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FLAVONOID GLYCOSIDES FROM NEEDLES OF *PINUS MASSONIANA**

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Key Word Index—*Pinus massoniana*; Pinaceae; needles; eriodictyol glucoside; *C*-methyl-dihydroflavonol, acylated dihydroflavonol glucoside; phenylacetic acid.

Abstract—Seven flavonoids have been isolated from *Pinus massoniana* needles and identified as taxifolin and its 3′-*O*-β-D-glucopyranoside, (+)-catechin, naringenin-7-*O*-β-D-glucopyranoside and three new flavonoid glycosides, 6-*C*-methylaromadendrin 7-*O*-β-D-glucopyranoside, taxifolin 3′-*O*-β-D-(6″-*O*-phenylacetyl)-glucopyranoside and eriodictyol 3′-*O*-β-D-glucopyranoside.

INTRODUCTION

Pinus massoniana is one of the most important pine species in China. Its needles may be used as additives in poultry and other livestock feeds [1]. During an investigation of low MW phenolic compounds from the needles, a series of monocyclic phenolic compounds, lignan glycosides and flavonoids were isolated and identified. The present paper reports the identification of the flavonoid glycosides from *P. massoniana* needles. A more detailed presentation of other phenolic compounds will be published elsewhere.

RESULTS AND DISCUSSION

From the ethyl acetate fraction of the water–acetone extract of the needles, seven flavonoids were isolated by subsequent chromatography on columns of Sephadex LH-20 and silica gel (230–400 mesh). Among the flavonoids, the major compounds taxifolin and its 3′-*O*-β-D-glucopyranoside, (+)-catechin, as well as naringenin 7-*O*-β-D-glucoside, were found in our previous studies on needles of *P. sylvestris* and/or *Picea abies* [2] and were identified by direct comparison with authentic samples (^{1}H NMR, $[\alpha]_D$, TLC). The other three flavonoid glycosides (**1**, **2** and **3**) do not seem to have been reported previously.

The first compound, **1**, crystallized from methanol as colourless plates, mp 164.5–166.5°, $[\alpha]_D^{25}$ −29.1°. Its UV λ_{max} at 291, 342 (sh) nm and a pair of doublets (each for one proton) at δ4.57 and 5.01 with J = 11.6 Hz in its ^{1}H NMR spectrum suggested it to be a dihydroflavonol or its glycoside. Apart from these two proton signals, the ^{1}H NMR spectrum clearly showed two doublets of four protons at δ6.82 and 7.36 with J = 8.7 Hz, typical of the A_2B_2 pattern for the B-ring, a singlet at δ6.3 and a singlet for an aromatic methyl group at δ2.05, indicating the presence of a *C*-methyl substituted A-ring.

On enzymatic hydrolysis, compound **1** gave a sugar, which was identified by GC and PC as glucose, and an aglycone **1a**. The UV spectrum of **1a** showed λ_{max}^{MeOH} at 294 nm and gave bathochromic shifts on addition of aluminium chloride and sodium acetate, indicating the presence of free 5- and 7-hydroxyl groups. The ^{1}H NMR of **1a** showed two doublets for vicinal protons at C-2 and C-3 and two doublets of A_2B_2 pattern for four protons in the B-ring. These signals were similar to those in the ^{1}H NMR spectrum of **1**, but the signals of the two singlets for the aryl methyl group and one proton at the C-6 or C-8 position shifted upfield to δ1.96 and 5.92, respectively. This implied that the sugar moiety was linked to the C-7 position in **1**. This linkage was also confirmed by comparison of the UV spectra of **1** and **1a** with diagnostic reagents. Thus, on addition of aluminium chloride, both spectra showed bathochromic shifts, but with sodium acetate only the spectrum of **1a** showed the bathochromic shift, indicating the presence of a free 5-hydroxyl group and an occupied 7-hydroxyl group in compound **1** [3]. Therefore compound **1** must be 6- or 8-*C*-methyl-aromadendrin 7-*O*-β-D-glucoside.

The position of the aromatic methyl group was determined by comparing the ^{13}C NMR spectra of aromadendrin, **1** and **1a** (Table 1). The assignments for aromadendrin were based on the ^{13}C NMR spectra of other dihydroflavonols and kaempferol in the literature [4, 5] with consideration of solvent effects. The signals of C-6 and C-8 in aromadendrin were obtained at δ97.46 and 96.40, but in compound **1** these signals were at δ105.69 and 95.43, indicating the presence of a methyl group at C-6. Similar results were reported for 6-methyl-taxifolin and 6-methyl-dihydromyricetin [6, 7]. The ^{13}C NMR spectrum of **1** also provided further confirmation for the glycosylation at the 7-hydroxyl group [8], and the chemical shifts for glucose carbons indicated its β-pyranose form by comparison with the ^{13}C NMR spectrum of methyl-β-D-glucopyranoside [9].

Thus, compound **1** was confirmed to be 6-methyl-

*Part 10 in the series "The Constituents of Conifer Needles". For Part 9 see Lundgren, L. N., Popoff, T. and Theander, O. (1982) *Acta. Chem. Scand. Sect. B* **36**, 695.

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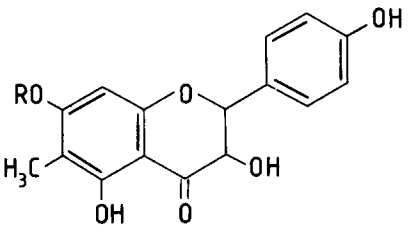
Table 1. ^{13}C NMR spectral data for aromadendrin, **1a** and **1** (CD_3OD, ppm)

C	Aromadendrin	1a	1	C	Aromadendrin	1a	1
2	85.00	84.89	85.21	2′	130.37	130.29	130.39
3	73 70	73.68	73.92	3′	116.26	116.12	116.23
4	198.41	198.39	199.40	4′	159.21	159.03	159.24
5	168.78	166.34	165.09	5′	116.26	116.12	116.23
6	97.46	105.69	108.21	6′	130.37	130.29	130.29
7	165.34	162.28	162.22	1″			101.41
8	96.40	95.43	95.37	2″			74.81
9	164.58	161.95	161.55	3″			78.17
10	101.93	101.55	103.14	4″			71.29
Me		6.96	7.37	5″			78.17
1′	129 37	129.39	129.31	6″			62.49



1 R= β-D-glucose
1a R=H

aromadendrin 7-*O*-*β*-D-glucopyranoside and the aglycone **1a** was 6-methylaromadendrin.

Compound **2**, $[\alpha]_D^{22}$ −21.6°, proved to be an acylated dihydroflavonol glycoside. Its ^1H NMR spectrum was similar to that of taxifolin 3′-*O*-*β*-D-glucopyranoside, except for a singlet of two protons at *δ* 3.55 and a multiplet of five protons around *δ* 7.16. On acetylation, **2** gave a heptaacetate (**2a**) with four aliphatic and three aromatic acetoxyl groups. After enzymatic hydrolysis, a mixture of two aromatic compounds was recovered from the ethyl acetate extract of the hydrolysate. These two compounds were identified after preparative TLC in chloroform–methanol–water (70:25:2). One of them was taxifolin, identified by the ^1H NMR spectrum and co-chromatography with the authentic sample in several solvent systems. The other compound turned out to be phenylacetic acid, which may be deduced from its ^1H NMR spectrum, mass spectrum and carbon signals in the ^{13}C NMR spectrum of **2** (see Table 2). The sugar moiety remaining in the hydrolysate after ethyl acetate extraction was identified as glucose by GC. Its carbon signals in the ^{13}C NMR spectrum of **2** and its anomeric proton signal in the ^1H NMR spectrum of **2** indicated the *β*-linkage and pyranose form of glucose in compound **2**. On mild alkaline hydrolysis of **2**, taxifolin 3′-*O*-*β*-D-glucopyranoside was isolated and identified by co-chromatography with an authentic sample.

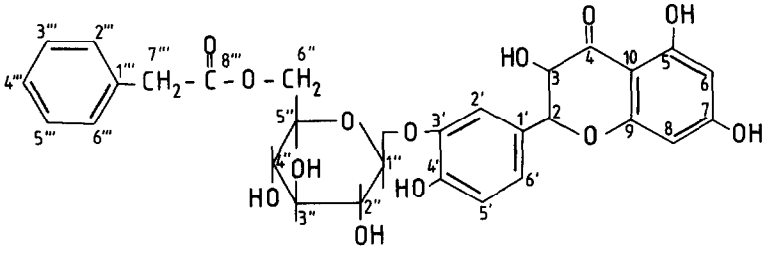
The site of acylation in compound **2** was determined by comparison of the ^{13}C NMR spectra of **2** and taxifolin 3′-*O*-*β*-glucoside. Their ^{13}C NMR spectral data and those of taxifolin are listed in Table 2. The assignments of the taxifolin carbon signals were based on literature data [6]. The ^{13}C NMR spectrum of taxifolin 3′-*O*-*β*-D-glucoside has not been reported in the literature and the assignments were made on the basis of the ^{13}C NMR spectrum of taxifolin and the known effect of 3′-*O*-glucosylation on flavonoids [8]. The introduction of glucose to the 3′-hydroxyl group of taxifolin caused the signals due to C-2′, C-4′, C-5′ and C-6′ to shift downfield by 2.39, 1.94, 1.03 and 3.82 ppm, respectively. The carbon chemical shifts of **2** remained the same as those of taxifolin 3′-*O*-*β*-D-glucoside, except for the signals due to C-5″, C-6″ of glucose and those of phenylacetic acid. The signal due to C-5″ moved upfield by 2.50 ppm and that of C-6″ downfield by 2.62 ppm, providing unambiguous evidence of the attachment of the phenylacetic acid to the 6″-hydroxyl group of glucose in **2**. Hence, compound **2** was taxifolin 3′-*O*-*β*-D-(6″-*O*-phenylacetyl)-glucopyranoside. This is the first reported acylated flavonoid glycoside with an acyl group of phenylacetic acid. The finding of a carbohydrate substituted with phenylacetic acid also appears to be unique.

Compound **3**, $[\alpha]_D^{22}$ −36.1°, was confirmed as a flavonone (or its glycoside) by its UV λ_{max} at 289 and 323 (sh) nm and ^1H NMR signals at *δ* 2.70 (*dd*), 3.15 (*dd*) for two protons and 5.33 (*dd*) for one proton. On acetylation, **3** gave the heptaacetate **3a** with three aromatic and four aliphatic acetoxyl groups. The chemical ionization mass spectrum (ammonia) of **3a** showed the peak *m/z* 762 $[M+NH_4]^+$. After enzymatic hydrolysis, compound **3** yielded glucose and an aglycone **3b**, which was identified as eriodictyol by comparison of its UV, mass and ^1H NMR spectra with literature data [3, 10].

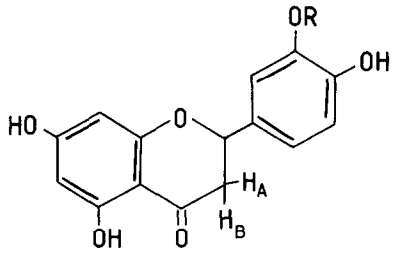
The position of the linkage of glucose to eriodictoyl was established by comparing the UV and ^1H NMR spectra of **3** and **3b**. Both absorption spectra showed bathochromic shifts on the addition of aluminium chloride, aluminium chloride–hydrochloric acid and sodium acetate. Thus, the 5- and 7-hydroxyl groups were free in **3** and glucose must be linked to the 3′- or 4′-hydroxyl group. The ^1H NMR spectrum of **3** showed that, as a result of glycosylation, the proton signal of C-2′ shifted most downfield in comparison with those of C-5′ and C-6′, thus indicating that the position of glycosylation was the 3′-hydroxyl group. Accordingly, compound **3** could be characterized as eriodictyol 3′-*O*-*β*-D-glucopyranoside, to our knowledge a new flavonone glycoside.

Table 2. ^{13}C NMR spectral data of taxifolin, taxifolin 3′-*O*-glucoside and 2 (CD_3OD, ppm)

C	Taxifolin	Taxifolin 3′-*O*-glucoside	2	C	Taxifolin	Taxifolin 3′-*O*-glucoside	2
2	85.10	84.81	84.97				
3	73.70	73.51	73.70	1″		104.15	104.34
4	198.33	198.27	198.17	2″		74.89	74.84
5	168.70	168.64	168.69	3″		77.68	77.50
6	97.40	97.49	97.54	4″		71 51	71.70
7	165.29	165.23	165.31	5″		78.31	75.81
8	96.38	96 46	96 46	6″		62 60	65.22
9	164.52	164.39	164.34	1‴			135.27
10	101.90	101.92	101.90	2‴			130 22
1′	129.96	130.02	130.02	3‴			129 44
2′	116.20	118.59	119.10	4‴			127 93
3′	146.32	146.49	146.43	5‴			129.44
4′	147.14	149.08	149.30	6‴			130.22
5′	116.01	117.04	117.17	7‴			41.77
6′	120.94	124.76	124.79	8‴			173.79



2



3 R= β-D-glucose

3b R=H

EXPERIMENTAL

Mps are corr. UV spectra were recorded on a double-beam spectrophotometer; NMR spectra at 90 MHz for ^1H NMR and 25.2 MHz for ^{13}C NMR with TMS as internal standard; mass spectra on a quadrupole instrument at 70 eV. TLC was performed on silica gel HF_{250} plates.

Plant material and extraction. Needles of *P. massoniana* were collected in July at the arboretum of Nanjing Technological College of Forestry, Nanjing, People's Republic of China. Air-dried needles (280 g, H_2O content 10.1%) were refluxed with Me_2CO for 30 min. After filtration, the needles were dried, milled and extracted in an ultrasonic bath at room temp. with Me_2CO (4 × 0.5 l.) and aq. 50% Me_2CO (4 × 0.5 l.) for 8 × 40 min. After removal of the precipitating waxes, the extracts were combined, concd to small vol. and extracted with $CHCl_3$ (3 × 0.5 l.) and EtOAc (3 × 0.5 l.). After evapn, the EtOAc extract residue (4.7 g) was dissolved in a small amount of 10% EtOH and separated on a Sephadex LH-20 column (50 × 3.5 cm), eluting with 10–96% EtOH. Fourteen fractions were collected. The 9–14th fractions were rechromatographed on silica gel (230–400 mesh) columns, eluting with MeCOEt–H_2O. The respective subfractions were refractionated on silica gel columns with $CHCl_3$–MeOH–H_2O (80:15:1, 70:25:2, etc.) to obtain crude subfractions of the above-mentioned flavonoids. Further purification by prep TLC afforded the chromatographically homogeneous compounds 1–3. Compound 1 (15 mg) was then crystallized from MeOH, 2 (38 mg) and 3 (45 mg) were amorphous.

Compound 1. Mp 164.5–166.5°, $[\alpha]_D^{25}$ −29.1° (MeOH; *c* 1.03); UV λ_{max}^{MeOH} nm: 291, 342 sh; λ_{max}^{NaOMe} nm: 291, 346 sh; λ_{max}^{NaOAc} nm: 291, 342 sh; $\lambda_{max}^{NaOAc-H_3BO_3}$ nm: 291, 342 sh; $\lambda_{max}^{AlCl_3}$ nm: 318, 394 sh, $\lambda_{max}^{AlCl_3-HCl}$ nm: 315, 388 sh. ^1H NMR (CD_3OD): δ2.05 (3H, *s*, C-6 Me), 3.3–4.0 (6H, *m*, H-2″ to H-6″), 4.58 (1H, *d*, *J* = 11.6 Hz, H-3), 5.01 (1H, *d*, *J* = 11.6 Hz, H-2), 6.29 (1H, *s*, H-8), 6.82 (2H, *d*, *J* = 8.7 Hz, H-3′, H-5′) 7.36 (2H, *d*, *J* = 8.7 Hz, H-2′, H-6′). The anomeric proton of glucose was hidden under the broad OH peak at *ca* δ4.9.

Hydrolysis of 1 (5 mg) was carried out with cellulase (Practical Grade, Type 1, Sigma) in H_2O at room temp. overnight. The hydrolysate was extracted with EtOAc (3 × 10 ml) to obtain the aglycone **1a** (2.8 mg). UV λ_{max}^{MeOH} nm: 294, 334 sh; λ_{max}^{NaOMe} nm: 267, 331; $\lambda_{max}^{AlCl_3}$ nm: 283 sh, 321, 386 sh; $\lambda_{max}^{AlCl_3-HCl}$ 317, 376 sh, λ_{max}^{NaOAc} nm: 284 sh, 332 $\lambda_{max}^{NaOAc-H_3BO_3}$ nm: 296, 333 sh. ^1H NMR (CD_3OD): δ1.96 (3H, *s*, C-6 Me), 4.52 (1H, *d*, *J* = 11.6 Hz, H-3), 4.95 (1H, *d*, *J* = 11.6 Hz, H-2), 5.92 (1H, *s*, H-8), 6.82 (2H, *d*, *J*

= 8.5 Hz, H-3', H-5'), 7.35 (2H, *d*, *J* = 8.5 Hz, H-2', H-6'). Glucose was identified in the aq. phase by GC and PC

Compound **2**. Amorphous, $[\alpha]_D^{22}$ −21.6° (MeOH; *c* 0.9). UV λ_{max}^{MeOH} nm. 208, 226 sh, 292, 323 sh. ^{1}H NMR (CD_3OD): δ3.3–4.3 (6H, *m*, H2″ to H-6″), 3 55 (2H, *s*, Ar–CH_2–), 4.52 (1H, *d*, *J* = 11 6 Hz, H-3), 4.95 (1H, *d*, *J* = 11.6 Hz, H-2), 5.84 (1H, *d*, *J* = 2 Hz, H-6), 5.86 (1H, *d*, *J* = 2 Hz, H-8), 6.92 (1H, *d*, *J* = 8.3 Hz, H-5'), 7.08–7.19 (6H, *m*, H-6' and H-2‴ to H-6‴), 7.36 (1H, *d*, *J* = 1.8 Hz, H-2'). The anomeric proton of glucose was hidden under the broad OH peak, and appeared at δ4.81 as a doublet, *J* = 7 Hz, by recording the ^{1}H NMR spectrum in CD_3OD at 50°

Acetylation (Ac_2O–pyridine) of **2** (8 mg) yielded the hepta-acetate **2a** (5 mg) after purification on TLC in Et_2O–EtOAc (1.1). ^{1}H NMR ($CDCl_3$): δ2.00, 2.02, 2.03, 2.09 (12H, 4*s*, 4 × OAc), 2.29, 2.30, 2.36 (9H, 3*s*, 3 × Ar-OAc), 3.60 (2H, *s*, Ar–CH_2–), 3.8–4.0 (1H, *m*, H-5″), 4.2–4.4 (2H, *m*, H-6″), 5.0–5.35 (4H, *m*, H-1″ to H-4″), 5 39 (1H, *d*, *J* = 11 5 Hz, H-3), 5.75 (1H, *d*, *J* = 11.5 Hz, H-2), 6.58 (1H, *d*, *J* = 2.2 Hz, H-6), 6.74 (1H, *d*, *J* = 2 2 Hz, H-8), 7.1–7.25 (8H, *m*, H-2', H-5', H-6' and H-2‴ to H-6‴)

Hydrolysis of **2** (10 mg) was performed with cellulase for 16 hr at room temp. The hydrolysate was extracted with EtOAc. After purification of the extract by TLC, two aromatic compounds, **2b** (5 mg) and **2c** (2 mg), were obtained. **2b** was identified as taxifolin by ^{1}H NMR and co-chromatography (TLC silica gel) in MeCOEt–H_2O, $CHCl_3$–MeOH–H_2O (80:15·1), EtOAc–MeOH–H_2O (100:15:10) with an authentic sample. **2c**. ^{1}H NMR (Me_2CO-d_6): δ3.63 (2H, *s*, H-7‴), 7 30 (5H, *m*, H-2‴ to H-6‴) MS (probe) *m/z* (rel. int.): 136 $[M]^+$ (32), 92 (19), 91 (100), 90 (4), 89 (3), 77 (2), 65 (19). Glucose was identified in the aq. phase by GC and PC.

Mild alkaline hydrolysis of **2** (5 mg) was carried out with 0 05 N NH_4OH in 50% MeOH at room temp. for 1 hr. After removal of NH_4OH *in vacuo* at 20°, taxifolin 3'-*O*-β-D-glucoside was identified in the hydrolysate by TLC with an authentic sample in the same solvents as mentioned above for co-chromatography of taxifolin.

Compound **3**. Amorphous, $[\alpha]_D^{22}$ −36.1° (MeOH; *c* 0.82). UV λ_{max}^{MeOH} nm: 205, 222 sh, 288, 323 sh; $\lambda_{max}^{AlCl_3}$ nm: 205, 224, 280 sh, 313, 368; $\lambda_{max}^{AlCl_3-HCl}$ nm: 224, 281 sh, 311, 364; λ_{max}^{NaOAc} nm· 280 sh, 325, 376 sh; $\lambda_{max}^{NaOAc-H_3BO_3}$ nm: 289, 324 sh. ^{1}H NMR (CD_3OD): δ2.73 (1H, *dd*, *J* = 3.4, 17.1 Hz, H-3_A), 3.15 (1H, *dd*, *J* = 12.2, 17.1 Hz, H-3_B), 3.3–4.0 (6H, *m*, H-2″ to H-6″), 5.33 (1H, *dd*, *J* = 3.4, 12.2 Hz, H-2), 5.87 (1H, *d*, *J* = 2.2 Hz, H-6), 5.91 (1H, *d*, *J* = 2.2 Hz, H-8), 6.87 (1H, *d*, *J* = 8.3 Hz, H-5'), 7.06 (1H, *dd*, *J* = 2 and 8.3 Hz, H-6'), 7.35 (1H, *d*, *J* = 2 Hz, H-2'). The anomeric proton of glucose was hidden under the MeOH OH peak; it appeared at δ4.79 as a doublet with *J* = 7 Hz, when the spectra were recorded at 45°.

Acetylation (Ac_2O–pyridine) of **3** (10 mg) gave the hepta-acetate **3a** (4 mg). CIMS (NH_3 probe) *m/z*: 762 $[M+NH_4]^+$. ^{1}H NMR ($CDCl_3$): δ2 0, 2.03, 2.05, 2.08 (12H, 4*s*, 4 × OAc), 2.28, 2.31, 2.39 (9H, 3*s*, 3 × Ph–OAc), 2.8–3.1 (2H, *m*, H-3), 3.8–4.4 (3H, *m*, H-5″, H-6″), 5.0–6.0 (5H, *m*, H-1″ to H-4″, H-2), 6.53 (1H, *d*, *J* = 2.2 Hz, H-6), 6.77 (1H, *d*, *J* = 2.2 Hz, H-8), 7.11 (3H, *s*, H-2', H-5', H-6').

Hydrolysis of **3** (12 mg) with cellulase at room temp. overnight followed by EtOAc extraction yielded the aglycone **3b** (5 mg), identified as eriodictyol (^{1}H NMR, MS and UV). Glucose was identified in the aq. phase by GC and PC.

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REFERENCES

1. Zhou, W. C., Que, M. Y., Zhao, X. C. and Yuan, Q. R. (1981) *Chemistry and Industry of Forest Products* **4**, 31.
2 Theander, O. (1982) *Svensk Papperstidning* **9**, 85.
3. Mabry, T. J , Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*. Springer, New York.
4. Harborne, J. B. and Mabry, T. J. (1982) *The Flavonoids* Chapman & Hall, London.
5. Markham, K. R. and Ternai, B. (1976) *Tetrahedron* **32**, 2607
6. Agrawal, P. K , Agrawal, S. K., Rastogi, R. P. and Österdahl, B. G. (1981) *Planta Med.* **43**, 82.
7 Agrawal, P. K , Agrawal, S. K. and Rastogi, R. P. (1980) *Phytochemistry* **19**, 893
8. Markham, K. R., Ternai, B., Starley, R , Geiger, H. and Mabry, T. J. (1978) *Tetrahedron* **34**, 1389.
9 Gorin, P A J and Mazurek, M (1975) *Can. J. Chem.* **53**, 1212.
10. Ishikura, N., Sugahava, K. and Kurosawa, K. (1979) *Z Naturforsch. Teil C* **34**, 628