and Shibamoto, 1980; Sekizawa and Shibamoto, 1980).

The presence of NTHZ in fried bacon is not altogether unexpected. Thiazolidine, the parent amine, has not been reported in foods; however, it has been obtained by a cysteamine–D-glucose–water Browning model system reaction (Sakaguchi and Shibamoto, 1978). The expected precursors for thiazolidine formation, cysteamine, and formaldehyde should be present in bacon. Cysteamine can result from the decarboxylation of cysteine and formaldehyde by the fragmentation of endogenous or added sugar and to a lesser extent the oxidation of pork lipids, both of which are known to produce carbonyl compounds. Nitrite used for curing could serve as the nitrosating precursor for NTHZ formation. The presence of structurally related *N*-nitroso-5-methyl-1,3-oxazolidine has been reported in one brand of cooling, cutting, and hydraulic fluid (Stephany et al., 1978). The nitrosamine was presumably formed by a similar reaction to that of NTHZ, in which 1-amino-2-propanol instead of cysteamine reacted with formaldehyde and nitrite.

In conclusion, we have evidence that the NTHZ is present in fried bacon, a product intentionally browned for consumption. However, most of the nitrosamine found in the mineral oil distillation method was artifactually formed when residual nitrite was present in the sample prior to analysis.

ACKNOWLEDGMENT

We thank E. L. Greenfield and T. Phillippo (USDA, FSIS) for fried bacon samples and the National Cancer Institute for the loan of a thermal energy analyzer under Contract NO1 CP 55715.

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Received for review September 28, 1981. Accepted April 2, 1982. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Phytotoxic Compounds from *Melilotus alba* (White Sweet Clover) and Isolation and Identification of Two New Flavonoids

Gilles F. Nicollier[1] and A. C. Thompson*

Coumarin derivatives and flavonoids from a hot MeOH extract of white sweet clover flowers (*Melilotus alba*) inhibited the growth of tomato and radish seedling roots. Studies indicate that ortho-substituted hydroxyphenyl aliphatic acids caused greater root growth inhibition than the corresponding para-substituted isomers and that the root growth inhibition increased as the length of the aliphatic chain increased. Blockage of the *o*-hydroxyl group eliminates the phytotoxic properties. Two new flavonoids were isolated: kaempferol 3-*O*-galactosyl-(1→6)-glucoside 7-*O*-rhamnorhamnoside (melitin) and quercetin 3-rhamnosyl-(1→6)-galactoside 7-*O*-rhamnoside (clovin). These compounds were characterized from MS, UV, ^{1}H NMR, and ^{13}C NMR spectra along with enzymatic hydrolysis. These flavonoids showed little phytotoxicity.

Previous chemical studies on *Melilotus alba* (white sweet clover) have dealt with coumarins and sugar derivatives of coumarins. Haskins and Gorz (1959) and Kosuge (1961) found that bound coumarin was present as coumarinic acid β-glucoside. Haskins and Gorz (1961) observed that the conversion of *trans*-coumarins to the cis form was photochemical rather than enzymatic. Huisman and Kosuge (1970) showed that coumarin and related compounds occur primarily as β-D-glucosides. He further demonstrated that in the reduction of *o*-(β-D-glucosyl)cinnamic acid to the corresponding hydrocinnamic acid the glucose remains attached, indicating free coumarin was not an important intermediate. Blaim and Preszlakowska (1969) analyzed the seed of sweet clover for glycosides and found evidence of a saponin. Torck et al. (1971) found robinin and assumed the presence of quercetin 3-rhamnogalactoside from sweet clover flowers. They tested these flavonoids against 14 bacteria and found the kaempferol showed the greatest antibacterial activity.

The phytotoxic properties of the water extract of sweet clover have been tested on the germination and growth of corn by McCalla and Duley (1948), who found variations

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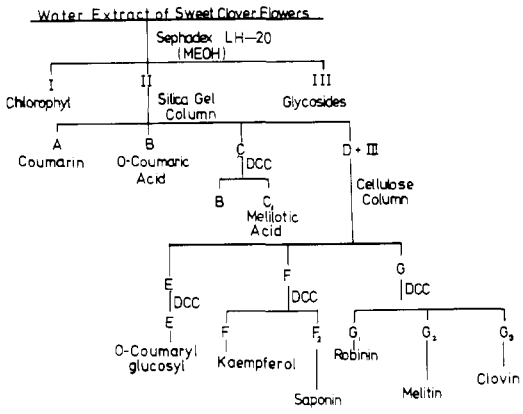

Figure 1. Schematic for the extraction of *M. alba* flowers.

in activity among the coumarins with respect to concentration. San Antonio (1952) found coumarin and its derivatives inhibitory to sweet clover growth.

In a survey of plants with potential allelopathic activity (unpublished experiments) both methanol and water extracts of white sweet clover showed strong growth inhibition of radish and tomato seedlings. In this article, some known chemical constituents of white sweet clover and their growth inhibition potential are described as well as the isolation and identification of two new flavonoids.

MATERIALS AND METHODS

Extraction of *M. alba*. Fresh plant material was collected and identified by the Botanical Institute of Mississippi State University. Fresh roots, leaves, and flowers were separated and finely ground in a blender with water and filtered, and the residue was suspended in methanol and heated at reflux for 2 h.

Fractionation of the Water Extract of the Flower. The water extract of flowers (600 g of fresh material) was freeze-dried, taken up in 20 mL/g MeOH, and fractionated by chromatography on a Sephadex LH-20 column (l = 40 cm; i.d. = 2.5 cm; solvent = MeOH) according to the scheme given in Figure 1. Three fractions were collected and bioassayed. Fraction 2, containing most of the activity, was chromatographed on a silica gel column (l = 40 cm; i.d. = 6 cm) with an elution gradient of benzene, benzene–EtOAc (1:1 v/v), and EtOAc–MeOH (1:1 v/v). Four fractions were collected.

Fraction A (100 mg) gave a white solid identified as coumarin by comparing the physical and chemical properties to those of the known compound. Fraction B (40 mg) was identified as *o*-coumaric acid by comparison of the TLC and UV properties with those of an authentic sample. Fraction C (15 mg) was separated by DCC (Droplet CounterCurrent apparatus, Tokyo Rikakikai, Nishikawa, Toyama-cho, Kanda, Chiyoda-ku, Tokyo, Japan) with the upper phase of $CHCl_3$–MeOH–H_2O (65:35:20 v/v/v) as the stationary phase and the lower phase as the moving phase to give *o*-coumaric acid (10 mg) and metilotic acid (*o*-hydroxyhydrocinnamic acid) (5 mg).

Fraction D (50 mg) containing the glycosides was combined with fraction 3 from the Sephadex LH-20 column and chromatographed on a cellulose column (l = 45 cm; i.d. = 6 cm) with the upper phase of the solvent mixture BuOH–AcOH–H_2O (4:1:5 v/v/v). Three fractions were collected from the cellulose column, and each separately was chromatographed by DCC with the lower phase of a mixture of $CHCl_3$–MeOH–H_2O–PrOH (5:6:4:1 v/v/v/v) as the moving phase and the upper phase as the stationary phase (72 DCC tubes). Silica gel TLC of the fraction obtained from the lower phase of the DCC system gave six subfractions, E_1, F_1, F_2, G_1, G_2, and G_3.

Hydrolysis and Sugar Analysis. The flavonoid glycosides were refluxed with 2 N HCl for 2 h, cooled, neutralized with $NaHCO_3$, taken to dryness, dissolved in pyridine, and TLC was performed on cellulose in the solvent system BuOH–Pyr–H_2O (9:5:4 v/v/v). The sugars were identified by comparing their R_f values to those of authentic sugars and quantitated by GLC of the aldononitrile acetates on a stainless steel column (1.8 m × 3.2 mm) packed with 3% OV-225 and 2.5% high-efficiency 9BP on Supelcoport (80–100 mesh) at 210 °C (Chen and McGinnis, 1981).

(*o*-Hydroxyphenyl)butyric Acid. Two grams of 4-(*o*-aminophenyl)butyric acid hydrochloride was converted to the 4-(*o*-hydroxyphenyl)butyric acid by diazotization with $NaNO_2$ followed by treatment with sulfuric acid by the method of Manske (1963) for the synthesis of *m*-nitrophenol from *m*-nitroaniline. At the end of the reaction, the solution was extracted with 200 mL of ether, the ether was evaporated, and the mixture was chromatographed on a silica gel column (l = 20 cm; i.d. = 1.5 cm) in the solvent system benzene–EtOAc (1:1 v/v). The product which ran with the solvent front was recrystalized from EtOH to give 70% yield: mp 61 °C; MS m/e (rel intensity) 180 (18, M^+), 162 (20), 120 (20), 117 (30), 107 (100), 91 (28), 79 (24), 77 (60), 65 (10).

Bioassay. Each fraction to be tested (2.5 mg, 100 ppm) was dissolved or dispersed in 25 mL of distilled water. Fifteen 10 × 10 cm squares of germination paper (Anchor Paper Co., St. Paul, MN) were soaked in the test solutions of the isolated compounds, and three of the 10 × 10 cm paper squares were placed in each of five 10 × 10 cm plastic disposable Petri dishes. Twelve radish seeds (*Raphanus sativum* L.) or tomato (*Lycopersicon asculentum*) seeds were placed in a 3 × 4 array on the paper in each dish. The dishes were incubated at 20 °C in a growth chamber for 96 h in the dark. At the end of the test, the dishes were removed and frozen immediately. The roots were measured to the nearest millimeter and the mean length and standard error of the mean determined. Each compound was assayed at 2, 25, 50, 75, and 100 ppm. Regression analysis of root growth vs. concentration showed the best fit to be a straight line.

RESULTS AND DISCUSSION

Identification. The structures of the flavonoids isolated and identified from white sweet clover are shown in Figure 2. Fractions A, B, and C (Figure 1) were identified as coumarin, coumaric acid, and melilotic acid, respectively, by comparing their UV, IR, and MS spectra with those of standard compounds.

Fraction E_1 (10 mg, mp 240 °C) when hydrolyzed gave *o*-coumaric acid and glucose. Fraction E_1 is therefore *o*-coumaryl glucoside.

Fraction F_1 (30 mg, mp 276 °C) had UV, MS, and ^1H NMR spectra identical to those of kaempferol. Fraction F_2 (200 mg, mp 296 °C) when hydrolyzed with 6 N HCl gave an unidentified triterpenoid (mp 250 °C). Neutralization of the acid hydrolysate followed by cellulose TLC gave rhamnose and glucose.

Fraction G_1 (50 mg, mp 250 °C) was identified as robinin after comparing the UV and ^1H NMR spectra of the Me_3Si derivative to those given for robinin by Mabry et al. (1970) and the ^{13}C NMR given for robinin by Wenkert and Gottlieb (1977).

Fraction G_2 (120 mg, mp 183 °C dec) had the following UV spectra: (MeOH) 349, 321 sh, 302 sh, and 266; ($AlCl_3$) 399, 353, 302 sh, and 275; ($AlCl_3$ plus HCl) 399, 347, 301 sh, 275, and 256 sh; (NaOMe) 401, 359, and 277; (NaOAc) 349, 323 sh, and 266. The shifts observed for G_2 with $AlCl_3$

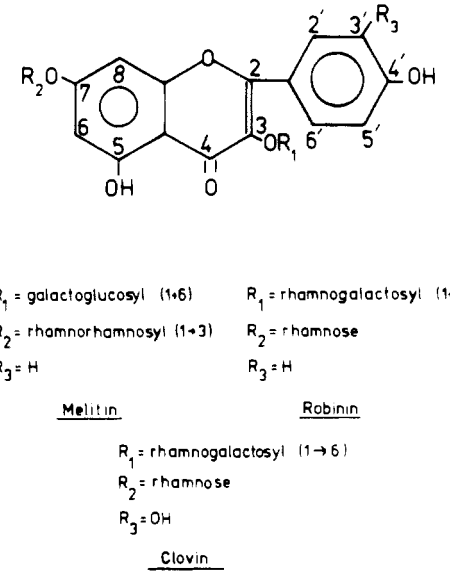

Figure 2. Chemical structures of compounds isolated from *M. alba* flowers.

and $AlCl_3$ plus HCl are identical with those of robinin (Mabry et al., 1970). However, in G_2 a bathochromic shift of 10 nm was observed in both bands I and II with NaOMe and a hypsochromic shift of 10 nm was observed in band I with NaOAc. These shifts are indicative of a free 4′-hydroxyl group. The difference in shifts with NaOMe and NaOAc for G_2 and robinin (G_1) is attributed to different sugars at the 3 and 7 positions. Hydrolysis of G_1 and G_2 gave kaempferol as demonstrated by identical UV shifts as those of authentic kaempferol. Cellulose TLC of the neutralized hydrolysate gave rhamnose, galactose, and glucose. The R_f values of G_1 and G_2 were 0.74 and 0.90, respectively, by cellulose TLC (2% AcOH), confirming the presence of more sugar in G_2 than in G_1. Hydrolysis of G_2 with β-glucosidase decreased the R_f on cellulose TLC (2% AcOH) from 0.90 to 0.39. Glucose and galactose were found in the hydrolysate by TLC. Subsequent hydrolysis with β-galactosidase did not further affect the R_f (0.39), but hydrolysis with β-rhamnosidase at the R_f 0.39 fraction gave kaempferol.

^1H NMR (Me_3Si-G_2, $CDCl_3$): 8.07–7.95 (dd, 2 H, H-2′ and H-6′, J = 2 Hz, J = 9 Hz); 6.9–6.8 (dd, 2 H, H-3′, H-5′, J = 2 Hz, J = 9 Hz); 6.75 (d, 1 H, H-8, J = 2 Hz); 6.37 (d, 1 H, H-6, J = 2 Hz); 5.8–5.74 (d, 1 H, H-1 glucosyl, J = 10 Hz); 5.25 (m, 1 H, H-1, 7-rhamnosyl); 4.75 [m, 1 H, H-1, 7-rhamnosyl-(1→3)]; 4.3 [m, 1 H, H-1, 3-galactosyl-(1→6)]; 3.95–3.40 (m, 18 H, 3-galactoglucosyl, 7-dirhamnosyl); 1.0 (m, 6 H, CH_3, rhamnosyl). The resonance value of 5.74 (J = 10 Hz) indicates a β configuration. The shift downfield from 4.3 ppm (normal) to 4.75 ppm indicates that there is a rhamnorhamnosyl linkage that is 1 → 3. Mabry et al. (1970) found that when rhamnose is attached at the 2 position of the glucosyl unit in neohesperidoside the resonance value is shifted downfield to 4.9 ppm.

The ^{13}C NMR (MeOD, 90° pulse, AT = 2.0431) assignments for G_2 (Table I) were made by comparing the carbon shifts to the ^{13}C NMR of robinin and flavonoids by Markham and Ternai (1976) and Yamasaki et al. (1977) (Table I). The interglycoside linkage between rhamnose and rhamnose is 3 → 1. The shift downfield of 10 ppm from 73.2 (normal value) to 84.9 is characteristic of the 3 position of the rhamnose and the downfield shift of 6 ppm from 61.3 to 67.1 ppm indicates that the other linkage glucose–galactose is at the 6 position of glucose. From these data, the structure of G_2 is kaempferol 3-*O*-galactosyl-(1→6)-glucoside, 7-*O*-rhamnosyl-(1→3)-

Table I. ^{13}C NMR Spectra of Clovin and Melitin Isolated from *M. alba*

compound	carbon no. and shifts (ppm from Me_4Si)															
	2	3	4	5	6	7	8	9	10	1′	2′	3′	4′	5′	6′
clovin	155.73	134.20	178.20	161.75	99.46	162.83	95.23	157.07	106.50	122.10	116.00	145.31	149.39	116.09	122.89
melitin	159.60	132.60	179.01	162.00	98.50	163.00	95.20	*a*	104.21	*a*	131.00	115.70	160.50	115.04	131.90

compound	sugar carbon no. and shifts (ppm from Me_4Si)															
	R″ C-1	Glc C-1	R-7 C-1	Gal C-1	Gal C-2	Gal C-3	Gal C-4	Gal C-5	Gal C-6	Glc C-2	Glc C-3	Glc C-4	Glc C-5	Glc C-6
clovin	101.18		101.12	103.90	72.37	74.31	69.17	74.80	66.62					
melitin	100.05	101.93	100.50	102.20	71.31	73.96	69.50	75.45[b]	61.92	74.17	77.65	69.90	75.80[b]	67.13

compound	sugar carbon no. and shifts (ppm from Me_4Si)													
	R″ C-2	R″ C-3	R″ C-4	R″ C-5	R″ C-6	R-7 C-2	R-7 C-3	R-7 C-4	R-7 C-5	R-7 C-6
clovin	71.36[b]	71.72[b]	73.10	69.27	18.13	70.87[b]	71.42	72.89[b]	70.70	18.13
melitin	72.44[b]	72.14[b]	73.69	69.73	21.34	71.78	84.90	72.14	70.78	21.34

[a] Cannot be assigned. [b] Can be reversed.

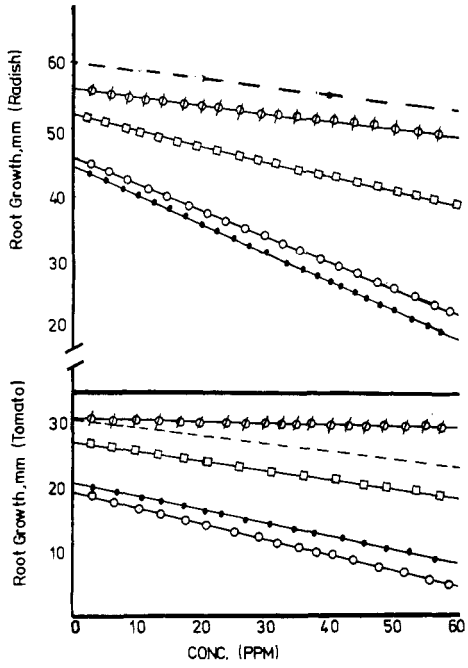

Figure 3. Linear regression plot of tomato and radish seedling root growth inhibition with various concentrations of chemical compounds from *M. alba* flowers. Robinin and melitin (---); kaempferol (∅); melilotic acid (●); *o*-coumaric acid (□); coumarin (○).

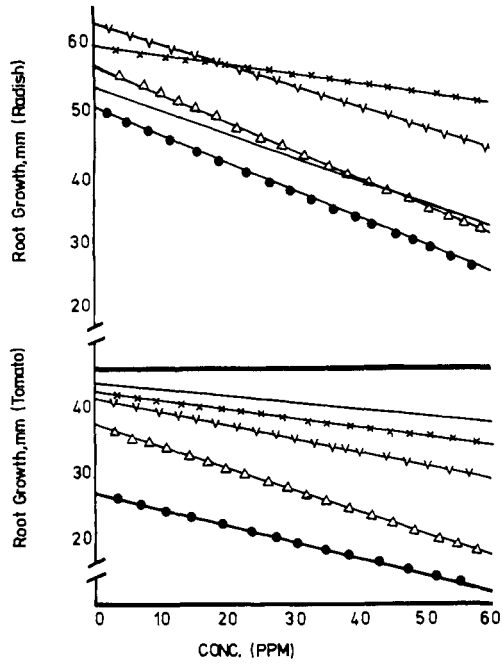

Figure 4. Linear regression plot of tomato and radish seedling root growth inhibition with varying concentrations of phenyl aliphatic acids. Benzoic acid (V); phenylacetic acid (×); 3-phenylpropanoic acid (△); 4-phenylbutanoic acid (●); *trans*-cinnamic acid (—).

rhamnoside, which is named melitin.

Fraction G_3 (30 mg, mp 192 °C) had the following UV spectra: (MeOH) 359, 304 sh, 257; ($AlCl_3$) 432, 360, 298 sh, 275; ($AlCl_3$ plus HCl) 406, 361, 298 sh, 270; (NaOMe) 396, 293 sh, 269, 242; (NaOAc) 427, 362, 294 sh, 257. The shifts observed for G_3 are indicative of free *o*-dihydroxyl groups at the 3′ and 4′ positions (bathochromic shift with $AlCl_3$). Addition of HCl to $AlCl_3$ decomposed the complex. Hydrolysis of G_3 with 2 N HCl gave an aglycon with identical UV shifts as those of an authentic sample of quercetin. The aqeuous phase of the acid hydrolysate gave on TLC rhamnose and galactose. Hydrolysis with β-galactosidase gave an incomplete hydrolysis, and subsequent hydrolysis with 2 N HCl gave rhamnose. The conclusion is that the rhamnogalactoside is attached to the 3 position and another rhamnose at the 7 position of the flavone. ^1H NMR of the Me_3Si derivative of G_3 ($CDCl_3$): 7.26 (m, 2 H, H-2′, H-6′); 6.8 (1 s, 1 H, H-5′); 6.7 (d, 1 H, H-8, J = 2 Hz); 6.3 (d, 1 H, H-6, J = 2 Hz); 5.6 (d, 1 H, H-1 galactosyl, J = 10 Hz); 5.21 (m, 1 H, H-1, 7-rhamnosyl); 4.3 (m, 1 H, H-1, 3-rhamnosyl). The shifts of the H-1 of the sugars are similar to those of robinin (G_1), which also confirms their position.

^{13}C NMR assignments of G_3 (Table I) were made by comparing to those of Markham and Ternai (1976) and Yamasaki et al. (1977). The assignment of the interglycoside linkage between rhamnose and galactose is on the same basis as that in robinin. Rhamnose is attached to the 6 position of the galactose (61.92 → 66.6 ppm). The weaker shielding observed in this linkage compared to that in G_2 is due to the increase of rotation and properties of the rhamnose moiety. From these data the structure of G_3 is quercetin 3-*O*-rhamnosyl-(1→6)-galactoside 7-*O*-rhamnoside, which is named clovin.

Figure 3 gives the tomato and radish seedling root growth inhibition caused by the major compounds isolated from white sweet clover. In both assays, coumarin and *o*-hydroxyhydrocinnamic acid (melilotic acid) showed the greatest activity. The root growth inhibiton was greatest in the radish assay, having a slope nearly twice that of the tomato assay. In both assays, *o*-coumaric acid showed intermediate activity, and the flavonoids had no activity.

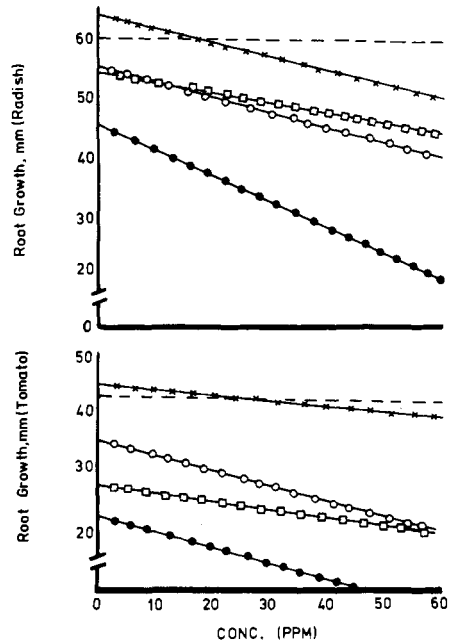

Figure 5. Linear regression plot of root growth inhibition of tomato and radish seedlings by *o*- and *p*-hydroxyphenyl aliphatic acids. *o*-Hydroxycinnamic acid, (*o*-hydroxyphenyl)acetic acid (□); melilotic acid, 2-(*o*-hydroxyphenyl)butanoic acid (●); *o*-hydroxybenzoic acid, (*p*-hydroxyphenyl)acetic acid (○); *p*-hydroxycinnamic acid, *p*-hydroxybenzoic acid, 3-(*p*-hydroxyphenyl)propanoic acid (×); *o*-coumaryl glucoside, water control (---).

The experimental data from the bioassay were subjected to regression curve fitting analysis (Ruckdeschel, 1980). The data best fit a linear expression.

In order to correlate structure–activity relationships, we conducted root growth assays with phenyl-substituted carboxylic acids (Figure 4). As the number of carbons

inserted between the phenyl and carboxyl group increased, root growth inhibition increased even though the rate of change (slope) was not significantly different among the acids. The root growth inhibition is not attributed to the acidity of the organic acids since the pH did not change for the acids at the concentrations studied. Therefore, the growth inhibition by these compounds can be due to their ability to interfere with enzyme activity. Williams (1963) has shown the effects of simple phenols, chlorogenic acid, and flavonoids against polygalacturonase activity and tissue macerating. Flavonoids like naringenin, found in dormant peach buds, antagonize the action of gibberellins (Phillips, 1962) as well as 5,4'-dihydroxy-7-methoxyflavonone isolated from *Betula verrucosa* (Popravko et al., 1974).

For establishment of the effect of hydroxyl substitution on the phenyl group to root growth inhibition, a series of *p*- and *o*-hydroxyphenyl aliphatic acids were tested (Figure 5). In each case, the ortho-substituted acids were more active than the corresponding para isomers. Blockage of the *o*-hydroxyl group eliminates the phytotoxic properties. The root growth inhibition properties of the coumarin-type compounds are due to the configuration of the molecule.

ACKNOWLEDGMENT

We are grateful to M. Burks for mass spectra data and M. Nicollier for her technical support.

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Received for review November 2, 1981. Accepted March 19, 1982. MAFES paper no. 5000. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

COMMUNICATIONS

Isolation and Identification of Phenolic Glucosides in Liquid Sugars from Cane Molasses

The major colored components of the orange-yellow liquid sugar from cane molasses have been isolated and fractionated with chromatographic techniques. The main components at low molecular weight have been identified as phenolic glucosides through enzymatic hydrolysis and GC-MS spectrometry.

Liquid sugars obtained from several cane molasses by ion-exchange demineralization and decolorization (Baldassarri, 1972) are always retaining a light yellow-orange color resistant to the usual forms of industrial refining. The purpose of our work was to characterize the minor components of the syrups from cane molasses and obtain more information to improve the industrial decolorization process.

Several colored components from cane and beet juices and sugars have been characterized in the past and classified in four major classes: plant pigments, poliphenolic compounds, caramels, and degradation products of sugars condensed with amino derivatives (Binkley, 1970; Tu, 1974). Methods used to investigate complex mixtures of colorants of cane sugar and juices have already been described; they include colorimetric determination of phenolics, separation by gel filtration for higher molecular weight compounds (Tu, 1974; Muro et al., 1974), precipitation with methanol, and sometimes solvent extraction (Farber et al., 1971). Colored components in raw cane sugar have also been studied by paper electrophoresis and identified as chlorogenic acid, cinnamic acids, and flavones (Farber and Carpenter, 1971; Carpenter et al., 1975). However, with know methods we did not obtain satisfactory recovery of colorants from our can molasses liquid sugars; due to the great sugar affinity they remained in the sugar even after decolorization processes with anionic macroporous resins. Therefore we carried out a new method of recovering highly hydrophilic colored components from sugar syrups, by absorption on nonionic polymers and elution with methanol. We describe here the isolation of the colorants, the fractionation on Sephadex G-10, the purification of the low molecular weight colored fraction by silica gel column chromatography, and their characterization by enzymatic hydrolysis and by GC-MS spectra of the silylated derivatives. In this way a new series of

0021-8561/82/1430-0764$01.25/0