Journal of Natural Products
Vol. 52, No. 4, pp. 875-878, Jul-Aug 1989

PLANT ANTIVIRAL AGENTS, VI.[1] ISOLATION OF ANTIVIRAL PHENOLIC GLUCOSIDES FROM *POPULUS* CULTIVAR BEAUPRE BY DROPLET COUNTER-CURRENT CHROMATOGRAPHY

L. VAN HOOF, J. TOTTÉ, J. CORTHOUT, L.A. PIETERS, F. MERTENS, D.A. VANDEN BERGHE, A.J. VLIETINCK,*

Faculty of Medicine, University of Antwerp, UIA, Universiteitsplein 1, B-2610 Antwerp, Belgium

R. DOMMISSE, and E. ESMANS

Laboratory of Organic Chemistry, University of Antwerp, RUCA, Groenenborgerlaan 171, B-2020 Antwerp, Belgium

ABSTRACT.—Bioassay-guided isolation of the 80% EtOH extract of the leaves of *Populus* cultivar Beaupré by means of dccc afforded four known phenolic glucosides. Salicin and salireposide were shown to be, at least partially, responsible for the antiviral activity of the extract against poliomyelitis and Semliki forest viruses.

The results of a broad screening program of higher plants for antimicrobial and antiviral activities showed that the 80% EtOH extracts from the leaves of several poplar trees exhibited pronounced anti-*Pseudomonas*, antidermatophyte, and antiviral properties (1,2).

The leaves of the poplar tree cultivar Beaupré (*Populus trichocarpa* V 235 × *Populus deltoides* S.1-173, formerly known as Unal 8 or S.910-2) (Salicaceae) have been selected for a more detailed investigation of their active principles. The genus *Populus*, and more generally the Salicaceae, contains a large number of phenolic glucosides, which have been thoroughly investigated by the research groups of I.A. Pearl (3,4) and H. Thieme (5,6). The preferred technique to separate and isolate the phenolic glucosides consisted of EtOAc extraction of the hot-H_2O-soluble portion of an EtOH extractive followed by polyamide chromatography employing step-gradient elution with H_2O and dilutions of EtOH (2). Identification of these compounds was mostly carried out by destructive chemical methods and 2D paper chromatography procedures (7). A few papers comment on the mass spectral behavior of phenolic glucosides after derivatization to their peracetylated derivatives (8).

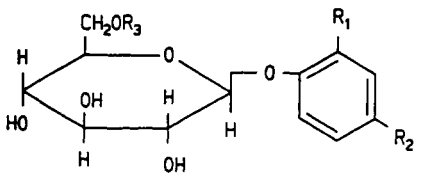
Recently, more modern methods such as hplc and gc procedures and ^1H- and ^{13}C-nmr spectroscopy have been used respectively for the quantitative and structural analyses of phenolic glucosides (9,10).

We have developed an appropiate and accurate dccc system for the bioassay-guided isolation of the antivirally active compounds from the leaves of the poplar tree cultivar Beaupré. The isolation was guided by following the antiviral activity against poliomyelitis virus.

The antivirally active EtOAc extract obtained as mentioned above was directly submitted to dccc. Using a solvent mixture of $CHCl_3$-MeOH-H_2O (7:13:8, upper layer) as mobile phase in the ascending mode we obtained four fractions, which readily afforded four crystalline but known phenolic glucosides: salicin [2-(hydroxymethyl)-phenyl-β-D-glucopyranoside] [**1**], salireposide [2-(benzoyloxymethyl)-4-hydroxy-phenyl-β-D-glucopyranoside] [**2**], trichocarpin [2-(benzyloxycarbonyl-4-hydroxy)-phenyl-β-D-glucopyranoside] [**3**], and populin [2-(hydroxymethyl)-phenyl-β-D-glucopyranoside-6′-benzoate] [**4**].

These compounds were tested in our chemotherapeutic battery (11). The re-

[1]For the previous part of this series see Van Hoof *et al.* (16).



	R_1	R_2	R_3
1	CH_2OH	H	H
2	$CH_2OCOC_6H_5$	OH	H
3	$COOCH_2C_6H_5$	OH	H
4	CH_2OH	H	COC_6H_5

sults of the antiviral testing of both the extracts and the isolated compounds are shown in Table 1. Salicin and salireposide were found to be active at 25 μg/ml against poliomyelitis virus, whereas the latter was active against Semliki forest virus at 50 μg/ml. Both compounds were inactive against all bacteria and fungi tested at concentrations as high as 500 μg/ml. Trichocarpin was only marginally active against the dermatophytes tested, including *Microsporum canis*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*, and was not active against the other test fungi, bacteria, or viruses. The fungistatic activity of trichocarpinin, the aglycone of trichocarpin, against *Aspergillus niger* (12), could not be confirmed for the glucoside. Populin was inactive against all test microorganisms.

In conclusion, our results have shown salicin and salireposide to be at least partially responsible for the antiviral properties of the 80% EtOH extract of *Populus* cultivar Beaupré leaves. Another or other substances, however, should be responsible for the pronounced anti-*Pseudomonas* and antidermatophyte activities of this extract.

EXPERIMENTAL

PLANT MATERIAL.—The leaves of the poplar tree cultivar Beaupré were harvested in August 1980, in the Rijksstation voor Plantenziekten at Merelbeke, Belgium. The hybridization between species of the sections Tacamahaca and Ageiros was achieved in 1961 at the Rijksstation voor Populierenteelt at Geraardsbergen, Belgium. Voucher specimens have been deposited at the herbarium of the National Botanical Garden of Belgium in Meise, Belgium.

GENERAL EXPERIMENTAL PROCEDURE.—Dccc was carried out on a Model A instrument containing 300 glass columns of 40 cm length and 0.2 mm i.d. (Tokyo Rikakikai, Tokyo, Japan) and equipped with a flow-through microcell Type 6 Optical Unit (250 μl hold up volume) connected to a uv monitor Model UA-5 and a multiplex expander Model 1133, all from Instrumentation Specialities Company (ISCO). The solvent system $CHCl_3$-MeOH-H_2O (7:13:8) was used in an ascending mode, and fractions of 13 ml were collected at a flow rate of 20 ml/h in an ISCO fraction collector Model 328, Golden Retriever. Uv monitoring was carried out at 280 nm,

TABLE 1. Antiviral Activity in vitro of *Populus* Fractions and Phenolic Glucosides **1–4**.

Compound	Virus				
	Coxsackie	Herpes simplex	Measles	Polio	Semliki forest
80% EtOH extract	1	1	1	10^2 (1/4)	10 (1/4)
EtOAc phase	1	1	1	10^3 (1/8)	10^2 (1/8)
Dccc fractions 10–13	1	1	1	10^2 (1/8)	1
Dccc fractions 17–21	1	1	1	10^3 (1/8)	10^3 (1/8)
Dccc fractions 23–26, 44–50	1	1	1	1	1
salicin [**1**]	1	1	1	10^2 (25)	1
salireposide [**2**]	1	1	1	10^3 (25)	10^3 (50)
trichocarpin [**3**]	1	1	1	1	1
populin [**4**]	1	1	1	1	1
3-methylquercetin	10^6 (5)	1	1	10^7 (5)	1

[a]Antiviral activity is expressed as the reduction factor of the viral titer (ratio of viral titer in the absence to that in the presence of the test fraction or compound). The dilution of the extract or the concentration of the test compound (in μg/ml) is given in parentheses. A reduction factor of 1 means no antiviral activity of the undiluted extract or the test compound at a concentration of 500 μg/ml.

whereas tlc monitoring was performed on Si gel plates (10 × 10 cm, F_{254} Merck, Darmstadt, FRG) with $CHCl_3$-MeOH (4:1) as eluting system and uv light at 254 nm and 366 nm and with 20% H_2SO_4 in EtOH as spray reagent, followed by heating during 10 min at 105°. Mp's were determined on a Büchi SMP-20 apparatus and were uncorrected.

Because ms of underivatized phenolic glucosides revealed no structural information using a direct insertion probe (8), we analyzed these compounds by desorption cims using NH_3 as the reagent gas. The following procedure was used: A solution (1 μl) of the product was placed upon a 60-μm tungsten wire by means of a microsyringe. After evaporation of the solvent, the wire was inserted in the plasma of the NH_3 reagent gas. The ion source was operated at a pressure of 0.1–0.2 Torr, and all heating connections to the source were interrupted. Then a current of 70 mA was sent through the wire, gradually rising to 400 mA at a rate of 9 mA/sec. During this heating procedure the quadrupole mass spectrometer was scanned at a peak integration time of 3 msec. As soon as a protonated molecular ion was detected, either on the screen of the computer terminal or on the oscilloscope of the mass spectrometer, the rate of heating of the tungsten wire was set at 1–2 mA/sec. Primary ionization of the NH_3 reagent gas was performed with 70 eV electrons at an emission current of 80 μA. All spectra were run on a Riber 10-10 quadrupole mass spectrometer equipped with a Sidar data system. All glucosides are characterized by $[M + NH_4]^+$ adducts and/or $[M + H]^+$ ions, accompanied by $[BH + H]^+$ and $[BH + NH_4]^+$ fragment ions for salireposide [**2**] and trichocarpin [**3**] and $[MH - H_2O]^+$ ions for populin [**4**] and salicin [**1**] when scanning a mass range from $m/z = 200$ to $m/z = 600$. B represents the aglycone fragment R-O.

ISOLATION AND IDENTIFICATION OF THE CHEMOTHERAPEUTICALLY ACTIVE COMPOUNDS.—Thawed leaves (500 g) were cut into small pieces and macerated in a Waring Blender with 80% EtOH (500 × 5 ml). All EtOH extracts were combined at a temperature not in excess of 40°. The residue was covered with H_2O (500 ml), stirred, heated on a steam bath, and allowed to stand overnight. The aqueous extract was filtered, concentrated to 100 ml under water pump pressure, and extracted seven times with EtOAc (100 ml). The EtOAc layers were combined and concentrated under reduced pressure to dryness. This phase was tested and exhibited the same chemotherapeutic activities as the EtOH extract. The residue was dissolved in equal volumes of the stationary and mobile phases of the abovementioned solvent system (lower and upper layers, respectively), submitted to the dccc columns, and chromatographed as described earlier.

Evaporation of fractions 10–13 and crystallization of the residue from aqueous EtOH afforded 153 mg (0.03%) salicin [**1**]: mp 199°; R_f 0.14 (violet); dcims *m/z* $[M + NH_4]^+$ 304 (100% rel. int.), $[M + H]^+$ 287 (7%) $[MH - H_2O]^+$ 269 (21%). In the same way fractions 17–21 afforded 270 mg (0.05%) salireposide [**2**]: mp 202°; R_f 0.22 (deep yellow); dcims *m/z* $[M + NH_4]^+$ 424 (100%), $[BH + H]^+$ 245 (15%), $[BH + NH_4]^+$ 262 (9%), B being the aglycone R-O. Fractions 23–26 afforded 213 mg (0.06%) trichocarpin [**3**]: mp 135–136°; R_f 0.25 (black); dcims *m/z* $[M + NH_4]^+$ 424 (46%), $[M + H]^+$ 407 (10%), $[BH + H]^+$ 245 (100%), $[BH + NH_4]^+$ 262 (69%). Finally, fractions 44–50 gave 115 mg (0.02%) populin [**4**]: mp 178–180°; R_f 0.34 (brown); dcims *m/z* $[M + NH_4]^+$ 407 (100%), $[M + H]^+$ 391 (53%), $[MH - H_2O]^+$ 373 (56%). Uv and ir data correspond well with literature data (12–14), whereas ^1H- and ^{13}C-nmr data were published elsewhere (10).

CHEMOTHERAPEUTIC EVALUATION.—*Microorganisms.*—The chemotherapeutic battery consisted of the same test bacteria, yeasts, and fungi as described earlier (11). The test viruses poliomyelitis type 1 and measles Edmonston A were plaque-purified, and herpes type 1 virus was isolated in our laboratory. Coxsackie B_2 virus was obtained from NIH, Bethesda, Maryland, and Semliki forest L_{10} was supplied by Dr. C.J. Bradish, Microbiology Research Establishment, Porton Down, Salisbury, UK.

Culture media.—Viruses were grown in HeLa or VERO cells. The tissue culture medium used was that described by Hronovsky supplemented as described previously (15). Viral titers were estimated by the 50% endpoint titration technique (EPTT) as described earlier (16). The viral titers were, 10^7 TCD_{50}/ml for poliomyelitis, Semliki forest, Coxsackie, and herpes viruses and 10^5 TCD_{50}/ml for measles.

Antiviral testing.—The antiviral testing was carried out by means of the previously described 50% endpoint titration technique (16,17). Monolayers of VERO cells in microtiter plates (Nunc, Denmark) were infected with 0.1 ml of serial tenfold dilutions of the virus suspension. The virus was allowed to absorb for 60 min at 37° after which 0.1 ml of serial twofold dilutions of extracts or compounds in tissue culture medium were added. The cultures were incubated at 37° and examined daily for cytopathogenic effect by light microscopy for at least 1 week. Virus control, tissue culture control, and product control were included in the test in order to determine the toxicity of extracts or compounds at each dilution. The antiviral activity of the extract or compound was determined as the reduction factor of the viral titer (ratio of the virus titer in the absence to that in the presence of the extract or com-

pound). 3-Methylquercetin was used as reference for the antipoliomyelitis activity (16).

ACKNOWLEDGMENTS

The authors are grateful to Dr. ir.R. Veldeman, Rijksstation voor Plantenziekten, Merelbeke, Belgium for supplying the plant material and to Prof. I.A. Pearl, Institute of Paper Chemistry, Appleton, Wisconsin, for the gift of authentic samples of phenolic glucosides. The authors are also pleased to acknowledge the Belgian National Fund for Scientific Research (NFWO) for financial support of the work under grant no. 3.003180.

LITERATURE CITED

1. L. Van Hoof, D.A. Vanden Berghe, and A.J. Vlietinck, *Biol. Plant.*, **22** (4), 265 (1980).
2. L. Van Hoof, B. Pycke, F. Mertens, J. Totté, and A.J. Vlietinck, *Planta Med.*, **42**, 127 (1981).
3. I.A. Pearl and S.F. Darling, *Can. J. Chem.*, **49**, 49 (1971).
4. I.A. Pearl and S.F. Darling, *J. Agric. Food Chem.*, **25**, 730 (1977).
5. H. Thieme, *Pharmazie*, **18**, 770 (1963).
6. H. Thieme, *Pharmazie*, **20**, 436 (1965).
7. H. Thieme and R. Benecke, *Pharmazie*, **25**, 780 (1970).
8. I.A. Pearl and S.F. Darling, *Phytochemistry*, **9**, 1277 (1970).
9. B. Meier, R. Julkunen-Tittio, J. Tahvanainen, and O. Sticher, *J. Chromatogr.*, **442**, 175 (1988).
10. R.A. Dommisse, L. Van Hoof, and A.J. Vlietinck, *Phytochemistry*, **25**, 1201 (1986).
11. P.C. Rwangabo, M. Claeys, L. Pieters, J. Corthout, D.A. Vanden Berghe, and A.J. Vlietinck, *J. Nat. Prod.*, **51**, 966 (1988).
12. V. Loeschke and H. Francksen, *Naturwissenschaften*, **51**, 140 (1964).
13. I.A. Pearl and S.F. Darling, *J. Org. Chem.*, **24**, 731 (1959).
14. I.A. Pearl, S.F. Darling, and O. Justman, *J. Org. Chem.*, **27**, 2685 (1962).
15. D.A. Vanden Berghe, M. Ieven, F. Mertens, A.J. Vlietinck, and E. Lammens, *Lloydia*, **41**, 463 (1978).
16. L. Van Hoof, D.A. Vanden Berghe, C.M. Hatfield, and A.J. Vlietinck, *Planta Med.*, 459 (1984).
17. D.A. Vanden Berghe, A.J. Vlietinck, and L. Van Hoof, *Bull. Inst. Pasteur, Paris*, **84**, 101 (1986).

Received 16 December 1988