*Biosci. Biotechnol. Biochem.*, **75** (12), 2418–2420, 2011

*Note*

# Enzymatic Synthesis of 4-Pentulosonate (4-Keto-D-pentonate) from D-Aldopentose and D-Pentonate by Two Different Pathways Using Membrane Enzymes of Acetic Acid Bacteria*

Osao ADACHI,[1,†] Roque A. HOURS,[2] Emiko SHINAGAWA,[3] Yoshihiko AKAKABE,[1] Toshiharu YAKUSHI,[1] and Kazunobu MATSUSHITA[1]

[1]*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*
[2]*Research and Development Center for Industrial Fermentation (CINDEFI; UNLP, CONICET La Plata), School of Science, La Plata National University, 47 y 115 (B1900ASH), La Plata, Argentina*
[3]*Department of Chemical and Biological Engineering, Ube National College of Technology, Tokiwadai, Ube 755-8555, Japan*

Received August 5, 2011; Accepted September 20, 2011; Online Publication, December 7, 2011
[doi:10.1271/bbb.110575]

**4-Keto-D-arabonate (D-*threo*-pent-4-ulosonate) and 4-keto-D-ribonate (D-*erythro*-pent-4-ulosonate) were prepared from D-arabinose and D-ribose by two successive reactions of membrane-bound enzymes, D-aldopentose 4-dehydrogenase and 4-keto-D-aldopentose 1-dehydrogenase of *Gluconobacter suboxydans* IFO 12528. Alternatively, they were prepared from D-arabonate and D-ribonate with another membrane-bound enzyme, D-pentonate 4-dehydrogenase. Analytical data confirmed the chemical structures of the 4-pentulosonates prepared. This is the first report of successful enzymatic synthesis of 4-pentulosonates.**

**Key words:** D-pentonate 4-dehydrogenase; D-aldopentose 4-dehydrogenase; 4-keto-D-aldopentose; 4-keto-D-aldopentose 1-dehydrogenase; 4-pentulosonate

In a previous study,[1)] 4-keto-D-arabonate (D-*threo*-pent-4-ulosonate, 4KAB) accumulation in a culture medium of *Gluconacetobacter liquefaciens* RCTMR 10 was confirmed as one of the metabolites derived from 2,5-diketo-D-gluconate (25DKA) in the D-glucose oxidizing system of acetic acid bacteria. Two sequential enzymes, 25DKA decarboxylase and 4-keto-D-arabinose 1-dehydrogenase, were necessary for 4KAB formation. Identification of 4KAB was the first finding of a novel sugar acid in carbohydrate chemistry. In our recent study,[2)] 4KAB was formed after C4-position specific oxidation of D-arabonate by a membrane-bound D-pentonate 4-dehydrogenase. As an alternative route for 4KAB formation, D-arabinose was oxidized to 4-keto-D-arabinose (4KAR) with a membrane-bound D-aldopentose 4-dehydrogenase. The putative 4KAR was further oxidized to 4KAB by 4-keto-D-aldopentose 1-dehydrogenase. Likewise, D-ribose was oxidized to 4-keto-D-ribonate (D-*erythro*-pent-4-ulosonate, 4KRN) *via* 4-keto-D-ribose (4KRB), similarly to the case of D-arabinose to 4KAB. Furthermore, D-ribonate was oxidized to 4KRN by the same membrane-bound enzyme, D-pentonate 4-dehydrogenase, in *G. suboxydans* IFO 12528. To confirm these observations, in this study, we attempted enzymatic synthesis of 4KAB from D-arabinose and D-arabonate, and of 4KRN from D-ribose and D-ribonate.

*G. suboxydans* IFO 12528 was grown on a medium containing 0.5% D-glucose, 2% Na-D-gluconate, 0.3% glycerol, 0.3% yeast extract (Oriental Yeast, Tokyo), and 0.2% polypeptone until microbial growth reached the early stationary phase. Preparation of the membrane fraction was done as described previously.[2)] A wet paste of precipitated membrane fraction was lyophilized (Taitec, VD-800F, Tokyo) and stored at $-20\,^{\circ}C$ until use. The reaction mixture contained 1 g of dried membrane fraction of *G. suboxydans* IFO 12528 and 300 mg of substrate in 100 mL of 10 mM acetate buffer. Acetate buffer pH 5.0 was used for D-aldopentose oxidation and acetate buffer pH 4.0 was used for D-pentonate oxidation. The reaction was carried out overnight at $30\,^{\circ}C$ under stirring. The reaction mixture was centrifuged at $10^5 \times g$ for 60 min to precipitate the membrane fraction. The clear supernatant was applied to a column of Dowex $1 \times 4$ ($1 \times 10$ cm, acetate form), and the non-adsorbed materials were passed through the column by washing the column with water. Elution of the column was done by a linear gradient concentration of acetic acid formed by 300 mL of water and 300 mL of 0.2 M acetic acid, and 10 mL fractions were collected. Elution was checked by TLC chromatography under conditions reported previously.[1)] Preliminary detection of a keto-compound that formed in the reaction mixture was done by TLC-chromatography by spraying an alkaline-ethanol solution of 2,3,5-triphenyltetrazolium chloride (TTC) over the TLC plate as described previously.[1,2)] The acetic acid in the pooled fractions was removed by evaporation under reduced pressure at $45\,^{\circ}C$. The resulting viscous liquid was neutralized with finely powdered $CaCO_3$. After the brown color was removed by the addition of activated charcoal, the solution was evaporated until crystals appeared.

* Part of this paper was presented at the 13th International Congress of Bacteriology and Applied Microbiology held in Sapporo, Japan, September 6–10, 2011, and at the 16th Japanese-German Workshop on Enzyme Technology held in Toyama, Japan, September 14–15, 2011.

† To whom correspondence should be addressed. Tel: +81-83-933-5857; Fax: +81-83-933-5859; E-mail: osao@yamaguchi-u.ac.jp

*Abbreviations*: 4KAB, 4-keto-D-arabonate; 4KAR, 4-keto-D-arabinose; 4KRB, 4-keto-D-ribose; 4KRN, 4-keto-D-ribonate

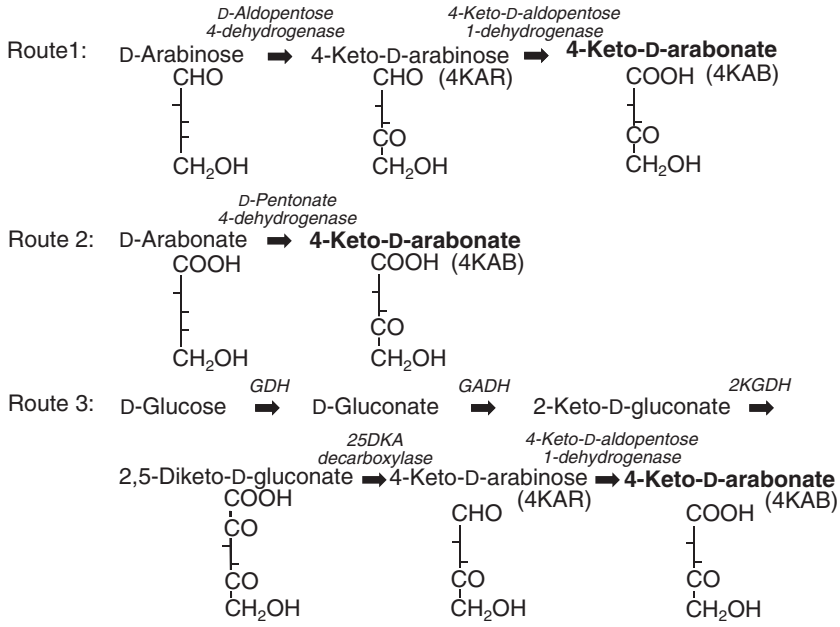


**Fig. 1.** Three Different Enzymatic Routes Found in Acetic Acid Bacteria Leading to the Formation of 4-Keto-D-arabonate (D-*threo*-Pent-4-ulosonate, 4KAB).

Route 1, 4KAB formation from D-arabinose; Route 2, 4KAB from D-arabonate; Route 3, 4KAB from D-glucose (previous studies[1,2]). GDH, quinoprotein D-glucose dehydrogenase; GADH, D-gluconate dehydrogenase; 2KGDH, 2-keto-D-gluconate dehydrogenase; 25DKA, 2,5-diketo-D-gluconate. All the enzymes that figured in individual reactions are indicated in italics.

D-Arabinose was incubated with the membrane fraction of *G. suboxydans* IFO 12528, and the supernatant of the reaction mixture was applied to a Dowex $1 \times 4$ column (acetate form). Only one TTC-positive spot, corresponding to 4KAB, appeared in the eluted chromatogram, but the putative 4KAR passed through the column and was found in the non-adsorbed fractions. The analytical data for 4KAB prepared from D-arabinose showed good coincidence to those for 4KAB.[1)] When D-arabonate was incubated with the membrane-fraction of *G. suboxydans* IFO 12528, only one TTC-positive spot appeared on a TLC plate, with an *Rf* value of 0.6. Chromatographic purification of the TTC-positive compound with a Dowex $1 \times 4$ column was done, and the compound came out from the column at an acetic acid concentration of 0.05–0.1 M. The compound was finally crystallized as Ca-salt.

The analytical data with the oxidation products from D-arabinose and D-arabonate are as follows: IR $\nu_{max}$ (KBr) $cm^{-1}$: 3375 (s), 1723 (s), 1611 (s), 1423 (m), 1306 (m), 1245 (m), 1113 (m), 1702 (m), 986 (w). $^1H$ NMR $\delta_H$ (400 MHz, $D_2O$): 4.46 (d, 1H, $J = 2.0$ Hz, H2), 4.57 (s, 1H, H5), 4.63 (s, 1H, H5), 4.69 (d, 1H, $J = 2.0$ Hz, H3). $^{13}C$ NMR $\delta_C$ (100 MHz, $D_2O$): 65.99 (C5), 73.05 (C2), 76.88 (C3), 177.13 (C1), 212.38 (C4). HRMS: calcd. $C_5H_7O_6$ for $(M^+ - H)$, 163.0243; found, 163.0240.

In addition to the presence of a hydroxy group (3375 $cm^{-1}$), the IR spectrum revealed the absorption of carbonyl groups (1723 $cm^{-1}$ for keto and 1611 $cm^{-1}$ for carboxy), as confirmed by the $^{13}C$ signals at 212.4 and 177.1 ppm. The H-H COSY spectrum revealed correlations between H-2 and H-3 and the presence of an isolated methylene group at C-5. The HMBC spectrum showed correlations of H-5 to C-4 and C-3, and correlation of H-2 to C-1. Based on these data, the oxidation product was identified as D-*threo*-pent-4-ulosonate (4-keto-D-arabonate, 4KAB). The two different routes of enzymatic synthesis of 4KAB starting from D-arabinose and D-arabonate gave entirely the same results which were identical to those of a previous study.[1)] With 4KAB formation from the D-glucose oxidizing system,[1,2)] three different pathways for 4KAB formation have thus been confirmed (Fig. 1).

As found in the previous study,[2)] D-ribose was oxidized to putative 4KRB by D-aldopentose 4-dehydrogenase in the membrane fraction of *G. suboxydans* IFO 12528, and then further oxidized to 4KRN by 4-keto-D-aldopentose 1-dehydrogenase. The putative 4KRB was not adsorbed into the Dowex $1 \times 4$ column under the conditions imposed and passed through the column together with other impurities. The formation of a single TTC-positive spot from Dowex $1 \times 4$ chromatography was found at an *Rf* value of 0.7 in the TLC chromatogram. It was at the same location as 4KRN. D-Ribonate was oxidized with the same membrane fraction under the conditions described above. After purification of the compound, the white crystals were analyzed.

The analytical data with the oxidation products from D-ribose and D-ribonate are as follows: IR $\nu_{max}$ (KBr) $cm^{-1}$: 3361 (s), 2903 (m), 1726 (s), 1598 (s), 1380 (m), 1289 (m), 1081 (m), 1028 (m), 991 (w), 954 (w). $^1H$ NMR $\delta_H$ (400 MHz, $D_2O$): 4.44 (d, 1H, $J = 2.8$ Hz, H2), 4.55 (s, 1H, H5), 4.56 (s, 1H, H5), 4.59 (d, 1H, $J = 2.8$ Hz, H3). $^{13}C$ NMR $\delta_C$ (100 MHz, $D_2O$): 66.21 (C5), 74.62 (C2), 77.88 (C3), 176.43 (C1), 211.13 (C4). HRMS: calcd. $C_5H_7O_6$ for $(M^+ - H)$, 163.0243; found, 163.0247.

In addition to the presence of a hydroxy group (3361 $cm^{-1}$), the IR spectrum revealed the absorption of carbonyl groups (1726 $cm^{-1}$ for keto and 1598 $cm^{-1}$ for carboxy), as confirmed by the $^{13}C$ signals at 211.1 and 176.4 ppm. The H-H COSY spectrum revealed correlations between H-2 and H-3 and the presence of an isolated methylene group at C-5. The HMBC spectrum

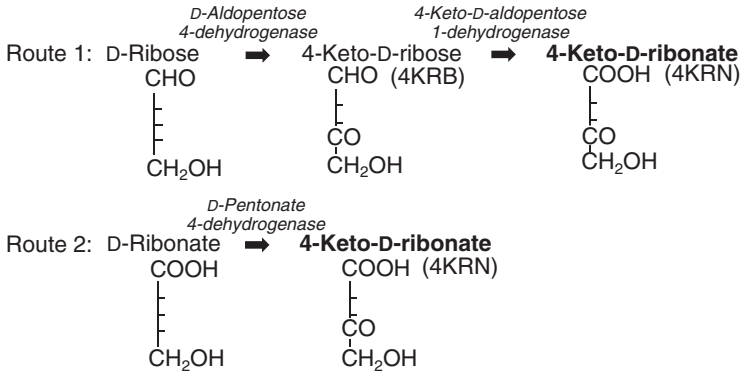


**Fig. 2.** Two Different Enzymatic Routes Found in Acetic Acid Bacteria Leading to the Formation of 4-Keto-D-ribonate (D-*erythro*-Pent-4-ulosonate, 4KRN).

Route 1, 4KRN formation from D-ribose; Route 2, 4KRN from D-ribonate. All the enzymes that figured in individual reactions are indicated in italics.

showed correlations of H-5 to C-4 and C-3, correlations of H-3 to C-4, C-2, and C-1, and correlations of H-2 to C-4, C-3, and C-1. Based on these data, the oxidation product was identified as D-*erythro*-pent-4-ulosonate (4-keto-D-ribonate, 4KRN). Thus, it was found in this study that 4KRN was formed by two different pathways, as shown in Fig. 2.

In addition to putative 4KRB, putative 4KAR remained to be purified, because they were not adsorbed into the Dowex $1 \times 4$ column under the conditions imposed. To identify these two putative compounds, purification of D-aldopentose 4-dehydrogenase is underway. We hope to indicate in future that 4KAR and 4KRB are direct oxidation products of D-arabinose and D-ribose respectively by the action of D-aldopentose 4-dehydrogenase. As preliminary information, D-aldopentose 4-dehydrogenase is not identical to D-pentonate 4-dehydrogenase in many respects, including catalytic and physicochemical properties.

## Acknowledgments

A part of this work was supported by the FY2010 Researcher Exchange Program between the Japan Society for the Promotion of Science (JSPS) and the Argentine National Council for Scientific and Technological Research (CONICET).

## References

1) Adachi O, Hours RA, Akakabe Y, Tanasupawat S, Pattaraporn Y, Shinagawa E, Yakushi T, and Matsushita K, *Biosci. Biotechnol. Biochem.*, **74**, 2555–2558 (2010).
2) Adachi O, Hours RA, Akakabe Y, Shinagawa E, Yakushi T, and Matsushita K, *Biosci. Biotechnol. Biochem.*, **75**, 1801–1806 (2011).