Daucane Phytoestrogens: A Structure−Activity Study

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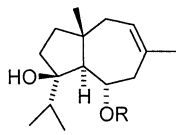
The estrogenic activity of a series of analogues of the daucane ester ferutinin (**1a**) modified at the acyl moiety was investigated in a yeast screen containing the human estrogen receptor α. Rather strict structure−activity relationships were observed. Thus, while the parent polyol (jaeschkeanadiol, **2a**) was inactive, the presence of a *p*-hydroxybenzoyl moiety was necessary for activity in the yeast screen. Homologation and vinylation were both detrimental for activity, as were methylation of the *p*-hydroxyl substituent and the introduction of oxygen functions on the adjacent carbons.

Plants from the genus *Ferula* (family Umbelliferae) have a long history of use in medicine, perfumery, and cuisine,[1] and their hormonal effects, anecdotically documented in medicinal and veterinarian practice,[2] have been confirmed by modern animal studies.[3] The active constituents are believed to be daucane alcohols esterified with aromatic acids, as exemplified by ferutinin (**1a**),[4] the *p*-hydroxybenzoyl ester of jaeschkeanadiol (= ferutinol, **2a**).[5] Ferutinin shows powerful estrogen activity both in binding and in functional assays[6] and has been developed in the former USSR as a dietary supplement to treat hot flashes and other menopausal-associated disorders.[7] High concentrations of ferutinin have also been reported in the roots of *F. hermonis* Boiss. ("Zallouh"),[8] a rare Middle-East species currently being promoted as a natural alternative to Viagra.[9] While structure−activity studies have been performed with various classes of naturally occurring estrogen mimics,[10] there has been no attempt to identify the structural requirements responsible for the estrogenic activity of daucane derivatives. This is most surprising, since the reported affinity of ferutinin for the estrogen receptors is higher than that of the well-known phytoestrogen genistein,[11] while a large variety of daucane derivatives is available from plants and microorganisms, providing access to a series of diversely functionalized templates.[12]

We report here the first structure−activity study on the estrogenic activity of daucane derivatives, taking ferutinin (**1a**) as a lead compound and focusing on its oxygenated aromatic acyl group. Variation of this moiety underlies the occurrence of a large variety of jaeschkeanadiol esters in plants from the genus *Ferula*.[13] Many of these compounds co-occur with ferutinin, but are difficult to obtain in pure form by isolation, and were therefore more conveniently synthesized from the parent diol **2a**.

Results and Discussion

Jaeschkeandiol (**2a**) was obtained by hydrolysis of ferutinin (**1a**), in turn isolated in ca. 1.9% yield from the roots of the nonpoisonous chemotype of giant fennel (*F. communis* L.) from Sardinia.[14] The acylation of **2a** with oxygenated aromatic acids was investigated under a variety of conditions. Reaction with acyl chlorides was sluggish with both methoxy- and acetoxy-substituted compounds. Several days and a large excess of reagents were necessary for completion, as exemplified by the trimethoxybenzoyl derivative **1j** (= palliferidine[15]), whose synthesis required prolonged heating of **2a** (7 days at 40 °C) with a large excess (20 equiv) of trimethoxybenzoyl chloride. A more general synthesis could be achieved directly from acetyl-protected phenolic acids using a DCC-mediated coupling under forced conditions (toluene, reflux). Chemoselective aminolysis of the acetoxy esters with pyrrolidine in dichloromethane was uneventful, eventually affording the final phenolic esters. The yield was sometimes poor due to the competitive formation of jaeschkeanadiol acetate (**2b**), the result of an acetyl-transfer reaction. The hindered nature of the secondary hydroxyl of **2a** and its involvement in a strong intramolecular hydrogen bonding with the tertiary hydroxyl presumably underlie the difficulties encountered in the esterification reaction.[16]



	R
2a	H
2b	Ac

The modifications pursued on the acyl moiety of ferutinin were homologation, bis-homologation, and vinylation of the benzoyl group (**1b**−**d**), as well as methylation, acetylation, and deletion of the phenolic hydroxyl (**1e**−**g**), and the introduction of further oxygenated functions adjacent to it (**1h**−**j**). Compounds combining modifications at the *ipso*-, *meta*-, and *para*-carbons of the 4-hydroxybenzoyl moiety of ferutinin were also prepared (**1k**−**o**). Table 1 summarizes the preparation of the library of jaeschkeanadiol esters and provides a key to the plethora of trivial names that have been assigned to some of these compounds.

The estrogenic activity of jaeschkeanadiol (**2a**) and its various 6-acyl derivatives was investigated in a yeast screen containing the human estrogen receptor alpha (ERα). A number of compounds showed little or no activity, including jaeschkeanadiol (**2a**), jaeschkeanadiol benzoate (**1g**), jaeschkeanadiol anisate (**1e**), and compounds **1i**−**o**. In contrast, ferutinin (**1a**) showed a high estrogenic activity

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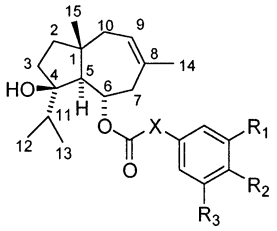
Table 1. Synthetic Yields, Trivial Names, and Estrogenic Activity Measured in an Estrogen-Inducible Yeast Assay for Ferutinin (**1a**) and Its Analogues **1b**−**1o**[a]



	R_1	R_2	R_3	X	yield[b]	trivial name	EC_{50} relative to estradiol (%)
1a	H	OH	H			ferutinin	0.5
1b	H	OH	H	CH_2	89% (B)		<0.0001
1c	H	OH	H	CH_2−CH_2	75% (B)		<0.0001
1d	H	OH	H	CH=CH (*E*)	42% (B)		<0.0001
1e	H	OMe	H		79% (A)	ferutidin[25]	<0.0001
1f	H	OAc	H		*c*	fersorin[26]	0.2
1g	H	H	H		69% (A)	teferidine[27]	inactive
1h	H	OH	OH		7% (B)	akiferidin[28]	<0.1
1i	H	OH	OMe		10% (B)	teferin[29]	inactive
1j	OMe	OMe	OMe		78% (A)	palliferidin[15]	inactive
1k	H	OH	OMe	CH_2	57% (B)		inactive
1l	H	OH	OH	CH_2−CH_2	48% (B)		inactive
1m	H	OH	OH	CH=CH (*E*)	66% (B)		inactive
1n	H	OH	OMe	CH=CH (*E*)	29% (B)		inactive
1o	OMe	OH	OMe	CH=CH (*E*)	51% (B)		inactive

[a] Compounds failing to produce a response in the yeast bioassay corresponding to 0.2 nM estradiol are marked as inactive. [b] Conditions A: acyl chloride−pyridine; Conditions B: acid, DCC, DMAP, toluene, room temperature for **1b** and **1c**; 90 °C for the remaining compounds. [c] Prepared by acetylation (Ac_2O, pyridine, room temperature) of ferutinin.

(approximately 200-fold lower than that of estradiol), closely followed by its acetate (**1f**, Table 1). Significant estrogenic activity was also detected in compound **1h** (<1/1000 compared to estradiol), and an indication of estrogenic activity was detected in compounds **1b**, **1c**, **1d**, and **1e** at the highest dose levels, representing, however, an activity < $1/10^6$ compared to estradiol. Ferutinin (**1a**) administered orally has also been reported to increase uterine weight in rats,[11] and our findings suggest that this compound must be considered as one of the most active phytoestrogens, on a par with 8-prenylnaringenin and coumestrol.[17]

The ER is quite promiscuous in its binding, but a general requirement for estrogen activity includes an aromatic ring bearing a hydroxyl which can act both as a hydrogen donor and a hydrogen acceptor.[18] Methylation of the phenolic hydroxyl significantly decreases the affinity for the ER, but several antiestrogens such as tamoxifen and clomiphene remain strong binders despite the lack of a phenolic moiety. Furthermore, extensive studies on natural products and xenochemicals have shown that two ring structures separated by two carbon atoms (steroidal and synthetic estrogens and diphenyl ethanes) have higher binding affinity than compounds with a single ring structure (e.g., alkylphenols, parabens) or two rings separated by one carbon atom alone.[19] These suggestions have been fully supported by crystallographic studies of ER−ligand complexes, which have indicated that the critical spacing of hydrophobic and hydrogen bond interactions plays a major role in binding affinity.[20] These structural requirements are consistent with the low or negligible estrogenic activity of the *p*-*O*-methylated analogues of ferutinin, but do not completely explain the high potency of the parent compound, where the phenolic moiety is connected by an ester, and not an ethylene tether, to a cyclic element.

Ferutinin (**1a**) strongly interacted with ERα and ERβ in competitive binding assays (Figures 1 and 2), showing an affinity approximately 10% of that of estradiol on both receptors and confirming a recent independent observation.[21] The relative binding affinity of **1f** was considerably lower (<1% of that of estradiol), suggesting that its strong activity in the yeast bioassay may reflect the conversion to a more active metabolite by hydrolysis of the phenolic acetate. In cotransfection experiments, ferutinin binding could inhibit the interaction of ERβ with TRAP220, while the interaction of ERα with this nuclear receptor coactivator remained unaffected.[21] The differential effect of ferutinin (**1a**) on ERα and ERβ may have a considerable significance for its biological activity in vivo. As a subtype-selective phytoestrogen, ferutinin may thus be useful as a selective estrogen receptor modulator (SERM), providing a new opportunity to build on natural product diversity to develop hormonal agents.

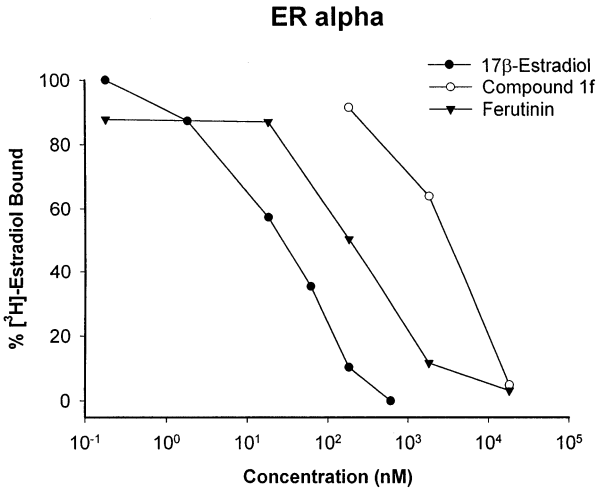


Figure 1. Competitive displacement of [2,4,6,7-^{3}H] 17β-estradiol from isolated ER alpha by 17β-estradiol, ferutinin (**1a**), and compound **1f** (fersorin).

Taken together, the results of these structure−activity studies show that the *p*-hydroxybenzoyl moiety is a key element of the ferutinin (**1a**) pharmacophore and that

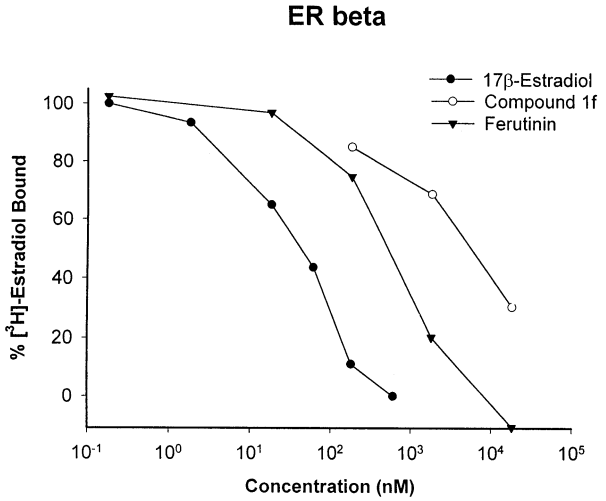


Figure 2. Competitive displacement of [2,4,6,7-^{3}H] 17β-estradiol from isolated ER beta by 17β-estradiol, ferutinin (**1a**), and compound **1f** (fersorin).

daucanes qualify as novel structure templates for estrogen receptor binding. *p*-Hydroxybenzoic acid is structurally related to preservatives used in food, cosmetics, and pharmaceuticals and has been shown to exert a very weak estrogenic activity.[22] Thus, the spectacular increase in estrogenic activity observed in ferutinin (**1a**) compared to *p*-hydroxybenzoic acid must be the result of the esterification to a daucane alcohol. Investigations aimed at the modification of the terpenoid moiety of ferutinin are in progress and will be reported in due course.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Shimadzu DR 8001 spectrophotometer. MS (EI, 70 eV) were taken on VG 7070 EQ spectrometers. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-300 spectrometer (300 and 75 MHz respectively). The solvent signals ($CHCl_3$/$CDCl_3$ 7.27/76.9 ppm) were used as internal reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. Silica gel 60 (70−230 mesh, Merck) was used for open-column chromatography. Phenolic acids were acetylated by treatment with Ac_2O (5 molar equiv) in pyridine and were purified by crystallization upon dilution of the reaction mixture with water or by column chromatography after extraction of the reaction mixture with EtOAc.[23] The known jaeskeanadiol esters **1d**,[24] **1e**,[25] **1f**,[26] **1g**,[27] **1h**,[28] **1i**,[29] and **1j**[15] were identified by comparison of their spectroscopic data (IR, ^{1}H NMR) with the reported data.

Plant Material and Isolation of Jaeskeanadiol. *F. communis* L. used in this investigation was collected in Senenghe (Oristano, Sardinia) in March 2000 and was identified by Prof. Mauro Ballero (Dipartimento di Scienze Botaniche, Università di Cagliari, Italy). A voucher specimen (612B) is deposited at the Dipartimento di Scienze Botaniche, Università di Cagliari. Ferutinin (**1a**) was isolated from an acetone extract of *F. communis*[14] and was hydrolyzed to jaeskeanadiol (**2a**) using the protocol developed by Prof. Sukh Dev and coworkers.[5]

Determination of the Estrogenic Activity in Vitro. All compounds were dissolved in ethanol, and a number of serial dilutions were prepared. Aliquots (20 μL) were added to individual wells in a 96-well plate, and the solvent was evaporated. Estrogenic activity was determined using an estrogen-inducible yeast screen (*Saccharomyces cerevisiae*) expressing the human estrogen receptor (ERα) and containing expression plasmids carrying estrogen-responsive sequences controlling the reporter gene lac-Z (encoding the enzyme β-galactosidase). Estrogenic activity was determined from the enzymatic hydrolysis of chlorophenol red β-D-galactopyranoside by monitoring the absorbance at 540 nm.[30]

Receptor Binding Experiments. Estrogen receptor binding activity was studied using recombinant human estrogen receptors α and β (ERα and ERβ) obtained from PanVera Corporation (Madison, WI).[17] Dilutions of the test compounds were incubated in 100 μL of buffer (10 mM Trizma preset crystals (pH 7.5), 10% glycerol, 2 mM D,L-dithiothreitol (DTT), 1 mg/mL bovine serum albumin) with 15 nM [2,4,6,7-^{3}H]-l7β-estradiol (84.0 Ci/mmol; Amersham Life Science, Amersham, UK) and ER (1.5 nM). The mixture was incubated overnight at 4 °C, and free and bound hormone were separated using 100 μL of 50% hydroxylapatite slurry (in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA). After three washes in buffer (ERα: 40 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM EGTA; ERβ: 40 mM Tris-HCl, pH 7.5), the slurry was extracted with two washes of 200 μL of ethanol, and the radioactivity in the extracts was determined.

Esterification of Jaeschkeanadiol. (1) With Acyl Chlorides. (Synthesis of 1j as an Example). To a solution of jaeschkeanadiol (**2a**, 256 mg, 1.1 molar equiv) in pyridine−CH_2Cl_2 (3:1, 13 mL) were added trimethoxybenzoyl chloride (4.97 g, 21.5 mmol, 20 molar equiv) and DMAP (ca. 10 mg). After stirring for 7 days at 40 °C, the reaction was worked up by dilution with $CHCl_3$ and washing with 1 N HCl, saturated aqueous $NaHCO_3$, and brine. After drying (Na_2SO_4) and removal of the solvent, the residue was purified by column chromatography (petroleum ether−EtOAc 8:2) to afford 364 mg of **1j** (78%).

(2) With Acetylated Phenolic Acids (Synthesis of 1m as an Example). To a suspension of diacetylcaffeic acid (183 mg, 0.69 mmol, 2.9 molar equiv) in dry toluene (3 mL) were sequentially added jaeschkeanadiol (**2a**, 60 mg; 0.24 mmol), DCC (137 mg; 0.66 mmol; 2.8 mol), and DMAP (31 mg; 0.25 mmol; 1 molar equiv). The reaction mixture was heated at 90 °C for 3 h and then worked up by dilution with EtOAc and washing with saturated aqueous $NaHCO_3$ and brine. After drying (Na_2SO_4) and removal of the solvent, the residue was suspended in Et_2O and left standing for 3 h at 5 °C. After filtration of the copious precipitate of dicyclohexylurea and evaporation of the solvent, the residue was purified by column chromatography (petroleum ether−EtOAc gradient, from 9:1 to 8:2) to afford 75 mg of the protected ester. The latter was dissolved in THF (1 mL) and treated with an excess of pyrrolidine (ca. 250 μL). After stirring at room temperature for 30 min, the reaction was worked up by dilution with EtOAc, and the organic phase was sequentially washed with 2 N HCl, saturated aqueous $NaHCO_3$, and brine. Removal of the solvent gave 48 mg (66%) of **1m** as an amorphous foam.

Jaeschkeanadiol *p*-hydroxyphenylacetate (1b): amorphous foam; IR (liquid film) ν_{max} 3375, 1710, 1615, 1597, 1516, 1447, 1379, 1262, 1150, 964 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (2H, AA′, acyl), 6.72 (2H, BB′, acyl), 5.48 (1H, br s, H-9), 4.92 (1H, dt, J = 10, 1 Hz, H-6), 3.52 (2H, AB, acyl), 2.35 (1H, t, J = 10 Hz, H-7a), 2.23 (1H, dd, J = 10, 1 Hz, H-7b), 1.79 (3H, br s, H-14), 0.98 (3H, s, H-15), 0.80−0.71 (each 3H, d, J = 6.7 Hz, H-12, H-13); HREIMS m/z 354.2186 [M − H_2O]$^+$ (5) (calcd for $C_{23}H_{32}O_4$ − H_2O, 354.2195).

Jaeschkeanadiol *p*-hydroxyphenylpropionate (1c): amorphous foam; IR (liquid film) ν_{max} 3384, 1709, 1615, 1597, 1516, 1265, 1225, 1170, 1152, 830 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.04 (2H, AA′, acyl), 6.76 (2H, BB′, acyl), 5.50 (1H, br s, H-9), 4.95 (1H, dt, J = 10, 1 Hz, H-6), 2.88 (2H, m, acyl), 2.59 (2H, m, acyl), 2.33 (1H, t, J = 10 Hz, H-7α), 2.12 (1H, dd, J = 10, 1 Hz, H-7β), 1.79 (3H, br s, H-14), 0.99 (3H, s, H-15), 0.87−0.85 (each 3H, d, J = 6.7, H-12, H-13); HREIMS m/z 368.2339 [M − H_2O]$^+$ (12) (calcd for $C_{24}H_{34}O_4$ − H_2O, 368.2351).

Jaeschkeanadiol homovanillate (1k): amorphous foam; IR (liquid film) ν_{max} 3420, 1725, 1603, 1516, 1464, 1454, 1433, 1273, 1152 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (1H, d, J = 8 Hz, acyl), 6.77 (1H, s, acyl), 6.73 (1H, d, J = 8 Hz, acyl), 5.75 (1H, br s, OH), 5.49 (1H, br s, H-9), 4.95 (1H, dt, J = 10, 1 Hz, H-6), 3.86 (3H, s, −OCH_3), 3.53 (2H, AB, acyl), 2.34 (1H, t, J = 10 Hz, H-7α), 2.19 (1H, dd, J = 10, 1 Hz, H-7β), 1.79 (3H, br s, H-14), 1.00 (3H, s, H-15), 0.79−0.71 (each 3H, d, J

= 6.7, H-12, H-13); HREIMS *m/z* 384.2315 $[M - H_2O]^+$ (11) (calcd for $C_{24}H_{34}O_5 - H_2O$, 384.2301).

Jaeschkeanadiol dihydrocaffeate (1l): amorphous foam; IR (liquid film) ν_{max} 3402, 1709, 1607, 1520, 1449, 1283, 1192, 1152, 1111 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 6.75 (1H, d, J = 8 Hz, acyl), 6.73 (1H, s, acyl), 6.60 (1H, d, J = 8 Hz, acyl), 6.41 (1H, br s, OH), 6.22 (1H, br s, OH), 5.49 (1H, br s, H-9), 4.94 (1H, dt, J = 10, 1 Hz, H-6), 2.85 (2H, m, acyl), 2.58 (2H, m, acyl), 2.33 (1H, t, J = 10 Hz, H-7α), 2.12 (1H, dd, J = 10, 1 Hz, H-7β), 1.79 (3H, br s, H-14), 0.98 (3H, s, H-15), 0.87−0.82 (each 3H, d, J = 6.7, H-12, H-13); HREIMS *m/z* 402.2400 $[M - H_2O]^+$ (6) (calcd for $C_{24}H_{34}O_5 - H_2O$, 402.2406).

Jaeschkeanadiol caffeate (1m): amorphous foam; IR (liquid film) ν_{max} 3370, 1682, 1632, 1603, 1516, 1447, 1383, 1275, 1181, 1115 1111 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.67 (1H, br s, OH), 7.68 (1H, d, J = 16 Hz, acyl), 7.17 (1H, s, acyl), 7.01 (1H, d, J = 8 Hz, acyl), 6.90 (1H, d, J = 8 Hz, acyl), 6.47 (1H, d, J = 16 Hz, acyl), 5.56 (1H, br s, H-9), 5.13 (1H, dt, J = 10, 1 Hz, H-6), 2.64 (1H, t, J = 10 Hz, H-7a), 2.27 (1H, dd, J = 10, 1 Hz, H-7β), 1.84 (3H, br s, H-14), 1.10 (3H, s, H-15), 0.93−0.91 (each 3H, d, J = 6.7, H-12, H-13); HREIMS *m/z* 382.2154 $[M - H_2O]^+$ (12) (calcd for $C_{24}H_{32}O_5 - H_2O$, 382.2144).

Jaeschkeanadiol ferulate (1n): amorphous foam; IR (liquid film) ν_{max} 3345, 1688, 1630, 1592, 1514, 1269, 1175, 1159, 1121, 1034 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.63 (1H, d, J = 16 Hz, acyl), 7.10 (1H, dd, J = 8, 1 Hz, acyl), 7.04 (1H, s, acyl), 6.94 (1H, d, J = 8 Hz, acyl), 6.25 (1H, d, J = 16 Hz, acyl), 6.09 (1H, s, OH), 5.55 (1H, br s, H-9), 5.15 (1H, dt, J = 10, 1 Hz, H-6), 3.95 (3H, s, $-OCH_3$), 2.50 (1H, t, J = 10 Hz, H-7α), 2.28 (1H, dd, J = 10, 1 Hz, H-7β), 1.83 (3H, br s, H-14), 1.13 (3H, s, H-15), 0.95−0.92 (each 3H, d, J = 6.7, H-12, H-13); HREIMS *m/z* 396.2309 $[M - H_2O]^+$ (3) (calcd for $C_{25}H_{34}O_5 - H_2O$, 396.2301).

Jaeschkeanadiol sinapinate (1o): amorphous foam; IR (liquid film) ν_{max} 3465, 1697, 1632, 1601, 1514, 1283, 1258, 1175, 1154, 1117 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.61 (1H, d, J = 16 Hz, acyl), 6.85 (2H, s, acyl), 6.26 (1H, d, J = 16 Hz, acyl), 5.84 (1H, s, OH), 5.55 (1H, br s, H-9), 5.16 (1H, dt, J = 10, 1 Hz, H-6), 3.94 (3H, s, $-OCH_3$), 2.51 (1H, t, J = 10 Hz, H-7a), 2.23 (1H, dd, J = 10, 1 Hz, H-7β), 1.83 (3H, br s, H-14), 1.08 (3H, s, H-15); 0.94−0.92 (each 3H, d, J = 6.7, H-12, H-13); HREIMS *m/z* 426.2411 $[M - H_2O]^+$ (9) (calcd for $C_{26}H_{36}O_6 - H_2O$, 426.2406).

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Supporting Information Available: ^{13}C NMR data for the unnatural jaeskeanadiol esters **1b**, **1c**, **1k**, **1l**, **1m**, **1n**, and **1o**. This material is available free of charge via the Internet at http://pubs.acs.org.

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