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BIOSYNTHESIS AND BIOLOGICAL ACTIVITY OF β-(5-METHYL-ISOXAZOLIN-3-ON-2-YL)ALANINE IN HIGHER PLANTS*

ISAMU MURAKOSHI, FUMIO IKEGAMI, TADAHIRO NISHIMURA and KAZUO TOMITA†

Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Chiba 260, Japan; †Agricultural Chemicals Research Laboratories, Sankyo Co., Ltd., Hiromachi 1-2-58, Shinagawa-ku, Tokyo 140, Japan

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Key Word Index—Biosynthesis, β-substituted alanine; β-(5-methylisoxazolin-3-on-2-yl)alanine; *O*-acetyl-L-serine; 3-hydroxy-5-methylisoxazole; Tachigaren; hymexazol; higher plants

Abstract—Enzyme preparations from *Leucaena* seedlings catalysed the formation of β-(5-methylisoxazolin-3-on-2-yl)alanine (MIA) by using 3-hydroxy-5-methylisoxazole (HMI) and *O*-acetyl-L-serine. Some properties of this enzyme are described. The β-substituted alanine synthases from *Pisum* and *Citrullus* seedlings could not catalyse the synthesis of MIA. The phytotoxic effect of HMI on rice seedlings is reduced by alanylation.

INTRODUCTION

3-Hydroxy-5-methylisoxazole (Tachigaren, hymexazol, HMI, **1**), a soil-fungicide, has been used for the control of soil-borne diseases, such as damping-off and *Fusarium* wilt of rice seedlings [1, 2].

In plants more than 60% HMI was metabolized into two glucosides [3] and its metabolic pathway was enzymatically demonstrated in several plants [4]. HMI and its *O*-glucoside (**3**) inhibited the germination and subsequent growth of rice seedlings, especially in the early stages of growth. *N*-Glucoside (**4**), on the other hand, did not affect growth or germination of rice seedlings [5]. Thus, the metabolites appeared to be less phytotoxic than the original chemical.

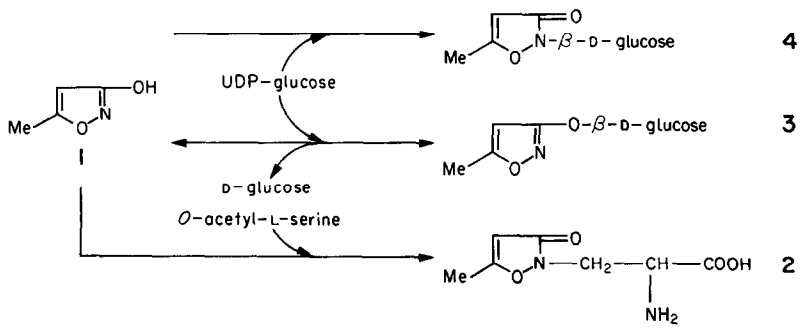
During our continuing study on the biosynthesis of heterocyclic β-substituted alanines in higher plants [6–13], we have demonstrated biosyntheses of β-isoxazolin-5-on-alanine derivatives [6, 7], β-(3-amino-1,2,4-triazol-1-yl)-L-alanine, a major metabolite of 3-amino-1,2,4-triazole [8] and other heterocyclic β-substituted alanines [9–13].

In this paper we have examined the formation of β-(5-methylisoxazolin-3-on-2-yl)alanine (MIA, **2**) from HMI and *O*-acetyl-L-serine (OAS) as a metabolite of HMI and its biological activity in higher plants.

* Parts of this work were presented at the 24th Annual Meeting of Kanto Branch of the Pharmaceutical Society of Japan at Tokyo, 18 October, 1980 (Meeting Abstracts, p. 79) and were included in the dissertation presented by F.I at Tokyo University, 1981 (in Japanese)

RESULTS AND DISCUSSION

The activity of MIA formation could be detected in the cell-free extracts of the seedlings of the Legumes *Leucaena leucocephala* and *Lathyrus maritimus* and the grasses *Coix Ma-yuen* and *Oryza sativa* used for enzyme sources. The most active enzyme preparations for MIA formation were obtained from *L. leucocephala* which was a rich source of L-mimosine synthase [12]. The activity in enzyme preparations from pea (*Pisum sativum*) and watermelon (*Citrullus vulgaris*) seedlings were negligible as shown in Fig. 1, while pea contains high enzyme activities of the



Scheme 1. Biosynthetic Pathways for β-(5-methylisoxazolin-3-on-2-yl)alanine (**2**), 3-β-D-glucopyranosyloxy-5-methylisoxazole (**3**) and 2-β-D-glucopyranosyl-5-methyl-4-isoxazolin-3-one (**4**) in higher plants.

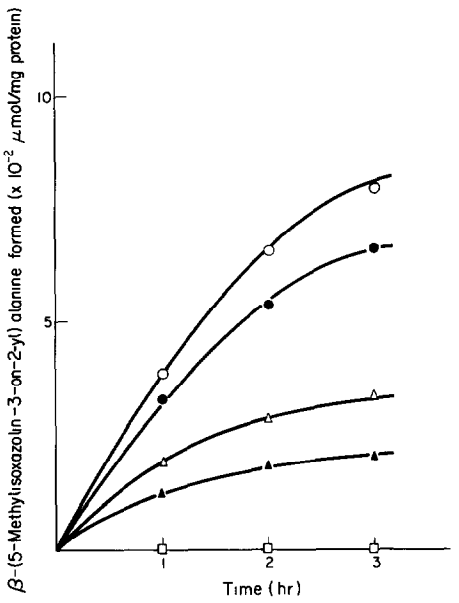


Fig. 1. Comparative specific activity for the β-(5-methylisoxazolin-3-on-2-yl)alanine formation by an enzyme from seedlings of (1) *Leucaena leucocephala* (○); (2) *Coix Ma-yuen* (●); (3) *Oryza sativa* (Δ); (4) *Lathyrus maritimus* (▲) and (5) *Pisum sativum*, *Citrullus vulgaris* and young leaves of *Datura metal* (□).

formation of β-isoxazolin-5-on-alanine derivatives [6, 7] and watermelon contains a high activity of β-(pyrazol-1-yl)-L-alanine synthase [11].

Under standard assay conditions, the enzyme clearly appears to be specific for OAS as a donor for the alanyl-moiety. No enzyme activity was detectable when OAS was substituted by *O*-acetyl-D-serine, *O*-phospho-L-serine, *O*-sulpho-L-serine or L-serine.

The effect of pH on the formation of MIA by the enzyme was investigated using 0.05 M K-Pi buffer. The enzyme exhibited a pH optimum of 7.5. The synthase activity for MIA was dependent upon the concentration of HMI used. A relatively low final concentration of HMI at around 75 mM was sufficient to give maximum rate of MIA formation in the presence of a fixed concentration (12.5 mM) of OAS. Higher concentrations of HMI progressively inhibit the enzymatic formation of MIA. This property is similar but less pronounced than the complete substrate inhibition shown by willardiine and isowillardiine synthases [9].

The phytotoxic effects of HMI and MIA on the germination and growth of rice seedlings were investigated (Table 1). HMI had an inhibitory effect on the root growth of rice seedlings at 100 μg/ml (1 mM), while it had no detectable effects at 1–10 μg/ml. MIA, on the other hand, did not limit the growth of germinated rice roots at 10–100 μg/ml (0.053–0.53 mM) and phytotoxic effects of MIA appeared less intense than HMI. These findings suggest that HMI undergoes a detoxication process in plants and shows a root growth promoting effect after alanylation to MIA. A similar metabolic detoxication by alanyl-substitution of a heterocyclic toxin 3-amino-1,2,4-triazole and by glucosyl-substitution of HMI at the ring-*N* of the original chemicals had been described before in plants, respectively [3, 14]. It seems likely that the formation of heterocyclic β-substituted alanines from unnatural chemicals is a protection mechanism of plants.

From the above results and from the earlier work in this laboratory, it can be suggested that the enzymes catalysing the formation of naturally occurring heterocyclic β-substituted alanines also catalyse the formation of MIA from HMI and OAS, because purified β-(pyrazol-1-yl)-L-alanine and L-mimosine synthases also catalyse the formation of β-(3-amino-1,2,4-triazol-1-yl)-L-alanine from 3-amino-1,2,4-triazole and OAS [11, 12].

When given to pea seedlings, HMI did not induce the formation of MIA, but there was an increase in the formation of the naturally occurring β-(3-isoxazolin-5-on-2-yl)alanine [F. Ikegami and F. Lambein, unpublished results].

EXPERIMENTAL

Plant materials. The seedlings of *Leucaena leucocephala*, *Coix Ma-yuen*, rice (*Oryza sativa*), *Lathyrus maritimus*, pea (*Pisum sativum*) and watermelon (*Citrullus vulgaris*) were grown in moistened vermiculite in the dark for 5–6 days at 26–28° and plants of *Datura metel* were raised in a greenhouse In some cases 10–14 days old seedlings of rice (greenish) were used. After harvest, the testas were removed and the seedlings and the young leaves were cooled at 0–4° for 30 min before enzyme extraction.

Chemicals. *O*-Acetyl-L-serine-[3-^{14}C] was synthesized in our laboratory from L-serine-[3-^{14}C] by a modification of the method of ref [15] and diluted with unlabelled OAS to obtain material of specific activity used in the different incubation

Table 1 Phytotoxic effect of β-(5-methylisoxazolin-3-on-2-yl)alanine (MIA) and 3-hydroxy-5-methylisoxazole (HMI) on the germination and subsequent growth of rice (*Oryza sativa*) seeds

Compound	Conc. (μg/ml)	Length of root (mm) 6 days	8 days
β-(5-Methylisoxazolin-3-on-2-yl)alanine	10	62	95
	100	67	94
3-Hydroxy-5-methylisoxazole	1	55	57
	10	50	62
	100	10	13
Control		50	54

The test was triplicated and the root length of the rice seedlings was measured after 6 days and 8 days. Mean of the root length and of triplicate tests are given. The test conditions were as described in the Experimental.

mixtures. HMI was synthesized by the method of ref. [1] and MIA was also synthesized biomimetically by the method of refs [16, 17]. All other chemicals used were of the highest commercial grade available.

Enzyme preparation. All operations were carried out at 0–4°. The enzyme preparations were obtained from the whole seedlings or young leaves as described before [6–13]. Protein was determined by the method of ref. [18].

Assay for MIA synthase activity. Reaction mixtures used to demonstrate the formation of MIA were incubated at 28° in a final vol. of 0.4 ml and contained OAS or OAS-[3-^{14}C] (12.5 mM, 1.25 mCi), HMI (75 mM) and 0.2 ml enzyme preparation containing 2–3 mg of the protein. The pH of the incubation mixtures was normally adjusted to pH 7.5 by 0.05 M K-Pi buffer. Reactions were terminated by the addition of 0.02 ml of 10% NH_4OH and were then allowed to stand for 30 min at room temp. The protein ppt. was removed by centrifugation and the clarified supernatant was examined chromatographically for the presence of MIA.

Identification of the reaction product as MIA. The formation of MIA was demonstrated by subjecting the supernatant solns to PC, using ninhydrin as chromogenic reagent. The product formed was identical with the authentic MIA in the following solvent systems: 1, *n*-BuOH–HOAc–H_2O (90:10:29); 2, pyridine–*n*-BuOH–H_2O–HOAc (10:5:5:1). The R_f values for MIA, OAS and L-serine in solvent 1 were 0.15, 0.21 and 0.12 respectively, in solvent 2: 0.36, 0.48 and 0.24 respectively. Further confirmation of the identity of the reaction product as MIA was obtained by using an automatic amino acid analyser (Shibata model AA-500, Tokyo). Under standard operating conditions (150 cm column, 50°, 0.2 M Na-citrate buffer, pH 3.25, flow rate 0.52 ml/min), MIA eluted at about 226 min (117–118 ml) from the column. However, since the MIA-peak partly overlapped with the OAS-peak (222 min), the reaction mixtures were allowed to stand at pH 9.5 for 30 min at room temp. before applying the samples to an amino acid analyser: by this procedure OAS was almost quantitatively rearranged into *N*-acetylserine. When unlabelled OAS was replaced in the reaction mixture with OAS-[3-^{14}C], radioactivity associated with each ninhydrin-positive substance on the paper chromatograms (in solvent 1) was determined using a gas-flow 4π radiochromatogram scanner (Aloka model PCS-4, Tokyo). Quantitative determinations of MIA were also made using the cadmium–ninhydrin reagent and the general method described in ref. [19].

Assay of the biological activity of MIA. Ten seeds of rice were respectively placed in Petri-dishes, in which 5 ml of MIA and HMI solns at the indicated concns (1, 10 and 100 μg/ml) was added. They were grown under a light intensity of 4000 lx for 6 days or 8 days at 28°. The test was triplicated and the root length of the rice seedlings was measured after 6 days and 8 days. Mean of the root length of respective ten seedlings and of triplicate test were used to measure the phytotoxic effects of MIA and HMI as compared with an untreated control sample (Table 1).

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