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Metabolism of 3,5-Di-*t*-butyl-4-methylphenyl N-Methylcarbamate, Terbucarb, on Isolated Rat Hepatocytes

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2,6-Di-t-butyl-4-methylphenyl N-methylcarbamate, Terbucarb, is a herbicide used for controlling crab- and goose-grass on golf courses in Japan. Terbucarb has been detected in drainage and runoff water from golf courses at ppb levels (Tsuji et al. 1991). Terbucarb belongs to phenylcarbamates family, and the alkyl substituted phenyl N-methylcarbamates are usually oxidized in mammals, insects, fungi and plants (Aizawa H. 1982). The chemical sites undergoing oxidation in the carbamates are mainly alkyl groups bound to the aromatic ring or N-methyl groups. The former group is oxidized to carboxylic acid via alcohol, and the latter group is converted to N-carbamate. Hydrolysis of the carbamate linkage is also another important metabolic reaction yielding phenolic compounds. In a previous study, we have demonstrated that the addition of Terbucarb to isolated rat hepatocytes caused acute cell death. Terbucarb's cytotoxicity was shown to be less than for its hydrolysis product, 2,6-di-t-butyl-4-methylphenol (BHT) (Nakagawa et al. 1994). In this study, we have investigated the metabolism of Terbucarb and BHT in isolated rat hepatocytes.

MATERIALS AND METHODS

Chemicals were purchased from the following companies: BHT, 2,6-di-t-butyl-4-hydroxymethylphenol(4-CH_2OH-BHT), 2,6-di-t-butyl-4-formylphenol (4-CHO-BHT) from Tokyo Chemical Industry (Tokyo, Japan); 2,6-di-t-butyl-4-carboxylphenol (4-COOH-BHT) from Aldrich (Milwaukee, USA); and Terbucarb was purchased from GL Science (Tokyo). 2,6-Di-t-butyl-4-hydroxymethylphenyl N-methylcarbamate (4-CH_2OH-Trebucarb), 2,6-di-t-butyl-4-formylphenyl N-methylcarbamate (4-CHO-Terbucarb) and 2,6-di-t-butyl-4-carboxylphenyl N-

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methylcarbamate (4-COOH-Terbucarb) were synthesized with methylisocyanate and corresponding phenols in dimethylformamide (DMF) by the method of Douch and Smith (1971). 2,6-Di-t-butyl-4-methylphenyl N-carbamate (NH_2-Terbucarb) and 2,6-di-t-butyl-4-carboxylphenyl N-carbamate (NH_2-4-COOH-Terbucarb) were prepared by the reaction of the chloroformate, prepared by the reaction of the corresponding phenol with phosgene in the presence of K_2CO_3, with NH_4OH solution in DMF (Cheng and Casida. 1973), and were purified with column chromatography. The synthesized compounds were identified by IR, ^{1}H-NMR, ^{13}C-NMR and GC-MS analysis, and those purities were more than 95%.

Male Fischer-344 rats (220 - 260 g) were used in all experiments. Hepatocytes were isolated by collagenase perfusion of liver as described by Moldéus et al., (1978) and were suspended at a final concentration of 10^6 cells/mL in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM Hepes and 0.1% albumin. Initial cell viability assessed by Trypan blue exclusion was typically 90%. All incubations were performed in rotating, round-bottomed flasks at 37° C under a continuous flow of humidified carbogen (95% O_2 and 5% CO_2). Reactions were initiated by the addition of Terbucarb or BHT dissolved in DMSO (final concentration less than 1%). The corresponding control groups received an equivalent volume of DMSO. Aliquots of cell suspensions were taken at indicated times for the determination of the metabolites of Terbucarb or BHT.

The cell suspension aliquots were deproteinized by the addition of an equivalent volume of acetonitorile and centrifuged. The metabolites in the supernatant were analyzed by reversed-phase HPLC, Jasco 800 series; column, TSK-gel ODS-120T, 4.6 mm i.d. x 250 mm (Tosoh) equipped with an UV absorbance detector at 270 nm. GC-MS analysis was performed with a GC, HP5890; col., DB-5, 0.25 mm i.d. x 15 m, film thickness, 0.25 μm; column head press., 40 kPa; inj. temp., 220° C; split-less injection; oven, 50° C for 1 min and increased from 50 to 250° C at 15° C/min; MS, Trio-1000; EI^+ at 70 eV. The supernatant (200 μL) was diluted with 10 mL of 20% NaCl solution and extracted twice with 2 mL of dichloromethane. The dichloromethane phase was dehydrated with sodium sulfate and then evaporated to dryness under the stream of nitrogen. The residue was dissolved in n-hexane. The metabolites containing a carboxyl group were analyzed as follows. The cell suspension (200 μL) was diluted with 5 mL of 20% NaCl solution and acidified to pH 2 with 1N HCl. The metabolites were extracted twice with 2 mL of diethylether. The ether phase was dehydrated with sodium sulfate and

evaporated to dryness under the stream of nitrogen. Derivatives were prepared by addition of 250 μL of 0.025% 9-anthryldiazomethane (ADAM) dissolved in acetone to the residues (Suzuki and Watanabe, 1991). The ADAM derivatives were analyzed by reversed- phase HPLC described above equipped with a fluorescence detector at excitation, 365 nm; emission, 412 nm. To determine the amounts of glucuronide or sulfate conjugates cell supernatants were first deproteinized and evaporated before treatment with β-glucuronidase (15000 units) or sulfatase (2 units) dissolved in 1 mL of 0.5 M acetate buffer (pH 5.0) at 37° C for 16 hr. The liberated aglycons were extracted and analyzed.

RESULT AND DISCUSSION

The materials found in peaks 1, 2, 3, 4, 8, 9 and 10 of the HPLC elution profile shown in Figure 1A were identified as Terbucarb, 4-CH_2OH-Terbucarb, 4-CHO-Terbucarb, 4-COOH-Terbucarb, 4-CH_2OH-BHT, 4-CHO-BHT, 4-COOH-BHT and NH_2-Terbucarb, respectively. The metabolites found in peaks 4, 6 and 10 on the HPLC elution profile shown in Figure 1B were identified as 4-COOH-Terbucarb, NH_2-4-COOH-Terbucarb and 4-COOH-BHT, respectively. The GC-MS chromatogram of the CH_2Cl_2 phase extracted from cell suspension with Terbucarb is shown in Figure 2A. The fragmentation patterns of chemically synthesized N-methylcarbamates and N-carbamates coincided with those of the corresponding phenols; BHT, Terbucarb and NH_2-Terbucarb having a common base peak ion at m/z 205 and fragment ions, which were m/z 220, 177, 161, 145, 105 and 57. 4-CHO-BHT and 4-CHO-Terbucarb appeared both at the base peak ion at m/z 219 which fragment ions at m/z 234, 191, 175, 159, 115 and 57. 4-CH_2OH-BHT and 4-CH_2OH-Terbucarb had a base peak ion at m/z 221 and fragment ions at m/z 236, 193, 161, 147, 115 and 57. In addition, the compounds identified by HPLC, NH_2-Terbucarb (peak 5) and BHT (peak 7) were detected. Furthermore, mass spectrum of peak 11 is shown in Figure 2C. Previously, Thompson et al (1987) have identified 6-t-butyl-2-[2-(1-hydroxy-2-methylpropyl)]-4-methylphenol as a metabolite derived from BHT by. This metabolite corresponds to metabolite No.12 (Figure 2D and 3) in our experiments. It is of interest that the fragmentation of peak 11 derived from Terbucarb were similar to that of peak 12. However, the material corresponding to peak 11 was not found in the CH_2Cl_2 extracts from cells incubated with BHT (Figure 2B). Mass fragmentations of Terbucarb and its metabolites at the 4-methyl group produced a low intensity molecular ion (>1%), which are similar to the corresponding phenols. Although the ester linkage group

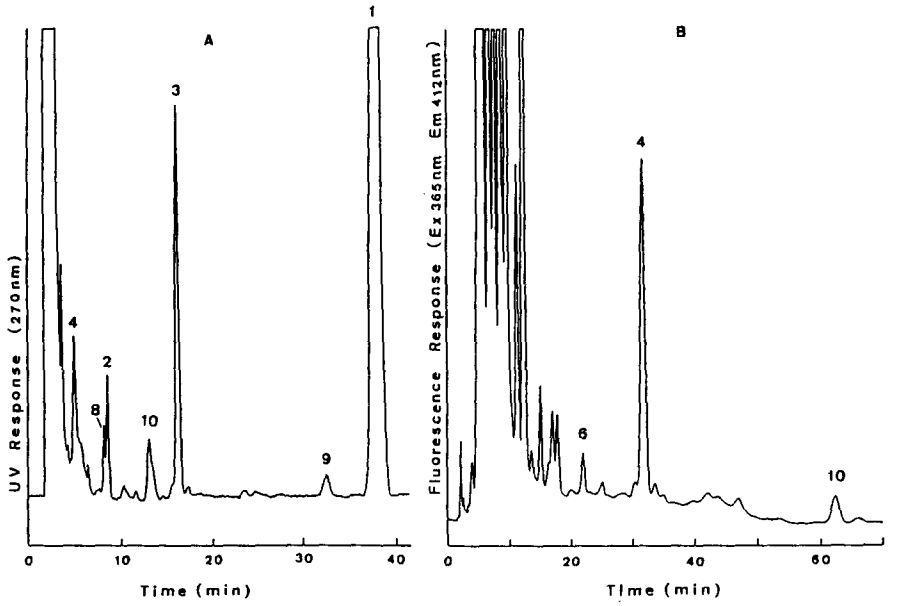

Figure 1. HPLC chromatograms of Terbucarb metabolites recovered from isolated rat hepatocytes. (A) UV detection, CH_3CN - 50 mM KH_2PO_4 (50 : 50, v/v) 1mL/min, 40° C; (B) Fluorescence detection, CH_3CN - H_2O (70 : 30, v/v) 1mL, 40° C; For further details see "Materials and Methods". Numbers indicated in the figure refer to structural formulas given in Figure 3.

could not be identified, these results suggest that a t-butyl group of Terbucarb was hydroxylated in rat hepatocytes.

To further investigate the metabolism of BHT and Terbucarb in rat hepatocytes, the changes in levels of several metabolites were examined by GC-MS with time (Table 1). Hydroxylation of the 4-methyl group was the major route of Terbucarb metabolism in isolated rat hepatocytes as evidenced by the large amount of 4-CH_2OH-Terbucarb formed in comparison to the other metabolites detected. The amounts of BHT, a hydrolysis product of Terbucarb, and its 4-methyl group oxidative product were detected in the range of 0.1 to 1.2 μM. The amounts of NH_2-Terbucarb, N-demethylation products of Terbucarb, and NH_2-4-COOH-Terbucarb were observed in the range of 0.1 to 0.8 μM. Following treatment of deproteinized/evaporated supernatants with β-glucuronidase or sulfatase the amounts of glucuronide or sulfate conjugates were determined. 4-COOH-Terbucarb and 4-CH_2OH-Terbucarb were not increased in the hydrolysate and the amounts of both compounds were less than 0.1 μM. O-Glucuronide and ester glucuronide conju-

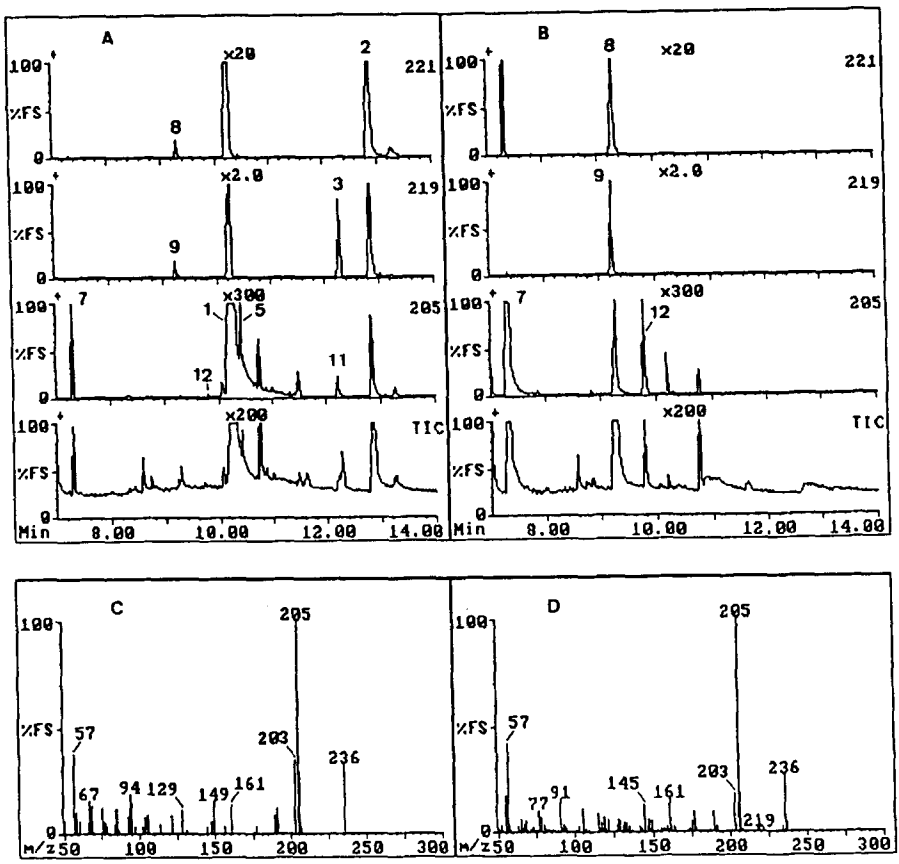

Figure 2. GC-MS chromatograms and mass spectra of Terbucarb (A,C) and BHT (B,D) metabolites recovered from isolated rat hepatocytes. For further details see "Materials and methods". Numbers see Figure 1.

gates were negligible amounts by hepatocyte. After hydrolysis by sulfatase, 1.7 and 1.6 μM free 4-CH_2OH-Terbucarb was recovered for cell suspensions incubated with 0.5 mM and 1 mM Terbucarb, respectively, after a 120 min. These results suggest that the major metabolic pathway of Terbucarb in isolated rat hepatocytes is via the oxidation of the 4-methyl group whereas N-demethylation, hydroxylation of the t-butyl group and hydrolysis of the ester linkage are minor metabolic pathways (See Figure 3). Phenyl carbamate pesticides such as BPMC (m-sec-butylphenyl N-methylcarbamate, Cheng and Casida. 1973), Tsumicide (m-tolyl N-methylcarbamate, Ohkawa et al. 1974), and MTBC (m-t-butylphenyl N-methylcarbamate, Douch and Smith. 1971) have been shown to be metabolized oxidatively at the alkyl substituent of the phenyl ring, N-demethylated and hydrolyzed to their corresponding phenols

Table 1. Metabolism of Terbucarb and BHT on isolated rat hepatocytes

No.[b] Metabolites	Concentration (μM)[a]							
	Terbucarb				BHT			
	Incubation Time (min)				Incubation Time (min)			
	0	30	60	120	0	30	60	120
1. Terbutcarb	495.0	470.0	448.0	422.5	-	-	-	-
	990.0	973.9	967.7	940.9	-	-	-	-
2. 4-CH_2OH-Terbucarb	ND	9.3	15.1	17.9	-	-	-	-
	ND	8.3	11.2	19.9	-	-	-	-
3. 4-CHO-Terbucarb	ND	0.6	0.9	1.0	-	-	-	-
	ND	0.4	0.6	0.9	-	-	-	-
4. 4-COOH-Terbucarb	ND	0.5	1.1	2.4	-	-	-	-
	ND	0.6	0.7	1.6	-	-	-	-
5. NH_2-Terbucarb	ND	0.4	0.5	0.5	-	-	-	-
	ND	0.1	ND	ND	-	-	-	-
6. NH_2-4-COOH-Terbucarb	ND	0.8	0.2	0.4	-	-	-	-
	ND	0.2	0.1	0.4	-	-	-	-
7. BHT	ND	0.1	0.2	0.2	499.0	459.0	428.5	367.5
	ND	0.1	0.2	0.2	995.0	955.7	946.9	904.0
8. 4-CH_2OH-BHT	ND	0.1	0.4	0.6	ND	24.1	32.6	33.2
	ND	ND	ND	ND	ND	21.6	22.7	19.0
9. 4-CHO-BHT	ND	0.1	0.2	0.3	ND	7.5	16.5	28.5
	ND	ND	ND	ND	ND	5.5	10.0	11.9
10. 4-COOH-BHT	ND	ND	ND	0.2	ND	2.1	3.8	6.8
	ND	ND	0.3	1.0	ND	2.2	3.6	4.1

[a]For each metabolite, the upper and lower dada are from incubations with 500 and 1000 μM, respectively. ND, less than 0.1 μM; -, not determined.
[b]Numbers indicated in this Table refer to structural formulas given in Figure 3.

by rat or mice liver microsomes in the presence of a NADPH generating system. In the same manner it is expected that Terbucarb will be metabolized by microsomal enzymes.

Comparative cytotoxicity between BHT and Terbucarb has been reported. In isolated rat hepatocytes, Terbucarb, which causes acute cell death accompanied by the inhibition of mitochondrial respiration was less toxic than BHT (Nakagawa et al. 1994). Metabolites of BHT have previously been identified by Thompson et al., (1987). In this study the 4-methyl group of BHT was oxidized to the corresponding alcohol, aldehyde, and carboxylic acid in isolated rat hepatocytes. Since in our study the total amount of these metabolites derived from 1 mM BHT in cell suspensions was less than with 0.5 mM BHT during the incubation period, it seems reasonable that BHT and/or its metabolites at high concentrations affect the function of microsomal enzymes. Nakagawa et al., (1983) suggested that the irreversible binding of BHT-quinone methide or 4-CH_2OH-BHT to sulfhydryl groups of microsomal protein affects the normal

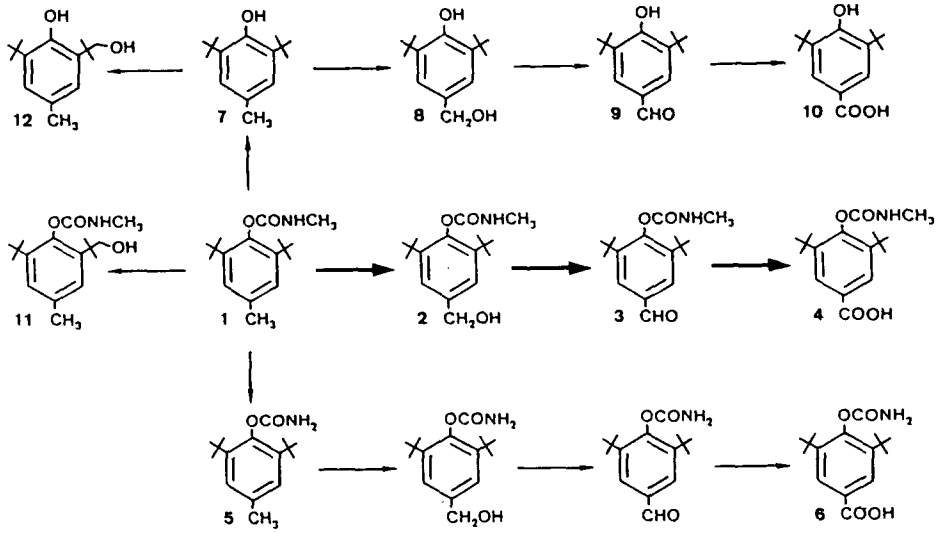

Figure 3. Proposed metablic pathway of Terbucarb on isolated rat hepatocytes.

physiological cellular function. In the present study, the formation of BHT derived from Terbucarb by hydrolysis was negligble compared with the amount of 4-CH_2OH- Terbucarb recovered during the same incubation period (Table 1). The hydroxy group of BHT is causally associated with the cytotoxicity and inhibition of mitochondrial respiration because substitution with a methylcarbamate group for the hydroxy group of BHT decreased toxicity (Nakagawa et al, 1994). Although the addition of Terbucarb (1 mM) to hepatocytes resulted in approximately 50% cell death at 180 min (Nakagawa et al, 1994), the addition of BHT (1 mM) caused 100% cell death only after 90 min (Table 1). Based on these results, it seems that the toxic effects of Terbucarb in hepatocytes differ from that of BHT. Futher investigative work will be necessary to determine the mechanism of Terbucarb-induced cytotoxicity.

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