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A NEW GLYCOSYLATED FLAVONOID, 7-*O*-α-L-RHAMNOPYRANOSYL-4'-*O*-RUTINOSYLAPIGENIN, IN THE EXUDATE FROM GERMINATING SEEDS OF *Sesbania rostrata**

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ABSTRACT

The title apigenin triglycoside was isolated (4 mg/6000 seeds) by reverse-phase column chromatography as the major u.v.-absorbing compound in the exudate of germinating seeds of *Sesbania rostrata*. The structure was assigned on the basis of u.v. spectra, f.a.b.-mass-spectral and 2D-n.m.r. data. The triglycoside was released continuously from the germinating seeds, but at a decreasing rate during the first two weeks.

INTRODUCTION

Compounds exuded by the roots play a role in the *Leguminosae–Rhizobium* symbiosis and genetic studies of the mechanism of rhizobial nodulation have been reviewed[1,2]. We have been interested in the nature of major products exuded by the roots and their role in the *Sesbania rostrata–Azorhizobium caulinodans* system[3].

Investigations[4–6] of the phenolic compounds exuded during the first days of the life of leguminosae plants revealed mostly polyphenolic aglycons. These molecules play a regulatory role in the symbiotic interaction of rhizobia with leguminous plants, some inducing expression of nodulation genes in *Rhizobium* spp.[5–7], some antagonizing this induction[7], and some being toxic to, for instance, *Proteus* and *Staphylococcus* spp.[8], or to *Azotobacter* and *Rhizobium* spp.[9]. The mechanism of the specific recognition of an appropriate plant root by a *Rhizobium* strain is still unclear[1,2].

*Dedicated to the late Robert W. Hedges.
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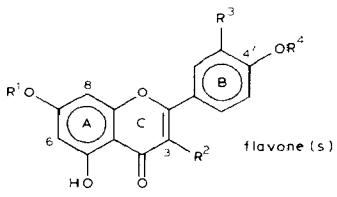
Fractionation of plant exudates showed that, in the symbiotic interaction of *R. meliloti* and alfalfa, the most active inducer of nodulation was luteolin (**2**), but other active components, probably also flavonoids, were also present[4]. That luteolin should be the inducer was surprising, since it is produced by plants of several families, most of which are not nodulated by rhizobia. However, subsequent studies showed that flavones (4′,7-dihydroxy derivatives), produced only by leguminosae, were active inducers[5] and that different leguminous plants produced different sets of flavonoid compounds[10].

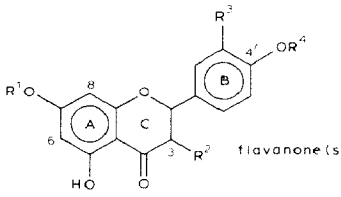
Each of these flavonoids was found as an aglycon, whereas the roots contained the corresponding glycosides, suggesting that hydrolysis took place during exudation[10]. Indeed, only flavonoid aglycons were found[4–6] in the methanol or ethanol extracts of 3-day germinating seeds. In these studies, the surrounding liquid was not analyzed but, when this was done subsequently, a mixture of inducers and antagonizers of nodulation was found and one of the inducers was 7-*O*-glucosylapigenin[7]. Early reports[11–13] suggested also the presence of glycosylated flavonoids with antibiotic properties in aqueous extracts of *Trifolium* seeds, namely, myricetin and its glycosides[11] (toxic to *R. leguminosarum*), rutin (**3**), and quercetin glycosides[9]. In the root exudates of 3-day-old *Vicia* seeds, none of the flavonoid aglycons, naringenin (**5**), eriodictyol (**7**), apigenin (**1**), or luteolin (**2**) could be detected, but active, unidentified inducers of nodulation were present[14].

Whether the balance between diffusible inhibitors of *Rhizobium* and inducers plays a role in the regulation of nodulation is not known, but glycosylated flavonoids may be involved in the process. Since nodulation is effective during the ten days following the germination of seeds[15], the appearance of u.v.-absorbing compounds in the exudate during that time was monitored and we now describe the isolation and characterization of the major compound from exudates of germinating seeds of *Sesbania rostrata*.



flavone (s)

1 $R^1 = R^2 = R^3 = R^4 = H$ (apigenin)
2 $R^1 = R^2 = R^4 = H, R^3 = OH$ (luteolin)
3 $R^1 = R^4 = H, R^2 = O$-rutinosyl, $R^3 = OH$ (rutin)
4 R^1 = rutinosyl, $R^2 = R^3 = H, R^4 = CH_3$ (linarin)



flavanone (s)

5 $R^1 = R^2 = R^3 = R^4 = H$ (naringenin)
6 R^1 = neohesperidosyl, $R^2 = R^3 = R^4 = H$ (naringin)
7 $R^1 = R^2 = R^4 = H, R^3 = OH$ (eriodictyol)
8 $R^1 = R^4 = H, R^2 = R^3 = OH$ (taxifolin)
9 R^1 = rutinosyl, $R^2 = H, R^3 = OH, R^4 = CH_3$ (hesperidin)

RESULTS AND DISCUSSION

In general, the coloured substances, released by plants, roots, or germinating seeds into the water layer during the first hours after immersion mask the detection

of actively secreted compounds. In order to overcome this problem, surface-sterilized *Sesbania rostrata* seeds were germinated in water and the secreted material was monitored as a function of time. Many fractions collected during the first few hours showed weak biological activity (induction or inhibition of nodulation genes or antibiotic effects) on *Azorhizobium caulinodans*, the symbiotic nodulating organism of this leguminous host plant[3]. Each day for two weeks, the water layer was analyzed by reversed-phase column chromatography. A major, rather hydrophilic, polyphenolic compound was detected which was released continuously from the germinating seeds but at a decreasing rate, and the compound exuded between 24 and 48 hours was isolated.

The purified compound (**11**) and also the aglycon apigenin (**1**) at μM–20 μM did not induce the nodulation genes of *Azorhizobium caulinodans*. The biological role of glycosylated plant factors and the corresponding genetic aspects for the bacteria will be reported elsewhere.

The structure 7-*O*-α-L-rhamnopyranosyl-4′-rutinosylapigenin was assigned to the above major u.v.-absorbing compound (**11**) on the basis of the following spectroscopic data.

U.v. spectra. — Compound **11** had λ_{max}^{MeOH} at 321 and 268 nm. The effects of various shift reagents, as indicated for flavonoid identification[16], and the u.v.-spectral data for closely related reference compounds[17–19] are shown in Table I. The λ_{max}^{MeOH} at 268 nm for **11** suggests a flavone with a 5- and 7-hydroxyl group (substituted or not) on ring A. The NaOMe-induced spectrum is diagnostic of the absence of vicinal hydroxyl groups, and there is no shoulder or band at 320–335 nm, indicative of substitution of the 7-hydroxyl group. A 49-nm bathochromic shift with 66% decrease in intensity of the band at 321 nm (1.25 o.d.) to that at 370 nm (0.42 o.d.) is typical for a substituted 3′- or 4′-hydroxyl function in ring B. The spectra induced by $AlCl_3$ and $AlCl_3$/HCl confirm the absence of unsubstituted vicinal hydroxyl groups. The additional peak appearing at 382 nm is indicative of an unsubstituted 5-hydroxyl group.

The band at 268 nm for **11** is unaffected by NaOAc, which confirmed the presence of a substituted 7-hydroxyl group in ring A.

TABLE I

U.V.-SPECTRAL DATA FOR ACACETIN[19], APIGENIN TRIGLYCOSIDES[19], AND **11**

Solvent	**11**[a]	*Acacetin triglycoside*[b]	*Apigenin triglycoside*[c]
MeOH	268, 321	269, 323	268, 332
+NaOMe[d]	245sh, 283, 368^{+}, 425sh^{++}	245sh, 291, 371^{+}	243sh, 270, 300sh, 349sh, 386*
+$AlCl_3$	277, 297, 335, 382	276, 299, 343, 376	230sh, 275, 299, 348, 379sh
+$AlCl_3$/HCl	278.5, 296, 330.5, 382.5	276, 298, 337, 376	275, 298, 341, 377sh
+NaOAc[d]	267, 312, 430^{+}	268, 325	257sh, 267, 352sh, 386
+NaOAc/H_3BO_3	267.5, 316	268, 326	267, 338

[a]7-*O*-α-L-Rhamnopyranosyl-4′-*O*-rutinosylapigenin. [b]Partially known structure[19]: 7-(di-*O*-rhamno, -*O*-gluco-syl)acacetin. [c]7-*O*-(2,4-di-*O*-α-rhamnopyranosyl-β-D-glucopyranosyl)apigenin[17–19]. [d]Key: +, decreased intensity (stable); ++, decreased intensity (unstable); *, increased intensity (stable).

F.a.b.-mass spectra and acid hydrolysis. — The f.a.b.-mass spectrum of **11** obtained using a glycerol matrix contained a weak peak for $(M + H)^+$ at *m/z* 725. The more abundant fragment ion at *m/z* 271 is indicative[20] of a trihydroxyflavone core. Ions of low abundance at *m/z* 563 and 579 could be the $(M + H)^+ - 162$ (hexose) or $- 146$ (deoxyhexose), respectively[20]. Treatment of **11** with 2M HCl in aqueous 50% methanol for 1 h at ~100° caused complete hydrolysis, but no appearance of biological activity. The aglycon flavonoid had a u.v. spectrum typical of, and co-chromatographed with, apigenin (**1**). Thus, **11** contains a trihydroxyflavone (mol. wt. 270) glycosylated with a hexose and two deoxyhexoses, *i.e.*, $C_{33}H_{40}O_{18}$ (mol. wt., 724).

^{1}H-N.m.r. spectra. — The resonances in the region for aromatic protons of **11** (Fig. 1a and Table II) accord with the apigenin (**1**) nucleus. The $J_{6,8}$ value of 2.1 Hz is typical for *meta*-protons, implying four substitution sites. Further, the AA'BB' spin pattern points to a second *para*-substituted phenyl ring. The singlet at δ 6.95 is assigned to H-3. In comparison with the values[21] for apigenin, the resonances of **11** are shifted 0.14–0.24 p.p.m. upfield, so that both aromatic rings must be substituted.

The presence of a sharp peak at δ 12.8 indicates[21] a hydrogen bond between HO-5 and the carboxyl function on C-4, which demonstrates that HO-5 is not substituted.

In the region (δ 4.50–5.50) for anomeric protons, there were three 1-proton resonances (δ 4.55, 4.98, and 5.56). Two of these resonances (δ 5.56 and 4.55) showed a $J_{1,2}$ value of ~1.7 Hz, indicating[22] O-1,2 to be diaxial. This finding, together with the two methyl doublets (δ 1.10 and 1.19), suggests the presence of two α-L-rhamnopyranosyl units (the L configurations are assumed). The coupling constants, $J_{2,4}$ ~3.5, $J_{3,4}$ ~$J_{4,5}$ ~9.5 Hz, accord with those for α-L-rhamnopyranose and its methyl glycoside in aqueous solution[22].

The resonance at δ 4.98 showed an apparent coupling constant of 7.2 Hz. Moreover, this doublet was disturbed possibly due to the degeneration of the spin system or by the collapse (or quasi collapse) of two neighbouring ring protons[23]. From the COSY 45 experiment (see below), it was deduced that H-2,3 and 4,5 are quasi-collapsed. The resonances of H-6 and H-6' can be assigned easily. Although only apparent coupling constants can be measured, a β-D-glucopyranosyl unit can be identified. Indeed, a value of 7.2 Hz for $J_{1,2}$ is usual for a diaxial relationship of H-1,2. The values of $J_{5,6}$ and $J_{5,6'}$ indicate[24] HO-4 to be equatorial. The value of the chemical shift of the resonance of H-3 agrees only with an axial position.

^{1}H–^{1}H Shift correlation (COSY) spectra. — The result of a COSY 45 experiment[25] is shown in Fig. 1. The patterns at δ 3.13 and 3.19 each integrate for one proton, that at δ 3.32 for three protons, and that at δ 3.43 for four protons. Because of its connectivity with one of the methyl groups, the pattern at δ 3.13 must be assigned to H-5 of the isolated α-L-rhamnopyranosyl moiety. The pattern at δ 3.13 shows two cross-peaks with one showing a connectivity with the complex pattern

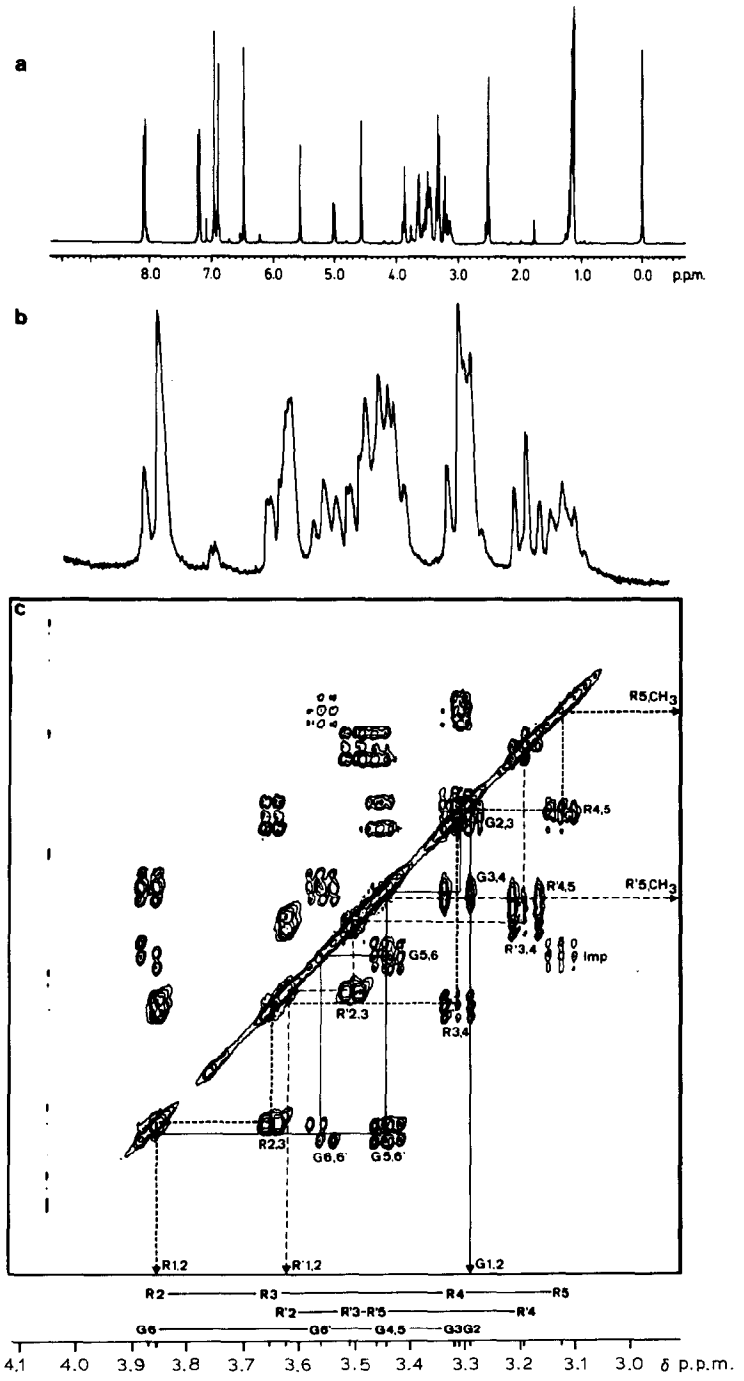


Fig. 1. (a) ^{1}H-N.m.r. spectrum (400 MHz) of the title compound (**11**) in $(CD_3)_2SO$ (+ CF_3COOH), (b) extension of the region δ 3.0–4.1, (c) contour plot of the COSY 45 experiment of the region δ 3.0–4.1; G, the β-D-glucopyranosyl unit; R', α-L-rhamnopyranosyl unit of the rutinosyl moiety; R, α-L-rhamnopyranosyl moiety on C-7.

TABLE II

^{1}H-N.M.R. DATA FOR THE APIGENIN NUCLEUS [$(CD_3)_2SO$, INTERNAL Me_4Si]

Atom	**11**	*Linarin* (**4**)	*Apigenin* (**1**)
H-3	6.95	6.94	6.77
H-6	6.46[a]	6.45	6.22
H-8	6.79	6.79	6.50
H-2′,5′	8.06	8.05	6.92
H-3′,5′	7.18	7.12	6.95

[a] $J_{6,8}$ 2.1 Hz.

centered at δ 3.32. As the connectivities between H-1,2,3,4 of the same unit are clear, the resonance of H-4 of this unit must be that at δ 3.33. The other weak connectivity is assigned to an impurity (in the F2 dimension, it is found at a somewhat lower field).

The triplet at δ 3.19 shows two cross-peaks, partially overlapping in the F1 dimension. The cross-peak at the higher frequency region is clearly related to H-3′ (assigned by following the cross-peaks between H-1′ and H-2′, and H-2′ and H-3′) of the α-L-rhamnopyranosyl unit in the rutinosyl moiety. The cross-peak at lower frequency in the F1 dimension agrees with the position of H-5′ as assigned by its cross-peak with the methyl group. In the F1 dimension, the position of the latter is found at a somewhat higher frequency than the cross-peak at δ 3.32. Consequently, all the resonances of the two α-L-rhamnopyranosyl moieties are assigned.

In the β-D-glucopyranosyl moiety, four resonances can be assigned directly, based on the connectivities between H-5, H-6, and H-6′, and between H-1 and H-2. However, the assignment of H-3 and H-4 is not straightforward, although the cross-peak close to the diagonal near δ 3.32 probably involves H-2 and H-3 of the β-D-glucopyranosyl unit. As the pattern at δ 3.32 integrates for three protons, all the resonances in the region are identified. Consequently, the resonance for H-4 must be that at δ 3.43. Unfortunately, although this analysis agrees with the integration of the patterns, no cross-peak between H-4 and H-5 was found close to the diagonal. That these protons are closely coupled is verified by the disturbance in the values of the coupling constants associated with H-6 and H-6′, due to the degeneration in the spin system caused by the overlap of the resonances of H-4 and H-5. Consequently, the resonances of H-2 and H-3 of the β-D-glucopyranosyl moiety collapse with that of H-4 of the isolated α-L-rhamnopyranosyl moiety at δ 3.32. The resonances of H-4,5 of the β-D-glucopyranosyl moiety overlap with those of H-3′,5′ of the α-L-rhamnopyranosyl part of the rutinosyl moiety.

Comparison with analogues. — Some reference data are given in Tables II and III. The resonances of H-1 of one of the α-L-rhamnopyranosyl units, methyl α-D-mannopyranoside, and the α-L-rhamnopyranosyl part of the rutinosyl moiety in linarin (**4**) are at δ 4.55. In contrast, the resonance of H-1 of the other α-L-

TABLE III

^{1}H-N.M.R. DATA FOR **11** AND RELATED COMPOUNDS [$(CD_3)_2SO$, INTERNAL Me_4Si]

Rutinosyl					*α-L-Rhamnopyranosyl*	
β-D-Glucopyranosyl		*Phenyl β-D-glucopyranoside*	*α-L-Rhamnopyranosyl*		**11**	p-*Nitrophenyl α-D-mannopyranoside*
11	*Linarin* (**4**)		**11**	*Linarin* (**4**)		
H-1 4.98 ($J_{1,2}$ 7.2)	5.07	4.85	4.55 ($J_{1,2}$ 1.7)	4.55	5.56 ($J_{1,2}$ 1.7)	5.58
H-2 3.28	3.26	3.14	3.63 ($J_{2,3}$ 3.5)	3.67	3.86 ($J_{2,3}$ 3.5)	3.86
H-3 3.32	3.31	3.27	3.51 ($J_{3,4}$ 9.5)	3.46	3.66 ($J_{3,4}$ 9.5)	3.68
H-4 3.43	3.14	3.23	3.19 ($J_{4,5}$ 9.5)	3.15	3.33	3.51
H-5 3.43 ($J_{5,6}$ ~1.0)	3.43	3.32	3.45	3.46	3.13	3.31
H-6 3.87 ($J_{5,6'}$ ~5.0)	3.85	3.69				3.58
H-6′ 3.56 ($J_{6,6'}$ ~10.0)	3.61	3.45				3.45
CH_3			1.10	1.09	1.19	

rhamnopyranosyl unit and *p*-nitrophenyl α-D-mannopyranoside are at δ 5.56–5.57. Thus, the latter α-L-rhamnopyranosyl unit is attached to position 7 or 4′ in apigenin (**1**), whereas the former is attached to the isolated α-L-rhamnopyranosyl unit or the β-D-glucopyranosyl unit. Moreover, the parallelism between the resonances of the ring protons in the α-L-rhamnopyranosyl unit in the rutinosyl moiety and in linarin (**4**) is striking. For the α-L-rhamnopyranosyl moiety attached to the apigenin (**1**) nucleus and *p*-nitrophenyl α-D-mannopyranoside, only the correspondence of H-2,3 is good. The difference for H-4 is to be expected on the basis of data for α-L-rhamnopyranose and α-D-mannopyranose and in their glycosides[22], but the difference for H-5 is unexpected.

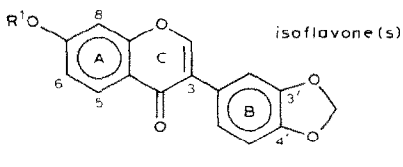
The similar chemical shifts of the resonances for H-1 in the β-D-glucopyranosyl unit in **11**, linarin (**4**), and phenyl β-D-glucopyranoside indicate that the β-D-glucopyranosyl unit in **11** is attached to apigenin.

Further, it can be deduced that one of the α-L-rhamnopyranosyl units in **11** is 6-linked to the β-D-glucopyranosyl unit. Indeed, the chemical shifts of the resonances of H-5,6,6′ of the β-D-glucopyranosyl unit in **11** and linarin (**4**) are identical and are 0.1–0.18 p.p.m. upfield of the corresponding resonances in phenyl β-D-glucopyranoside in accord with the results of a study of glucodisaccharides[26]. Thus, the disaccharide moiety in **11** is rutinose.

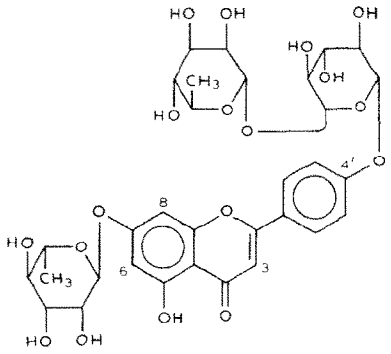
When the chemical shifts of the rutinosyl moiety of the resonances of **11** are compared with those of linarin (**4**), only three differences are seen, namely, H-1, 0.09; H-2, 0.04; and H-4, 0.29 p.p.m. downfield.

^{13}C-N.m.r. spectra. — The ^{13}C-n.m.r. data are given in Table IV. The ^{13}C resonances were assigned by comparison with reported data for apigenin[21] and the sugar moieties[27].

Since, for the apigenin moiety in **11**, the resonance for C-7 is shifted upfield by 0.76 p.p.m., and those of C-6 and C-8 are shifted downfield by 0.88 and 0.70 p.p.m., respectively, substitution at position 7 can be inferred[28]. Although the resonances of C-3′,5′ in **11** are shifted downfield by 0.45 p.p.m., the values are too small to be diagnostic for 4′-substitution. The resonance of C-1′ in naringenin (**5**) and taxifolin (**8**) show[27] a downfield shift of 1.5–2.3 p.p.m. if HO-4′ is substituted. Such an effect is seen also in **11**.



10 R¹ = rutinosyl (pseudobaptisin)



11

TABLE IV

^{13}C-N.M.R. DATA FOR **11** [$(CD_3)_2SO$, INTERNAL Me_4Si]

	Apigenin nucleus			*4'-O-Rutinosyl moiety*		*7-O-α-L-Rhamnopyranosyl moiety*
	11	*Apigenin* (**1**)		*β-D-Glucopyranosyl*	*α-L-Rhamnopyranosyl*	
C-2	160.67[a]	163.93	C-1	100.58	98.37	99.56
C-3	104.07	103.06	C-2	73.12	70.64	70.03
C-4	181.98	181.94	C-3	76.65	70.42	70.22
C-5	161.27[a]	161.69	C-4	69.77	71.92	71.54
C-6	99.95	99.07	C-5	75.77	68.40	69.92
C-7	163.56	164.32	C-6	66.63	17.18	17.18
C-8	94.98	94.18				
C-9	156.94	157.48				
C-10	105.38	105.34				
C-1′	123.89	121.41				
C-2′,6′	128.22	128.64				
C-3′,5′	116.62	116.17				
C-4′	161.07[a]	161.37				

[a]May be exchanged.

The ^{13}C resonances of the β-D-glucopyranosyl moiety in **11** can be assigned readily. In comparison with other derivatives containing a β-D-glucopyranose moiety, *e.g.*, naringin[27] (**6**) where HO-6 of the β-D-glucopyranosyl unit is not substituted, a downfield shift of 6 p.p.m. for the resonance of C-6 is seen. Likewise, the resonance of C-5 shows an upfield shift of 1.2 p.p.m. These facts agree with the general observations on sugars[27]. Thus, the conclusion, based on the ^1H-n.m.r. data, that the β-D-glucopyranosyl moiety in **11** is 6-substituted is verified. The chemical shift of the resonance of C-1 of the α-L-rhamnopyranosyl moiety in the rutinosyl part of **11** is found at slightly higher field (δ 98.37) in comparison with the corresponding data[27] for the rutinosyl moieties in rutin (**3**), hesperidin (**9**), and pseudobaptisin (**10**), and for α-L-rhamnopyranosides[18]. For the 4′-*O*-α-L-rhamnopyranosyl unit in **11**, the resonances for C-2,3,5 are similar (δ 70.64, δ 70.42, and δ 68.40). The ^{13}C resonances of the two α-L-rhamnopyranosyl units in **11** can be discriminated due to their different environments. The resonance for C-4 of the 4′-*O*-α-L-rhamnopyranosyl unit is found at δ 71.92, ~1 p.p.m. to higher field than expected. Such an upfield shift is also found[27] in rutin (**3**), which contains a 3-*O*-α-L-rhamnopyranosyl unit. The ^{13}C-n.m.r. data clearly indicate that only the β-D-glucopyranosyl unit of **11** is substituted, and the observed deviations of the ^1H-n.m.r. chemical shifts reflect a molecular environment different from those of the reference compounds.

2D-N.O.e. experiments. — In order to confirm the points of attachment of the sugar moieties to the apigenin nucleus in **11**, 2D-n.O.e. spectra with different mixing time were obtained since n.O.e. effects for solutions in methyl sulfoxide are weak[29].

With a mixing time of 120 ms, connectivities were revealed between the methyl of the α-L-rhamnopyranosyl unit of the rutinosyl moiety and H-3, H-3′ (or H-5′), and H-2′ (or H-6′) of the apigenin moiety and between the methyl group of the isolated α-L-rhamnopyranosyl unit and C-6 of the apigenin nucleus. With a mixing time of 240 ms, connectivities were revealed between H-1 of the isolated α-L-rhamnopyranosyl unit and H-8 of the apigenin nucleus, proving that HO-7 was substituted by an α-L-rhamnopyranosyl unit, between H-1 of the β-D-glucopyranosyl unit and H-3′ (or H-5′), proving the position of this sugar unit at position 4′, between the methyl of the α-L-rhamnopyranosyl unit of the rutinosyl moiety and H-3′ (or H-5′), and between H-6′ of the β-D-glucopyranosyl unit and H-3′ (or H-5′). With a mixing time of 480 ms, also connectivities between H-1′ of the α-L-rhamnopyranosyl unit of the rutinose moiety and H-2′(-6′), H-1 of the D-glucopyranosyl unit and H-3, and H-1 of the isolated α-L-rhamnopyranosyl unit with H-6 and H-8 were found.

Thus, **11** is 7-*O*-α-L-rhamnopyranosyl-4′-*O*-rutinosylapigenin (the absolute configurations of the sugar residues are assumed). The assignment of structure involved non-destructive methods and allows the maximum use of material for biological tests.

EXPERIMENTAL

General. — The flavonoids used in this study were commercial products.

U.v. spectra were recorded with a Shimadzu UV-160 double-beam spectrophotometer scanning at 60 nm/min. The shift reagents were used as described by Markham and Mabry[16]. The dried material was suspended in methanol (h.p.l.c. grade) to ~50μM.

F.a.b.-mass spectra were obtained with an AEI M902 double-focusing mass spectrometer equipped with an INCOS data system. The ion source was equipped with a M Scan atom gun. Methanol solutions of the compound acidified with aqueous 10% acetic acid (2 μL containing 1–10 μg) were dropped into glycerol on the stainless steel target of a direct insertion probe and bombarded with 9.5-kV argon atoms. Ions were accelerated from the source region at 4 kV and at a total scan time of 30 s. The spectra were calibrated against the standard perfluorokerosene and glycerol.

Acid hydrolysis[30] involved a solution of the compound (10 μg) in 2M HCl–MeOH (5 mL, 1:1), which was heated on a steam bath for 60 min and then concentrated to dryness. The residue was dissolved in the minimum volume of aqueous 10% acetonitrile containing 0.1% trifluoroacetic acid, and subjected to chromatography. Before and after hydrolysis, samples (1 mL) were made up to ~2μM in water for biological assay.

400-MHz ^{1}H-n.m.r. spectra were recorded for solutions in $(CD_3)_2SO$ (internal Me_4Si) at 22° with a Bruker AM-400 WB spectrometer fitted with an aspect 3000 computer (Max-Planck-Institut für Systemphysiologie, Dortmund, F.R.G.). Resolution enhancement of the spectra was achieved by Lorentz to Gaussian transformation.

^{1}H–^{1}H shift correlation (COSY) spectra were recorded by a two-pulse sequence, 90°–t_1–90°–acq. A 2048 × 8k data matrix was obtained with 32 scans for each experiment; no zero-filling was performed. Resolution enhancement in ω_2 and suppression of truncation artifacts in ω_1 were obtained by a Π/4-shifted sine-squared bell function in t_2 and t_1.

In the 2D-n.O.e. experiments, the mixing period was arbitrarily set to 80, 120, 240, 320, and 480 ms. A data matrix was obtained of 256 × 2k points, which was zero-filled to 1k × 4k prior to Fourier transformation. For resolution enhancement and suppression of truncation artifacts, a Π/4-shifted sine-squared bell function was applied in both dimensions. The 100.6-MHz ^{13}C-n.m.r. experiments (internal Me_4Si) were performed on the same spectrometer, using a Bruker broad-band VSP 10-mm probehead. Proton decoupling was done by a standard sequence (Waltz).

Isolation procedure. — Approximately 6000 *Sesbania rostrata* seeds were surface-sterilized as follows. After treatment for 1 h in conc. sulfuric acid, the seeds were placed, each time for 3 min, in aqueous calcium hypochlorite (0.5 g/L), aqueous 0.05% sodium dodecyl sulfate, and aqueous 0.5% $HgCl_2$ with washing in

between. The seeds (10 per mL) were germinated in water in the dark. The aqueous phase was replaced at 24-h intervals. The supernatant solution was acidified with trifluoroacetic acid (0.1%) and loaded on a PepRPC pre-packed column (10 × 100 mm; HR 10/10; Pharmacia) containing 15-μm silica particles with C-2 and C-18 alkyl side-chains. The column was pre-equilibrated with aqueous 0.1% trifluoroacetic acid and eluted at 2.8 mL/min, using a Pharmacia fast-protein liquid chromatography (FPLC) system equipped with the LCC 500 chromatographic programmer. A single-path u.v. monitor (214 nm) was used. The eluent contained increasing proportions of acetonitrile in aqueous 0.1% trifluoroacetic acid. The major peak was eluted at 12% of acetonitrile and the compound was purified to homogeneity by further chromatography. For preparative isolation, the aqueous phases between 24–48 h were combined and yielded 4 mg of pure **11**. Apigenin (Roth 5640, lot 0065410) was eluted in this system at 17% of acetonitrile.

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