ハロメチル-1,3,5-トリアジン系化合物の硝酸化成に及ぼす影響

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Original Article

Effects of Halomethyl-1, 3, 5-triazines on Nitrification

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To evaluate the effect of the halogenated methyl groups of 1, 3, 5-triazines on nitrification by nitrifying bacteria, twenty-six halomethyl-1, 3, 5-triazines were examined. Nitrification inhibition activity by the 1, 3, 5-triazines was determined through two experiments; *i.e.* The inhibition activities were measured for ammonia-oxidation to nitrate (NO_3^--N) in an upland soil and in the second one, for ammonia-oxidation to nitrite (NO_2^--N) by *Nitrosomonas europaea* ATCC 25978. The 1, 3, 5-triazines bearing trichloromethyl group(s) inhibited nitrification more strongly both in soil and in cell suspension of ATCC 25978 than other partially chlorinated methyl-1, 3, 5-triazines. The 1, 3, 5-triazines having tribromomethyl group(s) exhibited rather weaker nitrification inhibition in soil, although they indicated the strong inhibition in cell suspension. The halomethyl-1, 3, 5-triazines in this study inhibited ammonia-oxidation step, but did not inhibit hydroxylamine-oxidation step in the culture of ATCC 25978. This fact was found that the inhibitory target site of them may be on the ammonia-oxidation step from ammonium (NH_4^+-N) to hydroxylamine (NH_2OH), as we found for trichloromethyl-1, 3, 5-triazines in our previous study.

INTRODUCTION

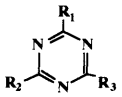
We have already reported the nitrification inhibitory activities of several trichloromethyl-1, 3, 5-triazines in upland soil and in *Nitrosomonas* cell cultures.[1-4] The trichloromethyl-1, 3, 5-triazines, those especially bearing an amino group as one of other two substituents, generally exhibit the strong inhibitory activities both in soil and *Nitrosomonas* cultures.[3,4] Furthermore, it has been indicated that the triazines possibly interfere with the ammonia-oxidizing step from ammonia (NH_4^+-N) to hydroxylamine (NH_2OH) in *Nitrosomonas* cells, inhibiting the ammonia monooxygenase.[1] However, the nitrification inhibitory activity of halomethyl-1, 3, 5-triazines, which have chlorinated methyl (*e.g.* $CHCl_2$ or CH_2Cl), brominated methyl (*e.g.* CBr_3) or fluorinated methyl (*e.g.* CF_3) groups, has not been fully investigated yet, although their nitrification activity in upland soil were reported.[2] In this study, twenty-six halomethyl-1, 3, 5-triazines were examined to evaluate their effect on the nitrification by nitrifying bacteria.

MATERIALS AND METHODS

1. Chemicals

2, 4, 6-Trimethyl-1, 3, 5-triazine (**1**) was synthesized by trimerization of ethyl acetimidate.[5] 2-Chloromethyl-4, 6-dimethyl-1, 3, 5-triazine (**2**) and 2-dichloromethyl-4, 6-dimethyl-1, 3, 5-triazine (**3**) were obtained through chlorination of 2, 4, 6-trimethyl-1, 3, 5-triazine (**1**) according to the method of Schaefer[6] and Schaefer & Ross.[7] 2-(Halo)methyl-4-methyl-6-trichloromethyl-1, 3, 5-triazines (**4**, **6**, **7** and **15**) and 2-halomethyl-4, 6-bis-(trichloromethyl)-1, 3, 5-triazines (**10**, **11** and **16**) were synthesized by condensation reaction of *N*-(acetimidoyl)-trichloroacetamidine and *N*-(trichloroacetimidoyl)trichloroacetamidine, respectively, with corresponding acid anhydrides.[8] 2, 4, 6-Trichloromethyl-1, 3, 5-triazine (**5**), 2, 4, 6-tris(dichloromethyl)-1, 3, 5-triazine (**9**) and 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (**12**) were synthesized by trimerization of corresponding nitriles (CH_2ClCN, $CHCl_2CN$ and CCl_3CN, respectively) in the presence of the Norton-Wakabayashi complex catalyst, *e.g.* $AlBr_3$-HCl, where the trimerization of CH_2ClCN

Table 1 1, 3, 5-Triazines assayed for nitrification-inhibitory activity in this study.



No.	R_1	R_2	R_3	Physical property
1	CH_3	CH_3	CH_3	mp 60°C (lit.[12] mp 57°C)
2	CH_3	CH_3	CH_2Cl	$n_{D28.0}$ 1.5003 (lit.[7] $n_{D25.0}$ 1.5032, bp 97–98°C)
3	CH_3	CH_3	$CHCl_2$	$n_{D28.0}$ 1.5167 (lit.[6] bp 117°C/18 mmHg)
4	CH_3	CH_3	CCl_3	mp 75–77°C (lit.[9] mp 72–73°C)
5	CH_2Cl	CH_2Cl	CH_2Cl	mp 77–78°C (lit.[5] mp 78–79°C)
6	CH_3	CH_2Cl	CCl_3	$n_{D28.0}$ 1.5388 (lit.[7] liquid)
7	CH_3	$CHCl_2$	CCl_3	$n_{D28.0}$ 1.5441 (lit.[7] liquid)
8	CH_3	CCl_3	CCl_3	mp 96–97°C (lit.[9] mp 96–97°C)
9	$CHCl_2$	$CHCl_2$	$CHCl_2$	mp 68°C (lit.[13] mp 68°C)
10	CH_2Cl	CCl_3	CCl_3	mp 60–63°C (lit.[7] mp 63–64°C)
11	$CHCl_2$	CCl_3	CCl_3	mp 39–41°C (lit.[14] mp 41–42°C)
12	CCl_3	CCl_3	CCl_3	mp 94–95°C (lit.[9] mp 92–93°C)
13	CF_3	CF_3	CF_3	bp 98°C (lit.[10] bp 98°C)
14	CBr_3	CBr_3	CBr_3	mp 157–159°C (lit.[7] mp 158–159°C)
15	CH_3	CF_3	CCl_3	bp 73–79°C/3 mmHg (lit.[8] bp 79–80°C/8 mmHg)
16	CF_3	CCl_3	CCl_3	mp 44–46°C (lit.[8] mp 44–46°C)
17	CF_3	CCl_3	CBr_3	mp 51–52°C
18	CCl_3	CCl_3	CBr_3	mp 90–93°C
19	CH_3	CF_3	NH_2	mp 103–104°C (lit.[11] mp 103–104°C)
20	CH_3	CCl_3	NH_2	mp 160–161°C (lit.[2] mp 160–161°C)
21	CF_3	CCl_3	NH_2	mp 115–118°C
22	CCl_3	CCl_3	NH_2	mp 165–167°C (lit.[2] mp 165–167°C)
23	CBr_3	CCl_3	NH_2	mp 173–178°C
24	CH_3	NH_2	NH_2	mp 274–276°C (lit.[15] mp 274–276°C)
25	CF_3	NH_2	NH_2	mp >300°C (lit.[16] mp 318–321°C)
26	CCl_3	NH_2	NH_2	mp 241–242°C (lit.[2] mp 235–236°C)
27	CBr_3	NH_2	NH_2	mp 200–203°C (lit.[17] mp 210°C)
28	NH_2	NH_2	NH_2	mp 347°C

was carried out in a sealed tube.[9] 2-Methyl-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (**8**) was prepared by cotrimerization of CCl_3CN with CH_3CN.[9] 2, 4, 6-Tris(trifluoromethyl)-1, 3, 5-triazine (**13**) was prepared by fluorination of 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (**12**) using the antimony fluorinating agent (SbF_3+SbCl_5) according to Norton.[10] Five tribromomethyl-1, 3, 5-triazines (**14**, **17**, **18**, **23** and **27**) were obtained by bromination of methyl group(s) of 1, 3, 5-triazines (**1**, **15**, **8**, **20** and **24**) respectively, with bromine in glacial acetic acid in the presence of CH_3COONa at 60–70°C.[7] 2-Amino-1, 3, 5-triazines (**20**, **21** and **22**) and 2, 4-diamino-1, 3, 5-triazines (**24**, **25** and **26**) were prepared by nucleophilic monoamination and diamination reaction, respectively, of corresponding trichloromethyl-1, 3, 5-triazines (**8**, **16** and **12**) using 28% ammonium hydroxide.[3,4] 2-Amino-4-methyl-6-trifluoromethyl-1, 3, 5-triazine (**19**) was similarly prepared by monoamination of 2-methyl-4-trichloromethyl-6-trifluoromethyl-1, 3, 5-triazine (**15**).[11] All reaction products were purified through recrystallization and/or column chromatography, and their structures were confirmed by IR-, NMR- and Mass spectroscopy and elementary analysis for C, H and N (also for halogen for some compounds). See Table 1 for physical data. Spectroscopical data of four new compounds (**17**, **18**, **21** and **23**) are also described in *1.1–1.4* below.

2, 4, 6-Triamino-1, 3, 5-triazine (**28**, melamine) was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Nitrapyrin was kindly provided by Dow Elanco Japan Ltd., Tokyo. Analytical grade chemicals for *Nitrosomonas* culture and other chemicals were purchased from Kanto Chemical Co., Inc., Tokyo, and Dojindo Laboratories, Kumamoto, Japan.

1.1 2-Tribromomethyl-4-trichloromethyl-6-trifluoromethyl-1, 3, 5-triazine (17)

mp: 51–52°C. Anal. Calcd. for $C_6Br_3Cl_3F_3N_3$: C, 13.94; N, 8.13. Found: C, 13.85; N, 8.08%. MS *m/z*: 513 (M^+), 494 (M^+-F), 478 (M^+-Cl), 438 (M^+-Br+4, base peak), 434 (M^+-Br), 399 ($M^+-Br-Cl$), 196 (CBr_2CN), 108 (CCl_2CN) and so on. Intensities of isotope peaks relative to the M^+-F ion for Br_3+Cl_3 were found; Calcd. (found): M^+-F, 16.5% (17.6%); M^+-F+2, 64.6% (64.6%); M^+-F+4, 100% (100%);

M^+-F+6, 77.8% (76.3%) and M^+-F+8, 31.9% (32.3%). IR ν_{max}^{KBr} cm^{-1}: 1557 (1, 3, 5-triazine ring). ^{13}C NMR $\delta_{TMS}^{CDCl_3}$ ppm: 31.9 (s, CBr_3), 93.4 (s, CCl_3), 177.8 (s, C-2), 176.6 (s, C-4), 118.0 (q, $^1J_{CF}=277$ Hz, CF_3), 167.4 (q, $^2J_{CF}=40$ Hz, C-6).

1.2 2-Tribromomethyl-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (18)

mp: 90–93°C. Anal. Calcd. for $C_6Br_3Cl_6N_3$: C, 12.72; N, 7.42. Found: C, 12.69; N, 7.21%. MS *m/z*: 561 (M^+), 526 (M^+-Cl), 486 (M^+-Br+4, base peak), 482 (M^+-Br), 447 ($M^+-Br-Cl$), 196 (CBr_2CN), 108 (CCl_2CN) and so on. Intensities of isotope peaks relative to the $M^+-Br-Cl$ ion for Br_2+Cl_5 were found; Calcd. (found): $M^+-Br-Cl$, 19.2% (20.4%); $M^+-Br-Cl+2$, 70.2% (68.9%); $M^+-Br-Cl+4$, 100% (100%); $M^+-Br-Cl+6$, 76.6% (77.0%); $M^+-Br-Cl+8$, 33.6% (34.1%) and $M^+-Br-Cl+10$, 8.6% (9.5%). IR ν_{max}^{KBr} cm^{-1}: 1524, 1559 (1, 3, 5-triazine ring). ^{13}C NMR $\delta_{TMS}^{CDCl_3}$ ppm: 32.7 (s, CBr_3), 93.7 (s, CCl_3), 177.5 (s, C-2), 176.3 (s, C-4 and C-6).

1.3 2-Amino-4-trichloromethyl-6-trifluoromethyl-1, 3, 5-triazine (21)

mp: 115–118°C. Anal. Calcd. for $C_5Cl_3F_3H_2N_4$: C, 21.34; H, 0.72; N, 19.91. Found: C, 21.38; H, 0.85; N, 19.82%. MS *m/z*: 280 (M^+), 261 (M^+-F), 245 (M^+-Cl, base peak), 108 (CCl_2CN) and so on. Intensities of isotope peaks relative to the M^+ ion for Cl_3 were found; Calcd. (found): M^+, 100% (100%); M^++2, 95.9% (94.8%); M^++4, 30.7% (29.8%) and M^++6, 3.3% (4.1%). IR ν_{max}^{KBr} cm^{-1}: 1524, 1559 (1, 3, 5-triazine ring). ^1H NMR $\delta_{TMS}^{CDCl_3}$ ppm: 6.18 (2H, bs, NH_2). ^{13}C NMR $\delta_{TMS}^{CDCl_3}$ ppm: 93.4 (s, CCl_3), 117.3 (q, $^1J_{CF}=277$ Hz, CF_3), 166.7 (s, C-2), 174.2 (s, C-4), 165.3 (q, $^2J_{CF}=38$ Hz, C-6).

1.4 2-Amino-4-tribromomethyl-6-trichloromethyl-1, 3, 5-triazine (23)

mp: 173–178°C. Anal. Calcd. for $C_5Br_3Cl_3H_2N_4$: C, 12.94; H, 0.43; N, 12.07. Found: C, 13.05; H, 0.40; N, 12.02%. MS *m/z*: 460 (M^+), 425 (M^+-Cl), 385 (M^+-Br+4, base peak), 381 (M^+-Br), 346 ($M^+-Br-Cl$), 196 (CBr_2CN), 108 (CCl_2CN) and so on. Intensities of isotope peaks relative to the M^+-Cl ion for Br_3+Cl_2 were found; Calcd. (found): M^+-Cl, 20.5% (21.1%); M^+-Cl+2, 73.4% (74.1%); M^+-Cl+4, 100% (100%); M^+-Cl+6, 63.8% (64.1%); M^+-Cl+8, 18.7% (19.3%) and $M^+-Cl+10$, 2.0% (2.7%). IR ν_{max}^{KBr} cm^{-1}: 1540 (1, 3, 5-triazine ring). ^1H NMR $\delta_{TMS}^{CDCl_3}$ ppm: 6.06 (2H, bs, NH_2). ^{13}C NMR $\delta_{TMS}^{CDCl_3}$ ppm: 36.1 (s, CBr_3), 94.8 (s, CCl_3), 168.1 (s, C-2), 175.9 (s, C-4), 174.9 (s, C-6).

2. Reaction of 2, 4, 6-Tris(trihalomethyl)-1, 3, 5-triazines with NH_4OH

A mixture of 10 mmol of 2, 4, 6-tris(trihalomethyl)-1, 3, 5-triazines (**12**, **13**, **14**, **16**, **17** and **18**) and 25 ml of 28% ammonium hydroxide was stirred for 3 hr under ice-cooling (0–5°C). The molar ratio (NH_4^+/triazine≒35) corresponded to the ratio used in the nitrification inhibitory assay for *Nitrosomonas*. The precipitates were then filtered, washed with water, dried and analyzed by Shimadzu LC-9A HPLC system (flow rate: 1 ml/min, detection: 254 nm). Senshu Pak ODS-1251-SK column (ϕ 4.6×250 mm) and acetonitrile–distilled water (8: 2, v/v) as mobilephase were used for analysis.

3. Reaction of Trihalomethyl-1, 3, 5-triazines with $(NH_4)_2SO_4$ Solution

A mixture of 500 mg of trihalomethyl-1, 3, 5-triazines (**12**, **13**, **14**, **16**, **17** and **18**) and 6 g of ammonium sulfate was added to 20 ml of phosphate buffer (pH 8.0; 0.2 M) and stirred for 6 hr at 35–40°C. The molar ratio (NH_4^+/triazine≒35) corresponded to the ratio used in the nitrification inhibitory assay for *Nitrosomonas*. Then, the solid materials were filtered, washed with water, dried and analyzed by HPLC mentioned in MATERIALS AND METHODS *2*. No nucleophic amination reaction was observed for all the seven trihalomethyl-1, 3, 5-triazines.

4. Evaluation of Nitrification-inhibitory Activity

4.1 Nitrification-inhibitory activity in soil

The stock soil treatment formulations (125 mg/*l*) were prepared by mixing 10 g of Celite 545 and 62.5 mg of each 1, 3, 5-triazine tested. The soil used was upland soil in the field of Tamagawa University.[1] In each wide mouthed culture flask, the soil (25 g), urea (16.5 mg) and the appropriate amount of the 1, 3, 5-triazine formulation were mixed well. After adjusting the soil moisture to 50% of the field capacity and pH to 6.8 using calcium carbonate, the flasks were covered with iron caps having 5 holes (2 mmϕ) and incubated in the dark at 28°C for 14 days. Then 100 ml of water was added to the flasks, and these were subsequently shaken for 1 hr. Nitrate (NO_3^--N) was determined by a nitrate ion-meter (Horiba Compact Ion-Meter C-141). The nitrification inhibitory index in soil was represented as pI_{50}(soil), the logarithm of the reciprocal molar concentration for 50% nitrification inhibition by the compound tested.

4.2 Determination of ammonia-oxidation and hydroxylamine-oxidation in Nitrosomonas cell culture

Ammonium sulfate and hydroxylamine hydrochloride were used as substrates for ammonia-oxidation and hydroxylamine-oxidation by *Nitrosomonas europaea* ATCC 25978, respectively, according to our method described previously.[4] Ten milligrams of each 1, 3, 5-triazine were emulsified in water with a few drops of Tween 20, a small amount of ethanol and a little amount of talc to make 5 ml of inhibitor solution. The cell suspension of ATCC 25978 precultured in the modified Lewis & Pramer's medium[1] was diluted with phosphate buffer to make 1/50 fold suspension. In a 2 ml test tube, 1 ml of each substrate solution, 1 ml of cell suspension and 10 μl of inhibitor solution were mixed, and this

mixture was allowed to stand at 37°C for 30 min. The nitrite concentration was determined by means of Griess-Ilosvay methods[18] at the start and after 30 min of incubation. This cell suspension converted the substrate into about 1 μg/ml of nitrite nitrogen during 30 min incubation. Nitrification inhibitory index in *Nitrosomonas* cell culture was represented as pI_{50}(cell), the logarithm of the reciprocal molar concentration for 50% nitrification inhibition by the compound tested.

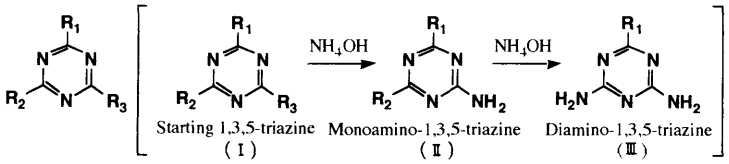
RESULTS AND DISCUSSION

1. *Reactivity of Trihalomethyl-1, 3, 5-triazines with Ammonia or Ammonium Sulfate*

It is reported that two trichloromethyl groups of 2, 4-bis(trichloromethyl)-6-substituted-1, 3, 5-triazines can be replaced stepwise with amino groups to produce 2, 4-diamino-6-substituted-1, 3, 5-triazines.[3] In this paper, reactivity of six trihalomethyl-1, 3, 5-triazines (**12**, **13**, **14**, **16**, **17** and **18**) with 28% ammonium hydroxide or ammonium sulfate was examined to explain a difference in nitrification inhibitory activities of the 1, 3, 5-triazine inhibitors between in soil and the culture of ATCC 25978. 2, 4, 6-Tris(trichloromethyl)-1, 3, 5-triazine (**12**) was used as the positive standard to compare reactivity of other five 1, 3, 5-triazines with ammonia. The stepwise nucleophilic amination was carried out at low temperature (0–10°C), because the rate of nucleophilic replacement of trichloromethyl groups attached to 1, 3, 5-triazine ring depends upon reaction temperature. Results are shown in Table 2. Although the 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (**12**) was completely converted into the monoamino derivative (**22**; 90%) and into the diamino-1, 3, 5-triazine (**26**; 10%) within 3 hr, no amination was observed in the reaction of 2, 4, 6-tris(trifluoromethyl)-1, 3, 5-triazine (**13**) with ammonia. However, 2, 4, 6-tris(tribromomethyl)-1, 3, 5-triazine (**14**) seemed to be more fast converted into its monoamino and diamino derivatives than 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (**12**), producing more the diamino derivative (**27**). In 2, 4-bis(trichloromethyl)-6-trifluoromethyl-1, 3, 5-triazines (**16**) and 2-tribromomethyl-4-trichloromethyl-6-trifluoromethyl-1, 3, 5-triazine (**17**), the trifluoromethyl group was not replaced by an amino group. Especially in the amination reaction of 2-tribromomethyl-4-trichloromethyl-6-trifluoromethyl-1, 3, 5-triazine (**17**) bearing three different trihalomethyl groups, it appeared that the tribromomethyl group was the first leaving group, and the trichloromethyl group was second in this 1, 3, 5-triazine. Thus, it may be concluded that the reactivity order of trihalomethyl groups of the 1, 3, 5-triazines examined in nucleophilic amination is: $CBr_3 > CCl_3 \gg CF_3$.

No amination reaction of the trihalomethyl-1, 3, 5-triazines (**12**, **13**, **14**, **16**, **17** and **18**) was observed in ammonium sulfate solution (molar ratio; NH_4^+/triazine≒35), corresponding to the condition in the nitrification inhibitory assay in *Nitrosomonas* cell culture (pH 8.0). Obviously a more alkaline condition is essen-

Table 2 Ammination of 2, 4, 6-tris(trihalomethyl)-1, 3, 5-triazines with NH_4OH.[a, b]



No.	I			II			III		
	R_1	R_2	R_3	R_1	R_2	R_3	R_1	R_2	R_3
12	CCl_3	CCl_3 [0]	CCl_3	CCl_3	CCl_3 [90]	NH_2	CCl_3	NH_2 [10]	NH_2
13	CF_3	CF_3 [100]	CF_3	CF_3	CF_3 [0]	NH_2	CF_3	NH_2 [0]	NH_2
14[c]	CBr_3	CBr_3 [0]	CBr_3	CBr_3	CBr_3 [34]	NH_2	CBr_3	NH_2 [32]	NH_2
16	CF_3	CCl_3 [0]	CCl_3	CF_3	CCl_3 [98]	NH_2	CF_3	NH_2 [2]	NH_2
17	CF_3	CCl_3 [0]	CBr_3	CF_3	CCl_3 [76]	NH_2	CF_3	NH_2 [24]	NH_2
18	CCl_3	CCl_3 [0]	CBr_3	CCl_3	CCl_3 [63]	NH_2	CCl_3	NH_2 [37]	NH_2

[a] More than 95% of 1, 3, 5-triazine derivatives were recovered in all experiments.

[b] Figures in parentheses for I, II and III indicate the molar selectivity (%) determined by HPLC.

[c] *ca.* 30% of 1, 3, 5-triazine (**14**) was furthermore converted into other 1, 3, 5-triazine derivatives including 2, 4-diamino-6-dibromomethyl-1, 3, 5-triazine and 2, 4, 6-triamino-1, 3, 5-triazine.

tial for the nucleophilic amination of the 1, 3, 5-triazines. Thus, pI_{50}(cell) values obtained in cell culture indicate the intrinsic pI_{50}-values of the 1, 3, 5-triazines administrated originally.

2. Nitrification Inhibitory Activity of Chlorinated Methyl-1, 3, 5-triazines in Soil and Nitrosomonas Cell Culture

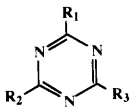
Effects of eleven chlorinated-methyl-1, 3, 5-triazines (**2**–**12**) on nitrification of urea in the upland soil from Tamagawa University's field was investigated. The nitrification activity of the soil was extremely strong and the urea applied as a nitrogen source was completely nitrified within 14 days as reported previously.[1] The pI_{50}(soil)-values for nitrate formation and the pI_{50}(cell)-values for nitrite formation were used as the nitrification-inhibitory indices. Results are shown in Table 3. 2-Methyl-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (**8**) was the most potent inhibitor in cell suspension, with index pI_{50}(cell) of 6.15. However, the strongest inhibitor, 2-chloro-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (**10**), in soil has a pI_{50}(soil) of 4.86, while the 1, 3, 5-triazine (**8**) had a pI_{50}(soil) of 4.30. This cause of different activity may be related to the different susceptibility between *N. europaea* ATCC 25978 and the nitrifying bacteria in soil, the different metabolic detoxifying of the 1, 3, 5-triazine in the soil used or the different adsorption of 1, 3, 5-triazine compounds in soil.[1]

Although the inhibitory activities (pI_{50}(soil) and pI_{50} (cell)) of 2, 4, 6-tris(dichloromethyl)-1, 3, 5-triazine (**9**) without trichloromethyl group was weaker than other 1, 3, 5-triazines bearing at least one trichloromethyl group, among the chlorinated methyl-1, 3, 5-triazines assayed, the 1, 3, 5-triazines bearing 4 to 7 chlorines showed the higher inhibitory activity both in soil and cell culture. These facts indicate that the trichloromethyl group(s) may be essential for the highly active 1, 3, 5-triazine nitrification inhibitors.

3. Nitrification Inhibitory Activity of Trihalomethyl-1, 3, 5-triazines in Soil and Nitrosomonas Cell Culture

After experiment of nitrification inhibition activity with 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (**12**) in soil, the metabolized 1, 3, 5-triazines extracted from the soil with acetone were analyzed by HPLC mentioned in MATERIALS AND METHODS *2*. As a result, 2, 4, 6-tris-(trichloromethyl)-1, 3, 5-triazine (**12**) changed to 1.1% of 2, 4-diamino-6-trichloromethyl-1, 3, 5-triazine (**26**), 89.3% of 2-amino-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (**22**), 1.6% of 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (**12**) and 8.0% of others. We thought that the 1, 3, 5-triazines having highly reactive substituents reacted with ammonia. Therewith we reported[3] that a combination of an amino group and a trichloromethyl group as substituents of the 1, 3, 5-triazines is favorable to the nitrification-inhibitory activity. Considering these prerequisites, effects of trihalomethyl-1, 3, 5-triazines on nitrification inhibitory activity were also investigated in this study. For this purpose we divided the trihalomethyl-1, 3, 5-triazines into 2, 4, 6-tris(trihalomethyl)-1, 3, 5-triazines (Group A ; **12**, **13** and **14**), 2-trihalomethyl-4, 6-bis(trichloromethyl)-1, 3, 5-triazines (Group B ; **12**, **16** and **18**), 2-trihalomethyl-4-amino-6-trichloromethyl-1, 3, 5-triazines (Group C ; **21**, **22** and **23**) and 2-trihalomethyl-4, 6-diamino-1, 3, 5-triazines (Group D ; **25**, **26** and **27**). Results are shown in Table 4. In Group A, trichloromethyl group and tribromomethyl group brought about a moderate nitrification-inhibitory activity both in soil and *Nitrosomonas* cell culture, indicating pI_{50}(soil) of 4.48

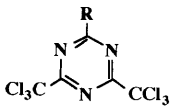
Table 3 pI_{50} (soil) and pI_{50} (cell) values of chlorinated methyl-1, 3, 5-triazines.

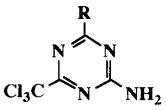


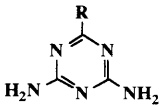
Compound	Number of chlorine atom	Number of CCl_3 group	R_1	R_2	R_3	pI_{50} (soil)	pI_{50} (cell)
1	0	0	CH_3	CH_3	CH_3	<3.00	<3.00
2	1	0	CH_3	CH_3	CH_2Cl	<3.00	<3.00
3	2	0	CH_3	CH_3	$CHCl_2$	3.36	4.14
5	3	0	CH_2Cl	CH_2Cl	CH_2Cl	4.05	3.12
9	6	0	$CHCl_2$	$CHCl_2$	$CHCl_2$	3.75	4.26
4	3	1	CH_3	CH_3	CCl_3	4.45	5.26
6	4	1	CH_3	CH_2Cl	CCl_3	4.77	5.94
7	5	1	CH_3	$CHCl_2$	CCl_3	4.79	5.88
8	6	2	CH_3	CCl_3	CCl_3	4.30	6.15
10	7	2	CH_2Cl	CCl_3	CCl_3	4.86	5.86
11	8	2	$CHCl_2$	CCl_3	CCl_3	4.76	5.43
12	9	3	CCl_3	CCl_3	CCl_3	4.48	4.60

Table 4 pI_{50}(soil) and pI_{50}(cell) values of trihalomethyl-1, 3, 5-triazines.


Group A


Group B


Group C


Group D

Group A				Group B			
R	No.	pI_{50} (soil)	pI_{50} (cell)[a]	R	No.	pI_{50} (soil)	pI_{50} (cell)[a]
CH_3	**1**	<3.00	<3.00	CH_3	**8**	4.30	6.15
CF_3	**13**	<3.00	<3.00	CF_3	**16**	4.19	4.50
CCl_3	**12**	4.48	4.60	CCl_3	**12**	4.48	4.60
CBr_3	**14**	4.49	5.91	CBr_3	**18**	4.32	6.10
NH_2	**28**	<3.00	<3.00				
Group C				**Group D**			
R	No.	pI_{50} (soil)	pI_{50} (cell)[a]	R	No.	pI_{50} (soil)	pI_{50} (cell)[a]
CH_3	**20**	5.17	5.31	CH_3	**24**	<3.00	<3.00
CF_3	**21**	4.02	5.73	CF_3	**25**	4.05	4.19
CCl_3	**22**	5.31	6.72	CCl_3	**26**	4.64	5.15
CBr_3	**23**	4.17	7.12	CBr_3	**27**	3.79	6.30

[a] Inhibition rate (%) $=\left(1-\frac{\text{Inhibitor (OD value at 30 min)}-\text{Inhibitor (OD value at 0 min)}}{\text{Blank (OD value at 30 min)}-\text{Blank (OD value at 0 min)}}\right)\times 100$.

and pI_{50}(cell) of 4.60 for 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (**12**), and pI_{50}(soil) of 4.49 and pI_{50}(cell) of 5.91 for 2, 4, 6-tris(tribromomethyl)-1, 3, 5-triazine (**14**), respectively. 2, 4, 6-Tris(trifluoromethyl)-1, 3, 5-triazine (**13**) as well as 2, 4, 6-trimethyl-1, 3, 5-triazine (**1**) and 2, 4, 6-triamino-1, 3, 5-triazine (**28**) exhibits no strong inhibitory activity. In Group B, 2-tribromomethyl-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (**18**) as well as the reference 2-methyl-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (**8**) exhibits a strong nitrification inhibition in cell culture, indicating pI_{50}(cell) of 6.10. The about 100-times less activity of these two compounds in soil (**8**: pI_{50}(soil)=4.30, **18**: pI_{50}(soil)=4.32) may be readily explained by their conversion to the 1, 3, 5-triazines of Group D (**24**, and **26** or **27**, respectively) in soil for 14 days. The same may be true for pI_{50}(soil) of 4.48 of 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (**12**), reflecting pI_{50}(soil) of 4.64 for 2, 4-diamino-6-trichloromethyl-1, 3, 5-triazine (**26**). Also in Group C, introduction of a tribromomethyl group produces an excellent nitrification-inhibitory activity in the cell culture (2-amino-4-trichloromethyl-6-tribromomethyl-1, 3, 5-triazine, **23**: pI_{50}(cell)=7.12), although the activity of this compound in soil is very weak, with a pI_{50}(soil) of 4.17. The instability of the trihalomethyl group may be connected with this decreased activity in soil, as considered in Group B. 2-Amino-4-methyl-6-trichloromethyl-1, 3, 5-triazine (**20**) and 2-amino-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (**22**) exhibit potent nitrification-inhibitory activities both in soil and cell culture, but changing from a trichloromethyl group to an amino group lowers the inhibitory activity somewhat. Anyway, the potent inhibitory-activity of the compounds in this Group C confirms that a combination of an amino group and trihalomethyl group(s) on the 1, 3, 5-triazine ring may produce potent nitrification inhibitors. In Group D, the two potent nitrification inhibitors in cell culture (trichloromethyl analogue (**26**), pI_{50}(cell)=5.15, and tribromomethyl analogue (**27**), pI_{50}(cell)=6.30), decrease their inhibitory activity to pI_{50}(soil) of 4.64 and pI_{50}(soil) of 3.79, respectively, although the trifluoromethyl analogue (**25**) indicates almost the same level of low activity both in cell culture and soil, with pI_{50}(cell) of 4.19 and pI_{50}(soil) of 4.05. This finding may be coincident with the instability order or reactivity order within the trihalomethyl group ($CBr_3 > CCl_3 \gg CF_3$) in ammonia solution, as discussed in Section *1*.

4. Target Site of Halomethyl-1, 3, 5-triazines in This Study

In our previous paper,[1] the target site of various trichloromethyl-1, 3, 5-triazine nitrification inhibitors has been reported to be on the ammonia-oxidation step from ammonia to hydroxylamine. All the halomethyl-1, 3, 5-triazines (**2**–**27**) in this paper were checked whether they were also inhibitors on the ammonia-oxidation step mentioned above. They inhibited nitrite production from ammonia in 1 to 50 ppm concentration, indicating 60 to 78% inhibition rates, while they exhibited no inhibition for nitrite production from hydroxylamine at 10 ppm (see Table 5). This result showed that the present halomethyl-1, 3, 5-triazines as well as the trichloromethyl-1, 3, 5-triazines in the previous paper[4] are the inhibitors of the ammonia-oxidation step from

Table 5 The effects of 1, 3, 5-triazines on ammonia- and hydroxylamine-oxidation in cell suspension of *Nitrosomonas europaea* ATCC 25978.

(a) The effect of 1, 3, 5-triazines on ammonia-oxidation in cell suspension.

No.	ppm	0 min OD value[a)]	30 min OD value[b)]	Inhibition rate (%)[d)]
Blank	—	0.02	1.36	0
Nitrapyrin[c)]	1	0.02	0.46	67.1
2	50	0.02	0.33	76.9
15	10	0.02	0.31	78.3
16	1	0.02	0.55	60.4
22	1	0.02	0.34	76.1

(b) The effect of 1, 3, 5-triazines on hydroxylamine-oxidation in cell suspension.

No.	ppm	0 min OD value[a)]	30 min OD value[b)]	Inhibition rate (%)[d)]
Blank	—	0.02	0.02	0
Nitrapyrin[c)]	10	0.02	0.02	0
2	10	0.02	0.02	0
15	10	0.02	0.02	0
16	10	0.02	0.02	0
22	10	0.02	0.02	0

[a)] OD value at the start of reaction indicates nitrite amount before adding inhibitor and substrate.

[b)] OD value after 30 min incubation with inhibitor and substrate.

[c)] 2-Chloro-6-trichloromethylpyridine.

[d)] Inhibition rate (%)

$$= \left(1 - \frac{\left(\text{Inhibitor (OD value at 30 min)} - \text{Inhibitor (OD value at 0 min)}\right)}{\left(\text{Blank (OD value at 30 min)} - \text{Blank (OD value at 0 min)}\right)}\right) \times 100.$$

ammonia to hydroxylamine, but not inhibitors of the hydroxylamine-oxidation step.

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要　　約

ハロメチル-1, 3, 5-トリアジン系化合物の硝酸化成に及ぼす影響

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宮本美子，徳山龍明，Johannes Willem Vonk
佐藤幸治，若林　攻

1, 3, 5-トリアジンのハロメチル置換基が硝化細菌の硝酸化成作用に及ぼす影響について調べることを目的として，26種のハロメチル-1, 3, 5-トリアジン系化合物を用いて試験を行なった．1, 3, 5-トリアジン系化合物の硝酸化成抑制効果は二つの実験方法によって測定した．畑土壌を用いたアンモニアから硝酸（NO_3^--N）への酸化と，*Nitrosomonas europaea* ATCC 25978の細胞懸濁液を用いたアンモニアから亜硝酸（NO_2^--N）への酸化を測定した．その結果，トリクロルメチル基を有する化合物は畑土壌と菌体の両方において，部分的に塩素化されたメチル基（CH_2Clおよび$CHCl_2$）をもつ化合物よりも強い硝酸化成抑制効果を示すことが判明した．トリブロモメチル基をもつ1, 3, 5-トリアジン系化合物は *N. europaea* ATCC 25978の細胞懸濁液では非常に強い抑制効果を示したが，土壌試験においては弱い抑制効果しか示さなかった．また，*N. europaea* ATCC 25978を用いた試験では基質として$(NH_4)_2SO_4$を用いた場合には抑制効果がみられたが，$NH_2OH \cdot HCl$を用いたときにはみられなかった．したがって，検討したすべてのハロメチル-1, 3, 5-トリアジン系化合物の作用点はアンモニアからヒドロキシルアミンへの酸化過程にあると推測された．