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RESEARCH ARTICLE

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# Oxidative Deamination of Hydrolyzed Fumonisin B<sub>1</sub> (AP<sub>1</sub>) by Cultures of *Exophiala spinifera*

Barbara A. Blackwell, Jacob T. Gilliam, Marc E. Savard, J. David Miller and Jonathan P. Duvick,

<sup>1</sup>Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Canada K1A 0C6

<sup>2</sup>Department of Crop Protection, Pioneer Hi-Bred, International, Inc., Box 552, Johnston, IA 50131 USA

<sup>3</sup>Ottawa-Carleton Chemistry Institute, Carleton University, Ottawa, Canada K1S 5B6

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**ABSTRACT** Fumonisin is a mycotoxin of world-wide distribution in maize infected by the fungus *Fusarium verticillioides*. They are highly toxic to certain livestock and are potential carcinogens. *Exophiala spinifera*, a black yeast fungus found on moldy maize kernels, was identified previously as capable of growing on fumonisin B<sub>1</sub> as a sole carbon source and thus is a potential source for fumonisin detoxifying enzymes. Pure cultures of *E. spinifera* transform fumonisin B<sub>1</sub> to the amino polyol AP<sub>1</sub> plus free tricarballic acid through the activity of a soluble extracellular esterase, and further transformation is evidenced by accumulation in culture supernatant of a less polar compound(s) lacking a fluorescamine-reactive amino group. A free amine is thought to be critical for biological activity of FB<sub>1</sub> or AP<sub>1</sub>. As a first step towards characterizing this amine-modifying activity, we investigated the biotransformation of AP<sub>1</sub> by *E. spinifera* liquid cultures that had been previously grown in liquid medium containing AP<sub>1</sub> as a sole carbon source. Accumulation of AP<sub>1</sub>-derived metabolites was monitored by thin-layer chromatography of culture supernatants, and product metabolites were purified and evaluated by mass spectrometry and nuclear magnetic resonance. Two products of treatment of purified AP<sub>1</sub> with cultures of *E. spinifera* are shown to be N-acetyl AP<sub>1</sub> and a new compound, 2-oxo-12,16-dimethyl-3,5,10,14,15-icosanepentol hemiketal (or 2-OP<sub>1</sub> hemiketal). Copyright © 1999 John Wiley & Sons, Ltd.

**Key words:** *Exophiala spinifera*; fumonisin B<sub>1</sub>; *Fusarium verticillioides*; N-acetyl AP<sub>1</sub>; 2-OP<sub>1</sub> hemiketal; maize

## INTRODUCTION

Fumonisin B<sub>1</sub> (FB<sub>1</sub>) was characterized from cultures of *Fusarium verticillioides* by Bezuidenhout *et al.* (1988) and has since been found as a common contaminant of corn (International Programme on Chemical Safety, 1998). *F. verticillioides* and a related species, *F. proliferatum* (which also produces FB<sub>1</sub>), are the most common fungi associated with maize (Shurtleff, 1980). These fungi cause *Fusarium* ear rot, one of the most important diseases in hot corn-growing areas.

FB<sub>1</sub> is toxic to most domestic animals, and epidemiological data suggest that human exposures to fumonisins result in esophageal cancer (IARC, 1993). The difficulty of breeding for resistance together with health, trade and economic problems associated with fumonisin contamination of corn makes them worthwhile targets for

strategies aimed at detoxification *in planta* (Karlovsky, 1999).

In a screening program to identify fumonisin-detoxifying microbes, a black yeast fungus capable of growing on fumonisin B<sub>1</sub> as a sole carbon source was isolated from moldy maize ears (Duvick *et al.*, 1994; 1998). This fungus, identified as *Exophiala spinifera*, produces fumonisin catabolizing enzymes when FB<sub>1</sub> or its hydrolysis product, AP<sub>1</sub>, are supplied as a sole carbon source in a mineral salts liquid medium. One of these enzymes is a secreted carboxylesterase which hydrolyzes the tricarballic side chains of FB<sub>1</sub> and related

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\*Correspondence to: J. P. Duvick, Department of Crop Protection, Pioneer Hi-Bred, International, Inc., Box 552, Johnston, IA 50131 USA.  
e-mail: duvickj@phibred.com

Received 24 June 1998; Accepted 21 March 1999.

compounds, giving rise to free tricarballylic acid and an aminopentol backbone similar to alkaline hydrolyzed FB<sub>1</sub> (AP<sub>1</sub>) (Duvick *et al.*, 1998). A cDNA that encodes fumonisin tricarballylate esterase activity has been cloned from *E. spinifera* and shown to function in transgenic maize (Duvick *et al.*, in preparation). When cultures of *E. spinifera* are grown on uniformly <sup>14</sup>C-labelled FB<sub>1</sub> (Blackwell *et al.*, 1994) or its alkaline-hydrolyzed product <sup>14</sup>C-AP<sub>1</sub>, as a sole carbon source, the backbone molecule represented by AP<sub>1</sub> is ultimately taken up by *E. spinifera* cells, and <sup>14</sup>CO<sub>2</sub> is generated, indicating that additional fumonisin catabolic enzymes are present in this fungus (Duvick *et al.*, 1998). Fumonisin esterase activity has also been detected in a culture of *Fusarium proliferatum* (Keller *et al.*, 1998).

While tricarballylic acid shows very little toxicity in mammalian and plant cell assays (Gelderblom *et al.*, 1993); Lamprecht *et al.*, 1994) the AP<sub>1</sub> backbone retains a portion of the toxicity of the intact fumonisin molecule (Norred *et al.*, 1997). Several studies employing fumonisin analogs suggest the amine group, along with the C-3 hydroxyl, is important for biological activity and toxicity of fumonisins (Abbas *et al.*, 1993); Gelderblom *et al.*, 1993); Voss *et al.*, 1996). All fumonisins share the same conformational structure from C-1 to C-3, yet differ in the presence or absence of hydroxyl functions at C-4 (McKenzie *et al.*, 1998), C-5, C-10 and C-15 (Musser *et al.*, 1995), supporting this premise. Attempts to modify fumonisin using crude or purified enzymes has been unsuccessful (Murphy *et al.*, 1996; Cawood *et al.*, 1994). A method employing high temperature coupling to sugars (Lu *et al.*, 1997; Howard *et al.*, 1998) may prove useful, but is of limited utility where raw grain is consumed. Therefore, the enzymatic removal of the amine group of fumonisin or its hydrolysis product could be considered an important alternative method of detoxification. In order to evaluate such a strategy, it is necessary to know the structure and biological activity of products of deamination of fumonisin. In this report we describe the structure and stereochemistry of 2-oxo-12,16-dimethyl-3,5,10,14,15-icosanepentol hemiketal (2-OP<sub>1</sub> 4), the product of oxidative deamination of AP<sub>1</sub> by the black yeast fungus *Exophiala spinifera*.

## MATERIALS AND METHODS

### Preparation of Hydrolysed FB<sub>1</sub> (AP<sub>1</sub>) Substrate

FB<sub>1</sub> was produced and purified as previously described (Blackwell *et al.*, 1994; Miller *et al.*, 1994). FB<sub>1</sub> (1.07 g) was treated with 25 ml of 2N KOH at 70°C for 2 h. After cooling, an insoluble pellet had formed. Extraction with EtOAc (3 × 20 ml) dissolved this pellet. The extract was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The residue was chromatographed on SiO<sub>2</sub> (100 g) with CHCl<sub>3</sub>/MeOH/NH<sub>4</sub>OH (60:30:2), collecting 17 ml fractions. The

product was found in fractions 7–11. The yield was 0.58 g AP<sub>1</sub> (97%). AP<sub>1</sub> was characterized by nuclear magnetic resonance (NMR) (Tables 1 and 2) and mass spectrometry (MS).

### Conversion of AP<sub>1</sub> by *E. spinifera* Cultures

Liquid cultures of *Exophiala spinifera* isolate 2141.10 were prepared from YPD agar plates (yeast extract 10 g, bacto-peptone 20 g, dextrose 0.5 gm, bacto-agar 15 g per liter of H<sub>2</sub>O). Aliquots (400–500 µl) of a water suspension of *E. spinifera* cells from YPD agar were spread uniformly onto 150 × 15 mm YPD agar plates with 4 mm sterile glass beads. The plates were incubated at room temperature for 6–7 days. The mycelia/conidia were transferred from the agar plates into mineral salts medium (MSM) (Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 9 g, KH<sub>2</sub>PO<sub>4</sub> 1.5 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g, NH<sub>4</sub>Cl 1.0 g, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.01 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.02 g per liter of distilled H<sub>2</sub>O, pH 4.5) and centrifuged at 5000 × g, 4°C, 10 min to pellet the cells. The cell pellet was rinsed once with 40 ml MSM and recentrifuged. The rinsed cell pellet was used to inoculate MSM at a 1:19 ratio of packed cells: MSM. The culture was supplemented with AP<sub>1</sub> (prepared as described in previous section) to a final concentration of 0.5–1.0 mg ml<sup>-1</sup> and incubated at 28°C, 100 rpm, in the dark to induce catabolic enzymes. The *in vivo* conversion of AP<sub>1</sub> to its metabolites was monitored using the thin layer chromatography (TLC) assay and the cultures were harvested when the disappearance of AP<sub>1</sub> was near completion, typically 30–40 h. The cultures were centrifuged at 14000 × g, 4°C, 10 min and the supernatant was used to extract the metabolites. To obtain additional AP<sub>1</sub> metabolites from the same culture, the cell pellet was rinsed once in MSM then resuspended to the same volume in MSM and supplemented again with AP<sub>1</sub> to 0.5–1.0 mg ml<sup>-1</sup>. The second set of cultures was incubated as before and monitored for disappearance of AP<sub>1</sub> from the medium, then harvested when AP<sub>1</sub> was no longer detectable in the culture fluid, typically 10–12 h.

The cultures were centrifuged at 14000 × g, 4°C, 20 min and the supernatants were filtered through 0.2 µm cellulose acetate membranes (Nalgene). The pH of the culture supernatants was adjusted to 7 with 5M NaOH and the metabolites were partitioned into an equal volume of ethyl acetate. The organic phase was removed and the aqueous phase was extracted twice more with ethyl acetate. The organic phases were pooled and evaporated under nitrogen until the volume was similar to the volume of the aqueous phase. When both phases were analyzed using reverse phase TLC, most of the detectable material was in the organic phase. Pooled organic phases were evaporated to dryness under nitrogen.

**Table 1.** 500 MHz <sup>1</sup>H NMR Assignments of AP<sub>1</sub> (**1**), NAc-AP<sub>1</sub> (**2**) and 2 $\alpha$ -OP<sub>1</sub> Hemiketal **4** (2 $\beta$ - in brackets) in CD<sub>3</sub>OD (ppm from TMS)

Position	<b>1</b>	<b>2</b>	<b>4</b>
<b>1</b>	1.06 (d, J <sub>1,2</sub> = 6.9)	1.12 (d, J <sub>1,2</sub> = 6.9)	1.33 (s)
<b>2</b>	2.72 (dt, J <sub>1,2</sub> = J <sub>2,3</sub> = 6.5)	3.88 (ddd, J <sub>1,2</sub> = 6.9; J <sub>2,3</sub> = 3.8, 13.4)	–
<b>3<math>\alpha</math></b>	3.52 (m)	3.78 (dt, J <sub>2,3</sub> = 3.8, 13.4; J <sub>3,4</sub> = 3.3, 9.8)	3.85 (3.88) (t, J <sub>3,4</sub> = 7.0)
<b>4<math>\alpha</math></b>	1.47–1.52	1.40–1.52	2.44 (2.25) (dt, J = 13.2, 7.0)
<b>4<math>\beta</math></b>	–	–	1.45 (1.69)
<b>5<math>\alpha</math></b>	3.79 (m)	3.77 (m)	3.92 (3.95) (dd, J = 3.1, 7.0)
<b>6</b>	1.38–1.45	1.40–1.50	1.40–1.50
<b>7,8</b>	1.33–1.52	1.3–1.5	1.3–1.5
<b>9</b>	1.45–1.50	1.40–1.50	1.40–1.50
<b>10</b>	3.66 (m)	3.65 (m)	3.64 (m)
<b>11</b>	1.57; 1.08	1.08; 1.58	1.10; 1.57
<b>12</b>	1.95 (m)	1.94 (m)	1.95 (m)
<b>13</b>	1.34; 1.49	1.38; 1.60	1.35; 1.47
<b>14</b>	3.64 (ddd, J <sub>14,15</sub> = 6.2; J <sub>14,13</sub> = 10.2, 2.2)	3.65 (m)	3.64 (m)
<b>15</b>	3.20 (t, J <sub>14,15</sub> = 6.2; J <sub>15,16</sub> = 5.8)	3.20 (t, J <sub>14,15</sub> = J <sub>15,16</sub> = 5.8)	3.20 (t)
<b>16</b>	1.69 (m)	1.68 (m)	1.68 (m)
<b>17</b>	1.13; 1.62	1.18; 1.62	1.12; 1.65
<b>18</b>	1.18–1.24	1.15–1.25	1.3–1.4
<b>19</b>	1.26–1.41	1.27–1.43	1.3–1.4
<b>20</b>	0.915 (t, J <sub>20,19</sub> = 7.2)	0.914 (t, J <sub>20,19</sub> = 7.3)	0.914 (t, J <sub>20,19</sub> = 7.2)
<b>21</b>	0.980 (d, J <sub>21,12</sub> = 6.7)	0.973 (d, J <sub>21,12</sub> = 6.7)	0.975 (d, J <sub>21,12</sub> = 6.7)
<b>22</b>	0.910 (d, J <sub>22,16</sub> = 6.9)	0.905 (d, J <sub>22,16</sub> = 6.9)	0.904 (d, J <sub>22,16</sub> = 6.8)
<b>CH<sub>3</sub></b>		1.94 (s)	

Filtered samples were spotted onto reverse phase TLC plates (Whatman KC18 Silica Gel, 200  $\mu$ m), air-dried, then developed in either MeOH:4% KCl (3:2) (Rottinghaus *et al.*, 1992) (solvent A) or acetonitrile:50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 3.3 (40:60) (solvent B). The separated compounds were visualized by spraying with 10% H<sub>2</sub>SO<sub>4</sub> (in EtOH), heated at 170 °C for 5–7 min, and examined under long wave UV light. The number of compounds, migration distances, and R<sub>f</sub> values were then determined.

Two samples of crude degradation products, representing two independent fermentations, were prepared by this method: one of 40 mg (sample 1) and one of 50.5 mg (sample 2) (dry weight estimates). These were identical when assayed by reverse phase TLC and showed approximately 7 or 8 compounds of which one was predominant. This compound appeared at R<sub>f</sub> = 0.12 in solvent A and at R<sub>f</sub> = 0.40 in solvent B. Residual AP<sub>1</sub> was present in both crude samples (R<sub>f</sub> = 0.22 for the former solvent system and R<sub>f</sub> = 0.30 for the latter, detected by spraying plates with 0.5% fluorescamine in acetonitrile.

### Purification of major products

Initial TLC chromatography of sample 1 on silica gel showed the best eluent to be 1:9 MeOH/CHCl<sub>3</sub> (solvent

C) This solvent system showed eight spots, some visible under UV at 254 nm, others not, but all reacting with the p-anisaldehyde reagent used for the visualization of fumonisins (and other amines). Addition of ammonia to the TLC eluent greatly reduced the mobility of some spots. Performing a 2-dimensional TLC of the sample using 1:9 MeOH/CHCl<sub>3</sub> in the first dimension and adding ammonia for the second dimension clearly showed that seven spots moved at the same R<sub>f</sub> with both solvents, one spot moved at half the speed in the ammonia-containing solvent and 3 or 4 spots stayed on the baseline in the ammonia-containing solvent despite having R<sub>f</sub>'s of 0.5 to 0.9 in the first dimension.

The sample was chromatographed on silica gel (30 g) with a succession of solvents in the following order: CHCl<sub>3</sub> (15 ml, fractions 1–3), 5% MeOH in CHCl<sub>3</sub> (20 ml, fractions 4–6), 10% MeOH in CHCl<sub>3</sub> (20 ml, fractions 7–9), 25% MeOH in CHCl<sub>3</sub> (35 ml, fractions 10–13), and 50% MeOH in CHCl<sub>3</sub> (20 ml, fractions 14–16). Fractions 7, 8 and 9 showed complex mixtures by TLC and were combined (39 mg). Fractions 11–13 showed only one spot and were combined (2 mg). This compound (**2**) was then characterized by NMR and MS. Fractions 15–16 showed one spot with a higher R<sub>f</sub> than expected, but could not be quantitated and no further characterization was performed.

**Table 2.** 125 MHz  $^{13}\text{C}$  NMR Assignments of AP<sub>1</sub> (1), NAc-AP<sub>1</sub> (2) and 2 $\alpha$ -OP<sub>1</sub> Hemiketal 4 (2 $\beta$ - in brackets) in CD<sub>3</sub>OD (ppm from TMS)

Position	1	2	4
1	19.12	17.13	16.77 (19.34)
2	52.77	51.15	110.60 (104.45)
3	74.05	71.50	77.39 (78.51)
4	41.91	41.91	40.86 (38.90)
5	69.17	69.15	77.94 (78.09)
6	39.65	39.64	37.14
7	26.89	26.86	26.86 (26.80)
8	26.91	26.89	27.36 (27.12)
9	39.30	39.22	39.59
10	70.05	70.11	70.02 (70.08)
11	44.66	44.70	44.65
12	26.99	27.05	27.12
13	41.61	41.59	41.58
14	70.47	70.55	70.50
15	80.78	80.81	80.80
16	35.93	35.97	35.95
17	31.74	31.79	31.76
18	30.66	30.64	30.65
19	24.19	24.18	24.18
20	14.53	14.48	14.49
21	21.44	21.42	21.42
22	16.82	16.79	16.79
C=O		172.9	
CH <sub>3</sub>		22.7	

The combined fractions 7,8 and 9 were rechromatographed on silica gel (30 g) with 5% MeOH in CHCl<sub>3</sub> (fractions 1–12), 10% MeOH in CHCl<sub>3</sub> (fractions 13–21) and 50% MeOH in CHCl<sub>3</sub> (fractions 22–23). All fractions were 5 ml. Fractions 4, 5 and 6 were combined (13.5 mg), Fractions 7–11 showing 2–3 compounds were combined (9.7 mg), and fractions 12–15 were combined (one major compounds with two small ones (8.6 mg)). Fractions 16–23 were combined and showed one major compound (2.9 mg) with an R<sub>f</sub> close to that of 2 (NAc-AP<sub>1</sub>). Since this compound (4) appeared to be relatively pure, it was also completely characterized by NMR and MS. These fractions were further checked by reverse-phase TLC in 40:60 MeCN/0.5 M KH<sub>2</sub>PO<sub>4</sub>, pH 3.5 to compare with the original crude sample. The TLC showed combined fractions 12–15 to consist of a mixture of poorly resolved compounds while fractions 16–23 contained one major compound whose R<sub>f</sub> corresponded to that earlier attributed to the main AP<sub>1</sub> metabolite. The other combined fractions showed mainly spots close to the baseline.

To check for possible effects of the protic solvent methanol on the compounds under study, an attempt was made to chromatograph sample 2 using ethyl acetate and hexane. However, this solvent system proved to be very poor at obtaining adequate resolution of the compounds and recoveries were low.

## NMR and Mass Spectrometry

The NMR spectra were recorded in methanol-d<sub>4</sub> (CD<sub>3</sub>OD) at 500.13 and 125.18 MHz respectively either on a Bruker AM500 operating at 303 °K using a 5 mm normal geometry probe or a Varian Inova 500 operating at 300 °K using a 5 mm inverse geometry Inova Bioprobe. Structural determinations were made using a combination of homonuclear (COSY) and heteronuclear (HETCOR, HMBC and HMQC) as well as NOESY spectroscopy. Routine Bruker or Varian software was used for all 2D NMR experiments. Chemical shifts are referenced to CD<sub>3</sub>OD at 3.3 ppm ( $^1\text{H}$ ) and 49.0 ppm ( $^{13}\text{C}$ ). Mass spectra were recorded either on a Finnegan MAT 312 operating in Fast Atom Bombardment (FAB) mode using glycerol as a matrix or a Perkin Elmer SCIEX API 100LC system using direct injection Chemical Ionization (DCI) mode.

## RESULTS

The NMR spectra of AP<sub>1</sub> (1) were fully characterized by 2DNMR methods (HMBC, HSQC, COSY), and compared favourably to those reported for the intact fumonisins (Savard and Blackwell, 1994). The assignments are reported in Tables 1 and 2. As expected, there are shifts in the resonances for both the protons and carbons associated with the loss of the tricarballylic side chains (positions 14 and 15) and concomitant shifts of those resonances due to the neighbouring positions C-16, C-13 and the methyl group at C-22. Surprisingly, however, there are also substantial changes in the resonances associated with the amino terminus. In the carbon spectrum, C-1 has shifted downfield by 3 ppm from 16 ppm to 19 ppm, C-2 has shifted slightly from 53.7 ppm to 52.8 ppm and C-3 has shifted from 70.3 ppm to 74.0 ppm. Changes of similar relative magnitude are noted in the proton spectrum for the resonances due to these three positions. From C-4 down the rest of the chain, however, the NMR resonances are very similar to those of FB<sub>1</sub>. Unlike FB<sub>1</sub> is also soluble in chloroform and there are solvent-related shifts from the spectra in CD<sub>3</sub>OD observed, particularly in the  $^{13}\text{C}$  spectrum and with the resonances C-2 to C-5 and C-13 to C-16 (Blackwell, unpublished data). These differences observed in the NMR spectrum may be due to configurational differences of the entire molecule in solution associated with the loss of the side chains. The FAB mass spectrum of AP<sub>1</sub> shows a M + 1 ion at 406 amu (for a molecular weight of 405), plus ions at 388, 352 and 478 amu associated with loss of one hydroxyl group (as H<sub>2</sub>O, -18 amu), loss of three hydroxyl groups and addition of glycerol respectively. The DCI mass spectrum also showed a M + 1 ion at 406 plus the sequential loss of five hydroxyl groups (-18 amu) at 388, 370, 352, 334

and 316, corresponding to those functions at C-15, 14, 10, 5 and 3.

The NMR spectra of the unpurified samples obtained from incubation of AP<sub>1</sub> with *E. spinifera* cultures were checked before further processing to ensure that no rearrangements occurred as a result of the purification procedure. The proton NMR spectrum of the first sample (40 mg) showed a complex mixture in which the main component was not AP<sub>1</sub>. However, there was some residual AP<sub>1</sub> as viewed from the H-1 signal at 1.08 ppm. The dominant compound appeared to have most of the correct resonances for AP<sub>1</sub>, i.e. H-3, 5, 10, 14 and 15, but H-1 and H-2 were shifted or missing. A new methyl resonance appeared at ~1.3 ppm. The methyl signals of H-20 and H-22 appeared to be unaffected, but the H-21 methyl was shifted slightly. The <sup>13</sup>C spectrum of this sample was more complex, but the dominant resonances could be assigned to the C-15 through to C-22 moiety. Other signals with reduced intensity could be assigned to C-7 through to C-11, as would be expected for a mixture of AP<sub>1</sub> and a closely related compound differing at the amino terminus. Resonances corresponding to C-1 and C-2 of AP<sub>1</sub> were clearly visible as ~20% of the intensity of the dominant resonances. The spectra of the second sample (50 mg) were slightly different from those of the first in that the sample appeared to be more complex and that the protons of the remaining CHOHs of C-5, 3 and 10 were shifted. The signals due to H-1 and H-2 of AP<sub>1</sub> were reduced and the new H-1 singlet at 1.3 ppm was again present. The carbon spectrum also showed this to be more of a mixture and there seemed to be a greater proportion of residual AP<sub>1</sub> in this sample.

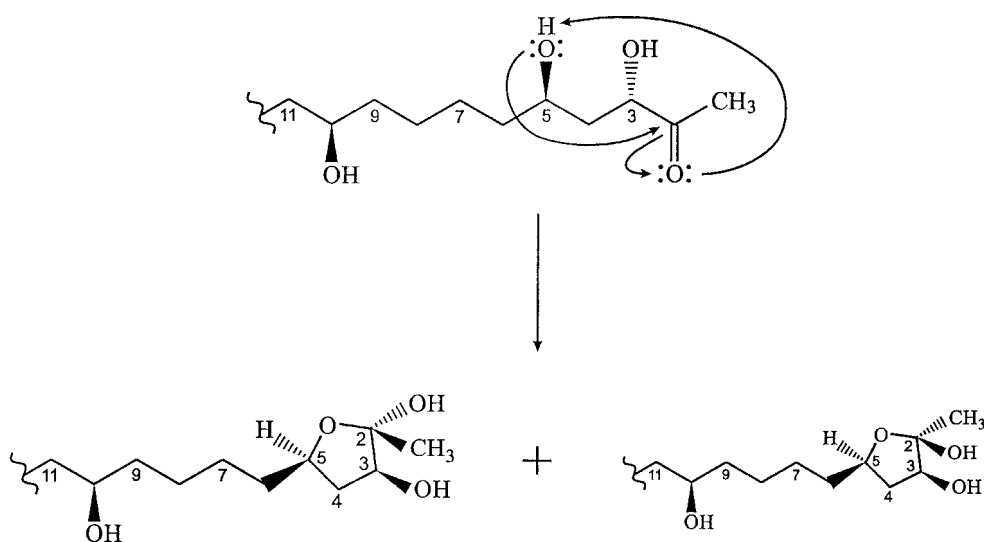
The NMR spectra of compound **2** were very similar to those of AP<sub>1</sub> (**1**). All the resonances of AP<sub>1</sub> were present in both <sup>1</sup>H and <sup>13</sup>C spectra with the exception that H-2 had shifted from 2.72 ppm to ~3.9 ppm, H-3 had shifted from 3.5 ppm to 3.8 ppm and H-1 had shifted from 1.06 ppm to 1.12 ppm. An acetate singlet appeared at 1.94 ppm. In the <sup>13</sup>C spectrum, acetate peaks appeared at 22.7 ppm and 173 ppm typical of an N-acetyl function and the resonances due to C-1, C-2 and C-3 were slightly shifted (Table 2). The DCI mass spectrum showed a M + 1 peak at 448 for a molecular ion of 447 and loss of four 18 amu units corresponding to loss of the four hydroxyl functions (430, 412, 394, 376 amu). An ion at 407 (M-41) was associated with the loss of an acetyl group as was the ion at 353 (loss of the acetyl (M-41) from the 394 ion). A small ion at 334 was associated with the loss of the final fifth hydroxyl function. The combined NMR and MS data are consistent with the structure of **2** being N-acetyl AP<sub>1</sub>. Thus, one of the reactions that *E. spinifera* accomplishes is acetylation of the amino function (Figure 1).

The proton spectrum of **4** was similar to that of AP<sub>1</sub>, except that H-2 was missing or shifted and the resonance

due to the methyl function at position 1 had shifted from a doublet at 1.06 ppm to a singlet at 1.33 ppm, indicating that C-2 was now quaternary. In spite of the fact that the presence of only one molecular ion was indicated by the mass spectrum (see below), the proton spectrum showed the presence of two compounds of similar structure in approximately 2:1 ratio which therefore must be isomers. Analysis of the COSY spectrum showed that there were two H-5 resonances at 3.92 and 3.95 (the minor component) ppm, shifted from 3.75 ppm in AP<sub>1</sub>, as well as two H-3 resonances at 3.85 (3.88) ppm shifted from 2.72 ppm in AP<sub>1</sub>. A corresponding pair of new multiplets appeared at 2.44 and 2.25 ppm. The combined information from the COSY and HMQC spectra showed that these multiplets are due to AB systems at 2.44 and 1.45 ppm (2.25 and 1.69 ppm for the minor component), which were assigned to C-4. All of the other resonances were virtually identical to those of AP<sub>1</sub> (see Table 1) with the exception of a small shift of the C-22 methyl resonance from 0.910 ppm to 0.904 ppm. The presence of two isomers was more easily observed in the <sup>13</sup>C spectrum. Resonances of full intensity and unshifted frequency from those of AP<sub>1</sub> were observed for positions C-11 through to C-22, while many of those due to C-1 to C-10 which were resolvable, were reduced in intensity and doubled. Two new quaternary resonances due to the major and minor components were observed at 110 and 104 ppm, consistent with a hemiketal structure and concomitant with the disappearance of C-2 (52 ppm). C-1 had shifted from 19.12 ppm to 16.77 ppm (19.34 ppm for the minor component). While the site of major structural difference seems to be centered at C-2, positions up to C-10 (see Table 2) were affected. The chemical shifts as well as the molecular weight of 404 amu were consistent with the presence of a ring structure. The long range correlation spectrum (HMBC) showed coupling of the H-1 methyl resonance at 1.33 to the carbon resonances at C-2 (110 and 104 ppm) and C-3 (77.39 and 78.51 ppm) confirming the hemiketal to be at position 2. This was further identified by the presence of a weak correlation between the H-5 resonance at 3.92 ppm and the C-2 at 110 ppm.

The DCI mass spectrum was somewhat misleading in that the apparent molecular ion was observed at 387 amu (M + 1) for an apparent molecular weight of 386 amu. However, in the light of the NMR data, closer inspection revealed a small ion at 405 amu (M + 1) for the correct molecular weight of 404 amu, which is one amu less than AP<sub>1</sub>. The loss of one unit is consistent with replacement of a proton and the NH<sub>2</sub> unit on C-2 by a carbonyl or two oxygens of an internal hemiketal. The loss of water (-18 amu) would be very easy from a hemiketal structure such as is proposed for **4**, giving rise to the base peak at 387 amu. Additional ions observed at 369, 351, 333, 315 and 297 correspond to the sequential loss of five more water





**Figure 2.** Possible mechanism for the cyclization of metabolite **3** to give rise to 2 $\alpha$ - and 2 $\beta$ -OP<sub>1</sub> hemiketal (**4**)

## DISCUSSION

Two AP<sub>1</sub> derivatives were identified in incubation mixtures of AP<sub>1</sub> with induced cultures of *E. spinifera*: N-acetyl AP<sub>1</sub> (**2**) (present in smaller amounts as determined by yield as well as indications from the initial NMR spectra of the crude incubation mixture) and a novel derivative, the hemiketal of 2-oxo-12, 16-dimethyl-3,5,10,14,15-icosanepentol, or 2-OP<sub>1</sub> hemiketal (**4**). Both derivatives lack a free amino function thought to be important for the biological activity of fumonisins. Whereas N-acetylated AP<sub>1</sub> could potentially revert to AP<sub>1</sub> through amide hydrolysis, the production of compound **4** is probably not reversible without enzymatic intervention.

Unlike other mycotoxins, degradation of FB<sub>1</sub> has not always resulted in the expected reduction of toxicity. AP<sub>1</sub> is less toxic *in vitro* (Abbas *et al.*, 1993) and in short-term carcinogenicity assays (Gelderblom *et al.*, 1993). However, AP<sub>1</sub> has renal toxicity comparable to that of FB<sub>1</sub> (Voss *et al.*, 1996). To that end, further enzymes are needed to degrade FB<sub>1</sub> to non-toxic fragments. The oxidative removal of the amino function at C-2 is consistent with oxidative deamination by an amine oxidase (EC 1.4.3) or amine dehydrogenase (EC 1.4.99). Such an enzyme would be a likely candidate for a companion enzyme to the fumonisin tricarballylate esterase previously described, predicted to result in the conversion of fumonisin to the polyol ketone or hemiketal **4** plus tricarballylic acid. Further studies of the toxicology and stability of compound **4** will be forthcoming with the availability of cell-free enzyme preparations that catalyze conversion of AP<sub>1</sub> to its amine-free product.

## ACKNOWLEDGEMENTS

The authors would like to thank John Nikiforuk for assistance in obtaining the NMR spectra.

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