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# IDENTITY OF THE PHYTOTOXIN STEMPHYLIN FROM *STEMPHYLIUM BOTRYOSUM* WITH ALTERSOLANOL A*

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**Key Word Index** -*Stemphylium botryosum*; Hyphomycetes; secondary metabolites; stemphylin; altersolanol A; dactylariol; macrosporin.

**Abstract** The structure of stemphylin, a phytotoxic compound previously isolated from the fungus *Stemphylium botryosum* v. *lactucum*, has been revised; it is identical to altersolanol A. Isolation of dactylariol and macrosporin from the fungus is also reported.

## INTRODUCTION

The fungus *Stemphylium botryosum* Wallr. v. *lactucum*, a destructive pathogen of lettuce, produces phytotoxic compounds which induce necrotic lesion areas on young leaves, in a linear relationship with concentration [1]. Isolation and characterization of one of the phytotoxins, a yellow 'chromone glucoside' called stemphylin was also reported [2]. The empirical formula $C_{17}H_{22}O_9$ and the structure 3-hydroxy-2,2-dimethyl-5-α-D-glycopyranoside-2,3-dihydrochromone was attributed to it on the basis of an ambiguous interpretation of the spectroscopic data.

The toxin, on hydrolysis with trifluoroacetic acid, led to two yellow compounds plus a third, identified as glucose only by chromatography [2]. Since the interpretation of the data appeared open to doubt, 3,5-dihydroxy-2,2-dimethylchroman-4-one was prepared by an unambiguous synthesis [3] and showed spectroscopic data incompatible with those of the aglycone moiety of stemphylin; thus the structure of the toxin was uncertain [4].

In this paper we report the isolation of stemphylin (**1**) from the fungus and the determination of its structure, which appears identical with that of the well-known altersolanol A [5, 6]; the phytotoxicity of the compound was tested according to the literature [2]. Dactylariol (**2**) and macrosporin (**3**) were also detected. Stemphyltoxins and stemphyperylenol from the same fungus have been recently characterized [7]. From the first cultures we isolated mainly the anthraquinones, whereas with repeated subculturing of the fungus, the production of the reduced perylenequinone stemphyltoxins increases significantly.

## RESULTS AND DISCUSSION

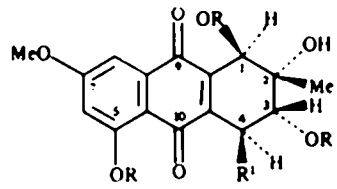
From the ethyl acetate extracts of the fungus culture grown on potato-dextrose-agar, a major metabolite (**1**) was isolated by silica gel column chromatography in the more polar fractions. After crystallization of **1** from acetone–hexane, orange crystals mp 218° were obtained; the molecular formula $C_{14}H_{16}O_8$ was established by field desorption mass spectroscopy (FD-MS, $M^+$, 336) and the UV spectrum showed $\lambda_{max}$ at 218, 268 and 428 nm (lit. [2] for stemphylin 218, 268 and 427.5 nm) and $[\alpha]_D^{20}$ −161° (*c* 0.1; MeOH); the IR spectrum in Nujol showed signals between 3300 and 3500 (OH), 1670 (unchelated CO) and 1640 $cm^{-1}$ (lit. [2], 1635 $cm^{-1}$, conjugated CO group).

The $^1H$ NMR spectrum measured in acetone-$d_6$–DMSO-$d_6$ (2:1) showed a pair of *meta* coupled aromatic signals (*d*, 1H each, centred at δ7.05, and 6.68; $J$ = 2.5 Hz), broadened signals from alcoholic hydroxyl groups at δ3.8 to 5.1, a singlet (1H, chelated OH) at δ12.9, an aromatic methoxyl group (3H, *s*, δ3.94), a singlet (3H, *s*, δ1.40) corresponding to an aliphatic methyl group; a broadened singlet (1H, δ4.57) and a pair of doublets (1H each, centred at δ4.78 and 3.9) are shifted downfield by acetylation. Acetylation of (**1**) in pyridine gave a tetraacetate (**1a**), $M^+$ = 504, whose properties (exchangeable alcoholic proton at δ3.04 in the $^1H$ NMR spectrum in $CDCl_3$) required the presence of a tertiary hydroxyl group. The $sp^2$ resonances in the $^{13}C$ NMR spectrum of **1a** in $CDCl_3$ indicated the presence of two carbonyl (C-9 and C-10; δ182.3 and 180.2), two methine (δ115.6 and 109.0), and 10 quaternary carbon atoms, while the $sp^3$ resonances were assigned to five methyl, one methoxyl, three methine oxygen bearing (δ74.6, 68.7 and 68.1) and one quaternary carbon atom (δ72.4).

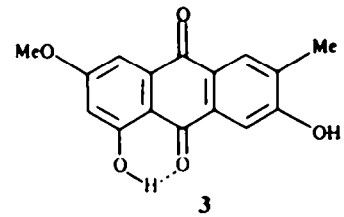
The similarity of the behaviour, and of the spectroscopic data (UV, IR and NMR), leave no doubt that compound **1** is stemphylin. On the other hand, on the basis of the above spectral data and for direct TLC comparison the structure of **1** appeared to be altersolanol A, a metabolite first isolated from *Alternaria solani* [5, 6]; furthermore altersolanol A tested for phytotoxicity on lettuce leaves according to [2] (see Experimental) induced brown necrotic lesions; moreover its toxicity was confirmed in other recent papers [8, 9].

During the chromatographic purification of altersolanol A, two other metabolites were separated and

* Part 17 in the series "Secondary Mould Metabolites". For Part 16 see ref. [7].



1 R = H, R¹ = OH
1a R = Ac, R¹ = OAc
2 R = H, R¹ = H



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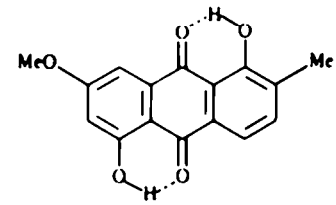

4

identified: dactylariol (2) and macrosporin (3); compound 2 was separated only by chromatography on RP-18 in poor yield.

We repeated on the mixture of 1, 2 the reaction with trifluoroacetic acid, according to [2], for the identification of the yellow products therein described; a few mgs of two main compounds 3 and 4 were isolated. Compound 3, needles from acetone, mp 303–305°, is identical to macrosporin extracted from the culture of the fungus, by comparison of TLC and spectroscopic data. The yellow compound 4, mp 172–175°, $M^+$, 284 had the formula $C_{16}H_{12}O_5$, confirmed by high resolution mass spectroscopy; the $^1H$ NMR spectrum showed the presence of two chelated hydroxyl groups, four aromatic protons, two *ortho* and two *meta* coupled, one methyl and one methoxy groups and we propose the structure 4 for this new unnatural anthraquinone. Evidently trifluoracetic acid dehydrates compound 2 to form the corresponding anthraquinones (3) and (4).

Altersolanol A (1) and macrosporin (3) lacked antibacterial or antifungal activity. Because of its similarity with dactylariol which induces measurable ATP catabolism in Ehrlich ascites tumour cells [10], 1 was tested for antitumour activity. Low activity was registered at lower doses, whereas at 40 mg/kg the compound caused the death of the treated animals.

## EXPERIMENTAL

Mps are uncorr. Flash chromatography was performed with Merck silica gel (0.040–0.063 mm) and TLC with Merck $HF_{254}$ silica gel. Unless otherwise indicated, the purity of products was checked by TLC, NMR and MS and deemed sufficient for the purpose of structural elucidation.

*Cultivation of the fungus and isolation of the metabolites.* *Stemphylium botryosum* Wallr. v. *lactucum* CBS 273.55 was supplied by the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. 50 Roux flasks of potato-glucose-agar, inoculated with a mycelium suspension were extracted twice with EtOAc, after 2 weeks growth. From 1.5 g of crude extract 100 mg of 1 and 5 mg of 3 were obtained by silica gel column chromatography in $CH_2Cl_2$–MeOH (9:1) followed by crystallization. The fractions containing the mixture of 1 and 2 were chromatographed with Si gel RP-18 (Merck) with acetone–$H_2O$ (2:1). Altersolanol A and dactylariol were identified by direct comparison with authentic samples.

*Aromatization of the mixture of 1 and 2 with TFA.* The mixture (150 mg approximately 50% of each compound) was reacted with TFA (5 ml) under $N_2$ according to [2]. The solvent was removed *in vacuo* and the residue was chromatographed on TLC using $CH_2Cl_2$–MeOH (15:1) as eluent to separate the two yellow compounds, macrosporin (3) and 4.

*2-Methyl-7-methoxy-1,5-dihydroxyanthraquinone* (4). Compound 4 (10 mg), yellow powder, had mp 172–175°, $\nu_{max}$ 1630 (chelated OH), and 1610 (conj. CO) cm$^{-1}$; UV, $\lambda_{max}$ 228, 257, 278 and 435 nm (ε29 100, 17 800, 16 000 and 10 600); *m/z* 284 ($M^+$); ($M^+$, 284.0667 ± 0.003; $C_{16}H_{12}O_5$ required 284.0684); NMR (90 MHz, $CDCl_3$–DMSO-$d_6$) δ2.32 (3H, *s*, Me-2), 3.95 (3H, *s*, OMe-7), 6.72 and 7.37 (2H, *d*, *J* = 2 Hz, H-6 and H-8), 7.55 and 7.78 (2H, *dd*, H-3 and H-4), 12.9 and 13.0 (2H, *s*, 2 chelated OH).

*Phytotoxicity test.* 1 (1 mg) dissolved in 70 μl MeOH was diluted with 10 ml of 0.05 M Tris–HCl buffer (pH 7.1). Lettuce leaves (cv. Trocadero) were cut from 3-week-old seedlings and placed in a transparent moist chamber. 5 μl of the test solution were applied on a puncture wound and the dishes were kept under diffuse light. After 16 hr the first symptoms of sunken necrotic brown lesions appeared, whilst the control did not show any symptom.

*Antifungal and antibacterial tests* were performed using paper disks (6 mm φ) containing 250 μg of 1 and 3. The disks were tested against *Bacillus cereus*, *B. subtilis*, *Saccharomyces cerevisiae*, *Aspergillus niger* and *Cladosporium cladosporioides* inoculated in suitable agar in Petri dishes. The antitumoural activity of 1 was tested on mice inoculated with $10^6$ leukemia cells P 388 and treated with 2.5, 10, 20 and 40 mg/kg; values of the activity were taken according to [10].

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## REFERENCES

1. Barash, I., Netzer, D., Nachmias, A. and Strobel, G. A. (1978) *Phytoparasitica* **6**, 95.
2. Barash, I., Karr, A. L. and Strobel, G. A. (1975) *Plant Physiol.* **55**, 646.
3. Starrat, A. N. and Stoessl, A. (1977) *Can. J. Chem.* **55**, 2360.
4. Strobel, G. A. (1982) *Annu. Rev. Biochem.* **51**, 3090.

5. Turner, W. B. and Aldridge, D. C. (1983) in *Fungal Metabolites II*, p. 145. Academic Press, London.
6. Stoessl, A. (1969) *Can. J. Chem.* **47**, 767.
7. Arnone, A., Assante, G., Merlini, L. and Nasini, G. (1986) *J. Chem. Soc. Perkin I* 525.
8. Holenstein, J. E. and Stoessl, A. (1983) *Phytopath. Z.* **108**, 143.
9. Suemitsu, R., Yamada, Y., Sano, T. and Yamashita, K. (1984) *Agr. Biol. Chem.* **48**, 2383.
10. Henderson, J. F., Zombor, G. and Miko, M. (1980) *Biochem. Pharmacol.* **29**, 2155.