Isolation of Podophyllotoxin from *Callitrus drummondii*

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Aqueous suspensions and extracts of the dried needles of *Callitrus drummondii* have been shown to be tumor inhibiting against Sarcoma 37 in mice. Hydrolysis of the water extract has given a good yield of podophyllotoxin which is a known tumor-inhibiting substance.

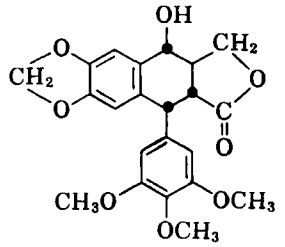
CALLITRUS DRUMMONDII (Parlat) F. Muell is an evergreen tree native to Australia where it is commonly known as Drummond's cypress pine. In a recent survey of conifers for tumor-damaging substances (1), the needles of *C. drummondii* were found to cause necrosis and hemmorrhage in Sarcoma 37 in mice. This study was initiated in an effort to isolate and characterize the principle responsible for this activity.

An initial test indicated that an aqueous suspension of the needles ground to a 100 mesh fineness was active at a level of 100 mg./Kg. against Sarcoma 37 in mice. Extracts of the needles made with ethyl acetate and ethanol were inactive in the same test at levels of 400 mg./Kg. and 750 mg./Kg., respectively. These results indicated that the active principle was highly water soluble, exhibiting a low solubility in nonpolar solvents. A preliminary extraction with water followed by lyophylization produced a residue with an activity of 50 mg./Kg., confirming the belief that the active principle was highly water soluble. Furthermore, the finding of some activity in a xylene defatting solution and the extent depending upon the time of exposure indicated that the active principle could be a glycoside. Partial hydrolysis during processing might well produce a xylene soluble aglycone.

Large-scale defatting and extraction with water followed by lyophylization produced an amorphous hygroscopic material.

Column chromatography of this material was attempted on several adsorbents, silica gel (2) proving to be the most satisfactory. Water eluents became increasingly more active as they became lighter in color. A maximum activity of 2 mg./Kg. was obtained for the light-colored lyophylized residue of the final eluents.

The ultraviolet adsorption of the active fraction showed a high intensity maximum at 290 mμ and a minimum at 260 mμ. The infrared spectrum indicated a very close resemblance to that of podophyllotoxin (I) from an authentic specimen.



(I)

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Hydrolysis of the combined active eluents with emulsin produced glucose, identified as the phenylhydrazone. The aglycone portion was recrystallized from benzene, m.p. 110–113°. This is close to the melting point reported for the hydrated form of podophyllotoxin (I) (3). Drying this hydrate gave a material (m.p. 180–182°) which gave no depression with authentic podophyllotoxin (I). The infrared and ultraviolet spectra were identical.

The finding of podophyllotoxin (I) in a conifer is not unusual. It has been reported in several *Juniperus* species (4, 5). The minimum effective dose for podophyllotoxin (I) against Sarcoma 37 in mice has been reported to be 2 mg./Kg. (4). This activity is consistent with the finding of the activity of the chromatographed fractions of *C. drummondii*. Although this fact does not preclude the possibility that activity may also be due to some other constituent present, it cannot be doubted that podophyllotoxin (I) is certainly largely responsible for the antitumor activity of *C. drummondii*.

EXPERIMENTAL

Plant Material.—The needles were collected in the fall of 1960 near Ravensthorpe, Western Australia, and dried prior to shipment. They were identified as *C. drummondii* (Parlat) F. Muell by T. C. Dunne, Director of Agriculture, Western Australia.

Preliminary Studies.—A quantity of needles were ground to 100 mesh fineness and subjected to antitumor screening.[1] The minimum effective dose of an aqueous suspension was found to be 100 mg./Kg. against Sarcoma 37 in mice. A second portion of ground needles was extracted with water, lyophylized, and the residue subjected to the same test. The M.E.D. was found to be 50 mg./Kg. Extracts of the ground needles with ethanol and ethyl acetate gave no activity at levels of 750 and 400 mg./Kg., respectively.

Extraction.—One-hundred grams of the ground needles were stirred for 5 minutes in 500 ml. of xylene then filtered and dried. The dried marc was vigorously stirred successively in four 1-L. portions of water. The combined aqueous filtrates were lyophylized to a light brown hygroscopic residue weighing 20 Gm.

Isolation of Podophyllotoxin (I) Glycoside.—The lyophylized residue, about 20 Gm., was placed on 1500 Gm. of hydrated silica gel. The column was eluted with water. Fractions were taken according to the intensity of color. The first fractions were dark in color and showed little tumor-damaging activity. As the eluents became lighter in color, the activity rose. The lyophylized residue from the final cuts yielded a light, fluffy, slightly yellow material having a M.E.D. of about 2 mg./Kg.

[1] All antitumor testing was conducted under the auspices of the Cancer Chemotherapy Section, National Institutes of Health, Bethesda, Md.

The ultraviolet absorption of the more active fractions showed a high intensity maximum at 290 mμ and a minimum at 260 mμ. The infrared absorption showed a broad hydroxyl band (3450 cm.$^{-1}$), a lactone (1740 cm.$^{-1}$), and an overall resemblance to a spectrum of podophyllotoxin (I).

Isolation of Podophyllotoxin(I).—The active lyophylized fractions were dissolved in water at pH 5, treated with half their weight of emulsin and allowed to stand 24 hours at 37°. The solutions were then extracted with chloroform in a continuous extractor. The chloroform was concentrated to dryness and the residue recrystallized from benzene, m.p. 110–113°. This melting point is in fair agreement with the value reported for podophyllotoxin (I) benzene hydrate (6, 7). The material was dried for 24 hours *in vacuo* at 100°, m.p. 180–182°. A mixed melting point with authentic podophyllotoxin (I) gave no depression and the infrared and ultraviolet spectra were identical.

Anal.—Calcd. for $C_{22}H_{22}O_8$: C, 63.75; H, 5.35; OCH_3, 22.47. Found: C, 63.88; H, 5.62; OCH_3, 22.18.

The yield of podophyllotoxin (I) from the dried needles based on spectrophotometric analysis was about 1.4 per cent.

The water solution from the chloroform extraction was treated with phenylhydrazone hydrochloride and sodium acetate. The resulting crystals of the phenylhydrazone gave no depression with the corresponding glucose derivative.

REFERENCES

(1) Fitzgerald, D. B., Hartwell, J. L., and Leiter, J., *J. Nat. Cancer Inst.*, **18**, 83(1957).
(2) Von Wartburg, A., Angliker, E., and Renz, J., *Helv. Chim. Acta*, **40**, 1331(1957).
(3) Hartwell, J. L., and Schrecker, A. W., *J. Am. Chem. Soc.*, **73**, 2909(1951).
(4) Hartwell, J. L., Johnson, J. M., Fitzgerald, D. B., and Belkin, M., *ibid.*, **75**, 235(1953).
(5) *Ibid.*, **75**, 2138(1953).
(6) Kofod, H., and Jorgensen, C., *Acta Chem. Scand.*, **9**, 347(1955).
(7) Schrecker, A. W., Hartwell, J. L., and Alford, W. C., *J. Org. Chem.*, **21**, 288(1956).

Synthesis and Antifungal Activity of Anilides of Salicylic Acid and *o*-Coumaric Acid

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A series of *o*-hydroxycinnamanilides and the corresponding series of salicylanilide derivatives were prepared and tested for antifungal activity. These compounds were investigated for the purpose of determining the effect of vinylogy and also the effect of substitution on the anilide rings. The results showed that all of the *o*-hydroxycinnamanilide derivatives had less antifungal activity than did their corresponding salicylanilide derivatives. Most of the compounds had some antifungal activity at the tested concentration of 0.5%; however, only two compounds had activity greater than salicylanilide. These compounds were the 3′-chloro- and the 4′-chloro-salicylanilides.

ALTHOUGH a large number of salicylanilide derivatives have been investigated for antifungal activity (1–10), the structural modifications have been generally limited to substitution on one or both of the aromatic rings. It was of interest to note that none of the reported investigations have been concerned with the vinylog derivatives, which are represented by the anilides of *o*-coumaric acid (*o*-hydroxycinnamanilides). Because of the principle of vinylogy (11), it appeared that such derivatives might possess activity similar to that of the related salicylanilide derivatives.

In this investigation a series of *o*-hydroxycinnamanilide derivatives and the corresponding series of salicylanilide derivatives were prepared and tested for antifungal activity. The compounds consisted of the free acids, the unsubstituted anilides, the 2′-, 3′-, and 4′-chloro-anilides, the 2′-, 3′-, and 4′-nitro-anilides and the 2′-, 3′-, and 4′-methyl-anilides. These derivatives not only provided the possibility of determining the effect of vinylogy but also the effect of substitution in varying positions on the anilide rings.

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EXPERIMENTAL

Synthesis of *o*-Hydroxycinnamanilides

***o*-Coumaric Acid.**—From 32.1 Gm. (0.22 mole) of coumarin treated with 400 ml. of 8% sodium hydroxide and 4 Gm. of yellow mercuric oxide according to the procedure of Seshadri and Rao (12), there was obtained 27.5 Gm. (76%) of coumaric acid having a m.p. of 208°. This material was used in the following reaction.

***o*-Acetoxycinnamic Acid (13).**—A mixture of 27.5 Gm. (0.17 mole) of *o*-coumaric acid, 7.0 Gm. (0.85 mole) of sodium acetate and 90 ml. (0.95 mole) of acetic anhydride was heated on a steam bath for 7 hours. After cooling to room temperature, the reaction mixture was poured into 1 liter of ice and water and allowed to stand overnight. The white crystalline material was separated and washed with cold water and dried in air to give 33.0 Gm. of product (94% yield). A sample after recrystallization from benzene gave m.p. 153–154°.

***o*-Acetoxycinnamoyl Chloride (14).**—A mixture of 10.3 Gm. (0.05 mole) of *o*-acetoxycinnamic acid, 11.8 Gm. (0.1 mole) of thionyl chloride and 15 ml. benzene was heated under reflux for $^1/_2$ hour and cooled to room temperature. The solvent and excess thionyl chloride was removed *in vacuo* with gentle heating. Upon standing, a white crystalline material resulted.

4′-Methyl-2-Hydroxycinnamanilide.—In a typical example, the above *o*-acetoxycinnamoyl chloride