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Thin-layer chromatographic fractionation of Ames mutagenic chlorosemiquinone in chlorinated 4-methylphenol solution

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Abstract

The diethyl ether extract from an aqueous solution of 4-methylphenol after treatment with chlorine was mutagenic to the Ames *Salmonella* test strain TA100 in the absence of liver homogenate. Gas chromatography–mass spectrometry (GC–MS) showed the occurrence of chlorinated products in the extract: chloro-4-methylphenols, chloro-4-methylquinones and chlorinated 4-methylphenol dimers. The diethyl ether extract was fractionated into several fractions by polyamide thin-layer chromatography (TLC). The fractionated components were examined for mutagenicity by means of Ames assays, and were identified by GC–MS. TLC fractionation of the extract revealed that two components present in the extract are mutagenic. GC–MS analysis indicated the presence of 2,6-dichloro-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one as the major mutagen and chlorinated 4-methylphenol dimers as minor mutagenic compounds in the chlorinated 4-methylphenol solution.

1. Introduction

Mutagenic and carcinogenic organic compounds found in source water [1] and drinking water [2] have recently caused concern as to their potential effects on human health. Mutagenicity in source waters has generally been attributed to contamination by industrial waste or agricultural run-off, and to a lesser extent to naturally occurring substances.

In comparison studies of raw water vs. finished water, Glatz et al. [3] and Maruoka and Yamanaka [4] suggested that chlorination may play a major role in the production of organic mutagens in potable water. Subsequent laboratory studies by Cheh et al. [5], in which a drinking water treatment process was simulated, clearly demonstrated that non-volatile mutagens were produced by chlorine disinfection. Several volatile compounds such as chloroacetones and chloropropenal, and chlorinated hydroxyfuranone (MX), exhibiting mutagenicity have been identified to be present in chlorinated waters [6–10], but many of the non-volatile mutagenic substances have not been fully characterized.

One class of compounds recognized as important pollutants in aquatic environment is phenols because of off-favour in water and their high toxicity. Therefore, it seems that mutagens are formed as a result of the reaction of chlorine with phenolic compounds that may be present in the water sources. Direct support for this hypothesis has been provided by Rapson et al. [11]

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and by our recent studies which demonstrated that cresol, naphthols and phenylphenols react with chlorine to form direct-acting mutagenic products [12–14]. We now present more detailed findings on the characteristics of the mutagens formed by the reactions of chlorine with 4-methylphenol in aqueous solution. 4-Methylphenol was chosen in this work because this compound has been used as an intermediate in the production of important industrial chemicals and also identified as being present in aquatic samples [15].

2. Experimental

2.1. Materials

4-Methylphenol, 2-chloro-4-methylphenol, 2,6-dichloro-4-methylphenol, Irgasan DP 300 (as a typical chlorinated phenoxyphenol) and trichloroacetic acid used for product identification and mutagenicity tests were commercial products. Standard solutions of these compounds both alone and as mixtures were prepared by dissolving the compounds in methanol and subsequent dilution. Chlorinated aqueous solution was prepared by diluting sodium hypochlorite solution (ca. 10% available Cl) (Nacalai Tesque, Kyoto Japan) with 0.1 *M* disodium hydrogenphosphate–sodium dihydrogenphosphate solution. The hypothclorite concentrations were determined by iodimetric titration. Polyamide 11 F_{254} (20 × 20 cm, thickness 0.15 mm) precoated thin-layer chromatographic (TLC) plates (Merk, Darmstad, Germany) were used to fractionate the diethyl ether extract.

2.2. Aqueous chlorination of 4-methylphenol and extraction of reaction mixture

Aqueous 4-methylphenol solution (0.5 mmol/l) was treated with 6 equiv. of chlorine (as HOCl) at 20°C for 1 h at pH 5. The unreacted chlorine was removed by addition of sodium thiosulfate solution. The reaction mixture was then acidified to pH 2 with 6 *M* hydrochloric acid before extraction with two 200-ml portions of diethyl ether. The extracts were dried over anhydrous sodium sulfate and concentrated under vacuum at 40°C to volumes suitable for polyamide TLC, GC and GC–MS analyses. Part of the diethyl ether extract was resuspended in 1 ml of dimethyl sulfoxide for mutagenicity assays.

2.3. Fractionation of mutagenic extract by TLC

Diethyl ether extract (about 50 mg) was fractionated into seven fractions by TLC on polyamide plates using chloroform as a developing solvent. The detection of spots on the plates was performed by UV irradiation. The plates were also sprayed with 0.5% bromocresol green solution in order to detect acidic products. The separated zones were scraped off by using special recovery tubes (Wako, Osaka, Japan) and the adsorbed substances were eluted with diethyl ether. The R_F values of the fractions and the amounts of sample recovered from each fraction are shown in Table 1. The recoveries (%, w/w) of individual fractions were calculated as [(amount of sample recovered)/(amount of sample applied)] · 100.

2.4. Product resolution and characterization

A Hitachi Model 263-30 gas chromatograph equipped with a flame ionization detector and a 2 m × 3 mm I.D. glass column packed with 2% OV-1 on Uniport HP (60–80 mesh) was programmed from 100 to 260°. The injector and detector temperatures were 260°C. The helium gas flow-rate was 30 ml/min. A Hitachi D-2500 chromato-integrator was used to determine the retention times and peak areas on the chromatograms.

A Hitachi M-80 combined mass spectrometer–gas chromatograph with a Hitachi M-003 data processing system was used for the qualitative analyses of samples under the following conditions: ion source temperature, 250°C; trap current, 70 μA; and electron energy, 70 eV. The column and chromatographic conditions were the same as described above.

The NMR spectra were measured on a JEOL JNM-FX100 (^{1}H, 100 MHz) instrument. Deuterochloroform was used as the solvent. The chemical shifts (δ values) are given in ppm downfield from tetramethylsilane.

2.5. Mutagenicity tests

The mutagenicity of the samples was tested according to the method of Ames et al. [16] with minor modifications. Liver homogenate (S9) was prepared from male Sprague–Dawley rats, which had been pretreated with polychlorinated biphenyls to activate enzymes. Addition of the S9 mix was conducted to confirm whether test compounds are direct-acting or indirect-acting mutagens which are required mammalian metabolic activation. *Salmonella typhimurium* strains TA98 and TA100 were used throughout the experiments. The samples were dissolved in dimethyl-sulfoxide and preincubated with a test strain at 30°C for 30 min (prior plating) with and without S9. A buffer was used when S9 was not employed. After addition of the test samples, the plates were incubated at 37°C for 2 days. The assay was performed in triplicate for each sample. The mutagenic activity is expressed as the mean value of revertants.

3. Results and discussion

A previous study [14] revealed that treatment with a high chlorine dose (20 equiv. of HOCl per mole of compound) of 4-methylphenol in neutral aqueous solution (pH 7) products a series of highly chlorinated compounds. They were chlorinated 4-methylphenols, chlorohydroxylated 4-methylquinone and polychlorinated 4-methyl-phenol dimers. Among these chlorinated compounds, polychlorinated 4-methylphenol dimers have also been shown to be mutagenic towards test strain TA100 without S9 mix. However, our recent studies demonstrated that aqueous chlorination of 4-methylphenol produces the mutagenicity, even at a low chlorine dose (5 equiv. of HOCl per mole of compound) in an acidic solution of pH 5. Since the mutagenic substances were not characterized in the previous work [14], further GC–MS studies combined with TLC fractionation of the mutagenic diethyl ether extract were performed again in this study.

3.1. TLC fractionation of mutagenic diethyl ether extract

A preliminary fractionation of the mutagenic diethyl ether extract from the chlorine-treated 4-methylphenol solution was performed by TLC using polyamide plates. The TLC results for the original extract and mutagenicity tests with strain TA100 in the absence of S9 are shown in Table 1 and presented graphically in Fig. 1.

When the original diethyl ether extract was fractionated on polyamide plates, the recovery in the fractions was approximately 75% (w/w) of the amount of sample applied, while the mutagenicity recovered from the plates was 60% of the overall activity applied (Table 1). These results indicate that mutagenic substances in the extract are converted into non-mutagenic materials during the TLC fractionations [17]. In addition, most of the mutagenic materials in the diethyl ether extracts were found to be concentrated in fraction 3 on the polyamide plate (Table 1 and Fig. 1). This suggests that the mutagenic materials in the extract are of intermediate polarity.

The dose–response curves of the samples are shown in Fig. 1. Among these components, fraction 3 showed the strongest mutagenic response, and its mutagenic potential was approximately 30–50 revertants/nmol (Fig. 1). This potential was nearly the same as those reported for 1,1,3-trichloroacetone and 2-chloropropenal, but much lower than that found for chlorinated hydroxyfuranone (MX), which have been cited as important mutagens in Kraft chlorination effluents [6–8]. Fractions 6 and 7 also exhibited mutagenicity, but the activity was very low (2 revertants/nmol) as compared with that of fraction 3. Most of the mutagenicity of the original diethyl ether extract obtained from the chlo-

Table 1
Results of TLC of the diethyl ether extract on polyamide with chloroform, and mutagenicity tests with strain TA100 in the absence of S9 for each fraction and standard compounds

Fraction No. and standard compound	R_F values	Amount of sample recovered		Net revertants		
		mg	% (w/w)[a]	Per μg	Per fraction[b] ($\times 10^4$)	(%)
Original extract	–	55	100	160	880	100
1	0.00–0.09	0.08	0.15	–	0.038	0.004
2	0.09–0.37	0.08	0.15	–	0.025	0.003
3	0.37–0.69	33.92	61.67	156	529	60.113
4	0.69–0.80	3.17	5.77		ND[c]	
5	0.80–0.93	0.05	0.04		ND	
6	0.93–0.96	0.17	0.30		0.016	0.002
7	0.96–1.00	3.73	2.79	6.24	2.325	0.264
4-Methylphenol	0.45–0.55				ND	
2,6-Dichloro-4-methylphenol	0.72–0.80				ND	
Irgasan DP 300	0.80–0.85				ND	
Trichloroacetic acid	0.00–0.20				ND	

[a] [(Amount of sample recovered)/(amount of sample applied)] · 100.
[b] Mutagenicity derived from the initial and linear portions of the dose–response curve for each sample (Fig. 1).
[c] Not detected in the tested range of 1–1000 μg per plate.

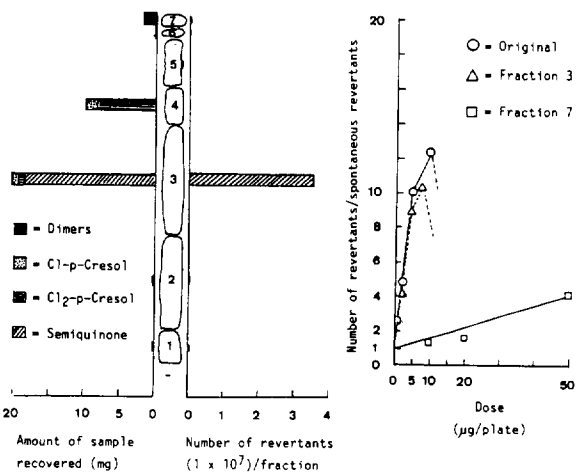

Fig. 1. Fractionation of a mutagenic diethyl ether extract of chlorine-treated 4-methylphenol solution by polyamide TLC (left), and mutagenicity in the original extract and polyamide TLC fractions 3 and 7 against *S. typhimurium* strain TA100 without S9 (right). For TLC conditions and mutagenicity tests, see Experimental. Each point on the dose–response curves (right) represents the average of triplicate determinations.

rinated 4-methylphenol solution was thus accounted for by fraction 3.

3.2. GC and GC–MS studies of the TLC fractions

In order to obtain further information on the mutagenic components present in the diethyl ether extract of chlorine-treated 4-methylphenol solution, the components (fractions 1–7) obtained by polyamide TLC were investigated by means of GC–MS and NMR. Fig. 2 shows the gas chromatograms (flame ionization detection) of the original extract and fractions 3, 4 and 7. The results of mutagenicity tests and GC determinations on the polyamide TLC fractionations are shown in Fig. 1.

2-Chloro-4-methylphenol and 2,6-dichloro-4-methylphenol, corresponding to peaks 1 and 2, respectively, in Fig. 2 were distributed in fractions 3 and 4, but these compounds exhibited no mutagenic response (Table 1). Polychlorinated 4-methylphenol dimers were also distributed in fractions 6 and 7 in increasing amount and

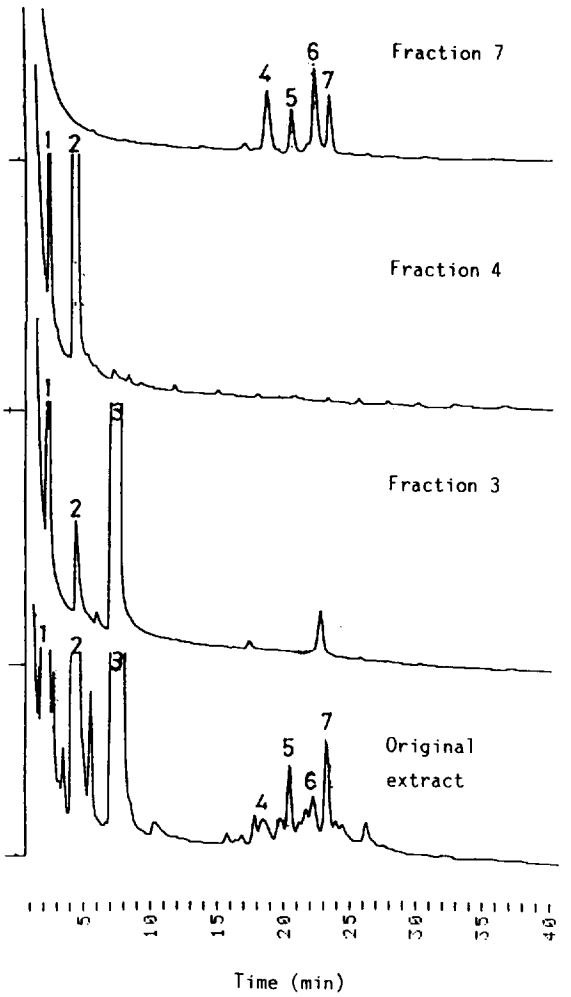

Fig. 2. Gas chromatograms (flame ionization detection) of the original extract and polyamide TLC fractions 3, 4 and 7. For main compounds, see text; for GC conditions, see Exerimental.

mutagenicity (Fig. 1). Fraction 7, with a weak mutagenic response (Fig. 1), consisted of a mixture of di-, tri- and tetrachlorinated 4-methylphenol dimers. These compounds have been shown previously [14] to be the mutagenic materials in the extract from the aqueous 4-methylphenol solution after treatment with a high chlorine dose at pH 7 for 1 h.

Fraction 3, with the highest mutagenic response (Fig. 1), consisted of an unknown compound corresponding to peak 3 in Fig. 2, and its purity was over 87% by GC determination with flame ionization detection. The gas chromatogram in Fig. 2 also shows that this compound is the major component in the original extract. Therefore, it seems that this compound, formed by the reaction of 4-methylphenol with chlorine in an acidic aqueous solution contributes to the mutagenicity of the chlorinated 4-methylphenol solution.

Fig. 3 shows the mass spectrum of the compound that corresponds to peak 3 in Fig. 2, with the highest mutagenic response. This compound gave the molecular ion $[M^+]$ at m/z 192 with two chlorine atoms and the most abundant fragment ions at m/z 177 $[M^+ - CH_3]$ and m/z 157 $[M^+ - Cl]$, indicating the occurrence of $C_7H_5Cl_2O_2$ in the highest mutagenic fraction 3. In addition, the NMR spectrum of this compound showed the disappearance of the hydroxyl proton signal and chemical shifts of the methylproton signals on the aromatic ring to high magnetic field, compared with those observed for 4-methylphenol and 2,6-dichloro-4-methylphenol (Fig. 4). On the basis of these MS and NMR interpretations for TLC fraction 3, the mutagenic compound present in the fraction 3

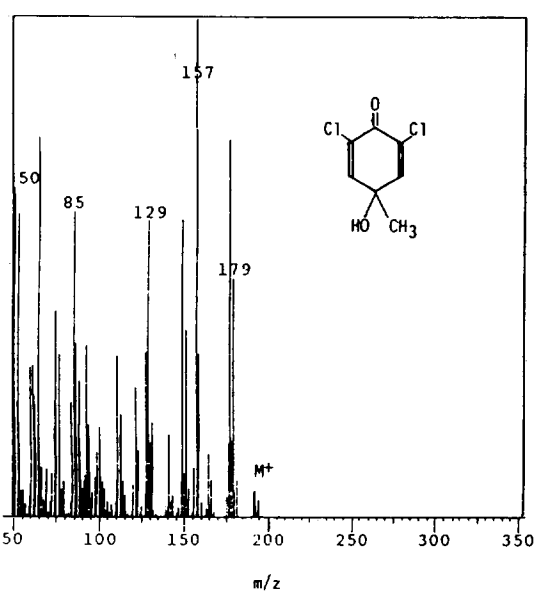

Fig. 3. Mass spectrum of compound corresponding to peak 3 in the chromatogram in Fig. 2.

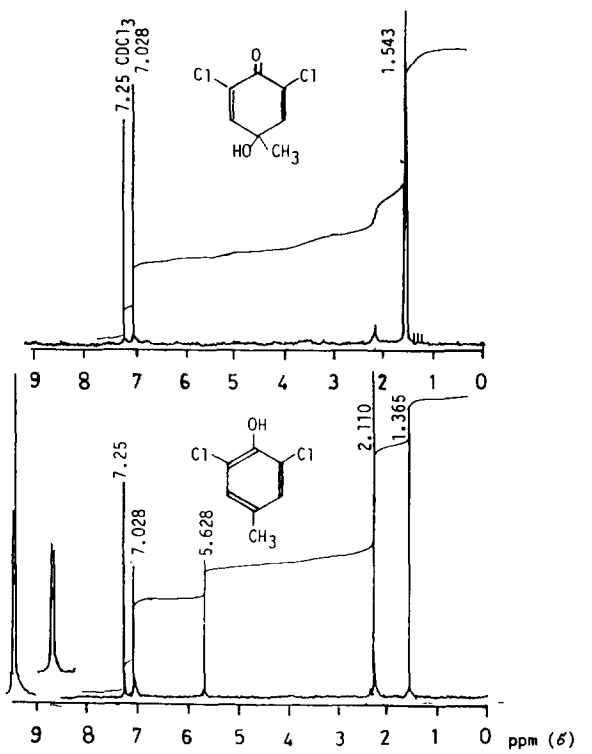

Fig. 4. Proton NMR spectra of compound present in the mutagenic fraction 3 in Fig. 1 and 2,6-dichloro-4-methylphenol standard.

may be 2,6-dichloro-4-hydroxy-4- methylcyclohexa-2,5-dien-1-one.

4. Conclusion

The compound responsible for the mutagenic response could be completely isolated from the diethyl ether-extractable products of the reaction of 4-methylphenol with hypochlorite in acidic aqueous solution (Table 1 and Fig. 1). The compound isolated was mutagenic in the TA100 strain without mammalian metabolic activation, and its mutagenic potential was approximately 30–50 revertants/nmol (Fig. 1). GC–MS and NMR studies of the mutagenic component isolated suggested that this compound is probably 2,6-dichloro-4-hydroxy 4-methylcyclohexa-2,5-dien-1-one (chlorosemiquinone, Figs. 3 and 4), which have yet to be identified. Therefore, subsequent investigations will be conducted to identify and determine the chlorosemiquinone in chlorine-treated waters, such as pulp mill effluents, industrial wastewater and drinking water.

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