Note

Action of Nitrile Hydratase from *Rhodococcus rhodochrous* IFO 15564 on Derivatives of 2,5-Anhydro-D-allononitrile

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The conversion of 2,5-anhydro-D-allononitrile derivatives by a nitrile hydratase from *Rhodococcus rhodochrous* IFO 15564 was studied. The activity of the enzyme was strongly affected by the steric bulkiness of the substituents at the 3-position of the substrates, and the corresponding amides were obtained in high yields from the nitriles with free hydroxyl groups at the 3- and 4-positions.

Key words: *Rhodococcus rhodochrous* IFO 15564; nitrile hydratase; 2,5-anhydro-D-allononitrile; 2,5-anhydro-D-allonamide; hydantocidin

Selective and mild hydrolyzing systems for nitriles by means of microorganisms recently became available for the production of fine chemicals. Few examples, however, have been reported on the application to nitriles with a carbohydrate framework. We were interested in the hydration of 2,5-anhydro-D-allononitriles 1 to amides 2, since 2a has been reported as the synthetic intermediate of nucleosides and hydantocidin.

Incubation of 1a with *Rhodococcus rhodochrous* IFO 15564 afforded a rather complex mixture of amides. It was observed that concomitant hydrolysis of the acetate protective groups (3-, 4-, and 6-positions) partially occurred during the incubation by an esterase in the microorganism. Subsequently, the crude mixture was benzoylated to elucidate which acetates had been hydrolyzed by the esterase. This workup also facilitated the separation of products to give known amide 2a (35%), 3-aceate 2b, and 4-aceate 2c (2b + 2c: 9%). This result indicated that hydrolysis of the 6-aceate had taken place faster than that of the 3-aceate and 4-aceate.

To simplify the situation, the 3-aceate and 4-aceate were substituted by benzoate, which was expected to be less resistant to the esterase-mediated hydrolysis. Nitrile 1e was prepared from D-ribose through the intermediates, a mixture of 3a and 3b, via regioselective lipase-catalyzed acetylation at the 6-position of ribose. To our disappointment, hydration of the nitrile became very slow in the case of benzoates 1b and 1e. The major product from 1e was the corresponding hydroxy nitrile, only the 6-aceate being hydrolyzed.

These results indicated the importance of sterical hindrance by the ester group at the 3-position. Indeed, the hydration of 1d and 1e, which had free hydroxyl groups at the 3- and 4-positions, proceeded smoothly; especially in the case of 6-aceate 1d, this hydration was extremely fast. Because of the shorter reaction time, the 6-aceate was scarcely affected, and thus amide 2d was obtained as the major product (87%) after benzoylation. Although the 6-benzoate in 1e retarded the hydration of the nitrile, desired product 2e was obtained in as high a yield as 80% after an acetylation workup. All the results are listed in the Table.

The effect of steric hindrance at the 3-position on the nitrile hydratase activity was further studied. The activity of nitrile hydratase was almost lost by introducing a 3,4-acetonide group (1f).

Table: Hydration of 2,5-Anhydro-D-allononitriles by *Rhodococcus rhodochrous* IFO 15564

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction time (h)</th>
<th>Nitrile</th>
<th>Amide (Yield %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>19</td>
<td></td>
<td>2a (35), 2b + 2c (9)</td>
</tr>
<tr>
<td>1b</td>
<td>24</td>
<td>1b (91)</td>
<td></td>
</tr>
<tr>
<td>1c</td>
<td>22</td>
<td>1b (59)</td>
<td>2a (3)</td>
</tr>
<tr>
<td>1d</td>
<td>2</td>
<td></td>
<td>2a (7), 2d (87)</td>
</tr>
<tr>
<td>1e</td>
<td>12</td>
<td></td>
<td>2e (80), 2f (5)</td>
</tr>
<tr>
<td>1f</td>
<td>72</td>
<td>1f (60)</td>
<td></td>
</tr>
</tbody>
</table>

* All reactions were carried out at 30°C.
^ Products were isolated after benzoylation.
" Products were isolated after acetylation.

Experimental

IR spectra were recorded as thin films for oils or in KBr discs for solids. 1H-NMR spectra were measured in chloroform-d with tetramethylsilane.
Hydration of 1a. A suspension of harvested cells of *Rhodococcus rhodochrous* IFO 15564T (0.1 g) in a phosphate buffer solution (0.1 M, pH 6.0, 10 ml) was added to nitrite 1a (17.7 mg, 0.062 mmol), and the mixture was stirred for 19 h at 30 °C. The cells were removed by centrifugation, and the resulting supernatant was lyophilized. The residue was benzoylated in dimethylformamide with benzoyl chloride. The product was subjected to silica gel preparative TLC (hexane ethyl acetate, 1:1, developed three times) to afford 2a (10.5 mg, 35%) and a mixture of 2b and 2c (2.5 mg, 9%). Analytical sample of 2a: oil, δ-H NMR: δ = 4.67, 4.77 (3H, m), 4.78 (1H, d, J = 3.9 Hz), 5.72 (1H, dd, J = 5.2, 6.0 Hz), 5.82 (1H, br, s), 5.96 (1H, dd, J = 3.9, 5.2 Hz), 7.03 (1H, br, s), 7.30-7.63 (9H, m), 7.86-8.10 (6H, m). Its NMR spectrum was in good accordance with that reported previously. The minor product was revealed to be a mixture of 3-acetate and 4-acetate by its NMR spectrum. 1H NMR: δ 2.09 and 2.15 (each s, total 3H), 4.30-4.70 (4H, m), 5.49-5.84 (3H, m), 6.80 (br, s), 6.90 (br, s). 7.34-7.55 (6H, m), 7.83-8.02 (4H, m).

5-O-Authoxy-1,2,3-tri-O-benzoyl-α-bifurancarboxamide (3a and 3b). A mixture of p-ribose (1.509 g, 10.1 mmol), 2,6-di-isobutylphenol (BHT, a catalytic amount), and immobilized *Candida antarctica* lipase (2.02 g) was heated in vinyl acetate (10 ml) and pyridine (15 ml) at 45 °C for 2 days under a slow, continuous flow of nitrogen. After removing the insoluble material by filtration through a Celite, the filtrate and washings were combined and concentrated in vacuo. The residue was benzoylated in the conventional manner, and after the workup, the product was purified by silica gel column chromatography (250 g). Elution with toluene ethyl acetate (30:1) afforded 3 (1.971 g, 39%) as an anemic mixture. This mixture was employed in the next step without further purification.

The second anemic mixture was separated by silica gel preparative TLC. The fast-moving isomer was assigned to a β-anomer, and the slow-moving isomer to a α-anomer by comparing the coupling constants between H-1 and H-2 with the reported values for tetra-O-acetyl-α-bifurancarboxylic

d 6.41, J = 4.3 Hz, for H-1 of the x-anomer, and δ 6.18, J = 8.8 Hz, for H-1 of the β-anomer. Fast-moving isomer (3a): R = 0.58 (toluene ethyl acetate, 9:1, developed twice). δ-H NMR: 1.35 (3H, s), 2.41 (1H, dd, J = 4.9, 12.2 Hz), 4.43 (1H, dd, J = 3.9, 12.2 Hz), 4.66 (1H, dd, J = 3.9, 4.9, 6.8 Hz), 5.82 (1H, dd, J = 4.9, 6.8 Hz), 5.85 (1H, d, J = 4.9 Hz), 6.57 (1H, s), 7.27-7.58 (9H, m), 7.83-8.05 (6H, m).

Slow-moving isomer (3b): R = 0.44 (the same solvent system). δ-H NMR: δ 2.12 (3H, s), 4.35 (1H, d, J = 3.9, 12.2 Hz), 4.42 (1H, dd, J = 3.9, 12.2 Hz), 4.71-4.73 (1H, m), 5.55 (1H, dd, J = 4.4, 6.8 Hz), 5.70 (1H, dd, J = 2.2, 6.8 Hz), 6.85 (1H, d, J = 4.4 Hz), 7.20-7.56 (9H, m), 7.76-8.01 (6H, m).

6-O-(2-acetoxy-4-hydroxyphenyl) α-bifurancarboxylate (1c). A mixture of 1b (322 mg, 0.638 mmol) was treated with methyloxylodiol and boron trifluoride etherate. After the workup, the product was purified by silica gel column chromatography (10 g). Elution with toluene ethyl acetate (1:1) afforded 1c (165 mg, 63%) as an oil, [α] D 20 +3.1, 3.1 (c 10.2, chloroform). IR νcm 1740, 1640, 1500, 1490, 1460, 1370, 1320, 1280, 1185, 1130, 1100, 1075, 1030, 965, 885, 815, 720, 670. δ-H NMR: δ 3.90 (3H, s), 4.27 (1H, dd, J = 2.9, 12.2 Hz), 4.55 (1H, dd, J = 2.9, 12.2 Hz), 4.59-4.62 (1H, m), 4.95 (1H, d, J = 4.4 Hz), 5.79 (1H, dd, J = 4.9, 5.4 Hz), 5.95 (1H, dd, J = 4.4, 9.4 Hz), 7.37-7.60 (6H, m), 7.93-7.96 (4H, m);

C NMR δ: 20.89, 62.68, 69.71, 72.01, 74.73, 81.32, 115.92, 128.21, 128.48, 128.59, 128.63, 128.79, 128.83, 129.77, 129.86, 132.86, 134.00, 164.90, 165.13, 170.44. Anal. Found: C, 64.27; H, 4.57; N, 3.53%. Calculated for C 34 H 21 N O 5: C, 64.48; H, 4.68; N, 3.42%.

Hydration of 1c. Nitric acid (100.0 mg, 0.246 mmol) was incubated with *Rhodococcus rhodochrous* for 22 h at 30 °C. After benzoylation and workup, the product was purified by silica gel column chromatography (20 g). Elution with hexane ethyl acetate (6:1:1) and subsequent preparative TLC (hexane ethyl acetate, 3:1) afforded 1d (67.8 mg, 59%) and 2a (3.8 mg, 3%).

6-O-(5-phenyl-2,3-anhydro-1,2-dimannuronic acid (1d). A solution of 1a (171 mg, 2.02 mmol) in ethanol (5 ml) was added to a saturated methanolic ammonia solution (20 ml) at 0 °C in an argon atmosphere. The mixture was stirred for 2 h at 0 °C, and then concentrated in vacuo while being kept at 0 °C. The residue was purified by silica gel column chromatography (37 g). Elution with hexane ethyl acetate (25:25:1) afforded 1d (456 mg, 91%) as an oil, [α] D 20 +18.9 (c 1.07, methanol). IR νcm 1740.