Bisucaberin biosynthesis: an adenylating domain of the BibC multi-enzyme catalyzes cyclodimerization of *N*-hydroxy-*N*-succinylcadaverine†

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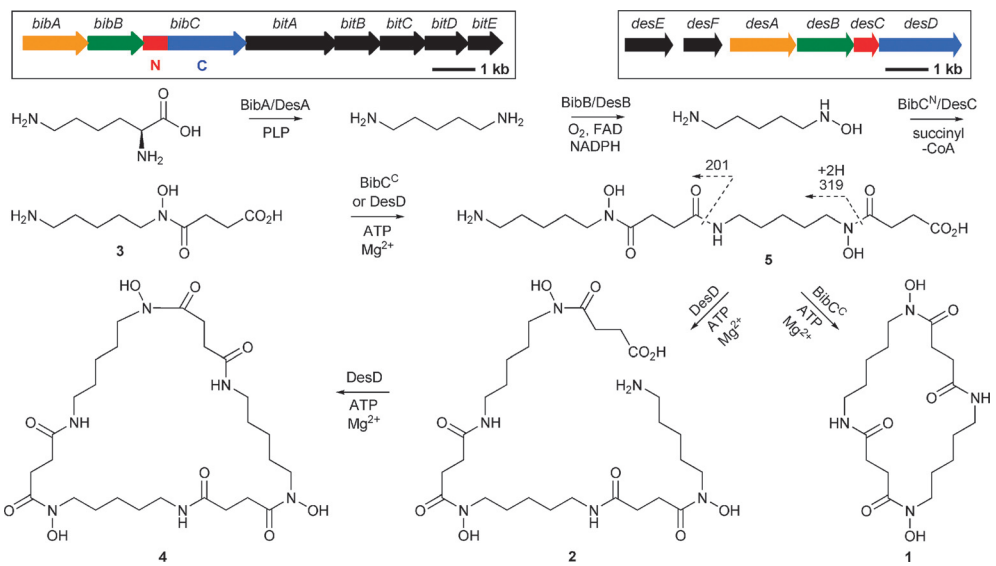
The bisucaberin biosynthetic gene cluster has been identified in *Vibrio salmonicida* and a domain from within the BibC multienzyme encoded by the cluster has been shown to catalyse ATP-dependent dimerisation and macrocyclisation of *N*-hydroxy-*N*-succinylcadaverine to form bisucaberin.

Macrocycles are key structural motifs of many bioactive natural products, including clinically-used antibacterials, antifungals, immunosuppressants and antiparasitics, as well as several iron-chelating siderophores that play vital roles in bacterial iron acquisition.[1,2] Several macrocyclic natural products are oligomeric and are composed of two or more identical or similar subunits joined together by ester, amide, thioester or carbon–carbon linkages.[1–6] Recently, it has become apparent that the key macrocyclization and oligomerization–macrocyclization reactions in the biosynthesis of many natural products are catalyzed by thioesterase (TE) domains of nonribosomal peptide synthetase (NRPS) and modular polyketide synthase (PKS) multienzymes.[1] These reactions proceed *via* a series of intermediates that are covalently attached to the multienzymes throughout the assembly process.[1]

Bisucaberin **1** is a dimeric macrocylic metabolite of the marine bacterium *Alteromonas haloplanktis* (Scheme 1).[7] It renders tumor cells susceptible to macrophage-mediated cytolysis and also causes direct cytostasis by specific inhibition of DNA synthesis.[7] The macrocyclic bis-hydroxamate structure of bisucaberin is highly preorganized for ferric iron binding; it has a much higher affinity for ferric iron than acyclic bis-hydroxamate ligands.[8] At low pH bisucaberin predominantly forms a 1 : 1 complex with ferric iron, whereas at neutral pH a monobridged 2 : 3 ligand : iron complex is the predominant species.[8] Bisucaberin may function as a siderophore in *A. haloplanktis*. However, this hypothesis remains to be verified.

Recently, bisucaberin has also been isolated from another marine bacterium, *Vibrio salmonicida*, the causative agent of cold-water vibriosis, a fatal septicaemia of farmed fish including Atlantic salmon, cod and rainbow trout.[9] Interestingly,



Scheme 1 Organization of the *bib-bit* and *des* gene clusters, and proposed roles of Bib and Des proteins in bisucaberin/desferrioxamine biosynthesis. Genes encoding proteins with analogous functions have the same colour. The structures of fragment ions observed in the ESI-MS/MS spectrum of **5** are indicated.

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another marine *Vibrio* species has been reported to produce the acyclic tris-hydroxamate desferrioxamine G1 **2**.[10] Bisucaberin **1** and desferrioxamine G1 **2** both consist of alternating succinic acid and *N*-hydroxycadaverine units linked by amide bonds (Scheme 1). However, **1** contains two molecules each of

succinic acid and *N*-hydroxycadaverine, whereas **2** contains three molecules of each of these units.

We recently showed that the *Streptomyces coelicolor* DesD enzyme catalyzes key trimerization and macrocyclization reactions in desferrioxamine biosynthesis.[2c] DesD is the first biochemically characterized member of a putative family of novel synthetases that catalyze oligomerization–macrocyclization reactions *via* a different mechanism to the TE domains of NRPSs and PKSs.[2c] It catalyzes ATP-dependent trimerization of *N*-hydroxy-*N*-succinylcadaverine (HSC) **3** to form desferrioxamine G1 **2** and subsequent ATP-dependent macrocyclization of **2** to form desferrioxamine E **4** (Scheme 1).[2c] We speculated that bisucaberin **1** may be assembled by a DesD homologue that catalyzes dimerization and subsequent macrocyclization of HSC **3**,[2c] rather than the trimerization–macrocylization of HSC catalyzed by DesD in the biosynthesis of desferrioxamine E **4**.

Sequencing of the *Vibrio salmonicida* LFI1238 genome is currently in progress at the Sanger Institute (http://www.sanger.ac.uk/Projects/V_salmonicida/). We used the TBLASTN algorithm to search the draft genome sequence of *V. salmonicida* for DesD homologues.[11] This analysis identified a putative gene, which we named *bibC* (*bi*sucaberin *b*iosynthesis), encoding a 819 amino acid protein predicted to contain two functional domains. The C-terminal domain (BibC^{C}) shows 44% identity and 63% similarity across a 603 amino acid overlap to DesD.[2] The N-terminal domain (BibC^{N}) shows 32% identity and 50% similarity over a 172 amino acid overlap to DesC, which has been proposed to catalyze the acylation of *N*-hydroxycadaverine with succinyl-CoA to form HSC **3** during desferrioxamine biosynthesis in *Streptomyces* species.[2] This analysis suggested that BibC is a multienzyme that catalyzes the formation of HSC **3**, followed by its dimerization and subsequent macrocyclization to form bisucaberin **1** (Scheme 1). We also analyzed the coding sequences flanking *bibC*. Two putative genes (*bibA* and *bibB*) upstream of *bibC* encode proteins that are similar to the DesA and DesB enzymes, respectively, and that are proposed to catalyze identical reactions in desferrioxamine and bisucaberin biosynthesis (Scheme 1).[2] Downstream of *bibC* we identified five putative genes which we designated *bitABCDE* (*bi*sucaberin *t*ransport) that encode homologs of proteins known to participate in the uptake of ferric-siderophore complexes in Gram-negative bacteria (TonB-dependent ferric-siderophore outer membrane receptor, putative ferric-siderophore periplasmic binding protein, 2 × permease components and an ATPase component of ferric-siderophore ABC transport systems, respectively) (Scheme 1).[12] Taken together these analyses suggest that the *bibABC-bitABCDE* gene cluster encodes all the proteins required for the biosynthesis of bisucaberin **1** and the uptake of its ferric complex(es). Intriguingly, two coding sequences flanking this gene cluster encode transposase homologs, suggesting that it may have been acquired by horizontal transfer into the genome of *V. salmonicida*.

To examine the involvement of the identified gene cluster in bisucaberin biosynthesis and test the hypothesis that BibC^{C} catalyzes the dimerization and subsequent macrocyclization of HSC **3**, we cloned nucleotides 601-2460 of the *bibC* coding sequence (encoding the C-terminal 619 amino acids of BibC corresponding to the BibC^{C} domain) into pET151 to create

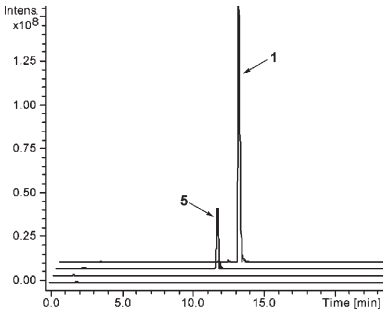


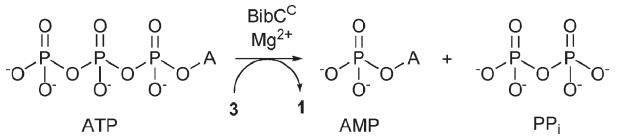
Fig. 1 Extracted ion chromatograms (EICs) from LC-MS analyses of the incubation of HSC **3** with His$_6$-BibC, ATP and Mg^{2+}. From top: EIC at m/z = 401; EIC at m/z = 419; EIC at m/z = 401 from negative control; EIC at m/z = 419 from negative control.

pNK003, which encodes a His$_6$-BibC^{C} fusion protein. The plasmid was introduced into *E. coli* BL21star(DE3) and His$_6$-BibC^{C} was overproduced as a soluble protein that was purified to homogeneity from cell-free extracts of the expression host by Ni-NTA chromatography and subsequent gel filtration. The gel formation indicated that His$_6$-BibC^{C} exists as a dimer in solution. The identity of the purified protein was confirmed by peptide mass fingerprinting.

Purified His$_6$-BibC^{C} was incubated with ATP, Mg^{2+} and synthetic HSC **3**.[2c] The reaction was stopped by addition of trichloroacetic acid and LC-MS analysis identified two compounds with retention times of 11.7 and 13.0 minutes and m/z = 419 and 401, respectively (Fig. 1), which were absent from control reactions containing His$_6$-BibC^{C} inactivated by boiling. The compound with m/z = 401 was isolated from a scaled-up incubation by semi-preparative HPLC. ESI-TOF-MS analysis yielded the molecular formula $C_{18}H_{33}N_4O_6^+$ for the m/z = 401 ion (calc.: 401.2395, found: 401.2397), consistent with structure **1** for this compound. ESI-MS/MS of the m/z = 401 ion produced a dominant fragment at m/z = 201, as previously reported for bisucaberin isolated from *V. Salmonicida*.[9] The ^{1}H NMR data for this compound were identical to those reported for synthetic and natural bisucaberin.[7,13] COSY, HSQC and HMBC experiments unambiguously confirmed that the isolated compound was bisucaberin **1**. Trace amounts of desferrioxamine E **4** were also observed during these analyses.

The compound with m/z = 419 was isolated from a scaled-up incubation that was stopped after 5 min. The molecular formula of this ion was deduced to be $C_{18}H_{35}N_4O_7^+$ (calc.: 419.2500, found: 419.2501) by ESI-TOF-MS analysis, consistent with structure **5** for this compound. Although the compound could not be isolated in sufficient amounts to characterize by NMR spectroscopy (because it is a transient intermediate in the conversion of **3** to **1**—see below), ESI-MS/MS analysis generated daughter ions with m/z = 319 and 201 from the m/z = 419 $[M + H]^+$ ion, clearly indicating that it is structurally related to **1** and providing strong evidence for structure **5** (Scheme 1).

To investigate whether **5** is an intermediate in the conversion of HSC **3** to biscucaberin **1**, we incubated HSC with purified His$_6$-BibC^{C}, Mg^{2+} and ATP for 2.5, 5, 7.5, 10, 20, 30, 45 and 60 min. The reactions were halted by addition of ferric chloride, which precipitated the enzyme and converted **1** and **5** to their



Scheme 2 Conversion of ATP to AMP and PP_i in the $BibC^C$-catalyzed assembly of bisucaberin **1** from HSC **3** (A = adenosine).

corresponding ferric complexes. The formation of the ferric complexes was confirmed by LC-DAD-MS; the complexes exhibited absorbance maxima at 471 nm and produced ions with m/z 454.3 and 472.3 in positive ion mode, consistent with the expected formation of 1 : 1 iron : ligand complexes.[8] The relative quantity of the ferric complexes of **1** and **5** in each incubation mixture was determined using HPLC monitoring absorbance at 471 nm. A plot of the concentration of each complex against time indicated that **5** is a transient intermediate in the conversion of HSC **3** to bisucaberin **1** (see ESI†). Compound **5** was unambiguously confirmed as an intermediate in the conversion of HSC **3** to bisucaberin **1** by incubating a purified sample of it with purified His_6-$BibC^C$, Mg^{2+} and ATP. LC-MS analyses of the ferric complexes that resulted from halting the reaction by addition of ferric chloride indicated that **5** had been completely converted to **1**. No conversion was observed in a control reaction using heat-inactivated enzyme.

Using coupled continuous assays for AMP and ADP, as well as for phosphate and pyrophosphate (PP_i), we showed that $BibC^C$ converts ATP to AMP and PP_i (Scheme 2 and ESI†), consistent with the formation of acyl adenylate intermediates during the assembly of bisucaberin **1** from HSC **3**.

Taken together, our data lead us to propose a mechanism for $BibC^C$-catalyzed biosynthesis of bisucaberin **1** from HSC **3**, as follows. A molecule of ATP and a molecule of HSC **3** bind to the active site of $BibC^C$ and react to form an adenylate. A second molecule of HSC binds to the active site and undergoes general base-mediated condensation with the adenylate to form **5**, which can dissociate from the active site. The PP_i and AMP formed by the adenylation and condensation reactions dissociate from the active site and are replaced by a second molecule of ATP. This reacts with bound **5** to form an adenylate that undergoes macrocyclization *via* general base-mediated intramolecular condensation of the amino group and the activated carboxyl group in the adenylate to form **1**, which is released along with a molecule of AMP and a molecule of PP_i from the active site of $BibC^C$. Alternative orders of substrate binding and intermediate/product release are also possible. Further experiments will be required to discriminate between these.

The findings reported here are significant for several reasons. They show that bisucaberin **1** and desferrioxamines are assembled by very similar NRPS-independent siderophore (NIS) biosynthetic pathways *via* the common intermediate HSC **3**. NIS pathways are responsible for the biosynthesis of a wide variety of structurally diverse iron-chelating natural products.[14] Autonomous synthetase enzymes showing no sequence similarity to NRPS adenylation domains or any other class of adenylate-forming enzyme catalyze key transformations in NIS pathways.[2a,14,15] The bisucaberin pathway elucidated here provides the first example of an NIS pathway in which such a synthetase is not an autonomous enzyme but a domain within a multienzyme, providing an unexpected parallel with nonribosomal peptide and polyketide biosynthesis, where multienzymes are also involved.[1] BLAST searches indicate that similar multienzymes to BibC are encoded by other NIS gene clusters in the database (see ESI†). Intriguingly, the DesD enzyme catalyzes selective trimerisation and subsequent macrocyclization of HSC **3** in desferrioxamine biosynthesis,[2a] whereas the $BibC^C$ domain catalyzes selective dimerization and subsequent macrocyclization of HSC to form bisucaberin **1**. Both enzymes employ a catalytic mechanism for oligomerization and macrocyclization that involves free intermediates. This mechanism is very different from the mechanism of the oligomerization and macrocyclization reactions catalyzed by thioesterase domains of NRPSs and PKSs, where all intermediates in the process remain covalently bound to the enzyme throughout.[1] The DesD enzyme and the $BibC^C$ domain share 66% sequence similarity, raising the intriguing question: how do two such similar enzymes control the selective formation of trimeric and dimeric macrocycles, respectively, from a common intermediate?

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