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Registry No. 1, 134-62-3; 2, 72236-22-7; 2 (acetyl derivative), 126926-37-2; 3, 72236-23-8; 3 (methyl ester), 126926-38-3; 4, 26819-07-8; 5, 105394-84-1; 5 (aldehyde), 126926-39-4; 6, 126926-33-8; 6 (methyl ester), 126926-40-7; 7, 618-47-3; 8, 126926-34-9; 8 (acetyl derivative), 126926-41-8; 9, 4481-28-1; 9 (methyl ester), 106748-24-7; 9 (ethyl ester), 78950-33-1; 10, 99-04-7; 11, 28286-79-5; 11 (methyl ester), 67853-03-6; 12, 121-91-5; 13, 126926-35-0; 13 (methyl ester), 126926-42-9; 14, 5448-35-1; *m*-formylbenzoic acid, 619-21-6; diethylamine, 109-89-7; *N,N*-diethyl-*m*-formylbenzamide, 126926-36-1; ethylamine, 75-04-7; *m*-toluoyl chloride, 1711-06-4; *m*-formylbenzoic acid, 619-21-6; dipropylamine, 142-84-7.

Flavonoids with Mosquito Larval Toxicity

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Culture broths of an unidentified species of *Streptomyces*, designated 85–88, showed toxicity to mosquito larvae that was traced to be due to three crystalline compounds, identified as tangeretin (**1**) (5,6,7,8,4′-pentamethoxyflavone), genistein (**2**) (5,7,4′-trihydroxyisoflavone), and daidzein (**3**) (7,4′-dihydroxyisoflavone). Activity was produced only when soybean meal was included in the culture medium, and at least compounds **2** and **3** appear to arise as a result of the hydrolysis of the corresponding glycosides known to be present in soybean. The acetates of **2** and **3** also showed activity, actually somewhat greater than that shown by the corresponding isoflavones.

INTRODUCTION

During the past several decades, limitations observed with many synthetic pesticides such as slow biodegradability and mammalian toxicity, including carcinogenic potential, have prompted an active search for pesticides from alternative sources such as plants and microorganisms. The rationale behind this search is based on the general expectation that natural products are at least readily biodegradable and that other factors such as

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potency or mammalian toxicity might be modulated through structural changes. Some outstanding examples of microbially derived pesticidal compounds include the avermectins (Burg et al., 1979) and piericidins (Tamura et al., 1963). In such a program directed toward finding pesticidally active compounds among microbial metabolites, we observed that culture broths of an unidentified species of *Streptomyces* showed significant activity in the mosquito larval assay. Characterization of the active principles and a brief description of their activity are the subjects of this paper.

MATERIALS AND METHODS

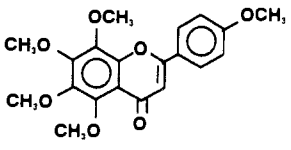
General Procedures. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The following conditions and instrumentation were used for obtaining the spectra described: UV, EtOH, Beckman 35; IR, KBr pellet, Beckman Acculab 3; NMR, $CDCl_3$, unless otherwise specified, with TMS as internal standard, Varian EM 390; MS, Kratos MS 80 RFA. Column chromatography was carried out by using silica gel (Merck <0.063 mm), and TLC was performed on silica gel (Merck, H60-P254-366).

The Organism and Its Culture. The organism designated as *Streptomyces* isolate 85–88 was deposited in the culture collection of the Plant Pathology Department, University of Florida, Gainesville, FL. It was grown in submerged culture in 4-L stirred glass jars containing 2 L of the medium consisting of glucose 1%, soybean meal 1.5%, distillers solubles 5%, dipotassium phosphate 0.3%, calcium carbonate 0.1%, and sodium chloride 0.1%. The culture was aerated at the rate of 1 volume of air per volume of liquid, agitated at the rate of 160 rpm, and maintained at a temperature of 26–28 °C for 72–96 h.

The organism was also grown in two other commercially available (Difco) media: nutrient broth and potato dextrose broth.

Isolation. The broths were tested for their activity against the mosquito larvae, and this activity was used to follow the course of fractionation.

The filtered broth (4 L) was extracted with *n*-butanol (2 L) and the extract concentrated azeotropically. The concentrate (200 mL) was extracted with ethyl acetate twice (100 mL each) and the concentrated extract chromatographed on silica gel (100 g) in benzene. Elution with 4% methanol in benzene gave a homogeneous fraction (TLC) which was crystallized from ether to give 1: yield, 95 mg; mp 152–153 °C; UV λ_{max} 275, 325 nm;



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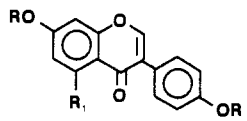

2, R=H, R_1=OH 4, R=$COCH_3$, R_1=O-$COCH_3$
3 R=R_1=H 5, R=$COCH_3$, R_1=H

IR, 1650, 1610, 1590 cm^{-1}; ^1H NMR δ 3.90 (s, 3 H, OCH_3), 3.93 (s, 6 H, 2 × OCH_3), 4.03, 4.10 (2 s, 6 H, 2 × OCH_3), 6.60 (s, 1 H, H-3), 7.08 (d, J = 9 Hz, 2 H, H-2′ and 6′), 7.9 (d, J = 9 Hz, 2 H, H-3′ and 5′); MS M^+ 372, 357 (100%), 240, 225, 197, 132. Anal. Calcd for $C_{20}H_{20}O_7$: C, 64.51; H, 5.41. Found: C, 64.58; H, 5.45. The ^1H NMR and mass spectral fragmentation were identical with those described in the literature (Talapatra et al., 1974) for tangeretin, thus confirming the identity of 1 with tangeretin.

Further elution of the column with 6% methanol in benzene gave the next fraction: yield, 22 mg, crystallized from ether/hexane (1:1); mp 290–292 °C. It was identical in all respects with a synthetic sample of genistein (2), which was prepared according to the method of Bradbury and White (1951).

The third band was obtained by elution with 8% methanol in benzene. It gave a crystalline compound: yield 25 mg, from ether; mp >295 °C (dec). It was identical with daidzein prepared by demethylation of formononetin, which in turn was synthesized as described by Markham et al. (1974).

For additional comparison of activity, two other isoflavones, 7-hydroxy-4′-methoxyisoflavone (formononetin) and 7,8-dihydroxy-4′-methoxyisoflavone (retusin), were prepared according to the methods described in the literature (Karmarkar, 1961; Markham et al., 1974; Jurd et al., 1972).

Acetylation. A mixture of 2 or 3 (0.1 g), pyridine (0.2 mL), and acetic anhydride (1 mL) was heated at 100 °C for 30 min. After the mixture was cooled and water was added, the solid that separated out was filtered after 30 min and crystallized from ether/hexane (1:1).

Compound 4, the acetate of 2, mp 200–202 °C, was identical with the acetate of genistein (Baker and Robinson, 1928).

Compound 5, the acetate of 3, mp 188–190 °C, was identical with the acetate of daidzein (Baker and Robinson, 1933).

Acetates of formononetin and retusin were also prepared as described above, and the properties agreed with those described in the literature (Karmarkar, 1961; Markham et al., 1974; Jurd et al., 1972).

Activity on Mosquito Larvae. The eggs of *Aedes aegypti* were received from the USDA Laboratories, Gainesville, FL. They were hatched and maintained as described by Hartzell et al. (1958). After 3–5 days at 30–32 °C, the larvae were ready for use in the assay. Samples of the compounds dissolved in DMSO were delivered into 18 × 150 mm test tubes. Concentrations were such that 0.1-mL aliquots contained, for example, 1, 0.2, or 0.04 mg which, when diluted with water (10 mL) gave final levels of 100, 20, or 4 ppm, respectively. After dilution with water, 10 larvae were transferred into each tube. Controls with distilled water and DMSO were similarly treated. The tubes were loosely plugged with cotton and incubated at 30–32 °C. After 24 h, the number dead in each tube was recorded, and a similar reading was also taken after 48 h. For a compound to be considered active, it must cause the death of at least 7 of 10 larvae after a 48-h incubation or sooner. The controls generally showed 0 or 1 death of 10. This criterion was used in testing the broths and fractions during the isolation. The purified compounds were tested over a concentration range in repeated tests. In those cases where more than one result is given for any concentration (Table I), the order of results in the 48-h column corresponds to that in the 24-h column; for example, 24 h, 5, 7, 10, and 48 h, 8, 10, 10, means that at 24 h 5 died and at 48 h 8 died in one test and that 7 died at 24 h and 10 died at 48 h and so on. The LD_{50} values were calculated from a graph relating the log concentration and the percent lethality using the values at the 48-h reading.

RESULTS

As outlined in the preceding section, the activity of the broth was found to be extractable into *n*-butanol, and chromatography of the concentrated extract on silica gel gave three crystalline compounds which, together, appeared to be responsible for the activity. Analytical and spectral data showed that compound 1 was 5,6,7,8,4′-pentamethoxyflavone (tangeretin), compound 2 5,7,4′-trihydroxyisoflavone (genistein), and compound 3 7,4′-dihydroxyisoflavone (daidzein). The two isoflavones as well as the two that were synthesized, formononetin and retusin, were converted to their corresponding acetates. The results of mosquito larval activity of these compounds are shown in Table I.

As seen from Table I, the methoxyflavone 1 and the hydroxyisoflavones 2, 3, and formononetin all exhibit significant activity against mosquito larvae. A possible indication of structural specificity is seen from the inactivity of retusin and the 3–8-fold increased activity of the acetates of 2 and 3 and of formononetin as compared with the corresponding hydroxyisoflavones.

DISCUSSION

Since occurrence of flavonoid compounds as metabolites of a *Streptomyces* is relatively rare, the possibility that these compounds might be artifacts, traceable to con-

Table I. Mosquito Larval Activity of the Flavonoids from *Streptomyces* 85-88 and Their Derivatives

compd	concn, ppm	no. dead/10 24 h	no. dead/10 48 h	LD_{50},[a] ppm
tangeretin (1)	100	8, 9, 7	9, 10, 9	7.5 (2×10^{-5} M)
	20	9, 7	10, 9	
	10	1, 3	6, 3	
	4	2	4	
genistein (2)	100	10	10	10.8 (4×10^{-5} M)
	50	10	10	
	20	10, 8, 10	10, 10, 10	
	10	3	5	
	4	1	3	
daidzein (3)	100	10	10	7.6 (3×10^{-5} M)
	50	9	10	
	20	5, 7, 10	8, 10, 10	
	10	0, 9	4, 9	
	4	1	2	
formononetin	100	10	10	10.7 (4×10^{-5} M)
	20	10	10	
	4	0	0	
retusin	100	0	0	not active
genistein triacetate (4)	50	10	10	4.0 (1×10^{-5} M)
	20	10	10	
	10	10, 10	10, 10	
	4	3	5	
daidzein diacetate (5)	20	9	10	2.0 (6×10^{-6} M)
	10	9, 10	10, 10	
	4	10	10	
	2	3	5	
formononetin acetate	100	8	8	2.5 (8×10^{-6} M)
	20	10	10	
	4	7	7	
	2	3	4	
retusin acetate	100	0	0	not active

[a] The LD_{50} values were determined by plotting the percent mortality against log concentration. Additional data will be necessary to obtain more accurate LD_{50} values. The quoted numbers in the table may be viewed as approximate values only for the LD_{50}.

stituents of the medium, must be established. This possibility is especially valid since soybean is known to contain the glycosides of genistein and daidzein (Walz, 1931). To prove this point, the organism was grown in two other media (nutrient broth and potato dextrose broth), and, in spite of good growth in these media, the broths showed little or no activity against the mosquito larvae; chromatography (TLC) of the extracts did not show the presence of **1**, **2**, or **3**. From the soybean flour used in the medium, the glycosides of genistein and daidzein were isolated, and acid hydrolysis gave **2** and **3**, respectively. Thus, it appears that the organism produces glycosidases which caused the hydrolysis of the glycosides present in the medium, yielding **2** and **3**.

Occurrence of flavonoids among microbial metabolites has been described in a few cases, although infrequently. In nearly all cases, the medium contained soybean meal or flour. Chimuza et al. (1975) reported the occurrence of isoflavones from a *Streptomyces* culture that was grown in a medium containing soybean meal. They did consider the possibility that the isoflavones might have been derived from precursors present in the medium, although no experiments were conducted to establish this point. The same is also true with the papers by Ganguly and Sarre (1970), Umezawa et al. (1975), Hazato et al. (1977), and Koenig et al. (1977), who used *Streptomyces*, *Micromonospora*, or some fungi grown in similar media. Finally, the isolation of isoflavonoids from mycobacteria described by Hudson and Bentley (1969) also belongs to the same category. The only flavonoid compound whose biosynthetic origin could be traced directly to the polyketide pathway appears to be chloroflavonin (Marchelli and Vining, 1973). Thus, although the origin of the isoflavones **2** and **3** in the present instance can be explained, the occurrence of tangeritin is still to be regarded as unusual as it is not traceable to any of the components of the medium.

Numerous flavone and isoflavone derivatives have been isolated from various parts of the soybean plant [*Glycine max* (L.) Merrill] (Dewick, 1988; Caballero et al., 1986; Ingham, 1983). Of these, the isoflavones have been reported to show some insecticidal activity to a mild degree but a much stronger antifungal activity. Two isoflavonoids, phaseol and afrormosin, were recently found in the leaves of a variety of the soybean plant resistant to the soybean looper, *Pseudoplusia includans*, and said to account for the resistance of the plant (Caballero, 1986). Many isoflavonoids are often considered phytoalexins induced by various damage-causing agents such as UV light, fungi, and mites in the plant. Noteworthy among these is coumestrol, which is induced by such agents in the soybean plant and which is also credited for the resistance of certain strains of this plant to the soybean looper (Ngo, 1984; Beggs et al., 1985; Dowd et al., 1986).

Although a mosquito larval assay such as this is commonly used as a prescreen for insecticidal activity, there have been no reports in the literature on such activity of the flavonoid compounds described here. The activity of the isoflavones is somewhat moderate, but that of the acetates such as **4** and **5** is not insignificant in comparison with many plant-derived compounds. The information provides a useful lead and is worthy of further pursuit.

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Registry No. 1, 481-53-8; 2, 446-72-0; 3, 486-66-8; 4, 5995-97-1; 5, 3682-01-7; formononetin, 485-72-3; retusin, 37816-19-6; formononetin acetate, 13293-49-7; retusin acetate, 37816-22-1.

Resistance of Chlorpyrifos to Enhanced Biodegradation in Soil

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Laboratory studies were conducted to determine whether the organophosphorus insecticide chlorpyrifos undergoes enhanced microbial degradation in soil. Repeated treatment of soils in the laboratory with chlorpyrifos did not alter the chlorpyrifos degradation rates or product distributions observed in four soils. Likewise, soils from three plots that received annual field applications of chlorpyrifos for 2–4 years did not develop an enhanced rate of chlorpyrifos degradation in laboratory degradation studies as compared to soils from untreated plots. Soils from fields in which a number of insecticides failed to control the target insect pests displayed short chlorpyrifos half-lives of between 4 and 9 days. The degradation of chlorpyrifos in these "problem" soils, which were highly alkaline (pH ≥8), was not microbially mediated and appeared to be a hydrolytic process. Accumulation or mineralization of the major chlorpyrifos hydrolysis product, 3,5,6-trichloro-2-pyridinol, was unrelated to the rate of chlorpyrifos hydrolysis observed. Results indicate that chlorpyrifos is not susceptible to enhanced microbial degradation and repeated chlorpyrifos application should have no effect on its persistence or efficacy.

INTRODUCTION

Enhanced microbial degradation is recognized as a specialized form of pesticide biodegradation in which an increased rate of pesticide degradation is associated with repeated application of that compound to soil. A microbial adaptation for pesticide catabolism is the cause of the accelerated rate of pesticide degradation, and it has been demonstrated that the soil bacterial populations involved utilize the pesticides or metabolites as carbon/energy or nutrient sources (Fournier et al., 1981; Karns et al., 1986; Racke and Coats, 1987; Tam et al., 1987; Mueller et al., 1989). The enhanced degradation of a number of phenoxyalkanoic and carbamothioate herbicides and carbamate and organophosphorus insecticides, primarily in soils used for corn production, has been demonstrated. Several excellent reviews of enhanced microbial pesticide degradation have recently appeared (Kaufman et al., 1985; Roeth, 1986; Suett and Walker, 1988; Sandmann et al., 1988; Felsot, 1989; Racke and Coats, 1990). The agricultural significance of enhanced degradation is that it may lead to a failure to control the target pest due to dramatically decreased pesticide persistence.

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