Constituents of a Cotton Bud

Formulation of a Boll Weevil Feeding Stimulant Mixture

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Highly active feeding (puncturing) stimulant mixtures were formulated for the boll weevil (*Anthonomus grandis* Boheman) from known cotton constituents, common metabolites, and compounds inducing primary mammalian sensations of taste and odor. Of 286 compounds bioassayed individually, 52 elicited substantial activity, and 14 of these had previously been reported in cotton. The insect was found to express preference for sweet, sour, and cooling taste properties, but odor preferences were difficult to establish. Compatability of the individually active components was necessary for formulation of active mixtures. An 8-component mixture of β-sitosterol, 15-pentadecanolide (15-hydroxypentadecanoic acid ξ-lactone), 1,8-cineole (1,8-epoxy-*p*-methane), *N*,*N*-dimethylaniline, vanillin, mannitol, rhamnose, and 0.1*M* phosphate buffer, pH 7.0, was superior to cottonseed oil and competitive with aqueous bud extract baits.

The recognition by screening and isolational work of a number of compounds that stimulate moderate feeding by the boll weevil, *Anthonomus grandis* Boheman, and the formulation from them of highly active mixtures are reported here. This insect—the most serious pest of cotton, *Gossypium* sp., in the United States—oviposits in the flower bud (square). Feeding by the developing larvae after egg hatch eventually results in abscission, and consequently, decreased yield of fruit. The observation by Keller *et al.* (1962) that aqueous bud extracts stimulated feeding by this insect precipitated the present investigation. Other pertinent entomological literature was reviewed by Hedin *et al.* (1966).

Isolational studies in the past five years (Hedin *et al.*, 1966; Struck *et al.*, 1968) indicated that no single compound in the bud evoked a full feeding (puncturing) stimulant response by the insect. Feeding activity was elicited with each of a series of successive solvent extracts of increasing polarity. Subsequent fractionation implicated several classes of compounds, but activity decreased or even disappeared when the pure components were isolated.

Since recombination or fortification of fractions by sugars and buffers often rejuvenated part of the activity, efforts were directed to formulating an active feeding mixture from known cotton constituents, common metabolites, and compounds that cause primary mammalian sensations of odor and taste.

The present report is believed to have significance because a number of constituents of cotton were shown to elicit substantial insect feeding, feeding activity was caused by diverse classes of compounds and required admixture for optimum response, and feeding activity was interpreted as a response to the major classes of mammalian odors and tastes.

EXPERIMENTAL

Insects and the Assay. A laboratory-adapted strain of unsexed 1- to 5-day-old adult weevils that had been reared in the laboratory on an artificial medium since eclosion were used. Best test responses were obtained with insects reared, fed, and handled as described previously by Hedin *et al.* (1966). Test materials in solution were applied to 37-mm. squares of Whatman No. 1 filter paper that were wrapped around cylindrical agar-water plugs and fastened with a paper staple and a straight pin. Ten insects were placed in a 100-mm. Petri dish with two agar plugs, one wrapped with paper impregnated with the test compound and the other with paper that was not impregnated. The dishes were placed in an incubator that was maintained at 28° C. and equipped with balanced lighting, and the weevils were allowed to feed for 4 hours. Then papers were unwrapped, and the punctures visible on the underside were counted. Each test was replicated three times each day on five or more days spaced over a minimum period of one month. The mean number of punctures on the blank was subtracted from the mean on the test plugs to give a positive or negative score, and this score was converted into an index (*T*/*S*) by multiplying by 100 and dividing by the response obtained to an aqueous extract of freeze-dehydrated cotton bud powder. The latter was prepared by boiling 3 grams with 100 ml. of water for 3 minutes and filtering. Normally, insects would make between 100 and 200 punctures on the plug impregnated with the aqueous bud extract but less than 20 on the control. Additional details of the test procedure are given by Hedin *et al.* (1966).

Compounds and Test Concentrations. Except for several flavonoids which were isolated from the cotton bud, all compounds were obtained from one of the following commercial sources and tested without further purification: K & K Laboratories, Inc., Eastman Organic Chemicals, Nutritional Biochemicals Corp., Matheson Coleman and Bell, Pfanstiehl Laboratories, Inc., J. T. Baker Chemical Co., California Corporation for Biochemical Research, and Chemical Procurement Laboratories. The gossypol acetate [1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphthalene)-8,8′-dicarboxaldehyde hexaacetate)] was purified by repeated precipitation from ether.

The feeding threshold for water extracts had been previously determined to be 15 μg. (total solids applied to paper square) with optimum responses requiring about 500

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μg. After a period of exploration, all compounds were tested initially at 0.5 mg. per ml. (100 μg.) applied to each paper. This test level is thereafter denoted 1X. Responses normally would be obtained at concentrations no higher than this if the compound were stimulatory. However, concentrations above and below this value, proceeding by half logs, were generally investigated if low or no activity was observed at the initial 1X concentration.

Isolation of Flavonoid Compounds. Kaempferol (3,4′,5,7-tetrahydroxyflavone), quercetin (3,3′,4′,5,7-pentahydroxyflavone), isoquercitrin (3,3′,4′,5,7-pentahydroxyflavone 3-β-D-glucopyranoside), quercimeritrin (3,3′,4′,5,7-pentahydroxyflavone 7-β-D-glucopyranoside), and quercetin-3′-glucoside (3,3′,4′,5,7-pentahydroxyflavone 3′-glucoside), all previously identified as present in cotton buds or flowers, were isolated by polyamide thin-layer and paper chromatography. Crystallization from water afforded sufficient quantities (100 to 500 mg.) for bioassay and for structural determination. These compounds were readily identified by comparison of their melting points, R_f values, spectral properties, products of acid and alkaline degradation, and aglycone-sugar ratios, where applicable, with published data (Harborne, 1958; Hayashi, 1962; Jurd, 1962; Parks, 1965; Sadykov, 1965). The structural determination of cyanidin-3-β-glucoside [3-(β-glucosyloxy)-3,3′,4′,5,7-pentahydroxyflavylium chloride], the only anthocyanin in *Gossypium hirsutum* L., was described by Hedin *et al.* (1967).

RESULTS AND DISCUSSION

Bioassay Data. The 286 test compounds were divided for presentation into three groups on the basis of acquisition and activity. The activity was expressed in terms of *T/S* (Struck *et al.*, 1968), a ratio of positive feeding value of the test to the feeding value of a standard aqueous extract of freeze-dehydrated bud powder (see Experimental). Several flavonoid compounds isolated from the cotton bud elicited moderate activity; these data are summarized in Table I. Compounds giving limited positive or negative activity (*T/S* values less than +30 or −30—i.e., 30% potency) are noted for archival purposes and are not otherwise reported. Compounds eliciting *T/S* values of greater than +30 or −30 were assigned to groups according to primary odor and taste and tabulated according to their activity (Tables II and III). Some compounds that exhibited an appreciably higher activity and were diluted from the 100-μg. level appear more than once. Compounds reported previously in cotton are identified with an asterisk (Minyard, 1967; Sadykov, 1965). Such listed compounds as the vitamins, minerals, and DNA-RNA precursors would also be expected to be present in cotton, but citations were not located.

Table I. Feeding Activity of the Major Flavonoid Compounds

Compound	Feeding Activity, *T/S*[a]	*T/S* plus *RP*[b]
Quercetin	44	43
Kaempferol	−8	33
Quercetin-3-glucoside	1	27
Quercetin-7-glucoside	32	43
Quercetin-3′-glucoside	52	56
Cyanidin-3-glucoside	−5	75
0.1*M* phosphate buffer, pH 7.0	32	..
Rhamnose	17	...
Rhamnose + phosphate buffer	39	...

[a] 100 μg. of each compound. [b] Plus 100 μg. of rhamnose in 0.2 ml. of 0.1*M* phosphate buffer, pH 7.0.

Bioassay of Flavonoid Components. During the isolation studies (Hedin *et al.*, 1966; Struck *et al.*, 1968), the highly active alcoholic and aqueous extracts of buds were found to contain larger amounts of flavonoids, but subsequent hydrolytic and chromatographic evidence showed that the major portion of the activity was retained when the flavonoids were removed. However, appreciable activity could be attributed to this class, therefore, the major components were isolated for bioassay. All except the anthocyanin, whose structure was established by Hedin (1967), had been previously reported in cotton (Minyard, 1967; Sadykov, 1965). These isolates were bioassayed with and without 0.1*M* phosphate buffer, pH 7.0, and rhamnose, which, on occasion, had fortified or stabilized the activity of the isolates, presumably because they ameliorated the bitter flavonoid taste. Rhamnose was used in preference to glucose, fructose, or sucrose, all of which elicit similar feeding activity individually, because it appeared to provide better fortification.

Quercetin, quercetin-7-glucoside (quercimeritrin), and quercetin-3′-glucoside were moderately active; however, rhamnose and phosphate buffer did not appreciably increase activity. Quercetin-3-glucoside (isoquercitrin) and kaempferol were inactive. Cyanidin-3-glucoside was not active alone but was appreciably synergized by rhamnose and phosphate. The activity of these compounds has some added significance because they are the first materials isolated from bud extracts that elicited activity.

Compounds with Limited Activity. *T/S* 21 to 30. Abietic acid; 3′-adenylic acid; ammonium phosphate, monobasic; *p*-aminobenzoic acid; 4-aminobutyric acid*; *p*-anistic acid, 1/10X; calcium cyclamate (calcium cyclohexanesulfamate); chlorogenic acid; β-cholestanol; cinnamaldehyde, 1/100X; *m*-coumaric acid (*m*-hydroxycinnamic acid); dipropylamine, 1/3X; epinephrine (3,4-dihydroxy-α-[(methylamino)methyl]benzyl alcohol; erythritol; glutaric acid*; monolaurin; guaiacol (*o*-methoxyphenol), 1/100X; hypoxanthine; inositol*; inosine; isocitric acid*; isovanillin (3-hydroxy-*p*-anisaldehyde); methyl-3-(methylthio)propionate, 1/100X; oleic acid*; oxalic acid*; protocatechuic acid; *o*-pyrocatechuic acid; pyruvic acid, 1/32X; α-resorcylic acid; salicylaldehyde; salicylic acid*; squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene); stearic acid*; tannins*; thymidine; tiglic acid, 1/32X; tryptophan*; tyrosine*; umbelliferone (7-hydroxycoumarin); *o*-vanillin (2-hydroxy-*m*-anisaldehyde).

T/S 11 to 20. Acetic acid*; alanine*; ammonium phosphate, dibasic; ammonium thiocyanate; β-amyrin* (olean-12-en-3β-ol); L-ascorbic acid*; asparagine*; aspartic acid*; benzoic acid; caffeic acid (3,4-dihydroxycinnamic acid); citric acid*; *s*-collidine (2,4,6-trimethylpyridine); coniferin; cytidine; dulcitol; ethyl propionate; eugenol (4-allyl-2-methoxyphenol), 1/100X; glucose*;

L-glutamic acid*; glycine*; guanosine 5′-diphosphate; kaempferol*; α-ketovaleric acid (2-oxovaleric acid), 1/100X; levulinic acid; maleic acid; *p*-methoxycinnamic acid; methyl-3-(methylthio)propionate; methyl sulfide; nicotinamide*; octacosane*; panthothenic acid; phloroglucinol; phthalic acid; piperidine; proline*; pyrogallol; β-resorcylic acid; rhamnetin (3,3′,4′,5-tetrahydroxy-7-methoxyflavone); rhamnose; riboflavin*; rutin*; saponins*; shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid); sodium glutamate; syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid); thiamine.

T/S 1 to 10. *cis*-Aconitic acid (1-propene-1,2,3-tricarboxylic acid); *p*-anisic acid (4-methoxybenzoic acid); ammonium phosphate, monobasic; D-(+)-catechin; catechol (1,2-dihydroxybenzene); *p*-coumaric acid; diethylamine; 3,5-dimethoxybenzoic acid; fructose; fructose

Table II. Flavor and Odor Assignments of Highly Positive Feeding Stimulants Arranged in Descending Order of Activity

Compound	Flavor	Odor	Molecular Wt.
	T/S OVER 60		
Gossypol* acetate	Sweet	None	477
Menthone 1/10X	Cooling	Pepperminty	154
1,8-Cineole 1/10X	Pungent/sweet	Camphoraceous	154
15-Pentadecanolide 1/100X	Bitter/sweet	Musky	240
Phytosterol(s), tech.	Sweet	None	(400)
	T/S 51 TO 60		
Benzylamine	Bitter/sweet	Pungent	107
Ferulic acid	Sweet	Floral	194
	T/S 41 TO 50		
α-Ketoglutaric acid*	Sour	None	146
α-Ketobutyric acid	Sour	Sweaty	88
Dihydroxyacetone	Sweet	Sweaty	90
1-Menthol 1/10X	Cooling	Pepperminty	156
Malonic acid*	Sour	None	104
Mandelic acid	Sour	Aromatic	152
Styrene glycol 1/10X	Pungent/sweet	Aromatic	138
Uric acid	Sweet	None	168
Vanillin* 1/3X	Biting	Floral	152
Ethyl methyl phenethyl carbinol 1/100X	Bitter	Floral	178
Maltol	Sweet/salty	Aromatic	126
	T/S 31 TO 40		
2-Aminoethanol	Burning/sweet	Pungent	61
Adipic acid	Sour	None	146
Anthranilic acid	Sour	Aromatic	137
α-Tocopherol	Sweet	None	431
Butylamine	Burning/sweet	Pungent	73
Calcium cyclamate 1/100X	Sweet	Aromatic	261
trans-Cinnamic acid	Pungent/sour	Aromatic	148
Cyclopentadecanone 1/10X	Sweet	Musky	224
N,N-Dimethylaniline	Pungent/sweet	Aromatic	121
Ethyl β-methyl-β-phenylglycidate 1/100X	Sour/sweet	Floral	206
Folic acid	Sweet	None	441
Formic acid*	Sour	Pungent	46
Gossypol (free)*	Sweet	None	519
Lactic acid*	Sour	Sweaty	90
Lanosterol	Sweet	Sweaty	427
l-Malic acid*	Sour	Aromatic	134
Mannitol	Sweet	None	182
Methionine	Bitter	Putrid	149
Phosphate buffer, pH 7.0	Salty	None	(132)
Phosphatidylserine	Salty	None	789
Pyruvic acid	Sour	Sweaty	88
Pyruvaldehyde	Pungent/sweet	Aromatic	72
Quercetin*	Bitter	None	302
β-Sitosterol*	Sweet	Sweaty	415
Succinic acid*	Sour	None	118
Terephthalic acid	Sour	None	166
Theobromine	Bitter	None	180
l-Thyroxine (Na)	Salty	None	798
Vanillic acid	Bitter/sweet	Floral	168
Valine*	Sweet/salty	None	117
Phytic acid	Sour	Sweaty	660

Table III. Flavor and Odor Assignments of Highly Negative (Feeding Deterrent) Compounds ($T/S > -30$) Arranged in Descending Order of Activity

Compound	Flavor	Odor	Molecular Wt.
	T/S BELOW −60		
Eugenol	Pungent/sweet	Aromatic	164
Heptanol	Bitter	Pungent	116
3-Hexen-1-ol	Bitter	Sweaty	98
Ribitol	Not ranked	None	152
Tiglic acid	Sour/sweet	Sweaty	100
	T/S −51 TO −60		
Ethyl methyl phenethyl carbinol	Bitter	Floral	178
Cinnamaldehyde	Pungent/bitter	Aromatic	132
γ-Undecalactone	Pungent/bitter	Sweaty	184
15-Pentadecanolide	Bitter/sweet	Musky	240
Styrene glycol	Pungent/sweet	Aromatic	138
	T/S −41 TO −50		
Cyclopentadecanone	Sweet	Musky	224
Ethyl β-methyl-β-phenylglycidate	Sour	Floral	206
2-Hexenal	Bitter	Sweaty	98
2-Methyl-1,4-naphthoquinone	Bitter/sweet	None	172
Saccharin	Sweet/bitter	None	183
Triethylamine	Bitter/pungent	Pungent	101
Salicin	Bitter	None	286
	T/S −31 TO −40		
p-Anisaldehyde	Burning/sweet	Aromatic	136
Benzaldehyde	Burning/bitter	Aromatic	106
Cholesterol stearate	Sweet	Not ranked	652
Erythrose	Sweet	None	120
Fumaric acid*	Sour	None	116
Gibberellic acid	Sour	None	346
Glycerol	Sweet	Not ranked	92
o-Methoxybenzoic acid	Bitter/sweet	Aromatic	152
Nicotine	Pungent	Putrid	162
Octyl acetate	Pungent/bitter	Pungent	172
Phytol	Sweet	Sweaty	296
α-Ketovaleric acid	Sour	Sweaty	102
Inosine 5′-monophosphate	Salty	None	348
Methone	Cooling	Pepperminty	154
Tartaric acid	Sour	None	150
Zingerone	Pungent	Aromatic	194

1,6-diphosphate; gallic acid; gentisic acid; gluconic acid; guanosine 5′-phosphate; hesperidin; *p*-hydroxybenzoic acid; octyl acetate, 1/32X; phenylalanine*; pyridoxine; resorcinol; rose oxide (1-*cis*-tetrahydro-4-methyl-2-(2-methylpropenyl)-pyran), 1/100X; threonine*; trehalose, 2,4,6-trihydroxybenzoic acid.

T/S 0 to −10. β-Aminoisobutyric acid (2-methyl-β-alanine); arginine*; *tert*-butylamine; caffeine, cholic acid; choline*; dibutylamine; 2,6-dimethyoxybenzoic acid; DNA; dulcin ((*p*-ethoxyphenyl)-urea); ethyl vanillin (3-ethoxy-4-hydroxybenzaldehyde); glutathione; 4-heptanol; hexanoic acid; hexatricontane*; lecithin; *m*-methoxybenzoic acid; DL-mevalonic acid lactone (3,5-dihydroxy-3-methylvaleric acid δ-lactone); mucic acid (galactaric acid); naringenin (4′,5,7-trihydroxyflavanone); oxalosuccinic acid (1-oxo-1,2,3-propanetricarboxylic acid); 15-pentadecanolide, 1/10X; sucrose*; 2,3,4-trihydroxybenzoic acid; valeric acid; veratraldehyde; vitamin B_{12}.

T/S −11 to −20. *p*-Aminobenzoic acid; amygdalin; calcium cyclamate; calcium gluconate; capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide); β-carotene*; cholesterol; *o*-coumaric acid (*trans*-*o*-hydroxycinnamic acid); creatine; digitonin; histidine*; *m*-hydroxybenzoic acid; indole; indole-3-acetic acid; isobutylamine; kinetin (*N*-furfuryladenine); lysine*; *m*-methoxycinnamic acid; *o*-methoxycinnamic acid; γ-nonalactone (4-hydroxynomanoic acid γ-lactone); oxalacetic acid; quinine; sodium guanylate; sorbitol (glucitol); stigmasterol; 3,4,5-trimethoxybenzoic acid; veratric acid; xanthurenic acid (4,8-dihydroxyquinaldic acid); *m*-xylene.

T/S −21 to −30. Adenosine; adenosine triphosphate; biotin; β-bisabolol*; butyric acid*; coumarin; creatinine; dihydroxyacetone(1,3-dihydroxy-2-propanone); 2,4-dimethoxybenzoic acid; diphenylamine; esculetin (6,7-dihydroxycoumarin); ethylenediamine; L-glutamine; guaiacol; guanine; hexylacetate; hippuric acid; α-ketobutyric acid (2-oxobutyric acid); maltol (3-hydroxy-2-methyl-4*H*-pyran-4-one); piperine; quassin; quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid); γ-resorcylic acid; rose oxide; sodium chloride; uracil; urea.

Analysis of Taste, Odor, and Structure-Activity of the More Active Compounds. Compounds eliciting *T/S* values greater than +30 or −30 were grouped according to primary odor and flavor and taste sensation. (Tables

II and III). These assignments were made with reference to the systems of Amoore and Venstrom (1966), Theimer and Davies (1967), and Kulka (1967). Candidate and reference compounds were dissolved in water with a small amount of ethanol, if necessary, and diluted to the bioassay concentration (100 p.p.m.). The odors of sweaty (Amoore, 1967) and aromatic, which probably possess a principal camphoraceous odor (Amoore, 1962), were added to the seven primary odors of Amoore. No deviation from Kulka's system for taste seemed indicated; however, a number of compounds possessed subliminal tastes; therefore, their secondary or subordinate taste was also recorded.

While it must be conceded that there is not universal agreement about the theoretical basis for these classifications, and that individual assignments made in this study may also be disputed, the major objective of this analysis was to determine whether the boll weevil responds to any or all mammalian tastes and odors in some consistent fashion. If so, human sensory evaluations could conceivably facilitate formulation of mixtures acceptable to insects.

The boll weevil appeared to prefer compounds with sweet, sour, and cooling tastes since the 15 most positively active compounds (stimulating) were so classified. However, no obvious correlation was established between odor and preference. Insect feeding appeared to be deterred by bitter substances since 9 of the 15 most negatively active compounds (repelling) were bitter. An additional 4 compounds that were considered pungent were also active negatively. Again, no correlation with odor was apparent.

To permit further inspection, the same compounds were tabulated by taste, odor, and chemical structure (Tables IV, V, and VI). In an attempt to recognize structure-activity correlations, it was recognized that widely diverse structures may possess similar odors or tastes (Amoore, 1962; Kulka, 1967; Theimer and Davies, 1967). All of the mammalian primary flavors and tastes elicited insect reactions. Stimulation was also obtained from all classes of odors except ethereal; however, informal responses to ethereal odors have been observed, since insects are slightly attracted to agar plugs impregnated with low concentrations of solvents such as ethyl ether, dichloromethane, and alcohols, but are not stimulated to feed.

Of the 43 most positive stimulatory compounds, 27 were graded as having a primary taste of sweet or sour (Table IV) and an additional 8 were given these grades for their after

Table IV. Highly Positive and Negative Components Classified on the Basis of Taste Sensation[a]

Classification	Negative Components		Positive Components		Negative Becoming Positive on Dilution[b]	
	Initial taste	Secondary taste	Initial taste	Secondary taste	Initial taste	Secondary taste
Sweet	5	5	14	7	1	3
Sour	5	0	13	1	1	0
Salty	1	0	3	2	0	0
Bitter	7	5	5	0	2	0
Cooling	0	0	1	0	1	0
Burning	2	0	2	0	0	0
Pungent	6	1	4	0	1	0
Biting	0	0	1	0	0	0
Not ranked	1	0	0	0	0	0
Total	27	11	43	10	6	3

[a] Tested at 100 p.p.m. or less if stated; compounds listed include those that had positive or negative T/S values of 30 or greater.
[b] From >-30 to $>+30$; these values not included in always positive and always negative categories.

Table V. Highly Positive and Negative Compounds Classified on the Basis of Primary Odors[a]

Primary Odor	Negative Components	Positive Components	Negative Becoming Positive on Dilution[b]
Camphoraceous	0	1	0
Pungent	3	4	0
Ethereal	0	0	0
Floral	0	3	2
Pepperminty	0	1	1
Musky	0	0	2
Putrid	1	1	0
Aromatic	6	8	1
Sweaty	6	7	0
Not ranked or none	11	18	0
Total	27	43	6

[a] Tested at 100 p.p.m. or less if stated; compounds listed include those that had positive or negative T/S values of 30 or greater.
[b] From >-30 to $>+30$; these values not included in always positive and always negative categories.

Table VI. Highly Positive and Negative Compounds Classified on the Basis of Structure

Structure Classification	Negative Components	Positive Components	Negative Becoming Positive on Dilution
Acid	5	16	0
Alcohol	3	8	1
Amide	1	4	1
Amine	3	6	0
Aromatic	4	3	0
Carbonyl	8	8	2
Ester	3	0	1
Ether	0	2	0
Mono- and sesquiterpenoid	0	2	1
Di- and triterpenoids and steroids	2	4	0
Lactone	2	0	0
Phenol	2	8	0
Polyhydroxy	6	2	1
Salts and zwitterions	0	4	0
Unsaturation	3	2	0
Total classifications	42	69	7
Total compounds	27	43	6

or subliminal tastes. Also 5 of the 6 compounds that were changed by dilution from highly negative to highly positive were graded sweet or sour. However, this apparent relationship must be reviewed with caution, because 10 of 27 highly negative compounds had primary ratings as sweet or sour, and 5 additional negative compounds were given these ratings for their after tastes. Furthermore, the test compounds, in particular, and natural products, in general, are probably biased toward these two categories. The insects also appeared to be favorably inclined toward saltiness since 5 positives and only 1 negative were recorded. Feeding seemed to be deterred by bitterness (12/27 *vs.* 5/43) and to a lesser extent by pungency (7/27 *vs.* 4/43). (12 of a total of 27 negative compounds had initial or after tastes of bitter compared with only 5 of 43 positive compounds.) Feeding was stimulated by compounds with floral odors (5/49 *vs.* 0/27) (Table V); they also fed voraciously on 1,8-cineole, menthone, and menthol (camphoraceous and pepperminty), but the absence of other test compounds in these categories precludes establishment of a correlation.

The only structure-activity correlations that were promising were phenolics (8/43 *vs.* 2/27) and acids (16/43 *vs.* 5/27) (Table VI).

Only two previous studies seem to be related to this work, and they deal with attractancy rather than feeding (puncturing) stimulation. Wright (1966) statistically analyzed USDA screening reports by Beroza and Green (1963) of compounds tested for attractancy with six insects including the boll weevil. Only secondary responses by the boll weevil to the test compounds had been reported by Beroza and Green. Wright correlated molecular vibrational frequencies and statistical attractivity frequencies with primary odors. He did not attempt to relate these primary odors to those sensed by mammals. Amoore *et al.* (1964) demonstrated that honey bees, *Apis mellifera* L., could discriminate between pepperminty and floral odors but had difficulty detecting a difference between two scents within the same group.

Flavor–Molecular Weight Interactions. Several preliminary efforts to correlate feeding activity with molecular weight were not promising. Plots of molecular weight *vs.* activity, with and without regard to structure—i.e., functional groups—suggested general increasing activity with size and possibly a favored range of sizes; however, the data were limited and conflicting. However, the subgrouping of data from Tables II, III, and IV according to arbitrary ranges of molecular size appeared to reveal several relationships not otherwise discernible (Table VIII).

While the boll weevil is both stimulated and deterred by sweet substances having molecular weights of 200 or below, sweet substances having molecular weights above 200 were consistently well accepted (11 of 13). Since most of these compounds were di- or triterpenoids or steroids, hydroxylated and much less sweet than the sugars, their activity may be associated with their predicted low rate of desorption. On the other hand, the most favored molecular size for sour and salty compounds was below 150: 11 of 15 acids gave positive responses, and of the four negative acids, two were apparently special cases (unsaturated); all the limited number of salty components were positive. Bitter deterrent compounds were concentrated in the 100 to 200 range; two hexenal compounds (MW 98) bolstered this group. Pungent compounds of intermediate size (MW 150 to 200) were most deterrent. Among the other categories, insufficient sample size precluded analysis.

Maxwell and Jenkins (1967) made an exhaustive review of biologically active substances in host plants affecting insect behavior. From these data a frequency table was prepared relating feeding stimulation and/or attractants and deterrents and/or repellents to molecular structure. The leading attractive compounds were: 8 acids attractive to 1 unattractive; monoterpene hydrocarbons, 9 to 2; di- or triterpenoids and steroids, 23 to one; esters and alcohols, each 7 to 0; and nucleotides and tannins, each 5 to 0. The leading repellent compounds were: 14 alkaloid glycosides unattractive to 1 attractive; and lactones, 7 to 1. Flavonoid, cyanogenic, and other unclassified glycosides were normally stimulatory, 17 to 6. The tastes of the stimulatory substances appeared to be sour, cooling, semisweet, and salty, and the stimulatory odors appeared to be floral, musky, pepperminty, and camphoraceous. Repellent substances appeared to have selected bitter tastes and strongly flavored (pungent?) characteristics afforded by the lactones. The similarity of these results to those that we observed with the boll weevil is striking.

Formulation of Feeding Active Mixtures. As anticipated, 11 of the compounds found to be most active ($T/S > 30$, Table II) have been reported present in cotton. Others from this list may also be present and/or are structurally related. In addition, 3 cotton flavonoids were demon-

Table VII. Interaction between Taste and Molecular Size on Insect Feeding[a]

	Response of Insects to Indicated Range of Molecular Weights														
	100			101 to 150			151 to 200			201 to 300			300		
Taste	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−
Sweet	4		2	4	1	2	5		4	1	3	1	7		1
Sour	4		1	7		3	2				1		1		1
Salty				3									2		1
Bitter			2	2		4	2	1	5		1	1	1		
Cooling							1	1							
Burning	2					2									
Pungent	1			2	1	2	1		5						
Biting							1								
Not ranked									1						
Totals	11	0	5	18	2	13	12	2	15	1	5	2	11	0	3

[a] Compounds eliciting feeding $>T/S$ +30, or $>T/S$ −30, or changing from highly negative to highly positive upon dilution.

strated to possess comparable activity. These observations corroborated isolational studies (Hedin *et al.*, 1966; Struck *et al.*, 1967) that feeding activity can be elicited with each of a series of successive solvent extractions of increasing polarity, but activity is decreased or even disappears with the isolation of pure components. The diversity of compounds giving secondary activity strongly suggested the need to use several representative or critical compounds in a formulation to obtain a full response.

Premises on which formulation could be based include an empirical combination of the more active components; a combination of active components representing all the primary flavors, or, alternatively, the preferred flavors; a combination of the more active components present in cotton; and all three combinations superimposed by an analysis of compatability.

With empirical combinations, there is a limit to the feasibility of statistical treatment of combinations; for example, a study of eight compounds at just one concentration in all possible combinations (1 to 8 components in each mixture) would require 256 bioassays with no allowance for replications. However, when screening of individual components had proceeded until a fairly large number of compounds were identified that would elicit some activity but that no single one would elicit a complete response, mixtures of 2 to 4 components were studied. Vanillin, which occasionally elicited strong feeding gave a somewhat increased and more consistent response in the presence of phosphate buffer, pH 7.0. However, attempts to fortify the mixture of vanillin and phosphate buffer with other active components did not always cause increased responses, and, in fact, some additions elicited negative responses. Similarly, with quercetin and quercetin glucosides, fortification was generally unhelpful. Since a compatability factor seemed operative, an analysis of the frequency with which individual compounds appeared in the most active mixtures was indicated.

Table VIII is a compilation of the frequency with which 17 selected active compounds were present in 330 empirical mixtures that were bioassayed over three months. Admittedly, other active compounds (see bioassay data section) might have been used.

Eighty per cent of the mixtures were feeding active; 47 and 21% gave T/S values of above 50 and 80, respectively. The average T/S for the 330 mixtures was 46, and an average of 4.8 compounds was employed in each mixture. β-Sitosterol, pentadecanolide, *N,N*-dimethylaniline, and 1,8-cineole appeared in mixtures having T/S values above 80 in 30% or more of the combinations in which they were included and in mixtures having T/S values > 50 in 60% or more of the combinations in which they were included. Gossypol, maltol, vanillin, calcium cyclamate, and mannitol also were frequently present in successful combinations. Methionine, uric acid, and particularly quercetin seemed to be poor in compatability. α-Ketoglutaric acid, despite its strong individual activity and the strong activity of acids in general, did not appear to improve the mixtures in which it was included. Since the cotton plant contains large quantities of organic acids, the showing of α-ketoglutaric acid here may be misleading.

Component Compatibility Analysis. To test the compatibility premise, an 8-component formulation (mixture A) was provided that included the four most promising compounds, β-sitosterol, pentadecanolide, *N,N*-dimethylaniline, and 1,8-cineole, plus two slightly less compatible compounds, vanillin and mannitol, plus rhamnose and pH 7 phosphate buffer, 0.1*M*. Each compound except the buffer was withheld in turn (Table IX). With mannitol absent, the mixture gave the best response; with rhamnose absent, it gave the poorest response. The variability of the bioassay results dictates caution in interpretation, but the over-all average T/S of 87 is a distinct improvement over the average (T/S 46) for the 330 mixtures formulated empirically.

The reality of the increase of stimulation produced by mixture A was supported by the averages from three similar compatability studies conducted with somewhat different compounds. Mixture B with vanillin, pentadecanolide,

Table VIII. Frequency of Occurrence of Individual Activity Components in 330 Test Mixtures

Compound	No. of Times Present in Mixtures Having T/S Values That Were				% Frequency of Presence in Mixtures Having T/S Values	
	Negative	Between 1 and 50	Between 51 and 80	Above 80	Above 50	Above 80
Calcium cyclamate	0	6	7	5	67	28
1,8-Cineole	14	20	27	26	61	30
N,N-Dimethylaniline	4	20	15	19	59	33
Gossypol acetate	9	29	31	25	60	27
α-Ketoglutaric acid	8	33	17	17	45	23
Maltol	4	10	3	7	42	29
Mannitol	19	47	46	38	56	25
Methionine	10	21	13	8	40	13
pH 6.0 phosphate buffer	4	9	8	10	58	32
pH 7.0 phosphate buffer	38	94	59	51	45	21
pH 8.0 phosphate buffer	2	12	20	12	69	26
Quercetin	15	10	4	2	19	6
15-Pentadecanolide	7	18	20	25	64	36
Rhamnose	49	116	82	64	47	21
β-Sitosterol	1	22	29	37	71	42
Uric acid	9	25	14	6	37	11
Vanillin	19	49	40	41	54	28
Total mixtures	51	123	85	71	47	21

Table IX. Insect Feeding on Variations of an 8-Component Formulation Selected for Compound Compatibility (Mixture A)

Variation of Mixture	Compounds[a] Present or Absent[b]								Av. T/S[d]
	β-Sistosterol	Pentadecanolide	*N,N*-Dimethylaniline	1,8-Cineole	Vanillin	Mannitol	Rhamnose	Phosphate buffer[c]	
1	+	+	+	+	+	+	+	+	90
2	0	+	+	+	+	+	+	+	82
3	+	0	+	+	+	+	+	+	83
4	+	+	0	+	+	+	+	+	95
5	+	+	+	0	+	+	+	+	76
6	+	+	+	+	0	+	+	+	80
7	+	+	+	+	+	0	+	+	119
8	+	+	+	+	+	+	0	+	70
									87 av.

[a] 31.6 μg. of each except pentadecanolide (0.316 μg.) applied to test paper.
[b] + = presence; 0 = absence.
[c] pH 7.0, 0.1 *M*.
[d] Av. of 9 replications tested over 3 days.

1/100X, β-sitosterol, *N,N*-dimethylaniline, rhamnose, mannitol, and phosphate buffer, pH 7.0, gave an average T/S of 69. Mixture C with β-sitosterol, vanillin, gossypol, methionine, mannitol, rhamnose, and phosphate buffer, pH 7.0, gave an average T/S of 69. Mixture D with α-ketoglutaric acid, pentadecanolide, β-sitosterol, vanillin, gossypol, rhamnose, mannitol, and phosphate buffer, pH 7.0, gave an average T/S of 73.

Precedence exists for inclusion in the mixture of at least four of the components. β-Sitosterol was found by Hamamura *et al.* (1962) to be a biting stimulant for the larval silkworm *Bombyx mori* L. Pentadecanolide was reported by McGovern *et al.* (1966) to extend the period of attraction of the Mediterranean fruit fly *Geratitis capitata* (Wiedemann) to trimedlure. Monoterpenes including cineole (Beroza and Green, 1963) are attracted to a broad spectrum of insects. Vanillin is also known to be mildly attractive to a fairly broad spectrum of insects (Beroza and Green, 1963; Wright, 1966). In addition, as noted, several of the components in the four mixtures have been reported present in cotton.

Each of the four mixtures was inspected to determine how each component contributed to the flavor profile. Several components contributed to sweetness, but others were apparently best accepted if only limited concentrations were included. While this selection of components admittedly was biased, the insect seemed to accept a mixture that was fairly bland but with interesting overtones, judged on the basis of mammalian flavor experience.

Comparison of Selected Synthetic Mixtures with Aqueous Bud Extracts and Cottonseed Oil. Previously, McLaughlin (1966) with aqueous bud extracts, and Daum *et al.* (1967) with expeller processed cottonseed oil formulations demonstrated feeding on these materials by the boll weevil, even in the presence of growing cotton. In both these studies, viscous gelating formulations containing dye markers were sprayed on the plant. In preliminary studies, synthetic mixtures, while highly active against water blanks, were only partially stimulatory in the presence of the aqueous extract of the cotton bud. Therefore, bioassay of the better synthetic mixtures in direct confrontation with cottonseed oil and with the bud extract seemed necessary to assess the true potency. The same 17 components that were present most often in active formulations (Table VIII) were therefore used to formulate 49 mixtures which were tested by direct preference against cottonseed oil on six separate days. Each was also assayed against a water blank.

In 37 of 49 bioassays, the mixtures elicited a greater number of feeding punctures than the oil and produced an over-all ratio of feeding punctures of mixtures to oil of 2.2 to 1 (Table X). Mixture A (Table IX) was preferred to cottonseed oil in 8 of 9 direct comparisons. Mixtures B, C, and D were preferred to cottonseed oil in 5 of 7, 6 of 8, and 5 of 6 direct comparisons, respectively. A related mixture of vanillin, β-sitosterol, *N,N*-dimethylaniline, pentadecanolide, and 0.1 *M* phosphate buffer was preferred in 8 of the remaining 12 direct comparisons.

Table X. Comparison of Boll Weevil Preference for Synthetic Mixtures and Cottonseed Oil[a]

Day of Test	No. of Bioassays	Mixture Preferred	Oil Preferred	Av. No. of Punctures on		Av. T/S of Mixture
				Mixture	Oil	
1	8	3	5	36	36	29
2	10	9	1	73	19	78
3	2	2	0	61	16	69
4	7	5	2	60	28	91
5	12	12	0	60	19	62
6	10	6	4	40	28	58
	49	37	12	55 av.	25 av.	64 av.

[a] Crude cottonseed oil, expeller processed.

In similar fashion, these and similar synthetic mixtures were compared with the aqueous extract of cotton buds. On five separate days over a three-week period, 37 bioassays were conducted in which insects could express a preference for the extract or for the synthetic formulations. The data were analyzed in the same fashion as before. In 22 of 37 bioassays, the mixtures caused more feeding punctures than the aqueous extract, and the over-all ratio of feeding punctures of mixtures to the extract was 1.2 to 1 (76 to 64). The average T/S of the mixtures was 82. Thus the formulation of synthetic mixtures that are as stimulatory as aqueous plant extracts appears to be feasible.

Although the mixtures did not elicit quite as many punctures as the extract in test-blank preference studies (T/S 82), they were slightly superior in this relatively limited (37 tests) comparison of direct preference. Again mixtures A, B, C, and D were the most competitive (14 of 21 were superior; 6 other related mixtures were preferred in 8 of 16 direct comparisons).

The data indicate that synthetic mixtures can be formulated which are superior to cottonseed oil for stimulation of puncturing and which are also competitive with water extracts. Probably the mixtures can be improved by further screening. Moreover, these mixtures may be used to synergize or fortify the activity of plant isolates that have often decreased in activity as fractionation was pursued. The application of these findings to field conditions is, of course, yet to be determined.

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