ORIGINAL ARTICLE

Reactivation of antibiosis in the entomogenous fungus *Chrysosporthe* sp. SNB-CN74

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***Chrysosporthe* sp. SNB-CN74 was isolated from a *Nasutitermes corniger* nest, and its ethyl acetate extract was found to exhibit very strong antibacterial activity. Two antibacterial metabolites were isolated, (–)-*R*-skyrin (2) and (+)-rugulosin A (3). Eventually, the fungus lost its antibiotic potential when subcultured, and the use of yeast extract induced the re-expression of these two antibiotics. Yeast extract possibly activated a cryptic pathway by mimicking the presence of an ecological competitor.**

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INTRODUCTION

It is urgent to find new antibiotic compounds because of increased pathogen resistance to the available antimicrobials. It is with this objective that we became interested in exploring new resources of natural substances, and in particular, social insect symbiotic microorganisms. According to the literature data and because insects, fungi and bacteria represent the most diverse and unknown groups of living organisms,¹ they should be considered as an innovative, ecologically relevant source of antimicrobial compounds.^{2–13} Indeed, eusociality should in principle favor the transmission of infectious pathogens (many individuals, promiscuity, frequent interactions and genetic homogeneity), and it has been observed that social insects have developed mutually beneficial associations with microorganisms that provide antimicrobial agents to the colonies, improving the fitness of the host insect. Several antimicrobial compounds have been isolated from insect symbiotic microorganisms.^{2,14} However, few studies have been conducted on termite symbionts.^{15–17} We have thus embarked into characterizing antimicrobial agents from Guianese termite symbionts in the search for compounds that may eventually be useful in human health.^{17,18} An ethyl acetate extract of a fungus isolated from a *Nasutitermes corniger* nest, identified as *Chrysosporthe* sp. SNB-CN74, was strongly antibacterial (MIC = 16 µg ml^{–1} on *Staphylococcus aureus* and *Escherichia coli*). However, the fungus lost its antibacterial potential when it was subcultured. The present article describes our efforts to recover the antibacterial activity of the fungus and to identify the compounds responsible for this activity.

RESULTS AND DISCUSSION

Characterization of antibacterial metabolites

Initially, the only active extract obtained was the extract used for the screening. With 75.7 mg, we undertook to isolate the active

metabolites by bioactivity-guided fractionation. Three compounds were isolated and characterized, and the antibacterial potential was supported by compounds 2 and 3 (Figure 1). Compound 1 was new to science. These metabolites have been identified by high-resolution MS, NMR and spectroscopic analyses.

Compound 1 was a colorless oil. The molecular formula C₁₅H₂₂O₃ was determined via high-resolution ESI time-of-flight mass MS analysis, which showed a pseudomolecular ion peak at *m/z* 251.1650 [M+H]⁺ (calculated (calcd) for C₁₅H₂₃O₃, 251.1647). This formula implied five degrees of unsaturation. The NMR spectrum in CDCl₃ suggested the presence of three olefinic protons at δ 5.21, 5.29 and 5.35 and four protons on oxygenated carbons at δ 3.19, 4.19, 4.32 and 4.53. Protons at δ 5.29 and 5.35, as well as those at δ 4.19 and 4.32 were linked to the same carbons (δ 115.4 and 64.0, respectively; Table 1). Two methyl groups were also present. The first one was a doublet at δ 1.01 and the second one was a singlet at δ 1.03. It was possible to describe a cyclohexane A-ring following the sequence of the COSY correlations along the left side of the molecule (1–15), and it was eventually found that compound 1 was an octahydronaphthalene based on the COSY and HMBC correlations between the two parts of the molecule (Figure 2). In particular, H-1ax correlated with H-8 and H-9, whereas in the HMBC, H-6 and the bridgehead methyl group H-14 both correlated with the central quaternary carbons C-5 and C-10. Finally, an isopropenyl substituent was elucidated within positions 12, 11 and 13, and this substituent was linked to carbon 7 based on the HMBC experiment.

Because the molecule contained three oxygens and had four carbons linked to oxygens, it remained to ascertain the position of one heterocycle to reach the right crude formulae. The H-6 chemical shift at δ 3.19 was rather upfield, a fact that suggested the presence of an epoxide between C-6 and C-7. The NMR spectrum was recorded in

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dimethyl sulfoxide *d*₆ (DMSO-*d*₆) to observe labile protons and their couplings with CH (Supplementary Table S1 in the Supplementary Information). Additional coupling was observed in H-8 and H-13, whereas H-6 remained singlet, thus confirming the presumed position of the additional unsaturation in the form of an oxiran ring. The analytical data are consistent with those described in the literature for epoxydecalins such as phomenone and the phomodecalins.^{19–21} Compound **1** has not been previously described in the literature and was given the trivial name phomenol after the structurally related phomenone.

Figure 1 Compounds isolated from *Chrysosporthe* sp. SNB-CN74.

Relative stereochemistry was determined upon examination of the coupling constants in the cyclohexane ring and the observation of the NOE correlations (Figure 3). H-3_{ax} had three large coupling constants, indicating that H-4 was axial. The methyl group at C-14 was therefore equatorial. The NOE correlations between H-15 and H-1_{ax}, and between H-3_{ax} and H-6 indicated that these protons were on the same side of the molecule. The stereochemistry at C-7 is governed by the oxiran ring and the stereochemistry at C-8 remained uncertain. However, the NOE correlations between H12_a and H-8, and between H12_a and H-6 may indicate that these three protons were on the same side of the molecule in the most stable conformer around the C-7–C-11 single bond. This assumption was confirmed by the NOE spectroscopy and the ROE spectroscopy experiments conducted in DMSO-*d*₆ with the observation of a correlation between H-8 and H-15 indicating that these protons are indeed on the same side of the molecule (Supplementary Table S1 in the Supplementary Information).

Compound **2** was identified as (–)-*R*-skyrin based on NMR, HRMS and optical rotation.^{22,23} (–)-*R*-Skyrin is less frequent than its enantiomer, although it has been already isolated in the past from fungal sources.^{24,25}

Compound **3** was identified as (+)-rugulosin A. The spectral data were identical to those already described in the literature.^{26,27}

Figure 2 Proton to carbon HMBC (dashed arrows) and proton to proton COSY (bold) correlations in **1**.

Table 1 NMR data for phomenol (**1**) in CDCl₃

Position	δ_C , type	δ_H (<i>J</i> in Hz)	COSY	HMBC ^a	NOESY
1	32.3, CH ₂	eq: 2.12 m ax: 2.20 m	1ax, 2eq, 2ax 1eq, 2eq, 2ax, 8, 9		1ax, 2eq, 9 1eq, 15
2	26.5, CH ₂	ax: 1.34 br. qt (<i>J</i> =13.4, 3.8) eq: 1.74 m	1eq, 2eq, 3eq 1eq, 1ax, 2ax, 3eq, 3ax	10	2eq, 3eq 1eq, 2ax, 3eq
3	30.3, CH ₂	ax: 1.39 br. qd (<i>J</i> =13.2, 3.1) eq: 1.56 m	2eq, 3eq, 4 2eq, 2ax, 3ax, 4 3eq, 3ax, 14	2	3eq, 14, 15 2eq, 2ax, 3ax, 14 14
4	36.7, CH	1.75 m			
5	38.3, C				
6	68.4, CH	3.19 s		5, 7, 10, 11, 15	12a, 14, 15
7	64.5, C				
8	67.0, CH	4.53 br. t (<i>J</i> =2.9)	1ax, 9	9, 10	12a
9	118.0, CH	5.21 br. t (<i>J</i> =2.4)	1ax, 8	1, 5, 7	1eq, 8
10	142.2, C				
11	146.0, C				
12	115.4, CH ₂	a: 5.29 d (<i>J</i> =1.2) b: 5.35 q (<i>J</i> =1.2)	12b 12a, 13a, 13b	7, 11, 13 7, 11, 13	6, 8, 12b 12a, 13a, 13b
13	64.0, CH ₂	a: 4.19 dd (<i>J</i> =12.9, 1.2) b: 4.32 dd (<i>J</i> =12.9, 1.2)	12b, 13b 12b, 13a	7, 11, 12 7, 11, 12	12b, 13b 12b, 13a
14	15.9, CH ₃	1.01 d (<i>J</i> =6.8)	4	3, 4, 5	3eq, 4, 6
15	16.3, CH ₃	1.03 s		5, 6, 10	1ax, 3ax, 6

Abbreviations: ax, axial; eq, equivalent.

^aHMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon.

Biological activity

All compounds isolated from *Chrysosporthe* sp. SNB-CN74 were tested on microbial human pathogens (Table 2). The cytotoxicities toward human non-cancer MRC5 cells were recorded to evaluate the antimicrobial selectivity. All compounds were inactive against fungi, but (–)-*R*-skyrin (2) and (+)-rugulosin A (3) were strongly antibacterial, with remarkable selectivity indexes. Interestingly, (–)-*R*-skyrin was more active than the positive control against the Gram-negative strain of *E. coli*, whereas (+)-rugulosin A was more efficient on the Gram-positive bacterium *S. aureus*.

Reinduction of the antibacterial compounds synthesis

After the discovery of its antibacterial potential and before the identification of the active metabolites, the fungus *Chrysosporthe* sp. SNB-CN74 was cultivated on a large scale to generate more crude material. The crude extract obtained from this large-scale cultivation was inactive. It was also colorless, whereas the active extract was dark orange. After isolating compounds 2 and 3 on the small scale, it was obvious that the two antibiotic metabolites were colored and were therefore absent in the inactive large-scale extract. This assumption was confirmed by the HPLC profiling, and (–)-*R*-skyrin and (+)-rugulosin A can thus be considered as activity markers of this specific strain that are visible to the naked eye. *Chrysosporthe* sp. SNB-CN74 was therefore an ideal strain to undertake metabolite re-expression studies. In one of the Petri dishes of the large-scale cultivation, a fungal contaminant was surrounded with an orange halo (Figure 4).

Although the outsider was a fungus, it was detected as a potential competitor and induced an antibiosis-like reaction of *Chrysosporthe* sp.

Figure 3 Key NOE correlations in 1.

SNB-CN74.^{28,29} There are many examples in the literature describing the discovery of fungal metabolites through epigenetic activation.³⁰ Here the intruder acted as an epigenetic key that opened a silent biosynthetic pathway. It was therefore sought to mimic this process with a fanciful outsider. SNB-CN74 culture recovered a deep orange coloration on potato dextrose agar (PDA) supplemented with 5 g l⁻¹ yeast autolysate. The ethyl acetate extract from this culture recovered the antibacterial activity of this initial extract (16 µg ml⁻¹). With a higher concentration of yeast autolysate (10 and 20 g l⁻¹), the extracts were light orange and were less active (64 and 256 µg ml⁻¹, respectively). HPLC profiling confirmed that compounds 2 and 3 were present both in the initial active extract and in the orange extract generated from the culture with 5 g l⁻¹ yeast autolysate (Figures 5a and c), and were absent in the inactive culture (Figure 5b, the peak of rugulosine (2) corresponds to a very small relative proportion of this metabolite, which has a strong specific absorbance at 210 nm; it was not detected with an evaporating light scattering detector).

In conclusion, this study of *Chrysosporthe* sp. SNB-CN74 has allowed us to isolate phomenol (1), as well as the two known antibacterial agents (–)-*R*-skyrin (2) and (+)-rugulosin A (3). These two metabolites have proven active on human pathogens, the first one being more efficient on Gram-negative *E. coli* and the second more active on Gram-positive *S. aureus*. The presence of these metabolites in the fungal extract was somewhat labile, and it was possible to reactivate antibiosis with yeast autolysate. The latter was presumably detected as

Figure 4 A fungal contaminant in *Chrysosporthe* sp. SNB-CN74 culture surrounded with an orange halo. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 2 MIC and SI of the isolated compounds

Strain	MIC (µg ml ⁻¹)				SI ^a		
	1	2	3	Pos.ctrl. ^b	1	2	3
<i>S. aureus</i> (ATCC 29213)	>256	16	≤0.5	0.5	—	2.3	≥102.4
<i>E. coli</i> (ATCC 25922)	>256	4	16	8	—	9.3	>3.2
<i>C. albicans</i> (ATCC 10231)	>256	>256	>256	4	—	—	—
<i>A. fumigatus</i> (SNB-AF1)	>256	>256	>256	0.5	—	—	—
<i>T. rubrum</i> (SNB-TR1)	128	>256	128	0.03	>0.4	—	>0.4
	IC50 (µM)						
MRC5	>204.5	69.3	>94.4	0.0005	—	—	—

Abbreviations: Pos. ctrl., positive control; SI, selectivity indexes.

^aSI = IC₅₀/MIC based on IC₅₀ measured on MRC5 cell line.

^bPositive controls: *S. aureus*: Oxacillin; *E. coli*: gentamicin; *C. albicans*: fluconazole; filamentous fungi: Itraconazole; MRC5: docetaxel.

Figure 5 UV traces (210 nm) of (a) initial active extract; (b) inactive extract from large-scale cultivation; (c) extract from culture with 5 g l⁻¹ yeast autolysate.

an environmental competitor by *Chrysosporthe* sp. SNB-CN74 and acted as an epigenetic modifier. This approach allowed the expression of a silent metabolic pathway and could open new opportunities in the discovery of original bioactive metabolites from fungi.

EXPERIMENTAL PROCEDURE

General experimental procedures

Optical rotation was measured using an Anton Paar MCP 300 polarimeter in a 100-mm long 350-μl cell. NMR spectra were recorded on a Bruker 500 MHz spectrometer (Bruker-Biospin, Billerica, MA, USA). Chemical shifts (δ) are reported as p.p.m. based on the TMS signal. High-resolution ESI time-of-flight mass MS measurements were performed using a Waters Acquity UPLC system with column bypass coupled to a Waters Micromass LCT Premier time-of-flight mass spectrometer equipped with an ESI (Waters, Milford, MA, USA). Analytical and preparative HPLCs were conducted with a Gilson system equipped with a 322 pumping device, a GX-271 fraction collector, a 171 diode array detector, and a prepELSI detector electrospray nebulizer. The columns used for these experiments included a Phenomenex Luna C18, 5 μm, 4.6 × 250 mm for analytical HPLC and a Sunfire (Waters) C18, 5 μm, 10 × 150 mm for preparative HPLC. The flow rate was set to 1 or 4 ml min⁻¹, respectively, using a linear gradient of H₂O mixed with an increasing proportion of CH₃CN. Both solvents were modified with 0.1% formic acid. All solvents were HPLC grade. PDA and yeast extract were purchased from Fluka Analytical (Sigma-Aldrich, St Louis, MO, USA).

Fungal material

The strain was isolated from a *Nasutitermes corniger* termite aerial nest sampled in Rémire-Montjoly, French Guiana in July 2011. *N. coniger* belong to the Termitidae family. *Nasutitermes* is the most frequent genus in French Guiana, representing more than half of the genera inventoried in the territory.³¹ *Nasutitermes* species are easily identified by the specific pear-shaped head of soldier individuals. The termites were collected from a dark brown arboreal nest built 1.5 m above ground. The nest was ~1-m high and 20-cm wide and was located in a seashore area (Supplementary Figure S11 in Supplementary Information). A piece of the nest matter was placed in suspension in sterile water, and this solution was spread on PDA medium. After 1 week of incubation at 25 °C, the first fungal hyphae were removed and transferred to another Petri dish. The colony was maintained in triplicate at -80 °C in glycerol-H₂O (50/50). A sample submitted for amplification and nuclear ribosomal internal transcribed spacer region ITS4 sequencing allowed for strain identification by NCBI sequence comparison. The sequence has been registered in the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>) under registry number KJ023737. Molecular analysis was performed externally by BACTUP, France.

Extraction and isolation

The *Chrysosporthe* sp. SNB-CN74 strain was cultivated on PDA at 26 °C for 15 days, initially on a small scale (6, 10-cm diameter Petri dishes) for the biological screening. The fungus and the culture medium were then transferred into an Erlenmeyer flask and macerated with EtOAc for 24 h. The organic solvent was collected by filtration, washed with H₂O in a separating funnel and evaporated, yielding 75.7 mg of extract. The crude extract was purified by preparative HPLC with H₂O/ACN (95/5 to 0/100 in 30 min, 0/100 during 10 min; flow rate 4 ml min⁻¹), which led to the isolation of phomenol (1, 2.1 mg, *t*_R = 14.50 min), (-)-*R*-skyrin (2, 1.9 mg, *t*_R = 24.83 min) and (+)-rugulosin A (3, 1.4 mg, *t*_R = 20.74 min).

Phomenol (1): colorless oil; [α]_D²⁵ + 23.3 (*c* 0.21, MeOH); ¹H-NMR (500 MHz, CDCl₃) see Table 1; ¹³C-NMR (125 MHz, CDCl₃) see Table 1; HRESIMS *m/z* 251.1650 [M+H]⁺ (calcd for C₁₅H₂₃O₃, 251.1647).

(-)-*R*-Skyrin (2): orange solid; [α]_D²⁵ - 105.6 (*c* 0.16, dioxan); ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.78 (2H, s, 8-OH/8'-OH), 12.02 (2H, s, 1-OH/1'-OH), 11.15 (2H, s, 6-OH/6'-OH), 7.28 (2H, s, H-4/H-4'), 7.15 (2H, s, H-2/H-2'), 6.73 (2H, s, H-7/H-7'), 2.33 (6H, s, 3-CH₃/3'-CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 189.7 (C-9/C-9'), 182.0 (C-10/C-10'), 164.3 (C-6/C-6'), 164.3 (C-8/C-8'), 161.2 (C-1/C-1'), 148.2 (C-3/C-3'), 133.2 (C-4a/C-4a'), 131.2 (C-10a/C-10a'), 123.6 (C-2/C-2'), 123.4 (C-5/C-5'), 120.2 (C-4/C-4'), 113.0 (C-9a/C-9a'), 109.1 (C-8a/C-8a'), 107.0 (C-7/C-7'), 21.2 (C-3-CH₃/C-3'-CH₃); HRESIMS *m/z* 539.0966 [M+H]⁺ (calcd for C₃₀H₁₉O₁₀, 539.0978).

(+)-Rugulosin A (3): yellow-orange solid; [α]_D²⁵ + 206.6 (*c* 0.18, dioxan); ¹H NMR (500 MHz, DMSO-*d*₆) δ 14.71 (2H, s, 8-OH/8'-OH), 11.40 (2H, s, 1-OH/1'-OH), 7.44 (2H, s, H-4/H-4'), 7.18 (2H, s, H-2/H-2'), 5.42 (2H, s, 6-OH/6'-OH), 4.38 (2H, t, *J* = 5.4 Hz, H-6/H-6'), 3.36 (2H, brs, H-5/H-5'), 2.76 (2H, d, *J* = 5.4 Hz, H-7/H-7'), 2.43 (6H, s, 3-CH₃/3'-CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 194.6 (C-10/C-10'), 186.0 (C-9/C-9'), 159.4 (C-1/C-1'), 147.4 (C-3/C-3'), 131.9 (C-4a/C-4a'), 123.9 (C-2/C-2'), 120.5 (C-4/C-4'), 114.0 (C-9a/C-9a'), 105.8 (C-8a/C-8a'), 68.5 (C-6/C-6'), 58.5 (C-7/C-7'), 55.6 (C-10a/C-10a'), 47.7 (C-5/C-5'), 21.4 (C-3-CH₃/C-3'-CH₃), n.d. (C-8/C-8'); HRESIMS *m/z* 543.1306 [M+H]⁺ (calcd for C₃₀H₂₃O₁₀, 543.1291).

Cultivation with yeast autolysate

Chrysosporthe sp. SNB-CN74 was cultivated on PDA supplemented with 5, 10 or 20 g l⁻¹ *Saccharomyces* yeast autolysate (Sigma-Aldrich). After 15 days at 26 °C, the whole fungus+PDA was macerated with ethyl acetate as described above.

Antibacterial assays

The species of human pathogenic microorganisms used in this study were the Gram-negative bacterium *E. coli* (ATCC 25922), the Gram-positive bacterium *S. aureus* (ATCC 29213), the yeast *Candida albicans* ATCC 10231 and two clinical isolates of filamentous fungi *Aspergillus fumigatus* SNB-AF1 and *Trichophyton rubrum* SNB-TR1. The extracts and pure compounds were tested according to the reference protocol of the European Committee on Antimicrobial Susceptibility Testing.^{32,33} The MIC was read after 18 h for *C. albicans*, after 5 days for filamentous fungi and after 24 h for the bacteria. All of the assays were conducted in duplicate. The extract obtained from the culture medium (blank) was tested in all assays and did not present significant activity.

Cytotoxic assays

The cytotoxicity assays were conducted with normal human lung fibroblast cells MRC5 (ATCC CCL-171) according to the procedure described by Tempête et al.³⁴

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)