Phytochemistry, Vol. 28, No. 9, pp. 2379–2383, 1989.
Printed in Great Britain.

0031-9422/89 $3.00 + 0.00

STRUCTURAL ANALYSIS OF A RHAMNOARABINOGALACTAN AND ARABINOGALACTANS WITH IMMUNO-STIMULATING ACTIVITY FROM *CALENDULA OFFICINALIS*

JADRANKA VARLJEN, ANDRAS LIPTÁK* and HILDEBERT WAGNER†

Institute of Chemistry and Biochemistry, Medicinal Faculty of Rijeka, Olge Ban 20, 51000 Rijeka, Yugoslavia; *Kossuth Lajos Tudomenyegyetem, Biokemia, Itezete, H-4010 Debrecen, Hungary; †Institute of Pharmaceutical Biology, University of Munich, D-8000 München 2, Karlstrasse 29, F.R.G.

(*Received* 4 *January* 1989)

Key Word Index—*Calendula officinalis*; Asteraceae; rhamnoarabinogalactan; arabinogalactans; plant polysaccharides; structural analysis; immunological activity.

Abstract—From the flowers of *Calendula officinalis*, three homogeneous polysaccharides were isolated by ethanol fractionation and gel filtration. Sugar and methylation analysis showed that all three polysaccharides contain a (1 → 3)-linked β-D-galactan backbone with branching points at C-6. The side chains, are composed of short α-Araf-(1 → 3)-Araf, α-L-Rhap-(1 → 3)-Araf or simple α-L-Araf units. Further characterization of the polysaccharides was performed by periodate oxidation and Smith degradation, acid hydrolysis and various ^{13}C NMR spectroscopic methods. The polysaccharides showed immunostimulating activity in several *in vitro* immunological test systems.

INTRODUCTION

Calendula officinalis L. is widely distributed in the Near East and in southern Europe. Alcoholic water extracts of the dried flowers are chiefly used externally in the form of ointments for wound healing. Anti-tumour activity of a polysaccharide fraction, isolated from *C. officinalis* was reported by Manolov *et al.* [1]. A polysaccharide mixture of the plant extract increased phagocytosis as determined by *in vitro* granulocyte and *in vivo* carbon clearance tests [2, 3]. To complete our earlier investigations, we separated the crude polysaccharide fraction into three homogeneous polysaccharides and report herein on the structure analysis of these pure polysaccharides.

RESULTS AND DISCUSSION

A crude polysaccharide fraction was obtained from ethylacetate and methanol pretreated dried flowers of *C. officinalis* by extraction with 0.5 M aqueous sodium hydroxide according to Caldes *et al.* [4], followed by precipitation with ethanol and treatment with trichloroacetic acid. On hydrolysis, the non-dialysable, freeze-dried *N*-free fraction gave rhamnose, arabinose, galactose and traces of xylose and glucose. The presence of uronic acid was disclosed by the carbazole test.

The polysaccharide mixture was further fractionated and purified by ethanolic precipitation followed by gel chromatography on Sephadex G-25. The purification steps gave three main fractions which proved to be homogeneous by gel chromatography. The first polysaccharide (PS-I) contained arabinose, rhamnose and galactose. The two other polysaccharides (PS-II and PS-III) were built up only from arabinose and galactose. The molar ratios of the monosaccharide components as determined by GC of the derived alditol acetates [5] are given in Table 1. The mean M_r of PS-I, PS-II and PS-III were determined as 15 000, 25 000 and 35 000 respectively, and the specific optical rotation as 1.3°, −22.7° and −38.6°.

Hakomori methylation [6] of the polysaccharides, followed by TFA acid hydrolysis and GC and GC-MS of the resultant partially methylated alditol acetates [7] gave the results shown in Table 2. The structures of the

Table 1. Monosaccharide composition of polysaccharides isolated from *C. officinalis* (molar ratios)

Sugar	PS-I	PS-II	PS-III
Arabinose	34.2	27.6	48.7
Galactose	41.0	72.4	51.4
Rhamnose	24.8	—	—

Table 2. Methylation analysis of polysaccharides isolated from. *C. officinalis* (molar ratios)

Partially methylated alditol-acetates*	PS-I	PS-II	PS-III
2,5-Me_2-Ara	19.8	9.1	20.1
2,3,5-Me_3-Ara	13.8	18.6	28.5
2,4-Me_2-Gal	36.0	18.3	29.0
2,4,6-Me_3-Gal	5.1	54.0	22.4
2,3,4-Me_3-Rha	26.3	—	—

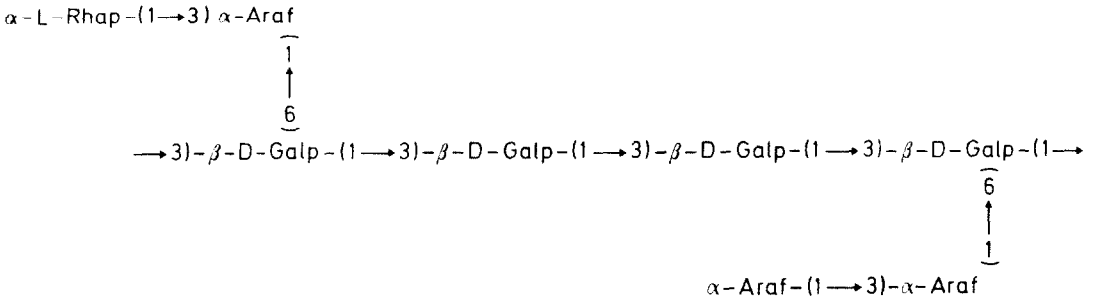
*Analysed by GC-MS.

Part 8 in the series, 'Immunologically Active Polysaccharides'.

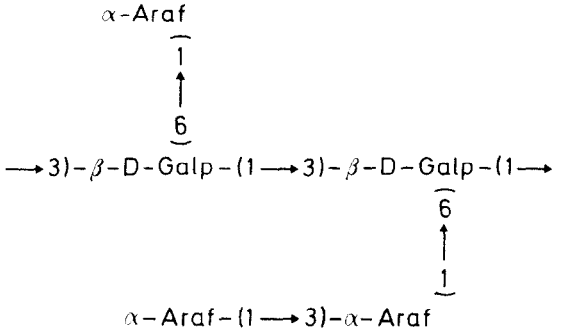
Table 3. ^{13}C NMR data of

	PS-I							
Sugar linkages	C-1	2	3	4	5	6	C-1	2
Araf-(1→	109.19	80.18	77.53	84.20	63.1	—	109.12	80.09
→3)-Araf-(1→	107.63	79.11	81.05	82.45	61.15	—	107.53	78.71
→3)-Galp-(1→	103.50	70.95	81.45	68.85	76.54	61.59	103.38	70.80
→3) →6) Galp-(1→	102.95	70.45	81.45	68.58	75.19	66.78	101.65	70.25
Rhap-(1→	101.73	71.65	71.31	72.87	70.02	16.67	—	—

PS I



PS II



methylated alditol acetates were confirmed by comparison with standard substances [8]. In the case of polysaccharide PS-I, the permethylation yielded the alditol acetates of 2,3,4-tri-*O*-methyl-rhamnose indicating the existence of non-reducing terminal rhamnopyranose side-units. Similarly, the incidence of 2,3,5-tri-*O*-methylarabinofuranose showed that the side-chains were partly terminated by arabinofuranoside units. The presence of the 2,4-di- and that of the 2,4,6-tri-*O*-methyl-galactose suggested the presence of (1→3)-bonded core-chains. Analysis of the methylated alditol acetates of PS-II and that of the PS-III showed clearly that they have very similar structures. In both cases only four methylethers could be detected, namely, 2,5-di-*O*-, 2,3,5-tri-*O*-methyl-arabinose, 2,4-di-*O*- and 2,4,6-tri-*O*-methyl-galactose. The large amount of 2,3,5-tri-*O*-methyl-arabinose measured in PS-III compared to PS-II suggested a highly branched structure. This assumption was verified by the different molar ratios of the 2,4,6-tri-*O*-methyl-galactose in the two polysaccharide samples.

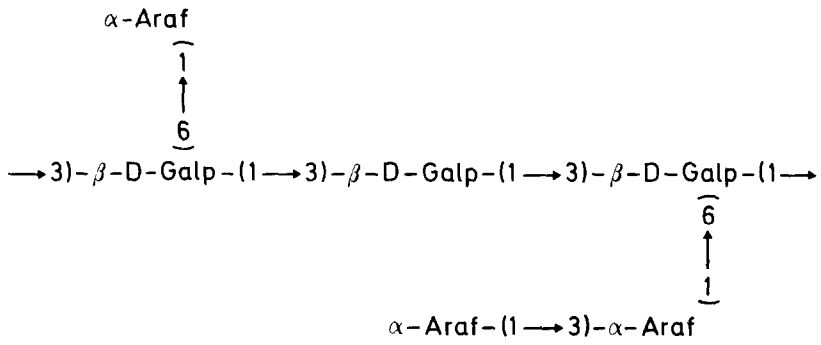
In another experiment, we subjected all polysaccharides to periodate oxidation, followed by Smith degradation [9]. The observed very low periodate consumption showed, that only the non-reducing chain-ends, the rhamnopyranose and the arabinofuranose units, were cleaved. Analysis of the products showed the presence of galactose and of a small amount of arabinose, whereas the rhamnose has completely disappeared. The results of these experiments were in good accord with the data of the methylation analysis and showed that both the galactose and the arabinose units were glycosylated in position 3, whereas the rhamnose units must be situated exclusively at the non-reducing ends of the side-chains.

To get more information about the bonding types and the sugar sequences of the three polysaccharides, they were hydrolysed under very mild conditions, and the dialysable and non-dialysable fractions investigated. The dialysable fraction from PS-I contained arabinose and rhamnose in a molar ratio of *ca* 4:1. In the non-dialysable fraction of hydrolysed PS-I, galactose and rhamnose were detected also in a ratio of 4:1. Methylation of the non-dialysable fraction, followed by acid hydrolysis and GC of the derived partially methylated alditol acetates gave 2,3,4-tri-*O*-methyl-rhamnose, 2,4-di-*O*-methyl-ga-

Calendula polysaccharides

Chemical shifts									
PS-II				PS-III					
3	4	5	6	C-1	2	3	4	5	6
76.71	84.04	62.91	—	109.34	81.02	76.87	84.02	63.41	—
80.09	82.38	61.02	—	107.67	81.02	81.20	82.45	61.40	
81.31	68.47	76.71	61.39	103.50	70.87	82.13	68.77	76.46	62.26
80.93	68.71	74.99	66.93	103.33	70.01	81.44	68.77	74.68	66.60
—	—	—	—	—	—	—	—	—	—

PS III



lactose, 2,4,6-tri-*O*-methyl-galactose and traces of 2,3,4,6-tetra-*O*-methyl-galactose. The ratio of the 2,3,4-tri-*O*-methyl-rhamnose to 2,4-di-*O*-methyl-galactose was nearly 1:1. These experiments clearly showed that PS I contains a (1→3)-linked core chain possessing a branching point in position 6 substituted either with an Araf-(1→3)-Araf-(1→ or Rhap-(1→3)-Araf-(1→ or a Araf-(1→ unit.

Similar treatments of PS-II and PS-III resulted in dialysable parts which contained exclusively arabinose, whereas the non-dialysable parts consisted of a galactan only. Methylation of this galactan gave 2,4,6-tri-*O*-methyl-galactose and the traces of 2,3,4,6-tetra-*O*-methyl-galactose. The extremely easy hydrolysability of the side-chains is in good agreement with the result of the methylation analysis of the intact polysaccharides, indicating that the arabinose building-blocks contain the sugar in the furanose form.

The ^{13}CNMR spectra of PSI-III support the structures derived from methylation analysis, periodate oxidation and partial hydrolysis. Determination of the heteronuclear coupling $^{3}J_{C1-H1}$ constants showed clearly that all galactopyranosyl units occur in a β-configuration (163 Hz), whereas the arabinofuranosyl residues must be present as α-anomers (174.6–173.6 Hz) and the rhamnopyranosyl units in PS-I with an α-configuration (≈ 172 Hz) [10]. INEPT or '*J*-echo' [11] experiments also indicated that the C-5 hydroxyls of the arabinopyranosyl units are unsubstituted, whereas the C-6-OH of the galactopyranosyl residues must be partly glycosylated. The very characteristic low field signals of the arabinose units were consistent with the presence of furanosidic forms.

Based on these results, it can be concluded that the backbone chains of all three polysaccharides are composed of β-(1→3)-linked galactopyranosyl residues possessing branching points at position C-6. The arabinofuranosyl residues appear to be attached to the backbone at position C-6 as α-(1→)-linked side-chains and as non-reducing terminal groups. The α-pyranosyl residues are attached either directly to the galactopyranosyl core at position C-6 or to the arabinofuranosyl side-chain at position 3 (see structure proposals for PS-I, PS-II and PS-III).

The arabinogalactans and rhamnoarabinogalactan of *C. offinalis* are structurally very similar to those arabinogalactans which were isolated earlier from the wood of European larch by Hirst and co-workers [12, 13]. Concerning the bonding type of the rhamnoarabinogalactan, a very similar polysaccharide was isolated from the wood sap of sugar maple [14, 15].

Immunological activity

All three polysaccharides were subjected to the *in vitro* granulocyte test, which was performed with human

granulocyte fractions according to the method described in [17]. PS-I enhanced the phagocytosis at a concentration of 10^{-5}–10^{-6} mg/ml between 40 and 57%. PS-II stimulated the phagocytosis at the same concentration range to about 20–30%, whereas PS-III, enhanced the phagocytosis index between 54 and 100%. The latter, therefore, is one of the most immunological active polysaccharide ever investigated in this test system.

EXPERIMENTAL

General and analytical methods. All evapns were performed under red. pres. at a temp. below 40°. TLC was performed on silica gel 60 F_{254} (Merck) with $CHCl_3$–MeOH–H_2O (8:5:1) as solvent and detection with aniline diphenylaminophosphoric acid (100°/10 min). FDE-GC was performed on a glass column (3 m × 2 mm) with 3% OV 225(A) on Chromosorb W-HP (80/200 mesh) at 215° for alditolacetates. GC/MS: Fractorap 2110, Carlo Erba and Kratos MFC500 for partially methylated alditolacetates on an OV-225-Chrompack 14024-capillary column (25 m, 0, 23 mm φ, 150–210°/5°/min or on an OV-1701 quartz-capillary column (30 mx 0.25 mm φ, 150–260°/3°/min, coupled with Varian MS CH 7/188 and Kratos MS 870 FRA, ^{13}C NMR spectra for D_2O solns were recorded at 20.15 MHz; chemical shifts are given in δ values. For M_r estimation, calibration was performed using dextrans of known M_r (T10, T40, T70, T110, Pharmacia Fine Chemicals).

Quantitative estimation of the monosaccharides was performed with an automatic sugar analyser (Biotronik ZA 51000). Optical rotations were measured in aq. soln using a Perkin-Elmer 241 polarimeter at 365 nm at 20°. The drug material was purchased at Fa. Klenk, Schweinfurth/Schwebheim in 1980.

Isolation of polysaccharides. Powdered flower material (1.3 kg) was extracted with EtOAc and MeOH under reflux in a Soxhlet apparatus for 4 days and the extracts discarded. The dried plant residue (915 g) was extracted twice with 0.5 M aq. NaOH (5 ml/g plant material) and allowed to stand overnight. After each extraction the residue was separated from the supernatant soln by filtration. To the combined extracts 3 vol. EtOH were added under stirring and the mixture kept for 24 hr at +4°. After decanting the supernatant soln, the brownish ppt. was centrifuged and resuspended in cold H_2O. TCA (15%) was added under stirring at 0°. The ppt. formed was again sepd by centrifugation and 4 vol. EtOH added to the supernatant. After storage for 48 hr and centrifugation, the ppt was resuspended in 2% aq. NaOAc. The soln was centrifuged to remove insoluble material, followed by addition of 0.5 vol. of EtOH to the supernatant. After standing 96 hr the supernatant soln was decanted and the ppt. collected by centrifugation. The supernatant soln was diluted with 1 vol. of EtOH and after standing for 96 hr the ppt. (Fraction PS-I) collected by centrifugation. Fraction PS-II was obtained by dilution of the supernatant with 2 vol. of EtOH and fraction PS-III collected after treatment of the foregoing supernatant with 4 vol. of EtOH. The crude polysaccharide fractions were separately dissolved in H_2O and dialysed against H_2O for 3 days and then lyophilized. Yields: 1.05 g PS-I, 0.55 g PS-II, 0.66 g PS-III.

Fractionation of polysaccharides by gel chromatography. 0.5 g portions of PS-I, PS-II and PS-III fractions were dissolved in H_2O, applied to a Sephadex G-25 column (2.5 × 60 cm) and eluted with H_2O. The fractions were monitored by phenol–H_2SO_4 reagent and measured at 480 nm. All fractions were further purified by Biogel-P-60 DEAE-Trisacryl M columns.

Analysis of sugars. Complete acid hydrolysis of the polysaccharides was achieved by treatment with 2 M TFA for 6 hr at 100°. The hydrolysates were then divided into two parts. One part was analysed using either TLC or a sugar analyser. The monosaccharides of the second part were converted into their corresponding alditol acetates for GC analysis.

Methylation analysis. Polysaccharides (5–10 mg) were dissolved in dry DMSO (5 ml) and were methylated with methylsulphinyl carbanion (2.5 ml) and MeI (2.5 ml) according to ref. [6]. After hydrolysis the methylated sugars were converted into their corresponding partially methylated alditol acetates using $NaBH_4$ reduction followed by acetylation (Ac_2O/pyridine) and analysed by GC and GC-MS.

Periodate oxidation and Smith degradation. Polysaccharides (5–10 mg) were oxidized with 0.01 M $NaIO_4$ (20 ml) at 4° in the dark for 5 days. The periodate consumption was determined spectrophotometrically [16]. At the end of the oxidation, ethylene glycol (0.4 ml) was added to destroy the excess of the reagent and the reaction mixture dialysed against H_2O for 48 hr. The non-dialysable fraction was lyophilized, dissolved in H_2O (10 ml) and reduced with $NaBH_4$ (4 mg) for 24 hr at room temp. Excess $NaBH_4$ was destroyed by addition of NaOAc and the reaction mixture dialysed again for 24 hr against H_2O. The non-dialysable fraction was hydrolysed with 2 M TFA and the monosaccharides obtained analysed as their corresponding alditol acetates by GC.

Partial acidic degradation of the polysaccharides. Polysaccharides (30–30 mg) were treated with 0.05 M TFA at 100° for 4 hr. After evapn of acid, the reaction mixture was dissolved in H_2O and dialysed against 100 ml of H_2O. Aliquots of water were evaporated and analysed for monosaccharides as their corresponding alditol acetates by GC. The non-dialysable part was divided into two parts and lyophylized. One part was hydrolysed with 2 M TFA and the monosaccharides analysed after converting them to alditol acetates by GC. The other part was methylated by the method of ref. [6], the methylated polysaccharide hydrolyzed and the partially methylated monosaccharides transformed into their corresponding alditol acetates and analysed by GC.

Acknowledgements—Dr I. Varljen (Lubliana) and Prof. Dr. A. Lipták (Debrecen) thank the DAAD and DFG for their fellowships.

REFERENCES

1. Manolov, T., Boyadzhiev, T. and Nikolov, P. (1964) *Eksp. Med. Morfol.* **3**, 41.
2. Wagner, H., Kreutzkamp, B. and Jurcic, K. (1985) *Planta Med.* 419.
3. Wagner, H., Proksch, A., Riess-Maurer, I., Vollmar, A., Odenthal, S., Stuppner, H., Jurcic, K., Le Turdu, M. and Fang, J. N. (1984) *Arzneim.-Forsch* **34**, 659.
4. Caldes, G., Prescott, B., Thomas, C. A. II and Baker, P. J. (1981) *J. Gen. Appl. Microbiol.* **27**, 157.
5. Albersheim, P., Nevins, J., English, P. D. and Karr, A. (1967) *Carbohydr. Res.* **5**, 340.
6. Hakomori, S.-I. (1964) *J. Biochem.* (Tokyo) **55**, 502.
7. Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. and Lönngren, J. (1976) *Chem. Commun.* (Stockholm University) **8**.
8. Lipták, A., Oláh, V. A., Varljen, J. and Wagner, H. (1989), *Carbohydr. Res.* (in press).
9. Abdel-Akher, M., Hamilton, J. K., Montgomery, R. and Smith, F. (1952) *J. Am. Chem. Soc.* **74**, 4970.
10. Bock, K., Lundt, I. and Pedersen, C. (1973) *Tetrahedron Letters* 1037.

11. Doddrell, D. M. and Pegg, D. T. (1980) *J. Am. Chem. Soc.* 102, 6388.
12. Campbell, W. G., Hurst, E. L. and Jones, J. K. N. (1948) *J. Chem. Soc.* 774.
13. Aspinall, G. O., Hirst, E. L. and Ramstad, E. (1958) *J. Chem. Soc.* 593.
14. Adams, G. A., Bishop, C. T. and McDonald, I. J. (1959) *Can. J. Biochem. Physiol.* **37**, 507.
15. Adams, G. A. and Bishop, C. T. (1960) *Can. J. Chem.* **38**, 2380.
16. Dixon, J. S. and Lipkin, D. (1954) *Anal. Chem.* **26**, 1092.
17. Brandt, L. (1967) *Scand. J. Haematol.* Suppl. 2.