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Purification and kinetic characterization of the essential condensation enzymes involved in prodiginine and tambjamine biosynthesis

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Abstract: Prodiginines and tambjamines are related families of bioactive alkaloid natural products with pharmaceutical potential. Both compound families result from a convergent biosynthetic pathway ending in the condensation of a conserved bipyrrole core with a variable partner. This reaction is performed by unique condensation enzymes, and has the potential to be manipulated to produce new pyrrolic compounds. We have purified and reconstituted the *in vitro* activity of the condensation enzymes- PigC and TamQ, from *Pseudoalteromonas* sp., involved respectively in prodiginine and tambjamine biosynthetic pathways. Kinetic analysis confirmed a Uni Uni Bi Uni ping-pong reaction sequence with competitive and uncompetitive substrate inhibition for PigC and TamQ respectively. The kinetic parameters of each enzyme provide insight into their differing substrate scope, and suggest that TamQ may have evolved a wide substrate tolerance that can be utilized for the production of novel prodiginines and tambjamine.

Introduction

Prodiginines and tambjamines are two structurally related classes of pigmented microbially-derived alkaloids, each boasting a range of clinically relevant bioactivities^[1–6]. Compounds in both families possess a bipyrrole core that interacts with biologically relevant ions, from which most of their bioactive properties emerge^[7,8]. The structural variation found in members of these family, such as macrocyclization and halogenation, alter the potency and specificity of their bioactivities^[9–11]. For this reason many studies have concentrated on chemical synthesis of analogues^[10,12,13], however, the

native bacterial producers have already evolved a simple biosynthetic mechanism to allow for straightforward generation of structural diversity^[4,14,15].

Representative examples of each molecule class are prodigiosin, originally isolated from *Serratia* sp.^[14], and tambjamine YP1 from *Pseudoalteromonas tunicata*^[5]. Both prodigiosin and tambjamine YP1 have been functionally linked to their respective biosynthetic gene clusters (BGCs) (Figure 1A)^[15,16].

The *pig* cluster, responsible for prodigiosin production in *Serratia* sp., has been extensively studied and a detailed overview of prodiginine biosynthesis can be found in this recent review^[17]. The final step in the biosynthetic scheme involves the enzyme-catalyzed coupling of a pathway specific monopyrrole, 2-methyl-3-amyl-pyrrole (MAP) in the case of prodigiosin, to the shared intermediate 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC)^[18,19]. Based on enzyme homology and preliminary gene inactivation experiments, the tambjamines are predicted to result from a similar overall biosynthetic pathway, wherein MBC condenses with a primary amine rather than a pyrrole in the final step^[15]. This final biosynthetic step is critical in the structural diversification of the two molecular families and developing a clear understanding of how the enzymes carry out substrate selection. This step not only allows for future engineering of new molecules but also illuminates how structural diversity has evolved in nature.

PigC is a condensation enzyme responsible for C-C bond formation in prodigiosin biosynthesis^[20], while, TamQ forms a N-C bond between MBC and *cis*-dodec-3-en-1-amine (DDEA) in the biosynthesis of tambjamine YP1 and tambjamine MYP1 in *P. tunicata* and *Pseudoalteromonas citrea* respectively (Figure 1B)^[15,18,21,22]. Although the two catalytic reactions result in differing atom connectivity, the terminal condensation enzymes are conserved with high sequence similarity in all prodiginine and tambjamine BGCs^[15,20,23,24]. Currently, the limited information available about this enzyme family predominantly arises from studies on PigC from prodigiosin biosynthesis. Production and purification of soluble PigC from *Serratia* sp. by heterologous expression in *Escherichia coli* has thus far proved elusive; therefore, previous *in vitro* studies were instead performed using a semi-purified cell lysate

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Additional figures for experimental procedures including protein purification, compound synthesis, and kinetic analysis. Full *pig* and *tam* gene cluster annotation.

[12,13,20,25]. In a similar manner, the tambjamine YP1 forming condensation reaction catalyzed by TamQ was recently probed using cell lysates from both *P. tunicata* and *P. citrea*, suggesting its analogous function to that of PigC [15,22].

Early studies of PigC indicated that it catalyzes an ATP-dependent reaction involving a catalytic histidine residue, identified through site-directed mutagenesis studies [20]. Analysis of domain homology, suggests the condensation enzymes have two distinct binding domains for ATP and MBC, with an arm bearing the catalytic histidine to transfer a phosphate between the two domains [16,20,26,27]. Chawrai *et al* proposed a reaction mechanism involving histidine-mediated phosphorylation of the aldehyde of MBC to facilitate a subsequent condensation reaction with MAP (Figure S1) [20]. Aldehydes are already reactive electrophilic groups that do not typically need phosphate activation to react with a nucleophile such as MAP. Therefore, we propose alternatively that MBC/MAP likely react together first, with phosphorylation of the resulting intermediate oxyanion generating a good leaving group that, along with rearomatization, drives formation of prodigiosin (Figure 1C). An analogous mechanism is seen in the carboxylation of biotin, in which phosphorylation also occurs on a tetrahedral intermediate after coupling of biotin and bicarbonate [28]. Discrimination between these two proposed mechanisms is outside the scope of this study, and future studies will be required to determine which is used by PigC (and TamQ).

To date there have been only limited investigations of the reactivity differences between PigC and TamQ. Thorough and rigorous kinetic characterization of the two homologous enzymes will not only provide insight into their chemical reactivity but will also shed light on the

divergent evolution of analogous biosynthetic pathways. This information can be used to guide future manipulation of this enzyme family to design and build new pyrrolic compounds. Furthermore, enzymes exhibiting innate substrate promiscuity can be utilized as simple alternatives to current bioengineering techniques for manipulation of biosynthetic pathways and production of novel bioactive compounds [29]. PigC has already exhibited considerable flexibility in substrate selection [12,13,30], demonstrating potential to enzymatically construct novel prodiginine compounds. TamQ has also recently been shown to form both prodiginines and tambjamines [22], and could serve as an additional tool to direct alternate bond connectivity for the preparation of more extensive analogue libraries. To achieve this goal, understanding the characteristics and limitations of this condensation enzyme family is essential. To date, a major challenge facing researchers attempting to study these condensation enzymes has been difficulties accessing pure and soluble protein, without which, very little meaningful mechanistic and kinetic analysis can be carried out.

In this work, we describe the first successful cloning and purification of soluble PigC from prodigiosin producer *Pseudoalteromonas rubra* DSM 6842, and TamQ from tambjamine MYP1 producer *Pseudoalteromonas citrea* DSM 8771 [4,21]. The catalytic properties of PigC and TamQ are directly compared using *in vitro* kinetic analysis of each enzyme with native and modified substrates.

Figure 1. (A) Biosynthetic gene clusters for prodigiosin production in *Pseudoalteromonas rubra*, and tambjamine YP1 production in *Pseudoalteromonas tunicata* [31]. (B) The terminal condensation reactions catalyzed by PigC and TamQ; "SA" indicates a saturated analogue at the red double bond. (C) Proposed PigC catalyzed condensation reaction mechanism.

Results and Discussion

There are multiple reports stating PigC is a membrane-associated protein with limited solubility or activity in solution when heterologously expressed in *E. coli* [20,25]. Most of these investigations used the PigC homologue from *Serratia* sp. for their experiments [20,25]. Multiple online transmembrane prediction tools provide conflicting results as to whether this homologue should be membrane-associated [20,25]. We postulated that perhaps previous expression attempts were negatively affected by incompatibility problems between the producer and host organisms. Thus, to obtain pure soluble protein for enzymatic assay of PigC and TamQ activity we chose instead to use homologues from *Pseudoalteromonas*. We predicted that the *Pseudoalteromonas* proteins might be more soluble as there are multiple reports of biosynthetic enzymes from the genus being successfully expressed in *E. coli* [32,33].

Multiple constructs were designed and tested for each enzyme and we ultimately found over-expression of PigC with a C-terminal His₆ tag in *E. coli* BL21(DE3) yielded soluble protein that was purified by nickel affinity chromatography (Supplemental Figure S2). TamQ was also successfully over-expressed in soluble form with N-terminal thioredoxin and His₆ tags (Supplemental Figure S3). To our knowledge this is the first account of the

successful purification of members in this ATP-dependent condensation enzyme family. In this work, there are likely several factors that contributed to the successful production of soluble protein including the identity of the source organism and the design of the protein expression constructs. Solubility of TamQ was greatly improved with the use of an N-terminal thioredoxin tag. A C-terminal His₆ tag enhanced the Ni-binding affinity of PigC allowing for greater protein yields. Ready access to purified soluble enzyme enabled a full kinetic analysis of PigC and TamQ and will allow for further *in vitro* characterization in future studies.

The activity of purified PigC was confirmed by incubating it with MBC, MAP, and ATP, resulting in the formation of prodigiosin as confirmed by UPLC-PDA-MS. Monitoring the increasing concentration of prodigiosin at an absorbance wavelength of 535 nm allowed for kinetic analysis of MBC and MAP as substrates for PigC. ATP concentration was held constant in the analysis to focus on the rate behaviour of MBC and MAP, which were analyzed by Michaelis-Menten plots (Figure 2).

We observed substrate inhibition of PigC at high concentrations of MAP, this is reflected in the Michaelis-Menten plots for MBC by an increase in the apparent *K_M*, along with a decrease in the apparent *V_{max}* (Figure 2). Further information was obtained upon replotting the kinetic data as Lineweaver-Burk plots. At low concentrations of MAP, the Lineweaver-Burk plots form a series of parallel lines with decreasing y-intercepts. This is characteristic of a Ter Bi (three

substrate, two product) ping-pong sequence, where MBC and MAP are the second and third substrates to bind the enzyme. The parallel lines indicate that the binding of MBC and MAP are independent of each other; as the amount of available substrate increases, proportionately so too do the apparent V_{max} and K_M . A more detailed description of kinetic analysis of Ter Bi Ping Pong reactions systems can be found in this reference [34]. As the concentration of MAP increases, the Lineweaver-Burk plots form a series of lines that intersect at the same y-intercept, reflecting competitive inhibition. The apparent K_M of MBC increases, as more substrate is needed to achieve the uninhibited reaction rate. Competitive inhibition is likely explained by the structural similarities between planar and aromatic MBC and MAP, thereby allowing MAP to compete for the MBC binding

pocket of PigC. Using the Ter Bi ping-pong reaction model with competitive substrate inhibition, the kinetic data were fit with Equation S1, producing the kinetic parameters in Figure 2. PigC has nearly identical apparent K_M values for MBC and MAP ($0.7 \pm 0.1 \mu\text{M}$ and $0.68 \pm 0.04 \mu\text{M}$, respectively). The K_i value of MAP at $10 \pm 3 \mu\text{M}$ (Figure 2) is approximately 10-fold greater than the K_M for MBC.

The Lineweaver-Burk regression patterns cannot distinguish the order in which MBC and MAP bind the enzyme, however, in this manuscript we have shown MBC binding first for simplicity. Note MBC would likely bind first in the event Chawrai's proposed mechanism is functional, whilst either MAP or MBC might bind first for our proposed alternative mechanism.

MBC K_M (μM)	MAP K_M (μM)	MAP K_i (μM)	k_{cat} (s^{-1})	MBC k_{cat}/K_M ($\times 10^5 \text{ M}^{-1} \text{ s}^{-1}$)	MAP k_{cat}/K_M ($\times 10^5 \text{ M}^{-1} \text{ s}^{-1}$)
0.7 ± 0.1	0.68 ± 0.04	10 ± 3	0.43 ± 0.06	6 ± 2	6 ± 1

Figure 2. (Left) Michaelis-Menten plots of MBC as a substrate for PigC at varying concentrations of MAP. (Right) Lineweaver-Burk double-reciprocal replots of MBC kinetic data separated into low (bottom, [MAP] 0.5-5 μM) and high concentrations of MAP (top, [MAP] = 10-200 μM). Pattern in Lineweaver-Burk plots display a ping-pong reaction sequence with competitive substrate inhibition by MAP, global kinetic analysis produced kinetic values shown beneath the plots.

After successful purification of TamQ, its catalytic activity was reconstituted *in vitro* using ATP, MBC and dodecylamine (DDA), the latter a mimic of the native substrate DDEA. The condensation of MBC and DDA by TamQ forms the known tambjamine derivative BE-18591 (Figure 1B) [1], production of which was confirmed by UPLC-PDA-MS and an increase of absorbance at 405 nm. Similar to PigC, TamQ was found to be ATP dependent; the concentration of this substrate

was again held constant in the kinetic analysis to focus on the kinetic parameters of MBC and DDA.

The Michaelis-Menten plots for MBC display potential substrate inhibition of TamQ by DDA (Figure 3), akin to the effect of MAP on PigC. And the corresponding Lineweaver-Burk plots indeed suggest inhibition, albeit in a different form. At low concentrations of DDA, the plots show the parallel line pattern consistent with a Ter Bi ping-pong mechanism as seen for PigC. However, at

higher DDA concentrations, the plots show a series of parallel lines with increasing y-intercepts, which reflects uncompetitive substrate inhibition. Therefore, a Ter Bi ping-pong reaction scheme with uncompetitive substrate inhibition was used as a model to fit the kinetic data for TamQ (Equations S2 and S3). TamQ has a lower apparent K_M value for DDA (K_M of $1.4 \pm 0.4 \mu\text{M}$) than MBC (K_M of $5 \pm 1 \mu\text{M}$). The apparent K_I value for DDA (K_I of $9 \pm 1 \mu\text{M}$) is greater than both K_M values.

Substrate inhibition by DDA is perhaps unexpected, as one cannot make the same structural justification as with MAP. An uncompetitive inhibition model suggests that DDA binds to TamQ in an unproductive manner once MBC has already bound to the active site. One potential explanation is that non-specific hydrophobic interactions between DDA and the enzyme slow the rate of catalysis and cause this inhibition.

MBC K_M (μM)	DDA K_M (μM)	DDA K_I (μM)	k_{cat} (s^{-1})	MBC k_{cat}/K_M ($\times 10^5 \text{ M}^{-1} \text{ s}^{-1}$)	DDA k_{cat}/K_M ($\times 10^5 \text{ M}^{-1} \text{ s}^{-1}$)
5 ± 1	1.4 ± 0.4	9 ± 1	0.037 ± 0.008	0.07 ± 0.02	0.29 ± 0.08

Figure 3. (Left) Michaelis-Menten plots of MBC as a substrate for TamQ at varying concentrations of DDA. (Right) Lineweaver-Burk double-reciprocal replots of MBC kinetic data separated into low (bottom, [DDA] = 0.5–5 μM) and high concentrations of DDA (top, [DDA] = 5–25 μM). The pattern in Lineweaver-Burk plots show a ping-pong reaction sequence with uncompetitive substrate inhibition by DDA, global kinetic analysis produced kinetic values shown beneath the plots.

The kinetic analysis of both PigC and TamQ reveals a Ter Bi ping-pong mechanism with two possible reaction sequences^[34]. The first option being a Uni Uni Bi Uni sequence, where ATP binds and then ADP leaves prior to MBC and MAP binding and then prodigiosin formation. The second option is a Bi Uni Uni Uni sequence, where ATP and MBC (or MAP) both bind before ADP is produced and then the second heteroaromatic substrate binds and finally prodigiosin is released^[34]. To determine if the presence of MBC (or MAP) is necessary for ATP dephosphorylation by the enzyme, PigC and TamQ were incubated with ATP in the absence of MBC (or MAP) and monitored for ADP

production. Both PigC and TamQ produced a significant level of ADP after incubation compared to the enzyme free control (Figure 4A). Release of ADP demonstrates that the enzyme can turnover ATP without requiring bound MBC (or MAP), consistent with a Uni Uni Bi Uni Ter Bi ping-pong scheme. However, these results do not affirm that MBC (or MAP) cannot bind before ATP, or that their binding would prevent ATP dephosphorylation. In Figure 4B, we have proposed reaction sequences for PigC and TamQ catalyzed condensation reactions to complement both the previously suggested mechanism (Figure S1) and our newly proposed mechanism (Figure 1C).

Figure 4. (A) Negative-mode MALDI-TOF spectra of ATP (red arrow, $[M-H]^- = 506.2$) incubated with and without each condensation enzyme to produce ADP (blue asterisk, $[M-H]^- = 426.2$). (B) Proposed Uni Uni Bi Uni ping-pong reaction sequences for PigC (top) and TamQ (bottom) catalyzed reactions, inhibition pathways are highlighted in red, PG is prodigiosin, TJ is tambjamine BE-18591

When we compare the efficiencies of PigC and TamQ with their common substrate MBC using their k_{cat}/K_M values, we can see that PigC is 100-fold more efficient with a k_{cat}/K_M of $6 \times 10^5 \pm 2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ versus TamQ's k_{cat}/K_M of $0.07 \times 10^5 \pm 0.02 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Figure 2 and Figure 3). This marked difference in efficiency is very interesting and we propose may result from an evolutionary divergence between the enzymes' biosynthetic pathways. PigC binds and condenses two pyrrolic compounds, allowing the binding pocket to be highly structurally specialized for planar heteroaromatics. By contrast, TamQ possesses much lower specificities for both MBC and DDA, perhaps due to these substrates being structurally dissimilar. The lower catalytic efficiency of TamQ is particularly noteworthy when considering a more nucleophilic substrate (amine vs pyrrole) is involved in the reaction.

We propose the changes to catalytic efficiency we measured for TamQ hint at an evolutionary relationship between the prodiginine and tambjamine BGCs and may also represent a key step to generate diversity in the biosynthesis of the broader natural product family. Should an ancestral version of the prodigiosin BGC lose the capacity to produce the MAP feedstock, mutations to the condensation enzyme that decrease its substrate specificity would provide the best chance at remodelling the enzyme to make alternative prodiginines and tambjamins. The amine condensation partner in tambjamine YP1 arises from simple modifications of a primary metabolite (dodecanoic acid) making it a good candidate for a co-opted substrate. In a similar manner, several other tambjamins, that are not currently linked to biosynthetic gene clusters, appear to incorporate decarboxylated amino acids as substrates for the

condensation reaction with MBC^[35–37]. Mutations of the condensation enzymes that result in continued prodiginine/tambjamine production would be favoured because these molecules are all highly bioactive and are likely conferring a competitive advantage to the producing organism.

Further support for our hypothesis can be found in studies of substrate scope published for both enzymes. PigC is already known to accept a range of modified heteroaromatic substrates^[30]. Intriguingly, Brass *et al.* recently described extensive promiscuity for TamQ^[22]. TamQ is reported to accept heteroaromatic MAP and MAP variants as substrates, in place of the native alkylamine substrates, allowing formation of products consistent with the mass and absorption spectra of the corresponding prodiginines^[22]. Surprisingly, at least one of these TamQ products was confirmed by NMR to have C-C bond connectivity, rather than the N-C bond made in TamQ's native product^[22]. TamQ's capacity to produce prodiginines aligns with our theory that the tambjamine BGC potentially evolved from the prodigiosin BGC. This could be due to an erosion of the enzyme's affinity for pyrrolic compounds allowing it to accept a wider range of nucleophilic coupling partners. Or, this divergence could have arisen after the producer lost the capacity to make MAP-like substrates, driving TamQ to become more promiscuous. Either way, the high levels of substrate promiscuity observed for TamQ suggest it may be possible to use this enzyme for biocatalytic synthesis of analogues and as a starting point for directed evolution of an enzyme with even greater chemical scope.

Finally to probe the relationship between condensation partner structure and enzyme activity further it would be valuable to investigate kinetic

properties of other condensation enzymes from the family including RedH, a PigC/TamQ homologue from the biosynthesis of undecylprodigiosin. This prodiginine is a tripyrrole much like prodigiosin, but bears a C11 alkyl chain on the pyrrole condensation partner, imparting additional tambjamine-like structural features. Obtaining structural information of these unique active sites would also provide insight into how the enzymes can mutate to catalyze different bond connectivity which, in turn could be useful for directed evolution or active site engineering.

Conclusions

By expressing enzymes from *Pseudoalteromonas* sp., we have reported herein the first purification of soluble condensation enzymes PigC and TamQ involved in the biosynthesis of prodiginines and tambjamins. Through kinetic characterization we have demonstrated the functional similarities between the mechanisms of PigC and TamQ, despite their formation of differing bond connectivity and we provide evidence of Uni Uni Bi Uni ping-pong reaction sequences. The differences between the k_{cat}/K_M values of substrates with PigC vs TamQ suggest that TamQ may have a wider substrate tolerance than PigC and the data leads us to propose the evolution of these diverse pathways may be traced by looking at the activity profile of the key terminal reaction. Future studies including X-ray crystal structures of these enzymes and extended substrate tests will fully elucidate their mechanism of action and the contribution of structural attributes involved in substrate binding that could help direct bioengineering of analogues.

Experimental Section

Plasmid Construction

The *pigC* gene was amplified from *Pseudoalteromonas rubra* DSM 6842 genomic DNA, and the *tamQ* gene was amplified from *Pseudoalteromonas citrea* DSM 8771 by polymerase chain reaction (PCR). The *pigC* amplicon was ligated into a linearized *pET-28a* vector, and *tamQ* was ligated into a linearized *pET-32a* using Gibson Assembly® (New England Biolabs). The resulting plasmids encode for PigC with a carboxy-terminal His₆ tag and TamQ with amino-terminal thioredoxin and His₆ tags.

Protein Expression and Purification

The *pigC* and *tamQ* constructs were transformed into *Escherichia coli* BL21(DE3) cells and protein expression was induced by adding a final concentration 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) and incubation at 15 °C and 160 rpm for 14–18 h.

The harvested cell pellet was resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 40 mM imidazole, pH 7.5) containing a cOmplete EDTA-free protease inhibitor cocktail tablet (Roche). The cells were lysed by emulsification. The soluble lysate was separated by centrifugation at 29 000 xg and 4 °C for 30 min and collected.

Protein was purified using a Pharmacia Biotech fast-protein liquid chromatography (FPLC) system. The lysate was loaded onto a 5-mL Ni Sepharose High-Performance resin and washed with 100 mL lysis buffer at a flow rate of 5 mL/min. The target protein was eluted with a 20-min gradient from 100% lysis buffer to 100% elution buffer (50 mM HEPES, 150 mM NaCl, 250 mM imidazole, pH 8.0). Fractions containing the protein of interest were collected and concentrated in storage buffer; buffer A (50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, pH 7.0) for TamQ and buffer A with 1 mM DTT for PigC. The resulting protein was quantified using the extinction coefficients for PigC (115 740 M⁻¹ cm⁻¹) and TamQ (142 670 M⁻¹ cm⁻¹), and stored at -80 °C.

PigC and TamQ Kinetic Assays

All kinetic assays for PigC and TamQ were performed in Corning™ flat-bottom clear polystyrene 96-well plates at a final volume of 200 μ L per reaction. Absorbance measurements were done using Molecular Devices Spectramax® M2e microplate reader, with SoftMax® Pro 5 Software.

The extinction coefficient of prodigiosin and tambjamine BE-18591 were measured using prodigiosin isolated and LC-purified from *P. rubra*, and synthesized tambjamine BE-18591. Each analyte was solubilized in assay buffer at a range of final concentrations and the absorption at 535 nm (prodigiosin) and 405 nm (BE-18591) were measured.

Kinetic reactions were performed in assay buffer with constant concentrations of ATP, and MAP (for PigC) or DDA (for TamQ), and increasing concentrations of MBC (0, 0.5, 2.5, 5, 10, 50, 100, and 200 μ M). Enzyme was introduced simultaneously to each well to a final concentration of 1 μ M. The activity of both PigC and TamQ were optimal at 40 °C. The production of prodigiosin by PigC was monitored at 535 nm; the production of BE-18591 by TamQ was monitored at 405 nm. Each reaction condition was done in triplicate and data were interpreted using MATLAB® R2018a.

PigC kinetic parameters were determined using Equation S1 to fit kinetic data from MBC as a substrate for PigC. TamQ kinetic parameters were determined using Equation S2 to fit kinetic data from MBC as a substrate for TamQ, and Equation S3 to fit kinetic data from DDA as a substrate for TamQ. The kinetic parameters reported are an average of the values from each experiment, and the standard deviation was taken as the error.

ADP Detection

To detect ADP, 150 μ L samples containing 30 μ M PigC or TamQ, in the presence or absence of 200 μ M ATP in assay buffer were incubated at 30 °C for 1 h. The reaction was stopped by addition of 50 μ L MeOH, clarified, and mixed 1:1 with a matrix solution of 10 mg/mL 9-aminoacridine in MeOH, the mass spectra were obtained in negative ion mode. Mass spectra

were measured using a Bruker FLEX series MALDI-TOF instrument.

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Keywords: biosynthesis, enzyme, enzyme kinetics, enzyme purification, natural products, prodiginine, tambjamine

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Purification and kinetic characterization of the essential condensation enzymes involved in prodiginine and tambjamine biosynthesis

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Additional figures for experimental procedure including protein purification, compound synthesis, and kinetic analysis. Full *pig* and *tam* gene cluster annotation.