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Isolation and Identification of Xanthomegnin, Viomellein, Rubrosulphin, and Viopurpurin as Metabolites of *Penicillium viridicatum*

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Four of the metabolites of *Penicillium viridicatum* 66-68-2 grown on rice cultures were isolated and identified as xanthomegnin, viomellein, rubrosulphin, and viopurpurin. Melting points, elemental analysis, and infrared, ultraviolet, and field desorption and electron impact mass spectra of the isolated compounds were consistent with values reported in the literature for these compounds. In addition, diacetate and triacetate derivatives were prepared, and the chemical and physical analyses of the derivatives were also consistent with literature data. Proton magnetic resonance spectroscopy and thin-layer chromatography were also used for the additional identification of selected compounds.

Penicillium viridicatum is a common contaminant of stored grain (18, 20) and is one of the fungi associated with a disease of corn known as "blue-eye" (11). Krogh et al. (12-14) have also established that some strains of *P. viridicatum* growing on barley produce ochratoxin A, citrinin, and oxalic acid and that these compounds can cause porcine nephrosis. Another strain of *P. viridicatum* produces viridicatumtoxin (9), the structure of which is now known (C. Kabuto, J. V. Silverton, T. Okiyama, U. Sankawa, R. D. Hutchison, P. S. Steyn, and R. Vlegaar, Chem. Commun., in press). The toxicity of a strain of *P. viridicatum* (Purdue University strain 66-68-2) that produces neither ochratoxin nor citrinin has been extensively investigated by Carlton et al. (3-6, 15-17). Feeding the killed fungal mats from liquid or solid fermentations to the rat produced ocular, scrotal, hepatic, and gastric lesions (15-17). Toxic effects were also demonstrated in mice, miniature swine, and guinea pigs (3-5).

The toxicity of *P. viridicatum* 66-68-2 did not appear to be caused by ochratoxin A, citrinin, or oxalic acid. Indeed, we could find no evidence of ochratoxin A, citrinin, or oxalic acid production by this strain. However, during our attempts to isolate and identify the compounds responsible for the observed toxicity, large amounts of yellow, red, and purple pigments were produced, as well as a bright greenish-yellow fluorescent compound that had been isolated from the same strain of mold by Wilson et al. (21, 22) and was shown to be brevianamide A. We have been able to show, mainly through the use of field desorption (FD) mass spectrometry and infrared (IR) spectroscopy, that four of the pigments are xanthomegnin, viomellein, rubrosulphin, and viopurpurin. The structures are shown in Fig. 1. Preliminary toxicity studies on these compounds are reported elsewhere (4).

MATERIALS AND METHODS

Production and isolation of metabolites. *P. viridicatum* 66-68-2 was grown at room temperature for 24 days in 19 2.8-liter Fernbach flasks containing sterile rice (500 g) and water (500 ml). The cultures from each flask were extracted twice with 1 liter of chloroform, and the extracts were filtered. The filtrate was concentrated in a rotary evaporator, and the concentrate was chromatographed on silica gel (500 g) contained in a glass column (50 cm by 5 cm, ID). The column was eluted with 8 liters of chloroform and 5 liters of acetone-chloroform (1:9, vol/vol). The chloroform extract (30 g, containing xanthomegnin-like compounds and oils) was further cleaned up as follows. The extract was placed on another silica gel column (500 g) and eluted with 5 liters of hexane (12 g of oily material removed) and then with 5 liters of chloroform (pigments removed). The chloroform solution was evaporated to 100 ml, 400 ml of hexane was added, and a red precipitate (15 g) resulted. The precipitate was dissolved in chloroform and purified by elution from a silica gel column (250 g) with 3 liters of benzene-chloroform-acetic acid (25:25:1, vol/vol/vol, 1.5 g of viomellein eluted), 3 liters of chloroform-acetic acid (50:1, vol/vol, 0.5 g of rubrosulphin, and 9 g of xanthomegnin eluted), and 3 liters of chloroform-acetic acid (9:1, vol/vol, 0.1 g of viomellein eluted). The chloroform-acetic acid (50:1) eluant was evaporated to a small volume and benzene was added; one compound crystallized (rubrosulphin), and one compound remained in solution (xanthomegnin). The acetone-chloroform eluant

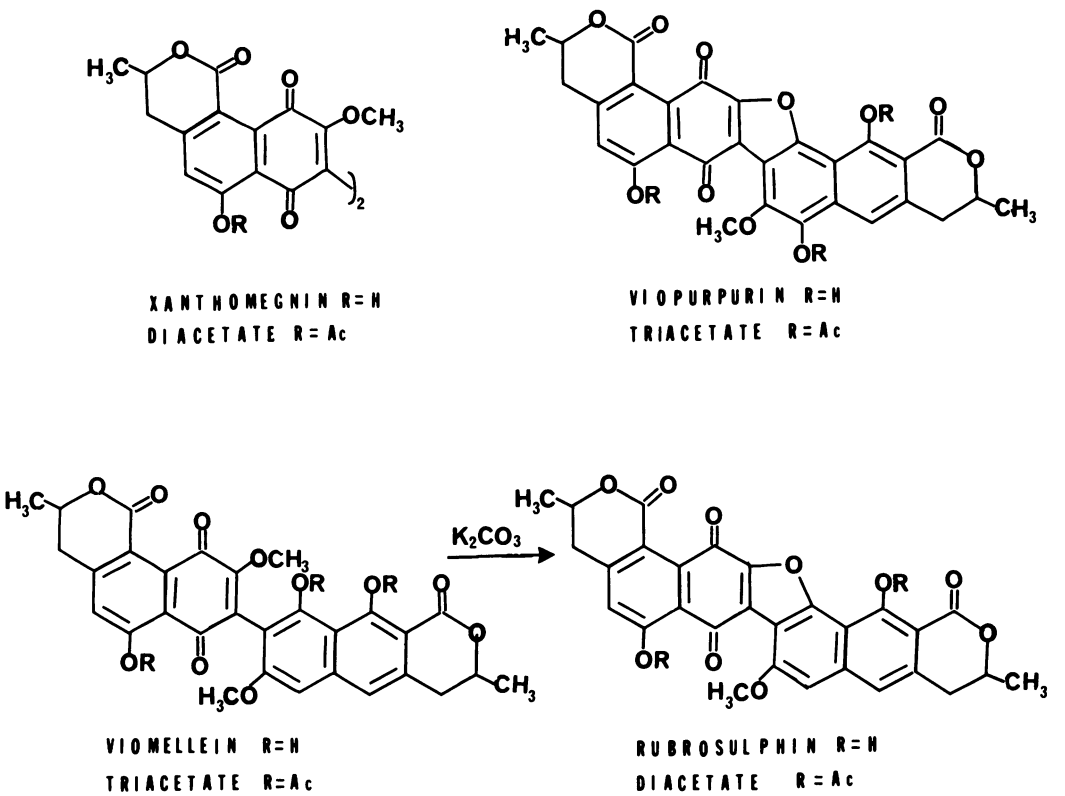

FIG. 1. *Structures of xanthomegnin, viopurpurin, viomellein, rubrosulphin, and their acetate derivatives.*

from the original silica gel column was not subjected to further cleanup (the eluant contained 15 g of brevianamide A and other greenish-yellow fluorescent compounds).

Physical and chemical analyses. Melting points were determined with a micromelting point apparatus (Kofler) and were uncorrected. Elemental analyses for carbon and hydrogen were performed on a elemental analyzer (Perkin-Elmer model 240). The ultraviolet (UV) spectra of the metabolites in the specified solvent were determined with a spectrophotometer (Cary model 14). IR spectra of the compounds were obtained on a spectrophotometer (Perkin-Elmer model 621) and KBr disks. The proton magnetic resonance spectra of the metabolites in $CDCl_3$ were recorded on a spectrophotometer (Varian model HA-100). Electron impact (EI) or FD mass spectra, depending on the characteristics of the individual compound, were determined with a mass spectrometer (Varian MAT CH-5DF) equipped with a combintion EI/field ionization/FD source.

In addition to the metabolites isolated from *P. viridicatum* 66-68-2 grown on rice cultures, the following were also prepared and analyzed by the chemical and physical techniques described above.

(i) Diacetates. These compounds were prepared by reaction of xanthomegnin (27 mg) or rubrosulphin (19 mg) with acetic anhydride (2 ml) in pyridine (10 drops) at room temperature for 24 h in the dark. Xanthomegnin diacetate was purified by silica gel chromatography with hexane-chloroform (1:1, vol/vol) as the eluant.

(ii) Triacetates. These compounds were prepared by reaction of viomellein (10 mg) or viopurpurin (3 mg) with acetic anhydride (1 ml) in pyridine (6 drops) at room temperature for 24 h in the dark.

(iii) Conversion of viomellein to rubrosulphin. Viomellein (20 mg) was added to anhydrous K_2CO_3 (3 g) in acetone (30 ml), and the mixture was refluxed for 22 h. The acetone was removed by rotary evaporation, and water (20 ml) was added to the blue residue. Concentrated HCl was slowly added until the solution was neutral to litmus, and the solution was then extracted with chloroform. A portion of the chloroform solution was applied to a silica gel thin-layer plate and developed with chloroform-methanol-acetic acid (90:5:5, vol/vol/vol); two spots were present, unreacted viomellein and a red compound with a lower R_f. Benzene was added to the chloroform, and the solvents were slowly evaporated in a rotary evaporator until small red crystals (12 mg) formed.

RESULTS

Physical and chemical analyses. (i) Brevianamide A. The IR and EI mass spectra of the brevianamide A isolated from *P. viridicatum* 66-68-2 were compared with those from an authentic sample isolated by Wilson (21, 22).

(ii) Xanthomegnin. Xanthomegnin crystallized from chloroform-benzene (1:2, vol/vol) as orange plates that decomposed without melting above 260°C. Chemical and physical analyses gave the following data: elemental analysis, found, C = 62.6%, H = 3.9% (calculated for $C_{30}H_{22}O_{12}$, C = 62.7%, H = 3.9%); UV absorptions, λ MeOH/maxima at 222, 264, and 380 nm (ε = 26,000, 19,300, and 7,900, respectively); IR absorption maxima, KBr, at 3,430 (broad), 1,721, 1,675, 1,616, 1,590 (shoulder), and 840 cm^{-1}; proton magnetic resonance shifts (δ) at 1.51 (CH_3), 3.02 (CH_2), 4.67 (CH), 7.50 (6H), and 4.11 (OCH_3); EI mass spectral peaks at *m/e* 574, 559, 544, 529, 527, 511, 500, 485, 483, 187, and 160. The IR and mass spectral data were the same as those reported for an authentic sample prepared by Just and Day (10).

(iii) Viomellein. Viomellein crystallized from chloroform-benzene (1:2, vol/vol) as reddish-brown plates that turned black without melting above 275°C. Chemical and physical analyses gave the following data: elemental analysis, found, C = 62.5%, H = 4.5% (calculated for $C_{30}H_{24}O_{11}$, C = 62.3%, H = 4.5%); UV absorptions, λ MeOH/maxima at 225, 264, and 395 nm (ε = 16,800, 20,200, and 8,200, respectively); IR absorption maxima, KBr, at 3,395, 2,980, 2,965, 1.730, 1,680, 1,640 (broad), and 1,590 cm^{-1}; FD mass spectrum showed a molecular ion peak at 560.

(iv) Rubrosulphin. Rubrosulphin crystallized from chloroform-benzene (1:2, vol/vol) as red plates that turned brown above 280°C. Chemical and physical analyses gave the following data: UV absorptions, λ chloroform/maxima at 280, 357, and 415 nm (ε = 31,500, 3,800, and 4,900, respectively); IR absorption maxima, KBr, at 3,278, 2,910, 2,845, 1,729, 1,670, 1,640, and 1,600 cm^{-1}; FD mass spectrum showed a molecular ion peak at 528.

The red crystals resulting from the conversion of viomellein to rubrosulphin had the same FD mass spectrum, IR, and UV spectra, and R_f on silica gel thin-layer chromatographic plates, using chloroform-methanol-acetic acid (90:5:5, vol/vol/vol) as a developing solvent, as the rubrosulphin isolated above.

(v) Viopurpurin. Viopurpurin crystallized from chloroform as purple plates, melting point (mp) >300°C. Chemical and physical analyses gave the following data: UV absorptions, λ chloroform/maxima at 272, 280, and 375 nm (ε = 37,600, 39,000, and 8,400, respectively); IR absorption maxima, KBr, at 3,420 (broad), 1,730, 1,670, 1,635, 1,600, 1,560, and 1,545 cm^{-1}; FD mass spectrum showed a molecular ion peak at 544.

(vi) Xanthomegnin diacetate. This compound crystallized from hexane-chloroform (1:1, vol/vol) as yellow-orange prisms (10 mg), mp 225 to 230°C, with the following properties: UV absorptions, λ MeOH/maxima at 262 and 345 nm (ε = 21,000 and 4,800, respectively); IR absorption maxima, KBr, at 1,776, 1,734, 1,728, 1,675, and 1,599 cm^{-1}; EI mass spectral peaks at *m/e* 658, 616, 574, 559, and 227.

(vii) Viomellein triacetate. This compound crystallized from benzene-chloroform (1:1, vol/vol) as yellow prisms (19 mg), mp 194 to 198°C, with the following properties: UV absorptions, λ MeOH/maxima at 212, 261, and 325 nm (ε = 8,200, 46,200, and 23,800, respectively); IR absorption maxima, KBr, at 1,775, 1,728, 1,725, 1,677, 1,628, and 1,602 cm^{-1}; FD mass spectrum showed a molecular ion peak at 686.

(viii) Rubrosulphin diacetate. This compound crystallized from benzene as orange prisms (19 mg), which turned red at 200 to 205°C, melted, and became purple at 290°C, with the following properties: UV absorptions, λ MeOH/maxima at 216, 275, and 320 (shoulder) (ε = 13,700, 21,700, and 4,400, respectively); IR absorption maxima, KBr, at 1,775, 1,725, 1,681, 1,627, 1,600, 1,570, and 880 cm^{-1}; FD mass spectrum showed a molecular ion peak at 612.

(ix) Viopurpurin triacetate. This compound crystallized from benzene-chloroform (2:1, vol/vol) as yellow prisms (2 mg), mp 209 to 213°C, and the liquid solidified to form a red-orange solid, mp 250°C; the following characteristics were found: UV absorptions, λ MeOH/maxima at 217, 270, and 277 (ε = 5,600, 9,200, and 9,200, respectively); IR absorption maxima, KBr, at 1,775, 1,728, 1,680, 1,628, and 1,601 cm^{-1}; FD mass spectrum showed a molecular ion peak at 670.

DISCUSSION

Xanthomegnin, viomellein, rubrosulphin, and viopurpurin were isolated from rice cultures of *P. viridicatum* (Purdue University strain 66-68-2); the presence of brevianamide A was confirmed.

The identity of xanthomegnin was established by the direct comparison of the mass and IR spectra with the spectra obtained for an authentic sample. The proton magnetic resonance and UV spectra, mp, and elemental analysis were also consistent with the structure of xanthomegnin. The diacetate of xanthomegnin was prepared, and its physical properties were consistent with literature data (10).

Viomellein and rubrosulphin were identified by comparison of the mass, IR, and UV spectra with data for these compounds and for their acetate derivatives published by Durley et al.

(8) and also by conversion of viomellein to rubrosulphin. Viomellein is converted to rubrosulphin simply by displacement of the methoxide ion at C2 of the quinone by the C4′ alkoxide ion (8).

Viomellein, rubrosulphin, and their acetates gave poor EI mass spectra, with small or nonexistent molecular ions for viomellein, rubrosulphin, and viomellein triacetates and the appearance of a large M + 2 peak for rubrosulphin diacetate caused by reduction of the quinone by water present in the spectrometer; this was previously reported by Durley et al. (8). However, good FD mass spectra were obtained for these four compounds. The major peaks observed were the molecular ions of 560 for viomellein, 528 for rubrosulphin, 686 for viomellein triacetate, and 612 for rubrosulphin diacetate.

Viopurpurin was identified by comparison of the UV and IR spectra with those reported in the literature (8). The IR spectrum of the triacetate matched that reported by Blank et al. (2). Viopurpurin and its triacetate gave poor EI mass spectra but good FD spectra, with major peaks at the molecular ions of 544 for viopurpurin and 670 for the triacetate. An ion at mass 672 (M + 2) was noted in the FD mass spectrum of the triacetate, probably due to the reduction of the quinone in the mass spectrometer.

Xanthomegnin had previously been isolated from the dermatophytes *Trichophyton rubrum* (23), *T. megnini* (1), and *T. violaceum* (19), and from *Aspergillus sulphureus* and *A. melleus* (8). Viopurpurin had previously been isolated from *T. violaceum* (19) and from *A. sulphureus* and *A. melleus*. Viomellein was previously isolated from *A. sulphureus* and *A. melleus* (8), and rubrosulphin was isolated from *A. sulphureus* (8). The occurrence of xanthomegnin and related compounds from three different genera indicates that the metabolites may be quite common and that the fungi, although not closely related, may have identical biosynthetic pathways. Ciegler et al. (7) are of the opinion that the six-membered lactone ring present in xanthomegnin and related compounds has potential carcinogenic activity. Indeed, six-membered lactone rings are present in numerous biologically active mold metabolites, including aflatoxin, ochratoxin, citreoviridin, and alternariol. Although xanthomegnin has been known since 1963, its biological activity had not been tested until recently (4). Since both xanthomegnin and viomellein produced the hepatic lesions typical of *P. viridicatum* 66-68-2 toxicoses, they may be the elusive toxins. We hope to be able to investigate further the toxicity of these compounds, as well as the possible synergism between xanthomegnin and viomellein, rubrosulphin, and viopurpurin and between the xanthomegnin class of compounds and other *P. viridicatum* metabolites such as brevianamide A. It is possible that, although each of the compounds alone is only moderately toxic, when combined they may be quite toxic. Another possibility is that the toxicity of *P. viridicatum* 66-68-2 may be due in part to a compound or compounds that have not yet been isolated and identified. We are continuing the isolation and structural elucidation of other *P. viridicatum* metabolites.

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