First synthesis, characterization, and evidence for the presence of hydroxycinnamic acid sulfate and glucuronide conjugates in human biological fluids as a result of coffee consumption†

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A systematic investigation of the human metabolism of hydroxycinnamic acid conjugates was carried out. A set of 24 potential human metabolites of coffee polyphenols has been chemically prepared, and used as analytical standards for unequivocal identifications. These included glucuronide conjugates and sulfate esters of caffeic, ferulic, isoferulic, *m*-coumaric and *p*-coumaric acids as well as their dihydro derivatives. A particular focus has been made on caffeic and 3,4-dihydroxyphenylpropionic acid derivatives, especially the sulfate conjugates, for which regioselective preparation was particularly challenging, and have so far never been identified as human metabolites. Ten out of the 24 synthesized conjugates have been identified in human plasma and/or urine after coffee consumption. A number of these conjugates were synthesized, characterized and detected as hydroxycinnamic acid metabolites for the first time. This was the case of dihydroisoferulic acid 3′-*O*-glucuronide, caffeic acid 3′-sulfate, as well as the sulfate and glucuronide derivatives of 3,4-dihydroxyphenylpropionic acid.

Introduction

With more than one billion drinkers over the world, coffee is one of the most popular beverages. Coffee consumption may be associated with several health benefits,[1,2] some of them related to the antioxidant activity of its phenolic constituents.[1,3] Coffee is one of the major sources of chlorogenic acids.[4,5] The chlorogenic acid compositions of commercial roasted[6] and brewed[7] coffees have been reported. On the other hand, green coffee beans contain the 3 isomers (3-, 4-, and 5-) of *O*-caffeoyl quinic acids, 5-*O*-feruloylquinic acid, and a number of di-*O*-caffeoylquinic acids (3,5-, 3,4-, and 4,5-).[8] Recently, our knowledge of the phytochemical composition of green coffee has been considerably increased by the use of LC-MS^n, leading to identification of complex mixtures of mono- and di-*O*-*p*-coumaroyl, caffeoyl, feruloyl, and/or dimethoxycinnamoyl quinic acid esters.[9,10] However, it is well known that the polyphenols contained in food are extensively metabolized by the human body. Thus, the real entities that the target cells or tissues will be exposed to after consumption are likely to be the metabolites, rather than the native forms present in plants. These metabolites could significantly contribute to the biological activity.[11] Indeed, chlorogenic acids are transformed during digestion and the first site of their human metabolism is the small intestine. Since the presence of an esterase activity has been detected in the human small intestine mucosa,[12,13] this results in the limited release of a first pool of free hydroxycinnamic acids. The second site of hydrolysis of chlorogenic acids is the colon, catalyzed by the colonic microflora.[14,15] The released caffeic acid is subsequently dehydroxylated into *m*-coumaric acid by the microflora.[16] Alternatively, caffeic acid may be reduced to 3,4-dihydroxyphenylpropionic acid (dihydrocaffeic acid), and subsequently dehydroxylated into 3-hydroxyphenylpropionic acid.[17] All the metabolites released in the digestive tract can be absorbed and further metabolized in the enterocyte and the liver by phase-II enzymes into their sulfated and/or glucuronidated conjugates. The absorption and the human metabolism of phenolic acids from coffee have not been studied in detail. Indeed, caffeic acid was found in plasma,[18] and caffeic acid and metabolites resulting from methylation and/or reduction were present in urine. These metabolites included ferulic, isoferulic, dihydroferulic, and dihydrocaffeic acids.[19,20] *p*-Coumaric acid was also identified in the urine of most subjects.[20] Since all these compounds have been identified after treatment of plasma or urine by β-glucuronidase and sulfatase, no identification of the original conjugates was carried out, and these were only suspected to be glucuronidated and/or sulfated derivatives. In contrast, Caco-2[21] and HepG2[22] cells have been clearly shown to produce hydroxycinnamic acid glucuronides and sulfates, and the conjugates identified by LC-MS. However, the technique was obviously not able to assign the structure of caffeic acid derivatives, where two different positions of conjugation are possible on the catechol ring. On the other hand, ferulic acid is metabolized in rats into two different monoglucuronides, one presumably conjugated at the phenolic hydroxyl of ferulic acid, and a second one presumably conjugated at the carboxylic acid function of ferulic acid.[23] Again, no distinction could be made by LC-MS between these two isomers. However, the exact knowledge of the position of conjugation is of

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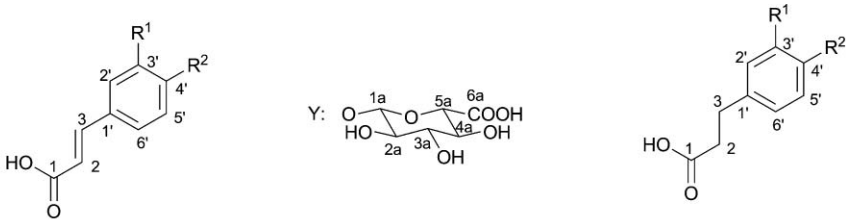
† Electronic supplementary information (ESI) available: Detailed synthetic strategies, procedures and characterization for compounds **1** to **16**, and **19** to **22** can be found in ESI part A. Purity data (HPLC control, HRMS, and/or quantification of sulfate esters) and NMR data (^{1}H and ^{13}C) of the entire set of standard conjugates **1–24** can be found in ESI part B. See DOI: 10.1039/c0ob00137f



1: R^1 = H, R^2 = Y: *p*-Coumaric acid 4'-*O*-β-D-glucuronide.
2: R^1 = Y, R^2 = H: *m*-Coumaric acid 3'-*O*-β-D-glucuronide.
3: R^1 = OCH_3, R^2 = Y: Ferulic acid 4'-*O*-β-D-glucuronide.
4: R^1 = Y, R^2 = OCH_3: Isoferulic acid 3'-*O*-β-D-glucuronide.
5: R^1 = Y, R^2 = OH: Caffeic acid 3'-*O*-β-D-glucuronide.
6: R^1 = OH, R^2 = Y: Caffeic acid 4'-*O*-β-D-glucuronide.
7: R^1 = H, R^2 = OSO_3^-: *p*-Coumaric acid 4'-sulphate.
8: R^1 = OSO_3^-, R^2 = H: *m*-Coumaric acid 3'-sulphate.
9: R^1 = OCH_3, R^2 = OSO_3^-: Ferulic acid 4'-sulphate.
10: R^1 = OSO_3^-, R^2 = OCH_3: Isoferulic acid 3'-sulphate.
11: R^1 = OSO_3^-, R^2 = OH: Caffeic acid 3'-sulphate.
12: R^1 = OH, R^2 = OSO_3^-: Caffeic acid 4'-sulphate.

13: R^1 = H, R^2 = Y:Dihydro-*p*-Coumaric acid 4'-*O*-β-D-glucuronide.
14: R^1 = Y, R^2 = H: Dihydro-*m*-Coumaric acid 3'-*O*-β-D-glucuronide.
15: R^1 = OCH_3, R^2 = Y: Dihydroferulic acid 4'-*O*-β-D-glucuronide.
16: R^1 = Y, R^2 = OCH_3: Dihydroisoferulic acid 3'-*O*-β-D-glucuronide.
17: R^1 = Y, R^2 = OH: Dihydrocaffeic acid 3'-*O*-β-D-glucuronide.
18: R^1 = OH, R^2 = Y: Dihydrocaffeic acid 4'-*O*-β-D-glucuronide.
19: R^1 = H, R^2 = OSO_3^-: Dihydro-*p*-coumaric acid 4'-sulphate.
20: R^1 = OSO_3^-, R^2 = H: Dihydro-*m*-coumaric acid 3'-suphate.
21: R^1 = OCH_3, R^2 = OSO_3^-: Dihydroferulic acid 4'-sulphate.
22: R^1 = OSO_3^-, R^2 = OCH_3: Dihydroisoferulic acid 3'-sulphate.
23: R^1 = OSO_3^-, R^2 = OH: Dihydrocaffeic acid 3'-sulphate.
24: R^1 = OH, R^2 = OSO_3^-: Dihydrocaffeic acid 4'-sulphate.

Fig. 1 Structures of the most probable circulating human hydroxycinnamic acid glucuronide **1–12**, and sulfate **13–24** conjugates.

the utmost important since this parameter is known to have an impact on the biological activity.[11,24]

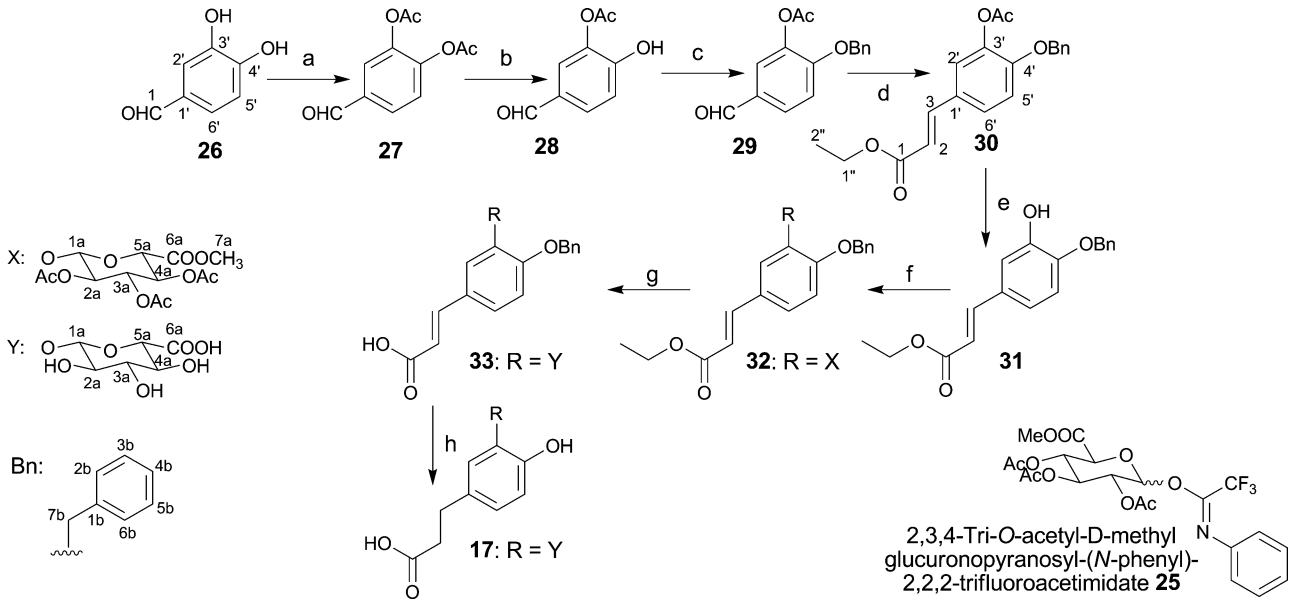
Furthermore, in the absence of chromatographic standards of known purity, all the previous identifications only remained qualitative and no quantification was made. All these examples clearly demonstrate the limitations of the LC-MS technology as a unique analytical tool. Beside the fact that we still do not have an exact picture of the nature of the human metabolites of coffee phenolics, only the synthesis of well characterized standard conjugates would allow the investigation of their contribution to the overall health effects of coffee, as well as their distribution in circulating fluids and tissues. Very few reports have appeared on the synthesis of animal and human polyphenol metabolites, and the subject has been reviewed by us.[25] The methods of synthesis of glucuronic acid conjugates have been improved tremendously due to the introduction of trichloroacetimidates as glycosyl donors, but in contrast, the synthesis of sulfate conjugates is still difficult. Even more problematic is the purification of phenolic sulfate conjugates, which remain frequently contaminated by some inorganic material, thus precluding determination of their exact purity. In the present paper, we report on the efficient synthesis of a set of 24 sulfated or glucuronidated conjugates of hydroxycinnamic and hydroxyphenylpropionic acids, as potential human metabolites of coffee phenolics. Furthermore, the purity of all conjugates including sulfates was determined. This allowed us to identify the circulating human phenolic acid metabolites resulting from coffee consumption.

Results and discussion

Considering i) the phenolic composition of green and brewed coffee, and ii) the previous metabolic studies related to coffee consumption, the metabolites that may potentially be present in human circulating fluids could be glucuronidated and sulfated conjugates of hydroxycinnamic and dihydro hydroxycinnamic (hydroxyphenylpropionic) acids. The structures of these potential metabolites **1–24** are displayed in Fig. 1. Hydroxycinnamic acid mono-*O*-glucuronides **1–4** have been synthesized by an improved procedure involving glucuronidation (using glucuronic acid donor **25**, see Scheme 1) of their ethyl ester precursors. The latter compounds have been prepared by the Horner–Wadsworth–Emmons reaction of the corresponding substituted hydroxybenzaldehydes with ethyl (triphenylphosphoranyliden)acetate. Catalytic hydrogenation of hydroxycinnamic acid glucuronides **1–4** yielded the corresponding dihydro derivatives **13–16**. Caffeic acid 3′- and 4′-*O*-glucuronides (**5** and **6**) have been regioselectively obtained for the first time by glucuronidation of ethyl 4′-acetyl- and ethyl 3′-acetyl caffeic acids, respectively. On the other hand, the sulfate conjugates **7–10** and **19–22** have been prepared by direct sulfation (with a sulfur trioxide-trimethylamine complex, in aqueous Na_2CO_3) of the corresponding hydroxycinnamic acids or their reduced forms. In the main body of this paper, only the synthetic strategy for the four caffeic- and dihydrocaffeic acid sulfates (**11**, **12**, **23**, and **24**), and of the two dihydrocaffeic acid glucuronides (**17** and **18**) is presented. These are newly characterized compounds, the presence of which has never been evidenced in human biological fluids. However the detailed synthetic strategies and preparations of the above mentioned compounds **1** to **10**, **13** to **16**, and **19** to **22** are displayed in the ESI of the paper (Part A; Schemes S1 to S3).†

For the preparation of dihydrocaffeic acid 3′-*O*-β-D-glucuronide, we used the benzyl group as protector (Scheme 1). The main advantage was to combine, in a single step, the benzyl deprotection and the reduction of the 2,3-double bond. Acetylation of 3′,4′-dihydroxybenzaldehyde **26** by acetyl chloride in the presence of DBU as a base yielded the desired compound **27** in 88% yield. The regioselective deacetylation of **27** using thiophenol and imidazole gave access to 3′-acetoxy-4′-hydroxybenzaldehyde **28** in 77% yield.

Compound **28** was benzylated under Mitsunobu conditions to give aldehyde **29** (97%), which was submitted to a Horner–Wadsworth–Emmons reaction, yielding the ethyl cinnamate **30** (89%). Deacetylation of **30** with sodium methoxide in dry methanol and dry CH_2Cl_2 yielded compound **31** (97%). The latter compound was glucuronidated at position 3′, using **25** as glucuronic acid donor, to give the protected glucuronide **32** (76%). The correct aromatic substitution of compound **32** was



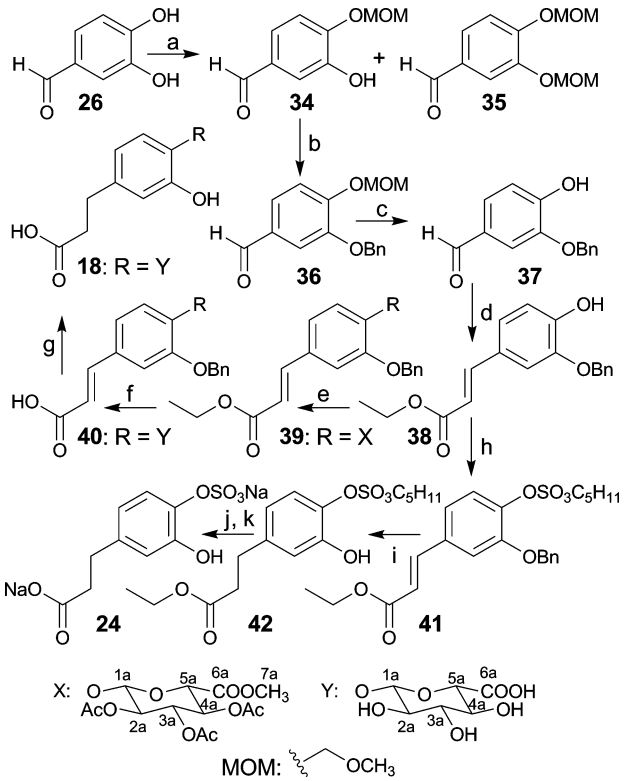
Scheme 1 The preparation of dihydrocaffeic acid 3′-*O*-β-D-glucuronide **17**. a) Acetyl chloride, DBU, CH_2Cl_2, 88%; b) Thiophenol, imidazole, NMP, 77%; c) Benzyl alcohol, triphenylphosphine, DEAD, 97%; d) Ethyl (triphenylphosphoranyliden)acetate, CH_2Cl_2, 89%; e) NaOMe, dry MeOH–dry CH_2Cl_2, 97%; f) 2,3,4-Triacetyl-D-methyl glucuronopyranosyl-(*N*-phenyl)-2,2,2-trifluoroacetimidate **25**, BF_3 etherate, CH_2Cl_2, 76%; g) NaOH, MeOH–H_2O, then Amberlite® IR-120, 73%; h) H_2, 5% Pd/C, MeOH–H_2O, 96%.

evidenced by the presence, on its long distance HMBC spectrum, of a correlation between the benzyl protons H7b at 5.11 ppm and C4′ at 151.42 ppm, as well as a correlation between the anomeric proton H1a at 5.09 ppm and C3′ at 146.12 ppm. Similar H7b/C4′ correlations, indicative of a 4′-benzylation, were detected on the HMBC spectra of compounds **29–31**. Saponification of compound **32** furnished 4′-*O*-benzylcaffeic acid 3′-*O*-β-D-glucuronide **33** (73%), which was hydrogenated to dihydrocaffeic acid 3′-*O*-β-D-glucuronide **17** in 96% yield.

The preparation of dihydrocaffeic acid 4′-*O*-β-D-glucuronide **18** required access to a 3′-*O*-benzylated precursor (Scheme 2). Our strategy was based on our previous observation that methoxymethylation of quercetin only affected the most acidic phenolic hydroxyl groups when *N*-ethyldiisopropylamine was used as a base.[26] Taking that into consideration, methoxymethylation of protocatechualdehyde **26** was expected to mainly affect the 4′-hydroxyl group (conjugated to the carbonyl). We found it more convenient to combine the three steps a–c of Scheme 2 into a one pot protocol. By manipulating and optimizing the extraction procedures, the side products of each reaction could be eliminated. In fact, reaction of **26** with chloromethyl methyl ether in presence of *N*-ethyldiisopropylamine, yielded 3′-hydroxy-4′-methoxymethoxybenzaldehyde **34**. Other compounds present in the reaction medium were unreacted 3′,4′-dihydroxybenzaldehyde **26**, and 3′,4′-bis(methoxymethoxy)benzaldehyde **35**. Extraction of the products of the reaction in dichloromethane under alkaline conditions led to the elimination of unreacted protocatechualdehyde **26**. The subsequent benzylation reaction (step b) was carried out on the mixture of **34** plus **35**. This resulted in the formation of 3′-benzyloxy-4′-methoxymethoxybenzaldehyde **36**, while of course **35** was not affected by this step. Hydrolysis of the MOM groups in step c led to a mixture of 3′-benzyloxy-4′-hydroxybenzaldehyde **37** and protocatechualdehyde **26** which, again, was easily eliminated by extraction of the products by ethyl acetate at alkaline pH. Final purification of **37** was carried out by VLC on silica and RP-18, with an overall yield of 42% from **26**. Neither on the basis of the melting points, nor on the analysis of the ^{1}H NMR data, a clear distinction could be made in the past between 3′-benzyloxy-4′-hydroxybenzaldehyde and its 4′-benzyloxy-3′-hydroxy isomer.[27] In compound **37**, however, 3′-*O*-benzylation was clearly evidenced by the presence of the long distance H7b/C3′ correlation on its HMBC spectrum. The remainder of Scheme 2 followed similar procedure as above, *i.e.* i) reaction of aldehyde **37** to cinnamic acid **38** (56%), ii) glucuronidation of **38** with activated sugar **25** to give the protected glucuronide **39** (73%), and iii) saponification of **39** to benzylated glucuronide **40** (78%). Again, hydrogenation of **40** led to both the removal of the 3′-*O*-benzyl protective group, and to the reduction of the 2,3-double bond, affording dihydrocaffeic acid 4′-*O*-β-D-glucuronide **18** in quantitative yield.

For the preparation of hydroxycinnamic acid sulfates, again access to regioselectively protected precursors was necessary. Furthermore, the protecting group had to be removed under conditions compatible with the stability of the sulfate ester bond.

The use of alkyl chlorosulfates in the synthesis of sulfate monoesters has recently been reported,[28] and 2,2,2-trichloroethyl chlorosulfate has been involved in the preparation of a sulfated phenolic alkaloid.[29] Among the main advantages of this approach, the sulfation reaction was compatible with the presence of a wide range of protecting groups. Thus, 3′-*O*-benzylcaffeate **38** was sulfated with neopentyl chlorosulfate,[28,30] to afford compound **41** in 86% yield. Reduction of **41** yielded compound **42** (90%). Elimination of the neopentyl (sodium azide), and ethyl ester (aq. NaOH) substituents yielded dihydrocaffeic acid 4′-sulfate **24** in 34% overall yield from **42**.

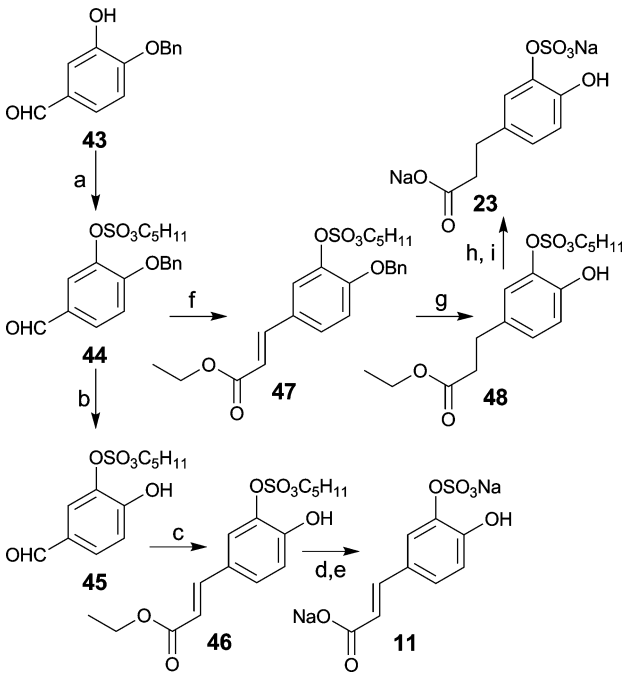


Scheme 2 The preparations of dihydrocaffeic acid 4′-*O*-β-D-glucuronide **18**, and of dihydrocaffeic acid 4′-sulfate **24**. a) Chloromethyl methyl ether, iPr_2NEt, THF; b) Benzyl bromide, K_2CO_3, MeCN; c) 37% HCl, MeOH; a + b + c: 42% d) Ethyl (triphenylphosphoranyliden)acetate, CH_2Cl_2–THF, 56%; e) Compound **25**, BF_3 etherate, CH_2Cl_2, 73%; f) NaOH, MeOH/H_2O, then Amberlite® IR-120, 78%; g) H_2, 5% Pd/C, MeOH–H_2O, 100%; h) Cl–SO_3–C_5H_{11}, Et_3N, DMAP, THF, 86%; i) H_2, 5% Pd/C, MeOH–H_2O, 90%; j) NaN_3, DMF; k) 1 N aq. NaOH then Amberlite® CG-50, j + k: 34%.

As the precursor of caffeic acid 3′-sulfate **11**, we used 4-benzyloxy-3-hydroxybenzaldehyde **43**.[31,32] The latter compound was sulfated with neopentyl chlorosulfate, to give the neopentyl sulfate ester **44** in 91% yield (Scheme 3). Attempts to debenzylate compound **44** using classical hydrogenation conditions (H_2 + 5% Pd/C), led to the reduction of the aldehyde functional group as well. However, under milder catalytic transfer hydrogenation conditions (cyclohexene + 10% Pd/C), only debenzylation took place, leaving both the aldehyde and the neopentyl sulfate ester groups intact. This yielded aldehyde **45** in excellent 94% yield.

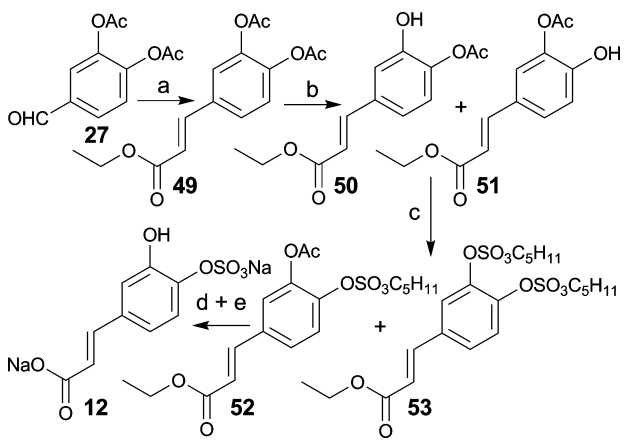
The latter compound was reacted with ethyl (triphenylphosphoranyliden)acetate, yielding the protected sulfated ethyl caffeate **46** in 83% yield. Elimination of the neopentyl (sodium azide), and the ethyl ester (aq. NaOH) groups, gave the desired caffeic acid 3′-sulfate **11** in 43% yield from **46**, and in the form of a 9/1 mixture of (*E*)- and (*Z*)-isomers.

Alternatively, aldehyde **44** was directly submitted to the Horner–Wadsworth–Emmons reaction, to give protected sulfated ethyl caffeate **47** in 86% yield (Scheme 3). The reduction of compound **47** directly afforded the debenzylated ethyl dihydrocaffeate **48** (quantitative yield), which was submitted to the usual deprotection sequence (NaN_3, followed by 1 N aq. NaOH), to give the desired dihydrocaffeic acid 3′-sulfate **23** in 70% yield from **48**.



Scheme 3 The preparations of caffeic acid 3′-sulfate **11**, and dihydrocaffeic acid 3′-sulfate **23**. a) Cl–SO_3–C_5H_{11}, DBU, CH_2Cl_2, 91%; b) Cyclohexene, 10% Pd/C, DMF, 94%; c) Ethyl (triphenylphosphoranyliden)acetate, CH_2Cl_2–THF, 83%; d) NaN_3, DMF; e) 1 N aq. NaOH then Amberlite® CG-50, d + e: 43%; f) Ethyl (triphenylphosphoranyliden)acetate, CH_2Cl_2–THF, 86%; g) H_2, 5% Pd/C, MeOH–H_2O, 100%; h) NaN_3, DMF; i) 1 N aq. NaOH then Amberlite® CG-50, h + i: 70%.

In the preparation of caffeic acid 4′-sulfate, we submitted 3′,4′-diacetoxycinnamic acid ethyl ester **49** (prepared in quantitative yield by the reaction of 3′,4′-diacetoxybenzaldehyde **27** with ethyl (triphenylphosphoranyliden)acetate) to deacetylation using thiophenol as acetyl acceptor (Scheme 4). This step turned out not to be regioselective, and a 1/1 mixture of mono deacetylated products **50** and **51** was obtained (overall mono deacetylation yield: 54%). However, during sulfation of the mixture of **50** and **51** with neopentyl chlorosulfate, partial acetate hydrolysis and/or rearrangement took place, and the 4′-neopentyl sulfate ester **52**



Scheme 4 The preparation of caffeic acid 4′-sulfate **12**. a) Ethyl (triphenylphosphoranyliden)acetate, CH_2Cl_2, 100%; b) Thiophenol, imidazole, NMP, 54% of **50** + **51**; c) Cl–SO_3–C_5H_{11}, Et_3N, DMAP, THF, 80% of **52**; d) NaN_3, DMF; e) 1 N aq. NaOH then Amberlite® CG-50, d + e: 67%.

Table 1 ^{13}C NMR sulfation shifts[a] for sulfate conjugates **7–12**, and **19–24**

Compound	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
7	–3.7	1.1	–4.6	4.4	–4.6	1.1
8	0.5	–5.2	3.8	–5	0.5	–3.9
9	–3.5	–0.3	–2.5	4.4	–4.8	1.5
10	0.9	–5.3	4	–2.6	–0.4	–3.7
11	–1.2	–6.4	4.6	–2.7	–1.6	–3.2
12	–7.4	–0.5	–3.6	6.9	–7.0	2.7
19	–5.7	0.8	–5.3	4.3	–5.3	0.8
20	–1	–5.2	3.9	–4.8	0.9	–4.1
21	–4.8	–0.2	–2.9	4.0	–5.6	0.8
22	1.1	–5.3	3.9	–2.9	–0.2	–4
23	–0.7	–7.3	3.8	–6.6	–2.8	–6.9
24	–8.3	–2.7	–9.3	3.9	–7.0	4.2

[a] Sulfation shift = δ non sulfated compound – δ corresponding sulfated conjugate.

was the major product of the reaction (80%), contaminated with a small amount of the 3′,4′-neopentyl disulfate ester **53**.

The latter contaminant was eliminated by MPLC on silica and was not further characterized. Finally treatment of pure **52** by sodium azide (removal of the neopentyl group), followed by 1 N aq. NaOH (hydrolysis of the ethyl ester), yielded caffeic acid 4′-sulfate **12** in 67% yield from **52**, again as a 9/1 mixture of (*E*)- and (*Z*)-isomers.

The identification of all the conjugates was based on their complete 1D and 2D NMR data, as well as HRMS analysis. In addition the structure of all the sulfated conjugates was confirmed on the basis of the carbon shifts, induced by sulfation, of the ^{13}C NMR spectra. In fact, when comparing the spectra of phenolic compounds with their sulfated analogues, the carbon bearing the sulfate group is shifted upfield, while in contrast the carbons *ortho* and *para* to this position are moved downfield.[33] The sulfation shifts measured for the complete panel of sulfate esters prepared in the course of this work are summarized in Table 1.

All the shifts were in accordance with the expected structures. The chromatographic purity of all the conjugates was determined by HPLC-UV detection. All conjugates were at least 94% pure (See Table S1 of ESI part B).† Sulfated conjugates, however, represented a special case. First the sulfate ester linkage may partially hydrolyze on storage, releasing the free hydroxycinnamic acid. On the other hand, the last step of the synthesis involved exposure to either sodium carbonate or sodium hydroxide and consequently the final sulfated conjugates were possibly contaminated with non UV-absorbing inorganic material. This inorganic salt was difficult to totally eliminate, even after purification by gel filtration and/or reversed phase chromatography. Furthermore we found out that the complete elimination of the inorganic material tended to increase the risk of degradation by hydrolysis of the sulfate ester bond. Thus, the presence of inorganic material played a stabilization role on the sulfate conjugates. HPLC analysis demonstrated that no free hydroxycinnamic acids were present in our samples (Figures S7–S12 and S19–S24 of ESI part B).† On the other hand examination of the NMR spectra of the sulfate conjugates demonstrated that they were also free of other organic contaminants (Figures S31–S36 and S43–S48 of ESI part B).† Thus, the exact amount of sulfate conjugate present in the samples was deduced after acid hydrolysis of an aliquot, followed by the quantification of the released aglycone. The results were expressed in μmol of sulfate conjugate per mg of sample. Depending on the conjugate, the quantification varied from 1.57 to 2.81 μmol/mg. The quantification enabled us to calculate the yields of the reactions: Hydroxycinnamic acid sulfates, 43–92% yield; dihydro hydroxycinnamic acid sulfates, 36–40%.

Previous studies on the metabolism of hydroxycinnamic acid derivatives have been carried out on rats. Furthermore, in a number of cases, the evidence for the presence of glucuronidated and/or sulfated metabolites was only indirect. For example both the glucuronide and sulfate conjugates of *m*-coumaric, dihydro-*m*-coumaric, ferulic, and dihydroferulic acids have been shown to be present in rat urine after administration of γ-oryzanol.[34] Ferulic acid sulfate, glucuronide and sulfoglucuronide have been shown to be metabolites of ferulic acid sugar esters in rats.[35,36] During the sole human trial previously performed, ferulic acid glucuronide or sulfate has been found in human urine after consumption of a pine bark extract.[37] However, in all these studies, no identification of the real circulating metabolites was made since the data was collected after enzymatic deconjugation using β-glucuronidase and/or aryl sulfatase.

A set of other identifications has been carried out online by chromatography and/or mass spectrometry techniques. When only one position of conjugation is available, the LC-MS identification of the metabolite was straightforward. In fact *p*-coumaric acid sulfate has been identified as an urinary metabolite of nonylphenol,[38] together with *p*-coumaric acid glucuronide, and dihydro-*p*-coumaric acid sulfate and glucuronide. Similarly, administration of ferulic or isoferulic acids led to the detection of their respective glucuronides in rat urine.[39] In contrast the identification of positional isomers was problematic. For example caffeic acid sulfates have been identified in rat urine by HPLC-MS^n after administration of caffeic acid,[39] but the position of sulfation was not determined. Similarly the two caffeic acid glucuronides have been identified in rat urine and plasma after dosing of caffeic acid. In the absence of proper standards, the identification of each isomer was only based on an unconvincing comparison of their relative chromatographic behavior with that of ferulic and isoferulic acids.[39]

The phenolic acid conjugates present in human plasma and in human urine collected 0–24 h after drinking coffee containing 412 μmol of chlorogenic acids were investigated. The results of peak co-elution with our 24 chromatographic standards were combined with LC-MS-MS data (Table 2). All the conjugates that could be detected in human plasma and urine after coffee consumption were caffeic acid derivatives, or compounds resulting from the methylation (ferulic and isoferulic), the reduction (dihydrocaffeic), or both methylation and reduction (dihydroferulic and dihydroisoferulic) of caffeic acid.

Four conjugates were present in human plasma, *i.e.* caffeic- and dihydrocaffeic acid 3′-sulfates (**11** and **23**), ferulic- and dihydroferulic acid 4′-sulfate (**9** and **11**). Ten conjugates were present in human urine, *i.e.* caffeic acid 3′- and 4′-sulfates (**11** and **12**), dihydrocaffeic 3′-*O*-glucuronide and 3′-sulfate (**17** and **23**), ferulic acid 4′-sulfate **9**, isoferulic acid 3′-sulfate **10**, dihydroferulic acid 4′-*O*-glucuronide and 4′-sulfate (**15** and **21**), isoferulic acid- and dihydroisoferulic acid 3′-*O*-glucuronides (**4** and **16**). No *p*-coumaric acid derivative was detected in plasma or urine, probably because of the low *p*-coumaroyl quinic acid content of coffee. On the other hand, dehydroxylation (by microflora in the colon) of caffeic acid to *m*-coumaric acid was a minor pathway since no

Table 2 HPLC-MS^n identification of chlorogenic acid derivatives and metabolites detected in the plasma and urine of human volunteers 0–24 h following the ingestion of 200 mL of instant coffee beverage

HPLC R_t (min)[a]	$[M - H]^-$ (m/z)[b]	Metabolite	Location
10.3	261	Dihydrocaffeic acid 3′-sulfate **23**	Plasma, Urine
11.1	357	Dihydrocaffeic acid 3′-*O*-glucuronide **17**	Urine
11.7	259	Caffeic acid 4′-sulfate **12**	Urine
12.1	275	Dihydroferulic acid 4′-sulfate **21**	Plasma, Urine
12.8	259	Caffeic acid 3′-sulfate **11**	Plasma, Urine
13.9	371	Dihydroferulic acid 4′-*O*-glucuronide **15**	Urine
14.5	273	Ferulic acid 4′-sulfate **9**	Plasma, Urine
16.9	273	Isoferulic acid 3′-sulfate **10**	Urine
17.7	371	Dihydroisoferulic acid 3′-*O*-glucuronide **16**	Urine
19.6	369	Isoferulic acid 3′-*O*-glucuronide **4**	Urine

[a] R_t, retention time. [b] $[M - H]^-$, negatively charged molecular ion.

m-coumaric derivatives were detected. Furthermore, in plasma, all phenolic acid conjugates were found in the sulfated form, although some glucuronides (4 out of 11 total) were found in urine. This implies that glucuronides are produced in lower quantities and that they are more rapidly cleared from the blood circulation.

Neither dihydroisoferulic acid 3′-glucuronide, nor dihydrocaffeic acid sulfates or dihydrocaffeic acid glucuronides have been previously identified. In the two latter cases, only one of the two possible isomers was present in human plasma and/or urine. Co-elution with our analytical standards unambiguously identified these compounds as dihydrocaffeic acid 3′-sulfate, and dihydrocaffeic acid 3′-glucuronide, respectively. Caffeic acid 3′-sulfate is also a novel compound and its 4′-sulfate analogue is now detected for the first time as human metabolites. Furthermore the exact order of elution of the two isomers was determined with accuracy. Finally both dihydroferulic acid 4′-sulfate and isoferulic acid 3′-sulfate were novel human metabolites as well.

Conclusion

This is the first time that such an extended panel of potential conjugates is produced and used as analytical standards for unequivocal identifications. Out of the 24 synthesized potential conjugates, 4 have never identified before in any animal or human circulating fluid, and are now evidenced for the first time to be hydroxycinnamic acid metabolites. One the other hand, 7 metabolites are novel conjugates in humans.

Experimental Section

Source of chemicals and materials

GF 92 glass fiber prefilters were purchased from Schleicher & Schuell (Dassel, Germany). Acetyl chloride, benzyl alcohol, diethyl azodicarboxylate (DEAD), 3,4-dihydroxybenzaldehyde, dry dichloromethane, dry THF, dry methanol, *N*-ethyldiisopropylamine, ethyl (triphenylphosphoranyliden)acetate, D-(+)-glucuronic acid γ-lactone, trifluoroacetic acid, 3-(3,4-dihydroxyphenyl)propionic acid, dry DMF, sodium azide, Pd/C 5 and 10%, cyclohexene, Amberlite® IR 120, Amberlite® CG 50, sodium carbonate, potassium carbonate, 1,8-diazabicyclo-[5,4,0]undec-7-ene (DBU), triethylamine (TEA), tetrabutylammonium bromide and benzyl bromide were from Fluka. Sulfur trioxide-trimethylamine complex was purchased from Lancaster. BF_3-etherate, chloromethyl methyl ether, tributyltin methoxide, and triphenylphosphine were obtained from Aldrich. 2,3,4-Tri-*O*-acetyl-D-methyl glucuronopyranosyl-(*N*-phenyl)-2,2,2-trifluoroacetimidate **25** was prepared by reaction of *N*-phenyl-2,2,2-trifluoroacetimidoyl chloride with methyl 2,3,4-tri-*O*-acetyl-D-glucuronate. The former compound was prepared in one step,[40] by heating a mixture of trifluoroacetic acid and aniline in carbon tetrachloride in the presence of triphenylphosphine and triethylamine. The latter compound was prepared in two steps by established procedures from D-(+)-glucuronic acid γ-lactone. This yielded first methyl 1,2,3,4-tetra-*O*-acetyl-β-D-glucuronate,[41] and secondly methyl 2,3,4-tri-*O*-acetyl-D-glucuronate after deacetylation with tributyltin methoxide.[42] 4-Benzyloxy-3-hydroxybenzaldehyde,[32] and neopentyl chlorosulfate,[28,30] were prepared according to published procedures.

General methods

Thin layer chromatography (TLC) was carried out on Silica 60 F254 (Merck), RP-18 F_{254s} and RP-18 WF_{254s} (Merck), DIOL F_{254s} (Merck) or CN F_{254s} plates. The detection was carried out by UV 254 nm or after spraying with Pancaldi reagent. Vacuum liquid chromatography (VLC) was performed on a 500 mL sintered glass filter (100 × 80 mm, P2 porosity) filled with 170 g of silica gel 60 (40–63 μM) Merck, and using a gradient of EtOAc in hexane. Alternatively, the filter was filled with an octadecyl-bonded phase (C_{18}) for flash chromatography (40 μM average particle diameter, Merck), using a gradient of MeOH in water as solvent. Column chromatography (CC) was performed on a 22 × 400 mm column, filled with 100 g of silica gel 60 (63–200 μM) Merck; or on a 22 × 30 mm column filled with 50 g of Sephadex® LH 20 (Amersham Bioscience), using MeOH–water 1/1 as solvent (the flow rate was 10 mL/10 min and fractions of 10 mL were collected); or on a 22 × 30 mm column filled with 20 g of Lichropep RP-18 Merck, using water (100 mL), and water–MeOH 9/1 (100 mL), successively. Medium pressure liquid chromatography (MPLC) was carried out on a Büchi apparatus comprising a 688 chromatographic pump, a 684 fraction collector, and equipped with a C 690 Sepacore® glass column (dimensions: 36 × 460 mm, volume 470 mL) plus a pre-column (volume 11 mL). The column plus the pre-column were filled with 222 g of silica gel 60 (40–63 μm), Merck. For elution, a gradient of EtOAc in hexane was used. Alternatively MPLC was

performed on a Biotage HPFC SP1 system, using normal silica gel or RP-18 manufactured columns (Biotage). The solvents were mixtures of hexane in EtOAc for normal phase and MeOH in water for reverse phase. The gradients were calculated by the software, on the basis of the TLC behavior. A multiwavelength detector and a fraction collector completed the equipment. High pressure liquid chromatography (HPLC) was carried out on a Agilent 1100 tower including a binary pump, an autosampler, a column oven, a UV detector (DAD 1040A), and a CC 250/4Nucleosil 100-5C_{18} column (Macherey Nagel). The flow rate was 1 mL/min, with an injection volume of 5 µL. The column was held at 40 °C and the UV detector set to 210, 254, and 280 nm. The following solvents were used. *Method A*: solvent A: 0.1% TFA in water; solvent B: MeCN; gradient from 10 to 100% B over 20 min; 5 min 100% B; from 0 to 90% A over 1 min; 4 min 90% A. *Method B*: solvent A: 0.1% TFA in water; solvent B: methanol–water (1/1); gradient from 20 to 50% B over 15 min; from 50 to 90% B over 10 min; 10 min 90% B. ^{1}H NMR (360.13 MHz) and ^{13}C NMR (90.56 MHz): The spectra were recorder on a Bruker DPX-360 spectrometer equipped with a broadband multinuclear z-gradient probehead. The chemical shifts (in ppm) were expressed with respect to tetramethylsilane (TMS) as an internal reference, and using $CDCl_3$ as solvent. The attribution of the signals was based on the complete interpretation of direct and long distance heteronuclear correlations. Liquid Chromatography-High-Resolution Mass Spectrometry (ESI–) was carried out on an Agilent-1200 series LC System including a binary pump SL, an autosampler, a diode-array detector and a thermostatted column compartment with an Agilent Eclipse XDB (44.6 × 150 mm, 5 µm) coupled with an Agilent 6210 time-of-flight mass spectrometer.

General procedure A: synthesis and purification of ethyl hydroxycinnamic acids.

In a 250 mL Erlen flask, one part of the desired hydroxybenzaldehyde was dissolved in dry CH_2Cl_2, or in a mixture of dry THF + dry CH_2Cl_2. One part of ethyl (triphenylphosphoranyliden)acetate was added and the reaction was allowed to take place at rt under stirring for 4 h. The medium was directly purified by VLC on silica, using a gradient of EtOAc in hexane as solvent.

General procedure B: synthesis and purification of protected hydroxycinnamic acid *O*-β-D-glucuronides

One part of hydroxycinnamic acid ethyl ester and 2 parts of 2,3,4-triacetyl-D-methyl glucuronopyranosyl-(*N*-phenyl)-2,2,2-trifluoroacetimidate **25** were dissolved in 10 mL of dry CH_2Cl_2. 0.30 parts of BF_3 etherate were added and the mixture was stirred at rt for 15 h. The medium was diluted with 50 mL of CH_2Cl_2, washed with 3 × 50 mL of H_2O and concentrated under reduced pressure. The liquid residue was purified by MPLC on silica using a gradient of EtOAc in hexane as solvent. The fractions containing the product were further purified by MPLC on RP-18, using a gradient of MeOH in 50% aqueous MeOH as solvent.

General procedure C: deprotection of the protected glucuronides and purification of hydroxycinnamic acid *O*-β-D-glucuronides

To solid protected hydroxycinnamic acid *O*-β-D-glucuronides were added 10 mL of 1 N aq. NaOH and 10 mL of MeOH. The resulting suspension was stirred at rt for 4 h. The medium was diluted with 40 mL of H_2O, cooled to 0 °C (ice bath) and neutralized (to pH 4.0–4.5) by addition of 4–6 g of Amberlite® IR-120. The resin was eliminated by filtration and washed with 3 × 50 mL of H_2O. The filtrate plus the three washings were mixed, concentrated under reduced pressure to a volume of about 5 mL (or lyophilized and the residue dissolved in 5 mL of H_2O), and directly purified by MPLC on RP-18 using a gradient of MeOH in H_2O as solvent.

General procedure D: reduction of hydroxycinnamic acids, or hydroxycinnamic acid glucuronides to 3-(hydroxyphenyl)propionic acids, or 3-(hydroxyphenyl)propionic acid glucuronides

To 100 mg of hydroxycinnamic acid or hydroxycinnamic acid glucuronide, dissolved in 5 mL of water plus 5 mL of MeOH, 10 mg of palladium (5%) on activated carbon were added. The suspension was submitted to hydrogenation for 2–4 h (until no hydrogen consumption). After filtration on GF 92 (Schleicher-Schuell, 421030) and evaporation of the solvent, the product was dried overnight under vacuum (0.01 mbar). If necessary, the residue was purified by MPLC on RP-18 using a gradient of MeOH in 1% aqueous AcOH as solvent.

General procedure E: synthesis of protected caffeic and 3-(3,4-dihydroxyphenyl)propionic acid 3′- and 4′-sulfates

A solution of 1 part of the starting material, 2 parts of DBU, and 2 parts of neopentyl chlorosulfate in dry dichloromethane (10 mL/mmol), or a solution of 1 part of the starting material, 2 parts of DMAP, 2.5 parts of TEA, and 2.5 parts of neopentyl chlorosulfate in dry THF (10 mL/mmol) was stirred at room temperature for 24 h under nitrogen atmosphere. The medium was directly purified by MPLC (Biotage) on silica, using a gradient of EtOAc in hexane as solvent.

General procedure F: deprotection of protected caffeic and 3-(3,4-dihydroxyphenyl)propionic acid 3′- and 4′-sulfates

A solution of 1 part of the protected acid, and 2 parts of sodium azide in DMF (3 mL/mmol of acid) was stirred at 60 °C for 24 h. The DMF was removed under high vacuum (1 mbar/40 °C) and methanol (2 mL/mmol of starting material) and 1.0 M NaOH (4 mL/mmol of starting material) were added to the residue. The solution was stirred at room temperature for 4 h. After dilution with water (5 to 10 times) the solution was brought to pH 6 to 6.5 with Ambertite® CG50. The resin was eliminated by filtration through a P zero sintered glass filter. The filtrate was filtered again though a GF 92 pre-filter and lyophilized. The filtrate was purified by MPLC (Biotage) on RP-18 using a gradient of MeOH in water as solvent. After evaporation of the methanol under reduced pressure, the water solution was freeze-dried.

General procedure G: quantification of the amount of sulfate conjugate in the isolated products

Before acid hydrolysis, the sulfate conjugates were analyzed by HPLC to check for the absence of free acids in the sample. 5–8 mg of hydroxycinnamic acid sulfate were hydrolyzed

to the corresponding acids in 1 mL of water, plus 1 mL of 6 M aq. HCl, for 1 h at room temperature in Pyrex tubes (SVL18/10 ml) under stirring (shaker). After evaporation (3H/50 °C/0.1 mbar/Jouan RC10.22/vacuum concentrator/centrifugal evaporator), the residue was taken in water–THF 1/1, and transferred into a 50 mL volumetric flask. A 20 µL aliquot of the solution was analyzed by HPLC and the free hydroxycinnamic acid quantified. A similar procedure was followed for the hydrolysis of 3-(hydroxyphenyl)propionic acid sulfates, except that due to the lower UV response of this class of compounds, samples of 10 mg were hydrolyzed. Calibration curves (4 points) were obtained by submitting 2, 5, 8, and 12 mg of hydroxycinnamic acid, or 5, 10, 15, or 20 mg of 3-(hydroxyphenyl)propionic acid to the same procedure. The results were expressed in µmol of sulfate conjugate/mg of sample.

3′,4′-Diacetoxybenzaldehyde (27)

In a 250 mL 3-neck round-bottom flask, equipped with a condenser and a dropping funnel, 4 g (28.96 mmol) of 3′,4′-dihydroxybenzaldehyde **26** were suspended in 40 mL of CH_2Cl_2. Acetyl chloride (6 mL, 84.46 mmol) was added dropwise under stirring to this suspension. On the other hand, 13 mL (87.01 mmol) of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were diluted with 20 mL of CH_2Cl_2 and introduced into the dropping funnel. The round-bottom flask was cooled to 0 °C (ice bath) and the apparatus was equilibrated under argon. The DBU solution was added to the 3,4-dihydroxybenzaldehyde suspension dropwise under stirring at 0 °C under inert atmosphere. After complete addition of the DBU, total dissolution of the solid was observed. The medium was removed from the ice bath and allowed to react at rt under stirring for 14 h. The CH_2Cl_2 medium was washed with 100 mL of 1 N aq. HCl and with 4 × 100 mL of H_2O, and evaporated under reduced pressure. The residue was dissolved in 5 mL of EtOAc and 5 mL of hexane were added. This solution was purified by VLC using a gradient of EtOAc in hexane as solvent. This yielded 5.6844 g (25.58 mmol, 88%) of compound **27**. TLC R_f = 0.46 (Silica 60 F254, Hexane–EtOAc 6/4). The ^{1}H-, and ^{13}C NMR spectral data of **27** were in accordance with published values.[43–45]

3′-Acetoxy-4′-hydroxybenzaldehyde (28)

3.00 g (13.56 mmol) of 3′,4′-diacetoxybenzaldehyde **27** were dissolved in 30 mL of THF. The solution was cooled to 0 °C in an ice bath and 350 mg (5.1307 mmol) of imidazole, followed by 1.65 mL (16.2 mmol) of thiophenol, were added. The reaction medium was removed from the ice bath and allowed to react at rt for 1.5 h under stirring. 100 mL of CH_2Cl_2 were added to the medium. The organic solution was washed with 100 mL of 1 N aq. HCl, and with 3 × 100 mL of H_2O. The CH_2Cl_2 extract was concentrated to constant volume under reduced pressure. The liquid residue was purified by MPLC on silica using a gradient of EtOAc in hexane as solvent. This afforded 1.8763 g (10.41 mmol, 77%) of compound **28**. The ^{1}H and ^{13}C NMR data for 3′-acetoxy-4′-hydroxybenzaldehyde in methanol-d_4 has been published.[46] The NMR data in $CDCl_3$ and acetone-d_6 are presented here for the first time. TLC R_f = 0.21 (Silica 60 F254, Hexane–EtOAc 6/4).^{1}H NMR (360 MHz, $CDCl_3$) δ 2.36 (s, 3H, 3′-OAc), 7.07 (d, 1H, J = 9.0 Hz, H5′), 7.66 (m, 2H, H2′+H6′), 9.82 (s, 1H, H1). ^{13}C NMR (90 MHz, $CDCl_3$) δ 20.9 (3′-OAc), 117.6 (C5′), 124.2 (C2′), 129.8 (C1′ + C6′), 138.7 (C3′), 153.5 (C4′), 169.2 (3′-OAc), 190.6 (C1). ^{1}H NMR (300 MHz, Acetone-d_6) δ 2.30 (s, 3H, 3′-OAc), 7.15 (d, 1H, J = 8.3 Hz, H5′), 7.63 (d, 1H, J = 2.0 Hz, H2′), 7.72 (dd, 1H, J = 8.3 and 2.0 Hz, H6′), 9.86 (s, 1H, H1). ^{13}C NMR (75 MHz, Acetone-d_6) δ 20.7 (3′-OAc), 118.0 (C5′), 125.0 (C2′), 130.2 (C6′), 130.6 (C1′), 140.0 (C3′), 155.6 (C4′), 169.3 (3′-OAc), 190.7 (C1).

3′-Acetoxy-4′-benzyloxybenzaldehyde (29)

1.7088 g (9.485 mmol) of 3′-acetoxy-4′-hydroxybenzaldehyde **28** were dissolved in 20 mL of dry THF. 3.23 g (12.31 mmol) of triphenylphosphine and 1.5 mL (14.5 mmol) of benzyl alcohol were added. The medium was cooled to 0 °C (ice bath), equilibrated under argon, and 6.6 mL (15.16 mmol) of a solution of 40% of diethyl azodicarboxylate (DEAD) in toluene were added in 15 min dropwise under stirring. The medium was allowed to warm up slowly to room temperature, and left to react at rt for 17 h. The medium was diluted with 100 mL of CH_2Cl_2 and the extract was washed with 100 mL of H_2O. The CH_2Cl_2 extract was purified by VLC using a gradient of EtOAc in hexane as solvent, to give 2.5 g (9.25 mmol, 97%) of compound **29**. TLC R_f = 0.37 (Silica 60 F254, Hexane–EtOAc 4/1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.29 (s, 3H, 3′-OAc), 5.18 (s, 2H, H7b), 7.10 (d, 1H, J = 8.5 Hz, H5′), 7.34–7.39 (m, 5H, H2b + H3b + H4b + H5b + H6b), 7.61 (d, 1H, J = 2.0 Hz, H2′), 7.71 (dd, 1H, J = 8.5 and 2.1 Hz, H6′), 9.85 (s, 1H, H1). ^{13}C NMR (75 MHz, $CDCl_3$) δ 20.5 (3′-OAc), 70.8 (C7b), 113.3 (C5′), 123.4 (C2′), 127.1 (C2b/6b), 128.3 (C4b), 128.7 (C3b/5b), 130.1 (C6′), 130.1 (C1′), 135.6 (C1b), 140.6 (C3′), 155.4 (C4′), 168.7 (3′-OAc), 190.1 (C1). The NMR data was in accordance with previously published information.[32]

3′-Acetoxy-4′-benzyloxycinnamic acid ethyl ester (30)

Procedure A was carried out using 2.00 g (7.4 mmol) of 3′-acetoxy-4′-benzyloxybenzaldehyde **29** in 10 mL of dry CH_2Cl_2, and 2.6 g (7.47 mmol) of ethyl (triphenylphosphoranyliden)acetate. This afforded, after purification, 2.2441 g (6.59 mmol; 89%) of novel compound **30**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (t, 3H, J = 7.1 Hz, H2″), 2.29 (s, 3H, 3′-OAc), 4.25 (q, 2H, J = 7.1 Hz, H1″), 5.12 (s, 2H, H7b), 6.29 (d, 1H, J = 16.0 Hz, H2), 6.98 (d, 1H, J = 8.5 Hz, H5′), 7.26 (d, 1H, J = 2.6 Hz, H2′), 7.32 (dd, 1H, J = 8.5 and 2.2 Hz, H6′), 7.31–7.38 (m, 5H, H2b + H3b + H4b + H5b + H6b), 7.59 (d, 1H, J = 16.0 Hz, H3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3 (C2′), 20.6 (3′-OAc), 60.4 (C1″), 70.7 (C7b), 113.8 (C5′), 117.0 (C2), 122.0 (C2′), 127.1 (C2b/6b), 127.5 (C6′), 127.9 (C1′), 128.1 (C4b), 128.6 (C3b/5b), 136.2 (C1b), 140.4 (C3′), 143.3 (C3), 151.9 (C4′), 167.0 (C1), 168.9 (3′-OAc).

Ethyl 4′-Benzyloxy-3′-hydroxycinnamate (31)

In a 100 mL Erlen flask, 401.4 mg (1.18 mmol) of 3′-acetoxy-4′-benzyloxycinnamic acid ethyl ester **30** were dissolved in 2 mL of dry CH_2Cl_2. 95.23 mg (1.76 mmol) of sodium methoxide, dissolved in 15 mL of dry MeOH were added, and the mixture was stirred at rt for 30 min. The medium was acidified by addition of 10 mL of 1 N aq. HCl, diluted with 50 mL of H_2O, and extracted with 50 mL of CH_2Cl_2. The CH_2Cl_2 extract was washed with 3 × 10 mL of H_2O, and evaporated under reduced pressure, to give 344.1 mg

(1.15 mmol; 97%) of compound **31**. TLC R_f = 0.49 (Silica 60 F254, Hexane–EtOAc 7/3). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 7.2 Hz, H2″), 4.25 (q, 2H, J = 7.2 Hz, H1″), 5.14 (s, 2H, H7b), 5.74 (s, 1H, 3′-OH), 6.28 (d, 1H, J = 15.9 Hz, H2), 6.90 (d, 1H, J = 8.3 Hz, H5′), 7.00 (dd, 1H, J = 8.3 and 2.0 Hz, H6′), 7.15 (d, 1H, J = 2.0 Hz, H2′), 7.36–7.42 (m, 5H, H2b + H3b + H4b + H5b + H6b), 7.58 (d, 1H, J = 15.9 Hz, H3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3 (C2″), 60.4 (C1″), 71.1 (C7b), 111.9 (C5′), 113.3 (C2′), 116.5 (C2), 121.6 (C6′), 127.8 (C2b/6b), 128.4 (C1′), 128.6 (C4b), 128.8 (C3b/5b), 135.8 (C1b), 144.3 (C3), 146.1 (C3′), 147.6 (C4′), 167.3 (C1). The ^{1}H NMR data was in accordance with the previously published one.[47]

2-Propenoic acid, 3-[4-benzyloxy-3-[(2,3,4-tri-*O*-acetyl-6-methyl-β-D-glucopyranurosyl)oxy]phenyl]-, ethyl ester (32)

Using procedure B, 211.3 mg (0.71 mmol) of 4′-benzyloxy-3′-hydroxycinnamic acid ethyl ester **31** were reacted with 0.716 g (1.42 mmol) of 2,3,4-triacetyl-D-methyl glucuronopyranosyl-(*N*-phenyl)-2,2,2-trifluoroacetimidate **25**,[48] and with 30 µL (0.24 mmol) of BF_3 etherate. This yielded, after purification by MPLC on silica and RP-18, 332.1 mg (0.54 mmol; 76%) of novel compound **32**. TLC R_f = 0.10 (Silica 60 F254, Hexane–EtOAc 7/3). ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 7.1 Hz, H2″), 1.78 (s, 3H, C$\underline{H_3}$–CO), 2.03 (s, 3H, C$\underline{H_3}$–CO), 2.04 (s, 3H, C$\underline{H_3}$–CO), 3.78 (s, 3H, H7a), 4.11 (d, 1H, J = 9.3 Hz, H5a), 4.25 (q, 2H, J = 7.1 Hz, H1″), 5.09 (m, 1H, H1a), 5.11 (s, 2H, H7b), 5.30–5.39 (m, 3H, H2a + H3a + H4a), 6.29 (d, 1H, J = 15.9 Hz, H2), 6.94 (d, 1H, J = 8.5 Hz, H5′), 7.21 (dd, 1H, J = 8.5 and 2.1 Hz, H6′), 7.31–7.41 (m, 5H, H2b + H3b + H4b + H5b + H6b), 7.42 (d, 1H, J = 2.1 Hz, H2′), 7.58 (d, 1H, J = 16.0 Hz, H3). ^{13}C NMR (90 MHz, $CDCl_3$) δ 14.3 (C2″), 20.3 (C$\underline{H_3}$–CO), 20.5 (C$\underline{H_3}$–CO), 20.6 (C$\underline{H_3}$–CO), 53.0 (C7a), 60.4 (C1′), 69.1 (C4a), 70.9 (C7b + C2a), 72.0 (C3a), 72.5 (C5a), 100.4 (C1a), 114.2 (C5′), 116.8 (C2), 119.6 (C2′), 125.6 (C6′), 127.4 (C2b/6b), 128.0 (C1′), 128.2 (C4b), 128.7 (C3b/5b), 136.2 (C1b), 143.7 (C3), 146.1 (C3′), 151.4 (C4′), 166.8 (C6a), 167.1 (C1), 169.3 (2 × CH$_3$–C$\underline{O}$), 170.1 (CH$_3$–C$\underline{O}$).

(*E*)-4′-*O*-Benzylcaffeic acid 3′-*O*-β-D-glucuronide (33)

Following procedure C, 314.9 mg (0.512 mmol) of 2-propenoic acid, 4-[4-benzyloxy-3-[(2,3,4-tri-*O*-acetyl-6-methyl-β-D-glucopyranurosyl)oxy]phenyl]-, ethyl ester **32** were treated with 10 mL of 1 N aq. NaOH. After acidification (6.3 g of wet Amberlite® IR-120) and purification by MPLC on RP-18, 167 mg of the novel (*E*)-4′-*O*-benzylcaffeic acid 3′-*O*-β-D-glucuronide **33** were isolated (yield: 154 mg, 0.373 mmol, 73%). HPLC R_t = 30.31 (method B). ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.31–3.45 (m, 3H, H2a + H3a + H4a), 3.98 (d, 1H, J = 9.6 Hz, H5a), 5.20 (s, 2H, H7b), 5.28 (d, 1H, J = 7.5 Hz, H1a), 6.42 (d, 1H, J = 15.9 Hz, H2), 7.06 (d, 1H, J = 8.5 Hz, H5′), 7.24 (dd, 1H, J = 8.5 and 1.8 Hz, H6′), 7.31 (m, 1H, H4b), 7.38 (m, 2H, H3b + H5b), 7.46–7.49 (m, 3H, H2′ + H2b+ H6b), 7.50 (d, 1H, J = 15.9 Hz, H3). ^{13}C NMR (90 MHz, DMSO-d_6) δ 69.7 (C7b), 71.2 (C4a), 72.8 (C2a), 75.3 (C5a), 76.0 (C3a), 99.5 (C1a), 114.5 (C5′), 114.7 (C2′), 117.0 (C2), 123.6 (C6′), 127.3 (C1′), 127.3 (C2b + C6b), 127.6 (C4b), 128.3 (C3b + C5b), 136.8 (C1b), 143.7 (C3), 146.5 (C3′), 149.8 (C4′), 167.7 (C1), 170.1 (C6a).

Dihydrocaffeic acid 3′-*O*-β-D-glucuronide (17)

145 mg 0.325 mmol) of (*E*)-4′-*O*-benzylcaffeic acid 3′-O-β-D-glucuronide **33** was dissolved in 20 mL of 50% aq. MeOH. 22 mg of 5% Pd/C were added and the compound was hydrogenated at rt and 1 atmosphere for 4 h under stirring. The solid was removed by filtration on a GF 92 glass fibre prefilter and the filter was washed with 2 × 5 mL of 50% aq. MeOH. The filtrate plus the two washings were mixed, concentrated under reduced pressure (removal of MeOH) and the resulting aqueous phase was lyophilized, to give 111.69 mg (0.312 mmol; 96%) of the novel compound dihydrocaffeic acid 3′-*O*-β-D-glucuronide **17**. TLC R_f = 0.29 (Silica 60 F254, BuOH–AcOH–water 4/1/1). HPLC R_t = 5.46 (method A; 99% purity at 280 nm). ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.44 (t, 2H, J = 7.5, H2), 2.69 (t, 2H, J = 7.6, H3), 3.27–3.42 (m, 3H, H2a + H3a + H4a), 3.83 (d, J = 9.5 Hz, 1H, H5a), 4.83 (d, 1H, J = 7.5 Hz, H1a), 6.71 (brs, 2H, H6′), 6.90 (brs, 1H, H5′). ^{13}C NMR (90 MHz, DMSO-d_6) δ 29.9 (C3), 35.6 (C2), 71.4 (C4a), 73.1 (C2a), 75.3 (C3a + C5a), 101.8 (C1a), 115.9 (C2′), 116.8 (C5′), 122.6 (C6′), 131.7 (C1′), 144.7 (C3′), 145.2 (C4′), 170.3 (C6a) 173.8 (C1). LC-HRMS [M − H]$^-$ calc for $C_{15}H_{17}O_{10}$: 357.0821; found: 357.0816.

3′-Benzyloxy-4′-hydroxybenzaldehyde (37) from 3′,4′-dihydroxybenzaldehyde (26) (3 steps)

Step 1: 3 g (21.72 mmol) of 3′,4′-dihydroxybenzaldehyde **26** were dissolved in 10 mL of THF. The solution was cooled in an ice bath. 4.5 mL (26.06 mmol) of *N*-ethyl diisopropylamine were added dropwise with stirring. Finally 2 mL (26.06 mmol) of chloromethyl methyl ether were added dropwise under stirring. The medium was removed from the ice bath and allowed to react at rt under stirring for 4 h. The medium was diluted with 50 mL of 10% aq. $KHCO_3$ and extracted with 2 × 50 mL of CH_2Cl_2. The CH_2Cl_2 extract was washed with 50 mL of 1 N aq. HCl, and 3 × 50 mL of H_2O. It was evaporated under reduced pressure, to give 3.7928 g of crude residue. TLC R_f = 0.51 (Silica 60 F254, Hexane–EtOAc 6/4).

Step 2: the 3.7928 g of crude 3′-hydroxy-4′-methoxy-methoxybenzaldehyde **34** were dissolved in 50 mL of MeCN. 3 g (21.72 mmol) of anhydrous K_2CO_3, 0.71 g (2.2 mmol) of tetrabutylammonium bromide, and 2.6 mL (21.72 mmol) of benzyl bromide were added, successively at rt under stirring. The medium was allowed to react at rt under stirring for 1 h. The medium was diluted with 100 mL of H_2O and extracted with 2 × 100 mL of EtOAc. The EtOAc extract was washed with 3 × 200 mL of H_2O and concentrated under reduced pressure, to give 5.33 g of crude product. TLC R_f = 0.09 (RP-18 F254s, MeOH–water 6/4).

Step 3: the 5.33 g of crude 3′-benzyloxy-4′-methoxy-methoxybenzaldehyde **36** were dissolved in 20 mL of MeOH and 2 mL of 37% HCl (fuming) were added dropwise under stirring. The medium was refluxed (oil bath 80 °C) under stirring for 1 h. The medium was diluted with 50 mL of 10% aq. $KHCO_3$ and extracted with 2 × 50 mL of EtOAc. The EtOAc extract was washed with 50 mL of 1 N aq. HCl, and 3 × 50 mL of H_2O. It was purified by VLC on silica, followed by VLC on RP-18, using gradients of EtOAc in hexane, and of MeOH in 20% aqueous MeOH as solvents, respectively. TLC R_f = 0.40 (RP-18 F254s, MeOH–water 7/3). Final yield in 3′-benzyloxy-4′-hydroxybenzaldehyde **37**:[49,50] 2.0898 g (9.16 mmol; 42% from **26**). ^{1}H NMR (300 MHz,

Acetone-d_6) δ 5.25 (s, 2H, H7b), 7.05 (d, 1H, J = 8.1 Hz, H5′), 7.34–7.42 (m, 3H, H3b + H4b + H5b), 7.47 (dd, 1H, J = 8.1 and 1.8 Hz, H6′), 7.53 (m, 2H, H2b/6b), 7.56 (d, 1H, J = 1.8 Hz, H2′), 9.81 (s, 1H, H1). ^{13}C NMR (75 MHz, Acetone-d_6) δ 71.4 (C7b), 112.6 (C2′), 116.4 (C5′), 127.1 (C6′), 128.9 (C4b + C2b/6b), 129.3 (C3b/5b), 130.6 (C1′), 137.5 (C1a), 148.0 (C3′), 153.8 (C4′), 191.0 (C1).

3′-Benzyloxy-4′-hydroxycinnamic acid ethyl ester (38)

Procedure A was carried out using 1.9 g (8.32 mmol) of 3′-benzyloxy-4′-hydroxybenzaldehyde **37** in 10 mL of dry THF + 10 mL of dry CH_2Cl_2, and 2.96 g (8.5 mmol) of ethyl (triphenylphosphoranyliden)acetate. This afforded, after purification by VLC on silica, 2.34 g of product. The compound was further purified by CC on silica using hexane–EtOAc 75/25 as solvent, to give 1.39 g (4.66 mmol; 56%) of compound **38**.[51] TLC R_f = 0.28 (Silica 60 F254, Hexane–EtOAc 2/1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (t, 3H, J = 7.1 Hz, H2″), 4.25 (q, 2H, J = 7.1 Hz, H1″), 5.13 (s, 2H, H7b), 5.92 (brs, 1H, 4′-OH), 6.26 (d, 1H, J = 15.9 Hz, H2), 6.94 (d, 1H, J = 8.2 Hz, H5′), 7.09 (m, 2H, H2′ + H6′), 7.37–7.43 (m, 5H, H2b + H3b + H4b + H5b + H6b), 7.59 (d, 1H, J = 15.9 Hz, H3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4 (C2″), 60.4 (C1″), 71.2 (C7b), 111.0 (C2′), 115.0 (C5′), 115.7 (C2), 123.2 (C6′), 127.0 (C1′), 127.8 (C2b/6b), 128.6 (C4b), 128.8 (C3b/5b), 135.8 (C1b), 144.6 (C3), 146.0 (C3′), 148.1 (C4′), 167.3 (C1).

2-Propenoic acid, 3-[3-benzyloxy-4-[(2,3,4-tri-*O*-acetyl-6-methyl-β-D-glucopyranurosyl)oxy]phenyl]-, ethyl ester (39)

Using procedure B, 400 mg (1.34 mmol) of 3-benzyloxy-4-hydroxycinnamic acid ethyl ester **38** were reacted with 1.5 g (2.96 mmol) of 2,3,4-triacetyl-D-methyl glucuronopyranosyl-(*N*-phenyl)-2,2,2-trifluoroacetimidate **25**,[48] and with 43 µL (0.43 mmol) of BF_3 etherate. This yielded, after purification by MPLC on silica and RP-18, 617 mg (0.982 mmol; 73%) of novel compound **39**. TLC R_f = 0.40 (Silica 60 F254, Hexane–EtOAc 1/1). ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 7.1 Hz, H2″), 1.80 (s, 3H, C$\underline{H}_3$–CO), 2.02 (s, 3H, C$\underline{H}_3$–CO), 2.04 (s, 3H, C$\underline{H}_3$–CO), 3.73 (s, 3H, H7a), 4.11 (m, 1H, H5a), 4.26 (q, 2H, J = 7.1 Hz, H1″), 5.09–5.15 (m, 3H, H7b + H1a), 5.31–5.36 (m, 3H, H2a + H3a + H4a), 6.30 (d, 1H, J = 15.9 Hz, H2), 7.10–7.45 (m, 8H, H2b + H3b + H4b + H5b + H6b + H2′ + H5′ + H6′), 7.59 (d, 1H, J = 16.0 Hz, H3). ^{13}C NMR (90 MHz, $CDCl_3$) δ 14.3 (C2″), 20.3 (C$\underline{H}_3$–CO), 20.5 (C$\underline{H}_3$–CO), 20.6 (C$\underline{H}_3$–CO), 53.0 (C7a), 60.5 (C1′), 69.1 (C4a), 70.9 (C7b), 71.1 (C2a), 71.8 (C3a), 72.6 (C5a), 99.9 (C1a), 113.7 (C2′), 117.7 (C2), 120.1 (C5′), 122.1 (C6′), 127.1 (C2b/6b), 128.0 (C1′), 128.2 (C4b), 128.7 (C3b/5b), 136.2 (C1b), 143.8 (C3), 146.1 (C3′), 151.4 (C4′), 166.8 (C6a), 167.1 (C1), 169.3 (2 × CH_3–C$\underline{O}$), 170.1 (CH_3–C$\underline{O}$).

(*E*)-3′-*O*-Benzylcaffeic acid 4′-*O*-β-D-glucuronide (40)

Following procedure C, 617 mg (0.98 mmol) of 2-propenoic acid, 3-[3-benzyloxy-4-[(2,3,4-tri-*O*-acetyl-6-methyl-β-D-glucopyranurosyl)oxy]phenyl]-, ethyl ester **39** were treated with 10 mL of 1 N aq. NaOH. After acidification (17 g of wet Amberlite® IR-120) and purification by MPLC on RP-18, 352 mg of the novel compound (*E*)-3′-*O*-benzylcaffeic acid 4′-*O*-β-D-glucuronide **40** were isolated (yield: 352 mg, 0.7643 mmol, 78%). HPLC R_t = 27.85 (method B). ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.30–3.45 (m, 3H, H2a + H3a + H4a), 3.89 (d, 1H, J = 9.6 Hz, H5a), 5.17 (d, 1H, J = 7.3 Hz, H1a), 5.20 (s, 2H, H7b), 6.43 (d, 1H, J = 16.0 Hz, H2), 7.12 (d, 1H, J = 8.6 Hz, H5′), 7.23 (dd, 1H, J = 8.5 and 1.6 Hz, H6′), 7.29–7.51 (m, 7H, H3 + H2′ + H2b+ H3b + H4b + H5b + H6b). ^{13}C NMR (90 MHz, DMSO-d_6) δ 69.9 (C7b), 71.2 (C4a), 72.8 (C2a), 75.3 (C5a), 75.9 (C3a), 99.7 (C1a), 113.7 (C2′), 115.8 (C5′), 117.4 (C2), 122.5 (C6′), 127.5 (C2b + C6b), 127.6 (C4b), 128.3 (C3b + C5b), 128.5 (C1′), 137.0 (C1b), 143.6 (C3), 148.1 (C3′), 148.3 (C4′), 167.7 (C1), 170.1 (C6a).

Dihydrocaffeic acid 4′-*O*-β-D-glucuronide (18)

Following procedure D, 300 mg (0.65 mmol) of compound **40** were hydrogenated for 5 h. This yielded after filtration and freeze-drying 240 mg (0.67 mmol; 100%) of the novel compound **18**. HPLC R_t = 5.23 (method A; 98% purity at 280 nm). ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.46 (t, 2H, J = 7.6, H2), 2.69 (t, 2H, J = 7.6, H3), 3.27–3.42 (m, 3H, H2a + H3a + H4a), 3.83 (d, 1H, J = 9.6 Hz, H5a), 4.78 (d, 1H, J = 7.4 Hz, H1a), 6.59 (dd, 1H, J = 8.3 and 2.0 Hz, H6′), 6.68 (d, 1H, J = 7.4 Hz, H2′), 6.90 (d, 1H, J = 8.3 Hz, H5′). ^{13}C NMR (90 MHz, DMSO-d_6) δ 29.7 (C3), 35.3 (C2), 71.3 (C4a), 73.0 (C2a), 75.1 (C5a), 75.3 (C3a), 102.1 (C1a), 115.9 (C2′), 116.7 (C5′), 118.8 (C6′), 135.9 (C1′), 143.1 (C4′), 146.7 (C3′), 170.0 (C6a) 173.7 (C1). LC-HRMS [M – H]$^-$ calc for $C_{15}H_{17}O_{10}$: 357.0821; found: 357.0824.

(*E*)-Ethyl 3-(3-(benzyloxy)-4-(pentyloxysulfonyloxy)phenyl)acrylate (41)

Following procedure E, 300 mg (1.0 mmol) of compound **38** were dissolved in 10 mL of THF. 220 µl (1.50 mmol) of TEA, 122 mg of DMAP (1.00 mmol), and 300 mg of neopentylchlorosulfate[28,30] (1.5 mmol) were added successively under stirring at rt and nitrogen atmosphere. After removal of the solvent under reduced pressure, the oily colored residue was purified by MPLC (Biotage 25 M) using a gradient of EtOAc in hexane as solvent. Yield: 401 mg (0.86 mmol; 86%) of novel compound **41**. TLC R_f = 0.61 (Silica 60 F254, Hexane–EtOAc 2/1). ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.85 (s, 9H, H1c), 1.34 (t, 3H, J = 7.13 Hz, H2″), 4.03 (s, 2H, H3c), 4.27 (q, 2H, J = 7.13 Hz, H1″), 5.14 (s, 2H, H7b), 6.38 (d, 1H, J = 16.0 Hz, H2), 7.14 (dd, 1H, J = 8.4 and 1.9 Hz, H6′), 7.19 (d, 1H, J = 1.9 Hz, H2′), 7.30–7.50 (m, 6H, H2b + H3b + H4b + H5b + H6b + H5′), 7.61 (d, 1H, J = 16.0 Hz, H2),. ^{13}C NMR (90 MHz, $CDCl_3$) δ 14.6 (C2″), 26.4 (C1c), 32.0 (C2c), 61.0 (C1″), 71.4 (C7b), 84.0 (C3c), 113.6 (C2′), 119.9 (C2), 121.5 (C6′), 123.8 (C5′), 128.1 (C2b/6b), 128.8 (C4b), 129.1 (C3b/5b), 134.9 (C1′), 135.9 (C1b), 140.6 (C4′), 143.4 (C3), 151.1 (C3′), 166.9 (C1).

Ethyl 3-(3-hydroxy-4-(pentyloxysulfonyloxy)phenyl)propanoate (42)

Following procedure D, 400 mg (0.87 mmol) of compound **41** were hydrogenated for 4 h. This yielded after filtration, evaporation and purification by MPLC (Biotage 25 M) using a gradient of EtOAc in hexane as solvent, 277 mg (0.77 mmol; 90%) of novel compound **42**. TLC R_f = 0.65 (Silica 60 F254, Hexane–EtOAc 2/1). ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.99 (s, 9 H, H1c), 1.24 (t, 3H, J = 7.13 Hz, H2″), 2.59 (t, 2H, J = 8.12 Hz, H2), 2.88 (t, 2H, J = 7.54 Hz, H3), 4.13 (q, 2H, J = 7.17 Hz, H1″), 4.16 (s, 2H, H3c), 6.71 (dd, 1H,

J = 8.36 and 2.16 Hz, H6′), 6.90(d, 1H, J = 2.12 Hz, H2′), 7.20 (d, 1H, J = 8.32 Hz, H5′). ^{13}C NMR (90 MHz, $CDCl_3$) δ 14.3 (C2″), 26.0 (C1c), 30.5 (C3), 31.9 (C2c), 35.6 (C2), 60.5 (C1″), 83.7 (C3c), 118.0 (C2′), 120.0 (C6′), 122.4 (C5′), 136.4 (C4′), 141.2 (C1′), 148.4 (C3′), 172.7 (C1).

Dihydrocaffeic acid 4′-sulfate (24)

Following procedure F, 277 mg (0.77 mmol) of compound **42** in 2 mL of dry DMF were reacted with 71 mg (1.09 mmol) of sodium azide and hydrolyzed in methanol and NaOH 1.0 M. This yielded, after purification by CC on Biotage RP-18, 165 mg (Quantification of the sulfate ester content following procedure G: 1.55 µmol/mg; 0.26 mmol; 34%) of novel compound **24**. HPLC R_t = 5.70 (method A). ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.14 (m, 2H, H2), 2.59 (m, 2H, H3), 6.23 (dd, 1H, J = 8.0 and 2.0 Hz, H6′), 6.50 (d, 1H, J = 2.0 Hz, H2′), 6.83 (d, 1H, J = 8.0 Hz, H5′). ^{13}C NMR (90 MHz, DMSO-d_6) δ 32.3 (C3), 40.1 (C2), 114.4 (C6′), 118.3 (C2′), 122.4 (C5′), 139.4 (C4′), 139.9 (C1′), 154.2 (C3′), 177.2 (C1). LC-HRMS $[M - H]^-$ calc for $C_9H_9O_7S$: 261.0069; found: 261.0108.

2-(Benzyloxy)-5-formylphenyl neopentyl sulfate (44)

Following procedure E, 1.14 g (5 mmol) of 4-(benzyloxy)-3-hydroxybenzaldehyde **43**,[31,32] were dissolved in 25 mL of CH_2Cl_2. 1.14 g (7.5 mmol; 1.12 mL) of DBU and 1.52 g (7.5 mmol) of neopentylchlorosulfate[28,30] were added successively under stirring at RT and nitrogen atmosphere. After removal of the solvent under reduced pressure, the oily colored residue was purified by MPLC (Biotage 40 M) using a gradient of EtOAc in hexane as solvent. Yield: 1.71 g (4.5 mmol; 91%) of novel compound **44**. TLC R_f = 0.36 (Silica 60 F254, Hexane–EtOAc 2/1). ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.89 (s, 9H, H1c), 4.06 (s, 2H, H3c), 5.23 (s, 2H, H7b), 7.18 (d, 1H, J = 8.55 Hz, H5′), 7.38–7.48 (m, 5H, H2b + H3b + H4b + H5b + H6b), 7.82 (dd, 1H, J = 8.55 and 2.0 Hz, H6′), 7.91 (d, 1H, J = 2.19 Hz, H2′). ^{13}C NMR (90 MHz, $CDCl_3$) δ 25.8 (C1c), 31.8 (C2c), 71.3 (C7b), 83.9 (C3c), 114.0 (C5′), 123.9 (C2′), 127.7 (C2b/6b), 128.7 (C4b), 128.8 (C3b/5b), 130.0 (C1′), 130.6 (C6′), 135.0 (C1b), 139.5 (C3′), 155.6 (C4′), 189.5 (C1).

5-Formyl-2-hydroxyphenyl neopentyl sulfate (45)

To avoid the reduction of the aldehyde group, procedure D was modified as follows. 1.08 g (2.85 mmol) of **44** was added to a suspension of 1.5 g (1.42 mmol) of Pd–C (10%, standard, unreduced, wet) and 4.6 mL of cyclohexene in 100 mL of DMF. The suspension was heated 3 h at 70 °C. After filtration on GF 92 and evaporation of the DMF, the resulting residue was dissolved in EtOAc and washed with water. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by MPLC (Biotage 40 M) using a gradient of EtOAc in hexane as solvent yielded 770 mg (2.67 mmol, 94%) of novel compound **45**. TLC R_f = 0.61 (Silica 60 F254, Hexane–EtOAc 1/1). ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.02 (s, 9H, H1c), 4.19 (s, 2H, H3c), 7.20 (d, J = 8.43 Hz, 1H, H5′), 7.77 (dd, 1H, J = 8.41 and 1.78 Hz, H6′), 7.87 (d, 1H, J = 1.7 Hz, H2′), 9.86 (s, 1H, H1). ^{13}C NMR (90 MHz, $CDCl_3$) δ 25.8 (C1c), 32.0 (C2c), 84.7 (C3c), 118.6 (*C5′), 123.7 (*C2′), 130.0 (C1′), 130.9 (C6′), 138.1 (C3′), 153.7 (C4′), 189.8 (C1).

(*E*)-Ethyl 3-(4-hydroxy-3-(neopentyloxysulfonyloxy)phenyl)acrylate (46)

Procedure A was carried out using 390 mg (1.35 mmol) of 5-formyl-2-hydroxyphenyl neopentyl sulfate **45** in 10 mL of dry CH_2Cl_2, and 0.5 g (1.43 mmol) of ethyl (triphenylphosphoranyliden)acetate. This afforded, after purification, 400 mg (1.12 mmol; 83%) of novel compound **46**. ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.03 (s, 9H, H1c), 1.35 (t, 3H, J = 7.13 Hz, H2″), 4.17 (s, 2H, H3c), 4.28 (q, 2H, J = 7.13 Hz, H1″), 6.34 (d, 1H, J = 16.0 Hz, H2), 7.09 (d, 1H, J = 8.5 Hz, H5′), 7.42 (dd, 1H, J = 8.5 and 1.8 Hz, H6′), 7.49 (d, 1H, J = 1.7 Hz, H2′), 7.60 (d, 1H, J = 16.0 Hz, H3). ^{13}C NMR (90 MHz, $CDCl_3$) δ 14.3 (C2″), 25.8 (C1c), 32.0 (C2c), 60.6 (C1″), 84.8 (C3c), 117.7 (*C5′), 118.7 (*C2′), 121.9 (*C2), 128.1 (C1′), 128.6 (C6′), 137.8 (C3′), 142.7 (C3), 149.4 (C4′), 166.9 (C1).

(*E*)- and (*Z*)-Caffeic acid 3′-sulfates (11a) and (11b)

Following procedure F, 400 mg (1.12 mmol) of compound **46** in 4 mL of dry DMF were reacted with 150 mg (2.2 mmol) of sodium azide and hydrolyzed in methanol and NaOH 1.0 M. This yielded, after purification by CC on Biotage RP-18, 210 mg (Quantification of the sulfate ester content following procedure G: 2.30 µmol/mg; 0.48 mmol; 43%) of compound **11** as a 9/1 mixture of (*E*)-**11a** and (*Z*)-**11b** forms. HPLC R_t = 5.50 (method A). ^{1}H NMR (360 MHz, DMSO-d_6) δ 5.75 (d, 0.1H, J = 12.8 Hz, H2 *Z* form), 5.96 (d, 0.1H, J = 12.8 Hz, H3 *Z* form), 6.16 (d, 0.9H, J = 15.8 Hz, H2 *E*-form), 6.71 (d, 0.1 H, J = 8.4 Hz, H5′ *Z* form), 6.78 (d, 0.9H, J = 8.3 Hz, H5′ *E*-form), 7.06 (d, 0.9H, J = 15.8 Hz, H3 *E*-form), 7.08 (dd, 0.9H, J = 8.3 and 1.9 Hz, H6′ *E*-form), 7.32 (d, 0.9H, J = 1.9 Hz, H2′ *E*-form), 7.36 (dd, 0.1H, J = 8.3 and 1.9 Hz, H6′ *Z* form), 7.49 (d, 0.1H, J = 1.9 Hz, H2′ *Z* form). ^{13}C NMR (90 MHz, DMSO-d_6) δ 117.2 (C5′), 120.9 (C2′), 124.2 (C6′), 125.3 (C2), 126.8 (C1′), 136.6 (C3), 140.8 (C3′), 150.7 (C4′), 171.7 (C1). LC-HRMS $[M - H]^-$ calc for $C_9H_7O_7S$: 258.9912; found: 258.9960. NMR data of compound **11** in D_2O has been previously reported, but only as a inseparable mixture with its 4′-*O*-sulfate isomer.[52] Thus compound **11** is now fully characterized for the first time.

(*E*)-Ethyl 3-(4-(benzyloxy)-3-(pentyloxysulfonyloxy)phenyl)acrylate (47)

Procedure A was carried out using 590 mg (1.56 mmol) of 2-(benzyloxy)-5-formylphenyl neopentyl sulfate **44** in 10 mL of dry CH_2Cl_2, and 0.6 g (1.72 mmol) of ethyl (triphenylphosphoranyliden)acetate. This afforded, after purification, 600 mg (1.34 mmol; 86%) of novel compound **47**. ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.87 (s, 9H, H1c), 1.33 (t, 3H, J = 7.12 Hz, H2″), 4.04 (s, 2H, H3c), 4.26 (q, 2H, J = 7.13 Hz, H1″), 5.15 (s, 2H, H7b), 6.33 (d, 1H, J = 15.99 Hz, H2), 7.05 (d, 1H, J = 8.57 Hz, H5′), 7.35–7.70 (m, 8H, H2b + H3b + H4b + H5b + H6b + H2′+ H6′ + H3).

Ethyl 3-(4-hydroxy-3-(pentyloxysulfonyloxy)phenyl)propanoate (48)

Following procedure D, 300 mg (0.67 mmol) of compound **47** were hydrogenated for 4 h. This yielded, after filtration, evaporation and drying under high vacuum, 240 mg (0.67 mmol; 100%) of novel compound **48**. TLC R_f = 0.49 (Silica 60 F254, Hexane–EtOAc 1/1). ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.01 (s, 9H, H1c), 1.23 (t,

3H, J = 7.13 Hz, H2″), 2.58 (t, 2H, J = 7.67 Hz, H2), 2.89 (t, 2H, J = 7.89 Hz, H3), 4.12 (q, 2H, J = 7.13 Hz, H1″), 4.13 (s, 2H, H3c), 6.97 (d, 1H, J = 8.36 Hz, H5′), 7.06 (dd, 1H, J = 8.32 and 2.12 Hz, H6′), 7.13 (d, 1H, J = 2.12 Hz, H2′). ^{13}C NMR (90 MHz, $CDCl_3$) δ 14.2 (C2″), 25.8 (C1c), 29.9 (*C3), 32.0 (C2c), 35.8 (*C2), 60.6 (C1″), 84.5 (C3c), 118.4 (*C5′), 122.1 (*C2′), 128.6 (C6′), 133.9 (C1′), 137.4 (*C3′), 145.9 (*C4′), 172.6 (C1).

Dihydrocaffeic acid 3′-sulfate (23)

Following procedure F, 240 mg (0.67 mmol) of compound **48** in 2 mL of dry DMF were reacted with 100 mg (1.5 mmol) of sodium azide and hydrolyzed in methanol and NaOH 1.0 M. This yielded, after purification by CC on Biotage RP-18, 270 mg (Quantification of the sulfate ester content following procedure G: 1.75 µmol/mg; 0.47 mmol; 70%) of novel compound **23**. HPLC R_t = 4.82 (method A). ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.20 (m, 2H, H2), 2.63 (m, 2H, H3), 6.67 (d, J = 8.18 Hz, 1H, H5′), 6.77 (dd, 1H, J = 8.18 and 2.12 Hz, H6′), 6.99 (d, 1H, J = 2.12 Hz, H2′). ^{13}C NMR (90 MHz, DMSO-d_6) δ 32.2 (C3), 40.8 (C2), 118.1 (C5′), 122.8 (C2′), 125.6 (C6′), 132.3 (C1′), 141.0 (C3′), 149.9 (C4′), 179.1 (C1). LC-HRMS $[M - H]^-$ calc for $C_9H_9O_7S$: 261.0069; found: 261.0115.

3′,4′-Diacetoxycinnamic acid ethyl ester (49)

Procedure A was carried out using 2.7154 g (12.22 mmol) of 3′,4′-diacetoxybenzaldehyde **27** in 20 mL of dry CH_2Cl_2, and 4.33 g (12.42 mmol) of ethyl (triphenylphosphoranyliden)acetate. This afforded, after purification, 3.9 g of compound **49**.[53] ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 7.1 Hz, H2″), 2.30 (s, 3H, OAc), 2.31 (s, 3H, OAc), 4.26 (q, 2H, J = 7.1 Hz, H1″), 6.38 (d, 1H, J = 16.0 Hz, H2), 7.22 (d, 1H, J = 8.4 Hz, H5′), 7.36 (d, 1H, J = 2.0 Hz, H2′), 7.40 (dd, 1H, J = 8.4 and 2.0 Hz, H6′), 7.62 (d, 1H, J = 16.0 Hz, H3). ^{13}C NMR (90 MHz, $CDCl_3$) δ 14.3 (C2″), 20.6 (OAc), 20.7 (OAc), 60.7 (C1″), 119.5 (C2), 122.7 (C2′), 123.9 (C5′), 126.4 (C6′), 133.4 (C1′), 142.4 (C3′), 142.6 (C3), 143.4 (C4′), 166.6 (C1), 168.0 (OAc), 168.1 (OAc).

4′-Acetoxy-3′-hydroxycinnamic acid ethyl ester (50) and 3′-acetoxy-4′-hydroxycinnamic acid ethyl ester (51)

1.5289 g (5.23 mmol) of 3′,4′-diacetoxycinnamic acid ethyl ester **49** were dissolved in 5 mL of 1-methyl-2-pyrrolidone (NMP). The solution was cooled to 0 °C in an ice bath and 135 mg (1.98 mmol) of imidazole, followed by 0.7 mL (6.85 mmol) of thiophenol were added. The reaction was allowed to take place at rt under stirring for 4 h. The reaction medium was directly purified by MPLC on silica using a gradient of EtOAc in hexane as solvent, affording 707.5 mg (2.83 mmol, 54%) of a mixture of 4′-acetoxy-3′-hydroxycinnamic acids ethyl ester **50**, and of 3′-acetoxy-4′-hydroxycinnamic acids ethyl ester **51** (in a ratio of about 1/1, according to NMR analysis). TLC R_f = 0.53 (Silica 60 F254, Hexane–EtOAc 3/2).

(*E*)-Ethyl 3-(3-(acetoxy)-4-(pentyloxysulfonyloxy)phenyl)acrylate (52)

Following procedure E, 125 mg (0.5 mmol) of the mixture of **50** and **51** were dissolved in 5 mL of THF. 150 µL (1.00 mmol) of TEA, 122 mg (1.00 mmol) of DMAP and 203 mg (1.0 mmol) of neopentylchlorosulfate[28,30] were added successively under stirring at RT and nitrogen atmosphere. After removal of the solvent under reduced pressure, the oily colored residue was purified by MPLC (Biotage 25 M) using a gradient of EtOAc in hexane as solvent. Yield: 168 mg (0.42 mmol; 80%) of novel compound **52**. TLC R_f = 0.55 (Silica 60 F254, Hexane–EtOAc 2/1). ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.01 (s, 9H, H1c), 1.34 (t, 3H, J = 7.13 Hz, H2″), 2.37 (s, 3H, $COC\underline{H}_3$), 4.11 (s, 2H, H3c), 4.27 (q, 2H, J = 7.13 Hz, H1″), 6.40 (d, 1H, J = 16.0 Hz, H2), 7.38 (d, 1H, J = 2.1 Hz, H2′), 7.42 (dd, 1H, J = 8.50 and 2.1 Hz, H6′), 7.47 (d, 1H, J = 8.5 Hz, H5′). ^{13}C NMR (90 MHz, $CDCl_3$) δ 14.60 (C2″), 21.0 ($CO\underline{C}H_3$), 26.2 (C1c), 32.3 (C2c), 61.1 (C1″), 84.4 (C3c), 120.7 (C2), 122.8 (C5′), 123.7 (C2′), 126.9 (C6′), 134.8 (C1′), 142.3 (C3), 142.7 (*C3′), 143.0 (*C4′), 166.7 (C1), 168.4 ($\underline{C}OCH_3$).

(*E*)- and (*Z*)-Caffeic acid 4′-sulfate (12a) and (12b)

Following procedure F, 168 mg (0.42 mmol) of compound **52** in 1 mL of dry DMF were reacted with 41 mg (0.65 mmol) of sodium azide and hydrolyzed in methanol and NaOH 1.0 M. This yielded, after purification by CC on Biotage RP-18, 148 mg (Quantification of the sulfate ester content following procedure G: 1.91 µmol/mg; 0.28 mmol; 67%) of compound **12** as a 9/1 mixture of (*E*)-**12a** and (*Z*)-**12b** forms. HPLC R_t = 5.29 (method A). ^{1}H NMR (360 MHz, DMSO-d_6) 5.81 (d, 0.1H, J = 12.9 Hz, H2 *Z*-form), 5.91 (d, 0.1H, J = 12.9 Hz, H3 *Z*-form), 6.24 (d, 0.9H, J = 15.8 Hz, H2 *E*-form), 6.87 (dd, 0.9H, J = 8.4 and 2.1 Hz, H6′ *E*-form), 6.95 (d, 0.9H, J = 2.1 Hz, H2′ *E*-form), 7.06 (d, 0.9H, J = 15.8 Hz, H3 *E*-form), 7.12 (d, 0.9H, J = 8.3 Hz, H5′ *E*-form). ^{13}C NMR (90 MHz, DMSO-d_6) 115.1 (C2′), 118.3 (C6′), 122.7 (C5′), 127.8 (C2), 133.0 (C1′), 136.1 (C3), 141.1 (C4′), 149.1 (C3′), 170.9 (C1). ^{1}H and ^{13}C NMR data of the (*E*)-isomer **12a** were in accordance with published values in DMSO-d_6.[54] LC-HRMS $[M - H]^-$ calc for $C_9H_7O_7S$: 258.9912; found: 258.9955.

Human intervention study design

Trial Design. The study and protocol were reviewed by Glasgow Royal Infirmary NHS Research Ethics Committee. Eleven healthy human volunteers followed a low polyphenol diet for 48 h prior the beginning of the study. On the night preceding the trial, the subjects stopped consuming any food and came to the trial unit at 8.30 am the next day in a fasted state and consumed 200 mL of an instant coffee containing 412 µmol of chlorogenic acids, and remained on a low polyphenol diet for 24 h. Plasma and urine were collected over the ensuing 24 h period and aliquots were stored at –80 °C prior to analysis.

Plasma and urine analyses. Plasma samples were extracted using a method developed by Day *et al.*,[55] and along with urine samples were analysed by HPLC-PDA-MS^n using a Surveyor HPLC with a PDA detector and a LCQ Duo ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, USA). Separations were performed at 40 °C using a SYNERGI 4 µm POLAR-RP 250 × 4.6 mm i.d. reverse phase column (Phenomenex, Macclesfield, UK). Injections were carried out with an autosampler maintained at 4 °C. The mobile phase comprising a 40 min, 5–16% gradient of acetonitrile in 0.5% aqueous acetic acid was pumped at a flow rate of 1 mL/min. The column eluate initially passed through the PDA detector and was then split,

with 0.3 mL/min^{-1} directed to the mass spectrometer operating in full scan negative ionisation mode (100–1000 m/z). Analyses were carried out using full scan, data dependent MS^2 scanning from m/z 100 to 1000. The tuning of the mass spectrometer was optimised by infusing a standard of ferulic acid, dissolved in the initial HPLC mobile phase, into the source at a flow rate of 0.3 mL/min. Capillary temperature was 300 °C, sheath gas and auxiliary gas were 80 and 60 units/min^{-1} respectively, and source voltage was 3.0 kV, and collision energy set at 35%.

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