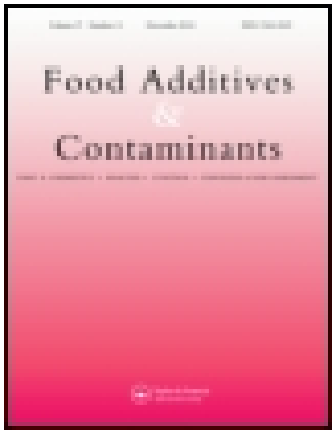

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Purification and full characterisation of citreoviridin produced by *Penicillium citreonigrum* in yeast extract sucrose (YES) medium

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The mycotoxin citreoviridin has been associated with the 'yellow rice' disease, which caused cardiac beriberi in Japan. In Brazil, the consumption of contaminated rice was suspected to be involved in a recent beriberi outbreak. In this work, citreoviridin was produced by *Penicillium citreonigrum*, cultivated in 500 ml yeast extract sucrose (YES) liquid medium for 8 days at 25ºC, and the toxin extracted with chloroform from the liquid medium and the mycelium. A total of 15.3 g of crude extract was obtained from 48 culture flasks, with an estimated citreoviridin contend of 5.54 g, 74.3% being present in the mycelia. Semi-preparative HPLC of the crude extract yielded 27.1% citreoviridin. The HPLC-purified citreoviridin fraction was fully characterised by UV/VIS, FT-IR, ^{1}H- and ^{13}C-NMR, LC-MS/MS and LC-MSD TOF, and purity confirmed by gravimetric analysis. Isocitreoviridin was also produced by *P. citreonigrum*, accounting for about 10% of the citreoviridin present in the crude extract, most transformed into citreoviridin after 10 months under freezing conditions protected from light. Citreoviridin was shown to be stable under the same conditions, although it can suffer isomerisation after a longer storage period. Isomerisation is a potential source of variability in toxicological studies and purity of the material should be checked before study initiation.

Keywords: *Penicillium citreonigrum*; citreoviridin; production; purification; characterisation

Introduction

Citreoviridin, a mycotoxin produced by several *Penicillium* species, was first isolated by Ueno (1970) during the investigation of 'yellow rice' disease in Japan, which caused acute cardiac beriberi (Uraguchi 1969). The rice was contaminated predominantly with one *Penicillium* species, later named *P. citreoviridide* Biorge (Ueno 1970), or *P. citreonigrum* (Pitt & Hockin 2009). Beriberi is mainly caused by thiamin (vitamin B_1) deficiency, affecting the cardiac system (wet beriberi and Shoshin beriberi) or the neural system (dry beriberi and Wernicke–Korsakoff syndrome) (Thurnham 2009). In animals, *P. citreoviride* ethanol extract caused the same neurotoxic symptoms as those observed in human acute cardiac beriberi (Uraguchi 1969). Citreoviridin (Figure 1) has also been associated with Keshan disease, an endemic cardiomyopathy that particularly affects children and young women in China and North Korea (Sun 2010; Wan et al. 2011; Yu et al. 2012). Additionally, it may be a risk factor for the development of atherosclerosis in some areas of China (Hou et al. 2013).

In May 2006, an outbreak of beriberi was reported in the state of Maranhão, Brazil, and by 2008, 1207 cases of the disease were reported, with 40 deaths (Padilha et al. 2011). Rice samples collected in the outbreak region were highly infested with many fungal species, including *P. citreonigrum*, with some samples being positive for citreoviridin (Rosa et al. 2010). Cases of beriberi are still being reported in the region, but its association with the consumption of rice contaminated with citreoviridin is not yet clear (Alves et al. 2010). Rice is a staple food in Brazil, and is produced in some areas of the country without the proper good agricultural practices that guarantee a safe product for consumption (Alves et al. 2010). Almeida et al. (2012) found 22.5% of the 129 rice samples collected from various Brazilian regions containing citreoviridin (≥ 0.9 µg kg^{-1}).

In vitro studies have been conducted to elucidate the involvement of citreoviridin in cardiac and other diseases. *In vitro* studies have shown that citreoviridin inhibits triphosphate adenosine (Linnett et al. 1978; Cataldi de Flombaum & Stoppani 1981; Sayood et al. 1989) and thiamine diphosphate, suggesting a relationship between anti-thiamine effect of citreoviridin and cardiac beriberi (Data & Ghosh 1981). Citreoviridin was shown to mediate cell apoptosis and to inhibit human umbilical vein endothelial cells growth (Hou et al. 2014) and to induce DNA damage in human liver-derived HepG2 cells, showing a possible genotoxicity (Bai et al. Forthcoming).

In vivo studies with this mycotoxin are, however, limited in the literature. Macroscopic analysis of hearts from rats treated daily with citreoviridin in the feed at 15 mg kg^{-1} bw for 8 weeks showed infiltration of lymphocytes and mononuclear cells, myofibril disintegration and cardiac myocyte granular degeneration (Liu et al.

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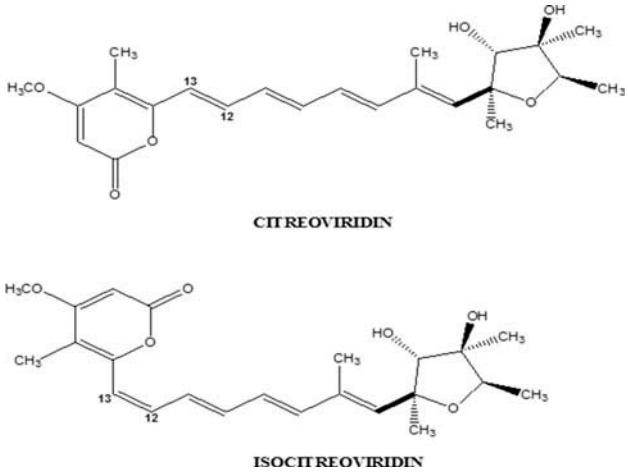

Figure 1. Chemical structures of citreoviridin (E) and isocitreoviridin (Z) ($C_{23}H_{30}O_6$).

2007; Hong et al. 2010). *In vivo* studies requires a significant amount of toxin, which are in general produced in-house.

Ueno (1970) evaluated the citreoviridin yield by *P. citreonigrum* grown in different culture media and environmental conditions. The crude extracts were subject to column chromatography and different fractions tested for lethality in mice. Datta and Ghosh (1981) conducted a similar study and investigated the effect of the purified toxin on the TPP-dependent liver transketolase in rats. Nagel et al. (1972) produced citreoviridin from *P. pulvillorum* grown in mould maize meal during the investigation of an outbreak of toxicogenic fungi in cereals in South African cereals.

However, the purity of the material in most studies is not always thoroughly checked, a challenge that may arrive as the toxin is unstable under light (Suh & Wilcox 1988). Commercially available citreoviridin is expensive (approximately US$140/mg), and although an organic synthetic pathway has been proposed, it was shown to be very lengthy (25 steps), with only 0.4% total yield (Suh & Wilcox 1988). The objectives of this work were to establish a protocol for production of citreoviridin by *P. citreonigrum*, purify it and fully characterise the material using state-of-art spectroscopic techniques.

Materials and methods

Fungal culture

Culture of *Penicillium citreonigrum* (ITAL 2313/09) was supplied in agar medium by Dr Marta Taniwaki (ITAL, Brazil). The identity of the culture was confirmed by the Fungal Molecular Genetics Laboratory (UEL, Brazil), using PCR through sequencing of the beta-tubulin gene and comparison with the nucleotide sequence from databases of the National Center for Biotechnology Information using the Basic Local Alignment Search Tool (Altschul et al. 1990). Subcultures of *P. citreonigrum* were maintained in malt extract agar and in yeast extract sucrose agar (YESA) media.

Toxin production

A YESA subculture plate was incubated for 7 days and the medium equally divided into six parts. Two parts were randomly taken to inoculate a yeast extract sucrose (YES) liquid medium (500 ml in 4 L Erlenmeyer flask), which is particularly useful for mycotoxin production by *Penicillium* genera (Singh et al. 1991). The YES culture flasks were cultivated in stationary mode, at 25°C, protected from light. In order to evaluate the best incubation time for toxin production, the culture was monitored for 15 days, and the production evaluated daily (extraction and quantification are described below).

Citreoviridin extraction

The content of the culture flask was filtered through a paper filter and citreoviridin was extracted separately from the liquid medium and the mycelium. The liquid medium was divided into two portions of 250 ml and each portion extracted twice with 20 ml of chloroform (Cromoline, P.A., 99.8%). The mycelium was fragmented and transferred to a beaker and chloroform was added until the mycelium surface was covered (about 100 ml). Mycelium extraction conditions were tested using sonication or agitation for 20 min. Although the results were not significantly different (t test $\alpha = 0.05$), sonication was slightly more efficient in extracting citreoviridin than agitation and was simpler and easier to operate under the laboratory routine. Efficiency of sonication extraction was tested at 10, 20, 30 and 40 min ($n = 3$ at each time), and showed to be significantly different among the groups (ANOVA test, $p < 0.05$), with the best results obtained for 20 and 30 min, which were not significantly different ($p > 0.05$, Tukey and Dunnett tests). Hence, 20 min of sonication was chosen for mycelium extraction. Figure 2 illustrates the procedure for citreoviridin production and extraction.

The liquid medium and mycelium extracts were filtered under anhydrous sodium sulfate, aliquots of 10 µl taken, evaporated, diluted in methanol and filtered through syringe filters (0.45 µm) to determine the citreoviridin content by HPLC-PDA. The combined extracts were then evaporated to dryness to obtain the crude toxin extract mass.

Citreoviridin quantification

Citreoviridin quantification was performed on a Shimadzu HPLC system equipped with a quaternary pump (LC-20AT), a degasser (DGU-20A), a system controller (CBM-20A), an

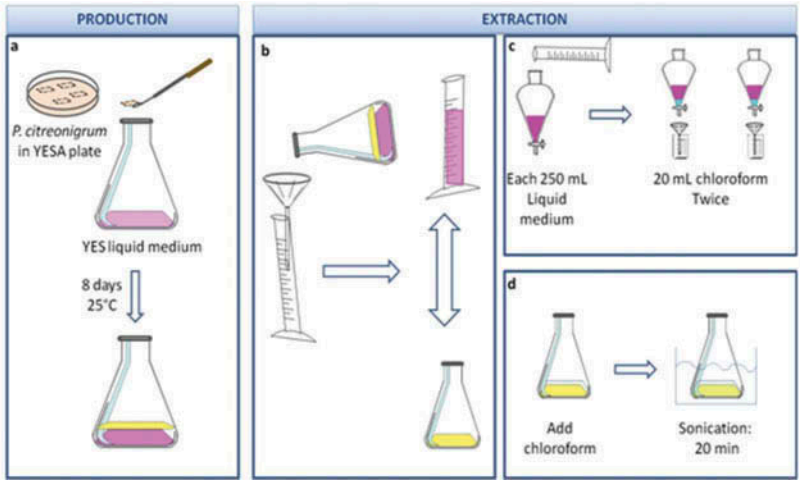

Figure 2. (colour online) Procedure for the production and extraction of citreoviridin.

autosampler (SIL-20A), a column oven (CTO-20A), and a photo-diode array detector (PDA SPD-M20A) (Kyoto, Japan). Chromatographic separations were performed in a Gemini C18 reversed-phase analytical column (150 × 4.6 mm, 5 µm) from Phenomenex (Torrance, CA, USA). The mobile phase consisted of methanol (Merk, HPLC grade, 99.9%):water with 1% of acetic acid (J.T. Baker, HPLC grade) (65:35) at a flow rate of 1.0 ml min^{-1}. The column oven temperature was set at 40°C. Injection volume was 10 µl and wavelength set at 385 nm.

Citreoviridin standard (5 mg; 97% purity, not verified further) was purchased from Enzo Life Sciences International Inc. (Farmingdale, NY, USA). Stock solution (50 µg ml^{-1}) was prepared in ethyl acetate (Merk, Darmstadt, Germany; HPLC grade, 99.8%), taking into consideration the declared purity of the commercial standard. Working solutions (2.5 µg ml^{-1}, in methanol) were used to prepare the standard curves (0.5–5.0 µg ml^{-1}, six calibration points), which were used to quantify the citreoviridin content thought the study.

Citreoviridin purification

The purification of the fungal extracts were performed in a Shimadzu HPLC-PDA system coupled to a FC-203B fraction collector (Gilson Middleton, WI, USA) using a Gemini C18 semi-preparative column (150 × 10 mm, 5 µm) from Phenomenex, at a flow rate of 5 ml min^{-1}. The column oven and mobile phase were as described above. The crude toxin extracts were diluted with the mobile phase (approximately 50 mg ml^{-1}; methanol: water with 1% of acetic acid – 65:35), and 1 ml of fungal extract injected into the system. The citreoviridin peak was monitored at 385 nm, five collected fractions were concentrated on a rotary evaporator and lyophilised for further characterisation.

Characterisation of the citreoviridin fraction purified by HPLC

Melting point (mp) was determined using an electrically heated apparatus from Logen Scientific (model LSIII plus, Serie 5294) coupled to a thermometer (UK BCR; –10 to 260°C). The instrument was calibrated using three substances (at least 99% purity) with known melting points: 4-nitrotoluene (Sigma-Aldrich; mp = 53.5°C), benzoic acid (Vetec, mp = 122.4°C) and salicylic acid (Isofar, mp = 159°C). The mp of each substance was determined three times, and the mean value was used to construct a calibration curve. Citreoviridin dissolved in methanol: water (65:35) was lyophilised (Liobras, modelo K105; less than –90°C, about 30 µHg) until a solid was obtained, and the mp determined three times.

Ultraviolet/visible spectroscopy analysis of the purified citreoviridin, in methanol, was performed on a Shimadzu UV/VIS 1650PC spectrophotometer (Kyoto, Japan). Molar absorptivity (ε) was determined for the main absorption wavelengths (201, 235, 285, 294, 378, 387 nm) at five citreoviridin concentrations (1, 2.5, 5, 10, 15 µg ml^{-1}), each measurement done in triplicate.

Nuclear magnetic resonance (NMR) spectrometric analysis was performed on a Varian Mercury Plus spectrometer (7.05 T) operating at 300 MHz for ^{1}H and at 75.46 MHz for ^{13}C. The analysis was conducted with purified citreoviridin dissolved in CD_3OD and in CD_2Cl_2, using TMS (Me_4Si) as internal reference. The ^{1}H- and ^{13}C-NMR chemical shifts are reported in parts per million (ppm) relative to TMS (^{1}H-NMR) and CD_3OD or $CDCl_3$ (^{13}C-NMR), coupling constants as J (Hz) and multiplicities as doublet, doublet of doublets, quartet and singlet. Field gradient mode was used in the two-dimensional experiments COSY (^{1}H-^{1}H homonuclear through-bond (J-coupling) correlations), HMQC and HMBC (^{1}H-^{13}C heteronuclear correlations) experiments.

Infrared (IR) spectrometric analysis was performed on a Varian 640 FTIR spectrometer with a KBr Beam splitter. The purified citreoviridin was dried at 130°C and prepared in KBr pellet. The spectrum was registered in the range of 400–4000 cm^{-1} with 2 cm^{-1} spectral resolution.

LC-MS analyses were performed in a Shimadzu LC-20AD liquid chromatographer from Shimadzu (Kyoto, Japan), coupled with an Applied Biosystems/MDS Sciex 4000 QTRAP MS mass spectrometer system (Foster City, CA, USA), with an electrospray interface (ESI). Chromatographic columns and conditions were as described for citreoviridin quantification. The parameters optimised for MRM were 56 V declustering potential, 13 V collision energy for *m/z* 315 and 33 V for *m/z* 139, and 10 V collision cell exit potential for both ions. Ion source parameters were 20 psi curtain gas, median collision gas, 4000 ion spray voltage, 600°C source temperature and 50 V ion source gas.

The exact mass analysis was performed on a LC-MSD TOF (Agilent 1100 Series; Santa Clara, CA, USA) coupled to an positive ESI source (3500 V capillary, 55 psig nebuliser, 12 l min^{-1} drying gas, 350°C gas temp, 120 V fragmentor and 60 V skimmer). Chromatographic separations were performed on a Zorbax Eclipse Plus C18 reversed-phase analytical column (150 × 4.6 mm, 3.5 µm) from Agilent. Mobile phase was methanol:water with 0.1% of formic acid (J.T. Baker, HPLC grade) (65:35) at a flow rate of 0.45 ml min^{-1}. The column oven temperature was set at 40°C and the injection volume was 5 µl.

Gravimetric analysis

A 10 ml aliquot of the purified citreoviridin solution (fraction 4) was transferred to a pre-weighed flask (analytical balance Bel Mark model 210A with a measuring uncertainty of 0.000086 for 20 g), the solvent dried under nitrogen at RT and the flask reweighed for toxin mass estimation. This procedure was repeated in triplicate. The citreoviridin content in the aliquot was quantified by HPLC-PDA against a citreoviridin curve made with the commercial standard (97% pure).

Results

Citreoviridin production

P. citreonigrum growth in YES medium and citreoviridin production were monitored in the liquid medium for 15 days. The fungus showed a fast growth, covering the entire medium surface (approximately 20 cm) after 4 days of incubation. Citreoviridin production increased from day 4, reaching a maximum on the 8th day, with further variation up to day 15 (Figure 3). Three culture flasks prepared in the same day (one batch) and incubated for 8 days at RT and protected from light, visually showed similar fungus growth. Liquid medium and mycelium from each flask were extracted and citreoviridin content determined. A total of 16 batches (three culture flasks each, 48 flasks total) were prepared over 16 weeks (not consecutive).

Table 1 summarises the results obtained from the 16 batches. Total crude extract masses (from liquid medium plus mycelium) varied from 210.9 to 2036 mg (mean of 955 mg), with a total of 15.3 g obtained from all batches. On average, 346.3 mg of citreoviridin were present in each culture medium batch (corresponding to 230.9 mg l^{-1} for a 500 ml liquid medium flask), with a mean yield of 44.8% per batch. A total of 5.54 g toxin was estimated to be present in the crude extract of all 16 batches, corresponding to a total yield of 36.2%. Almost three times more citreoviridin was estimated to be present in the mycelium compared with the liquid medium (mean of 85.7 and 29.7 mg per flask, respectively). A larger variation in toxin levels among the three flasks from each batch was also found in the mycelium (up to 125%).

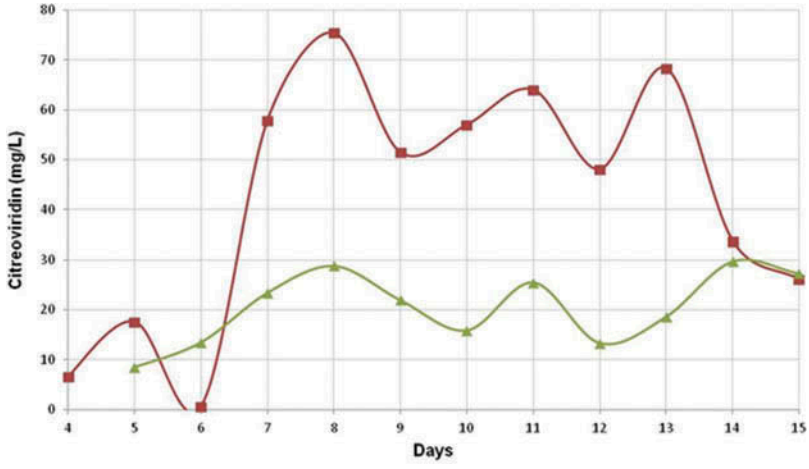

Figure 3. (colour online) Citreoviridin production by *Penicillium citreonigrum* cultivated in YES liquid medium at 25°C during 15 days.

Table 1. Production of citreoviridin by *P. citreonigrum* in YES medium for 8–9 days.

Batch	Total crude extract, mg	Total citreoviridin, mg[a]	Yield (%)	Citreoviridin, mg (RSD%)[b]	
				Liquid medium	Mycelium
1	653.4	389.3	59.6	37.7 (29.7)	92.1 (34.9)
2	210.9	173.7	82.4	21.3 (28.6)	36.6 (40.8)
3	469.1	421.8	89.9	40.7 (9.2)	99.9 (8.7)
4	1149.4	334.8	29.1	39.7 (1.1)	71.9 (18.8)
5	966.5	559.7	57.9	34.7 (10.4)	151.8 (15.4)
6	344.0	203.3	59.1	32.1 (16.1)	35.7 (19.4)
7	990.0	574.7	58.0	38.3 (9.0)	153.2 (15.4)
8	1431.7	338.2	23.6	29.6 (9.6)	83.1 (5.7)
9	1431.7	235.3	16.4	31.7 (4.1)	46.8 (44.2)
10	1089.3	128.6	11.8	14.5 (68.7)	28.3 (125.2)
11	1089.3	161.2	14.8	23.5 (10.4)	30.3 (69.3)
12	1272.3	316.2	24.9	24.8 (7.4)	80.6 (35.7)
13	2036.4	546.1	26.8	31.4 (2.8)	150.6 (6.4)
14	776.4	391.4	50.4	28.0 (3.7)	102.5 (5.7)
15	519.1	288.2	55.5	27.7 (7.6)	68.4 (18.9)
16	851.0	479.1	56.3	19.8 (10.9)	139.9 (16.5)
Mean (RSD, %)[c]	955.0 (49.0)	346.3 (41.7)	44.8 (53.8)	29.7 25.5)	85.7 (52.1)
Total, g[d]	15.28	5.54		1.43	4.11

Notes: [a]Total citreoviridin (liquid medium plus mycelium)*100/crude extract.
[b]Mean of three cultures flasks within each batch.
[c]Mean of all batches.
[d]Total from all batches

Purification

The chromatograms of the five fractions collected during the purification of the crude toxin extracts by semi-preparative HPLC are shown in Figure 4. Fraction 4 showed only the citreoviridin peak, and was considered the purified citreoviridin fraction. Fraction 2 was later identified to contain isocitreoviridin (Figure 1), and accounted for about 10% of the citreoviridin present in the crude extract. No other compound was found at significant concentration in any other fraction. Fractions 3 and 5 contained small amounts of citreoviridin, but they were not investigated further.

Fractions 4 collected from 243 injections of the crude extract (1 ml injections of a 50 mg ml^{-1} extract, about 12 g of the 15.3 g total crude extract obtained) were concentrated in rotary evaporator and lyophilised. A total of 3.25 g of purified citreoviridin (estimated by HPLC) were obtained, representing a yield of 27.1%.

Characterisation

The purified citreoviridin obtained from semi-preparative HPLC (fraction 4) was characterised by UV/VIS, FT-IR, ^{1}H- and ^{13}C-NMR spectroscopy, LC-MS/MS and LC-MSD TOF. The purified citreoviridin is a yellow-orange solid, with a determined melting point of 111ºC (corrected though calibration curve, $R^2 = 0.9979$) (Table 2), the same found by Ueno (1970). A UV/Vis spectrum of pure citreoviridin in methanol is shown in Figure 5. The absorption at 387 nm, near to visible, is characteristic of the toxin yellow colour and indicates the presence of double bonds in the extensive conjugations present in the molecule (Figure 1). Molar absorptivities (ε) were determined at five citreoviridin concentration (1–15 µg ml^{-1}). The calculated mean values (RSD < 3%) were (λ^{MeOH} (ε)) 285 (20 058), 294 (21 959), 378 (29 159) and 387 (31 590) (Table 3). Absorptivities obtained at 201 and 235 nm showed a large variation among the different concentrations (16 782–46 021 and 8077–13 427, respectively), and were not considered reliable.

FT-IR spectrum (Figure 6) of purified citreoviridin (fraction 4) showed the major absorptions at 3431 (OH), 2964 (CH_3), 2929 (CH), 1691(C = O), 1625 (C = C), 1457 and 1385 (CH_3), 1261, 1095 and 1020 (C − O), and 802 (C = C trisubstituted) cm^{-1}. The ^{13}C-NMR spectra of the purified citreoviridin and of the crude extract illustrate the efficiency of the purification process (Figures 7a and 7b). The proton spectrum (Figure 7c) confirmed the large coupling constants of the E double bonds of citreoviridin exhibited for C-8/C-9 ($J_{H\text{-}8/H\text{-}9}$ = 15.1 Hz), C-9/C-10 ($J_{H\text{-}9/H\text{-}10}$ = 9.0 Hz), C-10/C-11 ($J_{H\text{-}10/H\text{-}11}$ = 14.7 Hz), C-11/C-12 ($J_{H\text{-}11/H\text{-}12}$ = 11.1 Hz) and C-12/C-13 ($J_{H\text{-}12/H\text{-}13}$ = 15 Hz). Attached proton test and ^{13}C-NMR experiments, showed an acyl lactone group (C-18), one methoxyl (C-23), five methyls (C-1, C-19, C-20, C-21, C-22), two methines (C-2, C-4), four quartenary sp^2 carbons (C-7, C-14, C-15, C-16), eight tertiary sp^2 carbons (C-6, C-8, C-9, C-10, C-11, C-12, C-13, C-17), and two quartenary sp^3 carbons (C-3, C-5). The proton NMR

spectrum of citreoviridin in the aromatic region is very distinct from its isomer (Suh et al. 1996), so the identity of the toxin present in the purified fraction 4 is confirmed. Furthermore, if fraction 4 contained any isocitreoviridin, the ^{13}C-NMR would show additional carbons than what is expected for a pure compound. Both proton and ^{13}C-NMR spectra found for fraction 4 (Figure 7) are identical to what was published by Suh and Wilcox (1988) for citreoviridin and the data are shown in Table 4. All chemical shifts were confirmed by COSY, HMQC and HMBC experiments (data not shown).

LC-MS full-scan analysis of purified citreoviridin (fraction 4) showed only the citreoviridin peak at 7.14 min ([M + 1] at 403.2 *m/z*), with the major fragment ions at *m/z* 315.1 (100%), 139.0 (67.5%) and 297.1 (65.7%) (Figure 8); *m/z* 315 was obtained after a neutral loss of 88 da ($C_2H_8O_2$). The determination of the exact mass of the purified citreoviridin (theoretical mass of 402.2042) performed by LC-MSD TOF, showed the molecular ion $[M + H]^+$ 403.2112 *m/z*, with a calculated mass of 402.2039 g mol^{-1}, attributable to the molecular formula $C_{23}H_{30}O_6$ (accuracy of 0.74 ppm). LC-MSD TOF chromatogram of fraction 2 (Figure 4) showed the presence of a peak eluting before (at 5.2 min) the citreoviridin peak (at 6.8 min), but showing the same mass (Table 5), corresponding to its isomer isocitreoviridin (Figure 1).

The citreoviridin peak was not present in the first analyses of fraction 2 (Figure 4), but a isocitreoviridin: citreoviridin ratio of approximately 3:4 (estimated by the areas under the peaks) was observed when this fraction was reanalysed after being stored for about 10 months at

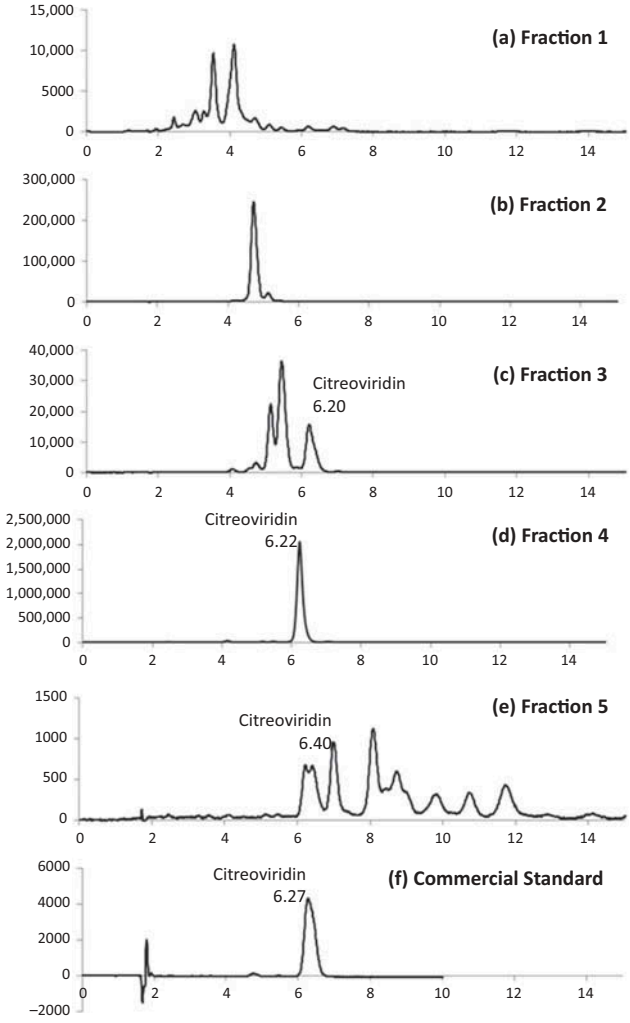

Figure 4. HPLC-PDA chromatograms (385 nm) of crude extract fractions obtained from a semi-preparative column. The chromatograms are in different scales.

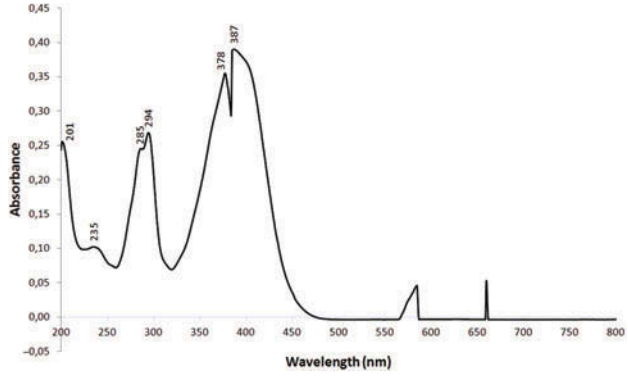

Figure 5. UV/Vis spectrum of the pure citreoviridin (5 µg ml^{-1} in methanol) (fraction 4).

Table 2. Melting point of purified citreoviridin (fraction 4).

Reagent	Fabricant (purity)	Literature melting point (ºC)[a]	Experimental melting point (ºC)[b]
4-Nitrotoluene	Sigma-Aldrich (99%)	53.5	56.5
Benzoic Acid	Vetec (99.5%)	122.4	137.7
Salicylic Acid	Isofar (99%)	159.0	173.3
Calibration curve[c]: $y = 1.1166x - 2.1444$ $R^2 = 0.9979$			
Citreoviridin	Experimental melting point[a]		122.8
	Corrected melting point		111.9

Note: [a]O'Neil et al. (2001).
[b]Mean of three lectures.
[c]Ordinary least squares method.

Table 3. Estimation of the molar absorptivity of purified citreoviridin (fraction 4).

Concentration		Molar absorptivity (ε)[a]					
µg ml^{-1}	Molar	201 nm	235 nm	285 nm	294 nm	378 nm	387 nm
1	2.49E–06	91 294.2	13 426.8	20 783.4	22 512.0	29 506.8	31 677.6
2.5	6.22E–06	46 021.0	10 500.2	20 180.4	22 045.7	29 297.8	31 549.0
5	1.24E–05	25 615.4	8610.8	19 907.4	21 836.6	28 855.6	31 468.6
10	2.49E–05	18 624.7	8076.2	19 460.8	21 422.6	28 409.3	31 167.1
15	3.73E–05	16 782.2	8077.5	19 960.6	21 981.4	29 705.1	32 090.3
	Mean	39 667.5	9738.3	20 058.5	21 959.6	29 154.9	31 590.5
	RSD	78.41	23.52	2.40	1.79	1.79	1.06

Note: [a]Molar absorptivity (ε) calculated with the mean of three lectures of absorbance in different wavelength. $\varepsilon = \frac{A}{c\ell}$, where A is absorbance (nm); c is concentration molar (mol l^{-1}); and ℓ is path length (cm).

–15°C in an amber flask (Figure 9), showing that the Z/E isomerisation at the C12-C13 double bound occurred (Figure 1). No E/Z isomerisation was observed (citreoviridin → isocitreoviridin) in the purified citreoviridin (fraction 4) after storage for the same period and conditions. However, an isocitreoviridin peak was observed in fraction 4 reanalysed 1 year later corresponding to about 4% of the citreoviridin (data not shown), in addition to an unknown compound that eluted between the two isomers (about 6% of citreoviridin absorbance). The commercial standard used in this study (97% purity) also showed the presence of a small amount of isocitreoviridin (about 2%).

Gravimetric analysis

The weighted mass of a dried 10 ml aliquot of a purified citreoviridin solution (fraction 4) was 0.0953 ± 0.0005 g ($n = 3$). The mass of citreoviridin in this solution estimated by HPLC-PAD was 0.1016 ± 0.0062 g ($n = 6$), taking into account the commercial standard purity. The values estimated by gravimetric and chromatographic methods were not statistically different ($p > 0.05$), and it was concluded that the purified citreoviridin (fraction 4) is 100% pure.

Discussion and conclusions

In this study, a protocol for the production of citreoviridin by *P. citreonigrum* in YES medium was established. Optimal toxin production was achieved after 8 days of incubation at 25°C, faster than what was reported by Ueno (1970) using Mannit, Czapeck, Glycerine–Czapeck, Waksman or Ushinsky medium. Even under the best conditions (20–24°C in Ushinsky medium), the highest citreoviridin production was obtained by the author only after 2 or 3 weeks of incubation. The large variation in toxin production found in the three culture flaks of some batches in this study was most likely due to the differences in the size of inoculums taken from the YESA subculture plate to inoculate each YES culture flask (Barberis et al. 2012). The toxin production levels obtained in this study (up to 383 mg l^{-1}) were similar to what was reported by Ueno (1970) using the Ushinsky media (380 mg l^{-1}). Using this same media, Datta and Ghosh (1981) extracted 2.6 g l^{-1} only from mycelium. Indeed, mycelium contained most of the toxin in the flask (about 74%), although there was a large variation in the amount of toxin extracted from each mycelium. The proportion of citreoviridin extracted from mycelia and the liquid medium seems to depend on the substrate. Ueno (1970) found the same amount of toxin in the liquid medium and mycelia of a Mannit culture, about three times more citreoviridin in the liquid medium of Waksman and Glycerin–Czapeck cultures, and mycelium from the Ushinsky culture contained over seven times more citreoviridin than the liquid medium.

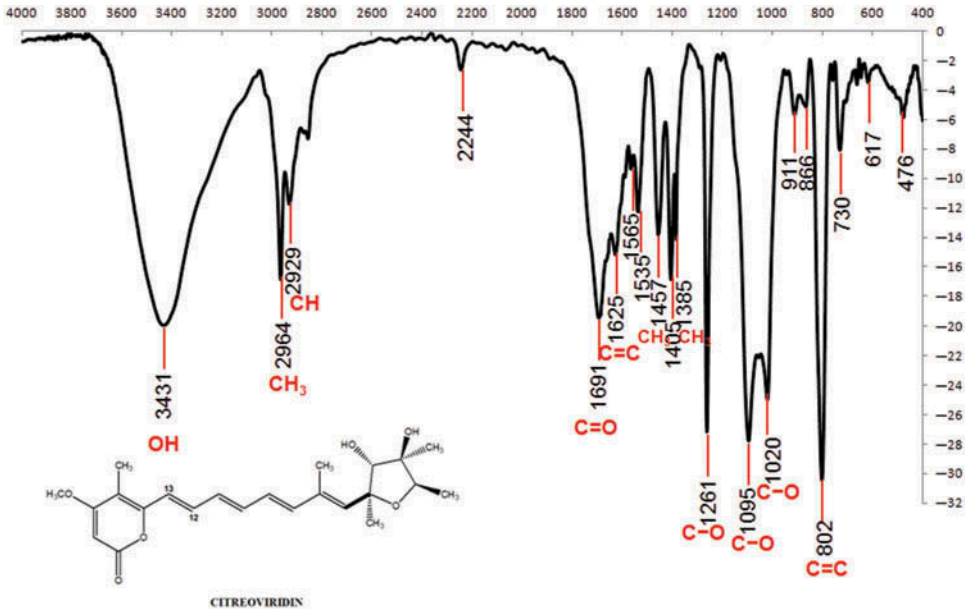

Figure 6. (colour online) Infrared spectrum of the purified citreoviridin (KBr pellet), also showing the absorbance by CO_2 from the air at 2244 nm (fraction 4).

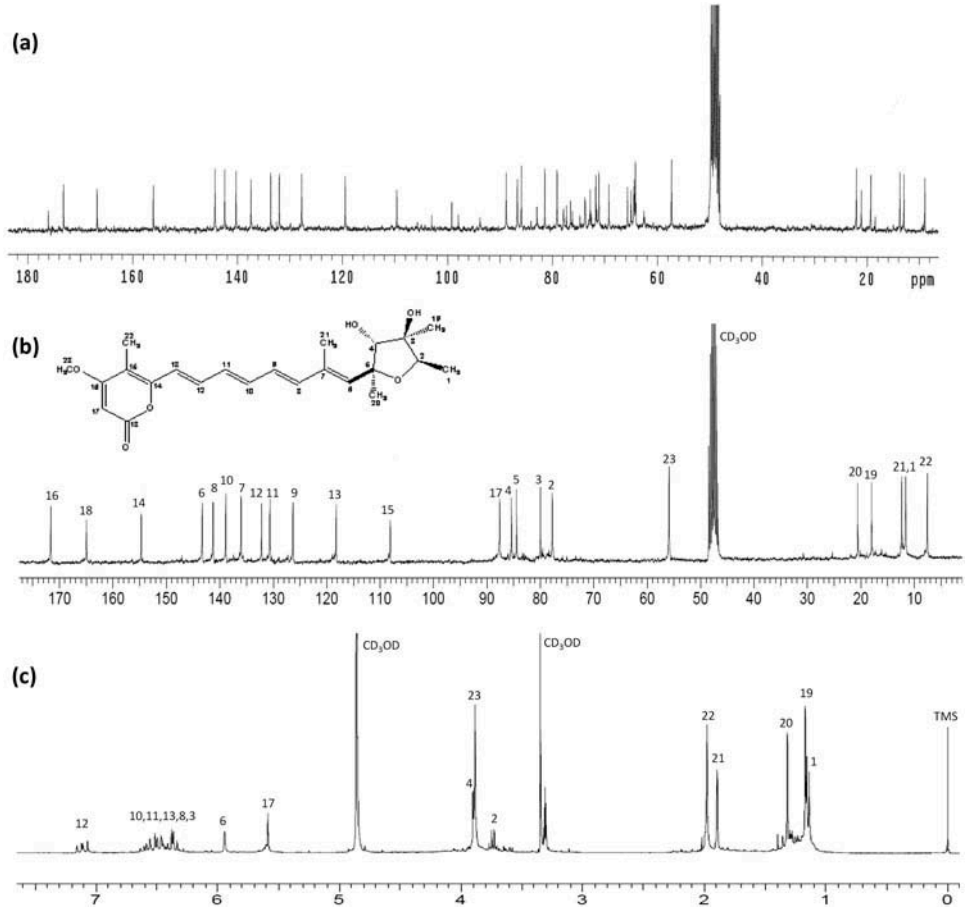

Figure 7. ^{13}C-NMR spectra (75.46 MHz) of crude toxin extract (a) and purified citreoviridin (fraction 4) in CD_3OD (b), and the ^{1}H-NMR spectra of purified citreoviridin (fraction 4) at 300 MHz in CD_3OD (c).

Table 4. NMR data of purified citreoviridin (fraction 4) dissolved in CD_3OD and CD_2Cl_2 and data from the literature (Suh & Wilcox 1988).

	δ_H (ppm) (*J*, Hz)			δ_C (ppm)		
Position	Experimental CD_3OD	Experimental CD_2Cl_2	Literature CD_2Cl_2	Experimental CD_3OD	Experimental CD_2Cl_2	Literature CD_2Cl_2
1	1.13d (6.3)	1.13d (6.3)	1.14d (6.3)	13.0	12.5	12.6
2	3.73q (6.3)	3.77q (6.3)	3.79q (6.3)	79.1	77.9	78.0
3	–	–	–	81.4	81.0	81.1
4	3.90s	3.93s	3.97s	86.8	86.3	86.2
5	–	–	–	85.9	84.4	84.4
6	5.94s	5.44s	5.44s	144.7	141.7	142.2
7	–	–	–	137.4	135.8	136.0
8	6.41d (15.0)	6.37d(15.9)	6.34d (15.2)	142.7	141.1	141.3
9	6.34dd (15.3; 9.0)	6.35dd (15.5; 9.6)	6.31dd (15.3; 9.3)	127.7	127.6	127.5
10	6.62dd (14.8; 9.0)	6.57dd (14.4; 9.9)	6.54dd (14.7; 9.4)	140.3	138.8	139.0
11	6.51dd (15.0; 11.1)	6.46dd (14.4; 11.0)	6.41dd (14.7; 11.2)	132.0	131.6	131.4
12	7.15dd (15.0; 11.2)	7.13dd (15.0; 11.0)	7.14dd (14.9; 11.2)	133.6	134.7	134.4
13	6.49d (15.0)	6.39d(15.0)	6.38d(15.1)	119.6	119.4	119.2
14	–	–	–	156.1	154.9	154.9
15	–	–	–	109.5	108.4	108.4
16	–	–	–	173.1	171.1	171.2
17	5.61s	5.30s	5.30s	88.9	89.0	88.8
18	–	–	–	166.5	163.7	163.9
19	1.16s	1.17s	1.18s	19.4	17.6	17.8
20	1.31s	1.33s	1.34s	22.0	21.5	21.5
21	1.90d (1.2)	1.91d (1.2)	1.92d (1.2)	13.7	13.7	13.6
22	1.99s	1.96s	1.96s	8.9	9.1	9.0
23	3.89s	3.83s	3.82s	57.3	56.7	56.7

Note: s, Singlet; d, doublet; q, quartet; dd, doublet of doublets.

Datta and Ghosh (1981) and Ueno (1970) found higher yields of purified toxin using the Ushinsky medium (38% and over 67%, respectively, calculated from the data provided) than that found in our work (27.1%). The purities of the toxins produced, however, were not reported in these studies, and it is possible that the amount of toxin reported to be produced was overestimated. This is the first time that citreoviridin has been purified by semi-preparative HPLC. Other studies have used liquid–liquid partition and silica-gel column chromatography (Ueno 1970; Nagel et al. 1972; Cole et al. 1981; Datta & Ghosh 1981; Steyn et al. 1982), purification techniques considered less efficient than semi-preparative HPLC.

Chemical characterisation of citreoviridin produced in fungal culture was performed over 30 years ago, and no single study showing complete spectrometric information obtained from state-of-the-art techniques was found in the literature. The UV/Vis spectrum for the toxin purified in this study was similar to what have been reported (Ueno 1970; Cole et al. 1981; Nielsen & Smedsgaard 2003). However, the ε values determined at the main wavelengths were 20–30% lower than what was reported by other authors (Ueno 1970; Nagel et al. 1972; Cole et al. 1981; Stubblefield et al. 1988). We could not determine a reliable ε at 201 and 235 nm because they varied greatly with concentration, although they had been reported elsewhere (14 000–15 000 and 10 000–11 000, respectively; Ueno 1970; Nagel et al. 1972; Cole et al. 1981; Stubblefield et al. 1988). It is important to emphasise that none of the previous studies gave detailed information on the determination of ε.

FT/IR for citreoviridin agreed with the data described previously (Ueno 1970; Nagel et al. 1972; Sakabe et al. 1977; Cole et al. 1981). ^{1}H- and ^{13}C-NMR data for citreoviridin in both solvent were also in good agreement, within the experimental error, with the data reported by Suh and Wilcox (1988) (CD_2Cl_2) and Steyn et al. (1982) ($CDCl_3$).

Mass spectrometric data obtained for the purified citreoviridin and the commercial standard showed the molecular ion peak [M + 1] at m/z 403.2. The main fragments at m/z 315 and 139 may be selected as quantification and confirmation ions, respectively, on a routine citreoviridin analysis in food matrices by LC-MS/MS. The m/z 315, formed after a neutral loss of 88 Da, is most likely due to the loss of $C_4H_8O_2$, which is possible via a cross-ring fragmentation through the five-membered ring (Figure 9). High-resolution MS confirmed the identity of

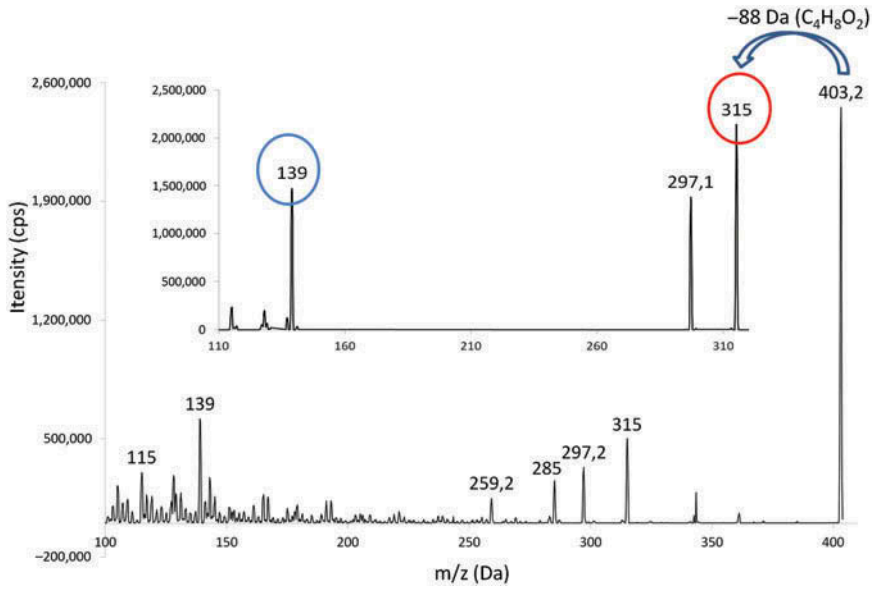

Figure 8. (colour online) Product ion obtained by full-scan LC-MS of the purified citreoviridin (fraction 4), ($[M + H]^+$ *m/z* 403.2. The inserted spectrum shows the main fragments: *m/z* 315, obtained after a neutral loss of 88 Da ($C_2H_8O_2$), and *m/z* 139.

Table 5. Measured mass of commercial standard, purified citreoviridin (fraction 4) and fraction 2 obtained by LC-MSD TOF, and mass accuracy attributed to molecular formula $C_{23}H_{30}O_6$ (theoretical mass 402.2042).

Material	Compound	Retention time (min)	$[M + H]^+$ *m/z*	Measured mass (g mol^{-1})	Mass accuracy (ppm)
Standard	Citreoviridin	6.8	403.2131	402.2059	4.22
Fraction 4	Citreoviridin	6.8	403.2112	402.2039	0.74
Fraction 2	Isocitreoviridin	5.2	403.2116	402.2043	0.24
	Citreoviridin	6.8	403.2119	402.2047	1.2

Note: Mass accuracy = [|measured mass-theoretical mass|*1 000 000]/theoretical mass.

citreoviridin, and of its isomer isocitreoviridin, also produced by *P. citreonigrum* under the conditions of the study. Nagel et al. (1972) showed that diluted solutions of citreoviridin or isocitreoviridin submitted to diffuse light with a catalytic amount of iodine at RT yielded a citreoviridin:isocitreoviridin mixture at a ratio of 7:3. Suh and Wilcox (1988) found that up to 40% of citreoviridin is transformed into isocitreoviridin when exposed for 400 minutes to incandescent, fluorescent or natural light. The isomer has also been reported in the citreoviridin standard in this study and by other authors (Linnett et al. 1978; Stubblefied et al. 1988; Suh et al. 1996). Some isomerisation of citreoviridin was observed in our study when the purified material was stored in methanol for over 1 year, even under frozen conditions and protected from light. It is important to emphasise that isocitreoviridin seems to have no toxic effect (Nagel et al. 1972; Sayood et al.

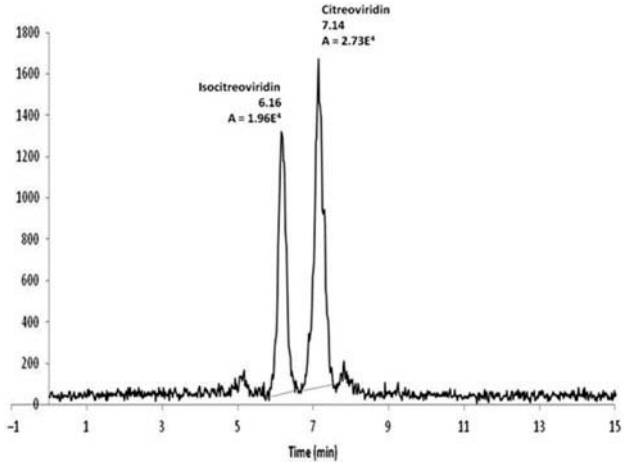

Figure 9. LC-MS full-scan chromatogram of fraction 2 stored for about 10 months at –15°C in an amber flask.

1989). Therefore, the isomerisation is a potential source of variability in toxicological studies and purity of the material should be checked before study initiation.

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