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A New Competitive Fluorescence Assay for the Detection of Patulin Toxin

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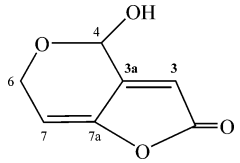
Patulin is a toxic secondary metabolite of a number of fungal species belonging to the genera *Penicillum* and *Aspergillus*. It has been mainly isolated from apples and apple products contaminated with the common storage-rot fungus of apples, *Penicillum expansum*, but it has also been extracted from rotten fruits, moldy feeds, and stored cheese. Human exposure to patulin can lead to serious health problems, and according to a long-term investigation in rats, the World Health Organization has set a tolerable weekly intake of 7 ppb body weight. The content of patulin in foods has been restricted to 50 ppb in many countries. Conventional analytical detection methods involve chromatographic analyses, such as HPLC, GC, and, more recently, techniques such as LC/MS and GC/MS. However, extensive protocols of sample cleanup are required prior to the analysis, and to accomplish it, expensive analytical instrumentation is necessary. An immunochemical analytical method, based on highly specific antigen−antibody interactions, would be desirable, offering several advantages compared to conventional techniques, i.e., low cost per sample, high selectivity, high sensitivity, and high throughput. In this paper, the synthesis of two new derivatives of patulin is described, along with their conjugation to the bovine serum albumin for the production of polyclonal antibodies. Finally, a fluorescence competitive immunoassay was developed for the *on-line* detection of patulin.

Patulin (Figure 1) is a toxic secondary metabolite of a number of fungal species belonging to the genera *Penicillum* and *Aspergillus*. It has been mainly isolated from apples and apple products contaminated with the common storage-rot fungus of apples, *Penicillum expansum*, but it has also been extracted from rotten fruits, moldy feeds, and stored cheese.[1−3] Although several mycotoxins occur in nature, very few are regularly found in fruits,



(**1**)

Figure 1. Chemical structure of patulin.

e.g., aflatoxins, ochratoxin A, patulin, and *Alternaria* toxins. Mycotoxins may remain in fruits even when the fungal mycelium has been removed; furthermore, the processing of fruits does not result in the complete removal of mycotoxins. Human exposure to mycotoxins occurs by ingestion of contaminated products and can lead to serious health problems, including immunosuppression and carcinogenesis.[4,5] In animal studies patulin has been shown to be acutely toxic[6,7] and carcinogenic and teratogenic.[8] One important aspect of patulin toxicity in vivo is an injury of the gastrointestinal tract including ulceration and inflammation of the stomach and intestine.[9] Recently, patulin has been shown to be genotoxic by causing oxidative damage of DNA,[10] and oxidative DNA base modifications have been considered to play a role in mutagenesis and cancer initiation.[11] Patulin is believed to exert its cytotoxic effects mainly by forming covalent adducts with essential cellular thiol groups in proteins and amino acids.[12] Indeed, from a chemical point of view patulin is a water-soluble unsaturated lactone, readily reactive toward thiol groups and also amino groups. The electrophilic properties of patulin and its reactivity toward several model nucleophiles, such as *N*-acetylcysteine and glutathione, have been elucidated.[13,14] Glutathione represents one of the most abundant cellular nucleophiles, and it

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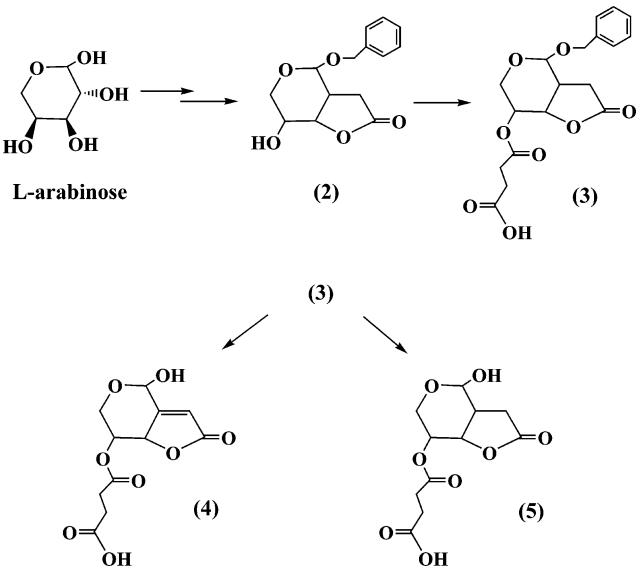
has been shown to spontaneously form covalent adducts with patulin.[13] The same reactivity is responsible for the ability of patulin to induce in vitro intra- and intermolecular protein cross-links involving cysteine residues and lysine and histidine side chains[15] and for the capability to inhibit the activity of several enzymes.[16,17] Patulin is also reported to be endowed with selective DNA-damaging activity.[18]

The widespread presence of fungi in the environment renders mycotoxins practically ubiquitous contaminants in food and feeds; therefore, one of the most effective measures to protect public health is to establish reasonable regulatory levels of these toxins on the basis of valid toxicological data. To ensure the safety and quality of foods, a number of regulatory authorities have decreed maximum residue levels of several mycotoxins in foods. In agreement with the results of a long-term investigation in rats,[19] the World Health Organization (WHO) has set a tolerable weekly intake of 7 ppb body weight. The content of patulin in foods has been restricted to 50 ppb in many countries: the EU has set a limit of 10 ppb in children's foods, but the objective is to reach 25 ppb patulin in apple-containing products.[20,21] A ready detection is the most prudent means to prevent the entry of patulin in commerce. Conventional analytical methods of detection involve chromatographic analyses, such as HPLC, GC,[22,23] and, more recently, techniques such as LC/MS and GC/MS.[24] However, extensive protocols of sample cleanup are required prior to the analysis, and expensive analytical instrumentation is necessary to accomplish it. Furthermore, the problem of precise quantitative determination of patulin and other mycotoxins by these methods is particularly important due to the high variability of the real matrixes and can be solved only by exploiting isotopically labeled internal standards. All these limitations make it difficult to extend the methods to detect patulin outside the laboratory. As an alternative, immunochemical analytical methods, for instance, enzyme-linked immunosorbent assay (ELISA), offer several advantages compared to conventional analysis, i.e., low cost per sample, high selectivity, high sensitivity, and high throughput. Extensive extraction and sample cleanup are not needed. Such methods have been shown to have a potential for the routine analysis of many mycotoxins.[25−27] However, no immunoassay has been developed yet for patulin detection. This is due in part to chemical reasons, since patulin is, like other nonproteinaceous toxins, a low molecular weight compound (MW = 154), and to develop antibodies, the use of a hapten−protein conjugate is necessary. In addition, patulin, unlike other toxins, is highly reactive toward thio-containing compounds and highly unstable under basic conditions.[28] This sensitivity may result in decomposition of patulin in the course of conjugation to the protein carrier. Furthermore, the exposed epitopes of the injected hapten−protein conjugate can be attacked by sulfydryl-containing compounds in the blood. For this reason few papers have appeared in the literature on the synthesis of patulin−protein conjugates and on the production of antibodies against this toxin, and the results are far from satisfactory in all cases.[29−31] A recent review on the research so far on patulin, from history and regulation to health effects and existing methods to detect the toxin in foods, has recently appeared.[32]

Scheme 1. Synthesis of Derivatives 4 and 5 from L-Arabinose



Here the authors describe the synthesis of two new patulin derivatives, **4** and **5** (Scheme 1), still maintaining the original skeleton of the natural toxin, but exhibiting a higher chemical stability, as well as their conjugation to the bovine serum albumin carrier protein for the production of polyclonal antibodies in rabbits. They also report on the purification of the produced antibodies by affinity chromatography on columns obtained by covalently immobilizing **4** and **5** on Sepharose resin. The titer of the specific antibodies was determined by means of indirect ELISA assays. Finally, tetramethylrhodamine isothiocyanate-labeled IgG's (IgG = immunoglobulin G) were prepared and used to develop a "hit and run" fluorescence immunoassay for patulin, based on the antibody competition between the immobilized patulin derivative and free added patulin. This technique was described for the detection of T-2 toxin[33] and more recently for the sensing of 2,4,6-trinitrotoluene in seawater.[34]

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MATERIALS AND METHODS

All reagents were of the highest commercially available quality and were used as received. L-Arabinose was purchased from Sigma. NMR spectra were recorded on Bruker WM-400, Varian Gemini 300, Varian Gemini 200, and Varian Inova 500 spectrometers. All chemical shifts are expressed in parts per million with respect to the residual solvent signal. For electrospray ionization spectroscopy mass spectrometry (ESI-MS) analyses, a Waters Micromass ZQ instrument, equipped with an electrospray source, was used in the positive and/or negative mode. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide (EDC), bovine serum albumin (BSA; fraction V), and ovalbumin (OVA; grade V) were purchased from Sigma. PURE1A Protein A Antibody Purification Kit was purchased from Sigma. Goat polyclonal to rabbit IgG−HRP conjugate (secondary antibody) was from Abcam. Affinity resin EAH Sepharose 4B was purchased from Amersham Biosciences. Nitrocellulose transfer membrane Protran from Schleicher & Schuell and ECL detection reagents from Amersham Biosciences were used in dot blot and Western blot experiments. Microplates (96-well), LockWell MaxiSorp from Nunc, 3,3′,5,5′-tetramethylbenzidine (TMB) enzyme substrate from Sigma, and a microplate reader, Multiskan EX from Thermo, were used for ELISA experiments. UV measurements (detection at $\lambda = 278$ nm) were carried out on a Varian Cary 50 Bio spectrophotometer. Tetramethylrhodamine isothiocyanate (TMRI) was purchased from Sigma. Fluorescence experiments were carried out on an ISS K2 fluorometer (ISS, Champaign, IL).

Synthesis and Characterization of Patulin Derivatives. *Synthesis of 4-{[4-(Benzyloxy)-2-oxo-2,6,7,7a-tetrahydro-4H-furo[3,2-c]pyran-7-yl]oxy}-4-oxobutanoic Acid (**3**)*. 4-(Benzyloxy)-7-hydroxy-7,7a-dihydro-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one (**2**; 350 mg, 1.37 mmol) was dissolved in 9 mL of dry THF and treated with 16.7 mg of DMAP (0.137 mmol) and 1.37 g of succinic anhydride (13.7 mmol), under stirring at room temperature. After 8 h, a second aliquot of succinic anhydride (685 mg, 6.85 mmol) was added and the reaction left for an additional 15 h. The reaction mixture was concentrated under reduced pressure, then redissolved in 25 mL of ethyl acetate, washed three times with water, and purified by silica gel chromatography (eluent system: acetone in $CHCl_3$, from 5% to 10%), which resulted in 330 mg (0.91 mmol, 68%) of **3** as a pure compound. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 7.38−7.33 (5H, complex signals, aromatic protons); 5.98 (1H, d, H-3); 5.70 (1H, s, H-4); 5.48 (1H, d, H-7a); 5.23 (1H, m, H-7); 4.81 (1H, dd, J = 11.7 Hz, CHHPh); 4.65 (1H, dd, J = 11.7 Hz, CHHPh); 4.05 (1H, d, J = 12.6 Hz, H-6); 3.90 (1H, dd, J = 12.6 Hz, H-6′); 2.62 (4H, m, H-10 and H-11). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 176.9 (C-9); 171.7 (C-2); 171.0 (C-12); 158.9 (C-3a); 136.1, 128.7, 128.4, and 128.1 (aromatic carbons); 115.0 (C-3); 93.3 (C-4); 77.6 (C-7a); 70.3 (C-7); 69.7 (CH_2Ph); 59.5 (C-6); 28.8 and 28.6 (C-10 and C-11). ESI-MS m/z calcd for $C_{18}H_{18}O_8$, 362.04; found (positive ions), 385.28 (M + Na^+), 401.25 (M + K^+).

*Synthesis of 4-[(4-Hydroxy-2-oxo-2,6,7,7a-tetrahydro-4H-furo[3,2-c]pyran-7-yl)oxy]-4-oxobutanoic Acid (**4**, P-ins-HS)*. To a solution of **3** (150 mg, 0.41 mmol) in dry DCM (10 mL) was slowly added $SnCl_4$ (150 μL) at room temperature. After 24 h an additional 75 μL of $SnCl_4$ was added and the reaction allowed to proceed for 24 h. The reaction mixture was poured into cold H_2O (20 mL) and diluted with $CHCl_3$ (5 mL). The organic layer was separated, and the aqueous phase was concentrated under reduced pressure, purified by silica gel chromatography (eluent system: MeOH in $CHCl_3$ from 20% to 30%), and passed through a DOWEX H^+ ion-exchange resin to obtain 75 mg (0.28 mmol, 67%) of pure **4**. ^{1}H NMR (500 MHz, D_2O): δ_H 6.25 (1H, s, H-3); 6.15 (1H, s, H-4); 5.62 (2H, overlapped signals, H-7 and H-7a); 4.36 (1H, d, J = 13.5 Hz, H-6,); 3.96 (1H, dd, J = 13.5 Hz and J = 13.0 Hz, H-6′); 2.67 − 2.57 (4H, m, 4 × H of butanoic acid), ^{13}C NMR (125 MHz, D_2O): δ_C 178.0 (C-4 of butanoic acid); 173.8 (C-1 of butanoic acid); 165.1 (C-2); 161.7 (C-3a); 114.5 (C-3); 89.0 (C-4); 77.1 (C-7a); 71.6 (C-7); 70.1 (C-6); 29.6 (C-2 and C-3 of butanoic acid). ESI-MS: m/z calcd for $C_{11}H_{12}O_8$, 272.05; found (positive ions), 255.15 (M − H_2O + H^+), 295.14 (M + Na^+), 313.10 (M + K^+).

*Synthesis of 4-[(4-Hydroxy-2-oxohexahydro-4H-furo[3,2-c]pyran-7-yl)oxy]-4-oxobutanoic Acid (**5**, P-sat-HS)*. To a solution of **3** (150 mg, 0.41 mmol) in 5 mL of MeOH was added a suspension of 150 mg of 10% Pd/C in MeOH (3 mL) under a flux of argon, and the mixture was hydrogenated at atmospheric pressure for 16 h. The reaction mixture was filtered through a Celite pad and concentrated under reduced pressure. The residue was taken up in 40 mL of H_2O and washed twice with DCM. The aqueous layer was concentrated to yield 90 mg (0.32 mmol, 80%) of pure **5**. ^{1}H NMR (400 MHz, D_2O): δ_H 5.32 (1H, m, H-7); 5.21 (1H, d, J = 3.2 Hz, H-4); 5.10 (1H, m, H-7a); 4.16 (1H, dd, J = 12.8 Hz and J = 2.4 Hz, H-6); 3.75 (1H, dd, J = 13.2 Hz and J = 1.6 Hz, H-6′); 2.80 − 2.60 (7H, overlapped signals, H-3; H-3a; 2CH_2 of butanoic acid). ^{13}C NMR (100 MHz, D_2O): δ_C 183.9 (C-1 of butanoic acid); 183.1 (C-4 of butanoic acid); 177.2 (C-2); 95.9 (C-4); 77.9 (C-7a); 70.1 (C-7); 62.1 (C-3a); 42.7 (C-3); 34.7 (C-2 of butanoic acid); 33.1 (C-3 of butanoic acid). ESI-MS: m/z calcd for $C_{11}H_{14}O_8$, 274.07; found (positive ions), 297.15 (M + Na^+), 313.12 (M + K^+).

Synthesis of BSA Conjugates (Antigens A and B). *Synthesis of P-Ins-HS−BSA Conjugate (Antigen A)*. To a solution of P-Ins-HS (2 mg, 0.0073 mmol) in 0.5 mL of MES buffer (0.1 M), pH 5, were added 0.2 mL of a solution of BSA (4 mg·mL^{-1}) in the same buffer and 0.1 mL of an EDC solution in H_2O (10 mg·mL^{-1}). The reaction mixture was incubated for 2 h at room temperature and then dialyzed against 0.5 L of PBS (0.01 M), NaCl (0.01 M), pH 7.4 (0.5 L, for 3 days with daily buffer changes). The concentration of the conjugate, spectrophotometrically determined at $\lambda = 278$ nm, was 2.8 mg·mL^{-1}.

Synthesis of P-Sat-HS−BSA Conjugate (Antigen B). To a solution of P-Sat-HS (1.5 mg, 0.0054 mmol) in Tris (pH 8)/dioxane, 1:1 (v/v, 0.4 mL), were added 20 μL (1.0 mg, 0.0054 mmol) of an EDC solution in H_2O (50 mg·mL^{-1}) and 0.5 mL of a BSA solution (8 mg·mL^{-1}) in PBS (0.1 M) at pH 7.4. After 2 h at room temperature, the reaction mixture was dialyzed against PBS (0.01 M), NaCl (0.01 M), pH 7.4 (0.5 L, for 3 days with daily buffer changes). The conjugate concentration determined spectrophotometrically at $\lambda = 278$ nm was 4.2 mg·mL^{-1}.

Antibody Production and Purification. *Antibody Production*. Two rabbits were immunized following a standard protocol by intradermal inoculation of a mixture of antigens A and B (0.5 mg each per rabbit). After the immunization period, the rabbits were

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sacrificed and the blood was centrifuged to separate blood cells from serum (SI1 from rabbit 1 and SI2 from rabbit 2).

IgG Purification. A 2.0 mL sample of antiserum (SI1 and SI2) was applied to a protein A column of the PURE1A Protein A Antibody Purification Kit, Sigma, and the IgG fraction was purified according to the manufacturer's instructions. Elution of proteins was monitored by absorbance at $\lambda = 278$ nm. The IgG fraction was eluted with glycine (0.1 M) at pH 2.8 and immediately buffered in Tris (1.0 M) at pH 8.0. Concentration on an Amicon XM50 membrane and dialysis against PBS (20 mM), pH 7.0, NaCl (50 mM) resulted in 4.0 mL of IgG1 and 4.0 mL of IgG2.

Affinity Column Preparation. Two affinity columns were obtained by conjugating derivatives P-Ins-HS and P-Sat-HS, respectively, to EAH Sepharose 4B as follows. A 1.0 mL sample of resin was washed with H_2O at pH 4.5 (20 mL), with NaCl (0.5 M) (20 mL), and again with H_2O at pH 4.5 (20 mL) and finally suspended in 2.0 mL of H_2O. The Sepharose resin was added to a solution of **4** or **5** (5 mg in 0.5 mL of H_2O at pH 4.5), and the resulting suspensions were gently shaken. The slurry was cooled to 0 °C, and EDC was added in two steps to a final concentration of 0.1 M (52 mg). After 12 h at 4 °C, the reaction mixture was taken to room temperature, and after an additional 4 h the resin was extensively washed with H_2O at pH 4.5 and then treated with 1.0 mL of AcOH (0.1 M) and 38 mg of EDC for 1 h at room temperature. The suspension was washed with H_2O at pH 4.5 (20 mL), acetate buffer (0.1 M) containing NaCl (0.5 M) (20 mL), pH 4.0, and PBS (0.1 M) containing NaCl (0.3 M), pH 7.4 (20 mL), and finally packed into a polystyrene column (2 mL, BIORAD).

Antibody Purification by Affinity Chromatography. For the affinity chromatography purification, a 2.0 mL aliquot of IgG (obtained from SI1 and SI2 serums) was applied dropwise to the affinity column prepared as described above. To eliminate unspecific antibodies, the column, before elution, was washed with three high-salt buffers: (1) PBS (0.01 M), NaCl (0.1 M), pH 7.0 (20 mL); (2) PBS (0.01 M), NaCl (0.5 M), pH 7.0 (20 mL); (3) PBS (0.01 M), NaCl (1.0 M), pH 7.0 (20 mL). At that point absorbance at $\lambda_{278\,nm}$ had fallen to 0.0. For the elution step glycine (0.1 M), pH 2.7 (2.5 mL), was applied to the column, and the eluate was collected in 0.5 mL fractions and monitored by absorbance measurements at $\lambda = 278$ nm.

The fractions containing the antibodies were collected, concentrated by means of a Centricon YM-3 membrane to a volume of 1.0 mL, and dialyzed against PBS (0.1 M), NaCl (0.1 M), pH 7.4. The concentration of the antibodies was spectrophotometrically determined by absorbance measurements at $\lambda = 278$ nm.

Synthesis of GlnBP Conjugates (P-sat-HS−GlnBP, P-ins-HS−GlnBP). To avoid interference by the carrier protein in the polyclonal antibody detection process, P-Ins-HS and P-Sat-HS were conjugated to the glutamine-binding protein from *Escherichia coli* (GlnBP), a bacterial protein different from the protein used to carry out the immunization. The following procedure was used: 2.5 mg (0.0092 mmol) of **4** or **5** was dissolved in 0.25 mL of MES buffer (0.1 M), pH 5. The solution was incubated at room temperature with 0.25 mL of a GlnBP solution (5 mg·mL^{-1}) in the same buffer and 0.1 mL of an aqueous solution of EDC (10 mg·mL^{-1}). After 5 h, the reaction mixture was dialyzed against PBS (0.01 M) containing NaCl (0.1 M) at pH 7.4 (0.5 L, 3 days with daily buffer changes).

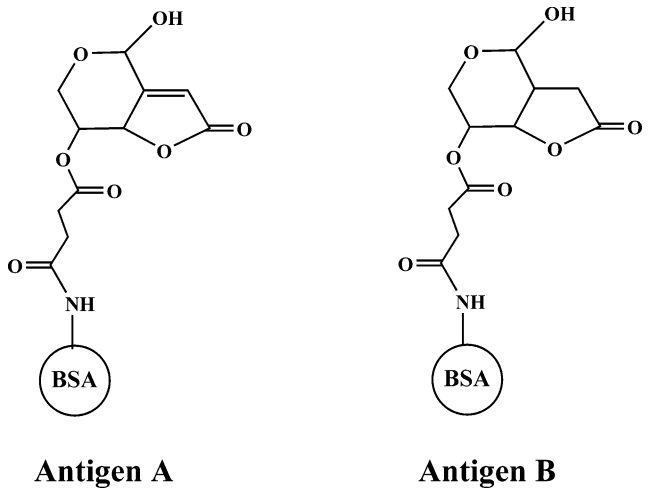

Figure 2. Schematic representation of the BSA conjugates used for the production of the rabbit anti-patulin antibodies.

Dot Blot Experiments. P-sat-HS−GlnBP, P-ins-HS−GlnBP, and GlnBP were spotted on four nitrocellulose membranes (10 and 1 μg of each antigen on each filter) and the spots allowed to dry. The filters were washed twice with PBS (0.1 M), NaCl (0.4 M) at pH 7.4 (PBS) and then incubated with the blocking buffer (PBS containing 5% skim milk, 0.2% Tween 20, and 0.05% Tryton, pH 7.4) for 1 h at room temperature. After two washings with PBS (0.1 M), NaCl (0.4 M), Tween (0.2%), and Tryton (0.05%) at pH 7.4 (PBS−TT) and one with PBS (10 min per washing), the filters were incubated with SI1, SI2, SPI1, and SPI2 (each diluted 1:250 in blocking buffer), respectively, for 1 h at room temperature. The filters were washed (twice with PBS−TT and one time with PBS, 10 min per washing) before being incubated with the secondary antibody (goat anti-rabbit HRP conjugate, 1:3000 in blocking buffer) for 1 h at room temperature. The filters were washed three times as described above and developed with the detection reagent ECL.

Western Blot Experiments. Proteins (BSA, P-Ins-HS−GlnBP or P-Sat-HS−GlnBP, and GlnBP, 10 μg each) were loaded, separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (12% SDS−PAGE), and then transferred overnight at 4 °C onto a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature by rocking in 50 mL of the blocking buffer (PBS containing 5% skim milk, 0.2% Tween 20, and 0.05% Tryton). After two washings with PBS−TT and one with PBS (10 min per washing), the filters were incubated with a-PiHS and a-PsHS, respectively (1:500 in the blocking buffer), for 1 h at room temperature. After two washings with PBS−TT and one with PBS (10 min per washing), the filters were incubated with secondary antibody (goat anti-rabbit HRP conjugate, 1:3000 in the blocking buffer) for 1 h at room temperature. The filters were washed three times as described above and then developed with the detection reagent ECL.

Antibody Titration. The antibody titer was determined by an indirect ELISA assay by the following general procedure. The antigens (P-Ins-HS−GlnBP or P-Sat-HS−GlnBP), in PBS (0.1 M), pH 7.4, were used to coat 96-well microplates, varying the concentration by a factor 3 from 11 to 1.7×10^{-3} μg·mL^{-1} (one column for every antigen concentration, 100 μL per well), overnight at 4 °C. Control wells were incubated for the same

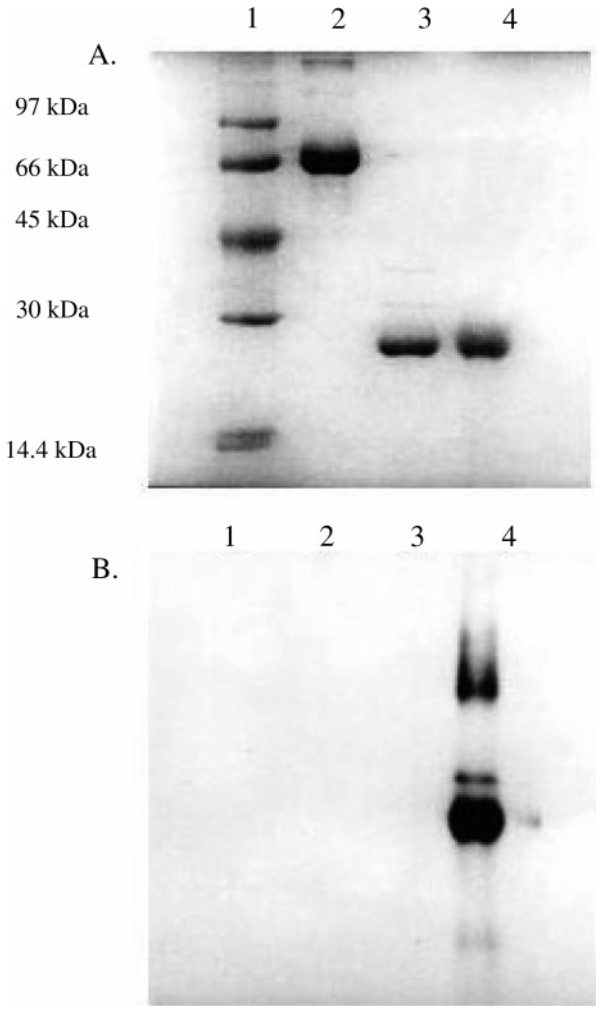

Figure 3. SDS−PAGE and Western blot analyses of the specific antibody anti- derivative **5**. SDS−PAGE stained with Coomassie Brillant Blue R-250 (A) and nitrocellulose filter (B) after incubation with αPsHS1 antibodies. Key: lane 1, molecular weight standards; lane 2, bovine serum albumin; lane 3, GlnBP; lane 4, P-Sat-HS−GlnBP.

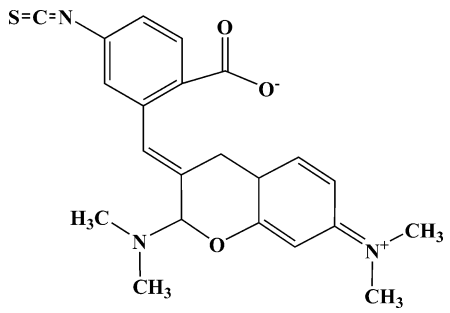

Figure 4. Chemical structure of tetramethylrhodamine isothiocyanate.

period with BSA in the same buffer. The wells were rinsed three times with PBS (0.1 M) containing 0.05% Tween (PBS−T), pH 7.4, and blocked by incubation for 2 h at room temperature with PBS−T containing BSA (1%) (100 μL each well). After three washings with PBS−T, serially diluted antibody, aPiHS1, aPsHS1, aPiHS2, or aPsHS2, was added to the wells, incubated at room temperature for 1 h, and then rinsed three times with PBS−T. Horseradish peroxidase-conjugated anti-rabbit IgG antibodies, diluted 1:4000 in PBS−T containing BSA (1%), were added to the wells (100 μL) and incubated for 1 h at room temperature. After three washings with PBS−T, the enzyme substrate TMB was added (100 μL per well), and the color reaction was quenched after 5 min by addition of 1 M H_2SO_4 (100 μL per well). The absorbance was measured at $\lambda = 450$ nm. The antibody titer was graphically determined by plotting the reciprocal of the antibody dilution vs absorbance for each dilution of antibodies. The titer was taken as the maximum antibody dilution able to give a reading of 0.1 absorbance unit. The following values were found: 1/25000 for aPsHS1 and aPsHS2 and 1/25000 for aPiHS1 and aPiHS2.

Synthesis of the IgG−TMRI Conjugate. IgG fractions from each rabbit serum were labeled with TMRI as follows. A 0.7 mL sample of IgG in buffer, $NaHCO_3$ (10 mM), at pH 9.0 (2.5 mg·mL^{-1}) was incubated with a solution of TMRI (0.15 mg·mL^{-1}) in DMF (50 μL)/$NaHCO_3$ (10 mM), pH 9.0 (450 μL), for 2 h at room temperature. The labeled antibodies were purified on a Sephadex G-25 column. The fractions containing labeled IgG were pooled and concentrated to a volume of 2.0 mL on a Centricon microconcentrator. The degree of labeling (DOL), as calculated from the absorbance values at $\lambda = 278$ and 550 nm, by applying a correction factor for label absorption at $\lambda = 278$ nm, was found to be 3.5.

Binding of IgG−TMRI to Patulin Derivatives on Affinity Columns and Detection of Free Patulin. The previously prepared affinity column was washed and equilibrated with the binding buffer PBS (0.1 M), NaCl (0.1 M), pH 7.4. A 1.5 mL sample of diluted IgG−TMRI (dilution 1:3 in binding buffer) was applied dropwise onto the resin. The column was washed with the binding buffer (20 mL), then with PBS (0.1 M), NaCl (0.5 M), pH 7.4 (20 mL), and finally with PBS (0.1 M), NaCl (1 M), pH 7.4, until the fluorescence signal was returned to the preloading level ($\lambda = 550$ nm excitation, $\lambda = 575$ nm emission). Samples of patulin (0, 1, 10, and 100 ppb) in 2.0 mL of binding buffer were applied to the column at room temperature and incubated for 10 min. At the end of the incubation period, the patulin−IgG−TMRI complex was eluted from the column and collected in 0.5 mL fractions. The fluorescence emission signal was measured at $\lambda = 575$ nm. The column was washed with 5.0 mL of the binding buffer and could be reused for the analysis of the successive patulin sample.

DISCUSSION AND RESULTS

Synthesis of Patulin Derivatives 4 and 5. The presence of a carrier protein conjugated to the toxin is required for the production of antibodies against patulin, which is, like other nonproteinaceous toxins, too small to elicit any immunological response. However, the conjugation reaction is hampered by the reactivity of patulin functional groups in the reaction conditions required to functionalize the OH group at the C-4 position (Figure 1). Indeed, from a chemical point of view patulin is a highly conjugated bicyclic lactone and, in general, very reactive at the C7 position under basic conditions and toward nucleophiles, especially thiol and amino groups.[13] Furthermore, even if the desired antigen could be obtained, the patulin epitopes would be sensitive, in vivo, to the SH-containing molecules in the blood, thus rendering the intact molecule unavailable and the recognition process disfavored, with no or poor production of antibodies. With the aim of reducing the reactivity of patulin toward nucleophiles, two new derivatives, **4** (P-Sat-HS) and **5** (P-Ins-HS) (Scheme 1),

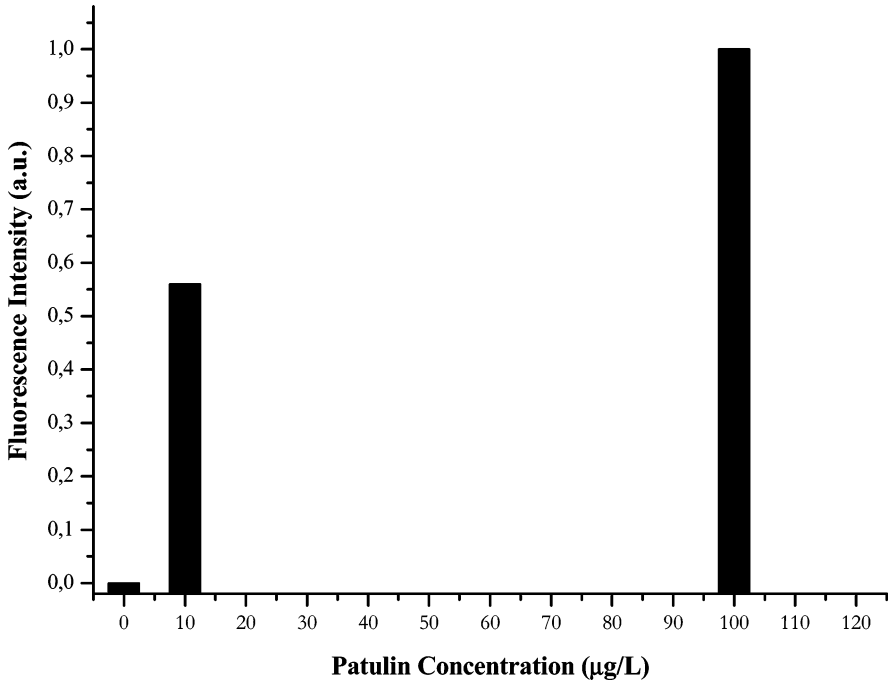

Figure 5. Fluorescence immunoassay for patulin detection. Fluorescence emission was recorded at $\lambda = 575$ nm and is representative of the IgG−TMRI eluted from the column after incubation of patulin. The fluorescence experiments were performed at room temperature; excitation was at 550 nm.

lacking the highly reactive C7−C7a double bond, were synthesized starting from compound **3** (Scheme 1). This in turn was obtained from derivative **2**, prepared from commercial L-arabinose by a previously described synthetic scheme.[35] Removal of the benzyl protecting group in **3** by treatment with $SnCl_4$ yielded compound **4**; conversely, catalytic hydrogenation of **3** nicely afforded compound **5**. In both cases the synthesis gave a mixture of diastereoisomers of **4** and **5**, which were not separated and used as such in the successive protein conjugation reactions.

Conjugation of Patulin Derivatives to the Bovine Serum Albumin. BSA was chosen as the carrier protein, and haptens **4** and **5** were conjugated to it by the water-soluble carbodiimide method, leading to antigens A and B (Figure 2). The efficiency of the conjugation reaction could not be determined because of the lack of a UV chromophore in the case of derivative **5** and because the maximum absorption at $\lambda = 278$ nm of **4** and BSA overlapped.

Production of Antibodies against the Patulin Derivatives. Both antigens A and B were used to immunize rabbits for the production of polyclonal antibodies against patulin, following a standard protocol. At the end of the immunization period, the presence of antibodies against patulin derivatives in serums (SI1 serum from rabbit 1 and SI2 serum from rabbit 2) was verified by dot blot assay.

To exclude false reactions due to anti-BSA antibodies present in the rabbit serums, a protein different from BSA was conjugated to the patulin derivatives P-Sat-HS and P-Ins-HS. For this purpose, the glutamine-binding protein (GlnBP) from *E. coli* was chosen. The GlnBP conjugates were prepared by the same procedures already used for BSA conjugates.

P-sat-HS−GlnBP, P-ins-HS−GlnBP, BSA as the positive control, and GlnBP as the negative one were spotted on four nitrocellulose membranes, and each membrane was incubated with immune antiserums (SI1 and SI2, 1:250 dilution) and preimmune serums from both rabbits (SPI1 and SPI2, respectively, from rabbit 1 and rabbit 2, dilution 1:250). After incubation of the HRP conjugate secondary antibody and development with ECL-specific reagent, preimmune serums showed no response, while SI1 and SI2 gave signals with both antigens P-sat-HS−GlnBP and P-ins-HS−GlnBP and with BSA but not with GlnBP (not shown).

Immunoglobulin Purification from Serums. The IgG fraction of each serum (IgG1 from rabbit 1 and IgG2 from rabbit 2) was isolated by the protein A column kit by standard procedures. After elution, the IgG fraction was concentrated and dialyzed against PBS (20 mM), NaCl (50 mM), pH 7.0.

Preparation of Affinity Columns and Purification of Specific Antibodies. Antibodies specific for either compound **4** or compound **5** were purified from the IgG fraction by affinity chromatography on one of two resins conjugated with compound **4** or **5**. After loading, the columns were washed with high-salt buffers to eliminate unspecific antibodies and eluted using a buffer at pH 2.7. The fractions containing the antibodies, as judged by monitoring the absorbance at $\lambda = 278$ nm, were pooled and tested against the patulin derivatives by Western blot. Only antibodies eluted by changing the pH conditions were effective in the recognition of compounds **4** and **5**, as shown in Figure 3 (aPsHS and aPiHS, respectively). These antibodies were used in the experiments of antibody titration (aPsHS1 and aPiHS1 from rabbit 1 and aPsHS2 and aPiHS2 from rabbit 2). To the best of our knowledge, this is the first time that polyclonal antibodies produced against patulin have been purified by affinity chromatography, due to the chemical stability of derivatives **4** and **5**, which allowed them to be conjugated to an NH-functionalized

(35) Bennett, M.; Gill, G. B.; Pattenden, G.; Shuker, A. J.; Stapleton, A. *J. Chem. Soc., Perkin Trans. 1* **1991**, 929−937.

resin. The affinity columns were also used to develop the fluorescence competitive immunoassay for the detection of patulin.

Specific Antibody Titration. The titer of purified antibodies was determined by indirect ELISA, by coating on the microplate wells several different concentrations of antigens P-sat-HS−GlnBP and P-ins-HS−GlnBP and by testing serially diluted aPsHS1 and aPsHS2 against P-sat-HS−GlnBP and P-ins-HS−GlnBP, respectively. Each experiment was performed in triplicate, and the results showed that the titer of antibodies, expressed as the reciprocal dilution giving 0.1 optical density (OD) unit at $\lambda = 450$ nm, was 25000 for all the tested antibodies (data not shown).

Patulin Detection by Affinity Chromatography with IgG−TMRI. A hit and run fluorescence immunoassay[33] was performed by labeling the IgG fraction with the fluorophore TMRI (Figure 4). The technique is based on the binding of fluorescence-labeled IgG to the derivative P-sat-HS or P-ins-HS, immobilized on a Sepharose 4B matrix, as previously described, followed by displacement of the TMRI-labeled antibodies by patulin. Displaced fluorescence antibodies are measured by fluorescence emission in the eluate and represent a measure of the amount of free patulin present in the sample. The labeling of the IgG fraction was achieved by incubating it with TMRI (previously dissolved in a mixture of DMF and binding buffer, pH 9.0). After purification of the labeled IgG by gel filtration, an aliquot of IgG−TMRI was applied to the column previously loaded with patulin derivative **4** or **5**. The column was washed with buffer, and the fluorescence emission was monitored at $\lambda = 575$ nm (excitation at $\lambda = 550$ nm). When the signal returned to the preloading IgG−TMRI level, samples of patulin (0, 10, and 100 $\mu g \cdot L^{-1}$) in 2.0 mL of binding buffer, PBS (0.1 M), NaCl (0.1 M), pH 7.4, were applied to the column and incubated for 10 min at room temperature: 0.5 mL fractions were collected, and the fluorescence emission was measured, providing a measure of the IgG−TMRI complex released and, in turn, the amount of free patulin present in the sample. Each experiment was performed in triplicate on each affinity column. Representative peaks of fluorescence are shown in Figure 5 for the experiments performed with the column on which P-Ins-HS was immobilized. Similar results were obtained for the derivative P-Sat-HS. A concentration of patulin of 10 $\mu g \cdot L^{-1}$ is easily detectable by this technique. To test the specificity of the method, the antibiotic neomycin was also incubated with the matrix−IgG−TMRI complex under the same conditions used for patulin. In this case, as expected, no fluorescence emission at $\lambda = 575$ nm was detected.

CONCLUSIONS

The polyclonal antibodies produced against the mycotoxin patulin, by means of two synthetic derivatives of the molecule conjugated to bovine serum albumin as the carrier protein, after affinity purification, showed a high titer for the recognition of the haptene. The immunoglobulins were successfully used to develop a competitive immunofluorescent assay for the detection of small quantities of patulin. The method was based on the binding of fluorescently labeled antibodies to the synthetic patulin derivative, covalently immobilized on a Sepharose matrix. The displacement of the antibodies by addition of free patulin was detected as a fluorescence signal at the emission wavelength of the fluorophore. The assay allowed the detection of 10 $\mu g/L$ patulin and thus represents a promising basis for the development of an alternative methodology compared to the analytical chromatographic techniques in use.

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