Novel β-Methoxyacrylates of the 9-Methoxystrobilurin and Oudemansin Classes Produced by the Basidiomycete *Favolaschia pustulosa*

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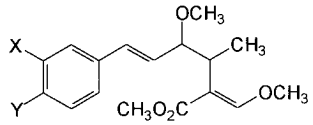
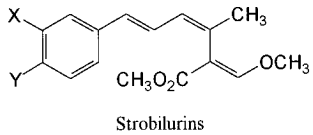
Submerged liquid cultures of the basidiomycete *Favolaschia pustulosa* (Xenova culture collection no. X27732) afforded the novel 9-methoxystrobilurin derivatives, 9-methoxystrobilurin L (**1**) and 9-methoxystrobilurin E (**2**), and the related oudemansin derivative, oudemansin L (**3**). Their structures were established by 2D NMR experiments. Compounds **1** and **3** possess a novel arrangement of two isoprenoid units fused to the aromatic nucleus. Both **1** and **2** have the *E,E,E*-configuration in the pentadienyl side chain as reported previously for 9-methoxystrobilurins. Compound **1** was cytotoxic to cells of the human B lymphoblastoid cell line (Jijoye), with an IC_{50} of 1.8 nM. This cytotoxicity was observed in a 5- day assay only and was not apparent after 2 days. Compound **1** showed some antibacterial activity against *Bacillus subtilis* (MIC = 0.9 μM) and antifungal activity against *Candida albicans* (MIC = 6 μM).

Natural product derivatives of β-methoxyacrylic acid include the strobilurin, oudemansin, and myxothiazol series produced by various fungi and myxobacteria.[1] The strobilurins, oudemansins, and myxothiazols have significant antifungal, insecticidal, antiviral, and antitumor activities, which they cause by binding to a specific site on cytochrome b and inhibiting mitochondrial respiration.[1,2] The fungicidal properties of these compounds have attracted much interest, and synthetic programs based on the β-methoxyacrylates have resulted in the development of broad-spectrum fungicides with potential agricultural use.[1]

The strobilurins and oudemansins are produced by a number of saprotrophic higher fungal species. These include the ascomycete *Bolinia (Camarops) lutea,* a basidiomycete from the family Crepidotaceae (*Crepidotus fulvotomentosus*), and several members of the basidiomycete family Tricholomataceae from the genera *Oudemansiella, Xerula* (formerly a subgenus of *Oudemansiella*), and *Strobilurus* (= *Pseudohiatula*).[1] The structure of strobilurin A was published in 1978[3,4] and was later confirmed by stereocontrolled synthesis.[5] The structure of the first oudemansin, later referred to as oudemansin A, was published 1 year later.[6] The oudemansins differ from the strobilurins in that the 9,10 double bond of the triene system in the side chain is reduced and bears a methoxyl substituent. The structures of the strobilurins and oudemansins are shown in generic form below. Two metabolites of an Ethiopian *Favolaschia* species have recently been identified as the first metabolites of a new series that links the strobilurins and oudemansins, the 9-methoxystrobilurins.[7] We have now isolated and identified two further 9-methoxystrobilurins, 9-methoxystrobilurin L (**1**) and 9-methoxystrobilurin E (**2**), and a novel oudemansin (**3**), oudemansin L, produced by *Favolaschia pustulosa* (Jungh.) Singer and report the details herein.



Oudemansins



Strobilurins

Results and Discussion

During the course of a screening program devoted to the search for new compounds with antiinflammatory and antiallergic properties, the CH_2Cl_2 and MeOH extracts of the biomass of submerged fermentations of a wood-rotting basidiomycete collected from a tropical forest were found to contain a series of active compounds. This isolate, derived from fruit-body tissue, had dikaryotic mycelium with clamped septa, which was consistent with that of the target species. This sample was identified from the dried poroid white fruit body as *Favolaschia pustulosa* (Jungh.) Singer, which is currently accommodated in the family Tricholomataceae. Initial activity in the absence of detectable cytotoxicity was determined in a 2-day bioassay for interleukin-4-dependent activation of cells of the human B lymphoblastoid cell line (Jijoye). Bioassay-directed fractionation of the extract led to the isolation of the 9-methoxystrobilurins L (**1**) and E (**2**) and oudemansin L (**3**). Subsequent investigation of **1** indicated cytotoxicity in longer-term (5 day) culture (IC_{50} = 1.8 nM) with the same human B lymphoblastoid cell line (Jijoye). Jijoye cells were seeded at 10^5/well or 5×10^5/well and cultured in the presence and absence of **1** at the concentrations indicated for 2 or 5 days. An LC_{50} value

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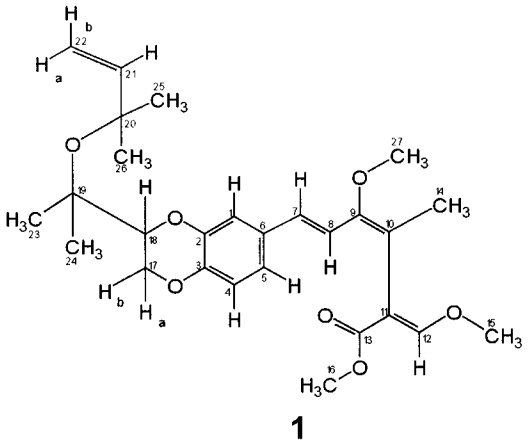

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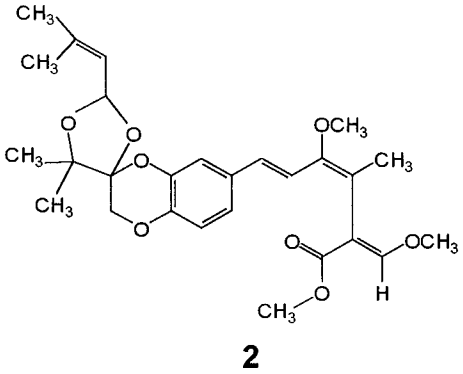

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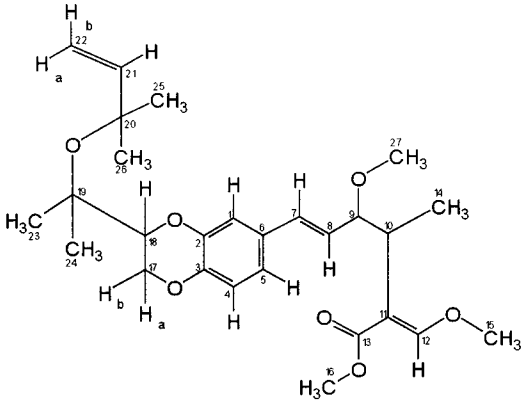

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(drug concentration resulting in 50% lethality) of 1.8 nM was obtained at 5 days' culture (Figure 1). These results are similar to the cytostatic properties reported for strobilurin E.[8]

MIC values against *Bacillus subtilis* and *Candida albicans* for **1** were 0.9 and 6 μM, respectively, as determined by the conventional serial dilution method using Sabouraud dextrose broth for fungi and nutrient agar for bacteria and incubating at 37 °C for 18 h.

Examination of the ^{1}H- and ^{13}C-NMR spectra of **1** revealed eight methyl groups, including signals characteristic of three methoxyl substituents; two methylene groups, one of which gave a signal at 114.9 ppm characteristic of unsaturation, and eight methine groups. The ^{13}C-NMR spectrum showed a resonance at 169.9 ppm, which indicated the presence of a single carbonyl group. There were three quaternary carbon signals at 154.3, 147.9, and 152.2 ppm, typical of unsaturated

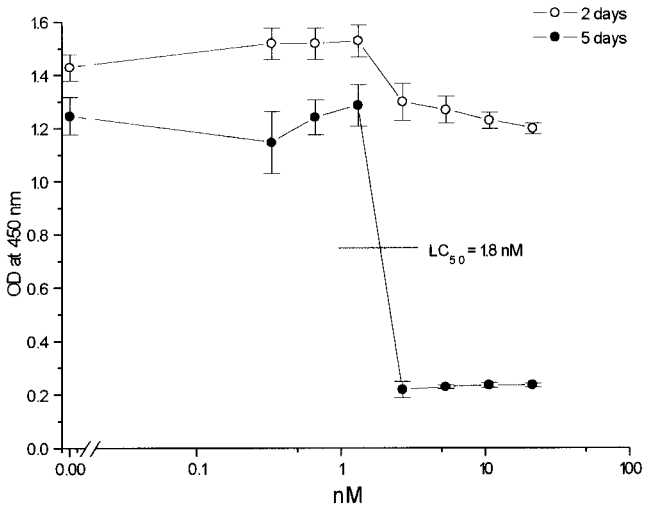

Figure 1. Cytotoxicity of **1** after 2 and 5 days in cultures of Jijoye cells. Cell viability was determined by metabolism of XTT.

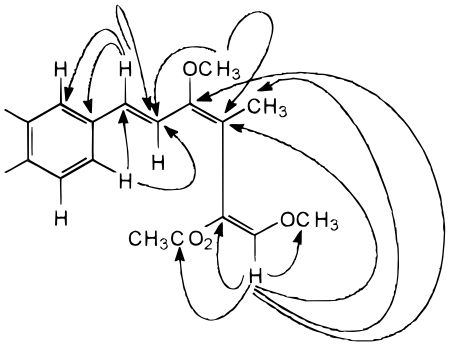

Figure 2. HMBC correlations for the β-methoxyacrylate fragment of **1**.

carbons bearing oxygen, and three further unsaturated quaternary carbon signals at 134.5, 119.6, and 111.9 ppm. The chemical shifts of the remaining two quaternary signals at 82.9 and 77.4 ppm also indicated attachment to oxygen. Closer inspection of the ^{1}H-NMR spectrum revealed signals for three aromatic protons, a doublet of doublets centered at 6.97 ppm, and two doublets centered at 6.84 and 6.80 ppm. Two doublets centered at 6.54 and 6.39 ppm showed a $J_{HH} = 15.0$ Hz indicating an isolated *trans* double bond. HMBC correlations linked the aromatic system, the *trans* double bond, and methoxyl substituents with all of the unsaturated quaternary carbons, identifying **1** as a 9-methoxystrobilurin derivative (Figure 2).

Comparison of the NMR spectra of **1** with those of previously identified strobilurins[8] confirmed that **1** did possess the methyl β-methoxyacrylate group linked at the α position to a substituted phenylpentadienyl unit, common to all known strobilurins. This was further confirmed by the HMBC correlations, particularly those of the methine proton at $\delta_H = 7.44$ ppm, $\delta_C = 161.6$ ppm (Figure 2). The presence of a strong cross peak between the methoxy proton signal at 3.62 ppm and the methyl proton signal at 1.85 ppm in the NOESY spectrum suggests that **1** has the same *E*,*E*,*E*-configuration as found for 9-methoxystrobilurins A and K.[7]

Examination of the COSY-45 spectrum of **1** indicated two further isolated spin systems. The first of these was equivalent to three protons consisting of two doublets of doublets at 4.20 and 4.0 ppm and an unresolved

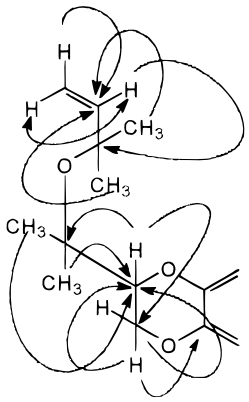

Figure 3. HMBC correlations for the isoprenoid fragment of **1**.

multiplet at 3.70 ppm. HMQC correlations allowed these signals to be assigned to the carbon resonances at 72.8 and 77.7 ppm. The chemical shift of these carbon signals, together with the shifts of the signals at 147.9 and 152.2 ppm, pointed to a dioxan ring fused to the aromatic system at positions 2 and 3, as in strobilurin E.[8] The second spin system consisted of three doublets of doublets centered at 5.94, 5.22, and 5.19 ppm, respectively. HMQC correlations linked the first of these proton signals to the carbon at 145.3 ppm and the other two to the carbon at 114.9 ppm, indicating a terminal methylene moiety. The remaining proton and carbon resonances were four methyl singlets at 28.2, 22.6, 26.4, and 26.8 ppm and two quaternary carbons at 82.9 and 77.4 ppm. Detailed examination of the HMBC correlations indicated that these two proton-spin systems, the methyl singlets and the quaternary carbons, were due to two isoprenoid units consisting of a 1,1-dimethyl-2-propenyloxy group and a 3,3-dimethylpropylene-1,2-dioxy group, linked by an oxygen bridge and fused to the aromatic ring as shown in Figure 3, thus establishing the structure of **1**. The stereochemistry at position 18 is currently undetermined.

Comparison of the NMR spectrum of **2** with the published data of strobilurin E revealed almost exact agreement in both the carbon and proton domains, except for the 9 position, which was methoxylated in **2**. HREIMS mass measurement of **2** confirmed its identity as 9-methoxystrobilurin E, which also has the *E,E,E*-configuration as confirmed by NOESY correlations.

The methoxyl substitution at the 9 position marks **1** and **2** as the third and fourth members of the 9-methoxystrobilurin class of β-methoxyacrylate antibiotics, all of which have been isolated from *Favolaschia* spp.

Compound **3** was isolated only in small amounts. The ^{1}H-NMR spectrum showed that the signal due to the H-8 proton was a doublet of doublets and that two extra signals, a doublet of doublets at 3.95 ppm and an unresolved multiplet at 2.15 ppm for H-9 and H-10, were now present. The methoxy protons at 3.62 ppm were shifted to 3.35 ppm, and the methyl singlet at 1.85 ppm appeared as a doublet at 1.48 ppm. All other signals were identical to the corresponding signals in **1**, confirming the proposed structure of **3** as a new oudemansin analogue of **1** in which the 9,10 double bond is reduced.

These new *F. pustulosa* metabolites are structurally among the most complex of the strobilurin and oudemansin series yet reported, alongside strobilurins D, E, and G and 9-methoxystrobilurin K, all of which possess two isoprenoid units fused in various ways to the central aromatic ring.

Experimental Section

General Experimental Procedures. UV-vis spectra were recorded on a Perkin−Elmer Lambda-17 UV-vis spectrophotometer. IR spectra were recorded on a Nicolet 5PC FTIR spectrophotometer using a Spectra Tech "collector" diffuse reflectance accessory. LREIMS and DCIMS were obtained on a VG Trio 3 triple quadrupole mass spectrometer. HREIMS were obtained on a Finnigan MAT 95 mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded at 308 K on a Bruker ACF 400 spectrometer at 400 MHz, and 100 MHz respectively. All chemical shifts (δ) are quoted in ppm referenced to an external TMS standard. Standard techniques were used to obtain DEPT, COSY-45, HMQC, HMBC, and NOESY spectra. In HMBC experiments the long-range coupling constants $^{3-5}J_{CH}$ was optimized for 5 Hz. Mixing times form 570 to 1200 ms were used in the NOESY experiments.

Fungal Material. *F. pustulosa* Jungh. Singer (Xenova culture collection no. X27732) was collected from rotting wood in a tropical forest.

Fermentation and Extraction. A cell suspension was obtained by adding 5 mL of a 0.1% Tween 80−10% glycerol solution to a well-grown slope culture of *F. pustulosa*. The cell suspension (1 mL) was used to inoculate a 250-mL conical flask containing 4 mL of seed medium (20 g/L malt extract, 20 g/L glucose, 1 g/L bacto peptone), which was incubated statically for 5 days at 25 °C (seed stage 1). Five 2-L conical flasks containing 300-mL seed medium each were inoculated with 7 mL of the culture from seed stage 1 and incubated statically at 25 °C for 16 days (seed stage 2). A 14-L fermenter containing 10 L of production medium (9.75 g/L 2-*N*-morpholinoethanesulfonic acid, 19.9 g/L sucrose, 14.51 g/L glutamate sodium salt, 0.25 g/L K_2HPO_4, 0.5 g/L KCl, 1 mL/L Tween 80, 2 mL/L of a 1% $MgSO_4$ solution, 2 mL/L of a 1% $CaCl_2$ solution, 20 mL/L of a vitamin mix, 5 mL/L trace element mix, pH = 6.0) was inoculated with the seed stage 2 cultures and incubated at 25 °C with an aeration rate of 0.5 vvm and 350 rpm agitation for 9 days. The culture was harvested via centrifugation, both biomass and liquor being retained. The biomass from this fermentation of *F. pustulosa* was freeze dried and sequentially extracted with 3 × 2 L of CH_2Cl_2 and 3 × 2 L of MeOH. The solvent extracts were combined, concentrated to dryness *in vacuo*, and resuspended in 15 mL of MeOH.

Cytotoxicity. Jijoye cell viability was determined as described by Roehm *et al.*[9] using a colorimetric assay based on metabolism of the tetrazolium salt, XTT, sodium 3′-((1-(phenylamino))-carbonyl)-3,4-tetrazolylbis-(4-methoxy-6-nitrobenzenesulfonic acid) hydrate. Dehydrogenase enzymes of metabolically active cells cleave XTT to yield a highly colored H_2O-soluble formazan product. Bioreduction of XTT is potentiated by addition of the electron-coupling agent phenazine methosulfate (PMS). XTT/PMS mixture is added to the test-cell suspension at the end of the assay period to final concentrations of 200 μg/mL of XTT and 25 μg/mL of PMS. After an additional 3 h of incubation at 37 °C, the OD of the culture wells were determined at 450 nm.

Under the conditions used, OD is proportional to cell concentration.

Purification. The cell extract was redissolved in MeOH and purified by reversed-phase HPLC on two Prep Nova-Pak HR C_{18} radial compression cartridge columns (40 × 100 mm, 6 μm particle size, 60 Å pore size, Waters WAT037704) connected in series in a Prep Pak holder assembly with extension (Waters) along with a Prep Nova-Pak HR C_{18} Guard-Pak insert (Waters, WAT037854). Elution was achieved with an isocratic mobile phase (70% MeCN:30% H_2O) applying UV detection at 220 nm. 9-Methoxystrobilurin L (**1**, 10 mg) was obtained as a colorless oil. Oudemansin L (**3**, 2 mg) was obtained as a white powder. 9-Methoxystrobilurin E (**2**, 100 mg) was obtained as a white powder.

9-Methoxystrobilurin L (1): UV λ_{max} (ϵ) 306 nm (23 600), 229 nm (23 000), 202 nm (44 000); IR (KBr) ν_{max} (cm^{-1}) 2977, 2933, 2852, 1708, 1627, 1569, 1502, 1495, 1434, 1417, 1328, 1368, 1265, 1195, 1126, 1066, 1202, 931, 862, 771; the terminal double bond at C21−22 gives rise to the bands at 1627, 1434, and 931 cm^{-1}; the α,β unsaturated ester gave three strong bands at 1708, 1265, and 1126 cm^{-1}; MS [DCI + (NH_3)] *m/z* 473 MH+, 441, 373, 305; (EI^+) *m/z* 472 $M^{\bullet+}$, 440, 372, 305; (ESI) *m/z* 473 MH^+, 441, 373, 305; HREIMS mass measurement obsd 472.2434, calcd for $C_{27}H_{36}O_7$ 472.24610; HREIMS mass measurement obsd 441.2245; calcd for $C_{26}H_{33}O_6$ 441.22771; ^1H NMR (CD_3OD, 400 MHz) δ 7.44 (1 H, s, H-12), 6.97 (1 H, dd, J = 2.1, 8.2 Hz, H-5), 6.84 (1 H, d, J = 2.1 Hz, H-1), 6.80 (1 H, d, J = 8.2 Hz, H-4), 6.54 (1 H, d, J = 15.0 Hz, H-7), 6.39 (1 H, d, J = 15 Hz, H-8), 5.90 (1 H, dd, J = 17.4, 10.0 Hz, H-21), 5.22 (1 H, dd, J = 18.0, 0.7 Hz, 22-Ha), 5.19 (1 H, dd, J = 10.0, 0.7 Hz, 22-Hb), 4.20 (1 H, dd, J = 12.3, 3.1 Hz, 17-Ha), 4.00 (1 H, dd, J = 12.4, 7.5 Hz, 17-Hb), 3.82 (3 H, s, H-15), 3.70 (1 H, m, H-18), 3.70 (3 H, s, H-16), 3.62 (3 H, s, H-27), 1.85 (3 H, s, H-14), 1.39 (3 H, s, H-23), 1.32 (3 H, s, H-25), 1.32 (3 H, s, H-26), 1.21 (3 H, s, H-24); ^{13}C NMR (CD_3OD, 100 MHz) δ 169.9 (s, C-13), 161.6 (d, C-12), 154.3 (s, C-9), 152.2 (s, C-3), 147.9 (s, C-2), 145.3 (d, C-21), 134.5 (s, C-6), 128.3 (d, C-7), 123.7 (d, C-5), 122.9 (d, C-1), 121.5 (d, C-8), 121.3 (d, C-4), 119.6 (s, C-10), 114.9 (t, C-22), 111.9 (s, C-11), 82.9 (s, C-19), 77.7 (d, C-18), 77.4 (s, C-20), 72.8 (t, C-17), 62.6 (q, C-15), 59.9 (q, C-27), 52.2 (q, C-16), 28.2 (q, C-23), 26.8 (q, C-26), 26.4 (q, C-25), 22.6 (q, C-24), 16.5 (q, C-14).

9-Methoxystrobilurin E (2): UV λ_{max} (ϵ) 318 nm (20 000), 226 nm (23 750), 199 nm (21 650); IR (KBr) ν_{max} (cm^{-1}) 2981, 2938, 2852, 1708, 1629, 1584, 1507, 1436, 1382, 1195, 1061, 1029, 966, 939, 910, 875, 769; MS [DCI + (NH_3)] *m/z* 487 MH^+, 403, 371, 319, 235; (EI^+) *m/z* 471, 319, 235; (ESI) *m/z* 487 MH^+, 319, 235; HREIMS mass measurement obsd 486.221, calcd for $C_{27}H_{34}O_8$ 486.2253; ^1H NMR (CD_3OD, 400 MHz) δ 7.44 (1 H, s, H-12), 6.97 (1 H, dd, J = 2.1, 8.2 Hz, H-5), 7.01 (1 H, d, J = 2.1 Hz, H-1), 6.91 (1 H, d, J = 8.2 Hz, H-4), 6.70 (1 H, d, J = 15.0 Hz, H-7), 6.54 (1 H, d, J = 15 Hz, H-8), 6.00 (1 H, d, J = 9.3, H-20), 5.30 (1 H, d, J = 9.3 Hz, H-21), 4.40 (1 H, d, J = 12.4, 17-Ha), 4.20 (1 H, d, J = 12.3 Hz, 17-Hb), 3.92 (3 H, s, H-15), 3.80 (3 H, s, H-16), 3.72 (3 H, s, H-27), 1.95 (3 H, s, H-14), 1.84 (3 H, s, H-23), 1.80 (3 H, s, H-24), 1.48 (3 H, s, H-25), 1.40 (3 H, s, H-26); ^{13}C NMR (CD_3OD, 100 MHz) δ 169.9 (s, C-13), 161.6 (d, C-12), 154.3 (s, C-9), 143.8 (s, C-2), 143.0 (s, C-22), 142.5 (s, C-3), 133.0 (s, C-6), 128.2 (d, C-7), 123.1 (d, C-21), 121.7 (d, C-5), 121.2 (d, C-8), 119.3 (s, C-10), 117.8 (d, C-4), 115.9 (d, C-1), 111.6 (s, C-11), 102.2 (s, C-18), 99.1 (d, C-20), 84.3 (s, C-19), 67.2 (t, C-17), 62.4 (q, C-15), 59.8 (q, C-27), 52.1 (q, C-16), 28.2 (q, C-23), 26.8 (q, C-26), 26.4 (q, C-25), 22.6 (q, C-24), 16.5 (q, C-14).

Oudemansin L (3): UV λ_{max} (ϵ) 306 nm (23 600), 229 nm (23 000), 202 nm (44 000). IR (KBr), ν_{max} (cm^{-1}) 2977, 2933, 1707, 1628, 1584, 1507, 1434, 1408, 1330, 1194, 1148, 1068, 1059, 1028, 965, 942, 910, 808, 772. The α, β unsaturated ester gives three strong bands at 1707, 1330, and 1148 cm^{-1}; MS [DCI + (NH_3)] *m/z* 475 $MH^{\bullet+}$; (EI^+) 474 $M^{\bullet+}$, HREIMS mass measurement obsd 474.223; calcd for $C_{27}H_{38}O_7$: 474.2253; ^1H NMR (CD_3-OD, 400 MHz) δ 7.44 (1 H, s, H-12), 6.97 (1 H, dd, J = 2.1, 8.2 Hz, H-5), 6.84 (1 H, d, J = 2.1 Hz, H-1), 6.80 (1 H, d, J = 8.2 Hz, H-4), 6.54 (1 H, d, J = 15.0 Hz, H-7), 5.94 (1 H, dd, J = 17.4, 10.0 Hz, H-21), 5.80 (1 H, dd, J = 15.0, 8.0 Hz, H-8), 5.22 (1 H, dd, J = 18.0, 0.7 Hz, 22-Ha), 5.19 (1 H, dd, J = 10.0, 0.7 Hz, 22-Hb), 4.20 (1 H, dd, J = 12.3, 3.1 Hz, 17-Ha), 4.00 (1 H, dd, J = 12.4, 7.5 Hz, 17-Hb), 3.95 (1 H, dd, J = 9.2, 8.0 Hz, H-9), 3.82 (3 H, s, H-15), 3.70 (3 H, s, H-16), 3.70 (1 H, m, H-18), 3.35 (3 H, s, H-27), 2.15 (1 H, m, H-10), 1.48 (3 H, d, J = 6.2 Hz, H-14), 1.39 (3 H, s, H-23), 1.32 (3 H, s, H-25), 1.32 (3 H, s, H-26), 1.21 (3 H, s, H-24).

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