Original Article

Characterization of very long chain fatty acid synthesis inhibition by ipfencarbazone

Tatsuya Kasahara,1,* Hiroshi Matsumoto,2 Hisakazu Hasegawa,3 Kohei Koyama3 and Takashi Takeuchi1

1 Hokko Chemical Industry Co., Ltd., 1–5–4 Nihonbashi Honcho, Chuo-ku, Tokyo 103–8341, Japan
2 Faculty of Life and Environmental Sciences, University of Tsukuba, 1–1 Tennodai, Tsukuba, Ibaraki 305–8572, Japan
3 Central Research Laboratories, Hokko Chemical Industry Co., Ltd., 2165 Toda, Atsugi-shi, Kanagawa 243–0023, Japan

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Ipfencarbazone exhibits excellent herbicidal activity against *Echinochloa* spp. and is safe for rice. The effects of ipfencarbazone on very long chain fatty acid (VLCFA) elongation in rice and late watergrass and its inhibitory mechanism were investigated in this study. Although ipfencarbazone inhibited VLCFA elongation in the microsomes prepared from late watergrass and rice at low concentrations, the inhibitory effect was higher in late watergrass than in rice. These results suggested that the primary site of action of ipfencarbazone is VLCFA elongase (VLCFAE) and ipfencarbazone has a differential affinity between the VLCFAEs of the plants. The inhibitory activity of ipfencarbazone became higher in proportion to pre-incubation period with the VLCFAE. The degree of inhibition did not decrease by dilution of the VLCFAE–ipfencarbazone complex. These results suggested that ipfencarbazone binds to the VLCFAE irreversibly.

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Keywords: ipfencarbazone, mode of action, very long chain fatty acid, selectivity, rice, late watergrass.

Electronic supplementary material: The online version of this article contains supplementary material (Supplemental Table S1), which is available at http://www.jstage.jst.go.jp/browse/jpestics/

Introduction

Late watergrass (*Echinochloa oryzicola* Vasing.) is one of the most troublesome weeds in paddy rice cultivation, and herbicides used on paddy rice must have high herbicidal activity against late watergrass but also be safe for rice. Ipfencarbazone (1-(2,4-dichlorophenyl)-2′,4′-difluoro-1,5-dihydro-N-isopropyl-5-oxo-4H-1,2,4-triazole-4-carboxanilide) is a new herbicide developed by Hokko Chemical Industry Co., Ltd. (Fig. 1). Ipfencarbazone has been reported to exhibit high herbicidal activity against *Echinochloa* spp. from pre-emergence to the 3-leaf stage while being safe for transplanted rice at a dose of 250 g a.i./ha.1,2) Symptoms of ipfencarbazone-treated *Echinochloa* are a reduction in the growth and number of tillers accompanied by a darkening green color and the twisting and unfurling of leaves.3) These symptoms are similar to those observed in plants treated with chloroacetamide herbicides, which inhibit very long chain fatty acid (VLCFA) elongation. Kondo et al. reported that ipfencarbazone decreased the content of fatty acids consisting of more than 20 carbon atoms in late watergrass (*E. oryzicola*).4) Since the structure of ipfencarbazone is similar to that of fentrazamide, which inhibits VLCFA elongation, the target site of ipfencarbazone is assumed to be the VLCFA elongation. However, neither the effect of ipfencarbazone on VLCFA elongation nor its inhibition mechanism has been sufficiently analyzed.

In this study, to further elucidate the inhibition mechanism of ipfencarbazone, its effect on VLCFA elongation in rice and late watergrass and its inhibitory mechanism of the VLCFA elongase (VLCFAE) were investigated.

Materials and Methods

1. Chemicals

Technical grade ipfencarbazone (purity 99.5%) was supplied by Hokko Chemical Industry Co., Ltd. (Tokyo, Japan). Stearoyl-CoA (C18:0) and arachidoyl-CoA (C20:0) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and [2-14C] malonyl-CoA

Fig. 1. Structure of ipfencarbazone
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(2.035 GBq/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). The other chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) except for those noted separately. The herbicides used in the study were dissolved in acetone to produce a stock solution (10⁻² M) and diluted with 10% acetone containing 1% dimethyl sulfoxide (DMSO) to the desired concentration just before use in the experiments.

2. Plant materials
Rice (Oryza sativa cv. Nipponbare) and late watergrass (E. oryzicola Vasing.) were used as test plants. Rice seeds were sterilized by soaking in 70% (v/v) ethanol solution for 5 min and then in 10% (v/v) hydrogen peroxide for 10 min. These seeds were then soaked in distilled water and germinated in the dark for 2 days at 30°C in an incubator. Late watergrass seeds were immersed in distilled water, and the seeds were allowed to absorb it under reduced pressure for 10 min. Thereafter, they were germinated for 2 days in an incubator at 30°C.

3. Effect of ipfencarbazone on the growth of plants in a hydroponic culture
The germinated seeds of late watergrass and rice were sown on a stainless mesh and grown up to the 2-leaf stage in 1/2 strength modified Kasugai nutrient solution⁶ in a growth chamber at 25°C/20°C (day/night cycle, 12 hr each). The composition of the nutrient solution is shown in Supplemental Table S1. The roots and base of the coleoptiles of these plants (ca. 1 cm) were immersed for 48 hr in a nutrient solution containing zero (untreated control), 0.005, 0.05, 0.5, or 5 µM ipfencarbazone. The final concentration of acetone in each solution was adjusted to 0.1%. After washing the root and base of the coleoptile with distilled water, the plants were grown in a nutrient solution without herbicide in the growth chamber. Root and leaf lengths were measured 5, 10, and 15 days after treatment, and the fresh weight (FW) was measured 15 days after treatment. The 50% effective concentrations (EC₅₀) for FWs of the plants were determined via probit analysis. The experiment was conducted in triplicate for each concentration.

4. Effect of ipfencarbazone on fatty acid elongation
Microsome preparation and the determination of fatty acid elongation were performed using a modification of the method of Takahashi et al.⁷ Germinated rice and late watergrass were sown on a stainless mesh and grown up to the 2-leaf stage in nutrient solution in the dark at 25°C/20°C (temperature cycle, 12 hr each). Etiolated rice and late watergrass seedlings (4 g FW) were ground using a polytron homogenizer in 40 mL of 100 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES)/KOH buffer (pH 7.2) containing 320 mM sucrose, 10 mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (diopotassium salt), 0.3 mM phenylmethylsulfonyl fluoride, and 1 g (25% of plant sample) of polyvinylpolypyrrolidone. The homogenate was filtered through three layers of Miracloth, and
the filtrate was centrifuged at 10,000g for 20 min using an SRX-201 centrifuge (TOMY SEIKO Co., Ltd., Tokyo, Japan). The supernatant was then centrifuged at 100,000g for 60 min using an LP-70K ultracentrifuge (Beckman Coulter Inc., CA, USA). The pelleted microsomal fraction obtained was resuspended in 2 mL of 100 mM HEPES/KOH buffer (pH 7.2) containing 0.1% Triton X-100, 1 mM DTT, and 1 mM MgCl₂. All preparation procedures were conducted at 4°C or on ice. The protein content of the microsome suspension was determined using a Bradford assay with bovine serum albumin as a standard. The microsomal suspensions were stored at −80°C until assay.

VLCFAE activity was determined by monitoring the incorporation of [2-14C] malonyl-CoA into the acyl-CoA. The assay solution contained 30 μL of microsomal suspension, 3 μL of 1, 10, 100, or 1000 mM herbicide, 5 μL of 20 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 5 μL of 20 mM reduced nicotinamide adenine dinucleotide (NADH), 5 μL of 2 mM stearoyl-CoA or arachidoyl-CoA, 5 μL of 0.2 mM [2-14C] malonyl-CoA and 7 μL of 100 mM HEPES-KOH buffer (pH 7.2). For the control, 3 μL of 10% acetone containing 0.1% DMSO was used instead of herbicide. The assay solution was pre-incubated at 30°C for 10 min before initiating the reaction by the addition of [2-14C] malonyl-CoA. The reaction was conducted for 30 min at 30°C and then terminated by adding 40 μL of 60% KOH. Reaction products were saponified for 30 min at 80°C and acidified with 280 μL of 6% HCl. The labeled fatty acids were extracted with 1080 μL of acetone/n-hexane (4:3, v/v) and the organic phase was washed with 200 μL of 6% HCl to remove non-reacted [2-14C] malonyl-CoA. The radioactivity in the organic phase was measured by a liquid scintillation counter (AccuFLEX LSC-7200, Hitachi Aloka Medical, Ltd., Tokyo, Japan). The rates of inhibition of VLCFA elongation were determined by comparing the amounts of incorporated labeled carbon with that of the control. The 50% inhibitory concentration (IC₅₀) for VLCFA elongation was determined via probit analysis. In addition, to analyze the inhibitory mechanism of ipfencarbazone, the inhibitory effect of ipfencarbazone on VLCFA elongation was determined after different pre-incubation periods (0 to 45 min).

5. Effect of enzyme–inhibitor complex dilution on the inhibition of VLCFA elongation

To obtain more information on the mechanism of ipfencarbazone binding to the VLCFAE, the effect of dilution of microsome–ipfencarbazone complex on inhibitory activity was evaluated. The dilution experiment was performed using a modification of the method described by Schmalfuß et al. A 60 μL microsomal suspension was incubated at 30°C for 10 min after adding 6 μL of zero or 10 mM ipfencarbazone solution. Then this mixture was divided into two equal aliquots, and one was diluted with 297 μL of 100 mM HEPES/KOH buffer (pH 7.2) containing 0.1% Triton X-100, 1 mM DTT, and 1 mM MgCl₂. After a further incubation at 30°C for 10 min, VLCFA elongation was initiated by adding the co-enzymes and substrates (arachidoyl-CoA) to 33 μL of the non-diluted and diluted solutions. The reaction was incubated for 20, 30, or 45 min at 30°C. The enzyme concentration in the dilution samples would be lower; therefore, to reduce of the reaction velocity, the reaction was conducted

| Herbicide concentration (μM) | Inhibition of VLCFA elongation (%) |
|------------------------------|----------------------------------|
| 0.05                         |                                  |
| 0.5                          |                                  |
| 5                            |                                  |
| 50                           |                                  |

Table 1. Inhibition of VLCFA elongation by ipfencarbazone

|                | C18:0 to C20:0 | C20:0 to C22:0 |
|----------------|----------------|----------------|
| IC₅₀ (μM)      |                |                |
| ORYSA          | 0.65           | 0.39           |
| ECHOR          | 0.21           | 0.06           |
| Ratio (ORYSA/ECHOR) | 3.16           | 6.88           |

a) ORYSA, Oryza sativa cv. Nipponbare, b) ECHOR, Echinochloa oryzicola Vasing.
for 20, 30, or 45 min at 30°C. After the termination of elongation, the extraction of labeled fatty acid and the measurement of its radioactivity were conducted according to the methods described above.

6. Statistical analysis
Statistical significance was determined using SPSS Statistics Version 24.0 (IBM Japan Corp., Tokyo, Japan). Tukey's test was conducted, and differences were considered significant at $p < 0.05$.

Results
1. Effect of ipfencarbazone on plant growth
Ipfencarbazone significantly inhibited the growth of the third leaf and completely suppressed the emergence of the fourth leaf of late watergrass at 0.5 µM or higher (Fig. 2Aa). Although ipfencarbazone significantly inhibited growth of the third and fourth leaves of rice at 5 µM, it did not affect fourth leaf emergence at any concentration (Fig. 2Ba). On the other hand, ipfencarbazone had only a slight inhibitory effect on the root growth of late watergrass and rice at all concentrations (Fig. 2Ab, Bb). As with leaf growth inhibition, ipfencarbazone decreased the FW of late watergrass more drastically than that of rice. The FW inhibition of late watergrass by ipfencarbazone was statistically significant at 0.05 µM or higher, whereas that of rice was statistically significant at 0.5 µM or higher (Fig. 2C). The $EC_{50}$ for FW in late watergrass and rice calculated using the probit analysis was 0.16 µM and 2.12 µM, respectively.

2. Effect of ipfencarbazone on fatty acid elongation
Ipfencarbazone inhibited VLCFA elongation (C18:0 to C20:0 and C20:0 to C22:0) in late watergrass microsomes in a concentration-dependent manner similar to cafenstrole, which is a known VLCFAE inhibitor6) (Fig. 3A, B). Furthermore, both herbicides inhibited elongation in late watergrass more strongly than in rice. More specifically, there was a significant difference in the inhibition of C18:0 to C20:0 elongation by ipfencarbazone between late watergrass and rice at 0.05 and 0.5 µM. The $IC_{50}$ for the elongation of late watergrass was approximately 3 times lower than that of rice (Table 1). Similarly, there was a significant difference in the inhibition of C20:0 to C22:0 elongation by ipfencarbazone between late watergrass and rice at all concentrations tested. The $IC_{50}$ for late watergrass was approximately 7 times lower than that for rice (Table 1).

Ipfencarbazone inhibition of VLCFA elongation tended to become stronger with the pre-incubation period (Fig. 4). Furthermore, the inhibitory activity of VLCFA elongation in ipfencarbazone–microsome complexes diluted 10 times with a buffer was nearly the same as that in the undiluted complex (Fig. 5).

Discussion
Previous studies suggested that ipfencarbazone exhibits positional selectivity between rice and late watergrass by forming...
a herbicide-treated layer on the soil surface regardless of soil texture or rate of water leakage. In this study, it was shown that ipfencarbazone strongly inhibits the growth of the third and fourth leaves in late watergrass but not in rice in a hydroponic culture (Fig. 2Aa, Ba). In addition, the EC₅₀ for the FW inhibition in late watergrass was approximately 13 times lower than that in rice. These results suggested that ipfencarbazone exhibits physiological and/or biochemical selectivity between rice and late watergrass.

Ipfencarbazone inhibited the incorporation of [2,14C] malonyl-CoA into stearoyl-CoA (C18:0) and arachidoyl-CoA (C20:0) in rice and late watergrass microsomes at a low concentration, similar to cafenstrole, a known VLCFAE inhibitor (Fig. 3A, B). Therefore, the primary site of action of ipfencarbazone was considered to be VLCFAE. Since the inhibitory activity of ipfencarbazone on VLCFA elongation (C18:0 to C20:0 and C20:0 to C22:0) was different between late watergrass and rice, the difference in sensitivity between rice and late watergrass observed in the hydroponic culture might be partially due to the differential affinity of ipfencarbazone between the VLCFAEs of the plants.

In higher plants, multiple isoforms of VLCFAE are located in the endoplasmic reticulum, and each of these VLCFAE catalyzes a different process of the VLCFA elongation. Furthermore, Trenkamp et al. reported that the target isoforms of VLCFAE are different among VLCFAE-inhibiting herbicides. Tanetani et al. suggested that the inhibition of VLCFA elongation is the sum of inhibitory activities for each VLCFAE in the endoplasmic reticulum. In this study, ipfencarbazone exhibited selectivity between rice and late watergrass at the target site level. Therefore, it was considered that the ipfencarbazone selectivity could be analyzed in more detail by evaluating its effect on each VLCFAE individually.

The inhibition mechanism of herbicides that inhibit VLCFAEs is still a matter of debate. Generally, the conventional VLCFAE-inhibiting herbicides are assumed to show irreversible inhibition by covalent binding with the thiol group of cysteine residue in the active site of the VLCFAE. In the irreversible inhibition, as the preincubation time of herbicides with enzymes increases, its inhibitory activity increases (time-dependent inhibition). However, Tanetani et al. reported that conventional VLCFAE-inhibiting herbicides exhibited time-independent inhibition against the VLCFAE in Italian ryegrass and suggested that the mode of inhibition of these herbicides is different between Arabidopsis thaliana and Italian ryegrass. Therefore, we investigated the mode of inhibition of ipfencarbazone against late watergrass and rice. VLCFAE inhibition by ipfencarbazone tended to increase with the pre-incubation period in late watergrass and rice (Fig. 4). Furthermore, the inhibitory activity of diluted microsome–ipfencarbazone complexes did not change (Fig. 5). These results suggest that ipfencarbazone inhibition of late watergrass and rice VLCFAEs is irreversible, as in the case with conventional VLCFAE-inhibiting herbicides. This irreversible inhibition might be due to highly electrophilic carbamoyl carbon in the structure of ipfencarbazone. Furthermore, the differential susceptibility between late watergrass and rice was not due to differences in the inhibition mechanism.

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References

1) T. Kido, H. Okita, M. Okamura, T. Takeuchi and K. Morita: J. Pestic. Sci. 41, 113–119 (2016).
2) M. Okamura, S. Kondo, Y. Honma, T. Takeuchi, I. Taketomi, T. Kido, H. Okita and K. Morita: Abstr. 33rd Annu. Meeting Pestic. Sci. Soc. Jpn., 104 (2008) (in Japanese).
3) T. Takeuchi: JAPR Journal 48, 132–137 (2014), (in Japanese).
4) S. Kondo, T. Kido, I. Nishida and H. Ohta: Abstr. 38th Annu. Meeting Pestic. Sci. Soc. Jpn., 104 (2013) (in Japanese).
5) Y. Ohta, K. Yamamoto and M. Deguchi: J. Soil Sci. Plant Nutr. 41, 19–26 (1970), (in Japanese).
6) H. Takahashi, A. Ohki, M. Kanzaki, A. Tanaka, Y. Sato, B. Matthes, P. Böger and K. Wakabayashi: Z. Naturforsch. 56c, 781–786 (2001).
7) J. Schmalfuß, B. Matthes, K. Knuth and P. Böger: Pestic. Biochem. Physiol. 67, 25–35 (2000).
8) T. Kasahara, T. Takeuchi, K. Koyama and S. Kuzuma: J. Pestic. Sci. 43, 255–260 (2018).
9) S. Trenkamp, W. Martin and K. Tietjen: Proc. Natl. Acad. Sci. U.S.A. 101, 11903–11908 (2004).
10) C. Lechelt-Kunze, R. C. Meissner, M. Drewes and K. Tietje: Pest Manag. Sci. 59, 847–856 (2003).
11) B. J. Blacklock and J. G. Jaworski: Biochem. Biophys. Res. Commun. 346, 583–590 (2006).
12) Y. Tanetani, K. Kaku, K. Kawai, T. Fujioka and T. Shimizu: Pestic. Biochem. Physiol. 95, 47–55 (2009).
13) P. Böger, B. Matthes and J. Schmalfuß: Pest Manag. Sci. 56, 497–508 (2000).
14) C. Eckermann, B. Matthes, M. Nimtz, V. Reiser, B. Lederer, P. Böger and J. Schröder: Phytochemistry 64, 1045–1054 (2003).
15) P. Böger: J. Pestic. Sci. 28, 324–329 (2003).
16) Y. Tanetani, T. Fujioka, J. Horita, K. Kaku and T. Shimizu: J. Pestic. Sci. 36, 357–362 (2011).
17) Y. Tanetani, T. Fujioka, K. Kaku and T. Shimizu: Regul. Plant Growth Dev. 47, 120–126 (2012) (in Japanese).