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INDUCIBLY-FORMED ISOFLAVONOIDS FROM LEAVES OF SOYBEAN

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Key Word Index—*Glycine max*; Leguminosae; soybean; *Pseudomonas pisi*; isoflavonoids; pterocarpans; isoflavone; phytoalexins; antibacterial activity.

Abstract—Isoformononetin, glyceollins I, II and III, and 2-isopentenyl-3,6a,9-trihydroxypterocarpan (glyceocarpin) accumulated in soybean (*Glycine max*) leaves after treatment with aqueous sodium iodoacetate or a cell suspension of the bacterium, *Pseudomonas pisi*. These compounds were also accompanied by two previously unreported pterocarpans, glyceofuran and its 9-*O*-methyl derivative. Glyceocarpin is described for the first time as a plant product.

INTRODUCTION

Four pterocarpan phytoalexins (glyceollins I–IV) have been isolated from cotyledons and hypocotyls of soybean, *Glycine max* following treatment with various chemical elicitors (e.g. aqueous $CuCl_2$) or mycelium of the fungus *Phytophthora megasperma* f. sp. *glycinea* (formerly *P. megasperma* var. *sojae* [1–5]. Cotyledons exposed to Cu ions additionally accumulate 3,6a,9-trihydroxypterocarpan (**1**), a compound with only slight fungitoxic activity [6]. Previous studies have also demonstrated that soybean leaves produce glyceollins I–III (**2**–**4**) as well as coumestrol (3,9-dihydroxycoumestan), daidzein (7,4′-dihydroxyisoflavone) and several other unidentified isoflavonoids, after inoculation with various non-pathogenic *Pseudomonas* spp. [7, 8]. We now report the isolation and characterisation of these additional compounds as isoformononetin (7-methoxy-4′-hydroxyisoflavone, **5**) and three novel pterocarpans for which we suggest the common names glyceofuran (**6**), 9-*O*-methylglyceofuran (**7**) and glyceocarpin (**8**).

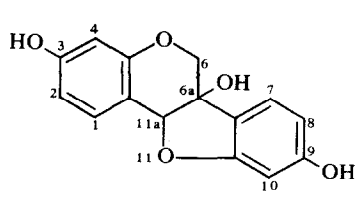
RESULTS AND DISCUSSION

Extracts of soybean leaves infiltrated with either *Pseudomonas pisi* or Na iodoacetate were chromatographed (Si gel TLC; $CHCl_3$–Me_2CO–conc NH_4OH) to give 4 fluorescence-quenching bands (see Experimental) not associated with comparable extracts of healthy (H_2O-treated) leaves. These zones were eluted and their components further purified to yield compounds **5**–**8** and the known *Glycine* phytoalexins, glyceollins I, II and III (**2**–**4**).

Compound **5** (M^+ 268) was readily identified as the rare isoflavone, isoformononetin (7-methoxy-4′-hydroxyisoflavone) by UV, MS and TLC comparison with a sample prepared via selective 7-*O*-methylation of authentic daidzein (see Experimental). Isoformononetin has previously been found in heartwood extracts of the leguminous tree, *Machaerium villosum* (Papilionoideae; tribe Dalbergieae) [9]. Direct comparison of the *Glycine* isoflavone and *Machaerium*-derived isoformononetin confirmed that the two compounds were identical.

Glyceofuran (**6**) had M^+ 354 ($C_{20}H_{18}O_6$) with major fragments at *m/e* 339 ($M^+ - Me$), 336 ($M^+ - H_2O$) and 318 ($M^+ - H_2O - H_2O$) and could be methylated to give a mono Me ether (M^+ 368) indistinguishable (UV, MS, TLC) from a second novel pterocarpan **7** (9-*O*-methylglyceofuran) isolated in small quantities from the bacteria-infiltrated leaves. The presence of a labile C-6a OH group was confirmed by acidic (conc HCl) dehydration to afford a pterocarpene (λ_{max} (nm) 346 and 362 sh), and by ^1H NMR analysis (see below and Table 1) which revealed the two expected doublets (H-6, 6′) at δ 4.20 and 4.24 [1, 2, 10]. Like edulin (neodulin) and neodunol from *Neorautanenia edulis* and *Pachyrrhizus erosus* [11, 12], the UV (EtOH) spectrum of **6** exhibited prominent maxima between 245 and 260 nm, a feature characteristic of pterocarpans with a 2,3-furano substituent.

Upon hydrogenation, glyceofuran afforded two phenolic isoflavans (M^+ 342 and 326) both of which showed MS ions at *m/e* 136 and 123 (attributable to a dihydroxylated aromatic [B] ring), these being accompanied by A-ring derived base peak fragments (*m/e* 207 and 191 respectively) each substituted with a modified dihydrofuran group. Apart from the 6/6′ protons, ^1H NMR analysis of **6** revealed a pattern of aromatic signals corresponding almost exactly with that observed for ring D of 3,6a,9-trihydroxypterocarpan and glyceollins I–IV (Table 1) [1, 2]. Assignment of the furanoid sidechain to C-2/C-3 was confirmed by the presence of a pair of one-proton singlets at δ 6.95 and 7.66 ascribed to the *para*-related aromatic protons H-1 and H-4. The signal at δ 6.63 showing slight (< 1 Hz) long-range coupling was assigned to the β-furanoid proton H-12, whilst the



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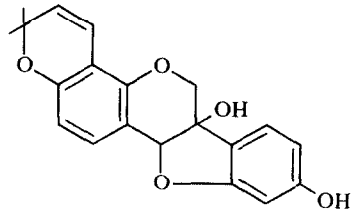

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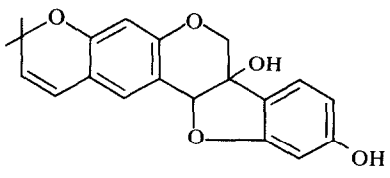

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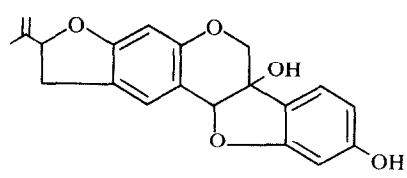

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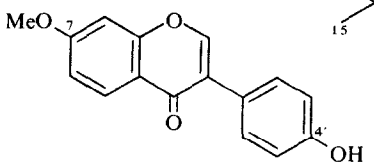

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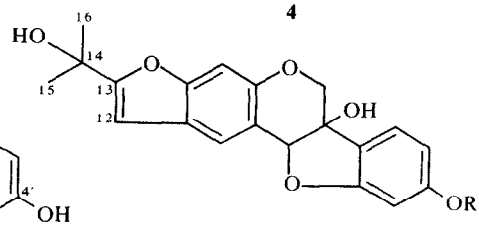
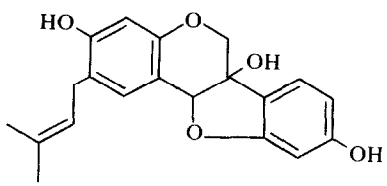


6 R = H
7 R = Me



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equivalent *gem*-Me groups appeared as a characteristically deshielded singlet (δ 1.68), a fact which thus allows the second non-aromatic OH group to be located at C-14 as in the comparable isoflavone, erythrinin C [13]. Both glyceofuran and its naturally occurring 9-*O*-Me ether have negative optical rotations and this, together with the above data, allows their structures to be fully defined as 6a*S*; 11a*S*—(**6**) and 6a*S*; 11a*S*—(**7**) respectively.

The fourth, very minor, *Glycine* isoflavonoid (glyceocarpin, **8**) had a neutral (EtOH) UV spectrum superimposable on that of glyceollin IV (2-isopentenyl-3-methoxy-6a,9-dihydroxypterocarpan) and, like 3,6a,9-trihydroxypterocarpan, developed an intense magenta colouration (λ_{max} 534 nm) in the presence of dilute (0.1 N) aqueous NaOH [6]. ^{1}H NMR analysis showed signals essentially co-incident with those previously reported for 2-isopentenyl-3,6a,9-trihydroxypterocarpan, a compound obtained as a major product when dimethylallyl pyrophosphate and 3,6a,9-trihydroxypterocarpan were incubated *in vitro* with a cell-free preparation from soybean cotyledons (see [14] and Experimental). Structure **8** for glyceocarpin was also supported by the MS which revealed the parent ion at *m/e* 340 and fragments (e.g. $M^+ - 18$ and $M^+ - 18 - 55/56$) similar to those previously reported [14]. Glyceocarpin has a negative optical rotation and is therefore assigned the 6a*S*; 11a*S* absolute configuration.

3,6a,9-Trihydroxypterocarpan, the 3 glyceollin isomers and glyceocarpin were found to exhibit antibacterial activity against *P. pisi* when bioassayed on TLC plates [15] at minimum spotted levels of *ca* 25 μg. In contrast, glyceofuran (100 μg) and isoformononetin (50 μg) lacked both antifungal (against *Cladosporium cucumerinum*) and antibacterial properties; glyceocarpin (30 μg) and 3,6a,9-trihydroxypterocarpan (50 μg) were only weakly antifungal.

The isolation of glyceocarpin from bacteria-inoculated and iodoacetate-treated soybean leaves supports the earlier cell-free experiments of Zähringer *et al.* [14] which suggested that this pterocarpan was a probable biosynthetic precursor of glyceollin II (**3**) and III (**4**). As might be expected, we have also observed that glyceocarpin accumulates in elicitor-treated soybean cotyledons which likewise synthesise pterocarpans **3** and **4**. In contrast, whereas 3,6a,9-trihydroxypterocarpan can be readily isolated from cotyledon extracts (see Experimental), we have failed to demonstrate its presence in soybean leaves. Accordingly, this compound would appear to be of little significance as a phytoalexin in the latter tissues despite its antibacterial activity which is comparable with that of the glyceollin isomers.

Glyceofuran was found to be significantly less antibiotic than glyceollins I–III; moreover, its accumulation in soybean leaves occurred after that of the

Table 1. ^{1}H NMR data for glyceollin II (**3**), glyceofuran (**6**) and glyceocarpin (**8**)

Proton	Glyceollin II		Glyceofuran		Glyceocarpin	
	δ	J(Hz)	δ	J(Hz)	δ	J(Hz)
H-1	7.15*s*		7.66*s*		7.16*s*	
H-4	6.21*s*		6.95*s*[b]		6.34*s*	
H-6	4.04*d*	12	4.20*d*	11	3.97*d*	11
H-6′	4.13*d*	12	4.24*d*	11	4.08*d*	11
H-7	7.21*d*	8	7.24*d*	8	7.19*d*	8
H-8	6.43*q*	8, 2	6.44*q*	8, 2	6.41*q*	8, 2
H-10	6.25*d*	2	6.24*d*	8	6.24*d*	2
H-11a	5.25*s*		5.47*s*		5.23*s*	
H-12	6.41*d*	10	6.63*d*	<1	3.28*d*[b]	7.5
H-13	5.65*d*	10	—		5.34*m*	7.5, 1.5
Me-15	1.37*s*		1.68*s*		1.73*s*[b]	
Me-16	1.40*s*					

s = singlet, *d* = doublet, *q* = quartet, *m* = multiplet, superscript *b* = broad signal.
Spectra were measured at 360 MHz in acetone-d_6 (TMS reference).

various glyceollin isomers (N. T. Keen, unpublished data). These observations suggest that **6** may possibly originate from glyceollin III (the major pterocarpan present in leaf extracts) as a result of either plant or bacterial metabolism. The latter possibility is highly unlikely, however, because large quantities of glyceofuran can be isolated from iodoacetate-treated leaves as well as from tissues inoculated with *P. pisi*. Other workers have recently demonstrated that two sesquiterpene phytoalexins (capsidiol and rishitin) are metabolised by plant cells, a process which in both cases results in hydroxylation of isopropenyl sidechains [16]. The conclusion that glyceofuran represents a plant turnover product is also consistent with the observations of Yoshikawa [3] which suggest that glyceollins may undergo constitutive metabolism in soybean tissues. It should be noted, however, that although relatively large amounts of glyceofuran accumulate in elicitor-treated soybean leaves, only small quantities can be recovered from the cotyledons (see Experimental). The significance of this differential accumulation remains to be explained.

EXPERIMENTAL

Induction and isolation of compounds **1–8** *from leaves and cotyledons.* In a typical expt, leaves of 10–14 day old soybean (*Glycine max* [L.] Merr. cv Harosoy) plants (grown as previously described [15]) were infiltrated [8] with *Pseudomonas pisi* (*ca* 8×10^7 cells/ml in sterile dist. H_2O). Alternatively, the leaves were infiltrated with a 1 mM soln of Na iodoacetate at *ca* pH 7. After a further 2–5 days growth, the leaves (100–150 g) were harvested and then extracted (8 hr) with 40% EtOH using the facilitated diffusion method reported earlier [8]. The resulting soln was filtered, reduced (*in vacuo*, 45°) to *ca* half vol., shaken with Et_2O (100–200 ml) and the aq. phase discarded. Si gel TLC (Merck, GF-254, layer thickness 0.375 mm) of the Et_2O extract using $CHCl_3$–Me_2CO–conc. NH_4OH (CAA) (50:50:1) gave fluorescence-quenching zones at R_f 0.71, 0.56 and 0.25, attributable to compounds **7**, **5** and **6** + **8** respectively. A mixture of glyceollins I–III was located at R_f 0.50. All 4 zones were eluted (Me_2CO) prior to HPLC or TLC purification. Glyceollins I–III were separated by HPLC on a Partisil (10 μg, Whatman) column (0.94 × 50 cm) eluted with hexanes *–*iso*-PrOH (50:4, flow rate 5 ml/min); the eluate was monitored using UV (254 nm) and refractive index detectors. Elution times for glyceollins I, II and III were *ca* 15, 16 and 17 min respectively. Leaf extracts typically contained glyceollins I–III in the ratio 1:3:6. The other *Glycine* isoflavonoids were normally purified by Si gel TLC as follows: (a) **5**, hexanes*–EtOAc–MeOH, 60:40:1 (R_f 0.26) followed by $CHCl_3$–MeOH, 100:3 (R_f 0.29†); (b) **6** + **8**, *n*-pentane–Et_2O–HOAc–MeOH (PEAM), 75:25:3:5 (**6**, R_f 0.16†; **8**, R_f 0.28†) or toluene–$CHCl_3$–Me_2CO (TCA), 40:25:35 (**6**, R_f 0.33; **8**, R_f 0.46); and (c) **7**, PEAM, 75:25:6:3 (R_f 0.48†). An expt with 108 g fr. wt of iodoacetate-infiltrated leaves (harvested after 5 days) gave 165 mg mixed glyceollin isomers, 66 mg glyceofuran, 2 mg glyceocarpin, 1 mg 9-*O*-methylglyceofuran, and <1 mg isoformononetin. 3,6a,9-Trihydroxypterocarpan was not detected.

Soybean cotyledons (8 days old) were wounded and then treated with cell wall suspensions of *Phytophthora megasperma* f. sp. *glycinea* (1 mg/ml H_2O) as described elsewhere [17]. Diffusates were recovered after 48 hr incubation, shaken with EtOAc and the extract then chromatographed (Si gel TLC) in CAA 40:60:1 to give glyceollins I–III (R_f 0.70), **6** + **8** (R_f 0.50) and **1** (R_f 0.30). Elution and further TLC in TCA 40:25:35 afforded pure **1** (R_f 0.47), **6** and **8**. A typical expt employing 100 g cotyledons yielded 27 mg mixed glyceollin isomers, 10 mg 3,6a,9-trihydroxypterocarpan, 1 mg glyceocarpin and <1 mg glyceofuran. 9-*O*-Methylglyceofuran and isoformononetin were not detected.

7-Methoxy-4′-hydroxyisoflavone **5** (*isoformononetin*). Diazotised *p*-nitroaniline, pale orange, λ_{max}^{MeOH} (nm): 212 (94%), 235 sh (90%), 249 (99%), 262 (100%), 307 sh (44%); $\lambda_{max}^{EtOH+NaOH}$ (nm) 212, 240, 247 sh, 283; the MeOH spectrum was unaffected by addition of NaOAc. MS *m/e* (rel. int.) 269 (18), 268 (M^+; 100), 267 (46), 266 (8), 152 (8), 151 (68), 150 (21), 122 (23),

* A commercial mixture of hexane isomers (bp 68–70°).

† R_f value on TLC plates (Merck, F-254) of layer thickness 0.25 mm.

118 (32), 107 (14). ^{1}H NMR (acetone-d_6; 360 MHz; TMS reference) δ 3.96 (OMe, *s*, 3H), 6.89 (H-3′,5′,*m*, J = 8.5 Hz, 2H), 7.05 (H-8, *s*, 1H), 7.07 (H-6, *q*, J = 7.5, 2 Hz, 1H), 7.48 (H-2′,6′, *m*, J = 9 Hz, 2H), 8.10 (H-5, *d*, J = 9 Hz, 1H), 8.21 (H-2, *s*, 1H). On TLC plates viewed under long wavelength UV light, isoformononetin exhibited an extremely faint blue fluorescence intensifying slightly upon fuming with NH_3. *Mono Me ether* (CH_2N_2; R_f 0.25, $CHCl_3$). λ_{max}^{MeOH} (nm) 211 (93%), 233 sh (89%), 249 (99%), 260 (100%), 307 sh (47%). MS *m/e* (rel. int.); 283 (19), 282 (M^+; 100), 281 (33), 267 (15), 150 (19), 149 (44), 132 (67). The above Me ether showed a pale blue fluorescence under long wavelength UV light unaffected by NH_3.

Preparation of isoformononetin. CH_2N_2 was bubbled through a soln of daidzein (7,4′-dihydroxyisoflavone, 1 mg) in $MeOH/CH_2Cl_2$ (4:1) for 90 sec. Work up and Si gel TLC ($CHCl_3$–MeOH, 50:3) gave isoformononetin (0.8 mg, R_f 0.43) together with traces of starting material (R_f 0.12). Isoformononetin (lower zone) could be separated from its isomer, formononetin (7-hydroxy-4′-methoxyisoflavone, upper zone) by TLC in *n*-pentane–Et_2O–HOAc (75:25:3, ×5).

Glyceofuran (**6**). Diazotised *p*-nitroaniline, orange. Colourless needles from Me_2CO–hexanes, mp 181° (dec.). λ_{max}^{EtOH} (nm); 226 sh (log ε = 4.24), 250 (4.08), 257 4.12), 287 sh (3.96), 293 (4.00), 306 sh (3.75); $\lambda_{max}^{EtOH+NaOH}$ (nm): 214, 250, 257 sh, 298; $\lambda_{max}^{EtOH+conc.HCl}$ (nm) (i) after 1 min: 211, 225 sh, 240 sh, 253, 275, 294, 305, 319, 346, 362 sh, (ii) after 10 min: 206, 226, 241 sh, 252, 276 sh, 293 sh, 302 sh, 319, 347, 355, 377 sh. MS *m/e* (rel. int.): 355 (6), 354 (M^+; 25), 339 ($M^+ - Me$; 21), 337 (11), 336 ($M^+ - H_2O$; 38), 335 (11), 322 (8), 321 ($M^+ - H_2O - Me$; 38), 320 (9), 319 (25), 318 ($M^+ - H_2O - H_2O$; 89), 317 (100), 311 (17), 277 (9), 159 (8), 123 (7). High resolution MS, M^+ 354.1096 ($C_{20}H_{18}O_6$). ^{1}H NMR, see Table 1. $[\alpha]_{589nm}$ − 242° (*ca* 0.3 mg in 1 ml MeOH). *Mono Me ether* **7** (CH_2N_2; R_f 0.36, $CHCl_3$). λ_{max}^{EtOH} (nm): 211 (100%), 225 sh (58%), 250 (27%), 257 (29%), 287 sh (22%), 293 (26%), 306 sh (17%); $\lambda_{max}^{EtOH+conc.HCl}$ (nm): (i) after 1 min: 210, 226 sh, 251, 258, 274 sh, 293, 306 sh, 319 sh, 346, 356, 376 sh, (ii) after 10 min: 208, 240 sh, 250, 258 sh, 278, 290 sh, 300 sh, 317, 345 sh, 356, 374 sh; the MeOH spectrum was unaffected by addition of aq. NaOH (0.1 N, 2 drops). MS *m/e* (rel. int.) 369 (15), 368 (M^+; 71), 354 (10), 353 ($M^+ - Me$; 56), 352 (5), 351 (14), 350 ($M^+ - H_2O$; 40), 340 (21), 335 ($M^+ - H_2O - Me$; 20), 333 (23), 332 ($M^+ - H_2O - H_2O$; 100), 331 (71), 326 (13), 325 (73), 323 (11), 322 (24), 317 (30), 316 (12).

Hydrogenation of glyceofuran. H_2 (Zn + HCl) was bubbled (30 min; room temp.) through a soln of **6** (*ca* 2 mg) in MeOH (5 ml) containing 10% Pd–C (5 mg). Work up and Si gel TLC ($CHCl_3$–MeOH, 20:3) gave two phenolic products designated 'Isoflavan 1' (R_f 0.56) and 'Isoflavan 2' (R_f 0.52).

'Isoflavan 1'. Diazotised *p*-nitroaniline, orange/yellow; Gibbs reagent, purple/blue. λ_{max}^{EtOH} (nm): 210 (100%), 230 sh (42%), 283 sh (16%), 289 (19%), 294 sh (18%), 299 sh (17%), 305 sh (13%); $\lambda_{max}^{EtOH+NaOH}$ (nm): 218, 246 sh, 296, 300, 306 sh. MS *m/e* (rel. int.) 327 (4), 326 (M^+; 23), 192 (12), 191 (100), 149 (15), 147 (12), 136 (16), 135 (9), 123 (19), 111 (11), 109 (10), 107 (11).

'Isoflavan 2'. Diazotised *p*-nitroaniline, orange/yellow; Gibbs reagent, purple/blue. λ_{max}^{EtOH} (nm): 213 (100%), 230 sh (67%), 283 sh (32%), 290 (39%), 294 sh (38%), 299 sh (35%), 305 sh (27%); $\lambda_{max}^{EtOH+NaOH}$ (nm): 218, 246 sh, 296, 300, 305 sh. MS *m/e* (rel. int.): 343 (6), 342 (M^+; 31), 309 (7), 208 (15), 207 (100), 191 (13), 189 (31), 171 (7), 161 (13), 149 (35), 148 (12), 147 (23), 136 (35), 135 (27), 123 (35), 107 (28).

9-O-*Methylglyceofuran* (**7**). UV and MS data as given for the mono Me ether of **6**. $[\alpha]_{589nm}$ − 247° (*ca* 0.2 mg in 1 ml MeOH).

Glyceocarpin (**8**). Diazotised *p*-nitroaniline, orange. λ_{max}^{EtOH} (nm): 214 (100%), 230 sh (64%), 282 sh (32%), 287 (34%), 292 sh (31%) (cf. glyceollin IV [2] λ_{max}^{EtOH} (nm): 281 sh, 286, 291 sh); $\lambda_{max}^{EtOH+conc.HCl}$ (nm) 213, 253 sh, 292, 330 sh, 340, 357; $\lambda_{max}^{EtOH+NaOH}$ (nm): 218, 247, 293. An intense magenta colouration (λ_{max} 534 nm) rapidly developed (*ca* 30 sec) in the presence of aq. NaOH. MS as lit. [14]. High resolution MS, M^+ 340.1312 ($C_{20}H_{20}O_5$). ^{1}H NMR, see Table 1. $[\alpha]_{589nm}$ − 236° (*ca* 0.1 mg in 1 ml MeOH).

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REFERENCES

1. Lyne, R. L., Mulheirn, L. J. and Leworthy, D. P. (1976) *J. Chem. Soc. Chem. Commun.* 497.
2. Lyne, R. L. and Mulheirn, L. J. (1978) *Tetrahedron Letters* 3127.
3. Yoshikawa, M. (1978) *Nature* **275,** 546.
4. Keen, N. T., Sims, J. J., Erwin, D. C., Rice, E. and Partridge, J. E. (1971) *Phytopathology* **61,** 1084.
5. Burden, R. S. and Bailey, J. A. (1975) *Phytochemistry* **14,** 1389.
6. Ingham, J. L. (1980) *Z. Naturforsch. Teil C* **35,** 384.
7. Keen, N. T. and Kennedy, B. W. (1974) *Physiol. Plant Pathol.* **4,** 173.
8. Keen, N. T. (1978) *Phytopathology* **68,** 1237.
9. Braga De Oliveira, A., Gottlieb, O. R. and Ollis, W. D. (1968) *An. Acad. Bras. Cienc.* **40,** 147.
10. Ingham, J. L. and Markham, K. R. (1980) *Phytochemistry* **19,** 1203.
11. Van Duuren, B. L. (1961) *J. Org. Chem.* **26,** 5013.
12. Ingham, J. L. (1979) *Z. Naturforsch. Teil C* **34,** 683.
13. Deshpande, V. H., Pendse, A. D. and Pendse, R. (1977) *Indian J. Chem.* **15B,** 205.
14. Zähringer, U., Ebel, J., Mulheirn, L. J., Lyne, R. L. and Grisebach, H. (1979) *FEBS Letters* **101,** 90.
15. Keen, N. T. and Ingham, J. L. *Z. Naturforsch. Teil C* (in press).
16. Ward, E. W. B., Stoessl, A. and Stothers, J. B. (1977) *Phytochemistry* **16,** 2024.
17. Keen, N. T. (1976) in *Biochemistry and Cytology of Plant–Parasite Interaction* (Tomiyama, K. *et al.*, eds.) p. 84. Kodansha, Tokyo.