J. Pesticide Sci. **27**, 141–144 (2002)

Note

Differential Inhibition of a Melanin Biosynthetic Enzyme Scytalone Dehydratase by Carpropamid, a Fungicide for Rice Blast Control, and Its Isomers

Takayuki MOTOYAMA, Shinzo KAGABU,† Masashi MATSUOKA† and Isamu YAMAGUCHI*

RIKEN (The Institute of Physical and Chemical Research) and RIKEN Plant Science Center, Saitama 351-0198, Japan
† *Department of Chemistry, Faculty of Education, Gifu University, Gifu 501-1193, Japan*

(Received October 15, 2001; Accepted December 14, 2001)

Key words: rice blast fungus *Pyricularia oryzae*, scytalone dehydratase, melanin biosynthesis, carpropamid isomer, tight-binding inhibitor.

INTRODUCTION

Carpropamid (1*RS*,3*SR*)-2,2-dichloro-*N*-[1-(4-chlorophenyl)ethyl]-1-ethyl-3-methylcyclopropanecarboxamide) is a potent agent against rice blast disease caused by *Pyricularia oryzae* (teleomorph: *Magnaporthe grisea*). The primary target of this fungicide is scytalone dehydratase (SD) in the melanin biosynthetic pathway of the rice blast fungus.[1–3] Carpropamid consists of four isomers, KTU3616A (1*R*, 3*S*, 1′*R*), KTU3616B (1*S*, 3*R*, 1′*R*), KTU3615A (1*S*, 3*R*, 1′*S*), and KTU3615B (1*R*, 3*S*, 1′*S*) (Fig. 1). An analysis of enzyme kinetics revealed that KTU3616B can inhibit SD at very low concentrations close to the enzyme concentration.[3] This finding suggested that KTU3616B is a tight-binding inhibitor. We discuss the relation between the inhibition strength and the stereomers, and determined the inhibition type by kinetic analysis.

MATERIALS AND METHODS

1. *Synthesis*

1.1. *Resolution of racemic 1-(4-chlorophenyl)ethylamine*

To a solution of 1-(4-chlorophenyl)ethylamine (78.33 g, 0.5 mol) in 500 ml of reagent-grade methanol was added (+)-tartaric acid (77.94 g, 0.5 mol) at 40°C in small portions over the course of 2 hr. After standing 48 hr at room temperature, the precipitated crops were filtered. The diastereomeric mixture of ammonium salts was subjected to repeated recrystallization from methanol until each fraction showed a constant specific optical rotation. The separated salt was treated with 20% aqueous NaOH and the liberated amine was extracted with ether. Drying the ether solution over KOH pellets, evaporating the ether and subsequent distillation of the residue at 130–140°C/20 mm Hg afforded the enantiomeric amine. Amine (A); yield 5.99 g (7.7%), $[\alpha]_D^{25}$ +22.3 (c=2.0, MeOH). Amine (B); yield 4.95 g (6.3%), $[\alpha]_D^{25}$ −20.4 (c=2.0, MeOH). The absolute configurations *R* and *S* were determined for amine (A) and (B), respectively by X-ray analysis of the product from amine (A).[4,5]

1.2 *Preparation and separation of a diastereomer mixture of KTU3616A and KTU3616B and KTU3615A and KTU3615B.*

To a solution of *R*-amine (1.57 g, 0.01 mol) and triethylamine (1.20 g, 0.01 mol) in 30 ml of toluene was added a solution of (1*RS*, 3*SR*)-2,2-dichloro-1-ethyl-3-methylcyclopropanecarbonyl chloride (2.15 g, 0.01 mol) in 15 ml of toluene dropwise over the course of 30 min. The reaction mixture was stirred for 9 hr at room temperature and subsequently at 40°C for an additional 1 hr. The precipitates were filtered off and washed with 30 ml of toluene. The combined filtrate was washed successively with 3% aqueous HCl, 3% aqueous NaOH and brine and dried over $MgSO_4$. The mixture was subjected to preparative TLC (SiO_2) with hexane/ethyl acetate (5: 1). Repeated TLC separated two components, each of which was recrystallized from methanol, and the purity was confirmed by HPLC. The component from the lower zone was confirmed as KTU3616B by X-ray analysis,[4,5] consequently the upper component should be KTU3616A. KTU3616A: yield 60 mg (1.8%), mp 135°C, $[\alpha]_D^{25}$ +40.5 (c=1.00, MeOH), ^{1}H NMR $\delta_{TMS}^{CDCl_3}$: 0.89 (3H, t, J=7.2 Hz, $CH_2\underline{CH_3}$), 1.19 (3H, d, J=6.6 Hz, cyclopropyl-$\underline{CH_3}$), 1.52 (3H, d, J=6.9 Hz, NHCH$\underline{CH_3}$), 1.55 (1H, dq, J=14.6/7.3 Hz, CH_{2a}), 1.91 (1H, dq, J=14.6/7.3 Hz, CH_{2b}), 2.21 (1H, q, J=6.6 Hz, cyclopropyl $\underline{H}$), 5.16 (1H, dd, J=7.3/6.9 Hz, NH$\underline{CH}$CH$_3$), 5.87 (1H, d, J=7.3 Hz, $\underline{NH}$), 7.26–7.34 (5H, m, phenyl). KTU3616B: yield 50 mg (1.5%), mp136 C, $[\alpha]_D^{25}$ +87.9 (c=1.00, MeOH), ^{1}H NMR $\delta_{TMS}^{CDCl_3}$: 0.99 (3H, t, J=7.3 Hz, $CH_2\underline{CH_3}$), 1.20 (3H, d, J=6.6 Hz, cyclopropyl-CH_3), 1.52 (3H, d, J=6.8 Hz, NHCH$\underline{CH_3}$), 1.57 (1H, dq, J=15.0/7.3 Hz, CH_{2a}), 1.94 (1H, dq, J=15.0, 7.3 Hz, CH_{2b}), 2.20 (1H, J=6.6 Hz, cyclopropyl $\underline{H}$), 5.17 (1H, dq, J=7.3/6.8 Hz, NH$\underline{CH}$CH$_3$), 5.88 (1H, d, J=7.3 Hz, $\underline{NH}$), 7.26–7.36 (5H, m, phenyl). KTU 3615A and 3615B were prepared and separated similarly. KTU3615A: mp 135°C, $[\alpha]_D^{25}$ −44.6 (c=0.20, MeOH). KTU3615B: mp 136°C, $[\alpha]_D^{25}$ −90.0 (c=0.20, MeOH). KTU3616, KTU3615, and NTN33853 are mixtures of these isomers (Fig. 1).

2. *SD Assay and Determination of the K_i Value for Chemicals*

Recombinant SD was prepared as described previously.[6] Scytalone was prepared from the agar culture of *Verticillium*

* To whom correspondence should be addressed.
E-mail: isyama@postman.riken.go.jp

dahliae strain *Brm-1* or from the cultured broth of carpropamid-treated *P. oryzae* strain P2 grown in YGPCa medium as described previously.[2] The activity of SD was measured with a Shimadzu UV-recording spectrophotometer (model UV-2500PC) at 30°C in 20 mM TES buffer (pH7.0) based on the decrease of UV absorption at 282 nm caused by a change of scytalone to 1,3,8-trihydroxynaphthalene. The reaction rates were calculated from the tangents of the progress curves at the initial stage of the reaction. $\Delta\varepsilon_{282}$ was $-7.9\ mM^{-1}cm^{-1}$ under these conditions. Concentrations of ethanol, used to dissolve scytalone and the chemicals, were less than 0.5% in all the reactions.

K_i values of all the isomers except KTU3615B were determined by fitting the enzymatic activity data to the following equation for competitive inhibitors:

$$v = V_{max}S/(S + K_m(1 + I/K_i))$$

where v is the velocity of the catalysis, V_{max} the maximum velocity, S the initial concentration of the substrate, K_m the Michaelis constant, and I the concentration of the inhibitor.

The K_i value of KTU3615B was determined by fitting the enzymatic activity data to the following equation for non-competitive inhibitors:

$$v = (V_{max}S/(1 + I/K_i))/(S + K_m)$$

where v is the velocity of catalysis, V_{max} the maximum velocity, S the initial concentration of the substrate, K_m the Michaelis constant, and I the concentration of the inhibitor.

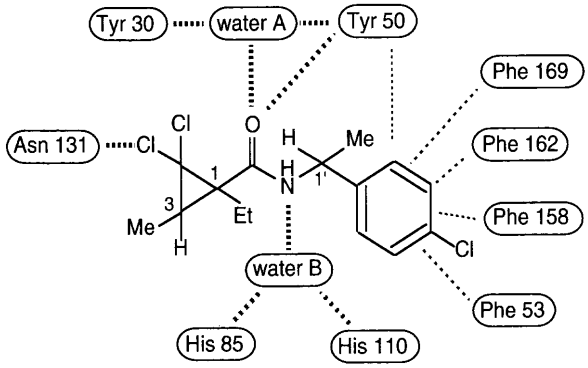
RESULTS AND DISCUSSION

1. *Chemicals*

Carpropamid was prepared based on the patent description.[7] Though carpropamid has three asymmetric carbon atoms, four diastereomers exist because the dichlorocyclopropane part is constructed from (*E*)-2-ethylcrotonate. We separated the stereoisomers by exhaustive TLC. The absolute configuration of each isomer was determined by referring to the X-ray analysis of the 1*S*,3*R*,1'*R*-enantiomer (KTU3616B)[4,5] and the corresponding specific rotations.

2. *Inhibition of SD*

We first analyzed the inhibition of SD by carpropamid-isomers (see Fig. 1 for structures) at 1 nM in reaction mixtures containing 1 nM SD and 40 μM scytalone (Table 1). KTU3616B, KTU3616, and NTN33853 significantly inhibited SD. No inhibition was observed by KTU3616A, KTU3615A, KTU3615B, KTU3615, and NTN36945. This suggests that KTU3616B alone is a tight-binding inhibitor among the eight isomers of carpropamid. On using a 50-fold higher concentration of inhibitor (50 nM) than in the initial experiment, we observed significant inhibition by KTU3616A, KTU3615A, KTU3615B, KTU3615, and NTN36945 (Table 1), indicating that KTU3616A, KTU3615A, KTU3615B, and some component of NTN36945 have considerable inhibitory activity against SD.



KTU3616A (1*R*, 3*S*, 1'*R*)
KTU3616B (1*S*, 3*R*, 1'*R*)
KTU3615A (1*S*, 3*R*, 1'*S*)
KTU3615B (1*R*, 3*S*, 1'*S*)
KTU3616 KTU3616A + KTU3616B
KTU3615 KTU3615A + KTU3615B
NTN33853 KTU3616A + KTU3616B+KTU3615A + KTU3615B
NTN36945 (1*R*, 3*R*, 1'R) + (1*S*, 3*S*, 1'R) + (1*R*, 3*R*, 1'S) + (1*S*, 3*S*, 1'S)

Fig. 1 Carpropamid, its isomers, and mixtures of isomers used in the present study. The major elements for the tight-binding inhibition of SD by carpropamid (KTU3616B) are also shown. Hydrogen bonds and van der Waals interactions are shown with thick and thin dotted lines, respectively.

Table 1 Inhibition of SD by isomers of carpropamid.

reagents	concentrations (nM)	activity (% of that without reagents)[a]
KTU3616A	50	31
KTU3616B	1	40
KTU3615A	50	54
KTU3615B	50	50
KTU3616	1	65
KTU3615	50	55
NTN33853	1	88
NTN36945	50	35

[a] 1 nM SD, 40 μM scytalone, 20 mM TES (pH 7.0), 30°C.

Table 2 Enzyme kinetics of the Inhibition of SD by isomers of carpropamid.

reagents	type of inhibition	K_i (nM)
KTU3616A	competitive	11±1
KTU3616B	competitive	0.10±0.01
KTU3615A	competitive	24±2
KTU3615B	not determined	>46±4

A Lineweaver-Burk plot of the SD activity in the presence of isomers showed that KTU3616A, KTU3616B, and KTU3615A are competitive inhibitors and that KTU3615B is

Table 3 Evaluation of contamination by tight-binding-inhibitors in the synthesized reagents.

reagents	concentrations (nM)	remaining activity (% of that without reagents)[a]				
		enzyme (nM)				
		0.5	1.0	2.0	4.0	8.0
KTU3616A	50	NT	31	NT	33	32
KTU3616B	1	34	40	57	78	88
KTU3615A	60	52	50	49	52	53
KTU3615B	50	NT	50	NT	81	88
NTN36945	50	NT	35	NT	37	39

[a] NT: not tested. 40 μM scytalone, 20 mM TES (pH 7.0), 30°C.

a noncompetitive inhibitor (data not shown). So, we determined K_i values for KTU3616A, KTU3616B, and KTU3615A by fitting the enzymatic activity data to the equation for competitive inhibitors. The K_i value for KTU3615B was calculated by fitting the activity data to the equation for noncompetitive inhibitors. The determined K_i values for KTU3616A, KTU3615A, and KTU3615B were more than 100-fold larger than the value for KTU3616B (Table 2).

Enzyme kinetic analysis showed that KTU3615B is a noncompetitive inhibitor. However, this finding is difficult to accept because all the other isomers are competitive inhibitors. In inappropriate enzyme kinetic experiments, where the concentrations of enzymes and inhibitors are similar, competitive tight-binding inhibitors often behave as if they are noncompetitive inhibitors in a Lineweaver-Burk plot.[8] In the present case, the kinetics data are valid if the KTU3615B preparation was pure, because a 50-fold excess of KTU3615B over the enzyme was used. However, if small amounts of tight-binding inhibitors such as KTU3616B contaminated the KTU3615B preparation, it could mislead to the conclusion that KTU3615B is a noncompetitive inhibitor. If the KTU3615B preparation was contaminated with a small amount of tight-binding inhibitors, the efficiency of inhibition should decrease when the enzyme concentrations are elevated because free tight-binding inhibitors can easily be removed from the solution by binding to the target enzymes. To clarify this point, we analyzed the dependence of the inhibition efficiency of KTU3616A, KTU3616B, KTU3615A, KTU3615B, and NTN36945 on enzyme concentrations (Table 3). The tight-binding inhibitor KTU3616B showed a clear dependence of inhibition efficacy on the enzyme concentrations as expected. KTU3616A, KTU3615A, and NTN36945 showed no such dependence. These findings suggest that the inhibition by KTU3616A, KTU3615A, and NTN36945 was not brought about by a contaminated tight-binding inhibitor. In contrast, the inhibition of SD by KTU3615B was dependent on enzyme concentration, indicating that the KTU3615B preparation was contaminated with a small amount of a tight-binding inhibitor, probably KTU3616B, and that the true K_i value for KTU3615B is larger than the determined value (Table 2). In summary, KTU3616B, a tight-binding competitive inhibitor, is one of the strongest small molecule inhibitors, while KTU3616A, KTU3615A, and KTU3615B showed more than 100-fold larger K_i values against SD than did KTU3616B, *i.e.* they are not tight-binding inhibitors.

A comparison of *in vivo* anti-blast efficacy between KTU3616B and KTU3616 showed that the two preparations have similar abilities to control the rice blast disease,[9] suggesting that KTU3616A and KTU3616B have a synergistic effect in rice blast control. However, the present *in vitro* data suggested that KTU3616B was about 2-fold more effective than KTU3616, indicating that KTU3616A and KTU3616B have little synergistic effect on the inhibition of SD. The synergy may be accomplished through the activity of KTU3616A and KTU3616B to induce blast-resistance in rice plants.[10]

In the crystal structure of the SD-carpropamid (KTU3616B) complex,[5] we identified hydrogen bonds and van der Waals interactions contributing to the tight-binding inhibition by carpropamid (Fig. 1). The interaction in the complex likely accounts for the reduced affinity of the KTU3616B isomers to SD. For instance, when KTU3616A sits in the active-site pocket in the same orientation as KTU3616B, the ethyl group of KTU3616A may compete with His-85 and weaken the water-mediated hydrogen bond between His-85 and the nitrogen atom of the isomer. The 1′-methyl group of KTU3615A seems to hinder the water-mediated hydrogen-bond network among the oxygen atom of the isomer, Tyr-30 and Tyr-50. KTU3615B may have a combination of the modes of interaction of KTU3616A and KTU3515A. Thus, the three-dimensional structure of the SD-carpropamid complex[5] and the binding affinity data for the carpropamid isomers presented here may be of importance in the design of more efficient anti-blast chemicals.

ACKNOWLEDGMENTS

We thank Dr. M. Nakasako of Keio University for valuable discussions. This study was supported by grants for the Bio-design and Bio-architect Research Program of RIKEN. It was also supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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要　　約

イネいもち病防除薬剤カルプロパミドとその異性体によるメラニン生合成酵素シタロン脱水酵素阻害

本山高幸，利部伸三，松岡将資，山口　勇

イネいもち病防除剤カルプロパミドは4種類の異性体，KTU3616A (1*R*, 3*S*, 1′*R*), KTU3616B (1*S*, 3*R*, 1′*R*), KTU3615A (1*S*, 3*R*, 1′*S*), KTU3615B (1*R*, 3*S*, 1′*S*) からなる．我々は既にこれらの中のKTU3616Bが強力にシタロン脱水酵素を阻害する強結合阻害剤（tight-binding inhibitor）であることを示していた．カルプロパミドとその立体異性体のグループにはKTU3616A, KTU3616B, KTU3615A, KTU3615B以外に4種類のジアステレオマーが存在する．今回，KTU3616B以外の7種の異性体について阻害の強さを解析したところ，いずれもtight-binding inhibitorではないことが明らかとなった．これらの内の3種の異性体（KTU3616A, KTU3615A, KTU3615B）は，KTU3616Bに対して100倍程度あるいはそれ以上の K_i 値を示した．すなわち強力な阻害作用を示すためには（1*S*, 3*R*, 1′R）の立体配置が必要なことが明らかとなった．