Production of Chloro-5-hydroxy-2-nitrobenzoic Acid through Chlorination of 3-Methyl-4-nitrophenol, a Typical Hydrolysate of Fenitrothion


*Department of Chemistry, Biotechnology, and Chemical Engineering, Graduate School of Science and Engineering, Kagoshima University, 1-21-40, Korimoto, Kagoshima, Kagoshima 890-0065, Japan
**Division of Natural Environment and Information, Faculty of Environment and Information Sciences, Yokohama National University, 79-7, Tokiwadai, Hodogaya, Yokohama 240-8501, Japan
***Department of Environmental Engineering, Graduate School of Engineering, Hokkaido University, N13W8, Sapporo 060-8628, Japan

Abstract
Fenitrothion \([O, O\text{-}dimethyl O\text{-}(3\text{-}methyl\text{-}4\text{-}nitrophenyl)phosphorothioate]\), a typical organophosphorus pesticide, is widely used as an insecticide in Japan. In this study, 3-methyl-4-nitrophenol (3M4NP), which is a typical hydrolysate of fenitrothion, was focused as a possible mutagen precursor because it is reported as a common contaminant in raw water for water works, and is capable of forming mutagens when it is chlorinated in water works. In order to examine molecular formulae of mutagens produced from 3M4NP, a chlorinated 3M4NP sample was analyzed with a high-resolution LC/MS. Several peaks were found in a base peak chromatogram. A peak of \(m/z\) 215.971 was focused to be analyzed, suggesting a formation of \(\text{C}_7\text{H}_4\text{O}_5\text{NCl}\) (mass error = 0.397 mmu or 1.837 ppm). To examine the chemical structure of the found substance, MS² experiments were conducted with a collision induced disassociation technique. Four product ions: \(m/z\) 198.9679, 187.9757, 170.9731 and 136.0043 which can be attributed to losses of \(\text{OH}\), \(\text{CO}\), \(\text{COOH}\) and \(\text{Cl}\) functional groups were observed. From these fragment ions and the structure of 3M4NP, the substance was extrapolated to be chloro-5-hydroxy-2-nitrobenzoic acid (\(\text{C}_5\text{H}_2\text{NB}\)). The mutagen formation potential (MFP) of 5-hydroxy-2-nitrobenzoic acid, which is considered to be a precursor of \(\text{C}_5\text{H}_2\text{NB}\), was tested and the MFP of 3,400 net rev./\(\mu\)mol was observed.

Keywords: accurate mass analysis, Ames test, collision induced disassociation, mutagen formation potential, mutagenicity

INTRODUCTION
Chlorination in water purification is indispensable for producing microbiologically safe drinking water. However, chlorine may produce some toxicants by reacting with organic matter. Typical chlorination by-products are trihalomethanes and haloacetates (Bull, R. J., 1981). They are often found in drinking water, so their toxicities have been investigated by many researchers. For instance, Giller et al. (1997) tested the mutagenicity of chloroacetate using the Ames Salmonella assay and reported that it has significant mutagenicity.

The Ames assay has been widely used to detect mutagens in environmental samples because of its minimal labor intensity and good accuracy. For example, the authors...
(Takanashi et al., 2009; Takanashi et al., 2011) tested the mutagenicity of Japanese tap water samples and reported that the Ames assay is more sensitive than the umu assay for detecting mutagens in chlorinated tap water, and that the mutagenicity in 2005 was lower than that in 1993. Due to concerns over mutagens in tap water, many researchers have tried to find major mutagens in tap water. A typical strong mutagen in chlorinated water is 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5H)-furanone (MX) (Wright et al., 2002). The mutagenic activity of MX is reported to be 5,600 – 8,200 net revertant colonies per µmol (net rev./µmol) (Romero et al., 1997), intriguing researcher’s interests on the contribution of the mutagenicity caused by MX to whole mutagenicity of chlorinated water sample. However, the contribution is generally reported to be 8 to 30% (Romero et al., 1997), which may show other major mutagens will be found in chlorinated tap water.

Pesticides are focused as possible precursors of mutagens in the present study. Pesticides are sprayed onto plants, weeds or soil for insect or weed control, and some of the pesticides will transfer to the water environment. These pesticides may undergo various reactions such as hydrolysis, photolysis and biodegradation in the water environment. Pesticides and their transformation products have been found in natural water (Kondo et al., 2012). Natural water containing these pesticides and their degradation products will often be chlorinated at water purification plants, and distributed as tap water.

Fenitrothion \([O, O\text{-dimethyl } O-(3\text{-methyl-4-nitrophenyl})\text{phosphorothioate}],\) a typical organophosphorus pesticide, is widely used as an insecticide in Japan, and is one of 102 pesticides listed in the “Complementary Items for Setting the Targets for Water Quality Management in Japan” document by the Ministry of Health, Labour and Welfare Japan. This document is a category to set water standards for tap water quality management. Fenitrothion itself does not possess mutagenicity (Matsushita et al., 2002), but the authors reported that fenitrothion chlorinated with sodium hypochlorite acid possesses the mutagenicity (Kishida et al., 2008; Kishida et al., 2010a). This report suggests that fenitrothion reacts with chlorine and some of the by-product will be mutagenic.

There are many studies (Weber et al., 2009; Abiru et al., 2011, Kameya et al., 2012) on by-products which are produced from fenitrothion in a natural environment, however, the information on the mutagenicity of the by-products are considerably limited. No mutagen produced from fenitrothion through chlorination has been found according to our knowledge. In the present study, 3-methyl-4-nitrophenol (153.0420 u), thereafter referred to as 3M4NP, was focused as a possible precursor of mutagens in chlorinated fenitrothion. 3M4NP is a typical hydrolysate of fenitrothion and reported that 3M4NP itself has no mutagenicity (Kishida et al., 2010b). In order to examine molecular formulae of mutagens produced from 3M4NP, chlorinated 3M4NP sample was analysed by an LC-ESI-Kingdon trap MS. A possible precursor of the identified substance was tested its mutagenicity and MFP with the Ames Salmonella assay.
MATERIALS AND METHODS

Chemicals
3M4NP (extra pure grade, Fig. 1(a)) were purchased from Tokyo Chemical Industries, Ltd. (Tokyo, Japan). Acetone (for RP/PCB anal.), dichloromethane (for RP/PCB anal.), dimethyl sulfoxide (DMSO, JIS special grade), methanol (super special grade), n-hexane (for RP/PCB anal.), sodium hypochlorite solution (practical grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5-hydroxy-2-nitrobenzoic acid (5H2NB, > 99%, Fig. 1(b)) was purchased from Alfa Aesar, Ltd. (Lancashire, UK).

Sample preparation
To prepare an aqueous solution of 10 mg-3M4NP/L, 25 mg of 3M4NP was dissolved into 1 mL of ethanol and 800 μL of the solution was added to 2 L of distilled water. The prepared sample solutions were stirred for 24 hours under light-shading at room temperature. After the pH of the test solutions was adjusted to 7.0 ± 0.1 with 0.1 M NaOH, 1.0 - 9.0 mol-Cl/mol-3M4NP of sodium hypochlorite was added to the solutions in order to measure the mutagen formation potential (MFP). The solutions were stirred for 24 hours under light-shading at 21°C. The pH after the chlorination were 8.5 - 9.8. The residual chlorine of the samples after chlorination was measured by the DPD (N,N-diethyl-p-phenylenediamine) colorimetric method. The mutagens produced by chlorination were concentrated 1,000 times with a Sep-Pak Plus CSP-800 cartridge (Nihon Waters K. K., Tokyo, Japan) for the Ames assay and a Sep-Pak Plus PS-II cartridge (Nihon Waters K. K., Tokyo, Japan) for the high-resolution LC/MS analysis. The residual chlorine was not quenched because it was confirmed that there was no effect of residual chlorine on the mutagenicity in the concentration procedure (Takanashi et al., 2001).

A high-resolution LC/MS analysis
An accurate mass analysis was performed using a Shimadzu Prominence HPLC system (Shimadzu Co., Kyoto, Japan) interfaced to a calibrated LTQ-Orbitrap XL mass spectrometer with an electrospray ionization (ESI) source (Thermo Fisher Scientific Inc., MA, USA). A Shim-pack FC-ODS (4.6 mm i.d.; 150 mm/L.; 3 μm, Shimadzu Co.) was used as a HPLC column. The gradient program for the mobile phase was as follows: started at acetonitrile:water at 30 : 70 (v/v), increasing linearly up to 99% acetonitrile over a period of 30 min, and then 99% acetonitrile for 20 min. The flow rate was 0.5 mL/min, and injection volume was 20 μL. Ammonium acetate (1 mM) was used as a

![Fig. 1 - Chemical structures of 3-methyl-4-nitrophenol (a) and 5-hydroxy-2-nitrobenzoic acid (b).](image-url)
modifier. The pH of the mobile phase was 6.94. Operation parameters were as follows:
ESI source voltage, –2.5 kV; sheath gas, 60 mL/min; auxiliary gas, 20 mL/min; sweep
gas, 0 mL/min; vaporizer temp., 350°C; and capillary temperature, 400°C. The ESI
source was operated in a negative ion mode. Samples were dissolved in acetonitrile.
Calibration of \( m/z \) was performed using a standard ESI-tuning-mixture. The scan ranges
in a general mass spectrometry and in a CID mass spectrometry were set to \( m/z \) 100 –
400 and 50 – 250. The scan resolution was set to 100,000 FWHM at \( m/z \) 400.

**AMES Salmonella mutagenicity assay**

The preincubation method of the Ames assay was adopted according to the guidebook
published by Japan’s Ministry of Health, Labour and Welfare (Ministry of Labour Japan,
1991). The assay was performed using *Salmonella typhimurium* TA100 strains without
metabolic activation (S9), and was performed with 3 or 4 dose steps using duplicate
plates for each step. Quadruplet plates were used for the negative control tests. The
mutagenicity and the MFP of the samples was evaluated as net revertant colonies per
µmol (net rev./µmol), which was calculated from the slope of their dose-response lines.
In order to confirm the strains’ specific activity, 4-nitroquinoline-1-oxide (4NQO) was
used as the positive control substance.

At 8,700 – 9,600 net rev./µg-4NQO, the strains’ specific activity was consistent through
all the runs. The negative control test results were also consistent, showing 142 – 157
rev./plate. From these results, all the Mutation Ratio (MR) values attained in the
different runs of the Ames assay could be compared with each other. The MR value was
calculated according to equation (1). In this study, test results with an MR value over
1.4 were deemed positive (significant), because quadruplet plates were used for the
negative control steps (Takanashi and Urano, 1998):

\[
MR = \frac{R_d}{R_0}
\]

where \( R_d \) is the maximum average number of revertant colonies within the linearly
responsive range (rev./plate), and \( R_0 \) is the average number of revertant colonies in the
spontaneous tests (rev./plate). Mutagenicity with the MR value under 1.4 was described
as N. D. in the present study.

**RESULTS AND DISCUSSION**

**Effect of chlorine dosage on the mutagen formation potential of 3-methyl-4-nitrophenol**

The effect of the chlorine dosage on the MFP of 3M4NP was examined to determine
what chlorine dosage shows the highest MFP, in order to create a chlorinated 3M4NP
sample to be used both for the Ames assay and the LC/MS analysis. The molar ratio of
chlorine to 3M4NP (Cl/3M4NP) was changed by varying the chlorine dosage applied to
the 3M4NP solution. The experimental results are illustrated in Fig. 2.

The MFP of 3M4NP with less than 1.6 mol-Cl/mol-3M4NP was insignificant, nevertheless the MFP was significant with greater than 2.1 mol-Cl/mol-3M4NP, which
indicates that 3M4NP has significant MFP. Residual chlorine was not determined until
1.6 mol-Cl/mol-3M4NP, which corresponded to the determination of the mutagenicity. By increasing the Cl/3M4NP, the residual chlorine concentration increased from greater than 2.1 mol-Cl/mol-3M4NP. As for the MFP, it increased until the Cl/3M4NP reached 3.9 mol-Cl/mol-3M4NP. The highest MFP, 2,600 net rev./µmol, was observed when the Cl/3M4NP was 3.9 mol-Cl/mol-3M4NP. The MFP decreased as the chlorine dosage increased at 3.9 mol-Cl/mol-3M4NP or greater. Thus, the sample prepared at 3.9 mol-Cl/mol-3M4NP was used for the following experiments.

Figure 2 indicates that excessive chlorine dosages decrease the MFP of 3M4NP. Detailed discussions are unable to be done because the reaction steps, the reaction rate constants and the equilibrium constants for producing the mutagens are unknown. It can be pointed out that excessive chlorine dosages may cause further decomposition of the produced mutagens.

Exploring chlorination by-products of 3-methyl-4-nitrophnol
Flow injection analyses (FIAs) were performed without the HPLC column in the LC line. A mass spectrum of a blank sample (acetonitrile, data not shown) was subtracted from the mass spectrum obtained for the chlorinated 3M4NP sample. The subtracted mass spectrum is shown in Fig. 3. Disappearance of a peak \(m/z\) 153.0420 indicates a complete degradation of 3M4NP through chlorination. As for abundant peaks, peaks of \(m/z\) 124.9572, 204.9469, 215.9709 and 223.9209 which can be attributed to chlorination by-products of 3M4NP, were analysed. From the FIA mass spectrum, however, it was unable to examine molecular formulae of the produced mutagens due to complicated mass peaks nearby with each other. Thus, LC chromatograms were drawn by the LC/MS analyses.
Extracted ion chromatograms (EIC) for the $m/z$ windows of 204.9465 – 204.9475, 215.9705 – 215.9715 and 223.9205 – 223.9215 u were shown in Fig. 4. Several peaks can be found for the identical $m/z$ values of 204.947, 215.971 and 223.921 u, but no significant peak was observed for $m/z$ 124.957 due to inadequate LC conditions (data not shown). From the obtained EIC, it is clear that each substance consisted of several isomers. For instance, the peak $m/z$ 215.971 in the FIA mass spectrum consisted of at least two isomeric peaks.

As a result of the LC separation, simple mass spectra which were able to analyse their molecular formulae were obtained. The accurate mass of $m/z$ 215.971 ion was focused to be analyzed in this study. Because of a fine separation and high intensity with the LC, a mass spectrum at retention time ($rt$) 13.39 min is shown in Fig. 5 (a). A formation of C$_7$H$_4$O$_5$NCl (mass error = 0.397 mmu or 1.837 ppm) was extrapolated under the assumptions of the atomic composition: $^{12}$C(6-7); $^1$H(0-10); $^{14}$N(1, 3, 5, 7); $^{16}$O(0-10); $^{35}$Cl(1). The compositions of carbon, chlorine and nitrogen were assumed based on the relative peak intensity of their isotopic atoms to the base peak as well as the nitrogen rule.

To examine the chemical structure of the found substance, MS$^2$ experiments were conducted with a collision induced disassociation technique. Four product ions: $m/z$ 198.9679, 187.9757, 170.9731 and 136.0043 which can be attributed to losses of OH, CO, COOH and Cl functional groups were observed as shown in Fig. 6. From these product ions, it was found that the explored substance has hydroxyl group and chlorine. It was unable to examine from the CID experimental results that the explored substance
has carboxyl group or formyl group because a CO loss and a COOH loss can be observed in the CID experimental results of both benzoic acid and hydroxy benzaldehyde (Taylor et al., 1994; Letzel et al., 1999; Lakshmikant et al., 2005). However, it can be extrapolated that the explored substance possesses carboxyl group because formyl group will be oxidized to carboxyl group with ease in the presence of hypochlorite acid or hypochlorite ion (Abramovici et al., 1985).

Consequently, the production of chloro-5-hydroxy-2-nitrobenzoic acid (C5H2NB) was extrapolated based on the CID experimental results and the chemical structure of 3M4NP. The broad peaks in the EIC for 215.9705 – 215.9715 in Fig. 4 support the production of C5H2NB because the pH of the mobile phase was 6.94 and the pKa of a similar compound to C5H2NB, 2-nitrobenzoic acid, is reported to be 2.47 at 293 K (Torsten et al., 2004).

Mutagenicity and MFP of 5-hydroxy-2-nitrobenzoic acid
The mutagenicity and the MFP of 5-hydroxy-2-nitrobenzoic acid (5H2NB) were examined instead of C5H2NB because the authors could not obtain any C5H2NB analogues. As shown in Fig. 7, the mutagenicity of 5H2NB was insignificant but the MFP of it was significant. The MFP of 5H2NB was 3,400 net rev./μmol, which was greater than that of 3M4NP, 2,700 net rev./μmol. This fact indicates that C5H2NB is one of the candidates of mutagen which will be produced from fenitrothion through chlorination.

Fig. 4 - Extracted ion chromatograms of chlorinated 3M4NP. Monitoring ion: m/z 204.9465 – 204.9475, 215.9705 – 215.9715 and 223.9205 – 223.9215.
Fig. 5 - An observed accurate mass spectrum for m/z 215.971 ion with some isotopic ions at rt 13.39 min (a) and a simulated mass spectrum of C7H4O5NCl (b).

Fig. 6 - A CID mass spectrum for m/z 215.9707.
CONCLUSION
A typical hydrolysate of fenitrothion, 3-methyl-4-nitrophenol, was chlorinated to explore a mutagenic chlorination by-product from fenitrothion. Based on the accurate mass analytical results and the MS\textsuperscript{2} experimental results, one of the mutagenic chlorination by-products was extrapolated to be chloro-5-hydroxy-2-nitrobenzoic acid (C5H2NB). The mutagen formation potential through chlorination of 5-hydroxy-2-nitrobenzoic acid was tested and the MFP of 3,400 net rev./µmol was observed, indicating that C5H2NB is one of the candidates of mutagen which will be produced from fenitrothion through chlorination.

ACKNOWLEDGEMENT
This research was supported by the Environment Research and Technology Development Fund (B-1104) of the Ministry of the Environment, Japan.

REFERENCES

Fig. 7 - Mutagenicity and MFP of 5-hydroxy-2-nitrobenzoic acid (5H2NB), MFP of 3-methyl-4-nitrophenol (3M4NP): mutagenicity of 5H2NB; MFP of 5H2NB; MFP of 3M4NP; Δ.


