**Mutasynthetic production of darobactin analogs**

*E. coli* strains for cloning and expression were grown in LB broth or on agar medium supplemented with appropriate antibiotics or supplements at 37° C or 30° C using standard working concentrations. Plasmid DNA was isolated using the innuPREP plasmid mini kit 2.0 (AnalytikJena, Jena, Germany) according to the manufacturer’s protocol. Genomic DNA was extracted using the innuPREP bacteriaDNA kit (AnalytikJena, Jena, Germany). PCR amplification for cloning purposes was performed using Q5 DNA polymerase (NEB Biolabs, New Brunswick, USA) according to the given instruction. Restriction digestion was performed using standard techniques and employing NEB enzymes (NEB Biolabs, New Brunswick, USA). DNA fragments were analysed on and excised from 1% or 2% TAE-agarose with GeneRuler 1kb Plus (ThermoFisher, Waltham, USA) as marker. DNA for cloning purposes was purified using the Zymoclean large fragment DNA recovery kit according to manufacturer’s instruction. DNA concentrations were determined photometrically with an Eppendorf BioSpectrometer (Eppendorf AG, Hamburg, Germany) using a 1 mm light path UV cuvette. DNA fragments to be fused by isothermal assembly were gel purified and fused using self-made isothermal assembly master mix (*Nat Methods* **2009**, *6*, 343–345) using NEB enzymes (NEB Biolabs, New Brunswick, USA). Assembled plasmids were transferred to *E. coli* cells using standard electroporation protocols (*Nature* **2019**, *576*, 459-464, *Metab Eng* **2021**, *66*, 123-136).

Construcion of pNBDaroMod for modification of the precursor peptide was performed by linearising pNB03 (*Nature* **2019**, *576*, 459-464) by PCR using 5’‑TCCCTTAACGTGAGTTTTCG-3’/ 5’-TTTTATAACCTCCTTAGAGCTCGAA-3’, amplification of truncated (3’ minus 50 nt) *darA* using 5’‑GCTCTAAGGAGGTTATAAAAATGCATAATACCTTAAATGAAACCGTTAAA-3’/ 5’-TAGGTTTATTGCTTAATTCGTTTAGTGCTT-3’, the lacZ spacer from pCRISPOMYCES-2 (5’‑CGAATTAAGCAATAAACCTAAAGTCTTCTCAGCCGCTACA-3’/ 5’‑ACCTGATGGGATAAGCTTTAATGTCTTCACCGGTGGAAAG-3’) and the rest of the *P. khanii* DSM3369 BGC using 5’-TAAAGCTTATCCCATCAGGTTATTT-3’/ 5’‑CGAAAACTCACGTTAAGGGATTACGCCGCGATGGTTTGTTTTATT-3’ and subsequent isothermal assembly of the plasmid. After transformation and selection on LBKan/Apra/IPTG/X-gal, blue colonies were picked and the correct assembly of the plasmid was corroborated by test restriction. AA modifications were designed *in silico* and ordered as complementary oligonucleotides with 4 nt overlap to the pNBDaroMod backbone. Oligonucleotides were annealed and assembled into pNBDaroMod using the protocol described in *ACS Synth Biol* **2015**, *4*, 723-728 and the resulting plasmids were transferred to *E. coli* BW25113 and selected on LBKan/Apra/IPTG/X-gal. White colonies were picked and grown in LBKan/IPTG for three days at 220 rpm and 30° C. The correct assembly of the plasmid was corroborated by UHPLC-MS profiling, *i.e.* detection of the expected product ion. For increased production titter, the modified BGCs were recloned into pRSF-duett using the primers 5’-GTATAAGAAGGAGATATACAATGCATAATACCTTAAATGA-3’/ 5’‑TGCTCAGCGGTGGCAGCAGCTTACGCCGCGATGGTTTGTT-3’ for all constructs to match the layout of pRSF-ADC5 and produced in *E. coli* Bap1 (*Metab Eng* **2021**, *66*, 123-136).