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Improved broad-spectrum antibiotics against Gram-negative pathogens *via* darobactin biosynthetic pathway engineering†

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The development of new antibiotics is imperative to fight increasing mortality rates connected to infections caused by multidrug-resistant (MDR) bacteria. In this context, Gram-negative pathogens listed in the WHO priority list are particularly problematic. Darobactin is a ribosomally produced and post-translationally modified bicyclic heptapeptide antibiotic selectively killing Gram-negative bacteria by targeting the outer membrane protein BamA. The native darobactin A producer *Photorhabdus kharii* HGB1456 shows very limited production under laboratory cultivation conditions. Herein, we present the design and heterologous expression of a synthetically engineered darobactin biosynthetic gene cluster (BGC) in *Escherichia coli* to reach an average darobactin A production titre of 13.4 mg L⁻¹. Rational design of *darA* variants, encoding the darobactin precursor peptide with altered core sequences, resulted in the production of 13 new 'non-natural' darobactin derivatives and 4 previously hypothetical natural darobactins. One of the non-natural compounds, darobactin 9, was more potent than darobactin A, and showed significantly improved activity especially against *Pseudomonas aeruginosa* (0.125 µg mL⁻¹) and *Acinetobacter baumannii* (1–2 µg mL⁻¹). Importantly, it also displayed superior activity against MDR clinical isolates of *E. coli* (1–2 µg mL⁻¹) and *Klebsiella pneumoniae* (1–4 µg mL⁻¹). Independent deletions of genes from the darobactin BGC showed that only *darA* and *darE*, encoding a radical forming S-adenosyl-L-methionine-dependent enzyme, are required for darobactin formation. Co-expression of two additional genes associated with the BGCs in hypothetical producer strains identified a proteolytic detoxification mechanism as a potential self-resistance strategy in native producers. Taken together, we describe a versatile heterologous darobactin platform allowing the production of unprecedented active derivatives in good yields, and we provide first experimental evidence for darobactin biosynthesis processes.

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Introduction

Increasing levels of antimicrobial resistance (AMR) is a threat for health care systems globally and it leads to increasing morbidity and mortality rates. Notably, multidrug-resistant (MDR) Gram-negative pathogens such as *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and also *Enterobacter* species cause difficult-to-treat nosocomial

infections and were consequently ranked with critical priority by the WHO.¹ Some MDR strains developed resistance towards almost all clinically approved antibiotics.² This exemplifies the need for the discovery and clinical development of new antibiotics to fight AMR-related infections in the future. Most of the recently FDA-approved antibiotics derive from well-known antibiotics classes such as the β-lactam/β-lactamase inhibitor combinations ceftolozane/tazobactam, ceftazidime/avibactam,³ meropenem/vaborbactam,⁴ imipenem-cilastatin (dipeptidase inhibitor)/relebactam,⁵ the aminoglycoside plazomicin,⁶ the tetracycline derivative eravacycline,⁷ and the siderophore cephalosporin cefiderocol,⁸ which make rapid resistance development likely because resistance mechanisms against congeners of these antibiotics classes already exist among pathogens.

As a result of this challenge, future antibiotics discovery campaigns should focus on antibiotics with novel scaffolds, molecular targets and modes of action,⁹ such as the cyclic peptidomimetic murepavidin, which targets the outer

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membrane (OM) biogenesis in Gram-negative bacteria by inhibiting the lipopolysaccharide transport protein D (LptD). Murepavidin is currently in preclinical development as precision antibiotic against *P. aeruginosa* to treat patients with cystic fibrosis.¹⁰ Another example is the benzamide compound TXA709, which is active against *Staphylococcus aureus* by affecting septum formation during cell division through the inhibition of FtsZ, a protein without homolog in eukaryotic cells.¹¹ Moreover, the *streptomyces*-derived griselimycins are highly active against *Mycobacterium tuberculosis* by inhibiting the new antimicrobial target DnaN, the DNA polymerase sliding clamp.¹² Other examples are the spiropyrimidintrione zoliflodacin and the linear polyaromatic peptide cystobactamid, which show broad-spectrum antibacterial activity, e.g. against *Neisseria gonorrhoeae* and MDR *Escherichia coli*, respectively, by inhibiting DNA gyrase at distinct binding sites compared to other gyrase-targeting antibiotics like the quinolones.¹³ However, the number of promising antibiotic lead structures with activity against Gram-negative pathogens currently in clinical development is limited. One key reason why it is so difficult to find novel antibiotics active against Gram-negative bacteria is their OM, which constitutes a major additional permeation barrier for most of the candidate compounds under development. Several new antibiotic classes currently under investigation avoid the issue of cell penetration since their molecular target is located in the OM itself. Among these antibiotics classes are the small molecule MRL-494,¹⁴ the ribosomally produced and post-translationally modified peptide (RiPP) darobactin,¹⁵ and a chimeric peptidomimetic combining the pharmacophores of murepavidin and polymyxin B₁.¹⁶

The antibiotic darobactin A was isolated from the bacterial nematode symbiont *Photorhabdus khanii* HGB1456 and it selectively kills Gram-negative bacteria such as *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *A. baumannii*.¹⁵ In addition, it showed no cytotoxic activity against a variety of human cell lines or symbiotic gut bacteria, including Gram-negative members belonging to the genus *Bacteroides*. Darobactin targets the β -barrel assembly machinery A protein (BamA)¹⁷ located in the OM. It was recently shown that darobactin A binds to the open form BamA lateral gate with high affinity by mimicking a β -strand in its BamA-bound binding pose.¹⁸ Consequently, the β -signal binding site of BamA is blocked, cognate substrate binding is inhibited and nascent OM proteins are not inserted into the OM. Darobactin A is a bicyclic heptapeptide and part of the RiPP secondary metabolite class characterized by the precursor peptide amino acid sequence W₁N₂W₃S₄K₅S₆F₇ (Fig. 1).¹⁵

The darobactin biosynthetic gene cluster (BGC) in *P. khanii* consists of six open reading frames (ORFs), *darABCDE* and a homolog of the global translation inhibitor *relE* from *E. coli*.^{15,19} The *darA* ORF encodes the propeptide sequence and *darBCD* encode a tripartite ABC-type trans-envelope exporter system, consisting of an ABC transporter permease (DarB), a membrane fusion protein (DarC) and an ATP binding domain (DarD). The *darE* gene encodes a radical *S*-adenosyl methionine (SAM) enzyme, which was proposed to catalyse two consecutive aryl-alkyl couplings between the aromatic parts of the

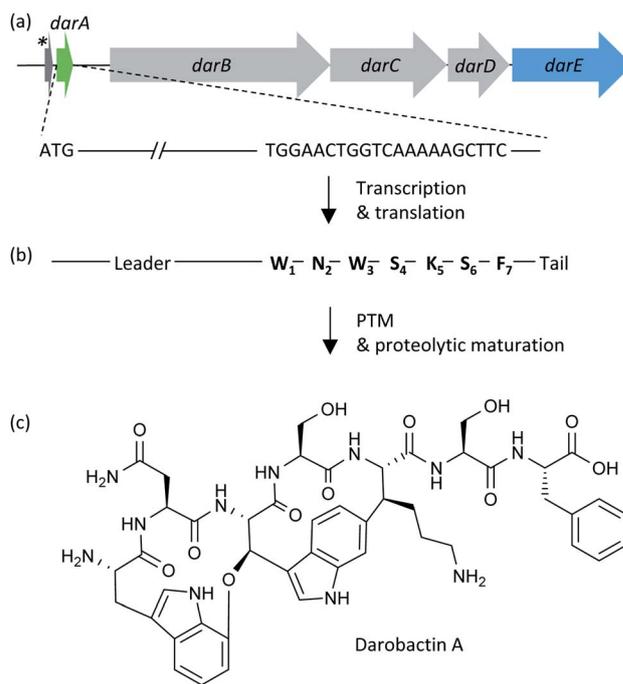


Fig. 1 (a) The biosynthetic gene cluster (BGC) from *Photorhabdus khanii* HGB1456 consisting of a homolog of the global translation inhibitor gene *relE* from *E. coli*¹⁹ (dark grey arrow labelled with asterisk), the darobactin propeptide encoding gene *darA* (green arrow), the three ABC-type trans-envelope exporter encoding genes *darBCD* (light grey arrows) and the radical SAM enzyme encoding gene *darE* (blue arrow). (b) Transcription and translation of *darA* leads to the synthesis of the darobactin propeptide, which consists of a leader, core (W₁N₂W₃S₄K₅S₆F₇) and tail sequence. Two cyclization post-translational modification (PTM) reactions and proteolytic maturation result in formation of darobactin. The proteolytic maturation was recently assumed to be performed by a peptidase from *E. coli*, which is not encoded by a gene from the darobactin BGC, or by a self-cleavage mechanism catalysed by the propeptide.²⁰ (c) Structural formula of the mature darobactin A.

tryptophan side chain leading to two ring formations. The first ring is formed by connection of the indole group of W₁ with C_β of W₃ via an aryl-alkylether bond and the second ring is formed by a carbon-carbon aryl-alkyl bond between the indole group of W₃ and C_β of K₅.¹⁵ The cyclisations were shown to give darobactin its β -strand mimic structure and are vital for the bioactivity, as the linear W₁N₂W₃S₄K₅S₆F₇ peptide did not bind BamA.^{15,18} A search in the National Centre for Biotechnology Information (NCBI) bacterial genome database using the darobactin propeptide sequence as query led to the identification of additional darobactin BGCs in *Photorhabdus*, *Yersinia*, *Vibrio*, and *Pseudoalteromonas* species.¹⁵ Two additional ORFs, *darF* and *darG*, encoding proteins with unknown functions, are located in the BGC from *Pseudoalteromonas luteoviolacea*.¹⁵ Moreover, Imai and co-workers proposed structures of the hypothetical darobactin derivatives B–E based on the darobactin A structure and the propeptide core sequences encoded in the respective *darA* homologs of those BGCs.¹⁵ Due to the low production titre ($\leq 3 \text{ mg L}^{-1}$) of darobactin A in its native producer strain, *darABCDE* were transferred into *E. coli*



E. coli.¹⁹ We placed the isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible *T7lac* promoter system²¹ (including the *lacI* repressor gene and *lacO* operator) upstream of *darA* intending to allow time-controlled gene expression and thus the production of darobactin. The constitutive *nptII* promoter²² was placed upstream of *darBCDE*, since we assumed that constitutive gene expression of the *darBCD*-encoded ABC-type trans-envelope exporter system increases the capability of our *E. coli* production strain to export darobactin prior to induction of the *darA* gene expression with IPTG and therefore can improve its self-resistance to darobactin. We inserted the *tD1* terminator sequence upstream of the *nptII* promoter to prevent *T7lac* promoter-dependent gene expression affecting the transcription of the *darBCDE* operon. Furthermore, we aimed to exclude that a potential native promoter located in the 396 bp long intergenic region between *darA* and *darBCDE* influences the gene expression of *darBCDE*. In addition to the natively present unique restriction endonuclease recognition sites (R-sites), we inserted several more R-sites at strategic locations allowing to remove the *relE*-like gene, exchange promoters or *darA* and to facilitate efficient sub cloning of the modified darobactin BGC into different vector systems (see Fig. 2 and ESI Table 2†).

The modified darobactin BGC DNA sequence was chemically synthesized in one part (8.5 kb), delivered in a pUC57 high-copy plasmid and cloned *via* restriction hydrolysis/DNA ligation into pNOSO (described in ESI†), which features a low-copy p15A origin of replication to allow for genetic stability of the resulting recombinant strain. The expression constructs were transformed into *E. coli* BL21 (DE3) and the heterologous production of darobactin A was initially confirmed by uHPLC-HRMS and MS² fragmentation after a small-scale batch cultivation (see

ESI†). After initial evaluation of different *E. coli* expression strains, cultivation media and expression constructs, the highest relative production based on the mass peak intensity was achieved in *E. coli* BL21 (DE3) pNOSO-relEdarABCDE using FM medium for cultivation (see ESI† and Fig. 3).

Furthermore, we observed peptide impurities in the Base Peak Chromatograms (BPCs) upon expression of high-copy number plasmids (see ESI, Fig. 2†), which would hamper darobactin purification. Thus, we favoured the pNOSO-based expression construct. Since removal of the *relE* homologue from the plasmid encoded BGC did not affect the darobactin production, we used pNOSO-darABCDE, lacking the *relE*-like gene, as basis for all following experiments.

Notably, darobactin A production levels were consistently higher if the *lac* promoter-regulated gene expression of the *T7* RNA polymerase, which is encoded in the genome of BL21 (DE3) and related *E. coli* strains, and the *T7lac* promoter on the plasmid remained non-induced as compared to induction with 0.1 mM IPTG. We assume that leaky *darA* gene expression by the *lac*-repressed *T7* promoter leads to a favourable amount of propeptide in the cell for optimal darobactin A production. This leaky expression might be caused by traces of lactose in the complex cultivation medium leading to slight induction of the *lac*-repressed genes. Higher *darA* gene expression levels caused by induction of the *lac*-regulated *T7* RNA polymerase expression and induction of the *T7lac* promoter with IPTG might result in metabolic overburdening of the *E. coli* cell. This result is not entirely consistent with observations made in parallel in the work by Wuisan *et al.*, in which an increasing *darA* to *darE* transcript ratio led to higher darobactin A production.²⁰ We assume that a certain *darA* : *darE* transcript ratio, which has to be determined in future experiments, is required for optimum darobactin A production. To investigate whether slight induction or repression of the *T7lac* promoter results in higher relative darobactin production, we supplemented the production broth with 10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM and 0.1 nM concentrations of IPTG or 0.5%, 1.5% and 5% of glucose. However, no production improvement with respect to non-induced *T7* polymerase and *darA* expression was achieved under any of the tested conditions (see ESI, Table 7†).

Subsequently, we performed a large-scale batch cultivation with *E. coli* BL21 (DE3) pNOSO-darABCDE omitting any IPTG induction to isolate darobactin A and confirm the structure and minimum inhibitory concentrations (MICs) published by Imai *et al.* for internal comparison with novel darobactins (see below).

Purification of darobactin A from the culture supernatant was done by adsorption of darobactin on XAD-16N resin followed by elution with 80% MeOH in H₂O and several steps of preparative and semi-preparative HPLC (see ESI†). The purified compound was used to calculate a linear regression based on the MS peak surface areas of different stock concentrations (see ESI†). On this basis, an absolute quantification of the darobactin A concentration in the fermentation broth of *E. coli* BL21 (DE3) pNOSO-darABCDE was performed, providing an average production rate of 13.4 (\pm 2.0) mg L⁻¹.

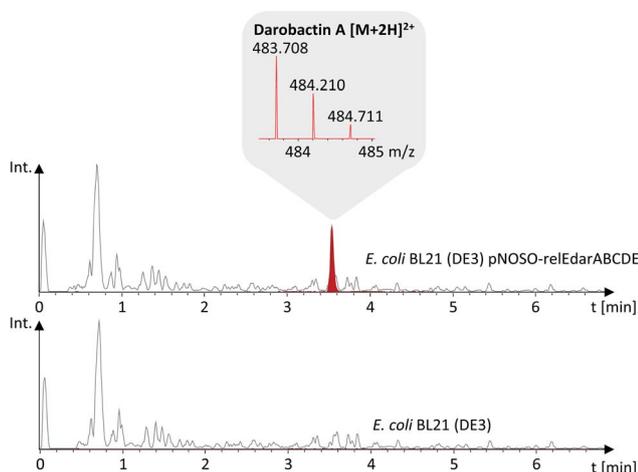


Fig. 3 Darobactin A production was confirmed by uHPLC-HRMS analysis of the *E. coli* pNOSO-relEdarABCDE fermentation broth supernatant. Darobactin A is visualized as extracted ion chromatogram (EIC) at 483.71 Da (red trace) represents the $[M + 2H]^{2+}$ MS peak, including the corresponding isotope pattern, which is shown in the grey field. The grey trace represents the base peak chromatogram (BPC) of the culture supernatant of *E. coli* BL21 (DE3) with and without the darobactin expression vector.



Production of natural and non-natural darobactin derivatives

Darobactin is a promising candidate for biosynthetic structure engineering attempts due to its excellent antibacterial activity profile, the hypothesised natural variability in its amino acid composition,¹⁵ and its presumed insensitivity towards amino acid mutations in its binding site.¹⁸ Thus, we investigated the plasticity of our heterologous production system aiming at the production of novel darobactin derivatives with improved antibacterial properties. To this end, we designed four DNA fragments *in silico* including the entire *darA* gene with an altered core sequence encoding the propeptides of the hypothetical natural darobactins B–E (Table 1 and Fig. 4).

The existence of these darobactin congeners was previously assumed based on the *in silico* analysis of darobactin propeptide sequences in the respective BGCs but has not yet been confirmed.¹⁵

After chemical synthesis of the DNA fragments containing the chimeric *darA* genes (named *darA*-B to *darA*-E), the native *darA* sequence was replaced with *darA*-B to -E, independently, in pNOSO-darABCDE using restriction hydrolysis/DNA ligation cloning to generate pNOSO-darABCDE-B to -E. Heterologous expression of the respective constructs in *E. coli* BL21 (DE3) resulted in the production of the previously *in silico* predicted native darobactins B–E, as confirmed by uHPLC-HRMS and MS² fragmentation analysis (see Table 1, ESI Fig. 4–7 and 23–26†).

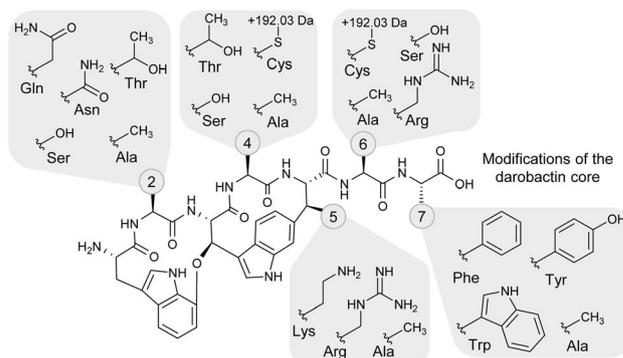


Fig. 4 Structural variation among the heterologously produced darobactin derivatives. The side chain moieties of the different amino acids in positions 2, 4–6 and 7 (see numbered circles) that were observed in the heterologously produced darobactin derivatives (see also Table 1) are shown in the grey-shaded areas. Cysteine side chains in positions 4 and 6 led to formation of an adduct with a mass shift of +191.026 Da and a potential adduct sum formula of C₆H₉NO₄S based on ChemCalc prediction (discussed in more detail in the text). Amino acid side chain moieties of derivatives, which were not observed upon expression of the respective pNOSO construct with altered *darA* sequence in *E. coli* BL21 (DE3), are not shown (darobactins 1, 5 and 7).

Based on the amino acid tolerability found at positions 2 and 4–6 in the natural darobactins A–E, we designed and cloned three new expression constructs (pNOSO-darABCDE-1, -2, and

Table 1 Production of darobactin derivatives after heterologous expression of the modified darobactin BGC with altered core peptide encoding *darA* sequences in *E. coli* BL21 (DE3). Changes in the *darA* core sequence and core peptide sequence with respect to the native (darobactin A) sequence are shown in bold. The calculated (calc.) and observed (obs.) exact mass ($[M + 2H]^{2+}$) for each derivative is given. For darobactin-related masses, which were different from the calculated masses and identified after MS² spectral networking analysis, the corresponding mass shifts are shown in italic. In case of darobactin 18, additionally the terminal *darA* core DNA sequence codon, which was responsible for the unplanned L-glutamine incorporation (see text), is highlighted in italic. UHPLC-HRMS chromatograms and MS² fragmentation data for all produced darobactin derivatives are shown in ESI Fig. 3–21 and 22–40, respectively

Darobactin	<i>darA</i> core DNA sequence	Core peptide	Calc. mass $[M + 2H]^{2+}$	Obs. mass $[M + 2H]^{2+}$	Mass deviation $[M + 2H]^{2+}$ (ppm)	Mass shift (Da)
A	TGG AAC TGG TCA AAA AGC TTC	W N W S K S F	483.7089	483.7095	1.24	
B	TGG AAC TGG ACC AAA CGA TTC	W N W T K R F	525.2512	525.2511	0.19	
C	TGG TCA TGG TCA AGA AGC TTC	W S W S R S F	484.2065	484.2066	0.21	
D	TGG AAC TGG TCA AGA AGC TTC	W N W S R S F	497.7119	497.7116	0.60	
E	TGG TCA TGG TCA AAA AGC TTC	W S W S K S F	470.2034	470.2037	0.64	
1	TGG TCA TGG ACC AAA CGA TTC	W S W T K R F	511.7458	Not observed		
2	TGG TCA TGG ACC AGA AGC TTC	W S W T R S F	491.2143	491.2146	0.61	
4	TGG AAC TGG ACC AAA AGC TTC	W N W T K S F	490.7167	490.7171	0.82	
5	TGG TGG TGG TCA AAA AGC TTC	W C W S K S F	478.1920	Not observed		
6	TGG AAC TGG TGC AAA AGC TTC	W N W C K S F	491.6974	587.2104		+191.026
7	TGG TGC TGG TGC AAA AGC TTC	W C W C K S F	486.1806	Not observed		
8	TGG AAC TGG TCA AAA TGC TTC	W N W S K C F	491.6974	587.2100		+191.0252
9	TGG AAC TGG TCA AAA AGC TGG	W N W S K S W	503.2123	503.2139	3.18	
10	TGG AAC TGG TCA AAA AGC TAC	W N W S K S Y	491.7063	491.7064	0.20	
11	TGG CAG TGG TCA AAA AGC TTC	W Q W S K S F	490.7167	490.7169	0.41	
12	TGG ACC TGG TCA AAA AGC TTC	W T W S K S F	477.2112	477.2115	0.63	
13	TGG GCA TGG TCA AAA AGC TTC	W A W S K S F	462.2059	462.206	0.22	
14	TGG AAC TGG GCA AAA AGC TTC	W N W A K S F	475.7114	475.7118	0.84	
15	TGG AAC TGG TCA GCA AGC TTC	W N W S A S F	455.1799	455.1802	0.66	
16	TGG AAC TGG TCA AAA GCT TTC	W N W S K A F	475.7114	475.7119	1.05	
17	TGG AAC TGG TCA AAA AGC GCA	W N W S K S A	445.6932	445.6936	0.90	
18	TGG AAC TGG TCA AAA AGC CAG	W N W S K S Q	410.1746/474.2039	474.2043	0.84	+128.0594
19	TGG AAC TGG TCA AAA	W N W S K	366.6586	Not observed		
20	TGG AAC TGG TCA	W N W S	303.6190	Not observed		
21	TGG AAC TGG	W N W	260.1023	Not observed		



-4) with altered core sequences, in which naturally occurring amino acids were combined in a non-natural manner.

For example, in darobactins B–E position 2 is L-serine or L-asparagine, position 4 is L-serine or L-threonine, position 5 is L-lysine or L-arginine and position 6 is L-serine or L-arginine. However, natural darobactins A–E do not cover all possible combinations. The *darA* core sequence in pNOSO-darABCDE-1, on the other hand, encodes the core peptide W₁S₂W₃T₄K₅R₆F₇ (named darobactin 1 here), representing one of the possible non-natural combinations of natural occurring amino acids. After heterologous expression of pNOSO-darABCDE-1, -2, and -4 in *E. coli* BL21 (DE3), production of the respective non-natural darobactins was confirmed by uHPLC-HRMS (see Table 1, ESI Fig. 8–10 and 27–29†).

Next, we aimed to investigate whether our heterologous production system also tolerates the exchange of natively occurring amino acids in positions 2 and 4–6 of the darobactin core peptide by amino acids natively not found in the respective positions of the darobactins A–E. Therefore, numerous *darA* variants (5–8 and 11–17) were designed *in silico*, in which the core sequences encode non-natural darobactin A derivatives containing L-glutamine (darobactin 11) or L-threonine (darobactin 12) in position 2, L-cysteine in positions 2, 4, 2 and 4, or 6 (darobactins 5, 6, 7, and 8, respectively), and L-alanine in positions 2 and 4–7 (darobactins 13–17, respectively). After chemical synthesis and subsequent cloning of the *darA* variants into pNOSO-darABCDE, generating pNOSO-darABCDE-5, -6 to -8, and -11 to -17, heterologous expression of the respective constructs in *E. coli* BL21 resulted in the production of darobactins 11–17 (see Table 1, ESI Fig. 14–20 and 33–39†), whereas darobactin-related but unexpected masses were detected after analysis *via* GNPS-based spectral networking²³ of the corresponding tandem MS upon expression of pNOSO-darABCDE-6 and -8 (*m/z* 587.209 and 587.212 instead of 491.697 [M + 2H]²⁺) (see Table 1, ESI Fig. 10, 11, 29 and 30†). Darobactin derivative production was confirmed by observation of the corresponding exact mass followed by analysis of the corresponding MS² spectrum that shows characteristic fragments for darobactin scaffolds (see ESI†). In case of expression of pNOSO-darABCDE-5, -7 and -13, no expected darobactins nor darobactin-related compounds were detected. This clearly shows that the chemical space to exchange amino acids at certain positions in darobactin is limited. For example, in position 2 the exchange of the naturally occurring amino acids L-asparagine and L-serine by the sterically and physicochemically similar amino acids L-glutamine and L-threonine was tolerated, whereas L-alanine was not accepted in this position. The attempt to introduce one L-cysteine at different positions either resulted in production of darobactin-related compounds with unexpected masses or, similar to our attempt involving two L-cysteine residues at the same time, to complete abolishment of the production. Since the unexpected masses were higher than the expected masses, we assume that the reactive cysteine side chain thiol group underwent chemical reactions leading to adduct formation. Based on the measured exact mass we observe a mass shift of +191.02 Da with respect to the expected darobactin 6 mass. ChemCalc²⁴ analysis predicted a potential

sum formula of C₆H₉NO₄S based on the exact mass of the adduct, taking into account the seven golden rules for heuristic sum formula prediction.²⁵ We assume that the presence of a sulphur makes adduct formation *via* a disulphide bond likely, but purification of darobactins 6 and 8 with subsequent structure elucidation is required to make a precise statement about the respective structures. However, we de-prioritized these compounds as they were produced in significantly lower amounts based on comparison of the MS peak area under the curve with respect to darobactin A (see Table 2).

Furthermore, L-alanine was successfully incorporated into the darobactin scaffold in case of darobactins 14–17 (see Table 1, ESI Fig. 17–20 and 36–39†). This was surprising, since the C-terminal amino acid (position 7) occurring in natural darobactins A–E was always L-phenylalanine. Still, replacement with L-alanine in case of darobactin 17 was found possible. The L-phenylalanine moiety is conserved in natural darobactins A–E at this position¹⁵ and was shown to bind BamA exactly where a conserved aromatic peptide side chain in the β-signal sequences of the cognate BamA substrates bind.¹⁸ Consequently, we hypothesise that an aromatic side chain moiety in position 7 is crucial for the bioactivity of darobactins and that further amino acid exchanges in this position against other aromatic amino acids might result in active darobactin variants. We therefore designed two new *darA* variants (9 and 10), encoding L-tryptophan and L-tyrosine in position 7, respectively. After cloning of *darA*-9 and *darA*-10 into pNOSO-darABCDE and heterologous expression of the respective constructs in *E. coli* BL21 (DE3), the production of darobactins 9 and 10 exhibiting a terminal L-tryptophan and L-tyrosine moiety, respectively, was confirmed by uHPLC-HRMS (see Table 1, ESI Fig. 12, 13, 31 and 32†).

As the heterologous production system tolerated the majority of amino acid exchanges in the darobactin precursor peptide, we subsequently aimed for a truncation of the core structure. This experiment might provide first insights into a possible structure–activity-relationship, which will be investigated in more detail in the future, the mechanism of tail sequence cleavage and pave the way for semi-synthesis on darobactin scaffolds. We designed four additional *darA* variants (18 to 21) in which the propeptide sequence length was reduced by one (*darA*-18) up to four (*darA*-21) amino acids, respectively, without removing the darobactin tail sequence. However, after cloning and heterologous expression of the generated constructs in *E. coli* BL21(DE3), we only observed production of one unexpected darobactin derivative (darobactin 18) upon expression of pNOSO-darABCDE-18, which was shown to carry a L-glutamine at position 7 (see Table 1, ESI Fig. 21 and 40†). We had expected the production of a truncated darobactin derivative, thus having a terminal L-serine in position 6. Interestingly, the L-glutamine found in position 7 is the first amino acid of the tail sequence. Truncation of the core sequence therefore seems to lead to a maturation process in which the first amino acid of the tail sequence is recognized as part of the core and is thus not cleaved off in the proteolytic process. The attempt to truncate the darobactin core sequence by two or more amino acids resulted in a complete loss of production (see Table 1).



Table 2 Estimation of antimicrobial activity and relative production titre of the darobactin derivatives with respect to darobactin A based on crude extracts (see ESI). The antimicrobial activity of each darobactin derivative-containing crude extract against *K. pneumoniae* DSM-30104, *P. aeruginosa* PAO1, *E. coli* ATCC25922 and *A. baumannii* DSM-30008 is given as concentration factor (100× concentration caused by the extraction process) divided by the lowest dilution factor (1 : 15–1 : 240 dilutions applied in the MIC assay) where growth inhibition was last observed. The lower the crude extract concentration factor at which growth inhibition of a certain pathogen was last observed, the stronger the antimicrobial activity. Weak activity is highlighted in light green colors (e.g. growth inhibition at 6.67× and 3.34× concentration of the crude extract) and strong activity is highlighted in green to dark-green colors (e.g. 1.67×–0.42× concentration), whereas “-” means no activity even at 6.67× concentration. Crude extract concentrations of 0.83× and 0.42× are synonymous with a total crude extract dilution of 1 : 1.2 and 1 : 2.4, respectively. The assignment of a concentration factor as measure of the antimicrobial activity is described in more detail in ESI (see ESI Fig. 41). The area under curve (AUC) ratio serves as measure of the production titre of the respective derivative in the crude extract based on the MS peak surface compared to the AUC of darobactin A. “Traces” means an AUC ratio below 0.01. It should be noted, however, that without purification of all congeners an exact quantification is not possible

Darobactin in crude extract	Concentration/dilution of crude extract at which full growth inhibition is observed				AUC ratio (derivative / Dar. A)
	<i>K. pneumoniae</i> DSM-30104	<i>P. aeruginosa</i> PAO1	<i>E. coli</i> ATCC25922	<i>A. baumannii</i> DSM-30008	
A	0.83x	0.42x	0.83x	1.67x	1
B	3.34x	1.67x	1.67x	1.67x	0.03
C	-	-	-	-	traces
D	-	3.34x	3.34x	6.67x	0.14
E	-	-	-	-	0.02
2	-	6.67x	6.67x	6.67x	0.06
4	1.67x	0.83x	0.83x	0.83x	0.69
6	6.67x	3.34x	3.34x	3.34x	0.06
8	-	6.67x	6.67x	6.67x	0.02
9	1.67x	1.67x	0.83x	0.83x	0.55
10	-	6.67x	-	-	0.41
11	6.67x	6.67x	6.67x	3.34x	0.07
12	-	6.67x	6.67x	6.67x	0.04
13	-	-	-	-	traces
14	1:1,2	1.67x	1:1,2	1.67x	0.82
15	-	-	-	-	traces
16	1.67x	1.67x	1.67x	1.67x	1.01
17	-	-	-	-	0.21
18	-	-	-	6.67x	0.26

Consequently, we assume that a core sequence of seven amino acids length is recognized by the so far unidentified protease (see below). Notably, in our experiment we did not remove the tail sequence from *darA*, but only removed one or several of the darobactin core sequence codons. In the contemporaneously performed work of Wuisan and co-workers a darobactin BGC with a *darA* variant without the tail sequence was heterologously expressed in *E. coli*, showing that darobactin A was still produced.²⁰ Future experiments, in which the entire tail

encoding sequence plus additional core amino acid encoding sequences are removed from *darA* might thus result in the formation of truncated darobactin congeners.

In summary, 18 natural and non-natural darobactin derivatives were successfully heterologously produced in *E. coli* BL21 (DE3) after expression of the darobactin BGC with different *darA* core sequences. Five out of the 18 produced derivatives were native darobactins (A–E), four of which (darobactins B–E) were not previously observed, and 11/19 were non-natural derivatives (1, 2, 4, 9–12, and 14–17), whereas the remaining three showed unexpected masses (6, 8, and 18).

Purification and antibacterial activity profiling of darobactin 9

Since the purification of highly water-soluble darobactin A was a time- and resource-intensive process, we assumed that purification of the newly produced derivatives to be similarly challenging. Thus, we devised a prioritization and preselection strategy enabling us to categorize the newly produced derivatives over others according to their anti-Gram-negative activity. Therefore, we performed MIC assays using a small panel of Gram-negative pathogens (*K. pneumoniae* DSM-30104, *P. aeruginosa* PAO1, *E. coli* ATCC25922 and *A. baumannii* DSM-30008) using crude extracts of all heterologous producer strains (Table 2). Each crude extract was tested in serial dilutions and the given values are the concentration factor for which no visible growth was observed. The assignment of the concentration factor and the interpretation of the estimated antimicrobial activity of the crude extract is described in more detail in the legend of Table 2 and in ESI (see ESI Fig. 41†).

However, we could not assign absolute quantities and exact concentrations of novel darobactins without isolating all of them. Thus, the concentration of the darobactin derivatives differed in each crude extract and this experiment does not allow direct comparison of the bioactivity observed for a certain darobactin derivative with the antibiotic activity of darobactin A. Nevertheless, we were able to de-prioritise darobactin derivatives for which no bioactivity was observed (darobactins C, E, 5, 7, 13, 15 and 17), even though this might be caused by low concentration of the respective compound in the crude extract. In order to further narrow down the pool of the remaining darobactin derivatives of interest and select a candidate for isolation and biological assessment based on the pure compound, we calculated the area under curve (AUC) of the respective darobactin derivative MS peaks (Table 2). As the darobactin derivatives differ in their ionisability in the ESI process, this analysis can only serve as a rough estimate of the amount of compound in each crude extract. For instance, darobactin B, in which the L-serine at amino acid position 6 was replaced by the well-ionisable L-arginine, showed a higher MS signal provided from the $[M + 3H]^{3+}$ ions than the MS signal provided from $[M + 2H]^{2+}$ ions, whereas $[M + 3H]^{3+}$ ions were only barely observed for darobactin A. Since we cannot predict the ionisation behaviour of the derivatives we cannot provide an absolute quantification of each congener. We therefore refrained from calculating mg L^{-1} values for each analog based on the absolute quantification of the darobactin A production



titre. The largest AUC values were calculated for darobactins 4, 9, 10, 14 and 16. Due to the challenges associated with isolation and purification of compounds for follow up experiments by NMR and bioactivity assays we therefore prioritized compounds likely to exhibit good activity at reasonable productivity.

Since L-phenylalanine is conserved in position 7 in native darobactins and an essential structural feature to mimic the β -signal of native BamA substrates,¹⁸ we were surprised that the extract containing darobactin 9, in which the L-phenylalanine was replaced by L-tryptophan, showed similar antibacterial activity to the darobactin A-containing sample (Table 2). On the contrary, the extract containing darobactin 10 with L-tyrosine in position 7 showed only weak activity despite its structural similarity to darobactin A and similar production level based on the AUC of the respective MS peaks. This indicates that the additional OH-group in L-tyrosine compared to L-phenylalanine negatively affects the interaction of darobactin with BamA, whereas expansion of the side chain π -system by introducing an indole moiety, as it is the case for L-tryptophan, potentially enables stronger aromatic interaction with the BamA target site. Since darobactin 9 was produced in similar quantities to darobactin A based on comparison of the respective AUCs, we classified darobactin 9 as promising starting point for further investigations and purified the compound from a large-scale production culture (see ESI†). The purified compound was used to determine the production titre of darobactin 9 in the fermentation broth of *E. coli* BL21 (DE3) pNOSO-darABCDE-9 (see ESI†), providing an average production rate of 2.9 (± 0.6) mg L⁻¹. The predicted structure (Fig. 5) was verified by acquisition of tandem MS data followed by acquisition of multidimensional NMR experiments. Next, we assessed the MICs of pure darobactin 9, showing that this derivative exhibits superior antibacterial activity against the strains of a larger pathogen panel being on average two-fold more potent than darobactin A (Table 3).

Encouragingly, most Gram-negative bacterial strains were inhibited by sub to low $\mu\text{g mL}^{-1}$ concentrations of darobactin 9, and the new derivative was particularly active against *E. coli* (0.125–2 $\mu\text{g mL}^{-1}$), *Citrobacter freundii* (0.5 $\mu\text{g mL}^{-1}$), *Enterobacter cloacae* (1 $\mu\text{g mL}^{-1}$), and *K. pneumoniae* (1–2 $\mu\text{g mL}^{-1}$). Among the species displaying high MICs for darobactin A, we observed significant improvements for darobactin 9, where MICs on *A. baumannii* of 1–2 $\mu\text{g mL}^{-1}$ and MICs on *P. aeruginosa*

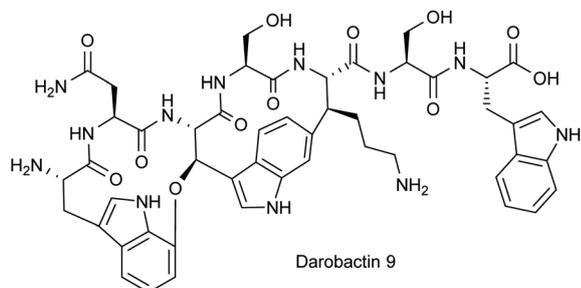


Fig. 5 Structural formula of darobactin 9 carrying a C-terminal L-tryptophan.

Table 3 Minimal inhibitory concentration (MIC in $\mu\text{g mL}^{-1}$) of darobactin 9 in an extended panel of clinically relevant Gram-negative pathogens. Darobactin A served as control. The heterologous darobactin producer strain *E. coli* BL21 (DE3) + BGC contains the pNOSO-darABCDE plasmid

Strain		Dar. 9	Dar. A
<i>E. coli</i>	BL21 (DE3)	0.25	0.5–1
	BL21 (DE3) + BGC	2–4	8–16
	ATCC-25922	1–2	2
	JW0451-2 (ΔacrB)	0.125	0.5
	K12 ΔtolC	0.125	0.5
	ECO24 ^a	1–2	8
	ECO25 ^a	1–2	8
<i>A. baumannii</i>	DSM-30007	2	16
	DSM-30008	1–2	4
<i>C. freundii</i>	DSM-30039	0.5	1
<i>E. cloacae</i>	DSM-30054	1	2
<i>K. pneumoniae</i>	DSM-30104	1–2	2–4
	KPN12 ^a	4	8
	KPN19 ^a	1	2
	KPN62 ^a	2	4
<i>P. aeruginosa</i>	PAO1	0.125	0.5–1
	PA14	2	16
	PA14 ΔmexAB	1	4
<i>P. mirabilis</i>	DSM-4479	32–64	64
<i>P. vulgaris</i>	DSM-2140	8	16
<i>S. marcescens</i>	DSM-30121	64	>64
<i>E. faecalis</i>	ATCC-29212	>64	>64
<i>S. aureus</i>	ATCC-29213	>64	>64

^a MDR clinical isolates (see ESI).

of 0.125–2 $\mu\text{g mL}^{-1}$ were obtained, which corresponds to an approximately 8-fold improvement over darobactin A. Darobactin 9 also gained some activity against *Proteus* spp. (8–64 $\mu\text{g mL}^{-1}$) and *Serratia marcescens* (64 $\mu\text{g mL}^{-1}$) both being gaps in the antibacterial spectrum of darobactin A. Intriguingly, darobactin 9 was equally active against recent clinical MDR isolates (see ESI†) and MICs were as low as 1–2 $\mu\text{g mL}^{-1}$ and 1–4 $\mu\text{g mL}^{-1}$ on *E. coli* and *K. pneumoniae*, respectively, further highlighting the potential of darobactins as new broad-spectrum and resistance-breaking antibiotics. Thus, darobactin 9 is a very promising starting point for further structure engineering attempts in the future in which we attempt to further engineer darobactin 9. Especially with respect to difficult-to-treat *A. baumannii* and *P. aeruginosa* infections, the improvement in MICs is highly encouraging for such efforts.

Targeted gene deletions and co-expression of *darF* and *darG*

Even though hypothetical biosynthetic functions were previously assigned to *darBCD*, encoding a tripartite ABC-type trans-envelope exporter, and *darE*, encoding a radical SAM enzyme proposed to catalyse both ring formations in darobactins, experimental confirmation has yet to be performed on a molecular basis. Thus, we conducted targeted, seamless gene deletions of *darB*, *darC*, *darD*, and *darE*, independently, with subsequent heterologous expression of the deletion constructs in *E. coli* BL21 (DE3). UHPLC-HRMS analysis of the fermentation broth supernatant (described in ESI†) showed that



deletions of *darB*, *darC* and *darD* led to a two- to four-fold decrease in darobactin A production with titres of *ca.* 6 mg L⁻¹, 5 mg L⁻¹ and 3 mg L⁻¹, respectively, compared to the control.

Given that the gene products of *darBCD* encode an efflux transporter machinery, which is presumably non-functional after deletion of one of its components, the removal of darobactin A from the cell is likely impaired, leading to lower extracellular concentrations as detected by mass spectrometry. Similar observations were made by Wuisan and co-workers, who measured 50% reduced extracellular darobactin A concentration upon heterologous expression of only *darA* and *darE* in *E. coli*, compared to the control strain expressing *darABCDE*.²⁰ Interestingly, the authors could rule out that darobactin A accumulated in the cell, since intracellular concentrations were also reduced when *darBCD* was not expressed.²⁰ Since darobactin production was still observed despite deletion of the efflux transporter co-located with the BGC, *E. coli* BL21 (DE3) might express a *darBCD* homolog located elsewhere in the genome that partly compensates for the loss of function. Consequently, we searched for *darBCD* homologs in the genome of *E. coli* BL21 (DE3) (GenBank accession CP001509) and found only *darD* homologs (ABC-transporter ATP-binding protein; approx. 30 hits $\geq 70\%$ pairwise identity), whereas no close *darB* and *darC* homologs were found. Nevertheless, we cannot exclude that another efflux system, such as AcrAB-TolC,²⁶ YdhE,²⁷ and MdtABC-TolC²⁸ MDR efflux transporters encoded in the *E. coli* BL21 (DE3) genome, is involved in the export of darobactin from the cell. Since *E. coli* strains with deletion of the *acrB* or *tolC* were significantly more sensitive towards darobactin A and darobactin 9 as compared to strains harbouring those genes (Table 3), we assume that involvement of both of these broad-spectrum efflux systems in darobactin export is likely. In parallel work, a cooperation of DarBCD with TolC, both being involved in the export of darobactin, was discussed.²⁰ However, deletion of *tolC* from the genome of the heterologous darobactin producer *E. coli* BAP-1 did not affect the ratio between intra- and extracellular darobactin concentrations.²⁰

Deletion of *darE* in our heterologous expression construct resulted in complete abolishment of darobactin A production, confirming its essential function in darobactin biosynthesis. To identify potential darobactin-related compounds in the fermentation broth, we performed a MS² spectral similarity analysis, but no darobactin-related compounds, such as truncated derivatives, monocyclised, or linear darobactin, were detected. Even though we are not able to confirm the involvement of DarE in the cyclisation reactions on an enzyme level *in vitro*, it is safe to assume that the previously hypothesised function is correct, because bicyclised darobactin was still produced after *darBCD* deletion and taking into account that *darA* encodes the darobactin propeptide, which was proven by Wuisan and coworkers.²⁰ Furthermore, alkyl-aryl couplings like the ones observed for darobactin are usually formed in radical reactions catalysed by radical-SAM-type enzymes or Cytochrome P450 enzymes, which supports the previous hypothesis.²⁹ As no further genes are located within the BGC, we

assume the involvement of other genes in the cyclization reactions to be unlikely since those genes would need to be located elsewhere in the genomes of both the native and heterologous producer strains.

Some of the darobactin BGCs found by genome mining in potential producer strains like *Pseudoalteromonas luteoviolacea* S4054 include two additional ORFs of unknown function, *darF* and *darG*. A BLAST (Basic Local Alignment Search Tool)³⁰ search in the non-redundant protein database (nr) using the protein sequence of DarF and DarG as query provided only hypothetical proteins of unknown function and ABC transporters as respective hits (see ESI Data†). HHpred³¹ analysis of DarF however, revealed homology to zinc-dependent metalloproteases (see ESI Data†), making it a potential candidate protease involved in the darobactin maturation process which was not yet described. Since *darF* is not part of the darobactin BGC in *P. khanii* HGB1456, many other native producer strains and the heterologous producer, a homolog would have to be located elsewhere in these genomes. In parallel work, Wuisan and coworkers speculated that the proteolytic maturation of the darobactin propeptide is indeed performed by a protease elsewhere in the genome of *E. coli* even though they could not rule out an autoproteolytic function of DarA itself.²⁰ Thus, we performed a BLAST search in the genomes of *P. khanii* HGB1456 and *E. coli* BL21 (DE3) using the DarF protein sequence as query, however without identification of potential homologs. To investigate the function of *darF* and *darG*, we co-expressed both genes under the regulation of the *nptII* promoter in combination with *darABCDE* in *E. coli* BL21 (DE3) and observed complete abolishment of darobactin A production. In order to investigate whether this effect is caused by combined co-expression of *darFG* or whether it can be related to *darF* or *darG* alone, we co-expressed both genes independently with *darABCDE*. While co-expression of *darG* only slightly decreased darobactin yields, co-expression of *darF* led to complete production abolishment. Since a spectral networking analysis did not identify any darobactin-related compounds upon expression of *darF*, we hypothesised that DarF acts as a protease degrading either the mature darobactin A itself or the corresponding propeptide, thus impeding formation of mature darobactin. To investigate this hypothesis, we heterologously expressed and purified DarF from *E. coli* BL21 (DE3) and incubated the enzyme with darobactin A or darobactin 9 *in vitro* (see ESI†). We indeed observed degradation of darobactins A and 9 after 12 h of incubation with DarF, whereas no degradation was seen in the control experiments without DarF (see ESI Fig. 49 and 50†). These observations supported that DarF is a darobactin-degrading enzyme. A possible function of DarF in *P. luteoviolacea* S4054 might be a feedback-regulated self-detoxification or self-resistance mechanism, in which high darobactin A concentrations lead to *darF* gene expression and consequently the degradation of darobactin A, thereby setting a limit to darobactin concentrations not harmful for the native producer. In our heterologous expression system, however, the potential feedback-regulated gene expression might be impaired, because *darF* is constitutively overexpressed under the regulation of the *nptII* promoter, which seems to lead to complete degradation of darobactin.



In summary, *darA* and *darE* are the only genes essential for darobactin production in *E. coli* BL21 (DE3), which was also shown in parallel work by Wuisan *et al.*²⁰ Co-expression of *darBCD* increases the production level of darobactin A by a factor of 2–4, whereas *darG* co-expression reduces the production level, as judged by comparison of the darobactin A mass peak. Since co-expression of *darF* led to complete abolishment of darobactin production and no homologs were identified in the genomes of *P. khanii* HGB1456 and *E. coli* BL21 (DE3), we rule out that DarF is the protease involved in the proteolytic maturation process of the compound, in which the leader peptide or tail sequence are removed. Both the mechanism of action of DarF and the proteolytic maturation mechanism of darobactin, performed by a so far unidentified protease enzyme in the native producer strain and a similar enzyme in *E. coli* BL21 (DE3) have to be investigated in future experiments.

Conclusions

In this work, we developed a versatile heterologous production platform for darobactin derivatives based on a darobactin BGC from *P. khanii* HGB1456 modified for the expression in *E. coli* BL21 (DE3). Excluding the previously published darobactin A,¹⁵ 17 new darobactin derivatives were produced after expression of *darA* variants encoding different core peptide sequences. Every position in the molecule was shown to be reasonably flexible for structure engineering by incorporation of different amino acids, except of the two L-tryptophans in positions 1 and 3, which are required for formation of the bicyclic darobactin core structure, proving the versatility of the presented system. Likewise, high substrate tolerance has been observed for biosynthetic enzymes in other RiPP pathways, *e.g.* in lanthipeptide, thiopeptide, lasso peptide and sactipeptide biosynthesis,³² underlining the potential of RiPP structure engineering to generate new antibiotics.

However, due to difficulties in the isolation and compound purification process that will have to be addressed in the future, we were so far only able to purify our most promising congener darobactin 9, which showed favourable activity against MDR clinical pathogens and significantly improved antibacterial activity towards *P. aeruginosa* and *K. pneumoniae* strains with respect to darobactin A. For the other darobactin derivatives, we relied on appearance of the corresponding mass peak after heterologous expression of the respective plasmid-based BGC, followed by comparison of the calculated to the measured exact mass and corresponding MS² fragmentation pattern to assign the predicted structures for the remaining derivatives not isolated so far. However, for the L-cysteine-containing derivatives we observed an unexpected mass shift of +191.02 Da that we were not able to account for, while the MS² spectrum indicates the molecule contains the darobactin core structure including the L-cysteine. It therefore seems likely that the sulphur nucleophile in the respective darobactin derivatives has reacted with a metabolite in the cell in order to detoxify the sulphur nucleophile. Nevertheless, future experiments will address the optimization of cultivation conditions and downstream purification steps, finally allowing characterization of the remaining

derivatives, which showed promising antibacterial activity in the crude extract-based MIC assay.

Even though the production titre of darobactin A in our heterologous host (>13 mg L⁻¹ in 3 days) was improved compared to the native producer strain *P. khanii* HGB1456, parallel work of Wuisan *et al.* reached a titre of >30 mg L⁻¹ in 2 days.²⁰ Nevertheless, the production titres of darobactin A and of a few derivatives achieved with our system is sufficient for isolation and purification, with the production resulted from leaky expression of *darA* by an uninduced *T7lac* promoter. Furthermore, it is unlikely that the codon usage in the biosynthetic genes responsible for darobactin production are optimal for translation in *E. coli*, since the GC content of the darobactin BGC is only 32% as compared to *E. coli* genome with about 50% GC content. As we did not perform codon optimization so far, it is likely that production improvement can be achieved on both transcriptional and translational level after further optimizations of the darobactin expression vector.

Moreover, determination of the MIC of darobactin A and darobactin 9 against the heterologous producer strain *E. coli* BL21 (DE3) pNOSO-darABCDE showed that growth inhibition occurred at concentrations of 8–16 and 2–4 μg mL⁻¹ (see Table 3), respectively, indicating that the maximum production titres of both compounds (>13 mg L⁻¹ darobactin A and <3 mg L⁻¹ darobactin 9) might be limited by self-resistance of the producer. In order to achieve industrial scale production titres, the self-resistance of the producer might be improved through targeted mutation of BamA in the genome, since it was previously shown that certain BamA mutations confer resistance towards darobactin A.¹⁵ As experiments that occurred in parallel to this work showed BamA mutations to lead to a significant loss in fitness and darobactin A productivity,²⁰ the co-expression of mutated BamA contemporaneously with native BamA might be an alternative route to be explored. Furthermore, utilizing a phylogenetically more distant heterologous producer strain, *e.g.* of Gram-positive or eukaryotic origin, could circumvent the self-resistance issue completely as these organisms do not rely on BamA.

Targeted gene deletions and the attempts to produce truncated darobactins provided a first insight into the biosynthesis of this compound class. Interestingly, only two genes, *darA* and *darE*, are required for the formation of darobactin, a result also observed in parallel work by Wuisan and coworkers.²⁰ Deletion of the *relE*-like gene, which is associated with the darobactin BGC in some of the native producer strains, had no impact on the darobactin A production in *E. coli* BL21 (DE3). However, as the gene expression of the *relE* homologue was still regulated by its native promoter, its expression might be weak or totally absent in *E. coli* BL21, which prevented us to elucidate its actual function and influence on darobactin biosynthesis. In future experiments, the *relE* homologue might be re-introduced into pNOSO-darABCDE downstream of *darBCDE* under the regulation of the *nptII* promoter for further investigation into its biological role. Moreover, despite the identification of *darF* and *darG* as genes associated with the darobactin BGC in several native producer strains, their role in darobactin biosynthesis was previously not discussed. Co-expression of *darF* and *darG* in



the heterologous producer and the *in vitro* investigation of purified DarF showed that DarF is a protease leading to degradation of darobactin, whereas the function of DarG remains unclear. We hypothesise that the expression of *darF* in the native producer strain might be feedback-regulated, thereby limiting the darobactin production. However, experimental evidence has to be provided in future experiments. The proteolytic maturation process of darobactin however, including the enzymes that are potentially involved, still remains unclear.

Taken together, this work provided experimental evidence for darobactin biosynthesis processes, which was in part also confirmed by parallel work by Wuisan *et al.*²⁰ Importantly, we achieved efficient structure engineering of darobactin and heterologous production of new and superior bioactive derivatives like darobactin 9 in good yields. Therefore, the work reported further advances the darobactin natural product class towards a potential use as an antibiotic drug.

Data availability

The accession data is referred to in the results section and the accession numbers are provided in ESI.†

Author contributions

Sebastian Groß: conceptualization (equal); methodology (supporting); project administration (equal); investigation (supporting); visualization (equal); writing – original draft (lead); writing – review & editing (equal). Fabian Panter: methodology (supporting); investigation (equal); formal analysis (lead); visualization (equal), writing – original draft (supporting); writing – review & editing (equal). Domen Pogorevc: conceptualization (equal), methodology (lead), project administration (equal), investigation (supporting), formal analysis (supporting). Carsten E. Seyfert: investigation (equal), formal analysis (supporting). Selina Deckarm: investigation (equal). Chantal D. Bader: methodology (supporting), investigation (supporting), formal analysis (supporting) Jennifer Herrmann: formal analysis (supporting), writing – original draft (supporting). Rolf Müller: conceptualization (equal); supervision; funding acquisition; writing – review & editing (equal).

Conflicts of interest

There are no conflicts to declare.

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