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Oxidative chemistry of hydroxytyrosol: isolation and characterisation of novel methanooxocinobenzodioxinone derivatives

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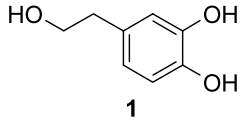
Abstract—Autoxidation or tyrosinase-catalysed oxidation of the natural antioxidant hydroxytyrosol (**1**) in phosphate buffer at pH 7.4 leads to the formation of two main regioisomeric products which could be isolated and identified as the novel methanooxocinobenzodioxinone derivatives **2a**,**b** by extensive spectral analysis.

Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol, **1**) is a potent natural antioxidant which is found both free and as component of the secoiridoid glycoside oleuropein in extra virgin olive oil,[1,2] in olive oil mill waste waters[3] as well as in olive drupes.[3,4] Over the past few years, interest in **1** has increased dramatically following recognition of its broad biological and pharmacological properties.[5]



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The role of **1** and related compounds in the known association between Mediterranean diet and the decreased incidence of coronary heart disease and cancer has also been repeatedly emphasised.[1] The recent development of a range of synthetic routes,[6–8] and the commercial availability of ^{14}C labelled derivative have enabled several studies on the absorption,[9] metabolism[10] and disposition[11] of **1**.

Despite the exponential growth of papers linking the health-beneficial effects of **1** to its potent antioxidant and free radical scavenging properties,[12] the oxidative behaviour of this compound has remained virtually uncharted and little is known about the products formed. Elucidation of this chemistry is relevant to the fate of **1** associated with its antioxidant action in vivo and to the chemical processes underlying quality deterioration of olive oil.

In this paper we report the identification and complete spectral characterisation of the major products formed by oxidation of **1** with different systems of biological relevance.

The oxidation of **1**[13] (1 mM) in 0.05 M phosphate buffer, pH 7.4, was initially investigated in the presence of mushroom tyrosinase, a copper-containing enzyme that catalyses the aerobic conversion of phenols and *o*-diphenols to *o*-quinones.[14] Under such conditions, the reaction proceeded smoothly with gradual darkening of the solution and eventual precipitation of a brownish pigment. After 1 h, HPLC† and TLC‡ analysis of the chloroform-extractable fraction showed the presence of a single major species with a chromophore at 282 nm. No other compound besides unreacted **1** (about 20%) could be observed in the aqueous and organic phases, the remainder of the mixture being apparently accounted for by chromatographically ill-

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† Analytical HPLC was conducted with an octadecylsilane column (5 μm, 4.6 mm×25.0 cm); eluent: 0.5% acetic acid/water (solvent A) and acetonitrile (solvent B) gradient as follows: 2% B, 5 min, from 2 to 70% B, 15 min; flow rate: 1.0 mL/min; UV detection at 280 nm.

‡ Analytical and preparative TLC was performed on 0.25 and 0.50 mm silica gel plates, respectively; eluent: chloroform/methanol 95:5 v/v.

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Table 1. Spectral data of compounds **2a**,**b**

Position number	**2a**		**2b**	
	^{1}H (mult. *J*, Hz)	^{13}C	^{1}H (mult. *J*, Hz)	^{13}C
1 (H_{ax}/H_{eq})	1.88 (ddd,12.4, 12.4, 5.6)/1.83 (m)	61.71	1.88 (ddd, 12.4, 12.4, 5.6)/1.83 (m)	61.71
2 (H_{ax}/H_{eq})	3.63 (ddd, 12.4, 12.4, 4.4)/4.15 (ddd, 12.4, 5.6, 1.6)	30.34	3.63 (ddd, 12.4, 12.4, 4.4)/4.15 (ddd, 12.4, 5.6, 1.6)	30.34
4		93.88		93.88
5		192.38		192.38
6	6.05	105.93	6.04	106.87
6a		168.12		168.12
7a		139.45[a]		138.19
8	6.96[b]	116.86	7.03 (d, 8.4)	116.44
9		134.14	6.89 (dd, 8.4, 2.0)	123.68
10	6.97[b]	125.86		136.42
11	6.97[b]	118.19	6.91 (d, 2.0)	118.51
11a		138.72[a]		140.13
12a		73.13		73.13
13 (H_{ax}/H_{eq})	2.40 (d, 12.0)/2.48 (dd, 12.0, 2.0)	43.26	2.40 (d, 12.0)/2.48 (dd, 12.0, 2.0)	43.26
-$\underline{CH_2}CH_2OH$	2.83 (t, 6.4)	38.30	2.83 (t, 6.4)	38.45
-$CH_2\underline{CH_2}OH$	3.86 (t, 6.4)	63.33	3.86 (t, 6.4)	63.28

[a] Interchangeable.
[b] Second order spin system.

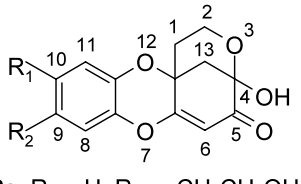
defined materials. Interestingly, the same product was observed as the main detectable species following autoxidation in aqueous phosphate buffer at pH 7.4 over 2 days, or oxidation with sodium periodate, whereas oxidation with potassium hexacyanoferrate or peroxidase/H_2O_2 led chiefly to dark polymeric material.

Based on these preliminary observations, the oxidation of **1** with tyrosinase was carried out on a larger scale (100 mg) and the product was obtained as an apparently homogeneous (TLC, HPLC) oily solid (20 mg) following TLC of the extractable fraction. Quite unexpectedly, scrutiny of the ^{1}H and ^{13}C NMR spectra revealed an intimate mixture of two closely related compounds in comparable amounts. Eventually, after several trials, suitable HPLC elution conditions were set up, which allowed separation of the two products in pure form (^{1}H NMR evidence) by repeated fractionation steps on a semipreparative reverse phase column.[§]

The EI-MS spectra of the products thus obtained were virtually identical and displayed a molecular ion peak at m/z 304 suggestive of dimeric structures.

The ^{1}H and ^{13}C NMR spectra were almost superimposable and displayed in the low field regions resonances for only one catechol-like ring system, along with a distinct 1H singlet at δ about 6, correlating with a carbon around δ 106. A carbonyl signal at δ 192 was also noted along with a deshielded C resonance around δ 168. Inspection of the high field region of the spectra revealed that the protons of one of the two hydroxyethyl chains experienced different chemical environments, reflecting adjacent stereocentres and, presumably, cyclisation.[15] Moreover, two geminally coupled proton signals at δ 2.48 and 2.40 (J=12.0 Hz) were distinguishable, giving a cross peak in the HMQC spectrum with a carbon resonance at δ around 43, on account of an additional CH_2 group. The signals at δ 2.48 exhibited also a long range coupling (J=2.0 Hz) with one of the CH_2 protons of the cyclised chain.

Based on these data, the products were formulated as the novel regioisomeric derivatives 4-hydroxy-9-(2-hydroxyethyl)-1,2-dihydro-4,12a-methanooxocino[4,5-*b*]-[1,4]benzodioxin-5-one (**2a**)[¶] and 4-hydroxy-10-(2-hydroxyethyl)-1,2-dihydro-4,12a-methanooxocino[4,5-*b*]-[1,4]benzodioxin-5-one (**2b**).[¶]



2a: R_1 = H, R_2 = -CH_2CH_2OH
2b: R_1 = -CH_2CH_2OH, R_2 = H

[§] An octadecylsilane column (10 μm, 10 mm×25.0 cm) was used; mobile phase was 0.5% aqueous acetic acid/acetonitrile 84/16 v/v flow rate: 3 mL/min.

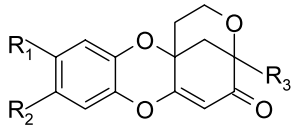
[¶] The compounds were eluted at R_T 76 min (**2a**) and 79 min (**2b**), R=0.7 under the elution conditions described in the previous footnote. HR EIMS: found m/z 304.3036 (**2a**) and m/z 304.3029 (**2b**); calculated for $C_{16}H_{16}O_6$: m/z 304.3023.

[‖] UV ($CHCl_3$) λ_{max}=251, 284 nm. ^{1}H NMR ($CDCl_3$), δ (ppm): 1.87 (2×1H, m), 1.95 (2×1H, m), 2.04 (2×3H, s), 2.18 (2×3H, s), 2.28 (2×1H, d, J=11.6 Hz), 2.89 (2×2H, t, J=7.0 Hz), 3.40 (2×1H, dd, J=11.6, 2.4 Hz), 3.73 (2×1H, ddd, J=12.4, 12.4, 4.4 Hz), 4.20–4.28 (2×3H, m), 6.06 (1H, s), 6.07 (1H, s), 6.86 (1H, dd, J=9.2, 2.2 Hz), 6.88 (1H, d, 2.2 Hz), 6.95 (3H, m), 7.02 (1H, d, J=9.2 Hz).

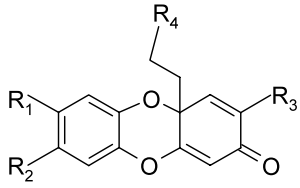
Assignment of ^{1}H and ^{13}C signals to each structure was not straightforward, due to the numerous overlappings and the very subtle chemical shift differences in the most favourable cases. NOESY experiments indicated for the more retained product on HPLC a cross peak between the resonance at δ 1.83 and δ 6.91, suggesting the structure of **2b**. The complete NMR assignments for **2a**,**b** are reported in Table 1.

Brief molecular mechanics calculations (MM+) for **2a** and **2b** indicated for both geometry optimised structures a near W-like conformation for the Heq-C1-C12a-C13-Heq bonds, which would account for the long range coupling (^{1}H–^{1}H COSY) between the signals at δ 2.48 and 1.83 (Fig. 1).

Upon acetylation, products **2a**,**b** yielded two types of acetylated derivatives which were formulated as **3a**,**b**[‖] and **4a**,**b**.[**]



3a: R_1 = H, R_2 = -$CH_2CH_2OCOCH_3$, R_3 = -$OCOCH_3$
3b: R_1 = -$CH_2CH_2OCOCH_3$, R_2 = H, R_3 = -$OCOCH_3$



4a: R_1 = H, R_2 = -$CH_2CH_2OCOCH_3$, R_3, R_4 = -$OCOCH_3$
4b: R_1 = -$CH_2CH_2OCOCH_3$, R_2 = H, R_3, R_4 = -$OCOCH_3$

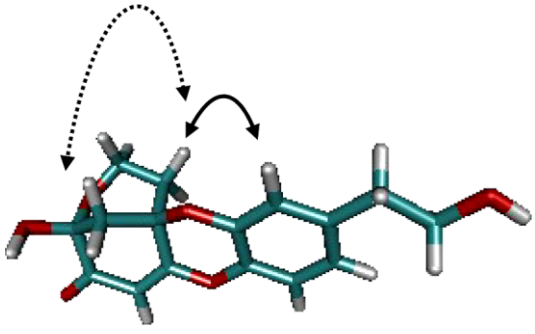


Figure 1. Energy minimised structure of **2b** (HyperChem™ 6.01): protons giving the diagnostic NOE contact (solid line) and long range coupling (dashed line) are highlighted.

[**] UV ($CHCl_3$) λ_{max} = 263, 320 nm. ^{1}H NMR ($CDCl_3$), δ (ppm): 1.99 (2×3H, s), 2.03 (3H, s), 2.04 (3H, s), 2.23 (2×2H, m), 2.30 (2×3H, s), 2.88 (2H, t, J = 7.0 Hz), 2.89 (2H, t, J = 7.0 Hz), 4.10 (2×2H, m), 4.24 (2H, t, J = 7.0 Hz), 4.25 (2H, t, J = 7.0 Hz), 6.02 (1H, s), 6.03 (1H, s), 6.61 (2×2H, s), 6.85 (1H, d, J = 2.2 Hz), 6.87 (1H, dd, J = 9.2, 2.2 Hz), 6.95 (3H, m), 7.03 (1H, d, J = 9.2 Hz).

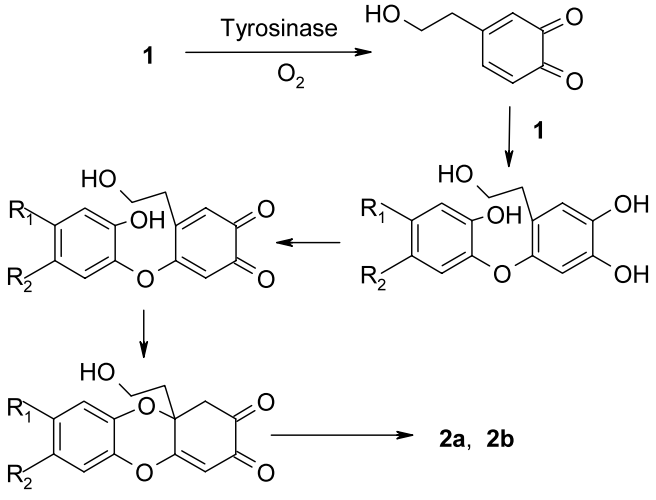
Structures **3a**,**b** displayed molecular ion peaks at m/z 388 in the EI-MS spectra and NMR spectra consistent with the diacetyl derivative of **2a**,**b**. The loss of the methanooxocine ring system and its replacement by a 2-acetoxy-2,5-cyclohexadienone moiety were clearly apparent in the case of **4a**,**b** (molecular ion peak at m/z 430).

Based on the known ability of tyrosinase to generate *o*-quinones, a plausible mechanistic scheme accounting for formation of the methanooxocinobenzodioxinone system by oxidation of **1** can be envisaged (Scheme 1).

The generation of an equimolar mixture of regioisomers would suggest that the *ortho* hydroxyl groups of **1** can bring the initial nucleophilic attack to **1** quinone with comparable efficiency.

Formation of dibenzo-1,4-dioxanes of the type **2** by oxidation of catechols is well documented,[16] and so is the generation of bicyclo[3.3.1]heterononanes following dimerisation.[17,18] This involves a cyclisation step that has been described in the case of catecholamine compounds bearing 2-aminoethyl residues but seems unprecedented for catechols with 2-hydroxyethyl side chains related to **1**.

In conclusion, oxidation of **1** under biomimetic conditions has been shown to yield as main products the dimers **2a**,**b**, featuring the peculiar methanooxocinobenzodioxinone ring system. Compounds **2a**,**b** are the first products isolated by oxidation of **1** and their characterisation fills a gap in the chemistry of this potent natural antioxidant, expanding the current panorama of heterocyclic compounds of potential biological relevance.



R_1 = H, R_2 = -CH_2CH_2OH, *route to* **2a**
R_1 = -CH_2CH_2OH, R_2 = H, *route to* **2b**

Scheme 1.

Acknowledgements

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