SUPPORTING INFORMATION

Reannotation of fly amanita L-DOPA dioxygenase gene enables its cloning and heterologous expression

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MATERIAL AND METHODS

General

All chemicals were purchased from Sigma-Aldrich, and used without further purification, unless otherwise stated. Solutions were prepared using deionized water (18.2 MΩ cm at 25 °C, TOC ≤ 4 ppb, Milli-Q, Millipore). All values are expressed as the mean ± standard deviation (SD) of three independent replicates.

Buffers

Sodium phosphate buffer (50 mM, pH 7.4) was prepared by dissolving the appropriate amount of phosphate sodium salt in water, and adjusting the pH to 7.4, with NaOH at 25 °C.

Sodium phosphate lysis buffer (50 mM, pH 7.4, 0.3 M NaCl) was prepared by supplementing the sodium phosphate buffer with imidazole (10 mM), phenylmethanesulfonyl fluoride (PMSF, 1 mM), β-mercaptoethanol (2 mM), and one tablet of cOmplete protease inhibitor cocktail (Roche).

Elution buffer was prepared by supplementing the sodium phosphate lysis buffer with a linear gradient of imidazole from 100 mM to 500 mM.

Standard compounds

Betalamic acid (HBt) was obtained according to the procedure described by Schliemann et al.,[1] with a few modifications. Fresh beetroot juice (200 mL) was paper-filtered and subjected to alkaline hydrolysis using NH_4OH (pH 11.4, 30 min, room temperature [rt]). Next, the solution was cooled in an ice bath, and the pH was adjusted to 3 by slow addition of concentrated HCl. HBt was extracted with ethyl acetate (50 mL, 2×), and subjected to chromatographic and spectrophotometric analyses.

L-Dopaxanthin was obtained as described by Schliemann et al.,[1] with minor modifications. HBt in ethyl acetate was obtained from hydrolyzed beetroot juice. The acid was partitioned into

water, and the residual organic solvent was evaporated under reduced pressure (80 mm Hg, 25 °C). L-DOPA (50 equiv.) was added to a solution of HBt (5 mL) in water at pH 10. Depletion of HBt, and concomitant appearance of L-dopaxanthin were monitored spectrophotometrically at 430 nm and 470 nm, respectively. After completion, HCl (*conc.*) was added slowly until a pH of 5 was reached. The product was purified by gel chromatography using Sephadex LH-20 as the stationary phase and water as the eluent. Final characterization was carried out using LC-HRMS.

Apoenzyme preparation

Sodium phosphate buffer (50 mM, pH 8.5) was treated with Chelex-100 (50 mg mL^{-1}) for 24 h, and filtered, and the pH was confirmed and adjusted when necessary (Ch-PB). Stock solutions of ascorbic acid (250 mM) and L-DOPA (5 mM) were prepared using Ch-PB. The stock solution of AmDODA in sodium phosphate buffer (1.12 mg mL^{-1}, 50 μM, 10 μL) was diluted 100-fold using Ch-PB and Chelex-100 (50 mg). pH of the supernatant was measured prior to use. The mixture was incubated at 4 °C and 450 rpm using an orbital mixer, and aliquots were taken every 30 min until the activity of the enzyme treated with Ch-PB was lost (3.5 5 h). Activity of the enzyme prepared with Ch-PB was compared to that of the negative control. All solutions were prepared using sodium phosphate buffer and incubated under the same conditions.

Kinetic modelling

To model the kinetics of 2,3-seco-DOPA, 4,5-seco-DOPA, betalamic acid, muscaflavin, L-dopaxanthin, and L-DOPA hygroaurin formation, the observed rate constants (k_{obs}) for the formation and/or consumption of these intermediates and products were calculated by non-linear fitting of the chromatographic data to mono- or biexponential functions using Origin 2016 software (OriginLab). Next, a minimal kinetic model was used to investigate the conversion of 1.0 mM L-DOPA into L-dopaxanthin and L-DOPA hygroaurin using COPASI v.4.22.[2] For simplicity, all elementary reactions were defined as unimolecular and irreversible, and the values of k_{obs} were

used to develop the model, ensuring that the resulting kinetic profile was qualitatively similar to the experimental data. The maximum theoretical concentration of each intermediate and product was divided by the maximum experimental absorption measured by HPLC-PDA analysis. The resulting factor was used to calculate the relative concentration of each species based on the experimental absorption. Changes in the concentration of each species over time were fitted to different kinetic models, and the first-order rate constants were calculated using the evolution programming method.

FIGURES

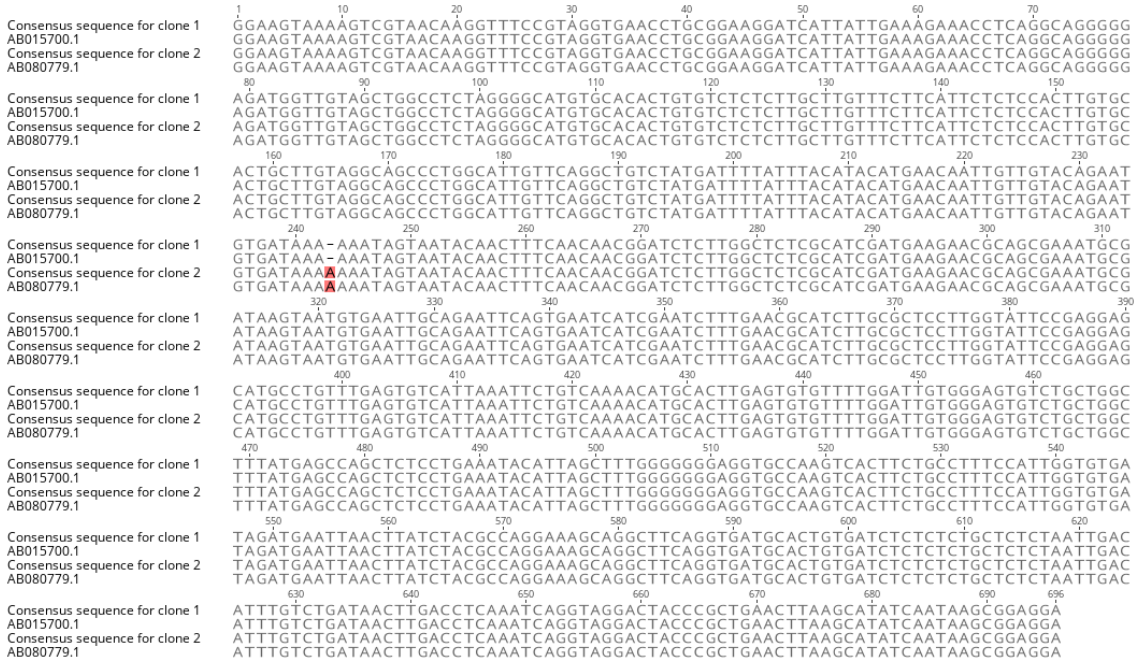

Figure S1. Multiple alignment between two consensus sequences obtained from the DNA sequencing of internal transcribed spacer elements (ITS1 and 2) of the nuclear rDNA cluster in the *A. muscaria* specimens collected in Brazil, and two *A. muscaria* ITS sequences deposited in the NCBI nucleotide database (GenBank AB015700.1 and AB080779.1).

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   1 acatgcgctt cgtcactcaa atatatccat tggtttcgcg acacgctggt tccgaaccca
  61 gagagtctcg cgactctctt cagaaattct cccaacttgc gttccttcaa caccccttg
 121 gtgcccgcgg cagaggactg cgacagttag ccatgacgga ttctttcatt cctctctgac
 181 gactatccat gcagaggcca ggattctgga ctatgctagt cctatcccac ctcatctcat
 241 gaaccttcct tctctacgcc agatgtcctt cgttgttcct ctttcgccgg gcagacgcac
 301 gttggcgcag tttcctcgag tggcatggtt cacgtttaca gcatatccaa gctttgattt
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 541 acgttcttct ctcgtttaga taggatcgag tatggtgcca agcttcgttg tatacagttc
 601 ttgggtaaac ggacgtcaga gatatattcg acaagcattt gcatcaattc tggtctggca
 661 cgaggcgact aagaggtagt ggggttagat tcatgaatca tagaggggca tgtatagcgt
 721 agctaaggat agcactggca gttccacagt tctatataat ccgtgatacg actctctcgt
 781 tcccctcaca tactaccatg tccaccaagc cagagactga ccttcaaact gtcctcgaca
 841 gcgaaatcaa gtttgtctag tttgtctcca ctagcaaaaa ctctgctgac ttgtgtacag
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1141 accaccagtc cttgaacaag tcccttgact ctcaaggttc ttatgagatc tgggttccgt
1201 ctgaaacgtt cgcttccgtg ttctcctact tgtgcatgaa cagagggaga ttaagcatcc
1261 ttgtgcatcc tttgacacgc gaagaactca gagaccatga aattcgtaat gcctggatag
1321 gaccctcttt cccactcaat ctcgccaacc taccgatcaa gagtgatgag atccctttgc
1381 aatatccaag cctcagtatg tcatctcacc actctctggc ggccctcagt gacaaattgt
1441 tgcagagctt gggtactcat cgacagcgca taagatgtca ttggaagaaa ggcggaaatt
1501 aggcgacgat atagaagcag tgcttagggg agagaaagag gcggccagag cgccccatcg
1561 agatgcatag agctacattc gattgtctat attgctactg acatagggta atgttggaat
1621 tttctgccc
```

Figure S2. DNA sequence for *dodA* (Hinz *et al.*, 1997; GenBank accession Y12886, NCBI). *dodA* exons are highlighted in blue and the inferred 3′-AG splice site, altered to GA in *A. muscaria* specimens analyzed, is highlighted in yellow. The start codon for 558-bp CDS is shown in red.

```
MVPSFVVYSSWVNGRQRYIRQAFASILFYIIRDTTLSFPSHTTMSTKPETDLQTVLDSEIKEWHFHIYFHQNNAAEHQ
AALELRDAVLRLRQDGAFVAVPLFRVNMDPMGPHPVGSYEIWVPSETFASVFSYLCMNRGRLSILVHPLTREELRDHE
IRNAWIGPSFPLNLANLPIKSDEIPLQYPSLKLGYSSTAHKMSLEERRKLGDDIEAVLRGEKEAARAPHRDA-
```

Figure S3. Amino acid sequence inferred for 228-aa AmDODA encoded by *dodA* (full sequence)[3] and the 185-aa AmDODA fragment (highlighted).

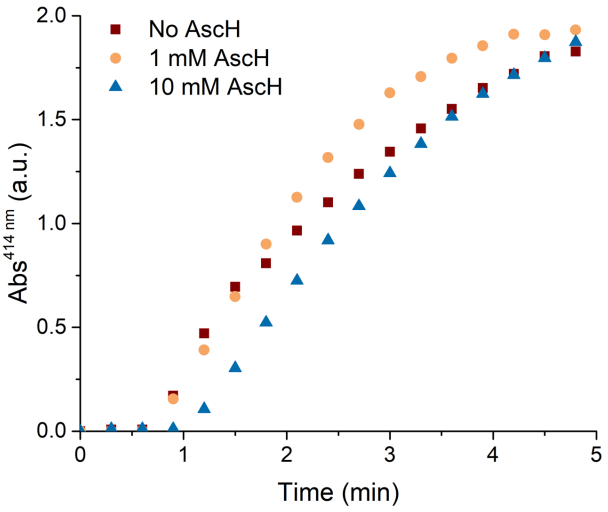

Figure S4. Effect of ascorbic acid (AscH) on the oxidative cleavage of L-DOPA catalyzed by AmDODA. Absorption at 414 nm of the conversion of L-DOPA (1 mM) by AmDODA (1 µM) in the presence or absence of 1 or 10 mmol L^{-1} AscH in sodium phosphate buffer (50 mM), pH 8.5.

Figure S5. Effect of the presence (left) or absence (right) of ascorbic acid (AscH) in the standard reaction without AmDODA. Picture credit: Dr. Douglas M. M. Soares.

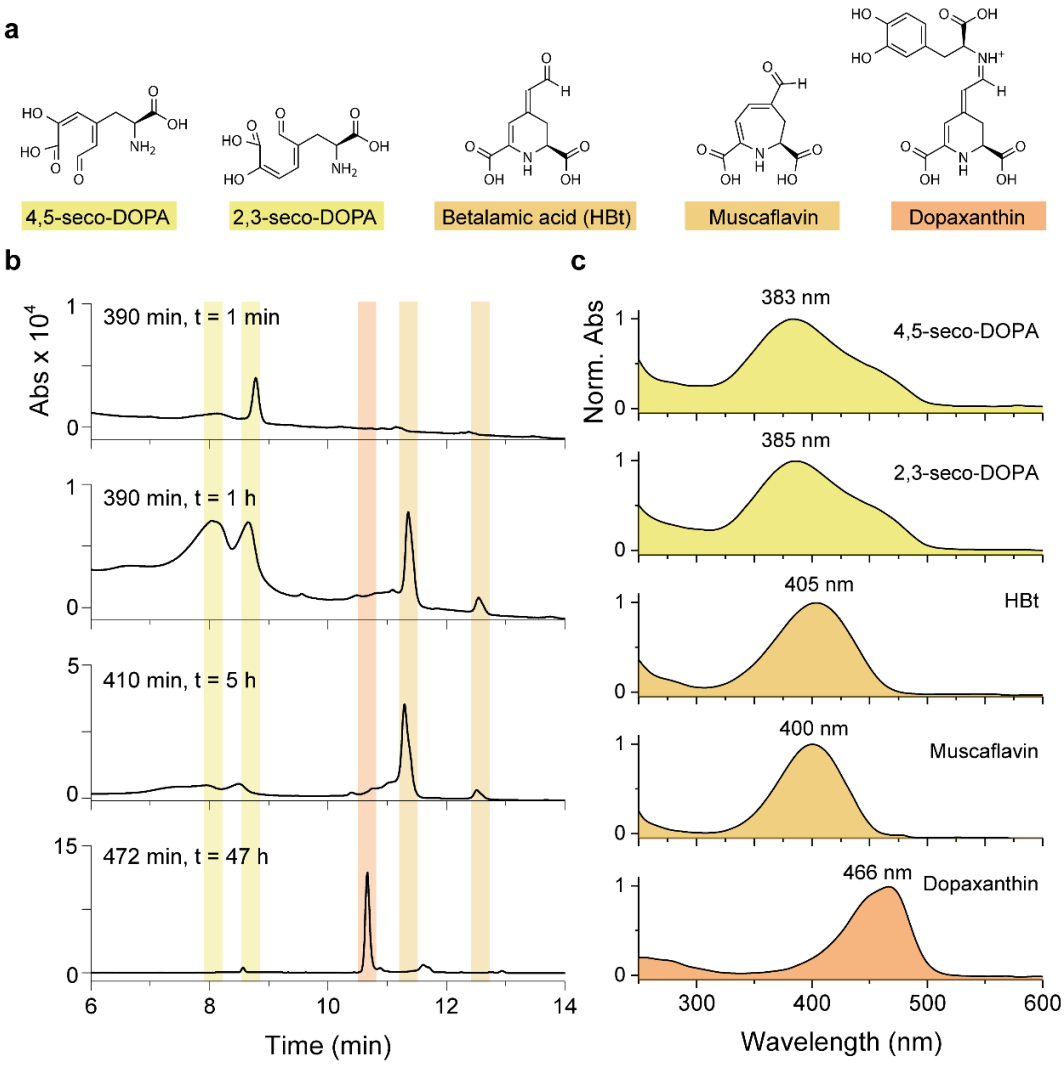

Figure S6. HPLC of 4,5-seco-DOPA and 2,3-seco-DOPA formed during oxidative cleavage of L-DOPA catalyzed by AmDODA, and the derived compounds (betalamic acid, muscaflavin and dopaxanthin). (a) Reaction products monitored by HPLC. (b) Chromatograms obtained at 390 nm (seco-DOPA), 410 nm (betalamic acid and muscaflavin), and 472 nm (dopaxanthin). Note that *t* corresponds to the time of injection after the reaction was triggered. (c) Spectra of the peaks of the reaction products with retention times: 8.1 min (383 nm, 4,5-seco-DOPA), 8.7 min (385 nm, 2,3-seco-DOPA), 10.7 min (466 nm, L-dopaxanthin), 11.3 min (405 nm, betalamic acid), 12.5 min (400 nm, muscaflavin). Reaction condition: [AmDODA] = 1 μM, [AscH] = 10 mM, [L-DOPA] = 2.5 mM, sodium phosphate buffer (50 mM), pH 8.5.

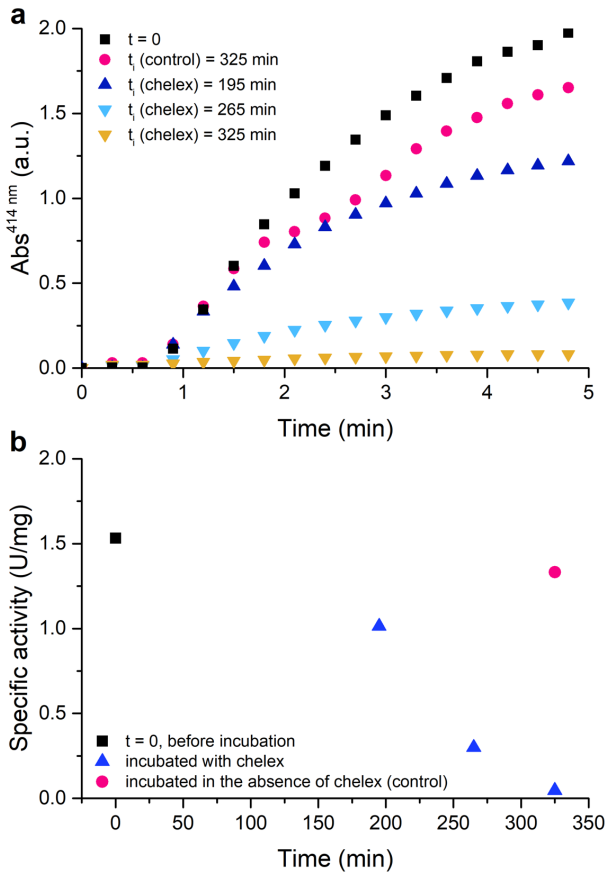

Figure S7. Effect of incubation with Chelex-100 on the oxidative cleavage of L-DOPA catalyzed by AmDODA. (a) Absorption at 414 nm for AmDODA (0.24 mg/mL) incubated at 4 °C under agitation (450 rpm), with or without Chelex-100, in sodium phosphate buffer (50 mM), pH 8.5. (b) Specific activity (U/mg) at the same conditions. Reaction condition: [AmDODA] = 1 μM, [AscH] = 10 mM, [L-DOPA] = 1 mM, sodium phosphate buffer (50 mM), pH 8.5.

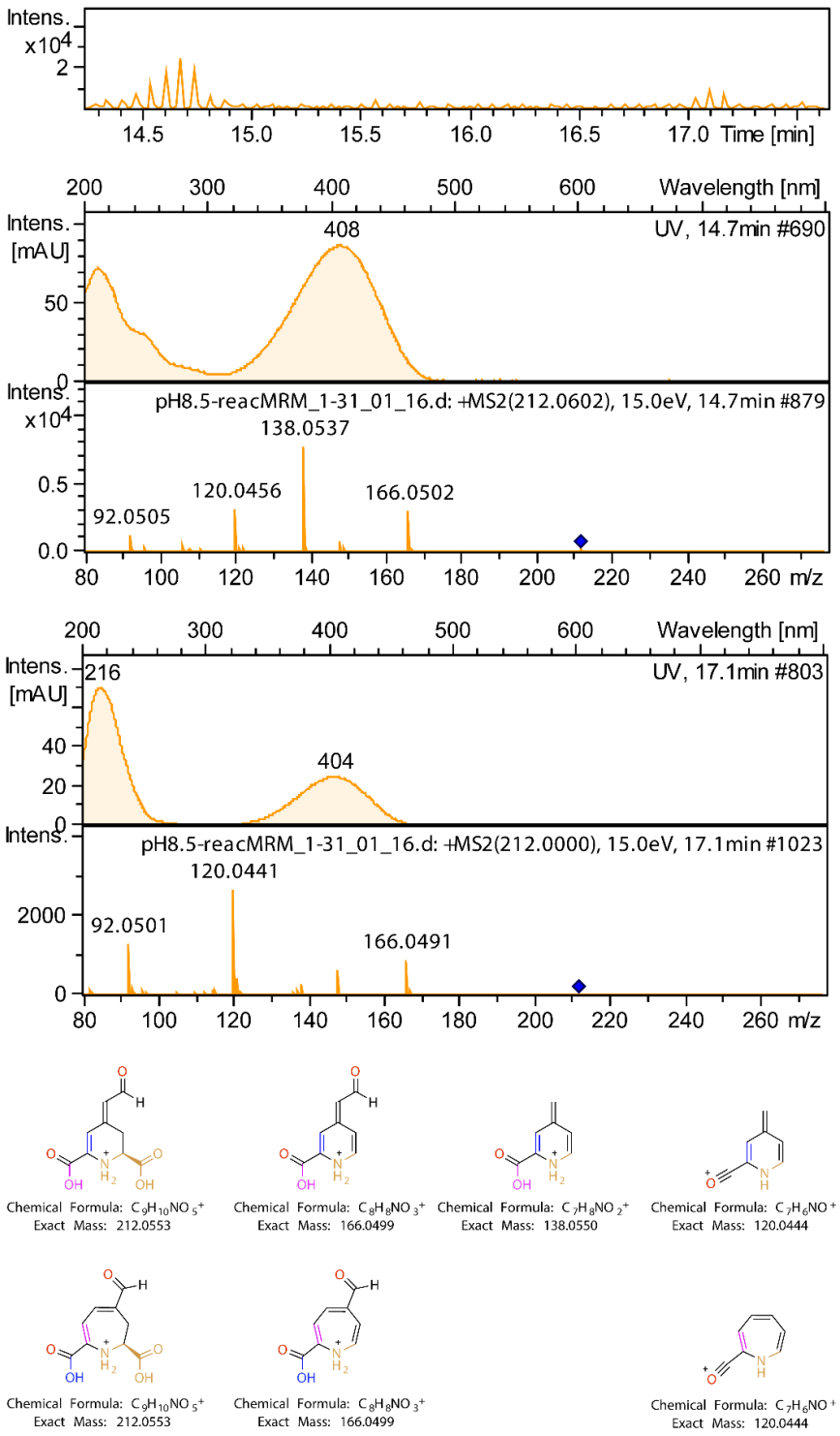

Figure S8. Comparison of HPLC-PDA-MS(ESI) and MS/MS analysis of the substances with retention time of 14.7 min and 17.1 min, which were characterized as betalamic acid and muscaflavin.

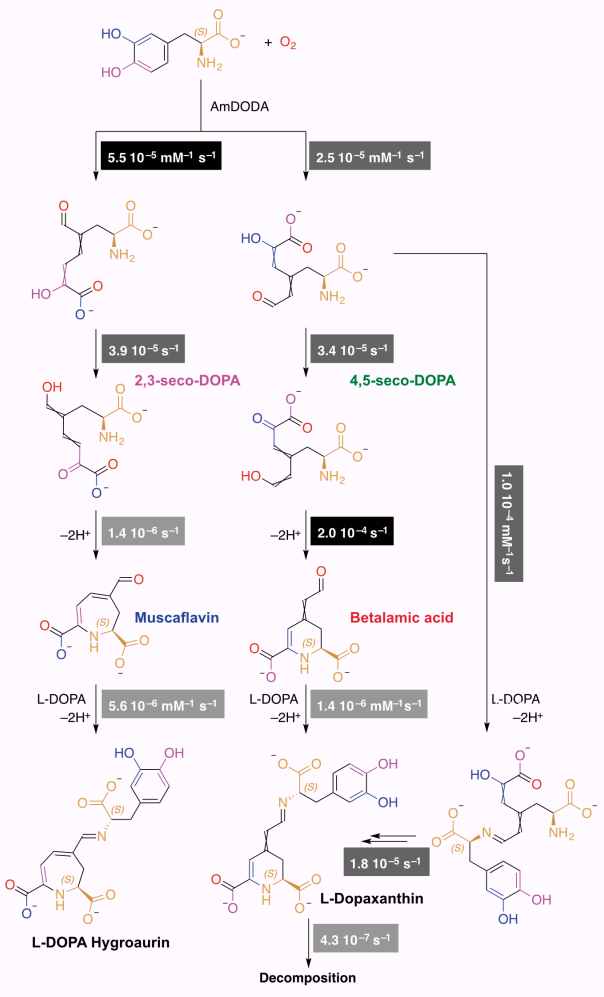

Figure S9. Kinetic modelling for the oxidative cleavage of L-DOPA by oxygen in the presence of AmDODA.

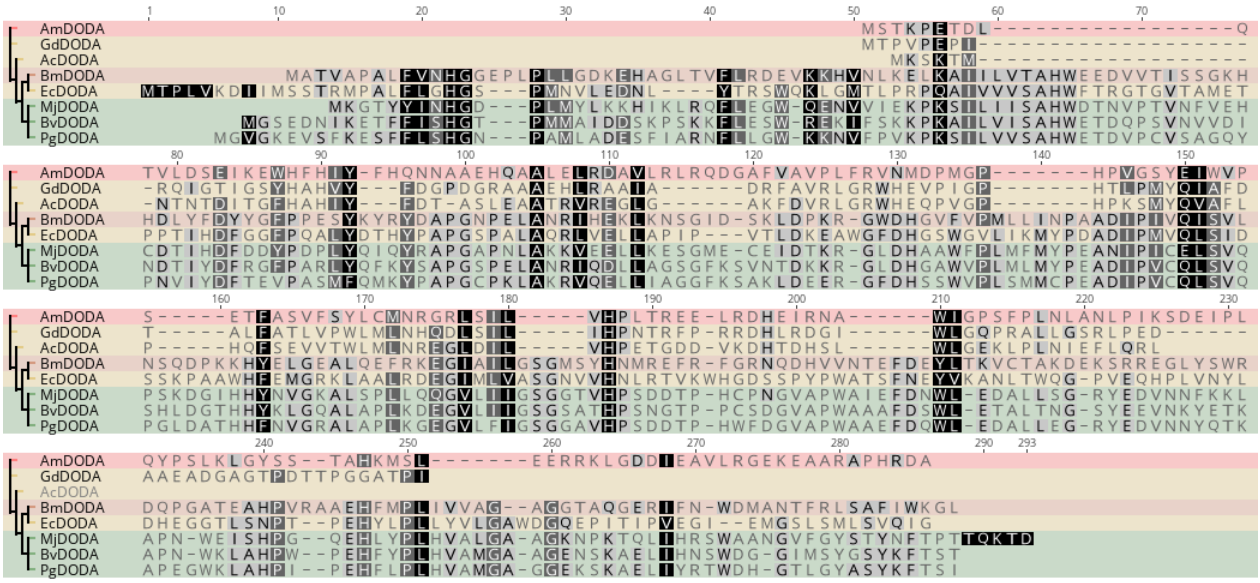

Figure S10. Multiple alignment of amino acid sequences of functionally characterized DODAs from fungi (*A. muscaria* – AmDODA), bacteria (*Gluconacetobacter diazotrophicus* – GdDODA, *Anabaena cylindrica* – AcDODA, *Escherichia coli* - EcDODA), insect (*Bombyx mori* – BmDODA) and plants (*Mirabilis jalapa* – MjDODA, *Beta vulgaris* – BvDODA and *Portulaca grandiflora* – PgDODA). Similarity of residues is indicated by colors: black for 100%, dark gray for 80-100%, and light gray for 60-80%. Not highlighted residues are less than 60% similar. The horizontal colors group sequences together according to similarity.

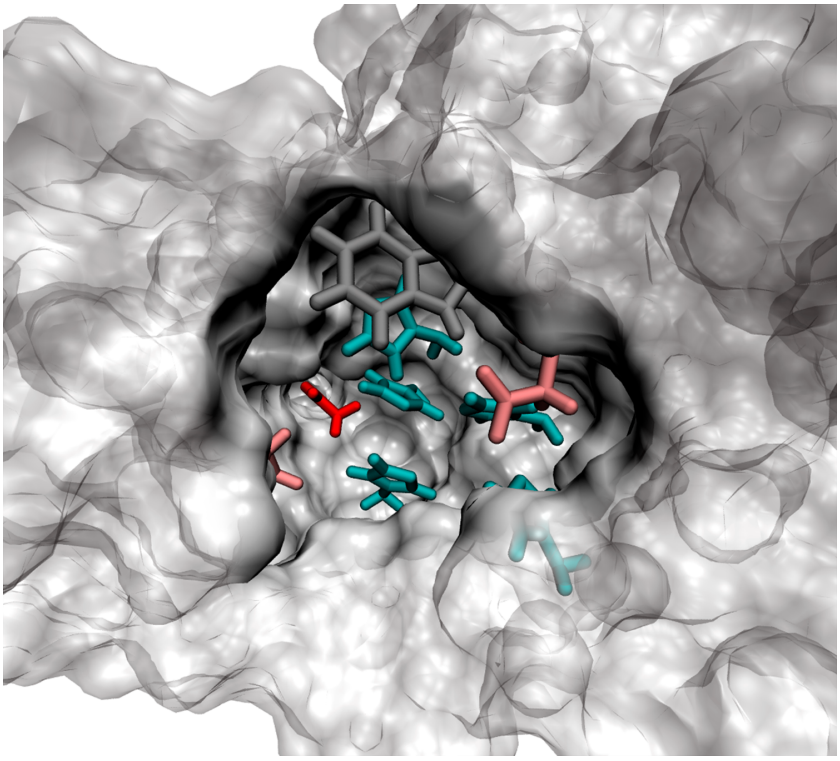

Figure S11. Catalytic pocket and amino acid residues located at regions I and II of AmDODA.

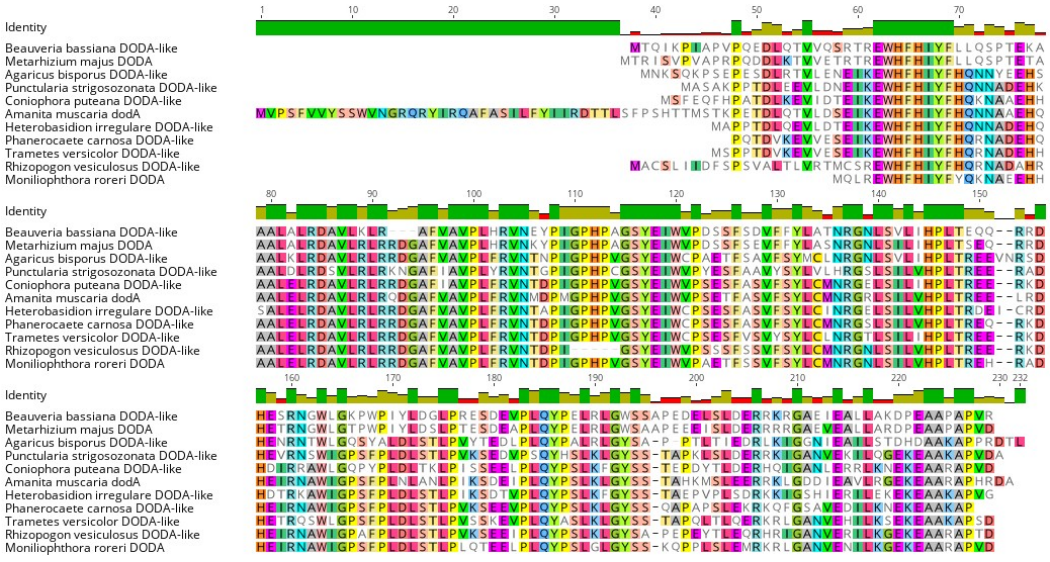

Figure S12. Multiple alignment of amino acid sequences of fungal L-DOPA-dioxygenases (MUSCLE, Geneious Prime v11.0.4).

TABLES

Table S1. Alignments showing the main-chain root-mean-square deviation (RMSD, in Å) below the diagonal, and the number of overlapping residues above the diagonal.

Protein	**1**	**2**	**3**	**4**
AmDODA_Hinz *et al.* (**1**)		121	99	106
AmDODA (**2**)	0.504		99	106
AcDODA (**3**)	1.010	0.994		99
GdDODA (**4**)	0.875	0.853	0.744	

Table S2. Homology models used for the metal cation binding sites of DODAs.

Rank	**Template**	**Confidence**	**Coverage**	**Seq. id.**	**E-value**	**z-score**
AmDODA, TM-score = 0.667						
1	2P8I_D	100	62.2	40.0	4.9E–51	85.540
2	2PEB_A	100	61.1	39.8	5.8E–50	82.898
GdDODA, TM-score = 0.790						
1	2P8I_D	100	79.6	45.2	9.7E–50	87.542
2	2PEB_A	100	79.6	43.4	2.2E–50	87.532
AcDODA, TM-score = 0.907						
1	2PEB_A	100	94.1	69.9	2.2E–51	84.713
2	2P8I_D	100	95.0	45.2	9.9E–51	82.726

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2. Hoops, S.; Sahle, S.; Gauges, R.; Lee, C.; Pahle, J.; Simus, N.; Singhal, M.; Xu, L.; Mendes, P.; Kummer, U., COPASI—a COmplex PAthway SImulator. *Bioinformatics* **2006,** *22* (24), 3067-3074.

3. Hinz, U. G.; Fivaz, J.; Girod, P. A.; Zrÿd, J. P., The gene coding for the DOPA dioxygenase involved in betalain biosynthesis in *Amanita muscaria* and its regulation. *Mol Gen Genet* **1997,** *256* (1), 1-6.