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Strain of *Fusarium oxysporum* Isolated from Almond Hulls Produces Styrene and 7-Methyl-1,3,5-cyclooctatriene as the Principal Volatile Components

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An isolated strain of *Fusarium oxysporum* from the hulls of *Prunus dulcis* (sweet almond) was found to produce relatively large quantities of the hydrocarbons styrene and two isomers of 7-methyl-1,3,5-cyclooctatriene (MCOT). Production of styrene and MCOT was reproduced on a small scale using potato dextrose agar as a growth medium and scaled up using 1 L of inoculated potato dextrose broth. The compounds were trapped as volatile organic compounds (VOCs) onto solid-phase microextraction (SPME) for small scale and Tenax for large scale and then isolated using standard high-performance liquid chromatography (HPLC) methods. Styrene was authenticated by a comparison to the retention times, fragmentation patterns, and calculated retention indices of a commercially available sample. The identity of MCOT was verified by a short chemical synthesis and a comparison of spectroscopic data to the isolated sample. A biosynthetic scheme of styrene is proposed on the basis of a ^{13}C-labeling study. This is the first report of MCOT isolated as a natural product.

KEYWORDS: Fungi; *Fusarium*; methyl cyclooctatriene; styrene; volatile

INTRODUCTION

Styrene is the principal monomer used for ubiquitous polystyrene plastics and resins. Industrially, 90% of styrene production is from benzene, which originates from the refining of crude oil, and ethylene, which is extracted from natural gas as ethane and then steam-cracked to provide ethylene. Benzene and ethylene are allowed to react in the presence of aluminum trichloride to form ethylbenzene, which undergoes high-temperature dehydrogenation in the presence of iron oxide to form the monomer, styrene. In 2006, the U.S. reportedly produced over 1.3×10^{10} pounds of styrene, with the states of Texas and Louisiana producing the greatest quantities (*1*).

Styrene is a common component of many foods, albeit in low concentrations (*2*); however, it is classified as a mutagen, and long-term exposure via inhalation has been reported to cause encephalopathy (*3*). However, benzene, the synthetic precursor of styrene, is recognized as a carcinogen (*4*), and ethylene, while industrially known as a flammable gas, is not classified as carcinogenic and, in fact, is a prevalent volatile organic compound (VOC) emitted from many plants in response to insect herbivory (*5*).

It is estimated that by 2025 85% of the world's energy needs will be dependent upon crude oil. In the year 2006, the U.S. imported 66% of its crude oil, and it is predicted that by 2025 that number will increase to 71%. Additionally, it is projected that by 2025 electricity requirements in the U.S. will increase by approximately 50% (*6*). The scientific community has begun in earnest to investigate alternative means of energy; alternatives that do not add to environmental hazards or human health concerns typically associated with energy (*7*).

Because of both the accidental release as well as the typical gaseous and effluent output during the manufacturing of styrene, many research efforts have focused on microbes that demonstrate the ability to eliminate styrene from the environment (*8, 9*). For example, species from the genera *Pseudomonas*, *Rhodococcus*, *Nocardia*, *Xanthobacter*, and *Enterobacter* have demonstrated the ability of bacteria to degrade styrene (*10*). Additionally, fungi have shown their ability to use styrene as a feedstock and thus may be used for purification of gaseous effluents containing styrene (*11*).

The fungus *Penicillium camemberti* has assisted in elucidation of the biosynthetic production of styrene, which is a downstream product of phenylalanine (*12*). In their work, Pagot et al. tentatively assigned the enzymes phenylalanine ammonia-lyase and cinnamic acid decarboxylase as being involved in the process of converting phenylalanine to styrene. Furthermore, because it is known that phenylalanine is a biosynthetic product of glucose (*13*), it is postulated that the feedstock for styrene in this case is glucose.

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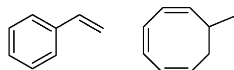

Figure 1. Chemical structures of styrene and MCOT.

Almonds, as well as most agricultural commodities, are known to have ambient microbes associated with them. The following is a sampling of the genera found during a study of the mycoflora of almonds: *Cladosporium*, *Penicillium*, *Aspergillus*, *Fusarium*, and *Trichoderma* (*14*). *Fusarium oxysporum* is a ubiquitous soil-borne fungus capable of causing wilt in several agricultural crops worldwide and is known to produce mycotoxins (*15, 16*). Conversely, *F. oxysporum* has been reported to produce compounds with antifungal (*17*) and anticancer (*18*) activities. Reports of volatiles from *F. oxysporum* strains are few, with one investigation reporting on the use of VOCs to distinguish between different strains of *F. oxysporum* (*19*); however, this investigation only used the qualitative differences of the GC traces to distinguish between species and did not report the identities of the VOCs. A second investigation reported styrene as one of numerous volatiles from *Fusarium coeruleum* (*20*). The investigation herein will be the first report of volatile styrene (**Figure 1**) from a *F. oxysporum* strain and in a major, isolable amount. Three isomers of 7-methyl-1,3,5-cyclooctatriene (MCOT), one of which is most likely due to thermal cyclization, identified by gas chromatography−mass spectrometry (GC−MS) only, have been reported to be present in the VOC bouquet of female gametes of marine brown algae (*21*); however, the authors state that the presence of the three MCOT isomers may be due to cyclization and/or rearrangement of the other various alkenes and cycloalkenes present in the mixture. Accordingly, this is the first report of MCOT (**Figure 1**) isomers as volatile components from a natural source. The procedure described in this report shows promise to supply the industrially important feedstock monomer, styrene, from a renewable source.

MATERIALS AND METHODS

General Experimental Procedures. VOCs were collected onto solid-phase microextraction (SPME) 100 μm polydimethylsiloxane (PDMS) fibers (Supelco, catalog number 504823, Bellefonte, PA). SPME fibers were conditioned via the suggested protocol of the manufacturer prior to use. VOCs were desorbed, separated, and identified using either a Hewlett-Packard (HP) 6890 Series gas chromatograph (GC) coupled to HP 5973 mass selective detector (MS) (Hewlett-Packard, Palo Alto, CA), or an Agilent Technologies 6890N GC coupled to a 5975B inert MS (Santa Clara, CA). DB-Wax (60 m, 0.320 mm inner diameter, 0.25 μm film, catalog number 123-7062) and DB-1 (60 m, 0.320 mm inner diameter, 0.25 μm film, catalog number 123-1062) GC columns were purchased from Agilent J&W Scientific (Santa Clara, CA) and conditioned via the suggested protocol of the manufacturer prior to use. Tenax was purchased from Chromatography Research Supplies, Inc. (Addison, IL) and conditioned with a helium flow (20 mL/min) while at 250 °C for 12 h. Diethyl ether (ACS grade), methanol (HPLC grade), *n*-pentane (HPLC grade), phenol, chloroform, isopropanol, Tris/EDTA (TE) solution, and lysis solution components were purchased from Fisher Scientific (Pittsburgh, PA, and Fair Lawn, NJ). Potato dextrose broth and agar were purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ). Diethyl ether was treated with ferrous sulfate, dried over sodium sulfate, and distilled over Ethanox 330 prior to use. Methanol and *n*-pentane were used without further purification. Styrene for comparison of retention times, RNase, isoamyl alcohol, and chloramphenicol were purchased from Aldrich (St. Louis, MO, and Milwaukee, WI) and analyzed/used without further purification. SPME sampling was performed via the PEST method (*22*). Semipreparative high-performance liquid chromatography (HPLC) was performed on a Hewlett-Packard 1100 Series HPLC with a fluorescence detector (Hewlett-Packard, Palo Alto, CA), using a YMC-Pack Pro C18 (250 × 10 mm, 5 μm, 12 nm) reversed-phase column (Supelco, Bellefonte, PA). Almonds, Nonpareil variety, were obtained from Paramount Farming Company (Bakersfield, CA).

Isolation of Styrene-Producing Fungal Strains. Almonds that underwent previous VOC analysis (*23*) were sliced into quarters and allowed to stand in their 12 L collection flask to accumulate fungi on their surfaces; ambient volatiles were directed into the appropriate effluent hood. The volatiles emitted were occasionally monitored for VOC composition using SPME and GC−MS for VOC collection and identification (*22*). Fungi were isolated from the hulls of almonds on which visible fungal growth was present. Almonds were washed individually in 25 mL of 0.05% Tween 80 and 30% glycerol by vortexing for 30 s in 50 mL conical tubes, and the resultant washes were stored at −20 °C. Almond washes were inoculated onto potato dextrose agar (PDA) for cultivation of fungal populations. For the analysis of volatile compounds produced by these fungal populations, almond washes (100 μL each) were inoculated into 25 mL screw-top Erlenmeyer flasks containing 10 mL of PDA and fitted with Teflon-lined septum screw caps. Cultures were incubated at 28 °C for 6 days with the screw caps loosened to allow for air exchange. Caps were then tightened to seal the flasks, and cultures were incubated for 24 h at 28 °C to allow for the accumulation of volatile compounds in the flask headspace.

From fungal populations (mixed cultures) that produced styrene, individual fungal colonies were isolated by spreading 100 μL of the corresponding almond wash onto PDA supplemented with 100 mg/L chloramphenicol and incubated for 2 days at 28 °C. Random colonies were transferred to individual PDA plates and incubated at 28 °C for 7 days to ensure culture purity. Pure cultures of individual fungal isolates were inoculated onto PDA in 25 mL screw-top Erlenmeyer flasks and incubated as described above but with the caps tightened for 1 min prior to VOC analyses. Three fungal isolates produced styrene under these conditions and were designated I3-4, I3-5, and I3-8. The styrene-producing *F. oxysporum* strain, fungal isolate I3-8, was deposited in the NRRL patent culture collection and assigned the accession number NRRL 50189.

Fungal isolates were identified by modified DNA sequence analysis (*24, 25*) and are described fully in the Supporting Information.

Volatile Organic Compound Collection, Small Scale. VOCs were collected in Teflon-capped 25 mL Erlenmeyer flasks containing the fungi inoculated onto PDA (see also Isolation of Styrene-Producing Fungal Strains). VOCs were allowed to accumulate for 24 h sans air flow prior to exposure to SPME fibers and immediately thermally desorbed onto the GC−MS injector port (permeation of VOCs in collection chamber, 24 h; exposure of VOCs to SPME fiber, 30 s; storage of VOCs on fiber, 10 s; thermal desorbtion of VOCs, 15 min). VOC samples were collected in duplicate, and each sample was separated and identified using two different GC methods.

Volatile Organic Compound Separation and Identification, Small Scale. GC−MS method for DB-1 column: injector temperature, 200 °C; splitless; inlet pressure, 11.78 psi; total flow, 14.5 mL/min; gas saver on; helium flow, 2.0 mL/min; average velocity, 36 cm/s; constant flow; initial temperature, 40 °C; hold time, 0 min; ramp 1, 4 °C/min; final temperature, 250 °C; hold time, 30 min; MSD source, 230 °C; MS Quad temperature, 150 °C; auxiliary temperature, 250 °C; solvent delay, 1 min. GC−MS method for DB-Wax column: injector temperature, 200 °C; splitless; inlet pressure, 17.82 psi; total flow, 35.6 mL/min; gas saver on; helium flow, 3.0 mL/min; average velocity, 44 cm/s; constant flow; initial temperature, 40 °C; hold time, 0 min; ramp 1, 4 °C/min; final temperature, 200 °C; hold time, 40 min; MSD source, 230 °C; MS Quad temperature, 150 °C; auxiliary temperature, 250 °C; solvent delay, 1 min. Mass fragmentation patterns were compared to Wiley275, NIST75K, or NIST05a libraries for tentative identification. Retention indices (RI) were calculated using a homologous series of *n*-alkanes for the corresponding columns and compared to literature values (*26, 27*). Styrene identification was further verified by comparison to authentic sample retention times and RI. Styrene retention time for DB-1 was 9.01−9.12 min, and styrene retention time for DB-Wax was 11.39−11.42 min. Calculated RI for DB-1 = 871, and calculated RI for DB-Wax = 1252.

Styrene Collection, Large Scale. A 12 L round-bottom flask (RBF), charged with 1 L of potato dextrose broth (PDB) inoculated with *F. oxysporum*, was kept static and at room temperature for a total of 1582 h. The experimental conditions for the inoculated broth scale-up were similar to a published method (*28*). The RBF was attached to a metal cap with Teflon gaskets and two ports: one for purified air input (6 mL/min) and one for VOC collection onto a Tenax column (25 g in a 3 × 20 cm glass tube fitted with a fritted filter). Aluminum foil was wrapped around the Tenax collection column. Occasional VOC analysis and relative styrene ratio determination were accomplished by shutting off air flow to the RBF for 3 min, removing the air flow line, sampling with SPME (*P*, 3 min; *E*, 1 min; *S*, 30 s; *T*, 15 min), and analyzing by GC−MS. A total of five Tenax columns were used for VOC collection: column 1, days 0−3 (69.7 h); column 2, days 3−7 (97.3 and 167.0 h); column 3, days 7−24 (405.9 and 572.9 h); column 4 (2 × 10 g Tenax columns in tandem), days 24−45 (509.6 and 1082.5 h); column 5, days 45−66 (499.3 and 1581.8 h). Absorbed volatiles were desorbed from the Tenax via a published protocol (*23*). Briefly, the Tenax medium was washed with *n*-pentane, and the *n*-pentane was concentrated via immersion of the RBF in a warm water bath and distilled until a mother liquor volume of ca. 100 mL.

Styrene Isolation, Semipreparative HPLC. Aliquots (100 μL) of the mother liquor were separated via HPLC: 80:20, MeOH/H_2O; flow, 1.5 mL/min; pressure, 70−72 bar; detector, excitation at 285 nm and emission at 385 nm. Commercial styrene was used to determine the retention time of the peak for collection. Pure styrene, with an elution time of 20.8 min, was collected into a darkened vial and stored in a refrigerator. Combined styrene fractions (ca. 10 runs) were transferred to a separatory funnel, and chilled pentane (10 mL) was added. The aqueous methanol layer was partitioned and then washed twice more with chilled pentane (2 × 10 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated *in vacuo* to a volume of approximately 0.5 mL (composition checked by GC−MS injection, 1 μL), and then a stream of nitrogen gas was passed over the vial to near dryness to afford pure styrene (1.7 mg), confirmed by SPME and GC−MS analysis, which matched all data of the authentic sample.

MCOT Isolation, Semipreparative HPLC. Aliquots (900 μL) of the mother liquor were separated via HPLC: 80:20, MeOH/H_2O 0−28.99 min; flow, 1.5 mL/min and then 99:1, MeOH/H_2O 29−42 min; flow, 2.0 mL/min; detector, excitation at 295 nm and emission at 395 nm. SPME headspace GC−MS was used to determine the retention time of the peak for collection. MCOT isomers, with an elution time of 38.5 min, were collected into a darkened vial and stored in a refrigerator. Combined fractions (ca. 7 runs) were transferred to a separatory funnel, and chilled pentane (10 mL) was added. The aqueous methanol layer was partitioned and then washed twice more with chilled pentane (2 × 10 mL). The combined organic layers were dried over anhydrous sodium sulfate prior to concentration via gentle distillation in a warm water bath. Attempts to concentrate to neat MCOT resulted in complete loss of product. Ultimately, characterization was performed via MS fragmentation patterns and high-resolution MS (HRMS), with MCOT concentrated in pentane. Electron impact mass spectrum (EIMS 70 eV) found: 51 (10), 52 (9), 78 (100), 79 (20), 91 (20), 105 (15), 120 (9). HREIMS: m/z 120.0941 (calcd for C_9H_{12}, 120.0939).

RESULTS AND DISCUSSION

VOC analyses of the moldy almonds revealed one sampling that exhibited an unusually large amount of styrene, nearly 90% of the relative abundance by GC−MS (**Figure 2A**). Steps were taken to ensure the slicing process (temporary storage container and cutting board) did not introduce styrene as a contaminant. Other VOCs noted in minor amounts from the almond fungal bouquet included 2-pentylfuran, a common fungus volatile (*29*), ethyl anisole, and other plant volatiles in trace amounts. Once the possibility of styrene being introduced as a contaminant was eliminated, work was initiated on duplicating the fungal growth on PDA. Several Petri dishes containing inoculated PDA were stacked into a VOC collection flask, and the volatile output was measured via SPME and GC−MS (**Figure 2B**). Styrene as the primary volatile component was successfully duplicated with butyrolactone as a minor contaminant. The source of butyrolactone was not investigated, because the VOCs were collected from a bouquet of fungi.

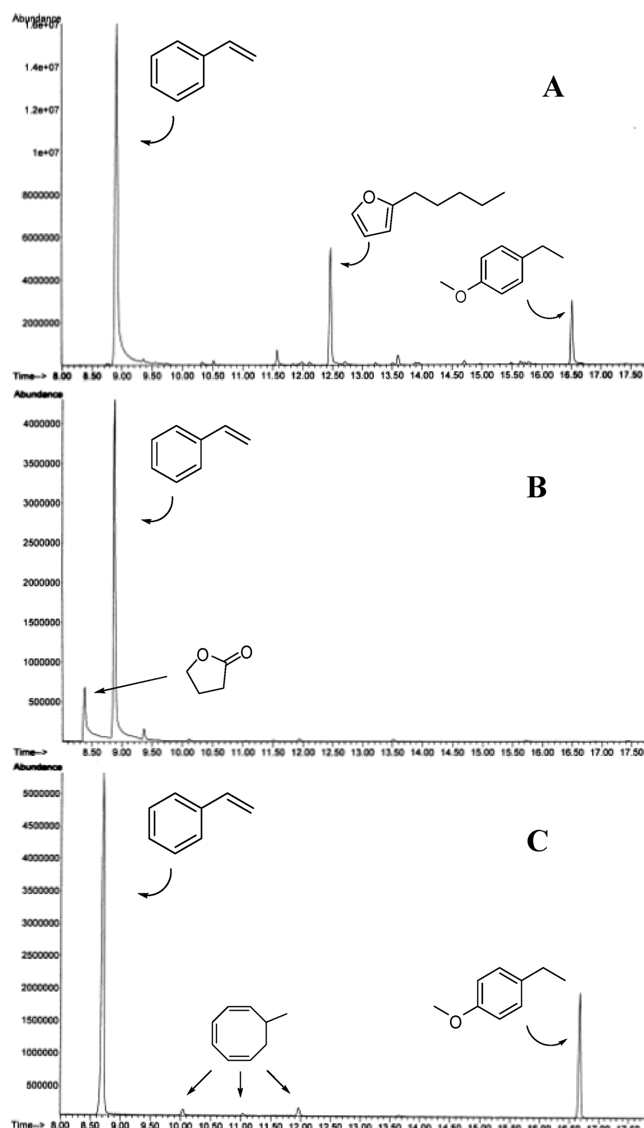

Figure 2. GC traces of VOCs from (A) fungal bouquet on almonds, (B) fungal bouquet on PDA, and (C) isolated fungus *F. oxysporum* strain on PDA.

With the production of styrene from a microbial source verified, investigation into the identity of the responsible microbe was undertaken. Using standard microbe isolation techniques (*24, 25*) combined with a styrene-generation-guided fractionation, the fungus *F. oxysporum* was isolated and identified as a potential candidate. During the isolation study, the fraction that displayed styrene growth (**Figure 2C**) was selected for fungus identification. Ethyl anisole was again present in minor amounts in addition to trace amounts of the three isomers of MCOT, which at the time of analysis were not being investigated. The slight difference in retention time noted in **Figure 2C** was due to a small change in the GC program. For authentication purposes commercially available styrene was injected to compare retention times as well as fragmentation patterns.

The next portion of the project set out to determine how long the styrene was being generated. Using the 25 mL Erlenmeyer flasks containing inoculated PDA, the VOC emission was

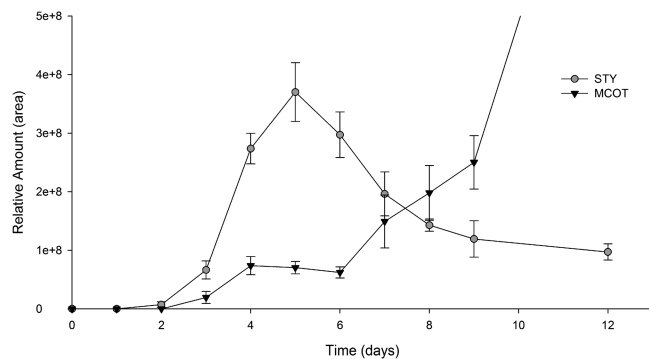

Figure 3. Graph of styrene and MCOT production from *F. oxysporum* grown on PDA over 12 days, small scale. Samples were monitored for styrene every 24 h by SPME.

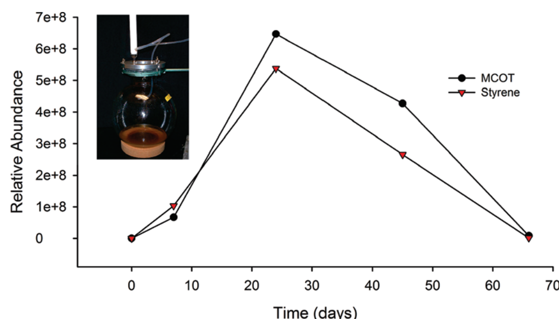

Figure 4. Relative abundance of large-scale styrene and MCOT production from *F. oxysporum* over a 66 day period. (Inset) Chamber used to grow *F. oxysporum* on a larger scale.

sampled by SPME every 24 h and analyzed by a rapid ramp GC−MS program. The graph in **Figure 3** shows the relative abundance of styrene versus time. Styrene was essentially the only component being generated for 3 days, after which, a second component was produced after 8 days in concurrence with a reduction of styrene production. A tentative assignment based on a relatively low qualitative hit from the fragmentation library indicated the second volatile to be an isomer of MCOT. This compound and its identification will be discussed in detail presently. Only one other investigation reporting styrene and another component from a fungal source has been published. Pinches and Apps reported on *Trichoderma* species that produced styrene and 1,3-pentadiene from PDB containing sorbic acid and/or cinnamic acid (*30*). The authors did not report the amounts of VOCs produced and did not mention whether or not other VOCs were noted.

Attention was then turned to whether the experiment could be scaled to a larger volume using PDB and at room temperature. The VOC composition was occasionally monitored for relative abundance of styrene and MCOT (**Figure 4**). For the first 11 days of VOC emission, the large scale essentially mimicked the small scale relative amounts of styrene and MCOT, with styrene being in greater quantities; however, after 11 days, MCOT exceeded and stayed above the amount of styrene generated. Near the time of VOC composition check near day 22, it appeared as if either the feedstock had started to run out or the fungus had reached the peak of its ability to generate the two compounds. At the time of investigation, it was not determined if the addition of glucose into the fungal broth while the experiment was running would maintain consistent VOC emission, and furthermore, the data from the experiments do not appear to show any direct correlation between the two VOCs, despite the small-scale experiment showing a decrease in styrene as the MCOT increased. The fungus initially grew on top of the broth along the sides and continued growth inward until it resembled a sheet of "thick, wavy cotton".

The volatiles were collected on a total of five Tenax "fractions"; the first cut, 0−3 days, coincided with days that no VOCs were generated. According to information from the small-scale VOC collection experiment, VOC production did not start until the third day. This observation was not applicable to the large scale, which did not start generating styrene until day 6. The second fraction, 3−7 days, was subjected to the usual method of desorbtion from the Tenax (*23*) and used for HPLC method development. For "fraction 2", diethyl ether was used to desorb the volatiles from the Tenax. It was thought that the radical inhibitors in ether would help stabilize the styrene from radical polymerization. However, when the ether mixture of fraction 2 was concentrated for semipreparative HPLC, the mixture underwent decomposition (the peak corresponding to styrene decreased over time). It was not determined if the styrene was reacting with MCOT, if the styrene was polymerizing, or if ether or its contaminants played a role. The information regarding the inability to concentrate the styrene mixture was duly noted, and the VOCs from fraction 3 were desorbed using pentane and kept dilute prior to injection on the HPLC. The styrene was isolated using standard natural product chemistry techniques; although these techniques were not the most efficient for this particular compound, it did provide pure styrene to demonstrate that it was isolable from the volatile mixture. By retracing the amount of styrene isolated against the relative abundances from the SPME and GC−MS (**Figure 4**), a crude approximation of 200−240 mg of styrene were generated from 1 L of inoculated broth over 66 days. This does not include any styrene that may have been in the aqueous broth. If the solubility of styrene in water (300 mg/L) (*31*) is taken at face value, ignoring the fungus and byproduct in the mixture, it can be surmised that an additional 300 mg of styrene could have been present. This idea was not explored at the time of investigation because the goal was to determine the amount of volatile styrene generated from the fungal broth.

With the ability to isolate styrene from the volatile mixture in hand, attention was turned to the isolation and identification of the second major component tentatively assigned as MCOT. During the HPLC experiments to isolate styrene, the second peak detected was also collected. Concentration of the *n*-pentane and subsequent GC−MS with a slow oven ramp provided three peaks, all with M^+ m/z of 120 amu and similar fragmentation patterns, varying only in the relative abundance of major ions. Authentication of these isomers required a short chemical synthesis using a modified method (*32*) and starting from commercially available 3-methyl-1,5-cyclooctadiene (see Figure SI-1 in the Supporting Information). The isomers were isolated as a mixture via semipreparative HPLC and compared to retention times and fragmentation patterns of the VOC collected from *F. oxysporum*. It is hypothesized that only two isomers of MCOT are generated by the fungus and that the third isomer is a result of a known thermal cyclization to the bicycle[4.2.0]octa-2,4-diene (*33*).

One final point of discussion is a feasible biosynthetic scheme for production of styrene from *F. oxysporum*. The food production of styrene has been attributed to numerous precursors (*2*), with glucose having been reported as one of the reactants (*34*). It seemed logical to implicate glucose, the major component of PDA and PDB, as the primary feedstock of styrene. To verify this, a modified potato dextrose broth using C-6 ^{13}C-labeled glucose was inoculated with *F. oxysporum* and the small-

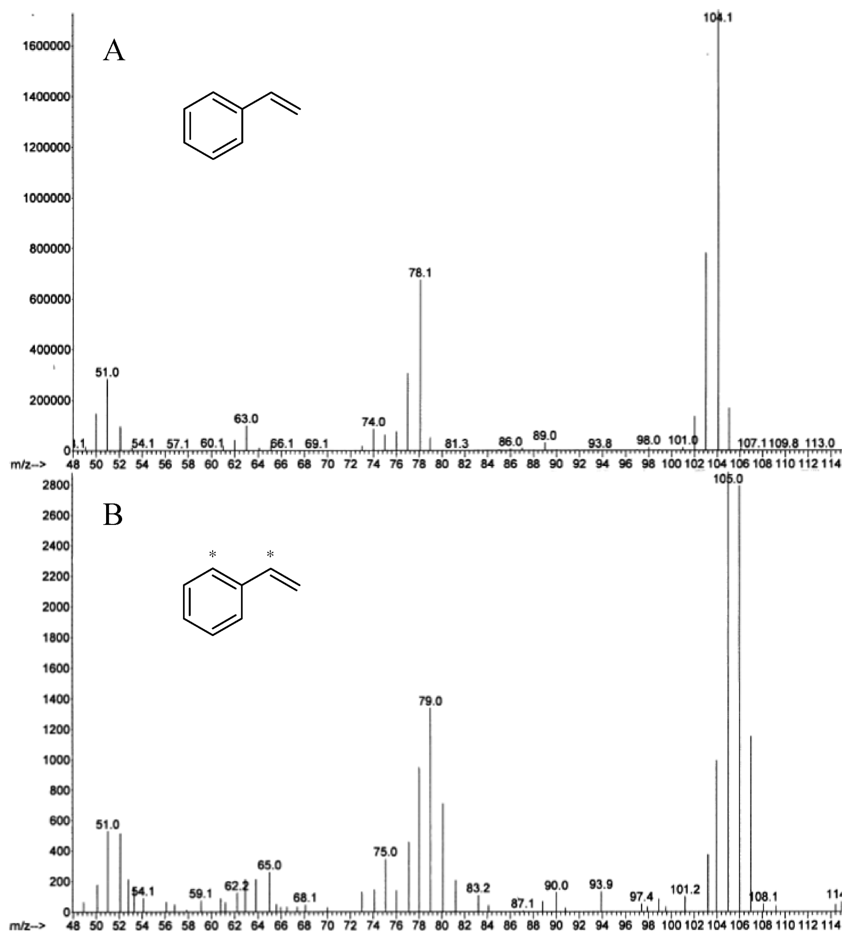

Figure 5. GC−MS fragmentation patterns for (A) unlabeled styrene and (B) ^{13}C-labeled styrene with probable positions of ^{13}C-labeling corresponding to a *m*/*z* of 106 amu shown as asterisks.

scale collection of VOCs was repeated (see the Supporting Information for detailed experimental procedures). The incorporation of the ^{13}C-labeled feedstock was determined by GC−MS. The fragmentation pattern for natural styrene (**Figure 5A**) shows the expected relative abundance of the *m/z* 104 (M^+) and 105 (M + 1) peaks. Assuming glucose undergoes glycolysis to form the intermediate phenylalanine via the shikimic pathway and then continues forward to form styrene, it was hypothesized that one ^{13}C-labeled glucose would be incorporated into the styrene at the *ortho* position on the aromatic ring (*12, 13*); however, inspection of the fragmentation pattern (**Figure 5B**) of the peak corresponding to styrene showed significant enhancement of the *m/z* 105 and 106 M^+ peaks, as well as an increase in the *m/z* 107 peak M^+, corresponding to incorporation of one, two, and three ^{13}C atoms, respectively. The approximate relative peak abundance of the *m/z* 104−107 peaks was 1:2:2:1. Accordingly, the normal *m/z* 78 fragment of styrene showed isotopic enrichment of the *m/z* 79 and 80 peaks in the enrichment experiment analogous to incorporation of two ^{13}C atoms into the aromatic ring.

Further investigation into a possible biosynthesis of styrene resulted in the proposed biosynthetic scheme outlined in **Figure 6**. During the pentose phosphate pathway, glycolysis of the labeled glucose results in two three-carbon units, glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate, which are in equilibrium with each other. This provides two available three-carbon units, of which one is labeled, available for conversion to phosphoenol pyruvate (PEP). GAP can also be converted to erythrose 4-phosphate (EAP), which eventually combines with PEP to form shikimic acid 3-phosphate (SAP).

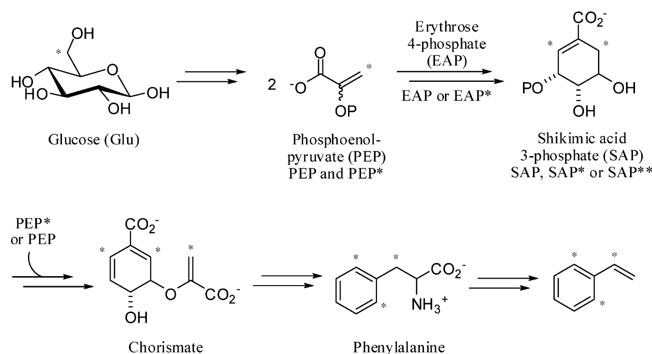

Figure 6. Proposed biosynthetic pathway of styrene by *F. oxysporum* showing probable positions of ^{13}C-labeling as asterisks. The pentose phosphate pathway converts glucose to EAP and PEP, which combine and proceed to the shikimic acid pathway providing phenylalanine, and then continues forward to form styrene. The pathway shows the maximum possible number of isotopic enrichments during the proposed biosynthetic scheme.

As a result of the downstream process, SAP can have zero, one, or two ^{13}C units incorporated. SAP undergoes further biosynthesis with PEP to provide the side chain of phenylalanine with one more possible ^{13}C incorporation for a final possible enrichment of three ^{13}C isotopes, resulting in the observed *m/z* 105, 106, and 107 peaks in **Figure 5B**.

Initially, only one ^{13}C-labeling experiment was performed (in duplicate) to determine if styrene would incorporate the isotopically enriched medium. Interestingly, MCOT was not observed during this experiment. It was hypothesized that,

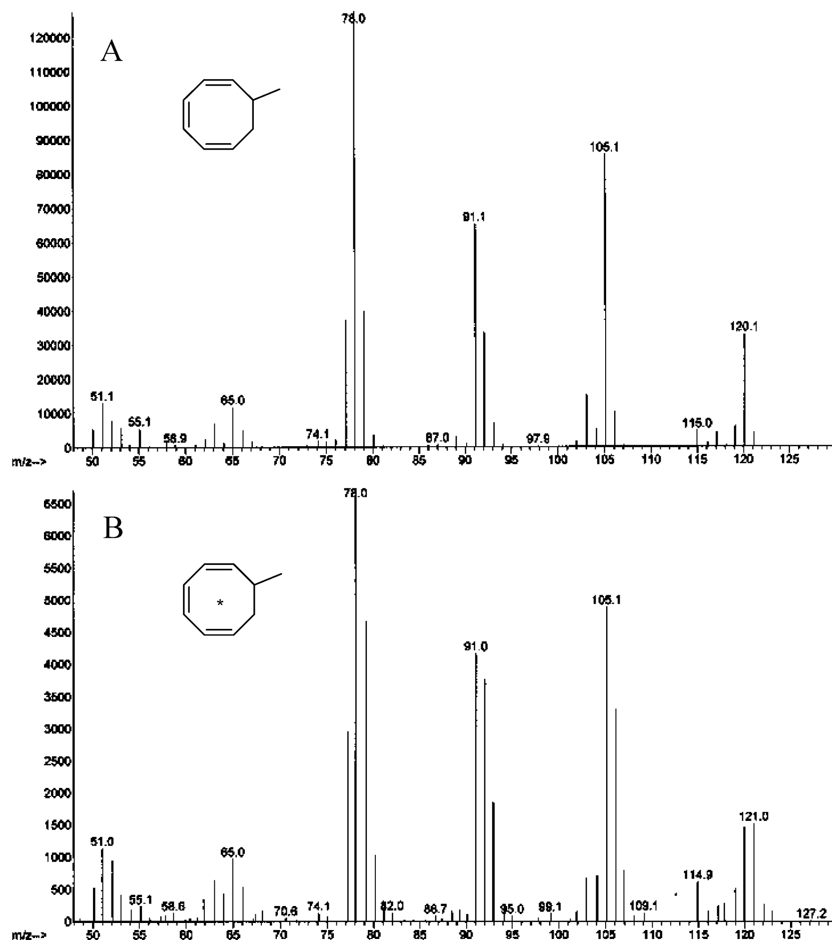

Figure 7. GC−MS fragmentation patterns for (A) unlabeled MCOT and (B) ^{13}C-labeled MCOT with fragments indicating the ^{13}C-labeled atom within the ring.

because of the small amount of glucose and the lapse between styrene versus MCOT generation, there was not enough feedstock to generate the MCOT. To test this hypothesis, a second experiment was performed, but with no ^{13}C-enriched glucose added until after the MCOT was seen by GC−MS (see the Supporting Information for detailed experimental procedures). The remaining ^{13}C-enriched glucose was added to the broth and the VOCs monitored on a daily basis. The small amount of ^{13}C-enriched glucose was able to sustain enriched MCOT biosynthesis for 2 days before diminishing. The resulting fragmentation pattern (**Figure 7**) showed that MCOT was able to incorporate one ^{13}C-enriched glucose demonstrated by a 1:1 ratio of *m*/*z* 120 (M^+ of MCOT) and 121 (M^+ of enriched MCOT) versus the normal relative ratio of 100:8.8 of $M^+/M + 1$ of nonenriched MCOT. The isotope appears to be incorporated within the ring, because the $M^+ - 15$ (M^+ less the methyl group) of *m*/*z* 105 shows a distinctive increase in the *m*/*z* 106 fragment. The location of the enriched position within the ring was not determined; however, once the methyl group for both the enriched and nonenriched MCOT molecules is lost, the fragmentation of the resultant cyclooctatriene cation is identical to a literature report (*35*).

Once optimized, the project shows promise to supply the ubiquitous, industrially important feedstock monomer, styrene with possible industrial applications, and the novel hydrocarbon, MCOT. There are numerous options that can be explored for all aspects of production, all of which can be performed with "green chemistry" alternatives; options include multiple sources of feedstock (leftover industrial and/or agricultural byproducts) or direct polymerization of styrene to eliminate isolation issues. Most importantly, it was demonstrated that (1) styrene was one of two metabolites produced in major amounts, (2) both styrene and MCOT were produced over a period of time from a finite amount of feedstock, (3) the fungal broth was scalable to a level that allowed both styrene and MCOT to be isolable from the volatile mixture, and (4) the scaled-up broth mixture could mimic a starting point for industrial production of styrene.

ABBREVIATIONS USED

EAP, erythrose 4-phospate; GAP, glyceraldehyde 3-phosphate; GC−MS, gas chromatography−mass spectroscopy; HPLC, high-performance liquid chromatography; HREIMS, high-resolution electron-impact mass spectroscopy; MCOT, 7-methyl-1,3,5-cyclooctatriene; NRRL, Northern Regional Research Lab; PDA, potato dextrose agar; PDB, potato dextrose broth; PDMS, polydimethylsiloxane; PEP, phosphoenol pyruvate; PEST, permeation/exposure/storage/thermal desorbtion; SAP, shikimic acid 3-phosphate; SPME, solid-phase microextraction; TE, Tris/EDTA; VOC, volatile organic compound.

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Supporting Information Available: Detailed materials and methods for fungus identification, styrene standard conditions,

styrene production over time, small scale; ^{13}C-enhanced experiments for styrene and MCOT; synthetic scheme for MCOT synthesis; comparison of retention times and fragmentation patterns of synthesized MCOT and natural MCOT; and the thermal isomerization of MCOT. This material is available free of charge via the Internet at http://pubs.acs.org.

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