Formation of β-Galactosyl Compounds of Arabinosylcytosine in Growing Culture of Sporobolomyces singularis

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Two new compounds of 1β-D-arabinofuranosylcytosine (ara-C) were found to be produced in a high yield in a culture filtrate of Sporobolomyces singularis, when grown on a medium containing lactose and ara-C. The compounds I and II were obtained as white needle crystals from the culture filtrate by preparative paper chromatography, gel-filtration on Sephadex G-10 and Toyopearl HW-40S, lyophilization, and ethanol treatment. The compounds I and II were identified as 3′-O-(β-D-galactopyranosyl)-ara-C and 3′-O-[β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl]-ara-C, respectively, on the basis of the various experimental results, viz., element analyses, UV, IR, 1H-, and 13C-NMR spectra, and products by hydrolysis with α- and β-galactosidases. Also, the yeast produced a large amount of 3′-O-β-galactosyl compounds of adenosine and inosine in the culture filtrate when grown on a medium containing lactose and their ribonucleosides.

A novel derivative of nucleoside, O-glycosyl-nucleoside, was first isolated and identified as 5′-O-α-glucosyl-adenosine by us from an incubation mixture containing maltose, adenosine, and a highly purified α-glucosidase from Aspergillus niger.1,2) Subsequently, 5′-O-β-galactosyl-inosine and several glycosyl nucleosides such as 5′-O-α-glucosyl-inosine, 5′-O-α-maltosyl-inosine, 5′-O-α-glucosyl-guanosine, and 5′-O-α-maltosyl-guanosine were isolated from the fermentation broth of Brevibacterium ammoniagenes3) and of a Bacillus sp.,4) respectively. Moreover, we showed that a crystalline β-galactosidase from Escherichia coli and a partially purified α-glucosidase from mature rice seeds transferred the glycosyl residues of o-nitrophenyl-β-D-galactopyranoside and of soluble starch to ribonucleosides, respectively, with the formation of not only 5′-O-glycosyl-ribonucleosides but also 2′ or 3′-O-glycosyl-ribonucleosides,5-8) and that these O-glycosyl-ribonucleosides were resistant to the actions of deaminase and nucleoside phosphorylase.5,6,8) But these glycosidases had low transglycosylation activities to ribonucleosides (the yields of O-glycosyl-ribonucleosides, 2–10%). Thereafter, Lichtenthaler et al. chemically synthesized these O-glycosyl-ribonucleosides, and also kindly presented 1H-, 13C-NMR, and chemical evidences that all of our enzymatically prepared 2′ or 3′-O-β-galactopyranosyl-ribonucleosides (adenosine, inosine, and uridine) were unequivocally assigned β-(1→3)-galactosidic linkages.9-12) Enzymatic formation of only either 3′-O-glycosyl-ribonucleoside or 2′-O-glycosyl-ribonucleoside without 5′-O-glycosyl-ribonucleoside-formation has not been hitherto observed.

1(β-D-Arabinofuranosyl)-cytosine (ara-C) is an important antitumor agent in experimental tumor systems or in the treatment of acute human myelogenous leukemia. Several derivatives of ara-C, such as cycloctydine,13) ara-C-5′-ester,14-16) N4-acetyl-ara-C,17,18) and 5′-(3-sulfophatidyl)-ara-C19) have been chemically synthesized. However, a glycosylated ara-C has been neither chemically nor biologically synthesized. Sporobolomyces singularis, when grown on a medium containing lactose, was found to use the glucose portion of the dissociachride as an energy source and to transfer the galactose moiety to unused lactose to form a trisaccharide [O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-D-glucose] and a tetrasaccharide [O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-D-glucose] (50% combined yield).20) In its growing cultures containing lactose and various sugar acceptors, the synthesis of β-galactopyranosyl dissociachrides took place, and the products of β-galactosyl transfer arose mainly from substitution of secondary rather than primary hydroxyl groups in the acceptor molecules.21) This paper describes a remarkable formation of a β-galactosyl-ara-C and a β-galactobiosyl-ara-C in growing culture of S. singularis, and their isolation and characterization.

Materials and Methods

Materials. Sporobolomyces singularis ATCC 24193 was obtained from the American Type Culture Collection, Maryland, U.S.A. Ara-C was obtained from Yamasa Shoyu Co., Ltd., Chiba. Crystalline β-galactosidase from Escherichia coli (Boehringer Mannheim Japan Co., Tokyo) and α-galactosidase from Mortierella vinacea (Seikagaku Kogyo Co., Tokyo) were purchased through commercial routes. β-Galactosidase from Aspergillus oryzae was kindly supplied from Kuhin Co., Tokyo. Other chemicals were of reagent grade and commercially available.

Culture. S. singularis was grown at 25°C in the dark with shaking on a culture medium (adjusted to pH 3.7) containing 5% lactose, 0.75% yeast extract, and 1.2% ara-C. Ara-C was sterilized separately, before inoculation. After a 6-day cultivation, a culture broth (1.3 liters) was centrifuged to remove the cells. The supernatant solution was used for the isolation of ara-C compounds.

Analyses. Assay of ara-C compounds. A suitable amount of the supernatant fluid

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was applied as a band on a Toyo filter paper No. 50 (40 × 40 cm), and developed twice by ascent in n-butanol-pyridine-water (6:4:4; v/v) (solvent A). After drying, bands of ara-C and its derivatives were detected on the chromatogram under an ultraviolet ray lamp (2557 Å, filter), cut out, and extracted with deionized water overnight at 37°C. The amount of ara-C compound in each extract, after the addition of HCl to give a concentration of 0.1 N, was measured by the optical density at 280 nm.

**Hydrolysis by α- and β-galactosidases.** The reaction mixture containing the isolated ara-C compound (4 mg), 5 units of enzyme, and buffer in a total volume of 1 ml was incubated for 20 h. Control experiments were done with ara-C (2.4 mg). Five hundredth m acetate buffer, pH 5.3, was used in the reaction mixture with Mortierella x-galactosidase at 25°C, 0.05 m phosphate buffer, pH 7.3, with E. coli β-galactosidase at 37°C, and 0.05 m phosphate buffer, pH 5.0, with A. oryzae β-galactosidase at 37°C. The amounts of ara-C and sugar (galactose) released were estimated by the above-mentioned assay method and the method of Nelson, respectively.

**Instrumental analyses.** Ultraviolet (UV) absorption spectra were measured with a Hitachi recording spectrophotometer model EPS-3T. Infra-red (IR) absorption spectra were measured in a KBr tablet with a Hitachi IR spectrophotometer model 260-30. 1H- and 13C-NMR spectra were taken on a Varian spectrometer model VX-500 at 500 MHz in D2O with sodium 3-(trimethylsilyl)-propionate sulfonate as internal standard. FAB-MS spectrometry was done on a VG mass spectrometer model 70 SE. [α]D25 was measured on a Nippon Bunko digital polarimeter model DIP-360.

**Results**

**Formation of new compounds of ara-C**

As shown in Fig. 1, the yeast produced two new compounds, I and II, which showed lower Rf values (I, 0.20; II, 0.13) than that of ara-C (0.42) on a paper chromatogram developed overnight with solvent A. The formation of both compounds commenced in the earlier stage of fermentation and their accumulation attained a maximum value after a 6-day incubation (yield being about 70% of ara-C added).

**Isolation of ara-C compounds I and II**

To the supernatant culture fluid (1.3 liters) of S. singularis which was cultivated for 6 days on a 5% lactose medium with 1.2% ara-C, after adjustment to pH 6.0, was added an equal volume of ethanol, and then the mixture was heated for 10 min in a boiling water bath, and centrifuged. The supernatant solution was concentrated below 30°C in vacuo. The concentrate (400 ml) was applied to the 1st preparative paper chromatography (PPC) with 400 sheets of Toyo filter paper No. 50 in solvent A, using 8 paper-developing boxes. Examination of the paper chromatogram developed twice for 2 days by ascent in solvent A showed that compounds I (0.60) and II (0.44) being slower than ara-C (1.00) in the relative rate of mobility, could be separated from glucose (0.84), galactose (0.74), and lactose (0.52) by PPC. Each of the absorption bands of compounds I and II was cut out and extracted with deionized water. Each extract, after concentration, was reapplied to the 2nd PPC with solvent A, to remove sugar contaminants. After appropriate sectioning, elution and concentration, each concentrate was subjected to the 1st gel filtration on Sephadex G-10 column (5.6 × 96 cm) and eluted with water. The fractions of each compound, after concentration, were applied to the 2nd gel filtration on Toyopearl HW-40s column (5.6 × 96 cm), eluted with water, and lyophilized. Each powdered preparation was dissolved in the mixture of ethanol and a very small amount of water, and insoluble materials were removed by filtration. To each filtrate, acetone was added until turbidity appeared, and each mixture was left in the refrigerator to give needle crystals. Each crystalline preparation of compounds I and II was recrystallized three times from the ethanol solution by the addition of acetone, and dried in vacuo on P2O5 (Yield: I, 1.12 g; II, 0.90 g). Their melting points and [α]D25 were 243–245°C, +97.8° (c = 3.74, H2O); II, 233–236°C, +107.7° (c = 2.60, H2O). FAB-MS data: I, [M + H]+ ion at 406; II, [M + H]+ ion at 568. The results of elementary analyses of the compounds were as follows. I, Found: C, 43.32; H, 5.50; N, 9.78%, Calcd. for C15H23N3O16: C, 44.45; H, 5.72; N, 10.37%. II, Found: C, 43.11, H, 5.56; N, 6.92%, Calcd. for C21H33N3O15: C, 44.45; H, 5.86; N, 7.40%.

**Identification of compounds I and II**

Rf values (I, 0.20, 0.25, 0.01; II, 0.13, 0.19, 0.01) of the isolated crystalline compounds I and II on PPC in three solvent systems [solvent A, n-butanol-acetic acid-water (2:1:1, v/v), and n-butanol saturated with water] were different from those of ara-C (0.42, 0.48, 0.12), and cytosine (0.36, 0.49, 0.16). On enzymatic hydrolysis with E. coli β-galactosidase, compound I was completely hydrolyzed to a sugar and a UV absorption substance, which showed Rf values identical to those of ara-C on paper chromatograms. The sugar component in the hydrolysate was confirmed as galactose on a kieselgel 60 plate developed with a solvent system of n-propanol-2% NH4OH (2:1, v/v) and also by a high performance liquid chromatography using a Waters carbohydrate analysis column developed with acetonitrile-water (90:10, v/v). Compound II was readily hydrolyzed by A. oryzae β-galactosidase, and very weakly by E. coli β-galactosidase. The molar ratio of ara-C and galactose liberated was around 1:1 in compound I and 1:2 in compound II. Mortierella x-galactosidase had no effect on compounds I and II. No reducing activity for compounds I and II was found by the Nelson method. UV absorption spectra of compounds I and II in 0.01 N HCl, 0.1 N HCl, deionized water, and 0.01 N NaOH showed the position of maxima and minima in the resemblance with those of ara-C. The examination of 1H-NMR spectra of ara-C, compounds I and II (in D2O, ppm) showed the following signals (Fig.

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**Fig. 1.** Formation of β-Galactosyl Compounds of Arabinosylcytosine during Fermentation.

Fermentation was done at 25°C with the culture medium (adjusted to pH 3.7) containing 5% lactose, 0.75% yeast extract, and 1.2% ara-C in the dark on a reciprocal shaker. Ara-C, ••; compound I, ○○; compound II, ○○○.
2). Ara-C: signals of H-5'A (3.91, 1H, dd, J=12.5 and 3.3 Hz), H-5'B (3.82, 1H, dd, J=12.5 and 5.9 Hz), H-4' (4.00, 1H, ddd, J=5.8, 5.4, and 3.3 Hz), H-3' (4.11, 1H, dd, J=5.3 and 4.0 Hz), and H-2' (4.39, 1H, dd, J=4.8 and 4.1 Hz), one anomic proton signal (H-1') of β-arabinosidic linkage (6.18, 1H, d, J=4.9 Hz) in the arabinose moiety, and two methyne signals of H-5 (6.02, 1H, d, J=7.6 Hz), and H-6 (7.79, 1H, d, J=7.6 Hz) in the cytosine moiety. I: signals of ring protons of the sugar moiety (3.5-4.0 ppm, 8H), signals of H-5'A (3.92, 1H, dd, J=12.4 and 3.6 Hz), H-5'B (3.85, 1H, dd, J=12.4 and 5.7 Hz), H-4' (4.16, 1H, ddd, J=5.6, 5.0, and 3.6 Hz), H-3' (4.22, 1H, dd, J=4.9 and 3.4 Hz), and H-2' (4.63, 1H, dd, J=4.7 and 3.4 Hz), one anomic proton signal (H-1') of β-arabinosidic linkage (6.19, 1H, d, J=4.8 Hz) in the arabinose moiety, one anomic proton signal (H-1'') of β-galactosidic linkage (4.49, 1H, d, J=7.8 Hz), and two methyne signals of H-5 (6.02, 1H, d, J=7.5 Hz) and H-6 (7.79, 1H, d, J=7.6 Hz)
in the cytosine moiety. II: signals of ring protons of the sugar moiety (3.5-4.0 ppm, 1H), signals of H-5' (3.92, 1H, dd, J=12.0 and 3.6 Hz), H-5'B (3.85, 1H, dd, J=11.7 and 5.5 Hz), H-4' (4.16, 1H, dd, J=5.5, 5.0, and 3.5 Hz), H-3' (4.22, 1H, dd, J=5.0 and 3.2 Hz), and H-2' (4.60, 1H, dd, J=5.1 and 2.2 Hz), one anomeric proton signal (H-1') of β-arabinofuranoside linkage (6.18, 1H, d, J=4.8 Hz) in the arabinose moiety, and two anomeric proton signals (H-1'' and H-1'''') of β-galactosidic linkage (4.52, 1H, d, J=7.9 Hz) and a β-galactosidic linkage of a non-reducing residue in the galactobiosyl moiety (4.60, 1H, d, J=8.0 Hz), and two methine signals of H-5 (6.02, 1H, d, J=7.6 Hz) and H-6 (7.79, 1H, d, J=7.5 Hz) in the cytosine moiety. These data suggested that compounds I and II were β-D-galactopyranosyl-ara-C and β-D-galactobiosyl-ara-C, respectively.

Their IR spectra showed absorption bands at 3400 to 3200 (alcoholic hydroxyl group in sugar moiety, O-H), 2900 to 2850 (C-H), 1660-1620 (C=O), 1100 to 1000 (sugar hydroxyl group, C=O), and 880 (β-D-galactopyranosyl linkage) cm⁻¹ (Fig. 3). To confirm their fine structures, carbon-13 chemical shifts of compounds I and II in D₂O were compared with those of ara-C (Table I). Signal assignments were based upon the data reported for methyl-β-D-galactopyranoside,²³) β-D-arabinofuranoside,²⁴) and cytidine²⁵) in D₂O. Comparing the arabinose carbons of compound I with those of the parent nucleoside (ara-C), it was clearly apparent that the galactosyl residue was linked to O-3', since the appended C-3' signal was displaced downfield by a sizable 6.5 ppm with only smaller effects on the other arabinose carbons. There were negligible differences in cytosine carbons between ara-C and compound I. In compound II, carbon-13 chemical shifts of the arabinose moiety were very similar to those of the compound I. The formation of compound II by galactosylation of compound I caused a large chemical shift of C-4'' in galactose carbon which was linked to the arabinose moiety. From these results, it was apparent that the galactosylation site in compound II (galactosylated compound I) was present at C-3' in the arabinose moiety and also at C-4'' in galactose carbons linked to the arabinose moiety. Thus, compounds I and II were identified as O-β-D-galactopyranosyl(1→3)-O-β-D-arabinofuranosyl(1→1)-cytosine [3'-O-(β-D-galactopyranosyl)-ara-C] and O-β-D-galactopyranosyl(1→4)-O-β-D-galactopyranosyl(1→3)-O-β-D-arabinofuranosyl(1→1)-cytosine [3'-O-[β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl]-ara-C], respectively.

Formation of β-galactosyl compounds of other ribonucleosides during fermentation

S. singularis, when grown on a culture medium (adjusted to pH 3.7) containing lactose, yeast extract, and nucleoside (such as adenosine and inosine), produced 3'-O-(β-D-galactopyranosyl)-adenosine and 3'-O-(β-D-galactopyranosyl)-inosine, and their galactobiosyl compounds (Table II). These β-galactosyl compounds were identified by comparing with the characteristics of 3'-O-(β-D-galactopyranosyl)-adenosine and 3'-O-(β-D-galactopyranosyl)-inosine synthesized by the action of E. coli β-galactosidase.

Table I. ¹³C-NMR Chemical Shifts of Compounds I and II, and Related Compounds (in D₂O)

<table>
<thead>
<tr>
<th>Carbon No.</th>
<th>Ara-C</th>
<th>Compound I</th>
<th>Compound II</th>
<th>Methyl-β-D-galactopyranoside*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>158.32</td>
<td>158.28</td>
<td>158.11</td>
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<tr>
<td>C-4</td>
<td>167.09</td>
<td>167.10</td>
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<td>C-5</td>
<td>96.23</td>
<td>96.22</td>
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<td>C-6</td>
<td>143.57</td>
<td>143.67</td>
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<tr>
<td>Arabinose</td>
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<tr>
<td>C-1'</td>
<td>86.85</td>
<td>87.03</td>
<td>87.10</td>
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<tr>
<td>C-2'</td>
<td>76.39</td>
<td>76.30</td>
<td>76.07</td>
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<tr>
<td>C-3'</td>
<td>76.26</td>
<td>82.76</td>
<td>82.80</td>
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<tr>
<td>C-4'</td>
<td>84.64</td>
<td>84.90</td>
<td>84.97</td>
<td></td>
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<tr>
<td>C-5'</td>
<td>61.64</td>
<td>61.68</td>
<td>61.58</td>
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<tr>
<td>Transferred galactose carbon</td>
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<tr>
<td>C-1''</td>
<td>103.91</td>
<td>103.87</td>
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<tr>
<td>C-2''</td>
<td>71.60</td>
<td>72.00</td>
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<tr>
<td>C-3''</td>
<td>73.48</td>
<td>73.71</td>
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<tr>
<td>C-4''</td>
<td>69.46</td>
<td>77.84</td>
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<tr>
<td>C-5''</td>
<td>75.14</td>
<td>75.05</td>
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<tr>
<td>C-6''</td>
<td>61.97</td>
<td>61.68</td>
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<tr>
<td>O-Me</td>
<td>105.16</td>
<td>104.53</td>
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* Reference 23.

Table II. Formation of β-Galactosyl Compounds of Adenosine and Inosine during Fermentation

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Days of cultivation</th>
<th>Nucleoside remaining</th>
<th>β-Galactosyl compound formed</th>
<th>β-Galactobiosyl compound formed</th>
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<tbody>
<tr>
<td>Adenosine</td>
<td>2</td>
<td>1451</td>
<td>400</td>
<td>0</td>
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<tr>
<td>Inosine</td>
<td>6</td>
<td>1246</td>
<td>420</td>
<td>182</td>
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<tr>
<td>Adenosine</td>
<td>10</td>
<td>1513</td>
<td>333</td>
<td>0</td>
</tr>
<tr>
<td>Inosine</td>
<td>10</td>
<td>1425</td>
<td>370</td>
<td>35</td>
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</table>
| Medium: 5% lactose and 3.7% nucleoside

Medium: 5% lactose and 3.7% nucleoside.

Fig. 3. Infra-Red Spectra of Compounds I and II (KBr pellet).
Discussion

It has been already pointed out by us that a highly purified α-glucosidase preparation from A. niger and a partially purified α-glucosidase preparation from mature rice seeds catalyzed the transfer of α-glucosyl residue from maltose and soluble starch to 5'-hydroxymethyl groups of adenosine and inosine, and the resultant compounds formed were 5'-O-(β-D-galactopyranosyl)-adenosine and 5'-O-(β-D-galactopyranosyl)-inosine, respectively. Brevisbacterium ammoniagenes and a Bacillus sp. produced only 5'-glycosyl-ribonucleosides in fermentation broths. On the other hand, a crystalline β-galactosidase preparation from E. coli transferred the galactosyl residue from α-nitrophenyl-β-D-galactopyranoside to both 5'-hydroxymethyl- and 2' or 3'-hydroxygroups of ribonucleosides to form 5'-O-(β-D-galactopyranosyl)-adenosine, 2' or 3'-O-(β-D-galactopyranosyl)-inosine, 5'-O-(β-D-galactopyranosyl)-uridine, and 2' or 3'-O-(β-D-galactopyranosyl)-uridine. The chemical structures of these 2' or 3'-O-β-galactopyranosyl-ribonucleoside preprations formed on enzymatic galactosylation by us were unequivocally assigned to be 3'-O-β-galactopyranosyl-ribonucleosides, using NMR analyses and chemical synthesis by Lichtenthaler et al. Moreover, not only two new sugar compounds, 3'-O-(β-D-galactopyranosyl)-ara-C and 3'-O-(β-D-galactopyranosyl)-(1→4)-O-β-D-galactopyranosyl)-ara-C, but also 3'-O-β-D-galactopyranosyl-adenosine and 3'-O-β-D-galactopyranosyl-inosine have been found by us to be formed in a high yield in a growing culture of S. singularis. There is no report on the enzymatic formation of 2'-O-glycosyl-ribonucleosides. The enzyme catalyzing the synthesis of 3'-O-β-galactosyl-ara-C and 3'-O-β-galactobiosyl-ara-C remains to be investigated.

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