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CLOVER CONSTITUENTS

Isolation of Daphnoretin from Ladino Clover

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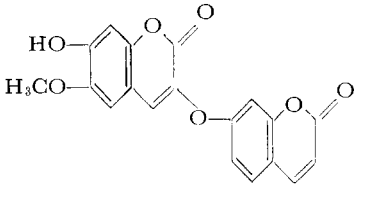
The isolation of the novel coumarin, daphnoretin, from ladino clover is described. The clover extract was purified by solvent distribution followed by column chromatography. Identity was confirmed by the preparation of derivatives and by comparison of their mixed chromatograms, melting points, and ultraviolet absorption spectra with those of authentic samples.

IN THE COURSE of isolating saponin from ladino clover (*8*), an ether extract was prepared from which a crystalline monomethoxyphenol, $C_{19}H_{12}O_7$, has now been isolated. The formation of monoacyl and monoalkyl derivatives indicated the presence of only one phenolic hydroxyl group. The fluorescence of the compound (blue in ultraviolet light) and its λ_{max} (343 mμ) suggested a coumarin structure. The possible presence of two lactone groupings, as in dicoumarol, was indicated by an unusually wide band at 1710 cm.$^{-1}$ in the infrared spectrum of the phenol.

While this investigation was in progress, Tschesche *et al.* (*7*) reported the isolation of a novel dicoumarin from daphne, daphnoretin(I).



The ultraviolet spectra of daphnoretin and its acetate were identical with that of the ladino clover phenol (Figure 1), and the melting points of the phenols and their derivatives were in close agreement. Direct chromatographic, mixed melting point, and infrared comparisons of the two phenols confirmed their identities.

The final step in the biosynthesis of this compound may well involve the formation of the dicoumarin structure by means of the oxygen bridge. It is of considerable interest that two plants of such widely different species should contain this unusual compound.

Most coumarins have been shown to have a number of physiological effects on animals. Dicoumarol was found to be the hemorrhagic factor in sweet clover by Campbell and Link (*4*). Bose and Sen (*3*) found that ayapin and ayapinin were the hemostatic principles in the leaves of *Enpatorium ayapana*, and Iida (*5*) found that fraxin caused par-

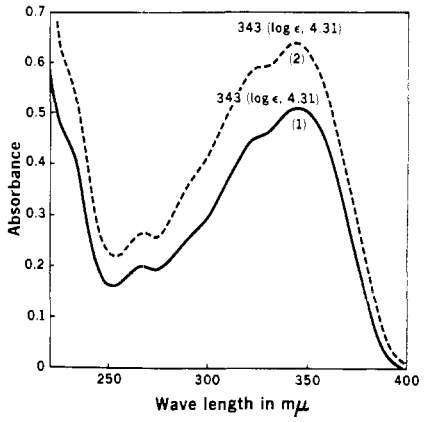


Figure 1. Ultraviolet absorption spectra of ladino phenol "B" (1) and daphnoretin (2) in ethanol

alysis of the central nervous system on intravenous injection. Coumestrol, the estrogenlike coumarin derivative isolated from ladino clover at this laboratory (*1*) has been found to increase the weight gain of sheep (*6*). Daphnoretin, a novel coumarin, may also have important physiological properties. The authors plan to prepare a sufficient quantity of this new coumarin to evaluate its possible significance to crop utilization, animal feeding, or nutrition.

Experimental

Isolation. Freshly cut ladino clover (150 kg.) was extracted with 360 liters of 95% ethanol. The alcohol solution was concentrated and distributed between ether and water. From the aqueous phase crystalline saponin was isolated. Details of this procedure have been previously described (*8*). The ether phase from the saponin study was the starting material for the present investigation.

The ether solution was concentrated to yield a green waxy solid (5100 grams). The solid was dissolved in 64 liters of 95% ethanol. Aliquots of this alcohol solution were then treated as follows: 600 ml. of alcohol solution, 400 ml. of water, and 200 ml. of 70° C. benzene were shaken in a 2-liter separatory funnel. The two layers were allowed to separate and the alcohol phases drawn off. No further work was done on the combined benzene phases. The combined alcohol phases were concentrated to 19 liters and extracted in 1-liter portions with equal volumes of diethyl ether. Sufficient alcohol was added to each portion to break the emulsion. The ether phases were drawn off, and the aqueous phases were re-extracted in the same manner seven times. Concentration of the combined ether phases gave about 500 grams of solids. These solids were purified a second time by distribution with alcohol, water, and benzene using the same ratios of solids to solvent as above. The benzene phases were added to the benzene phases from the first distribution. The combined alcohol phases were concentrated to 2 liters and extracted with eight 500-ml. portions of diethyl ether. The ether extracts were combined and concentrated to dryness. The residue was added to 100 grams of silica gel–celite (1:1). The dry blended mixture was added to the top of a column of silica gel 8 inches in height by $1^1/_2$ inches in diameter. The column was developed first with 10 liters of Skellysolve B, followed by 2 liters of a mixture of diethyl ether and Skellysolve B (1:9). The Skellysolve B fraction was discarded. The diethyl ether: Skellysolve B fraction was concentrated to 100 ml. Upon standing, yellow crystals formed. Recrystallization from methanol yielded light yellow rods (1.2 grams), m.p. 244° C., showing no depression with an authentic sample of daphnoretin. Calculated for $C_{19}H_{12}O_7$: C, 64.77; H, 3.41; OCH_3, 8.81. Found: C, 64.9, 65.0; H, 3.58, 3.61; OCH_3, 8.62, 8.53.

Ultraviolet. $\lambda_{max.}$ 343 mμ (log ε, 4.31), identical with an authentic sample.

Infrared. 3340 (hydroxyl); 1710 (δ-lactone); 615, 1520, 1450, 1420, 1380 (aromatic ring); 1270 $cm.^{-1}$ (ether linkage).

Color Tests. Alcoholic ferric chloride—light green; nitric acid—red; viewed under ultraviolet light plus ammonia vapor—bright blue; ferric chloride plus hydroxylamine—purple; magnesium plus hydrochloric acid—no color.

Titration with Alkali. Saponification of 50 mg. of the parent compound by refluxing with 0.1*N* potassium hydroxide resulted in the consumption of two equivalents of alkali, and gave an equivalent weight of approximately 150.

Alkaline Fusion. A few milligrams of the crystals were fused with about 200 mg. of potassium hydroxide and one drop of water. After acidification and extraction with diethyl ether, aliquots were spotted on sheets of Whatman No. 1 paper with and without known compounds. Development by a previously described procedure (*2*) revealed only a single spot corresponding to 2,4-dihydroxybenzoic acid. The failure to see a spot corresponding to 2,4-dihydroxy-5-methoxybenzoic acid on the chromatograms is probably due to strong alkaline fusion conditions used.

Monoacetate. The acetate derivative at this laboratory melted at 235°–36° C. [lit. m.p. 235°–37° C. (*7*)].

Calculated for $C_{21}H_{14}O_8$: C, 63.96; H, 3.58; OCH_3, 7.86; CH_3CO, 10.9. Found: C, 63.9; H, 3.69; OCH_3, 7.20; CH_3CO, 11.2.

Methyl Ether. The methyl ether derivative prepared at this laboratory melted at 230°–32° C. [lit. m.p. 231°–33° C. (*7*)].

Calculated for $C_{20}H_{14}O_7$: C, 65.57; H, 3.85; OCH_3, 16.9. Found: C, 65.4; H, 3.98; OCH_3, 16.7.

Benzoate. The parent compound, 50 mg., was refluxed for 10 minutes with 2 ml. of pyridine and 1 ml. of benzoyl chloride. The mixture was left at room temperature for $2^1/_2$ hours, then poured into ice water. The solids were collected and crystallized from acetone-methanol to give 42 mg. of crystals, m.p. 224°–25° C.

Calculated for $C_{26}H_{16}O_8$: C, 68.4; H, 3.51; OCH_3, 6.80. Found: C, 68.4; H, 3.61; OCH_3, 6.63.

Benzyl Ether. Fifty milligrams of the parent compound, 1 ml. of benzyl chloride, 70 mg. of potassium iodide, and 250 mg. of potassium carbonate were refluxed for $5^1/_2$ hours. The mixture was filtered and the filtrate concentrated and diluted with methanol. The benzyl ether crystallized as needles, 60 mg., m.p. 241°–43° C. Calculated for $C_{26}H_{18}O_7$: C, 70.6; H, 4.07; OCH_3, 7.01. Found: C, 70.6; H, 4.17; OCH_3, 6.84.

Attempt to Prepare *Ortho*-Methoxy Methyl Ester Derivative. A previously described procedure (*2*) using dimethyl sulfate and 10% potassium hydroxide failed to produce the *ortho*-methoxy methyl ester. The reaction was followed on a chromatoplate. The starting compound seemed to form several products which proved difficult to crystallize.

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