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High-Yielding Synthesis of Methyl Orthoformate-Protected Hydroxytyrosol and Its Use in Preparation of Hydroxytyrosyl Acetate

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Abstract: The new methyl orthoformate of the powerful antioxidant hydroxytyrosol (or 2-(3,4-dihydroxyphenyl)ethanol) has been synthesized by a two-step high yielding procedure. The protection stabilizes hydroxytyrosol against fast oxidation and allows both easy chromatographic purification and long term storage. The protective group is hydrolyzed over pH = 10 and below pH = 5, thus allowing the release of the active principle under physiological conditions. The use of the methyl orthoformate-protected hydroxytyrosol allows the preparation of protected hydroxytyrosyl esters, like the acetate herein reported, by selective esterification of the alcoholic function. The subsequent quantitative deprotection under non-aqueous and mild conditions affords the hydroxytyrosyl acetate in high yields.

Keywords: Hydroxytyrosol, antioxidants, hydroxytyrosyl esters, protective groups.

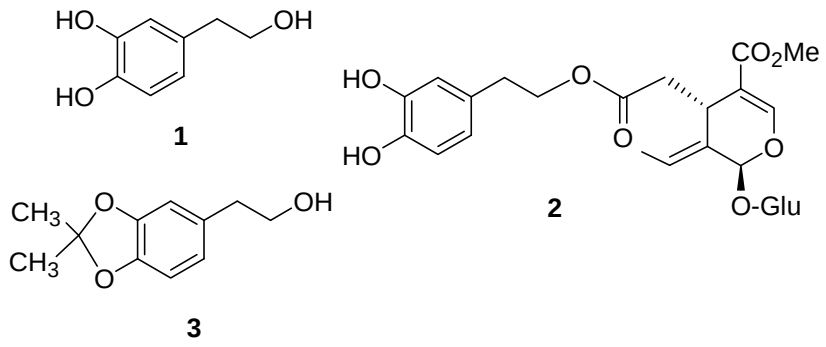
Introduction

Hydroxytyrosol **1** (2-(3,4-dihydroxyphenyl)ethanol or 3,4-DHPEA) [1] is the hydrolysis product of the glycoside oleuropein (**2**, Figure 1), an antifeedant secondary metabolite [2], present in considerable

amount in all parts of olive trees [3]. Compound **1** shows many interesting biological activities. It has antimicrobial [4] and anticancer (fat related) activity [5] and, mainly, protects cells against oxidative stress [6], by reducing the hearth disease pathogenesis [7].

In view of the above biological properties, it is not surprising that the preparation of pure hydroxytyrosol, to be used as a dietary supplement or as a stabilizer in foods and cosmetic preparations, has been the subject of many patents [8] and articles [9].

Figure 1. Structure of hydroxytyrosol **1** oleuropein **2** and hydroxytyrosol acetonide **3**.



However, manipulation of **1** suffers from two difficulties. First, hydroxytyrosol is such a good antioxidant that it undergoes quick oxidation in the air, particularly on silica gel and in alkaline medium, [9b] to afford a black polymeric material. This behaviour does not allow either long-term storage, chromatographic purification or easy recovery of **1** from the abundant natural glycoside **2**.

The second problem is concerned with the selective esterification of the primary hydroxyl in **1**. Preparation of hydroxytyrosyl esters is of interest because the introduction of the ester function results in higher solubility in lipophilic environments, without any loss of antioxidant activity [10]. However, due to the competition between alcoholic and phenolic hydroxyls, the simple use of acyl chlorides results in mixtures of mono- di- and tri-esterified derivatives [9a], unless laborious protection of the catechol function as benzyl ethers [10c] or cerium-catalyzed conditions [11] are applied. Better results have been reported under patented transesterification conditions [10a,b] but experimental details are not given.

On this basis, we have been engaged in searching for suitable and easy removable protective groups able to stabilize the catechol function and to allow regioselective esterification. Recently, we reported the novel acetonide **3** as a protected form of **1** [12]. The acetonide **3** can be directly obtained from natural oleuropein **2** in good yields (76%) and is stable both over silica and after two months exposition to the air and light.

However, the high intrinsic stability of the acetonide group can be regarded as troublesome if the aim is to deprotect the cathecol function under very mild conditions, as required if protected hydroxytyrosol was used as an antioxidant additive in foods and cosmetics or for the synthesis of hydroxytyrosyl esters.

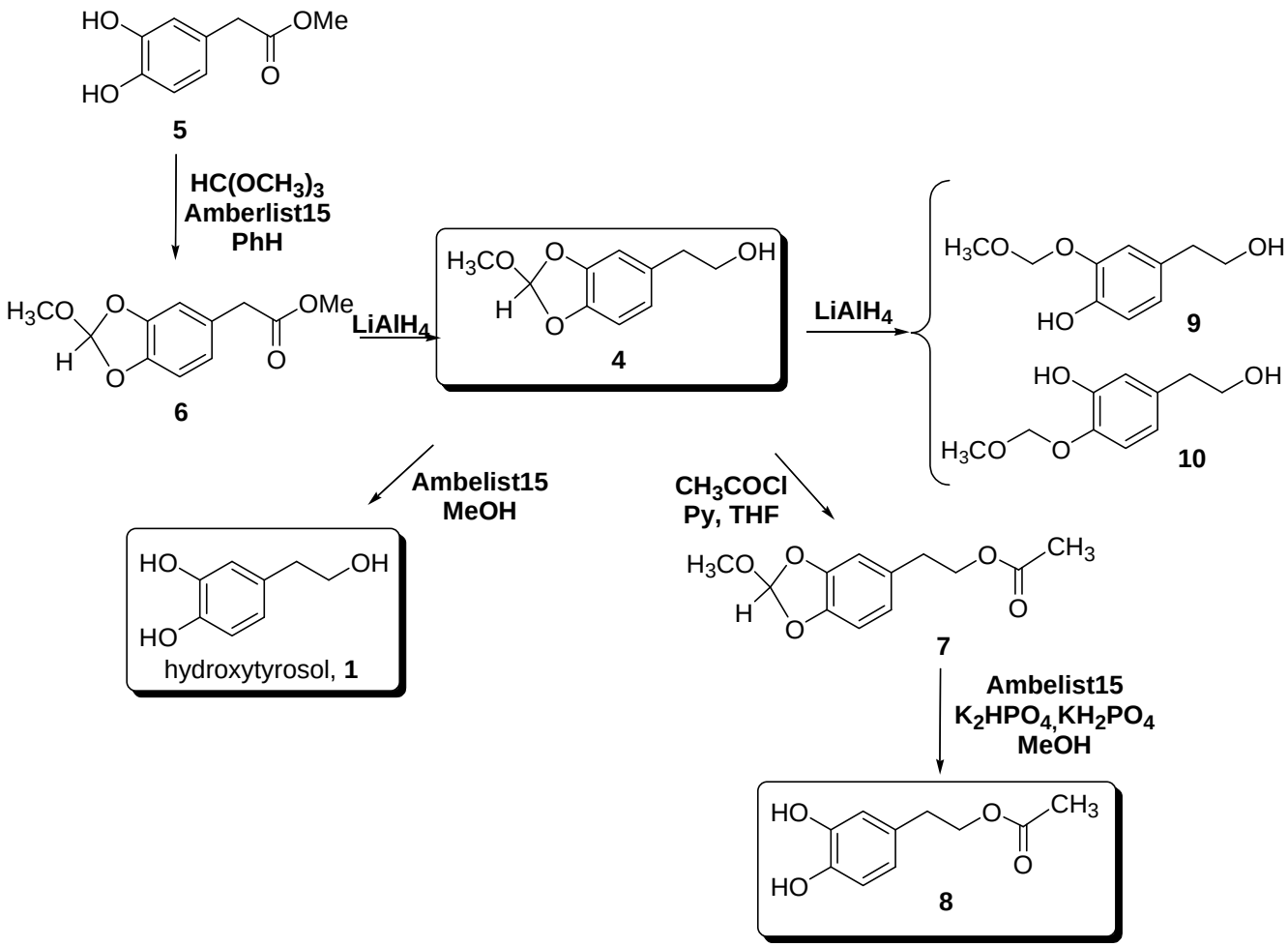
Therefore, we chose the unprecedented methyl orthoformate moiety (see compound **4** in Figure 2) as an easy removable protective group to be tested for the ability both to stabilize hydroxytyrosol against oxidation and to allow a high yielding synthesis of acetate **8**, regarded as a model molecule for

other hydroxytyrosyl esters. We report here the synthesis, properties and conditions of protection and removal of methyl-orthoformate **4** together with its use in the preparation of the acetate **8**.

Results and Discussion

Synthesis of methyl orthoformate **4** was performed, as depicted in Figure 2, following the same route successfully adopted for the acetonide **3** [12]. First experiments to obtain compound **6** through the installation of the methyl orthoformate protection in methyl ester **5** were carried out under transesterification conditions identical to those described for the preparation of corresponding acetonide **3** [12]. However, when the substrate **5** and trimethyl orthoformate (TMO) were reacted in chloroform as solvent and camphorsulfonic acid as a catalyst, only small amounts of the desired orthoformate were detected in the reaction mixture. In the hypothesis that this negative result could be the result on the unfavorable transesterification equilibrium, the reaction was forced to product by removing the produced methanol. The use of benzene as solvent, Amberlist® 15 as a catalyst and a Dean-Stark apparatus filled with molecular sieves to adsorb methanol, resulted in the quantitative production of the methyl orthoformate **6**.

Figure 2. Synthesis of hydroxytyrosyl methyl orthoformate **4**, hydroxytyrosol **1** and hydroxytyrosyl acetate **8**.

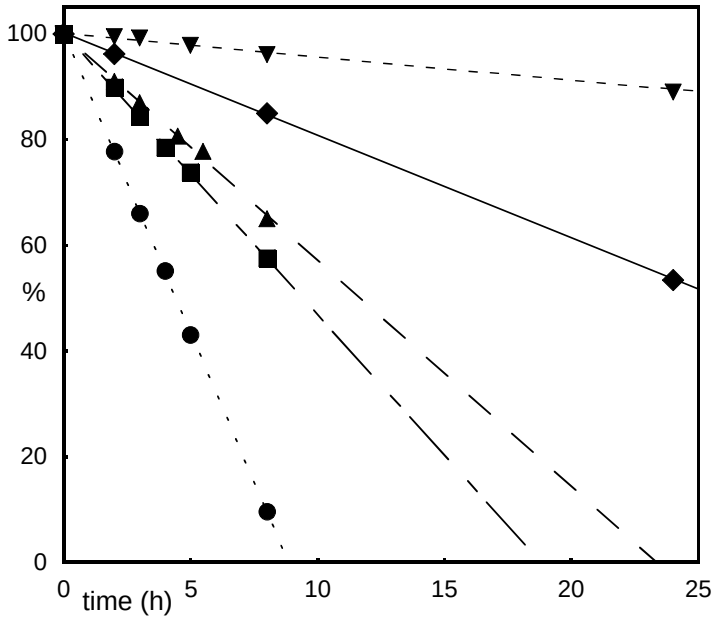


Subsequent reduction of the ester moiety to give alcohol **4** required only a careful control of the substrate/reagent stoichiometry in order to avoid the formation of overreduction products **9** and **10**, not isolated and identified by their GC-MS spectra. Indeed, the use of 1:1 substrate/reagent molar ratio allowed a high yielding recovery of the desired protected hydroxytyrosol **4**.

As expected, protection as an orthoformic ester stabilized hydroxytyrosol against oxidation but the orthoformate group proved to be less stable than the previously studied acetonide group. In fact, this protection did not survive on commercial silica. However, chromatographic purification of **4** was still possible if pre-washed silica was used (see Experimental Section). Moreover, quantitative protection removal under non aqueous conditions was easily obtained after a short treatment with the acid resin Amberlist® 15 in methanol.

Therefore, in order to apply the orthoformate protection both to the recovery of **4** from the glycoside oleuropein and to the synthesis of hydroxytyrosyl esters, we studied the hydrolytic behaviour of **4** at 25°C at various pHs. The experiments were carried out measuring the decrease of the substrate and the contemporary formation of hydroxytyrosol **1** by HPLC. Since preliminary experiments, carried out at pH= 14 and 12, showed complete consumption of **4** after 0.5 h and 1 h, respectively, accurate measurements were made only for pHs ≤ 10. Results are resumed in Figure 3, where only the percent decrease of substrate is shown.

Figure 3. Acid hydrolysis of **4** at 25°C and at various pHs. ● pH = 3; ■ pH = 4.5; ▲ pH = 5.5; ▼ pH = 6.5; ♦ pH = 10.



As clearly evidenced in Figure 3 and in contrast to the high stability of the acetonide **3** [12], the range of stability of the orthoformate protection is very narrow, ranging between pH=10 ($t_{1/2}$ = 25.9 h) and pH= 6.5 ($t_{1/2}$ = 112.9 h). This feature ruled out any possibility to obtain **4** from oleuropein by applying the same protocol successfully used to get the acetonide **3**. On the contrary, the easy hydrolysis of **4** below pH = 5 seemed to be very useful for the selective esterification of

hydroxytyrosol at the primary alcoholic function. On these bases, we choose the hydroxytyrosyl acetate **8** as a model molecule and experimented how to get it by using **4** as a precursor.

Quantitative acetylation of hydroxytyrosol methyl-orthoformate **4** was easily carried out, with routine methods, by simply reacting the substrate in tetrahydrofuran (THF) with one equivalent of a mixture of pyridine and acetyl chloride in 1:1 molar ratio. Subsequent chromatographic purification over pre-washed silica gave the desired protected hydroxytyrosyl acetate **7** in 94% yield. In spite of the above reported easy hydrolysis of the orthoformate moiety in **4**, deprotection of **7** was unexpectedly troublesome. Trial experiments showed that, under acid catalyzed methanolic transesterification, deprotection took always place together with the concomitant and undesired formation of free hydroxytyrosol. In the hypothesis that the deprotected acidic catechol groups could catalyze the subsequent ester methanolysis, the reaction was carried out by adding a phosphate buffer (pH = 7.2). This resulted in selective deprotection with formation of hydroxytyrosyl acetate **8** in 87% overall yield, with respect to the starting methyl orthoformate **4**. In order to avoid loss of product by easy oxidation, the lipofilic resin Sephadex® LH-20 was used for chromatographic purification. In conclusion, in view of the high yield and the purity (99% *via* HPLC) of the obtained hydroxytyrosyl acetate **8**, this procedure represents a new alternative route towards hydroxytyrosyl esters.

Experimental Section

General

Silica gel 60 F254 plates and silica gel 60 were purchased from Fluka, Switzerland. All solvents and chemicals were obtained from Aldrich Chemical Co., UK. Trimethyl orthoformate (TMO) and acetyl chloride were used directly after distillation. (3,4-Dihydroxyphenyl)acetic acid methyl ester (**5**) was obtained from the corresponding acid as previously reported [13]. When specified, solvents were dried over opportune drying agents [14] and then distilled. Pre-washed silica gel refers to silica gel (Fluka, 70-230 mesh) washed with 0.1N HCl and rinsed with hot distilled water until a negative test for chlorides. Petroleum ether used for chromatographic separations is the 40-60°C fraction. HPLC analyses were performed on a TSP Spectra Series P200 apparatus equipped with a Thermo Hypersil BDS C_{18} column (250 x 4.6 mm, 5μ) at λ= 280nm. Elutions were carried out at 1mL/min flow rate by using a H_2O/AcCN mixture: (90:10) for the first minute, then a 20 minutes gradient to pure MeCN. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ (99.8% in deuterium) using a Gemini 200 spectrometer. All chemical shifts are expressed in parts per million (δ scale) and are referenced to either the residual protons or carbon of the solvent. FT-IR spectra were recorded in $CHCl_3$ on a Brucker Vector 22 spectrometer. GC/MS analyses were obtained on a FISONS GC 8000 gas chromatograph equipped with a capillary column (Supelco Equity 5, 30 m-long, ID 0.25 mm, film thickness 0.25 μm) and coupled with a FISONS MD800 mass detector. The following elution program was used: injection port temperature 250°C, MS temperature 280°C, oven program: 50°C 2 min, ramp at 10°C min^{-1} to 250°C, hold at 250°C for 8 min. HRMS were recorded with Micromass Q-TOF *micro* Mass Spectrometer (Waters). Only the spectral data of new compounds are reported here.

2-(2-Methoxybenzo[1,3]dioxol-5-yl)-acetic acid methyl ester (**6**). A suspension of Amberlist® 15 (hydrogen form, 400 mg) and 4 Å powdered molecular sieves (500 mg) in dry benzene (45 mL) was placed in a flask equipped with a Dean-Stark apparatus and magnetic stirring. A solution of (3,4-dihydroxyphenyl)acetic acid methyl ester (**5**, 3.16 g, 17.36 mmol) in freshly distilled trimethyl orthoformate (10 mL) was added and the mixture was refluxed while stirring for 6 h under argon atmosphere in the dark. After Amberlist® and molecular sieves filtration, the resulting solution was evaporated to dryness under reduced pressure. The residue was suspended in $NaHCO_3$ (saturated solution) and extracted three times with diethyl ether. The collected organic extracts were dried over dry Na_2SO_4 and evaporated *in vacuo* to give the orthoformate **6** (3.718 g, 16.60 mmol, yield 96%) clean enough (97% *via* GC/MS and HPLC) to be directly used in the next step. An analytical sample was obtained after column chromatography on pre-washed silica (20:1) by eluting with petroleum ether/AcOEt (9:1). Spectroscopic properties were as follows. GC-MS *m/z* (%): 224 (M^+, 40), 193 (40), 165 (100), 137 (27), 105 (21), 77 (26); ^{1}H-NMR δ/ppm (200 MHz, $CDCl_3$): 6.84 (s, 1H, $CH(OR)_3$), 6.83 (d, 1H, J=1.5 Hz, Ph-H^6), 6.82 (d, 1H, J= 8.0 Hz, Ph-H^3), 6.75 (dd, 1H, J= 8.0, 1.5 Hz, Ph-C^4), 3.7 (s, 3H, -$COOCH_3$), 3.6 (s, 2H, Ph- CH_2), 3.4 (s, 3H, (orthoformate-OCH_3); ^{13}C-NMR (50 MHz, $CDCl_3$): δ=172.00 (C=O), 146.16, 145.15, 127.6, 122.4, 109.7, 107.8 (aromatic carbons), 119.2 ($\underline{C}(OR_3)$, 52.0 ($COO\underline{C}H_3$), 49.9 (orthoformate-$O\underline{C}H_3$), 40.72 ($Ph\underline{C}H_2$-); IR (ν_{max}, cm^{-1}, $CHCl_3$): 3020, 2846, 1735, 1499, 1463, 1143, 1039; HRMS: found 224.0682, $C_{11}H_{12}O_5$ requires 224.068.

2.2-(2-Methoxybenzo[1,3]dioxol-5-yl)-ethanol (**4**). $LiAlH_4$ (632 mg, 16.6 mmol) was added to a solution of the ester **6** (3.50 g, 15.6 mmol) in anhydrous THF (500 mL) and the suspension was refluxed for 2 h under argon atmosphere in the dark. At the end, the mixture was cooled in an ice-bath and excess hydride was cautiously decomposed first by adding wet Et_2O in small portions and then water, until the formation of a white precipitate. After removal of the precipitate by filtration under reduced pressure, the solution was dried over Na_2SO_4 and evaporated *in vacuo* to obtain **4** (2.93 g, 15.0 mmol, yield 96 %) clean enough (97% *via* GC/MS and HPLC) to be directly used in the next step. An analytical sample was obtained after chromatography over pre-washed silica gel (40:1) by eluting with petroleum ether/AcOEt (8:2). Samples of **4** were found unchanged by HPLC and GC/MS analyses after one month in the refrigerator. Spectroscopic properties were as follows: GC-MS *m/z* (%): 196 (M^+ , 29), 165 (100), 137 (20), 105 (14), 77 (25); ^{1}H-NMR δ/ppm (200 MHz, $CDCl_3$): 6.82 (s, 1H, $CH\underline{(}OR)_3$), 6.81 (d, 1H, J = 7.8 Hz, Ph-H^3), 6.77 (d, 1H, J = 1.4 Hz, Ph-H^6), 6.71 (dd, 2H, J = 7.8, 1.4 Hz, Ph-H^4), 3.81 (t, 2H, J = 6.5 Hz, CH_2O), 3.40 (s, 3H, orthoformate-OCH_3), 2.79 (t, 2H, J = 6.5 Hz, Ph-CH_2); ^{13}C-NMR (50 MHz, $CDCl_3$): δ=146.3, 144.7, 132.3, 121.9, 108.9, 109.0 (aromatic carbons), 119.2 ($\underline{C}(OR_3)$, 63.7 ($COO\underline{C}H_3$); 50.0 (orthoformate-$O\underline{C}H_3$); 38.9 (Ph-$\underline{C}H_2$); IR (ν_{max}, cm^{-1}, $CHCl_3$): 3670, 3450, 3011, 2889, 1496, 1442, 1140, 1039; HRMS: found 196.0739, $C_{10}H_{12}O_4$ requires 196.074.

Effect of pH on the hydrolysis of **4**. A standard solution was prepared by dissolving **4** (392 mg, 2 mmol) in EtOH (5 mL) and small portions (30 μL) of this solution were added, under argon atmosphere and in the dark, to buffered solutions (1 mL) at pHs= 10.0 ($NaHCO_3$/NaOH buffer), 6.5 (citric acid/NaOH buffer), 5.5 (acetic acid/NaOH buffer), 4.5 (citric acid/NaOH buffer) and pH=3 (10^{-3} M HCl) respectively, to reach a 12 mM final concentration of **4**. Hydrolyses were carried out at 25 °C and were monitored by collecting, time by time, small samples of the proper solution, neutralizing to

pH=7 (0.01M HCl or solid $NaHCO_3$) and analyzing by HPLC the percent decrease of **4** with respect to the sum of the residual **4** and the formed **1**. Results are shown in Figure 3 as average of three independent runs.

*Hydroxytyrosol **1** from **4** through protection removal.* Amberlist® 15 (50 mg) was added to a solution of **4** (100 mg, 0.51 mmol) in MeOH (5 mL) and the suspension was refluxed for 1h under stirring while monitoring the reaction progress by HPLC. At the end, the resin was removed by filtration and the resulting solution was evaporated under reduced pressure to leave **1** (75 mg, 0.39 mmol, yield 97%, purity 99% *via* HPLC and GC/MS). Spectroscopic data were super imposable with those of a pure standard of **1**.

Acetic acid 2-(2-methoxy-benzo[1,3]dioxol-5-yl)-ethyl ester (**7**). A solution of hydroxytyrosol methylorthoformate **4** (157 mg, 0.80 mmol) in dry tetrahydrofuran (THF, 4 mL) was transferred by a syringe into a flask equipped with a rubber injection septum, magnetic stirring and argon atmosphere. Dry pyridine (1.5 equivalents, 95 μL) and freshly distilled acetyl chloride (1.5 equivalents, 83 μL) were added and the mixture was stirred at room temperature for 16 h. Excess acetyl chloride was destroyed by adding methanol (1 mL) and the solution was neutralized with $NaHCO_3$ (saturated solution). Organic solvents were removed under reduced pressure, the resulting aqueous suspension was extracted three times with ethyl acetate and the collected organic phases were dried over dry Na_2SO_4 and evaporated *in vacuo* to afford a crude residue that was purified over pre-washed silica gel (40:1) by eluting with petroleum ether/AcOEt (8:2) to afford pure **7** (175 mg, 0.74 mmol, yield 94 %). Spectroscopic properties were as follows: GC-MS *m/z* (%): 238 (M^+,4), 207 (16), 178 (100), 165 (23), 147 (62), 135 (30), 77 (28), 43 (57); ^{1}H-NMR δ/ppm (200 MHz, $CDCl_3$): 6.83 (s,1H, $CH(OR)_3$), 6.80 (d, 1H, J = 7.9 Hz, PhH^3), 6.76 (d, 1H, J = 1.5 Hz, PhH^6), 6.70 (dd, 1H, J = 7.9, 1.5 Hz, PhH^4), 4.22 (t, 2H, J =7.0 Hz, CH_2OAc), 3.40 (s, 3H, orthoformate-OCH_3), 2.86 (t, 2H, J = 7.0 Hz, Ph -CH_2), 2.04 (s, 3H, CH_3CO); ^{13}C-NMR (50 MHz, $CDCl_3$): δ=171.0 (C=O); 146.2, 144.8, 131.7, 121.9, 108.9, 107.9 (aromatic carbons), 119.2 ($\underline{C}(OR_3)$, 65.0 ($COO\underline{C}H_3$); 50.0 (orthoformate-$\underline{C}H_3$); 34.9 (Ph-$\underline{C}H_2$); 20.9 (CO-$\underline{C}H_3$); IR (cm^{-1}, $CHCl_3$): 3033, 2853, 1732, 1499, 1444, 1253, 1036; HRMS: found 238.0841, $C_{12}H_{14}O_5$ requires 238.084.

Acetic acid 2-(3,4-dihydroxy-phenyl)-ethyl ester (**8**). Protected ester **7** (53 mg, 0.22 mmol) was added to a suspension of Amberlist® 15 (106 mg), K_2HPO_4 (38 mg, 0.22 mmol) and KH_2PO_4 (30 mg, 0.22 mmol) in 3 mL of dry methanol and the mixture was refluxed under argon atmosphere in the dark for 4h. HPLC analysis showed near complete conversion of the substrate and only traces of hydroxytyrosol. The suspension was filtered and the liquid phase was evaporated *in vacuo*; the residue was redissolved in ethyl acetate, filtered and evaporated to leave a crude (42 mg) that was purified over Sephadex® LH-20 (40:1) by eluting with hexane: ethyl acetate gradients (from 9:1 to 7:3) to give pure hydroxytyrosyl acetate **8** (38 mg, 0.19 mmol, yield 93%). Spectroscopic data were coherent with those reported in the literature [10c].

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Sample Availability: Contact the authors.