
| pET-41-K-*abhemB<sub>wt</sub>* | Expression vector; *attB1*, *attB2*, Amp<sup>R</sup>, T7 promoter, *lacI*, pBR322 ori, N-6×His, MBP, gene *hemB* cloned into *attR1* and *attR2* sites of pET-41-K | Gateway cloning                          | This study      |
| pET 300-*abkatG*    | Expression vector; *attB1*, *attB2*, Amp<sup>R</sup>, *lacI*, T7 promoter, pBR322 ori, N-6×His, gene *katG* (*A. baumannii*) cloned into *attR1* and *attR2* sites of pET 300 | Gateway cloning                          | This study      |
| pET 300-*eckatG*    | Expression vector; *attB1*, *attB2*, Amp<sup>R</sup>, *lacI*, T7 promoter, pBR322 ori, N-6×His, gene *katG* (*E. coli*) cloned into *attR1* and *attR2* sites of pET 300 | Gateway cloning                          | This study      |
| pET MBP-1a          | Modified pET-24d; T7 promoter, *lacI*, pBR322 ori, Kan<sup>R</sup>, N-6×His, MBP, TEV site, MCS | -                                           | EMBL            |
| pET MBP-1a-*abhemB<sub>wt</sub>* | Expression vector; T7 promoter, *lacI*, pBR322 ori, Kan<sup>R</sup>, N-6×His, MBP, TEV site, MCS, gene *hemB* cloned into MCS (Ncol, Npml) | Restriction enzyme-based cloning and ligation | This study      |
| pET MBP-1a-abhemB<sub>P241S</sub> | Expression vector; T7 promoter, lacI, pBR322 ori, Kan<sup>R</sup>, N-6×His, MBP, TEV site, MCS, gene hemB(P241S) cloned into MCS (NcoI, NotI) | QuikChange site-directed mutagenesis | This study |
|---|---|---|---|
| pVRL2 | E. coli-Acinetobacter species shuttle vector, araC-P<sub>BAD</sub> arabinose-inducible expression cassette; Gen<sup>R</sup>, MCS, origin of replication for A. baumannii (oriAb). | - | Lucidi et al.<sup>13</sup> |
| pVRL2-abhemB<sub>wt</sub> | Expression vector, araC-P<sub>BAD</sub> arabinose-inducible expression cassette; Gen<sup>R</sup>, MCS, oriAB, gene hemB was cloned into MCS (EcoRI, XbaI) | Restriction enzyme-based cloning and ligation | This study |
| pVRL2-abhemB<sub>P241S</sub> | Expression vector, araC-P<sub>BAD</sub> arabinose-inducible expression cassette; Gen<sup>R</sup>, MCS, oriAB, gene hemB(P241S) was cloned into MCS (EcoRI, XbaI) | Restriction enzyme-based cloning and ligation | This study |

**Gateway cloning**

For the recombinant expression of porphobilinogen synthase (or 5-aminolevulinic acid dehydratase (PbgS)) (gene: hemB, UniProt ID: D0C9T7) wild type, catalase-peroxidase (gene: katG, UniProt ID: D0CAQ1) wild type from A. baumannii ATCC19606 (taxid: 575584; AbCAT) and catalase-peroxidase (gene: katG, UniProt ID: P13029) wild type from E. coli K12 (taxid: 83333; EcCAT), the Invitrogen™ Gateway™ cloning system (Thermo Fisher Scientific) was used. The following primers containing the required attB recombination sites were designed (fwd. = forward, rev. = reverse):

| Primer name | Sequence (5’ – 3’) |
|---|---|
| fwd. attB1 hemB | ggggacaagtgtcataaaaaagcaggttt ATGGGGCTACAATACAGCAGT |
The polymerase chain reaction (PCR) was carried out in a CFX96 Real-time System in combination with a C1000 Thermal Cycler (BioRad). An overnight culture of *A. baumannii* ATCC19606 or *E. coli* K12 was used as DNA template. The reaction mixtures contained 10 µL GC or HF buffer (*NEB*), 1 µL dNTP (10 mM), 2.5 µL fwd. primer (10 µM), 2.5 µL rev. primer (10 µM), 1 µL overnight culture, 0.5 µL Phusion® High Fidelity DNA polymerase (*NEB*) and 32.5 µL ddH₂O. After initial denaturation (98 °C, 1 min 50 s), the mixtures underwent 34 cycles of denaturation (98 °C, 10 s), annealing (60 °C, 30 s) and extension (72 °C, 60 s), before a final extension (72 °C, 3 min). The PCR products were purified by the MicroElute® Cycle-Pure Kit (*OMEGA Bio-Tek*) and finally the DNA concentration was measured on an Infinite® M200 Pro microplate reader using a NanoQuant Plate™ (*Tecan Group Ltd.*).

In the BP-reaction the PCR-product is incorporated in the donor vector of choice, here pDONR 207 was used. For this, 1 µL of the PCR-product (100-150 ng), 1 µL of the vector pDONR 207 (150 ng), 6 µL TE-buffer and 2 µL Gateway® BP Clonase® II enzyme mix (*Invitrogen*) were mixed and incubated for 4 h at RT. For transformation, the BP-solution was added to 200 µL of chemically competent *E. coli* TOP10 cells (*Invitrogen*) and incubated for 15 – 30 min on ice. After a 40 – 45 s heat shock at 42 °C the cells were put on ice for another 5 min. Afterwards, the cells were grown for 1 h in 500 µL SOC medium (2% tryptone, 0.5 % yeast extract, 10 mM NaCl, 2,5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ und 20 mM glucose) at 37 °C, plated on LB agar plates supplemented with Gen (7.5 mg/L) and incubated at 37 °C overnight. Single colonies were picked and grown in 5 mL LB medium containing Gen (7.5 mg/L) for 15 h at 37 °C. The plasmid DNA of the cells was isolated using the E.Z.N.A.® Plasmid DNA mini Kit I (*OMEGA Bio-Tek*) according to the manufacturer’s protocol and the final DNA concentration was measured as described above. The sequences of resulting plasmids pDONR 207-*abhemB*ₜₜ, pDONR 207-*abkatG* and pDONR 207-*eckatG* were verified via Sanger sequencing (*GATC Biotech AG*).
For the LR-reaction, pDONR 207-\textit{abhemB}\textsubscript{wt}, pDONR 207-\textit{abkatG} or pDONR 207-\textit{eckatG} (100 – 150 ng) were mixed with destination vectors (150 ng) (Supplementary Table 8) and TE buffer was added to a final volume of 8 µL. Afterwards, 2 µL Gateway® LR Clonase® II enzyme mix (Invitrogen) was added and mixtures were incubated for 2 h at RT. The transformation into chemically competent \textit{E. coli} TOP10 cells (Invitrogen), the isolation of resulting plasmids and verification of their sequences was done as already described for the BP-reaction using the appropriate antibiotic.

### Supplementary Table 8

| Destination vector | Tags         | Selection       |
|--------------------|--------------|-----------------|
| pET 300            | N-6×His      | Ampicillin (100 mg/L) |
| pDest 17           | N-6×His      | Ampicillin (100 mg/L) |
| pET- 41-K          | N-6×His-MBP  | Kanamycin (25 mg/L) |

Chemically competent \textit{E. coli} expression strains (\textit{E. coli} BL 21 (DE3) and \textit{E. coli} Lemo21 (DE3)) were transformed with the respective expression plasmid (100 ng) following the protocol as described in the BP-reaction using the appropriate antibiotic or antibiotic combinations (Supplementary Table 9).

### Supplementary Table 9

| Vectors   | Expression strain   | Selection                                   | Genes          |
|-----------|---------------------|---------------------------------------------|----------------|
| pET 300   | \textit{E. coli} BL21(DE3) | Ampicillin (100 mg/L)                      | \textit{abkatG, eckatG} |
| pDest 17  | \textit{E. coli} BL21(DE3) | Ampicillin (100 mg/L)                      | \textit{hemB}   |
|           | \textit{E. coli} Lemo21(DE3) | Ampicillin (100 mg/L), Chloramphenicol (30 mg/L) | \textit{hemB}   |
| pET- 41K  | \textit{E. coli} BL21(DE3) | Kanamycin (25 mg/L)                        | \textit{hemB}   |
|           | \textit{E. coli} Lemo21(DE3) | Kanamycin (25 mg/L), Chloramphenicol (30 mg/L) | \textit{hemB}   |
Restriction enzyme-based cloning and ligation

Cloning of pET MBP-1a-αhemB<sub>wt</sub> and pET MBP-1a-αhemB<sub>P241S</sub>

In order to install a TEV-cleavage site between the maltose binding protein (MBP) and PbgS, vector pET MBP-1a was used and <i>hemB</i> was cloned into this plasmid using standard techniques based on PCR, restriction digest and ligation.

For amplification of <i>hemB</i> wt, PCR was performed as described in section “Gateway cloning” using the primers indicated in Supplementary Table 10 and an annealing temperature of 63 °C. After purification, the PCR product as well as plasmid pET MBP-1a (each 1 µg) was digested in CutSmart buffer (<i>NEB</i>) at 37 °C for 60 min using 10 units of the restriction enzymes <i>Nco</i>-HF and <i>Not</i>-HF, each. The digested PCR product was purified and DNA concentration was measured as described above. The digested vector was purified by agarose gel electrophoresis on a 1% agarose gel. After extraction using a gel extraction kit (<i>VWR</i>, catalog no. 101318-972) according to the manufacturer´s instructions, 50 ng of the purified vector and 50 ng of the purified insert were ligated using 1 µL Quick Ligase (<i>NEB</i>) in 10 µL of Quick Ligase buffer (<i>NEB</i>) in a total volume of 20 µL. The ligation mixture was incubated at 25 °C for 10 min. The transformation in chemically competent <i>E. coli</i> TOP10 cells (<i>Invitrogen</i>), the isolation of pET MBP-1a-αhemB<sub>wt</sub> and the verification of the sequence was performed as already described in section “Gateway cloning” using kanamycin (25 mg/L).

| Primer name       | Sequence (5’ – 3’)                      |
|-------------------|----------------------------------------|
| fwd. MBP-TEV-hemB | TAAGCA CCATGG CA ATGGGGCTACAATACAGCAGT |
| rev. MBP-TEV-hemB | TGCTTA GCGGCCGC TTAGTTCATTTCCCTGAGTTTTTCAGC |
| fwd. <i>hemB</i> P241S | GCATCACGGAACGAACCATAGAAGCTAGACGCAT |
| rev. <i>hemB</i> P241S | ATGCGTCTAGCTTCTATGGTTCGTTCCGTGATGC |

To exchange proline 241 for serine in PbgS, the QuikChange site-directed mutagenesis system (<i>Agilent</i>) was used. The two mismatching primers (fwd. <i>hemB</i> P241S, rev. <i>hemB</i> P241S) were
designed with the QuikChange Primer Design program (Agilent) and were used for PCR (Supplementary Table 10). The plasmid pET MBP-1a-abhemB mt served as DNA template for the mismatch PCR reaction. According to Phusion manufacturer’s protocol, 50 µL PCR reaction mixture were prepared containing 32 µL ddH₂O, 10 µL GC buffer (NEB), 1.5 µL fwd. and 1.5 µL rev. primer (10 µM), 1.0 µL dNTPs (10 mM), 1.5 µL DMSO and 2.0 µL template DNA (50 ng/µL). Finally, 0.5 µL Phusion® High Fidelity DNA polymerase (NEB) were added and the PCR reaction was subsequently performed. After initial denaturation (98 °C, 3 min), the mixtures underwent 34 cycles of denaturation (95 °C, 45 s), annealing (60 °C, 30 s) and extension (72 °C, 4 min), before a final extension (72 °C, 7 min). The unmutated parental DNA was removed by digestion of the PCR mixture with the endonuclease DpnI (NEB), which is specific for methylated and hemimethylated DNA. Therefore, 1.0 µL DpnI (NEB) as well as 1.0 µL CutSmart® buffer (NEB) was added to 8 µL of PCR product and the digestion mixture was incubated for 3 h at 37 °C. The transformation of PCR product into E. coli XL1-Blue competent cells, isolation of the resulting plasmid pET MBP-1a-abhemB P241S and verification of the mutation P241S was performed as already described in section “Gateway cloning” using kanamycin (25 mg/L).

Cloning of pVRL2-abhemB wt and pVRL2-abhemB P241S

The vector pVRL2, which was shown to be suitable for gene cloning and expression in Acinetobacter species, was generously provided by Paolo Visca. The genes hemB wt and hemB P241S were cloned into the pVRL2 expression vector. The genes hemB wt and hemB P241S were amplified by PCR using pET MBP-1a-abhemB mt and pET MBP-1a-abhemB P241S as templates, respectively. PCR was performed as described in section “Gateway cloning” using the primers indicated in Supplementary Table 11 and an annealing temperature of 63 °C. PCR products and vector pVRL2 were digested using EcoRI-HF and XbaI and purified as described in section “Cloning of pET MBP-1a-abhemB mt and pET MBP-1a-abhemB P241S”. Next, 50 ng of the purified vector and 45 ng of the purified inserts were ligated using 2 µL T4 Ligase (NEB) in T4 Ligase buffer (NEB) in a total volume of 20 µL. The ligation mixture was incubated at RT for 4 h. Transformation into TOP10 chemically competent E. coli (Invitrogen) and the isolation of the plasmids was performed as already described in section “Gateway cloning” using Gen (10 mg/L). The plasmid sequence was verified by GeneWiz using pVRL2 sequencing primer (Supplementary Table 11).
Supplementary Table 11 | Primers used for cloning and sequencing of hemB wt and hemB P241S into the pVRL2 vector (fwd. = forward, rev. = reverse). Restriction sites of EcoRI and XbaI are underlined.

| Primer name                      | Sequence (5’–3’)                      |
|----------------------------------|---------------------------------------|
| fwd. pVRL2 hemB                  | TAAGCA GAATTC ATGGGGCTACAATACAGC       |
| rev. pVRL2 hemB                  | TGCTTA TCTAGA TTAGTTCATTTCCTGAGTTTTTCAGC |
| fwd. pVRL2 sequencing             | CAACTCTCTACTGTTTCTCCAT                 |
| rev. pVRL2 sequencing             | GACGTTGTAAACGACG                      |

Overexpression and purification of catalases-peroxidases (AbCAT, EcCAT)

To overexpress CATs, a protocol published by Di Gennaro et al. was used with modifications.\(^\text{14}\) E. coli BL21(DE3) harbouring either pET300-akatG or pET300-eckatG were grown at 37 °C overnight in 20 mL LB medium supplemented with ampicillin (100 mg/L). The next day, 10 mL of the overnight culture were transferred into 1 L LB medium containing ampicillin (100 mg/L) and bacteria were grown at 37 °C to an OD\(_{600}\) of 0.4. Then, the gene expression was induced by the addition of IPTG (1 mM) as well as FeCl\(_2\) (250 μM) and the cells were grown for 24 h at 30 °C. Afterwards, bacteria were harvested (6,000 xg, 4 °C, 30 min) and washed with PBS. The pellets were then resuspended in 20 mL His-lysis buffer (20 mM Tris/HCl, 10 mM imidazole, 150 mM NaCl, 2 mM β-Mercaptoethanol, 0.2% (v/v) NP-40, pH 8.0 in ddH\(_2\)O) and lysed by sonication using the following protocol twice: 7 min at 30% intensity, 3 min at 80% intensity (Sonopuls HD 2070 ultrasonic rod, Bandelin electronic GmbH). Soluble and insoluble fractions were separated by centrifugation (20,000 xg, 4 °C, 30 min), the soluble fraction was loaded on a 50 mL Superloop (GE Healthcare) and injected into an Äkta Purifier 10 System equipped with UV-detector (UPC-900, P-900, Box-900, Frac-950, GE Healthcare). For affinity chromatography a His-Trap HP 5 mL column (GE Healthcare) was used, which was equilibrated with wash buffer 1 (20 mM Tris/HCl, 10 mM imidazole, 150 mM NaCl, 2 mM β-mercaptoethanol, pH 8.0 in ddH\(_2\)O; 5 CV). The column was washed with wash buffer 1 (8 CV), wash buffer 2 (20 mM Tris/HCl, 10 mM imidazole, 1 mM NaCl, 2 mM β-mercaptoethanol, pH 8.0 in ddH\(_2\)O; 8 CV) and wash buffer 3 (20 mM Tris/HCl, 40 mM imidazole, 150 mM NaCl, 2 mM β-mercaptoethanol, pH 8.0 in ddH\(_2\)O; 8 CV) and proteins were eluted with elution buffer (20 mM Tris/HCl, 500 mM imidazole, 150 mM NaCl, pH 8.0 in ddH\(_2\)O). The protein-containing fractions were pooled and dialyzed in CAT storage buffer (20 mM Tris/HCl, 2 mM Na\(_2\)EDTA, pH 7.0 in ddH\(_2\)O) overnight, aliquotted and stored at −80 °C. Protein
concentrations were determined on an Infinite® M200 Pro microplate reader using a NanoQuant Plate™ (Tecan Group Ltd.) by measuring the absorption at 280 nm. Extinction coefficients of 156,870 and 144,840 M⁻¹cm⁻¹ were used for AbCAT and EcCAT, respectively.

**Catalase activity assay**

The catalase activity assay kit (catalog no. K773-100, BioVision) was used and the assay was performed according to manufacturer’s protocol with slight modifications. Hydroxylamine was used as a positive control for CAT inhibition. CAT (25 nM final concentration) was pre-incubated with either Xan or Hydroxylamine for 30 min at RT and the reaction was started by the addition of H₂O₂ (16 µM final concentration). After 15 min at RT, 10 µL of the stop solution and 50 µL of the develop mix (47.7 µL assay buffer, 0.3 µL OxiRed probe, 2 µL HRP) were added to terminate the reaction. After incubation for 10 min, the fluorescence signal (λₑₓ. = 535 nm, λₑᵐ. = 587 nm) was measured. Experiments were performed in three independent experiments with two technical replicates each.

**Generation and sequencing of Xan resistant *A. baumannii* ATCC19606**

**Resistant development assay**

Resistant development assay is based on a procedure previously published by Le et al. and Bogdanovich et al. with slight modifications. For resistance development by sequential passaging, an overnight culture of *A. baumannii* ATCC19606 in cation-adjusted Mueller-Hinton broth (MHII) was adjusted to an OD₆₀₀ of 2.0 and subsequently diluted 1:100 into MHII medium (1 mL) containing various concentrations of Xan (1% DMSO final concentration) or Cip as positive control as well as DMSO (1%) or 0.1 N HCl as growth controls. Bacteria were incubated (37 °C, 200 rpm) and passaged in 20 - 24 h intervals in the presence of various concentrations of Xan or Cip (0.25, 0.5, 1, 2, 4 x MIC). The culture of the second highest concentration that allowed visible growth was adjusted to an OD₆₀₀ of 2.0 and subsequently diluted 1:100 into fresh MHII media (1 mL) containing different concentrations of the respective antimicrobial agent (0.25, 0.5, 1, 2, 4 x MIC). If a shift in MIC levels was observed, the concentrations of the respective antimicrobial were adjusted accordingly for the subsequent passage. This serial passaging was repeated for 10 days and in three independent biological replicates. The growth control, which contained only DMSO, was additionally passaged as a negative control to identify naturally occurring mutations. For this purpose, the growth control was adjusted each day to an OD₆₀₀ of 2.0 and diluted 1:100 into MHII medium (1 mL) containing 1% DMSO.
Preparation of isolated clones for whole genome sequencing

For sequencing, clones of Xan resistant mutants and of the negative control (DMSO) were isolated. For this purpose, culture tubes with 1 mL of MHII media containing either 25 µM Xan (for resistant mutants) or DMSO (for negative control) were inoculated using the cryostocks of day 11 and incubated overnight (37 °C, 200 rpm). On the next day, bacteria (120 µL) were harvested (7,000 xg, 10 min, 4 °C), washed once with MHII media to remove excess of Xan and resuspended in 20 µL LB medium. Bacterial suspensions were plated on either LB agar plates supplemented with Xan (8 µM, for resistant mutants) or on LB agar plates (negative control), which were incubated overnight at 37 °C. Three colonies of each independent biological experiment (A-1, A-2, A-3 (replicate A), B-1, B-2, B-3 (replicate B), C-1, C-2, C-3 (replicate C)) and of the negative control were isolated and were subjected to genome sequencing.

Whole genome sequencing and processing

Total bacterial DNA was extracted and purified from A. baumannii ATCC19606 (wt) and derivatives using the DNeasy Blood & Tissue kit (Qiagen). DNA sequencing libraries were prepared using the NEBNext UltraII FS DNA Library kit, and the genomic DNAs were sequenced using MiSeq Reagent Kits v3 (2x300bp) on an Illumina MiSeq.

Whole-genome sequencing data are available on the SRA repository under Bioproject number PRJNA639720 (https://www.ncbi.nlm.nih.gov/sra/PRJNA639720) with Biosample accession numbers SAMN15248516-SAMN15248528.

Briefly, reads were trimmed based on quality with a Phred quality score cut-off of 30 and reads shorter than 50 nucleotides were discarded. Processed reads were assembled using SPAdes (version 3.11.1) de novo genome assembler software in careful mode. Contigs with a length lower than 200 bp or average coverage lower than 15 were discarded. Average contig coverage was determined by mapping processed reads to the assembled contigs utilizing Bowtie2 (version 2.3.2) and the per base coverage was determined using SAMtools (version 1.8).

Processed reads of derivatives were mapped to the ATCC19606 genome assembly using Bowtie2. Variants were called using mpileup part of BCFtools (version 1.8). Variants were filtered out by a minimum SNP and INDEL distance of 10 bp, variant quality of 30, coverage of 50, mapping quality of 40 and a Z-score of 1.96.

All assemblies were annotated utilizing Prokka genome annotation software (version 1.4).
Overexpression and purification of PbgS(wt) in *E. coli*

Overnight cultures of *E. coli* expression strains, whose preparation is described in the section "Cloning", were grown (37 °C, 200 rpm) under respective antibiotic selection. On the next day, 20 mL LB medium containing appropriate antibiotics were inoculated (1:100) and cells were grown (37 °C, 200 rpm) until an OD$_{600}$ of 0.4 – 0.8 was reached. Protein expression was induced by the addition of 400 µM IPTG and cells were incubated at indicated temperature for 20 h at 150 rpm. On the next day, 5 mL of bacterial culture was harvested by centrifugation (15,000 ×g, 1 min, 4 °C) and cells were resuspended in 200 µL lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0). Lysozyme was added to a final concentration of 1 mg/ml followed by incubation on ice for 30 min. Cells were lysed by gently vortexing and lysate was cleared by centrifugation (15,000 ×g, 10 min, 4 °C). The supernatant was transferred into a fresh tube, 20 µL of a 50% slurry of Ni-NTA (Qiagen) were added and mixed gently for 30 min at 4 °C. Resins were washed twice with 200 µL wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0) by centrifugation (1,000 ×g, 10 s, 4 °C) and proteins were eluted with 3 × 20 µL elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0). Next, 50 µL of each collected fraction were mixed with one equivalent of 2× Laemmli buffer and analyzed by SDS-PAGE with Coomassie-staining as already described in section “Gel-based ABPP”.

**Generation of *A. baumannii* (pVRL2-ahhemBwt) and (pVRL2-ahhemB$_{P241S}$)**

Competent *A. baumannii* cells were prepared for electrotransformation as previously described. Briefly, an overnight culture of *A. baumannii* ATCC19606 was used to inoculate 50 mL of pre-warmed LB medium (1:100) and the bacteria were grown for 24 h (37 °C, 200 rpm). Cells were harvested by centrifugation (3,000 ×g, 15 min, 4 °C), washed twice with sterile 10% glycerol (25 mL) at RT, and suspended in 10% glycerol (1.5 mL). Bacterial suspension was split into 50 µL aliquots, which were stored at -80 °C until they were used.

Electroporation was performed in 2 mm electroporation cuvettes (Gene Pulser; BioRad) using 300 ng/µL of plasmids pVRL2-ahhemB$_{wt}$ and pVRL2-ahhemB$_{P241S}$ as well as 50 µL aliquots of competent *A. baumannii* cells. After pulsing (2.5 kV, 200 Ω, 25 µF), cells were immediately recovered in 1 mL of pre-warmed SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$ und 20 mM glucose) and incubated at 37 °C for 3 h. Transformants were selected on LB agar plates supplemented with Gen (100 mg/L). On the next day, 5 mL LB medium supplemented with Gen (100 mg/L) were inoculated with single colonies and cryostocks were prepared using 600 µL of overnight culture and 400 µL of sterile glycerol.
Overexpression of PbgS(wt) and PbgS(P241S) in A. baumannii ATCC19606

Overnight cultures of A. baumannii (pVRL2-abhemB<sub>wt</sub>), A. baumannii (pVRL2-abhemB<sub>P241S</sub>) and A. baumannii (wt) (as control) in LB medium supplemented with Gen (100 mg/L, only for A. baumannii harbouring pVRL2 plasmids) were diluted 1:100 into LB medium containing Gen (100 mg/L, only for A. baumannii harbouring pVRL2 plasmids) and incubated (37 °C, 200 rpm) until an OD<sub>600</sub> of 0.7 - 0.8 was reached. Protein expression was induced by adding arabinose (20% in sterile LB medium) to a final concentration of 0.5% and bacteria were incubated for 3.5 h (37 °C, 200 rpm). Bacterial suspensions equivalent to 200 mL with an OD<sub>600</sub> of 2.0 were harvested (7,000 × g, 30 min, 4 °C) and washed with PBS. Pellets were resuspended in 20 mL PbgS activity buffer (100 mM Tris/HCl, 1 mM MgCl<sub>2</sub>, pH 8.5 in ddH<sub>2</sub>O), lysed by sonication (6 × 45 s, 80% intensity, 30 s break on ice, Sonopuls HD 2070 ultrasonic rod, Bandelin electronic GmbH) and cell lysate was cleared by centrifugation (20,000 × g, 30 min, 4 °C). The supernatants were transferred into new tubes, kept on ice and were directly used for the PbgS activity assay as well as for full proteome analysis to quantify overexpressed PbgS(wt) and PbgS(P241S).

PbgS activity assay

PbgS activity assay was performed as described by Lentz et al. with slight modifications. The assay was performed in a 96 well-plate (transparent Nunc 96-well flat bottom, Thermo Fisher Scientific). Into each well, 45 µL of cell lysate (prepared as described in “Overexpression of PbgS(wt) and PbgS(P241S) in A. baumannii ATCC19606”) were added and equilibrated at 37 °C for 20 min. To determine K<sub>m</sub> and v<sub>max</sub>, varying concentrations of 5-aminolevulinic acid (5-ALA; 5 µL of 100× stock in ddH<sub>2</sub>O) were added, reaction mixtures were incubated at 37 °C without shaking and stopped after various time points by the addition of 200 µL of modified Ehrlich’s Reagent (1 g p-dimethylaminobenzaldehyde in 42 mL acetic acid, 12 mL perchloric acid and 7.3 mL 12% trichloroacetic acid). After another 10 min incubation, the absorption was measured at 555 nm with an Infinite® M200 Pro microplate reader (Tecan Group Ltd.). Samples without 5-ALA added was used as a negative control and the lysate of A. baumannii (wt) (without plasmid) was used to subtract the basal PbgS activity level. For each substrate concentration, the linear range from the progress curves was used to determine the reaction velocity (slope). Michaelis Menten constants K<sub>m</sub> and v<sub>max</sub> were calculated with GraphPad Prism (version 5.03) using non-linear regression. The assay was performed in three independent experiments with two technical replicates each.

In order to test PbgS inhibition by Xan, 0.5 µL of Xan (100 µM final concentration with 1% DMSO) were added to 44.5 µL cell lysate and pre-incubated for 30 min before the activity assay was started by the addition of 5-ALA (100 µM final concentration). The reaction was incubated at 37 °C.
without shaking for 30 min. The reaction was stopped and the absorption was measured as described above. The assay was performed in two independent experiments with two technical replicates each.

**Full proteome analysis**

*A. baumannii* ATCC19606 (wt), *A. baumannii* (pVRL2-abhemB<sub>wt</sub> and *A. baumannii* (pVRL2-abhemB<sub>P241S</sub>)

A volume of 500 µL of the cell lysates prepared in “Overexpression of PbgS(wt) and PbgS(P241S) in *A. baumannii* ATCC19606”, corresponding to protein amounts of 500 µg, 500 µg and 250 µg of *A. baumannii* (pVRL2-abhemB<sub>wt</sub>), *A. baumannii* (pVRL2-abhemB<sub>P241S</sub>) and *A. baumannii* (wt), respectively, was directly used to precipitate proteins with ice-cold acetone (4 volumes) at -20 °C overnight. Afterwards, the experimental procedure was analogous to “preparative ABPP in *A. baumannii* and *E. coli* with XP”, but without affinity enrichment on avidin beads, using 10 µL (0.5 µg/µL; sequencing grade, modified, *Promega*) trypsin for digestion. The experiment was performed in n = 2 independent experiments.

**Xan** treatment of *A. baumannii* ATCC19606 (wt)

*A. baumannii* ATCC19606 overnight cultures were diluted 1:100 in 20 mL LB medium containing Xan (125 nM, 1% DMSO final concentration) or DMSO (1%). Bacteria were incubated (37 °C, 200 rpm) until an OD<sub>600</sub> of 2.0, harvested (6,000 xg, 4 °C, 15 min) and pellets were washed with PBS. Cell pellets were resuspended in 0.4% (w/v) SDS in PBS (1 mL) and lysed (3 × 6,500 rpm, 30 s, with 30 s cooling breaks, liquid nitrogen cooling; Precellys Ceramic Kit CK01L, 2.0 mL tubes; Precellys 24 Homogenizer, *Bertin Technologies*). The supernatants were clarified by centrifugation (20,000 xg, 30 min, 4 °C) and cell lysates corresponding to protein amount of 250 µg were precipitated with ice-cold acetone (4 volumes) at -20 °C overnight, centrifuged (16,900 xg, 4 °C, 15 min) and the pellets were washed twice with ice-cold methanol (1 mL). Protein pellets were air dried and dissolved in 200 µL denaturation buffer (7 M urea, 2 M thiourea in 0.1 M pH 7.5 Tris/HCl-buffer). Upon reduction with TCEP (5 mM) at 37 °C for 1 h, proteins were alkylated using IAA (10 mM) at 25 °C for 30 min and samples were quenched with DTT (10 mM) at RT for 30 min. Enzymatic digestion using 1 µL Lys-C (0.5 ng/µL, MS-grade, *Wako*) was first carried out at RT for 2 h, upon which samples were diluted with 600 µL TEAB buffer (50 mM in ddH<sub>2</sub>O) and digested with 10 µL trypsin (0.5 µg/µL; sequencing grade, modified, *Promega*) at 37 °C overnight under continuous shaking (450 rpm). On the next day, the experimental procedure was analogous to “preparative ABPP in *A. baumannii* and *E. coli* with XP” as described above. The experiment was performed in n = 4 independent experiments.
**Xan** resistant *A. baumannii* ATCC19606 mutants

For overnight cultures of three **Xan** resistant *A. baumannii* mutants (A-3, B-3, C-3), 5 mL of LB medium supplemented with 500 nM **Xan** were inoculated. Additionally, 5 mL overnight culture of *A. baumannii* ATCC19606 (wt) was prepared in LB medium. On the next day, overnight cultures were diluted 1:100 in 20 mL LB medium and bacteria were incubated (37 °C, 200 rpm) until an OD_{600} of 2.0 was reached. Bacteria were harvested (6,000 xg, 4 °C, 15 min) and cell pellets were washed with PBS. Afterwards, the experimental procedure was analogous to **“Xan treatment of A. baumannii ATCC19606 (wt)”**. The experiment was performed in n = 4 independent experiments.

**LC-MS/MS analysis**

Before MS measurements, the lyophilized peptides were resolved in 1% FA to get a final concentration of 1-5 µg/µL and samples were filtered as described in section “Gel-free Activity-based protein profiling (ABPP) and Affinity-based protein profiling (A/BPP)”.

Samples were analyzed with an UltiMate 3000 nano HPLC system *(Dionex)* using an Acclaim C18 PepMap100 (75 µm ID × 2 cm) trap column and an Aurora Series Emitter Column with Gen2 nanoZero fitting (75 µm ID × 25 cm, 1.6 µm FSC C18) separation column (both heated to 40 °C) coupled to an Orbitrap Fusion *(Thermo Fisher)* in EASY-spray setting. Samples were loaded on the trap column and washed with 0.1% TFA, then transferred to the analytical column (buffer A: H_{2}O with 0.1% FA, buffer B: ACN with 0.1% FA, flow 400 nL/min, gradient 5 to 22% buffer B in 115 min, then to 32% buffer B in 10 min, then to 90% buffer B in 10 min and hold 90% buffer B for 10 min, then to 5% buffer B in 0.1 min and hold 5% buffer B for 9.9 min). Orbitrap Fusion was operated in a TOP10 data dependent mode. Full scan acquisition was performed in the orbitrap at a resolution of 120,000 and an AGC target of 2e^{5} (maximum injection time of 50 ms) in a scan range of 300–1,500 m/z. Monoisotopic precursor selection as well as dynamic exclusion (exclusion duration: 60 s) was enabled. Precursors with charge states of >1 and intensities greater than 5e^{3} were selected for fragmentation. Isolation was performed in the quadrupole using a window of 1.6 m/z. Precursors were collected to an AGC target of 1e^{4} (maximum injection time of 35 ms) and acquisition was performed at a scan range of 120–2,000 m/z. Fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 30%) and detected in the ion trap operating in rapid mode.
MS data analysis

Raw files were analyzed using MaxQuant software (version 1.6.2.10) with the Andromeda search engine. The settings were applied as described in section “Gel-free Activity-based protein profiling (ABPP) and Affinity-based protein profiling (AfBPP)”. Searches were performed against the UniProt database for A. baumannii ATCC19606 (taxid: 575584, 29.06.2020). For comparison of LFQ intensities of overexpressed PbgS(wt) and PbgS(P241S) the FASTA file was modified to contain a generic PbgS P241W, in order to exclude mutation-site containing peptides from quantification. Statistical analysis of the data was performed using Perseus (version 1.6.13.0.). Putative contaminants, reverse peptides and peptides only identified by site were deleted. LFQ intensities were log2-transformed and data was filtered for two valid values in at least one group (for full proteome analysis of A. baumannii (pVRL2-abhemBwt), A. baumannii (pVRL2-abhemBP241S) and A. baumannii (wt)), for three valid values in each group (for full proteome analysis of Xan treatment of A. baumannii ATCC19606 (wt)) and for three valid values in at least one group (for full proteome analysis of Xan resistant A. baumannii ATCC19606 mutants) and a missing value imputation was performed over the total matrix. For statistical evaluation, \(-\log_{10}(P\text{ values})\) were obtained by a two-sided two sample Student’s \(t\)-test.

UV-Vis spectroscopy

The assay was performed in a transparent 96-well plate (transparent Nunc 96-well flat bottom, Thermo Fisher Scientific) and the final volume was 200 µL. Hemin (20 µM final concentration) was incubated with various concentrations of Xan (2% DMSO final concentration), XP (2% DMSO final concentration), resveratrol (Res) or DMSO (2%) in buffer h (200 mM HEPES, pH 7.0) for 1 h at RT in the dark without shaking. Afterwards, UV-Vis spectrum was recorded with an Infinite® M200 Pro microplate reader (Tecan Group Ltd.). The respective compound, Xan, XP or Res, in buffer h served as blank control. The assay was performed in three independent experiments with two technical replicates each.

Experiments to study the interaction between XanDME and hemin as well as Xan and PPIX need to be performed in DMSO / 200 mM HEPES (pH 7.0) (1:1, v/v) due to the poor solubility of XanDME and PPIX followed by incubation at 70°C for 30 min.

Hemin decomposition

The glutathione (GSH)-mediated destruction assay of hemin was performed as previously described by Wright \textit{et al.} with slight modifications. The assay was performed in a 96-well plate.
(transparent Nunc 96-well flat bottom, *Thermo Fisher Scientific*) with a final volume of 200 µL per well. Hemin (20 µM final concentration) was pre-incubated with various concentrations of Xan (1% DMSO final concentration) in buffer h (200 mM HEPES, pH 7.0) for 15 min at 37 °C without shaking. DMSO (1%) was included as positive control for hemin decomposition and a sample without GSH addition was used as a negative control. The assay was started by the addition of GSH to a final concentration of 2 mM and the absorption of hemin at the Soret band (400 nm) was measured with an Infinite® M200 Pro microplate reader (*Tecan Group Ltd.*). To determine hemin decomposition, the decrease in absorption over a period of 7.5 min was calculated and normalized to DMSO-treated samples. The assay was performed in three independent experiment with three technical replicates each.

The corresponding experiment using the XanDME was performed in DMSO / 200 mM HEPES (pH 7.0) (1:1, v/v) due to the poor solubility of the compound. Incubation was performed at 70°C for 25 min. All other steps were performed analogously to the experiment with Xan.

**Measuring regulatory heme (RH)**

Experiments to measure regulatory heme (RH) was performed as described by Atamna *et al.* with slight modifications.  

**Preparing stock solution of apoHRP**

Briefly, 8 mg holoHRP were dissolved in cold ddH₂O (2 mL) and heme was extracted by the addition of 40 mL acid acetone (1 mL concentrated HCl (36%) in 40 mL acetone). The pellet was then collected by centrifugation (2,000 × g, 2 min), and was dissolved in PBS (2 mL). The cycle of extraction was repeated twice and the concentration of apoHRP was determined as described in section “Overexpression and purification of catalases-peroxidases (*AbCAT*, *EcCAT*)” using a molar extinction coefficient at 280 nm of 20,000 M⁻¹cm⁻¹. A stock solution of apoHRP (100 µM) was prepared in PBS and stored in aliquots at -20 °C. To exclude residual HRP activity, the peroxidase TMB substrate solution (*Thermo Fisher Scientific*) was used as described in the following section.

**In vitro apoHRP reconstitutions activity assay**

The final assay volume of the reconstitution reactions was 100 µL and PBS was used as buffer. Hemin (5 nM final concentration) was pre-incubated with various concentrations of Xan (1% DMSO final concentration) for 5 – 10 min at RT without shaking in the dark. DMSO (1%) was included as a positive control for reconstitution of the active holoHRP. Afterwards, apoHRP was added to a final concentration of 5 µM and incubated for 10 min at 4 °C without shaking. ApoHRP without hemin added was used as a blank control. To measure the resulting holoHRP activity,
10 µL aliquots were transferred from the reconstitution reaction to a transparent 96-well plate (transparent Nunc 96-well flat bottom, *Thermo Fisher Scientific*), 100 µL TMB substrate solution (*Thermo Fisher Scientific*) were added to the aliquots and absorbance was measured at 650 nm with an Infinite® M200 Pro microplate reader (*Tecan Group Ltd.*). The assay was performed in four independent experiments with two technical replicates each.

To exclude an inhibition of the active holoHRP by Xan, apohRP (5 nM final concentration) was pre-incubated with hemin (5 nM final concentration) for 10 min at 4 °C in the dark. Afterwards, Xan was added in various concentrations (1% DMSO final concentration) and reconstitution reactions were incubated for 5 – 10 min at RT in the dark. DMSO (1%) was included as a negative control for inhibition. ApoHRP without hemin added was used as a blank control. The resulting holoHRP activity was measured as described above. The assay was performed in three independent experiments with two technical replicates each.

**In vivo apohRP reconstitution activity assay**

An overnight culture of *A. baumannii* ATCC19606 was diluted 1:100 into LB medium and incubated (37 °C, 200 rpm) until an OD₆₀₀ of 1.0 was reached. The bacterial suspension was diluted to an OD₆₀₀ of 0.4 and 5 mL aliquots were prepared containing various concentrations of Xan (1% DMSO final concentration) or DMSO (1%). After incubation for 30 min (37 °C, 200 rpm), bacteria were harvested (7,000 ×g, 15 min, 4 °C), washed with PBS and were resuspended in 500 µL PBS. Cells were lysed by sonication (3 × 25 s, 75% intensity; Sonopuls HD 2070 ultrasonic rod, *Bandelin electronic GmbH*) with cooling breaks on ice. Lysate was cleared by centrifugation (20,000 ×g, 30 min, 4 °C), protein concentration was measured using the Pierce BCA Protein assay kit (*Thermo Fisher Scientific, Pierce Biotechnology*) and sample concentration was adjusted to 0.15 µg/µL. This lysate was directly used to measure regulatory heme by the reconstitution assay. The final volume of this assay was 100 µL and PBS was used as buffer. Cell lysate (2.5 µg protein) was pre-incubated with apohRP (10 µM final concentration) for 10 min at 4 °C. Lysate without apohRP added was used as a blank control. The resulting holoHRP activity was measured as described above. The assay was performed in three independent experiments with four technical replicates each.

**Porphyrin quantification**

The porphyrin extraction was performed as previously described by Mancini *et al.* with slight modifications.²⁵ An overnight culture of *A. baumannii* ATCC19606 was diluted 1:100 into LB medium and incubated (37 °C, 200 rpm) until an OD₆₀₀ of 1.0 was reached. The bacterial suspension was diluted to an OD₆₀₀ of 0.4 and 20 mL aliquots were prepared containing 1 µM Xan
(1% DMSO final concentration) or DMSO (1%). After incubation for 1 h (37 °C, 200 rpm), bacterial suspensions equivalent to 20 mL with an OD_{600} of 0.5 were harvested (7,000 ×g, 30 min, 4 °C) and washed twice with PBS. Cell pellets were resuspended in 1 mL EtOAc / acetic acid (3:1, v/v) and lysed by sonication (3 × 25 s, 75% intensity; Sonopuls HD 2070 ultrasonic rod, Bandelin electronic GmbH) with cooling breaks on ice. Cell debris was removed by centrifugation (18,000 ×g, 20 min, 4 °C) and supernatant was transferred to a new Eppendorf tube. The organic phase was washed twice with H₂O (1 mL), transferred to a new Eppendorf tube and 3 M HCl (100 µL) was added to water-solubilize porphyrins. In order to quantify porphyrins, 100 µL of the aqueous phase were transferred into a black flat bottom 96-well plate (Greiner) and the fluorescence spectrum (λ_{ex.} = 406 nm, λ_{em.} = 550 – 750 nm) was recorded with an Infinite® M200 Pro microplate reader (Tecan Group Ltd.). The experiment was performed in six independent experiments.

**Time-kill assays**

To determine the levels of killing by Xan, time-kill experiments were performed as described previously by Le et al.¹⁵

An overnight culture of *A. baumannii* ATCC19606 was diluted 1:100 into LB medium and bacteria were grown to mid-exponential phase (OD_{600} of 0.3 - 0.7; 37 °C and 200 rpm). Subsequently, cells were diluted to 1 × 10⁷ CFU/mL in LB medium and were split into 3 mL aliquots into culture tubes containing Xan (4 µM, 1% DMSO final concentration), DMSO (1%), thiourea (TU) (150 mM) or TU (150 mM) and Xan (4 µM). Cells were incubated (37 °C, 200 rpm) and serial dilutions were plated on LB agar plates at indicated time points for the determination of viable cells (CFU/mL). Experiments were performed in n = 4 (DMSO), n = 3 (Xan) and n = 2 (TU as well as TU and Xan (4 µM)) independent biological experiments and CFU enumeration was conducted in four replicates each.

**Checkerboard assay**

Overnight cultures of *A. baumannii* ATCC19606 and *A. baumannii* AB5075 were diluted 1:10,000 into LB medium and were directly used for testing. Various dilutions of Xan and Gen were prepared and 1 µL of the respective 100× stock was pipetted in a 96 well-plate (transparent Nunc 96-well flat bottom, Thermo Fisher Scientific). Two- to four-fold the MIC of each antibiotic was used as the highest concentration tested. The resulting checkerboard contains each combination of the two test molecules. Each well was inoculated with 99 µL of the diluted bacterial suspension, and the plates were incubated for 24 h (37 °C, 200 rpm). The lowest concentration in the dilution series at which no growth of bacteria could be observed by eye was defined as the MIC. In order to quantify
the interactions between the antibiotics, the following equation was used to determine the fractional inhibitory concentration (FIC) index:

$$\Sigma\text{FIC} = \text{FIC(Xan)} + \text{FIC(Gen)},$$

where FIC(Xan) is the MIC of Xan in the combination/MIC of Xan alone, and FIC(Gen) is the MIC of Gen in the combination/MIC of Gen alone. The combination is considered synergistic when the $$\Sigma\text{FIC}$$ is $$\leq 0.5$$, indifferent when the $$\Sigma\text{FIC}$$ is $$> 0.5 \sim 4$$, and antagonistic when the $$\Sigma\text{FIC} > 4$$. FICs were determined in three ($A.\ baumannii$ ATCC19606) and two ($A.\ baumannii$ AB5075) independent experiments.

**MTT assay**

HeLa cells were grown in DMEM medium ($Sigma\ Aldrich$) with 10% FBS ($Sigma\ Aldrich$) and 2 mM glutamine ($Sigma\ Aldrich$) in 5% CO$_2$ at 37 °C.

For splitting or passaging, the medium was removed, and cells were washed with 10 mL of PBS. Afterwards, the cells were incubated with 1 mL of Accutase® for 10 min at 37 °C until full detachment. Then, 10 mL of the respective medium were added, mixed thoroughly and 1 mL of this cell-solution was transferred into a new flask. The cell-solution was diluted with medium to a volume of 10 mL.

To evaluate the cytotoxicity, HeLa cells were seeded in 96-well plates at a concentration of 4,000 cells per cavity. After 24 hours the growth medium was removed by suction and 100 µL medium (without FBS) with either DMSO (1%) or Xan (10 µM, 1% DMSO final concentration) was added. After 24 h exposure at 37 °C and 5% CO$_2$ 20 µL Thiazolyl blue tetrazolium bromide (MTT, 5 mg/mL in PBS, $Sigma\ Aldrich$) were added to the cells and incubated for 4 h to allow the MTT to be metabolized. The medium was removed by suction and the resulting formazan dissolved in 200 µL DMSO. The optical density was measured with an Infinite® M200 Pro microplate reader ($Tecan\ Group\ Ltd.$) at 570 nm and background was subtracted at 630 nm. Absorption values were normalized to DMSO control. Each data set represents six replicates obtained from three independent experiments.
Chemical synthesis

Compound synthesis materials

Reagents and solvents were purchased from commercial suppliers (*Sigma Aldrich* Co. LLC, *Thermo Fisher Scientific* Inc., *Merck* KGaA, *TCI Europe* GmbH and *Alfa Aesar* GmbH) and used as received. Reaction grade or anhydrous solvents (max. 0.01% water content, stored over molecular sieve under an argon atmosphere) were used for all reactions. TLC was performed on precoated silica gel plates (60 F-254, 0.25 mm, *Merck* KGaA) with detection by UV (λ = 254 and/or 366 nm) and/or by coloration using potassium permanganate (KMnO₄; 3.0 g KMnO₄, 20 g K₂CO₃ and 5 mL 5% NaOH in 300 mL ddH₂O) stain and subsequent heat treatment. Flash chromatography was performed on silica gel 60 (0.035 – 0.070 mm, mesh 60 Å, *Merck* KGaA). The solvent compositions reported for all chromatographic separations are on a volume/volume (v/v) basis. Common solvents for chromatography [n-hexane (Hex), ethyl acetate (EtOAc), dichloromethane (DCM) and methanol (MeOH)] were distilled prior to use.

Compound characterization methods

¹H- and proton-decoupled ¹³C-NMR spectra were recorded at the indicated temperature either on a Bruker AVHD-500 or on a Bruker AV-II-500 equipped with cryo probe head. Chemical shifts are reported in delta (δ) units in parts per million (ppm) relative to residual solvent signals [deuterated acetone (acetone-d₆) δ H = 2.05 ppm and δ C = 29.84, 206.26 ppm]. The following abbreviations were used for the assignment of signals: s – singlet, d – doublet, t – triplet, m – multiplet. Coupling constants J are given in Hertz [Hz], HR-MS spectra were recorded in the ESI mode on a LTQ-FT Ultra (FT-ICR-MS, *Thermo Fisher Scientific*) coupled with an UltiMate 3000 HPLC system (*Thermo Fisher Scientific*).
Synthesis of 4,4’-((1Z,3Z)-2,3-diisocyanobuta-1,3-diene-1,4-diyl)diphenol (**Xan**)

**Xan** was prepared in 10 steps from commercially available 4-hydroxybenzaldehyde by following the approach published by Tatsuta *et al.*\textsuperscript{27,28} The NMR and HRMS data of **Xan** match with the reported ones.

![Chemical structure of Xan](image)

Compound 10 (20.0 mg, 38.7 µmol, 1.0 eq.) was dissolved in dry THF (1 mL) and the reaction mixture was cooled to 0 °C. Then, tetra-**n**-butylammonium fluoride (TBAF) (1 M in THF) (77.4 µL, 77.4 µmol, 2.0 eq.) was slowly added dropwise. The reaction solution was stirred for 10 min at 0 °C and the solution was directly purified by column chromatography (DCM:MeOH = 20:1 + 1% acetic acid) to give **Xan** (8.80 mg, 30.5 µmol, 79%) as a yellow powder.

**TLC** (Hex:EtOAc = 2:1): *R*\textsubscript{f} = 0.27 [UV, KMnO\textsubscript{4}].

**\textsuperscript{1}H-NMR** (500 MHz, Acetone-d\textsubscript{6}): δ [ppm] = 9.17 (s, 2H), 7.83 (d, *J* = 8.7 Hz, 4H), 7.08 (s, 2H), 7.00 (d, *J* = 8.7 Hz, 4H).

**\textsuperscript{13}C-NMR** (126 MHz, Acetone-d\textsubscript{6}): δ [ppm] = 175.0, 160.5, 132.9, 128.4, 124.9, 116.9, 116.6.

**ESI-HRMS** (*m/z*): [M–H\textsuperscript{+}] calcd. for C\textsubscript{18}H\textsubscript{11}N\textsubscript{2}O\textsubscript{2}, 287.0826; found, 287.0826.
Synthesis of 4,4’-((1Z,3Z)-2,3-diisocyanobuta-1,3-diene-1,4-diyl)bis(methoxybenzene) (XanDME)

**Xan** (30.0 mg, 104 µmol, 1.0 eq) was dissolved in dry acetone (7 mL), the suspension was cooled to 0 ºC and K₂CO₃ (30.2 mg, 219 µmol, 2.1 eq.) as well as methyl iodide (13.6 µL, 31.0 mg, 219 µmol, 2.1 eq.) were added. The reaction mixture was stirred at 50 ºC and the product formation was controlled via ESI-MS. After 2 h the reaction solution was concentrated under reduced pressure and the residue was purified by column chromatography (Hex:EtOAc = 10:1) to obtain **XanDME** (7.90 mg, 25.0 µmol, 24%) as green crystals.

**TLC** (Hex:EtOAc = 5:1): *Rf* = 0.46 [UV, KMnO₄].

**¹H-NMR** (500 MHz, Acetone-d₆): δ [ppm] = 7.90 (d, *J* = 8.8 Hz, 4H), 7.14 (s, 2H), 7.10 (d, *J* = 8.8 Hz, 4H), 3.90 (s, 6H).

**¹³C-NMR** (126 MHz, Acetone-d₆): δ [ppm] = 175.1, 162.3, 132.6, 128.4, 125.7, 117.0, 115.3, 55.9.

**ESI-HRMS** (*m/z*): [M+H⁺] calcd. for C₂₀H₁₇N₂O₂, 317.1285; found, 317.1283.

The NMR and HRMS data of **XanDME** match with published data.²⁸
Synthesis of \(4-((1Z,3Z)-2,3\text{-diisocyano-4-}(\text{4-prop-2-yn-1-}yloxy)phenyl)\text{buta-1,3-dien-1-yl)}\)phenol (XP)

A protocol published by Lee et al. was used with slight modifications.\(^{29}\) \text{Xan} (17.0 mg, 59.0 µmol, 1.0 eq) was dissolved in dry acetone (5 mL), the suspension was cooled to 0 °C and \(K_2\text{CO}_3\) (8.15 mg, 59.0 µmol, 1.0 eq.) was added. Then, propargylic bromide solution (9.2 M in toluene) (6.41 µL, 59.0 µmol, 1.0 eq.) was added, the reaction mixture was stirred at 50 °C and the product formation was controlled via ESI-MS. After 2.5 h the reaction solution was concentrated under reduced pressure and the residue was purified twice by column chromatography (DCM:MeOH = 20:1) to obtain \text{XP} (3.60 mg, 11.0 µmol, 19%) as a light yellow powder. \text{Xan} (13.9 mg, 48.2 µmol, 82%) was re-isolated as a yellow powder.

\text{TLC} (DCM:MeOH = 20:1): \(R_I = 0.18\) [UV, K\text{MnO}_4].

\text{\(^1H-NMR\)} (500 MHz, Acetone-\(d_6\)): \(\delta\) [ppm] = 9.12 (s, 1H), 7.92 (d, \(J = 8.8\) Hz, 2H), 7.84 (d, \(J = 8.8\) Hz, 2H), 7.17 (d, \(J = 8.8\) Hz, 2H), 7.14 (s, 1H), 7.12 (s, 1H), 7.01 (d, \(J = 8.8\) Hz, 2H), 4.91 (d, \(J = 2.4\) Hz, 2H), 3.15 (t, \(J = 2.4\) Hz, 1H).

\text{\(^{13}C-NMR\)} (126 MHz, Acetone-\(d_6\)): \(\delta\) [ppm] = 175.2, 175.1, 160.6, 160.1, 133.0, 132.6, 128.9, 128.0, 126.6, 124.8, 117.7, 116.9, 116.4, 116.3, 79.3, 77.6, 56.5.

\text{ESI-HRMS} (m/z): [M-H\(^+\)] calcd. for \(C_{21}H_{13}N_2O_2\), 325.0983; found, 325.0982.
Synthesis of 4-((1Z,3Z)-4-(4-(2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethoxy)phenyl)-2,3-diisocyanobuta-1,3-dien-1-yl)phenol (XPP)

Compound 2 was synthesized as previously described.²

![Reaction Scheme]

The protocol for XP was used with slight modifications. Xan (35.5 mg, 123 µmol, 1.0 eq) was dissolved in dry acetone (10 mL), the suspension was cooled to 0 °C and K₂CO₃ (17.0 mg, 123 µmol, 1.0 eq.) was added. Then, compound 2 (36.0 mg, 123 µmol, 1.0 eq.) was added, the reaction mixture was stirred at 50 °C and the product formation was controlled via ESI-MS. After 8 h the reaction solution was concentrated under reduced pressure and the residue was purified by column chromatography (Hex:acetone = 6:1) to give XPP (11.2 mg, 27.4 µmol, 22%) as a light yellow powder. Xan (17.3 mg, 60.0 µmol, 49%) was re-isolated.

**TLC** (Hex:acetone = 4:1): Rₜ = 0.24 [UV, KMnO₄].

**¹H-NMR** (500 MHz, Acetone-δ₆): δ [ppm] = 9.11 (s, 1H), 7.90 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H), 7.14 – 7.08 (m, 4H), 7.00 (d, J = 8.8 Hz, 2H), 4.03 (t, J = 6.2 Hz, 2H), 2.4 (t, J = 2.7 Hz, 1H), 2.11 (td, J = 7.5, 2.7 Hz, 2H), 1.97 (t, J = 6.2 Hz, 2H), 1.75 (t, J = 7.5 Hz, 2H).

**¹³C-NMR** (126 MHz, Acetone-δ₆): δ [ppm] = 175.0, 175.0, 161.0, 160.5, 132.9, 132.6, 128.7, 128.0, 126.0, 124.7, 117.3, 116.8, 116.3, 115.8, 83.6, 70.6, 63.7, 33.3, 33.3, 27.5, 13.6.

**ESI-HRMS** (m/z): [M-H⁺] calcd. for C₂₅H₁₉N₄O₂, 407.1513; found, 407.1512.
Xanthocillin (Xan)

$^1$H-spectrum

$^{13}$C-spectrum
$^1$H-spectrum

Xanthocillin Dimethyl ether (XanDME)

$^{13}$C-spectrum

206-2 Acetone

208-8 Acetone
$^1$H-spectrum

Xanthocillin probe (XP)

$^{13}$C-spectrum

298.7 Acetone
Xanthocillin photoprobe (KPP)

$^1$H-spectrum

$^{13}$C-spectrum

206.2 Aromatic

207.0

$^1$H-spectrum

$^{13}$C-spectrum
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