Isolation of Rice Weevil Feeding Inhibitors Uncinatone and Pectolinarigenin from *Clerodendron siphonenthus*

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Two feeding inhibitors, a diterpene hydroquinone [7,11-dihydroxy-3,4,9,11b-tetramethyl-1,8,9,11b-tetrahydrophenanthro[3,2-*b*]furan-6(2*H*)-one (uncinatone, I)] and a flavone [5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one (pectolinarigenin, II)], were isolated from *Clerodendron siphonenthus*, identified from their physical and spectral evidence. It was found that I and II inhibit the feeding of adult *Sitophilus oryzae*.

Clerodendron tricotomum, a representative member of the Verbenaceae family, is reported to possess feeding deterrent activity against the larvae of *Prodonia litura* due to the presence of clerodendrin A and clerodendrin B in the leaves (Kato et al., 1972). Later, Hosozawa et al. (1974) isolated a weak antifeedant, phytol, from *Clerodendron japonica*, a new antifeedant, 3-epicaryoptin, from *Clerodendron calamitosum*, and clerodendrin A from *Clerodendron cryptophyllum*. Because only a few species of this genus have so far yielded novel and structurally interesting feeding inhibitors, it was thought worthwhile to screen additional species. A preliminary study showed that the petroleum ether extract of *Clerodendron siphonenthus*, which grows abundantly in the plains of India, had absolute antifeeding activity at a concentration of 2.5% against the 5th instar larvae of *Diacrisia obliqua* by the leaf-disc test. Therefore, it seemed reasonable to assume that this plant contains feeding inhibitors.

EXPERIMENTAL SECTION

Extraction. Dry stems and leaves of *C. siphonenthus*, collected from M/S United Chemicals and Allied Products, Calcutta, where a voucher specimen has been preserved, were cut into pieces and powdered with a mechanical grinder. The powdered plant material was extracted with petroleum ether (60–80 °C) in a Soxhlet apparatus for 30 h. After complete removal of petroleum ether, a crude extract was obtained, which was made into desired concentrations (w/v) with chloroform and used for feeding deterrent activity.

Isolation and Identification of Feeding Inhibitors. The petroleum ether extract of *C. siphonenthus* (15 g in petroleum ether) was placed onto a silica gel chromatographic column (5 cm × 100 cm) and eluted successively with petroleum ether (fraction 1), petroleum ether–benzene (1:1) (fraction 2), benzene (fraction 3), and benzene–ethyl acetate (9:1) (fraction 4). After removal of solvents from each fraction, oily residues were obtained that were then subjected to thin-layer chromatography (Figure 1) on 20 cm × 20 cm glass plates coated with silica gel G at a thickness of 0.25 mm and eluted with benzene–ethyl acetate (9:1) and the feeding inhibitory test was performed with these fractions.

Fraction 2 was then rechromatographed over a column (2 cm × 40 cm) of silica gel. Elution with petroleum ether–benzene (1:1) gave an orange yellow solid that was purified by preparative thin-layer chromatography over silica gel (R_f 0.65) by elution with benzene–ethyl acetate (9:1). A compound crystallized from petroleum ether–chloroform as orange yellow needles, mp 214–16 °C, and analyzed for $C_{20}H_{22}O_4$ (M^+ 326). A negative Shinoda test for flavone coupled with the UV absorption maxima [λ_{max} (EtOH) 222, 284, 300, 340, 384 nm] indicated the compound to be a diterpene hydroquinone (Burnell et al., 1972). The ^1H NMR spectrum signaled for two vinylic methyls [δ 1.90 (s, 6 H)], one tertiary methyl [δ 1.58 (s, 3 H)], and an α-methyldihydrobenzofuran ring [δ 1.55 (d, J = 4.5 Hz, sec methyl), 5.10 (methine proton), 3.40 (dd, J = 7.5, 9 Hz), 2.88 (dd, J = 7, 12 Hz, methylene protons)]. Furthermore, the UV and ^1H NMR data were found to be in good agreement with those reported for the diterpene hydroquinone (uncinatone, I), occurring only in an African species, viz., *Clerodendron uncinatum* (Dorsaz et al., 1985). Moreover, the IR spectrum was found to be identical with that of an authentic sample.

Similarly, fraction 4 was rechromatographed over a column (2 cm × 40 cm) of silica gel. The benzene eluates afforded a greenish yellow solid that crystallized from petroleum ether–ethyl acetate as light yellow needles, mp 212 °C, and analyzed for $C_{17}H_{14}O_6$ (M^+ 314). A positive Shinoda test together with the UV spectrum [λ_{max} (EtOH) 265, 297, 333 nm] suggested the compound to be a flavone derivative (Markham and Mabry, 1975). Functional group analysis revealed the presence of two methoxy groups [δ 3.88, 4.05 (s, 3 H each)] and one chelated phenolic OH (14 δ, D_2O exchangeable) as indicated by the ^1H NMR spectrum and a conjugated carbonyl function at 1630 cm^{-1} and a free phenolic OH appearing as a sharp band at 3490 cm^{-1} in the IR spectrum. The ^1H NMR spectrum disclosed the presence of typical A_2B_2 system of H-2′, H-6′, H-3′, and H-5′ at δ 7.05 and 7.85 (each d, J = 8.5 Hz; Herz and Sumi, 1964). An intermediate retro-Diels–Alder fragment at m/e 132 suggested the location of one methoxy at the 4′-position of the B ring. The location of another methoxy in ring A was indicated by the appearance of a peak at m/e 167 that could arise due to loss of a methyl group from the intermediate retro-Diels–Alder fragment at m/e 182. That the flavone had a free phenolic OH at C-7 was suggested by its solubility in aqueous Na_2CO_3 (Herz and Sumi, 1964). Moreover, the spectral properties of this compound showed close resemblance to those reported for pectolinarigenin (Subramanian and Nair, 1972). On the basis of spectral data, this flavone was identified as 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one (pectolinarigenin, II).

Antifeedant Test. The insect used was the adult rice weevil, *Sitophilus oryzae* (Linn.), which was reared on wheat grains. The sample solutions of desired concentrations (w/v) were made by dissolving the crude petroleum ether extract, its constituent fractions, and pure compounds in chloroform. The sample solution (0.34 mL of each concentration) was applied to 20 selected sound grains of wheat in a small glass vial. After application of

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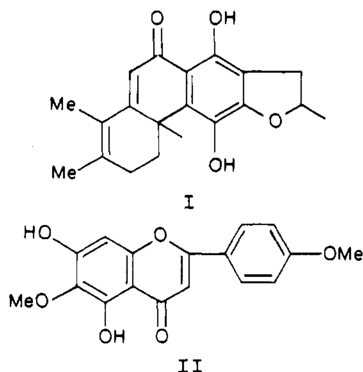

the sample to the grains, sufficient time (3 h) was allowed for evaporation of the solvent. Into each glass vial containing 20 grains were placed 10 freshly emerged mixed sex (5 ♀; 5 ♂) adults of *S. oryzae*. For controls, grains were treated with respective solvents only. To determine the feeding inhibitory activity, five replications of each concentration were used and observations were made after 24 h on the number of grains damaged in both sample- and solvent-treated glass vials. The samples were considered to have strong feeding inhibitory activity if less than 20% of the grains were damaged relative to those of control (Wada and Munakata, 1968).

RESULTS AND DISCUSSION

The feeding inhibitory activity of the crude extract of *C. siphonenthus*, its constituent fractions, and pure compounds against the adult *S. oryzae*, as summarized in Table I, indicated that the sample-treated grains were not consumed at either 2.5% or 5%, while 8.33% food damage was observed in control-treated grains. Apparently, the petroleum ether extract contained substances having feeding inhibitory activity. Further, the fraction that eluted with petroleum ether (fraction 1) was of oily consistency (R_f 0.9) and had absolute antifeeding activity only at 5%. Fraction 2 gave three spots on TLC (R_f 0.65, 0.50, 0.45) and exhibited strong feeding deterrent property at 2.5% and 5%. Fraction 3 showed two main spots (R_f 0.50, 0.45) and gave absolute antifeeding activity at 5%, while fraction 4 displayed one main spot (R_f 0.40) and possessed strong feeding inhibitory activity at both the concentrations. Since fractions 2 and 4 had strong feeding deterrent activity at 2.5% and 5% (Table I), chemical investigations were pursued only with these fractions. Subsequently, two colored compounds were isolated from these active fractions, and one was identified as 7,11-dihydroxy-3,4,9,11b-tetramethyl-1,8,9,11b-tetrahydrophenanthrol[3,2-*b*]furan-6(2*H*)-one (uncinatone, I), the occurrence of which is being reported for the second time from a *Clerodendron* species. Another compound, mp 212 °C, was identified as 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one (pectolinarigenin, II). The latter appears to be the first report of the occurrence of pectolinarigenin (II) in *C. siphonenthus*.

To determine the threshold concentration of feeding deterrent property, serial chloroform dilutions of pure uncinatone (I) and pectolinarigenin (II) were tested with adult *S. oryzae*. The feeding ratios (the damaged food of the sample-treated grains expressed as a percentage of the control-treated grains) for both the compounds varied from O to 20%, indicating the strong feeding inhibitory activity against the adult *S. oryzae* at concentrations of 250 and 500 ppm. At 100 and 50 ppm the feeding ratios were 25–41.61% for uncinatone (I), thus showing slight antifeeding activity, while pectolinarigenin (II) did not exhibit any activity at 50 and 100 ppm. The threshold concentrations, as calculated by plotting feeding ratio against log of concentration, were found to be 147.75 and 302.91 ppm for uncinatone (I) and pectolinarigenin (II), respectively. It is pertinent to mention that clerodendrin A and clerodendrin B inhibit feeding of the larvae of a number of insect species, including *Calospilos mirands*, the European cornborer *Ostrinia nubilalis* at a concentration of 5000 ppm, and the oriental tussock moth at 1000 ppm (Kato et al., 1972). Wada and Munkata (1968) reported that isoboldine isolated from *Cocculus trilobus* showed feeding inhibitory activity against tobacco cutworm at 200 ppm. The activities of I and II compared very favorably with those of Clerodendrin A and B and isoboldine.

Furthermore, fraction 1, which contained oily substances, had been shown to contain no uncinatone (I) and pectolinarigenin (II) by TLC but this fraction still possessed activity at 5%. Similarly, fraction 3, which was shown to contain a triterpene and sterol (not identified), also possessed deterrent property at higher concentrations. Therefore, it seems reasonable to assume that both uncinatone (I) and pectolinarigenin (II) in cooperation with some other substances that may occur in fraction 1 and

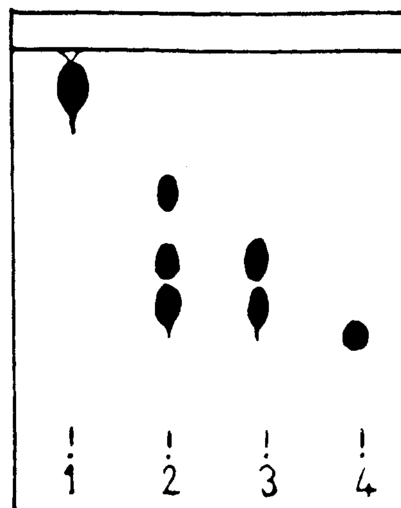

Figure 1. Thin-layer chromatogram of the various fractions. Developing solvent benzene–ethyl acetate (9:1).

Table I. Feeding Inhibitory Activity of the Crude Extract, Its Constituent Fractions, and Pure Compounds against the Adult *S. oryzae*

		% food damaged			
sample	concn	control (A)	sample (B)	feeding ratio: [B]/[A] × 100	act.[a]
crude extract	5%	8.3	0	0	++
	2.5%	8.3	0	0	++
fraction 1	5%	8.3	0	0	++
	2.5%	8.3	3.3	40.0	+
fraction 2	5%	8.3	0	0	++
	2.5%	8.3	1.3	15.5	++
fraction 3	5%	8.3	0	0	++
	2.5%	8.3	3.3	40.0	+
fraction 4	5%	8.3	0	0	++
	2.5%	8.3	0	0	++
uncinatone (I)	500 ppm	20.0	0	0	++
	250 ppm	20.0	1.7	8.3	++
	100 ppm	20.0	5.0	25.0	+
	500 ppm	20.0	8.3	41.6	+
pectolinarigenin (II)	500 ppm	16.7	1.7	9.9	++
	250 ppm	16.7	3.3	20.0	++
	100 ppm	16.7	8.3	50.0	–
	50 ppm	16.7	11.7	70.0	–

[a] Key: ++ = strong feeding inhibitory activity (B = 0–20% of A); + = slight feeding inhibitory activity (B = 20–50% of A); – = no feeding inhibitory activity (B = 50% over of A).

fraction 3 would act as resistance factors in inhibiting insect feeding.

ACKNOWLEDGMENT

We are grateful to the ICAR, New Delhi, for financial assistance and to Dr. M. R. Ghosh, Department of Agricultural Entomology, BCKV, for helpful advice and for performing the entomological screening.

Registry No. I, 99624-92-7; II, 520-12-7.

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Received for review August 27, 1987. Revised manuscript received March 14, 1988. Accepted July 15, 1988.

Development of *s*-Triazine Anticytokinins and Their Quantitative Structure–Activity Relationship

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A new, non-adenylate series of anticytokinins, N^2-substituted 2-amino-4-chloro-6-(ethylamino)-*s*-triazines, has been developed. The activity in terms of the I_{50} value of the most potent members was (0.3–0.5) $\times 10^{-6}$ M when examined by the tobacco (*Nicotiana tabacum* L.) callus assay in the presence of 0.05 $\times 10^{-6}$ M kinetin. The design of the molecule was made on the basis of insight into the active structure obtained from a cytokinin receptor map drawn previously. Quantitative analysis of their structure–activity relationship showed that the mode of their binding to the receptor was in important ways the same as for previously known anticytokinins, the structure of which resembles the adenylate cytokinins.

Five structural classes of anticytokinins, 7-substituted 3-methylpyrazolo[4,3-*d*]pyrimidines (Hecht el al., 1971; Skoog et al., 1973), 4-substituted 7-(β-D-ribofuranosyl)-(Iwamura et al., 1974, 1975), 4-substituted 2-methyl-(Iwamura et al., 1979a), and 4-substituted 2-(methylthio)pyrrolo[2,3-*d*]pyrimidines (Skoog et al., 1975), and 4-substituted 2-(methylthio)pyrido[2,3-*d*]pyrimidines (Iwamura et al., 1979b), have been developed in the past 15 years, and they have been used to study the biochemical mechanisms of their agonists, cytokinins (Hamaguchi et al., 1985; Iwamura et al., 1979a; Skoog et al., 1973; Tanimoto and Harada, 1982–1984, 1986). All of them are immediately similar in structure to a naturally occurring class of cytokinins, N^6-substituted adenines. Anticytokinins with a structural resemblance to diphenylureas, another class of cytokinins, or a nonadenylate structure, are not yet known.

The quantitative analysis of the structure-activity relationships of N^6-substituted adenine and diphenylurea cytokinins and anticytokinin-active pyrrolo- and pyrido-[2,3-*d*]pyrimidines has helped us to draw a cytokinin receptor map (Iwamura et al., 1980, 1983, 1985) that shows visually the framework of the receptor or receptor cavity into which an active compound should be accommodated and also the difference in the binding modes of agonists and antagonists. We selected from among all *s*-triazine structures non-adenylate candidates that may fit the receptor and exhibit activity, we hope an antagonistic one. The structures of N^2-substituted 2-amino-4-chloro-6-(ethylamino)-*s*-triazines thus prepared and tested are shown in Figure 1, together with the cytokinins and anticytokinins mentioned above. Figure 2 reproduces the receptor map.

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MATERIALS AND METHODS

Chemicals. The preparation of compounds **1**–**6**, **8**, **10**–**14**, and **16**–**18** was previously reported (Mitsutake et al. 1986). Triazines **7**, **9**, and **19** were synthesized by the reaction of 2,6-dichloro-4-(ethylamino)-*s*-triazine (Thurston et al., 1951) with *n*-decyl-, 2-methoxyethyl-, and 4-phenylbenzylamines, respectively, in the presence of $NaHCO_3$ in water at about 50 °C. N^2-(*n*-Decyl)-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (**7**): mp 162–163 °C. Anal. Calcd for $C_{15}H_{28}N_5Cl$: C, 57.40; H, 8.99; N, 22.31. Found: C, 57.60; H, 9.22; N, 22.59. N^2-(2-Methoxyethyl)-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (**9**): mp 172 °C. Anal. Calcd for $C_8H_{14}N_5OCl$: C, 41.47; H, 6.09; N, 30.23. Found: C, 41.71; H, 6.23; N, 30.27. N^2-(4-phenylbenzyl)-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (**19**): mp 240 °C. Anal. Calcd. for $C_{18}H_{18}N_5Cl$: C, 63.62; H, 5.34; N, 20.61. Found: C, 63.82; H, 5.19; N, 20.39. N^2-Phenyl-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (**15**) was prepared by the reaction of ethylamine with 2,6-dichloro-4-anilino-*s*-triazine produced from cyanuric chloride

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