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METABOLISM OF PHASEOLLIN BY *SEPTORIA NODORUM* AND OTHER NON-PATHOGENS OF *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; *Septoria nodorum*; phaseollin; phytoalexin; metabolism; ^{13}C NMR.

Abstract—Phaseollin is metabolised by cultures of *Septoria nodorum*, a non-pathogen of bean, into *cis* and *trans* isomers of 12,13-dihydrodihydroxyphaseollin. These products are much less fungitoxic than phaseollin which suggests that the capacity to detoxify phytoalexins is not confined to pathogenic fungi.

INTRODUCTION

Different fungi exhibit differing degrees of pathogenicity towards a given host plant and it has been suggested that this may be related to their varying capacities to metabolise and detroxify induced antifungal compounds (phytoalexins). Studies on a French bean phytoalexin, phaseollin, have already shown that this compound can be metabolised by different biochemical pathways. Thus phaseollin was converted to 6a-hydroxyphaseollin and 6a,7-dihydroxyphaseollin by *Colletotrichum lindemuthianum* [1], to 1a-hydroxyphaseollone by *Fusarium solani* f.sp. *phaseoli* [2] and to phaseollinisoflavan by *Stemphyllium botryosum* [3]. We have now extended this study to include several other fungal species and *Septoria nodorum* was found to convert phaseollin into two previously undescribed metabolites. The present paper summarises these findings and presents spectroscopic data from which the structures and relative stereochemistries of the metabolites have been deduced.

RESULTS AND DISCUSSION

Eleven species of fungi were incubated with 2 μg of phaseollin per ml for 20 hr. Only 3 fungi, all species of *Colletotrichum*, caused the disappearance of more than 90% of the phaseollin (see Table 1). Two other species, *Septoria nodorum* and *Alternaria brassicicola* reduced the level of phaseollin by 40%, while the remainder caused only slight reductions.

When cultures of *S. nodorum* were incubated with 8 μg of phaseollin per ml the phytoalexin was also metabolised. Examination of the extract of the culture by TLC revealed that two compounds had been produced during the period of incubation. These were absent in cultures not supplied with phaseollin. Time-course studies (Fig. 1) showed that the level of phaseollin was low after incubation for 24 hr, during which period the level of both metabolites increased. However, levels of these metabolites did not increase during incubation for a further 24 hr. The compound which ran to a higher R_f on TLC is hereafter referred to as metabolite 1 and the compound of lower R_f as metabolite 2.

Preliminary investigations showed that metabolites 1 and 2 had very similar properties. Thus both gave a yellow colour with diazotised *p*-nitroaniline and the UV spectra (λ_{max}^{EtOH} 287, 282 nm, *sh*, λ_{max}^{NaOEt} 294 nm) were identical. The mass spectra were also similar with a prominent molecular ion at *m/e* 356 and precise mass measurements confirmed that the metabolites were isomers of molecular formula $C_{20}H_{20}O_6$.

Proton NMR studies of the metabolites (Table 2) indicated the removal of two olefinic resonances (at δ 5.53 and 6.45) from phaseollin and their replacement by two OCH methine resonances in each case. The two new methine protons are coupled with each other, J = 4.5 Hz in metabolite 1, J = 7.2 Hz in metabolite 2,

Table 1. Metabolism of phaseollin by facultative fungi

	Weight of phaseollin extracted (% of control)*
Colletotrichum lindemuthianum	< 10
Colletotrichum lagenarium	< 10
Colletotrichum gleosporiodes†	< 10
Colletotrichum coffeanum†	97 ± 1.0
Septoria nodorum	61 ± 1.5
Alternaria brassicicola	54 ± 10
Cladosporium cucumerinum	76 ± 6
Ascochyta fabae	72 ± 11
Fusarium culmorum	98 ± 11
Aspergillus niger	77 ± 15
Botrytis cinerea	91

* Measured amounts of phaseollin (approximately 200 μg) were added in 2 ml EtOH to 100 ml of each culture. Triplicate flasks were extracted immediately and 24 hr later. The amount of phaseollin obtained after 24 hr is expressed as a percentage of that obtained immediately. The amount of phaseollin obtained immediately varied between 148 and 189 μg.

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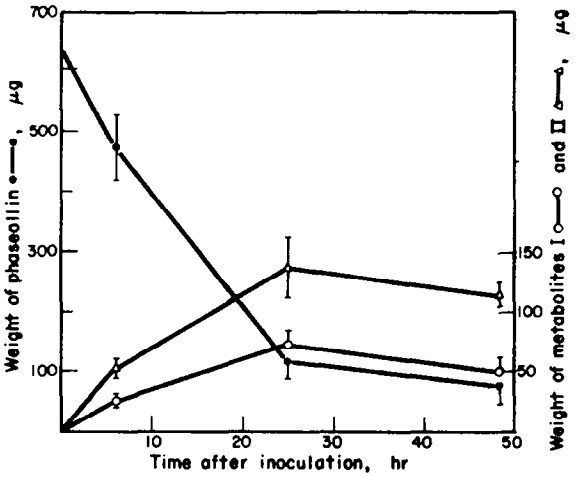


Fig. 1. Metabolism of phaseollin by *Septoria nodorum*.

and together with the increase in molecular weight of 34, this is in keeping with reduction and dihydroxylation of the C_{12}–C_{13} double bond of phaseollin. The characteristics ABMX pattern for the central heterocyclic ring system of phaseollin [4] was also observed for the metabolites.

The carbon-13 spectra (Table 3) are also in accord with the proposed structures for metabolites 1 and 2. Each spectrum consisted of four groups of lines: (a) a set of four lines assigned to oxygenated sp^2 carbons between 153 and 160 ppm; (b) a set of eight lines (including a pair overlapping in metabolite 2 at 109 ppm) associated with unsubstituted or carbon-substituted sp^2 carbons between 103 and 133 ppm; (c) a group of five lines between 62 and 80 ppm due to oxygenated methylene and methine carbons; (d) a group of three lines between 20 and 40 ppm due to non-oxygenated saturated carbon atoms.

The carbon-13 spectrum of phaseollin also contained four groups of lines. However, it differed from those of metabolites 1 and 2 in three respects: (a) in addition to the eight lines between 103 and 133 ppm, two further lines were found in this region, which intensity measurements indicated to be directly protonated (short T_1 + NOE[5]); (b) only three lines were found in the 60–80 ppm region; (c) instead of three lines at high field, there was one line at the same shift (*ca* 40 ppm) as lines in the spectra of metabolites 1 and 2 together with a single doubly intense line at 28 ppm in place of the two separate resonances exhibited by the metabolites. Chemical shift considerations indicate that this is fully in accord with hydroxylation of phaseollin at C-12 and C-13 resulting in (a) removal of two lines at 130.4 and 117.1 ppm from the phaseollin spectrum (b) addition of two lines in the 60–80 ppm region due to two OCH methine carbons and (c) differentiation of the two methyl groups (axial and equatorial) at the C-14 carbon following saturation of the E-ring of phaseollin. Chemical shift correlations are shown in Table 3.

Table 3. Assignment of chemical shifts in the proton noise-decoupled ^{13}C NMR spectra*

	Phaseollin	Metabolite 1	Metabolite 2
C-10a†	159.7	159.0	159.0
C-9†	157.7	159.0	159.0
C-4a†	156.3	157.1	156.9
C-3†	154.5	153.9	153.8
C-1	133.1	132.6	132.4
C-13	130.4	—	—
C-7	125.0	124.7	124.9
C-6b	120.5	118.8	119.0
C-12	117.1	—	—
C-11b	112.7	112.2	112.2
C-2	110.4	109.8	109.8
C-8	109.1	109.0	109.0
C-10	106.5	107.8	109.0
C-4	103.9	103.2	103.2
C-11a	79.8	79.3	79.4
C-14	76.6	77.5	78.2
C-13	—	71.3	75.1
C-6	67.1	66.5	67.1
C-12	—	62.0	66.3
C-6a	40.5	39.7	39.4
C-14a	28.0	25.2	25.7
C-14a'	28.0	21.8	20.0

* Shifts in ppm downfield from TMS, CD_3COCD_3 solvent.
† Tentative assignments only.

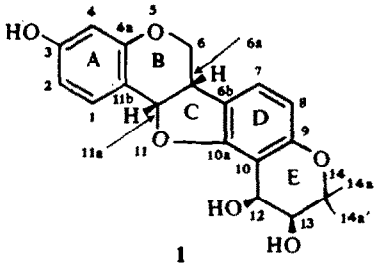
Given the gross structure of the two metabolites, it remains to detail the stereochemistry of the 1,2-dihydroxy system at C-12 and C-13. The four possible stereoisomers are shown below.

Consideration of Dreiding models of all four isomers shows that the differences in ^{13}C and ^1H spectral parameters of metabolites 1 and 2 are most unlikely to arise as the result of isomerism of the *exo–endo* type. That is to say, the metabolites are not **1** and **2** or **3** and **4**, differing only in respect of the orientation of A/B ring pair to the remaining C/D/E system. This is particularly clear from the *J*-values for the C-12 and C-13 methine protons. The stereochemistry of the B/C ring function is most unlikely to affect the conformation of the E ring and the *J*-values should therefore be the same for the isomer pairs **1/2** or **3/4**. As stated earlier, these *J*-values are quite different in the two metabolites and point rather to a

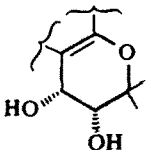
Table 2. Assignment of chemical shifts in the PMR spectra*

	H-1	H-2	H-4	H-6	H-6a	H-7	H-8	H-11a	H-12	H-13	$(Me)_2$
Phaseollin	7.35	6.58	6.35	4.22 3.55	3.55	6.95	6.35	5.55	6.45	5.53	1.45
Metabolite 1	7.32	6.50	6.40	4.20 3.60	3.60	7.03	6.40	5.58	5.00	3.75	1.21
Metabolite 2	7.26	6.50	6.35	4.20 3.40	3.40	6.98	6.35	5.48	4.80	3.60	1.20

* Shifts in δ ppm, $CDCl_3$ solvent.



1



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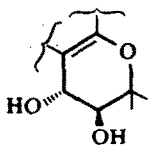

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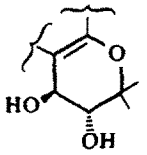
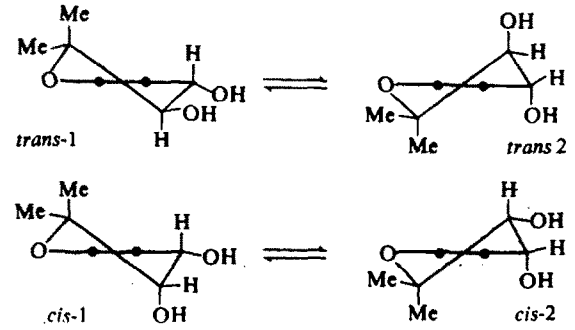


4

pair of isomers **1/3** or **2/4**, i.e. a *cis–trans* glycol pair.

Although the available information does not allow a distinction to be made between the **1/3** and **2/4** pairings, it is possible to specify which isomer has the *cis* and which the *trans* configurations.



trans-1 *trans* 2

cis-1 *cis*-2

If the conformations of ring E are examined it can be seen that *trans*-1 should predominate (two equatorial OH groups, no 1-3 OM/Me interaction). This gives a dihedral angle for the protons of *ca* 150°. In the case of the *cis* isomer it is more difficult to distinguish the conformers in energy terms, although *cis*-2 will have a 1.3 OH/Me interaction and so *cis*-1 should be slightly more stable. In either case, however, the dihedral angle between H-12 and H-13 is 30° and so this does not affect the calculations. The heavy oxygenation will serve to reduce the coupling values in both cases [6, 7] and furthermore, in the case of the *cis* isomer, each conformation allows one of the C-OH bands to lie in an antiperiplanar relationship to the plane of the 'coupling path' and this should further reduce J in the *cis*-isomer [8]. The *cis*-geometry can, therefore, be assigned to metabolite 1 and the *trans* geometry to metabolite 2, the measured coupling constants being respectively 4.5 and 7.2 Hz.

The ^{13}C spectrum provides further evidence for these assignments. Firstly on the basis of chemical shifts, *cis*-1,2-glycols of six-membered systems [9] show C-1 and C-2 chemical shifts smaller than those of their *trans*-isomers and here the chemical shifts of C-12 and C-13 in metabolite 1 are significantly lower than the corresponding shifts in metabolite 2. More reliable evidence, however, rests on the chemical shifts of the *gem*-dimethyl groups on C-14. In phaseollin the only difference between these is their disposition with respect to the ring A/B plane since ring E is planar. This is not sufficient to lead to any chemical shift difference and hence a single resonance at 28.0 ppm is observed. In both metabolite 1 and metabolite 2 both methyl carbon-resonances are shifted upfield. However, although the two equatorial methyls [10] (25.2 for 1, 25.7 for 2) undergo very similar shifts relative to the methyls in phaseollin, the axial methyl of metabolite 1 is shielded by about 2 ppm less than that of metabolite 2. The reason for this lies in the nearly equal shielding of the equatorial and axial methyls by the 13-OH in *2* (conformation *trans*-1, with the OH lying between the methyls) but a smaller degree of shielding of the axial methyl in 1 (conformation *cis*-1 with *trans*-periplanar axial Me and 13-OH).

It can, therefore, be concluded that metabolite 1 has either structure **1** or **2**, and metabolite 2 has either structure **3** or **4**.

The metabolites produced from phaseollin by the non-pathogen *Septoria nodorum* thus differ from those produced by the pathogenic fungus *Colletotrichum lindemuthianum*. With this latter fungus 6a-hydroxyphaseollin (metabolite 1) and 6a,7-dihydroxyphaseollin (metabolite 2) are formed [1]. In order to assess whether such conversions can be related to pathogenicity, it is necessary to assess the toxicity of the metabolites. Their effect, compared with phaseollin, on the growth of one-day-old sporelings of both *Septoria nodorum* and *Colletotrichum lindemuthianum* is shown in Table 4. It can be seen that phaseollin was extremely toxic, completely preventing growth at a level of 10 μg per ml. The metabolites of *Colletotrichum lindemuthianum* were less active, particularly metabolite 2 which only prevented growth of *Colletotrichum lindemuthianum* at 100 μg per ml and did not prevent growth of *Septoria nodorum*. The *Septoria* metabolites had no effect on either fungus. The results show that metabolism of phaseollin by both fungi leads to a significant detoxification, demonstrating that non-pathogens can detoxify phaseollin. Extensive loss of phaseollin also occurred in cultures of the non pathogens *Colletotrichum laganarium* and *Colletotrichum gleosporiode* (Table 1). Whether detoxification of phaseollin by *Colletotrichum lindemuthianum* plays any part in the disease process must await the results of *in vivo* studies.

EXPERIMENTAL

Fungi were grown in 100 ml sucrose–casein hydrolysate medium [11] for 5 to 7 days. Phaseollin, obtained from virus-infected hypocotyls, was added in EtOH. The amount of phaseol-

Table 4. Effect of phaseollin and its metabolites on fungal growth

	C. lindemuthianum	*S. nodorum*
Colletotrichum metabolite 1	50*	100
Colletotrichum metabolite 2	100	> 100
Septoria metabolite 1	> 100	> 100
Septoria metabolite 2	> 100	> 100
Phaseollin	10	10

* Values indicate the lowest levels (μg/ml agar medium) which completely prevented growth of 1-day-old sporelings. Concentrations were calculated from UV data using the extinction coefficient of phaseollin.

lin present immediately and after incubation was measured [1]. Fungitoxicity was measured using 1-day-old sporelings [12]. TLC was performed on Merck Si gel F254 plates in $CHCl_3$–EtOH (20:1). Metabolite 1 had an R_f of 0.23 while metabolite 2 had an R_f of 0.16. Using this technique, 15 mg of metabolite 1 and 25 mg of metabolite 2 were obtained from cultures which had received a total of 200 mg phaseollin. Carbon-13 spectra were obtained using a JEOL PS-100 spectrometer equipped with PFT-100 pulse Fourier transform and SD-HC heteronuclear spin-decoupling units used in conjunction with a JEC-6 computer. Spectra were obtained at 25.15 MHz using a 15.36 MHz phase-locked deuterium resonance as internal lock. Samples were run in hexadeutereoacetone with internal TMS as standard using Wilmad microcells inside 10 mm sample tubes. For phaseollin and metabolite 2 20 mg samples were used and 19 × 1000 transients were block-averaged. In the case of metabolite 1 only a 9 mg sample was available and in this case 37 × 1000 transients were collected. The spectral width in each case was 6.25 KHz and 4096 data points were used to give a resolution of *ca* 0.12 ppm. In the case of phaseollin the *gem*-dimethyl signal was partially obscured by the solvent DC_3 resonance and the presence and singlet nature of the signal was confirmed by re-running the spectrum in $CDCl_3$. Proton NMR spectra were obtained in $CDCl_3$. Mass spectra were obtained using a MS-902 spectrometer with a direct insertion probe. Metabolite 1 exhibited ions at *m/e* 357 (15%), 356 (75), 339 (20), 338 (100), 321 (63), 309 (17), 284 (21), 267 (27) and 147 (13). Metabolite 2 had a very similar spectrum with ions at *m/e* 357 (20), 356 (96), 339 (23), 338 (100), 321 (88), 309 (25), 284 (27), 267 (37), 147 (22). Precise mass measurements were performed using peak-matching methods. Values obtained for metabolites 1 and 2 were 356.128440 and 356.124217 respectively. Calculated value for $C_{20}H_{20}O_6$ is 356.125978.

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REFERENCES

1. Burden, R. S., Bailey, J. A. and Vincent, G. G. (1974) *Phytochemistry* **13**, 1789.
2. Heuvel, J. van den, VanEtten, H. D., Serum, J. W., Coffen, D. L. and Williams, T. H. (1974) *Phytochemistry* **13**, 1129.
3. Heath, M. C. and Higgins, V. J. (1973) *Physiol. Plant Pathol.* **3**, 107.
4. Perrin, Dawn R., Whittle, C. P. and Batterham, T. J. (1972) *Tetrahedron Letters* 1673.
5. Noggle, J. N. and Schirmer, R. E. (1971) *The Nuclear Overhauser Effect*. Academic Press, New York.
6. Waugh, J. S. and Castellano, S. (1961) *J. Chem. Phys.* **35**, 1900.
7. Banwell, G. N. and Sheppard, N. (1962) *Disc. Farad, Soc.* No. **34**, 115.
8. Williams, D. H. and Bhacca, N. S. (1964) *J. Am. Chem. Soc.* **86**, 2742.
9. Perlin, A. S. and Koch, H. J. (1970) *Can. J. Chem.* **48**, 2639.
10. Dalling, D. K. and Grant, D. M. (1967) *J. Am. Chem. Soc.* **89**, 6612.
11. Skipp, R. A. and Deverall, B. J. (1973) *Physiol. Plant. Pathol.* **3**, 299.
12. Skipp, R. A. and Bailey, J. A. (1976) *Physiol. Plant. Pathol.* **9**, 253.