(Merck) with 3 solvent systems consisting of ethanol-acetic acid-water (65:1:34), 1-propanol-water (7:3), and 1-propanol-34% ammonium hydroxide (7:3), or on precoated cellulose plates (Funakoshi) with a solvent system of 1-butanol-acetic acid-water (60:15:25), the purified arsenic compound gave a single spot in each system. The spot, which was positive to iodine vapor and the Dragendorff reagent but negative to ninhydrin, appeared at an Rf value identical with that of arsenobetaine. The homogeneity of the purified compound was further evidenced by electrophoresis on cellulose acetate strips (Sartorius); with a buffer system of pyridine-acetic acid-water (1:10:89; pH 3.6), its mobility was the same as that of arsenobetaine.

The purified arsenic compound exhibited no characteristic UV absorption band. Its ^{1}H-NMR spectrum (100 MHz, D_2O) gave 2 signals at δ 1.87 (singlet, 9 H) and 3.30 (singlet, 2 H). The field desorption mass spectrum showed a molecular ion peak at m/z 179 and a base peak at m/z 135 (M^+-CO_2). These spectral data coincided well with those reported for arsenobetaine[3, 7, 12].

Judging from these results, the purified arsenic compound from the shrimp *S. lucens* was identified as arsenobetaine $(CH_3)_3A_s^+CH_2COO^-$. As mentioned above, about 90% of the total arsenic in the shrimp was found in the water-soluble fraction. In addition, 90% of the arsenic in the water-soluble fraction was adsorbed by Dowex 50 × 2 (H^+ form) and it was attributed to arsenobetaine because no arsenic compounds other than arsenobetaine were detected in any steps of the succeeding purification procedure. Therefore, arsenobetaine seemed to account for approximately 80% of the total arsenic of the shrimp.

Fukui et al.[11] previously suggested the presence of an arsenobetaine-containing oligopeptide in the shrimp. However, the suggestion should now be accepted with doubt because their final preparation was apparently contaminated with a large amount of impurities and its arsenic content was as low as 0.092%. Actually, just when we started this work, Norin et al.[12] also reported from the behavior of the compounds in TLC and electrophoresis that not an arsenobetaine-containing oligopeptide but arsenobetaine and arsenocholine were present in shrimps whose scientific names were not given. Our results prove the presence of arsenobetaine in the shrimp *S. lucens* and support those of Norin et al. We could not, however, detect arsenocholine in the shrimp. It seems reasonably safe to assume that, apart from arsenocholine, arsenobetaine is a common arsenic compound in shrimps.

All the marine animals in which the presence of arsenobetaine has been reported so far are carnivores. The present study, together with that of Norin et al.[12], confirmed the presence of arsenobetaine in shrimps which are typical non-carnivores and plankton-feeders. This finding is very interesting from the point of view of the marine ecosystem. Although the arsenic in marine animals in higher trophic levels is present chiefly in organic forms such as arsenobetaine, they cannot transform inorganic arsenic, which is the major form of arsenic in sea water, into organic arsenic in their own bodies[13]. It is very likely, therefore, that the inorganic arsenic in sea water is first incorporated and metabolized to organic arsenic compounds such as arsenobetaine and its precursor by plankton. The shrimp and other plankton-feeders will accumulate arsenobetaine directly from the plankton or will incorporate a precursor from them and convert it to arsenobetaine. Finally, carnivorous animals will get arsenobetaine from their food, including shrimps. Another pathway, from arsenosugars found in the brown kelp to arsenobetaine in marine animals, has also been suggested by Edmonds et al.[14]. For more detailed discussion on the arsenic cycle in the marine ecosystem it will be necessary to elucidate the chemical forms of arsenic in a wide variety of marine animals in connection with feeding habits.

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2 The National Research Council, Arsenic, National Academy of Sciences, Washington, D.C. 1977.
3 Edmonds, J.S., Francesconi, K.A., Cannon, J.R., Raston, C.L., Skelton, B.W., and White, A.H., Tetrahedron Lett. *1977*, 1543.
4 Kurosawa, S., Yasuda, K., Taguchi, M., Yamazaki, S., Toda, S., Morita, M., Uehiro, T., and Fuwa, K., Agric. biol. Chem. *44* (1980) 1993.
5 Cannon, J.R., Edmonds, J.S., Francesconi, K.A., Raston, C.L., Saunders, J.B., Skelton, B.W., and White, A.H., Aust. J. Chem. *34* (1981) 787.
6 Edmonds, J.S., and Francesconi, K.A., Chemosphere *10* (1981) 1041.
7 Shiomi, K., Shinagawa, A., Yamanaka, H., and Kikuchi, T., Bull. Jap. Soc. scient. Fish. *49* (1983) 79.
8 Shiomi, K., Shinagawa, A., Azuma, M., Yamanaka, H., and Kikuchi, T., Comp. Biochem. Physiol. *74C* (1983) 393.
9 Edmonds, J.S., and Francesconi, K.A., J. chem. Soc. Perkin Trans. *1* (1983) 2375.
10 Edmonds, J.S., Francesconi, K.A., Healy, P.C., and White, A.H., J. chem. Soc. Perkin Trans. *1* (1982) 2989.
11 Fukui, S., Hirayama, T., Nohara, M., and Sakagami, Y., J. Fd-Hyg. Soc. Japan *22* (1981) 513.
12 Norin, H., and Christakopoulos, A., Chemosphere *11* (1982) 287.
13 Maeda, S., and Takeshita, T., Kagaku no Ryoiki (J. Japan. Chem.) *36* (1982) 686.
14 Edmonds, J.S., Francesconi, K.A., and Hansen, J.A., Experientia *38* (1982) 643.

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Alteichin: an unusual phytotoxin from *Alternaria eichorniae*, a fungal pathogen of water hyacinth[1]

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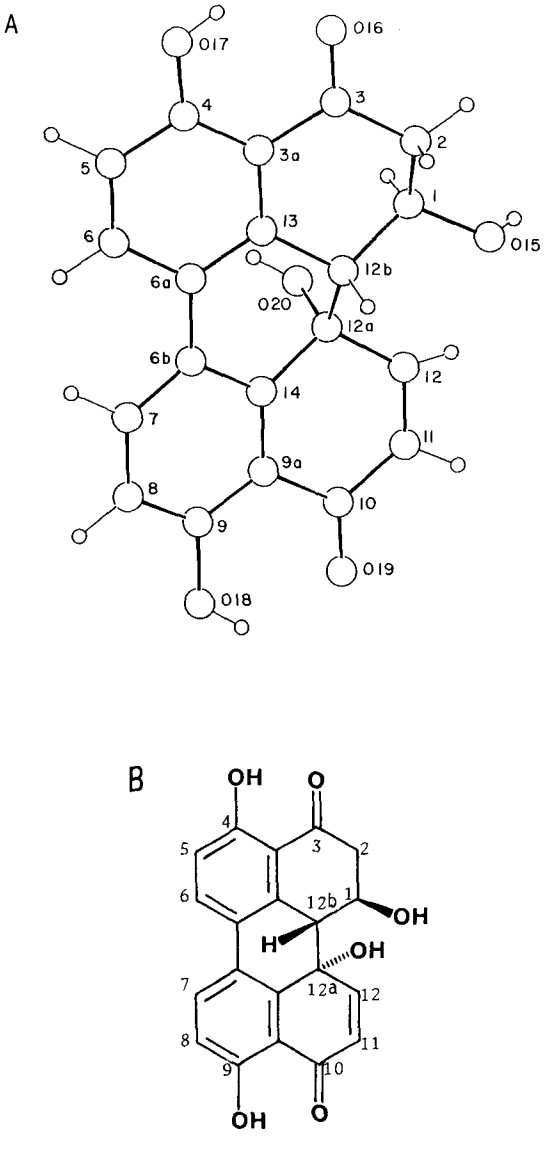
Summary. The phytopathogenic fungus *Alternaria eichorniae* attacks water hyacinth, an economically significant aquatic weed. The novel phytotoxin alteichin was isolated from liquid cultures of this fungus and its structure was deduced by X-ray crystallographic analysis. Altheichin is a doubly hydrated form of 4,9-dihydroxy perylene-3,10-quinone. A single step dehydration of alteichin to anhydroalteichin is catalyzed both by acid and by a crude enzyme preparation from water hyacinth.
Key words. Fungus, phytopathogenic; *Alternaria eichorniae;* phytotoxins; altheichin.

Although the vast majority of well described phytopathogenic microorganisms are parasitic on crop plants, weeds are also attacked by various fungi, bacteria, and viruses. There is considerable current interest in the use of plant pathogenic microbes as agents for the biological control of certain economically important weeds[3]. One potential approach to control weeds is to use phytotoxins or their derivatives for direct application to the noxious plant. Alternatively, a study of the chemistry of phytotoxins may provide useful structural information for the synthesis of novel herbicides. A necessary prelude to these approaches is the isolation, characterization and biological testing of potentially phytotoxic metabolites.

Water hyacinth (*Eichornia crassipes* (Mart.) Solms (Pontederiaceae)) is a perennial, herbaceous, aquatic plant native to the Amazon basin. It is one of the world's most troublesome weeds occurring predominantly in the tropics and subtropics but extending to latitudes 40° N and 45° S[4]. In some areas water hyacinth is a serious problem in paddy crops such as rice and taro, but its ability to block formerly navigable waterways, irrigation canals and drainage ditches is also a significant problem. *Alternaria eichorniae* Nag Raj and Ponnappa is the etiological agent of blight of water hyacinth[5]. Symptoms of this disease are necrotic leaf spots varying in size from small flecks to 6 cm in diameter. Severe infection, in the form of several large spots or numerous small ones, culminates in the premature death of the leaf[5]. Because of this form of symptom expression, the narrow host range of the fungus, and the evidence they obtained for its production of phytotoxic metabolites, Nag Raj and Ponnappa[5] nominated *A. eichorniae* as a possible agent for biocontrol of water hyacinth. Earlier workers described bostrycin, a reduced anthraquinone, from cultures of *A. eichorniae*, but it reportedly lacked phytotoxicity[6]. In a subsequent report other workers observed non-specific phytotoxicity for bostrycin when applied to leaves of a range of plant species[7]. We have confirmed this later report. It seemed likely to us that there were still other phytotoxic metabolites of *A. eichorniae* to be isolated. We grew *A. eichorniae* in liquid shake culture in a modified Czapek-Dox medium for 3–4 weeks[8]. Chloroform extracts of culture filtrates were subjected to thin layer chromatography (TLC) on silica gel using solvent system (a) acetonitrite:water 9:1 v/v as developing solvent. The yellow band appearing at R_f 0.85 was eluted with ethanol and further purified by TLC in solvent system (b), n-pentane: diethyl ether: acetic acid 20:80:3 to afford alteichin R_f 0.58. Another phytotoxin with an R_f 0.48 also appeared in the second TLC system. It should be noted that under these culture conditions bostrycin was not detected. When *A. eichorniae* was grown in potato-dextrose broth shake culture neither alteichin nor bostrycin were detectable. However, still cultures of the pathogen on the Czapek-Dox broth medium yielded altheichin, but not bostrycin. After elution of alteichin from silica gel plates with ethanol, it was further purified by crystallization from ethanol: H_2O (10:90) solutions. The crystals melted at ≥ 350 °C (decomposition) and the yield was circa 10 mg/l.



A computer generated perspective drawing of the final X-ray model of alteichin (A), and the chemical formula of alteichin (B); no absolute configuration is implied.

The structure of alteichin was determined by crystallographic techniques. Preliminary X-ray photographs displayed monoclinic symmetry, and accurate lattice constants of a = 10.352(2), b = 13.336(3), c = 12.353(3) Å, and β = 114.00(2)° were determined. Systematic extinctions and the presumed presence of a chiral object were uniquely accommodated by space group $P2_1$ with 2 molecules of $C_{20}H_{14}O_6$ forming the asymmetric unit. All unique diffraction maxima with $2\theta \leq 114°$ were collected using an ω-scan technique and graphite monochromated CuKα radiation (1.54178Å). After correction for Lorentz polarization and background effects 1878 (80%) reflections were judged observed. ($|F_o| \geq 3\sigma(F_o)$). Solution by direct methods proved very troublesome but eventually a reasonable phasing model was achieved[9]. Block diagonal least squares refinements with 53 anisotropic atoms, 2 molecules of alteichin and a water of crystallization, and 28 isotropic hydrogens have converged to a standard crystallographic residual of 0.07. Figure A is a computer generated perspective drawing of the final X-ray model of alteichin. The absolute configuration ($[\alpha]_D = +90°$) was not determined so the enantiomer shown is an arbitrary choice. Alteichin is (1R*, 12aS*, 12bR*)-1,2,11,12,12a,12b-hexahydro-1,4,9,12a-tetrahydroxyperylene-3,10-quinone, a doubly hydrated derivative of 4,9-dihydroxyperylene-3,10-quinone. The hydroxy at C12a is in a pseudoaxial position while the hydroxy at C1 is pseudoequatorial.

The 300 MHz 'H NMR spectrum in CD_3COCD_3 is fully consistent with the structure in figure A although much of the spectrum is not first order. The phenolic hydroxyls appear as singlets at δ 12.4 and 12.5 and exchange very slowly. The aromatic protons H6, H7 and H12 all appear as overlapped doublets at approximately 8.0. Our tentative assignments are: δ8.05 (d, H6 or H7, J δ8.7 Hz), δ8.04 (d, H12, J ~ 10 Hz),

δ8.01 (d, H6 or H7, J δ8.7 Hz). The remaining aromatic protons H5 and H8 appear as doublets at 6.95 (d, J ~ 8.7 Hz) and 7.04 (d, J δ8.7 Hz). H11 appears as a doublet at 6.31 with J ~ 10z. The proton on C1, and H1 appears as a triplet of doublets with equal *trans* coupling of approximately 10 Hz, and a *cis* coupling constant of approximately 5 Hz at ~ δ4.74. The proton α to the carbonyl and *cis* to the hydroxyl, H2β, appears at δ3.26 as a triplet with essentially identical *trans* and geminal coupling constants of roughly 10 Hz. The remaining 2 protons, H2α and H12b, are a complex 6 line pattern at δ3.01. Selectively decoupling experiments support these assignments. The major peaks in the mass spectrum (chemical ionization, reagent gas methane) are also consistent with the proposed structure. They are m/z 343, ($M+H^+-H_2O$), (22.51%), and a base peak of m/z 315 from a further loss of H_2O. The compound has UV absorption maxima at 254, 285, and 260 with log ε values of 3.68, 3.53 and 2.95, respectively.

Alteichin, at concentration of 1, 5, and 10 µg per 10 µl droplet of 2% ethanol applied to the puncture wound of a water hyacinth leaf produced a necrotic fleck within 12 h. The fleck grew to 3–4 mm in diameter after 5 days and possessed a chlorotic halo. The size of the lesion that developed was a function of alteichin concentration. The lesions resembled those caused by water hyacinth infected by *A. eichorniae*. The toxin, at similar concentrations, also caused necrotic lesions on tomato, Canada thistle, wheat, sunflower, and barley leaves. A more sophisticated bioassay test was performed using the protoplasts of cucumber and the technique of Strange et al.[10]. In this assay, alteichin had mean LD_{50} value of 280 µg/ml for 3 experiments. Conceivably, the production of alteichin by *A. eichorniae* could occur during the plant disease process ultimately leading to some of the characteristic disease symptoms. However, we were unable to detect alteichin in chloroform-methanol-H_2O extracts of blighted water hyacinths. This is not surprising since alteichin is unstable in acidic conditions and is easily transformed to anhydroalteichin, presumably by dehydration from the 12a, 12b positions, and eventually to the water insoluble compound, 4,9-dihydroxyyperylene-3,10-quinone. The addition of 2 µl of concentrated HCl to 1 ml of a 0.5 mM ethanolic solution of alteichin resulted in its complete disappearance, as measured chromatographically, after 12 h at 23 °C. The resulting acid treated product had absorption maxima at 390, 370, 315, and 260 nm which is consistent with the formation of anhydroalteichin. It had R_f values of 0.52 and 0.45 in solvent systems a and b, respectively, and thus behaves differently than the unknown toxin previously mentioned. It, too, is phytotoxic when placed on leaves of water hyacinth and to the same extent as alteichin.

In addition, we have evidence that water hyacinth possesses an enzyme capable of converting alteichin to anhydroalteichin. A freshly prepared acetone powder extract of water hyacinth leaves containing 300 µg protein/ml converted 30% of a 0.5 mM solution of alteichin to anhydroalteichin in 20 h at 23 °C. The reaction was carried out in 5 mM acetate buffer pH 5.1. The conversion of substrate to product was measured by the difference UV spectra after extraction of both the boiled enzyme preparation and the active enzyme preparation with an equal volume of chloroform. Furthermore, the product possessed the same R_f values in solvent systems a and b as anhydroalteichin. The difference spectrum between enzyme treated alteichin and boiled enzyme control possessed identical UV absorbance maxima as anhydroalteichin indicating that one enzymically mediated dehydration had occurred. Thus, it is not completely clear whether alteichin, anhydroalteichin, or both are actually acting as the phytotoxic principle. However, the conversion of the more water soluble alteichin to anhydroalteichin and ultimately to 4,9-dihydroxyperylene-3, 10-quinone may well be the clue to the mechanism by which alteichin causes plant cell death. This quinone itself is known as a fungal metabolite[11] and phytotoxicity has been previously demonstrated for some perylene quinones[12], such as phleichrome[13] and cercosporin[14]. Other quinones are known to be phytotoxic, including juglone, a product of walnut trees[15] which has recently been reported to also be a fungal metabolite[16].

Alteichin may act directly on some site in the plant cell similar to that of cercosporin which causes structural changes in plant membranes[17]. On the other hand, alteichin, once converted to the quinone form could conceivably function as an electron trap, an alkylating agent or an intercalator.

Note added in proof: Since the submission of this manuscript a recent paper by Okuno et al. in Tetrahedron Lett. *24* (1984) 5653 has described similar compounds.

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2 Present address: ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568.

3 Charudattan, R., and Walker, H.L., Eds, Biological Control of Weeds with Plant Pathogens. Eds John Wiley, New York 1982.
4 Holm, L.G., Plunknett, D.L., Pancho, J.V., and Herberger, J.P., in: The World's Worst Weeds: Distribution and Biology. The University Press of Hawaii, Honolulu 1977.
5 Nag Raj, T.R., and Ponnappa, K.M., Trans. Br. Mycol. Soc. *55* (1970) 123.
6 Stevens, K.L., Badar-Ud-Din, A.A., and Admad, M., Phytochemistry *18* (1979) 1579.
7 Charudattan, R., and Rao, K.V., Appl. envir. Microbiol. *43* (1982) 846.
8 *Alternaria eichorniae* was obtained from the American Type culture collection (number 22255). One 1 batches of modified Czapek-Dox liquid medium [D.J. Robeson, G.R. Gray and G.A. Strobel, Phytochemistry *21* (1982) 2359] in 2 L Erlenmeyer flasks were inoculated with a 5-mm^2 agar plug from a culture of *A. eichorniae* maintained on V-8 juice agar and incubated at 25 ± 1 °C on a gyratory shaker at 200 rpm.
9 Crystallographic programs are described in E. Arnold and J. Clardy, J. Am. chem. Soc. *103* (1981) 1243. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre and are also available from J.C.
10 Strange, R.N., Pippard, D.J., and Strobel, G.A., Physiol. Pl. Path. *20* (1982) 359.
11 Anderson, J.M., and Murray, J., Chemy Ind. (1956) 376.
12 Allport, D.C., and Bu'Lock, J.D., J. chem. Soc. (1960) 654. Bu'Lock, J.D., Essays in Biosynthesis and Microbial Development, p.7ff. Wiley, New York 1967. Barbetta, M., Casnati, G., and Ricca, A., Rc. Ist. lomb. Sci. Lett. A *101* (1967) 75. Lousberg, R.J.J.Ch., Salemink, C.A., Weiss, U., and Batterham, T.J., J. chem. Soc. *C* (1969) 1219. Lousberg, R.J.J.Ch., Salemink, C.A., and Weiss, U., J. chem. Soc. *C* (1970) 2159.
13 Yoshihara, T., Shimanuki, T., Araki, T., and Sakamura, S., Agric. biol. Chem. *39* (1975) 1683.
14 Assante, G., Locci, R., Camarda, L., Merlini, L., and Nasini, G., Phytochemistry *16* (1977) 143.
15 Soderquist, C.J., J. chem. Educ. *50* (1973) 782.
16 Stipanovic, R.D., and Bell, A.A., Mycologia *69* (1977) 164.
17 Paub, M.E., and Briggs, S.P., Pl. Physiol. *71* (1983) 763.

0014-4754/84/111248-03$1.50 + 0.20/0