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Regiospecific hydroxylation of 3-(methylaminomethyl)pyridine to 5-(methylaminomethyl)-2(1*H*)-pyridinone by *Arthrobacter ureafaciens*

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Abstract

Microbial production of a 6-hydroxy-3-pyridylmethyl compound from 3-pyridylmethyl compound was investigated. The hydroxylation of 3-(methylaminomethyl)pyridine to 5-(methylaminomethyl)-2(1*H*)-pyridinone, tautomer of 2-hydroxy-5-(methylaminomethyl)pyridine, by resting cells of *Arthrobacter ureafaciens* JCM3873 was found to proceed regio- and chemo-selectively with an almost quantitative yield. The addition of molybdate ion and nicotine as an inducer to the culture medium was required for the preparation of cells containing high hydroxylation activity. The optimal temperature and pH for the hydroxylation by using resting cells were 35°C and around 7, respectively. This hydroxylation enzyme does undergo inhibition by the substrate. The inhibitory effect could be eliminated by stepwise feeding of the substrate. Under adequate conditions, 23 mg/ml of 5-(methylaminomethyl)-2(1*H*)-pyridinone was produced with a molar yield of nearly 100% from 3-(methylaminomethyl)pyridine.

Keywords: *Arthrobacter ureafaciens*; Hydroxylation; 5-(Methylaminomethyl)-2(1*H*)-pyridinone; 5-(Aminomethyl)-2(1*H*)-pyridinone; Pyridinone derivatives

1. Introduction

Many pyridine derivatives are used as important intermediates for the preparation of agrochemicals and pharmaceuticals. Recently, 6-chloro-3-pyridylmethylamino compounds are increasingly receiving attention as important intermediates for the synthesis of new insecticides, e.g. acetamiprid (NI-25, [1]), imidacloprid [2], nitenpyram [3]. However, it is troublesome to synthesize regioselectively 2,5-disubstituted pyridines described above by chemical methods. For example, 6-hydroxynicotinic acid was prepared from malic acid with 5 steps in a low yield [4,5]. In contrast to chemical methods, nicotinic acid degradation pathway by microorganisms has been reported to proceed by way of the initial formation of 6-hydroxynicotinic acid [6]. One of nicotine degradation pathways by microorganisms is also through 6-hydroxynicotine [7]. These suggest that it is possible to prepare a 6-hydroxy-3-substituted pyridine from 3-substituted pyridine by an enzymatic method.

Therefore, we used 3-pyridylmethyl com-

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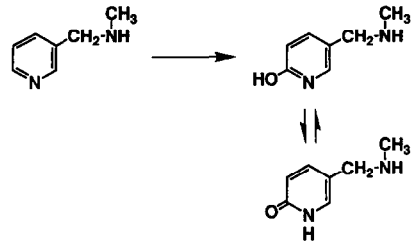

Fig. 1. Microbial hydroxylation of MAP to HMAP.

pounds as substrates, and surveyed the hydroxylation activity of 3-pyridylmethyl compounds at position 6 by various microorganisms to prepare 6-hydroxy-3-pyridylmethyl compounds which can be converted chemically to 6-chloro-3-pyridylmethylamino compounds.

This paper deals with the regio- and chemoselective hydroxylation of 3-(methylaminomethyl)pyridine (MAP) to 5-(methylaminomethyl)-2(1*H*)-pyridinone (HMAP), tautomer of 2-hydroxy-5-(methylaminomethyl)pyridine, by *Arthrobacter ureafaciens* JCM3873 (Fig. 1).

2. Materials and methods

2.1. Chemicals

MAP was prepared from 3-(chloromethyl)pyridine hydrochloride and methylamine. *N*-(Pyridylmethyl)acetamide was prepared from 3-(aminomethyl)pyridine and acetyl chloride. *N*-(Pyridylmethyl)-*p*-toluenesulfonamide was prepared from 3-(aminomethyl)pyridine and *p*-toluenesulfonyl chloride. 3-Pyridylmethyl acetate was prepared from 3-pyridinemethanol and acetyl chloride. Methyl 3-pyridylmethyl ether was prepared from 3-(chloromethyl)pyridine hydrochloride and sodium methoxide. Other chemicals were the best available commercial products.

2.2. Microorganism, media and cultivation conditions

A. ureafaciens JCM3873 was obtained from Japan Collection of Microorganisms (Japan).

Medium I contained 10 g of K_2HPO_4, 4 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 1 g of yeast extract, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.04 g of $MnSO_4 \cdot 4\text{–}6H_2O$, and 2 mg of $FeSO_4 \cdot 7H_2O$ per 1 of distilled water. The pH was adjusted to 7.0. Medium II was medium I supplemented with 4 g of nicotine.

One loopful of cells of *A. ureafaciens* JCM3873 subcultured on a Luria–Bertani medium agar plate [8] was inoculated into 20 ml of medium II in a 100 ml flask, followed by aerobic cultivation at 30°C for 20 h. The intact cells harvested from the cultured broth were used as an enzyme source unless otherwise stated.

2.3. Identification of reaction products

HMAP was isolated as its hydrochloride salt from a reaction mixture as described in the text. A part of the isolated HMAP hydrochloride was converted to HMAP for the identification.

The ^{13}C-NMR chemical shifts of the reaction product were compared with those of hydroxyl-

Table 1
Comparison of ^{13}C-NMR chemical shifts of the reaction product with those of hydroxylated MAP

		Chemical shifts (ppm) of heterocyclic carbons				
		C2	C3	C4	C5	C6
Reaction product	(Obsd.)	133.3	117.2	142.3	119.6	162.4
6-hydroxylated MAP	(Calcd.)	134.2	118.2	141.7	120.3	162.9
2-hydroxylated MAP	(Calcd.)	161.3	133.1	141.7	105.4	135.8
MAP (substrate)	(Obsd.)	147.8	136.0	135.6	123.2	149.4

The ^{13}C-NMR spectra were obtained in DMSO-d_6. The ^{13}C-NMR chemical shifts of 2- and 6-hydroxylated MAP were calculated from the chemical shifts of MAP and 2-hydroxypyridine by using the additivity.

ated MAP which was calculated from the chemical shifts of MAP and 2-hydroxypyridine by using the additivity. As is shown in Table 1, the chemical shifts of the reaction product were in fair agreement with those of 6-hydroxylated MAP. Other spectral data follow: ^{1}H NMR (DMSO-d_6) δ 7.41 (dd, 1H, $J = 2.4$, 9.6 Hz), 7.23 (d, 1H, $J = 2.4$ Hz), 6.31 (d, 1H, $J = 9.6$ Hz), 3.34 (s, 2H), 2.19 (s, 3H); mass spectrum, m/z 138 (M^+), 123, 108, 80; exact mass calcd. for $C_7H_{10}N_2O$ 138.0791, obsd. 138.0782. From these results, the reaction product was confirmed to be HMAP.

When 3-(aminomethyl)pyridine was used as the substrate, 5-(aminomethyl)-2(1*H*)-pyridinone was isolated from a reaction mixture. The structure of this product was similarly confirmed by comparing its spectra with those reported [9].

2.4. Analytical methods

HMAP produced and MAP remaining in the reaction mixture were separated by thin-layer chromatography in the solvent system of 2-propanol–28% ammonia solution–water (9:1:2, by volume), and they were detected by a method using potassium permanganate alkaline solution as the color-producing reagent. HMAP and MAP were measured by high performance liquid chromatography by using a TSK gel ODS-80Ts column (4.6 mm ID × 150 mm, Tosoh, Japan). The mobile phase of PIC reagent B6 (Waters, USA) was used at a flow rate of 1 ml/min. The eluate was monitored at 263 nm.

3. Results

3.1. Substrate specificity of A. ureafaciens JCM3873

The substrate specificity of *A. ureafaciens* JCM3873 was examined by using the resting cells. Among 3-substituted pyridines tested, MAP was efficiently hydroxylated and 3-(aminomethyl)pyridine was slightly hydroxylated as shown in Table 2. Therefore, MAP was more suitable substrate and was used for further experiments.

Table 2
Substrate specificity of *A. ureafaciens* JCM3873

3-Substituent	Relative activity
$-CH_2OH$	0
$-CH_2NH_2$	17
$-CH_2NHCH_3$	100
$-CH_2NHAc$	0
$-CH_2NHTos$	0
$-CH_2OCH_3$	0

A. ureafaciens JCM3873 was cultured in medium II containing 0.25 mg/l of $Na_2MoO_4 \cdot H_2O$ at 30°C for 20 h. The cells harvested from 5 ml of the culture broth were added to 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 100 mM of 3-substituted pyridine, and then incubated at 30°C for 5 h with shaking.

3.2. Establishment of the effective medium for the preparation of cells containing high HMAP-producing activity

3.2.1. Effect of pyridine-related compounds on the formation of HMAP-producing enzyme

The effect of pyridine-related compounds on the formation of HMAP-producing enzyme was examined by using medium I as basal medium. The results were shown in Table 3. The HMAP-producing activity was scarcely ob-

Table 3
Effects of pyridine-related compounds on the formation of HMAP-producing enzyme

Pyridine-related compounds	Relative activity
none	12
HMAP	18
6-hydroxynicotinic acid	100
2-hydroxypyridine	46
acetylcholine chloride	21
nicotine	369

A. ureafaciens JCM3873 was cultured in medium I containing 2 g/l of pyridine-related compound at 30°C for 20 h. The cells harvested from 10 ml of the culture broth were added to 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 50 mM of MAP, and then incubated at 30°C for 20 h with shaking.

tained with the medium I containing 3-substituted pyridine such as MAP, nicotinic acid, or 3-cyanopyridine. The cells cultured in medium I containing 2-hydroxypyridine derivatives, which are similar in structure to the reaction product, or acetylcholine chloride showed low HMAP-producing activity. The cells cultured with nicotine showed high HMAP-producing activity. Thus, this enzyme activity was completely inducible and the best inducer was found to be nicotine. It can be seen from Fig. 2 that the optimal concentration of nicotine was around 1 g/l.

3.2.2. *Effect of molybdate ion*

Molybdate ion at first was found to have remarkable promoting effect on the HMAP production when the effect of various metal ions was examined by using the intact cells cultured in medium II (data not shown). So the effect of MoO_4^{2-} ion on the cultivation of *A. ureafaciens* JCM3873 was examined by using medium I containing 2 g/l of nicotine as basal medium. As can be seen from Fig. 3, the HMAP-producing activity and the growth of *A. ureafaciens* JCM3873 was increased by the addition of Na_2MoO_4, and the activity was nearly parallel to the growth. This suggests that MoO_4^{2-} ion also affects the stimulation of the growth of the cells containing HMAP-producing activity. It can be seen from Fig. 3 that the effectual concentration of MoO_4^{2-} ion was above 1 μM (0.25 mg/l as $Na_2MoO_4 \cdot H_2O$). The cells grown in the medium supplemented with above 1 μM of MoO_4^{2-} ion did not show the remarkable promoting effect of MoO_4^{2-} ion on the resting cells reaction.

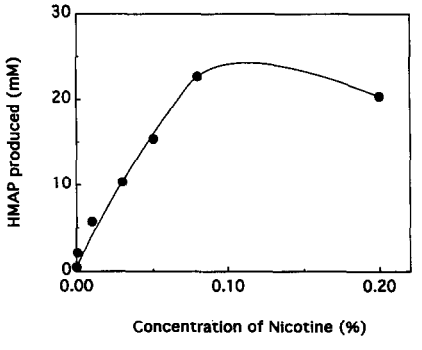

Fig. 2. Induction of HMAP-producing enzyme by nicotine. *A. ureafaciens* JCM3873 was cultured in medium I containing nicotine at the indicated concentrations at 30°C for 20 h. The cells harvested from 5 ml of the culture broth were added to 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 50 mM of MAP and 0.1 mM of Na_2MoO_4. The reactions were done at 30°C for 5 h with shaking.

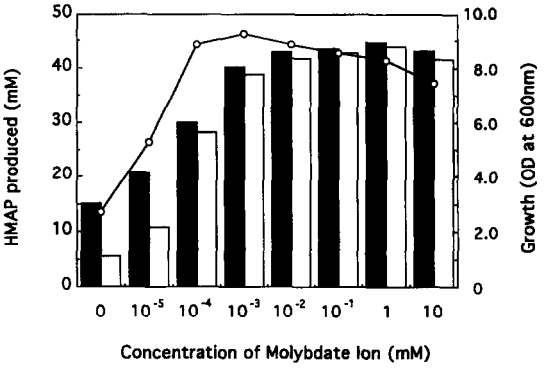

Fig. 3. Effect of molybdate ion. *A. ureafaciens* JCM3873 was cultured in medium I containing 2 g/l of nicotine and $Na_2MoO_4 \cdot H_2O$ at the indicated concentrations at 30°C for 20 h. Growth was expressed as the optical density at 600 nm. The reaction mixture (total volume 1 ml) contained 50 mM of MAP, the cells harvested from 5 ml of the culture broth, and (or not) 0.1 mM of Na_2MoO_4 in 0.1 M sodium phosphate buffer (pH 7.0). The reactions were done at 30°C for 5 h with shaking. Symbols: ■, HMAP produced in the presence of 0.1 mM of Na_2MoO_4; □. HMAP produced in the absence of 0.1 mM of Na_2MoO_4; ○, growth.

3.3. *Establishment of the optimal reaction conditions for HMAP production by resting cells*

3.3.1. *Effect of temperature and pH*

The effects of temperature and pH on HMAP production were examined by using the resting cells. As is shown in Fig. 4, the optimal temperature and pH for HMAP production were 35°C and around 7, respectively.

3.3.2. *Effect of substrate concentration*

The effect of the substrate, MAP, on HMAP production was examined by using the resting cells. As can be seen from Fig. 5, the amount of HMAP produced was decreased in the presence of high concentrations of MAP. This indicates that HMAP-producing enzyme has undergone

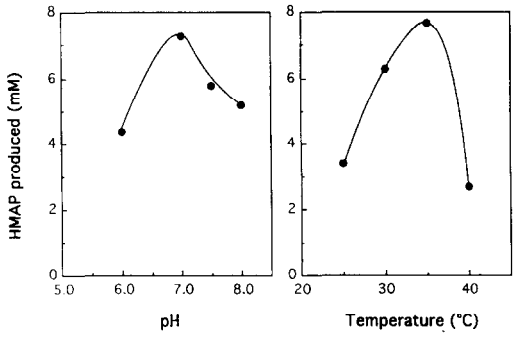

Fig. 4. Effects of temperature and pH on HMAP production by resting cells of *A. ureafaciens* JCM3873. *A. ureafaciens* JCM3873 was cultured in medium II at 30°C for 20 h. The cells harvested from 5 ml of the culture broth were added to 1 ml of 0.1 M sodium phosphate buffer containing 50 mM of MAP. (A) The reactions were done at 30°C for 5 h in 0.1 M sodium phosphate buffers with shaking. (B) The reactions were done in 0.1 M sodium phosphate buffer (pH 7.0) at 25–40°C for 5 h with shaking.

inhibition by the substrate. This inhibitory effect could be eliminated by stepwise feeding of the substrate.

3.4. Repetition of resting cells reaction by the reused cells

In order to overcome a substrate inhibition, the resting cells reactions by the reused cells were carried out. The reactions were repeated in 100 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 100 mM of MAP at 30°C. The results were shown in Table 4. In this case, the cells were utilizable about 3 times, though the third reaction required more time to complete the reaction.

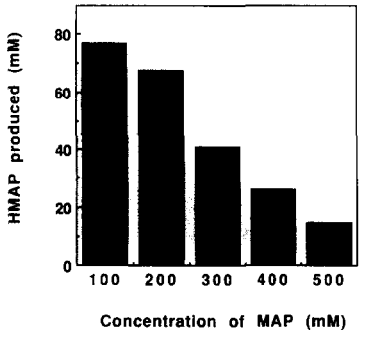

Fig. 5. Effects of the MAP concentration for HMAP production by resting cells of *A. ureafaciens* JCM3873. *A. ureafaciens* JCM3873 was cultured in medium II at 30°C for 20 h. The cells harvested from 20 ml of the culture broth were added to 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing MAP at the indicated concentrations. The reactions were done at 30°C for 42 h with shaking.

Table 4
Repetition of resting cells reaction by the reused cells

Reaction	Reaction time (h)	Yield (%)
1st	20	> 99
2nd	23	> 99
3rd	48	96

The cells (2.6 g as wet weight) cultured in medium I containing 2 g/l of nicotine at 30°C for 20 h were added to 100 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 100 mM of MAP and 0.1 mM of Na_2MoO_4, and then incubated at 30°C with rotary shaking (120 rpm). After completion of the reaction, the cells were harvested from the reaction mixture and were used for the next reaction.

3.5. Production of HMAP from MAP added successively by resting cells

A typical production of HMAP from MAP added successively by the resting cells is illustrated in Fig. 6. The cells grown in medium I containing 2 g/l of nicotine and 0.25 mg/l of $Na_2MoO_4 \cdot H_2O$ at 30°C for 20 h were harvested by centrifugation. The washed cells (0.6 g as wet weight) were added to 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 100 mM of MAP, and then incubated at 30°C

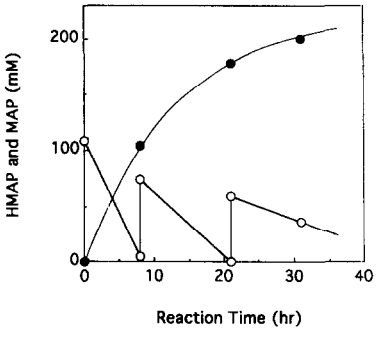

Fig. 6. Production of HMAP from MAP added successively by *A. ureafaciens* JCM3873. The cultivation of *A. ureafaciens* JCM3873 and the reaction were done as described in text. Symbols: ●, HMAP; ○, MAP.

with shaking. After incubation for 8 h, 500 mM of MAP solution 2 ml was added. After another incubation for 13 h, the concentration of HMAP produced reached 23 mg/ml, with a molar yield of nearly 100%. The further addition of MAP brought about the slowing down of the reaction rate.

3.6. Isolation of HMAP as its hydrochloride salt

After the completion of a reaction, cells were removed from the reaction mixture by centrifugation. The solution (537 ml) contained 14.0 g of HMAP hydrochloride. The solution was evaporated to dryness under reduced pressure. The residue was dissolved in 600 ml of aqueous ammoniacal 2-propanol (vide supra) and the resulting insoluble material was removed by filtration. The filtrate was concentrated to about 50 ml and its pH was adjusted to 7 with conc. HCl. The solution was further concentrated under reduced pressure. The resulting white crystal was separated by filtration, washed with ethanol, and vacuum-dried over phosphorus pentoxide. The yield of HMAP hydrochloride was 8.3 g (59%). The concentration of the filtrate gave a second crop of 4.3 g. Total yield of HMAP hydrochloride was 12.6 g (90%).

4. Discussion

The hydroxylation of MAP to HMAP by *A. ureafaciens* JCM3873 proceeded regio- and chemo-selectively with an almost quantitative yield.

Similar enzymatic hydroxylations of nicotinic acid [10–12] and 3-cyanopyridine [13] to the corresponding 6-hydroxy compounds have been reported. However, these enzymatic hydroxylations are limited to 3-substituted pyridines having electron-withdrawing group, the hydroxylation of 3-substituted pyridine having electron-releasing group such as MAP has not previously been reported except for a metabolic pathway of nicotine. This, therefore, is the first report on a synthetic hydroxylation of 3-substituted pyridine having an electron-releasing group.

The HMAP-producing activity was induced strongly by nicotine. 2-Hydroxypyridine derivatives and acetylcholine chloride, which is not a pyridine derivative, acted slightly as an inducer. This finding is interesting in studying a structure of the repressor which controls the formation of HMAP-producing enzyme.

On the HMAP production by the intact cells cultured in medium II, MoO_4^{2-} ion had a remarkable promoting effect. The cells grown in the medium supplemented with above 1 μM of MoO_4^{2-} ion did not show the remarkable promoting effect of MoO_4^{2-} ion on the resting cells reaction. This suggests that the content of MoO_4^{2-} ion in medium II is not enough to form HMAP-producing enzyme, and molybdenum-deficient enzymes were partly produced.

The HMAP-producing enzyme is presumed to be nicotine dehydrogenase (EC 1.5.99.4) involved in the nicotine degradation pathway through 6-hydroxynicotine, because the enzyme was induced strongly by nicotine and it also required MoO_4^{2-} ion for the activity [14–16]. If so, the production of HMAP with a quantitative yield is explicable as given below: nicotine is gradually degraded by nicotine dehydrogenase, followed by both 6-hydroxy-L-nicotine oxidase (EC 1.5.3.5) and 6-hydroxy-D-nicotine oxidase (EC 1.5.3.6) in one of nicotine degradation pathways. On the other hand, HMAP produced is not degraded by the second enzymes for reasons of structure.

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