ChemComm

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Journal:	*ChemComm*
Manuscript ID	CC-COM-06-2020-004171.R2
Article Type:	Communication

COMMUNICATION

Precursor-directed biosynthesis of catechol compounds in *Acinetobacter bouvetii* DSM 14964

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Genome mining for VibH homologs reveals several species of *Acinetobacter* with a gene cluster that putatively encodes the biosynthesis of catechol siderophores with an amine core. *A. bouvetii* DSM 14964 produces three novel biscatechol siderophores: propanochelin (1), butanochelin (2), and pentanochelin (3). This strain has a relaxed specificity for the amine substrate, allowing for the biosynthesis of a variety of non-natural siderophore analogs by precursor directed biosynthesis. Of potential synthetic utility, *A. bouvetii* DSM 14964 condenses 2,3-dihydroxybenzoic acid (2,3-DHB) to allylamine and propargylamine, producing catecholic compounds which bind iron(III) and may be further modified via thiol-ene or azide-alkyne click chemistry.

Many bacteria acquire iron from their environment by producing small-molecule, high-affinity iron(III) chelators called siderophores. Siderophores containing the catechol 2,3-dihydroxybenzoic acid, a common ligand in siderophores, are synthesized by nonribosomal peptide synthetases (NRPSs), large multi-domain enzymes that function in an assembly-line manner. 2,3-DHB is synthesized in three steps from chorismate, activated by a 2,3-DHB–AMP ligase, and transferred onto an aryl carrier protein (ArCP).[1] A condensation domain then catalyzes amide bond formation between 2,3-DHB and an amine, most often a NRPS-bound amino acid. VibH of vibriobactin (Fig. 1) biosynthesis is an unusual standalone condensation domain that condenses ArCP-bound 2,3-DHB and norspermidine, forming the amide linkage.[2, 3] Several other siderophore biosyntheses feature VibH-like enzymes putatively responsible for the condensation of 2,3-DHB to diamines and polyamines, including brucebactin (Fig. 1), fluvibactin, and agrobactin, among others.[4-6]

Precursor directed biosynthesis (PDB) was one of the first methods used to generate novel NRPS products.[7] Non-natural substrates, when added to the growth medium of a bacterial culture, are incorporated into the final molecule by the native biosynthetic pathway.[8-10] PDB can access novel secondary metabolites while bypassing the complexities of synthetic chemistry or synthetic biology. Cleto and Lu combined PDB and heterologous expression to generate a variety of siderophore analogs, demonstrating the versatility of VibH by producing several non-natural 2,3-DHB-diamine and 2,3-DHB-polyamine conjugates.[11] However, the reactivity of VibH is limited to installing a single DHB moiety and no bis-catechol product was reported.[2, 11] In contrast, the VibH homolog involved in the biosynthesis of brucebactin (Fig. 1) twice adds 2,3-DHB to spermidine.[6] Similarly the unsequenced strain *Acinetobacter soli* MTCC 5918, which produces acinetoamonabactin (Fig. 1) may also possess a homolog of VibH capable of condensing three 2,3-DHB moieties to tris(aminomethyl)methylamine, a

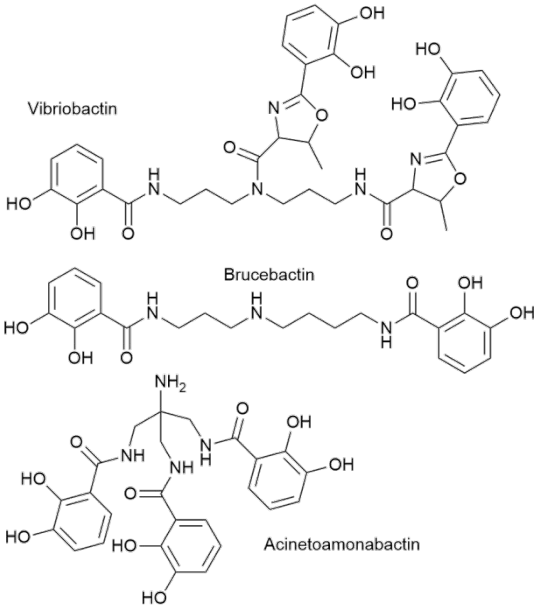

Fig. 1. Structures of vibriobactin (based on norspermidine), brucebactin (based on spermidine), and acinetoamonabactin (based on tris(aminomethyl)-methylamine).

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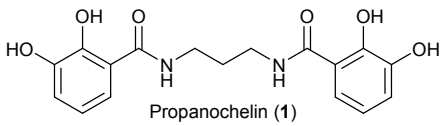
Electronic Supplementary Information (ESI) available. See DOI: 10.1039/x0xx00000x

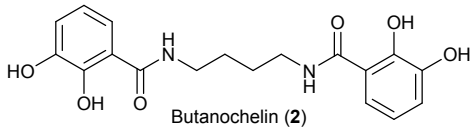
new polyamine scaffold in siderophores.[12] Catecholic compounds have been shown to adhere to surfaces in aqueous conditions,[13 14] as well as to promote the adhesion of *E. coli* to titania via the siderophore enterobactin.[15] Thus a VibH homolog capable of iterative 2,3-DHB conjugation is an attractive candidate for further PDB investigations directed towards biosynthesis of new catechol materials.

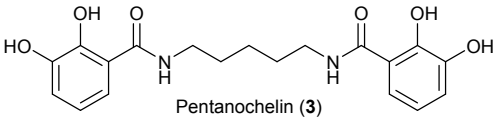
Using VibH as a genomic handle, we identified several *Acinetobacter* species that could potentially produce polyamine-based catechol siderophores similar to acinetoamonabactin (Fig. 1). We report herein three novel biscatechol siderophores present in the low-iron culture supernatant of *A. bouvetii* DSM 14964, named propanochelin (**1**), butanochelin (**2**), and pentanochelin (**3**). Precursor directed biosynthesis demonstrates that *A. bouvetii* DSM 14964 can incorporate non-natural amines *in vivo* to produce a variety of siderophore analogs. We found that PDB using *A. bouvetii* DSM 14964 is particularly effective at condensing 2,3-DHB to propargylamine and allylamine, forming catechol products which would be suitable for click-directed chemical modifications in the design of potential new wet adhesive catechol materials.

VibH homologs were found in *Acinetobacter* genomes by searching the UniProtKB protein database with phmmer.[16] Filtering the results to standalone condensation domains eliminated traditional condensation domains that are generally fused to other NRPS domains. Homologs were found in *A. bouvetii*, *A. larvae, A. gandensis*, *A. piscicola*, *A. pragensis, A. puyangensis*, and several unspecified strains (Table S1). Each VibH homolog is part of an identical gene cluster, which contains genes putatively encoding 2,3-DHB biosynthesis and incorporation enzymes, a TonB-dependent outer membrane receptor protein, a Fe(III)-siderophore reductase, and a PLP-dependent enzyme annotated as a Orn/Lys decarboxylase (Fig. 2, Table S2). Together, these genes are predicted to encode for the biosynthesis and utilization of a catechol siderophore with a diamine backbone.

To identify the natural catechol siderophores putatively produced by the VibH homolog AbsH, *Acinetobacter bouvetii* DSM 14964 was cultured in a low-iron medium (See SI for culture and isolation conditions). The methanolic supernatant extract contained three peaks with a UV-Vis absorbance band near 310 nm and an ESI-MS^E fragment of 137 m/z, each







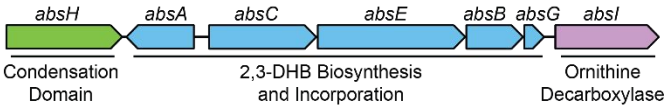


Fig. 2. The biosynthetic gene cluster in A. bouvetii DSM 14964 putatively responsible for siderophore production. The cluster is drawn to scale; arrows represent the direction of transcription. A full description of the gene cluster can be found in Table S2.

consistent with catechol-containing compounds (Figs. 3, S1, and S2). The three peaks had base molecular ions at 347, 361, and 375 *m/z*, respectively, suggesting a suite of compounds each differing by a methylene unit (Figs. S3-S5). Each is capable of binding Fe(III), as indicated by ions corresponding to $[L+Fe]^+$ and $[L_2+Fe]^+$ (Figs. S3-S5). The observed masses and fragmentation patterns are consistent with biscatechol siderophores based on diaminopropane, putrescine, and cadaverine; herein named propanochelin (**1**), butanochelin (**2**), and pentanochelin (**3**), respectively.

The flexibility and fidelity of the biosynthesis pathway was investigated by precursor directed biosynthesis (Table S3). The ratios of the three natural siderophores could be shifted by the addition of diaminopropane, putrescine, or cadaverine to the growth medium, although diaminopropane had only a slight effect on production of propanochelin, **1** (Fig. 3). No 2,3-DHB-diamine single addition product was identified by ESI-MS. In contrast, supplementation with ethylenediamine resulted in

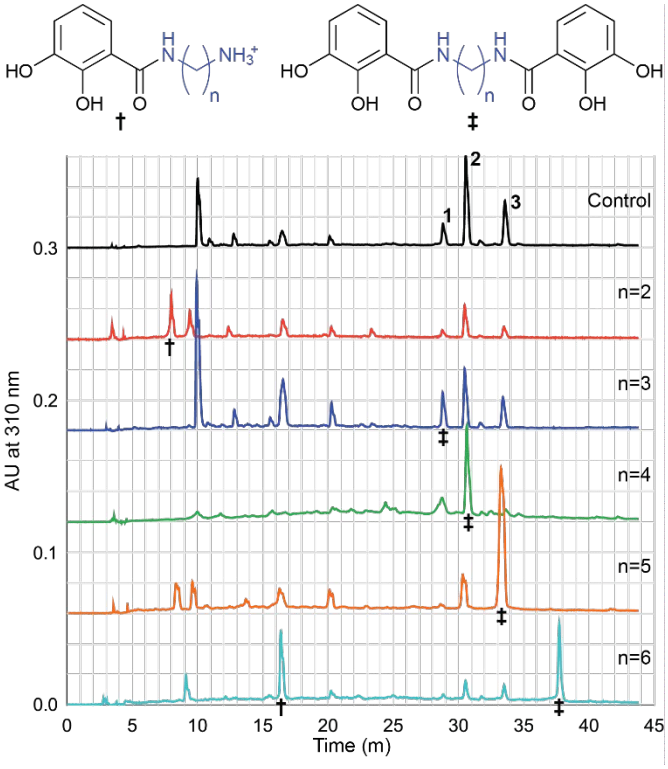


Fig. 3. Precursor directed biosynthesis of natural and unnatural diamine conjugates in growth of *A. bouvetii* DSM 14964. Peaks corresponding to mono-2,3-DHB (†) and di-2,3-DHB (‡) conjugates are marked. The methanol eluent from the XAD column of 72 h cultures were monitored by HPLC-UV/Vis at 310 nm to detect catechol-containing compounds. Casamino acid minimal medium (control) was supplemented with diamines (1 mM final concentration for each) prior to inoculation. The di-2,3-DHB conjugates for n=3, n=4, and n=5 correspond to the natural siderophores **1**, **2**, and **3**, respectively. Conjugate identities were confirmed by UPLC/MS (Figs. S3-S7).

only the single 2,3-DHB addition product, while 1,6-

diaminohexane resulted in a mixture of single and double 2,3-DHB addition products (Figs. 3, S6, and S7). Thus, the biosynthetic pathway can accommodate a wider variety of diamines for the addition of the first 2,3-DHB than the second 2,3-DHB.

Next we investigated whether functionalized amines could be accepted as substrates in the biosynthetic pathway of *A. bouvetii* DSM 14964 (Table S3). No condensation products were formed with the amino acids L-diaminobutyric acid, L-ornithine, or L-lysine; however, the addition of L-Orn caused an increase in butanochelin (**2**) production (Fig. 4). Therefore, the PLP-dependent enzyme AbsI is likely responsible for the decarboxylation of ornithine to putrescine. The polyamine diethylenetriamine is an isostere of cadaverine (1,5-diaminopentane) and was likewise incorporated as the biscatechol product **4** (Figs. 5 and S8-S13). Norspermidine (1 mM final concentration) and the branched polyamine tris(2-aminoethyl)amine (tren; 1 mM) both inhibited detectible growth of *A. bouvetii* DSM 14964. Histidine (1 mM) was not incorporated, and instead partially inhibited natural siderophore production (Fig. S14).

Propargylamine showed the most efficient coupling of any of the amines tested, nearly abolishing native siderophore production in favor of the 2,3-DHB-propargylamine conjugate **5** (Figs. 5 and S15-S18). The 2,3-DHB-allylamine conjugate **6** was also produced in greater quantities than the natural siderophore (Figs. 5 and S19-S22). Increasing the medium concentration of allylamine to 10 mM suppressed native compounds to levels similar to 1 mM propargylamine; however, the production of the desired conjugate **6** also decreased relative to 1 mM allylamine addition.

In sum, the genomes of several species of *Acinetobacter* were found to contain homologs of *vibH*, alongside biosynthetic genes putatively encoding the biosynthesis of catechol siderophores with an amine core. *A. bouvetii* DSM 14964 was found to produce three novel biscatechol siderophores: propanochelin (**1**), butanochelin (**2**), and pentanochelin (**3**). The amide bond-forming enzyme, putatively assigned as AbsH, is able to append a second 2,3-DHB molecule to diamines and polyamines, albeit with less flexibility than the first addition. Precursor directed biosynthesis revealed that the biosynthetic machinery has a relaxed specificity for the diamine substrate, allowing for the biosynthesis of a variety of non-natural siderophore analogs. Of particular significance, *A. bouvetii* DSM 14964 condenses 2,3-DHB to allylamine and propargylamine, producing catecholic compounds which bind iron(III) and may be further modified via thiol-ene or azide-alkyne click chemistry.[17,18] Propargylamine was particularly well incorporated, outcompeting the natural diamine substrates at 1 mM concentration. With the exceptions of tren and norspermidine, growth of *A. bouvetii* DSM 14964 was not significantly affected by amine supplementation. Future enzymatic studies *in vitro* or in a heterologous system will provide further evidence that AbsH is responsible for amide bond formation. AbsH and other VibH homologs may prove useful in the synthesis of catechol compounds tailored for robust adhesion in salty aqueous conditions, such as those with

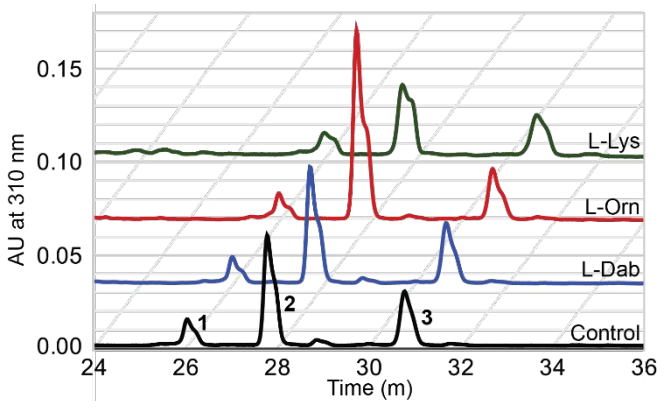


Fig. 4. HPLC trace of catechol-containing products resulting from addition of L-Lys, L-Orn and L-Dab to cultures of *A. bouvetii* DSM 14964. The methanol eluent from the XAD column of 72 h cultures were monitored by HPLC-UV/Vis at 310 nm to detect catecholic compounds. Peaks corresponding to natural siderophores **1**, **2**, and **3** are labeled. Casamino acid minimal medium (control) was supplemented with the indicated amino acids (1 mM final concentration for each) prior to inoculation.

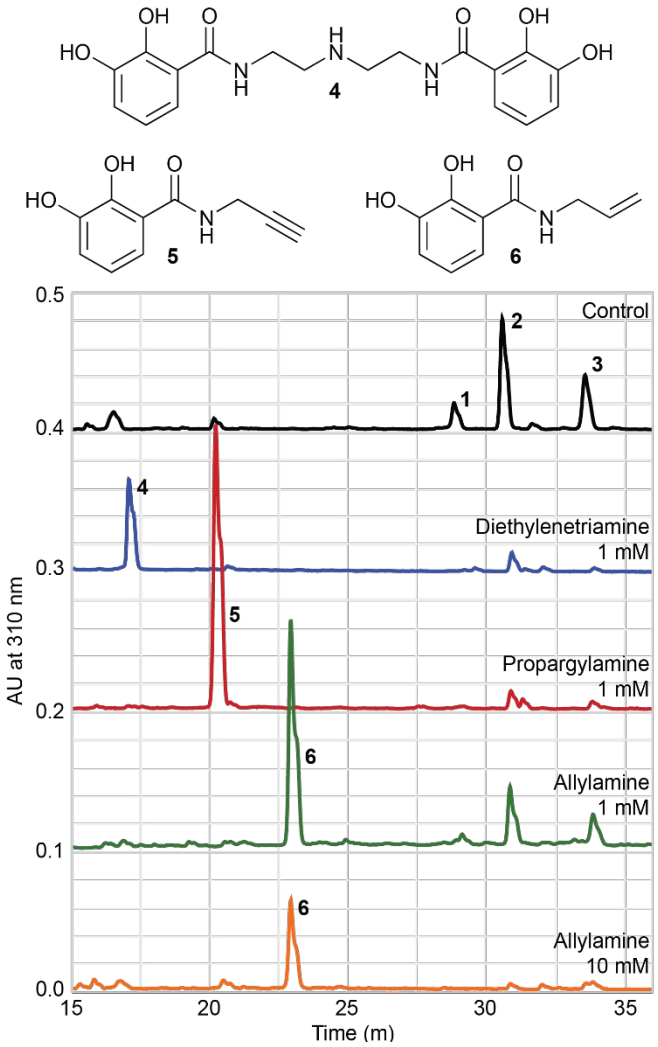


Fig. 5. HPLC trace of catechol-containing products resulting from addition of diethylaminetriamine, allylamine, and propargylamine to cultures of *A. bouvetii* DSM 14964. The methanol eluent from the XAD column of 72 h cultures were monitored by HPLC-UV/Vis at 310 nm, indicative of catecholic compounds. Peaks corresponding to natural siderophores **1**, **2**, and **3** are labeled. Conjugate identities were confirmed by MS and NMR analyses (Figs. S8-S13 and S15-S19). Casamino acid minimal medium (control) was supplemented with the indicated amines prior to inoculation.

attendant amine functionalities mimicking mussel foot proteins.[14, 19]

Support from NSF Grant CHE-1710761 is gratefully acknowledged, along with the U.S. Army Research Office accomplished under cooperative agreement W911NF-19-2-0026 for the Institute for Collaborative Biotechnologies. This work made use of the UCSB Mass Spectrometry Facility, as well as the Materials Research Laboratory Shared Experimental Facilities supported by the Materials Research Science and Engineering Centers Program of the NSF (Grant DMR-1121053). This work was supported in part by NSF Major Research Instrumentation award MRI-1920299, for magnetic resonance instrumentation. We thank D. Uchenik and R. Behrens for mass spectrometry support and H. Zhou for NMR support.

Conflicts of interest

There are no conflicts to declare.

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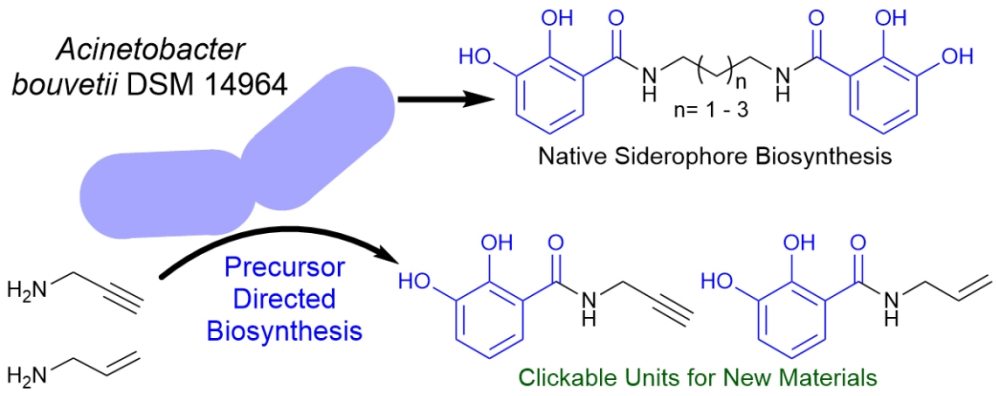
Acinetobacter bouvetii DSM 14964
Native Siderophore Biosynthesis
n= 1 - 3
Precursor Directed Biosynthesis
Clickable Units for New Materials