OL Organic Letters

pubs.acs.org/OrgLett Letter

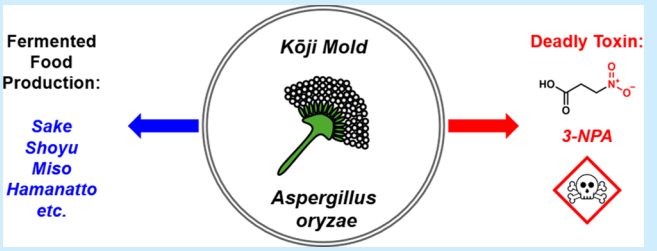
How Fungi Biosynthesize 3-Nitropropanoic Acid: The Simplest yet Lethal Mycotoxin

Colin W. Johnson, Masao Ohashi,* and Yi Tang*

Cite This: *Org. Lett.* 2024, 26, 3158−3163 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: We uncovered the biosynthetic pathway of the lethal mycotoxin 3-nitropropanoic acid (3-NPA) from koji mold *Aspergillus oryzae*. The biosynthetic gene cluster (BGC) of 3-NPA, which encodes an amine oxidase and a decarboxylase, is conserved in many fungi used in food processing, although most of the strains have not been reported to produce 3-NPA. Our discovery will lead to efforts that improve the safety profiles of these indispensable microorganisms in making food, alcoholic beverages, and seasoning.



Mycotoxins are toxic small secondary metabolites produced by molds (fungi) and can accumulate in food and crops, where they pose health hazards to humans and animals.[1] According to USDA, mycotoxins are estimated to impact 25% of the world's crops and cost US agriculture approximately $1 billion each year.[2] More than 300 mycotoxins have been found to be persistent contaminants of food and agricultural commodities worldwide, including those highlighted by WHO,[3] FDA,[4] and CDC[5] (Figure 1A): trichothecenes, fumonisins, and zearalenone from *Fusarium* spp.; citrinin (**2**) from *Penicillium* spp., *Monascus* spp., and *Aspergillus* spp.; aflatoxins (**3**) from *Aspergillus flavus*; ergotamine (**4**) from *Claviceps purpurae*; ochratoxins (**5**) from *Aspergillus* spp. and *Penicillium* spp.; and 3-nitropropanoic acid (3-NPA, **1**) from *Aspergillus oryzae* and other fungi.[6,7] Extensive efforts toward characterization of biosynthetic gene clusters (BGCs) of mycotoxins have been undertaken in recent decades.[8,9] The BGCs of many mycotoxins have been identified, especially those containing polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), or terpene synthases (TSs) (Figure 1A).

In contrast, the BGC of 3-NPA (**1**), a deadly neurotoxic nitroalkane found in numerous leguminous plants and fungi,[10] has remained elusive. **1** is known as an antimetabolite of succinate and irreversibly inhibits succinate dehydrogenase and disrupts mitochondrial oxidative phosphorylation.[5,11] While the first report of **1** was in 1920,[12] the structure wasn't elucidated until 1949.[13,14] There are significant public health concerns stemming from the ability of industrially relevant molds (*A. flavus*[15−17] and *A. oryzae*[18−20]) used in agriculture, food, and enzyme fermentation to produce **1**.[21] In particular, *A. oryzae* is a major organism in the production of fermented food processes[22] including shoyu (fermented wheat and soybeans), miso (rice and soybean), and hamanatto (soybeans). Additionally, modern biotechnology processes including the commercial production of enzymes and flavoring agents such as nucleotides and monosodium glutamate also rely on *A. oryzae* fermentation.[21] Cases of food contaminated with **1** causing human deaths are well documented,[23] with deaths as recent as 2021 by *Arthrinium* spp. (Figure 2A).[5] Therefore, there is urgency to identify genes involved in the biosynthesis of **1**.

Isotope-labeling studies of **1** from fungi and plants established the biosynthetic origins are distinct (Figures 1B and 1D).[24−27] Whereas the plant pathway starts with malonic acid (**6**) and proceeds via *N*-hydroxy-*β*-alanine (**7**), fungi initiate the pathway to **1** with L-aspartate (**8**). Baxter et al. proposed the iterative oxidation of **8** to generate (*S*)-nitrosuccinic acid (**9**), followed by decarboxylation to **1** (Figure 1D).[28,29] In reexamining this proposal, we noted the oxidation of *α*-amine of **8** to *α*-nitro of **9** is identical to the first step of cremeomycin biosynthesis in bacteria, in which a flavin-dependent monooxygenase CreE catalyzes the successive oxidization.[30] **9** then serves as a substrate for the lyase CreD, which liberates nitrous acid for N−N bond formation in cremeomycin (Figure 1C). Characterization of FzmM, a close CreE homologue from the fosfazinomycin BGC, led to detection of **1**, which was attributed to the nonenzymatic decarboxylation of **9** to **1**.[31] Based on these prior studies, we hypothesized that fungi that produce **1** could encode a CreE-like enzyme.

Received: February 28, 2024
Revised: April 1, 2024
Accepted: April 3, 2024
Published: April 8, 2024

https://doi.org/10.1021/acs.orglett.4c00758
Org. Lett. 2024, 26, 3158−3163

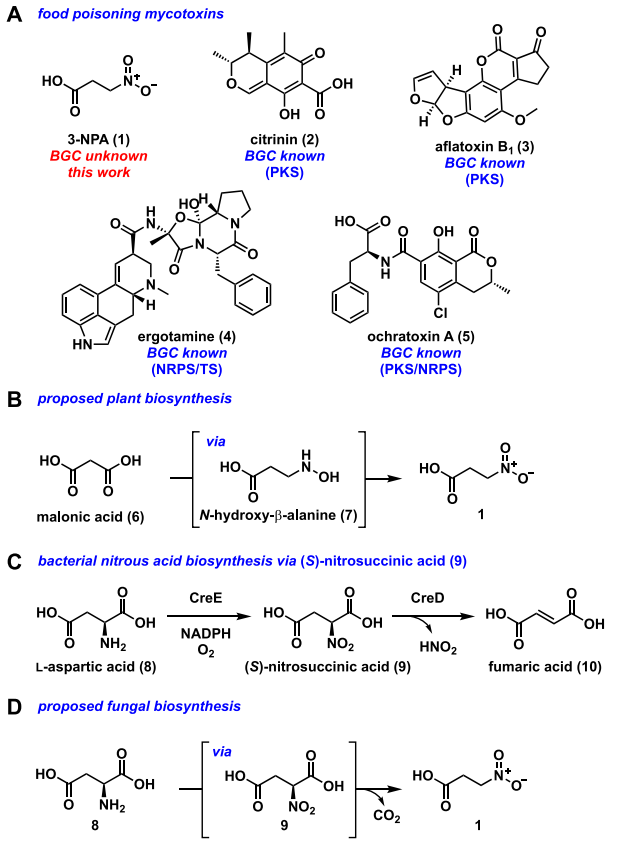


Figure 1. 3-NPA is a lethal mycotoxin. (**A**) Mycotoxins with relevance to human health and identified BGC. (**B**) The proposed biosynthesis of **1** in plants starting from malonic acid. (**C**) The bacterial pathway of nitrite production through (*S*)-nitrosuccinic acid. (**D**) The proposed biosynthesis of **1** via (*S*)-nitrosuccinic acid.

To test the hypothesis, we searched for a homologue of CreE in *A. oryzae* and identified a gene encoding a putative flavoenzyme (NpaA) with ∼55% identity to CreE. We found CreE/NpaA homologues are widely present in fungi (Figure S1). This suggests one or more horizontal gene transfer event(s) of CreE/NpaA homologues between bacteria and fungi could have occurred. Immediately adjacent to *npaA* is a gene encoding a putative decarboxylase (NpaB) annotated as a 4-carboxymuconolactone decarboxylase (Figure 2A).[32] The functions of these enzymes are in line with the oxidation−decarboxylation sequence proposed for the biosynthesis of **1** (Figure 1D). The juxtaposition of these two genes is not only conserved across all known fungal producers of **1**, but also present in fungi not reported as producers (Figure 2A and Figure S2). In addition, species in *Metarhizium* and *Hypoxylon* genera, both not previously reported producers of **1**, contain an additional conserved gene in the cluster, NpaC, which is putatively annotated as a nitronate monooxygenase (NMO) (Figure 2A and Figure S3).[33] Based on this predicted function, NpaC was hypothesized to catalyze the degradation of **1** as a means of resistance, or to metabolize **1** into nitrogen and carbon sources.

The role of *npa* BGC, in particular *npaA*, was first investigated via genetic knockout in *A. oryzae* NSAR1.[34] *A. oryzae* NSAR1 is auxotrophic for nitrate (*niaD*−), adenine (*adeA*−), methionine (*sC*−), and arginine (*argB*−) and is derived from *A. oryzae* RIB40[35] that is often used as a model for strains of *A. oryzae* used in commercial processes. *A. oryzae* NSAR1 was cultured in Nakamura medium for the detection of **1**, based on a previous report of titers as high as ∼1.2 g/L of **1** when *A. oryzae* ATCC12892 was grown in this medium.[18] The low molecular weight and hydrophilicity of **1** complicate detection using conventional reversed-phase LC/MS instrumentation. As a result, detection of **1** was achieved through derivatization with 3-nitrophenylhydrazine (3-NPH) to increase MS sensitivity and the retention on the reverse-phase column.[36] Following validation and optimization of this procedure using the commercial 3-NPA standard (Figure S5),[36] 3-NPH-**1** (Figures S19 and S20 and Table S4) was successfully detected by LC/MS from NSAR1 after 6 days of culturing in Nakamura medium, with an approximate titer of ∼4 mg/L (Figure 2B,i and Figure S10). To directly implicate NpaA in the biosynthesis of **1**, a bipartite knockout cassette

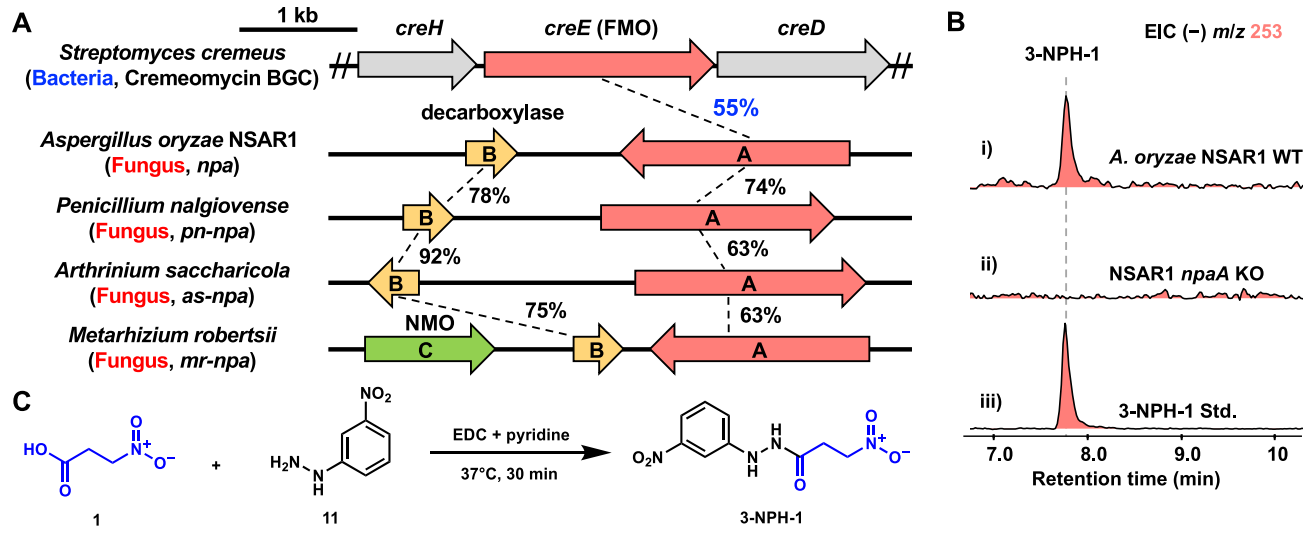


Figure 2. Biosynthesis of **1** by a dedicated biosynthetic gene cluster in fungi. (**A**) Putative BGCs of **1** are not only found in all known producers of **1** such as *A. oryzae* and *A. saccharicola* but also in fungi not reported to produce **1**, such as *P. nalgiovense* and *M. robertsii* (see the Supporting Information). (**B**) LC/MS detection of 3-NPH-**1** from *A. oryzae* WT and abolished detection in the *npaA*-KO strain. (**C**) The synthetic scheme of 3-NPH-**1** using EDC as the condensation reagent.

https://doi.org/10.1021/acs.orglett.4c00758
Org. Lett. 2024, 26, 3158−3163

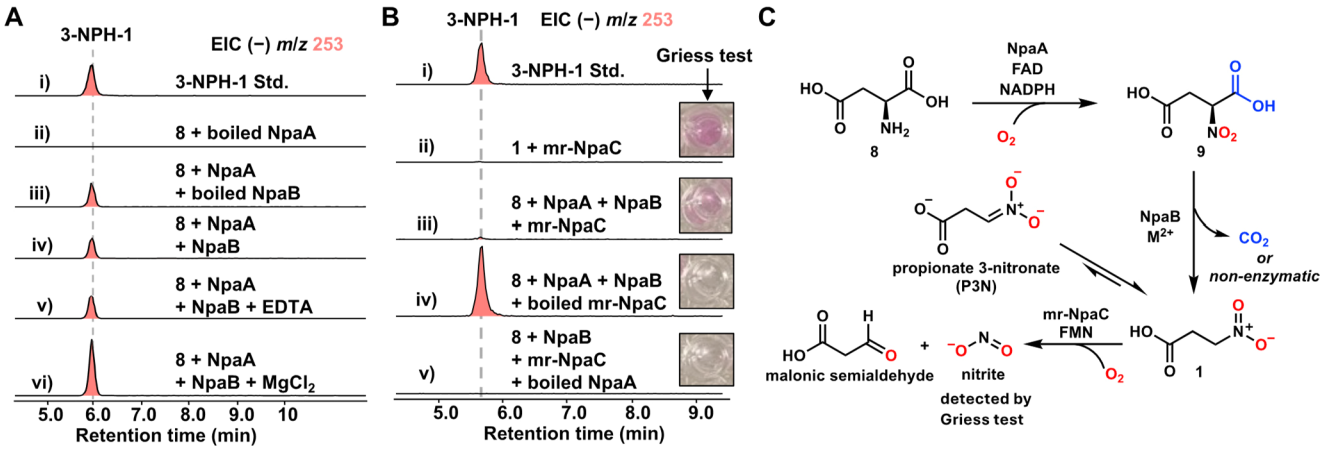


Figure 3. Biochemical characterization of NpaA, NpaB, and mr-NpaC. (**A**) In vitro characterization of NpaA and NpaB. (**B**) Detection of nitrite from the in vitro reaction of mr-NpaC with **1**. Pinkish color in the wells represents the detection of nitrite by the Griess reaction. (**C**) The proposed biosynthetic and degradation pathway of **1**.

was constructed with homology flanking the target site in *npaA*, using *argB* (restoring the Arg auxotrophy) as a selectable marker. Colonies subjected to two sequential passages of selection were then sequenced to genetically verify successful *npaA* knockout via *argB* integration (Figures S6−S8). The production of **1** was completely abolished in the *npaA*Δ::*argB* knockout strain even after prolonged culturing (>24 days) (Figure 2B,ii).

NpaA was next expressed and purified from BL21 Star (DE3) (Figure S9). Purified enzyme displayed the characteristic yellow color of flavin-dependent enzymes. Cofactor copurified with NpaA was identified as FAD by LC/MS analysis of the boiled enzyme supernatant (Figure S11). The use of FAD as the cofactor instead of FMN is in agreement with the bacterial homologue CreE.[30] To assay the activity of NpaA, the purified enzyme was mixed with L-aspartate (**8**) and NADPH, followed by derivatization with 3-NPH and LC/MS analysis. As shown in Figure 3A, 3-NPH-**1** can be detected directly from this mixture within 15 min of incubation. Alternative amino acid substrates such as L-glutamate and D-aspartate were not accepted by NpaA as a substrate, showing the strict substrate specificity toward L-aspartate. While NADH can be used as a cofactor for reduction of the flavin cofactor, the efficiency is much lower than that of NADPH as determined by the yield of 3-NPH-**1** (Figure S12). Collectively, these knockout and biochemical data pinpoint the role of NpaA as (*S*)-nitrosuccinate synthase in the oxidation of **8** to **9**, which can undergo decarboxylation, with the α-nitro group serving as the electron sink.

While NpaA alone is sufficient to produce **1** under in vitro assay conditions, the putative decarboxylase NpaB colocalized with NpaA could accelerate the decarboxylation of **9** to **1**. NpaB is predicted to be a member of the 4-carboxymuconolactone decarboxylase family, which consists of the bacterial decarboxylase that catalyzes the cofactor- and oxygen-independent decarboxylation of β-carboxymuconolactone to β-ketoadipate enol lactone in the β-ketoadipate pathway.[32] NpaB may facilitate decarboxylation of **9** in a metal-dependent fashion similar to known nonredox decarboxylases.[37] NpaB was expressed and purified from *E. coli* BL21(DE3) (Figure S9). The enzyme was then dialyzed against an EDTA-containing buffer to remove any divalent metal ions to become apo-NpaB. A series of combined assays of NpaA and NpaB with and without divalent metal were performed to monitor the production of 3-NPH-**1**. Although the background nonenzymatic decarboxylation of **9** to **1** is also accelerated by divalent metal ions such as Zn^{2+}, Mn^{2+}, and Mg^{2+} (Figure S14),[38] the production of **1** is the highest when NpaA is paired with NpaB in the presence of $MgCl_2$ (Figure 3A,iv−vi and Figure S13). This notable increase in the level of **1** suggests that NpaB is likely the dedicated decarboxylase in the biosynthesis of **1**, using Mg^{2+} as a Lewis acid.

Next, we investigated the role of the conserved putative nitronate monooxygenase (NMO) in *Metarhizium* (mr-NpaC) and *Hypoxylon* genera (Figure 2A). NMOs are FMN-dependent enzymes that use molecular oxygen to oxidize anionic nitronates or neutral nitroalkanes to the corresponding carbonyl compounds and nitrite (Figure S16).[33] Enzymes capable of catalyzing the oxidation of **1** and/or its anionic form propionate-3-nitronate (P3N) have been discovered from bacteria, plants, and fungi.[39] Studies suggest NMOs play important roles not only in detoxifing the toxic nitronate but also in allowing the plants and microorganisms to utilize the toxin as a source of carbon and nitrogen.[39] Although the sequence identity between mr-NpaC and the characterized fungal NMO from *Neurospora crassa* is moderate (34%) (Figure S17), NpaC may play a similar role in the metabolism of **1**.

To test this possibility, mr-NpaC from *Metarhizium robertsii* was expressed and purified from *E. coli* BL21(DE3) (Figure S9). In agreement with reported fungal NMOs,[39] mr-NpaC was copurified with FMN as judged by LC/MS detection of FMN from denatured mr-NpaC (Figure S15). Upon addition of **1** directly to the reaction mixture containing mr-NpaC, **1** was rapidly consumed as determined through the disappearance of 3-NPH-**1** (Figure 3B,ii and Figure S18), while the consumption was not observed in boiled mr-NpaC control. The formation of nitrite was confirmed by Griess test, as the quenched reaction showed a characteristic pink color following formation of a diazonium salt (Figure 3B,ii). The same conclusions can be drawn from the combined, three-enzyme assay of NpaA, NpaB, and mr-NpaC. Whereas no **1** can be detected in the presence of an active mr-NpaC and the Griess test was positive (Figure 3B,iii), adding denatured mr-NpaC led to accumulation of **1** and a negative Griess test (Figure 3B,iv). As a negative control, adding denatured NpaA led to no

https://doi.org/10.1021/acs.orglett.4c00758
Org. Lett. 2024, 26, 3158−3163

detectable **1** as well as a negative Griess test (Figure 3B,v). In total, these data confirmed mr-NpaC is indeed a NMO that catalyzes the oxidation of **1** (and P3N) to nitrite and malonic semialdehyde (Figure 3C). While it is possible that mr-NpaC serves as a self-protection mechanism against accumulation of **1** in the producing host, the more likely scenario may be the three enzymes representing an alternative catabolic pathway of aspartate to generate readily metabolizable nitrogen and carbon sources.

Our identification of the biosynthetic pathway of **1** showed minimally that only one enzyme, NpaA, is required to make a simple yet deadly mycotoxin. The addition of NpaB can accelerate the decarboxylation step and may play a more important role in the biosynthesis of **1** in vivo. Prior difficulties in finding the genes for **1** may be due to having the simplest structure among mycotoxins, which makes searching for responsible BGCs using bioinformatics tools difficult. As most of these tools rely on well-studied core enzymes, such as PKSs, NRPSs, and TSs, BGCs that do not encode such core enzymes are challenging to find. To leverage knowledge from bacterial pathways in fungal biosynthesis, we used bacterial (*S*)-nitrosuccinate synthase CreE as a query to identify the *npa* genes for producing **1** in *A. oryzae*. While such examples of "cross-kingdom genome mining" for secondary metabolism BGCs are still limited, this approach could be fruitful to identify the BGCs of metabolites that are not biosynthesized from canonical core enzymes.[40] One example of this approach was the recent identification of a fungal Pictet-Spenglerase (PS) using the bacterial PS McbB from the marinacarboline biosynthesis.[41]

There are significant implications to finally establishing the gene−metabolite link of **1**. In East Asia, mycotoxins produced by koji mold *A. oryzae* are especially concerning since many Asian foods and seasonings including soy sauce and miso are produced from the fermentation of ingredients with *A. oryzae*.[20,21,35] The FDA maintains a requirement of commercial food additives produced by *A. oryzae* to have levels of mycotoxin, including that of **1**, to be below a threshold.[21,42] BGCs of most mycotoxins produced by *A. oryzae* such as kojic acid,[43] cyclopiazonic acid,[44] and aspirochlorine[45] have been identified, allowing development of modern industrial *A. oryzae* strains with deletions of selected BGCs.[21] In this work, the genetic inactivation of *npaA* abolished the production of **1**, and the resulting strain can therefore be considered a safer strain to use for industrial purposes. The *npaA*−*npaB* pair appears to be widely conserved in many fungi not reported to produce **1** (Figure 2A and Figure S2). Among them, *Penicillium nalgiovense* and *Aspergillus melleus* stand out. *P. nalgiovense* has been used to make mold-fermented meat sausages such as salami, while the traditional production of dried bonito, so-called katsuobushi, involves fermentation using *Aspergillus* spp. such as *A. melleus*.[46] While the detection of **1** in mold-fermented sausages and katsuobushi has not been reported to our knowledge, the presence of *npaA*−*npaB* certainly suggests that the fungi are capable of producing **1** (Figure 2A and Figure S2). For such strains, we recommend revisiting the transcriptomes and metabolomes based on results reported here to ensure food safety.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.4c00758.

Detailed experimental details and spectroscopic data (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Masao Ohashi − *Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, California 90095, United States;* orcid.org/0000-0001-7103-541X; Email: gph422001@ucla.edu

Yi Tang − *Department of Chemistry and Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, California 90095, United States;* orcid.org/0000-0003-1597-0141; Email: yitang@ucla.edu

Author

Colin W. Johnson − *Department of Chemistry, University of California, Los Angeles, California 90095, United States;* orcid.org/0000-0003-1092-1172

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.orglett.4c00758

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported by NIGMS R35GM118056 to Y.T. C.W.J. was a research fellow of the BioPacific Program supported by the NSF cooperative agreement (DMR-1933487).

■ REFERENCES

(1) Moretti, A.; Logrieco, A. F.; Susca, A. Mycotoxins: An Underhand Food Problem. In *Mycotoxigenic Fungi: Methods and Protocols*; Moretti, A., Susca, A., Eds.; Methods in Molecular Biology; Springer: New York, 2017; pp 3−12.

(2) USDA ARS. What are Mycotoxins? https://www.ars.usda.gov/midwest-area/peoria-il/national-center-for-agricultural-utilization-research/mycotoxin-prevention-and-applied-microbiology-research/docs/what-are-mycotoxins/.

(3) Mycotoxins. https://www.who.int/news-room/fact-sheets/detail/mycotoxins.

(4) Chemical Hazards. https://www.fda.gov/animal-veterinary/biological-chemical-and-physical-contaminants-animal-food/chemical-hazards#Mycotoxins.

(5) Birkelund, T.; Johansen, R. F.; Illum, D. G.; Dyrskog, S. E.; Østergaard, J. A.; Falconer, T. M.; Andersen, C.; Fridholm, H.; Overballe-Petersen, S.; Jensen, J. S. Fatal 3-Nitropropionic Acid Poisoning after Consuming Coconut Water. *Emerg. Infect. Dis.* **2021**, *27* (1), 278−280.

(6) Habauzit, D.; Lemée, P.; Fessard, V. MycoCentral: An Innovative Database to Compile Information on Mycotoxins and Facilitate Hazard Prediction. *Food Control* **2024**, *159*, 110273.

(7) El-Sayed, R. A.; Jebur, A. B.; Kang, W.; El-Demerdash, F. M. An Overview on the Major Mycotoxins in Food Products: Characteristics, Toxicity, and Analysis. *J. Future Foods* **2022**, *2* (2), 91−102.
(8) Ferrara, M.; Perrone, G.; Gallo, A. Recent Advances in Biosynthesis and Regulatory Mechanisms of Principal Mycotoxins. *Curr. Opin. Food Sci.* **2022**, *48*, 100923.
(9) Kolawole, O.; Meneely, J.; Petchkongkaew, A.; Elliott, C. A Review of Mycotoxin Biosynthetic Pathways: Associated Genes and Their Expressions under the Influence of Climatic Factors. *Fungal Biol. Rev.* **2021**, *37*, 8−26.
(10) Parry, R.; Nishino, S.; Spain, J. Naturally-Occurring Nitro Compounds. *Nat. Prod. Rep.* **2011**, *28* (1), 152−167.
(11) Alston, T. A.; Mela, L.; Bright, H. J. 3-Nitropropionate, the Toxic Substance of Indigofera, Is a Suicide Inactivator of Succinate Dehydrogenase. *Proc. Natl. Acad. Sci. U. S. A.* **1977**, *74* (9), 3767−3771.
(12) Gorter, K. Hiptagin, a new glucoside from Hiptage medablota. *Gaertu Bull. Jordin Bot Buitenzorg.* **1920**, *2*, 187−202.
(13) Morris, M. P.; Pagán, C.; Warmke, H. E. Hiptagenic Acid, a Toxic Component of Indigofera Endecaphylla. *Science* **1954**, *119* (3088), 322−323.
(14) Carter, C. L.; Mcchesney, W. J. Hiptagenic Acid Identified as β-Nitropropionic Acid. *Nature* **1949**, *164* (4170), 575−576.
(15) Bush, M. T.; Touster, O.; Brockman, J. E. The Production of β-Nitropropionic Acid by a Strain of Aspergillus Flavus. *J. Biol. Chem.* **1951**, *188* (2), 685−693.
(16) Bush, M. T.; Goth, A. Flavicin: An Antibacterial Substance Produced by an Aspergillus Flavus. *J. Pharmacol. Exp. Ther.* **1943**, *78* (2), 164−169.
(17) Marshall, K. C.; Alexander, M. Nitrification by Aspergillus Flavus. *J. Bacteriol.* **1962**, *83* (3), 572−578.
(18) Iwasaki, T.; Kosikowski, F. V. Production of β-Nitropropionic Acid in Foods. *J. Food Sci.* **1973**, *38* (7), 1162−1165.
(19) Penel, A. J.; Kosikowski, F. V. Beta-Nitropropionic Acid Production by Aspergillus Oryzae in Selected High Protein and Carbohydrate-Rich Foods. *J. Food Prot.* **1990**, *53* (4), 321−323.
(20) Deng, S.; Pomraning, K. R.; Bohutskyi, P.; Magnuson, J. K. Draft Genome Sequence of Aspergillus Oryzae ATCC 12892. *Genome Announc.* **2018**, *6* (18), No. e00251-18.
(21) Frisvad, J. C.; Møller, L. L. H.; Larsen, T. O.; Kumar, R.; Arnau, J. Safety of the Fungal Workhorses of Industrial Biotechnology: Update on the Mycotoxin and Secondary Metabolite Potential of Aspergillus Niger, Aspergillus Oryzae, and Trichoderma Reesei. *Appl. Microbiol. Biotechnol.* **2018**, *102* (22), 9481−9515.
(22) Hesseltine, C. W.; Wang, H. L. Traditional Fermented Foods. *Biotechnol. Bioeng.* **1967**, *9* (3), 275−288.
(23) Hamilton, B. F.; Gould, D. H.; Gustine, D. L. History of 3-Nitropropionic Acid. In *Mitochondrial Inhibitors and Neurodegenerative Disorders*; Sanberg, P. R., Nishino, H., Borlongan, C. V., Eds.; Contemporary Neuroscience; Humana Press: Totowa, NJ, 2000; pp 21−33.
(24) Hylin, J. W.; Matsumoto, H. The Biosynthesis of 3-Nitropropanoic Acid by Penicillium Atrovenetum. *Arch. Biochem. Biophys.* **1961**, *93* (3), 542−545.
(25) Shaw, P. D.; McCloskey, J. A. Biosynthesis of Nitro Compounds. II. Studies on Potential Precursors for the Nitro Group of β-Nitropropionic Acid*. *Biochemistry* **1967**, *6* (7), 2247−2253.
(26) Shaw, P. D.; Wang, N. Biosynthesis of Nitro Compounds i. *J. Bacteriol.* **1964**, *88* (6), 1629−1635.
(27) Candlish, E.; La Croix, L. J.; Unrau, A. M. Biosynthesis of 3-Nitropropionic Acid in Creeping Indigo (Indigofera Spicata). *Biochemistry* **1969**, *8* (1), 182−186.
(28) Baxter, R. L.; Hanley, A. B.; Chan, H. W.-S.; Greenwood, S. L.; Abbot, E. M.; McFarlane, I. J.; Milne, K. Fungal Biosynthesis of 3-Nitropropanoic Acid. *J. Chem. Soc., Perkin Trans.* **1992**, *1* (19), 2495−2502.
(29) Baxter, R. L.; Smith, S. L.; Martin, J. R.; Hanley, A. B. The Fungal Biosynthesis of 3-Nitropropionic Acid: Is the Decarboxylation of L-Nitrosuccinate an Enzymatic Reaction? *J. Chem. Soc., Perkin Trans.* **1994**, *1* (16), 2297−2299.
(30) Sugai, Y.; Katsuyama, Y.; Ohnishi, Y. A Nitrous Acid Biosynthetic Pathway for Diazo Group Formation in Bacteria. *Nat. Chem. Biol.* **2016**, *12* (2), 73−75.
(31) Huang, Z.; Wang, K.-K. A.; van der Donk, W. A. New Insights into the Biosynthesis of Fosfazinomycin. *Chem. Sci.* **2016**, *7* (8), 5219−5223.
(32) Parke, D. Structural Comparison of γ-Carboxymuconolactone Decarboxylase and Muconolactone Isomerase from Pseudomonas Putida. *Biochim. Biophys. Acta, Protein Struct.* **1979**, *578* (1), 145−154.
(33) Gadda, G.; Francis, K. Nitronate Monooxygenase, a Model for Anionic Flavin Semiquinone Intermediates in Oxidative Catalysis. *Arch. Biochem. Biophys.* **2010**, *493* (1), 53−61.
(34) Jin, F. J.; Maruyama, J.; Juvvadi, P. R.; Arioka, M.; Kitamoto, K. Development of a Novel Quadruple Auxotrophic Host Transformation System by argB Gene Disruption Using adeA Gene and Exploiting Adenine Auxotrophy in Aspergillus Oryzae. *FEMS Microbiol. Lett.* **2004**, *239* (1), 79−85.
(35) Machida, M.; Asai, K.; Sano, M.; Tanaka, T.; Kumagai, T.; Terai, G.; Kusumoto, K.-I.; Arima, T.; Akita, O.; Kashiwagi, Y.; Abe, K.; Gomi, K.; Horiuchi, H.; Kitamoto, K.; Kobayashi, T.; Takeuchi, M.; Denning, D. W.; Galagan, J. E.; Nierman, W. C.; Yu, J.; Archer, D. B.; Bennett, J. W.; Bhatnagar, D.; Cleveland, T. E.; Fedorova, N. D.; Gotoh, O.; Horikawa, H.; Hosoyama, A.; Ichinomiya, M.; Igarashi, R.; Iwashita, K.; Juvvadi, P. R.; Kato, M.; Kato, Y.; Kin, T.; Kokubun, A.; Maeda, H.; Maeyama, N.; Maruyama, J.; Nagasaki, H.; Nakajima, T.; Oda, K.; Okada, K.; Paulsen, I.; Sakamoto, K.; Sawano, T.; Takahashi, M.; Takase, K.; Terabayashi, Y.; Wortman, J. R.; Yamada, O.; Yamagata, Y.; Anazawa, H.; Hata, Y.; Koide, Y.; Komori, T.; Koyama, Y.; Minetoki, T.; Suharnan, S.; Tanaka, A.; Isono, K.; Kuhara, S.; Ogasawara, N.; Kikuchi, H. Genome Sequencing and Analysis of Aspergillus Oryzae. *Nature* **2005**, *438* (7071), 1157−1161.
(36) Meng, X.; Pang, H.; Sun, F.; Jin, X.; Wang, B.; Yao, K.; Yao, L.; Wang, L.; Hu, Z. Simultaneous 3-Nitrophenylhydrazine Derivatization Strategy of Carbonyl, Carboxyl and Phosphoryl Submetabolome for LC-MS/MS-Based Targeted Metabolomics with Improved Sensitivity and Coverage. *Anal. Chem.* **2021**, *93* (29), 10075−10083.
(37) Sheng, X.; Himo, F. Mechanisms of Metal-Dependent Non-Redox Decarboxylases from Quantum Chemical Calculations. *Comput. Struct. Biotechnol. J.* **2021**, *19*, 3176−3186.
(38) Kubala, G.; Martell, A. E. Zinc(II)-, Aluminum(III)-, and Copper(II)-Catalyzed Decarboxylation of 2-Oxalopropionic Acid. *J. Am. Chem. Soc.* **1982**, *104* (24), 6602−6609.
(39) Francis, K.; Smitherman, C.; Nishino, S. F.; Spain, J. C.; Gadda, G. The Biochemistry of the Metabolic Poison Propionate 3-Nitronate and Its Conjugate Acid, 3-Nitropropionate. *IUBMB Life* **2013**, *65* (9), 759−768.
(40) Gilchrist, C. L. M.; Li, H.; Chooi, Y.-H. Panning for Gold in Mould: Can We Increase the Odds for Fungal Genome Mining? *Org. Biomol. Chem.* **2018**, *16* (10), 1620−1626.
(41) Li, X.-L.; Sun, Y.; Yin, Y.; Zhan, S.; Wang, C. A Bacterial-like Pictet−Spenglerase Drives the Evolution of Fungi to Produce β-Carboline Glycosides Together with Separate Genes. *Proc. Natl. Acad. Sci. U. S. A.* **2023**, *120* (30), No. e2303327120.
(42) GRAS Notices. https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=1039.
(43) Terabayashi, Y.; Sano, M.; Yamane, N.; Marui, J.; Tamano, K.; Sagara, J.; Dohmoto, M.; Oda, K.; Ohshima, E.; Tachibana, K.; Higa, Y.; Ohashi, S.; Koike, H.; Machida, M. Identification and Characterization of Genes Responsible for Biosynthesis of Kojic Acid, an Industrially Important Compound from Aspergillus Oryzae. *Fungal Genet.Biol.* **2010**, *47* (12), 953−961.
(44) Liu, X.; Walsh, C. T. Cyclopiazonic Acid Biosynthesis in Aspergillus Sp.: Characterization of a Reductase-like R* Domain in Cyclopiazonate Synthetase That Forms and Releases Cyclo-Acetoacetyl-l-Tryptophan. *Biochemistry* **2009**, *48* (36), 8746−8757.
(45) Chankhamjon, P.; Boettger-Schmidt, D.; Scherlach, K.; Urbansky, B.; Lackner, G.; Kalb, D.; Dahse, H.-M.; Hoffmeister, D.;

https://doi.org/10.1021/acs.orglett.4c00758
Org. Lett. 2024, 26, 3158−3163

Hertweck, C. Biosynthesis of the Halogenated Mycotoxin Aspirochlorine in Koji Mold Involves a Cryptic Amino Acid Conversion. *Angew. Chem., Int. Ed.* **2014**, *53* (49), 13409−13413.
(46) Nout, M. J. R.; Aidoo, K. E. Asian Fungal Fermented Food. In *Industrial Applications*; Osiewacz, H. D., Ed.; The Mycota; Springer: Berlin, Heidelberg, 2002; pp 23−47.

https://doi.org/10.1021/acs.orglett.4c00758
Org. Lett. 2024, 26, 3158−3163