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Natural anti-HIV agents—part I: (+)-demethoxyepiexcelsin and verticillatol from *Litsea verticillata*

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Abstract

The eudesmane sesquiterpenoid, verticillatol (**1**), as well as the lignan, (+)-5′-demethoxyepiexcelsin (**2**), and a known lignan, (+)-epiexcelsin (**3**), were isolated from *Litsea verticillata* Hance. Lignan **2** showed moderate anti-HIV activity with an IC_{50} value of 16.4 μg/ml (42.7 μM), while the known lignan **3** was inactive up to a concentration of 20 μg/ml (48.3 μM). Compound **1** demonstrated weak activity with an IC_{50} value of 34.5 μg/ml (144.7 μM) while being devoid of cytotoxicity at 20 μg/ml. The structures were elucidated by 1D and 2D NMR spectroscopy, and the absolute configuration of the new sesquiterpenoid was determined by the generation of Mosher esters.

Keywords: *Litsea verticillata*; Lauraceae; Verticillatol; Sesquiterpene; (+)-5′-Demethoxyepiexcelsin; Lignan; Sesquiterpene; 2D NMR; Absolute structure; Anti-HIV activity; HOG.R5; Reporter cell line; Green fluorescent protein

1. Introduction

As part of our International Cooperative Biodiversity Group (ICBG) group research project, we examined plants collected at the Cuc Phuong National Park, Vietnam, for anti-HIV, antimalarial, anticancer, and anti-tuberculosis activities (Soejarto et al., 1999). *Litsea verticillata* Hance (Lauraceae) was found to be one of the most promising leads against the human immunodeficiency virus (HIV). Since the phytochemical profile of this plant has not been reported previously, it was selected for fractionation in an attempt to identify any anti-HIV constituents. The MeOH extract from the dried leaves and twigs was partitioned with $CHCl_3$ to yield a mixture, which was then subjected to Si gel chromatography to afford a sesquiterpene and two lignans. The structure of the first isolate was elucidated as a new eudesmane sesquiterpene, and given the name of verticillatol (**1**). Of the two lignans, one was determined to be (+)-5′-demethoxyepiexcelsin (**2**), and the second lignan was established as the known compound (+)-epiexcelsin (**3**) (Russell and Fenemore, 1973). The bioassay-directed isolation, structural elucidation, and anti-HIV activity of these compounds are reported herein.

2. Results and discussion

Compound **1**, $[\alpha]_D^{25}$ −41.2°, was shown to have a molecular formula of $C_{15}H_{26}O_2$ according to HREIMS ($[M]^+$ m/z 238.1927). The IR spectrum revealed an absorption band at ν_{max} 3432 cm^{-1} that was ascribed to a hydroxyl group. From the ^1H and ^{13}C NMR, and DEPT spectra, **1** was deduced to contain a tertiary methyl (δ 1.15), an isopropyl (δ_H 0.89, 0.90 and 1.43; δ_C 20.1, 20.2 and 33.3), an oxy-methine (δ_H 4.73), an exo-methylene (δ_H 4.87 and 4.93; δ_C 107.1 and 153.8), a quaternary oxy-carbon (δ_C 75.3), five methylenes (δ 24.8, 31.0, 31.0, 32.2, 34.7), one methine (δ 38.7), and a

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quaternary carbon (δ 43.3). Based on the above features, **1** was deduced to be a sesquiterpenoid. The ^{1}H–^{1}H COSY and HMBC NMR spectral data further defined **1** as an eudesmane sesquiterpenoid. The single secondary hydroxyl in **1** was assigned to C-1 due to the presence of ^3J correlations between C-1 (δ_C 72.2) and Me-15 (δ_H 1.15), H-1 (δ_H 4.73) and C-3 (δ_C 31.0), and H-1 and C-9 (δ_C 31.0), while the tertiary hydroxyl was assigned to C-5 due to the ^3J correlations between C-5 (δ_C 75.3) and H_2-14 (δ_H 4.87 and 4.93).

The relative stereochemistry of **1** was determined by means of a NOESY experiment. The NOEs of H-1 (δ_H 4.73) to H-9β (δ_H 2.25), and of H-9β to H-7β (δ_H 2.07) revealed both hydroxyls at C-1 and the isopropyl group at C-7 to be α-orientated. The NOEs between Me-14 (δ_H 1.15) and H-6α (δ_H 1.70), and the NOEs between H-14 and H-8α (δ_H 1.70) assigned the methyl group at C-10 as α. The relative configuration of the hydroxyl group at C-5 was determined based on the following considerations. In the NOESY spectrum of **1**, one of the H_2-14 was observed to have correlations with both protons at C-6. If the hydroxyl group at C-5 was of the β-orientation, a steric conformation with minimized energy showed that the space interval between H-14 and H_2-6 would be too great to generate any NOEs between these protons. However, in the case of an α-orientation, the NOEs between these protons would occur as expected. Therefore, the hydroxyl at C-5 was assigned the β-orientation, which was consistent with published reports for sesquiterpenes having similar structures (Su et al., 2000).

To establish the absolute configuration of **1**, the method of Mosher was applied (Dale and Mosher, 1973; Ohtani et al., 1991). Following treatment of **1** with (*S*)- and (*R*)-α-methoxy-α-trifluoromethyl-phenylacetyl chlorides (MTPA-Cl), respectively, the mono-(*R*)-ester (**1b**) and the mono-(*S*)-ester (**1a**) derivatives at C-1 were obtained (see Materials and Methods section). Analysis of the $\Delta_{H(S\text{-}R)}$ data showed a negative chemical shift distribution for the protons in ring-A and a positive chemical shift distribution for the protons in ring-B (Fig. 1). Consequently, the configuration of C-1 was determined to be *S*. The structure of **1** was, therefore, determined to be (−)-1*S*,5*R*,7*R*,10*R*-eudesm-4(14)-en-1α,5β-diol, and assigned the trivial name of verticillatol.

(+)-5′-Demethoxyepiexcelsin (**2**) was isolated as colorless crystals with a molecular formula of $C_{21}H_{20}O_7$ by HRFABMS ([M]$^+$ *m*/*z* 384.1221). The ^{1}H and ^{13}C NMR spectroscopic data of **2** revealed the presence of a piperonyl group [δ_H 6.87 (1H, *brs*), 6.83 (1H, *d*), 6.78 (1H, *d*), and 5.95 (2H, *brs*); and δ_C 147.9 (*s*), 147.2 (*s*), 135.0 (*s*), 119.5 (*d*), 108.1 (*d*), 106.5 (*d*), and 101.0 (*t*)] and a 3-methoxy-4, 5-methylenedioxyphenyl [δ_H 6.59 (1H, *brs*), 6.52 (1H, *brs*), 5.98 (2H, *brs*), and 3.86 (3H, *s*, OMe); and δ_C 148.8 (*s*), 143.5 (*s*), 134.1 (*s*), 132.9 (*s*), 104.8 (*d*), 101.4 (*t*), 99.8 (*d*), and 56.6 (*q*)]. The remaining

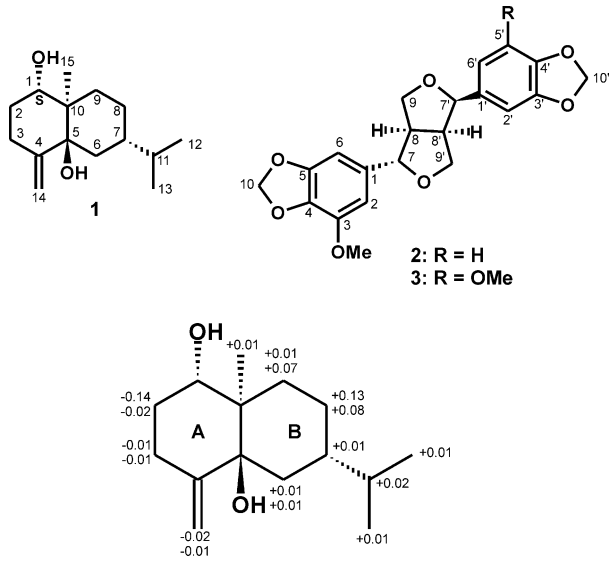

Fig. 1. Structures of compounds **1**–**3**, and $\Delta\delta$ (in ppm) values (δ_S–δ_R) for (*R*)- and (*S*)-MTPA esters of compound **1**.

$C_6H_8O_2$ portion of the molecule was then determined to be a 3,7-dioxabicyclo-[3,3,0]-octane unit according to spectral data interpretation and biogenetic considerations. Through an analysis of the HMBC spectrum of **2**, the 3-methoxy-4, 5-methylenedioxyphenyl unit was assigned to C-7 of one of the tetrahydrofuran group of the dioxabicyclo-[3,3,0]-octane unit and the piperonyl group was assigned to C-7′ of the second tetrahydrofuran unit. The upfield chemical shift of C-7 (δ_C 82.0) and C-8 (δ_C 50.1) and the downfield chemical shift of C-7′ (δ_C 87.6) and C-8′ (δ_C 54.5) strongly suggested that the 3-methoxy-4, 5-methylenedioxyphenyl and the piperonyl units were oriented in the α- and β- positions, respectively. Further evidence for this assignment was supported by observation of the coupling pattern of H-7 (J = 4.9 Hz) and H-7′ (J = 7.0 Hz) (Pelter et al., 1976). (+)-Demethoxyexcelsin, a compound with the same molecular formula but a different stereochemistry has been reported (Russell and Fenemore, 1973; De Carvalho et al., 1987). The comparison of NMR spectral data of **2** with those reported for (+)-demethoxyexcelsin clearly indicated a different stereo configuration at C-7 for these two compounds. Furthermore, the known compound, (+)-epiexcelsin (**3**), which is structurally similar to **2**, was isolated from the adjacent chromatographic fraction. The similarities between the ^{1}H and ^{13}C NMR spectra of **2** and **3** suggested that these isolates have identical stereochemistries. Accordingly, the structure of **2** was elucidated as (+)-5′-demethoxy epiexcelsin.

Many compounds of plant origin have been identified that inhibit different stages in the replication cycle of the HIV. Lignans have been known to interfere with the processes of integration (Eich et al., 1996) and reverse transcription (Rimando et al., 1994; Fujihashi et al.,

1995; Chen et al., 1996; Hara et al., 1997). The tetrahydronaphthalene-type lignans have also been reported to exhibit selective inhibitory activities towards herpes simplex virus and cytomegalovirus (Markkanen et al., 1981; MacRae et al., 1989). Due to the diversity of structures that can arise from the dimerization of two phenylpropanoid units, it is easy to rationalize the multiplicity of antiviral targets that are associated with this class of compounds.

During the initial screen, the total chloroform extract of *Litsea verticillata* inhibited HIV-1 replication by 50% at a concentration of 20 μg/ml with minimal toxicity (90% cell viability). Consequently, the isolates **1**, **2** and **3** were tested for in vitro inhibitory effects against HIV replication in HOG.R5 cells. The data are listed in Table 1. Among these compounds, only the tetrahydrofurofuran lignan, (+)-5′-demethoxyepiexcelsin (**2**), demonstrated anti-HIV-1 activity with an IC_{50} value of 16.4 μg/ml (42.7 μM). The associated selectivity index (SI) value of 1.4 was considered unfavorable for the further development of the compound. Compound **3** is a related lignan that differed from **2** only by the presence of a methoxy group at position 5′. Compound **3** lacked inhibitory activity against HIV-1 replication; a property that could be attributable to the 5′-methoxy group and the reduced solubility of **3** compared with **2**. The eudesmane sesquiterpenoid (**1**) demonstrated weak activity against HIV-1 [IC_{50} = 34.5 μg/ml (144.7 μM)] which was notable because of the complete lack of toxicity up to a concentration of 20 μg/ml. The concentration at which **1** mediates a 50% cytotoxic response (CC_{50}) could not be determined because of the limited quantity of material isolated. Thus far, there have been no reports on the anti-HIV activity of this class of sesquiterpenoids in the literature. The plant kingdom should be explored further for these chemotypes as new leads for anti-HIV drug development before an assessment can be made regarding their potential for clinical development.

3. Experimental

3.1. General

Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. IR spectra were recorded on a Jasco FT/IR-410 spectrometer as a film on a KBr plate. 1D and 2D NMR spectra were recorded on a Brüker DRX-500 MHz spectrometer. Chemical shifts (δ) were expressed in ppm with reference to internal TMS. All NMR experiments were obtained by using standard pulse sequences supplied by the vendor. Column chromatography was carried out on Si gel (200–400 mesh, Natland International Corporation). Thin-layer chromatography was performed on Whatman glass-backed plates coated with 0.25 mm layers of Si gel 60. EIMS and HREIMS spectra were recorded on a Finnigan Mat 95 spectrometer.

3.2. Plant material

The initial collection of leaf, twig and flowerbud samples (SVA-0001) of *Litsea verticillata* Hance (Lauraceae) were made at the Cuc Phuong National Park (CPNP) on 22 November 1998, and was documented by voucher specimens Soejarto et al. *10352*. A large amount of the plant sample (SVA-0001, 4.5 kg, voucher specimens Soejarto et al. 11003) was subsequently recollected at the same site at CPNP on 17 November 1999, for complete isolation work. Duplicate voucher specimens of both collections have been deposited at the herbaria of CPNP, Institute of Ecology and Biological Resources (IEBR at National Center for Science and Technology, Hanoi), and the Field Museum of Natural History (Chicago, IL, USA).

3.3. Assay for the inhibition of HIV infectivity with HOG.R5 cells

A reporter cell line for quantitating HIV-1 replication was developed using HOS (human osteosarcoma) cells rendered susceptible to HIV-1 infection by the transfection of genes for CD4 and CCR5, the coreceptor utilized by macrophage-tropic (R5) HIV-1 isolates (Tan et al., 1997). This microtiter assay is based on the transactivation of a stably-integrated HIV-1 LTR-green fluorescent protein (GFP) transcription unit. Upon HIV-1 entry into these HOS target cells, Tat expression increases the HIV LTR-directed transcription of the GFP gene as demonstrated by the enhanced fluorescence of detergent lysates of infected cells relative to lysates of uninfected controls.

HOG.R5 cells were plated in 96-well microtiter plates at a density of 4000 per well in DMEM (100 μl) containing 10% fetal bovine serum, 4 μM L-glutamine, 100

Table 1
Anti-HIV activity and cytotoxicity of compounds (**1**–**3**) isolated from *Litsea verticillata*

Compound	Name selectivity	Cytotoxicity to HOG.R5 [CC_{50}; μg/ml (μM)]	Anti-HIV activity (IC_{50})	Index
1	Verticillatol	Nontoxic at 20 μg/ml	34.5 (144.7)	–
2	(+)-5′-Demethoxyepiexcelsin	23 (59.8)	16.4 (42.7)	1.4
3	(+)-Epiexcelsin	Nontoxic at 20 μg/ml	Inactive	–

units/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37 °C for 24 h prior to each assay, during which time cells generally reached 20% confluence. The anti-HIV activity of pure compounds and plant extracts was assessed by the addition of test agents to cells in triplicate just before the addition of virus (HIV-1_{IIIB}, 5 ng p24/ml final inoculum concentration). Relevant controls consisting of infected cells which had not been treated with the test material were included, and infected cultures were incubated for 4 days. At the end of the assay, the media were completely removed and 200 μl of 0.5% (v/v) Nonidet P-40 in phosphate buffered saline (PBS) were added to each well. The contents were mixed by repeated pipeting, and then transferred to black U-bottom plates (Dynex) designed specially for fluorometric applications. GFP fluorescence signal was quantitated as relative fluorescence units (RFUs) at excitation and emission wavelengths of 485 nm and 535 nm, respectively, using the Packard FluoroCount™ fluorometer interfaced with a computer equipped with the Packard PlateReader v3.0 software for data acquisition and analysis. The background fluorescence due to basal GFP expression in uninfected cells was subtracted from the fluorescence signal of all infected cultures, after which the percent remaining fluorescence output (% control infection,%CI) of test wells was determined relative to those of untreated but infected control cultures. The median inhibitory concentration (IC_{50}) was computed from a linearly regressed dose–response plot of% control fluorescence versus concentration or log concentration of compound, utilizing at least five concentrations of each test agent. The positive control compound used was 3TC (Lamivudine) which had an IC_{50} value of approximately 1.2 μM in the HOG.R5 system utilizing the assay conditions described above. This nucleoside reverse transcriptase inhibitor and the virus stock of HIV-1_{IIIB}/H9 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

3.4. Assay for cytotoxicity

The toxicity of pure compounds and plant extracts to HOG.R5 cells in 96-well microtiter plates was evaluated in parallel assays where virus was omitted. Untreated cells served as controls. A decrease in the basal level of fluorescence of cell lysates coupled with microscopic evidence of cell death in the presence of the test agent were indicative of an adverse effect on cell growth. Only compound concentrations that were devoid of cellular toxicity were employed for generating useful dose responses.

3.5. Extraction and isolation

The dried and milled leaves and twigs (4.5 kg) were extracted with MeOH, and the extract was subsequently defatted with *n*-hexane and partitioned with $CHCl_3$. The $CHCl_3$-soluble fraction (93.0 g) was applied to a Si gel column (400 g), which was then developed by gradient elution with petroleum ether and increasing concentrations of Me_2CO to afford 16 fractions. Fraction 6 was applied further to a Si gel column and eluted with petroleum ether/EtOAc to afford compound **1** (13.0 mg). Work-up of fractions 7 and 8 yielded (+)-5′-demethoxyepiexcelsin (**2,** 39.0 mg) and (+)-epiexcelsin (**3**, 852.8 mg), respectively.

3.6. Verticillatol (*1*)

Colorless oil, $[\alpha]_D^{25}$ −41.2° ($CHCl_3$; *c* 0.13). IR ν_{max} (film): 3432, 2932, 2869, 1016 cm^{-1}. ^1H NMR (pyridine-d_5, *J* in Hz): δ 4.93, 4.87 (1H each, *brs*, H_2-14), 4.73 (1H, *dd*, *J* = 11.5, 4.9, H-1β), 3.18 (1H, *td*, *J* = 13.6, 5.6, H-3α), 2.25 (1H, *m*, H-9β), 2.18 (1H, *m*, H-3β), 2.17 (1H, *m*, H-9α), 2.15 (1H, *m*, H-2β), 2.07 (1H, *m*, H-7), 1.99 (1H, *m*, 2α-H), 1.82 (1H, *dd*, *J* = 12.7, 2.7, H-6β), 1.70 (1H, *brt*, *J* = 12.7, H-6α), 1.70 (1H, *brt*, *J* = 12.7, H-8α), 1.43 (1H, *m*, H-11), 1.38 (1H, *m*, H-8β), 1.15 (3H, *s*, Me-15), 0.90, 0.89 (3H each, *d*, *J* = 6.0, Me-12 and-13). ^{13}C NMR (pyridine-d_5): δ 72.2 (*d*, C-1), 32.2 (*t*, C-2), 31.0 (*t*, C-3), 153.8 (*s*, C-4), 75.3 (*s*, C-5), 34.7 (*t*, C-6), 38.7 (*d*, C-7), 24.8 (*t*, C-8), 31.0 (*t*, C-9), 43.3 (*s*, C-10), 33.3 (*d*, C-11), 20.1 and 20.2 (each *q*, C-12 and C-13), 107.1 (*t*, C-14), 13.3 (*q*, C-15). EIMS: *m*/*z* 238 (51), 220 (26), 205 (100), 182 (17), 178 (33), 160 (69), 141 (40), 131 (20), 116 (21), 105 (29), 91 (38), 77 (40), 63 (35), 51 (26), 35 (39), 21 (32), 18 (42). HREIMS: *m*/*z* 238.1927 $[M]^+$ (calcd for $C_{15}H_{26}O_2$: 238.1933).

3.7. Preparation of (*S*)-MTPA ester of *1*

To a solution of 2.0 mg of **1** in 0.5 ml of dry pyridine was added sequentially 4-(dimethylamino) pyridine (0.2 mg) and (*R*)-(−)-α- MTPA chloride (10 mg). The mixture was allowed to react overnight under N_2 at room temperature, after which 5 ml of $CHCl_3$ (pre-washed first with water, and then with 5% $NaHCO_3$ solution) were added. The mixture was then passed through a disposable pipet (0.6×5 cm) packed with Si gel and eluted with 5 ml of $CHCl_3$ to yield (*S*)-Mosher ester of compound **1** as a colorless oil: ^1H NMR (pyridine-d_5): δ 6.07 (1H, *dd*, *J* = 11.8, 4.9, H-1β), 4.90, 4.83 (1H each, *brs*, H_2-14), 3.07 (1H, *m*, H-3α), 2.25 (1H, *m*, H-9β), 2.15 (1H, *m*, H-3β), 2.18 (1H, *m*, H-9α), 2.15 (1H, *m*, H-2β), 2.01 (1H, *m*, H-7), 1.76 (1H, *dd*, *J* = 12.7, 2.7, H-6β), 1.70 (1H, *m*, 2α-H), 1.58 (1H, *m*, H-6α), 1.58 (1H, *m*, H-8α), 1.39 (1H, *m*, H-11), 1.22 (1H, *m*, H-8β), 0.94 (3H, *s*, Me-15), 0.85, 0.83 (3H each, *d*, *J* = 6.0, Me-12 and Me-13).

3.8. Preparation of (*R*)-MTPA ester of *1*

Treatment of **1** with (*S*)-(−)-α- MTPA chloride as described above for the preparation of the (*S*) Mosher ester yielded the (*R*)-Mosher ester of **1** as a colorless oil:

^{1}H NMR (pyridine-d_5): δ 6.09 (1H, *dd*, $J=11.7$, 4.9, H-1β), 4.92, 4.84 (1H each, *brs*, H_2-14), 3.07 (1H, *m*, H-3α), 2.18 (1H, *m*, H-9β), 2.16 (1H, *m*, H-3β), 2.17 (1H, *m*, H-9α), 2.17 (1H, *m*, H-2β), 2.00 (1H, *m*, H-7), 1.84 (1H, *m*, 2α-H), 1.76 (1H, *dd*, $J=12.7$, 2.7, H-6β), 1.57 (1H, *m*, H-6α), 1.47 (1H, *m*, H-8α), 1.37 (1H, *m*, H-11), 1.14 (1H, *m*, H-8β), 0.93 (3H, *s*, Me-15), 0.84, 0.82 (3H each, *d*, $J=6.0$, Me-12 and Me-13).

3.9. (+)-5′-Demethoxyepiexcelsin (**2**)

Colorless crystal from Me_2CO, $[\alpha]_D^{20}$ +116.3° ($CHCl_3$; *c* 1.35). ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.87 (1H, *brs*, H-2′), 6.83 (1H, *d*, $J=8.1$, H-6′), 6.78 (1H, *d*, $J=7.8$, H-5′), 6.59 (1H, *brs*, H-2), 6.52 (1H, *brs*, H-6), 5.98 (2H, *brs*, H_2-10), 5.95 (2H, *brs*, H_2-10′), 4.82 (1H, *d*, $J=4.9$, H-7), 4.40 (1H, *d*, $J=7.0$, H-7′), 4.10 (1H, *d*, $J=9.4$, H-9), 3.86 (3H, *s*, 3-OMe), 3.84 (1H, overlap, H-9), 3.84 (1H, overlap, H-9′), 3.32 (1H, overlap, H-9′), 3.32 (1H, overlap, H-8), 2.86 (1H, *brq*, $J=6.8$, H-8′). ^{13}C NMR: δ 132.9 (*s*, C-1), 104.8 (*d*, C-2), 143.5 (*s*, C-3), 134.1 (*s*, C-4), 148.8 (*s*, C-5), 99.8 (*d*, C-6), 82.0 (*d*, C-7), 50.1 (*d*, C-8), 70.9 (*t*, C-9), 101.4 (*t*, C-10), 56.6 (*q*, OMe), 135.0 (*s*, C-1′), 106.5 (*d*, C-2′), 147.2 (*s*, C-3′), 147.9 (*s*, C-4′), 108.1 (*d*, C-5′), 119.5 (*d*, C-6′), 87.6 (*d*, C-7′), 54.5 (*d*, C-8′), 69.6 (*t*, C-9′), 101.0 (*t*, C-10′). CIMS: *m*/*z* 402 $[M+NH_3]^+$ (100), 385 $[M+1]^+$ (68), 367 (13), 263 (16), 233 (15), 203 (3), 181 (3). HRFABMS: *m*/*z* 384.1221 $[M]^+$ (calc. for $C_{21}H_{20}O_7$: 384.1209).

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