Extradiol cleavage of L-DOPA as strategy for natural product biosynthesis

Paige A. Jones,[1] Kameron L. Klugh,[2] Nyjah Johnson,[1]
Larryn W. Peterson[2] and Keri L. Colabroy[1]

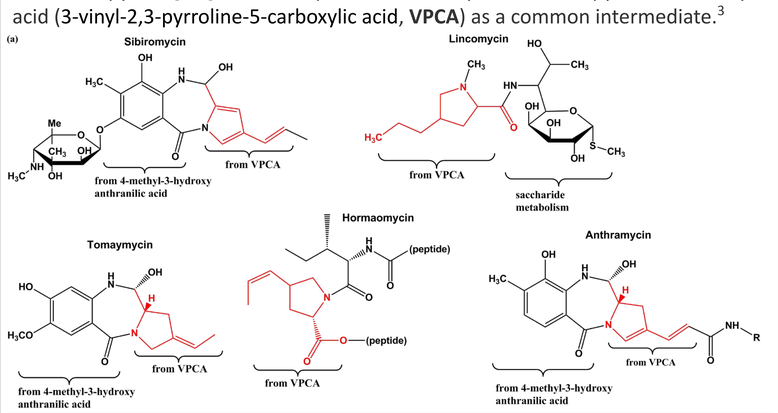
[1]Department of Chemistry, Muhlenberg College, Allentown, PA, 18104
[2]Department of Chemistry, Rhodes College, Memphis, TN, 38112

Abstract

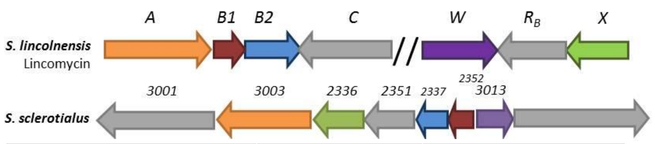
L-DOPA dioxygenase was first discovered as part of a biosynthetic gene cluster to the natural product antibiotic, lincomycin. Within the larger biosynthetic context, L-DOPA dioxygenase is part of a mini-pathway to the synthon 3-vinyl-2,3-pyrroline-5-carboxylic acid (VPCA). This synthon is elaborated and embedded within the final product structure of lincomycin, and also within the natural product structures of anthramycin, sibiromycin, tomaymycin and hormaomycin. Using the VPCA mini-pathway as a starting point, we searched sequence space to identify 1) novel natural product pathways containing a VPCA synthon and 2) instances of L-DOPA dioxygenase homologs that were not associated with a known VPCA containing natural product pathway, and that might provide new sources of natural product diversity and answer questions about the evolutionary origins of L-DOPA dioxygenase. Using bioinformatic methods, including PSI-BLAST, genome context analysis and structurally informed multiple-sequence alignment, we identified a variety of known and novel natural product pathways that utilize an L-DOPA dioxygenase - many with the context of a VPCA synthon. Representative examples from these pathways were isolated and tested for iron binding and L-DOPA dioxygenase activity.

Making Natural Products from Tyrosine and L-DOPA

The metabolic pathways for the production of lincomycin, hormaomycin and the antitumor pyrrolo[1,4]benzodiazepines share a vinyl substituted pyrroline carboxylic acid (3-vinyl-2,3-pyrroline-5-carboxylic acid, **VPCA**) as a common intermediate.[3]



Biosynthesis of VPCA requires a three-enzyme mini-pathway. The heme-dependent L-tyrosine hydroxylase (i.e. LmbB2) is a peroxidase that specifically monohydroxylates tyrosine,[3] while the non-heme Fe(II) dependent L-DOPA dioxygenase (i.e. LmbB1 from *S. lincolnensis*;[1] SsDDO from *S. sclerotialus*[2]) is a member of the vicinal-oxygen-chelate (VOC) superfamily. In the third step, the dioxygenase product undergoes a C-C bond cleavage reaction catalyzed by a gamma-glutamyl transferase homolog (i.e. LmbA).[4] This mini-pathway demonstrates the use of chemistry typically associated with natural product degradation in order to build a compact, functionalized building block for larger, bioactive molecules. We were interested in the potential use of the VPCA mini-pathway (in whole or in part) to biosynthesize other natural products.



Assigned/annotated function	*S. lincolnensis* gene	*S. sclerotialus* gene
gamma-glutyl transferase	lmbA (CAA55746.1)	WP_030623003.1
L-DOPA dioxygenase	lmbB1 (CAA55747.1	WP_051872352.1
L-tyrosine hydroxylase	lmbB2 (CAA55748.1)	WP_051872337.1
O-methyltransferase	lmbW (CAA55769.1)	WP_030623013.1
PhzF phenazine biosynthetic protein	lmbX (CAA55771.1)	WP_051872336.1

Five known natural products contain a VPCA synthon in their structures. L-DOPA dioxygenase is the second step to VPCA as shown in (b). The functionalized pyrroline carboxylic acid (i.e. VPCA) is further modified and incorporated into each natural product scaffold.

Methods

Basic Local Alignment Search Tool (BLAST)

BLAST was used to compare protein sequences in genomic databases in order to locate homologs of L-DOPA dioxygenase (i.e. LmbB1[1] and SsDDO[2]) in other organisms. Position-Specific Iterated BLAST (PSI-BLAST) was used find more distantly related homologs and organisms.

Genome Context Analysis (GCA)

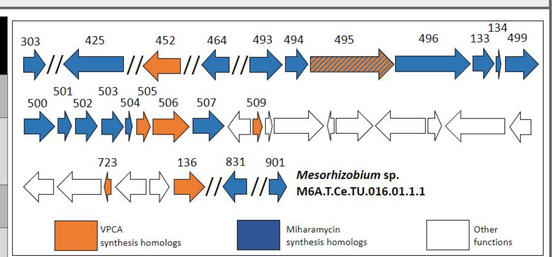
Once homologs were identified, GCA was used to look at proteins surrounding the putative LmbB1 homolog to 1) identify elements of a putative VPCA pathway and 2) other genes that might indicate a natural product biosynthetic pathway. Multiple sequence alignment and reverse-BLAST was used to confirm function.

Biochemical Characterization

Representative examples of L-DOPA dioxygenase homologs were synthesized as codon-optimized genes for expression in *E. coli*. The proteins were purified, reconstituted with active site Fe(II) and assessed for activity on L-DOPA and structural analogs in order to determine substrate preference.

Identification of L-DOPA Derived Synthons Embedded within Cryptic Biosynthetic Pathways

Annotated/Predicted Function	*Mesorhizobium* sp. M6A.T.Ce.TU.016.01.1.1 Gene
Aromatic ring-hydroxylating dioxygenase subunit alpha *(XylX homolog)*	WP_127288**452**.1
Amino acid adenylation domain-containing protein *(Mhr8 homolog, Mhr9 homolog,* ***LmbC homolog****)*	WP_127288**495**.1
SDR family NAD(P)-dependent oxidoreductase *(XylL homolog)*	WP_127288**505**.1
Gfo/Idh/MocA family oxidoreductase *(dihydrodiol dehydrogenase)*	WP_127288**506**.1
VOC family protein ***(LmbB1 homolog)***	WP_127288**509**.1
Rieske (2Fe-2S) protein	WP_023797**723**.1
Hybrid-cluster NAD(P)-dependent oxidoreductase *(XylZ homolog)*	WP_185928**136**.1



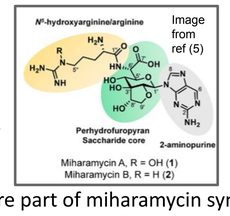


Image from ref (5)

The genome diagram of *Mesorhizobium* sp. M6A.T.Ce.TU.016.01.1.1 (taxid: 2496783) shows VPCA biosynthetic genes colored in orange interspersed among genes that are part of miharamycin synthesis[5] in blue. There is no L-tyrosine hydroxylase homolog, but L-DOPA may be synthesized via dihydroxylation of an alternative aromatic precursor.

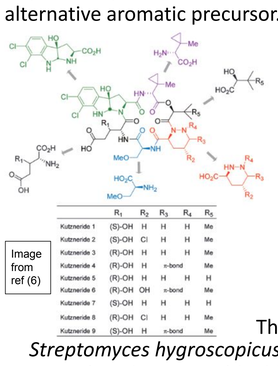
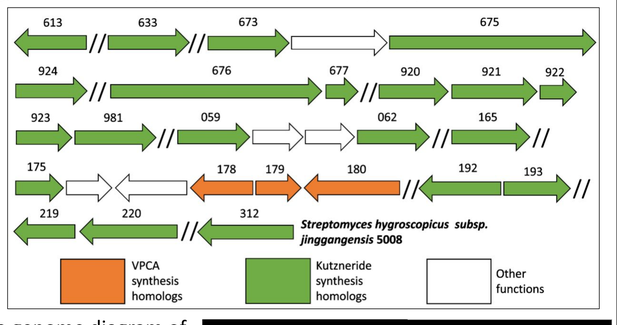


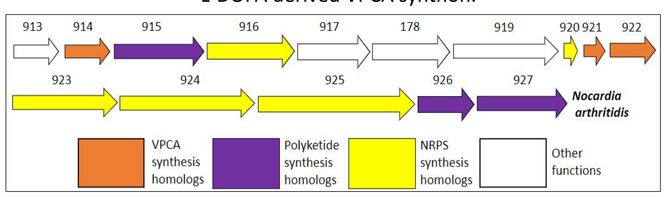
Image from ref (6)

	R_1	R_2	R_3	R_4	R_5
Kutzneride 1	(S)-OH	H	H	H	Me
Kutzneride 2	(S)-OH	Cl	H	H	Me
Kutzneride 3	(R)-OH	H	H	H	Me
Kutzneride 4	(R)-OH	H	=bond		Me
Kutzneride 5	(R)-OH	H	H	H	H
Kutzneride 6	(R)-OH	OH	=bond		Me
Kutzneride 7	(S)-OH	H	H	H	H
Kutzneride 8	(R)-OH	Cl	H	H	Me
Kutzneride 9	(S)-OH	H	=bond		Me



The genome diagram of *Streptomyces hygroscopicus subsp. jinggangensis 5008* (taxid: 1133850) highlights genes similar to those of kutzneride biosynthesis[6] in *Kutzneria* sp.744 (taxid: 345341) alongside genes used in the VPCA mini-pathway, which implies that *S. h. jinggangensis* could make a kutzneride derivative containing an L-DOPA derived VPCA synthon.

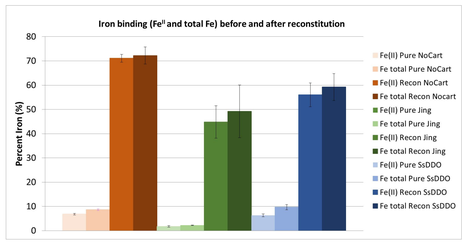
Annotated/Predicted Function	*Streptomyces hygroscopicus subsp. jinggangensis 5008* Gene
L-tyrosine hydroxylase ***(LmbB2 homolog)***	WP_014677**178**.1
VOC family protein ***(LmbB1 homolog)***	WP_014677**179**.1
Gamma-glutamyltransferase family protein ***(LmbA homolog)***	WP_014677**180**.1



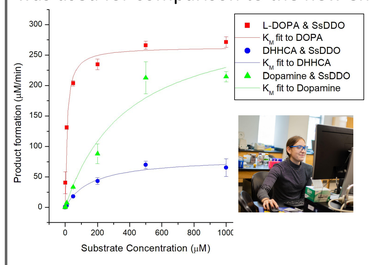
Annotated/Predicted Function	*Nocardia arthritidis* Gene	Annotated/Predicted Function	*Nocardia arthritidis* Gene
Proline hydroxylase	WP_167472**913**.1	L-tyrosine hydroxylase ***(LmbB2 homolog)***	WP_167472**922**.1
LLM class flavin-dependent oxidoreductase ***(LmbY homolog)***	WP_167472**914**.1	Non-ribosomal peptide synthetase	WP_167472**923**.1
Acetolactate synthase large subunit	WP_167472**915**.1	Non-ribosomal peptide synthetase	WP_167472**924**.1
Condensation domain-containing protein	WP_167472**916**.1	Non-ribosomal peptide synthetase	WP_167472**925**.1
MbtH family NRPS accessory protein	WP_167472**920**.1	Ketoacyl-ACP synthase III family protein	WP_167472**926**.1
L-DOPA dioxygenase ***(LmbB1 homolog)***	WP_167472**921**.1	Acyl-CoA ligase (AMP-forming)	WP_167472**927**.1

The genome diagram of *Nocardia arthritidis* (taxid: 1210067) highlights genes with homology to non-ribosomally biosynthesized peptide natural products (NRPS), and genes associated with polyketide biosynthesis,[7] alongside genes from the VPCA mini-pathway (in orange). This analysis implies there is a cryptic pathway in this organism that contains a VPCA synthon.

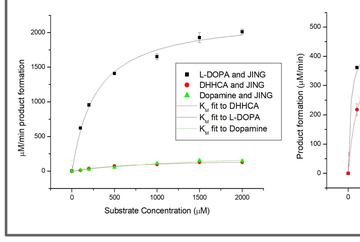
Biochemical Characterization of New L-DOPA Dioxygenase Homologs

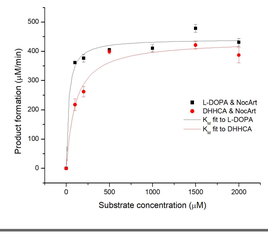


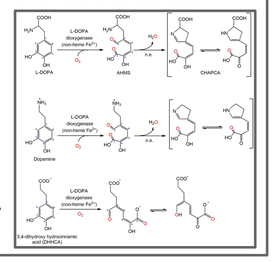
In order to assess the substrate preference for the new L-DOPA dioxygenase homologs from *S. hygroscopicus jinggangensis* (JING) and *Nocardia arthritidis* (NocArt), the enzymes were purified and reconstituted with Fe(II), which is essential for activity.[1] Then, enzymatic activity was examined for the following substrates: 3,4-dihydroxy phenylalanine (L-DOPA) and analogs: Dopamine and 3,4-dihydroxy hydrocinnamic acid (DHHCA). We used UV-Vis to measure formation of the dioxygenase product. SsDDO[2] was used for comparison to the new enzymes, JING and NocArt.



Steady-state kinetics indicate that SsDDO is most active on L-DOPA, with weaker activity on Dopamine and DHHCA. JING shows a clear preference for L-DOPA, while NocArt has similar activity on L-DOPA and DHHCA. These results indicate that our predicted function for these proteins is plausible, and that NocArt may have more substrate flexibility or L-DOPA may not be the native substrate.







Conclusion

L-DOPA dioxygenase was first characterized within a biosynthetic mini-pathway to the synthon VPCA, which is embedded and functionalized within the structures of five known natural products: lincomycin, hormaomycin, sibiromycin, anthramycin and tomaymycin.

When we examine the genomic context surrounding L-DOPA dioxygenase homologs in *S. hygroscopicus jinggangensis,* we find L-DOPA dioxygenase and other VPCA synthesis homologs interspersed among homologs of kutzneride biosynthesis, a pathway first characterized from *Kutzneria*.[6] In the same way, the genomic context of the L-DOPA dioxygenase homolog from *Mesorhizobium* shows the L-DOPA dioxygenase is co-located with homologs of miharamycin synthesis, a pathway first characterized from *Streptomyces miharaensis*.[5] Lastly, we also propose that the L-DOPA dioxygenase of *N. arthritidis* colocated with other VPCA biosynthesis genes and embedded within an, as yet uncharacterized, biosynthetic gene cluster. To support these predictions, the L-DOPA dioxygenase homologs from *S. hygroscopicus jinggangensis* (JING) and *Nocardia arthritidis* (NocArt) were purified and reconstituted. Enzymatic activity assays showed robust activity on L-DOPA. These observations support the assignments we have made, and taken together, our data suggest that *Mesorhizobium* and *S. hygroscopicus jinggangensis* are biosynthesizing kutzneride and miharamycin derivatives respectively, with an L-DOPA/VPCA derived synthon. The L-DOPA dioxygenase homolog from *Nocardia arthritidis* is likely embedded within a cryptic biosynthetic pathway to a natural product with NRPS and PKS[7] derived structural elements.

References

(1) Colabroy, K. L., Horwitz, A. D., Basciano, V. R., Fu, Y., Travitz, K. M., Robinson, M. K., Shimanski, B. A., and Hoffmann, T. W. (2019) A New Way of Belonging: Active-Site Investigation of L-DOPA Dioxygenase, a VOC Family Enzyme from Lincomycin Biosynthesis. *Biochemistry 58*, 4794–4798.
(2) Wang, Y., Shin, I., Fu, Y., Colabroy, K. L., and Liu, A. (2019) *Biochemistry 58*, 5339-5350.
(3) Colabroy, K. L. (2016) Tearing down to build up: Metalloenzymes in the biosynthesis lincomycin, hormaomycin and the pyrrolo [1,4]benzodiazepines. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1864*, 724–737.
(4) Zhong, G., Zhao, Q., Zhang, Q., and Liu, W. (2017) 4-alkyl- L -(Dehydro)proline biosynthesis in actinobacteria involves N -terminal nucleophile-hydrolase activity of γ-glutamyltranspeptidase homolog for C-C bond cleavage. *Nature Communications 8*, 16109.
(5) Romo, A. J., Shiraishi, T., Ikeuchi, H., Lin, G.-M., Geng, Y., Lee, Y.-H., Liem, P. H., Ma, T., Ogasawara, Y., Shin-Ya, K., Nishiyama, M., Kuzuyama, T., and Liu, H.-W. (2019) The Amipurimycin and Miharamycin Biosynthetic Gene Clusters: Unraveling the Origins of 2-Aminopurinyl Peptidyl Nucleoside Antibiotics. *J Am Chem Soc 141*, 14152–14159.
(6) Fujimori, D. G., Hrvatin, S., Neumann, C. S., Strieker, M., Marahiel, M. A., and Walsh, C. T. (2007) Cloning and characterization of the biosynthetic gene cluster for kutznerides. *Proc Natl Acad Sci U S A 104*, 16498–16503.
(7) Herisse, M., Ishida, K., Porter, J. L., Howden, B., Hertweck, C., Stinear, T. P., and Pidot, S. J. (2020) Identification and Mobilization of a Cryptic Antibiotic Biosynthesis Gene Locus from a Human-Pathogenic Nocardia Isolate. *ACS Chem. Biol. 15*, 1161–1168.

Acknowledgements

NSF CHE 1708237 (K.L.C.) 1708234 (L.W.P.)
Snow Du (Muhlenberg '21), Lily Yoza (Muhlenberg '23)