dryness, and added to solid nutrients, vitamins, and agar components of a meridic artificial diet[6]. Newly-molted larvae of the 4 species of aforementioned insects were placed singly on portions of the diet in plastic vials. Daily observations on growth and molting were made; larval weights were determined after 10 days (equivalent in time to 4th instar control larvae). These feeding experiments showed that sendanin inhibits growth in the 4 tested species. Thus, ED_{50}-values for growth inhibition ranged from 9 to 60 ppm, with pink bollworm being the most sensitive and *Heliothis* complex the least (table 1).

At least a part of this growth inhibitory activity of sendanin can be attributed to an 'antifeedant' effect (table 2). Unlike the artificial diet bioassay, this antifeedant effect was determined in a 'choice' situation by confining either fall armyworm or cotton bollworm with both cotton leaf disks treated with sendanin dissolved in acetone and cotton leaf disks treated only with acetone[11]. Scoring of this antifeedant bioassay was done by visually estimating the amount of each leaf disk eaten after 48 h. The scores were then reported as PC_{95} (95% protective concentration) values. Thus, fall armyworm ($PC_{95} = 6.2$ µg/disk) is seen to be about 6 times more sensitive than cotton bollworm ($PC_{95} = 37.9$ µg/disk) in the antifeedant or choice bioassay. This result is similar in pattern to that found in the feeding or no-choice bioassay.

1 Insects were kindly supplied by the agencies of the USDA in Tifton, Ga, Phoenix, Az, and Brownsville, Tx. Authentic sample of sendanin was kindly provided by Prof. M. Ochi. The authors thank J.C. James for the CD measurement.
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Study of the metabolites of *Phyllosticta maydis*. I. Isolation and partial identification

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Summary. Various metabolites of the fungus *Phyllosticta maydis* have been isolated, particularly mevalonolactone. The most interesting of these compounds, which is highly toxic to corn seeds *(Zea mays)*, and which is probably a complex polyol, is the subject of the present report.

Among corn pathogens, *Phyllosticta maydis* Arny and Nelson presents, as does *Helminthosporium maydis* Nisikado and Myake race T, a relative specificity for plants with sterile male 'Texas' cytoplasm[2].

A filtrate of a *P. maydis* culture produces the same overall effects as that of a *H. maydis* culture on isolated mitochondria from Texas plants; inhibition of the coupling of respiration to ATP synthesis, inhibition of malate and α-ketoglutarate oxidations, stimulation of the oxidation of exogenous NADH, and mitochondrial swelling. Characteristic lesions are produced when the culture filtrates of these 2 fungi are applied to the foliar stalks of corn plants. The response obtained with the *H. maydis* extract, however, is the more rapid of the two.

This apparent similarity of action suggests that the toxic compounds of the 2 fungi are related. On the other hand, it is known that plant resistance to the 2 pathogenic agents is not always parallel, since mutagenesis has led to the creation of corn resistant to *H. maydis* race T[3]. Similarly, mitochondria resistant to *H. maydis* are not necessarily resistant to *P. maydis* and *vice-versa*. In parallel with ongoing genetic resistance studies, we have undertaken the characterization of metabolites produced by *P. maydis* in comparison with those produced by *H. maydis*[4].

Materials and methods. The metabolites of *P. maydis* were studied by extracting cultures grown in a modified Fries medium with glucose (30 g/l) and yeast extract (1 g/l) as sole carbon sources. Extractions of mitochondria and tests on corn leaves have been described elsewhere[3,5].

Extraction and isolation. 10 l of culture medium and the corresponding mycelium yielded 11.2 g of a water-soluble thick yellow oil, after isobutanol extraction and reduction of the aqueous phase to 0.1 its original volume. Isobutanol is preferred to the commonly used ethyl acetate, since the latter generates artifacts, regardless of its purity[4]. The resulting oil was dissolved in 30 ml of distilled water and underwent a continuous extraction by diethyl ether for 24 h. After evaporation of the ether, 4.8 g of a yellow oil was obtained, which was carefully neutralized with a cold 0.1 N solution of NaOH. A 2nd continuous extraction with ether yielded the neutral fraction (2.66 g), which was particularly active with Texas cytoplasm. Aqueous metabolite solutions which were not extractable with ether had no effect in the test system.

Spectroscopy. Proton NMR-spectra were obtained with a Varian T.60 spectrometer. The ^{13}C NMR-spectra were recorded with a Varian CFT.20 operating at 20 MHz in Fourier transformed mode. Samples were dissolved in $CDCl_3$ containing 1% TMS as internal standard. IR-spectra were obtained with a Perkin-Elmer 257 spectrometer. Samples were dissolved in CCl_4. Microanalyses were performed at the Central Microanalysis Laboratory, C.N.R.S., Gif-sur-Yvette, France. Mass-spectra were recorded with a ZAB 2.F spectrometer at the Physical Chemistry Institute of the 'Ecole Polytechnique' of Lausanne, Switzerland.

Results. Identification of metabolites. 1. Acid fraction. The last aqueous residue obtained was acidified with dilute cold 1N HCl. Organic acids were then continuously extracted with diethyl ether. A partially crystallized magma was obtained, which was then diluted in a minimum volume of

boiling isopropyl ether. Crystals were isolated from this solution, m.p. 179–180 °C, corresponding to succinic acid (identification by comparison with an authentic sample).

The analysis of spectroscopic data, particularly those of ^{13}C NMR-spectra of methyl esters obtained by diazomethane action on the total acid fraction, demonstrated the esters of oleic, linoleic, lactic and succinic acids. TLC on HF 254 silica gel led to the identification and isolation of a very small quantity (4 mg) of a fluorescent component identified as methyl 5-hydroxymethylfuroate, previously synthesized[4]. Mass spectrometry of all the methyl esters obtained confirmed previous identifications (table 1).

The acid fraction represented about half (2.1–2.2 g) the total metabolites extracted, whose predominant components were succinic, oleic, linoleic, palmitic and stearic acids.

2. Neutral fraction. a) Inactive metabolites. The neutral fraction (2.6–2.7 g) was composed of a major product which distilled colorless at 175–180 °C under atmospheric pressure. Its spectroscopic data are summarized in table 2.

This compound corresponds to meso-2,3-butanediol by comparison with an authentic sample; the racemic geometric isomer is characterized in ^{13}C NMR by 72.5 and 19.3 ppm. The corresponding diacetate was characterized by the following ^{13}C data: 170.4, 71.4, 21.2 and 15.1 ppm. The 2,3-butanediol was inactive towards isolated mitochondria.

Table 1

$M^{+}\cdot$	Methylic esters
256	$CH_3(CH_2)_{13}COOCH_3$
270	$CH_3(CH_2)_{14}COOCH_3$, methyl palmitate
298	$CH_3(CH_2)_{16}COOCH_3$, methyl stearate
294	$CH_3(CH_2)_3(CH_2CH=CH)_2(CH_2)_7COOCH_3$, methyl linoleate
296	$CH_3(CH_2)_7CH=CH(CH_2)_7COOCH_3$, methyl oleate
312	$CH_3(CH_2)_{17}COOCH_3$
340	$CH_3(CH_2)_{19}COOCH_3$
336 and 338	unsaturated esters, homologues of 294 and 296

Table 2

IR CCl_4, cm^{-1}	^{1}H NMR $CDCl_3$, TMS	^{13}C NMR $CDCl_3$, TMS
3620, 3590, 1450	doublet at 1.12 ppm	
1375, 1250, 1045	J = 6.5 Hz	70.9 ppm d.
	quadruplet at 3.78 ppm	
	J = 6.5 Hz	16.8 ppm q.
	large singlet at 3 ppm	
	Ratio of corresponding surfaces: 3/1/1	

Table 3

δ ppm ($CDCl_3$ + 1% TMS)	Multiplicity	Probable structure
171.0	singlet	C=O
68.0	singlet	HO–C–
66.3	triplet	–CH_2OH or –CH_2OCO
44.8	triplet	CH_2CO–
35.2	triplet	CH_2–CH_2–OH
29.7	quadruplet	CH_3CO or CH_3–N

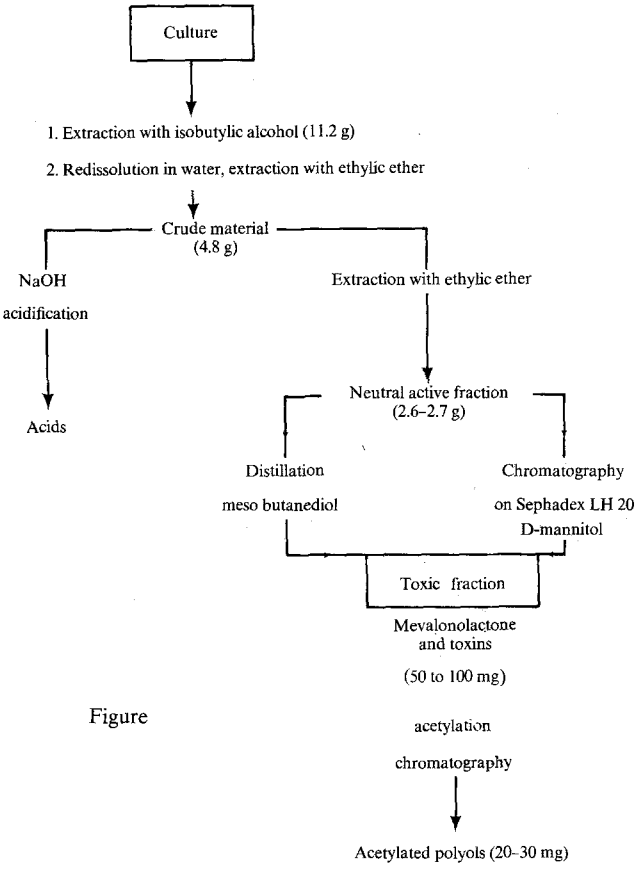
Acetylmethylcarbinol was detected by ^{13}C NMR-spectroscopy. Diacetyl is probably included in the neutral fraction, since the relationship between these 2 compounds and butanediol has been established[6].

Fatty acid esters of butanediol were also demonstrated with ^{13}C NMR, but in low quantities (total of 150 mg), i.e. about 6% of the total, whereas the neutral fraction of *H. maydis* extracts was shown to contain 55–65% triglycerides[4]. The elimination of the diol and its derivatives under 10^{-2} mm of Hg yielded a small quantity of residue which was highly active towards isolated mitochondria. This fraction was also isolated after Sephadex LH 20 chromatography of the neutral fraction, which also yielded 30 mg of a compound crystallized in fine needles, m.p. 167–168.5 °C, which was water soluble. Comparison with an authentic sample showed this compound to be D-mannitol.

The above active fraction, which remains unchanged after diazomethane action, presents an IR-spectrum characterized by intense absorptions at 3500–3300, 1740 and 1660 cm^{-1} (amide or urea). No notable absorption was observed, however, between 1510 and 1570 cm^{-1} (amide band II). TLC on HF 254 silica gel and the various spectroscopic data of the active fraction indicate that it corresponds to a complex mixture in which a major component predominates. This component is characterized by the following ^{13}C NMR-data (table 3).

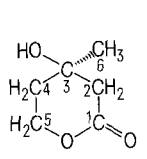
Molecular mass determinations of the mixture with electron impact or chemical ionization mass spectrometry did not yield conclusive results as a result of its complexity.

The spectroscopic data of the isolated metabolite apparently correspond to those of mevalonolactone, which was thus synthesized[10]. Another working hypothesis, based on the study of ^{13}C NMR chemical shifts of the crude toxin, accounting for the IR-absorption at 1660 cm^{-1}, was also considered. A substance responding to these spectra data



Figure

could be the amide **2**, whose synthesis will be described in a forthcoming publication. It is known that *H. carbonum* toxins are polyamides[7]. The comparison of the data obtained, especially those of ^{13}C NMR, leads to the affirmation that mevalonolactone **1** is present in the neutral fraction. ^{13}C NMR ($CDCl_3$), ppm: 171.4 (s) C_1, 67.9 (s) C_3, 66.3 (t) C_5, 44.7 (t) C_2, 35.8 (t) C_4, 29.5 (q) C_6.



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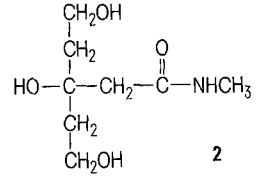

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This molecule was inactive towards mitochondria and so the cytotoxicity of the neutral fraction must, by elimination, be attributed to another compound(s) present in this fraction.

b) Active metabolites or toxins. The figure summarizes the isolation schema of the active fraction.

Following diazomethane treatment and chromatography on Sephadex LH 20, the active fraction was again chromatographed on HF 254 silica gel buffered with $NaHCO_3$. An initial development with pure ether eliminated residual fatty acid esters and mevalonolactone. A 2nd development with methanol: ether (1/9 v/v) eluted other higly polar components soluble in chloroform. These compounds stimulated NADH oxidation by isolated Texas mitochondria (table 4).

Fraction IV (table 4) contained the majority of toxic components. Its IR-spectrum was dominated by a very intense absorption at 3500-3300 cm^{-1}. Low absorptions at 1660 and 1710 cm^{-1} probably reflect the presence of minor contaminants. The elemental composition of this fraction confirmed the presence of nitrogen compounds. It was verified that these compounds were not artifacts introduced by the yeast extract in the culture medium.

The *P. maydis* toxins were acetylated with acetic anhydride in pyridine and benzene under nitrogen and were chromatographed on silica gel. The toxin and these derivatives furnished ^{13}C NMR-spectra which demonstrated certain structural groups (table 5).

As in the spectra of *H. maydis* toxins, this strain, supplied by O.C. Yoder, yielded polyketols with structures different from those described by Kono and Daly[8,9]; these spectra did not demonstrate resonances at 209.8 and 210.4 ppm. The absence of signals between 160 and 209 ppm led to the consideration of *P. maydis* toxins being linear polyols or hydroxyethers. The structural analogy between *H. maydis* and *P. maydis* toxins is seen after comparing the various spectroscopic data. The hypothesis of a linear polyol seems probable, since ^1H NMR-spectra of the different toxic fractions and their acetates, recorded at 400 Hz, confirmed the structural relationship. In both cases, terminal methyl groups appeared in the form of a triplet towards 0.90 ppm, a methylene peak was centered at 1.26 ppm, acetate methyls were found at 2.05-2.10 ppm and the proton peak was found at the foot of the acetates towards 4.90-4.95 ppm.

The toxins of these 2 fungi would thus be related, with the exception of slight differences in the degree of oxidation of the hydrocarbon skeleton.

The acetylated derivatives of the toxic fraction are currently being studied by chemical ionization: negative ions by electron capture and field desorption followed by collisions with the molecular ion. The mass spectrometry of the acetylated polyol from *P. maydis*, obtained by negative ionization, demonstrated a series of ions at m/z = 1380, 1440 and 1500, if the latter value is taken as an indication of molecular mass and if the linear polyacetate hypothesis is admitted, this compound may be represented as: $CH_3(CH_2)_{33}-(CHOAc)_{14}-CH_3$ which corresponds to the elemental composition: C = 61.6%, H = 8.53%.

The same analysis of the acetylated toxic fraction after chromatography: C = 60.3%, H = 8.2%, is a relatively good verification of this hypothesis, since the toxic mixture is fairly complex.

In addition, electron capture negative chemical ionization mass-spectrometry of the acetylated and chromatographed toxic fractions of *P. maydis* and *H. maydis* indicates that they share the ion 822. This indication could serve to corroborate the previous conclusion deduced from ^{13}C and ^1H-NMR-data, i.e. that the 2 families of toxins are related. Although this hypothesis is quite tempting, the presence of contaminants necessitates confirmation and justifies the continuation of this mass-spectrometry study in order to define further the identity of the major toxin by additional

Table 4

	Fraction tested	Toxic activity measured as coefficient of stimulation of NADH oxidation[4]
I	Crude culture filtrate	+ +
II	Active isolated fractions after Sephadex LH.20, HF 254 silica gel (eluted with CH_3OH: ether (9/1)	
	Upper half of plate	+ +
	Lower half of plate	+ + +
III	Meso-2,3-butanediol	inactive
IV	Active fraction obtained after elimination of acids and neutrals, HF 254 silica gel chromatography	+ + + +
V	Mevalonolactone *1*	inactive
VI	Above active fraction after acetylation	inactive

10 µl of fraction IV, i.e. 0.2 µg/ml, provoked symptoms on Texas cytoplasm leaves which were characteristic of toxicity. There was no effect on plants with normal (N) or Charrua (C) cytoplasm.

Table 5

δ ppm ($CDCl_3$ + 1% TMS) Toxic fraction	Acetylated and chromatographed toxins		Probable structure
	170.6	s	C = O acetates carbonyls
	170.4	s	2 distinct groups
72.2	70.7	d	−CHOAc−, 2 distinct groups
68.1	68.0	d	of secondary carbons bearing oxygen with several resonances each
45.3	39.9	t	
43.5	38.4	t	−CHOAc−CH_2−CHOAc−
39.5			
38.0	34.0	t	−CHOAc−CH_2CH_2−CHOAc−
35.8			
31.9	31.4	t	
29.7	29.6	t	
29.2	24.6	t	
24.0	23.9	t	methylenic chain
23.6	22.4	t	
23.1			
22.6	21.2	q	methyl acetates
	21.1	q	
19.4	19.9	t	−CHOAc−$CH_2CH_2CH_2$−CHOAc−
14.1	13.9	q	CH_3−CH_2

structural data. A more elaborate structural model of this compound(s) would be only speculative at present.
Finally, the relationship between the 2 families of toxins is a particularly constructive working hypothesis, since it leads to the better understanding of their respective toxicities.

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Dimethyloxarsylethanol from anaerobic decomposition of brown kelp *(Ecklonia radiata)*: A likely precursor of arsenobetaine in marine fauna[1]

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Summary. The novel arsenic compound, dimethyloxarsylethanol, has been isolated from anaerobically incubated *Ecklonia radiata.* It is proposed that this compound has a key position in the biosynthesis of arsenobetaine.

Arsenic is present as arsenobetaine (fig., e) at levels up to 40 ppm in a range of marine animals used as human food and taken from nonpolluted waters[2-5]. It has been shown that arsenic-containing sugars (fig., a) are present in the brown kelp *Ecklonia radiata* and that tnese are a possible source of arsenobetaine in the western rock lobster, *Panulirus cygnus,* and the school whiting, *Sillago bassensis,* associated with the nearshore waters of Western Australia supporting *Ecklonia*[6]. The conversion of the arsenic-containing sugars to arsenobetaine requires the cleavage of the C_3–C_4 bond of the sugar residue with subsequent oxidation at the C_4 position, and reduction and further methylation at the arsenic atom. Thus both reducing and oxidizing conditions are necessary and if cleavage of the C_3–C_4 bond occurs under reducing conditions (as found in kelp bed sediments or in large beach deposits of kelp) an accumulation of dimethyloxarsylethanol (fig., b) or arsenocholine (fig., d) might be expected. The latter would be produced if reduction and methylation of the arsenic atom occurs under the same reducing conditions. We here report the isolation of dimethyloxarsylethanol from anaerobically incubated *Ecklonia.* The position of this compound as a key intermediate in the biosynthesis of arsenobetaine is indicated in the figure.

Freshly collected *Ecklonia* (50 g) was incubated at room temperature under argon with unfiltered seawater (1 l) and sand (10 g) collected from the same site as the *Ecklonia.* The incubation flask was shaken in the dark for 11 days. Sulphide was first detected after 72 h and continued to be produced until the end of the incubation period. Methane was not detected. After a further 30 days the contents of the fermentation flask, smelling strongly of H_2S, were filtered and the filtrate boiled to dryness and the residue (60 g) extracted with methanol. Atomic absorption spectrometric analysis revealed the bulk of the arsenic (> 200 µg) in the filtrate and subsequently in the methanol extract. Only a trace of arsenic (< 10 µg) remained in the decomposed fragments of *Ecklonia.* TLC examination (n-butanol, acetic acid, water; 60: 15: 25; cellulose) of the crude methanol extract demonstrated the presence of a single major arsenic compound (R_f 0.65) and a trace of arsenic-containing material at R_f 0.2 (the value expected for unchanged arsenic-containing sugars). The major arsenical in the methanol extract was isolated by the following procedure: The methanol was evaporated and the solid residue (14 g) dissolved in water and extracted with phenol. The arsenic compound was recovered from the phenol by dilution with ether and extraction with water. This technique had previously been used to great advantage in the isolation of arsenobetaine from animal tissue[2]. After concentration, the aqueous solution (containing 200 µg As in 400 mg total solids) was twice subjected to gel filtration chromatography (Sephadex LH20, MeOH) to yield 200 µg As in 12 mg total solids. The arsenic compound was further purified by TLC (n-butanol, acetic acid, water; 60: 15: 25; cellulose; R_f 0.63) to yield a white solid (200 µg As in about 500 µg total weight). It was identified as dimethyloxarsylethanol by comparison of its 80 MHz ^1H NMR-spectrum, mass-spectrum and TLC behavior with those of a synthetic specimen. Dimethyloxarsylethanol, prepared by a modification of the method used by Wigren[7,8] to synthesize unsymmetrical dialkylarsyl compounds, crystallized from methanol/acetone as needles (m.p. 148–149.5 °C) which contained: C, 28.54%; H, 6.85%. $C_4H_{11}O_2As$ requires: C, 28.93%; H, 6.63%. IR: ν_{max} (film) 3080, 2800, 1473, 1350, 1264, 1077 s, 1008 s, 920, 893, 870 s, 640, 620 cm^{-1}. NMR (80 MHz ^1H, D_2O) δ 3.90, t, J 6.25 Hz, 2H, $AsCH_2$ CH_2 OH; 2.36, t, J 6.25 Hz, 2H, As CH_2 CH_2; 1.72, s, 6H, $(CH_3)_2$ As. MS (electron impact, 200°/35 eV) 165 (4.5%), 136 (52.5), 123 (13.1), 122 (49.1), 121 (36.8), 107 (100), 105 (9.4), 103 (10.2), 93 (41.1), 91 (35.1), 89 (24.8).

The conversion of arsenic-containing sugars to dimethyloxarsylethanol could occur through the metabolic activity of a number of anaerobic bacterial groups. Under anaerobic conditions, the complete microbial degradation of organic compounds is a sequential process with partial degradation by different microorganisms at each step. Fermentative bacteria utilize complex organic substrates releasing sim-