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BIOLOGY OF METALS

Tolypocladin – a new metal-chelating 2-aza-anthraquinone from *Tolypocladium inflatum*

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Summary. A new chelator of di- and trivalent cations (tolypocladin) was isolated from the mycelium of *Tolypocladium inflatum* DSM 915. The structure has been determined by NMR methods as 3-methyl-5,6(7),8-trihydroxy-2-aza-anthraquinone. The ultraviolet, visible and fluorescence spectral properties of some metal complexes (in methanol) are described. The compound forms water-soluble fluorescent aluminium complexes. Its production is dependent on zinc ions in the medium. It serves as an endogenous hydrogen acceptor under oxygen limitation in the producing strain, *T. inflatum*.

Key words: Tolypocladin – New 2-aza-anthraquinone – Metal-chelating properties – Metabolite of *Tolypocladium inflatum* – Regulation of production by zinc ions – Role as endogenous electron acceptor

Introduction

Fungi are known to produce numerous pigmented metabolites amongst which anthraquinone structures have frequently been observed (Turner and Aldridge 1983). These microorganisms are even capable of producing heterologous 2-aza-anthraquinones such as bostrycoidin (II) (Arsenault 1965; Steyn et al. 1979; Parisot et al. 1989). Moreover, the biological role of such metabolites, either as metal-chelating agents or constituents of a particular respiratory chain, has been subject to discussion (Medentsev et al. 1989). In particular, the biological importance of pigment production for the producing microbe itself is not clear; it could be, for instance, as a scavenger of trace elements or as a detoxifying agent for high heavy metal concentrations.

In the course of a screening program aimed at coloured quinoid substances of fungal origin, we detected a mutant strain of cyclosporin-producing *Tolypocladium inflatum* DSM 915 (Dreyfuss et al. 1976; obtained from *Deutsche Sammlung für Mikroorganismen*, Göttingen, FRG) which grows on surface agar as a lawn of strongly pigmented brownish-violet biomass.

Here, we report the isolation of this pigment, structure elucidation and complex-forming properties of the new metabolite which has been given the name tolypocladin (Fig. 1, I). Furthermore, regulation of tolypocladin production by the fungus and use of the pigment as an electron acceptor is discussed.

Materials and methods

Pigment production and isolation. Tolypocladium inflatum (DSM 915), mutant strain WB 6/5, was maintained on agar slants made of 36 g/l sucrose, 27 g/l D-glucose, 7.0 g/l $(NH_4)_2SO_4$, 0.15 g/l KH_2PO_4, 0.21 g/l K_2HPO_4, 0.009 g/l $MgSO_4 \cdot 2H_2O$, 0.02 g/l $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.01 g/l $ZnSO_4 \cdot 2H_2O$, 0.002 g/l $CaSO_4 \cdot 5H_2O$, 10 g/l $CaCO_3$, pH 5.3, solidified with 20 g/l agar; and grown on the same medium (except for agar) in liquid culture for 4 days to produce an inoculum. From this inoculum, 0.5 l was used to inoculate a 20-l fermentor containing the same liquid medium. After 7 days of cultivation at 25° C (impeller speed 300 rpm; 0.5 l sterile air/fermentor volume) the brownish biomass was separated from 100 l broth and resuspended overnight in 5 l acetone containing 2% oxalic acid. The acetone extract was evaporated to dryness and the residue treated with 1 l $CHCl_3$. Metabolite I was extracted with 1 l 1 M $NaCO_3$, and the $CHCl_3$ phase was discarded. The water phase was adjusted to pH 3 with HCl and extracted three times with 1 l $CHCl_3$. The combined $CHCl_3$ extracts were dried and evaporated to 100 ml volume yielding 2 g of nearly pure I as a brownish-violet precipitate. The supernatant solution contained two minor components with similar but unknown structures. Further purification of I was achieved by silica gel chromatography (0.063–0.2 mm, acetone/$CHCl_3$, 3:1, with 2% oxalic acid) and subsequent column chromatography on Sephadex LH-20 (in methanol).

Spectroscopic analyses. Proton NMR spectra were determined at 400 MHz and ^{13}C-NMR spectra at 100 MHz with a Varian XL-400 FT instrument. Electron impact mass spectra were recorded with a Jeol JMS-D100 instrument, ultraviolet and visible spectra (in methanolic solution) with a Specord UV-VIS instrument (VEB Carl Zeiss Jena), and fluorescence spectra (in methanolic solution) with a Shimadzu spectrofluorimeter type RF-540.

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Thin-layer chromatography. All purification steps were checked by thin-layer chromatography on silica gel sheets (Silufol sheets, Kavalier, CSR) soaked with 5% oxalic acid in methanol and dried at room temperature. The solvent was acetone/$CHCl_3$ (2:1, by vol.).

Measurement of pigment concentration in the mycelium. A 10-ml culture was centrifuged and the mycelium sediment resuspended in the same volume of acetone containing 50 g oxalic acid/l. After standing at ambient temperature for 20 h, the supernatant was measured at 500 nm against water. Then, the absorbance at 500 nm was taken as a relative measure of pigment production.

Measurements of complex formation. The pigment (2.7 mg) was dissolved in 300 µl hot dimethylformamide and 0.7 ml methanol. Each 100 µl of this solution was diluted with methanol and methanolic solutions of metal salts to give the appropriate concentrations. Unless otherwise stated the metals were added in trimolar excess to the pigment solution.

Results and discussion

Isolation and properties of tolypocladin (I)

The new metabolite I was extracted from the mycelium and purified to homogeneity by common chromatographic procedures. Its concentration amounted to approximately 2 mg/g dry mycelium. Two minor components with unknown structure but very similar spectral and complex-forming properties were coproduced.

Tolypocladin (I) is a reddish-brown amorphous powder with m.p. >320° C (decomposition). The R_f value is 0.35–0.40 on silica gel sheets (Silufol, Kavalier, CSFR). It dissolves well in dimethylformamide and dimethylsulfoxide but only moderately in methanol, acetone, and chloroform. Moreover, it is insoluble in *n*-hexane and water <pH 7, but soluble in water >pH 9.

Elucidation of structure

The ^1H,^{13}C-NMR and MS spectroscopic data suggest that the new metabolite I is a close relative of bostrycoidin (II) (Parisot et al. 1989), probably its 6-*O*-demethyl derivative, 3-methyl-5,6,8-trihydroxy-2-aza-anthraquinone (Fig. 1).

EI-MS direct inlet (200° C): *m/z* 271.0475 (M^+) found; 271.0481 calcd for $C_{14}H_9NO_5$. Characteristic fragmentations: *m/z* 243 ($M^+ - CO$); 228 ($M^+ - CO—CH_3$); 215 ($M^+ - 2CO$); 201 ($M^+ - C_3H_2O_2$); 197 ($M^+ - 2CO—H_2O$); 169 ($M^+ - H_2O—3CO$); 145 ($M^+ - C_3H_2O_4$). ^1H-NMR (400 MHz, $(C^2H_3)_2SO$, 25° C): δ/ppm = 2.70 (s, CH_3); 6.69 (s, H-6 or H-7); 7.88 (s, H-4); 9.23 (s, H-1); 11.55 broad (OH); 12.77 (OH); 13.32 (OH).

^{13}C-NMR (100 MHz, $(C^2H_3)_2SO$, 25° C): δ/ppm = 25.05; 105.38; 110.69; 113.29; 118.12; 124.61; 138.71; 148.37; 150.36; 157.76; 161.04; 165.33; 183.22; 186.27.

Although the chemical shift data of I assigned to C-5, C-6, and C-7-H are similar to the corresponding data of 5-deoxy-6-*O*-demethyl-bostrycoidin (Parisot, personal communication), the final assignment of the OH group to either carbon 6 or 7 will need further elucidation, e.g. by X-ray investigation. But taking into account the presumed biosynthesis of such compounds (Parisot et al. 1989), attachement of the OH group at C-6 seems more likely than to C-7 (Fig. 1).

Electron spectral properties and complex formation

Metabolite I displays characteristic infrared, visible and ultraviolet spectral properties.

Infrared spectrum. λ_{max}/nm (in KBr) = 630, 645, 690, 705, 740, 755, 760, 790, 805, 810, 850, 925, 1050, 1120, 1140, 1170, 1210, 1300, 1360, 1405, 1450, 1455, 1500, 1515, 1535, 1550, 1575, 1610 (C=O; *p*-quinone H-bonded), 2320, 3000, 3400, 3710, 3810.

Ultraviolet and visible spectrum. Acid form of I (in methanol): λ_{max}/nm = 505 (ε = 3.5 μM^{-1} cm^{-1}), 545 and 460 (shoulders), 320 and <250 (strong absorption). Trisodium salt of I (in methanol): λ_{max}/nm = 560, 525, 420 and 290 (Fig. 2a).

The addition of di- or trivalent metal ions in trimolar excess with respect to the ligand I yielded characteristic bathochromic shifts of the electron spectral pattern due to formation of complex salts under weak acidic conditions (Fig. 2a–c). Both divalent and earth alkaline ions such as Be^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+}, and heavy metals such as Fe^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} (all ions added as chlorides or sulfates) caused colour changes of the methanolic solution of I (Fig. 2b). Fig. 2c demonstrates with Zn^{2+} ions that spectral absorption intensity of these complexes increased when the ratio of metal/I was increased from 1:1 to 1:4.

Trivalent cations such as Fe^{3+} ($FeCl_3$), Al^{3+} ($AlCl_3$) and Ga^{3+} [$Ga(NO_3)_3$] also displayed shifts of electron spectral properties due to complex formation. But these complexes were unstable in aqueous solution as well as under alkaline conditions. Thus, metal hydroxides and the ligand I were precipitated when the methanolic solution of complexes was diluted with water. An excep-

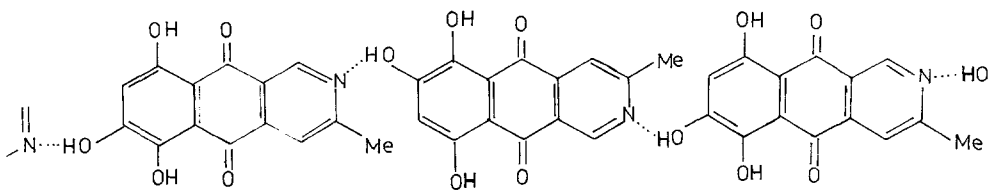

Fig. 1. Chemical structures of tolypocladin (I), bostrycoidin (II; from *Fusarium* species), and possible formation of H-bonded oligomers of tolypocladin (III)

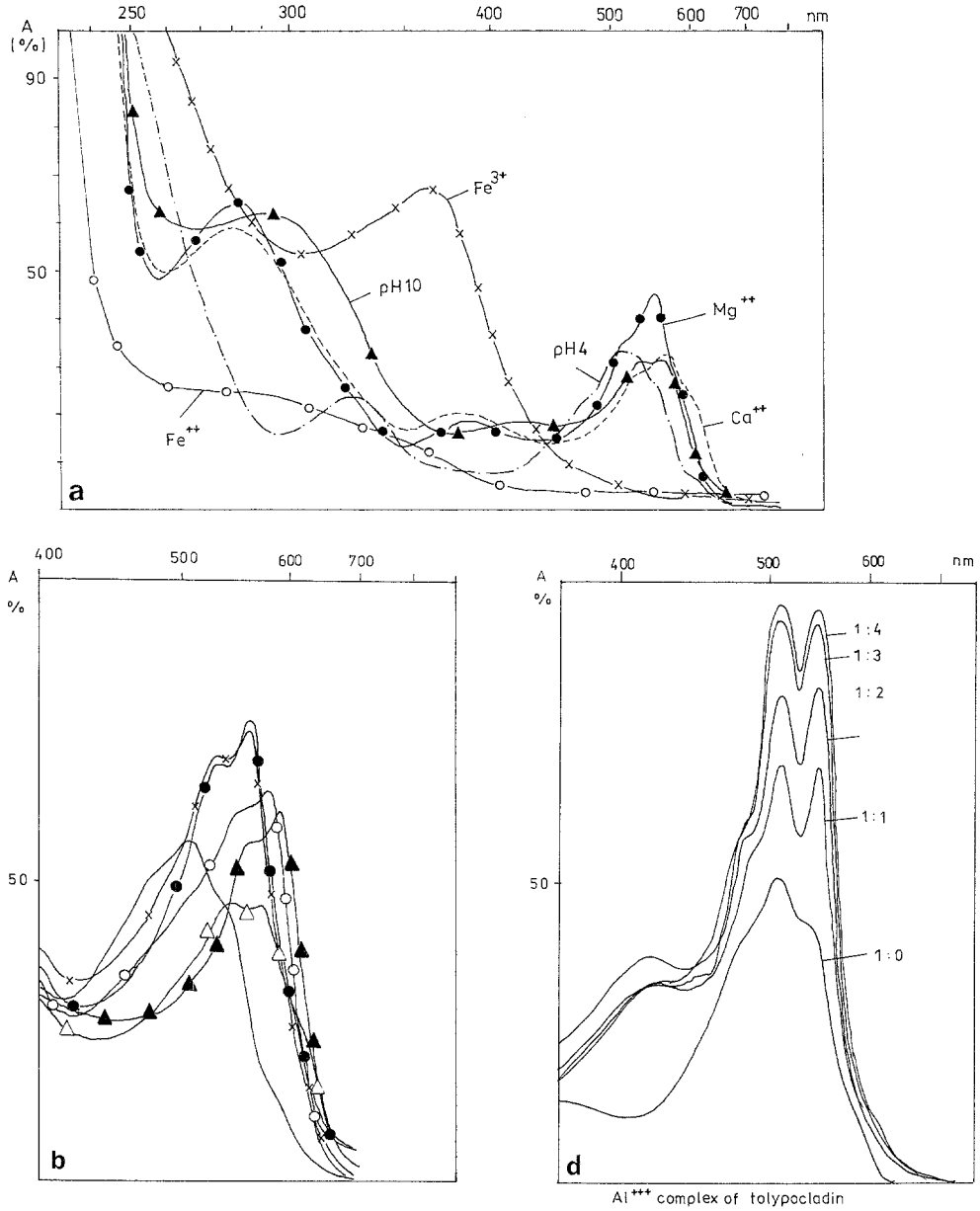

Fig. 2. Spectral properties of I and its metal complexes in methanolic solution (0.1 mM). **(a)** Ultraviolet/visible spectra of I and selected complexes. (— · —) I acid, pH 4; (▲) trisodium salt of I, pH 9; (●) Mg^{2+} complex; (×) Fe^{3+} complex; (○) Fe^{2+} complex; (– – –) Ca^{2+} complex. **(b)** Visible spectrum of metal complexes of I (0.2 mM in methanol). (—) I acid, pH 4; (●) Mn^{2+} complex; (×) Zn^{2+} complex; (○) Cu^{2+} complex; (▲) Ni^{2+} complex; (△) Co^{2+} complex. **(c)** Visible spectrum of I (0.2 mM) with increasing amounts of Zn^{2+} in molar ratios I/Zn^{2+} of 1:0, 1:1; 1:2; 1:3; 1:4; T = tolypocladin. **(d)** Visible spectra of I and its complex with aluminium (in methanol) T: tolypocladin (I). Ratios of I/Al^{3+}: 1:0; 1:1; 1:2; 1:3; 1:4

tion from this rule was the aluminium complex which was stable at pH 1–6.8 in water.

This particular property led us to study the dependence of complex formation on aluminium concentration in more detail. An increase of the ratio Al^{3+} to tolypocladin (I) from 0:1 to 1:1 brought about a colour change of the methanolic solution from reddish to violet (Fig. 3a). Addition of more $AlCl_3$ (ratio Al^{3+}/I=4:1) caused a further shift to reddish-violet which became more pronounced when the Al^{3+} concentration was further increased (Fig. 2d). Gallium was capable or replacing aluminium to yield the same spectral changes.

The flurorescence spectra of I (in methanol) and its aluminium complex (Fig. 3a, b) show that the rather weak intrinsic fluorescence emission at 410 nm of ligand I (excitation at 305 nm) disappeared in the presence of increasing amounts of aluminium chloride. Moreover, formation of the strongly fluorescent Al^{3+}-tolypocladin complex, documented in Fig. 3b, shows that excitation at 520 nm caused intensive light emission with λ_{max} 550–565 nm. Remarkably, λ_{max} of fluorescence shifted to lower wavelengths with increasing Al^{3+} concentration. To yield maximum fluorescence, a molar ratio of Al^{3+}/I of 4:1 is necessary. Further increase of aluminium concentration only slightly improved the quantum yield of fluorescence. Bostrycoidin (II, Fig. 1) forms similar fluorescent complexes with Al^{3+} possessing equivalent spectral properties (excitation 520 nm; emission 590 nm) but the full fluorescence of the Al^{3+} – bostrycoidin complex was achieved at a molar ratio of Al^{3+}/II of 1:1. This feature, as well as metal-concentration-dependent changes of ultraviolet/visible spectral intensity of complexes of I, could be explained by the strongly acidic character of the phenolic C6-OH group which could give rise to the formation of molecular associations in methanolic solution (Fig. 1, III). The formation of different complexes could thus be achieved through the addition of increasing metal concentrations. The strong fluorescence of the 2:1 to 4:1 Al^{3+}-I complexes has been observed within a pH range of 1–6.8. Below pH 1 the fluorescence was deleted, probably due to protonation of the pyridine ring.

Metabolite I forms fluroescent gallium and beryllium complexes with properties comparable to Al^{3+} complexes while solutions of other complex salts were not fluorescent. In the case of beryllium, a molar ratio of beryllium/tolypocladin (I) of at least 2:1 was necessary to obtain full fluorescence but for gallium a molar ratio of 4:1 was needed for maximum fluorescence emission. It is interesting that tolypocladin and bostrycoidin form water-soluble fluorescent aluminium complexes as this phenomenon has been demonstrated so far for few natural compounds, such as morin.

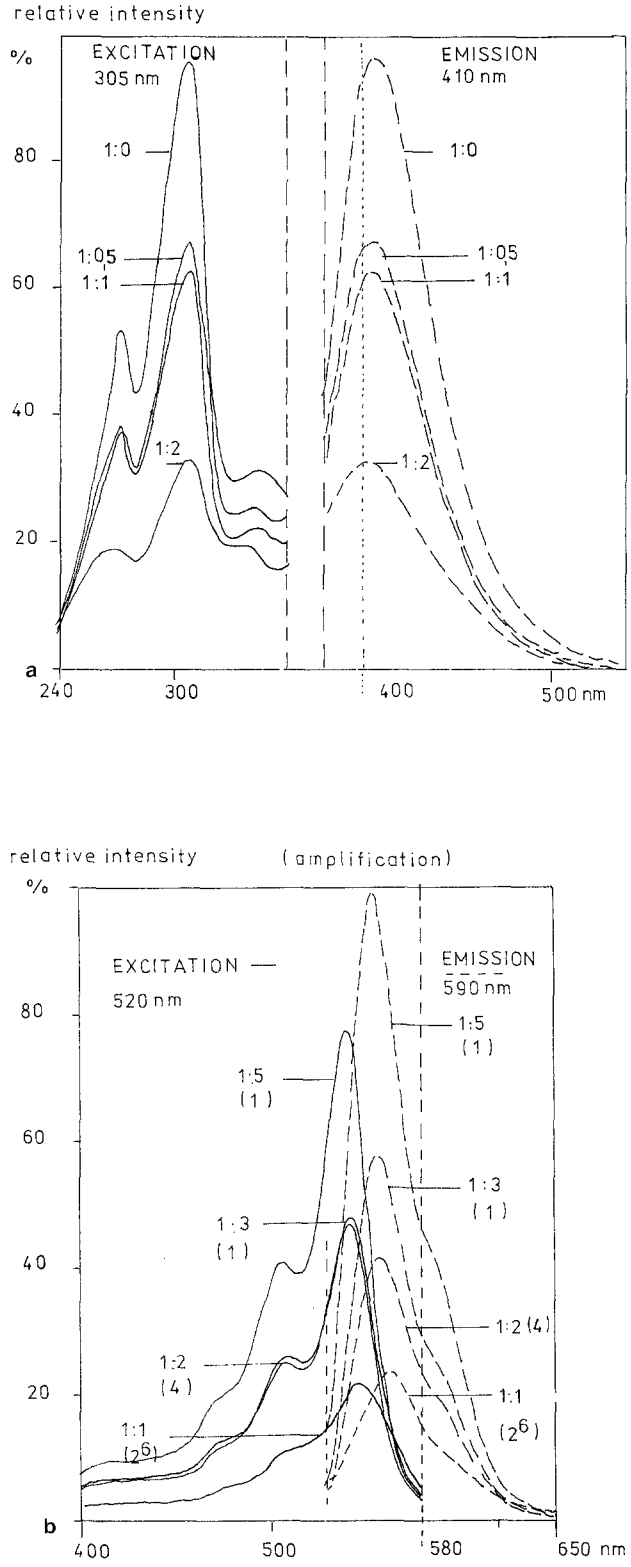

Fig. 3. Fluorescence spectra of tolypocladin (I) and its aluminium complex (in methanol). **(a)** Decrease of fluorescence (excitation 305 nm; emission 410 nm) of tolypocladin in the presence of increasing amounts of Al^{3+}. Molar ratios of I (0.02 mM) to $AlCl_3$ were 1:0, 1:0.25, 1:0.5, 1:1, 1:2, 1:4 (the spectra were amplified tenfold). **(b)** Increase of fluorescence due to Al^{3+} complex formation of I (excitation 540 nm; emission 550–565 nm). Molar ratios I/Al^{3+} 1:1, 1:2, 1:3 and 1:5 with the amplifications in parentheses on the curves

Regulation of pigment production by Tolypocladium inflatum *and the possible biological role of tolypocladin*

As has recently been shown (Parisot et al. 1989), formation of bostrycoidin (6-*O*-methyl tolypocladin) by *Fu-*

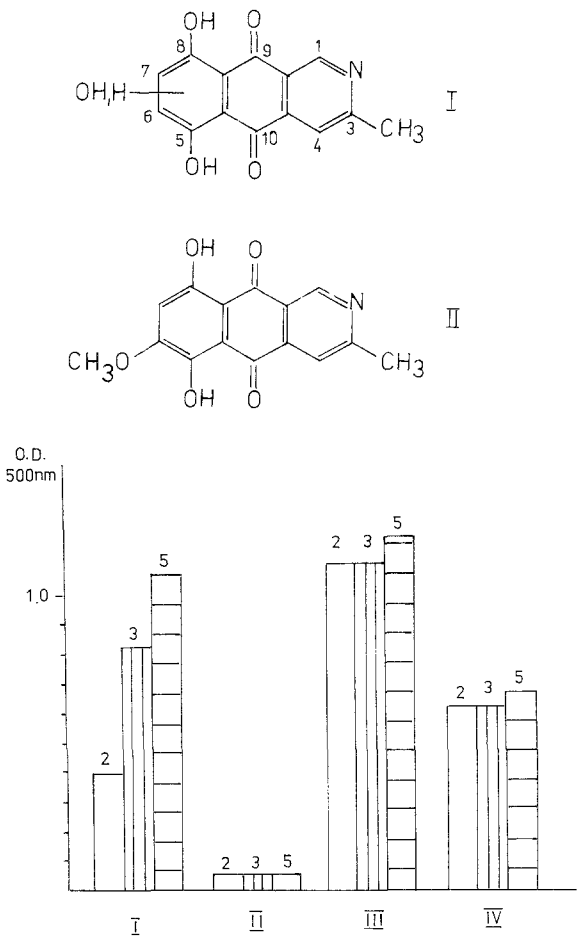

Fig. 4. Effect of medium composition on pigment production by *Tolypocladium inflatum*. (I) Normal medium as shown in Material and methods; (II) the same medium but supplied with 7 g peptone/l instead of $(NH_4)_2SO_4$; (III) the same medium as in II but additionally 14 g $(NH_4)_2SO_4$; (IV) the same medium as in II but additionally 21 g $(NH_4)_2SO_4$/l. (2, 3 and 5 refer to age of culture in days)

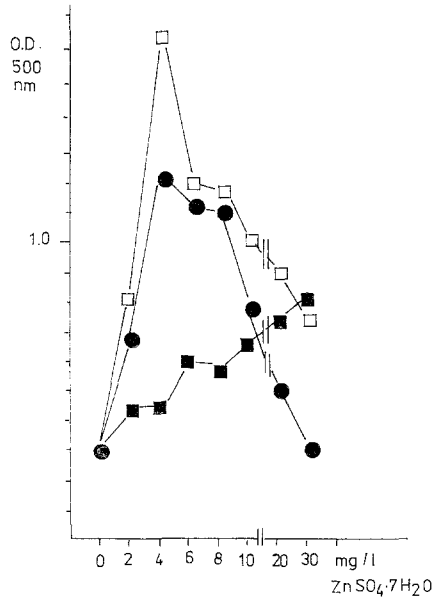

Fig. 5. Effects of increasing Zn^{2+} and NH_4^+ concentrations on pigment production by *T. inflatum*. 7th day cultures. (—□—) Medium as shown in Materials and methods except for zinc; (○) the same medium but with 14 g $(NH_4)_2SO_4$/l; (■) the same medium but 21 g $(NH_4)_2SO_4$/l

sarium species starts from fusarubin which is then oxidized to an intermediate that is convertible non-enzymatically to bostrycoidin in the presence of NH_3. The results of nutrient shift experiments with *T. inflatum* are consistent with this idea (Fig. 4). When $(NH_4)_2SO_4$ was replaced by peptone, no pigment production was observed. Addition of $(NH_4)_2SO_4$ to the peptone medium restored tolypocladin production, but higher NH_4^+ concentration repressed pigment production without concomitant decrease of mycelium dry mass (cf. Lee et al. 1989). Of the trace elements added to the medium, only zinc seemed to play a crucial role in the regulation of pigment production (Fig. 5). With 4–8 mg $ZnSO_4 \cdot 7H_2O$/l, pigment production was greatly stimulated. But when the optimum zinc concentration was exceeded, production of I was reduced due to growth inhibition. With 21 g $(NH_4)_2SO_4$/l in the medium, a higher zinc concentration was necessary to antagonize the nitrogen catabolite repression of pigment production. Obviously, tolypocladin can be used by the producer strain during growth as an endogenous electron sink when oxygen limitation suddenly occurs. When aeration of 48–96 h in fermentor or shake flask cultures was interrupted for 6–10 h, the reddish mycelium turned greenish. Extraction with methanol of this type of mycelium under fully anaerobic conditions (N_2 gas) yielded a greenish solution showing characteristic light absorption at 352 and 370 nm. In the presence of traces of oxygen, the solution immediately turned reddish to give the visible spectrum characteristic of tolypocladin. The same spectrum was recorded when I was reduced with 0.1 mol HCl/l methanol and elemental zinc.

By trapping the reducing equivalents under oxygen limitation, the fungus *Tolypocladium inflatum* could probably maintain its obligate aerobic metabolism for some time. Induction of pigment production in the presence of zinc ions, forming lipophilic complexes, could indicate that there is a detoxification mechanism for this trace element. Tolypocladin does not possess any inhibitory properties against Gram-positive bacteria (*Bacillus subtilis* ATCC 6633) or other fungi (*Phoma* spec., *Penicillium* spec.).

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