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STEREOCHEMISTRY AND MECHANISM OF THE GDP-MANNOSE DEHYDRATASE REACTION*

PHILIP J. OTHS, ROBERT M. MAYER†, AND HEINZ G. FLOSS‡
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)
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ABSTRACT

The reaction catalyzed by bacterial GDP-mannose dehydratase (E.C. 4.2.1.47), the conversion of GDP-D-mannose to GDP-4-keto-6-deoxymannose (GDP-6-deoxy-D-*lyxo*-hexos-4-ulose), was studied with (6*R*)- and (6*S*)-GDP-D-[4-$^2H_1$,6-^{3}H]mannose. Conversion of these stereospecifically labeled substrates in the presence of excess unlabeled GDP-mannose into the 4-keto-6-deoxy derivatives followed by Kuhn–Roth oxidation gave acetic acid samples which were subjected to configurational analysis of the isotopically chiral methyl group. The observed F values of 64 for the material from the (6*S*) substrate and 31 for that from the (6*R*) isomer, corresponding to 48% e.e. *R* and 66% e.e. *S* configuration, respectively, of the methyl group indicate that (a) the oxidoreductase reaction involves transfer of H-4 to C-6, (b) the transfer is predominantly intramolecular, and (c) the transfer is stereospecific, H-4 replacing the C-6 hydroxyl group with inversion of configuration. A mechanism for the reaction is proposed on the basis of these results.

INTRODUCTION

The *de novo* synthesis of the deoxyhexose, L-fucose, has been demonstrated in bacteria[1], plants[2], and mammalian tissue[3]. It takes place at the sugar nucleotide level, the first step of the biosynthesis being the formation of GDP-6-deoxyhexos-4-ulose ("GDP-4-keto-6-deoxyhexose") from GDP-D-mannose. This first pathway-specific reaction is catalyzed by the enzyme GDP-mannose dehydratase (E.C. 4.2.1.47). Subsequent steps in the formation of GDP-L-fucose involve stereospecific reduction at C-4 and epimerization at C-3 and C-5 of the hexose moiety[4]. The reaction catalyzed by GDP-mannose dehydratase is irreversible and is accompanied by replacement of the C-5 hydrogen of the hexose moiety by a solvent proton. It resembles the reactions catalyzed by other hexose nucleotide dehydratases which have been studied in more detail, *e.g.*, the CDP-D-glucose-, UDP-D-glucose-, and

*This paper is dedicated to the memory of Professor Robert M. Mayer.
†Deceased October 6th, 1989.
‡Present address: Department of Chemistry BG-10, University of Washington, Seattle, WA 98195, U.S.A.

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TDP-D-glucose-utilizing enzymes[5]. In both TDP-glucose and CDP-glucose oxidoreductase, NAD^+ has been identified as a cofactor which mediates a transfer of H-4 of the sugar to C-6 (ref. 5). This hydride transfer is strictly intramolecular[6], because the cofactor is bound very tightly to the active site of the enzyme and does not exchange with free pyridine nucleotide during the catalytic cycle. With (6*R*)- and (6*S*)-TDP-D-[4-$^2H_1$,6-^{3}H]glucose and the dehydratase from *E. coli* it was shown[7] that the hydride migration is stereospecific, H-4 replacing the 6-OH group with inversion of configuration. The same is true for the TDP-glucose dehydratase in several streptomycetes[8–10].

In the present work, the methodology employed with the glucose nucleotides was used to gain further insight into the mechanism of the GDP-mannose dehydratase reaction.

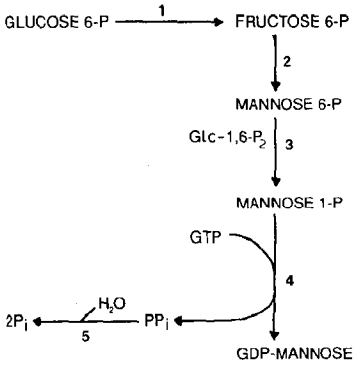
RESULTS AND DISCUSSION

The objective of our study was to elucidate in a single set of experiments three mechanistic aspects of the enzymic conversion of GDP-D-mannose into GDP-6-deoxy-D-*lyxo*-hexos-4-ulose ("GDP-4-keto-6-deoxymannose"). The three questions are: (A) Does the reaction involve a migration of H-4 of the hexose to carbon 6? (B) If so, is this hydrogen migration intramolecular? (C) Is the migration of H-4 to C-6 stereospecific and what is its steric course?

All three questions could be answered using specially prepared samples of GDP-D-mannose, stereospecifically labeled with tritium at C-6 in either the *R* or *S* configuration and with deuterium at C-4, in such a way that every tritiated molecule also contained deuterium. If these two samples of GDP-mannose were converted into GDP-4-keto-6-deoxymannose in the presence of a large excess of unlabeled GDP-mannose, they would generate a chiral methyl group in the enzyme reaction product if H-4 migrates to C-6, but only if the migration is strictly intramolecular, because a methyl group is only chiral if ^{1}H, ^{2}H and ^{3}H are present in the same molecule. Comparison of the configuration of this chiral methyl group with the configuration of the substrate would reveal the steric course of the reaction.

The requisite samples of (6*R*)- and (6*S*)-GDP-D-[4-$^2H_1$,6-^{3}H]mannose were synthesized as outlined in Scheme 1, from the correspondingly labeled samples of D-glucose 6-phosphate, which were available from earlier work[7].

The conversion of glucose 6-phosphate to GDP-mannose using a crude baker's yeast system has been described in the past[11], but in our hands this system failed to produce GDP-mannose in any significant yield. Baker's yeast samples from a wide variety of sources were tried, all with equally unsuccessful results. Brewer's yeast, however, was able to convert glucose 6-phosphate into GDP-mannose, with the ale formulation obtained from Red Star giving the highest yield of any brewer's yeast tried. The radiochromatogram of the products of the conversion of [U-^{14}C]glucose 6-phosphate into GDP-[U-^{14}C]mannose is shown in Fig. 1. To confirm that the sugar of the guanosine nucleotide is mannose, the material which cochromatographed with authentic GDP-mannose was hydrolyzed and



Scheme 1.

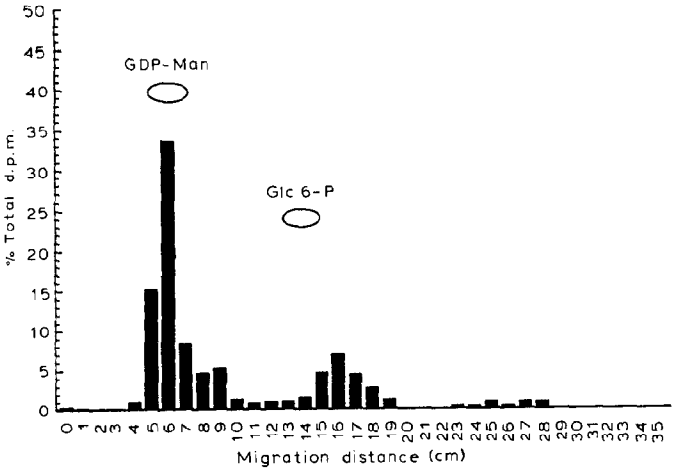


Fig. 1. Chromatographic profile of the products obtained from the reaction of [U-^{14}C]glucose 6-P, GTP, and the desalted brewer's yeast enzyme preparation. The reaction was carried out using the following amounts of reagents: 270 000 c.p.m. of D-[U-^{14}C]glucose 6-P (specific activity 280 μCi/μmol); glucose 1,6-P_2, 0.015 μmol; $MgCl_2$, 1.5 μmol; GTP, 0.50 μmol; and brewer's yeast enzyme preparation, 0.30 mg of protein, in a buffer consisting of 0.02M Tris and 1mM dithiothreitol in a volume of 56 μL, final pH 7.86. Incubation was for 45 min at 25°. The chromatogram was produced by taking a small aliquot (about 5 μL) of the reaction mixture, spotting it onto a prewashed sheet of Whatman 3MM paper, and developing in solvent system 1. The solvent front ran to 45 cm in 13 h at room temperature. The distribution of radioactivity was determined as described in the Experimental Section.

chromatographed in solvent system 2. The radioactivity distribution on the chromatogram, shown in Fig. 2, indicates that virtually all the radioactive material in the original GDP-mannose band is hydrolyzable with dilute acid, and hence is a sugar nucleotide, and that the sugar liberated in the hydrolysis comigrates with authentic mannose.

Thus, the brewer's yeast cell-free extract is capable of carrying out the conversion shown in Scheme 1.

The (6*R*) and (6*S*) isomers of GDP-D-[4-$^2H_1$,6-^{3}H]mannose were then prepared under identical conditions from (6*R*)- and (6*S*)-D-[4-$^2H_1$,6-^{3}H]glucose 6-phosphate with the brewer's yeast enzyme system. The products were isolated by chromatographing the reaction mixtures in solvent system 1 and eluting the regions containing the radioactive peaks that comigrated with authentic GDP-mannose.

GDP-mannose dehydratase was isolated from an unidentified soil bacterium following a previously described procedure[12]. Incubation of this enzyme preparation with (6*R*)- and (6*S*)-GDP-D-[4-$^2H_1$,6-^{3}H]mannose in the presence of a 150-fold and 200-fold excess of unlabeled GDP-mannose, respectively, was followed by Kuhn–Roth oxidation of the entire reaction mixture. Since the Kuhn–Roth procedure yields acetic acid specifically only from carbon-bound methyl groups, the only possible source of tritiated acetic acid in the system was C-5 and C-6 of the GDP-4-keto-6-deoxymannose generated in the enzyme reaction. The acetic acid samples, formed in radiochemical yields of 90% and 72%, respectively, were analyzed for the chirality of the methyl group by the enzymatic method of Cornforth, Arigoni, and coworkers[13–15] and gave F values of 31 and 64, respectively*. These values correspond to 66% e.e. *S* configuration for the methyl group generated from the substrate of (6*R*) configuration, and 48% e.e. *R* configuration for the methyl group from the (6*S*) isomer of GDP mannose†.

The above results indicate that the GDP-mannose dehydratase reaction does indeed involve a hydrogen migration from C-4 to C-6 of the hexose. This hydrogen transfer is stereospecific and its stereochemistry conforms to that established earlier for TDP-glucose dehydratase[7]. The migrating hydrogen replaces the 6-hydroxy group with inversion of configuration at C-6. Since the reactions were carried out in the presence of excess unlabeled substrate, the formation of chiral methyl groups also shows that the hydrogen migration must be predominantly intramolecular. Although no cofactor has been identified for GDP-mannose dehydratase, the above results are consistent with, albeit do not prove, migration of H-4 to C-6 as a hydride mediated by an enzyme-bound pyridine nucleotide, as in the glucose nucleotide

*This procedure involes the enzymatic conversion of acetate via acetyl-CoA into malate, which is then equilibrated with fumarase to wash out tritium from the *pro-2R* position. An isotope effect in the malate synthase reaction leads to an uneven tritium distribution between the two methylene hydrogens of malate, which is analyzed in the fumarase reaction. The F value[15] is the percentage tritium retention in the fumarase reaction; F = 79 ≅ 100% e.e. *R*, F = 21 ≅ 100% e.e. *S*, F ≈ 50 ≅ racemic.

†The determination of F values is subject to a relatively large error, so that the estimate of the % e.e. of chiral methyl groups is only accurate to about ±10%. The values for the present enantiomeric pair are within the error range of the assay.

dehydratase[5]. The stereochemical purity of the methyl groups generated with the bacterial GDP-mannose dehydratase is lower than that of the methyl groups of TDP-6-deoxy-D-*xylo*-hexos-4-ulose ("TDP-4-keto-6-deoxyglucose") generated with *E. coli* TDP-glucose dehydratase from (6*R*)- and (6*S*)-TDP-D-[4-$^2H_1$,6-^3H]glucose, which had been synthesized from the same glucose 6-phosphate samples. The methyl groups in those earlier experiments had 82% e.e. *S* and 88% e.e. *R* configuration, respectively[7]. As a likely explanation it is suggested that the degree of intramolecularity of the hydrogen transfer is not as high in the GDP-mannose dehydratase reaction as in the reaction catalyzed by TDP-glucose dehydratase. The reduced cofactor in GDP-mannose dehydratase may be somewhat less tightly bound than in TDP-glucose dehydratase, allowing it to dissociate occasionally from the enzyme during a catalytic cycle and to be replaced by another, unlabeled cofactor molecule from the unbound, cellular pool.

The observation of predominantly *intramolecular* hydrogen migration implies that the transfer of H-4 to C-6 must be suprafacial. This stereochemical inference together with the observed inversion at C-6 suggests a plausible model for the conformation of the mannose moiety of the substrate in the enzyme active site. The

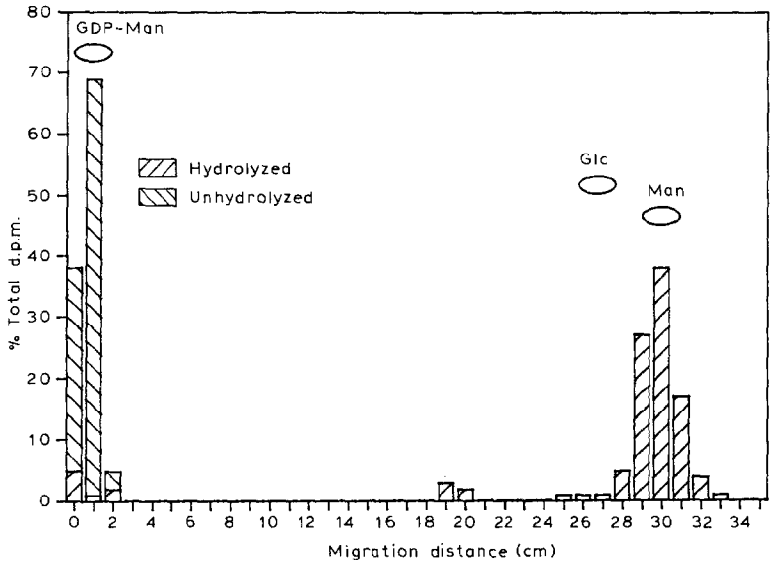


Fig. 2. Chromatographic profile of the hydrolysis product of the enzymatically generated GDP-[U-^{14}C]mannose. The material in the radioactive peak found in the region 4–8 cm of the chromatogram shown in Fig. 1 was eluted and one aliquot subjected to acid hydrolysis in 0.1M HCl for 15 min at 100°. The entire contents of the reaction tube was spotted on Whatman 3MM paper, as was the other aliquot of the unhydrolyzed material, and the chromatogram developed for 15 h in solvent system 2. The distribution of radioactivity was determined as described in the Experimental Section. Standards of glucose and mannose were spotted alongside the lanes containing radioactivity and visualized by the silver staining technique.

cofactor must be situated on the face of the sugar ring on which H-4 is located in an axial position. The 6-hydroxy group is displayed on the opposite face, *syn* to the axial H-5. Two basic groups on the enzyme may assist in the reaction, one in proximity to the 4-hydroxyl group and one near H-5 and the 6-OH group. Two scenarios for the events during a catalytic cycle are depicted in Fig. 3. Following dehydrogenation at C-4, one mechanism (Fig. 3A) envisions *syn* elimination of water between C-5 and C-6, a stereochemistry precedented in other examples, *e.g.*, dehydroquinate dehydratase[16], followed by hydride reduction of the polarized C-C double bond. The other proposal (Fig. 3B) pictures enolization of the 4-keto group with proton transfer from C-5 to the 6-OH group, followed by a concerted displacement at C-6. As in the case of the glucose nucleotide dehydratase, no distinction

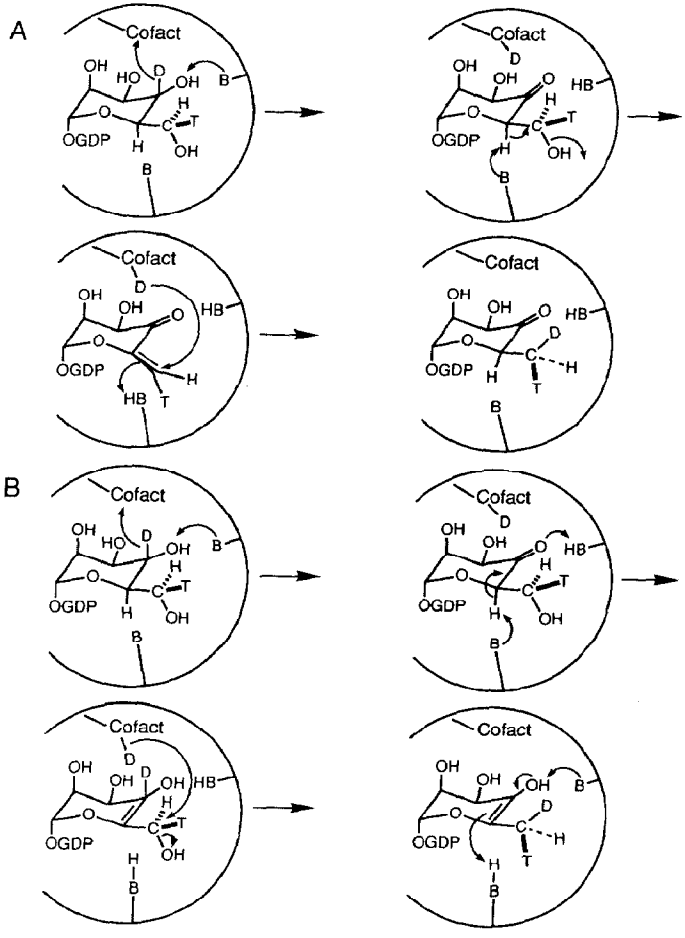


Fig. 3. Alternative schemes rationalizing the stereochemical course of the reaction catalyzed by GDP-mannose dehydratase: A, mechanism involving a 4-keto-5,6-ene intermediate; B, mechanism involving a concerted displacement of the 6-OH group by hydride.

can be made between these two versions of the reaction mechanism on the basis of the presently available data.

EXPERIMENTAL

Materials. — The source of GDP-mannose dehydratase was an unidentified soil bacterium[12], ATCC 19241, obtained from the American Type Culture Collection in lyophilized form. Red Star ale brewer's yeast packaged in lyophilized form was used as the source of yeast enzymes. The following compounds were obtained from Sigma: α-D-glucopyranose 1,6-bisphosphate cyclohexylammonium salt, guanosine 5′-triphosphate sodium salt (type 3), guanosine 5′-diphospho-D-mannose sodium salt (type 1), D-glucose 6-phosphate sodium salt, and L-rhamnose. D-[U-^{14}C]Glucose 6-phosphate (specific activity 280 mCi/mmol) was obtained from Amersham Corp., and (6*R*)- and (6*S*)-D-[4-$^{2}H_1$,6-^{3}H]glucose 6-phosphate were previously obtained from (2*R*,3*S*)- and (2*R*,3*R*)-3-phospho-[3-^{3}H]glycerate[17], respectively. Ammonium sulfate, ultra-grade, was purchased from Schwartz/Mann Biotech, Cleveland, Ohio. All other reagents used were of reagent grade or the highest grade commercially available.

General methods. — Paper chromatography was carried-out descendingly in a pre-equilibrated chromatography tank using Whatman 3MM paper washed with 1.0M citric acid and then with distilled water. Solvent systems for chromatography were: 1, 1:2.5 M ammonium acetate, pH 3.8–95% ethanol; 2, 5:12:4 pyridine–ethyl acetate–water. All solvent systems were prepared fresh immediately before use. Reducing sugars were visualized by the silver stain method[18], and nucleotide sugars and nucleotides were detected with the aid of a u.v. lamp. The hexose 6-phosphates were visualized using the Wade–Morgan method[19].

Radioactivity was measured by liquid scintillation counting in a Beckman LS 4500 spectrometer. The scintillation fluid was prepared by dissolving 16.08 g of 2,5-diphenyloxazole (PPO), and 0.402 g of 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP) in 4 L of toluene. Samples were counted using 10 mL of scintillation fluid. Counting efficiency in the analysis of the radioactive paper strips from paper chromatograms was determined using an external standard supplied with the instrument, and counting efficiencies for samples in solution were obtained with internal standards of tritiated or ^{14}C-labeled toluene. The radioactivity distribution on paper chromatograms was determined by cutting 1 cm-long by 2 cm-wide strips along the length of the chromatogram and counting each in 10 mL of scintillation fluid.

Protein determinations were done by the method of Warburg and Christian[20] in a Zeiss PMQ 2 single beam spectrophotometer using quartz cuvettes of 1 cm path length.

Kuhn–Roth oxidations were carried out as described earlier[21] and the resulting acetic acid samples were purified by subjecting them to steam-distillation in a Wiesenberger apparatus[21] at an alkaline pH to remove neutral and basic impurities,

followed by steam distillation at an acidic pH. The configuration and stereochemical purity of acetic acid was determined by the method of Cornforth *et al.*[13] and Arigoni and co-workers[14], using a procedure routinely employed in this laboratory[15].

*Preparation of (6R)- and (6S)-GDP-*D*-[4-$^2H_1$,6-^3H]mannose.* — The following operations were carried out at 0–4°; the yeast enzyme preparation obtained was used immediately. One packet (7 g) of brewer's ale yeast was suspended in 24 mL of a 0.05M solution of K_2HPO_4, 6mM in dithiothreitol, and passed through a prechilled French pressure cell twice at a pressure of 12 000–14 000 psi. All centrifugation steps were carried out at 15 000 × *g* for 20 min. The broken-cell slurry was centrifuged, and to 17 mL of supernatant fluid 6.5 mL of a 10% streptomycin sulfate solution in the above K_2HPO_4 buffer was added dropwise with stirring. The mixture was centrifuged and the supernatant fluid brought to 35–40% ammonium-sulfate saturation by adding solid $(NH_4)_2SO_4$ slowly with stirring. The solution was stirred for another 20 min and centrifuged. The supernatant fluid was passed through cotton to remove any floating particulate matter, and then brought to 70% ammonium sulfate saturation. The solution was stirred and centrifuged as described above, and the pellet was redissolved in a minimal amount of 0.05M Tris buffer, pH 7.5, 4mM in dithiothreitol. The solution was desalted by forced passage through a column of Bio-Gel P-2 (200–400 mesh), prepared by swelling the gel in the same buffer and loading 1.0 mL of gel into a tuberculin syringe fitted with a cotton plug. The column was placed in a bench-top centrifuge and spun dry of buffer. Sixty μL (protein concentration 30–60 mg/mL) of the redissolved pellet was loaded onto the column and spun through the gel. The resulting desalted extract was then used in the preparation of GDP-mannose from glucose 6-phosphate and GTP.

For the conversion of (6*R*)- and (6*S*)-D-[4-^{2}H,6-$^3H_1$]glucose 6-phosphate to the corresponding GDP-mannose derivatives, the following reagents were placed in a 1 × 7.5 cm plastic test tube: $MgCl_2$, 2.5 μmol; GTP, 0.7 μmol; glucose 1,6-bisphosphate, 0.05 μmol; 250 000 d.p.m. (6*R*)- or (6*S*)-D-[4-$^2H_1$,6-^{3}H]glucose 6-phosphate (specific activity 100 μCi/μmol); and desalted yeast extract, 0.45 mg protein, all in a total volume of 120 μL of 0.03M Tris buffer, 2.5mM in dithiothreitol, final pH 7.80. The reaction mixtures were incubated for 45 min at room temperature, at which time an additional portion of yeast extract (0.45 mg protein) was added, and incubation was continued for 15 more min. The reaction mixture was briefly placed in a 100° heating-block, the precipitated protein centrifuged off, and the supernatant fluid spotted on paper and chromatographed with glucose 6-phosphate and GDP-mannose markers in solvent system 1. The radioactive material that ran with authentic GDP-mannose was eluted with water. Its identity as GDP-mannose was further confirmed by subjecting an aliquot of the product of a duplicate reaction with [U-^{14}C]glucose 6-phosphate to hydrolysis with 0.1M HCl for 10 min at 100°, and chromatographing the hydrolyzate together with glucose and mannose markers in solvent system 2.

GDP-mannose dehydratase reaction. — GDP-mannose dehydratase activity was obtained from a crude cell-free extract of the soil bacterium grown on a medium described by Markovitz and Sylvan[22]. The cells were harvested one hour into lag phase, washed twice with a 0.05M K_2HPO_4–NaH_2PO_4 buffer, pH 7.5, and centrifuged at 12 000 × *g* for 10 min. All subsequent steps were carried out at 0–4°. The cell pellet was suspended in the same phosphate buffer made 4mM in dithiothreitol and passed through a prechilled French pressure cell at 13 000 psi. After centrifugation of the broken-cell suspension, the supernatant fluid was brought to 45% ammonium-sulfate saturation. The precipitated protein was removed by centrifugation and the supernatant fluid brought to 80% ammonium-sulfate saturation. The pellet obtained after centrifugation of the 45–80% ammonium sulfate cut was resuspended in a minimal amount of buffer to give protein concentrations of 20–30 mg/mL. Such preparations could be stored frozen for periods up to two weeks with minimal loss of activity.

GDP-mannose dehydratase activity in the cell-free extracts was assayed by a procedure similar to the one used by Markovitz[12] as follows: 0.025M KH_2PO_4, 0.025M Na_2HPO_4 (final pH 7.50), 4mM dithiothreitol, 12mM EDTA, 1.3mM GDP-D-mannose, and 3 μL of cell-free extract in a total volume of 75 μL were incubated for 45 min at 37°. After heat denaturation, 700 μL of 0.10M NaOH was added and the mixture allowed to react for 20 min at 37°, at which time the absorbance at 318 nm due to the enolate anion of GDP-4-keto-6-deoxymannose ($\varepsilon = 5000M^{-1}.cm^{-1}$) was measured against a blank prepared by substituting heat-denatured enzyme in the above procedure. Typical active preparations gave absorbance readings of 0.2–0.4.

For the first experiment with a chirally labeled sample the reaction mixture, in a 1 × 7.5 cm culture tube, contained: 0.05M sodium–potassium phosphate buffer, pH 7.5, 13mM in EDTA and 3mM in dithiothreitol; 4.30mM unlabeled GDP-D-mannose; 31 000 d.p.m. (6*R*)-GDP-D-[4-$^2H_1$,6-^3H]mannose; and 40 μL of crude GDP-mannose dehydratase corresponding to 0.30 O.D. units, in a total volume of 560 μL. The incubation was carried out for 25 min at 37°, after which time an additional 20 μL of enzyme was added and the incubation continued for 35 more min. The mixture was subjected to 100° for 10 sec to stop the reaction, then stored at −70°. The (6*S*) isomer was converted into the 4-keto-6-deoxy derivative under similar conditions, with the following changes: The total reaction volume was 435 μL, 20 000 d.p.m. of the radioactive substrate was used, the concentration of unlabeled GDP-mannose was 2.30mM, the activity of the enzyme corresponded to 0.20 O.D. units, and the incubation time after the second enzyme addition was increased to 1 h.

Both reaction mixtures were subsequently oxidized, without isolation of the GDP-4-keto-6-deoxymannose, under Kuhn–Roth conditions, using 3 mL of oxidation solution[21], to give 28 500 d.p.m. (90%) and 14 500 d.p.m. (72%) of tritiated acetate from the (6*R*) and the (6*S*) isomer, respectively, for chirality analysis.

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