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Conversion of the Mycotoxin Patulin to the Less Toxic Desoxypatulinic Acid by the Biocontrol Yeast *Rhodosporidium kratochvilovae* Strain LS11

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Supporting Information

ABSTRACT: The infection of stored apples by the fungus *Penicillium expansum* causes the contamination of fruits and fruit-derived products with the mycotoxin patulin, which is a major issue in food safety. Fungal attack can be prevented by beneficial microorganisms, so-called biocontrol agents. Previous time-course thin layer chromatography analyses showed that the aerobic incubation of patulin with the biocontrol yeast *Rhodosporidium kratochvilovae* strain LS11 leads to the disappearance of the mycotoxin spot and the parallel emergence of two new spots, one of which disappears over time. In this work, we analyzed the biodegradation of patulin effected by LS11 through HPLC. The more stable of the two compounds was purified and characterized by nuclear magnetic resonance as desoxypatulinic acid, whose formation was also quantitated in patulin degradation experiments. After *R. kratochvilovae* LS11 had been incubated in the presence of ^{13}C-labeled patulin, label was traced to desoxypatulinic acid, thus proving that this compound derives from the metabolization of patulin by the yeast. Desoxypatulinic acid was much less toxic than patulin to human lymphocytes and, in contrast to patulin, did not react in vitro with the thiol-bearing tripeptide glutathione. The lower toxicity of desoxypatulinic acid is proposed to be a consequence of the hydrolysis of the lactone ring and the loss of functional groups that react with thiol groups. The formation of desoxypatulinic acid from patulin represents a novel biodegradation pathway that is also a detoxification process.

KEYWORDS: patulin, metabolization, desoxypatulinic acid, detoxification, biocontrol yeast

INTRODUCTION

Patulin is a mycotoxin that is synthesized by a number of fungi. It is a polyketide secondary metabolite, which represents a serious health hazard and is a major issue for food safety.[1] Its major producer, the fungus *Penicillium expansum* Link., is an important postharvest pathogen of apples and pears and causes the accumulation of patulin in infected pome fruits. This leads to the contamination with patulin in pome fruit-derived juices and baby foods, consumed by children, who have a low tolerance level to patulin.[2] Patulin has diverse toxic effects such as immunosuppression, embryo and maternal toxicity in mice, and genotoxicity to cultured human cells.[3−5] The mechanism of patulin toxicity has been proposed to be due to its reactivity with thiol-bearing molecules such as the important cellular antioxidant glutathione.[6,7] Acute symptoms caused by patulin in humans are gastritis and nausea.[2] Therefore, many countries, including the U.S. and those of the European Union, have established maximum tolerable levels of patulin contamination for fruit-derived products.[8,9]

The most effective way of avoiding mycotoxin contamination is to stop the fruit from being attacked by mycotoxin-producing fungi, through the appropriate handling of fruit and the treatment with fungicides.[2] Fungicide treatments cannot completely prevent infection by *P. expansum*. Furthermore, selecting healthy fruits on an industrial scale does not ensure the absence of patulin in the final product.[10] Detoxification of mycotoxins (i.e., the conversion or degradation of mycotoxins to less toxic compounds) in food for human consumption has recently gained interest, with particular focus on mycotoxins present in beverages that derive from crops that are susceptible to the attack of mycotoxigenic pathogens.[11,12]

Over the past two decades, research interest on preventive strategies such as the biocontrol of postharvest pathogenic fungi by utilizing harmless bacteria and yeasts has steadily increased, as has public concern regarding fungicide residues in food. Some biocontrol agents have been developed for commercial use.[13,14]

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The mechanisms of action of biocontrol agents have been examined, with the final goal to understand how biocontrol agents operate and to improve their efficacy.[13,15−17] Preharvest application of biocontrol agents for control of postharvest diseases has been proposed and tested.[18] Interestingly, some biocontrol agents appear to have both the ability to prevent fungal infections on fruit and the potential for degrading the mycotoxins that are produced by these same fungi. Strains of *Aureobasidium pullulans* effectively protected wine-grapes from infection by *Aspergillus carbonarius* in the vineyard and prevented their subsequent contamination with ochratoxin A. They were also able to degrade the mycotoxin to the less toxic compound ochratoxin α in synthetic medium and fresh grape must.[19] Another biocontrol yeast, strain LS11 of the basidiomycete *Rhodosporidium kratochvilovae* [a strain that was formerly designated *Rhodotorula glutinis*, but was renamed *R. kratochvilovae* following the analysis of the sequence data of the internal transcribed spacer (ITS) region (unpublished data)], can prevent apples from the attack of postharvest rot fungi[20] and lower patulin contamination in stored fruit.[21] Less patulin accumulated in apples that had been pretreated with the yeast and infected by *P. expansum* than in non-yeast-treated, infected fruits.[21] Furthermore, strain LS11 can resist very high concentrations of patulin (500 μg/mL) and is able to metabolize this mycotoxin under aerobic conditions, leading to the formation of two products with R_f (retention factor) 0.46 and R_f 0.38, as detected by TLC.[21] The compound(s) with R_f 0.46 appears to be more stable and is most likely the major final product(s) of the degradation process, because it is present at the end of the degradation process, when the other product is no longer detectable (unpublished data). Preliminary observations indicated that the two biodegradation products were less toxic than patulin to *Escherichia coli* (unpublished data). However, their toxicity to eukaryotic cells, such as human cells, is unknown.

Metabolization of patulin to other unknown compounds has been reported previously. Several research groups also observed that strains of *Saccharomyces cerevisiae* were able to cause the disappearance of patulin from fermented apple juice over time.[22,23] In recent years, the degradation products have been identified in the case of the biodegradation carried out by the fermentative yeast *Saccharomyces cerevisiae* in anaerobic conditions.[12] The final products are two different isomers of ascladiol, *E*-ascladiol and *Z*-ascladiol. *E*-Ascladiol is the predominant degradation compound. Not much data exist on the toxicity of ascladiol. It is allegedly less toxic than patulin but still retains a quarter of the toxicity of patulin, and can be considered a mycotoxin in itself.[24] The results of TLC analyses suggest that the major product of aerobic patulin degradation by the biocontrol yeast *R. kratochvilovae* LS11 has an R_f value of 0.46, which is distinct from that of ascladiol.[21,25] Therefore, a novel pathway could be responsible for the metabolization of patulin by *R. kratochvilovae* LS11.

In this work, we analyzed by HPLC the metabolization of patulin operated by *R. kratochvilovae* LS11, characterized by NMR the major metabolite produced, and analyzed by HREIMS isotope incorporation into this compound following incubation of strain LS11 with ^{13}C-labeled patulin, to provide unequivocal evidence that the produced metabolite derives from the biodegradation of patulin by the yeast. Furthermore, we confirmed that a detoxification had occurred by comparing the toxicity of the new compound to that of patulin in a bioassay based on cultured human lymphocytes.

MATERIALS AND METHODS

Materials. A commercial standard of patulin was purchased from Sigma-Aldrich (Milan, Italy). Immediately prior to use, weighed aliquots of the mycotoxin were dissolved in water that had been acidified with acetic acid (pH 4). All solvents employed were double-distilled or HPLC grade. All solvents and reagents were obtained from Sigma−Aldrich (Milan, Italy). Sodium [1-^{13}C] acetate (isotopic purity 99 atom % ^{13}C) was obtained from the Euriso-Top Co. (Saint Aubin, France).

All microbial stains used in this study were obtained from the culture collection of the Dipartimento di S.A.V.A., Università del Molise. Cells of the yeast *R. kratochvilovae* LS11, originally isolated from an olive tree, were maintained viable in 20% glycerol at −80 °C. Cultures of *P. expansum* FS-7 were preserved on potato dextrose agar (PDA, 200 g of potatoes, 20 g of D-glucose, and 20 g of agar per liter of distilled water) on slant tubes at 4 °C.

Growth of *R. kratochvilovae* LS11 in the Presence of Unlabeled Patulin. For the analysis of patulin metabolization by *R. kratochvilovae* LS11, cells of the yeast were scraped from a Petri dish, added to 50 mL of Lilly-Barnett medium (LiBa, 10.0 g of D-glucose, 2.0 g of L-asparagine, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 mg of $FeSO_4 \cdot 7H_2O$, 8.7 mg of $ZnSO_4 \cdot 7H_2O$, 3.0 mg of $MnSO_4 \cdot H_2O$, 0.1 mg of biotin, and 0.1 mg of thiamine per liter of medium),[26] and incubated at 23 °C on a rotary shaker at 150 rpm for 24−36 h. The culture was centrifuged for 20 min at 6000 rpm, the cell concentrations were adjusted to 1.0×10^5 CFU/mL, corresponding to values of 0.01 optical density (OD) at 595 nm, and the cells were transferred to three flasks, each containing 50 mL of LiBa supplemented with 150 μg/mL patulin. Three flasks each containing 50 mL of LiBa with 150 μg/mL patulin, and not inoculated with the yeast, were used as the control. All of the flasks were incubated on a rotary shaker at 150 rpm and at 23 °C for 72 h. At 0, 24, 48, and 72 h, samples of 200 μL were withdrawn from each flask for subsequent HPLC analyses of crude samples. The same experiments were also carried out in macerated browning apple tissue, supplemented with patulin, and inoculated with *R. kratochvilovae* LS11 as previously described.[21] The only difference from the previously reported biodegradation procedure[21] was the volume of liquid used and the application of gentle shaking in the present experiments.

For NMR analysis of the major biodegradation product of patulin by *R. kratochvilovae* LS11, yeast cells were grown as described above, but after centrifugation they were transferred to a single flask containing 50 mL of LiBa with 250 μg/mL patulin. During the course of the incubation, the decrease in intensity of the spot corresponding to patulin and the increased intensity of two spots with R_f 0.46 and R_f 0.38, which appeared during incubation, were monitored by TLC. Forty microliter aliquots of culture filtrate, which had previously been extracted with ethyl acetate and concentrated,[21,25] were applied on 0.25 mm thick plates (Merck Kiesegel 60 F_{254}, Darmstadt, Germany), and chromatography was performed by using toluene−ethyl acetate−formic acid 5:4:1 (v/v/v) as the solvent system. TLC results were first observed under UV light and then visualized by spraying with a 0.5% solution of 3-methyl-2-benzothiazolinone hydrazone (Sigma-Aldrich, Milan, Italy) (w/v) and heating at 120 °C for 15 min. The biodegradation reaction was stopped after 96 h, at which time the patulin spot and the one having R_f 0.38 could no longer be detected. The culture was centrifuged, cells were discarded, and the supernatant was lyophilized and then dissolved in acidified water (pH 4) prior to purification of the major biodegradation product.

HPLC Analyses of Patulin Metabolization by *R. kratochvilovae* LS11. HPLC analyses were performed as previously described[27] with slight modifications. The HPLC apparatus was a Dionex (Sunnyvale, CA) analytical system consisting of a P680 solvent delivery system and a 20 μL injector loop (Rheodyne, Cotati). The UVD170 detector (Dionex, Sunnyvale) set at 276 nm was connected to a data integration system (Dionex Chromeleon Version 6.6). The column used

was a 250 mm × 4.6 mm i.d., 5 μm, Agilent Zorbax C18 (Agilent technologies Italia s.p.a, Milan, Italy). The mobile phase was acidified water (with 1% acetic acid v/v) and methanol 95:5 (v/v) with a flow rate of 1 mL/min and a total run of 18 min. Standard serial solutions of patulin in acidified water were injected, and peak areas were determined to generate a standard curve for quantitative analyses. Quantitation of the major metabolite of patulin metabolization was also carried out as described for patulin, following purification and characterization as desoxypatulinic acid. The quantity of desoxypatulinic acid was determined by using a calibration curve with injected amounts, ranging from 0.02 to 0.4 μg. The linear fit was: $y = 63.145x - 0.0521$ [Pearson's coefficient (R^2) = 1]. The limit of quantitation (LOQ) was 20 ng (1 μg/mL), and the limit of detection (LOD) was 6.7 ng (0.333 μg/mL), with signal/noise (S/N) ratios of 9 and 3, respectively. The experiments were performed three times, and each experiment consisted of three replicates. Data from the experiments were pooled, because they were similar in the three repetitions, and expressed as μg/mL of patulin or desoxypatulinic acid ± standard deviation.

Characterization of the Major Biodegradation Product of Patulin. Purification of the patulin degradation product was carried out using an HPLC apparatus series 200 Perkin-Elmer, equipped with a binary pump and a UV/vis 785 A detector set at 276 nm. The column used was a 250 × 4.6 mm i.d., 5 μ, Phenomenex Luna C_{18} 100 A (Torrance, CA). The mobile phase and flow were the same as for HPLC analyses. Twenty aliquots of 100 μL were injected with an autosampler (Perkin-Elmer, USA), and fractions of 500 μL each from the peak with retention time of 15.2 min were collected in the dark. Twenty fractions were pooled, and the solvent was evaporated under a N_2 stream in a preweighed glass vial. After being dried, the weight of the purified sample was determined to 2.20 mg, and it was stored at −20 °C.

Prior to NMR analysis, both this sample and a sample of patulin were dissolved in D_2O with 0.17 M deuterated acetic acid with pH adjusted to 4.0 by the addition of potassium carbonate. NMR spectra were performed at 300 K on an AVANCE AQS600 spectrometer (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany) operating at the proton frequency of 600.13 MHz. The ^{1}H spectra were referred to the residual ^{1}H signal of CHD_2COOD set at 2.08 ppm. ^{13}C spectra were referred to the ^{13}C methyl carbon of residual CHD_2COOD set at 21.4 ppm.

2D NMR experiments, that is, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC, were performed using the same experimental conditions as previously reported by Mannina et al.;[28] the delay for the evolution of long-range couplings in ^{1}H−^{13}C HMBC experiments was 80 ms.

Mass spectrum was recorded by GC−MS with a VG-Autospec instrument. Calculated mass for $C_7H_8O_4$ $[M]^+$ was 156.0423, while the one we found experimentally was 156.0415.

Isotope Incorporation Studies. Labeled patulin was produced as follows: *P. expansum* strain FS-7 was grown at 25 °C, at 150 rpm, in four Erlenmeyer flasks (500 mL) each containing 300 mL of potato dextrose broth (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 time interval that was identified as the optimum one, following a time-course study on the in vitro formation of patulin), the mycelia from the four flasks were transferred into the same number of new Erlenmeyer flasks (500 mL), each containing 300 mL of PDB medium, diluted as above. A filter-sterilized (by using 0.22 μm filters) aqueous solution of sodium [1-^{13}C] acetate was added at a final concentration of 300 μg/mL. Three days after addition of the labeled precursor, the culture medium and mycelia were separated by filtration. The broth was saturated with NaCl and extracted three times with ethyl acetate. The organic extract was washed three times with H_2O, dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (Merck, Darmstadt, Germany), by using toluene/ethyl acetate/formic acid 5:4:1 (v/v/v) as the solvent system, to afford 55 mg of labeled patulin.

A suspension of fresh cells of *R. kratochvilovae* LS11 (1.0×10^5 CFU/mL) was added to an Erlenmeyer flask (500 mL) containing 200 mL of Lilly-Barnett medium and 150 μg/mL of labeled patulin. The yeast was incubated for 4 days at 23 °C, at 150 rpm. At this time point, the culture medium and the yeast cells were separated by centrifugation for 20 min at 6000 rpm. The medium was acidified to pH 2 with an aqueous solution of HCl 2 M, saturated with NaCl, and extracted three times with ethyl acetate. The ethyl acetate extract was washed three times with H_2O, dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Purification of labeled desoxypatulinic acid was carried out as described for labeled patulin, to afford 26 mg of this pure compound. The isotopic composition of patulin and desoxypatulinic acid was calculated by GC-HREIMS on VG-Autospec spectrometer following correction for natural abundance levels, which were determined experimentally using authentic and unlabeled standard compounds.

Effect of Patulin and Desoxypatulinic Acid on Cultured Human Lymphocytes. Peripheral lymphocytes were withdrawn from two healthy nonsmoking males belonging to AVIS (Italian Association of Voluntary Blood donors) who were less than 40 years old. Immediately after withdrawal, lymphocytes were separated from whole blood using a density gradient (Histopaque 1077 Sigma-Aldrich), and cultured at a concentration of 2×10^6 cells/mL in 5 mL (i.e., 4×10^5 cells/mL) of RPMI 1640 medium (Sigma-Aldrich) supplemented with 15% fetal calf serum, 1% phytohemagglutinin, 1% penicillin−streptomycin solution (v/v), and 1 mM L-glutamine (Sigma-Aldrich). The cultures were incubated at 37 °C in a humid atmosphere in the presence of 5% CO_2 (v/v). After 48 h, lymphocyte cultures were treated with patulin or with desoxypatulinic acid (the purified major product of patulin metabolization by the yeast, which had been identified by NMR analyses). Both desoxypatulinic acid and patulin were individually dissolved in sterile double-distilled water and added to lymphocyte cultures to obtain final concentrations of 0.1, 0.5, 1, 5, 10, 50, and 100 μM. Cell concentrations were measured as a function of time by trypan blue-exclusion test. Cell aliquots withdrawn from each culture at 3 and 24 h from the beginning of the experiment were mixed with an equal volume of trypan blue solution (diluted at 0.8 mM in PBS) for 2 min at room temperature, and the cells that stained positive were counted under a light microscope. Living cells with membrane integrity do not take up trypan blue, while dead cells with damaged membranes stain blue due to the uptake of trypan blue. The percentage of live lymphocytes was determined as the ratio of the number of nontrypan blue (negative) cells (i.e., living cells) to the total number of cells, that is, the sum of trypan blue (positive) cells (i.e., dead cells) and living cells.[29,30] Results were expressed as the percentage of viable cells in treated cultures, which was compared to that of untreated control cultures. Each treatment was performed in duplicate with separate cultures of cells from two donors (i.e., four cultures were setup for each treatment). The experiment was performed twice. Only the results from one experiment are reported because the outcome was similar in both cases.

Fate of Patulin and Desoxypatulinic Acid in the Presence of Glutathione in Vitro. These experiments were performed following the method described by Pfeiffer et al.[31] with slight modifications. Patulin or desoxypatulinic acid (10 mM) was incubated with a 5-fold molar excess of GSH (50 mM) in 67 mM potassium phosphate buffer, pH 7, at 37 °C for 24 h. Controls were patulin and desoxypatulinic acid incubated in buffer under the same conditions as above in the absence of GSH. Aliquots of 100 μL were taken from the incubation mixtures at 0, 1/12 (i.e., 5 min), 1, 6, and 24 h, acidified with 10 μL of formic acid, and analyzed for the presence of patulin and desoxypatulinic acid by HPLC. Analyses were performed using a Shimadzu LC10 HPLC instrument

(Shimadzu, Kyoto, Japan) equipped with a photodiode array detector. Separation was carried out using the same column and the same initial mobile phase [acidified water (with 1% acetic acid v/v) and methanol 95:5 (v/v) with a flow rate of 1 mL/min] as those used for patulin and desoxypatulinic acid analysis in the biodegradation experiments, with the following linear gradient: 5% methanol for 20 min, 5−100% methanol in 40 min.

Statistical Analysis. Data from all of the experiments reported in this study were analyzed with the SPSS program (SPSS Inc., Chicago, IL, release 15 for Windows). Data obtained from the three experiments of patulin metabolization by *R. kratochvilovae* LS11 were similar, so they were pooled prior to statistical analysis. Likewise, the results from the three experiments of the in vitro binding of patulin and desoxypatulinic acid to glutathione were pooled because the data were similar. Results from the experiments of patulin metabolization were expressed as μg/mL of patulin or desoxypatulinic acid ± standard deviation (n = 9). Those assessing the fate of patulin and desoxypatulinic acid in the presence of glutathione were expressed as millimolar concentrations of the two compounds ± standard deviation (n = 9). The results were analyzed by Student's t test ($P < 0.001$). Results of the effect of patulin and desoxypatulinic acid on cultured human lymphocytes were expressed as percentage of viable cells ± standard deviation in treated cultures (n = 8). Percentages were compared to those of the untreated control by Student's t test (at $P < 0.05$).

RESULTS AND DISCUSSION

Analytical HPLC chromatograms demonstrating the typical fate of patulin after 0, 24, 48, and 72 h of in vitro incubation with the yeast *R. kratochvilovae* LS11 are presented in Figure 1A−D. In the presence of the yeast, the concentration of patulin progressively decreased, and only 3.7 ± 1.2 μg/mL of the mycotoxin could be detected 72 h after the beginning of the experiment. Conversely, approximately the same concentration of patulin added to the noninoculated growth medium (control) at the beginning of the experiment (150 μg/mL) was recorded at the same time point. Interestingly, in the presence of the yeast, a peak with Rt 14.70 min appeared over the first time interval (0−24 h) (Figure 1B) and progressively increased with time paralleling the decrease of patulin (Figure 1C,D). This new peak corresponds to desoxypatulinic acid, as shown by NMR (Figure 2A), and to the spot in thin layer chromatography analyses with the R_f 0.46 (data not shown).[21] The concentration of desoxypatulinic acid progressively increased and reached the value of 87.0 ± 11.7 μg/mL at 72 h. Similar results were obtained when patulin was incubated with strain LS11 in browning and macerated apple tissue (data not shown).[21] The quantitative analyses of patulin and desoxypatulinic acid show that the yeast metabolizes patulin almost completely within 72 h and confirm its ability to resist high patulin concentrations.[21] Because patulin is a broad-spectrum toxin and antifungal agent, it is potentially lethal also to *R. kratochvilovae* LS11, which may convert it to desoxypatulinic acid to protect itself against the toxin. Scott et al. reported that 46 μg/mL of this compound was not toxic to a number of bacteria and yeasts, whose growth was completely inhibited by patulin under the same conditions.[32] In fruit wounds, the basidiomycetous yeast *R. kratochvilovae* LS11 can utilize its capacity to convert patulin to desoxypatulinic acid to protect itself and the fruit from one of the weapons of the green mold fungus.

Desoxypatulinic acid was identified as the major compound deriving from patulin biodegradation in vitro by LS11 following purification and subsequent NMR analysis. The chromatographic conditions for HPLC quantitative analyses of patulin were similar to those used for the purification of desoxypatulinic acid and subsequent NMR analysis. A yield of 2.20 mg of desoxypatulinic acid was obtained and used for NMR analysis and structural characterization.

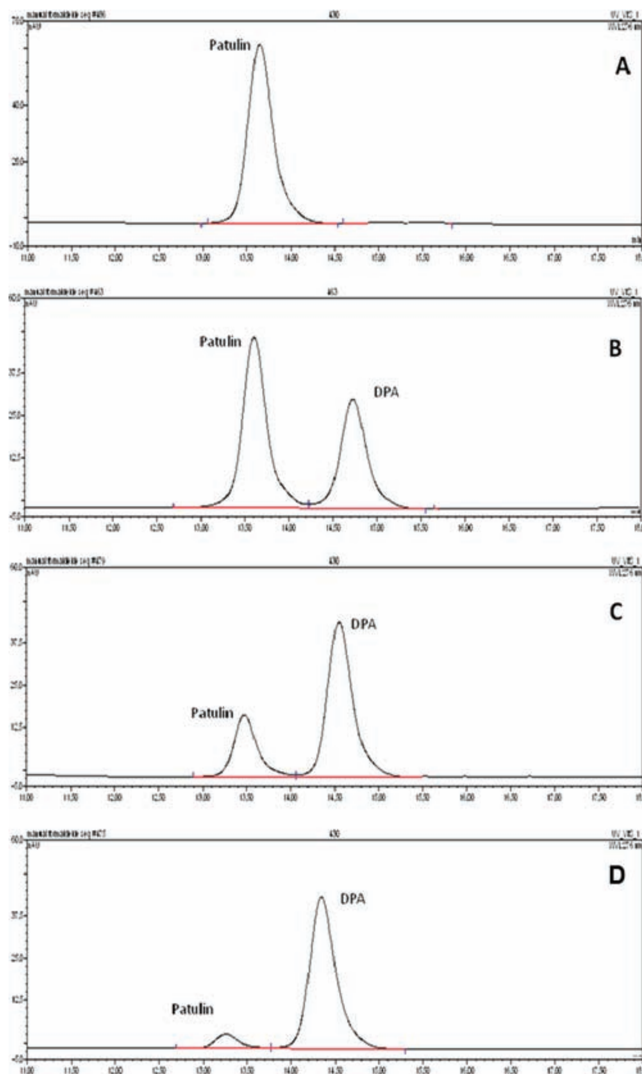

Figure 1. Time-course HPLC chromatograms of in vitro patulin degradation and production of desoxypatulinic acid (DPA) by the biocontrol yeast *Rhodosporidium kratochvilovae* strain LS11 following incubation for 0 (A), 24 (B), 48 (C), and 72 h (D).

The assignment of ^{1}H and ^{13}C NMR spectra of patulin in D_2O/acetic acid is reported in Table 1. The high-resolution mass spectrum of the major biodegradation product indicated a molecular formula $C_7H_8O_4$. To determine its molecular structure, a complete NMR study, including 1D and 2D experiments, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC (Figure 2C), was performed. The ^{1}H spectrum of this stable metabolite shows the presence of three CH_2 groups labeled as 2, 3, and 7 and one CH group labeled as 6. The CH_2-2 at 4.61 ppm and the CH_2-3 at 2.72 ppm correlate in COSY experiment and therefore belong to a CH_2−CH_2 fragment, while CH_2-7 is a doublet with a very small coupling constant, indicating the absence of the vicinal CH_x groups.

The ^{1}H/^{13}C chemical shifts of CH_2-2 group in this metabolite are similar to chemical shifts of CH_2-6 of patulin (Figure 2A,B), suggesting a structural similarity (O−CH_2), but the multiplicity

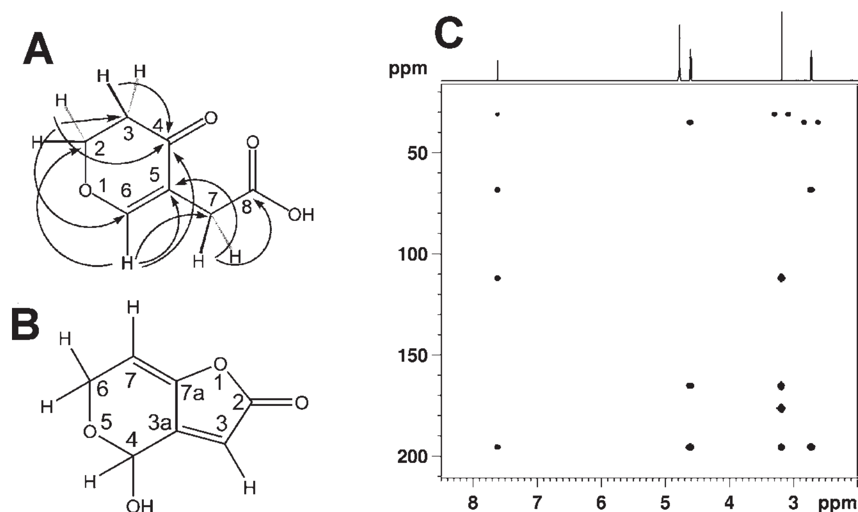

Figure 2. Structures of (A) desoxypatulinic acid (arrows indicate key long-range correlations in HMBC spectrum) and (B) patulin. (C) $^{1}H-^{13}C$ HMBC spectrum of desoxypatulinic acid in D_2O/acetic acid at 27 °C.

Table 1. ^{1}H and ^{13}C Assignments of Patulin and Desoxypatulinic Acid in D_2O/Acetic Acid

patulin				desoxypatulinic acid			
group	^{1}H (ppm)	multiplicity: *J*, (Hz)	^{13}C (ppm)	group	^{1}H (ppm)	multiplicity: *J*, (Hz)	^{13}C (ppm)
CH (3,7)	6.20−6.22	m	110.2	CH (6)	7.62	t: <0.7	164.7
CH (4)	6.14	s	88.3	CH_2 (2)	4.61	t: 7.1	67.9
CH_2 (6, 6′)	4.70	dd: 17.5, 3.0	59.5	CH_2 (3)	2.72	t: 7.1	34.6
	4.53	dd: 17.5, 3.8					
C=O (2)			172.2	CH_2 (7)	3.18	d: <0.7	30.5
C (7a)			145.9	C=O (4)			195.6
C (3a)			151.3	C=C (5)			111.8
				COOH (8)			176.5

of ^{1}H signals is different. Geminal protons of the patulin CH_2-6 group are not chemically equivalent (Table 1), being close to the asymmetric C-4 carbon atom, whereas the protons of the CH_2-2 group of the biodegradation product of patulin are equivalent, indicating the transformation of chiral CH(OH)-4 group into a nonchiral one.

The carbon skeleton of the patulin biodegradation product was unequivocally assigned using long-range $^{1}H-^{13}C$ correlations (Figure 2C). Long-range contacts between protons of CH_2-2 and carbon C-6 and between proton CH-6 and carbon C-2 in the HMBC spectrum, together with diagnostic chemical shifts, suggested that the CH(OH)-4 group of patulin was transformed into a CH= (6) group in this metabolite. The signals of the COOH (8) and C=O (4) groups in the ^{13}C NMR spectrum indicate the opening of the lactone ring. These results and the $^{1}H-^{13}C$ long-range correlations of protons with three quaternary carbons in the HMBC experiment (Figure 2A) enabled us to identify the metabolite as desoxypatulinic acid. The corresponding NMR assignments for desoxypatulinic acid are presented in Table 1. This compound had previously been isolated by Scott et al. from *Penicillium patulum*.[32] The spectroscopic data of the metabolite resulting from patulin biodegradation agreed with those published in the literature for desoxypatulinic acid.[32] The present study is the first to report the production of desoxypatulinic acid by a microorganism other than *Penicillium* sp., as a biodegradation product of patulin.

Isotope incorporation experiments show that biodegradation of ^{13}C-labeled patulin by *R. kratochvilovae* LS11 led to the emergence of ^{13}C-labeled desoxypatulinic acid, the major biodegradation product, with a similar distribution of label as in the original ^{13}C-labeled patulin. The specific ^{13}C incorporation into patulin and desoxypatulinic acid was analyzed by HREIMS. This analytical method involves obtaining the spectra of the labeled compound and the unlabeled analogue under conditions as identical as possible. The isotopic composition of the labeled compound is determined by subtracting the relative abundance ratios of the labeled and unlabeled species in the ratio found for the unlabeled compound.

GC−MS analysis of patulin revealed that up to three ^{13}C labels were incorporated in 0.3% of the molecules, following administration of sodium [1-^{13}C] acetate to *P. expansum* FS-7, whereas 14.7% and 1.5% of the molecules carried one and two ^{13}C atom(s), respectively. Isotope incorporation was also detected in desoxypatulinic acid, following incubation of *R. kratochvilovae* LS11 with ^{13}C-labeled patulin. Most importantly, a similar percentage of the molecules of desoxypatulinic acid (14.4%) carried one ^{13}C label. However, no incorporation of more than one ^{13}C atom was detected in desoxypatulinic acid

Table 2. Effect of Different Concentrations of Patulin (PAT) and Desoxypatulinic Acid (DPA) on the Viability of Cultured Human Lymphocytes

treatment	duration of treatment	
	viable cells (%), 3 h	viable cells (%), 24 h
	PAT (μM)	
0.1	94.00 ± 1.41	92.50 ± 2.12
0.5	92.00 ± 4.24	89.50 ± 3.54
1	89.00 ± 14.14	68.00 ± 7.07[a]
5	78.50 ± 12.02	46.00 ± 9.90[a]
10	78.00 ± 1.41[a]	38.00 ± 14.14[a]
50	77.00 ± 4.24[a]	30.00 ± 11.10[a]
100	66.50 ± 9.21[a]	25.00 ± 5.66[a]
	DPA (μM)	
0.1	95.50 ± 0.71	93.50 ± 2.12
0.5	94.50 ± 0.71	92.00 ± 1.41
1	93.00 ± 1.41	90.00 ± 1.41
5	91.00 ± 7.07	88.00 ± 2.83
10	90.00 ± 5.56	87.50 ± 4.95
50	89.00 ± 8.49	74.50 ± 4.95[a]
100	87.50 ± 7.78	45.00 ± 4.24[a]
control	95.50 ± 2.12	96.00 ± 1.41

[a] Significantly different from control according to Student's test ($P < 0.05$).

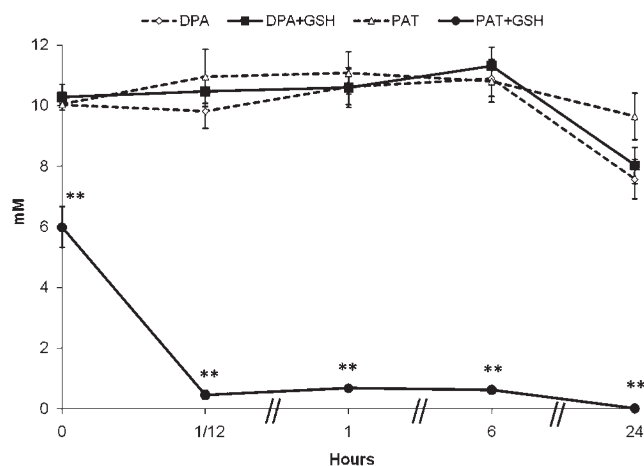

Figure 3. Time-course of the persistence of 10 mM patulin (PAT) and 10 mM desoxypatulinic acid (DPA), following incubation for 24 h at 37 °C and pH 7 in the presence of 50 mM of the thiol-bearing tripeptide glutathione (GSH). Values (mM) are the means ± standard deviation ($n = 9$, using three measurements in three experiments). Symbols (**) indicate highly significant differences ($P < 0.001$) between the resulting PAT and DPA concentrations in the presence of GSH at the same time points, when analyzed by Student's t test.

because of the low relative intensities of the isotopic peaks in the HREIMS spectrum for the labeled and unlabeled compound. These results prove that desoxypatulinic acid derives from metabolization of patulin by the yeast.

Table 2 shows the percentage of viable human lymphocytes following incubation with 0.1−100 μM of patulin or desoxypatulinic acid for 3 and 24 h. The harvested cells were counted. The percentage of viable cells in control cultures was similar at both time points. Treatment with patulin caused a significant decrease in the number of viable cells in a time- and dose-dependent manner. The most rapid patulin-induced effect on lymphocyte viability was observed after 3 h of incubation with patulin at 10 μM, when a decrease of viable cells slightly above 20% was recorded. The lowest patulin concentration that had a significant effect on the viability of lymphocytes was 1 μM, which caused a decrease of approximately 30% of viable cells after 24 h. The most dramatic effect was recorded after 24 h of incubation and with the highest concentration of patulin, that is, 100 μM, which caused a decrease of 70% in lymphocyte viability. The observed cytotoxicity of patulin in the present study agrees with that observed by other authors on other cell lines.[6,33] Conversely, desoxypatulinic acid, on the other hand, was not cytotoxic to lymphocytes following 3 h of incubation, for the whole range of concentrations used. Desoxypatulinic acid was slightly toxic only at the highest concentrations tested (50 and 100 μM) and over the longest period of incubation (24 h).

The mechanism of patulin toxicity is usually attributed to its reactivity to thiol groups, which are present in cysteine, glutathione, and in cellular enzymes that contain thiol groups in their active sites. Patulin thus destroys and inactivates these compounds and blocks the active sites of enzymes containing sulfhydryl groups.[7,34] Within the patulin molecule, carbon 3, 4, or 7 has been demonstrated to bind covalently to thiol groups.[6,7] The biotransformation of patulin into desoxypatulinic acid by *R. kratochvilovae* LS11 leads to the loss of the hydroxyl group at C-4 of patulin, thus hindering the nucleophilic substitution at the corresponding carbon in the molecule of desoxypatulinic acid (C6). Desoxypatulinic acid could possibly undergo a Michael-like addition of thiol groups at C-6 because of the presence of an α,β-unsaturated carbonyl system. However, reports show that similar α,β-unsaturated ketones do not add thiol nucleophiles to this position, possibly because of deactivation by the ring oxygen.[34]

The proposed inability of desoxypatulinic acid to react with thiol groups is supported by the results reported in Figure 3, showing the fate of patulin and desoxypatulinic acid in the presence of glutathione at 37 °C and pH 7. In these experiments, the mycotoxin reacted so rapidly with glutathione that even at time 0 the concentration of patulin in the presence of glutathione was lower than in the respective control (i.e., patulin in the absence of glutathione). After 5 min and until the end of the experiment, very low concentrations of patulin were present in the reaction buffer, whereas in the absence of glutathione the concentration of the mycotoxin remained unchanged at all time points. The concentration of desoxypatulinic acid, in contrast to that of patulin, remained almost unaffected over time in the presence of glutathione. A slight decrease of desoxypatulinic acid concentrations was only detected at 24 h, both in the presence and in the absence of glutathione.

The absence of thiol-binding activity of desoxypatulinic acid could provide an explanation for the low toxicity of this compound. The low toxicity of desoxypatulinic acid could be due to the loss of the hemiacetal group[35] and the hydrolysis of the lactone ring. The well-known instability of patulin in alkaline conditions, which leads to loss of biological activity,[36] is a consequence of the opening of the lactone ring, suggesting that the lactone component of patulin is essential to its toxicity. Moreover, desoxypatulinic acid is more hydrophilic than patulin, and the increased polarity would facilitate its excretion from the body after ingestion.

The ascomycetous yeast *Saccharomyces cerevisiae* is known to metabolize patulin to *E*-ascladiol under anaerobic conditions. This explains why patulin contamination in fermented drinks such as cider is usually lower than in juices and other nonfermented pome fruit-based products.[12,22,23] *E*-Ascladiol has no hemiacetal group (which would probably account for its slightly lower toxicity as compared to patulin), but retains the integrity of the lactone ring in its molecular structure. It is in itself considered to be a mycotoxin, although with less acute toxicity than patulin.[24] The biodegradation of patulin by *R. kratochvilovae* LS11 to desoxypatulinic acid has the additional advantage that it is an aerobic process. From a practical perspective, an aerobic degradation pathway is advantageous for the development of a large-scale apple juice detoxification process, because costly removal of oxygen becomes unnecessary.

The present study shows that a biocontrol yeast, *R. kratochvilovae* LS11, forms a novel degradation product, desoxypatulinic acid, from the mycotoxin patulin. Desoxypatulinic acid is much less toxic than patulin. This conversion by strain LS11 can therefore be considered to be a detoxification process. Future studies will include toxicity and genotoxicity tests of desoxypatulinic acid to cultured human cells, and the elucidation of the degradation pathway. The future identification of the enzymes involved in the degradation process will enable the development of tools for a more effective prevention and diagnosis of patulin contamination in pome fruit-derived products.

ASSOCIATED CONTENT

Supporting Information. Figure 1: COSY of desoxypatulinic acid. Figure 2: $^{1}H-^{13}C$ HSQC of desoxypatulinic acid. This material is available free of charge via the Internet at http://pubs.acs.org.

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