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First chemical synthesis report of an anthocyanin metabolite with in vivo occurrence: cyanidin-4′-*O*-methyl-3-glucoside

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ABSTRACT

Anthocyanins are natural polyphenolic compounds with important biological properties. In humans , these compounds are metabolized into different derivatives namely methyl, glucuronyl, and sulfate conjugates. Among these, cyanidin-4′-*O*-methyl-3-glucoside, already detected in vivo, seems to be an interesting metabolite to be used as standard for biological studies. The lack of suitable standards is a major drawback in biological studies. Bearing this in mind, this work describes a strategy for the chemical synthesis of cyanidin-4′-*O*-methyl-3-glucoside **9**, which involved in the synthesis of the 'Western' and 'Eastern' molecules, namely 2,4-diacetoxy-6-hydroxybenzaldehyde **2** and 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)-3′-benzyloxy-4′-methoxyacetophenone **8**, respectively. The final step consisted in the Robinson's acidic aldol condensation between **2** and **8** molecules, which after the respective deprotections yield product **9**.

Anthocyanins are naturally occurring polyphenols belonging to the group of flavonoid compounds widely found in plants. These pigments are responsible for the color of many flowers, leaves, seeds, fruits, and vegetables ranging from red to purple and blue.[1]

It is well known that in aqueous solution the color of anthocyanins is pH dependent: at very low pH, they are essentially present in the red flavylium cation form (AH^+); when the pH is raised the flavylium cation rapidly undergoes proton transfer reactions leading to the formation of blue quinonoidal bases (A and A^-) and simultaneously, but more slowly, the flavylium cation leads to the formation of colorless hemiketal (B) in the hydration reaction; the hemiketal species further undergoes a tautomerization reaction to give the pale yellow *cis*-chalcone (C_c) which isomerises to the *trans*-chalcone (C_t).[2,3] The anthocyanic pigments have shown potential application as dyes for food[4] and also for organic solar-sensitized cells.[5] In vitro and in vivo studies have demonstrated that anthocyanins may offer potential beneficial effects to human health because of their biological properties such as anti-aging, anticancer, anti-inflammation, anti-infection, and diabetes. Epidemiological evidence suggests that the ingestion of high proportions of anthocyanins in the diet may contribute to a lower risk of cardiovascular events such as hypertension and stroke.[6–8]

Because of the potential benefits of these compounds, it is crucial to understand their bioavailability, that is, absorption, metabolism, and excretion. In the human organism, anthocyanins are metabolized to different derivatives (glucuronides, methylethers, and sulfates), which are further found in urine and plasma, and should have different biological effects from their precursors.[9–12] This enterohepatic recycling opens a new field of interest that remains a challenge: the biological properties of anthocyanin metabolites. To evaluate their activity, it is important to have sufficient quantities of these molecules which cannot be obtained commercially or by isolation from biological samples. Some anthocyanin metabolites (methylated, glucuronides, and glutathione adducts from cyanidin and delphinidin-3-glucosides) were recently obtained through enzymatic synthesis but, the yields are poor and the synthesis expensive.[13] Among those metabolites, cyanidin-3-glucoside (cy-3-glc) has already been detected, in vivo, as a methylated product in both the 4′ and 3′ positions.[14] 3′-*O*-methylation yields the natural pigment peonidin-3-glucoside, whereas the methylated form at the 4′ position of ring B has never been identified in nature.

Bearing this in mind, the goal of this work was to develop a strategy for chemical synthesis of cyanidin-4′-*O*-methyl-3-glucoside.

Known synthetic routes to obtain 3-deoxyanthocyanidins and other flavylium pigments result from the construction of the C-ring by cyclization which involves the coupling together of two halves, the so-called 'Eastern' and 'Western' portions of the molecule. The methods are based on an acid-mediated condensation either between a protected salicylaldehyde derivative and a substituted acetophenone (Pratt and Robinson, 1922)[15] or a phenolic derivative and an aryl ethynyl ketone (Johnson and Melhuish, 1947).[16]

Due to the inherent instability of anthocyanins under neutral and basic conditions, only few syntheses have been reported to

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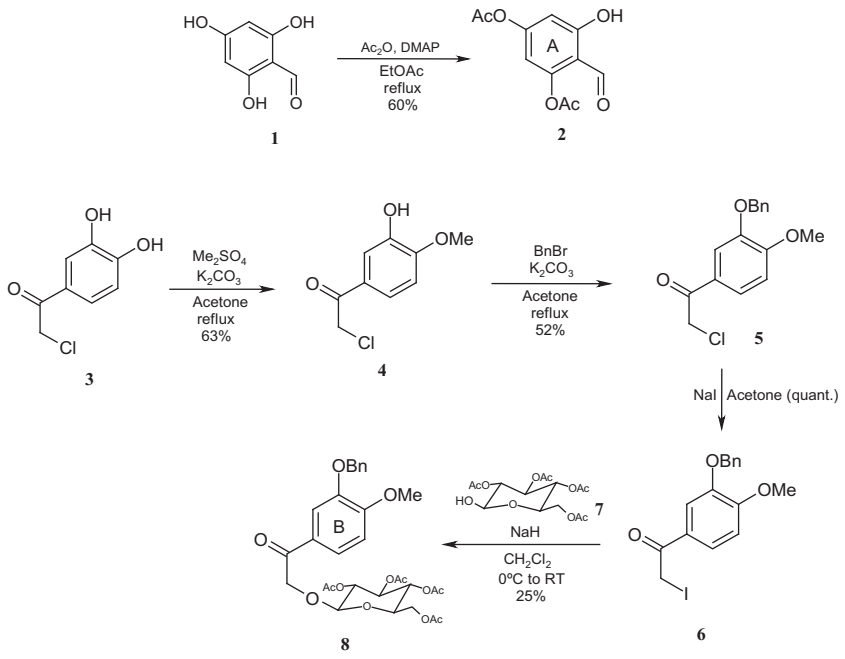
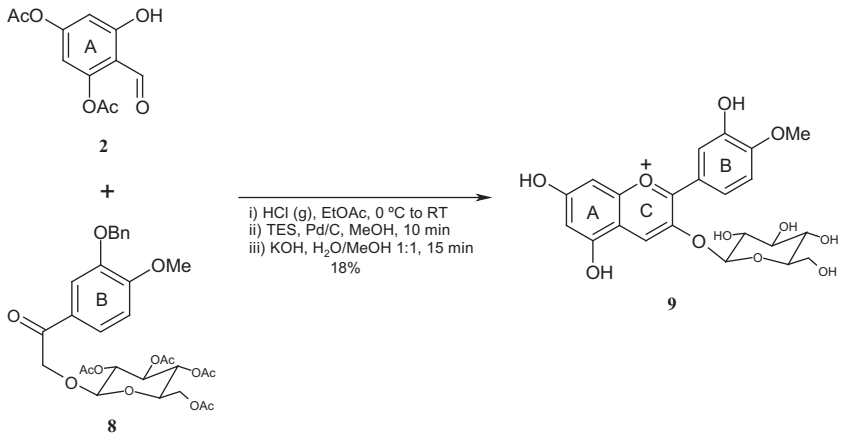


Scheme 1. Synthesis schemes of 'Western' **2** and 'Eastern' **8** molecules.



Scheme 2. Synthesis scheme of cyanidin-4′-*O*-methyl-3-glucoside **9**: (i) acidic aldol condensation between **2** and **8** molecules and respective deprotections: (ii) debenzylation and (iii) deacetylation.

date.[17–21] Among them, Robinson's acidic aldol condensation remains efficient until date and has subsequently been further developed.[22] Although the method is classical, it has never been driven towards the synthesis of the above referred anthocyanin derivatives.

Overall, the strategy adopted involved firstly the preparation of the A and B rings, the so-called 'Western' **2** and 'Eastern' **8** parts of the molecule, respectively (Scheme 1).

The 'Western' molecule was prepared from acetylation of 2,4,6-trihydroxybenzaldehyde **1** to produce the corresponding 2,4-diacetate **2** in a yield of 60% after column chromatography and recrystallization.[21]

The construction of the 'Eastern' part consisted in four steps starting from the reagent 2-chloro-3′,4′-dihydroxyacetophenone **3**. In the first step, derivatization of the C-4′ hydroxyl to a methoxy group was accomplished using 1 equiv of dimethyl sulfate and 1 equiv of potassium carbonate in refluxing acetone. Flash column chromatography of the residue (CH_2Cl_2/acetone 10:1) gave **4**[23] as white crystals (63% yield). A regioselective monomethylation in C-4′ was achieved with success.[24–26] Next, the protection of C-3′ hydroxyl group of **4** was performed by benzylation with benzyl bromide/potassium carbonate in refluxing acetone giving the product **5**[27] as white crystals with 52% yield, after column chromatography (CH_2Cl_2/acetone 20:1).[28] The next reaction consisted in the replacement of chloride atom for a better leaving group such as bromo or iodide. Therefore, the halide exchange reaction was accomplished by treatment of **5** with excess of sodium iodide in dry acetone at room temperature. The product **6**[29] was obtained as yellow crystals and the reaction revealed to be quantitative. The final step of the construction of the 'Eastern' molecule deals with the glycosylation of **6** using β-D-glucose-2,3,4,6-tetraacetate **7** (prepared from the hydrolysis of acetobromoglucose[30]) in the presence of sodium hydride as the base in dry dichloromethane at room temperature. The resulting dark oil was purified by column chromatography (CH_2Cl_2/acetone 10:1) to give product **8**[31] as a pale yellow solid in a modest yield of 25%.

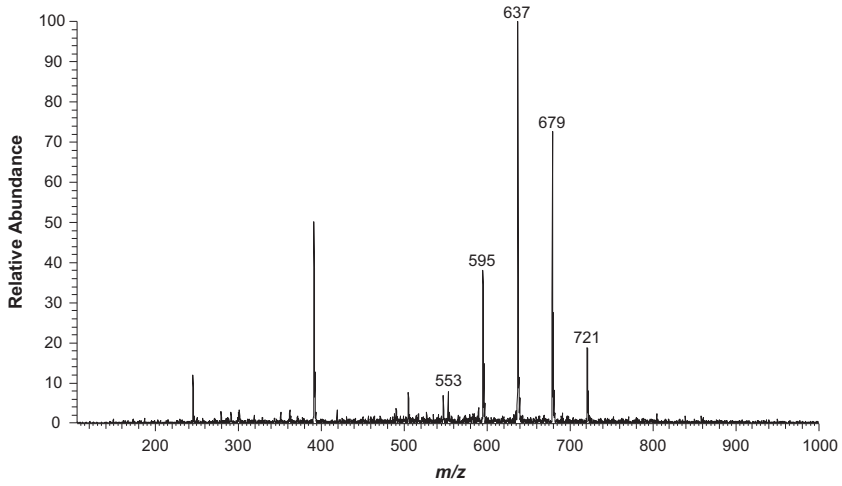


Figure 1. Full MS spectrum of the reaction between **2** and **8** molecules recorded in positive ion mode.

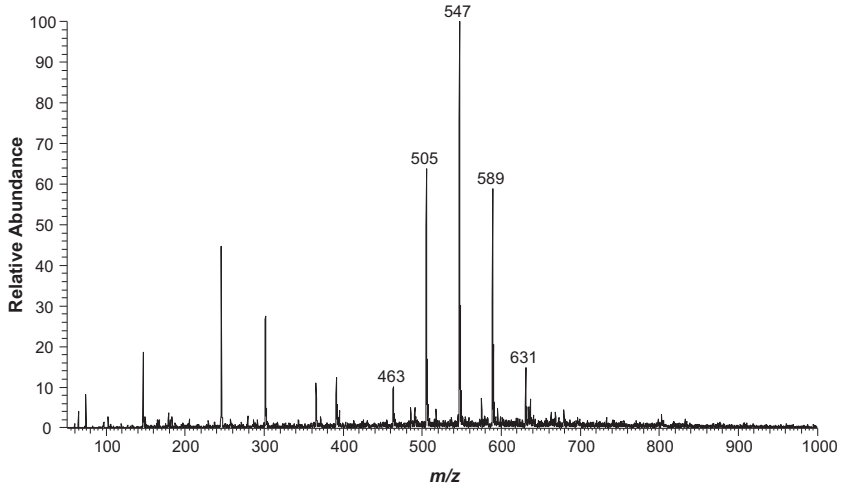


Figure 2. Full MS spectrum of the debenzylation reaction recorded in positive ion mode.

Finally, the aldol-type condensation between **2** and **8** compounds generates the C-ring of the final compound **9**. The reaction was performed in dry EtOAc promoted by anhydrous HCl (g) (Scheme 2).

The mixture was stirred from 0 °C to room temperature (RT) until starting materials disappeared and a red color developed. Direct injection in mass spectrometry (ESI-MS[32]) showed that the coupling had occurred as well as a partial deacetylation giving a mixture of glycosylated flavylium ions (Fig. 1).

Molecular ions $[M]^+$ at m/z 721, 679, 637, and 595 correspond to the glycosylated flavylium compounds with different degrees of acetylation. Traces of cyanidin-3′-benzyloxy-4′-methoxy-3-glucoside (without any acetyl protecting group, $[M]^+$ m/z 553) were also detected in the solution. MS^2 of all molecular ions gave rise to the ion $[M]^+$ at m/z 391, which corresponds to the aglycone molecule (loss of glucoside residue with different number of acetyl groups). MS^3 fragmentation originated molecular mass of m/z 300 which agrees with the loss of benzyl group ($[MS^2-91]^+$).

Removal of the protecting groups was followed and it was found that the order of cleavage is important to optimize the yield of the product. Removal of the benzyl groups first followed by the acetate groups gives best product yields. Therefore, debenzylation was performed first adding triethylsilane to palladium–charcoal

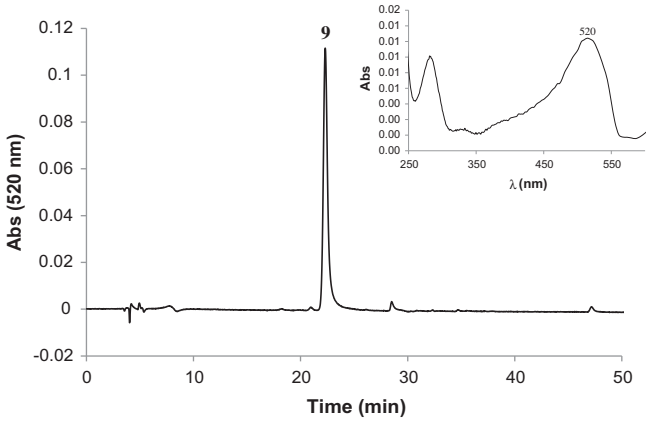


Figure 3. HPLC chromatogram recorded at 520 nm of the mixture after debenzylation and deacetylation procedures. Inset: UV–vis spectrum of cyanidin-4′-*O*-methyl-3-glucoside **9**.

catalyst which generates H_2 in situ. This method has been proved to be rapid and efficient for reduction of multiple bonds, azides, imines, and nitro groups, as well as benzyl and allyl group

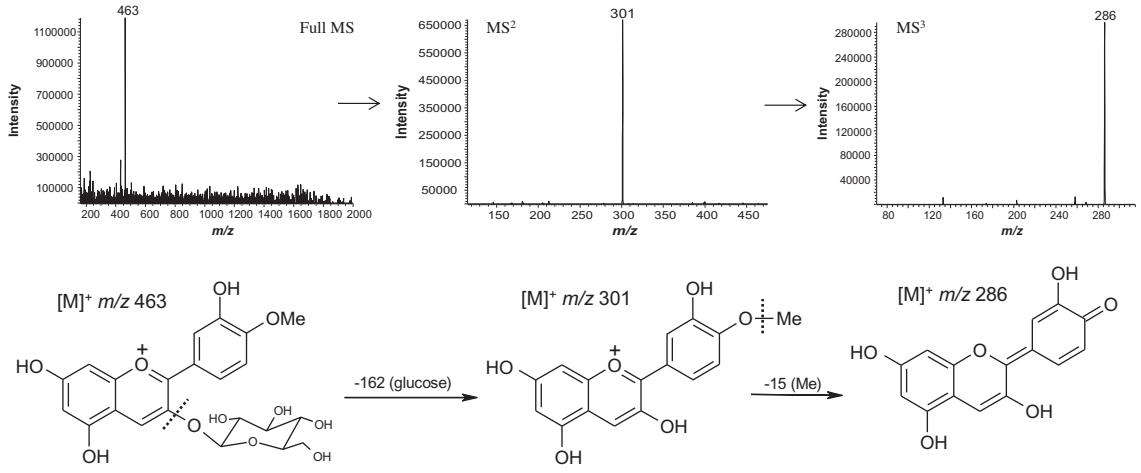


Figure 4. MS spectra of compound **9** (Full MS, MS^2, and MS^3) and respective fragmentation patterns recorded in positive ion mode.

deprotection under mild and neutral conditions.[33] After 10 min at RT, the catalyst was filtered and the mixture analyzed in mass spectrometry by direct injection (Fig. 2).

The full MS spectrum showed the molecular ions $[M]^+$ at *m*/*z* 631, 589, 547, and 505 which corresponds to the glycosylated flavylium compounds without the benzyl group. All these molecular ions produce the MS^2 ions at *m*/*z* 301, which corresponds to the cyanidin-4′-methylated aglycone molecule. The signal $[M]^+$ at *m*/*z* 463 revealed that the presence of the final compound, cyanidin-4′-methoxy-3-glucoside **9** (without any protecting group) was already detected in the solution at this stage.

Complete deacetylation was then performed by hydrolysis with aqueous KOH in MeOH (15 min, RT). After acidification (aqueous HCl 1 M), the aqueous solution was filtered and extracted (ethyl acetate) to remove the majority of KCl and traces of toluene, and the formation of cyanidin-4′-*O*-methyl-3-glucoside **9** was further checked by HPLC-DAD[34] (Fig. 3).

The UV–vis spectrum of peak **9** (λ_{max} 520 nm) agrees with the one already reported.[13] Further LC-DAD/ESI-MS analysis of the mixture in positive ion mode revealed that the chromatographic peak **9** showed a molecular ion $[M]^+$ at *m*/*z* 463, which agrees with the flavylium cation of cyanidin-3-glucoside methylated derivative. MS^2 fragmentation of this ion yielded one peak $[M-162]^+$ at *m*/*z* 301, corresponding to the methylated aglycone (loss of glucose residue). MS^3 fragment was detected $[M-162-15]^+$ at *m*/*z* 286, which corresponds with the loss of the methyl group (Fig. 4).

The aqueous fraction was eluted on a silica gel C18-reversed phase to remove the acetic acid and the desired compound was recovered in acidic MeOH. The final purification was made by column chromatography using TSK Toyopearl HW-40 (S) gel (150 mm × 16 mm i.d.). The metabolite was eluted with 10% aqueous methanol acidified with 2% HCl at a flow rate of 0.8 mL/min. After removal of methanol on a rotary evaporator, the compound was freeze–dried. The product **9**[35] was obtained as a dark red powder in a satisfactory yield of 18%. The identity and purity of cyanidin-4′-*O*-methyl-3-glucoside was confirmed by NMR analyses.

This work reports for the first time a full chemical synthesis strategy to produce an anthocyanin metabolite, namely cyanidin-4′-*O*-methyl-3-glucoside with a purity of 99.9% (by HPLC). This methodology allowed obtaining this metabolite in better yields rather than by doing enzymatic synthesis. This synthesis approach will allow obtaining other in vivo anthocyanin metabolites (delphinidin, petunidin) conjugated with different groups (methyl, glucuronyl) which will be very useful as standards for bioavailability and biological studies.

Acknowledgments

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23. *Data for* **4**: ^{1}H NMR (400.14 MHz, $CDCl_3$), δ (ppm): 7.56 (d, J = 2.2 Hz, 1H), 7.53 (dd, J = 2.2, 8.4 Hz, 1H), 6.91 (d, J = 8.4 Hz, 1H), 4.64 (s, 2H, CH_2Cl), 3.97 (s, 3H, OCH_3). ^{13}C NMR (100.62 MHz, $CDCl_3$), δ (ppm): 189.8 (C=O), 151.3 (C), 145.7 (C), 127.9 (C), 122.3 (CH), 114.5 (CH), 110.2 (CH), 56.1 (OCH_3), 45.7 (CH_2Cl); ESI-MS *m*/*z*: 201 $[M+H]^+$, 199 $[M-H]^-$.
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27. *Data for* **5**: ^{1}H NMR (400.14 MHz, $CDCl_3$), δ (ppm): 7.60–7.25 (m, 7H), 6.93 (d, J = 8.9 Hz, 1H), 5.19 (s, 2H, CH_2Bn), 4.61 (s, 2H, CH_2Cl), 3.96 (s, 3H, OCH_3). ^{13}C NMR (100.62 MHz, $CDCl_3$), δ (ppm): 189.8 (C=O), 154.7 (C), 148.3 (C), 136.3 (C), 128.6 (2CH), 128.1 (CH), 127.5 (2CH), 127.2 (C), 123.6 (CH), 113.1 (CH), 110.6 (CH), 70.9 (CH_2Bn), 56.1 (OCH_3), 45.5 (CH_2Cl); ESI-MS *m*/*z*: 291 $[M+H]^+$.

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29. *Data for* ***6***: ^{1}H NMR (400.14 MHz, $CDCl_3$), δ (ppm): 7.63 (dd, J = 2.1, 8.4 Hz, 1H), 7.59 (d, J = 2.1, 1H), 7.49–7.29 (m, 5H, Bn), 6.92 (d, J = 8.4 Hz, 1H), 5.19 (s, 2H, CH_2Bn), 4.27 (s, 2H, CH_2I), 3.96 (s, 3H, OCH_3). ^{13}C NMR (100.62 MHz, $CDCl_3$), δ (ppm): 191.6 (C=O), 154.5 (C), 148.4 (C), 136.4 (C), 128.7 (2CH), 128.1 (CH), 127.5 (2CH), 126.4 (C), 124.2 (CH), 113.5 (CH), 110.5 (CH), 71.0 (CH_2Bn), 56.1 (OCH_3), 1.3 (CH_2I); ESI-MS m/z: 383 $[M+H]^+$.
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31. *Data for* ***8***: ^{1}H NMR (400.14 MHz, $CDCl_3$), δ (ppm): 7.56–7.29 (m, 7H), 6.90 (d, J = 8.9 Hz, 1H), 5.22 (t, J = 9.5 Hz, 1H), 5.18 (s, 2H, CH_2Bn), 5.12–5.04 (m, 2H), 4.89–4.75 (m, 2H), 4.67 (d, J = 7.9 Hz, 1H), 4.23 (dd, J = 4.6, 12.4 Hz, 1H), 4.15–4.08 (m, 2H, CH_2Oglc), 3.94 (s, 3H, OCH_3), 2.07 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.00 (s, 6H, 2 × Ac); ^{13}C NMR (100.62 MHz, $CDCl_3$), δ (ppm): 193.3 (C=O), 170.6 (C=O), 170.1 (C=O), 169.7 (C=O), 169.4 (C=O), 154.4 (C), 148.3 (C), 136.4 (C), 128.6 (2CH), 128.1 (CH), 127.7 (C), 127.5 (2CH), 123.2 (CH), 112.6 (CH), 110.6 (CH), 100.2 (CH), 72.6 (CH_2Oglc), 71.9 (CH), 71.0 (CH_2Bn), 70.9 (CH), 70.3 (CH), 68.3 (CH), 61.7 (CH_2), 56.1 (OCH_3), 20.6 (4 × CH_3, Ac); ESI-MS m/z: 603 $[M+H]^+$, 625 $[M+Na]^+$.
32. LC-DAD/ESI-MS: LC-DAD/ESI/MS analyses were performed on a Finnigan Surveyor series liquid chromatograph equipped with Finnigan LCQ (Finnigan Corp., S. J., Calif., USA) mass detector and an API source using an ESI interface. The samples were analyzed on a reversed-phase column (150 × 4.6 mm, 5 μm, C18) at 25 °C using the same eluents, gradients, and flow rates referred for HPLC analysis. The capillary voltage was 4 V and the capillary temperature 275 °C. Spectra were recorded in positive and negative ion modes between m/z 120 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass (MS), a zoom scan of the most intense ion in the first scan (MS^2), and a MS–MS of the most intense ion using relative collision energy of 30 and 60 (MS^3).
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34. *HPLC–DAD*: HPLC analyses were performed on a Merck–Hitachi L-7100 (Merck, D., Germany) apparatus with a 150 × 4.6 mm i.d. reversed-phase ODS C18 column (Merck, Darmstadt) at 25 °C; detection was carried out using a L-7450A diode array detector (DAD). The eluents were (A) H_2O/HCOOH (9:1) and (B) CH_3CN. The gradient consisted of 10–35% B for 50 min at a flow rate of 0.5 mL/min. The column was washed with 100% B during 10 min and then stabilized with the initial conditions during another 10 min.
35. *Cyanidin-4′-O-methyl-3-glucoside* (**9**): 2,4-Diacetoxy-6-hydroxybenzaldehyde **2** (7.9 mg, 0.0332 mmol) and 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)-3′-benzyloxy-4′-methoxyacetophenone **8** (20 mg, 0.0332 mmol) were dissolved in dry EtOAc (1.5 mL) and anhydrous HCl (g) was bubbled through the solution. The reaction was stirred from 0 °C to RT and a red color gradually developed. The mixture was then stirred until all the starting materials were consumed as monitored by TLC. No purification was undertaken at this stage. The solvent was evaporated and the residue dissolved in MeOH. Debenzylation was performed using 10 equiv of triethylsilane in 20% Pd/C at RT for 10 min. The catalyst was removed by filtration. Complete deacetylation was performed with KOH (3 equiv) in MeOH/H_2O 1:1. The mixture was stirred at RT for 15 min and then carefully acidified to pH 1 with HCl 1 M. After MeOH evaporation, the aqueous solution was filtered to remove the majority of KCl precipitate and further extracted with ethyl acetate to remove traces of toluene. The aqueous fraction was eluted on silica gel C18-reversed phase to remove the acetic acid and the desired compound was recovered in acidic MeOH. The final purification was made by column chromatography using a TSK Toyopearl HW-40 (S) gel (150 × 16 mm i.d.). The metabolite was eluted with 10% aqueous methanol acidified with 2% HCl at a flow rate of 0.8 mL/min. The fraction collection was made upon visual detection of the red band. After removal of methanol on a rotary evaporator and freeze–drying, compound **9** was obtained as a red solid (3 mg, 18%). ^{1}H NMR (600.13 MHz, CD_3OD/TFA 98:2), δ (ppm): 9.10 (s, 1H), 8.36 (dd, J = 2.3, 8.8 Hz, 1H), 8.02 (d, J = 2.3 Hz, 1H), 7.21 (d, J = 8.8 Hz, 1H), 6.93 (d, J = 1.6 Hz, 1H), 6.68 (d, J = 1.6 Hz, 1H), 5.31 (d, J = 7.7 Hz, 1H), 4.04 (s, 3H, OCH_3), 3.91 (dd, J = 2.2, 12.1 Hz, 1H), 3.72–3.65 (m, 2H), 3.56 (m, 1H), 3.54 (t, J = 9.1 Hz, 1H), 3.42 (t, J = 9.4 Hz, 1H). ^{13}C NMR (125.77 MHz, CD_3OD/TFA 98:2), δ (ppm): 169.6 (C), 162.7 (C), 156.7 (C), 156.6 (C),154.7 (C), 147.1 (C), 144.4 (C), 136.7 (CH), 127.9 (CH), 121.1 (C), 116.4 (CH), 112.7 (C), 111.5 (CH), 102.4 (CH), 102.1 (CH), 93.8 (CH), 77.5 (CH), 76.7 (CH), 73.4 (CH), 69.7 (CH), 61.0 (CH_2), 55.5 (OCH_3); LC-DAD/ESI-MS m/z: 463 $[M]^+$.