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Hydrolyzable Tannins with the Hexahydroxydiphenoyl Unit and the *m*-Depsidic Link: HPLC-DAD-MS Identification and Model Synthesis

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This study was designed to develop efficient analytical tools for the difficult HPLC-DAD-MS identification of hydrolyzable tannins in natural tissue extracts. Throughout the study of the spectroscopic characteristics of properly synthesized stereodefined standards, it was observed that the UV−vis spectra of compounds with the *m*-depsidic link showed a characteristic shoulder at 300 nm, consistent with the simple glucogalloyl esters, whereas compounds with the hexahydroxydiphenoyl (HHDP) unit gave a diagnostic fragmentation pattern, caused by a spontaneous lactonization in the mass spectrometer. These observations were confirmed by HPLC-DAD-MS analyses of tannic acid and raspberry extracts, which are rich in hydrolyzable tannins with the *m*-depsidic link and the HHDP unit, respectively.

KEYWORDS: Polyphenols; gallotannins; ellagitannins; tannic acid; berries

INTRODUCTION

Hydrolyzable tannins are secondary metabolites that most generally are found in nature as multiple esters of gallic acid with glucose. They represent a biologically relevant group of polyphenols, which have garnered growing interest from consumers, food manufacturers, and pharmaceutical industries. On the basis of their structural characteristics, they are classified into simple esters, depside metabolites, hexahydroxydiphenoyl esters (HHDP), or ellagitannins, and oligomers formed by oxidative coupling of monomers (*1*, *2*). More than 500 glucogalloyl molecules from more than 20 plant families have been identified, from the very simple 1-monogalloyl-β-glucose (glucogallin), with a molecular weight 332, to complex polymers with molecular weights of over 4000 (*1*, *2*). The qualitative and quantitative determination of glucogalloyl derivatives in natural matrices is a difficult task even for modern analytical techniques (*3*, *4*). In addition, the lack of pure and structurally defined commercially available standards of hydrolyzable tannins render the analyses complex. This study develops a stereodefined synthesis of glucogalloyl compounds that allow an easy entry to the HHDP unit C−C and the *m*-depsidic link. The spectrometric and spectroscopic characteristics (UV−vis, MS, and NMR) of these synthetic standards were used to provide information useful for the identification of hydrolyzable tannins in natural tissue extracts.

MATERIALS AND METHODS

Material. Methanol (MeOH), tetrahydrofuran (THF), petroleum ether (PE), ethyl acetate (EtOAc), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), *n*-hexane, benzene, and diethyl ether (Et_2O) were from Carlo Erba (Carlo Erba Reagenti SpA, Milano, Italy). Methyl gallate, CH_3-CN, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), acetic acid, 10% Pd/C, Na_2SO_4, $CDCl_3$, CD_3OD, H_3PO_3, $Pb(AcO)_4$, $(NH_4)_6MoO_4$, $Ce(SO_4)_2$, H_2SO_4, thionyl chloride, pyridine, gallic acid, and tannic acid were from Sigma-Aldrich (St. Louis, MO). Acetonitrile (HPLC grade) and formic acid were purchased from Aldrich Co. Inc. (Milwaukee, WI).

NMR and MS Analysis. ^{1}H NMR spectra at 200 and 400 MHz and ^{13}C NMR spectra at 50 and 100 MHz were obtained on a Varian Gemini 2000 spectrometer and a Varian Mercury Plus 400 spectrometer, respectively. For the NMR experiments $CDCl_3$ and CD_3OD were used as solvents. Chemical shifts are reported in parts per million (ppm) relative to the solvent nondeuterated residue. The following abbreviations are used: s, singlet; d, doublet; t, triplet; and m, multiplet. High-resolution mass spectrometry HRMS (ESI) was measured on an LTQ Orbitrap-Ultimate 3000-(Finnigan LTQ), operated at the Interdepartmental Centre of Mass Spectrometry (CISM, Florence, Italy).

HPLC Analysis. HPLC-DAD analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies, Paolo Alto, CA). A Luna C18(2), 250 × 4.60 mm, 5 μm (Phenomenex, Torrance, CA), column was used, operating at 27 °C. The mobile phase was a multistep linear solvent gradient system

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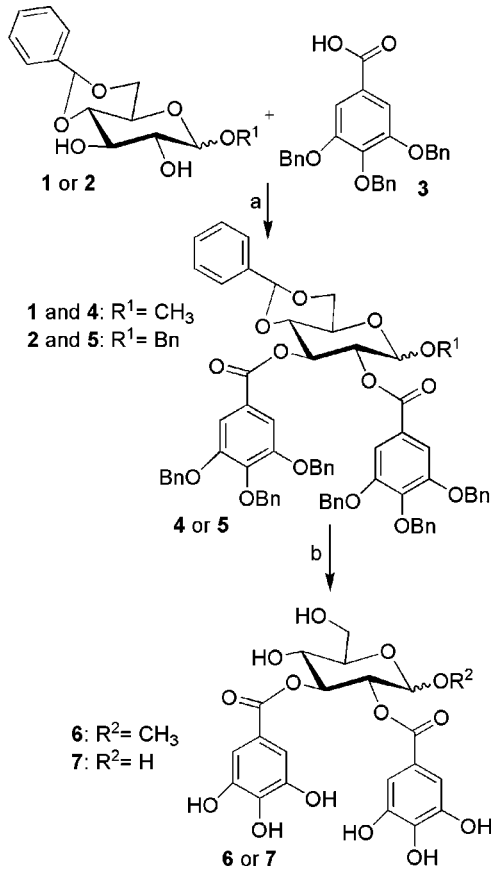

Figure 1. Synthesis of compounds **7** and **6**. Reagents and conditions: (a) DCC, DMAP, DMAP·HCl, CH_2Cl_2, reflux, 19 h, 80% for **4** and 60% for **5**; and (b) Pd/C, H_2, THF, 24 h, 73% for **6** and 73% for **7**.

'consisting of (A) H_2O (adjusted to pH 3.2 by HCOOH) and (B) CH_3-CN and a flow rate of 0.8 mL/min. The elution profile was as follows: 2 min 100% A, then solvent B was increased first to 20% in 8 min, then to 50% in 10 min, linear for 5 min, and subsequently increased to 100% in 5 min, with 10 min for equilibration. HPLC-MS analyses were performed using a HP 1100L liquid chromatograph linked to a HP 1100 MSD mass spectrometer with an API/ESI interface (Agilent Technologies). The mass spectrometer operating conditions were as follows: gas temperature, 350 °C; nitrogen flow rate, 10.5 L/min; nebulizer pressure, 40 psi; quadrupole temperature, 40 °C; and capillary voltage, 3500 V. The orthogonal position of the nebulizer with respect to the capillary inlet enabled the use of the same conditions as for HPLC-DAD analysis. The mass spectrometer was operated in negative mode; and the fragmentor was set at 120 eV for the standard and the synthetic products and at 120 and 200 eV for the raspberry extracts.

Organic Synthesis. Solvents were purchased and dried according to usual laboratory techniques. Unless otherwise noted, all air- and moisture-sensitive reactions were performed under a nitrogen atmosphere. Analytical thin-layer chromatography (TLC) was conducted on glass-backed silica gel Durasil-25-UV254 plates (Macherey-Nagel, Germany**)** with detection by UV light and charring with vanillin or Pancaldi [$(NH_4)_6MoO_4$, $Ce(SO_4)_2$, H_2SO_4, H_2O] reagents. Flash chromatography was carried out on 60M silica gel (Macherey-Nagel).

*Esterification General Procedure (**Figures 1** and **2**).* A mixture of the properly protected glucose (1.0 equiv) and gallic acid (1.1 equiv per hydroxyl), DMAP (0.5 equiv per hydroxyl), and DMAP·HCl (0.5 equiv per hydroxyl) was refluxed in dry CH_2Cl_2 for 1 h, then DCC (1.1 equiv per hydroxyl) was added, and the mixture was reacted for 16−19 h at room temperature. The reaction was cooled at −18 °C for 30 min and filtered through a pad of Celite. The organic phase was washed with 3% HCl, brine, and H_2O, dried over Na_2SO_4, concentrated, and purified by flash chromatography (*5*).

*Hydrogenation/Deprotection General Procedure (**Figures 1** and **2**).* A mixture of the benzylated glucogalloyl derivative was hydrogenated over 10% Pd/C with 1 atm of H_2, in distilled THF. The hydrogenation was repeated twice to complete the deprotection. The gray-green glassy solid obtained was triturated with three portions of Et_2O and three portions of hexanes and purified by flash chromatography (*5*).

*2,3-Di(3,4,5-tribenzyloxy)galloyl-O-methylglucose (**4**).* By use of the general procedure of esterification, the acid **3** (1.40 g, 0.32 mmol), DMAP (0.26 g, 0.21 mmol), DMAP·HCl (0.34 g, 0.21 mmol), methyl glucoside **1** (0.30 g, 0.11 mmol), and DCC (0.66 g, 0.32 mmol) were coupled to afford, after flash chromatography (PE/EtOAc, 5:1, 3:1, and 2:1), 956 mg of a mixture of α- and *β*-anomer of **4**, and two small fractions of the single anomers, that were used for their identification (overall yield = 80%). α-Anomer: ^{1}H NMR ($CDCl_3$, 400 MHz), *δ* 3.45 (s, 3H, OCH_3), 3.82−3.87 (m, 1H, H_5), 3.89−4.07 (m, 2H, H_6), 4.39 (dd, *J* = 10.0 and 4.8 Hz, 1H, H_4), 5.00−5.14 (m, 12H, CH_2 and 1H, H_2), 5.24 (d, *J* = 4.0 Hz, 1H, H_1), 5.57 (s, 1H, H_7), 6.03 (t, *J* = 10.0 Hz, 1H, H_3), 7.18−7.50 (m, 39H, CH_{phenol}). *β*-Anomer: ^{1}H NMR ($CDCl_3$, 200 MHz), *δ* 3.53 (s, 3H, OCH_3), 3.82−3.87 (m, 1H, H_5), 4.08−4.11 (m, 2H, H_6), 4.43−4.49 (m, 1H, H_4), 5.00−5.14 (m, 12H, CH_2 and 1H, H_1), 5.37−5.48 (m, 1H, H_2), 5.55 (s, 1H, H_7), 5.75−578 (m, 1H, H_3), 7.18−7.50 (m, 39H, CH_{phenol}).

*2,3-Di(3,4,5-tribenzyloxy)galloyl-O-benzylglucose (**5**).* By use of the general procedure of esterification, 3,4,5-tribenzyloxygallic acid **3** (0.84 g, 1.91 mmol), DMAP (0.17 g, 0.954 mmol), DMAP·HCl (0.15 g, 0.954 mmol), benzyl glucoside **2** (0.31 g, 0.868 mmol), and DCC (0.40 g, 1.91 mmol) were coupled to afford, after flash chromatography (PEE/EtOAc/CH_2Cl_2, 5:1:1), 627 mg of a mixture of the two anomers of **5** (yield 60%): ^{1}H NMR ($CDCl_3$, 400 MHz), *δ* 3.69 (dt, *J* = 9.6 and 4.8 Hz, 1H, $H_{5\beta}$), 3.81−3.97 (m, 4H, $H_{6\alpha+\beta}$), 4.18 (dt, *J* = 10.0 and 4.8 Hz, 1H, $H_{5\alpha}$), 4.32 (dd, *J* = 10.0 and 4.8 Hz, 1H, $H_{4\alpha}$), 4.48 (dd, *J* = 10.8 and 4.8 Hz, 1H, $H_{4\beta}$), 4.60 (d, *J* = 12.4 Hz, 1H, CH_2Bn_α), 4.66 (d, *J* = 12.4 Hz, 1H, CH_2Bn_β), 4.80 (d, *J* = 12.4 Hz, 1H, CH_2Bn_α), 4.82 (d, *J* = 8.0 Hz, 1H, $H_{1\beta}$), 4.92 (d, *J* = 12.4 Hz, 1H, CH_2Bn_β), 4.93−5.11 (m, 24H, $CH_2Bn_{\alpha+\beta}$), 5.15 (dd, *J* = 10.0 and 4.0 Hz, 1H, $H_{2\alpha}$), 5.36 (d, *J* = 4.0 Hz, 1H, $H_{1\alpha}$) 5.52 (dd, *J* = 8.0 and 1.6 Hz, 1H, H_2), 5.56 (s, 1H, $CHBn_\beta$), 5.57 (s, 1H, $CHBn_\alpha$), 5.92 (t, *J* = 9.6 Hz, 1H, $H_{3\beta}$), 6.08 (t, *J* = 10.0 Hz, 1H, $H_{3\alpha}$), 7.04−7.39 (m, 88H, CH_{phenol}).

*2,3-Digalloyl-O-methylglucose (**6**).* The general hydrogenation procedure applied to glucogalloyl derivative **4** (780 mg, 0.69 mmol) afforded, after chromatography (PE/THF/MeOH, 6:3:1), 321 mg of 2,3-digalloyl-*O*-methylglucose **6** (yield 73%): MS, *m*/*z* (%) 497 (100, [M − H]$^-$), 345 (62), 169 (59), 125 (25); ^{1}H NMR (CD_3OD, 400 MHz), *β*, *δ* 3.54 (s, 3H, OCH_3), 3.65−3.71 (m, 1H, H_5), 3.76−3.84 (m, 2H, H_6), 4.67 (d, *J* = 8.0 Hz, 1H, H_1), 4.91 (s, 8H, OH), 5.11 (dd, *J* = 8.0 and 9.6 Hz, 1H, H_2), 5.42 (dd, *J* = 9.2 and 9.6 Hz, 1H, H_3), 6.98 (s, 2H, H_{phenol}), 7.02 (s, 2H, H_{phenol}); α, *δ* 3.47 (s, 3H, OCH_3), 3.62−3.71 (m, 2H, H_6), 3.76−3.79 (m, 1H, H_5), 4.28 (td, *J* = 0.8 and 6.4 Hz, 1H, H_4), 4.90 (s, 8H, OH), 5.01−5.07 (m, 2H, H_{1+2}), 5.65−5.72 (m, 1H, H_3), 7.00 (s, 2H, H_{phenol}), 7.06 (s, 2H, CH_{phenol}); ^{13}C NMR (CD_3OD, 100 MHz), *β*, *δ* 57.3, 62.3, 69.8, 73.4, 77.0, 78.1, 103.2, 110.26, 110.32, 120.9, 121.1, 139.8, 139.9, 146.29, 146.34, 167.2, 167.9; α, *δ* 55.6, 67.9, 69.8, 73.6, 74.3, 98.6, 110.3, 110.4, 120.6, 121.4, 140.0, 140.1, 146.3, 146.4, 167.6, 168.2.

*2,3-Digalloyl-O-glucose (**7**).* The general hydrogenation procedure applied to glucogalloyl derivative **5** (230 mg, 0.19 mmol) afforded, after chromatography (PE/THF/MeOH, 6:3:1), 67 mg of 2,3-digalloyl-*O*-glucose **7** (yield 73%): MS, *m*/*z* (%) 483 (100, [M − H]$^-$), 313 (*10*), 169 (95); ^{1}H NMR (CD_3OD, 400 MHz), *δ* 3.47−3.54 (m, 1H, $H_{5\beta}$), 3.70−3.94 (m, 6H, $H_{6\alpha,5\alpha,6\beta,4\beta}$), 3.96−4.02 (m, 1H, $H_{4\beta}$), 4.89 (d, *J* = 8.0 Hz, 1H, $H_{1\beta}$), 4.97 (dd, *J* = 10.4 and 3.6 Hz, 1H, $H_{2\alpha}$), 5.05 (dd, *J* = 9.6 and 8.0 Hz, 1H, $H_{2\beta}$), 5.37 (at, 1H, $H_{3\beta}$), 5.42 (d, *J* = 3.8 Hz, 1H, $H_{1\alpha}$), 5.73 (at, 1H, $H_{3\alpha}$), 6.97 (s, 1H, $H_{phenol-\beta}$), 7.00 (s, 1H, $H_{phenol-\beta}$), 7.00 (s, 1H, $H_{phenol-\alpha}$), 7.04 (s, 1H, $H_{phenol-\alpha}$).

*2,3-Di[3-tert-butyldimethylsilyloxy-4,5-diphenylmethylenedioxybenzoyl]benzyl-O-glucose (**9**).* By use of the general procedure of esterification, 3-*tert*-butyldimethylsilyloxy-4,5-diphenylmethylenedioxybenzoic acid **8** (757 mg, 1.55 mmol), DMAP (920 mg, 0.75 mmol), DMAP·HCl (120 mg, 0.75 mmol), benzyl-*O*-glucoside (200 mg, 0.74 mmol), and DCC (309 mg, 1.55 mmol) were coupled to afford, after flash chromatography (PE/EtOAc/CH_2Cl_2, 10:1:1), 265 mg of the derivative **9** as a mixture of anomers **(**yield = 31%): ^{1}H NMR ($CDCl_3$, 400 MHz), *δ* 0.09−0.15 [m, 24H, $Si(CH_3)_2$], 0.94−0.99 [m, 36H, $C(CH_3)_3$], 3.63

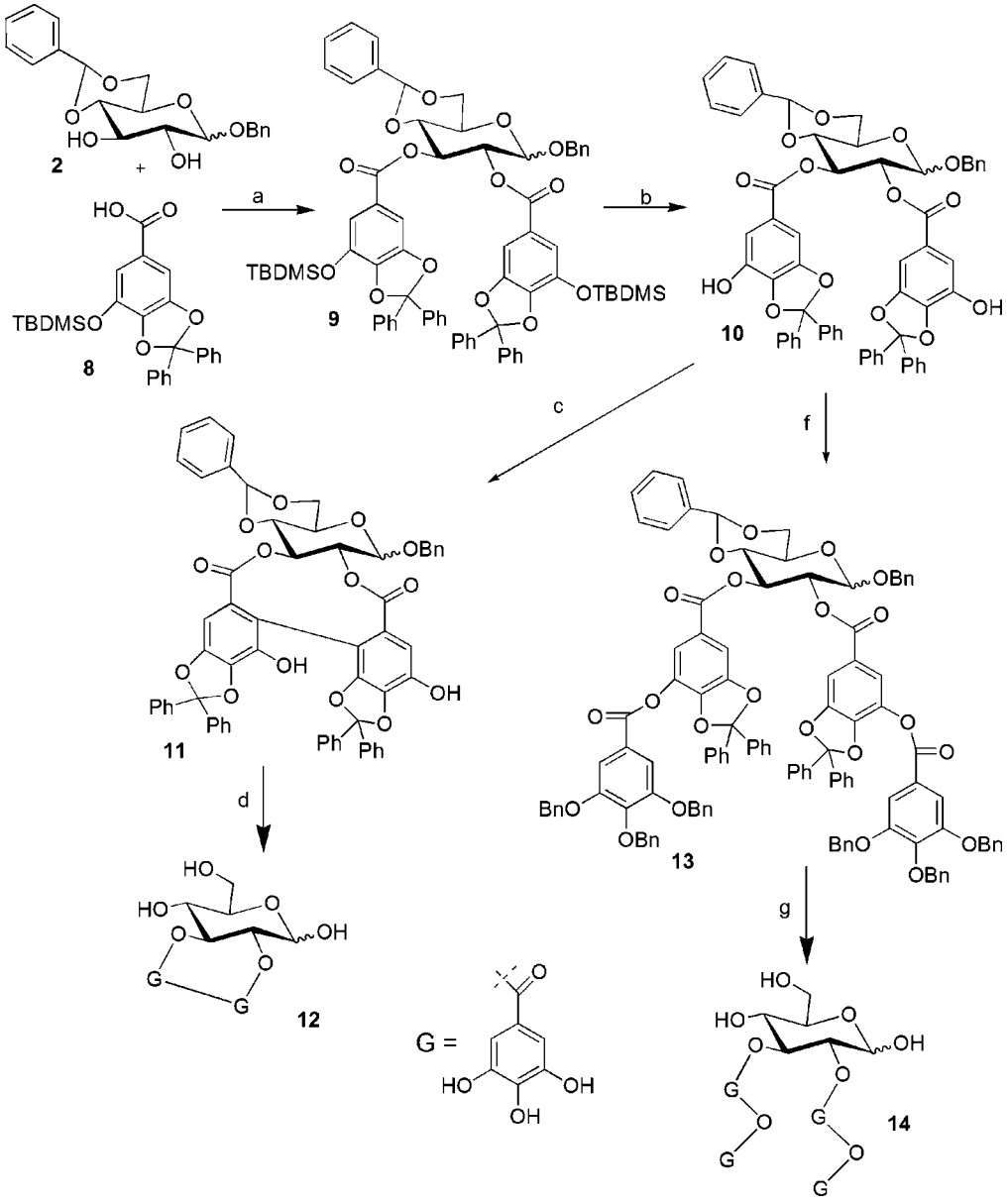

Figure 2. Synthesis of the glucogalloyl derivatives with the HHDP unit and the *m*-depsidic link. Reagents and conditions: (a) DCC, DMAP, DMAP·HCl, CH_2Cl_2, reflux, 19 h, 31%; (b) TBAF/AcOH, THF, room temperature, 5 h, 79%; (c) pyridine, $Pb(AcO)_4$, CH_2Cl_2, –20 °C, 31 min, 25%; (d) Pd/C, H_2, THF, 24 h, 45%; (e) tribenzyloxybenzoyl chloride, DMAP, pyridine, 60 °C, 48 h, 21%; and (g) Pd/C, H_2, THF, 24 h, 40%.

(dt, J = 9.6 and 4.8 Hz, 1H, $H_{5\beta}$), 3.77−3.91 (m, 4H, $H_{6\alpha+\beta}$), 4.11 (dt, J = 10.0 and 4.8 Hz, 1H, $H_{5\alpha}$), 4.27 (dd, J = 10.0 and 4.8 Hz, 1H, $H_{4\alpha}$), 4.43 (dd, J = 10.8 and 4.8 Hz, 1H, $H_{4\beta}$), 4.55 (d, J = 12.0 Hz, 1H, $CH_2Ph_{z\alpha}$), 4.63 (d, J = 12.4 Hz, 1H, CH_2Ph_β), 4.75 (d, J = 8.0 Hz, 1H, $H_{1\beta}$), 4.76 (d, J = 12.0 Hz, 1H, CH_2Ph_α), 4.88 (d, J = 12.4 Hz, 1H, CH_2Ph_β), 5.12 (dd, J = 10.0 and 4.0 Hz, 1H, $H_{2\alpha}$), 5.28 (d, J = 4.0 Hz, 1H, $H_{1\alpha}$), 5.52 (dd, J = 9.2 and 1.2 Hz, 1H, H_2), 5.52 (s, 1H, $CHPh_\beta$), 5.53 (s, 1H, $CHPh_\alpha$), 5.61 (t, J = 9.6 Hz, 1H, $H_{3\beta}$), 5.99 (t, J = 9.6 Hz, 1H, $H_{3\alpha}$), 7.04−7.39 (m, 88H, CH_{phenol}).

*2,3-Di[3-hydroxy-4,5-diphenylmethylenedioxybenzoyl]-benzyl-O-glucose (**10**).* To a solution of 2,3-di[3-*tert*-butyldimethylsilyloxy-4,5-diphenylmethylenedioxybenzoyl]benzyl-*O*-glucose (625 mg, 0.217 mmol) in 10 mL of THF buffered with acetic acid (0.062 mL, 1.087 mmol) was added a solution of TBAF·$3H_2O$ (343 mg, 1.087 mmol) in 5 mL of THF at room temperature. The reaction mixture was stirred at room temperature for 2 h, then diluted with 100 mL of ethyl acetate, and washed with 1 M aqueous H_3PO_4, and brine. Purification by flash chromatography (PEE/EtOAc/CH_2Cl_2, 4:1:1) allowed the isolation of 170 mg of the 2,3-di[3-hydroxy-4,5-diphenylmethylenedioxybenzoyl]-benzyl-*O*-glucose **10** (yield = 79%) as a white solid: ^{1}H NMR ($CDCl_3$, 400 MHz), δ 3.62 (dt, J = 9.6 and 4.8 Hz, 1H, $H_{5\beta}$), 3.77−3.90 (m, 4H, $H_{6\alpha+\beta}$), 4.10 (dt, J = 10.0 and 4.8 Hz, 1H, $H_{5\alpha}$), 4.27 (dd, J = 10.4 e 4.8 Hz, 1H, $H_{4\alpha}$), 4.42 (dd, J = 10.4 and 4.8 Hz, 1H, $H_{4\beta}$), 4.54 (d, J = 12.4 Hz, 1H, CH_2Ph_α), 4.62 (d, J = 12.4 Hz, 1H, CH_2Ph_β), 4.74 (d, J = 12.4 Hz, 1H, CH_2Ph_α), 4.74(d, J = 8.0 Hz, 1H, $H_{1\beta}$), 4.86 (d, J = 12.4 Hz, 1H, CH_2Ph_β), 5.07 (dd, J = 10.0 and 4.0 Hz, 1H, $H_{2\alpha}$), 5.28 (d, J = 4.0 Hz, 1H, $H_{1\alpha}$), 5.40 (dd, J = 9.6 and 1.2 Hz, 1H, H_2), 5.50 (s, 1H, $CHPh_\beta$), 5.53 (s, 1H, $CHPh_\alpha$), 5.56 (t, J = 9.6 Hz, 1H, $H_{3\beta}$), 5.99 (t, J = 9.6 Hz, 1H, $H_{3\alpha}$), 7.04−7.39 (m, 88H, CH_{phenol}).

*Hexahydroxydiphenoyl Derivative **11**.* To a solution of 2,3-di[3-hydroxy-4,5-diphenylmethylenedioxybenzoyl]benzyl-*O*-glucose **10** (41 mg, 0.041 mmol) and pyridine (0.013 mL, 0.165 mmol) in 10 mL of CH_2Cl_2 distillate kept at −20 °C was added dropwise a solution of $Pb(AcO)_4$ (20 mg, 0.045 mmol) in 5 mL of CH_2Cl_2 over 10 min. The resulting cloudy light orange reaction mixture was stirred at −20 °C for 30 min and at room temperature for 2 h and then poured into 10 mL of saturated aqueous 1 M H_3PO_4, washed with brine (4 × 10 mL), dried over Na_2SO_4, filtered, and concentrated to afford a light orange oil. Purification of the residue by flash column chromatography (PE/EtOAc, 4:1:1) furnished 10 mg of diphenyl derivative **11** (yield = 25%): ^{1}H NMR ($CDCl_3$, 400 MHz), δ 3.41 (m, 1H, $H_{5\beta}$), 3.77−3.90 (m, 4H, $H_{6\alpha+\beta}$), 4.02 (td, J = 10.0 and 4.8 Hz, 1H, $H_{5\alpha}$), 4.23 (dt, J =

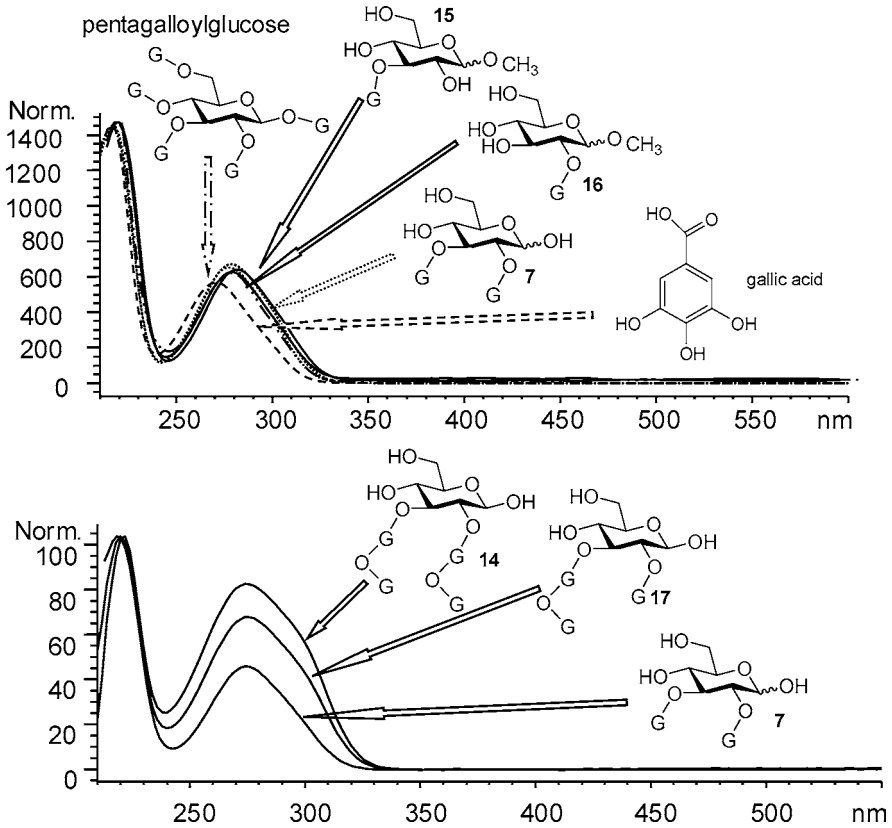

Figure 3. On-line UV–vis spectra of gallic acid, mono-, di-, and pentagalloylglucose, and glucogalloyl synthetic derivatives with and without the *m*-depsidic link.

10.0 and 4.4 Hz, 1H, $H_{4\alpha}$), 4.37−4.42 (m, 1H, $H_{4\beta}$), 4.79−4.96 (m, 4H, $CH_2Ph_{\alpha+\beta}$), 4.80 (d, J = 8.0 Hz, 1H, $H_{1\beta}$), 5.02−5.06 (m, 1H, $H_{2\alpha}$), 5.15−5.23 (m, 1H, $H_{1\alpha}$), 5.28−5.36 (m, 1H, H_2), 5.54 (s, 1H, $CHPh_\beta$), 5.60 (s, 1H, $CHPh_\alpha$), 5.63−5.70 (m, 1H, $H_{3\alpha+\beta}$), 66.2−6.81 (m, 4H, CH_{phenol}), 5.99 (t, J = 9.6 Hz, 1H, $H_{3\alpha}$), 7.02−7.68 (m, 66H, CH_{phenol}).

Hexahydroxydiphenoyl Derivative ***12***. The general hydrogenation procedure applied to glucogalloyl derivative **11** (10 mg, 0.01 mmol) afforded 3 mg of the hexahydroxydiphenoyl compound **12** (yield = 45%): MS, *m*/*z* (%) 481 (20, $[M - H]^-$), 301 (100), 275 (20); ^{1}H NMR (CD_3OD, 400 MHz), δ 3.42−3.50 (m, 1H, $H_{5\beta}$), 3.68−3.96 (m, 7H, $H_{6\alpha,5\alpha,6\beta,4\beta,4\alpha}$), 4.73 (dd, J = 9.6 and 8.0 Hz, 1H, $H_{2\beta}$), 4.99 (d, J = 8.0 Hz, 1H, $H_{1\beta}$), 5.28−4.96 (dd, J = 3.6 and 9.6 Hz, 1H, $H_{2\alpha}$), 5.03 (t, J = 9.6 Hz, 1H, $H_{3\beta}$), 5.32 (d, J = 3.6 Hz, 1H, $H_{1\alpha}$), 5.36 (t, J = 9.6 Hz, 1H, $H_{3\alpha}$), 6.58 (s, 1H, CH_{phenol}), 6.59 (s, 1H, CH_{phenol}), 6.65 (s, 1H, CH_{phenol}), 6.66 (s, 1H, CH_{phenol}).

2,3-Di[3-(3,4,5-tribenzyloxygalloyl)-4,5-diphenylmethylenedioxybenzoyl]benzyl-O-glucose (***13***). To a stirred solution of 3,4,5-tribenzyloxybenzoic acid **3** (5.00 g, 1.14 mmol) and thionyl chloride (2.71 g, 22.8 mmol) in benzene (10 mL) was added pyridine (9 mg, 0.11 mmol). The mixture was refluxed for 1.5 h at 73 °C. To the resulting solution was added petroleum ether (50 mL) followed by cooling in ice. The crude precipitate was recrystallized from *n*-hexane to give 3.05 g of 3,4,5-tribenzyloxybenzoyl chloride as a colorless powder (yield = 61%). To a solution of the 2,3-di[3-hydroxy-4,5-diphenylmethylenedioxybenzoyl]benzyl-*O*-glucose **10** (129 mg, 0.13 mmol) and DMAP (0.046 g, 0.39 mmol) in 5 mL of dry pyridine was added dropwise a solution of freshly prepared 3,4,5-tribenzyloxybenzoyl chloride (180 mg, 0.13 mmol) in 5 mL of dry pyridine. The reaction was stirred under a nitrogen atmosphere for 18 h at room temperature and for 48 h at 60 °C. The mixture was diluted with 50 mL of CH_2Cl_2, washed with a 1 M aqueous solution of H_3PO_4 (2 × 10 mL) and brine (4 × 10 mL), concentrated, and purified by flash column chromatography (PE/EtOAc/CH_2Cl_2, 5:1:1) to give 50 mg of 2,3-di[3-(3,4,5-tribenzyloxygalloyl)-4,5-diphenylmethylenedioxybenzoyl]benzyl-*O*-glucose **13** (yield = 21%): ^{1}H NMR ($CDCl_3$, 200 MHz), δ 3.31−3.41 (m, 1H, $H_{5\beta}$), 3.45−3.98 (m, 4H, $H_{6\alpha+\beta}$), 4.10−4.20 (m, 1H, $H_{5\alpha}$), 4.22−4.34 (m, 1H, $H_{4\alpha}$), 4.39−4.50 (m, 1H, $H_{4\beta}$), 4.52−4.95 (m, 5H, $H_{1\beta,}$ $CH_2Ph_{\alpha+\beta}$), 5.01−5.23 (m, 24H, CH_2Ph) 5.25−5.35 (m, 1H, $H_{2\alpha}$), 5.38−5.49 (m, 1H, $H_{1\alpha}$), 5.51−5.59 (m, 3H, $H_2CHPh_{\alpha+\beta}$), 5.60−5.70 (m, 1H, $H_{3\beta}$), 5.92−6.09 (m, 1H, $H_{3\alpha}$), 7.01−7.85 (m, 126H, CH_{phenol}).

2,3-Di(3-galloyl-4,5-dihydroxybenzoyl)-O-glucose (***14***)*.:* Glucogalloyl derivative **13** (25 mg, 0.03 mmol) was hydrogenated following the general procedure to afford 5 mg of the depsidic glucogalloyl derivative **14** (yield = 40%): ^{1}H NMR (CD_3OD, 400 MHz), δ 3.49−3.56 (m, 1H, $H_{5\beta}$), 3.71−3.95 (m, 6H, $H_{6\alpha,5\alpha,6\beta,4\beta}$), 3.97−4.02 (m, 2H, $H_{4\beta+\alpha}$), 4.98−5.02 (m, 1H, $H_{1\beta}$), 5.03−5.11 (m, 1H, $H_{2\alpha}$), 534−5.48 (m, 3H, $H_{2\beta,3\beta,1\alpha}$), 5.75 (at, 1H, $H_{3\alpha}$), 7.20 (s, 4H, H_{phenol}), 7.21 (s, 4H, H_{phenol}), 7.22−7.25 (m, 3H, H_{phenol}), 7.28 (d, J = 2.4 Hz, 1H, H_{phenol}), 7.31 (d, J = 2.0 Hz, 1H, H_{phenol}), 7.33−7.35 (m, 2H, H_{phenol}), 7.38 (d, J = 1.6 Hz, 1H, H_{phenol}). HRMS (ESI): calcd for $C_{34}H_{29}O_{22}$ $(M + H)^+$, 789.1150; found, 789.1153.

*2-Galloyl-*α*-methyl-O-glucose* (***15***)*:* ^{1}H NMR (CD_3OD, 400 MHz), β, δ 3.38 (s, 3H, OCH_3), 3.58−3.76 (m, 3H, H_{5+6}), 3.86 (dd, J = 2.4 and 12.0 Hz, 1H, H_4), 3.93 (at, 1H, H_3), 4.77 (dd, J = 4.0 and 10.4

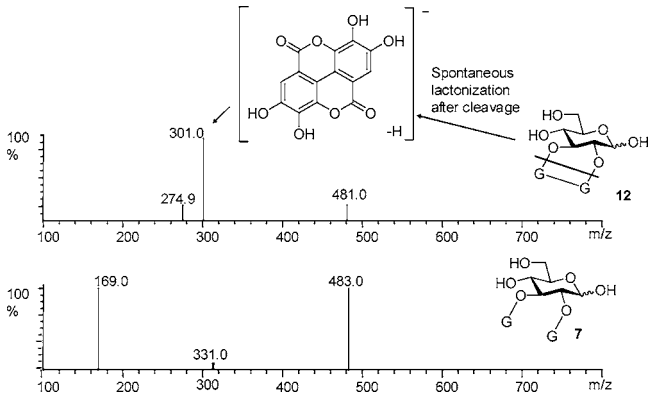

Figure 4. Difference ESI-MS (negative ionization) fragmentation pattern between the HHDP compound **12** and its simple digalloyl glucose analogue **7**.

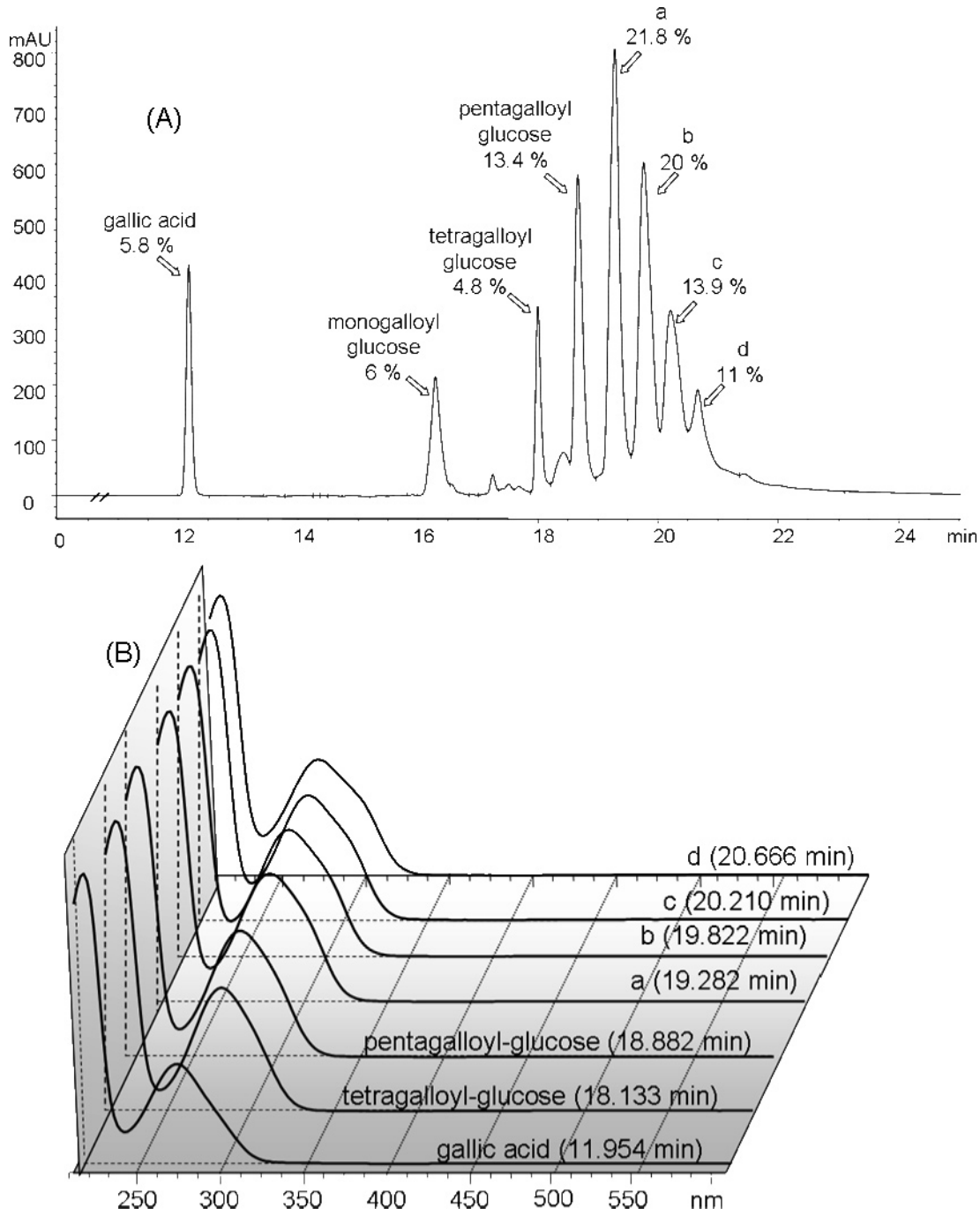

Figure 5. (**A**) HPLC-DAD profile of tannic acid on reverse phase chromatography at 280 nm. (**B**) On-line UV–vis spectra of tannic acid major components ($[M - H]^-$) *m*/*z* a, 1091; b,1243; c, 1395; d, 1547.

Hz, 1H, H_2), 4.93 (d, J = 4.0 Hz, 1H, H_1), 7.11 (s, 2H, H_{phenol}); ^{13}C NMR (CD_3OD, 100 MHz), δ 56.4, 63.5, 72.8, 73.5, 74,4 75.9, 99.5, 111.2, 122.1, 140.8, 147.3, 168.9. HRMS (ESI): calcd for $C_{14}H_{19}O_{10}$ $(M + H)^+$, 347.0978; found, 347.0973.

*3-Galloyl-α-methyl-O-glucose (**16**):* ^1H NMR (CD_3OD, 400 MHz), δ 3.46 (s, 3H, OCH_3), 3.55−3.75 (m, 4H, $H_{2,5,6}$), 3.86 (dd, J = 2.4 and 12.0 Hz, 1H, H_4), 4.75 (d, J = 3.6 Hz, 1H, H_1), 5.33 (at, 1H, H_3), 7.12 (s, 2H, H_{phenol}); ^{13}C NMR (CD_3OD, 100 MHz), δ 56.6, 62.4, 69.1, 69.9, 72.0, 77,2, 101.3, 110.7, 121.9, 129.9, 132.4, 146.4, 168.5. HRMS (ESI), calcd for $C_{14}H_{19}O_{10}$ $(M + H)^+$, 347.0978; found, 347.0971

Sample Preparation. A sample of 2 g of fresh raspberries, obtained from the local market, was extracted with 20 mL of ethanol/water (70:30) for 18 h at room temperature. The mixture was filtered; the extract was concentrated, rinsed with a solution of ethanol/water (70:30) to a final volume of 2 mL, and directly analyzed by HPLC-DAD-MS. One milligram of each standard, commercial tannic acid, and synthetic compound was separately dissolved in 1 mL of ethanol/water (70:30) and directly analyzed by HPLC-DAD-MS.

RESULTS AND DISCUSSION

The preparation of derivatives **6**, **7**, **12**, and **14**, used in this study is depicted in **Figures 1** and **2**. Whether in the target compounds no distinction was required for the gallic acid's hydroxyl groups, the three OH groups were protected as tribenzyl ether (compound **3**) (*6*). When the *m*-depsidic link or the HHDP unit was mandatory in the final target, an orthogonal double protection, with a silyl ether and a benzylidene ring (i.e., compound **8**), was chosen (*5*). As far as the sugar counterpart was concerned, the methyl and benzyl glycosides **1** and **2** were used as starting materials, respectively (*5*, *7*). The esterification,

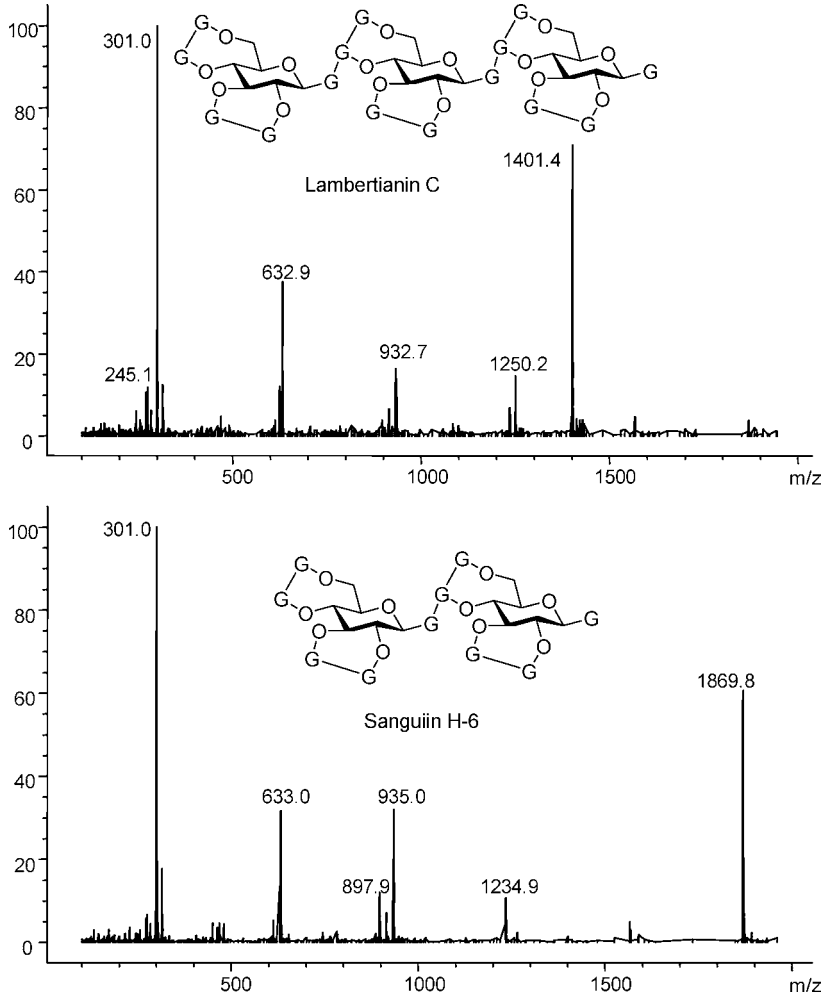

Figure 6. Fragmentation pattern of sanguiin H-6 (MW 1870, $[M - H]^-$) *m*/*z* 1869) and lambertianin C (MW 2804, $[M - 2H[^{-2}$ *m*/*z* 1401).

between the galloyl acids and the glucose moieties, was carried out using DCC in the presence of DMAP and DMAP·HCl, to give the compounds **4** and **5** (*5*). Deprotection by catalytic hydrogenation allowed the isolation of digalloyl derivatives **6** and **7** (**Figure 1**) (*5*). Partial esterification of sugar derivative **1** with galloyl derivative **3** allowed the formation of small quantities of monogalloyl derivatives, which after deprotection furnished the isolation of 2-monogalloyl-*O*-methyl-glucose **15** and 3-monogalloyl-*O*-methyl-glucose **16** (**Figure 3**). The esterification between the galloyl derivative **8** and the glucose derivative **1** furnished the digalloyl compound **9**, the silyl ether protection groups of which were selectively cleaved to give the compound **10**, the key molecule for the synthesis of the HHDP unit C−C and depsidic link (**Figure 2**) (*5*, *8*). The HHDP unit C−C link was established by oxidation with $Pb(OAc)_4$ at low temperature, followed by hydrogenation to give ellagic compound **12** (**Figure 2**) (*8*). The esterification of compound **10** with tribenzyloxy galloyl chloride afforded the depsidic-containing derivative **13**, which after hydrogenation furnished the desired glucogalloyl compound **14** (**Figure 2**) (*6*). The identification and the importance of natural gallotannins with the depsidic link have been known since the early 1900s (*1*, *2*, *9*), but, to the best of our knowledge, this is the first time that a synthetic method for their preparation is reported.

Through the analytical characterization of these synthetic glucogalloyl compounds several important pieces of information were obtained. Upon esterification on the glucose core, the absorption maximum of gallic acid experienced a bathochromic shift of 10−12 nm without a change in the shape of the spectrum. The UV−vis profile of the digalloylglucose was identical with that of the monogalloyl and pentagalloyl derivatives and very similar to that of gallic acid at the same concentration. The presence of one *m*-depsidic link caused an auxochromic effect due to the conjugation of the two galloyl chromophores, and, more significantly, a shoulder at 300 nm that became even more obvious and larger when two *m*-depsidic links were present (**Figure 3**). This characteristic could probably be related to a nonbonding to antibonding transition (n to π^*), which requires less energy with respect to the bonding to antibonding transition (π to π^*) and, in association, with the increase of ester groups in the same chromophore. Additional evidence for the presence of a *m*-depsidic link arose from the analysis of the ^{1}H NMR spectra as such an ester bond causes a convincing deshielding of all of the aromatic protons. For example, in the depsidic compound **14** all galloyl aromatic protons appear between 7.19 and 7.39 ppm, whereas glucogalloyl derivative **7** showed two groups of aromatic protons at 6.99 and 7.04 ppm, respectively.

To date, the identification of this group of compounds has required complex isolation procedures or expensive HPLC-NMR techniques, given that the mass spectrum is not sufficient to provide characterization. This peculiarity of the UV−vis spectrum of glucogalloyl compounds with a depsidic link has never been reported before and could be crucial for the determination and identification of the *m*-depsidic link in extracts of natural tissues by HPLC-DAD-MS on-line analysis. When the derivative **14** was dissolved in a pH 3.2 methanol/water solution, hydrolysis of the *m*-depsidic link occurred to give small

amounts of methyl gallate and glucogalloyl compounds with one depsidic link (e.g., derivative **17**, **Figure 3**), which were identified by HPLC-DAD-MS analysis. Therefore, the extraction by acidic alcohol solutions, or any other kind of acid treatment, should be avoided when the identification and quantification in natural matrix of depsidic links containing gallotannis are required.

Also in this case of glucogalloyl derivatives with the HHDP unit, the ^{1}H NMR spectra can support the attribution of the presence of a C−C link. In fact, the residual protons of the joined aromatic rings experience a clear shielding with respect to corresponding protons in simple galloyl esters. For example, in compound **12** the aromatic protons appear at 6.58 and 6.66 ppm, roughly 0.3 ppm downfield from those of the corresponding simple glucogalloyl compound **7**. Interesting information was also obtained from the MS spectrum of the diphenoyl unit containing compound **12**. **Figure 4** shows the MS spectra of a digalloylglucose with and without the HHDP unit C−C link (**12** and **7**, respectively). In both cases the deprotonated molecular ions are present (*m*/*z* 481 for **12** and *m*/*z* 483 for **7**), but the two compounds show completely different fragmentation patterns. In fact, the mass spectrum of compound **7**, without the C−C link, shows fragments at *m*/*z* 313 (loss of a galloyl moiety) and at *m*/*z* 169 (gallic acid deprotonated ion). These fragments are absent in the case of compound **12**, which shows the fragment ion at *m*/*z* 301, derived from the cleavage of both ester links with a spontaneous lactonization to ellagic acid negative ion.

Studies carried out on HPLC-MS analysis of natural tissue extracts rich in ellagitannin have suggested its formation through the spontaneous lactonization described above (*10*, *11*). However, this finding has never been reported before for synthetic ellagitannin.

To verify the identified characteristics, matrices rich in hydrolyzable tannins, known to contain *m*-depsidic links and HHDP units, were analyzed. As far as the *m*-depsidic link is concerned, tannic acid was used, which is widely utilized in food manufacturing and biological research and is an interesting example to validate this observation (*12*−*15*). Various works identify tannic acid as a single compound, yet different interpretations exist from report to report (*12*−*15*). Even the *Merck Index* refers to carilagin (1,3,6-trigalloylglucose with a HHDP C−C link between the 3- and 6-galloyl groups) under the name of tannic acid (*16*). Commercially available tannic acid samples contain variable amounts of more than 10 compounds. Among these components there are gallic acid, methyl gallate, and mono-, di-, tri-, tetra-, and pentagalloyl glucose, as well as bigger compounds with one, two, three, four, or even more *m*-depsidic links (*17*, *18*). As a result of the HPLC-DAD-MS analysis (**Figure 5**) of tannic acid's different components, the observations arising from the synthetic glucogalloyl derivatives with the *m*-depsidic link were confirmed. Also in the case tannic acid's components, with the increase of the molecular weight, we observed an increase of the shoulder at 300 nm, with respect to the UV−vis spectra of the simple glucogalloyl derivatives, as expected because of the increase in the number of *m*-depsidic links. The *m*/*z* 301 fragment was not detected in any of tannic acid's components, which were analyzed via HPLC-DAD-MS. On the other hand, to confirm the diagnostic fragmentation pattern observed in the synthetic glucogalloyl compounds containing the HHDP unit, we analyzed the extracts deriving from raspberries, which are rich in such hydrolyzable tannins (*10*, *11*). As expected, and in agreement with previously published data (*10*, *11*), the fragmentation pattern of the two major ellagitannins of raspberries, sanguiin H-6 and lambertianin C, confirmed the characteristic fragmentation of the HHDP unit, because in both cases the ion at *m*/*z* 301 represents the more abundant peak of the mass spectra (**Figure 6**).

In conclusion, this work reports the preparation of differently substituted glucogalloyl derivatives, including compounds with the HHDP unit or *m*-depsidic link, and their use to establish a novel analytical protocol for the identification of hydrolyzable tannins in a natural matrix.

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