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Covalently linked anthocyanin–flavonol pigments from blue *Agapanthus* flowers

S.J. Bloor*, R. Falshaw

Industrial Research Ltd, P. O. Box 31-310, Lower Hutt, New Zealand

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Abstract

The structures of the two major anthocyanins in blue *Agapanthus* flowers have been determined to be a *p*-coumaroylated delphinidin diglycoside attached to a flavonol triglycoside via a succinic acid diester link. The structure has been determined unambiguously through degradation studies, glycosidic analysis and NMR experiments. These compounds represent unique examples of anthocyanin pigments where both types of co-pigment, an aromatic acyl group and a flavonoid co-pigment, are attached covalently to the anthocyanin.

Keywords: *Agapanthus praecox* sp. *orientalis*; African lily; Liliaceae; [6‴-*O*-(delphinidin 3-*O*-(6″-*O*-*p*-coumaroyl-glucoside) 7-*O*-glucosyl)] [6⁗-*O*-(kaempferol 3-*O*-glucoside, 7-*O*-xyloside 4′-*O*-glucosyl)]succinate; Delphinidin 3,7 diglucoside; Kaempferol 3,4′-diglucoside 7-xyloside; Kaempferol 3,7,4′-triglucoside; Succinate diester; Blue flower colour

1. Introduction

Blue flowers are of considerable interest to flower colour chemists. More often than not the blue colour arises from an anthocyanin in a pH 5–7 environment stabilised by interaction with a colourless co-pigment (Goto & Kondo, 1991; Bloor, 1997, 1999). This stabilisation can be through intramolecular interaction of the anthocyanin with an attached aromatic acyl group or intermolecular interaction between an anthocyanin and a co-pigment derived from the flavonoid pathway (such as a flavone or flavonol). Metal ions may also be involved.

Agapanthus sp. or African lilies present a striking summer display of white or blue flowers and the blue flowers have yielded a pigment showing another variation on this anthocyanin–co-pigment theme. We became interested in this flower when our initial studies showed the pigment mixture from the blue flowers was remarkably stable and had unusually high molecular weight.

2. Results and discussion

The two major anthocyanin pigments, **1** and **2**, were purified by a series of chromatography steps involving initial clean-up of the extract with Diaion HP-20, followed by CC using Sephadex G-25, cellulose, RP and finally Toyopearl HW-40. The Sephadex G-25 step was very useful in partitioning these high MW anthocyanins from other metabolites. The UV–Vis spectra showed the presence of significant absorption in the 300–350 nm region indicating a high degree of aromatic acylation.

The mass spectrum of **1** showed a molecular ion at m/z 1597. HPLC analysis of the acid hydrolysis product mixture from **1** or **2** showed the presence of *p*-coumaric acid, kaempferol and delphinidin. Base hydrolysis of **1** gave three UV-absorbing units, a delphinidin glycoside, *p*-coumaric acid and a flavonol glycoside, **3**. Similar treatment of the second major anthocyanin, **2**, gave the same delphinidin glycoside

* Corresponding author. Tel.: +64-4-569-0576; fax: +64-4-569-0055.

E-mail address: s.bloor@irl.cri.nz (S.J. Bloor).

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and *p*-coumaric acid but yielded a different flavonol glycoside, **4**. The flavonol glycosides, **3** and **4**, were also present in significant amounts in fresh extracts of the flower tissue. These two flavonols were purified and shown, by analysis of NMR spectra, to be kaempferol 3,4′-diglucoside 7-xyloside (**3**) and kaempferol 3,4′,7-triglucoside (**4**). The structure of **3**, a new triglycoside, was deduced by comparison with reported NMR data for **4** (Yoshida, Saito & Kadoya, 1987). A NOE between the xylose H-1 and H-6,8 of the kaempferol confirmed the placement of the xylose at the 7 position. The mass spectrum of the second anthocyanin, **2**, (*m*/*z* 1628 i.e. 30 more than **1**) showed that the xyloside/glucoside interchange was probably the only difference between the two pigments.

The delphinidin glycoside derived from base hydrolysis of either **1** or **2** was almost certainly delphinidin 3,7-diglucoside. It showed co-chromatography (HPLC, TLC) with one of the pigments in a mixture derived from partial degradation of *Felicia* anthocyanin (delphinidin 3-rutinoside 7-(6-*O*-malonylglucoside)) (Bloor, 1999), and was clearly different (HPLC, on-line spectrum, TLC) from delphinidin 3-rutinoside or the more common diglucoside, delphinidin 3,5-diglucoside.

Thus, pigments **1** and **2** must be assembled from an anthocyanin and a flavonol with a covalent linkage. The relative ease with which this covalent linkage could be broken suggested the involvement of an aliphatic diacid. The most likely linkage was a succinic acid unit as 100 mass units remained after subtraction of the flavonol glycoside molecular weight, a *p*-coumaroyl group and a delphinidin diglucoside, from the molecular mass of **1** (and allowing for linkage).

Although a certain amount of structural information could be obtained from the NMR spectra of the purified pigments, we resorted to degradation studies to cleave the molecules into more easily studied sections. Partial acid hydrolysis of a crude mixture of **1** and **2** gave a mixture of two main anthocyanins, **5** and **6** (see structure), as well as the two flavonol glycosides. The HPLC on-line UV–Vis spectra of **5** and **6** showed they had similar spectra and had clearly retained the *p*-coumaric acid group. NMR and mass spectra of purified **5** and **6** revealed **6** to be the succinylated version of **5**. The structure of **5** was determined to be delphinidin 3-(6-*p*-coumaroylglucoside) 7- glucoside. The key anomeric proton signal of the acylated glucose was shown to be that on the 3-sugar by an NOE with the delphinidin H-4 and linked via a TOCSY experiment to the C-6 methylene protons which resonated at 4.5–4.4 ppm due to acylation by the *p*-coumaric acid. In **6**, both glucose 6-hydroxyls appeared to be acylated. Signals due to an extra methylene unit were present in the same region as the first and could be linked (TOCSY) to the anomeric proton of the glucose at the delphinidin C-7 position (NOE with H-6, H-8). Thus, the pigments **1** and **2** are delphinidin 3-*O*-(6-*O*-*p*-coumaroyl glucoside) 7-*O*-(6-succinyl glucoside) linked to kaempferol-3,4′-diglucoside 7-xyloside and kaempferol-3,7,4′-triglucoside, respectively.

There only remained the question of the site on the flavonol glycoside to which the succinyl group was attached. Once again some degradation studies revealed which sugar of the flavonol triglycoside is involved. The relative rates of acid hydrolysis of sugars attached to flavonols has been well studied (Harborne, 1965). Under normal conditions a 3-sugar can be selectively cleaved in the presence of 7 or 4′ substituents. Mild acid hydrolysis of the two *Agapanthus* flavonols **3** and **4** showed this was true for **4**, i.e. mild acid treatment yielded kaempferol 7, 4′-diglucoside, but for **3** the 7- xylose and the 3-glucoside are lost at about the same rate so mild hydrolysis yields kaempferol-4′-glucoside. This selectivity allowed the determination of the linkage sugar. Mild acid hydrolysis of the anthocyanin **2** gave as an initial hydrolysis product — an anthocyanin with an extra absorption peak at 370 nm (from HPLC on-line spectra) characteristic of the anthocyanin linked to a flavonol lacking a substituent at 3-OH (Mabry, Markham & Thomas, 1970). Further hydrolysis yielded **5** and **6** and kaempferol 7,4′ diglucoside. Therefore, the linkage is via the 7 or 4′ sugar. The mixture of products from mild hydrolysis of the major pigment **1** was more complex. However, it was

Table 1
NMR data for **1**

	^{1}H shift (ppm, *m*, *J*)		^{13}C (ppm)[a]	NOE
Delphinidin	H-4	8.60, *s*	133.5	Glc-A H-1
	H-2′,6′	7.72, *s*	113.3	
	H-6	6.45, *d*, 2	104.5	Glc-B H-1
	H-8	6.77, *d*, 2	95.5	Glc-B H-1
p-Coumaroyl	H-7	7.33, *d*, 16	146.8	
	H-8	6.16, *d*, 16	114.6	
	H-3,5	6.69, *d*, 8.5	116.6	
	H-2,6	7.16, *d*, 8.5	131.1	
Kaempferol	H-6	6.35, *d*, 2	101.0	xyl H-1
	H-8	6.39, *d*, 2	96.0	xyl H-1
	H-2′,6′	8.13, *d*, 9	132.0	
	H-3′,5′	7.11, *d*, 9	117.5	Glc-C H-1
Glc-A	H-1	5.39, *d*, 7.6	102.1	
	H-6	4.55, *d*; 4.41, *dd*	64.2	
Glc-B	H-1	4.85, *d*, 7.8	101.5	
	H-6	4.38, *d*; 4.27, *dd*	65.2	
Glc-C	H-1	4.91, *d* (obsc)		
	H-6	4.61, *d*; 3.83, *dd*	65.5	
Glc-D	H-1	5.22, *d*, 7.6	103.7	
	H-6	3.66, 3.48	62.4	
Xyl	H-1	4.79, *d*, 6.6	101.8	
	H-5	3.98, 3.36	66.8	

[a] Only those carbons connected to protons of interest are presented. These carbons shifts were determined from a HMQC experiment.

clear from relative retention times and on-line spectral data that products consistent with the anthocyanin being attached to the flavonol via a 4′-sugar were present, ie. three such products, due to loss of 7-xylose or 3-glucose or both sugars, were seen and the later two had the additional absorption at 370 nm. These results can only be explained if the succinyl group is linked to the 4′-glucose of the kaempferol glycoside. The relative rates of hydrolysis are 3-glucose > 7-xylose > hydrolysis of succinyl ester bond > 4′-glucose. This assignment of linking sugar was supported by data from NMR experiments on the major pigment, **1** (see Table 1). An NOE between one of the glucose anomeric protons and the kaempferol H-3′,5′ placed this glucose at the 4′ position of the kaempferol. This proton could then be connected using a TOCSY experiment to the two C-6 protons (3.83 and 4.61 ppm). These protons are attached (HMQC) to a carbon resonating at 65.5 ppm, a shift consistent with succinyl acylation. The other signals in the spectrum of **1** are very similar to those seen in the NMR spectra of the separate parts, **6** and **3**, of the molecule.

Thus, the structures of **1** and **2** are the succinate diesters of delphinidin 3-(6-*p*-coumaroylglucoside) 7-glucoside and kaempferol 3,4′-diglucoside 7-xyloside or kaempferol 3,4′,7-triglucoside respectively, linked via the 6 positions of the glucoses at the 7 position of the anthocyanin and 4′ position of the flavonol.

Further confirmation of the structure of **1** was provided by chemical analysis of the glycosidic components. The types of sugar units present were determined by constituent sugar analysis of **1** (by gc/ms analysis of alditol acetate derivatives). Only xylose and glucose were detected, as expected. The molar ratio was 1:3.3, close to that anticipated for structure **1**. The position of glycosidic and certain other substituents, such as sulfate ester groups, can be determined by methylation analysis. This method has been used previously to determine the position of glycoside branching on a triglycoside from *Daucus carota* L. anthocyanin (Glässgen, Hofmann, Emmerling, Neumann & Seitz, 1992). However, under the traditional, basic conditions used for methylation, acyl groups are cleaved so no information on the substitution position of such groups can be obtained. Traditional methylation analysis of anthocyanin **1** produced predominantly T-Glc (i.e. 1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl glucitol, 75 mol%) corresponding to terminal glucose, as expected. T-Xyl (i.e. 1,5-di-*O*-acetyl-1-deuterio-2,3,4-tri-*O*-methyl xylitol, 12 mol%) corresponding to terminal xylose was also detected.

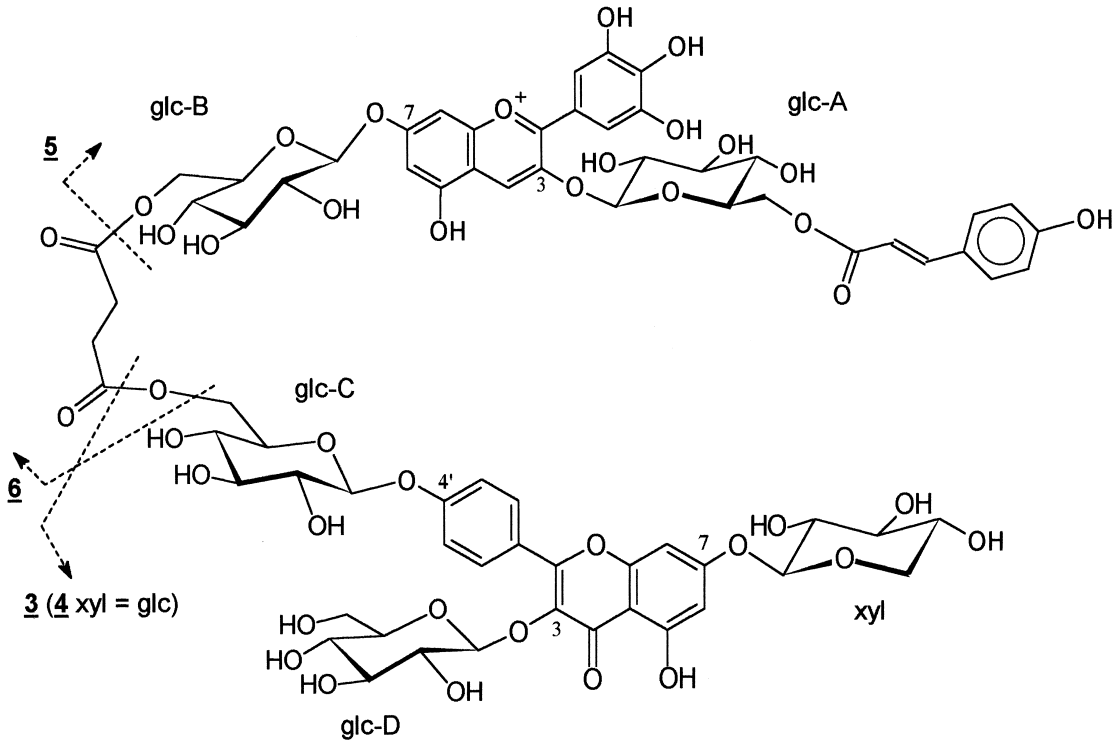
Methylation can be undertaken under conditions where acyl groups are stable (Prehm, 1980). This "neutral" methylation technique was also applied to **1**. In this case, the predominant species obtained was 6-Glc (44 mol%). This would indicate acyl substitution at the 6-position of some of the glucose units. The other species detected were T-Xyl (19 mol%), T-Glc (27 mol%), and small amounts of 2-Glc (5 mol%) and 2,6-Glc (i.e. 1,2,5,6-tetra-*O*-acetyl-1-deuterio-3,4-di-*O*-methyl glucitol, 5 mol%). The proportion of T-Glc relative to 6-Glc was higher than expected for structure **1** (actual ratio 1:1.6, cf. theoretical value of 1:3). Additional T-Glc may arise from partial cleavage of the succinate diester even under the "neutral" conditions used. Evidence for this was obtained by neutral methylation of cyanidin 3,5-diglucoside 6″,6″ malyl diester from carnation flowers (Bloor, 1998). This species should yield only 6-Glc after "neutral" methylation but instead both T-Glc and 6-Glc were obtained, in a ratio of 1:1.4. This indicates that one half of the diester is cleaved even under "neutral" methylation conditions. If the results for **1** are adjusted to allow for this, then the ratio of T-Glc:6-Glc is 1:3.5 close to that expected for the structure shown for **1**. The major species observed by this analysis support the structure of **1** obtained above from NMR and degradation studies. The presence of minor amount of other species may be due to structural variability or contamination in the sample.

These compounds represent unique examples of anthocyanin pigments where both types of co-pigment, an aromatic acyl group and a flavonoid co-pigment, are attached covalently to the anthocyanin. Previously only one compound where an anthocyanin is attached to a flavonoid co-pigment has been reported (Toki, Saito, Limura, Suzuki & Honda, 1994), although evidence exists for the occurrence of these anthocyanins in blue lupin (Takeda, Harborne & Waterman, 1993) and orchids (Strack, Busch & Klein, 1989).

3. Experimental

3.1. General

NMR experiments were run at 500 MHz or at 300 MHz (75 MHz for ^{13}C). Anthocyanin samples were dissolved in 2% CF_3COOD in CD_3OD. Flavonols were run in DMSO-d_6. Mass spectra were obtained using a VG 70-250S (FAB) or VG Platform II (Electrospray) instrument. RP HPLC analyses were performed as described previously (Bloor, 1997) using a Waters 600 solvent delivery system coupled to a Waters 994 PDA detector. GC/EIMS was performed using a Hewlett-Packard MSD 5970 instrument fitted with an HP Ultra-2 column (2 m × 0.20 mm) at 100°C (3 min), 35°C/min to 180°C (2 min), 50°C/min (15 min), and electron impact voltage of 70 eV. Helium was used as the carrier gas as 50 ml/min and 8 psi head pressure.



Structure of Agapanthus pigment 1 (2, xyl = glc)

3.2. Isolation of anthocyanins and flavonols

Freshly picked blue petals of *Agapanthus praecox* sp. *orientalis* "blue" from roadside plantings in Wellington, NZ, were extracted by soaking in 0.1% aq. TFA. The filtered extracts were absorbed onto a large Diaion HP-20 column, washed with 0.1% aq. TFA and the pigment mixture then eluted with MeOH–TFA–H_2O (80:0.1:19.9), and freeze-dried after evaporation of the MeOH. The flavonols were separated from the anthocyanin mixture using Avicel CC: flavonols are eluted first with *t*-BuOH:HOAc:H_2O (3:1:1), followed by the anthocyanins with HOAc:H_2O (15:85). The flavonols were further purified using RP CC with a Merck Lobar® column. The anthocyanins were passed through a Sephadex G-25 column, eluted with MeOH–TFA–H_2O (30:0.1:69.9). The major pigment fraction was freeze-dried and further separated using RP CC (Merck Lobar®, CH_3CN–TFA–H_2O (15:0.2:84.8)). Final purification was achieved with Toyopearl HW-40F gel CC using MeOH–TFA–H_2O (40:0.1:59.9).

3.3. Degradation experiments

Partial acid hydrolysis was performed by dissolving samples in 1N TFA and heating on a steam bath. Aliquots were removed after 1, 2, 5 and 10 min and immediately frozen. Base hydrolysis was performed using 2N NaOH in the absence of air for 1 h. Samples were analysed by HPLC.

3.4. Glycosidic analysis.

Methylated samples were prepared from **1** or cyanidin 3,5-diglucoside 6′,6″ malyl diester from carnation flowers (Bloor, 1998), using basic (Stevenson & Furneaux, 1991) or neutral (Prehm, 1980) methylation conditions. Native anthocyanin **1** (0.05 mg) or these methylated samples were hydrolysed in sealed tubes with aqueous TFA (2 M, 0.25 ml) for 1 h at 120°C. After cooling, the solvent was evaporated at 40°C in a stream of dry air. Two portions of toluene (~0.5 ml) were added and the samples evaporated to dryness to remove residual traces of acid.

Reduction was performed by adding aqueous $NaBD_4$ (0.25 ml, 15 mg/ml in aqueous 1M NH_4OH) to the samples followed by heating at 50°C for 1 h. After cooling, acetone (0.25 ml) was added to quench excess reductant and the samples were evaporated to dryness in a stream of dry air at 40°C. CH_3CN (~0.4 ml) was then added and evaporated. The resulting alditols were acetylated using glacial HOAc (0.04 ml), EtOAc (0.2 ml), Ac_2O (0.6 ml) and $HClO_4$ (60%, 0.023 ml). After 15 min at room temperature, water (2

ml) and 1-methylimidazole (0.04 ml) were added to decompose the excess Ac_2O. The mixture was extracted with CH_2Cl_2 (1 ml), and the organic layer was washed with water (4 ml), aqueous Na_2CO_3 (0.5 M, 4 ml) and water (4 ml). Each time, the phases were separated by low speed centrifugation and the aqueous phase drawn off and discarded. The organic layer was then removed to a clean tube and evaporated at 40°C under a stream of dry air. The residue was dried by addition and evaporation of CH_3CN (~0.4 ml) evaporated at 40°C under a stream of dry air. The resulting (partially methylated) alditol acetates were dissolved in acetone (0.05 ml). Derivatised samples were analysed by GC/EIMS. Partially methylated alditol acetates were identified by comparison of their retention times with those of authentic standards and mass spectral data (Carpita & Shea, 1988).

3.5. (6‴-O-(Delphinidin 3-O-(6″-O-p-coumaroyl-glucoside) 7-O-glucosyl)) (6⁗-O-(kaempferol 3-O-glucoside, 7-O-xyloside, 4′-O-glucosyl))succinate, **1**

UV–Vis (HPLC on-line) 274, 283, 313, 541 nm, $E_{313/541} = 1.23$. LR FABMS(+) 1598 (MH^+), 1619 (M + Na). ESMS 1597.6 ($C_{72}H_{77}O_{41}$ = 1597.39). ^{1}H-NMR and ^{13}C-NMR data, see Table 1.

3.6. (6‴-O-(Delphinidin 3-O-(6″-O-p-coumaroyl-glucoside) 7-O-glucosyl)) (6⁗-O-(kaempferol 3,7-di-O-glucoside, 4′-O-glucosyl))succinate, **2**

UV–Vis (HPLC on-line) 274, 283, 313, 541 nm, $E_{313/541} = 1.20$. ESMS 1627.6.

3.7. Kaempferol 3,4′-di-O-glucoside-7-O-xyloside, **3**

UVλ_{max}(MeOH) 270, 322, 345; +NaOMe 284, 380; +$AlCl_3$ 274, 300, 342; +$AlCl_3$–HCl 278, 300, 338; +NaOAc 270, 322, 345. ESMS 742.8 ($C_{32}H_{38}O_{20}$ = 742.2). ^{1}H-NMR; 8.20 (2H, *d*, *J* = 8.9 Hz, H-2′,6′),7.20 (2H, *d*, *J* = 8.9 Hz, H-3′,5′), 6.84 (*d*, *J* = 1.7 Hz, H-8), 6.45 (*d*, *J* = 1.7 Hz, H-6), 5.49 (*d*, *J* = 7.1 Hz, H-1 of 3-glc), 5.11 (*m*, H-1 of xyl), 5.04 (*d*, *J* = 6.9 Hz, H-1 of 4′-glc). ^{13}C-NMR; 177.8 (C-4), 162.8 (C-7), 160.9 (C-5), 159.4 (C-4′), 156.1 (C-9), 156.1 (C-2), 134.1 (C-3), 130.7 (C-2′,6′), 123.6 (C-1′), 115.8 (C-3′,5′), 105.9 (C-10), 99.4 (C-6), 94.5 (C-8); sugars: glc-3; 100.0, 73.3, 76.5, 69.7,77.1, 60.9. xyl-7; 100.8, 74.2, 75.3, 69.2, 65.8. glc-4′; 100.2, 73.0, 76.6, 70.0, 77.6, 60.7.

3.8. Delphinidin 3-O-(6″-O-p-coumaroyl-glucoside) 7-O-glucoside, **5**

UV–Vis (HPLC on-line) 283, 310(*sh*), 528 nm, $E_{283/528} = 1.0$. ESMS 773.1 ($C_{36}H_{37}O_{19}$ = 773.2). ^{1}H-NMR; 8.80 (*s*, H-4), 7.82 (2H, *s*, H-2′,6′), 7.40 (*d*, *J* = 16 Hz, *p*-coum H-7), 7.26 (2H, *d*, *J* = 8.6 Hz, *p*-coum H-2,6), 7.15 (*s*, H-8), 6.77 (2H, *d*, *J* = 8.6 Hz, *p*-coum H-3,5), 6.63 (*s*, H-6), 6.24 (*d*, *J* = 16 Hz, *p*-coum H-8), 5.44 (*d*, *J* = 7.8 Hz, glc-3 H-1), 5.06 (*d*, *J* = 5.8 Hz, glc-7 H-1), 4.53 (*dd*, *J* = 12, 1.7 Hz, glc-3 H-6a), 4.39 (*dd*, *J* = 12, 8 Hz, glc-3 H-6b).

3.9. Delphinidin 3-O-(6″-O-p-coumaroyl-glucoside) 7-O-(6‴-O-succinyl-glucoside), **6**

UV–Vis (HPLC on-line) 283, 310(*sh*), 526 $E_{283/526}$ = 1. ESMS 873.1. ^{1}H-NMR; 8.83 (*s*, H-4), 7.82 (2H, *s*, H-2′,6′), 7.39 (*d*, *J* = 16 Hz, *p*-coum H-7), 7.26 (2H, *d*, *J* = 8.6 Hz, *p*-coum H-2,6), 7.16 (*s*, H-8), 6.79 (2H, *d*, *J* = 8.6 Hz, *p*-coum H-3,5), 6.57 (*s*, H-6), 6.23 (*d*, *J* = 16 Hz, *p*-coum H-8), 5.46 (*d*, *J* = 7.8 Hz, glc-3 H-1), 5.18 (*d*, *J* = 5.8 Hz, glc-7 H-1), 4.6-4.4 (3H, *m*, glc-3 H-6a,6b, glc-7 H-6a), 4.34 (*dd*, *J* = 12, 8 Hz, glc-7 H-6b), 2.60 and 2.46 (each 2H, *m*, succinyl CH_2–CH_2).

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