Biosynthesis of Trichothecenes: Oxygenation Steps Post-trichodiene

L. O. Zamir,[*a,b] **K. A. Devor,**[a] **N. Morin**[a] **and F. Sauriol**[b]

[a] *Université du Québec, Institut Armand-Frappier, 531, boul. des Prairies, C.P. 100, Laval-des-Rapides, Laval, Québec H7V 1B7, Canada*

[b] *McGill University, 801 Sherbrooke St. West, Montreal, Québec H3H 3K6, Canada*

Two dioxygenated derivatives of trichodiene **1** have been isolated from *Fusarium culmorum* cultures treated with the inhibitor ancymidol and characterized as 9,10-trichoene-12,13-diol **2** and 12,13-epoxy-9,10-trichoene-2-ol **3**; radiolabelling experiments demonstrate that **2** is a dead-end metabolite whereas **3** is incorporated into both 3-acetyldeoxynivalenol **4** and sambucinol **7**.

Trichothecenes are of particular interest because unlike other mycotoxins they are also antifungal,[1] antileukaemic[2] and phytotoxic.[1] *Fusarium culmorum* (HLX-1503) produces two major metabolites:[3] 3-acetyldeoxynivalenol **4** and sambucinol **7**. Isotrichodermin **5** was proved to be a good precursor to **4** but not to **7**.[4] On the other hand, 12,13-epoxytrichothec-9-ene **6** was shown to be incorporated into **7** *via* an intermediate 3-deoxysambucinol[4] but not to **5** or **4**. Very recently,[5] a post-trichodiene intermediate: isotrichodiol **8** was detected and characterized in *F. culmorum* cultures treated with the inhibitor xanthotoxin. Radiolabelled isotrichodiol[5] was incorporated† into **4**, **5**, **6** and other epoxytrichothecenes.

In this communication, we report the detection and characterisation of two new dioxygenated derivatives of trichodiene. 12,13-Epoxy-9,10-trichoene-2-ol **3** was proved to be a precursor to trichothecenes. 9,10-Trichoene-12,13-diol **2** was a dead-end metabolite which was not metabolized to the end products **4** and **7**.

The kinetic pulse-labelling method[6] was used to detect plausible intermediates to trichothecenes. We fed (3*RS*)-[2-^{14}C]mevalonate to *F. culmorum* cultures and followed the formation of the radiolabelled metabolites with time and an HPLC peak with a retention time in the vicinity of trichodiene (R_t: 88.8 min, trichodiene: R_t 112.9 min)[7]‡ behaved like a transient intermediate. It started to be formed ten minutes after the feeding of (3*RS*)-[2-^{14}C]mevalonate and decreased in amount as the end-products (**4**, **7**) accumulated. When the level of this plausible transient intermediate is at a minimum (24 h), metabolites **4** and **7** have almost reached their maximum. In order to prove rigorously that this plausible transient intermediate is indeed a precursor to trichothecenes we purified this radiolabelled intermediate and found that it consisted of two compounds (**a***, **b***) in unequal amounts. In our search for inhibitors to trichothecene biosynthesis,[8] we noticed that ancymidol (0.30 mmol dm^{-3}) triggered the accumulation in *F. culmorum* of these two compounds (**a**, **b**). The unlabelled compounds (**a**, **b**) were added to part of their radiolabelled counterparts (**a***, **b***) as markers and purified to homogeneity by: (*i*) repetitive HPLC aided by a radioactivity detector; (*ii*) TLC and Bioscan radioactivity scanner analysis; (*iii*) acetylation with [^{2}H$_6$]acetic anhydride, which changed the retention times of both compounds (acyl-**a***: R_t 65.4 min; acyl-**b***: R_t 61.8 min) and (*iv*) deacetylation and regeneration of the original natural products, with the original retention times (49.3 min).§ The purified samples were analysed by spectroscopic techniques¶ and characterized as 9,10-trichoene-12,13-diol **2** and 12,13-epoxy-9,10 trichoene-2-ol **3**. The radiolabelled compounds (**a***, **b***) were purified as described previously and fed to cultures of *F. culmorum*. The radiolabelled compound **a*** which has been characterized as **3**

---

† The percentage incorporation of isotrichodiol into 3-acetyldeoxynivalenol, calculated using equation (1), from the values given in reference 11 was ~6%. This result is in agreement with the levels of incorproation of trichodiene[7] and of 12,13-epoxy-9,10-trichoen-2-ol (this work) into 3-acetyldeoxynivalenol. In reference 5, isotrichodiol was reported to be 31–79% incorporated into trichothecenes but this is due to an error in the calculation of incorporations. Indeed, the authors§ utilized the ratios of the specific activities of product and precursor as a measure of percentage incorporation according to equation (2). This is inaccurate since the incorporation would then depend on the amount of unlabelled product synthesized: a radiolabelled precursor fed to two flasks containing production cultures would be appreciably differently incorporated into the product than if fed to 100 flasks. On the other hand, when one wants to prove rigorously that a precursor is incorporated into a product, it has to be purified to constant *specific* radioactivity (dpm mg^{-1} or dpm mmol^{-1}).

$$\frac{\text{Total radioactivity in 3-ADN dpm}}{\text{Radioactivity of precursor fed dpm}} \times 100 \qquad (1)$$

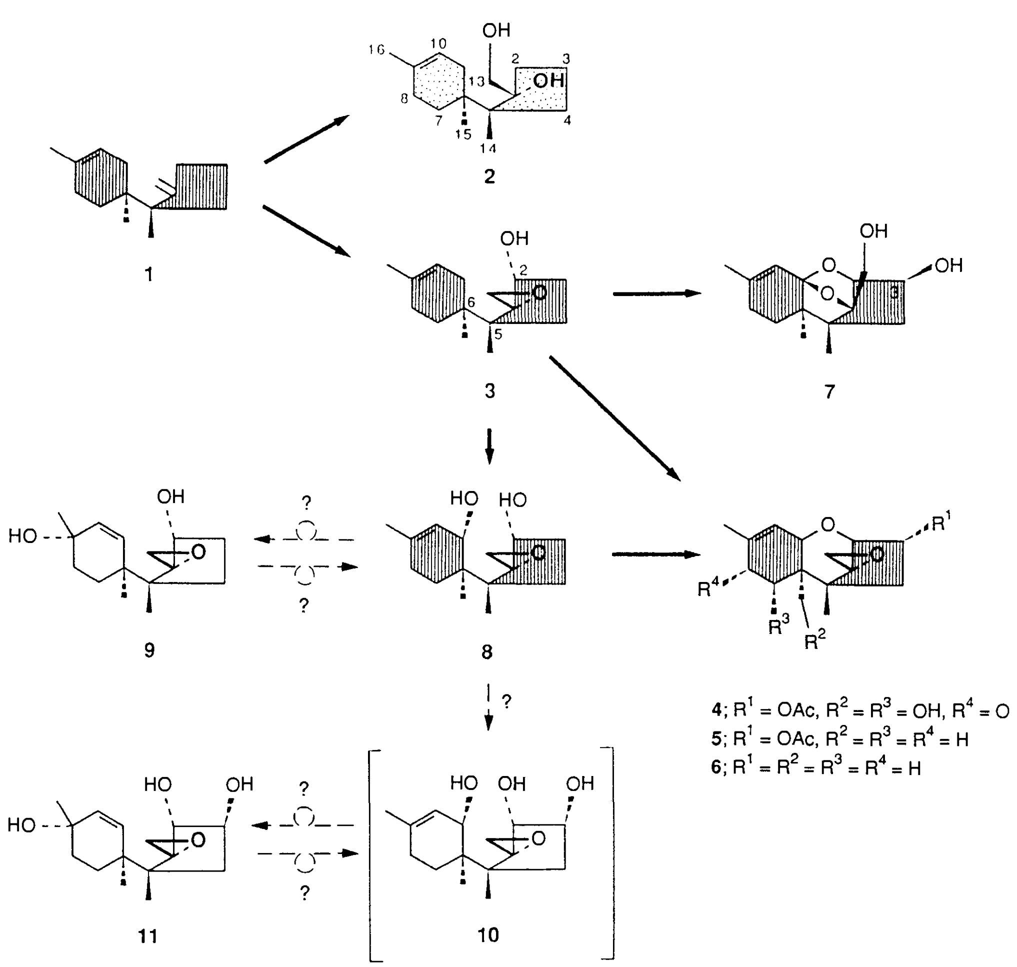
$$\frac{\text{Specific activity of product dpm mmol}^{-1}}{\text{Specific activity of precursor dpm mmol}^{-1}} \times 100 \qquad (2)$$

‡ HPLC conditions for the isolation of radiolabelled (**a*** : **b***, 60 : 40): 2 × ODS-2 analytical columns, eluted using 1 ml min^{-1}: 0–15 min, 15% methanol; 15–65 min, 15–75% methanol; 65–95 min, 75% methanol; 95–105 min, 75–99.9% methanol; 105–115 min, 99.9% methanol.

§ HPLC conditions for the purification of the mixture (**a***, **b***) (R_t 49.3 min) by acetylation (acyl-**2**, R_t 61.8 min; acyl-**3**, R_t 65.4 min) separation and deacetylation (R_t: 49.3 min): 2 × ODS-2 analytical columns, eluted using 1 ml min^{-1}: 0–30 min, 74% methanol, 26% water; 30–70 min, 74–99.9% methanol.

¶ The [^{2}H$_3$]acetate derivatives of 9,10-trichoene-12,13-diol (at C-13) and of 12,13-epoxy-9,10-trichoene-2-ol (at C-2) and the nonacetylated compounds were characterized by ^{1}H and ^{13}C NMR, COSY, NOED, DEPT and mass spectrometry. The NMR values obtained were in agreement with the related derivatives such as isotrichodiol, trichodiol or trichotriol. The NOE difference experiments run on the [^{2}H$_3$]acetate derivative of 12,13-epoxy-9,10-trichoene-2-ol have allowed us to assign the relative stereochemistry of the substituents, in particular the relationship between the methyl at C-14, the methylene epoxide and H-2. Upon preirradiating the methyl group (C-14) (δ 0.813), an increase in intensity was observed for one of the methylene protons on the epoxide (H-13A at δ 3.24). Upon irradiating the other methyl group (C-15) (δ 0.952) there was no increase in intensity on the methylene protons on the epoxide. Upon preirradiating the other methylene proton (H-13B at δ 2.915 ppm) an increase in intensities was observed in the other methylene proton (H-13A) as well as in H-2. This result established the *cis* relationship between the methyl at C-14, the methylene epoxide and H-2.

The NMR spectra were obtained on a Varian XL-300 spectrometer equipped with proton probe and 5 mm broadband probe, at 300 MHz for ^{1}H NMR for samples (1–2 mg) in CDCl$_3$ at ambient temperature (22 °C). For routine ^{1}H NMR, a 45° pulse was used with an acquisition time of 4 s. The data were zero filled and resolution enhanced. The COSY spectra were acquired using 256 increments in the evolution time (t_1) each with four repetitions. The 2D matrix was 1024 × 1024 data points after processing. The data were pseudo-echo-shaped and symmetrized prior to plotting. For ^{13}C NMR a 40° pulse was used with an acquisition time of 0.97 s. The solvent (CDCl$_3$), used as an internal reference, was set at 77.0 ppm. Line broadening processing was used only with very dilute solution. The DEPT experiment was performed using a 2 s repetition delay. The 90° pulse provided by the decoupler was 65 μs.



Scheme 1 Biosynthesis of the trichothecenes: 3-acetyldeoxynivalenol **4**, isotrichodermin **5**, 12,13-epoxytrichothec-9-ene **6** and sambucinol **7**. The shading differentiates between proven precursors to trichothecenes (vertical lines), dead-end metabolite (dotted), postulated precursors (unshaded) which have been found in other microorganisms and in brackets plausible precursor which is yet unknown.

was efficiently[||] incorporated[†] into the trichothecenes **4** and **7**. On the other hand, metabolite **2** was neither incorporated into the trichothecenes **4** nor into **7**.[||]

We can conclude that the only oxygenated derivatives of trichodiene which have been characterized as precursors to trichothecenes biosynthesis are the metabolites **3** (this work) and **8**.[5] On the other hand, compound **2** is a dead-end metabolite. Two related structures trichodiol **9** and trichotriol **11** have been detected in other fungal species (*Trichothecium roseum*[9] and *Fusarium sporotrichioides*,[10] respectively). It is possible that **9** is also involved in the biosynthesis of trichothecenes. Indeed, the rearrangement interconverting trichodiol **9** and isotrichodiol **8** could be enzymatic and reminiscent of the chorismate isochorismate reaction.[11,12] Similarly, we could envisage the next oxidation step leading to a putative intermediate **10** which would have the same biological rearrangement to trichotriol **11**. The compounds **10** and **11** would only be precursors to isotrichodermin and 3-acetyldeoxynivalenol but not to the sambucinol series.[4]

We thank the Natural Sciences and Engineering Research Council of Canada for support of this work *via* operating and strategic grants to L. O. Z. and a university undergraduate research award to N. M.

Received, 6th March 1991; Com. 1/01060C

[||] A representative feeding experiment is described: a culture of *F. culmorum* (HLX-1503) (kindly supplied by D. Miller and R. Greenhalgh, Agriculture Canada) was grown in a shaker on a rich medium for 3 days and then transferred to a production medium for 48 h as previously described.[6] [2-^{14}C]Mevalonate-derived-**3** and [2-^{14}C]mevalonate-derived-**2** were added separately (10^4 dpm each). After 5 days of incubation on a shaker (220 rpm) in the dark, at 25 °C, the trichothecenes **4** and **7** were isolated and purified by HPLC. The incorporation of **3** into **4** and **7** was in the order of 5–7%. On the other hand **2** was not incorporated into **4** or **7**.

References

1 A. Z. Joffe, in *Fusarium species: their biology and toxicology*, Wiley, New York, 1986.
2 R. Hromas, B. Barlogie, D. Swartzendruler and B. Drewinko, *Cancer Res.*, 1983, **43**, 1135.
3 L. O. Zamir, K. A. Devor, Y. Nadeau and F. Sauriol, *J. Biol. Chem.*, 1987, **262**, 15 354.
4 L. O. Zamir, K. A. Devor, A. Nikolakakis and F. Sauriol, *J. Biol. Chem.*, 1990, **265**, 6713.
5 A. R. Hesketh, L. Gledhill, D. C. Marsh, B. W. Bycroft, P. M. Dewick and J. Gilbert, *J. Chem. Soc., Chem. Commun.*, 1990, 1184.
6 L. O. Zamir and K. A. Devor, *J. Biol. Chem.*, 1987, **262**, 15 348.
7 L. O. Zamir, M. J. Gauthier, K. A. Devor, Y. Nadeau and F. Sauriol, *J. Chem. Soc., Chem. Commun.*, 1989, 598.
8 L. O. Zamir, A. Nikolakakis, K. Devor, F. Vairinhos and F. Sauriol, *J. Agric. Food Chem.*, 1991, submitted.
9 S. Nozoe and Y. Machida, *Tetrahedron*, 1972, **28**, 5105.
10 S. P. McCormick, S. L. Taylor, R. D. Plattner and M. N. Beremand, *Appl. Environ. Microbiol.*, 1989, **55**, 2195.
11 J. Liu, N. Quinn, G. A. Berchtold and C. T. Walsh, *Biochemistry*, 1990, **29**, 1417.
12 L. O. Zamir, K. A. Devor, R. A. Jensen, R. Tiberio, F. Sauriol and O. Mamer, *Can. J. Microbiol.*, 1991, **37**, 276.