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Analytical Methods

Isolation and structure elucidation of novel phenolic constituents from *Sorbus domestica* fruits

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ABSTRACT

In the framework of the detailed phytochemical analysis of *Sorbus domestica* fruits at several maturity stages and additively to the phenolic compounds elucidated by LC-DAD-MS (ESI+), ten more, novel phenolic compounds were isolated after preparative work and their structure elucidation was achieved with UV–vis, NMR (^{1}H, ^{13}C, COSY, HSQC, HMBC, NOESY, TOCSY, ROESY), LC-DAD-MS (ESI+) and HR-NanoESI-QqTOF-MS/MS. The novel compounds belong to the categories of hydroxybenzoic acid derivatives (Compounds **1, 2, 3**), polyphenolic phenylpropanoid derivative (Compound **4**), quercetin glycosides (Compounds **5, 6**), flavanol glycoside (Compound **7**), quercetin dimmer (Compound **8**) and biphenyls (Compounds **9, 10**). Their structures were established as: Vannilic acid 4-O-α-L-rhamnoside **(1)**, protocatechuic acid anhydrite **(2)**, trivanilloyl-(1,3,4-trihydroxybenzol) ester **(3),** 3-{4-(bis[4-hydroxy-3-(5-hydroxypentanoyloxy) phenyl) methoxy]-3,5-dihydroxy phenyl} propanoic acid **(4),** quercetin 3-O-β-D-glucopyranosyl(1‴→2″)-α-L-rhamnosyl(1⁗→3‴)-α-L-rhamnosyl(1⁗⁗→3⁗)-α-L-arabinofuranoside, quercetin 3-O-α-L-rhamnosyl(1‴→3″)-β-D-glucopyranoside **(6)**, 5,7,3′,6′-tetrahydroxyflavanol 7-O-β-D-glucopyranoside **(7),** (7-O-4‴, 4′O-7″) quercetin dimmer **(8),** [2,2′-dihydroxy, 4-(propionic acid hexyl ester), 4′-(propionic acid heptyl ester)] biphenyl **(9)** and [2,6,2′,6′-tetrahydroxy, 4,4′-bis-(propionic acid hexyl ester)] biphenyl **(10).**

1. Introduction

Sorbus domestica fruits are traditionally used as an antioxidant agent (Termentzi, Kefalas, & Kokkalou, 2006), as well as a remedy against long term diabetic complications (Termentzi, Alexiou, Demopoulos, & Kokkalou, in press). It is known from oral depositions that the fruit is only consumed as traditional medicine in a well matured, nearly rotted, stage, thus for comparison reasons five different fruit categories were tested for their biological activities, as well as for their phenolic content. Previous detailed LC-DAD-MS (ESI+) showed that generally fruits are rich in flavonols (aglycons, glycosides and esters), mainly quecetin and secondary kaempherol derivatives, hydroxybenzoic acids (vanillic and protocatechuic acid) and hydrocinnamic acids and derivatives, such as chlorogenic acid, that characterize the phenolic content (Termentzi, Kefalas, & Kokkalou, 2008).

Apart from the compounds, whose structure was totally elucidated from the LC-DAD-MS (ESI+) data, nine more phenolic compounds were isolated after preparative scale work and their structures were elucidated also by means of UV–vis, NMR and HR-NanoESI-QqTOF-MS/MS. Furthermore, a quercetin dimmer found at the LC-DAD chromatogram in the crude extract was isolated and its structure was confirmed also by NMR spectrometry. All these compounds are novel and mentioned for the first time in literature. The elucidation of these compounds could not be realized only by the LC-DAD-MS (ESI+) system, either because the data obtained from this system were not enough for their elucidation, since their structures were novel and complicated, or because due to very small quantities they were not detectable at the conditions applied.

2. Materials and methods

2.1. Plant material and extraction procedure

Sorbus domestica fruits were collected from the mountain region of Rodopi (northern Greece) in September 2003 and processed to give five fruit categories: (A) Unripe fruits (yellow colour). (B) Well matured on tree (brown color). (C) Collected unripe and matured for one week in dark, at room temperature. (D) As in C, but prolonged maturation at three weeks (dark brown color), form consumed by the local population. (E) Sterilized pulp from well matured fruits (disposed at local drugstores). All five fruit samples were directly and exhaustively extracted with methanol at soxhlet apparatus and the extracts were evaporated under vacuum to dry-

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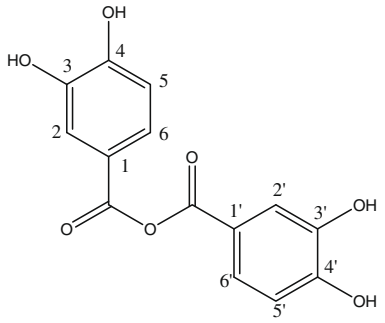
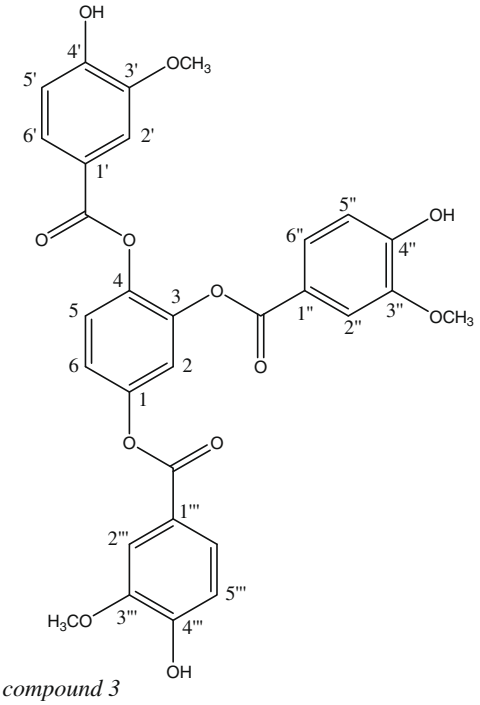
ness. The extracts were then partitioned with solvents of increasing polarity in order to simplify the analysis: dichloromethane, diethyl ether, ethyl acetate, butanol and water (Termentzi et al., 2008).

2.2. Isolation of the compounds

Compounds 1, 2, 3, 4: The diethyl ether fraction of fruits matured for one week at room temperature (dry weight 230 mg) was chromatographed on a normal phase polyamide column 25 × 2.2 cm with elution solvents at a gradient program: Hexane → CH_2Cl_2 → MeOH. The polarity of the elution solvent was gradually increased from 100% hexane to 100% CH_2Cl_2 and continously to 100% MeOH by 5%. Fractions of 20 ml volume were collected.



compound 2



compound 3

Fractions 40–45, eluted at CH_2Cl_2:MeOH ratio 35:65, were merged and after evaporation the dry remaining was chromatographed on a Sephadex LH-20 column (25 × 1 cm) using MeOH as the elution solvent and fractions of 2 ml volume were collected. Fractions 10–14 of this second column were merged, since Cellulose TLC analysis showed the existence of two compounds of deep purple fluorescence. The mixture was evaporated and chromatographed on a prep-HPLC system in order to separate the two compounds. The system involves an HPLC Pump SSI 222C coupled with a Gradient SSI 232C and a Detector UV–vis SSI. Clarity Lite programme was used for the analysis of the chromatograms. A reverse phase preparative column C-18 Macherey-Nagel SP 250/10, 25 × 1 cm, Nucleosil 100-7, 7 μm particle size was used at a flow rate of 4.5 ml/min. Water with 10% AcOH (A) and Methanol (B) were used as solvent for a 140 min program: 0–5 min, 90% A and 10% B; 20 min, 100% B; 30 min, 100% B. The UV detector was set at 260 nm. Compound **1** had Rt 2.90 min and after evaporation the dry weight was 2.5 mg. On the other had compound **3** had a much more lipophilic behavior and was an intense peek at Rt = 22.00 min.

Fractions 144–175 of the initial polyamide column, eluted at CH_2Cl_2:MeOH ratio 5:95, were merged and chromatographed on a Sephadex LH-20 column (25 × 1 cm) using MeOH as the elution solvent and fractions of 2 ml volume were collected. After chromatographic control on TLC cellulose plates, fractions 1–4 were merged. After evaporation of the solvent, the exact weight of the compound **4** was 4.5 mg. The substance was difficult to dissolve in MeOH and DMSO, while it was resolvable in $CHCl_3$. Fractions 9–12 of the second column were also merged and TLC chromatogram showed that the mixture contained only one compound. The final dry weight of compound **2** was 3.5 mg.

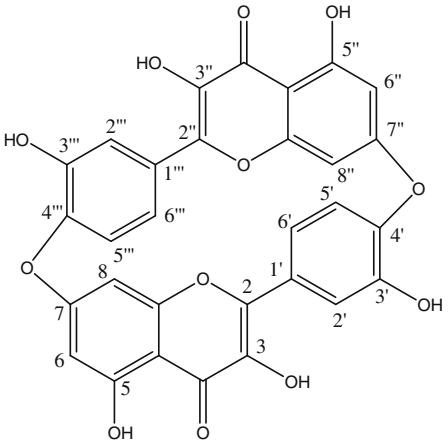
Compounds 5, 7: The *n*-butanolic fraction of the unripe fruit category was chromatographed on a MPLC polyamide phase column, 27 × 2.7 cm with gradient elution with H_2O → MeOH. The polarity of the elution solvent was gradually decreased from 100% H_2O to 100% MeOH by 5%. 50 ml volume fractions were collected.

Fractions 11–14, eluted at H_2O:MeOH ratio 90:10, were merged and chromatographed on a second Sephadex LH-20 column (20 × 1 cm) with MeOH as elution solvent and fractions of 2 ml volume were collected. Fractions 13–17 were merged, and after evaporation compound 7 was collected as yellow powder, 1.2 mg.

Additionally fractions 272–323 from the first polyamide column, eluted at H_2O:MeOH ratio 45:55, were merged and were rechromatographed on a Sephadex LH-20 column (20 × 1 cm) with MeOH and fractions of 2 ml volume were collected. Fractions 5–15 from the sephadex column were merged and then chromatographed again on the prep-HPLC with the conditions described for compounds **1, 3.** The UV detector was set at 350 nm. Compound **5** was collected at Rt.3.00 min and had 3.6 mg dry weight.

Compound 6: The dry *n*-butanolic extract of the fruit pulp was chromatographed on a Sephadex LH-20 column 40 × 2 cm with MeOH. Fractions of 3 ml volume were collected and fractions 25–27 were recromatographed on a second Sephadex LH-20 column 50 × 1 cm with MeOH. Fractions of 2 ml volume were collected and finally 2.5 mg of compound **6** were isolated from fractions 6–7 as a yellow powder.

Compound 8: The diethyl ether fraction of fruits well matured at room temperature was chromatographed on a polyamide column 25 × 2.2 cm with a gradient elution program: Hexane → CH_2Cl_2 → MeOH; The polarity of the elution solvent was gradually increased from 100% hexane to 100% CH_2Cl_2 and continously to 100% MeOH by 5%; fractions of 20 ml volume were collected. Fractions 325–346, eluted at CH_2Cl_2:MeOH ratio 5:95, were merged and chromatographed on a Sephadex LH-20 column 25 × 1 cm with MeOH. Fractions of 2 ml volume were collected. Fractions 3–7 were collected and chromatographed again on the prep-HPLC system with the conditions described for compounds **1, 3.** Compound **8** was isolated at Rt = 22.18 min as yellow powder of 2.9 mg dry weight.



compound 8

Compounds 9, 10: The two novel biphenyls were isolated only from fruits well matured on tree (B). The isolation was realized by 60 sequential injections of 5 mg dry weight of the ethyl acetate fraction diluted in 1 ml of methanol at the prep-HPLC apparatus, which was described before. A reverse phase preparative column C-18 Macherey-Nagel SP 250/10, 25 × 1 cm, Nucleosil 100-7, 7 μm particle size was used at a flow rate of 4.5 ml/min. Water with 10% AcOH (A) and Methanol (B) were used as solvent for a 140 min program: 0–10 min, 90% A and 10% B; 100 min, 100% B; 140 min, 100% B. The UV detector was set at 280 nm. Substance **9** was collected at 110 min and after evaporation was obtained as white needles, dry weight 1.3 mg. Substance **10** was eluted as an intense peak at 115 min and also obtained after evaporation as white needles, dry weight 5.2 mg.

2.3. Acidic hydrolysis and GC analysis

For the acidic hydrolysis of the flavonoid glycosides 1 mg of each compound were dissolved in 5 ml MeOH 3% HCl in a 25 ml volume flask and the dilution is heated in a waterbath for 60 min. The solvent is then evaporated until dryness and re-dissolved in a minimum volume of H_2O. The samples were then filtered for the removal of the insoluble particles. The filtrate was then evaporated and until dryness with nitrogen gas. The sugars were then converted to their trimethylsilyl derivatives using BSTFA containing 1% TMCS (20–25 μL) and pyridine (10 μL) for 3 h at 70 °C. Immediately before GC analysis, derivatized extracts were evaporated to dryness using nitrogen gas and redissolved in 20–25 μL of hexane for the injection.

The GC analysis of the samples, as well as the standard sugars was realized on a Shimadzu GC-2010 gas chromatographer according to Medeiros & Simoneit (2007). A capillary column DB5-MS 30 m × 0.25 mm, film thickness of 0.25 μm, carrier gas helium, flow rate 1.3 mL/min. Column temperature program: injection at 65 °C, hold for 2 min, temperature increase of 6 °C/min to 300 °C, isothermal hold at 300 °C for 15 min.

2.4. UV spectra

UV spectra were recorded in methanol using a Hitachi U-2000 spectrometer.

2.5. Nano-ESI-QqTOF MS–MS analysis

Full scan and MS/MS experiments for compounds **4, 9** and **10** were performed using an orthogonal geometry hybrid quadrupole-TOF mass spectrometer retrofitted with a nanoelectrospray source (nano-ESI QqTOF MS/MS, QSTAR XL, Applied Biosystems, Toronto, Canada) in positive ion mode. A volume of 100 μL was aliquoted from each sample and then mixed with 50 μL of 60% ACN and 40% water containing 0.5% formic acid. The samples were syringe infused with the syringe pump retrofitted onto the nano-electrospray source (Applied Biosystems – MDS Sciex) and connected to a 8 μm tip × 360 μm OD × 75 μm ID PicoTip™ nano-electrospray emitter with coating 1P-4P (New Objective, Dingoes, NJ). An in-line MicroFilter™ (Upchurch Scientific, Oak Harbor, WA) was connected between the syringe pump outlet tubing and the emitter in order to minimize exposure of particulate matter originating from the samples. The infusion flow rate was at 50 nL/min in order maximize ionization efficiency given that no heating was used for the nano-electrospray process. A mass window of 0.3 Da was used for the precursor ion when generating the MS–MS spectra. The MS–MS signal accumulation time was dependent on the S/N ratio of the weakest product ion set at 50:1 and the signal intensity of the strongest product ion not to exceed 1000 cts so as to not cause signal saturation effects. For both substances, full spectral MS experiments were conducted. Additionally, product ion MS–MS experiments of the reaction intermediates and products were performed with collision energy (CE) generally chosen to maintain about 10% of the precursor ion.

2.6. LC-DAD-MS (ESI+)

MS spectra for compounds **1, 5, 6, 7, 8** were recorded on LC-DAD-MS (ESI+) apparatus. The system includes a Finnigan MAT Spectra System P4000pump coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. The analysis was performed on a 125 × 2 mm Superspher 100-4 RP-18 column (Macherey-Nagel, 4 lm particle size) at a flow rate of 0.33 ml/min. The detection was monitored at 290, 340 and 365 nm. The MS–ESI(+) spectroscopy at a probe temperature of 450 °C, probe voltage of 4.9 kV and at 12, 20 and 80 eV in the mass analyzer. The following gradient program was used for all samples: (A) AcOH (2%) and (B) MeOH, 90% A for 2 min, 0% A at 37 min, 0% A at 42 min, 90% A at 45 min and 90% A at 50 min. The data were processed using the Xcalibur 1.2 software.

2.7. NMR spectra

One and two dimensional NMR spectra were recorded on BRUKER AVANCE II 600 MHz and VARIAN 300 MHz spectrometer. MeOD-d_4, DMSO-d_6 and $CDCl_3$ were used as solvents. Experimental data were processed using MestRe-C s/w.

Compound 1: *Vannilic acid 4-O-α-L-rhamnoside.* White powder (MeOH); UV (MeOH) λ_{max} nm: 258, 294; LC-DAD-MS (ESI+), 12 eV: Rt = 2.18 min, *m/z* (Rel. Int.): 315 $[M+H]^+$ (20), 151 [M-rhamnose+H]$^+$ (100), 109 [M-(rhamnose-H_2O)-COOH-CH_3+H]$^+$ (85). ^{1}H NMR (300 MHz, MeOD-*d4*): δ (ppm): 7.41 (1H, d, J = 1.5 Hz, H-2), 7.40 (1H, dd, J = 8.1 and 1.5 Hz, H-6), 6.77 (1H, d, J = 8.1 Hz, H-5), 4.82 (1H, bs, H-1′), 3.87 (1H, dd, J = 9.3 and 2.5 Hz, H-3′), 3.82 (3H, s, H–OCH_3), 3.20-3.60 (3H, m, H-2′,4′,5′), 0.88 (3H, d, J = 6.9, H–Me); 13C NMR (75 MHz, MeOD-*d4*) δ (ppm): 170.1 (C–COOH), 149.0 (C-4), 145.2 (C-3), 124.6 (C-6), 123.9 (C-1), 116.3 (C-2), 114.0 (C-5), 99.8 (C-1′), 75.5 (C-3′), 74.2 (C-2′), 73.8 (C-4′), 67.1 (C-5′), 18.5 (C-6′).

Compound 2: *Protocateuic acid anhydrite.* White powder (MeOH); UV (MeOH) λ_{max} nm: 258, 294; ^{1}H NMR (600 MHz, MeOD-*d4*) δ (ppm): 7.44 (2H, d, J = 1.5 Hz, H-2, H-2′), 7.40 (2H, dd, J = 8.2 and 1.5 Hz, H-6, H-6′), 6.76 (2H, d, J = 8.2 Hz, H-5, H-5′); ^{13}C NMR (150 MHz, MeOD-*d4*) δ (ppm): 169.8 (C–COOR),

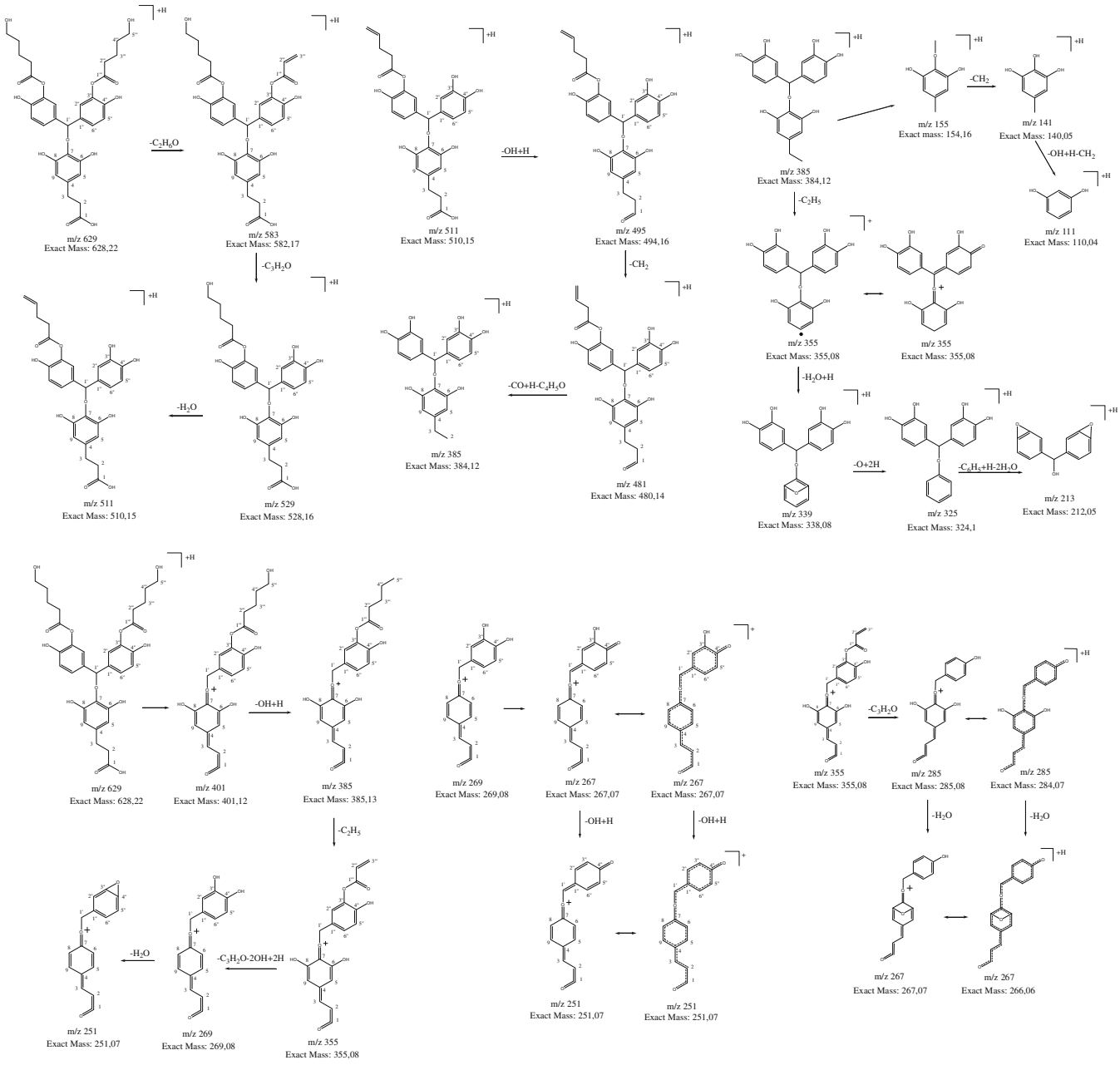


Fig. 1. MS-MS fragmentation mechanism of 3-{4-(bis[4-hydroxy-3-(5-hydroxypentanoyl- oxy)phenyl)methoxy]-3,5-dihydroxyphenyl}propanoic acid (**Compound 4**). Parent and daughter ions.

169.6 (C–COOR′), 148.8 (C-4, C-4′), 144.1 (C-3, C-3′), 124.2 (C-6, C-6′), 121.9 (C-1, C-1′), 116.2 (C-2, C-2′), 114.2 (C-5, C-5′).

Compound 3: *Trivanilloyl-(1,3,4-trihydroxybenzol) ester*. White powder (MeOH); UV (MeOH) λ_{max} nm: 258, 294; ^{1}H NMR (600 MHz, MeOD-*d4*): δ (ppm): 7.41 (3H, d, *J*=1.8 Hz, H-2′, H-2″, H-2‴), 7.40 (3H, dd, *J* = 8.2 and 1.8 Hz, H-6′, H-6″, H-6‴), 7.32 (1H, dd, *J* = 1.5 and 8.1 Hz, H-6), 7.22 (1H, d, *J* = 1.5, H-2), 6.79 (3H, d, *J* = 8.2 Hz, H-5′, H-5″, H-5‴), 6.58 (1H, d, *J* = 8.1, H-5), 3.82 (6H, s, H–OCH$_3$); ^{13}C NMR (150 MHz, MeOD-*d4*) δ (ppm): 166.2 (COO‴), 165.8 (COO′ and COO″), 148.9 (C-4‴), 148.8 (C-4′, C-4″), 146.1 (C-1), 145.9 (C-3‴), 145.1 (C-3′, C-3″), 140.2 (C-4), 138.8 (C-3), 125.1 (C-6‴), 124.6 (C-6′, C-6″), 124.2 (C-1‴), 123.9 (C-1′, C-1″), 118.0 (C-6), 117.8 (C-5), 116.8 (C-2‴), 116.1 (C-2′, C-2″), 114.1 (C-5‴), 113.9 (C-5′, C-5″), 113.6 (C-2).

Compound 4: 3-*{4-(bis[4-hydroxy-3-(5-hydroxypentanoyloxy) phenyl)methoxy]-3,5-dihydroxy phenyl} propanoic acid*: White powder (CHCl$_3$); UV (MeOH) λ_{max} nm: 280, 320sh; HR-NanoESI-QqTOF-MS/MS (positive mode) *m*/*z* (Rel. Int.): 629.2221 [M+H]$^+$ (25) (calc. for 629.2251), Main fragments: see Fig. 1; ^{1}H NMR (300 MHz, CDCl$_3$) δ (ppm): 7.53 (2H, d, *J* = 8.7 Hz, H-5″), 7.35 (2H, d, *J* = 2.7 Hz, H-2″), 7.13 (2H, dd, *J* = 8.7 and 2.7 Hz, H-6″), 6.97 (2H, s, H-5 and H-9), 5.06 (1H, bs, H-1′), 4.08 (4H, m, H-5‴), 2.84 (2H, t, *J* = 8.1, H-3), 2.60 (2H, t, *J* = 8.1, H-2), 2.31 (4H, m, H-2‴), 1.62 (4H, m, H-3‴), 1.59 (4H, m, H-4‴); ^{13}C NMR (75 MHz, CDCl$_3$) δ (ppm): 173.9 (C-1‴), 173 (C-1), 152.3 (C-6, C-8), 147.6 (C-4″), 147.2 (C-3″), 138.7 (C-1″), 130.3 (C-4), 124.8 (C-5, C-9), 124.6 (C-2″), 124.1 (C-6″), 118.2 (C-5″), 86.7 (C-1′), 62.4 (C-5‴), 36.3 (C-2), 34.3 (C-2‴), 30.8 (C-4), 27.5 (C-4‴), 25.0 (C-3‴).

Compound 5: *Quercetin 3-O-β-D-glucopyranosyl (1‴→2″)-α-L-rhamnosyl(1⁗→3‴)-α-L-rhamnosyl(1⁗′→3⁗)-α-L-arabinofuranoside*. UV (MeOH) λ_{max} nm: 256, 265sh, 300sh, 356; LC-DAD-MS (ESI+), Rt.4.87 min, 12 eV, *m*/*z* (Rel. Int.): 998 [M+H]$^+$ (100), 757

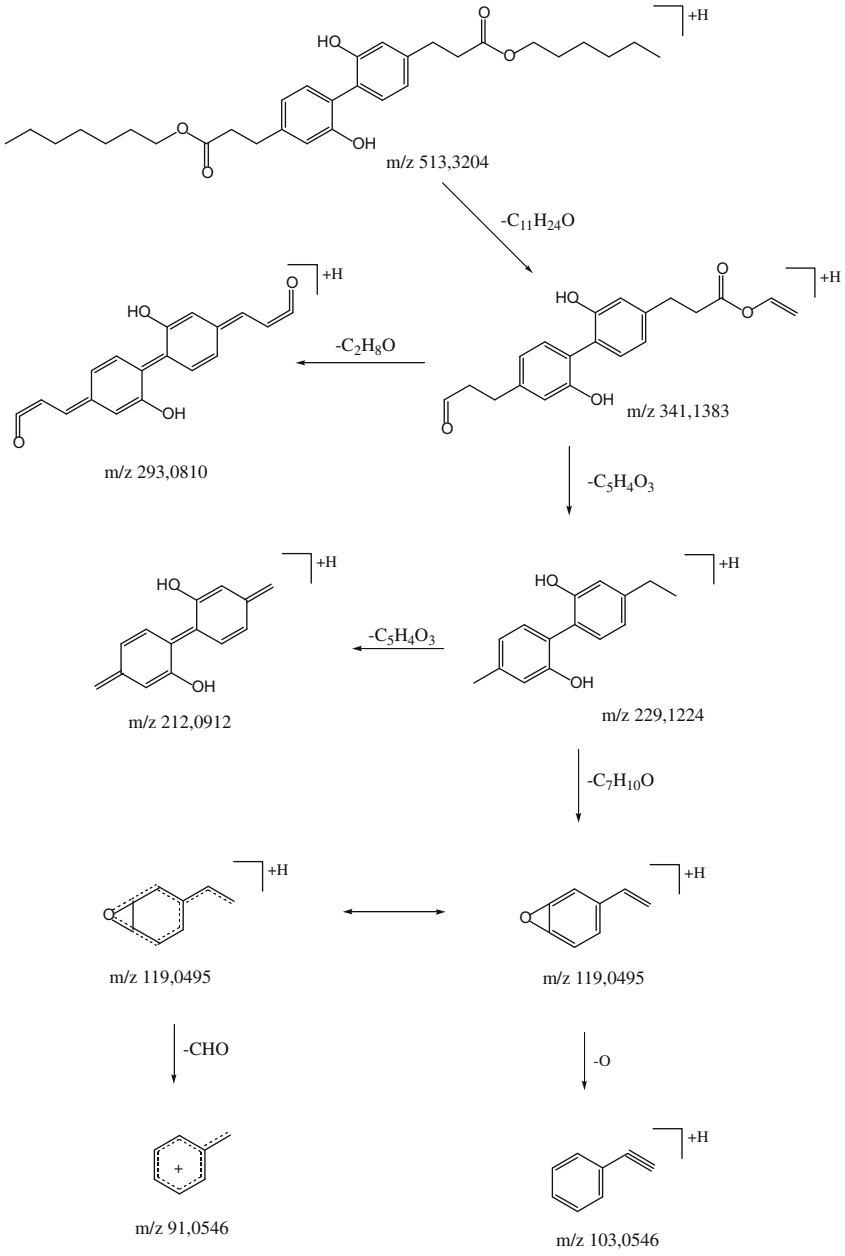


Fig. 2. MS/MS fragmentation mechanism of [2,2′-dihydroxy, 4-(propionic acid hexyl ester), 4′-(propionic acid heptyl ester)] biphenyl (**Compound 9**).

[M-(arabinose-H_2O)+H]$^+$ (15), 611 [756-(rhamnose-H_2O)+H]$^+$ (18), 465 [610-(rhamnose-H_2O)+H]$^+$ (13), 303 [464-(glucose-H_2O)+H]$^+$ or [A+H]$^+$ (17); ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 7.81 (1H, dd, J = 2.1 and 8.4 Hz, H-6′), 7.49 (1H, d, J = 2.1 Hz, H-2′), 6.75 (1H, d, J = 8.4 Hz, H-5′), 5.89 (1H, d, J = 2.4 Hz, H-8), 5.74 (1H, d, J = 2.4 Hz H-6), 5.70 (1H, d, J = 7 Hz, H-1″), 4.91 (1H, bs, H-1‴), 4.55 (1H, bs, H-1⁗), 4.24 (1H, bs, H-1⁗′), 3.84 (1H, m, H-3″), 3.82 (2H, m, H-3⁗′, H-4⁗′), 3.74 (1H, m, H-2″), 3.71 (1H, m, H-6a″), 3.68 (1H, bs, H-2⁗′), 3.65 (3H, m, H-4″, H-2‴, H-2⁗′), 3.52 (1H, m, H-5″), 3.49 (1H, m, H-6b″), 3.41 (2H, bs, H-5⁗′), 3.38 (2H, m, H-5‴, H-5⁗′), 3.25 (4H, m, H-3‴, H-4‴, H-3⁗′, H4⁗′), 1.06 (3H, d, J = 6Hz, H-6‴), 1.05 (3H, d, J = 6Hz, H-6⁗′); 13C NMR (150 MHz, DMSO-d_6) δ (ppm): 178.9 (C-4), 165.5 (C-7), 162.3 (C-5), 156.7 (C-9), 156.0 (C-2), 133.9 (C-3), 104.1 (C-10), 102.5 (C-1″), 99.8 (C-1‴), 99.6 (C-1⁗′), 99.0 (C-6), 93.5 (C-8), 81.1 (C-2″), 80.2 (C-3‴), 80.0 (C-3⁗′), 78.4 (C-3⁗′), 77.5 (C-3″), 76.4 (C-5″), 73.4 (C-4⁗′), 72.2 (C-2‴), 72.0 (C-2⁗′), 70.8 (C-4″), 70.6 (C-4‴), 70.5 (C-4⁗′), 70.1 (C-2⁗′), 68.9 (C-5‴), 68.9 (C-5⁗′), 65.6 (C-5⁗′), 61.1 (C-6″), 17.6 (C-6‴), 17.3 (C-6⁗′).

Compound 6: *Quercetin 3-O-α-L-rhamnosyl(1‴→3″)-β-D-glucopyranoside*. UV (MeOH) λ_{max} nm: 256, 265sh, 300sh, 356; LC-DAD-MS (ESI+), Rt.8.06 min, 20 & 80 eV, *m/z*: 611 [M+H]$^+$, 633 [M+Nα]$^+$, 449 [M-(glucose-H_2O)+H]$^+$, 303 [M-(glucose-H_2O)-(rhamnose-H_2O)+H]$^+$;^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 7.55 (1H, dd, J = 2.1 and 8.4 Hz, H-6′), 7.47 (1H, d, J = 2.1 Hz, H-2′), 6.74 (1H, d, J = 8.4 Hz, H-5′), 6.35 (1H, d, J = 2.1 Hz, H-8), 6.15 (1H, d, J = 2.1 Hz H-6), 5.19 (1H, bs, H-1″), 4.40 (1H, d, J = 8.0, H-1‴), 3.91 (1H, m, H-2″), 3.75 (1H, m, H-3‴), 3.73 (1H, m, H-3″), 3.63 (1H, m, H-2‴), 3.59 (2H, m, H-4‴, H-6a‴), 3.57 (2H, m, H-5‴, H-6b‴), 3.32 (1H, m, H-4″), 3.25 (1H, m, H-5″), 1.01 (3H, d, J = 6.0, H-Me″); 13C NMR (150 MHz, DMSO-d_6) δ (ppm): 178.6 (C-4), 165.3 (C-7), 162.0 (C-5), 156.7 (C-9), 156.5 (C-2), 133.7 (C-3), 104.1 (C-10), 99.1 (C-6), 93.8 (C-8), 103.8 (C-1‴), 100.9 (C-1″), 80.2 (C-3″), 76.7 (C-3‴), 76.2

(C-5‴), 74.5 (C-2‴), 70.6 (C-4″), 69.4 (C-4‴), 69.1 (C-2″), 69.0 (C-5″), 60.8 (C-6‴), 17.8 (C-6″).

Compound 7: *5,7,3′,6′-tetrahydroxyflavanol 7-O-β-D-glucopyranoside*. UV (MeOH) λ_{max} nm: 294, 335sh; HR-NanoESI-QqTOF-MS/MS (positive mode) m/z: 467.1195 $[M+H]^+$ (calc. for 467.1183); LC-DAD-MS (ESI+), Rt.8.06 min, 20 & 80 eV, *m/z* (Rel. Int.): 467 $[M+H]^+$, 489 $[M+N\alpha]^+$, 305 $[A+H]^+$, 451 $[M-OH+H]^+$, 473 $[450+N\alpha]^+$, 289 $[M-OH-(glucose-H_2O)+H]^+$, 235 $[A-C_3H_2O_2+H]^+$, 289 $[A-O+H]^+$, 271 $[A-O-H_2O+H]^+$, 162 $[270-C_6H_6O_2+2H]^+$, 243 $[270-CO+H]^+$, 165 (RDA); ^{1}H NMR (600 MHz, MeOD-d_4) δ (ppm): 6.95 (1H, d, J = 1.8 Hz, H-6′), 6.83 (1H, dd, J = 1.8 and 8.4 Hz, H-4′), 6.78 (1H, d, J = 8.4 Hz, H-3′), 6.21 (1H, d, J = 2.4 Hz, H-8), 6.19 (1H, d, J = 2.4 Hz H-6), 4.95 (1H, d, J = 7.2 Hz H-1″), 4.95 (1H, d, J = 11.4, H-2), 4.55 (1H, d, J = 11.4, H-3), 3.64 (1H, m, H-3″) 3.43 (1H, d, J = 7.2 Hz H-2″), 3.24-3.89 (4H, m, H-4″,5″,6″); ^{13}C NMR (150 MHz, MeOD-d_4) δ (ppm): 198.5 (C-4), 166.8 (C-7), 164.7 (C-5), 163.8 (C-9), 146.1 (C-5′), 145.5 (C-2′), 102.1 (C-1″), 101.6 (C-10), 130.2 (C-1′), 116.3 (C-6′), 114.7 (C-4′), 114.6 (C-3′), 97.3 (C-8), 96.2 (C-6), 86.8 (C-2), 77.6 (C-5″), 76.5 (C-3″), 74.7 (C-2″), 74.5 (C-3), 69.4 (C-4″), 61.0 (C-6″).

Compound 8: *(7-O-4‴, 4′O-7″) quercetin dimmer*. UV–vis (MeOH) λ_{max} nm: 252, 354; LC-DAD-MS (ESI+), Rt.15.30 min, 20 & 80 eV, *m/z*: 569 $[M+H]^+$, 591 $[M+N\alpha]^+$, 285 $[Quercetin-OH]^+$, 307 $[285+Na]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 7.59 (2H, d, J = 2.1 Hz, H-2′, H-2‴), 7.45 (2H, dd, J = 2.1 and 8.4 Hz, H-6′, H-6‴), 6.75 (2H, d, J = 8.4 Hz, H-5′, H-5‴), 6.58 (2H, d, J = 1.2 Hz, H-8, H-8″), 6.39 (2H, d, J = 1.2 Hz H-6, H-6″); ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 180.9 (C-4, C-4″), 175.2 (C-7, C-7″), 162.2 (C-5, C-5″), 155.8 (C-9, C-9″), 146.0 (C-3′, C-3‴), 144.8 (C-4′, C-4‴), 144.6 (C-2, C-2″), 137.9 (C-3, C-3″), 122.1 (C-6′, C-6‴), 120.7 (C-1′, C-1‴), 119.4 (C-5′, C-5‴), 117.9 (C-2′, C-2‴), 109.9 (C-10, C-10″), 95.3 (C-6, C-6″), 90.1 (C-8, C-8″).

Compound 9: *[2,2′-dihydroxy, 4-(propionic acid hexyl ester), 4′-(propionic acid heptyl ester)] biphenyl*. White powder (MeOH); UV (MeOH) λ_{max} nm: 278, 282sh; HR-NanoESI-QqTOF-MS/MS (positive mode) *m/z* (Rel. Int.): 513.3204 $[M+H]^+$ (25) (calc. for 513.3244), 469.2580 $[M-C_3H_8+H]^+$ (34), 457.2580 $[M-4CH_2+H]^+$ (55), 439.2475 $[M-4CH_2-H_2O+H]^+$ (44), 341.1383 $[M-C_{11}H_{24}O+H]^+$ (23), 323.1278 $[M-C_{11}H_{24}O-H_2O+H]^+$ (48), 293.0810 $[(M-C_{11}H_{24}O+H)-C_2H_8O]^+$ (69), 229.1224 $[(M-C_{11}H_{24}O+H)-C_5H_4O_3]^+$

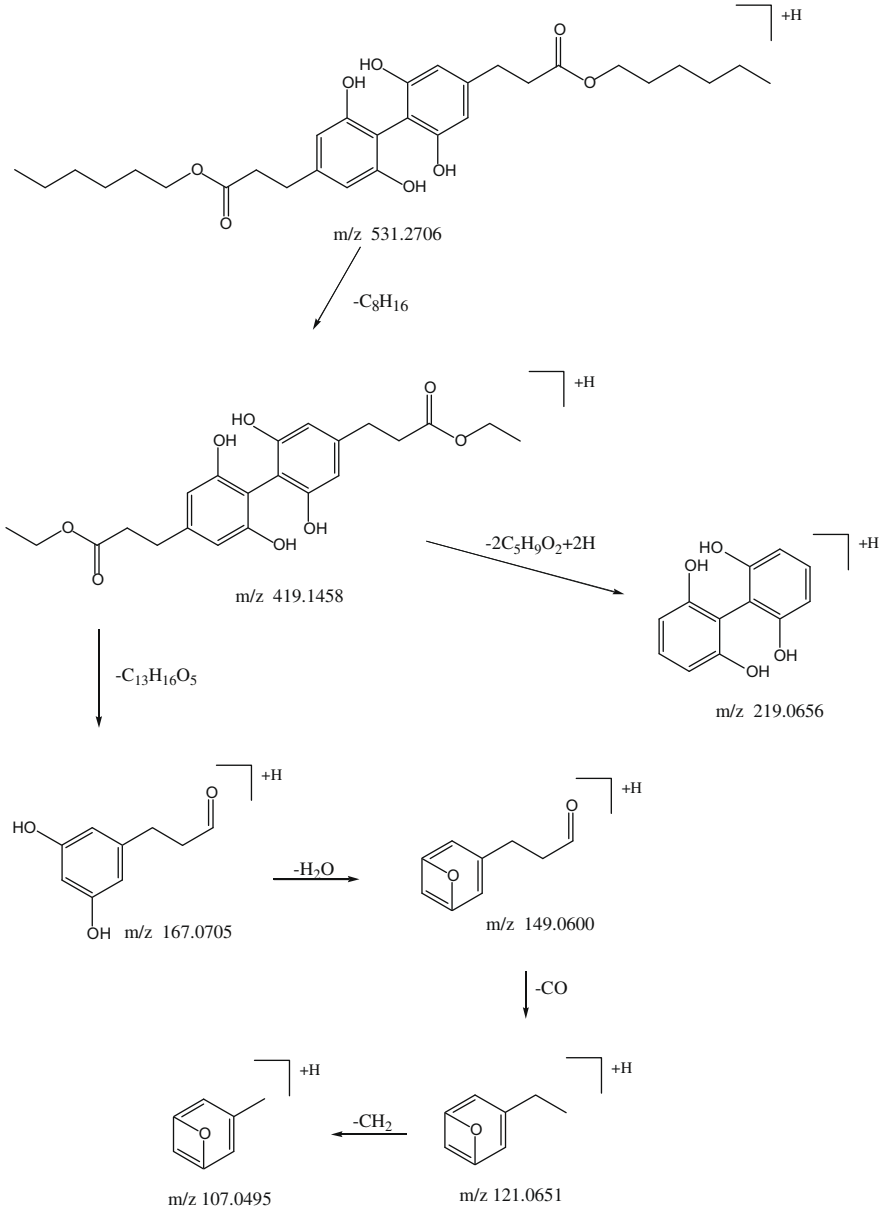


Fig. 3. MS/MS fragmentation mechanism of [2,6,2′,6′-tetrahydroxy, 4,4′-bis-(propionic acid hexyl ester)] biphenyl (**Compound 10**).

(76) (Fig. 2); ^{1}H NMR (600 MHz, MeOD-d_4) δ (ppm): 7.00 (2H, d, J = 2.0 Hz, H-3), 6.82 (2H, dd, J = 2.0 and 8.0 Hz, H-5), 6.62 (2H, d, J = 8 Hz, H-6), 4.03 (4H, t, J = 6.8 Hz, H-10), 2.80 (4H, t, J = 7.4 Hz, H-7), 2.56 (4H, t, J = 7.4 Hz, H-8), 1.59 (4H, t, J = 6.8 Hz, H-11), 1.33 (4H, m, H-12), 1.30 (4H, m, H-13), 1.27 (4H, m, H-14), 1.27 (2H, m, H-15′), 0.89 (6H, t, J = 7.0 Hz, H-15, H-16′); ^{13}C NMR (150 MHz, MeOD-d_4) δ (ppm): 173.8 (C-9), 154.3 (C-2), 135.4 (C-4), 130.4 (C-1), 126.1 (C-3), 125.9 (C-5), 115.6 (C-6), 64.2 (C-10), 36.1 (C-8), 31.4 (C-7), 29.4 (C-12), 29.2 (C-13), 28.9 (C-11), 25.6 (C-14), 22.4 (C-15′), 13.1 (C-15, C-16′).

Compound 10: *[2,6,2′,6′-tetrahydroxy, 4,4′-bis-(propionic acid hexyl ester)] biphenyl*. White powder (MeOH); UV (MeOH) λ_{max} nm: 274; HR-NanoESI-QqTOF-MS/MS (positive mode) m/z (Rel. Int.): 531.2706 $[M+H]^+$ (10) (calc. for 531.2734), 475.2082 $[M-C_4H_8+H]^+$ (20), 419.1458 $[M-C_8H_{16}+H]^+$ (85), 219.0656 $[(M-C_8H_{16}+H)-2C_5H_9O_2+2H]$ (75) (Fig. 3); ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 6.95 (4H, s, H-3, H-5), 6.71 (H–OH), 4.02 (4H, t, J = 6.0 Hz, H-10), 2.81 (4H, t, J = 7.5 Hz, H-7), 2.55 (4H, t, J = 7.5 Hz, H-8), 1.56 (4H, t, J = 6.0 Hz, H-11), 1.30 (4H, m, H-13), 1.27 (4H, m, H-12), 1.25 (4H, m, H-14), 0.89 (6H, t, J = 6.6 Hz, H-15); ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 173.3 (C-7), 152.7 (C-2, C-6), 131.8 (C-4), 124.8 (C-3, C-5), 109.8 (C-1), 64.5 (C-10), 36.3 (C-8), 32.0 (C-7), 29.5 (C-11, C-12), 26.0 (C-13), 22.8 (C-14), 14.6 (C-15).

3. Results and discussion

3.1. Phenolic acids

Compound 1: *Vannilic acid 4-O-α-L-rhamnoside*. The UV–vis spectrum showed λ_{max} at 258 and 294 nm, which is characteristic of vanillic acid (Waldron, Parr, Ng, & Ralph, 1996). The aromatic region of the ^{1}H NMR spectrum displayed an ABX system for H-2, H-5 and H-6 at δ_H 7.41 (d, J = 1.5 Hz), δ_H 6.77 (d, J = 8.1 Hz) and δ_H 7.40 (dd, J = 8.1, 1.5 Hz) respectively. one H-^{1}H COSY spectrum affirms those correlations. The methoxyl protons are a singlet at δ_H 3.82. After acid hydrolysis, the sugar was identified as L-rhamnose, while the broad singlet displayed by the anomeric proton was indicative of an α-configuration (δ_H 4.82). HMBC spectrum affirms the glycosylation position, since there is correlation signal between rhamnose H-1′ and C-4 of the vanilloyl moiety (δ_C 145.66). Additively the upfield resonance position of the carboxylic carbon compared to esters, confirms that the carboxylic group is not esterified (Baderscheider & Winterhalter, 2001; Conrad et al., 2001; Braham, Mighri, Ben Jannet, Matthew, & Abreu, 2005). Rhamnosyl H-3′ is a dd at δ_H 3.87 (J = 9.3 & J = 2.5) (Markham, Geiger, & Jaggy, 1992). The rest of the sugar protons are resonated at chemical shifts δ_H 3.20–3.60 ppm. Finally, the rhamnose methyl protons are a characteristic doublet at δ_H 0.88 ($J_{(5'/6')}$ = 6.9). The MS spectrum of the compound confirmed the structure of the compound and was recorded at 12 eV at the LC-DAD-MS (ESI+) system. The retention time was Rt 2.18 min, indicating glycosylation, compared to the aglycon vanillic acid (Rt 2.34 min). Molecular ion is $[M+H]^+$ = 315 and the base peak at 151amu corresponds to the vanilloyl moiety, after the rhamnose detachment. Peak at 109 corresponds to [vanillic acid–COOH–CH_3] (Maffei Facino, Carini, Aldini, & De Angelis, 1997).

Compound 2: *Protocateuic acid anhydrite*. The UV–vis spectrum is typical of a *para*-, *meta*-bisubstituted hydroxylbenzoic acid with λ_{max} at 258 and 294 nm. The aromatic region of the ^{1}H NMR spectrum displayed two protocatechuic resonance patterns (two ABX systems) with slight differentiations between the respective chemical shifts. HSQC and HMBC confirm that each signal corresponds to two protons with slight chemical shift differentiations, while the respective carbons seem to resonance at the same chemical shifts. The exception is the carboxylic carbons that slightly differentiate. This is also affirmed from the ^{13}C spectrum. The connection of the two protocatehuic moieties is realized by the detachment of one water moiety from the two carboxyl groups. This conclusion arises after comparison with the simple protocatechuic NMR spectra. Thus, compared to the protocatechuic acid carbons′ chemical shifts, C-1 resonates more upfield, while the hydroxylated carbons do not show important differences. Furthermore, the carboxyl carbons also resonate more upfield. These differences, in comparison to previous references result to the conclusion that the compound is a protocatehuic acid anhydrite (Dini, Tenore, & Dini, 2004).

Compound 3: *Trivanilloyl-(1,3,4-trihydroxybenzol) ester*. The UV–vis absorbance is typical of meta-, para- bisubstituted hydroxybenzoic acids, as mentioned for the previous compounds (Waldron et al., 1996). However, the compound seems to have a more lipophilic behaviour compared to a protocatechuic or vanillic acid, since it was isolated from a reverse phase prep-HPLC programme at MeOH as an elution solvent. ^{1}H NMR spectrum confirmed this assumption displaying three main peaks, each one integrated for three protons, and three secondary peaks, each one integrated for one proton. The three main peaks are representative of an ABX system of the vanillic acid. The aromatic methoxyl protons are a singlet at 3.82 ppm. The ^{1}H NMR spectrum pattern confirms the fact that the compound is a polyphenolic vanillic acid derivative. The integration ratio between the vanilloyl protons and the aromatic protons that correspond to the lower peaks at the ^{1}H NMR spectrum is 1:3. The peaks of the lower intensity correspond to the aromatic protons of 1,3,4-trihydroxy benzene: H-2 [δ_H 7.22 (bs)], H-5 [δ_H 6.58 (d), J = 8.1], H-6 [δ_H 7.32 (d), J = 8.1]. 1H-^{1}H COSY confirms the coupling between H-5 and H-6. HMBC spectrum shows correlation signals between H-2 and the carboxyl

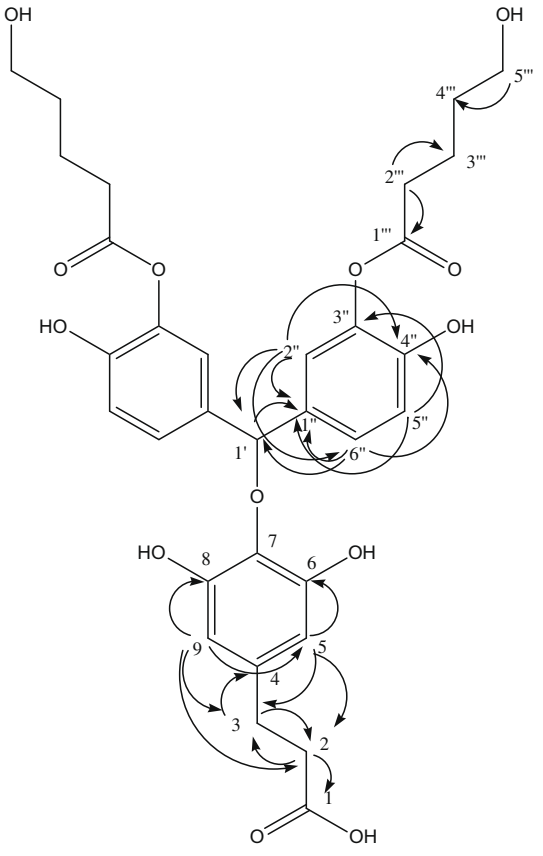


Fig. 4. HMBC correlation signals for 3-{4-(bis[4-hydroxy-3-(5-hydroxypentanoyloxy) phenyl)methoxy]-3,5-dihydroxyphenyl}propanoic acid (**Compound 4**).

carbon COO′ (δ_C 165.8), H-5 and the carboxyl carbon COO‴ (δ_C 166.2) and H-2 and the carboxyl carbons COO″ and COO‴ (δ_C 165.8 and 166.2 respectively). That confirms that the three vanillic acid etherify the three aromatic hydroxyls of 1,3,4-trihydroxy benzene.

Compound 4: *3-{4-(bis[4-hydroxy-3-(5-hydroxypentanoyloxy)phenyl)methoxy]-3,5-dihydroxyphenyl}propanoic acid*. The structure elucidation was realized by comparison of the chromatographic data obtained from NMR and HR/MS-MS spectra. All NMR experiments were carried out with $CDCl_3$. H-2 and H-3 of the dihydrosinapoyl moiety at the ^{1}H NMR spectrum are two triplets with chemical shifts δ_H 2.60 and δ_H 2.84 respectively ($J_{2/3}$ = 8.1). Their coupling is also affirmed from the ^{1}H-^{1}H COSY spectrum. HSQC spectrum shows that the chemical shifts of the respective carbons are δ_C 36.3 and δ_C 30.8 ppm. HMBC confirms this arrangement since there are correlation signals between H-2 and C-3 and the carboxylic carbon C-1 (Fig. 4); additively correlation signals between H-3 and C-2 and C-4 of the aromatic ring. Equivalent aromatic protons H-5 and H-9, are a singlet at δ_H 6.97. Those protons correlate at the HMBC spectrum with hydroxylated carbons C-6 and C-8 (Fig. 4). Proton H-1′ is a broad singlet at δ_H 5.06. H-1′ correlates with the aromatic C-1″ carbons at the HMBC spectrum, confirming the proposal structure, while the same spectrum affirms the chemical shift of C-1′ at δ_C 123.30 ppm (Fig. 4). The aromatic region of the ^{1}H NMR spectrum displayed an ABX system for H-2″, H-5″ and H-6″ at δ_H 7.35 (d, $J_{3''/5''}$ = 2.7), δ_H 7.53 (d, $J_{6''/5''}$ = 8.7) and δ_H 7.13 (dd) respectively. Proton resonances are slightly shifted downfield compared to relative hydroxylated patterns, probably due to the methylenic substitution at 1″ position. The arrangement of the system is further confirmed from ^{1}H-^{1}H COSY signals. The carbon resonances of C-2″, C-5″ and C-6″ were assigned by the use of HSQC spectrum. HMBC confirms the intended structure with the correlations between H-2″ and C-1″, C-3″, C-6″, as well as with the methynic C-1′ (Fig. 4). Furthermore there are correlations between H-6″ and C-1″, C-2″, C-4″ and C-1′. Finally, H-5″ correlates with C-1″, C-3″ and C-4″. Additionally, HMBC spectrum gives the information about the chemical shifts of the deprotonated carbons C-1″, C-3″ and C-4″ (Fig. 1). The aliphatic protons H-2‴, H-3‴ and H-4‴ are multiplets at chemical shifts δ_H 2.32, 1.62 & 1.59 ppm, respectively. Protons at position 5″ are more deshielded (δ_H 4.08 ppm), due to vicinity with the hydroxyl group of the aliphatic chain. ^{1}H-^{1}H COSY spectrum gives the couplings between the neighbor protons of the aliphatic chain, while HMBC signals confirm this arrangement. Additively the same spectrum confirms the arrangement giving correlation signal between H-2‴ and C-1‴ of the carboxylic group.

The exact molecular weight confirmed the intended structure and the fragments obtained from the MS–MS spectrum led to the proposed fragmentation mechanism (Fig. 1).

3.2. Flavonoids

Compound 5: *Quercetin 3-O-β-D-glucopyranosyl (1‴→2″)-α-L-rhamnosyl(1⁗→ 3‴)-α-L-rhamnosyl(1⁗→3⁗)-α-L-arabinofuranoside*. UV–vis spectrum of compound **6** indicated a 3-O-glycosilated quercetin (Mabry, Markham, & Thomas, 1970). ^{1}H and ^{13}C NMR spectra, as well as two dimensional ^{1}H-^{1}H COSY, HSQC and HMBC, well defined a quercetin glycosilated at the 3-OH: H-6 [δ_H 5.74(d), J = 2.4], H-8 [δ_H 5.89(d), J = 2.4], H-2′ [δ_H 7.49(d), J = 2.1], H-5′ [δ_H 6.75(d), J = 8.4], H-6′ [δ_H 7.81, J = 2.1, 8.4]. (Bennini, Chulia, Kaouadji, & Thomasson, 1992). However, ^{1}H NMR spectrum showed four anomeric protons at δ_H 5.70 (d, J = 7), δ_H 4.91 (bs), δ_H 4.55 (bs) and δ_H 4.24 (bs). The corresponding anomeric carbons were located at δ_C 102.5, On acid hydrolysis the compound afforded D-glucose, L-rhamnose and L-arabinofuranose, which were identified by co-injection as their trimethylsilyl derivatives on GC–MS with authentic samples. The MS spectrum of the compound, recorded on the LC-DAD-MS system at a positive mode displayed the molecular ion at $[M+H]^+$ = 889. The elution of the compound at the Rp-18 column at Rt = 4.87 min, shows the hydrophilic character of the molecule. The gradual fragmentation shows initially the detachment of a pentose $[M-(pentose-H_2O)+H]^+$ = 757, then of a rhamnose moiety $[756-(rhamnose-H_2O)+H]^+$ = 611, continually of another rhamnose moiety $[610-(rhamnose-H_2O)+H]^+$ = 465 and finally of an hexose $[464-(hexose-H_2O)+H]^+$ = 303, where the aglycon moiety is settled. In comparison to these data, it is concluded that the aglycon is glycosilated on the 3-OH group by four sugars in order: 3-O-D-glucose-O-L-rhamnose-O-L-rhamnose-O-L-arabinose. The coupling constant of the anomeric proton of the glucose unit (J = 7 Hz) indicated β-configuration, while the broad singlets displayed for the anomeric protons of the rhamnosyl (4.91 & 4.55 ppm) and the arabinofuranosyl units (4.24 ppm) showed α-configuration. The downfield shift of C-2″ by +7 ppm at δ_C 81.1, established by the HMBC spectrum, indicated that the first rhamnose unit has a 1‴→2″ connection to glucose. This is further confirmed by the HMBC correltion signal between H-1‴ and C-2″. The downfield shift of C-3‴ and C-3⁗ at 80.2 and 80 ppm respectively indicate that the rest of the sugars have a rhamnose (1⁗→3‴)rhamnose (1⁗→3⁗)apiose configuration. This is further confirmed with the HMBC correlation signals between H-1⁗ and C-3‴ and H-1⁗ and C-3⁗. The two rhamnose methyl groups are two doublets at δ_H 1.06 and δ_H 1.05 respectively with coupling constant J = 6Hz. 1H-^{1}H COSY, HMBC and HSQC spectra helped to the establishment of the rest proton and carbon chemical shifts.

Compound 6: *Quercetin 3-O-α-L-rhamnosyl(1‴→3″)-β-D-glucopyranoside*. UV–vis and NMR spectra of compound **6** show a quercetin aglycon, glycosylated on the 3-OH group, as described for compound **5**. 1H NMR spectrum also displayed two anomeric proton signals at δ_H 5.19 (bs) and δ_H 4.40 (d, J = 8.0). Acid hydrolysis and GC analysis of the trimehtylsilylated sugars showed the presence of an L-rhamnose and a D-glucopyranose moiety. The MS spectrum at the LC-DAD-MS system at positive mode confirmed the double glycositation with peaks at 611 = $[M+H]^+$, 449 = $[M-(glucose-H_2O)+H]^+$ and 303 = $[M-(glucose-H_2O)-(rhamnose-H_2O)+H]^+$. The broad singlet displayed by the anomeric proton of rhamnose was indicative of an *a*-configuration, whereas the coupling constant of the glucose unit (J = 8.0) indicated its β-configuration. The relative chemical shifts of the two anomeric protons indicate that rhamnose is the inner and glucose the terminal sugar. This is further evidenced by the HMBC correlation signal between C-3 and the rhamnose H-1″. In the NOESY spectrum a cross peak was observed between the terminal glucose anomeric proton and H-3″ of the rhamnose moiety (δ_H 3.72 ppm). Furthermore C-3″ of the inner rhamnose unit is downfield shifted by almost 10 ppm at δ_C 80.2 ppm, compared to an unsubstituted rhamnose. All these data indicate that the sugar unit has an α-L-rhamnosyl(1‴→3″)-β-D-glucopyranosyl configuration. Rhamnose methyl sugars are a doublet at δ_H 1.01 (J = 6.0). This is the first report about 3-O-α-L-rhamnosyl(1‴→3″)-β-D-glucopyranosyl glycosylation of quercetin.

Compound 7: *5,7,3′,6′-tetrahydroxyflavanol 7-O-β-D-glucopyranoside*. UV–vis spectrum for compound 7 displayed a main absorbance at λmax 294 nm with a shoulder at 335 nm. According to references (Mabry et al., 1970) this absorbance is indicative of a dihydroflavonol. The aromatic region of the ^{1}H NMR spectrum exhibited three signals at δ_H 6.95 (1H, d, J = 1.8), δ_H 6.78 (1H, d, J = 8.4) and δ_H 6.83 (1H, dd, J = 1.8, 8.4) that correspond to H-6′, H-3′ and H-4′ of the B ring respectively, due to 2,5-disubstitution. At the aromatic region there is also a typical *meta*-coupled pattern for H-6 and H-8 protons (δ_H 6.21 and δ_H 6.19, d, J = 2.4). H-2 and H-3 are two doublets at δ_H 4.95 and δ_H 4.55 respectively. The large coupling constant between these protons (J = 11.4) reveals them

to be *trans* to each other, leading to relative configuration 2*R*, 3*R* (Sinkkonen, Liimatainen, Karonen, & Pihlaja, 2005). HSQC and ^{1}H-^{1}H COSY confirmed that flavanol configuration. Additionally, a doublet at δ_H 4.95 with coupling constant J = 7.2 is observed and is attributed to the anomeric proton of a sugar. This pattern is well adapted to a β-D-glucopyranose. The glucose unit is further confirmed from the molecular weight of the molecule $[M+H]^+$ = 467. Main peak at the MS spectrum is also the ion at 305amu, which corresponds to the aglycon after the fragmentation of the glucose moiety.

^{13}C NMR spectrum, as well as HSQC and HMBC correlation signals confirmed the chemical shifts of C-2 and C-3 and C-4 at δ_C 86.8, 74.5 and 198.5 ppm respectively. The relatively upfield shift C-3 and the downfield shift of C-2 and C-4, compared to the glycosilated aglycon at the 3-position, indicates that hydroxyl group at position 3 is not glycosilated (Mendez, Hasegawa, Bilia, & Morelli, 1994; Sinkkonen et al., 2005). However, the upfield shift of C-7 at δ_C 166.6, compared to the aglycon, together with the correlation signals at the HMBC spectrum between C-7 and H-1″ confirm that 7-OH is glycosilated (Mendez et al., 1994). The glycosilation position is also affirmed by the anomeric proton chemical shift (Sang et al., 2002).

MS spectra at 80 eV confirmed the main fragments of the aglycon that are given for the first time. The fragments are in agreement with those obtained from the HR-NanoESI-QqTOF-MS/MS spectrum, where the exact molecular weight of the compound was confirmed at 466.1104amu.

Compound 8: *(7-O-4‴, 4′O-7″) quercetin dimmer*. Preparative work led to the isolation of compound **8**, pre-detected as a main constituent in the chromatogram of the diethyl ether extract of fruits well matured at room temperature. The compound has LC-DAD-MS (ESI+) characteristics that indicate a quercetin dimmer, formed after the detachment of two H_2O moieties of the two monomers, as described before (Termentzi et al., 2008). The retention time on the reverse phase column (Rt = 15.30 min), as well as the UV–vis spectrum (λmax 252, 354 nm with absence of a shoulder at 300 nm and a significant diminish of the intensity of Band I) indicate that hydroxyl group at position 7- as well as the acidic 4′-OH should be the connection positions of the two molecules.

NMR spectra confirmed this conformation. ^{1}H NMR spectrum displayed a typical quercetin pattern, but with slight upfield or downfield shifts, compared to the quercetin monomer, which was also tested for comparison reasons. Thus, H-6 and H-8 of A ring resonate more downfield compared to the monomer at δ_H 6.39 and δ_H 6.58, shifted by +0.23 and +0.22 ppm respectively. On the other hand protons of B ring resonate slightly upfield compared to quercetin: δ_H 7.59 (H-2′, −0.06 ppm), δ_H 6.76 (H-5′, −0.10 ppm) and δ_H 7.45 (H-6′, −0.07 ppm). HSQC and HMBC spectra indicated the chemical shifts of the molecule carbons. The intense downfield shift of C-7 (+11.4 ppm) and the smaller upfield shift of C-6 and C-8 (−4.1 and −4.5 ppm respectively) indicate that 7-OH is the first conjunction position of the two monomers. Concerning the B-ring, C-4′ is slightly upfield shifted, −2.9 ppm at δ_C 144.8 ppm. C-2′, C-3′, C-5′ and C-6′ are slightly downfield shifted compared to the quercetin monomer. HMBC signals between C-4′ and H-8, as well as between C-7 and H-5′ confirm that the unification of the two monomers is 7-O-4‴ and 4′-O-7″. This is further affirmed from the NOE long-range couplings of H-8 with H-5′ and H-6′. All these data completely agree with those obtained from the UV–vis and MS spectra, confirming that this new quercetin dimmer is (7-O-4‴, 4′O-7″) bisquercetin.

3.3. Biphenyls

Maloideae is a subfamily of the economically important Rosaceae species and includes many fruit tress, such as *Malus*, *Pyrus*, *Prunus* and *Sorbus* species. The phytoalexins of Maloideae are biphenyls and dibenzofurans (Kokubun & Harborne, 1995) and their pathogen-induced accumulation is unique to the Maloideae plants (Liu, Beuerle, Klundt, & Beerhues, 2004). Several biphenyl

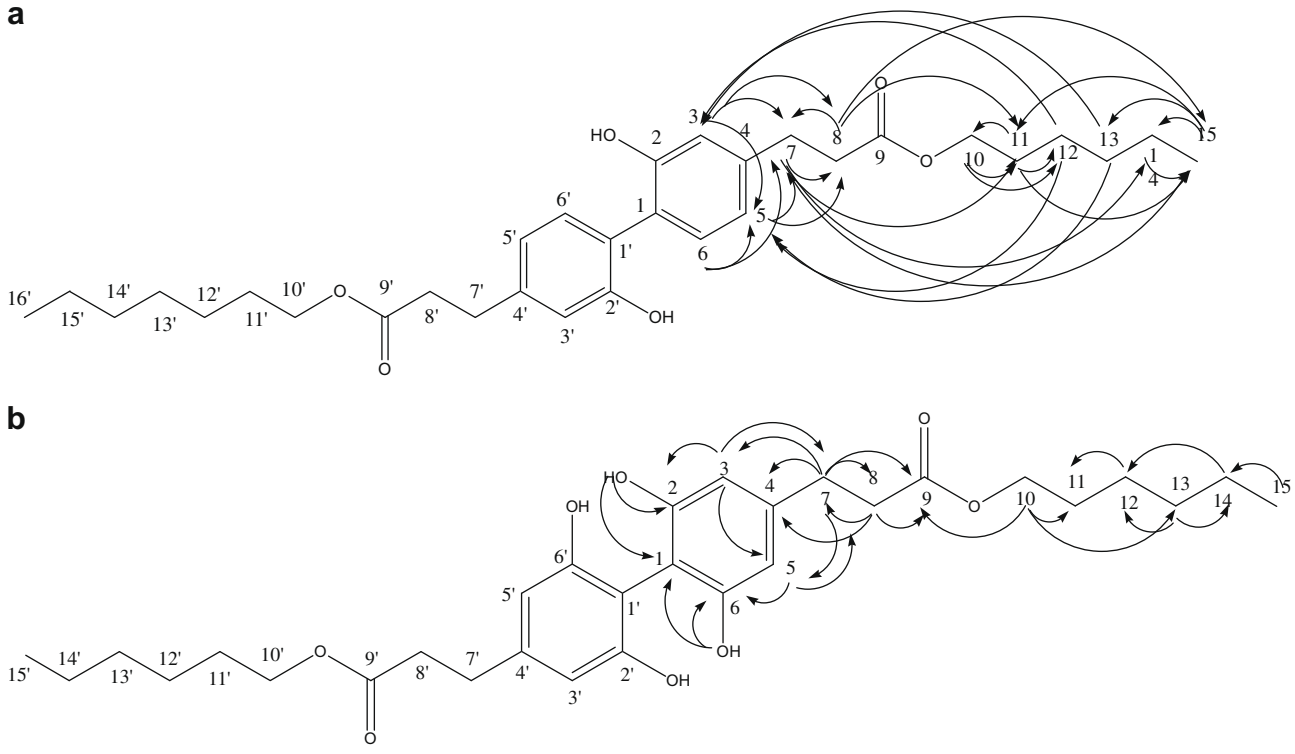


Fig. 5. (a) ROE spectrum correlation signals for [2,2′-dihydroxy, 4-(propionic acid hexyl ester), 4′-(propionic acid heptyl ester)] biphenyl (**Compound 9**). (b) HMBC spectrum correlation signals for [2,6,2′,6′-tetrahydroxy, 4,4′-bis-(propionic acid hexyl ester)] biphenyl (**Compound 10**).

compounds have been isolated in the past from the sapwood, heartwood, leaves and tissues cultures of several *Sorbus* and other Maloideae species (Borejsza-Wysocki, Lester, Attygalle, & Hrazdina, 1999; Kokubun & Harborne, 1994; Kokubun & Harborne, 1995; Kokubun, Harborne, Eagles, & Waterman, 1995). Aucuparin was the first one isolated from the heartwood of *Sorbus aucuparia* (Erdtman, Eriksson, Norin, & Forsen S., 1963). Biphenyls from these species' fruits have never been isolated before. The two novel biphenyls were isolated only from fruits well matured on tree, while they were totally absent from all other fruit categories, indicating the fact that their synthesis is inducted by stress conditions and probably microbial infection.

Compound 9: *[2,2′-dihydroxy, 4-(propionic acid hexyl ester), 4′-(propionic acid heptyl ester)] biphenyl*. The NMR spectra (MeOD-*d4*) of compound **9** had a simple resonance pattern, which, in comparison to the exact molecular weight, 512.3166, indicated the fact that the molecule was symmetrical with one methylene difference in the two aliphatic chains. The aromatic region of the ^{1}H NMR spectrum displayed an ABX system for H-3, H-6 and H-5 at δ_H 7.0 (d, J = 2.0 Hz), δ_H 6.62 (d, J = 8.0 Hz) and δ_H 6.82 (dd, J = 8.0, 2.0 Hz) respectively. This system is further confirmed by the ^{1}H-^{1}H COSY and TOCSY correlations. The ^{13}C NMR spectrum supports the existence of the aromatic ring showing six carbon peaks at the aromatic region. The carbon resonances of C-3, C-5 and C-6 were assigned by the use of HSQC spectrum. Carbon resonance at δ_C 154.3, in combination with the HMBC correlation with proton H-6, confirmed the substitution by a hydroxyl group in position 2. The relatively shielded resonance of carbon 1 at δ_C 130.4, established by the HMBC correlation with proton H-6, is indicative of the presence of another aromatic ring attached to this carbon atom in correlation to the presence of the hydroxylated carbon C-2 (Kokubun et al., 1995). C-4 which bonds on an aliphatic chain resonates more downfield at δ_C 135.4. The attachment of the chain to the C-4 position of the aromatic ring is further supported by HMBC correlations between proton H-7 and carbon atoms C-4 and C-3.

The protons of the propionyl chains that bond on the aromatic rings appear at the ^{1}H NMR spectrum as two triplets [δ_H 2.56 (J = 7.5 Hz) and δ_H 2.81 (J = 7.5 Hz)] that correspond to H-8 and H-7 respectively and ^{1}H-^{1}H COSY, as well as TOCSY correlations, confirm the coupling between these protons. HSQC spectrum attributes the carbon resonance at δ_C 37.5 to C-8 and the more shielded peak at δ_C 31.8 to C-7 (Tsevegsuren et al., 2007). The propionyl phenyl configuration was further affirmed with characteristic connectivities of H-7 to δ_C 135.4 (C-4), 126.1 (C-3), 125.9 (C-5), 36.1 (C-8), carboxylic carbon 173.8 (C-9) and of H-8 to δ_C 173.8 (C-9), 31.4 (C-7), 135.4 (C-4) in the HMBC spectrum. ROE correlations between proton H-7 of the akyl chain and aromatic protons H-3 and H-5, as well as between H-8 and H-3, support additionally the proposed configuration (Fig. 5a). The presence of C-9 carboxylic carbon resonating at δ_C 173.8 was established by HMBC correlations with both H-8 and H-7 as well as with the adjacent protons H-10.

Protons 10 and 11 of the alkyl chain are triplets in the ^{1}H NMR spectrum at δ_H 4.03 (J = 6.8 Hz) and δ_H 1.59 (J = 6.8 Hz) respectively. The corresponding carbon resonances were assigned through the HSQC spectrum. HMBC correlations between H-10 and C-11, as well as H-11 and C-10, C-12, further confirm the chemical shifts. Hydrogens 12, 13 and 14, as well as 12′, 13′, 14′ and 15′, are overlapping at the aliphatic region of ^{1}H NMR spectrum, giving a featureless broad resonance H-(12-14) and H-(12′-15′) at δ_H 1.3. ^{1}H-^{1}H COSY correlations establish the coupling between protons H-10 and H-11 as well as between H-11 and H-12. TOCSY correlations also confirm the connectivity between H-10, H-11 and H-12. Additionally, proton H-10 shows HMBC correlations with carbons C-11 and C-12. Regarding the number of -CH_2- groups of the alkyl chain, this was determined by the methylene resonances at δ_H 1.3 from the HSQC spectrum. Thus, the 2D traces led to the unambiguous assignment of the aliphatic carbon resonances. The methyl protons 15 and 16′ appear as a triplet at [δ_H 0.89 (J = 7.0 Hz)] showing COSY and TOCSY correlations with the resonance peak corresponding to H-(12-14) and H-(12′-15′). The HMBC correlations though, between the methyl protons and the C-14 and C-13 resonances confirm that the alkyl chain is well-defined.

Interestingly, ROE correlations between the aliphatic, propionyl and aromatic protons give information about spatial configuration of the molecule. Specifically, H-8 correlates with H-11, H-15 and H-7 with H-14 and H-15, while aromatic hydrogens H-3 and H-5 correlate with the aliphatic H-12 and H-13 (Fig. 5a).

MS/MS experiment gave the fragments of the compound confirming the structure.

Compound 10: *[2,6,2′,6′-tetrahydroxy, 4,4′-bis-(propionic acid hexyl ester)] biphenyl*. The compound was eluted nearly five minutes later than compound **9** on a RP-18 HPLC column, due to the existence of four active aromatic hydroxyl groups, which prolong the withholding inside the column. The exact molecular weight of the compound (530.2656) in comparison to 1D and 2D NMR spectra (DMSO-*d6*) confirm the symmetrical pattern of the molecule. The ^{1}H NMR pattern is identical to the profile of substance 9 with the exception of the aromatic region. Hence, two singlets appear, resonating at δ_H 6.89 and δ_H 6.73, respectively. These are attributed to aromatic protons H-3/H-5 and phenolic hydroxyl groups at positions 2/6 accordingly. The rationale for the presence of the hydroxyl groups at positions 2 and 6 of the aromatic ring is based on the HMBC correlations between the propionyl chain protons H-7 with aromatic carbons C-3/C-5 as well as the HMBC correlation between protons H-3/H-5 with carbons C-7 and C-8 (Fig. 5b). Additional confirmation is provided through the HMBC 2D traces which correlate hydroxyl protons 2/6 with carbon C-1 and atoms C-2/6 while further informative are the correlation signals between protons H-3/H-5 with C-2/C-6 (Fig. 5b). The deshielded resonance of carbons C-2 and C-6 at δ_C 152.70 confirms hydroxylation, while C-3 and C-5 are at δ_C 124.78. At the ^{13}C NMR spectra, C-1 is at δ_C 109.8. That chemical shift is confirmed by the correlation of this carbon with the hydroxyl protons at the 2D HMBC spectrum (Fig. 5b). The relatively shielded resonance for C-1, further confirms the double aromatic hydroxylation at 2 and 6 positions, as well as the dimeric pattern of the compound (Hussein, Ayoub, & Nawwar, 2003).

The rest of the observed correlations and chemical shifts are similar with those recorded for substance 9 which has previously been described. Additionally NOE experiments gave the same information about the spatial configuration of the molecule, as for Compound **9**.

The MS/MS analysis gave the parent and daughter fragments, further confirming the structure (Fig. 3).

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