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Article

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New Prodigiosin Derivatives obtained by Mutasynthesis in *Pseudomonas putida*

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Supporting information for this article is given via a link at the end of the document.

ABSTRACT

The deeply red-colored natural compound prodigiosin is a representative of the prodiginine alkaloid family, which possesses bioactivities as antimicrobial, antitumor and antimalarial agents. Various bacteria including the opportunistic human pathogen *Serratia marcescens* and different members of the *Streptomycetaceae* and *Pseudoalteromonadaceae* produce prodiginines. In addition, these microbes generally accumulate many structurally related alkaloids making efficient prodiginine synthesis and purification difficult and expensive. Furthermore, it is known that structurally different natural prodiginine variants display differential bioactivities. In the herein described mutasynthesis approach, 13 different derivatives of prodigiosin were obtained utilizing the GRAS (generally recognized as safe) classified strain *Pseudomonas putida* KT2440. Genetic engineering of the prodigiosin pathway together with incorporation of synthetic intermediates thus resulted in the formation of a so far unprecedented structural diversity of new prodiginine derivatives in *P. putida*. Furthermore, the formed products allow reliable conclusions regarding the substrate specificity of PigC, the final condensing enzyme in the prodigiosin biosynthesis pathway of *S. marcescens*. The biological activity of prodigiosin towards modulation of autophagy was preserved in prodiginine derivatives. One prodiginine derivative displayed more potent autophagy inhibitory activity than the parent compound or the synthetic clinical candidate obatoclax.

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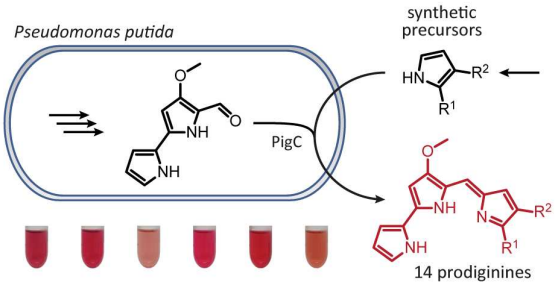
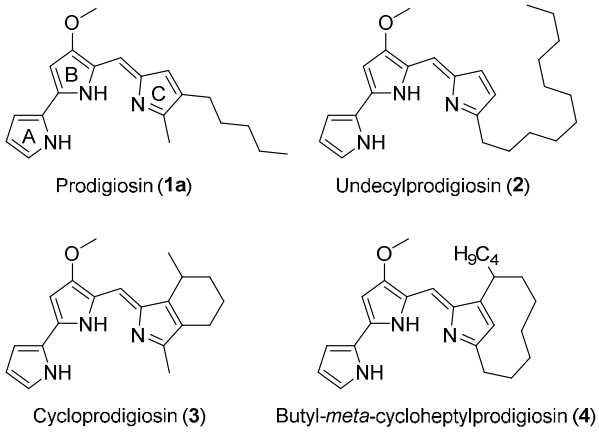


Keywords
Antibiotics, antitumor agents, metabolic engineering, mutasynthesis, natural product production, autophagy activator, prodiginine

INTRODUCTION

Natural products are a precious source for the development of new drugs. Prodiginines are a class of red-colored microbial pigments, which exhibit several important pharmacological properties including antitumor, antimalarial and antimicrobial activities against pathogens such as *Salmonella typhi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[1-3] Furthermore, immunosuppressive and antifungal activities have been demonstrated for prodiginines.[4] Prodigiosin, as well as the synthetic derivative obatoclax mesylate (GX15-070), have been shown to modulate autophagy.[5] Autophagy is a cellular recycling process which is used by cells to dispose of cellular toxic waste such as aggregated proteins or damaged organelles as well as to survive during starvation periods.[6] Modulation of autophagy is discussed as a promising therapeutic approach for many diseases like neurodegenerative disorders, metabolic disease or cancer. Autophagy is a highly dynamic process. The hallmark of autophagy is the generation of autophagosomes, small vesicles that receive input from the endocytic pathway. In the last step of autophagy, autophagosomes fuse with lysosomes thus enabling the degradation of cargo and recycling of nutrients. Weakly basic amine-containing drugs like prodigiosin have been shown to be sequestrated into lysosomes and induce their alkalization.[7] Thereby, the compounds interfere with autophagy by blocking the last step in the recycling process.

In the main, prodiginines are naturally produced from amino acids and acetate building blocks by *Serratia*, *Hahella*, *Vibrio*, *Pseudoalteromonas*, and *Streptomyces* species.[8, 9] Contrary to the large number of natural producers, there is only a minor chemical diversity in the presently discovered prodiginines. They can be generally classified into two groups, namely compounds with *n*-alkyl residues like prodigiosin (**1a**) or undecylprodigiosin (**2**) and cyclized derivatives like cycloprodigiosin (**3**) and butyl-*meta*-cycloheptylprodigiosin (**4**) (Scheme 1).[1] Total chemical syntheses of various prodiginines and derivatives thereof have been reported, but they are typically associated with high synthetic cost and low overall yields of multistep synthetic routes.[10] Therefore, attention has been drawn to exploitation of prodiginine biosynthesis. All prodiginines are biologically synthesized in a bifurcated pathway producing the bipyrrole 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC, **5**; A+B-ring) and a monopyrrole (e.g. 2-methyl-3-amylpyrrole, MAP, **6a**; C-ring).[8, 11] These two intermediates are finally condensed to the tripyrrolic natural compound (compare Scheme 1). Cyclic variants are then generated from alkyl residue prodiginines by a subsequent oxidative cyclization catalyzed by non heme iron oxidases.[12]



Scheme 1. Examples of natural prodiginine derivatives biosynthetically produced in *Serratia*, *Hahella*, *Vibrio*, *Pseudoalteromonas* and *Streptomyces* species.

The MBC biosynthesis is highly conserved in all producing species, whereas the pathways of the monopyrrole intermediates are different in terms of both, substitution pattern and functional

moiety. Hence, the monopyrroles are responsible for the natural diversity of prodiginines. Accordingly, previous studies reported flexible substrate recognition of the condensing enzyme PigC derived from the prodigiosin biosynthetic pathway within *Serratia marcescens* towards analogs of MAP (**6a**) as well as MBC (**5**). For example, MAP could successfully be replaced by 2,4-dimethylpyrrole (**7**) and 2,4-dimethyl-3-ethylpyrrole (**8**), whereas also derivatives of MBC altered in the aromatic A-ring were widely accepted by PigC [11, 13, 14] These results highlight the possibility of using PigC for mutasynthesis approaches to gain access to new prodigiosin (**1a**) derivatives. Therefore, we envisioned that a combination of chemical synthesis of MAP (**6a**) derivatives and their subsequent biocatalytic conversion would constitute a highly appealing strategy to create diversity in the C-ring of prodiginines and to determine the substrate specificity of PigC.

Mutasynthesis comprises the feeding of a precursor analog to a mutant strain, which carries a block in the biosynthesis towards an early key intermediate.[15, 16] The artificial precursor (mutasynthon) thus can be fed into the recombinant pathway thereby generating (non-natural) derivatives. The use of a mutasynthesis approach, commonly in homologous hosts, has been successfully reported for instance for the generation of ansamitocin, rapamycin, vancomycin, and platensimycin analogs.[17-22] Previously, we have demonstrated an effective heterologous prodigiosin production in the GRAS-certified strain *P. putida* KT2440 by expression of the *S. marcescens pig* gene cluster (*P. putida* pig-r2) and the straightforward product recovery from culture broth using polyurethane (PU) foam cubes as an adsorbent for prodiginines.[23] Consequently, a mutasynthesis approach encompassing a MAP (**6a**) deficient, but still MBC (**5**) producing mutant of this safe recombinant production strain presents a fast and cost efficient alternative to chemical synthesis of prodigiosin (**1a**) derivatives. Furthermore, the approach offers a potential model system to characterize the substrate spectra of condensing enzyme PigC and the production of new and more complex prodiginines with altered bioactivities.

RESULTS AND DISCUSSION

Precursor acceptance of prodigiosin production strain

As a pretest for our mutasynthetic endeavors, the import into the cells and the acceptance of precursors by PigC was first proven in a precursor-directed biosynthesis. Therefore, both, the natural precursor MAP (**6a**) and derived pyrroles **6e** as well as **6f** were synthesized via a *Trofimov* reaction (for details see Supporting Information S6) and fed to the prodigiosin (**1a**) producing strain *P. putida* pig-r2.[23-25] Supplementation of MAP (**6a**) in ethanol resulted in a 52% higher production of prodigiosin, as measured by means of photometric absorption (Figure S2), whereas the MAP derivatives lead to a mixture of the natural (**1a**) and unnatural product (**1e** or **1f**), respectively (for LC-MS traces see Figure S27 and S28). The results indicate that pyrrole precursors are imported and the condensing enzyme PigC accepts minor changes of the precursor structure.

Construction of knockout strain and development of mutasynthesis

Next, we aimed at establishing an effective mutasynthesis setup. To this end, we constructed a mutant strain (*P. putida* pig-r2 Δ*pigD*) blocked in the very first step of the monopyrrole biosynthesis (Figure 1A and B). Therefore, the mutant strain is unable to produce the monopyrrole MAP (**6a**), while the pathway of MBC (**5**) and the condensing enzyme PigC remain unaffected. In contrast to *P. putida* pig-r2, which formed deeply red cultures, the phenotype of *P. putida* pig-r2 Δ*pigD* remained colorless (Figure 1C) and and cells accumulated the intermediate MBC (**5**) as demonstrated by HPLC analysis (Figure S1). The biosynthesis of prodigiosin (**1a**) could be restored by feeding of exogenous MAP (**6a**) to this MAP-deficient mutant (Figure 1C). Using the natural precursor **6a**, the optimal reaction conditions for the incorporation of supplemented precursors into the prodiginine were investigated concerning the co-solvent (DMSO) and the effective precursor concentration. A final DMSO concentration of 2% (v/v), which proved to be nontoxic, and a precursor concentration of 1.25 mM (analytical scale) and 0.5 mM (preparative scale) were utilized for further mutasynthesis approaches, respectively (Figure S29 and S30).

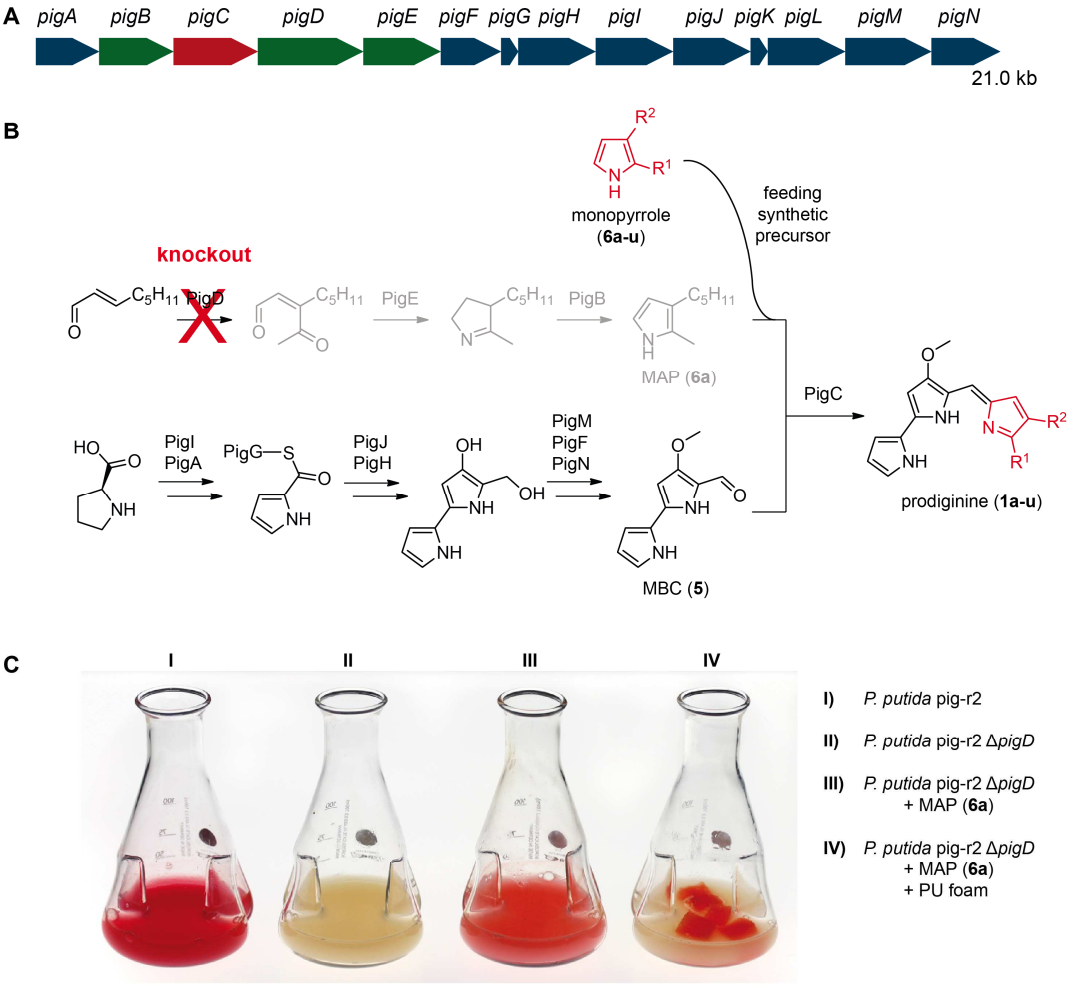
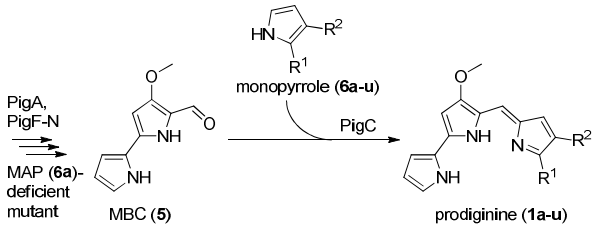


Figure 1. Strategy for the mutasynthesis approach. **A)** Prodigiosin *pig* gene cluster of *S. marcescens* (blue: genes for MBC (**5**) biosynthesis; green: genes for MAP (**6a**) biosynthesis; red: condensing enzyme). **B)** *P. putida* pig-r2 Δ*pigD* strain is blocked in the very first step of the MAP (**6a**) biosynthesis by knockout of *pigD*. Different monopyrrole derivatives (**6a-u**) were supplemented to this knockout strain. **C)** Phenotypes of the prodigiosin (**1a**) producing strain *P. putida* pig-r2 (I) and the MAP (**6a**) deficient strain *P. putida* pig-r2 Δ*pigD* (II). The red phenotype could be re-established by feeding of exogenous MAP (**6a**) (III). The produced prodigiosin (**1a**) is adsorbed from culture broth by polyurethane (PU) foam cubes (IV).

In vivo exploration of substrate acceptance in mutasynthesis

The substrate acceptance range of *P. putida* pig-r2 Δ*pigD* and the condensing enzyme PigC was exploited by mutasynthesis *in vivo* with different synthetic precursor derivatives (Table 1) (for synthesis details see Supporting Information S6).

Table 1. Mutasynthesis with the MAP (**6a**)-deficient mutant *P. putida* pig-r2 Δ*pigD*.



entry	substrate	R^1	R^2	product
1	**6b**	methyl	H	**1b**
2	**6c**	methyl	methyl	**1c**
3	**6d**	methyl	*n*-propyl	**1d**
4	**6e**	methyl	*n*-butyl	**1e**
5	**6a**	methyl	*n*-pentyl	**1a**
6	**6f**	methyl	*n*-hexyl	**1f**
7	**6g**	methyl	*n*-octyl	**1g**
8	**6h**	methyl	*n*-decyl	**1h**
9	**6i**	methyl	*n*-dodecyl	**1i**
10	**6j**	H	ethyl	**1j**
11	**6k**	H	*n*-propyl	**1k**
12	**6l**	H	*n*-pentyl	**1l**
13	**6m**	H	*n*-hexyl	**1m**
14	**6n**	H	*n*-octyl	**1n**
15	**6o**	H	*n*-undecyl	**1o**
16	**6p**	ethyl	*n*-pentyl	**1p**
17	**6q**	*n*-butyl	*n*-propyl	**1q**
18	**6r**	*n*-pentyl	*n*-butyl	**1r**
19	**6s**	*n*-hexyl	*n*-pentyl	**1s**
20	**6t**	methyl	2-propenyl	**1t**
21	**6u**	methyl	4-pentenyl	**1u**

In the first series of mutasynthesis approaches, the influence of the alkyl chain length in 3-position of the pyrrole was investigated by feeding monopyrroles **6a–i** to the mutant strain *P. putida* pig-r2 Δ*pigD*. By analogy to the natural precursor MAP (**6a**), the methyl group in 2-position remained untouched. As shown in Figure 2, the mutasynthons could be incorporated by *P. putida* and were widely accepted by PigC resulting in the formation of seven red-colored prodiginines (**1a**, **1c–h,** for LC-MS traces see Supporting Information S11) including the natural prodigiosin. Among these, prodiginines **1c** and **1h**, where monopyrrole moieties differed most from the natural precursor MAP, could only be detected in traces by LC-MS and the extracts showed only minor coloration. Clearly, the results demonstrate that the substrate spectrum of PigC in mutasynthesis is limited by the alkyl chain length in 3-position of the monopyrrole precursor (R^2 in Table 1); however, the presence of an alkyl chain in this position is indispensable because addition of precursor **6b** did not result in prodiginine formation. Based on these results, a second series of mutasynthesis was intended to investigate the importance of the methyl group in 2-position (R^1 in Table 1) by using demethylated precursors **6j–o**. As shown in Figure 2, the acceptance of these monopyrroles by *P. putida* and PigC decreased drastically in comparison to **6a–i**. Prodiginine formation could only be detected after supplementation with mutasynthons **6l–n** (Figure 2, for LC-MS traces see Supporting Information S11), which are closely related to the naturally occurring precursor MAP (**6a**) in terms of 2-position chain length. These results clearly indicate the importance of a methyl group present at this position of the precursor. The combination of the results obtained from experimental series one and two led us to devise series three altering both, the 2- and 3-position. Here, elongation of the

essential methyl group, together with an alteration of the alkyl chain in 3-position, was investigated by feeding pyrroles **6p–s**.[26] Remarkably, in contrast to the flexibility in 3-position, an elongation of the alkyl chain length exceeding methyl in 2-position was almost impossible as only precursor **6p** was tolerated in mutasynthesis. The product of pyrrole **6q**, prodiginine **1q**, could again only be detected in traces by LC-MS. Finally, series 4 of experiments was conducted to further enhance the functionalization of prodiginines. Here, the terminal alkene-pyrroles **6t** and **6u** were fed as mutasynthons as a proof of concept for functional diversification and could successfully be incorporated in analogy to pyrroles **6d** and **6a**.

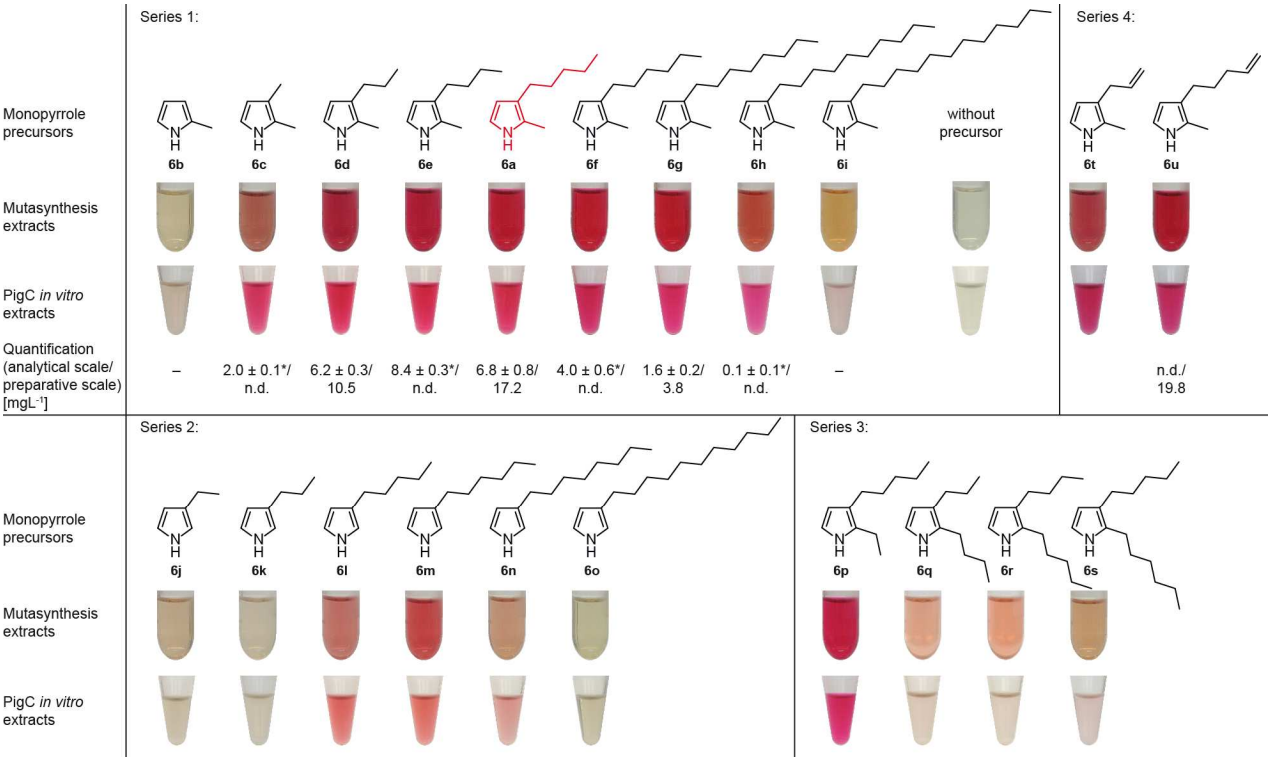


Figure 2. Mutasynthesis *in vivo* and *in vitro* of prodignines using differently substituted monopyrrole precursors. Ethanolic extracts are shown obtained from *in vivo* mutasynthesis experiments and *in vitro* biotransformations catalyzed by isolated PigC with monopyrroles **6a-u**. The structure of natural precursor MAP (**6a**) is highlighted in red. Four experimental series were carried out using monopyrrole precursors with different alkylation patterns in positions 2 and/or 3. [n.d.: not determined; *: quantification relies on predicted extinction coefficient (see Methods)]

In vitro substrate acceptance of condensation enzyme PigC

The mutasynthesis data shown in Figure 2 indicate that the length of the alkyl chains of the mutasynthons is important for their recognition by the condensing enzyme PigC. Additionally, we decided to develop a cell-free assay with isolated PigC where direct substrate access is granted to exclude putative physiological effects, e.g. caused by hampered cellular incorporation of particular precursors, and to further verify the substrate spectrum of the condensing enzyme. To this end, protein extracts were subjected to an appropriate *in vitro* assay using MBC with the substrates **6a-u**. As indicated by an overall matching pattern of *in vivo* and *in vitro* coloration (i.e. product formation) in Figure 2, the substrate spectrum of PigC is in accordance with the spectrum observed in mutasynthesis (for LC-MS traces see Supporting Information S13). Thus, the here observed boundaries in prodigiosin diversification during mutasynthesis are not due to a limitation of substrate access, but can be clearly correlated to the substrate acceptance of the condensing enzyme.

Preparative scale production of prodiginines in mutasynthesis

The mutasynthetic strategy was proven successful for most fed precursors in analytical scale. Furthermore, preparative scale (500 mL) reactions feeding the natural precursor MAP (**6a**) and the structurally related alkene-pyrrole precursor **6u** were performed to assess feasibility. In these approaches, the previously reported product recovery with PU foam was applied. The respective yields were 17.2 mgL^{-1} (53.2 µmol, 11%) of prodigiosin (**1a**) and 19.8 mgL^{-1} (61.6 µmol, 12%) of prodiginine **1u**. In comparison to these product titers, the strain *P. putida* pig-r2 is able to produce 48 mgL^{-1} (148.4 µmol) prodigiosin at the same cultivation conditions. The lower yields might be a consequence of decomposition of the fed precursors in aerated aqueous medium or reduced accumulation of the aldehyde intermediate MBC (**5**) in the knockout strain. In addition to these well-accepted precursors, mutasynthons **6d** and **6g** were also applied in preparative scale reactions to investigate the limitation in production regarding a shorter and a longer alkyl moiety in 3-position. The respective yields were 10.5 mgL^{-1} (35.5 µmol, 7%) of prodiginine **1d** and 3.8 mgL^{-1} (10.4 µmol, 2%) of prodiginine **1g**. Nevertheless, in contrast to a total synthesis, the mutasynthesis is able to provide an effective alternative method for the rapid production of prodiginines based on easily synthesizable precursors without the need of expensive catalysts or starting materials which are obligatory in the time consuming synthesis of MBC (**5**).

Biological evaluation of prodiginines

Several bioactivities, including the modulation of autophagy, are known for prodigiosin (**1a**) as well as obatoclax mesylate (GX15-070, **9**, Figure 3D), a synthetic derivative investigated in several phase II clinical trials.[27-30] To investigate the influence of the alkyl chain length in 3-position (C-ring) towards autophagy, the four prodiginines (**1a**, **1d**, **1g** and **1u**), produced in the preparative scale reactions, were tested for their ability to modulate eGFP-LC3 puncta, an autophagosomal marker. After 3 h incubation of eGFP-LC3-MCF7 cells with prodiginines, a clear accumulation of eGFP positive autophagosomes was detected (Figure 3A and B). Compound **1d** showed the most potent autophagosome accumulation with an EC_{50} of approximately 150 nM, which was about two-fold more potent than prodigiosin and obatoclax mesylate (for EC_{50} values of **1a**, **1d**, **1g**, **1u** and obatoclax mesylate see Table S3). On protein level, derivative **1d** caused the accumulation of lipidated LC3 (LC3-II) in relation to the Actin control, as expected for a compound that inhibits the autophagosome lysosome fusion (Figure 3C). This occurred to a much greater extent than with the known fusion inhibitor chloroquine (CQ) as verified under both fed and starved conditions. Inhibition of autophagic flux was also confirmed, as **1d** inhibited autophagic degradation of protein p62, a known autophagy substrate, after induction of autophagy through starvation.[31] Compound **1d** also caused growth inhibition in a dose-dependent manner, which resulted in cell death through induction of apoptosis (Figure 3E and F). Both were assessed using live-cell imaging of MCF7 cells incubated with a caspase 3/7 selective probe. It is interesting to note that while **1d** inhibited autophagy more potently than Obatoclax, the effect on the induction of apoptosis was less pronounced at equivalent concentrations (Figure 3F, 0.11 µM). This is presumably because Obatoclax has been optimized for inhibition of Bcl-2, an anti-apoptotic protein, and thus the pro-apoptotic activity is likely to have multiple origins.

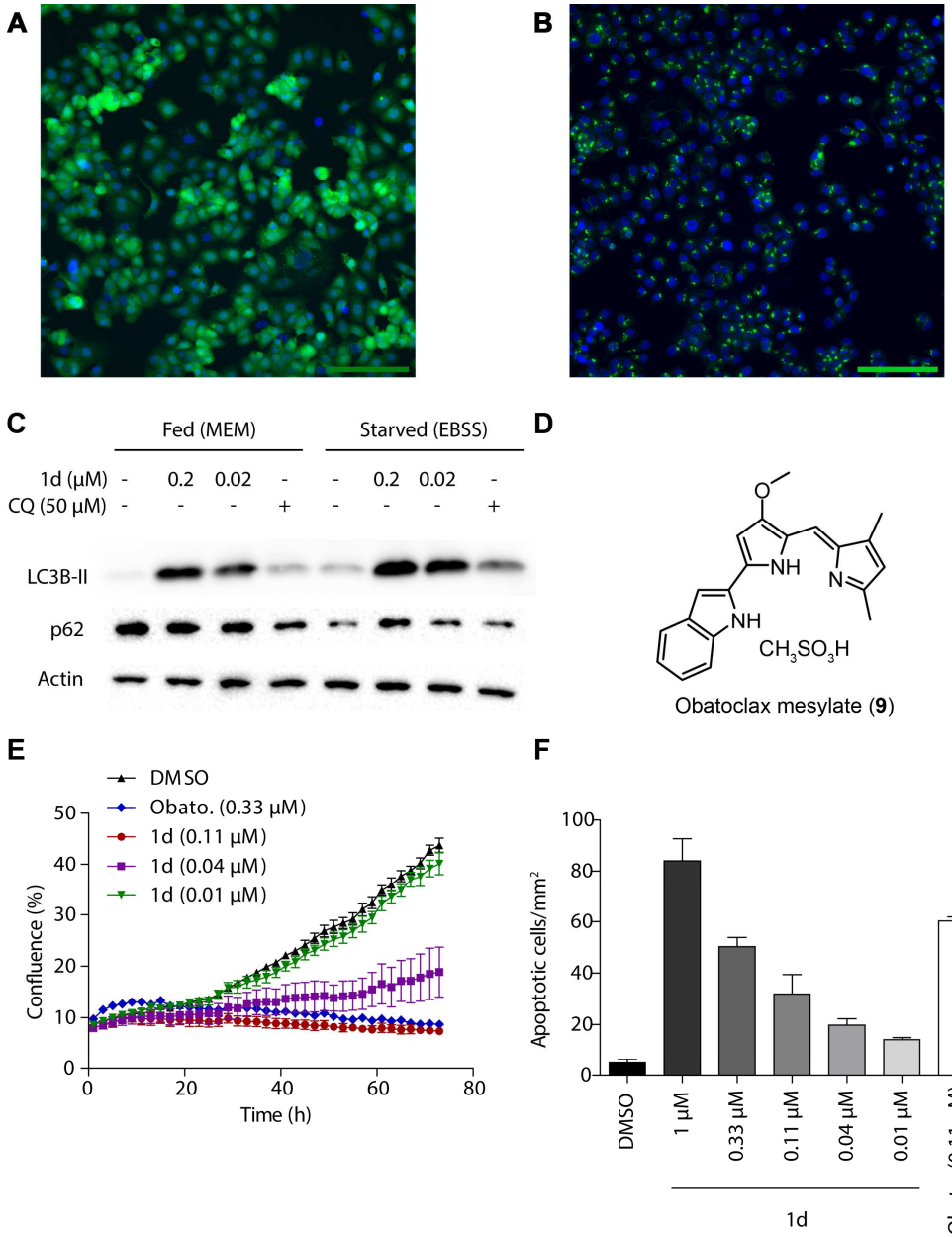


Figure 3. Accumulation of autophagosomes in eGFP-LC3-MCF7 cells upon treatment with prodiginine **1d**. Whereas DMSO treated cells (**A**) show a diffuse eGFP-LC3 staining, (**B**) 0.3 µM **1d** treatment for 3 h leads to accumulation of autophagosomes (green: GFP fluorescence; blue: nuclei detection with Hoechst; scale bar = 150 µM). **C)** Treatment of eGFP-LC3-MCF7 cells with **1d** for 3 h resulted in increased LC3-II levels and decreased p62 degradation after starvation-induced autophagy. Using DMSO as a negative and obatoclax mesylate (**9**, **D**, Obato.) as a positive control, the influence of treatment with different doses of compound **1d** on cell growth (measured as % confluence over 72 h) (**E**), and on occurrence of apoptosis (after 24 h of treatment) (**F**) were determined by live-cell imaging and automated image analysis using the Incucyte Zoom. Shown data represent mean values and respective standard deviations (n = 3).

In summary, we have successfully explored the potential of a mutasynthesis approach to create diverse prodiginines. Beside the natural product prodigiosin (**1a**), four known and nine new derivatives of this pharmacologically promising class of compounds could be obtained by feeding pyrrole precursors to an accordingly engineered *P. putida* KT2440 mutant strain. By alteration of the monopyrrole alkylation pattern, the precursor substrate spectrum of the condensing enzyme PigC was characterized for the first time and was proven in an *in vitro* biotransformation in addition to the

mutasynthesis. Although the here presented results indicate a restricted acceptance of pyrrole precursors by PigC, this study offers a potential model system for the application and investigation of other condensing enzymes in *P. putida*. The potential of the mutasynthesis approach was further demonstrated by applying preparative scale reactions in combination with product recovery during cell cultivation using a cost efficient PU foam-based procedure. This provided the possibility to access four prodiginines for testing their efficacy against autophagy in eGFP-LC3-MCF7 cells. All prodiginines caused accumulation of autophagosomes. Prodiginine **1d** was the most potent of the series and inhibited autophagic flux as well as cell growth, leading to cell death *via* apoptosis. Indicating promising perspectives, the acceptance of alkene-pyrrole precursors establishes the access to prodiginine building blocks for semisynthesis purposes and therefore offers high potential for the production of new biologically active compounds.

METHODS

Oligonucleotides used in this study are listed in Table S1.

Bacterial strains and culture conditions: *Escherichia coli* strains DH5α, S17-1 and BL21(DE3) (applied for cloning, conjugation and gene expression) were cultivated on LB-agar plates or under constant shaking (120 rpm) at 25–37 °C in LB (lysogeny broth) liquid medium (LB medium (Luria/Miller), Carl Roth®, Karlsruhe, Germany: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) or TB (terrific broth) liquid medium (Carl Roth®, Karlsruhe, Germany: 12 g/L casein, 24 g/L yeast extract, 12.54 g/L K_2HPO_4, 2,31 g/L KH_2PO_4, 4 mL/L glycerol).[32-34] Standard conditions for *Pseudomonas putida* prodigiosin production strains (*P. putida* pig-r2) and *P. putida* pig-r2 Δ*pigD* mutant strains were cultivation on LB-agar plates or in liquid LB or TB medium under constant shaking (120 rpm) at 28 °C. Cultivation media were prepared using distilled water and sterilized by autoclaving. Antibiotics were added to the culture medium to the following final concentrations [µg/mL]: *E. coli*: 50 (kanamycin, Kan); *P. putida*: 25 (gentamicin, Gm) 80 (streptomycin, Sm).

DNA manipulation equipment: Enzymes for DNA manipulation and PCR-reagents were obtained from *ThermoFisher Scientific*, *Walkham*, *USA*, and oligonucleotides from *Sigma-Aldrich*, *Steinheim*, *Germany*, and *Eurofins Genomics GmbH*, *Ebersberg*, *Germany*. PCR reactions were performed in 100 µL thin-wall tubes using a TProfessional Basic thermocycler (*Biometra, Göttingen, Germany)*. DNA amounts in plasmid samples were determined via a NanoDrop 2000c (*Thermo Fischer Scientific)* spectrophotometer. Commercially available kits were applied for DNA isolation and purification. DNA sequencing was conducted at *GATC Biotech*.

Construction of *P. putida* pig-r2 Δ*pigD* mutant strain

The MAP biosynthesis deficient *pigD* deletion mutant strain was constructed based on the constitutive prodigiosin production strain *P. putida* pig-r2 described in Domröse, Klein *et al*.[23] A part of *pigD* was exchanged for the streptomycin resistance gene *aadA* via homologous recombination using the vector pSUP202-pigCaadApigD.

The vector was generated in two steps: First, a 4.6 kb *Eco*72I fragment of vector pUC19-pig containing parts of *pigC* and *pigD* was ligated with *Sca*I hydrolyzed vector pSUP202 (6.5 kb), resulting in pSUP202-pigCD (11.1 kb).[23, 35] In the second step, a partial deletion in *pigD* was realized by *Mau*BI/*Asi*SI restriction hydrolysis, removing a 294 bp part of *pigD*, and subsequent ligation with a 0.9 kb *Mau*BI/*Asi*SI fragment of the promoterless *aadA* gene. The *aadA* gene was amplified by PCR from pHP45omega vector as DNA template using oligonucleotide primers #3 and #4. The PCR reaction contained 1 U Phusion® High-Fidelity DNA Polymerase, 100 ng plasmid, 200 µM of each dNTP and 200 nM forward and reverse primer in 50 µL High-Fidelity buffer. The applied temperature protocol was 30 s 98 °C, 35 cycles of 10 s 98 °C and 20 s 72 °C, a final step of 10 min at 72 °C and cooling to 10 °C. Correct vector assembly was verified by sequencing using primers #1, #2, #5 and #6.

The resulting mutagenesis vector pSUP202-pigCaadApigD was transformed into *E. coli* S17-1 and further transferred to *P. putida* pig-r2 via conjugation. The *pigC-aadA-pigE* cassette was designed to incorporate the promoterless *aadA* gene in the same orientation as *pig* genes instead of *pigD* in the *pig* gene cluster. As in *P. putida* pig-r2 expression of *pig* genes is constitutively driven by an intrinsic

promoter,[23] integration of the *aadA* gene at this locus should drive its expression likewise and confer corresponding resistance to streptomycin. Since the pSUP202 derivative represents a suicide vector in *P. putida*, clones with integrated *aadA* gene could be directly selected on LB_{Sm} agar plates. Potential streptomycin resistant *P. putida* pig-r2 Δ*pigD* mutant strains were first identified by lack of the red phenotype of pig-r2 due to disruption of prodigiosin biosynthesis, and secondly confirmed by colony PCR using different combinations of oligonucleotides. Primers #7 and #8 are located in the *aadA* gene while #9 binds upstream in *pigC* and #10 downstream in *pigE*, both outside the homologous regions. Thus PCR products of the pairs #7/#9 (1.4 kb) and #8/#10 (3 kb) confirm both the presence of *aadA* and its correct orientation.

Mutasynthesis experiments

Analytical scale: Reactions were performed in 96 x 2000 µL deep-well-plates (conical bottom, solvent resistant) using a final volume of 750 µL containing TB liquid medium, 1.25 mM pyrrole precursor and 2% (v/v) DMSO. In detail: The TB_{Sm} liquid medium was inoculated with an overnight culture of *P. putida* pig-r2 Δ*pigD* to an OD of 0.02. The pyrrole precursor was initially dissolved in DMSO and added to the culture medium to achieve the final concentration. The plates were sealed with AeraSeal™ breathable sealing films (*Excel Scientific, Victorville, USA*). The mutasynthesis proceeded at 25 °C and 1000 rpm for 24 h. The cells containing the prodiginines were harvested by centrifugation, the supernatant discarded and the cells extracted with 300 µL of acidified ethanol (4% (v/v) 1N HCl in ethanol). The extraction was supported using an ultrasonic bath for 5 min. Afterwards the cell debris was removed by centrifugation and extracts of three samples were combined. For further LC-MS analysis, ethanol was removed under reduced pressure, 200 µL water was added and the prodiginines extracted with dichloromethane (2 x 200 µL). Evaporation of dichloromethane and addition of methanol provided the desired crude extracts for LC-MS analysis.

Preparative scale: The reactions were performed either in a baffled 100 mL flask or 3 L *Fernbach* flask using a final volume of 20 mL or 500 mL TB liquid medium, 1.25 mM (for 20 mL scale) or 0.5 mM (for 500 mL scale) pyrrole precursor and 2% (v/v) DMSO. Polyurethane (PU) foam cubes (*Bornewasser, Göllheim, Germany*: Softpur, 25 kg/m^{3}density, 4 kPa compression hardness), each approximately 1 cm^3, were added as an adsorbent for the *in situ* recovery of prodiginines (275 mg or 5 g).[23] In detail: The TB_{Sm} liquid medium was inoculated with an overnight culture of *P. putida* pig-r2 Δ*pigD* to an OD of 0.02. The pyrrole precursor was initially dissolved in DMSO and added to the culture medium to achieve the final concentration. After the addition of the PU foam cubes, the tops of the shaking flasks were covered with aluminum foil and the mutasynthesis proceeded at 25 °C and 125 rpm for 24 h. The PU foam cubes of the 20 mL scale were wrung out and extracted with diethyl ether (10 mL) by washing in a beaker. The extraction of prodiginines from the PU foam cubes of the 500 mL cultures was performed with a *Soxhlet* extractor: The foam cubes were recovered from the cell culture by sieving, wrung out and charged into a 250 mL *Soxhlet* apparatus fitted with a reflux condenser and a 500 mL round bottom flask filled with 300 mL of diethyl ether. The sample was continuously extracted by heating with the chosen solvent. After extraction, the organic layer was washed with water, dried over $MgSO_4$ and the solvent was removed under reduced pressure providing the desired crude extracts for analysis.

Plasmid construction for *pigC* expression

The *pigC* gene was PCR amplified from pUC19-pig vector as DNA template using Gibson Assembly® primers #11 and #12. The PCR reaction contained 0.4 µL Phire Hot Start II DNA polymerase, ≈ 30 ng plasmid, 200 µM of each dNTP and 500 nM forward and reverse primer, 0.6 µL DMSO in 20 µL buffer. The applied temperature protocol was 30 sec at 98 °C, 35 cycles of 10 sec at 98 °C, 30 sec at 69.5 °C and 1 min at 72 °C, a final step of 10 min at 72 °C and cooling to 10 °C. Amplification was confirmed by agarose gel electrophoresis. The final vector was assembled via the Gibson Assembly® protocol by using the PCR product and a previously linearized pET28a(+) vector (digested with *Nde*I and *Xho*I).[36] The obtained vector is referred to as pPIGC. The vector was applied for transformation of *E. coli* DH5α cells by standard heat-shock procedure using chemically-competent *E. coli* DH5α cells.[37] Colonies of transformants grew overnight on LB_{Kan} agar-plates at 37 °C. The presence of the desired vector was tested by colony PCR and agarose gel electrophoresis which enabled clone selection for

cultivation in LB_{Kan} medium and plasmid isolation. The same conditions as mentioned before were applied for the *pigC* gene amplification in the colony PCR.

Expression of the *pigC* gene

For recombinant, heterologous expression of *pigC*, chemically competent cells of *E. coli* BL21(DE3) were transformed with the pPIGC vector and selected on LB_{Kan} agar-plates. In a baffled 3 L *Fernbach* flask, 500 mL TB_{Kan} medium was inoculated with 1% (v/v) of an overnight culture. The culture was incubated to an OD_{600} of about 0.6 at 37 °C (120 rpm), induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubated for additional 18 h at 25 °C (120 rpm). Cells were harvested by centrifugation at 4 °C and stored at -20 °C.

PigC *in vitro* assay

Frozen cells (1 g) were thawed by suspension in potassium phosphate buffer (KP_i buffer, 50 mM, pH 7.0) and disrupted using a *SONOPULS Ultrasonic* homogenizer (*Bandelin, Berlin, Germany*) for 2 times 5 min and 5 cycles. The cell debris was removed by centrifugation. The assay solution contained 440 µL cell-free supernatant, 25 µL of a pyrrole-precursor (**6a-u**) solution in DMSO (20 mM), 25 µL of MBC-precursor (**5**) solution in DMSO (20 mM) and 10 µL of an ATP·Na_2 solution in water (62.5 mM). The mixture was shaken in a 1.5 mL reaction tube at 300 rpm and 30 °C for 4 h. Afterwards, the supernatant was discarded after centrifugation and the produced prodiginine extracted by the addition of 300 µL of ethanol. Ethanolic extracts were separated by centrifugation, transferred to a new reaction tube and the solvent was removed under reduced pressure. The production of prodiginines was analyzed by LC-MS. To exclude a self-condensation of MAP (**6a**) and MBC (**5**), the assay was carried out without the addition of cell-free supernatant. Furthermore, a second negative control was performed without the addition of ATP.

Quantification of prodiginines

Prodiginines were quantified either by absorption in acidified ethanol (4% v/v of 1 N HCl) or by mass after column chromatography. For prodigiosin (**1a**), the previously reported molar extinction coefficient (ε_{535} = 139,800 ± 5,100 $M^{-1}cm^{-1}$) was used.[23] The molar extinction coefficients of prodiginines **1d** and **1g** were determined from the slope by plotting the absorption of the pigment versus the concentration at the absorption maximum (535 nm) in acidified ethanol (4% v/v of 1 N HCl): ε_{535}(**1d**) = 125,776 ± 6,450 $M^{-1}cm^{-1}$, ε_{535}(**1g**) = 189,904 ± 2,950 $M^{-1}cm^{-1}$. The extinction coefficients of other 2-methyl-3-alkyl-prodiginines were predicted by a linear fit after plotting the above mentioned extinction coefficients versus the chain length in 3-position (for details see Supporting Information S14).

Evaluation of biological activity of prodiginines

Stable (eGFP-LC3)-MCF7 breast cancer cells were kindly provided by Georgios Konstantinidis. Cells were cultured in MEM containing 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 0.01 mg/ml human insulin and 0.2 mg/ml G418. (eGFP-LC3)-MCF7 cells (4,000 cells/well) were seeded in 384 well plates (*Greiner Bio-One, Kremsmünster, Austria*). The next day, compounds were added using Echo dispenser (*Labcyte, Sunnyvale, USA*). After three hours incubation at 37 °C and 5% CO_2, cells were fixed by addition of 25 µL formaldehyde in phosphate buffered saline (PBS; 4.6% final concentration) and simultaneous nuclear staining was performed with 1:500 Hoechst (Stock 1 mg/mL) for 20 min at RT. Fixed cells were washed three times with 1x PBS using plate washer ELX405 (*BioTek, Winooski, USA*). For visualization, 4 pictures/well were acquired using ImageXpress Micro XL (*Molecular Devices, Sunnyvale, USA*) at 20x magnification and analyzed with the granularity algorithm of MetaXpress Software (*Molecular Devices*). Dose-response analysis was carried out starting from 10 µM using a three-fold dilution curve over eight steps. EC_{50} calculations were performed using Quattro Workflow software (*quattro research GmbH, Planegg, Germany*).

Antibodies

Anti-p62/SQSTM1 was purchased from MBL international (Cat# PM045, *Woburn, USA*) and used at 1:10,000. Anti-LC3B was obtained from Cell Signaling Technology (Cat# 2775, *Cambridge, UK*) and used at 1:1,000. Anti-beta-actin was purchased from abcam (Cat# ab8227, *Cambridge, UK*) and used at 1:10,000. Goat anti-rabbit-HRP was purchased from Pierce (*ThermoFisher Scientific*, *Walkham*, *USA*, cat# 31460) and used at a dilution of 1:10,000.

Western blotting

200,000 (eGFP-LC3)-MCF7 cells in 2 mL media were seeded in 6-well plates and incubated (37 °C, 5% CO_2) overnight. The media was removed and the cells were washed with 1x PBS, before adding test compounds at the required concentrations in EBSS (starvation medium) or MEM (full medium). Cells were incubated (37 °C, 5% CO_2) for 3 h before removing the media, washing with 1x PBS, and lysing in SDS loading buffer without bromophenol blue. Protein concentrations were determined using the DC Assay (*Bio-Rad* Laboratories*, Hercules, USA*) according to the manufacturer's instructions. SDS-PAGE was carried out using 15% polyacrylamide gels run at a constant voltage of 80 V for 15 min followed by 120 V for approximately 1.5 h. Semi-dry transfer onto a polyvinylidene difluoride (PVDF) membrane was performed at 25 V for 50 min. Membranes were blocked in 5% milk in Tris-buffered saline with 0.1% Tween20 (TBST) for 1 h at room temperature. The membrane was incubated with the primary antibody in blocking buffer overnight at 4 °C. After washing with TBST (3 x 5 min), the membrane was incubated with the secondary antibody in blocking buffer for one hour at room temperature. Signals were visualized using the SuperSignal West Pico Chemiluminescent Substrate or the SuperSignal West Femto Maximum Sensitivity Substrate (*ThermoFisher Scientific*, *Walkham*, *USA*) on a Li-COR Odyssey Fc.

Confluence and apoptosis measurement using IncuCyte™ Zoom

4,000 (eGFP-LC3)-MCF7 cells in 100 µL medium were seeded in a clear flat-bottomed 96-well plate and incubated overnight. The media was removed gently and replaced with 100 µL of MEM containing 5 µM IncuCyte™ Kinetic Caspase-3/7 Apoptosis Assay Reagent (Cat#4440). To this were added test compounds at a concentration of 6x the desired final concentration in 20 µL MEM. Cells were incubated at 37 °C for 72 h in the Incucyte Zoom. Images were acquired at 10x magnification in the phase and green channel every 2 h. Images were analysed using automated image analysis, where the key parameters were confluence (%) and Caspase 3/7 positive cells (cells/mm^2).

ASSOCIATED CONTENT

Supporting Information

Supplementary methods describe the chemical synthesis and characterization of molecules used in this study. Supporting Information contains additional tables and figures based on the descriptions provided in the text. This material is available free of charge on the ACS publication website http://pubs.acs.org.

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Author Contributions

J.P. and K.-E.J. developed the idea for the mutasynthesis approach. A.S.K., A.D., T.C., T.D., and A.L. designed the experiments. A.S.K. performed the chemical syntheses, the mutasynthesis (analytical scale), the *in vitro* biotransformation and analytics. A.S.K. and H.U.C.B. performed the mutasynthesis (preparative scale). A.D. and A.L. performed the construction of the knockout strain. P.B. performed the LC-MS analysis. L.L. and S.S. performed the physiological experiments and analysis. A.S.K., A.L., A.D., T.C., P.B., T.D., S.S., K.-E.J. and J.P. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

EBSS, Earle´s balanced salt solution (starvation medium); MAP, 2-methyl-3-amylpyrrole; MBC, 4-methoxy-2,2'-bipyrrole-5-carbaldehyde; MEM, minimum essential medium (full medium); PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; PU, polyurethane

REFERENCES

[1] Williamson, N. R., Fineran, P. C., Leeper, F. J., and Salmond, G. P. C. (2006) The biosynthesis and regulation of bacterial prodiginines, *Nat. Rev. Microbiol. 4*, 887-899.

[2] Papireddy, K., Smilkstein, M., Kelly, J. X., Shweta, Salem, S. M., Alhamadsheh, M., Haynes, S. W., Challis, G. L., and Reynolds, K. A. (2011) Antimalarial activity of natural and synthetic Prodiginines, *J. Med. Chem. 54*, 5296-5306.

[3] Perez-Tomas, R., Montaner, B., Llagostera, R., and Soto-Cerrato, V. (2003) The prodigiosins, proapoptotic drugs with anticancer properties, *Biochem. Pharmacol. 66*, 1447-1452.

[4] Stankovic, N., Senerovic, L., Ilic-Tomic, T., Vasiljevic, B., and Nikodinovic-Runic, J. (2014) Properties and applications of undecylprodigiosin and other bacterial prodigiosins, *Appl. Microbiol. Biotechnol. 98*, 3841-3858.

[5] Stamelos, V. A., Fisher, N., Bamrah, H., Voisey, C., Price, J. C., Farrell, W. E., Redman, C. W., and Richardson, A. (2016) The BH3 mimetic obatoclax accumulates in lysosomes and causes their alkalinization, *PloS one 11*, e0150696.

[6] Rubinsztein, D. C., Codogno, P., and Levine, B. (2012) Autophagy modulation as a potential therapeutic target for diverse diseases, *Nat. Rev. Drug. Discov. 11*, 709-730.

[7] Kaufmann, A. M., and Krise, J. P. (2007) Lysosomal sequestration of amine-containing drugs: Analysis and therapeutic implications, *J. Pharm. Sci. 96*, 729-746.

[8] Fürstner, A. (2003) Chemie und Biologie des Roseophilins und der Prodigiosin-Alkaloide: 2500 Jahre im Überblick, *Angew. Chem. 115*, 3706-3728; *Angew. Chem. Int. Ed. 42*, 3582-3603.

[9] Hu, D. X., Withall, D. M., Challis, G. L., and Thomson, R. J. (2016) Structure, chemical synthesis, and biosynthesis of prodiginine natural products, *Chem. Rev. 116*, 7818-7853.

[10] Nisha, Kumar, K., and Kumar, V. (2015) Prodigiosin alkaloids: recent advancements in total synthesis and their biological potential, *RSC Adv. 5*, 10899-10920.

[11] Williamson, N. R., Simonsen, H. T., Ahmed, R. A. A., Goldet, G., Slater, H., Woodley, L., Leeper, F. J., and Salmond, G. P. C. (2005) Biosynthesis of the red antibiotic, prodigiosin, in *Serratia*: identification of a novel 2-methyl-3-*n*-amyl-pyrrole (MAP) assembly pathway, definition of the terminal condensing enzyme, and implications for undecylprodigiosin biosynthesis in *Streptomyces*, *Mol. Microbiol. 56*, 971-989.

[12] Bruner, S. D. (2011) Enzyme catalysis C-H activation is a Reiske business, *Nat. Chem. 3*, 342-343.

[13] Chawrai, S. R., Williamson, N. R., Mahendiran, T., Salmond, G. P. C., and Leeper, F. J. (2012) Characterisation of PigC and HapC, the prodigiosin synthetases from *Serratia sp* and *Hahella chejuensis* with potential for biocatalytic production of anticancer agents, *Chem. Sci. 3*, 447-454.

[14] Chawrai, S. R., Williamson, N. R., Salmond, G. P. C., and Leeper, F. J. (2008) Chemoenzymatic synthesis of prodigiosin analogues-exploring the substrate specificity of PigC, *Chem. Commun.,* 1862-1864.

[15] Kirschning, A., Taft, F., and Knobloch, T. (2007) Total synthesis approaches to natural product derivatives based on the combination of chemical synthesis and metabolic engineering, *Org. Biomol. Chem. 5*, 3245-3259.

[16] Kennedy, J. (2008) Mutasynthesis, chemobiosynthesis, and back to semi-synthesis: combining synthetic chemistry and biosynthetic engineering for diversifying natural products, *Nat. Prod. Rep. 25*, 25-34.

[17] Taft, F., Brunjes, M., Floss, H. G., Czempinski, N., Grond, S., Sasse, F., and Kirschning, A. (2008) Highly active ansamitocin derivatives: mutasynthesis using an AHBA-blocked mutant, *ChemBioChem 9*, 1057-1060.

[18] Gregory, M. A., Petkovic, H., Lill, R. E., Moss, S. J., Wilkinson, B., Gaisser, S., Leadlay, P. F., and Sheridan, R. M. (2005) Mutasynthesis of rapamycin analogues through the manipulation of a gene governing starter unit biosynthesis, *Angew. Chem. Int. Ed. 44*, 4757-4760; *Angew. Chem. 117*, 4835-4838.

[19] Weist, S., Kittel, C., Bischoff, D., Bister, B., Pfeifer, V., Nicholson, G. J., Wohlleben, W., and Süssmuth, R. D. (2004) Mutasynthesis of glycopeptide antibiotics: Variations of vancomycin's AB-ring amino acid 3,5-dihydroxyphenylglycine, *J. Am. Chem. Soc. 126*, 5942-5943.

[20] Boecker, S., Zobel, S., Meyer, V., and Süssmuth, R. D. (2016) Rational biosynthetic approaches for the production of new-to-nature compounds in fungi, *Fungal Genet. Biol. 89*, 89-101.

[21] Bilyk, O., and Luzhetskyy, A. (2016) Metabolic engineering of natural product biosynthesis in actinobacteria, *Curr. Opin. Biotechnol. 42*, 98-107.

[22] Dong, L. B., Rudolf, J. D., and Shen, B. (2016) A Mutasynthetic library of platensimycin and platencin Analogues, *Org. Lett. 18*, 4606-4609.

[23] Domröse, A., Klein, A. S., Hage-Hülsmann, J., Thies, S., Svensson, V., Classen, T., Pietruszka, J., Jaeger, K.-E., Drepper, T., and Loeschcke, A. (2015) Efficient recombinant production of prodigiosin in *Pseudomonas putida*, *Front. Microbiol. 6*, 972.

[24] Trofimov, B. A., Mikhaleva, A. I., Vasilev, A. N., Korostova, S. Y., and Shevchenko, S. G. (1985) Pyrroles from Ketoximes and Acetylene .29. Synthesis of Alkylpyrroles from Dialkylketoximes and Dichloroethane in KOH-DMSO systems, *Khim. Geterotsiklicheskikh Soedin.*, 59-63.

[25] Trofimov, B. A., Mikhaleva, A. b. I., Ivanov, A. V., Shcherbakova, V. S., and Ushakov, I. A. (2015) Expedient one-pot synthesis of pyrroles from ketones, hydroxylamine, and 1,2-dichloroethane, *Tetrahedron 71*, 124-128.

[26] The synthesis of pure 2-butyl-3-pentylpyrrole via the Trofimov reaction is not possible. The [3,3] sigmatropic rearrangement does not show any regioselectivity using 5-undecanonoxime. Therefore the reaction provided a mixture of the desired product and 2-hexyl 3-propylpyrrole.

[27] Schimmer, A. D., Raza, A., Carter, T. H., Claxton, D., Erba, H., DeAngelo, D. J., Tallman, M. S., Goard, C., and Borthakur, G. (2014) A multicenter phase I/II study of obatoclax mesylate administered as a 3- or 24-hour infusion in older patients with previously untreated acute myeloid leukemia, *PLoS one 9*, e108694.

[28] Langer, C. J., Albert, I., Ross, H. J., Kovacs, P., Blakely, L. J., Pajkos, G., Somfay, A., Zatloukal, P., Kazarnowicz, A., Moezi, M. M., Schreeder, M. T., Schnyder, J., Ao-Baslock, A., Pathak, A. K., and Berger, M. S. (2014) Randomized phase II study of carboplatin and etoposide with or without obatoclax mesylate in extensive-stage small cell lung cancer, *Lung Cancer 85*, 420-428.

[29] Oki, Y., Copeland, A., Hagemeister, F., Fayad, L. E., Fanale, M., Romaguera, J., and Younes, A. (2012) Experience with obatoclax mesylate (GX15-070), a small molecule pan–Bcl-2 family antagonist in patients with relapsed or refractory classical Hodgkin lymphoma, *Blood 119*, 2171-2172.

[30] Arellano, M. L., Borthakur, G., Berger, M., Luer, J., and Raza, A. (2014) A phase II, multicenter, open-label study of obatoclax mesylate in patients with previously untreated myelodysplastic syndromes with anemia or thrombocytopenia, *Clin. Lymphoma Myeloma Leuk. 14*, 534-539.

[31] Laraia, L., Ohsawa, K., Konstantinidis, G., Robke, L., Wu, Y.-W., Kumar, K., and Waldmann, H. (2017) Discovery of novel cinchona-alkaloid-inspired oxazatwistane autophagy inhibitors, *Angew. Chem. Int. Ed. 56*, 2145-2150; *Angew. Chem. 129*, 2177-2182.

[32] Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids, *J. Mol. Biol. 166*, 557-580.

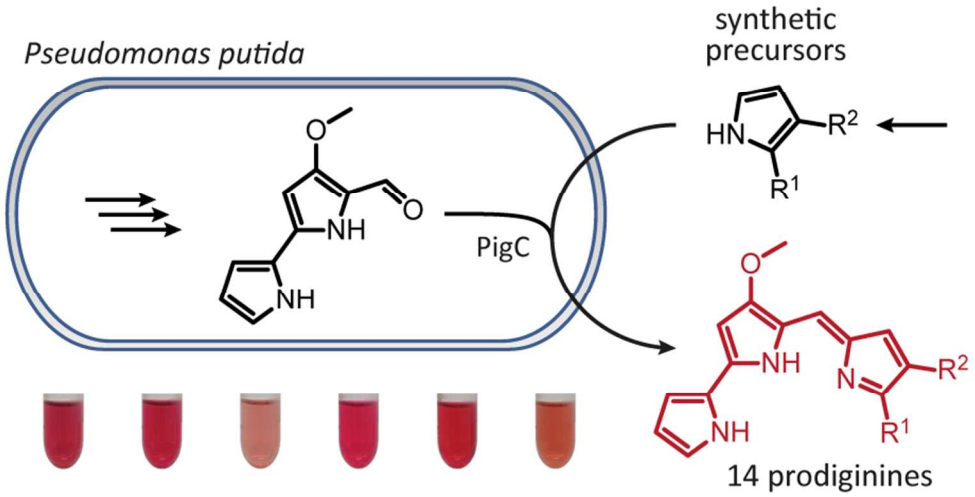
[33] Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in gram negative bacteria, *Nat. Biotech. 1*, 784-791.

[34] Studier, F. W., and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, *J. Mol. Biol. 189*, 113-130.

[35] Loeschcke, A., Markert, A., Wilhelm, S., Wirtz, A., Rosenau, F., Jaeger, K. E., and Drepper, T. (2013) TREX: A universal tool for the transfer and expression of biosynthetic pathways in bacteria, *ACS Synth. Biol. 2*, 22-33.

[36] Gibson, D. G. (2011) Chapter fifteen - enzymatic assembly of overlapping DNA fragments, In *Method Enzymol.* (Christopher, V., Ed.), pp 349-361, Academic Press.

[37] Mülhardt, C. (2008) *Der Experimentator: Molekularbiologie / Genomics*, Spektrum Akademischer Verlag.



TOC-Graphic

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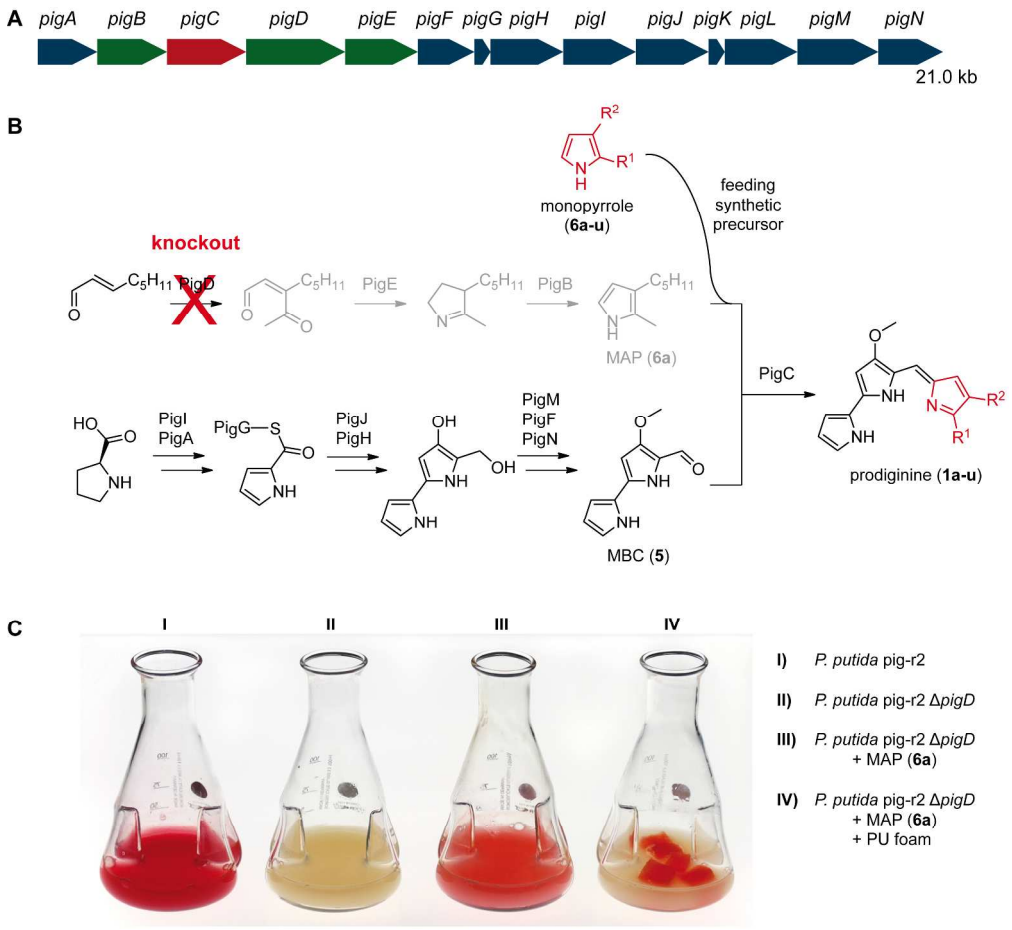

Figure 1

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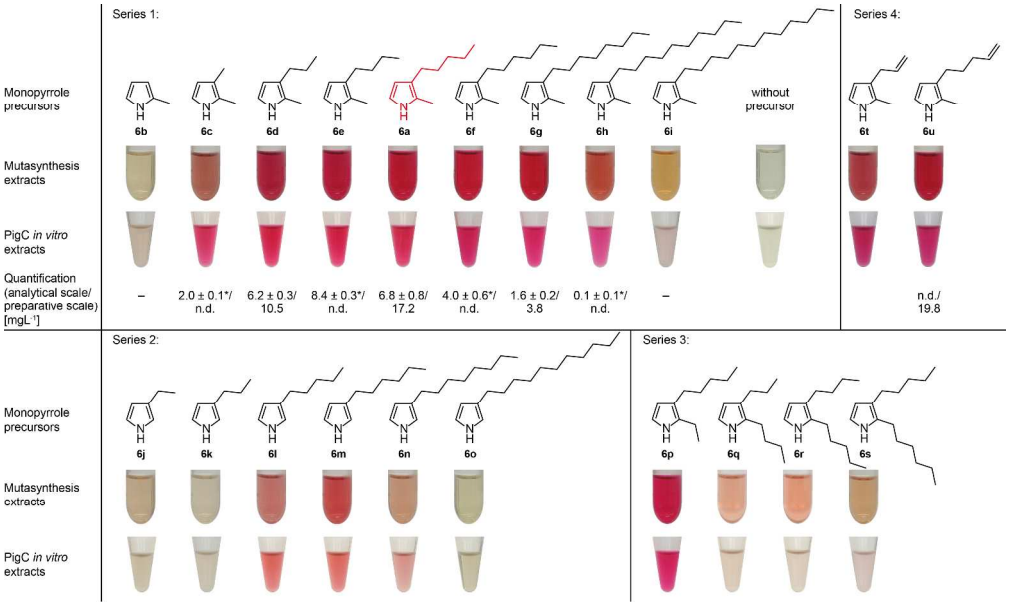

Figure 2

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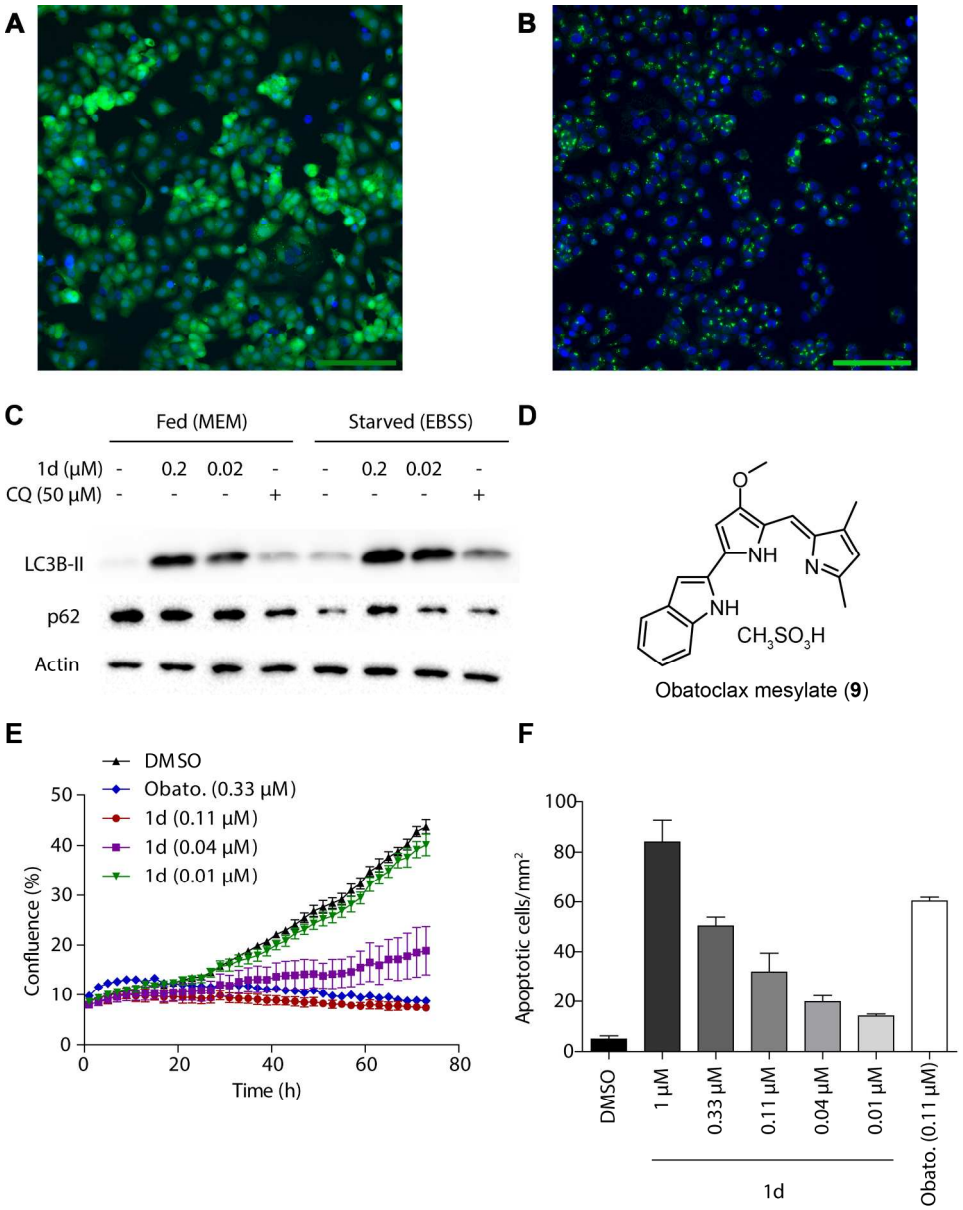

Figure 3

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