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Flavonol pentaglycosides of *Cordyla* (Leguminosae: Papilionoideae: Swartzieae): Distribution and taxonomic implications

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ABSTRACT

A survey of foliar flavonoids in the swartzioid legume genus *Cordyla* s.l. revealed that three species, *C. haraka*, *C. pinnata* and *C. richardii*, were rich in flavonol pentaglycosides. Their structures were elucidated by spectroscopic and chemical methods as the 3-*O*-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranosides of quercetin and kaempferol (cordylasins A and B, respectively). These compounds were not found in the remaining species, *C. africana*, *C. densiflora*, *C. madagascariensis* (two subspecies) and *C. somalensis*, which exhibited different profiles of flavonoid glycosides. The distribution of flavonol pentaglycosides in *Cordyla* s.l. does not support a recent proposal to place both *C. haraka* and *C. madagascariensis* in the genus *Dupuya* [Kirkbride, J.H., 2005. *Dupuya*, a new genus of Malagasy legumes (Fabaceae). Novon 15, 305–314]. The generic relationship between *Cordyla* s.l. and *Mildbraediodendron* is also reassessed on the basis of chemical characters, as the *O*-linked tetrasaccharide that characterises cordylasins A and B is the same as that found in mildbraedin (kaempferol 3-*O*-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside), the main foliar flavonoid of *Mildbraediodendron excelsum*. Mildbraedin itself was found to be a minor constituent of leaflet extracts of *C. haraka*, *C. pinnata* and *C. richardii*, and a major constituent of *C. somalensis*.

1. Introduction

The genus *Cordyla* Lour. (Leguminosae: subfamily Papilionoideae: tribe Swartzieae) as traditionally circumscribed comprises seven species of tree or shrub found in tropical Africa (five species) and Madagascar (two species) (Ireland, 2005). In a recent study, Kirkbride (2005) proposed that the Madagascan species, both of which are endemic to the island, should be placed in the new genus *Dupuya* J.H. Kirkbride based on the analysis of morphological characters, although aside from the differences in androecium (staminodes are present in the flowers of *Dupuya*, but absent in *Cordyla*) and seed characteristics, "*Cordyla* and *Dupuya* are so similar morphologically that they probably are sister genera". Chemical characters have also proved valuable for generic and species delimitation in legumes (Harborne et al., 1971; Hegnauer and Hegnauer, 1994, 1996, 2001; Wink and Mohamed, 2003), but relatively little has been published on the phytochemistry of *Cordyla* sensu lato, with reports limited to those on isoflavonoids from the heartwood of *C. africana* Lour. (Campbell et al., 1969; Campbell and Tannock, 1973), and cassane diterpenoids from the fruits of *C. madagascariensis* R.Vig. subsp. *madagascariensis* (Hou et al., 2008). However, work on the monospecific genus *Mildbraediodendron* Harms, which is a close generic relative of *Cordyla*, revealed that the major phenolic component of the leaves of *Mildbraediodendron excelsum* Harms was a flavonol glycoside (mildbraedin, **4**) characterised by a novel *O*-linked branched tetrasaccharide at C-3 of kaempferol (Veitch et al., 2005). Here we extend this survey of the flavonoid chemistry of swartzioid legumes to the genus *Cordyla* s.l., revealing the presence of two unique flavonol pentaglycosides, cordylasins A (**1**) and B (**2**), in three of the seven species. The structural determination of these compounds is described, and their distribution as chemical characters discussed in the context of *Cordyla* and the recently published new genus *Dupuya*. The generic relationship between *Cordyla* and *Mildbraediodendron* is also reexamined on the basis of their flavonol glycoside content, with particular reference to the structures of cordylasins A (**1**) and B (**2**), and mildbraedin (**4**).

2. Results and discussion

2.1. Characterisation of flavonol pentaglycosides *1* and *2*

LC-ESI-MS analysis of MeOH–H_2O (1:1) extracts of leaflet material from seven species of *Cordyla* s.l. (one of which has two subspecies) revealed the presence of two highly glycosylated flavonoids (**1** and **2**) in three of them. The earlier eluting compound (**1**) had a UV spectrum (255, 266sh, 355 nm) typical of a quercetin *O*-glycoside

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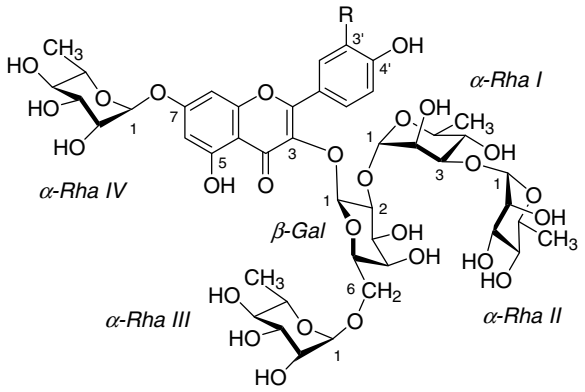

1 R = OH; Cordylasin A
2 R = H; Cordylasin B

(Markham, 1982). This gave a protonated molecule at *m*/*z* 1049 $[M+H]^+$, and protonated fragments following MS2 (MS/MS) at *m*/*z* 903, 757, 611, 449 and 303, consistent with a glycosidic profile of four deoxyhexose residues and one hexose residue (Section 3.5). The UV spectrum (265, 349 nm) of the second compound (**2**) was characteristic of a kaempferol *O*-glycoside (Markham, 1982). A protonated molecule at *m*/*z* 1033 $[M+H]^+$, and MS2 fragments at *m*/*z* 887, 741, 595, 433 and 287 (all 16 a.m.u. less than those of **1**), suggested that **2** was similarly glycosylated (Section 3.6). In both cases, the UV spectra of the compounds resembled those of flavonols *O*-glycosylated at C-3 and C-7 (broad valley between bands I and II with no shoulder at ca. 300 nm) rather than solely at C-3 (sharp valley between bands I and II with a shoulder at ca. 300 nm). Following MS2 of $[M+Na]^+$, both compounds showed losses of deoxyhexose and (deoxyhexose + aglycone), indicating that one of the *O*-glycosyl groups was deoxyhexose, while the single loss of deoxyhexose from $[M-H]^-$ suggested that this substitution was at C-7 (Kite et al., 2007). These preliminary observations indicated that **1** and **2** were glycosides of quercetin and kaempferol, respectively, each comprising five sugar residues and glycosylated at both C-3 and C-7, with a deoxyhexose substituted at the latter position. Although detected in leaflet material of three species of *Cordyla* s.l., **1** and **2** were present in approximately equal amounts as the major phenolic components of MeOH–H_2O (1:1) extracts of *C. pinnata* (A.Rich.) Milne-Redh. Extract clean-up on a Sep-Pak C-18 column followed by semi-preparative HPLC afforded the compounds as yellow solids (Section 3.3), the structures of which were determined using high-resolution MS and NMR.

The 1D ^{1}H NMR spectrum of **1** comprised only aromatic and glycosidic proton resonances. For the former, the *meta*-coupled resonances at δ_H 6.46 (1H, *d*, *J* = 2.2 Hz, δ_C 100.6) and 6.72 (1H, *d*, *J* = 2.2 Hz, δ_C 95.7) were assigned to H-6 and H-8 of the flavonoid A-ring, respectively. The remaining aromatic resonances at δ_H 7.70 (1H, *d*, *J* = 2.2 Hz, δ_C 117.5), 6.90 (1H, *d*, *J* = 8.5 Hz, δ_C 116.4) and 7.61 (1H, *dd*, *J* = 8.5, 2.2 Hz, δ_C 123.2) were assigned to H-2′, H-5′ and H-6′, respectively, of the B-ring. Analysis of correlations in HSQC and HMBC spectra gave the full assignment of the ^{1}H and ^{13}C resonances of the aglycone of **1**, which was confirmed to be quercetin. Evidence for *O*-glycosylation at both C-3 and C-7 was afforded in the first instance by comparison of chemical shift values with those for quercetin itself, using NMR data acquired in the same solvent (Fossen and Andersen, 2006). Thus chemical shift changes for C-2 (+11.1 ppm), C-3 (−2.2 ppm) and C-4 (+2.4 ppm) were typical of 3-*O*-glycosylation (Agrawal, 1989), while the downfield shifted H-6 (+0.19 ppm) and H-8 (+0.25) resonances were as expected for 7-*O*-glycosylation (Markham and Geiger, 1994). Long-range correlations in the HMBC spectrum between the anomeric proton resonances at δ_H 5.68 (1H, *d*, *J* = 7.8 Hz, δ_C 101.1) and C-3 of the aglycone (δ_C 135.0), and between δ_H 5.55 (1H, *d*, *J* = 1.8 Hz, δ_C 100.1) and C-7 of the aglycone (δ_C 163.6), gave proof of 3- and 7-*O*-glycosylation, respectively. ROE connectivities were detected between the anomeric proton of the 7-*O*-linked sugar residue (δ_H 5.55) and both H-6 and H-8. Three further anomeric proton resonances corresponding to *O*-linked sugars were noted in the 1D ^{1}H NMR spectrum at δ_H 5.21 (1H, *d*, *J* = 1.7 Hz, δ_C 102.7), 5.02 (1H, *d*, *J* = 1.8 Hz, δ_C 104.2) and 4.54 (1H, *d*, *J* = 1.8 Hz, δ_C 102.0). The remaining sugar resonances appeared between 3.26 and 4.11 ppm, with the notable exception of four methyl doublets (*J* = 6.2 or 6.3 Hz) at δ_H 1.30, 1.26, 1.18 and 0.96 ppm (δ_C 18.1, 18.2, 18.1 and 17.5 ppm, respectively).

Full assignment of the ^{1}H and ^{13}C resonances of the five sugar residues of **1** was achieved using a combination of 1D selective ROE, COSY, TOCSY, HSQC and HMBC data (Table 1). These assignments, together with the multiplicities and coupling constants for the proton resonances, indicated that the primary sugar *O*-linked at C-3 (corresponding to the anomeric proton δ_H 5.68) was β-galactopyranose (Duus et al., 2000; Markham and Geiger, 1994). The four deoxyhexose sugar residues were similarly identified as α-rhamnopyranose. The strategy for the sequential assignment of these α-Rha residues relied upon connectivities in COSY (generally H-1 to H-3/H-4 and 6-CH_3 to H-5/H-4), TOCSY (giving the crucial 6-CH_3 to H-1 correlation at a mixing time of 100 ms) and HMBC (6-CH_3 to C-4 and C-5; H-1 to C-2, C-3 and C-5). The absolute configurations of the constituent monosaccharides of **1** released on acid hydrolysis were determined as D-Gal and L-Rha (Section 3.4).

The interglycosidic linkages of the intact flavonol glycoside were determined from ROE and HMBC data. Long-range correlations from Gal H-2 to C-1 of Rha I at δ_C 102.7, from H-1 of Rha I to Gal C-2 at δ_C 77.6 (both HMBC) and between Gal H-2 and H-1 of Rha I (ROE), indicated that the primary sugar was 2-*O*-linked to Rha I. A 6-*O*-linkage from Gal to Rha III was characterised by correlations from Gal 6-CH_2 to C-1 of Rha III at δ_C 102.0, from H-1 of Rha III to Gal C-6 at δ_C 67.3 (both HMBC) and between H-1 of Rha III and Gal 6-CH_2 (ROE). The downfield shifted resonance of C-3 of Rha I (2-*O*-linked to Gal) at δ_C 80.3 suggested that a further Rha residue was linked there. This was confirmed by the long-range correlations from H-3 of Rha I to C-1 of Rha II at δ_C 104.2, from H-1 of Rha II to C-3 of Rha I (both HMBC) and between H-1 of Rha II and H-3 of Rha I (ROE). These data demonstrated that a branched tetrasaccharide was *O*-linked at C-3 in addition to the monosaccharide (Rha IV) *O*-linked at C-7. Compound **1** was therefore identified as quercetin 3-*O*-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (cordylasin A). Confirmation of the molecular formula of $C_{45}H_{60}O_{28}$ was by HRESI-MS (Section 3.5).

The 1D ^{1}H NMR spectrum of **2** was similar to that of **1,** except for the aromatic resonances corresponding to the protons of the flavonoid B-ring, which appeared instead at δ_H 8.09 (2H, *d*, *J* = 9.0 Hz, δ_C 132.4) and 6.91 (2H, *d*, *J* = 9.0 Hz, δ_C 116.5). These corresponded to H-2′/6′ and H-3′/5′ of kaempferol, the remaining resonances of which were assigned using HSQC and HMBC data (Table 1). The distinctive resonances of the five anomeric and four Rha 6-CH_3 protons were almost superimposable between the ^{1}H NMR spectra of **1** and **2**. Nevertheless, the glycosidic components of **2** were characterised independently as described for **1**. Analysis of a complementary set of 1D and 2D NMR spectra for **2** revealed that the ^{1}H and ^{13}C resonance assignments of the sugars were near-identical to those of **1** (Table 1), and the same pattern of long-range

Table 1
^{1}H and ^{13}C NMR spectral data for flavonol pentaglycosides **1** and **2** (CD_3OD, 30 °C)

	Atom	**1**		**2**	
		δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C
Aglycone	2		159.1		159.4
	3		135.0		134.9
	4		179.7		179.7
	5		163.1		163.1
	6	6.46 *d* (2.2)	100.6	6.47 *d* (2.1)	100.6
	7		163.6		163.6
	8	6.72 *d* (2.2)	95.7	6.73 *d* (2.2)	95.8
	9		158.1		158.1
	10		107.7		107.6
	1′		123.3		122.9
	2′	7.70 *d* (2.2)	117.5	8.09 *d* (9.0)	132.4
	3′		146.1	6.91 *d* (9.0)	116.5
	4′		150.0		161.8
	5′	6.90 *d* (8.5)	116.4	6.91 *d* (9.0)	116.5
	6′	7.61 *dd* (8.5, 2.2)	123.2	8.09 *d* (9.0)	132.4
3-*O*-β-Gal	1	5.68 *d* (7.8)	101.1	5.64 *d* (7.8)	100.9
	2	3.97 *dd* (9.3, 7.8)	77.6	3.95 *dd* (9.5, 7.8)	77.8
	3	3.74 *dd* (9.5, 3.4)	75.8	3.72 *dd* (9.5, 3.4)	75.8
	4	3.80 *dd* (3.5, 1.0)	71.0	3.77 *dd* (3.4, 1.0)	70.9
	5	3.67 *m*	75.5	3.64 *m*	75.6
	6	3.74 *m*, 3.49 *m*	67.3	3.72 *m*, 3.46 *m*	67.4
2^{Gal}-*O*-α-Rha (I)	1	5.21 *d* (1.7)	102.7	5.21 *d* (1.7)	102.7
	2	4.11 *dd* (3.3, 1.8)	72.3	4.10 *dd* (3.3, 1.8)	72.4
	3	3.88 *dd* (9.7, 3.2)	80.3	3.88 *dd* (9.6, 3.3)	80.3
	4	3.45 '*t*' (9.7)	73.3	3.46 *m*	73.4
	5	4.08 *dd* (9.7, 6.2)	70.2	4.09 *dd* (9.5, 6.2)	70.2
	6	0.96 *d* (6.2)	17.5	0.97 *d* (6.2)	17.7
3^{Rhal}-*O*-α-Rha (II)	1	5.02 *d* (1.8)	104.2	5.03 *d* (1.8)	104.2
	2	3.96 *dd* (3.4, 1.8)	72.3	3.97 *dd* (3.4, 1.8)	72.3
	3	3.77 *dd* (9.6, 3.4)	72.4	3.77 *dd* (9.5, 3.4)	72.4
	4	3.40 '*t*' (9.5)	74.3	3.40 '*t*' (9.5)	74.3
	5	3.84 *dd* (9.5, 6.2)	70.2	3.84 *dd* (9.5, 6.2)	70.2
	6	1.30 *d* (6.3)	18.1	1.31 *d* (6.3)	18.1
6^{Gal}-*O*-α-Rha (III)	1	4.54 *d* (1.8)	102.0	4.52 *d* (1.7)	102.0
	2	3.54 *dd* (3.4, 1.8)	72.2	3.53 *dd* (3.4, 1.7)	72.2
	3	3.49 *br d* (9.5)	72.2	3.48 *dd* (9.6, 3.5)	72.4
	4	3.26 '*t*' (9.4)	74.0	3.25 '*t*' (9.5)	74.0
	5	3.52 *dd* (9.5, 6.2)	69.8	3.51 *dd* (9.5, 6.2)	69.8
	6	1.18 *d* (6.2)	18.1	1.17 *d* (6.3)	18.0
7-*O*-α-Rha (IV)	1	5.55 *d* (1.8)	100.1	5.56 *d* (1.8)	100.1
	2	4.02 *dd* (3.5, 1.8)	71.8	4.02 *dd* (3.4, 1.8)	71.8
	3	3.83 *dd* (9.4, 3.4)	72.2	3.83 *dd* (9.5, 3.4)	72.2
	4	3.48 '*t*' (9.4)	73.8	3.48 '*t*' (9.5)	73.8
	5	3.61 *dd* (9.5, 6.1)	71.4	3.62 *dd* (9.6, 6.2)	71.4
	6	1.26 *d* (6.2)	18.2	1.26 *d* (6.1)	18.2

connectivities in HMBC and ROE data was observed. These indicated that the sugar residues, sites of glycosylation and interglycosidic linkages were all as found in **1**. Determination of the absolute configurations of the constituent monosaccharides of **2** released on acid hydrolysis gave D-Gal and L-Rha (Section 3.4). Thus **2** was identified as kaempferol 3-*O*-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (cordylasin B). Confirmation of the molecular formula of $C_{45}H_{60}O_{27}$ was by HRESI-MS (Section 3.6).

According to recent surveys, the structures of more than 1500 flavonol glycosides have now been determined (Williams, 2006; Veitch and Grayer, 2008). However, pentaglycosides are rarely reported, with only four examples recorded to date. In the case of montbretins A and B, isolated from the corms of *Crocosmia crocosmiiflora* (Iridaceae), the glycosidic components comprised an acylated linear trisaccharide and a disaccharide *O*-linked at C-3 and C-4′ of myricetin, respectively (Asada et al., 1988). More recently, Shrestha et al. (2006) described two identically glycosylated derivatives of kaempferol and quercetin with an *O*-linked acylated linear trisaccharide at C-3 and an *O*-linked disaccharide at C-7, from the aerial parts of *Aconitum naviculare* (Ranunculaceae). Cordylasins A (**1**) and B (**2**) are thus the first flavonol pentaglycosides to be reported with an *O*-linked branched tetrasaccharide at C-3 and an *O*-linked monosaccharide at C-7.

2.2. Detection and distribution of flavonol pentaglycosides in Cordyla

The flavonoid glycosides of MeOH–H_2O (1:1) extracts of leaflet samples of *Cordyla* s.l. (see Table 4 for details) were analysed by LC-ESI-MS and HPLC coupled to diode-array detection (LC-UV). Cordylasins A (**1**) and B (**2**) were only detected in *C. haraka* Capuron, *C. pinnata* and *C. richardii* Planch. ex Milne-Redh. In *C. pinnata*, the two compounds were almost equally abundant, each comprising approximately 1% by weight of the dried leaflet (Section 3.3). However, in *C. haraka* (represented by three accessions) and *C. richardii*, the kaempferol pentaglycoside **2** was more abundant than its quercetin analogue (**1**). Analysis of the 1D ^{1}H NMR spectra of the CD_3OD-soluble portions of the dried MeOH–H_2O (1:1) extracts of these species confirmed that **2** was the main phenolic component in two accessions of *C. haraka* (*Du Puy et al. M375* and *McPherson 14735*), and *C. richardii*. The third accession of *C. haraka* (*J.-N. Labat et al. 3419*) contained approximately equal amounts of **2** and the corresponding tetraglycoside, kaempferol 3-*O*-α-rhamnopyranosyl(1→2)[α-rhamnopyranosyl(1→6)]-β-galactopyranoside-7-*O*-α-rhamnopyranoside (**6**). These data indicate that flavonol pentaglycosides accumulate in the leaflets of all three species.

A third flavonol pentaglycoside which eluted before cordylasin A (**1**) in LC-ESI-MS and LC-UV analyses (Table 2) was detected as a minor component in *C. pinnata* and *C. richardii*. This had a protonated molecule at *m*/*z* 1065 $[M+H]^+$, and protonated fragments on MS2 at *m*/*z* 919, 773, 627, 465 and 319 (all 16 a.m.u. greater than those of **1**). Serial MS of the protonated molecule (MS3; *m*/*z* 1065→319) identified the aglycone as myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone). This compound is likely to be the myricetin analogue of **1** and **2**, but its low concentration in the extracts precluded full analysis by NMR. Two late-eluting minor components detected by LC-ESI-MS (t_R 30.0 and 32.8 min) in one accession of *C. haraka* (*McPherson 14735*) had UV spectra (266, 331 nm) typical of kaempferol glycosides acylated by hydroxycinnamic acids (Llorach et al., 2003). Both had deprotonated molecules at *m*/*z* 1207 $[M-H]^-$, and the MS2 (*m*/*z* 1207) spectra showed a base ion at 885 $[(M-H)-(146+176)]^-$ and a low abundance ion at *m*/*z* 1061 $[(M-H)-146]^-$. Their MS3 (*m*/*z* 1061→885) spectra were similar to that of mildbraedin (**4**). These data suggested that the compounds were derivatives of cordylasin B (**2**) with an additional feruloyl substituent. The predominant loss of a feruloylrhamnosyl group from the deprotonated molecule, and the presence, in the MS2 spectra of the protonated molecule, of an ion at *m*/*z* 609 $[(\text{kaempferol} + \text{feruloylrhamnosyl})+H]^+$, indicate that the site of feruloyl substitution is likely to be on the 7-*O*-rhamnopyranosyl residue, where three positions are available (2-OH, 3-OH and 4-OH).

2.3. Flavonoid profiles of Cordyla s.l.

The remaining flavonoid constituents of *Cordyla* s.l. were identified from the analysis of LC-ESI-MS and LC-UV data together with reference to authentic standards, as summarised in Table 2. These comprised mainly kaempferol and quercetin glycosides *O*-linked at C-3 or C-3 and C-7. Galactose predominated as the primary sugar at C-3 in all species, and the only other sugar present was rhamnose, which was found *O*-linked to the primary sugar or C-7 or both. The glycosidic chains of tri- and tetrasaccharides *O*-linked at C-3 were branched, with C-2 and C-6 of the primary sugar as substitution sites. Several different flavonoid profiles were

Table 2
Chromatographic and structural data for flavonol glycosides present in the leaves of *Cordyla* s.l. and *Mildbraediodendron*[a]

Number[b]	t_R (min)	M_r	Agly.	C-3	C-7	Det.
1[c]	11.6	1048	Q	3-*O*-α-Rha*p*-(1→3)-α-Rha*p*-(1→2)[α-Rha*p*-(1→6)]-β-Gal*p*	7-*O*-α-Rha*p*	NMR
2[c]	13.5	1032	K	3-*O*-α-Rha*p*-(1→3)-α-Rha*p*-(1→2)[α-Rha*p*-(1→6)]-β-Gal*p*	7-*O*-α-Rha*p*	NMR
3	16.1	902	Q	3-*O*-α-Rha*p*-(1→3)-α-Rha*p*-(1→2)[α-Rha*p*-(1→6)]-β-Gal*p*		MS
4	19.1	886	K	3-*O*-α-Rha*p*-(1→3)-α-Rha*p*-(1→2)[α-Rha*p*-(1→6)]-β-Gal*p*		NMR
5	11.8	902	Q	3-*O*-α-Rha*p*-(1→2)[α-Rha*p*-(1→6)]-β-Gal*p*	7-*O*-α-Rha*p*	MS
6	13.8	886	K	3-*O*-α-Rha*p*-(1→2)[α-Rha*p*-(1→6)]-β-Gal*p*	7-*O*-α-Rha*p*	NMR
7	16.2	756	Q	3-*O*-α-Rha*p*-(1→2)[α-Rha*p*-(1→6)]-β-Gal*p*		MS
8	19.4	740	K	3-*O*-α-Rha*p*-(1→2)[α-Rha*p*-(1→6)]-β-Gal*p*		NMR
9	20.7	740	K	3-*O*-α-Rha*p*-(1→6)-β-Gal*p*	7-*O*-α-Rha*p*	Std.
10	21.5	610	Q	3-*O*-α-Rha*p*-(1→6)-β-Gal*p*		NMR
11	24.6	594	K	3-*O*-α-Rha*p*-(1→6)-β-Gal*p*		Std.
12	21.2	464	Q	3-*O*-β-Gal*p*		NMR

[a] HPLC retention times (t_R in LC-ESI-MS analyses), M_r, aglycone identity as kaempferol (K) or quercetin (Q), *O*-linked glycosides at C-3 and C-7, and identification method (Det.) are given (Std. = standard). NMR determinations; **1**, **2**, **10** and **12** (this work), **4** (Veitch et al., 2005), **6** and **8** (Kite et al., 2007). MS determinations; **3** (Veitch et al., 2005), **5** and **7** (Kite et al., 2007; Glc analogues); note that the anomeric configurations of sugars are assumed to be those found in naturally occurring flavonol glycosides.
[b] Glucosylated analogues of **7**, **8** and **11** elute at t_R 16.8, 19.9 and 26.0 min, respectively.
[c] A further pentaglycoside tentatively identified as the myricetin analogue of **1** and **2** was detected by LC-ESI-MS (t_R 10.0 min) as a minor component of *C. pinnata* and *C. richardii* (Section 2.2).

recognised from the analysis (Table 3), in addition to that corresponding to species containing mainly flavonol pentaglycosides (*C. haraka, C. pinnata* and *C. richardii*; Type I). Although *C. somalensis* J.B. Gillett (Type II) contained no pentaglycosides, the tetraglycoside mildbraedin (**4**) and its quercetin analogue (**3**) were major components together with the 3-*O*-robinobioside (**10**) and 3-*O*-galactoside (**12**) of quercetin. A third group comprising *C. africana* and *C. densiflora* Milne-Redh. (Type III) had kaempferol 3-*O*-α-rhamnopyranosyl(1→2)[α-rhamnopyranosyl(1→6)]-β-galactopyranoside (**8**) as the major component. No higher glycosides were detected and the minor components comprised 3-*O*-diglycosides of both quercetin and kaempferol. Quercetin 3-*O*-α-rhamnopyranosyl(1→2)[α-rhamnopyranosyl(1→6)]-β-galactopyranoside (**7**) was also a minor component in *C. densiflora*, but occurred only at low levels in *C. africana*. In these species, flavonol glycosides with Gal as the primary sugar at C-3 were accompanied by smaller amounts of their glucosylated analogues.

The flavonoid profiles of the two subspecies of *C. madagascariensis* (Type IV), represented by two accessions of *C. madagascariensis* subsp. *madagascariensis* and three of *C. madagascariensis* subsp. *tamarindoides* (Capuron) Du Puy & Labat (Table 4), were more variable, but had several features distinguishing them from the other *Cordyla* species. The flavonol pentaglycosides of the Type I species were absent, as were tetraglycosides such as mildbraedin (e.g. in Type II), in which a tetrasaccharide is *O*-linked at C-3. However, tetraglycosides characterised by an *O*-linked trisaccharide at C-3 and an *O*-linked-monosaccharide at C-7 were detected, notably kaempferol 3-*O*-α-rhamnopyranosyl(1→2)[α-rhamnopyranosyl(1→6)]-β-galactopyranoside-7-*O*-α-rhamnopyranoside (**6**), which was a major component of *C. madagascar-*

Table 3
Distribution of flavonoid glycosides in the leaves of *Cordyla* s.l. and *Mildbraediodendron*[a]

Species (accession)	Penta[b]		Tetra				Tri			Di		Mono	Others	
	4:1	4:1	4:0	4:0	3:1	3:1	3:0	3:0	2:1	2:0	2:0			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**AFG**	**FCG**
Type I														
C. haraka (1)	○	●		○		○			○					
C. haraka (2)	○	●		○					○		○		○	
C. haraka (3)	○	●		○	○	●			○					
C. pinnata	●	●	○	○						○		○		
C. richardii	○	●	○	○										
Type II														
C. somalensis			●	●				○		●	○	●		
Type III[c]														
C. africana								●			○			
C. densiflora							○	●		●	○			
Type IV														
C. madagascariensis subsp. *madagascariensis* (1)					○	●			●				○	○
C. madagascariensis subsp. *madagascariensis* (2)						○			○	○		○	○	○
C. madagascariensis subsp. *tamarindoides* (1)					○	●			○	○		○	○	○
C. madagascariensis subsp. *tamarindoides* (2)							○	●		○		●	○	○
C. madagascariensis subsp. *tamarindoides* (3)							○	●		○	○		○	○
Other taxon														
Mildbraediodendron excelsum				●				○						○

[a] Detection by LC-ESI-MS and LC-UV (all species). For specimen details see Table 4. The filled (●) and open circle (○) symbols represent major and minor components, respectively, according to LC-UV chromatograms extracted at 350 nm (all species), and 1D ^{1}H NMR (major components of Type I species only).
[b] For flavonol glycosides **1**–**12** see Table 2. The pairs of numbers separated by colons indicate the number of *O*-linked saccharides at C-3 and C-7. AFG = acylated flavonol glycosides, FCG = flavone *C*-glycosides.
[c] The glucosylated analogues of **7**, **8** and **11** were detected in these species at lower concentrations. Kaempferol 3-*O*-α-Rha*p*-(1→2)-β-Hex*p* (Hex = Gal and Glc) were also detected as minor components in *C. africana*, again with the Gal analogue predominating.

Table 4
Details of herbarium specimens of *Cordyla* s.l. and *Mildbraediodendron* used in the survey

Species	Collector reference	Origin	Collection date (dd/mm/yyyy)	Specimen number
Cordyla				
C. africana Lour.	Verdcourt 5274	Kenya	10/04/1978	BI-16082
C. densiflora Milne-Redh.	Ruffo 933	Tanzania	06/10/1973	BI-16083
C. haraka Capuron (1)[a]	Du Puy et al. M375	Madagascar	16/11/1989	BI-16084
C. haraka Capuron (2)[a]	McPherson 14735	Madagascar	20/12/1989	BI-07165
C. haraka Capuron (3)	J.-N. Labat et al. 3419	Madagascar	25/10/2001	BI-16998
C. madagascariensis R.Vig. subsp. *madagascariensis* (1)[a]	Du Puy et al. M361	Madagascar	20/10/1989	BI-16085
C. madagascariensis R.Vig. subsp. *madagascariensis* (2)[a]	Du Puy et al. M297	Madagascar	18/07/1989	BI-16999
C. madagascariensis R.Vig. subsp. *tamarindoides* (Capuron) Du Puy & Labat (1)[a]	Du Puy et al. M755	Madagascar	24/03/1994	BI-16093
C. madagascariensis R.Vig. subsp. *tamarindoides* (Capuron) Du Puy & Labat (2)[a]	Du Puy et al. M267	Madagascar	10/06/1989	BI-17000
C. madagascariensis R.Vig. subsp. *tamarindoides* (Capuron) Du Puy & Labat (3)[a]	Service Forestier 12731	Madagascar	18/12/1954	BI-17001
C. pinnata (A.Rich.) Milne-Redh.	Sanou & van Slageren, MSLS-1363	Burkina Faso	07/05/2003	BI-16086
C. richardii Planch. ex Milne-Redh.	Friis & Vollesen 1186	S. Sudan	13/03/1982	BI-16087
C. somalensis J.B.Gillett	Fagg & Styles 16	Somalia	20/09/1987	BI-16088
Mildbraediodendron				
M. excelsum Harms	1996-2364 (Cheek 8350)[b]	(Cameroon)	(05/1996)	BI-16089

[a] Specimen examined by Kirkbride (2005).
[b] RBG Kew living collection, specimen grown from seed (collector reference, origin and collection date in parentheses).

iensis subsp. *madagascariensis* (*Du Puy et al. M361*) and *C. madagascariensis* subsp. *tamarindoides* (*Du Puy et al. M755*). Both accessions of *C. madagascariensis* subsp. *madagascariensis* contained **9** (kaempferol 3-*O*-α-rhamnopyranosyl(1→6)-β-galactopyranoside-7-*O*-α-rhamnopyranoside; robinin), whereas kaempferol 3-*O*-α-rhamnopyranosyl(1→2)[α-rhamnopyranosyl(1→6)]-β-galactopyranoside (**8**) was observed in accessions of *C. madagascariensis* subsp. *tamarindoides* (major component of *Du Puy et al. M267* and *Service Forestier 12731*). Flavone *C*-glycosides, notably vicenin-2 (apigenin 6,8-di-*C*-β-glucopyranoside), were present in all accessions of *C. madagascariensis* subsp. *tamarindoides*, and *C. madagascariensis* subsp. *madagascariensis*. They were not found in any of the other species of *Cordyla* examined here. All five accessions of *C. madagascariensis* contained acylated flavonol glycosides as minor components, which were recognised from their distinctive UV spectra (band II at 268 nm; band I at 315–330 nm) (Llorach et al., 2003). Preliminary MS data suggest that these comprise both coumaroyl and feruloyl derivatives of kaempferol tri- or tetraglycosides, however, further analysis was not possible due to their low concentration in the extracts.

2.4. Cordyla or Dupuya?

The recent segregation of the endemic Madagascan species of *Cordyla* into the new genus *Dupuya* was made by Kirkbride (2005) on the basis of morphological characters. However, the present study is the first in which a complete survey of *Cordyla* s.l. has been made using chemical characters. Given the particular interest in *C. haraka* Capuron (= *Dupuya haraka* (Capuron) J.H. Kirkbride) and the two subspecies of *C. madagascariensis* R.Vig. (= *Dupuya madagascariensis* (R.Vig.) J.H. Kirkbride) placed in *Dupuya*, several accessions of each taxon were analysed. Of these, two of the three specimens of *C. haraka* and all the specimens of *C. madagascariensis* subsp. *madagascariensis* and *C. madagascariensis* subsp. *tamarindoides* were the same as those examined by Kirkbride (2005), as indicated in Table 4. The differences in the flavonoid profiles of leaf material from these taxa are striking. As Table 3 shows, all three accessions of *C. haraka* accumulate the previously undescribed flavonol pentaglycoside, cordylasin B (**2**), and also contain small amounts of the related cordylasin A (**1**). Similar profiles were obtained for *C. pinnata* and *C. richardii*. However, these compounds were not detected in any of the accessions of *C. madagascariensis*, which presented a different profile of flavonoid glycosides (Section 2.3). In this context it is important to emphasise that there is no evidence for degradation of flavonoid glycosides during storage under herbarium conditions, as demonstrated by a study in which the flavonoid profiles of a leaf sample of *M. excelsum* collected in 1928 and one under cultivation, were found to be the same, each containing the flavonol tetraglycoside mildbraedin (**4**) as the major component (Veitch et al., 2005). The chemical data summarised in Table 3 do not support a close relationship between *C. haraka* and *C. madagascariensis*, suggesting instead that *C. haraka* should be allied with *C. pinnata* and *C. richardii*. As such, the segregation of both *C. haraka* and *C. madagascariensis* into *Dupuya* is questionable. Although the flavonoid profiles of the five *C. madagascariensis* accessions (Table 3) exhibit some distinctive features, such as the presence of flavone *C*-glycosides and acylated flavonol glycosides, these relate to minor components only. Thus it is difficult to make a strong case based on the flavonoid chemistry alone for *C. madagascariensis* to be transferred from *Cordyla* to a separate genus. Segregating endemic Madagascan taxa at the genus level based on morphology alone has shown to be premature in the legume tribe Indigofereae (Du Puy et al., 1994). More recent molecular studies of the genus *Indigofera* and allies by Schrire et al. (2003) conclusively demonstrate that the morphologically distinctive *Vaughania* (containing 11 species all restricted to Madagascar) is nested within *Indigofera* and as a result all eleven species are now being transferred back into *Indigofera* (B.D. Schrire, personal communication). It is likewise possible that future molecular studies will show that *Dupuya* should be returned to *Cordyla* in support of the phytochemical findings. Morphology diverges significantly on islands isolated over long geological time (e.g., MacArthur and Wilson, 2001) and this is especially true for the legume flora on Madagascar, so the fact that the two Madagascan species are morphologically distinct from all the other species on the African mainland is not surprising. Further studies, including molecular analyses, of *Cordyla* s.l. and related genera, will be necessary to resolve the question of whether to recognise *Cordyla* s.l. as one or more genera (see also Section 2.5).

2.5. Cordyla and Mildbraediodendron

Cordylasins A (**1**) and B (**2**), the flavonol pentaglycosides of *C. haraka*, *C. pinnata* and *C. richardii* are closely related structurally to the kaempferol tetraglycoside, mildbraedin (**4**), the major flavonoid component of the leaves of *M. excelsum* (Veitch et al., 2005). Mildbraedin was also detected as a minor component in *C. haraka*

(all accessions), *C. pinnata* and *C. richardii*, and as a major component in *C. somalensis* (Table 3). All these compounds are distinguished by the presence of the same *O*-linked tetrasaccharide at C-3 of the flavonol aglycone, namely *O*-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranose. This is also presumed to be present in the quercetin analogue (**3**) of mildbraedin detected as a minor component in *C. pinnata* and *C. richardii* (Tables 2 and 3). At present there are no additional reports of this tetrasaccharide in combination with any natural product in the literature. Thus its presence in the flavonoid glycosides of four of the seven species of *Cordyla* s.l. and *Mildbraediodendron* supports the close relationship between these African genera suggested in previous work (Herendeen, 1995; Kirkbride, 2005; Pennington et al., 2001). According to the latter study, which is based on sequence analysis of the chloroplast *trn*L intron, *Cordyla* and *Mildbraediodendron* are part of the Aldinoid clade of swartzioid legumes, a group which also includes the South American genera *Aldina* and *Amburana*. Within this group, *Cordyla* was placed as sister to *Aldina*, rather than *Mildbraediodendron*. However, only one species from each of the four genera was sequenced in the study of Pennington et al. (2001), and in particular, *Cordyla* was represented by *C. madagascariensis* subsp. *tamarindoides* (*Du Puy et al. M755*), a specimen also examined in the present study, but which does not contain either cordylasins A and B, or mildbraedin. Herendeen's analysis of morphological characters placed *Cordyla* and *Mildbraediodendron* in the same clade (Herendeen, 1995), but with a more distant relationship to *Aldina* and *Amburana* than that suggested by Pennington et al. (2001). The present results indicate that a majority of species of *Cordyla* s.l. share a similar flavonoid chemistry with *Mildbraediodendron*, based on the presence of the structurally related derivatives, cordylasins A (**1**) and B (**2**), and mildbraedin (**4**). These compounds, which are readily detected in LC-ESI-MS analyses, are excellent markers even where source material is limited. A wider survey of their distribution may therefore be useful to further investigate relationships in the Aldinoid clade, particularly between the African (*Cordyla*, *Mildbraediodendron*) and South American (*Aldina*, *Amburana*) swartzioid genera. It may also indicate whether the two subspecies of *C. madagascariensis* have flavonoid profiles that are more similar to *Aldina* than *Mildbraediodendron*.

3. Experimental

3.1. General instrumentation

NMR spectra were acquired in CD_3OD at 30 °C on a Bruker Avance II+ 700 MHz instrument equipped with a TCI-cryoprobe or a Bruker Avance 400 MHz instrument. Standard pulse sequences and parameters were used to obtain 1D ^1H, 1D ^{13}C, 1D site selective ROE, COSY, TOCSY, HSQC and HMBC spectra. Chemical shift referencing was carried out with respect to TMS at 0.00 ppm.

LC-ESI-MS/MS analysis was performed using a Thermo-Finnigan system consisting of 'Surveyor' autosampler, pumps and PDA connected to an 'LCQ Classic' ion trap mass spectrometer fitted with an ESI source. Chromatography of 10 μl injections was performed on a Phenomenex Luna C18(2) column (150 mm × 4.6 mm i.d., 5 μm particle size) using a 1 ml/min linear mobile phase gradient of 20–50% aq. MeOH (containing 1% HOAc) in 30 min followed by a MeOH wash. The flow to the ESI source was reduced to 0.2 ml/min by a splitter and the source was operated using the manufacturer's standard conditions in positive and negative modes in separate analyses. Data dependent MS2 spectra were obtained automatically from the most abundant ions observed in MS1, and the two most abundant product ions were subject to MS3 analysis, also automatically. Accurate mass measurements were performed on a Finnigan MAT900 XLT mass spectrometer in positive ESI mode. LC-UV analysis (HPLC coupled to diode-array detection; analytical scale) was carried out using a Waters system (LC600 pump and controller, 996 photodiode array detector and 717 Plus autosampler) with a Merck LiChrospher 100RP-18 column (250 × 4.0 mm i.d.; 5 μm particle size) operating at 30 °C and a flow rate of 1.0 ml/min. Gradient elution was as above, except that 5% HOAc was used.

3.2. Plant material

Leaflet samples were obtained from Herbarium material deposited at the Royal Botanic Gardens, Kew (K). Information on the species studied, collector number, geographical origin and date of collection is given in Table 4.

3.3. Sample preparation and isolation of pentaglycosides

Leaflet material from each accession listed in Table 4 (typically 20–300 mg) was ground to a powder in a pestle and mortar and extracted in MeOH–H_2O (1:1) to give solutions of 50 mg/ml concentration (referred to dry wt). Samples were extracted for 24 h at room temp., following which solid residues were removed by microcentrifugation and the supernatants passed through nylon acrodisc filters (0.45 μm, Fisher Scientific) prior to LC-ESI-MS and LC-UV analysis. Subsequently, the extracts of *C. haraka* (3 accessions) and *C. richardii* were taken to dryness, and the CD_3OD-soluble portions analysed directly by NMR.

Quantities of the flavonol pentaglycosides **1** and **2** sufficient for NMR analysis were obtained from ground leaflet material of *C. pinnata*, 430 mg of which was extracted in 8.6 ml MeOH–H_2O (1:1) for 24 h at room temp. After microcentrifugation, the supernatant was passed through a nylon acrodisc filter before sample clean-up on a Sep-Pak C-18 column as described previously (Veitch et al., 2003). A flavonoid-rich fraction (4.5 ml) eluted from the latter with MeOH–H_2O (1:1) was taken to dryness under a stream of N_2, redissolved in 1 ml MeOH, and filtered through a nylon acrodisc filter prior to semi-preparative HPLC. This was carried out on a Waters system comprising a LC600 pump and controller, 996 photodiode array detector and 717 Plus autosampler. A Merck LiChrospher 100RP-18 column (250 × 10.0 mm i.d.; 5 μm particle size) operating at 30 °C and a flow rate of 4.5 ml/min was used. The gradient elution program was based on A = MeOH and B = H_2O, with A = 20% at t = 0 min; A = 50% at t = 20 min; A = 50% at t = 25 min and A = 20% at t = 26 min. Repetitive isolation of flavonoids eluting at t_R = 13.5, 14.9 and 20.9 min gave **1** (3.8 mg), **2** (3.6 mg), and a mixture of two minor components (0.8 mg), respectively, all as yellow solids. NMR analysis revealed the latter to comprise quercetin 3-*O*-β-galactopyranoside and quercetin 3-*O*-α-rhamnopyranosyl(1→6)-β-galactopyranoside (approx. 1:1).

3.4. Sugar analysis

Acid hydrolysis of **1** and **2** (0.5 mg of each in 20 μl MeOH) was carried out by standard procedures (0.5 ml 2 M HCl, 100 °C, 1.5 h). After cooling, particulates were spun down by microcentrifugation and the supernatant removed and dried under a stream of N_2. The absolute configurations of the constituent monosaccharides of **1** and **2** released by acid hydrolysis were determined from GC-MS analysis of their trimethylsilylated thiazolidine derivatives, which were prepared using the method of Ito et al. (2004). Conditions for GC were: capillary column, DB5-MS (30 m × 0.25 mm × 0.25 μm), oven temp. programme, 180–300 °C at 6 °C/min; injection temp., 350 °C; carrier gas, He at 1 ml/min. Both **1** and **2** gave L-rhamnose and D-galactose, t_R = 10.2 and 12.3 min, respectively (identical to authentic standards).

3.5. Quercetin 3-O-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside-7-O-α-L-rhamnopyranoside (cordylasin A) (**1**)

UV (LC-PDA) λ_{max} nm: 255, 266sh, 355; ^{1}H and ^{13}C NMR: see Table 1; LC-ESI-MS *m/z*: 1049 $[M+H]^+$; Ion trap MS/MS of *m/z* 1049 $[M+H]^+$, *m/z* (rel. int.): 903 $[(M+H)–Rha]^+$ (29), 757 $[(M+H)–(2 \times Rha)]^+$ (46), 611 $[(M+H)–(3 \times Rha)]^+$ (31), 595 (27), 465 $[(M+H)–(4 \times Rha)]^+$ (6), 449 $[(M+H)–(3 \times Rha)–Gal]^+$ (100), 303 $[(M+H)–(4 \times Rha)–Gal]^+$ (17); HRESIMS *m/z*: 1049.3350 $[M+H]^+$ (calc. for $C_{45}H_{61}O_{28}$, 1049.3344).

3.6. Kaempferol 3-O-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside-7-O–α-L-rhamnopyranoside (cordylasin B) (**2**)

UV (LC-PDA) λ_{max} nm: 265, 349; ^{1}H and ^{13}C NMR: see Table 1; LC-ESI-MS *m/z*: 1033 $[M+H]^+$; Ion trap MS/MS of *m/z* 1033 $[M+H]^+$, *m/z* (rel. int.): 887 $[(M+H)–Rha]^+$ (43), 741 $[(M+H)–(2 \times Rha)]^+$ (49), 595 $[(M+H)–(3 \times Rha)]^+$ (34), 579 (27), 449 $[(M+H)–(4 \times Rha)]^+$ (6), 433 $[(M+H)–(3 \times Rha)–Gal]^+$ (100), 287 $[(M+H)–(4 \times Rha)–Gal]^+$ (5); HRESIMS *m/z*: 1033.3392 $[M+H]^+$ (calc. for $C_{45}H_{61}O_{27}$, 1033.3395).

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