cells of each strain, membranes were prepared and methylene blue–dependent H_2 uptake activity was measured, methylene blue being an excellent electron acceptor for purified rhizobial hydrogenase (*13*) (Table 2). By this criterion, free-living cells of the mutant strains contained significantly more hydrogenase activity than the parent strain. Bacteroids of the oxygen-insensitive strains contained five to six times as much H_2-activating enzyme as bacteroids of the wild-type strain (Table 2).

The isolation of *R. japonicum* mutant strains that synthesize large amounts of hydrogenase as bacteroids in soybean nodules is of potential agricultural significance. As strains of *R. japonicum* with enhanced nitrogenase activity are developed (*14*), it may be helpful to increase their hydrogenase activity correspondingly. In addition, the method we used to select oxygen-insensitive mutants may be useful for increasing the hydrogen-oxidizing activity of *Rhizobium* strains having naturally low H_2 uptake activity.

Mutant strains that express hydrogen-oxidizing activity in the presence of high oxygen also should be useful in answering some basic questions concerning the regulatory role of oxygen in *Rhizobium*. Symbiotic characteristics of *Rhizobium* that are expressed in free-living culture only when the cells are subjected to low oxygen include elevated heme-synthesizing activity (*15*) and differentiation into bacteroid-like morphology (*16*). In addition, hydrogenase (*5, 6*), nitrogenase (*17*), and cytochromes similar to those detected in bacteroids (*15, 18*) are expressed by free-living cells only under low oxygen.

The mutant strains that have higher hydrogenase activity in culture also have higher activity in nodules, suggesting that common elements are involved in regulating the H_2 uptake system in free-living cells and bacteroids.

DAVID MERBERG
ROBERT J. MAIER*

Department of Biology,
Johns Hopkins University,
Baltimore, Maryland 21218

References and Notes

1. S. L. Albrecht, R. J. Maier, F. J. Hanus, S. A. Russell, D. W. Emerich, H. J. Evans, *Science* **203**, 1255 (1979).
2. R. M. Zablotowicz, S. A. Russell, H. J. Evans, *Agron. J.* **72**, 555 (1980).
3. J. E. Lepo, R. Hickok, M. Cantrell, S. A. Russell, H. J. Evans, *J. Bacteriol.* **146**, 614 (1981).
4. F. J. Hanus, R. J. Maier, H. J. Evans, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1788 (1979).
5. J. E. Lepo, F. J. Hanus, H. J. Evans, *J. Bacteriol.* **141**, 664 (1980).
6. R. J. Maier, F. J. Hanus, H. J. Evans, *J. Bacteriol.* **137**, 824 (1979); R. J. Maier, N. E. R. Campbell, F. J. Hanus, F. B. Simpson, S. A. Russell, H. J. Evans, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3258 (1978).
7. R. J. Maier, J. R. Postgate, H. J. Evans, *Nature (London)* **276**, 494 (1978).
8. R. J. Maier, *J. Bacteriol.* **145**, 533 (1981).
9. ——— and D. M. Merberg, *ibid.* **150**, 161 (1982).
10. P. E. Bishop, J. G. Guevara, J. A. Engelke, H. J. Evans, *Plant. Physiol.* **57**, 542 (1976).
11. M. R. O'Brian and R. J. Maier, *J. Bacteriol.* **152**, 422 (1982).
12. G. Eisbrenner and H. J. Evans, *ibid.* **149**, 1005 (1982).
13. D. J. Arp and R. H. Burris, *Biochim. Biophys. Acta* **570**, 221 (1979).
14. R. J. Maier and W. J. Brill, *Science* **201**, 448 (1978).
15. Y. J. Avissar and K. R. Nadler, *J. Bacteriol.* **135**, 782 (1979).
16. C. E. Pankhurst and A. S. Craig, *J. Gen. Microbiol.* **106**, 207 (1978).
17. R. L. Robson and J. R. Postgate, *Annu. Rev. Microbiol.* **34**, 183 (1980); D. L. Keister and W. R. Evans, *J. Bacteriol.* **129**, 149 (1976); D. B. Scott, H. Hennecke, S. T. Lim, *Biochim. Biophys. Acta* **565**, 365 (1979).
18. R. M. Daniel and C. A. Appleby, *Biochim. Biophys. Acta* **275**, 347 (1972).
19. R. Wang, F. P. Healy, J. Myers, *J. Plant Physiol.* **48**, 108 (1971).
20. J. M. Vincent, *A Manual for the Practical Study of Root-Nodule Bacteria*. (International Biological Programme, Blackwell, Oxford, 1970), pp. 86–90.
21. R. J. Maier and S. Mutaftschiev, *J. Biol. Chem.* **257**, 2092 (1982).
22. Supported by grant 59-2243-0-1-435-0 from the United States Department of Agriculture. This is contribution 1201 from the Department of Biology, Johns Hopkins University.
* To whom correspondence should be addressed.

29 November 1982; revised 28 January 1983

Crystallization and X-ray Analysis of Stemphyloxin I, a Phytotoxin from *Stemphylium botryosum*

Abstract. *Certain isolates of the plant-pathogenic fungus* Stemphylium botryosum *produce a phytotoxin, stemphyloxin I. This toxin ($C_{21}H_{34}O_6$) was crystallized and its structure was determined by x-ray crystallography to be a β-ketoaldehyde* trans-*Decalin. This compound is a highly unusual natural product. Iron (Fe^{3+}) controls production of toxin by this fungus. Furthermore, iron reacts with the toxin to yield a colored product which aids in its detection on chromatograms and in its quantitative estimation by colorimetry.*

Phytopathogenic fungi are commonly able to produce some or all of the disease symptoms in their respective host plants by means of one or more phytotoxins that they produce (*1*). The foliage blight disease of tomato caused by *Stemphylium botryosum* walr. f. sp. *lycopersici* is no exception (*2*). Culture filtrates of this fungal pathogen have been reported to contain a phytotoxin, stemphyloxin I, that is capable of producing the necrotic and chlorotic blighting symptoms associated with this disease (*3*). Stemphyloxin I is bioassayed by placement of a few microliters onto a puncture wound made on a leaf blade. After 14 hours, measurements are made on the size of the necrotic lesion that develops. Stemphyloxin I at concentrations between 10 and 250 μg/ml exhibits differential toxicity toward various plants, with tomato being the most sensitive and no lesions being produced on barley (*Hordeum sativum* L.) (*3*). One can obtain a more sensitive measure of toxin activity by following the incorporation of ^{14}C-labeled amino acids into protein. White or green tomato cell suspensions are completely suppressed in amino acid incorporation in the presence of 1 μ*M* toxin (*3*).

Few compounds that are classified as phytotoxins have ever been crystallized and subsequently subjected to structural analysis by x-ray crystallography (*1*). We have recently obtained crystals of stemphyloxin I suitable for x-ray analysis. Stemphyloxin I (80 mg) was prepared by silica gel H column chromatography (*3*). The toxin preparation was dried under N_2 and dissolved in 1 ml of 70 percent ethanol. The transparent yellowish solution was transferred onto a watch glass (50 mm in diameter), which was placed in an open petri dish. Distilled water was added up to a final ethanol concentration of 60 percent, and then 50 μl of xylene was added. Crystals appeared during incubation of this ethanolic solution at 4°C for 16 hours (*4*). The crystals were dried under N_2, transferred to a stoppered vial, and kept at 4°C until subjected to x-ray analysis (*5*). They were birefringent under the polarizing microscope and stable for at least 9 months at 4°C. A phasing model was achieved, after some difficulty with the use of direct methods (*6*). Block-diagonal, least-squares refinements with anisotropic heavy atoms and isotropic hydrogens have converged to a standard crystallographic residual of 0.0505 (*7*).

Stemphyloxin I is a highly functionalized β-ketoaldehyde *trans*-Decalin (Fig. 1). It does not closely resemble previously reported metabolites of the plant kingdom. Furthermore, we know of no other reported phytotoxin that has a β-ketoaldehyde functionality. In fact, β-ketoaldehydes are quite rare among natural products, although aliphatic enolized β-ketoaldehydes are major constituents used for defense by the termite *Rhinotermes hispidus* (*8*).

Although the structure of stemphyloxin I is highly substituted with methyl groups, it does not seem to obey the rule

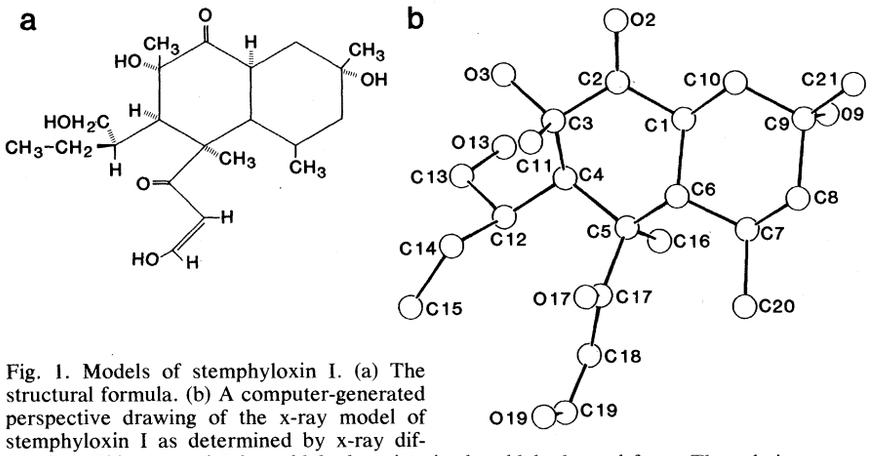

Fig. 1. Models of stemphyloxin I. (a) The structural formula. (b) A computer-generated perspective drawing of the x-ray model of stemphyloxin I as determined by x-ray diffraction. This unusual β-ketoaldehyde exists in the aldehyde-enol form. The relative stereochemical designators are (1S*), (3S*), (4R*), (5R*), (6S*), (7R*), (9R*), and (12R*). Hydrogens are omitted for clarity, and no absolute stereochemistry is implied.

that isoprene units are precursors. Instead, propionyl or acetyl units, or both, through their coenzyme A intermediates are assumed to be the most likely precursors (*9*).

The presence of the enolic β-ketoaldehyde group and the adjacent hydroxyl group on carbon-13 suggest that stemphyloxin I may act as a chelating agent (Fig. 1). It is apparently for this reason that ethanolic solutions of $FeCl_3$ form a red reaction product with the phytotoxin with a maximal absorbance at 480 nm. This reaction has been used for the detection of stemphyloxin I on chromatograms and its quantitative estimation by spectrophotometry (Fig. 2).

Under the standard conditions of culture (*3*), the production of the phytotoxin begins early (5 to 6 days) and quickly becomes proportional to the growth rate of the fungus (Fig. 2). Interestingly, however, the production of stemphyloxin is iron-regulated. The amount of this phytotoxin that is produced is proportional to $[Fe^{3+}]$ in the culture medium, with the optimum at $[Fe^{3+}] = 2$ mg/liter. Therefore, Fe^{3+} was added to the medium in order to optimize toxin production.

Thus far, stemphyloxin I has been found in only nine isolates of *S. botryosum* f. sp. *lycopersici*. These fungi were all originally isolated from tomato plants showing classical symptoms of foliage blight obtained from widely scattered vegetable-growing locations in Israel ranging from Achyhood in the north to Beer Sheva in the south. Other representative *Stemphylium* spp. examined from which stemphyloxin I could not be isolated include the following: *S. botryosum* ATCC (American Type Culture Collection) 26881, *S. botryosum* ATCC 14319, *S. botryosum* f. sp. *lactuca*, *S. botryosum* f. sp. *alfalfa*, *S. loti*, *S. sarcinaeforme*, *S. lancipes*, *S. vesicarium*, and *S. aspercilatum*. Two Israeli isolates of *S. botryosum* f. sp. *lycopersici*, one from Mivchor and the other from Chvat Shalem, did not produce stemphyloxin I.

At this point it is not clear what particular evolutionary advantage exists in those Israeli isolates of *S. botryosum* f. sp. *lycopersici* that produce stemphyloxin I. Conceivably its virulence is enhanced by virtue of its ability to produce stemphyloxin I. Although the structure of the toxin is totally different from any known siderophore (*10*), its interaction with Fe^{3+} suggests that it may interfere with the iron metabolism of the infected plant.

Knowledge of the structure of stemphyloxin will aid in studies of its biosynthesis, mode of action, and genetics. Secondary metabolites with structures related to that of stemphyloxin I, with or without phytotoxic activity, will probably be found in other species of *Stemphylium* and closely related fungal genera such as *Alternaria* and *Helminthosporium*. Recently, betaenones A and B, β-ketoaldehyde polyketide phytotoxins differing only slightly in structure from stemphyloxin I, have been isolated from the phytopathogenic fungus *Phoma betae* (*11*).

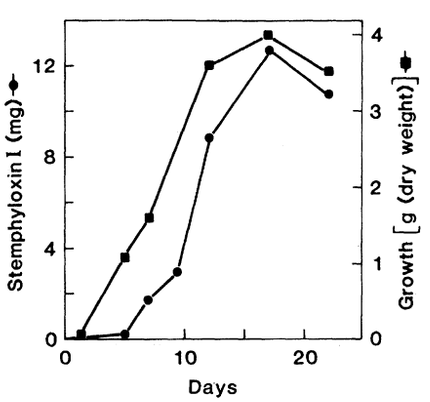

Fig. 2. Production of stemphyloxin I in culture filtrates as a function of time. The *S. botryosum* f. sp. *lycopersici* was grown in a synthetic medium, and stemphyloxin I was extracted with chloroform and purified as described by Rotem *et al.* (*2*). The stemphyloxin I determination was carried out by the following colorimetric procedure: 50 to 250 μg of pure toxin was dissolved in 50 μl of ethanol. After the addition of 50 μl of 5 percent ethanolic $FeCl_3$, the volume was adjusted to 1 ml and the color was read at 480 nm. Linearity was obtained between 50 and 500 μg of stemphyloxin I.

ISAAC BARASH
SHULAMIT MANULIS
Department of Botany, University of Tel Aviv, Tel Aviv, Israel
YOEL KASHMAN
Department of Chemistry, University of Tel Aviv
JAMES P. SPRINGER
Merck Institute for Therapeutic Research, Rahway, New Jersey 07065
MARIE H. M. CHEN
JON CLARDY
Department of Chemistry, Cornell University, Ithaca, New York 14853
GARY A. STROBEL
Department of Plant Pathology, Montana State University, Bozeman 59717

References and Notes

1. G. A. Strobel, *Annu. Rev. Biochem.* **51**, 309 (1982).
2. J. Rotem, Y. Cohen, I. Wahl, *Can. J. Plant Sci.* **46**, 265 (1966).
3. I. Barash, G. Pupkin, D. Netzer, Y. Kashman, *Plant Physiol.* **69**, 23 (1982).
4. Although most of the crystals were needle-shaped and more than 0.2 mm long, a few were considerably thicker and reached a width of 0.1 mm. The largest crystals were gently moved to the edge of the watch glass with a needle. They were rinsed with a few drops of 50, 55, and 60 percent ethanol, respectively, until the yellowish color disappeared. Because of the solubility of the crystals in the highest ethanol concentration, the rinsing procedure was carried out rapidly and carefully.
5. Stemphyloxin I crystallized in space group *P*1 with $a = 8.589(5)$ Å, $b = 11.840(4)$ Å, $c = 12.665(6)$ Å, $\alpha = 64.18(5)°$, $\beta = 77.53(6)°$, and $\gamma = 77.91(6)°$; two molecules of composition $C_{21}H_{34}O_6$ and one C_2H_5OH of crystallization formed the asymmetric unit. All unique diffraction maxima with $2\theta \leq 153°$ (θ is the Bragg angle) were collected with a θ-2θ scan and graphite-monochromated CuKα (1.54178 Å) radiation. After correction for Lorentz, polarization, and background effects, 3067 (61 percent) were judged observed.
6. Crystallographic programs have been described by E. Arnold and J. Clardy [*J. Am. Chem. Soc.* **103**, 1243 (1981)].
7. Crystallographic data have been deposited with the Cambridge Crystallographic Data File.
8. G. S. Prestwich and M. S. Collins, *Tetrahedron Lett.* **2**, 5001 (1980).
9. We are currently testing this hypothesis, using ^{13}C- and ^{14}C-labeled acetate and propionate.
10. J. B. Neilands, *Annu. Rev. Biochem.* **50**, 715 (1981).
11. Information was provided by A. Ichihara, Department of Agricultural Chemistry, Hokkaido University Sapporo 060, Japan (A. Ichihara, in preparation).

12. Supported in part by grants 1.31.79 from the United States–Israel Binational Agricultural Research and Development fund to I.B. and G.A.S., NIH grant CA 24487 to J.C., a Herman Frasch grant to G.A.S., and the Montana Agricultural Experiment Station.

15 November 1982; revised 28 January 1983