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A new cytotoxic tetralone derivative from *Humicola grisea*, a filamentous fungus from wood in the southeastern lagoon of New Caledonia

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Abstract—Humicolone, a new phenolic tetralone in acetal form, was isolated, together with a known phenolic tetralone, from cultures of *Humicola grisea* Traaen, a hyphomycete taken from drifting wood in the southeastern lagoon of New Caledonia. Humicolone, determined from MTPA esters to have absolute configuration 1′*R*,3*S*,4*S*, showed cytotoxicity on KB cell lines, with IC_{50} values between 1 and 5 ppm. Another unidentified filamentous fungus, also from drifting wood in a nearby New Caledonian marine area, gave phosphatidyl cholines and, unusually for fungi, a bisabolane sesquiterpene. © 2002 Published by Elsevier Science Ltd.

1. Introduction

The taxonomy of filamentous fungi is still largely based on morphological and ecological observations, although increasing attention is paid to molecular data in assessing their phylogeny.[1] As concerns fungal species from the sea, the main differentiation is traditionally between marine-adapted and true-marine fungi.[2] In the former group are placed genera also known from land, although it is recognized that full definition is at the species level. Characteristic of the second group are lignicolous species exclusive of the sea.

Secondary metabolites isolated from the culture of fungal strains obtained from the sea have been recently reviewed.[2,3] Since then, growing attention has brought to light many metabolites in all biogenetic classes, mostly already known from terrestrial fungi.

2. Results and discussion

A new phenolic tetralone in acetal form, humicolone (**1**), endowed with KB cytotoxic activity, was isolated alongside a minor, known, phenolic analogue (**2**),[4] from cultures of *Humicola grisea* Traaen, a hyphomycete taken from drifting wood in the Baie of Prony, southeastern lagoon of New Caledonia. From HR-EI-MS measurements, humicolone (**1**) gave the composition $C_{12}H_{12}O_4$, supporting four unsaturations and three cycles. The 1,2,3-trisubstituted benzene moiety was identified from a characteristic ^{1}H NMR ABX pattern. The OR group at C-5 and the (O═C)R group at C-9 were suggested by characteristic patterns of arene proton chemical shifts (*ortho*/*para* shielding by OR and larger *ortho* than *para* deshielding by (O═C)R). The carbonyl group is also supported by the δ_C=194.82 s. Lack of further aromatic signals in the ^{13}C NMR spectrum pointed to the presence of the two saturated rings. C-3 and C-4 in the cyclohexanone ring were revealed as two deshielded signals, δ_C=69.88 d (HMQC coupled to δ_H=4.17 ddd) and δ_C=78.03 d (HMQC coupled to δ_H 4.90 d), respectively, while particularly revealing as to the 3,4-O-disubstitution of the cyclohexanone was the coupling of H-3 with H-4 (9.1 Hz) and the further coupling of H-3 with the two diastereotopic, deshielded, protons at C-2 (HMQC coupled with the δ_C=44.11 t). The acetal ring is supported by δ_H 1.61 q for Me-2′, coupled to the δ_C 20.68 q; this is confirmed by a 5.2 Hz coupling of the C-2′ methyl group with H-1′. These data, and the lack of a signal for loss of water from the molecular ion in both EI and ESI mass spectra, are also compatible with the isomeric structure bearing acetalization at the O–C-3/O–C-4 positions. However, the presence of a free phenolic group was in contrast with the observation of unchanged UV spectra on addition of a stoichiometric amount of KOH. Unequivocal support to structure **1** was finally given by three key

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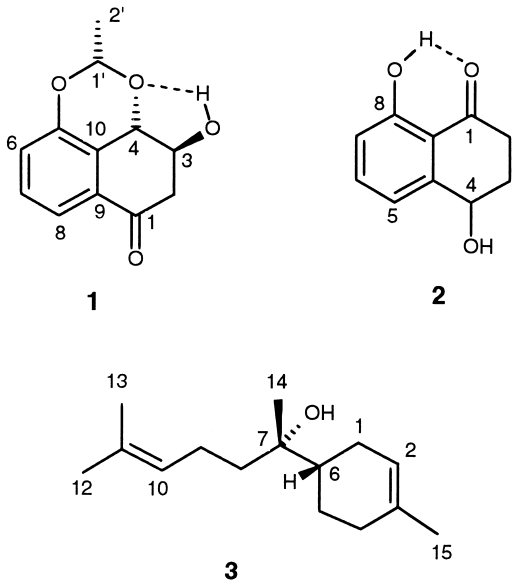

observations: (i) the high-field resonance of the hydroxyl proton (δ=2.75 d) in dried $CDCl_3$, coupled with H-3 (J=0.6 Hz); (ii) a weak heteronuclear coupling (J^3) between C-1′ and the proton at C-4, and (iii) resonance of Me-1′ at δ_H=1.61 d, which is better in accordance with a six-membered than a five-membered acetal ring. Preferential loss of acetaldehyde over water in both EI and ESI mass spectra can be attributed to the extensively conjugated system that is generated (Fig. 1 and Section 4).

Structure **1** fully defines the stereochemistry of humicolone too. The relative configurations at C-3 and C-4 are supported by both the coupling pattern of the cyclohexanone protons, which could be nicely simulated by MM3 calculations (Table 1). Moreover, an intense positive nOe at H-4 on irradiation at H1′ allowed us to establish the relative configuration at C-1′. In agreement, on irradiation at Me-2′, no nOe was evoked. MM3 calculations also suggest that the conformation of **1** with half chair cyclohexanone ring (Fig. 2) is less strained, by as much as 5 kcal mol^{-1}, than the one obtained by flipping the 2,3 bond (C-4 below the paper plane in representation **1**), which would force eclipsing of the two couples of substituents at C-3 and C-4. In the half chair conformation of humicolone, the distance between the hydroxyl proton and O-4 is 2.4 Å, implying a strong hydrogen bond. Appearance of the hydroxyl proton as a sharp d in ^{1}H NMR spectra suggests that this hydrogen bond actually occurs. The absolute configuration **1** for humicolone rests on the homogeneous trend of $\delta_S-\delta_R$ data for MTPA esters (Fig. 3, in a conformation supported by MM3 calculations), showing that the β face of the tricyclic system experiences the diamagnetic shift of the ester phenyl group more strongly than the α face does.

For the minor analogue **2**, previously described in both enantiomeric forms as a phytotoxin,[4] we observed from ^{1}H NMR spectra that the protons at C-3 and C-4 undergo rapid axial/equatorial exchange, corresponding to two lowest-strain conformations, where the protons at C-3 and C-4 are perfectly staggered. Thus, in spite of a strong hydrogen bond between the phenolic proton and the carbonyl oxygen, the C-1/C-8 portion in tetralone **2** is far less rigid than the corresponding C-4/C-5 portion of humicolone (**1**). In the latter, the staggered conformation with C-4 down cannot be attained. MM3 calculations (Table 2) for **2** are in good agreement with these observations.

Antibiotic activity towards *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus* was noticed for the AcOEt extracts from *H. grisea* cultures (Section 4). However, this activity was not due to humicolone (**1**), which showed instead appreciable cytotoxicity on KB cell lines, with IC_{50} values comprised between 1 and 5 ppm.

Another fungus, 793M/212-2, failing to sporulate, defied identification. In culture, unusually for fungi, it gave a bisabolane sesquiterpene, (−)-(6*S*,7*R*)-α-bisabolol (**3**), already known as a plant product,[5] and also obtained by

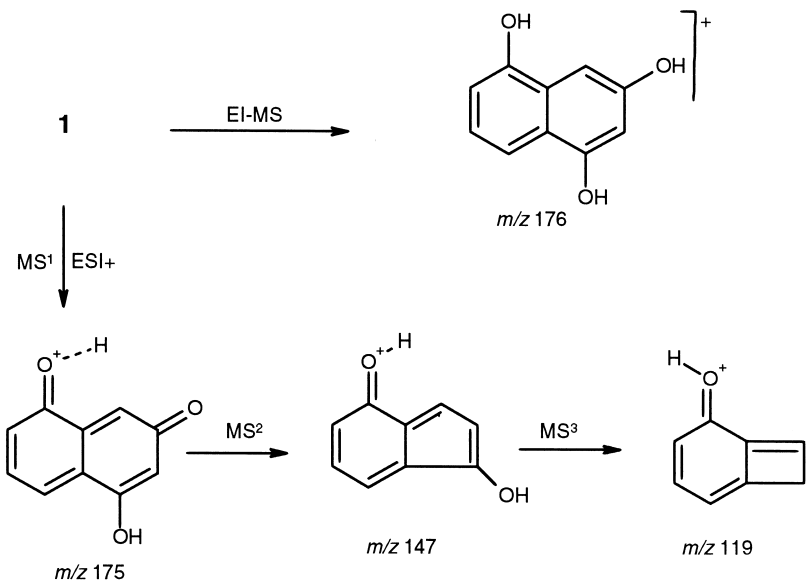

Figure 1. Mass fragmentation pattern of humicolone (**1**) in EI- and ESI ionization methods.

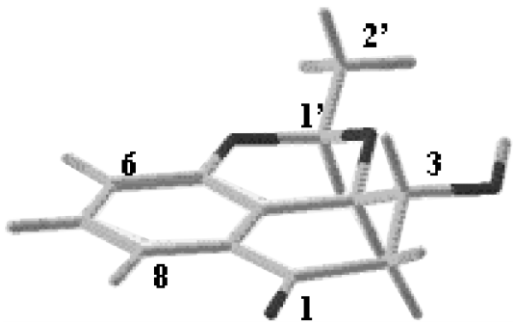

Figure 2. Strain-energy minimized conformation of compound **1**.

Table 1. Experimental and MM3 calculated proton couplings for humicolone **1**

Proton coupling	Experimental value	Calculated value
H2β–H3α	12.2	11
H2α–H3α	5.1	5.4
H3α–H4β	9.1	9

total synthesis.[6] A mixture of phospholipids, mainly comprised of phosphatidylcholines bearing various acyl groups at the glycerol moiety, was also obtained from the culture broth. Although these phosphatidylcholines proved amenable to chromatographic separation, extensive migration and oxidation of C=C double bonds occurred at −20°C in the dark in a few months, before ESIMS experiments could be carried out.

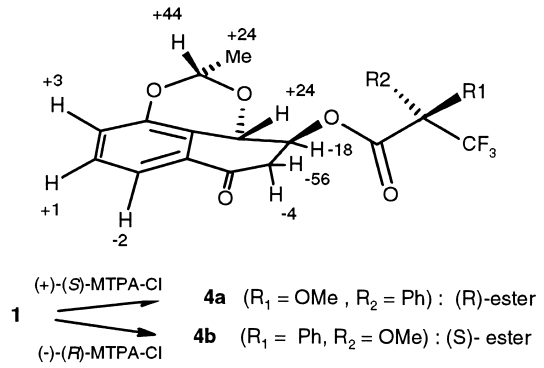

Figure 3. ^{1}H NMR resonance differences (Hz) between (*S*)-MTPA ester **4b** and (*R*)-MTPA ester **4a**.

3. Conclusions

The variability of strains of *H. grisea* Traaen is noticeable. Described also in seawater,[7] *H. grisea*, as a terrestrial soil mould, is known for both cellulases and a macrolide antibiotic identical with radicicol.[8] It has also been reported that mycelial extracts of *H. grisea* show toxic effects against brine shrimps.[9]

The main message from our work is the noticeable cytotoxic activity of humicolone (**1**) against human cancer cell lines. Though of low molecular weight, humicolone bears three chiral centers that stimulate interest in the total synthesis of a variety of diastereomers, hopefully endowed of a better cytotoxic profile than the natural product.

4. Experimental

4.1. General procedures

^{1}H and ^{13}C NMR 1D and 2D spectra were recorded on a Varian XL-300 spectrometer operating at 299.94 and 75.43 MHz, respectively; δ values are given in ppm, with respect to Me_4Si as an internal standard, and J values are given in Hz. ESI-MS experiments were performed in positive ion mode on a Bruker Esquire-LC™ ion trap spectrometer via an electrospray interface as either direct infusion or LC-ESI-MS. In the latter case, it was coupled with a HP Mod. 1100 liquid chromatograph bearing a 250×4 mm column packed with 5 μm Merck LiChrospher RP-18 under UV monitoring at λ=254 nm and solvent flux 1 mL min^{-1}, 7:3 split for UV and ESI detectors. EI-MS experiments were performed with a Kratos MS-80 mass spectrometer, equipped with a home-built computerized data system. Polarimetric data were obtained with a Jasco-DIP-181 apparatus, reporting $[\alpha]_D$ in dm^{-1} deg mL g^{-1}. CD data were obtained from a Jasco-J-710 spectropolarimeter, and give as $\Delta\varepsilon$ (λ) (λ_{max} in nm, ε in mol^{-1} L cm^{-1}). Molecular mechanics calculations were carried out by the computer program PCMODEL 7.0, Serena Software, Bloomington, Indiana.

4.2. Isolation and culturing of the fungi and isolation of the metabolites

H. grisea Traaen (880M/272) was isolated in January 1998 from a deeply parasitized piece of wood, drifting at a depth of 4–5 m in the Baie of Prony, southeastern coast of New Caledonia. The piece of wood was placed in a moist chamber for some days, which allowed isolation of the

Table 2. Experimental and MM3 calculated proton couplings for compound **2**

Proton coupling	Experimental value[a] (Hz)	Calculated value –OH eq.	Calculated value –OH axial
H2α–H3β	8.3	2.5	13.6
H2α–H3α	4.8	3.8	4.2
H2β–H3β	4.8	4.1	4.4
H2β–H3α	8.3	13.6	2.3
H3β–H4β	3.6	4.3	2.4
H3α–H4β	7.3	11.3	3.8

[a] Experimental values show a rapid equilibration between the two calculated conformations.

fungus from the wood core. The culture was carried out on a solidified agar medium made with potato extract (125 g of pressed potatoes with 1 L of seawater, 10 g of glucose, 1MU of penicillin G, 1 MU of colimycin), to which 15 g of agar–agar were finally added. During the culture an aerial white mycelium was formed, which gave rise to a brown–green pigment and abundant black spores. Young colonies showed an aerial white mycelium, next characteristically olivaceous, lighter towards the margin, darker in reverse. Numerous brown, globose aleurioconidia (10–17 μm) were produced after a few days of incubation at 25°C. Rare obovate phialoconidia (3×2 μm) were observed. No growth was obtained at temperatures above 37°C.

The other fungus (793M/212-2) was isolated in August 1997 from drifting wood at the bottom of the Baie de Sainte Marie in a mangrove area in the urban zone of Nouméa, southeastern New Caledonia. Treated and cultured as the other fungus above, it grew up slowly as a gray felt, giving rise to a black pigment. The process was not affected by the addition of cellulose. Neither illumination periods, nor scarification could induce sporulation, which prevented taxonomic identification.

With both fungi, the cultures were carried out repeatedly in the above medium in Erlenmeyer flasks for 4 days at 25°C under orbital shaking at 100 rpm, alternating periods of illumination and dark. With both fungi, the cultured mass was homogenized and filtered, and the filtrate was AcOEt extracted, while the mycelium was extracted first with petroleum ether, then MeOH, and the latter extract was partitioned between CH_2Cl_2 and H_2O. The AcOEt extract of the fifth culture of *H. grisea* was subjected to flash chromatography on Merck Si60 with *n*-hexane/AcOEt gradient elution, collecting five fractions of 30 mL each. Fraction 3 was subjected to preparative HPLC (Merck LiChrosphere, *n*-hexane/*i*-PrOH 91:9, 5 mL min^{-1}) getting humicolone (**1**), t_R=11.0 min, 2.0 mg, and compound **2**, t_R=15.4 min, 0.2 mg. The AcOEt extract of the unidentified 793M/212-2 fungus was subjected to flash chromatography on Merck Si60 with *n*-hexane/AcOEt gradient elution; fraction 7 gave crude (−)-(6*S*,7*R*)-α-bisabolol (**3**), which was further purified by HPLC on a Merck LiChrosphere column with *n*-hexane/*i*-PrOH 95:5, 5 mL min^{-1}, giving pure **3**, t_R=7.6 min, 1.3 mg.

4.2.1. Humicolone (1). UV (MeOH); λ_{max}=207, ε=96000; λ_{max}=253, ε=12000; λ_{max}=310, ε=3700. CD (MeOH): λ_{max}=253, $\Delta\varepsilon$=+5.5, λ_{max}=301, $\Delta\varepsilon$=1.5. $[\alpha]_D^{20}$=+8.1, $[\alpha]_{365}^{20}$=+41 (*c*=0.3, MeOH); δ_H (300 MHz, $CDCl_3$): 7.53 (1H, dd, *J*(8,7)=8.3 Hz, *J*(8,6)=1.2 Hz, H8), 7.31 (1H, dd *J*(7,6)=8.3 Hz, *J*(7,8)=8.3 Hz, H7), 7.04 (1H, dd, *J*(6,7)=8.3 Hz, *J*(6,8)=1.2 Hz, H6), 5.51 (1H, q, *J*(1′,Me)=5.1 Hz, H1′), 4.90 (1H, d, *J*(4β,3α)=9.1 Hz, H4β), 4.17 (1H, dddd, *J*(3α,OH)=0.6 Hz, *J*(3α,2β)=12.1 Hz, *J*(3α,2α)=5.1 Hz, *J*(3α,4β)=9.1 Hz, H3α), 3.08 (1H, dd, *J*(2α,3α)=5.1 Hz, *J*(2β,2α)=17.4 Hz, H2α), 2.75 (1H, d, *J*(OH,3α)=0.6 Hz, *OH*–C(3). 2.68 (1H, dd, *J*(2β,3α)=12.1 Hz, *J*(2β,2α)=17.4 Hz, H2β); 1.62 (3H, d, *J*(Me,1′)=5.1 Hz, Me-1′). nOe enhancements were observed on the Me–1′ and H4β signals on irradiation at 5.51 ppm, on the H-1′ and H2β signals on irradiation at 4.90 ppm, and on the H2α signal on irradiation at 4.17 ppm. EI-MS: 220 (55, $M^{+\cdot}$); 176 (81, $[M-MeCHO]^+$); 147 (22); 134 (100). HR-EI-MS: 220.0732, 0.002 ($C_{12}H_{12}O_4$; calcd 220.0735). ESI-MS (positive ion detection): 221 ($M+H^+$); 243 ($M+Na^+$). MS experiments: 221|175|147|119.

4.2.2. Compound 2 (4,8-dihydroxy-3,4-dihydro-2*H*-naphthalen-1-one). δ_H (300 MHz, $CDCl_3$): 12.42 (1H, s, *OH*–C(8), 7.49 (1H, dd, *J*(6,5)=8.3 Hz, *J*(6,7)=7.5 Hz, H6), 7.01 (1H, dd *J*(7,6)=7.5 Hz, *J*(7,5)=1.2 Hz, H7), 6.92 (1H, dd, *J*(5,6)=8.3 Hz, *J*(5,7)=1.2 Hz, H5), 4.92 (1H, dd, *J*(4β,3α)=7.3 Hz, *J*(4β,3β)=3.6 Hz, H4β), 3.00 (1H, ddd, *J*(2β,3β)=4.8 Hz, *J*(2β,3α)=8.3 Hz, *J*(2β,2α)=17.7 Hz, H2β), 2.65 (1H, ddd, *J*(2α,3β)=8.3 Hz, *J*(2α,3α)=4.8 Hz, *J*(2β,2α)=17.7 Hz, H2α), 2.34 (1H, dddd, *J*(3β,2α)=8.3 Hz, *J*(3β,2β)=4.8 Hz, *J*(3β,4β)=3.6 Hz, *J*(3α,3β)=12.0 Hz, H3β), 2.19 (1H, dddd, *J*(3α,2α)=4.8 Hz, *J*(3α,2β)=8.3 Hz, *J*(3α,4β)=7.3 Hz, *J*(3α,3β)=12.0 Hz, H3α), EI-MS: 178 (30, $M^{+\cdot}$); 160 (5, $[M-H_2O]^+$); 150 (9); 141 (10); 121(26). HR-EI-MS: 178.0637, 0.002 ($C_{10}H_{10}O_3$; calcd 178.0630).

4.2.3. (−)-(6*S*,7*R*)-α-Bisabolol (3). $[\alpha]_D^{20}$=−27.0 (*c*=0.1, MeOH), (lit. $[\alpha]_D^{20}$=−55.7[5]). The ^1H NMR spectra proved superimposable to those for natural (−) bisabolol reported by Schwarz et al.[6] EI-MS: 204 (47, $[M-H_2O]^+$); 161 (19); 121 (34); 119 (93), 109 (100), 95 (36), 93 (52), 69 (94).

4.3. Formation of MTPA esters of 1

4.3.1. (*R*)-MTPA ester 4a. To a solution of **1** (0.3 mg) and a catalytic amount of dimethylaminopyridine in 0.3 mL of dry pyridine were added 5 mol equiv. of (+)-(*S*)-MTPA-Cl. The resulting solution was allowed to stand at room temperature for 24 h and then 0.5 mL of sat. aq. $CuSO_4$ solution were added and the mixture was percolated through a Whatman phase-separation filter. The filtrate was evaporated and the residue subjected to Merck Lichrolut Si60 with *n*-hexane/ethyl acetate gradient elution obtaining pure **4a** (0.3 mg). UV (MeOH): λ_{max}=221, ε=96000; λ_{max}=255, ε=12000; λ_{max}=303, ε=3600. CD (MeOH): $\lambda_{max=}$257, $\Delta\varepsilon$=+4.6); δ_H (300 MHz, $CDCl_3$): 7.30–7.60 (5H, series of m, aromatic H′s), 7.57 (1H, dd, *J*(8,7)=8.3 Hz, *J*(8,6)=1.2 Hz, H8), 7.35 (1H, dd *J*(7,6)=8.3 Hz, *J*(7,8)=8.3 Hz, H7), 7.08 (1H, dd, *J*(6,7)=8.3 Hz, *J*(6,8)=1.2 Hz, H6), 5.62 (1H, ddd, *J*(3α,2β)=12.1 Hz, *J*(3α,2α)=5.2 Hz, *J*(3α,4β)=9.6 Hz, H3α), 5.37 (1H, q, *J*(1′,Me)=5.3 Hz, H1′), 5.18 (1H, d, *J*(4β,3α)=9.6 Hz, H4β), 3.59 (3H, s, O*Me*), 3.18 (1H, dd, *J*(2α,3α)=5.2 Hz, *J*(2β,2α)=17.0 Hz, H2α), 2.77 (1H, dd, *J*(2β,3α)=12.1 Hz, *J*(2β,2α)=17.0 Hz, H2β); 1.52 (3H, d, *J*(Me,1′)=5.3 Hz, *Me*-1′). ESI-MS (positive ion detection): 459 (100%, $M+Na^+$).

4.3.2. (*S*)-MTPA ester 4b. By the same procedure above, using 5 mol equiv. of (−)-(*R*)-MTPA-Cl, 0.3 mg of pure **4b** were obtained. UV (MeOH): λ_{max}=221, ε=96000; λ_{max}=255, ε=12000; λ_{max}=314, ε=3600. CD (MeOH): λ_{max}=254, $\Delta\varepsilon$=+2.0; δ_H (300 MHz, $CDCl_3$): 7.30–7.60 (5H, series of m, aromatic H′s), 7.57 (1H, dd, *J*(8,7)=8.3 Hz, *J*(8,6)=1.2 Hz, H8), 7.35 (1H, dd *J*(7,6)=8.3 Hz,

J(7,8)=8.3 Hz, H7), 7.09 (1H, dd, J(6,7)=8.3 Hz, J(6,8)=1.2 Hz, H6), 5.56 (1H, ddd, J(3α,2β)=12.1 Hz, J(3α,2α)=5.2 Hz, J(3α,4β)=9.6 Hz, H3α), 5.48 (1H, q, J(1′,Me)=5.3 Hz, H1′), 5.18 (1H, d, J(4β,3α)=9.6 Hz, H4β), 3.64 (3H, s, O*Me*), 3.17 (1H, dd, J(2α,3α)=5.2 Hz, J(2β,2α)=17.0 Hz, H2α), 2.58 (1H, dd, J(2β,3α)=12.1 Hz, J(2β,2α)=17.0 Hz, H2β); 1.60 (3H, d, J(Me,1′)=5.3 Hz, *Me*-1′). ESI-MS (positive ion detection): 459 (100%, $M+Na^+$).

4.4. Bioassays

Antimicrobial and cytotoxicity assays (KB) were carried out by standard techniques. The AcOEt extract of *H. grisea* showed antibiotic activity on *S. aureus*, *E. coli*, and *C. albicans*. This activity was not due to **1**, which showed instead cytotoxicity on KB cell lines with IC_{50} values between 1 and 5 ppm.

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