Antifungal Acylcyclopentenediones from Fruiting Bodies of *Hygrophorus chrysodon*

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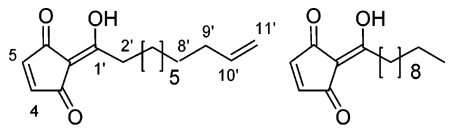
Chrysotriones A (**1**) and B (**2**), two new 2-acylcyclopentene-1,3-dione derivatives, were isolated from the fruiting bodies of the Basidiomycete *Hygrophorus chrysodon*, and their structures were established by spectroscopic data and synthesis of compound **2**. They represent the first examples of 2-acylcyclopentene-1,3-diones found in mushrooms and suggest an unprecedented biosynthetic pathway, starting from fatty acid derivatives. Initial tests indicated interesting antifungal activity of chrysotriones against *Fusarium verticillioides*, one of the common worldwide pathogens of cultivated plants.

In our search for new prototype bioactive agents from higher mushrooms (Basidiomycetes),[1] we examined the chemical contents of *Hygrophorus chrysodon* (Batsch.: Fr.) Fr. (Hygrophoraceae).[2] This is an ectomycorrhizal fungal species, growing solitary, scattered to gregarious in mixed conifer-hardwood woods, in particular in the presence of *Fagus* trees, and fruiting from late fall to mid-winter. It is easily recognized by a white, viscid cap and stipe, both decorated with golden yellow granules, hence the ancient Greek name "chrysodon", and by widely spaced, waxy, white decurrent gills. Our field observations indicated that fruiting bodies of *H. chrysodon* are hardly ever attacked by parasitic fungi in the wild, in accordance with moderate activity of an EtOAc extract against *Fusarium verticillioides* (Sacc.) Nirenberg. This fungal species is a widely distributed mitosporic pathogen, able to cause corn (*Zea mays* L.) seedling blight and root, stalk, ear, and seed rot.[3] Some strains of *F. verticillioides* have also been isolated from rice plants affected by the bakanae disease in Northern Italy.[4] *Fusarium* infection on rice, in addition to its contribution to crop losses, can affect the quality and the health of seeds.[4] Both the ecological observations and the antifungal activity prompted us to look for their chemical basis.

Fungicidal 4-oxo-2-alkenoic fatty acids were recently isolated from *H. discoxanthus* (Fr.) Rea and from *H. eburneus* (Bull.: Fr.) Fr.,[1,5] and related cyclopentenone derivatives were isolated from different *Hygrophorus* species.[6,7] Other secondary metabolites found in extracts of *Hygrophorus* species include common fungal sterols such as ergosterol and oxidized derivatives, volatile monoterpenes,[8] a ceramide,[9] volatile indole derivatives,[10] and the pigments muscaflavine and hygrophoric acid.[11] With the exception of hygrophoric acid,[11] other secondary metabolites of *H. chrysodon* and their biological activities remain unknown.

Extraction of fresh fruiting bodies of *H. chrysodon* was performed with EtOAc at −20 °C, to prevent undesired enzymatic reactions; subsequent antifungal bioassay-guided fractionation of the crude extract by chromatographic separations on reversed-phase C-18 columns afforded chrysotriones A (**1**) and B (**2**), 0.026% and 0.03%, respectively. In addition, ubiquitous fungal long-chain fatty acids and their methyl derivatives were isolated. Compounds **1** and **2** were detected on TLC plates as pale yellow spots, UV active, exhibiting a yellow-orange color after spraying with a sulfovanillin solution followed by heating. The UV spectra of **1** and **2** were almost superimposable, showing an intense absorption band near 270 nm attributable to a $\pi \rightarrow \pi^*$ transition of a conjugated carbonyl group. The presence of unsaturated carbonyl groups in the two structures was further corroborated by intense absorption peaks near 1655 and 1615 cm^{-1} in the IR spectra.



1 **2**

The negative ESIMS of **1** showed a pseudomolecular ion at *m*/*z* 261 $[M - H]^-$, which, in accordance with carbon and proton counting from the NMR spectra, corresponded to the molecular formula $C_{16}H_{22}O_3$. In the upfield portion of the ^1H NMR spectrum of **1**, the sp^3 methylene proton resonances comprised a broad signal at δ 1.20−1.30, integrating for 10H, a distorted triplet (J = 7.0 Hz) for 2H at δ 2.81, a quintuplet (J = 7.0 Hz) for 2H at δ 1.68, and a distorted quartet (J = 7.0 Hz) for 2H at δ 2.07. A terminal double bond was identified by the signals from the three spin system at δ 5.86 (1H, ddt, J = 17.2, 10.1, 7.0 Hz), 4.92 (1H, dtd, J = 17.2, 1.8, 1.5 Hz), and 5.02 (1H, dtd, J = 10.1, 1.8, 1.5 Hz), while two quaternary carbons at δ 183.3 and 104.7 in the ^{13}C NMR spectrum were attributed to an enol moiety. These signals, along with consistent COSY and HMBC spectra, revealed that compound **1** contained an unbranched methylene chain ($-C_8H_{16}-$) attached to the terminal vinyl group on one side and to the enol unit at the other end.

Two olefinic proton signals at δ 6.88 (1H, d, J = 6.1 Hz) and 6.98 (1H, d, J = 6.1 Hz) showed correlation with carbon signals at δ 146.4 and 142.3, respectively, in the HSQC spectrum; in addition, they both displayed long-range correlations with two ketone carbonyls at δ 201.9 and 192.7 and with the enol carbon at δ 104.7 in the HMBC spectrum. On the whole, the spectroscopic data of chrysotrione A (**1**) were thus consistent with the structure of a 1,3-cyclopentenedione unit linked at C-2 to the enol-alkyl chain. The strong intramolecular H-bond resulting from chelation between the enolic proton and one carbonyl was indicated by a broad signal at about δ 12.5, exchangeable with D_2O, in the ^1H NMR spectrum of **1**, and by a broad band extending from 3500 to 2800 cm^{-1} in the IR spectrum.

2-Acylcyclopent-4-ene-1,3-diones of type **1** show an interesting series of tautomeric enol−enol equilibria, which have been described in seminal studies by Forsén and collaborators.[12] In summary, NMR studies have suggested the presence of four different equilibrating tautomeric enols, **a**−**d** (Figure 1).[13,14] The interconversion between the "internal" tautomers **a** ⇌ **b** and **c** ⇌ **d** is a relatively fast process on the NMR time scale at room

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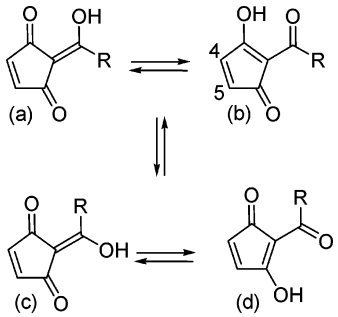

Figure 1. Tautomerism in 2-acylcyclopent-4-ene-1,3-dione.

Table 1. Antifungal Activity of Chrysotriones A and B against *Fusarium verticillioides*, Compared with Ciclopirox[a]

compound	50 μg	100 μg	300 μg
1	3	10	35
2	3	15	18
ciclopirox	25		

[a] Diameter of the inhibition areas (mm) in the plate diffusion assay (50 to 300 μg of tested compound on 6 mm filter disk).

temperature, causing no splitting of the NMR signals. By contrast, the interconversion between "external" tautomers **a**,**b** ⇌ **c**,**d** is a comparatively slow process and gives rise to the NMR nonequivalence of the protons and carbons in positions 4 and 5. However, acceleration of the interconversion of identical "external" tautomers eliminates the NMR nonequivalence and leads to the signal averaging.

In accordance with this behavior, we definitely assigned structure **1** to chrysotrione A, since the AB quartet attributed to the two cyclopentene protons in the ^{1}H NMR spectrum nicely collapsed to a narrow singlet on addition of a trace of Et_3N. Similarly, the signals of the two carbonyl carbons of **1** coalesced to a slightly broad peak at δ 197.3 on addition of the base, due to the almost complete averaging of the spectrum.

The structure of chrysotrione B (**2**) was then easily derived from that of **1**, taking into account the pseudomolecular ion peak at *m*/*z* 263 [M − H]$^-$ in the negative ion ESIMS, corresponding to the molecular formula $C_{16}H_{24}O_3$, and the absence of the signals of the terminal propenyl unit in the NMR spectra, which were replaced by those of a terminal propyl group. The remaining ^{1}H and ^{13}C NMR signals were almost superimposable to those of **1**, proving the structure **2** for chrysotrione B.

To have larger amounts for biological tests, compound **2** was synthesized in 16% yield by condensation of 4-cyclopentene-1,3-dione with undecanoyl chloride in the presence of excess anhydrous $AlCl_3$.

The results of the in vitro antifungal activity of **1** and **2** against *Fusarium verticillioides* are reported in Table 1. The test was carried out using the agar diffusion method, which involved the addition of the sample to agar plates that were inoculated with the test organism by means of impregnated paper disks;[15] ciclopirox, a well-known broad-spectrum antifungal agent, was used as the reference compound. Compounds **1** and **2** both showed similar moderate antifungal activity, roughly one-sixth that of cyclopirox.

A handful of naturally occurring acylcyclopentenediones related to chrysotriones **1** and **2** are known, and their distribution is limited to a few plant species.[16−21] Chrysotriones represent, therefore, the first examples of this class of secondary metabolites found in mushrooms. Different from **1** and **2**, plant cyclopentenediones are, however, mono- or disubstituted on the cyclopentene double bond by methoxy or methyl groups, reflecting the supposed biogenetic derivation from the appropriate acylphloroglucinols through a ring-contraction reaction.[22,23] Only aliphatic intermediates seem, instead, to be involved in the biogenesis of chrysotriones. The structural

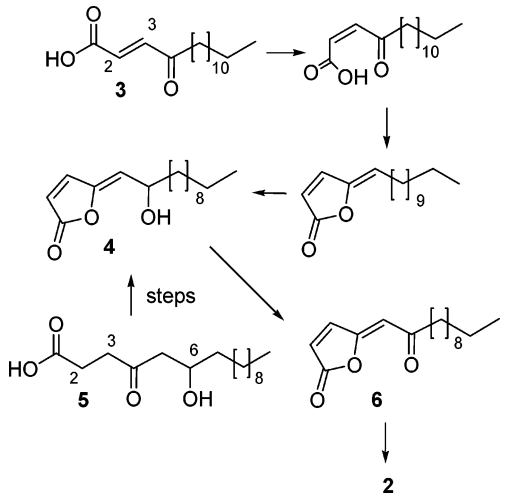

Figure 2. Hypothetical biogenetic pathway to chrysotrione B (**2**).

similarities of compounds **1** and **2** with the antibiotics pentenomycins may indicate a 1,4-α-glucan as precursor of the cyclopentenone moiety, as one referee has suggested; however, **1** and **2** more likely derive from oxidation of a C_{16} fatty acid. Actually, they are strictly related to 4-oxo-2-alkenoic fatty acids and hygrophorones recently isolated from various *Hygrophorus* species,[1,5−7] which suggests a possible biogenetic pathway (Figure 2). Thus, the known (*E*)-4-oxohexadec-2-enoic acid (**3**),[5] although not found in *H. chrysodon*, might well be the precursor of **2** through a series of simple condensation and oxidation steps. On the other hand, butenolide **4** is still unknown, but hygrophorone G,[12] the corresponding C_{18} homologue, has been isolated from *H. personii*.[6] Alternatively, oxidation of the C-6 position of the chain may precede desaturation at C-2/C-3, as suggested by the oxidation pattern of acid **5**, recently isolated from *H. discoxanthus*.[1] Moreover, the hypothetical rearrangement of 4-ylidenebutenolides of type **6** to **2** has been realized in vitro, albeit under strong basic conditions.[24,25]

Examining the literature on the whole, one can conclude that each *Hygrophorus* species is characterized by its own pattern of oxidized C_{16}−C_{22} fatty acid derivatives, which, therefore, can be considered significant chemotaxonomic markers. Moreover, thanks to their antifungal and bactericidal properties,[1,5,6] they likely function as "chemical deterrents" constituting a chemical defense system that protects *Hygrophorus* fruiting bodies against parasites and predators.

Experimental Section

General Experimental Procedures. IR spectra were recorded on an FT-IR Perkin-Elmer Paragon 1000 PC spectrometer as neat films on NaCl disks. UV spectra were obtained in spectrometer grade $CHCl_3$ from a Jasco V-550 spectrophotometer. ^{1}H and ^{13}C NMR spectra were determined in CD_2Cl_2 on a Bruker CXP 300 spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C), respectively. ^{1}H and ^{13}C chemical shifts (δ, ppm) are relative to residual $CHCl_3$ signals [δ_H 7.26; δ_C (central line of t) 77.1, respectively]. 2D NMR spectra (COSY, HSQC, HMBC) were recorded by using standard pulse sequences. Coupling constants (*J*) are reported in Hz. ESIMS experiments were carried out using a Finnigan LCQ Advantage MS 1.4 spectrometer, equipped with the Xcalibur 1.4 software. High-resolution ESI mass spectra were determined on a Bruker Apex II FT-ICR mass spectrometer. TLC was performed on sheets precoated with silica gel F_{254} (Polygram) or with RP-18 F_{254} (Merck, Germany). Compounds were visualized under UV light (254 and 366 nm) and by spraying with a 0.5% solution of vanillin in H_2SO_4−EtOH (4:1), followed by heating. Preparative column chromatography was carried out on LiChroprep RP-18 (25−40 μm, Merck). Reagent grade solvents, redistilled just before use, were employed for extraction; HPLC grade solvents were used for chromatographic separations. 4-Cyclopentene-1,3-dione and undecanoyl chloride were purchased from Aldrich. Ciclopirox was obtained from

A/S Rosco, Taastrupgaardsvej 30 DK-2630 Taastrup, Denmark. A strain of *Fusarium verticillioides* (Sacc.) Nirenberg (strain PAT VEG 18 F, Mycology Section, University of Pavia, Italy) was isolated from a rice plant cv. Augusto, cultivated in Vercelli (Italy).

Fungal Material. Fresh fruiting bodies of *H. chrysodon* (Batsch.: Fr.) Fr. were collected in October 2005 in a mixed conifer and beech woods near Brallo, in the province of Pavia, Italy, at an altitude of 1050 m. The mushroom was identified by one of the authors (M.C.), and a frozen voucher specimen (HC-1) has been deposited at the Dipartimento di Chimica Organica, University of Pavia, Italy.

Extraction and Isolation. Fruiting bodies (57 g) were frozen at −20 °C in the same day of collection, minced, and extracted with EtOAc (3 × 0.5 L) for 1 h at −20 °C. The light yellow EtOAc solution was dried with Na_2SO_4 and concentrated to dryness in vacuo at 25 °C to produce an oily residue (323 mg), which was separated by column chromatography on a RP-18 column (50 g). Elution was performed with a gradient of MeOH−H_2O, starting from a 6:1, v/v, mixture and increasing MeOH regularly every 50 mL, until a final mixture of 8:1 v/v. Eight fractions (A1−A8) of 25 mL each were collected. The column was then washed with MeOH (100 mL) to give fraction A9. Fractions A3 and A4 were pooled together, and after evaporation, the residue (35.5 mg) was further separated on a RP-18 column (30 g), eluted with a gradient of MeOH−H_2O as above, to give 10 fractions of 10 mL each. Evaporation of fraction B4 afforded 15 mg of pure chrysotrione A (**1**). Evaporation of fraction A5 afforded 17 mg of pure chrysotrione B (**2**). Almost pure linoleic acid (47 mg), oleic acid (38 mg), and methyl linoleate (16 mg) were obtained by evaporation of fractions A6, A7, and A8, respectively.

Chrysotrione A (1): pale yellow crystals; mp 38−40 °C; UV (EtOH) λ_{max} (log ϵ) 229 (3.92), 271 (4.21) nm; IR (KBr pellet) 3076, 2927, 2855, 1721, 1657, 1618, 1206, 857 cm^{-1}; ^1H NMR (CD_2Cl_2, 300 MHz) δ 12.5 (1H, br, OH-1′), 6.98 (1H, d, J = 6.1 Hz, H-5), 6.88 (1H, d, J = 6.1 Hz, H-4), 5.86 (1H, ddt, J = 17.2, 10.1, 7.0 Hz, H-10′), 5.02 (1H, dtd, J = 17.2, 1.8, 1.5 Hz, H-11′*Z*), 4.92 (1H, dtd, J = 10.1, 1.8, 1.5 Hz, H-11′*E*), 2.81 (2H, t, J = 7.0 Hz, H_2-2′), 2.07 (2H, q, J = 7.0 Hz, H_2-9′), 1.68 (2H, quint, J = 7.0 Hz, H_2-3′), 1.20−1.50 (10H, m, H_2-4′-H_2-8′); ^{13}C NMR (CD_2Cl_2, 75 MHz) δ 201.9 (C, C-1), 192.7 (C, C-3), 183.3 (C, C-1′), 146.4 (CH, C-4), 142.3 (CH, C-5), 140.1 (CH, C-10′), 114.6 (CH_2, C-11′), 104.7 (C, C-2), 34.5 (CH_2, C-9′), 32.3 (CH_2, C-2′), 30.0, 30,0, 29.9, 29.8, 29.7 (5 × CH_2, C-4′−C-8′), 27.1 (CH_2, C-3′); negative HRESIMS *m*/*z* 261.1494 $[M - H]^-$ (calcd for $C_{16}H_{21}O_3^-$, 261.1491).

Chrysotrione B (2): yellow crystals; mp 39−41 °C; UV (EtOH) λ_{max} (log ϵ) 225 (4.12), 268 (4.22) nm; IR (KBr pellet) 3172, 2955, 2918, 2849, 1708, 1653, 1607, 1165, 855 cm^{-1}; ^1H NMR (CD_2Cl_2, 300 MHz) δ 12.5 (1H, br, OH-1′), 6.97 (1H, d, J = 6.1 Hz, H-5), 6.87 (1H, d, J = 6.1 Hz, H-4), 2.81 (2H, t, J = 7.0 Hz, H_2-2′), 1.68 (2H, quint, J = 7.0 Hz, H_2-3′), 1.20−1.50 (14H, m, H_2-4′−H_2-10′), 0.91 (3H, t, J = 7.5 Hz, H_3-11′); ^{13}C NMR (CD_2Cl_2, 75 MHz) δ 201.9 (C, C-1), 192.7 (C, C-3), 183.3 (C, C-1′), 146.4 (CH, C-4), 142.3 (CH, C-5), 104.7 (C, C-2), 32.7 (CH_2, C-9′), 32.3 (CH_2, C-2′), 30.3, 30.2, 30.1, 30.0, 29.9 (5 × CH_2, C-4′−C-8′), 27.1 (C-3′), 23.4 (CH_2, C-10′), 14.6 (CH_3, C-11′); negative HRESIMS *m*/*z* 263.1649 $[M - H]^-$ (calcd for $C_{16}H_{23}O_3^-$, 263.1647).

Synthesis of Chrysotrione B (2). Dry $AlCl_3$ (1 g, 7.5 mmol) was added in one portion to 4-cyclopentene-1,3-dione (300 mg, 3.1 mmol) dissolved in anhydrous CH_2Cl_2 (20 mL) at rt under an argon atmosphere. To the resulting whitish suspension was added, via canula, undecanoyl chloride (1.06 g, 5.2 mmol) in anhydrous CH_2Cl_2 (15 mL). The reaction mixture slowly became strongly red colored. After 4 h of stirring, the reaction mixture was poured into a saturated aqueous solution of sodium/potassium tartrate (40 mL). The organic phase was separated, and the aqueous layer was extracted with Et_2O (4 × 50 mL). The combined organic phases were dried over Na_2SO_4 and evaporated to afford an oily residue (1.5 g), which was separated by column chromatography on a RP-18 column (50 g). Elution with a mixture of MeOH−H_2O, 6:1 v/v, gave a sample (132 mg, 16%) identical (TLC mobility, IR, ^1H and ^{13}C NMR spectra) with the natural chrysotrione B (**2**).

Antifungal Assay. Antifungal activity was evaluated against a strain of *Fusarium verticillioides* (Sacc.) Nirenberg using the agar diffusion method according to a procedure described in the literature.[15] Ciclopirox was employed as the compound for positive control.

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References and Notes

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