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RESEARCH ARTICLE

Creation of an engineered amide synthetase biocatalyst by the rational separation of a two-step nitrile synthetase.

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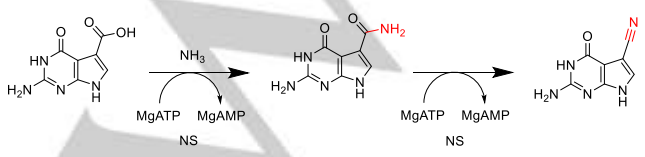
Abstract: The synthesis of amides through acid and amine coupling is one of the most commonly-used reactions in medicinal chemistry, yet still requires atom-inefficient coupling reagents. There is a current demand to develop greener, biocatalytic approaches to amide bond formation. The nitriles synthetase (NSs) enzymes are a small family of ATP-dependent enzymes which catalyse the transformation of a carboxylic acid into the corresponding nitrile via an amide intermediate. The *B. subtilis* QueC (BsQueC) is a NS involved in the synthesis of 7-cyano-7-deazaguanine (CDG) natural products. Through sequence homology and structural analysis of BsQueC we identified three highly-conserved residues, which could potentially play important roles in NS substrate binding and catalysis. Rational engineering led to the creation of a NS K163A/R204A biocatalyst that converts the CDG acid into the primary amide, but does not proceed to the nitrile. This study suggests that NSs could be further developed for coupling agent-free, amide-forming biocatalysts.

Introduction

The nitrile synthetases (NSs, also known as 7-cyano-7-deazaguanine synthases, EC 6.3.4.20) is a small family of enzymes that catalyze the conversion of 7-carboxy-7-deazaguanine (CDG) into 7-cyano-7-deazaguanine ($PreQ_0$). They play a key role in the biosynthetic pathways of nucleoside natural products such as the modified tRNA base queuosine and the antibiotic toyocamycin.[1] The best studied members of the NS family are the QueC enzymes from *Bacillus subtilis* (BsQueC) and *Geobacillus kaustophilus* (GkQueC) and the ToyM from *Streptomyces rimosus* (SrToyM).[2] The NS family is Mg^{2+} adenosine triphosphate (MgATP)-dependent and was shown to proceed via the formation of the corresponding amide intermediate, 7-amido-7-deazaguanine (ADG).[3] Ammonia has been shown to be the nitrogen source of the reaction and the overall reaction catalysed by NS is shown in Scheme 1.



Scheme 1. The two-step nitrile synthetase (NS) reaction scheme. In the first step the CDG acid substrate is first transformed into the ADG amide intermediate, using one equivalent of MgATP. In the second step, the ADG is then converted into the $PreQ_0$ nitrile using a second equivalent of MgATP.

Interestingly, these enzymes also bind a Zn^{2+} cation; however, this metal ion is thought to be "structural" and not involved in the chemical mechanism.[2] The reaction has been proposed to proceed via a mechanism that involves the formation of two deazaguanine adenylate intermediates that sequentially activate both the acid and then the amide to form the final $PreQ_0$ nitrile product, as shown in Figure S1.[2] Evidence supporting this mechanism has come from a combination of ^{31}P ATP NMR studies of GkQueC and ^{13}C ATP analysis of SrToyM.[2-3] Thus, the conversion of CDG acid to $PreQ_0$ nitrile uses two molar equivalents of ATP and both parts of the reaction (the conversion of CDG to ADG and the conversion of ADG to $PreQ_0$) are thought to follow similar mechanisms. However, little is known about the catalytic residues in the NS active site. In fact, it is not known whether these enzymes have one active site where both the amide-bond formation and the subsequent dehydration to the nitrile take place, or two distinct active sites, each specific for one part of the two-step reaction. Winkler and co-workers postulated that an aromatic residue such as a tyrosine may be involved in binding CDG through π-stacking interactions while an aspartic acid residue could direct the substrate through binding the primary and secondary amines of the CDG substrate pyrimidine ring.[2] More data is required to support this hypothesis and to date, little is known about this class of enzymes. This is surprising since they could have useful biocatalytic applications in the syntheses of commercially-relevant amide- or nitrile-containing compounds.[2-3]

Amide synthesis was recognized as a critical unmet need at the American Chemical Society (ACS) Green Chemistry Institute Pharmaceutical Roundtable held in 2016.[4] Amide synthesis is atom inefficient and relies on expensive coupling reagents, yet it is one of the most used reactions in medicinal chemistry.[5] Hence the drive towards greener, more sustainable processes.[6] Biocatalysts that generate amides are of growing importance across many fields including medicinal and polymer chemistry.[7] Lipases such as Cal-B have been used for decades as versatile amide bond-forming biocatalysts.[8] More recently, enzymes such as the ATP-dependent amide ligases (ANLs) provide an alternative, coupling-free method to prepare amides from acid and amine building blocks.[9] Another route from acid to amide can be carried out by biocatalysts from the carboxylic acid reductase (CAR) family. [10-12] The CARs contain three domains (adenylation, carrier protein and thioester reduction) and an elegant example from Flitsch and colleagues designed a CAR mutant where the carrier protein domain had been inactivated. This mutant CAR was able to convert a range of acids and amines to amides and

was used in the preparation of the anticonvulsant drug ilepcimide. [13] More recently Winn et al., used a coronafacic acid ligase (CfaL) to prepare a very broad library and medicinally-useful amides. The determination of the crystal structure allowed rational mutagenesis to engineer improved CfaL variants. [14]

The aim of this work was to generate an engineered amide synthetase (eAmS) biocatalyst from a NS enzyme and for this we chose BsQueC. This could be achieved by separating the two parts of the NS-catalysed reaction; retaining the first step, the formation of the ADG amide from CDG, and stopping the subsequent conversion of this intermediate to the $PreQ_0$ nitrile product. This engineering of the BsQueC biocatalyst requires subtle changes that will affect only one function of the enzyme, without causing complete inactivation of the whole process. We successfully achieved this by both structural and sequence analysis, combined with mutagenesis of three key residues. The designed BsQueC-eAmS biocatalyst was used to prepare milligram quantities of ADG from CDG. This study not only generated a useful biocatalyst but also lays the foundation for the engineering of the NS family.

Results and Discussion

Generation of the wild-type NS.

Recombinant QueC from *B. subtilis* (BsQueC, UNIPROT Code: O31675) was used as the biocatalyst in this study. The optimised expression protocol involves expressing the *B. subtilis* QueC gene in the *E. coli* BL21 (DE3) strain for 5 hours at 30°C after induction with 0.5 mM IPTG and with 0.1 mM $ZnSO_4$, as a zinc source (see Experimental Section). The enzyme was then purified by immobilised metal affinity chromatography (Ni^{2+} IMAC) using a 40-500 mM imidazole gradient. The enzyme eluted at 220 mM imidazole and SDS-PAGE analysis showed traces of a few higher molecular weight proteins (Figure S4). The protein was then purified by size exclusion chromatography (SEC) using a Superdex S200 HR column. The protein eluted at 66 mL. Using the column calibration data, this suggests that BsQueC eluted as a hexamer (Determined Mwt of the complex from S200 calibration curve: 164 kDa; Mwt of BsQueC hexamer: 159 kDa). The molecular weight of a BsQueC monomer, determined by ESI-MS+ was found to be 26535.89 ± 0.73 Da, in good agreement with the predicted mass (26535.16 Da) (Figure S4).

Optimisation of BsQueC reaction conditions.

The CDG substrate was prepared as by Gangjee *et al.* with a few modifications (See Experimental Section).[15] The reaction was monitored by RP-HPLC with a C18 column observing product formation at 254 nm and the retention times for the substrate and product were CDG (19.5 min), $PreQ_0$.(20.6 min). The calibration curves for CDG and $PreQ_0$ are shown in Figure S5. The resulting optimal conditions for the biocatalysed reactions are as follows: CDG (0.5 mM), ATP (1 mM), ammonium sulfate (50 mM), $MgCl_2$ (50 mM), Tris (100 mM, pH 7.0), Dithiothreitol (1 mM), 37°C for 5 hours. The reaction is then stopped by addition of trichloroacetic acid (5%).

We then carried out a time-dependent analysis (Figure 1), stopping the reaction between 0-300 mins. After 10 mins we observed the formation of the $PreQ_0$ nitrile product and a large peak at 18.3 min eluting before the substrate and product peaks. We assigned this peak to the ADG amide intermediate since this order of elution (ADG, PreQ0 and CDG) matched that observed by Nelp and Bandarian in their study of SrToyM, a homolog of the BsQueC with 28% sequence identity (Figure S4).[3] We also noted the appearance of the ADG peak immediately after initiating the reaction (0 min) which suggests amide formation is rapid. After 30 mins the CDG substrate was all but consumed, with only peaks remaining for the ADG intermediate and $PreQ_0$ nitrile. Moreover, at 60 mins the substrate CDG was no longer present, the ADG intermediate had decreased, and the $PreQ_0$ nitrile product had increased relative to the 30 min reaction. Finally, after 300 mins, no ADG amide intermediate was present and only the $PreQ_0$ nitrile product was observed.

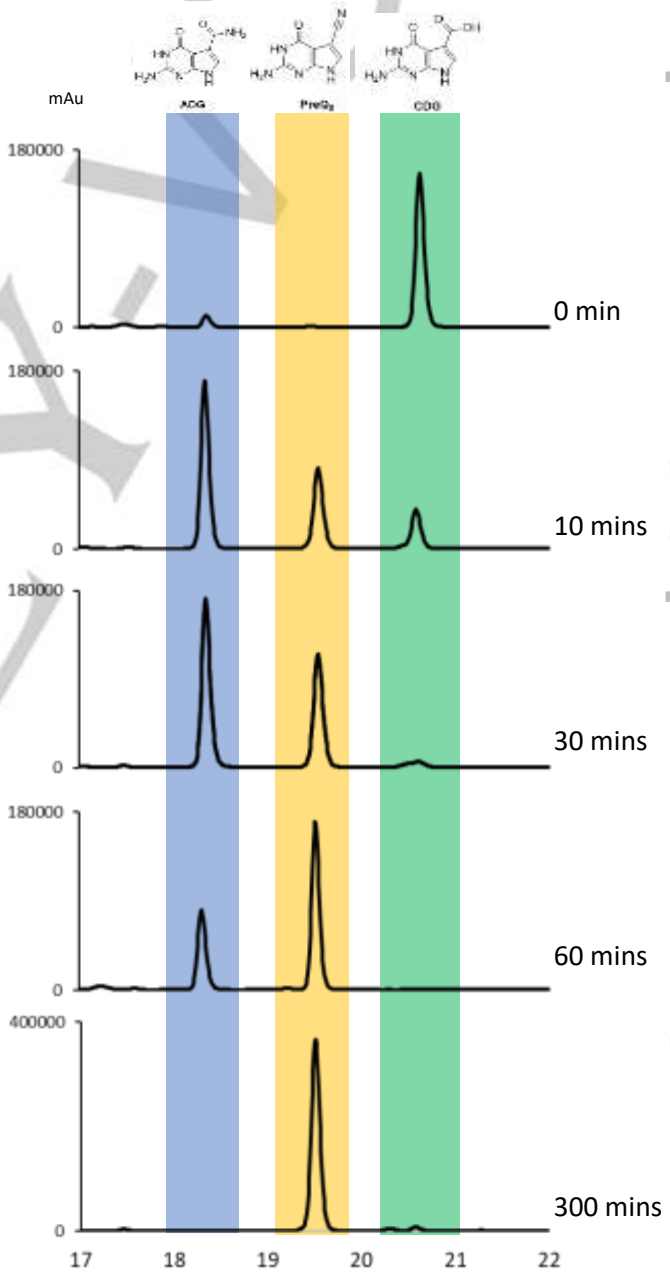


Figure 1. Time-dependant study of the NS-catalysed reaction. Experimental conditions: BsQueC (2mg/mL), 0.5 mM CDG, 1 mM ATP, 50 mM MgCl2, 50 mM ammonium sulfate in 100 mM TRIS buffer (pH 7.0), 1 mM DTT at 37°C for 0-300 mins.

BsQueC engineering.

Having confirmed that, in the presence of MgATP and NH_3, the wild type BsQueC could catalyse the conversion of CDG to $PreQ_0$ via an ADG amide intermediate, we set out to engineer the enzyme to block the second part of the reaction. To locate the BsQueC active site, we used the BsQueC crystal structure (PDB code: 3BL5) that was previously determined by Cicmil and coworkers.[16] This would have been made easier if this structure had been determined with substrate or product ligands bound at the active site, but to date this is the only structure of BsQueC with small molecules bound – a Mg^{2+} cation and a phosphate (Figure 2A). Nevertheless, these two ligands provided a good guide to the location of the active site as both Mg^{2+} and phosphate-containing substrates are required for the reaction. The Mg^{2+} ion is bound by S11 and S16 and the phosphate interacts with a phosphate-binding loop (residues 10 to 20).[11] In particular, the phosphate is a useful surrogate for the triphosphate of ATP and the CDG-adenylate intermediate. In a recent study, Winkler and colleagues also used the X-ray structure to build a model of the BsQueC-AMP complex and predicted a series of residues that maybe involved in ATP binding (Q39, R97, N98 and T119).[2] They also suggested that the amines of CDG would interact with the side chains of D125 or D131 and the CDG ring would π-stack with Y129 or Y1872. To date, the residues that bind the substrate NH_3 and those that interact with the key CDG-adenylate are unknown. Our task was to retain the catalytic residues to enable the BsQueC to generate, then release, the amide.

To aid in the identification of potential residues to target, we have also used a sequence alignment of eight NS enzymes (both QueC and ToyM) from various organisms (shown in Figure S6). We noted all four cysteines (C186, C195, C198, C201) that bind Zn^{2+} are conserved. Similarly, as one would expect, the residues involved in Mg^{2+} and phosphate binding are also identical across all eight sequences. We were struck by three residues (K163, Y187 and R204) which are 100% conserved and located in close proximity (within 7 Å) of the phosphate ligand. Positively charged side chains such as Lys, Arg or His are involved in the stabilisation of many enzymes that generate an adenylate intermediate. Examples include BirA[17], acetyl-CoA synthetase[18] or firefly luciferase[19] and have been reviewed by Naismith[20] and Gulick[21]. Such enzymes can undergo large conformational changes during the catalytic cycle as they bind the substrates, catalyse formation of the adenylate intermediate and PP_i, then react with the incoming nucleophile. In the case of BsQueC these would be the CDG-adenylate and NH_3 respectively.

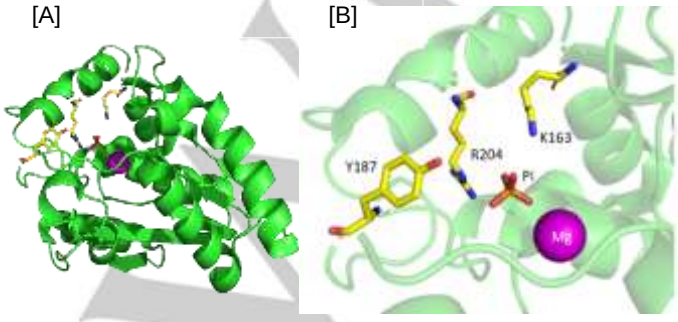


Figure 2. Crystal structure of **[A]** the BsQueC monomer (PDB code: 3BL5). **[B]** A zoomed-in view on the Mg^{2+} (pink sphere) and the phosphate ions. The three residues targeted (K163, Y187 and R204) are shown in stick form.

Thus we made three mutants K163A, Y187A and R204A. Each BsQueC variant was soluble and was isolated in a similar way to the wild type enzyme. To assess the impact of the mutations on the NS activity, each mutant was incubated under the standard conditions for 5 hrs and the products were analysed by HPLC (Figure S8). We ranked the ability of the mutants to catalyse the CDG to ADG amide to $PreQ_0$ product conversion relative to the wild type BsQueC. We noted that the BsQueC Y187A mutant converted nearly all of the CDG to the $PreQ_0$ without any ADG amide in a similar way to the wild type biocatalyst. This suggests that this residue is not essential for the NS-catalysed reaction. In contrast, both the BsQueC K163A and R204A converted almost all of the CDG into $PreQ_0$ but we also noted the presence of the ADG amide intermediate. This suggested that these residues played a catalytic role in the second step of the reaction; the formation of the $PreQ_0$ nitrile from the ADG. To fully exploit this discovery we combined these two mutants and prepared the double mutant BsQueC K163A/R204A. When this double mutant was incubated under the standard reactions conditions for 5 hours we were pleased to observe the complete conversion (>99%) of CDG into the ADG amide with no observed $PreQ_0$ product (Figure 3). We conclude that our rational engineering has delivered a novel biocatalyst, which we named BsQueC-eAmS (engineered AmS).

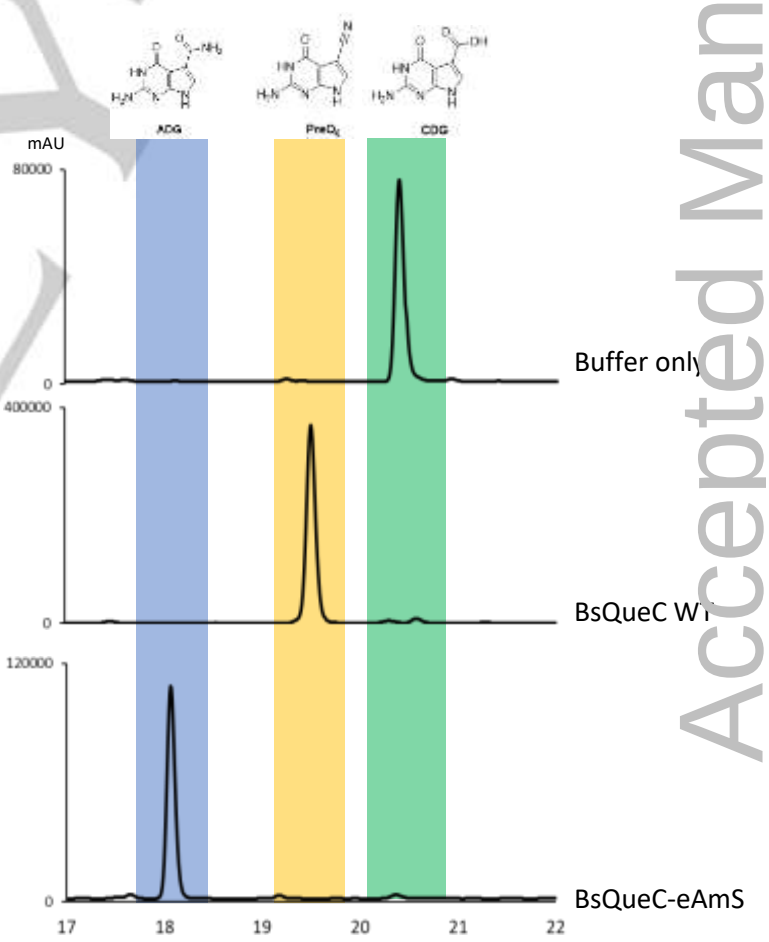


Figure 3. HPLC chromatograms of the NS reaction for BsQueC WT and BsQueC-eAmS. Experimental conditions: BsQueC (2mg/mL), 0.5 mM CDG, 1 mM ATP, 50 mM MgCl2, 50 mM ammonium sulfate in 100 mM TRIS buffer (pH 7.0), 1 mM DTT at 37°C for 300 mins.

Preparative-scale generation of ADG using BsQueC-eAmS. With the new BsQueC-eAmS in hand, we tested whether it could act as a synthetically useful biocatalyst for the preparation of the ADG. Previously, the synthesis of ADG was achieved but with two recombinant enzymes, a NS (SrToyM) to convert CDG into PreQ0 and then a nitrile hydratase complex (SrToyJKL) was used to revert the conversion of $PreQ_0$ to ADG.[3] Within the complex, SrToyJ is the Co^{2+}-dependent nitrile hydratase.[22] This reaction was carried out with the SrToyJKL complex (200 µM) on a 100 mg scale of $PreQ_0$ in 20% DMSO and incubated for 2 days at room temperature but no yield of ADG was reported.[3] In contrast we incubated the BsQueC-eAmS (0.25 mg/mL, 80 µM) with 1 mM of CDG (65 mg), ATP (386 mg, 0.7 mmol, 2 mM, 2 molar equivalents), ammonium sulfate (50 mM) and magnesium chloride (50 mM). We found that the reaction had to be carried out at quite diluted concentrations (in 350 mL buffer) to avoid precipitation of ATP and enzyme. After incubation for 2 hours, >99% conversion of CDG was observed by RP-HPLC. The product was filtered and recovered alongside some precipitated biocatalyst. The product was then extracted and 9 mg ADG (14% isolated yield) was found to be >95 % pure by HPLC and NMR (Figure S9). Despite the high % conversion, significant losses were down to the dilute work up and could be further optimised. Interestingly, by detailed analysis of the ^{1}H NMR, we noted that the two protons of the primary amide have different chemical shifts, with a chemical shift difference of ~2.5 ppm. This suggested that one of the two protons of the primary amide undergoes intramolecular H-bonding interaction with the deazaguanine carboxyl, which causes this proton to be less shielded. Now that we have biosynthesized, isolated and fully characterised ADG, we were able to confirm that the peak observed on RP-HPLC at 18.3 mins corresponds indeed to ADG, as we hypothesized.

Conclusion

The NS BsQueC is an unusual, ATP-dependent enzyme that catalyses the conversion of an acid to a nitrile via an amide intermediate using a two-adenylate mechanism. Previous studies on the related homolog SrToyM suggested that these enzymes are amide synthetase that evolved NS activity by using adenylation chemistry to act on the amide intermediate. This gain in function allowed QueC and ToyM to fulfil roles in the biosynthetic pathways of nitrile-containing natural products. To explore the NS family further and begin to understand how the two-step pathway originated required us to separate the two reactions. In effect we have reverse evolved the enzyme. This work represents the first successful engineering of a NS enzyme and uncovers the residues involved in the unusual amide dehydratase activity of the enzyme. Using a previously determined crystal structure of BsQueC and sequence alignments, we were able to locate three residues which could potentially be involved in catalysis. We have found that two of them (K163 and R204) were important as significant losses in amide dehydratase activity were observed. This suggests that these residues are not involved in the first step of the reaction, rather they play a role in the second amide to nitrile conversion. Generating a double mutant, which combined both mutations, finally led to the complete disappearance of the amide dehydratase activity, allowing this engineered enzyme to join the amide synthetase family. These observations weaken the hypothesis that the two reaction steps proceed via an analogous mechanism. However, as the residues involved in the amide dehydratase activity of the enzyme have been uncovered, saturation mutagenesis of these could be carried out to probe their role in catalysis. Nucleoside-based drugs continue to be attractive medicinal targets. A recent highlight is the nucleoside analog islatravir which is an HIV reverse transcriptase translocation inhibitor. Scientists at Merck and Codexis have developed a nine-enzyme cascade for the manufacture of islatravir at a kilogram scale. [23] The route was constructed using native and engineered biocatalysts and auxiliary enzymes, and it is hoped that such approaches will play an increasing role in synthetic strategies. [24]

Experimental Section

General

All reagents, chemicals and media were purchased from Sigma-Aldrich, Thermo Fisher Scientific, Alfa Aesar, Fluorochem or Bio-Rad unless otherwise stated. All primers were purchased from Sigma-Aldrich and competent cells, pET plasmids and restriction enzymes from New England Biolabs (NEB), Novagen, Promega and Life Technologies. Protein purification columns were purchased from GE Healthcare. HPLC columns were purchased from Phenomenex.

BsQueC expression

A single colony of BL21 (DE3) *E. coli* cells containing the expression pET-28a/BsQueC plasmid was used to inoculate 250 1nm (OD_{600}) was then recorded, and the pre-culture was diluted in 1 L fresh LB + appropriate antibiotic to an OD_{600} = 0.15. The new culture was allowed to grow to an OD_{600} = 0.6-0.9 at 37°C. After which IPTG (to a final concentration of 0.1 mM) and $ZnSO_2$ (to a final concentration of 0.1 mM) were added to induce protein expression. The cultures were shaken further for 5 hours at 30°C. The bacterial cells were harvested by centrifugation in a ThermoScientific Multifuge centrifuge at 7000 rpm for 7 min at 4°C. The cell pellet was resuspended in a minimum amount of PBS and further centrifuged at 3500 rpm for 15 mins at 4°C. The supernatant was discarded, and the cell pellets, containing the protein of interest was stored at -20°C.

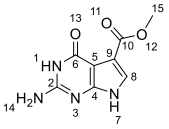
BsQueC purification

Cells were resuspended in 500 mM KCl, 40 mM imidazole (pH = 7.5) and lysed by sonication for 15 cycles (30 seconds on, 30 seconds off). DNase (0.2 mg) was added and the lysed cell suspension was centrifuged (12000 rpm, 45 mins). The supernatant was filtered with a 0.45 µm filter and injected on a 1 mL HisTrap HP column attached to an AKTA Start. The column was washed with 10 column volumes (CVs) of 500 mM KCl, 40 mM imidazole (pH = 7.5) and the protein was eluted with a 40-500 mM imidazole gradient for 10 CVs at a flow rate of 1 mL/min, monitoring at 280 nm. The fractions that contained the protein were then pooled and concentrated to 5 mL using a Vivaspin 20 (GE Healthcare, MW cut-off = 10 kDa). The protein was then injected onto a pre-equilibrated (1 CV, 100 mM Tris, 1 mM 1,4-Dithiothreitol (DTT), pH = 7.0) HiPrep 16/600 Superdex 200. The recombinant enzyme was eluted at a flow rate of 1 mL/min, monitoring at 280 nm. The purity of recombinant proteins was analysed by 12% SDS-PAGE. Fractions containing the protein were combined. The enzyme was concentrated to 4 mg/mL, flash-frozen in liquid nitrogen and stored at -80°C.

BsQueC site directed mutagenesis

Primers were constructed following the overlapping primers method.[25] The sequence and melting temperatures (T_m) of the site-directed mutagenesis primers are shown on Table S1. PCR reactions were prepared on ice and consisted of: template DNA (150 ng), forward primer (10 µM), reverse primer (10 µM), dNTP mix (10 mM), 5x Phusion HF reaction buffer (10 µL), Pfu DNA polymerase enzyme (1.0 µL) and deionised water to a final volume of 50 µL. Initial denaturation was carried at 98°C for 2 minutes before 30 cycles of denaturation (98°C for 1 minute), annealing (T_m - 5°C for 30 seconds) and extension (72°C for 2 minutes). Final extension was carried at 72°C for 5 minutes. 15 µL of the reaction was analysed by gel electrophoresis to check for correct amplification. Amplified PCR products were mixed with CutSmart buffer (4 µL) and Dpn1 enzyme (1.5 µL). The reaction was incubated at 37°C overnight. 5 µL of the mix were used to transform C2987 cells on LB Agar.

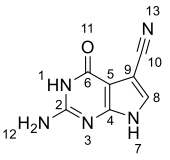
Organic synthesis



One-pot synthesis of CDG-methyl ester: To methyl formate (3.20 g, 58 mmol, 5.8 eq.) in THF (25 mL) cooled to 0°C were subsequently added sodium methoxide (0.72 g, 13 mmol, 1.3 eq.) and methyl chloroacetate (1.09 g, 10 mmol, 1.0 eq). The reaction was stirred overnight at RT. Deionised water (25 mL) was added to stop the reaction and quench excess sodium methoxide. Excess methyl formate and THF were removed under reduced pressure and 2,4-diamino-6-hydroxypyrimidine (1.20 g, 10 mmol, 1.0 eq.) was subsequently added and the mixture was refluxed for 1 hour. The product precipitated upon cooling and was recovered by filtration. After THF washings, the product was obtained (0.92 g, 3.7 mmol, 47% yield) with a purity exceeding 95%. The NMR data were in good agreement with the published structure.[15]

^{1}H NMR (600 Hz, dDMSO): δ = 11.63 (1H, s, N1-H), 10.35 (1H, br s, N7-H), 7.35 (1H, d, J = 3Hz, C8-H), 6.49 (2H, br s, N14-H_2), 2.80 (3H, s, C15-H_3).

^{13}C NMR (600 Hz, dDMSO): δ = 163.18 (C10), 155.88 (C6), 152.80 (C2), 152.72 (C4), 122.15 (C8), 110.47 (C9), 99.14 (C5), 53.62 (C15).

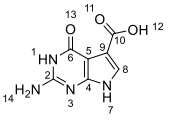


One-pot synthesis of PreQ$_0$: To methyl formate (3.20 g, 58 mmol, 5.8 eq.) in THF (25 mL) cooled to 0°C were subsequently added sodium methoxide (0.72 g, 13 mmol, 1.3 eq.) and chloroacetonitrile (0.76 g, 10 mmol, 1.0 eq). The reaction was stirred overnight at RT. Deionised water (25 mL) was added to stop the reaction and quench excess sodium methoxide. Excess methyl formate and THF were removed under reduced pressure and 2,4-diamino-6-hydroxypyrimidine (1.20 g, 10 mmol, 1.0 eq.) was subsequently added and the mixture was refluxed for 1 hour. The product precipitated upon cooling and was recovered by filtration. After THF washings, the product was obtained (1.12 g, 6.4 mmol, 64% yield) with a purity exceeding 95%. The NMR data were in good agreement with the published analysi.[26]

^{1}H NMR (600 Hz, dDMSO): δ = 11.95 (1H, br s, N1-H), 10.70 (1H, br s, N7-H), 7.58 (1H, s, C8-H), 6.37 (2H, br s, N12-H_2).

^{13}C NMR (600 Hz, dDMSO): δ = 158.09 (C6), 154.26 (C2), 152.16 (C4), 128.40 (C8), 116.37 (C10), 99.19 (C5), 86.00 (C9).

MS (ESI, [M+H$^+$]): *m/z* = 176.0558 (theoretical *m/z* ($C_7H_5N_5O$) = 176.0572).



Synthesis of CDG from CDG-methyl ester: To a suspension of CDG-methyl ester (0.92 g, 3.7 mmol, 1.0 eq.) in 1,4-dioxane (10 mL) was added HCl (37%, 10 mL, excess). The mixture was refluxed for 5 hours. The precipitate formed upon cooling was filtered, washed with copious amounts of THF and dried. The brown solid (0.67 g, 2.8 mmol, 81%) was confirmed to be the product at purity > 95%.

^{1}H NMR (600 Hz, dDMSO): δ = 12.04 (1H, s, N1-H), 11.51 (1H, br s, N7-H), 7.45 (1H, d, J = 3 Hz, C8-H), 6.58 (2H, br s, N14-H_2).

^{13}C NMR (600 Hz, dDMSO): δ = 163.48 (C10), 161.86 (C6), 153.55 (C2), 152.74 (C4), 126.30 (C8), 110.98 (C9), 96.82 (C5).

MS (ESI, [M+H$^+$]): *m/z* = 195.0509 (theoretical *m/z* ($C_7H_6O_3N_4$) = 195.0518).

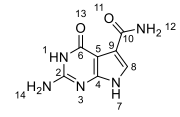
Optimised BsQueC-catalysed NS reaction

To a solution of BsQueC in 100 mM Tris buffer, 1 mM 1,4-Dithiothreitol, pH = 7.0 (2 mg/mL) were added $MgCl_2$ (50 mM), ammonium sulfate (50 mM), ATP (1 mM) and CDG (0.5 mM). The mixture was then stirred at 280 rpm for 5 hours at 37°C.

HPLC analysis

Samples of the reaction mixture (100 µL) were removed and 100 % trichloroacetic acid (5 µL) was added to stop the reaction. The samples were centrifuged at 13000 rpm for 2 mins and 10 µL of the supernatant was injected onto a Phenomenex Luna 5u C18 HPLC column. Products were eluted with water + 0.1% TFA for 5 minutes followed by a 17 minute gradient to 20% MeCN + 0.1% TFA. The column was then washed for 1 minute with 50% MeCN + 0.1% TFA/50% water + 0.1% TFA before re-equilibrating the column with 100% water + 0.1% for 5 minutes. The analytes were detected by a UV detector at 254 nm.

Preparative scale synthesis of ADG using the BsQueC eAMS biocatalyst



To a 350 mL solution of BsQueC eAMS (0.25 mg/mL) in 100 mM Tris buffer, 1 mM 1,4-Dithiothreitol, pH = 7.0 were subsequently added $MgCl_2$ (50 mM, excess), ammonium sulfate (50 mM, excess), ATP (386 mg, 0.7 mmol, 2 mM, 2 eq.) and CDG (67 mg, 0.35 mmol, 1 mM). CDG was added last to initiate the reaction and avoid ATP precipitation. The reaction was shaken at 280 rpm for 2 hours at 37°C and the cloudy solution was filtered through Whatman paper (11 µm). The recovered solid was redissolved in DMSO (20 mL) and filtered through a centrifugal spin column (Vivaspin 20 (GE Healthcare, MW cut-off = 10 kDa) to remove any remaining biocatalyst. The filtrate was retained and the DMSO was removed under high vacuum (5 days) to recover a brown oil. The ADG was recovered from the oil by the addition of THF (~20 ml) which caused the precipitation of a brown solid which was recovered by filtration on Whatman paper (11 µm). The brown solid (9 mg, 0.05 mmol, 14%) was confirmed to be ADG by ^{1}H NMR, ^{13}C NMR and the purity (>95%) determined by RP-HPLC (ret. time 18.3 min). The NMR data were in good agreement with the published analysis.[26]

^{1}H NMR (600 Hz, dDMSO): δ = 11.57 (1H, s, N1-H), 10.68 (1H, br s, N7-H), 9.55 (1H, s, N12-H), 7.21 (1H, d, J = 3 Hz, C8-H), 6.97 (1H, s, N12-H), 6.37 (2H, br s, N14-H_2).

^{13}C NMR (600 Hz, dDMSO): δ = 164.75 (C10), 160.76 (C6), 153.20 (C2), 153.06 (C4), 123.43 (C9), 115.08 (C8), 96.47 (C5).

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Keywords: Biocatalysis • amide synthetase • nitrile synthetase • enzyme engineering • rational design

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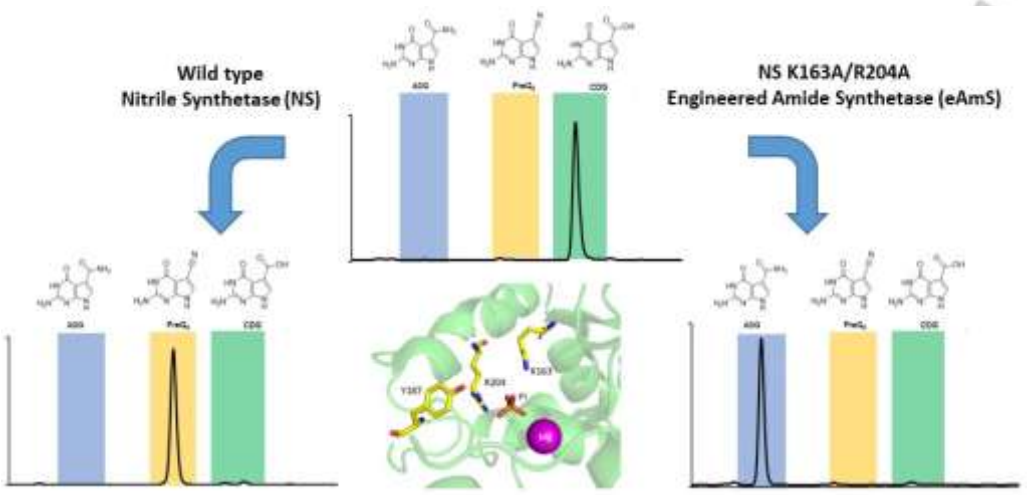

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Rational engineering of the ATP-dependent biocatalyst QueC converts a nitrile synthetase to an amide synthetase.

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