NMR Spectra of Flavone Di-C-glycosides from *Apometzgeria pubescens* and the Detection of Rotational Isomerism in 8-C-Hexosylflavones

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^{1}H and ^{13}C NMR spectra are documented for the novel flavone-6,8-di-C-glycosides isolated from the liverwort, *Apometzgeria pubescens*. The existence of rotational isomerism evident from these spectra is shown to be general for 8-C-monohexosylflavones and of possible diagnostic value.

Introduction

The number of new flavonoid C-glycosides reported from plant sources has increased rapidly in the past 10 years [1, 2]. Recently, a wide variety of unusual and often unique flavone C-glycosides has been isolated from liverworts [3] and their structures have generally been well supported with spectroscopic evidence. However, preliminary data only has so far appeared in support of five recently reported flavone-C-glycosides from *Apometzgeria pubescens* [4, 5]. The present paper describes the isolation in quantity of these components and documents for the first time, their full ^{1}H and ^{13}C NMR spectra, and their distribution in nature. As a result of compiling these spectra, characteristics apparently diagnostic for 8-C-hexosylflavones have become evident.

Results and Discussion

The five flavone-di-C-glycosides previously isolated in small quantity from *Apometzgeria pubescens* and for which full NMR data have not yet appeared are: tricetin-6,8-di-C-glucopyranoside (**1**), tricetin-6-C-arabinopyranoside-8-C-glucopyranoside (**2**), apigenin-6,8-di-C-arabinopyranoside (**3**), tricin-6,8-di-C-arabinopyranoside (**4**), and apometzgerin (3′,4′-di-O-methyltricetin)-6,8-di-C-arabinopyranoside (**5**) [4, 5]. All are unusual and several are unique. Thus **1** has previously been found only in the liverworts *Metzgeria furcata* [6], *Plagiochila asplenioides* [7], and *Radula* [8]; **2** has been detected (by the authors) in *Metzgeria furcata* and some *Radula* species; **3** has been found in *Hymenophyton* [9], *Takakia* [10], *Melilotus alba* [11], *Angiopteris* [12] and *Hoya lacunosa* [13]; and **4** and **5** are new.

Full ^{13}C and ^{1}H NMR spectra for compounds **1**–**5** are presented in Tables I and II together with selected reference spectra. The spectra are all consistent with the structures previously proposed, but in addition, the ^{1}H NMR spectra define the C-glucosyl moieties to be β-linked ($J_{1,2}$ for H-1″ = 10 Hz) and the C-arabinosyl moieties to be α-linked ($J_{1,2}$ for H-1″ = 9.5 Hz) [14]. Several spectra unexpectedly exhibited extensive doubling of signals. In Table I this phenomenon is evident in the spectra of **1** and **2** both of which contain 8-C-hexosyl substituents, but is not shown by compounds **3**, **4** and **5** all of which have 8-C-arabinosyl substituents. That this phenomenon is a general characteristic of 8-C-monohexosylflavones was revealed when a wider range of available (20 MHz) spectra was examined. Of these spectra, those showing no signs of signal doubling included the apigenin C-glycosides, isovitexin, schaftoside, neoschaftoside and apigenin-6-C-xyloside-8-C-arabinoside; the luteolin glycosides, carlinoside, isoorientin-2″-O-glucoside and neocarlinoside; and tricetin 6-C-glucoside and tricetin-6-C-glucoside-8-C-arabinoside, all of which lack an 8-C-hexosyl substituent. Apart from that of **2**, the only 20 MHz spectrum found which exhibited doubling was that of vitexin-7-O-glucoside (C-2′6′ and C-6 signals only), the spectra of vitexin-2″-O-rhamnoside and orientin-2″-O-glucoside exhibiting only doubtful doubling. It is possible that the resolution normally obtained at 20 MHz may be insufficient to ensure that signal doubling, when present, is always revealed. For this

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reason 100 MHz ^{13}C NMR spectra were recorded for two further 8-C-glucosylflavones, lucenin-2 (luteolin-6,8-di-C-glucoside) and stellarin-2 (chrysoeriol-6,8-di-C-glucoside) and for vitexin-2″-O-rhamnoside. Both lucenin-2 and stellarin-2 showed extensive signal doubling of both the aromatic and sugar carbon signals, but this was not evident with vitexin-2″-O-rhamnoside.

The above observations suggest that in flavones, interaction occurs between a C-linked(β?)-monohexose at C-8 and the B-ring. Since 8-C-pentopyranosides do not exhibit this phenomenon, the primary hydroxyl group of the hexose would appear to be the function interacting with the B-ring. This results in restricted rotation of the B-ring and/or the hexose, giving rise to a mixture of two NMR distinguishable isomers (see *e.g.* [15]). The presence of an additional sugar at the 2″-position however complicates this situation, since a 2″-O-rhamnosyl substituent apparently locks the 8-C-hexosyl unit in a position from which it cannot interact with the B-ring.

Rotational isomers such as those proposed above should be interconvertible at higher temperatures and indeed, with lucenin-2 and stellarin-2 for which extensively doubled signals were obtained at 25 °C, no sign of doubling was evident when their spectra were measured at 90 °C. This provides confirmation of the existence of rotational isomers and also explains why the 90 °C spectra presented in a recent widely used compilation [16] consistently fail to show signal doubling. Further confirmation derives from the observation that signal doubling is not observed in the spectra of 8-C-hexosides in which the B-ring is moved away from possible steric interaction with the glycosyl moiety, as in the 8-C-glucosylisoflavones puerarin and mirificin (puerarin-6″-O-apiofuranoside). It is thus evident that the presence of rotational isomers can be detected by ^{13}C NMR spectroscopy at 100 MHz (and generally also at 20 MHz). Such information is clearly of potential diagnostic value in the structure elucidation of any flavone C-glycoside which contains a C-linked monohexose (with no 2″-O-linked sugar).

The ^{1}H NMR spectra also reflect the presence of these rotational isomers in approximately the same ratios (see Tables I and II). It has long been thought that the anomalous H-2′6′ signals in the spectrum of the TMS-ether of vitexin [17] compared with those of isovitexin might indicate some interaction between the 8-C-hexose and the B-ring, and Besson *et al.* [18] made passing reference to the possible existence of 'rotamers' when reporting doubled anomeric proton signals in the spectrum of the PDM-ether of corymboside (apigenin-6-C-arabinoside-8-C-galactoside). In the present study (Table II) total signal doubling was observed in the spectra of underivatized **1** and **2** when measured at ambient temperature in d_6-DMSO at 400 MHz, whereas the spectra of **3** and **5** (and vitexin-2″-O-rhamnoside) measured under identical conditions show no doubling. Doubling was also absent from the high field spectra of the 8-C-glucosylisoflavones puerarin and mirificin. Reference to other available spectra indicates that rotational isomerism may also be evident in 80 MHz spectra. For example the spectrum of tricin-6-C-arabinoside-8-C-glucoside reveals a doubled H-2′,6′ signal and noticeable irregularities are visible in the H-2′,6′ signals of the TMS-ethers of isoschaftoside, vicenin-2 (and at 60 MHz, vitexin, [17]). In contrast, the spectra (TMS ethers) of flavones lacking an 8-linked hexose, *e.g.* schaftoside, tricin-6-C-glucoside-8-C-arabinoside (and the 60 MHz spectrum of isovitexin [17]) are normal. As with the ^{13}C NMR spectra, spectrum irregularities disappear at elevated temperatures and this was demonstrated with the TMS-ether of **1** which was determined both at 30 °C and 145 °C.

In conclusion, it is evident from the above results that ^{13}C or ^{1}H NMR spectra, when measured at ambient temperature and (preferably) at high field, can provide evidence of value for determining the site of attachment of C-linked hexoses in flavone C-glycosides (after removal of any 2″-O-linked sugars).

Materials and Methods

General

See reference [19].

Plant material

See reference [4].

Extraction and isolation

Air-dried plants (110 g) were carefully cleaned and ground in a Waring Blendor with $CHCl_3$ and subsequently with 80% aq. EtOH, 80% aq. MeOH and 50% aq. MeOH. The isolation of the individual compounds was achieved by repeated, combined CC and PC (often overrun). Purified compounds were

Table I. ^{13}C NMR spectra of *Apometzgeria pubescens* flavonoids **1**–**5** and reference compounds*.

	Tricetin 6-C-glucoside [19]	Tricetin 6,8-di-C-glucoside (**1**)+	Tricetin 6-C-arabinoside 8-C-glucoside (**2**)+	Apigenin 6-C-glucoside 8-C-arabinoside [16]	Apigenin 6,8-di-C-arabinoside (**3**)	Tricin 6,8-di-C-arabinoside (**4**)	Apometzgerin 6,8-di-C-arabinoside (**5**)
C-2	164.1[a]	164.3, 164.9	165.9, 165.7	164.2	165.9	163.9	164.3
C-3	103.0	102.8, 102.9	104.6[c], 104.2[c]	102.6	103.6	103.3[a]	103.2[a]
C-4	182.0	182.5	183.7	182.5	183.8	182.5	183.6
C-5	160.9	158.9, 160.2	159.6, 160.5	159.6	160.0	159.5	160.3
C-6	109.0	107.8, 109.4	109.7, 108.5	108.5	108.3	109.2	108.9
C-7	163.7[a]	161.2, 162.0	163.1, 162.4	161.6[a]	162.7	162.6	163.1
C-8	93.7	104.2[a], 105.5[a]	104.2[c], 105.5[c]	104.5	104.9	104.1[a]	104.0[a]
C-9	156.5	154.3, 155.4	156, 156.7	154.5	155	154.2	155
C-10	103.5	103.4[a], 103.6[a]	105.1[c]	103.6	103.6	103.6[a]	105.0[a]
C-1′	120.5	120.9, 121.4	122.1, 122.4	121.4	122.6	120.9	126.8
C-2′	105.8	106.3[c], 106.8[c]	107.3[a], 107.9[a]	129.3	130.4	104.8	103.2[a]
C-3′	146.6	146.7	147.2	116.2	117.3	148.3	154.5
C-4′	138.1	138.2, 138.3	139.1	161.3[a]	161.6	139.8	140.6
C-5′	146.6	146.7	147.2	116.2	117.3	148.3	151.8
C-6′	105.8	106.3[c], 106.8[c]	107.3[a], 107.9[a]	129.3	130.4	104.8	105.0[a]
G-1	73.3	75.4, 74.5, 73.8, 73.6	74.9, 74.5	73.7			
G-2	70.8[b]	72.3[b], 72.1[b], 71.1[b]	72.5, 72.4	71.1			
G-3	79.2	79.5, 79.3, 78.2, 78.2	79.6, 79.4, 79.2	78.8			
G-4	70.4[b]	70.8[b], 69.7[b], 69.5[b]	70.9, 70.7	70.2			
G-5	81.7	82.5, 82.1, 81.8, 81.3	82.8, 82.3	81.5			
G-6	61.7	62.2, 61.7, 60.6, 60.2	62.9, 61.9	61.0			
A-1			75.2	74.6	75.5[a], 74.9[a]	74.9[b], 74.1[b]	74.5[b]
A-2			69.8[b]	68.9[b]	70.0	68.8[c], 68.5[c]	69.3
A-3			75.6	75.1	75.8[a], 75.5[a]	75.5[b], 74.9[b]	75.6[b], 75.0[b]
A-4			69.9[b]	69.2[b]	70.0	69.3[c], 69.1[c]	69.3
A-5			71.8	71.1	72.0, 71.6	70.8, 70.5	71.1, 70.8
OCH_3						56.4	60.5(4′), 56.5(3′)

* Assignments bearing the same superscript are interchangeable.
+ Approximate rotamer ratios: **1**, 1:0.95; **2**, 1:0.55; (stellarin-2, 1:0.8; lucenin-2, 1:0.6).

Table II. ^{1}H NMR spectra of *Apometzgeria pubescens* flavonoids **1**–**5**.

	Tricetin 6,8-di-C-glucoside (**1**)+	Tricetin 6-C-arabinoside 8-C-glucoside (**2**)+	Apigenin 6,8-di-C-arabinoside (**3**)	Tricin 6,8-di-C-arabinoside (**4**)	Apometzgerin 6,8-di-C-arabinoside (**5**)
H-3	6.57 s, 6.54 s	6.59 s, 6.55 s	6.83 s	6.94 s	6.88 s
H-2′,6′	7.08 s, 6.99 s	7.07 s, 6.93 s	8.20 br.d. (J=8 Hz)	7.38 s	7.22 br.s.
H-3′,5′			6.91 d (J=8.8 Hz)		
H-1″/H-1‴	4.97 d (J= 9.7 Hz) 4.79 d (J=10.1 Hz) 4.74 d (J= 9.8 Hz) 4.65 d (J= 9.6 Hz)	4.87 d (J= 9.2 Hz) 4.73 d (J=10.0 Hz) 4.69 d (J= 9.6 Hz) 4.62 d (J= 9.6 Hz)	4.70 d (J=9.5 Hz) 4.66 d (J=9.5 Hz)	4.83 d (J=9.5 Hz) 4.58 d (J=9.5 Hz)	4.76 d (J=9.5 Hz) 4.58 d (J=9.5 Hz)
Methoxyl				3.89 s	3.88 s, 3.73 s
5-OH	13.77 s, 13.69 s	13.89 s, 13.63 s	>10	>10	13.25 s

+ Approximate rotamer ratios: **1**, 1:0.95; **2**, 1:0.5.

crystallized from MeOH–H_2O mixtures. Approximate yields:

tricetin 6,8-di-C-glucoside: 1130 mg;
tricetin 6-C-arabinoside-8-C-glucoside: 300 mg;
tricetin 6,8-di-C-arabinoside: 110 mg;
apometzgerin 6,8-di-C-arabinoside: 180 mg;
apigenin 6,8-di-C-arabinoside: 20 mg.

Chromatography

CC: microcrystalline cellulose (Avicel, Merck) with solvents 3% HOAc to 10% HOAc; Sephadex LH-20 (Pharmacia) with solvents 70% aq. MeOH to 100% MeOH. PC: Whatman 3MM with solvents BAW and n-pentanol:HOAc:H_2O (2:1:1).

^{13}C NMR spectroscopy

Conditions used for measurement of spectra in Table I: **1**, 100 MHz, d_6-DMSO, ambient temp.; **2**, 20 MHz, d_6-DMSO:H_2O (1:1), 30 °C; **3**, 20 MHz, d_6-DMSO:H_2O (1:1), 30 °C; **4**, 20 MHz, d_6-DMSO, 30 °C; **5**, 100 MHz, d_6-DMSO, ambient temp. Conditions used for measurement of stellarin-2: 100 MHz, d_6-DMSO, 25 °C and 90 °C, for lucenin-2: 100 MHz, d_6-DMSO, 25 °C and 80 °C, for vitexin-2″-O-rhamnoside: 100 MHz, d_6-DMSO, 25 °C, and for puerarin and mirificin: 20 MHz, d_6-DMSO, ambient temp.

^1H NMR spectroscopy

Conditions used for measurement of spectra in Table II: **1**, 400 MHz, d_6-DMSO, ambient temp.; **2**, 400 MHz, d_6-DMSO, ambient temp.; **3**, 200 MHz, d_6-DMSO, ambient temp., checked for signal doubling using 400 MHz; **4**, 80 MHz, d_6-DMSO, 30 °C; **5**, 200 MHz, d_6-DMSO, ambient temp., checked for signal doubling using 400 MHz; vitexin-2″-O-rhamnoside, 400 MHz, d_6-DMSO, 25 °C, and for puerarin and mirificin, 200 MHz, d_6-DMSO, ambient temp. The TMS-ether of compound **1** was prepared as described in ref. [19] and the spectra determined at 80 MHz in d_6-DMSO at 30 °C, 110 °C and 145 °C.

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