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Hybrubins: Bipyrrole Tetramic Acids Obtained by Crosstalk between a Truncated Undecylprodigiosin Pathway and Heterologous Tetramic Acid Biosynthetic Genes

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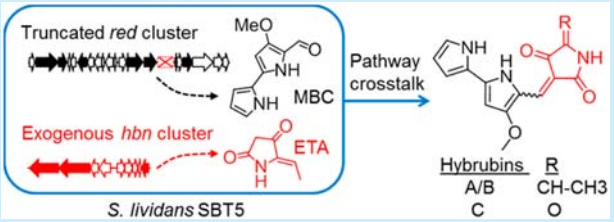
Supporting Information

ABSTRACT: Heterologous expression of bacterial artificial chromosome (BAC) clones from the genomic library of *Streptomyces variabilis* Snt24 in *Streptomyces lividans* SBT5 which carried a truncated undecylprodigiosin biosynthetic gene cluster led to the identification of hybrubins A–C. The hybrubins represent a new carbon skeleton in which a tetramic acid moiety is fused to a 2,2′-dipyrrole building block. Gene knockout experiments confirmed that hybrubins are derived from two convergent biosynthetic pathways including the remaining genomic *red* genes of *S. lividans* SBT5 as well as the BAC encoded *hbn* genes for the production of 5-ethylidenetetramic acid. A possible biosynthetic pathway was also proposed.



Prodiginines are a group of red-colored tripyrrole natural products including undecylprodigiosin (**1**),[1] its cyclic derivative streptorubin B,[2] and prodigiosin (**2**).[3] Despite their wide range of biological activities, including uses as antibacterial, -fungal, and -malarial agents,[4] prodiginines are best known for their cytotoxic and immunosuppressive potentials that supposedly manifest themselves via different modes of action.[5] Moreover, synthetic prodigiosin analogues like PNU-156804[6] and Bcl2-inhibitor obatoclax (GX15-070)[7] are being evaluated in phase I/II clinical trials for potential applications in the treatment of different types of cancer.[5b,8]

The biosynthesis of **1** in *Streptomyces coelicolor* relies on the key intermediates 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC) and 2-undecylpyrrole (Scheme 1).[9] Initially, L-proline (ring A) is activated by RedM and loaded onto the PCP RedO.[10] Oxidation by RedW is followed by transfer of the resulting pyrrole-2-carboxyl moiety to RedX and condensation with RedN-bound malonate.[10,11] Subsequently, L-serine is incorporated under cyclization to the second pyrrole ring (ring B) by the oxoamine synthase domain of RedN.[11] Oxidation by RedV (as proposed) and methylation by RedI complete the assembly of MBC.[12] The 2-undecylpyrrole building block (ring C) is obtained from acetyl-CoA, malonyl-CoA, and glycine by a PKS/NRPS machinery (RedK/L/P/Q/R).[13] RedH catalyzes the condensation of MBC and 2-undecylpyrrole to yield **1**.[14] In the last step, streptorubin B is formed by RedG-catalyzed carbocyclization.[12b] Interestingly, production of **2** by the *pig* gene cluster of *Serratia marescens* follows a similar convergent biosynthetic logic.[12a] In both pathways, rings A and B originate from the common bipyrrole intermediate MBC. However, this building block is then connected with 2-methyl-3-amylpyrrole by PigC for the biosynthesis of **2**.[12a]

The remarkable bioactivities of the prodiginines have promoted a multidisciplinary search for new analogues of **1** and **2**. For example, a mutasynthetic approach utilized the MBC production defective mutants *S. coelicolor* Δ*redM*[14] and *S. marescens pigH*::57 bp.[15] Prodiginine production by these strains is dependent on supplementation of exogenous MBC. Subsequently, feeding of synthetic MBC analogues allowed for production of derivatives of **1** and **2** under replacement of ring A with a phenyl, indolyl, furyl, or thienyl group.[14,15] Interestingly, the relatively broad substrate specificity of PigC even allowed for the incorporation of the non-native substrate 2,4-dimethylpyrrole as a substitute for ring C, which ultimately led to the enzyme catalyzed production of obatclax.[15]

Streptomyces lividans is a close relative of *S. coelicolor* that also produces the red pigments **1** and streptorubin B.[16] Recently, the Δ*redKL* mutants *S. lividans* SBT5 and SBT18 have been described.[17] SBT5 lacks the ability to produce 2-undecylpyrrole but still contains all genes for the biosynthesis of MBC, and SBT18 is an SBT5-derived strain containing an additional copy of the global regulator gene cassette $afsRS_{cla}$ (Figure S1). In this study, we complemented the Δ*redKL* mutant *S. lividans* SBT5 with a genomic bacterial artificial chromosome (BAC) library of

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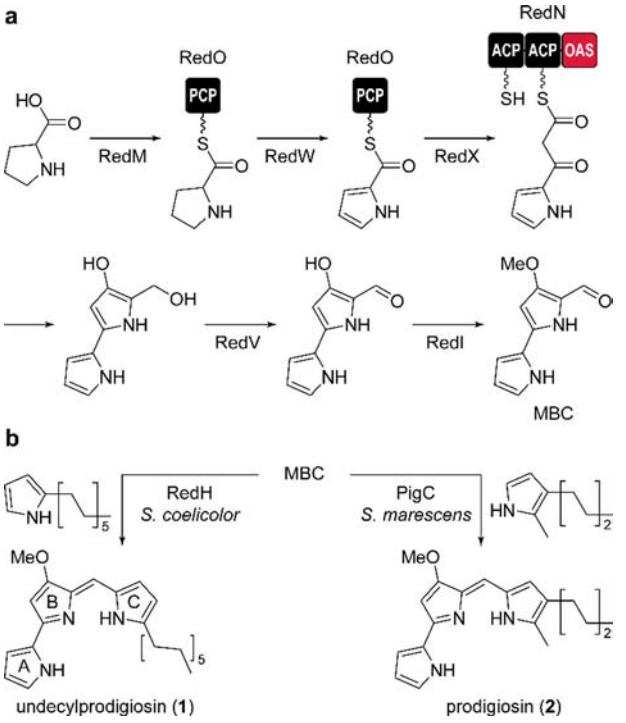
Scheme 1. Biosynthesis of (a) the Prodiginine Precursor MBC and (b) 1 in *S. coelicolor* and 2 in *S. marescens*



Streptomyces variabilis Snt24, a strain recently isolated from Shennongjia (Eastern Hubei, China) forest soil, to screen for exconjugants that could restore red pigmentation by production of hybrid secondary metabolites.[18] This effort led to the identification of the hybrubins which constitute a new group of prodiginine analogues in which ring C is replaced by a tetramic acid moiety. These "non-natural" natural products are hybrids of the *red* pathway from *S. lividans* and a tetramic acid pathway from *S. variabilis* Snt24.

A genomic BAC library of *S. variabilis* Snt24 with 576 clones and an average insert size of 100 kb was constructed according to a standard protocol (cf. the SI for details).[19] The complete BAC library was transferred into *S. lividans* SBT5, and the resulting exconjugants were grown for 7 d on several test media at 30 °C. Accumulation of a red pigment, indicative of prodiginine production, was observed for six exconjugants (BAC clones 2B5, 3E4, 3G4, 4H8, 5H5, and 6D1) on R3 medium. The six BAC clones were subsequently introduced into *S. lividans* SBT18, and all six BAC clones conferred red pigmentation to the exconjugants of *S. lividans* SBT18 as well (Figure 1A). Comparative metabolic profiling of *S. lividans* SBT18/BAC by HPLC revealed three distinct peaks (A–C) that were neither present in a vector control strain nor in *S. variabilis* Snt24 (Figure 1B).

The three peaks were purified from an *S. lividans* SBT18/6D1 fermentation culture and named hybrubin A–C. HR-ESI-MS analysis (Figure S2) revealed molecular formulas of $C_{16}H_{15}N_3O_3$ (hybrubins A and B) and $C_{14}H_{11}N_3O_4$ (hybrubin C). The ^{1}H NMR data of hybrubin A contained two sets of signals at a ratio of 2:1 (A_1: major component; A_2: minor component). Each set consisted of signals for 15 protons, including six aromatic/olefinic protons, two methyl groups, and three exchangeable protons. The ^{13}C NMR data featured eight quaternary carbons, six methine groups, and two methyl groups for both isomers. Comparison of the NMR spectra with literature data for prodiginines, revealed a common dipyrrole moiety and the signals for this fragment were assigned accordingly (ring A and B). Furthermore, COSY, HSQC, and HMBC data allowed for the elucidation of the remaining fragment as 5-ethylidenetetramic acid. HMBC signals supported that a methine group bridged rings B and C (Figure 1C and Figures S3 and S4). The NMR data of hybrubin B showed two isomers (B_1 and B_2) at a ratio of 1:1 akin to the spectra of A_1 and A_2 (Figure 1C and Figures S3 and S5). Theoretically, there are four diastereomers for the proposed structure of hybrubins A and B that differ in the configuration of the C6′-

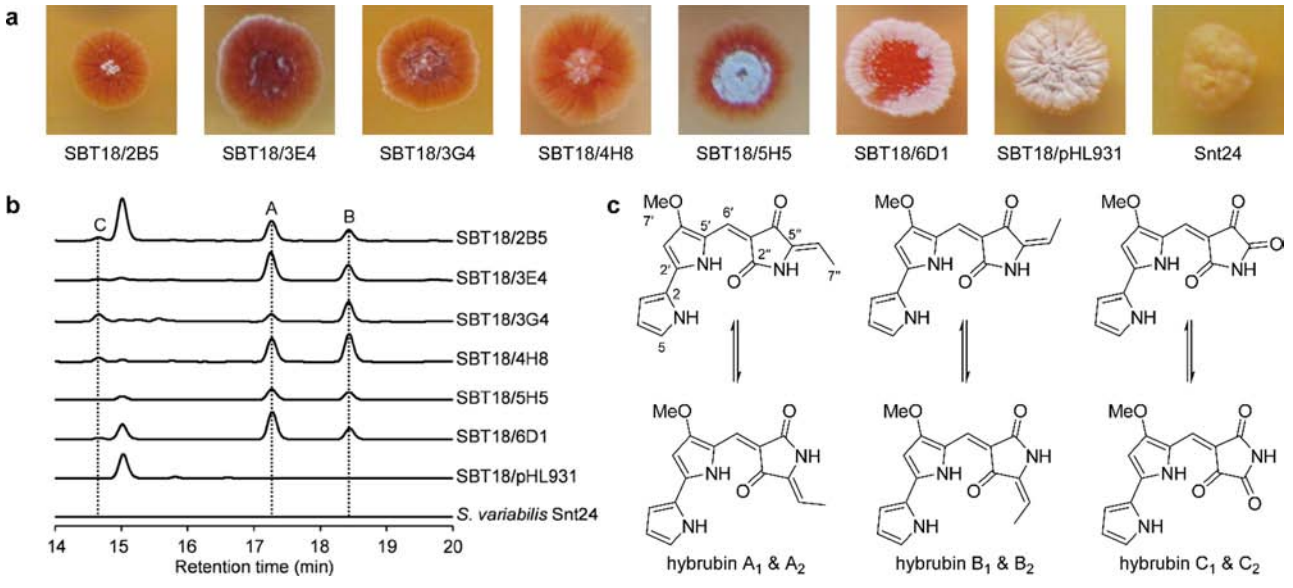


Figure 1. Discovery and characterization of hybrubins. (A) Red pigmentation of *S. lividans* SBT18/BAC colonies on R3 medium. (B) Comparative metabolic profiling of *S. lividans* SBT18/BAC exconjugants by HPLC (detection at 500 nm). The compound eluting at 15 min is not related to the hybrubins. The ratio of hybrubins A–C varied for different batches of fermentations. Hybrubin C could not be detected in SBT18/5H5. (C) Structures of hybrubins A–C.

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C3″ and the C5″-C6″ double bonds. NOE correlations in the NOESY spectra were used to assign the (ZΔ5″,6″)-configuration for the major diastereomer A_1 (1″-H-7″-H) and the (EΔ5″,6″)-configuration for B_1 (1″-H-6″-H). Two sets of signals were found for hybrubin C at a ratio of 3.5:1 (C_1: major component; C_2: minor component) (Figure 1C and Figures S3 and S6). An additional carbonyl group and the lack of signals for an ethylidene group designated hybrubin C as the 5-oxo analogue of hybrubins A and B (Figure 1C). The occurrence of multiple sets of peaks in the NMR spectra of prodiginines[5a] and tetramic acids[20] has been reported in the literature. If the interconversion between two isomers is slow enough, for example, because it requires rotation of a C−C bond,[20] a second set of peaks arises in the respective NMR spectra. Therefore, the second diastereomer (A_2/B_2/C_2) observed in the NMR spectra of each hybrubin possibly represents the respective *E*/*Z* isomer regarding the C6′−C3″ bond. The ^{1}H NMR and ^{13}C NMR data of hybrubins A_1/A_2, B_1/B_2, and C_1/C_2 are summarized in Tables S2−S4 respectively.

Sequencing of the hybrubin producing BAC clone 6D1 gave a 115 kb DNA sequence. Judging from restriction analysis with *Bam*HI and *Pvu*II, the six pigmentation-conferring BAC clones shared a ca. 41 kb overlapping region encompassing 32 genes (Figure S7). To localize the hybrubin gene cluster, BAC 6D1 was truncated by restriction digests with *Asc*I (pHZZL1, 40 kb of the overlapping DNA region), *Sca*I (pHZZL2, 21 kb), *Eco*RI (pHZZL3, 13 kb), and *Bam*HI (pHZZL4, 5 kb) (Figure S8). All plasmids except for pHZZL4 were able to confer hybrubin production to *S. lividans* SBT18 (Figure S9). Therefore, the genes involved in the hybrubin biosynthesis had to be located within the 13 kb fragment of pHZZL3. pHZZL3 encodes nine open reading frames *hbnA-H* (Figure 2A, Table S5) including a

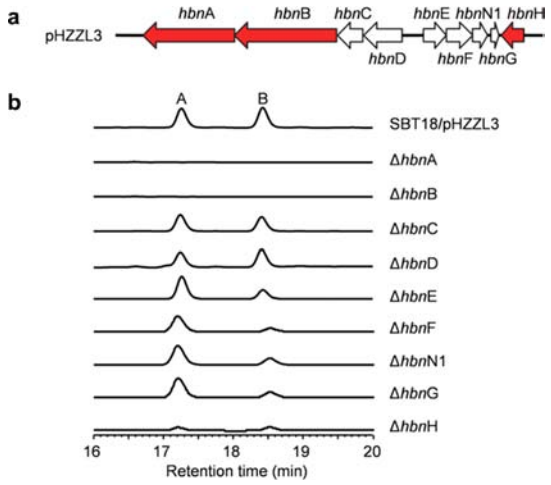


Figure 2. Analysis of the *hbn* gene cluster. (A) Organization of *hbnA*−*hbnH* on the hybrubin production conferring plasmid pHZZL3. (B) HPLC analysis of single gene deletion mutants.

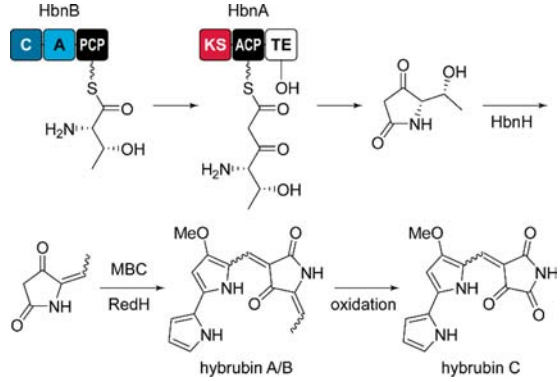
PKS (*hbnA*, domain architecture: KS-ACP-TE) and an NRPS (*hbnB*, C-A-PCP) with a predicted adenylation domain specificity for L-threonine.[21] Additionally, genes for two ketoreductases (*hbnC*, *hbnE*), an oxoamine synthase (*hbnD*), a short-chain dehydrogenase/reductase (*hbnF*), a dehydratase (*hbnN1*), a discrete acyl carrier protein (*hbnG*), and a putative hydrolase (*hbnH*) are found on pHZZL3.

Analogous to the prodiginine biosynthesis, hybrubins could be the product of a convergent biosynthetic pathway. The bipyrrole building block MBC would likely be provided by the genome-encoded *red* gene cluster of the host strain, whereas the assembly of 5-ethylidenetetramic acid would rely on some of the BAC-encoded Hbn proteins. To test this hypothesis, pHZZL3 was transferred to the MBC production impaired strain *S. lividans* CXF28 (Δ*redMN*, Figure S1). No hybrubin could be detected in a crude extract of the *S. lividans* CXF28/pHZZL3 exconjugant (Figure S9) which confirmed the crucial role of the *red* pathway for the hybrubin biosynthesis. To determine the genes for the biosynthesis of the tetramic acid building block, nine in-frame deletion mutations (Δ*hbnA*−Δ*hbnH*) of pHZZL3 were constructed (Figure S10−S12) and transferred to *S. lividans* SBT18. Hybrubin production was completely abolished in the Δ*hbnA* and Δ*hbnB* mutants and significantly impaired in the Δ*hbnH* mutant (Figure 2), which designated these three genes responsible for the biosynthesis of 5-ethylidenetetramic acid.

Ring closure in the biosynthesis of tetramic acids proceeds in many cases by Dieckmann cyclization. Four groups of enzymes are known for this reaction, in particular (i) the redox-incompetent reductase* (R*) domain of fungal tetramate synthases,[22] (ii) the TE domain of bacterial polycyclic tetramate macrolactam synthases,[23] (iii) two proteins PyrD3 and PyrD4 of the pyrroindomycin gene cluster,[24] and (iv) a recently discovered dedicated Dieckmann cyclase.[25] However, homologues of neither of the above-mentioned proteins are encoded in the 13 kb *hbn* gene cluster. Although, a TE domain is found within HbnA, phylogenetic analysis revealed that it is not located in the same clade as the Dieckmann cyclase TE domains (Figure S13). Instead, the closest neighbor of HbnA-TE is TycC-TE, which catalyzes the macrolactamization in the tyrocidine pathway of *Corallococcus coralloides*.[26]

A hybrubin biosynthetic pathway was proposed combining the bioinformatic analysis with experimental data (Scheme 2):

Scheme 2. Proposed Hybrubin Biosynthetic Pathway



Activation of L-threonine by HbnB-A and loading onto HbnB-PCP is followed by HbnA-KS-catalyzed condensation to a malonate extender unit to give HbnA-ACP-tethered 5-hydroxy-4-amino-3-oxohexanoate. As HbnA is lacking an AT domain, the loading of malonyl-CoA to HbnA-ACP needs to be accomplished by an acyltranferase from the host acting in trans.[27] Subsequently, HbnA-TE catalyzes the lactamization under release of 5-(1-hydroxyethyl)tetramic acid. Dehydration by HbnH would finish the assembly of 5-ethylidenetetramic acid. The residual amounts of hybrubins found in the Δ*hbnH* mutant could be explained by other enzymes substituting HbnH for the dehydration in the mutant, although slow

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nonenzymatic loss of water also remains a possibility. The dipyrrole building block MBC is synthesized by the genome-encoded *red* gene cluster (Scheme 1). RedH might condensate the two building blocks to yield hybrubins A and B, given the previously established promiscuity of the enzyme.[14] Finally, hybrubin C arises by further oxidation of hybrubins A and B.

In summary, the hybrubins were identified by heterologous expression of BAC clones from an *S. variabilis* Snt24 genomic library in the 2-undecylpyrrole production-deficient MBC producer *S. lividans* SBT5. The convergent hybrubin biosynthetic pathway utilizes both the genome-encoded truncated *red* gene cluster of SBT5 as well as the BAC-encoded *hbn* gene cluster originating from *S. variabilis*. To our knowledge, hybrubins are the first examples of chimeras combining the 2,2′-dipyrrole and the tetramic acid moiety. Both compound classes, prodiginines and tetramic acids, are rich sources of bioactive natural products despite the lack of obvious cytotoxicity or antibacterial activity of the hybrubins in initial bioactivity tests. The identification of the *hbn* gene cluster paves the way for the production of a variety of new hybrubin analogues with diverse tetramic acid substituents for bioactivity tests. These could be obtained in a combinatoric approach by expression of other (partial) tetramic acid biosynthetic gene clusters in *S. lividans* SBT5.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03609.

Experimental procedures and spectroscopic data for all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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