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Penicillium digitatum Metabolites on Synthetic Media and Citrus Fruits

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Penicillium digitatum has been cultured on citrus fruits and yeast extract sucrose agar media (YES). Cultivation of fungal cultures on solid medium allowed the isolation of two novel tryptoquivaline-like metabolites, tryptoquialanine A (**1**) and tryptoquialanine B (**2**), also biosynthesized on citrus fruits. Their structural elucidation is described on the basis of their spectroscopic data, including those from 2D NMR experiments. The analysis of the biomass sterols led to the identification of **8**−**12**. Fungal infection on the natural substrates induced the release of citrus monoterpenes together with fungal volatiles. The host−pathogen interaction in nature and the possible biological role of citrus volatiles are also discussed.

KEYWORDS: Citrus fruits; *Penicillium digitatum*; quivaline metabolites; tryptoquivaline; *P. digitatum* sterols; phenylacetic acid derivatives; volatile metabolites

INTRODUCTION

P. digitatum grows on the surface of postharvested citrus fruits producing a characteristic powdery olive-colored conidia and is commonly known as green-mold. This pathogen is of main concern as it is responsible for 90% of citrus losses due to diseases occurring during the storage period, and it causes serious damages in commerce (*1*). Most of the research works on *P. digitatum* focus on treatments against the infection symptoms, and literature about fungal metabolites is scarce (*2*, 3). To extend the study of *P. digitatum* metabolites and their possible role in the toxigenesis, as well as to know more about medium−pathogen relation, the microorganism has been cultured on natural and synthetic media. Several metabolites have been isolated and studied by NMR.

MATERIALS AND METHODS

General Experimental Procedures. NMR spectra were recorded in DMSO on a Bruker DRX 600, at 600.13 MHz for ^{1}H and 150.92 MHz for ^{13}C according to Larsen et al. (*4*). The chemical shifts are given relative to DMSO, 2.50 ppm for H^{1} and 39.5 ppm for C^{13} (**Table 1**). The circular dichroism (CD) spectra were measured on a JASCO J-710 spectropolarimeter and the UV spectra were measured on a Hewlett-Packard 8452A diode array spectrophotometer. Analytical HPLC conditions were similar to those given by Smedsgaard (*5*), and retention indices (RI) of fungal metabolites were calculated according to Frisvad and Thrane (*6*). EIMS for the study of citrus volatiles were obtained from a Hewlett-Packard 5972A mass spectrometer using an ionizing voltage of 70 eV, coupled to a Hewlett-Packard 5890A gas chromatograph.

Preparation of TMS Derivatives and Analysis by GC−MS. TMS derivatives were obtained according to the procedure previously described (*7*). The samples (1 μL) were injected onto a 25 m × 0.32 mm i.d. HP-1 methylsilicone capillary column with a He flow rate of 0.6 mL min^{-1}. The temperature program for the study of sterols and oxygenated triterpenes went from 120 °C to 220 °C at 5 °C min^{-1}, from 220 °C to 280 °C at 3 °C min^{-1}, and 10-min hold at 280 °C. Identification of the sterols and oxygenated triterpenes from *P. digitatum* biomass has been made by interpretation of the characteristic mode of fragmentation of their TMS-ethers when subjected to GC−MS (*7*−*9*), and by comparison of RI and mass spectra with those from standards.

Fungal Material and Fermentation. *P. digitatum* IBT 10051 and IBT 21830 isolates, (ex Mandarin), were obtained from the fungal culture collection (IBT) at BioCentrum-DTU, at The Technical University of Denmark.

P. digitatum Cultured on YES Liquid Medium. IBT 10051 was cultured on YES liquid medium, (20 g of yeast extract and 150 g of sucrose in 1000 mL of distilled water). The fungus was inoculated into 50 500-mL Erlenmeyer flasks (100 mL of YES medium per flask) and grown in the dark with shaking at 24 °C for 15 days.

P. digitatum Cultured on YES Solid Medium. IBT 21830 was cultured for 14 days at 25 °C in the dark, as three-point mass inoculation on 200 YES agar plates (*2*).

P. digitatum Cultured on Citrus Fruits. IBT 21830 was cultured on citrus fruits (Valencia oranges) for production of both volatile and nonvolatile metabolites. The citrus fruits were inoculated at several points and cultivated at 25 °C.

Collection of Volatile Metabolites. Volatile metabolites were collected by diffusive sampling onto Tenax TA and analyzed by GC−

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Table 1. NMR Data of Tryptoquialanine A (**1**) in DMSO-d_6

no	^{13}C	^{1}H (mult., J = Hz)	HMBC	NOESY
2	84.0	5.23 (sa)	H-13, 16-NOH	H-13b
3	85.6		H-2, H-5, H-13	
4	134.4		H-6, H-8	
5	125.5	7.91 (dd, 8.5, 1.1)	H-7	H-6, H-12, H-13a
6	125.2	7.31 (ddd, 8.5, 6.8, 1.8)	H-8	H-5, H-7
7	131.5	7.52 (ddd, 7.9, 6.8, 1.1)	H-5	H-6, H-8
8	114.8	7.52 (dd, 7.9, 1.8)	H-6	H-7
9	137.2		H-5, H-7	
11	170.0		H-12, H-13	
12	54.5	5.92	H-13	H-5, H-13a, H-27
13	34.3	3.13 (dd, 13.4, 10.0) 3.08 (dd, 13.4, 10.0)	H-2, H-12	
14	170.8		H-2, H-29, H-30	
15	70.7		16-NOH, H-29, H-30	
-NO*H*		7.95 (sa)		
18	160.5		H-12, H-20	
19	119.9		H-21, H-23	
20	126.3	8.18 (ddd, 8.1, 1.5, 0.5)	H-22	H-21
21	128.1	7.64 (td, 8.1, 1.1)	H-23	H-20, H-22
22	135.6	7.94 (td, 8.1, 1.5)	H-20	H-21, H-23
23	127.5	7.77 (ddd, 8.1, 1.1, 0.5)	H-21	H-22
24	146.0		H-20, H-22	
26	154.9		H-12, H-27, H-28	
27	68.5	6.31 (q, 6.3)	H-28	H-12
CH_3*C*OO-	170.1		C*H*$_3$COO, H-27	
CH$_3$COO-	20.7	2.08 (s)		
28	18.7	1.70 (d, 6.3)		
29	22.9	1.32 (s)	H-30	
30	16.8	1.35 (s)	H-29	

MS according to Larsen and Frisvad (*10*) except that citrus fruits were placed in glass exicators fitted with cotton stoppers, instead of in Petri dishes.

Extraction and Separation. *P. digitatum Cultured on YES Liquid Medium*. After the incubation period of IBT 10051 the fungal mycelium was removed by vacuum filtration, lyophilized, and weighed, giving 14.207 g of dried biomass per L of culture. Dried mycelium (71 g) was ground and extracted first with $CHCl_3$ (4 × 1.5 L) to give 7.814 g of chloroform extract, and then extracted with MeOH (4 × 1.5 L), yielding 60.380 g. The chloroform fraction (6 g) was chromatographed over an Al_2O_3 column, using hexane/MTBE mixtures of increasing polarity. Sterol esters (270 mg) were eluted with hexane/MTBE (80:20), whereas free sterols (75 mg), were eluted with mixtures of hexane/MTBE (50:50 to 30:70). Sterol esters were saponified, and the nonsaponifiable fraction was derivatized and analyzed by GC−MS together with the free sterols. Cholesterol (**8**), ergosta-7,22-dien-3β-OH (**9**), ergosta-7,22,24(28)-trien-3β-OH (**10**), episterol (**11**), and eburicol (**12**) were identified in the sterol ester fraction, whereas **8** and **12** were found in the free sterol and oxygenated triterpene fraction.

P. digitatum Cultured on YES Solid Medium. Agar plates (200) cultured with IBT 21830 were extracted repeatedly with EtOAc (3 L) to give a crude extract (8 g). This extract was partitioned between MeOH/H_2O and heptane (50:50) to give 4.7 g of polar fraction. This polar extract was subjected to vacuum liquid chromatography on silica (40 g), eluting with mixtures of solvents of increasing polarity (heptane, heptane/EtOAc, EtOAc/EtOH, and EtOH; 75 mL per run). As a result the polar extract was separated into five fractions. The second fraction was partitioned again with $CHCl_3$/H_2O, giving 1.7 g of organic extract, which was further separated on a 440 × 37 mm, 40−63-μm Merck Lichroprep Si column, using a gradient of heptane/EtOAc (50:50 to 0:100 in 30 min), with a mobile phase at 40 mL/min flow rate, giving 9 fractions. The heptane/EtOAc, 20:80 fraction (127 mg), was purified on the same column, using a gradient of heptane/EtOAc (70:30 to 0:100 in 30 min) with 13 mL/min flow rate. The resulting fraction, rich in **1** and **2,** was finally purified on a 25 mm × 100 mm, 6-μm, Waters Prep Nova-Pack HR silica column, running with heptane/EtOAc, 65:35 at 2 mL/min flow rate. As a result, compounds **1** (25 mg) and **2** (13 mg) were isolated in pure form. The heptane/EtOAc, 30:70 fraction (585 mg), was finally separated on a 25 × 330 mm, 40−63-μm Merck Lichroprep Si column, using a gradient of heptane/EtOAc (70:30 to 0:100 in 40 min) with a mobile phase at 20 mL/min flow rate, to give 37.7 mg of 2-hydroxyphenylacetic acid methyl ester (**5**), 88.2 mg of **6**, and 22.5 mg of phenylacetic acid (**7**).

P. digitatum Cultured on Citrus Fruits. Fungal spores and mycelium were scraped off the highly molded citrus fruits with a knife and transferred into a glass vial in which they were extracted with 2 mL of MeOH. This extract was filtered through a 0.45-μm filter and then analyzed by HPLC−DAD according to Smedsgaard (*5*).

Tryptoquialanine A (1). HREIMS m/z (rel intensity) found 518.1801 (100) [calcd for $C_{27}H_{26}O_7N_4$, 518.1801 (M^+)]. $[\alpha]^{22}_D = 0°$ (c 0.09, EtOH). UV λ_{max} (EtOH) nm (log ∈) 222 (4.48), 225 (4.49), 273 (sh 3.90), 289 (sh 3.69), 298 (sh 3.54), 310 (sh 3.39). CD (EtOH, c 0.02), Δ∈ (λ nm) 215 (+13.15), 228 (−4.24), 240 (−2.12), 252 (−4.45), 290 (0.00). NMR, **Table 1**.

Tryptoquialanine B (2). HREIMS m/z (rel intensity) found 504.1645 (100) [calcd for $C_{26}H_{24}O_7N_4$, 504.1645 (M^+)]. $[\alpha]^{22}_D = +57.1°$ (c 0.14, EtOH). UV λ_{max} (EtOH) nm (log ∈) 220 (4.48), 225 (4.49), 272 (sh 3.88), 286 (sh 3.67), 296 (sh 3.53), 311 (sh 3.43). CD (EtOH, c 0.03), Δ∈ (λ nm) 216 (+11.13), 234 (−2.07), 236 (−1.13), 252 (−4.70), 312 (0.00). NMR **Table 2**.

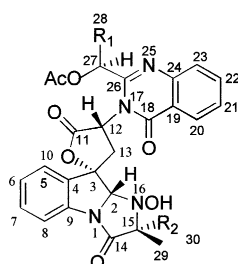
RESULTS AND DISCUSSION

To find out more about the influence of the medium on the production of fungal metabolites, *P. digitatum* was cultured on solid medium and on its natural substrates, i.e., citrus fruits. Analytical HPLC and GC−MS studies showed that fungal extracts obtained from *P. digitatum* on YES solid medium had a composition similar to that of those derived from the culture of the fungus on citrus fruits, revealing the presence of metabolites with UV spectra characteristic of tryptoquivalines in both extracts. Looking for the isolation of these compounds, the fungal extracts on YES solid medium were subjected to consecutive VLC and HPLC chromatographic separations, allowing the isolation of **1** and **2** in pure form (**Figure 1**). The structural elucidation of the novel compounds was made on the basis of their spectroscopic data.

A molecular weight of 518 amu for **1**, different from any of the known tryptoquivalines, suggested the presence of a new

Table 2. NMR Data of Tryptoquialanine B (**2**) in DMSO-d_6

no.	^{13}C	^{1}H (mult., J = Hz)	HMBC	NOESY
2	88.4	5.42 (s)	13H, 15H, 16-NO*H*	H-13b
3	84.2		2H, 5H, 13H	
4	134.3		H-6, H-8	
5	125.4	7.92 (da, 7.6)	H-7	H-6, H-12, H-13a
6	125.4	7.32 (ta, 7.6)	H-8	H-5, H-7
7	131.5	7.54 (ta, 7.6)	H-5	H-6, H-8
8	115.0	7.50 (da, 7.6)	H-6	H-7
9	137.3		H-5, H-7	
11	169.9		H-12, H-13	
12	54.4	5.92 (t, 10.0)	H-13	H-5, H-13a, H-27
13	33.5	3.14 (dd, 13.9, 10.0) 3.04 (dd, 13.9, 10.0)	H-2, H-12	
14	170.0		H-15, H-29	
15	67.4	4.08 (q, 7.1)	16-NO*H*, 29H,	H-29
-NO*H*		8.02 (sa)		
18	160.6		H-12, H-20. H-23	
19	119.9		H-21, H-23	
20	126.3	8.17 (da, 8.0)	H-22	H-21
21	128.1	7.64 (ta, 7.6)	H-23	H-20, H-22
22	135.7	7.94 (ta, 7.6)	H-20	H-21, H-23
23	127.4	7.77 (da, 8.0)	H-21	H-22
24	146.0		H-20, H-22	
26	154.9		H-12, H-27, H-28	
27	68.4	6.29 (q, 6.4)	H-28	H-12, H-28
CH_3*C*OO-	170.2		H-27, C*H*$_3$COO-	
CH_3COO-	20.7	2.09 (s)	H-27	
28	18.6	1.69 (d, 6.4)	H-27	H-27
29	10.8	1.44 (d, 7.1)	H-15	H-15



	R$_1$	R$_2$
1	CH_3	CH_3
2	CH_3	H
3	$CH(CH_3)_2$	CH_3
4	$CH(CH_3)_2$	H

Figure 1. Chemical structure of tryptoquialanine A (**1**), tryptoquialanine B (**2**), tryptoquivaline A (**3**), and nortryptoquivaline (**4**).

compound and the molecular formula was established as $C_{27}H_{26}O_7N_4$ by HREIMS. The ^{1}H NMR spectrum of **1** showed three singlet and one doublet methyl groups, one methylene function, three methine groups resonating between δ_H 5.2 and 6.3, and signals corresponding to nine protons from δ_H 7.3 to 8.2.

The H−H COSY spectrum confirmed the coupling between the hydrogens at δ_H 6.31 (q, 1H, J = 6.3, H-27) and δ_H 1.70 (d, 3H, J = 6.3, 28-CH_3), as well as the presence of a structural sequence −CH−CH$_2$− responsible for the ABX system observed in the ^{1}H NMR spectrum (12-C*H* and 13-CH_2). HMBC correlations between H-12 and H-13 with C-11, a carbonyl lactone group, showed the structural sequence −CH−CH$_2$− to be close to the latter. A detailed analysis of the H−H COSY spectrum revealed the presence of two 1,2-disubstituted benzene rings. Long-range correlations between a quaternary oxygenated carbon (C-3) and H-5, a proton from an aromatic ring, as well as between C-3 and 13-CH_2, supported the presence of a tryptophan moiety including a spiro-γ-lactone ring between C-11 and C-3. Signals characteristic of an imine functionality (C-26) and an α,β-unsaturated carbonyl group (C-18 and H-20), suggested the other benzene ring to come from an anthranilic moiety involved in a quinazoline structure. The link between this structure and the mentioned methines functions could be inferred by HMBC correlations (C-26 to H-12 and H-27, and C-18 to H-12).

Information about the connection with the rest of the molecule was revealed by a long-range coupling between C-2, a tertiary carbon at δ_C 85.6, and 13-CH_2. This carbon also exhibited HMBC correlations with a hydrogen resonating like a singlet signal at δ_H 8.02, signal which could be attributed to a hydroxylamine (16-NO*H*). Likewise, a quaternary carbon bearing two *gem*-dimethyl groups, (C-15), also showed long-range coupling with the proton of the hydroxylamine. The latter information together with HMBC correlations observed from a lactone carbonyl group (C-11) to H-2 and the hydrogens of the geminal dimethyl groups (C-29 and C-30) allowed the elucidation of the remaining part of the molecule.

From the above data, the structure **1** was established for tryptoquialanine A. This structure is very similar to the one of tryptoquivaline A (**3**), a tremorgenic mycotoxin from *Aspergillus* species (*11*, *12*). NOE correlations observed between H-2 and H-13$_b$, between H-5, H-12, and H-13$_a$, and between H-12 and H-27, indicated the same relative configuration as that of tryptoquivaline A. CD data showed a negative Cotton effect with an inflection point at λ = 224 nm, the characteristic maximum of the lactone chromophore (*12*). According to the Legrand−Bocourt rule, the sign of the Cotton effect is consistent with a C-12 (*R*) configuration, and confirms the same absolute configuration as the known tryptoquivalines, except for the tryptoquivaline epimers at C-12, possible artifacts (*12*, *14*).

With respect to the compound **2** (the other major tryptoquivaline-like metabolite isolated from *P. digitatum*) all its spectroscopic data were similar to those of tryptoquialanine A, except that the resonances associated with the geminal dimethyl

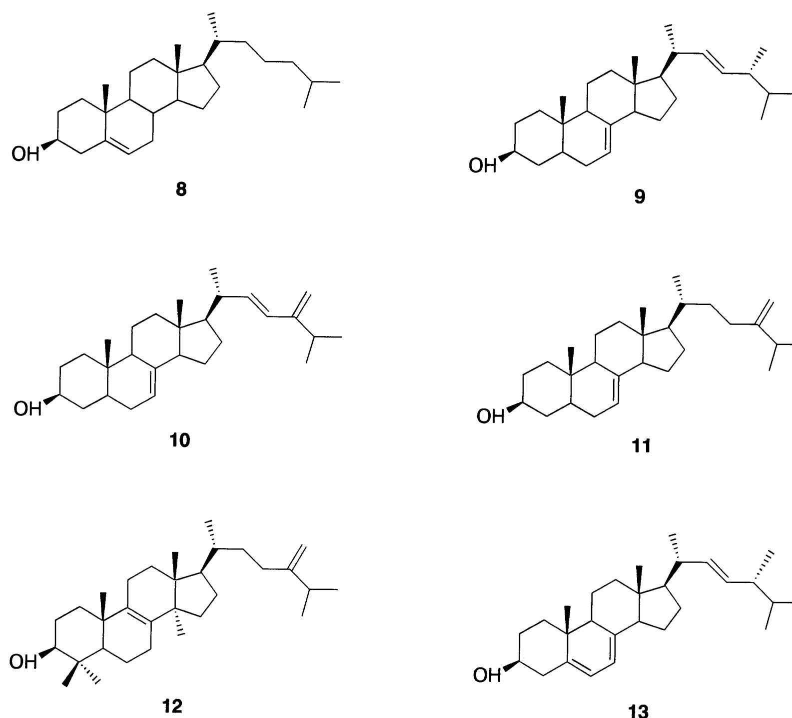


Figure 2. Sterols and oxygenated triterpenes identified in *Penicillium digitatum* biomass: cholesterol (**8**), ergosta-7,22-dien-3β-OH (**9**), ergosta-7,22,-24(28)-trien-3β-OH (**10**), episterol (**11**), and eburicol (**12**).

group in the latter, were replaced by those of a secondary methyl function at C-15 ($_{H}$ 1.44 d, J = 7.1 Hz, H-29; 4.08 q, J = 7.1 Hz, H-15). On that basis, the structure **2** was proposed for tryptoquialanine B. The CD data of **2** were similar to those of **1** suggesting identical absolute configuration at C-2, C-3, C-12, and C-27. A number of chemical, chiroptical, and spectroscopic experiments, including X-ray diffraction, have shown that except for epimerization at C-12, the corresponding centers of asymmetry in the known tryptoquivalines are identical. On that basis we assume that the absolute configuration at C-15 is (*S*) as in nortryptoquivaline (*14*). This assumption is consitent with the incorporation of L-alanine.

The structures of the previously described tryptoquivalines, isolated from *A. clavatus* and *A. fumigatus*, have been proposed to derive biogenetically from four amino acids: tryptophan, anthranilic acid, valine, and alanine (*15*). The structural elucidation of compounds **1** and **2** shows a difference in the secondary metabolism of the mentioned fungi, as *Aspergillus* species seem to incorporate valine in the bond with anthranilic acid, whereas *P. digitatum* introduces alanine.

The study of *P. digitatum* biomass allowed the identification of **8**−**12** (**Figure 2**, and showed that episterol (**11**), instead of ergosterol (**13**), is the main *P. digitatum* sterol. This peculiarity, together with the detection of ions revealing the presence of other unknown sterols in the mycelium of *P. digitatum,* points out that further studies should be done considering the inherent interest of these metabolites. Sterols are important constituents of biological membranes, as they have been shown to play critical roles in normal membrane structure and function (*16*, *17*), as well as in the cell cycle. They are essential for fungal development, thus constituting a useful tool for the design of new antifungal strategies (*18*, *19*). Moreover sterols with important biological activities have been described (*20*).

Because of interest in the relationship of *P. digitatum* and the medium, and citrus−pathogen interaction, volatile compounds in the atmosphere surrounding citrus fruits infected by *P. digitatum* were studied. To differentiate between citrus volatiles and fungal volatiles, and to explore the similarities between the stress situation provoked by mechanical damage of citrus fruits, and the one due to fungal infection, volatiles surrounding intact healthy oranges, mechanically damaged oranges, and infected oranges were compared. The major volatile metabolites released from undamaged oranges were known citrus sesquiterpenes such as valencene, together with relatively small amounts of monoterpenes such us limonene. When the orange peel was damaged with a knife a very different pattern of terpenes could be observed. High amounts of limonene and other known citrus monoterpenes, such as β-phellandrene, β-myrcene, and 3-carene, were released instead of sesquiterpenes. This result is consistent with previous studies in which injured fruits have been shown to release a much greater amount of terpene peel oil constituents than healthy ones (*21*, *22*).

The pattern of volatile metabolites released from oranges contaminated with *P. digitatum* resembled the situation in which the citrus fruit was mechanically damaged. However, together with limonene and other monoterpenes a number of more volatile compounds such as ethanol, methyl acetate, and ethyl acetate, and an unknown volatile compound likely to have a molecular weight of 114 amu, were major components. Effluent sniffing revealed that the above-mentioned unknown compound was the major impact odor substance responsible for the characteristic moldy odor experienced from cultures of *P. digitatum*, when growing on both synthetic and natural substrates. This unknown compound was not detected during the screening of production of volatile metabolites from a large number of *Penicillium* species (*23*), which indicates it to be a unique chemical marker of *P. digitatum*.

The effects of volatile terpene essential oil components on fungal development have been studied previously. As a result, some of the substances produced by citrus fruits, were shown to facilitate fungal infection. This is the case of limonene, present in the flavedo tissue and in the atmosphere surrounding both damaged and undamaged oranges (*24*, *25*). On the other hand, a positive correlation between the monoterpene and sesquiter-

pene content of a citrus essential oil, and its inhibition effect on the growth of *P. digitatum* and *P. italicum* was observed (*26*). Furthermore the fungistatic properties of the oxygenated monoterpene citral are well-known (*27*, *28*).

These results suggest that host−pathogen interaction is a complex phenomenon under the influence of opposing effects. However, antifungal tests of citrus essential oils against *P. digitatum* and *P. italicum* have provided very good results (*26*), supporting a defensive role for the substances involved. Therefore, volatiles from citrus essential oils could be mainly considered as a natural defense barrier.

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