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Plant Constituents biologically Active to Insects. I. Feeding Stimulants for the Larvae of the Yellow Butterfly, *Eurema hecabe mandarina*. (1)

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Biting responses were obtained in the larvae of the yellow butterfly, *Eurema hecabe mandarina* de l'Orza, to four chemical constituents of their host plant, *Lespedeza cuneata* G. Don, namely, D-fructose (II), *myo*-inositol (III), D-pinitol (IV), and 6,8-di-*C*-pentosylapigenin (V).

Keywords——feeding stimulants; biting factors; *Eurema hecabe mandarina*; *Lespedeza cuneata*; D-pinitol; D-fructose; *myo*-inositol; D-glucose; 6,8-di-*C*-pentosylapigenin; amino acids

It has now known that plants contain numerous biologically active substances to insects; such as hormone-like active substances, attractants, feeding stimulants, antifeedants, repellents.[2–10]

As a part of works to isolate biologically active substances to insects from plants, the present paper describes the observations on feeding stimulants for larvae of the yellow butterfly, *Eurema hecabe mandarina* de l' Orza, which have been briefly reported in a preliminary form.[11] The presence of three stimulants, attractants, biting and swallowing factors, in the preferential selection of food by insects was first reported in *Bombyx mori* by Hamamura.[7,12] Subsequently one of present authors, K. Takaishi, has suggested the presence of three stimulants for *Luehdorfia japonica* Leech.[13] From these reports it is considered that feeding stimulants for lepidopterous insects comprize biting factor and swallowing factor. However, among the relatively many works of the feeding stimulants the studies on such two factors have been seldom reported. Assuming the presence of such two stimulants, we studied on the feeding stimulants of the yellow butterfly larvae. This paper presents primarily biting factors.

1) Location: *a) 2-10-65, Kawai, Matsubara, Osaka, 580, Japan*; *b) Kowakae, Higashiosaka, Osaka, 577, Japan.*
2) K. Nakanishi, M. Koreeda, S. Sasaki, M.L. Chang, and H.Y. Hsu, *Chem. Commun.*, **1966**, 915.
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12) *a)* Y. Hamamura, *Nature* (London), **183**, 1746 (1959); *b)* Y. Hamamura, K. Hayashiya, and K. Naito, *ibid.*, **190**, 880 (1961); *c)* Y. Hamamura, K. Hayashiya, K. Naito, K. Matsuura, and J. Nishida, *ibid.*, **194**, 754 (1962).
13) K. Takaishi, K. Yamamoto, and Y. Kawahara, *Yakugaku Zasshi*, **89**, 1144 (1969).

Results and Discussion

The larvae of the yellow butterfly accept as food the leaves of only a limited number of leguminous plant species. A favored host plant, *Lespedeza cuneata* G. Don (Japanese name "Medohagi"), was used for isolation of the feeding stimulants.

As a first step, fresh leaves of their host plant were extracted with several solvents and fractionated as shown in Chart 1.

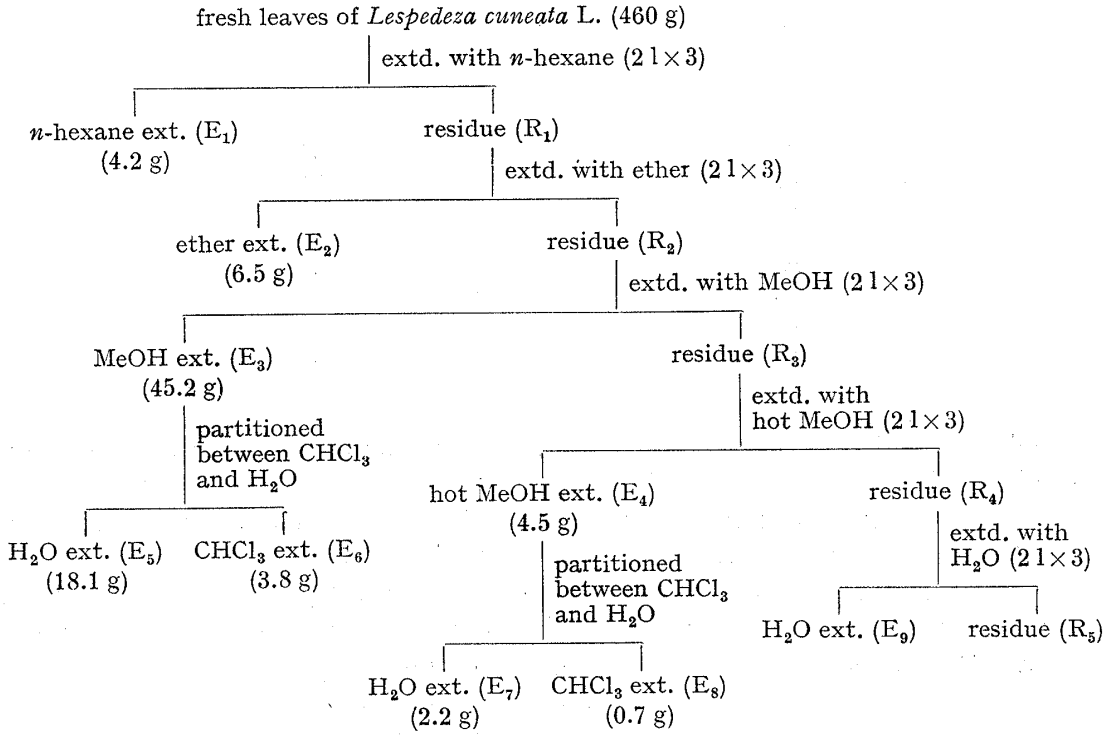

Chart 1

Biting responses of *E. hecabe mandarina* larvae to the obtained extracts and residues were tested. The technique carried out in *Bombyx mori* by Hamamura[12b] was used in some of the preliminary biting tests. However, it did not lend itself to determination of biting responses of the yellow butterfly larvae because the responses were not strong and the reproducibility of data was poor. These difficulties were overcome by moistening filter papers treated with test substances and representing numerically the degree of biting behavior observed for 3 minutes as shown in Table I.

Table I. The Degrees of Biting Responses represented numerically

Degrees	Biting responses
0	No response
1	Slight biting action
2	Strong but discontinued biting action
3	Strong and continued biting action
4	Strong and continued biting action along with shaking the head up and down as feeding on the leaves of the host plant

In the biting tests to the extracted residues of the host plant leaves it was difficult to moisten them and to observe in detail biting behavior because the individual leaves of them were fairly small in size. Therefore, only in this case the method of Hamamura was used. In the preliminary tests to the residues it was presumed that biting stimulants were contained

Table II. Biting Responses of *E. hecabe mandarina* Larvae to the Extracts and Residues of *L. cuneata* Leaves

Residue and extract	No. of larvae ex 5 biting	Degree of biting response
R_1	5	
R_2	3	
R_3	5	
R_4	0	
R_5	0	
H_2O (control)		0
E_1		0.2
E_2		0.2
E_3		2.0
E_4		1.4
E_5		2.5
E_6		0.3
E_7		2.2
E_8		0
E_9		0.1

in methanol extract (E_3). This was also confirmed in biting tests to extracts. These results are shown in Table II.

Moreover, it became clear from Table II that biting stimulants were contained in the two water-soluble fractions (E_5, E_7), which were obtained by partitioning between water and chloroform from methanol and hot methanol extracts (E_3, E_4). On the other hand, the stimulating effect of several fractions on feeding was investigated by a modified method of Hamamura's[12b] to determine whether the effective extracts (E_5, E_7) as the biting stimulants would induce practically the feeding responses of the butterfly larvae. The amount of feeding was evaluated on the basis of the total number of frass pellets produced by 5 larvae, which were placed individually on agar diets containing dried leaf powder or fractions of their host plant (Table III).

Table III. Feeding Responses of *E. hecabe mandarina* Larvae to Fractions of *L. cuneata* Leaves

Fraction	Total no. of frass pellets	Fraction	Total no. of frass pellets
None	14.5±5.5	R_2	80.3±4.6
1% Agar	14.7±1.2	R_3	29.0±2.0
Fresh leaves	156.0±1.5	R_4	17.1±3.1
Dried leaf powder	125.9±3.8	E_5	13.2±2.2
R_1	82.8±4.5	E_5+R_3	66.6±1.8
R_1+E_1	79.3±5.2	E_5+R_4	62.7±2.0

Positive feeding responses were obtained with the residue R_1 and R_2, although the effectiveness was slightly depressed when compared with the dried leaf powder. On the other hand, each of R_3, R_4 and E_5 alone did not exert the apparent stimulating effect on feeding. However, E_5 elicited a feeding response when combined with R_3 or R_4. These results suggest that E_5 contains the constituents which induce biting and R_4 contains the constituents which induce swallowing.

It is expected that the commonly naturally-occurring sugars and amino acids are contained in the effective water-soluble extract (E_5).

Analysis of sugars in E_5 was made by paper partition (PPC) and gas–liquid chromatography (GLC). Its results evidenced the presence of D-glucose (I), D-fructose (II), and inositol (III) in E_5. These sugars were also present in E_7. These carbohydrates were tested and the results are shown in Table IV.

TABLE IV. Biting and Feeding Responses of the Larvae to Sugars and Flavonoids

Compound	Concentration (%)	Degree of biting responses	Total no. of frass pellets
Control		0	16.5±3.3
D-Glucose (I)	0.1	0.5	
	1.0	0.1	39.5±3.5
	10.0	0.2	
D-Fructose (II)	0.01	0.2	
	0.1	1.2	
	1.0	0.9	
	10.0	1.6	74.7±2.3
	20.0	1.1	
	40.0	0.7	
myo-Inositol (III)	0.01	0.3	
	0.1	0.3	
	1.0	1.1	54.6±8.3
	10.0	0.1	
D-Pinitol (IV)	1.0	0.8	
	10.0	1.7	76.6±0.1
	20.0	1.5	
Mixture of II and III	10.0, 1.0	1.5	74.5±3.5
Mixture of I and II	1.0, 10.0	1.4	72.2±2.3
Mixture of II and IV	5.0, 5.0		91.4±3.0
Mixture of I, II, III, leucine, isoleucine, glycine and tryptophan	1.0, 10.0, 1.0, 0.5, 1.0, 5.0, 1.0		67.3±3.2
Mixture of I, II, III, IV, leucine, isoleucine, glycine and tryptophan	1.0, 5.0, 1.0, 5.0, 0.5, 1.0, 5.0, 1.0		56.5±4.2
6,8-Di-*C*-pentosylapigenin (V)	10.0	1.0	

It became clear that two of the sugars, II and III, are quite effective on biting but I is of low or negligible effectiveness. Although a satisfactory ranking of the relative effectiveness of the sugars was not possible, it seemed that II at a concentration of 10 percent was more effective than III at a concentration of 1 percent. The effectiveness of II as biting stimulants was not increased by the addition of I and/or III. These effective sugars on biting proved also to stimulate feeding (Table IV).

On the other hand, analysis of amino acids in E_5 was made by amino acid analyzer. Its result evidenced the presence of leucine, isoleucine, glycine, asparagine, valine, tyrosine and tryptophan. The biting and feeding responses to these amino acids were tested but they proved to be of low or negligible effectiveness. Also, the feeding responses of sugars were not increased by the addition of these amino acids (Table IV). Although other amino acids, alanine, glutamic acid, methionine and phenylalanine which did not occur in E_5 were tested, none of them were effective.

The above sugars undoubtedly stimulate biting and yet it seems that these are less effective than E_5. This suggests the presence of other substances for the full expression of the extract's stimulating effect. This simple assumption had us separate E_5.

It was made by several chromatographic methods as shown in Chart 2, under monitoring by the following method. The biting response to each fraction of column chromatography was tested and the effective fractions were analyzed with PPC. A sheet of chromatographic strip paper developed by ascending method using *n*-BuOH–AcOH–H_2O (4: 1: 2) mixture as solvent was cut in two at its center. A sheet of cut paper was sprayed with distilled water and set upright turning up the cut end, on which the larvae were placed and then biting tests carried out. The data permitted us to detect the active spot to biting response.

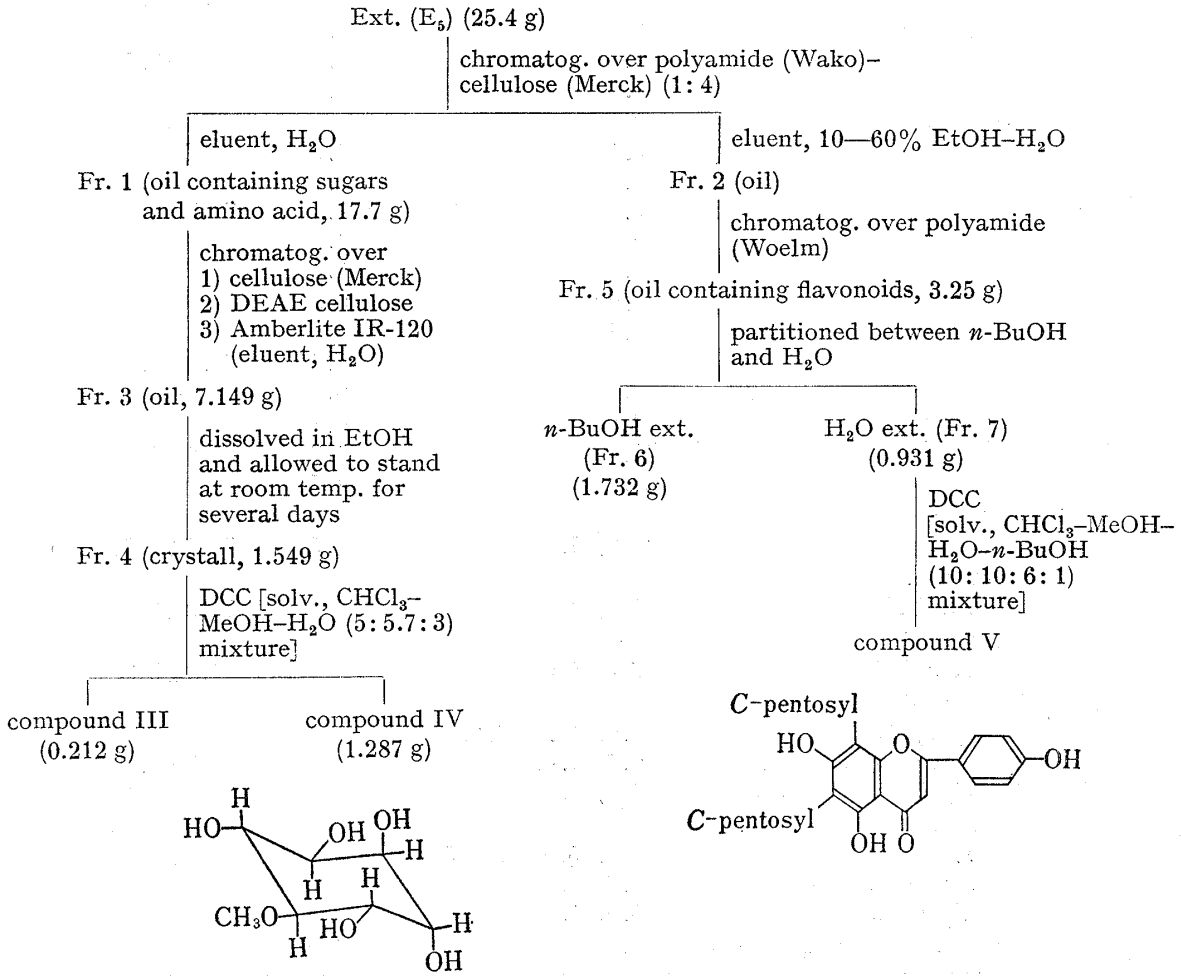

Chart 2

This experiment indicated that the effective substances are contained in two fractions as follows; the fraction (Fr. 3) given as an oil from an ion-exchange resin (Amberlite IR-120) column and the fraction (Fr. 5) given as a yellow oil from a polyamide column.

The former was dissolved in ethanol–water and the solution left for several days to give needles, which was recrystallized from ethanol–water. PPC of the crystals showed the presence of two constituents (Rf_1 0.34 and 0.20). Therefore, it was subjected to droplet countercurrent chromatography (DCC) using $CHCl_3$–MeOH–H_2O (5: 5.7: 3) mixture to separate two constituents.

The first compound (IV), mp 185—188°, Rf_1 0.34, obtained from the initial fractions as needles, was identified as D-pinitol[14] by its proton magnetic resonance (PMR) and carbon

14) A.B. Anderson, D.L. MacDonald, and H.O.L. Fischer, *J. Am. Chem. Soc.*, 74, 1479 (1952).

magnetic resonance (CMR) spectral analysis, PMR spectral analysis of the acetate and direct comparison with an authentic sample.

The second compound (III), mp 224—227°, Rf_1 0.20, obtained chromatographically in conjunction with IV as optically inactive needles, was identified as *myo*-inositol by comparison with an authentic sample.

IV was tested and proved to stimulate biting, and feeding on the presence of R_4. The feeding responses of IV were not increased by the addition of II, III or amino acids contained in E_5 (Table IV).

On the other hand, the another effective fraction (Fr. 5) separated by polyamide chromatography contained several flavonoids, of which the component (V) of Rf_1 0.39 proved to be effective as a biting stimulant. The compound (V), mp 235—238°, was isolated from Fr. 5 by partitioning between *n*-BuOH and H_2O, followed by DCC using $CHCl_3$–MeOH–H_2O–*n*-BuOH (10: 10: 6: 1) mixture.

Its coloration with various diagnostic reagents established it as a flavone. The shifts of the ultraviolet (UV) spectra by the addition of various reagents[15] indicated an apigenin derivative with free hydroxyl groups at the 4′,5 and 7 positions and acid hydrolysis produced no sugar nor isomerization to other products. The PMR spectrum of the trimethylsilyl (TMS) ethers showed the presence of twelve protons of the sugar moiety and the absence of the protons at the 6 and 8 positions. As signals (doublets) of two benzylic protons overlap, two sugars are likely to be identical. The mass spectrum (MS) of the permethylated derivative exhibited a molecular ion peak at *m/e* 660. Thus acid hydrolysis, PMR, UV and mass spectral data indicated that V is 6,8-di-*C*-pentosylapigenin.[16] This compound proved to stimulate biting (Table IV).

Hamamura has reported β-sitosterol as one of the biting factors of *Bombyx mori*.[12b] As it widely occurs among plants, its occurrence in *L. cuneata* would be also expected. GLC was used to determine whether β-sitosterol is present in sterol-containing fraction (E_1) of *L. cuneata*. Sterols in E_1 were run on OV-1 after the preparation of TMS ethers. This experiment showed the presence of β-sitosterol[17] in addition to stigmasterol and campesterol in E_1. Our result suggests that β-sitosterol does not induce the biting response to the yellow butterfly larvae, since E_1 containing it does not stimulate biting.

Experimental

Materials——i) Plants: The leaves of *L. cuneata* served as food of the yellow butterfly larvae and used for extracting the feeding stimulants of the larvae were collected in the field of Gakuenmae, Nara-shi or grown at our medicobotanical garden.

ii) Insects: The yellow butterflies, collected at the mount of Nijo, were allowed to lay their eggs and the larvae were reared on the fresh leaves of *L. cuneata* at 25°, 14 hr illumination in our laboratory. The larvae, reached the 32nd-hour of the 5th instar, were used for the present experiments.

Bioassay Procedure——i) Biting Test: The larvae starved for 4 hr were placed individually on filter papers which were dipped in the 5% extract of solution and sprayed with distilled water after evaporation of the solvent. Their biting behavior to the filter papers was observed for 3 minutes. The degrees of biting response were represented numerically as shown in Table I. Each result represents the average amount of biting responses in 10 larvae.

ii) Feeding Test: The starved larvae were placed individually on the following test diets; (1) diet prepared by incorporating dried leaf powder or residues (0.32 g) in 1 ml of 1% agar; (2) diet prepared by incorporating the extract (0.12 g) and R_3 or R_4 (0.26 g) in 1 ml of 1% agar. The feeding responses to the various diets were estimated by the total number of frass pellets produced by 5 larvae in 20 hr at 25°. Each

15) T.J. Mabry, K.R. Markham, and M.B. Thomas, "The Systematic Identification of Flavonoids," Springer, New York, 1970.
16) M.L. Bouillant, J. Favre-Bonvin, and J. Chopin, *Phytochemistry*, **14**, 2267 (1975).
17) The occurrence of this compound was presented by Prof. S. Matsuura *et al.* at the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1977.

test was repeated four times using fresh diet and a new group of larvae each time. Each result represents the average of four determinations.

Spectral Measurement——PMR spectra were taken with a Hitachi R 40 (90 MHz) spectrometer. Chemical shifts are given in parts per million (δ) downfield from TMS as an internal standard. The following abbreviations are used; s=singlet, d=doublet. The optical rotation was measured with a JASCO DIP-181 digital polarimeter.

Chromatography——PPC was conducted by ascending method using the following system: Rf_1, Toyo Roshi No. 50, *n*-BuOH–AcOH–H_2O (4: 1: 2); Rf_2, Toyo Roshi No. 51, *n*-BuOH–AcOH–H_2O (4: 1: 5, upper layer); Rf_3, Toyo Roshi No. 50, 10% AcOH; Rf_4, Toyo Roshi No. 50, 15% AcOH; Rf_5, Toyo Roshi No. 50, *t*-BuOH–AcOH–H_2O (3: 1: 1). Sugars and flavonoids were respectively detected with aniline–phthalic acid or silver nitrate and aluminum chloride. GLC were run on a Hitachi Model 063 equipped with flame ionization detector. Two stainless columns were used: I (2 m×3 mmϕ) for sterol determination was packed with 1.5% OV-101 on 80—100 mesh Chromosorb W (HP) and operated at 260° with a nitrogen carrier gas flow rate of 30 ml/min. Injector and detector temperatures were 340° and 350°, respectively; and II (2 m×3 mmϕ) for sugar determination was packed with 2% OV-1 on 80—100 mesh Chromosorb W (AW-DMCS) and operated at 170° with a nitrogen carrier gas flow rate of 30 ml/min. Injector and detector temperatures were 240° and 270°, respectively. Retention times are given in minutes. DCC was carried out with a chromatograph equipped with five hundred of glass tubes (60 cm×2 mmϕ) packed with the upper layer (stationary phase) of $CHCl_3$–MeOH–H_2O (5: 5.7: 3) or $CHCl_3$–MeOH–H_2O–*n*-BuOH (10: 10: 6: 1). The flow rate of moving phase (lower layer) was 0.21 ml/min and fractions of 15 ml were collected.

Extraction and Fractionation——Fresh leaves of *Lespedeza cuneata* L. were extracted with several solvents as shown in Chart 1. The extract (E_5) was fractionated, under monitoring by biting tests and PPC, as shown in Chart 2.

Analysis of Sterols——E_1 (10 mg) was trimethylsilylated by heating with N-trimethylsilylimidazole (TMSI) (200 μl) at 100° for 1 hr. The solution of the TMS ethers (1 μl) was injected into the gas chromatograph. The following sterols were detected; β-sitosterol, t_R 18.2; stigmasterol, t_R 15.6; campesterol, t_R 15.1.

Analysis of Sugars——E_5 and E_7 were analyzed independently by GLC and PPC. The extract (10 mg), after trimethylsilylated by shaking with TMSI (20 μl) and trimethylchlorosilane (TMCS) (20 μl) in pyridine (100 μl), was injected into the gas chromatograph. As a result, the following sugars were detected in both extracts; D-pinitol (IV), t_R 8.36, Rf_1 0.34; D-fructose (II), t_R 7.84, Rf_1 0.36, Rf_2 0.25; D-glucose (I), t_R 10.6 (α), 16.7 (β), Rf_1 0.32, Rf_2 0.21; *myo*-inositol (III), t_R 25.5, Rf_1 0.20, Rf_2 0.13.

***myo*-Inositol (III)**——Colorless needles, mp 224—227° (from EtOH–H_2O). It was identified by the comparison of IR and GLC with an authentic sample.

D-Pinitol (IV)——Colorless needles, mp 185—188° (from EtOH–H_2O). It was identified as D-pinitol by mixed melting point and comparison of IR spectrum with an authentic specimen. Treatment with a mixture (1: 1) of Ac_2O and pyridine at room temperature overnight gave a penta-acetate as colorless needles, mp 98—99° (from 80% EtOH).

6,8-Di-*C*-pentosylapigenin (V)——Yellow needles, mp 235—238° (from 50% MeOH–H_2O), $[\alpha]_D^{25}$ +48.3° (c=0.52, MeOH). Rf_1 0.39, Rf_3 0.40, Rf_4 0.49, Rf_5 0.24. It gave a dark brown color with ferric chloride and a red color with magnesium-hydrochloric acid. Hydrolysis, conducted by heating with 2 N hydrochloric acid for 5 hr, left it paper chromatographically unchanged. Its TMS ethers were prepared by the previously described procedure.[18] PMR (CCl_4) δ: 2.90—4.10 (10H, m, CH–OSi, CH–O–C), 4.56 (2H, d, J=9 Hz, 1″-H), 6.47 (1H, s, 3-H), 7.00 (2H, d, J=8 Hz, 3′-H, 5′-H), 8.07 (2H, d, J=8 Hz, 2′-H, 6′-H). MS of its permethylated derivative m/e: 660 (M^+).[16]

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18) T.J. Mabry, J. Kagan, and H. Rösler, *Phytochemistry*, **4**, 177 (1965).