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Stereoselective biosynthesis of chloroarylpropane diols by the basidiomycete *Bjerkandera adusta*: exploring the roles of amino acids, pyruvate, glycerol and phenyl acetyl carbinol

Peter James Silk [a,*], John B. Macaulay [b]

[a] *Natural Resources Canada, Canadian Forest Service, Atlantic Forestry Centre, P.O. Box 4000, 1350 Regent Street, Fredericton, NB, Canada E3B 5P7*
[b] *RPC, 921 College Hill Road, Fredericton, NB, Canada E3B 6Z9*

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Abstract

Bjerkandera adusta produces many chlorometabolites including chlorinated anisyl metabolites (CAMs) and 1-arylpropane-1,2-diols (**1**, **2**, **3**, **4**) as idiophasic metabolic products of L-phenylalanine. These diols are stereoselectively biosynthesized from a C_7-unit (benzylic, from L-phenylalanine) and a C_2-unit, of unknown origin, as predominantly erythro (1**R**,2**S**) enantiomers. 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 $^2H_2$ in the C3 side chain) 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 arylpropane diol biosynthesis. Incubation of *B. adusta* with synthetic [2-^{2}H$_1$,2-^{18}O]-glycerol showed that neither ^{2}H nor ^{18}O were incorporated in the α-ketols or diols. The oxygen atom on the C2 of the ketols/diols, therefore, does not appear to come from the oxygen atom on the 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. Labeled α-ketol, phenyl acetyl carbinol (**5**) (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 (**6**) (ring-d_5 and 2,3-$^{13}C_2$); in all other metabolites produced, the 2,3-$^{13}C_2$ label is washed out. Incubation of the fungus with 4-fluorobenzaldehyde (**13**) produces a pooling of predominantly erythro (1**R**,2**S**) 1-(4′-fluorophenyl)-1,2-propane diol (**18** as diacetate) (through the corresponding α-ketols **16**, **17**). Blocking the para-position with fluorine thus appears to prevent ring oxygenation and also chlorination, forcing the conclusion that para-ring oxygenation precedes meta-chlorination.

Keywords: Chlorometabolite; Ketol; Phenyl acetyl carbinol; Chloroarylpropane diol; Pyruvate decarboxylase; Biosynthesis; Glycerol; Pyruvate; Stable isotope; *Bjerkandera adusta*

1. Introduction

The production of chlorometabolites by basidiomycetes is well documented [1,2]. The biochemical processes leading to their production, however, are not well elaborated and the chlorinating enzyme(s) (and, therefore, the chlorinating mechanisms) remain obscure.

The white rot basidiomycete *Bjerkandera adusta* biosynthesizes many chlorometabolites [2], including chloro-1-arylpropane-1,2-diols [3–6] that are produced from L-phenylalanine during idiophasic metabolism and that are stereoselectively biosynthesized from a C_7-unit (ring+benzylic carbon) and a C_2-unit as predominantly erythro (1**R**,2**S**) enantiomers [6].

Previous stable isotope labeling experiments have indicated that the aromatic aldehydes benzaldehyde, 4-hy-

* Corresponding author. Tel.: +1 (506) 451-6084; Fax: +1 (506) 452-3828.
E-mail address: psilk@rogers.com (P.J. Silk).

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droxybenzaldehyde and 4-methoxybenzaldehyde are the likely C_7-unit precursors in the purported carboligation reaction that leads to chloroarylpropane diol biosynthesis. These aldehydes are all stereoselectively incorporated into the corresponding 1-arylpropane-1,2-diols, including the chloro-analogs and the corresponding α-ketols (phenyl acetyl carbinols (PACs) and 2-hydroxyl-propiophenones (2-HPPs)), the presumed precursors of the diols [7–9]. The source of the C_2-unit remains to be determined.

The metabolic role of the ketols/diols, if any, also remains to be elucidated. We have postulated [9] that they may act as substrates for the chlorination/hydroxylation enzymes yet to be identified in chlorometabolite-producing white rot fungi and, therefore, in the biosynthesis of chlorinated anisyl metabolites (CAMs). They may also be important intermediates in CAM aldehyde-alcohol recycling (substrates for aryl alcohol oxidase (AAO) which generate hydrogen peroxide for the peroxidases) [2,6].

In this paper, using stable isotope labeling techniques, we present evidence which determines the origin of the C_2-unit and the possible role of the α-ketol intermediates.

2. Materials and methods

2.1. 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 received from Cambridge Isotope 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%) was used without further purification; all solvents were of spectral grade.

[2-^{2}H$_1$,2-^{18}O]-glycerol was prepared following the procedure of Schaffrath et al. [10] which was by isotope exchange of the carbonyl oxygen of dihydroxyacetone (Aldrich, 97%) with [^{18}O]-water (Aldrich, 95% ^{18}O) under acidic conditions followed by reduction in methanol with NaB^2H_4. In ca. 50% yield (120 mg), the final product contained ca. 68 atom% ^{18}O and >90 atom% ^{2}H as determined by gas chromatographic/mass spectrometric (GC/MS) analysis of the triacetate derivative.

PAC with ring protons replaced by deuterium (d_5) and the 2,3-propyl carbons ^{13}C labeled, was synthesized 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) following the procedure of Crout et al. [11]. Sodium pyruvate (2,3-$^{13}C_2$) (60 mg) was stirred into a 10 ml standard phosphate buffer in a 15 ml conical vial (pH 5.9) to which was added 3.6 mg $MgSO_4$ and 0.07 mg of co-carboxylase (thiamine diphosphate, TDP; Sigma) predissolved in 50 ul deionized water. Benzaldehyde (d_5) was then slowly added (13 mg) by syringe. PDC was then added (14.9 U) supplied as a suspension in 3.2 M $(NH_4)_2SO_4$ (pH 6.5). The vial was capped, wrapped in foil and stirred. The progress of the C_2-homologation reaction was followed by analysis of aliquots by capillary GC. After 16 h, no further formation of the α-ketol was detected and the products were extracted into ethyl acetate and dried over anhydrous Na_2SO_4. Rotoevaporation and solvent exchange to *n*-hexane followed and the product was cleaned up on a silica gel 60 (Caledon, 70–230 mesh) column (2.5 g) to remove residual benzaldehyde. This was eluted with 10% diethyl ether/hexane with the α-ketol quantitatively eluting with pure diethyl ether. Acetylation of a portion of this ketol with acetic anhydride/pyridine (Aldrich) and GC/MS analysis showed pure PAC with label ring-d_5 and both the 2,3-C in the propyl side chain ^{13}C labeled; the EI (+) mass spectrum was characteristic of the labeled product (M^+ *m/z* 199). No traces of benzaldehyde (d_5) were detected (ca. 97% pure; ca 50% yield) and the product also appeared to be of high chiral purity (>90% ee) by α-cyclodextrin column chromatography of the acetylated derivative. The product was assigned as 1**R**-PAC since the stereochemistry of this product was previously determined using this enzymatic procedure [11].

2.2. 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°C.

2.3. 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 (Sigma), 0.2 g peptone (Difco), 0.2 g yeast extract (Difco), 0.2 g KH_2PO_4 (Aldrich) and 0.1 g $MgSO_4$ (Caledon) in 100 ml distilled water. The mixture was autoclaved and 1 mg thiamine hydrochloride (Sigma) in 50 ul water was filter sterilized and added to the cooled medium. The measured (colorimetry) chloride content was 40–46 mg l^{-1}. The cultures were grown aerobically and maintained static in the dark at 22–25°C. Cultures were generally established with a thick mycelial mat (ca. 10 days) prior to the addition of labeled substrates

with cultures being harvested after 10 days post-addition except where noted.

Media were inoculated with a 5 mm agar plug taken from the growing edge of a fresh culture. All experiments were performed in at least duplicate; glycerol experiments were performed in triplicate. Each culture was carried out in a 250 ml Erlenmeyer flask with 50 ml of medium in each replicate. Labeled materials were dissolved in water at 20–40 mg per 50 ml medium except for the labeled synthetic PAC which was dissolved (5 mg) in ethanol (1 ml) prior to adding to the culture medium, L-serine at the 100 mg level and pyruvate (2,3-$^{13}C_2$), which was cultured at the 200 mg level. Glycerol (d_5) was added at 20, 40, 60 and 200 mg levels all being harvested 10 days post-addition with the 200 mg dose being extracted after 5 days; 4-fluorobenzaldehyde was incubated in duplicate at the 20 mg level both alone and in the presence of glycerol (d_5). Incubation with synthetic [2-$^2H_1$,^{18}O]glycerol was carried out at the 100 mg level.

2.4. Extraction of fungal cultures

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

2.5. Instrumental analysis

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 used for most analyses was Supelcowax-10 (30 m, 0.25 mm ID, 0.25 um film thickness), temperature programmed from 50°C at a rate of 20°C min^{-1} to 250°C with injection temperature set at 250°C. Some extracts were analyzed 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°C, held for 5 min and then at 5°C min^{-1} to 220°C.

Retention time and EI (+) mass fragmentation patterns

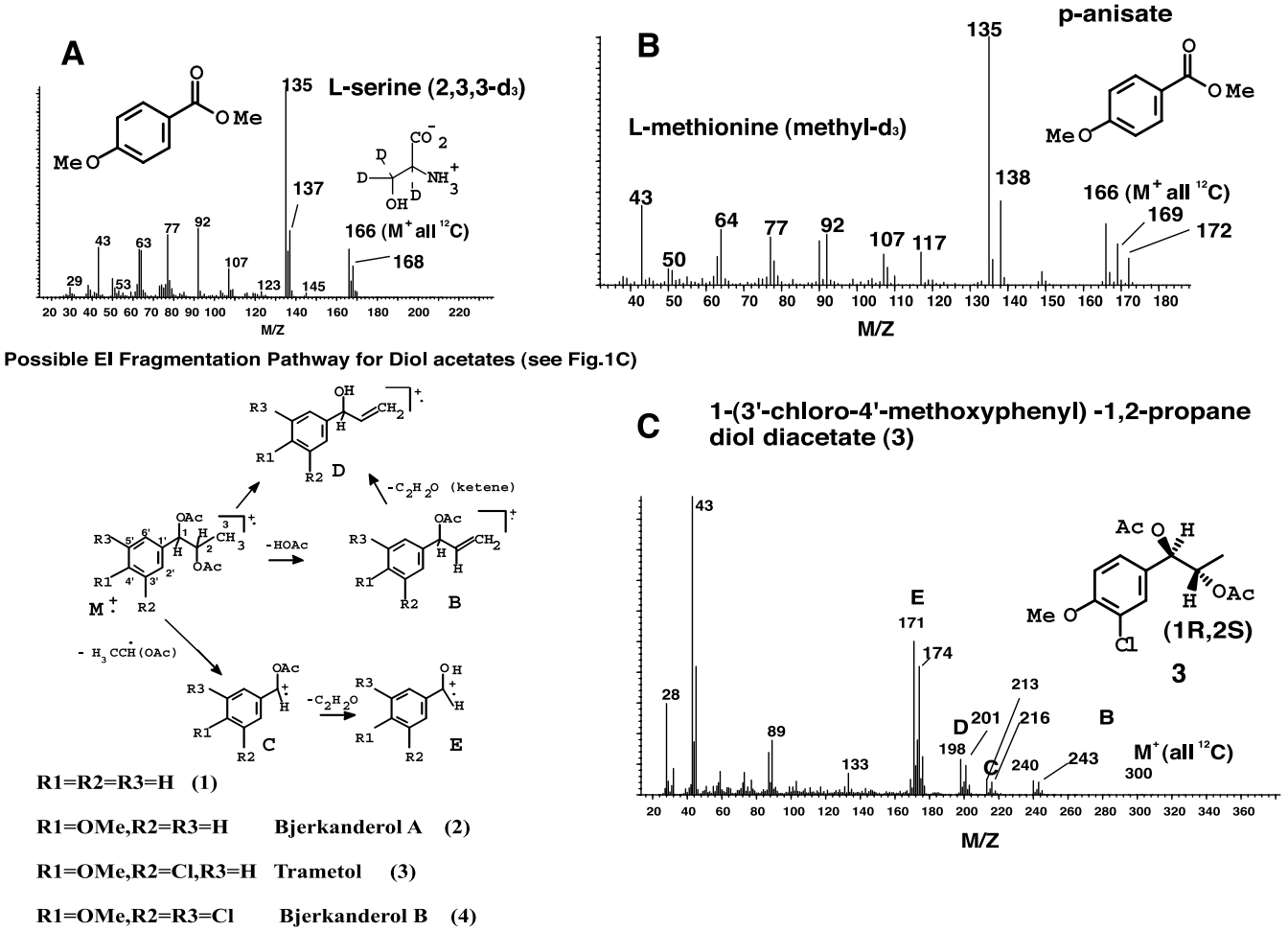

Fig. 1. GC/MS analysis of extracts of cultures of *B. adusta* incubated with A: L-serine (2,3,3-d_3) and B: L-methionine (methyl-d_3) illustrating methylation of *p*-anisate and C: Trametol (**3**) (shown as the diacetate); the EI mass spectral fragmentation pathway of the diol diacetates is also illustrated.

were used to identify compounds by comparison with authentic material, characterized synthetics or those made by yeast biomimetic synthesis previously characterized [6].

3. Results

3.1. Incorporation of amino acids

None of the amino acids cultured with *B. adusta*, at the 4–10 mM levels tested, produced any significant label into the 2,3-propane carbons of the 1-arylpropane-1,2-diols. However, both glycine (2-^{13}C) (data not shown) and L-serine (2,3,3-d_3) did, independently and effectively, label the methyl groups on the methylated ring hydroxyls and the carboxylate methyl in, for example, *p*-anisate. In Fig. 1A we illustrate this with *p*-anisate showing methoxyl methyl and carboxylate methyl both labeled ($^2H_2$ from L-serine). L-methionine (methyl-d_3) very effectively achieved the same result with label (d_3) entering ring-*O*-methylation and in the carboxylate ester methyl in *p*-anisate (Fig. 1B) and, by illustration, the ring *O*-methoxyl methyl in Trametol (**3**; Fig. 1C). Note the incorporation of isotopes (d_3) in the ring *O*-methyls in all the characteristic fragments (**B**, **C**, **D** and **E**; see postulated fragmentation pathway, Fig. 1). Experiments with labeled L-alanine (2,3,3,3-d_4) failed to show any significant label in any metabolites when cultured at this level (< 10 mM), although traces of deuterium were noted associated with the propane carbons of the diols but not in methyl groups observed in the biomethylation pathway.

L-serine (2,3,3-d_3), at 40 mM in culture, showed not only evidence of entry into the biomethylation pathway but also deuterium ($^2H_2$) appeared in the C3 side chain of all diol diacetates (of **1**, **2**, **3**, **4**) as evidenced (data not shown) in all the C_9 fragments (**B** and **D**, Fig. 1). Specifically, deuterium label appears in the C_9 fragment of PAC, a product which does not manifest the simultaneous complication of biomethylation and, therefore, facilitating mass spectral interpretation. Regio-specific deuterium location by EI MS alone, can often be complex and inconclusive.

3.2. Other possible C_2-units

The isotopically labeled compounds pyruvate (2,3-$^{13}C_2$; sodium), acetate (1,2-$^{13}C_2$; sodium), acetaldehyde (d_4), and ethanol (ethyl-d_5) failed to incorporate any significant label(s) at the 4–10 mM levels into the ketols, diols or, indeed, any other secondary metabolites. Pyruvate (2,3-$^{13}C_2$) at the 40 mM level, however, significantly labeled the 2,3-propane carbons both in the α-ketols and diols but did not appear to enter the biomethylation pathway. This is illustrated in Fig. 2. The upper figure shows 1-(4′-methoxyphenyl)-1-hydroxy-2-propanone (acetylated) with the 2,3-propane carbons ^{13}C labeled. Similarly, the middle

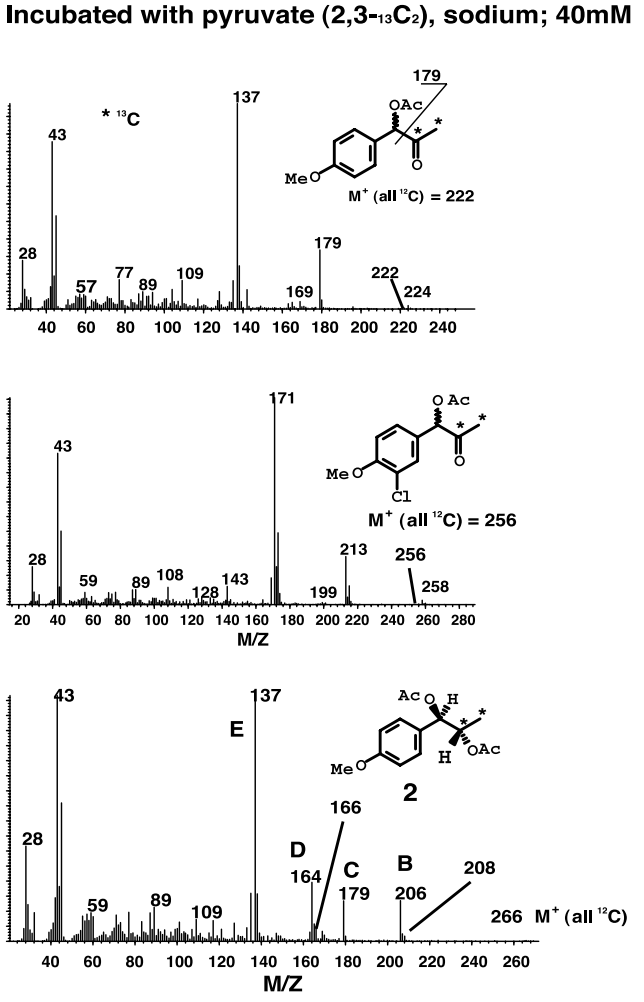

Fig. 2. GC/MS analysis of extracts of *B. adusta* incubated with pyruvate (2,3-$^{13}C_2$, sodium) at ca. 40 mM. Upper: EI mass spectrum of 4′-methoxy PAC (acetylated) showing 2,3-$^{13}C_2$ insertion into the propane side chain. Middle: EI mass spectrum of 3′-chloro-4′-methoxy PAC (acetylated) showing 2,3-$^{13}C_2$ incorporation into the propane side chain. Lower: EI mass spectrum of 1-(4′-methoxyphenyl)-1,2-propane diol (diacetate) showing incorporation of 2,3-$^{13}C_2$ into the propane side chain.

figure illustrates the 3′-chloro-derivative of the same α-ketol with the molecular ion at 256 atomic mass units (AMU) clearly augmented by 2 AMU showing the $^{13}C_2$ incorporation. The lower figure is the EI mass spectrum of 1-(4′-methoxyphenyl)-1,2-propane diol (diacetate) illustrating the $^{13}C_2$ incorporation into the 2,3-propane carbons of the diol from the 2,3-$^{13}C_2$-pyruvate.

3.3. Glycerol and glucose incorporation

Deuterium from deuterated glycerol (1,1,2,3,3-d_5) was incorporated into *B. adusta* secondary metabolites. It appeared to do this in two distinct ways under these conditions with this labeled intermediate.

Firstly, ring-*O*-methylation occurred in which populations of mono- and di-deuterated methyls appeared in,

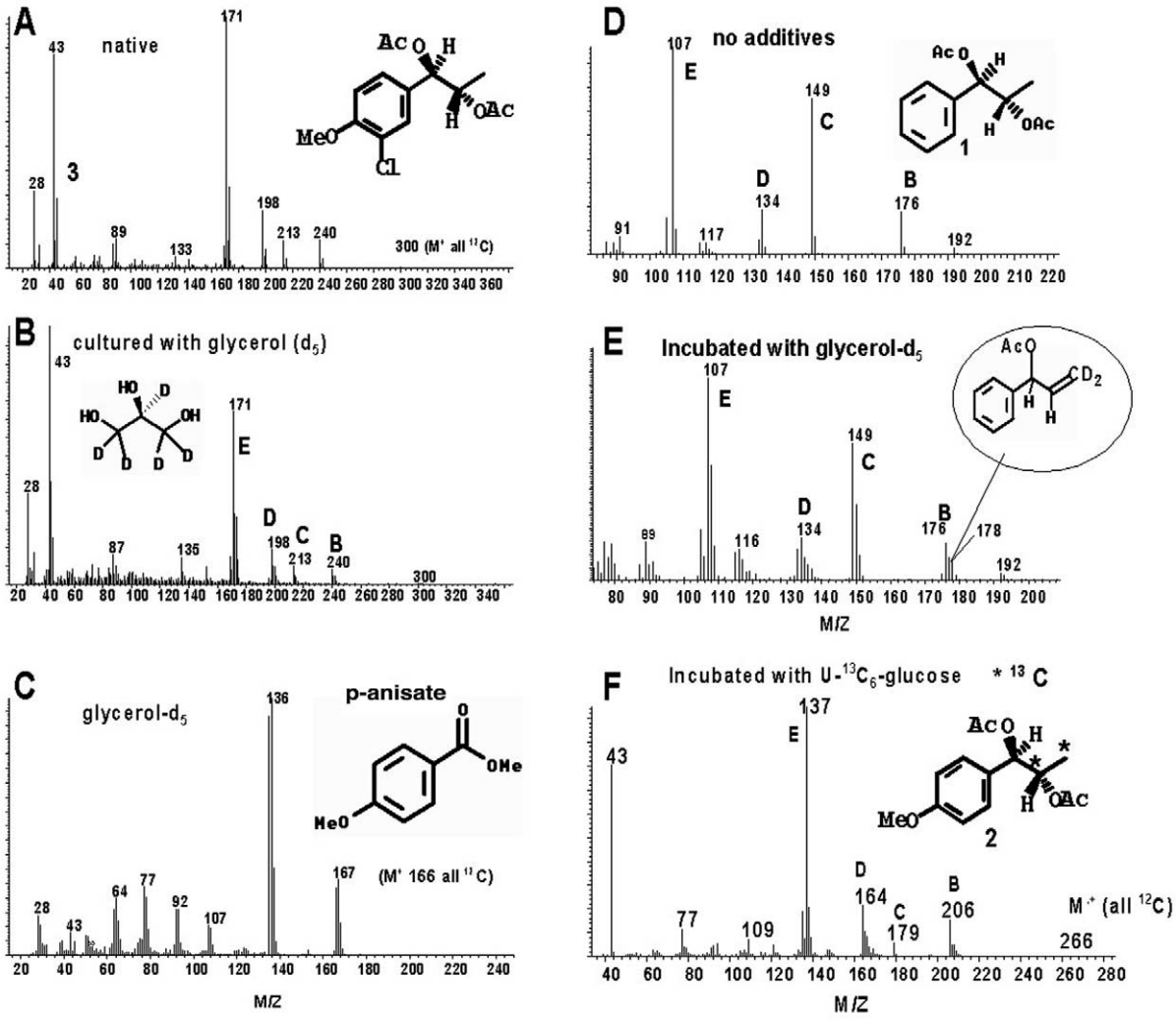

Fig. 3. GC/MS analysis of extracts of cultures of *B. adusta* showing Trametol incorporation with no additives (A) and incubated with glycerol (1,1,2,3,3-d_5) (B) and glycerol (d_5) into *p*-anisate (C). PAC formation with no additives (D); PAC formation with glycerol (1,1,2,3,3,-d_5; 60 mg) (E) indicating incorporation of deuterium (C3-$^2H_2$) likely on C3; Bjerkanderol A formed by incubation with U-$^{13}C_6$-glucose (F); (EI mass spectra).

for example, *p*-anisate (Fig. 3C) and also, for example, in characteristic **B**, **C**, **D** and **E** fragments (see Fig. 3) in Trametol (**3**) diacetate (Fig. 3B; compare to the native compound, Fig. 3A).

Secondly, glycerol (d_5) also appeared to label, with deuterium, likely the C3 carbon on the propane chain of the arylpropane diols. Fig. 3E illustrates the C_9 fragments of the EI (+) mass spectrum of PAC (**1**; Fig. 1). The mass spectrum is not complicated by the simultaneous ring-*O*-methylation as in the other diols. The characteristic C_9 fragments, which contain the phenyl ring and all three propane carbons (see Fig. 3D,E), point to populations of molecules having both one and two deuterium atoms in the C3 propane carbon of each of the C_9 fragments **D** and **B** (*m/z* 134 and 176, respectively). Although incorporation is low (ca. 5%) the level appears to be significant. In addition, the C_7 fragments **E** and **C** (*m/z* 107 and 149, respectively) also appeared to show a population of molecules which have incorporated a significant level (ca. 8%) of a deuterium atom on the benzylic carbon (Fig. 3E) possibly through enzyme catalyzed tautomerism at the ketol stage, ion rearrangements of the diol diacetates in the ion source of the mass spectrometer or during acid treatment of the extracts.

Analysis of the products of the incubation of [2-$^2H_1$, 2-^{18}O]glycerol with *B. adusta* showed that no deuterated products were detected. Determination of incorporation of ^{18}O proved complex but also could not be detected in ketol or diol products. The acetate derivatives readily lose acetic acid (see proposed fragmentation pathway, Fig. 1) under EI (+) conditions which would include the ^{18}O on C2 (these diacetates do not display molecular ions). Direct analysis of the underivatized extracts gave no useful data either. Acetonide formation by acid catalyzed (*p*-toluene sulfonic acid) treatment with refluxing acetone worked well with standards but the derivatization of the extract

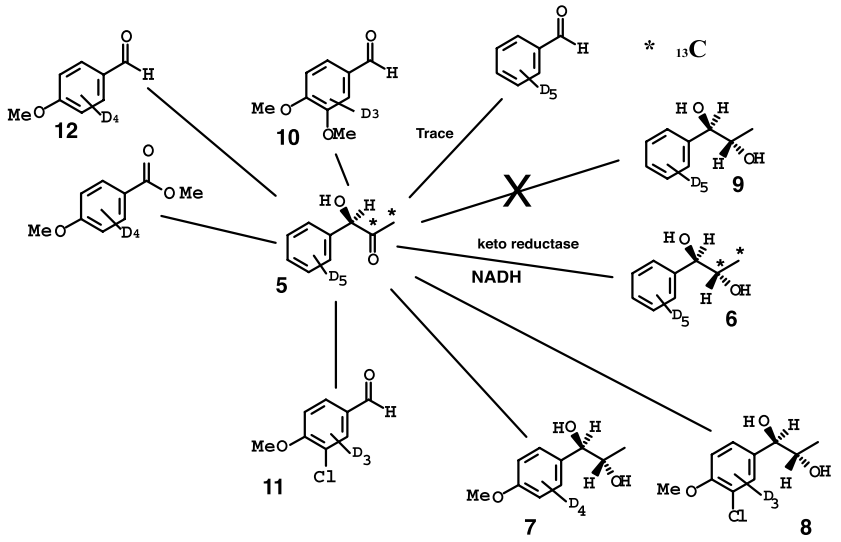

Fig. 4. Products determined from the GC/MS analysis (as acetates) of extracts from the incubation of labeled PAC (ring-d_5, 2,3-^{13}C) (**5**) with *B. adusta*.

was very complex and not diagnostic. Although further work is required to get more useful data with this labeled substrate, no ^{18}O or ^{2}H could be detected in any of the α-ketols or diols. Glycerol does, however, provide a convenient entry point in which to insert a label(s) to aid in determining the source and biochemical processing leading to the C_2-unit.

Incubation of *B. adusta* with U-$^{13}C_6$-D-glucose produced a metabolic motif that lends strong support to the thesis that the glycolytic pathway is the source of the 2,3-propane carbons in the diol side chain, offering further explanation to incorporation through glycerol and pyruvate. This is illustrated in Fig. 3F (lower) which indicates $^{13}C_2$ inclusion into the C_9 fragments in the EI mass spectra of the diol diacetate of (**2**). Incorporation of deuterium onto ring carbons is also noted in all metabolites in trace amounts likely by entry through the shikimate pathway en route to L-phenylalanine.

3.4. Labeled PAC degradation

The ring (d_5) and C2,C3-^{13}C-labeled PAC (**5**) was readily degraded by *B. adusta* to all of the predicted downstream products (Fig. 4). Extracts were examined on both the Supelcowax-10 and the α-Dex 20 columns. A stereospecific reduction to the erythro (1**R**,2**S**) diol(s) occurred with the corresponding diol containing both the ring-d_5 and the 2,3-$^{13}C_2$ labels (**6**). This $^{13}C_2$ label, however, was not detected in any other metabolites and was, therefore, 'washed out'. The deuterated (d_4) Bjerkanderol A (**7**) and (d_3) Trametol (**8**) were also produced as erythro (1**R**,2**S**) enantiomers (>90% ee). Of significance was the absence of deuterated (d_5) phenyl diol (**9**) from the extract. Veratraldehyde (d_3) (**10**) was detected in significant quantities as were 3-chloro-4-methoxybenzaldehyde (d_3) (**11**) and anisaldehyde (d_4) (**12**) (see Fig. 4). Only traces of benzaldehyde (d_5) were detected.

3.5. 4-Fluorobenzaldehyde processing

Incubation of 4-fluorobenzaldehyde (**13**) with *B. adusta* showed that this compound did not appear to alter secondary metabolic processes because all the usual secondary metabolites were produced in expected quantities.

However, several fluorometabolites were also detected. Apart from unreacted 4-fluorobenzaldehyde (**13**), 4-fluorobenzoic acid (methyl ester) (**14**) and 4-fluorobenzyl alcohol (acetylated) (**15**) were both detected. Both fluoro-α-ketols were also produced in small amounts. This is illustrated in Fig. 5 with mass spectra of acetylated 4-fluoro-PAC (**17**) (upper) and acetylated 1-(4′-fluorophenyl)-2-hydroxy-1-propanone (**16**) (middle). More importantly, significant and much larger amounts of (acetylated) erythro (plus ca. 1% threo) 1-(4′-fluorophenyl)-1,2-propane diol (1**R**, 2**S**) (**18**) were produced as illustrated in the mass spectrum in Fig. 5 (lower) and the chromatogram of the extract. No other ring substituted fluoromethoxyl, chlorofluoro or chlorofluoromethoxyl products (α-ketols or diols) were detected.

4. Discussion

The arylpropane diols, including the chloro-analogs, are formed from L-phenylalanine [6] but $^{13}C_9$-L-phenylalanine experiments indicated label in only benzylic and ring carbons, supporting a biosynthesis of these arylpropane diols by *B. adusta* from ligasing C_7- and C_2-units. Previous data [9] support the thesis that indeed the C_7-unit is benzaldehyde and/or 4-hydroxy/4-methoxybenzaldehyde.

The stereoselective nature of the biosynthesis of these diols and the presence of the corresponding (precursor) α-ketols, indicates that enzyme systems analogous to PDC [11,12], which plays an important role in glycolysis (α-keto acid decarboxylases), and/or phenyl glyoxalate de-

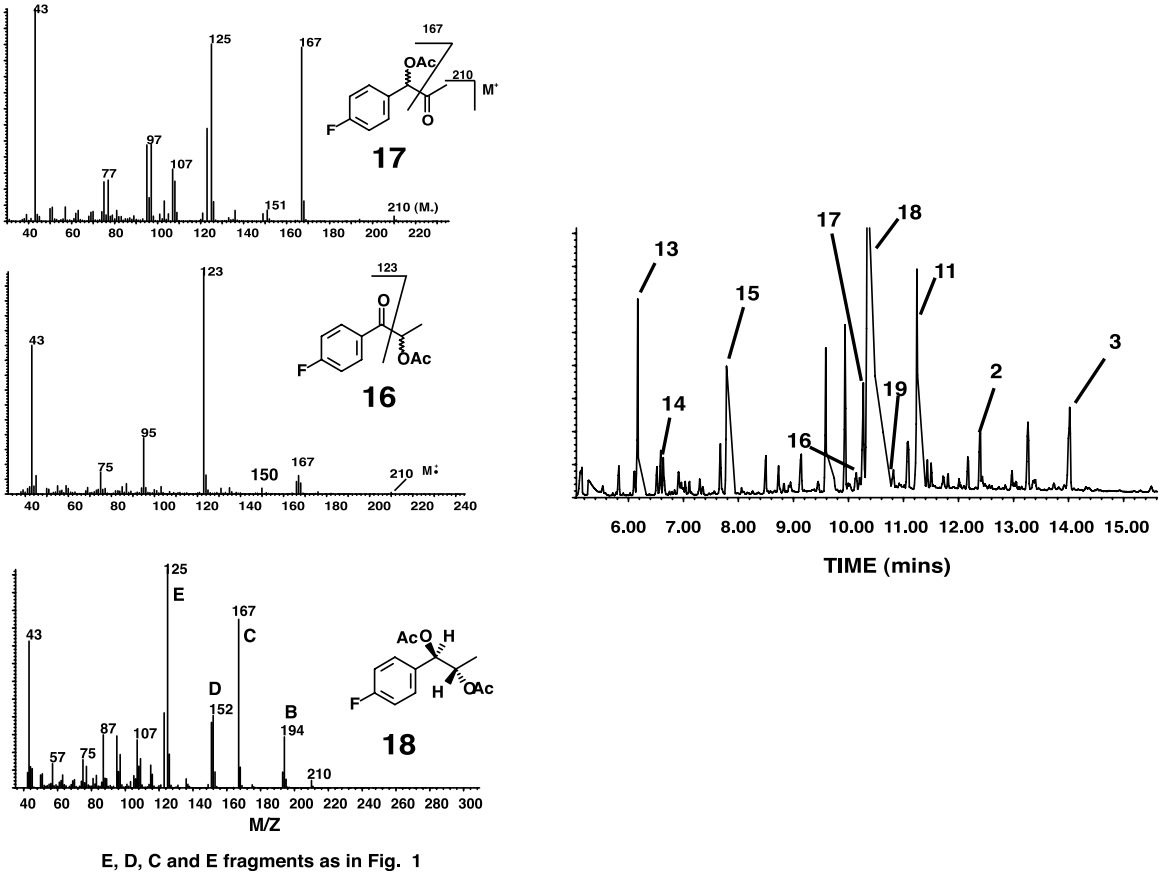

Fig. 5. Incubation of 4-fluorobenzaldehyde with *B. adusta*: Upper: EI mass spectrum of 4-fluoro-PAC (acetylated) (**17**). Middle: EI mass spectrum of 1-(4′-fluorophenyl)-2-hydroxy-1-propanone (acetylated) (**16**). Lower: EI mass spectrum of erythro 1-(4′-fluorophenyl)-1,2-propane diol (diacetate) (**18**). Chromatogram: GC/MS analysis of an extract of *B. adusta* incubated with 4-fluorobenzaldehyde (**13**); 4-fluorobenzoic acid, methyl ester (**14**); 4-fluorobenzyl alcohol acetate (**15**); (**16**), (**17**), (**18**) as in this figure; (**19**) threo diastereomer of (**18**); (**2**), (**3**), (**11**) as in Fig. 4 except non-deuterated.

carboxylase [13,14], an enzyme involved in the mandelate pathway, are likely present in *B. adusta*, with TDP as cofactor and pyruvate as the source of the C_2-unit (among several possible C_2-units). Although this thesis made intuitive sense from the literature, it is not entirely supported from our present database since only labeled pyruvate at high levels, and not acetaldehyde, enters the pathway and effectively ^{13}C labels C2 and C3 (from (2,3-$^{13}C_2$)-pyruvate) of the arylpropane diols under these conditions.

Glycerol, however, also appears to effectively enter the pathway (Fig. 6A). This compound is situated between glucose (which also labels the C_2-unit) and pyruvate on the glycolytic pathway, giving further support that pyruvate is the C_2-unit precursor. Pyruvate, as a labeled intermediate, however, may not be able to efficiently enter the pathway at low levels because of the very tight channeling of intermediates cascading toward the C_2-homologation reaction (see [15]). On this basis the label may be washed out very rapidly in the recycling process and the label, therefore, does not pool in the final product at a high level. It also cannot be concluded that glycerol is the intermediate either and further work is needed particularly on the processing of glycerol by *B. adusta*. Glycerol itself is not, chemically or biochemically, sufficiently reactive to undergo the carbon–carbon bond breaking reaction that would lead to a C_2-unit. We suggest, like others [16,17], that it is much more likely that the reactive species are the triose intermediates of glycolysis, dihydroxyacetone-1-phosphate or 3-phosphoglyceraldehyde from glycerol. Glycerol is likely processed as illustrated in Fig. 6A wherein it is converted, via glycerol-3-phosphate, to pyruvate and then, mediated by TDP, to carbons 2 and 3 of the propane side chain of the α-ketols. All but two deuterium atoms (residing on the pyruvate methyl), from the glycerol-d_5 employed, would be eliminated, supporting our present data.

Glycerol also can be converted by NAD^+/glutamate to 3-phospho-serine then to serine, which can form pyruvate via the action of PLP/serine dehydratase and enter the C_2-homologation pathway, but with the oxygen on C2 of glycerol being then lost in the transamination step. This is the more likely process supported by our present data on the processing of synthetic [2-^{2}H,2-^{18}O]glycerol. In addition, L-serine thus formed can readily, via action of serine hydroxy methyl transferase (SHMT), be converted to methylene (releasing glycine) and then methyl-tetrahydrofolate, on to biosynthesize L-methionine, explaining why glycerol can also enter the biomethylation pathway

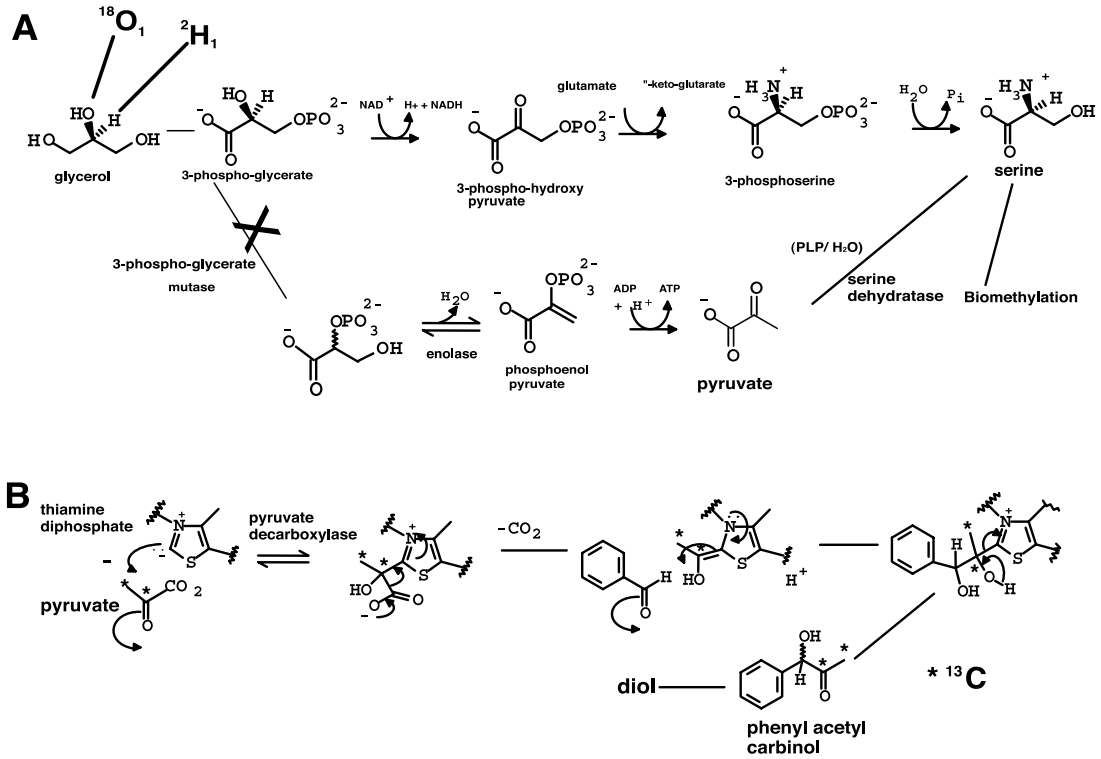

Fig. 6. A: Postulated entry of glycerol into the glycolytic pathway and B: speculated PAC formation. A: Conversion of 3-phosphoglycerate to serine and postulated entry into the methylation pathway and pyruvate formation. B: Speculated pathway of pyruvate addition to benzaldehyde via PDC catalyzed by TDP to form PAC; note the labeling pattern of the 2,3-$^{13}C_2$-carbons; only the deprotonated thiazolium ring of TDP is depicted.

with this fungus (Fig. 6A). L-methionine is then either processed through SAM or methyl chloride by phenol-*O*-methyl transferases (see [18,19]). The processing of both glycerol and pyruvate is intriguing but the stereochemical mechanism of their utilization remains to be fully elaborated.

The processing of 4-fluorobenzaldehyde to the corresponding α-ketols and pooling (illustrated in the chromatogram of Fig. 5) of large amounts of the corresponding fluorophenyl diol through stereospecific reduction, is revealing. Obviously, the C_2-homologation reaction has occurred with this substrate. When 4-fluorobenzaldehyde was concurrently incubated with glycerol (d_5; data not shown), the outer carbon (C3) appeared to be deuterium labeled. Further ring substitution of the fluorophenyl ring did not occur in that no hydroxylation/chlorination appeared in any fluoro products. Thus, 4-fluorination, unlike 2-fluorination [20] effectively blocks para-hydroxylation, which also then apparently blocks chlorination. These data thus force the conclusion that ring para-oxygenation/hydroxylation must precede (or occur simultaneously with) ring meta-chlorination.

The stereochemical processing of the labeled PAC (**5**) indicates that the α-ketols are involved in arylpropane diol biosynthesis and are very likely involved in CAM recycling, with evidence that the C_2-homologation reaction and para-hydroxylation are important to these processes and, therefore, in the physiology of the fungus. These data also supply evidence that the α-ketols and/or the diols are important substrates for the ring hydroxylation/chlorination enzymes since these processes occur downstream of PAC with recycling of the C_2-unit.

NADH-dependent halogenases, rather than chloroperoxidases (not detected in basidiomycetes; [2]), have been described in chlorometabolite biosynthesis [21], imparting substrate and regio-selectivity to the chlorinating enzyme (suggested by our data). This enzyme, an FAD-OOH-halogenase complex, is postulated to oxygenate the aromatic ring via an epoxide (arene oxide) activating the organic substrate. The epoxide is then opened by a nucleophilic attack (enzyme catalyzed) by chloride anion. The α-ketol(s) and/or diols may then be the enzyme substrate(s) of the halogenase complex.

In summary, we conclude that α-ketols are important intermediates in arylpropane diol biosynthesis and, therefore, in CAM aldehyde-alcohol cycling, as well as being substrates for the chlorinating enzyme(s). Fig. 6B illustrates the process by which we speculate that the 2,3-$^{13}C_2$-carbons of pyruvate, after decarboxylation, are incorporated into the PAC via a TDP catalyzed PDC mediated nucleophilic attack of the stabilized acyl anion on benzaldehyde (the C_7-unit; [9]) leading to the α-ketol. Stereospecific reduction [7,8] leads to the (1**R**,2**S**) diols. The discovery of stereospecific α-ketol/diol formation in a chlorometabolite-forming basidiomycete is an exciting new finding and may have important implications in fu-

ture biosynthetic potential of these ligninolytic organisms. Some enzymes in *B. adusta* metabolism have been characterized [22] but those involved in α-ketol/diol formation and chlorination remain to be isolated.

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