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Proteus mirabilis Dehydrogenates Aldonates and Aldarates with an (*R*)-Configured α-Carbon Atom to the Corresponding 2-Oxocarboxylates

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Abstract—Resting cells of *Proteus mirabilis* effectively dehydrogenate aldonates and aldarates to the corresponding 2-oxocarboxylates (Figure 2). The prerequisite is an (*R*)-configured α-carbon atom next to the carboxylate group. The oxidation reagent is dimethylsulfoxide and the electron mediator is anthraquinone-2,6-disulfonate (Figure 1). The reactions mostly proceed quantitatively in concentrations up to 0.5 M. The two enzymes necessary for the dehydrogenation, (2*R*)-hydroxycarboxylate viologen oxidoreductase and dimethylsulfoxide reductase, are present in *P. mirabilis* in high activities. Nine aldonates have been dehydrogenated to 2-glyculosonates (2-oxoaldonates) and two aldarates to α-oxo aldarates. As shown with lactobionate and 6-phospho-D-gluconate, derivatives of aldonates can be dehydrogenated too. The apparent K_m values of the substrates are often < 1 mM. The products were isolated as sodium or potassium salts with yields between 65 and 98 % and characterized. D-*xylo*-Hex-2-ulosonate obtained from D-gulonate was converted to D-ascorbic acid.

Introduction

Biocatalyzed redox reactions on a preparative scale can be conducted by enzymes or whole cells. On a preparative scale much fewer enzymatic dehydrogenations than reductions are described.[1a-c] Using redox enzymes requires coenzyme regeneration. Many methods have been reported for the pyridine nucleotides.[2a,b] So far only the regeneration of NADH is a generally solved problem. The regeneration of NADPH and the oxidized pyridine nucleotides NAD(P) is often more complicated. Furthermore, dehydrogenations with pyridine nucleotide dependent enzymes are often restricted by thermodynamic reasons or inhibition phenomena by which the elegance of biocatalysis is seriously hampered.

Reductions with resting or growing microbial cells have mostly been conducted with yeasts.[3a-c] These systems are often not very effective considering the ratio of the amount of biocatalyst used to product formed,[4] and are not always of satisfactory stereoselectivity.[5] Dehydrogenations with yeasts or other microorganisms have not been conducted very often. Nevertheless, they can be very valuable as can be seen from the vitamin C synthesis.[6]

(2*R*)-Hydroxycarboxylate viologen oxidoreductase (HVOR), detected by us in *P. mirabilis* and *P. vulgaris* sometime ago[7a,b] catalyzes the following reversible reaction (1):

$$RCOCOO^- + Med_{red} \rightleftharpoons (R)\text{-}RCHOHCOO^- + Med_{ox} \quad (1)$$

Dedicated to Prof. J. B. Jones on the occasion of his 60th birthday.

The use of this pyridine nucleotide independent enzyme activity, in the form of whole resting cells together with suitable artificial mediators, for reductions or dehydrogenations on a preparative scale has been demonstrated in various papers.[4,7a-e,8] In the form of a short note, some of the results described here have been published too.[9]

The molybdenum containing HVOR present in various *Proteus* species[7a,f] for which the natural electron mediator is not known, is useful for the following reasons: (i) it has an extremely broad substrate specificity for reversibly reducing 2-oxocarboxylates (eq. 1). Also 2-oxocarboxylates with one or two conjugated carbon–carbon double bonds, an additional carboxylate or carbonyl group are effectively reduced in a complete regio- and stereospecific manner.[7a,c,d] The same is true for the carbonyl group of N-acetylneuraminic acid.[9] (ii) The enzyme reacts with many different artificial electron carriers as cosubstrates varying in their redox potential by more than 600 mV.[7e,9] Therefore, depending on the redox potential of the artificial mediator (eq. 1) the equilibrium constants (K) of reaction (1) can be changed by more than 20 orders of magnitude. Taking eq. 1 and the relation $\Delta G^\circ = -nFE^{\circ\prime} = -RT \ln K$ for the mediators methylviologen ($E^{\circ\prime}$ = –440 mV) and dichlorophenol–indophenol ($E^{\circ\prime}$ = 217 mV) the equilibrium constants K of reaction (1) are 7.5×10^{14} and 4.6×10^{-8}, respectively, calculated for $n = 2$ at pH 7 and 298 Kelvin. (iii) If the mediators are not protonated in the reduced and oxidized form as is the case with viologens, also the pH value under which the reaction is conducted has a very pronounced influence on the actual equilibrium constant K' = $K/[H^+]^2$. The situation becomes more complicated if the reduced form of the mediator is dissociated partially into an anionic form and protons. (iv) Reactions up to 0.6 M

substrate concentrations can be carried out because there is generally no serious substrate or product inhibition.[7c,8]

If reaction (1) is conducted with benzylviologen (BV) $E^{\circ\prime}$ = –330 mV (in the literature values for $E^{\circ\prime}$ differ from –311 mV up to –363 mV[10]) as the mediator at pH 6 the 2-oxocarboxylates will be completely reduced since the equilibrium constant for the reduction of pyruvate as an example is 8 x 10^6. At pH 8.5 with anthraquinone-2,6-disulfonate (AQDS) ($E^{\circ\prime}$ = –184 mV) the equilibrium constant of reaction (1) is about 6 orders of magnitude smaller than with benzylviologen and dehydrogenations of (2*R*)-hydroxy acids can be conducted quantitatively only when the reduced quinone is effectively reoxidized for instance by reaction (2):

$$\text{DMSO} + \text{AQDS–H}_2 \rightleftharpoons \text{DMS} + \text{H}_2\text{O} + \text{AQDS} \qquad (2)$$

The pair dimethylsulfoxide/dimethylsulfide (DMSO/DMS) has an $E^{\circ\prime}$ = +160 mV. The ΔG of reaction (2) is –25.1 kJ/mol at pH 8.5 and 25 °C corresponding to a K = 2.5 x 10^4. The combination of reaction (1) and (2) with AQDS as mediator is easily possible since *P. mirabilis* and *P. vulgaris* possess a DMSO reductase. Its activity depends on the growth conditions.[7e,8] By running reaction (1) from the right hand side to the left using 1 mM AQDS, up to 0.65 M (*R*)-lactate can be completely dehydrogenated with DMSO according to Figure 1.[8]

As recently briefly announced, *P. mirabilis* is generally useful for the dehydrogenation of polyhydroxycarboxylates such as aldonates and aldarates, if an α-carbon atom to the carboxylate group is of (*R*)-configuration.[9] A series of such dehydrogenations and the partial characterisation of the products is presented in this paper.

Some methods are known for the preparation of 2-glyculosonates (2-oxoaldonates).[6,11a–c] The catalytic dehydrogenation of α-carbon atoms of aldonates from the hexose series to 2-glyculosonates shows a pronounced time optimum and up to 5 or 6 by-products.[11b] A fermentative process is described by which a *Pseudomonas* species converts glucose to D-*arabino*-hex-2-ulosonate (2-oxo-D-gluconate) in a 0.32 M solution, which was converted to iso-vitamin C.[12] As recently shown by the group of Wong[11c] with the synthesis of 3-deoxy-D-*manno*-2-octulosonic acid (KDO), 2-glyculosonates can also be prepared by aldolase catalyzed reactions. Important is the preparation of L-*xylo*-hex-2-ulosonate (2-oxo-L-gulonate), which is converted to L-ascorbic acid.[6]

Results and Discussion

Figure 1 shows the type of, and Figure 2 the actually, conducted reactions. The HVOR dehydrogenates (*R*)-configured α-hydroxycarboxylates to the 2-oxocarboxylates and transfers the electrons to AQDS, which in turn reduces the DMSO reductase. The latter is reoxidized by reducing DMSO to DMS. Due to its boiling point of 38 °C and low solubility in water it leaves the system.

That HVOR is the enzyme in *P. mirabilis* which dehydrogenates the aldonates was checked using purified HVOR in tests described below. There seems to be no other enzyme present catalyzing the dehydrogenation of aldonates in the presence of an artificial electron acceptor since after electrophoresis of a crude bacterial extract only such spots reacting with an antibody against HVOR also showed the dehydrogenation of an aldonate. This was indicated by dipping the gels after electrophoretic separation under exclusion of oxygen into a solution of D-gluconate and oxidized carboxamido-methylviologen (CAV^{2+}) ($E^{\circ\prime}$ = –296 mV). The appearance of blue spots on the electrophoretic gels (not shown) due to the formation of $CAV^{+\cdot}$ is indicative for the dehydrogenation (reaction (1) running from right to left).

The apparent K_m values of the substrates are mostly < 1 mM (Table 1). They were determined with crude extract of *P. mirabilis* to which the substrates were added as salts at pH 8.5. The apparent V_{max} values are 2–13 % of about 5 U for (*R*)-lactate dehydrogenation.[7e,8] But the absolute values are still rather high when compared with redox enzyme activities of many other microorganisms for rather simple substrates. Nakamura *et al.*[5] report for β-oxoester reductases in yeasts, specific activities of about ≤ 0.02 U/mg protein.

The aldonates (**1–9**) and aldarates (**10**, **11**) (Table 1) have been dehydrogenated to 2-oxo acids shown in Table 2. If **1** is dehydrogenated (exp. 1) most of the substrate disappears but no D-*arabino*-hex-2-ulosonate is formed. The addition of low concentrations of ethylenediamine tetraacetate

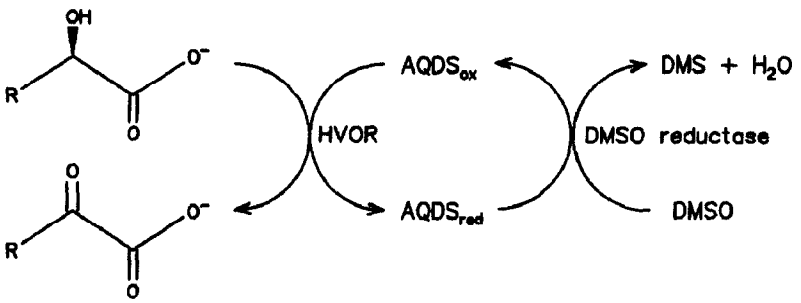

Figure 1. Dehydrogenation of carbohydrate derivatives containing an (*R*)-configured carbon atom in α-position to a carboxylate group and the regeneration of the reduced form of the artificial mediator anthraquinone-2,6-disulfonate by reduction of dimethylsulfoxide catalyzed by (2*R*)-hydroxycarboxylate viologen oxidoreductase and dimethylsulfoxide reductase present in *P. mirabilis*.

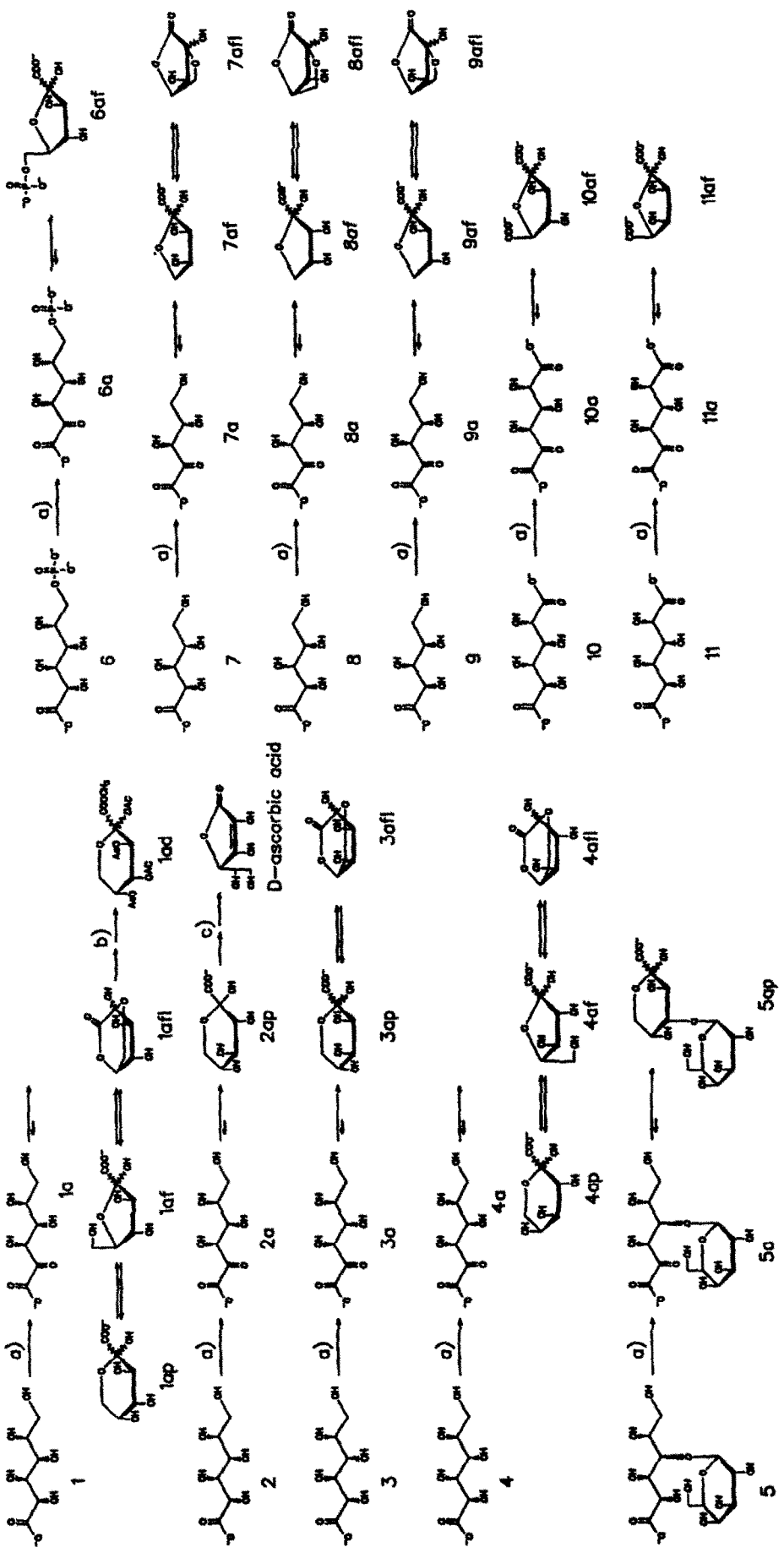

Figure 2. The structures indicated here are listed with their NMR data in Table 3. p Indicates pyranose, f furanose and fl furanose/lactone forms; a. is the dehydrogenation by *P. mirabilis*; b. prepared according to l.c. 23; c. prepared according to l.c. 24.

(EDTA) changes this drastically as can be seen from experiment 2 (Table 2). EDTA was also essential in the production of pyruvate from (*R*)-lactate.[8] In the latter case we applied it for complexing ferrous ions, which are essential for the activation of the pyruvate formate lyase, converting pyruvate to acetyl-CoA and formate.[13]

As shown in Table 2 the productivity numbers (PN = mmol product per kg dry biocatalyst and hour[4]) are between 140 and 760. The yields of isolated products vary between 65 and 98 %. The yields of conversion are in the most cases above 95 %. An exception is **11a** which was formed only up to 79 % with the lowest PN. The comparison of **1a**, **5a** and **6a** shows that rather large substituents are tolerated. A negatively charged group in the form of a phosphate residue diminishes the rate of product formation more than a bulky group such as a galactopyranosido residue. The aldarates are more slowly converted than the aldonates.

HVOR is not able to dehydrogenate L-gluconate and D-mannonate. This can be understood since both aldonates possess only (*S*)-configured α-hydroxycarboxylate arrangements. Also not dehydrogenated are aldoses and uronates with an (*R*)-configured α-carbon atom adjacent to the carbonyl group but an (*S*)-configured carbon atom adjacent to the carboxylate group. This was seen e.g. with D-glucuronate and D-*arabino*-furanosiduronate, which were not converted. But as expected L-arabinuronate (**12**) was dehydrogenated under the conditions of the optical test. The time course of the dehydrogenation of a 0.2 M solution of **1** to **1a** and in other experiments of **2**, **3** and **4** to **2a**, **3a** and **4a**, respectively is shown in Figure 3.

Figure 4 shows the consumption of **6** agreeing rather well with that of DMSO indicating that only one alcohol group is dehydrogenated and the progress of the reaction could also be measured by the amount of dimethylsulfide leaving the system.

The ^{13}C-NMR signals of the products are shown in Table 3.

None of the substrates showed a peak near δ = 100 (not shown), but such a signal is present around 100 ppm in the pyranose or furanose forms of the products **1a** to **11a** and is typical for the carbonyl group formed by dehydrogenation, present in an acetal structure.[15] The various ^{13}C-NMR signals shown in Table 3 were grouped according to their size and by comparison with other structures (Figure 2). For example phosphate **6a** and the pentose derivatives **7a**, **8a** and **9a** are not able to form a pyranose ring. They all show signals about 2–7 units above 100 ppm. **1a** in the form of its tetraacetate methyl

Table 1. Apparent K_m values determined with crude extracts of *P. mirabilis* at 38 °C and pH 8.5. The data were calculated according to Eadie and Hofstee.[14a,b] The substrates were tested in the presence of 6 mM AQDS. The K_m values for the mediators were determined with 50 mM **1** as the substrate.

Substrate	K_m (mM)	V_{max} (U/mg)
D-lactate	0.68	5.10
D-gluconate (1)	0.98	0.20
D-gulonate (2)	0.64	0.15
D-galactonate (3)	0.48	0.25
L-mannonate (4)	0.92	0.20
lactobionate (5)	0.66	0.15
6-phospho-D-gluconate (6)	0.64	0.20
L-arabinonate (7)	2.03	0.30
D-ribonate (8)	0.30	0.65
D-xylonate (9)	1.27	0.15
D-glucarate (10)	1.68	0.40
galactarate (11)	0.42	0.10
L-arabinuronate (12)	1.46	0.15
$AQDS_{ox}$	0.19	0.50
CAV^{2+}	0.025	0.25
BV^{2+}	0.042	0.35
(DMSO)[a)]	0.66	0.45

a) Kinetic data for the DMSO reductase were determined with 0.6 mM reduced AQDS.

Table 2. Isolated products prepared according to Figure 1 and 2 with *P. mirabilis*.

Exp-No.	Products	Conversion [%]	Yield as isolated products [%]	PN
1	D-*arabino*-hex-2-ulosonate (1a)[a]	92	< 1	-
2	D-*arabino*-hex-2-ulosonate (1a)	96	85	760
3	D-*xylo*-hex-2-ulosonate (2a)	97	95	740
4	D-*lyxo*-hex-2-ulosonate (3a)	99	98	650
5	L-*arabino*-hex-2-ulosonate (4a)	98	88	210
6	4-O-(ß-D-galactopyranosido)-D-*arabino*-hex-2-ulosonate (5a)	99	70	480
7	6-phospho-D-*arabino*-hex-2-ulosonate (6a)	99	98	250
8	L-*erythro*-pent-2-ulosonate (7a)	98	65	280
9	D-*erythro*-pent-2-ulosonate (8a)	99	71	690
10	D-*threo*-pent-2-ulosonate (9a)	99	78	270
11	2-oxo-D-glucarate (10a)	99	91	270
12	2-oxogalactarate (11a)	79	70	140

a) Without EDTA most of D-gluconate and D-*arabino*-hex-2-ulosonate is converted to acetate (see text).

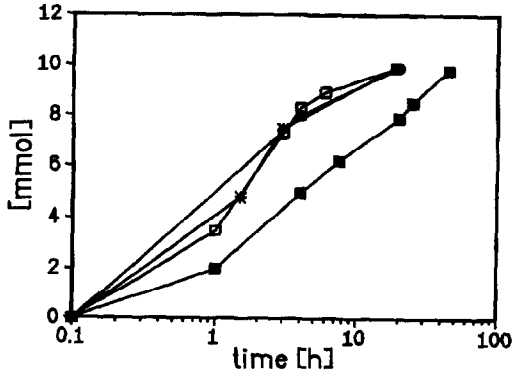

Figure 3. Time course of the dehydrogenation of D-gluconate (○), D-gulonate (□), D-galactonate (*), and L-mannonate (■). A total volume of 50 mL water (no buffer) containing 10 mmol aldonate, 15 mmol DMSO, 0.05 mmol AQDS, 0.25 mmol EDTA, 5 x 10^{-5} mmol tetracycline and 0.8 g *P. mirabilis* (dry weight) was adjusted to pH 9.0 and kept at this value by adding 2 N NaOH with a pH-stat. Temperature 38 °C.

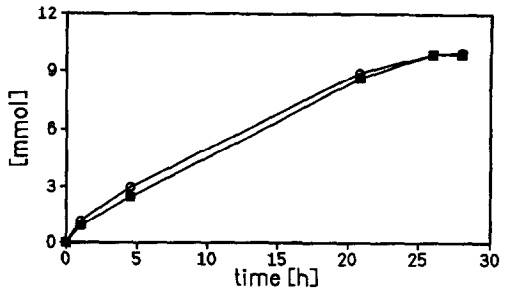

Figure 4. Dehydrogenation of **6** to **6a** under conditions given for Figure 3. Consumption of **6** (○) and dimethylsulfoxide (■).

ester derivative (**1ad**) and **2a–5a** show additional values between 99 and 100 ppm. These seem to be characteristic for pyranose forms. The ^{13}C-NMR spectrum obtained from commercially available **1a** was identical with that prepared by us. Whether **1a** is an α- or β-form was not decided. Other forms of **1a**, **3a**, **4a** and **7a–9a** present in aqueous solution seem to be lactones without a signal near 100 ppm but one at about 85 ppm. The products **1a–3a** differ by the forms present in aqueous solution. The **1a** tetraacetate methyl ester derivative (**1ad**) exists only in the pyranose configuration. The proportions of pyranose and furanose forms seem to depend on the configuration of the carbon atoms C-3 to C-5. For **2a** only one tautomeric form could be observed by ^{13}C-NMR spectroscopy.

According to the literature, α-pyranose derivates show two typical IR absorption bands at 915 cm^{-1} and 847 cm^{-1}. The β-anomers show only one band.[16] The IR spectrum of **2a** showed clearly these two bands (not shown).

The presence of 2 or 3 epimers in solution causes a complex mutarotation behavior. Table 4 gives an impression, **2a** stays rather constant. This is in accordance with the fact that only one form can be seen by ^{13}C-NMR spectroscopy. However, to analyze and to discuss this bevavior is not the purpose of this paper.

Table 3. ^{13}C NMR data of the products **1a–11a** in $^{2}H_2O$ recorded at 360 MHz. Chemical shifts are given in ppm relative to an internal sodium 3-(trimethylsilyl)-propanesulfonate standard at 25 °C.

Products	chemical shifts in ppm					
	C-1	C-2	----C-3 - C-5----			C-6
1a:						
pyranose form	177.3	99.9	72.7	72.0	72.0	67.0
furanose form	178.1	102.3	83.6	80.9	76.9	64.7
lactone form	182.8	85.3	85.0	78.2	75.0	63.9
1ad: tetra-acetate Me ester:						
pyranose form	[a]	96.1	67.8	67.7	66.6	63.6
2a:						
pyranose form	177.1	99.5	76.8	75.2	72.1	65.3
3a:						
pyranose form	177.7	99.7	74.2	73.7	68.9	65.2
lactone form	182.3	83.9	83.4	76.5	73.5	63.8
4a:						
pyranose form	177.3	99.9	72.7	72.0	72.0	67.0
furanose form	177.9	102.3	83.6	80.9	77.0	64.8
lactone form	182.5	85.3	85.0	78.2	75.1	63.9
5a:						
pyranose form	177.0	99.8	73.5	70.4	69.6	66.6
(β-D-galactopyranosido	103.4	80.1	78.0	75.3	71.4	63.8)
6a:[b]						
furanose form 1	178.2	102.3	82.8	80.8	77.1	66.9
furanose form 2	178.0	107.1	82.9	79.3	77.3	66.9
10a:[c]						
furanose form 1	180.8	107.5	84.3	82.9	77.9	178.1
furanose form 2	180.8	103.2	83.4	80.9	80.5	179.4
11a:[c]						
furanose form 1	178.5	107.3	84.3	80.5	75.5	177.2
furanose form 2	178.5	103.7	84.2	76.4	76.0	177.9
D-ascorbic acid from 2a	175.9	158.1	120.6	78.9	71.6	64.8
	C-1	C-2	--C-3--	--C-4--	C-5	
7a:						
furanose form 1	178.9	103.0	77.3	75.2	72.5	
furanose form 2	178.6	107.0	79.5	75.3	73.8	
lactone form	178.8	85.9	77.8	77.3	65.2	

Table 3. *Continued*

8a:					
furanose form 1	178.9	103.0	76.3	75.2	72.5
furanose form 2	178.6	107.2	79.4	75.3	73.7
lactone form	178.7	85.9	77.8	77.3	65.2
9a:					
furanose form	179.4	103.8	78.1	75.3	73.2
lactone form[d]	178.8	85.9	77.8	77.3	65.2

a) Measured in $CDCl_3$. The values for C-1 and the carbonyl groups of the acetyl residues are not well separated in the area of 170 ppm. A signal at 53.6 ppm shows the methyl group; b) the ^{31}P-NMR of the starting material **6** showed one value $\delta = 7.96$. In the aqueous solution of **6a** two peaks appeared at $\delta = 7.28$ and 8.04 in a ratio of about 7:1. Phosphoric acid was used as an external standard; c) C-1 and C-6 cannot be differentiated with certainty; d) the crystalline product immediately after solubilization.

Table 4. Optical rotation measurements of various products usually as sodium or potassium salts in water, 5 min and 20 h after dissolving and afterwards for 20 h in phosphate buffer pH 7.0.

Product	Salt	pH[a]	$[\alpha]^{25}_{[D]}$ 5 min	20 h[b]	20 h[c]
1a[d]	Na	8.2	-64.6	-63.5	-130.7
2a[e]	Na	6.7	+22.6	+17.3	+25.1
3a[f]	Na	9.1	-85.4	-7.5	-14.6
4a	Na	7.5	+47.5	+50.0	+97.0
5a	Na	5.9	-36.4	-42.1	-79.3
6a	Na	7.6	-10.1	-7.6	-2.9
7a	K	7.1	+13.5	-96.6	+23.6
8a	Na	8.6	-72.6	-212.4	-27.8
9a	K	5.5	-10.4	-6.5	-8.3
10a	Na	7.1	-37.8	-231.5	-0.8
11a	K	6.4	-42.5	-85.9	-19.8
D-Ascorbic acid[g]		2.6	-34.1	-23.4	-187.1

a) Products dissolved in water (c = 2.0, of **8a** = 1.2); b) after the first measurement the solution was kept at 4 °C; c) one volume of the original solution dissolved in 2 volumes of 0.3 M potassium phosphate buffer pH 7.0. All solutions of **1a–11a** showed a pH of 7.0. The ascorbate solution showed a pH of 6.0; d) $[\alpha]_D^{20}$ -83.0 ° → -75.2 ° after 30 min (c = 4.5, H_2O);[17] e) $[\alpha]_D^{24}$ -24.4 ° (c = 1.8 H_2O) for the L-enantiomer;[18] f) $[\alpha]_D^{20}$ -6.7 ° (c = 1.2, H_2O);[18] g) $[\alpha]_D^{21}$ -22 ° (c = 2.0, H_2O).[20]

Experimental Section

General procedures

High-performance liquid chromatography analysis was carried out on a TEAP=Si 100 column (triethylammonium–polyol, 5 µm, 4.6 x 250 mm) from Serva (Heidelberg, FRG). The samples were eluted with 20 mM potassium phosphate buffer pH 3.3 or 3.5 with 10 % acetonitrile at a flow rate of 1 mL min^{-1}. The refractive index and UV absorption were simultaneously recorded. Most ^{13}C-NMR spectra were recorded at 360 MHz on a Bruker AM 360 spectrometer (Karlsruhe, FRG). Chemical shifts are given in ppm relative to internal sodium 3-(trimethylsilyl)-propanesulfonate ($(CH_3)_3Si(CH_2)_3SO_3Na$) standard at 25 °C. Optical rotation was measured in water or in the presence of a phosphate buffer on a Perkin–Elmer digital polarimeter type 241 ML (Ueberlingen, FRG) at room temperature (23 °C). IR spectra were obtained in potassium bromide on a Perkin–Elmer 157 G (Ueberlingen, FRG). Melting points as well as decomposition points are not corrected.

Cell growth

Proteus mirabilis (DSM 30115) was obtained from Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG) and was grown with racemic lactate and DMSO as described.[7e] The cells were harvested by centrifugation, washed with 5 mM EDTA in 10 mM Tris–HCl buffer pH 8.5 and stored as wet packed cells under an atmosphere of nitrogen at –18 °C.

Kinetic measurements

Preparation of crude extracts, determination of enzyme activities and kinetic studies by initial rate measurements were conducted as described.[7a,e] One unit (U) of enzyme activity oxidized 1 µmol substrate per min. K_m Values were determined according to l.c.[14a,b]

Chemicals

Chemicals of the highest quality available were purchased form Aldrich (Steinheim, FRG), Sigma (Deisenhofen, FRG), Fluka (Neu-Ulm, FRG) and Merck (Darmstadt, FRG). Solvents were dried according to the usual procedures and distilled before use. Commercially unavailable aldonates were synthesized by oxidation of the corresponding aldoses and isolated as potassium salts.[21] L- and D-Arabinuronate were synthesized according to l.c.[22]

Typical dehydrogenation experiments

D-*Arabino*-hex-2-ulosonate sodium salt monohydrate **1a**. Sodium D-gluconate **1** (10 mmol), DMSO (15 mmol), AQDS (0.05 mmol), EDTA (0.25 mmol) and tetracycline (5×10^{-5} mmol) were dissolved in 50 mL deoxygenated deionized water and *P. mirabilis* cells (0.8 g dry weight) were added. The pH of the buffer-free solution was held constant at 9.0 during the reaction by a pH-stat delivering 2 N sodium hydroxide. The mixture was stirred under an atmosphere of nitrogen at 38 °C. The course of the reaction was monitored by HPLC. At the end of the reaction (~ 98 % conversion), the pH of the mixture was brought to 3.5 with perchloric acid, the protein precipitate was removed by centrifugation, the supernatant was treated with active charcoal and filtrated. The free acid was isolated by ion exchange chromatography on a 10 x 210 mm Dowex-50W (20–50 mesh) column in the H^+ form (Sigma Deisenhofen, FRG). The pale yellow solution was then brought to pH 8.0 with sodium hydroxide and lyophilized. The residue was taken up in 200 mL methanol containing 2 % water and stirred for 1 h at room temperature. The sparingly soluble sodium salt was collected by vacuum filtration and washed with cold methanol and acetone. The resulting white amorphous hygroscopic product (8.8 mmol, 88 % from D-gluconate) was vacuum dried and stored over potassium hydroxide at 4 °C. The NMR spectrum in ^2H_2O is identical with that of an authentic sample from Aldrich. A small portion was converted to methyl 2,3,4,5-tetra-O-acetyl-D-*arabino*-hex-2-ulosonate **1ad**.[23]

Starting from D-gulonic-γ-lactone the salt was formed and a 0.5 M solution converted to D-*xylo*-hex-2-ulosonate sodium salt monohydrate (**2a**) in the same manner as described for the preparation of **1a**. Part of it was converted to methyl-D-*xylo*-hex-2-ulosonate and this further to D-*threo*-hex-2-enono-1,4-lactone (D-ascorbic acid) as described.[24] The ^1H and ^{13}C-NMR spectra in ^2H_2O are identical to those of an authentic sample of L-ascorbic acid.

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Table 5. Decomposition points and elemental analysis of various products after isolation and recrystallisation from methanol with a trace of water.

Product	Dec. p.	Elemental analysis			
		Calculated		Found	
	°C	C %	H %	C %	H %
1a $C_6H_9O_7Na \times 1/2\ H_2O$	120–124	32.01	4.48	31.54	4.31
2a $C_6H_9O_7Na \times 1\ H_2O$	131	30.78	4.73	30.18	4.83
3a $C_6H_9O_7 \times Na \times 1\ H_2O$	121	30.78	4.73	30.07	4.62
4a $C_6H_9O_7 \times Na \times 1/2\ H_2O$	118	32.01	4.48	32.32	4.49
5a $C_{12}H_{19}O_{12} \times Na \times 3\ H_2O$	128	33.34	5.83	33.32	6.08
7a $C_5H_7O_6K$	105–108	29.70	3.46	29.43	3.76
8a $C_5H_7O_6Na \times 1H_2O$	105	29.42	4.44	29.44	4.23
10a $C_6H_8O_{10}Na_2 \times 1\ H_2O$	129	22.51	3.15	22.01	3.33
D-ascorbic acid[a)]	137	40.92	4.58	40.98	4.63

a) Crystals from a syrup, mp not corrected.

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