ANTIMICROBIAL AGENTS FROM HIGHER PLANTS. ANTIMICROBIAL ISOFLAVANOIDS AND RELATED SUBSTANCES FROM *GLYCYRRHIZA GLABRA* L. VAR. *TYPICA*

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ABSTRACT.—Bioassay-directed fractionation of *Glycyrrhiza glabra* L. var. *typica* resulted in the isolation and characterization of glabridin (**1**), glabrol (**2**), glabrene (**3**), 3-hydroxyglabrol (**4**), 4'-*O*-methylglabridin (**5**), 3'-methoxyglabridin (**6**), formononetin (**7**), phaseollinisoflavan (**8**), hispaglabridin A (**9**), hispaglabridin B (**13**), salicylic acid and *O*-acetyl salicylic acid. Of these, hispaglabridin A, hispaglabridin B, 4'-*O*-methylglabridin, glabridin, glabrol and 3-hydroxyglabrol possess significant antimicrobial activity *in vitro*; hispaglabridin A, hispaglabridin B, 3'-methoxyglabridin, 4'-*O*-methylglabridin 3-hydroxyglabrol, phaseollinisoflavan, salicylic acid, and *O*, acetyl salicylic acid are newly found in *Glycyrrhiza* sp.; and hispaglabridin A, hispaglabridin B, 3'-methoxyglabridin, 4'-*O*-methylglabridin, and 3-hydroxyglabrol are new to the literature and their structures are proposed herein.

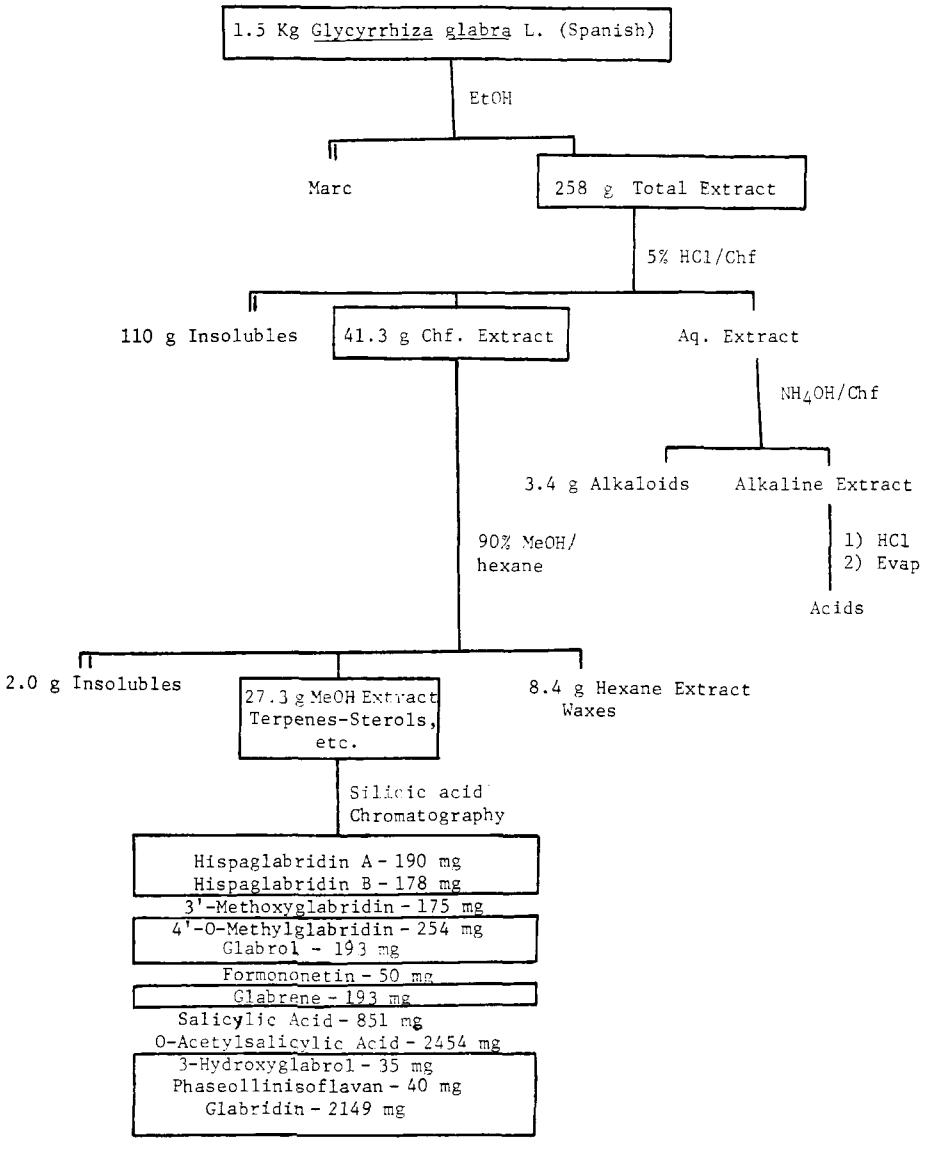
In a screening program devised to detect antimicrobial activity in higher plant extracts (1), ethanolic extracts of the powdered roots of commercial *Glycyrrhiza glabra* L. var. *typica* (known in Commerce as Spanish licorice) showed reproducible antimicrobial activity *in vitro* against *Staphylococcus aureus* (ATCC 13709), *Mycobacterium smegmatis* (ATCC 607) and *Candida albicans* (ATCC 10231). Systematic fractionation was undertaken in order to isolate and structurally characterize the active constituents, and this paper records our findings. A brief, preliminary account of a portion of this work has appeared (2).

DISCUSSION

Glycyrrhiza glabra is an economically valuable plant grown in temperate climate regions because of its flavoring and antiulcer principles. Accordingly, it has been much studied, even in quite recent years (3–16), however, not from the vantage point of antimicrobial activity. It has been our experience that bioassay-directed fractionation of apparently thoroughly studied species (*e.g.*, *Ptelea trifoliata* (17, 18)) frequently leads to the isolation of new, active substances. Accordingly, when extracts of *G. glabra* var. *typica* (Spanish licorice)—but not var. *glandulifera* (Russian licorice)—were repeatedly active in our screen, this work was undertaken.

Following the gross fractionation scheme developed for this kind of work (1), illustrated in scheme 1, the majority of the biological activity was found to reside in the neutral terpene-steroid fraction. Column chromatography over silicic acid produced numerous bioactive fractions from which a number of known and new compounds could be separated in pure form and characterized.

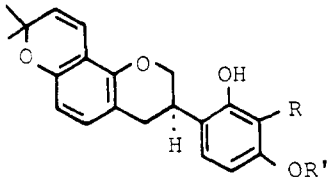
The identification of previously known substances was straightforward and depended upon spectroscopic analysis, comparison of physical constants, preparation of derivatives, and, in a few cases, comparison with authentic samples. In this manner, the presence of glabridin (**1**) (6), glabrene (**3**) (7), glabrol (**2**) (6) and formononetin (**7**) (11, 19) in *Glycyrrhiza* extracts was confirmed. In addition, phaseollinisoflavan (**8**), previously known as a phytoalexin from *Phaseolus vulgaris*



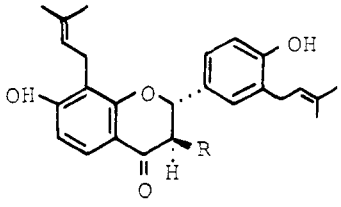
Scheme 1

SCHEME 1. Bioassay directed fractionation of *G. glabra*. Antimicrobially active materials are enclosed in boxes.

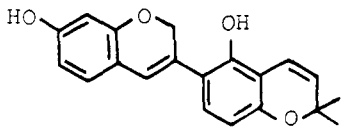
infected with tobacco necrosis virus (20); salicylic acid; and *O*-acetyl salicylic acid, all new to this species, were identified. Curiously, *O*-acetyl salicyclic acid seems not to have been encountered directly in nature before. The quantity present (0.15%) in the plant is relatively small, but its presence in extracts (licorice) might lead to some therapeutic effect.



(1), R = R' = H
(5), R = H; R' = CH_3
(6), R = OCH_3; R' = H



(2), R = H
(4), R = OH



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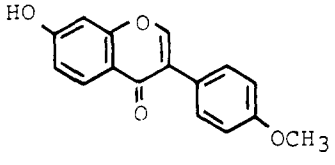

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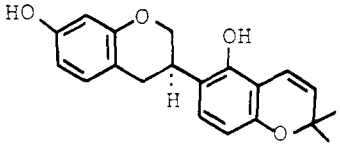
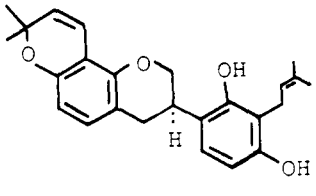


(8)

Hispaglabridin A (**9**) (2), $C_{25}H_{28}O_4$ (anal.; $M^+=392$), is optically active and possesses a uv spectrum which closely resembles that of glabridin (**1**), suggesting the presence of a common chromophore. The infrared spectrum contained absorption bands attributable to OH (3500 and 3350 cm^{-1}), olefinic (1632), and aromatic double bonds.



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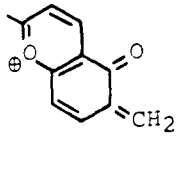

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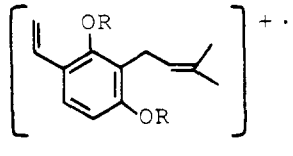


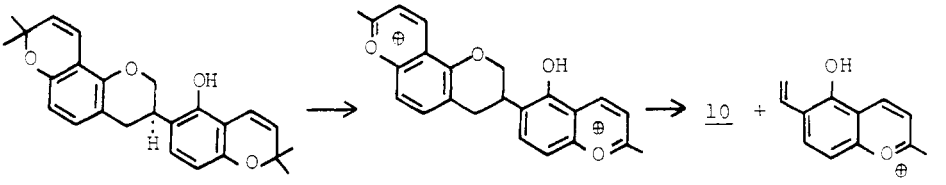
(11), R = H
(12), R = CH_3

The mass spectra of isoflavans such as glabridin readily lose 15 atomic mass units from the molecule ion by expulsion of one of the *gem*-dimethyl groups from the chromene ring. There follows a pronounced *retro* Diels-Alder fragmentation which, in

the case of hispaglabridin A, was particularly revealing. In addition to the base peak at *m/e* 173 (**10**), which is also found in the spectrum of glabridin, a companion peak was found at *m/e* 204 (**11**). This requires the presence of a C_5H_9 unit not present in glabridin. The presence of a dimethylallyl moiety was readily seen in the pmr spectrum as two vinyl methyl groups (1.75 and 1.81δ) and an A_2M system (3.42δ,2H,*d*,J=7Hz; and 5.25δ,1H,*t*,*J*=7Hz). Two phenolic groups were suggested by the presence of D_2O exchangeable protons at 4.87 and 5.42δ. This was confirmed by the formation of a di *O*-methyl ether by heating with $(CH_3)_2SO_4$ and K_2CO_3 in acetone and, in particular, the presence of RDA peaks at *m/e* 173 (**10**) and 232 (**12**) in the mass spectrum. At least one of the phenolic groups has a free *para*-position as shown by a positive Gibbs test (purple blue).

That all of the substituents on the non-fused benzenoid ring were vicinal was seen from the pmr in which the aromatic hydrogens were seen as two pairs of partially overlapping *ortho*-coupled, AB pairs at 6.32 (1H,d,*J*=8Hz), 6.35 (1H,d,*J*=8Hz) and 6.78δ (2H,d,*J*=8Hz). Considering these factors and the close similarity of its spectral properties with those of glabridin, structure **9** was considered most likely for hispaglabridin A. This was confirmed by reaction of glabridin with 3-methyl-but-2-en-l-ol in the presence of $BF_3{\cdot}Et_2O$ (22). Four products were seen on tlc. These were separated by silica gel chromatography, and one of the monoprenylated products was identical (ir,tlc,pmr,ms and ord (2)) with **9**.

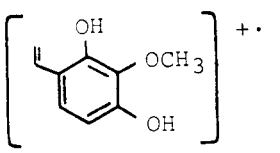
Hispaglabridin B (**13**), $C_{25}H_{26}O_4$, was very similar in its general properties to **9** and contained two hydrogens less, leading to the suspicion that it might be a cyclized version containing two *gem*-dimethylchromene rings. Only a single phenolic OH group was present, as shown by monomethyl ether formation, and the requisite mass spectrum shifts. The pmr spectrum showed a 12-proton methyl singlet at δ1.40, and the AB doublets for the olefinic protons encompassed 4-hydrogens and consisted of a slightly separated pair of doublets. Furthermore, the mass spectrum showed an intense (38%) doubly-charged ion at *m/2e* 180 (**14**) corresponding to the loss of the two methyl groups. The *retro* Diels-Alder peaks were at *m/e* 173 (base, **10**), as before, and at *m/e* 187 (**15**, 67%) in agreement with this assignment. A positive (blue) Gibbs test requires that the ring closure not have taken place on the phenolic hydroxyl possessing a free *p*-position. This hypothesis was confirmed by pyridine catalyzed reaction of glabridin and 3-hydroxy-3-methyl-1,1-dimethoxybutane, which led to hispaglabridin B (ir,tlc,ms and ord (2)). The direction of closure is doubtless dictated by the avoidance of a *peri*-buttressing effect, which would arise on closure to the phenolic OH at C-2'.



(13) (14) (15)

3'-Methoxyglabridin (**6**), $C_{21}H_{22}O_5$, possesses an uv spectrum closely similar to that of glabridin (**1**), as shown clearly by its formula and its pmr spectrum (3.78δ,3H,*s*,OCH_3), differed from it by possession of a methoxy group which,

from the mass spectral fragmentation to **10** and **16** (*m/e* 166), was located in the pendant benzene ring. The pmr signals for the aromatic hydrogens consisted of two sets of *ortho*-coupled AB pairs requiring that the three oxygen functions (**6** is converted to a trimethyl ether with Me_2SO_4) be attached to consecutive carbons starting with C–2'. The methoxy group was judged to be at C–3' because **6** failed to show the substantial uv bathochromic shift with $AlCl_3$ reagent which is characteristic of catechols (23).



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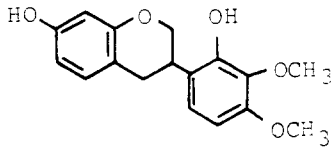

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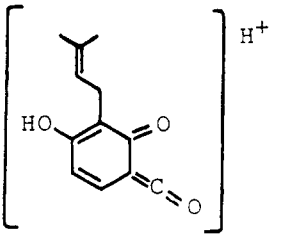
The oxygenation pattern of 3'-methoxyglabridin (**6**) has precedent in *G. glabra* constituents as the oxygenated isoflavan (**17**) has been isolated recently from the leaves as a phytoalexin (14).

The structure of 4'-*O*-methylglabridin (**5**) was easily established from its spectroscopic properties and the reaction of glabridin with etherial diazomethane, which proceeded somewhat sluggishly, to produce a mixture of **5** and di *O*-methylglabridin. The *O*-methylation product of **5** was identical to di *O*-methylglabridin. It seems likely that the less hindered phenolic OH group at C–4' of **5** should react the fastest; in any case, **5** gives a positive (blue) Gibbs test in agreement with the structure assigned to it.

Compounds **5**, **6**, **9** and **13** all belong to the same absolute configurational family (3*R*) based upon comparison of their ord spectra with published values (24, 25) and comparison with the spectra of samples synthesized from **1** of known absolute configuration. In the original paper (20), no assignment of stereochemistry was made to phaseollinisoflavan. Based upon our ord measurements, the absolute configuration depicted in structure **8** is assigned.

3-Hydroxyglabrol (**4**), $C_{25}H_{28}O_5$, possessed spectral properties rather similar to those of glabrol (**2**). The additional oxygen atom could be placed unambiguously at C–3 based upon the following considerations. In the mass spectrum, the pair of *retro* Diels-Alder peaks at *m/e* 205 (56%, **18**) and 204 (15%, **19**) indicated to which portion of the molecule the OH to be located was attached (26). The ir spectrum showed a chelated C=O group at 1652 cm^{-1} which shifted to 1710 cm^{-1} in the triacetate. The uv spectrum underwent a substantial bathochromic shift upon addition of $AlCl_3$ (282 to 317 mm) and $AlCl_3$–HCl (317 to 283 nm) (27). These results are in agreement with assignment of the OH group to C–3, where it can chelate to the carbonyl group. The pmr spectrum showed a two-proton pair of doublets at δ4.54 and 5.05 (J=12Hz) characteristic for antiperiplanar *trans* H–2 and H–3 resonances (28). The corresponding signals were shifted to δ5.33 and 5.71 in the triacetate. In the cmr spectrum of **4**, the signal for C–3 was seen at δ73.4 (*d*), as expected (29 30). The absolute configuration was assigned to be 2*R*:3*R* by comparison with published spectra for 3-hydroxyflavanones (26, 31).

The antimicrobial potencies of these agents in an *in vitro* agar dilution-streak test is given in the table. Attention is drawn to the rather potent activity of



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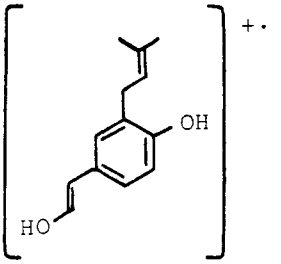

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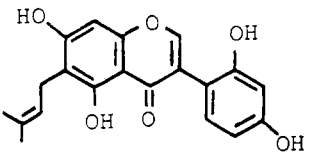
glabrol against *Staphylococcus aureus* and *Mycobacterium smegmatis*. This work appears to be the first in which antimicrobial activity is described for isoflavans and suggests that a systematic study would be worthwhile. Among similar higher plant secondary metabolites, the closest previous reports of antimicrobial activity appears to be those of 5,7,2',4'-tetrahydroxy-6-(3,3-dimethylallyl)-isoflavone (**20**) from *Lupinus luteus* (32), sophoraisoflavanone A (**21**) from *Sophora tomentosa* (34), dichamanetin (**22**) and related substances from *Uvaria chamae* P. Beauv. (35), and cajanone (**23**) from *Cajanus cajan* Mill sp. (36).



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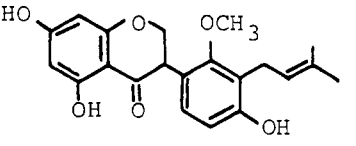

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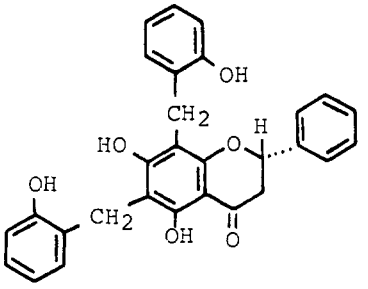

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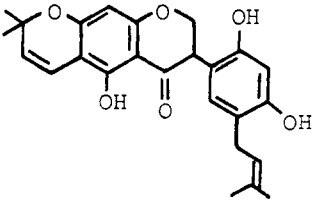

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EXPERIMENTAL[2]

PLANT MATERIAL.—The roots of *Glycyrrhiza glabra* L. var. *typica* (Spanish licorice) used in this study were purchased from S. B. Penick and Company in 1971 and were ground before extraction.

[2]Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Uv spectra were recorded on a Cary model 14 or 219 recording spectrophotometer; ir spectra on a Beckman model IR-33 spectrometer; pmr spectra on a Varian model EM360 spectrophotometer with TMS as internal standard; cmr spectra on a Bruker WP80 spectrophotometer; mass spectra on a Varian CH-5B mass spectrometer; ord on a Cary-model 60 CD/ORD and microanalyses with a Hewlett-Packard model 185B analyzer.

TABLE 1. *In vitro* antimicrobial activity of *G. glabra* principles.

Substance	Minimum Inhibitory Concentration (mcg/ml) Organism No.[1]					
	1	2	3	4	5	6
Glabridin (**1**)	6.25	i	i	i	6.25	25
Glabrol (**2**)	1.56	i	i	i	1.56	i
Glabrene (**3**)	25	i	i	i	25	i
3-Hydroxyglabrol (**4**)	6.25	i	i	i	6.25	i
4'-*O*-methylglabridin (**5**)	6.25	i	i	i	3.12	i
3'-Methoxyglabridin (**6**)	50	i	i	i	i	i
Formononetin (**7**)	i	i	i	i	i	i
Phaseollinisoflavan (**8**)	25	i	i	i	12.5	i
Hispaglabridin A (**9**)	3.12	i	i	i	3.12	i
Hispaglabridin B (**13**)	6.25	i	i	i	3.12	i
Streptomycin Sulfate	5	5	50	2.5	1.25	i

[1]1=*Staphylococcus aureus* (ATCC 13709), 2=*Escherichia coli* (ATCC 9637), 3=*Salmonella gallinarum* (ATCC 9184), 4=*Klebsiella penumoniae* (ATCC 10031), 5=*Mycobacterium smegmatis* (ATCC 607), 6=*Candida albicans* (ATCC 10231), i=no inhibition at 100 mcg/ml.

EXTRACTION, FRACTIONATION AND COLUMN CHROMATOGRAPHY.—Ten 150 g portions of the dried and ground roots were extracted for 20–24 hrs with 20 liters of absolute ethanol in a Soxhlet extractor. Concentration of the combined extracts under reduced pressure at 40° gave 258 g of a dark-brown residue. The residue was suspended in 2 liters of 5% hydrochloric acid solution and extracted with 7 x 3.5 liters of chloroform.

The combined chloroform layer was back washed with 5% HCl solution and dried over sodium sulfate. Filtration and evaporation of the chloroform fraction produced 41.3 g of a crude residue which showed activity against *Staphylococcus aureus* (ATCC 13709) and *Mycobacterium smegmatis* (ATCC 607) at 100 μg/ml[3] and *Candida albicans* (ATCC 10231) at 1,000 μg/ml when tested by means of an agar-dilution streak method (1).

The aqueous acid layer was made alkaline with concentrated ammonium hydroxide solution (to pH 9) and extracted with chloroform. Evaporation of the dried chloroform layer produced 3.4 g of a brown oily residue which was antibacterially inactive. The aqueous alkaline layer was acidified with hydrochloric acid, evaporated to dryness, and found to be antibacterially inactive. Some material (110 g) was insoluble in the chloroform and 5% hydrochloric acid layers and was found, also, to be antibacterially inactive.

The bioactive residue (41.3 g) was dissolved in 700 ml of 90% methanol and extracted with 5 x 1,000 ml of *n*-hexane. Evaporation of the dried *n*-hexane fraction produced 8.4 g of inert material. Some dark brown material (2.0 g) was insoluble in both layers and was found to be weakly active (= at 1,000 μg/ml) against only *M. smegmatis*. Evaporation of the methanol phase produced 27.3 g of a bioactive (100 μg/ml *vs.* three organisms) residue. This fractionation method has been described previously (1).

The bioactive residue (27.3 g) was dissolved in 25 ml of chloroform, and the chloroform solution was applied to a silicic acid (AR 100 mesh, Mallinckrodt) column (7.0 x 66 cm, 1.2 kg) packed in chloroform. Elution with 1.5 liters of chloroform resulted in a fraction containing a negligible amount of oil. Further elution with an additional 3.1 liters of chloroform, 3.5 liters of 0.5% methanol in chloroform, 4.5 liters of 1% methanol in chloroform, 4 liters of 2% methanol in chloroform, and 3.5 liters of 4% methanol in chloroform yielded a number of semicrystalline fractions. Fractions of 50 ml each were collected with the aid of a fraction collector. The antibacterial activity of each fraction was determined, and it was found that activity was spread over most of the fractions eluted with chloroform and 0.5%, 1%, and 2% methanol in chloroform.

ISOLATION OF HISPAGLABRIDIN A (**9**).—The semicrystalline residue (347 mg) from fractions 30–39, on crystallization from cyclohexane, gave 190 mg of hispaglabridin A as white needles, mp 132–133°; $[\alpha]^{25}D$ −8.23 (C=2.43, $CHCl_3$); positive Gibbs test (purple-blue); ir (KBr) νmax 3500, 3350, 2920, 1632, 1610, 1582, 1500, and 1472 cm^{-1}; pmr ($CDCl_3$, 100 mHz) δ1.38 (s, 6H, $C(CH_3)_2$), 1.75 (s, 3H, CH_3), 1.81 (s, 3H, CH_3), 2.85 (dd, 1H, J=6Hz, J<1Hz, A of ABMXX'),

[3]Lowest level in the screening test.

2.88 (dd, 1H, J=10Hz, J<1Hz, B of ABMXX'), 3.30–3.50 (m, 1H, M of ABMXX'), 3.42 (br. d., 2H, J=7Hz, CH_2-CHC$(CH_3)_2$), 3.97 (t, 1H, J=10Hz, X of AMBXX'), 4.36 (br. dd, 1H, J=10 and 4Hz, X' of ABMXX'), 4.87 (s, 1H, exchange D_2O), 5.24 (br. t., 1H, J=7Hz, CH_2-CHC$(CH_3)_2$), 5.42 (s, 1H, exchanges D_2O), 5.51 (d, 1H, J=10Hz, HβC=), 6.32 (d, 1H, J=8Hz, HC=), 6.35 (d, 1H, J=8Hz, HC=), 6.65 (d, 1H, J=10Hz, HαC=), and 6.78 (d, 2H, J=8Hz, 2xHC=); uv λmax (MeOH) 281 nm (log ϵ 4.05), 290 infl. (3.95), and 312 (3.41); ms, m/e 392 (M^-, 23%), 377 (76%), 204 (5%), 189 (30%), 187 (39%), 174 (23%), 173 (100%), and 147 (19%); ord (C=0.000036, MeOH), $[\phi]_{365}$ 0, $[\phi]_{295}$ +2725, $[\phi]_{289}$ 0, $[\phi]_{275}$ −7625, $[\phi]_{265}$ −1850.

Anal. Calcd. for $C_{25}H_{28}O_4$: C, 76.50; H, 7.19. Found: C, 76.47; H, 7.39.

ISOLATION OF HISPAGLABRIDIN B (**13**).—Chromatography of the residue (289 mg) from fractions 40–49 on silica gel (230–400 mesh, 13 g) using 5% ether in benzene afforded 178 mg of pure, amorphous hispaglabridin B; $[\alpha]^{25}D$ −25.7 (C=2.35, $CHCl_3$); positive Gibbs test (blue); ir (KBr) νmax 3400, 1635, 1605, 1583, 1497 and 1475 cm^{-1}; pmr ($CDCl_3$, 60 mHz) δ1.40 (s, 12H, 2=$(CH_3)_2$), 2.83 (2H, AB of ABMXX'), 3.3–3.5 (1H, M of ABMXX'), 3.97 (1H, X of ABMXX'), 4.33 (1H, X' of ABMXX'), 5.00 (s, 1H, exchanges D_2O), 5.47 (d, 1H, J=9.5 Hz, HβC=), 5.50 (d, 1H, J=10 Hz, HβC=), 6.13 (d, 1H, J=8 Hz, HC=), 6.28 (d, 1H, J=8 Hz, HC=), 6.57 (d, 1H, J=9.5 Hz, HαC=), 6.60 (d, 1H, J=10 Hz, HαC=), 6.71 (d, 1H, J=8 Hz, HC=), and 6.74 (d, 1H, J=8 Hz, HC=); uv λ max (MeOH) 280 nm (log ϵ 4.17), 290 infl. (4.11) and 309 (3.67); ms, m/e 390 (M^+, 29%), 375 (100%), 202 (2%), 189 (29%), 187 (67%), 180 (38%), 173 (100%) and 147 (17%); ord (C=0.000043, MeOH), $[\phi]_{310}$ −8350, $[\phi]_{300}$ −10875, $[\phi]_{292}$ 0, $[\phi]_{280}$ +14500, and $[\phi]_{264}$ 0.

Anal. Calcd. for $C_{25}H_{24}O_4$: C, 76.88; H, 6.71. Found: C, 77.16; H, 7.21.

ISOLATION OF 3'-METHOXYGLABRIDIN (**6**).—Chromatography of the residue (326 mg) from fractions 50–60 on silica gel (230–400 mesh, 15 g) with 5% ether in benzene eluent afforded 175 mg of 3'-methoxyglabridin, mp 104–105° (from cyclohexane); $[\alpha]^{25}D$ +10.28 (C=1.41, $CHCl_3$); positive Gibbs test (deep marine blue); ir (KBr) max ν 3480, 2980, 2940, 2850, 1630, 1610, 1580, 1508 and 1476 cm^{-1}; pmr ($CDCl_3$, 100 mHz) δ1.38 (s, 6H, C$(CH_3)_2$), 2.87 (1H, A of ABMXX'), 2.91 (1H, B of ABMXX'), ca. 3.5 (m, of M of ABMXX'), 3.78 (s, 3H, OCH_3), 4.02 (1H, X of ABMXX'), 4.37 (1H, X' of ABMXX'), 5.51 (d, 1H, J=10 Hz, HβC=), 5.58 (s, 2H, 2 x OH, D_2O exchangeable), 6.34 (d, 1H, J=8 Hz, HC=), 6.38 (d, 1H, J=8.5 Hz, HC=), 6.58 (d, 1H, J=8.5 Hz, HC=), 6.65 (d, 1H, J=10 Hz, HαC=), 6.78 (d, 1H, J=8 Hz, HC=); uv λ max (MeOH) 280 nm (log ν 3.98), 289 infl. (3.89), and 311 (3.36) (uv maxima were not shifted by an addition of excess $AlCl_3$ solution.); ms, m/e 354 (M^+, 23%), 339 (78%), 189 (10%), 187 (29%), 173 (100%), 166 (5%), 153 (18%) and 147 (6%); ord (C=0.0000217, MeOH) $[\phi]_{363}$ 0, $[\phi]_{296}$ +3250, $[\phi]_{290}$ 0, $[\phi]_{279}$ +5700, $[\phi]_{270}$ 0, $[\phi]_{250}$ −5225, $[\phi]_{230}$ −8975, $[\phi]_{240}$ 0.

Anal. Calcd. for $C_{21}H_{22}O_5$: C, 71.19; H, 6.25. Found: C, 71.21; H, 6.42.

ISOLATION OF 4'-*O*-METHYLGLABRIDIN (**5**).—Chromatography of the residue (601 mg) from fractions 61–86 (eluted with 0.5% methanol in chloroform) on silica gel (230–400 mesh, 15 g) using 3% ether in benzene produced 254 mg of 4'-*O*-methylglabridin as white needles on crystallization from cyclohexane, mp 120–121°; $[\alpha]^{28}D$+10.2 (C=1.04, $CHCl_3$); positive Gibbs test (blue); ir (KBr) ν max 3410, 2980, 2920, 2860, 1615, 1600, 1583 and 1515 cm^{-1}; pmr ($CDCl_3$, 100 mHz) δ1.40 (s, 6H, C$(CH_3)_2$), 290 (2H, AB of ABMXX'), 3.3–3.6 (1H, M of ABMXX'), 3.70 s, 3H, OCH_3), 4.05 (1H, X of ABMXX'), 4.38 (1H, X' of ABMXX'), 5.20 (s, 1H, exchanges D_2O), 5.50 (d, 1H, J=10 Hz, HβC=), 6.29 (d, 1H, J=2Hz, HC=), 6.32 (d, 1H, J=8Hz, HC=), 6.41 (dd, 1H, J=2 and 8Hz, HC=), 6.61 (d, 1H, J=10Hz, HαC=), 6.77 (d, 1H, J=8Hz, HC=), and 6.96 (d, 1H, J=8Hz, HC=); uv λ max (MeOH) 281 nm (log ϵ 4.07), 290 sh (3.96) and 312 (3.34); ms, m/e 338 (M^+, 25%), 323 (91%), 187 (32%), 173 (100%), 150 (12%), 149 (22%) and 137 (15%); ord (C=0.000048, MeOH), $[\phi]_{310}$ +3525, $[\phi]_{296}$ +5275, $[\phi]_{284}$ 0, $[\phi]_{274}$ −1400 and $[\phi]_{264}$ 0.

Anal. Calcd. for $C_{21}H_{22}O_4$: C, 74.53; H, 6.55. Found: C, 74.47; H, 6.51.

ISOLATION OF GLABROL (**2**).—Column chromatography of the residue (1,577 mg) from fractions 144–151 on silica gel with 10% ether in benzene eluent gave 560 mg of glabrol. The identity was confirmed by a direct comparison with an authentic sample of glabrol (mmp, tlc behavior, ir (KBr) and mass spectra).

ISOLATION OF FORMONONETIN (**7**).—The semi-crystalline residue (485 mg) from fractions 173–178 was washed with chloroform, and the chloroform-insoluble material was recrystallized from ethanol to give 50 mg of colorless rosettes, mp 255–256° (reported, 257–258.5° (11)), which was identical with formononetin as shown by uv, ir, ms, pmr, mp, and the properties of its methyl ether.

ISOLATION OF GLABRENE (**3**).—Column chromatography of the mother liquor residue (310 mg) from fractions 173–178 (from which 50 mg of formononetin was isolated by crystallization); and the residue (652 mg) from fractions 179–186 on silica gel using 20% ether in benzene as the solvent gave 193 mg of slightly brownish granules, mp 200–201° (reported, 198–202° (7)), on crystallization from chloroform-cyclohexane. The compound was identified as glabrene by mp, uv, ir, pmr, ms and the properties of its diacetate, mp 149–150° (reported, 148–150° (7)).

ISOLATION OF SALICYLIC ACID AND *O*-ACETYL SALICYLIC ACID.—The semicrystalline residue from fractions 179–292 produced 688 mg of colorless needles of salicylic acid, mp 157–158°, and 2,254 mg of *O*-acetyl salicylic acid as white needles, mp 138°, on repeated fractional crystallization from benzene and chromatography of the mother liquors on a silica gel column with benzene-ether (8:2) eluent. The identification was confirmed by a direct comparison with salicylic acid (Merck Co.) and *O*-acetyl salicylic acid (Matheson, Coleman & Bell) by mmp, tlc, ir, pmr and ms analyses.

ISOLATION OF 3-HYDROXYGLABROL (**4**).—The mother liquor residue (*ca.* 800 mg) from fractions 197–207, from which 843 mg of *O*-acetyl salicylic acid had been isolated by crystallization, was chromatographed on a silica gel column (30 g), using benzene-ether (8:2) as an eluent; 7 ml fractions were collected. Subfractions 4–11 and 12–18 gave an additional 163 mg of salicylic acid and 200 mg of *O*-acetylsalicylic acid, respectively, on crystallization from benzene. The residue from subfractions 23–32 (106 mg) was chromatographed on a preparative silica gel plate (Brinkman, 0.5 mm) using benzene-ethyl acetate (7:3) to obtain 35 mg of white crystals, mp 117–119°. In a preliminary study with 800 g of the plant material and a more tortuous process, a previous sample (44 mg) had been isolated by purification of the 90% methanol soluble bioactive residue (17.5 g) by silica acid and sephadex LH-20 columns. The data reported was collected at different times on different samples: $[\alpha]^{20}D$ −17.65 (C=1.02, $CHCl_3$) ir (KBr, ν max 3300 (broad), 1652, 1590 and 1505 cm^{-1}; pmr ($CDCl_3$ −acetone d-6 (95:5), 60 MHz), δ1.64 (br. s, 6H, $(CH_3)_2C$), 1.75 (br. s, 6H$(CH_3)_2C$), 3.34 (br. d, 4H, J=7Hz, 2 x $CH_2CHC(CH_3)_2$), 4.54 (d, 1H, J=12Hz, H-3), 5.05 (d, 1H, J=12Hz, H-2), 5.32 (m, 2H, 2 x $CHC(CH_3)_2$), 6.57 (d, 1H, J=9Hz, H-6), 6.73 (d, 1H, J=9Hz, H-5'), 7.3 (m, 2H, H-2' and H-6'), and 7.70 (d, 1H, J=9Hz, H-5); cmr (acetone-d6), δ17.4 (q, 2 x CH_3), 22.1 (t, Ar–CH_2–), 25.3 (q, 2 x CH_3), 28.6 (t, ArCH_2–), 73.4 (d, C-3), 84.4 (d, C-2), 110.5 (d), 112.1 (s), 114.8 (d), 116.7 (s), 122.3 (d), 123.2 (d), 126.1 (d), 126.9 (d), 127.8 (s), 128.9 (s), 129.7 (d), 131.4 (s), 135.7 (s), 155.6 (s), 161.6 (s), 162.4 (s), 193.2 (s, C=0); uv λ max (MeOH) 282 nm (log ε 4.22), 312 sh (3.92), (MeOH/$AlCl_3$), 283 (4.07), 317 (4.17), (MeOH/NaOMe), 247 (4.30), 284 (4.05), and 338 (4.20), (MeOH/$AlCl_3$+HCl), 283 (4.20), 315 (3.90); ms, *m/e* 408 (M^+, 2%), 407 (M^+−1.8%) 379 (29%), 205 (56%), 204 (15%), 189 (4%), 175 (10%) and 149 (100%); ord (C=0.000166, MeOH), $[\phi]_{362}$ 0, $[\phi]_{350}$−2450, $[\phi]_{344}$ +3550, $[\phi]_{337}$ 0, $[\phi]_{330}$ −16200, $[\phi]_{315}$ −30500, $[\phi]_{302}$ =0, $[\phi]_{296}$ −19650, $[\phi]_{284}$+42275, $[\phi]_{276}$−16700, $[\phi]_{266}$ +11800, $[\phi]_{250}$ −15250, $[\phi]_{235}$ +32450, $[\phi]_{228}$ +19675.

Anal. Calcd. for $C_{25}H_{28}O_5$: C, 73.50; H, 6.90. Found: C, 73.37; H, 6.60.

ISOLATION OF PHASEOLLINISOFLAVAN (**8**).—The 90% methanol soluble bioactive residue (17.5 g), obtained from a preliminary study with 800 g of plant material, was chromatographed on a silica acid column (645 g) by elution with chloroform (4,400 ml) and 1% methanol in chloroform (5,300 ml). A pool (2.8 g) was made of an initial 2,420 ml of the 1% methanol in chloroform fractions and rechromatographed on a sephadex LH-20 column (115 g) by elution with chloroform (3,700 ml), 1% methanol in chloroform (3,610 ml), 2% methanol in chloroform (4,270 ml), and 4% methanol in chloroform (4,510 ml). Chromatography of the residue (148 mg) from the initial 700 ml of the 2% methanol in chloroform eluent on a small silica gel column with benzene-ether (9:1) eluent produced 40 mg of pure amorphous material. The material was identified as phaseollinisoflavan by a direct comparison with an authentic sample (tlc, co-tlc, ir, uv, pmr, ms). The spectral properties of its dimethyl ether are identical to those reported for phaseollinisoflavan (6). Ord (C=0.0006, MeOH, $[\phi]_{363}$ 0, $[\phi]_{340}$ −810, $[\phi]_{313}$ 0, $[\phi]_{309}$ +270, $[\phi]_{295}$ +1625, $[\phi]_{290}$ 0, $[\phi]_{280}$ −4325, $[\phi]_{270}$ −2150, $[\phi]_{266}$ −550).

ISOLATION OF GLABRIDIN (**1**).—One semicrystalline residue (4,649 mg) from fractions 245–279 produced 2,149 mg of white crystals, mp 155–156°, after repeated fractional crystallization from benzene and purification by chromatography of the mother liquor residue on a silica gel column with benzene-ethyl acetate (8:2) eluent. The material was identical with glabridin (reported mp 154–155° (6)) as shown by mmp, ir, pmr, ms, and the properties of its diacetate, mp 165–166° (reported mp, 164–166° (6)), and its dimethyl ether, mp 110–111° (reported mp, 109–111° (6)).

DIMETHYL HISPAGLABRIDIN A.—Potassium carbonate (50 mg) was suspended in a stirred acetone solution of hispaglabridin A (40 mg). Dimethyl sulfate (0.1 ml) was added to this suspension. After refluxing for 4 hours, the reaction mixture was diluted with water and extracted with chloroform. The combined chloroform fraction was dried over sodium sulfate and evaporated under reduced pressure, and the residue was chromatographed over a silica gel column with chloroform-benzene (1:1) as eluent to give 36 mg of the *O*-dimethyl ether which was pure by tlc; ir (KBr) ν max 2980, 2930, 2860, 1632, 1595, 1475, 1370, 1265, and 1080 cm^{-1}; nmr ($CDCl_3$, 60 mHz) δ1.38 (s, 6H, $C(CH_3)_2$), 1.63 (br. s, 3H, CH_3), 1.72 (s, 3H, CH_3), 2.78 (2H, AB of ABMXX'), 3.33 (br. d, 2H, J=7Hz, $CH_2CHC(CH_3)_2$), 3.10–3.50 (1H, M of ABMXX'), 3.67 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 3.83 (1H, X of ABMXX'), 4.25 (1H, X' of ABMXX'), 5.10 (br. t, 1H, J=7Hz, $CH_2CHC(CH_3)_2$), 5.42 (d, 1H, J=10Hz, $H\beta C$=), 6.13–6.87 (aromatic 4H and $H\alpha C$=); uv λ max (MeOH) 279 nm (log ε 4.12) 289 sh (3.77) and 310 (3.21); ms, *m/e* 420 (M^+, 32%), 405 (100%), 390 (43%), 232 (5%), 187 (24%) and 173 (63%).

METHYL HISPAGLABRIDIN B.—Treatment of hispaglabridin B (40 mg) with dimethyl sulfate and potassium carbonate in a manner similar to that described for 9 afforded 35 mg of the *O*-methylether which was pure by tlc; ir (KBr) ν max 2970, 2925, 2850, 1635, 1592, 1490, 1475, 1370, 1270, 1080 cm $^{-1}$; nmr ($CDCl_3$, 60 mHz) δ1.37 (s, 12H, 2xC(CH_3)$_2$), 2.85 (2H, AB of ABMXX'), *ca.* 3.4 (1H, M of ABMXX'), 3.74 (s, 3H, OCH_3), 3.98 (1H, X of ABMXX'), 4.33 (1H, X' of ABMXX'), 5.47 (d, 2H, J=10Hz, 2x$H\beta$C=), 6.18–6.87 (aromatic 4H and 2x$H\alpha$C=); uv λ max (MeOH) 279 nm (log ϵ 4.13) 289 sh (4.12) and 310 (3.24); ms, *m/e* 404 (M^+, 27%), 389 (100%), 216 (5%), 201 (33%), 188 (12%), 187 (58%), 173 (64%).

METHYLATION OF 3'-METHOXYGLABRIDIN (**6**).—Treatment of 3'-methoxyglabridin (50 mg) with dimethyl sulfate and potassium carbonate in the manner previously described yielded a residue which was crystallized from cyclohexane to give 40 mg of dimethyl-3'-methoxyglabridin as white crystals, mp 127°; ir (KBr) ν max 2970, 2940, 2900, 2840, 1635, 1590, 1580, 1490, 1455, 1280, 1080 cm^{-1}; pmr (C_6D_6, 60 mHz) δ1.33 (s, 6H, C(CH_3)$_2$), 2.67 (2H, AB of ABMXX'), 3.31 (s, 3H, OCH_3), 3.56 (s, 3H, OCH_3), 3.66 (s, 3H, OCH_3), 3.20–3.70 (1H, M of ABMXX'), 3.78 (1H, X of ABMXX'), 4.23 (1H, X' of ABMXX'), 5.23 (d, 1H, J=10Hz, $H\beta$C=), 6.10–7.10 (aromatic 4H and $H\alpha$C=); uv λ max (MeOH), 278 nm (log ϵ 3.99), 289 infl. (3.61) and 311 (3.16); ms, *m/e* 382 (M^+, 23%), 367 (100%), 194 (10%), 188 (4%), 187 (3%), 181 (30%), 179 (10%), 173 (99%).

Anal. Calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85. Found: C, 71.88; H, 6.80.

ACETYLATION OF 3-HYDROXYGLABROL (**4**).—Five ml of acetic anhydride was added to 5 ml of pyridine solution of 3-hydroxyglabrol (40 mg). The solution was stirred for 22 hrs at room temperature. The reaction mixture was diluted with ice-cold water and extracted with chloroform. The combined chloroform layer was dried over Na_2SO_4 and evaporated to give 50 mg of residue. Crystallization of the residue from ethanol gave 53 mg of white needles, mp 92–93°; ir (KBr) ν max 1760, 1710, 1600 and 1520 cm^{-1}; pmr ($CDCl_3$, 60 mHz) δ1.56 (s, 3H, $-CH_3$), 1.70 (m, 9H, 3 x CH_3), 2.02 (s, 3H, $-\overset{O}{\overset{\|}{C}}-CH_3$), 2.30 (s, 6H, 2X$-\overset{O}{\overset{\|}{C}}-CH_3$), 3.24 (br. d, 4H, J=7Hz, 2XCH_2CHC(CH_3)$_2$), *ca.* 5.10 (m, 2H, 2XC*H*C(CH_3)$_2$), 5.33 (d, 1H, J=12Hz, H-3), 5.71 (d, 1H, J=12Hz, H-2), 6.75 (d, 1H, J=9Hz, H-6), 7.01 (d, 1H, J=9Hz, H-5'), *ca.* 7.3 (m, 2H, H-2' and H-6') and 7.73 (d, 1H, J=9Hz, H-5); ms, *m/e* 534 (M^+, 3%), 533 (M^+-1, 8%), 491 (3%), 473 (9%), 390 (10%), 288 (3.5%), 247 (80%), 246 (35%), 205 (75%), 204 (100%), and 149 (47%).

Anal. Calcd. for $C_{31}H_{34}O_8$: C, 69.65; H, 6.41. Found: C, 69.50; H, 6.42.

CONVERSION OF GLABRIDIN (**1**) TO 4'-*O*-METHYLGLABRIDIN.—Glabridin (50 mg) in chloroform was treated with ethereal-diazomethane for 6 hours at room temperature. The solvent and excess diazomethane were removed by evaporation and the residue, on chromatography over silica gel using benzene-ether (95:5), provided the dimethyl and monomethyl ethers in the ratio one to two. The monomethyl ether, mp 119–120°, was identical with the isolated 4'-*O*-methylglabridin as shown by tlc, mmp, ir, pmr and ms.

METHYLATION OF 4'-*O*-METHYLGLABRIDIN (**5**) TO GIVE DI-*O*-METHYGLABRIDIN.—Treatment of 4'-*O*-methylglabridin with dimethyl sulfate and potassium carbonate in a manner previously stated yielded di-*O*-methylglabridin which crystallized as white needles from ethanol, mp 109° (reported 109–111° (6)). The methyl ether was identical with glabridin dimethyl ether (prepared by the same manner as reported in the preceding section) as shown by mmp, tlc, ir, pmr, and ms.

CONVERSION OF GLABRIDIN TO HISPAGLABRIDIN A.—Glabridin (300 mg) was dissolved in 6 ml of dioxane and heated to 50°. To this solution, 80 mg of 3-methyl-2-buten-1-ol (Aldrich Chem. Co.) and 0.5 ml of $BF_3.Et_2O$ dissolved in 6 ml of dioxane were added over 30 min (22). The solution was stirred for 30 min, cooled, diluted with ether, and then extracted with water several times. The combined water layers were backwashed with ether, and the combined ether layers were then dried over sodium sulfate and evaporated. The residue (a mixture of five compounds by tlc analyses, one of which was the starting compound) was chromatographed on a silica gel column with chloroform. One of the isolated monoisopentenylated materials (15 mg) was found to be identical (tlc, uv, ir, pmr, ms and ord) with **9**.

CONVERSION OF GLABRIDIN TO HISPAGLABRIDIN B.—To a stirred solution of glabridin (486 mg, 1.5 mmole) in pyridine (300 mg) at 160°, 3-hydroxy-3-methyl-1,1-dimethoxybutane (222 mg, 1.5 mmole), prepared by the action of MeMgCl on acetoacetaldehyde dimethyl acetal, was added dropwise, and the heating was continued for 5 hrs (33). The reaction mixture was dried with the aid of high vacuum and chromatographed on a silica gel column with chloroform as eluent. Fractions of 6 ml each were collected. Rechromatography of the residue from fractions 16–33 on a silica gel column with chloroform as eluent provided a compound (29 mg) which was identical with hispaglabridin B (tlc, uv, ir, pmr, ms and ord).

ACKNOWLEDGMENTS

The authors are pleased to acknowledge the NIH for partial financial support of this work under grants AI-13155 and GM 07775. We are grateful to Professors T. Kinoshita, S. Shibata and J. A. Bailey for provision of authentic samples.

Received 24 May, 1979.

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