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Detection and assay of secondary metabolites of *Penicillium vermiculatum* DANG

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Abstract

Thin-layer chromatography on silica gel plates with detection at 254 and 365 nm, spraying with vanillin–sulphuric acid, ethanol ammonia, aqueous iron(III) chloride or ethanolic potassium hydroxide is suggested for rapid detection of (−)-vermiculin, (−)-vermiculinic and vermiculic acids, (−)-vermistatin, (−)-mitorubrinol, vermilutin, (±)-dehydroaltenusin, (+)-vermixocin A, (−)-vermixocin B, funiculosic acid and 2-methylsorbic acid, metabolites of *Penicillium vermiculatum* DANG. High-performance liquid chromatography on a Separon SGX C_{18} compact glass column eluted with methanol–water (pH 3) mixtures with detection at various wavelengths was used for assaying these compounds in cultivation media and extracts during their processing.

1. Introduction

Recently, vermiculin, a sixteen-membered macrodiolide, was isolated from the cultivation medium of *Penicillium vermiculatum* DANG in our laboratory [1]. Because this diolide showed promising pharmacological, especially antimicrobial, cytotoxic and immunoregulatory, activities, greater amounts of this compound were required for further biological evaluation. In the course of a detailed study of the microbial-producing strain and elaboration of pilot-plant technology for vermiculin preparation, we isolated further metabolities of *P. vermiculatum*: the phthalidopyranone (−)-vermistatin [2], the azaphilone (−)-mitorubrinol [3], the xanthone vermilutin [4], the lactone (±)-dehydroaltenusin [5], the 5*H*,7*H*-dibenzo[*c*,*f*][1,5]dioxocine derivatives (+)-vermixocin A and B [6], the phthalaldehydic funiculosic acid [3] and aliphatic 2-methylsorbic acid [7]. We also identified vermiculinic and vermiculic acid, intermediates in vermiculin biosynthesis [8].

P. vermiculatum is the imperfect (conidial) state of *Talaromyces flavus* (Klöcker) Stolk and Samson, the commonly occurring soil fungus, producing secondary metabolites other than those mentioned above [9–11]. Mycotoxins of *T. flavus* and of other microbial strains have been examined by thin-layer chromatography (TLC) [12] and high-performance liquid chromatography (HPLC) [13]. As only the determination of vermiculin [14,15] and mitorubrinol [15] has been published so far, we developed a method for the identification and determination of all known metabolites of *P. vermiculatum*.

2. Experimental

2.1. Apparatus

The HPLC equipment (Laboratory Instruments, Prague, Czech Republic) consisted of an

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HPP 5001 pump, an LCI 30 injector with a 3-μl loop, an LCD 2040 variable-wavelength UV detector and a CI-105 integrator.

The columns were glass cartridges (compact glass column) (150 × 3 mm I.D.) packed with Separon SGX C_{18}, 7 μm (Tessek, Prague, Czech Republic).

Silufol UV-254 plates (10 × 10 cm) (Kavalier, Votice, Czech Republic) were used for TLC.

UV spectra were recorded with a Specord 40 M spectrophotometer (Zeiss, Jena, Germany).

2.2. Chemicals and reagents

All the metabolites were isolated and purified in our laboratory. They were identified by physico-chemical (m.p., optical rotation) and spectral (UV, IR, MS and NMR) data.

All chemicals were of analytical-reagent grade (Lachema Brno, Czech Republic).

Vanillin–sulphuric acid reagent (D_3) was prepared by dissolving vanillin (0.5 g) in concentrated sulphuric acid (5 ml) and diluting to 100 ml with ethanol. After spraying, TLC plates were heated at 105°C for 5 min.

Aqueous Iron(III) chloride reagent (D_4) was prepared by dissolving $FeCl_3 \cdot 6H_2O$ (1.0 g) in 100 ml of water.

Ethanolic ammonia solution (D_5) was prepared by dissolving ammonia solution (5 ml) in and diluting to 100 ml with ethanol.

Ethanolic potassium hydroxide solution (D_6) was prepared by dissolving KOH (5 g) in 100 ml of ethanol. After spraying, the plates were heated at 105°C for 5 min.

2.3. Determination of vermiculin in a technical-grade product

A solution of vermiculin (5.0 mg in 25.0 ml of acetonitrile) was injected on to the column. The mobile phase was methanol–water (35:65) (pH 3, adjusted with phosphoric acid) at a flow-rate of 0.3 ml/min and the detection wavelength was 230 nm.

2.4. Determination of organic acids in cultivation medium of P. vermiculatum

A 20.0-g amount of medium acidified with 1 *M* hydrochloric acid (1 ml) was extracted with chloroform (three times, 15 ml each), the combined organic extracts were re-extracted with 0.1 *M* sodium hydrogecarbonate solution (twice, 15 ml each), the combined aqueous solutions were diluted to 50.0 ml with water and aliquots of the solution was injected on to the chromatographic column. The mobile phase was methanol–water (50:50) (pH 3) at a flow-rate of 0.6 ml/min and the detection wavelength was 240 nm. The efficiency of organic acid extraction was established with a sample spiked with 2-methylsorbic, vermiculinic and vermiculic acid (75 μg/ml each) and was 96.5%, 99.2% and 98.2%, respectively.

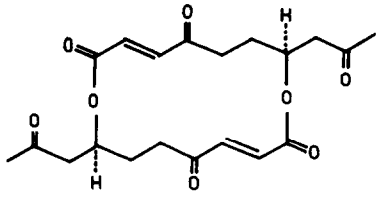
2.5. Determination of vermixocin A in cultivation medium containing whey

A 20.0-g amount of medium was extracted with ethyl acetate (3 times, 15 ml each), the combined extracts were evaporated to dryness, the residue was dissolved in methanol and the solution was diluted to 25.0 ml with methanol. The mobile phase was methanol–water (55:45) at a flow-rate of 0.6 ml/min and the detection wavelength was 230 nm.

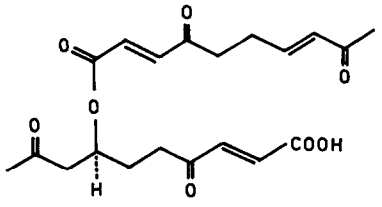
3. Results and discussion

So far, we have isolated and structurally characterized (−)-vermiculin, (−)-vermistatin, (−)-mitorubrinol, vermilutin, (±)-dehydroaltenusin, vermixocins, funiculosic acid and 2-methylsorbic acid (Fig. 1) in cultivation media of *P. vermiculatum*.

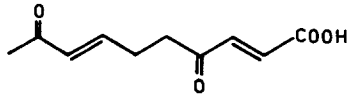
A combination of TLC R_F values with specific colours developed after spraying with appropriate reagents was used for the rapid detection of these metabolites (Table 1). Vermiculin and its precursors, vermiculinic acid and vermiculic acid gave brown spots only with vanillin reagent and ethanolic potassium hydroxide after heating for 5



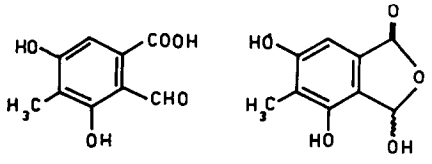
VERMICULIN VM



VERMICULINIC ACID V20

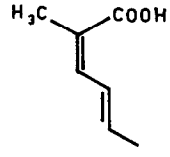


VERMICULIC ACID V10

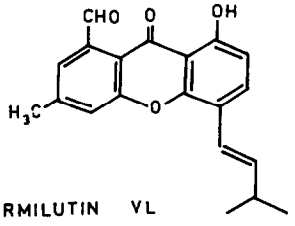


FAa FAb

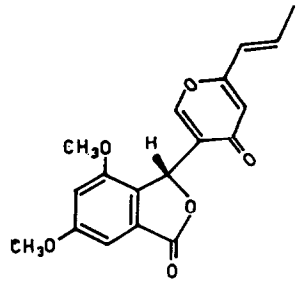
FUNICULOSIC ACID FA



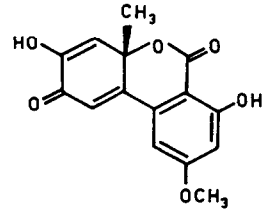
METHYLSORBIC ACID MA



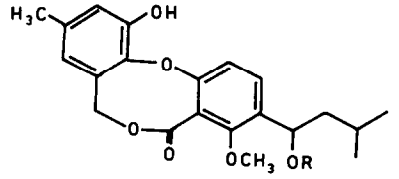
VERMILUTIN VL



VERMISTATIN VS

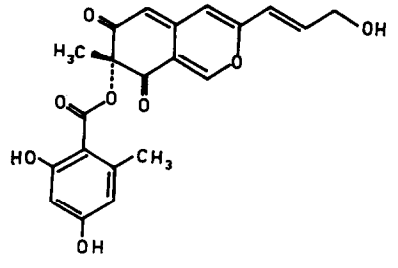


DEHYDROALTENUSIN DH



R: H VERMIXOCIN A VXA

R: $COCH_3$ VERMIXOCIN B VXB



MITORUBRINOL MT

Fig. 1. Metabolites of *Penicillium vermiculatum* DANG.

Table 1
TLC data for *P. vermiculatum* metabolites

Compound	R_F in system[a]			Detection[b]					
	S_1	S_2	S_3	D_1	D_2	D_3	D_4	D_5	D_6
Vermilutin	0.99	0.97	0.99	+	+	G	Y	–	R
Dehydroaltenusin	0.94	0.46	0.73	+	+	R	O	B	B
Vermixocin B	0.94	0.48	0.78	+	–	G	–	–	–
Vermistatin	0.91	0.33	0.56	+	+	–	–	–	Y
Vermilutin	0.88	0.26	0.36	+	–	B	–	–	B
Vermiculinic acid	0.80	0.15	0.27	+	–	B	–	–	B
Methylsorbic acid	0.79	0.60	0.71	+	+	V	–	–	B
Vermixocin A	0.68	0.23	0.37	+	–	G	–	–	–
Vermiculic acid	0.63	0.09	0.20	+	–	B	–	–	B
Funiculosic acid	0.44	0.13	0.17	+	+	–	V	–	Y
Mitorubrinol	0.41	0.08	0.10	+	+	B	Y	R	R

[a] S_1 = chloroform–methanol (9:1); S_2 = benzene–methanol (9:1); S_3 = chloroform–2-propanol (19:1).
[b] D_1 = 254 nm; D_2 = 365 nm; D_3 = vanillin–sulphuric acid; D_4 = iron(III) chloride; D_5 = ethanolic ammonia; D_6 = ethanolic potassium hydroxide. Colours: B = brown; G = green; O = orange; R = red; V = violet; Y = yellow.

min. The red colour of mitorubrinol with ammonia is a specific reaction of azaphilones [16]. A remarkable observation was the green colour of biogenetically related vermixocins and the xanthone vermilutin with vanillin reagent. Vermilutin, dehydroaltenusin, funiculosic acid and mitorubrinol gave also characteristic coloured

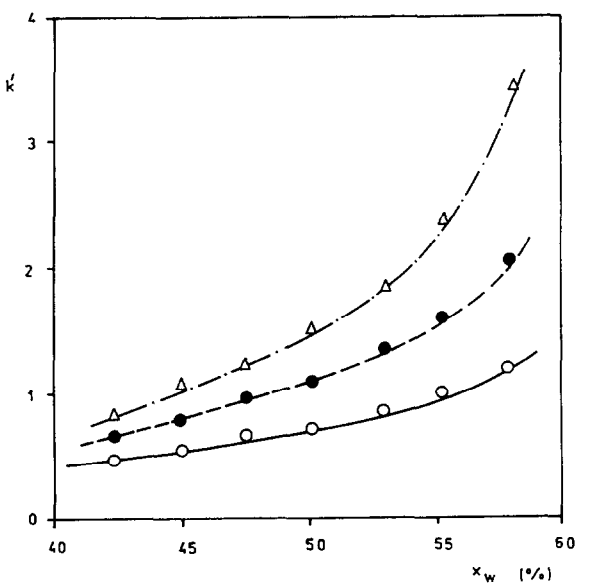


Fig. 2. Plot of capacity factor (k') and volume percentage of water in the mobile phase (x_w). ○ = Vermiculic acid; ● = vermiculin; △ = vermiculinic acid. Column, 150 × 3 mm I.D., Separon SGX C_{18} (7 μm); mobile phase, methanol–water (pH 3); flow-rate, 0.4 ml/min.

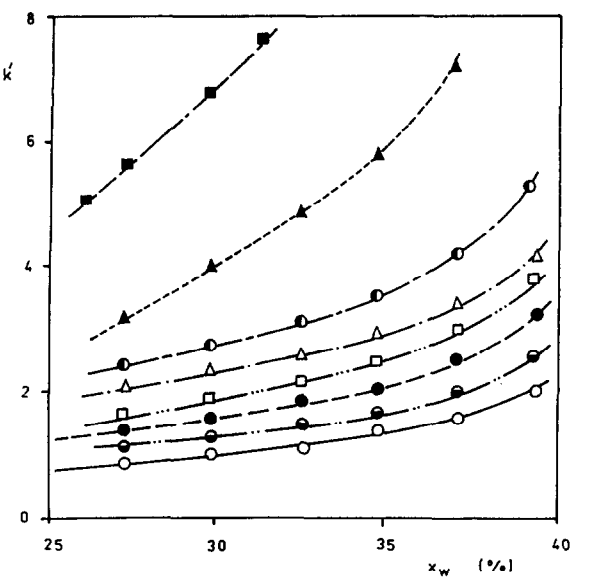


Fig. 3. Plot of capacity factor (k') and volume percentage of water in the mobile phase, x_w. ○ = Dehydroaltenusin; ◒ = funiculosic acid; ● = vermilutin; □ = mitorubrinol; △ = methylsorbic acid; ◐ = vermistatin; ▲ = vermixocin A; ■ = vermixocin B. Column, 150 × 3 mm I.D., Separon SGX C_{18} (7 μm); mobile phase, methanol–water (pH 3); flow-rate, 0.4 ml/min.

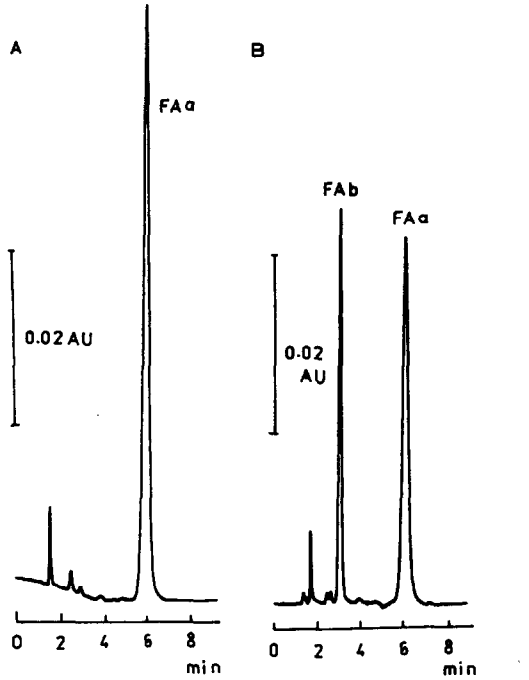


Fig. 4. Chromatograms of funiculosic acid: (A) fresh solution; (B) solution refluxed for 1 h at pH 2. FAa = phthaladehydic form; FAb = phthalidic form. Column, 150 × 3 mm I.D., Separon SGX C_{18} (7 μm); mobile phase, methanol–water (55:45) (pH 3); flow-rate, 0.5 ml/min; detection wavelength, 230 nm.

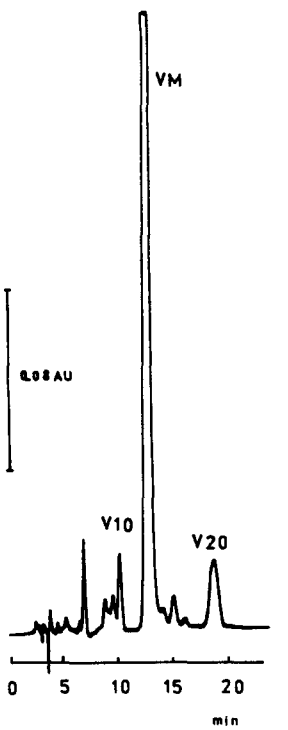


Fig. 5. Chromatogram of *P. vermiculatum* cultivation medium harvested in 144 h. V10 = vermiculic acid; VM = vermiculin; V20 = vermiculinic acid. Column, 150 × 3 mm I.D., Separon SGX C_{18} (7 μm); mobile phase, methanol–water (35:65) (pH 3); flow-rate, 0.4 ml/min; detection wavelength, 230 nm.

spots with iron(III) chloride solution (Table 1).

HPLC on an RP-18 column was used for the determination of the metabolites of *P. vermiculatum*. According to the capacity factors (Figs. 2 and 3), the compounds studied can be divided into three groups. The first group,

Table 2
UV bands of studied metabolites

Compound	M_r	λ (nm) [ε(m^2/mol)][a]
Vermilutin	392.4	230 (616)
Mitorubrinol	398.4	216 (3455), 238 (1860), 267 (3456), 306 (1827), 346 (3056)
Vermistatin	328.3	220 (2240), 239 (646), 262 (1024)
Vermilutin	322.2	226 (3645), 248 (2830), 272 (4056), 350 (80), 398 (256)
Dehydroaltenusin	288.3	220 (1967), 241 (976), 252 (990), 276 (378), 298 (540)
Vermixocin A	372.4	220 (6891), 258 (53), 281 (144)
Vermixocin B	414.4	220 (6899), 257 (55), 283 (143)
Funiculosic acid	196.2	233 (1547), 267 (336), 297 (1168)
Methylsorbic acid	126.2	259 (594)
Vermiculinic acid	392.4	230 (720)
Vermiculic acid	196.2	230 (920)

[a] ε = Molar absorptivity (values in parentheses).

characterized by the highest retention, consists of the dioxocine derivatives vermixocin A and B. Vermistatin, vermilutin, methylsorbic acid, mitorubrinol, funiculosic acid and dehydroaltenusin belong to the second group. These compounds can also be discriminated by varying the pH of the mobile phase owing to the acidic character of two metabolites. Funiculosic acid occurs in phthalaldehydic (FAa) and hydroxyphthalidic (FAb) forms (Fig. 4), as was confirmed also by ^{1}H NMR spectrometry [3]. Equilibrium between the two forms occurs after a long period in solution at neutral pH, but after only 1 h in acidic solution under reflux.

The third group is constituted by vermiculin, vermiculic acid and vermiculinic acid. Surprisingly, vermiculin, which has no polar functions in its structure, is little retained by the C_{18} phase. A chromatogram of the cultivation medium of *P. vermiculatum* optimized for vermiculin production is shown in Fig. 5 and that optimized for vermistatin biosynthesis in Fig. 6. All these compounds rarely occurred in one cultivation;

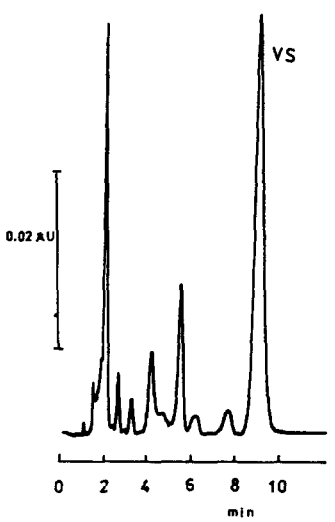


Fig. 6. Chromatogram of extract of cultivation medium optimized for vermistatin production. VS = vermistatin. Column, 150 × 3 mm I.D., Separon SGX C_{18} (7 μm); mobile phase methanol–water (60:40) (pH 3); flow-rate, 0.5 ml/min; detection wavelength, 230 nm.

vermiculin, vermiculinic acid and vermiculin acid, vermilutin, vermixocins, methylsorbic acid funiculosic acid and mitorubrinol are metabolites of *P. vermiculatum* cultivated on a sucrose medium, vermistatin is produced on glucose, only vermixocin A is biosynthesized on a medium containing whey and dehydroaltenusin is a metabolite of a *P. vermiculatum* mutant grown on sucrose. This last compound exhibited the greatest tailing, comparable to that of some flavonoids.

These diverse structures have different UV spectra (Table 2), which can be used for their identification and selective detection in complex mixtures.

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