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ORIGINAL PAPER

Biotransformation of methyl *tert*-butyl ether by human cytochrome P450 2A6

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Abstract Methyl tert-butyl ether (MTBE) is widely used as gasoline oxygenate and octane number enhancer for more complete combustion in order to reduce the air pollution caused by motor vehicle exhaust. The possible adverse effects of MTBE on human health are of major public concern. However, information on the metabolism of MTBE in human tissues is scarce. The present study demonstrates that human cytochrome P450 2A6 is able to metabolize MTBE to tert-butyl alcohol (TBA), a major circulating metabolite and marker for exposure to MTBE. As CYP2A6 is known to be constitutively expressed in human livers, we infer that it may play a significant role in metabolism of gasoline ethers in liver tissue.

Keywords Biotransformation · MTBE · Cytochrome P-450 · Metabolism · HS-GC/MS · Formaldehyde · TBA

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Introduction

Methyl *tert*-butyl ether (MTBE) is the most widely used fuel oxygenate added to gasoline in order to enhance octane ratings and improve combustion efficiency, thus reducing air pollution caused by emissions from motor vehicles. Despite the air quality benefits provided by MTBE, its extensive use in the last two decades has led to widespread groundwater pollution, caused by accidental releases from fuel storage and distribution systems, so that over only a few years of extensive use, MTBE has become one of the most frequently detected underground water pollutants (Bruijn et al. 2005). The severe impact of MTBE on groundwater is linked to its high mobility into the subsurface, caused by (a) high water solubility, that is in the range 5200–5400 mg l^{-1} from gasoline and as high as 50000 mg l^{-1} from the pure phase liquid (US EPA 2004), (b) low volatility and (c) low sorption to soil (Shaffer and Uchrin 1997; Zogorski et al. 1997; Fischer et al. 2004).

MTBE toxicity and potential carcinogenicity were a matter of long debate until 2002, when an EU risk assessment report concluded that exposure to MTBE is not expected to have any harmful impact on human health, the atmosphere and the environment (Hansen et al. 2002). Moreover, water remediation for drinking purposes is necessary when MTBE taste and odor thresholds, of 2.0 and 2.5 ppb, respectively, (US EPA 2000), are exceeded. The present guideline limit established by the US Environmental Protection

Agency (EPA) is 20–40 μg l^{-1} for MTBE in drinking water (Jacobs et al. 2001). In Canada, as in Europe (Hansen et al. 2002) and many states within the USA, e.g., California, the threshold is accepted as 15 ppb, while it is 10 ppb in some others, e.g., New York. In addition, some authorities, such as the state of California, have set a secondary (non-enforcible) maximum concentration level of 5 ppb for aesthetic protection. As a result, the remediation of MTBE-impacted aquifers has become an active area of research.

Oxidation is one of the most important reactions for the metabolism of biotic substrates and many enzymes are known to catalyze various types of oxidation. In the past three decades, one of the major goals of bioinorganic chemistry of monooxygenase enzymes was elucidation of the "oxidized intermediate" in reactions of natural enzymes and in metalloporphyrins (McLain et al. 2000; Montellano 1995; Traylor et al. 1995; Shilov and Shteinman 1999; Sono et al. 1996; Yoshizawa et al. 1998). Amongst such oxidizing enzymes, the heme-containing monooxygenase cytochrome P-450 has been most extensively studied (Coon and White 1980). Cytochromes P450 (CYP) are heme proteins that catalyse numerous oxidations of natural and foreign substrates and in this way metabolize and detoxify xenobiotics (Snyder 2000; Oberdorster et al. 1998). Humans have been estimated to have at least 53 different CYP genes and 24 pseudogenes, one of them is CYP2A6. The CYP2A6 protein has been detected in liver (Yun et al. 1991), where it constitutes about 4% of total CYP content (Shimada et al. 1994; Imaoka et al. 1996). CYP2A6 catalyzes the metabolism of the gasoline additive MTBE (Hong et al. 1999a, b) and some pharmaceuticals, including halothane and losigamone (Raunio et al. 1995). The blood levels in exposed people of MTBE and *tert*-butyl alcohol (TBA), a major circulating metabolite and a suitable marker for MTBE exposure, as well as the pharmacokinetics data have been reported (Clayton Environmental Consultants 1991; Prah et al. 1994; Johanson et al. 1995; Cain et al. 1996). Previously, Brady et al. (1990) reported the metabolism of MTBE by rat liver microsomes in which the involvement of cytochrome P450 enzymes was implicated. However, the metabolism of MTBE in human tissues and the enzymes involved were unknown. This information is important in our understanding of the health effects of MTBE in humans, and is critical in assessing the human relevance of the pharmacokinetics and toxicity data obtained from animal studies.

In this study, we investigated the ability of commercially available, purified CYP2A6 enzyme to metabolize the gasoline oxygenate, MTBE, in order to gain information on biotransformation kinetics and by-products, and in order to propose a reasonable mechanism. Using HPLC (for monitoring aldehydes and ketones) and HS-GC-MS (for TBA, alcohols and other intermediates), we were able to confirm previous work (Hong et al. 1997a, b; Le Gal et al. 2001) and to supply definite proof for the suggested pathway.

Materials and methods

Chemicals

MTBE (99.8% pure) and TBA (99.5% pure) were purchased from Aldrich Chemical Co. A 37% w/v stock formaldehyde solution was purchased from Merck Chemical Co. Electrolytes, $MgCl_2$ and KCl were analytical grade supplied from Merck company and were used without further treatment. All other chemical used were of reagent grade and obtained from standard supplies. Human CYP2A6 enzyme (Product no. C4735) was purchased from Aldrich Chemical Co (Saint Louis, Missouri 63103, USA). Double distilled water (DDW) was used for sample preparation. Helium and nitrogen (ultra pure carrier grade) were obtained from Roham Gas Company (Tehran, Iran).

Identification of pathway intermediates

MTBE degradation products were identified and quantified after various reaction times by headspace gas chromatography/mass spectrometry (HS-GC/MS). Formation of TBA, formaldehyde and some other intermediates were also determined by HS-GC/MS. Static headspace analysis was performed using a CTC-CombiPAL autosampler (Bender and Hobein, Zurich, Switzerland) mounted on top of a GC/MS system. The autosampler was equipped with a heatable CTC agitator for incubation and shaking, and a robotic arm.

Table 1 Headspace conditions

Syringe temperature: 71°C	Plunger fill speed: 100 µl s^{-1}
Agitator temperature: 70°C	Pre-injection delay: 4 s
Sample incubation time: 20 min	Plunger injection speed: 250 µl/s
Agitator speed: 500 rpm	Syringe flush time: 120 s
Agitation cycle: 2 s on, 4 s off	Sample volume, 10 ml in 20 ml vial

To prevent the carry over of analytes, we used a heated flushing station for conditioning of the HS needle and reconditioning after each analysis. Both the gas station and the heated flushing station were flushed with nitrogen. The syringe body was held in the syringe adapter heater. 20 ml vials sealed with screw top caps with PTFE/silicon septa were used. Parameters of the instrument are shown in Table 1. A salt content of 30 (% w/v) was chosen for the quantitative determination of target analytes.

The GC-MS analysis was performed using a Varian (CP-3800 series) gas chromatograph equipped with a mass-selective detector (Varian, quadrupole 1200) and a factor-four, VF-5 ms fused-silica capillary column with a 30 m × 0.25 mm i.d. and 250 µm film thickness (Varian) was used. The GC conditions were as follows: inlet temperature, 250°C; inlet mode, split operation with split ratio 1:25. The initial oven temperature was 50°C, which was raised to 100°C at a rate of 5°C/min and then to 275°C at a rate of 20°C/min. The final temperature was maintained for 1.75 min and the total run time was 20 min. Helium, at a constant flow rate of 1.5 ml/min was used as the carrier gas. Mass spectra were obtained at 70 eV in the electron impact ionization mode; the spectrometer was operated in the full scan mode over the mass range from m/z 15 to 110. The source, transfer line and quadrupole temperatures were maintained at 200, 250 and 200°C, respectively. Total ion current chromatograms were acquired and processed using Workstation data analysis software (Varian). To increase sensitivity, the selected ion monitoring (SIM) mode of EI was applied in quantitative analysis. The most abundant ion was used as the quantified ion. In Table 2, some analytical conditions of MTBE and methyl ethyl ketone (MEK, internal standard) by GC/MS with SIM mode are shown. All GC/MS quantifications made in this study were based on the relative peak area of analytes to the internal standard from the average of three replicate measurements.

Table 2 Analytical conditions of MTBE and methyl ethyl ketone by GC/MS with SIM

Compound	Molecular weight (g/mol)	Retention time (min)	Quantification ions (m/z)
MTBE	88	1.85	73
Methyl ethyl ketone	73	2.6	43

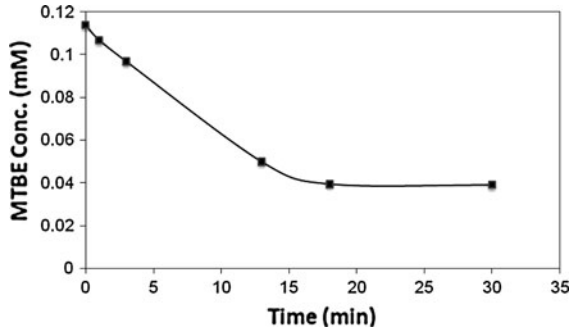

Fig. 1 Degradation of MTBE by CYP-450. 20 pmol CYP-450 was incubated with 10 mg of MTBE per liter at 60°C. MTBE concentration was determined by HS-GC/MS

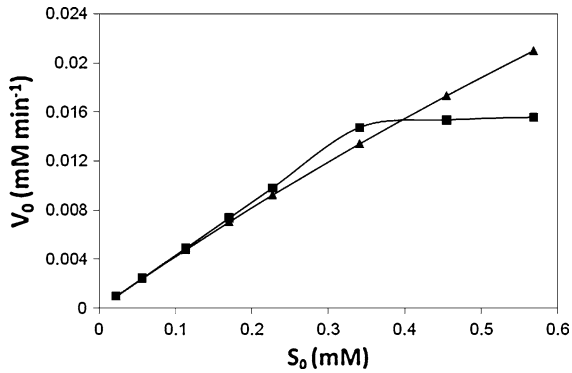

Fig. 2 Initial rates of the enzyme catalyzed reaction (V_0) against concentrations of substrate (S_0)

The HPLC system consisted of a Model 600E pump (Waters Associates) controlled by a Model 7725i manual injection valve (Rheodyne, Cocati, CA, USA) equipped with a 20 µl sample loop and SPD-6AV UV–visible detector (Shimadzu, Kyoto, Japan) was used for monitoring some aldehydes and ketones. Data were collected and integrated using the Maxima 820 software package (Waters Associates). Separations were carried out at room temperature using a Nova-

pak C_{18} column (300 × 3.9 mm I.D.) and a mixture of acetonitrile–water (35:65 v/v) as the mobile phase. The formaldehyde was analyzed as 2,4-dinitrophenylhydrazone (DNP) derivatives as previously described (Hong et al. 1997a, b). 2,4-dinitrophenylhydrazones were analyzed after extraction and dilution with acetonitrile. Eluates were also detected at 365 nm.

Metabolism of MTBE by CYP 2A6

1.0 nmol of cytochrome P450 isozyme in 0.5 ml of 100 mM Tris–HCL buffer, pH 7.5 was supplied from Sigma-Aldrich Co. (Missouri, USA). In order to minimize freeze–thaw cycles, 10 µl (20 picomol enzyme) aliquots were prepared and stored at −70°C until used. According to supplier recommendations, the product is stable for at least 24 months and generally, 80% or more of the catalytic activity is retained after 6 freeze–thaw cycles.

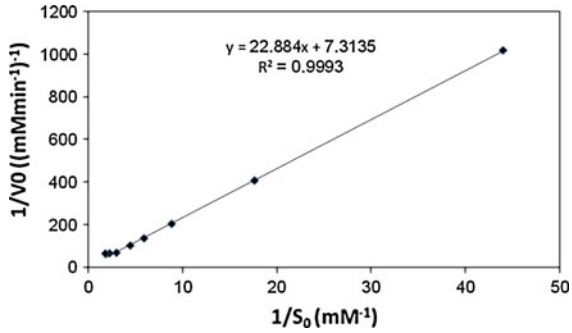

Fig. 3 $1/V_0$ (V_0 initial rates of the enzyme catalyzed reaction) versus $1/S_0$ (S_0 concentrations of substrate) according to Lineweaver–Burk equation

Incubation reactions (0.5 ml final volume in 5 ml tubes) contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 1 mM NADPH and 10 µl microsomal proteins (containing 20 picomol CYP 2A6). Incubation was carried out in sealed headspace vials and the reaction was initiated, after 5 min pre-incubation at 37°C, by injecting MTBE dissolved

Table 3 Biodegradation kinetic parameters for some enzymes and liver microsomes

No.	Enzyme	Kcat (nM min^{-1}) nM^{-1}	K_m (mM)	V_{max}/K_m (min^{-1})	Ref.
1	Cyp 2A6	3425	3.13	0.04	This study
2	Human liver microsomal protein (CYP 2A6 + CYP 3A4)	1620 ± 860	0.25 ± 0.17	4.7 ± 2.1	Le Gal et al. (2001)
3	Liver male microsomes from 4-months old P450 2E1 konckout mice	1000 ± 300	–	–	Hong et al. (1999a, b)
4	Liver female microsomes from 4-months old P450 2E1 konckout mice	1050 ± 175	–	–	Hong et al. (1999a, b)
5	Liver male microsomes from 8-months old P450 2E1 konckout mice	1700 ± 150	–	–	Hong et al. (1999a, b)
6	Liver female microsomes from 8-months old P450 2E1 konckout mice	1200 ± 750	–	–	Hong et al. (1999a, b)
7	CYP 2E1 knockout ($2E1^{-/-}$)	1350 ± 425	–	–	Hong et al. (1999a, b)
8	Human liver microsomes	312 ± 28	–	–	Hong et al. (1997a, b)
9	Rat liver microsomes	700 ± 35	–	–	Hong et al. (1997a, b)
10	Mouse liver microsomes	720 ± 72		–	Hong et al. (1997a, b)
11	CYP 2A6	6.1	–	–	Hong et al. (1997a, b)
12	CYP 2E1	0.7	–	–	Hong et al. (1997a, b)

Unit of Kcat for microsomal proteins (Refs. of 2, 3, 4, 5, 6, 7, 8, 9, 10) is in nM min^{-1} mg^{-1}

Unit of Vmax/Km for Ref. 2 is µl min^{-1} mg^{-1}

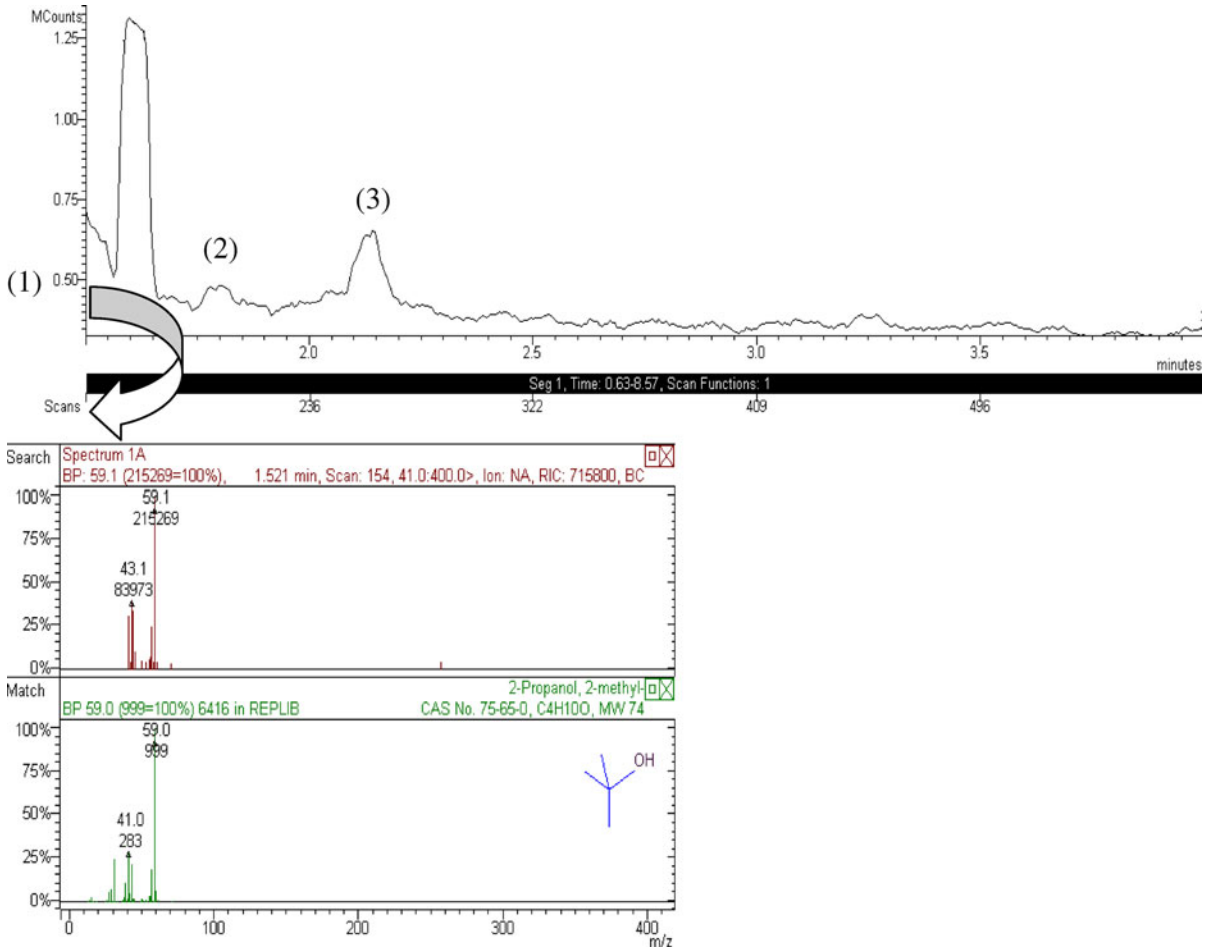

Fig. 4 Headspace GC spectra of MTBE degradation by CYP 2A6: (*1*) TBA; (*2*) 2-methyl-2-hydroxy-1-propanol and (*3*) 2-hydroxy isobutyric acid

in buffer through the septum at concentrations of 0.5–10 mM. After 30 min incubation at 37°C, the reaction was terminated with 150 µl perchloric acid 0.3 M for the HPLC and HS-GC/MS analysis. A blank assay was run without substrate for each sample. Aldehyde DNP derivatives were formed by adding 100 µl 2,4-dinitrophenylhydrazine dissolved in HCl 6 N. The samples were mixed for 20 min, centrifuged for 5 min and then analyzed by HPLC. The mean recovery of aldehydes as DNP derivatives was greater than 90%.

Kinetic analysis

For kinetic studies, the metabolic rates were determined at eight different substrate concentrations ranging from 0.02 to 0.6 mM and the determinations were repeated at least two times to ensure data reproducibility. The Michaelis–Menten kinetic parameters V_{max} and K_m were determined and the initial estimated values for these parameters were used to obtain efficiency of the enzyme (V_{max}/K_m).

Results and discussion

Kinetic analysis of MTBE biodegradation in closed system

In biochemistry, we are interested in rates of enzyme catalyzed reactions for understanding metabolic pathways and the mechanism of catalysis of an enzyme.

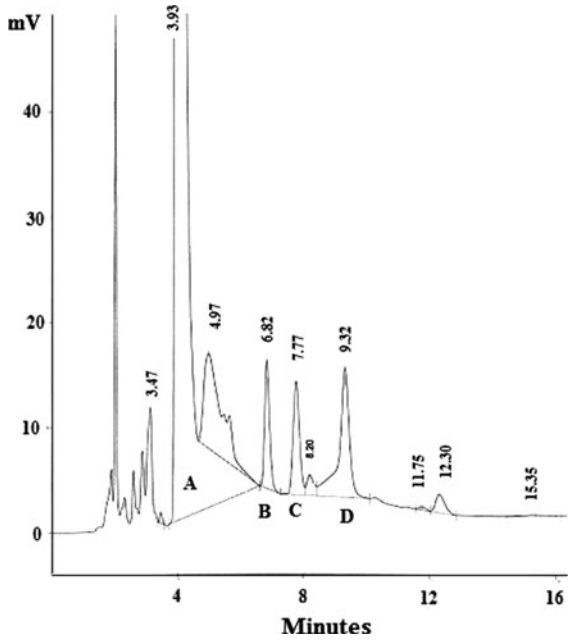

Fig. 5 HPLC analysis of MTBE degradation by CYP 2A6. Chromatographic peaks were identified by their retention times (*RT*): (*A*) DNPH (RT = 3.93), (*B*) formaldehyde (RT = 6.82), (*C*) acetaldehyde (RT = 7.77) and (*D*) acetone (RT = 9.32)

When 20 pmol CYP-450 was incubated with 0.114 mM MTBE at 37°C with different termination times from zero to 30 min, degradation of MTBE was initiated without a significant lag period (Fig. 1). As it can be seen from Fig. 1, most of MTBE is degraded up to 13 min. Considering this time, several concentrations of substrate (S_0) were studied and initial rates of the enzyme catalyzed reaction (V_0) were plotted against S_0 (Fig. 2).

Here it is easy to see the saturation of the enzyme at high S_0 where the initial velocity approaches a limiting value. According to Lineweaver–Burk equation and by plotting $1/V_0$ versus $1/S_0$ (Fig. 3), V_{max} was calculated as 0.137 mM min^{-1} Kcat as 3425 (nM min^{-1}) nM^{-1} and K_m as 3.13 mM. So the efficiency of the enzyme is $V_{max}/K_m = 0.044$ min^{-1}. Table 3 summarizes the kinetic parameter evaluation results for MTBE degradation in the presence of the some enzymes. As can be seen, the present study demonstrates that CYP 2A6 possesses a high activity towards the oxidative metabolism of MTBE.

Proposed pathway for MTBE degradation by CYP 2A6

To identify MTBE degradation products and to determine a pathway for MTBE degradation (Fig. 6), CYP-450 was incubated with MTBE and degradation was monitored by HS-GC/MS and HPLC. A representative GC/MS chromatogram for the metabolic profile of MTBE in the presence of CYP 2A6 is shown in Fig. 4. Initial experiments demonstrated that the first measureable degradation product of MTBE was TBA. In addition to TBA, HS-GC/MS results suggested that *tert*-butoxy methanol, 2-methyl-2-hydroxy-1-propanol and 2-hydroxy isobutyric acid were the key intermediate products.

HPLC analysis of the culture media during the disappearance of MTBE revealed the presence of formaldehyde, acetone and acetaldehyde (Fig. 5). Also an unknown peak at 4.97 min was observed in Fig. 5. Although we were not able to assay the peak by a certified reference material but an idea to explain the peak can be oxidizing 2-methyl-2-hydroxy-1-propanol to 2-methyl-2-hydroxy-1-propionaldehyde.

According to identified molecular structures, the possible relation of these metabolites could be speculated as Fig. 6. It is noted that some of the chemicals found in low concentrations, such as acetaldehyde (derived from HPLC analysis), may be artifacts of the isolated enzyme CYP 2A6.

Conclusions

CYP2A6, which is constitutively expressed in human livers, was used for the study of MTBE metabolism. Kinetic studies demonstrate that CYP 2A6 possesses a high activity towards the oxidative metabolism of MTBE and plays a significant role in the metabolism of MTBE. HS-GC/MS and HPLC were used for identification of MTBE degradation products. The results suggested that the main intermediate were TBA, formaldehyde, *tert*-butoxy methanol, 2-methyl-2-hydroxy-1-propanol, 2-hydroxy isobutyric acid, acetone and acetaldehyde, so a pathway for MTBE degradation was proposed.

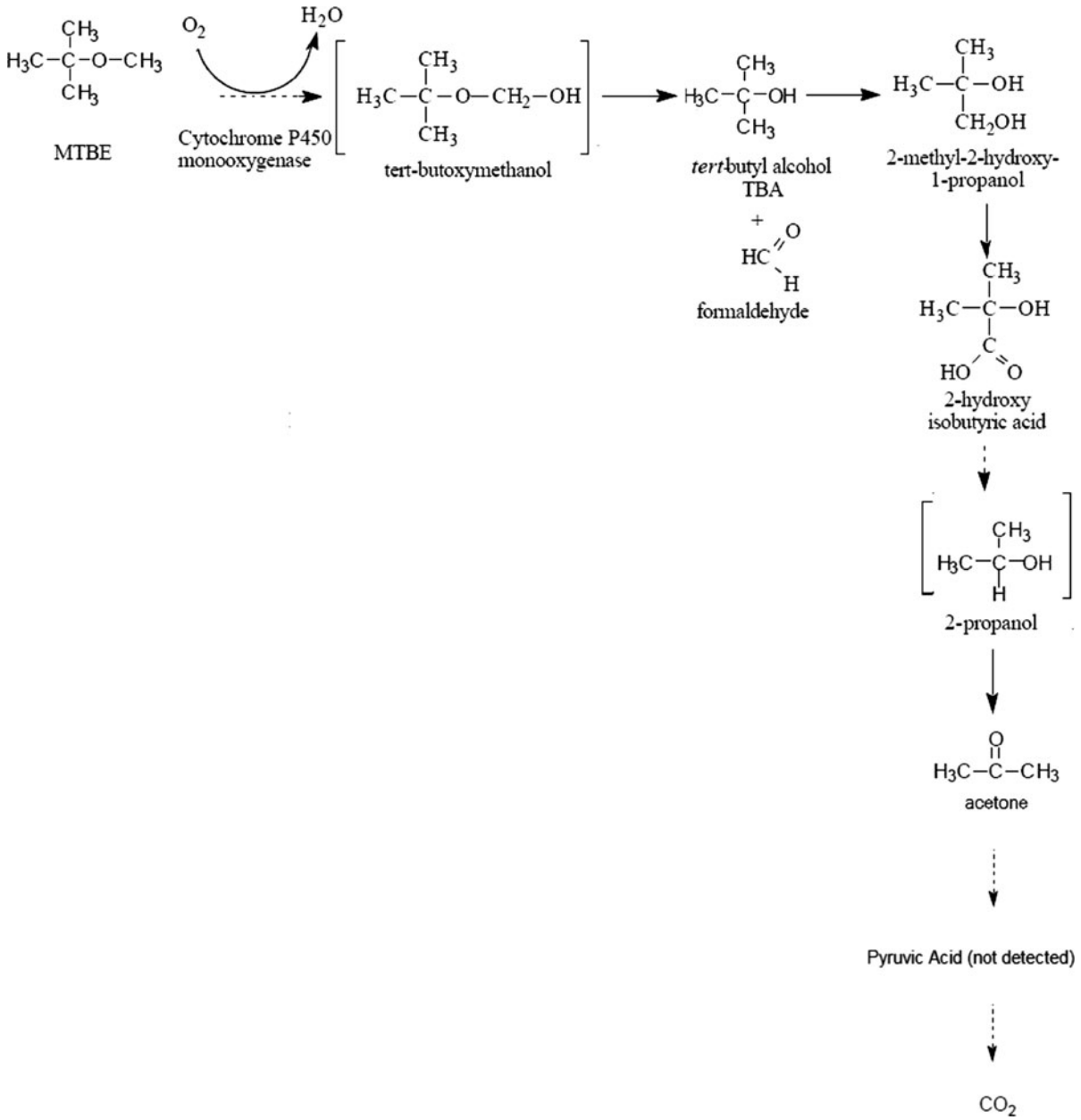

Fig. 6 Proposed pathways for the degradation of MTBE by CYP-450 which were derived from analysis of intermediates of MTBE degradation by HS-GC/MS and HPLC

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