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Mildbraedin, a novel kaempferol tetraglycoside from the tropical forest legume *Mildbraediodendron excelsum*

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Abstract—Aqueous methanol extracts of the leaves of *Mildbraediodendron excelsum* yielded a novel flavonol glycoside characterized by an O-linked branched tetrasaccharide. The structure of the compound was determined by spectroscopic methods to be kaempferol 3-*O*-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside. This previously unrecorded natural product was the major phenolic component of leaf material obtained both from a living specimen of the plant and a historic collection made in the field in 1928.

Mildbraediodendron excelsum Harms (Leguminosae: tribe Swartzieae) is a tall buttressed tree native to tropical forests of western central Africa. Its distribution extends from Ghana and Cameroon to the western borders of Uganda, where it finds some use as a timber for heavy construction work.[1,2] Little is known of the chemistry of this species, with the exception of a single report on isoflavonoids isolated from the heartwood.[3] Among the herbarium collections of the Royal Botanic Gardens at Kew (RBG, Kew) is a specimen of *M. excelsum* collected by Johannes Mildbraed in Cameroon in 1928 (*Mildbraed 10643*).[4] The opportunity to analyze a fragment of leaf material from this historic specimen revealed the presence of a novel glycoside of kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) as the main phenolic constituent. The equivalent analytical profile of leaf material taken from a living specimen of *M. excelsum* grown under glasshouse conditions closely resembled that of the historic sample, both presenting the same kaempferol glycoside as the major component. Here, we report on the isolation and structural elucidation of this new natural product (**1**).

Ground dried leaf material of *M. excelsum*, sourced from both living (65 mg) and herbarium (14 mg) specimens, was extracted in 10 mL 50% aqueous MeOH for 24 h at room temperature. After removal of solid residues the extracts were dried, redissolved in 50% aqueous MeOH to 5 mg/mL concentration and passed through nylon acrodisc filters (0.45 μm, Fisher Scientific). Analytical HPLC with diode-array detection was carried out with a Merck LiChrospher 100RP-18 (250 × 4.0 mm i.d.; 5 μm particle size) column operating under gradient conditions (solvent A = MeOH–HOAc–H_2O (18:1:1), solvent B = HOAc–H_2O (2:98); A = 25% at $t = 0$ min; A = 100% at $t = 20$ min; A = 100% at $t = 25$ min and A = 25% at $t = 26$ min) at a flow rate of 1 mL/min and at 30 °C. The main component in each extract eluted at $t_R = 11.9$ min (**1**), and displayed a UV spectrum (λ_{max} 266, 348 nm) typical of 3-*O*-glycosides of the flavonol, kaempferol.[5] Further analysis by LC-APCI-MS, using methods described previously,[6] confirmed that the same kaempferol glycoside was present in both samples. This gave a protonated molecule at *m*/*z* 887 $[M+H]^+$, and protonated fragments at *m*/*z* 741, 595, 449 and 287. The latter were interpreted in terms of the loss of three deoxyhexose sugar residues and one hexose sugar residue. MS–MS of the protonated aglycone at *m*/*z* 287 gave a product ion spectrum matching that of kaempferol. These preliminary results indicated that **1** was a kaempferol glycoside bearing an O-linked tetrasaccharide at C-3 of the aglycone.

Quantities of compound **1** sufficient for NMR analysis were obtained from a 50% aqueous MeOH extract (200 mL) of 16.6 g ground dried leaves of *M. excelsum*

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(RBG, Kew living collections accession number 1996-2364; seed from *Cheek 8350*).[7] Following filtration and concentration, a flavonoid-rich fraction equivalent to 270 mg dried residue was obtained by use of Sep-Pak C18 columns for extract clean-up, as described previously.[8] The residue was redissolved in a minimum quantity of 50% aqueous MeOH and subjected to column chromatography on Sephadex LH-20 (400 × 16 mm i.d.; successive elution with 210 mL 50%, 70 mL 70% and 70 mL 100% MeOH; fraction size 10 mL). The compound of interest (monitored by analytical HPLC) eluted in fractions 7–10 and was further purified by semi-preparative HPLC on a Merck LiChrospher 100RP-18 (250 × 10.0 mm i.d.; 5 μm particle size) column, using a simple gradient method with MeOH and H_2O as solvents. This yielded **1** as a yellow amorphous solid (6 mg), which recrystallized from MeOH as yellow crystals, mp 221–223 °C, $[\alpha]_D^{22}$ −113.2 (*c* 0.2, MeOH). NMR data for **1** were acquired in DMSO-d_6 at 37 °C on Varian 600 MHz, 500 MHz or Bruker Avance 400 MHz instruments. Standard pulse sequences and parameters were used to obtain 1D ^{1}H, 1D ^{13}C, 1D selective ROESY and TOCSY, DQF-COSY, HSQC and HMBC spectra. Chemical shift references were obtained from the solvent resonances of DMSO-d_6 at δ_H 2.50 and δ_C 39.5, relative to TMS. High-resolution ESI-MS data were obtained on a Bruker Apex II instrument operating in the positive mode.

The 1D ^{1}H NMR spectrum of **1** comprised aromatic and glycosidic proton resonances together with a broad downfield-shifted exchangeable resonance at δ 12.63 (1H, s) typical of the hydrogen-bonded 5-OH group of flavonoids.[9] Two *meta*-coupled proton resonances at δ 6.14 (1H, d, $J = 1.8$ Hz, δ_C 99.0) and 6.35 (1H, d, $J = 1.8$ Hz, δ_C 93.7) were characteristic for H-6 and H-8 of a flavonoid A-ring, respectively.[9] Similarly, the coupled resonances at δ 8.03 (2H, d, $J = 8.9$ Hz, δ_C 130.5) and 6.86 (2H, d, $J = 8.9$ Hz, δ_C 114.9) were typical of H-2′/6′ and H-3′/5′ of a flavonoid B-ring, respectively.[9] Full assignment of the ^{1}H and ^{13}C NMR spectral resonances for the aglycone moiety of **1** (Table 1) followed through use of long-range correlations in HMBC spectra. These confirmed that the compound was a 3-*O*-glycoside of kaempferol. The expected pattern of chemical shift changes for C-2 (+9.0 ppm), C-3 (−3.2 ppm) and C-4 (+1.0 ppm), with respect to kaempferol, was noted as evidence in support of 3-*O*-glycosylation.[10] More compelling, however, was the long-range correlation detected in the HMBC spectrum between an anomeric sugar proton resonance at δ 5.60 (1H, d, $J = 7.7$ Hz, δ_C 98.7) and C-3 of the aglycone (δ_C 132.4). Three additional anomeric proton resonances corresponding to O-linked sugars were found in the 1D ^{1}H spectrum at δ 5.02 (1H, d, $J = 1.8$ Hz, δ_C 100.7), 4.78 (1H, d, $J = 1.8$ Hz, δ_C 102.3) and 4.37 (1H, d, $J = 1.8$ Hz, δ_C 100.0). The remaining sugar resonances appeared as a series of overlapping multiplets between 3.09 and 3.84 ppm, with the exception of three distinctive methyl resonances at δ 1.15 (3H, d, $J = 6.2$ Hz, δ_C 17.8), 1.05 (3H, d, $J = 6.2$ Hz, δ_C 17.7) and 0.78 (3H, d, $J = 6.2$ Hz, δ_C 17.0). The ^{1}H and ^{13}C resonances of each of the four sugar residues were assigned using 1D selective TOCSY and ROESY, DQF-COSY, HSQC and HMBC spectra (Table 1). These allowed them to be identified as β-galactopyranose (corresponding to the anomeric proton at δ 5.60) and three α-rhamnopyranose sugars.[9,11] Assignment of the α-Rha resonances was aided by HMBC connectivities observed from 6-CH_3 to C-4 and C-5 and from H-1 to C-2, C-3 and C-5, in each case. Also significant in this respect were ^{1}H–^{1}H correlations in DQF-COSY spectra from H-1 to H-2 and sequentially from 6-CH_3 to H-4. The configurations of the anomeric carbons of Gal and Rha were assigned as β and α, respectively, based on the magnitudes of the corresponding $^3J_{H\text{-}1,H\text{-}2}$ coupling constants.[9,11] The absolute configurations of the constituent monosaccharides of **1** released by acid hydrolysis were determined to be D-Gal and L-Rha by GC–MS analysis of their trimethylsilylated thiazolidine

Table 1. ^{1}H and ^{13}C NMR spectral data for **1** (DMSO-d_6, 37 °C)

	^{1}H (δ) mult. (*J* in Hz)	^{13}C (δ)
Kaempferol		
2		155.8
3		132.4
4		176.9
5		161.1
6	6.14 d (1.8)	99.0
7		165.6
8	6.35 d (1.8)	93.7
9		156.4
10		103.2
1′		120.9
2′, 6′	8.03 d (8.9)	130.5
3′, 5′	6.86 d (8.9)	114.9
4′		159.7
5-OH	12.63 br s	
3-O-β-Gal		
1	5.60 d (7.7)	98.7
2	3.76 dd (9.5, 7.7)	75.3
3	3.63 dd (9.5, 3.2)	73.6
4	3.59 m	68.4
5	3.56 m	73.2
6	3.57 m, 3.23 m	65.0
2^{Gal}-O-α-Rha (I)		
1	5.02 d (1.8)	100.7
2	3.84 m	70.2
3	3.55 dd (9.5, 3.4)	78.6
4	3.26 t (9.3)	70.8
5	3.82 dd (9.6, 6.2)	68.2
6	0.78 d (6.2)	17.0
3^{RhaI}-O-α-Rha (II)		
1	4.78 d (1.8)	102.3
2	3.75 m	70.3
3	3.55 dd (9.5, 3.6)	70.5
4	3.20 t (9.3)	72.2
5	3.69 dd (9.3, 6.2)	68.2
6	1.15 d (6.2)	17.8
6^{Gal}-O-α-Rha (III)		
1	4.37 d (1.8)	100.0
2	3.37 m	70.3
3	3.30 dd (9.3, 3.3)	70.5
4	3.09 t (9.4)	71.8
5	3.36 dd (9.4, 6.2)	68.1
6	1.05 d (6.2)	17.7

derivatives.[12] These were prepared according to the method of Ito et al.,[13] and analyzed under the following GC conditions: capillary column, DB5-MS (30 m × 0.25 mm × 0.25 μm), oven temperature programme, 180–300 °C at 6 °C/min; injection temperature, 250 °C; carrier gas, He at 1 mL/min; L-rhamnose and D-galactose, $t_R = 10.4$ and 12.5 min, respectively (identical to authentic standards). The acid hydrolysis of **1** was carried out by standard procedures.[5]

The interglycosidic linkages of the intact flavonol glycoside were characterized from HMBC and ROE data. Confirmation of the primary sugar as β-Gal was given by the long-range correlation in the HMBC spectrum of **1** from Gal H-1 to C-3 of the aglycone, as noted above. Further correlations from Gal H-2 to C-1 of Rha I at δ_C 100.7 and from H-1 of Rha I to Gal C-2 at δ_C 75.3 indicated that the primary sugar was O-linked at C-2 to Rha I. Similarly, correlations from Gal 6-CH_2 to C-1 of Rha III at δ_C 100.0 and from H-1 of Rha III to Gal C-6 at δ_C 65.0 specified an additional O-linkage to Rha III at C-6. These linkages to the primary sugar were supported by the downfield shifts of Gal C-2 and C-6 with respect to their counterparts in kaempferol 3-*O*-β-D-galactopyranoside.[10] The remaining α-rhamnopyranose (Rha II in Fig. 1) was found to be O-linked at C-3 of Rha I, based on the long-range correlations from H-3 of this sugar residue to C-1 of Rha II at δ_C 102.3 and from H-1 of Rha II to the downfield shifted C-3 resonance of Rha I at δ_C 78.6. Further evidence for these interglycosidic linkages was provided by ROE connectivities detected between H-1 of Rha I and Gal H-2, H-1 of Rha II and H-3 of Rha I, and H-1 of Rha III and Gal 6-CH_2. Compound **1** was therefore identified as kaempferol 3-*O*-α-L-rhamnopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside (mildbraedin), a novel flavonol glycoside that has not been reported previously (Fig. 1). Confirmation of the molecular formula of $C_{39}H_{50}O_{23}$ was obtained by HRESI-MS (*m*/*z* 909.2644 $[M+Na]^+$, Δ + 0.9 mmu).

A second example of a flavonol glycoside bearing an O-linked tetrasaccharide was detected as a minor component of the leaf extract of *M. excelsum* obtained from the specimen collected by Mildbraed in 1928. Eluting at $t_R = 10.5$ min (1.4 min earlier than **1**), this component had a UV spectrum (λ_{max} 256, 266 sh, 355 nm) typical of 3-*O*-glycosides of the flavonol, quercetin (3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one).[5] LC-APCI-MS analysis gave a protonated molecule at *m*/*z* 903 $[M+H]^+$, and protonated fragments at *m*/*z* 757, 611, 465 and 303. MS–MS of the protonated aglycone at *m*/*z* 303 gave a product ion spectrum matching that of quercetin. The evidence available indicated that this compound was the quercetin analogue of **1** (Fig. 1), but further characterization could not be carried out because of the restricted availability of the source material. The same compound was not detected in the equivalent analytical profile of the *M. excelsum* extract sourced from the living specimen. This subtle difference in flavonoid content between glasshouse-grown and field specimens may be related to the higher levels of UV-B light experienced by the latter. Enhanced protection against UV-B and free radical damage has been demonstrated for flavonoids with *ortho*-dihydroxy (e.g., quercetin), as opposed to monohydroxy (e.g., kaempferol) substitution, in the B-ring.[14]

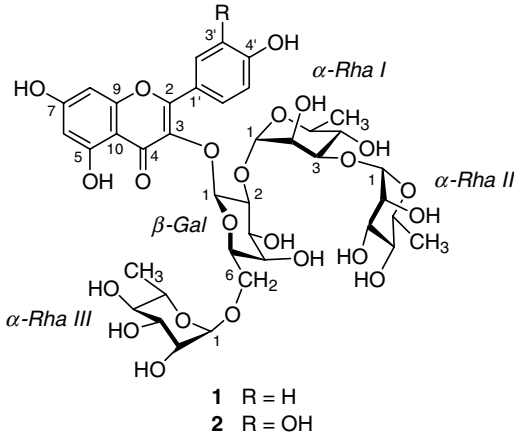

Figure 1. Flavonol tetraglycosides from *Mildbraediodendron excelsum*.

Flavonol *O*-glycosides are relatively common constituents of flowering plants and more than 1400 examples are known.[15] Nevertheless, fewer than 20 flavonol glycosides bearing an O-linked tetrasaccharide have been reported in the literature.[15,16] These invariably comprise branched tetrasaccharides O-linked to the flavonol aglycone at C-3, or more rarely, C-7. Of the five different branched tetrasaccharides found as 3-*O*-glycosides of kaempferol,[15–18] all contain β-Gal as the primary sugar with an α-Rha residue O-linked to Gal C-6 ('robinobioside core'). The trisaccharide motif of *O*-α-Rha(1→2)[α-Rha(1→6)]-β-Gal common to four of these branched tetrasaccharides is also found in mildbraedin (**1**). However, the tetrasaccharide that characterizes the latter compound has not been reported previously, either as a constituent of a flavonol glycoside or as a natural product in its own right. Thus, the flavonol glycoside mildbraedin appears to be an excellent chemical marker for *Mildbraediodendron excelsum*, and one which is valid for both historic and living collections of this species. The remarkable long-term stability of mildbraedin present in the specimen of *M. excelsum* dating from 1928 underlines the potential value of analysis of fragments of plant material from herbarium collections, particularly when it is difficult to obtain living material for study. The genus *Mildbraediodendron* contains only a single species, but its phylogenetic position with respect to other legume genera is of taxonomic interest.[19] Its closest generic relatives are currently considered to be *Cordyla* (Africa) and *Aldina* (S. America), which, together with *Amburana* (S. America), form the so-called Aldinoid clade of tribe Swartzieae, a group in need of further study. Targeted surveys by LC-UV-MS of the distribution of mildbraedin and its analogues may therefore offer valuable data towards the resolution of these generic relationships.

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