Studies on the degradation of recalcitrant environmental pollutants by white-rot fungi

THESIS

STUDIES ON THE DEGRADATION OF RECALCITRANT ENVIRONMENTAL POLLUTANTS BY WHITE-ROT FUNGI

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THESIS

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白色腐朽菌による難分解性環境汚染物質の分解に関する研究

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Chapter I
Introduction

1. White-rot fungi

Lignin, the most abundant aromatic biopolymer on earth, is extremely recalcitrant to degradation. Lignin is a heterogeneous, random, phenylpropanoid polymer that constitutes 20-30% of woody plant cell walls. By linking to both hemicellulose and cellulose, it creates a barrier to any solutions or enzymes and prevents the penetration of lignocellulolytic enzymes into the interior lignocellulosic structure. White-rot basidiomycetous fungi are the only known microorganisms that are capable of degrading lignin extensively to CO_2 and H_2O (Kirk and Farrell, 1987).

Many white-rot fungi produce multiple extracellular enzymes to degrade lignin polymers. These are often referred to as lignin modifying enzymes (LMEs), and they are lignin peroxidase (LiP), manganese peroxidases (MnP), and laccase. Moreover, a hybrid-type peroxidase of LiP and MnP, versatile peroxidases (VP), was discovered in several fungi. The peroxidases are heme-containing enzymes with catalytic cycles that involve the activation by H_2O_2 and substrate reduction of compound I and compound II intermediates. LiP was first discovered in *Phanerochaete chrysosporium*, have the unique ability to catalyze oxidative cleavage of C-C bonds and ether (C-O-C) bonds in non-phenolic aromatic substrates of high redox potential (Schoemaker et al., 1994). MnP is able to oxidize Mn(II) to Mn(III) that it chelates, acting as a diffusing oxidizer (Wariishi et al., 1992). VPs are hybrids of LiP and MnP with a bifunctional characteristic. Laccases are multi-copper-containing proteins that catalyze the oxidation of phenolic substrates with concomitant reduction of molecular oxygen to water (Wong, 2009).

In addition, cytochrome P450 monooxygenases involved in anthracene metabolism by the white-rot fungi *P. chrysosporium* were identified by comprehensive screening of both catalytic potentials and transcriptomic profiling (Yadav and Loper, 2000; Yadav et al., 2003). Cytochrome P450 enzymes are heme-thiolate proteins that are known to catalyze the metabolism of a variety of exogenous and endogenous compounds in prokaryotes and eukaryotes. The typical eukaryotic P450 monoxygenase system contains a P450 monooxygenase and a P450 oxidoreductase, both of which are normally membraneassociated. The whole genome sequence has revealed that the P450 monooxygenase

system of *P. chrysosporium*, a lower eukaryotic organism, comprises of ~150 P450 monooxygenases and a P450 oxidoreductase (Martinez et al., 2004). It have been reported that the presence of cytochrome P450 enzymes and P450 enzymes are also involved in the degradation of organic compounds.

2. Biodegradation of environmental pollutions by white-rot fungi

Research on white-rot fungi for environmental biotechnology has been conducted for more than 20 years. The concept for the development of environmental technology using white rot fungus was proposed in 1980s (Bumpus and Aust, 1987; Tien, 1987; Aust, 1990). The first published report on the degradation of chemicals by white-rot fungi demonstrated that these fungi degrade DDT, polychlorinated biphenyls (PCBs), lindane, dioxin, and benzo(α)pyrene (Bumpus et al., 1985).

2.1. Pesticides

Pesticides are chemical compounds used to: kill, repel or control pests to protect crops before and after harvest; influence the life processes of plants; destroy weeds or prevent their growth; preserve plant products. The chemical pesticides present problems of toxicity and persistence.

Organochlorine insecticides such as 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), lindane, and the aldrins (aldrin, dieldrin, endosulfan) have been manufactured and applied in vast quantities since the 1940s. Organochlorines are persistent in the environment, and as a result of biomagnification through the food chain. White-rot fungi have been shown as capable of mineralizing DDT. Three strains of *P. chrysosporium* plus *Pleurotus ostreatus*, *Phellinus weirii*, and *Trametes versicolor* were able to mineralize 5.3-13.5% of added ^{14}C-radiolabeled DDT, dicofol, and methoxychlor over 30 days under ligninolytic growth conditions (Bumpus and Aust, 1987). A model for white-rot fungal pesticide degradation has recently been proposed, in which distinct hydrolytic and oxidative (including cytochrome P450 mediated) pathways are involved, on the basis of metabolite analysis of *P. chrysosporium* degradation of endosulfan (Kullman and Matsumura, 1996). And degradation of other pesticides was summarized by Pointing, 2001.

2.2. Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) consist of 209 different congeners, a family of compounds with a wide range of industrial application in heat transfers fluids, dielectric fluids, hydraulic fluids, flame retardants,

plasticizers, solvent extenders, and organic diluents. These compounds are produced by chlorination of biphenyl, and many different congeners are produced which vary in their degree of substitution. They are environmental pollutants that accumulate in the food chain due to their high lipophilicity and low biotransformation rate (Safe, 1992).

A lot of studies have shown that white-rot fungi including *Coriolopsis polyzona*, *P. chrysosporium*, *P. ostreatus*, and *T. versicolor* are all capable of significant PCB (Zeddel et al., 1993; Yadav et al., 1995; Novotny et al., 1997). In the mineralization experiment in which $^{14}CO_2$ is recovered from the universally labeled ^{14}C parent congener, ^{14}C-labeled PCBs show that *C. polyzona* (Vyas et al., 1994), *P. chrysosporium* (Dietrich et al., 1995), and *T. versicolor* (Beaudette et al. 2000) are capable of mineralizing PCBs, but the exact role of LMEs in this process is not clear. Kamei et al. have reported that fungal cytochrome P450 is involved in the hydroxylation of PCBs (Kamei et al., 2006).

2.3. Polycyclic aromatic hydrocarbons

A wide variety of polycyclic aromatic hydrocarbons (PAHs) are found in the environment due to the incomplete combustion of organic matter, emission sources, automobile exhaust, stationary matter, domestic matter, and area source matter (Finlayson-Pitts and Pitts, 1997). These compounds are benzene homologues formed from the fusion of four or more benzene rings, present huge problems of toxicity and persistence in the environment.

White-rot fungi have been extensively exploited for their ability to transform PAHs in the environment. Several studies have shown that diverse white-rot fungi are capable of PAH mineralization, and that rates of mineralization correlate with the production of LMEs (Field et al., 1992; Sack et al., 1997). Extracellular preparations of LiP from *P. chrysosporium* were among the first to be shown as capable of PAH oxidation (Haemmerli et al., 1986; Hammel et al., 1986; Bumpus, 1989). Furthermore, enzymatic mineralization of PAHs of crude MnP from MnP crude preparation of *Nematoloma forwardii* was descripted by Sack et al., 1997. Purified laccase from *T. versicolor* has been shown to oxidize a range of 3-5 ring PAHs (Collins et al., 1996; Majcherczyk et al., 1998). Despite apparent non-LME mediated PAHs mineralization by some white-rot fungi grown under non-ligninolytic conditions (e.g. by *P. chrysosporium*, Dhawale et al., 1992), there is substantial and conclusive evidence that ligninolytic enzymes are involved in PAHs mineralization

by white-rot fungi. A cytochrome P450 monooxygenase mediated PAHs transformation reaction is also thought to occur in certain white-rot fungi (Bezalel et al., 1997).

3. Research purposes

The white-rot fungus *P. sordida* YK-624, isolated from rotted wood, showed much higher ligninolytic activity and selectivity than *P. chrysosporium* or *T. versicolor*. In the present, *P. sordida* YK-624 was used for the degradation of recalcitrant environmental pollutions.

In chapter II, degradation of aflatoxin B_1 (AFB) and acetamiprid (ACE), which are contained in contaminated rices, was demonstrated. AFB is a potent mycotoxin with mutagenic, carcinogenic, teratogenic, hepatotoxic, and immunosuppressive properties. It is a frequent contaminant of many food products and one of the most potent naturally occurring mutagens and carcinogens known. In section 1, detoxification of AFB by MnP from the white-rot fungus *P. sordida* YK-624 was investigated. ACE belongs to the neonicotinoid class of systemic broad-spectrum insecticides, which are the most highly effective and largest-selling insecticides word-wide for crop protection. In section 2, we examined the elimination of ACE by *P. sordida* YK-624.

In chapter III, the effective removal of endocrine disrupting compounds (EDCs) by lignin peroxidase from *P. sordida* YK-624 was investigated. The major extracellular ligninolytic enzymes of this strain are MnP and LiP. Particularly, this strain produces 2 novel LiP (YK-LiP1 and YK-LiP2), and these enzymes can degrade lignin model compounds more effectively than LiP from *P. chrysosporium* (Pc-LiP H8) (Sugiura et al., 2003; Hirai et al., 2005). In this chapter, YK-LiP1 was applied to the removal of EDCs, and the removal properties were compared with Pc-LiP H8. Moreover, the structures of metabolites from bisphenol A (BPA) and *p*-*t*-octylphenol were determined to clarify the removal mechanism of EDCs by YK-LiP1.

In chapter Ⅳ, the metabolism of BPA by *P. sordida* YK-624 was investigated. BPA is one of the representative compounds of the endocrine disrupting compounds group and the highest volume chemicals produced worldwide. As a result, BPA is often detected in many soil and water environments. In section 1, we examined the removal of BPA by *P. sordida* YK-624 under ligninolytic condition. The metabolites from BPA were detected and a metabolic pathway for the metabolism of BPA by *P. sordida* YK-624 was proposed. In section 2, the removal of BPA by *P.*

sordida YK-624 under non-ligninolytic condition in which ligninolytic enzymes are not produced was investigated. And the metabolite from BPA was detected and the metabolic pathway of BPA by *P. sordida* YK-624 was proposed.

Chapter II

Detoxification of AFB and ACE by *P. sordida* YK-624 or ligninolytic enzymes

Section 1
Detoxification of AFB by MnP from the white-rot fungus *P. sordida* YK-624

1.1. Introduction

The human diet contains a wide variety of natural carcinogens that are present in foods as the result of contaminated raw materials or produced during the processing and/or cooking of foods. Aflatoxins, a group of potent mycotoxins with mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive properties, are of particular importance because of their adverse effects on animal and human health (Lewis et al., 2005). Aflatoxins are produced as secondary metabolites of fungal strains (*Aspergillus flavus*, *A. parasiticus* and *A. nomius*) that grow on a variety of food and feed commodities during growth, harvest, storage, and transportation of these products (Peltonen et al., 2001; Jiang et al., 2005). AFB, (Fig. 1), the most toxic aflatoxins, is of particular interest because it is a frequent contaminant of many food products and one of the most potent naturally occurring mutagens and carcinogens known (Teniola et al., 2005).

Recently, the degradation of AFB by fungal laccases has been reported (Alberts et al., 2009). However, no degradation product was detected, and the degradation mechanism was not clear.

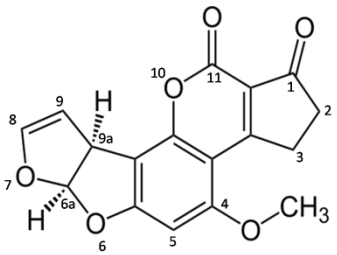


Fig. 1 Structure of AFB

The present study, the degradation of AFB by MnP from *P. sordida* YK-624 was demonstrated, and the degradation mechanism was discussed by the detection of these metabolites.

1.2. Materials and methods

1.2.1. Fungus

P. sordida YK-624 (ATCC 90872), was used in this study. The fungus was maintained on potato dextrose agar (PDA) slants at 4°C.

1.2.2. Chemicals

AFB was purchased from Wako Pure Chemical Industries, Japan. The umu test with umulac AT (Protein Purify Ltd. Japan) was used for the assay of mutagenic activity. All other chemicals were also extra-pure

grade, obtained from commercial sources, and used without further purification.

1.2.3. MnP preparation and determination of MnP activity

MnP from *P. sordida* YK-624 was prepared and purified with the methods described by Kondo et al. (Kondo et al., 1994).

MnP activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol to coerulignone (470=49.6 $mM^{-1}cm^{-1}$). The reaction mixture (1 mL) contained 2,6-dimethoxyphenol (1 mM), $MnSO_4$ (1 mM), and H_2O_2 (0.2 mM) in 50 mM malonate buffer (pH 4.5). One katal (kat) of MnP activity is defined as the amount of enzyme producing 1 mol of product per second.

1.2.4. MnP treatment of AFB

For reactions with MnP, 1 ml reaction mixture consisted of 5 nkat MnP, 10 μL of 1 mM AFB in 10% dimethylsulfoxide (DMSO), 1 mM $MnSO_4$, 0.1% Tween 80, 4 nkat glucose oxidase, and 2.5 mM glucose in 50 mM malonate buffer (pH 4.5). Reactions (n=3) were carried out at 30°C, 150 rpm, for 24 hours. For some experiments, the amount of MnP (1-20 nkat) and the reaction time (1-48 hours) were changed, and no Tween 80 was added to the reaction mixture to clarify the effect of Tween 80 on AFB degradation by MnP. The amount of AFB was determined by high-performance liquid chromatography (HPLC) analysis. The analytical conditions were as follows: column, Wakosil-II 5C18HG (4.6 mm x 150 mm, Wako Pure Chemical Industries, Japan); mobile phase, 40% MeOH aq.; flow rate, 0.5 mL min^{-1}; UV wavelength, 365 nm.

1.2.5. Mutagenic activity of AFB

The umu test with umulac AT was used for the assay of mutagenic activity of AFB. The test was performed by employing *Salmonella typhimurium* TA1535 and S9 liver homogenate. The TA1535 is the strain which was constructed by subcloning the bacterial *O*-acethyltransferase gene into a plasmid vector pACYC184 and inducing the plasmid into the original strain *S. typhimurium* TA1535/pSK1002 strain harbouring an *umuC*'–'*lacZ* fusion gene (Oda et al., 1995). In the assay system (n=3), 10 μL of test sample, 10 μL of S9mix (which is a metabolic activation system based on S9 liver homogenate), and 100 μL of bacterial culture were mixed, and incubated at 37°C for 2 hours. After incubation, 100 μL of X-Gal solution was added to each well, and incubated at 37°C for 1 hour. The reaction was stopped by the addition of SDS/DMSO solution, and the absorbance at 600 nm of the reaction mixture was measured by a

plate reader. Relative mutagenic activity (%) was defined as the percentage of β-galactosidase activity of enzyme-treated AFB compared with that of untreated AFB.

1.2.6. Metabolism experiment

AFB (final concentration; 160 μM) was incubated at 30°C for 48 hours in 100 mL reaction mixture consisted of 750 nkat MnP, 1 mM $MnSO_4$, 0.1% Tween 80, 600 nkat glucose oxidase, and 2.5 mM glucose in 50 mM malonate buffer (pH 4.5). The reaction mixture was extracted two times with 100 mL ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate, and then and evaporated to a state of dryness. The concentrate was applied to HPLC analysis to purify the metabolite from AFB with the method described above. The purified metabolite was analyzed by HR-ESI-MS and ^{1}H NMR. The HR-ESI-MS data were measured by a JMS-T100LC mass spectrometer. ^{1}H NMR spectra were recorded by a Jeol lambda-500 spectrometer at 500 MHz. Chemical shifts are expressed in relative to sodium 3-trimethylsilyl propionate as an external standard.

1.3. Results

1.3.1. Elimination of AFB by MnP from *P. sordida* YK-624

In our previous reports, the elimination of a wide range of aromatic compounds by ligninolytic enzymes from white-rot fungi were demonstrated (Tsutsumi et al., 2001; Suzuki et al., 2003; Hirai et al., 2005; Tamagawa et al. 2005; Tamagawa et al. 2006; Tamagawa et al. 2007; Mizuno et al. 2009). Therefore, MnP from *P. sordida* YK-624 was used to the degradation of difuranocoumarin derivates, AFB. The reaction mixture containing 5 nkat MnP decreased the level of AFB by 73.3% after a 24-h treatment (Fig. 2). Therefore, the degradation of AFB was examined by MnP in detail. Firstly, the effect of MnP activity on the degradation of AFB was determined. As shown in Fig. 2, 27.1% of AFB was degraded by the reaction mixture containing 1 nkat MnP, and the maximum degradation (73.3%) was observed in the reaction mixture containing 5 nkat MnP.

MnP has been reported to oxidize nonphenolic compounds in the presence of unsaturated fatty acid such as Tween 80 although the enzyme alone cannot oxidize such compounds (Bao et al., 1994). So, the effect of Tween 80 on the elimination of AFB by MnP was determined. The elimination of AFB was accelerated by the addition of Tween 80 (Fig. 2).

Figure 3 shows the time course for AFB elimination by MnP. AFB was drastically decreased after a 4-h treatment, and 86% of AFB was eliminated after a 48-h treatment.

1.3.2. Removal of mutagenic activity of AFB

The greatest concern for the biodegradation of environmental pollutions should be focused on the removal of its toxicity. Therefore, the mutagenic activity of AFB was measured by the umu test with a metabolic activation system S9mix because the toxicity of AFB is mediated by the cytochrome P450 enzyme in animal livers (Eaton and Gallagher, 1994). As shown in Fig. 4, AFB indicated much higher mutagenic activity than 2-aminoantracene (2-AA) such as a well-known mutagen. Figure 5 showed the change of mutagenic activity of AFB by MnP treatment. 49.4% of the mutagenic activity was removed by the treatment with 5 nkat MnP, and the addition of 20 nkat MnP was decreased by 69.2% of the mutagenic activity.

1.3.3. Identification of a metabolite from AFB by MnP treatment

To detect metabolites from AFB by MnP treatment, the concentrate of the reaction mixture was applied to HPLC analysis (Fig. 6). A metabolite was detected at a retention time of 10.5 min, while AFB1 was detected at a retention time of 32.8 min (Fig.6). The metabolite was fractionated and purified by HPLC, and analyzed by ^{1}H-NMR and HR-ESI-MS. The metabolite was measured by ^{1}H-NMR analysis in CD_3OD solution. In the ^{1}H-NMR spectrum, the C8 and C9 proton signals of the metabolite was clearly observed in the upper field, as compared AFB. The molecular formula was determined as $C_{17}H_{14}O_8$ by HR-ESI-MS m/z 345.0579 $[M-H]^-$ (calcd. for $C_{17}H_{13}O_8$, 345.06104), suggesting that the molecular mass was 346 (Fig. 7). The metabolite with a mass 34 was greater than the molecular ion of AFB. These results indicate that AFB was transformed to AFB-8,9-dihydrodiol.

1.4. Discussion

The extracellular ligninolytic enzymes produced by white-rot fungi are nonspecific and nonstereoselective enzymes that are capable of degrading lignin as well as a range of recalcitrant pollutants, and this capability of the enzymes gives them great application potential in the environment field (Asgher et al., 2008). In the present study, the degradation of AFB by MnP from *P. sordida* YK-624 was determined. MnP reaction system degraded approximately 70% of AFB after 24-h

treatment as shown in Fig. 2. AFB was also degraded by MnP without Tween 80 (Fig. 2). Mn(III), which is produced by MnP, cannot oxidize AFB directly since AFB doesn't possess phenolic hydroxyl group. Under the addition of Tween 80, lipid-derived peroxy radicals are produced in the reaction mixture containing MnP (Bao et al., 1994), and it is suggested that the peroxy radicals directly oxidize AFB. On the other hand, formate radical and superoxide anion radical are generated in the MnP reaction mixture without Tween 80 (Khindaria et al., 1994). It is thought that formate radical and superoxide anion radical are involved in the degradation of AFB by MnP without Tween 80.

In the AFB-degradation experiment by MnP, AFB-8,9-dihydrodiol was detected as a metabolite. The same metabolite has been detected in metabolism of AFB by some animal (Wu et al., 2009). It is reported that AFB-8,9-dihydrodiol is produced by the hydrolysis of AFB-8,9-epoxide in some animal, and that the AFB-8,9-epoxide is formed by the oxidation of the 8,9-vinyl bond by the microsomal cytochrome P450 system (Kuilman et al., 2000). The similar reactions (epoxidation of AFB and hydrolysis of AFB-8,9-epoxide) probably occur in AFB degradation by MnP reaction system. Fig. 8 shows the proposed mechanism of AFB-degradation by MnP. The 8,9-vinyl bond of AFB is oxidized by the peroxy radicals of Tween 80 and/or formate radical and superoxide anion radical, and then is spontaneously hydrolyzed to AFB-8,9-dihydrodiol.

The greatest concern for the biodegradation of environmental pollutions should be focused on the removal of its toxicity. In the present study, the degradation and detoxification of AFB by MnP were demonstrated (Figs. 2 and 5). Indeed, AFB-8,9-dihydrodiol is a lower toxic compound than AFB since AFB-8,9-dihydrodiol can rearrange, forming a reactive dialdehyde configuration that can bind to primary amine groups in proteins by Schiff base reactions (Fig. 9, Sabbioni et al. 1987), thus preventing the formation of DNA adducts which can lead to mutations. This is the first report that MnP can remove the mutagenic activity of AFB effectively, and that AFB is transformed to AFB-8,9-dihydrodiol. By using this system, the bioremediation of AFB-contaminated foods is possible.

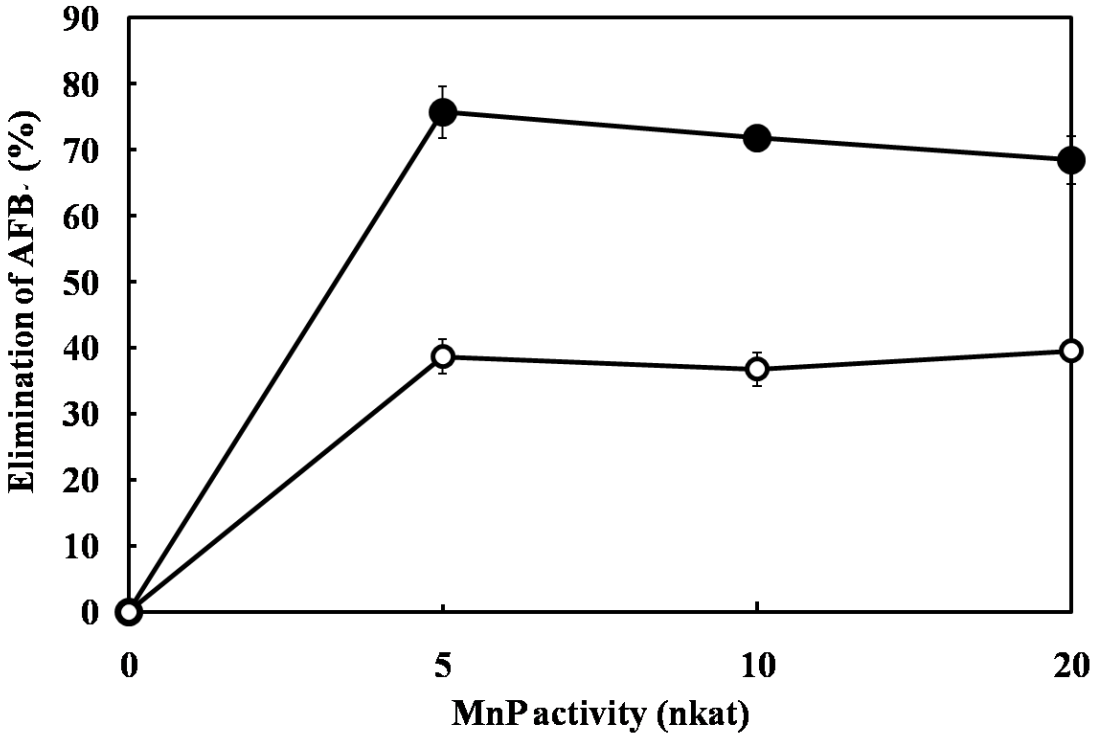


Fig. 2 Elimination of AFB in the presence of different activities of MnP. Closed circles, with Tween 80, open circles, without Tween 80. Values are means ± SD of triplicate samples.

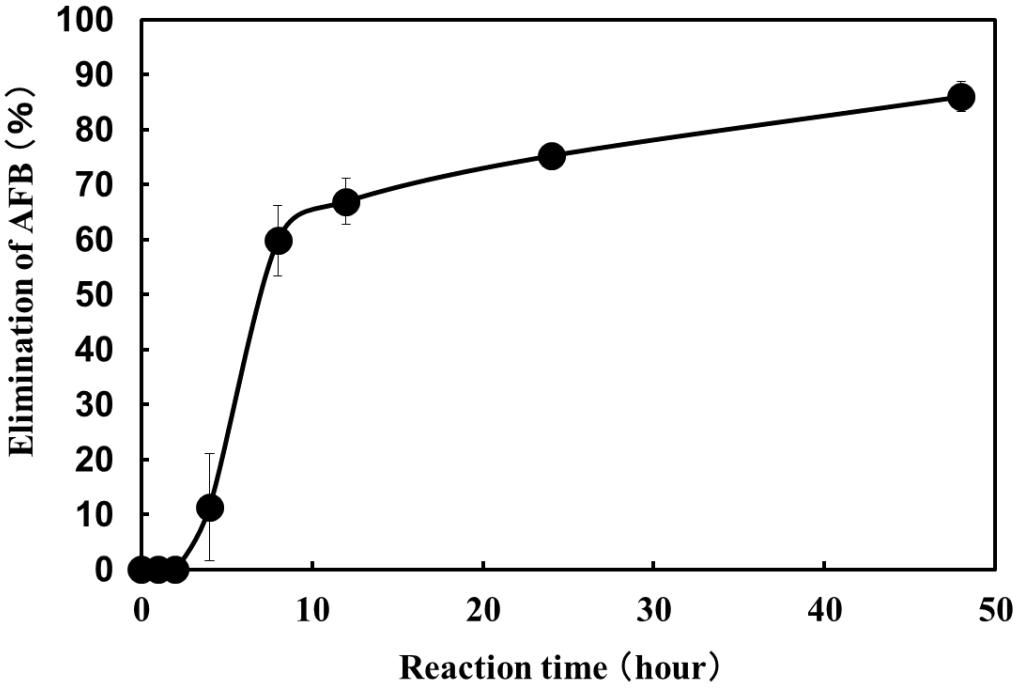


Fig. 3 Time course for AFB elimination by MnP. Values are means ± SD of triplicate samples.

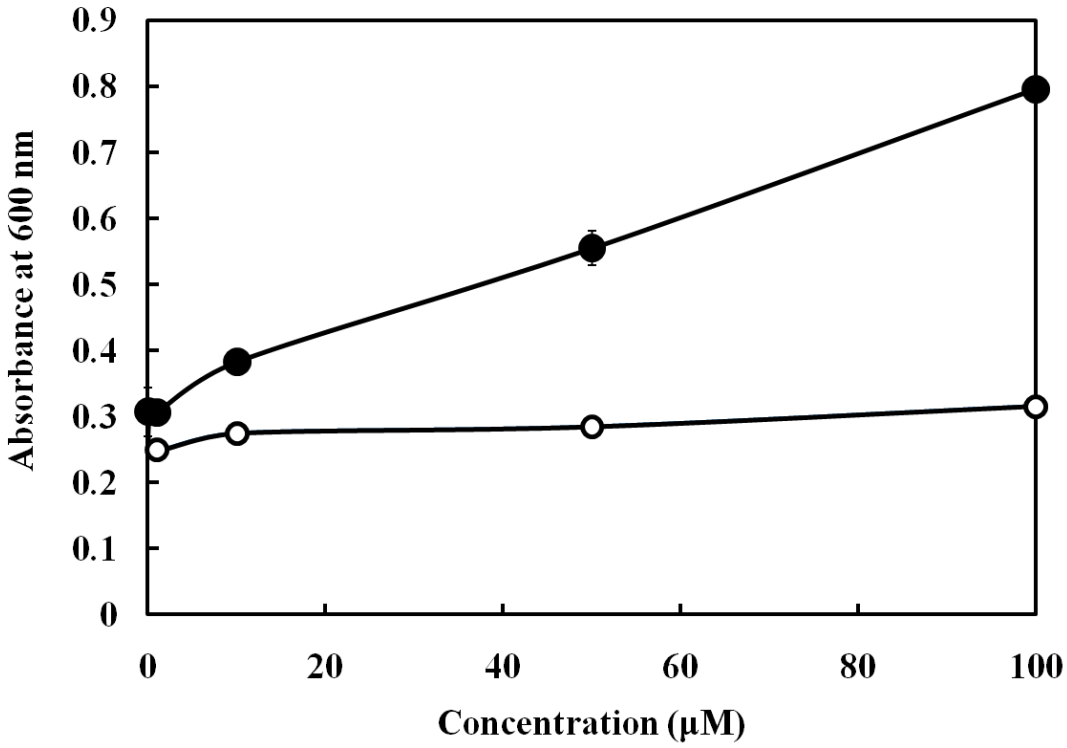


Fig. 4 Mutagenic activity of AFB in the umu test. Closed circles, AFB; open circles, 2-aminoanthracene. Values are means ± SD of triplicate samples.

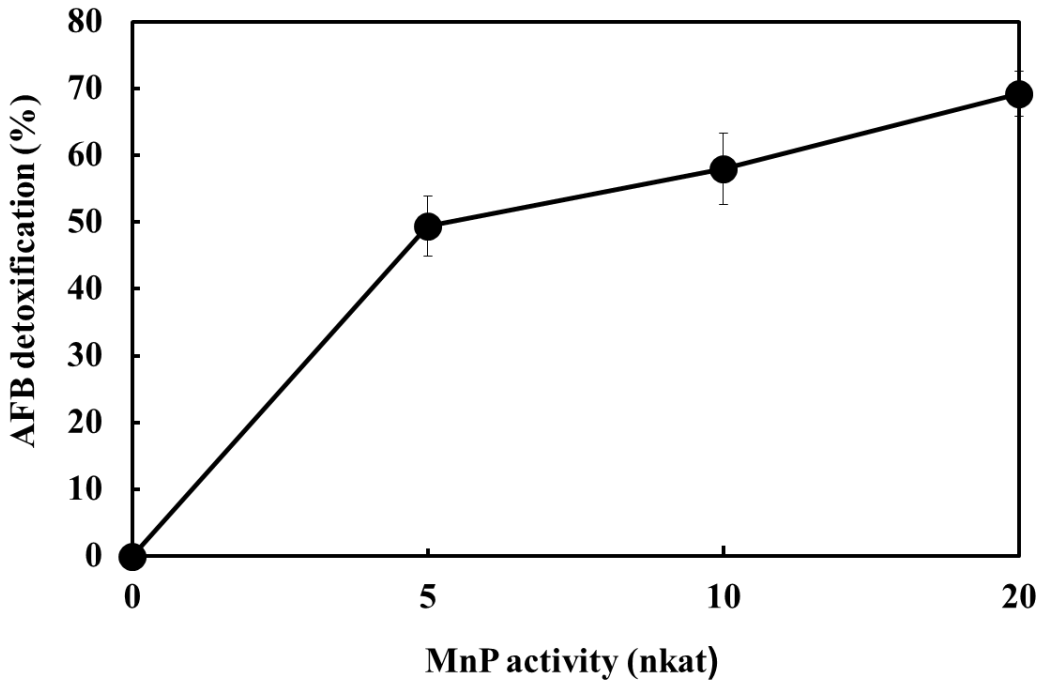


Fig. 5 MnP decrease the mutagenic activity of AFB. Values are means ± SD of triplicate samples.

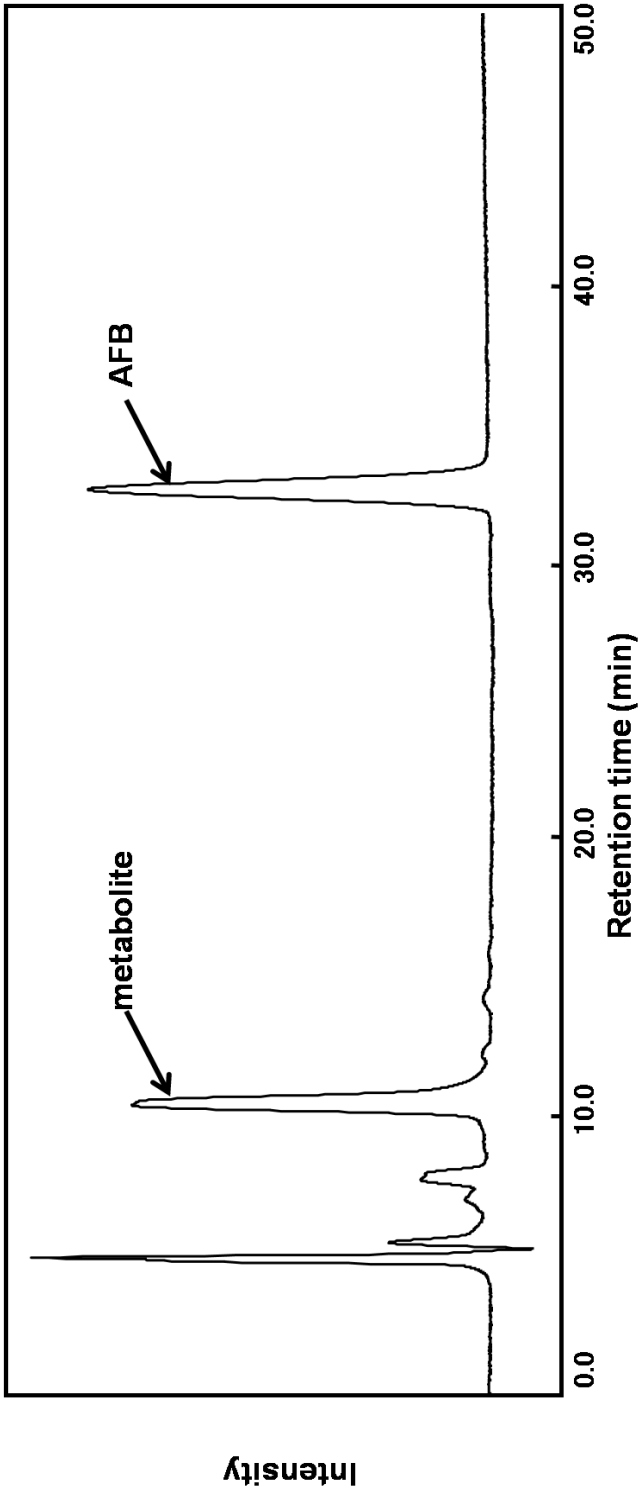


Fig. 6 Detection of the AFB metabolite by HPLC.

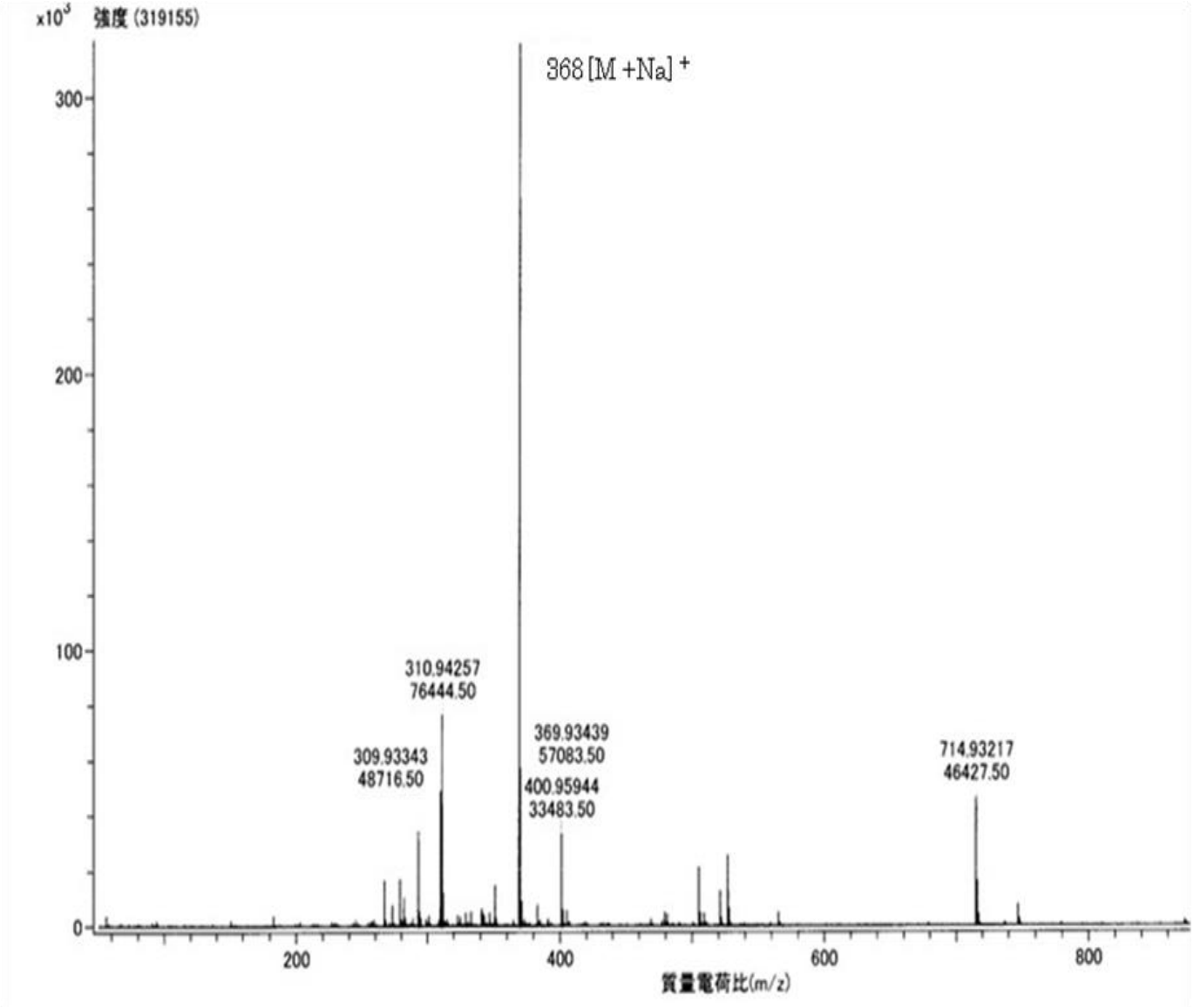


Fig. 7 ESI-MS spectrum of AFB metabolite.

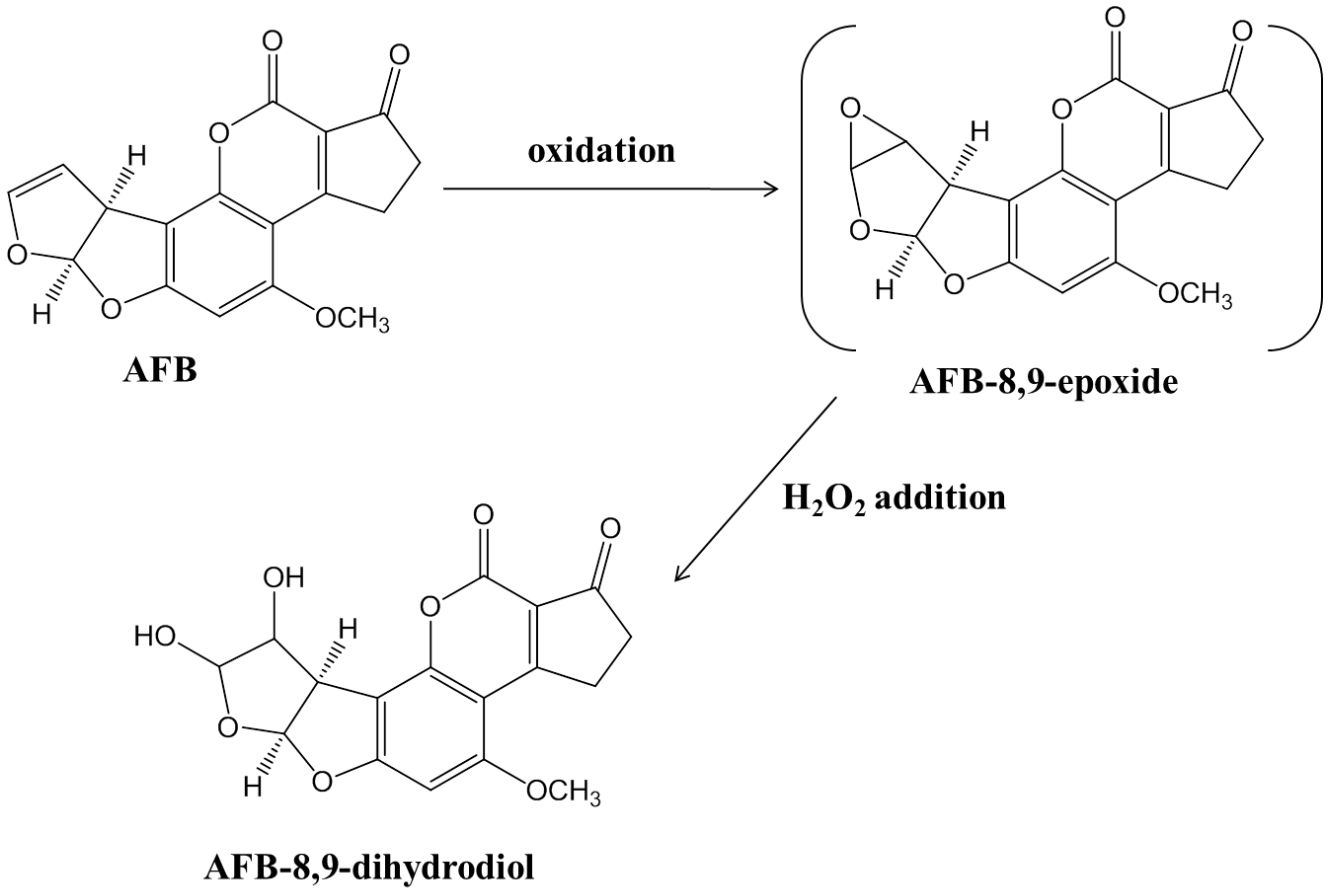


Fig. 8 Proposed mechanism of AFB oxidation by MnP.

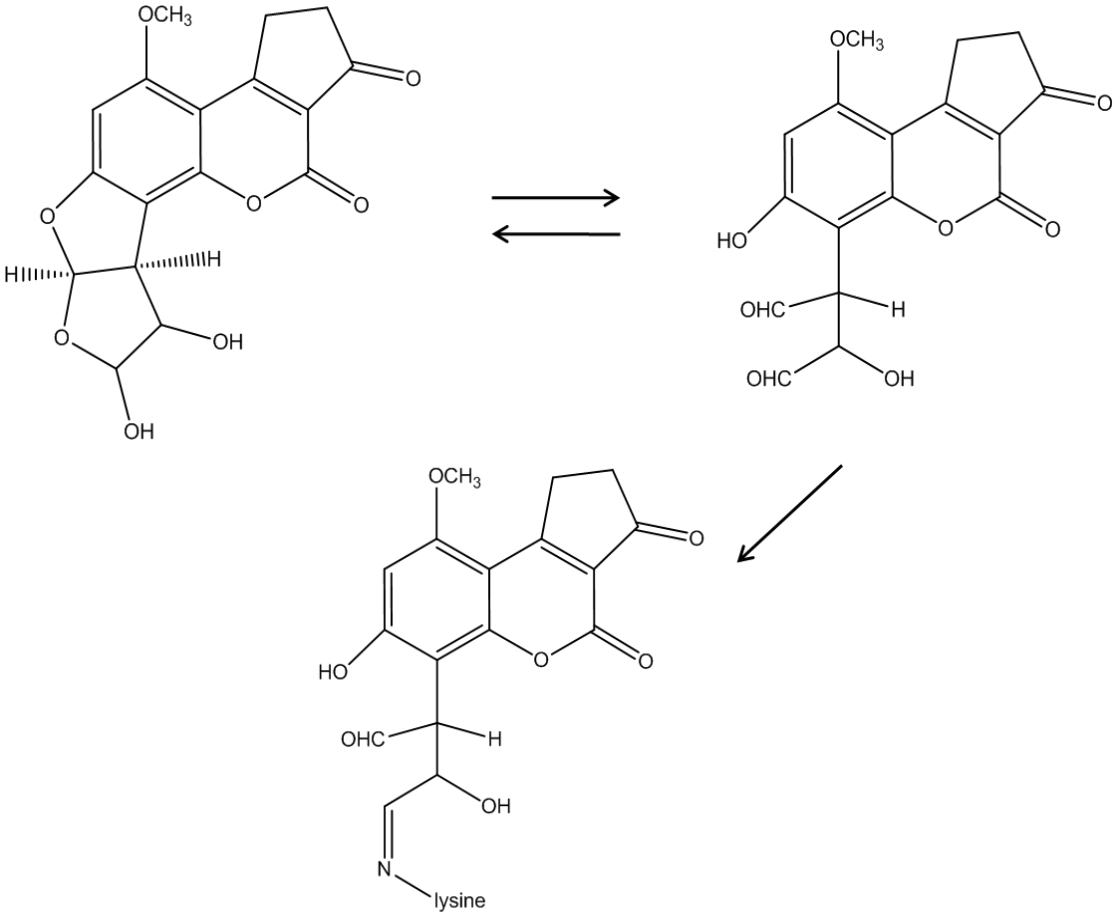


Fig. 9 Proposed pathway of lysine-AFB adduct formation.

Section 2
Biotransformation of ACE by the white-rot fungus *P. sordida* YK-624

2.1. Introduction

Neonicotinoid insecticides, which are one of the most important classes of commercial insecticides worldwide, are systemic in plants and animals and are used to manage crop pests and control fleas on cats and dogs (Arther et al., 1997; Jacobs et al., 1997). ACE, (Fig. 10) belongs to the class of chloronicotinyl neonicotinoid insecticides and is used to control Hemiptera, particularly aphids, Thysanoptera, and Lepidoptera, on a wide range of crop species (Mateu-Sanchez et al., 2003; Tokieda et al., 1997). In recent years, ACE residues in crops are receiving considerable attention due to their potential toxicity to humans (Pramanik et al., 2006; Sanyal et al., 2008), and methods for the biotransformation of neonicotinoids are being actively researched. A number of microorganisms that are capable of degrading neonicotinoids have been identified, including the yeast *Rhodotorula mucilaginosa* strain IM-2, which is able to hydrolyze ACE and thiacloprid, and the bacterium *Stenotrophomonas maltophilia* CGMCC 1.1788, which is able to hydroxylate imidacloprid and *N*-demethylate ACE (Chen et al., 2008;Dai et al., 2006, 2010).

Here, the elimination of ACE by *P. sordida* YK-624 was examined in order to develop a bioremediation system for ACE-contaminated foods. The metabolite from ACE was detected and a metabolic pathway for the metabolism of ACE by *P. sordida* YK-624 was proposed. This represents the first report describing the biotransformation of ACE by a white-rot fungus.

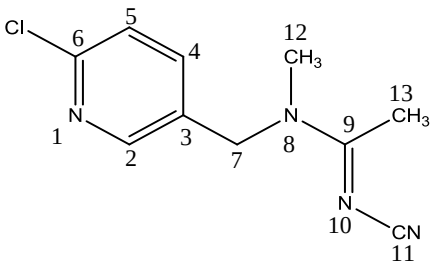


Fig. 10 Structure of ACE.

2.2. Materials and methods

2.2.1. Fungal strain and culture conditions

P. sordida YK-624 (ATCC 90872) was used in the present study. The fungus was maintained on PDA slants at 4°C.

2.2.2. Chemicals

ACE and piperonyl butoxide (PB) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were analytical pure grade and were used without further purification.

2.2.3. Fungal treatment of ACE

Nitrogen-limited (NL) Kirk medium described by Tien and Kirk (1988, Table 1) and potato dextrose broth (PDB) medium (4% potato starch, 2% dextrose, pH 4.5) were used for ACE elimination experiments. The fungus was incubated on a PDA plate at 30°C for 3 days, and then 10-mm-diameter disks were punched out from the growing edge of the mycelium. Two disks were each placed into a 100-mL Erlenmeyer flask containing 10 mL of liquid medium (NL or PDB medium, pH 4.5). After statically incubating the flasks at 30°C for 7 days, 100 μL of 1 mM ACE (final concentration, 10 μM) was added to the cultures, which were then further incubated for 5, 10, 15, and 20 days (each in triplicate). The culture was filtrated with a 0.2-μm membrane filter, and the filtrate was then subjected to HPLC for the quantification of ACE under the following conditions: column, Wakosil-II 5C18HG (4.6×150 mm); mobile phase, 30% aqueous MeOH; flow rate, 0.5 mL min^{-1}; and UV wavelength, 246 nm.

2.2.4. Metabolite identification

Inoculated cultures (5 L NL medium) of *P. sordida* YK-624 were prepared under the conditions described above. After static incubation at 30°C for 7 days, 5 mL of 100 mM ACE (final concentration, 100 μM) was added to these cultures. The cultures were further incubated for 15 days and then filtrated with a 0.2-μm membrane filter. Distilled water (100 mL) was added to the filtrate after evaporation to dryness, and the resulting solution was extracted twice with 100 mL EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was cleaned on a silica gel flash column chromatography (silica gel 60 N, φ40×600 mm) and eluted with dichloromethane/EtOAc/MeOH (10/0/0, 9/1/0, 8/2/0, 7/3/0, 5/5/0, 3/7/0, 0/10/0, 0/9/1, 0/8/2, 0/7/3, 0/5/5, and 0/0/10; v/v/v) to obtain 14 fractions. Each fraction was analyzed by thin-layer chromatography (TLC), HPLC, high-resolution electrospray ionization mass spectra (HR-ESI-MS), and ^{1}H nuclear magnetic resonance (NMR). Silica gel plates (Merck F254; Merck, Darmstadt, Germany) and silica gel 60N (Merck 100–200 mesh; Merck) were used for analytical TLC and flash column chromatography. The metabolite was further separated by HPLC (column, Develosil

C30-UG-5; Nomura Chemistry, Seto, Japan) using 70% MeOH. The purified metabolite was analyzed by HR-ESI-MS and NMR, including correlation spectroscopy, heteronuclear multiple quantum coherence, and heteronuclear multiple-bond connectivity (HMBC) spectroscopies. The HR-ESI-MS data were measured using a JMS-T100LC mass spectrometer. ^{1}H NMR spectra were recorded using a Jeol Lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz.

2.2.5. Cytochrome P450 inhibitor experiment

After preculturing *P. sordida* YK-624 for 5 days, ACE (final concentration, 10 μM) and the cytochrome P450 inhibitor PB (final concentration, 0, 0.01, and 0.1 mM) were added to cultures. The cultures were further incubated for 5, 10, and 15 days, and each culture was then subjected to HPLC for the quantification of ACE, as described above (column, Wakosil-II 5C18HG; mobile phase, 30% aqueous MeOH; flow rate, 0.5 mL min^{-1}; and UV wavelength, 246 nm). All experiments were performed in triplicate.

2.3. Results

2.3.1. Elimination of ACE by *P. sordida* YK-624

In the present study, NL and PDB media were used for the elimination experiment of ACE. When *P. sordida* YK-624 was cultured in the NL medium, ACE was reduced by approximately 45% after 20 days of incubation (Fig. 11). In the PDB medium, the eliminated concentration of ACE was approximately 28% after 20 days of incubation. Moreover, purified LiP and MnP from *P. sordida* YK-624 could not degrade ACE (data not shown). These results suggested that *P. sordida* YK-624 had a higher ability for the elimination of ACE in the NL medium and that ligninolytic enzymes (LiP and MnP) were not involved in the elimination of ACE, as ACE was also eliminated under the non-ligninolytic conditions provided by the PDB medium.

2.3.2. Identification of the metabolite from ACE

As shown in Fig. 12, the main metabolite was detected in the HPLC analysis of a 10-day NL culture fluid inoculated with *P. sordida* YK-624, and minor metabolites were hardly detected. To determine the structure of the metabolite produced during the degradation of ACE, 15-day cultures of *P. sordida* YK-624 in NL medium supplemented with 100 μM ACE were subjected to TLC and HPLC. The purified metabolite was then

subjected to HR-ESI-MS analysis, which yielded a molecular ion at m/z 231.0428 $[M+Na]^+$ (calculated for $C_9H_9ClN_4Na$, 231.0413), indicating that the molecular formula of this compound was $C_9H_9ClN_4$. This formula suggested that the metabolite might be a demethylated form of ACE. The structure of the purified metabolite was further characterized by NMR analyses (Figs. 13-15). Table 2 lists the chemical-shift assignment data for the metabolite of ACE. HMBC correlations (Fig. 15) (H-7/C-2, H-7/C-3, H-7/C-4; H-7/C-9, H-13/C-9) confirmed that the metabolite was *N*-demethylated ACE, (*E*)-N^1-[(6-chloro-3-pyridyl)-methyl]-N^2-cyano-acetamidine, which is commonly known as IM 2-1 (Fig. 16).

2.3.3. Effect of cytochrome P450 inhibitors

The effect of cytochrome P450 inhibitors on the elimination of ACE by *P. sordida* YK-624 was investigated using PB. The elimination rate of ACE after the addition of 0.01 and 0.1 mM PB into cultures of *P. sordida* YK-624 is shown in Fig. 17. In contrast to the elimination rate of ACE in cultures without PB, markedly lower elimination activity of ACE was observed in cultures containing PB. In the presence of 0.01 and 0.1 mM PB, only 16% and 7% of ACE were eliminated after 15 days of incubation, respectively, compared to 45% in cultures lacking PB.

2.4. Discussion

White-rot fungi are capable of degrading a wide variety of recalcitrant aromatic compounds, including polymeric lignin and environmentally persistent pollutants. However, prior to the present study, the degradation of neonicotinoid insecticides by white-rot fungi had not been reported. Here, the elimination of ACE from liquid cultures of the white-rot fungus *P. sordida* YK-624 was demonstrated. Under ligninolytic and non-ligninolytic conditions, 45% and 30% of ACE, respectively, were eliminated after 15 days of incubation (Fig. 11). Although the fungal growth in the PDB media is much faster than in the NL media, the elimination rate of ACE in the PDB media was almost the same or lower than in the NL medium. Moreover, no decrease was observed in the PDB medium containing 10 μM ACE without fungal inoculation for 20 days. These results suggest that ACE was biotransformed to other compounds by the fungal treatment, not by abiotic elimination or adsorption on biomass.

The generation of the ACE metabolite IM 2-1 by *P. sordida* YK-624 was detected (Fig. 12). The metabolic pathway of ACE has been studied

in honeybee, mice, spinach, and soil bacteria (Tokieda et al., 1999; Brunet et al., 2005; Ford and Casida, 2006, 2008). In these studies, the main metabolic pathway involved the *N*-demethylation of ACE to yield IM 2-1. In honeybee, the nitromethylene bond of IM 2-1 might be subsequently oxidized to yield 6-chloronicotinic acid (Brunet et al., 2005). In addition to the metabolite IM 2-1, other compounds were also detected by HPLC and TLC analyses of *P. sordida* YK-624 culture supernatant. However, the structures of these additional metabolites could not be determined due to their low concentrations.

The lipophilic *N*-methyl group of neonicotinoids plays an important role in the bioefficacy of these insecticides, with the loss of this group reducing the insecticidal activity by more than tenfold or even leading to complete inactivation (Chen et al., 2008). Although ACE is reported to have a relatively high toxicity in the honeybee, with an LD_{50} value of 7.1 μg/bee, the metabolite IM 2-1 resulted in no mortality at 50 μg/bee (Iwasa et al., 2004). Since the metabolite IM 2-1 has lower toxicity than ACE, the detoxification of ACE is possible using *P. sordida* YK-624.

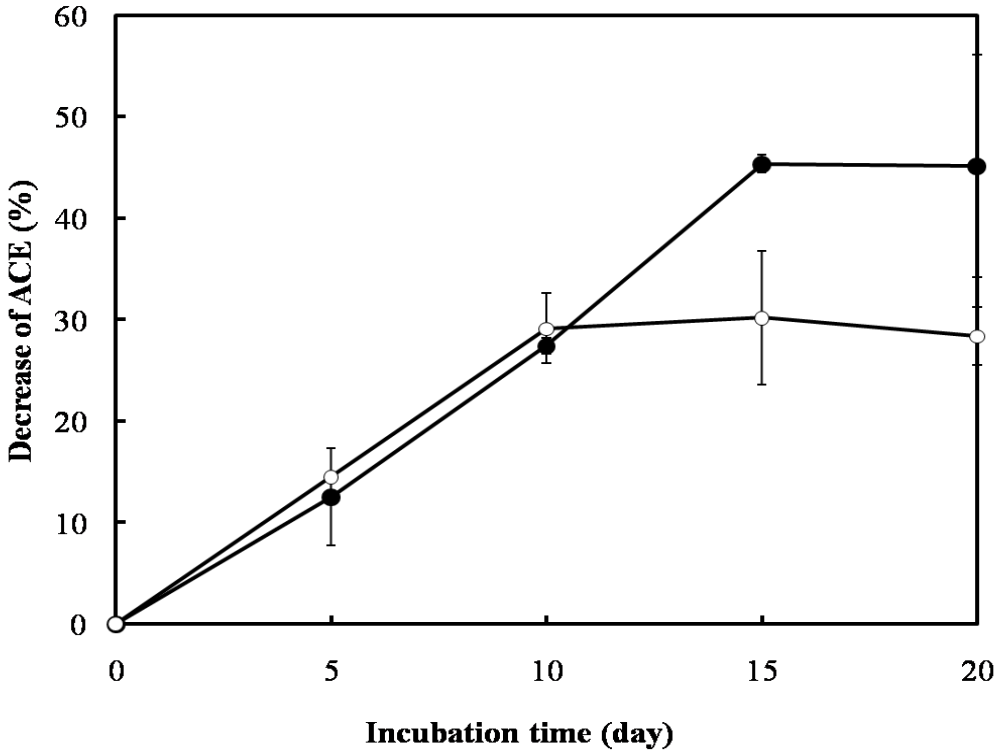


Fig. 11 Time course for ACE elimination by *P. sordida* YK-624. Closed circles, NL medium; open circles, PDB medium. Values are the means ± SD of triplicate samples

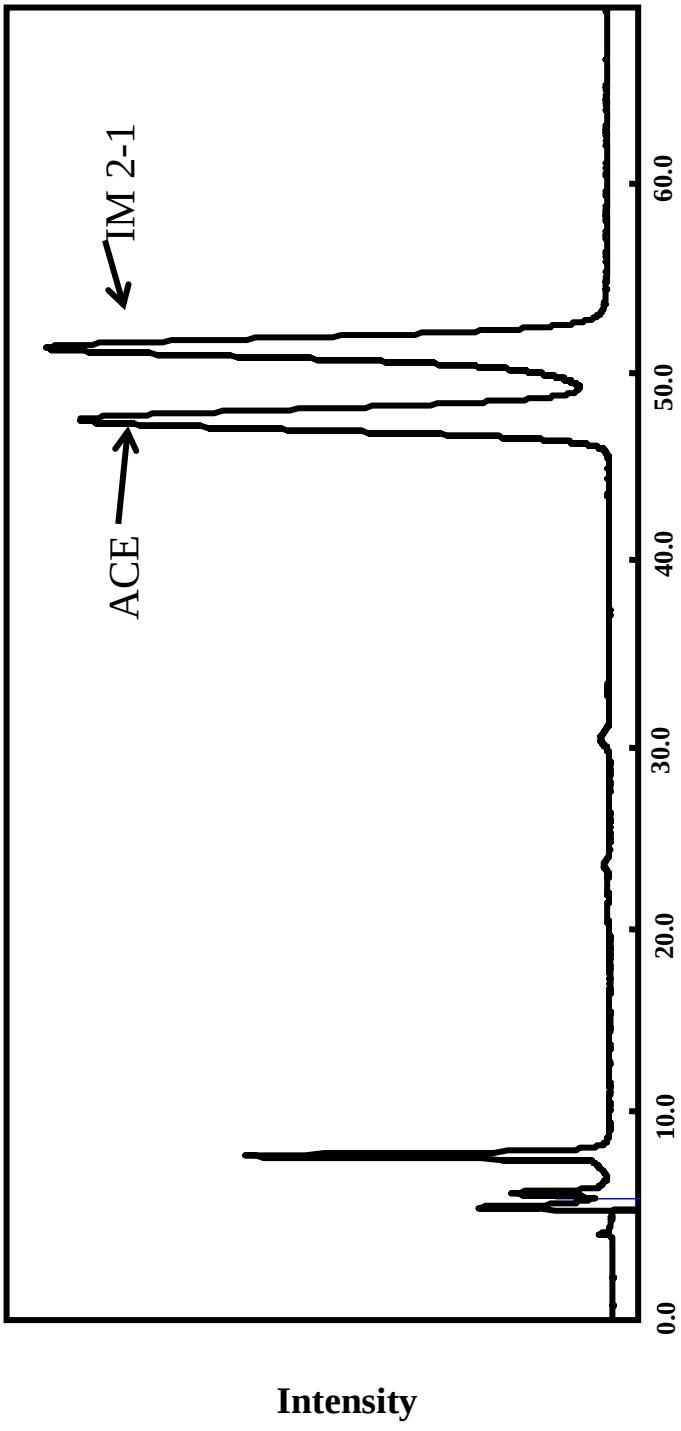


Fig. 12 Detection of the ACE metabolite in the 10-day NL culture fluid by HPLC.

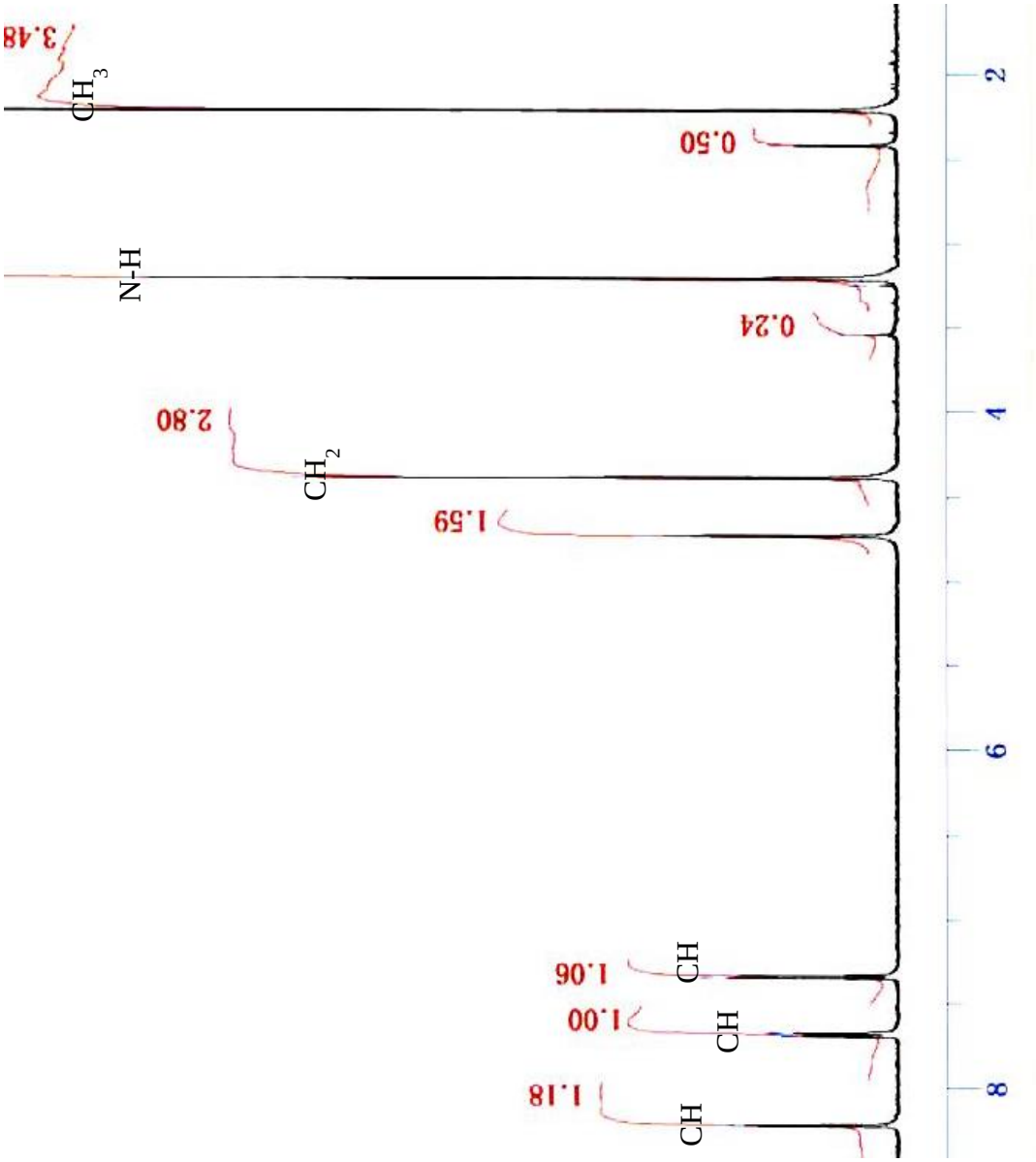


Fig. 13 ^{1}H NMR spectrum of the ACE metabolite

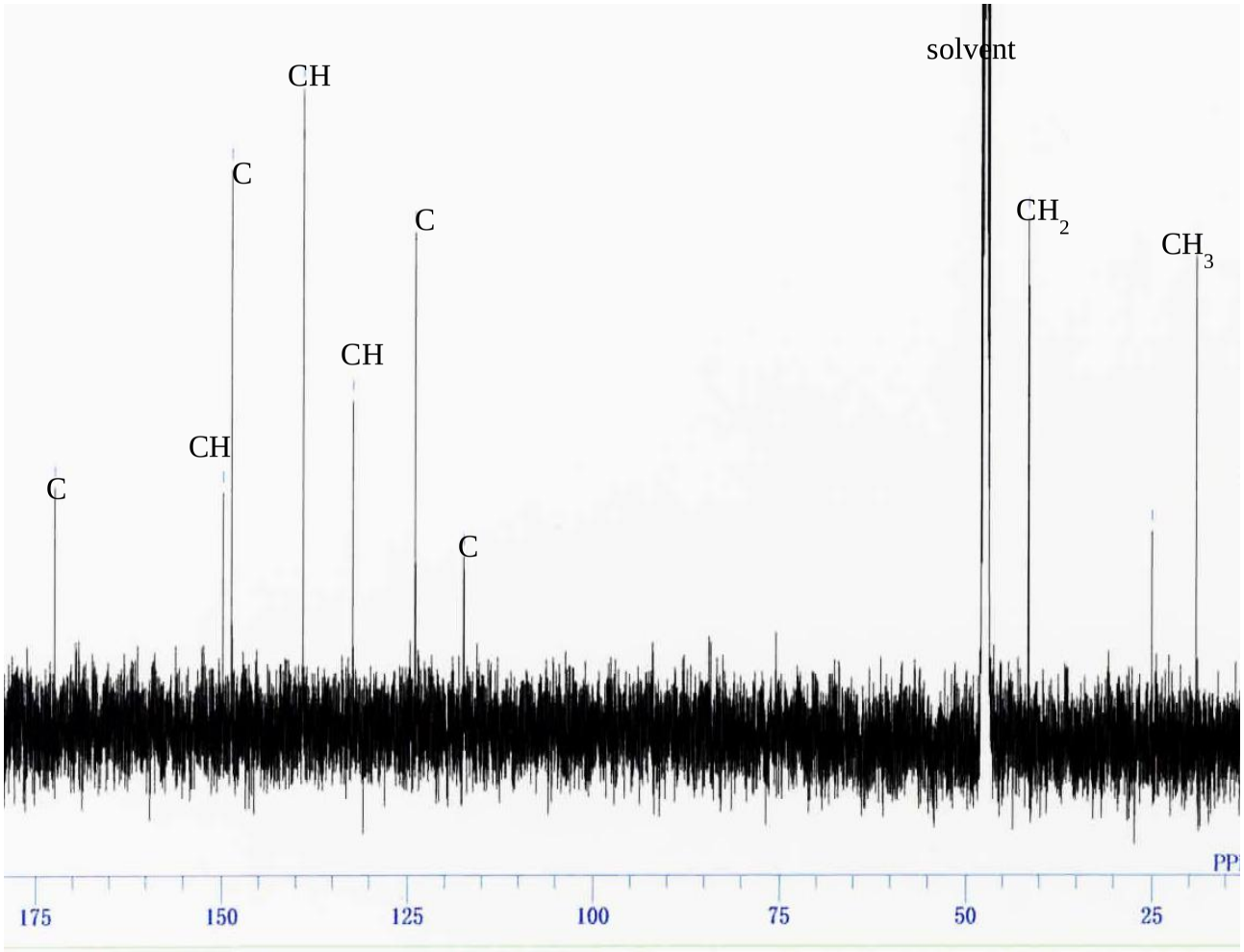


Fig. 14 ^{13}C NMR spectrum of the ACE metabolite (CD_3OD).

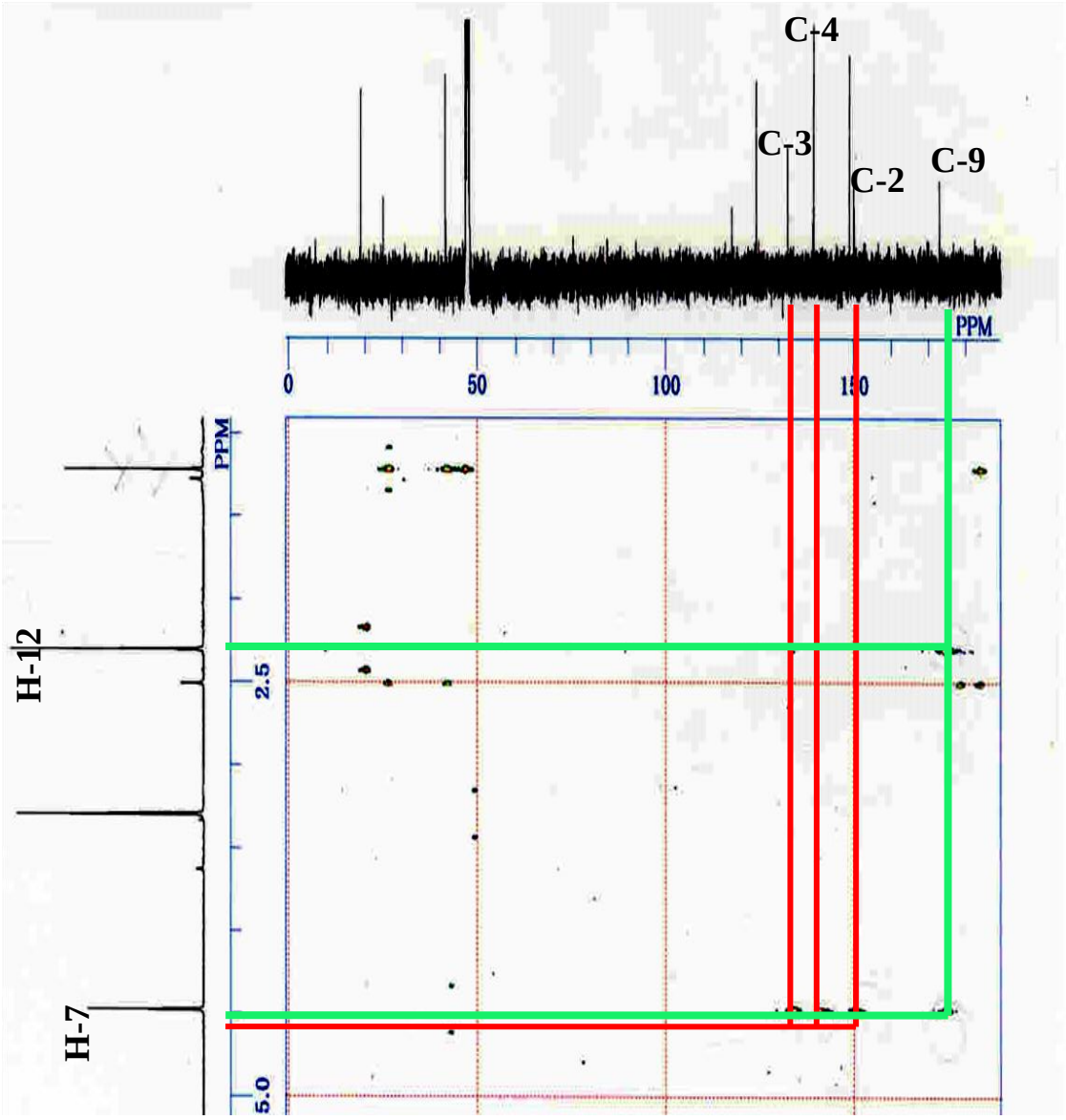


Fig. 15 HMBC spectrum of the ACE metabolite (CD_3OD).

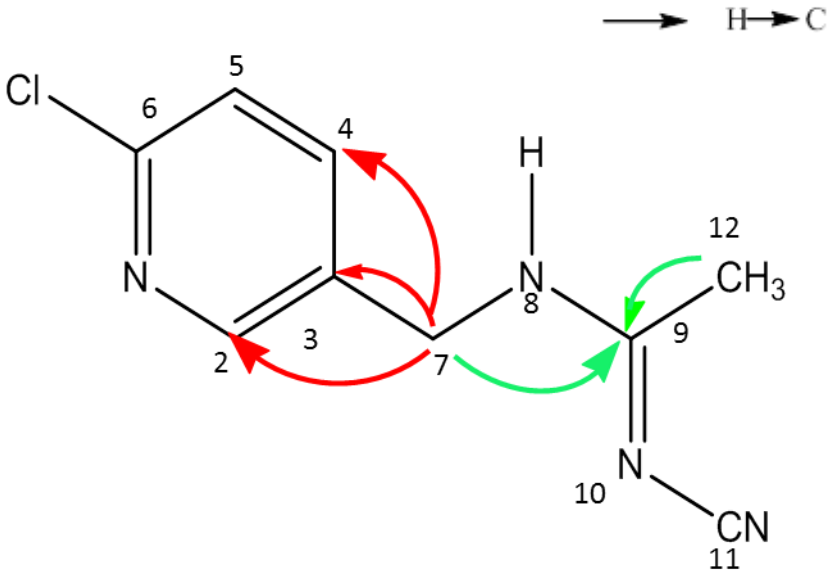


Fig. 16 HMBC correlations of the identified ACE metabolite.

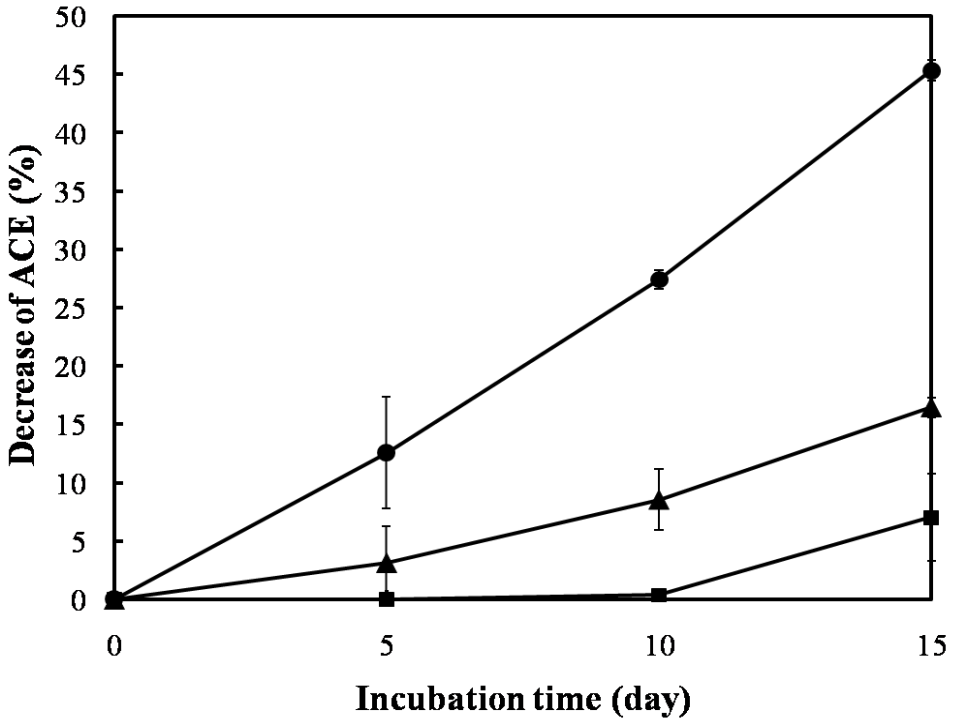


Fig. 17 Effect of the cytochrome P450 inhibitor PB on the elimination of ACE by *P. sordida* YK-624 in NL medium. Circles, without PB; triangles, 0.01 mM PB; squares, 0.1 mM PB. Values are the means ± SD of triplicate samples

Table 1 The composition of Kirk medium.

Kirk medium（for 1L、pH 4.5）	
glucose	10 g
Ammonium tartrate	0.221 g
2,2-dimethylsuccinic acid	2.92 g
Kirk salts solution	100 ml

Kirk salts solution (for 1L、pH 4.5)	
KH_2PO_4	20 g
$MgSO_4 \cdot 7H_2O$	5 g
$CaCl_2 \cdot 2H_2O$	1.3 g
Thiamine · HCl	10 mg
Kirk trace elements solution	16.7 ml

Kirk trace elements solution (for 1L、pH 4.5)	
Nitrilotriace	9 g
$MgSO_4 \cdot 7H_2O$	3 g
$MnSO_4 \cdot H_2O$	4.2 g
NaCl	6 g
$FeSO_4 \cdot 7H_2O$	0.6 g
$CoSO_4 \cdot 7H_2O$	1.1 g
$ZnSO_4 \cdot 7H_2O$	1.1 g
$CaCl_2 \cdot 2H_2O$	0.6 g
$CuSO_4 \cdot 5H_2O$	60 mg
$AlK(SO_4)_2 \cdot 12H_2O$	0.11 g
H_3BO_3	60 mg
$Na_2MoO_4 \cdot 2H_2O$	70 mg

Table 2 NMR data for the metabolite of ACE in CD_3OD.

Position	^1H δ_H (mult, *J* in Hz)	^{13}C δ_C
2	8.22 (d 2.6)	149.9
3	-	124.0
4	7.69 (dd 8.2, 2.6)	139.2
5	7.34 (d 8.2)	132.4
6	-	148.7
7	4.39 (s)	41.6
9	-	172.5
11	-	117.5
12	2.21 (s)	19.1

Chapter III

Effective removal of ECDs by LiP from *P. sordida* YK-624

1. Introduction

Considerable concern has recently been expressed over the possibility that some man-made chemicals mimic the effects of hormones and may adversely affect reproduction in wildlife and humans (Colborn et al., 1993). Certain chemicals have been known to affect the reproductive systems of various animals including human beings, and the compounds mimicking or interfering with the action of endogenous gonadal steroid hormones have been named EDCs (Colborn et al., 1994). The occurrence of EDCs in the aquatic environment has generated worldwide interest because these chemicals can cause feminization of fish as well as interfere with the reproduction and development of other aquatic organisms (Purdom et al. 1994; Harries et al. 1996; Larsson et al. 1999).

Various natural and synthetic chemical compounds have been identified as EDCs; including pharmaceuticals, pesticides, industrial chemicals, and heavy metals (Giesy et al., 2002). The elimination of ECDs by white-rot fungi or their LMEs was summarized by Cabana et al., 2007. The alkylphenolic EDCs nonylphenol, bisphenol A and triclosan, the phthalic acid esters dibutylphthalate, diethylphthalate and di-(2-ethylhexyl)phthalate, the natural estrogens estrone, 17β-estradiol (E_2), estriol and 17α-ethynylestradiol and the phytoestrogens genistein and β-sitosterol have been shown to be eliminated by several fungi and LMEs. White-rot fungi have manifested a highly efficient removal of EDCs in aqueous media and soil matrices using both LME and non LME-systems. The ligninolytic system of white-rot fungi could also be used for the elimination of several EDCs and the associated hormone-mimicking activity.

Typical EDCs of anthropogenic origin with estrogen-like action include *p*-*t*-octylphenol (OP) and bisphenol A (BPA). Natural estrogens, i.e., E_2 and estrone (E_1), and synthetic estrogen, i.e., ethynylestradiol (EE_2) (Fig. 18), are excreted into wastewater by humans and mammals mainly through their urine. The effluent concentrations of estrogens typically range from a few ng L^{-1} to a few tens of ng L^{-1} (Johnson and Sumpter, 2001; Khanal et al., 2006), but even these amounts are often high enough to cause endocrine-disrupting effects in some aquatic species such as trouts (Thorpe et al., 2001) and minnows (Panter et al., 2000). Estrogenic activities of estrogens are two or three orders of magnitude higher than those of EDCs such as BPA (Routledge and Sumpter, 1996; Tanaka et al., 2001).

In recent years, ligninolytic enzymes such as MnP and laccase were shown to be effective in removing the estrogenic activities of BPA, nonylphenol (Tsutsumi et al., 2001), 4-tert-octylphenol (Tamagawa et al., 2007), and steroidal hormones (Suzuki et al., 2003; Tamagawa et al., 2006). However, the detail mechanisms on the detoxification of these compounds are still unknown.

The major extracellular ligninolytic enzymes of *P. sordida* are MnP (Hirai et al., 1994) and LiP (Machii et al., 2004). Particularly, this strain produces 2 novel LiP (YK-LiP1 and YK-LiP2), and these enzymes degrade lignin model compounds more effectively than LiP from *P. chrysosporium* (Pc-LiP H8) (Sugiura et al., 2003; Hirai et al., 2005). In the present study, YK-LiP1 was applied to the removal of EDCs, and the removal properties were compaed with Pc-LiP H8. Moreover, the structures of metabolites from BPA and OP were determined to clarify the removal mechanism of EDCs by YK-LiP1.

2. Materials and methods

2.1. Fungus

P. sordida YK-624 (ATCC 90872) and *P. chrysosporium* ME446 were used in this study. These fungi were maintained on PDA slants at 4°C.

2.2. Chemicals

BPA, E_1, E_2 and EE_2 were purchased from Tokyo Chemical Industry, Tokyo, Japan. OP was obtained from Wako Pure Chemical Industries, Osaka, Japan. All other chemicals were extra-pure grade and were used without further purification.

2.3. LiP preparation and determination of LiP activity

YK-LiP1 from *P. sordida* YK-624 and Pc-LiP (isozyme H8) from *P. chrysosporium* were prepared and purified as described by Sugiura et al. (2003) and Wariishi and Gold (1990), respectively. LiP activity was measured by monitoring the oxidation of veratryl alcohol (VA) to veratraldehyde ($\varepsilon 310=9.3\ mM^{-1}cm^{-1}$). The reaction mixture (1 ml) contained VA (1 mM) and H_2O_2 (0.2 mM) in 20 mM succinate buffer (pH 3.0). One katal (kat) was defined as the amount of enzyme producing 1 mol of product per second.

2.4. LiP treatment of EDCs

LiP reactions were performed in 1 ml of reaction mixture containing 2 nkat each LiP, 100 μM EDCs, and 100 μM H_2O_2 in 20 mM

succinate, pH 3.0. Reactions were performed in triplicate for 24 h at 30°C and mixing at 150 rpm. The amount of EDCs was determined by HPLC under the following conditions: column, Wakosil-II 5C18HG (4.6 mm x 150 mm); mobile phase, 50% aqueous acetonitrile containing 0.1% acetic acid (E_1, E_2, EE_2, and BPA) or 80% aqueous methanol containing 0.1% acetic acid (OP); flow rate, 1.0 ml/min; detection wavelength, 275 nm (BPA) , 277 nm (OP), or 285 nm (E_1, E_2, and EE_2).

2.5. Estrogenic activity of E_1, E_2, or EE_2 treated with LiP

The estrogenic activities of E_1, E_2, or EE_2 before and after LiP treatment were evaluated by an in vitro screening test for chemicals with hormonal activities that used the yeast two-hybrid estrogenic assay system, developed by Nishikawa et al. (1999). The concentrations of E_1, E_2, and EE_2 before enzymatic treatment were 1 μM in the assay system (2.5 μl of reaction mixture containing 100 μM E_1, E_2, or EE_2 added to 50 μl of yeast culture and 200 μl of SD medium). Relative estrogenic activity (%) was defined as the percentage of β-galactosidase activity of enzyme-treated E_1, E_2, or EE_2 compared to that of untreated E_1, E_2, or EE_2.

2.6. Metabolism experiments

OP or BPA (final concentration 100 μM) were incubated at 30°C for 24 h in a 100-mL reaction mixture containing 200 nkat YK-LiP1 and 200 μM H_2O_2 in 20 mM succinic acid buffer, pH 3.0. The reaction mixtures were extracted 3 times with equal volume of EtOAc. The EtOAc extract were dried over anhydrous sodium sulfate and then evaporated to dryness. The concentrates were analyzed by TLC, HPLC, HR-ESI-MS and NMR. Silica gel plates (Merck F254, Merck, Darmstadt, Germany) was used for analytical TLC. The metabolite of OP or BPA was further separated by HPLC (column: Wakosil-II 5C18HG) by 80% aqueous methanol containing 0.1% acetic acid or 70% aqueous methanol containing 0.1% acetic acid, respectively. The purified metabolites were then analyzed by HR-ESI-MS and NMR including COSY, HMQC, and HMBC experiments. The HR-ESI-MS data were measured by a JMS-T100LC mass spectrometer. ^{1}H NMR spectra were recorded by a Jeol lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz.

3. Results

3.1. Degradation of EDCs by YK-LiP1 from *P. sordida* YK-624

YK-LiP1 from *P. sordida* YK-624 can degrade dimeric lignin model compounds more effectively than Pc-LiP (Sugiura et al., 2003; Hirai et al., 2005). Therefore, YK-LiP1 was applied to the removal of EDCs in the present study.

After a 24 h reaction using 2 nkat each LiP, the elimination of EDCs was determined (Fig. 19). YK-LiP1 effectively removed OP, BPA, E_1, E_2, and EE_2. Particularly, OP, BPA, E_2, and EE_2 were disappeared almost completely in the reaction mixture containing YK-LiP1 whereas Pc-LiP removed OP, BPA, E_1, E_2, and EE_2 by 46.1%, 52.5%, 23.9%, 38.2%, and 45.0%, respectively.

3.2. Removal of estrogenic activity of E_1, E_2, and EE_2

Because removal of toxicity is essential for the biodegradation of environmental pollutants, the estrogenic activities of E_1, E_2, and EE_2 treated with each LiP were examined since these EDCs shows much higher estrogenic activities that OP or BPA (Tsutsumi et al., 2001; Suzuki et al., 2003; Tamagawa et al., 2007). Although the estrogenic activity of E_1 treated with YK-LiP1 was hardly decreased, treatments of E_2 and EE_2 by YK-LiP1 reduced the estrogenic activities by 72.6% and 82.6%, respectively (Fig. 20). On the other hands, estrogenic activities of E_1, E_2, and EE_2 treated with Pc-LiP were not reduced.

3.3. Identification of the metabolites from OP and BPA

The metabolites were detected in the analysis of TLC and HPLC (data not shown). The HR-ESI-MS data for the metabolite of OP, which yielded a molecular ion at *m/z* 409.3084 $[M-H]^-$ (calculated for $C_{28}H_{41}O_2$, 409.3061), indicated that the molecular formula of this compound was $C_{28}H_{42}O_2$ (Fig. 21). This formula suggested that the metabolite might be a dimer of OP. The structure of the purified metabolite was further characterized by NMR analyses (Figs. 22-27, Table 3). The ^{13}C NMR and ^1H NMR spectra indicated that the metabolite of OP had four carbon atoms, one methylene, two methyls and 1,2,4 substituted benzene (Figs. 22, 23). The 1,1’,3,3’-tetramethylbutyl moiety was indicated by HMBC correlations (Fig. 27) (H-4,4’, H-2,2’/C-3,3’, H-1’-CH_3, H2,2’/C-1,1’). In addition, HMBC correlations (H-1’-CH_3, H-2,2’, H3,3’/C-5,5’, H-4,4’,H-6,6’/C-1,1’) confirmed the metabolite was 5,5’-bis(1,1’,3,3’-tetramethylbutyl)-[1,1’-biphenyl]-2,2’-diol (Fig. 28).

The HR-ESI-MS data for the metabolite of BPA, which yielded a molecular ion at *m/z* 453.2062 $[M-H]^-$ (calculated for $C_{30}H_{30}O_4$, 453.2058), indicated that the molecular formula of this compound was

$C_{30}H_{31}O_4$ (Fig. 29). This formula suggested that the metabolite might be a dimer of BPA. The structure of the purified metabolite was further characterized by NMR analyses (Figs. 30-35, Table 4). The ^{13}C NMR and ^{1}H NMR spectra indicated that the metabolite of BPA had six carbon atoms, two methyls, five methylenes, 1,2,4 substituted benzene and 1,4 substituted benzene (Figs. 30, 31). HMBC correlations (Fig. 35) (H-2',3'-methyl/C-1, C-2, C-5, H-3/C-1, C-2, C-5) confirmed the metabolite was 5,5'-bis-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,2'-diol (Fig. 36). The metabolites are novel compounds.

1.4. Discussion

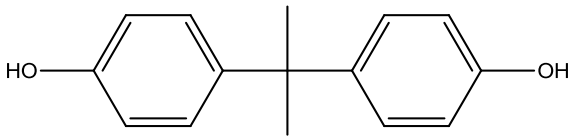
The extracellular ligninolytic enzymes produced by white-rot fungi are nonspecific and nonstereoselective enzymes that can degrade not only lignin but also a range of recalcitrant pollutants, making them of great interest for the removal of environmental contamination (Asgher et al., 2008). In the present study, YK-LiP1, which degrade dimeric lignin model compounds more effectively that Pc-LiP (Sugiura et al., 2003; Hirai et al., 2005), was applied to the elimination of EDCs. Five EDCs (OP, BPA, E_1, E_2, and EE_2) were eliminated by YK-LiP1 more effectively than Pc-LiP, and OP and BPA were disappeared almost completely in the reaction mixture after a 24 h treatment, as shown in Fig. 19. These results indicate that YK-LiP1 have a higher affinity than Pc-LiP for these phenolic compounds which were relatively high molecular weight (M.W. 206-296). Huang et al. reported that the activity of Pc-LiP toward various phenols is very low (Huang et al., 2003).

The removal of estrogenic activity is the most important goal for the biodegradation of EDCs. Estrogenic activities of E_1, E_2, and EE_2 shows much higher estrogenic activities than OP or BPA (Tsutsumi et al., 2001; Suzuki et al., 2003; Tamagawa et al., 2007). Therefore, the estrogenic activities of E_1, E_2, or EE_2 before and after LiP treatment were assayed using the yeast two-hybrid estrogenic assay system. This system is developed and is based on the ligand-dependent interaction between the nuclear hormone receptor and its coactivator (Nishikawa et al., 1999). Fig. 20 demonstrates that YK-LiP1 reduced the estrogenic activities of E_2 and EE_2 but Pc-LiP could not.

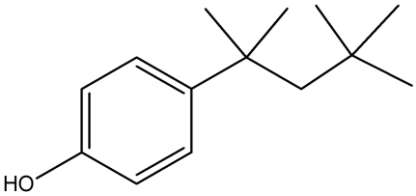
The removal of EDCs' estrogenic activities by ligninolytic enzymes from white rot fungi (MnP and lassace) has been reported (Tsutsumi et al.,

2001; Suzuki et al., 2003; Tamagawa et al., 2007). However, there have been no studies focusing on the metabolic product of these five ECDs by ligninolytic enzymes from white rot fungi. The structures of E_1, E_2, and EE_2 are too complex to identify, so the metabolites of OP and BPA were attempted to identify. Our current results suggest that a dimer was generated as a metabolite from OP or BPA by YK-LiP1. The formation of phenoxy radical might be followed by phenolic hydroxyl of OP was one-electron oxidized, and the radical was transferred to the para position, the radical polymerization thus a dimer of OP was generated. BPA occurs a similar reaction was demonstrated, the removal of the ECDs' estrogenic activities might be due to polymerization brought about by enzymatic oxidation was proposed.

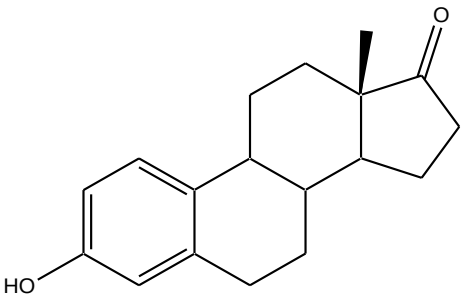
In summary, YK-LiP1 from white rot fungi *P. sordida* YK-624 could effectively eliminate these five ECDs (OP, BPA, E_1, E_2, and EE_2) and remove its estrogenic activity. To our knowledge, this is the first study describing the metabolites of OP and BPA, and proposed the detoxification process of ECDs by YK-LiP1 from *P. sordida* YK-624.



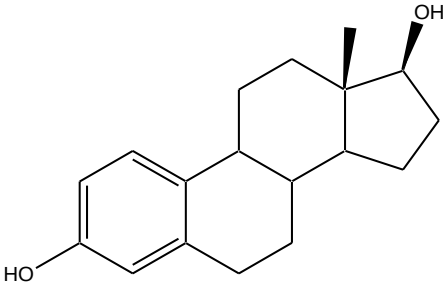
bisphenol A　(BPA)



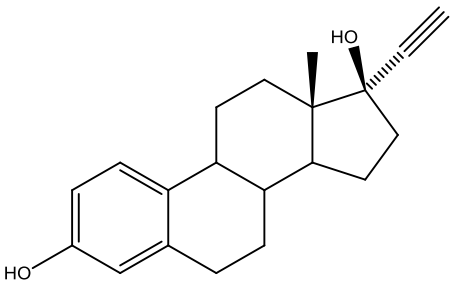
p-*t*-octylphenol　(OP)



estrone　(E_1)



17β-estradiol　(E_2)



ethinylestradiol　(EE_2)

Fig. 18 Structures of BPA, OP, E_1, E_2 and EE_2.

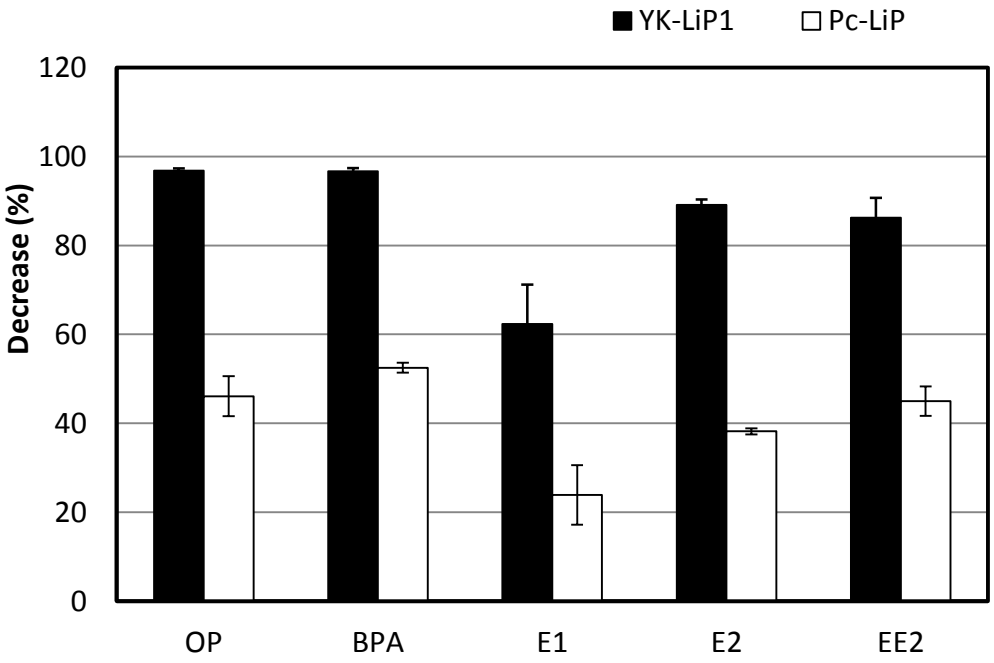


Fig. 19 Decrease of BPA, OP, E_1, E_2 and EE_2 by YK-LiP1 and Pc-LiP. Reactions contained 2 nkat each LiP, 100 μM EDCs, and 100 μM H_2O_2 in 20 mM succinate, pH 3.0. Reactions were performed for 24 h at 30℃ and mixing at 150 r.p.m. Values are means ± SD of triplicate samples.

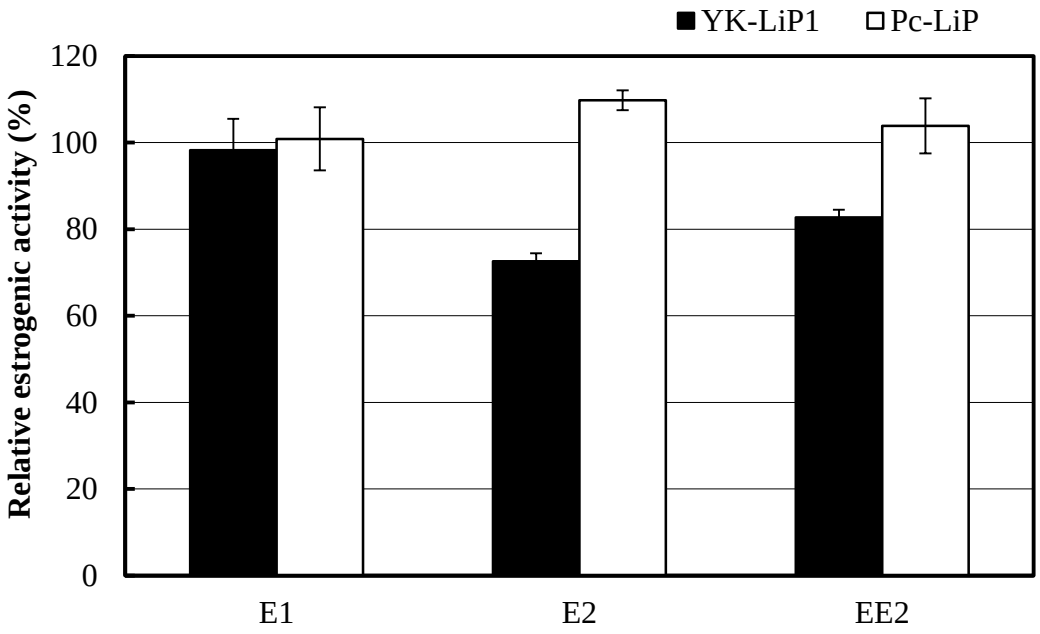


Fig. 20 Removal of estrogenic activities of E_1, E_2 and EE_2 by YK-LiP1 and Pc-LiP. Values are means ± SD of triplicate samples.

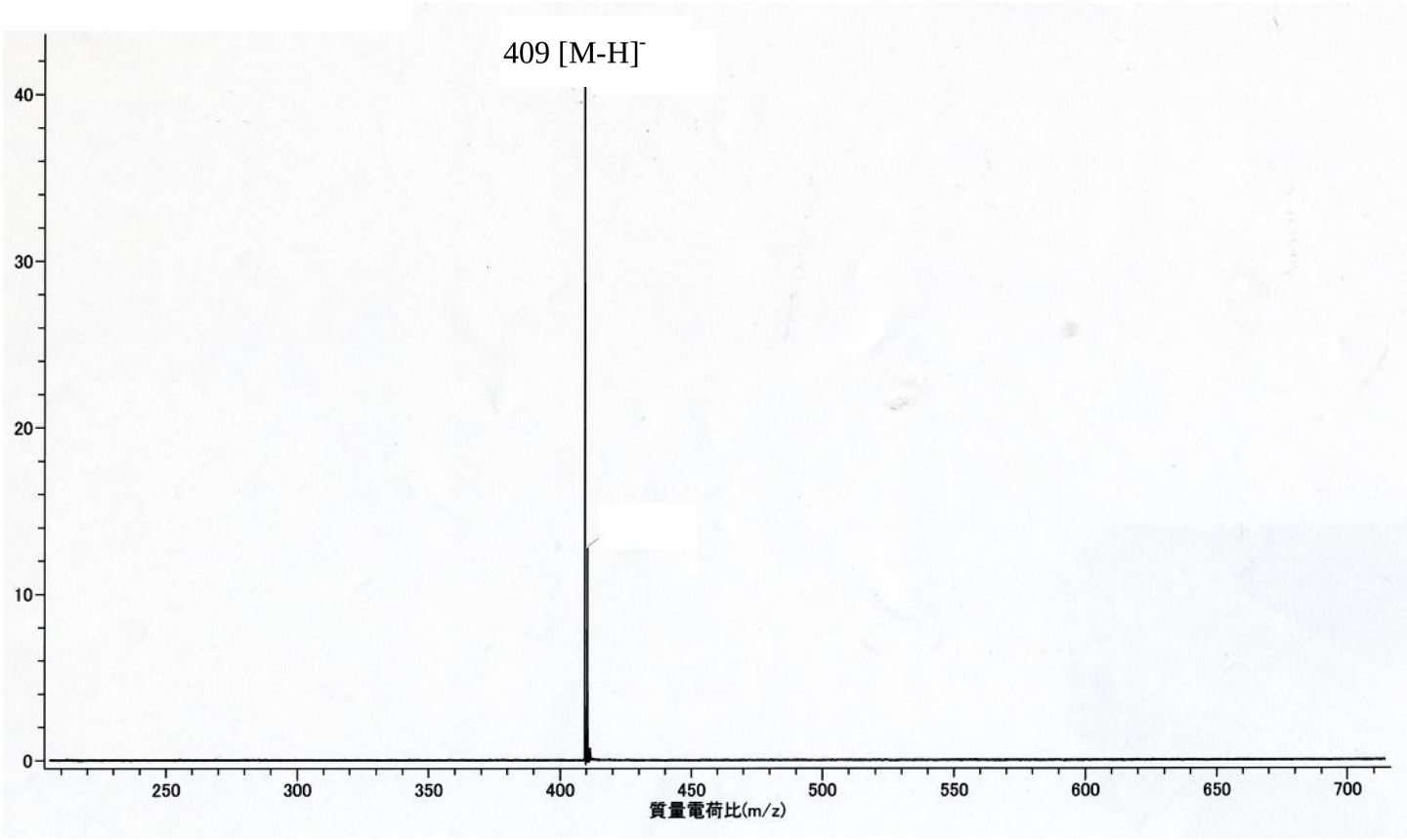


Fig. 21 ESI-MS spectrum of OP metabolite.

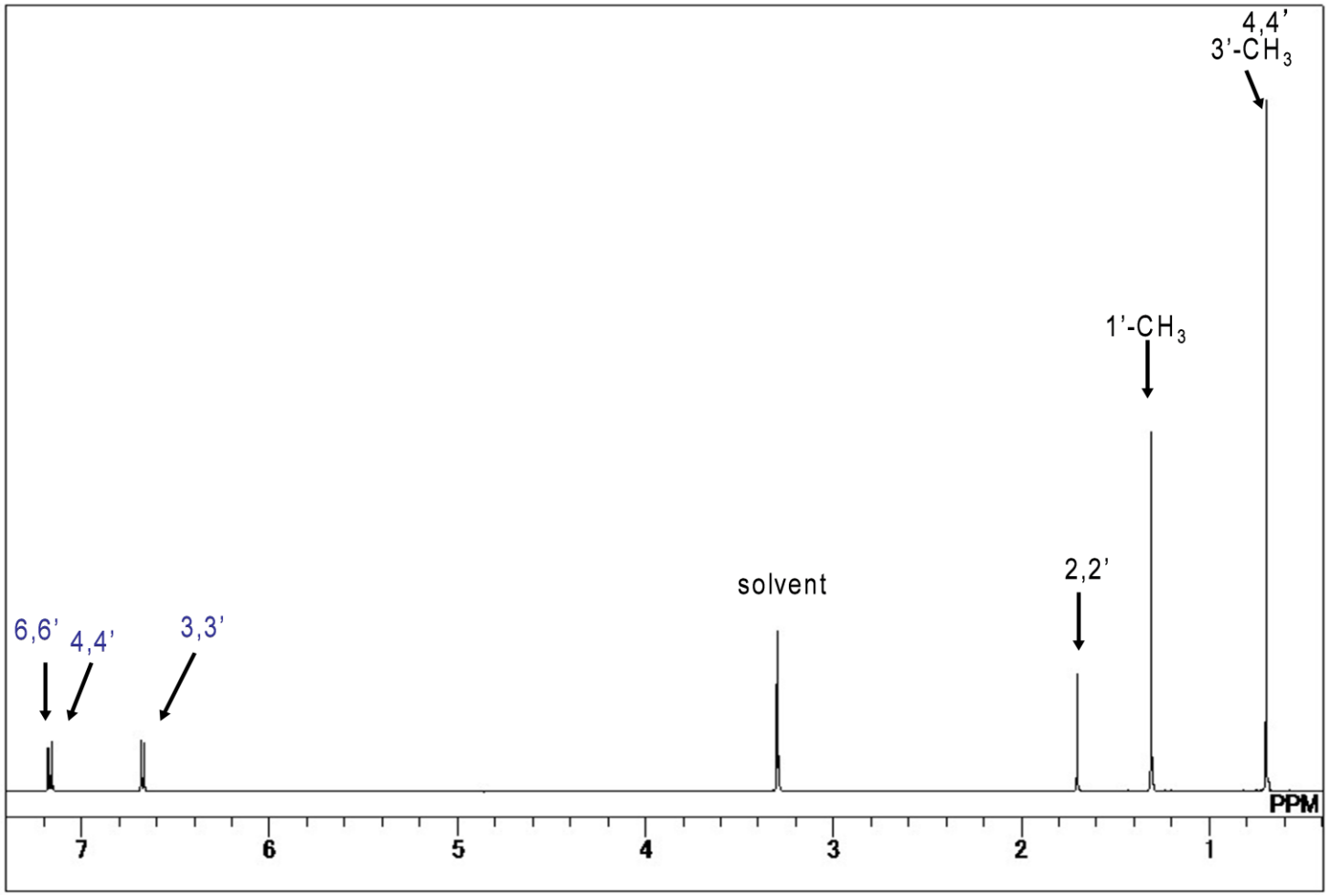


Fig. 22 ^{1}H NMR spectrum of OP metabolite (CD_3OD).

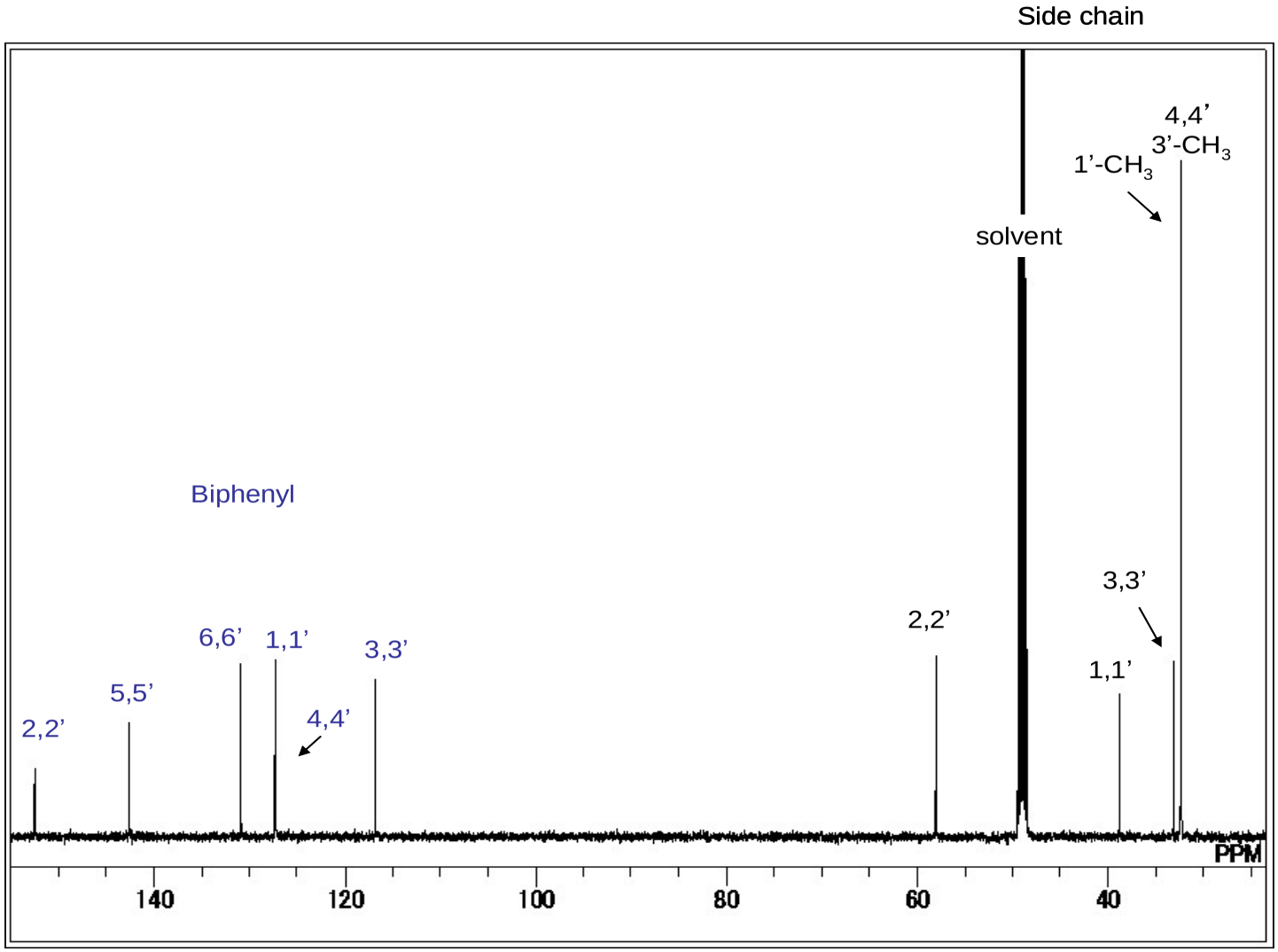


Fig. 23 ^{13}C NMR spectrum of OP metabolite (CD_3OD).

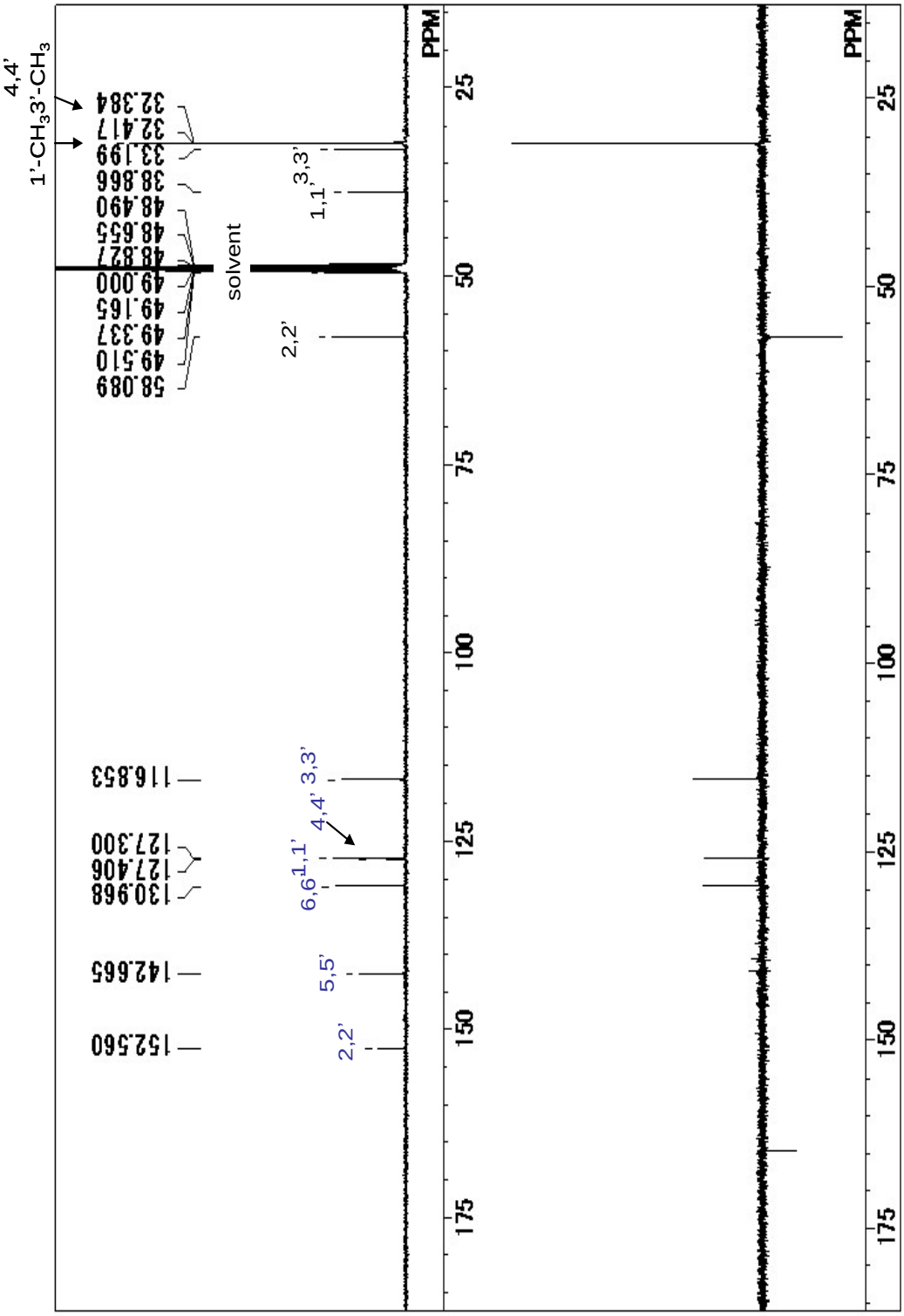


Fig. 24 DEPT spectrum of OP metabolite (CD_3OD).

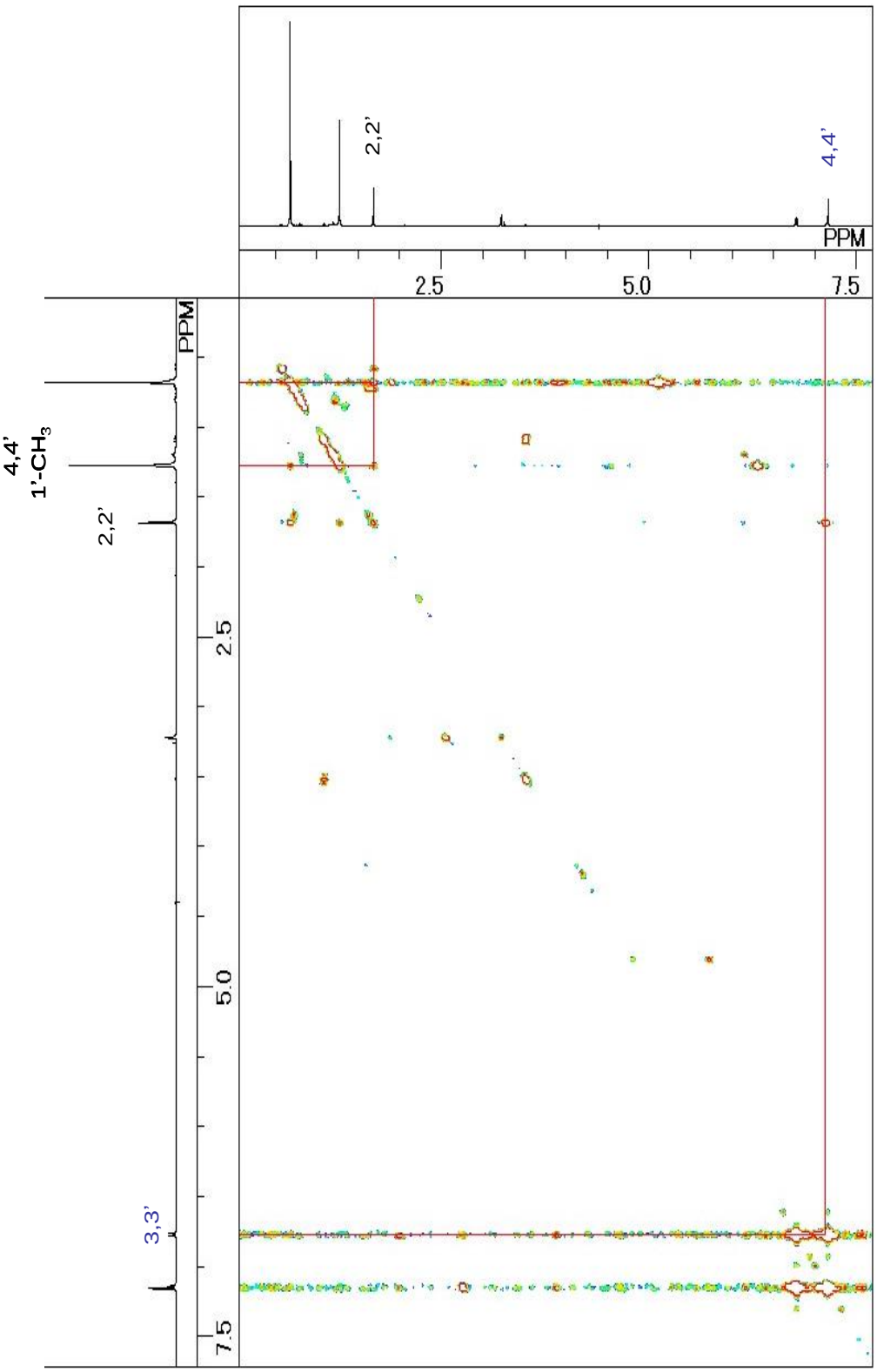


Fig. 25 COSY spectrum of OP metabolite (CD_3OD).

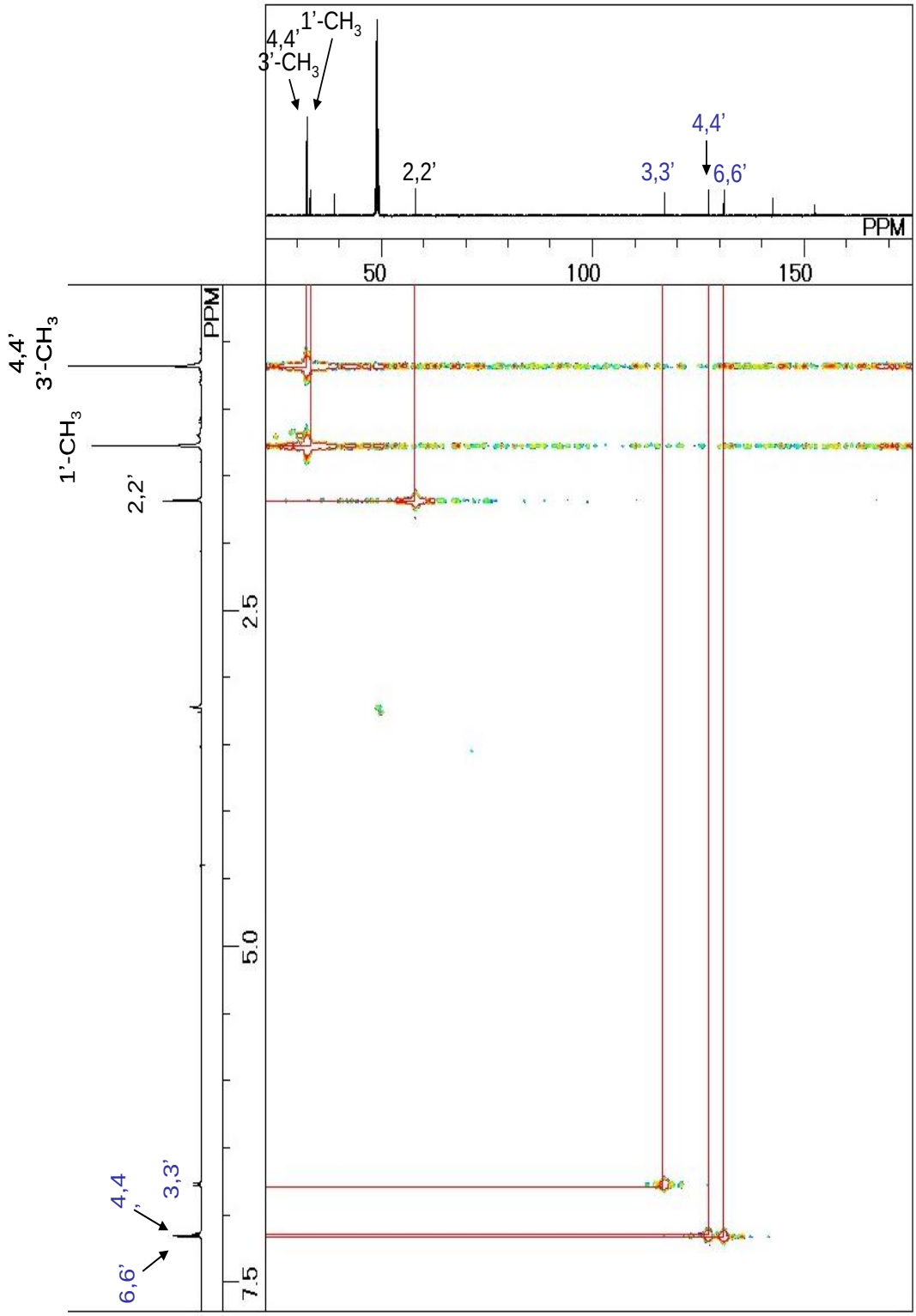


Fig. 26 HMQC spectrum of OP metabolite (CD_3OD).

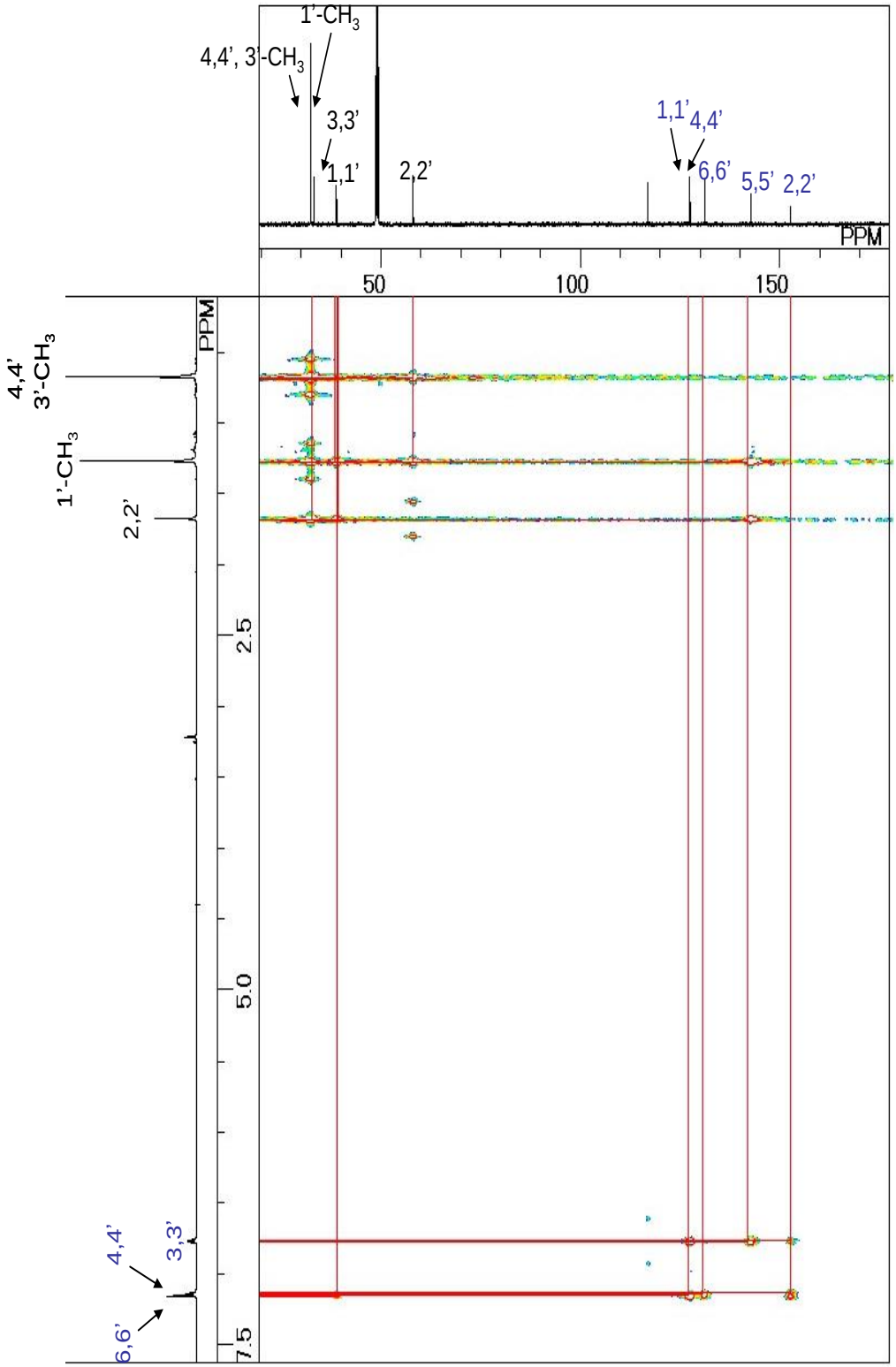


Fig. 27 HMBC spectrum of OP metabolite (CD_3OD).

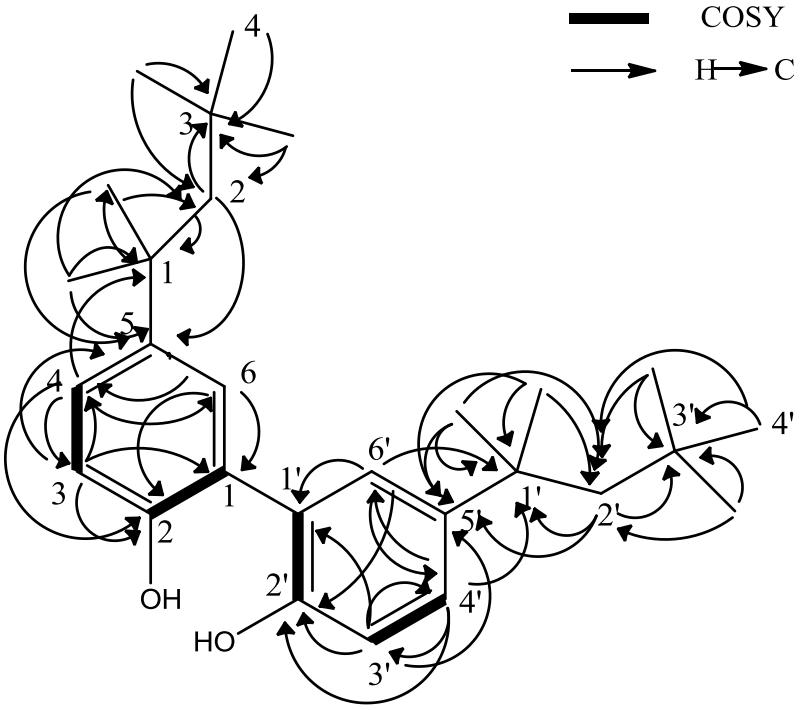


Fig. 28 COSY and HMBC correlations for the OP metabolite.

Table 3 NMR data for the metabolite of OP in CD_3OD.

Position	^1H δ_H (mult, *J* in Hz)	^{13}C δ_C
[Biphenyl]		
1, 1’	-	127.4
2, 2’	-	152.6
3, 3’	6.77 (d, 8.5)	116.8
4, 4’	7.14 (dd, 8.5, 2.4)	127.3
5, 5’	-	142.6
6, 6’	7.16 (d, 2.4)	130.9
[Side chain]		
1, 1’	-	38.9
2, 2’	1.69 (s)	58.1
3, 3’	-	33.2
4, 4’	0.69 (s)	32.4
1’-CH_3	1.28 (s)	32.4
3’-CH_3	0.69 (s)	32.4

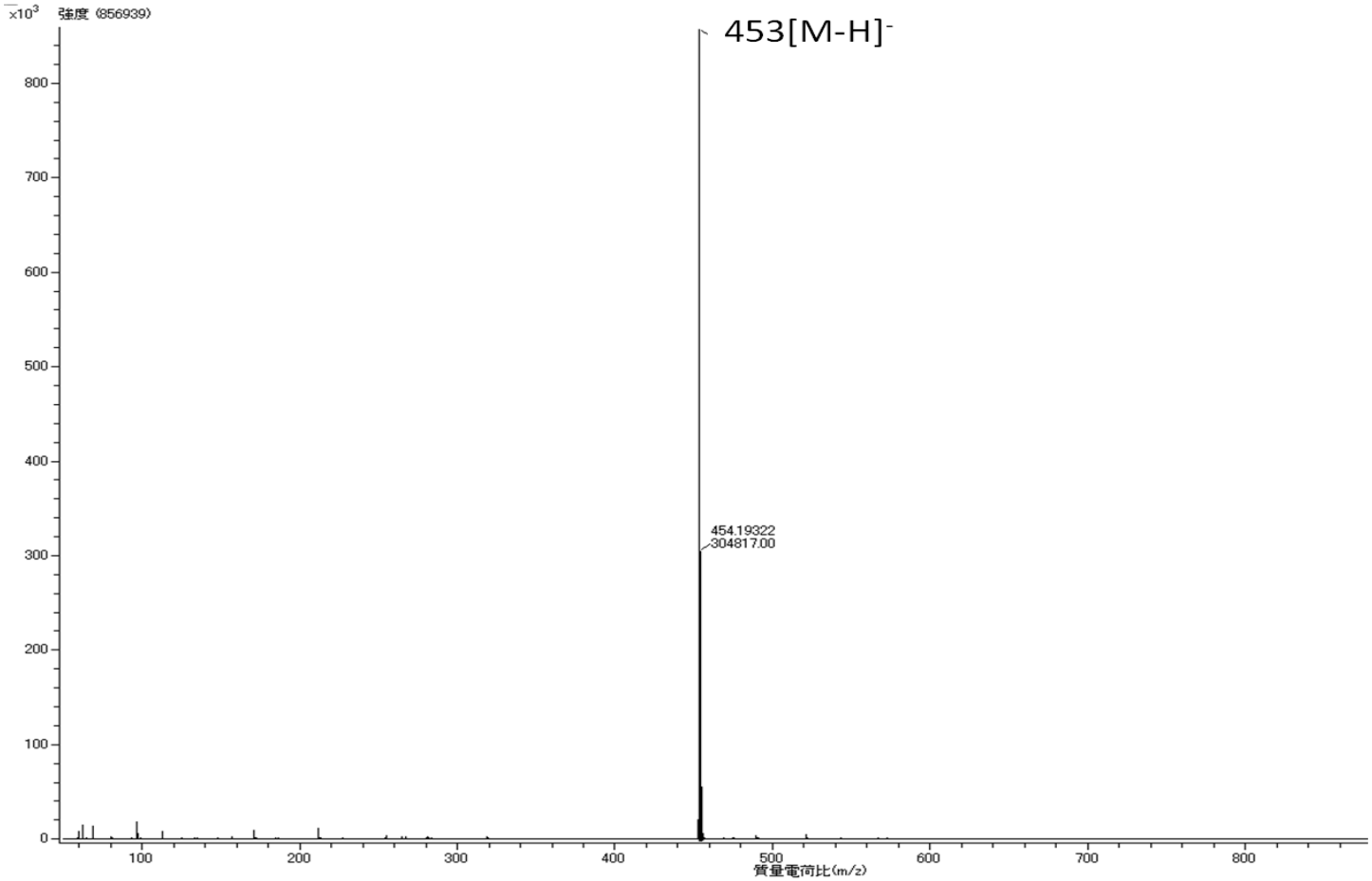


Fig. 29 ESI-MS spectrum of BPA metabolite.

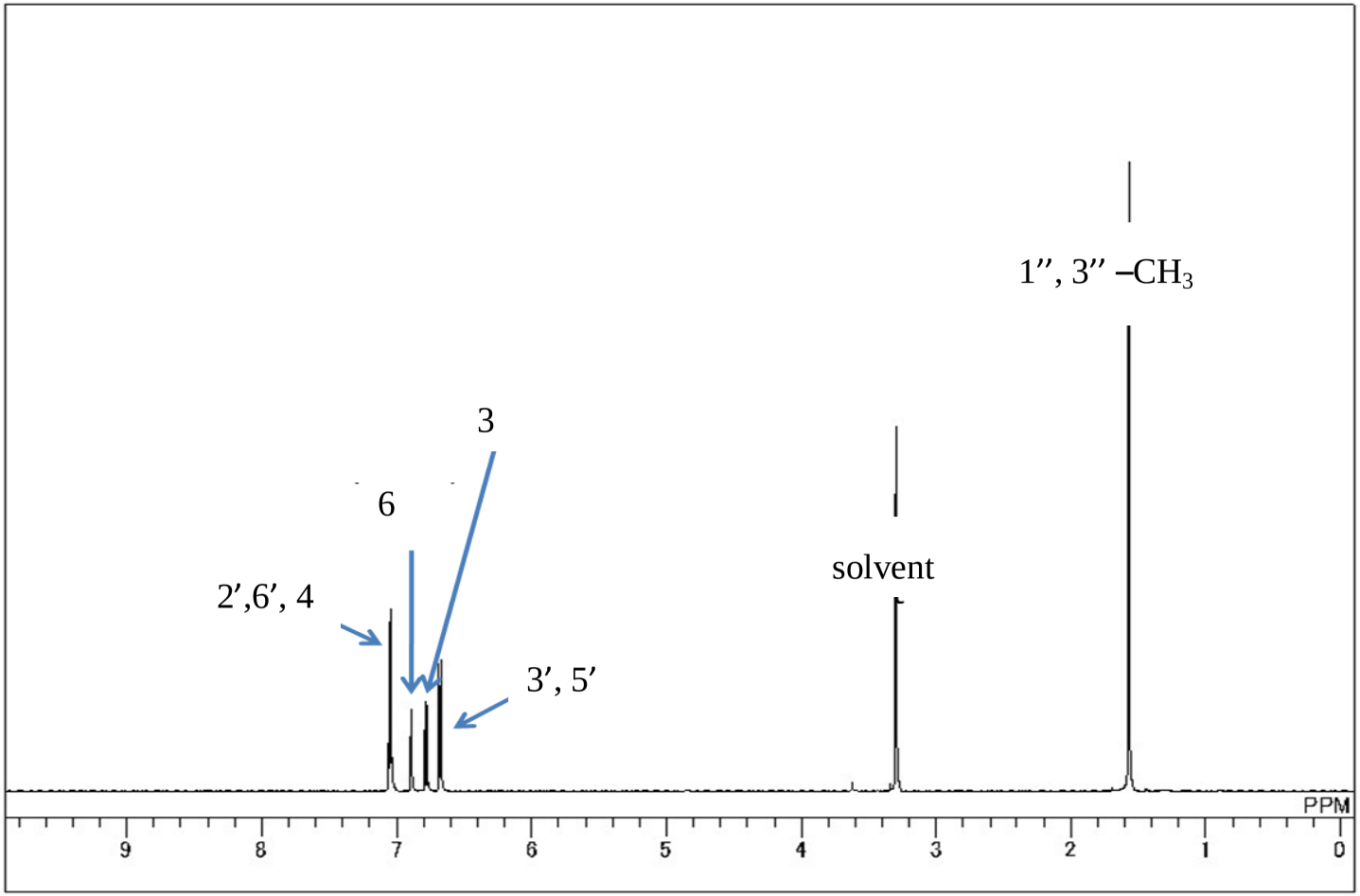


Fig. 30 ^{1}H NMR spectrum of BPA metabolite (CD_3OD).

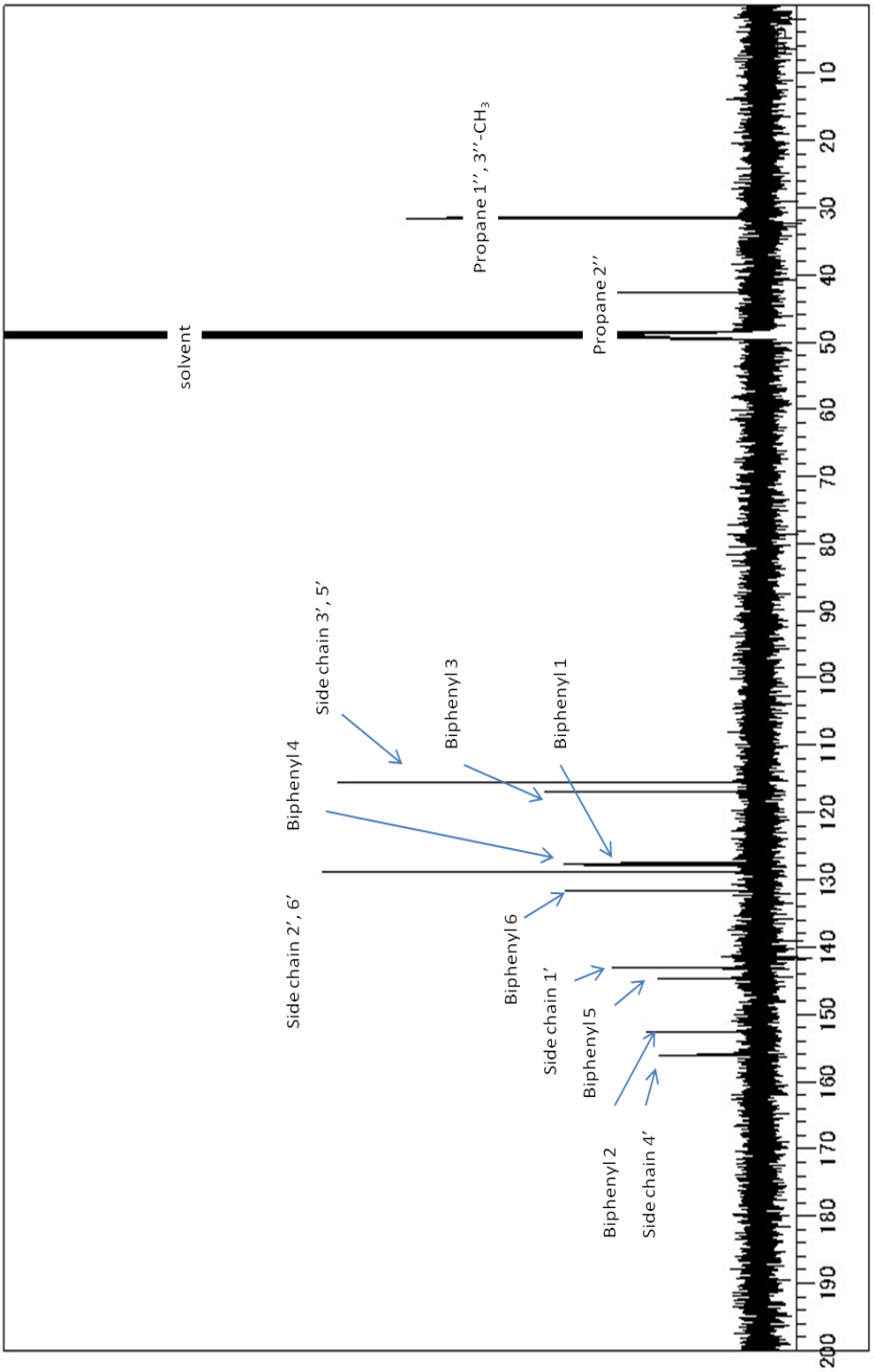


Fig. 31 ^{13}C NMR spectrum of BPA metabolite (CD_3OD).

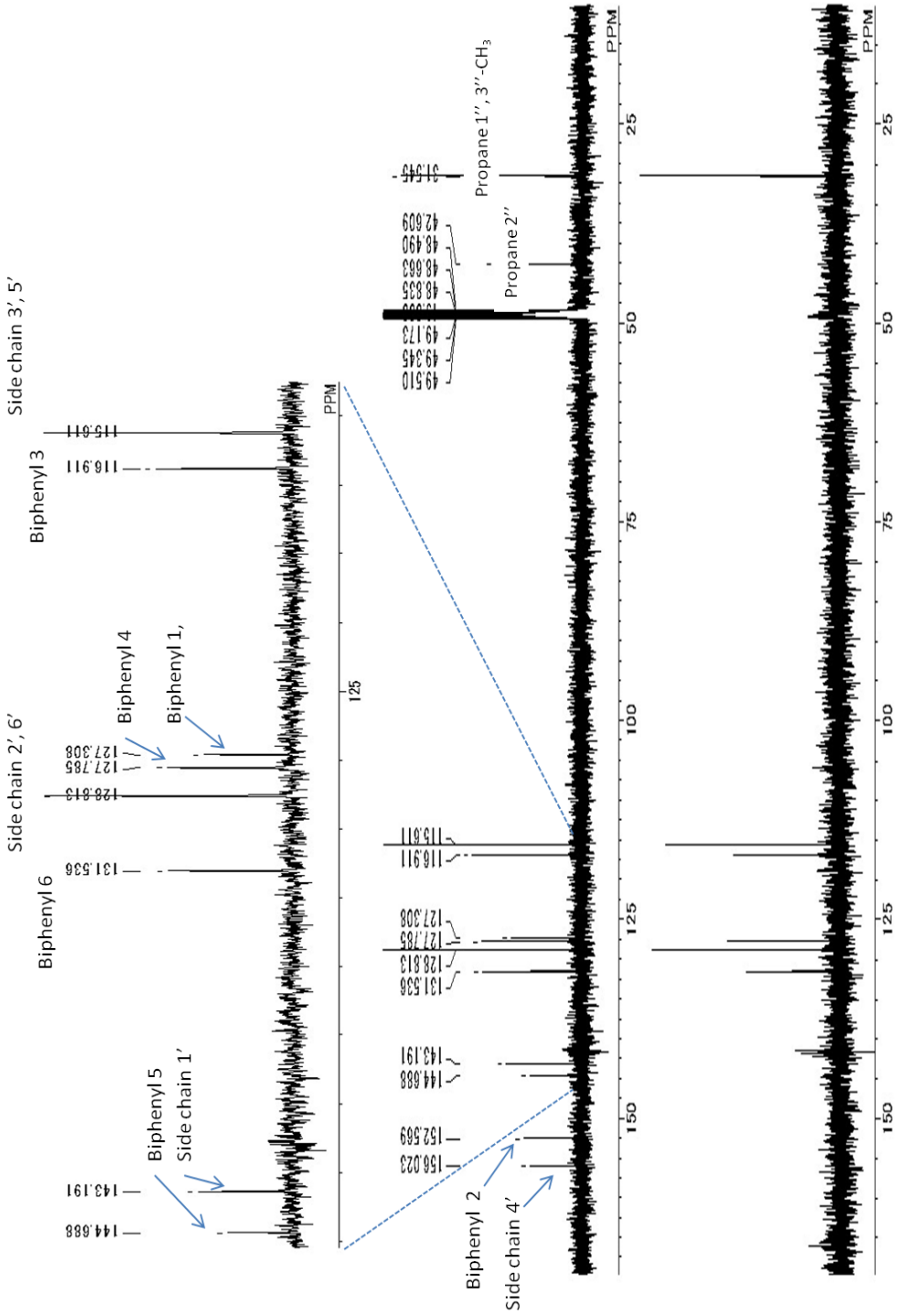


Fig. 32 DEPT spectrum of BPA metabolite (CD_3OD).

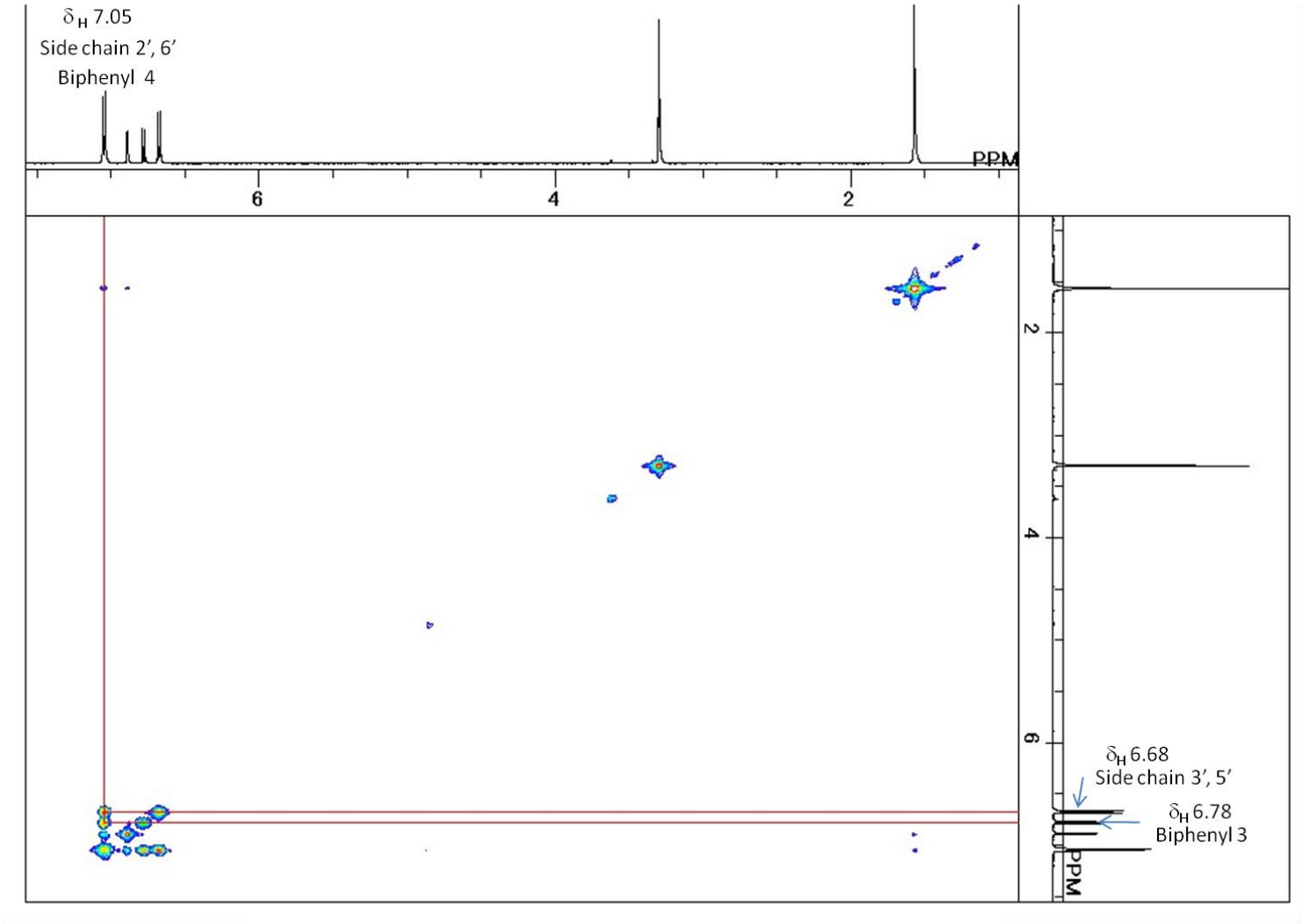


Fig. 33 COSY spectrum of BPA metabolite (CD_3OD).

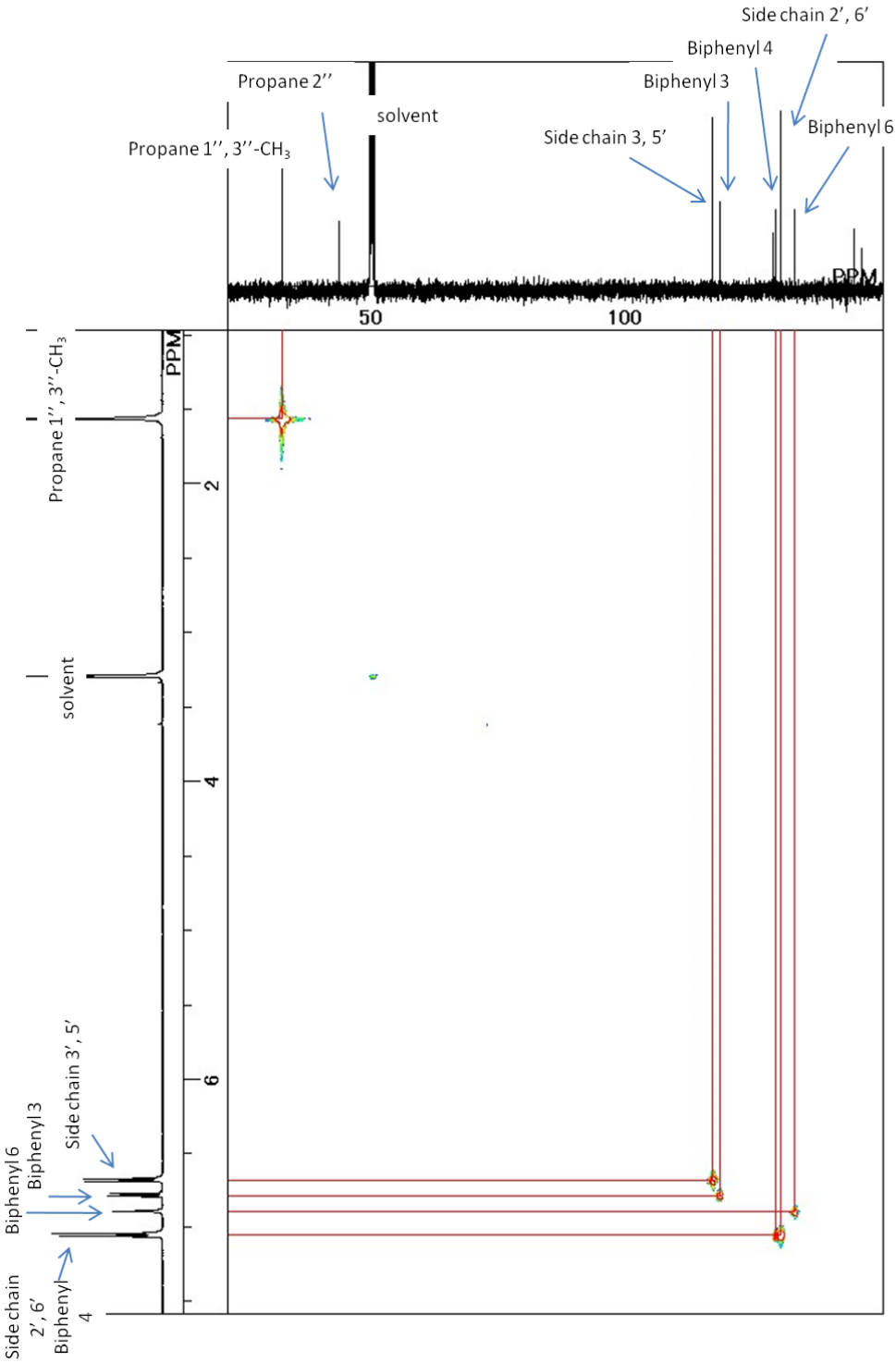


Fig. 34 HMQC spectrum of BPA metabolite (CD_3OD).

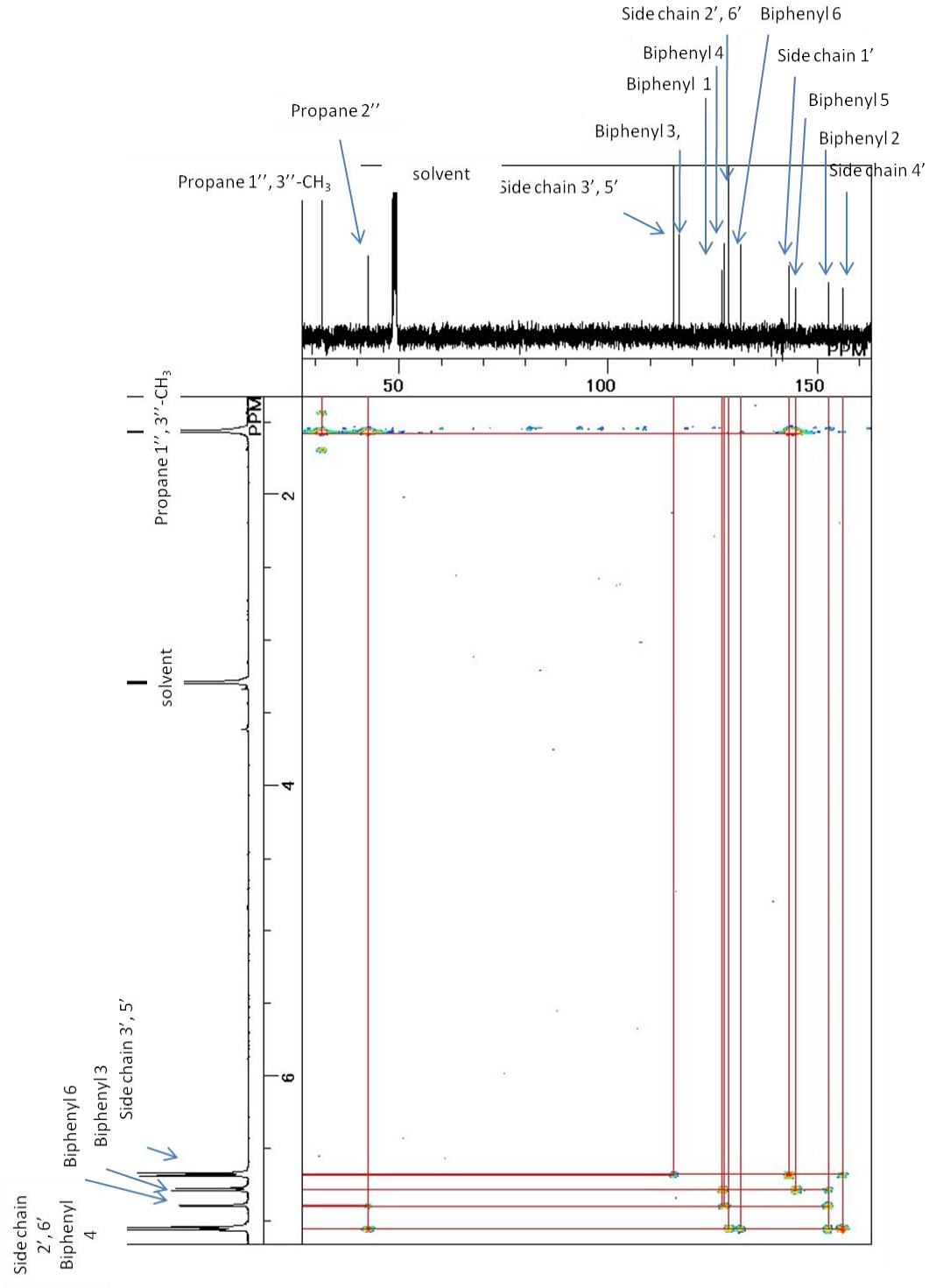


Fig. 35 HMBC spectrum of BPA metabolite (CD_3OD).

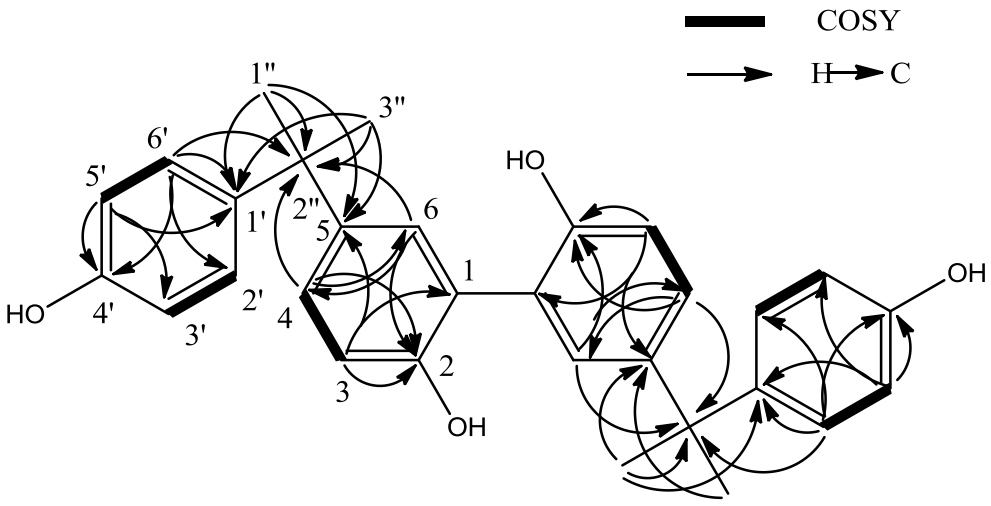


Fig. 36 COSY and HMBC correlations for the BPA metabolite.

Table 4 NMR data for the BPA metabolite in CD_3OD.

Position	^{1}H δ_H (mult, *J* in Hz)	^{13}C δ_C
1	-	127.3
2	-	152.7
3	6.78 (d, 8.2)	116.9
4	7.05 (dd, 8.2, 2.4)	127.8
5		144.7
6	6.89 (d, 2.4)	131.5
1’	-	143.2
4’	-	156.2
2’, 6’	7.05 (d, 8.6)	125.8
3’, 5’	6.68 (d, 8.6)	115.6
2’’	-	42.6
1’’, 3’’	1.57 (s)	31.5

Chapter Ⅳ

Metabolism of BPA by the white-rot fungus *P. sordida* YK-624

Section 1
Dimerization of BPA by the white-rot fungus *P. sordida* YK-624 under ligninolytic condition

1.1. Introduction

EDCs are organic compounds widely used, which are ubiquitous in the environment. Many synthetic chemicals released into the environment, such as plasticizers, pesticides, antifouling paints, raw materials for polymer and hormonal pharmaceuticals, have been identified to be potent EDCs and their environmental occurrence and ecotoxicological effects are being actively studied in recent years (Campbell et al., 2006; Matozzo et al., 2008). One of the representative compounds of the EDCs group is bisphenol A (2,2-bis(4-hydroxyphenyl)propane; BPA). BPA, a monomer component of polycarbonate plastics, is used in many consumer products, including lacquers applied as food-can linings and dental composite fillings and sealants (Brotons et al., 1995; Olea et al., 1996). Many countries throughout the world have large production capacities for BPA, especially Germany, the Netherlands, the USA and Japan. BPA is one of the highest volume chemicals produced worldwide, with an estimated production of 5 million tons in 2010 (Ballesteros-Gómez et al., 2009). As a result, BPA is often detected in many soil and water environments (Kitada et al., 2008; Matsumura et al., 2009).

In recent years, the degradation of EDCs by ligninolytic enzymes has attracted the attention of many researchers (Hirano et al., 2000; Suzuki et al., 2003; Saito et al., 2004; Tamagawa et al., 2006). In the previous section, the removal of BPA by lignin peroxidase from *P. sordida* YK-624 was also demonstrated (Wang et al., 2012b). However, degradation of BPA using white-rot fungi is also limited (Chai et al. 2005; Shin et al., 2007) and the detail mechanisms are still unknown. In the present study, the removal of BPA by *P. sordida* YK-624 under ligninolytic condition was examined. The metabolites from BPA were also detected and a metabolic pathway for the metabolism of BPA by *P. sordida* YK-624 was proposed.

1.2. Materials and methods

1.2.1. Fungi

P. sordida YK-624 (ATCC 90872) was used in the present study (Hirai et al.,1994). The fungus was maintained on PDA slants at 4°C.

1.2.2. Chemicals

BPA was purchased from Alfa Aesar, A Johnson Matthey Company (Lancashire, United Kingdom). All other chemicals were analytical pure grade and were used without further purification.

1.2.3. Elimination experiments

Nitrogen-limited (NL) Kirk medium described by Tien and Kirk (1988) was used for BPA elimination experiments. The fungus was incubated on a PDA plate at 30°C for 3 days, and then 10 mm-diameter disks were punched out from the growing edge of mycelium. Two disks were each placed into a 100-mL Erlenmeyer flask containing 10 mL of NL medium. After statically incubating the flasks at 30°C for 4 days, 100 μL of 10 or 100 mM BPA/DMSO were added to the cultures (final concentration at 0.1 or 1 mM, respectively), and then further incubated for 24, 48, 72 hours (each in triplicate). The culture was filtrated with a 0.2-μm membrane filter, and the filtrate was then subjected to HPLC for the quantification of BPA under the following conditions: column, Wakosil-II 5C18HG (4.6 x 150 mm); mobile phase, 60% MeOH in 1% acetic acid aq.; flow rate, 1 mL min^{-1}; and UV wavelength, 277 nm.

1.2.4. Metabolism experiment

Inoculated cultures (1 L of NL medium) of *P. sordida* YK-624 were prepared under the conditions described above. After static incubation at 30 °C for 4 days, BPA (final concentration, 1 mM) as added to these cultures. The cultures were further incubated for 24 hours and then homogenized 2 times by Waring blender for 30 seconds with low speed. The cell suspensions were extracted 3 times with equal amount of EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was cleaned on a silica gel chromatography (silica gel 60N, φ40 × 600 mm) and eluted with dichloromethane/acetone/MeOH (9/1/0, 8/2/0, 5/5/0, 0/7/3, 0/5/5, and 0/0/10; vol/vol/vol) to obtain 11 fractions. Each fraction was analyzed by TLC. Silica gel plates (Merck F254; Merck, Darmstadt, Germany) were used for analytical TLC. The metabolite was further separated by HPLC (column: Wakosil-II 5C18HG Prep, 50×250 mm) using 60% MeOH. The purified metabolite was analyzed by HR-ESI-MS and NMR, including DEPT, COSY, HMQC, and HMBC experiments. The HR-ESI-MS data were measured using a JMS-T100LC mass spectrometer. ^{1}H NMR spectra were recorded using a Jeol Lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz.

1.3. Results

1.3.1. Elimination of BPA by *P. sordida* YK-624 under ligninolytic condition

The removal of BPA by lignin peroxidase from hyper lignin-degrading fungus *P. sordida* YK-624 has been investigated (Wang et al. 2012a). In the present study, NL media were used for the elimination experiment of BPA. After preculturing by *P. sordida* YK-624, BPA (final concentration, 1 mM or 0.1 mM) was added to cultures. After a 24 h treatment, 60% or 100% of BPA were eliminated, respectively (Fig. 37).

1.3.2. Identification of the metabolites from BPA

The metabolites of BPA were attempted to identify, and two metabolites were detected in the analysis of TLC and HPLC (data not shown). Molecular formula of compound 1 was determined as $C_{30}H_{30}O_4$ by HR-ESI-MS, *m/z* 453.2062[M-H]$^-$(calcd. for $C_{30}H_{29}O_4$, 453.2022, Fig. 29). This formula suggested that the metabolite might be a dimer of BPA. The complete assignment of all the protons and carbons was accomplished by DEPT, HMQC, COSY and HMBC experiments (Figs. 30-35, Table 4). The structure of compound 1 was identified as 5,5’-bis-[1-(4-hydroxy-phenyl)1-methyl-ethyl]-biphenyl-2,2’-diol (Fig. 36). Compound 1 was the same as the metabolite which has been detected by the treatment of BPA by lignin peroxidase from hyper lignin-degrading fungus *P. sordida* YK-624, the estrogenic activity of the metabolite was lower than that of BPA has been reported (Wang et al. 2012a). Molecular formula of compound 2 was the same to compound 1 (Fig. 38). The complete assignment of all the protons and carbons was accomplished by DEPT, HMQC, COSY and HMBC experiments (Figs. 39-44, Table 5). The DEPT experiment indicated the presence of 4 methyls, 9 methylenes, and 11 quaternary carbons (Fig. 41). Compound 2 also had 1,2,4-trisubstituted phenyl group and 1,4-substituted group. The complete structure was determined by interpretating HMBC correlations (H-1’’’, 3’’’/phenol C-4’, C-2’’, hydroxyphenyl C-1’; H-1’’, 3’’/phenol C-4, C-2’’, hydroxyphenyl C-1), and the connection to phenol C-1 was not determined. The structure of compound 2 was identified as 4-(2-(4-hydroxy-phenyl)propan-2-yl)-2-(4-(2-(4-hydroxyphenyl) propan-2-yl) phenoxy)phenol (Fig. 45). This is the first time that compound 2 is chemically identified.

1.4. Discussion

The removal of EDCs' estrogenic activities by ligninolytic enzymes from white-rot fungi has been reported (Suzuki et al., 2003; Tamagawa et al., 2006; Wang et al., 2012a). However, there have been no studies focusing on the metabolic product of these ECDs by fungi. The formation of compound 1 and 2 by HPLC was assayed. However, trace amounts of two metabolites have detected. This result suggests that further radical coupling would occur during the fungal treatments. Tsutsumi et al. have reported that BPA tetramers were formed by manganese peroxidase treatment (2001). Similarly, it is thought that BPA tetramers are formed as main products by fungal treatment, following the dimerization of BPA.

The estrogenic activity of more than 500 chemicals were tested by the yeast two-hybrid system (Nishihara et al., 2000), deducing that most of the compounds which have a phenol ring with substitution of a bulky moiety at the *ortho*-position reduces the activity. The estrogenic activity of two metabolites might be reduced because the radical coupling occurred at the *ortho*-position.

Compound 1 was completely disappeared after 48 h treatment under ligninolytic condition (data not shown). It is possible that compound 2 is also removed under the condition. Furthermore, a proteomic differential display technique was utilized to study cellular responses of this fungus exposed to BPA, and both BPA and BPA polymers were not incorporated into intracellular region under ligninolytic condition since proteome map of cytosolic fraction of this fungus grown in the presence of BPA is same as that grown in the absence of BPA (data not shown). Therefore, the proposed pathway was described as: BPA is oxidized to these phenoxy radicals by ligninolytic enzymes, and BPA dimers are formed by radical couplings at extracellular region without the incorporation into the cell of *P. sordida* YK-624 (Fig. 46). Moreover, BPA dimer is converted to BPA tetramer, and then, the toxicity is decreased by the polymerization of BPA.

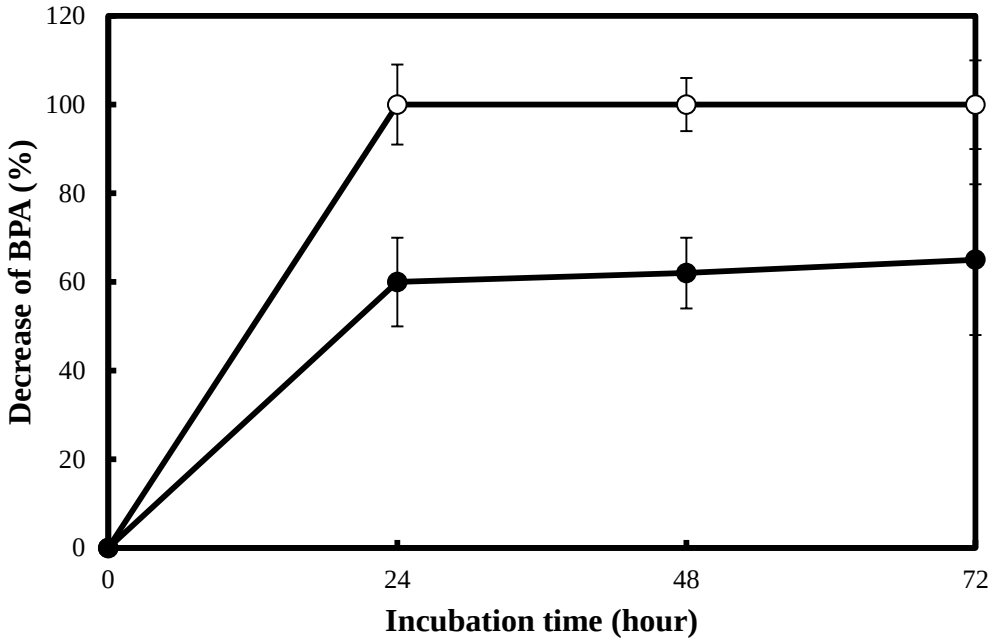


Fig. 37 Time course for BPA elimination by *P. sordida* YK-624. *Closed circles* 1 mM BPA, *open circles* 0.1 mM. Values are the means±SD of triplicate samples

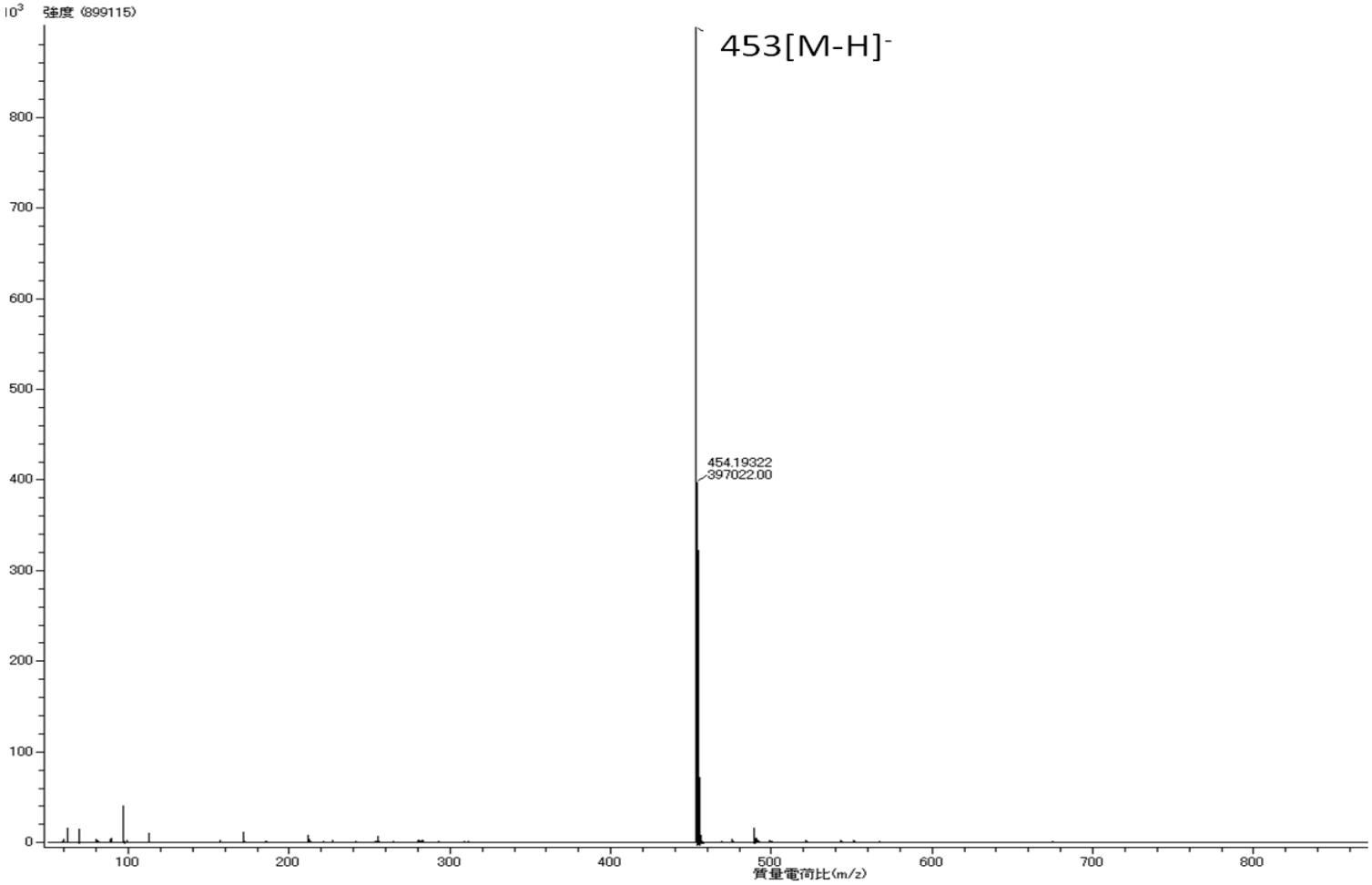


Fig. 38 ESI-MS spectrum of compound 2.

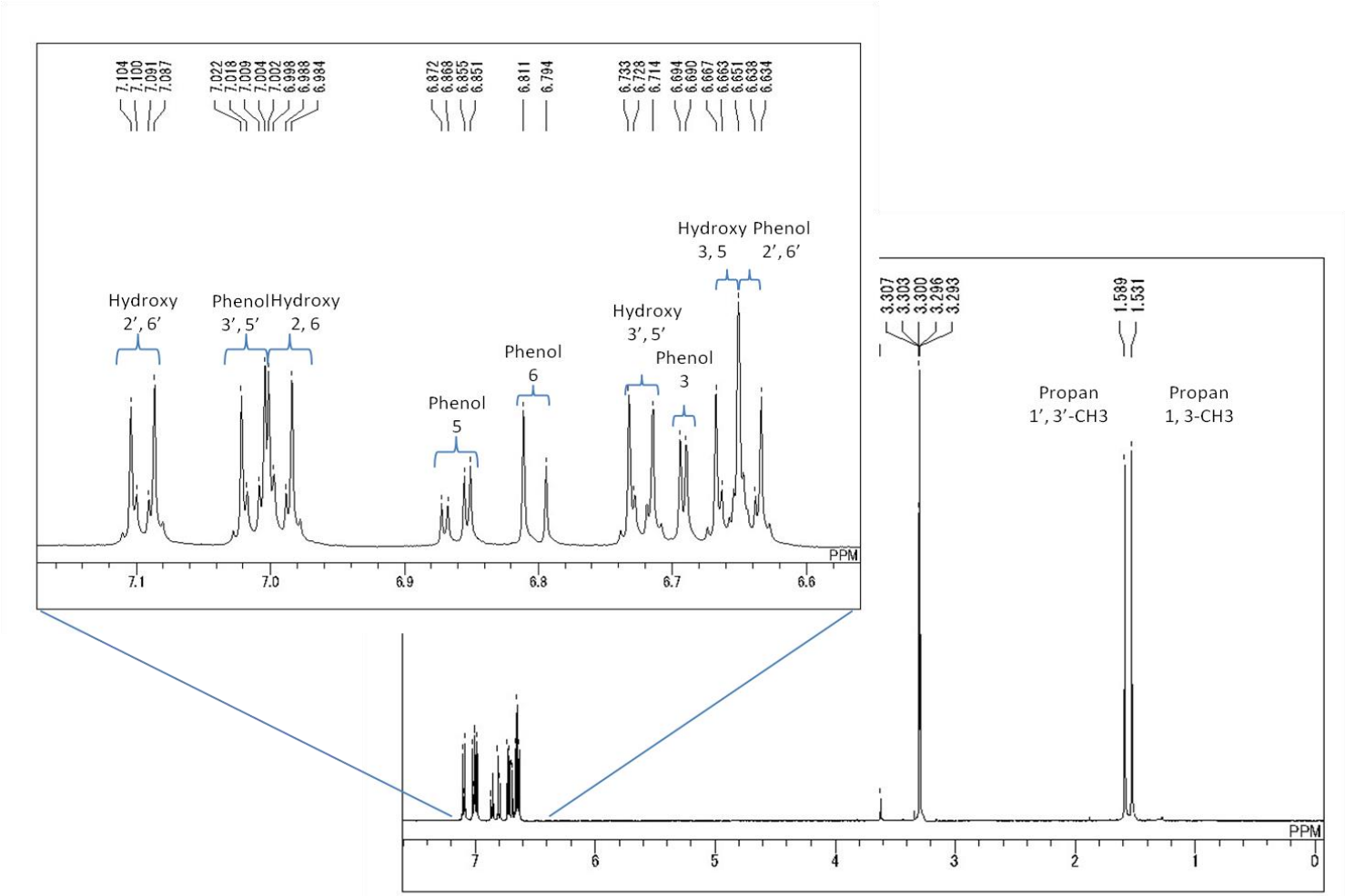


Fig. 39 ^{1}H NMR spectrum of compound 2 (CD_3OD).

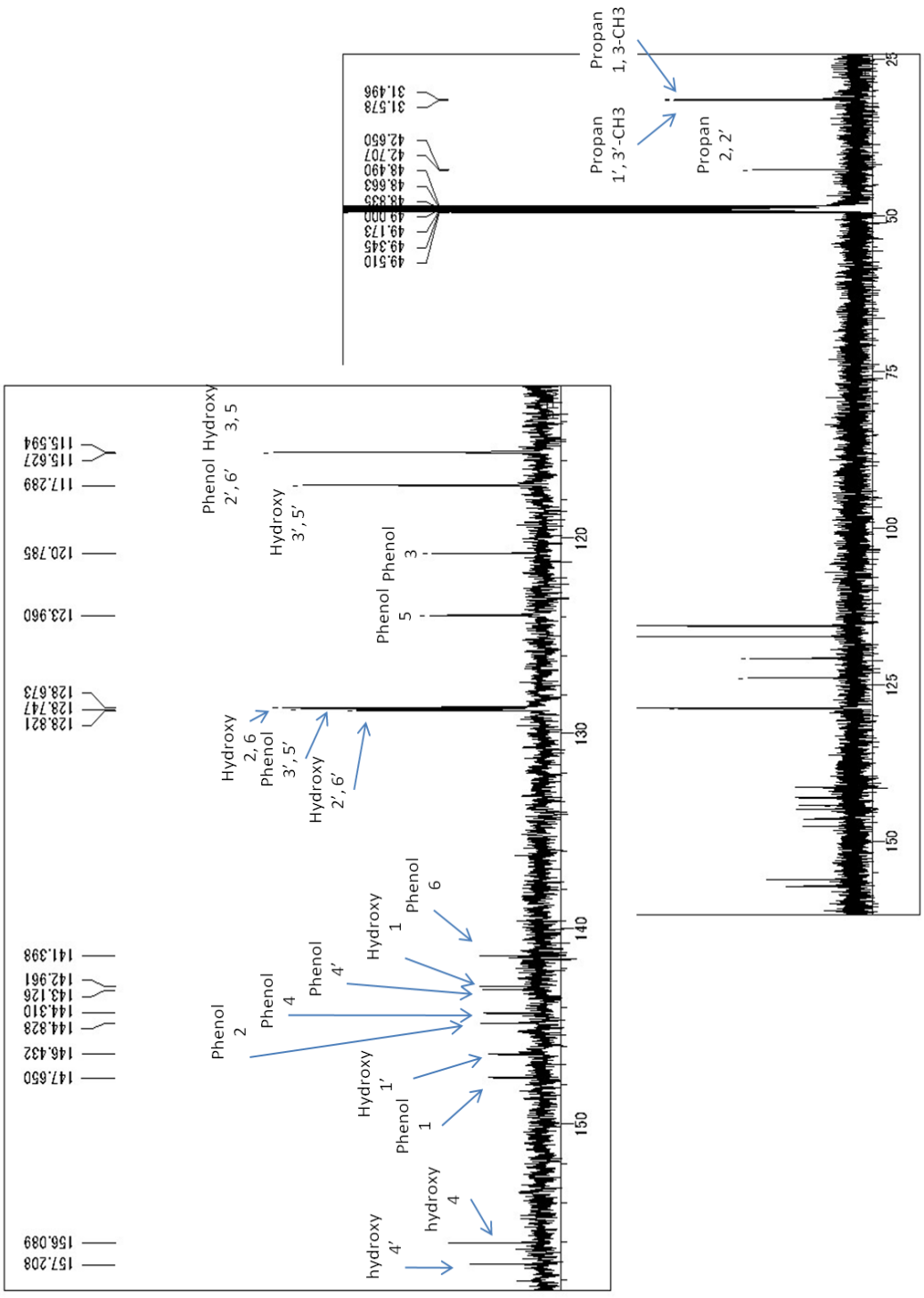


Fig. 40 ^{13}C NMR spectrum of compound 2 (CD_3OD).

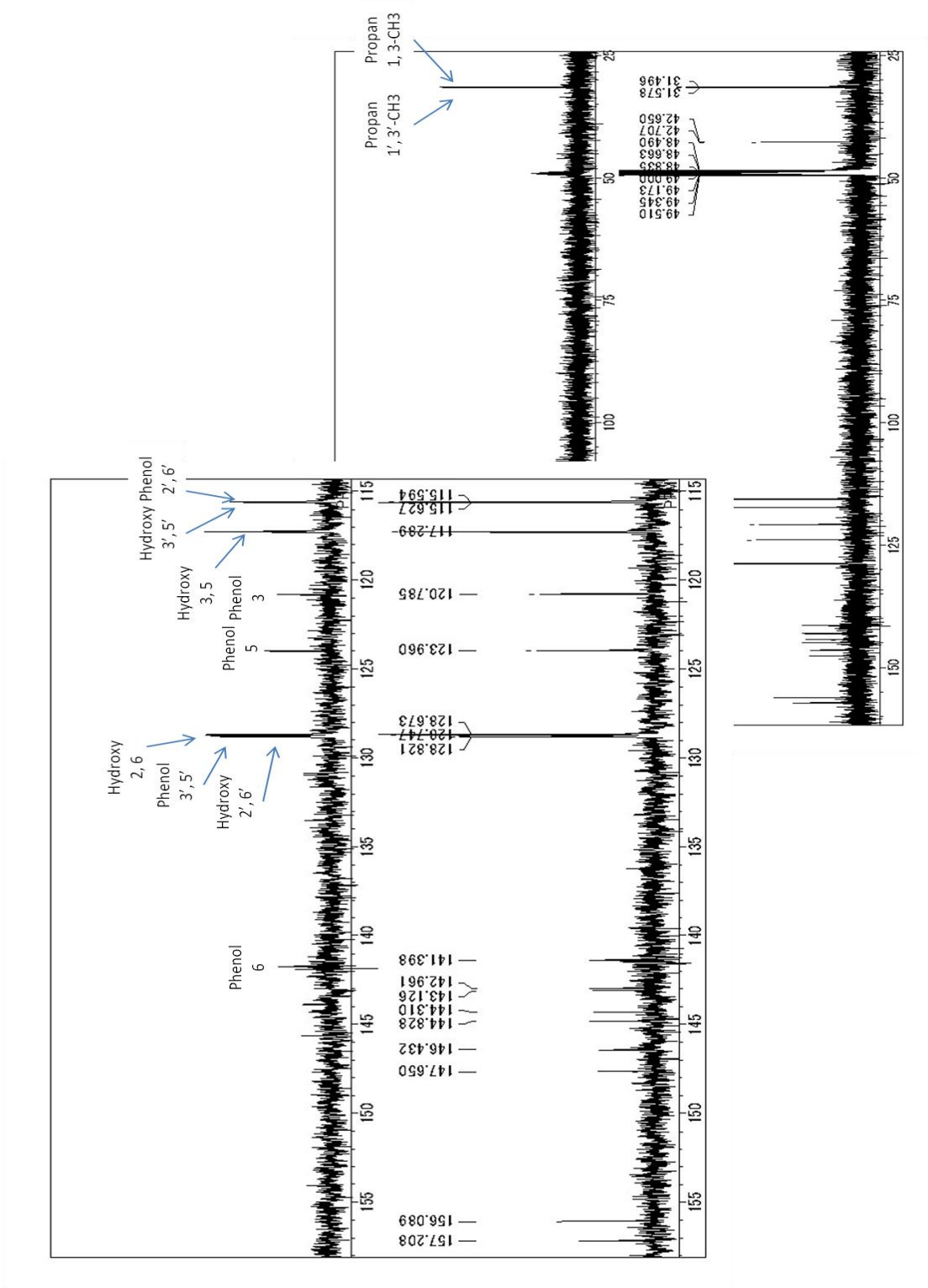


Fig. 41 DEPT spectrum of compound 2 (CD_3OD).

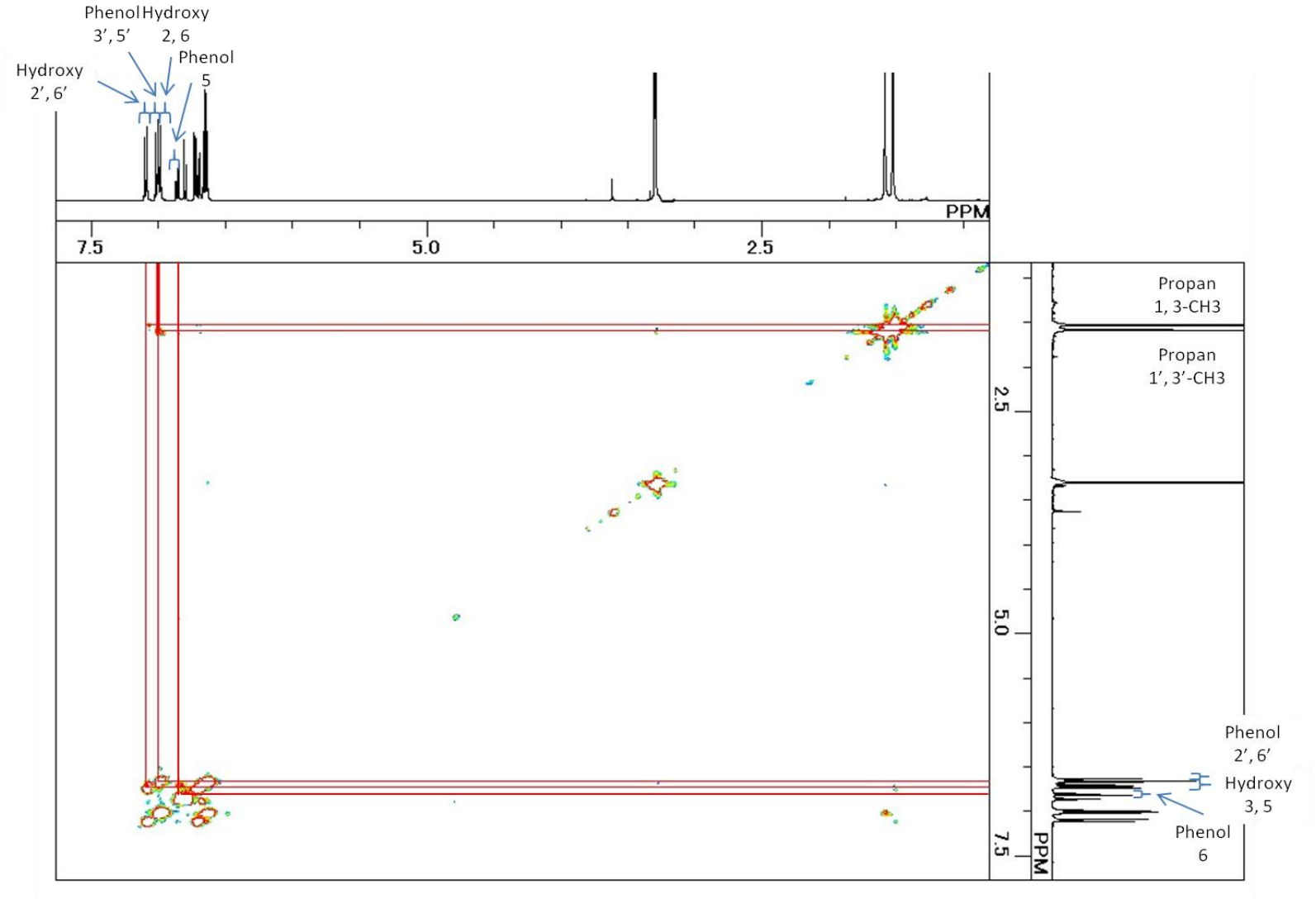


Fig. 42 COSY spectrum of compound 2 (CD_3OD).

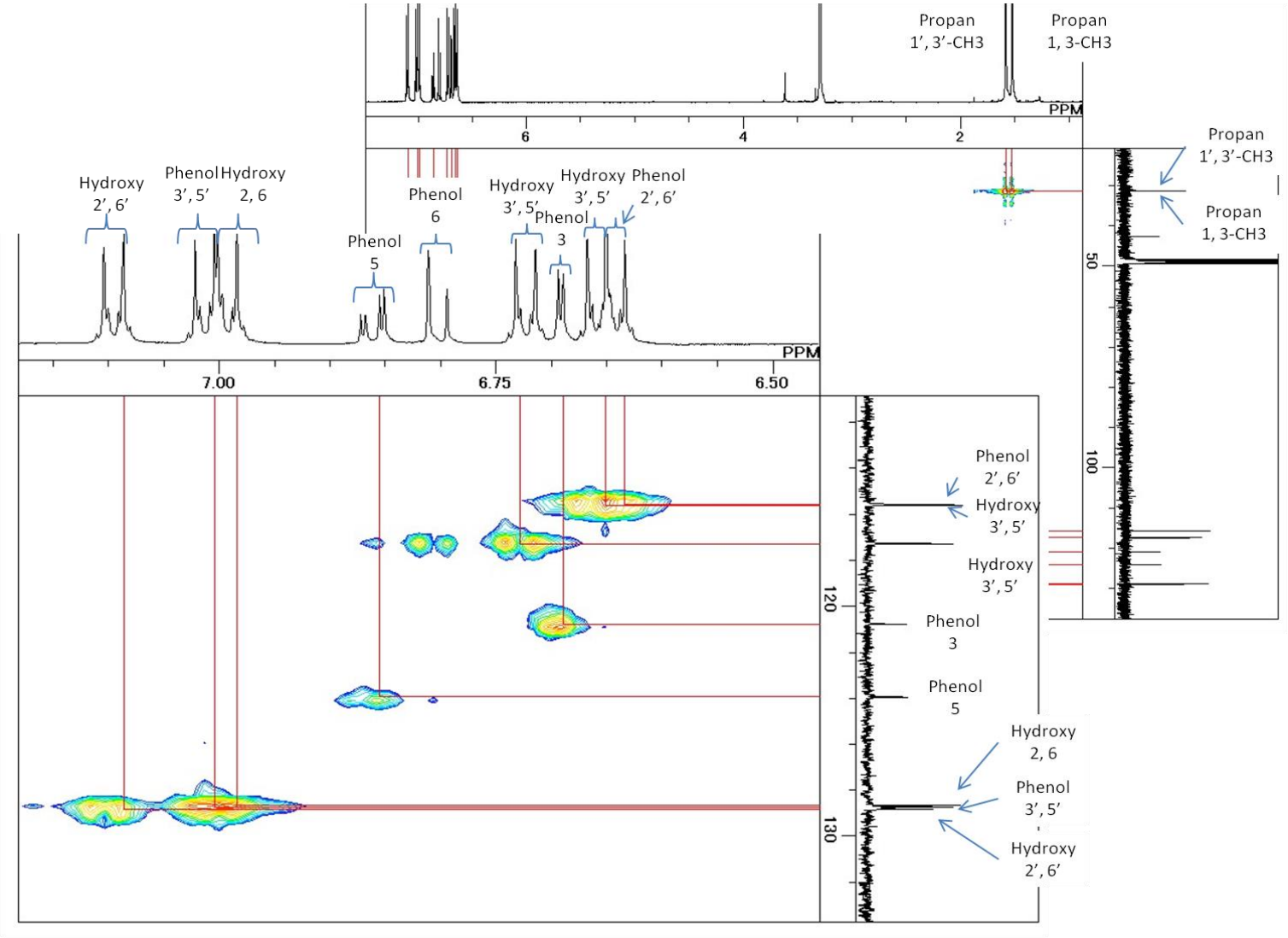


Fig. 43 HMQC spectrum of compound 2 (CD_3OD).

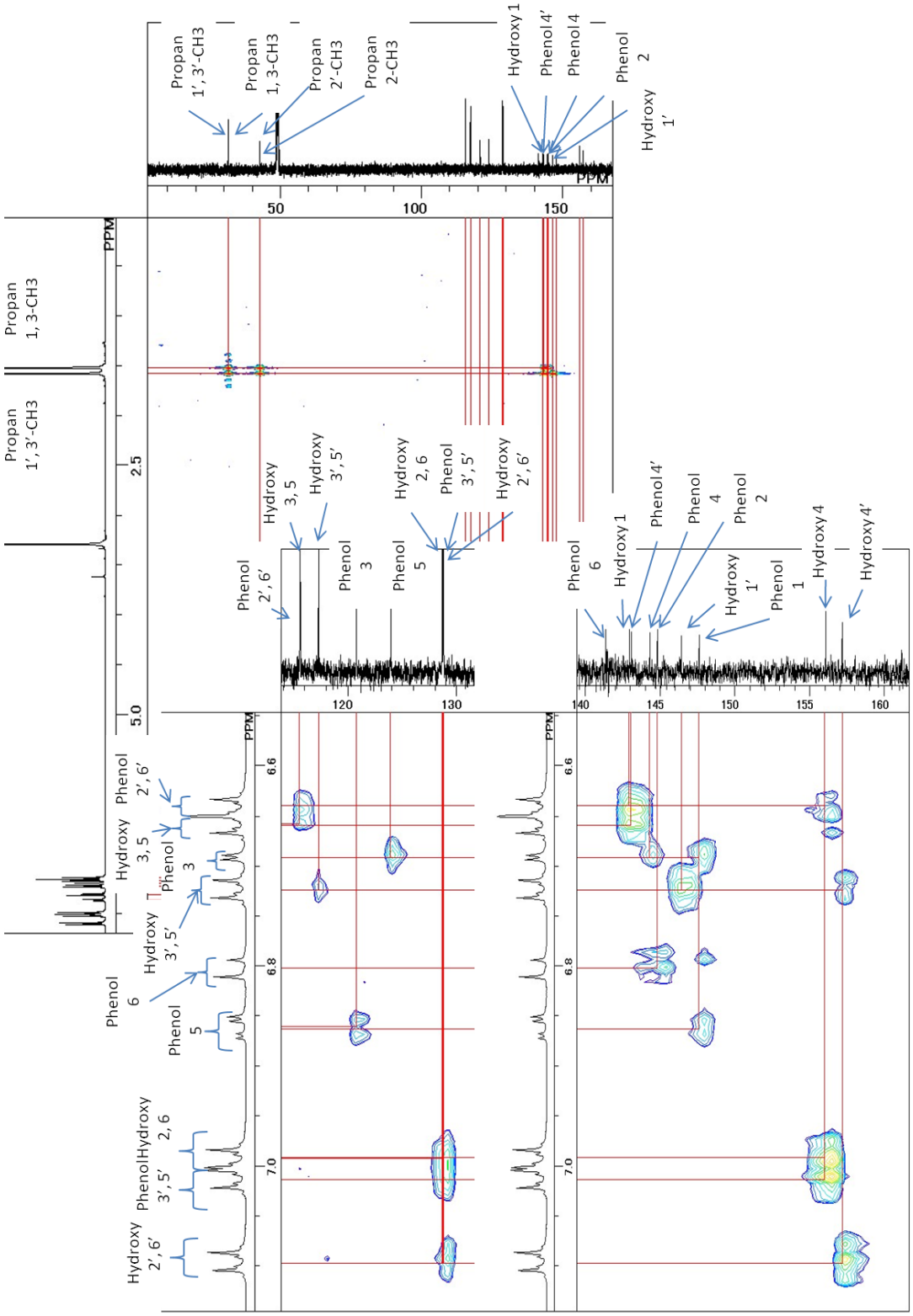


Fig. 44 HMBC spectrum of compound 2 (CD_3OD).

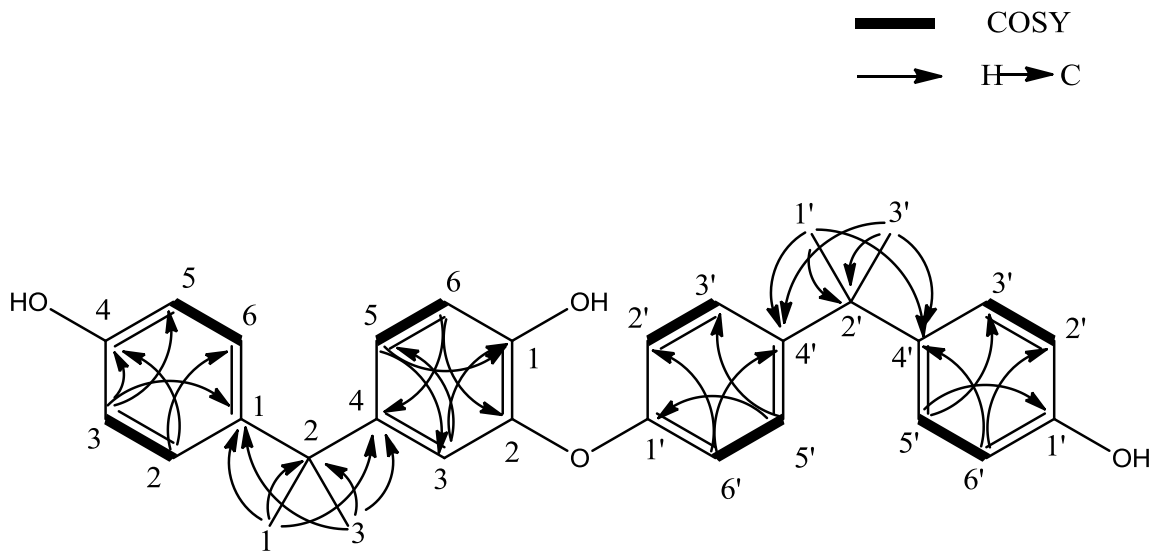


Fig. 45 COSY and HMBC correlations for compound 2.

Table 5 NMR data for the compound 2 in CD_3OD.

Position	^{1}H	^{13}C
	δ_H (mult, *J* in Hz)	δ_C
[phenol]		
1	-	147.7
2	-	144.8
3	6.69 (d, 2.0)	120.8
4	-	144.3
5	6.86 (dd, 8.5, 2.0)	124.0
6	6.80 (d, 8.5)	141.4
1’	-	156.1
2’, 6’	6.64 (d, 8.5)	115.6
3’, 5’	7.01 (d, 8.5)	128.8
4’	-	143.1
[hydroxyphenyl]		
1	-	143.0
2,6	6.99 (d, 8.5)	128.7
3,5	6.66 (d, 8.5)	115.6
4	-	156.1
1’	-	146.5
2’, 6’	7.10 (d, 8.5)	128.9
3’, 5’	6.72 (d, 8.5)	117.3
4’	-	157.2
1’’, 3’’	1.53 (s)	31.5
2’’	-	42.9
1’’’, 3’’’	1.59 (s)	31.6
2’’’	-	42.8

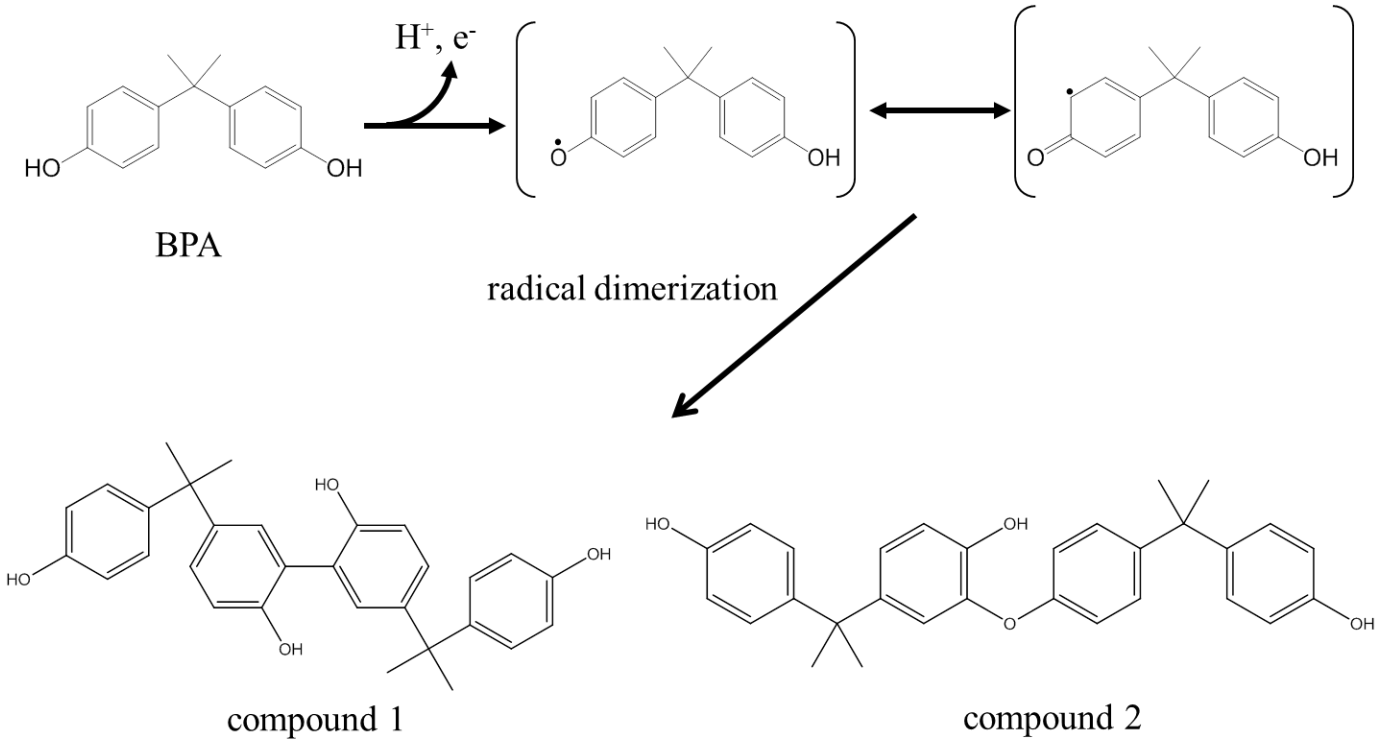


Fig. 46 Proposed mechanism of BPA by *P. sordida* YK-624 under ligninolytic condition.

Section 2
Metabolism of BPA by *P. sordida* YK-624 under non-ligninolytic condition

2.1. Introduction

The degradation of EDCs by ligninolytic enzymes has attracted the attention of many researchers (Hirano et al., 2000; Suzuki et al., 2003; Saito et al., 2004; Tamagawa et al., 2006). The degradation of BPA using fungi is also reported (Chai et al., 2005; Shin et al., 2007) but the detail mechanisms are still unknown. In the previous section, the removal of BPA by lignin peroxidase from *P. sordida* YK-624 was also demonstrated (Wang et al., 2012b) and *P. sordida* YK-624 under ligninolytic condition (Wang et al., 2013). In previous reports, BPA dimers have been formed as metabolites by radical couplings of BPA phenoxy radical generated by ligninolytic enzymes. Unfortunately, these BPA dimers are possible to be decomposed to toxic BPA by various reactions. Therefore, the transformation techniques of BPA without polymerization are necessary. In the present study, the removal of BPA by *P. sordida* YK-624 was examined under non-ligninolytic condition which hardly oxidize BPA to BPA phenoxy radical. The metabolite from BPA was also detected and the metabolic pathway of BPA by *P. sordida* YK-624 was proposed.

2.2. Materials and methods

2.2.1. Fungi and chemicals

P. sordida YK-624 (ATCC 90872) was used for the present study (Hirai et al., 1994). The fungus was maintained on PDA slants at 4 °C.

BPA was purchased from Alfa Aesar, A Johnson Matthey (Lancashire, United Kingdom). All other chemicals were of analytical-grade purity and were used without further purification.

2.2.2. Transformation of BPA

PDB medium which is a non-lignolytic (no production of lignolytic enzymes) condition was used for BPA transformation experiments. The fungus was incubated on a PDA plate at 30 °C for 3 d, and then 10 mm-diameter disks were punched out from the growing edge of mycelium. Two disks were each placed into a 100-mL Erlenmeyer flask containing 10 mL of PDB medium. After statically incubating the flasks at 30°C for 3 d, 100 μL of 100 mM BPA/DMSO were added to the cultures (final concentration at 1 mM), and then further incubated for 0-7

d (each in triplicate). The internal standard material was added to the culture, and then the culture was homogenized with 20 mL of acetone by using a Polytron PT1200E (Kinematica, Canada). The homogenate was filtrated and then evaporated to dryness. The residue was analyzed by HPLC for the quantification of BPA under the following conditions: column, Wakosil-II 5C18HG (4.6×150 mm); mobile phase, 10 to 100% MeOH gradient for 60 min; flow rate, 1 mL min^{-1}; and UV wavelength, 277 nm.

2.2.3. Cytochrome P450 inhibitor experiment

After preculturing *P. sordida* YK-624 for 3 d, BPA (final concentration at 1 mM) and PB (final concentration, 0, 0.1 and 1 mM) were added to cultures. The cultures were further incubated for 1-4 d, and each culture was then subjected to HPLC for the quantification of BPA, as described above.

2.2.4. Metabolism experiment of BPA

Inoculated cultures of *P. sordida* YK-624 were prepared under the PDB medium. After static incubation at 30 °C for 3 d, BPA (final concentration, 1 mM) was added to these cultures. These cultures were further incubated for 7 d and then filtrated with membrane filters (pore size; 0.2 μm). The resulting solution was extracted 3 times with equal amount of EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was cleaned on a TLC, to obtain 3 fractions. Each fraction was analyzed by TLC (aluminum sheets). Silica gel plates (Merck F254; Merck, Darmstadt, Germany) were used for TLC. The metabolite was further separated by HPLC (column: CAPCELL PAK C18 AQ; 20×250 mm, Shiseido, Japan) using 10 to 100% MeOH gradient. The purified metabolite was analyzed by HR-ESI-MS and NMR, including distorsionless enhancement by DEPT, COSY, HMQC, and HMBC experiments, respectively. The HR-ESI-MS data were measured using a JMS-T100LC mass spectrometer. ^{1}H NMR spectra were recorded using a Jeol Lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz.

2.2.5. Synthesis of hydroxy-BPA

A quantity of 5 g of *p*-hydroxyacetophenone was stirred with 25 mL pyridine until fully dissolved. After the addition of 12.5 mL acetic anhydride, the reaction mixture was stirred for 16 h at room temperature. The solution then was extracted with EtOAc. The organic layer was

washed with H_2O, and then purified by silica gel column chromatography with 30% EtOAc in hexane to obtain 4-acetylphenyl acetate (6 g, 33.6 mmol). A quantity of 21.05 g of methyltriphenyl-
phosphonium bromide in 227.6 mL anhydrous tetrahydrofuran was added dropwise, under argon, at 0 °C in the presence of 35 mL of *n*-butyllithium. The solution turned orange as the addition proceeded. After stirring at 0 °C for an additional half hour, a solution of 5.69 g 4-acetylphenyl acetate in 75.68 mL anhydrous tetrahydrofuran was added dropwise; the resulting mixture was warmed to room temperature and stirred for 36 h. The solution then was extracted with EtOAc. The organic layer was washed with H_2O, and then purified by silica gel column chromatography with a 10% EtOAc in hexane to obtain 4-isopropenylphenol (1.73 g, 12.89 mmol). A quantity of 1.67 g of catechol, together with 6.75 mL of benzene- methanol (MeOH) (90:10) and 0.05 mL of 36% HCl, was stirred at 30 °C. A solution of 1.73 g 4-isopropenylphenol in 7.61 mL benzene-MeOH (90:10) was added dropwise over 1 h, and stirred for 5 h at 30 °C. The solution then was cooled to room temperature, 1.11 mL 1 M NaOH was added, extracted with EtOAc. The organic layer was washed with brine, and then purified by silica gel column chromatography with a 20% EtOAc in hexane to obtain hydroxy-BPA (1.72 g, 7.05 mmol, 20.21%). ^{1}H NMR (500 MHz, CD_3OD) yielded the following signals: 1.54 (s, OMe), 6.57 (dd, H-5), 6.62 (d, H-3), 6.66 (dd, H-6, 3’’, 5’’), 7.02 (dd, H-2’’, 6’’), ^{13}C-NMR (500 MHz, CD_3OD): 31.6 (OMe), 42.5 (C-2’), 115.5 (C-6, 3’’, 5’’), 115.6 (C-3), 118.9 (C-5), 128.7 (C-2’’, 6’’), 143.5 (C-4), 143.7 (C-1), 144.4 (C-1’’), 145.4 (C-2), 155.7 (C-4’’). High-resolution electrospray ionization mass spectra (HR-ESI-MS, negative mode) of the final product was detected at 243.1021, consistent with the calculated value of 243.1015 for $C_{15}H_{15}O_3$ $[M-H]^-$.

2.2.6. Elimination of hydroxy-BPA

PDB, was used for hydroxy-BPA metabolism experiments. The fungus was incubated on a PDA plate at 30 °C for 3 d, and then 10 mm-diameter disks were punched out from the growing edge of mycelium. Two disks were placed into each 100-mL Erlenmeyer flask containing 10 mL of PDB medium. After static incubation of the flasks at 30 °C for 3 d, 100 μL of 30 mM hydroxy-BPA (in DMSO) were added to each flask (to a final hydroxy-BPA concentration of 0.3 mM), and the cultures were further incubated (in triplicate) for up to 7 d. An internal standard was added to each culture, and the cultures were homogenized

with 20 mL of acetone by using a Polytron PT1200E. The homogenate was filtered and then evaporated to dryness. The residue was analyzed by HPLC for the quantification of hydroxy-BPA under the following conditions: column, Wakosil-II 5C18HG (4.6×150 mm); mobile phase, 10 to 100% MeOH gradient for 60 min; flow rate, 1 mL min^{-1}; and UV wavelength, 277 nm.

2.2.7. Metabolism experiment of hydroxy-BPA

Inoculated cultures (total 5 L PDB medium) of *P. sordida* YK-624 were prepared under the conditions described above. After static incubation at 30 °C for 3 d, 5 mL of 300 mM hydroxy-BPA (to a final concentration of 0.3 mM) was added to these cultures. The cultures were further incubated for 7 d and evaporated to about 250 mL. The remaining cultures were extracted 4 times with an equal amount of EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was separated by HPLC (column: C30-UG-15/30; 50×500 mm; Nomura Chemical, Seto, Japan) using 70% MeOH to obtain 5 fractions. Each fraction was analyzed by TLC and HPLC (column: C30-UG-5; 4.6×250 mm; Nomura Chemical, Seto, Japan). Silica gel plates (Merck F254; Merck, Darmstadt, Germany) were used for analytical TLC. The metabolites were further separated by HPLC (column: C30-UG-5; 20× 250 mm; Nomura Chemical, Seto, Japan) using 70% MeOH. The purified metabolites were analyzed by HR-ESI-MS and NMR, including distorsionless enhancement by DEPT, COSY, HMQC, and HMBC experiments. The HR-ESI-MS data were measured using a JMS-T100LC mass spectrometer. ^{1}H-NMR spectra were recorded using a Jeol Lambda-500 spectrometer at 500 MHz, while ^{13}C-NMR spectra were recorded on the same instrument at 125 MHz.

2.2.8. Quantitative analysis

After preculturing *P. sordida* YK-624 under the conditions described in *Section 2.2.3.* for 3 d, 100 μL of 50 mM BPA in DMSO were added to the cultures (to a final concentration of 0.5 mM hydroxy-BPA), which were then further incubated for up to 7 d (each in triplicate). An internal standard was added to each culture, and then the culture was homogenized with 20 mL of acetone by using a Polytron PT1200E. The homogenate was filtered and then evaporated to dryness. The residue was analyzed by HPLC for the quantification of BPA and its metabolites under the following conditions: column, Wakosil-II 5C18HG

(4.6×150 mm); mobile phase, 10 to 100% MeOH gradient for 60 min; flow rate, 1 mL min^{-1}; and UV wavelength, 277 nm.

2.2.9. Membrane estrogen receptor competition assay

Cultures of HEK231 cells were stably transfected with a construct encoding the membrane estrogen receptor (GPR30) and estrogen receptor competition studies were carried out as described before (Thomas and Dong, 2006). Briefly, human HEK293 cells stably transfected with the human GPR30-encoding construct were cultured in Dulbecco's modification of Eagle's medium/Ham's F-12 medium with 5% fetal bovine serum and 100 μg mL^{-1} of G418 in 150-mm diameter plates. The medium was replaced every 1-2 d and the cells that reached 80% confluence after 3 d were used in the experiments.

Procedures for the preparation of plasma membrane fractions and assay of steroid membrane receptors were performed as previously described (Tokumoto et al., 2007) with the following modifications. [^{3}H] E_2 was assayed with binding to plasma membranes of HEK293 cells transfected with a construct encoding goldfish mPR Presoaking with Tween 80 was omitted. Radio-labeled steroid [2, 4, 6, 7-^{3}H]E_2 ([^{3}H]E_2, ~89 Ci $mmol^{-1}$) was purchased from PerkinElmer Life & Analytical Sciences (Waltham, MA). The competitive binding assay tubes contained [^{3}H]E_2 at 4 nM and competitors at concentrations ranging from 1 nM to 10 μM. After incubation (30 min, 4°C) with the membrane fractions, the reaction was stopped by filtration (Whatman GF/B filters). Following washing of the filters, bound radioactivity was measured by scintillation counting. The displacement of [^{3}H]E_2 binding by the competitors was expressed as a percentage of the maximum specific binding of E_2

2.3. Results

2.3.1. Eliminations of BPA by *P. sordida* YK-624

In the present study, PDB medium was used for the transformation experiment of BPA. When *P. sordida* YK-624 was cultured in PDB medium, BPA was decreased by 51, 71, 83% after 1, 4, and 7 d of incubation, respectively (Fig. 47). These results suggested that *P. sordida* YK-624 had the ability for the transformation of BPA under the non-ligninolytic conditions such as PDB medium.

2.3.2. Identification of the metabolites from BPA

To determine the metabolite(s) produced during the degradation of BPA, 7-d cultures of *P. sordida* YK-624 in PDB medium was

supplemented with 0.5 mM BPA were subjected to TLC and HPLC. From this analysis, one potential BPA metabolite was detected (data not shown). The purified metabolite was then subjected to high-resolution electrospray ionization mass spectrometry analysis, which yielded a molecular ion at *m/z* 243.1015 $[M-H]^-$ (calculated for $C_{15}H_{15}O_3$, 243.1021), indicating that the molecular formula of this compound was $C_{15}H_{16}O_3$ (Fig. 48). This formula suggested that the metabolite might be a hydroxylation form of BPA. The structure of the purified metabolite was further characterized by NMR analyses (Figs. 49-54). Table 6 lists the chemical-shift assignment data for the metabolite of BPA. HMBC (H-3/C-2', H-5/C-2') confirmed that the metabolite was 4-(2-(4-hydroxyphenyl)propan-2-yl)benzene-1,2-diol, which hydroxylated at the *ortho*-position of BPA (Fig. 55).

2.3.3. Effect of cytochrome P450 inhibitor

The effect of the cytochrome P450 inhibitor on the transformation of BPA by *P. sordida* YK-624 was investigated using PB. The transformation rate of BPA after the addition of 0.1 and 1 mM PB into cultures of *P. sordida* YK-624 is shown in Fig. 56. In contrast to the transformation rate of BPA in cultures without PB, markedly lower transformation activity of BPA was observed in cultures containing PB. In the presence of 0.1 and 1 mM PB, 63 and 38% of BPA were eliminated after 4 d of incubation, respectively, compared to 70% in cultures lacking PB.

2.3.4. Eliminations of hydroxy-BPA

When *P. sordida* YK-624 was cultured in PDB medium, hydroxy-BPA levels fell by 66% after 7 d of incubation (Fig. 57). These results suggested that *P. sordida* YK-624 can metabolize hydroxy-BPA under non-ligninolytic condition.

2.3.5. Identification of the metabolites from hydroxy-BPA

To determine the structure of metabolites produced during the transformation of hydroxy-BPA, 7-day cultures of *P. sordida* YK-624 in PDB medium supplemented with 0.3 mM BPA were subjected to TLC and HPLC (Fig. 58); two major BPA metabolites were detected. Using HR-ESI-MS, the molecular formula of compound I was determined as $C_{16}H_{18}O_3$, with an *m/z* 281.1133$[M+Na]^+$ (calculated for $C_{16}H_{18}NaO_3$, 281.1154). This formula suggested that a hydroxy group of hydroxy-BPA had been converted to a methoxy moiety. The NMR spectra of compound 4 were roughly in accordance with the hydroxy-BPA (Figs. 59-63). Table

7 lists the chemical-shift assignment data for compound 4. HMBC correlations (2-OCH_3/C-2) confirmed that compound 4 was methylated hydroxy-BPA, 4-(2-(4-hydroxyphenyl)propan-2-yl)-2-methoxyphenol (methoxy-BPA, Fig. 64). Using HR-ESI-MS, the molecular formula of compound 5 was determined as $C_{17}H_{20}O_3$, with an *m/z* 295.1289$[M+Na]^+$(calculated for $C_{17}H_{20}NaO_3$, 295.1310, Fig. 65). This formula suggested that the metabolite might be a dimethoxy form of hydroxy-BPA. The complete assignment of all the protons and carbons was accomplished by DEPT, HMQC, COSY, and HMBC analyses (Figs. 66-71, Table 8). HMBC correlations (3-OCH_3/C-3, 4-OCH_3/C-4) confirmed that compound 5 was 4-(2-(3, 4-dimethoxyphenyl)propan-2-yl)phenol (dimethoxy-BPA, Fig. 72).

2.3.6. Quantity of the metabolites from BPA by *P. sordida* YK-624 under non-ligninolytic condition

To evaluate the transformation of BPA by *P. sordida* YK-624 under non-ligninolytic condition, the time course of BPA metabolism was investigated (Fig. 73). Decreases in the concentration of BPA were detected starting at 2 d, with quantitative conversion to hydroxy-BPA. Most of the decreased BPA was transformed to hydroxy-BPA, indicating hydroxy-BPA was the main product. Subsequently, the methoxy-BPA and the dimethoxy-BPA forms were detected at low concentrations starting at 4 d, with levels remaining at or below 4 μmol L^{-1} by 7 d.

2.3.7. Steroid binding assay of BPA and the metabolites

The estrogenic activities of BPA metabolites were assayed using a steroid binding assay. BPA exhibited significant competition with estrogen, with a IC_{50} of 1.8 μM. Affinities of hydroxy-BPA, methoxy-BPA, and dimethoxy-BPA were reduced 67, 100, and 83% (respectively) compared to that of the parent compound (Fig. 74).

4. Discussion

Biotransformation of BPA has also been reported by many researchers. More recently, the removal of BPA by *P. sordida* YK-624 under ligninolytic condition has been reported (Wang et al., 2013). BPA dimers are formed by radical couplings at extracellular region without the incorporation into the cell of *P. sordida* YK-624. However, BPA dimers may be returned to BPA by various reactions. BPA glucuronide is the major metabolite of BPA in rat hepatocytes (Nakagawa and Tayama, 2000; Pritchett et al., 2002). In plants, cells of *Eucalyptus perriniana* and

Portulaca oleracea cv. metabolize BPA to its hydroxyl products (Hamada et al., 2002; Watanabe et al., 2012). In fungi, BPA is metabolized to BPA-*O*-β-D-glucopyranoside by *Aspergillus fumigatus* (Yim et al., 2003). In the present study, hydroxy-BPA was detected as a metabolite of BPA.

As noted above, the metabolism of BPA has been studied by many researchers. Based on the results of the present study, a novel metabolic pathway is proposed for BPA degradation by *P. sordida* YK-624 under non-ligninolytic condition (Fig. 75). Firstly, the generation of a BPA metabolite that was hydroxylated at the *ortho*-position of BPA (Wang et al., 2013b). Watanabe et al. (2012) suggested polyphenol oxidases were likely to contribute to BPA hydroxylation by plants of the *Portulaca* genus. *P. sordida* YK-624, a white-rot fungus, produces lignin peroxidases and manganese peroxidase as ligninolytic enzymes, but does not exhibit laccase activity. The functional diversity of cytochrome P450s in white-rot fungi has been studied because cytochrome P450s play an important role on degrading a wide variety of recalcitrant aromatic compounds (Kamei et al., 2005; Xiao et al., 2011; Wang et al., 2012a). Hata et al. (2010) suggested that the hydroxylation catalyzed by cytochrome P450 in *P. sordida* YK-624 might be involved in the elimination and detoxification of diclofenac and mefenamic acid. Our previous study showed that the transformation of BPA was inhibited by PB, which is a common inhibitor of cytochrome P450s, under non-ligninolytic conditions (Wang et al., 2013b). In the present study, hydroxy-BPA was further metabolized by this fungus, generating two additional methylated metabolites. *O*-methyltransferases are involved in the biosynthesis of veratryl alcohol (VA), which is a secondary metabolite produced by many species of white-rot fungi (Harper et al., 1996). Identification of 3-*O*-methyltransferase and 4-*O*-methyltransferase activities has been reported in *P. chrysosporium* (Coulter et al., 1993; Jeffers et al., 1997). Moreover, *P. sordida* YK-624 also produces VA under ligninolytic and non-ligninolytic conditions (data not shown). Hence, these methylations of hydroxy-BPA might be mediated in *P. sordida* YK-624 by intracellular *O*-methyltransferases.

Due to the high production volumes, the toxicity of BPA has been intensively studied since the 1970s. Estrogenic activity of BPA was first reported in 1993 (Krishnan et al., 1993). The steroid binding assay was used to evaluate the estrogenic activity of BPA and BPA metabolite in the present study. BPA showed significant competition with IC_{50} of 1.8 μM.

Just a few affinities for binding to the membrane estrogen receptor were detected in hydroxy-BPA (Fig. 74). Since the metabolite hydroxy-BPA has lower estrogenic toxicity than BPA, diminishing environmental estrogenic activity of BPA is possible using *P. sordida* YK-624.

In this report, *P. sordida* YK-624 can metabolize BPA to yield derivatives with reduced estrogenic activity. Based on the structures of these metabolites, the proposed mechanism is as follows: BPA is monooxygenated (by cytochrome P450) to form hydroxy-BPA; this primary metabolite then is methylated to generated two novel compounds, methoxy-BPA and dimethoxy-BPA (Fig. 75). To the best of our knowledge, this work represents the first elucidation of the hydroxylation and then methylation of BPA by a white-rot fungus.

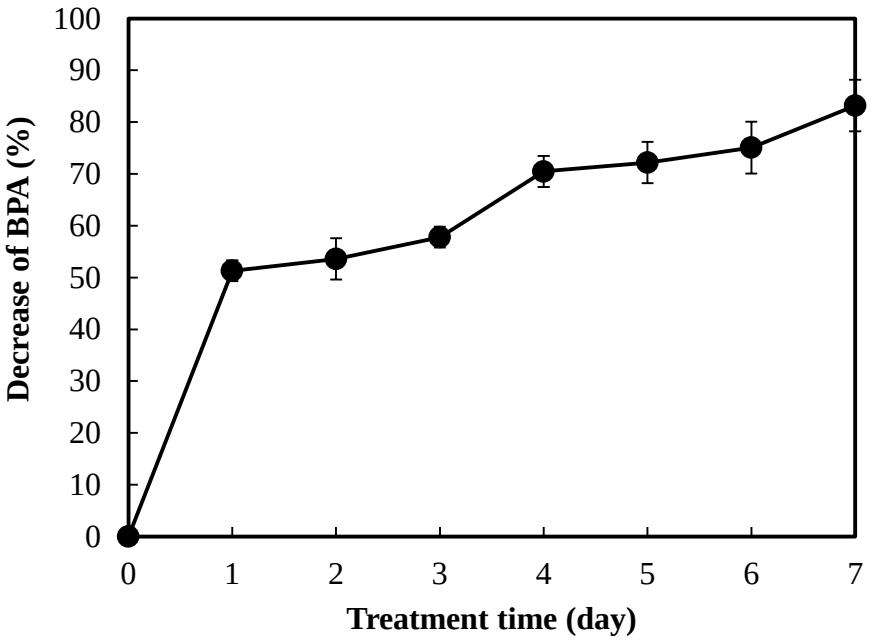


Fig. 47 Time course for BPA transformation by *P. sordida* YK-624 under non-ligninolytic condition. Values are the means ± SD of triplicate samples.

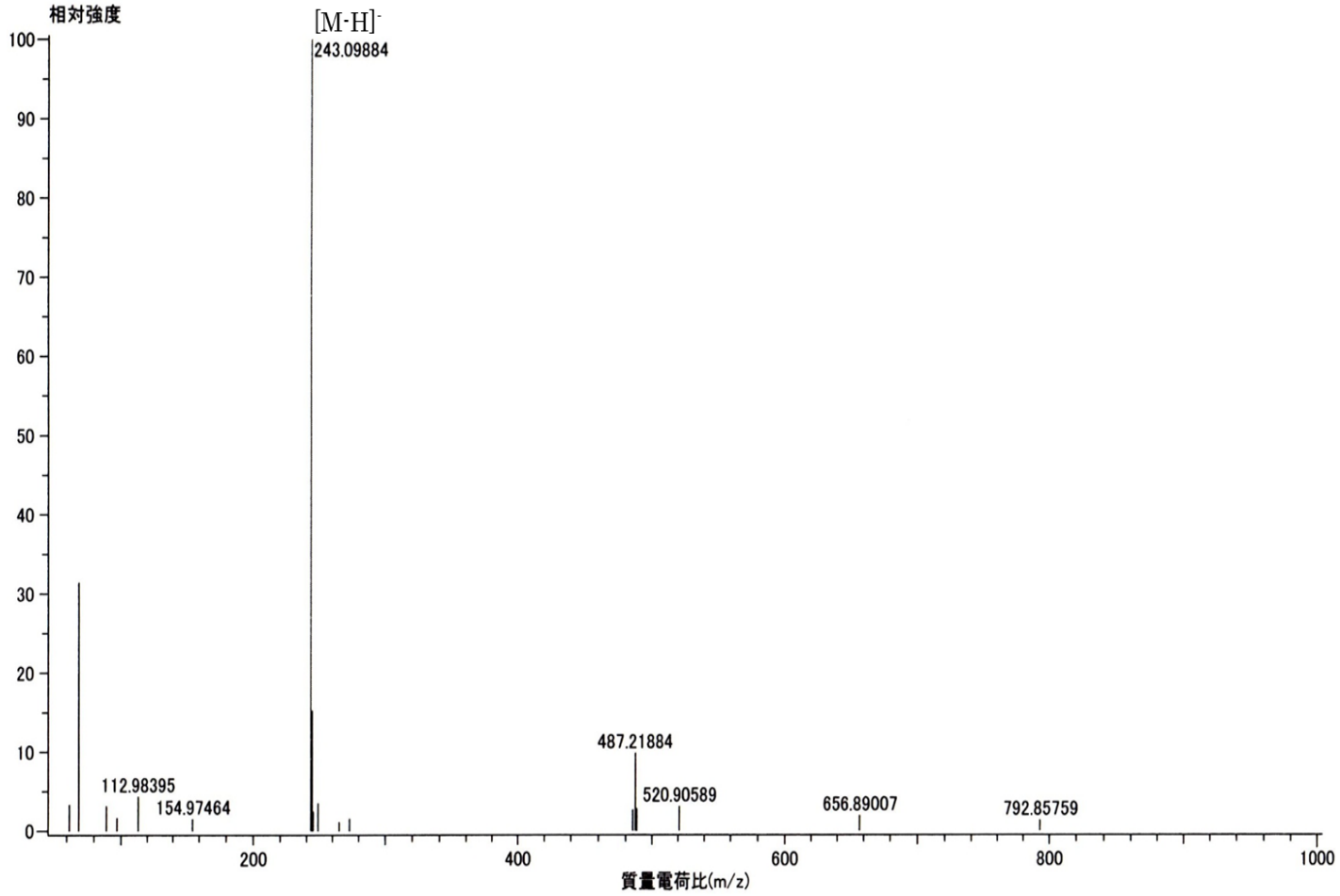


Fig. 48 ESI-MS spectrum of compound 3.

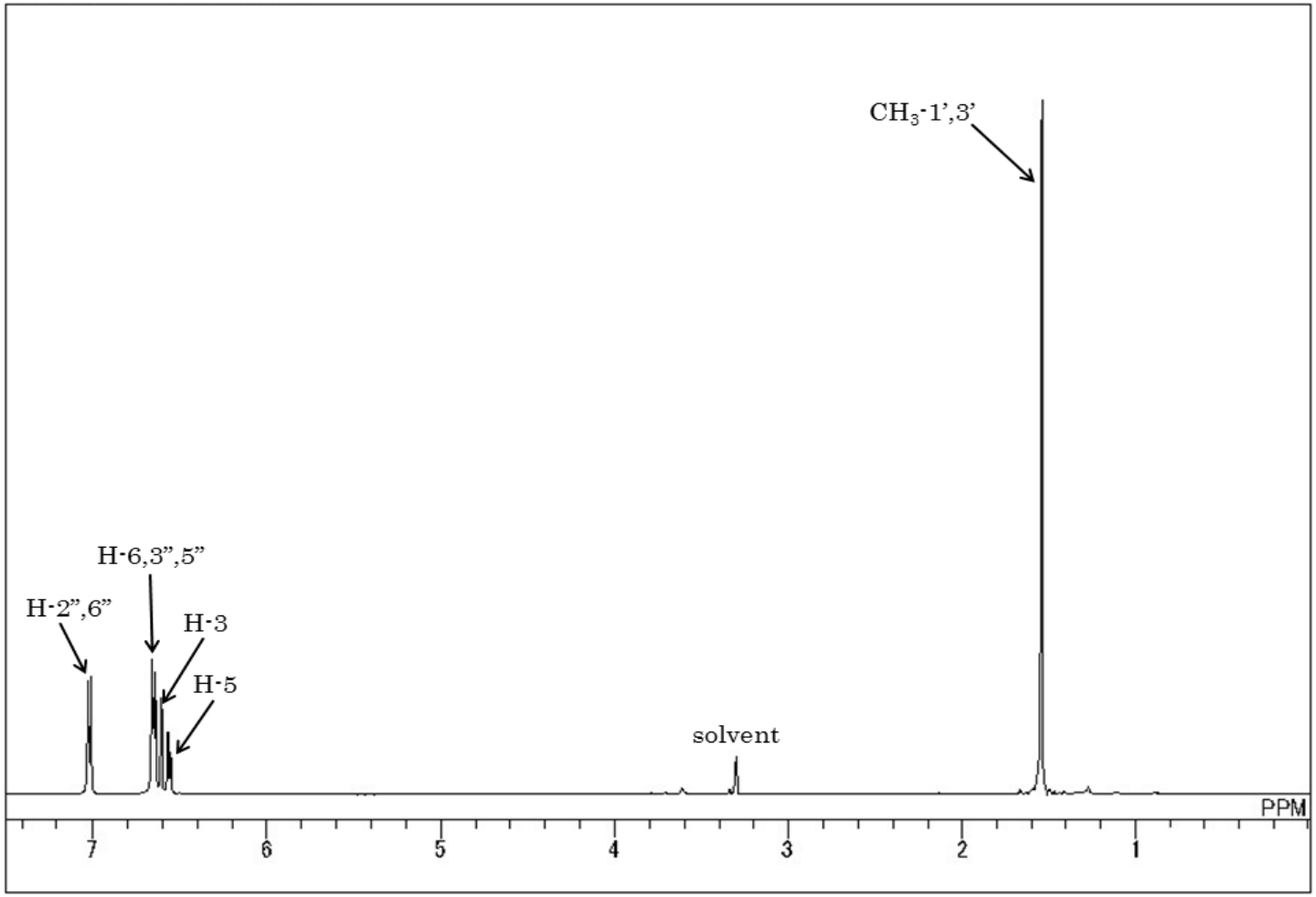


Fig. 49 ^{1}H NMR spectrum of compound 3 (CD_3OD).

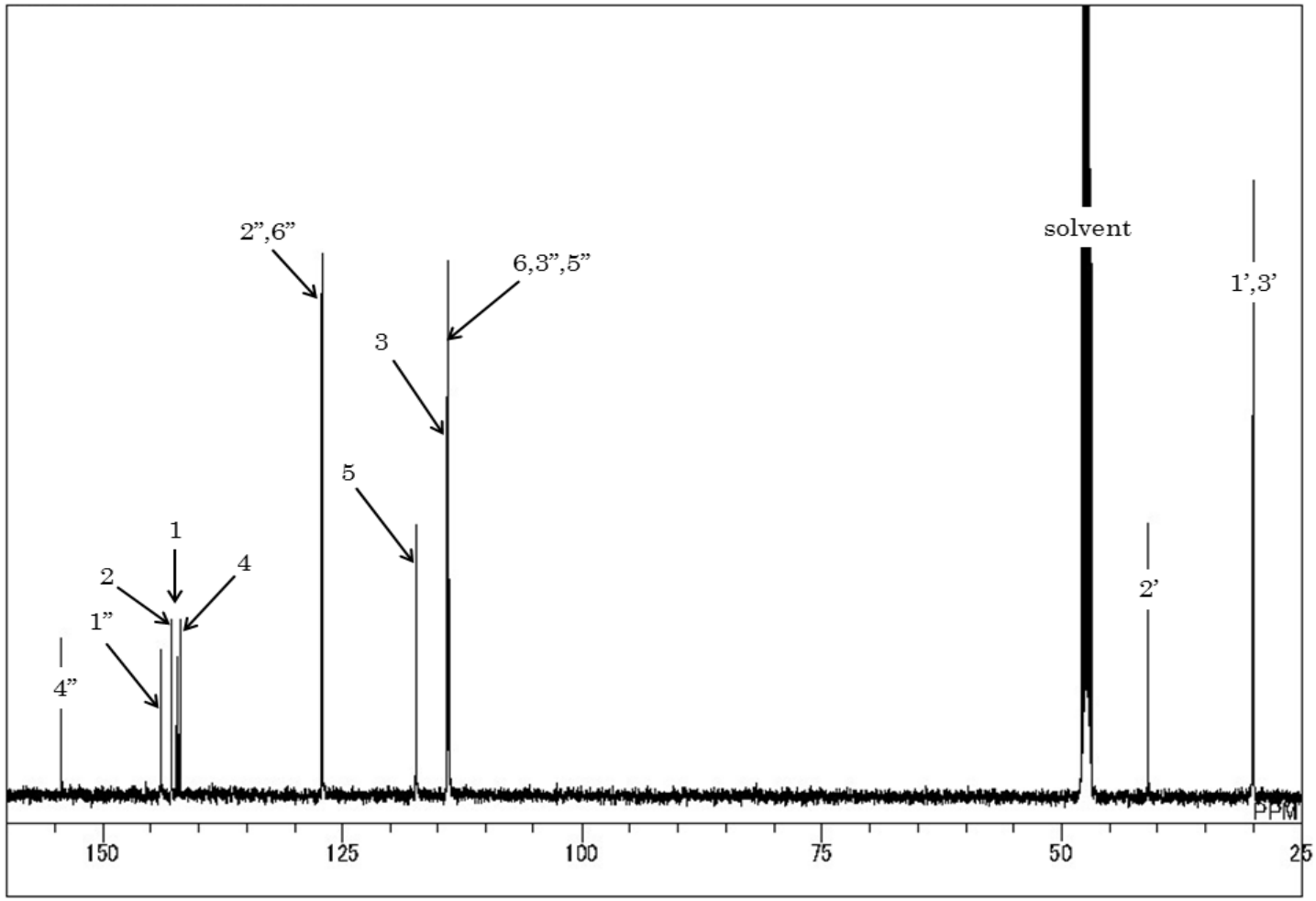


Fig. 50 ^{13}C NMR spectrum of compound 3 (CD_3OD).

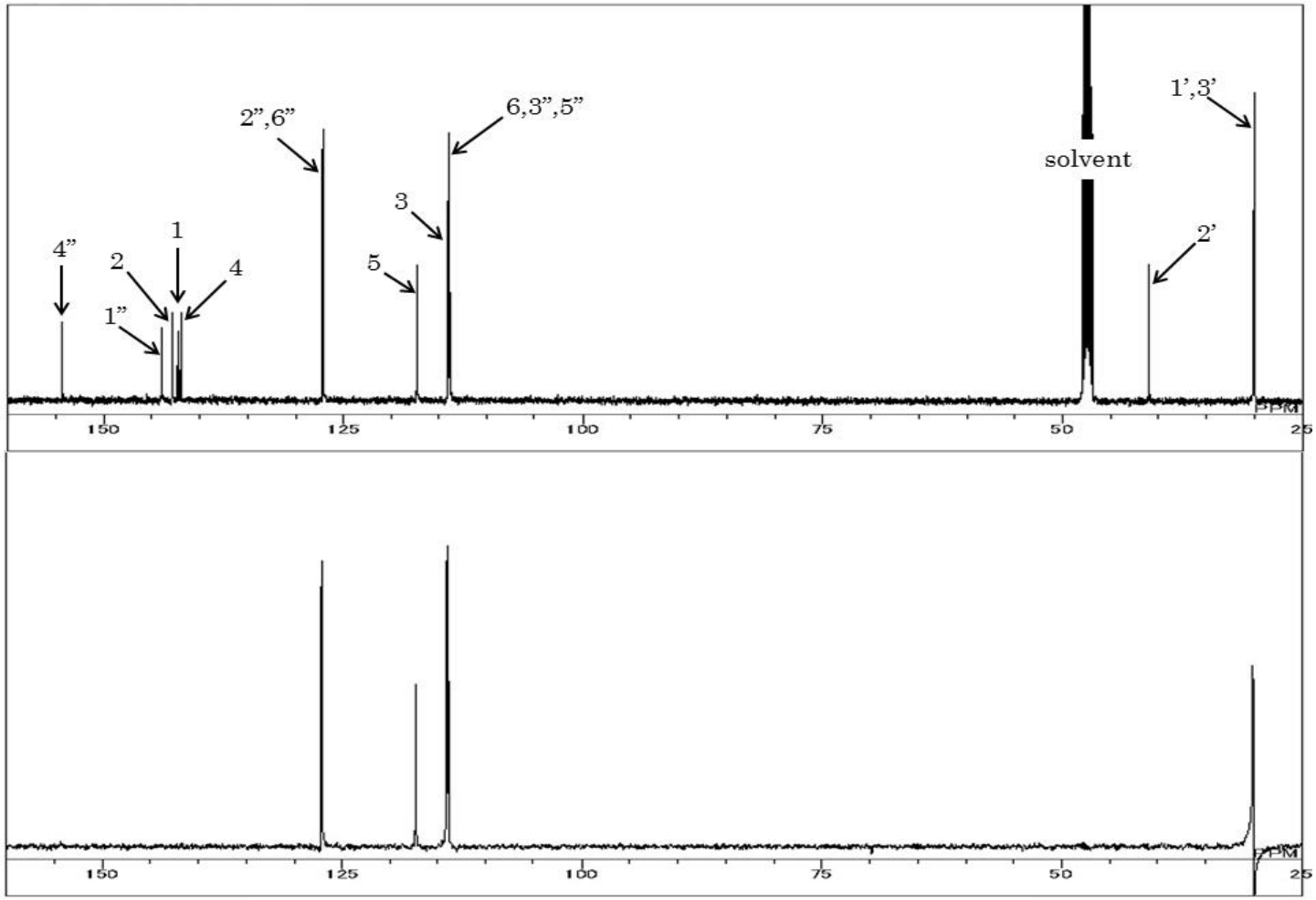


Fig. 51 DEPT spectrum of compound 3 (CD_3OD).

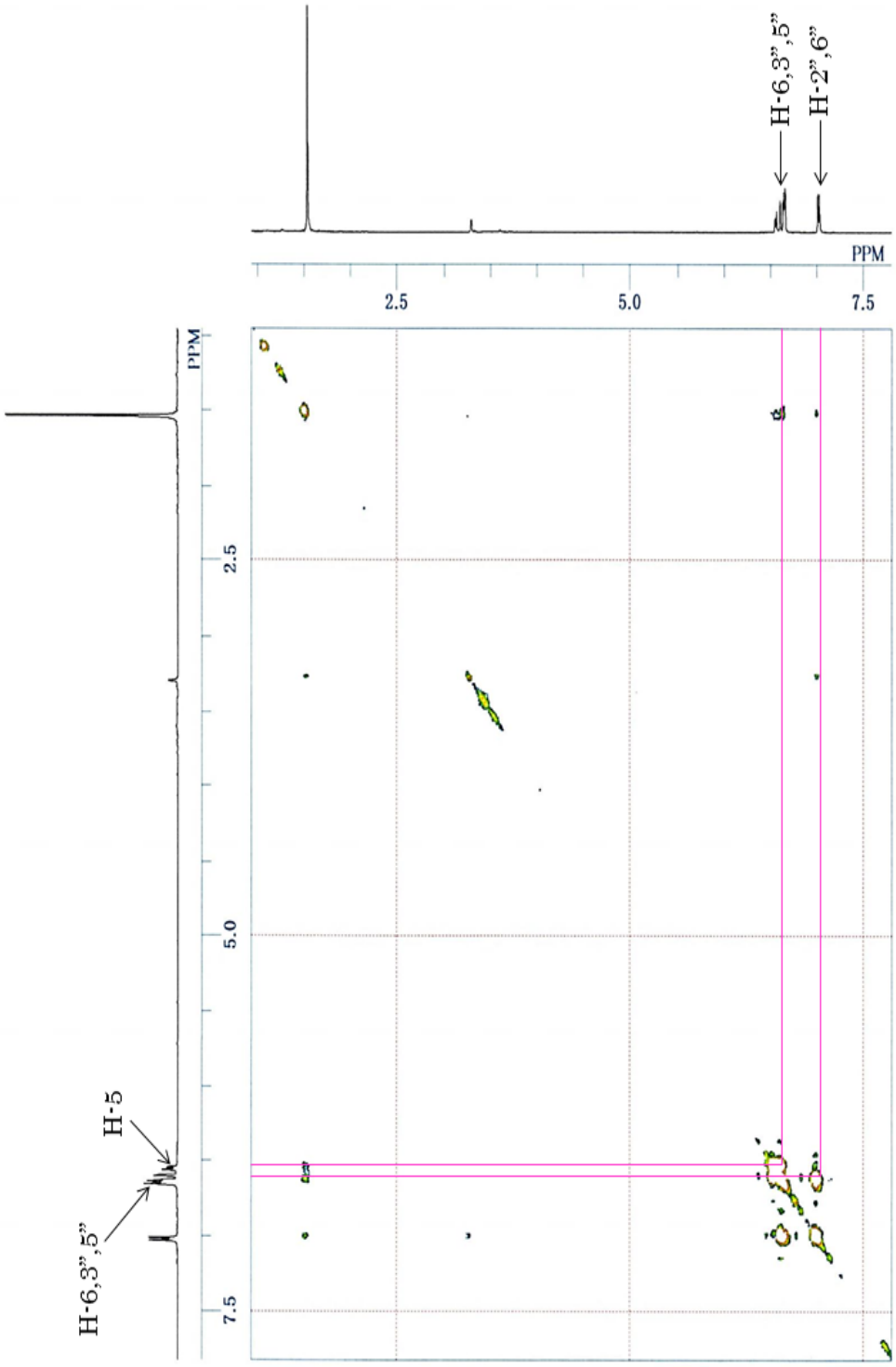


Fig. 52 COSY spectrum of compound 3 (CD_3OD).

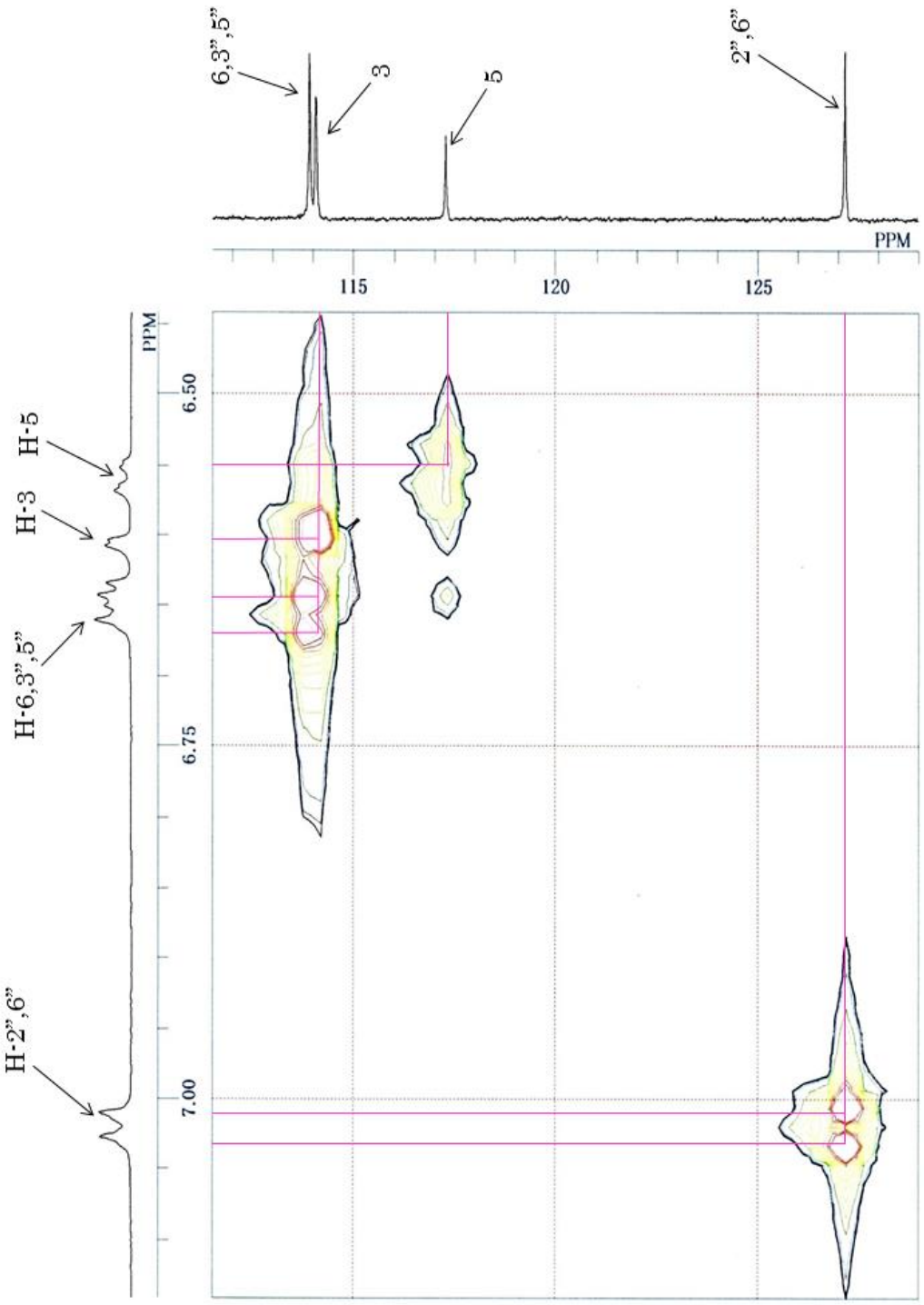


Fig. 53 HMQC spectrum of compound 3 (CD_3OD).

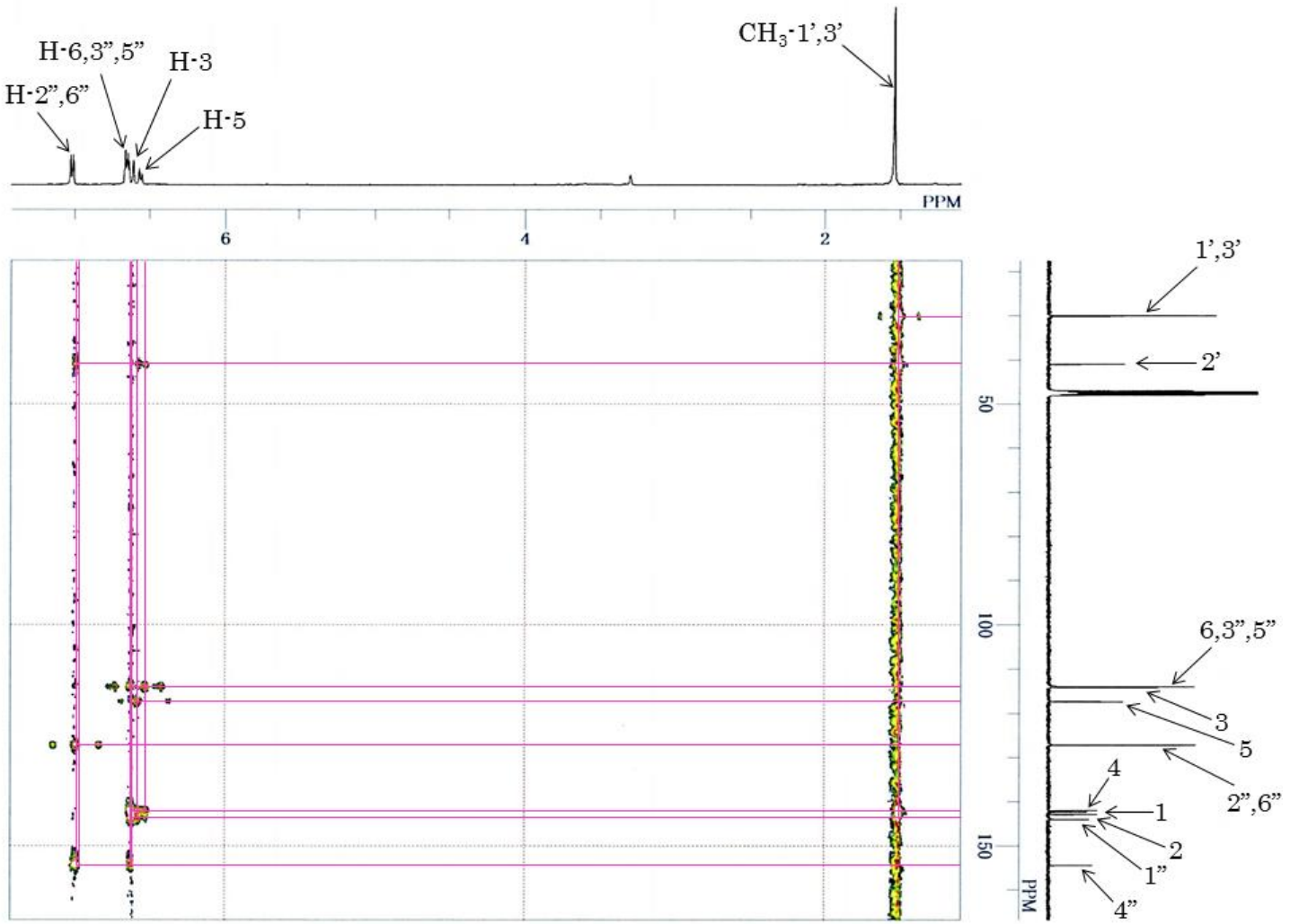
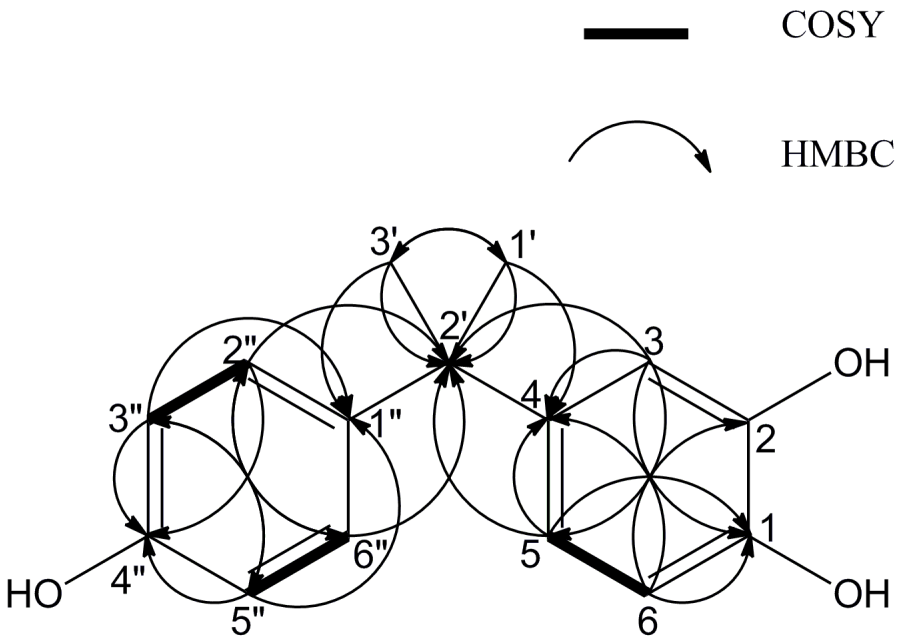


Fig. 54 HMBC spectrum of compound 3 (CD_3OD).



4-(2-(4-hydroxyphenyl)propan-2-yl)benzene-1,2-diol

Fig. 55 COSY and HMBC correlations for compound 3.

Table 6 ^{1}H and^{13}C NMR data for hydroxy-BPA (in CD_3OD).

Position	^{1}H	^{13}C
	δ_H (mult, *J* in Hz)	δ_C
1	-	142.3
2	-	142.9
3	6.61 (d, 2.2)	114.1
4	-	141.9
5	6.56 (dd, 7.9, 2.2)	117.3
6	6.64 (d, 7.9)	113.9
1’’	-	143.9
2’’	7.02 (d, 9.0)	127.2
3’’	6.65 (d, 8.5)	113.9
4’’	-	154.3
5’’	6.65 (d, 8.5)	113.9
6’’	7.02 (d, 9.0)	127.2
1’-CH_3	1.54 (s)	30.1
2’	-	41.0
3’-CH_3	1.54 (s)	30.0

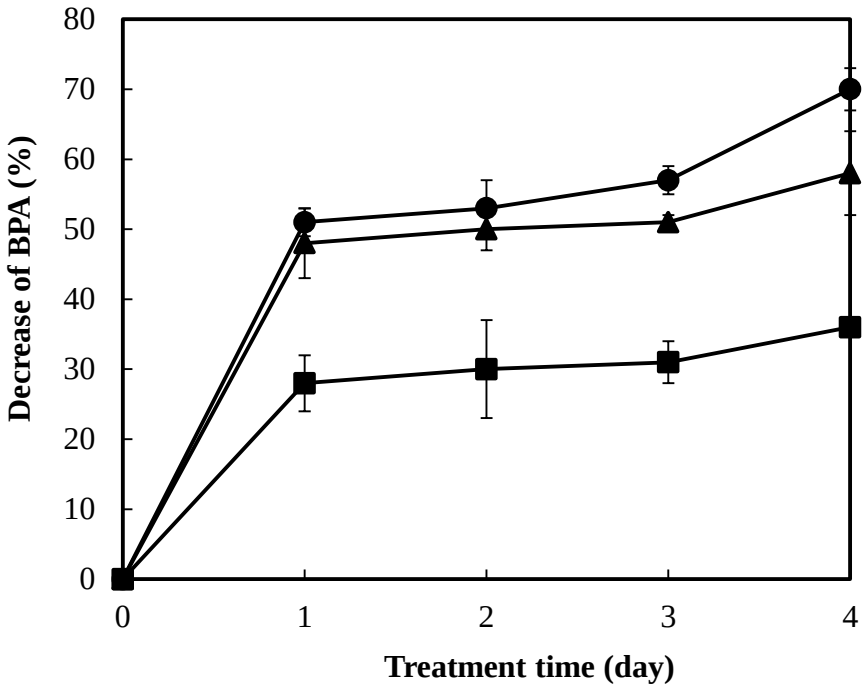


Fig. 56 Effect of cytochrome P450 inhibitor PB on the transformation of BPA by *P. sordida* YK-624. ● without PB, ▲ 0.1 mM PB, ■ 1 mM PB. Values are the means ± SD of triplicate samples.

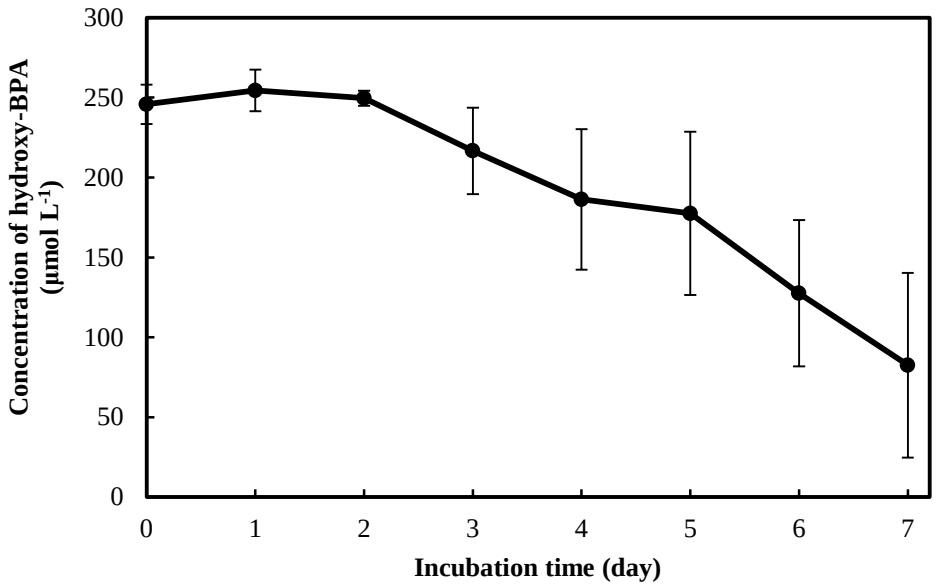


Fig. 57 Time course of hydroxy-BPA metabolism by *P. sordida* YK-624 under non-ligninolytic condition. Values are the means±SD of triplicate samples.

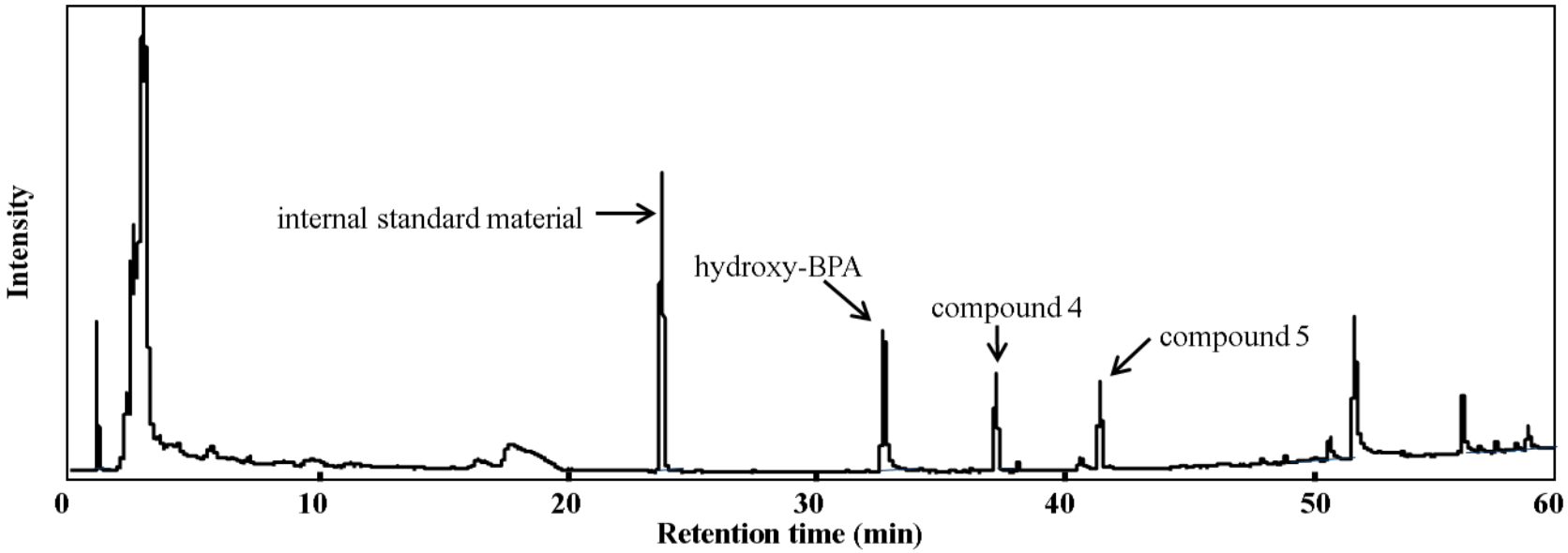


Fig. 58 Detection of the hydroxy-BPA metabolite by HPLC.

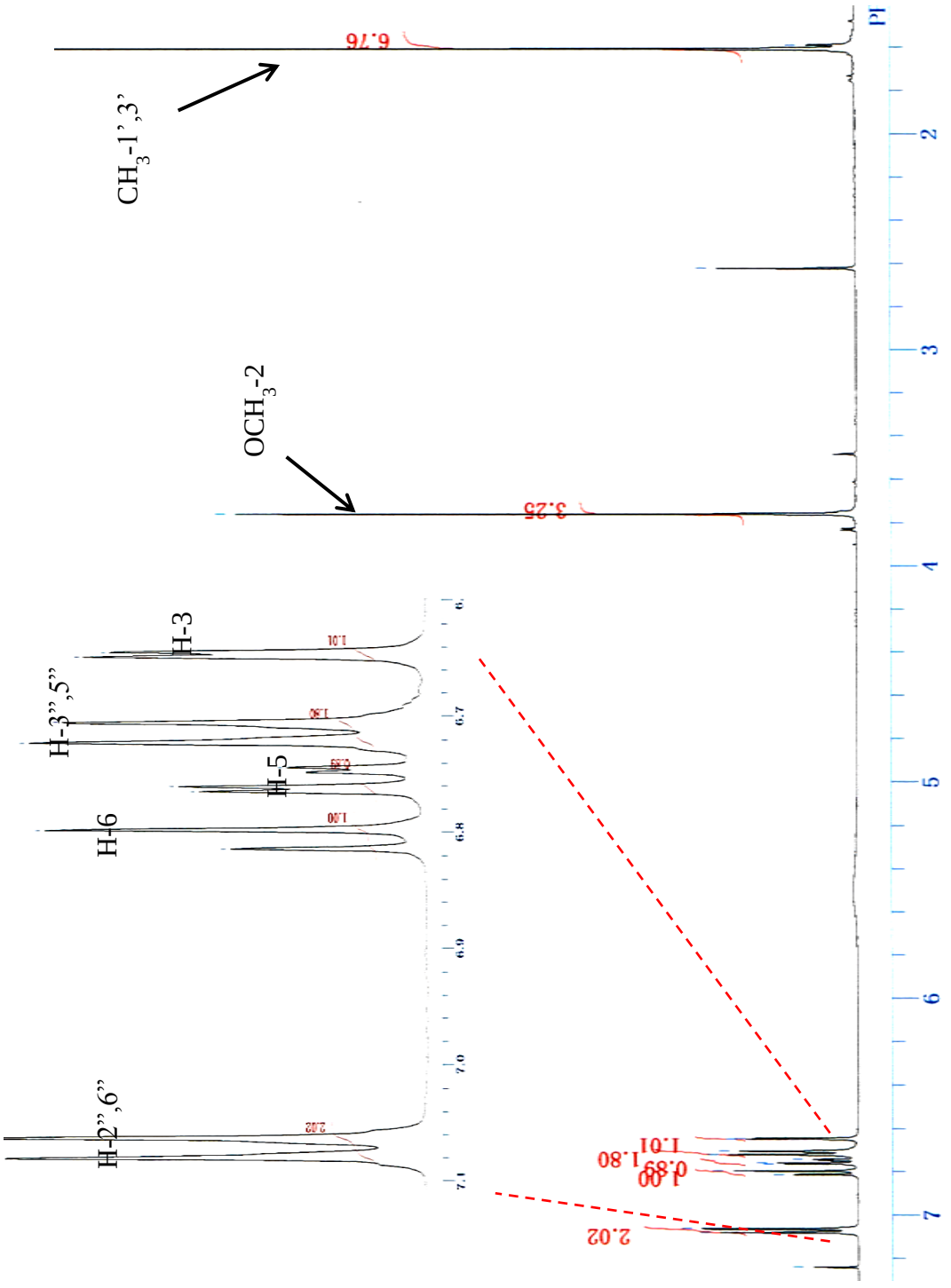


Fig. 59 ^{1}H NMR spectrum of compound 4 ($CDCl_3$).

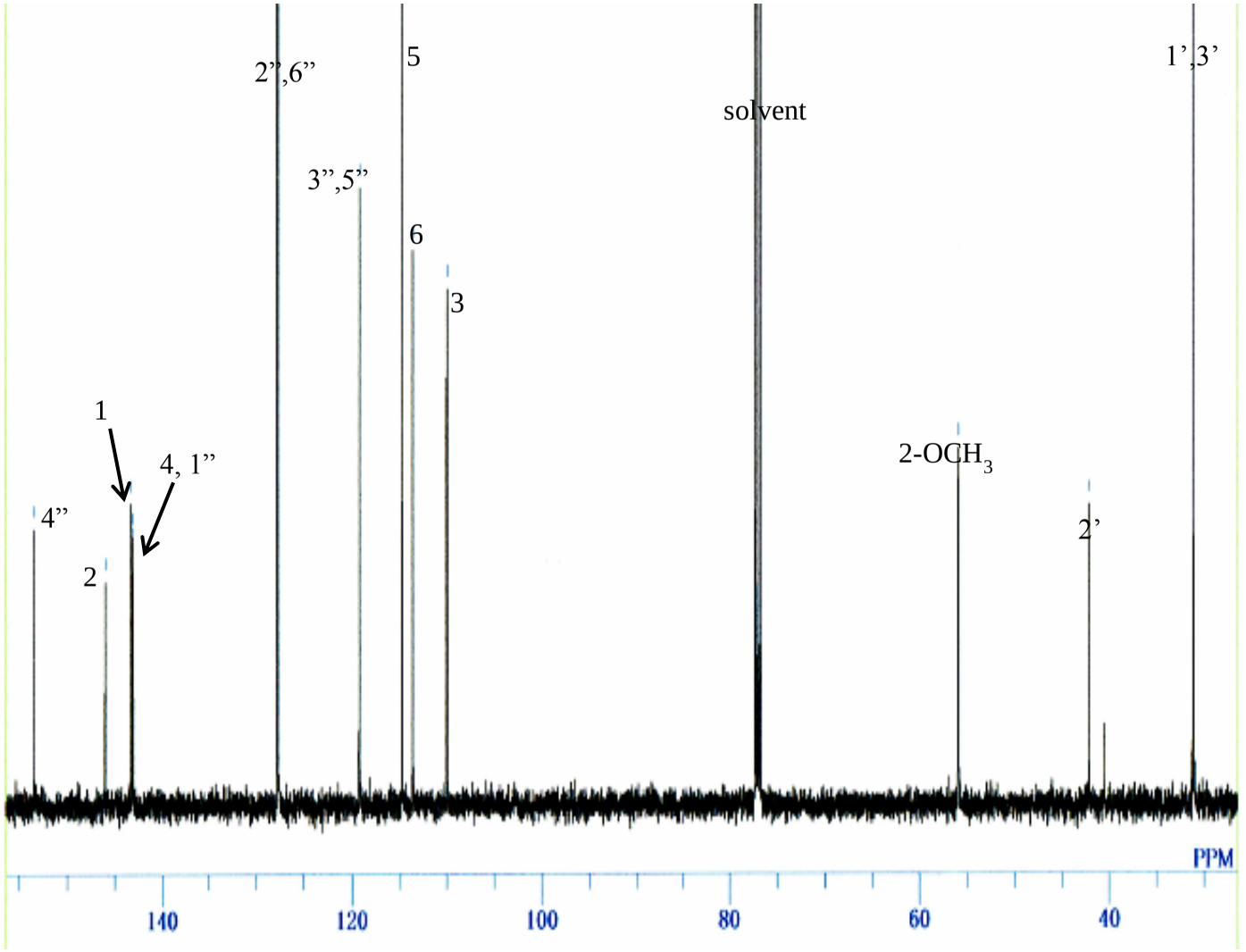


Fig. 60 ^{13}C NMR spectrum of compound 4 ($CDCl_3$).

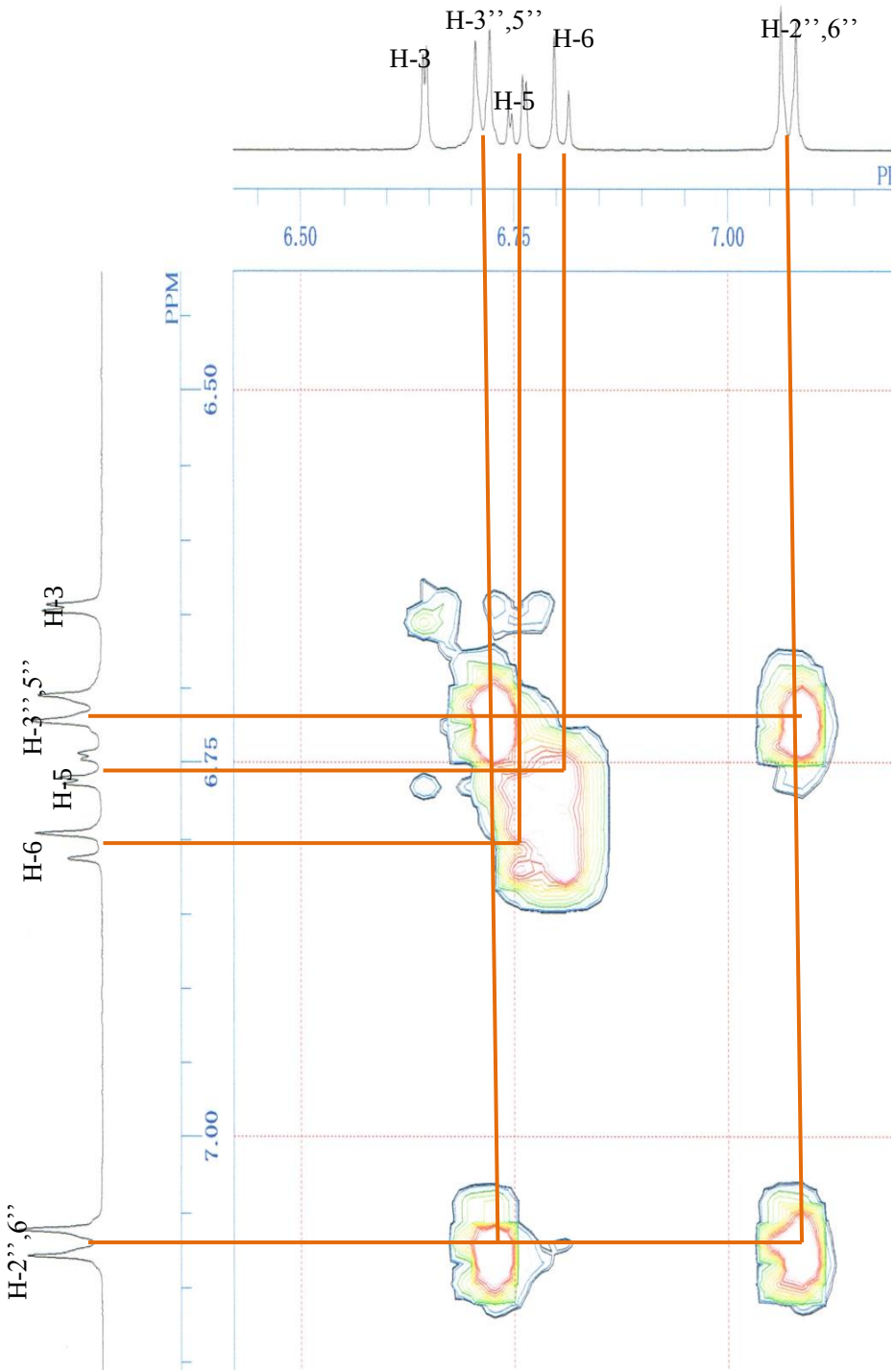


Fig. 61 COSY spectrum of compound 4 ($CDCl_3$).

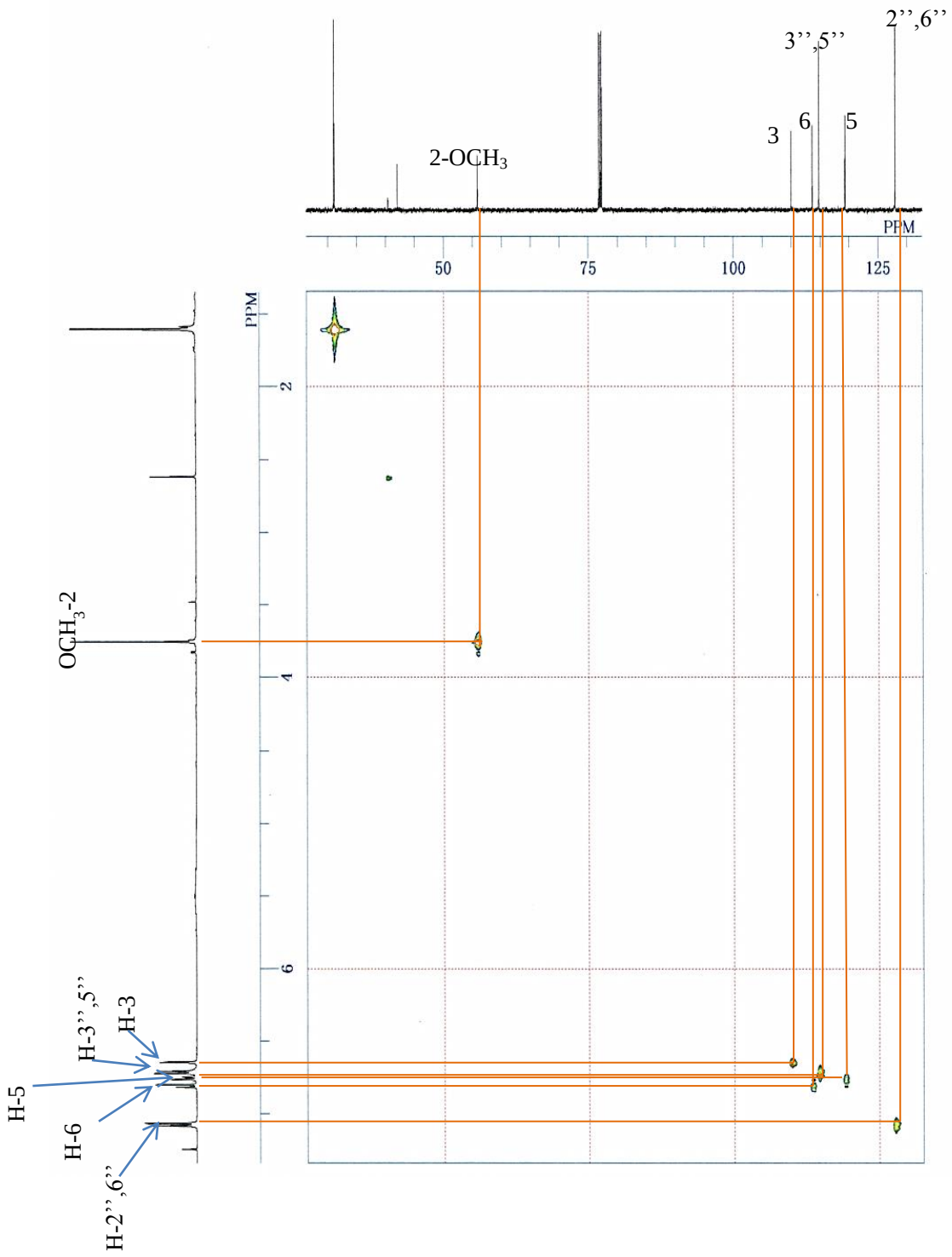


Fig. 62 HMQC spectrum of compound 4 ($CDCl_3$).

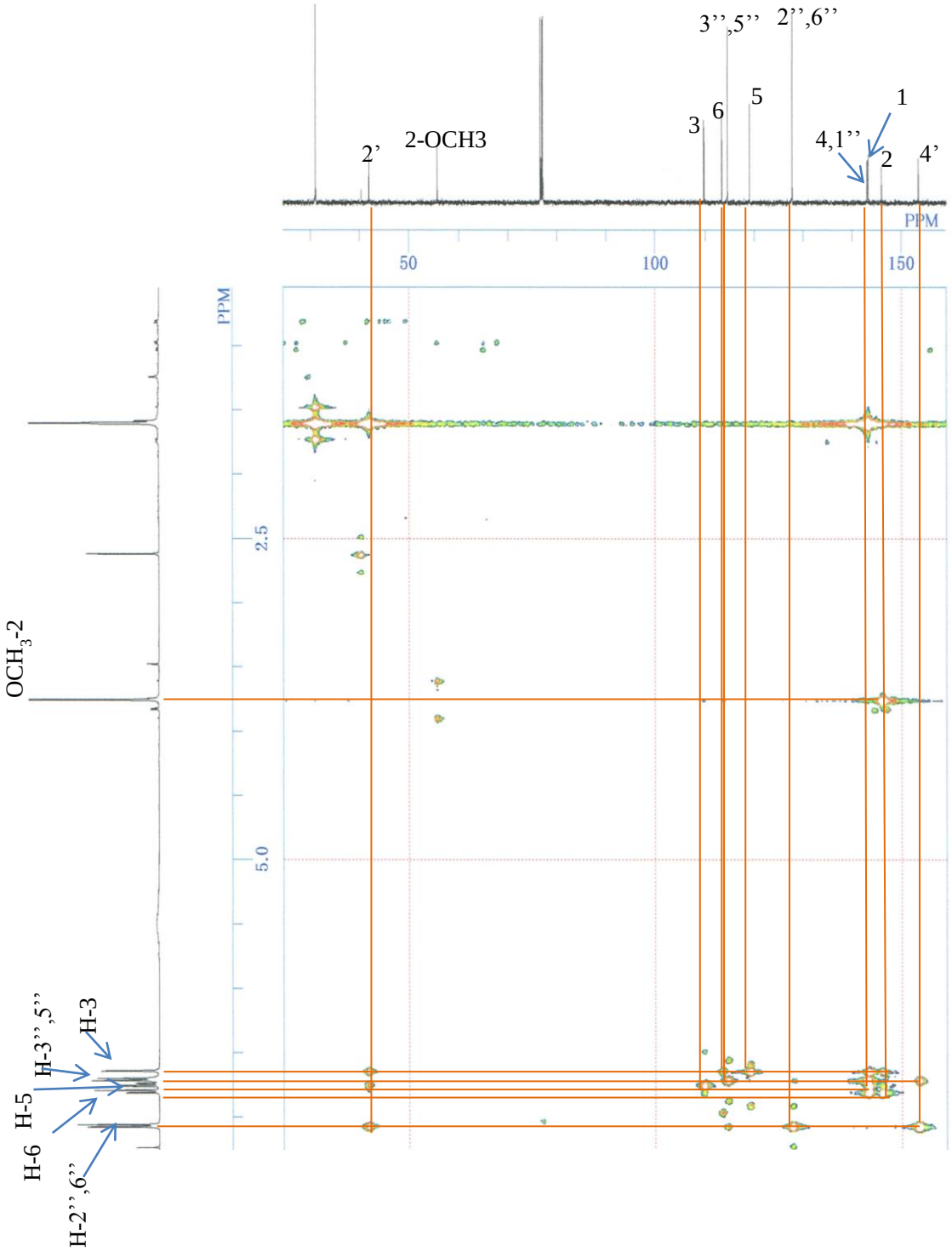
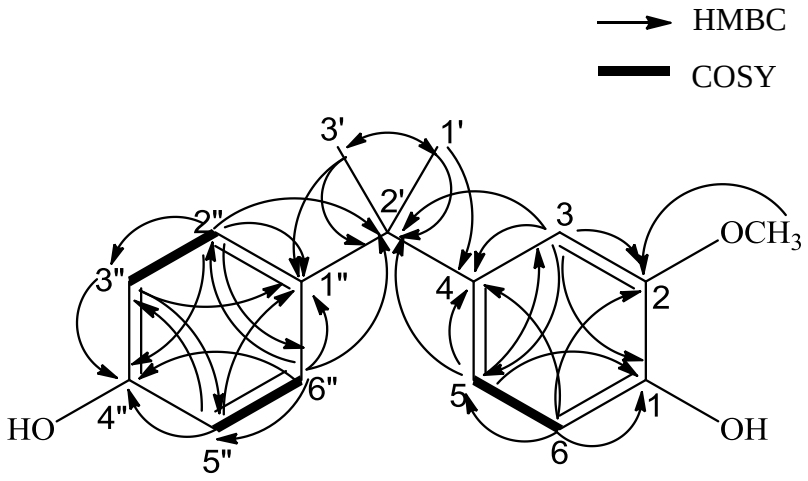


Fig. 63 HMBC spectrum of compound 4 ($CDCl_3$).



4-(2-(4-hydroxyphenyl)propan-2-yl)-2-methoxyphenol (methoxy-BPA)

Fig. 64 COSY and HMBC correlations for compound 4.

Table 7 ^{1}H and ^{13}C NMR data for methoxy-BPA (in $CDCl_3$)

Position	^{1}H	^{13}C
	δ_H (mult, *J* in Hz)	δ_C
1	-	143.4
2	-	146.0
3	6.65 (d, 2.0)	110.0
4	-	143.1
5	6.76 (dd, 10.0, 2.0)	119.3
6	6.80 (d, 8.0)	113.6
2-OCH_3	3.76 (s)	55.9
1''	-	143.1
2''	7.07 (d, 9.0)	127.9
3''	6.72 (d, 9.0)	114.7
4''	-	153.4
5''	6.72 (d, 9.0)	114.7
6''	7.07 (d, 9.0)	127.9
1'-CH_3	1.61 (s)	31.1
2'	-	42.0
3'-CH_3	1.61 (s)	31.1

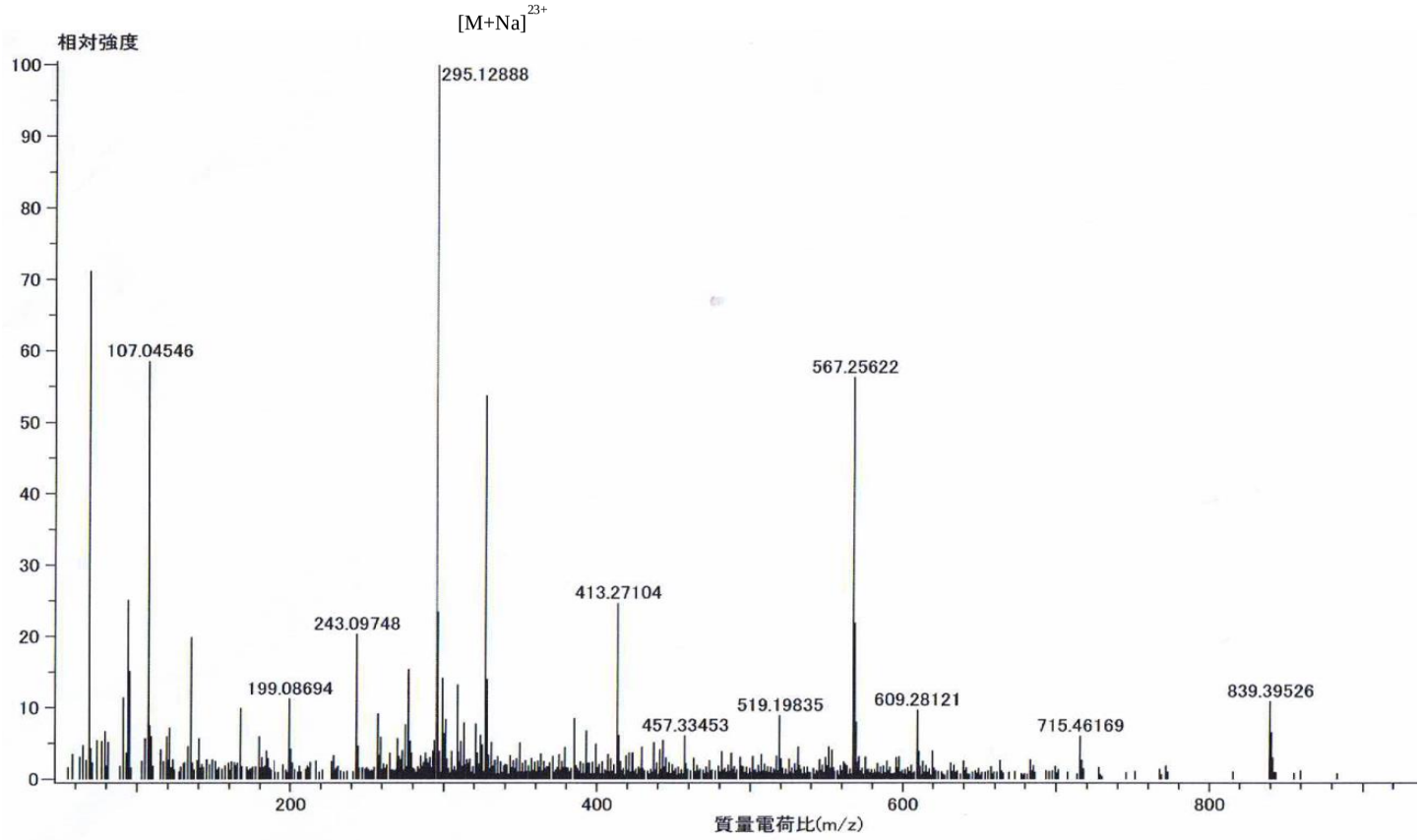


Fig. 65 ESI-MS spectrum of compound 5.

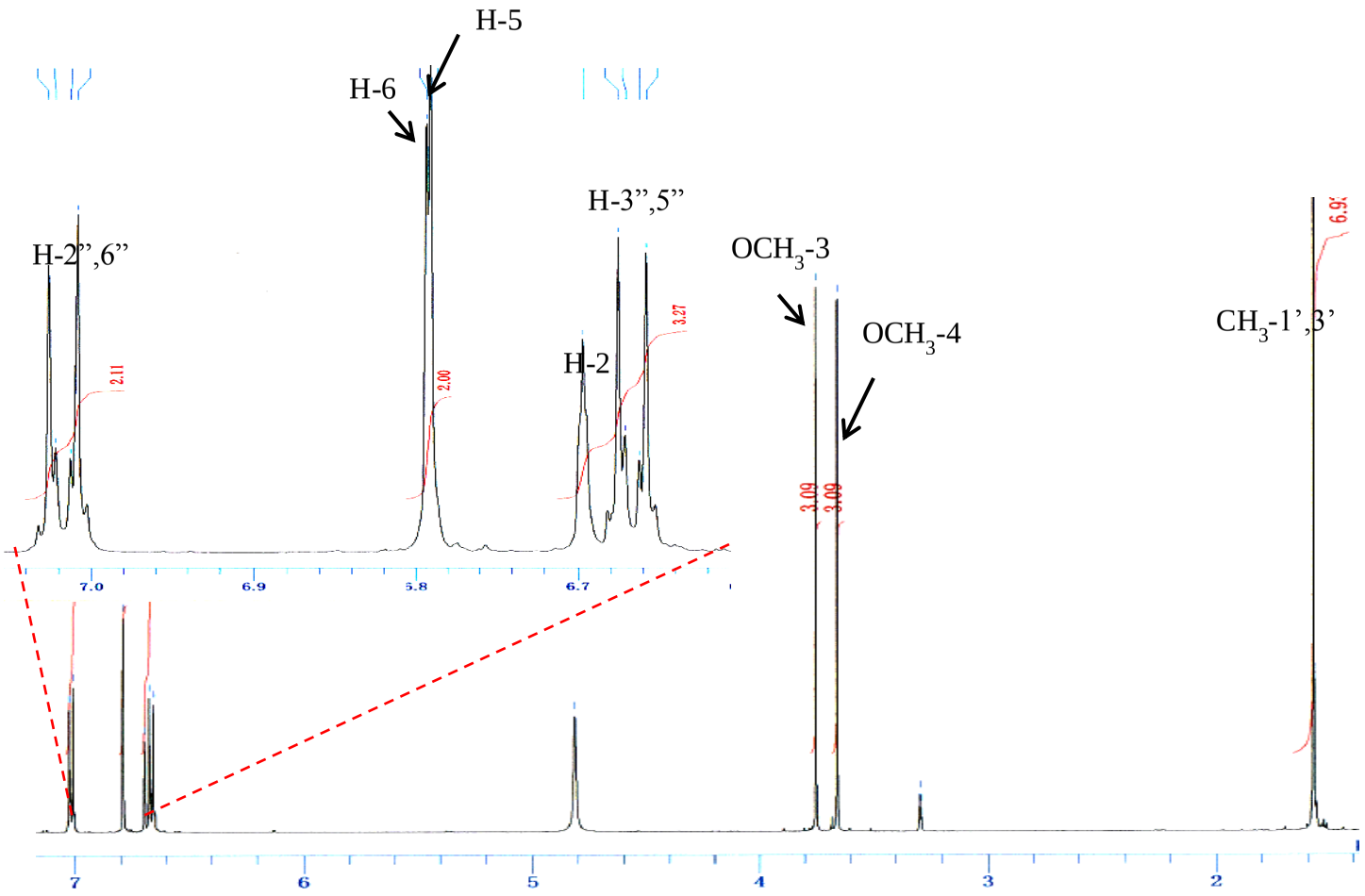


Fig. 66 ^{1}H NMR spectrum of compound 5 (CD_3OD).

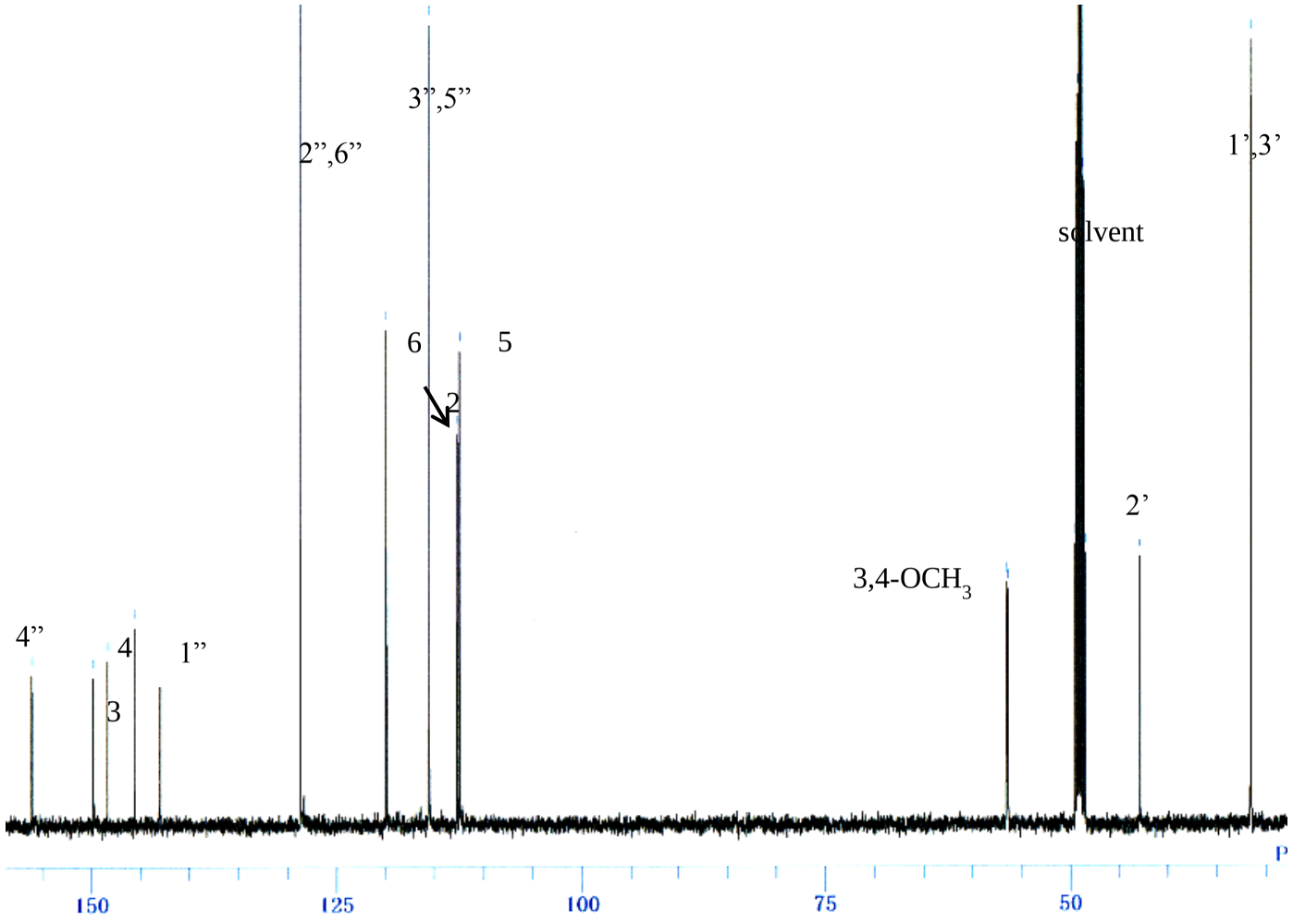


Fig. 67 ^{13}C NMR spectrum of compound 5 (CD_3OD).

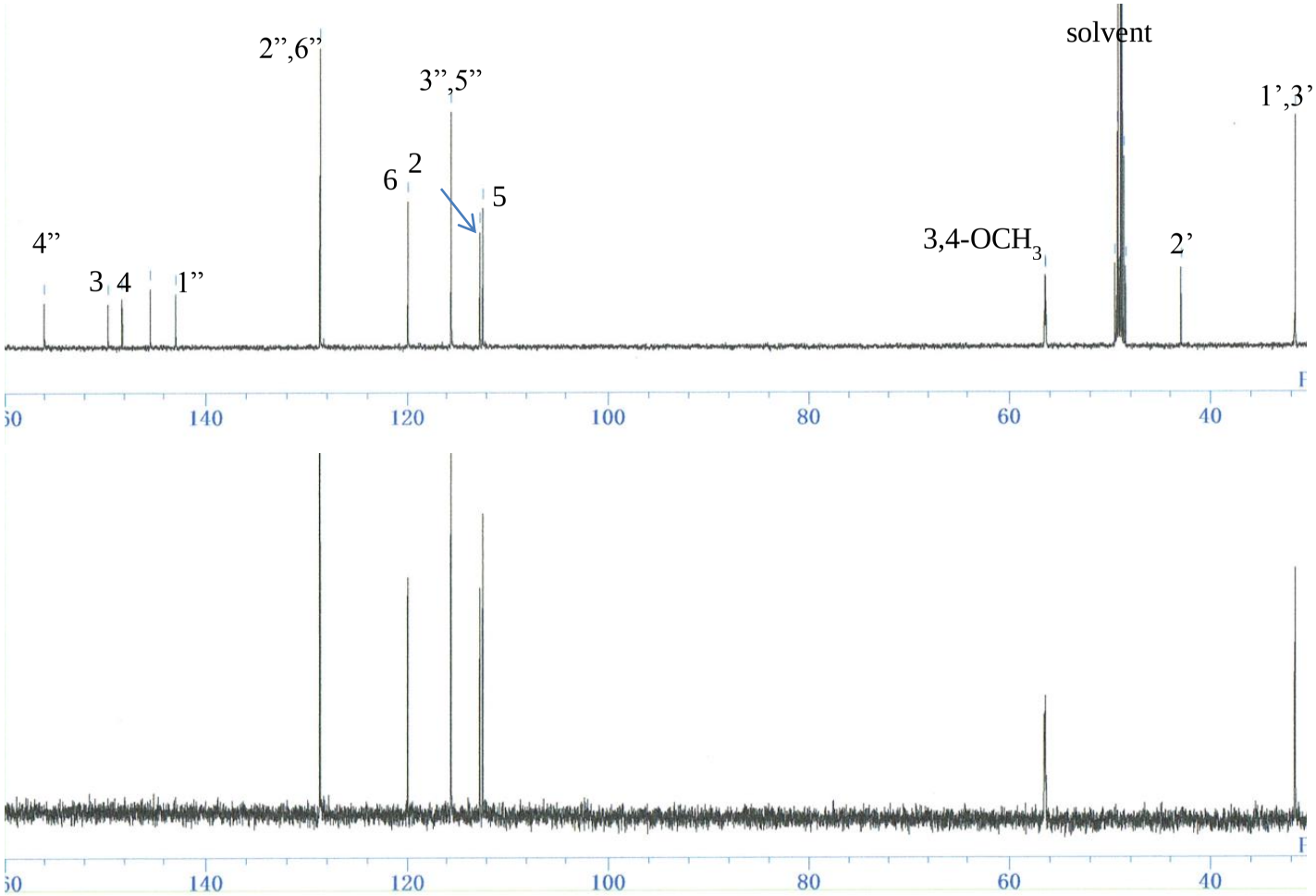


Fig. 68 DEPT spectrum of compound 5 (CD_3OD).

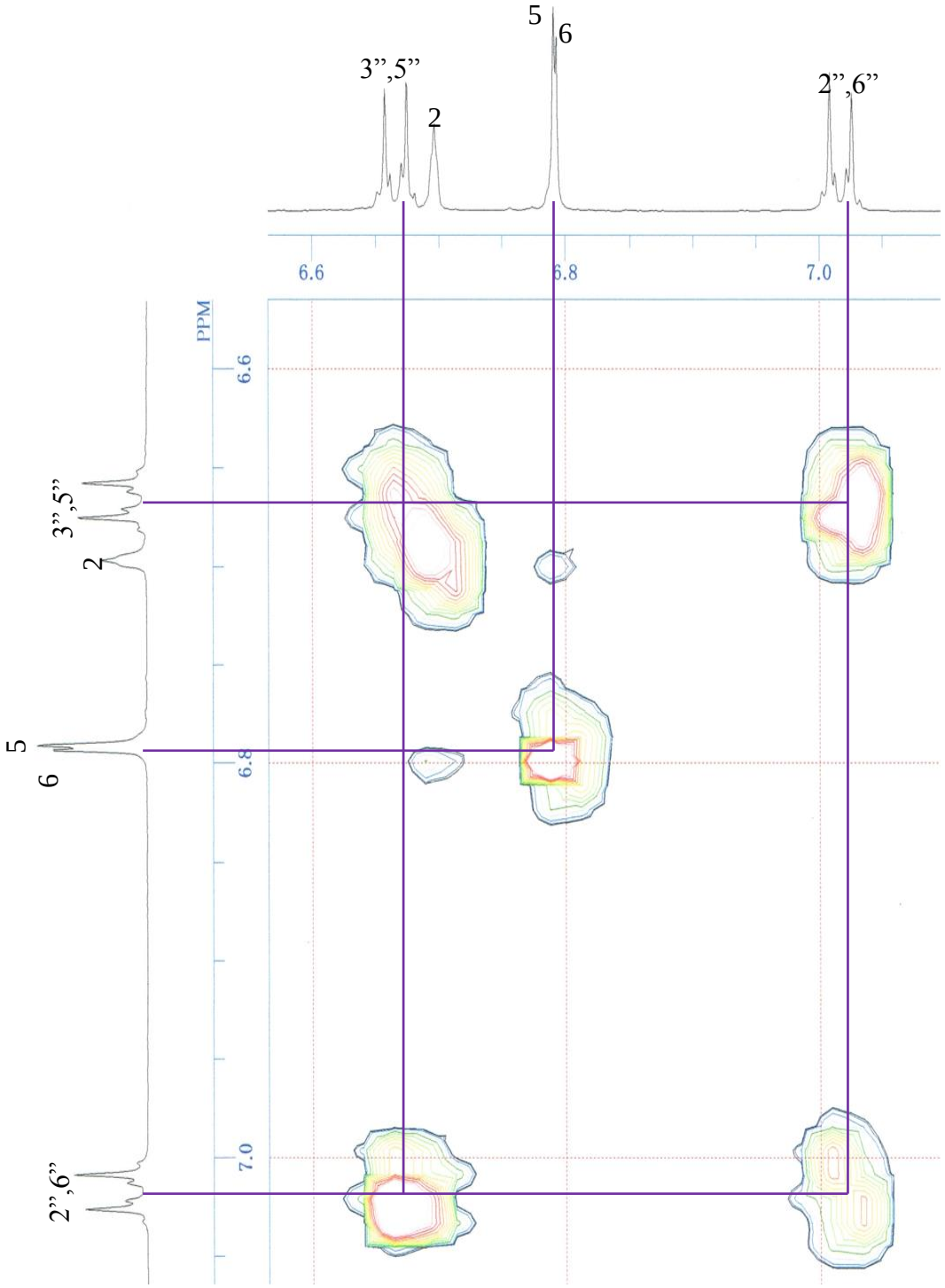


Fig. 69 COSY spectrum of compound 5 (CD_3OD).

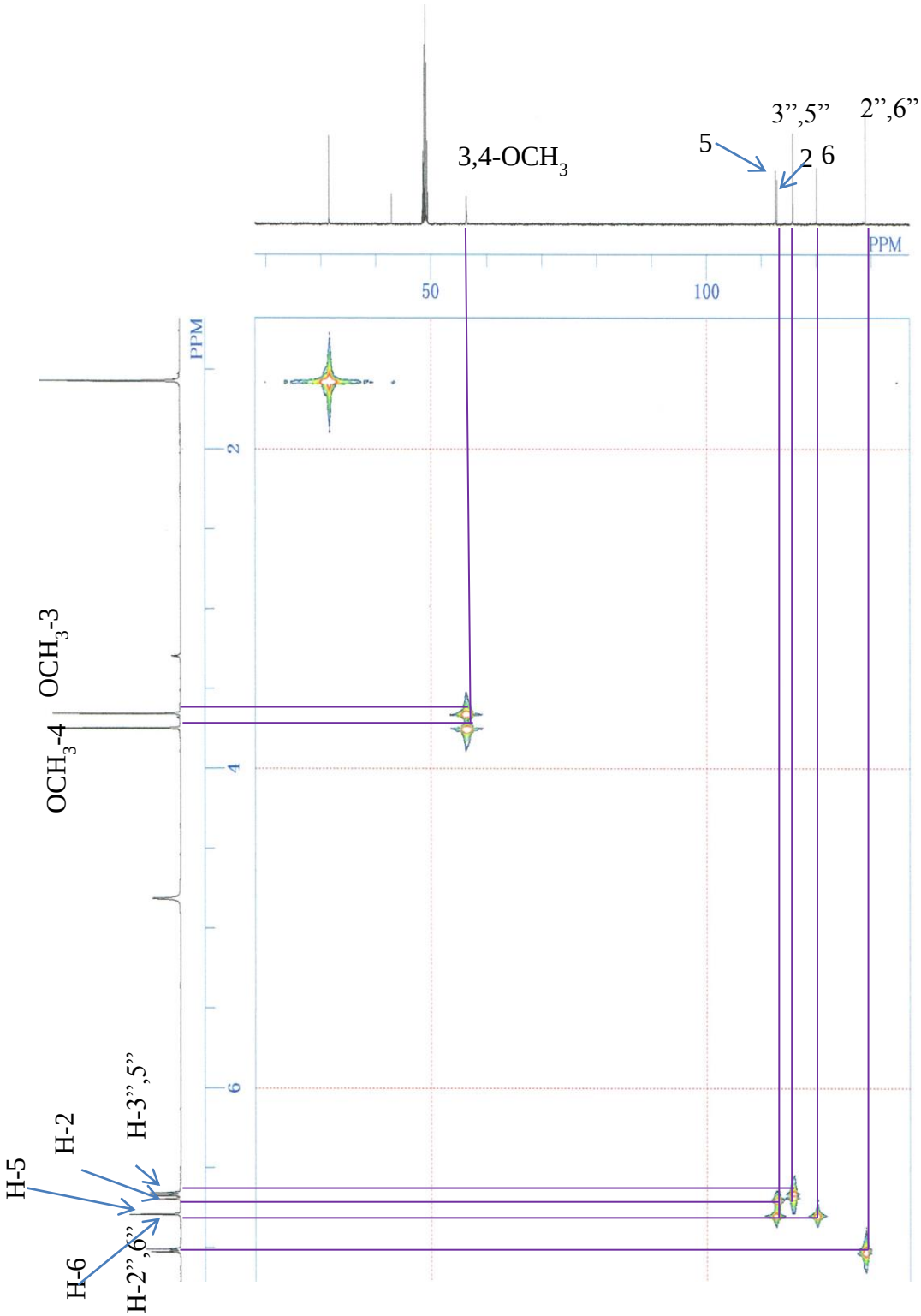


Fig. 70 HMQC spectrum of compound 5 (CD_3OD).

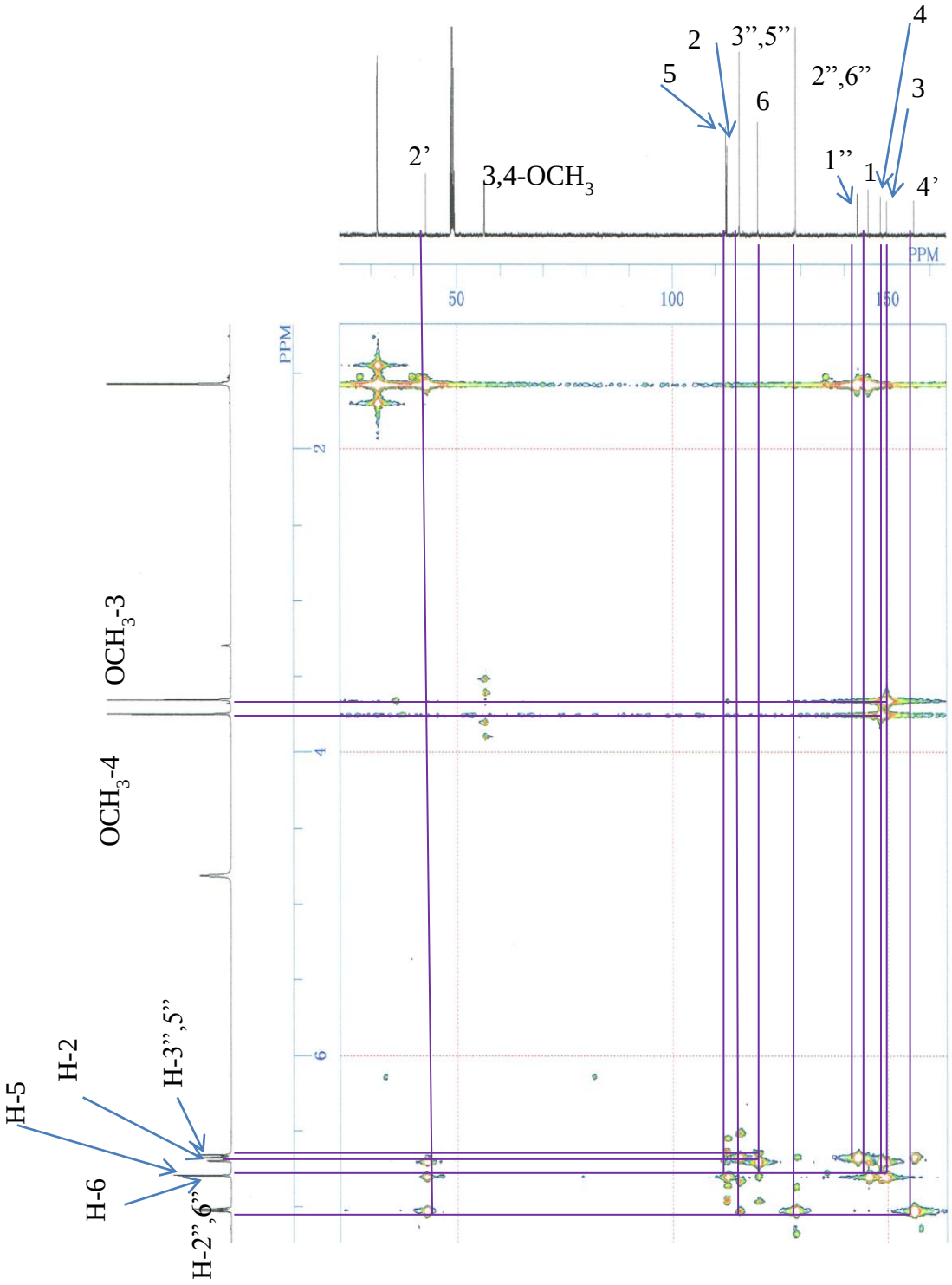
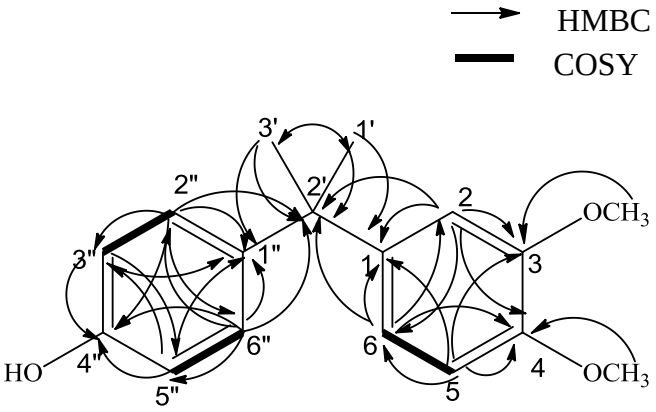


Fig. 71 HMBC spectrum of compound 5 (CD_3OD).



4-(2-(3,4-dimethoxyphenyl)propan-2-yl)phenol (dimethoxy-BPA)

Fig. 72 COSY and HMBC correlations for compound 5.

Table 8 ^{1}H and ^{13}C NMR data for dimethoxy-BPA (in CD_3OD).

Position	^{1}H	^{13}C
	δ_H (mult, *J* in Hz)	δ_C
1	-	145.6
2	6.70	112.7
3	-	149.8
4	-	148.3
5	6.79	112.5
6	6.79	119.9
3-OCH_3	3.67 (s)	56.5
4-OCH_3	3.75 (s)	56.4
1''	-	143.0
2''	7.02 (dd, 6.5, 2.0)	128.7
3''	6.66 (dd, 6.0, 2.0)	115.6
4''	-	156.0
5''	6.66 (dd, 6.0, 2.0)	115.6
6''	7.02 (dd, 6.5, 2.0)	128.7
1'-CH_3	1.58 (s)	31.6
2'	-	42.9
3'-CH_3	1.58 (s)	31.6

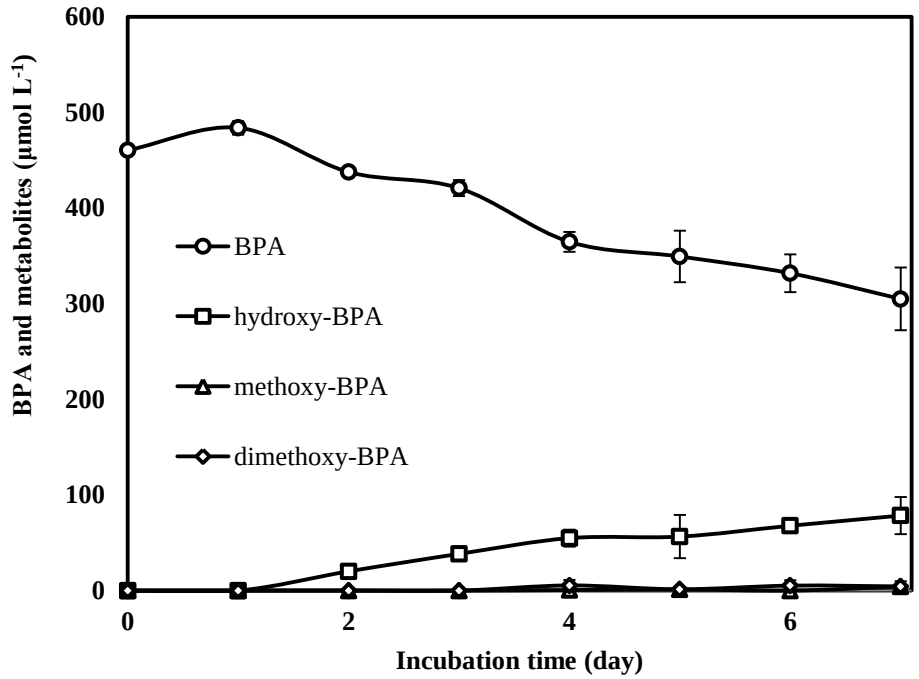


Fig. 73 Time course for BPA elimination and metabolic transformation by *P. sordida* YK-624 under non-ligninolytic condition. Values are the means±SD of triplicate samples.

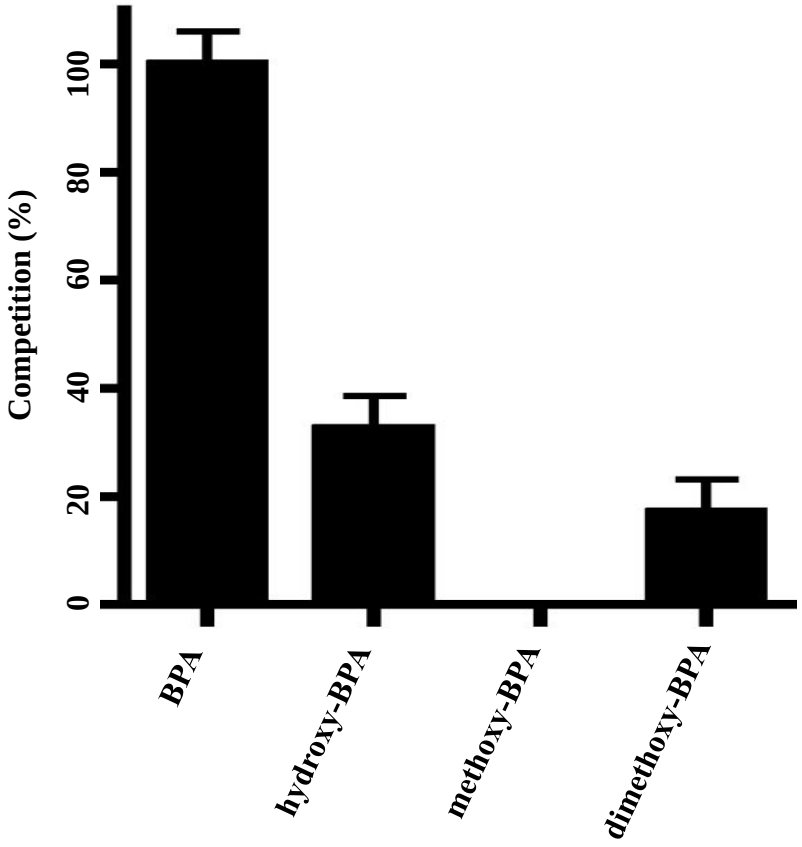


Fig. 74 Competition by BPA and three metabolites for binding to the membrane estrogen receptor. Samples were incubated with 4 nM [^{3}H]E_2 and 10 μM competitor. Competition rate (%) is represented as relative value of the displacement of [^{3}H]E_2 binding by BPA set as 100 %.

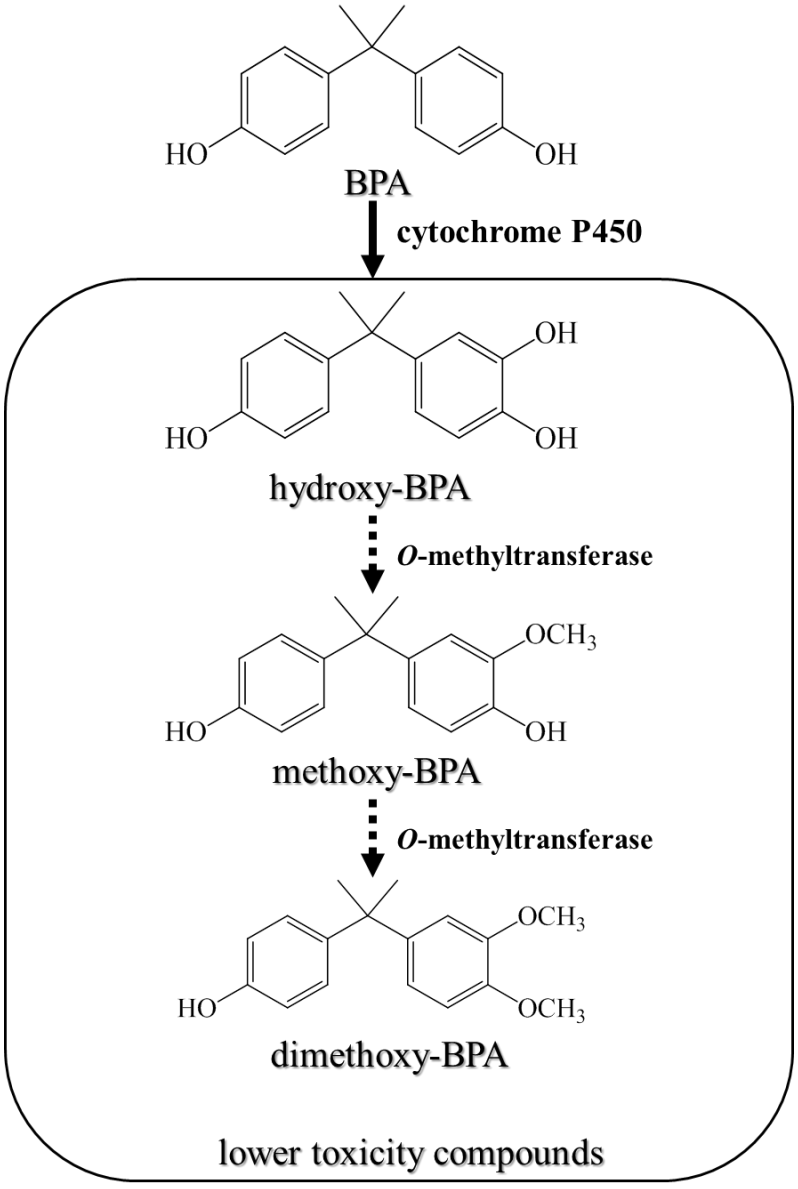


Fig. 75 Proposed mechanism of BPA transformation by *P. sordida* YK-624 under non-ligninolytic condition.

Conclusion

Since the 1980s when the composition of ligninolytic system in white-rot fungi has been elucidated, this group of fungi attracted considerable attention. Evidence for the ability of white-rot fungi to transform or mineralize a range of organopollutants has been reported. In the present study, the biodegradation of mycotoxin AFB, ACE as a neonicotinoid insecticide, and endocrine disrupting compounds by the white-rot fungus *P. sordida* YK-624 was investigated.

In chapter II, the detoxification of AFB and ACE by the *P. sordida* YK-624 was demonstrated. In section 1, MnP was able to degrade AFB, and the maximum degradation (86.0%) of AFB was observed by the reaction mixture containing 5 nkat MnP for 48 h treatment. The addition of Tween 80 accelerated the AFB degradation by MnP. Using umu test, it was also confirmed that MnP substantially removed the mutagenic activity of AFB. It became clear that MnP reaction system can transform AFB to AFB-8,9-dihydrodiol by ^{1}H NMR and HR-ESI-MS analyses of a metabolite. These results suggest that AFB was oxidized to AFB-8,9-epoxide, then hydrolyzed to AFB-8,9-dihydrodiol. In section 2, Under ligninolytic and non-ligninolytic conditions, 45% and 30% of ACE were eliminated, respectively, after 15 days of incubation. HR-ESI-MS and NMR analyses of a metabolite identified in the culture supernatant suggested that ACE was *N*-demethylated to (E)-N^{1}-[(6-chloro-3-pyridyl) -methyl]-N^{2}-cyano-acetamidine, which has a much lower toxicity than ACE. In addition, the effect of the cytochrome P450 inhibitor PB on the elimination of ACE was investigated. Cytochrome P450 is involved in the *N*-demethylation of ACE by *P. sordida* YK-624 since the elimination rate of ACE was markedly reduced by the addition of either 0.01 and 0.1 mM PB to culture medium.

In chapter III, The removal of EDCs by YK-LiP1 was investigated. Five endocrine disruptors, OP, BPA, E_1, E_2 and EE_2 were eliminated by YK-LiP1 more effectively than Pc-LiP, and OP and BPA were disappeared almost completely in the reaction mixture containing YK-LiP1 after a 24-h treatment. Particularly, the removal of estrogenic activities of E_2 and EE_2, which show much higher estrogenic activities than other EDCs such as BPA and OP, were removed following 24-h treatment with YK-LiP1. Moreover, 5,5’-bis(1,1,3,3-tetramethylbutyl) -[1,1’-biphenyl]-2,2’-diol and 5,5’-bis-[1-(4- hydroxy-phenyl)-1-methyl

-ethyl]-biphenyl-2,2’-diol were identified as the main metabolite from OP or BPA, respectively. These results suggest that YK-LiP1 is highly effective in removing of EDCs by the oxidative polymerization of these compounds.

In chapter Ⅳ, the metabolism of BPA by the white-rot fungus *P. sordida* YK-624 was demonstrated. In section 1, both 1 mM and 0.1 mM BPA were effectively decreased within a 24 h treatment and two metabolites were detected. Two metabolites (5,5’-bis-[1-(4-hydroxy -phenyl)1-methy-ethyl]-biphenyl-2,2’-diol and 4-(2-(4-hydroxy-phenyl) propan-2-yl)-2-(4-(2-(4-hydroxyphenyl)propan-2-yl)phenoxy)phenol) were identified by ESI-MS and NMR analysis. In section 2, we reported the conversion of BPA to 4-(2-(4-hydroxyphenyl)propan-2-yl)benzene -1,2-diol (hydroxy-BPA) by hyper lignin-degrading fungus *P. sordida* YK-624 under non-ligninolytic condition. The hydroxy-BPA was synthesized, and approximately 66% of hydroxy-BPA was degraded after 7 d of incubation. HR-ESI-MS and NMR analyses of the metabolites isolated from the culture broth indicated that hydroxy-BPA was metabolized to 4-(2-(4-hydroxyphenyl)propan-2-yl)-2-methoxyphenol (methoxy-BPA) and to 4-(2-(3,4-dimethoxyphenyl)propan-2-yl)phenol (dimethoxy-BPA) by sequential methylation events. These metabolites showed reduced estrogenic activity compared to BPA.

It is clear that *P. sordida* YK-624 show potential for possible use in bioremediation of a range of environmental pollutants.

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