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Anthocyanins from red flowers of *Camellia* cultivar 'Dalicha'

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ABSTRACT

Five anthocyanins, cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*Z*)-*p*-coumaroyl)-β-galactopyranoside (**2**), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-*p*-coumaroyl)-β-galactopyranoside (**3**), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-caffeoyl)-β-galactopyranoside (**4**), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-acetyl)-β-galactopyranoside (**5**), and cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-acetyl)-β-glucopyranoside (**6**), together with the known cyanidin 3-*O*-(2-*O*-β-xylopyranosyl)-β-galactopyranoside (**1**), were isolated from red flowers of *Camellia* cultivar 'Dalicha' (*Camellia reticulata*) by chromatography using open columns. Their structures were subsequently determined on the basis of spectroscopic analyses, i.e., ^{1}H NMR, ^{13}C NMR, HMQC, HMBC, HR ESI-MS and UV–vis.

1. Introduction

'Dalicha' (Tali camellia) has the largest flowers, up to 22 cm in diameter, of all the Yunnan camellias. It also has large thick leaves, strong axillary bud growth, and a loose branching pattern. It is commonly cultivated in the cities of Kunming, Chuxiong, Dali, and Tengchong, Yunnan Province, China. The flowering season is from early January to late March. There are several large 'Dalicha' trees more than 100 years old in Chuxiong, Dali, and Tengchong (Feng et al., 1986). It is believed that the wild species, *Camellia reticulata*, generated 'Dalicha' as a naturally occurring variety, described as '*C. reticulata* cv. Queen of Dali' (Ming 2000).

Hayashi and Abe (1953) were the first to report the isolation of petal anthocyanins in the genus *Camellia*. Cyanidin and delphinidin 3-*O*-β-glucoside, and 3-*O*-(6-*O*-(*E*)-*p*-coumaroyl)-β-glucoside were isolated from red flowers of *C. hiemalis*, *C. japonica*, and *C. sasanqua* (Saito et al., 1987). Recently, delphinidin 3-*O*-β-glucoside, delphinidin 3-*O*-β-galactoside, and delphinidin 3-*O*-(6-*O*-(*E*)-*p*-coumaroyl)-β-galactoside were identified in red leaves of Benibana-cha (*C. sinensis*) (Terahara et al., 2001). Sakata et al., first found cyanidin 3-galactoside in *C. japonica* ssp. *rusticana* (1986) and predicted the presence of various kinds of anthocyanins based on results of TLC and HPLC (1988). Lately, we have reported the known ten and seven anthocyanins isolated from the red flowers of wild species of *C. reticulata* (Li et al., 2007) and *C. saluenensis* (Li et al., 2008), respectively. Despite these efforts, the chemical taxonomy of the genus *Camellia* is still unclear, and the anthocyanin composition of 'Dalicha' has yet to be reported.

In this paper, we have focused on the isolation and the elucidation of the structure of five new anthocyanins from red flowers of 'Dalicha' using chemical and spectroscopic methods. Since the floral anthocyanins are considered to be the key products that can provide the information on the plant taxonomic distribution, it is thus required completely to clarify floral anthocyanins of *Camellia* species. Despite the former efforts, little is known for anthocyanin distribution of the camellia flowers. Furthermore, according to our recent examinations, floral flavonoids could also provide the basis for the plant chemotaxonomy based on the principal component and cluster analyses (Wang et al., 2001, 2004). In addition, the distribution of anthocyanins in the red flowers of 'Dalicha' was examined by HPLC, and the biogenesis of floral anthocyanins is discussed here for the first time.

2. Results and discussion

The HPLC analysis revealed the presence of several major and minor anthocyanins in 'Dalicha' flowers (Fig. 2). Among them, six anthocyanins (**1**–**6**) were isolated as amorphous powders of acetic acid salts, using repeated open column chromatography (CC) with reversed phase gels such as MCI-gel CHP-20P, Sephadex LH-20, and ODS gel, with a combination of 5% AcOH–H_2O and 5% AcOH–MeOH as elution solvents (Fig. 1).

In order to determine the chemical structure of these anthocyanins, ^{1}H NMR, ^{13}C NMR, high resolution (HR) ESI-MS, and acidic and alkaline hydrolyses were performed. For NMR measurements,

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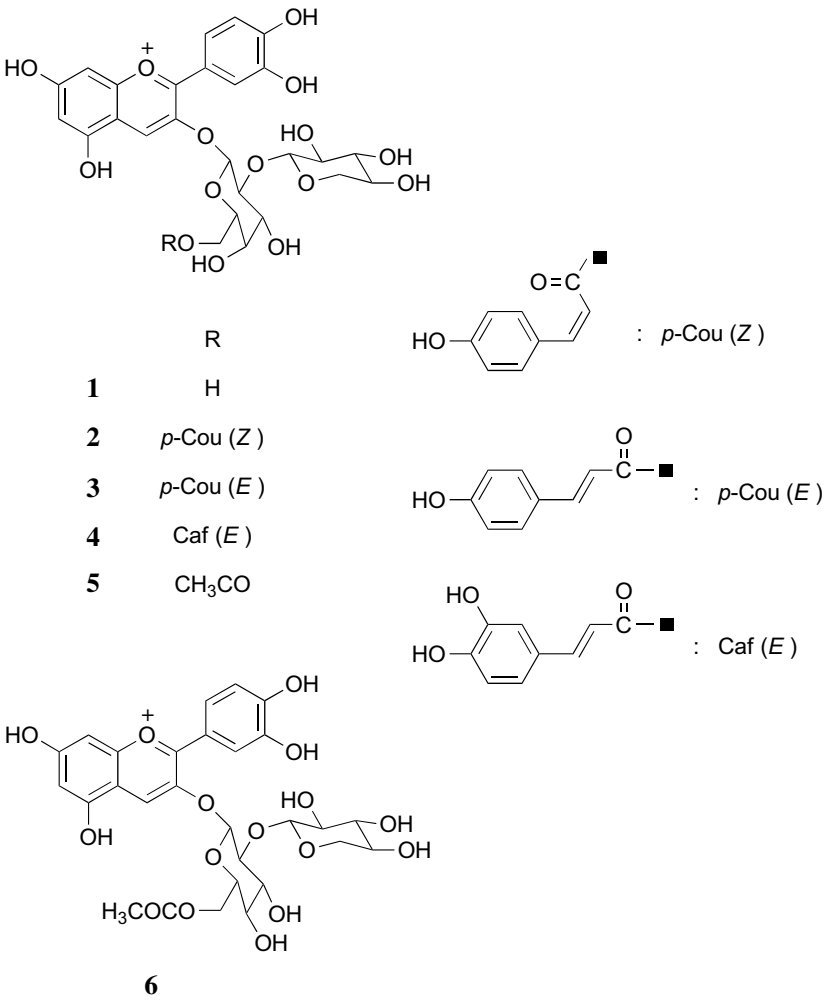

Fig. 1. Structure of anthocyanins isolated from red flowers of 'Dalicha'.

long-range correlation spectra such as the 2D field-gradient heteronuclear multiple quantum correlation (FG-HMQC) spectrum and the heteronuclear multiple bond correlation (HMBC) spectrum were recorded to investigate the linkages between the aglycone, sugars, and acyl units.

The six isolated anthocyanins are: cyanidin 3-*O*-(2-*O*-β-xylopyranosyl)-β-galactopyranoside (**1**), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*Z*)-*p*-coumaroyl)-β-galactopyranoside (**2**), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-*p*-coumaroyl)-β-galactopyranoside (**3**), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-caffeoyl)-β-galactopyranoside (**4**), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-acetyl)-β-galactopyranoside (**5**), and cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-acetyl)-β-glucopyranoside (**6**). Pigment **1** was first found by Harborne (1963), and matches the data of Jarman and Crowden (1974) and Terahara et al. (1992). Pigment **1** was also isolated lately from the red flowers of the wild *C. reticulata* (Li et al., 2007). Pigments **2–6** are novel anthocyanins, and their isolations as well as the elucidation of their structure are presented here for the first time (Fig. 1). Their chemical structures were completely determined through the following procedures and observations.

The ^{1}H NMR spectrum of **2** showed in the downfield area three proton singlet signals at δ 8.64 (*s*, H-4), δ 6.56 (*d*, *J* = 2.0 Hz, H-6), and δ 6.62 (*d*, *J* = 2.0 Hz, H-8) arising from the flavilium A- and C-rings (Table 1). Furthermore, the three protons at δ 7.97 (*d*, *J* = 2.0 Hz, H-2′), δ 7.02 (*d*, *J* = 8.9 Hz, H-5′), and δ 8.19 (*dd*, *J* = 8.9, 2.0 Hz, H-6′) are due to the ABX-type signals arising from the flavilium B-ring, suggesting that the structure is a cyanidin core. Two anomeric protons were found at δ 4.74 (*d*, *J* = 7.6 Hz) and δ 5.45 (*d*, *J* = 7.6 Hz). In the ^{13}C NMR spectrum of **2**, six carbon signals at δ 100.7 (C″-1), 80.3 (downfield shift, C″-2), 75.3 (C″-5), 74.9 (C″-3), 70.1 (C″-4) and 64.3 (C″-6) were observed, suggesting that the sugar is a hexose (Table 2). Furthermore, due to the small coupling constant at δ 4.00 (*br. d*, *J* = 3.4 Hz) of the hexose H-4, the sugar is assumed to be galactose. In addition, five carbon signals were observed at δ 106.4 (C‴-1), 77.9 (C‴-3), 75.3 (C‴-2), 71.0 (C‴-4), and 67.3 (C‴-5), suggesting the presence of a pentose sugar. The pentose was deduced to be a pyranose, given the chemical shift of the oxyomethylene carbon signal at δ 67.3 (C‴-5). Since, the hexose and pentose were assumed, respectively, to be galactose and either xylose or arabinose based on the assignment of proton and ^{13}C NMR spectroscopic signals, acidic and alkaline hydrolyses were performed to confirm our assumptions. When subject to HCl and NaOMe hydrolyses, **2** gave cyanidin, galactose, xylose and methyl (*Z*)-*p*-coumaroate.

In the ^{1}H NMR spectrum, the A_2B_2-type aromatic signals at δ 6.47 (*d*, *J* = 8.9 Hz, 2H) and 7.29 (*d*, *J* = 8.9 Hz, 2H) as well as each doublet signal at δ 5.70 (*d*, *J* = 13.0 Hz) and 6.52 (*d*, *J* = 13.0 Hz) were observed. The HMQC spectrum indicates that the A_2B_2-type aromatic signals are coupled with ^{13}C resonances at δ 115.8 and 133.5, and that the doublets are coupled with signals at δ 115.7 and 144.4, respectively. Also, the ^{13}C NMR spectrum revealed three

Table 1
^{1}H NMR spectroscopic data for anthocyanins **1–6** (δ and J, in acidified CD_3OD)

Pigments	1	2	3[a]	4	5	6
Aglycone						
	Cyanidin					
4	8.82 *s*	8.64 *s*	8.82 *s*	8.83 *s*	8.87 *s*	8.76 *s*
6	6.58 *br.s*	6.56 *d* 2.0	6.71 *br.s*	6.50 *br.s*	6.65 *d* 1.8	6.63 *d* 1.9
8	6.80 *br.s*	6.62 *d* 2.0	6.90 *br.s*	6.78 *br.s*	6.89 *d* 1.8	6.82 *d* 1.9
2′	7.88 *d* 2.0	7.97 *d* 2.0	8.00 *d* 2.0	8.02 *d* 2.0	8.03 *d* 2.4	7.90 *d* 2.2
5′	6.90 *d* 8.7	7.02 *d* 8.9	7.03 *d* 8.9	7.01 *d* 8.8	7.02 *d* 8.7	6.95 *d* 8.7
6′	8.16 *dd* 8.7,2.0	8.19 *dd* 8.9,2.0	8.33 *dd* 8.9,2.0	8.24 *dd* 8.8,2.0	8.28 *dd* 8.7, 2.4	8.20 *dd* 8.7,2.2
Sugars						
	Galactose					Glucose
1″	5.39 *d* 7.5	5.45 *d* 7.6	5.61 *d* 7.5	5.43 *d* 7.5	5.41 *d* 7.7	5.42 *d* 7.7
2″	4.26 *dd* 9.2,7.5	4.28 *dd* 8.9,7.6	4.18 *dd* 9.2,7.5	4.27 *dd* 9.1,7.5	4.25 *dd* 9.4,7.7	3.98 *dd* 8.9,7.7
3″	3.97 *dd* 9.2,3.2	3.97 *dd* 8.9,3.4	3.86 *dd* 9.2,2.7	3.95 *dd* 9.1,2.7	3.93 *dd* 9.4,3.3	3.83 *dd* 9.1,8.9
4″	4.01 *br.d* 3.2	4.00 *br.d* 3.4	3.89 *br.d* 2.7	4.02 *br.d* 3.1	3.97 *dd* 3.3,0.7	3.49 *dd* 9.7,9.1
5″	3.89 *br.dd* 8.2,3.2	4.15 *br.dd* 8.9,3.4	4.23 *br.dd* 8.2,3.4	4.14 *br.dd* 8.9,3.3	4.11 *ddd* 8.6,3.3,0.7	3.86 *ddd* 9.7,7.3,1.9
6″a	3.84 *dd* 11.8,8.2	4.76 *dd* 11.7,8.9	4.32 *dd* 11.6,8.2	4.56 *dd* 11.6,8.9	4.36 *dd* 11.8,8.6	4.52 *dd* 12.2 1.9
6″b	3.82 *dd* 11.8,3.2	4.20 *dd* 11.7,3.4	4.25 *dd* 11.6,3.4	4.31 *dd* 11.6,3.3	4.28 *dd* 11.8,3.3	4.21 *dd* 12.2 7.3
	Xylose					
1‴	4.78 *d* 7.6	4.74 *d* 7.6	4.66 *d* 7.5	4.74 *d* 7.5	4.74 *d* 7.7	4.82 *d* 7.7
2‴	3.22 *dd* 8.5,7.6	3.23 *dd* 8.2,7.6	3.01 *dd* 8.2,7.5	3.24 *dd* 8.2,7.5	3.18 *dd* 8.2,7.7	3.22 *dd* 8.9,7.7
3‴	3.37 *dd* 8.9,8.5	3.36 *dd* 8.9,8.2	3.13 *dd* 8.9,8.2	3.35 *dd* 8.9,8.2	3.33 *dd* 8.9,8.2	3.35 *dd* 9.1,8.9
4‴	3.46 *ddd* 10.2,8.9,5.4	3.45 *ddd* 10.3,8.9,5.5	3.26 *ddd* 10.3,8.9,5.4	3.44 *ddd* 10.3,8.9,5.3	3.41 *ddd* 10.3,8.9,5.4	3.46 *ddd* 10.3,9.1,5.3
5‴a	3.73 *dd* 11.5,5.4	3.76 *dd* 11.7,5.5	3.52 *dd* 11.6,5.4	3.73 *dd* 11.8,5.3	3.68 *dd* 11.5,5.4	3.78 *dd* 11.5,5.3
5‴b	3.10 *dd* 11.5,10.2	3.12 *dd* 11.7,10.3	2.99 *dd* 11.6,10.3	3.11 *dd* 11.8,10.3	3.06 *dd* 11.5,10.3	3.12 *dd* 11.5,10.3
Acyl groups						
		Z-*p*-coumaroyl	*E*-*p*-coumaroyl	*E*-caffeoyl	Acetyl	Acetyl
2		7.29 *d* 8.9	7.42 *d* 8.5	6.88 br.*s*	2.04 *s*	2.04 *s*
3		6.47 *d* 8.9	6.81 *d* 8.5			
5		6.47 *d* 8.9	6.81 *d* 8.5	6.75 *m*		
6		7.29 *d* 8.9	7.42 *d* 8.5	6.75 *m*		
α		5.70 *d* 13.0	6.30 *d* 15.8	6.18 *d* 15.9		
β		6.52 *d* 13.0	7.46 *d* 15.8	7.34 *d* 15.9		

br.: broad, *s*: singlet, *d*: doublet, *dd*: double doublet, *m*: multiplet.
[a] δ and J: In acidified DMSO-d_6.

additional quaternary carbons at δ 127.2, 159.9, and 168.3 (COO), suggesting that they have a *cis*-*p*-coumaroyl group (Inami et al., 1996). The configuration of the double bond can be easily distinguished by the coupling constant of the olefinic protons of the typical AB-type spectrum, i.e., J = ca. 13 Hz for the *cis*-configuration and ca. 16 Hz for the *trans*-configuration (Nakatani et al., 1995; Silverstein and Webster 1998; Fossen et al., 2005).

The HMBC spectrum indicated that the anomeric proton of the galactose (δ 5.45, *d*, J = 7.6 Hz) has a correlation with the C-3 carbon of cyanidin (δ 145.1) and the anomeric carbon at δ 106.4 has a correlation with the H-2 proton of the galactose moiety (δ 4.28, *dd*, J = 8.9, 7.6 Hz). The quaternary carbon at δ 168.3 has a correlation with the H-6″ proton of the galactose moiety (δ 4.76, *dd*, J = 11.7, 8.9 Hz). Based on these spectroscopic data, we deduced that galactose is attached to the 3-position of cyanidin and xylose is attached to the 2-position of the galactose, and *cis*-*p*-coumaric acid is attached to the 6-position of the galactose as an ester. The anomeric protons at δ 4.74 (*d*, J = 7.6 Hz) of xylose and δ 5.45 (*d*, J = 7.6 Hz) of the galactose were both found to have a β-configuration according to their coupling constant. The HR ESI-MS of **2** gave a molecular ion peak at m/z 727.5258 $[M]^+$, which is in good agreement with the calculated value for $C_{35}H_{35}O_{17}$. Based on these spectroscopic analyses and hydrolysis, we concluded that pigment **2** is cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*Z*)-*p*-coumaroyl)-β-galactopyranoside (**2**).

Pigment **3** was obtained as a reddish amorphous powder. Its ^{1}H NMR spectrum showed signals analogous to those found in **2** with the only difference being the *trans*-configuration for the *p*-coumaroyl group due to the doublet signals at δ 6.30 and 7.46 (each 1H, *d*, J = 15.8 Hz). Acidic and alkaline hydrolyses were performed to confirm the aglycone, sugar and phenolic acid composition of **3**, and hydrolysis gave cyanidin, galactose, xylose and methyl (*E*)-*p*-coumaroate, respectively. The HR ESI-MS of **3** gave a molecular ion peak at m/z 727.5553 $[M]^+$, which was in good agreement with the calculated mass for $C_{35}H_{35}O_{17}$. Based on these spectroscopic observations, and acidic and alkaline hydrolyses, **3** was identified as cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-*p*-coumaroyl)-β-galactopyranoside (**3**).

Pigment **4** was obtained as a reddish amorphous powder. The ^{1}H NMR spectrum showed almost identical resonances to those for compound **3**, except for a set of ABX-type aromatic signals at δ 6.88 (*br. s*, 1H) and 6.75 (*m*, 2H), and a set of doublet resonances at δ 6.18 and 7.34 (each 1H, *d*, J = 15.9 Hz) that were different. The HMQC spectrum showed that the ABX-type aromatic signals are coupled with ^{13}C-resonances at δ 115.5, 116.5, and 122.9, and the doublets are correlated with signals at δ 114.7 and 147.3, respectively. Furthermore, the ^{13}C NMR spectrum established that there are four additional quaternary carbons at δ 127.7, 146.8, 149.7 and 169.1 (COO). Taking into account all of these spectroscopic data, pigment **4** consists of pigment **1** coupled with an *E*-caffeoyl group (Chirol and Jay, 1995). The *E*-caffeoyl group is concluded to be attached to the 6-position of the galactose moiety due to the downfield shift observed in the ^{1}H NMR spectrum at δ 4.56 and 4.31 (each 1H, *dd*). The HR ESI-MS of **4** gave a molecular ion peak at m/z 743.5669 $[M]^+$, which is in good agreement with the calculated mass for $C_{35}H_{35}O_{18.}$ Based on these observations, we concluded that **4** is cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-caffeoyl)-β-galactopyranoside (**4**).

Pigment **5** was obtained as a reddish amorphous powder. In its ^{1}H NMR spectrum, **5** showed similar signals to **1** (Table 1). However, in the high magnetic field area, a singlet at δ 2.04 (*s*, 3H) was coupled with the ^{13}C-signals at δ 20.8 in the HMQC spectrum.

Table 2
^{13}C NMR spectroscopic data for anthocyanins **1–6** (δ, in acidified CD_3OD)

Pigments	1	2	3[a]	4	5	6
Aglycone						
	Cyanidin					
2	163.3	163.4	162.5	164.1	164.2	164.0
3	145.2	145.1	144.6	145.0	145.4	145.1
4	135.5	134.5	134.3	136.0	135.3	135.6
5	159.1	159.1	158.3	159.5	159.1	159.1
6	103.5	103.4	103.4	103.7	103.4	103.5
7	170.3	170.3	169.1	170.5	170.4	170.6
8	95.2	95.1	95.0	95.2	95.2	95.3
9	157.2	157.1	156.6	157.5	157.6	157.5
10	113.1	113.1	112.5	113.1	113.1	113.0
1′	121.1	121.2	120.6	121.3	121.3	121.2
2′	118.2	118.7	118.4	118.7	118.7	118.4
3′	147.3	147.5	147.2	147.5	147.6	147.6
4′	155.8	155.9	155.5	155.9	156.0	156.1
5′	117.4	117.5	117.5	117.5	117.5	117.5
6′	128.9	128.7	128.5	128.7	128.8	129.1
Sugars						
	Galactose					Glucose
1″	101.8	100.7	99.7	101.4	101.7	101.4
2″	79.8	80.3	79.3	80.2	79.9	81.4
3″	75.2	74.9	74.2	74.8	74.9	78.1
4″	70.2	70.1	69.0	70.1	70.2	71.2
5″	77.8	75.3	74.1	75.1	75.2	76.1
6″	62.4	64.3	64.7	64.5	65.2	64.8
	Xylose					
1‴	105.9	106.4	105.7	106.3	106.1	105.8
2‴	75.9	75.3	75.3	75.9	75.9	75.8
3‴	77.9	77.9	77.6	77.9	77.9	78.0
4‴	71.0	71.0	70.4	71.1	71.0	71.1
5‴	67.3	67.3	67.1	67.3	67.3	67.4
Acyl groups						
		C(*Z*)	C(*E*)	Caf(*E*)	Ac	Ac
1		127.2	126.0	127.7		
2		133.5	131.3	115.5	20.8	20.8
3		115.8	116.8	149.7		
4		159.9	161.0	146.8		
5		115.8	116.8	116.5		
6		133.5	131.3	122.9		
α		115.7	113.6	114.7		
β		144.4	146.1	147.3		
C=O		168.3	167.7	169.1	172.9	172.9

C(*Z*): *Z*-*p*-coumaroyl; C(*E*): *E*-*p*-coumaroyl; Caf(*E*): *E*-cafeoyl; Ac: acetyl.
[a] δ: In acidified DMSO-d_6.

Furthermore, the ^{13}C-NMR spectrum shows that there is an additional quaternary carbon at δ 172.9 (COO). The acyl group is considered to be an acetyl group (Hosokawa et al., 1995; Fossen et al., 1997), and it is attached to the 6-position of galactose due to the downfield shift at δ 4.36 and 4.28 (each 1H, *dd*). The HR ESI-MS of **5** gave a molecular ion peak at *m*/*z* 623.3951 $[M]^+$, which is in good agreement with the calculated mass for $C_{28}H_{31}O_{16}$. Taking into account all of the spectroscopic data, we concluded that **5** is cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-acetyl)-β-galactopyranoside (**5**).

Pigment **6** was obtained as a reddish amorphous powder. Its ^{1}H NMR spectrum showed similar signals to those observed in pigment **5** (Table 1), the only difference being the 4″ and 3″-positions of the hexose moiety due to the triplet resonances at δ 3.49 (*dd*, *J* = 9.7,9.1 Hz, H-4″) and 3.83 (*dd*, *J* = 9.1,8.9 Hz, H-3″). The HMQC spectrum indicated that these signals are coupled with ^{13}C-resonances at δ 71.2 and 78.1. 2N HCl hydrolysis of **6** gave cyanidin, glucose and xylose. The HR ESI-MS of **6** gave a molecular ion at *m*/*z* 623.3868 $[M]^+$, which is in good agreement with the calculated mass for $C_{28}H_{31}O_{16}$. Taking into account all of the spectral data and acidic hydrolysis, we concluded that **6** is cyanidin 3-*O*-(2-β-xylopyranosyl-6-*O*-acetyl)-β-glucopyranoside (**6**).

A characteristic feature of the pigmentation in 'Dalicha' is the biogenesis of anthocyanins. The constitution of anthocyanins in the flowers of 'Dalicha' was determined by HPLC analysis, and at least twelve peaks were detected (Fig. 2). Except pigments **1**–**6**, known pigments, cyanidin 3-*O*-(2-*O*-β-xylopyranosyl)-β-glucopyranoside (Cy3GX) (Robinson and Robinson, 1931; Cabrita and Andersen, 1999; Du et al., 2004), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*Z*)-*p*-coumaroyl)-β-glucopyranoside (Cy3GXZ) (Inami et al., 1996; Nakatani et al., 1994), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-*p*-coumaroyl)-β-glucopyranoside (Cy3GXE) (Nakatani et al., 1995), cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-caffeoyl)-β-glucopyranoside (Cy3GXC) (Chirol and Jay, 1995), cyanidin 3,5-di-*O*-β-glucopyranoside (Cy3G5G) (Mas et al., 2000) and cyanidin 3-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (Cy3G5GE) (Goto et al., 1978) were detected (Fig. 2). The known sambubiosides, Cy3GXZ, Cy3GXE, Cy3GXC, were isolated from the red flowers of the wild *C. reticulata*, and the known 3,5-diglucosides, Cy3G5G, Cy3G5GE, were isolated from the red flowers of the wild *C. saluenensis* lately (Li et al., 2007, 2008). Thus, it can be said that there are three types of glycosylation, namely, glucosylation, galactosylation and xylosylation, and there are four kinds of aromatic acid-acylation, namely, (*Z*)-*p*-coumaroyl, (*E*)-*p*-coumaroyl, (*E*)-caffeoyl and acetyl derivatives, found

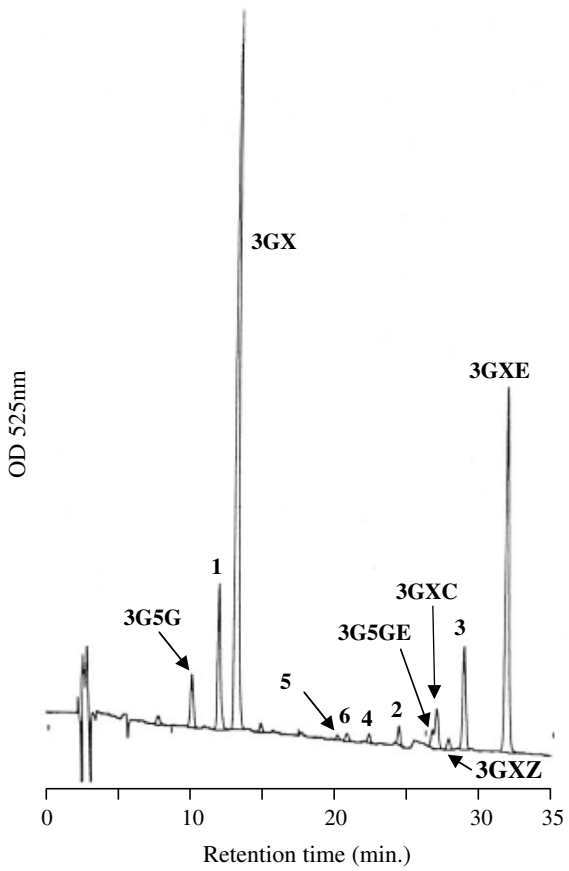

Fig. 2. HPLC chromatogram of an extract of 'Dalicha' flowers showing the relative abundance of compounds **1–6** compared to anthocyanins previously reported from the other *Camellia* species. Fresh petals of the 'Dalicha' were collected from 150 years old tree at Yong-An, Chuxiong City, Yunnan Province, China, in February 2002. Key to peaks: 3GX = cyanidin 3-*O*-(2-*O*-β-xylopyranosyl)-β-glucopyranoside, 3GXC = cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-caffeoyl)-β-glucopyranoside, 3GXZ = cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*Z*)-*p*-coumaroyl)-β-glucopyranoside, 3GXE = cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-*p*-coumaroyl)-β-glucopyranoside, 3G5G = cyanidin 3,5-di-*O*-β-glucopyranoside, and 3G5GE = cyanidin 3-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-glucopyranoside)-5-*O*-β-glucopyranoside.

in 'Dalicha' (Fig. 3). It should be noted that the acylation by acetic acid was firstly reported in red flowers of 'Dalicha' of the genus *Camellia*.

HPLC chromatography of 'Dalicha' flowers showed that there are at least 12 anthocyanins, and the major pigments are Cy3GX, Cy3GXE, **1** and **3** (Fig. 2). Cy3GX-series anthocyanins were also detected in the red flowers of the wild species *C. reticulata*, and the major pigments are also Cy3GX, Cy3GXE and **1** (Li et al., 2007). That is, *C. reticulata* lacks the pigment **3**. This suggests that the wild species *C. reticulata* is a closely-related parent of 'Dalicha' from a chemotaxonomical perspective, and that acylated anthocyanins play an important role in environmental adaptation of *C. reticulata* species and cultivars. We also believe that our findings could provide the basis for future chemotaxonomy of *Camellia* species and cultivars, and could provide for qualitative and quantitative analyses of many *Camellia* species and cultivars based on floral anthocyanin composition.

3. Concluding remarks

Pigments **2**–**6** from 'Dalicha' flowers are concluded to be novel anthocyanins, and their isolations as well as the elucidation of their structure are presented here for the first time. According to the structures of anthocyanins, there are three types of glycosylation, namely, glucosylation, galactosylation and xylosylation, and there are four kinds of aromatic acid-acylation, namely, (*Z*)-*p*-coumaroyl, (*E*)-*p*-coumaroyl, (*E*)-caffeoyl and acetyl derivatives, found in 'Dalicha'. The acylation of cyanidin glycosides by acetic acid was firstly reported in red flowers of 'Dalicha' of the genus *Camellia*. HPLC chromatography of 'Dalicha' flowers was compared to that of the wild species *C. reticulata*, and we suggest that *C. reticulata* and 'Dalicha' might be consanguineous. The data obtained here might be utilized for the future investigation that could provide the chemotaxonomy of all the red flowers of the genus *Camellia*.

4. Experimental

4.1. General procedures

Details of the instruments and chromatographic conditions used in this study were essentially the same as described in the previous report (Li et al., 2007, 2008). The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a NMR spectrometer (JEOL JNM-ECA600, JEOL, Japan, at the Venture Business Laboratory, Kagoshima University) and chemical shifts were expressed on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard (Tables 1 and 2). The solvents for NMR measurements were used with a combination of methanol-d_4 + 0.03% TMS (CD_3OD) or dimethylsulfoxide-d_6 (DMSO-d_6) and trifluoroacetic acid-*d* in a ratio of 9:1. For verifying the purity of each anthocyanin, HPLC (JASCO GULLIVER SERIES, Japan) was conducted at 525 nm with a TSK gel ODS-80Ts QA column (4.5 mm i.d. × 150 mm, Tosoh) at 40 °C. The flow rate was 0.8 ml min^{-1} with a linear flow gradient elution for 35 min, where solvent B (1.5% H_3PO_4-20% HCOOH-25% MeCN-5% tetrahydrofuran (THF) in H_2O, v/v) was increased linearly from 18% to 70% in solvent A (1.5% H_3PO_4 in H_2O, v/v). TLC was performed on precoated Kiesel gel 60 F_{245} (Merck) with a benzene–HCO_2Et–HCO_2H–H_2O system as described previously (Hashimoto et al., 2002). Briefly, combinations of around 4:1 to 1:4 of the following two solvent systems were used: solvent A, benzene–HCO_2Et–HCO_2H (1:7:1); and solvent B, HCO_2Et–HCO_2H–H_2O (3:1:1). UV–vis spectra were recorded on an MPS-2000 spectrophotometer (Shimadzu Co., Ltd, Japan) in 0.01% HCl–MeOH. HR ESI-MS was measured on a MAT900XL (Finnigan Inc., Japan; at the United Graduate School of Agricultural Sciences, Kagoshima University) in 10% AcOH–MeOH without a matrix in a positive mode.

4.2. Extraction and isolation

Fresh petals of 'Dalicha' were collected from a 150-year-old tree at Yong-An, Chuxiong City, Yunnan Province, China, in February 2002 and 2005. The petals were dipped into boiling water for 4–6 s to destroy polyphenol oxidase enzymatic activity, and were dried at room temperature (Sakata, 1988). The dried petals (ca. 3.5 kg) were immersed in AcOH–MeOH (1:1, v/v) (ca. 14 L) overnight (ca. 48 h) and the extracted solution were filtered. The extraction was repeated twice. The crude extracted solution was evaporated under reduced pressure to dryness, and the dried residue was preserved in a refrigerator at −20 °C. The crude extract was subjected to MCI gel CHP 20P CC with 5% AcOH–H_2O with increasing amounts of 5% AcOH–MeOH (from 0, 10, 20, to 100% of 5% AcOH–MeOH) to afford fractions. The fractions were further subjected to Sephadex LH-20 CC and ODS-gel CC repeatedly with 5% AcOH–H_2O and an increasing amounts of 5% AcOH–MeOH (from 0%, 5%, 10%, 15%, 20%, to 100% of 5% AcOH–MeOH) to furnish six reddish amorphous powders: **1**, 50 mg; **2**, 30 mg; **3**, 45 mg; **4**, 35 mg; **5**, 25 mg; **6**, 25 mg.

For HPLC analysis, fresh petals of 'Dalicha' were collected in Chuxiong, February 2002. Dried petals (45.8 mg) were extracted with 10 mL of an acidic solution (70% MeOH-2% HCO_2H-1% CF_3CO_2H-27% H_2O, v/v%) (Hashimoto et al., 2002), and twelve anthocyanins were detected in the extract by HPLC. Their retention times (min) in the HPLC chromatogram were as follows: 3G5G, 10.2; pigment **1**, 12.1; 3GX, 13.3; pigment **5**, 20.3; pigment **6**, 20.9; pigment **4**, 22.4; pigment **2**, 24.5; 3G5GE, 26.8; 3GXC, 27.1; 3GXZ, 27.9; pigment **3**, 29.0 and 3GXE, 31.9 (Fig. 2). The pigments, 3G5G and 3G5GE, were authenticated in comparison with the retention times of those isolated from the wild *C. saluenensis*, and the pigments, 3GX, 3GXC, 3GXZ and 3GXE, were authenticated in comparison with the retention times of those isolated from the wild *C. reticulata*.

4.3. Hydrolysis

Each anthocyanin (ca. 2 mg) was dissolved in 2 N HCl (ca. 4 mL) and the mixture was heated at 95 °C for 2 h. The hydrolyzed mixture was directly compared by TLC with the authentic sugars (glucose, galactose, mannose, rhamnose, xylose, fructose, fucose and arabinose). The following solvent systems for TLC were used for distinguishing sugars; BAW: *n*-BuOH–AcOH–H_2O (4:1:2), and

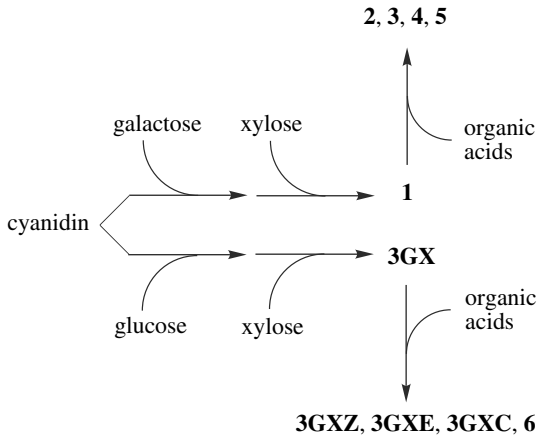

Fig. 3. Possible biogenesis of anthocyanins in red flowers of 'Dalicha'. Organic acids are referred to (*Z*)-*p*-coumaric, (*E*)-*p*-coumaric, (*E*)-caffeic and acetic acids.

$CHCl_3$–MeOH–H_2O (7:3:0.5), and the detection of sugars was achieved by spraying with 5% H_2SO_4, followed by heating. Acidic hydrolysis of pigments **1**–**5** gave cyanidin, xylose, and galactose, and pigment **6** gave cyanidin, xylose, and glucose.

Pigments **2**–**4** (ca. 1.5 mg) were treated with NaOMe in MeOH (0.3 mL) at room temperature. The hydrolysate was neutralized with 2 N HCl and the methyl esters, i.e., methyl (*Z*)-*p*-coumaroate, methyl (*E*)-*p*-coumaroate, and methyl (*E*)-caffeoate were detected by TLC with a benzene–EtOAc (9:1) solvent system, and the *Rf* values of each methyl ester were 0.266, 0.241, and 0.216, respectively.

4.4. HR ESI-MS and UV–vis data

Cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*Z*)-*p*-coumaroyl)-β-galactopyranoside (**2**). HR ESI-MS *m*/*z* 727.5258 $[M]^+$ (calc. for $C_{35}H_{35}O_{17}$ 727.5374). UV–vis λ_{max} 0.01% HCl–MeOH (nm) ($\log\varepsilon$): 284 (4.41), 311 (4.23), 534 (4.48); $AlCl_3$–MeOH: 289 (4.32), 311 (4.31), 405 (3.80), 574 (4.57). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

Cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-*p*-coumaroyl)-β-galactopyranoside (**3**). HR ESI-MS *m*/*z* 727.5553 $[M]^+$ (calc. for $C_{35}H_{35}O_{17}$ 727.5374). UV–vis λ_{max} 0.01% HCl–MeOH (nm) ($\log\varepsilon$): 284 (4.24), 312 (4.12), 531 (4.25); $AlCl_3$–MeOH: 290 (4.18), 313 (4.20), 407 (3.46), 571 (4.33). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

Cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-(*E*)-caffeoyl)-β-galactopyranoside (**4**). HR ESI-MS *m*/*z* 743.5669 $[M]^+$ (calc. for $C_{35}H_{35}O_{18}$ 743.5323). UV–vis λ_{max} 0.01% HCl–MeOH (nm) ($\log\varepsilon$): 284 (4.32), 331 (4.18), 531 (4.40); $AlCl_3$–MeOH: 289 (4.12), 315 (4.12), 355 (4.18), 567 (4.48). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

Cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-acetyl)-β-galactopyranoside (**5**). HR ESI-MS *m*/*z* 623.3951 $[M]^+$ (calc. for $C_{28}H_{31}O_{16}$ 623.4412). UV–vis λ_{max} 0.01% HCl–MeOH (nm) ($\log\varepsilon$): 283 (4.14), 335 (3.55), 381 (3.56), 531 (4.36); $AlCl_3$–MeOH: 287 (3.97), 312 (3.79), 403 (3.63), 572 (4.43). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

Cyanidin 3-*O*-(2-*O*-β-xylopyranosyl-6-*O*-acetyl)-β-glucopyranoside (**6**). HR ESI-MS *m*/*z* 623.3868 $[M]^+$ (calc. for $C_{28}H_{31}O_{16}$ 623.4412). UV–vis λ_{max} 0.01% HCl–MeOH (nm) ($\log\varepsilon$): 283 (4.22), 336 (3.56), 382 (3.65), 531 (4.45); $AlCl_3$–MeOH: 286 (4.09), 313 (3.89), 409 (3.71), 570 (4.52). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

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References

Cabrita, L., Andersen, Q.M., 1999. Anthocyanins in blue berries of *Vaccinium padifolium*. Phytochemistry 52, 1693–1696.

Chirol, N., Jay, M., 1995. Acylated anthocyanins from flowers of *Begonia*. Phytochemistry 40, 275–277.

Du, Q., Jerz, G., Winterhalter, P., 2004. Isolation of two anthocyanin sambubiosides from bilberry (*Vaccinium myrtillus*) by high-speed counter-current chromatography. J. Chromatog. A 1045, 59–63.

Feng, G.M., Xia, L.F., Zhu, X.H., 1986. 'Dalicha'. In: Feng, G.M., Xia, L.F., Zhu, X.H. (Eds.), Yunnan Camellias of China. Science Press, Beijing, China, p. 98.

Fossen, T., Rayyan, S., Holmberg, M.H., Nateland, H.S., Andersen, Q.M., 2005. Acylated anthocyanins from leaves of *Oxalis triangularis*. Phytochemistry 66, 1133–1140.

Fossen, T., Larsen, Å., Andersen, Q.M., 1997. Anthocyanins from flowers and leaves of *Nymphaea* × *Marliacea* cultivars. Phytochemistry 48, 823–827.

Goto, T., Takase, S., Kondo, T., 1978. PMR spectra of natural acylated anthocyanins, determination of stereostructure of awobanin, shisonin and violanin. Tetrahedron Lett. 27, 2413–2416.

Hayashi, K., Abe, Y., 1953. Studien über anthocyane, XXIII. Papierchromatographische übersicht der anthocyane im pfanzenreich I. Misc. Rep. Res. Inst. Resour. Tokyo 29, 1–8.

Harborne, J.B., 1963. Plant polyphenols IX. The glycosidic pattern of anthocyanin pigments. Phytochemistry 2, 85–97.

Hashimoto, F., Tanaka, M., Maeda, H., Fukuda, S., Shimizu, K., Sakata, Y., 2002. Changes in flower coloration and sepal anthocyanins of cyanic *Delphinium* cultivars during flowering. Biosci. Biotechnol. Biochem. 66, 1652–1659.

Hosokawa, K., Fukunaka, Y., Fukushi, E., Kawabata, J., 1995. Five acylated pelargonidin glucosides in the red flowers of *Hyacinthus orientalis*. Phytochemisty 40, 567–571.

Inami, O., Tamura, I., Kikuzaki, H., Nakatani, N., 1996. Stability of anthocyanins of *Sambucus canadensis* and *Sambucus nigra*. J. Agric. Food Chem. 44, 3090–3096.

Jarman, S.J., Crowden, R.K., 1974. Anthocyanins in the Epacridaceae. Phytochemistry 13, 743–750.

Li, J.-B., Hashimoto, F., Shimizu, K., Sakata, Y., 2007. Anthocyanins from red flowers of *Camellia reticulata* Lindl. Biosci. Biotechnol. Biochem. 71, 2833–2836.

Li, J.-B., Hashimoto, F., Shimizu, K., Sakata, Y., 2008. Anthocyanins from the red flowers of *Camellia saluenensis* Stapf ex Bean. J. Jpn. Soc. Hort. Sci. 77, 75–79.

Mas, T., Susperregui, J., Berké, B., Chéze, C., Moreau, S., Nuhrich, A., Vercauteren, J., 2000. DNA triplex stabilization property of natural anthocyanins. Phytochemistry 53, 679–687.

Ming, T.L., 2000. cv. Queen of Dali. In: Ming, T.L. (Ed.), Monograph of the Genus *Camellia*. Yunnan Science and Technology Press, Kunming, PR China, p. 39.

Nakatani, N., Kikuzaki, H., Hikida, J., Ohba, M., Inami, O., Tamura, I., 1995. Acylated anthocyanins from fruits of *Sambucus canadensis*. Phytochemistry 38, 755–757.

Nakatani, N., Kikuzaki, H., Inami, O., Kinoshita, H., Tamura, I., 1994. Cyanidin-3-*Z*-*p*-coumaroyl-sambubiosides and method for synthesizing them. Japan Patent H06-54766.

Robinson, G.M., Robinson, N.W., 1931. CLXXXII. A survey of anthocyanins. I. Biochem. J. 25, 1687–1705.

Saito, N., Yokoi, M., Yamaji, M., Honda, T., 1987. Cyanidin 3-*p*-coumaroylglucoside in *Camellia* species and cultivars. Phytochemistry 26, 2761–2762.

Sakata, Y., Arisumi, K., 1986. Cyanidin 3-galactoside, a new anthocyanin from *Camellia japonica* ssp. *rusticana* (Honda) Kitamura and its occurrence in the garden forms of *Camellia* of Japanese origin. J. Jpn. Soc. Hort. Sci. 55, 82–88.

Sakata, Y., 1988. Studies on the flower colours in the genus *Camellia*, with special reference to phylogenies of the genus. Bull. Fac. Agric. Kagoshima Univ. 38, 9–62.

Silverstein, R.M., Webster, F.X., 1998. Proton magnetic resonance spectrometry. In: Silverstein, R.M., Webster, F.X. (Eds.), Spectrometric Identification of Organic Compounds, sixth ed. John Wiley & Sons, Inc., New York, Chichester, Brisbane, Singapore, Toronto, pp. 144–216.

Terahara, N., Takeda, Y., Nesumi, A., Honda, T., 2001. Anthocyanins from red flower tea (Benibana-cha), *Camellia sinensis*. Phytochemistry 56, 359–361.

Terahara, N., Saito, N., Toki, K., Sakata, Y., Honda, T., 1992. Cyanidin 3-lathyroside from berries of *Fatsia japonica*. Phytochemistry 31, 1446–1448.

Wang, L.-S., Hashimoto, F., Shiraishi, A., Aoki, N., Li, J.-J., Shimizu, K., Sakata, Y., 2001. Phenetics in tree peony species from China by flower pigment cluster analysis. J. Plant Res. 114, 213–221.

Wang, L.-S., Hashimoto, F., Shiraishi, A., Aoki, N., Li, J.-J., Sakata, Y., 2004. Chemical taxonomy of the Xibei tree peony from China by floral pigmentation. J. Plant Res. 117, 47–55.