APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2002, p. 1524–1533 Vol. 68, No. 4
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.4.1524–1533.2002

Uric Acid Is a Genuine Metabolite of *Penicillium cyclopium* and Stimulates the Expression of Alkaloid Biosynthesis in This Fungus†

Florian Helbig, Jörg Steighardt, and Werner Roos*

Department of Cellular Physiology, Institute of Pharmaceutical Biology, University of Halle, Halle, Germany

Received 13 August 2001/Accepted 27 January 2002

On searching for endogenous, low-molecular-weight effectors of benzodiazepine alkaloid biosynthesis in *Penicillium cyclopium* uric acid was isolated from ethanolic or autoclaved mycelial extracts of this fungus. The isolation was based on a three-step high-pressure liquid chromatography procedure guided by a microplate bioassay, and uric acid was identified by mass spectrometry and the uricase reaction. Conidiospore suspensions that were treated with this compound during the early phase of outgrowth developed emerged cultures with an enhanced rate of alkaloid production. Uric acid treatment did not increase the in vitro measurable activity of the rate-limiting biosynthetic enzyme, cyclopeptine synthetase. However, these cultures displayed a reduced rate of uptake of the alkaloid precursor L-phenylalanine into the vacuoles of the hyphal cells as assayed in situ. It is suggested that the depressed capacity of vacuolar uptake caused by the contact of outgrowing spores with uric acid liberated from hyphal cells results in an enhanced availability of the precursor L-phenylalanine in the cytoplasm and thus accounts at least in part for the increase in alkaloid production.

Fungal cultures provide suitable eukaryotic research objects of low complexity for studies on diffusible signal molecules that either function within programs of cell specialization (12, 44) or are involved in symbiotic or defense relationships (15, 17). Such investigations may reveal interesting homologies or differences with signal steps of either prokaryotes (3, 19) or higher organisms (39, 46) and thus can aim at understanding evolutionary aspects of signal transfer as well as secondary metabolism (17, 20, 43).

For several pheromones (e.g., **a**- and α-factor in *Saccharomyces*, peptides a1 and a2 in *Ustilago*) and morphogenic factors (e.g., cyclic AMP, DIF-1, and discadenin in *Dictyostelium*), it is established that these primary signals are transferred via networks of protein phosphorylation into distinct patterns of gene activation (for reviews, see references 2, 5, 6, and 7).

Much less is known about the identity and mode of action of signal compounds that act on the expression and specific activity of distinct branches of secondary metabolism in fungi. Among the few examples are long-known stimulatory effects of precursors like that of α-aminoadipic acid and lysine on the expression of β-lactam biosynthesis in *Penicillium* or *Aspergillus* (24, 31), of methionine on the transcription of genes encoding enzymes of the cephalosporin biosynthesis in *Acremonium* (42), and of end products like the benzodiazepine alkaloid cyclopenin in *Penicillium cyclopium*, which stimulates enzyme activities involved in the biosynthesis of its precursor amino acids (35).

The present paper reports on the identification of uric acid in *Penicillium cyclopium* and its functioning as a stimulator of the biosynthesis of benzodiazepine alkaloids in the same fungus. These alkaloids are formed after a phase of intensive hyphal growth and the expression of the biosynthetic enzymes is coordinated with other events of cell specialization like the formation of conidia (29, 25, 36). It has long been argued that heat-stable, low-molecular-weight (low-MW) signal molecules are involved in the expression of alkaloid biosynthesis as mycelial extracts obtained from this fungus and added to outgrowing conidiospores caused an elevated rate of alkaloid biosynthesis in cultures developing from these conidia (4, 47).

Benzodiazepines are a group of secondary products that occur in some filamentous fungi and actinomycetes (26, 36). Among the natural benzodiazepines and related alkaloids are compounds of increasing pharmacological and therapeutic interest like asperlicin, an antagonist of the human hormone cholecystokinin (11) and 3-*O*-methyl viridicatin, a strong inhibitor of the tumor necrosis factor alpha (TNF-α)-induced replication of human immunodeficiency virus (HIV) (18).

The main alkaloids of *Penicillium cyclopium* are cyclopenin and cyclopenol (Fig. 1). They can be converted into the quinolines viridicatin and viridicatol, respectively, by the enzyme cyclopenase present in the conidia (45). Both cyclopenin/cyclopenol and viridicatin/viridicatol are of ecological significance due to their phytotoxic and antimicrobial properties (8, 41).

MATERIALS AND METHODS

Chemicals. All chemicals used were of analytical grade, and the solvents were of high-pressure liquid chromatography (HPLC) grade. Enzymes were purchased from Boehringer, Mannheim, Germany; U-[^{14}C]L-phenylalanine was from Amersham Buchler, Braunschweig, Germany.

Fungal culture. *Penicillium cyclopium* Westling, strain SM 72a, was cultured at 24°C in a minimal medium containing sucrose, glucose, and ammonium tartrate as C and N sources, and the pH was adjusted to 5.5 (32). The strain was maintained on agar slants, from which conidiospores were scraped off and used for inoculating either submerged or emerged growing cultures.

Submerged mycelium was produced in 500-ml Erlenmeyer flasks containing 150 ml of nutrient solution inoculated with 5×10^7 spores/ml on gyrotary shakers

* Corresponding author. Mailing address: Department of Cellular Physiology, Institute of Pharmaceutical Biology, University of Halle, 06120 Halle, Germany. Phone: 0345 5525010. Fax: 0345 5527006. E-mail: roos@pharmazie.uni-halle.de.

† Dedicated to Martin Luckner on the occasion of his retirement.

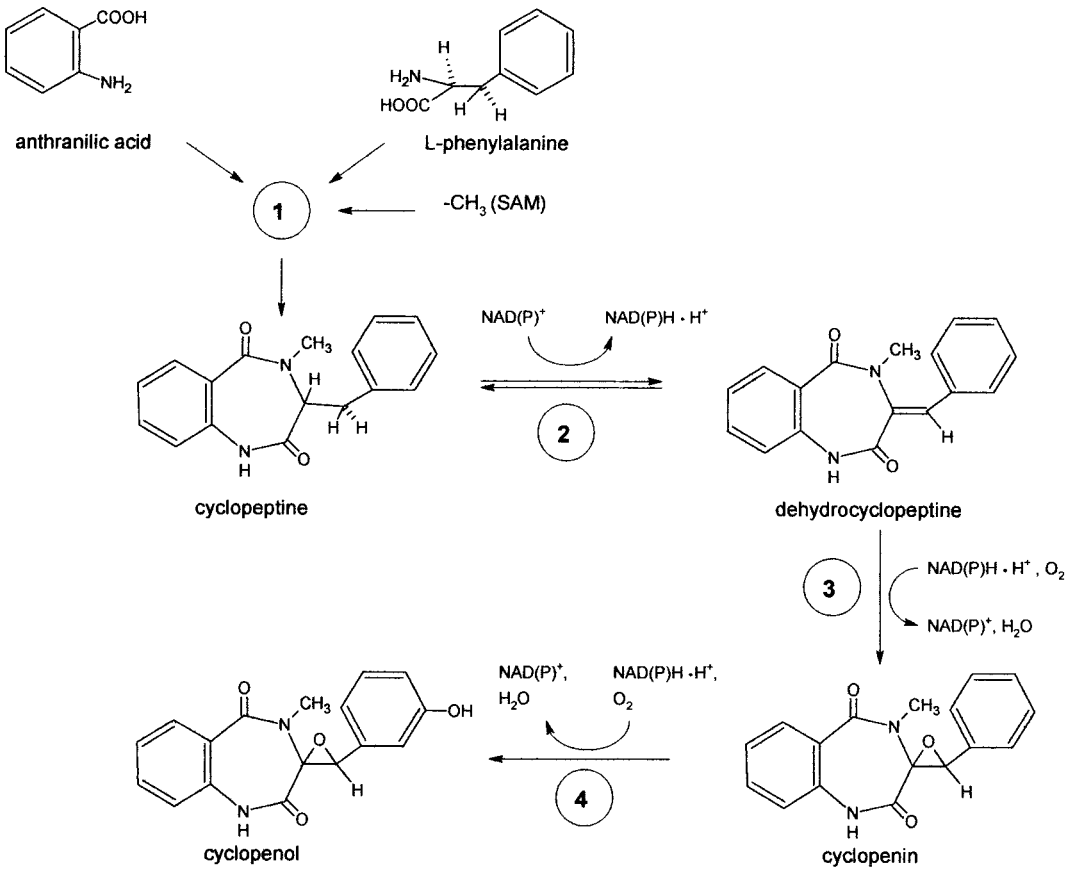

FIG. 1. Biosynthesis of benzodiazepine alkaloids in *P. cyclopium* (according to Luckner 1980). Enzyme activities involved are: 1, cyclopeptine synthetase; 2, cyclopeptine dehydrogenase; 3, dehydrocyclopeptine epoxidase; and 4, cyclopenin *m*-hydroxylase. SAM, *S*-adenosylmethionine.

at 120 rpm over 48 h of culture. Emerged cultures were grown in petri dishes of 14-cm diameter (50 ml of culture liquid, 8×10^7spores/ml).

Microplate assay of alkaloid production. Conidial suspensions in culture liquid (about 6×10^7spores/ml) were passed through a glass filter of about 50-μm pore diameter, divided into 250-μl portions, and cultured in a 96-well microplate with a filter bottom (MultiScreen; Millipore, Bedford, Mass.).

Solutions (50 μl) containing potential effectors of alkaloid production (mycelial extract, HPLC fractions, or uric acid at the specified concentrations, pH adjusted to 5.5) were added at the time of inoculation.

The minicultures that had developed during 7 days of emerged growth at 24°C were filtered by suction (microplate handler Event; Millipore), and 100 μl of the filtrates was transferred to a heat-resistant microplate (PS-type; Greiner, Hamburg, Germany) for the colorimetric determination of external benzodiazepine alkaloid content. This assay is based on an acid-catalyzed conversion of the benzodiazepines to quinolines that form a colored complex with Fe(III) (34). For this purpose, 100 μl of a ferric citrate reagent made from 120 g of $Fe(NH_4)_2SO_4$, 105 g of citric acid monohydrate, 100 ml of 37% HCl, and 900 ml of distilled water was added to each well, the plate was tightly covered by a Duraseal foil, heated to 80°C for 2 h, and cooled to room temperature. The optical density at 595 nm (OD_{595}) was read in a microplate reader (Bio-Rad 450) immediately after adding the reagent and after heating, and the difference was taken as a measure of benzodiazepine alkaloid content. The assay was calibrated with solutions of cyclopenin in culture medium. The alkaloid content of the mycelia was determined with the same procedure in supernatants obtained after overnight extraction from hyphal disks (about 1 ml of dimethyl sulfoxide per 5 mg of dry mass) at 50°C, followed by 10 min of centrifugation at 10,000 × *g*.

Preparation of mycelial extracts. Submerged-grown mycelium was suspended in distilled water (about 150 g of fresh weight [fwt]/liter), autoclaved at 121°C for 30 min, cooled to room temperature, and filtered through a 100-mesh nylon filter. The filtrate was concentrated on a rotary evaporator and then lyophilized. The lyophilisate was dissolved in water and ultrafiltered through Amicon YM membranes of 10-kDa cutoff, and the resulting filtrate was ultrafiltered again through YM membranes at 3-kDa cutoff. Then 1 ml of filtrate was equivalent to 50 mg of extracted dry mass. Alternatively, mycelia were extracted with ethanol at low temperature in order to avoid potential artifacts formed by autoclaving. For this purpose hyphal cells were collected by suction, washed three times with water, and immersed in 80% (vol/vol) ethanol (60 g of fwt/liter). After 2 h of gentle stirring at 4°C and subsequent centrifugation (5,000 × *g*, 4°C), the supernatant was filtered using a Millipore express unit (0.22-μm pore size) and finally lyophilized.

HPLC of mycelial extracts. The 3-kDa ultrafiltrate was subjected to a sequence of HPLC separations performed on a Gynkotek HPLC system equipped with a UV/VIS diode array detector.

(i) Cation exchange chromatography. The applied volume was 5 ml, the stationary phase was Nucleosil 100 SA, 20 μm (Muder & Wochele, Berlin), the column was 250 by 32 mm; the flow rate was 9.5 ml/min; eluent A was HCl at pH 3.0; and eluent B was NaCl at 1 M, used at the following gradient: 0, 0, 9, 100, 100, and 0% B at 0, 5, 25, 30, 50, and 55 min, respectively.

(ii) Reversed phase chromatography. The 9.50-ml fractions obtained in the first step were neutralized with NaOH, vacuum dried, dissolved in 300 μl of water and centrifuged (10,000 × *g*, 10 min); applied volume, 250 μl; stationary phase, silica gel RP 8 (Nucleosil C_8, 10 μm, VDS Optilab); column, 250 by 8 mm; flow rate, 5 ml/min; eluent A, 0.1% (wt/wt) tetrahydrofuran; eluent B, acetonitrile at the following gradient: 0, 0, 50, 50, 100, 100, and 0% at 0, 15, 25, 30, 40, 50, and 55 min, respectively.

(iii) Ion exclusion chromatography. The 10-ml fractions obtained in the second step were brought to dryness, dissolved in 100 μl of 14 mM H_2SO_4, and centrifuged (10,000 × *g*, 10 min); applied volume, 100 μl; stationary phase, Nucleogel ION 300 OA (Macherey & Nagel, Düren, Germany); column, 300 by 7.8 mm; flow rate, 320 μl/min; eluent, 14 mM H_2SO_4, isocratic; temperature, 75°C.

(iv) Desalting. Desalting of fractions obtained in either step was done by reversed phase HPLC; stationary phase, RP C_{18}, 5 μm (Macherey & Nagel); column, 250 by 4.6 mm; flow rate, 1 ml/min; eluent A, water; eluent B, acetonitrile at the following gradient: 0, 0, 100, 100, and 0% at 0, 7, 11, 15, and 18 min, respectively.

HPLC-coupled ESI-MS. Probes were applied to a reversed phase HPLC column (LiChrospher 100 RP 18 5 μm, 2 by 100 mm) and eluted isocratically at 0.2 ml/min with an acetonitrile-water mixture (1:9, vol/vol) on a constMetric 4100 HPLC instrument (Thermo Separation Products). Fractions were processed to a Finnigan TSQ 700 mass spectrometer and analyzed under the following conditions: electrospray voltage, 4.5 kV; nitrogen sheath gas; detection at 25 eV (negative ions) and −25 eV (positive ions); argon pressure, 0.27 Pa. EI-MS data were obtained from individual samples at an ionization energy of 70 eV.

Uricase assay of uric acid content. Purified samples (200 μl; HPLC fractions, uric acid, or allantoin solutions used as calibration standards) were mixed with 100 μl of a solution containing 10 mg of uricase (EC 1.7.3.3; specific activity, 5 to 10 U/mg of protein) per ml of phosphate buffer (pH 8.5, 50 mM) and incubated for 20 min at room temperature. The reaction products were separated by reversed phase HPLC, stationary phase: silica gel RP 8 (Nucleosil C_8, 10 μm, VDS Optilab), isocratic elution with acetate buffer (pH 4.2, 50 mM) at 1 ml/min and 30 °C. Identification of peaks was assisted by diode array spectra. Allantoin was quantified via the height of its OD_{214} peak (retention time, 2.65 min), which was calibrated to concentration with solutions of authentic allantoin.

Crude samples (mycelial extracts) were lyophilized and resuspended to 100 mg dry weight (dwt)/ml of sodium carbonate buffer, pH 10.0. After removal of insoluble material by filtration through Millipore Ultrafree microcentrifuge filters (0.22-μm cutoff), 4 to 20 μl of the filtrate was mixed with 380 μl of the same buffer containing 100 μM lucigenin. After 4 min, 20 μl of the aforementioned uricase solution was added, and the concentration of H_2O_2 produced was monitored luminometrically over 3 to 5 min (Clinilumat; Fa. Berthold), integration time 10 s. The method was calibrated via internal standards, and uric acid concentrations were calculated based on the initial rate of lucigenin oxidation.

Cyclopeptine synthetase assay and HPLC of benzodiazepine alkaloids. Emerged grown, 6-day-old mycelium (about 150 cm^2 of culture area) was separated from the culture liquid, the conidiospores were brushed off, and the hyphal pads were rinsed with water, immediately frozen in dry ice, and lyophilized. The lyophilisate was ground with dry ice and then stirred at 4°C in Tris-HCl buffer (pH 7.5), 200 mM, containing phenylmethylsulfonyl fluoride (PMSF), 50 mg/liter, and 2-mercaptoethanol, 50 μl/liter. After centrifugation (10,000 × *g*, 15 min) the supernatant was subjected to $(NH_4)_2SO_4$ fractionation: the first precipitation occurred at 35% saturation with $(NH_4)_2SO_4$, and the second sediment was obtained with the first supernatant brought to 65% saturation. Precipitations at 0°C over 45 min were collected by centrifugation at 12,000 × *g* for 20 min.

The second sediment was dissolved in Tris-HCl buffer (pH 7.5, 50 mM, containing 10% sucrose [wt/vol]), and 100 μl of this preparation was assayed for its ability to synthesize cyclopeptine. The reaction mix contained 1 mM anthranilic acid, 1 mM *S*-adenosylmethionine, 1 mM U-[^{14}C]L-Phe, 1 mM dithiothreitol (DTT), 10 mM KCl, 2 mM ATP, 2 mM ADP, 4 mM $MgSO_4$, 1 mM phosphoenolpyruvate, and 100 μkat of pyruvate kinase in a total volume of 120 μl. (The last four components constitute an ATP regenerating system that proved to maintain the ATP level above 1 mM.) After 20 min of incubation at 35°C, the reaction was stopped by adding 50 μl of acetone, and the mixture was centrifuged for 20 min at 12,000 × *g*. Then 10 μl of the supernatant was subjected to reversed phase HPLC; stationary phase, Nucleosil 120-5C18 (Macherey & Nagel); flow rate, 1 ml/min at 30°C; eluent A, water; eluent B, acetonitrile, used at the following gradient: 0, 0, 35, 50, 100, 100, 0, and 0% at 0, 1, 4, 12, 15, 19, 21, and 30 min, respectively.

Detection and quantification of the produced [^{14}C]cyclopeptine were done by simultaneous monitoring of radioactivity and OD_{235}, respectively; for this purpose a flowthrough detector (Berthold, Germany) was attached to the HPLC system.

For the separation of cyclopenin, cyclopenol, and dehydrocyclopeptine, these alkaloids were extracted from culture filtrates and mycelia with ethylacetate. After evaporating the solvent in vacuum, the residue was dissolved in acetonitrile and subjected to the same HPLC procedure as described above. Quantification was based on OD_{235} and authentic alkaloids were used as standards.

Determination of phenylalanine and anthranilic acid content in the culture. Ethanolic mycelial extracts were prepared as described above and lyophilized, and the residue was dissolved in citrate buffer, 10 mM, pH 2.2. Samples were applied to an amino acid analyzer LC 3000 (Biotronic), separated on cation exchange resins (divinylbenzene-polystyrene-copolymer, 125 by 4 mm), and peaks were detected by postcolumn derivatization with ninhydrin. Quantification of phenylalanine and anthranilic acid via the respective peak areas was based on comparison with the authentic compounds added as internal standards.

Assay of vacuolar Phe transport. The plasma membrane of hyphal cells was permeabilized by nystatin treatment (5 μg of nystatin/ml for 6 min) as described elsewhere in detail (33). Permeabilized hyphae were resuspended (4 mg fwt/ml) in 60 mmol of sodium morpholineethanesulfonic acid (Na MES) buffer, pH 7.0, with 0.4 mol sorbitol, 0.15 mol KCl, and 1 mmol MgATP per liter. Intact hyphae (used in reference experiments) were resuspended in 60 mmol maleate buffer, pH 3.5, per liter. The suspension was gently aerated through a glass sinter funnel, and after 1 min unlabeled L-Phe (final concentration 50 μmol/liter) together with U-[^{14}C]-L-Phe (final concentration, 1 μmol/liter) and effectors were added. Labeled L-Phe accumulated in the vacuoles was determined in 5-ml samples that were rapidly injected into 10 ml of the same buffer kept on ice, sucked onto cellulose nitrate filters (1.2-μm cutoff), and washed with 15 ml of this buffer (intact cells) or of 0.7 mol sorbitol/liter (permeabilized cells) at 0°C. The cell pellet was then immersed in 2 ml of 80% (vol/vol) ethanol, shaken for 2 h, centrifuged at 5,000 × *g* for 10 min, and radioactivity was counted in 1 ml of the supernatant by a liquid scintillation counter (Hewlett Packard LS).

In order to correct for ATP-independent uptake or binding, blank experiments were run without MgATP. Furthermore, the different pH optima of transport by in situ vacuoles and intact hyphae (around 7.0 and 3.5, respectively [33]) were routinely used as a criterion to check the efficiency of plasma membrane permeabilization. The rate of ATP-independent uptake at pH 3.5 compared to the uptake in the presence of MgATP at pH 7.0 can be roughly considered as the share of nonpermeabilized hyphal cells. This percentage was usually below 5%; suspensions with a lower degree of permeabilization were discarded.

At least 90% of the radioactivity extracted by 80% ethanol from intact cells after 80 min of incubation with U-[^{14}C]L-Phe were chromatographically identical with phenylalanine, as confirmed by reversed phase HPLC amino acid analysis.

Protease treatments were performed according to the manufacturer's instructions.

RESULTS

Screening for signal molecules of alkaloid formation. Emerged cultures of *Penicillium cyclopium* react with an accelerated onset of alkaloid formation if low-MW compounds obtained by autoclaving mycelial suspensions were added to the conidiospores at the time of inoculation. As shown in Fig. 2, the mycelial extract causes an increased extent of alkaloid formation in batch cultures of 4 to 9 days postinoculation (p.i.), whereas control cultures reach the same maximum alkaloid content only 1 to 2 days later. The separation and identification of signal molecules that selectively stimulated the expression of alkaloid biosynthesis without influencing growth rate or sporulation (termed alkaloid-stimulating activity in the following) proved difficult due to the presence of a multitude of compounds with either stimulating or inhibitory effects on alkaloid formation and overlapping influences on the development of conidia and hyphal growth (4, 47).

Under the conditions used, only emerged-grown mycelia or conidiospore suspensions produced benzodiazepine alkaloids, while low-MW fractions with alkaloid-stimulating activity could be extracted from both emerged- and submerged-grown mycelia. Isolation of effector molecules started from submerged-grown cultures because of their higher growth rate and the absence of conidiospores whose pigments otherwise complicated separation. An essential part of the purification protocol was a newly developed microplate-based bioassay that allowed a high throughput of samples to be tested for their influence on alkaloid production of emerged-grown minicultures (see Materials and Methods).

A <3-kDa ultrafiltrate of an aqueous mycelial extract was subjected to a sequence of three HPLC runs. Cation exchange HPLC resulted in one major fraction with alkaloid-stimulating activity that was separated from most but not all of the growth-

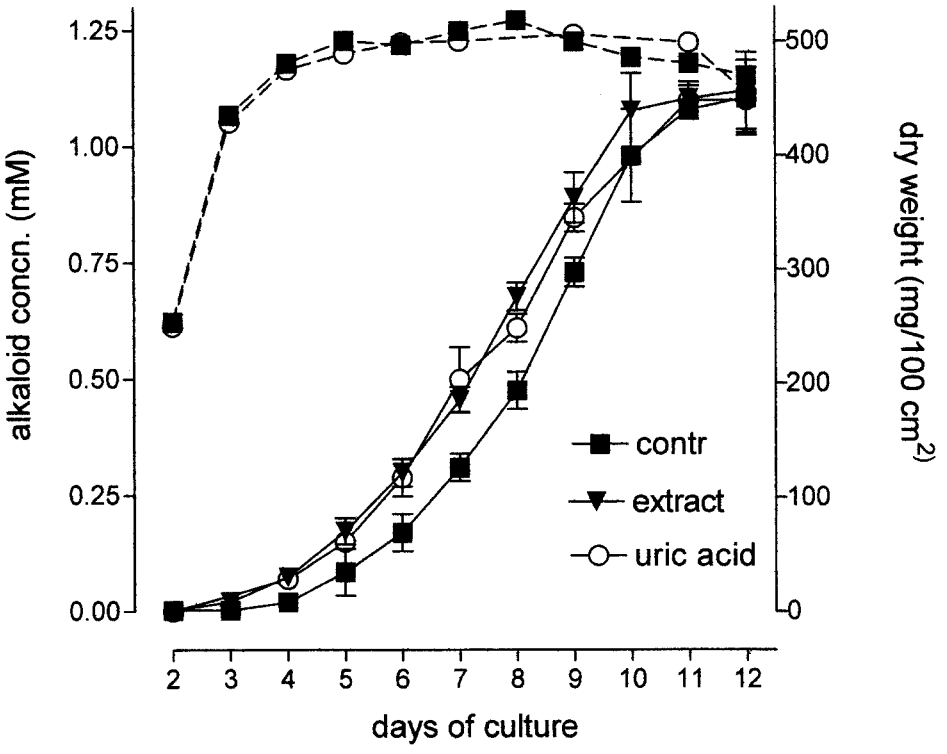

FIG. 2. Alkaloid concentration and biomass during the development of emerged cultures under the influence of mycelial extract or uric acid. Mycelial extract (3-kDa ultrafiltrate, diluted 1:4 with culture liquid; final uric acid concentration, 1.1 μM) or uric acid (final concentration, 1 μM) was added immediately after inoculation. At the times indicated, 100 μl of culture filtrate was withdrawn and spotted on microplates, and the alkaloid content was determined colorimetrically as described for the microplate assay (see Materials and Methods). Data are means with SD for three different emerged cultures inoculated with the same conidiospore suspension. Alkaloid concentrations (solid lines, left axis) are corrected for differences in the biomass that were observed only between cultures treated with mycelial extract and other cultures. Dry mass: dotted lines, right axis (SD ≤10%).

stimulating activity. This fraction on subsequent reversed phase chromatography yielded two active peaks that did not stimulate growth. Both peaks could be further separated by ion exclusion HPLC, which yielded fractions with alkaloid-stimulating but also -inhibiting activity (Fig. 3). The most active fraction (termed I2-6) was finally purified (desalted) by reversed phase HPLC and yielded a homogeneous peak at OD_{254}.

As a further indication of purity, this fraction contained no peptide compounds (which were present in most other fractions), as indicated by the absence of 9-fluorenylmethyl-chloroformate- or *N*,*N*-dimethylamino naphthylsulfonyl-reactive amino acids after acid hydrolysis or after protease treatment (proteinase K, endoproteinase Glu-C, carboxypeptidase P, and acylaminoacid peptidase). Neither of these protease treatments reduced the alkaloid-stimulating activity of the low-MW ultrafiltrates.

Identification of uric acid. ESI-mass spectrometry of the purified fraction I2-6 yielded a mole peak at $m/z = 168$ (167 at negative, 169 at positive ionization) that gave rise to daughter ions of $m/z = 124$, 96, and 42.5. Further fragment ions appeared at $m/z = 83$, 69, 60, and 44. A comparison of these data with the MS fragmentation patterns of authentic compounds yielded a high degree of coincidence with uric acid.

Authentic uric acid that was added to fraction I2-6 comigrated during reversed phase HPLC with the aforementioned peak measurable at OD_{254}. The identification of uric acid was confirmed by using the high specificity of the uricase enzymatic reaction. During incubation of the above fraction with this enzyme, the product allantoin appeared in the reaction mixture, while the original peak disappeared (shown by reversed-phase HPLC, Fig. 4).

Once identified, uric acid could be quantified via the uricase reaction in the crude, ultrafiltered extracts of hyphal cells. Autoclaving and ethanol extraction at 4°C yielded identical uric acid contents, and thus an artifactual formation of this compound by heat treatment of the fungal biomass can be excluded. The enzymatic assay in the crude ultrafiltrates (obtained from either extract) resulted in 18 μg of uric acid per g (dry weight) of the extracted mycelium. Similar contents were found in ethanol extracts of submerged (i.e., non-alkaloid-producing) or of emerged (i.e., alkaloid-producing) cultures. Our multistep isolation procedure yielded 1.4 μg of uric acid per g (dry weight), thus indicating a final yield of around 8% of the pure compound.

Uric acid stimulates the expression of alkaloid biosynthesis. Several lines of evidence indicate that uric acid acts as a positive signal on the expression of alkaloid biosynthesis if it is present externally during an early stage of culture development.

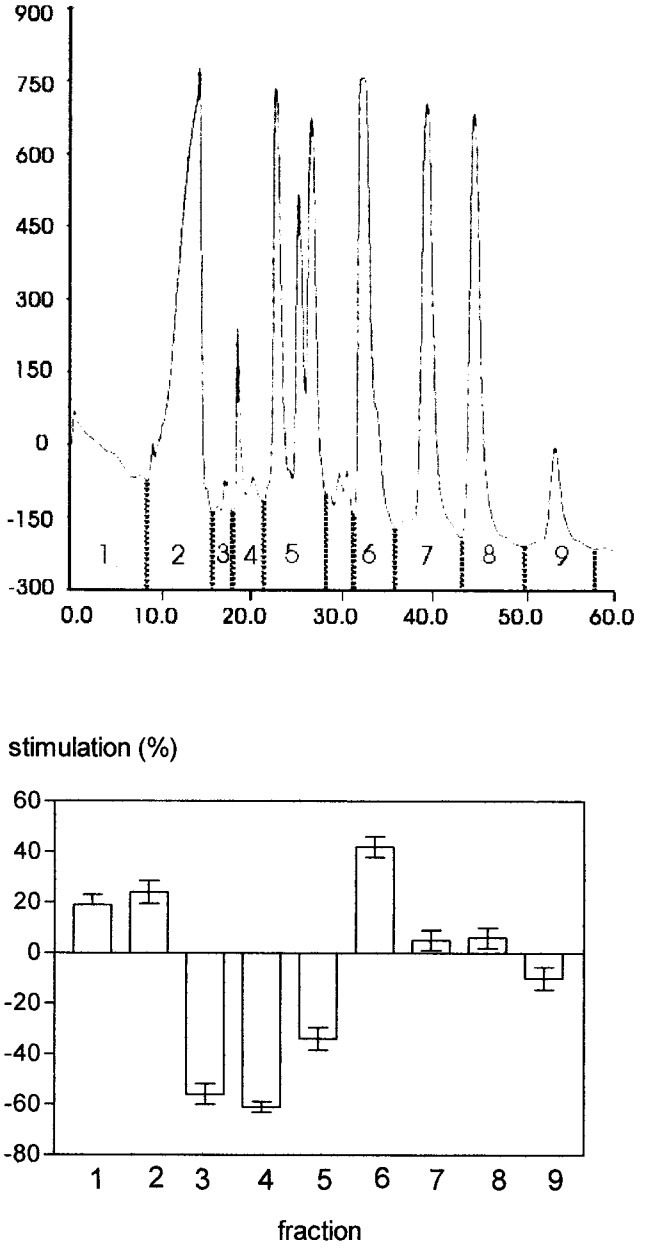

FIG. 3. Ion exclusion HPLC of compounds affecting the expression of alkaloid biosynthesis. A peak fraction with the main alkaloid-stimulating activity that had previously been obtained by reversed phase HPLC was separated on a Nucleogel ion exclusion column. Upper panel: a typical elution profile. Ordinate: OD_{220}; abscissa, retention time (minutes); numbers indicate fractions that were collected and screened for their influence on alkaloid formation. Lower panel: activities of the fractions indicated above, as measured by the microplate assay for the expression of alkaloid biosynthesis (see Materials and Methods for details).

Uric acid, fraction I2-6, and the crude mycelial extract all exerted an alkaloid-stimulating activity if one of these effectors was present in the external medium during a period of competence in the first 24 h of culture development (Fig. 5). Within this time window, a minimum length of effector contact of 1 h was sufficient to give rise to cultures with an enhanced rate of alkaloid production, as shown by rapid filtering and resuspending of the effector-treated conidia in fresh culture liquid (Fig. 5). During the 1-h period of incubation, the uric acid content of a 1 μM bathing solution was not significantly diminished, indicating that no substantial uptake or metabolization of this compound was required for the observed stimulation of alkaloid productivity. This is in line with earlier findings showing that nutrient solutions containing the ultrafiltrate could be used repeatedly without losing the alkaloid- stimulating activity.

Uricase treatment totally abolished the alkaloid-stimulating activity of fraction I2-6 (Fig. 5).

The uric acid content of fraction I2-6 was around 0.77 mmol/liter (assayed by uricase treatment; see Materials and Methods). Addition of this fraction to the culture liquid of emerged cultures at an estimated final concentration of 1 μM caused about 40% stimulation of total alkaloid production and a 20% stimulation of the external alkaloid content. This stimulation was fairly identical with that exerted by uric acid at the same final concentration (Fig. 5 and 6).

On a large scale, the alkaloid-stimulating activity of uric acid shows a bimodal concentration dependence (Fig. 6). At 1 μM, a maximum stimulation of about 42% was observed, whereas concentrations in the range between 2 and 100 μmol/liter displayed little or no effect. Surprisingly, 1 mM uric acid triggered a stimulation of alkaloid production similar to that seen with 1 μM. The same tendency is true for the extracellular alkaloids. The uric acid concentration of our crude ultrafiltrate was around 5 μM, as determined by the uricase assay, and its final concentration in the routine test was diluted to about 1 μM (Fig. 2). As the volume of mycelial extract prepared from a given biomass was adjusted to roughly equal the liquid of a culture that had produced the same biomass, it appears that concentrations in the micromolar range are responsible for the alkaloid-stimulating activity of the mycelial extracts. Pretreatment of conidiospores with 1 μM uric acid had no effect on the growth rate (Fig. 2) or on the external pH of the developing test cultures.

Seen together, the above data strongly suggest that a signaling effect exerted by external uric acid in the early phase of culture development is responsible for the alkaloid-stimulating activity of the most potent low-MW fraction isolated from *P. cyclopium* cells.

Search for potential targets of uric acid in the stimulation of alkaloid biosynthesis. (i) Cyclopeptine synthetase, the first committed enzyme of the biosynthetic chain. The initial step of alkaloid biosynthesis is catalyzed by the enzyme cyclopeptine synthetase, a multienzyme complex that catalyzes the ATP-dependent formation of two peptide bonds between phenylalanine and anthranilic acid, together with the methylation of the NH_2 group of Phe (Fig. 1). This reaction represents the most ATP-consuming step of alkaloid formation, as it includes the chemical activation of both precursor amino acids via aminoacyl adenylates (14). The formation of cyclopeptine most probably limits the overall rate of cyclopenin biosynthesis, as this intermediate was found in alkaloid-producing cultures only in minute amounts (<0.1% of total alkaloids) compared to the subsequent metabolites dehydrocyclopeptine (1 to 2%) and cyclopenin/cyclopenol (100%). In our experiments, the cyclopeptine content, if detectable at all, did not measurably in-

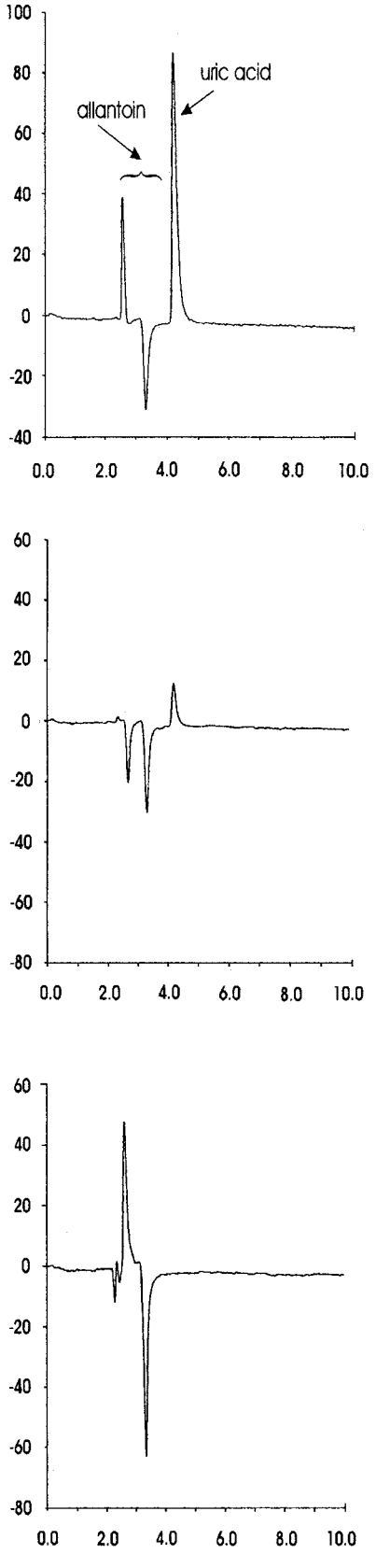

FIG. 4. Conversion of fraction I2-6 by uricase and separation of uric acid and allantoin by reversed phase HPLC. Upper panel: separation of authentic uric acid and allantoin (reference). Middle panel: fraction I2-6, 30 s after adding uricase (some allantoin has already been formed). Lower panel: fraction I2-6 incubated with uricase for 20 min (all uric acid disappeared, only allantoin is present). Uricase treatment was done under the conditions used for the uric acid assay (see Materials and Methods). Elution profiles were recorded at OD_{214} (ordinate, instrument units). Abscissa: retention time in minutes. The peak indicative of uric acid also appeared at 294 nm; at this wavelength, allantoin shows no absorbance.

crease in cultures derived from uric acid- or I2-6-treated conidia compared to control cultures.

In the present study, the activity of cyclopeptine synthetase was assayed by monitoring the accumulation of the end product (see Materials and Methods). As shown in Table 1, the in vitro measurable activity of this enzyme was not significantly enhanced in cultures derived from uric acid- or I2-6-treated spores compared to control cultures. Furthermore, the presence of uric acid during the in vitro assay did not influence the rate of cyclopeptine formation by this enzyme. Thus, the stimulatory effect of uric acid on alkaloid formation is unlikely to involve an increased amount or altered catalytic capacity of the rate limiting biosynthetic enzyme.

(ii)Vacuolar transport of the precursor phenylalanine. On searching for alternative targets of uric acid in the stimulation of alkaloid formation, the availability of amino acid precursors for the biosynthetic process was investigated. The total amount of precursor amino acids that was extractable with ethanol from 7-day-old mycelia was not increased in cultures treated with 1 μM uric acid: their Phe content ranged between 80 and 72% of the level found in standard cultures (about 3 ng/mg of protein), whereas in the case of anthranilic acid, only trace amounts were detectable (around 0.1 ng/mg of protein) that showed no significant difference between uric acid-treated and standard cultures. While these total contents would not reflect shifts of local cytoplasmic precursor concentrations, we attempted to get indications of such changes from assays of intracellular Phe transport.

From earlier experiments with *Penicillium cyclopium* it is known that the precursor Phe is highly concentrated in the vacuole (30, 33) and is channeled from this central pool to the site of biosynthesis in the cytoplasm (28). In contrast, anthranilic acid is not accumulated in the vacuole but needs to be supplied from cytoplasmic sources. This has been established in conidiospores whose plasma membrane was selectively permeabilized for micromolecules by polyene treatment, which left the vacuole functionally intact and allowed studies of vacuolar accumulation and alkaloid formation in situ. These cells were able to accumulate Phe but not anthranilic acid, and the accumulated Phe was incorporated into alkaloids only after anthranilic acid was supplied in the medium (30).

The short phase of alkaloid biosynthesis shown by conidiospores (within 3 h after resuspending in water) was not influenced by the mycelial extract (H. Pechstädt and W. Roos, unpublished data). In hyphal cells, the plasma membrane can likewise be permeabilized for micromolecules by nystatin treatment, and the vacuolar transport measured in situ was shown to maintain regulatory mechanisms that otherwise could be lost during the isolation of the vacuole (33). Such experiments have revealed that the extravacuolar concentration of purine

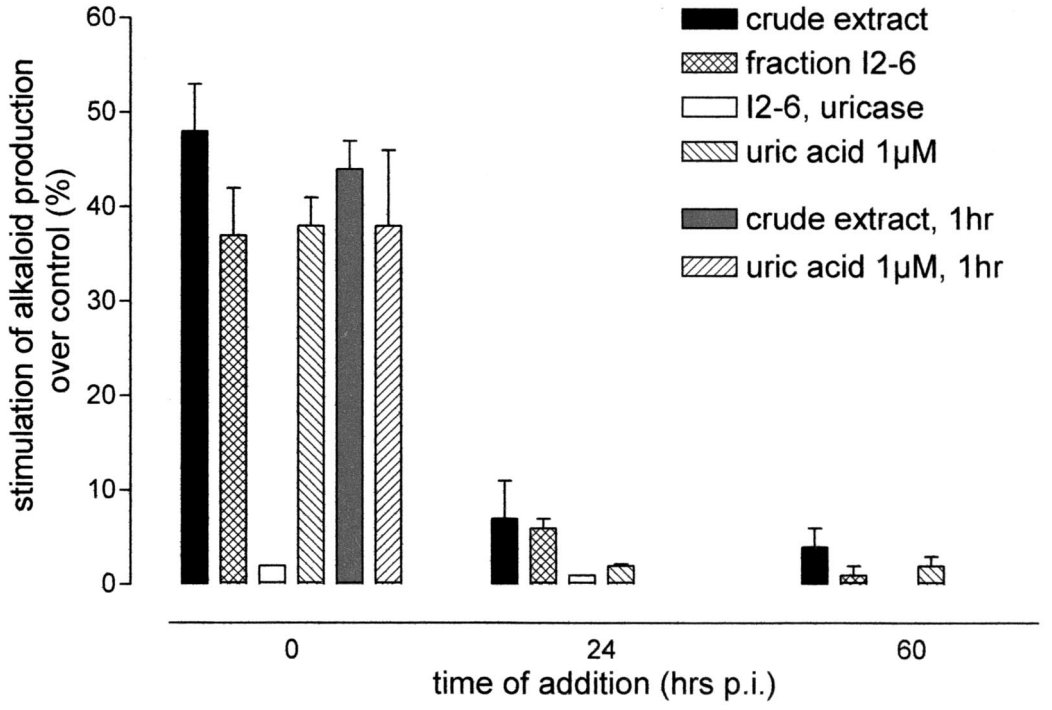

FIG. 5. Stimulation of alkaloid biosynthesis of emerged-grown cultures by mycelial extract, fraction I2-6, or uric acid. Conidial suspensions in 300 μl of culture liquid were grown in microplate compartments. At the times indicated they received the 3-kDa ultrafiltrate, fraction I2-6, 1 μM uric acid, or fraction I2-6 after uricase treatment, as marked. Mycelial extract and fraction I2-6 were diluted to 1 μM uric acid. The mycelial extract added represented low-MW material extracted from 9.3 mg (dry weight); the final uric acid content was 1 μM. Fraction I2-6 was also present at a final concentration of 1 μM uric acid. Uricase treatment was done under the conditions used for the uric acid assay (see Materials and Methods). Additional columns of the time-zero experiment (termed 1 h) represent minicultures grown from conidiospores that were treated for 1 h with either mycelial extract or 1 μM uric acid, filtered, and resuspended in fresh culture liquid prior to incubation in the microtiter plates. Total alkaloid content was assayed after 8 days of culture as described in Materials and Methods. All data are means with SD, $n = 8$ wells.

and pyrimidine nucleotides specifically regulates the efflux of preaccumulated phenylalanine into the cytoplasm (33, 38). The minimum structural requirement for this control function was found to be a noncyclic mononucleotide, i.e., nucleic acid bases and their analogs were inactive. It was therefore not unexpected to find that uric acid in concentrations between 1 μM and 1 mM did not influence the release of preaccumulated Phe from the vacuoles in situ (38).

In the present study, we investigated the effects of uric acid treatment on the uptake of radiolabeled L-Phe into the vacuoles. While the presence of extravacuolar uric acid during the uptake assay had no effect on Phe transport (data not shown), vacuoles of hyphal cells that had developed from uric acid-treated conidia displayed a depressed rate of phenylalanine uptake compared to vacuoles of standard cultures (Fig. 7). Hence it appears that contact of this compound with conidiospores within the phase of spore outgrowth exerts a negative influence on the expression of vacuolar transport capacity for L-Phe.

It is known that in *Penicillium cyclopium* the capacity of vacuolar accumulation strongly influences the size of the cytoplasmic pool and the availability of this amino acid in the cytosol, as shown by comparing efflux rates of preaccumulated Phe in intact cells and vacuoles in situ (33). A reduced activity of Phe uptake into the vacuole might therefore result in a higher availability of this alkaloid precursor at the cytoplasmic sites of biosynthesis and thus allow an enhanced rate of alkaloid formation. Such an idea is consistent with the finding that the inhibition of vacuolar Phe transport was caused by the same concentrations of uric acid (around 1 μM and around 1 mM, see Fig. 7) that stimulated alkaloid formation (Fig. 6).

DISCUSSION

Uric acid has long been known as a central product of purine degradation of higher animals that is excreted by humans, birds, and some reptiles or further degraded by other species (21, 40). In several fungi it has been characterized as a strong inducer of enzymes of purine catabolism, namely, xanthine oxidase and urate oxidase in *Aspergillus nidulans* (37), *Neurospora crassa* (27), and *Schizosaccharomyces* spp. (13), which is consistent with the ability of saprophytes to use uric acid as a substrate, e.g., *Histoplasma* (23) and *Hyphomyces* (22) spp. It is now well established that the expression of several structural genes involved in purine uptake and catabolism is subject to uric acid induction and mediated by the product of the positive regulatory gene *uaY* (9, 10, 16), including posttranslational modifications of the degrading enzymes (1).

The data presented here add two new facets to the biological functions of this many-sided molecule. (i) Uric acid is a genuine constituent of *P. cyclopium*, i.e., it is produced in the fungal cell itself. The mass spectrometric identification of uric

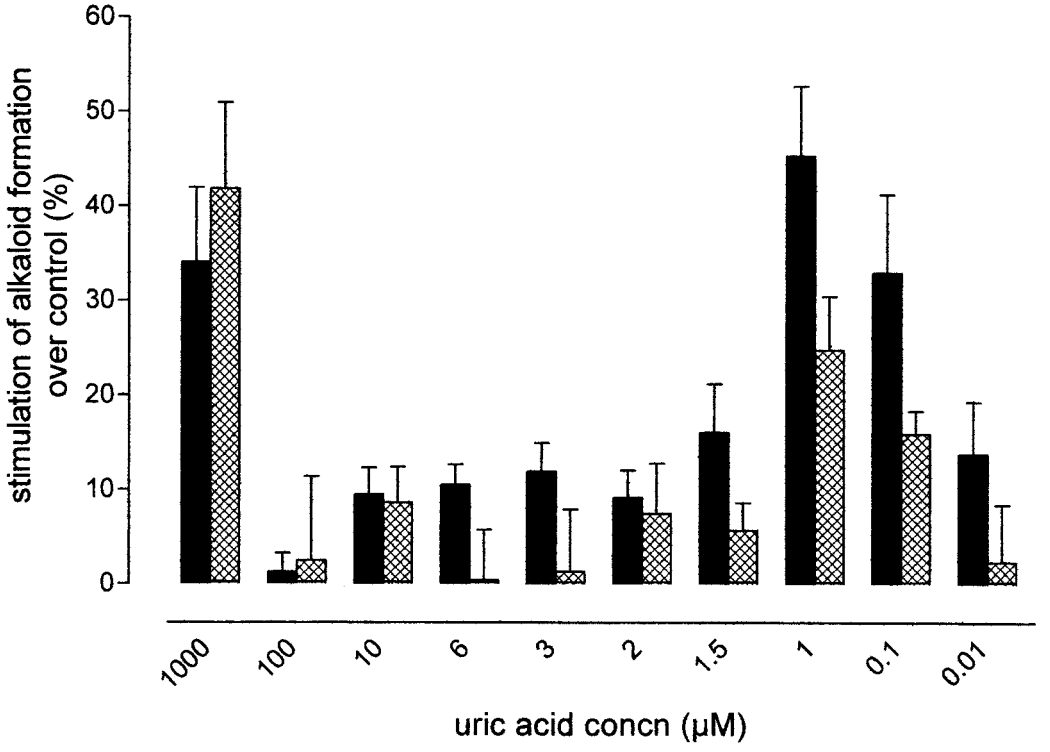

FIG. 6. Concentration dependence of uric acid stimulatory effects on alkaloid biosynthesis in emerged-grown cultures. Minicultures in 300 μl of culture liquid were grown in microplate compartments and received the indicated concentrations of uric acid at the time of inoculation. External alkaloid (hatched columns) was assayed in the individual culture filtrates after 7 days of emerged culture; in addition, the mycelial alkaloid was determined (see Materials and Methods). Total alkaloid (solid columns) represents the sum of mycelial plus external alkaloid. All data are related to the same amount of dry weight and are means with SD, $n = 14$ wells.

acid isolated via HPLC as well as the high selectivity of the uricase assay allow little doubt about the chemical identity of the isolated molecule. The detection of similar contents of this compound both in aqueous autoclaved cell suspensions and in ethanolic extracts obtained at low temperature confirms that substantial amounts accumulate in the mycelia (around 18 μg, i.e., 0.11 μmol per g [dry weight]).

(ii) A relatively short contact of outgrowing conidiospores with uric acid accelerates the onset of alkaloid production during the following development of emerged cultures. This effect could clearly be separated from the growth-promoting activity of other constituents of the crude mycelial extract. One attractive candidate for the molecular processes leading to

TABLE 1. Activity of cyclopeptine synthetase measured in vitro[a]

Expt/culture type	Enzyme activity (pkat/mg of protein) of uric acid concn (μM):					
	0	0.1	1	10	100	1,000
A/cultures developed from uric acid-treated spores	1.40	1.50	1.45	1.30	1.45	1.40
B/control cultures, uric acid present in enzyme assay	1.20	1.15	1.10	1.15	1.20	1.20

[a] Data are means with SD obtained from five emerged cultures inoculated with the same conidiospore suspension. Experiments A and B were performed with two different sets of cultures that were inoculated with different spore suspensions. SD, <6.2% (experiment A) and <9.2% (experiment B). Within each experiment, the data presented did not differ significantly (*t* test, $P = 95\%$).

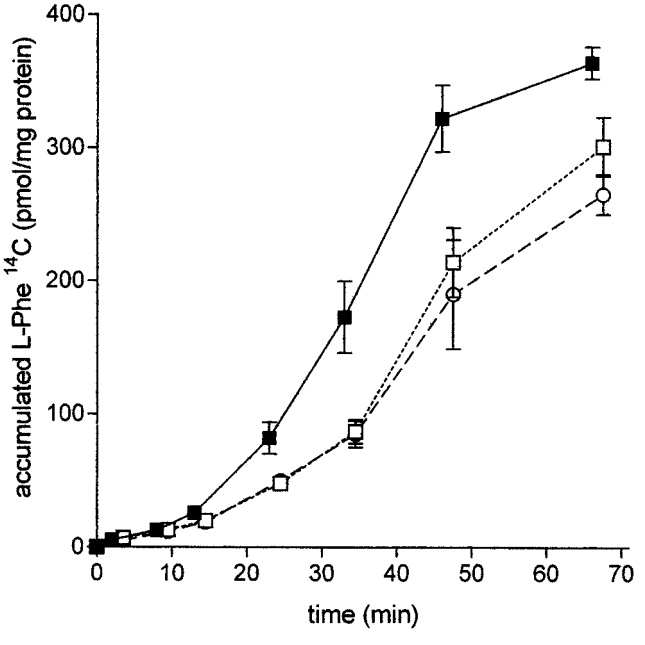

FIG. 7. Time course of L-Phe uptake by permeabilized hyphal cells. At the time of inoculation, submerged cultures received uric acid at 1 μM (□) or 1 mM (○) or no addition (■). After 48 h of growth, cells were harvested and selectively permeabilized by nystatin treatment, and the vacuolar transport of U-[^{14}C]L-Phe was assayed as described in Materials and Methods. Data are means with SD obtained from three different cell suspensions.

enhanced alkaloid biosynthesis is the expression of vacuolar amino acid transport systems. Uric acid acts as a modulator of intracellular amino acid transport by lowering the expression of the vacuolar accumulation capacity for L-phenylalanine at both of its alkaloid-stimulating concentrations. For this effect, the direct presence of uric acid at the vacuolar surface is not necessary, as shown by the lack of direct effects of this compound in both uptake and efflux assays in situ.

Since the net uptake of L-Phe into the vacuole is one of the processes that control the cytoplasmic level of this precursor amino acid (30, 33), reducing the uptake rate might well increase the cytoplasmic concentration of precursors and thus allow higher rates of alkaloid formation. The importance of local precursor supply is underlined by our finding that the rate-limiting enzyme activity of the alkaloid pathway (cyclopeptine synthetase) measured under saturating substrate concentrations in vitro was not changed by uric acid treatment. Of course, as not all cellular processes that influence precursor supply and alkaloid formation have been investigated, other potential sites of influence of uric acid on alkaloid biosynthesis also need to be considered. Among them should be, e.g., the impact of uric acid on the supply of the other precursors, i.e., anthranilic acid and *S*-adenosylmethionine.

The molecular details behind the newly found effects of uric acid appear as an attractive goal of further research not only in the present organism, but also in related peptide-producing fungi. For instance, one major question is whether the alkaloid-stimulating effect of uric acid is a consequence of the induction of purine-metabolizing enzymes and transporters (as known from *A. nidulans*) and the resulting metabolic changes, or whether it represents a novel, direct influence of this molecule on the expression of amino acid transport systems at the vacuolar membrane. The complex concentration dependence of the alkaloid-stimulatory effect (see Fig. 6) argues for more than one point of interaction of uric acid with the fungal secondary metabolism.

(iii) An intriguing biological feature of uric acid lies in the fact that this molecule needs to be present outside the conidia during an early phase of culture development in order to stimulate alkaloid formation. Under natural conditions that typically include discontinuous availability of nutrients and phases of strong starvation, the outgrowth phase of conidiospores proceeds in close neighborhood to the decay of the hyphal cells. In emerged batch cultures of *P. cyclopium*, during the later stages of development the hyphal mat is covered by a layer of conidia that obviously fell off the conidial chains carried by the conidiophorous cells. These spores are likely to come in contact with uric acid liberated from deceased hyphal cells. Thus, in the mycelium developing from these conidia, the ability for alkaloid formation should be induced or at least enhanced by uric acid pretreatment.

Although local concentrations of uric acid in the conidial layer of the emerged cultures are hard to determine, it can be roughly estimated that a hyphal mass of around 10 mg (dry weight) contains uric acid that would allow 1 ml of a 1 μM solution, i.e., a concentration that influences vacuolar Phe uptake and alkaloid biosynthesis. Whether the alkaloid stimulation shown experimentally by 1 mM uric acid (the concentration which is close to its solubility) can be of real significance in the fungal culture cannot yet be decided, especially because 1 μM and 1 mM uric acid show the same inhibitory effect on vacuolar Phe transport (Fig. 7).

(iv) Finally, it should be recognized that uric acid is not the only micromolecular effector of alkaloid biosynthesis present in mycelial extracts. Other positive-acting components are indicated by a difference between the alkaloid-stimulating activity of uric acid and of the <3-kDa ultrafiltrate; the latter was usually about 30% higher than the prediction, based on its uric acid content. Furthermore, during the course of HPLC fractionation, the presence of extract constituents with negative effects on alkaloid production became apparent (cf. the effects of fractions 3, 4, and 5 in Fig. 3). The strong inhibitory activity of these hitherto unknown compounds suggests that the actual rate of alkaloid biosynthesis is under the control of both positively and negatively acting low-MW regulators.

ACKNOWLEDGMENTS

The help of H. Kluge, Institute of Nutritional Sciences of this university, with the amino acid analysis is gratefully acknowledged. We thank Gabriele Danders and Kathrin Thomasch for expert technical assistance.

The work was supported by the Fonds der Chemischen Industrie.

REFERENCES

1. **Amrani, L., G. Cecchetto, C. Scazzocchio, and A. Glatigny.** 1999. The hxB gene, necessary for the posttranslational activation of purine hydroxylases in *Aspergillus nidulans*, is independently controlled by the purine utilization and the nicotinate utilization transcriptional activating systems. Mol. Microbiol. **31:**1065–1073.
2. **Bardwell, L., J. G. Cook, C. J. Inouye, and J. Thorner.** 1994. Signal propagation and regulation in the mating pheromone response pathway of the yeast *Saccharomyces cerevisiae.* Dev. Biol. **166:**363–379.
3. **Beppu, T.** 1995. Signal transduction and secondary metabolism: prospects for controlling productivity. TibTech **13:**264–269.
4. **Bley, B.** 1991. Isolation and amino acid composition of hormone-like signal molecules of secondary metabolism from *Penicillium cyclopium* WESTLING. Ph.D. thesis. Martin-Luther-University, Halle, Germany.
5. **Blusch, J., S. Alexander, and W. Nellen.** 1995. Multiple signal transduction pathways regulate discoidin I gene expression in *Dictyostelium discoideum*. Differentiation **58:**253–260.
6. **Burdine, V., and M. Clarke.** 1995. Genetic and physiologic modulation of the prestarvation response in *Dictyostelium discoideum.* Mol. Biol. Cell **6:**311–325.
7. **Cotter, D. A., T. W. Sands, K. J. Virdy, M. J. North, G. Klein, and M. Satre.** 1992. Patterning of development in *Dictyostelium discoideum*: factors regulating growth, differentiation, spore dormancy, and germination. Biochem. Cell Biol. **70:**892–919.
8. **Cutler, H. G., F. G. Crumley, R. H. Cox, J. M. Wells, and J. R. Cole.** 1984. The biological properties of cyclopenin and cyclopenol. Plant Cell Physiol. **25:**257–263.
9. **Diallinas, G., J. Valdez, V. Sophianopoulou, A. Rosa, and C. Scazzocchio.** 1998. Chimeric purine transporters of *Aspergillus nidulans* define a domain critical for function and specificity conserved in bacterial, plant and metazoan homologues. EMBO J. **17:**3827–3837.
10. **Diallinas, G., L. Gorfinkiel, H. N. Arst, G. Cecchetto, and C. Scazzocchio.** 1994. Genetic and molecular characterization of purine permease genes of *Aspergillus-nidulans* reveals a novel family of transporters conserved in prokaryotes and eukaryotes. Folia Microbiol. **39:**513–524.
11. **Dunlop, J.** 1998. CCK receptor antagonists. Gen. Pharmacol. **31:**519–524.
12. **Dyer, P. S., D. S. Ingram, and K. Johnstone.** 1992. The control of sexual morphogenesis in the ascomycotina. Biol. Rev. **67:**421–458.
13. **Fluri, R., and J. R. Kinghorn.** 1985. Induction control of purine catabolism in *Schizosaccharomyces pombe*. Curr. Genet. **9:**573–578.
14. **Gerlach, M., N. Schwelle, W. Lerbs, and M. Luckner.** 1985. Enzymatic synthesis of cyclopeptine intermediates in *Penicillium cyclopium*. Phytochemistry **24:**1935–1939.
15. **Gloer, J. B.** 1995. The chemistry of fungal antagonism and defense. Can. J. Bot. **73:**S1265–S1274.
16. **Gorfinkiel, L., G. Diallinas, and C. Scazzocchio.** 1993. Sequence and regulation of the uapA gene encoding a uric acid-xanthine permease in the fungus *Aspergillus nidulans*. J. Biol. Chem. **268:**23376–23381.
17. **Haslam, E.** 1995. Secondary metabolism—evolution and function: products or processes? Chemoecology 5/**6:**89–95.
18. **Heguy, A., P. Cai, P. Meyn, D. Houck, S. Russo, R. Michitsch, C. Pearce, B.**

Katz, G. Bringmann, D. Feineis, D. L. Taylor, and A. S. Tyms. 1998. Isolation and characterization of the fungal metabolite 3-O-methylviridicatin as an inhibitor of tumour necrosis factor alpha-induced human immunodeficiency virus replication. Antivir. Chem. Chemother. **9:**149–155.

19. **Horinouchi, S., and T. Beppu.** 1992. Autoregulatory factors and communication in actinomycetes. Annu. Rev. Microbiol. **46:**377–398.
20. **John, G.** 1999. 1998. Warkany lecture: Signaling pathways in development. Teratology. **60:**226–239.
21. **Kelley, W. N., and I. M. Weiner (ed.).** 1978. Uric acid. Springer, New York, N.Y.
22. **Kon, Y., Y. Dobashi, and H. Katsura.** 1976. The characteristics of uricase production by a Hyphomycetes isolated from the excrements of *Cettia diphone cantans*. II. The effectiveness of purines and pyrimidines as substrates. Yakugaku Zasshi. **96:**277–283.
23. **Lockwood, G. F., and R. G. Garrison.** 1968. Role of uric acid in the ecology of *Histoplasma capsulatum*. Mycopathol. Mycol. Appl. **35:**377–388.
24. **Lu, Y., R. L. Mach, K. Affenzeller, and C. P. Kubicek.** 1992. Regulation of alpha-aminoadipate reductase from *Penicillium chrysogenum* in relation to the flux from alpha-aminoadipate into penicillin biosynthesis. Can. J. Microbiol. **38:**758–763.
25. **Luckner, M., W. Lerbs, and W. Roos.** 1985. The integration of cyclopeninpenicyclopenol biosynthesis into the developmental program of *Penicillium cyclopium*, p. 133–156. *In* H. Kleinkauf, H. v. Döhren, H. Domauer, G. Nesemann, (ed.), Regulation of secondary metabolite formation. VCH Verlagsgesellschaft, Weinheim, Gwermany.
26. **Luckner, M.** 1980. Alkaloid biosynthesis in *Penicillium cyclopium*—does it reflect general features of secondary metabolism? J. Nat. Prod. **43:**31–40.
27. **Lyon, E. S., and R. H. Garrett.** 1978. Regulation purification and properties of xanthine dehydrogenase EC 1.2.1.37 in *Neurospora crassa*. J. Biol. Chem. **253:**2604–2614.
28. **Nover, L., W. Lerbs, W. Müller, and M. Luckner.** 1979. Channelling of exogenous phenylalanine to the sites of storage and the sites of alkaloid and protein biosynthesis in *Penicillium cyclopium*. Biochim. Biophys. Acta **584:** 270–283.
29. **Nover, L., and M. Luckner.** 1974. Expression of secondary metabolism as part of the differentiation processes during the idiophase development of *Penicillium cyclopium* WESTLING. Biochem. Physiol. Pflanzen **166:**293–305.
30. **Pechstädt, H.** 1990. Transport and compartmentation of precursor-amino acids of secondary metabolism in spores of *Penicillium cyclopium* WESTLING. Ph.D. thesis. Martin-Luther-University, Halle, Germany.
31. **Penalva, M. A., R. T. Rowlands, and G. Turner.** 1998. The optimization of penicillin biosynthesis in fungi. Trends Biotechnol. **16:**483–489.
32. **Pönitz, J., and W. Roos.** 1994. A glucose-activated electron transfer system in the plasma membrane stimulates the H+-ATPase in *Penicillium cyclopium*. J. Bacteriol. **176:**5429–5438.
33. **Roos, W., R. Schulze, and J. Steighardt.** 1997. Dynamic compartmentation of vacuolar amino acids in *Penicillium cyclopium*. J. Biol. Chem. **272:**15849–15855.
34. **Roos, W., and M. Luckner.** 1977. ATP-dependent permeability of membrane barriers in *Penicillium cyclopium* WESTLING. Biochem. Physiol. Pflanzen. **171:**127–138.
35. **Roos, W., and H. P. Schmauder.** 1989. Positive feedback effect of benzodiazepine alkaloids on enzymes of the aromatic pathway. FEMS Microbiol. Lett. **59:**27–30.
36. **Roos, W.** 1990. Benzodiazepine alkaloids, p. 93–97. *In* The alkaloids, vol. 39. Academic Press, New York, N.Y.
37. **Scazzocchio, C., and A. J. Darlington.** 1968. Induction and repression of the enzymes of purine breakdown in *Aspergillus nidulans*. Biochim. Biophys. Acta **166:**557–568.
38. **Steighardt, J., K. Meyer and W. Roos.** 2000. Selective regulatory effects of purine and pyrimidine nucleotides on vacuolar transport of amino acids. Biochim. Biophys. Acta **497:**321–327.
39. **Stone, M. J., and D. H. Williams.** 1992. On the evolution of functional secondary metabolites (natural products). Mol. Microbiol. **6:**29–34.
40. **Stryer, L. (ed.).** 1990. Biochemistry. Spektrum, Heidelberg, Germany.
41. **Taniguchi, M., and Y. Satomura.** 1970. Isolation of viridicatin from *Penicillium crustosum*, and physiological activity of viridicatin and its 3-carboxymethylene derivative on microorganisms and plants. J. Agric. Biol. Chem. **34:**506–510.
42. **Velasco, J., S. Gutierrez, F. J. Fernandez, A. T. Marcos, C. Arenos, and J. F. Martin.** 1994. Exogenous methionine increases levels of mRNAs transcribed from *pcbAB*, *pcbC*, and *cefEF* genes, encoding enzymes of the cephalosporin biosynthetic pathway, in *Acremonium chrysogenum*. J. Bacteriol. **176:**985–991.
43. **Vining, L. C.** 1990. Functions of secondary metabolites. Annu. Rev. Microbiol. **44:**395–427.
44. **Williams, J. G.** 1988. The role of diffusible molecules in regulating the cellular differentiation of *Dictyostelium discoideum*. Development **103:**11–16.
45. **Wilson, S., I. Schmidt, W. Roos, W. Fürst, and M. Luckner.** 1974. Quantitative Bestimmung des Enzyms Cyclopenase in Konidiosporen von *Penicillium cyclopium* WESTLING und *P. viridicatum* WESTLING. Zeitschr. Allg. Mikrobiol. **14:**515–523.
46. **Zakel-Mavric, M., T. Kastelic-Suhadolc, A. Plemenitas, T. L. Rizner, and I. Belic.** 1995. Steroid hormone signalling system and fungi. Comp. Biochem. Physiol. **112B:**637–642.
47. **Zendem, Z., S. Khalil, and M. Luckner.** 1982. P-factor, a developmental hormone of *Penicillium cyclopium*. Phytochemistry **21:**789–842.