Chemoenzymatic synthesis of prodigiosin analogues—exploring the substrate specificity of PigC†

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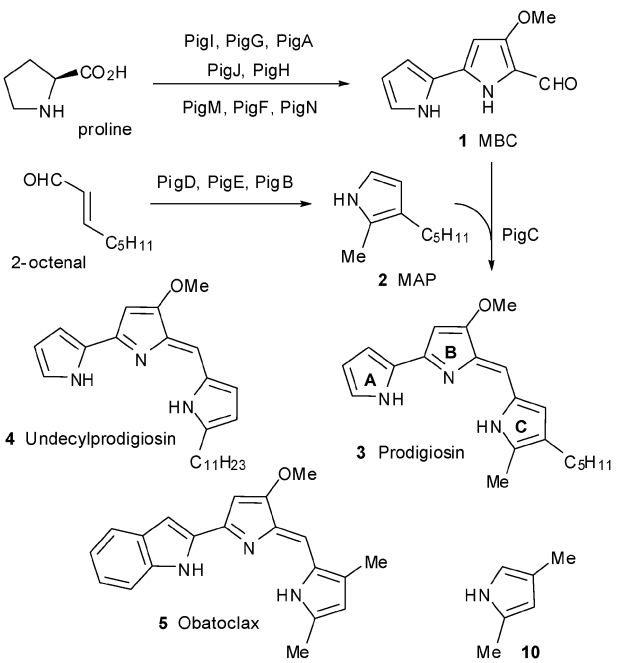
Received (in Cambridge, UK) 14th December 2007, Accepted 15th January 2008
First published as an Advance Article on the web 5th February 2008
DOI: 10.1039/b719353j

Analogues of prodigiosin, a tripyrrolic pigment produced by *Serratia* species with potent immunosuppressive and anticancer activities, have been produced by feeding synthetic analogues of the normal precursor MBC to mutants of *Serratia* sp. ATCC 39006 or to engineered strains of *Escherichia coli*; in this way it has been shown that the prodigiosin synthesising enzyme, PigC, has a relaxed substrate-specificity.

Prodigiosin **3** is a tripyrrolic secondary metabolite produced by *Serratia marcescens* as well as some other bacteria. It, and the organisms that produce it, are intensely red and as a result *S. marcescens* has a long and colourful history.[1,2] More recently it has been found that prodigiosin has potent immunosuppressive and anticancer activities. The immunosuppression is effected by a mechanism different from that of existing immunosuppressive drugs and a combination of a prodigiosin analogue with existing drugs is reported to be more effective than the existing drugs alone.[3] Prodigiosin appears to cause apoptosis in tumour cells at levels that do not affect healthy cells, though how this happens is not yet entirely clear.[4] A close analogue of prodigiosin named obatoclax **5** is currently in clinical trials for the treatment of various cancers.[5]

The biosynthesis of prodigiosin was studied in the 1960's and 70's by incorporation of isotopically labelled compounds[6] and it was proposed that separate biosynthetic pathways lead to two late-stage intermediates, 3-methoxy-5,5′-bipyrrole-2-carbaldehyde (MBC, **1**) and 2-methyl-3-amylpyrrole (MAP, **2**), which are condensed together in the final step to form prodigiosin (Scheme 1). We have recently discovered and sequenced the gene cluster for prodigiosin biosynthesis in *S. marcescens*[7] and have proposed a complete biosynthetic pathway based on studies of mutants in which each of the genes in turn has been disrupted.[8] From these studies it was clear that the biosynthesis of MAP involves just three genes, PigD, PigE and PigB in that order, and the final condensation is catalysed by PigC. The other genes in the cluster are involved in the biosynthesis of MBC. A similar prodiginine, called undecylprodigiosin **4**, is made by *Streptomyces coelicolor* and the genes for its biosynthesis have also been sequenced.[9] MBC is also an intermediate in *S. coelicolor* and that organism has homologues of all the *pig* genes involved in MBC biosynthesis. Mutations of the corresponding *S. coelicolor* genes have confirmed their role[10] and the functions of many of the enzymes of MBC biosynthesis have now been demonstrated biochemically both from *Streptomyces*[11] and *Serratia*.[12] In contrast, the pathway to the undecylpyrrole precursor of **4** is quite different to the pathway to MAP 2.[2,8]

As prodigiosin analogues such as **5** have such potentially valuable biological activity, we were interested to discover whether they could be made by enzymic means using the condensing enzyme PigC. A short synthesis of MBC **1**, shown in Scheme 2, has recently been published.[13] In the work that we report here, this route was used to make not only MBC **1** but also, by modifying the boronic acid used in the Suzuki coupling step, a series of analogues of MBC in which pyrrole



Scheme 1 Structures of prodigiosin and related compounds and outline of the biosynthesis of prodigiosin.

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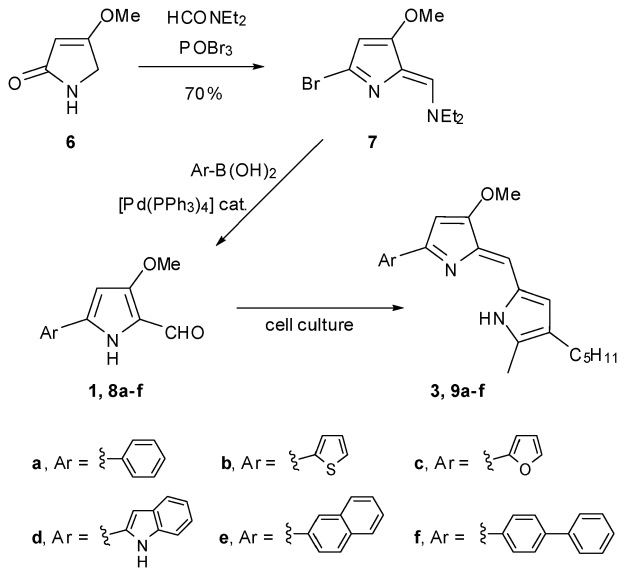
† Electronic supplementary information (ESI) available: Representative experimental details for the synthetic chemistry, spectroscopic data for compounds **8a–f**, experimental details for the feeding experiments, photographs of the agar plates from the feeding experiments, representative LC-MS chromatograms for **2**, **3** and **9a–d**. See DOI: 10.1039/b719353j



Scheme 2 Synthesis of MBC **1** and analogues **8a–f** and their conversion to prodigiosin **3** and analogues **9** (see Table 1). In the synthesis of **1** and **8d** the arylboronic acid was protected on nitrogen with a Boc group.

ring A is replaced by other ring systems, both carbocyclic **8a,e,f** and heterocyclic **8b–d**. In general the yield of the desired product was good to excellent (see Table 1), although it was in some cases necessary to vary the reaction conditions to obtain these yields.†

To test whether the synthetic MBC **1** and its analogues **8** could be used *in vivo* by the condensing enzyme PigC, two types of organism were used. Firstly the previously described[8] *pigM*Δ and *pigH57*Δ mutant strains of *Serratia* sp. ATCC 39006, which cannot make MBC and so are unpigmented, were grown in a streak on agar gel. Solutions of **1** or **8a–f** in DMSO were then spotted beside the streak. Only if the MBC analogue can be accepted as a substrate by PigC will pigment production occur. When synthetic MBC **1** was used rapid pigmentation occurred, observable by eye within minutes. With the phenyl-, thienyl- and furyl- analogues **8a–c** clear pigmentation was observed after an hour and with the indolyl analogue **8d** pigmentation could be observed within 24 h.† No pigmentation was observed with the naphthyl and biphenyl analogues **8e** and **f**. As a negative control the same experiments were repeated with mutant strains unable to make MAP 2 and in no case was any pigment production observed.

To confirm the identity of the pigment observed in the above experiments, the *pigH57*Δ mutant strain was grown in liquid medium (LB-sorbitol) and then supplemented with each of the MBC analogues (see supplementary material for details†). After further incubation overnight, each culture was centrifuged, the cell pellet was extracted with acidic EtOH and the pigment produced characterised by its UV–visible spectrum and LC-MS analysis. This experiment gave results very much in line with those above: MBC **1** and analogues **8a–d** all gave strong pigmentation when supplied at 100 μM concentration (Fig. 1 and Table 1) and clearly visible pigmentation at 10 μM. The other two analogues **8e** and **f** showed no more pigmentation than the blank. The UV–visible spectra of the extracted pigments showed an absorption maximum at 534 nm for prodigiosin **3** itself, as expected, and significantly shifted wavelengths for the various analogues, varying from 506 nm for the phenyl analogue **9a** to 551 nm for the indolyl analogue **9d**. By LC-MS also, prodigiosin **3** and its analogues **9a–d** were all observed (in addition to recovered MBC analogue in each case) as single peaks with the correct mass.

The second type of organism used was the previously described[8] *Escherichia coli* strain DH5α-pNRW73. The plasmid in this strain is pUC19 containing the genes for expression of PigB to PigE from *S. marcescens* ATCC 274, encoding all the enzymes in the cluster that are involved in the production of MAP 2 plus the condensing enzyme PigC. It was first shown by LC-MS that this strain does produce MAP, which was found mostly in the supernatant rather than the cell pellet.

It is significant that this strain does produce MAP because it shows not only that the enzymes are all expressed satisfactorily in active form but also that all the required substrates and cofactors are available in *E. coli*. The starting material for this pathway is proposed to be 2-octenal but it is not known how

Table 1 Yields in the syntheses of the MBC analogues, **1** and **8a–f**, and results of their incubation with the *Serratia* 39006 *pig*HΔ and *E. coli* DH5α-pNRW73 mutants

MBC analogue	Yield from **7**	Conc.[a]/μM	λ_{max} of extract[c]	$A_{\lambda max}$ of extract[d]	LC-MS[b] (M + H^+)
MBC **1**	78%	100	534	10.18	191, 324
		10		*3.84*	
Phenyl- **8a**	79%	100	506	4.60	202, 335
		10		*2.64*	
2-Thienyl- **8b**	88%	100	519	5.81	208, 341
		10		*2.85*	
2-Furyl- **8c**	95%	100	521	4.11	192, 325
		10		*1.58*	
2-Indolyl- **8d**	96%[e]	100	551	1.12	241, 374
2-Indolyl- **8d** + 2,4-DMP **10**		100/100	543	1.38	241, 374, 318
2-Naphthyl- **8e**	81%	100	—	0.62	252, n.d.
p-Biphenylyl- **8f**	82%	100	—	0.58	278, n.d.
None	—	—		0.74	n.d., n.d.

[a] Concentration of the analogue in the culture medium. Entries in normal typeface employed the *Serratia* strain and those in italics used the *E. coli* strain. [b] *m*/*z* values observed for the main peaks, the first number corresponds to the MBC analogue **8** and the second (and third) to the prodigiosin analogue **9** (and **5**); n.d. = not detected. [c] λ_{max} of peak between 500 and 600 nm. [d] Absorbance at the λ_{max} value or at 534 nm in cases where no peak was detected. [e] Combined yield of **8d** and its Boc-derivative.

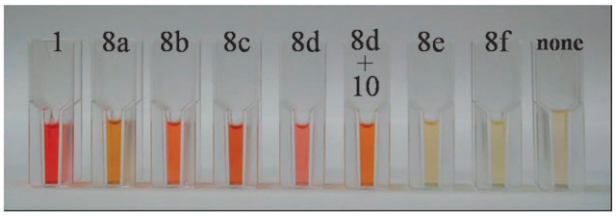


Fig. 1 Extracts from *Serratia* 39006 *pigH57*Δ mutant after supplementation with the MBC analogue indicated at 100 μM.

this is formed in *Serratia* and it was not certain that it would be present in *E. coli*.

This strain was grown in liquid culture and then supplemented with MBC **1** or analogues **8a–c** in the same way as the *Serratia* strain had been. The results were similar except that the pigment production was higher, so a lower concentration of MBC analogue (10 μM instead of 100 μM) could be used for the quantification of pigment production (Table 1). In the LC-MS spectra of the extracts it appeared that the amount of unrelated metabolites extracted from the *E. coli* strain was much lower and the spectra obtained after feeding analogues **8a–c** showed just two main peaks, one for the MBC analogue itself and one for the corresponding prodigiosin analogue **9a–c**.†

In view of the fact that PigC can utilise the indolyl analogue of MBC **8d** as a substrate to make prodigiosin analogue **9d**, we wondered whether the enzyme could also use 2,4-dimethylpyrrole **10** as its monopyrrolic substrate, thus making obatoclax **5**. Accordingly, the liquid culture of the *Serratia pigH57*Δ mutant strain was supplemented with both **10** and **8d** (100 μM each). As this strain continues to make MAP 2, the production of prodigiosin analogue **9d** was still observed but in addition a peak appeared at shorter retention time that showed the correct mass for obatoclax **5**.† The MS ion-currents for the two peaks suggested the ratio of **9d** to **5** was about 5 : 1.

It is clear that the prodigiosin synthetase, PigC, has a relatively relaxed substrate specificity. It is able to utilise substrates with a variety of monocyclic rings in place of the pyrrolic ring A of its natural substrate. Hence the pyrrolic N–H, though helpful, is not essential. Substrate analogues with a bicyclic ring system in place of ring A were considerably worse as substrates and indeed no reaction could be detected with the naphthyl and biphenyl analogues **8e** and **f**. However with the indolyl analogue **8d** some pigment formation could be detected, which again shows that the presence of the N–H is a factor in the substrate specificity.

With regard to the monopyrrolic precursor, which supplies ring C of prodigiosin, we have shown that, as well as MAP **2**, 2,4-dimethylpyrrole can also be accepted as a substrate and thus enzymic formation of obatoclax is possible. Furthermore it has previously been shown that 2,4-dimethyl-3-ethylpyrrole can restore pigment product to a mutant deficient in MAP biosynthesis.[8,14] Therefore the relaxed substrate specificity of PigC also extends to its monopyrrolic substrate.

It should be noted that the failure of **8e** and **f** to produce any pigment is not necessarily because they cannot be accepted as substrates by PigC, though this is very likely. Other possible reasons are that they do not reach the site of prodigiosin production within the cells or that they are rapidly degraded if they do enter the cells. We plan to test this point and measure the kinetic parameters for the MBC analogues that are substrates in experiments with purified PigC.

In conclusion, we have shown that PigC has the capability of making a range of prodiginines, although at a slower rate than with its natural substrate MBC. However, it may well be possible to improve its rate of reaction with unnatural substrates and expand or change its substrate specificity by mutagenesis. Thus PigC could be a key enzyme in combinatorial biosynthesis approaches for the formation of new prodiginines, which may have valuable pharmaceutical properties.

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