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MASS SPECTROMETRY IN THE STRUCTURAL DETERMINATION OF FLAVONOL TRIGLYCOSIDES FROM *VINCA MAJOR*

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Key Word Index—*Vinca major*; Apocynaceae; leaves; flavonol glycosides; kaempferol 3-*O*-(6″-*O*-rhamnosyl)-galactoside 7-*O*-glucoside; chlorogenic acid; mass spectrometry.

Abstract—Chlorogenic acid, robinin and a flavonol triglycoside were isolated from the leaves of *Vinca major*. The structure of the triglycoside was determined to be kaempferol 3-*O*-(6″-*O*-rhamnopyranosyl)-galactopyranoside 7-*O*-glucopyranoside by fast atom bombardment, electron impact and negative ion desorption chemical ionization mass spectrometry.

INTRODUCTION

In previous papers, we reported that EI-mass spectrometry was a very useful tool for structural elucidation of flavonoid glycosides, but the investigation was carried out on methyl ether, acetyl and trimethylsilyl derivatives [1–3]. We showed that fast atom bombardment (FAB) and desorption chemical ionization (DCI) mass spectrometry provided very useful information (e.g. the M_r, the aglycone and the sugar moiety) that helped in the structural elucidation of unmodified flavonoid glycosides [4, 5]. We reported the distribution of flavonoid glycosides in the Apocynaceae using gas chromatography/mass spectrometry [6]. The results indicated the presence of unknown flavonoid glycosides in the leaves of *Vinca major*. We have now carried out the isolation of these compounds. This paper describes the application of EI, DCI, and FAB mass spectrometry for the structural determination of flavonoid triglycosides isolated from *V. major*.

RESULTS AND DISCUSSION

Extraction was carried out as described in the previous paper [7]. The *n*-butanol extract was subjected to silica gel column chromatography using a methanol–chloroform gradient to afford compounds **1**–**3**.

Compounds **1** (colourless powder, $C_{16}H_{18}O_9 \cdot 1/2H_2O$, mp 204–207°) and **2** (yellow needles, $C_{33}H_{40}O_{19}$, mp 250–254°) were identified as chlorogenic acid (**1**) and robinin (**2**), respectively, by direct comparison with authentic samples [8, 9].

Compound **3** was recrystallized from ethanol plus water to give yellow needles, $C_{33}H_{40}O_{20} \cdot 5H_2O$, mp 230–235°, which reacted positively in the Hg–HCl, Zn–HCl and ferric chloride tests. Hydrolysis produced kaempferol, rhamnose, glucose and galactose. Acid hydrolysis of permethylated **3** with diazomethane gave **3a**. The EI-mass spectrum of trimethylsilylated **3a** showed the presence of peaks due to the $[M]^{+\cdot}$, the $[M-15]^+$ and the $[M-104\ (TMSiO+Me)]^{+\cdot}$ ions at *m/z* 458 (1.6%), 443 (100.0%) and 354. It also showed the presence of an ion at *m/z* 135 due to the B-ring [10]. In addition the spectrum of **3a** showed ions due to $[M-46]^{+\cdot}$ as a medium intensity peak, but no ions corresponding to $[M-1]^+$. This fact showed the presence of a methyl group at the C-5 position [11]. These results indicated that **3** was kaempferol 3,7-*O*-glycosides.

The negative ion DCI-mass spectrum of **3** (Fig. 1) showed the presence of ions due to the aglycone, the loss of the sugar moiety from the molecule and the sugar moiety. The ion *m/z* 143 and 126 were formed from the hexose moiety and those at *m/z* 145 and 163 from the deoxyhexose ion. But, in this spectrum there was no indication of the presence of the $[M]^+$.

The position of the linkage between deoxyhexose and hexose in **3** was confirmed by comparison of the results of the negative ion DCI mass spectra with those of neohesperidin and hesperidin as described in a previous paper [4]. The presence of an ion at *m/z* 290 indicated that the sugar moiety consisted of deoxyhexose and hexose, and that deoxyhexose should be linked by a glycosidic bond at the C-6 position of the hexose. Therefore, *m/z* 290 ions are formed by the loss of water from 6-rhamnosylglucose or 6-rhamnosylgalactose.

The positive ion FAB-mass spectrum of **3** (Fig. 2) showed the presence of ions due to the aglycone plus proton (base peak), the quasi-$[M]^+$ ($[M+H]^+$) and the loss of sugar moieties from the $[M]^+$. The presence of ions at *m/z* 595 and 611 indicated that the terminal sugars were deoxyhexose (rhamnose) and hexose. Also the relative intensity (6.1%) of the *m/z* 449 ion was greater than that of the ion *m/z* 595 (0.8%). This result indicated that **3** was kaempferol 3-*O*-(6″-rhamnosyl) glucoside 7-*O*-galactoside or kaempferol 3-*O*-(6″-rhamnosyl) galactoside 7-*O*-glucoside.

The EI-mass spectrum of acetylated **3** (**3c**) showed the presence of ions due to the sugar moieties and the aglycone plus the acetylated hexose moiety (Fig. 3). The fragmentation pattern was similar to that of the acetate of manghaslin 7-*O*-glucoside (unpublished data). The ions at *m/z* 273 and 331 supported the presence of the terminal hexose and deoxyhexose as described above. In addition, the presence of ions at *m/z* 169 and 111 indicated that the

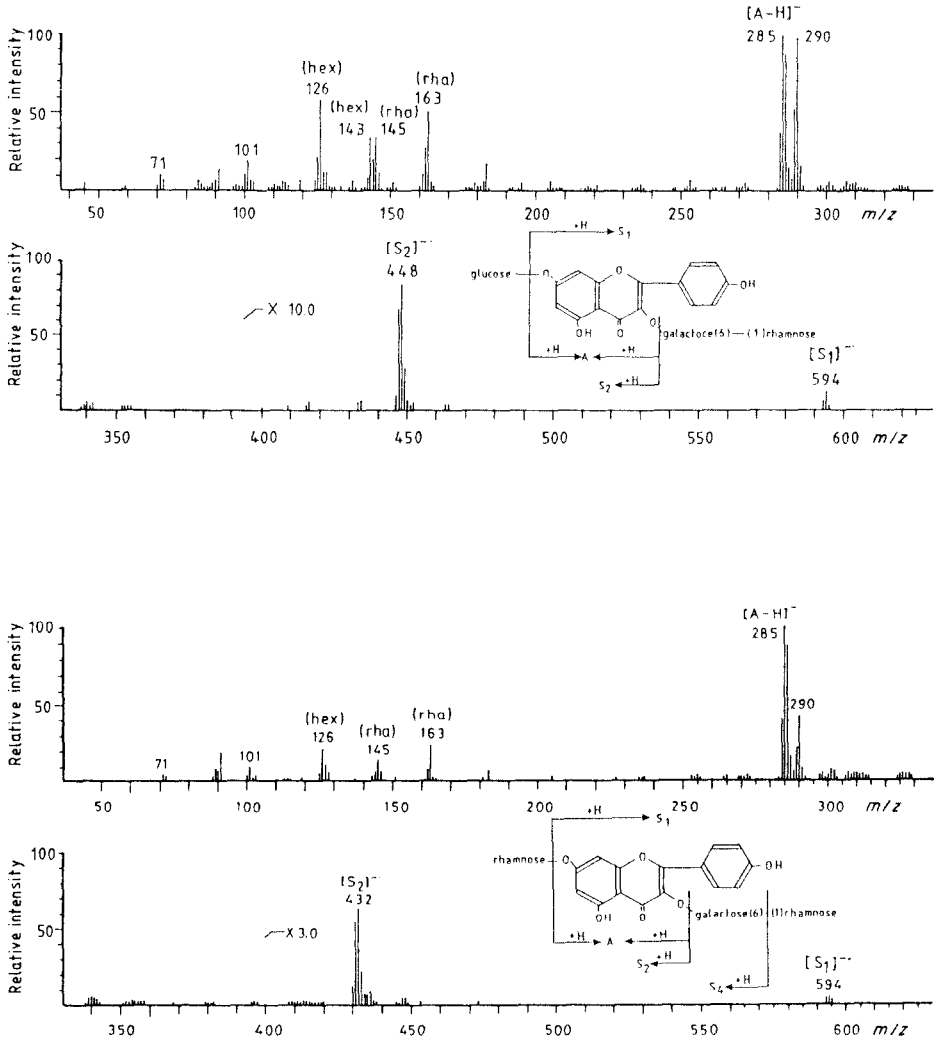

Fig. 1. Negative ion DCl-mass spectra of kaempferol 3-*O*-(6″-*O*-rhamnopyranosyl)galactopyranoside 7-*O*-glucopyranoside (**3**) and kaempferol 3-*O*-(6″-*O*-rhamnopyranosyl)galactopyranoside 7-*O*-rhamnopyranoside (**2**). (hex: galactose or glucose moiety).

rings of the sugar moieties were hexopyranose and deoxyhexopyranose types [12].

Acetolysis of **3c** in a deuterium medium gave the following compounds: 1-*O*-trideuterioacetyl 2,3,4,6-tetra-*O*-acetylglucopranose, 1,6-di-*O*-trideuterioacetyl 2,3,4-*O*-tri-*O*-acetylgalactopranose and 1-*O*-trideuterioacetyl 2,3,4-tri-*O*-acetylrhamnopranose. These compounds were identified by the shift of mass numbers due to the deuterium effect described in previous papers 12, unpublished data. Thus, **3** was identified as kaempferol 3-*O*-robinobioside 7-*O*-glucopyranoside [13].

The structure was finally proved as follows. Enzymatic hydrolysis of **3** with β-glucosidase gave glucose and **3b** (kaempferol 3-*O*-diglycoside); glucose was identified by chromatographic methods. Compound **3b** was permethylated with diazomethane and then hydrolysed with 3% sulphuric acid. In the reaction mixture was identified 5,7,4-tri-*O*-methylkaempferol, galactose and rhamnose using and GC and GC-mass spectrometry. In addition, the ^{13}C NMR of **3** was similar to that of robinin (**2**).

EXPERIMENTAL

NMR spectra were run at 90 MHz in DMSO-d_6 with TMS as int. std. EIMS was recorded using a direct inlet, electron energy 70 eV, ion source temp 320°. FABMS were measured using Ar or Xe, ion gun at 7 or 6 kV, matrix: glycerol or thioglycerol. Negative ion DCIMS were recorded in the pressure range of 10^{-3} to 10^{-2} torr. For our expts, CH_4 was used as reagent gas at a source pressure of 4×10^{-3} torr. The compounds were dissolved in MeOH and between 1 and 100 ng placed on a W wire at the end of the desorption probe. This filament was introduced into the centre of the ion source and heated from 50 to 1000° in 20 sec by applying a current of 50–400 mA at a rate of 10 mA per sec. The ion source temp was kept at 200°, the electron energy at 90 eV.

Plant material. Vinca major collected at Faculty of Pharmacy, Meijo University (Japan) in August 1984 as crushed and extracted with MeOH at 100° and the extract treated as described in ref. [7]. The BuOH extracts were subjected to CC over silica gel using $CHCl_3$–MeOH elution gradient. The fraction eluted

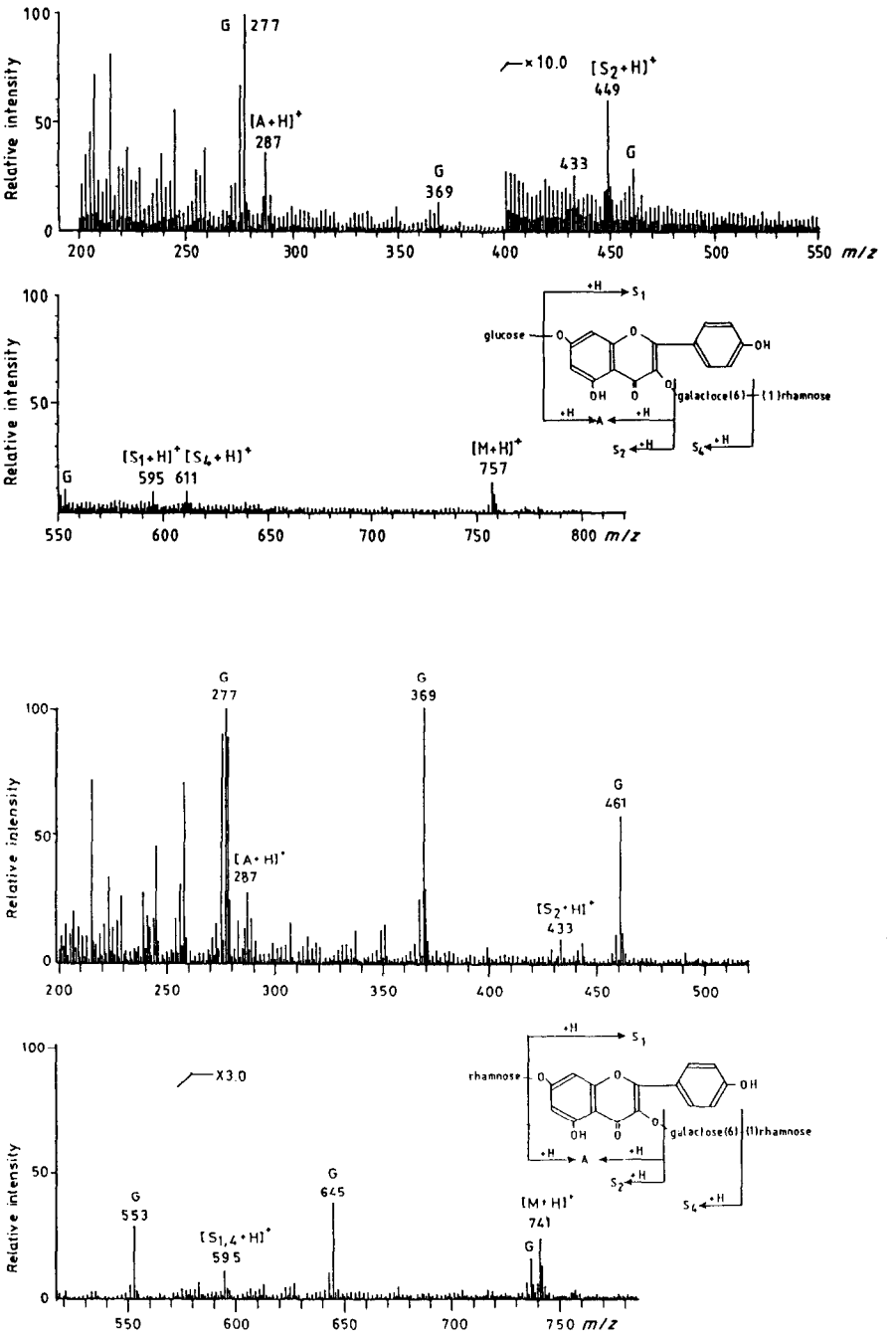

Fig. 2. Positive ion FAB-mass spectra of kaempferol 3-*O*-(6″-*O*-rhamnopyranosyl)galactopyranoside 7-*O*-glucopyranoside (**3**) and kaempferol 3-*O*-(6″-*O*-rhamnopyranosyl)galactopyranoside 7-*O*-rhamnopyranoside (**2**).

with MeOH–$CHCl_3$ (3:7) was rechromatographed on Sephadex LH-20 using a H_2O–EtOH gradient. The high polarity fractions were rechromatographed over silica gel in a $CHCl_3$–MeOH gradient to give 12.5 mg of (**1**), 15.5 mg of (**2**) and 10.5 mg of (**3**).

Chlorogenic acid (**1**). Colourless powder, dark green to the $FeCl_3$ reaction, negative to the HCl–Mg reaction. mp 204–207°. IR ν_{max}^{KBr} cm^{-1} 3400 (OH), 1732 (C=O), 1695 (C=O), 1632 (C=O), 1608 (C=C), 1522, 1282, 1180, 1040 (C–O). UV λ (in EtOH) nm (logε): 235 (sh) (3.85), 245 (3.88), 301 (3.99), 328 (4.12). EIMS (*m/z*): 354 $[M]^{+\cdot}$, 180 $[C_9H_8O_4]^{+\cdot}$, 163 $[C_9H_7O_3]^+$. ^1H NMR δ (in $CDCl_3$): 2.10 (4H, *br*, quinic acid 2,6-H), 3.60–3.90 (1H, *m*, quinic acid 4-H), 4.10–4.40 (1H, *m*, quinic acid 3-H), 5.10–5.60 (1H, *m*, quinic acid 5-H), 6.23 (1H, *d*, $J = 16.0$ Hz, –CH=CH–CO), 6.70–6.90 (2H, aromatic 5,6-H), 7.03 (1H, *s*, aromatic 3H), 7.57 (1H, *d*, $J = 16.0$ Hz. –CH=CH–CO). Calcd $C_{16}H_{18}O_9 \cdot 1/2H_2O$: C, 52.90; H, 5.27. Found: C, 53.05; H, 4.97. Identified by comparison of IR, ^1H NMR and EIMS spectra with authentic material.

Kaempferol 3-O-(6″-O-*rhamnosyl*)*galactoside* 7-O-*rhamnoside* (*robinin*) (**2**). Pale yellow powder, brown colour with the

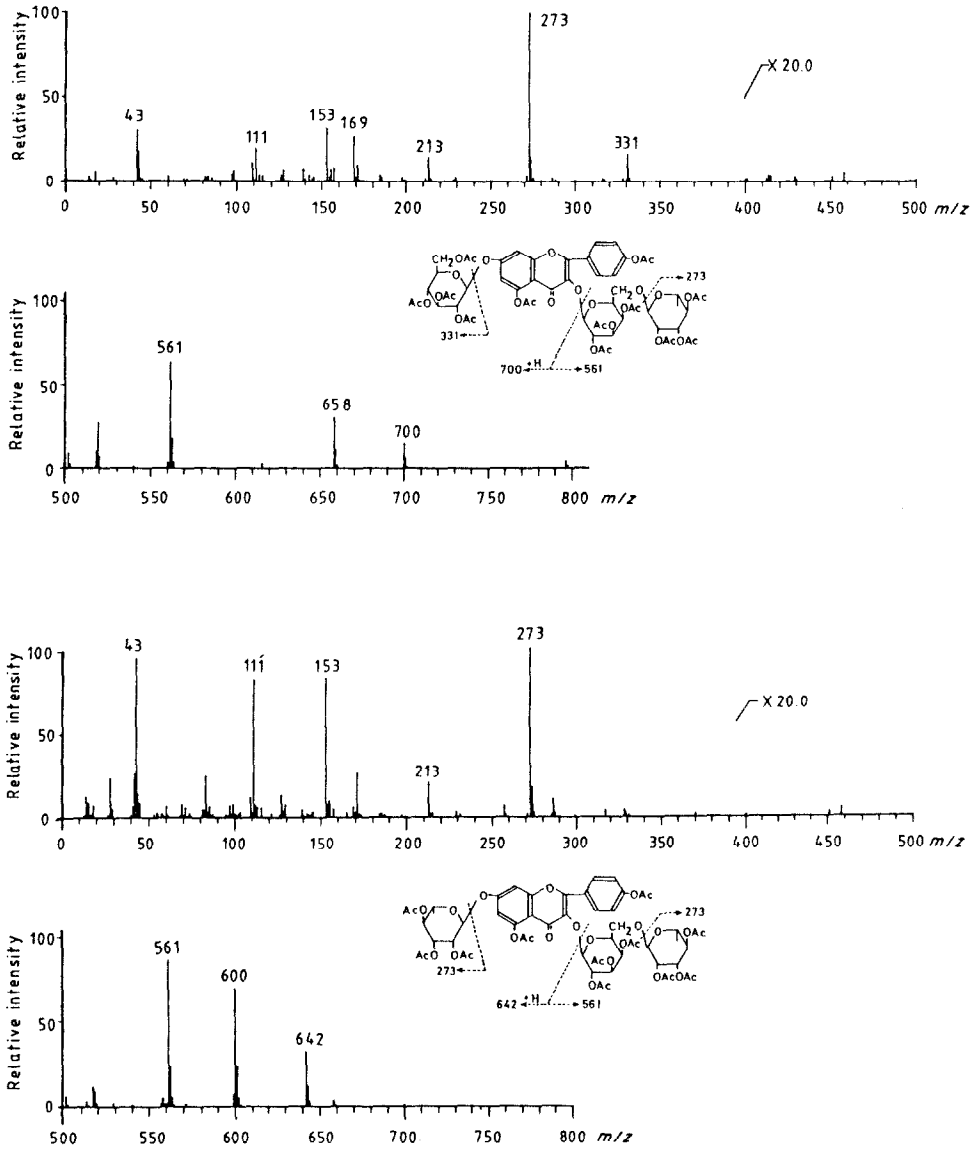

Fig. 3. EI-Mass spectra of kaempferol 3-*O*-(6″-*O*-rhamnopyranosyl)galactopyranoside 7-*O*-glucopyranoside peracetate (**3c**) and kaempferol 3-*O*-(6″-*O*-rhamnopyranosyl)galactopyranoside 7-*O*-rhamnopyranoside peracetate (**2c**).

$FeCl_3$ reaction, red. colour with the HCl–Mg reaction, red. colour with the HCl–Zn reaction. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2916 (CH), 1656 (C=O), 1590 (C=C), 1064 (C–O). UV λ (in EtOH) nm (logε): 265, 310 (sh), 350. UV λ (EtOH + AcONa) nm: 265, 315 (sh), 360, 400 (sh). UV λ (EtOH + $AlCl_3$) nm: 274, 302, 355, 395. ^{1}H NMR δ (in CD_3OD): 1.15–1.29 (4 H, *m*, rhamnosyl-Me × 2), 2.95–5.65 (*m*, gly-H), 6.45 (1H, *d*, J = 2.0 Hz, aromatic-H 3), 6.75 (1H, *d*, J = 2.0 Hz, aromatic H-8), 6.88 (2H, *d*, J = 9.2 Hz, aromatic 2′,6′-H), 8.11 (2H, *d*, J = 9.2 Hz, aromatic 3′,5′-H). ^{13}C NMR δ (in DMSO-d_6): 177.6 (C-4), 161.6 (C-7), 160.8 (C-5), 160.0 (C-4′), 157.0 (C-9), 155.9 (C-2), 133.5 (C-3), 131.0 (C-2′,6′), 120.7 (C-1′), 115.1 (C-3′,5′), 105.6 (C-10), 99.3 (C-6), 94.6 (C-8), sugar moiety; 105.5, 101.9, 99.3, 98.4, 73.6, 72.9, 71.9, 71.5, 70.3, 68.1, 65.0, 17.7. Calcd $C_{33}H_{40}O_{19}$: C, 53.50; H, 5.45. Found: C, 53.45; H, 5.47. Identified by comparison of IR, ^{1}H NMR and EIMS spectra with authentic material.

Hydrolysis of **2**. A soln of **2** (3 mg) in 3%H_2SO_4 was heated at 100° for 3 hr. The ppt. was sepd, washed with H_2O, and recrystallized from aq MeOH to give yellow needles, mp. 300°, UV λ (in EtOH) nm: 254 (sh), 268, 368, IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 2900 (CH), 1656 (C=O). The filtrate was treated in the usual way, and then examined by GC using a glass column (2 m × 3 mm) packed with 3% OV-17 on Shimalite-W(80–100 mesh)], column temp. 150–220° (3°/min), N_2 (30 ml/min), inj. and det. temp 280°, R_t (min): 5.3, 7.0 (TMSi derivative of L-rhamnose, 2 mol), R_t: 12.5, 14.0 (TMSi derivative of D-galactose, 1 mol).

Acetylation of **2**. Compound **2** (*ca* 5 mg) was treated with Ac_2O and pyridine. The product was purified by prep. TLC ($CHCl_3$) to give the peracetylated derivative **2** (**2c**), mp 147–149°. Colourless powder, negative to the $FeCl_3$ reaction, red colour with the HCl–Mg reaction, red colour with the HCl–Zn reaction. IR ν_{max}^{KBr} cm^{-1}: 2910 (CH), 1760 (MeCO), 1640 (C=O), 1600 (C=C), 1230, 1080 (C–O). ^{1}H NMR δ ($CDCl_3$): 1.00–1.30 (6 H, *d*, rhamnosyl-Me × 2), 1.90–2.20 (27H, each *s*, alphatic MeCO × 9), 2.29 (3H, *s*, aromatic MeCO), 2.41 (3H, *s*, aromatic MeCO), 2.86–5.55 (*m*, gly-H), 6.70 (1H, *d*, J = 2.2 Hz, aromatic 8-H), 7.05 (1H, *d*, J

= 2.2 Hz, aromatic 6-H), 7.15 (2H, *d*, *J* = 9.2 Hz, aromatic 2′,6′-H), 8.01 (2H, *d*, *J* = 9.2 Hz, aromatic 3′,5′-H).

Acetolysis of **2c**. The acetate (**2c**) was subjected to acetolysis with CD_3CO_2D (10), $(CD_3CO)_2O$(10) and D_2SO_4 (1) for two days. The reaction mixt. was extracted with EtOAc. The ext was washed with H_2O and evapd to dryness *in vacuo*. The residue was examined by GC/MS using a glass column (2 m × 3 mm) packed with 3% ECNSS-M on Gas chrom-Q(80–100 mesh) column temp 180°, He (50 ml/min), inj and det. temp 280°. R_t (min): 10.2 (1,6-di-*O*-trideuterioacetyl 2,3,4-tri-*O*-acetylgalactose). EIMS (*m/z*): 245 $[C_{11}H_{11}D_3O_6]^+$, 203 $[C_9H_9D_3O_5]^{+\cdot}$, 172 $[C_8H_6D_3O_4]^+$, 157 $[C_7H_9O_4]^+$, 143 $[C_7H_8O_3]^+$, 115 $[C_5H_7O_3]^+$, 98 $[C_5H_6O_2]^{+\cdot}$.

Methylation of **2**. Compound 2 (2 mg) was treated with excess CH_2N_2. A colourless powder was obtained (negative to the $FeCl_3$ reaction) which was then hydrolysed with 3% H_2SO_4 soln. The reaction mixt. was extracted with EtOAc. The EtOAc layer was washed with H_2O and evapd to dryness *in vacuo*. The residue was purified by prep. TLC ($CHCl_3$) to give the methylated derivative **2** (**2a**). **2a**: EIMS *m/z* (%): 314 (100.0) $[C_{17}H_{14}O_6]^{+\cdot}$, 285 (17.0) $[C_{16}H_{13}O_5]^+$, 268 (10.9) $[C_{16}H_{12}O_4]^{+\cdot}$, 135 (31.3) $[C_8H_7O_2]^+$. ^{1}H NMR δ (in $CDCl_3$): 3.85, 3.99 (6H, *s*, aromatic 4′, 7-OMe), 6.40 (1H, *d*, *J* = 2.2 Hz, aromatic 6-H), 6.57 (1H, *d*, *J* = 2.2 Hz, aromatic 8-H), 7.05 (2H, *d*, *J* = 9.2 Hz, aromatic 3′,5′-H), 8.16 (2H, *d*, *J* = 9.2 Hz, aromatic 2′,6′-H). **2a** was then silylated with TMS-PZ + TMS-HT (1:1, v/v, Tokyo kasei) and then examined by GCMS using an OV-17 column, 260°, He (30 ml/min), inj temp. 310° and separator temp 320°. R_t (min): 32.2 (TMSi derivative of 5,7,4′-tri-*O*-methyl kaempferol), EIMS *m/z* (%): 458 [M = $C_{23}H_{30}O_6Si_2]^{+\cdot}$, 443 $[M-15]^+$ (100), 354 $[M-15-TMSiO]^{+\cdot}$, 135 $[C_8H_7O_2]^+$.

Kaempferol-3-O-(6″-O-rhamnosyl)galactoside 7-O-glucopyranoside (**3**). Pale yellow powder, brown colour with the $FeCl_3$ reaction, red. colour with the HCl–Mg reaction and red. colour with the HCl–Zn reaction. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2920 (CH), 1660 (C=O), 1600 (C=C), 1078 (C–O). UV λ (in EtOH) nm: 265, 310 (sh), 350. UV λ (EtOH + AcONa) nm: 265, 315 (sh), 360, 400 (sh). UV λ (EtOH + $AlCl_3$) nm: 274, 302, 355, 395. ^{1}H NMR δ (in CD_3OD): 1.18 (3H, *m*, rhamnosyl-Me), 3.04–5.65 (*m*, gly-H), 6.21 (1H, *d*, *J* = 2.2 Hz, aromatic-H 6), 6.75 (1H, *d*, *J* = 2.2 Hz, aromatic H-8), 6.87 (2H, *d*, *J* = 9.2 Hz, aromatic 2′,6′-H), 8.08 (2H, *d*, *J* = 9.2 Hz, aromatic 3′,5′-H). ^{13}C NMR δ (in DMSO-d_6): 177.6 (C-4), 162.8 (C-7), 160.8 (C-5), 160.0 (C-4′), 157.2 (C-9), 156.0 (C-2), 133.5 (C-3), 131.0 (C-2′,6′), 120.7 (C-1′), 115.1 (C-3′,5′), 105.6 (C-10), 99.3 (C-6), 94.1 (C-8), sugar moiety; 100.0, (glu-1), 73.0 (glu-2), 76.3 (glu-3), 69.7 (glu-4), 77.0 (glu-5), 60.7 (glu-6), 101.8 (gal-1), 71.1 (gal-2), 73.6 (gal-3), 68.2 (gal-4), 73.0 (gal-5), 65.2 (gal-6), 100.0 (rham-1), 71.1 (rham-2,3) 71.8 (rham-4), 68.2 (rham-5), 17.8 (rham-6). FAB- and DCIMS see Figs 1 and 2. Calcd $C_{33}H_{40}O_{20}\cdot 5H_2O$: C, 46.79; H, 5.95. Found: C, 46.75; H, 5.83.

Hydrolysis of **3**. A soln of **3** (1 mg) in 3% H_2SO_4 was treated in the usual way. The residue was identified as kaempferol. The filtrate was treated in the usual way, and then examined by GC as described above. GC: R_t (min): 5.3, 7.0 (TMSi derivative of L-rhamnose, 1 mol), R_t: 12.5, 14.0 (TMSi derivative of D-galactose, 1 mol), R_t: 13.5, 14.7 (TMSi derivative of D-glucose, 1 mol).

Acetylation of **3**. Compound **3** (*ca* 5 mg) was treated with Ac_2O and pyridine. The product was purified by prep. TLC ($CHCl_3$) to give the peracetylated derivative of **3** (**3c**), mp 131–135°. Colourless powder, negative to the $FeCl_3$ reaction, red colour with the HCl–Mg reaction, red. colour with the HCl–Zn reaction. IR ν_{max}^{KBr} cm^{-1}: 2905 (CH), 1760 (MeCO), 1640 (C=O), 1605 (C=C), 1230, 1075 (C–O). ^{1}H-NMR δ ($CDCl_3$): 1.05 (3H, *d*, rhamnosyl-Me), 1.90–2.20 (30H, each *s*, alphatic MeCO × 10), 2.29 (3H, *s*, aromatic MeCO), 2.41 (3H, *s*, aromatic MeCO), 2.85–5.80 (*m*, gly-H), 6.70 (1H, *d*, *J* = 2.2 Hz, aromatic 8-H), 7.05 (1H, *d*, *J* = 2.2 Hz, aromatic 6-H), 7.15 (2H, *d*, *J* = 9.2 Hz, aromatic 2′,6′-H), 8.01 (2H, *d*, *J* = 9.2 Hz, aromatic 3′,5′-H). DCIMS see Fig. 3.

Acetolysis of **3c**. The acetate (**3c**) was subjected to acetolysis with CD_3CO_2D (10), $(CD_3CO)_2O$(10) and D_2SO_4 (1) for two days. The reaction mixt. was extd with EtOAc. The extract was washed with H_2O and evapd to dryness *in vacuo*. The residue was examined by GC/MS. Rhamnose and galactose were identified as for **2c**.

Methylation of **3**. Compound **3** (3 mg) was treated with excess CH_2N_2 to yield a colourless powder (negative to the $FeCl_3$ reaction) which was then hydrolysed with 3% H_2SO_4 soln. The reaction mixt was treated as described above. The ^{1}H NMR and EIMS data of **3a** were identical to those of **2a**. **3a** was silylated with TMS-PZ (1) + TMS-HT(1) and then examined by GC/MS. EIMS *m/z* (%): 458 $[M = C_{23}H_{30}O_6Si_2]^{+\cdot}$, 443 $[M-15]^+$ (100), 354 $[M-15-TMSiO]^{+\cdot}$, 135 $[C_8H_7O_2]^+$. The aq layer was treated in the usual way and then examined by GC as described above.

Enzymatic hydrolysis of **3**. A soln of **3** (2 mg) which was treated at room temp. with β-glucosidase for 7 days. The reaction mixt was extracted with BuOH. The BuOH layer washed with H_2O, evapd to dryness *in vacuo* to gave **3b**. **3b** was then treated with excess CH_2N_2. The reaction mixt. gave a negative $FeCl_3$ reaction and was hydrolysed as described above to give 5,7,4′-tri-*O*-methylkaempferol (3*d*), mp 143–145°. ^{1}H NMR (δ): 3.88–3.97 (9H, *s*, aromatic 4′,5,7-OMe), 6.36 (1H, *d*, *J* = 2.0 Hz, aromatic 6-H), 6.54 (1H, *d*, *J* = 2.0 Hz, aromatic 8-H), 7.02 (2H, *d*, *J* = 9.2 Hz, aromatic 3′,5′-H), 8.16 (2H, *d*, *J* = 9.2 Hz, aromatic 2′,6′-H). MS *m/z*: 328 $[M]^+$. Identical to authentic sample of 5,7,4-tri-*O*-methylkaempferol (UV, GC/MS of TMSi derivative).

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