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New Oxidized 4-Oxo Fatty Acids from *Hygrophorus discoxanthus*

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Dedicated to the memory of Professor Ivano Morelli.

The results are reported from the first investigation of the secondary metabolites of the basidiomycete *Hygrophorus discoxanthus* (Fr.) Rea. Five new oxidized 4-oxo fatty acids (C_{16}, C_{18}) were isolated from the fruiting bodies and their structures established on the basis of their spectroscopic data and an ozonolysis experiment. Preliminary data indicate a moderate fungicidal activity, suggesting a possible function of these acids as chemical deterrents against mushroom parasites and predators.

Keywords: *Hygrophorus discoxanthus*, Basidiomycetes, 4-oxo-fatty acids, fungicidal activities.

In a search for new prototype (bioactive) agents from higher mushrooms (Basidiomycetes) [1], we were attracted by the species *Hygrophorus discoxanthus* (Fr.) Rea (fam. Hygrophoraceae) [2]. This is a mycorrhizal fungal species, growing solitary, scattered to gregarious in hardwood forests, particularly in the presence of *Fagus* trees, and fruiting in the fall. It is easily recognized by a whitish, viscid cap, with an ochreous-brown border, hence the name, and by the widely spaced, cream colored decurrent gills, turning rust-colored on rubbing. Our own field observations revealed that the fruiting bodies of *H. discoxanthus* are hardly ever attacked by either insects or parasitic fungi.

Fungicidal 4-oxo-2-alkenoic fatty acids were recently isolated from *H. eburneus* (Bull.: Fr.) Fr. [3], and related cyclopentenone and cyclopentenedione derivatives were found in the extracts of various *Hygrophorus* species [4,5]. In addition to the common fungal sterol ergosterol and derivatives, the aroma components of various *Hygrophorus* species were investigated by GC-MS [6]; a ceramide was reported from a Chinese *Hygrophorus* species [7], malodorous indole derivatives were isolated from *H. paupertinus* A. H. Smith & Hesler [8], while muscaflavine and hygrophoric acid were identified as pigments of some *Hygrophorus* fruiting bodies [9]. No investigation of the secondary metabolites of *H. discoxanthus* has yet appeared in the literature. Along with the ecological observations, this prompted a study of the chemical constituents of this mushroom.

To prevent undesired enzymatic reactions, the fresh fruiting bodies were frozen after collection and extracted with EtOAc at –20°C. The crude extract was subsequently partitioned between *n*-hexane and MeCN, and the residue from the more polar layer was separated by chromatography on multiple reverse-phase C-18 columns to give acids **1**-**5**. Remarkably, these compounds exhibit brown-ochreous spots on C-18 TLC-plates sprayed with a sulfovanillin solution, followed by heating, and are thus well differentiable from the fungal ubiquitous oleic and linoleic acids, and methyl linoleate, of similar chromatographic polarity, which are detected as purple spots with the same reagent. In addition, TLC-spots of compounds **1** and **2** respond to UV light (fluorescence quenching at 254 nm).

Acids **1-5** (C_{16} or C_{18}) can be divided between those (**1-2**) presenting an *δ,ε*-unsaturated *γ*-oxocrotonate partial structure and those (**3-5**) containing a chetol system (Figure 1). Additionally, some compounds possess either an internal *Z*-configured double bond or a terminal one. Compounds **3-5** are optically active. Acid **1** was obtained as a whitish sticky solid. The UV spectrum showed an intense absorption band at λ_{max} = 234 nm (Log ε = 4.34) attributable to a $\pi \rightarrow \pi^*$ transition of a conjugated keto group, which was corroborated by an intense absorption peak at about 1666 cm^{-1} in the IR spectrum. On the other hand, an IR broad band extending from 3600 to 2800 cm^{-1}, along with a strong band at 1693 cm^{-1} revealed the presence of an unsaturated carboxylic acid. These attributions were firmly confirmed by the signals at δ 170.2 and δ 188.1 in the ^{13}C NMR spectrum of **1**, belonging to an unsaturated carboxylic group and an unsaturated carbonyl group, respectively. The negative ion ESI mass spectrum showed an ion at *m/z* 291 $[M-H]^-$ which, in accordance with data obtained from the NMR spectra, corresponded to the molecular formula $C_{18}H_{28}O_3$.

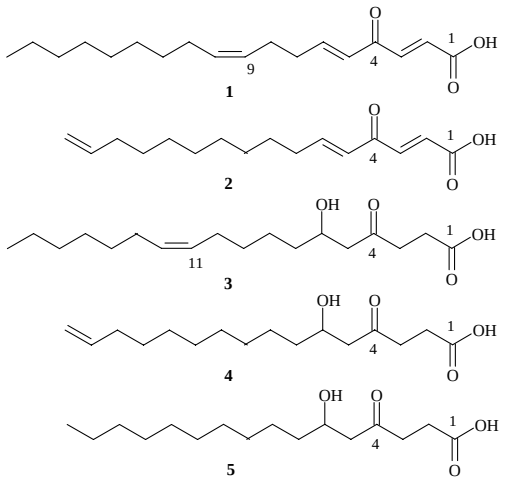

Figure 1: Acids **1-5** isolated from *Hygrophorus discoxanthus*.

The upfield portion of the ^1H NMR spectrum of compound **1** was typical of a long chain unsaturated fatty acid, as indicated by the distorted triplet at δ 0.88, integrating for 3H, attributable to the ω1 methyl group, a broad signal at δ 1.10–1.45, integrating for 12H, assignable to the ω2–ω7 methylene protons, and a distorted quartet at δ 2.05 typical of an allylic methylene group (C-11). COSY and HMBC (Figure 2) correlations proved that this group was linked to a 1,2-disubstituted double bond, whose carbon signals were found at δ 127.1 and 131.6, respectively. The corresponding protons resonated as well separated doublets of triplets at δ 5.30 and δ 5.42, respectively, and showed a mutual vicinal coupling constant of 10.3 Hz, indicative of a *Z*-configured double bond. Comparison of these data with the literature [10] showed that the structure of compound **1** corresponds, from C-9 to C-18, to that of oleic acid. The remaining eight carbons were assembled as a *δ,ε*-unsaturated γ-oxocrotonate unit, attached to C-9 by a C_2 linker, on the basis of the following NMR information. The proton doublets at δ 6.75 and δ 7.48 (1H each, J_{AB} = 15.7 Hz), which showed HSQC correlations with the carbon signals at δ 129.8 and δ 139.7, respectively, and HMBC correlations (Figure 2) with the signals at δ 170.2 and δ 188.1, indicated an *E*-configured double bond positioned between the carboxylic and the carbonyl group. The carbon signal of the ketone displayed additional HMBC cross peaks with two other olefinic methine resonances at δ 6.39 and δ 7.06 (1H each, vicinal coupling J_{AB} = 15.9 Hz) constituting an *E*-configured double bond, which was joined to C-9 through a CH_2CH_2 group. These two methylenes gave rise to two, well-resolved distorted quartets at δ 2.27 (H_2-8) and δ 2.38 (H_2-7), respectively, which showed two and three bond HMBC correlations with both C-6 and C-9 (Figure 2).

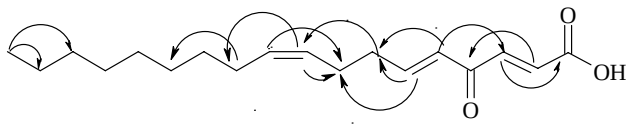

Figure 2: Selected HMBC correlations of compound **1**.

The spectral data of compound **2** were closely related to **1**, the most significant difference being the lack of evidence for an internal non-conjugated double bond. In fact, the UV absorption band at λ_{max} = 235 nm, along with the IR peaks at 1690 and 1664 cm^{-1}, and the almost superimposable patterns of the ^1H- and ^{13}C NMR signals for the C(1)-C(6) moiety clearly proved that acid **2** contains the same *E,E*-configured *δ,ε*-unsaturated *γ*-oxocrotonate unit as compound **1**. From the mass spectral data, the length of the fatty acid chain in compound **2** could be determined as C_{16}, while the terminal double bond was identified by the signals from the three spin system at δ 5.83 (1H, *ddt*, *J* = 17.0, 10.3, 6.7 Hz), δ 4.95 (1H, *dtd*, *J* = 10.3, 1.8, 1.5 Hz), and δ 5.02 (1H, *dtd*, *J* = 17.0, 1.8, 1.5 Hz).

The molecular formula $C_{18}H_{32}O_4$ of compound **3** was deduced from the ion at *m/z* 311 $[M-H]^-$ in the negative ion ESI spectrum, combined with the

protons and carbons counted from the NMR spectra. Remarkably, the ^{1}H NMR spectrum of **3** did not contain the characteristic signals of the cross conjugated dienone system of **1** and **2**; instead, three overlapping multiplets, each integrating for 2H, were found between δ 2.60-2.82, and were attributed to three different methylene groups adjacent to either saturated carbonyl or carboxylic groups. This assignment was confirmed by the resonances at δ 177.4 and 209.7 in the ^{13}C NMR spectrum, attributed to the carbons of a carboxylic acid and a saturated ketone, respectively. In addition, a broad multiplet at δ 4.10 (1H), which was correlated to a carbon at δ 67.7 in the HSQC spectrum, was firmly assigned to a secondary alcohol. The presence of an internal, non-conjugated, disubstituted olefin was demonstrated by an end absorption band at λ_{max} = 218 nm in the UV spectrum, along with the ^{13}C NMR signals of two methines at δ 129.3 and 130.1, which were correlated to an NMR signal at δ 5.25-5.45 in the HSQC spectrum. The AB coupling constant of 10.5 Hz of these two protons proved the *Z*-configuration of the double bond. A homonuclear COSY experiment, and two and three bonds HMBC correlations (Figure 3) allowed establishment of the 1,4-relationship of the carboxylic group with the ketone, and the 1,3-relationship of the hydroxyl and carbonyl groups.

NMR data alone left the position of the internal double bond undetermined. Therefore, compound **3** was exposed to ozone and, after work-up, the crude reaction mixture was directly subjected to GC analysis. Comparison with an authentic sample revealed heptanal to be formed by ozonolysis of olefin **3**. From all results, the structure of compound **3** was established as (*Z*)-6-hydroxy-4-oxo-octadec-11-enoic acid.

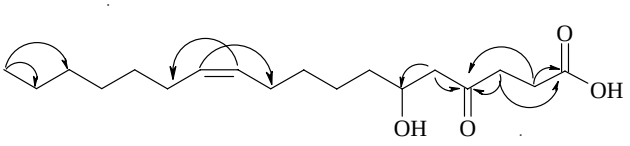

Figure 3: Selected HMBC correlations of compound **3**.

The NMR data of compounds **4** and **5** were closely related to **3** as regards to the 6-hydroxy-4-oxo-carboxylic acid [C(1)–C(6)] unit. In contrast, other than compound **3**, the acids **4** and **5** did not show the signals of an internal double bond. Instead, in the ^{1}H NMR spectrum of compound **4**, the pattern of signals from a three spin system at δ 4.95, 4.99, and 5.83, almost identical to that of acid **2** (see above) were due to a terminal double bond. On the other hand, compound **5** contains a fully saturated fatty acid-like chain, as indicated, in the ^{1}H NMR spectrum, by the characteristic distorted triplet (J = 6.8 Hz) at δ 0.88, assigned to the terminal methyl group, and by a broad peak at δ 1.20-1.60, assigned to the methylenes in the chain. From the mass spectral data, the length of the chain in both compounds **4** and **5** could be determined as C_{16}, thus permitting assignment of the structure of 6-hydroxy-4-oxo-hexadec-15-enoic acid to **4**, and of 6-hydroxy-4-oxo-hexadecanoic acid to **5**.

The absolute configuration of carbinols **3**-**5** has yet to be determined. Compounds **1**-**5** have never been isolated from a natural source; acid **5** was obtained previously as a racemate by synthesis [11].

A preliminary qualitative test indicated that acids **1** and **2** are moderately fungicidal against the phytopathogenic fungus *Cladosporium cucumerinum* Ell. et Arth..

The structures **1**-**5** are closely related to other oxidized C_{16}-C_{22} fatty acids and their derivatives recently isolated from a few *Hygrophorus* species [3-5], for which hypothetical biogenetic relationships have been proposed [3,5]. A rare feature of all these structures is the oxidation to a ketone of the C-4 of the parent fatty acid; a few compounds show an additional site-specific oxidation at C-6, which the optically active alcohols **3**-**5** indicate to occur under enzyme control. Indeed, 6-hydroxy-4-oxo-carboxylic acids like **3**-**5** are, to our knowledge, unprecedented in nature. They can be considered advanced biogenetic precursors of hygrophorones F[12] and G[12] [4a]. Examining the literature data, it was concluded that each *Hygrophorus* species is characterized by its own pattern of oxidized C_{16}-C_{22} fatty acid derivatives, which may thus be considered a significant chemotaxonomic marker. Moreover, due to the fungicidal and bactericidal properties [3-5], these metabolites likely function as “chemical deterrents”, protecting *Hygrophorus* fruiting bodies against the attack of parasites and predators.

Experimental

General experimental procedures: Optical rotations were determined on a Perkin-Elmer 241 polarimeter; IR spectra were recorded on an FT-IR Perkin Elmer Paragon 1000 PC spectrometer as neat films on NaCl

discs. UV spectra were obtained in spectrometer grade $CHCl_3$ from a Jasco V-550 spectrophotometer. ^1H and ^{13}C NMR spectra were determined in $CDCl_3$ on a Bruker CXP 300 spectrometer operating at 300 MHz (^1H) and 75 MHz (^{13}C), respectively. ^1H 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. The abbreviation s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad are used throughout; 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 either 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. GC analysis was performed with a Perkin Elmer Autosystem gaschromatograph.

Fungal material: Fresh fruiting bodies of *Hygrophorus discoxanthus* (Batsch.: Fr.) Fr. were collected on 16 October 2005 in a mixed conifer and beech wood 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 has been deposited at the Dipartimento di Chimica Organica, University of Pavia, Italy.

Extraction and isolation: Fruiting bodies (750 g) were frozen at –20 °C, minced, and extracted at –20 °C with EtOAc (3 x 1.5 L), followed by MeOH–H_2O (4:1, 1 L), and H_2O (1 L) at 0°C. The light yellow EtOAc solution was concentrated to dryness *in vacuo* at <30 °C to produce an oily residue (2.1 g), which was partitioned between MeCN (0.5 L) and *n*-hexane (0.5 L). Evaporation of the two layers gave crude residues of 1.02 g and 1.08 g, respectively. Acids **1**-**5** were contained in the MeCN extract (TLC: R_f = 0.55-0.70; RP-18 F_{254}, solvent system: MeCN-H_2O, 7:1 v/v), which was subjected to column chromatography on a LiChroprep RP-18 column (100 g). Elution was performed with a gradient of MeCN-H_2O, starting from a mixture 1:1, v/v, and increasing MeCN regularly every 100 mL, until a final mixture of MeCN-H_2O, 10:1, v/v. The column was then washed with MeCN (100 mL), followed by Me_2CO (100 mL). Thirty-four fractions (A1-A34), of 35 mL each, were collected. Fraction A9 gave acid **4** (12 mg, 1.6 10^{-3} % of fresh fruiting bodies), fraction A13 gave acid **5** (25 mg, 3.2 10^{-3} %), and fraction A14 afforded acid **3** (34 mg, 4.6 10^{-3} %). Linoleic acid (36 mg, 4.8 10^{-3} %), oleic acid (107 mg, 14 10^{-3} %), and methyl linoleate (37 mg, 4.9 10^{-3} %) were obtained by evaporation of fractions A23, A25, and A27, respectively. Fraction A15 (76 mg) was further separated on a LiChroprep RP-18 column (20 g) eluted with a gradient of MeCN-H_2O, starting from a mixture 1:1, v/v, and increasing MeCN regularly every 50 mL, until a final mixture of MeCN-H_2O, 10:1, v/v. Thirteen fractions (B1-B13), of 40 mL each, were collected. Acid **2** (10 mg, 1.4 10^{-3} %) was isolated by evaporation of fraction B7. Fractions A19 and A20 were pooled together and the residue (110 mg) was further separated on a LiChroprep RP-18 column (20 g) eluted with a gradient of MeCN-H_2O, starting from a mixture 1:1, v/v, and increasing MeCN regularly every 50 mL, until a final mixture of MeCN-H_2O, 10:1, v/v; 14 fractions (C1-C14), each of 35 mL, were collected. Fraction C5 (61 mg) afforded compound **1** (10 mg, 1.4 10^{-3} %) on successive separation on a LiChroprep RP-18 column (15 g) eluted with MeOH-H_2O, 4:1, v/v.

(2*E*, 5*E*, 9*Z*)-4-Oxo-octadeca-2,5,9-trienoic acid (**1**)

Whitish sticky solid.
Rf: 0.45 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-2800, 3090, 3050, 2920, 2852, 1693, 1666, 1613, 1278, 1216, 1000, 975, 950 cm^{-1}.
UV/Vis λ_{max} ($CHCl_3$) nm (log ε): 234 (4.34).
^1H NMR: 0.88 (3H, t, *J* = 6.8 Hz, Me), 1.10-1.45 (12H, brs H_2-12–H_2-17), 2.05 (2H, q, *J* = 6.8 Hz, H_2-11), 2.27 (2H, distorted q, *J* = 7.0 Hz, H_2-8), 2.38 (2H, distorted q, *J* = 7.0 Hz, H_2-7), 5.30 (1H, dd, *J* = 10.3, 6.5 Hz, H-9), 5.42 (1H, dd, *J* = 10.3, 6.5 Hz, H-10), 6.39 (1H, d, *J* = 15.9 Hz, H-5), 6.75 (1H, d, *J* = 15.7 Hz, H-3), 7.06 (1H, dt, *J* = 15.9, 6.7 Hz, H-6), 7.48 (1H, d, *J* = 15.7 Hz, H-2).
^{13}C NMR: 13.9 (CH_3, C-18), 22.5 (CH_2, C-17), 25.5 (CH_2, C-8), 27.2 (CH_2, C-11), 29.2, 29.3, 29.4, 29.5 ($4CH_2$, C-12, C-13, C-14, C-15), 31.6 (CH_2, C-16), 32.8 (CH_2, C-7), 127.1 (CH, C-9), 129.4 (CH, C-5),

129.8 (CH, C-3), 131.6 (CH, C-10), 139.7 (CH, C-2), 150.8 (CH, C-6), 170.2 (C, C-1), 188.1 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{18}H_{27}O_3$ 291.1960, found 291.1962.

(2*E*, 5*E*)-4-Oxo-hexadeca-2,5,15-trienoic acid (**2**)

Whitish sticky solid.
Rf : 0.5 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-3200, 3050, 2923, 2851, 1690, 1664, 1625, 1279, 1215, 1000, 915 cm^{-1}.
UV/Vis λ_{max} ($CHCl_3$) nm (log ε): 235 (4.19).
^{1}H NMR: 1.30-1.65 (12H, brs H_2-8–H_2-13), 2.06 (2H, q, *J* = 6.8 Hz, H_2-14), 2.31 (2H, q, *J* = 7.2 Hz, H_2-7), 4.95 (1H, dtd, *J* = 10.3, 1.8, 1.5 Hz, H-16*E*), 5.02 (1H, dtd, *J* = 17.0, 1.8, 1.5 Hz, H-16*Z*), 5.83 (1H, ddt, *J* = 17.0, 10.3, 6.7 Hz, H-15), 6.39 (1H, d, *J* = 15.9 Hz, H-5), 6.75 (1H, d, *J* = 15.7 Hz, H-3), 7.06 (1H, dt, *J* = 15.9, 6.7 Hz, H-6), 7.48 (1H, d, *J* = 15.7 Hz, H-2).
^{13}C NMR: 27.8, 28.7, 28.9, 29.2, 29.3, 29.4 (6 x CH_2, C-8, C-9, C-10, C-11, C-12, C-13), 32.8 (CH_2, C-7), 33.7 (CH_2, C-14), 114.0 (CH_2, C-16), 129.2 (CH, C-5), 129.7 (CH, C-3), 139.0 (CH, C-15), 139.7 (CH, C-2), 151.7 (CH, C-6), 169.5 (C, C-1), 188.2 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{16}H_{23}O_3$: 263.1647; found: 263.1649.

(*Z*)-6-Hydroxy-4-oxo-octadec-11-enoic acid (**3**)

Whitish sticky solid.
$[\alpha]_D^{25}$: -340º (*c* = 10 mg/mL, $CHCl_3$).
Rf: 0.6 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-3200, 3010, 2928, 2856, 1713, 1406, 1260, 1201, 1100 cm^{-1}.
^{1}H NMR: 0.88 (3H, t, *J* = 6.8 Hz, Me), 1.20-1.65 (14H, brs, H_2-14–H_2-17, H_2-7– H_2-9), 2.05 (4H, q, *J* = 7.0 Hz, H_2-10, H_2-13), 2.60-2.82 (6H, m, H_2-2, H_2-3, H_2-5), 4.10 (1H, brm, H-6), 5.25-5.45 (2H, m, H-11, H-12).
^{13}C NMR: 14.0 (CH_3, C-18), 22.5 (CH_2, C-17), 25.0, 27.0, 27.1, 28.9, 29.5, 29.6 (6 x CH_2, C-8, C-9, C-10, C-13, C-14, C-15), 27.4 (CH_2, C-2), 31.7 (CH_2, C-16), 36.3 (CH_2, C-7), 37.5 (CH_2, C-3), 49.1 (CH_2, C-5), 67.7 (CH, C-6), 129.3, 130.1 (2 x CH, C-11, C-12), 177.4 (C, C-1), 209.7 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{18}H_{31}O_4$ 311.2222, found 311.2225.

6-Hydroxy-4-oxo-hexadec-15-enoic acid (**4**)

Whitish sticky solid.
$[\alpha]_D^{25}$: -109º (*c* = 11 mg/mL, $CHCl_3$).
Rf: 0.7 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-3200, 3010, 2917, 2850, 1702, 1412, 1250, 1080, 1000, 913 cm^{-1}.
^{1}H NMR: 1.25-1.65 (14H, brs, H_2-7–H_2-13), 2.04 (2H, q, *J* = 6.7 Hz, H_2-14), 2.60-2.80 (6H, m, H_2-2, H_2-3, H_2-5), 4.10 (1H, brm, H-6), 4.95 (1H, dtd, *J* = 10.3, 1.8, 1.5 Hz, H-16*E*), 4.99 (1H, dtd, *J* = 17.0, 1.8, 1.5 Hz, H-16*Z*), 5.83 (1H, ddt, *J* = 17.0, 10.3, 6.7 Hz, H-15).
^{13}C NMR: 27.4 (CH_2, C-2), 25.3, 28.8, 29.0, 29.2, 29.3, 29.4 (6 x CH_2 C-8, C-9, C-10, C-11, C-12, C-13), 33.7 (CH_2, C-14), 36.3 (CH_2, C-7), 37.6 (CH_2, C-3), 49.1 (CH_2, C-5), 67.8 (CH, C-6), 114.0 (CH_2, C-16), 139.1 (CH, C-15), 177.1 (C, C-1), 209.7 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{16}H_{27}O_4$ 283.1909, found 283.1911.

6-Hydroxy-4-oxo-hexadecanoic acid (**5**)

Whitish sticky solid.
$[\alpha]_D^{25}$: -95º (*c* = 10 mg/mL, $CHCl_3$).
Rf: 0.65 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-3200, 2920, 2855, 1710, 1415, 1255 cm^{-1}.
^{1}H NMR: 0.88 (3H, t, *J* = 6.8 Hz, Me), 1.20-1.60 (18H, brs, H_2-7–H_2-15), 2.60-2.80 (6H, m, H_2-2, H_2-3, H_2-5), 4.10 (1H, brm, H-6).
^{13}C NMR: 14.0 (CH_3, C-16), 27.6 (CH_2, C-2), 22.8-29.6 (8 x CH_2, C-8, C-9, C-10, C-11, C-12, C-13, C-14, C-15), 36.3 (CH_2, C-7), 37.6 (CH_2, C-3), 49.1 (CH_2, C-5), 67.7 (CH, C-6), 177.2 (C, C-1), 209.5 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{16}H_{29}O_4$ 285.2066, found 285.2064.

Ozonolysis of acid 3: A saturated solution of O_3 in CH_2Cl_2-MeOH, 4:1 v/v, was added to compound **3** (3 mg) dissolved in CH_2Cl_2, (0.5 mL) at –78°C. The reaction was quenched after 3 h by adding excess Me_2S and the mixture was left at –20°C overnight. A sample was directly analyzed by GC under the following conditions: column HP-5 (25 m×0.25 mm, 0.33 μm film thickness), injection temperature 250°C, detector (FID) temperature 280°C, carrier gas nitrogen, flow rate 1.27 mL/min, constant flow mode, split splitless injection, ratio 1:35, column temperature program: 40°C for 5 min, then raised to 100°C at a rate of 2°C/min, then raised to 280°C at a rate of 10°C/min, then isothermal at 280°C for 5 min. Enrichment of the peak eluted at 9.69 min with an authentic sample of heptanal, confirmed its identity.

Fungicidal activity: A simple test, adapted from the literature [4a, 12], was carried out to reveal the

possible fungicidal activity of compounds **1**-**5**. Five solutions of compounds **1**-**5** in MeOH, each containing approximately 20 μg of substance, were spotted on F_{254} Merck silica gel plastic sheets, which were sprayed with a conidal suspension of *Cladosporium cucumerinum* Ell. et Arth spores in a glucose mineral medium (Czapek broth). The plates were then incubated at 25°C in the dark in a wet chamber (> 95% humidity) for 5 days, when they were overgrown with a dark gray colored mycelium. White spots (inhibition zones), signaling fungicidal activity, were found, in particular in correspondence with compounds **1** and **2**; they were about eight times smaller than the inhibition area of the reference compound pseudomycin A (20 μg).

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