Mode of Action of Novel 2-(Benzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazine Herbicides: Inhibition of Photosynthetic Electron Transport and Binding Studies

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Novel 2-(benzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazines have the same 1,3,5-triazine skeleton as atrazine, although some of them, for example, 2-(3-chlorobenzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazine [pI_{50}(spinach) = 7.21], show a >3 times stronger photosynthetic electron transport inhibitory activity than atrazine [pI_{50}(spinach) = 6.72]. The new triazines have only one amino group at the triazine ring, and their molecular shapes are different from atrazine. The replacement of the bound [^{14}C]atrazine by 1,3,5-triazines was tested to determine whether the novel 1,3,5-triazine analogues exhibit the same binding pattern at the D1-protein as atrazine. It was found that [^{14}C]atrazine bound to the D1-protein was replaced by the triazine tested by a clearly competitive interaction. Obviously, the novel 1,3,5-triazines are attached to the same binding niche as atrazine.

Keywords: *(Benzylamino)-1,3,5-triazines; D1-protein; binding partners; replacement; atrazine-resistant Chenopodium*

INTRODUCTION

Before herbicides are introduced into the market, determination of their mechanisms of action of becomes important not only to maintain environmental safety but also to facilitate registration. Photosynthetic electron transport (PET) inhibitors, such as simazine, atrazine, or diuron, bind to a specific site at the D1-protein, which plays an important role in photosystem II (PS-II) (Tischer and Strotmann, 1977). It is known that [^{14}C]atrazine bound to thylakoids is readily replaced competitively by diuron, indicating the same binding niche for atrazine and diuron. Because a cross-resistance for atrazine-resistant plants was never observed between atrazine and diuron, they were considered to have different binding partners (amino acid residues) in the same binding niche (Thiel and Böger, 1984; Hirschberg et al., 1984).

Novel 2-(benzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazines have the same 1,3,5-triazine skeleton as atrazine, although some of them, for example, 2-(3-chlorobenzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazine [pI_{50}(spinach) = 7.21], showed a >3 times stronger PET inhibitory activity with spinach thylakoids than atrazine [pI_{50}(spinach) = 6.72] (Kuboyama et al., 1999; Koizumi et al., 1998). The PET inhibitory activity may be caused by a binding to the D1-protein similar to that of atrazine. Interestingly, the new triazines dealt with in this paper have only one amino group at the triazine ring, and their molecular shape is quite different from that of atrazine. The binding pattern of the new triazines should be evaluated, and we used [^{14}C]-atrazine to confirm by replacement assays whether the new triazines have the same binding site as atrazine.

MATERIALS AND METHODS

Chemicals. Five 2-(benzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazines (**1**−**5**) were synthesized by the nucleophilic amination reaction of 2-methyl-4-(trichloromethyl)-6-(trifluoromethyl)-1,3,5-triazine with appropriate benzylamines (Kuboyama et al., 1998). The structures of these compounds were confirmed by IR, NMR, and mass spectroscopy. In Table 1 structures and melting points are shown together with PET inhibitory activity [pI_{50}(spinach)]. All compounds were dissolved in ethanol for both binding assay and determination of PET inhibitory activities, and the ethanol concentration was kept below 1% in the final reaction medium. [^{14}C]Atrazine (ring-UL-^{14}C) was purchased from Sigma Chemical Co., St. Louis, MO (specific radioactivity = 695.6 kBq/μmol). Other analytical grade chemicals and fine chemicals such as buffers were purchased from Kanto Chemical Co., Inc., Tokyo, Japan, or Sigma Chemical Co.

Preparation of Spinach Thylakoids. Spinach was purchased at the local market. Thylakoid preparation from spinach (*Spinacia oleracea*) leaves was performed according to the procedure of Böger (1993). After removal of the midribs, the leaves were homogenized in 50 mM tricine [*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine]−NaOH (pH 8.0) containing 0.4 M sucrose, 10 mM NaCl, and 5 mM $MgCl_2$ using a cooking mixer. The homogenate was filtered through eight

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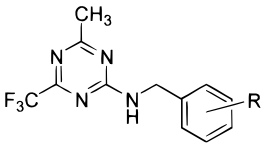
10.1021/jf990216o CCC: $18.00
Published on Web 09/25/1999

Table 1. PET Inhibitory Activities and K_i Values of 2-(Benzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazines with Spinach Thylakoids



no.	R	mp (°C)	pI_{50}(spinach)[a]	K_i (M)
1	3-Cl	58−61	7.21	0.18×10^{-7}
2	4-Cl	74−76	6.98	0.30×10^{-7}
3	4-Br	88−90	6.94	0.36×10^{-7}
4	H	45−47	6.85	1.08×10^{-7}
5	2-Cl	79−82	5.95	3.93×10^{-7}
	atrazine	171−174	6.72	

[a] pI_{50}(spinach) = −log I_{50}(spinach): the negative logarithms of the molar concentration of the compounds that produced a 50% PET inhibition.

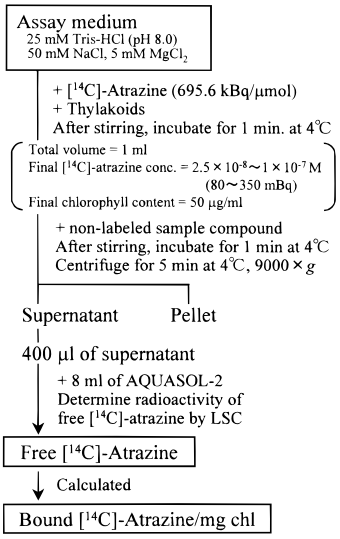


Figure 1. Replacement test using spinach thylakoids

layers of cheesecloth and centrifuged for 1 min at 4000*g* at 4 °C. The pellet was resuspended in the above-mentioned medium, and its chlorophyll content was adjusted to 1 mg of chlorophyll (chl)/mL (Arnon, 1949).

PET Inhibition by Compounds Using Spinach Thylakoids. This was carried out according to the method of Kuboyama et al. (1998). Photosynthetic electron transport was determined with the system $H_2O \rightarrow$ ferricyanide, uncoupled by NH_4Cl. The reaction mixture contained 0.1 M sucrose, 50 mM tricine (pH 8.0), 5 mM $MgCl_2$, 1 mM NH_4Cl, and 1 mM potassium ferricyanide. The freshly prepared spinach thylakoid suspension was added to give a final concentration of 15 μg of chl/mL. The measurement was performed at 20 °C for 20 s in white light with 35000 lx, and the final solvent concentration for the compounds added was kept below 1% (v/v). Oxygen was measured with the oxygen electrode (Rank Brothers, Botisham, Cambridge, U.K.,). Data of PET inhibition are presented as pI_{50} values, the negative logarithms of the molar concentration at which the compounds produced a 50% inhibition.

Replacement of Bound ^{14}C-Labeled Atrazine. The replacement experiment of [^{14}C]atrazine was carried out at 4 °C in a cold room, with some modification of the method of Böger [see the procedure in Figure 1 and Böger (1982)]. Herbicide binding was performed in 1.5 mL reaction tubes (Eppendorf, Hamburg, Germany). The reaction mixture of 1 mL final volume contained 50 mM NaCl, 25 mM Tris-HCl [tris-(hydroxymethyl)aminomethane-HCl] (pH 8.0), 5 mM $MgCl_2$, and 2.5×10^{-8} to 1×10^{-7} M [^{14}C]atrazine (≒ 85−350 mBq). [^{14}C]Atrazine was added to the reaction mixture as ethanol solution. Next, spinach thylakoids were added to the reaction mixture, yielding a chlorophyll concentration of 50 μg/mL. After 15 s of stirring with a vortex mixer, the mixture was allowed to stand for 1 min. The 1,3,5-triazines tested (compounds **1**−**5**) were then added to the mixture at a final concentration of $(1-2) \times 10^{-7}$ or $(1-2) \times 10^{-6}$ M, and the mixture was stirred for 15 s and again kept for 1 min. Total volume of the reaction mixture was 1 mL, and ethanol concentration was <1% (v/v). The replacement reaction was stopped by centrifugation for 5 min at 9000*g*. Four hundred microliter aliquots of the clear supernatant and 8 mL of cocktail (Aquasol-2, Packard Instrument Co., Meriden, CT) were taken for scintillation counting in an LSC-5100 instrument (Aloka Co., Ltd., Tokyo, Japan) to determine the amount of free unbound [^{14}C]atrazine. The bound atrazine was calculated by the difference between free [^{14}C]atrazine in the supernatant and the starting amount of [^{14}C]atrazine in the assay sample.

Postemergence Phytotoxic Activities against Atrazine-Resistant and Wild-Type *Chenopodium*. Compound **3** was evaluated against both the atrazine-resistant mutant and the wild-type *Chenopodium album*. The postemergence test was performed after the study of Kuboyama et al. (1998). Atrazine-resistant (2.2−2.4 leaf stage) and wild-type (2.5−2.7 leaf stage) *Chenopodium* were grown in the greenhouse under a postemergence treatment of 25, 50, 100, or 3200 g of active ingredient (ai)/1000 m^2 for 12 days. According to the extent of injury of *Chenopodium*, the herbicidal potency was assessed by a score of 0−5, where 5 indicates complete kill and zero indicates no effects.

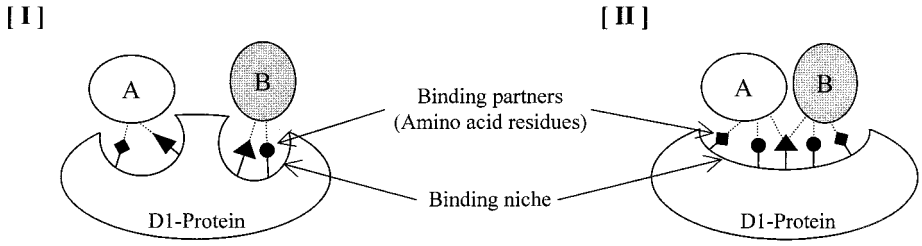
PET Inhibitory Activities of Compound 3 Using Atrazine-Resistant and Wild-Type *Chenopodium* Thylakoids. *C. album* seeds were provided by Dr. J. van Rensen, Wageningen, The Netherlands. Plants were grown in the greenhouse to ∼15 cm size. Thylakoids were prepared as described by van Rensen et al. (1977). The leaves were homogenized in a mixer with the ice-cold homogenate medium containing 20 mM tricine−NaOH (pH 7.8), 0.4 M sorbitol, 10 mM NaCl, 2 mM sodium ascorbate, and 2 mg/mL bovine serum albumin. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 30 s at 500*g* to precipitate debris. The supernatant was centrifuged again for 8 min at 1000*g* to precipitate thylakoids. The pellet was resuspended in the same medium for preparation, and its chlorophyll content was adjusted to 2 mg/mL. PET inhibition was tested in the system $H_2O \rightarrow$ ferricyanide, uncoupled by NH_4Cl using the oxygen electrode as for determination of PET inhibition with spinach thylakoids. The reaction mixture contained 50 mM tricine−NaOH (pH 7.6), 0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM NH_4Cl, 1 mM potassium ferricyanide, and *Chenopodium* thylakoids in a final concentration of 25 μg of chl/mL.

RESULTS AND DISCUSSION

Binding Niche of Novel 1,3,5-Triazines. The herbicidal activity of novel 2-(benzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazines has already been reported in our previous papers, indicating that some of their analogues, for example, 2-(4-bromobenzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazine, exhibited a stronger herbicidal activity in both pre- and postemergence applications than atrazine (Kuboyama et al., 1998; Koizumi et al., 1998).

At first we checked the PET inhibitory activity of the 1,3,5-triazine analogues. As shown by Table 1 the 1,3,5-triazine analogues exhibited a stronger PET inhibitory activity than atrazine, except the 2-chlorobenzyl analogue (**5**). Accordingly, the novel 1,3,5-triazine analogues were confirmed to be PET-inhibiting compounds presumably targeting the D1-protein like atrazine.

The replacement of bound [^{14}C]atrazine by 1,3,5-triazines was determined to demonstrate whether the 1,3,5-triazine analogues have the same binding pattern



A: Atrazine, B: 1,3,5-Triazine

[I] If compounds A and B bind to the different binding partners in different binding niches, they may not show competitive replacement.

[II] If compounds A and B bind to the same binding partners (amino acid residues) of the same binding niche, or the two compounds use common binding partners in part only, a competitive replacement is observed.

Figure 2. Model of binding niche and binding partners at the D1-protein.

Table 2. Increase of the Free [^{14}C]Atrazine by Addition of the 1,3,5-Triazine Compound 3[a]

1,3,5-triazine **3** (M)	free atrazine in assay (μM)	% free atrazine
0 (control)	22.8	45.6
1.0×10^{-7}	32.8	65.6
1.5×10^{-7}	36.7	73.4
2.0×10^{-7}	40.0	80.0

[a] Condition: [^{14}C]atrazine concentration = 5×10^{-8} M.

at the D1-protein as atrazine. This was done with 2-(4-bromobenzylamino)-4-methyl-6-(trifluoromethyl)-1,3,5-triazine (**3**) as a representative of the novel 1,3,5-triazine PET inhibitors. If atrazine and the 1,3,5-triazine (**3**), which both inhibit the D1-protein, bind to different binding niches, they should not show competitive mutual replacement (see Figure 2I). If atrazine and the 1,3,5-triazine (**3**) are attached to binding partners (amino acid residues) of the same niche, they should be replaced in a competitive mutual fashion. Competition should also show up when only some of the interacting amino acid residues of the same binding niche are different for atrazine and the analogues of this study (see Figure 2II).

The amounts of the free [^{14}C]atrazine in the reaction medium of the replacement experiment increased with the addition of triazine (**3**) as shown in Table 2, indicating that the bound [^{14}C]atrazine was replaced by triazine (**3**). Amounts of free atrazine (liberated and unbound atrazine) were determined after four fixed concentrations of the 1,3,5-triazine (**3**) had been added. The double-reciprocal plot of the amount of free [^{14}C]-atrazine versus the amount of bound [^{14}C]atrazine was calculated by linear regression (see Figure 3I). As demonstrated, spinach thylakoids exhibited replacement of [^{14}C]atrazine by the 1,3,5-triazine (**3**), which is clearly a competitive type of inhibition because a common intersection on the *y*-axis is observed. Also, with other 1,3,5-triazines (**1**, **2**, **4**, and **5**), the same competitive inhibition was found (data not shown). Accordingly, we conclude that the novel 1,3,5-triazines tested bind to same niche as atrazine.

The value of the intersection on the *y*-axis is ~0.5 mg (=500 nmol) of chl/nmol of triazine, which implies the following results: (1) The above ratio indicates that 1 molecule of atrazine binds to 1 molecule of D1-protein, because PS-II with the reaction center D1-protein has ~660 chlorophyll molecules per photosystem of higher plants (Siggel et al., 1971). (2) Accordingly, we conclude that the 1,3,5-triazines **1**−**5** bind to the D1-protein with a molar ratio of 1:1.

The order of K_i values found by the slopes of the double-reciprocal plots (see Figure 3II) was as follows: triazine (**1**) ($K_i = 0.18 \times 10^{-7}$ M) < triazine (**2**) ($K_i = 0.30 \times 10^{-7}$ M) < triazine (**3**) ($K_i = 0.36 \times 10^{-7}$ M) < triazine (**4**) ($K_i = 1.08 \times 10^{-7}$ M) < triazine (**5**) ($K_i = 3.93 \times 10^{-7}$ M). The bound atrazine is replaced best by the compound that has the smallest K_i value. That is, compound **1** binds best to the D1-protein.

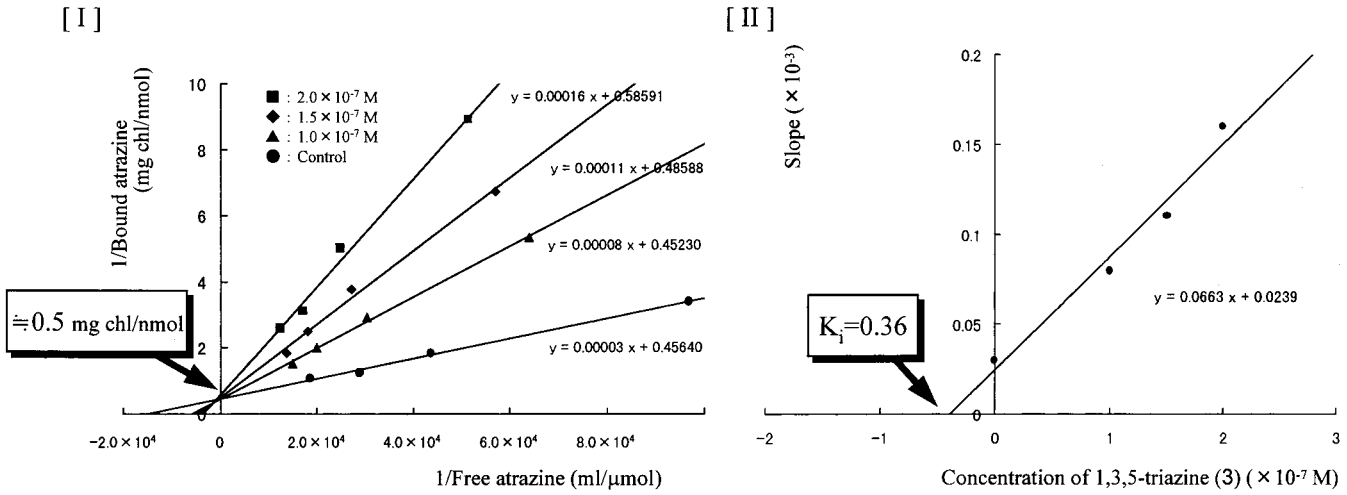


Figure 3. Double-reciprocal plot using 1,3,5-triazine compound **3** and labeled atrazine (I). In (II) the K_i for the triazine compound is determined by the slope of the regression line of part I.

Table 3. Postemergence Phytotoxic Activity (I) and PET Inhibitory Activity (II) of the Triazine Compound 3 and Atrazine against *C. album*

(I) Postemergence Phytotoxic Activity[a]

compound	dose (g of ai/10 acre)	phytotoxic activity[b] resistant type	phytotoxic activity[b] wild type
3	25	4	5
	50	5	5
	100	5	5
atrazine	25	1	5
	50	1	5
	100	1	5
	3200	1	5

(II) PET Inhibitory Activity against *Chenopodium* Thylakoids

compound	pI_{50}(*Chenopodium*)[c] resistant type	pI_{50}(*Chenopodium*)[c] wild type	I_{50} ratio R/W
3	7.43	7.34	0.8
atrazine	4.21	6.72	324

[a] Formulation, 10% WP; evaluation, after 12 days. [b] Herbicidal activities were visually evaluated by the following rating score: to *Chenopodium*, 5, 91−100% control (complete kill); 4, 76−90% control; 3, 51−75% control; 2, 26−50% control; 1, 1−25% control. [c] pI_{50}(*Chenopodium*) = −log I_{50}(*Chenopodium*): the negative logarithms of the molar concentration of the compounds that produce a 50% inhibition.

The order of PET inhibition of the 1,3,5-triazine (**1**−**5**) with spinach thylakoids was as follows (see Table 1): triazine (**1**) (pI_{50} = 7.21) > triazine (**2**) (pI_{50} = 6.98) > triazine (**3**) (pI_{50} = 6.94) > triazine (**4**) (pI_{50} = 6.85) > triazine (**5**) (pI_{50} = 5.95). This finding correlates the binding of the 1,3,5-triazine with the PET inhibitory activity of the 1,3,5-triazines.

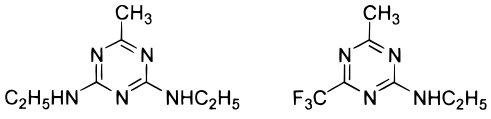
$$pI_{50}(\text{spinach}) = -0.864pK_i + 12.649$$
$$(n = 5,\ r = 0.944,\ s = 0.185)$$

Binding Partners of the Novel 1,3,5-Triazines. It is not yet clear from the results mentioned above whether binding partners of the novel 1,3,5-triazines tested are identical with those of atrazine. To clarify this problem, we examined the postemergence phytotoxic activity of 1,3,5-triazine **3**, with strong herbicidal activity and high PET inhibition, in greenhouse pot tests using atrazine-resistant (>100-fold resistant versus the wild-type) *C. album* on which Pfister and Arntzen (1979) and its wild-type (Koizumi et al., 1998). As shown in Table 3I, triazine **3** exhibited a strong herbicidal activity with a comparatively low dose (25 g of ai/1000 m^2) not only against wild-type *Chenopodium* but also against atrazine-resistant *Chenopodium*. In contrast, atrazine-resistant *Chenopodium* could not be killed by atrazine even with a dose far exceeding a use rate of 3200 g of ai/1000 m^2, although atrazine (25 g of ai/1000 m^2) completely killed wild-type *Chenopodium*. Thus, triazine **3** does not indicate a cross-resistance against atrazine-resistant *Chenopodium*.

Furthermore, the PET inhibitory activities of the novel 1,3,5-triazine compound **3** and atrazine were evaluated by using thylakoids isolated from wild-type and atrazine-resistant *Chenopodium* (Table 3II). The atrazine-resistant *Chenopodium* was ∼300-fold resistant to atrazine versus the wild-type [atrazine pI_{50}(wild-type *Chenopodium*) = 6.72 and pI_{50}(atrazine-resistant *Chenopodium*) = 4.21]. Triazine **3** showed a strong PET inhibitory activity against both wild-type and atrazine-resistant mutant [pI_{50}(wild-type *Chenopodium*) = 7.34 and pI_{50}(atrazine-resistant *Chenopodium*) = 7.43 respectively], indicating lack of cross-resistance of atrazine-resistant biotypes to the compound [the I_{50} ratio of triazine (**3** (atrazine-resistant *Chenopodium*)/I_{50}(wild-type *Chenopodium*) is ∼0.8]. Also, the PET inhibitory activity of triazine **3** was found to be ∼1000 times stronger than that of the atrazine-resistant form. Both pot test and PET inhibition reveal the absence of cross-resistance between atrazine and the 1,3,5-triazines. This implies that the latter do not interact with serine 264, a crucial amino acid of the D1-protein that interacts with atrazine. Change of this amino acid residue to glycine causes resistance to atrazine in the *Chenopodium* mutant (Hirschberg et al., 1984). In the D1-protein binding niche additional binding partners for the 1,3,5-triazines may be shared with those for atrazine. This can be clarified only with other mutants in which the respective amino acid residues have been altered.

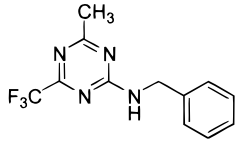
It seems a little surprising that no monoamino-1,3,5-triazines have been found active since the original discovery of herbicidal activity of bis(alkylamino)-1,3,5-triazines by Gysin and Knüsli (1954). Indeed, quantitative structure−activity correlations (QSAR) as introduced by Hansch and Fujita (1964) have been carried out only with bis(amino)-1,3,5-triazines (Gabbott, 1969; Soskic et al., 1989; Shimizu et al., 1988; Morita et al., 1987; Omokawa et al., 1989). These authors never tried an extrapolation of alkylamino to other functional groups.

In the beginning of this study, replacement of the ethyl group to the trifluoromethyl group for Gysin's original 2,4-diethylamino-6-methyl-1,3,5-triazine [pI_{50}(spinach) = 4.75] gave rather less active PET inhibition



pI_{50}(spinach)=4.75

pI_{50}(spinach)=4.69



pI_{50}(spinach)=6.85

[pI_{50}(spinach) = 4.69] (Ohki et al., 1997; Ohki, 1997). However, further substitution of the ethylamino group of Gysin's compound to a benzylamino substituent surprisingly yielded a PET inhibition ∼100 times stronger [compound **4** pI_{50}(spinach) = 6.85] compared with the that of the original lead. Furthermore, introduction of electron-negative group(s) at the benzene ring apparently brings about more active PET inhibition than the compound **4**. QSAR studies on the novel monoamino-1,3,5-triazines are under way in our laboratories.

ACKNOWLEDGMENT

We are indebted to Dr. Hiroyuki Watanabe, Mitsubishi Chemical Industries, Ltd., M.Sc. Shinpei Ohki, Tamagawa University, and Dr. J. J. S. van Rensen, Wageningen Agricultural University.

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Received for review March 8, 1999. Revised manuscript received July 26, 1999. Accepted August 2, 1999. This work was supported by the Monbusho International Scientific Research Program: University-to-University Cooperation Research from the Ministry of Education, Science, Sports and Culture of Japan.

JF990216O