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STRUCTURE AND ANTIBACTERIAL ACTIVITY OF PLANTAMAJOSIDE, A CAFFEIC ACID SUGAR ESTER FROM *PLANTAGO MAJOR* SUBSP. *MAJOR*

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Key Word Index—*Plantago major* subsp *major*, Plantaginaceae, caffeic acid sugar ester, caffeoyl glycoside, phenylpropanoid glycoside; plantamajoside, plant pathogenic bacteria

Abstract—The structure of plantamajoside, a phenylpropanoid glycoside isolated from *Plantago major* subsp. *major*, is deduced from chemical, spectral and other physical evidence, to be 3,4-dihydroxy-β-phenethyl-*O*-β-D-glucopyranosyl-(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside. The Minimum Inhibitory Concentration value has been evaluated for seven plant pathogenic bacteria and for *E. coli* (ML 30) and *S. aureus* (502 A) after preliminary investigations by the agar diffusion method.

INTRODUCTION

The compound 3,4-dihydroxy-β-phenethyl-*O*-β-D-glucopyranosyl (1→3)-4-*O*-caffeoyl-β-D-glucopyranoside has very recently been isolated, for the first time, from a callus of *Rehmannia glutinosa* (Scrophulariaceae) [1], but no trivial name was given to the compound. The same compound, designated as compound A, was found earlier as a chemotaxonomic marker in two subspecies of *Plantago major*, *P major* subsp. *major* and *P major* subsp *pleiosperma* [2]. We now show that compound A is **1**. In recent publication, *P major* was screened for polymorphism for this compound in relation to protection against slugs [3].

During recent years, several caffeic acid sugar esters have been described, the most common being verbascoside (**2**), first isolated in 1963 from *Verbascum sinuatum* (Scrophulariaceae) [4]. This compound was later shown to be identical with acteoside, isolated from *Syringa vulgaris* (Oleaceae) [5] and with kusaginin isolated from *Clerodendron trichotomum* (Verbenaceae) [6]. Caffeic acid sugar esters have been shown to have antibacterial [7,8], antifungal [9, 10] antiviral activity [11, 12] and selective inhibition of 5-lipoxygenase (from the leucotriene biosynthesis) [13, 14]. These compounds may also be of interest in plant pathology as natural plant protective agents [8, 15] and as repellents against herbivores, e.g. cereal aphids [16] and slugs and snails [17]. In the present work, the antibacterial activity of **1**, for which we suggest the name plantamajoside is determined against seven plant pathogenic bacteria and against *E. coli* and *S aureus*

RESULTS AND DISCUSSION

Compound A was obtained as an amorphous powder, with the elementary composition $C_{29}H_{36}O_{16}$ after extraction from leaves of *Plantago major* subsp. *major* and purification by column chromatography (CC). It was shown to be identical to 3,4-dihydroxy-β-phenethyl-*O*-β-D-glucopyranosyl (1 → 3)-4-*O*-caffeoyl-β-D-glucopyranoside (**1**) as described by Shoyama *et al.* [1]. Thus, the FABMS confirmed the M_r, all spectra were associated with other major fragments (see Experimental). Furthermore, both the UV and the IR spectrum closely resembled that of **1** With regard to the NMR data, the ^{13}C NMR spectrum of A was correlated with those reported for known compounds containing two monosaccharides, i e forsythiaside [1], verbascoside [18] and 3,4-dihydroxy-β-phenethyl-*O*-β-glucopyranosyl-(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside [1] The ^{13}C NMR data and their assignments are shown in Table 1. The attachment of the caffeoyl moiety at the C-4 carbon of the inner glucose is supported by the ^{13}C NMR since the chemical shift is the same for all four compounds. The attatchment of the outer glucose moiety at the C-3 carbon of the inner glucose is identical to the structure found in verbascoside (chemical shift $\delta = 83.1$ ppm) and in contrast to forsythiaside where the rhamnose moiety is bounded to the C-6 carbon of inner glucose ($\delta = 75\ 6$ ppm). Only signals corresponding to two monosaccharides were found, which is in agreement with the elementary analysis and with FABMS. In conclusion, the ^{13}C NMR spectrum was comparable with that described by Shoyama *et al* [1] for **1**, thus proving the presence of the dihydroxy-β-phenethyl moiety The ^{1}H NMR spectrum exhibited no signals at δ1 10–1.23 as described for forsythiaside by Nishibe *et al* [7] and for verbascoside respectively by Kitagawa *et al.* [18], this indicates that no free Me-groups are present in agreement with earlier observations for compound A [2] This was in agreement with the results of the hydrolysis, too, since no rhamnose was found (see below) The aromatic protons of **1** are resolved in two ABX systems, one belonging to the caffeic acid moiety substitution, the other to the 3,4-dihydroxy-phenethyl part

Table 1 ^{13}C NMR chemical shifts of caffeic acid sugar esters

Compound		Forsythiaside† [1]	Verbascoside† [18]	2* [1]	1†	3‡
3 4-Dihydroxyphenethyl moiety	1	130 3	131 4	130 2	130 3	136.6
	2	116 5	116 2	116 5	114 0	121 7
	3	146 4	145 9	146 3	144 8	141 6
	4	145 4	144 4	145 5	143 4	142 7
	5	117 4	117 0	117 4	115 4	122 1
	6	120 5	121 1	120 4	120 1	126 2
	7	36 1	36 3	36 1	35 3	34 3
	8	72 5	72 0	71 1	73 8	70 9
Caffeoyl moiety	1′	126 7	127 5	127 0	126 5	131 9
	2′	115 7	115 2	115 8	115 2	123 0
	3′	146 9	146 6	147 0	145 5	140 8
	4′	150 4	149 5	150 4	148 4	142 7
	5′	116 5	116 4	116 7	116 0	125 4
	6′	122 1	123 0	122 2	122 0	122 8
	7′	149 5	147 8	147 5	146 2	141 2
	8′	114 6	114 6	115 1	114 2	117 3
	9′	167 0	168 2	167 1	167 3	163 8
Glucose (inner) (Glu-1)	1	104 5	104 0	103 9	102 7	99 6
	2	74 6	75 8	76 2	74 8	71 6
	3	75 7	81 5	84 8	83 1	77 5
	4	71 4	70 2	70 5	69 7	68 7
	5	75 0	75 8	76 2	74 5	72 1
	6	67 5	62 2	62 0	61 2	61 4
Rhamnose	1	102 4	102 8			
	2	72 0	72 0			
	3	72.4	72 0			
	4	73 8	73.7			
	5	69 8	70 2			
	6	18 5	18 2			
Glucose (Glu)	1			106 6	104 5	99 8
	2			74 6	71 0	69 9
	3			78 2	76 4	74 6
	4			71 4	70 0	68 1
	5			78 0	76 6	76 0
	6			62 5	61 1	60 9
CO(Ac) (10 C)						163 8–169 6
Me (Ac) (10 C)						19 3– 19 8

^{13}C NMR chemical shifts of plantamajoside (**1**), 3,4-dihydroxy-β-phenethyl-*O*-β-D-glucopyranosyl-(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside (**2**) described by Shoyama *et al* [1], acetate of plantamajoside (**3**), forsythiaside and verbascoside

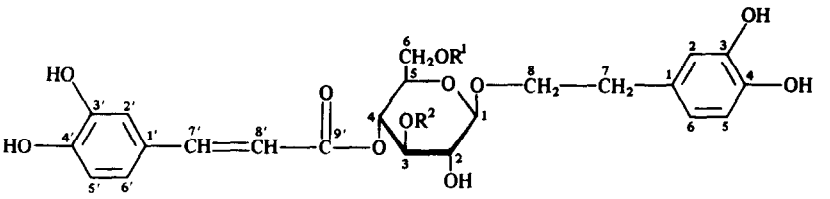
*In pyridine-d_5

†In CD_3OD

‡In $CDCl_3$

The α-CH_2 and the β-CH_2 protons in the aliphatic part of the dihydroxy-β-phenethyl moiety presents two pairs of interacting nonequivalent protons, seen as two broad triplets at 2 80 and 3.80 ppm respectively, a A_2B_2 system resembling that of an A_2X_2 due to the fact that $\Delta\nu AB \gg AB$ The H-7′ proton and the H-8′ proton from the caffeoyl moiety gave two doublets at δ7.61 and 6.34 respectively ($J = 16$ Hz) in both cases proving a *trans* configuration around the C=C The two anomeric protons (H-1 inner glucose, δ4 45) and (H-1 outer glucose, δ4.59) are both doublets with $J = 12$ Hz and hence in accordance with a β-configuration in both cases All these results suggest A is **1** as described by Shoyama *et al.* [1]

Enzymatic hydrolysis with β-glucosidase in water yielded desrhamnosylacteoside, D-glucose and an unidentified product. Upon further enzymatic hydrolysis with β-glucuronidase, caffeic acid was identified In order to establish the absolute configuration of the inner glucose, it was subjected to mild acid hydrolysis and the results compared with a similar treatment of forsythiaside **3** Desrhamnosylacteoside and D-glucose (in addition to traces of caffeic acid) were identified from compound A



1 R^1 = H, R^2 = Glc
2 R^1 = H, R^2 = Rha
3 R^1 = Rha, R^2 = H

The desrhamnosylacteoside was identified through its cochromatography (TLC, HPLC) with the desrhamnosylacteoside formed from forsythiaside upon this treatment [1], and through its similar fluorescence. The desrhamnosylacteoside formed was then isolated by prep. TLC (see Experimental) and shown to be devoid of any free glucose. Further acid hydrolysis was performed by treatment with 1 M HCl [1]. After a subsequent alkaline hydrolyse [1], D-glucose and caffeic acid was identified. The absolute configuration of the glucose in desrhamnosylacteoside has never been definitely proved before. We suggest the name plantamajoside for this natural plant constituent

Antibacterial activity

The antibacterial activity of plantamajoside, forsythiaside and chlorogenic acid were tested by the agar diffusion method and by a Minimum Inhibitory Concentration method as described by Ravn *et al.* [8]. In the agar diffusion assay, plantamajoside generally showed the largest inhibitory zones when compared to forsythiaside and chlorogenic acid. However, plantamajoside and forsythiaside showed nearly the same inhibition against the bacteria but generally not as good an inhibition as chlorogenic acid when the MIC-method was used (see Table 2). Compared with the result described by Ravn *et al.* [8], plantamajoside is less inhibitory than the free acids, caffeic acid, ferulic acid, rosmarinic acid and esculetin.

EXPERIMENTAL

^{1}H NMR spectra were recorded at 90 MHz and chemical shifts are given in (ppm) relative to TMS as int. standard. ^{13}C NMR spectra were recorded with TMS int. standard. FABMS spectra were taken using Ar atoms at 8 keV. Positive ion FAB-spectra were sampled on a Varian SS 200 data system, calibrated in the electron impact mode. HPLC-analysis were performed on the following system: a 100 mm × 4 mm ID Nucleosil® 100–5 C 18 analytical column with a 30 × 4 mm i.d. Nucleosil® 120–5 C 18 precolumn. A flow-rate of 2.0 ml/min of the eluent H_2O–MeOH–HOAc 150.50·1 was maintained. Selective detection at 330 nm TLC system: Precoated thin layer chromatography plates, silica gel 60 F_{254} (Merck) with the upper phase of *n*BuOH–HOAc–H_2O BAW (4:1·5) as solvents. Blue fluorescent spot (exitation at $\lambda = 366$ nm), spray dil. $FeCl_3$ or 2M H_2SO_4 (2 min at 105°) or 0·1% naphtoresorcionl 2M H_2SO_4 (1 1) (sugar identification) CC on a Sephadex LH-20 column (Phamacia) K 50/100, 66–91 × 5 cm with redest. H_2O as eluent; a LKB 2120 varioperpex® II-pump, a LKB Bromma 2138 UVICORDS, filter 279 nm; a LKB Bromma 2210 2-channel recorder connected with a LKB Bromma 2070 Ultro RAC® II (6 min = 17.3 ml).

Plant material, test bacteria, chemicals and media Leaves from *Plantago major* L subsp *major* were kindly supplied by Dr Per

Table 2 Minimum Inhibitory Concentration (MIC) in mg/ml and mM

Compound	1		2		3	
Concentration. Micro-organism:	mg/ml	mM	mg/ml	mM	mg/ml	mM
Corynebacterium rathayi	>2·5	>3.9	>2·5	>4·0	1.5	4.3
Corynebacterium fascians	2·0	3·1	1.5	2.4	1·0	2.9
Corynebacterium sepedonicum	>2.5	>3·9	1.0	1.6	1·0	2.9
Agrobacterium tumefaciens	>2.5	>3.9	>2.5	>4·0	2·0	5.7
Erwinia carotovora var *carotovora*	1·0	1·6	1.0	1.6	2.5	7.2
Xanthomonas pelargonii	>2.5	>3·9	>2·5	>4·0	1.5	4·3
Pseudomonas syringae	>2·5	>3·9	>2.5	>4.0	2.0	5.7
Staphylococcus aureus 502A	2.0	3·1	1.5	2.4	>2.5	>7.2
Escherichia coli Ml 30	>2.5	>3·9	>2.5	>4·0	2.5	7.2

The MIC-values produced in beef extract peptone bouillon as described by Ravn *et al.* [8]. **1** = plantamajoside, **2** = forsythiaside, **3** = chlorogenic acid

Mølgaard who also undertook the identification The plants were grown in the greenhouse at the Royal Danish School of Pharmacy Voucher specimens of the plants are deposited at the Royal Danish School of Pharmacy, Department of Pharmacognosy and Botany (voucher No 84–13, 20–8 1984)

The bacteria used were as follows *Corynebacterium rathayi* (Smith) Dowson No 1019 (RVAU), *C fascians* (Tilford) Dowson No 2155 (RVAU), *C sepedonicum* (Spieckermann & Kotthoff) Skaptason & Burkholder No 3A (RCPP), *Staphylococcus aureus* Rosenbach No 502A. (GSI), *Agrobacterium tumefaciens* (Smith & Townsend) Conn No 2165 (RVAU), *Erwinia carotovora* var *carotovora* (Jones) Dye No 135 (RCPP), *Xanthomonas pelargonii* (Brown) Starr & Burkholder No 310 (RCPP), *Pseudomonas syringae* van Hall No 126 (RCPP) and *Escherichia coli* (Migula) Castellani & Chalmers No ML30 (RDSP) Sources are RVAU = The Royal Veterinary and Agricultural University, Dept Plant Pathology, Denmark. RCPP = Research Centre for Plant Protection, 2800 Lyngby, Denmark, RDSP = Royal Danish School of Pharmacy, Dept of Microbiology Denmark, GSI = Government Serum Institute, Copenhagen, Denmark

The chlorogenic acid was obtained from Merck Schuchardt BDR (820319), forsythiaside was isolated from fruits of *Forsythia suspensa* Vahl, the fruits were obtained as a gift from Professor Sansei Nishibe, Japan The procedure was essentially the same as that for plantamajoside

The media used for inoculation of the bacteria were beef extract peptone bouillon (BPB) (0 8% Bacto Nutrient Broth, Difco in H_2O) Beef extract peptone agar (BPA) Merck (0 8% beef extract, 1% peptone, Difco, 0 5% NaCl, 1 2% Bacto agar, in water, pH = 7 5) 5 ml in 90 mm plast petri dishes were used for the agar diffusion method

Isolation of plantamajoside 100 g of crushed dried leaves from *Plantago major* subsp *major* was extracted with 500 ml MeOH in 24 hr at 40° The ppt was filtered off and the extract was evapd *in vacuo* at 40 Three more extractions were made and the four extracts were pooled and evapd in vacuum at 40° The dried extract was dissolved in 80–90 ml MeOH, filtered, H_2O was added and the extract was lyophilized to give a powder (14 57 g) 5 00 g of the lyophilized powder was dissolved in 5 ml H_2O, centrifuged and made up to 10 ml with H_2O Finally, the extract was filtered before the purification of plantamajoside by CC as described above Every 5th or 10th fractions was tested by HPLC and on a special TLC plate (silica gel 60 F_{254} (Merck) wetted with dil $FeCl_3$ and dried (blue or green spot), the fractions containing **1** were pooled and lyophilized

Plantamajoside An amorphous powder mp 158–162° (uncorr), $[\alpha]_D^{19.2}$ −42 47, $[\alpha]_D^{24.8}$ −43 88 (MeOH, *c* 0 47), UV λ_{max}^{MeOH} nm 220 4, 247 6, 292 4, 332 4, IR ν_{max}^{KBr}, 3350 cm^{-1} (*br*, OH) 1685 cm^{-1} (conjugated C = O), 1625 cm^{-1} [C(7′) = C(8′)], 1600 cm^{-1}, 1515 cm^{-1} (arom ring), 850, 810 cm^{-1} (1,3,4-tri subst arom ring) Anal calcd for $C_{29}H_{36}O_{16}$ C 54 37, H 5 66 Found C 52 60, H 5 71, FDMS *m/z* 641 $[M+1]^+$, 679 $[M+K]^+$, 773 $[M+Cs]^+$ ^1H NMR (in $CDCl_3$) δ 2 80 and δ 3 80 (both 2H, *t*, H-7, H-8 phe), δ 4 45 (1H, *d*, *J* = 12 Hz ~ H-1 glu-*i*), δ 4 59 (1H, *d*, *J* = 12 Hz H-1 ~ glu), δ6 34 (1H, *d*, *J* = 16 Hz ~ H-8′) δ 7 61 (1H, *d*, *J* = 16 Hz ~ H-7′) δ 6 52 [1H, *d*, *J* = 2 Hz ~ H-2 (phe)] δ 6 65 [1H, *d*, *J* = 10 Hz ~ H-5 (phe)] δ6 72 [1H, *d*, *J* = 9 Hz ~ H-6 (phe)] δ6 68 [1H, *d*, *J* = 10 Hz ~ H-5′] δ 7 03 (1H, *dd*, *J* = 2 Hz, *J* = 10 Hz ~ H-6′) ^{13}C NMR (see Table 1). Acetate (prepd via Ac_2O–pyridine), an amorphous powder mp 97–100° (uncorr) ^1H NMR (in CD_3OD) δ 1 78, 1 94, 2 00, 2 06 (15 H, each s, alcoholic MeCO), δ 2 28 (12H, s, phenolic MeCO–) δ 2 86 [2H, *t*, *J* = 16–17 Hz ~ H-7 (phe)] δ4 35 (1H, *d*, *J* = 10 Hz H-1 ~ glu-*i*) δ4 55 (1H, *d*, *J* = 10 Hz ~ H-1 glu), δ6 32 (1H, *d*, *J* = 16 Hz ~ H-8′) δ 7 63 (1H, *d*, *J* = 16 Hz ~ H-7′) δ6 59 [1H, *d*, *J* = 4 Hz, ~ H-2 (phe)], δ 7 04 [1Hz, *d*, *J* = 6 Hz ~ H-5 (phe)] δ = 7 05 [1H, *d*, *J* = 6 Hz ~ H-6 (phe)] δ 7 19 (1H, *d*, *J* = 2 Hz ~ H-2′), δ 7 31 (1H, *d*, *d*, *J* = 2 Hz, *J* = 14 Hz ~ H-6′) ^{13}C NMR see Table 1

Enzymatical hydrolysis of **1** 20 mg plantamajoside **1** was dissolved in 1 ml H_2O and 5 μl of a solution (4 5 mg/ml β-glucosidase (from almonds) No G-8625 type II, Sigma in H_2O) was added The solution was left at 37 and at suitable times, the solution was investigated by TLC, HPLC and Clinistix (Ames) (D-glucose oxidase/peroxidase test for D-glucose) Still after 24 hr **1** was seen (R_f 0 7 (TLC), R_t = 5 (HPLC)) further desrhamnosylacteoside (R_f 0 8 (TLC), R_t = 5 (HPLC)), D-glucose (R_f 0 3 (TLC)), + D-glucose, clinistix) and an unknown hydrolysis product (R_f 0 4 (TLC), R_t = 10 (HPLC)) were found Further hydrolysis was made by β-glucuronidase (*Helix pomatia*) (Sigma, same condition as above), and after 24 hr, a large amount of caffeic acid (R_f 0 9 (TLC), R_t = 2 (HPLC)) was found

Mild acid hydrolysis of **1** *and of forsythiaside* Forsythiaside **2** (3 mg) and **1** (4 mg) were each treated with 0 3 M HCl at 95°, at a suitable time the solutions were investigated by TLC, HPLC and after basification by Clinistix (Ames) After 3 hr the solution of forsythiaside showed still the original compound (R_f 0 67 (TLC), R_f 9 (HPLC)) further desrhamnosylacteoside, (R_f 0 8 (TLC), R_t 5 (HPLC)) caffeic acid (R_f 0 9 (TLC), R_t 2 (HPLC)) and D-glucose (trace) (+ D-glucose, Clinistix) The solution of **1** gave still the original compound R_f 0 7 (TLC), R_t 5 (HPLC), desrhamnosylacteoside (R_f 0 8 (TLC), R_t 5 (HPLC)), caffeic acid (R_f 0 9 (TLC), R_t 2 (HPLC)) and D-glucose (+ D-glucose, Clinistix)

Acid hydrolysis of **1** *followed by alkaline hydrolysis* Desrhamnosylacteoside was isolated after enzymatic and mild acid hydrolysis of **1** respectively by prep TLC (plates silica gel 60 F_{254} Merck art 5626 10 × 20 cm, 0 25 mm, eluent as above) The compound was extracted from zone [R_f 0 8 (TLC)] with MeOH, H_2O was added and the solution was lyophilized Desrhamnosylacteoside was now dissolved in 1 M HCl and hydrolysed at 20° in 24 hr Besides desrhamnosylacteoside [R_f 0 8 (TLC), R_t 5 (HPLC)], caffeic acid (R_f 0 9 (TLC), R_t 2 (HPLC)) and a hydrolysis product (R_f 0 4 (TLC), R_t 10 (HPLC)) were seen The solution was now basified to 1 M with NaOH and heated for 3 hr at 50° The solution was tested and D-glucose found (R_f 0 3 (TLC)), spray 0 1% naphtoresorcinol 2 M H_2SO_4 (1 1), + D-glucose, Clinistix)

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