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Cytochrome P450-catalysed L-tryptophan nitration in thaxtomin phytotoxin biosynthesis

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Abstract

Thaxtomin phytotoxins produced by plant-pathogenic *Streptomyces* species contain a nitro group that is essential for phytotoxicity. The *N,N*-dimethyldiketopiperazine core of thaxtomins is assembled from L-phenylalanine and L-4-nitrotryptophan by a nonribosomal peptide synthetase and nitric oxide synthase-generated NO is incorporated into the nitro group, but the biosynthesis of the non-proteinogenic amino acid L-4-nitrotryptophan is unclear. Here we report that TxtE, a unique cytochrome P450, catalyzes L-tryptophan nitration using NO and O₂.

Thaxtomin A exerts its phytotoxicity by inhibiting cellulose biosynthesis in plants¹. Conserved pathogenicity islands on the chromosomes of *Streptomyces turgidiscabies*, *Streptomyces scabies* and *Streptomyces acidiscabies* contain the thaxtomin biosynthetic gene cluster (Fig. 1a)². The *txtD* gene within this gene cluster encodes a nitric oxide synthase (NOS) that generates NO from L-arginine (Fig. 1b)³. Deletion of *txtD* in *S. turgidiscabies* severely attenuates thaxtomin A production³. A ¹⁵N label from the guanidino group of L-arginine is specifically incorporated into the nitro group of thaxtomin A and NO donors boost thaxtomin production in the *txtD* mutant, indicating that part of the nitro group

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S.M.B., G.L.C., J.A.K., E.G.J., and R.L. designed the research. S.M.B., J.A.K., E.G.J., L.S., P.R.A. and B.P. performed the research. S.M.B., G.L.C., L.S., R.L., J.A.K., E. G. J., S.B.K., D.M.G. and B.R.C., analysed data. S.M.B., G.L.C., J.A.K., E.G.J, R.L. and B.R.C., wrote the paper.

Competing financial interests

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is derived from NO^{3,4}. The *txtA* and *txtB* genes encode nonribosomal peptide synthetases that are implicated in the assembly of the thaxtomin *N,N*-dimethyldiketopiperazine core **1** from the unique non-proteinogenic amino acid L-4-nitrotryptophan **2**, L-phenylalanine and S-adenosyl-L-methionine, using ATP and Mg²⁺ as cofactors⁵. However, the mechanism for biosynthesis of L-4-nitrotryptophan remains to be elucidated. Nitro groups in natural products usually result from oxidation of an amino group⁶. While biomimetic syntheses and feeding experiments with labeled precursors implicate direct nitration in the biosynthesis of two metabolites⁶, a natural product biosynthetic enzyme that is capable of direct nitration has never been reported. Peroxidases and globins can catalyze nitration of L-tryptophan in the presence of NO⁻²/H₂O₂ and NO/O₂, respectively, but the reactions are unselective, resulting in several nitrotryptophan regioisomers⁷. Similarly, in *Deinococcus radiodurans* a NOS in partnership with an adjacently-encoded t-RNA synthetase can catalyze regioselective nitration of L-tryptophan. However, the catalytic efficiency of this transformation is very low⁸. In both cases, the biological significance of the reactions is unknown; it is unclear whether nitration is anything more than an adventitious side reaction. Here we report that *txtE* encodes a unique cytochrome P450 (CYP) that catalyzes direct regiospecific 4-nitration of L-tryptophan, the first committed step in thaxtomin A biosynthesis, using NO and O₂ as substrates and spinach ferredoxin (Fd) and ferredoxin reductase (Fr) as surrogate electron donors.

CYPs are ubiquitous heme-dependent enzymes that activate molecular oxygen to catalyze a vast array of oxidative transformations including epoxidation, C- and N-hydroxylation and oxidative dealkylation^{9,10}. Coordination of NO to the heme iron atom causes reversible inhibition of CYPs. Subsequent reaction with O₂ forms reactive nitrogen species that nitrate amino acid residues such as tyrosine, irreversibly inhibiting enzyme activity⁷. The inhibition of CYP monooxygenases by NO significantly affects signal transduction, cellular inflammation, neurodegeneration, and drug metabolism^{11,12}. P450nor (CYP55A1) from *Fusarium oxysporum* is an interesting exception, because it reduces bound NO to N₂O using electrons from NADH¹³.

We hypothesized that *txtE* and *txtD* are together responsible for 4-nitration of L-tryptophan in thaxtomin A biosynthesis. Thus, we expected the genes encoding these enzymes to be co-transcribed. S1 nuclease protection analysis confirmed co-transcription of *txtE* and *txtD* (Supplementary Methods; Supplementary Fig. 12). No additional protected RNA transcripts were observed, suggesting that *txtD* transcription is solely dependent on the *txtE* promoter. The transcriptional start site of *txtE* is located 232 bp upstream of the translational start site. The predicted DNA binding regions for the transcription factor are both shifted upstream of the -10 and -35 positions. A comparison of the *txtE* promoter region with other previously-characterized *Streptomyces* promoters revealed a potential core promoter motif CANNAT and a putative -35 core promoter motif, GNTTNC¹⁴. A 9 bp inverted repeat in the vicinity of the -10 and -35 regions in *S. turgidiscabies* (Supplementary Fig. 12) may indicate an additional level of transcriptional regulation of *txtE*.

To determine if *txtE* is required for thaxtomin biosynthesis, *txtE* was deleted from *S. turgidiscabies* Car8 using marker-exchange mutagenesis. Thaxtomin A production was abolished in the $\Delta txtE$ strain and partially restored by complementation with *txtE* expressed under the control of the thiostrepton-inducible *tipA* promoter in plasmid pIJ8600*txtE* (Supplementary Results; Supplementary Table 1). Partial restoration of thaxtomin A production in the $\Delta txtE$ pIJ8600*txtE* strain and improved complementation with addition of NO donors is consistent with a polar effect on *txtD* (Table 1). Reverse-transcriptase PCR was used to confirm the absence of *txtD* expression in *S. turgidiscabies* $\Delta txtE$. The low level of thaxtomin A production in the strain lacking *txtD* is likely due to the production of NO by

nitrate/nitrite reductases in *S. turgidiscabies*. In order to assess the individual contribution of TxtE to thaxtomin A biosynthesis, *txtD* was introduced into *S. turgidiscabies* $\Delta txtE$ using pIJ8600txtD³. Ectopic expression of *txtD* did not restore thaxtomin A production nor did addition of NO donors, indicating that deletion of *txtE* alone is responsible for the loss of thaxtomin A production in this strain (Table 1 and Supplementary Table 1).

To further analyze the respective roles of TxtD and TxtE in thaxtomin A production, thiostrepton-inducible *txtE* and *txtD* were introduced into *S. turgidiscabies* Car8 and the ability of these constructs to modulate thaxtomin A production was examined. Overexpression of *txtE* in the wild type strain resulted in a 34% increase in thaxtomin A production (Supplementary Table 1), suggesting that TxtE is a rate-limiting enzyme in its biosynthesis. In contrast, overexpression of *txtD* in wild type resulted in a 23% decrease in thaxtomin A production, consistent with NO cytotoxicity.

Since cultures of the $\Delta txtE$ strain did not accumulate non-nitrated thaxtomin derivatives, we hypothesized that TxtE-catalyzed 4-nitration of L-tryptophan is the first committed step in thaxtomin A biosynthesis. Addition of L-4-nitrotryptophan to cultures of the $\Delta txtE$ strain resulted in the restoration of thaxtomin A production (Table 1). This result is consistent with the hypothesis that L-4-nitrotryptophan is a biosynthetic intermediate utilized by the nonribosomal peptide synthetase TxtB for thaxtomin A biosynthesis (Fig. 1b)⁵.

To directly examine the role of TxtE in L-tryptophan nitration we cloned and overexpressed *txtE* in *E. coli*. Attempted overproduction of TxtE from *S. turgidiscabies* was not successful. Thus, we chose to work with the ortholog from *S. scabies* 87-22 (94% similarity, 87% identity). Recombinant TxtE was produced as an N-terminal His₆-tagged fusion protein, which was purified to homogeneity by nickel affinity and gel filtration chromatography (Supplementary Fig. 3). The latter indicated that the protein exists as a monomer in solution. Purified TxtE was analysed by UV-Vis spectroscopy (Supplementary Fig. 4). The Soret band at 447 nm in the ferrous minus ferrous-CO difference spectrum of TxtE confirmed that it is a CYP (Supplementary Fig. 4)¹⁵.

The substrate specificity of TxtE was examined by investigating changes in the UV-Vis spectrum upon titration with different amino acids. Characteristic type I binding spectra were observed (Supplementary Fig. 5) in the presence of L-tryptophan, arising from conversion of the heme iron atom from low to high spin upon substrate binding and resulting in a λ_{\max} shift from 417 nm to 390 nm¹⁶. We estimated that the dissociation constant for the L-tryptophan-TxtE complex is $60 \pm 6 \mu\text{M}$ from a plot of the difference in absorbance at 390 nm and 420 nm with increasing L-tryptophan concentrations (Supplementary Fig. 5). No shift in λ_{\max} was observed with D-tryptophan suggesting that TxtE is stereospecific.

Assuming that, like many other bacterial CYPs, TxtE requires the electron transfer enzymes Fd and Fr for activity, we incubated TxtE with spinach Fd and Fr, NADPH, the NO donor 2-(N,N-diethylamino)-diazeneolate 2-oxide (DEANO), and L-tryptophan. The reaction was carried out in air at room temperature for 90 minutes, over which time the solution became yellow. LC-MS analyses identified a product, absent from control reactions, with m/z 250 and a λ_{\max} at 400 nm, consistent with L-4-nitrotryptophan (Fig. 2a and Supplementary Fig. 6)⁸. The reaction was scaled up and the product was purified from the reaction by HPLC. The retention time, the ESI-MS/MS spectrum and the aromatic region of the ¹H NMR spectrum of this compound were identical to those for synthetic DL-4-nitrotryptophan (Supplementary Methods; Supplementary Figs. 7 and 8). No products with m/z corresponding to hydroxylated tryptophan derivatives were observed in either the enzymatic reaction or the control reaction lacking DEANO, suggesting that TxtE is not capable of hydroxylating L-tryptophan. Furthermore, compounds with m/z corresponding to 4-

nitrosotryptophan, a potential intermediate in the nitration of L-tryptophan, were not detected in the enzymatic reaction.

CYPs usually utilize O₂ as a co-substrate. O₂ binds to the iron atom of the ferrous heme resulting from reduction of the ferric enzyme-substrate complex by Fd/Fr (using electrons from NADPH). The resulting ferrous-O₂/ferric-superoxide complex undergoes further reduction and dehydration to form a ferryl complex (Fe(IV)=O porphyrin cation radical; often referred to as “compound I”) that oxidizes the substrate¹⁰. To examine the involvement of O₂ in the TxtE-catalyzed nitration reaction, we incubated the enzyme with L-tryptophan, NADPH, DEANO and spinach Fd/Fr under an ¹⁸O₂ atmosphere. High resolution MS analysis of the resulting L-4-nitrotryptophan showed that it was >90% labeled with a single ¹⁸O atom (Fig. 2b). No labeling of L-4-nitrotryptophan was observed in a control reaction in which ¹⁸O₂ was replaced with air (Fig. 2b), or H₂¹⁸O (Supplementary Fig. 9). Moreover, no turnover of L-tryptophan to L-4-nitrotryptophan was observed when air was excluded from the reaction (Supplementary Fig. 10). These data are consistent with the utilization of O₂ as a co-substrate by TxtE.

Taken together, our results prompt us to propose a catalytic mechanism for the formation of potential nitrating species, based on the well-established catalytic mechanism of CYP monooxygenases (Figure 2c)^{9,10}. L-tryptophan binds to the active site of TxtE, triggering loss of the water ligand from the ferric heme, resulting in the observed spin state change. This promotes reduction of the heme to its ferrous form by Fr/Fd and O₂ binds to the heme iron atom, forming a ferric-superoxide complex. Reaction of NO with the ferric-superoxide complex affords a ferric-peroxynitrite complex, which can undergo homolytic cleavage to yield NO₂ and an Fe(IV)=O species (often referred to as “compound II”). Nitration of the bound L-tryptophan could then occur via NO₂ addition and compound II-mediated hydrogen atom abstraction, resulting in formation of an Fe(III)-OH species (or vice versa). An alternative mechanism for nitration involving protonation-triggered heterolytic cleavage of the ferric-peroxynitrite complex to yield NO₂⁺ and an Fe(III)-OH species, followed by classical electrophilic aromatic substitution, is also possible. Irrespective of the nitration mechanism, protonation of the Fe(III)-OH species formed is required to regenerate the Fe(III)-OH₂ resting state of the enzyme. The observed incorporation of predominantly a single oxygen atom from ¹⁸O₂ into L-4-nitrotryptophan is consistent with all of the mechanistic scenarios outlined above and further experiments will be required to discriminate between them.

In conclusion, TxtE is a unique new member of the CYP superfamily that catalyzes regioselective 4-nitration of L-tryptophan using NO and O₂. Sequence alignments with several structurally-characterized CYPs indicate differences in several functionally-important regions of TxtE (Supplementary Fig. 2), providing clues to the origin of its dramatically different catalytic activity. TxtE may prove to be a useful nitration biocatalyst, given that in organic synthesis direct nitration of indoles is often carried out under harsh conditions and lacks regioselectivity¹⁷⁻¹⁹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Scheible WR, et al. *Plant Cell*. 2003; 15:1781–1794. [PubMed: 12897252]
2. Kers JA, et al. *Mol. Microbiol.* 2005; 55:1025–1033. [PubMed: 15686551]
3. Kers JA, et al. *Nature*. 2004; 429:79–82. [PubMed: 15129284]
4. Wach MJ, Kers JA, Krasnoff SB, Loria R, Gibson DM. *Nitric Oxide*. 2005; 12:46–53. [PubMed: 15631947]
5. Johnson EG, et al. *Mol. Microbiol.* 2009; 73:409–418. [PubMed: 19570136]
6. Winkler R, Hertweck C. *ChemBioChem*. 2007; 8:973–977. [PubMed: 17477464]
7. Roncone R, Barbieri M, Monzani E, Casella L. *Coord. Chem. Rev.* 2006; 250:1286–1293.
8. Buddha MR, et al. *J. Biol. Chem.* 2004; 279:49567–49570. [PubMed: 15466862]
9. Sono M, Roach MP, Coulter ED, Dawson JH. *Chem. Rev.* 1996; 96:2841–2887. [PubMed: 11848843]
10. Denisov IG, Makris TM, Sligar SG, Schlichting I. *Chem. Rev.* 2005; 105:2253–2277. [PubMed: 15941214]
11. Quaroni LG, et al. *Biochemistry*. 2004; 43:16416–16431. [PubMed: 15610036]
12. Franke A, et al. *J. Am. Chem. Soc.* 2004; 126:4181–4191. [PubMed: 15053607]
13. Nakahara K, et al. *J. Biol. Chem.* 1993; 268:8350–8355. [PubMed: 8463342]
14. Bourn WR, Babb B. *Nucleic Acids Res.* 1995; 23:3696–3703. [PubMed: 7478999]
15. Omura T, Sato R. *J. Biol. Chem.* 1964; 239:2379–2385. [PubMed: 14209972]
16. Schenkman JB, Remmer H, Estabrook RW. *Mol. Pharmacol.* 1967; 3:113–123.
17. Ishii Y, et al. *J. Pharm. Biomed. Anal.* 2007; 44:150–159. [PubMed: 17287102]
18. Ottoni O, Cruz R, Krammer NH. *Tetrahedron Lett.* 1999; 40:1117–1120.
19. Osborne AS, Som P, Metcalf JL, Phillips RS. *Bioorg. Med. Chem. Lett.* 2008; 18:5750–5752. [PubMed: 18851915]

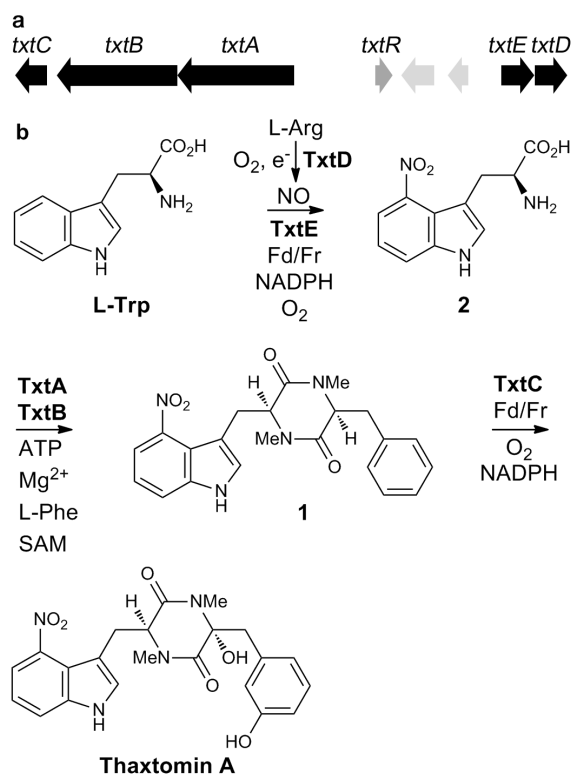


Figure 1. Thaxtomin biosynthesis. **a:** Organization of the thaxtomin biosynthetic gene cluster in *S. turgidiscabies*. Biosynthetic genes are in black; *txtR*, which encodes a cellobiose-responsive pathway specific activator, is in dark gray; two genes encoding putative transposases are in light gray. The *txtAB* genes encode nonribosomal peptide synthetases. The *txtC* and *txtE* genes encode CYPs. The *txtD* gene encodes a NOS that produces NO from L-arginine. **b:** Proposed pathway for thaxtomin A biosynthesis.

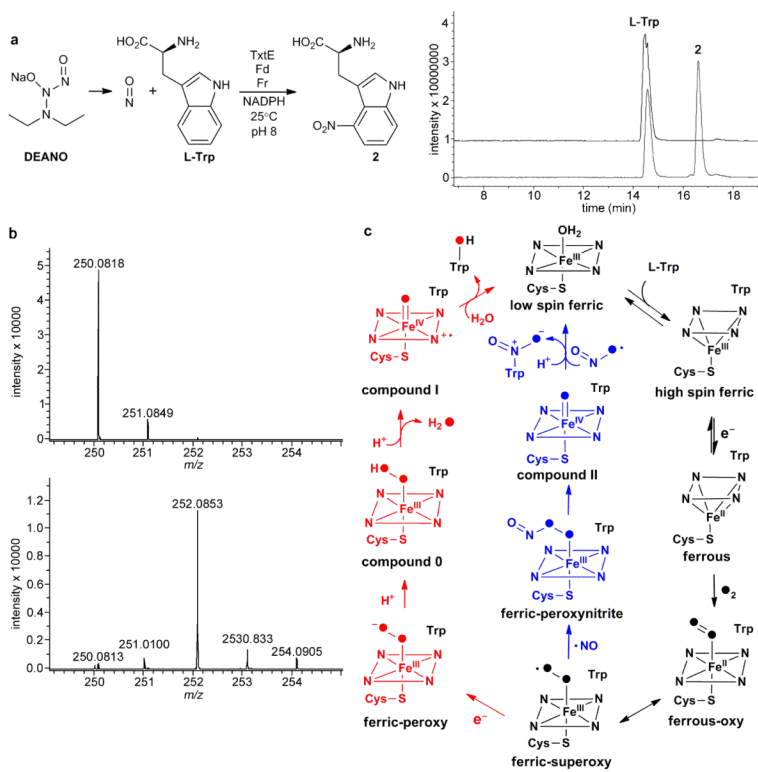


Figure 2. Characterization of purified recombinant TxtE. **a:** Extracted ion chromatograms at $m/z = 204$ and 250 (right) from LC-MS analyses of the enzymatic nitration reaction (left). The bottom chromatogram is from the enzymatic reaction and the top chromatogram is from a negative control containing boiled TxtE. **b:** High resolution mass spectra of L-4-nitrotryptophan obtained from incubation of L-tryptophan with TxtE, DEANO, ferredoxin, ferredoxin reductase and NADPH in air (top spectrum), and in an $^{18}\text{O}_2$ atmosphere (bottom spectrum). The $m/z = 250.0818$ ion corresponds to $[\text{M}+\text{H}]^+$ for L-4-nitrotryptophan. An ion corresponding to $[\text{M}+\text{H}]^+$ for L-4-nitrotryptophan containing a single ^{18}O label (m/z calculated for $\text{C}_{11}\text{H}_{12}\text{N}_3\text{O}^+_{4}$: 252.0865; found: 252.0853) predominates (>90%) in the bottom spectrum **c:** Comparison of the proposed mechanism of TxtE-catalyzed nitration and the mechanism of CYP-catalyzed hydroxylation. Intermediates that differ in CYP-catalyzed hydroxylation and nitration are highlighted in red and blue, respectively. Common intermediates are black. The atoms of O_2 are filled to illustrate their fate in the reactions.

Table 1

Thaxtomin A production by *S. turgidiscabies* strains Car8 (wild type), $\Delta txtD$, $\Delta txtE$ and the complemented $txtE$ mutant, $\Delta txtEpIJ8600txtE$, at 7 days after addition of DEANO (1 mM) or 15 μ g of L-4-nitrotryptophan (4-NO₂Trp), at 3 days after inoculation.

Strain	Thaxtomin A (μ g culture ⁻¹) ^a			
	- DEANO	+ DEANO	- 4-NO ₂ Trp	+ 4-NO ₂ Trp ^c
No inoculum	ND	ND	-	-
Car8	33.0 \pm 3.5	18.9 \pm 0.8	24.9 \pm 0.5	34.0 \pm 0.6 (34.2)
$\Delta txtD$	0.15 \pm 0.01	14.3 \pm 1.0	-	-
$\Delta txtE$	ND	ND	ND	8.0 \pm 0.2 (30.1)
$\Delta txtEpIJ8600txtE$ ^b	0.4 \pm 0.1	2.4 \pm 0.4	-	-

^a mean \pm standard deviation, n=6. ND = not detected

^b complementation construct induced by addition of 10 μ g/ml thiostrepton

^c numbers in parentheses are the percentage of added 4-NO₂Trp converted to thaxtomin A