International Biodeterioration & Biodegradation 92 (2014) 36–40

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Novel phosphorylation of aldrin-*trans*-diol by dieldrin-degrading fungus *Mucor racemosus* strain DDF

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ARTICLE INFO

Article history:
Received 24 February 2014
Received in revised form
1 April 2014
Accepted 4 April 2014
Available online 10 May 2014

Keywords:
Bioremediation
Dieldrin
Fungus
LC-ESI-MS
HR-FAB-MS
Phosphate conjugation

ABSTRACT

Dieldrin is one of the most persistent organic pollutants, and its oxidative degradation pathways by aerobic microorganisms to 6,7-*trans*-dihydroxydihydroaldrin (otherwise known as aldrin-*trans*-diol) and 9-hydroxydieldrin are well documented. The dieldrin-degrading fungus, *Mucor racemosus* strain DDF, can decrease dieldrin levels with simultaneous production of a small amount of aldrin-*trans*-diol. A reduction in the levels of aldrin-*trans*-diol by strain DDF has also been observed. Based on these results, it has been suggested that strain DDF transforms dieldrin to more polar compounds via aldrin-*trans*-diol. We have conducted a study to identify the metabolites arising from aldrin-*trans*-diol. The results showed that strain DDF gave reduced levels of aldrin-*trans*-diol and also produced unknown metabolites. Ultra performance liquid chromatography-electrospray ionization-mass spectroscopy (UPLC-ESI-MS) analysis indicated the metabolites to be either sulfated- or phosphorylated- derivatives of aldrin-*trans*-diol, but with the metabolites retaining six chlorine atoms. Therefore, the candidate derivatives were synthesized and the retention times of the natural metabolite and the synthetic phosphate were compared. As a result of a co-injection experiment, the metabolites were determined to be aldrin-*trans*-diol *exo*- and *endo*-phosphates. These results were also supported by high-resolution-fast atom bombardment-mass spectrometry (HR-FAB-MS) of the natural metabolite ($\Delta = 0.63$ ppm). Phosphorylation of aldrin-*trans*-diol is the first reported example of phosphate conjugation in microorganisms.

1. Introduction

Dieldrin, *rel*-(1*R*,4*S*,4a*S*,5*R*,5*R*,7*R*,8*S*,8a*R*)-6,7-epoxy-1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-1,4:5,8-dimethanonaphtha lene, a synthetic organochlorine insecticide, is one of the most persistent organic pollutants (POPs). Dieldrin was developed as an insecticide in the 1940s and used extensively throughout the world from the 1950s to the early 1970s until its use was prohibited because of the high level of toxicity and persistence in the environment. This led to dieldrin being classified as a POP. Residual levels of dieldrin in upland soils still represent a significant environmental problem because they can be absorbed by agricultural products such as cucurbitaceous plants (Hashimoto, 2005).

To date, a variety of different microorganisms causing degradation of POPs have been reported, including substrate-mineralizing bacteria against γ-hexachlorocyclohexane (Senoo and Wada, 1989; Nagata et al., 1994) and hexachlorobenzene (Takagi et al., 2009). Dieldrin-degrading bacteria (Wedemeyer, 1968; Matsumoto et al., 2008; Sakakibara et al., 2011), and a variety of different fungi (Matsumura and Boush, 1967; Kataoka et al., 2010), including wood-rotting fungi (Kamei et al., 2010; Xiao et al., 2011a) have also been reported for the degradation of POPs and with the formation of some co-metabolic species. Pathways resulting from co-metabolism generally lead to the formation of multiple compounds. Previously reported studies on dieldrin metabolites resulting from degradation by microorganisms under aerobic conditions have only reported the occurrence of

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http://dx.doi.org/10.1016/j.ibiod.2014.04.005
0964-8305/

hydroxylated metabolites, including aldrin-*trans*-diol (Matsumura and Boush, 1967; Wedemeyer, 1968), 9-hydroxydieldrin (Kamei et al., 2010; Xiao et al., 2011a), monohydroxy 6,7-dihydroxy aldrin (Xiao et al., 2011a), and dihydroxy dieldrin (Xiao et al., 2011a).

A dieldrin-degrading fungus, *Mucor racemosus* strain DDF, was recently isolated, and resulted in the detection of only a small amount of aldrin-*trans*-diol as a metabolite (Kataoka et al., 2010). These results suggested the occurrence of other downstream metabolites and metabolic pathways. With this in mind, in the current study, we have attempted to detect and identify the downstream metabolites of aldrin-*trans*-diol, by incubating strain DDF with aldrin-*trans*-diol as the initial and only substrate.

2. Materials and methods

2.1. Chemicals

Aldrin-*trans*-diol [*rel*-(1*R*,4*S*,4a*S*,5*R*,5*R*,7*R*,8*S*,8a*R*)-1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-1,4:5,8-dimethanonaphthalene-6,7-diol] and dihydrochlorodene dicarboxylic acid [DHCDS; *rel*-(1*R*,3*S*,3a*R*,4 *R*,7*S*,7a*S*)-4,5,6,7,8,8-hexachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene-1,3-dicarboxylic acid] were synthesized according to the procedures of Bedford and Harrod (1972) and Sakakibara et al. (2011), respectively.

2.2. Instrumentation for synthetic studies

Fourier transform-infrared spectra were recorded as films on a Jasco 4100 spectrometer (ATR, Zn–Se; Jasco, Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Varian System 600 (600 MHz) and 400-MR (400 MHz) spectrometers (Varian, Palo Alto, CA, USA). Mass spectra were measured by high-resolution-fast atom bombardment-mass spectrometry (HR-FAB-MS) using a JMS-700 spectrometer (Jeol, Tokyo, Japan). Microwave reaction chemistries were performed in a Discover microwave system (CEM, Buckingham, UK).

2.3. Synthesis of sulfated aldrin-trans-diols

A mixture of aldrin-*trans*-diol (20.0 mg, 0.0501 mmol), SO_3•Py (8.0 mg, 0.050 mmol), and Na_2SO_4 (40 mg, 0.28 mmol) in *N,N*-dimethylformamide (DMF; 1.2 mL) was stirred at 110 °C for 2 h under microwave irradiation. The mixture was concentrated *in vacuo*, and the residue was purified by preparative TLC (Merck RP-18 F_{254F}, 0.25 mm thickness; MeOH/H_2O = 1:1, R_f 0.30) to give the sulfate sodium salt (*ca.* 1:1 6-*exo*/7-*endo*-mixture, 11.0 mg, 0.0220 mmol, 43.8%) as an amorphous solid. IR ν: 3500 (vs), 2970 (m), 1662 (m), 1600 (m), 1258 (s), 1225 (s), 1060 (m), 998 (m), 830 (w) cm^{-1}. ^{1}H NMR (600 MHz, CD_3OD) δ (aldrin-*trans*-diol 6-*exo*-sulfate sodium salt): 1.42 (1H, d, J = 13.2 Hz, H-9$_{anti}$), 1.45 (1H, d, J = 13.2 Hz, H-9$_{syn}$), 2.42 (1H, d, J = 4.0 Hz, H-8), 2.70 (1H, d, J = 7.9 Hz, H-4a), 2.76 (1H, s, H-5), 3.42 (1H, d, J = 7.9 Hz, H-8a), 3.82 (1H, s, H-6), 3.97 (1H, d, J = 4.0 Hz, H-7); δ (7-*endo*-sulfate sodium salt): 1.43 (1H, d, J = 12.9 Hz, H-9$_{anti}$), 1.55 (1H, d, J = 12.9 Hz, H-9$_{syn}$), 2.24 (1H, s, H-5), 2.66 (1H, d, J = 7.6 Hz, H-4a), 2.79 (1H, d, J = 4.0 Hz, H-8), 3.33 (1H, d, J = 7.6 Hz, H-8a), 3.48 (1H, s, H-6), 4.29 (1H, d, J = 4.0 Hz, H-7). FAB(−)-MS *m/z*: 475 [M(^{35}Cl$_6$)−Na]$^-$, 497 [M(^{35}Cl$_6$)−H]$^-$, 523. FAB(+)-MS *m/z*: 499 [M(^{35}Cl$_6$)+H]$^+$, 521 [M(^{35}Cl$_6$)+Na]$^+$, 544 [M(^{35}Cl$_6$)−H+2Na]$^+$. HR-FAB(+)-MS *m/z*: 520.8102 (calcd. for $C_{12}H_9{}^{35}Cl_6O_5Na_2S$ [M(^{35}Cl$_6$)+Na]$^+$, 520.8097). Further purification by preparative TLC (MeOH/H_2O = 1:1) afforded the 6-*exo*-sulfate sodium salt containing 17% of the 7-*endo*-sulfate sodium salt.

2.4. Synthesis of phosphorylated aldrin-trans-diol

A stirred mixture of aldrin-*trans*-diol (40 mg, 0.10 mmol), dimethylaminopropyl (ethyl) carbodiimide hydrochloride (EDCI·HCl; 58 mg, 0.30 mmol), H_3PO_4 (15 mg, 0.15 mmol), and 4-dimethylaminopyridine (DMAP; 37 mg, 0.30 mmol) in DMF (2 mL) was heated at 130 °C for 12 h. The mixture was then cooled to ambient temperature before being concentrated *in vacuo* to give the crude product as a residue. This was then purified by preparative TLC (Merck Kieselgel F_{254F}, 0.5 mm thickness; $CHCl_3$/MeOH = 3:1, R_f 0.35) to give aldrin-*trans*-diol 6-*exo*-phosphate (7.6 mg, 0.016 mmol, 16%) as an amorphous solid. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.41 (1H, d, J = 13.2 Hz, H-9), 1.93 (1H, d, J = 13.2 Hz, H-9), 2.44 (1H, s, H-5), 2.65 (1H, s, H-4a), 3.34 (1H, s), 3.56 (1H, m), 3.67 (1H, m), 4.23 (1H, dd, J = 5.2, 2.0 Hz, H-6). HR-FAB(−)-MS *m/z*: 456.8278 (calcd. for $C_{12}H_8{}^{35}Cl_6O_4P$ [M(^{35}Cl$_6$)−H_2O−H]$^-$, 456.8291).

2.5. Fungus and culture conditions

M. racemosus strain DDF was grown on a potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plate at 25 °C for 5 days. Pre-cultured fungal disks (6 mm diameter) were inoculated statically into 10 mL of a modified Czapek yeast liquid medium (glucose 10 g; $MgSO_4·7H_2O$ 0.5 g; $NaNO_3$ 2.0 g; $FeSO_4·4H_2O$ 0.01 g; K_2HPO_4 1.0 g; and Bacto yeast extract 0.5 g L^{-1}) in a sterilized 100-mL Erlenmeyer flask at 25 °C in the absence of light. The flasks were sealed with a glass stopper.

2.6. Analytical conditions for ultra performance liquid chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS)

The concentration of aldrin-*trans*-diol was determined using an ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a Micromass Quattro micro API tandem quadrupole system (Waters). Separations were performed with an ACQUITY UPLC BEH C18 column (Waters; 1.7 μm particle size, 2.1 × 100 mm) at 40 °C. The pump was set in isocratic mode at a flow rate of 0.3 mL min^{-1} using a mobile phase of acetonitrile and 0.2% acetic acid solution (45:55 v/v) for aldrin-*trans*-diol, DHCDS, sulfated aldrin *trans*-diols, and unknown-metabolites. Additionally, a mobile phase (acetonitrile and 0.2% acetic acid, 30:70 v/v) was used for separation of the unknown metabolite. Mass spectrometric analyses of the compounds were performed with a Z-spray source with electrospray ionization (ESI) using the selected ion monitoring (SIM) mode. The mass spectra of aldrin-*trans*-diol at *m/z* = 456.8 [M + CH_3COO]$^-$, DHCDS at *m/z* = 428.8 [M−H]$^-$, and the unknown-metabolite containing six chlorines at *m/z* = 476.8 were determined using a cone voltage of 35 V in negative ion mode. Total ion chromatography (TIC) mode was used for detection in negative and positive ion modes over the mass range, *m/z* 300–500. The ESI-MS analyses were performed with a nebulization flow rate of 100 L h^{-1} and a desolvation gas (N_2) flow rate of 500 L h^{-1}. The source and desolvation temperatures were set at 100 and 350 °C, respectively, and the capillary voltage was 3.5 kV. The UPLC and ESI-MS systems were controlled using MassLynx 4.1 software (Waters).

2.7. Analytical conditions for high-performance liquid chromatography (HPLC)

The synthetic aldrin-*trans*-diol 6-*exo*-phosphate and natural extract were analyzed using an HPLC system (Shimadzu, Kyoto, Japan) equipped with a LC-10AD pump (Shimadzu) and a SPD-10A detector (254 nm; Shimadzu). Acetonitrile was used as the eluent.