Partial Characterization of Antitumor Tannin Isolated from *Calycogonium squamulosum* (Melastomataceae)

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Abstract □ The active antitumor tannin from *Calycogonium squamulosum* was isolated and determined to be a tri-*O*-galloyl-D-glucose.

Keyphrases □ Tannins, antitumor—isolated from *Calycogonium squamulosum*, identified as a tri-*O*-galloyl-D-glucose □ *Calycogonium squamulosum*—isolation of antitumor tannin, partial characterization as a tri-*O*-galloyl-D-glucose □ Tri-*O*-galloyl-D-glucose—isolated from *Calycogonium squamulosum*, determined to be the antitumor agent □ Antitumor tannins—isolated from *Calycogonium squamulosum*, characterized as a tri-*O*-galloyl-D-glucose

Previously it was reported that the antitumor activity elicited by extracts of *Calycogonium squamulosum* against the Walker 256 (IM) carcinosarcoma could be attributed to the tannin present in the plant (1). Hartwell and Abbott (2) pointed out that, in their experience, all antitumor tannins have been shown either to be highly toxic or to possess a low therapeutic index. Thus, for the most part, work on tannin antitumor agents has not progressed in the programs sponsored by the Drug Research and Development Branch of the National Cancer Institute.

To understand better the mechanism of tannin antitumor activity and the toxicity of this class of compounds, the structures of the active entities should be elaborated. To date, the only antitumor tannin studied from a structure point-of-view has been that derived from *Rumex hymenosepalus*, which was shown to be a polymeric leucoanthocyanidin (3).

The purpose of the present investigation was to characterize the antitumor tannin isolated from *C. squamulosum*.

EXPERIMENTAL

Plant Material—The aerial parts of *C. squamulosum* (Melastomataceae) were used in this study[1].

Isolation of Tannin—A sample of milled plant material was fractionated as previously reported (1), and the tannin was isolated according to the method of Wall *et al.* (4).

Antitumor Activity of Tannin—A sample of the isolated tannin gave a T/C of 32% at 100 mg./kg. against the Walker 256 (IM) carcinosarcoma using established protocols (5). A T/C of ≤42% is considered active in this system.

Hydrolysis of Tannin Fraction—A sample (250 mg.) of the tannin was dissolved in 25 ml. of aqueous sulfuric acid (5% v/v), forming a clear, amber solution, which was heated under reflux for 18 hr. After cooling to room temperature, the mixture was filtered to yield a clear, light-yellow solution. This solution showed the properties of a hydrolyzable tannin, since phlobaphenes, which form under these conditions with condensed tannins, were not apparent (6). The acidic hydrolysate was made neutral to litmus paper with ammonium hydroxide and diluted to 200 ml. with 95% ethanol to precipitate the ammonium sulfate formed during the neutralization procedure. The precipitate was removed by filtration and washed with hot ethanol, and the filtrate was heated *in vacuo* at 50° to remove the ethanol. The residue was then frozen and lyophilized.

TLC of Tannin Fraction—Samples of unhydrolyzed tannin, hydrolyzed tannin, gallic acid, pyrogallol, and ferulic acid were subjected to TLC analysis on replicate silica gel G plates, using a solvent mixture of chloroform–ethyl acetate–formic acid (5:4:1) for phenols and *n*-butanol–acetic acid–water (4:1:1) for sugars. The chromatograms were sprayed with 1% $FeCl_3$ in methanol to reveal the phenolic components and with *p*-anisidine to reveal the sugars.

GLC of Hydrolyzed Tannin—A sample (23.6 mg.) of the tannin was hydrolyzed as previously described and the hydrolysate was dissolved in 3.0 ml. of anhydrous, freshly distilled pyridine, followed by the addition of 0.60 ml. of hexamethyldisilazane and 0.30 ml. of trimethylchlorosilane. The mixture was shaken for 30 sec., followed by centrifugation. The supernate was taken for analysis. A 1.0-μl. (2.94 mcg. of hydrolyzed tannin) sample of the supernate was injected into the gas chromatograph[2], which was equipped with a U-shaped glass column, 0.64 cm. o.d. × 1.83 m. (0.25 in. o.d. × 6 ft.), containing 3% SE-30 on Chromosorb W (80–100 mesh) as the stationary phase. The injection port was maintained at 250°, and the column temperature was programmed to hold at 85° for 5 min. after injection of the sample and then to give a linear rise in temperature of 10°/min. up to 260°. Nitrogen was used as the mobile phase at a flow rate of 60 ml./min. (25 p.s.i.), and the effluent gases were monitored by a hydrogen flame-ionization detector operating at 200°.

In addition to the tannin hydrolysate, standard solutions of gallic acid and glucose were similarly prepared for GLC determination.

DISCUSSION

Hydrolysis of the antitumor tannin from *C. squamulosum*, followed by TLC examination, revealed the presence of gallic acid and glucose. Subsequent GLC examination of the hydrolysate, in comparison with standard solutions of gallic acid and glucose, revealed that a ratio of 3 moles of gallic acid was present for each mole of D-glucose. This suggests a partial characterization of the tannin present in *C. squamulosum* to be a tri-*O*-galloyl-D-glucose. The complete structure of this tannin will be the subject of a future investigation.

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[1] The plant material was collected at Pico del Oeste, Puerto Rico, under the supervision of Dr. R. A. Howard, Arnold Arboretum, Harvard University. A voucher specimen of the plant material collected was identified by Dr. Howard and deposited at the Herbarium of the Arnold Arboretum.

[2] Biomedical gas chromatograph, model 400, F & M Scientific Corp., Avondale, Pa.

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Synthesis of Site-Directed Chelating Agents I: Pteridine Carboxaldehyde Thiosemicarbazones

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Abstract □ Several 6-formylpteridine thiosemicarbazones and semicarbazones were synthesized as potential inhibitors of the enzyme dihydrofolate reductase. The most active derivative in this series was 2,4-diaminopteridine-6-carboxaldehyde semicarbazone, which caused 50% inhibition of dihydrofolate reductase of human liver at a concentration of 1.4×10^{-7} *M*.

Keyphrases □ Pteridine carboxaldehyde thiosemicarbazones—synthesized and tested as site-directed chelating agents of dihydrofolate reductase □ 6-Formylpteridine thiosemicarbazones and semicarbazones—synthesized and tested as site-directed chelating agents of dihydrofolate reductase □ Dihydrofolate reductase—synthesis and testing of pteridine carboxaldehyde thiosemicarbazones as potential inhibitors

Thiosemicarbazones of heterocyclic carboxaldehydes with the formyl group alpha to a heteroaromatic ring nitrogen have been shown to possess both antineoplastic and antiviral activities (1–5); several different heteroaromatic ring systems are active carcinostatic agents with such potency correlating with the inhibition of the synthesis of DNA (6–8). The site of the metabolic lesion on the DNA biosynthetic pathways, as elucidated with 1-formylisoquinoline thiosemicarbazone and 2-formylpyridine thiosemicarbazone, two members of the most potent heterocyclic ring systems, is at the level of the conversion of ribonucleotides to deoxyribonucleotides (9–11). A study of the mechanism of action of 1-formylisoquinoline thiosemicarbazone and 2-formylpyridine thiosemicarbazone indicated that they inhibit the enzyme ribonucleoside diphosphate reductase either by binding to the target enzyme through chelation of iron in a metal-containing form of the enzyme or by initially forming an iron chelate of the inhibitors which interacts with the enzyme (9, 11).

In an effort to orient the chelating potential of the formyl thiosemicarbazone side chain positioned alpha to a heteroaromatic ring nitrogen atom to a vulnerable enzymatic site of neoplastic cells other than ribonucleoside diphosphate reductase, several pteridine thiosemicarbazones were synthesized. The pteridine nucleus was selected to direct the formyl thiosemicarbazone portion of the molecule to the enzyme dihydrofolate reductase. Although dihydrofolate reductase has not been shown to be a metal-containing enzyme, Hakala and Suolinna (12) showed that the enzyme from neoplastic cells is inactivated by the metal-chelating agents *o*-phenanthroline and ethylenediaminetetraacetate.

EXPERIMENTAL

Enzymatic Studies—Dihydrofolate reductase was prepared to homogeneity from human liver[1]. Enzymatic activity was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm. which results from the conversion of NADPH and dihydrofolate to $NADP^+$ and tetrahydrofolate (13).

2,4-Diamino-6-formylpteridine Semicarbazone—2,4-Diaminopteridine-6-carboxaldehyde (0.19 g.) was dissolved in 50 ml. of water containing 1 ml. of concentrated hydrochloric acid. Semicarbazide (0.1 g.) was dissolved separately in 10 ml. of water, and the two solutions were mixed and warmed for 15 min. Sodium acetate (1 g.) was added and the mixture was stirred. The resulting compound was filtered, washed with hot water and ethanol, and dried. The compound did not melt but slowly decomposed above 300°.

For analytical purposes, the compound was isolated as its hydrochloride salt.

Anal.—Calc. for $C_8H_9N_9O \cdot HCl$: C, 33.86; H, 3.17; N, 44.44. Found: C, 33.36; H, 3.12; N, 43.95.

Thiosemicarbazone—The thiosemicarbazone derivative of 2,4-diaminopteridine-6-carboxaldehyde was synthesized as described above except that thiosemicarbazide replaced semicarbazide and was analyzed as its hydrochloride. The compound did not melt but slowly decomposed above 300°.

Anal.—Calc. for $C_8H_9N_9S \cdot HCl$: C, 32.05; H, 3.34; N, 42.07; S, 10.68. Found: C, 31.65; H, 3.30; N, 41.35; S, 10.49.

2-Amino-4-hydroxy-6-formylpteridine Semicarbazone—2-Amino-4-hydroxypteridine-6-carboxaldehyde (0.19 g.) was dissolved in 5 ml. of water by the addition of the minimum amount of 1 *N* sodium

[1] Performed by Dr. David R. Makulu and Dr. Joseph R. Bertino, who donated the enzyme for these studies.