

Isotopic Labeling Studies Reveal the Patulin Detoxification Pathway by the Biocontrol Yeast *Rhodotorula kratochvilovae* LS11

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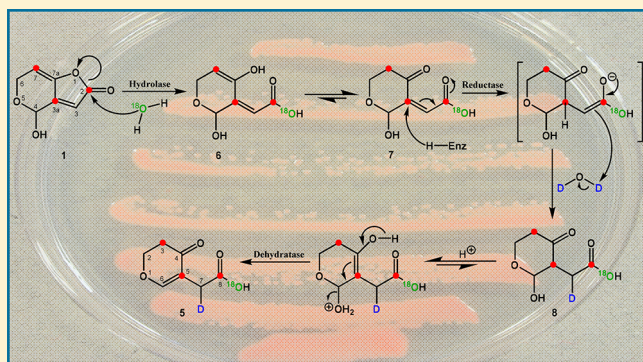
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Supporting Information

ABSTRACT: Patulin (**1**) is a mycotoxin contaminant in fruit and vegetable products worldwide. Biocontrol agents, such as the yeast *Rhodotorula kratochvilovae* strain LS11, can reduce patulin (**1**) contamination in food. *R. kratochvilovae* LS11 converts patulin (**1**) into desoxypatulinic acid (DPA) (**5**), which is less cytotoxic than the mycotoxin (**1**) to *in vitro* human lymphocytes. In the present study, we report our investigations into the pathway of degradation of patulin (**1**) to DPA (**5**) by *R. kratochvilovae*. Isotopic labeling experiments revealed that **5** derives from patulin (**1**) through the hydrolysis of the γ -lactone ring and subsequent enzymatic modifications. The ability of patulin (**1**) and DPA (**5**) to cause genetic damage was also investigated by the cytokinesis-block micronucleus cytome assay on *in vitro* human lymphocytes. Patulin (**1**) was demonstrated to cause much higher chromosomal damage than DPA (**5**).



Patulin (**1**) is a well-known mycotoxin, biosynthesized by species belonging to the genera *Penicillium*, *Aspergillus*, *Byssoschlamys* (anamorph *Paecilomyces*), *Gymnoascus*, *Paecilomyces*, and *Scopulariopsis*.^{1,2} It commonly occurs in fruits and in some other foods that have been attacked by these fungi. Patulin's (**1**) most noteworthy producer is *P. expansum* Link. (Trichocomaceae family), which causes blue mold of apples during postharvest storage.³

Patulin (**1**) is toxic to humans and animals, as well as to bacteria, yeasts, and plants.^{3–18} In mammals, exposure to **1** can induce genotoxicity, embryotoxicity, immunotoxicity, and neurotoxicity.^{3,7–18} Thus, human exposure to low levels of **1** is considered a major concern for public health. The Joint FAO/WHO Expert Committee on Food Additives has recommended a maximum tolerable daily intake of 0.4 $\mu\text{g/kg}$ body weight/day.¹⁹ In 2006 the European Commission set a maximum permitted level of 50 $\mu\text{g/L}$ for fruit juice and 10 $\mu\text{g/kg}$ in products for children.²⁰

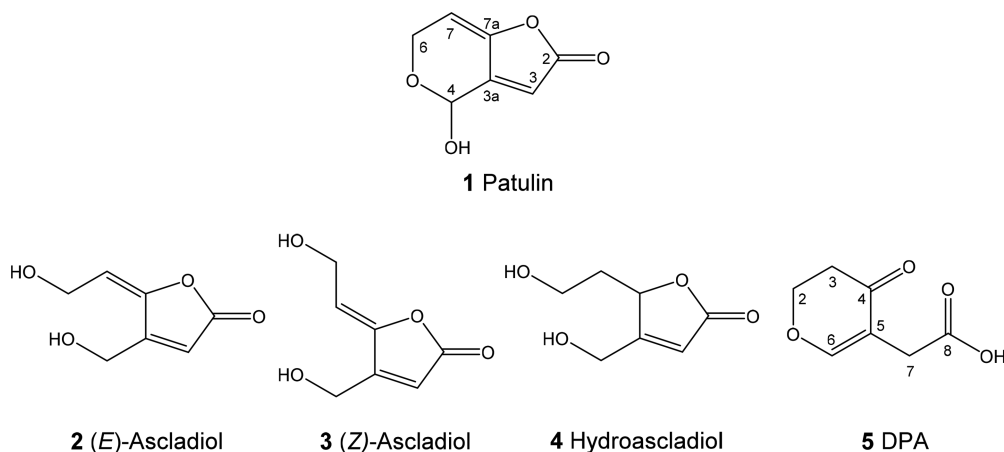
Several postharvest control strategies have been implemented to prevent, reduce, or eliminate patulin (**1**)

contamination of foodstuffs, including the use of chemical and physical methods.^{3,21–24} In recent years, biocontrol agents have emerged as a promising method to prevent infection of stored fruits by *P. expansum* and, as a consequence, eliminate patulin (**1**) contamination in fruit derivatives.^{25,26}

Various microbial species, including biocontrol agents, exhibited the ability to degrade patulin (**1**). *Saccharomyces cerevisiae* degraded **1** to (E)- and (Z)-ascladiol (**2**, **3**) during alcoholic fermentation of apple juice for cider production.²⁷ More recently, it was reported that the bacteria *Gluconobacter oxydans*²⁸ and *Lactobacillus plantarum*,²⁹ the marine yeast *Kodamea ohmeri*,³⁰ and the terrestrial yeasts *Candida guilliermondii*³¹ and *Pichia caribbica*³² also transformed **1** into **2** and **3**. An additional metabolite, hydroascladiol (**4**), was formed in a cell-free supernatant of a culture of *L. plantarum* supplemented with **1**.²⁹ (Z)-Ascladiol (**3**) has been reported to be the product of nonenzymatic isomerization of (E)-ascladiol

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Chart 1



(2) catalyzed by sulfhydryl compounds, such as homocysteine, cysteine, and glutathione or dithiothreitol.³³

In previous studies, we observed that the biocontrol yeast *Rhodotorula kratochvilovae* LS11 (basionym *Rhodospiridium kratochvilovae*, Sporidiobolaceae family) metabolized **1**, generating a new degradation product, desoxypatulenic acid (DPA, **5**).³⁴ Studies carried out with another basidiomycete yeast allowed us to demonstrate that *Sporobolomyces* sp. IAM 13481 was also able to convert **1** to **5**, in addition to **2** and **3**.³⁵ In a further report, a phylogenetically related yeast species, *Rhodospiridium paludigenum* (syn. *Rhodotorula paludigena*), was demonstrated to produce a biodegradation product of **1**, which was putatively identified as DPA (**5**).³⁶

Cytotoxicities of patulin degradation products have been investigated. (E)- and (Z)-ascladiol (**2**, **3**), unlike **1**, were devoid of cytotoxic effects on human cell lines of liver, kidney, intestine, and immune system.³⁷ In addition, **2** did not produce cytotoxicity in an *ex vivo* porcine intestinal model tissue.³⁸ On the other hand, DPA (**5**) was also much less cytotoxic than **1** to *in vitro* human lymphocytes³⁴ and hepatic cell lines.³⁶ These conversions by microbial species can therefore be considered detoxification processes of the mycotoxin (**1**).

Previous isotopic labeling experiments with ¹³C-labeled patulin (**1**) have allowed us to demonstrate that DPA (**5**) is derived from the metabolism of **1** by *R. kratochvilovae* strain LS11.³⁴ However, the biodegradation mechanism by which this conversion occurs is still unknown. In order to elucidate this detoxification pathway, we followed herein the fate of several isotopic labels during the biodegradation process of **1** into **5** by the strain LS11. For this purpose, we prepared two ¹³C-labeled analogues of **1** through administering sodium [^{1-¹³C}]- and [^{1,2-¹³C}]-acetates, separately, to resting cell cultures of *P. expansum* FS-7. We incubated the strain LS11 with these isotopically labeled analogues of **1** or with unlabeled **1** in the presence of either H₂O, ²H₂O, or H₂¹⁸O to follow the degradation of the mycotoxin (**1**) by the yeast into DPA (**5**). Furthermore, to gain a better understanding of the toxicity of both patulin (**1**) and DPA (**5**), we evaluated and compared the chromosomal damage and the cytotoxic effects induced by the two compounds in cultured human lymphocytes by using the cytokinesis-block micronucleus cytome (CBMN cyt) assay.³⁹

RESULTS AND DISCUSSION

Patulin (**1**) was labeled with ¹³C by pulse feeding sodium [^{1-¹³C}]- and [^{1,2-¹³C}]-acetates, separately, to two series of

resting cell cultures of *P. expansum* FS-7. A time-course study was carried out to determine the optimal time for feeding the isotopically labeled acetate to enable maximum production of highly labeled metabolite. As a result of this time-course study for the production of patulin (**1**), four-day-old resting cell cultures of *P. expansum* were fed with the above-mentioned ¹³C-labeled sodium acetates. Extraction and purification of the fermentation broths led to the isolation of two ¹³C-labeled analogues of patulin (**1**), which were analyzed by means of ¹³C NMR spectroscopy.

When *P. expansum* was fed with sodium [^{1-¹³C}]-acetate, significant enrichments with ¹³C were observed at C-2, C-3a, and C-7 of patulin (**1**), while the feeding experiment with sodium [^{1,2-¹³C}]-acetate led to enhanced signals for C-2, C-3, and C-6 and enhanced and spin–spin coupled signals between C-3a and C-4 and between C-7 and C-7a (Figure 1, Table 1),

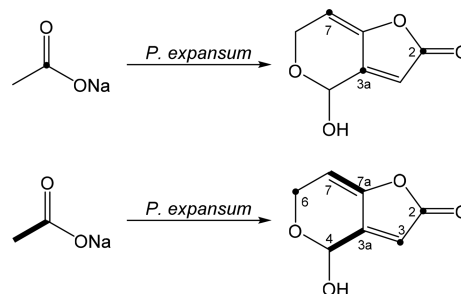


Figure 1. Labeling pattern resulting in patulin (**1**) after feeding *Penicillium expansum* FS-7 with sodium [^{1-¹³C}]- and [^{1,2-¹³C}]-acetates.

indicating that these positions resulted from the intact incorporation of two acetates. These findings are in agreement with the ¹³C incorporation detected in patulin (**1**) by HREIMS after administration of sodium [^{1-¹³C}]-acetate to *P. expansum* that we previously reported, where up to three ¹³C labels were incorporated in 0.3% of the molecules.³⁴

The ¹³C-labeled analogues of patulin (**1**) obtained in the above-mentioned experiments were subsequently administered to *R. kratochvilovae* LS11 cultures in order to follow the fate of the isotopic label during the metabolism of the compounds into DPA (**5**). The resulting labeled DPAs (**5**) were extracted after 3 days at acidic pH, purified, and analyzed by ¹³C NMR spectroscopy. Feeding experiment with the ¹³C-labeled

Table 1. Isotopic Enrichment of Carbons and Coupling Constants in Patulin (1) after Feeding *Penicillium expansum* FS-7 with Sodium [1-¹³C]- or [1,2-¹³C₂]-Acetates

carbon atom (N)	δ_C (CD ₃ OD)	atom % ¹³ C excess ^a	
		[1- ¹³ C]-acetate	[1,2- ¹³ C ₂]-acetate
2	170.8	3.23	enriched
3	111.0	−0.22	enriched
3a	153.5	3.36	56.8
4	89.9	0.22	56.8
6	60.3	0.00	enriched
7	109.6	3.36	84.9
7a	147.9	−0.07	84.9

^aAtom % ¹³C excess = $\{(R_N/R_{N(UL)} \times 1.1) - 1.1\}$, where R_N is the ratio of the peak intensity at N-position in labeled compound calculated on the basis of the peak intensity at the C-6-position. Similarly, $R_{N(UL)}$ is the ratio of the peak intensity at N-position in unlabeled compound. Value 1.1 is the theoretical ¹³C natural abundance (atom %).

analogue of patulin (1) from sodium [1-¹³C]-acetate led to DPA (5) with enhanced signals at carbons C-3, C-5, and C-8. When strain LS11 was fed with labeled patulin (1) deriving from sodium [1,2-¹³C₂]-acetate, the ¹³C NMR spectrum of DPA (5) showed the incorporation of two intact C₂ units for the carbon pairs C-3/C-4 and C-5/C-6. In addition, ¹³C enrichments at positions C-2, C-7, and C-8 were observed (Figure 2, Table 2). This pattern of observed couplings and

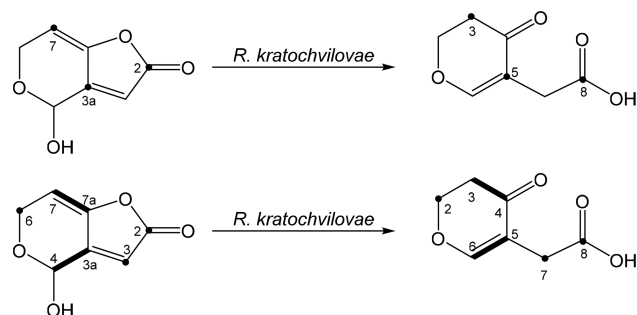


Figure 2. Labeling pattern resulting in DPA (5) after feeding *Rhodotorula kratochvilovae* LS11 with molecules of patulin (1) deriving from cultures of *Penicillium expansum* FS-7 fed with sodium [1-¹³C]- or [1,2-¹³C₂]-acetate.

enrichments suggests that DPA (5) derives from the metabolism of patulin (1) by the yeast through the biodegradation pathway shown in Scheme 1.

In order to explore the hydrolysis of the patulin (1) lactone ring, an experiment was conducted in which the ¹³C-labeled mycotoxin (1) from sodium [1-¹³C]-acetate was biodegraded by *R. kratochvilovae* LS11 in a culture medium containing 25% (v/v) of H₂¹⁸O. ¹⁸O does not possess a magnetic moment and therefore is not detectable by NMR. However, it can be indirectly detected by ¹³C NMR spectroscopy through the isotope-induced shift in the carbon atom directly attached to ¹⁸O.⁴⁰ The low levels of incorporation of isotopically labeled water achievable in biosynthetic studies can be difficult to observe. To enhance this, it is standard practice to carry out the labeling study on material that is ¹³C enriched in the position to be oxygenated in order to make the indirect detection of ¹⁸O more sensitive. In this experiment, labeling of DPA (5) was achieved by simultaneously incorporating ¹³C

Table 2. Isotopic Enrichment of Carbons and Coupling Constants in DPA (5) after Feeding *Rhodotorula kratochvilovae* LS11 with Molecules of Patulin (1) Deriving from Cultures of *Penicillium expansum* FS-7 Fed with Sodium [1-¹³C]- or [1,2-¹³C₂]-Acetate

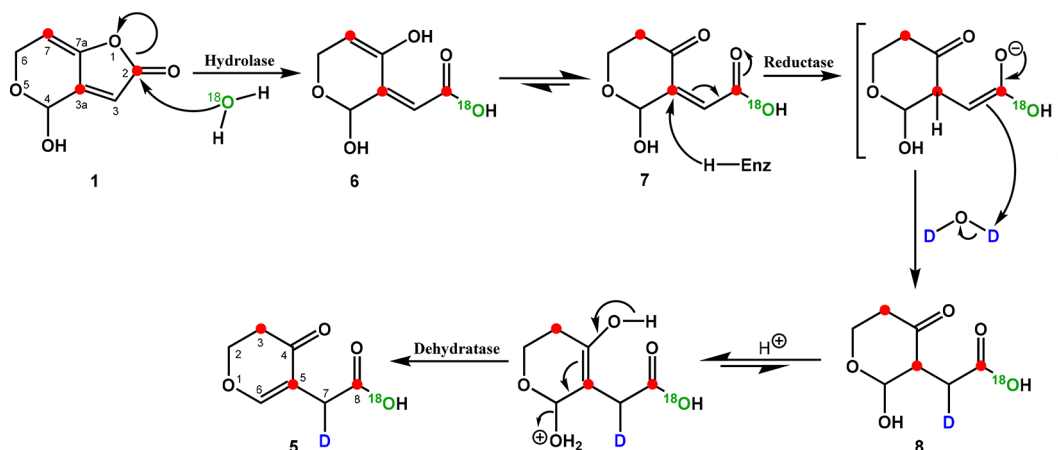
carbon atom (N)	δ_C (CD ₃ OD)	atom % ¹³ C excess ^a	
		[1- ¹³ C]-acetate	[1,2- ¹³ C ₂]-acetate
2	69.5	0	enriched
3	36.9	1.51	40.0
4	193.8	−0.61	40.0
5	114.4	5.12	71.4
6	164.4	−0.68	71.4
7	32.1	−0.49	enriched
8	176.2	4.46	enriched

^aAtom % ¹³C excess = $\{(R_N/R_{N(UL)} \times 1.1) - 1.1\}$, where R_N is the ratio of the peak intensity at N-position in labeled compound calculated on the basis of the peak intensity at the C-2-position. Similarly, $R_{N(UL)}$ is the ratio of the peak intensity at N-position in unlabeled compound. Value 1.1 is the theoretical ¹³C natural abundance (atom %).

from labeled patulin (1) derived from [1-¹³C]-acetate feeding, and ¹⁸O from H₂¹⁸O present in the culture medium. The ¹³C NMR spectrum of the resultant DPA (5) showed an isotopically shifted resonance 0.024 ppm for C-8, which is in the expected order of magnitude for ¹³C attached by a simple bond to ¹⁸O (Figure 3).⁴¹ This result clearly confirms the hydrolysis of the patulin (1) lactone ring by the water within the medium, leading to enol 6 outlined in Scheme 1, which is then rapidly isomerized into the more stable keto form 7.

The hydrolysis of patulin (1) could be catalyzed under slightly acidic or basic conditions. In order to investigate the role of LS11 in the hydrolysis of patulin (1), the pH of the yeast culture medium was measured at the beginning (pH 4.5) and at the end (pH 7.4) of the biodegradation experiment. Since at the beginning of the biodegradation process the culture medium had an acidic pH, patulin (1) was dissolved in water at pH 2 and stirred for 1 week at room temperature. After this time period, there was no evidence of any transformation product as detected by TLC analysis. In addition, patulin (1) was dissolved in water at pH 8 and also stirred for 1 week at room temperature, without degradation products being observed by TLC. These results, which are in agreement with those previously published on the stability of patulin (1) at acidic pH,^{42,43} suggest that the hydroxy group bound to C-8 in DPA (5) derives from medium water by the action of a hitherto unidentified hydrolase (Scheme 1).

The subsequent experimental step was aimed at confirming the reduction and subsequent dehydration of the hydrolysis product (7) of patulin (1) to yield DPA (5). For this purpose, an experiment of patulin (1) biodegradation by *R. kratochvilovae* LS11 was carried out in a culture medium containing 25% (v/v) of ²H₂O. The ²H NMR spectrum of the resultant DPA (5) showed only one signal at δ 3.00 ppm, corresponding to D-7, as a consequence of the reduction of the double bond C-5/C-7 by the conjugate addition of a hydride to the α,β -unsaturated acid 7 and subsequent dehydration of the resultant intermediate 8, as shown in Scheme 1. Recent studies have shown that 1 produces a marked increase, compared to the control, of the short-chain dehydrogenase–reductase protein, as well as the level of expression of the

Scheme 1. Degradation Pathway of Patulin (1) to DPA (5) by *Rhodotorula kratochvilovae* LS11^a

^aLabeling pattern resulting from experiments of LS11 incubation with H₂¹⁸O, ²H₂O, and ¹³C-labeled patulin (1) deriving from sodium [1-¹³C]-acetate.

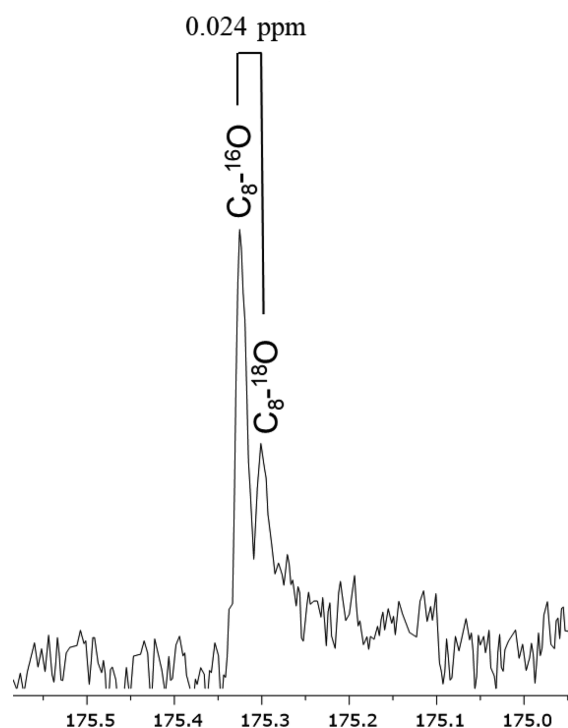


Figure 3. Region of the ¹³C NMR spectrum of DPA (5) showing the ¹⁸O isotope-induced shift in the signal for C-8 after incubation of *Rhodotorula kratochvilovae* LS11 in a culture medium containing H₂¹⁸O (25% v/v) and ¹³C-labeled patulin (1), deriving from sodium [1-¹³C]-acetate.

corresponding gene in *R. mucilaginosa*.⁴⁴ This finding reinforces the reduction step proposed in the detoxification pathway of patulin (1).

Taken together, all these results are consistent with the patulin (1) biodegradation pathway proposed in Scheme 1, according to which the $\alpha,\beta,\gamma,\delta$ -unsaturated γ -lactone ring of the mycotoxin 1 is hydrolyzed by the action of a hitherto unidentified hydrolase to afford the intermediate 6, which is rapidly isomerized to 7, probably without the need for any enzyme. Subsequent enzymatic reduction of the double bond C-5/C-7, through Michael-type reaction, and dehydration of

the resultant intermediate 7 by the action of an unidentified dehydratase lead to DPA (5).

Previously, we have reported that DPA (5) was much less cytotoxic than patulin (1) to *in vitro* human lymphocytes, probably as a consequence of the loss of the hemiacetal group and the hydrolysis of the lactone ring.³⁴ Patulin (1), unlike DPA (5), reacts with the thiol group present in the antioxidant tripeptide glutathione (GSH) and in enzymes, thus blocking their biological activity.^{34,45} Since patulin (1) is toxic to yeasts, they need to detoxify it in order to thrive. It has been observed that in *Saccharomyces cerevisiae* 12786 and *Sporobolomyces* sp. IAM 13481, the patulin (1) biodegradation process occurred in two steps, where the first involved an inhibition of growth and conditioning of cells to the toxic environment, and the second involved resumed microbial growth and successive biotransformation of patulin (1).^{46,47} As observed with the above-mentioned yeast, *R. kratochvilovae* needs to detoxify patulin (1). Therefore, it can be stated that the pathway depicted in Scheme 1 shows the process of detoxification of patulin (1) to DPA (5) by *R. kratochvilovae*.

In order to have a better understanding of the toxic effects of patulin (1) and DPA (5), the CBMN cyt assay was performed on *in vitro* human lymphocytes. This assay is a comprehensive system for measuring DNA damage, cytostasis, and cytotoxicity. DNA damage events were scored specifically in once-divided binucleated (BN) cells and include (a) MNi, a biomarker of chromosome breakage and/or whole chromosome loss, (b) NPBs, a biomarker of DNA misrepair and/or telomere end-fusions, and (c) nuclear buds (NBUDs), a biomarker of the elimination of amplified DNA and/or DNA repair complexes. Cytostatic effects were measured via the proportion of mono-, bi-, tri-, and tetranucleated cells, and cytotoxicity effects via necrotic and/or apoptotic cell ratios.³⁹

Patulin (1) and DPA (5) were obtained as described in the Experimental Section and dissolved in sterile double-distilled water for treatment of human lymphocyte cultures. Colcemid (COL) and ethyl methanesulfonate (EMS) served as the standard positive controls. All lymphocyte cultures were treated for 24 h with these compounds.

As shown in Table 3, patulin (1) at the highest concentration tested (10 μ M) induced a high percentage of necrotic cells (more than 20%), while at the dose of 5 μ M significantly decreased the nuclear division index (NDI)

Table 3. Mean (\pm SD) Frequencies of MNi, NPBs, and BUDs in BN Cells and NDI in Human Lymphocytes Treated *in Vitro* with Patulin (1) or DPA (5)

treatment (μ M)		MNi/1000 BN cells	NPBs/1000 BN cells	BUDs/1000 BN cells	NDI
patulin (1)	0.1	5.3 \pm 1.00	1.3 \pm 0.58	1.0 \pm 1.00	1.7 \pm 0.03
	0.5	12.3 \pm 2.08 ^{a,b}	1.3 \pm 0.58	4.7 \pm 1.53 ^a	1.7 \pm 0.04
	1.0	14.7 \pm 2.08 ^{a,b}	1.7 \pm 0.58	6.7 \pm 2.08 ^{a,b}	1.6 \pm 0.03
	5.0	toxic	toxic	toxic	toxic
	10.0	toxic	toxic	toxic	toxic
DPA (5)	0.1	5.0 \pm 1.00	1.0 \pm 1.00	1.3 \pm 0.58	1.8 \pm 0.03
	0.5	6.3 \pm 1.53	1.0 \pm 1.00	2.0 \pm 1.00	1.7 \pm 0.03
	1.0	7.3 \pm 1.15	1.0 \pm 1.00	2.3 \pm 1.15	1.6 \pm 0.03
	5.0	9.0 \pm 1.00 ^a	1.3 \pm 0.58	4.7 \pm 1.52 ^a	1.6 \pm 0.03
	10.0	9.7 \pm 1.15 ^a	1.7 \pm 0.58	6.3 \pm 1.52 ^a	1.6 \pm 0.04
solvent control (double-distilled water)		5.3 \pm 1.55	1.3 \pm 0.58	1.3 \pm 0.58	1.8 \pm 0.08
EMS (240 μ g/mL)		13.3 \pm 1.53 ^a	8.7 \pm 1.52	7.3 \pm 2.52	1.6 \pm 0.04
COL (0.02 μ g/mL)		20.3 \pm 2.08 ^a	1.7 \pm 0.58	2.3 \pm 1.15	1.7 \pm 0.03

^aStatistically significant as compared to the solvent control ($P < 0.05$). ^bStatistically significant as compared to DPA treatment ($P < 0.05$).

(–35%) with respect to the solvent control (sterile double-distilled water). These cytotoxic effects prevented a sufficient recovery of BN cells to allow chromosomal damage analysis. Patulin (1) also induced a significant increase of MNi frequency; the maximum increase observed at 1 μ M was about 3-fold higher than the corresponding control value. The increase of MNi was concentration dependent. No significant increase in the frequency of NPBs with respect to the solvent control was recorded in the range of 0.10–1 μ M concentrations of patulin (1), whereas the frequencies of BUDs were significantly higher at doses of 0.5 and 1.0 μ M. These results were consistent with previous observations made in both human and mammalian cells^{9–12} and confirm the ability of patulin (1) to cause chromosomal damage and to interfere with DNA. As expected, we recorded a significant increase in MNi frequency with respect to the solvent control in the treatment with COL. Moreover, the treatment with EMS induced a significant increase in the frequencies of all three DNA damage events as compared to those observed in the solvent control.

Interestingly, DPA (5) did not induce any cytotoxic effect in the concentration range of 0.1–10 μ M; thus analysis of the three DNA damage events could be performed in the lymphocyte cultures. The treatment with DPA (5) exhibited significant differences as compared to the treatment with patulin (1) for MNi and BUDs. No elevated level of either MNi and BUDs was observed in lymphocytes treated with 0.5–1.0 μ M 5, and the frequencies recorded were significantly lower than those induced by patulin (1) in the same concentration range. Only the highest concentrations of 5 (5 and 10 μ M) induced significant differences as compared to the solvent control for MNi and BUDs. On the other hand, treatment with DPA (5) at any concentration did not increase the frequency of NPBs.

The α,β -unsaturated carbonyl group is commonly recognized as an alert for mutagenicity due to its reactivity with cellular nucleophilic amines and thiols.⁴⁸ Patulin (1), a $\alpha,\beta,\gamma,\delta$ -unsaturated γ -lactone, could display its cytotoxic and chromosome-damaging effects by forming covalent adducts with these types of compounds, since small lactones are known to act as alkylating agents.⁴⁹ It has been suggested that 1 can interact irreversibly with nucleophilic groups of DNA, leading to chromosomal damage and cell cycle disturbance.¹⁴ Recently, Pfenning et al.⁵⁰ reported that patulin (1) reacts with

nucleophilic centers of DNA bases, both in the absence and in the presence of GSH, to yield several mono- and disubstituted DNA base adducts as well as mixed patulin–GSH–nucleobase adducts. The initial step for obtaining these diadducts is a Michael-like addition of the thiol group of GSH or the amine group of the DNA bases to the δ position of the unsaturated lactone moiety of patulin (1) to yield a reactive enol-lactone, which can add nucleophiles of DNA bases to the carbonyl carbon of the mycotoxin (1). Formation of patulin–nucleobase diadducts could contribute to the increase in MNi and BUD observed in cells treated with patulin (1). On the other hand, formation of patulin–GSH and patulin–GSH–nucleobase di- or triadducts could cause indirect DNA damage by depletion of the intracellular pool of GSH and antioxidant enzymes (superoxide dismutase and catalase)⁵¹ and subsequent increase of ROS^{16,52} and of lipid peroxidation products.⁵³

At a molecular level, DPA (5) is a carboxylic acid whose structure contains an α,β -unsaturated ketone, and the addition of nucleophiles to C-6 of 5 through a Michael-type addition reaction could be expected. However, it was shown that DPA (5) does not react with GSH.³⁴ In addition, it has also been reported that the adducts resulting from the reactions of patulin (1) with 4-bromothiophenol, *N*-acetylcysteine, or GSH, whose structures are similar to 5, did not undergo further addition of sulfur-containing nucleophiles.^{45,54} The lack of reactivity of DPA (5) could be due to the mesomeric effect + M produced by ring oxygen on the C-6 of 5, which renders this carbon, in contrast to C-7 of patulin (1), insufficiently electrophilic for Michael-type additions by nucleobases or cellular thiols. The electrophilic structure of DPA (5) could therefore explain the lower genotoxicity of this patulin (1) degradation and detoxification product.

In conclusion, the isotopic labeling experiments reported here have enabled us to propose a detoxification pathway of patulin (1) to DPA (5) by the yeast *R. kratochvilovae* LS11, which involves the hydrolysis of the γ -lactone ring of 1 and subsequent reduction of the double bond C-5/C-7 and dehydration of the resultant intermediate 7. It has also been shown that DPA (5) causes much less chromosomal damage than patulin (1) on human lymphocytes *in vitro*, possibly due, as indicated above, to its lack of reactivity with cellular nucleophilic amines and thiols as a consequence of the deactivation of its carbon C-6 by the oxygen ring.

The biodegradation pathway elucidated sets the foundation for identifying the microbial genes involved in the various enzymatic steps. As a result, new strategies for monitoring or reducing the levels of patulin (1) in food can be developed.

EXPERIMENTAL SECTION

General Experimental Procedures. ^1H and ^{13}C NMR spectra were recorded on Agilent 400 and 600 MHz spectrometers using CD_3OH as solvent (Merck, Darmstadt, Germany). TLC was performed on Merck Kiesegel 60 F_{254} , 0.25 mm thick. Silica gel 60PF $_{254}$ (60–100 mesh, Merck) was used for column chromatography.

Reagents and Labeled Precursors. All reagents were purchased from Sigma-Aldrich (Milan, Italy). Sodium [$1\text{-}^{13}\text{C}$]-acetate (isotopic purity 99 atom % ^{13}C), sodium [$1,2\text{-}^{13}\text{C}_2$]-acetate (99 atom % ^{13}C), and H_2^{18}O (97 atom % ^{18}O) were obtained from Euriso-Top (Saint Aubin, France). D_2O (99.9 atom % D) was obtained from Merck (Darmstadt, Germany). Both isotope-labeled sodium acetates were dissolved in water and sterilized through 0.22 μm Millex GP (Millipore) filters before being added to the flasks containing the culture medium for microbial growth. H_2^{18}O and D_2O were filter-sterilized prior to use.

Microorganisms. All microbial strains used in this study were obtained from the culture collection of the Dipartimento Agricoltura, Ambiente, Alimenti, Università del Molise, Italy. Conidial stock suspensions of *Penicillium expansum* FS-7 were maintained viable in 80% glycerol at -40°C . Cells of the yeast *Rhodotorula kratochvilovae* LS11, originally isolated from an olive tree, were preserved on modified PDA medium (200 g of potatoes, 20 g of D-glucose, and 20 g of agar per liter of distilled water) at 4°C .

Preparation of Labeled Patulin. *P. expansum* strain FS-7 was cultivated at 25°C and 150 rpm in Erlenmeyer flasks (500 mL) containing 300 mL of PDB medium (200 g of potatoes and 20 g of D-glucose per liter of distilled water) diluted with H_2O to a final ratio of 1:10. Each flask was inoculated with fresh conidia to a final concentration of 4×10^6 conidia/mL. After 4 days of incubation, the mycelia were transferred into the same number of Erlenmeyer flasks (500 mL), each containing 300 mL of PDB medium diluted as above and a filter-sterilized aqueous solution of the labeled precursor. Three days after administration of the precursor, the culture medium and mycelia were separated by filtration. The broth was saturated with NaCl and extracted with ethyl acetate ($\times 3$). The organic extract was washed with H_2O ($\times 3$) and dried over anhydrous Na_2SO_4 , and the solvent evaporated under reduced pressure.³⁴ The residue was purified by column chromatography on Si gel with an increasing gradient of ethyl acetate in *n*-hexane as eluent. Fractions containing patulin (1) were detected by TLC, collected, and analyzed by ^1H NMR. The signals observed in the ^1H NMR spectrum were coincident with those shown by the commercial sample.

Labeled Patuline (1) from Sodium [$1\text{-}^{13}\text{C}$]-Acetate. In accordance with the above procedure, four flasks inoculated with *P. expansum* FS-7 were fed with a filter-sterilized solution of sodium [$1\text{-}^{13}\text{C}$]-acetate in H_2O to a final concentration of 300 ppm. Extraction of the broth yielded a crude extract (120.2 mg), which was purified as described in the general method to afford 55.0 mg of labeled patulin (1). The isotope incorporations are shown in Table 1.

Labeled Patulin (1) from Sodium [$1,2\text{-}^{13}\text{C}_2$]-Acetate. A filter-sterilized aqueous solution of sodium [$1,2\text{-}^{13}\text{C}_2$]-acetate was added to one flask inoculated with *P. expansum* to a final concentration of 233 ppm. Extraction of the broth yielded a crude extract (35.9 mg), which was purified as described above to yield 20.7 mg of labeled patulin (1). The coupling constants are shown in Table 1.

Biodegradation of Patulin (1) by *R. kratochvilovae* LS11. A suspension of *R. kratochvilovae* LS11 cells (1.0×10^5 CFU/mL) obtained from a 2–3-day-old culture on PDA was added to a 500 mL Erlenmeyer flask containing 200 mL of Lilly-Barnett medium (10.0 g of D-glucose, 2.0 g of L-asparagine, 1.0 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 8.7 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.0 mg of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mg of biotin, and 0.1 mg of thiamine per liter of

medium) supplemented with 150 ppm of patulin (1). The yeast was incubated at 25°C and 150 rpm. The biodegradation was monitored on a daily basis by TLC, using toluene–ethyl acetate–formic acid 5:4:1 (v/v/v) as the eluent system. After complete biodegradation of patulin (1), the culture medium and the yeast cells were separated by centrifugation for 20 min at 6000 rpm. The broth was acidified to pH 2 with an aqueous solution of 2 M HCl, saturated with NaCl, and extracted with ethyl acetate ($\times 3$). The EtOAc extract was washed with H_2O ($\times 3$) and dried over anhydrous Na_2SO_4 , and the solvent evaporated under reduced pressure. The residue was purified by chromatography on a Si gel column, using toluene–ethyl acetate–formic acid, 5:4:1 (v/v/v), as the solvent system. Fractions containing DPA (5) were detected by TLC, collected, and analyzed by ^1H NMR. The spectrum achieved was identical to that shown by a real sample.

Biodegradation of Labeled Patulin (1) Deriving from Sodium [$1\text{-}^{13}\text{C}$]-Acetate. *R. kratochvilovae* LS11 was grown for 4 days in the presence of labeled patulin (1) from the feeding of *P. expansum* with sodium [$1\text{-}^{13}\text{C}$]-acetate in accordance with the above procedure. Extraction of the broth yielded a crude extract (26.6 mg), which was purified as described above to afford 18.0 mg of labeled DPA (5). The relative incorporations are shown in Table 2.

Biodegradation of Labeled Patulin (1) Deriving from Sodium [$1,2\text{-}^{13}\text{C}_2$]-Acetate. The yeast was grown for 3 days in the presence of labeled patulin (1) from the feeding of *P. expansum* with sodium [$1,2\text{-}^{13}\text{C}_2$]-acetate (see above). Extraction of the broth yielded a crude extract (26.1 mg), which was purified as above to obtain 10.2 mg of labeled DPA (5). The coupling constants are reported in Table 2.

Biodegradation of Patulin (1) in H_2^{18}O . A 50 mL Erlenmeyer flask, containing 20 mL of Lilly-Barnett medium in 25% of H_2^{18}O and supplemented with 150 ppm of labeled patulin (1) obtained from the feeding of *P. expansum* with sodium [$1\text{-}^{13}\text{C}$]-acetate, was inoculated with a suspension of *R. kratochvilovae* LS11 cells (1.0×10^5 CFU/mL). The yeast was incubated as described above for 6 days. Extraction of the broth yielded a crude extract (3.4 mg), which was purified as above to afford 1.2 mg of labeled DPA (5).

Biodegradation of Patulin (1) in $^2\text{H}_2\text{O}$. A suspension of *R. kratochvilovae* LS11 cells (1.0×10^5 CFU/mL) was added to an Erlenmeyer flask (100 mL) containing 40 mL of Lilly-Barnett medium in 25% of D_2O supplemented with 150 ppm of unlabeled patulin (1). The yeast was incubated as described above for 5 days. Extraction of the broth yielded a crude extract (6.1 mg), which was purified as above to afford 3.2 mg of labeled DPA (5).

Isolation of Unlabeled Patulin (1) and DPA (5). *P. expansum* strain FS-7 was cultivated at 25°C and 150 rpm in six Erlenmeyer flasks (500 mL), each containing 300 mL of PDB medium diluted with H_2O to a final ratio of 1:10. Each flask was inoculated with fresh conidia to a final concentration of 4×10^6 conidia/mL. After 4 days of incubation, the culture medium and mycelia were separated by filtration. The broth was extracted and purified using the methodology described above for the isolation of labeled patulin (1), which provided 128.0 mg of 1.

Four Erlenmeyer flasks (500 mL), each containing 200 mL of Lilly-Barnett medium supplemented with 150 ppm of 1, were inoculated with a suspension of *R. kratochvilovae* LS11 (1.0×10^5 CFU/mL) cells from a 2–3-day-old culture on PDA. The yeast was incubated for 4 days at 25°C and 150 rpm. At this time point, the culture medium and the yeast cells were separated by centrifugation. The medium was extracted and purified in accordance with the methodology described above for the isolation of labeled DPA (5), which yielded 34.0 mg of 5.

Block Micronucleus Cytome Assay. The assay was set up using blood samples from three healthy, nonsmoking adult males who were less than 40 years old, as provided by AVIS (Italian Association of Voluntary Blood Donors). Lymphocytes were separated from whole blood using a density gradient (Histopaque 1077), washed twice in RPMI 1640 medium, and then cultured at a concentration of 2×10^6 cells in 5 mL of RPMI 1640 medium (4×10^5 /mL) supplemented with 15% fetal calf serum (FCS) (v/v), 0.5% phytohemagglutinin (v/v), 1% penicillin–streptomycin solution (v/v), and 1 mM L-glutamine

solution. The cultures were incubated at 37 °C in a humid atmosphere in the presence of 5% CO₂.⁵⁵

After 44 h of incubation, all cultures were supplemented with cytochalasin-B (Cyt B) at a final concentration of 6 µg/mL. After 48 h, lymphocyte cultures were treated with an aqueous solution of patulin (1) or DPA (5) in sterile double-distilled water to obtain final concentrations of 0.10, 0.50, 1, 5, and 10 µM. Sterile double-distilled water was used as the solvent control, whereas positive controls were EMS (240 µg/mL), an alkylating agent, and COL (0.02 µg/mL), a microtubule-disrupting tubulin-binding agent.

At the end of the 72 h incubation period, the lymphocytes were collected and treated with a mild hypotonic treatment (RPMI 1640 medium–H₂O, 1:2, supplemented with 2% FCS) for 2 min and then fixed in ice-cold methanol–acetic acid (1:1). The slides were prepared by cytocentrifugation, air-dried, and stained with May Grunwald-Giemsa.⁵⁶ All were coded prior to scoring. Scoring was done by using a Zeiss Axioplan light microscope at 1000× magnification under oil immersion. MNi, NPBs, and BUDs were scored according to the criteria described by Fenech et al.⁵⁷ by examining at least 2000 BN cells for each concentration of the compounds under assessment (1000 for each culture). The frequencies of MNi, NPBs, or BUDs in 1000 BN cells were then calculated for each treatment. Cell-cycle parameters were evaluated by classifying 1000 cells according to the number of nuclei. The nuclear division index was calculated according to eq 1, where M1 through M4 indicate the number of cells with 1–4 nuclei and N indicates the total number of scored cells.⁵⁶ Necrotic and apoptotic cells were scored according to the guidelines reported by Fenech et al.⁵⁷ So, 1000 cells were counted and scored as either mononucleated, binucleated, trinucleated, tetranucleated, apoptotic, or necrotic, and percentages of the different types of cells were calculated. Assays with high % of necrotic or apoptotic or few BN cells were classified as “toxic”.

$$\text{NDI} = \frac{\text{M1} + 2\text{M2} + 3\text{M3} + 4\text{M4}}{\text{N}} \quad (1)$$

The experiments were performed in triplicate, and each concentration was tested in two parallel cultures. Data from the three experiments were pooled.

Statistical Analysis. All values from toxicity experiments are means ± SD. Student's *t*-test was used for comparing the mean of a specific treatment with a control mean. *P* ≤ 0.05 was considered as statistically significant.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00539.

¹H and ¹³C NMR spectra for patulin (1) and ¹H, ¹³C, and ²H NMR spectra for DPA (5) (PDF)

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Notes

The authors declare no competing financial interest.

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