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MYCOSPORINE GLUTAMINE AND RELATED MYCOSPORINES IN THE FUNGUS *PYRONEMA OMPHALODES*

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Key Word Index—*Pyronema omphalodes; Glomerella cingulata; Helvella leucomelaneae*; Ascomycetes; fungi; mycosporines; mycosporine glutamine; mycosporine glutamic acid; HPLC.

Abstract—The structure of mycosporine glutamine, a new compound, has been established and its presence demonstrated in two fungi *Pyronema omphalodes* and *Glomerella cingulata*. Mycosporine glutamic acid has been isolated from *Helvella leucomelaneae*. Co-occurrence of normycosporine glutamine, mycosporine glutamine and glucosylmycosporine glutaminol has been demonstrated in the fungus *P. omphalodes*. A biosynthetic pathway is proposed. Mycosporines have been compared by HPLC.

INTRODUCTION

The generic name mycosporine has been given [1] to water soluble UV-absorbing fungal metabolites, whose structures contain a substituted cyclohexenone linked to an amino acid or the corresponding amino alcohol. Their distribution and biological properties have been reviewed [2, 3].

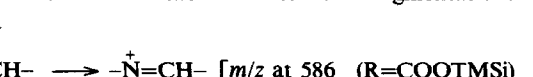
Two chromophores (Table 1) have been described, differentiating mycosporines *sensu stricto* (2-OMe, UV_{max} at 310 nm) from normycosporines (2-OH, UV_{max} at 320 nm). Within both groups, the structures vary according to the amino moiety. In fungal mycosporines the only amino acids involved are serine and its corresponding α-amino alcohol, serinol (as in **5a** (Fig. 2.) the first mycosporine [1, 4]) and the related pairs glutamine–glutaminol and glutamic acid–glutamicol (γ-amino δ-hydroxyvaleric acid) (Table 1)*. Normycosporine glutamine (**1a**) has been reported only once, as the major UV absorbing substance in the fungus *P. omphalodes* [5, 6]. In this paper, we report the co-occurrence of several mycosporines in this fungus. Among them, we describe mycosporine glutamine (gln) (**2a**), a new compound, which completes the series of known mycosporines. All compounds were compared by HPLC.

RESULTS

Good vegetative growth of the ascomycete *P. omphalodes* was obtained on synthetic medium (A), but sexual reproduction required medium B, depleted in C and N nutrients, and strict conditions of light and temperature [7]. Sexual fruiting bodies (apothecia) are formed by several asci where the ascospores differentiate. These are ejected when mature. The crude aqueous extract of the fruiting mycelium (mycelium + apothecia) showed UV absorbance at 320 nm due to the major component, normycosporine gln (**1a**) [normycosporine glu (**1b**) is a by-product of the extraction process]. However, the crude extract from ejected (mature) ascospores showed a UV_{max} at 310 nm due to other mycosporines. Extracts from the vegetative mycelium did not show any absorbance at these wavelengths. The absence of normycosporine was obvious from the elution pattern (Fig. 1) through the cationic resin Dowex 50 W × 8. From the crude fruiting mycelium extract, absorbance at 310 nm was found in two minor fractions (about 4% of the whole mycosporine pool): fractions I and III eluted before and after normycosporine respectively. By HPLC, these fractions were found qualitatively identical with the I′ and III′ fractions from the ascospore extract each yielding two mycosporine peaks.

Fraction I

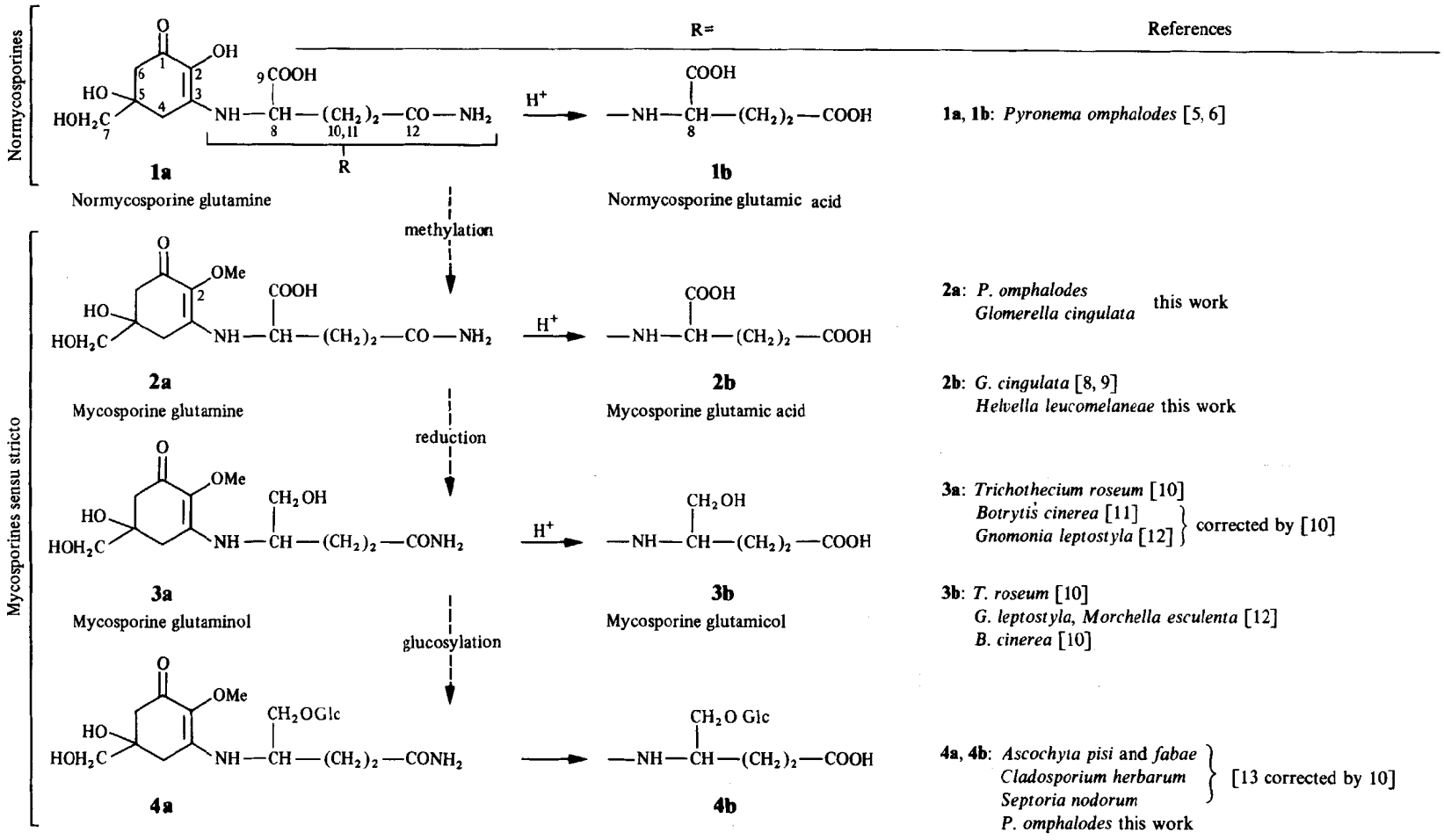
The lack of retention of both mycosporines of this fraction on cationic exchange resin suggests the presence of an acidic function in these molecules. Study of the mass spectra of the products of the trimethylsilylation of the whole fraction (see below about GC/MS of mycosporines derivatives) confirmed that it was a mixture of two homologous compounds; two peaks of higher mass at *m/z* 604 and 605 were followed by other homologous ions which differ also by one mass unit. Especially, ions at *m/z* 586 and 587, corresponding to the TMSi derivatives $\mathbf{6}_{2a}$ and $\mathbf{6}_{2b}$ (Fig. 2), were abundant. These last showed the characteristic fragmentation:



[*m/z* at 586 (R=COOTMSi) and 587–COOTMSi] supporting the presence of an α acidic group in both compounds. We thus had good evidence that Fraction I was a mixture of mycosporine gln (**2a**) (MS peak at 604) and mycosporine glu (**2b**) (MS peak at 605). Mycosporine gln, the major component, is a new compound but mycosporine glu had been previously

*Mycosporines with other amino moieties have been isolated from marine organisms, see [3] for references.

Table 1. Mycosporine structures



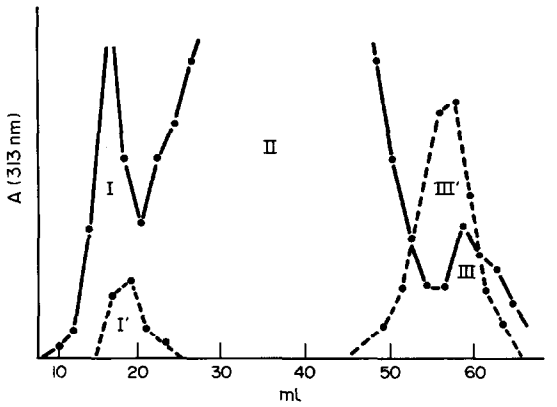


Fig. 1. Elution pattern (H_2O) on Dowex 50 W × 8 (H^+) of crude aqueous extracts of: ●——● the total fruiting mycelium and ●- - -● ejected ascospores de *P. omphalodes*.

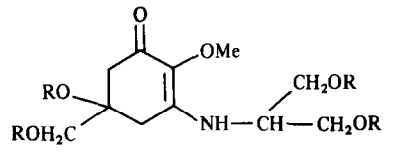
isolated and described [8] as constituent of *G. cingulata* conidia.

In order to obtain a sample of **2b**, the culture of a strain of *G. cingulata* and the extraction of its mycosporine were carried out as described [8]. We observed, by HPLC of the crude extract, that the native mycosporine in this fungus was identical with the mycosporine gln (**2a**) of *P. omphalodes* and not with **2b**. Mass spectral study of the purified (preparative HPLC) compound: FAB^+, MH^+ at *m/z* 317; EI, peak of higher mass at *m/z* 280.1057 (Calc. for $C_{13}H_{16}O_5N_2$ 280.1059) $M - 2H_2O$; GC/MS coupling of the TMSi derivative **7** (X = NH, R = COOTMSi) (Fig. 2) giving a peak at *m/z* 496 followed by the fragment at *m/z* 379 (496 − COOTMSi), confirmed both the structure of the compound and its occurrence in both fungi. We conclude that, perhaps, **2b** might constitute an artifact produced during extraction and(or) purification from *G. cingulata* by Young and Patterson, although we worked with a different strain from that examined by these authors.

Native mycosporine glu (**2b**) was further isolated, free of **2a**, as a constituent of the crude extract of the carpophore of *Helvella leucomelaneae*, collected *in natura*. MS study: FAB^+ MH^+ at *m/z* 318, and HPLC properties (see further) confirmed the structure. This compound was also obtained, at room temperature, from **2a**, in slightly acidic aqueous solution.

Fraction III

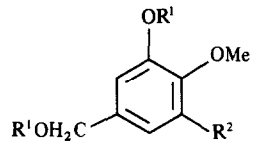
The minor compound of this fraction, as supported by the mass spectrum of its TMSi derivative (peak of higher mass at *m/z* 573 [10]) and its HPLC properties was found



5

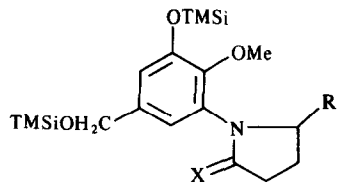
	R
5a	H
5b	Ac
5c	TMSi

Mycosporine serinol and derivatives



6

	R^1	R^2
6$_{2a}$	TMSi	—NH—CH(COOTMSi)—$(CH_2)_2$CONHTMSi
6$_{2b}$	TMSi	—NH—CH(COOTMSi)—$(CH_2)_2$COOTMSi
6$_{3a}$	TMSi	—NH—CH(CH_2OTMSi)—$(CH_2)_2$CONHTMSi
6$_{3b}$	TMSi	—NH—CH(CH_2OTMSi)—$(CH_2)_2$COOTMSi



7

X = O or NH
R = COOTMSi or CH_2OTMSi
or CH_2OTMSiglucosyl

Fig. 2. Mycosporine derivatives.

to be mycosporine glutamicol. The structure was confirmed by comparison with authentic samples obtained from *Gnomonia leptostyla* [12] and by hydrolysis of **3a** isolated from *Trichothecium roseum* [10].

The main product of Fraction III appeared to be a glycoside from its HPLC properties. Acidic hydrolysis (Table 2) yielded at first the aglycone **3a** and glucose (GC of the TMSi derivative) then the γ acidic compound **3b**. Finally, the structure was confirmed by ^{1}H NMR and HPLC cochromatography with the glucoside of mycosporine glutaminol (**4a**) first isolated from *Ascochyta fabae* [10, 13]. In this work, [10] ^{13}C and ^{1}H NMR showed that, in **4a**, the glucosidic linkage is α.

Mycosporines where the N moiety is an amino acid (types **1** and **2**) are more unstable than the amino alcohol mycosporines (types **3** and **4**) and normycosporines (type **1**) even more than type **2**. In each group, the glutamine (-ol) derivative (type **a**) was always easily hydrolysed to the glutamic (-ol) compound. *In vivo*, *Ascochyta fabae* yielded **4a** (its own mycosporine) from **3b** added to the culture medium* [14, 15]. Up to now, in the laboratory (not *in natura*) type **a** were the more widespread mycosporines from fungal cultures.

Comparison between mycosporines by HPLC

On reversed phase C_{18} columns mycosporines can be separated by water or buffers at different pH. Normycosporines were not recovered. From these data (Table 3), we observe that pH changes cause important R_t changes to the acidic mycosporines (**b**), which are delayed by an acidic solvent; amido mycosporines (a) R_t are only slightly advanced.

GC/MS study of mycosporine derivatives

In previous work [1], acetylated derivatives were used to elucidate the structure of mycosporine serinol **5a**. The tetraacetyl derivative **5b** was never obtained; dehydradation always occurs and leads to the aromatized derivative (type **6**). TMSi derivatives were found more suitable because the 'native' derivative **5c** can be obtained although usually mixed with secondary TMSi compound of type **6**. From mycosporines of type **1** to **4**, three kinds of reactions can occur during both derivatization and GC analysis: (i) aromatization then TMSi leading to **6**, (ii) aromatization, amide hydrolysis and TMSi ($\mathbf{6}_{2b}$, $\mathbf{6}_{3b}$), (iii) aromatization, TMSi and formation of one of the ring forms **7**. Relative abundance of these compounds is also linked to the nature of the solvent used for derivatization: acetonitrile is better than pyridine to avoid γ amidolyse of **a** compounds. In these latter mycosporines, the NH_2 group has always afforded a single or no TMSi group. By GC, R_t decreases from **6a** to **6b** then **7**. TMSi derivatives of glucosylmycosporines are insufficiently volatile and do not show any peak on GC. However, their MS [10, 13] can be obtained using direct insertion of the TMSi mixture.

Table 2. Acid hydrolysis of **4a** (0.5 N HCl, 100°C)

Time (mn)	Compound %		
	4a	**3a**	**3b**
0	90	5	5
5	45	30	15
15	5	50	25
25	2	40	40
40	0	10	70
50	0	8	70

*In dark cultures where *A. fabae* does not, normally, produce mycosporine.

Table 3. HPLC data of mycosporines

Compounds	R_t (min) RP 18 reversed phase* H_2O	RP 18 reversed phase* 0.1% HOAc	W_3 Beckmann resin† 2% HOAc
1a	—	—	5'50
2a	6'	3'10	2'40
2b	2'	5'10	1'30
3a	7'	4'40	24'40
3b	2'20	7'10	11'40
4a	8'40	5'20	4'30
4b	2'20	8'40	2'20
5a	5'	4'	12'40

*7 μ, length 25 cm, 1.5 ml/min.
†Length 30 cm, 2 ml/min.

DISCUSSION

The speculation that there is a biogenetic relationship between different mycosporines is confirmed by the discovery in the same fungus of both normycosporine **1a** and of the glucoside **4a**. From this, it can be postulated for mycosporines an order of biogenetic advancement which is symbolized by their progressive chemical stability. The beginning of this pathway might be normycosporine since this was found in the primordia but not in the spores. In previous work [3, 5] we demonstrated that production of **1a** decreases as the culture is ripening and it is tempting to assume that 2-*O*-methylation occurs during ascospore formation. So, mycosporine gln **2a** might constitute the second step of the pathway. However, the 4% ratio of mycosporines in spores fails to explain the total disappearance of normycosporine. In the next step, that leads to the reduced structure **3a**, the molecule is stabilized by further glycosylation. Compounds **4** accumulate, without any degradation in spores. This latter step has been demonstrated by Pittet *et al.* [16] using the soluble enzymatic fraction from conidia of *Ascochyta fabae*. Other steps along the pathway are under investigation.

EXPERIMENTAL

Materials and culture media. Strains of *Pyronema omphalodes* (Bull ex. Fr.) Fuckel and *Glomerella cingulata* (Stoneman) Spaulding and Schrenk were purchased from Centraal bureau voor Schimmelcultures PO Box 273 3740 AG Baarn Holland. Carpophores of *Helvella leucomelaneae* (Pers.) Nannf. were collected (in February 1983) on our campus. Culture media: (A), *P. omphalodes* (vegetative mycelium) and *G. cingulata* (conidia), pH 7, $NaNO_3$ 2 g, KH_2PO_4 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, glucose 20 g, asparagine 1.5 g, agar 15 g,

distilled H_2O 1000 ml. (B) *P. omphalodes* (apothecia) KH_2PO_4 1 g, $MgSO_4$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01 g, NH_4NO_3 0.2 g, maltose 3.4 g, biotine 10 μg, thiamine, HCl 200 μg, oligoelements sol. [17] 0.1 ml, agar 15 g, H_2O 1000 ml, pH 7. Carrot-orange medium, *G. cingulata* (conidia) [9].

Extraction and purification of mycosporines. Mycelia and fruiting bodies of *P. omphalodes* or *G. cingulata* were scraped off the agar surfaces and suspended in cold 50% EtOH; after 12 hr at 4°, the mycelium was filtered and the filtrate concd to dryness in vacuo at 20°. Ejected spores were extracted by washing the Petri dish lids. Carpophores of *H. leucomelaneae* were first frozen then extracted by H_2O at room temp. Aq. soln of the crude extracts of *P. omphalodes* first were passed through AG IX8 (OH^-) resin (200–400 mesh Biorad) eluted with H_2O, then several times if needed, on a chilled Beckmann W_3 HPLC column in 2% HOAc. *G. cingulata* and *H. leucomelaneae* mycosporines were only purified by HPLC.

Amidolysis of **2a** *and* **3a**. Cationic resin Dowex 50 W × 8 (about 5 g wet) was suspended into soln of **2a** or **3a** (about 50 to 100 μg) at 40°. By HPLC we observed a progressive and total (after 20 hr) conversion of **2a** and **3a**, respectively, into **2b** and **3b**.

GC/MS of TMSi derivatives. Acetonitrile, $CHCl_3$ or pyridine–BSTFA + 1% TMCS (1 : 1). GC: OV101, 25 m column, 120–180° (4°/min), He 1 ml min. MS FABS: ZAB (VG) glycerol matrice.

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