Antimicrobial Compounds of Fungi Vectored by *Clusia* spp. (Clusiaceae) Pollinating Bees

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The production of antimicrobial compounds by fungi associated with *Clusia* spp. pollinating bees (*Trigona* sp., Trigonini) was investigated in order to approach natural mechanisms of microbial density control within nest environment. By using a bioassay-guided approach based on bioautography and minimal inhibitory concentration (MIC), known α,β-dehydrocurvularin and curvularin were isolated from *Curvularia eragrostidis* (CCT 5634) and *Curvularia pallescens* (CCT 5654), and known cochlioquinone A and isocochlioquinone A were isolated from *Drechslera dematioidea* (CCT 5631).

Key words: Trigona, Dehydrocurvularin, Cochlioquinone

Introduction

Modern chemical ecology pollination studies have been concerned with the identification of molecules in charge of attraction of pollinators and with the primary function of pollinator rewards. From this perspective, previous studies regarding neo-tropical genus *Clusia* (Clusiaceae) and related pollinating bees have contributed with chemical knowledge on both *Clusia* spp. floral volatiles (Nogueira *et al.*, 2001) and floral resins (Porto *et al.*, 2000; de Oliveira *et al.*, 1999).

Nest-building pollinator bees such as *Trigona* spp. (Trigonini) have used floral resins of *Clusia* spp., rich in polymerizable benzophenone derivatives (Porto *et al.*, 2000; de Oliveira *et al.*, 1999), as a waterproof construction material (Bittrich and Amaral, 1996). Additionally, a putative reason leading bees to *Clusia* spp. floral resins have been sustained by their antimicrobial activity demonstrated *in vitro* against bacilli, yeast, and Gram-positive bacteria (Porto *et al.*, 2000; Lokvam and Braddock, 1999). However, most opportunistic nest pathogens have been related to Fungi (Roubik, 1989), against which *Clusia* spp. floral resins have not presented relevant *in vitro* growth inhibitory activity (Porto *et al.*, 2000; Lokvam and Braddock, 1999). Considering this evidence, the present authors have initiated a search for antimicrobial compounds that would be possibly involved with keeping fungal density in nests of *Clusia* spp. pollinating bees within acceptable limits.

Since microbial ecosystems are ruled, at least in part, by their own-generated bioactive compounds (Gloer, 1995), in the present approach the antimicrobial activity of secondary metabolites of fungi associated with *Clusia* spp. pollinating *Trigona* sp. are investigated.

Experimental

General

Malt extract, Müller Hinton and microbiological agar were purchased from Merck (Darmstadt, Germany). MTT [3-(4,5-dimethyl-2-tiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Merck, Darmstadt, Germany) was used as living cell indicator. Thin layer chromatography (TLC) analyses employed silica gel 60-F_{254} on aluminum sheets (Merck, Darmstadt, Germany). Preparative TLC (TLCP) separations employed glass plates coated (1 mm) with (1:1) silica gel G and silica gel PF_{254} (Merck, Darmstadt, Germany). Column chromatography purifications were performed on silica gel 60, 230–400 mesh (Merck, Darmstadt, Ger-

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many). Sample derivatization for gas chromatography employed N,O-bis-trimethylsilyl-trifluoroacetamide-BSTFA (Merck, Darmstadt, Germany). The distilled organic solvents hexane (Hex), ethyl acetate (EtOAc) and methanol (MeOH) were supplied by the UNICAMP-Chemistry Institute solvent pilot plant. Nuclear magnetic resonance analyses employed deuterochloroform ($CDCl_3$; CIL, Andover, MA, USA).

Fungi isolation

Trigona sp. (Trigonini) bees were trapped into sterile plastic bags while collecting *Clusia* sp. floral resins at Santa Cecília farm, Agronomic Institute of Campinas, Campinas, SP, Brazil. The bees were cut into pieces and placed on Petri dishes containing fungi isolation medium (malt extract agar 20 g, chloranphenicol 100 mg, distilled water 1 l) and incubated at 28 °C for 72 h. The fungal colonies were successively replicated on biomalt plates (malt extract agar 20 g, distilled water 1 l) in order to get pure cultures for taxonomical examination. Fifteen fungal strains were identified by mycologists from the Fundação Tropical de Pesquisas *André Tosello*, Campinas, SP, Brazil. Vouchers were deposited at the Coleção de Culturas *Tosello* (CCT) as following: *Aspergillus niger* CCT 5559, *Aspergillus* sp. CCT 5660, *Curvularia* sp. CCT 5629, *Curvularia lunata* CCT 5628, *Curvularia eragrostidis* CCT 5634, *Curvularia pallescens* CCT 5654, *Drechslera dematioidea* CCT 5631, *Drechslera halodes* CCT 5636, *Nodulisporium* sp. CCT 5552, *Trichoderma* sp. CCT 5551, and not yet identified strains CCT 5630, CCT 5632, CCT 5635, CCT 5553 and CCT 5661.

Cultivation and extraction

Fungi were seeded and incubated on biomalt plates for 7 days, at 28 °C. Out of these, mycelial mat plugs (1 cm diameter) were cut and transferred to flasks (125 ml) containing sterile grounded corn (corn-water, 10 g:30 ml), which were incubated for 14 days, at 28 °C. All cultures were extracted with EtOAc-MeOH (1:1, 50 ml) and filtered. The organic solvents were evaporated to give residues, which were further extracted with EtOAc (1:1, v/v, 2 ×). The combined organic layers were dried over anhydrous Na_2SO_4 and vacuum-concentrated. A control extract (culture medium only) was prepared as described above.

Bioautography

Aliquots (150 μg) of the crude fungal culture extracts were spotted on silica gel TLC plates and eluted with Hex-EtOAc (1:1). After evaporation of the organic solvents the TLC plates were placed into sterile Petri dishes (15 × 90 mm) and flooded with 20 ml of Müller Hinton (8 g/l) – agar (20 g/l) seeded in 1% with an aqueous microorganism suspension (10^8 cells/ml) (*Apergillus niger* CCT 1435, *Bacillus subtilis* CCT 0089, *Candida albicans* CCT 0776, *Escherichia coli* CCT 5050, and *Staphylococcus aureus* CCT 4295). The plates were incubated at 30 °C for 24 h (bacteria) or 48 h (fungus/yeast), and then flooded with 10 ml of microbiological agar (10 g/l) containing 0.05% of MTT. Cell growth inhibition (antimicrobial) compounds were detected as yellowish TLC spots on a deep blue back ground, allowing the chromatographic retention factors (R_f) observation.

Minimal inhibitory concentration – MIC

a) of bioautography detected spots

The bioautography selected fungal extracts were submitted to TLCP. Compounds of interest were localized by chromatographic Rf, scraped out of the plates and extracted with EtOAc/MeOH (1:1). Solvents were evaporated and the residues were suspended (4 mg/ml) in H_2O-DMSO (20%) prior to successive dilutions into a 96-well microtiter plate. Finally an aliquot (100 μl) of culture medium (bacteria: Müller Hinton, 8 g/l; fungi: Malt Extract, 20 g/l) containing 1% of an aqueous microorganism suspension (10^8 cells/ml; *B. subtilis* CCT 0089, *S. aureus* CCT 4295; *A. niger* CCT 1435) was added to the wells, leading bioactive compound fractions to final concentrations of 1000, 500, 250, 125, 62.5, and 31.2 μg/ml. Positive controls were prepared by substituting test fraction by either chloranphenicol or cyclopiroxolamina, and negative controls were prepared only with aqueous-DMSO plus inoculum. The plates were incubated at 30 °C, for 24/48 h. Aliquots (50 μl) of aqueous MMT (0.05%) were added to the wells, and reduction of the tetrazolium salt (yellow) to

formazan (blue) by living cells was observed within 30 min. All tests were run in duplicates.

b) of isolated compounds

Aqueous DMSO suspensions of the pure isolated bioactive compounds were prepared and tested as described above for TLCP extracted bands (Table I).

Bioactive compound isolation

Both crude extracts of *C. eragrostidis* and *C. pallescens* were submitted to flash silica gel column chromatography (1:30, extract:silica) under elution with hexane and increasing amounts of EtOAc to give compounds **1** and **2**.

E-Dehydrocurvularin (**1**): colorless crystals (*C. eragrostidis*: 0.15 mg/g culture EtOAc crude extract; *C. pallescens* 0.26 mg/g culture EtOAc crude extract); $[\alpha]_D^{20}$ – 64.9° (*c* 1,0, MeOH) and RMN ^{1}H, RMN ^{13}C, and MS as previously published (Arai *et al.*, 1989; Hyeon *et al.*, 1976). CD (MeOH) $\Delta\varepsilon_{MAX}$ = – 4.79 (235 nm, *c* 1.7 × 10^{-3} mol/l).

Curvularin (**2**): colorless flakes (*C. eragrostidis*: 0.19 mg/g culture EtOAc crude extract; *C. pallescens* 0.22 mg/g culture EtOAc crude extract); $[\alpha]_D^{20}$ – 44.9° (*c* 1,0, MeOH) and RMN ^{1}H, RMN ^{13}C, MS and CD as previously published (Arai *et al.*, 1989; Hyeon *et al.*, 1976).

The crude extract of *D. dematioidea* was first submitted to flash silicagel column chromatography (1:30, extract:silica) to give the bioactive compounds detected by bioautography, together in a single chromatographic fraction (3.3 mg/g culture EtOAc crude extract). A part of this bioactive fraction was eluted on TLCP-$AgNO_3$ (5%) with benzene-EtOAc (9:1, 3 ×) to give compound **3**, and another part was acetylated (acetic anhydride/pyridine, 2:1) and submitted to flash silicagel column chromatography (1:30, extract:silica) with hexane and increasing amounts of EtOAc to give compound **4**.

Cochlioquinone A (**3**): yellow solid (0.4 mg/g culture EtOAc crude extract); $[\alpha]_D^{25}$ + 205° (*c* 1.0, CH_3CN), CD (0.9 × 10^{-3} mol/l, CH_3CN) nm ($\Delta\varepsilon_{MAX}$) = 380 (+ 6.54), 315 (– 0.84), 278 (+ 10.0) and all remaining physical and spectroscopic data as previously published (Miyagawa *et al.*, 1994).

Isocochlioquinone A – diacetyl derivative – (**4**): yellow oil (0.5 mg/g culture EtOAc crude extract); $[\alpha]_D^{25}$ + 47° (*c* 1.0, CH_3CN), [Miyagawa *et al.*, 1994: neat natural product: $[\alpha]_D^{24}$ +65°, *c* 0.1, EtOH]; CD (1.6 × 10^{-3} mol/l, CH_3CN) nm ($\Delta\varepsilon_{MAX}$) = 348 (+ 6.2), 320 (– 7.7), 262 (+ 10.17), 236 (+ 5.90), 219 (+ 34.05); ^{1}H NMR (499.882 MHz, $CDCl_3$ δ 0.00 TMS) δ 6.61 (s, 1 H, H-11), 5.09 (dd, 1 H, H-4), 3.32 (qtd, 1 H, *J* = 7.4, H-5), 3.23 (dd, 1 H, *J* = 12.0; 2.4, H-21), 3.11 (dd, 1 H, *J* = 11.7; 3.5, H-17), 2.65 (s, 1 H, H-13, 2.59 (m, 1 H, H-19a), 2.36 (s, 3 H, CH_3-32), 2.33 (s, 3 H, CH_3-34), 2.00 (m, 2 H, H-15), 1.99 (s, 3 H, CH_3-30), 1.70 (m, 2 H, H-20), 1.75 (m, 1 H, H-16a), 1.63 (m, 1 H, H-3), 1.60 (m, 1 H, H-16b), 1.41 (s, 3 H, CH_3-26), 1.22 (m, 1 H, H-19b), 1.19 (s, 3 H, CH_3-23), 1.18 (s, 3 H, CH_3-24), 1.18 (d, 3 H, *J* = 7.0; CH_3-27), 1.14 (m, 2 H, H-2), 1.09 (s, 3 H, CH_3-25), 0.92 (t, 3 H, *J* = 7.4; CH_3-1), 0.90 (d, 3 H, *J* = 6.7; CH_3-28); ^{13}C NMR (125.695 MHz, $CDCl_3$ δ 0.00 TMS) δ 190.2 (C-12), 170.5 (C-29), 169.5 (C-31), 168.0 (C-33), 151.1 (C-10), 146.4 (C-8), 143.3 (C-6), 135.8 (C-7), 113.8 (C-11), 113.6 (C-9), 85.5 (C-21), 83.5 (C-17), 83.1 (C-14), 78.2 (C-4), 71.9 (C-22), 60.4 (C-13), 37.4 (C-15), 36.9 (C-19), 36.2 (C-3), 35.2 (C-18), 34.4 (C-5), 26.5 (C-2), 25.9 (C-24), 24.8 (C-16), 23.6 (C-23), 21.3 (C-20), 21.2 (C-26), 21.1 (C-32), 20.6 (C-30), 20.3 (C-34), 17..8 (C-27), 13.1 (C-28), 12.2 (C-25), 11.5 (C-1); EI-MS 70 eV *m/z* (%) 616 ($M^{+\cdot}$, 0), 601 (1.5), 574 (10), 532 (100), 472 (60), 457 (85), 179 (34).

Gas chromatography-mass spectrometry – GC-MS

GC-MS analyses were performed using an Agilent GC-MS 5973 (Palo Alto, CA, USA) assembly equipped with a HP-5 cross-linked fused silica capillary column (25 m × 0.32 mm × 0.25 μm). Helium was used as carrier gas at 38 cm/s. The column total flow rate was 1 ml/min. General temperature conditions were: split/splitless injector at 280 °C, transfer line at 280 °C, source 230 °C, and column temperature program of 80 °C–310 °C at 10 °C/min. Mass detection limits were 50–700 Da. Samples were reacted with BSTFA-pyridine (1:1, v/v) at room temperature for 30 min before analyses.

Nuclear magnetic resonance – NMR

^{1}H NMR (499.882 MHz) and ^{13}C NMR (125.695 MHz) were acquired in $CDCl_3$ with a Varian INOVA 500 (Palo Alto, CA, USA) spectrometer, using tetramethylsilane (TMS) as internal reference (δ 0.0). Methyl, methylene, methine, and car-

bon non-bonded to hydrogen were discriminated by ^{13}C NMR DEPT 135° and DEPT 90° experiments. Heteronuclear $^{1}H \times ^{13}C$ correlations at one and multiple bonds were discriminated by 2D NMR spectroscopy (HSQC and HMBC experiments).

Results and Discussion

Fifteen filamentous fungi were isolated from *Trigona* sp. bees and further cultivated for production of secondary metabolites. After 14 days incubation on corn bran, the EtOAc extracts of the cultures were prepared and screened for antibiotics.

As a first qualitative approach, the fungal culture EtOAc extracts were submitted to bioautography against *S. aureus*, *E. coli*, *B. subtilis*, *C. albicans*, and *A. niger.* Antimicrobial compounds were detected against *S. aureus*, *B. subtilis* and *A. niger* in the culture extracts of *Curvularia* sp., *C. eragrostidis*, *C. pallescens*, *D. dematioidea*, and strains CCT 5632, CCT 5635, CCT 5661, and CCT 5553.

As a quantitative, second approach, the bioautographically selected compounds were accessed by TLCP and submitted to MIC essays. However, most isolated TLCP fractions were not significantly active in comparison to positive controls (cloramphenicol/cyclopiroxolamina). Such samples comprised compounds of R_f = 0.0 (Hex-AcOEt, 1:1), which after silylation (BSTFA-pyridine, 1:1) and GC-MS analyses were characterized as mixtures of alditols (*e. g.*, manitol), monosaccharides and disacharides, due to mass spectra with typical ions (*m/z* 205/217 or *m/z* 204/217, base peaks) of silylated polyols. Their bioautography-inhibitory effect were tentatively attributed to high osmolarity. On the other hand, samples expressing a moderate activity, namely compounds of R_f = 0.5 (Hex-AcOEt, 1:1) from *C. eragrostidis*, *C. pallescens*, and *Curvularia* sp. culture extracts, and of R_f = 0.4 (Hex-AcOEt, 1:1) from *D. dematioidea*, were directed to purification.

Curvularin and α,β-dehydrocurvularin. The EtOAc crude extracts of *C. eragrostidis* and *C. pallescens* cultures were fractionated by flash silica

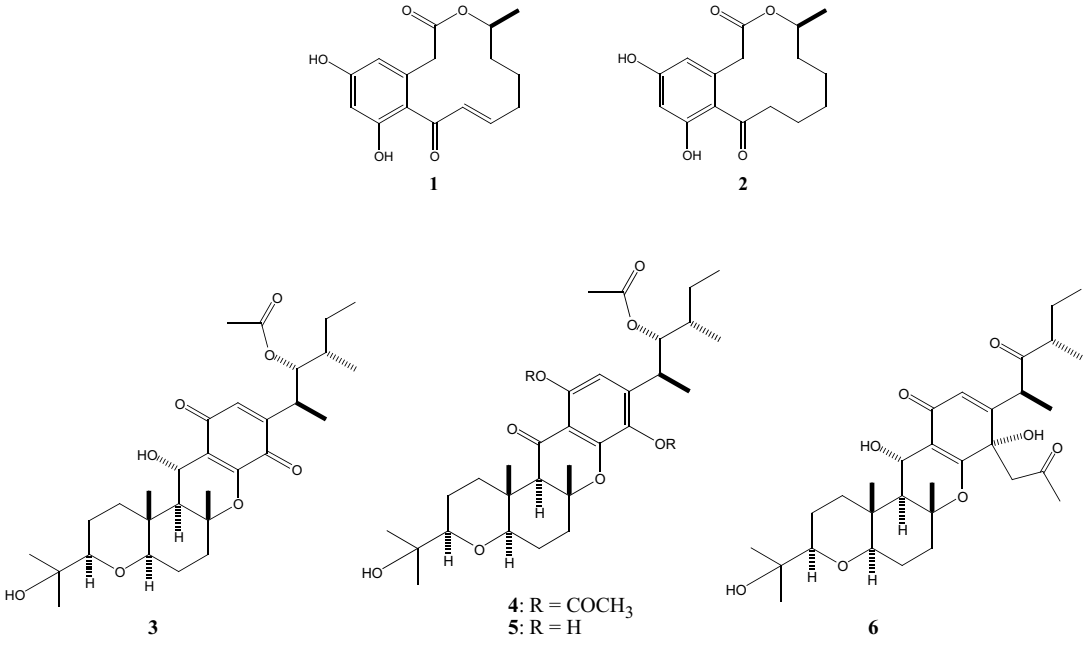

Fig. 1. Fungal bioactive compounds: (**1**) dehydrocurvularin, (**2**) curvularin, (**3**) cochlioquinone A, (**4**) isocochlioquinone A bis-acetyl derivative, (**5**) isocochlioquinone A, and (**6**) cochlioquinol.

Table I. Minimal inhibitory concentration (MIC) of isolated compounds.

Compound	MIC (ppm)		
	S. aureus	*B. subtilis*	*A. niger*
Dehydrocurvularin	> 500	> 250	> 1000
Curvularin	> 1000	> 1000	> 1000
Cochlioquinone A	> 15	> 15	> 250
Isocochlioquinone A (acetylated)	> 1000	> 1000	> 1000
Chloramphenicol*	> 4	> 4	–
Cyclopiroxolamina*	–	–	< 10

* Standard antimicrobial compounds used as positive controls.

gel column chromatography to yield compounds **1** and **2** which (Fig. 1), on TLC (Hex-EtOAc, 1:1), showed R_f = 0.5 and 0.4, respectively. The physical and spectral data (MS, RMN, $[\alpha]_D$, CD) agreed with those previously reported (Arai *et al.*, 1989; Hyeon *et al.*, 1976) for curvularin (**2**) and α,β-dehydrocurvularin (**1**).

MIC assays of **1** and **2** against *A. niger*, *S. aureus*, and *B. subtilis* revealed compound **1** as the only one endowed of antimicrobial activity, although of little expression when compared to chloramphenicol (Table I). The ability to trigger different biological responses toward cell development is a fact which has been noted for **1** and **2** by several authors (Kobayashi *et al.*, 1988; Hyeon *et al.*, 1976), who independently described **1** as a stronger cell growth inhibitory compound. Biosynthetic studies have supported that **1** is a polyketide product, which is excreted from the cells and reduced to **2** by extracelullar enzymes (Liu *et al.*, 1998). Indeed, it was observed in a parallel experiment that (data not shown) the concentration of **2** in the culture medium increases as concentration of **1** diminishes. All the data hitherto provided indicate compound **1** as a cell growth regulator.

Cochlioquinone A and Isocochlioquinone A. The EtOAc crude extract of the *D. dematioidea* culture was submitted to flash silica gel column chromatography. Among the extract fractions, the one comprising a mixture of yellow compounds of TLC R_f (Hex-EtOAc, 1:1) = 0.4 was selected and further purified to yield compounds **3** and **4** (Fig. 1), the later as an acetylated derivative (see experimental). The physical and spectral data obtained (MS, RMN, $[\alpha]_D$) provided the identification of cochlioquinone A (**3**) and isocochlioquinone A (bis-acetyl derivative, **4**) by comparison with data from the literature (Miyagawa *et al.*, 1994).

MIC assays against *A. niger*, *S. aureus*, and *B. subtilis* showed a dramatic potency difference between **3** and **4** (Table I), and then suggested that the quinone moiety plays a relevant role in antimicrobial activity. Similar observations have been previously assigned in regard to root growth inhibition of Italian ryegrass by **3** in comparison to cochlioquinol (**6**, Fig. 1) (Lim *et al.*, 1996), and in regard to root growth inhibition of finger millet and rice plants by **3** in comparison to isocochlioquinone A (**5**) (Miyagawa *et al.*, 1994). The mechanism of action of **3** toward cell growth inhibition have not yet been approached. However, it has been demonstrated that **3** interferes in cell signal transduction systems by inhibiting diacylglycerol kinase (Ogawara *et al.*, 1994).

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