Electronic supplementary information

A colourimetric high-throughput screening system for directed evolution of prodigiosin ligase PigC

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Material and Methods

Chemical synthesis of precursor and reference compounds

Pyrrolic compounds (Figure S1; PigC substrates (4-methoxy-2,2'bipyrrole-5-carbaldehyde (MBC, **2**); 2,3 dimethyl-1*H*-pyrrole (diMe-pyrrole, **3b**); 2-methyl-3-propyl-1*H*-pyrrole (**3c**); 3-amyl-2-methyl-1*H*-pyrrole (MAP, **3a**); 3-decyl-2-methyl-1*H*-pyrrole (**3d**); 3-ethyl-2-propyl-1*H*-pyrrole (**3e**); 4,5,6,7,8,9-hexahydro-1*H*-cycloocta[*b*]pyrrole (**3f**), 2-methyl-3-pentenyl-1*H*-pyrrole (**3g**), prodigiosin (**1a**), and prodiginine **1c** {(*Z*)-5-[(4-ethyl-5-propyl-2*H*-pyrrol-2-ylidene)methyl]-4-methoxy-1*H*,1'*H*-2,2'-bipyrrole} were synthesised chemically as described before[1–3]. Prodiginine **1b** {4-methoxy-5-[(4,5-dimethyl-2*H*-pyrrol-2-yliden)methyl]-1*H*,1'*H*-2,2'-bipyrrole x HCl} was synthesised as reference substance as described below.

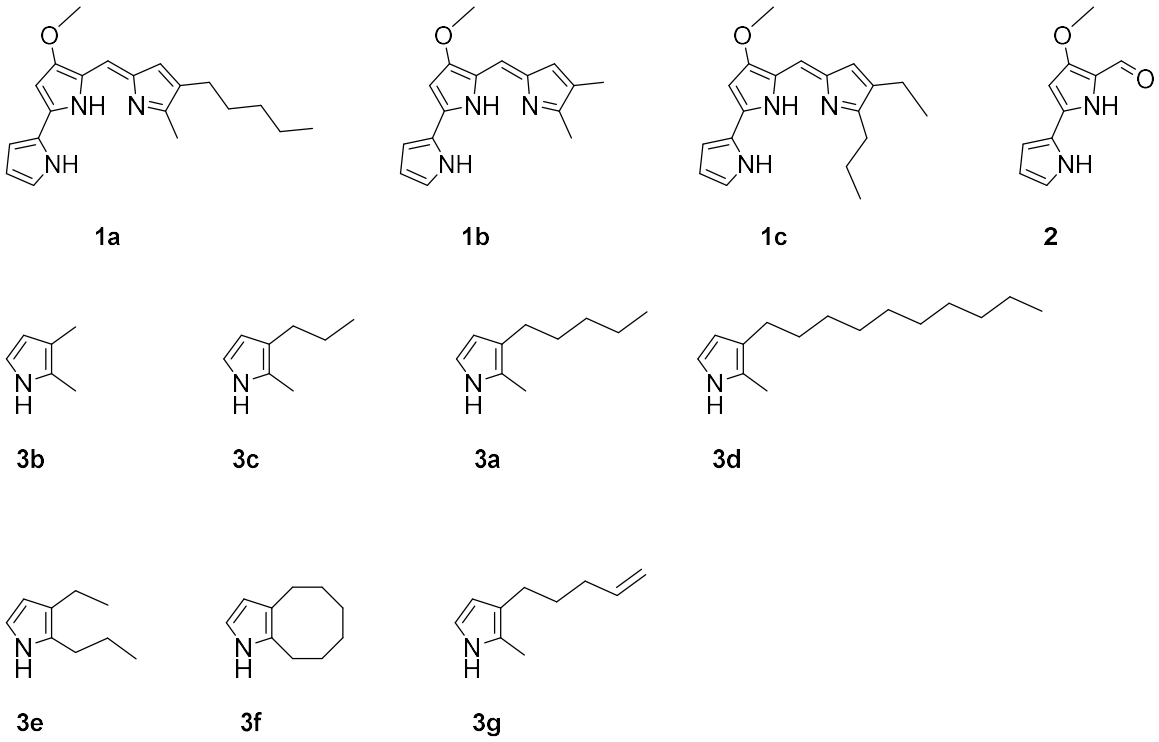
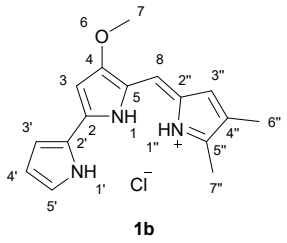


Figure S1: Overview of pyrrolic compounds synthesised in this study.

5-[(4,5-Dimethyl-2*H*-pyrrol-2-yliden)methyl]-4-methoxy-1*H*,1'*H*-2,2'-bipyrrole·HCl (1b)



1b

The reaction was performed according to the general procedure as described in *Brass et al*[3]. The reaction was performed in a *Schlenk* flask under nitrogen atmosphere. *tert*-butyloxycarbonyl-4-methoxy-2,2'-bipyrrole-5-carbaldehyde (Boc-MBC, 1.00 equiv, 0.34 mmol, 100 mg) and 2,3-dimethyl-1*H*-pyrrole (**3c**, 1.50 equiv., 0.52 mmol, 49.2 mg) were dissolved in 5 mL dry methanol. At 0 °C a 1.25% (*v*/*v*) solution of HCl in methanol (1.30 equiv, 0.45 mmol, 1.57 mL) was added, and the solution was stirred for 15 min. Upon addition of HCl in methanol, the solution turned immediately dark red. The solution was stirred for an additional 1 h at room temperature. The reaction was stopped by addition of a few drops ammonia solution [25% (*w*/*w*)] at 0 °C until the reaction mixture turned from red to orange. The reaction mixture was extracted with dichloromethane (3 x 10 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure. Prodiginine **1b** was purified *via* chromatography on silica with dichloromethane/ammonia in methanol (7 N) [0.5%–2.5% (*v*/*v*) gradient] and followed by chromatography on neutral aluminium oxide with *n*-pentane/EtOAc (gradient from 90:10 to 50:50). Precipitation as hydrochloride provided prodiginine **1b** (28.9 mg, 11 µmol, 31%) as dark red solid. The UV/Vis absorbance spectrum (Tecan Infinite M200 Pro plate reader) of prodiginine **1b** (Figure S2) in acidified ethanol [4% (*v*/*v*) 1 N HCl] showed a maximum at 536 nm.

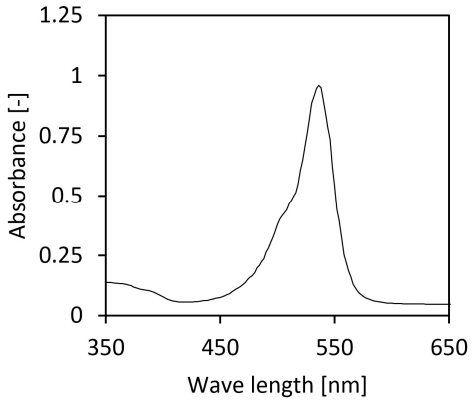


Figure S2: UV/Vis absorbance spectrum of diMe-prodiginine (**1b**, 50 µM) in 100 µL acidified ethanol [4% (*v*/*v*) 1 N HCl)] in a 96-well plate.

NMR spectroscopy: ^1H- and ^{13}C-NMR spectra were measured in an Advance/DRX 600 nuclear magnetic resonance spectrometer (Bruker, Billerica, US-Massachusetts) at ambient temperature in $CDCl_3$ at 600 and 151 MHz, respectively. The chemical shifts are given in ppm relative to tetramethylsilane [^1H: δ($SiMe_4$) = 0.00 ppm] as an internal standard or relative to the solvent [^1H: δ($CDCl_3$) = 7.26 ppm; ^{13}C: δ($CDCl_3$) = 77.16 ppm]. Signals were assigned by means of DEPT-135° Puls-, ^1H-^1H-COSY-, ^1H-^{13}C-HSQC- und ^1H-^{13}C-HMBC-experiments; splitting patterns are given as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad singlet (brs) plus coupling constants (*J*) are reported in Hz.

^{1}H-NMR (600 MHz, $CDCl_3$): δ [ppm] = 2.01 (s, 3H, 6″-H), 2.50 (s, 3H, 7″-H), 3.95 (s, 3H, 7-H), 6.03 (d, $^{4}J_{3,1}$ = 2.0 Hz, 1H, 3-H), 6.31 (m_c, 1H, 4′-H), 6.62 (m_c, 1H, 3″-H), 6.86 (s, 1H, 8-H), 6.87 (m_c, 1H, 3′-H), 7.19 (m_c, 1H, 5′-H), 12.52 (brs, 1H, 1′-NH), 12.61 (brs, 1H, 1-NH), 12.65 (brs, 1H, 1″-NH); **^{13}C-NMR** (151 MHz, $CDCl_3$): δ [ppm] = 10.7 (C-6″), 12.4 (C-7″), 58.8 (C-7), 93.0 (C-3), 111.8 (C-4′), 116.0 (C-8), 117.2 (C-3′), 120.8 (C-5), 122.3 (C-2′), 123.0 (C-4″), 125.1 (C-2″), 126.9 (C-5′), 129.3 (C-3″), 147.1 (C-5″), 147.8 (C-2), 165.9 (C-4); **IR** (ATR-Film): $\tilde{\nu}$ [1/cm] = 2922, 1725, 1634, 1602, 1575, 1545, 1511, 1449, 1412, 1366, 1249, 1135, 1066, 1042, 954, 887, 837, 803, 744, 708, 651, 611, 594, 542, 465; **HRMS** (ESI-FTMS, positive-Ion): Calculated for $C_{16}H_{17}N_3O$ $(M + H)^+$ = 268.1444, found = 268.1446.

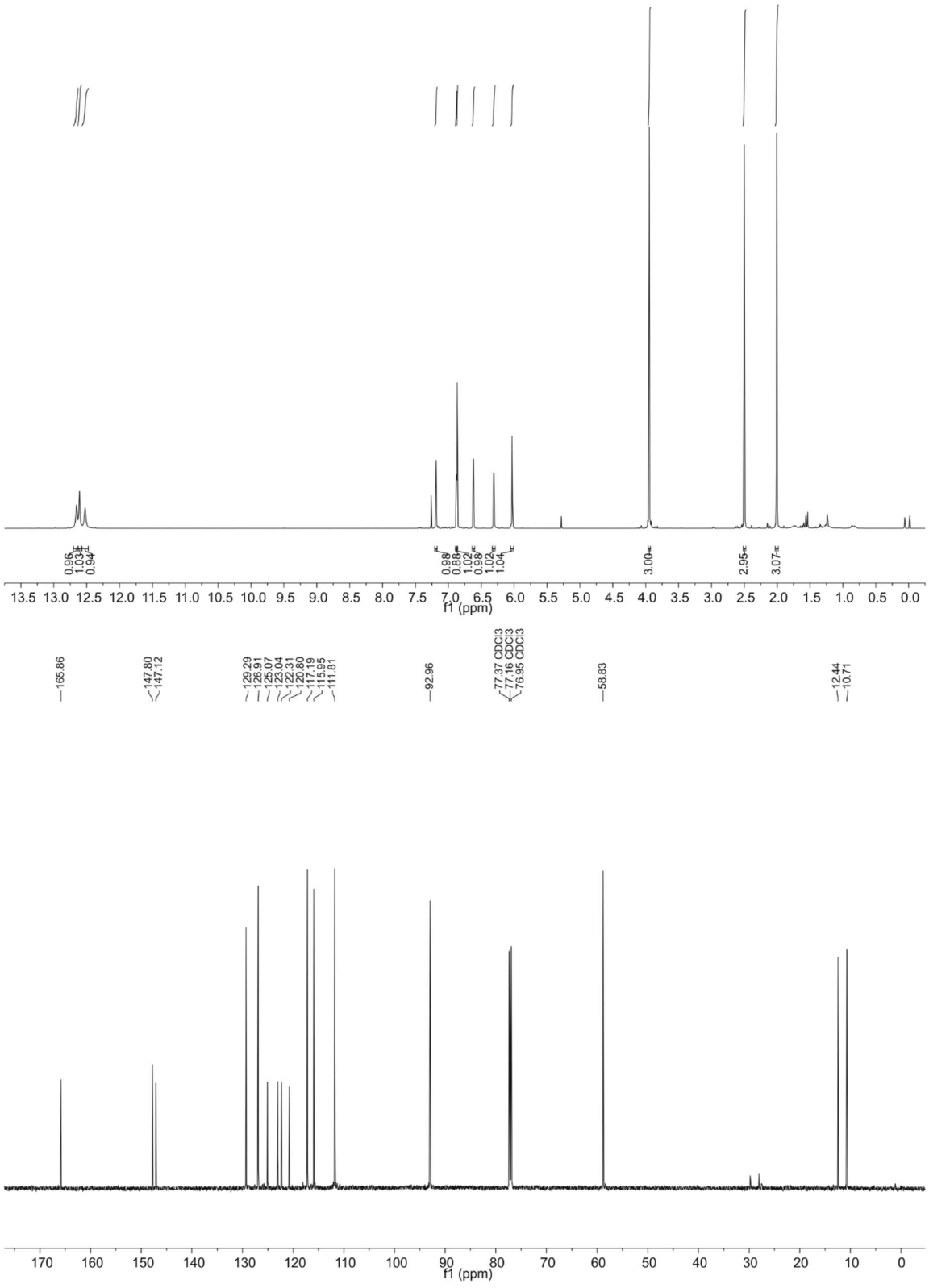


Figure S3. ^{1}H- and ^{13}C-NMR-spectra of **1b** in $CDCl_3$ (600 MHz/151 MHz).

Chemicals

All chemicals were purchased from AppliChem (Darmstadt, Germany), Sigma-Aldrich (Hamburg, Germany), Carl Roth (Karlsruhe, Germany), or Merck (Darmstadt, Germany), if not stated otherwise.

Bacterial strains and culture conditions

Escherichia coli strains NEB5alpha (New England Biolabs, Ipswich, UK), DH5α and BL21(DE3) (applied for cloning and gene expression) and *Pseudomonas putida* KT2440[4] were cultivated on LB agar plates or under constant shaking (150 rpm) at 37 °C (30 °C for KT2440) in LB liquid medium (lysogeny broth by Luria/Miller; 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) or ZYM 5052 autoinduction media for PigC expression in BL21 (10 g/L yeast extract, 20 g/L tryptone, 0.5 g/L glucose, 2 g/L alpha-lactose, 30 g/L glycerol, 2.55 g/L Na_2HPO_4, 3.4 g/L KH_2PO_4, 2.68 g/L NH_4Cl, 0.71 g/L Na_2SO_4, 0.49 g/L $MgSO_4$ x 7 H_2O). In all selective culture media 50 µg/mL kanamycin sulfate was added. All strains were stored in tubes or 96-well masterplates as glycerol stocks with 25% glycerol (*w*/*v*) at -80 °C.

Molecular biology

Enzymes for DNA processing (restriction/ligation/amplification) and PCR-reagents were obtained from New England Biolabs, and salt-free oligonucleotides from Eurofins Genomics (Ebersberg, Germany). Nucloetides (dNTPs) were purchased from Fermentas, Waltham, US-Massachusetts). PfuS polymerase and PfuS buffer were produced in-house. PCR reactions were performed in 100 µL thin-wall tubes using a Mastercycler® nexus (Eppendorf, Hamburg, Germany). DNA amounts in plasmid samples were determined via NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific, Waltham, US-Massachusetts). Commercially available kits (Qiagen, Hilden and Macherey-Nagel, Düren, Germany) were applied for DNA isolation and purification. DNA sequencing was conducted at Microsynth Seqlab (Göttingen, Germany) or Eurofins Genomics (Ebersberg, Germany).

Generation of PigC epPCR libraries in *P. putida* KT2440

For expression of PigC wild type (UniProt ID Q5W252) and variants in *Pseudomonas putida* KT2440, a *P. putida* codon optimised *pigC* (*optpigC*) sequence based on the *pigC* sequence of *Serratia marcescens* W838 (DSM No. 12487) has been synthesised (Thermo Fisher Scientific, Waltham, US-Massachusetts) and integrated into the low-copy shuttle vector pVLT33[5] (Kan^R, *Ptac* promotor, *lacI*) by PLICing[6]. The following phosphorothioated primers were used for amplification of the pigC insert sequence and pVLT33 vector sequence (5'→3'): PLICing_optpigC_fwd G*A*A*T*T*C*G*A*G*C*T*C*G*G*AATGCCCGTTACGCCTG, PLICing_optpigC_rev G*T*C*G*A*C*T*C*T*A*G*A*G*G*TCAGCCGTCGGCAC, PLICing_pVLT33_fwd C*C*G*A*G*C*T*C*G*A*A*T*T*C*TGTTTCCTGTGTG, PLICing_pVLT33_rev C*C*T*C*T*A*G*A*G*T*C*G*A*C*CTGCAGGCATGC, (*) indicate phosphorothioate nucleotides. The PCR reaction for amplification of pVLT33 vector and *pigC* wild type insert DNA was set up with 5 U PfuS polymerase, 500 µM forward and reverse primer, 20 ng of linearised template DNA (vector template pVLT33: KpnI, insert template pVLT33::*optpigC*: HindIII), 200 nM of each dNTP and 0.8 M betaine, in a final volume of 50 µL in PfuS buffer (10 mM Tris-HCl pH 8.9, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100). The PCR temperature protocol for

pVLT33 vector amplification was i) 3 min 94 °C, ii) 25 cycles of 30 s 94 °C, 30 s 61 °C, and 5 min 72 °C, and iii) 10 min 72 °C, before iv) cooling to 4 °C for storage. For *optpigC* insert amplification, the annealing temperature was adjusted to 58 °C and the elongation time reduced to 3 min. Error-prone PCR (epPCR) reactions for mutagenesis of *pigC* insert DNA contained 5 U Taq polymerase, 500 µM forward and reverse primer, 20 ng pVLT33::*optpigC* template DNA, 200 nM of each dNTP and 50, 75 or 100 µM $MnCl_2$, respectively, in a final volume of 50 µL in Taq buffer (50 mM Tris-HCl pH 8.7, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 1.5 mM $MgCl_2$). For epPCR, the temperature protocol of *optpigC* insert amplification was used (see above). After PCR, the template DNA was digested by addition of 20 U of DpnI to the PCR mix and incubation at 37 °C for 2-3 h. PCR products were then purified using a PCR Purification Kit (Qiagen, Hilden, Germany).

For transformation, 50 µL of chemically competent NEB5alpha cells (New England Biolabs, Ipswich, US-Massachussetts) were transformed with 1 µL of the PLICing reaction (ca. 150 ng DNA) by heat shock following the supplier's instructions. Resulting epPCR library colonies were pooled by resuspension in LB, from which plasmids were isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany), and transferred to *P. putida* KT2440 by electroporation[7] to obtain colonies for PigC screening. KT2440 colonies for screening were grown on nitrocellulose filter membranes (Protran® nitrocellulose membrane circles - BA85, pore size 0.45 µm, diam. 82 mm, Whatman, Maidstone, UK) placed onto the agar surface of LB agar plates containing 0.5 mM IPTG and kanamycin.

Agar plate prescreening system

For identification of active PigC clones in KT2440 colonies of a PigC mutagenesis library, nitrocellulose membranes with KT2440 colonies of a PigC library on the surface were transferred onto a filter paper (Whatman 543, hardened ashless, diam. 90 mm, Maidstone, UK) soaked with 1 mL substrate solution (250 µM MBC (**2**)/diMe-pyrrole (**3b**), 5 mM ascorbic acid in selective LB medium with 0.5 mM IPTG). Colonies were incubated with substrates at room temperature for 10 min to yield clear distinction between red active PigC colonies and colourless inactive PigC colonies. After incubation, the nitrocellulose membrane was transferred back to the original agar plate to avoid desiccation. Only red and active colonies were picked for screening in 96-well plates.

96-well plate PigC screening assay

Single red coloured colonies of a PigC library in *P. putida* KT2440 were transferred directly from nitrocellulose membranes of the prescreening plates into 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) filled with 150 µL LB medium (50 µg/mL kanamycin) with sterile toothpicks. Each 96-well microtiter plate (MTP) contained replicas of empty vector as negative control (*P. putida* KT2440 pVLT33), wild type PigC as positive control (*P. putida* KT2440 pVLT33::*optpigC*), and LB medium without inoculum as sterility control. Cultivation was performed in an MTP shaker (Multitron II; Infors, Einsbach, Germany) at 30 °C, 900 rpm, and 70% humidity. All MTPs were sealed with sterile gas permeable membranes (AeraSeal™ film, Excel Scientific, Victorville, US-California). MTP overnight precultures (150 µL LB, 50 µg/mL kanamycin, 18 h) from single colonies were used for inoculation of MTP expression cultures (150 µL LB, 50 µg/mL kanamycin, 0.5 mM IPTG). After 6 h of expression (30 °C, 900 rpm, 70% humidity), a

final concentration of 5 mM ascorbic acid (sterile filtered 0.5 M stock solution), and 100 µM of both pyrrolic PigC substrates [4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC, **2**) and the respective monopyrrole; stock solution of 5 mM in DMSO] were added to all wells. For substrate acceptance profiles, 50 µM of substrates **3a**, **3b**, **3c** and **3d** (instead of 100 µM) were added to biological triplicates in MTP cultures, respectively. After overnight incubation (16 h), cells and insoluble hydrophobic prodiginines were harvested by centrifugation (15 min, 3220×g, 4 °C), the media supernatant was decanted, and the pellets were resuspended in 150 µL acidified ethanol (4% (*v*/*v*) 1 N HCl) by pipetting for extraction of prodiginines. After removal of cell debris (15 min, 3220×g, 4 °C), 100 µL ethanolic supernatant per well were transferred to a flat bottom MTP. Finally, the absorbance at 536 nm (absorbance maximum of prodiginine UV/Vis spectrum) was measured in a photometric plate reader (Infinite M200 Pro, Tecan, Männedorf, Switzerland).

Expression and purification of PigC wild type and variants

For purification and kinetic assessment, PigC and variants have been expressed in *E. coli* BL21 (DE3). DNA sequences of PigC wild type and variants have been cloned in pET28a(+) applying homologous recombination[8]. Insert and vector backbone were amplified using the following primers: HR_opt pigC_fwd CGCGGCAGCCACATATGAACCCGACCCTGG; HR_opt pigC_rev GGTGGTGGTGCCTCGAGTCAGCCGTCGGCACG; HR_pET28a_fwd CGTGCCGACGGCTGACTCGAGGCACCACCACC; HR_pET28a_rev CCAGGGTCGGGTTCATATGTGGCTGCCGCG and PCR conditions: 5 U PfuS polymerase, 500 µM forward and reverse primer, 20 ng of template DNA, 200 nM of each dNTP and 0.8 M betaine, in a final volume of 50 µL in PfuS buffer (10 mM Tris-HCl pH 8.9, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100). The PCR protocol for pVLT33 vector amplification was i) 3 min 94 °C, ii) 25 cycles of 30 s 94 °C, 30 s 56 °C, and 3 min 72 °C, and iii) 1 min 72 °C, before iv) cooling to 4 °C for storage. Insert and vector fragments were mixed in a molar ratio of 2:1, and chemically competent *E. coli* BL21(DE3) cells were transformed by heat shock (45 s, 42 °C) with 100-200 ng of insert/vector mixture. For cell recovery, SOC media was added up to 1 mL, and cells incubated for 1 h at 37 °C and 250 rpm, before being transferred to selective LB agar plates. Plasmids from candidate clones were tested by *Eco*RI restriction (10 U *Eco*RI, 400-500 ng plasmid, 1x NEB CutSmart buffer, in a total volume of 10 µL for 2 h at 37 °C).

PigC wild type and variants were expressed in 200 mL ZYM 5052 autoinduction media in 1 L flasks (2 h at 37 °C and 250 rpm, 16-18 h at 18 °C and 250 rpm). For PigC characterization, the BL21 membrane fraction containing PigC has been purified by ultracentrifugation as published before with slight modifications[3]: Expression cultures were harvested (15 min, 4000 xg, 4 °C), and 4 g wet cell weight resuspended in 20 mL 100 mM Tris/HCl (pH 7.5). Cells were lysed by sonication (5 min, 30 s/30 s pulse/pause, 40% amplitude) and cell debris pelleted (15 min, 18,500 xg, 4 °C). After ultracentrifugation of the cell-free lysate supernatant (60 min, 100,000 xg, 4 °C), the membrane fraction pellet containing PigC was resuspended in 3.5 mL ice cold 100 mM Tris/HCl (pH 7.5), and used for kinetic characterization.

Kinetic characterization of PigC variants

For kinetic characterization, a 96-well plate based assay was applied[3,9]. The assay solution in each well contained 100 mM Tris/HCl buffer (pH 7.5), 200 µM ATPxNa$_2$, 0-200 µM MBC (**2**), 0-200 µM pyrrole **3b**/**3e**, 5% (*v*/*v*) DMSO as cosolvent and 50 µL resuspended membrane fraction (**3b**: 1:3 dilution, **3e**: 1:2 dilution in 100 mM Tris/HCl). The reaction was started by addition of the diluted membrane fraction. The absorbance at 536 nm was immediately measured as blank (CLARIOstar Plus Microplate Reader, BMG Labtech, Ortenberg, Germany) and then every 30 s for 30 min at 30 °C. From raw absorbance data at 536 nm, prodiginine concentrations were calculated by means of the standard curves of the respective prodiginines **1b** and **1c** in assay buffer (Figure S5a and b). Initial reaction rates [µM/min] were plotted against time, and kinetic parameters K_M [µM] and v_{max} [µM/min] determined by linear regression. For determination of specific activities [U/mg$_{PigC}$; U = µmol$_{substrate}$/(min^{-1}*mg$_{PigC}^{-1}$)], PigC concentrations in the membrane fractions were taken into account. Data evaluation was performed with the software OriginPro 9.1.0 (OriginLab, Northampton, MA, USA) and non-linear regression by using the standard Michaelis-Menten equation and Levenberg-Marquardt iteration algorithm. For substrate **3e**, an adapted fit function for Michaelis-Menten kinetics with substrate inhibition was applied[3]:

$$v = \frac{v_{max}[S]}{K_M + [S](1 + \frac{[S]}{K_i})}$$

Equation 1: Fit function for Michaelis Menten kinetics with substrate excess inhibition (substrate **3e**)

DNA Sequence of codon optimized *pigC* for *Pseudomonas putida*

>opt pigC (wild type)

ATGAACCCGACCCTGGTGGTGGAACTGAGCGGTGACAAGACCCTGGAACCGCACCGCCTGGGTGGCAAGGCCCACAGC
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CGTGCCGACGGCTGA

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GCGACCCAACTGATCAACTCGGGTGACACCCTGATCCTGGACGGCGACAGCGGCACCGTCATCATCCAACGTGGCGAG
CGTGCCGACGGCTGA

Determination of assay standard deviation (CV%)

The assay coefficient of variance (CV% = [standard deviation ÷ (mean) × 100%]) was determined for substrates **3d–g** additional to pyrrole substrate diMe-pyrrole (**3b**) in one 96-well plate each with 95 clones of wild type PigC and one well of sterility control. To quantify the substrate-specific background absorbance, one 96-well plate of empty vector (pVLT33) clones was screened with the corresponding substrates **3d–g** and MBC under the same assay conditions. Figure S4 shows the apparent (raw absorbance data) and true (raw data subtracted by the average empty vector absorbance background plus CV% [empty vector, n=95]) for substrates **3d–g**, which are also summarised in Table S1.

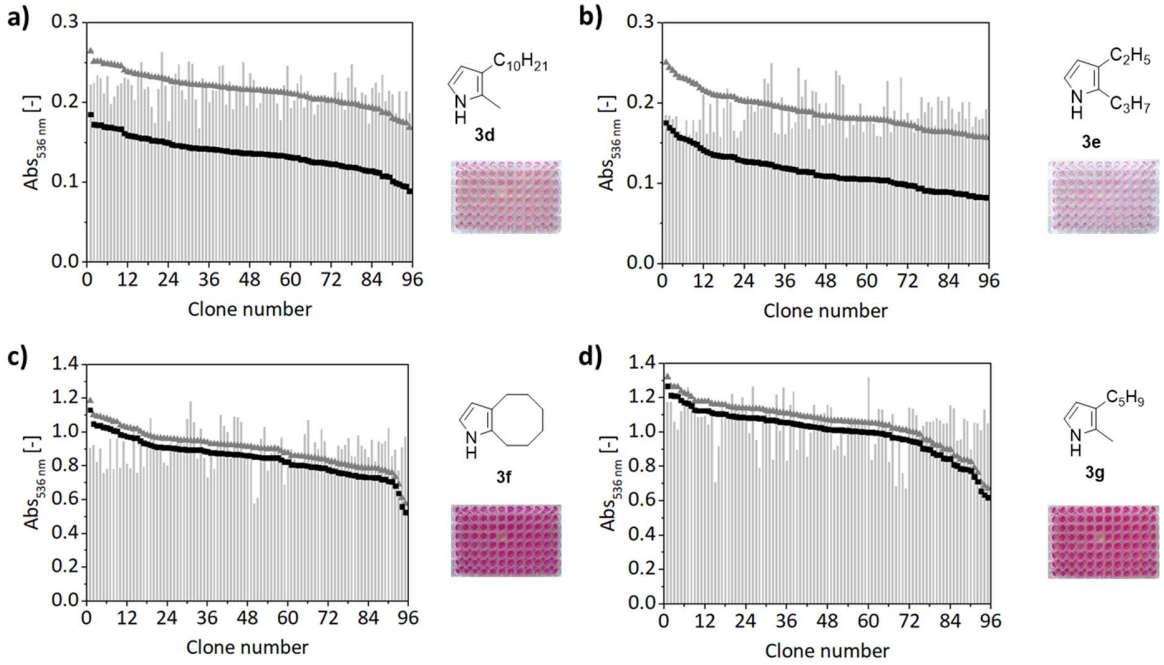

Figure S4: Standard deviations of the ProdEvlolve 96-well assay with pyrroles **3d–g** and MBC (**2**) [100 µM]. Apparent (▲) and true (▪) absorbance data after empty vector background subtraction **a)** 3-Decyl-2-methyl-1*H*-pyrrole (**3d**). **b)** 3-Ethyl-2-propyl-1*H*-pyrrole (**3e**). **c)** 4,5,6,7,8,9-hexahydro-1*H*-cycloocta[*b*]pyrrole (**3f**). **d)** 2-Methyl-3-pentenyl-1*H*-pyrrole (**3g**). The grey columns in the background show absorbance values sorted by position in the 96-well plate (A1-H12). The pictures show the pink colour intensities of the ethanolic extracts in 96-well plates, which correspond to the absorbance at 536 nm.

Table S1: Standard deviations of the ProdEvolve 96-well plate assay with substrates **3b**, **3d-g**.

Monopyrrole substrate	**$Abs_{563\ nm}$ [-]** (raw data)	**CV% [apparent]** (raw data)	**$Abs_{563\ nm}$ [-] [true]** (background subtraction)	**CV% [true]** (background subtraction)
3b	0.73 ± 0.07	9.7%	0.68 ± 0.07	10.5%
3d	0.22 ± 0.02	9.2%	0.07 ± 0.01	14.5%
3e	0.19 ± 0.02	12.5%	0.11 ± 0.02	19.4%
3f	0.90 ± 0.11	11.7%	0.85 ± 0.11	12.4%
3g	1.05 ± 0.13	12.2%	1.00 ± 0.11	12.9%

Standard curve of prodiginines **1b** and **1c** in buffer

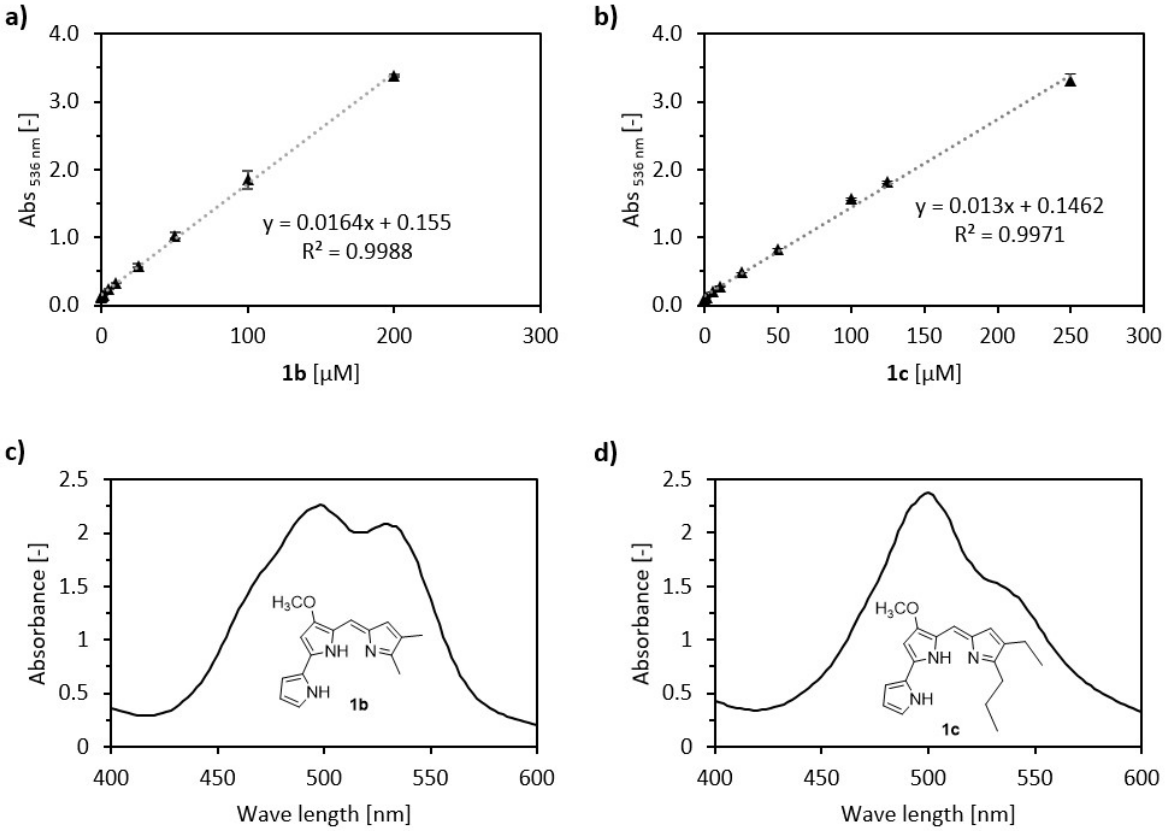

Figure S5: **a)** Linear detection range of diMe-prodiginine (**1b**) and **b)** prodiginine **1c** at 536 nm in 100 mM Tris/HCl buffer [pH 7.5, 200 µM $ATPxNa_2$, 5% (*v*/*v*) DMSO, and 25% (*v*/*v*) *E. coli* BL21(DE3) membrane fraction] in a 96-well plate in 200 µL volume. Linearity was achieved between 1 and 200 µM. **c)** UV/Vis absorbance spectrum of 100 µM diMe-prodiginine (**1b**) and **d)** prodiginine **1c** in 100 mM Tris/HCl buffer at pH 7.5. The absorbance was measured in a CLARIOstar Plus Microplate Reader.

Determination of PigC concentration in *E. coli* BL21(DE3) membrane fractions

Samples of isolated *E. coli* BL21(DE3) membrane fraction were prepared with an Experion Pro260 Analysis Kit (Bio-Rad Laboratories, Inc., Hercules, US-California) following the supplier's instructions. The PigC concentration was determined in an Experion Automated Electrophoresis System. PigC concentrations were finally calculated from the fluorescence area of the respective PigC peak in the electropherogram divided by the fluorescence area of an internal standard (250 ng/µL bovine serum albumin (BSA), 66.5 kDa) that was added to every sample. Figure S5 shows an electropherogram (a) for determination of PigC concentration in *E. coli* BL21(DE3) membrane fraction and the calculated PigC concentrations (b).

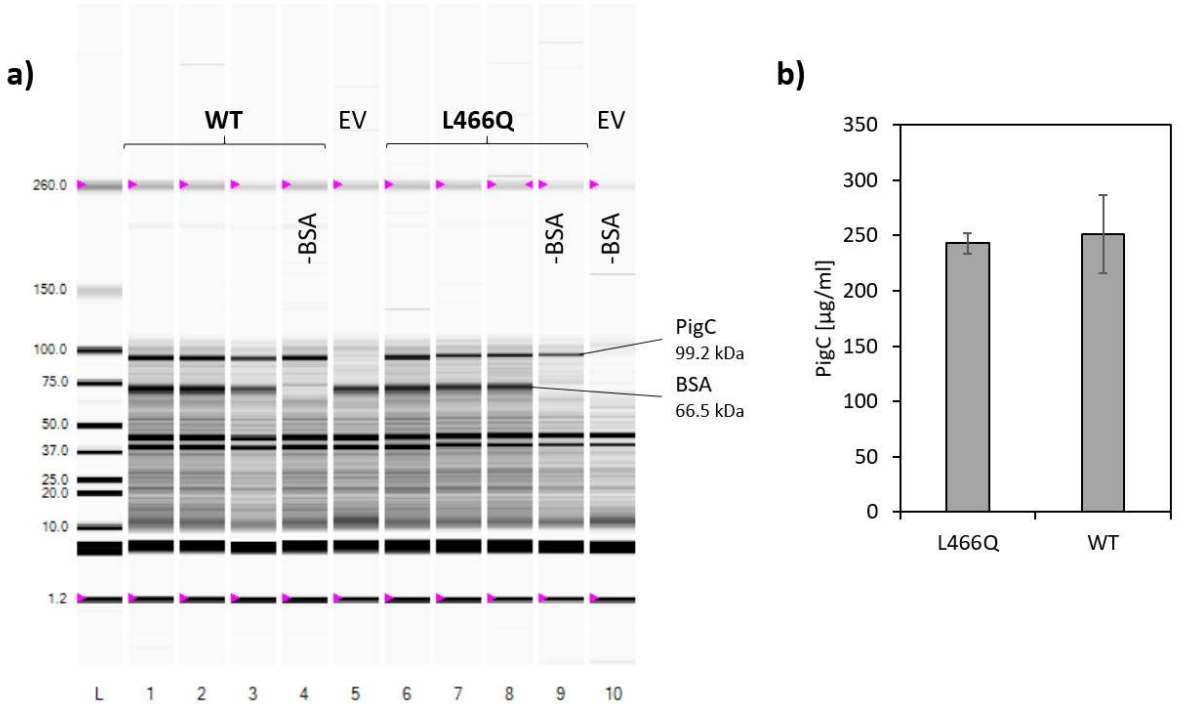

Figure S6: PigC concentration in *E. coli* BL21(DE3) undiluted membrane fraction. **a)** Electropherogram of an automated gel electrophoresis. **Lane 1–3** PigC wild type (WT) membrane fraction with internal BSA standard (66.5 kDa, 250 ng/µL), **Lane 4** PigC wild type membrane fraction control without internal BSA standard, **Lane 5** Empty vector (EV) membrane fraction, **Lane 6–8** Variant L466Q membrane fraction with internal BSA standard (250 ng/µL), **Lane 9** Variant L466Q membrane fraction without internal BSA standard, **Lane 10** Empty vector [EV, pET28a(+)] membrane fraction with internal BSA standard. The pink triangles point out the system upper (250 kDa) and lower (1.2 kDa) markers. **B)** PigC concentration in diluted membrane fractions of *E. coli* BL21(DE3) containing PigC variant L466Q and wild type, which were taken for kinetic characterization.

Kinetic characterization of L466Q and PigC wild type with substrate **3e**

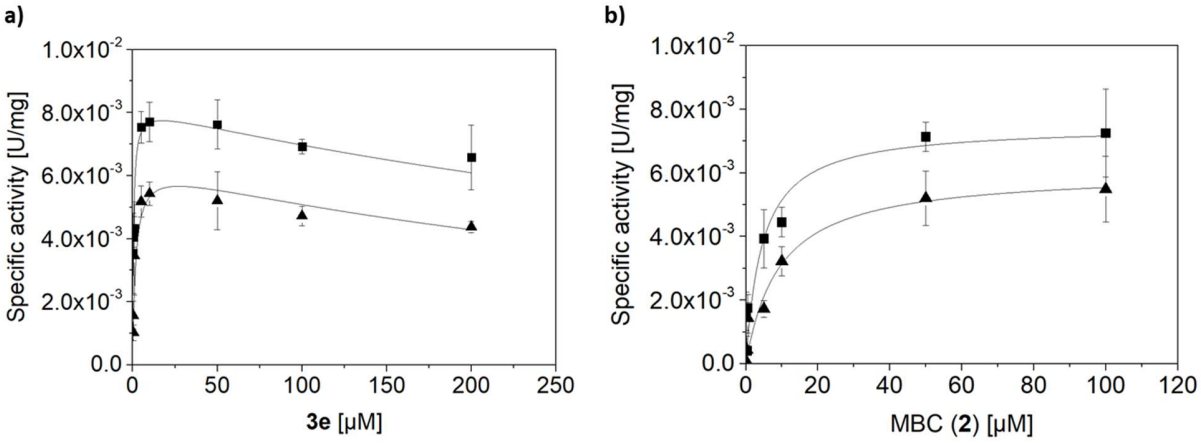

Figure S7: Michaelis-Menten plot (substrate excess inhibition, fitted with equation S1) of PigC wild type (▲) and variant L466Q (▪) in diluted *E. coli* BL21(DE3) membrane fraction: specific activity [U/mg_{PigC}] with 0–200 µM pyrrole **3e** (left) / 0–200 µM MBC (**2**, right).

Table S2: Kinetic parameters of PigC wild type (WT) and variant L466Q in *E. coli* BL21(DE3) membrane fraction.

PigC	K_M (3e) [µM]	K_M (MBC, 2) [µM]	k_{cat} [min^{-1}]	k_{cat}/K_M (3e) [$L/(mmol^{-1}*s^{-1})$]	K_i [µM]
WT	1.1 ± 0.3	8.1 ± 3.0	0.60 ± 0.14	9.5 ± 2.3	436.3 ± 230.5
L466Q	0.4 ± 0.1	4.7 ± 1.3	0.77 ± 0.08	29.6 ± 3.2	975.1 ± 838.5

PigC half-life time at 30 °C

For evaluation of PigC stability, the half-life time at 30 °C (decrease in initial activity) of PigC wild type and variant L466Q in *E. coli* BL21(DE3) membrane fraction has been determined. For this, 1 mL aliquots of PigC membrane fraction (1:3 dilution in 100 mM Tris/HCl, pH 7.5) have been incubated in a 1.5 mL reaction tube at 30 °C and 600 rpm for 1 h. The initial PigC activity has been determined first immediately after isolation of the membrane fraction (t_0), then every 5–10 min (Figure S8).

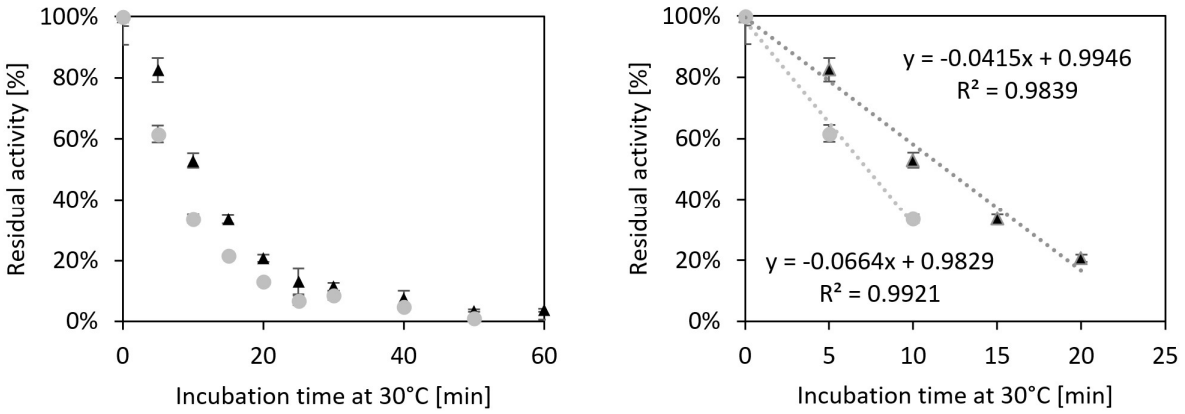

Figure S8: Half-life time of PigC wild type (circles) and variant L466Q (triangles) at 30 °C. By linear regression, the half-life time (incubation time reducing PigC residual activity by 50%) was determined (6.6 min for wild type, 11.2 min for L466Q).

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