Stereoselective Biosynthesis of Chiral chloroarylpropane diols by the basidiomycete Bjerkandera adusta

Peter Silk[1], John B Macaulay[2]

[1]Natural Resources Canada
[2]RPC, Fredericton

Introduction

The natural production of chlorometabolites by basidiomycetes is well documented [1,2] . These metabolites have important physiological functions as methyl donors, antibiotics and as substrates for H_2O_2-generating oxidases and are not "biological accidents". [2]

The biochemical processes leading to their production, however, are not well elaborated and the chlorinating enzymes(s)/substrates and, therefore, the chlorinating mechanisms remain to be elucidated. Chlorinated aromatics of natural origin can find their way into environmental compartments where they can, on microbial modification, become humus-bound, or, by oxidative coupling, form chlorinated dioxin-like structures.

The white rot basidiomycete *Bjerkandera adusta* biosynthesizes many metabolites [2] including chloro-1-arylpropane-1,2-diols [3,4,5,6] that are produced from L-phenylalanine during idiophasic metabolism and that are stereoselectively biosynthesized from a C_7-unit (benzylic), derived from phenylalanine, and a C_2-unit as predominantly the chiral *erythro* (1***R***,2***S***) enantiomers [6]. Stable isotope labeling experiments have shown that L-phenylalanine and benzaldehyde, 4-hydroxy- and 4-methoxybenzaldehydes derived from this amino acid, are the C_7-unit precursors in the carboligation reaction that leads to chloroarylpropanediol biosynthesis. These aldehydes are all stereoselectively incorporated into the corresponding 1-arylpropane-1,2-diols including the chloro analogues and the corresponding α-ketols (phenyl acetyl carbinols (PAC's)), the precursors of the diols [7,8,9].

The metabolic role for the diols and ketols is unknown but they may play a role as substrates for the chlorination enzyme(s) yet to be identified in chlorometabolite-producing white rot fungi and, therefore, in the biosynthesis of chlorinated anisyl metabolites (CAM's). They may also be important intermediates in CAM aldehyde-alcohol recycling (substrates for aryl alcohol oxidase (AAO) which generate hydrogen peroxide for the peroxidases in ligninolytic activity) [2,6].

This study identifies the source of the C_2-unit using stable isotope labeling techniques involving incubation experiments with suitable precursors and gas chromatography/mass spectrometry (GC/MS) enabling a mechanism for the formation of these natural chiral chlorinated ketols and diols to be postulated.

Materials and Methods

Sources of Cultures

B. adusta (DAOM 215869), isolated from a fruit body on an *Ulmus americana* stump (Cautley, PQ, Canada, 12 October, 1992, J. H. Ginns), deposited in CCFC (Agriculture and Agri-Food Canada, Ottawa), was used throughout. Cultures were maintained on sterile malt/agar/yeast slants at 4^{o}C.

Chemicals

The amino acids glycine (2- ^{13}C;99%;CLM-136), L- serine (2,3,3-d_3;98%; DLM- 582), L-methionine (methyl- d_3;98%;

DLM- 431) and L-alanine (2,3,3,3-d_4;98%; DLM-250) were obtained and used as is from Cambridge Isotopes Laboratories Inc.(CIL). Sodium pyruvate (2,3-$^{13}C_2$;98%; CLM-3507), sodium acetate (1,2-$^{13}C_2$;99%;CLM-440), glycerol (1,1,2,3,3- d_5;99%;DLM-1229), acetaldehyde (d_4;99%;DLM-112), ethanol (ethyl- d_5; 98%;DLM-413), D-glucose (U-$^{13}C_6$;99%;CLM 1396) and benzaldehyde (ring-d_5;98%; DLM-465) were also obtained from CIL and used without further purification; 4-fluorobenzaldehyde (Aldrich;98% pure) was used without further purification. [2-$^2H_1$,2-^{18}O]-glycerol was prepared following the procedure of Schaffrath et al. (2001) [10] which was by isotope exchange of the carbonyl oxygen of dihydroxyacetone with [^{18}O]-water under acidic conditions followed by reduction in methanol with NaB^2H_4. In ca. 50% yield the final product contained 68 atom% ^{18}O and >90 atom% ^2H as determined by GC/MS analysis of the triacetate derivative. Phenyl acetyl carbinol (PAC) with ring protons replaced by deuterium (d_5) and the 2,3-propyl carbons ^{13}C- labeled, was synthesised [11] by coupling benzaldehyde (ring-d_5) with pyruvate (sodium)(2,3-$^{13}C_2$) with the enzyme pyruvate decarboxylase (PDC)[EC 4.1.1.1 2-oxo- acid carboxy-lyase from Baker's Yeast; Sigma]. The product was assigned as 1***R***-phenyl acetyl carbinol since the stereochemistry of this product was previously determined [11] using this enzymatic procedure

Culture Media

B. adusta was grown in static liquid cultures to which was added, during idiophase, various isotopically-labeled compounds. The liquid culture media used throughout contained 1.0 g D-(+)-glucose, 0.2 g peptone, 0.2 g yeast extract, 0.2 g KH_2PO_4 and 0.1 g $MgSO_4$ in 100 ml distilled water. The mixture was autoclaved and 1 mg thiamine hydrochloride in 50 :l water was filter-sterilized and added to the cooled medium.

Extraction of fungal cultures

Cultures (10-12 days post-inoculation) were filtered to remove mycelial mats (Whatman #1), acidified with 0.5M H_2SO_4 to pH 2.0 and immediately extracted with ethyl acetate (3 x 100 ml). The combined extracts were dried over anhydrous Na_2SO_4, concentrated, acetylated with acetic anhydride/ pyridine and analysed by GC/MS. Cultures to which no labeled compounds were added and uninoculated media served as controls.

Yeast Biomimetic syntheses of alpha-ketols and diols

The biomimetic ability of fermenting Baker's yeast, *Saccharomyces cerevisiae*, to produce α-ketols and diols from aromatic aldehydes with high diastereo/enantioselectivity was employed. This C_2-homologation reaction produces *erythro* isomers with 1***R***,2***S*** configurations; the diols are produced from reduction of the alpha-ketols formed from aromatic aldehyde and a C_2-unit. The enzyme involved in the C_2-homologation has been shown to be pyruvate decarboxylase (PDC) having thiamine diphosphate (TDP) as cofactor [11]. The aromatic aldehydes used were benzaldehyde, p-anisaldehyde, 3-chloro-4-methoxybenzaldehyde and 3,5-dichloro-4-methoxybenzaldehyde. Each aldehyde was subjected to whole yeast fermentation with D-(+)-glucose to produce mixtures of the corresponding benzylic alcohol, α-ketols, and diols. Each mixture was then analysed by GC/MS both before and after acetylation.

Instrumental analyses

All GC/MS analyses were performed on a Hewlett-Packard 5890 II GC/5971 MSD in the electron impact mode at 70 eV. Injections were made in the splitless mode with Helium as carrier gas. The capillary column was Supelcowax-10 (30 m, 0.25 mm ID, 0.25 um film thickness), temperature programmed from 50^{o}C at 20^{o}C/min. to 250^{o}C with injection temperature at 250^{o}C. Some extracts were analysed on an α-cyclodextrin column to effect chiral separations (Supelco, 30 m, 0.2 mm ID, 0.25 um film thickness; α-Dex 20), temperature programmed from 50^{o}C, held for 5 mins. then at 5^{o}C/min. to 220^{o}C.

Retention time and EI(+) mass fragmentation patterns were used to identify compounds by comparison with

authentic material, characterized synthetics, or those made by yeast biomimetic synthesis previously characterized. [6]

Results and Discussion [6,9,12]

GC/MS analysis of culture extracts on the Supelcowax-10 and the α-Dex columns shows that *B. adusta* produces 1-phenyl, 1-anisyl, 1-(3'-chloro-4'-methoxy) and 1-(3',5'-dichloro-4'-methoxy)-propane-1,2-diols , predominantly as *erythro* diastereomers with 1***R***,2***S*** absolute configurations through their corresponding 1***R***-ketols. This was verified by comparing products produced by the yeast biomimetic route. Previous work [6] using cultures supplied with $^{13}C_9$-L-phenylalanine had shown that all products were derived from L-phenylalanine but that the propane diols and ketols were labeled only in the ring and benzylic carbon (carbon 1) and carbons 2 and 3 were unlabeled, suggesting a stereoselective re-synthesis from a C_7-unit and a C_2-unit, likely aromatic aldehyde and decarboxylated pyruvate , respectively.

Of the labeled amino acids tested as possible C_2-units, at the 4-10 mM level, none were found to efficiently label the 2,3-propane carbons of the diols. However, glycine (2-^{13}C), L-serine (2,3,3-d_3) and L-methionine (methyl-d_3) entered the biomethylation pathway. Neither pyruvate (2,3-$^{13}C_2$), acetate (1,2-$^{13}C_2$), acetaldehyde (d_4) nor ethanol (ethyl-d_5) labeled the 2,3-propane carbons of the diols at the 4-10 mM level. Pyruvate (2,3-$^{13}C_2$) and L-serine (2,3,3-d_3) (which also entered the biomethylation pathway) did, however, effectively label the 2,3-propane carbons of the α-ketols and diols at the 40 mM level as evidenced by mass spectrometry.

Glycerol (1,1,2,3,3- d_5) also appeared to label one of the 2,3-propane carbons (ca. 5% as $^2H_2$ on C3) as suggested by mass spectrometric data and also entered the biomethylation pathway, likely via amino acid synthesis. Glycerol (through pyruvate), therefore, likely supplies C2 and C3 of the propane side-chain with arylpropanediol biosynthesis. Incubation of *B. adusta* with synthetic [2-$^2H_1$, 2-^{18}O]-glycerol showed that neither ^{2}H nor ^{18}O were incorporated in the ketols or diols. The oxygen atom on the C2 of the ketols/diols, therefore, does not appear to come from C2 of glycerol. Glycerol, however, can readily form L-serine (which can then form pyruvate via PLP/serine dehydratase and involve transamination washing out the ^{18}O label and providing the oxygen from water), and can then go on to label the C_2-unit. [10]

Labeled α-ketol, phenyl acetyl carbinol (1***R***-PAC; ring-d_5, 2,3- $^{13}C_2$ propane) cultured with *B. adusta* leads to stereospecific reduction to the (1***R***,2***S***)-diol (ring-d_5 and 2,3-$^{13}C_2$); in all other metabolites produced, the 2,3-$^{13}C_2$ label was washed out.

Incubation of the fungus with 4-fluorobenzaldehyde produces a build-up of predominantly *erythro* (1***R***,2***S***) 1-(4'-fluorophenyl)-1,2- propane diol (as diacetate) (through the corresponding α-ketols). Blocking the *para*-position with fluorine thus prevents ring oxygenation and also chlorination, forcing the conclusion that *para*-ring oxygenation precedes *meta*-chlorination. [12]

We conclude that the α-ketols are important intermediates in aryl propane diol biosynthesis and are likely substrates for the chlorinating enzyme. Pyruvate is the likely source of the C_2-unit via a TDP mediated PDC-catalysed reaction with aromatic aldehyde leading to the 1***R***-α-ketols and, by stereospecific reduction, to the (1***R***,2***S***)-diols. [7,8]

Acknowledgements

Funding for this work was provided by the Canadian Chlorine Coordinating Committee (C4) and the Canadian Chemical Producers Association (CCPA). Support from Natural Resources Canada, Fredericton, NB to PS is acknowledged.

References

1. de Jong, E., Field, J. A., Spinnler, H. E., Wijnberg, J. B. P. A. and de Bont, J. A. M.. (1994) Significant biogenesis of chlorinated aromatics by fungi in natural environments. Appl. Environ. Microbiol. 60, 264-270.

2. de Jong, E., Field, J. A. (1997) Sulfur tuft and turkey tail: biosynthesis and biodegradation of organohalogens by basidiomycetes. Annu. Rev. Microbiol. 51, 375-414.

3. Swarts, H. J., Verhagen, F. J. M., Field, J. A., Wijnberg, J. B. P. A. (1998) Identification and synthesis of Novel Chlorinated p-Anisylpropanoid Metabolites from Bjerkandera species. J. Nat. Prod. 61, 1110-1114.

4. Swarts, H. J., Verhagen, F. J. M., Field, J. A., Wijnberg, J. B. P. A. (1996) Novel chlorometabolites produced by Bjerkandera species. Phytochemistry 42, 1699-1701.

5. Levy, L. M., Cabrera, G. M., Wright, J. E., Seldes, A. M. (2000) Bioactive Metabolites Produced by Fungal Cultures. Molecules. 5, 354-355.7.

6..Silk, P. J., Aubry, C., Lonergan, G. C., Macaulay, J. B. (2001) Chlorometabolite production by the ecologically important white rot fungus Bjerkandera adusta. Chemosphere 44, 1603-1616.

7. Hage, A., Petra, D. G. I., Field, J. A., Schipper, D., Wijnberg, J., Kamer, P. C. J., Reek, J. N. H., van Leeuwen, P., Wever, R. and Schoemaker, H. E. (2001a) Asymmetric reduction of ketones via whole cell bioconversions and transfer hydrogenation: complementary approaches. Tetrahedron: Asymmetry 12, 1025-1034.

8. Hage, A., Schoemaker, H. E. and Field, J. A. (2001b) Optimization of stereoselectiveketone reduction by the white-rot fungus Merulius tremellosus ONO991. Appl. Microbiol. Biotechnol. 57, 79-84.

9. Silk P. J. and Macaulay, J.B. (2003) Stereospecific Biosynthesis of Chloroaryl propane diols by the Basidiomycete Bjerkandera adusta , Chemosphere, 52, 503-512.

10. Schaffrath, C., Murphy, C., Hamilton, J.T.G., O'Hagan, D. (2001) Biosynthesis of fluoroacetate and 4-fluorothreonine in Streptomyces cattleya. Incorporation of oxygen-18 from [2-$^{2}H_{1}$, 2-^{18}O]-glycerol and the role of serine metabolites in fluoroacetaldehyde biosynthesis. J. Chem. Soc. Perkin Trans. 1, 3100-3105.

11. Crout, D. H. G., Hutchinson, D. W., Miyagoshi, M. (1991) Studies on pyruvate decarboxylase: acyloin formation from aliphatic, aromatic and heterocyclic aldehydes. J. Chem. Soc. Perkin Trans. 1, 1329-1334.

12. Silk, P. J. and Macaulay, J.B. (2003) Stereoselective biosynthesis of chloroarylpropanediols by the basidiomycete Bjerkandera adusta: exploring the roles of amino acids, pyruvate, glycerol and phenyl acetyl carbinol. FEMS Microbiology Letters, 228: 11-19.