Low-molecular-weight Components of Olive Oil Mill Waste-waters

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A new lignan 1-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-(3-acetyl-4-hydroxy-5-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane, the secoiridoid 2H-pyran-4-acetic acid, 3-hydroxymethyl-2,3-dihydro-5-(methoxycarbonyl)-2-methyl-, methyl ester, the phenylglycoside 4-[β-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-1,4-dihydroxy-2-methoxybenzene and the lactone 3-[1-(hydroxymethyl)-1-propenyl] 3-glutarolactone were isolated and identified on the basis of spectroscopic data including two-dimensional NMR, as components of olive oil mill waste-waters. The known aromatic compounds catechol, 4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, tyrosol, hydroxytyrosol, 2-(4-hydroxy-3-methoxy)phenylethanol, 2-(3,4-dihydroxy)phenyl-1,2-ethanediol, p-coumaric acid, caffeic acid, ferulic acid, sinapic acid, 1-O-[2-(3,4-dihydroxy)phenylethyl]-[3,4-dihydroxy]phenyl-1,2-ethanediol, 1-O-[2-(4hydroxy)phenylethyl]-[3,4-dihydroxy]phenyl-1,2-ethanediol, d (+)-erythro-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantrol, p-hydroxybenzenethiol-β-D-glucopyranoside, 2-(3,4-dihydroxyphenylethanol 3-glucopyranoside, and 2-(3,4-dihydroxyphenylethanol 3-glucopyranoside were also confirmed as constituents of the waste-waters. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: Phenols; lignan, secoiridoid; phenylglycoside; olive oil mill waste-waters.

INTRODUCTION

From an economical point of view, olive is the most important fruit tree in the Mediterranean basin, and Italy, Spain, Greece, Tunisia and Morocco are the leading olive oil producers in that area. The olive oil manufacturing process generates an aqueous effluent, referred to as olive oil mill waste-waters (OMW), which has significant polluting properties as indicated by high biological oxygen demand (BOD) (100 kg/m³) and chemical oxygen demand (COD) (200 kg/m³), marked acidity, high concentrations of mineral salts and a high content of toxic aromatic compounds (Borja , 1992; Capasso, 1997; Arienzo and Capasso, 2000).

The annual disposal of several million m³/year of OMW (800,000 m³/year in Italy alone; Mulinacci et al., 2001) poses a major environmental problem for agriculture in the Mediterranean area. OMW are usually released in the soil, but several studies have reported that this practice may be the cause of environmental pollution in the soil (Paredes et al., 1986; Rodriguez et al., 1988; Capasso et al., 1992) as well as in ground and surface waters (DellaGreca et al., 2001). All previous studies indicate that the major toxicity is due to phenolic components. Whilst catechol (1), tyrosol (2) and hydroxytyrosol (3) have been repeatedly found as components of OMW, the presence of other phenolic derivatives apparently depends on the ripeness of the olives, the environment of the olive cultivars, storage time and extraction procedures. In a recent study (DellaGreca et al., 2001), the waste-waters from a Ligurian mill were fractionated by ultrafiltration, nanofiltration and reverse osmosis. Along with 1–3, aromatic compounds 4–17 from the reverse osmosis fraction were isolated and identified (Fig. 1). The present paper reports on investigations performed on the nanofiltered fraction of the same effluent in order to isolate and characterize further low-molecular weight phenols from OMW.

EXPERIMENTAL

General experimental procedures. All reagents were of HPLC grade (Romil, Cambridge, UK) except for ethyl acetate and chloroform, which were of analytical grade. NMR spectra were recorded in 99.8% deuterio-methanol, 99.9% deuterio-chloroform or 99.5% deuterium oxide solutions and were obtained using a Varian (Palo Alto, CA, USA) Inova 500 instrument (1H-NMR at 500.13 MHz; 13C-NMR at 125.75 MHz) with TMS as internal standard. Homonuclear 1H connectivities were determined using two-dimensional (2D) COSY. One-bond heteronuclear 1H-13C connectivities were determined by a two-dimensional proton-detected HMQC experiment (optimised for J 1H-13C = 140 Hz); two- and three-bond 1H-13C connectivities were determined by a two-dimensional proton-detected HMBC experiment (optimised for J 1H-13C = 7 Hz). Low-resolution MS (LREIMS) spectra were determined with a Hewlett Packard (Palo Alto, CA, USA) HP 6890 spectrometer equipped with an MS 5973 N detector.

Analytical TLC was performed on Merck (Darmstadt, Germany) Kieselgel 60 F 254 plates (0.2 mm layer thickness). Spots were visualised by UV light or by spraying with sulphuric acid:acetic acid:water (1:20:4) followed by heating for 5 min at 110°C. Preparative HPLC were

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compounds 21 and 22 were isolated by fractionation on a flash-silica gel column eluted with chloroform:acetone (8:2). The NFW fraction, after extraction with ethyl acetate, was concentrated under reduced pressure and chromatographed on Amberlite XAD-4, eluted first with water (300 mL) and subsequently with methanol (300 mL). The methanol eluate was submitted to flash chromatography, eluting with chloroform:methanol:water (13:7:2), to yield compounds 23 and 24 (Fig. 2) together with several phenols which had been previously detected in the organic phase.

Sugar analysis. A sample of compound 23 (1 mg) was methylated with methyl iodide and dimethylsulphinyl anion as described by Hakomori (1964). The crude reaction product was filtered on a C18 Sep-Pak cartridge (Waters, Milford, MA, USA) that had been previously washed with ethanol (20 mL), acetonitrile (2 mL) and water (10 mL). Fractions were sequentially eluted with water (50 mL), water:acetonitrile (4:1) (8 mL), acetonitrile (2 mL) and ethanol (4 mL). The last two fractions were pooled and evaporated to give the methylated disaccharide, which was hydrolysed with 2 M trifluoroacetic acid. The partially methylated products were reduced with deuterated sodium borohydride, acetylated and analysed by GC-MS.

RESULTS AND DISCUSSION

Various low-molecular weight phenols were isolated and identified from the OMW fraction obtained by fractionation. The known natural products (1–20b) were identified by the analysis of their spectral data and by comparison with literature values, mainly 1H-NMR and 13C-NMR chemical shifts, reported for 1–15 (DellaGreca et al., 2001), 16 and 17 (DellaGreca et al., 2002).
et al., 2000). **18** (DellaGreca et al., 1998), **19** (Limiroli et al., 1996), and **20a** and **20b** (Bianco et al., 1998).

**1-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-(3-acetyl-4-hydroxy-5-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (21)**

EIMS (m/z 432 [M]+; C_{22}H_{24}O_{9}): 1H-NMR (CDCl_{3}) δ_{1} 1.73 (3H, s, Me), 3.33 (1H, m, H-5), 3.82 (1H, dd, J = 3.2 and 8.1 Hz, H-4), 3.92 (3H, s, OMe), 3.95 (3H, s, OMe), 4.28 (1H, d, J = 7.9 Hz, H-8), 4.43 (1H, dd, J = 3.2 and 8.1 Hz, H-4'), 4.45 (1H, d, J = 7.9 Hz, H-8'), 4.77 (1H, d, J = 3.6 Hz, H-6), 5.10 (1H, s, H-2), 6.8–7.1 (5H, aromatic Hs): 13C-NMR δ_{1} 121.6 (C-6), 121.7 (C-5), 109.0 (C-2), 146.3 (C-3), 145.8 (C-4), 114.4 (C-2), 114.0 (C-6), 86.0 (C-7), 59.0 (C-8), 69.9 (C-9), 119.5 (C-1'), 109.0 (C-2'), 146.3 (C-3'), 145.8 (C-4'), 111.7 (C-5'), 121.6 (C-6'), 87.2 (C-7), 97.4 (C-8'), 75.4 (C-9'), 169.6 (CO), 56.1 (C-OMe), 56.1 (C-OMe), 21.0 (C-Me).

The LREIMS showed a molecular ion at m/z 432 and the inverse-gated 13C-NMR spectrum showed twenty carbon signals that integrated for 22 carbons. These data were consistent with the molecular formula C_{22}H_{24}O_{9}. The 1H-NMR spectrum, measured in deuterio-chloroform, showed the aromatic H-2, H-5, H-6, H-2' and H-6' protons as overlapped signals in the 6.84–6.95 ppm range. Further, the spectrum showed the H-7 proton as a singlet at δ_{1} 5.10, the H-9 protons as two doublets at δ_{1} 4.25 and 4.45, the H-7' proton as a doublet at δ_{1} 4.77, the H-8' as a multiplet at δ_{1} 3.33, and the H-9' protons as two double doublets at δ_{1} 4.43 and 3.82, together with two methoxy methyls at δ_{1} 3.89 and 3.92 and one methyl at δ_{1} 1.73. In the 1H spectrum measured in deuterio-methanol, the aromatic meta coupled H-2' and H-6' protons (J = 1.8 Hz) were split at δ_{1} 7.01 and 6.95 indicating the presence of an asymmetrical 1,3,4,5 tetrasubstituted benzene, whereas the signals in the 6.8–6.95 ppm range were typical of a 1,3,4 aromatic ring (Deyama et al., 1987). In the 1H-1H COSY experiment, H-8' was correlated with the methylene H-9' and the methine H-7'. These data indicated that compound 21 was a typical furo-furanoid lignan (Deyama et al., 1987), in which one of the two aromatic rings carried an acetyl group as confirmed by the values in the 13C-NMR spectrum at δ_{1} 169.6 and 21.0. On the basis of the HMOC spectrum, the protons have been correlated to the corresponding carbons, while the entire structure has been assigned on the basis of an HMBC spectrum. In accordance with the assigned structure, the methyl protons at δ_{1} 1.73 yielded an NOE with the aromatic proton H-2'.

**2H-Pyran-4-acetic acid,3-hydroxymethyl-2,3-dihydro-5-(methoxycarbonyl)-2-methyl, methyl ester (22)**

EIMS (m/z 258 [M]+; C_{12}H_{18}O_{6}): 1H-NMR (CDCl_{3}) δ_{1} 7.56 (1H, s, H-6), 4.26 (1H, m, H-2), 3.57 (1H, dd, J = 6.4 and 11.6 Hz, H-8), 3.49 (1H, dd, J = 4.8 and 11.6 Hz, H-8), 2.83 (1H, m, H-4), 2.70 (2H, m, H-9), 1.74 (1H, m, H-3), 1.38 (3H, s, J = 6.8 Hz, CH{sub 3}), 3.68 (3H, s, 11-OMe), 3.65 (3H, s, 2'-OMe): 13C-NMR δ_{1} 75.8 (C-2), 46.7 (C-3),...
31.3 (C-4), 110.2 (C-5), 157.9 (C-6), 21.1 (C-7), 63.9 (C-8), 39.9 (C-9), 171.0 (C-10), 176.0 (C-1′), 53.5 (C-11-OMe), 53.1 (C-2′-OMe).

The LREIMS and the $^{13}$C-NMR data defined the molecular formula $\text{C}_{12}\text{H}_{18}\text{O}_{6}$ for compound 22. The $^1$H-NMR spectrum showed the H-6 olefinic proton as a singlet at $\delta_1 7.56$, the methine H-3, H-4 and H-2 protons as multiplets at $\delta_1 1.74$, 2.83 and 4.26, the methylene H-8 protons as two doublets at $\delta_1 3.49$ and 3.57, the methylene H-9 protons as a multiplet at $\delta_1 2.70$, the H-7 methyl protons as a doublet at $\delta_1 1.38$, and the two methoxyl methyls H-11 and H-13 at $\delta_1 3.68$ and 3.65. Taking into account that in the COSY experiment the correlations between H-2/H-7, H-2/H-3, H-3/H-8, H-3/H-9, H-8/C-2, H-8/C-4, H-4/C-3, H-4/C-2, H-4/C-9, H-6/C-5, H-6/C-10, H-11/C-10, H-9/C12, H-9/C4 supported the assignment of the hydroxymethyl group at C-3, the methoxycarbonyl group at C-5 and the acetic acid methyl ester at C-4.

$4\text{-}[\beta\text{-n-Xylopyranosyl}(1 \rightarrow 6)]\beta\text{-n-glucopyranosyl-1,4-dihydroxy-2-methoxy benzene (23) }$

EIMS ($m/z$ 434 [M]$^+$; $\text{C}_{15}\text{H}_{20}\text{O}_{13}$): $^1$H-NMR (D$_2$O) $\delta_1 6.75$ (1H, d, $J = 2.5$ Hz, H-3), 6.70 (1H, d, $J = 8.0$ Hz, H-5), 6.40 (1H, d, $J = 2.5$ and 8.0 Hz, H-5), 3.68 (3H, s, 2-OMe), 4.29 (1H, d, $J = 7.6$ Hz, H-1′), 3.07 (1H, $t, J = 7.6$ Hz, H-2′), 3.17 (1H, $t, J = 7.6$ Hz, H-3′), 3.70 (1H, m, H-4′), 3.41 (1H, $m, H-5′$), 3.94 (2H, $d, J = 10.6$, H-6′), 4.90 (1H, $d, J = 7.6$ Hz, H-1′), 3.29-3.33 (2H, H-2′ and H-5′), 3.60 (1H, $m, H-3′$), 3.65-3.70 (1H, $m, H-4′$), 3.00 (1H, $t, J = 7.6$ Hz, H-5′)$^\text{13}$C-NMR $\delta_1 150.1$ (C-4′), 147.7 (C-2), 140.4 (C-1), 115.4 (C-6), 108.6 (C-5), 102.9 (C-3), 55.8 (2-OMe), 103.1 (C-1′), 75.0 (C-13-C′), 75.2 (C-5′), 75.2 (C-5′), 69.0 (C-6′), 100.7 (C-1′), 75.0 (C-2′), 68.8 (C-3′), 67.9 (C-4′), 64.7 (C-5′).

The positive FAB MS of compound 23 showed the parent peak at $m/z$ 457 [M + Na]$^+$ which, together with the elemental analysis, defined the molecular formula as $\text{C}_{15}\text{H}_{20}\text{O}_{13}$. The $^1$H-NMR spectrum in the aromatic region showed three protons as a doublet at $\delta 6.75$ ($J = 2.5$ Hz), a doublet at $\delta 6.70$ ($J = 8.0$ Hz), and a double doublet at $\delta 6.60$ ($J = 2.5$ and 8.0 Hz), assigned to positions 2, 6 and 5 of a trisubstituted benzene. Also detectable were other protons which overlapped in the 3.8-3.0 ppm range, and two anomic doublets at $\delta 4.25$ and 4.90. Acid hydrolysis of 23 gave two sugars which were isolated by HPLC and identified as D-glucose and D-xylene by GC analysis (Leontine et al., 1978). The GC-MS identification of 1,5-di-O-acetyl-2,3,4-tri-O-methylxylitol and of 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol following the methylation analysis confirmed the 1 → 6 linkage between glucose and xylene and the couplings of 7.6 Hz for both the anomic protons defined the $\beta$ configuration of the two sugars.

3-[1-(Hydroxymethyl)-1-propenyl] $\delta$-glutarolactone (24)

EIMS ($m/z$ 184 [M]$^+$; $\text{C}_{12}\text{H}_{20}\text{O}_{4}$): $^1$H-NMR $\delta_1 5.62$ (1H, $q, J = 6.0$ Hz, H-8), 4.82 (1H, $d, J = 12.5$ Hz, H-7), 4.53 (1H, $d, J = 12.5$ Hz, H-7′), 3.30 (1H, $m$, H-3), 2.69 (1H, $dd, J = 7.5$ and 15.5 Hz, H-2), 2.75 (1H, $dd, J = 7.5$ and 15.5 Hz, H-2′), 2.47 (1H, $dd, J = 9.0$ and 15.0 Hz, H-4), 2.19 (1H, $dd, J = 10.5$ and 15.0 Hz, H-4′), 1.70 (3H, $d, J = 6.0$ Hz, H-9)$^\text{13}$C-NMR $\delta_1 179.2$ (C-5′), 176.6 (C-1′), 136.0 (C-6′), 125.6 (C-8′), 74.0 (C-7′), 44.0 (C-2′), 32.3 (C-3′), 14.2 (C-9′).

The molecular formula of $\text{C}_{12}\text{H}_{20}\text{O}_{4}$ for compound 24 was based on the MS and $^1$H-NMR data. The $^1$H-NMR spectrum showed the H-8 olefinic proton as a quartet at $\delta 5.65$ coupled with the H-9 doublet methyl at $\delta 1.74$. The H-3 multiplet at $\delta 3.33$ coupled with the H-4 methylene at $\delta 2.47$ and 2.19 and with the H-2 methylene at $\delta 2.69$ and 2.75; the H-7 methylene as two doublets at $\delta 4.50$ and 4.80 was also detected. In the HMBC spectrum the H-3, H-7, and H-8 protons were correlated with the quaternary C-6 carbon. The H-8 had a cross peak with the C-9 carbon, the H-2 proton showed correlation with the C-1 carbonyl carbon at $\delta 176.6$, and the H-4 protons were hetero-correlated with the C-5 carbon at $\delta 179.2$. This molecule derives from breaking of the aglycone part of oleuropeine (Gil et al., 1998).

In the present study the new lignan 21, the seco-iridoid 22, the phenylglycoside 23 and the lactone 24 have been isolated and identified as components of olive oil mill waste-waters along with the previously described aromatic compounds 20a, 21a, 20b, Erythro-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantrol (18), 2(3,4-dihydroxyphenyl)ethanol 3β-n-glucopyranoside (20a) and 2(3,4-dihydroxyphenyl)ethanol 4β-n-glucopyranoside (20b) have been isolated for the first time in olive oil mill waste-waters. The structures were established on the basis of spectroscopic data, including two-dimensional NMR analyses. It is reasonable to conclude, in agreement with the most recent literature in the field, that a detailed knowledge of all compounds to be found in OMW, as well as further studies on their phytotoxicity, is an important step for future developments in solving environmental problems related to the disposal of OMW.

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