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Biogenesis of Lung-Toxic Furans Produced during Microbial Infection of Sweet Potatoes (*Ipomoea batatas*)

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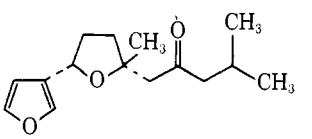
Abstract: Certain pathogenic fungi are able to convert a furanosesquiterpenoid stress metabolite of the sweet potato, 4-hydroxymyoporone (**6**), to the lung-toxic furans **2–5**. Isolation of radioactive **2** and **3** from incubations of ^{14}C-**6** with the fungus *F. solani* demonstrated that **6** can serve as a precursor to the lung toxins. A closely related fungus, *F. oxysporum*, could also convert **6** to **2–5** but less effectively. Compounds **2–5** could not be detected when *C. fimbriata*, another common fungal contaminant of sweet potatoes, was incubated with **6**.

Introduction

The discovery that the aflatoxins are frequent causative agents in toxicoses related to infection of food and feeds by the fungus *Aspergillus flavus* has led to increased concern about the effects of fungal contamination of foodstuffs.[2a] The possibility that the host plant may be producing potentially toxic phytoalexins or stress metabolites in response to fungal infection is also apparent.[2b] Stress metabolites produced by the sweet potato (*Ipomoea batatas*) have been subjects of investigation for many years. These include a group of furanosesquiterpenes, the classic example being ipomeamarone (**1**).[2c]

The toxicity of **1** and similar furanosesquiterpenes is directed primarily toward the liver. The LD_{50} values in mice for the hepatotoxic compounds are in the 200–300 mg/kg range;[2c] neither the site of attack nor the potency of these toxins is exceptional. Another group of more toxic furan compounds, **2–5**, has been isolated from mold-damaged sweet potatoes. Not only are these compounds more potent toxins than **1** (LD_{50} 20–70 mg/kg), but they are unusual in that they exhibit a striking toxicity for the lungs characterized primarily by severe pulmonary edema.[3] Lung-toxic effects of this sort have been noted in disease outbreaks in which cattle were fed culled sweet potatoes infected with fungi.[4]



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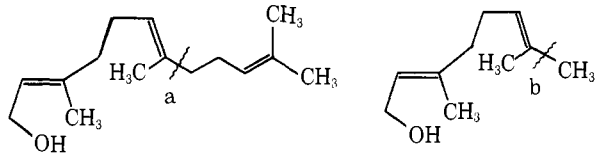
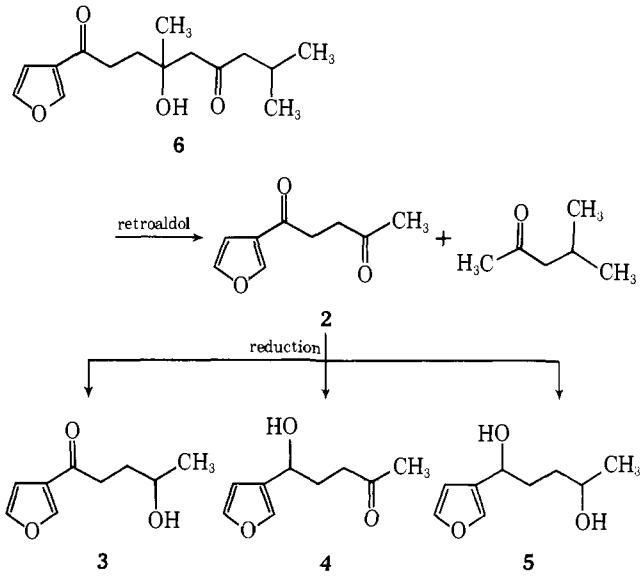
As part of our investigation of these toxins we have been

interested in the biogenesis of the compounds and the circumstances under which they are formed. While it is evident that compounds like ipomeamarone are unrearranged sesquiterpenes, it is not clear whether **2–5** arise by degradation of sesquiterpenes (a) or monoterpenes (b) or by some other route.



The isolation of 4-hydroxymyoporone (**6**) from mold-damaged sweet potatoes has recently been reported.[5] Sesquiterpene **6** is an attractive potential precursor of **2–5** in that a



retroaldol reaction would convert it to **2**. Reduction of **2** to **3–5** should take place easily in biological systems.

We would now like to describe experiments demonstrating the role of **6** in the biogenesis of **2–5**.

Experimental Section

High-pressure liquid chromatography (HPLC) was carried out on a Waters Associates Model ALC 202 liquid chromatograph. Two 1 ft × ⅜ in. μ-Porasil columns in series or a 16 ft × ⅛ in. Corasil II column were used with the solvent and flow rate indicated. The UV detector response was determined to be linear for 10 to 50-μg samples of ipomeanine and 4-ipomeanol by injecting known concentrations of each and measuring the peak area by triangulation. Values for dilution factors are the average of two–three determinations. Thin-layer chromatography was carried out on Bakerflex silica gel 1B-F plates using the solvent indicated. The compounds were detected by fluorescence quenching and by Ehrlich's reagent.[6] Ipomeanine reacts with Ehrlich's reagent only weakly unless the plates are sprayed first with ammonium hydroxide.

A Beckman Model LS100 instrument was used for scintillation counting using the wide ^{14}C window. Samples were counted for 10 min or for 10^4 counts. Counting efficiency for each sample was determined by adding an aliquot of [^{14}C]toluene (New England Nuclear) and recounting. NMR spectra were obtained using a JEOL JMN-MH-100 spectrometer. Mass spectra were obtained using a Finnigan Series 3200F GC/MS system. Gas chromatography was carried out on a Hewlett Packard Model 5750 gas chromatograph using a 8 ft × ¼ in. 12% OV101 on 100/120 Chrom Q glass column at 160 °C. Trimethylsilyl ether formation (silylation) was carried out using TriSil BSA (Pierce Chemical Co.). All solutions and vessels used in the incubations were autoclaved at 120 °C for 15 min before use.

Bioproduction of [^{14}C]-4-Hydroxymyoporone ([^{14}C]-6). In a typical procedure, sweet potatoes (2 kg, surface sterilized with Clorox) were cut into 75-mm slices, dipped into 15% mannitol solution, and placed in Teflon-lined pans on paper toweling saturated with 1% mercuric chloride solution.

A solution of 0.5 mCi sodium [2-^{14}C]acetate (New England Nuclear) in 50 mL of water and additional mercuric chloride solution was distributed among the slices. The pans were covered and allowed to stand at room temperature for 5 days. The slices were then homogenized in 95% ethanol in a stainless steel blender. The suspension was filtered through glass wool, and the solids were washed with additional ethanol. The combined filtrate was evaporated under reduced pressure, and the residue was extracted with ethyl acetate. The resulting solution was dried with magnesium sulfate and concentrated under reduced pressure. The residue (10 g) was partitioned on silica gel (50 g) using an ether–hexane gradient. The 4-hydroxymyoporone-rich fraction (0.75 g) was eluted with a 1:3 ether–hexane mixture. 4-Hydroxymyoporone (0.083 g) was isolated from the fraction by HPLC (μ-Porasil; ether–pentane, 4:6, 1.5 mL/min; two passes). A 5-μL aliquot of the purified material was rechromatographed on the μ-Porasil columns and the 4-hydroxymyoporone peak collected as three fractions: [wt (mg), sp act (dpm/mg)] 2.091, 69 700; 2.057, 69 000; 0.760, 67 000. The spectral characteristics of the isolated material were identical with those described earlier.[5]

Bioproduction of [^{14}C]Ipomeamarone ([^{14}C]-1). Material (1.5 g) eluting in 1–2% ether in hexane from several of the [^{14}C]-4-hydroxymyoporone bioproduction runs described above was pooled. The semicarbazone was formed and recrystallized repeatedly from carbon tetrachloride–hexane. The specific activity of the material from the last three recrystallizations was determined [wt (mg), sp act (dpm/mg)] 2.524, 57 200; 0.988, 58 200; 1.335, 57 400. The semicarbazone (860 mg) was heated under reflux with 20 mL of 5% oxalic acid in 1:1 water–acetone. After 30 min most of the acetone was removed under reduced pressure, and the aqueous suspension was extracted with ether. The ether solution was dried with magnesium sulfate and concentrated.

The residue was filtered through a silica gel column using 1:19 ether–hexane as solvent to give 590 mg (84% recovery) of [^{14}C]-**1**. The spectral characteristics of the compound were identical with those described earlier, in particular the ^1H NMR showed no evidence of epimerization of the tetrahydrofuran carbons.[7]

Incubation of Furanosesquiterpenes with *Fusarium solani*. Run 4. Mycelial mats of the fungus pathogen *Fusarium solani* (formerly *F. javanicum*) were grown statically in 500-mL Erlenmeyer flasks on Czapek medium (200 mL) containing 10% sweet potato infusion. After 2 weeks the mats were separated from the medium and washed several times with distilled water. [^{14}C]-4-Hydroxymyoporone (49 mg, 1.83×10^7 dpm/mmol) and unlabeled **1** (50 mg) suspended in 20 mL of distilled water with Tween 80 was distributed between two mats; enough distilled water was added to give the original volume. The flasks were allowed to stand at room temperature. After 7 days the contents were extracted with ethyl acetate; the ethyl acetate was removed under reduced pressure after drying with magnesium sulfate. HPLC (μ-Porasil; ether–methylene chloride, 1:3; 1 mL/min) of the residue (44 mg) allowed separation of **3** and a fraction enriched in **2** and **6**. The latter fraction was rechromatographed (μ-Porasil ether–methylene chloride, 1:7; 1 mL/min) to allow individual isolation of **2** and **6** (15 mg).

The isolated **3** was diluted with 9.4 mg of unlabeled 4-ipomeanol. The dilution factor was determined by adjusting the volume of the isolate to 1 mL and injecting aliquots on the HPLC (Corasil II; ether, isooctane, 1:1; 1.5 mL/min). Measuring peak areas before and after addition of unlabeled **3** gave a dilution factor of 13.4. Diluted **3** was derivatized as the semicarbazone which was recrystallized from 2-propanol. The specific activity of the semicarbazone after four recrystallizations was 3880 dpm/mg. The specific activity of undiluted **3** semicarbazone was 5.19×10^4 dpm/mg or 1.17×10^7 dpm/mmol.

The isolated ipomeanine was diluted with 8.4 mg of unlabeled **2**; the dilution factor was determined to be 3.25 (Corasil II; ether, isooctane, 1:3; 1.5 mL/min). The specific activity of diluted **2** after three recrystallizations from ether–pentane was 20 700 dpm/mg. The specific activity of the undiluted ipomeanine was 6.74×10^4 dpm/mg or 1.12×10^7 dpm/mmol.

Other incubations with *F. solani* were carried out in a similar manner. In two runs (runs 1 and 2 in Table I), no unlabeled **1** was added. In one run (run 5, Table I), unlabeled **6** and [^{14}C]-**1** were used. In another run, unlabeled **6** was incubated with a *F. solani* mat which

had been previously autoclaved. Analysis by TLC (ethyl acetate–hexane, 4:6) indicated that no reaction had taken place.

Ipomeanine (10 mg) was incubated with a *F. solani* mat for 5 days. Analysis by TLC (methanol–benzene, 1:9) indicated reduction of **2** to **3, 4,** and **5.** The identity of the products was verified by silylation, followed by gas chromatography. Retention times of the silylated products were identical with authentic samples of silyl **3, 4,** and **5.**

Incubation of Furanosesquiterpenes with *Fusarium oxysporum*. Unlabeled 4-hydroxymyoporone was incubated with mycelial mats of *F. oxysporum* in a manner similar to that described for run 4 with *F. solani*. The presence of ipomeanine (0.6 mg) and 4-ipomeanol (0.2 mg) in the extract was established by HPLC (Corasil II, same conditions as above). The HPLC peaks were collected and subjected to GC–MS. Mass spectra of ipomeanine (major peaks at *m/e* 166, 151, 124, and 95) and silyated 4-ipomeanol (major peaks at *m/e* 240, 225, 207, 117, 115, and 95) were identical with those of authentic samples.

Incubation of Furanosesquiterpenes with *Ceratocystis fimbriata*. Unlabeled 4-hydroxymyoporone was incubated with mycelial mats of *C. fimbriata* in a manner similar to that described for run 4 with *F. solani*. After 5 days TLC (ethyl acetate–hexane, 4:6) showed no conversion to Ehrlich-positive products.

Ipomeanine (10 mg) was incubated with *C. fimbriata* for 7 days. Analysis by TLC (methanol–benzene, 1:9) indicated reduction of **2** to **3, 4,** and **5.** The identity of the products was verified by silylation, followed by gas chromatography. Retention times of the silylated products were identical with authentic samples of silyl **3, 4,** and **5.**

Chemical Degradation of [^{14}C]-4-Hydroxymyoporone. [^{14}C]-4-Hydroxymyoporone (16 mg, 1.83×10^7 dpm/mmol) was treated with 0.5% sodium hydroxide in methanol–water, 1:1, at room temperature. After 30 min the reaction mixture was extracted with ether. The ether solution was washed with brine, dried with magnesium sulfate, and concentrated. Ipomeanine was isolated by HPLC (μ-Porasil; methylene chloride–ether, 1:7, 1 mL/min) and diluted with 9.6 mg of unlabeled **2.** The dilution factor, 11.0, was determined as described above. Specific activity of the ipomeanine after three recrystallizations from ether–pentane was 6410 dpm/mg. The specific activity of undiluted **2** was 7.05×10^4 dpm/mg or 1.17×10^7 dpm/mmol. The retroaldol reaction was carried out on the 0.692×10^7 dpm/mmol activity [^{14}C]-**6;** the resulting ipomeanine was determined to have a specific activity of 2.51×10^4 dpm/mg or 4.17×10^6 dpm/mmol.

Results

The ^{14}C-labeled furanosesquiterpenes, **1** and **6,** used in this work were obtained by stimulating sweet potato slices with mercuric chloride in the presence of [2-^{14}C]acetate. 4-Hydroxymyoporone was obtained by silica gel chromatography, followed by HPLC. Since a suitable solid derivative for **6** could not be found, the radiochemical homogeneity of the isolate was determined by reinjecting a portion on the HPLC and collecting the peak in three fractions. The specific activity of the fractions collected for each of the two samples used in this work was determined to be $1.83\ (\pm 0.04) \times 10^7$ dpm/mmol and $0.692\ (\pm 0.015) \times 10^7$ dpm/mmol (±SE). The lack of a significant variation in the specific activity through the peak was taken as demonstration of the radiochemical homogeneity of the sample. Ipomeamarone was pooled from several bioproduction runs. Pure material was obtained by silica gel chromatography, followed by derivatization as the semicarbazone, recrystallization to constant specific activity (1.77×10^7 dpm/mmol), and finally regeneration.

Incubations of the furanosesquiterpenes were carried out with fungi which are commonly found on damaged sweet potatoes. The substrate concentration approximated the maximal concentration of **6** that had been observed in damaged sweet potatoes. The incubation time was chosen to give maximum production of ipomeanine (**2**) and 4-ipomeanol (**3**); longer times result in production of more 1,4-ipomeadiol (**5**) and unidentified products. Production of **2** and **3** was chosen for study since they are the most potent lung toxins. They are also more readily assayed than **4** and **5** because they exhibit a UV maximum at about 250 nm, making detection, quantitation, and isolation by HPLC especially facile using a 254-nm UV detector.

Table I. Incubation of Furanosesquiterpenes with *F. solani*

	Specific activity, 10^7 dpm/mmol			
	Compound			
Run	**6**	**1**	**2**	**3**
1	0.692	*a*	0.35 (0.50)[b]	*c*
2	1.83	*a*	1.0 (0.57)	*c*
3	0.692	0.0[d]	0.42 (0.60)	0.39 (0.57)
4	1.83	0.0[d]	1.1 (0.61)	1.2 (0.64)
5	0.0[d]	1.77	0.01	0.01

[a] No ipomeamarone added. [b] Number in parentheses is the ratio of specific activities of the product and [^{14}C]-**6.** [c] No **3** was isolated. [d] Unlabeled material added.

The results of five incubations with *F. solani* are described in Table I. When [^{14}C]-**6** alone was added to the fungal mats (runs 1 and 2), only ipomeanine was produced in isolable quantity. Addition of unlabeled **1** to the incubation mixture (runs 3 and 4) stimulated formation of **2** and **3** and both products could then be isolated. However, **1** is apparently not a precursor of **2** and **3;** run 5 showed that no more than about 1% of the **2** and **3** which formed could have arisen from **1.** Even the small amount of incorporation that was observed may represent trace impurities in **2** and **3.** The amount of incorporation of radioactivity from **1** into **2** and **3** represents only 29 and 6 cpm above background in the counted samples. The conclusion to be reached from these experiments is that **6,** but not **1,** can serve as a precursor of **2** and **3** and that the latter retained 0.58 ± 0.04 of the isotopic label present in **6.**

A portion of both the high and low specific activity [^{14}C]-**6** was subjected to base-catalyzed retroaldol reaction to ascertain the distribution of the ^{14}C label. Ipomeanine obtained from the retroaldol reactions had 0.64 and 0.60 of the specific activity of the corresponding [^{14}C]-**6.**

The ability of *F. solani* to reduce **2** to **3, 4,** and **5** was confirmed. Ipomeanine, when incubated with the fungus for 5 days, was partly reduced to the other metabolites.

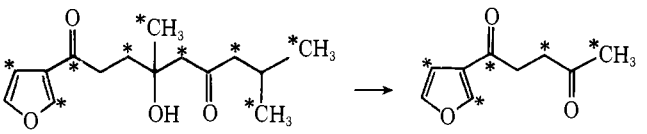
Fusarium oxysporum, a fungus which is closely related to *F. solani*, could also effect degradation of **6,** but it was only about 20% as effective in producing **2–5** as *F. solani* under the conditions used.

Ceratocystis fimbriata, the fungus which has been used in much of the work on the stress metabolites of sweet potatoes, did not degrade **6** to **2–5.** However, it was confirmed that *C. fimbriata* could reduce **2** to **3, 4,** and **5.**

Discussion

Incubations of 4-hydroxymyoporone (**6**) with *F. solani* clearly demonstrate that this sesquiterpene can serve as a precursor of **2–5.** This result provides an answer to the puzzle of why the hepatotoxins are produced under a variety of conditions, but the pulmonary toxins are only occasionally detected on mold-damaged sweet potatoes. Compounds **2** and **3** are produced efficiently (10 to 15% conversion) from **6** and show no significant dilution of label.

The ratio of the specific activity of **2** and **3** to **6** in runs 1–4 as well as in the chemical degradation of **6** center about 0.60. Nine carbons of a sesquiterpene should be labeled by [2-^{14}C]acetate and five of these remain in the part corresponding to ipomeanine upon retroaldol reaction. Thus, with completely uniform labeling one expects the specific activities of **2** and **3** to be 0.56 that of **6.** The fact that the theoretical and experimental values are comparable implies that no appreciable pools of terpene precursors were present in the sweet potato prior to



* = ^{14}C from 2-^{14}C-acetate

stimulation with mercuric chloride and supports the contention that **2** (and **3**) arises directly from **6** and not by some indirect route involving extensive degradation followed by resynthesis.

The presence of other furanosesquiterpenes, for example **1**, seems to have a stimulatory effect on *F. solani*, resulting in the production of greater quantities of **2** and **3** from **6**. In runs 3 and 4 where **1** was added, five to ten times more conversion of **6** to **2** and **3** took place. The role of stress metabolites as a defense mechanism against pathogens has been postulated.[2b] It may be that stimulation of metabolizing systems in the fungus by the presence of the furanosesquiterpenes is similar to the stimulation of metabolic processes in animals by some xenobiotics.

Although ipomeamarone might serve as a precursor of the lung toxins by a process involving opening of the tetrahydrofuran ring followed by a retroaldol reaction, **1** is not metabolized by *F. solani* in this manner to any great extent. Runs 3 and 4 show no significant dilution of the label in **2** and **3**. Run 5 with [^{14}C]-**1** and unlabeled **6** indicates that no more than about 1% of the product could come from **1**. Although negative incorporation experiments are usually considered equivocal due to the uncertainty of whether or not the labeled compound is able to diffuse to the site of metabolism, it should be pointed out that in the natural state **1** would have to diffuse from the site of synthesis in the plant to the site of metabolism in the fungus. It seems probable that the fungus lacks the enzymes required to convert **1** into **2–5**.

The observation that *F. oxysporum*, although closely related morphologically to *F. solani*, is not as efficient in producing **2** and **3** mirrors results from our earlier studies on bioproduction of lung toxins in sweet potatoes. *F. oxysporum* is only about 20% as effective in carrying out the retroaldol reaction as *F. solani* under the incubations used in this work. Of the organisms isolated from mold-damaged sweet potatoes we have found only *F. solani* and *F. oxysporum* to produce the lung toxins. *Ceratocystis fimbriata*, a common fungal contaminant of sweet potatoes, does not cause production of the lung toxins in the sweet potato, nor does it have the ability to carry out the retroaldol reaction when incubated with **6**. Although the retroaldol reaction is a simple one chemically, it does not occur spontaneously under metabolic conditions and only certain fungi can effect the reaction.

The reported[8] isolation of **2** from *C. fimbriata* infected sweet potatoes seems to be in conflict with our finding that *C. fimbriata* does not produce the lung toxins either upon incubation with **6** or when grown on live sweet potato tissue. Indeed, since the carbonyl groups are easily reduced by both the fungus and the sweet potato, all of the lung toxins would be expected to be present if **2** were available. The explanation for this inconsistency may be a thermal or base-catalyzed retroaldol reaction of **6** occurring during the multiple distillations that had been used to isolate ipomeanine.[8] Neutral suspensions of **6** in water are stable to autoclaving (120 °C, 15 min) but we have found measurable amounts of **2** formed during gas chromatography of **6** at 160 °C.

The biogenesis of the lung edemagenic agents **2–5** is a complex process. Chemical and physical damage as well as many invading organisms can stimulate the sweet potato to produce furanosesquiterpenoid stress metabolites, but the lung toxins are formed only when certain fungi are also present. These toxins are not produced by the sweet potato but are the result of fungal catabolism of a stress metabolite formed by the host plant.

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References and Notes

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