ENZYMATIC and CHEMICAL PREPARATION of 5-AMINO-4-CHLORO-2-(2,3-DIHYDROXYPHEN-1-YL)-3(2H)-PYRIDAZINONE

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SUMMARY

A hypothetical intermediate of the microbial degradation of pyrazon, 5-amino-4-chloro-2(2,3-dihydroxyphen-1-yl)-3(2H)-pyridazinone, was prepared by enzymatic and chemical treatment of 5-amino-4-chloro-2(2,3-dihydroxy-cyclohexa-4,6-dien-1-yl)-pyridazinone. The properties of the metabolite are described.

INTRODUCTION

The degradation of the herbicide pyrazon by bacteria has been described recently (1). The catechol compound (II) supposed to be an intermediate of this degradation was not detected in the growth medium. In this communication we report the preparation of 5-amino-4-chloro-2(2,3-dihydroxyphen-1-yl)-3(2H)-pyridazinone (II), both by chemical and enzymatic conversion of 5-amino-4-chloro-2(2,3-dihydroxycyclohexa-4,6-dien-1-yl)-3(2H)-pyridazinone (I), a metabolite of pyrazon which can be isolated from the medium.

Materials and Methods

The bacteria used in this study have been described by Fröhner et al. (2). The bacteria were grown in a minimal salts medium containing 600 ppm pyrazon as described previously (1).

1. Isolation of 5-amino-4-chloro-2(2,3-dihydroxycyclohexa-4,6-dien-1-yl)-3(2H)-pyridazinone (I)

Abbreviations:

I = 5-amino-4-chloro-2(2,3-dihydroxycyclohexa-4,6-dien-1-yl)-3(2H)-pyridazinone

II = 5-amino-4-chloro-2(2,3-dihydroxyphen-1-yl)-3(2H)-pyridazinone

III= 5-amino-4-chloro-3(2H)-pyridazinone

IV = 5-amino-4-chloro-2(2,3-dihydroxycyclohex-6-en-1-yl)-3(2H)-pyridazinone

Strain E of the bacteria was inoculated into an Erlenmeyer flask containing 50 ml of the pyrazon medium and grown on a rotary shaker for 2 days. The cell suspension was transferred to a flask containing 1 l of the medium and shaken for another 36 hours. Then the cells were removed by centrifugation. The solution was evaporated to dryness under reduced pressure. The residue was extracted repeatedly with ethyl acetate. The solvent was removed and the residue was dissolved in about 20 ml of ethanol and applied to a column (75 x 6,5 cm) of Sephadex LH 20. The column was developped with ethanol-water (1:1, v/v). The fractions containing I were evaporated to dryness. The raw material was recrystallized from ethanol yielding 25 mg of I per 1 medium.

2. Enzymatic preparation of 5-amino-4-chloro-2(2,3-dihydroxyphen-1-yl)-3(2H)-pyridazinone (II)

10 mg of I, 40 uM of NAD^+, 10 ml of crude extract prepared as described previously (1) and 25 ml of 0,1 m phosphate puffer (pH 7,0) were incubated at 25° for 1 hour. Then the solution was concentrated to a volume of 4 ml and applied to a column (40 x 4 cm) of Sephadex G 10. The column was eluted with water. The fractions containing II were detected by thin layer chromatography, collected, and concentrated. The concentrated solution was extracted with ethyl acetate. The organic extract was evaporated to dryness to leave 4 mg of a solid residue. No recrystallisation was necessary.

3. Chemical preparation of 5-amino-4-chloro-2(2,3-dihydroxyphen-1-yl)-3(2H)-pyridazinone (II)

40 mg I were dissolved in 10 ml of water and refluxed with 4 mg Pd/C for 20 minutes. The catalyst was filtered and the solution was allowed to cool. Immediately II precipitated as white crystals. The crystals were collected on a Büchner funnel yielding 6,5 mg of II; the supernatant still containing some residual II was applied to a column of Sephadex G 10 and eluted with water. The fractions containing II were detected by thin layer chromatography, collected, concentrated and extracted with ethyl acetate. The organic extract was evaporated to dryness to leave another 2 mg of II.

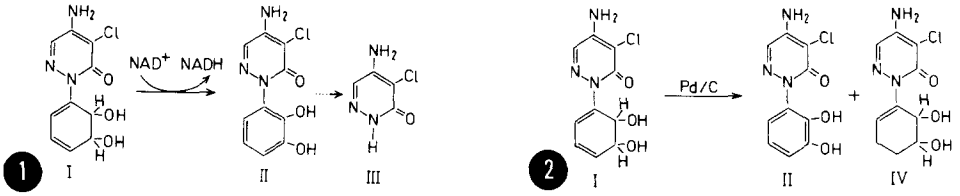

Fig. 1

Enzymatic conversion of 5-amino-4-chloro-2(2,3-dihydroxy-cyclohexa-4,6-dien-1-yl)-3(2H)-pyridazinone (I)

Fig. 2

Disproportionation of 5-amino-4-chloro-2(2,3-dihydroxy-cyclohexa-4,6-dien-1-yl)-3(2H)-pyridazinone (I)

Results

A crude cell extract of the pyrazon-degrading bacteria when incubated with I led to the formation of II (Fig. 1). In addition 5-amino-4-chloro-3(2H)-pyridazinone (III) was formed. We found that only a defined ratio of I to NAD^+ to crude extract led to an optimal result.

The highest yield of II was obtained in a reaction mixture containing 10 mg I, 40 uM NAD^+, 10 ml crude extract, and 25 ml 0,1 m phosphate buffer (pH7). Chromatography of the reaction mixture on Sephadex G 10 showed the formation of the following products:

3,1 mg of unchanged I, 4 mg of II, and 0,8 mg of III.

When the fractions containing II were concentrated almost to dryness, the colorless solution became brown; to prevent this the concentrated solution was extracted with ethyl acetate. When the organic phase was evaporated no discoloration took place; II precipitated as white crystals.

At pH7 the UV spectrum of II showed maxima of absorption at 280 nm and at 225 nm, respectively. The addition of NaOH to pH 13 caused a characteristic phenolate shift which was finished after 45 minutes. The results of CD and mass spectrometry provide strong evidence for the proposed structure of II.

Further evidence is given by the result of chemical treatment of I. As is well-known, cyclohexadiene systems disproportionate

under appropriate conditions into the aromatic and the partly or completely hydrogenated compound (3). Refluxing of I with Pd/C in water for 20 minutes leads to four products:

II, which precipitates during cooling of the solution,
IV, which is the second main product,
and two by-products.

From its mass spectrum and NMR spectrum IV can be assigned the structure as shown in Fig. 2. The data of UV and mass spectra of the compounds prepared by chemical and the enzymatic synthesis are identical. The result that II can be metabolized by crude extract may be taken as evidence for the intermediate character of II.

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