Dicitrinol, a Citrinin Dimer, Produced by *Penicillium janthinellum*

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A citrinin dimer, named dicitrinol (**3**), was isolated from extracts obtained from a rice cultivation of *Penicillium janthinellum*, a fungus found in fruits of *Melia azedarach*. Dicitrinol (**3**) was isolated by classical chromatographic procedures, and identified by MS, and 1D- and 2D-NMR data. A biosynthetic scheme is proposed for this new dimerization of citrinin. Dicitrinol (**3**) was shown to be slightly weaker than citrinin regarding bacterial inhibition.

Introduction. – Citrinin (**1**; *Fig.*) is produced by various fungi, especially by those from *Penicillium* genera, and it is a well-known contaminant of a number of agricultural products [1][2]. This fungal metabolite has been demonstrated to possess hepato-nephrotoxic, *Gram*-positive bacteria-antibiotic properties, along with a number of other chronic toxic effects [3][4]. Citrinin dimers, usually named citrinin H1 (**2**) and H2, pennicitrinones A–D, penicitrinol A, and dicitrins A–D, have been found in *P. notatum* and *P. citrinum* extracts obtained from solid cultivation [5–8]. Most of these dimers are differentiated by further modifications at ring B of one of the citrinin units (*e.g.*, **2**). This kind of dimerizations (bonding two citrinin molecules with posterior modifications) has been evidenced also during studies of citrinin detoxification *via* thermal degradation, which was shown to depend on the presence of H_2O [6]. A heterocyclic *Diels–Alder* reaction was suggested as the mechanism during these processes, resulting in analogs of citrinin H1 (**2**) [6]. However, it is still to be established

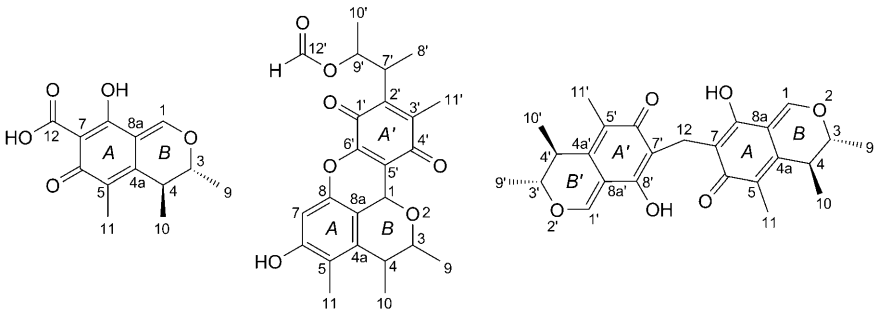

Figure. *Polyketides isolated from the fungus biomass produced by* P. janthinellum

whether some of these citrinin dimers are natural compounds or artifacts produced from citrinin during extraction and isolation procedures. Although, in most cases, citrinin degradation results in decrease of toxicity, some of the products can be even more cytotoxic than the parent compound [9]. This implies that it is important to extend the knowledge on citrinin dimers.

Among our collection of fungi obtained from apparently healthy organs of *Melia azedarach* [10] and *Murraya paniculata*, some of the *Penicillium* fungi have consistently produced a great structural diversity of bioactive polyketides [11], when cultivated in different growing medium, along with alkaloids, amides, and modified steroids [12][13]. The polyketides found so far in these fungi are formed mainly of anthraquinone and citrinin derivatives, including the dimer citrinin H1 (**2**) [11][13]. Here, we report the identification and the biological activity of a new citrin dimer, **3**, produced by one of these *Penicillium* species, identified as *P. janthinellum*, cultivated on sterilized white corn.

Results and Discussion. – 1. *Isolation and Structure Elucidation. P. janthinellum* produced citrinin (**1**) in relatively good yield, when cultivated on white corn (*ca.* 18 mg/g extract), along with the anthraquinones citreorosein, questinol, emodin, and janthinone [13]. The antimicrobial and leishmanicidal activities were evaluated for these compounds. Citrinin (**1**) showed very good results on inhibition of growth of *Leishmania mexicana* [13]. This motivated us to search for minor citrinin analogs in the extracts produced by this *Penicillium* species.

A minor fraction obtained during fractionation of the crude extract, from which the anthraquinones and citrin were obtained, was submitted to preparative TLC on silica gel, and the metabolite **3** was obtained as a yellow powder, with almost the same color as citrinin, but showing a little smaller R_f on TLC. The UV/VIS spectrum of compound **3** was almost identical to the one obtained for citrinin (**1**), and their IR spectra differed only slightly. The mass spectrum of **3**, obtained by atmosphere-pressure chemical ionization (APCI) in the negative-ion mode, displayed an abundant $[M-H]^-$ ion peak at *m*/*z* 423, corresponding to approximately twice the molecular weight of citrinin ($C_{13}H_{12}O_5$, 250 Da; $[M-H]^-$ at *m*/*z* 249). The ^{1}H-NMR spectrum of **3** exhibited signals at almost the same positions as that of **1**, but they appeared in duplicate or poorly resolved. Thus, the ^{1}H-NMR spectrum of **3**, analyzed with the aid of 2D-NMR data (COSY and HMBC) and compared with that of citrinin (**1**; *Table 1*), showed signals for six Me groups (δ(H) 1.21, 1.29 (3*d*, 7.0/6.7, Me(9)/Me(9′)[1])); 1.12, 1.17 (2*d*, 7.2/7.4, Me(10)/Me(10′)); and 1.95, 1.97 (2*s*, Me(11)/Me(11′))); signals of H–C(3) and H–C(3′) appeared as *quadruplet*s of broad lines at δ(H) 4.56 ($J = 6.7$); H–C(4) and H–C(4′) signals formed two *quadruplet*s with *quintet* appearance at δ(H) 2.82 and 2.86 ($J = 7.0$ Hz); and signals of both H–C(1) and H–C(1′) appeared at δ(H) 7.88 (*s*). The latter NMR signal (H–C(1)/H–C(1′)) is shielded when compared with that of H–C(1) for citrinin (δ(H) 8.25 ppm), probably due to the absence of extended conjugation of the C(1)=C(8a) bond with the C(12)=O group. This effect is also observed in the ^{13}C-NMR spectrum (see *Table 1*).

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[1]) Numbering as indicated in the *Figure*. For systematic names, *cf.* the *Exper. Part.*

Table 1. *NMR Data of* **1** *and* **3** ($CDCl_3$, 400 for ^1H and 100 MHz for ^{13}C)[1])

Position	**1**		**3**		
	δ(C)	δ(H)	δ(C)	δ(H)	HMBC (H → C)
1	162.9	8.25 (*s*)	157.1	7.88 (*s*)	C(3), C(4a), C(8), C(8a)
3	81.7	4.79 (*q*, *J* = 6.7)	80.3	4.56 (*q*, *J* = 7.0)	C(4a), C(1), C(9)
4	34.5	3.00 (*q*, *J* = 7.1)	34.7	2.82 (*q*, *J* = 7.0)	C(4a), C(5), C(9)
4a	139.2		136.0		
5	122.9		125.7		
6	183.6		187.3		
7	100.7		114.8		
8	177.1		163.1		
8a	107.3		107.7		
9	18.3	1.35 (*d*, *J* = 6.7)	18.2	1.21 (*d*, *J* = 7.0)	C(3), C(4)
10	18.1	1.23 (*d*, *J* = 7.1)	18.6	1.12 (*d*, *J* = 7.2)	C(3), C(4), C(4a)
11	9.3	2.02 (*s*)	10.1	1.95 (*s*)	C(4a), C(5), C(6)
12	174.4		18.3	3.49 (*d*, *J* = 15.3, H_a),	C(6), C(7), C(8), C(6′), C(7′), C(8′)
				3.58 (*d*, *J* = 15.3, H_b)	C(6), C(7), C(8), C(6′), C(7′), C(8′)
1′			156.7	7.88 (*s*)	C(3′), C(4′a), C(8′), C(8′a)
3′			80.2	4.56 (*q*, *J* = 6.7)	C(4′a), C(1′), C(9′)
4′			34.6	2.86 (*quint.*, *J* = 7.0)	C(4′a), C(5′), C(9′)
4′a			135.6		
5′			125.5		
6′			187.3		
7′			114.7		
8′			162.9		
8′a			107.6		
9′			18.0	1.29 (*d*, *J* = 6.7)	C(3′), C(4′)
10′			18.7	1.17 (*d*, *J* = 7.4)	C(3′), C(4′), C(4′a)
11′			10.0	1.97 (*s*)	C(4′a), C(5′), C(6′)
HO–C(8)		15.9 (*s*)		12.38 (br. *s*)	n.d.[a])
HO–C(8′)				12.38 (br. *s*)	n.d.
HO–C(12)		15.3 (*s*)			

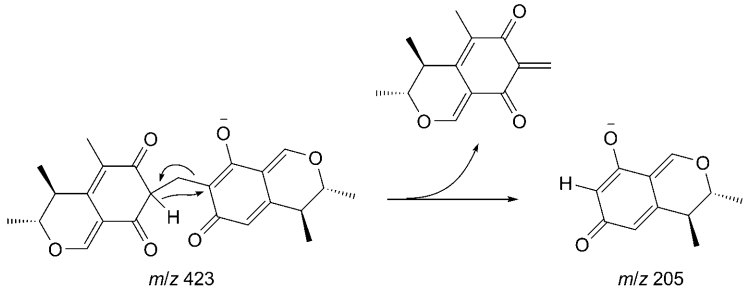
[a]) n.d.: Not detected.

The complex ^1H signals for H_a–C(12) (3.49 (*d*, *J* = 15.3)) and H_b–C(12) (3.58 (*d*, *J* = 15.3) forming an *AB* system in the NMR spectrum of **3** are HSQC-correlated with a shielded ^{13}C resonance at δ(C) 18.3, and both show multiple-bond correlations (HMBC) with C(6) and C(6′) (δ(C) 187.3/187.3), C(7) and C(7′) (δ(C) 114.8/114.7), and C(8) and C(8′) (δ(C) 163.1/162.9). The chemical shift for C(12) (18.3 ppm) is in agreement with other δ(C) values for a CH_2 group in a similar structure [14].

The MS/MS spectrum of **3**, obtained with the ion corresponding to the peak at *m*/*z* 423 as precursor, which was accelerated at 20 eV, displays only one product-ion peak at *m*/*z* 205. This fragmentation can be considered equivalent to a *retro-Michael* reaction (*Scheme 1*), similar to that demonstrated for dicoumarol, also a CH_2 bridged bis(β-hydroxy-β-keto) compound [15], in solution.

Other compounds containing symmetrical bis(β-hydroxy-β-keto) moieties joined by a CH_2 bridge provide exactly the same ^1H- and ^{13}C-NMR data for the two parts [16].

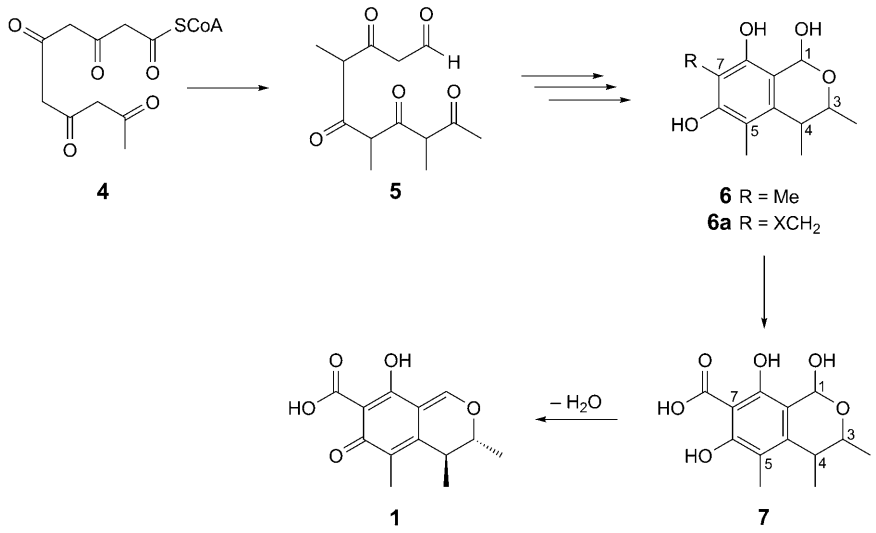
Scheme 1. *Proposed Fragmentation Pathway of the Precursor Ion Formed of Dicitrinol* (**3**) *under ESI* (neg.) *and Set to Collide with Ar* (CID) *to form the base peak at* m/z *205*



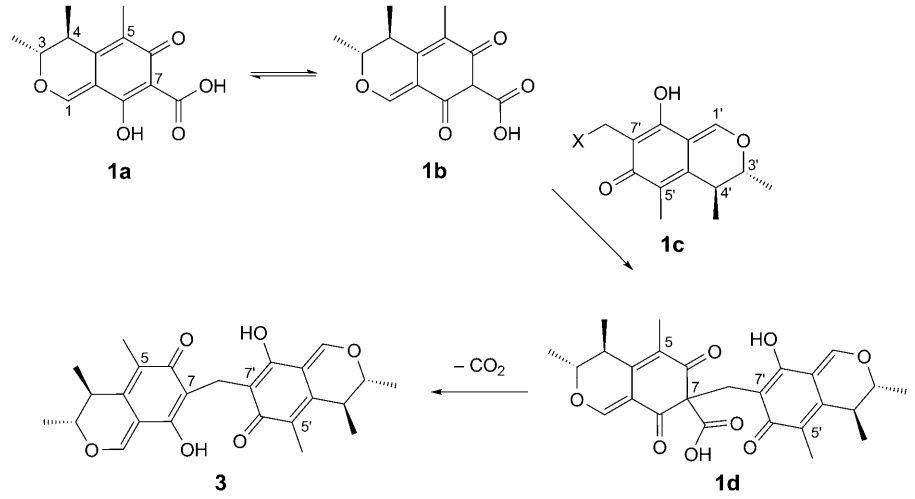
Therefore, the partial duplication of signals observed for **3** cannot be explained by a possible restriction of rotation at C(7)–C(12) or C(7′)–C(12) bonds. But even coumarol, which contains a stable aromatic ring and is a bis(β-hydroxy ester), can exist as various tautomers under certain conditions [17]. It is assumed that, in dicitrinol (**3**), a bis(β-hydroxy ketone) tautomer is rather likely.

2. *Biosynthetic Considerations.* An accepted biosynthetic pathway to citrin (**1**) (*Scheme 2*) begins with a pentaketide polymethylation at C(4), C(6), and C(8) forming **5**, which cyclizes to the aromatic lactol **6**. Oxigenation of one of these Me groups and dehydration results in the natural product citrinin (**1**) [18]. The formation of dicitrinol (**3**) can be rationalized (*Scheme 3*) by a nucleophilic substitution of **1b**, a keto–enol equilibration product of citrinin, with an eletrophilic citrinin derivative, **1c**. The resulting product **1d** readily undergoes decarboxylation to form **3**. The eletrophile **1c**

Scheme 2. *Biosynthetic Pathway to Citrinin* (**1**) [18]



Scheme 3. *Proposed Biosynthetic Route to Dicitrinol* (**3**)



may be formed from **6a** in early steps of the biosynthesis of **1** (*Scheme 2*) during the conversion of **6** to **7**.

3. *Antimicrobial Activity.* The antibacterial activity of dicitrinol (**3**) was examined in the presence of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, and the results are compared with those obtained for citrinin (**1**) under the same conditions (*Table 2*). In general, dicitrinol is less active than citrinin, except against *E. coli*, which stops growing in a medium containing 31.25 μg/ml of **3**.

Table 2. *Growth Behavior of Bacteria in the Presence of Different Concentrations of Polyketides Produced by* P. janthinellum

Concentration c [μg ml^{-1}] of test compounds	*E. coli*		*P. aeruginosa*		*B. subtilis*	
	1	**3**	**1**	**3**	**1**	**3**
500.00	=[a])	–[b])	=	–	–	–
250.00	+[c])	–	=	–	–	–
125.00	+	–	=	–	–	–
62.50	+	–	–	+	–	+
31.25	+	–	+	+	–	+
15.63	+	+	+	+	+	+
7.81	+	+	+	+	+	+

[a]) =: Bactericidal effect (no growth in subculture). [b]) –: Bacteriostatic effect (growth in subculture). [c]) +: Growth like in the negative control.

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Experimental Part

General. Column chromatography (CC): commercial silica gel (SiO_2; 230–400 mesh; *AnalytiCals Carlo Erba*). TLC: Home-made (SiO_2; 5–15 μm; *Sorbent Technologies*). UV Spectra: *Hewlett Packard 8452-A* spectrophotometer; in CH_2Cl_2 soln.; λ_{max} [nm]. IR Spectra: *Bomen MB-102* spectrophotometer; in KBr pellets; in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker DRX-400* spectrometer; at 400 and 100 MHz, resp.; in $CDCl_3$ soln.; δ in ppm; J in Hz. Low-resolution (LR) MS: *Waters QuattroLC*; APCI; negative-ion mode; in m/z.

Fungal Material. P. janthinellum was obtained from the collection of the Laboratório de Bioquímica Micromolecular (LaBioMMi) of the Chemistry Department at Universidade Federal de São Carlos. This collection contains isolates recently obtained from *Melia azedarach*. *P. janthinellum* is characterized by the No. LaBioMMi-018.

Production, Extraction, and Isolation. Forty-five *Erlenmeyer* flasks (500 ml), containing 90 g of white corn ('*Yoki*') and 75 ml of dist. H_2O per flask, were autoclaved twice at 121° for 40 min. A small disc of the PDA (Potato-Dextrose Agar) medium from the *Petri* dish containing mycelium of *P. janthinellum* was transferred under sterile conditions to 42 *Erlenmeyer* flasks containing sterilized corn. Three flasks were put aside for control purposes. After 20 d of growth at 25°, MeOH (200 ml) was added to each of the flasks, which were allowed to stand for 5 h, and then were filtered by gravity. The MeOH was evaporated under reduced pressure, producing a yellowish residue (14.2 g). Part of this residue (10.0 g) was subjected to a low-pressure SiO_2 CC eluted with hexane, AcOEt, and MeOH gradient. *Citrinin* (180.2 mg; **1**) crystallized from a MeOH soln. of a fraction eluted with AcOEt/MeOH 96:04 from the first SiO_2 CC. The medium-polarity fractions eluted with AcOEt were repeatedly subjected to CC (SiO_2; hexane/AcOEt/MeOH 60:35:05, isocratic) and prep. TLC ($CHCl_3$/MeOH 95:05), and the polyketides were finally purified and identified as *dicitrinol* (**3**; 6.9 mg), and as four hydroxyanthraquinones [13].

Citrinin (=*4,6-Dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H-*isochromene-7-carboxylic Acid*; **1**). Yellow needles. M.p. 158–163° (MeOH/H_2O). UV (CH_2Cl_2): 333 (3.73). IR (KBr): 3417, 2974, 2922, 1670, 1635, 1491, 1377, 1265, 818. ^1H- and ^{13}C-NMR ($CDCl_3$): see *Table 1*. ESI-MS (Daughter ions; 20 eV): 249 (21, $[M-H]^-$), 231 (10), 205 (100), 177 (61), 161 (36), 105 (21).

Dicitrinol (=*7,7′-Methanediylbis(3,4-dihydro-8-hydroxy-3,4,5-trimethyl-6*H-*isochromen-6-one)*; **3**). Yellow powder. M.p. 210° (dec.). UV (CH_2Cl_2): 328 (4.19). IR (KBr): 3448, 2969, 2928, 1723, 1638, 1475, 1451, 1220, 809. ^1H- and ^{13}C-NMR ($CDCl_3$): see *Table 1*. APCI-MS (Daughter ions, 20 eV): 423.4 (27, $[M-H]^-$), 205.1 (100).

Antibacterial Bioassay. The susceptibilities of microorganisms to the test polyketides were determined by microbroth dilution assay as recommended in [19] by the Subcommittee on Antifungal Susceptibility Testing of the US National Committee for Clinical Laboratory Standards (NCCLS), and in the same way as reported before.

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