JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

A Convenient Synthesis of Hydroxytyrosol Monosulfate Metabolites

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Supporting Information

ABSTRACT: The growing interest in the bioactivity of natural polyphenols and of their metabolites requires metabolites to be used in bioassays and as standards in research protocols. We report here on the synthesis of several hydroxytyrosol metabolite monosulfates achieved using a simplified protocol with improved yields. A synthetic solution based on avoidance of high temperature conditions during the synthesis and of low pressure conditions during purification has been established. Monosulfates of several phenolic compounds, namely, hydroxytyrosol, hydroxytyrosol acetate, homovanillyl alcohol, homovanillyl alcohol acetate, homovanillic acid, ferulic acid, and 3,4-dihydroxyphenylethanoic acid, were efficiently synthesized in 1−2 steps in good yield and isolated using simple procedures. The proposed protocol was shown to be relatively rapid, efficient, cheap, and widely applicable to a number of catechol scaffolds.

KEYWORDS: *polyphenols, olive oil, sulfates, hydroxytyrosol, homovanillyl alcohol, hydroxytyrosol acetate, metabolites*

INTRODUCTION

Virgin olive oil (VOO) is the major source of fat in the Mediterranean diet, and a number of studies indicate that phenolic compounds, a minor constituent of VOO, are a contributory factor to the healthy Mediterranean diet.[1−3] Over the past decade, many human intervention studies have been carried out in order to determine the exact bioefficacy of several different subclasses of olive oil polyphenols as protection against chronic degenerative diseases[4] and long-term clinical intervention trials have provided evidence that the phenolic compounds of virgin olive oil contribute to protecting humans against lipid oxidation in a dose-dependent way.[5,6]

The most common phenols found in olives include phenolic alcohols, such as hydroxytyrosol (compound **1**, 3,4-dihydroxyphenylethanol, 3,4-DHPEA or Hy) and tyrosol (4-hydroxyphenylethanol, 4-HPEA or Ty), secoiridoids, flavonoids, and lignans. Secoiridoids are the major class of phenols found in olives, with glycoside oleuropein (OL), one of the most important secoiridoids, making up to 14% of the dry weight of olives. Secoiridoid glycosides such as oleuropein are hydrolyzed during VOO extraction by β-glucosidases. Therefore, the major phenolic compounds found in VOO are the dialdehydic form of elenolic acid linked to Hy (3,4-DHPEA-EDA), the oleuropein aglycon (3,4-DHPEA-EA), and the dialdehydic form of elenolic acid linked to Ty (4-HPEA-EDA).[7] These compounds represent as much as 80% of the total phenolic fraction.[8]

Although there have been a large number of studies investigating the in vitro antioxidant properties of VOO phenolics, as well as their protective effects against cell injury, the biological properties of these phenols in vivo depends on the extent to which they are absorbed and metabolized. Olive oil polyphenols undergo extensive metabolism in the intestines and liver and are thus chiefly found in biological fluids as phase II metabolites.[9,10] Bioavailability studies have shown that Hy is efficiently absorbed in the small intestine[11] and, together with its acetate, glucuronide and sulfate conjugates, homovanillyl alcohol and its glucuronide and sulfate conjugates, it can be found in human urine and plasma after olive oil consumption (Figure 1).[9,10,12−15] Until recently, there were very few studies on the bioavailability for secoiridoids, except for oleuropein. However, there is now considerable evidence showing that OL aglycons are bioavailable. After VOO consumption, Hy concentration increases more than expected if only the Hy present in the oil was absorbed, and it is proportional to the total phenol content in the oil.[11] An initial study of 3,4-DHPEA-EDA and 3,4-DHPEA-EA bioavailability showed that both secoiridoid compounds are efficiently absorbed by Caco-2 cells and by the rat intestine and that they are hydrolyzed with the production of hydroxytyrosol and its metabolites (Figure 1).[16] In a recent study with the aim of identifying biomarkers for olive oil consumption after a 3-week dietary intervention with phenol-enriched olive oils as part of a randomized, double-blind, crossover, and controlled nutrition intervention trial, two olive oils were assessed, one virgin olive oil (VOO) and a second one phenol-enriched with its own phenolic compounds (FVOO).[10] The metabolites hydroxytyrosol sulfate (Figure 1, compounds **3**/**4**) and hydroxytyrosol acetate sulfate (Figure 1, compounds **5**/**6**) were shown to be the metabolites most suitable for monitoring FVOO intake compliance, which in addition to a significant post-treatment increase in both urine and plasma demonstrated a significant change compared to VOO in urine.[10]

Metabolic sulfation of compounds, or *O*-sulfonation, takes place in the cytosol when one of the sulfotransferases from the 3′-phosphoadenosine-5′-phosphosulfate (PAPS) donates the activated sulfonate group (SO_3) to an acceptor alcohol, phenol, or amine group.[17]This gives the molecule an anionic character,

Received: September 3, 2015
Revised: October 12, 2015
Accepted: October 13, 2015

DOI: 10.1021/acs.jafc.5b04307
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

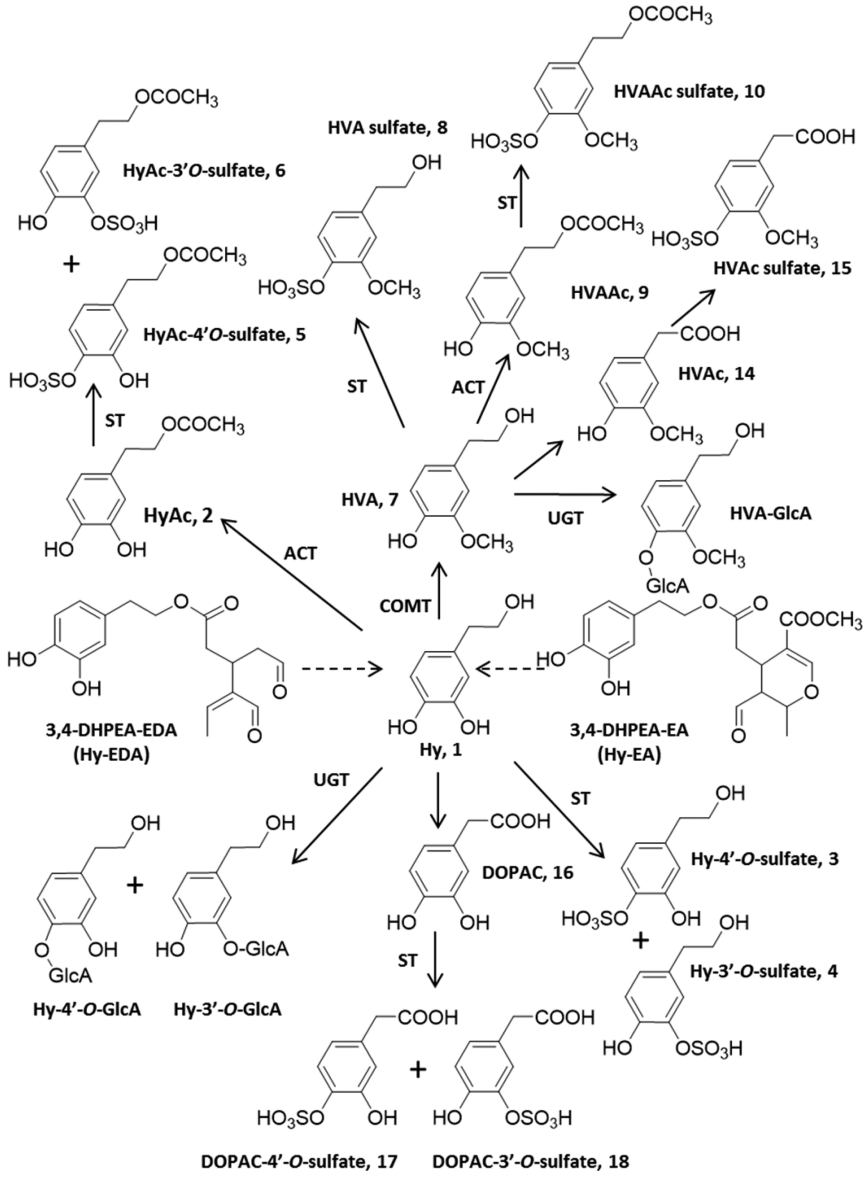


Figure 1. Olive oil polyphenol metabolites already identified in bioavailability studies. Arrows may represent a direct or an indirect metabolic pathway.

which improves its excretion properties to avoid potential adverse effects. However, sulfation is also an important means of regulating the bioactivity of certain molecules. For example, there is growing evidence that intracellular sulfation and desulfation play an important role in regulating the availability of active steroid hormones near the target sites.[18] In fact, the role of phase II conjugates as temporary deposits, from which hydroxytyrosol and its phase I metabolites can be regenerated by sulfatases and glucuronidases, has been attracting much attention recently.[19,20] Therefore, the potential health benefits of Hy and its derivatives may be attributable to both parent compounds together with their phase I and phase II metabolites, although it is still unknown which components play a major role in this protection. Investigations into these topics require pure metabolites to be used in bioactivity assays and as standards, but these metabolites are not widely available commercially and are expensive. The aim of this paper is therefore to report on the synthesis of the most important olive oil polyphenol metabolites in animals and humans, i.e., hydroxytyrosol and its phase II monosulfate metabolites (Figure 1, Table 1).

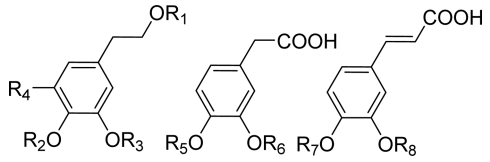
MATERIALS AND METHODS

All chemicals were obtained from chemical suppliers and used without further purification, unless otherwise noted. All reactions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 plates F_{254}, and detected by iodine. Products were purified by flash chromatography with silica gel 60 (200−400 mesh). NMR spectra were recorded on 400 MHz NMR equipment at room temperature for solutions in CD_3OD. Chemical shifts are referred to the solvent signal and are expressed in ppm. 2D NMR experiments (COSY, TOCSY, ROESY, and HMQC) were carried out when necessary to assign the corresponding signals of the compounds.

High resolution mass spectra were recorded on Hewlett-Packard HP5988A. ESI-MS analyses were performed on a Finnigan LCQ DECA XP MAX detector, equipped with an API source, using an electrospray ionization (ESI) probe. The capillary temperature and voltage used were 180 °C and 3 V, respectively, and spectra were obtained in negative ion mode. When the molecular ion was detected, the MS2 spectrum was obtained using a relative energy collision of 27.

DOI: 10.1021/acs.jafc.5b04307
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 1. Initial Polyphenols and Synthesized Compounds (in Bold Italics)



compound	R_1	R_2	R_3	R_4
1	H	H	H	H
2	$COCH_3$	H	H	H
3	H	SO_3H	H	H
4	H	H	SO_3H	H
5	$COCH_3$	SO_3H	H	H
6	$COCH_3$	H	SO_3H	H
7	H	H	CH_3	H
8	H	SO_3H	CH_3	H
9	$COCH_3$	H	CH_3	H
10	$COCH_3$	SO_3H	CH_3	H
11	SO_3H	H	H	H
12	$COCH_3$	SO_3H	SO_3H	H
13	$COCH_3$	H	H	SO_3H

compound	R_5	R_6
14	H	CH_3
15	SO_3H	CH_3
16	H	H
17	SO_3H	H
18	H	SO_3H

compound	R_7	R_8
19	H	CH_3
20	SO_3H	CH_3

Phenolic Compounds. Hydroxytyrosol (Hy, **1**) and hydroxytyrosol acetate (HyAc, **2**) were purchased from Seprox Biotech (Madrid, Spain). Homovanillyl alcohol acetate (HVAAc, **9**) was synthesized from homovanillyl alcohol (HVA, **7**) (Sigma-Aldrich) according to the procedure of Bernini et al.[21] Homovanillic acid (HVAc, **14**), ferulic acid (**19**), and 3,4-dihydroxyphenylacetic acid (DOPAC, **16**) were obtained from Sigma-Aldrich.

Synthesis of Monosulfates (Table 1). *General Procedure.* Phenolic compounds (1.0 equiv) and sulfur trioxide−pyridine complex, SO_3·Py (2.0 equiv), were placed in a round flask and dissolved in 10 mL of dry dioxane in an ice bath. The mixture was left under magnetic stirring for 15 min at 0 °C under argon and then closed and stored at −20 °C for 24 h. After this period, 5 mL of water was added and the mixture immediately neutralized with diethylamine (DEA). The mixture was washed with ethyl ether, and the aqueous phase was evaporated. The crude extract was purified by flash chromatography on silica gel to afford monohydrogenosulfates (η = 20−30%) and a mixture of monohydrogenosulfates and their DEA salts (η = 50−60%).

*4-(2-Ethanoyloxyethyl)-2-hydroxyphenyl Hydrogenosulfate (**5**) and 5-(2-Ethanoyloxyethyl)-2-hydroxyphenyl Hydrogenosulfate (**6**) and Their Sodium Salts.* Hydroxytyrosol acetate, **2** (300 mg, 1.55 mmol), and SO_3·Py (3.10 mmol) were dissolved in 6 mL of dioxane and submitted to sulfation conditions. TLC (ethyl acetate:methanol, 9:1) showed the formation of a major product and complete consumption of the initial material. Solvents were removed, and the crude extract was purified by flash chromatography. Fractions containing the desired product were evaporated to afford a mixture of two isomers (**5** and **6**, in the proportion of 6:4) (130 mg, 31%) as a pale cream-colored solid and a mixture of the two isomers and their DEA salts (320 mg). The mixture of DEA salts was then dissolved in 2 mL of water, applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g), and eluted with water. Fractions containing the desired product were lyophilized to afford a mixture of sodium salts of **5** and **6** as a white solid (268 mg, 58%). Isomer **5**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.24 (d, 1H, J = 8.2 Hz, H_{arom}), 6.83 (d, 1H, J = 2.1 Hz, H_{arom}), 6.73 (dd, 1H, ^4J = 2.1 Hz, ^3J = 8.2, H_{arom}), 4.28 (t, 2H, J = 6.8 Hz, CH_2OAc), 2.89 (t, 2H, J = 6.8 Hz, Ar−CH_2), 2.06 (s, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 173.0, 150.4, 140.0, 137.5, 124.1, 121.3, 118.8, 66.3, 35.5, 20.8. Isomer **6**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.23 (d, 1H, J = 2.9 Hz, H_{arom}), 6.86 (d, 1H, J = 8.2 Hz, H_{arom}), 6.95 (dd, 1H, ^4J = 2.1 Hz, ^3J = 8.2, H_{arom}), 4.25 (t, 2H, J = 6.8 Hz, CH_2OAc), 2.87 (t, 2H, J = 6.8 Hz, Ar−CH_2), 2.05 (s, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 172.9, 149.0, 141.2, 130.9, 127.5, 124.6, 118.2, 66.2, 35.1, 20.8; HRMS (EI) calcd for $[M - H]^-$ 275.0198, found 275.0201; MS fragmentation pattern $[M - H]^-$ = 275, $[M - SO_3H - H]^-$ = 195, $[2M - H]^-$ = 551.

*2-(3-Hydroxy-4-hydroxysulfonyloxyphenyl)ethanol (**3**) and 2-(4-Hydroxy-3-hydroxysulfonyloxyphenyl)ethanol (**4**), Sodium Salts.* A mixture of compound **5** and **6** DEA salts (200 mg, 0.72 mmol) and K_2CO_3 (190 mg, 1.4 mmol) was dissolved in methanol (5 mL). The reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated, and 10 mL of water was added and then acidified with Amberlit IR 120 H^+ resin. After filtration, the water phase was washed with diethyl ether and immediately neutralized with DEA. The water phase was concentrated to 2 mL, applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g), and eluted with water. Fractions containing the desired product were lyophilized to afford a mixture of sodium salts of **3** and **4** as a cream-colored solid (142 mg, 78%). Isomer **3**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.22 (d, 1H, J = 8.4 Hz, H_{arom}), 6.82 (d, 1H, J = 2.4 Hz, H_{arom}), 6.71 (dd, 1H, ^4J = 2.4 Hz, ^3J = 8.4, H_{arom}), 3.76 (t, 2H, J = 7.2 Hz, CH_2O), 2.78 (t, 2H, J = 7.2 Hz, Ar−CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 150.4, 139.8, 138.6, 124.0, 121.3, 118.9, 64.2, 39.8. Isomer **4**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.21 (d, 1H, J = 2.0 Hz, H_{arom}), 6.95 (dd, 1H, ^4J = 2.0 Hz, ^3J = 8.4, H_{arom}), (d, 1H, J = 8.4 Hz, H_{arom}), 3.75 (t, 2H, J = 7.2 Hz, CH_2OH), 2.77 (t, 2H, J = 7.2 Hz, Ar−CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 148.9, 141.1, 131.9, 127.7, 124.5, 118.2, 64.3, 39.4; HRMS (EI) calcd for $[M - H]^-$ 233.0101, found 233.0098; MS fragmentation pattern $[M - H]^-$ = 233, $[M - SO_3H - H]^-$ = 153, $[M - SO_3 - CH_2 - OH - H]^-$ = 123.

*4-(2-Ethanoyloxyethyl)-2-methoxyphenyl Hydrogenosulfate (**10**).* Homovanillyl alcohol acetate, **9** (300 mg, 1.4 mmol), and SO_3·Py (2.8 mmol) were dissolved in 6 mL of dioxane and submitted to sulfation conditions. TLC (ethyl acetate:methanol, 9:1) showed the formation of a major product and complete consumption of the initial material. Solvents were removed, and the crude extract was purified by flash chromatography. Fractions containing the desired product were evaporated to afford compound **10** (120 mg, 25%) as a white solid and a mixture of compound **10** and its DEA salt (260 mg). The mixture of DEA salts was then dissolved in 2 mL of water, applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g), and eluted with water. Fractions containing the desired product were lyophilized to afford the sodium salt of **10** as a white solid (238 mg, 54%): ^{1}H NMR (400 MHz, CD_3OD) δ 7.42 (d, 1H, J = 8.2 Hz, H_{arom}), 6.96 (d, 1H, J = 2.0 Hz, H_{arom}), 6.82 (dd, 1H, ^4J = 2.0 Hz, ^3J = 8.2 H_{arom}), 4.30 (t, J = 6.8 Hz, 2H, CH_2OAc), 2.94 (t, J = 6.8 Hz, 2H, Ar−CH_2), 3.89 (s, 3H, O−CH_3); ^{13}C NMR (100 MHz, CD3OD) δ 172.9, 153.2, 141.6, 137.1, 123.7, 121.9, 114.9, 66.2, 56.6, 35.8, 20.8; HRMS (EI) calcd for $[M - H]^-$ 289.0399, found 275.0402; MS fragmentation pattern $[M - H]^-$ = 289, $[M - SO_3H - H]^-$ = 209, $[M - SO_3 - CH_3 - CO_2 - H]^-$ = 149.

*2-(4-Hydroxysulfonyloxy-3-methoxyphenyl)ethanol (**8**), Sodium Salt.* Compound **10** sodium salt (200 mg, 0.69 mmol) and K_2CO_3 (190 mg, 1.4 mmol) was dissolved in MeOH (5 mL). The reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated, and 10 mL of water was added and then acidified with Amberlit IR 120 H^+ resin. After filtration, the water phase was washed with ethyl ether and immediately neutralized with DEA. The water phase was concentrated to 2 mL, applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g), and eluted with water. Fractions containing the desired product were lyophilized to afford the sodium salt of **8** as a white solid (142 mg, 78%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.43 (d, 1H, J = 8.2 Hz, H_{arom}), 7.05 (d, 1H, J

DOI: 10.1021/acs.jafc.5b04307
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= 2.0 Hz, H_{arom}), 6.82 (dd, 1H, ^4J = 2.2 Hz, ^3J = 8.2, H_{arom}), 3.87 (s, 3H, OCH_3), 3,75 (t, 2H, J = 7.0 Hz, CH_2OH), 2.74 (t, 2H, J = 7.0 Hz, Ar−CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 172.8, 153.1, 141.6, 137.1, 123.6, 121.9, 114.9, 66.1, 57.1, 39.7; HRMS (EI) calcd for $[M - H]^-$ 247.0299, found 275.0303; MS fragmentation pattern $[M - H]^-$ = 247, $[M - SO_3H - H]^-$ = 167.

*2-(3,4-Dihydroxyphenyl)ethyl Hydrogenosulfate (**11**).* Hydroxytyrosol, **1** (300 mg, 1.95 mmol), and SO_3·Py (3.1 mmol) were dissolved in 6 mL of dioxane and submitted to sulfation conditions. TLC (ethyl acetate:methanol, 9:2.5) showed the formation of a major product and complete consumption of the initial material. Solvents were removed, and the crude extract was purified by flash chromatography. Fractions containing the desired product were evaporated to afford compound **11** (115 mg, 25%) as a white solid and a mixture of compound **11** and its DEA salt (285 mg). The mixture of DEA salts was then dissolved in 2 mL of water, applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g), and eluted with water. Fractions containing the desired product were lyophilized to afford the sodium salt of **11** as a pale cream-colored solid (248 mg, 52%): ^{1}H NMR (400 MHz, CD_3OD) δ 6.74 (d, 1H, J = 2.1 Hz, H_{arom}), 6.72 (d, 1H, J = 8.0 Hz, H_{arom}), 6.61 (dd, 1H, ^4J = 2.1 Hz, ^3J = 8.0, H_{arom}), 4.15 (t, 2H, J = 7.3 Hz, CH_2OSO_3H), 2.87 (t, 2H, J = 7.3 Hz, Ar−CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 146.1, 144.8, 130.8, 121.3, 117.1, 116.3, 70.1, 36.2; HRMS (EI) calcd for $[M - H]^-$ 233.0100, found 233.0098; MS fragmentation pattern $[M - H]^-$ = 233, $[M - SO_3 - H]^-$ = 153, $[O-SO_3-H]^-$ = 97, $[2M + Na - H]^-$ = 489.

*4-(2-Ethanoyloxyethyl)phenyl 1,2-Dihydrogenosulfate (**12**).* Hydroxytyrosol acetate, **2** (300 mg, 1.55 mmol), and SO_3·Py (12 mmol) were dissolved in 10 mL of dioxane and submitted to sulfation conditions. Solvents were removed, and the crude extract was purified by flash chromatography (ethyl acetate:methanol, 9:2.5). Fractions containing the desired product were evaporated to afford compound **12** (184 mg, 33%) as a white-colored solid: ^{1}H NMR (400 MHz, CD_3OD) δ 7.54 (d, 1H, J = 8.4 Hz, H_{arom}), 7.53 (d, 1H, J = 1.9 Hz, H_{arom}), 7.04 (dd, 1H, ^4J = 1.9 Hz, ^3J = 8.4, H_{arom}), 4.27 (t, 2H, J = 6.8 Hz, CH_2OAc), 2.93 (t, 2H, J = 6.8 Hz, Ar−CH_2), 2.05 (s, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 172.9, 145.1, 143.8, 136.9, 126.7, 123.8, 123.3, 66.0, 33.4; HRMS (EI) calcd for $[M - H]^-$ 354.9801, found 354.9806; MS fragmentation pattern $[M - H]^-$ = 355, $[M - SO_3 - H]^-$ = 275.

*2-(3-Methoxy-4-hydroxysulfonyloxyphenyl)ethanoic Acid (**15**).* 4-Hydroxy-3-methoxyphenylethanoic acid, **14** (300 mg, 1.64 mmol), and SO_3·Py (3 mmol) were dissolved in 6 mL of dioxane and submitted to sulfation conditions. Solvents were removed, and the crude extract was purified by flash chromatography (ethyl acetate:methanol, 9:1). Fractions containing the desired product were evaporated to afford compound **15** (115 mg, 27%) as a white-colored solid: ^{1}H NMR (400 MHz, CD_3OD) δ 7.43 (d, 1H, J = 8.2 Hz, H_{arom}), 7.02 (d, 1H, J = 2.0 Hz, H_{arom}), 6.87 (dd, 1H, ^4J = 2.0 Hz, ^3J = 8.2, H_{arom}), 3.87 (s, 3H, CH_3O), 3.59 (s, 2H, Ar−CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 175.6, 153.1, 141.9, 133.8, 123.6, 122.4, 115.4, 56.6, 42.6; HRMS (EI) calcd for $[M - H]^-$ 261,0101, found 261,0105; MS fragmentation pattern $[M - H]^-$ = 261, $[M - CO_2 - H]^-$ = 217, $[M - SO_3 - H]^-$ = 181.

*2-(3-Hydroxy-4-hydroxysulfonyloxyphenyl)ethanoic Acid (**17**) and 2-(4-Hydroxy-3-hydroxysulfonyloxyphenyl)ethanoic Acid (**18**).* 3,4-Dihydroxyphenylethanoic acid, **16** (300 mg, 1,78 mmol), and SO_3·Py (3,5 mmol) were dissolved in 6 mL of dioxane and submitted to sulfation conditions. TLC (ethyl acetate:methanol, 9:2.5) showed the formation of a major product. Solvents were removed, and the crude extract was purified by flash chromatography. Fractions containing a mixture of the desired products were evaporated to afford compounds **17** and **18** (6:4) (115 mg, 26%) as a white-colored solid. Isomer **17**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.22 (d, 1H, J = 8.2 Hz, H_{arom}), 6.88 (d, 1H, J = 2.1 Hz, H_{arom}), 6.76 (dd, 1H, ^4J = 2.1 Hz, ^3J = 8.2, H_{arom}), 3.50 (s, 2H, Ar−CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 176.5, 150.5, 140.5, 135.1, 124.2, 122.0, 119.5, 43.7. Isomer **18**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.25 (d, 1H, J = 2.1 Hz, H_{arom}), 6.99 (dd, 1H, ^4J = 2.1 Hz, ^3J = 8.2, H_{arom}), 6.85 (d, 1H, J = 8.2 Hz, H_{arom}), 3.50 (s, 2H, Ar−CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 176.5, 149.5, 141.3, 128.5, 128.2, 125.3, 118.4, 42.7; HRMS (EI) calcd for $[M - H]^-$ 246.9921, found 246.9926; MS fragmentation pattern $[M - H]^-$ = 247, $[M - CO_2 - H]^-$ = 203, $[M - SO_3 - H]^-$ = 167, $[M - CO_2 - SO_3 - H]^-$ = 123.

*(E)-3-(4-Hydroxysulfonyloxy-3-methoxyphenyl)prop-2-enoic Acid (**20**).* Ferulic acid, **19** (300 mg, 1.54 mmol), and SO_3·Py (3.1 mmol) were dissolved in 6 mL of dioxane and submitted to sulfation conditions. Solvents were removed, and the crude extract was purified by flash chromatography (ethyl acetate:methanol, 9:2.5). Fractions containing the desired product were evaporated to afford compound **20** (95 mg, 23%) as a pale cream-colored solid: ^{1}H NMR (400 MHz, CD_3OD) δ 7.64 (d, 1H, J = 16.0 Hz, Ar−CH) 7.53 (d, 1H, J = 8.3 Hz, H_{arom}), 7.27 (d, 1H, J = 2.0 Hz, H_{arom}), 7.17 (dd, 1H, ^4J = 2.0 Hz, ^3J = 8.3, H_{arom}), 6.45 (d, 1H, J = 16.0 Hz, Ar−CH-CH), 3.90 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 170.8, 153.3, 145.7, 144.9, 133.2, 123.6, 122.1, 119.2, 113.1, 56.7; HRMS (EI) calcd for $[M - H]^-$ 273.0102, found 273.0106; MS fragmentation pattern $[M - H]^-$ = 273, $[M - SO_3 - H]^-$ = 193.

RESULTS AND DISCUSSION

Although the sulfation reaction has a long history and seems to be accomplished in one step, sulfated small molecules are difficult to chemically synthesize as their physicochemical properties are radically changed by the introduction of a sulfate group, making them water-soluble and therefore difficult to isolate in a pure form. Typical problems include the presence of sulfating complexes, the required proportion of which is usually high, the presence of inorganic salts, the lability of sulfate groups to acidic conditions and high temperatures,[22,23] and the need for purification protocols with expensive reagents and preparative RP-HPLC.[24−26]

There are a number of reported procedures for the sulfation of catechols.[27−31] The majority of these perform sulfation according to the method described by Kawai et al.,[31] using sulfur trioxide pyridine complex as a sulfating agent in excess pyridine at 80 °C. However, this procedure leads to a mixture of unreacted catechol with sulfates and has low reaction yields, and purification is relatively difficult and expensive. A number of strategies have therefore been developed to convert hydroxyl groups to sulfate diesters, creating a protected sulfate monoester that can then be deprotected at a later point in time. However, this approach always involves two steps, and the formation of sulfate diesters and the deprotection steps yield moderate yields. Furthermore, the deprotection conditions are incompatible with the synthesis of most aryl sulfate monoesters.[22,32]

Recently a microwave-based protocol has been developed to increase the sulfation rate of phenolic structures.[33] It was thought that microwaves would likely induce significant rate increases because the sulfated intermediates would couple to microwaves through ionic conduction. Optimal results were achieved by using SO_3−amine complex (−NR_3 or −Py, where R = Me or Et) at approximately 6−10 times molar proportion per phenolic group in the presence of microwaves at 100 °C for 10−20 min. The method is especially suitable for isolating small amounts of the persulfated products, and it may be possible to scale up. Since most of the natural metabolites are monosulfates, however, it is important to avoid disulfated products as they are difficult to separate from the monosulfated compounds. As a result, protection of one of the catechol groups needs to be carried out before the sulfation reaction. This limitation led us to seek an alternative approach to sulfation, which should be relatively rapid, efficient, cheap, and widely applicable to a number of catechol scaffolds.

DOI: 10.1021/acs.jafc.5b04307
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As the sulfating agent in this work, we started by using chlorosulfonic acid in ethyl ether, which allows for milder reaction conditions and shorter reaction times with respect to amine sulfate complexes. Chlorosulfonic acid has been successfully used for the sulfation of daidzein and other phenols and polyphenols such as quercetin, genistein, and equol.[34] However, in none of our attempts could we obtain the sulfated compound. In fact, after 24 h at room temperature with a ratio $ClSO_3H$/phenol of 8, only the 2,3-dihydroxy-5-(2-hydroxyethyl)benzenesulfonic acid was obtained (η = 32% after purification, please see Supporting Information, compound **13**, Spectra A).

Scaffolds based on phenolic structures and containing more acidic −OH groups seem to be more efficiently sulfated with SO_3 complexes with weaker bases such as pyridine and DMF, which suggests that the partial positive charge on the sulfur atom of the SO_3 complex would be greater than that of the SO_3·DEA or TEA complexes.[35] It is thus likely that the nucleophilic attack on the SO_3 complex would be more favorable and result in higher yields. Therefore, we decided to perform the direct sulfation of hydroxytyrosol acetate with SO_3·Py complex in dioxane using the conventional conditions reported in the literature:[36] temperature of 40 °C and a proportion of phenol/SO_3 of 8. TLC analysis showed the formation of a major product after 15 min at room temperature. However, when heated at 40 °C for 2 h according to the method described in the literature,[36] we observed that the product disappeared. After 5 h of reaction (entry 1, Table 2) and following the workup described in Materials and Methods, only compound **13** was again obtained. These results are in accordance with early works performed by Cerfontain et al. in the 1980s.[37,38] They observed that the initial reaction of 1-naphthol, 2,6-dialkylphenols, 2,6-dichlorophenol, phenol, and 2,6-dimethylanisole with SO_3 in an aprotic solvent was sulfation with formation of hydrogen sulfates. However, by increasing the temperature, the initial phenyl hydrogen sulfate was slowly converted into the phenolsulfonic acids via O-desulfonation and subsequent C-sulfonation if the phenol was in excess and via C-sulfonation and subsequent O-desulfonation if the SO_3 was in excess. This probably happened in this first attempt (entry 1, Figure 2). Therefore, after addition of reactants (Table 2, entry 2), the mixture was kept at −20 °C, and a complete consumption of the initial material was observed after 20 h.

Table 2. Reaction Conditions

entry	initial reagent	ratio phenol: SO_3·Py	T (°C)	t (h)	η (%)[a]	product
1	**2**	1:8	0/50	0.25/5	65	**13**
2	**2**	1:8	0/−20	0.25/20	33	**12**
3	**2**	1:2	0/−20	0.25/20	31	**5** and **6**
					58	Na^+ salts of **5** and **6**

[a]After purification.

The resulting acidic reaction mixture was then neutralized with DEA to avoid sulfate decomposition during the solvent evaporation. In fact, the decomposition of aryl sulfates to alcohol and sulfur trioxide appears to proceed, at pH < 4, via a rapid acid-catalyzed reaction where the leaving group is protonated. It is interesting to note that the rate of acid-catalyzed solvolysis of arylsulfates is subject to acceleration in systems of low water content. For example, the rate of the acid-catalyzed solvolysis of methyl sulfate is increased by a factor of 10^7 in changing from pure water to 1.9% water/dioxane.[39] This unusual dioxane−water effect is in opposite direction to those commonly observed for most hydrolytic reactions. The most likely mechanism thus involves a rate determining unimolecular sulfur−oxygen bond fission with the elimination of sulfur trioxide (Figure 3).[40] During solvent evaporation, SO_3 is eliminated from the mixture, shifting the reaction toward the decomposition. We have found that this decomposition during solvent evaporation can be avoided by previously using a stronger base than pyridine to neutralize the acidic mixture. Therefore, DEA was used, and this amine was chosen since its excess could also be easily removed by evaporation.

After solvent evaporation the crude extract was purified by flash chromatography on silica gel using polar organic solvents. During this purification and probably because of the acidic character of silica gel, TLC analysis of the collected fractions showed the presence of two spots partially overlapped corresponding to hydrogenosulfates and sulfates. Therefore, during this purification, two sets of fractions could be obtained: one with only hydrogenosulfates and another one corresponding to a mixture of hydrogenosulfates and their salts. Fractions containing only hydrogenosulfates were evaporated to dryness. Apparently, hydrogenosulfates showed much more stability in the organic solvents used (ethyl acetate/methanol) than in dioxane/water.

In this second attempt (Table 2, entry 2), still with the proportion between the phenol and the SO_3·Py complex of 8, the only product obtained was the dihydrogenosulfate **12** and its salts. Therefore, this ratio was decreased to 2 (Table 2, entry 3), and finally only monohydrogenosulfates were obtained. Under these conditions, there was a preference for the sulfation at the 4-hydroxyl group (~60%), whatever the initial catechol being sulfated. Moreover, only monohydrogenosulfates were obtained in a one-step reaction, without the need to protect one of the hydroxyl groups at the aromatic ring. After solvent evaporation the crude extract was purified by flash chromatography on silica gel. Again, two sets of fractions could be obtained: one with only hydrogenosulfates and another one corresponding to a mixture of hydrogenosulfates and their salts. Fractions containing DEA salts were evaporated, dissolved in 2 mL of water, applied to a column of cation-exchange resin, and eluted with water. This allowed the exchange between DEA and the physiological cation Na^+. The experimental conditions used for the sulfation reaction as well as their isolated yields are summarized in Table 2.

The same reaction conditions were then applied to compounds **9** (homovanillyl alcohol acetate), **14** (homovanillic acid), **16** (3,4-dihydroxyphenyl acetic acid, DOPAC), and **19** (ferulic acid) with similar results.

In the first attempt to sulfate the hydroxytyrosol molecule, the same conditions used above were applied. As expected, the aliphatic hydroxyl group was preferred and sulfated, and only the 2-(3,4-dihydroxyphenyl)ethyl hydrogenosulfate was obtained. Therefore, another strategy was needed in order to obtain these monohydrogenosulfates.

Using the DEA salts of compounds **5** and **6**, acetyl deprotection was carried out using K_2CO_3 in methanol to obtain the mixture of monosulfated hydroxytyrosol derivatives **3** and **4**. Solvent was then removed and water added, followed by neutralization with Amberlite IR 120 H^+ resin. This procedure permitted the K_2CO_3 removal from the mixture by

DOI: 10.1021/acs.jafc.5b04307
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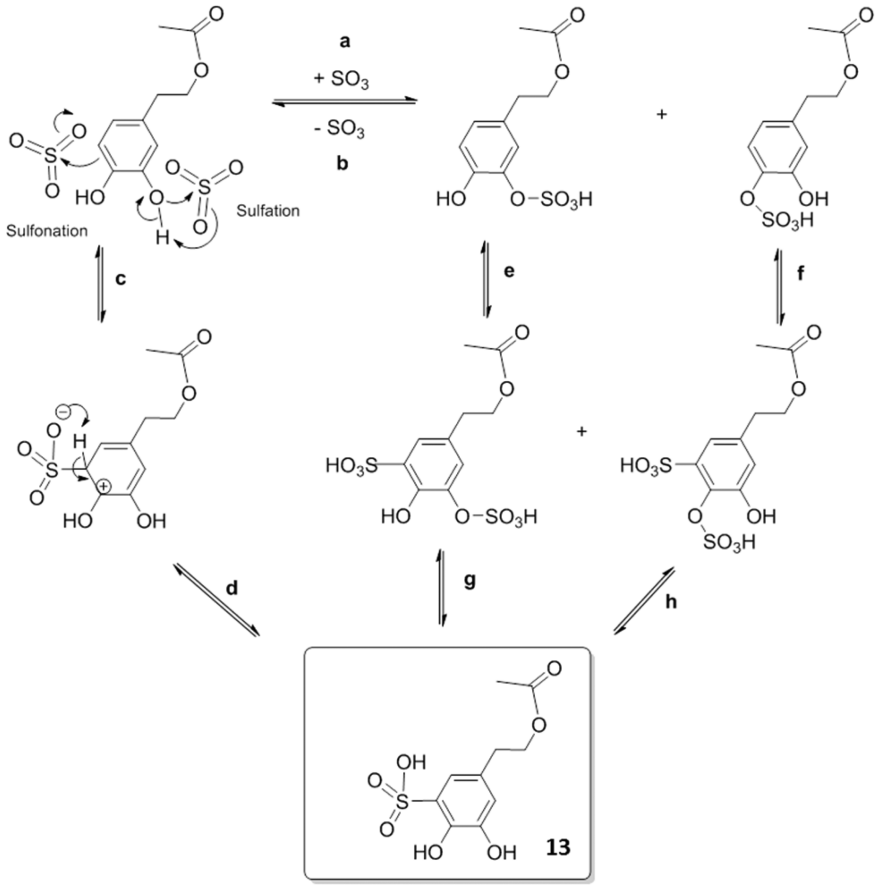

Figure 2. Phenol sulfonic acid formation by O-desulfonation. (b, g, h) O-protodesulfonations. (c) Slow reaction of the arene C═C with SO_3 to give a resonance stabilized hydroxy-carbocation. (d) Fast loss of H^+ (intramolecular elimination) from the carbocation to restore the aromatic system. (c + d, e, f) Ring-sulfonation on the C3 position ascribed to steric hindrance reasons. Hydroxytyrosol acetate used as an example.

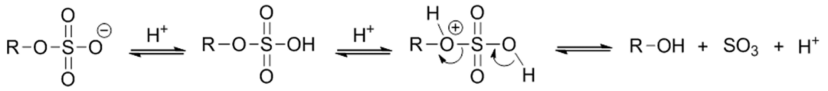

Figure 3. Sulfur−oxygen bond fission with the elimination of sulfur trioxide in acidic conditions.

the formation of CO_2. The aqueous phase was then washed with ethyl ether (to remove the formed acetic acid) and quickly neutralized again with DEA. After concentration, the mixture was then applied to a column of cation-exchange resin and eluted with water. Once more, this allowed the exchange between DEA and the physiological cation Na^+. The same procedure was then performed for the synthesis of compound **8** with similar results.

In conclusion, an efficient chemical method suitable for the synthesis of mono-O-sulfate metabolic conjugates of several simple phenolic compounds has been developed. The strategy adopted is based on mild conditions of reaction: avoidance of high temperature conditions during the synthesis and of low pressure conditions during purification. Ten monosulfates have been synthesized in 1−2 steps in a good yield and using simple, cheap, and fast procedures. These compounds can be used as standards for the determination of the metabolic profile of hydroxytyrosol in various species, including humans. It is foreseen that the proposed protocol will be useful for the synthesis of conjugates of other compounds based on the catechol scaffold.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b04307.

^{1}H- and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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DOI: 10.1021/acs.jafc.5b04307
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