Occurrence and Characteristics of Amino Alcohols and Cyclohexenone. Components of Fungal Mycosporines

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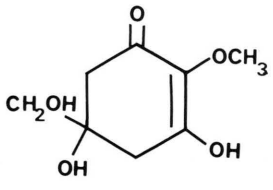
After determination of hydrolysis conditions which allow the best recovery of the mycosporine components (cyclohexenone **1** and the amino alcohols **2** and **3**), these compounds have been searched into mycelia of several fungi. In some of them, **1** was unambiguously detected, although in minute quantities, whilst the unknown natural **2** and **3** could not be identified. So, two alternative pathways of mycosporine biogenesis are under investigation.

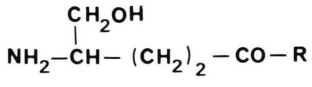
Introduction

During our research on secondary metabolites linked to the reproduction of fungi, we have shown that the fungal mycosporines resulted, from a structural point of view, with the association of a cyclohexenone and of an amino acid or, most often, with its corresponding amino alcohol [1, 2] (Fig. 1). In our last communication [2], we proposed a pathway of structural filiation leading from the normycosporine glutamine to the glucoside of the mycosporine glutaminol, through the following reactions: methylation (2-OH), reduction of the 8-COOH to primary alcohol and glycosylation of the latter. It remains to be determined if these different reactions occur from the precursors themselves, cyclohexenone (**1**) and amino acids (gln, glu, ser) or from the mycosporine amino acid themselves. The first way of approaching this question is to look for the presence, *in vivo,* of these precursors.

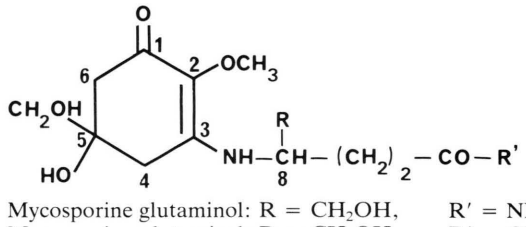
In other respects, the origin of the cyclohexenone – acetate or shikimate pathway – is still unknown. Before to undertake a biogenetic study with the help of radioactive precursors, it is necessary to control the hydrolysis conditions of the mycosporines in order to know the amount of radioactivity in the cyclohexenone and amined moieties. We have therefore carried out research of the optimum hydrolysis conditions (a break of the C-N bond) allowing the maximum recovery of both component fractions. Having separated, purified and characterized the cyclohexenone and the two amino alcohols, glutaminol (**2**) and glutamicol (**3**), unknown molecules *in natura,* we have searched for them in the extracts of different species of fungi. This paper deals with the results of this research.



Cyclohexenone: **1**



Glutaminol: R = NH_2 : **2**
Glutamicol: R = OH : **3**



Mycosporine glutaminol: R = CH_2OH, R′ = NH_2 : **4**
Mycosporine glutamicol: R = CH_2OH, R′ = OH : **5**
Mycosporine glu : R = COOH, R′ = OH : **6**

Fig. 1. Structures of several fungal mycosporines and their components.

Experimental

Production, extraction and purification of mycosporines

Mycosporine glutaminol

The mycosporine glutaminol (**4**) has been obtained from a mass-producing cultivation of *Trichothecium*

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roseum (Pers.) Link ex Gray, Deuteromycete, put into 100 boxes of Roux, containing 150 ml of liquid medium: Composition ($g \cdot l^{-1}$): glucose (10); asparagine (2.6); KH_2PO_4 (1.75); $MgSO_4$,7 H_2O (0.75); vitamines – biotine and thiamine (25 $\mu g \cdot l^{-1}$ each) – and trace elements (Zn, Fe, Cu, Mn, Mo). These boxes have been placed in a thermostated room to 20 ± 1 °C in alternating light and darkness (12/12 hrs; 2500 $ergs \cdot cm^2 \cdot s^{-1}$). At the end of 15 days (time corresponding to the maximum mycosporine production), the sporulating mycelium was separated from the broth by filtration on a crucible, washed and then extracted by boiling 50% EtOH for 2 min. After elimination of the sugars and other low molecular weight substances extracted by the boiling alcoholic solution, the dry residual mycelium extracted weighted 73 g. The amount of mycosporine extracted was 1.48 g($E^{1\%}_{1\,cm}$ = 890) (2% of dry weight). The mycosporines **4** and **5** represented respectively 85 and 15% of the total absorbance at 310 nm. The crude extract, concentrated to dryness and taken-up again by H_2O, was filtered through an anionic resin, Dowex AG 1X8, 50–100 mesh, under OH^- form. The mycosporines were not fixed and, after concentration to dryness, the eluate was submitted to a semi-preparative HPLC on cationic resin, Dowex 50 WX8, 200–400 mesh (H^+ form). By elution with H_2O (flow 10 $ml \cdot min^{-1}$) and detection at 313 nm, a first non-absorbing fraction, containing sugars and other neutral molecules, then two successive fractions with **4** and **5** compounds were obtained. By cooling down the column to 4 °C, the transformation of **4** to **5** [2, 3] was considerably limited; the purification course was followed by GC; **4** generally needed an additional purification by semipreparative HPLC on a NH_2 bonded column eluted with 0.1% HOAc.

Table I. HPLC data of mycosporines and hydrolysis products.

Columns	R_t						
	Compounds						
	Gln[a]	**2**[a]	Glu[a]	**3**[a]	**4**	**5**	**1**
RP_{18}[b]	6.55	7.30	11.20	11.00			
C_{18}[c]					5.30	7.00	
NH_2[d]					4.00	18.20	14.10

[a] As OPA derivatives, detection at 340 nm.
[b] 7 µm, H_2O-ACN-THF 75:25:0.1; flow: 1 $ml \cdot min^{-1}$.
[c] 10 µm, 1% AcOH; flow: 1 $ml \cdot min^{-1}$.
[d] C_{18} bonded NH_2, 10 µm, 0.05% HOAc; flow: 1 $ml \cdot min^{-1}$.

Transformation of **4** into **5**

It was easy to obtain the mycosporine glutamicol (**5**) by transforming **4** on a cationic resin, Dowex 50 WX8, 50–100 mesh (H^+); the absorption in batch of **4** was total after 15 min if sufficiently stirred; by maintaining the mixture at 40 °C, without stirring for 2 hrs, **5** was recovered with a yield of 80–90%. **5** was then purified on C_{18} column, eluted with 0.01% HOAc and then on a bonded NH_2 column with 0.1% HOAc. The extraction and purification of other mycosporines have been carried out according to the procedure already described [2, 4].

Kinetic of the mycosporine hydrolysis – General procedure of study

Solutions of 0.01 or 0.013 M HCl containing 1 to 2 $mg \cdot ml^{-1}$ of mycosporines (mycosporines-gln, glu, serinol, **4** and **5**) have been brought to ebullition under reflux. At T_0, then at regular intervals, an aliquot of the reactional mixture was quantitatively analyzed by HPLC, according the following procedure: Bonded NH_2 column, 10 µm, Injection: 10 µl of hydrolysate after eventual dilution. Elution: 0.05% (flow 1 $ml \cdot min^{-1}$) or 0.01% of HOAc (flow: 1.5 $ml \cdot min^{-1}$). Detection: at 280 nm for **1** and at 313 nm for the mycosporine; if necessary, both types of products can be detected at 280 nm. For the mycosporine-glu, the preceeding column has been replaced by 2 small columns (0.4, 2 cm), filled with cationic resin Durrum, put in series; elution: 5% HOAc (1 $ml \cdot min^{-1}$; detection: 313 nm). The hydrolysate of **5** was stirred in with Dowex resin 50 WX8, 100–200 mesh, during 30 min. A GC control revealed the fixation of **3** on the resin, whilst **1** stayed in supernatant. After concentration, **1** was purified on the semi-preparative NH_2 bonded column (0.05% HOAc, 4 $ml \cdot min^{-1}$ detection 280 nm). **3** was eluted from the cationic resin by N NH_4OH: the first alcaline fraction closed up **3**, characterized by HPLC under derivatized forms. To obtain **2**, the hydrolysis reaction of **4** must be stopped after 70 min under the above defined conditions. Then, a mixture of **1**, **4**, **5**, **3**, **2**, was obtained. To avoid the deamination of **2**, Dowex cationic resin was shunted and the derivatization (OPA or Dansyl) was directly carried out on the hydrolysate.

Detection and preparation of amino acids and amino alcohols

The amino alcohols **2** and **3**, gln and glu, coming from the hydrolysis of the mycosporines have been detected and purified as OPA or Dansyl derivatives.

OPA derivatives

54 mg of *o*-phtalaldehyde was dissolved in 1 ml of MeOH and 9 ml of borate buffer prepared according to [5]. 100 µl of mercaptoethanol was then added; after stirring, 10 µl of hydrolysate was added to 20 or 30 µl of reactive thus prepared. The OPA derivatives are unstable: their purification has been accomplished by HPLC 2 min after reaction: RP_{18} or C_{18} (Microbondapak) column: 30 or 40 µl of OPA sample; elution: H_2O-ACN-HOAc-THF 70-25-5-0.1; flow: 1 ml·min^{-1}; detection UV at 340 nm.

Dansyl derivatives

The sample was dissolved in 0.1 ml of 0.2 M carbonate buffer (pH 9.7), to which was added 0.1 ml of 1% dansyl chloride in acetone. The reactional mixture was left for 35 min at 40 °C; the dansylated products remained stable at the darkness. Purification by HPLC on RP_{18} or C_{18} columns; injection: 20 to 40 µl; elution H_2O-ACN-AcOH 69:30:1; flow 1 to 2 ml·min^{-1}; detection at 254 nm.

Trimethylsilyl derivatives

The purity of mycosporines and of their hydrolysis products has been checked by GC after formation of TMSi derivatives obtained in ACN (25 µl) by addition of BSTFA (25 µl) containing 1% of TMCS, for 24 h at room temperature.

*Spectroscopic properties of **1**, **2**, and **3***

ε of the cyclohexenone **1**

The specific absorbance of **1** has been measured by dosage in NMR: MeOH has been added to a solution in H_2O of **1**, with volume and concentration strictly determined; in a second attempt MeOH was replaced by ACN. The ratio of the areas of the methyl groups of each solvent added and of **1** has allowed us to determine the molar concentration; from its UV absorbance at 268 nm (pH 5) (H_2O), the ε of **1** has been calculated.

Cyclohexenone (**1**)

$\lambda_{max.\ nm(\varepsilon)}^{H_2O\ (pH\ 5)}$: 268 (15700); + OH^- : 292 nm (25100); ^1H NMR, 350 MHz, D_2O, ppm/TMS : OCH_3 : 3.76, 3 H, s; CH_2OH : 3.67, 2 H, s. 2 CH : 3.0, 2 H, d, J = 17 Hz; 2 CH : 2.68, 2 H, d, J = 17 Hz. MS of TMSi **1** (EI, 70 eV): *m/z* (rel. int%) at 404 M^+ (1), 389 M-15 (3), 374 (2), 314 M-90 (6), 301 (19), 299 (9), 73 (100). GC/MS (CPSil 5.50 m, ∅ : 0.25 mm), isothermal 120 (5 min), temp. programmed 120 to 320 at 5 °C min^{-1}. Two peaks have been obtained: R_t : 28.30 min (aromatized form, no MS) and 31.30 min : 404 M^+ (2), 389 M-15 (6), 374 (2), 314 (14), 299 (2), 287 (1), 259 (3), 73 (100).

Dansyl glutaminol (Dansyl **2**)

MS (EI, 70 eV): *m/z* (%) at 365 M^+ (6), 348 M-NH_3 (100), 250 (7), 235 (5), 186 (8), 171 (99), 154 (16), 127 (16), 115 (8), 85 (16).

Dansyl glutamicol (Dansyl **3**)

MS (EI, 70 eV): *m/z* (%) at 348 M-H_2O (37), 250 (53), 171 (100), 155 (28), 154 (21), 115 (20), 85 (16).

Results and Discussion

Adjusting the hydrolysis conditions to assure the optimum recovery of the mycosporine components

This adjustment has essentially been accomplished with **4**.

Optimum substrate concentration

The studies carried out with different concentrations showed that the content of mycosporine has a considerable influence on the rate of **1** recovery and that working with concentrated solutions must be avoided; the most satisfactory results have been obtained with solutions of concentration equal or inferior to ca. 2 mg·ml^{-1}.

Optimum H^+ concentration

A solution of **4**, with a concentration of 0.8 mg·ml^{-1} has been submitted to hydrolysis with different H^+ concentrations: 10^{-7} (H_2O), 10^{-3}, 10^{-2}, 10^{-1} (diluted HCl), at boiling point. At pH 7 as at pH 3, there was no hydrolysis after 120 min, but, over the same length of time, the hydrolysis was effective to 95% at pH 2 (0.01 M HCl). With concentrations higher than 0.02 mol·l^{-1}, and always at boil-

ing point, the hydrolysis took place very quickly but with almost total disappearance of the cyclohexenone. So, further experiments have been carried out with 0.01 to 0.02 M HCl, at boiling point because, below 100 °C, the hydrolysis time is notably larger.

Hydrolysis of the different mycosporines

In the specified conditions the hydrolysis of the amino mycosporines was almost total (95 to 98%) after 4 hrs, as shown in Fig. 2a relative to hydrolysis of **5**, which indicate a reaction of the first order. The hydrolysis of **4** involves a process a little more complex: in Fig. 2b, the transitory appearance of **5** and, above all, the transformation of **2** into **3** can be observed; the well-known process of deamination of the amide group occurred easily in this acid medium. The hydrolysis of the mycosporine glu (**6**) (Fig. 2c), occurred more quickly: 94% disappearance at the end of 1 h, total disappearance after 1½ h, with a maximum production of cyclohexenone after 1 h. This instability with regard to reduced compound, already observed in other works [2, 6] can be explained by the reactional mechanism proposed by Ito and Hirata [6]. Finally, after hydrolysis of 375 mg of **5** in 190 ml of 0.02 M HCl, cyclohexenone **1** has been obtained with a yield of ca. 12% (27 mg after purification by HPLC). It is possible that a part of **1** was decomposed during this reaction; we observed, in the course of hydrolysis, the appearance of an other product, having a single peak to 280 nm, presenting a bathochromic shift to 296 nm in the alcaline medium.

Characterization and occurrence in vivo of mycosporines precursors

The spectroscopic and chromatographic properties of mycosporine components, **1**, **2** and **3** are described under the experimental. The cyclohexenone **1** has already been obtained by Ito and Hirata [6] following a mild hydrolysis of mycosporine-glycine isolated from a Zoanthid, *Palythoa tuberculosa*. **1** has also been isolated, in very low quantities, from purified extracts of eggs, from the fish *Auxis thazard* by Chioccara *et al.* [7]. With the precise knowledge of its R_t in HPLC, we are able to check the existence, as natural molecule into fungi, of this compound; we have detected and characterized it, from the carpophores of *Helvella leucomelaneae*, collected *in natura* and immediately freezed. We checked that the watery extract, during the thawing, contained the cyclohexenone **1**, in very small quantities with regard to the mycosporine-glu, characteristic of this species. We have, also, found it in the extracts of

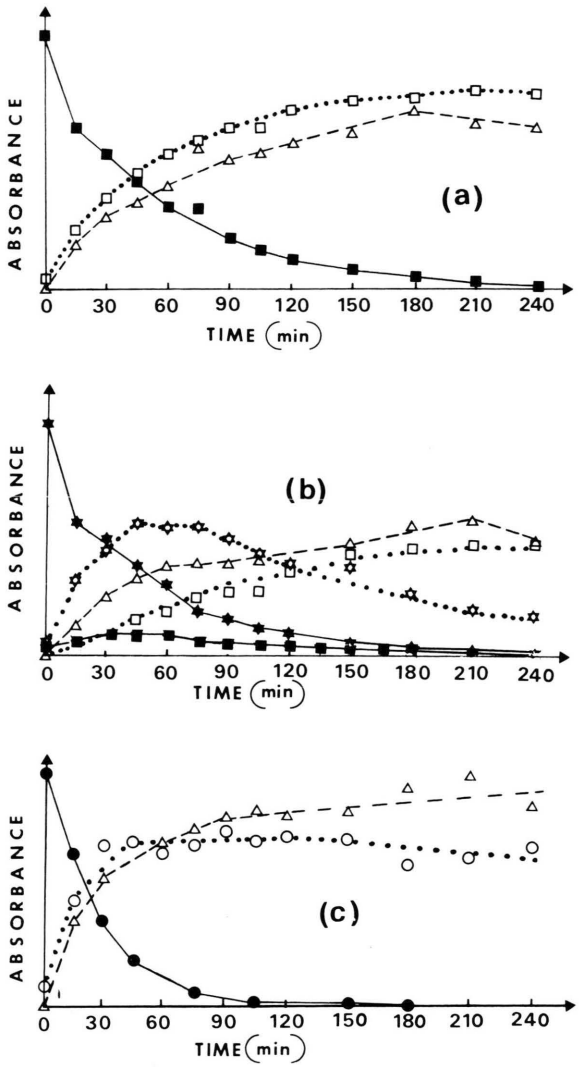

Fig. 2. Hydrolysis kinetics, under reflux, with 0.01 M HCl of several mycosporines (ca. 2 mg·ml^{-1}): (a) mycosporine glutamicol (**5**); (b) mycosporine glutaminol (**4**); (c) mycosporine glutamic acid (**6**).

■ ■ ■ ■ ■ (**5**);
★ ★ ★ ★ ★ ★ ★ ★ ★ (**4**);
● ● ● ● ● (**6**);
△ △ △ △ △ (**1**);
☆ ☆ ☆ ☆ ☆ ☆ ☆ ☆ ☆ (**2**);
□ □ □ □ □ □ (**3**);
○ ○ ○ ○ ○ (glutamic acid).

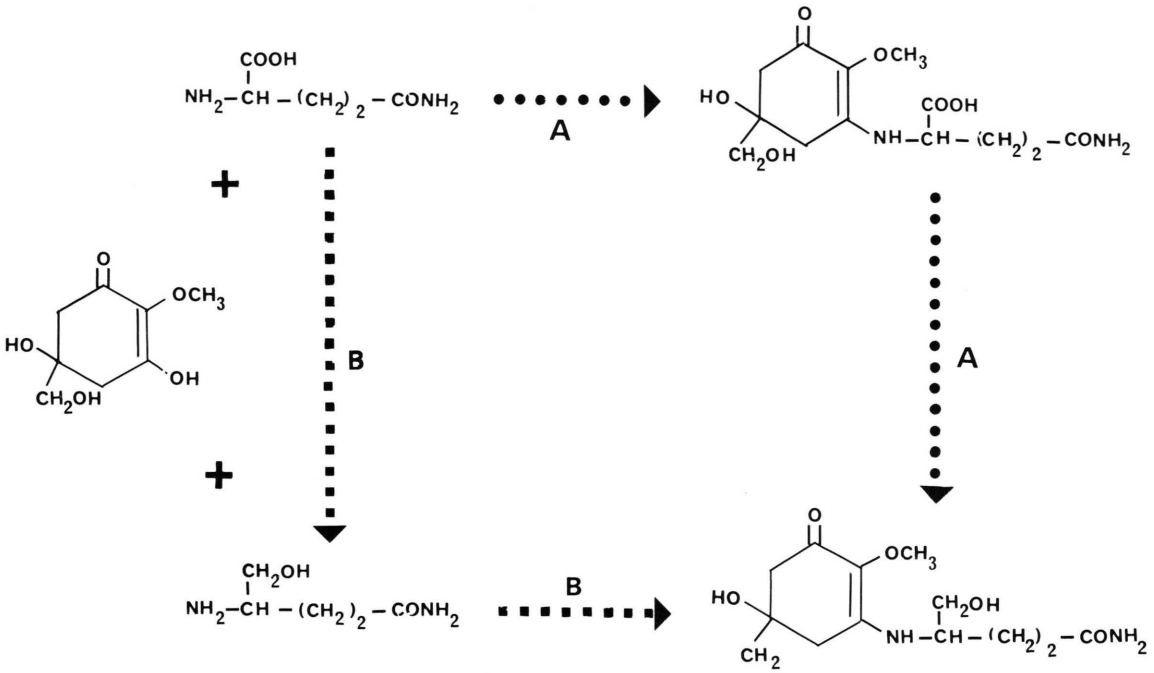

Fig. 3. Two alternative pathways for mycosporine glutaminol biogenesis.

Trichothecium roseum corresponding to the 3rd, 4th and 5th day of cultivation, during which the synthesis of mycosporine begins. Beyond the 6th day, it becomes a lot more difficult to put it into evidence. The research of amino alcohols in the mycelia of varied species: *Botrytis cinerea, Cladosporium herbarum* or *Trichothecium roseum,* as OPA or dansyl derivatives, does not permit a positive conclusion as to their occurrence as free molecules, especially into *T. roseum* where mycosporine-glutaminol represents ca. 2% of dry weight of sporulating mycelium. That could signify that the reduction of the glutamine only occurs after joining it with **1**, or that the two precursors **1** and **2** are synthesized in the same cell compartment and immediately react together to give **4**. The biogenetic work, now undertaken, must allow us to choose between the A or B routes preferentially adopted by *T. roseum.*

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