Conversion of Phaseollin to Phaseollinisoflavan by Stemphylium botryosum

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The authors wish to thank J. A. Bailey of Wye College, Ashford, England, for the sample of phaseollinisoflavan; and R. Lazier of the University of Western Ontario for determining the mass spectra.

Accepted for publication 20 August 1973.

ABSTRACT

A compound formed from the pterocarpanoid phytoalexin, phaseollin, by the activity of the fungus *Stemphylium botryosum* was identified as phaseollinisoflavan on the basis of chromatographic comparisons and by ultraviolet absorption, nuclear magnetic resonance, and mass spectra.

Phytopathology 64:105-107

The alfalfa pathogen *Stemphylium botryosum* Wallr. has been shown to alter phaseollin (Fig. 1-I), a pterocarpanoid phytoalexin produced by beans (*Phaseolus vulgaris* L.) (3). The first detectable conversion product, termed "Phas I", was a phenolic compound which was as inhibitory to fungal growth as phaseollin. Phas I itself appeared to be broken down to noninhibitory compounds upon further incubation with *S. botryosum* mycelium. This report pertains to the chemical structure of Phas I. Such information is needed, not only to understand the mechanism by which *S. botryosum* alters phaseollin and possibly other pterocarpanoid phytoalexins, but also to delineate the relationship between the structure of isoflavanoid compounds and their antifungal activity.

The ultraviolet (UV) absorption spectrum of Phas I corresponded closely to the published maxima for phaseollinisoflavan (Fig. 1-II), a compound recently isolated from beans infected with tobacco necrosis virus or with *Colletotrichum lindemuthianum* (1, 2). The nuclear magnetic resonance (NMR) spectrum of Phas I also, and independently, suggested that the compound was phaseollinisoflavan. An authentic sample of the latter was therefore secured from J. A. Bailey, A.R.C. Plant Growth Substances and Systemic Fungicide Unit, Wye College, Ashford, England, for a direct comparison with our substance.

METHODS AND RESULTS. – Phas I was obtained by large scale application of the methods described previously (3) in which phaseollin was added (about 10μg/ml) to germinated spores of *S. botryosum* growing in Czapek - Dox broth. Twenty-four hr after the addition of phaseollin, the cultures were filtered and Phas I was isolated from the culture filtrate and mycelium by solvent extraction and thin-layer chromatography as described previously (3). The final purification step was passage of the Phas I fraction through a Sephadex LH-20 column using 70% ethanol as the elutant.

Both Phas I and phaseollinisoflavan exhibited UV absorption maxima (recorded on Bausch and Lomb Spectronic 505) at 279, ca. 287 (slight inflexion) and 310 (shoulder) nm in neutral ethanol, and both gave a reversible shift to 291 and 336 nm in alkaline (NaOH) ethanol.

Phas I and phaseollinisoflavan were chromatographed in the three thin-layer chromatography solvent systems previously described [Table 1, in (3)]. The two specimens were indistinguishable by R_f values in all three solvents, were of identical appearance when viewed under ultraviolet light, and gave the same orange color with,

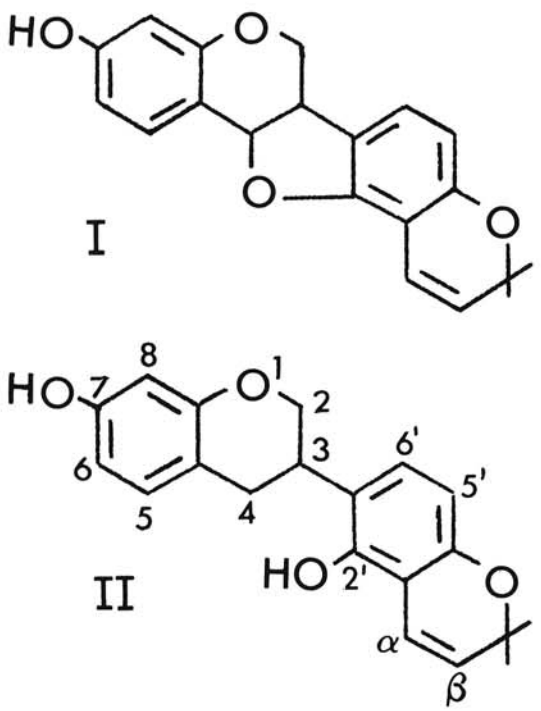

Fig. 1-I, II. Structures of **I)** phaseollin, and **II)** phaseollinisoflavan.

diazotized nitroaniline reagent.

Gas-liquid chromatography on a Pye Series 105 Chromatograph was also used to compare the trimethylsilyl ether (TMS) derivatives of the two specimens. The column used was 3% SE 30 on Chromosorb, W,AW/DMCS, 80- to 100-mesh with a column temperature of 225 C and nitrogen carrier gas flow of 100 ml/min. The TMS derivatives were prepared by adding 20 μl of Tri-Sil (Pierce Chemical Company) to 10 μg of each specimen. Four μl of the preparation were injected. The retention time for both was 13.0 min and on co-chromatography only one peak occurred. The retention time for TMS-phaseollin on the same column was 10.3 min. A compound which was isolated from diffusate obtained from *S. botryosum*- inoculated bean pods and which was suggested (3) to be Phas I produced in vivo also had the same retention time as phaseollinisoflavan.

Nuclear magnetic resonance spectra were determined with a Varian A60A spectrometer equipped with a C1024 time-averaging computer. Chemical shifts (δ) in deuteriochloroform, in parts per million from tetramethylsilane as internal reference, were measured at the geometric center of multiplets. The spectra obtained for Phas I (ca. 2 mg, 36 scans time averaged) were identical, in all significant details, with those of phaseollinisoflavan (6 mg, 9 scans). Assignments (δ, multiplicity, splittings in Hz, protons) were: 6.91, d, 8, H-5; 6.83, d, 8, H-6'; 6.7-6.3, m, -, H-5', H-6, H-8, H_α; 5.67, d,, 10, H_β; 4.35, d X d, 10 X 4, $H\text{-}2_{eq}$; 4.02, d X d (appearing as a triplet), 10 X 10, $H\text{-}2_{ax}$; 3.3, m, -H-3; 2.93, d (broad), 7.5, $H\text{-}4_{eq}$, $H\text{-}4_{ax}$; 1.42, s, -, *gem*-dimethyl.

Mass spectra were taken on a Varian M-66 spectrometer at 70 eV, with the solid sample probe at 100 C. Both specimens exhibited the molecular ion and simple fragmentation patter previously reported (2) for phaseollinisoflavan.

DISCUSSION. – It appears that the first step by *S. botryosum* in converting phaseollin to non-toxic compounds is a reductive ring opening to form the 2'-hydroxyisoflavan phaseollinisoflavan. Although such a change does not seem to have been previously reported for a biological system, chemically 2'-hydroxyisoflavans are readily produced from pterocarpans by hydrogenolysis (5), and Wong (5) proposed that the formation of isoflavans in plants happens by such a step. Recent research has shown that *S. botryosum* has the ability to change other pterocarpans in the same manner. *S. botryosum* alters medicarpin (demethylhomopterocarpin), a phytoalexin from alfalfa and red clover, much more readily than phaseollin (4) and the initial product formed has been identified as the 2'-hydroxyisoflavan, vestitol (P. Steiner and R. L. Millar, *personal communication). S. botryosum* also readily alters maackiain, a phytoalexin from red clover, and spectrophotometric and chromatographic (TLC and GLC) comparisons indicate that the initial product is dihydromaackiain (dihydroxymethylenedioxyisoflavan) (V. J. Higgins, *unpublished).* The possibility that this fungus also converts pisatin (3) to an isoflavan derivative is also being investigated.

These results suggest that care must be taken in interpreting those results where both a pterocarpan and the corresponding isoflavan are isolated from infected tissues. It must be determined whether the isoflavan is a direct product of the host or whether it is produced by the fungus from the pterocarpan. One approach to this problem is to show that the fungus is unable to make this conversion. Alternately, formation of the compounds can be induced with chemicals or viruses rather than with fungi and Burden et al. (2) have used the latter method to show that phaseollinisoflavan can be formed in bean tissue, together with phaseollin, without microbial intervention.

Detoxification of phaseollin by *Fusarium solani* f. sp. *phaseoli*, a pathogen of bean, has also been reported (6) but the main product isolated was not phaseollinisoflavan. This product was considerably less fungitoxic to *Fusarium* than phaseollinisoflavan was to *S. botryosum.*

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