



## Alternariol: evidence for biosynthesis via norlichexanthone

E. E. STINSON, W. B. WISE, R. A. MOREAU, A. J. JUREWICZ, and P. E. PFEFFER. *Can. J. Chem.* **64**, 1590 (1986).

*Alternaria* molds produce numerous mycotoxins including many  $\alpha$ -dibenzopyrones such as alternariol (AOH) and related polyketides. AOH, presumed to be the initial  $\alpha$ -dibenzopyrone produced, has for 20 years been considered to be biosynthesized from a single polyketide chain in a single step reaction. The present study presents evidence that the reaction may proceed through an intermediate, norlichexanthone (NLX). Bond cleavage and rearrangement of NLX to form AOH may be similar to aflatoxin B<sub>1</sub> formation from sterigmatocystin. The 2-D INADEQUATE experiment was used to assign the <sup>13</sup>C spectrum of AOH and to distinguish between possible mechanisms by which AOH may be synthesized from 1-<sup>13</sup>C and 2-<sup>13</sup>C acetates via NLX.

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Les moisissures *Alternaria* produisent diverses mycotoxines, y compris plusieurs  $\alpha$ -dibenzopyrones, comme l'alternariol (AOH) et des polycétides apparentés. Il est supposé que le AOH est l' $\alpha$ -dibenzopyrone qui est produite initialement et, depuis plus de 20 ans, on a considéré que ce composé est produit par une biosynthèse impliquant une seule chaîne polycétide réagissant au cours d'une seule réaction. Dans le présent travail, on présente des données suggérant que le réaction procède peut-être par le biais d'un intermédiaire, la norlichexanthone (NLX). Il est possible que le bris de la liaison et la transposition de la NLX pour former le AOH se produisent d'une façon semblable à ce qui se produit lors de la formation de l'aflatoxine B<sub>1</sub> à partir de la stérigmatocystine. On a fait appel à des expériences de 2-D INADEQUATE pour attribuer le spectre rmn du <sup>13</sup>C du AOH et pour distinguer entre les mécanismes possibles pour la synthèse du AOH à partir d'acétates marqués au <sup>13</sup>C dans les positions 1 et 2 et par le biais de la NLX.

[Traduit par la revue]

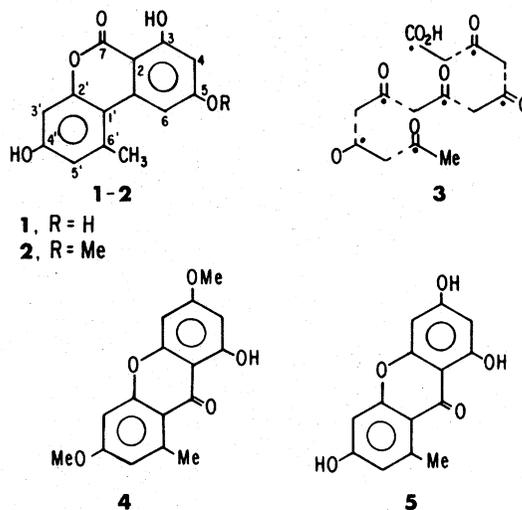
The *Alternaria*, a group of molds frequently involved in food spoilage, produce a wide variety of metabolites including the mycotoxins alternariol (AOH) (1) and alternariol monomethyl ether (AME) (2). These have been identified in food products contaminated by *Alternaria* (1). We became interested in the biosynthesis of these substances during the course of an investigation into the structure of fungal polyketide synthetases. The soluble enzyme responsible for the production of AOH seemed a good candidate for this study because of what then appeared to be the direct nature of the reaction. The synthesis of AOH had been reported to involve assembling a heptaketide chain, aromatizing, and release of the AOH in a single step. The pattern given for assembly of AOH and AME from 1-<sup>14</sup>C acetate is shown in 3 (2, 4). A procedure was given for partial purification of the enzyme. The resulting solution also possessed O-methyltransferase activity capable of forming AME from AOH by a transmethylation reaction involving S-adenosyl methionine (SAM) (2, 3).

We report results of the present study, which indicate that NLX is a precursor of AOH.

### Results and discussion

During our investigation, repeated attempts to duplicate the purification procedure for AOH synthetase were unsuccessful (5, 6). Using a cell-free extract prepared from a high AOH-producing strain, NRRL 6434, of *A. tenuis*, the species employed in the earlier biosynthetic studies, no production of AOH could be confirmed upon addition of <sup>14</sup>C labeled Ac-CoA and Mal-CoA to the cell-free extract.

However, we observed incorporation of <sup>14</sup>C Ac-CoA and Mal-CoA into materials on numerous zones of the tlc (thin-layer chromatography) plate, with heavy <sup>14</sup>C incorporation into a zone immediately above AOH. The same cell-free extract also catalyzed the formation of large amounts of <sup>14</sup>C AME in the



presence of AOH and <sup>14</sup>C SAM (as Gatenbeck had observed for his preparation), although whole cells of this strain of the fungus produce mainly AOH, not AME. Our inability to confirm the formation of AOH led us to consider the possibility that the previous group may actually have reported the formation of some other component that could not be separated from AOH by the methods then available.

Our detection method for AOH used the high resolution power of tlc and the automated linear analyzer for <sup>14</sup>C detection instead of the less selective paper chromatography used by the previous group (2).

Also, the previous group relied upon repeated recrystallization of product, which had been diluted with a large excess of exogenous AOH, to constant specific activity as confirmation that the counts were in the AOH rather than in some impurity. They used H<sub>2</sub>O-EtOH mixtures as solvent, although it is known that recrystallization from solvent pairs often leads to impure material. In fact, it had been reported previously that recryst-

<sup>1</sup> Author to whom correspondence may be addressed.

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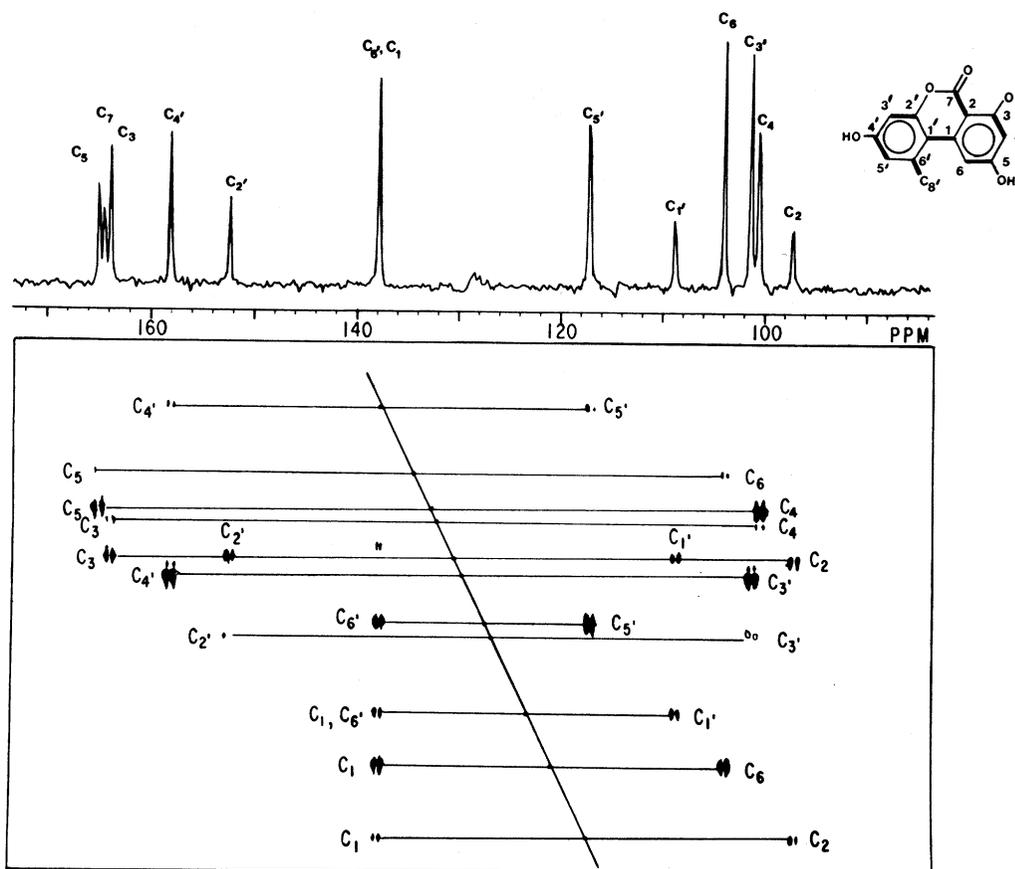


FIG. 1. 100-MHz  $^{13}\text{C}$  2-D INADEQUATE contour plot for a 0.95 M solution of alternariol (biosynthetically prepared from equal amounts of  $[1-^{13}\text{C}]$  and  $[2-^{13}\text{C}]$  acetates). Total acquisition time, 84 h. 1-D nmr shown above.

tallization from this solvent mixture ( $\text{H}_2\text{O}$ - $\text{EtOH}$ ) failed to remove a colored impurity from AOH (7). This was also our experience, as tlc examination disclosed that careful precipitation of AOH by the described method actually resulted in retention of a number of congeners.

An alternative mechanism for AOH biosynthesis has been suggested (1), which would proceed through the rearrangement of xanthone, in analogy to a well-documented step in aflatoxin biosynthesis (8). This notion is strongly supported by the recent isolation from *Penicillium notatum* of lichenxanthone (4) as a congener of AME (9). The same general mechanism, oxidative cleavage of an aromatic ring followed by rotation of the fragmented aryl structure, has also been reported in the formation of the  $\alpha$ -pyrone structure of chartreusin in the *Streptomyces* (10). These and other considerations led us to consider norlichexanthone, NLX (5), as a possible precursor.

#### Incorporation of norlichexanthone (NLX)

Particular care was taken with the purification procedure used to establish incorporation of norlichexanthone into AOH since it appears that the radioactivity measured in the earlier work (2, 3) may not have been associated with alternariol.

$^{14}\text{C}$ -labeled norlichexanthone was added to a stationary culture of *Alternaria tenuis* shortly after vigorous growth had ceased (Day 10). Previous experiments in our laboratory had shown that this was the period for optimal incorporation of  $^{14}\text{C}$ -labeled acetate into AOH. The mycelia were harvested after a suitable incubation period (Days 10-12), and extracted with ethyl acetate. AOH was isolated from this mycelial extract.

Two different solvent systems were used in succession for flash chromatography, and a normal and a reverse phase system were used for final purification by preparative tlc. Purification of the metabolite to constant specific radioactivity by these procedures indicated 3.8% incorporation of NLX into AOH in vivo.

#### $^{13}\text{C}$ spectral assignment of alternariol

As a prerequisite for investigation of the biosynthetic pathway to the production of AOH via NLX, it was necessary to establish an unambiguous assignment of the complete  $^{13}\text{C}$  spectrum of this molecule. The  $^{13}\text{C}$  shifts of alternariol have previously been only partly assigned (12), but we were able to make a full assignment by means of 2-D  $^{13}\text{C}$  INADEQUATE nmr (13). To this end, we used alternariol produced by *Alternaria tenuis* grown on a medium supplemented with 1- $^{13}\text{C}$  and 2- $^{13}\text{C}$  acetate in equal amounts (Fig. 1). It was anticipated that  $^{13}\text{C}$ - $^{13}\text{C}$  coupling would be observed with greater probability (and thus the signal enhancement would be greater) between C1 and C2 of each pair of adjacent acetate residues, beginning at C6'-C5'. A similar phenomenon has been observed with 1-D  $^{13}\text{C}$  nmr (14). The starting point in assigning the signals from the aromatic carbons was the signal of the ring carbon C6' (138.3 ppm) directly bonded to the C8' methyl group at 25.3 ppm. This signal was identified by the proton coupled  $^{13}\text{C}$  spectra (not shown), which showed a quartet (5.8 Hz) at this location. In addition, long range 3-bond coupling was observed from the  $\text{CH}_3$  group to the signal with split resonances (due to this geminal proton; 160 Hz) centered at 117.6 ppm, which was thus identified as C5'.

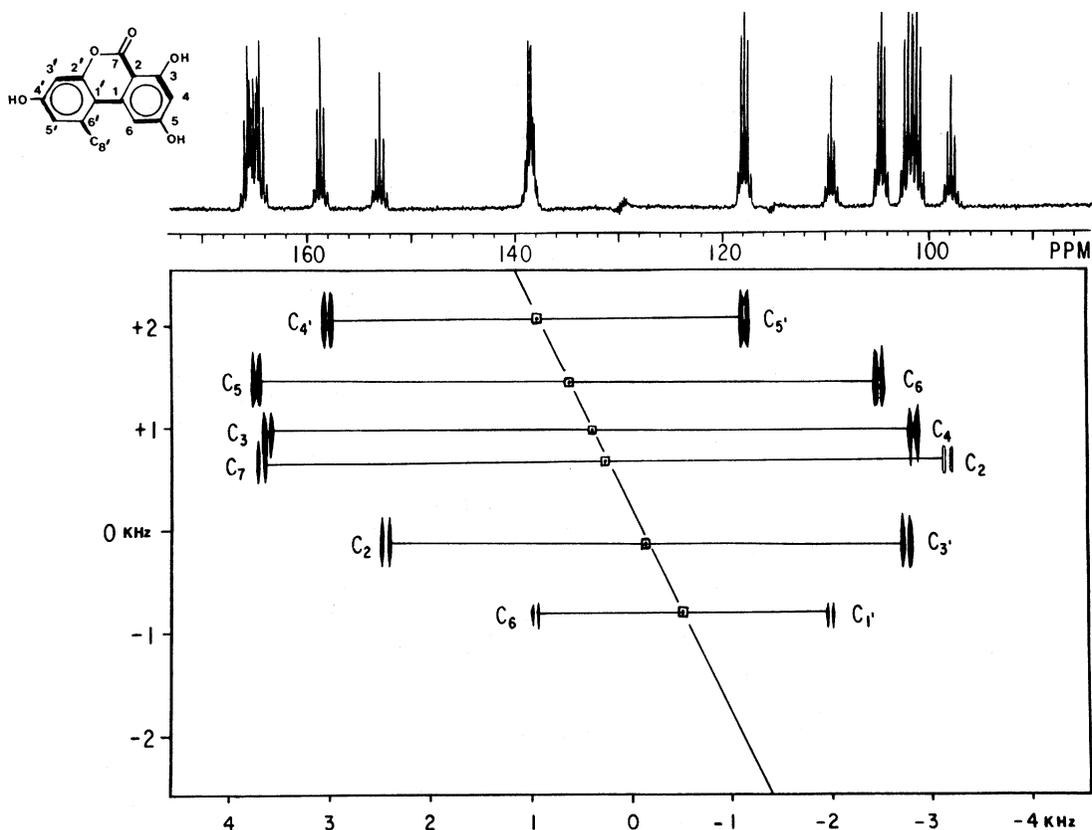


Fig. 2. 100-MHz  $^{13}\text{C}$  2-D INADEQUATE contour plot for a 0.47 M solution of alternariol (biosynthetically prepared from  $[1,2\text{-}^{13}\text{C}]$  acetate). Only the 5-kHz region in the vicinity of  $F_1 = 0$  is shown. Total acquisition time from this spectrum was 38 h. 1-D nmr shown above.

The resulting 2-D contour plot (Fig. 1) of connectivities (one-dimensional spectrum displayed above) shows the expected lower intensity of response for connectivities between those carbon pairs originating from intact acetate units. The dynamic range difference between these and the enriched  $^{13}\text{C}$ - $^{13}\text{C}$  coupled carbons occurring at the junction between adjacent acetate units is not large enough to pose any observational difficulty and thus we were able to proceed with a full assignment of carbon signals from this single experiment. The longer spin-lattice relaxation times of the quaternary carbon pairs ( $\text{C}2'\text{-C}1'$ ,  $\text{C}6'\text{-C}1'$ ,  $\text{C}1\text{-C}2$ ) contributed to the lower spectral responses for these resonances.

#### Possible mechanism for the biosynthesis of alternariol

Table 1 lists the full spectral assignment for alternariol.

Utilizing these assignments, an nmr experiment that examined AOH enriched using  $2\text{-}^{13}\text{C}$  acetate established that the biosynthetic pathway for AOH starts at the  $\text{C}8'$  and proceeds through the molecule with uniform enrichment of  $^{13}\text{C}$  at alternate carbons located at  $8'$ ,  $5'$ ,  $3'$ ,  $1'$ ,  $6$ ,  $4$ , and  $2$ . This experiment agrees with the alternating pattern found with  $1\text{-}^{14}\text{C}$  acetate (4) and presented in 3.

To determine whether the biosynthesis of AOH involves cleavage of acetate units, we examined the nmr spectra of AOH synthesized by *Alternaria tenuis* when grown on medium containing  $1,2\text{-}^{13}\text{C}$  acetates. The results of this experiment are clearly illustrated by the 2-D INADEQUATE contour plot shown in Fig. 2. As is evident, every carbon pair in this spectrum is coupled, which shows retention of intact acetate

TABLE 1. Nuclear magnetic resonance spectral assignment for AOH

Assignment	Shift $\sigma^a$	Multiplicity <sup>b</sup>	$\sigma^a$ 2-D connectivities
C-8'	25.3	Quartet	— <sup>c</sup>
C-6'	138.3	Singlet	109.0, 117.6
C-5'	117.6	Doublet	138.3, 158.4
C-4'	158.4	Singlet	117.6, 101.6
C-3'	101.6	Doublet	158.4, 152.6
C-2'	152.6	Singlet	101.6, 109.0
C-1'	109.0	Singlet	138.3, 152.6, 138.1
C-1	138.1	Singlet	104.4, 97.4, 109.0
C-2	97.4	Singlet	138.1, 164.7
C-3	164.1	Singlet	97.4, 100.9
C-4	100.9	Doublet	164.1, 165.5
C-5	165.5	Singlet	100.9, 104.4
C-6	104.4	Doublet	165.5, 138.1
C-7	164.7	Singlet	97.4 <sup>d</sup>

<sup>a</sup>Shift expressed in ppm relative to the solvent, DMSO, assigned a value of 39.5 ppm.

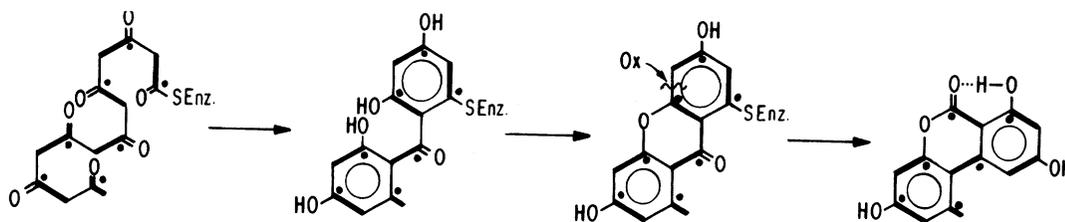
<sup>b</sup>Large one-bond C—H coupling only.

<sup>c</sup>Not in the spectral range.

<sup>d</sup>Assigned on basis of  $1,2\text{-}^{13}\text{C}$  INADEQUATE experiment.

units throughout the molecule. This pattern of incorporation also agrees with 3. This experiment gave no indication of a mixture of coupling patterns for AOH, indicating that no randomization had occurred.

No evidence that would indicate uncoupled carbon or a randomized pattern was observed in any of our  $^{13}\text{C}$  nmr experiments. This would imply that the biosynthesis is under



SCHEME 1

close steric control by the enzyme that prevents free rotation of aryl groups. The high rate of incorporation of NLX into AOH that is described in the Experimental probably indicates that NLX (or a very similar compound) is on the main biosynthetic route for AOH.

Scheme 1 presents a biosynthetic mechanism for biosynthesis of AOH that meets the above criteria. In this scheme, the point of attachment between the enzyme and the polyketide is shown as the active end of the polyketide chain, although the attachment could have migrated or could even involve several points of contact. The polyketide chain is assembled on the surface of the enzyme in a configuration that facilitated the formation of NLX. After rupture of the aromatic phloroglucinol ring, limited rotation of the aryl fragment and ring closure would produce the coupling pattern observed in AOH. This mechanism may require a second enzyme for oxidative cleavage of the aromatic ring. A reversible "retro-aldol" cleavage would result in destruction of an aromatic ring (which would be unlikely from free energy considerations), while oxidative fission of aromatic groups is widespread in the fungi (15, 16).

Other heptaketide assembly patterns and reactions can be suggested in addition to the mechanism shown in Scheme 1. For example, schemes can be devised in which (1) the phloroglucinol portion of the polyketide is assembled in a counterclockwise pattern, or (2) the polyketide chain could be assembled in the benzophenone configuration reported for griseofulvin biosynthesis (where NLX is also produced) (17). These mechanisms are more complex than that shown in Scheme 1 as they all require controlled rotation of aromatic groups to result in the observed AOH coupling pattern.

### Experimental

Solvents were reagent "distilled in glass" quality. The yeast extract and potato dextrose agar were from Difco Laboratories (Detroit, Mich.). Analytical tlc was on scored, precoated plates fluorescent under 254 nm ("Uniplat", Silica Gel GF, 250  $\mu$ m, Analtech, 75 Blue Hen Drive, Newark, DE 19711). Column chromatography was by the "flash chromatography" method (11) using the Ace Glass apparatus (Vineland, NJ) and 230–400 mesh silica gel (Merck, grade 60; Aldrich Chemical Co., Milwaukee, WI 53201). Sodium [ $1-^{13}\text{C}$ ] and [ $1,2-^{13}\text{C}$ ] acetate, 99%  $^{13}\text{C}$ , and deuterated solvents were purchased from KOR (Cambridge, MA). Sodium [ $2-^{13}\text{C}$ ] acetate, 99%, was purchased from Stohler Isotope Chemicals (Waltham, MA). Evaporations were done at room temperature or under a  $\text{N}_2$  stream. Mass spectra were obtained using the direct injection probe (DIP) mode of the Hewlett-Packard 5885 mass spectrometer. The infrared spectra were obtained using a 237B Perkin-Elmer spectrophotometer and KBr discs. Authentic samples of norlichexanthone, both natural carbon and  $^{14}\text{C}$ -labeled at the ketone carbon (30 000 cpm/mg), were kindly supplied by Dr. Constance M. Harris, Vanderbilt University, Nashville, TN.

#### *Fungal cultures and production of labeled mycotoxin*

The culture of *Alternaria tenuis* NRRL 6434, a strain with high production of AOH and a simple metabolic pattern that facilitated purification, was obtained from the Northern Regional Research Center, USDA/ARS, Peoria, IL. A single spore isolate from this strain

was maintained on potato/dextrose agar. Fresh cultures of this isolate were started at frequent intervals, and portions of well sporulated 14–18 day old mycelia were used to inoculate 250-mL Erlenmeyer flasks each containing 60 mL of modified Czapek-Dox (MCD) medium. This medium contained 40 g glucose, 1.0 g yeast extract, 1.0 g  $\text{NaNO}_3$ , 1.0 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g  $\text{NH}_4\text{Cl}$ , 0.25 g  $\text{KCl}$ , 0.25 g  $\text{NaCl}$ , 0.01 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.01 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , made up to 1 L with  $\text{H}_2\text{O}$  (18). These flasks were then incubated as stationary cultures in the dark at 25°C. Spiking with precursors was started on the 12th day and continued until the 16th day. The precursors were added by injecting the solutions into the MCD media beneath the mycelial cap. The flasks were then gently rotated without submerging any portion of the cap to mix the added precursor with the media. The acetates were added as 50 mg/0.2 mL aqueous solution per day and the NLX was added as 0.167 mg NLX/0.02 mL absolute EtOH per day. The mycelial cap was removed intact on the 17th day, and the cap was extracted three times by homogenizing with 30 mL MeOH for 1 min in a Waring Blender and filtering. The extracts were combined and 50 mL  $\text{H}_2\text{O}$  was added. The solution was then extracted 3 times with 50 mL  $\text{CHCl}_3$ . The combined  $\text{CHCl}_3$  solutions were concentrated by evaporation and the AOH was isolated and purified by flash chromatography. The column was developed first with 1 L of  $\text{CHCl}_3$  to remove fast moving materials, and then developed with 1 L  $\text{CHCl}_3$  solutions containing 2, 5, and 10% MeOH. The AOH emerged in the 2 and 5% MeOH eluates. The AOH was then repurified by flash chromatography using a hexane–EtOAc gradient. The AOH emerged at 25% EtOAc–hexane.

In a typical run, the yield of AOH obtained from 5 flasks was 307 mg. For unknown reasons, the yield of AOH and incorporation of precursors was highly variable. For most acetate runs under these conditions, incorporation into AOH was between 3 and 7% as determined by ms (mass spectra). The identity and purity of NLX and AOH were determined by ir, ms, and tlc with 5 solvent systems: toluene–glacial HOAc, 9:1;  $\text{CHCl}_3$ –1.6% EtOH;  $\text{CH}_2\text{Cl}_2$ –acetone, 95:5; toluene–EtOAc– $\text{HCO}_2\text{H}$ , 6:3:1; and  $(\text{Et})_2\text{O}$ –hexane–HOAc, 8:2:0.5. The yield of purified AOH from the flash column during the NLX incorporation experiment was 76.5 mg.

Thin-layer chromatography used 250- $\mu$ m "Uniplates" (Analtech) using Silica Gel GF for normal phase and Silica Gel RPS for reversed phase tlc. The respective solvents were toluene–ethyl acetate– $\text{H}_2\text{O}$  (6:3:1, v/v basis) and  $\text{H}_2\text{O}$ –methanol (65:35, v/v).  $^{14}\text{C}$  activity on channeled tlc plates was detected by use of the tlc linear analyzer (Model LB 27 C, Berthold Analytical Instruments, 28 Charron Ave., Nashua, NH). Samples of the AOH obtained from the flash chromatographic columns were further purified by 3 preparative tlc purifications using each of these systems. Specific activity was determined by calculating  $^{14}\text{C}$  activity (determined on a Beckman LS 8100 scintillation counter with "Aqualol" scintillation cocktail, New England Nuclear, Boston, MA) vs. peak height at 280 nm (Beckman Model 35 Spectrophotometer). The specific activity was constant after the first purification with each system.

#### *Nuclear magnetic resonance parameters*

Carbon-13 nmr spectra were measured in a 5-mm probe at 100.4 MHz using a JEOL GX-400 spectrometer system that included a 9.4 T Oxford narrow bore magnet and DEC LSI 11/23 data system. All data processing was with the JEOL Plexus software, version 2. Broadband  $^1\text{H}$  decoupling was accomplished through the use of Waugh-type sequences (19, 20), and proton coupled  $^{13}\text{C}$  spectra were measured

with grated decoupling in order to retain the nuclear Overhauser enhancements.

The two-dimensional studies were performed using the "INADEQUATE" pulse sequence proposed by Mareci and Freeman (21), where the final "read" pulse is allowed to have an arbitrary flip angle:

$$90^\circ(X) - \tau - 180^\circ(Y) - \tau - 90^\circ(X) - t_1 - \alpha(X)$$

$\tau$  was chosen to optimize conversion in double-quantum coherence in the usual manner,  $\tau = (4J_{CC})^{-1}$ , and the echo components emphasized by selecting  $\alpha = 135^\circ$ .

The 2-D INADEQUATE spectrum of alternariol (prepared utilizing  $[1-^{13}\text{C}]$  and  $[2-^{13}\text{C}]$  acetate) was obtained by processing an initial data matrix ( $t_1 \times t_2$ ) of  $128 \times 1024$  points, representing spectra widths ( $F_1 \times F_2$ ) of  $5 \text{ kHz} \times 9 \text{ kHz}$ . The  $\pi/2$  pulse width was  $11.4 \mu\text{s}$ , and  $\tau$  was  $4.386 \text{ ms}$  corresponding to  $J_{CC} = 57 \text{ Hz}$ . An overall recycle delay of  $4.5 \text{ s}$  was used to acquire 512 scans for each value of the incremented delay  $t_1$ . The 1-D spectrum reproduced above the 2-D contour plot was produced by transforming 1 K data acquired in a 9-kHz window using a 17.5 Hz broadening factor.

Finally, the 2-D data spectrum of alternariol (prepared utilizing  $[1,2-^{13}\text{C}]$  acetate) was obtained by processing an initial data matrix ( $t_1 \times t_2$ ) of  $512 \times 1024$  points representing spectral widths ( $F_1 \times F_2$ ) of  $100 \text{ kHz} \times 9 \text{ kHz}$ . Other parameters were the same as in the previous experiment; however, only 64 scans were acquired at each  $t_1$  value. The attached 1-D spectrum was prepared from a 32 K transform of data acquired in the same 9-kHz window, and a broadening factor of 2 Hz was used in data processing.

All 2-D data sets were double Fourier transformed utilizing trapezoidal window functions to approximate the sine-bell functions frequently used to improve the appearance of two-dimensional contour plots.

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