# Antimycobacterial Coumarins from the Sardinian Giant Fennel (*Ferula communis*)

Giovanni Appendino,*,† Enrico Mercalli,‡ Nicola Fuzzati,‡ Lolita Arnoldi,‡ Michael Stavri,§ Simon Gibbons,*,§ Mauro Ballero,⊥ and Andrea Maxia⊥

*Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche, Università del Piemonte Orientale, Viale Ferrucci 33, 28100 Novara, Italy, Indena S.p.A., Via Don Minzoni 6, 20090 Settala (MI), Italy, Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, U.K., and Dipartimento di Scienze Botaniche, Università di Cagliari, Viale Fra Ignazio 13, 09124 Cagliari, Italy*

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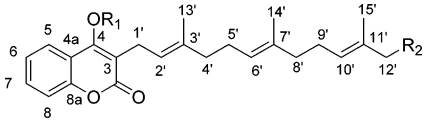
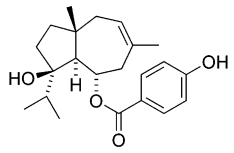
The structure of a new prenylated coumarin (*E*-ω-benzoyloxyferulenol, **1b**) from the Sardinian giant fennel (*Ferula communis*) has been confirmed by synthesis. The parent compound ferulenol (**1a**) showed submicromolar antimycobacterial activity, which was partly retained in **1b** and in the simplified synthetic analogue **3**, but diminished in its ω-hydroxy and ω-acetoxy derivatives (**1c** and **1d**, respectively). The outstanding activity of **1a**, its low toxicity, and the evidence for definite structure−activity relationships make this prenylated 4-hydroxycoumarin an interesting antibacterial chemotype worth further investigation.

The presence of two distinct chemotypes of giant fennel (*Ferula communis* L.; Apiaceae) has complicated the study of ferulosis, a hemorrhagic and often lethal intoxication of livestock pasturing in areas of Sardinia infested with this invasive species.[1] On the other hand, this chemical polymorphism has given phytochemists a unique opportunity to obtain large amounts of structurally unrelated bioactive compounds from the same species. Thus, while the poisonous chemotype is an excellent source of ferulenol (**1a**),[2] a hemorrhagic prenylated coumarin that shows also paclitaxel (Taxol) mimicry,[3] the potent phytoestrogen ferutinin (**2**) can be obtained in large amounts from the nonpoisonous chemotype.[4] Remarkably, the two chemotypes occupy distinct enclaves, and an extensive investigation failed to detect mixed populations.[5] The basis of this behavior remains unknown, but preliminary studies have shown distinct genetic diversities between the two chemotypes, which are otherwise morphologically indistinguishable.[6]

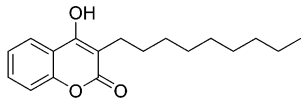
During the development of an expeditious HPLC procedure to fingerprint the two chemotypes, we noticed the presence of a minor compound having chromatographic behavior and a UV spectrum similar to those of ferulenol but with a higher molecular weight (+104 amu), corresponding to the introduction of a benzoyloxy group. From the mass fragmentation pattern, this moiety was located on one of the two ω-carbons of the prenyl group, but the low natural abundance and the close chromatographic behavior compared to ferulenol (**1a**), a major constituent of the extract, prevented its direct isolation.[7] To solve this matter, we planned to synthesize the *E*-isomer of the alleged structure (**1b**) from *E*-ω-hydroxyferulenol (**1c**), a compound easily available by isolation,[2] and compare the chromatographic and spectroscopic properties (UV, MS) of the synthetic compound with those of the unknown chromatographic peak.




| | $R_1$ | $R_2$ |
|---|---|---|
| **1a** | H | H |
| **1b** | H | OBz |
| **1c** | H | OH |
| **1d** | H | OAc |
| **1e** | Bz | OBz |




**2**




**3**

Diastereomerically pure *E*-ω-hydroxyferulenol (**1c**) was obtained, in 1.05% yield, along with ferulenol (**1a,** 0.55%) and its ω-acetoxy derivative (**1d**, 0.20%),[8] from a sample of giant fennel collected in southern Sardinia. The presence of *E,Z*-mixtures of ω-oxygenated ferulenols has been previously reported,[8] but in the sample under investigation, both ω-acetoxy and ω-hydroxyferulenol were obtained in a diastereomerically pure *E*-form. To prepare a compound with the alleged formula of the unknown peak, compound **1c** was treated with an excess of benzoyl chloride to afford the dibenzoate **1e**, which was then subjected to chemoselective deprotection. Opening of the lactone ring and decarboxylation to a prenylacetophenone[9] were not observed when a transamidation rather than a hydrolysis reaction was employed. Pyrrolidine is the standard base for the chemoselective transamidation of phenolic esters, but in our case, the two reaction products (**1b** and *N*-

* To whom correspondence should be addressed. Tel: +39 0321 375744 (G.A.); +44 207 753 5913 (S.G.). Fax: +39 0321 375631 (G.A.); +44 207 753 5909 (S.G.). E-mail: appendino@pharm.unipmn.it (G.A.); simon.gibbons@ulsop.ac.uk (S.G.).
† Università del Piemonte Orientale.
‡ Indena S.p.A.
§ University of London.
⊥ Università di Cagliari.

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**Table 1.** Minimum Inhibitory Concentrations (MIC, $\mu$g/mL) against Fast-Growing *Mycobacterium* Species of the Natural Coumarins **1a**−**1d**

| species | ferulenol (**1a**) | **1b** | **1c** | **1d** | ethambutol | isoniazid |
|---|---|---|---|---|---|---|
| *M. fortuitum* | 2 | 16 | 64 | 32 | 8 | 0.5 |
| *M. phlei* | 2 | 2 | 16 | 8 | 2 | 4 |
| *M. aurum* | 2 | 8 | 32 | 16 | 0.5 | 2 |
| *M. smegmatis* | 0.5 | 2 | 16 | 8 | 0.5 | 1 |

benzoylpyrrolidine) had very similar chromatographic mobility in a variety of solvents and could not be separated. The problem was solved by replacing pyrrolidine with ethylendiamine, which eventually afforded **1b** in overall 56% yield from **1c**. The NMR spectra confirmed the structure **1b** for the semisynthetic benzoate. Thus, when compared with *E*-$\omega$-hydroxyferulenol (**1c**), a downfield shift ($\Delta\delta$ = +0.70) was observed for the allylic oxymethylene protons (H-12′a,b) in the $^1$H NMR spectrum and diagnostic $\alpha$- and $\gamma$-downfield (C-12′, C-10′; $\Delta\delta$ = 1.6 and 3.5, respectively) and $\beta$-upfield (C-11, $\Delta\delta$ = 4.1) shifts in the $^{13}$C NMR spectrum.[10] Semisynthetic **1b** had a chromatographic mobility and UV and MS spectra indistinguishable from those of the unknown peak, suggesting that the two compounds are identical.

The antitubercular activity of ferulenol (**1a**) has been reported in preliminary form.[11] We have confirmed this antimycobacterial activity (Table 1), discovering excellent potency toward four strains of fast-growing mycobacteria, including *Mycobacterium fortuitum* ATCC 6841, which has been shown to be of use as an alternative screening model for potential antitubercular drugs.[12] The 12′-hydroxy-, 12′-benzoyloxy-, and 12′-acetoxy derivatives of ferulenol were also evaluated using these fast-growing strains. Introduction of a hydroxyl or an acetoxyl at position C-12′ (**1c** and **1d**, respectively) caused a marked reduction of potency, whereas a benzoyloxy group at C-12′ was better tolerated, fully restoring the antibacterial activity of the parent compound against *M. phlei* and *M. smegmatis* and, to a lesser extent, *M. fortuitum* and *M. aurum*. These observations indicate that a large and lipophilic group at C-12′ of ferulenol is tolerated.

Although ferulenol (**1a**) shows in vivo anticoagulant activity, its acute toxicity is low compared to warfarin.[13] Indeed, rather than with the presence of ferulenol, the toxicity of giant fennel seems better correlated with that of its $\omega$-oxygenated derivatives. Thus, ferulenol could be detected in large amounts in samples of giant fennel coming from areas (Sicily, Crete, and continental Greece) where ferulosis is rare or unknown,[14] while the presence of $\omega$-oxygenated derivatives is the hallmark of the plants coming from Sardinia and Morocco, where this intoxication is still widespread in livestock.[1,15]

Binding to tubulin seemingly underlies the cytotoxic activity of ferulenol (**1a**),[3] while as a 4-hydroxycoumarin derivative, its anticoagulant properties are due to inhibition of the enzyme vitamin K epoxide reductase.[16] Since neither protein is expressed in mycobacteria, a third and yet unknown antimicrobial target for ferulenol may exist, with possible divergences in the structure−activity requirements of the various molecular targets. In this context, it is interesting to remark that the structure of ferulenol combines two structural elements, the enolized $\beta$-dicarbonyl and the farnesyl moieties, individually present in two classes of antibacterial compounds.[17,18] Novobiocin, a coumarin that has limited use as a treatment for infections caused by Gram-positive bacteria, particularly resistant *Staphylococcus aureus* strains, also possesses a hydroxyl at C-4 and a substituent at C-3. This compound has been shown to inhibit mycobacterial DNA gyrase,[19] and it does not seem unreasonable to assume as a starting working hypothesis that ferulenol also exerts antimycobacterial effects via this mechanism.

When compared to the therapeutically used antimycobacterials such as isoniazid and ethambutol, the activity of ferulenol (**1a**) is promising, validating further work to evaluate the effects of its structural modification. Remarkably, antitubercular activity was substantially retained in a simpler analogue, 3-nonyl-4-hydroxycoumarin (**3**) (MIC = 3.5 $\mu$g/mL against *M. fortuitum*), a compound easily available by synthesis.[20] This observation shows that the isoprenoid features of the substituent at C-3 (methyl branches and double bonds) are redundant for the antimicrobial activity of ferulenol and suggests that this compound can serve as a basis for an analogue program aimed at defining the critical structure for antitubercular activity and its dissection from cytotoxic and anticoagulant properties.

## Experimental Section

**General Experimental Procedures.** IR spectra were obtained on a Shimadzu DR 8001 spectrophotometer. The $^1$H and $^{13}$C NMR spectra were recorded at room temperature on a Varian INOVA 300 spectrometer, operating at 300 MHz for $^1$H and 75 MHz for $^{13}$C. Chemical shifts were referenced to the residual solvent signal. HRMS were recorded on a VG Prospect (Fisons) mass spectrometer. Mass spectral analyses were performed with a Finnigan MAT LCQ ion trap sammspectrometer equipped with both ESI and APCI interfaces. HPLC-DAD analyses were obtained on a system made up by a SpectraSYSTEM AS 3000 automatic sample injector module and a SpectraSYSTEM UV6000LP diode array detector (DAD). Silica gel 60 (70−230 mesh) was used for gravity column chromatography.

**Plant Material.** *F. communis* L. was collected in October 2001 near Cagliari, Sardinia, Italy, and identified by M.B. A voucher specimen (612A) is deposited at the Dipartimento di Scienze Botaniche, Università di Cagliari.

**HPLC-DAD-UV Analysis of Giant Fennel Extracts.** Powdered, dried roots (0.5 g) were treated with MeOH (150 mL) and extracted under sonication. After filtration, the extracts were analyzed on a Zorbax XDB column (250 × 4.6 mm) with a binary eluant system [0.1% HCOOH in water (solvent A) and 0.1% HCOOH in acetonitrile (solvent B)], starting with 55% A for 5 min and moving then to a linear gradient, from 55% A to 10% A in 40 min. The flow rate was 1.0 mL/min, and the injection volume 10 $\mu$L. For ESIMS detection, the following parameters were employed: (a) positive mode: capillary temp 250 °C; sheath gas flow 90 units; auxiliary gas flow 60 units; source voltage 4.8 kV; capillary voltage 19 kV; (b) negative mode: capillary temp 290 °C; sheath gas flow 80 units; auxiliary gas flow 40 units; source voltage 6.0 kV; capillary voltage 30 kV; vaporizer temperature 350 °C. The unknown peak identified as **1b** had a retention time of 36 min and UV maxima at 282 and 310 nm and presented an intense ion at *m/z* 365, attributable to the loss of benzoic acid from the pseudomolecular ion at *m/z* 487.

**Isolation of $\omega$-Hydroxyferulenol.** A sample (450 g) of roots was powdered and extracted with acetone (3 × 3 L). The pooled extracts were evaporated to give 38 g of a brownish syrup, which was fractionated by gravity column chromatography (100 g silica gel, petroleum ether−EtOAc gradient) to give ferulenol (**1a**) (petroleum ether−EtOAc (9:1), 2.23 g, 0.55%), *E*-$\omega$-acetoxyferulenol (**1d**) (petroleum ether−EtOAc 8:2, 0.82 g, 0.20%), and *E*-$\omega$-acetoxyferulenol (**1c**) (petroleum ether−EtOAc (7:3), 4.2 g, 1.05%), identified by comparison ($^1$H NMR, TLC) with authentic standards.[8]

**Synthesis of *E*-$\omega$-Benzoyloxyferulenol (1b) from *E*-$\omega$-Hydroxyferulenol (1c).** (a) To a solution of **1c** (1.0 g, 2.6 mmol) in toluene (8 mL) were added benzoic acid (950 mg, 7.8 mmol, 3 molar equiv), dicyclohexylcarbodiimide (DCC) (1.6 g,

7.8 mmol, 3 molar equiv), and 4-(dimethylamino)pyridine (DMAP) (79 mg, 0.65 mmol, 0.25 molar equiv). After stirring overnight at room temperature, the reaction was worked up by filtration over alumina (ca. 5 g) to remove dicyclohexyl urea and the excess acid, and evaporation. The residue was further purified by filtration over silica gel (20 g) using petroleum ether−EtOAc (9:1) as eluant, to afford 1.3 g (82%) of crude **1e**. (b) To a solution of **1e** (583 mg, 0.96 mmol) in toluene (6 mL) was added an excess of ethylendiamine (320 $\mu$L, 288 mg, 4.8 mmol, 5 molar equiv), resulting in the formation of an orange color. After stirring at room temperature for 20 min, the reaction was worked up by dilution with EtOAc (10 mL) and washing with 2 N $H_2SO_4$ and brine. After drying ($Na_2SO_4$) and evaporation, the residue was purified by gravity column chromatography on silica gel (13 g, petroleum ether−EtOAc (9:1) as eluant), to afford 319 mg of **1b** (68% from **1d**, overall 56% from **1c**).

***E*-$\omega$-Benzoyloxyferulenol (1b):** colorless syrup; UV (EtOH) $\lambda_{max}$ (log $\epsilon$) 282 (2.70), 310 (2.69) nm; IR (liquid film) $\nu_{max}$ 3300−2900 (broad), 1670, 1609, 1568, 1203, 1190, 1109, 1056, 757 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 8.03 (2H, Bz-AA′), 7.60 (1H, Bz-C), 7.50 2H, Bz-BB′), 7.94 (1H, dd, $J$ = 7.9, 1.5 Hz, H-5), 7.60 (1H, m, H-7), 7.34 1H, t, $J$ = 8.6 Hz, H-6), 7.32 (1H, m, H-8), 5.21 (1H, td, $J$ = 8.4, 1.3 Hz, H-2′), 5.84 (1H, td, $J$ = 8.2, 1.3 Hz, H-10′), 5.11 (1H, td, $J$ = 8.0, 1.2 Hz, H-6′), 4.68 (2H, s, H-12′), 3.32 (2H, d, $J$ = 7.0 Hz, H-1′), 2.10 (2H, m, H-5′), 2.10 (2H, m, H-9′), 2.03 (2H, m, H-8′), 2.01 (2H, m, H-4′), 1.80 (3H, d, $J$ = 1.0 Hz, H-13′), 1.69 (3H, d, $J$ = 0.9 Hz, H-15′), 1.59 (3H, d, $J$ = 1.0 Hz, H-14′); $^{13}C$ NMR (75 MHz, MeOH-$d_4$) $\delta$ 166.7 (s, Bz), 165.0 (s, C-2), 160.7 (s, C-4), 152.4 (s, C-8a), 136.3 (s, C-3′), 134.3 (s, C-7′), 133.0 (d, Bz), 131.5 (d, C-7), 130.4 (s, C-11′), 130.1 (s, Bz), 129.4 (d, Bz), 129.3 (d, C-10′), 128.4 (d, Bz), 124.6 (d, C-6′), 124.0 (d, C-6), 123.0 (d, C-5), 121.0 (d, C-2′), 116.8 (s, C-4a), 116.3 (d, C-8), 105.3 (s, C-3), 70.4 (t, C-12′), 39.6 (t, C-4′), 38.9 (t, C-8′), 26.2 (t, C-9′), 26.1 (t, C-5′), 22.5 (t, C-1′), 15.3 (q, C-13′), 15.0 (q, C-14′), 12.9 (q, C-15′); HREIMS *m/z* 486.2419 $[M]^+$ (calcd for $C_{31}H_{34}O_5$, 486.2406); APCI+ MS-MS *m/z* 365 (35), 309 (100), 283 (80), 217 (45), 203 (49), 191 (55), 175 (32).

**Synthesis of 3-Nonyl-4-hydroxycoumarin.** To a cooled (ice bath) solution of 4-hydroxycoumarin (50 g, 30.8 mmol) in pyridine−piperidine (10:1, 800 mL) was added dropwise nonanoyl chloride (57.7 mL, 56.5 g, 32.1 mmol, 1.05 equiv) for ca. 5 min. At the end of the addition, the cooling bath was removed and the solution was allowed to warm to room temperature for 15 min and then refluxed for 48 h. After cooling, the reaction mixture was poured into crushed ice (ca. 200 g), and the orange suspension was acidified (pH 2) with 2 N $H_2SO_4$. The precipitate was collected by filtration, washed with water until neutral, dried, and crystallized from hot EtOH to afford 28.1 g (30%) of 3-nonanoyl-4-hydroxycoumarin. A portion of the latter (5 g, 26.3 mmol) was suspended in acetic acid (50 mL), heated to 100 °C (oil bath), and magnetically stirred until a clear solution was obtained. At this point, $NaBH_3CN$ (2.1 g, 33.8 mmol, 1.3 molar equiv) was added in small portions for ca. 10 min. At the end of the addition, the oil bath was removed, and the solution was stirred to room temperature for an additional 50 min. The reaction was then worked up by the addition of water, causing the formation of a bulky precipitate that was collected by filtration and repeatedly washed with water to afford 4.3 g (overall 27% from 4-hydroxycoumarin) of **3** as a white powder: mp 104 °C; IR (KBr) $\nu_{max}$ 3196, 1674, 1610, 1568, 1199, 1099, 1053, 914, 752 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 7.95 (1H, dd, $J$ = 7.9, 1.5 Hz, H-5), 7.54 (1H, m, H-7), 7.34 (2H, m, H-6 and H-8), 2.67 (1H, t, $J$ = 7.6 Hz, H-1′), 1.61 (2H, m, H-2′), 1.40−1.21 (12H, m, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′), 0.85 (3H, t, $J$ = 7.3 Hz, H-9); $^{13}C$ NMR (50 MHz, $CDCl_3$) $\delta$ 164.6 (s, C-2), 160.0 (s, C-4), 152.1 (s, C-8a), 131.4 (d, C-7), 124.0 (d, C-5 and C-6), 116.4 (d, C-8), 115.8 (s, C-4a), 105.6 (s, C-3), 31.7, 29.2, 28.4, 23.9, 22.5 (t, C-3′, C-4′, C-5′, C-6′, C-7′, C-8′), 14.0 (q, C-9′); CIMS (isobutanol) *m/z* 289 $[M + 1]^+$ $[C_{18}H_{24}O_3 + H]^+$.

**Biological Assays.** *Mycobacterium fortuitum* ATCC 6841 was obtained from Dr. Peter Lambert, Aston University, Birmingham, UK. *Mycobacterium smegmatis* ATCC 14468, *Mycobacterium phlei* ATCC 11758, and *Mycobacterium aurum* Pasteur Institute 104482 were obtained from Dr. Veronique Seidel, The School of Pharmacy, University of London. Bacteria were maintained on Columbia blood agar (Oxoid) supplemented with 5% defibrinated horse blood (Oxoid). This assay comprised a standard MIC determination[21,22] of test compound in $Ca^{2+}$ and $Mg^{2+}$ adjusted Mueller-Hinton broth (MHB). Compounds **1a**−**1d** were dissolved in DMSO and diluted out into MHB, to give a starting concentration of 512 $\mu$g/mL, which was then diluted across a 96-well microtiter plate in a 2-fold serial dilution to give a final concentration range from 512 to 1 $\mu$g/mL. Bacterial inocula equivalent to the 0.5 McFarland turbidity standard were prepared in normal saline and diluted to give a final inoculum density of $5 \times 10^5$ cfu/mL. The inoculum (125 $\mu$L) was added to all wells, and microtiter plates were incubated at 37 °C for 72 h for *M. fortuitum, M. smegmatis*, and *M. phlei*. For *M. aurum*, the plate was incubated for 120 h. The MIC was recorded as the lowest concentration at which no bacterial growth was observed. This was facilitated by the addition of 20 $\mu$L of MTT (Sigma 10 mg/mL in MeOH) to each well and incubation at 37 °C for 20 min, where bacterial growth was indicated by a blue coloration. Appropriate DMSO, growth, and sterile controls were carried out. Ethambutol and isoniazid were used as positive controls.

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