Original Article

Mode of Action and QSAR Studies of Nitrification Inhibitors : Effect of Trichloromethyl-1,3,5-triazines on Ammonia-oxidizing Bacteria

Iwao TAKAHASHI, Shinpei OHKI, Manabu MURAKAMI, Satoko TAKAGI,*
Yukiharu SATO, Johannes Willem VONK* and Ko WAKABAYASHI

Graduate School of Agricultural Science, Tamagawa University, Tamagawa-gakuen, Machida 194, Japan

*Department of Biology, TNO Institute of Environmental Sciences, Schoemakerstraat 97,
P.O. Box 6011, 2600 JA Delft, The Netherlands

(Received July 5, 1996 ; Accepted November 5, 1996)

The effects of trichloromethyl-1,3,5-triazines on the ammonia oxidizing activity of the nitrifying bacteria *Nitrosomonas europaea* ATCC 25978 and *Nitrosomonas* sp. TK 794 were investigated. Trichloromethyl-1,3,5-triazines inhibited nitrite production in the ammonia oxidation step, but showed no inhibition to the hydroxylamine oxidation step of both ATCC 25978 and TK 794. The ammonium oxidation by ATCC 25978 was affected more strongly than that by TK 794. These results suggest that the trichloromethyl-1,3,5-triazines may inhibit the ammonia monooxygenase of *Nitrosomonas* spp. Quantitative structure activity relations (QSAR) studies were carried out between the nitrification inhibitory activity (pI_{50}) values on both strains and a hydrophobic parameter (log octanol-water partition coefficient, $\log P$) by means of regression analysis. The optimum $\log P$ of trichloromethyl-1,3,5-triazines for both ATCC 25978 and TK 794 was calculated as about 4.

INTRODUCTION

In soil ammonia is mostly oxidized to nitrate via hydroxylamine and nitrite. This nitrification process is carried out by ammonia-oxidizing bacteria such as *Nitrosomonas* which oxidizes NH_4^+ to NO_2^- through NH_2OH , and nitrite-oxidizing bacteria such as *Nitrobacter* which oxidizes NO_2^- to NO_3^- .^{1,2)} If the nitrification process proceeds rapidly, too much ammonia fertilizer is needed to maintain the soil fertility. Moreover, nitrate may leach into the ground water causing pollution.¹⁾ Due to reduction of nitrate, the amount of the stratospheric ozone depletion gas N_2O increases in the atmosphere.¹⁾ Therefore, it is important to develop nitrification inhibitors, which slow down the process of nitrification.

In 1969 Wakabayashi & Okuzu reported the nitrification inhibitory activity of 2-amino-4-methyl-6-trichloromethyl-1,3,5-triazine.³⁻⁶⁾ Recently Murakami *et al.*⁷⁾ and Okano *et al.*⁸⁾ systematically synthesized a large number of 1,3,5-triazine type nitrification inhibitors and found a good correlation between nitrification inhibitory activity in soil and the hydrophobic parameter, log octanol-water partition coefficient ($\log P$) of the triazines synthesized. Takagi *et al.*⁹⁾

using *Nitrosomonas europaea* ATCC 25978 and *Nitrobacter agilis* ATCC 14123, concluded that some of these compounds inhibited the ammonia oxidizing process but not the nitrite oxidation. It is well known that the oxidation process of ammonia to nitrite involves oxidation of ammonia by ammonia monooxygenase (AMO) and of hydroxylamine by hydroxylamine oxidoreductase (HAO).^{1,10)} It is the purpose of this paper to determine which of the above oxidation steps is inhibited by the trichloromethyl-1,3,5-triazine nitrification inhibitors.

Furthermore, we carried out a quantitative structure-activity relationship (QSAR) study between the inhibitory activity and the hydrophobicity of these triazines, to design new highly active trichloromethyl-1,3,5-triazine nitrification inhibitors and to predict their activity precisely.

MATERIALS AND METHODS

1. Chemicals

2-Substituted-4,6-bis(trichloromethyl)-1,3,5-triazines (**1-4** and **20**) were synthesized by (co)trimerization of trichloroacetonitrile with appropriate nitriles or methylthiocyanate according to the published method.⁷⁾ 2-Alkoxy-4,6-bis(trichloromethyl)-1,3,5-triazines (**17**,

18 and 36), 2-alkoxy-4-methylthio-6-trichloromethyl-1, 3, 5-triazines (51 and 52) and 2-alkoxy-4-phenyl-6-trichloromethyl-1, 3, 5-triazines (44, 45 and 46) were prepared through nucleophilic substitution of 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (1), 2-methylthio-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (20) and 2-phenyl-4, 6-bis(trichloromethyl)-1, 3, 5-triazine (4), respectively, with appropriate alcohols in the presence of triethylamine.^{7,8)} 2-Aryloxy-4-substituted-6-trichloromethyl-1, 3, 5-triazines (19 and 37) were prepared by the reaction of 2-chloro-4-substituted-6-trichloromethyl-1, 3, 5-triazines with appropriate phenols in the presence of triethylamine.⁸⁾ 2, 4-Bis(isopropoxy)-6-methylthio-1, 3, 5-triazine was synthesized by the reaction of methylmercaptan with 2-chloro-4, 6-bis(isopropoxy)-1, 3, 5-triazine, which is obtained from cyanuric chloride and isopropyl alcohol. Monoamination of 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (1) and 2-substituted-4, 6-bis(trichloromethyl)-1, 3, 5-triazines gave 2-amino-4, 6-bis(trichloromethyl)-1, 3, 5-triazines (5-16) and 2-amino-4-substituted-6-trichloromethyl-1, 3, 5-triazines (21-35, 38-43 and 48-50), respectively.^{7,8)} 2, 4-Diamino-6-trichloromethyl-1, 3, 5-triazine (47) was obtained through diamination reaction of 2, 4, 6-tris(trichloromethyl)-1, 3, 5-triazine (1) with excess ammonia water.⁷⁾ All reaction products were purified by recrystallization and/or column chromatography, and their structures were confirmed by IR-, NMR-, Mass-spectroscopy and elementary analysis for C, H and N (also for halogen for some compounds). See Table 1 for melting points. Nitrapyrin was kindly provided by Dow Elanco Japan Limited, Tokyo, Japan. Chemicals for *Nitrosomonas* cultivation were purchased from Kanto Chemical Co., Inc., Tokyo, Japan, and fine chemicals and buffers from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan, and Dojindo Laboratories, Kumamoto, Japan.

2. Preparation of *Nitrosomonas* Cell Suspensions

Nitrosomonas europaea ATCC 25978 (ATCC 25978) and *N. sp.* TK 794 (TK 794) were used as ammonia-oxidizing bacteria. TK 794 was recently isolated from soil by Takahashi *et al.*¹¹⁾ and has different characteristics, *e.g.*, distribution of intracytoplasmic membrane, optimum phosphate concentration for growth and resistance for antibiotics, from ATCC 25978.

ATCC 25978 was cultured in Lewis & Pramer's medium consisting of 2.5 g of $(\text{NH}_4)_2\text{SO}_4$, 13.5 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.7 g of KH_2PO_4 , 0.5 g of NaHCO_3 , 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 mg of CaCl_2 , 1.0 mg of Fe(III)EDTA and 0.5 mg of Cresol Red per liter.¹²⁾ The medium was adjusted to pH 8.0-8.2 with 0.1 N NaOH solution and autoclaved.

TK 794 was cultured in modified Lewis & Pramer's medium consisting of 2.5 g of $(\text{NH}_4)_2\text{SO}_4$, 9.45 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.49 g of KH_2PO_4 , 0.5 g of NaHCO_3 ,

0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 mg of CaCl_2 , 1.0 mg of Fe(III)EDTA , 0.1 mg of Cu(II)EDTA and 0.5 mg of Cresol Red per liter.¹¹⁾ The medium was adjusted to pH 8.0-8.2 with 0.1 N NaOH solution and autoclaved.

Both strains were incubated in 500 ml Erlenmeyer flasks containing 100 ml of above media. The flasks were shaken at 200 rpm in the dark at 29°C for 7-10 days. Four hundred milliliters of culture were centrifuged at $8600 \times g$ for 15 min at 4°C and the supernatant was thrown away. Then the cells were washed twice with sterilized 0.02 M phosphate buffer, pH 8.0 (P-buffer) by centrifugation at $7000 \times g$ for 15 min at 4°C. The washed harvested wet cells were kept in 5 ml of P-buffer.

3. Determination of Nitrification Inhibitory Activity

Ammonium sulfate (100 μg nitrogen per milliliter in P-buffer) and hydroxylamine hydrochloride (100 μg nitrogen per milliliter in P-buffer) were used as the substrate for the ammonia oxidation step and the hydroxylamine oxidation step of *Nitrosomonas*, respectively. Ten milligrams of 1, 3, 5-triazines or nitrapyrin (as control) were emulsified in water with a few drops of Tween 20 and ethanol, and a small amount of talc by homogenizing vigorously for 30 min in a mortar and then diluted in water to make 5 ml of a test solution. The cell suspension in 5 ml of P-buffer was diluted 50 fold with P-buffer for assay. The rate of the nitrite nitrogen (NO_2^- -N) formation in this cell suspension was about 1 $\mu\text{g}/\text{ml} \cdot 30 \text{ min}$. Into a 2 ml test tube, 1 ml of each substrate solution and 10 μl of the prepared test solution were added, and then 1 ml of the diluted cell suspension was added. The test tube was incubated in a water bath at 37°C for 30 min without shaking.

The nitrite concentration in the reaction solution was determined at start and after 30 min incubation (30 min reaction solution) by means of the Griess-Ilosvay method.¹³⁾

Inhibitory activity was calculated by using Eqs. (1) and (2).

Formation of NO_2^- -N

$$= (\text{Amount of } \text{NO}_2^- \text{-N after 30 min}) - (\text{Amount of } \text{NO}_2^- \text{-N at start}) \quad (1)$$

NO_2^- -N formation (%) =

$$100 \times \left(1 - \frac{\text{Formation of } \text{NO}_2^- \text{-N with inhibitor}}{\text{Formation of } \text{NO}_2^- \text{-N without inhibitor}} \right) \quad (2)$$

The molar concentration of inhibitor which shows 50% inhibition (molar I_{50}) of the formation of NO_2^- -N without inhibitor was estimated by probit analysis. The inhibitory indices for the ATCC 25978 and the TK 794 were expressed as $\text{p}I_{50}$ (AT) and $\text{p}I_{50}$ (TK), the negative logarithm of the each molar I_{50} , respectively (Table 1).

4. QSAR for 1, 3, 5-Triazine Nitrification Inhibitors

Regression analysis were performed between pI_{50} values obtained from ATCC 25978 or TK 794 and hydrophobic parameters ($\log P$) determined by Okano *et al.*⁸⁾

The $\log P$ values of 1, 3, 5-triazine derivatives was estimated by a semi-empirical method based upon the additive property of the π value. As a first step the $\log P$ ($\log P-1$) values of twelve 2, 4, 6-trisubstituted-1, 3, 5-triazines were determined by the HPLC method using an ODS column (Inertsil ODS 4.6×250 mm) and methanol-water (8 : 2) as the mobile phase.¹⁴⁾ From $\log P-1$ values we estimated the $\log P$ values for 1, 3, 5-triazine ($\log P=2.69$) using the π (R_1), π (R_2) and π (R_3) values of Nys & Rekker¹⁵⁾ according to the Eq. (3). Using $\log P=2.69$, we calculated $\log P$ values of all 1, 3, 5-triazines in Table 1 according to the Eq. (3).

$$\log P \left(\text{N} \begin{array}{c} \diagup \text{N} \diagdown \\ \diagdown \text{N} \diagup \end{array} \right) + \pi(R_1) + \pi(R_2) + \pi(R_3) = \log P \left(\text{N} \begin{array}{c} \text{R}_1 \diagup \text{N} \diagdown \\ \text{R}_2 \diagdown \text{N} \diagup \text{R}_3 \end{array} \right) \quad (3)$$

RESULTS AND DISCUSSION

1. Effect of Trichloromethyl-1, 3, 5-triazine Nitrification Inhibitors on Ammonia or Hydroxylamine Oxidation in Cell Suspensions of *Nitrosomonas*

The oxidation process of ammonia to nitrite is composed of two sequential steps in *Nitrosomonas*. The first is an ammonia oxidation step which is catalyzed by AMO and the second is a hydroxylamine oxidation step

catalyzed by HAO. It is very difficult to discriminate directly whether the trichloromethyl-1, 3, 5-triazines act on AMO or HAO, because the activity of AMO is lost during isolation,¹⁶⁾ and HAO has a complex structure containing several subunits.^{17, 18)} In order to determine the target step of trichloromethyl-1, 3, 5-triazines nitrification inhibitors, a convenient assay system using intact cells was introduced. *Nitrosomonas* cells were incubated together with inhibitors and substrate of each enzyme for 30 min, and the amount of nitrite nitrogen produced in the medium was determined by the Griess-Ilosvay method.¹³⁾ A quantitative determination of hydroxylamine¹⁹⁾ in the medium was not attempted.

In the oxidation step of hydroxylamine, nitrite nitrogen production was not decreased by any trichloromethyl-1, 3, 5-triazines (data not shown). This indicates that the oxidation of hydroxylamine is not inhibited by trichloromethyl-1, 3, 5-triazines. Trichloromethyl-1, 3, 5-triazines reduced the nitrite nitrogen production in the ammonia oxidation step (Table 1). These results indicate that trichloromethyl-1, 3, 5-triazines and nitrapyrin, the reference nitrification inhibitor, inhibit the ammonia oxidation step. This suggests that the trichloromethyl-1, 3, 5-triazines may inhibit the AMO in *Nitrosomonas* spp. To confirm our conclusion, further experiments on the enzyme level are needed. The trichloromethyl-1, 3, 5-triazines are promising as nitrification inhibitors in agriculture, because they cause no accumulation of hydroxylamine which may be hazardous to crop plants.

Table 1 Nitrification inhibitory activity (pI_{50}) and $\log P$ values of trichloromethyl-1, 3, 5-triazines.

No.	R_1	R_2	mp (°C)	$\log P$	ATCC 25978			TK 794		
					Dose (mg/kg)	NO_2^- -N formation (%)	pI_{50} value	Dose (mg/kg)	NO_2^- -N formation (%)	pI_{50} value
Blank	—	—	—	—	—	100.0	—	—	100.0	—
Control	Nitrapyrin	—	60	—	1.0	30.1	5.72	5.0	22.9	5.54
1	CCl_3	CCl_3	94–95	3.71	—	—	—	25.0	50.0	3.52
2	CH_3	CCl_3	96–97	4.07	0.5	47.2	6.15	0.5	65.0	5.79
3	$(\text{CH}_2)_3\text{CH}_3$	CCl_3	Oil	5.65	1.0	29.5	6.21	1.0	33.6	5.71
4	C_6H_5	CCl_3	97–98	5.26	—	—	—	200.0	65.8	3.11
5	NH_2	CCl_3	154–158	2.45	—	—	—	1.0	56.2	5.35
6	NHCH_3	CCl_3	107–110	3.14	0.1	69.6	6.23	—	—	—
7	NHCH_2CH_3	CCl_3	79–81	3.68	0.1	52.7	6.59	—	—	—
8	$\text{NH}(\text{CH}_2)_2\text{CH}_3$	CCl_3	45–47	4.19	0.1	60.0	6.66	—	—	—
9	$\text{NH}(\text{CH}_2)_3\text{CH}_3$	CCl_3	Oil	4.72	0.1	72.4	6.01	—	—	—
10	$\text{NH}(\text{CH}_2)_4\text{CH}_3$	CCl_3	Oil	5.25	0.1	67.1	5.91	—	—	—
11	$\text{NH}(\text{CH}_2)_5\text{CH}_3$	CCl_3	Oil	5.79	1.0	42.7	5.78	—	—	—
12	$\text{NH}(\text{CH}_2)_9\text{CH}_3$	CCl_3	Oil	7.90	10.0	36.8	4.98	—	—	—
13	$\text{NH}(\text{CH}_2)_{11}\text{CH}_3$	CCl_3	Oil	8.96	5.0	50.0	5.24	5.0	55.2	3.90
14	$\text{NH}(\text{CH}_2)_{15}\text{CH}_3$	CCl_3	Oil	11.05	200.0	52.8	2.88	500.0	52.9	<3.00
15	NHC_6H_5	CCl_3	112–114	4.33	—	—	—	0.5	55.0	5.70
16	$\text{N}(\text{CH}_3)_2$	CCl_3	Oil	3.70	—	—	—	0.5	60.5	5.67
17	OCH_3	CCl_3	45–46	3.59	0.1	27.4	6.34	1.0	47.8	5.57

Table 1 (Continued.)

No.	R ₁	R ₂	mp (°C)	log P	ATCC 25978			TK 794		
					Dose (mg/kg)	NO ₂ ⁻ -N formation (%)	pI ₅₀ value	Dose (mg/kg)	NO ₂ ⁻ -N formation (%)	pI ₅₀ value
18	OCH ₂ CH ₃	CCl ₃	Oil	4.14	1.0	27.3	6.36	1.0	25.8	5.89
19	O-C ₆ H ₃ -4-F-2-Cl	CCl ₃	Oil	5.74	0.5	52.2	5.96	1.0	57.8	5.49
20	SCH ₃	CCl ₃	71-72	4.21	0.5	18.7	6.73	0.5	41.3	6.03
21	NH ₂	CH ₃	160-161	2.82	—	—	—	5.0	42.1	4.96
22	NH(CH ₂) ₂ CH ₃	CH ₃	75-76	4.56	1.0	42.9	5.70	—	—	—
23	NH(CH ₂) ₃ CH ₃	CH ₃	49-51	5.08	0.1	79.5	5.82	—	—	—
24	NH(CH ₂) ₄ CH ₃	CH ₃	Oil	5.61	0.1	61.5	6.30	—	—	—
25	NH(CH ₂) ₅ CH ₃	CH ₃	34-35	6.13	0.1	54.5	6.38	—	—	—
26	NH(CH ₂) ₆ CH ₃	CH ₃	46-47	6.66	0.1	44.9	6.42	—	—	—
27	NH(CH ₂) ₈ CH ₃	CH ₃	46-49	7.72	1.0	59.0	5.52	5.0	55.0	4.65
28	NH(CH ₂) ₉ CH ₃	CH ₃	36.5-37	8.27	10.0	56.2	5.04	50.0	50.0	3.89
29	NH(CH ₂) ₁₁ CH ₃	CH ₃	43-45	9.30	10.0	69.4	4.34	500.0	33.2	3.22
30	NH(CH ₂) ₁₅ CH ₃	CH ₃	60-62	11.40	400.0	45.2	3.05	500.0	81.0	<3.00
31	N(CH ₃) ₂	CH ₃	58-60	4.07	—	—	—	1.0	58.7	5.44
32	N(CH ₃)C ₄ H ₉	CH ₃	Oil	5.65	0.1	59.5	6.23	—	—	—
33	N(C ₂ H ₅)C ₄ H ₉	CH ₃	Oil	6.17	0.1	52.9	6.02	—	—	—
34	N(C ₄ H ₉) ₂	CH ₃	Oil	7.23	1.0	56.2	5.68	1.0	56.8	5.39
35	NHC ₆ H ₅	CH ₃	107-108	4.70	0.1	66.0	6.30	1.0	39.0	5.78
36	OCH ₃	CH ₃	Oil	3.98	—	—	—	0.5	32.0	5.69
37	O-C ₆ H ₄ -4-Cl	CH ₃	110-113	5.94	1.0	36.1	6.13	1.0	47.0	5.45
38	NH ₂	(CH ₂) ₃ CH ₃	117-118	4.40	1.0	29.0	5.92	1.0	39.7	5.31
39	NH ₂	C ₆ H ₅	175-176	4.02	1.0	20.8	6.44	5.0	49.7	5.70
40	NHCH ₃	C ₆ H ₅	69-70	4.70	0.1	53.2	6.43	0.5	57.1	5.96
41	NHCH ₂ CH ₃	C ₆ H ₅	160-162	5.23	0.1	45.3	6.42	0.5	47.8	5.91
42	NH(CH ₂) ₂ CH ₃	C ₆ H ₅	Oil	5.75	0.1	50.1	6.41	0.5	58.1	5.60
43	N(CH ₃) ₂	C ₆ H ₅	166-168	5.40	1.0	40.1	5.77	5.0	43.0	4.95
44	OCH ₃	C ₆ H ₅	128-130	5.17	0.1	31.4	6.64	1.0	39.0	5.67
45	OCH ₂ CH ₃	C ₆ H ₅	75-77	5.70	1.0	31.4	5.98	1.0	51.2	5.15
46	O(CH ₂) ₂ CH ₃	C ₆ H ₅	53-55	6.22	1.0	48.5	5.47	5.0	47.9	4.92
47	NH ₂	NH ₂	235-236	1.21	2.0	63.2	5.15	4.0	40.0	4.76
48	OCH ₃	NH ₂	176-178	2.36	0.1	36.1	6.31	0.5	29.5	5.60
49	OCH ₃	NHCH ₂ CH ₃	50-51	3.57	0.1	49.1	6.48	0.1	56.8	5.94
50	NH ₂	SCH ₃	172-174	2.96	0.5	65.5	5.90	5.0	27.0	5.40
51	OCH ₂ CH ₃	SCH ₃	Oil	4.64	0.1	64.8	6.17	1.0	60.0	5.48
52	O(CH ₂) ₂ CH ₃	SCH ₃	Oil	5.17	0.1	47.6	6.59	1.0	60.0	6.00

All components tested did not inhibit nitrite formation from hydroxylamine oxidation step by both strains, at 10 mg/kg concentration.

2. Qualitative Relation between Structure and Nitrification Inhibitory Activity of Trichloromethyl-1, 3, 5-triazines

In our previous study^{7,8)} a QSAR study of the trichloromethyl-1, 3, 5-triazines has been done using pI₅₀ values from nitrate formation in soil. In this paper, a more precise structure-activity relationship (SAR) was performed using pI₅₀ values obtained from the inhibition of the first ammonia oxidation step.

Various trichloromethyl-1, 3, 5-triazines and their nitrification inhibitory activity are listed in Table 1. The SAR study of trichloromethyl-1, 3, 5-triazines was carried out with the pI₅₀ (AT) values. The nitrification inhibitory activity was quite different among the trichloromethyl-1, 3, 5-triazines. The pI₅₀ (AT) values of the highly active trichloromethyl-1, 3, 5-triazines were

above 6.00. Compounds **8** and **20** were the most potent nitrification inhibitors, compounds **7**, **17**, **18**, **25**, **26**, **39**, **40-42**, **44**, **48**, **49** and **52** were also active, followed by **2**, **3**, **6**, **9**, **24**, **32**, **33**, **35**, **37** and **51**. Compounds **10**, **11**, **13**, **19**, **22**, **23**, **27**, **28**, **34**, **38**, **43**, **45-47** and **50** were moderately active with pI₅₀ values of 5.00-6.00. Nitrapyrin belonged to this group. Little activity, with pI₅₀ values lower than 5.00, was observed with compounds **12**, **14**, **29** and **30**, with the compounds **14** and **30** at the low end. The highly active trichloromethyl-1, 3, 5-triazines appear to have other two substituents selected from alkyl, aryl, alkoxy, aryloxy, alkylthio or amino group on 1, 3, 5-triazine ring. However, the trichloromethyl-1, 3, 5-triazines with moderately and little activity have also the structural characteristics similar to the highly active trichloromethyl-1, 3, 5-triazines. It should be noted that

introduction of a short chain alkylated amino group or an alkoxy group to the trichloromethyl-1, 3, 5-triazines improves the inhibitory activity (see pI_{50} value of compounds 6-9, 17, 18, 24-26, 32, 33, 40-42, 44, 48, 49, 51 and 52). However, introduction of a long chain (C_8 - C_{16}) alkylamino group produced less active compounds (12-14, 27-29 and 30). This fact may indicate that some suitable hydrophobicity is necessary for improving the inhibitory activity of trichloromethyl-1, 3, 5-triazine nitrification inhibitors. It can be tentatively summarized that an suitable combination consisted of a trichloromethyl group, a short chain alkylamino group and a phenyl or an alkoxy group in the three substituents of 1, 3, 5-triazine ring produces the active trichloromethyl-1, 3, 5-triazine nitrification inhibitors. Anyway, it is difficult to explain the correlation between substituents on 1, 3, 5-triazine ring and nitrification inhibitory activity, through the qualitative consideration. Therefore, the QSAR study using physicochemical parameter may be essential to design new and more active trichloromethyl-1, 3, 5-triazine nitrification inhibitors.

The SAR pattern of 1, 3, 5-triazine nitrification inhibitors was also examined qualitatively with pI_{50} (TK) values. The pI_{50} (TK) values are somewhat lower than the pI_{50} (AT) values. Introduction of a trichloromethyl group, a short chain alkylated amino group and a phenyl or an alkoxy group to the 1, 3, 5-triazine ring improves the inhibitory activity, too. The activity-activity correlation between pI_{50} (AT) values and pI_{50} (TK) values is analyzed in the following Eq. (4).

$$pI_{50} \text{ (ATCC 25978)} = 0.757 (\pm 0.052) pI_{50} \text{ (TK 794)} + 1.969 (\pm 0.277) \quad (4)$$

where $n=28$, $r=0.945$, $s=0.187$ and $F_{1,26}=215.87$.

In Eq. (4), n stands for number of data, r the correlation coefficient, s the standard deviation and F value of the F -test ($p < 0.01$), respectively. The figures in parentheses are 99% confidence intervals. This equation indicates that ATCC 25978 is more sensitive to the 1, 3, 5-triazines assayed in this study than TK 794. This higher sensitivity of ATCC 25978 to the 1, 3, 5-triazine inhibitors compared with TK 794 may be explained by that the thick lamellar structure of the cell membrane¹¹⁾ of TK 794 prevents absorption of the inhibitors into cells. Also, the susceptibility of AMO which is considered as the target enzyme of the inhibitors may differ between the both strains of *Nitrosomonas* spp.

3. Quantitative Structure-activity Relationship (QSAR) of Trichloromethyl-1, 3, 5-triazine Nitrification Inhibitors

In our previous study on the QSAR of trichloromethyl-1, 3, 5-triazine derivatives for their nitrification inhibitory activity in soil, the hydrophobicity constant ($\log P$) has been found to be a major parameter deter-

mining the inhibitory activity of trichloromethyl-1, 3, 5-triazines.^{7,8)} The nitrification inhibitory activity expressed by pI_{50} values in this paper related to the $\log P$ values of the compounds as mentioned in section 2. Therefore, QSAR analysis was performed using nitrification inhibitory activities, pI_{50} (AT) values and pI_{50} (TK) values, and the hydrophobic parameter, $\log P$. Two parabolic equations, (5) and (6), expressed a good correlation. Compounds 1 and 4 are eliminated from calculation to obtain Eq. (6), because they are readily converted into inactive ($pI_{50} < 2.00$) melamine and 2, 4-diamino-6-phenyl-1, 3, 5-triazine, respectively, in the presence of excess ammonia.

$$pI_{50} \text{ (ATCC 25978)} = -0.071 (\pm 0.007) (\log P)^2 + 0.618 (\pm 0.096) \log P + 4.930 (\pm 0.296) \quad (5)$$

where $n=44$, $r=0.931$, $s=0.306$ and $F_{2,41}=133.10$.

$$pI_{50} \text{ (TK 794)} = -0.096 (\pm 0.011) (\log P)^2 + 0.831 (\pm 0.118) \log P + 3.890 (\pm 0.309) \quad (6)$$

where $n=34$, $r=0.909$, $s=0.277$ and $F_{2,31}=73.45$.

The Eqs. (5) and (6) indicate that the hydrophobic parameter, $\log P$, plays a significant role in determining the nitrification activities in cell suspension and that optimum $\log P$ values exist for the activities of trichloromethyl-1, 3, 5-triazines assayed in this study because of the negativity of square terms in the parabolic equations (5) and (6). The optimum $\log P$ values in the Eqs. (5) and (6) were calculated as 4.35 and 4.33, respectively. Accordingly, activity of trichloromethyl-1, 3, 5-triazine nitrification inhibitors become at a maximum when the $\log P$ value is about 4. By using this value it will become possible to design highly active trichloromethyl-1, 3, 5-triazine nitrification inhibitors. This finding also indicates that the nitrification inhibitory activity of trichloromethyl-1, 3, 5-triazines relates to permeability into cell membrane or so-called hydrophobic binding of the inhibitors to the target site.

The pI_{50} (AT) value of 2, 4-bis(isopropoxy)-6-methylthio-1, 3, 5-triazine having no trichloromethyl group was found to be 5.91, indicating good fitness with the pI_{50} value (6.10) calculated using $\log P$ (5.91) and Eq. (5). This fact support that the nitrification inhibitory activity of 1, 3, 5-triazine inhibitors relates with hydrophobic parameter $\log P$. Design and synthetic studies of trichloromethyl-1, 3, 5-triazine derivatives using QSAR is presently investigated further, in order to develop newer trichloromethyl-1, 3, 5-triazine nitrification inhibitors.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Dr. Tatsuki Tokuyama and Dr. Reiji Takahashi, Nihon University, and Dr. Yoshiko

Miyamoto, Kitasato University, and Prof. Dr. Toshio Fujita, Fujitsu Kansai Systems Laboratory, for many helpful discussions.

This work was supported by a Grant-in-Aid for General Scientific Research (B) from the Ministry of Education, Science and Culture of Japan. J. W. Vonk was granted a stay at Tamagawa University by Japanese Society for the Promotion of Science (JSPS).

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要 約

硝化抑制剤の作用機構と QSAR 研究：アンモニア酸化細菌に対するトリクロロメチル-1,3,5-トリアジン系化合物の硝化抑制効果

高橋 巖, 大氣新平, 村上 学, 高木聡子
佐藤幸治, Johannes Willem VONK, 若林 攻

硝化細菌 *Nitrosomonas europaea* ATCC 25978 と *N. sp.* TK 794 の硝化作用に対するトリクロロメチル-1,3,5-トリアジン系化合物の硝化抑制効果を検討した。トリクロロメチル-1,3,5-トリアジン系化合物はアンモニア酸化段階で亜硝酸塩の生成を抑制したが、ヒドロキシルアミン酸化段階は抑制しなかった。以上の結果からトリクロロメチル-1,3,5-トリアジン系化合物の標的酵素はアンモニアモノオキシゲナーゼと考えられる。QSAR の結果、トリクロロメチル-1,3,5-トリアジン系化合物の最適 log *P* 値は約 4 である。