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New prodigiosin derivatives – chemoenzymatic synthesis and physiological evaluation against cisplatin-resistant cancer cells†

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Prodigiosin and its derivatives from the prodiginine family are a natural class of secondary metabolite alkaloids of bacterial origin. They are well known for multifarious biological activities against a broad range of bacteria, pathogenic fungi, parasites, and several cancer cell lines. Biosynthesis of natural derivatives is based on a converging route with a final ATP- and enzyme-dependent condensation reaction between the bipyrrole precursor MBC and miscellaneously substituted monopyrroles. Although these ligating enzymes have been recognised for promiscuity regarding monopyrroles, minor studies were exerted to investigate promiscuity for MBC derivatives. To overcome the current lack of structural knowledge, we synthesised six 5′-*n*-alkyl derivatives of MBC and validated their suitability for condensation with monopyrroles by the ligating enzymes PigC, TreaP, and TamQ to probe their active site experimentally. Moreover, chemically synthesised prodiginines with 5-*n*-alkylation on the A-ring were subjected to systematic cell viability screening with the urothelial cancer cell lines RT-112 (cisplatin-sensitive) and RT-112res (cisplatin-resistant) to fathom the effect of electron-donating substituents on cytotoxicity. Alongside an overall broad acceptance of short- and medium-chain alkylated MBC derivatives by the enzymes PigC, TreaP, and TamQ, we identified the A-ring substituted prodiginines with methyl substituents as superior anticancer agents against cisplatin-resistant RT-112res after 72 h (15.7–18.8 nM) compared to prodigiosin (41.1 nM) and the former phase II clinical candidate obatoclax mesylate (36.0 nM).

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Introduction

Prodigiosin (**1**) is a deep red coloured natural pigment of bacterial origin and eponym for the generic family term of prodiginine alkaloids. During the last 70 years, the family of achiral and chiral prodiginines of natural and synthetic provenance has constantly grown and has been first under investigation for structural elucidation, but then more and more become of interest for the development of total synthesis strategies and for its manifold biological activities.[1–11] Common for all prodiginines is a congeneric conjugated scaffold of A-, B-, and C-ring pyrroles and decorations on the ring systems. Prodigiosin (**1**) was once identified due to its striking colourful appearance,[11] but nowadays its derivatives and their cytotoxic properties have raised scientific and clinical attention.[12,13] The basic tripyrrolic core structure is able to bind divalent metal cations to induce oxidative stress. When binding Cu^{II}, prodigiosin was shown to efficiently cause copper-promoted single- and double-strand breaks of DNA.[14–17] Moreover, protonated prodiginines are allowed to passively diffuse across biologic membranes, symporting the chloride counter ion. The co-transport implicates uncoupling and depletion of the proton gradient that is essential to acquire energy in the form of adenosine triphosphate (ATP) or, more generally, to maintain the proton motive force and associated biological processes.[15,18–22] Albeit prodigiosin and prodiginine alkaloids exhibit a broad range of activities against bacteria, protozoa, pathogenic fungi, plants, and nematodes,[23–26] prodiginines were well recognised for their activity against the malaria parasite *Plasmodium falciparum*.[27–29] Furthermore, prodiginines feature anti-tumour activity against several human cancer cell lines by induction of apoptosis, showing relatively low effects against non-malignant tissue.[30–34] The diversity of reported biophysical properties and biological

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activities adumbrate the great challenge, which cells are facing after prodigiosin (**1**) treatment. Presumably, the relatively low doses being necessary to observe lethal biological activity are a consequence of blurring multidimensional modes of action.

Biosynthesis and condensing enzymes

With an increasing number of natural prodiginines and related natural products, the quantity of known bacterial gene clusters involved in their biosynthesis has constantly risen. To date, numerous clusters among diverse bacterial species, such as *pig* (*Serratia marcescens*, prodigiosin **1**),[35] *red* (*Streptomyces coelicolor*, undecylprodigiosin and streptorubin B),[36] *trea* (*Pseudoalteromonas citrea*, tambjamine MYP1 **2**),[37] and mar (marine *Streptomyces* sp., marineosin A) have been identified by genome mining and analyses of mutant strains (Fig. 1, bottom).[38–42] Despite the structural diversity of prodiginine-related natural products, their biosynthesis resorts to clustered genes, encoding for non-ribosomal peptide synthetases (NRPS) of type II.[36,43] Using the synthesis of prodigiosin (**1**) as representative example, the assembly employs a convergent route with two key precursors, namely the bipyrrole part (4-methoxy-2,2′-bipyrrole-5-carbaldehyde, MBC **3a**) and the monopyrrole part (2-methyl-3-amylpyrrole, MAP **4a**), which are consolidated in an ATP-catalysed condensation reaction to give the conjugated tripyrrole scaffold of prodigiosin (**1**) (Fig. 1, top).[35,44–46] For further in-depth elaboration on (bio)synthesis of MBC, MAP, the included enzymatic steps, and prodiginine-related natural products, consultation of the review from Hu *et al.* is recommended.[47]

The enzymes, being responsible for the C–C-bond forming condensation reaction of monopyrroles and MBC, belong to the class of ligases (EC 6.4). Here, the enzymes PigC, RedH, TreaP, and MarH from afore mentioned gene clusters take on the role of ligases in the corresponding pathways. Unfortunately, crystallisation attempts of these enzymes did not lead to fruitful insights into structure-based mechanistic peculiarities. And yet, approximations of the structure were achieved by homology modelling and docking studies,[48,49] while mechanistic intricacies were elucidated by kinetic studies.[50]

A-ring modifications of prodiginine-related natural products

The known natural prodiginine and connatural tambjamine derivatives featuring aliphatic substitutions on the A-ring are consistently belonging to the class of macrocycles, except the oddly protruding 2-(*p*-hydroxybenzyl)prodigiosin (**5**) from the marine bacterium *Pseudoalteromonas rubra* (Fig. 1, bottom).[51] The formation of macrocyclic prodiginines is explicitly owing to late stage oxidative carbocyclisation of long chain alkyl chains on the prodiginine C-ring, including a carbon-centred radical intermediate.[52,53] Because of this cyclisation, either formation of C- to A-ring (a) or C- to B-ring (b) bridging macrocycles is observed or formation of C-ring internal chiral cycles (c) catalysed. Prominent examples for those enzymes are the RedG homologue MarG involved in marineosin biosynthesis (b) or the Rieske oxygenase RedG in the making of streptorubin B (c).[53]

For the C- to A-ring bridged carbomacrocycle cyclononylprodigiosin (**6**) the biosynthetic *non* gene cluster with the RedG-homologue NonG has been assigned (a).[54] However, the upstream condensation of bipyrrole and monopyrrole exclusively relies on utilisation of MBC as bipyrrole condensation partner, meaning that no naturally occurring A-ring substituted MBC derivatives have been found to date. In case of A-ring modified 2-(*p*-hydroxybenzyl) prodigiosin (**5**), no proposal on biosynthesis and related genes, based on experimental evidence, has been published.[55] Another well-known example of a prodigiosin-

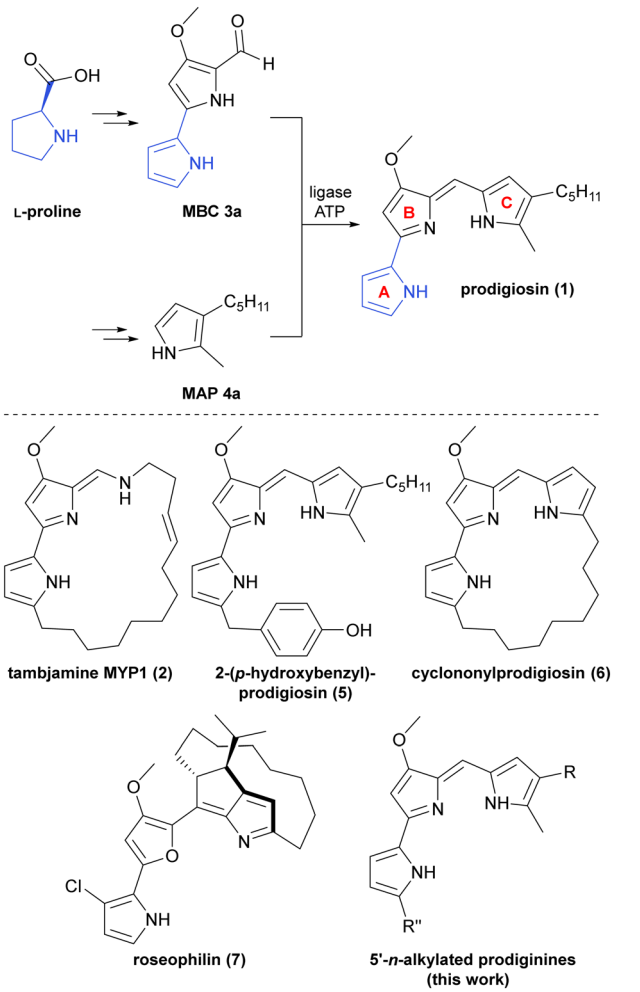


Fig. 1 Top: Shortened biosynthesis of prodigiosin (**1**) by the related *pig* genes from *S. marcescens*. The final condensation step of MBC **3** and MAP **4a** is catalysed by a ligase (here PigC) in an ATP-consuming manner. The fate of atoms from the initial precursor L-proline in the intermediate MBC **3** and the natural product prodigiosin (**1**) is highlighted in blue. Rings (A-, B-, and C-) of the prodiginine scaffold are accentuated in red. Bottom: Examples from the class of prodigiosin alkaloids and connatural natural products, illustrating the structural diversity.

related natural product is roseophilin (7) from *Streptomyces griseoviridis*, which shows 3-chlorination of the A-ring, substitution of the B-ring pyrrolyl moiety by furyl and a chiral macrocyclic C-ring.

Although the chlorination pattern implies utilisation of a chlorinated MBC analogue, comparable to the biosynthesis of pyoluteurin,[56] an adequate precursor and identification of the responsible halogenating enzyme is still missing. Late stage halogenation of dechlororoseophilin could be plausible, too. Indeed, the lack in discovery of substituted A-ring MBC derivatives from natural sources is not surprising, as the A-ring is built from the amino acid L-proline (*cf.* Fig. 1, top). Within the prodigiosin *pig* biosynthesis cluster of *S. marcescens* and undecylprodigiosin *red* gene cluster in *S. coelicolor*, the early steps of transformation include PigI/RedM-catalysed activation of proline as thioester and the subsequent oxidation of the pyrrolidine core by PigI/RedA to give acylated 1*H*-pyrrole.[35,36,47] In other words, a prerequisite for natural A-ring variation on MBC derivatives would be the acceptance of alternative canonic amino acids by PigI/RedA during the initial steps in biosynthesis or functionalisation of the A-ring pyrrole in successive steps. Nevertheless, no experimental proof has been furnished for existence of this kind of A-ring substituted MBC so far.

Shifting the focus from the bipyrrole MBC **3a** to the monopyrrole unit, promiscuity of condensing enzymes, exemplified by PigC from *S. marcescens*, is well-known for monopyrroles with cyclic and acyclic aliphatic side chains and the C-ring moiety of prodiginines.[57–59] Contemporaneously, little evidence was provided in the context of MBC derivatives being suitable substrates, yet. Haynes *et al.* attempted investigation of RedH-catalysed condensation of a dechlororoseophilin-inspired MBC analogue in combination with 2-undecylpyrrole, but did not observe any activity based on product formation. Since their study was conducted as feeding experiment with the mutant strain *S. coelicolor* W39, limited phase transfer across the bacterial membrane or lacking eligibility as RedH substrate were deduced as potential cause.[60] Chawrai *et al.* pioneered demonstration of accepted aryl substituted A-ring derivatives of MBC (*e.g.* thienyl-, furyl-, phenyl-) by PigC,[46] but since then, no studies were performed beyond that. Even though broad accessibility of A-ring substituted prodiginines by biosynthetic methods is not given from a today's perspective, those derivatives can be assessed by means of total synthesis. Melvin *et al.* attributed a negative effect on copper-promoted DNA cleavage, when the A-ring was either substituted with electron-withdrawing substituents in 5-position or replaced by aryl residues other than pyrrolyl.[15] Similar effects were observed by D'Alessio *et al.* with focus on cytotoxicity in extensive structure–activity-relationship studies (SAR).[61] At the same time, both studies denoted positive effects by adding electron-donating alkyl substitutions in 5-position of the A-ring,[15,61] providing substantial evidence of value to further analyse the consequences of A-ring associated alkylations. In the present study, we aim at expanding the knowledge about acceptance of A-ring *n*-alkylated MBC derivatives by prodiginine ligases to probe the active site of investigated enzymes experimentally and systematically infer the effects on prodiginine cytotoxicity against cisplatin-sensitive and -resistant urothelial carcinoma cell lines.

Results and discussion

Synthesis of prodiginine precursors

Prodiginine ligases exploit the bipyrrole MBC **3a** and derivatives of 1*H*-monopyrroles as substrates for their ATP-catalysed reaction mechanism.[62,63] Thus, the chemical synthesis of monopyrroles was approached using the Trofimov procedure, which allows conversion of oximes to 2,3-dialkylated 1*H*-pyrroles.[57,64] Starting from commercially available ketones, namely octan-2-one (**8a**), hexan-2-one (**8b**), and decan-2-one (**8c**), the respective oximes **9a–c** were synthesised in yields of ≥98% by refluxing the ketones with hydroxylamine hydrochloride and sodium acetate in EtOH/H_2O (4 : 1) (Fig. 2). As conversions were near quantitative, no further purification was needed and the *E*/*Z* mixtures could be used straightaway for the subsequent Trofimov reaction. The latter allows cyclisation of alkylated oximes in the presence of 1,2-dichloroethane under superbasic reaction conditions that can be obtained by mixing DMSO, ground potassium hydroxide and a small amount of H_2O.[64] The electron-rich pyrroles are highly sensitive towards oxygen and light. Consequently, in addition to inert reaction conditions we identified utilisation of thoroughly degassed and dried DMSO as key requirement for a reliable outcome of the reaction. Only with degassed solvent, the application of reaction temperatures of 90–100 °C over a prolonged period led to a minimal amount of polymerisation during the reaction and yielded 52–54% of pyrroles **4a–c** (Fig. 2).

Apparently, the use of such synthetic schemes that enable both, use of the products as substrates for chemical synthesis of reference compounds and as potential substrates for ligating enzymes are inevitable for our approach. Based on the biomimetic two-step sequence devised by Dairi *et al.* for gaining access to the natural prodigiosin and tambjamine precursor 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (**3a**, MBC),[65] we identified an adapted route to make 5′-*n*-alkylated

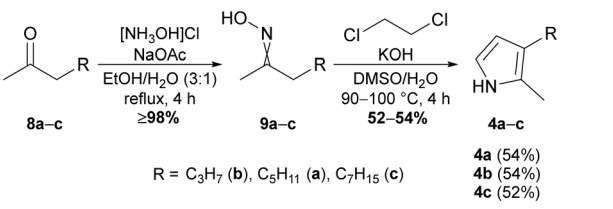


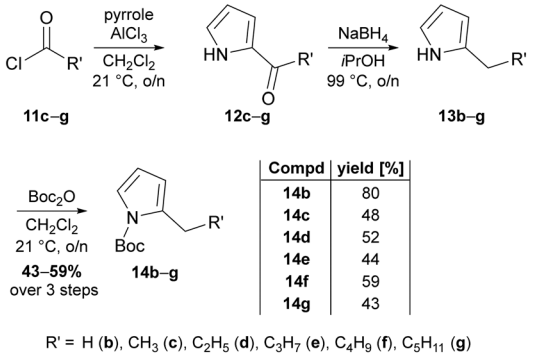
Fig. 2 Synthesis scheme of 2,3-dialkylated 1*H*-pyrroles **4a–c** from ketones **8a–c** and intermediate oximes **9a–c** *via* Trofimov pyrrole synthesis.

derivatives of MBC accessible. Although other positions for MBC alkylation might be of interest as well, we focused on the 5′-position for this fundamental study. The devised synthetic route is predicated on the haloformylation of 4-methoxy-3-pyrrolin-2-one to give bromide **10**, followed by a palladium-catalysed Suzuki–Miyaura cross-coupling of bromide **10** with 5-*n*-alkylated pyrrole-2-boronic acids. The fundament for the desired boronic acids were 2-alkylated *N*-Boc-protected 1*H*-pyrroles, which were afforded by a consecutive Friedel–Crafts acylation of 1*H*-pyrrole with acyl chlorides **11c–g** under usage of aluminium trichloride as Lewis acid.[66] Reduction of the 2-acylation products **12c–g** with sodium borohydride in iPrOH and final *N*-protection of the 2-alkyl-pyrroles **13b–g** with Boc-anhydride yielded the target compounds **14b–g** (Fig. 3).[1] In this reaction sequence, we were able to reduce the purification effort due to usage of crude reaction products and exclusive purification of the Boc-protected products (**14b–g**) by ordinary flash column chromatography. By this means, the *N*-Boc-2-alkylated pyrroles were synthesised in yields of 43–59%.

Since boronic acids are more reactive than their related boronic acid pinacol or MIDA esters,[68] we aimed for the synthesis of free pyrrole-2-boronic acids from *N*-Boc-2-alkylated pyrroles for the cross-coupling reaction with bromide **10**. Admittedly, 2-substituted boronic acids of *N*-heterocycles, such as pyrroles or pyridines, are delicate and significantly lacking storage stability.[69] Thus, utilisation of crude boronic acids from pyrrole borylation reactions was considered more reliable rather than isolation of pure boronic acids. Indeed, the crude products of the borylation sequence, which comprises deprotonation of pyrroles **14b–g** with lithium tetramethylpiperidide, borylation with trimethyl borate and lastly deprotection of the methyl ester with aqueous hydrochloric acid were ideal starting materials. Catalysed by palladium acetate, SPhos-assisted Suzuki–Miyaura cross-coupling in degassed *n*-butanol/H_2O (4 : 1) and with potassium phosphate as base allowed C–C coupling between pyrrole-2-boronic acids **15b–g** and bromide **10** under mild reaction conditions (Fig. 4). The reactions typically gave mixtures of Boc-protected and deprotected carbaldehydes. Full deprotection of crude reaction was rendered by refluxing in 2,2,2-trifluoroethanol to provide the desired products after filtration. Resulting from this sequence, the 5′-*n*-alkylated MBC derivatives **3b–g** were prepared in 52–80% yield (Fig. 4). Unsubstituted MBC **3a** was synthesised according to the published literature procedure.[65]

Synthesis of prodiginines

Although the alkylated MBC derivatives were primarily synthesised for systematic characterisation of the prodigiosin ligases substrate scope, they were also eligible for chemical prodiginine synthesis. Therefore, carbaldehydes **3a–g** and pyrroles **4a–c** were subjected to a condensation reaction under acid catalysis with hydrochloric acid to yield the



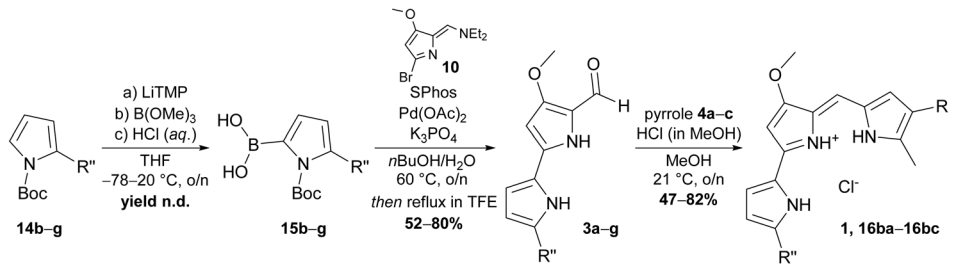
Compd	yield [%]
14b	80
14c	48
14d	52
14e	44
14f	59
14g	43

Fig. 3 Synthesis scheme of 2-*n*-alkylated *N*-Boc-pyrroles **14b–g**. 2-Methyl-1*H*-pyrrole (**13b**) was purchased from commercial suppliers. Intermediates were not purified and the yield over three steps determined after final purification of Boc-protected 2-alkylated pyrroles. For further information regarding systematic nomenclature, please see ref. 67.

corresponding prodiginines **16ba–bc** in yields of 47–82% (Fig. 4). The hydrochlorides of prodigiosin (**1**) and novel prodiginines **16ba–bc** generated thereby could be used as synthetic references for enzymatic condensation reactions, prodiginine quantification, toxicity screening, and were accessible in purities of up to 99.9%.

Absorption spectra and molar extinction coefficients

Prodiginines are well known to display solvent-dependent absorption properties, which greatly vary with pH.[70] To ensure that the molecule of interest is uniformly protonated, extinction coefficients and absorption spectra are typically recorded in acidified ethanol in which the purple pigment prodigiosin exhibits an absorption maximum at 535 nm.[70,71] By adding a 5-*n*-alkyl substitution on the A-ring, the absorption maximum was increased to 545–547 nm, noticeable in a visible small shift from pink to purple in solution. With pentyl-substitution on the C-ring, variation in A-ring substitution greatly enhanced the molar extinction coefficient with increasing chain length, based on the inductive electron-donating effect of the attached alkyl substituents (Fig. S2A, Table S2, ESI†). For prodigiosin (**1**) a molar extinction coefficient of 139 800 M^{-1} cm^{-1} (535 nm) was reported in the literature.[71] The A-ring methyl-derivative (**16ba**), however, already presented an extinction coefficient of 163 233 M^{-1} cm^{-1} (546 nm), followed by the ethyl- and propyl-derivative with 177 092 M^{-1} cm^{-1} (**16ca**, 546 nm) and 181 692 M^{-1} cm^{-1} (**16da**, 546 nm), respectively. From butyl- to hexyl-substitutions **16ea–ga**, no further increase of significance was observed and the molar extinction coefficients seemed to reach a plateau around 190 000 M^{-1} cm^{-1} at 547 nm (Fig. S2A, ESI†). Interestingly, molar extinction coefficients at 535 nm ranged from 126 508 M^{-1} cm^{-1} to 138 200 M^{-1} cm^{-1}, showing less chain length-dependent behaviour and solely fluctuations in the magnitude of standard deviations (Fig. S2B, ESI†). With



R = C_3H_7 (**16bb**), C_5H_{11} (**16ba, 16ca, 16da, 16ea, 16fa, 16ga**), C_7H_{15} (**1, 16bc**)
R" = H (**3a**), CH_3 (**14b, 15b, 3b**), C_2H_5 (**14c, 15c, 3c**), C_3H_7 (**14d, 15d, 3d**), C_4H_9 (**14e, 15e, 3e**), C_5H_{11} (**14f, 15f, 3f**), C_6H_{13} (**14g, 15g, 3g**)

compd	R"	yield [%]
3b	CH_3	60
3c	C_2H_5	52
3d	C_3H_7	69
3e	C_4H_9	75
3f	C_5H_{11}	75
3g	C_6H_{13}	80

compd	R	R"	yield [%]
1	C_5H_{11}	H	68
16ba	C_5H_{11}	CH_3	82
16ca	C_5H_{11}	C_2H_5	80
16da	C_5H_{11}	C_3H_7	58
16ea	C_5H_{11}	C_4H_9	73
16fa	C_5H_{11}	C_5H_{11}	47
16ga	C_5H_{11}	C_6H_{13}	48
16bb	C_3H_7	CH_3	66
16bc	C_7H_{15}	CH_3	80

Fig. 4 Synthesis scheme of *n*-alkylated A-ring derivatives of MBC (**3b–g**) and prodigiosin (**16ba–bc**) from *N*-Boc-pyrrole-2-boronic acid precursors (**15b–g**). Substituted prodiginines **16** were numbered in a modular fashion (**16**R″R), including the substitution pattern of the MBC derivative (**3a–g**, R″) and the monopyrrole (**4a–c**, R). For example, prodiginine **16ba** was synthesised from MBC **3b** and pyrrole **4a**, prodiginine **16bb** was synthesised from MBC **3b** and pyrrole **4b**. Abbreviations: n.d. – not determined; compd – compound; LiTMP – lithium 2,2,6,6-tetramethylpiperidide; SPhos – 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl; TFE – 2,2,2-trifluoroethanol.

constant methyl-substitution on the A-ring and variation in C-ring chain length, a linear correlation was observable at 535 nm for prodiginines **16ba**, **16bb**, and **16bc** allowing approximation of molar extinction coefficients (Table S3, ESI†) based on the experimental coefficients and the slope of linear regression (Fig. S2C, ESI†). In this way, we were able to quantify even prodiginines whose references had not been synthesised by chemical means.

Prodigiosin ligase substrate scope for A-ring substituted MBC

Chawrai *et al.* showed that prodigiosin derivatives with alternative aromatic A-rings are accepted by the ligase PigC from *S. marcescens*. Enzymatic activity was still present when the A-ring pyrrole moiety was either substituted by 2-phenyl-, 2-thienyl-, 2-furyl-, or 2-indolyl-residues. No activity was seen for napthyl- and biphenyl-substitution instead.[46] Their findings prompted us to investigate alkyl-substituted pyrrole derivatives more thoroughly, as they were not characterised as suited substrates for PigC and, moreover, with TreaP and TamQ two new ligating enzymes from *Pseudoalteromonadaceae* were found since then.[59]

In our experiment, we tested the three ligases PigC, TreaP, and TamQ for their substrate acceptance of A-ring substituted alkyl-derivatives **3b–g** of MBC and MBC **3a** itself and quantified the extracted prodiginines in LC-MS measurements with the corresponding extinction coefficients, which had been determined experimentally or by approximation (*vide supra*). As we used *Escherichia coli* cell lysate after expression of the corresponding genes rather than purified enzymes, *E. coli* harbouring the pET28a(+) empty vector was used as control. Unusual for this experimental setup in comparison to earlier studies on prodiginine ligases was the two-dimensional testing, varying MBC derivatives and monopyrroles at the same time (Fig. 5). We would like to emphasise that quantification was normalised to the amount of cells and not to the amount of enzyme being used in the experiment. Thus, differences in expression efficiencies for the analysed ligases might have an impact on absolute prodiginine titres but should not affect the relative acceptance of A-ring substituted MBC derivatives for each enzyme (Fig. 6A). Firstly, we validated whether prodiginines are readily formed in a catalyst-free environment, containing pyrroles, MBC, ATP, and buffer. It was found that no background reaction takes place (control reactions 1–6, ESI,† Fig. S111–S116) and that the ligating enzymes are needed for catalysis of the condensation reaction. Secondly, we performed reactions with enzyme containing cell lysates and ATP in buffer but without pyrroles and MBC to exclude formation of prodiginines from cell metabolites within the lysate. Again, no prodiginines were traced by coupled LC-MS measurements, proving that all four components (ligating enzyme, MBC, pyrrole, and ATP) are needed for their formation (negative controls, ESI,† Fig. S107–S110).

During the first experiments, the most prominent enzyme PigC showed high acceptance of methyl- and unsubstituted MBC (**3b** and **3a**) in combination with the short-chain monopyrrole **4b**, whereat the natural precursor MBC **3a** turned out to be the favoured bipyrrole, yielding 25.9 mg L^{-1} *versus* 16.7 mg L^{-1} of prodiginine extracts for methyl-MBC **3b**. Other MBC derivatives were not converted to the related prodiginines in noteworthy amounts. In contrast, TamQ, which is naturally involved in tambjamine biosynthesis, apparently converted all derivatives including butyl-MBC **3e**, but not pentyl- and hexyl-MBC **3f** and **3g**. Here, the obtained product titres of prodiginines derived from short-chain MBC derivatives **3a**, **3b**, and **3c** were rather similar in a range from 10–12 mg L^{-1}. The acceptance of MBC derivatives for TreaP was limited to C0–C3-substituted MBC **3a–d** and resulted in considerably lower titres (<3.6 mg L^{-1}) than PigC or TamQ. However, TreaP showed highest relative product concentrations for the ethyl-substituted MBC **3c**. With

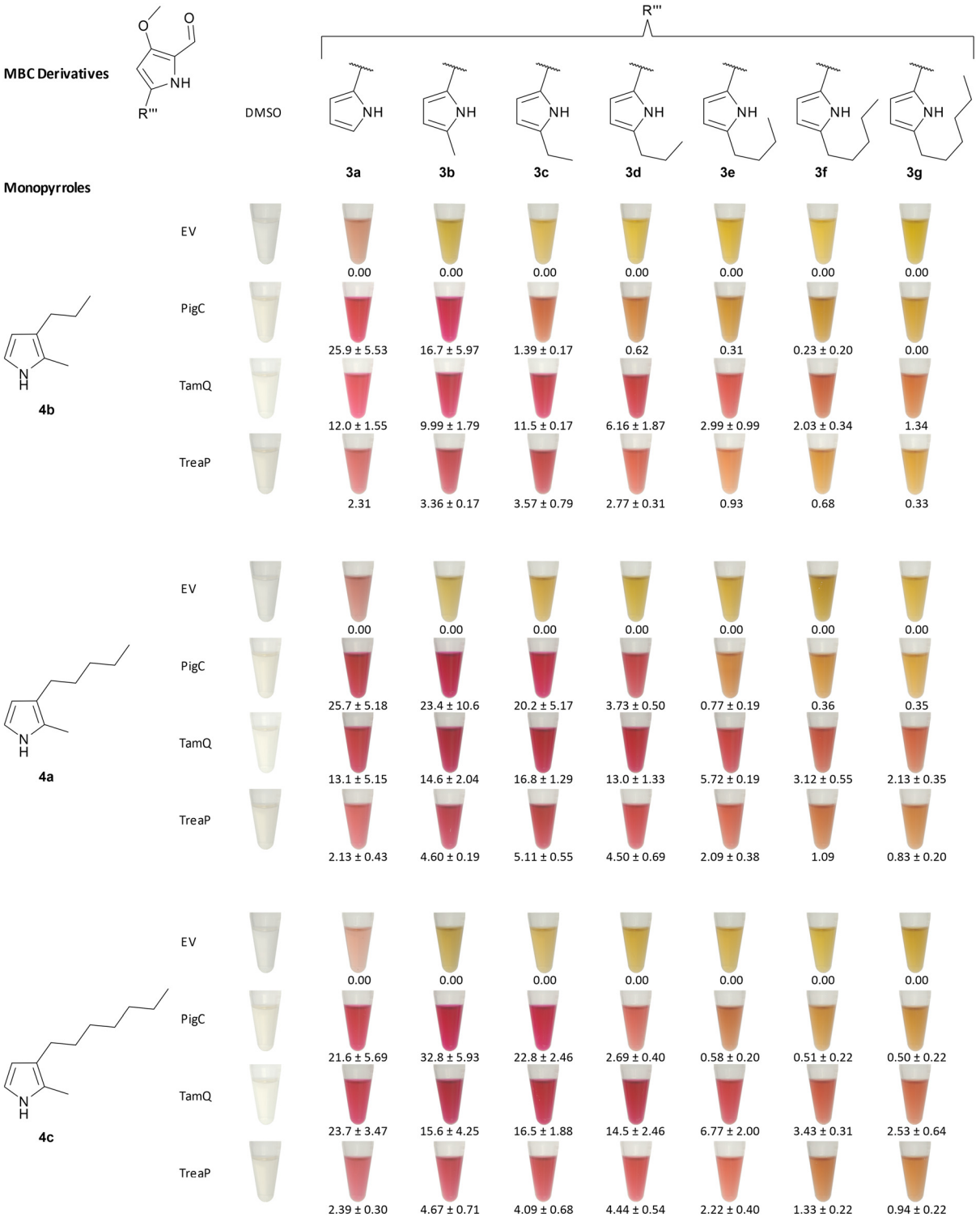


Fig. 5 Substrate spectrum of the prodiginine ligases PigC, TamQ, and TreaP for combinations of monopyrroles **4a**–**c**, MBC **3a** and 5-*n*-alkylated MBC derivatives **3b**–**g**. Cell lysates of *E. coli* BL21 with corresponding ligases in KP_i buffer were treated with pyrroles (1 mM), MBC (1 mM), and ATP (1.25 mM) and incubated at 30 °C for 4 h. Cell lysate of *E. coli* BL21 pET28a(+) was used as negative control. A red colouration of the solutions indicates the formation of products. Photographic record was employed to document the organic extracts in acidic ethanol. Product titres (mg L^{-1}) were determined by LC-MS measurements and are given below the corresponding tubes. Abbreviations: EV – empty vector.

increasing chain length of the monopyrrole all enzymes appeared to possess greater substrate promiscuity and conversions. For both, the medium- and long-chain monopyrroles **4a** and **4c**, the MBC substrate spectrum for PigC from *S. marcescens* was expanded to propyl-MBC **3d** but also to higher prodiginine titres. Again, unsubstituted MBC

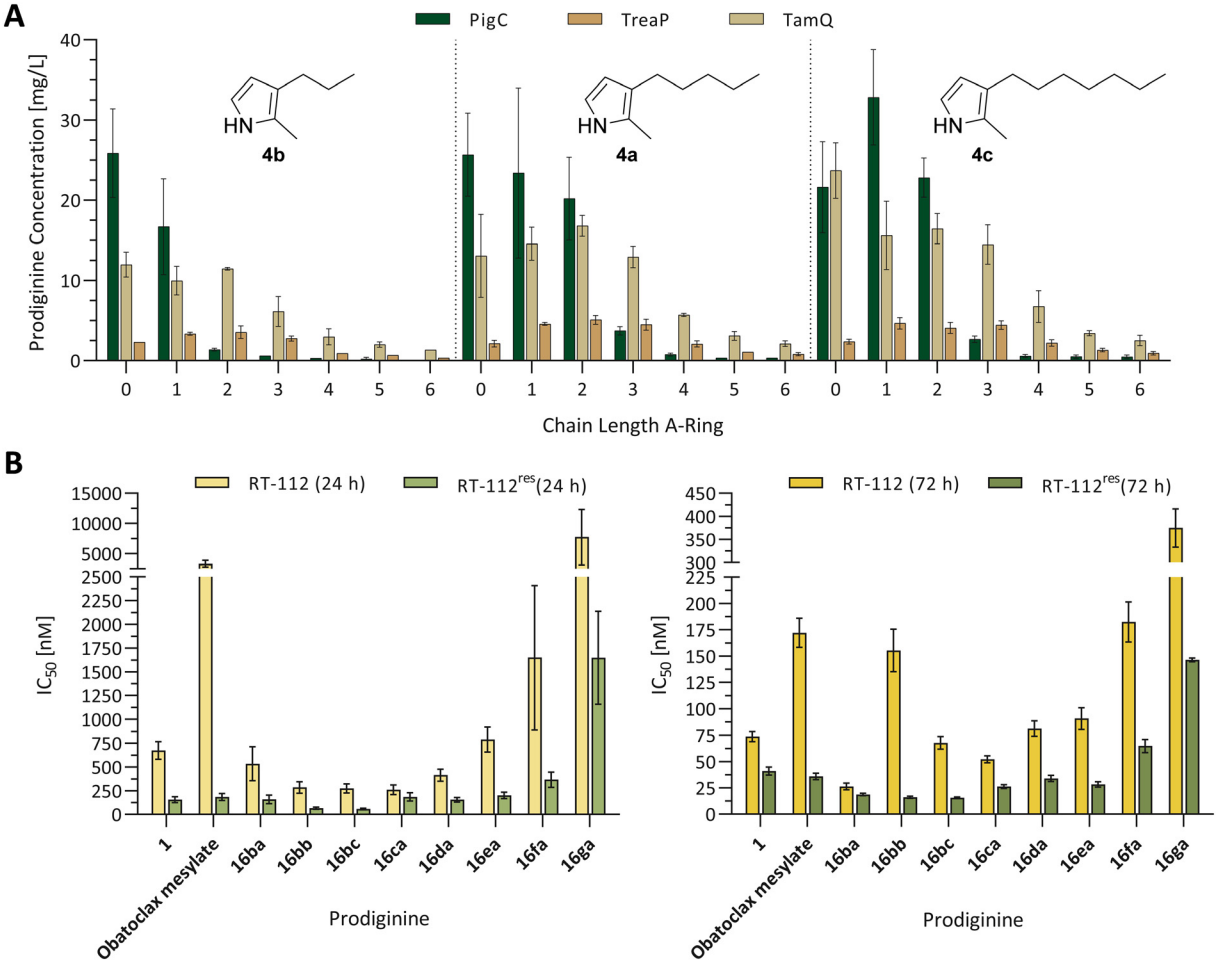


Fig. 6 (A) Quantification of prodiginines from *in vitro* reactions with the prodiginine ligases PigC, TamQ, and TreaP with varying chain lengths on the A- and C-ring. Methanolic extracts of biocatalytic reactions between monopyrroles **4a–c** and 5-alkyl MBC derivatives **3a–g** were subjected to coupled LC-MS measurements and the amount of substance quantified from the corresponding UV absorption at 535 nm and the prodiginine extinction coefficients. The chain length of alkylated MBC is depicted on the *x*-axis of the bar diagram, the used monopyrrole is shown in the diagram. Since all values for the empty vector control were zero, the data is not shown, but plotted raw data can be found in the supporting information (Fig. S3†). Each experiment was performed in triplicates and the standard error is given. (B) Cell viability of urothelial cancer cell lines RT-112 (cisplatin sensitive) and RT-112res (cisplatin resistant) in the presence of prodigiosin (**1**), obatoclax mesylate, and chemically synthesised prodiginines (**16ba–ga**) after 24 h (left) and 72 h (right). For improved reading and resolution of data sets, the scales of ordinates for the 24 h and 72 h diagrams were adjusted. Experiments were performed in triplicates and cell survival was analysed using the MTT assay. Error bars are representing the 95% confidence interval. Plotted raw data and dose–response fits for determination of IC_{50} values can be found in the supporting information (plots and fits: Fig. S117–S119, fitted IC_{50} values: Table S4†).

3a was apparently converted best, but only for the medium-chain monopyrrole **4a** at 25.7 mg L^{-1}. With the long-chain monopyrrole **4c**, the non-natural methyl-MBC derivative **3b** was identified as ideal partner for condensation, providing the related product in a titre of 32.8 mg L^{-1}. Supported by previous findings regarding the enzymes TamQ and TreaP,[59] either preferentially converted long-chain monopyrroles to the corresponding prodiginines, at which TamQ in total exhibited the broadest acceptance of 5-alkyl-substituted MBC derivatives. Even though MBC **3a** is the common prodiginine and tambjamine precursor in nature for all three enzymes under investigation, TreaP and TamQ shared a similar trend of MBC derivative promiscuity for the medium-chain monopyrrole **4a**, showing highest product titres for ethyl-MBC **3c** with 5.1 mg L^{-1} and 16.8 mg L^{-1}, respectively. The long-chain monopyrrole **4c** was best accepted in combination with methyl-MBC **3b** by PigC, providing the highest prodiginine product titre of 32.8 mg L^{-1} in our experiment.

From the contingent of tested enzymes, PigC was in fact the least versatile enzyme, demonstrated by tight restraints in terms of tolerating alkyl-substitution on the MBC part. In contrast, TamQ was identified as most profitable catalyst for substitutions on the monopyrrole and the A-ring of MBC. It is hypothesised that multiple reasons contribute to the observed acceptance pattern of combinations for monopyrroles and MBC. As the monopyrrole serves as nucleophile in the proposed mechanism (devised for PigC), reviewed by Hu *et al.*,[47] it is believed that shorter chain

length may result in a higher degree of freedoms and flexibility of side chains, less tight binding of the pyrrole core within the enzyme pocket, potentially leading to decreased or depleted reaction rates. In addition, short chain pyrroles are lacking additional activation, which is contributed by the electron-donating effect of alkyl-chains. Significance of the latter can be seen from the drastic effect of increasing chain-length on prodiginine extinction coefficients. For the alkyl-chain variation on MBC derivatives, we assume steric reasons as principal force behind the lack of conversion with increased number of methylene groups within the alkyl chain. Electronic contributions are judged rather implausible, as the condensation chemistry between monopyrrole and MBC is taking place at the distal part of the MBC derivatives, namely the carbaldehyde.

Evaluation of cytotoxicity for A-ring substituted prodiginines

To assess the impact of 5-*n*-alkyl substitutions on cytotoxic properties of chemically synthesised prodiginines, the urothelial carcinoma cell lines RT-112 (cisplatin sensitive) and RT-112res (cisplatin resistant) were selected for evaluation.[72–74] Cisplatin-based therapy is the standard of care for this kind of carcinomas, however, miscellaneous mechanisms are involved in emerging resistance against cisplatin, such as autophagy and apoptosis.[72,75–78] Prodigiosin (**1**) has been shown to display cytotoxic activity at nanomolar concentrations against both, cisplatin sensitive and resistant cell lines, and was thus judged as potential drug candidate for treatment of cisplatin resistant urothelial carcinomas.[30] Thus, prodigiosin (**1**) and the former phase II clinical candidate obatoclax mesylate were selected as benchmark compounds.[12,13,79,80] In order to assort the impact of A-ring associated alkyl substitutions on prodiginine derivatives, the two RT-112 cell lines were subjected to cell viability monitoring after 24 h and 72 h, using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Fig. 6B, data and dose–response fits in the supporting information). For the lead compound prodigiosin (**1**), inhibitory concentrations (IC_{50}) of 675 nM and 157 nM were traced after 24 h and were further reduced to 73.8 nM and 41.1 nM after 72 h for RT-112 and RT-112res, respectively. In contrast, obatoclax mesylate exhibited relatively low cytotoxic activity in RT-112 cells after 24 h of incubation. With 3327 nM against RT-112 and 184 nM against RT-112res after 24 h, obatoclax was acting similarly to the least cytotoxic long-chain derivatives **16ea–ga**. With increased incubation time of 72 h, improved cytotoxicity of 172 nM (RT-112) and 36.0 nM (RT-112res) were achieved, judging obatoclax significantly less effective against RT-112 than prodigiosin (**1**) and of similar value against RT-112res.

In the following, prodiginines with altered A-ring alkyl substitution, but constant C-ring substitution of prodigiosin (**1**) that originated from chemical synthesis were investigated for their cytotoxic effects. Simple addition of an electron donating methyl-group in prodiginine **16ba** resulted in a strong increase of cytotoxicity against both cell lines. With a final IC_{50} of 26.4 nM for RT-112 and 18.8 nM for RT-112res after 72 h, an up to 2.8-fold increase in cytotoxicity was observed. However, IC_{50} values after 24 h initially resembled the cytotoxicity of prodigiosin (**1**) itself. Interestingly, further chain elongation on the A-ring of prodiginines **16ca–ga** led to constant loss of biological activity with each step of elongation. Especially the transition from pentyl to hexyl substitution on the A-ring displayed a turning point in cytotoxicity and was accompanied by a significant loss of biological activity, independent from the cell line and time point of analysis. Aside from the effect of A-ring substitution with invariable C-ring substitution pattern of compounds **16ba–ga**, we chose the most cytotoxic methyl-substituted prodiginine **16ba** as starting point and scrutinised which effect a substitution in 4-position of the C-ring chain toward longer or shorter alkyl residues might have. All analysed prodiginines (**16ba**, **16bb**, and **16bc**) with methyl substitution on the A-ring appeared to have unspoiled high cytotoxicity against the cisplatin resistant subtype RT-112res after 72 h when the C-ring was modulated. With IC_{50} values ranging between 15.7 nM (**16bc**) and 18.8 nM (**16ba**), the impact was rather negligible. Nonetheless, the tested derivatives were shown to act differently after 24 h, as prodiginine **16ba** with C5-chain on the C-ring revealed slightly higher inhibitory concentrations against both cell lines than observed for derivatives **16bb** and **16bc**. Summarising the presented data from Fig. 6B, an additional methyl group in 5-position of the A-ring boosted the cytotoxicity against both, cisplatin sensitive and resistant urothelial carcinoma cell lines. In addition to this increased performance, we found prodiginine **16ba** to display the highest cytotoxicity against both cell lines after 72 h (RT-112: 26.4 nM, RT-112res: 18.8 nM). Concurrently, prodiginines **16bb** and **16bc** were outstandingly fast in unleashing their cytotoxic potential, having substantial improvements of toxicity after 24 h in comparison to derivative **16ba**, the mother compound prodigiosin (**1**) and the former phase II clinical candidate obatoclax mesylate. The tripyrrole core structure of prodigiosin (**1**) and its substituted clinical relative obatoclax is well known to inhibit the cellular process of autophagy,[30,57,81,82] which is essential to recycle or degrade cell organelles or anomalous proteins (misfolded, aggregated, or damaged).[30] At the same time, prodigiosin (**1**) was shown to induce apoptosis, an ensemble of signalling pathways of programmed cell death.[33,34,83] In the past, the mechanistic cause for autophagy inhibition and induction of apoptosis in prodigiosin-treated cancer cell lines was accounted to the capability of H^+/Cl^- symport across the membranes of lysosomes or other eukaryotic cell compartments and the cell cycle arrest.[18,20,21,84]

The ion pair of protonated prodigiosin and the chloride counter ion is lipophilic and tight enough to penetrate the membrane and diffuse across the permeability barrier.[11] A possible explanation for the increased cytotoxicity of prodiginines with short-chain electron-donating alkyl substituents on the A-ring could be a tighter bond between

the B-ring nitrogen atom and the proton from the hydrochloride complex, increasing the pK_a value and facilitating the trespass of the charged complex through the membrane. A further increase of the A-ring chain length could potentially result in a more lipophilic character for the resulting prodiginine, raising the risk of functioning as membrane anchor and attaching derivatives to the membrane rather than diffusing across, thereby reducing the cytotoxic potential. In fact, our results regarding A-ring substitution are in agreement with earlier studies on alkyl substitution on the C-ring, where short-chain propyl substitution was characterised by drastically stronger autophagy inhibition than for the long-chain octyl variant.[57]

Conclusions

Prodiginine ligating enzymes are known to exhibit substrate promiscuity on C-ring monopyrroles and MBC derivatives with aromatic A-ring substitution. However, reliable structural information for these enzymes in free or substrate-bound state are still lacking. Whilst enzyme structures remain unclear, probing of the active site and testing of suitable substrate combinations is limited to means of trial and error. We were able to transfer the previously gained knowledge of enzyme promiscuity in a systematic screening on 5′-*n*-alkylated MBC derivatives, showing broad substrate acceptance for both key precursors of the convergent biosynthesis route. While PigC only accepted short-chained MBC derivatives, TamQ and TreaP displayed fewer restraints, also for longer alkyl chains. During cell viability screening of A-ring substituted prodiginines in cisplatin-sensitive and -resistant urothelial carcinoma cell lines, methyl-substituted derivatives **16ba**, **16bb**, and **16bc** were cytotoxic at low nanomolar concentrations, and thereby up to 2.6-times more potent than prodigiosin (**1**) or obatoclax mesylate against the cisplatin-resistant cell line RT-112res after 72 h. Consequently, these derivatives are suggested as potential lead compounds for future structural optimisation in terms of cytotoxicity, drug administration and drug metabolism and could be potential candidates for treatment of cisplatin resistant carcinomas.

Experimental

Synthetic procedures

General considerations. All reactions were carried out under nitrogen or argon in pre-dried glassware using Schlenk technique. Organic solvents were acquired in technical grade and distilled prior to use. Dried solvents (CH_2Cl_2, THF) were obtained from a MB-SPS 800 drying apparatus (M. Braun Inertgas-Systeme GmbH) and by storage over activated molecular sieve for >72 h (iPrOH, DMSO). If stated in the synthetic instructions, solvents were degassed using the freeze–pump–thaw procedure (3×). 2,2,6,6-Tetramethylpiperidine was refluxed for 4 h over CaH_2 and then distilled under normal pressure and stored over 4 Å molecular sieve. For flash chromatography Macherey-Nagel silica gel 60 M (40–63 μm) was employed. Synthesised compounds were stored at −20 °C under argon. Further specifications and information on instrumentation can be found in the supporting information. Obatoclax mesylate (≥98%, HPLC) was purchased from Sigma Aldrich (Merck).

General procedure for the synthesis of MBC derivatives 3b–g. A Schlenk flask with magnetic stirring bar is sequentially charged with $Pd(OAc)_2$ (5.0 mol%), SPhos (6.0 mol%), bromide **10** (1.00 eq.), Boc-protected pyrrole boronic acid **15b–g** (3.00 eq.) and evacuated/N_2-refilled three times. Degassed *n*-butanol is added and the mixture stirred at 20 °C until homogeneous. A 1.45 M solution of K_3PO_4 (2.00 eq.) in degassed water is added to give an *n*-butanol/water ratio of 4 : 1. The reaction vessel is lowered into a pre-heated 60 °C bath and the orange solution is stirred under argon overnight. The solvent is evaporated, 50 mL of water are added to the slurry and the mixture is then extracted with EtOAc (3 × 50 mL). Combined organic phases are washed with brine (2 × 25 mL), dried over $MgSO_4$, filtered over degreased cotton wool and the solvent is removed. The resulting solid is recovered by filtration and washed with cold distilled *n*-pentane. The green solid is refluxed for 6 h at 90 °C in 45 eq. 2,2,2-trifluoroethanol. Evaporation of the solvent and trituration of the remaining solid in *n*-pentane provides the product as green solid that is recovered by filtration and washed with distilled *n*-pentane and Et_2O.

4-Methoxy-5′-methyl-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (3b). Following the general procedure for the synthesis of MBC derivatives, bromide **10** (800 mg, 3.09 mmol, 1.00 eq.), boronic acid **15b** (2.60 g, 9.26 mmol, 3.00 eq.), SPhos (76.0 mg, 0.19 mmol, 6 mol%), $Pd(OAc)_2$ (34.7 mg, 0.15 mmol, 5 mol%), and K_3PO_4 (1.31 g, 6.17 mmol, 2.00 eq.) are converted to 379 mg (1.86 mmol, 60%) of 4-methoxy-5′-methyl-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (**3b**) after 15 h reaction time. The product is obtained as green powder. δ^1H (600 MHz, DMSO) 2.22 (3 H, s, 6′-H), 3.82 (3 H, s, 10-H), 5.77–5.84 (1 H, m, 4′-H), 6.16–6.23 (1 H, m, 3-H), 6.58–6.66 (1 H, m, 3′-H), 9.26 (1 H, s, 7-H), 11.00 (1 H, s, 1′-NH), 11.27 (1 H, s, 1-NH). δ^{13}C (151 MHz, DMSO) 12.7 (C-6′), 57.7 (C-10), 90.3 (C-3), 107.6 (C-4′), 108.8 (C-3′), 117.2 (C-5), 122.0 (C-2′), 130.1 (C-5′), 133.5 (C-2), 158.8 (C-4), 171.1 (C-6). FT-IR (neat, cm^{-1}): 3254, 3205, 3127, 3071, 2954, 2837, 1585, 1546, 1514, 1426, 1357, 1333, 1296, 1280, 1254, 1203, 1177, 1164, 1033, 1018, 1000, 991, 972, 833, 790, 770, 697, 653, 624, 589, 482. T_m: 250–275 °C (decomposition) (*n*-pentane). HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{11}H_{13}N_2O_2$ 205.0973, found 205.0973. t_R (LC-MS method): 7.54 min.

5′-Ethyl-4-methoxy-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (3c). Following the general procedure for the synthesis of MBC derivatives, bromide **10** (850 mg, 3.28 mmol, 1.00 eq.), boronic acid **15c** (2.35 g, 9.84 mmol, 3.00 eq.), SPhos (80.8 mg, 0.20 mmol, 6 mol%), $Pd(OAc)_2$ (36.8 mg, 0.16 mmol, 5 mol%), and K_3PO_4 (1.39 g, 6.56 mmol, 2.00 eq.) are converted to 375 mg (1.72 mmol, 52%) of 5′-ethyl-4-methoxy-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (**3c**) after 14 h reaction time. The product is obtained as green powder.

δ^1H (300 MHz, DMSO) 1.19 (3 H, q, 7′-H), 2.53–2.65 (2 H, m, 6′-H), 3.67–3.92 (3 H, m, 10-H), 5.66–5.97 (1 H, m, 4′-H), 6.08–6.35 (1 H, m, 3-H), 6.49–6.78 (1 H, m, 3′-H), 9.26 (1 H, d, 7-H), 10.95 (1 H, s, 1′-NH), 11.27 (1 H, s, 1-NH). δ^{13}C (76 MHz, DMSO) 13.7 (C-7′), 20.4 (C-6′), 57.7 (C-10), 90.4 (C-3), 106.2 (C-4′), 108.7 (C-3′), 117.2 (C-5), 122.0 (C-2′), 133.6 (C-2), 136.6 (C-5′), 158.8 (C-4), 171.1 (C-6). FT-IR (neat, cm^{-1}): 3250, 3197, 2972, 2833, 1593, 1553, 1513, 1445, 1422, 1357, 1302, 1284, 1248, 1199, 1175, 1161, 1147, 1039, 1014, 990, 832, 766, 740, 698, 588, 484. T_m: 243.7–245.5 °C (*n*-pentane). HRMS-ESI (*m*/*z*): [M + H]$^+$ calculated for $C_{12}H_{15}N_2O_2$ 219.1128, found 219.1132. t_R (LC-MS method): 7.93 min.

4-Methoxy-5′-propyl-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (3d). Following the general procedure for the synthesis of MBC derivatives, bromide **10** (800 mg, 3.09 mmol, 1.00 eq.), boronic acid **15d** (2.34 g, 9.26 mmol, 3.00 eq.), SPhos (76.0 mg, 0.19 mmol, 6 mol%), $Pd(OAc)_2$ (34.7 mg, 0.15 mmol, 5 mol%), and K_3PO_4 (1.31 g, 6.17 mmol, 2.00 eq.) are converted to 497 mg (2.14 mmol, 69%) of 4-methoxy-5′-propyl-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (**3d**) after 17 h reaction time. The product is obtained as green powder. δ^1H (600 MHz, DMSO) 0.91 (3 H, t, *J* = 7.3 Hz, 8′-H), 1.60 (2 H, h, *J* = 7.4 Hz, 7′-H), 2.53 (2 H, t, *J* = 7.5 Hz, 6′-H), 3.83 (3 H, s, 10-H), 5.81–5.86 (1 H, m, 4′-H), 6.22 (1 H, s, 3-H), 6.57–6.65 (1 H, m, 3′-H), 9.26 (1 H, s, 7-H), 10.94 (1 H, s, 1′-NH), 11.27 (1 H, s, 1-NH). δ^{13}C (151 MHz, DMSO) 13.6 (C-8′), 22.4 (C-7′), 29.2 (C-6′), 57.7 (C-10), 90.4 (C-3), 106.8 (C-4′), 108.6 (C-3′), 117.1 (C-5), 121.9 (C-2′), 133.5 (C-2), 135.0 (C-5′), 158.7 (C-4), 171.1 (C-6). FT-IR (neat, cm^{-1}): 3253, 3201, 3135, 3071, 3008, 2956, 2929, 2870, 2381, 1593, 1553, 1516, 1462, 1447, 1421, 1356, 1343, 1302, 1248, 1224, 1199, 1176, 1161, 1041, 1016, 986, 831, 769, 692, 664, 624, 587, 484, 453. T_m: 224.7–226.8 °C (*n*-pentane). HRMS-ESI (*m*/*z*): [M + H]$^+$ calculated for $C_{13}H_{17}N_2O_2$ 233.1285, found 233.1286. t_R (LC-MS method): 8.23 min.

5′-Butyl-4-methoxy-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (3e). Following the general procedure for the synthesis of MBC derivatives, bromide **10** (700 mg, 2.70 mmol, 1.00 eq.), boronic acid **15e** (2.17 g, 8.10 mmol, 3.00 eq.), SPhos (66.5 mg, 0.16 mmol, 6 mol%), $Pd(OAc)_2$ (30.3 mg, 0.14 mmol, 5 mol%), and K_3PO_4 (1.15 g, 5.40 mmol, 2.00 eq.) are converted to 499 mg (2.03 mmol, 75%) of 5′-butyl-4-methoxy-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (**3e**) after 17 h reaction time. The product is obtained as green powder. δ^1H (300 MHz, DMSO) 0.90 (3 H, t, *J* = 7.3 Hz, 9′-H), 1.25–1.40 (2 H, m, 8′-H), 1.57 (2 H, p, *J* = 7.6 Hz, 7′-H), 2.55 (2 H, t, *J* = 8.1 Hz, 6′-H), 3.83 (3 H, s, 10-H), 5.80–5.86 (1 H, m, 4′-H), 6.22 (1 H, t, 3-H), 6.57–6.65 (1 H, m, 3′-H), 9.26 (1 H, s, 7-H), 10.93 (1 H, s, 1′-NH), 11.26 (1 H, s, 1-NH). δ^{13}C (76 MHz, DMSO) 13.7 (C-9′), 21.8 (C-8′), 26.9 (C-6′), 31.3 (C-7′), 57.7 (C-10), 90.4 (C-3), 106.8 (C-4′), 108.7 (C-3′), 117.1 (C-5), 121.9 (C-2′), 133.5 (C-2), 135.2 (C-5′), 158.8 (C-4), 171.1 (C-6). FT-IR (neat, cm^{-1}): 3257, 3208, 3002, 2952, 2928, 2856, 2835, 1596, 1553, 1516, 1427, 1361, 1342, 1303, 1243, 1198, 1163, 1038, 1024, 985, 834, 782, 741, 724, 691, 667, 587, 488. T_m: 203.5–205.6 °C (*n*-pentane). HRMS-ESI (*m*/*z*): [M + H]$^+$ calculated for $C_{14}H_{19}N_2O_2$ 247.1441, found 247.1446. t_R (LC-MS method): 8.56 min.

4-Methoxy-5′-pentyl-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (3f). Following the general procedure for the synthesis of MBC derivatives, bromide **10** (800 mg, 3.09 mmol, 1.00 eq.), boronic acid **15f** (2.60 g, 9.26 mmol, 3.00 eq.), SPhos (76.0 mg, 0.19 mmol, 6 mol%), $Pd(OAc)_2$ (34.7 mg, 0.15 mmol, 5 mol%), and K_3PO_4 (1.31 g, 6.17 mmol, 2.00 eq.) are converted to 605 mg (2.33 mmol, 75%) of 4-methoxy-5′-pentyl-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (**3f**) after 15 h reaction time. The product is obtained as green powder. δ^1H (600 MHz, DMSO) 0.87 (3 H, t, *J* = 6.9 Hz, 10′-H), 1.30 (4 H, tt, *J* = 7.3 Hz, 8′-H, 9′-H), 1.58 (2 H, p, *J* = 7.5 Hz, 7′-H), 2.54 (2 H, t, *J* = 7.7 Hz, 6′-H), 3.82 (3 H, s, 10-H), 5.78–5.88 (1 H, m, 4′-H), 6.14–6.27 (1 H, m, 3), 6.56–6.68 (1 H, m, 3′-H), 9.25 (1 H, s, 7-H), 10.93 (1 H, s, 1′-NH), 11.26 (1 H, s, 1-NH). δ^{13}C (151 MHz, DMSO) 14.4 (C-10′), 22.4 (C-9′), 27.6 (C-6′), 29.3 (C-7′), 31.4 (C-8′), 58.2 (C-10), 90.9 (C-3), 107.3 (C-4′), 109.2 (C-3′), 117.7 (C-5), 122.4 (C-2′), 134.0 (C-2), 135.7 (C-5′), 159.3 (C-4), 171.6 (C-6). FT-IR (neat, cm^{-1}): 3255, 3204, 3105, 3071, 2953, 2929, 2839, 1599, 1554, 1518, 1428, 1361, 1340, 1304, 1287, 1255, 1231, 1202, 1163, 1034, 1023, 987, 833, 781, 742, 724, 692, 667, 588, 490, 454. T_m: 196.2–199.5 °C (*n*-pentane). HRMS-ESI (*m*/*z*): [M + H]$^+$ calculated for $C_{15}H_{21}N_2O_2$ 261.1598, found 261.1601. t_R (LC-MS method): 8.78 min.

5′-Hexyl-4-methoxy-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (3g). Following the general procedure for the synthesis of MBC derivatives, bromide **10** (800 mg, 3.09 mmol, 1.00 eq.), boronic acid **15g** (2.73 g, 9.26 mmol, 3.00 eq.), SPhos (76.0 mg, 0.19 mmol, 6 mol%), $Pd(OAc)_2$ (34.7 mg, 0.15 mmol, 5 mol%), and K_3PO_4 (1.31 g, 6.17 mmol, 2.00 eq.) are converted to 678 mg (2.47 mmol, 80%) of 5′-hexyl-4-methoxy-1*H*,1′*H*-[2,2′-bipyrrole]-5-carbaldehyde (**3g**) after 14 h reaction time. The product is obtained as green powder. δ^1H (600 MHz, DMSO) 0.85 (3 H, t, 11′-H), 1.23–1.34 (6 H, m, 8′-H, 9′-H, 10′-H), 1.58 (2 H, p, *J* = 7.4 Hz, 7′-H), 2.54 (2 H, t, *J* = 7.7 Hz, 6′-H), 3.83 (3 H, s, 10-H), 5.78–5.87 (1 H, m, 4′-H), 6.19–6.23 (1 H, m, 3-H), 6.57–6.65 (1 H, m, 3′-H), 9.26 (1 H, s, 7-H), 10.93 (1 H, s, 1′-NH), 11.26 (1 H, s, 1-NH). δ^{13}C (151 MHz, DMSO) 13.9 (C-11′), 22.0 (C-10′), 27.2 (C-6′), 28.3 (C-8′), 29.1 (C-7′), 31.0 (C-9′), 57.7 (C-10), 90.4 (C-3), 106.8 (C-4′), 108.7 (C-3′), 117.1 (C-5), 121.9 (C-2′), 133.5 (C-2), 135.2 (C-5′), 158.7 (C-4), 171.1 (C-6). FT-IR (neat, cm^{-1}): 3223, 3117, 2954, 2924, 2855, 1664, 1464, 1458, 1377, 1368, 1258, 1217, 1168, 1113, 994, 956, 943, 830, 823, 724, 651, 614. T_m: 171.8–174.6 °C (*n*-pentane). HRMS-ESI (*m*/*z*): [M + H]$^+$ calculated for $C_{16}H_{23}N_2O_2$ 275.1754, found 275.1758. t_R (LC-MS method): 9.01 min.

General procedure for the synthesis of 1*H*-pyrroles 4a–c. Reactions were performed according to an earlier published procedure with modifications.[57] In detail, a mixture of oxime **9a–c** (1.00 eq.), pestled KOH (5.00 eq.), and water (13.7 μL mmol^{-1} oxime) is added to a three-necked flask under N_2 atmosphere. Degassed and dried DMSO (1.92 mL mmol^{-1} oxime) is added under nitrogen atmosphere. The reaction is refluxed at 100 °C and a solution of 1,2-dichloroethane (3.50 eq.) in degassed DMSO (0.21 mL mmol^{-1} oxime) is added with a syringe pump over 2 h. A second batch KOH (5.00 eq.)

is added under ice cooling after the first hour of 1,2-dichloroethane addition (dichloroethane-feeding is paused during KOH addition) before the dichloroethane addition is continued for further 1 h. Once the addition of dichloroethane is completed, the reaction is refluxed for 2 h. The reaction is cooled to 0 °C and ice water is added for quenching. Extraction is performed with Et_2O (3 × 100 mL). Combined organic phases are dried over $MgSO_4$, filtered over degreased cotton wool and the solvent removed *in vacuo*. Chromatographic purification with petholeum ether/CH_2Cl_2 + 1% (*v*/*v*) triethylamine on silica provides the product as yellow to orange oil.

2-Methyl-3-pentyl-1*H*-pyrrole (4a). Octan-2-one oxime (**9a**, 12.0 g, 83.8 mmol, 1.00 eq.), 1,2-dichloroethane (23.2 mL, 293 mmol, 3.50 eq.), and KOH (47.0 g, 838 mmol, 10.0 eq.) are converted to 6.89 g (45.6 mmol, 54%) 2-methyl-3-pentyl-1*H*-pyrrole (**4a**). The product is obtained as yellow volatile oil after purification with petroleum ether/CH_2Cl_2 (90 : 10) + 1% triethylamine. δ^1H (600 MHz, $CDCl_3$) 0.90 (3 H, t, J = 6.7 Hz, 5″-H), 1.28–1.38 (4 H, m, 3″-H, 4″-H), 1.54 (2 H, p, J = 7.4 Hz, 2″-H), 2.19 (3 H, s, 1′-H), 2.39 (2 H, t, J = 7.7 Hz, 1″-H), 6.02 (1 H, t, J = 2.8 Hz, 4-H), 6.60 (1 H, t, J = 2.7 Hz, 5-H), 7.70 (1 H, brs, 1-NH). δ^{13}C (151 MHz, $CDCl_3$) 11.2 (C-1′), 14.3 (C-5″), 22.8 (C-4″), 26.0 (C-1″), 31.2 (C-2″), 31.9 (C-3″), 109.0 (C-4), 114.9 (C-5), 119.9 (C-3), 123.3 (C-2). FT-IR (neat, cm^{-1}): 3376, 2956, 2923, 2854, 1464, 1444, 1378, 1246, 1107, 1064, 954, 901, 831, 771, 670, 551. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{10}H_{18}N$ 152.1434, found 152.1434. t_R (LC-MS method): 8.52 min.

2-Methyl-3-propyl-1*H*-pyrrole (4b). Following the general procedure for the synthesis of 1*H*-pyrroles, hexan-2-one oxime (**9b**, 12.0 g, 104 mmol, 1.00 eq.), 1,2-dichloroethane (28.8 mL, 365 mmol, 3.50 eq.), and KOH (58.5 g, 1.04 mol, 10.0 eq.) are converted to 6.99 g (56.7 mmol, 54%) 2-methyl-3-propyl-1*H*-pyrrole (**4b**). The product is obtained as yellow volatile oil after purification with petroleum ether/CH_2Cl_2 (85 : 15) + 1% triethylamine. δ^1H (600 MHz, $CDCl_3$) 0.95 (3 H, t, J = 7.3 Hz, 3″-H), 1.56 (2 H, h, J = 7.5 Hz, 2″-H), 2.19 (3 H, s, 1′-H), 2.37 (2 H, t, J = 7.6 Hz, 1″-H), 6.02 (1 H, t, J = 2.7 Hz, 4-H), 6.60 (1 H, t, J = 2.7 Hz, 5-H), 7.70 (1 H, brs, 1-NH). δ^{13}C (151 MHz, $CDCl_3$) 11.2 (C-1′), 14.2 (C-3″), 24.6 (C-2″), 28.2 (C-1″), 109.0 (C-4), 114.9 (C-5), 119.7 (C-3), 123.4 (C-2). FT-IR (neat, cm^{-1}): 3378, 2956, 2925, 2869, 1464, 1455, 1376, 1249, 1106, 1066, 955, 904, 889, 832, 801, 712, 663, 549. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_8H_{14}N$ 124.1121, found 124.1120. t_R (LC-MS method): 7.90 min.

3-Heptyl-2-methyl-1*H*-pyrrole (4c). Decan-2-one oxime (**9c**, 7.50 g, 43.8 mmol, 1.00 eq.), 1,2-dichloroethane (12.1 mL, 153 mmol, 3.50 eq.), and KOH (24.6 g, 438 mmol, 10.0 eq.) are converted to 4.11 g (22.9 mmol, 52%) 3-heptyl-2-methyl-1*H*-pyrrole (**4c**). The product is obtained as yellow volatile oil after purification with petroleum ether/CH_2Cl_2 (85 : 15) + 1% triethylamine. δ^1H (600 MHz, $CDCl_3$) 0.89 (3 H, t, 7″-H), 1.23–1.38 (8 H, m, 3″-H, 4″-H, 5″-H, 6″-H), 1.48–1.58 (2 H, m, 2″-H), 2.19 (3 H, s, 1′-H), 2.39 (2 H, t, 1″-H), 6.02 (1 H, t, J = 2.8 Hz, 4-H), 6.60 (1 H, t, J = 2.7 Hz, 5-H), 7.71 (1 H, brs, 1-NH). δ^{13}C (151 MHz, $CDCl_3$) 11.2 (C-1′), 14.3 (C-7″), 22.8 (C-6″), 26.1 (C-1″), 29.4 (C-3″), 29.7 (C-4″), 31.5 (C-2″), 32.1 (C-5″), 109.0 (C-4), 114.9 (C-5), 119.9 (C-3), 123.3 (C-2). FT-IR (neat, cm^{-1}): 3485, 3377, 2955, 2923, 2871, 1583, 1463, 1445, 1377, 1272, 1247, 1144, 1108, 1064, 953, 901, 831, 711, 668, 646, 634, 548, 485, 468. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{12}H_{22}N$ 180.1747, found 180.1749. t_R (LC-MS method): 9.01 min.

General procedure for the synthesis of (*E*/*Z*)-oximes 9a–c. The synthesis of oximes was performed following a published procedure of Mo *et al.*[85] To a 500 mL round bottom flask charged with a stir bar, sodium acetate (2.00 eq.) and hydroxylamine hydrochloride (1.50 eq.) are added a solution of the ketone (1.00 eq., 0.30 M) in EtOH/water (4 : 1). The reaction mixture is then heated for 4 h to reflux. The mixture is cooled to room temperature and excess of EtOH is removed under reduced pressure. Water (100 mL) is added to the crude mixture and the resulting aqueous solution is extracted with EtOAc (3 × 150 mL). The combined organic layer is washed with saturated $NaHCO_3$ (2 × 100 mL) and water (2 × 100 mL), dried over $MgSO_4$, filtered over degreased cotton wool, and concentrated *in vacuo*. The oxime products are obtained in quantitative yields as volatile oils and used without further purification.

(*E*/*Z*)-Octan-2-one oxime (9a). Following the general procedure for the synthesis of oximes, octan-2-one (**8a**, 12.0 g, 93.6 mmol, 1.00 eq.), hydroxylamine hydrochloride (9.76 g, 140 mmol, 1.50 eq.), and sodium acetate (15.36 g, 187.2 mmol, 2.00 eq.) are converted to 13.35 g (93.21 mmol, >99%) (*E*/*Z*)-octan-2-one oxime (**9a**) as colourless oil. *E*/*Z* ratio: 76:24 (NMR). δ^1H (600 MHz, $CDCl_3$, *E*-isomer) 0.872 (3 H, t, 8-H), 1.22–1.36 (6 H, m, 5-H, 6-H, 7-H), 1.45–1.53 (2 H, m, 4-H), 1.87 (3 H, s, 1-H), 2.12–2.24 (2 H, m, 3-H), 8.58 (1 H, brs, 10-H). δ^{13}C (151 MHz, $CDCl_3$, *E*-isomer) 13.5 (C-1), 14.2 (C-8), 22.7 (C-7), 26.4 (C-4), 29.0 (C-5), 31.70 (C-6), 35.9 (C-3), 158.9 (C-2). δ^1H (600 MHz, $CDCl_3$, *Z*-isomer) 0.879 (3 H, t, 8′-H), 1.22–1.36 (6 H, m, 5′-H, 6′-H, 7′-H), 1.45–1.53 (2 H, m, 4′-H), 1.86 (3 H, s, 1′-H), 2.32–2.41 (2 H, m, 3′-H), 8.58 (1 H, brs, 10′-H). δ^{13}C (151 MHz, $CDCl_3$, *Z*-isomer) 14.2 (C-8′), 19.9 (C-1′), 22.7 (C-7), 25.6 (C-4′), 28.8 (C-3′), 29.5 (C-5′), 31.73 (C-6′), 159.3 (C-2′). FT-IR (neat, cm^{-1}): 3224, 3120, 2955, 2926, 2858, 1664, 1466, 1458, 1376, 1369, 1261, 1247, 1179, 1111, 1031, 954, 943, 828, 796, 742, 726, 651, 613. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_8H_{18}NO$ 144.1383, found 144.1383.

(*E*/*Z*)-Hexan-2-one oxime (9b). Following the general procedure for the synthesis of oximes, hexan-2-one (**8b**, 15.0 g, 150 mmol, 1.00 eq.), hydroxylamine hydrochloride (15.7 g, 226 mmol, 1.50 eq.), and sodium acetate (24.6 g, 300 mmol, 2.00 eq.) are converted to 16.9 g (147 mmol, 98%) (*E*/*Z*)-hexan-2-one oxime (**9b**) as pale yellow oil. *E*/*Z* ratio: 73 : 27 (NMR). δ^1H (600 MHz, $CDCl_3$, *E*-isomer) 0.90 (3 H, t, J = 7.4 Hz, 6-H), 1.32 (2 H, h, J = 7.3 Hz, 5-H), 1.44–1.52 (2 H, m, 4-H), 1.87 (3 H, s, 1-H), 2.15–2.23 (2 H, m, 3-H), 9.09 (1 H, brs, 8-H). δ^{13}C (151 MHz, $CDCl_3$, *E*-isomer) 13.5 (C-1), 13.9 (C-6), 22.4 (C-5), 28.5 (C-4), 35.6 (C-3), 158.8 (C-2). δ^1H (600 MHz, $CDCl_3$, *Z*-isomer) 0.92 (3 H, t, J = 7.30 Hz, 6′-H), 1.36 (2

H, h, J = 7.4 Hz, 5′-H), 1.44–1.52 (2 H, m, 4′-H), 1.85 (3 H, s, 1′-H), 2.34–2.40 (2 H, m, 3′-H), 9.09 (1 H, brs, 8′-H). δ^{13}C (151 MHz, $CDCl_3$, *Z*-isomer) 14.0 (C-6′), 20.0 (C-1′), 23.0 (C-5′), 27.8 (C-4′), 28.5 (C-3′), 159.2 (C-2′). FT-IR (neat, cm^{-1}): 3222, 3118, 2957, 2928, 2873, 2862, 1664, 1467, 1433, 1368, 1331, 1262, 1207, 1105, 1010, 951, 919, 878, 823, 742, 648, 613. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_6H_{14}NO$ 116.1070, found 116.1071.

(*E*/*Z*)-Decan-2-one oxime (9c). Following the general procedure for the synthesis of oximes, decan-2-one (**8c**, 7.50 g, 48.0 mmol, 1.00 eq.), hydroxylamine hydrochloride (5.00 g, 72.0 mmol, 1.50 eq.), and sodium acetate (7.88 g, 96.1 mmol, 2.00 eq.) are converted to 8.22 g (48.0 mmol, >99%) (*E*/*Z*)-decan-2-one oxime (**9c**) as colourless oil. *E*/*Z* ratio: 76 : 24 (NMR). δ^1H (600 MHz, $CDCl_3$, *E*-isomer) 0.871 (3 H, t, 10-H), 1.19–1.35 (10 H, m, 5-H, 6-H, 7-H, 8-H, 9-H), 1.49 (2 H, p, 4-H), 1.88 (3 H, s, 1-H), 2.14–2.20 (2 H, m, 3-H), 8.14 (1 H, brs, 12-H). δ^{13}C (151 MHz, $CDCl_3$, *E*-isomer) 159.0 (C-2), 35.9 (C-3), 32.0 (C-8), 29.5 (C-6), 29.33 (C-5), 29.32 (C-7), 26.4 (C-4), 22.8 (C-9), 14.2 (C-10), 13.5 (C-1). δ^1H (600 MHz, $CDCl_3$, *Z*-isomer) 0.874 (3 H, t, 10′-H), 1.19–1.35 (10 H, m, 5′-H, 6′-H, 7′-H, 8′-H, 9′-H), 1.49 (2 H, p, 4′-H), 1.86 (3 H, s, 1′-H), 2.33–2.38 (2 H, m, 3′-H), 8.14 (1 H, brs, 12′-H). δ^{13}C (151 MHz, $CDCl_3$, *Z*-isomer) 159.4 (C-2′), 32.0 (C-8′), 29.9 (C-6′), 29.5 (C-5′), 29.32 (C-7′), 28.7 (C-3′), 25.6 (C-4′), 22.8 (C-9′), 19.9 (C-1′), 14.2 (C-10′). FT-IR (neat, cm^{-1}): 3223, 3117, 2954, 2924, 2855, 1664, 1464, 1458, 1377, 1368, 1258, 1217, 1168, 1113, 994, 956, 943, 830, 823, 724, 651, 614. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{10}H_{22}NO$ 172.1696, found 172.1699.

General procedure for the synthesis of Boc-protected pyrroles 14b–g

Acylation of 1H-pyrrole. The 2-acylation of 1*H*-pyrrole was performed according to the protocol of Ono *et al.*[66] In a Schlenk flask under N_2 atmosphere the acid chloride **11c–g** (1.00 eq.) is dissolved in dry CH_2Cl_2 (3.74 mL mmol^{-1} acid chloride) and cooled to 0 °C. Aluminium trichloride (1.20 eq.) is added slowly to give a yellow suspension and the mixture is allowed to warm to 24 °C over 30 min. The solution is cooled to 0 °C again and a solution of 1*H*-pyrrole (1.10 eq.) in dry CH_2Cl_2 (0.42 mL mmol^{-1} acid chloride) is transferred slowly to the acid chloride solution. The reaction is stirred for further 1 h at 0 °C, then slowly thawed to 21 °C and stirred overnight. A saturated solution of NH_4Cl is used at 0 °C for reaction quenching. The phases are separated, the aqueous phase subsequently extracted with CH_2Cl_2 (3 × 150 mL) and the combined organic phases washed with saturated $NaHCO_3$ (2 × 150 mL) and brine (1 × 150 mL). Drying over $MgSO_4$, filtration over degreased cotton wool and solvent removal *in vacuo* provides the crude acylation product **12c–g** as brown oil, which is used without further purification and contains mostly 2-acylated 1*H*-pyrrole.

Reduction of acylated 1H-pyrrole. The reduction of 2-acylated 1*H*-pyrroles to 2-alkyl-1*H*-pyrroles was performed according to the protocol of Fürstner *et al.*[1] Under N_2 atmosphere sodium borohydride (2.80 eq.) is suspended in dry isopropyl alcohol (0.90 mL mmol^{-1} acylated pyrrole) and cooled to 0 °C. The crude acylated pyrrole **12c–g** (1.00 eq.) is dissolved in dry isopropyl alcohol (3.15 mL mmol^{-1} acylated pyrrole), precooled to 0 °C and added to the sodium borohydride suspension at 0 °C. Afterwards, the solution is refluxed overnight at 99 °C. The reaction is quenched with water at 0 °C, additional water (300 mL) is added and the product extracted with MTBE (3 × 200 mL). Merged organic phases are washed with brine (2 × 200 mL), dried over $MgSO_4$, and filtered over degreased cotton wool. After solvent removal, the oily brown crude product, which contains mostly 2-alkylated 1*H*-pyrrole **13c–g**, is used without further purification.

Boc-protection of alkylated 1H-pyrrole. Boc-protection of 2-alkyl-1*H*-pyrroles was realised according to the protocol of Fürstner *et al.*[1] In a Schlenk flask under N_2 atmosphere crude alkylated 1*H*-pyrrole **13c–g** or commercially available 2-methyl-1*H*-pyrrole (**13b**) (1.00 eq.) and DMAP (0.10 eq.) are dissolved in dry CH_2Cl_2 (1.42 mL mmol^{-1} alkylated pyrrole). A solution of Boc_2O (1.20 eq.) in dry CH_2Cl_2 (2.37 mL mmol^{-1} alkylated pyrrole) is added to the prior solution at ambient temperature and the reaction is stirred overnight. The solvent is removed under reduced pressure and the residue purified by chromatography on silica using *n*-pentane/MTBE (99 : 1). The Boc-protected 2-alkylated 1*H*-pyrroles **14b–g** are afforded as colourless to yellow liquids.

1-*tert*-Butyloxycarbonyl 2-methyl-1*H*-pyrrole (14b). In a Schlenk flask under N_2 atmosphere are 2-methyl-1*H*-pyrrole (**13b**, 3.03 g, 37.4 mmol, 1.40 eq.) and DMAP (326 mg, 2.67 mmol, 0.10 eq.) dissolved in 20 mL dry CH_2Cl_2. A solution of Boc_2O (5.83 g, 26.7 mmol, 1.00 eq.) in CH_2Cl_2 (100 mL) is added to the pyrrole at 20 °C, turning instantly yellow, and the reaction stirred for 18 h at 21 °C. The solvent is removed under reduced pressure and the orange residue purified by chromatography on silica using *n*-pentane/MTBE (98 : 2), yielding 3.88 g (21.39 mmol, 80%) of a colourless liquid. δ^1H (600 MHz, $CDCl_3$) 1.59 (9 H, s, 10-H, 11-H, 12-H), 2.43 (3 H, d, J = 1.1 Hz, 1′-H), 5.92 (1 H, tt, J = 1.9 Hz, J = 1.0 Hz, 3-H), 6.06 (1 H, t, J = 3.3 Hz, 4-H), 7.18 (1 H, dd, J = 3.4 Hz, J = 1.8 Hz, 5). δ^{13}C (151 MHz, $CDCl_3$) 15.6 (C-1′), 28.2 (C-10, C-11, C-12), 83.4 (C-9), 110.0 (C-4), 111.9 (C-3), 120.7 (C-5), 131.7 (C-2), 149.9 (C-6). FT-IR (neat, cm^{-1}): 2979, 2928, 1737, 1497, 1396, 1370, 1329, 1308, 1254, 1237, 1170, 1127, 1065, 983, 883, 851, 798, 772, 715, 676, 597, 552. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{10}H_{16}NO_2$ 182.1176, found 182.1176.

1-*tert*-Butyloxycarbonyl 2-ethyl-1*H*-pyrrole (14c). Following the general procedure for the synthesis of Boc-protected pyrroles, 11.8 g (60.3 mmol, 48% over three steps) 1-*tert*-butyloxycarbonyl 2-ethyl-1*H*-pyrrole (**14c**) was synthesised from acetyl chloride (**11c**, 9.95 g, 127 mmol, 1.00 eq.) and obtained as pale yellow liquid. δ^1H (600 MHz, $CDCl_3$) 1.23 (3 H, t, J = 7.4 Hz, 2″-H), 1.59 (9 H, s, 10-H, 11-H, 12-H), 2.87 (2 H, qd, J = 7.4 Hz, 1′-H), 5.96 (1 H, tt, J = 3.0, 3-H), 6.08 (1 H, t, J 3.3, 4-H), 7.19 (1 H, dd, J = 3.4 Hz, 5-H). δ^{13}C (151 MHz, $CDCl_3$) 13.4 (C-2″), 22.4 (C-1′), 28.2 (C-10, C-11, C-12), 83.4 (C-9), 110.0 (C-3), 110.0 (C-4), 120.9 (C-5), 138.2 (C-2), 149.7 (C-6). FT-IR (neat, cm^{-1}): 2973, 2934, 2877, 1739, 1497, 1479,

1458, 1409, 1395, 1369, 1326, 1293, 1256, 1233, 1167, 1132, 1124, 1066, 1058, 1047, 1015, 977, 953, 882, 853, 840, 814, 772, 719. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{11}H_{18}NO_2$ 196.1332, found 196.1331.

1-*tert*-Butyloxycarbonyl 2-propyl-1*H*-pyrrole (14d). Following the general procedure for the synthesis of Boc-protected pyrroles, 12.5 g (59.8 mmol, 52% over three steps) 1-*tert*-butyloxycarbonyl 2-propyl-1*H*-pyrrole (**14d**) was synthesised from propanoic acid chloride (**11d**, 10.7 g, 115 mmol, 1.00 eq.) and obtained as colourless liquid. δ^1H (600 MHz, $CDCl_3$) 0.98 (3 H, t, *J* = 7.4 Hz, 3′-H), 1.59 (9 H, s, 10-H, 11-H, 12-H), 1.64 (2 H, h, *J* = 7.4 Hz, 2′-H), 2.75–2.87 (2 H, m, 1′-H), 5.95 (1 H, ddt, *J* = 3.0 Hz, *J* = 2.0 Hz, 3-H), 6.08 (1 H, t, *J* = 3.3 Hz, 4-H), 7.19 (1 H, dd, *J* = 3.4 Hz, *J* = 1.8 Hz, 5-H). δ^{13}C (151 MHz, $CDCl_3$) 14.1 (C-3′), 22.3 (C-2′), 28.2 (C-10, C-11, C-12), 31.1 (C-1′), 83.3 (C-9), 110.0 (C-4), 111.0 (C-3), 120.9 (C-5), 136.5 (C-2), 149.7 (C-6). FT-IR (neat, cm^{-1}): 3006, 2961, 2933, 2873, 1739, 1495, 1479, 1457, 1436, 1407, 1394, 1369, 1327, 1318, 1254, 1169, 1127, 1060, 1010, 972, 907, 895, 884, 849, 801, 772, 716, 599, 495. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{12}H_{20}NO_2$ 210.1489, found 210.1488.

1-*tert*-Butyloxycarbonyl 2-butyl-1*H*-pyrrole (14e). Following the general procedure for the synthesis of Boc-protected pyrroles, 9.55 g (42.8 mmol, 44% over three steps) 1-*tert*-butyloxycarbonyl 2-butyl-1*H*-pyrrole (**14e**) was synthesised from butanoic acid chloride (**11e**, 10.3 g, 96.5 mmol, 1.00 eq.) and obtained as pale yellow oil. δ^1H (600 MHz, $CDCl_3$) 0.94 (3 H, t, *J* = 7.4 Hz, 4′-H), 1.40 (2 H, h, *J* = 7.4 Hz, 3′-H), 1.59 (9 H, s, 10-H, 11-H, 12-H), 1.56–1.63 (2 H, m, 2′-H), 2.80–2.88 (2 H, m), 5.95 (1 H, td, *J* = 1.9 Hz, 3-H), 6.07 (1 H, t, *J* = 3.3 Hz, 4-H), 7.19 (1 H, dd, *J* = 3.4 Hz, *J* = 1.8 Hz, 5-H). δ^{13}C (151 MHz, $CDCl_3$) 14.2 (C-4′), 22.7 (C-3′), 28.2 (C-10, C-11, C-12), 28.8 (C-1′), 31.2 (C-2′), 83.3 (C-9), 110.0 (C-4), 110.8 (C-3), 120.9 (C-5), 136.7 (C-2), 149.7 (C-6). FT-IR (neat, cm^{-1}): 2958, 2932, 2872, 2863, 1737, 1495, 1478, 1458, 1407, 1394, 1369, 1325, 1315, 1254, 1235, 1221, 1166, 1123, 1059, 1013, 999, 883, 852, 804, 772, 714, 599. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{13}H_{22}NO_2$ 224.1645, found 224.1641.

1-*tert*-Butyloxycarbonyl 2-pentyl-1*H*-pyrrole (14f). Following the general procedure for the synthesis of Boc-protected pyrroles, 16.9 g (71.1 mmol, 59% over three steps) 1-*tert*-butyloxycarbonyl 2-pentyl-1*H*-pyrrole (**14f**) was synthesised from pentanoic acid chloride (**11f**, 14.5 g, 120 mmol, 1.00 eq.) and obtained as colourless oil. δ^1H (600 MHz, $CDCl_3$) 0.84–0.97 (3 H, m, 5′-H), 1.36 (4 H, ddd, *J* = 7.1 Hz, 3′-H, 4′-H), 1.59 (9 H, s), 1.62 (2 H, q, *J* = 7.7 Hz, 2′-H), 2.76–2.88 (2 H, m, 1′-H), 5.95 (1 H, ddt, *J* = 3.0 Hz, *J* = 2.0 Hz, 3-H), 6.07 (1 H, t, *J* = 3.3 Hz, 4-H), 7.19 (1 H, dd, *J* = 3.4 Hz, *J* = 1.8 Hz, 5-H). δ^{13}C (151 MHz, $CDCl_3$) 14.2 (C-5′), 22.7 (C-4′), 28.2 (C-10, C-11, C-12), 28.8 (C-2′), 29.0 (C-1′), 31.8 (C-3′), 83.3 (C-9), 110.0 (C-4), 110.8 (C-3), 120.9 (C-5), 136.7 (C-2), 149.7 (C-6). FT-IR (neat, cm^{-1}): 2957, 2931, 2871, 2861, 1738, 1495, 1478, 1458, 1408, 1394, 1369, 1326, 1317, 1254, 1235, 1166, 1125, 1059, 1010, 963, 883, 851, 844, 800, 772, 715, 599, 560, 497, 461. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{14}H_{24}NO_2$ 238.1802, found 238.1801.

1-*tert*-Butyloxycarbonyl 2-hexyl-1*H*-pyrrole (14g). Following the general procedure for the synthesis of Boc-protected pyrroles, 12.1 g (48.2 mmol, 43% over three steps) 1-*tert*-butyloxycarbonyl 2-hexyl-1*H*-pyrrole (**14g**) was synthesised from hexanoic acid chloride (**11g**, 15.0 g, 111 mmol, 1.00 eq.) and obtained as colourless oil. δ^1H (600 MHz, $CDCl_3$) 0.83–0.94 (3 H, m, 6′-H), 1.24–1.35 (4 H, m, 4′-H, 5′-H), 1.34–1.42 (2 H, m, 3′-H), 1.57–1.64 (2 H, m, 2′-H), 1.59 (9 H, s, 10-H, 11-H, 12-H), 2.77–2.86 (2 H, m, 1′-H), 5.95 (1 H, td, *J* = 1.9 Hz, 3-H), 6.07 (1 H, t, *J* = 3.3 Hz, 4-H), 7.19 (1 H, dd, *J* = 3.4 Hz, *J* = 1.8 Hz, 5-H). δ^{13}C (151 MHz, $CDCl_3$) 14.2 (C-6′), 22.8 (C-5′), 28.2 (C-10, C-11, C-12), 29.1 (C-3′), 29.3 (C-2′), 31.9 (C-4′), 83.3 (C-9), 110.0 (C-4), 110.8 (C-3), 120.9 (C-5), 136.7 (C-2), 149.7 (C-6). FT-IR (neat, cm^{-1}): 2956, 2927, 2858, 1740, 1495, 1478, 1458, 1407, 1394, 1369, 1327, 1319, 1253, 1234, 1165, 1127, 1061, 1008, 887, 851, 803, 772, 715. HRMS-ESI (*m*/*z*): $[M + H]^+$ calculated for $C_{15}H_{26}NO_2$ 252.1958, found 252.1959.

General procedure for the synthesis of pyrrole-2-boronic acids 15b–g. Following the procedure of Cai and Snider, Boc-protected pyrrole-2-boronic acids were accessed.[86] In a Schlenk flask under N_2 atmosphere *n*-butyl lithium (2.10 eq., 2.5 M in hexane) is added dropwise to a solution of distilled 2,2,6,6-tetramethylpiperidine (2.00 eq.) in dry THF (2.36 mL $mmol^{-1}$ pyrrole) at −78 °C. After stirring for 15 min the mixture is allowed to warm to 0 °C over 30 min and then cooled again to −78 °C. In a three necked flask is Boc-2-alkyl-1*H*-pyrrole **14b–g** (1.00 eq.) dissolved in dry THF (4.00 mL $mmol^{-1}$ pyrrole) and cooled to −78 °C. The LiTMP is then transferred dropwise to the pyrrolic solution in THF with a transfer cannula. The internal temperature is constantly kept below −70 °C and the rate adjusted if needed. The reaction mixture is stirred for 2 h at −78 °C, before trimethyl borate (3.00 eq.) in THF (1.07 mL $mmol^{-1}$ pyrrole) is added slowly. The solution is stirred for 15 min at −78 °C, then 30 min at 0 °C, and finally at 21 °C overnight. The reaction is quenched at 0 °C by dropwise addition of 0.20 M HCl (aq.) (2.10 eq.). The aqueous phase is extracted with Et_2O (3 × 50 mL). Merged organic phases are washed with water (100 mL), brine (100 mL), and dried over $MgSO_4$. The solvent is almost completely removed by evaporation at room temperature (water bath at 20 °C). The residue in Et_2O/THF is tansferred to a Schlenk flask and residual solvent is removed *in vacuo* while stirring to prevent bumping and the crude product is recovered as viscous orange solid or oil. The delicate crude product is used without further purification for the next synthetic step on the same day.

NMR data of crude boronic acids

(1-(*tert*-Butoxycarbonyl)-5-methyl-1*H*-pyrrol-2-yl)boronic acid (15b). δ^1H (300 MHz, $CDCl_3$) 7.09 (2 H, brs, 7-H, 8-H), 7.00 (1 H, d, *J* = 3.3 Hz, 3-H), 6.01 (1 H, dd, *J* = 3.2 Hz, 4-H), 2.42 (3 H, s, 1′-H), 1.62 (9 H, s, 13-H, 14-H, 15-H). δ^{13}C (76 MHz, $CDCl_3$) 153.4 (C-9), 137.8 (C-5), 127.6 (C-3), 113.8 (C-4), 85.9 (C-12), 28.2 (C-13, C-14, C-15), 17.8 (C-1′). δ^{11}B (96 MHz, $CDCl_3$) 26.5.

(1-(*tert*-Butoxycarbonyl)-5-ethyl-1*H*-pyrrol-2-yl)boronic acid (15c). δ^1H (300 MHz, $CDCl_3$) 7.01 (1 H, d, J = 3.4 Hz, 1-H), 6.66 (2 H, brs, H-14, H-15), 6.06 (1 H, dt, J = 3.4 Hz, 2-H), 2.83 (2 H, q, J = 7.5 Hz, 16-H), 1.63 (9 H, s, 10-H, 11-H, 12-H), 1.27–1.19 (3 H, m, 18-H). δ^{13}C (76 MHz, $CDCl_3$) 153.4 (C-6), 144.1 (C-3), 127.5 (C-1), 111.8 (C-2), 85.9 (C-9), 28.1 (C-10, C-11, C-12), 24.1 (C-16), 13.9 (C-18). δ^{11}B (96 MHz, $CDCl_3$) 25.8.

(1-(*tert*-Butoxycarbonyl)-5-propyl-1*H*-pyrrol-2-yl)boronic acid (15d). δ^1H (300 MHz, $CDCl_3$) 7.00 (1 H, d, J = 3.3 Hz, 1-H), 6.04 (1 H, d, J = 3.3 Hz, 2-H), 2.77 (2 H, t, J = 7.8 Hz, 16-H), 1.67–1.58 (11 H, m, H-13, H-14, H-15, H-18), 0.97 (3 H, t, J = 7.5 Hz, 19-H). δ^{13}C (76 MHz, $CDCl_3$) 153.3 (C-6), 142.2 (C-3), 127.2 (C-1), 112.8 (C-2), 85.8 (C-12), 32.8 (C-16), 28.0 (C-13, C-14, C-15), 22.7 (C-18), 13.9 (C-19). δ^{11}B (96 MHz, $CDCl_3$) 25.0.

(1-(*tert*-Butoxycarbonyl)-5-butyl-1*H*-pyrrol-2-yl)boronic acid (15e). δ^1H (300 MHz, $CDCl_3$) 7.00 (1 H, d, J = 3.3 Hz, 1-H), 6.71 (2 H, brs, 14-H, 15-H), 6.04 (1 H, d, J = 3.3 Hz, 2-H), 2.80 (2 H, t, J = 7.6 Hz, 16-H), 1.63 (9 H, s, 10-H, 11-H, 12-H), 1.62–1.52 (2 H, m, 18-H), 1.47–1.33 (2 H, m, 19-H), 0.94 (3 H, t, J = 7.3 Hz, 20-H). δ^{13}C (76 MHz, $CDCl_3$) 153.4 (C-6), 142.6 (C-3), 127.4 (C-1), 112.6 (C-2), 85.9 (C-9), 31.6 (C-18), 30.6 (C-16), 28.1 (C-10, C-11, C-12), 22.7 (C-19), 14.1 (C-20). δ^{11}B (96 MHz, $CDCl_3$) 25.5.

(1-(*tert*-Butoxycarbonyl)-5-pentyl-1*H*-pyrrol-2-yl)boronic acid (15f). δ^1H (300 MHz, $CDCl_3$) 7.35 (2 H, brs, 7-H, 8-H), 7.02 (1 H, d, J = 3.4 Hz, 3-H), 6.04 (1 H, d, J = 3.4 Hz, 4-H), 2.79 (2 H, t, J = 7.7 Hz, 1′-H), 1.68–1.51 (11 H, m, 2′-H, 13-H, 14-H, 15-H), 1.40–1.28 (4 H, m, 3′-H, 4′-H), 0.96–0.85 (3 H, m_c, 5′-H). δ^{13}C (76 MHz, $CDCl_3$) 153.4 (C-9), 142.6 (C-5), 127.5 (C-3), 112.7 (C-4), 85.9 (C-12), 31.8 (C-3′), 30.9 (C-1′), 29.2 (C-2′), 28.1 (C-13, C-14, C-15), 22.7 (C-4′), 14.2 (C-5′). δ^{11}B (96 MHz, $CDCl_3$) 23.8.

(1-(*tert*-Butoxycarbonyl)-5-hexyl-1*H*-pyrrol-2-yl)boronic acid (15g). δ^1H (300 MHz, $CDCl_3$) 7.00 (1 H, d, J = 3.3 Hz, 1-H), 6.04 (1 H, d, J = 3.4 Hz, 2-H), 2.79 (2 H, t, J = 7.6 Hz, 16-H), 1.63 (9 H, s, 13-H, 14-H, 15-H), 1.62–1.53 (2 H, m, 18-H), 1.43–1.22 (6 H, m, 19-H, 20-H, 21-H), 0.94–0.83 (3 H, m_c, 22-H). δ^{13}C (76 MHz, $CDCl_3$) 153.4 (C-6), 142.6 (C-3), 127.4 (C-1), 112.6 (C-2), 85.8 (C-12), 31.9 (C-20), 30.9 (C-16), 29.5 (C-18), 29.3 (C-19), 28.1 (C-13, C-14, C-15), 22.7 (C-21), 14.2 (C-22). δ^{11}B (96 MHz, $CDCl_3$) 25.5.

General procedure for the synthesis of prodiginines 16ba–bc. MBC and pyrrole derivatives were transformed to prodiginines under acid catalysis as performed by Boger and Patel.[87] An MBC derivative **3a–g** (1.00 eq.) is dissolved in MeOH (10 mM solution). After addition of 1*H*-pyrrole **4a–c** (2.00 eq.), the solution is cooled to 0 °C. After 15 min of stirring, 1.25 M HCl in MeOH (1.80 eq.) is added dropwise. With completed HCl addition, the reaction is thawed and then stirred at 21 °C overnight. Water is added and the mixture then quenched with 25% NH_3 (aq.). The product is extracted with CH_2Cl_2 (3 × 50 mL). Merged organic phases are dried over $MgSO_4$ and filtered over degreased cotton wool. After removal of the solvent, the residue is chromatographed on silica with CH_2Cl_2 and 0.7% 7 N NH_3 in MeOH. Product containing fractions are merged and chromatographed on silica with *n*-pentane/CH_2Cl_2 (60 : 40) and 4% 7 N NH_3 in MeOH to remove unconverted MBC precursor. Evaporation of the solvent and acidification with 1 M HCl in Et_2O yields a purple film that is precipitated by repeated addition of petroleum ether. A deep purple amorphous solid is obtained.

(*Z*)-4′-Methoxy-5-methyl-5′-((5-methyl-4-propyl-1*H*-pyrrol-2-yl)methylene)-1*H*,5′*H*-[2,2′-bipyrrol]-1′-ium chloride (16bb). Following the general procedure for the synthesis of prodiginines, carbaldehyde **3b** (54.0 mg, 0.26 mmol, 1.00 eq.), pyrrole **4b** (65.2 mg, 0.53 mmol, 2.00 eq.) and HCl in MeOH (381 µL, 0.48 mmol, 1.80 eq.) were converted to 60.1 mg (0.17 mmol, 66%, 95.7% ± 0.85% purity by qNMR) of a deep purple amorphous solid. δ^1H (300 MHz, $CDCl_3$) 0.92 (3 H, t, J = 7.3 Hz, 9″-H), 1.54 (2 H, h, J = 7.4, 8″-H), 2.34 (2 H, t, J = 7.5 Hz, 7″-H), 2.43 (3 H, s, 6-H), 2.50 (3 H, s, 6″-H), 3.94 (3 H, s, 7′-H), 5.97 (1 H, d, 3′-H), 6.03 (1 H, t, 4-H), 6.59 (1 H, d, 3″-H), 6.82 (2 H, d, 3-H, 6′-H), 12.38 (1 H, brs, 1′-NH), 12.44–12.63 (2 H, brs, 1-NH, 1″-NH). δ^{13}C (76 MHz, $CDCl_3$) 12.3 (C-6″), 13.6 (C-6), 13.9 (C-9″), 23.5 (C-8″), 27.5 (C-7″), 58.7 (C-7′), 92.6 (C-3′), 111.4 (C-4), 114.6 (C-6′), 118.9 (C-3), 121.0 (C-2), 121.3 (C-5′), 124.9 (C-2″), 127.2 (C-3″), 127.5 (C-4″), 139.6 (C-5), 145.1 (C-5″), 147.7 (C-2′), 165.7 (C-4′). FT-IR (neat, cm^{-1}): 3221, 3164, 3143, 3115, 3115, 3105, 3066, 3010, 2958, 2914, 2873, 2859, 2854, 2818, 1635, 1608, 1552, 1537, 1517, 1493, 1441, 1415, 1403, 1370, 1338, 1252, 1208, 1184, 1159, 1128, 1083, 1045, 995, 980, 967, 909, 891, 884, 867, 844, 823, 792, 769, 752, 743, 732, 668, 647, 638, 621, 548, 478. T_m: 158.6–160.5 °C (petroleum ether). HRMS-ESI (*m*/*z*): $[M–Cl]^+$ calculated for $C_{19}H_{24}N_3O$ 310.1914, found 310.1911. t_R (LC-MS method): 7.56 min.

(*Z*)-4′-Methoxy-5-methyl-5′-((5-methyl-4-pentyl-1*H*-pyrrol-2-yl)methylene)-1*H*,5′*H*-[2,2′-bipyrrol]-1′-ium chloride (16ba). Following the general procedure for the synthesis of prodiginines, carbaldehyde **3b** (100 mg, 0.49 mmol, 1.00 eq.), pyrrole **4a** (148 mg, 0.98 mmol, 2.00 eq.) and HCl in MeOH (705 µL, 0.88 mmol, 1.80 eq.) were converted to 150 mg (0.40 mmol, 82%, 89.5% ± 0.94% purity by qNMR) of a deep purple amorphous solid. δ^1H (300 MHz, $CDCl_3$) 0.80–0.94 (4 H, m, 11″-H), 1.30 (4 H, qq, 9″-H, 10″-H), 1.53 (2 H, p, J = 7.4 Hz, 8″-H), 2.38 (2 H, t, J = 7.6 Hz, 7″-H), 2.45 (3 H, s, 6-H), 2.52 (3 H, s, 6″-H), 3.97 (3 H, s, 7′-H), 6.00 (1 H, d, J 1.9, 3′-H), 6.03–6.11 (1 H, m, 4-H), 6.61 (1 H, d, 3″-H), 6.81–6.87 (2 H, m, 3-H, 6′-H), 12.44 (1 H, brs, 1′-NH), 12.54 (2 H, brs, 1-NH, 1″-NH). δ^{13}C (76 MHz, $CDCl_3$) 12.4 (C-6″), 13.7 (C-6), 14.2 (C-11″), 22.6 (C-10″), 25.5 (C-7″), 30.1 (C-8″), 31.6 (C-9″), 58.8 (C-7′), 92.6 (C-3′), 111.4 (C-4), 114.7 (C-6′), 118.9 (C-3), 121.1 (C-2), 121.3 (C-5′), 125.0 (C-2″), 127.3 (C-3″), 127.8 (C-4″), 139.7 (C-5), 145.3 (C-5″), 147.7 (C-2′), 165.8 (C-4′). FT-IR (neat, cm^{-1}): 3171, 3141, 3101, 3067, 2954, 2922, 2855, 1630, 1605, 1579, 1538, 1493, 1447, 1414, 1402, 1348, 1286, 1253, 1207, 1151, 1130, 1099, 1078, 1037, 994, 965, 899, 882, 841, 805, 785, 768, 732, 698, 666, 643, 623, 550, 502. T_m: 77.6–80.1 °C (petroleum ether). HRMS-ESI (*m*/*z*): $[M–Cl]^+$ calculated for $C_{21}H_{28}N_3O$ 338.2227, found 338.2228. t_R (LC-MS method): 7.83 min.

(*Z*)-4′-Methoxy-5-methyl-5′-((5-methyl-4-heptyl-1*H*-pyrrol-2-yl)methylene)-1*H*,5′*H*-[2,2′-bipyrrol]-1′-ium chloride (16bc). Following the general procedure for the synthesis of prodiginines, carbaldehyde **3b** (50.0 mg, 0.24 mmol, 1.00 eq.), pyrrole **4c** (87.8 mg, 0.49 mmol, 2.00 eq.) and HCl in MeOH (353 μL, 0.44 mmol, 1.80 eq.) were converted to 79.0 mg (0.20 mmol, 80%, 97.3% ± 0.87% purity by qNMR) of a deep purple amorphous solid. δ^1H (300 MHz, $CDCl_3$) 0.79–0.92 (3 H, m, 13″-H), 1.17–1.36 (8 H, m, 9″-H, 10″-H, 11″-H, 12″-H), 1.51 (2 H, p, J = 7.3 Hz, 8″-H), 2.36 (2 H, t, J = 7.5 Hz, 7″-H), 2.43 (3 H, s, 6-H), 2.50 (3 H, s, 6″-H), 3.94 (3 H, s, 7′-H), 5.97 (1 H, d, 3′-H), 6.03 (1 H, dd, 4-H), 6.59 (1 H, d, 3″-H), 6.78–6.84 (2 H, m, 3-H, 6′-H), 12.38 (1 H, brs, 1′-NH), 12.50 (2 H, brs, 1-NH, 1″-NH). δ^{13}C (76 MHz, $CDCl_3$) 12.3 (C-6″), 13.6 (C-6), 14.2 (C-13″), 22.8 (C-12″), 25.5 (C-7″), 29.2 (C-9″), 29.3 (C-10″), 30.3 (C-8″), 31.9 (C-11″), 58.7 (C-7′), 92.6 (C-3′), 111.4 (C-4), 114.6 (C-6′), 118.9 (C-3), 121.0 (C-2), 121.2 (C-5′), 125.0 (C-2″), 127.2 (C-3″), 127.7 (C-4″), 139.6 (C-5), 145.1 (C-5″), 147.6 (C-2′), 165.7 (C-4′). FT-IR (neat, cm^{-1}): 3223, 3171, 3144, 3116, 3073, 3062, 2953, 2921, 2851, 1629, 1605, 1581, 1540, 1516, 1494, 1447, 1403, 1369, 1347, 1289, 1257, 1205, 1174, 1089, 1042, 976, 887, 841, 829, 818, 810, 792, 779, 765, 741, 726, 705, 683, 661, 642, 605, 543, 517, 505, 476. T_m: 120.5–122.6 °C (petroleum ether). HRMS-ESI (*m*/*z*): $[M–Cl]^+$ calculated for $C_{23}H_{32}N_3O$ 366.2540, found 366.2546. t_R (LC-MS method): 7.95 min.

(*Z*)-5-Ethyl-4′-methoxy-5′-((5-methyl-4-pentyl-1*H*-pyrrol-2-yl)methylene)-1*H*,5′*H*-[2,2′-bipyrrol]-1′-ium chloride (16ca). Following the general procedure for the synthesis of prodiginines, carbaldehyde **3c** (50.0 mg, 0.24 mmol, 1.00 eq.), pyrrole **4a** (87.8 mg, 0.49 mmol, 2.00 eq.) and HCl in MeOH (353 μL, 0.44 mmol, 1.80 eq.) were converted to 79.0 mg (0.20 mmol, 80%, 94.6% ± 0.59% purity by qNMR) of a deep purple amorphous solid. δ^1H (300 MHz, $CDCl_3$) 0.83–0.92 (3 H, m, 11″-H), 1.21–1.36 (4 H, m, 9″-H, 10″-H), 1.38 (3 H, t, J = 7.6 Hz, 7-H), 1.46–1.59 (2 H, m, 8″-H), 2.37 (2 H, t, 7″-H), 2.51 (3 H, s, 6″-H), 2.80 (2 H, q, J = 7.6 Hz, 6-H), 3.96 (3 H, s, 7′-H), 6.00 (1 H, d, J 1.9, 3′-H), 6.09 (1 H, dd, 4-H), 6.61 (1 H, d, 3″-H), 6.79–6.89 (2 H, m, 3-H, 6′-H), 12.49 (2 H, brs, 1-NH, 1′-NH), 12.58 (1 H, brs, 1″-NH). δ^{13}C (76 MHz, $CDCl_3$) 12.3 (C-6″), 13.4 (C-7), 14.2 (C-11″), 21.6 (C-6), 22.6 (C-10″), 25.5 (C-7″), 30.0 (C-8″), 31.6 (C-9″), 58.7 (C-7′), 92.6 (C-3′), 110.0 (C-4), 114.6 (C-6′), 118.7 (C-3), 121.1 (C-2), 121.3 (C-5′), 125.0 (C-2″), 127.3 (C-3″), 127.8 (C-4″), 145.2 (C-5″), 146.0 (C-5), 147.8 (C-2′), 165.7 (C-4′). FT-IR (neat, cm^{-1}): 3221, 3171, 3145, 3118, 3063, 2950, 2923, 2853, 1632, 1606, 1580, 1547, 1540, 1493, 1447, 1402, 1375, 1360, 1308, 1290, 1257, 1202, 1174, 1146, 1077, 1058, 1043, 1004, 985, 969, 887, 841, 820, 807, 785, 764, 737, 648, 626, 610. T_m: 111.9–117.3 °C (petroleum ether). HRMS-ESI (*m*/*z*): $[M–Cl]^+$ calculated for $C_{22}H_{30}N_3O$ 352.2383, found 352.2387. t_R (LC-MS method): 7.92 min.

(*Z*)-4′-Methoxy-5-propyl-5′-((5-methyl-4-pentyl-1*H*-pyrrol-2-yl)methylene)-1*H*,5′*H*-[2,2′-bipyrrol]-1′-ium chloride (16da). Following the general procedure for the synthesis of prodiginines, carbaldehyde **3d** (116 mg, 0.50 mmol, 1.00 eq.), pyrrole **4a** (151 mg, 1.00 mmol, 2.00 eq.) and HCl in MeOH (719 μL, 0.90 mmol, 1.80 eq.) were converted to 116 mg (0.29 mmol, 58%, 99.5% ± 0.94% purity by qNMR) of a deep purple amorphous solid. δ^1H (300 MHz, $CDCl_3$) 0.84–0.93 (3 H, m, 11″-H), 0.99 (3 H, t, J = 7.3 Hz, 8-H), 1.22–1.38 (4 H, m, 9″-H, 10″-H), 1.52 (2 H, p, J = 7.4 Hz, 8″-H), 1.82 (2 H, h, J = 7.4 Hz, 7-H), 2.38 (2 H, t, J = 7.6 Hz, 7″-H), 2.52 (3 H, s, 6″-H), 2.75 (2 H, t, J = 7.6 Hz, 6-H), 3.98 (3 H, s, 7′-H), 6.01 (1 H, d, 3′-H), 6.10 (1 H, dd, 4-H), 6.62 (1 H, d, 3″-H), 6.83–6.88 (2 H, m, 3-H, 6′-H), 12.51 (2 H, brs, 1-NH, 1′-NH), 12.59 (1 H, brs, 1″-NH). δ^{13}C (76 MHz, $CDCl_3$) 12.4 (C-6″), 14.0 (C-8), 14.2 (C-11″), 22.6 (C-7), 22.7 (C-10″), 25.5 (C-7″), 30.1 (C-8″), 30.4 (C-6), 31.6 (C-9″), 58.8 (C-7′), 92.6 (C-3′), 110.7 (C-4), 114.7 (C-6′), 118.7 (C-3), 121.1 (C-2), 121.3 (C-5′), 125.0 (C-2″), 127.3 (C-3″), 127.8 (C-4″), 144.7 (C-5), 145.3 (C-5″), 147.8 (C-2′), 165.7 (C-4′). FT-IR (neat, cm^{-1}): 3170, 3144, 3111, 3010, 2961, 2927, 2869, 2857, 1632, 1605, 1577, 1537, 1519, 1495, 1463, 1447, 1407, 1384, 1346, 1284, 1251, 1207, 1187, 1151, 1131, 1065, 1044, 998, 972, 955, 891, 883, 836, 816, 806, 786, 728, 695, 677, 655, 623, 545, 505, 477. T_m: 105.7–107.5 °C (petroleum ether). HRMS-ESI (*m*/*z*): $[M–Cl]^+$ calculated for $C_{23}H_{32}N_3O$ 366.2540, found 366.2542. t_R (LC-MS method): 8.02 min.

(*Z*)-5-Butyl-4′-methoxy-5′-((5-methyl-4-pentyl-1*H*-pyrrol-2-yl)methylene)-1*H*,5′*H*-[2,2′-bipyrrol]-1′-ium chloride (16ea). Following the general procedure for the synthesis of prodiginines, carbaldehyde **3e** (120 mg, 0.49 mmol, 1.00 eq.), pyrrole **4a** (147 mg, 0.97 mmol, 2.00 eq.) and HCl in MeOH (702 μL, 0.88 mmol, 1.80 eq.) were converted to 148 mg (0.36 mmol, 73%, 99.9% ± 0.40% purity by qNMR) of a deep purple amorphous solid. δ^1H (300 MHz, $CDCl_3$) 0.84–0.92 (3 H, m, 11″-H), 0.95 (3 H, t, 9-H), 1.23–1.35 (4 H, m, 9″-H, 10″-H), 1.35–1.45 (2 H, m, 8-H), 1.46–1.60 (3 H, m, 8″-H), 1.69–1.83 (2 H, m, 7-H), 2.38 (2 H, t, 7″-H), 2.52 (3 H, s, 6″-H), 2.77 (2 H, t, 6-H), 3.98 (3 H, s, 7′-H), 6.01 (1 H, d, 3′-H), 6.10 (1 H, dd, 4-H), 6.62 (1 H, d, 3″-H), 6.85 (2 H, d, 3-H, 6′-H), 12.51 (2 H, brs, 1-NH, 1′-NH), 12.59 (1 H, brs, 1″-NH). δ^{13}C (76 MHz, $CDCl_3$) 12.4 (C-6″), 13.9 (C-9), 14.2 (C-11″), 22.5 (C-8), 22.7 (C-10″), 25.5 (C-7″), 28.0 (C-6), 30.1 (C-8″), 31.3 (C-7), 31.6 (C-9″), 58.8 (C-7′), 92.6 (C-3′), 110.6 (C-4), 114.6 (C-6′), 118.7 (C-3), 121.0 (C-2), 121.3 (C-5′), 125.0 (C-2″), 127.3 (C-3″), 127.8 (C-4″), 145.0 (C-5), 145.3 (C-5″), 147.8 (C-2′), 165.7 (C-4′). FT-IR (neat, cm^{-1}): 3170, 3142, 3109, 3008, 2960, 2926, 2856, 1632, 1605, 1577, 1536, 1495, 1464, 1452, 1406, 1350, 1299, 1257, 1240, 1208, 1186, 1151, 1131, 1066, 1043, 1000, 972, 956, 891, 882, 837, 816, 806, 785, 732, 695, 675, 655, 545, 505, 475. T_m: 96.7–100.7 °C (petroleum ether). HRMS-ESI (*m*/*z*): $[M–Cl]^+$ calculated for $C_{24}H_{34}N_3O$ 380.2696, found 380.2704. t_R (LC-MS method): 8.11 min.

(*Z*)-4′-Methoxy-5-pentyl-5′-((5-methyl-4-pentyl-1*H*-pyrrol-2-yl)methylene)-1*H*,5′*H*-[2,2′-bipyrrol]-1′-ium chloride (16fa). Following the general procedure for the synthesis of prodiginines, carbaldehyde **3f** (130 mg, 0.50 mmol, 1.00 eq.), pyrrole **4a** (151 mg, 1.00 mmol, 2.00 eq.) and HCl in MeOH (719 μL, 0.90 mmol, 1.80 eq.) were converted to 100 mg (0.23 mmol, 47%, 99.9% ± 0.78% purity by qNMR) of a deep purple amorphous solid. δ^1H (300 MHz, $CDCl_3$) 0.82–0.95 (6 H, m, 10-H, 11″-H), 1.21–1.43 (8 H, m, 8-H, 9-H, 9″-H, 10″-H), 1.45–

1.61 (2 H, p, J = 7.3 Hz, 8″-H), 1.70–1.85 (2 H, m, 7-H), 2.38 (2 H, t, J = 7.6 Hz, 7″-H), 2.52 (3 H, s, 6″-H), 2.77 (2 H, t, 6-H), 3.98 (3 H, s, 7′-H), 6.01 (1 H, d, 3′-H), 6.10 (1 H, dd, 4-H), 6.62 (1 H, d, 3″-H), 6.83–6.88 (2 H, m, 3-H, 6′-H), 12.51 (2 H, brs, 1-NH, 1′-NH), 12.60 (1 H, brs, 1″-NH). δ^{13}C (76 MHz, $CDCl_3$) 12.4 (C-6″), 14.2 (C-10, C-11″), 22.5 (C-9), 22.7 (C-10″), 25.5 (C-7″), 28.3 (C-6), 28.9 (C-7), 30.1 (C-8″), 31.6 (C-8, C-9″), 58.8 (C-7′), 92.6 (C-3′), 110.6 (C-4), 114.6 (C-6′), 118.7 (C-3), 121.0 (C-2), 121.3 (C-5′), 125.0 (C-2″), 127.3 (C-3″), 127.8 (C-4″), 145.0 (C-5), 145.3 (C-5″), 147.8 (C-2′), 165.7 (C-4′). FT-IR (neat, cm^{-1}): 3171, 3144, 3111, 3058, 3015, 2952, 2925, 2870, 2854, 1632, 1606, 1578, 1537, 1495, 1464, 1451, 1406, 1383, 1349, 1322, 1285, 1254, 1208, 1186, 1045, 971, 955, 891, 882, 836, 816, 805, 789, 731, 708, 696, 685, 675, 656, 622, 546, 505, 471. T_m: 98.0–99.7 °C (petroleum ether). HRMS-ESI (m/z): $[M–Cl]^+$ calculated for $C_{25}H_{36}N_3O$ 394.2853, found 394.2855. t_R (LC-MS method): 8.09 min.

(*Z*)-5-Hexyl-4′-methoxy-5′-((5-methyl-4-pentyl-1*H*-pyrrol-2-yl) methylene)-1*H*,5′*H*-[2,2′-bipyrrol]-1′-ium chloride (16ga). Following the general procedure for the synthesis of prodiginines, carbaldehyde **3g** (140 mg, 0.51 mmol, 1.00 eq.), pyrrole **4a** (154 mg, 1.02 mmol, 2.00 eq.) and HCl in MeOH (735 μL, 0.92 mmol, 1.80 eq.) were converted to 109 mg (0.25 mmol, 48%, 99.3% ± 3.73% purity by qNMR) of a deep purple amorphous solid. δ^1H (300 MHz, $CDCl_3$) 0.81–0.93 (6 H, m, 11-H, 11″-H), 1.20–1.42 (10 H, m, 8-H, 9-H, 9″-H, 10-H, 10”-H), 1.52 (2 H, p, J = 7.4 Hz, 8″-H), 1.77 (2 H, p, J = 7.5 Hz, 7-H), 2.37 (2 H, t, J = 7.6 Hz, 7″-H), 2.51 (3 H, s, 6”-H), 2.75 (2 H, t, J = 7.7 Hz, 6-H), 3.96 (3 H, s, 7′-H), 5.99 (1 H, d, 3′-H), 6.08 (1 H, dd, 4-H), 6.60 (1 H, d, 3″-H), 6.84 (2 H, d, 3-H, 6′-H), 12.47 (2 H, brs, 1-NH, 1′-NH), 12.57 (1 H, brs, 1″-NH). δ^{13}C (76 MHz, $CDCl_3$) 12.3 (C-6″), 14.2 (C-11, C-11″), 22.7 (C-10, C-10″), 25.4 (C-7″), 28.3 (C-6), 29.1 (C-7, C-8), 30.0 (C-8″), 31.6 (C-9, C-9″), 58.7 (C-7′), 92.6 (C-3′), 110.6 (C-4), 114.5 (C-6′), 118.7 (C-3), 121.0 (C-2), 121.3 (C-5′), 125.0 (C-2″), 127.2 (C-3″), 127.7 (C-4″), 144.9 (C-5), 145.1 (C-5″), 147.7 (C-2′), 165.7 (C-4′). FT-IR (neat, cm^{-1}): 3171, 3144, 3115, 3072, 3015, 2953, 2919, 2868, 2855, 1636, 1613, 1580, 1549, 1539, 1497, 1466, 1450, 1421, 1407, 1386, 1362, 1333, 1293, 1263, 1253, 1215, 1204, 1185, 1157, 1129, 1101, 1092, 1044, 991, 978, 969, 891, 843, 817, 808, 782, 767, 735, 709, 694, 653, 625, 614, 553, 497, 456. T_m: 96.6–98.7 °C (petroleum ether). HRMS-ESI (m/z): $[M–Cl]^+$ calculated for $C_{26}H_{38}N_3O$ 408.3009, found 408.3017. t_R (LC-MS method): 8.30 min.

Determination of molar extinction coefficients

Prodiginines were weighed in and dissolved in acidic EtOH (+ 4% 1 M HCl) to give a 10 mM solution. By means of serial 1 : 10 dilutions, the stock was diluted to 10 μM in acidic EtOH. Firstly, the absorption spectra of 10 μM solutions was collected on a Shimadzu UV-1800 UV Spectrophotometer, using the following instrument settings: 20 °C, 200–800 nm, 1.0 nm increment, Hellma Analytics quartz glass cuvette SUPRASIL QS (d = 10 mm light path length).

Secondly, from the diluted 10 μM stock, three concentrations were prepared in acidic EtOH, namely 1 μM, 3 μM and, 5 μM. The extinction was measured at 20 °C for each concentration at 535 nm, 545 nm and at the wavelength of maximum absorption, if the wavelength varied from the two aforementioned and the solvent background was subtracted on the instrument. The procedure was repeated three times for each compound. Resulting from the Beer–Lambert law ($E = \varepsilon \cdot c \cdot d$), the molar extinction E was plotted against the molar concentration c and the molar extinction coefficient ε determined from the slope of a linear regression curve using Origin 2019 (ESI†).

Biologic procedures

Chemically competent cells of *E. coli* BL21 (DE3) were transformed with of pET28a(+)-derived plasmids using the heat-shock protocol. In detail, 50 μL of cells were thawed on ice for 10 min and 1 μL of plasmid DNA was added. Incubation on ice for 30 min was followed by a heat shock at 42 °C for 30 s. The cells were again incubated on ice for 10 min and 700 μL lysogeny broth (LB) medium was added to allow cell proliferation at 37 °C for 1.5 h. Finally, the cells were plated on LB agar, containing 50 μg mL^{-1} kanamycin, and grown overnight at 37 °C.

Protein production of prodiginine ligating enzymes

Single colonies of transformed *E. coli* BL21 (DE3) with pET28a(+) (empty vector, EV), pPigC_3, pET28a(+)::*tamQ* or pET28a(+)::*treaP* were used to inoculate 50 mL of LB media (50 μg mL^{-1} kanamycin) in a 250 mL Erlenmeyer flask and incubated overnight at 37 °C and 130 RPM. The precultures were used to inoculate 2 × 1 L of terrific broth (TB) media (50 μg mL^{-1} kanamycin) in 3 L baffled flasks to an optical density at 600 nm (OD_{600}) of 0.05 and then cultivated at 37 °C and 130 RPM. At an OD_{600} of 0.6–0.9, the cultures were cooled at room temperature for 15 min and then induced by addition of 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were then transferred to 25 °C and incubated overnight at 130 RPM. Cells were harvested by centrifugation (15 min, 4.500 RPM, 4 °C), the supernatant discarded and dry pellets stored at −20 °C until further use.

Cell lines and cell culture

RT-112 and RT-112^{res} cells (kindly provided by Margaretha A. Skowron, Michèle J. Hoffmann, and Günter Niegisch; Department of Urology, Medical Faculty and University Hospital Düsseldorf, Heinrich Heine University Düsseldorf) were cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Sigma-Aldrich), 4.5 g L^{-1} D-glucose, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Thermo Fisher Scientific). The cells were cultivated and treated at 37 °C and 5% CO_2 in a humidified atmosphere. RT-112^{res} cells have been previously described.[73] Briefly, for the generation of this cisplatin-resistant cell line, RT-112 cells were treated with

increasing dosages of cisplatin over several months. During cell cultivation 12 μg mL^{-1} cisplatin (NeoCorp, Pawtucket, RI, USA) was added to the media of RT-112^{res} cells with every passage.

Cell viability assay

Viability of RT-112 and RT-112^{res} cells was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. RT-122 and RT-112^{res} cells were seeded in 96-well plates with a density of 2.5×10^3 or 5.0×10^3 cells per well, respectively. One day after seeding, cells were treated with prodigiosin (**1**) or derivatives **16ba–bc** for 24 or 72 h. 5 μM staurosporine was used as a positive control and 0.1% DMSO (PanReac AppliChem, Darmstadt, Germany) was used as a solvent control. After the incubation time, thiazolyl blue was added to the cells and they were incubated at 37 °C and 5% CO_2 in a humidified atmosphere for 45 min. After removal of the MTT-containing medium, 100 μL DMSO were added per well for extraction of the formazan. Absorbance was measured at 570 nm and 650 nm (reference) with a microplate reader (BioTek, Synergy Mx). After subtraction of the reference signal and the mean value of the positive control from each value, the mean of the absorbance of the solvent control was set as 100% and relative viability was calculated for each sample. All IC_{50} values were calculated using GraphPad Prism 7.01.

In vitro assay with prodiginine ligating enzymes

Cells of *E. coli* BL21 (DE3) with the desired pET expression vector were resuspended after expression of the corresponding ligating enzyme in 50 mM potassium phosphate buffer, pH 7.0 (KP_i) to a final mass concentration of 0.2 g mL^{-1} and cooled on ice. To disrupt the cells, sonication on ice was performed (40% amplitude, 3 × 5 min with 5 min rest on ice between each cycle, 0.5 s pulse and 0.5 s rest per pulse cycle).

The 1*H*-pyrroles **4a–c**, as well as the MBC derivatives **3a–g** were freshly dissolved in DMSO to a final concentration of 20 mM. For ATP, a 62.5 mM stock solution in water was prepared from the disodium salt Na_2-ATP × 3 H_2O.

In a 1.5 mL reaction tube, 10 μL of 62.5 mM ATP, 25 μL of 20 mM MBC, and 25 μL of 20 mM pyrrole were mixed. Subsequently, the reaction was initiated by supplementation with 440 μL of homogenous sonicated cells in KP_i buffer with a concentration of 0.20 g mL^{-1} (cell debris was not removed, as the enzymes are attached to the inner membrane). After carefully inverting the tubes, the reactions were incubated at 30 °C and 300 RPM for a total reaction time of 4 h. Afterwards, the analytical reactions were centrifuged (20 300 rcf, 20 min, 4 °C) and the supernatant was disposed. Depending on the further utilisation of samples, two workup procedures were used as follows.

In vitro assay – workup for LC-MS measurements

The cell debris was resuspended in 400 μL of MeOH, however, the resuspension process was assisted by an ultrasound bath. Centrifugation (20 300 rcf, 20 min, 4 °C) was used to remove cell debris and the supernatant transferred into a new reaction tube. MeOH was evaporated in a vacuum centrifuge at 45 °C and the residue was taken up in 200 μL water. Extraction with CH_2Cl_2 (2 × 200 μL) and successive evaporation of the solvent provided a residue, which was dissolved in 200 μL MeOH, filtered through a 0.45 μm syringe filter and subjected to LC-MS chromatography.

In vitro assay – workup for photographic documentation

The cell debris was resuspended in 300 μL acidic EtOH (+ 4% 1 M HCl) and the resuspension process assisted by an ultrasound bath. Centrifugation (20 300 rcf, 20 min, 4 °C) was used to remove cell debris and the supernatant was then transferred into a new 1.5 mL reaction tube for documentation (ESI†).

LC-MS parameters

Coupled LC-MS measurements were performed on a Thermo Scientific UltiMate 3000 UHPLC instrument with an Atlantis T3 3 μm, 3 × 100 mm column (Waters) and an ISQ-ES mass spectrometer. Sample volumes of 5 μL were injected at a temperature of 30 °C and a flow rate of 0.60 mL min^{-1}. UV detection was realised at 535 nm *via* photo diode array detector. Gradient elution with MeOH + 0.1% formic acid (solvent A) and Millipore water + 0.1% formic acid (solvent B) allowed separation of prodiginines from MBC and pyrrole precursors. Elution profile: −5–0 min with 10% solvent A, 0–4 min with 10–60% solvent A, 4–6 min with 60–100% solvent A, 6–13 min with 100% solvent A. The following parameters were used for the ISQ-MS detection – mode: positive, vaporiser temperature: 282 °C, ITT temperature: 300 °C, source voltage positive ions: 3000 V, source voltage negative ions: −2000 V, sheath gas pressure: 49.9 psig, aux gas pressure: 5.7 psig, sweep gas pressure: 0.5 psig, mass area 10–1000, CID 20.

Quantification of prodiginines from LC chromatograms

Based on the measured and approximated molar extinction coefficients for chemically synthesised prodiginine references, quantification of prodiginines in methanolic extracts from *in vitro* assays was performed. Therefore, eqn (1.2), devised by Torsi *et al.*, was deployed.[88]

$$c\left[\mu\text{mol}\,\mu\text{L}^{-1}\right] = \frac{n[\mu\text{mol}]}{V_{\text{inj}}[\mu\text{L}]} \tag{1.1}$$

with

$$n[\text{mol}] = E[\text{AU min}]\cdot F\left[\text{L min}^{-1}\right]\cdot\frac{1}{\varepsilon[\text{M}^{-1}\,\text{cm}^{-1}]\cdot d[\text{cm}]} \tag{1.2}$$

c: prodiginine concentration in the methanolic extract
n: mount of substance applied to chromatography
V_{inj}: injected volume applied to chromatography
E: integrated extinction at 535 nm
F: chromatographic flow rate
ε: molar extinction coefficient at 535 nm
d: flow cell path length

The UV traces at 535 nm and the extracted ion chromatograms (EIC) of the appropriate *m*/*z* ratio for the proposed products were generated from the total ion chromatograms (TIC). EIC spectra were used to validate the estimated product masses and UV absorbance at 535 nm was utilised to calculate the corresponding peak areas by integration. For quantification of prodiginines from the methanolic extracts, the mean value of absorbance from triplicates was used to determine the amount of substance that had been injected to the chromatographic system (eqn (1.2)), based on the extinction coefficients, which had been determined experimentally or by approximation. From the results of eqn (1.2), the concentrations in methanolic extracts were calculated by eqn (1.1).

Abbreviations

Compd	Compound
LiTMP	Lithium 2,2,6,6-tetramethylpiperidide
SPhos	2-Dicyclohexylphosphino-2′,6′-dimethoxybiphenyl
TFE	2,2,2-Trifluoroethanol
t_R	Retention time
LB	Lysogeny broth
TB	Terrific broth
KP_i	Potassium phosphate
IC_{50}	Half maximal inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
o/n	Overnight

Author contributions

Tim Moritz Weber: conceptualisation, methodology, investigation, writing – original draft, writing – review & editing, visualisation, supervision, project administration, funding acquisition; Alexandra Leyens: investigation; Lena Berning: investigation, visualisation, writing – original draft; Björn Stork: writing – review & editing, supervision, funding acquisition; Jörg Pietruszka: conceptualisation, writing – review & editing, supervision, funding acquisition, project administration.

Conflicts of interest

There are no conflicts to declare.

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