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Studies in Fructofuranose Chemistry.

Ronald Joseph Voll

Louisiana State University and Agricultural & Mechanical College

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Studies in fructofuranose chemistry

Voll, Ronald Joseph, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1991
STUDIES IN FRUCTOFURANOSE CHEMISTRY

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in
The Department of Chemistry

by
Ronald Joseph Voll
B.S., Louisiana State University, 1983
August 1991
DEDICATION

To Vickie and Frances who, together now, keep me going.

Vickie for the last 14 years.

Frances for the last almost 18 months.

Thanks!
ACKNOWLEDGEMENTS

To my research father, Dr. Ezzat S. Younathan, I can not give enough thanks. He has kept after me to do better in all my endeavors. I hope I have lived up to his expectations.

To my long time friend and colleague Dr. Theodore A. W. Koerner, Jr., thank you for introducing me to carbohydrate chemistry and all its details. It has been a productive set of years. I hope we can continue.

A special thanks to Dr. J. W. Robinson, my minor professor, for his advise and sense of humor. He was right all those years ago when he conveyed to me that it only gets more complicated as you get older. I did not really appreciate this until lately.

Thanks go to the members of my committees: Drs. N. H. Fischer, B. J. Hales, N. R. Kestner, and S. F. Watkins, for taking the time to guide me during these last years.

To all my collaborators in research, thanks for including me in the projects. The results have been good.

Thanks go to all my laboratory partners in graduate school and before. I shall try to keep you in my thoughts in later life. It has been a varied and expanding experience.
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ABSTRACT

The glycolytic pathway enzyme phosphofructokinase (E.C. 2.7.1.11) has as its substrate and activator two different carbohydrate phosphate esters. Both are derivatives of D-fructose in the furanose ring form. This dissertation is divided into two chapters: the first deals with the conformational specificity of the active site and the second the structure of the activator. The compound 2,5-anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol was designed to test the active site. The synthesis utilized a phase-transfer cyclo-dialkylation of a vicinal diol to yield a trans-fused 2,5,8-trioxabicyclo[4.3.0]nonane system. The final product was shown to exist in a locked conformation in solution by temperature dependent n.m.r. experiments which showed no line shape change. The data indicate that the five-membered ring is locked by the trans-fused six-membered 1,4-dioxane ring into a twist $^{4T_3}$ conformation. A single crystal X-ray study was carried out. The crystalline product exists in an ideal twist conformation with a pseudorotation angle of 0° and amplitude of 47.2° in agreement with the n.m.r. results. The compound, as the monophosphate, is intended to verify the linear plot of substrate efficacy index versus $\beta^{-4T_3}$ concentration observed with several ketose 6-phosphates.
Chapter two describes detailed n.m.r. studies of the activator molecule, D-fructofuranose 2,6-bisphosphate, which allowed the unequivocal assignment of all the proton, carbon and phosphorus resonances. Several unexpected chemical shift values and coupling constants were obtained. The unusual near-gauche orientations of C-1 and C-3 to P-2, obtained by molecular mechanics calculations, can explain their small vicinal coupling constants in contrast to the expected larger value seen for C-5 to P-6. Reduction did not affect the n.m.r. spectrum substantiating that C-2 is phosphorylated. Oxidation yielded an unstable intermediate which decomposed by a beta elimination mechanism involving the phosphate group on C-6. These data establish unequivocally the $^1$H, $^{13}$C and $^{31}$P assignments and explain the observed anomalous shifts. Moreover, they establish that the activator of fructose 6-phosphate 1-kinase is the $\beta$-anomer of the $^4T_3$ conformer of D-fructose 2,6-bisphosphate. The conclusion from both investigations is that both the active site and the activation site of phosphofructokinase seem to prefer the $^4T_3$ conformer of these fructofuranose ligands.
CHAPTER ONE

SYNTHESIS, X-RAY CRYSTAL AND SOLUTION STRUCTURES OF
2,5-ANHYDRO-3,4-\(\text{O-}(1,2\text{-ETHANEDIYL})\)-D-MANNITOL:
A LOCKED \(4_T^3\) FURANOSE CONFORMER

Introduction to Chapter One

This chapter consists of a paper ready for submission to the journal Carbohydrate Research with the following authors: R. J. Voll, F. R. Fronczek, D. Vargas, and E. S. Younathan from the Departments of Biochemistry and Chemistry. This paper reports the successful synthesis of a carbohydrate locked in a twisted conformation. The structure of the compound was investigated by a combination of techniques. The overall objective of this synthesis was to prepare a system which would be conformationally locked in a manner such that the easily obtainable monophosphate would be a substrate for the glycolytic enzyme phosphofructokinase. This enzyme has been shown to prefer one particular conformation of its substrate which exists in rapid equilibrium with two other conformations. The authors have been able to lock an analogue of the natural substrate in one conformation and the first author is reporting the research in this dissertation.
Introduction

During the past two decades, investigators in this laboratory have carried out studies on phosphofructokinase (E.C. 2.7.1.11) from rabbit muscle, which is one of the key regulatory enzymes of the glycolytic pathway. Through the use of structurally locked analogues of the \( \alpha \)- and \( \beta \)-forms of its carbohydrate substrate, it was established that the enzyme acts on the \( \beta \)-form. In specific terms, it was found that 2,5-anhydro-\( \Delta \)-mannitol 6-phosphate (an analogue of the \( \beta \)-form of \( \Delta \)-fructose 6-phosphate) was a good alternate substrate, whereas 2,5-anhydro-\( \Delta \)-glucitol 6-phosphate (an analogue of the \( \alpha \)-form) was a competitive inhibitor for this enzyme. In addition, the kinetic properties of the epimers of \( \Delta \)-fructose 6-phosphate were investigated. These substituted tetrahydrofuran rings may exist in twenty different conformers each with a different stability and concentration in solution. Based upon n.m.r data and estimated interaction energies, the conformational composition of these systems was calculated. Out of the twenty possible conformers, only three are thermodynamically favored. These three major conformers of the \( \Delta \)-ketohexose 6-phosphates are the \( ^4T_3 \), the \( ^0T_2 \), and the \( ^0T_5 \). The plot of the logarithm of the substrate efficacy index \( \frac{V_{\text{max}}}{K_m} \) versus the logarithm of the amount of each conformer was observed to be linear.
only in the case of the $\beta-4T_3$ conformer. Therefore, it was concluded that the $4T_3$ conformer of $\beta-D$-fructose 6-phosphate is the true substrate of rabbit muscle phosphofructokinase$^{1,3,1,4}$. In order to further study this effect, the authors have selected 2,5-anhydro-$D$-mannitol (2) as a candidate for modification in an attempt to create a system locked as the $4T_3$ conformer in solution. It was deemed worthwhile to test the monophosphate of such a locked conformer as a substrate for phosphofructokinase. It should be noted that 2 exists, on average, as the $4T_3$ conformer in solution and deviates only slightly from the ideal $4T_3$ in the crystalline state$^{1,5}$. The trans disposition of the hydroxy groups on carbons 3 and 4 and their equatorial orientation in the predominant $4T_3$ conformer should allow the formation of a trans-fused 1,4-dioxane ring. This paper reports a procedure for the synthesis of the twist-locked hexitol 2,5-anhydro-3,4-$O$-(1,2-ethanediyl)-$D$-mannitol (1) as well as its X-ray crystal and solution structures.
Experimental

General Methods. --- N.m.r. spectra were recorded with a Bruker AMX-500 spectrometer. N.m.r. simulations were performed on an ASPECT 3000 minicomputer using PANIC (1985 version, Bruker Instrument Co.). T.l.c. was performed using HPTLC plates (Analtech) and benzene-methanol, 24:1, as solvent.

2,5-Anhydro-3,4-\(\alpha\)-(1,2-ethanediyl)-1,6-di-\(\beta\)-trityl-D-mannitol (4). --- To a solution of 3.24 g of 2,5-anhydro-1,6-di-\(\beta\)-trityl-D-mannitol (3)\(^1\,6\) in 20 ml of 1,2-dibromoethane was added 20 ml of a 50% NaOH solution and 150 mg of benzyltriethylammonium chloride\(^1\,7\). The flask was connected to a bubbler since the slightly exothermic reaction released a gas as a byproduct (probably vinyl bromide). The mixture was stirred rapidly at room temperature for 24 hours. The mixture turned a red-brown color with considerable suspended materials. T.l.c analysis indicated the formation of a new fast-running component (\(R_f = 0.75\), \(R_f\) of 3 = 0.35). An additional 10 ml of 1,2-dibromoethane and 10 ml of 50% NaOH solution were added and the reaction allowed to proceed for a further 48 hours. The reaction mixture was then diluted with 100 ml of cold water and extracted with 100 ml of diethyl ether. The organic layer was washed with water, dried over sodium sulfate, and concentrated to a brown foamy residue (2.16 g). Filtration of a diethyl
ether solution of the crude residue through a short column of silica gel removed the very polar components from the mixture. The product crystallized away from the starting material upon cooling of an ethanol solution to give 4 (0.63 g, 18%), m.p. 168-170°C.

$^1$H-N.m.r. data (C$_6$D$_6$): an AA'BB'XX'YY' eight spin system, an independent CC'DD' four spin system and an aromatic system,

$\delta$ 3.23 (m, CC', 2H, J$_{7ax,7eq} = J_{8ax,8eq} = -12$ Hz, J$_{7ax,8ax} = 12$ Hz, J$_{7ax,8eq} = J_{7eq,8ax} = 3$ Hz, H$_{-7ax,8ax}$),

3.28 (m, DD', 2H, J$_{7eq,8eq} = 1$ Hz, H$_{-7eq,8eq}$),

3.46 (dd, AA', 2H, J$_{1,1'} = J_{6,6'} = -10$ Hz, J$_{1,2} = J_{5,6} = 5$ Hz, H$_{-1,6}$),

3.61 (dd, BB', 2H, J$_{1',2} = J_{5',6'} = 3$ Hz, H$_{-1',6'}$),

3.77 (m, YY', 2H, J$_{2,3} = J_{4,5} = 9.5$ Hz, J$_{3,4} = 9$ Hz, H$_{-3,4}$),

4.32 (m, XX', 2H, H$_{-2,5}$), 7.00-7.80 (m, 30H, ArH).

2,5-Anhydro-3,4-0-(1,2-ethanediyl)-D-mannitol (1).

--- The addition of 0.50 g of 2,5-anhydro-3,4-0-(1,2-ethanediyl)-1,6-di-O-trityl-D-mannitol (4) to 5 ml of 90% trifluoroacetic acid resulted in a bright yellow solution$^{1,8}$. After 1 hour, at room temperature, the clear solution was diluted with 10 ml of water, filtered to remove the triphenylmethanol byproduct, and concentrated to dryness. The residue was suspended in water, extracted with diethyl ether three times, and the aqueous layer was concentrated to dryness. The residue was crystallized
from ethanol to give 1 (0.10 g, 70 %), m.p. 150-152°C.

N.m.r. data (D₂O): ¹H, an AA'BB'XX'YY' eight spin system
and an independent CC/DD' four spin system, Karplus
equation derived torsion angle in square brackets [],
X-ray derived torsion angle in curved brackets {},

δ 3.43 (dd, AA', 2H, J₁₁,₁₁ = J₆,₆' = -13 Hz,
J₁₂ = J₅,₆ = 4 Hz [55°] {64°}, H-1,6),
3.53 (m, YY', 2H, J₂₂,₋,₁ = J₄,₅ = 9.5 Hz [155°] {165°},
J₃,₋ = 9 Hz [150°] {178°}, H-3,4),
3.55 (dd, BB', 2H, J₁',₁₂ = J₅,₆' = 3 Hz [50°] {58°},
H-1',6'),
3.60 (m, CC', 2H, J₇ax,7eq = J₈ax,8eq = -12 Hz,
J₇ax,8ax = 12 Hz [170°] {175°},
J₇ax,8eq = J₇eq,8ax = 3 Hz [55°] {55°}, H-7ax,8ax)
3.69 (m, XX', 2H, H-2,5),
3.72 (m, DD', 2H, J₇eq,8eq = 1 Hz [70°] {75°},
H-7eq,8eq);

¹³C, δ 63.8, 69.9 (C-1,6,7,8), 79.4, 80.6 (C-2,3,4,5).

Crystal Structure Determination. --- Slow evaporation of
an ethanol solution of 1 yielded needles suitable for
X-ray study. A colorless needle fragment was used for
data collection on an Enraf-Nonius CAD-4 diffractometer
with CuKα radiation and a graphite monochromator; cell
dimensions were obtained from setting angles of 25
reflections having 25° < θ < 30°. The unit-cell constants
are shown in Table 1.1. Data collection was by ω-2θ scans;
one hemisphere of data reflections having $2 < \theta < 75^\circ$, $h = 0$ to 5, $k = -17$ to 17, $l = -16$ to 16, was measured and corrected for background, Lorentz, and polarization effects. Absorption corrections were based on $\psi$ scans. Standard reflections 200, 080, and 004 exhibited no decrease in intensity. The space group was determined from systematic absences $hkl$ with $h+k$ odd and 001 with $l$ odd. Redundant data were averaged, to yield 907 unique data. All but 13 had $I > 3\sigma(I)$ and were used in the refinement.

The structure was solved using direct methods and refined by full-matrix least squares based on $F$ with weights $w = 4F_o^2 \left[ \sigma^2(I) + (0.02 F_o^2)^2 \right]^{-1}$. The atomic scattering factors were taken from the International Tables\textsuperscript{1,9}. Non-hydrogen atoms were refined anisotropically; H atoms were located from difference maps and refined isotropically. The largest $\Delta/\sigma$ was 0.01 on the final cycle, the maximum residual electron density was 0.50 e/A\textsuperscript{3} on the C3--C3' bond, and extinction coefficient was $5.1(2) \times 10^{-5}$. The programs used were MULTAN80\textsuperscript{1,10}, SDP/VAX\textsuperscript{1,11}, PLUTO78\textsuperscript{1,12}, and ORTEP\textsuperscript{1,13}. Atomic coordinates and equivalent isotropic thermal parameters are given in Table 1.2. The anisotropic thermal parameters and the observed and calculated structure-amplitudes are listed in Tables 1.6 and 1.7 will be deposited with, and can be obtained from Elsevier
Science Publishers. Refinement of the mirror-image structure under identical conditions yielded $R = 0.0321$, $R_w = 0.0452$, $S = 2.823$. 
Table 1.1 Crystal Data For
2,5-Anhydro-3,4-O-(1,2-Ethanediyl)-D-Mannitol.

<table>
<thead>
<tr>
<th></th>
<th>C₈H₁₄O₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>C₈H₁₄O₅</td>
</tr>
<tr>
<td>Formula weight</td>
<td>190.2</td>
</tr>
<tr>
<td>Cell Constants</td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>4.7252 (6)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>14.0364 (12)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>13.268 (2)</td>
</tr>
<tr>
<td>Volume (Å³)</td>
<td>880.0 (3)</td>
</tr>
<tr>
<td>Z (molecules/cell)</td>
<td>4</td>
</tr>
<tr>
<td>Density calculated (g/cm³)</td>
<td>1.435</td>
</tr>
<tr>
<td>µ (CuKα) (cm⁻¹)</td>
<td>9.8</td>
</tr>
<tr>
<td>Space group</td>
<td>C222₁</td>
</tr>
<tr>
<td>Crystal size (mm)</td>
<td>0.20 x 0.25 x 0.35</td>
</tr>
<tr>
<td>λ (CuKα) (Å)</td>
<td>1.54184</td>
</tr>
<tr>
<td>Minimum transmission</td>
<td>0.9016</td>
</tr>
<tr>
<td>Reflections measured</td>
<td>2185</td>
</tr>
<tr>
<td>Unique data</td>
<td>907</td>
</tr>
<tr>
<td>I &gt; 3σ (I)</td>
<td>894</td>
</tr>
<tr>
<td>R</td>
<td>0.0319</td>
</tr>
<tr>
<td>Rw</td>
<td>0.0446</td>
</tr>
<tr>
<td>S (89 variables)</td>
<td>2.785</td>
</tr>
</tbody>
</table>
Table 1.2

Atomic Coordinates and Equivalent Isotropic Thermal Parameters

For 2,5-Anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol.

<table>
<thead>
<tr>
<th>Atom</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>B(A^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1</td>
<td>0.2640(2)</td>
<td>0.8644(6)</td>
<td>0.44458(4)</td>
<td>3.54(2)</td>
</tr>
<tr>
<td>O2</td>
<td>0.000</td>
<td>0.88193(7)</td>
<td>0.250</td>
<td>3.06(2)</td>
</tr>
<tr>
<td>O3</td>
<td>-0.1307(2)</td>
<td>0.64611(5)</td>
<td>0.34845(4)</td>
<td>2.97(1)</td>
</tr>
<tr>
<td>Cl</td>
<td>-0.0325(2)</td>
<td>0.86487(7)</td>
<td>0.43221(6)</td>
<td>2.76(2)</td>
</tr>
<tr>
<td>C2</td>
<td>-0.1110(2)</td>
<td>0.82298(8)</td>
<td>0.33060(6)</td>
<td>2.42(1)</td>
</tr>
<tr>
<td>C3</td>
<td>0.0105(2)</td>
<td>0.72628(7)</td>
<td>0.30689(5)</td>
<td>2.12(1)</td>
</tr>
<tr>
<td>C7</td>
<td>0.0047(3)</td>
<td>0.56222(7)</td>
<td>0.30726(6)</td>
<td>3.52(2)</td>
</tr>
<tr>
<td>H10</td>
<td>0.291(4)</td>
<td>0.866(1)</td>
<td>0.509(1)</td>
<td>4.5(3)*</td>
</tr>
<tr>
<td>H1a</td>
<td>-0.129(2)</td>
<td>0.821(1)</td>
<td>0.4837(7)</td>
<td>2.2(2)*</td>
</tr>
<tr>
<td>H1b</td>
<td>-0.100(2)</td>
<td>0.932(1)</td>
<td>0.4349(9)</td>
<td>3.4(2)*</td>
</tr>
<tr>
<td>H2</td>
<td>-0.323(3)</td>
<td>0.824(1)</td>
<td>0.3305(9)</td>
<td>3.8(3)*</td>
</tr>
<tr>
<td>H3</td>
<td>0.206(2)</td>
<td>0.728(1)</td>
<td>0.3255(8)</td>
<td>2.6(2)*</td>
</tr>
<tr>
<td>H7a</td>
<td>-0.113(2)</td>
<td>0.509(1)</td>
<td>0.333(1)</td>
<td>5.4(4)*</td>
</tr>
<tr>
<td>H7b</td>
<td>0.211(3)</td>
<td>0.565(1)</td>
<td>0.3284(9)</td>
<td>3.5(2)*</td>
</tr>
</tbody>
</table>

Starred atoms were refined isotropically.

The equivalent isotropic thermal parameter, for atoms refined anisotropically, is defined by the equation:

\[
\frac{4}{3}[a^2B_{11} + b^2B_{22} + c^2B_{33} + abB_{12} \cos \gamma
\]

\[
+ acB_{13} \cos \beta + bcB_{23} \cos \alpha]
\]
Results and Discussion

2,5-Anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol (1) was prepared from 2,5-anhydro-D-mannitol (2) by a three step procedure (Figure 1.1). The tetrol was first di-O-tritylated to the amorphous 2,5-anhydro-1,6-di-O-trityl-D-mannitol (3)\(^{1.6}\), which was then di-O-alkylated utilizing 1,2-dibromoethane and sodium hydroxide under phase-transfer conditions in a manner similar to the use of 1,2-dichloroethane\(^{1.7}\). However, an attempt to use 1,2-dichloroethane instead of 1,2-dibromoethane produced none of the desired product as evidenced by t.l.c. The resulting 2,5-anhydro-3,4-O-(1,2-ethanediyl)-1,6-di-O-trityl-D-mannitol (4) was detritylated using aqueous trifluoroacetic acid\(^{1.8}\) to produce 1.

In order to verify the structure of this substituted, strained, trans-fused 2,5,8-trioxabicyclo[4.3.0]nonane system, a single crystal X-ray diffraction study was performed on 1. The molecular structure and atomic numbering are illustrated in Figure 1.2. The D form of this compound was assumed based on the configuration of the starting material. Refinement of the mirror-image structure yielded slightly higher R values, and thus supports the overall D assignment. The crystal structure determination confirms that the molecule has the manno configuration which results in all nearest neighbor
Figure 1.1
Reaction Pathway From Compound 2 to Compound 1.
Figure 1.2
Molecular Structure and Atomic Numbering
Of 2,5-Anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol.
dispositions being trans. A perspective view from the 1,4-dioxane ring side of the molecule is shown in Figure 1.3. Bond lengths are given in Table 1.3 and bond angles are listed in Table 1.4. Torsion angles are listed in Table 1.5.

The molecule lies on a crystallographic twofold axis and all the bond lengths and angles are symmetrical about the C₂ molecular symmetry axis (through O₂, through the midpoint of the C₃--C₃' bond, and through the midpoint of the C₇--C₇' bond in Figure 1.2). Crystallographic symmetry requires that a different numbering system be utilized for the X-ray results as compared to the numbering used in Figure 1.1. The corresponding set of labels are: C₃' = C₄, C₂' = C₅, C₁' = C₆, C₇' = C₈, O₃' = O₄, O₁' = O₆ and similarly for the attached hydrogen atoms. In general, the bond angles of ₁ follow the trends of those of ₂ except for the smaller C₃--C₃'--O₃' and C₃'--C₃--O₃ angles in ₁ (110.9°) as compared to ₂ (113.6° and 114.7°) and the larger C₂--C₃--O₃ and C₂'--C₃'--O₃' angles in ₁ (117.0°) as compared to ₂ (111.6° and 110.5°). This is a result of the fusion of the six-membered ring to this edge of the five-membered ring. The bond lengths of ₁ are similar to the corresponding ones of ₂.

As expected ₁ has a five-membered ring conformation in the same region of the pseudorotation itinerary as the
Figure 1.3
Perspective View
Of 2,5-Anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol.
Table 1.3

Bond Lengths

For 2,5-Anhydro-3,4-\(\alpha\)-(1,2-ethanediyl)-\(\beta\)-mannitol.

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Numbers in parentheses are estimated standard deviations in the least significant digits.
Table 1.4
Bond Angles
For 2,5-Anhydro-3,4-\(\beta\)-(1,2-ethanediyl)-D-mannitol.

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<td>C7'</td>
<td>O3'</td>
<td>C3'</td>
<td>59.82 (0.77)</td>
</tr>
<tr>
<td>C2'</td>
<td>C1'</td>
<td>O1'</td>
<td>H10'</td>
<td>-156.78 (1.15)</td>
</tr>
<tr>
<td>H1a'</td>
<td>C1'</td>
<td>O1'</td>
<td>H10'</td>
<td>-41.43 (1.35)</td>
</tr>
<tr>
<td>H1b'</td>
<td>C1'</td>
<td>O1'</td>
<td>H10'</td>
<td>84.28 (1.35)</td>
</tr>
</tbody>
</table>
parent compound 2, which was shown to exist in a slightly distorted $^4T_3$ conformation with pseudorotation angle of $-11.7^\circ$ and amplitude of 38.8$^\circ$ 1.5. The addition of the six-membered 1,4-dioxane ring in a trans disposition, as dictated by the manno stereochemistry of the parent compound, forces the five-membered ring into an ideal $^4T_3$ conformation in the crystal state with a pseudorotation angle of 0.0$^\circ$ and amplitude of 47.2$^\circ$ 1.14. The atoms C3 and C3' of 1 are each located 0.3771 (8) Å out of the plane defined by atoms C2, O2, and C2'. Both the exocyclic hydroxymethyl groups adopt the $+\text{gauche}$ disposition which results in the placement of H2 between the hydrogens on C1 in the crystalline state.

The solution structure of 1 was also examined by $^1$H- n.m.r. in deuterium oxide (Figure 1.4). While the expected six sets of signals were observed and some of the coupling constants were obtainable by inspection, a simulation of the molecule was required to obtain the remaining coupling constants. The system was treated as an eight spin system consisting of H1, H1', H2, H3, H4, H5, H6 and H6', and a separate four spin system consisting of H7$_{ax}$, H7$_{eq}$, H8$_{ax}$, and H8$_{eq}$ (see Figure 1.1 for numbering). The results of these simulations are shown in Figures 1.5 and 1.6 separately and overlayed in Figure 1.7. The coupling constants thus derived were used in a modified Karplus equation to calculate the
Figure 1.4

$^1$H N.m.r. Spectrum

Of 2,5-Anhydro-3,4-$\beta$-(1,2-ethanediyl)-D-mannitol.
Figure 1.5

PANIC Simulation of the AA'BB'XX'YY' Eight Spin Part
Of 2,5-Anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol.
Figure 1.6

PANIC Simulation of the CC'DD' Four Spin Part
Of 2,5-Anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol.
Figure 1.7
Overlay of PANIC Simulations of
the AA'BB'XX'YY' Eight Spin Part and
the CC'DD' Four Spin Part
Of 2,5-Anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol.
Agreement between the X-ray torsion angles and the n.m.r. derived torsion angles of 1 was excellent. The only major deviation was in the value for the H3—C3—C4—H4 torsion angle. This may be due to an inaccuracy in the Karplus equation parameters utilized. In any event, the agreement between the solid state data and the solution data, at room temperature, indicates that in both cases the molecule is locked in the $^4T_3$ conformation. As concerns the exocyclic hydroxymethyl group hydrogens, they have coupling constants to the ring hydrogen which indicate rotation around the connecting bond, but the major conformation is the same $+\text{gauche}$ disposition as observed in the X-ray results.

The signals for the axial and equatorial hydrogens of 1 were separated by 0.12 p.p.m. at room temperature. A study of the temperature dependency of the spectra was also performed. Spectra at 25°C, 40°C, 60°C and 80°C were obtained. All were identical in line shape and signal patterns indicating that no change in coupling occurred over this temperature range. The only effect of the change in temperature was a differential change in
relative chemical shifts of the six sets of signals such that the best resolution of the signals was obtained at 60°C. The expected results for a system not locked in conformation would be some coalescing of the signals. In this analysis the axial and equatorial hydrogens are the most sensitive indicators of conformational change. In fact no change in their relative chemical shift occurred over this temperature range. The couplings between the hydrogens on the exocyclic hydroxymethyl group and the ring hydrogen did not change. This was expected since they are already in free rotation and only a low temperature study might have changed their values. Thus, it can be concluded that 1 is in fact locked in a twisted conformation (4T₃) in solution in the same form as that observed in the X-ray crystal structure. Studies are underway to use this compound for testing the linear plot of efficacy index versus β-4T₃ concentration observed with the ketose 6-phosphates acting as substrates for phosphofructokinase.
References


CHAPTER TWO

TWO-DIMENSIONAL $^1$H-, $^{13}$C-, and $^{31}$P- NUCLEAR MAGNETIC RESONANCE AND MOLECULAR MECHANICS INVESTIGATION OF D-FRUCTOSE 2,6-BISPHOSPHATE

Introduction to Chapter Two

The structure of an organic compound is classically accepted as conclusively demonstrated when a total unequivocal synthesis has been developed. Because of advances in techniques, three procedures are now used to establish unequivocally the structure of an organic compound. These include: (a) total synthesis with rigorous characterization of all intermediates, (b) determination of the X-ray crystal structure of the compound and (c) determination of the multinuclear nuclear magnetic resonance spectrum of the compound in solution. The nuclear magnetic resonance procedure has been greatly facilitated by advances in two-dimensional nuclear magnetic resonance spectroscopy. Specific assignments of all the observable signals of a metabolite open up new research avenues such as the study of binding interactions with other molecules. A combination of two-dimensional nuclear magnetic resonance spectroscopy and chemical reactions was utilized to establish unequivocally the structure of a carbohydrate phosphate of major biological importance.
The authors have reported the data obtained on fructose 2,6-bisphosphate, a recently discovered carbohydrate modulator of the regulatory enzyme phosphofructokinase, using the nuclear magnetic resonance spectroscopy approach. Future work is planned to pursue the other approaches to further characterize this important metabolic regulator at a later date. The results of this research have been published in the journal Carbohydrate Research. Permission was obtained from the publisher of Carbohydrate Research to include it in this dissertation as indicated by the letter on the following page. A copy of the entire paper constitutes the main body of this chapter. The full reference to this paper is:

"Two-Dimensional \(^1\text{H}-, \(^{13}\text{C}-, \text{ and } \(^{31}\text{P- Nuclear Magnetic Resonance and Molecular Mechanics Investigation of D-Fructose 2,6-Bisphosphate}\)"

Dear Sir,

I am the first author on the two papers listed below which have been published in your journal Carbohydrate Research. They are:


I would like to have your written permission to include them as part of my Ph.D. dissertation which I plan to complete in May 1991.

If you have no objection to this, please send me a letter which I can submit to my graduate committee if the need arises.

If any further information is needed, I am reachable at:
(504)-388-2157 and by FAX # (504)-388-5321. Thank you very kindly for your courtesy.

Sincerely yours,

Ronald J. Voll

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Two-dimensional $^1$H-, $^{13}$C-, and $^{31}$P-nuclear magnetic resonance and molecular-mechanics investigation of D-fructose 2,6-bisphosphate

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**ABSTRACT**

Two-dimensional nuclear magnetic resonance studies have been carried out to assign unequivocally all the proton, carbon, and phosphorus resonances of D-fructofuranose 2,6-bisphosphate (1) and to verify its structure using a 400-MHz spectrometer. Several unexpected chemical-shift values and coupling constants were obtained. Molecular mechanics calculations (Sybyl) carried out to minimize the conformational energy of 1 yield $\phi_{C1\cdot P2} = +84^\circ$, $\phi_{C3\cdot P2} = -155^\circ$, and $\phi_{C5\cdot P4} = +175^\circ$. Thus the unusual near-gauche orientations of C-1 and C-3 to P-2 in I can explain their small vicinal coupling constants ($J_{C1\cdot P2} = 1.2$, and $J_{C3\cdot P2} = 3.8$ Hz), in contrast to the expected larger value seen for $J_{C5\cdot P4}$, namely, 6.9 Hz. Treatment of a sample of this compound with sodium borohydride did not affect its nuclear magnetic resonance spectrum, substantiating that O-2 is phosphorylated. Oxidation with sodium periodate yielded an intermediate which decomposed by a $\beta$-elimination mechanism involving the 6-phosphate group. These data establish unequivocally the $^1$H, $^{13}$C, and $^{31}$P assignments and explain the observed anomalous shifts. Moreover they indicate that the activator of fructose 6-phosphate 1-kinase is the $\beta$ anomer of the $T$, conformer of D-fructose 2,6-bisphosphate.

**INTRODUCTION**

In 1980, the isolation of a low-molecular-weight compound from rat liver which stimulated the activity of D-fructose 6-phosphate 1-kinase was reported. The activator was identified as D-fructose 2,6-bisphosphate (1), based on its $^{13}$C-n.m.r. spectra and our assignments for carbon signals of D-fructose 6-phosphate (2) and D-fructose 1,6-bisphosphate (3). Independently and simultaneously, the same structure (1) of the activator was reported by two other groups. A synthetic compound showing the same enzymic effects as the natural modulator was synthesized by these three groups, starting with 3 and $N,N'$-dicyclohexylcarboimide. This new modulator turned out to be one of the most important discoveries in the area of carbohydrate metabolism in Eukaryotes during the past decade and may have a role in the pathogenesis of diabetes mellitus. Its metabolic role has been reviewed and its enzymic stereospecificity has been reported.

We have observed certain anomalies in the $^{13}$C spectra of this compound as

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reported in the literature\textsuperscript{5,7,13}. Moreover, because of the sterically nonspecific method of its synthesis, it was deemed appropriate to carry out further work to ascertain its structure. This paper reports detailed one-dimensional (1-D) and two-dimensional (2-D) n.m.r. studies, and molecular-mechanics calculations, as well as chemical studies on 1. Complete and verified assignments of the \textsuperscript{1}H, \textsuperscript{13}C, and \textsuperscript{31}P chemical shifts are given.

**EXPERIMENTAL**

**Materials.** — \textalpha-Fructose 2,6-bisphosphate (1), sodium salt, was purchased from Sigma Chemical Co., St. Louis. This material was found by 1-D proton-n.m.r. analysis to have some volatile impurities, which were decreased considerably after repeated lyophilization of its D\textsubscript{2}O solution.

**Methods.** — N.m.r.-spectral studies were performed using a 0.6\texttimes\textsuperscript{m} solution of 1 in D\textsubscript{2}O at pH 8.3. The sample was stored at 5° when not in use. Data were acquired on a Bruker AM400 instrument at ambient probe temperature [\textsuperscript{1}H (400.1 MHz), \textsuperscript{13}C (100.6 MHz), and \textsuperscript{31}P (162.0 MHz)]. Three types of 2-D n.m.r. experiments were carried out. The homonuclear scalar correlation spectrum (COSY) was obtained according to the procedure of Aue \textit{et al.}\textsuperscript{14}. The distortionless enhancement by polarization transfer (DEPT) spectrum was prepared according to Doddrell \textit{et al.}\textsuperscript{15}. The heteronuclear COSY experiments were carried out according to Maudsley and Ernst\textsuperscript{16} and Bax and Morris\textsuperscript{17}. Representative spectral parameters are as follows: For the spectrum of Fig. 2, the 256\texttimes\textsuperscript{1} experiments were performed with 32 scans and 2K data points in t\textsubscript{2}. Data were zero-filled once in the \textit{t}\textsubscript{1} dimension and were matrix-processed in the magnitude mode using sine-bell window functions in both dimensions. Digital resolution along \textit{t}\textsubscript{0} and \textit{t}\textsubscript{2} was 1.34 and 10.4 Hz, respectively. For the spectrum of Fig. 3, the 256\texttimes\textsuperscript{2} data matrix (NS = 16) was processed in the magnitude mode, using sine-bell functions in both directions. Digital resolution along \textit{t}\textsubscript{1} and \textit{t}\textsubscript{2} was 3.32 and 1.59 Hz, respectively.

**Chemical studies.** — These were performed using a 0.1\texttimes\textsuperscript{m} solution of 1 in a 10\% D\textsubscript{2}O–H\textsubscript{2}O mixture. The reactions were monitored at the \textsuperscript{13}C frequency [50.3 MHz] on a Bruker WP200 instrument operating in the proton-decoupled mode at ambient probe temperature. The sodium borohydride treatment consisted of the addition of the solid reagent (7 equivalents of hydride) to the solution at 0°, with reaction allowed to proceed for 3 h at 5° before the spectrum was acquired. This is an adequate time for at least some of any free carbonyl group to be reduced. The sodium periodate treatment consisted of the addition of a solution of the reagent (5 equivalents) to the solution of 1 at 25° and the reaction allowed to proceed for 30 min before the spectrum was acquired. The sample was then stored for one month at 5° for later acquisition of spectra in order to study the final product composition. Control experiments with 2,5-anhydro-\textalpha-mannitol and its 1,6-bisphosphate (4) showed that these conditions were adequate to oxidize a trans-vicinal glycol unit and to cause a \textbeta-elimination of the phosphate group.

**Molecular mechanics.** — Studies of the conformation of 1 were done by using a structure built-up from tetrahydrofuran which was obtained from the Cambridge database. The absolute stereochemistry of the resulting structure was checked at each
chiral carbon atom. The conformational analysis was conducted while fixing the furanose ring in the \( ^4T_1 \) (d) conformation, as this ring conformation has been observed for all 2-hexuloses in their X-ray crystal structures, and was shown to be the enzymically active conformer in certain cases\(^{1,18,19} \). Both molecular building and conformational analyses were carried out using commercial software (Tripos Sybyl 3.5 and 5.1) for force-field calculations. The conformational search was done in two stages: the first involving the simultaneous rotation about bonds O-2-C-2, C-1-C-2, and C-3-O-3. Each bond was rotated in 5° increments from 0 to 360°, while fixing the geometry of the rest of the molecule. Initially, the phosphate groups were assumed not to be protonated, and the lowest conformational energy without charges was calculated. Subsequently, a charge of \(-0.66\) was added to each phosphate non-ester oxygen, the Pullman electrostatic charges calculated\(^{20} \), and the conformational search reinitiated. After the lowest energy was found for the three bonds around the P-2 phosphate, a similar conformational analysis about O-6-C-6 and C-6-C-5 was carried out.

RESULTS AND DISCUSSION

Two-dimensional n.m.r. studies. — Proton J-connectivities and subspectra. Shown in Fig. 1 are the 1-D highdigital resolution and the 2-D COSY spectra of 1. Integration of the 1-D spectrum reveals a total of seven non-exchangeable protons. The proton J-connectivities shown in the COSY spectrum reveal two subspectra. These are a downfield five-proton AHM(XZ) spin-system and an upfield two-proton AB spin-system. As has been previously pointed out\(^ {21} \), such a 5 + 2 pair of subspectra is characteristic of 2-hexuloses. The tightly-coupled M(XZ) part of the five-spin subspectrum is also very characteristic of 2-hexuloses and their derivatives\(^ {22} \). Both the \( J_{AH} \) and \( J_{HM} \) vicinal coupling-constants of the AHM(XY) system are seen to be large (> 5 Hz). Thus, the orientation of both A to H and H to M must be closely trans-periplanar and the stereochemistry of the 2-hexulose must be either d-arabino or l-arabino\(^ {23} \). Given the structure of the starting material (3), 1 must be a d-arabino-2-hexulose. Thus, the constitution and configuration of the monosaccharide core of 1 have remained intact during synthesis from d-fructofuranose 1,6-bisphosphate (3).

Carbon protonation, connectivities, and subspectra. The 1-D \(^{13} \)C spectrum of 1, which indicates that it contains six carbons, is partially shown in Fig. 2. Determination of the protonation of these six carbons through a DEPT experiment (not shown) reveals that the two upfield carbons are methylenes, the most downfield carbon is unprotonated, and the remaining three carbons are methines. In order to establish the proton-carbon J-connectivities of the protonated carbons of 1, a 2-D heteronuclear COSY experiment was carried out (Fig. 2). This study unambiguously connected the carbon resonances at 78.48, 76.54, 81.07, and 66.55 p.p.m. to the AHM(XY) proton subspectrum and the carbon resonance at 63.79 p.p.m. to the AB proton subspectrum. In all cases, the protonation of each carbon was correctly accounted for by the number of \(^1\)H–\(^{13} \)C connectivities. Thus, the AHM(XY) proton subspectrum must arise from a \(-\text{CH–CH–CH–CH} –\) fragment in 1, which for a 2-hexulose can only be the structure
Fig. 1. 1-D 'H and 2-D scalar 'H–'H shift-correlated n.m.r. spectrum of D-fructose 2,6-bisphosphate.

associated with the last four carbons of the molecule (C-3 through C-6). Likewise, the AB subspectrum must arise from the C-1–CH$_2$– fragment and the unprotonated carbon (104.84 p.p.m.) must be the C-2 anomeric carbon. The extreme downfield shift of this anomeric carbon indicates that 1 has a β-fructofuranose ring. Having thus allocated all seven protons and six carbons to these three structural fragments, complete proton and carbon resonance assignments can be made (Table I). These assignments are confirmed by their similarity with the analogous carbon and proton resonances in other 2-hexuloses (ketohexoses) and related 2,5-anhydrohexitols, especially 4.

Phosphorus resonances and couplings. Having established that the carbon and proton skeleton of 1 are unchanged from its precursor 3, it is clear that the novelty of 1
lies in the redispersion of its phosphate groups. Integration of the 1-D $^{31}\text{P}$ spectrum of 1 (Fig. 3, $^{31}\text{P}$-axis) shows two resonances at 0.35 and 4.77 p.p.m. In order to establish the proton–phosphorus connectivity of these two phosphorus nuclei, a 2-D heteronuclear COSY experiment was carried out (Fig. 3). This study showed that the $^{31}\text{P}$ nucleus at 4.77 p.p.m. coupled strongly ($J > 5$ Hz) to two protons, H-6a and H-6b, and the $^{31}\text{P}$ nucleus at 0.35 p.p.m. coupled strongly to no protons. Also seen were weak couplings ($J < 2$ Hz) for the downfield $^{31}\text{P}$ nucleus to H-5 and for the upfield nucleus to H-3. The finding that the downfield $^{31}\text{P}$ is strongly coupled to the H-6 protons indicates that the resonance at 4.77 p.p.m. arises from a C-6 phosphorylated hydroxymethyl group. The failure to find any proton–phosphorus coupling to H-4 rules out any 4,6-cyclic phosphate diester.

The finding that the upfield $^{31}\text{P}$ nucleus fails to manifest any strong coupling is

TABLE I

<p>| N.m.r. chemical-shift data and assignments of 1 |</p>
<table>
<thead>
<tr>
<th>$^1\text{H}$</th>
<th>$^13\text{C}$</th>
<th>$^{31}\text{P}$</th>
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<tr>
<td>3.95</td>
<td>H-4</td>
<td>104.84 C-2</td>
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<td>3.80</td>
<td>H-3</td>
<td>81.07 C-5</td>
</tr>
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<td>H-1b</td>
<td>78.48 C-3</td>
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<td>3.72</td>
<td>H-6b</td>
<td>76.54 C-4</td>
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<td>3.71</td>
<td>H-5</td>
<td>66.55 C-6</td>
</tr>
<tr>
<td>3.69</td>
<td>H-6a</td>
<td>63.79 C-1</td>
</tr>
<tr>
<td>3.62</td>
<td>H-1a</td>
<td></td>
</tr>
</tbody>
</table>

The observable $^1\text{H}^\leftrightarrow^1\text{H}$ spin-couplings (in Hz) are:

$^1J_{H-1b,H-6a} = 12.7$, $^1J_{H-3,H-4} = 7.9$, $^1J_{H-6a,H-5} = 6.7$.

The observable $^1\text{H}^\leftrightarrow^{31}\text{P}$ spin-couplings (in Hz) are:

$^3J_{H-6b,P-6}$ and $^3J_{H-6a,P-6} = 5.5$.

$^3J_{H-5,P-6} = 1.1$, $^3J_{H-3,P-2}$, and $^3J_{H-1b,P-2} < 0.1$.

The $^{13}\text{C}^\leftrightarrow^{31}\text{P}$ spin-couplings (in Hz) are:

$^5J_{C-2,P-2} = 6.4$, $^5J_{C-3,P-2} = 4.2$.

$^5J_{C-1,P-2} = 1.2$, $^5J_{C-3,P-2} = 3.8$, and $^5J_{C-3,P-4} = 6.9$.

* In p.p.m. with internal 1,4-dioxane at 3.53 p.p.m. * In p.p.m. with internal 1,4-dioxane at 67.40 p.p.m. * In p.p.m. with respect to phosphoric acid as external standard. P-2 and P-6 are the phosphates attached to C-2 and C-6 respectively.
very significant. Since vicinal ($J_{HP}$) coupling should always be greater than 2 Hz, its absence indicates that the upfield $^{31}$P nucleus is linked to a carbon that fails to present a vicinal proton for $^{31}$P coupling. This circumstance can only arise if the linkage carbon is unprotonated. Only one carbon is unprotonated, namely the anomeric carbon C-2. Thus, the upfield $^{31}$P resonance must be ester linked to C-2. The failure to find any other proton-phosphorus couplings in Fig. 3 rules out any 1,2- or 2,3-cyclic phosphate diesters. Thus, it may be concluded that all 2-D n.m.r. data are consistent with the assigned structure of the activator, namely $\beta$-D-fructofuranose 2,6-bisphosphate.

Chemical studies. — Borohydride reduction. The first chemical study involved the addition of one equivalent of sodium borohydride to the solution of 1 in an attempt to reduce any free anomeric carbon present in the molecule. The initial $^{13}$C spectra before the addition contained only the six signals of the six carbons in the molecule, with splittings caused by the phosphate ester groups. After the treatment, the resulting spectra showed no change in any of the original signals and no new signals were observed. These results indicate that the hemiacetal group of the molecule is blocked by a base-stable group. This information is consistent with the O-2 phosphorylation of 1.

Periodate oxidation. The second chemical study involved the addition of one equivalent of sodium periodate to a fresh solution of 1 in an attempt to oxidize any free vicinal glycol unit or equivalent group present in the molecule. The $^{13}$C spectrum before the addition was the same as already described. The resulting spectra showed several new peaks in the methylene region and in the anomeric region. In particular a new peak appeared at $\sim$ 110 p.p.m., indicating the creation of a new hemiacetal carbon. Another
spectrum was acquired after an extended period (see Experimental) which showed signals for vinyl carbons, indicating that a β-elimination of the phosphate group on C-6 had occurred. These results show that a vicinal glycol unit is present in 1 and that the molecule also contains a leaving group beta to one of the carbonyl groups of the cleavage product. Such a structural fragment is present in the d-fructofuranose 2,6-bisphosphate structure, but not in other possible structures such as the 3,6-bisphosphate. In summary, the chemical studies corroborate the findings obtained from the 2-D n.m.r. studies.

Unexpected couplings. — The fact that 1 is phosphorylated at C-6 is confirmed by the observed $^{13}$C-$^3$P couplings. Both $^2J_{C_6,P_6} = 4.2$ and $^2J_{C_5,P_6} = 6.9$ Hz are equal to the values expected from studies of many ketohexose phosphates. However, the conclusion that 1 is phosphorylated at C-2 yields $^{13}$C-$^3$P couplings that are anomalous. Thus the observed $^2J_{C_2,P_2} = 6.4$ Hz is 2 Hz larger than the value reported for other ketohexose phosphates. Moreover, the vicinal couplings $^3J_{C_1,P_2} = 1.2$ and $^3J_{C_3,P_2} = 3.8$ Hz are at least 5 and 2 Hz smaller, respectively, than expected. The finding that $^2J_{C_2,P_2}$ is larger than expected can be explained as due to the effect of the C-2 carbon lying within the fructofuranose ring, adjacent to the ring oxygen. No $^3J_{C,P}$ has previously been reported in such an environment. The unexpected $^3J_{C,P}$ values are more problematic and may only be explained if the P-2-C-1 and P-2-C-3 dihedral angles are unusual. This turned out to be the case (see later).
Another coupling problem that results from the assignment of a 2,6-bisphosphate structure (1) to the activator is that it means the \(^1H-^{31}P\) coupling at H-3 and H-5 must result from a four-bond or long-range coupling. Such long-range couplings are certainly possible; however, it is not clear why the two protons at H-1 do not also participate in such coupling. In order to explain the unexpected P-2 vicinal couplings, as well as the weak \(^4J\) proton–phosphorus couplings observed at H-3 and H-5 but not at the H-1 protons, we undertook a molecular-mechanics study of the \(\beta\)-D-fructofuranose 2,6-bisphosphate structure.

**Molecular-mechanics studies.** — Shown in Fig. 4 is the lowest energy conformation of \(\beta\)-D-fructofuranose 2,6-bisphosphate (1), as determined by molecular-mechanics calculations. The calculated dihedral angles of pertinent fragments of 1 are presented in Table II for comparison with n.m.r. coupling constants. These angles have been sequentially numbered in Table II for convenience in reference.

Considering the problem of the unexpected \(^{13}C-^{31}P\) vicinal coupling-constants, it is seen that the pertinent angles 3 and 6 are in fact different from the \(\sim 180^\circ\) angle expected for POCC dihedral angles\(^3\) and actually observed for angle 1 of 1. This difference is seen even more clearly for the angles computed after introducing the Pullman charges in the calculation. Angles 1, 3 and 6 are then seen to be +175, +84, and \(-155^\circ\), respectively, and therefore closely follow a Karplus relation with vicinal \(^{13}C-^{31}P\) couplings of 6.9, 1.2, and 3.8 Hz, respectively. Thus, the unexpected \(^{13}C-^{31}P\) vicinal couplings may be rationalized as resulting from deviations from trans-periplanarity in POCC dihedral angles, a situation unavoidable with 2-phosphorylation, but previously not encountered in ketohexose phosphates\(^3\).

Concerning the problem of selective \(^1H-^{31}P\) long-range coupling, it is important to recall the established requirement for such coupling\(^27\), namely that it is confined to a

### TABLE II

Dihedral angles present in the lowest energy conformation of \(\beta\)-D-fructofuranose 2,6-bisphosphate obtained from molecular-mechanics calculations, and comparison with observed \(^{31}P\) couplings of 1

<table>
<thead>
<tr>
<th>Angle No.</th>
<th>Four atom fragment</th>
<th>Dihedral angle(^a)</th>
<th>Observed coupling constants (Hz)</th>
<th>(^{13}C-^{31}P) (J)</th>
<th>(^{1H-^{31}P}) (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P-6, O-6, C-6, C-5</td>
<td>+175(^a)</td>
<td>6.9</td>
<td>(&lt;2^a)</td>
<td>(&lt;2^a)</td>
</tr>
<tr>
<td>2</td>
<td>O-6, C-6, C-5, H-5</td>
<td>-174(^a)</td>
<td>1.2</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>3</td>
<td>P-2, O-2, C-2, C-1</td>
<td>+69(^a) (+84(^a))</td>
<td>1.2</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>4</td>
<td>O-2, C-2, C-1, H-1a</td>
<td>-80(^a)</td>
<td>3.8</td>
<td>(-153^a)</td>
<td>(-153^a)</td>
</tr>
<tr>
<td>5</td>
<td>O-2, C-2, C-3, H-1b</td>
<td>+160(^a)</td>
<td>1.1</td>
<td>(-153^a)</td>
<td>(-153^a)</td>
</tr>
<tr>
<td>6</td>
<td>P-2, O-2, C-2, C-3</td>
<td>-170(^a) (-155(^a))</td>
<td>3.8</td>
<td>(-153^a)</td>
<td>(-153^a)</td>
</tr>
<tr>
<td>7</td>
<td>O-2, C-2, C-3, H-3</td>
<td>-153(^a)</td>
<td>1.1</td>
<td>(-153^a)</td>
<td>(-153^a)</td>
</tr>
</tbody>
</table>

\(^a\) Angles calculated using force-field equations (Sybyl 3.5). Angles in parentheses were calculated using Pullman charges (Sybyl 5.1). Angles are accurate to \(+/−3^\circ\), as calculations were carried out at \(5^\circ\) increments. \(^a\) Coupling is clearly present in the \(^1H-^{31}P\) COSY experiment (Fig. 3), however, accurate measure of \(^4J\) is not possible due to overlapping resonances in the high-resolution proton spectrum.
Fig. 4. The lowest-energy conformation of β-D-fructose 2,6-bisphosphate obtained through Sybyl force-field calculations.

near-planar zig-zag configuration for the five atoms involved. Such a "W-configuration" is in fact calculated for the P-2, O-2, C-2, C-3, H-3 fragment, since both angles 6 and 7 are within 30° or less of trans-periplanarity (−170 and −153°, respectively). On the other hand, no "W-configuration" is calculated for P-2, O-2, C-1, H-1a, or H-1b with at least angle 3 for both being very non-planar (+84°). Thus, the long-range ¹H-³¹P coupling seen for H-3, but not H-1a and H-1b, can be explained by the unique orientation between P-2 and H-3 that is found in the lowest energy conformation of β-D-fructofuranose 2,6-bisphosphate. In fact, this "W-configuration" is so difficult to create that the observation of ²H₁₃P₂ coupling can be used as a confirmation of the β-anomeric configuration of 1. It is impossible to create a "W-configuration" between P-2 and H-3 in the α-anomer (²H₁₃P₂ would be observed in this case). Finally, it should be noted in Table II that both angles 1 and 2 are calculated to be within 10° or less of trans-periplanarity (+175 and −174°, respectively). Thus, the long-range coupling observed between H-5 and P-6 may also be rationalized in terms of the "W-configuration".

In conclusion, this investigation of the structure of 1 using 2-D n.m.r. techniques confirms the previously proposed β-D-fructofuranose 2,6-bisphosphate structure. The anomalous ¹³C-³¹P couplings have been rationalized through molecular-mechanics studies that show these couplings to be expected for the β-D-fructofuranose ⁴T₃ 2,6-bisphosphate structure.

ACKNOWLEDGMENT

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REFERENCES

22 T. A. W. Koerner, Jr. (1975), Ph.D. Dissertation, Louisiana State University, Baton Rouge.
CONCLUSIONS

The glycolytic enzyme phosphofructokinase has as its physiological substrate, as its physiological product, and as a recently discovered activator three different carbohydrate phosphate esters. Structurally all of these metabolites are derivatives of D-fructose in the furanose ring form. The chemistry of these fructofuranose systems is the focus of this dissertation.

The active site specificity of phosphofructokinase is the theme of the first part of this dissertation. Phosphofructokinase has as its natural substrate D-fructose 6-phosphate. Based upon earlier studies from this laboratory the physiological form of the substrate seems to be the $^4T_3$ conformer of the $\beta$-D-fructofuranose 6-phosphate. The conclusion that only one conformer, out of a predicted three major ones, is preferred was based upon a linearity of logarithm of the substrate efficacy index $V_{\text{max}}/K_m$ versus the logarithm of the concentration of the $\beta$-$^4T_3$ conformer for four ketose 6-phosphates tested as alternate substrates of the enzyme. No other conformer gave such a linear relationship. This conformer is not the exclusive one in solution and further investigations were needed to verify the proposal that it is the preferred conformer acted upon by phosphofructokinase. As regards the activator site of phosphofructokinase, the assumption that it also prefers the $^4T_3$ conformer of
the activator is an attractive proposal which was examined in the course of this work.

In the first study, which was directed toward the active site of phosphofructokinase, the author has designed and synthesized a compound which was expected to be locked in the $^{4}\text{T}_3$ conformation in solution and suitable for later enzymatic studies. The starting material for this synthesis was 2,5-anhydro-D-mannitol. In this laboratory, the mono-phosphate derivative of this starting material has been found to be a substrate for phosphofructokinase and provides the efficacy index reference value. The desired compound, namely 2,5-anhydro-3,4-O-(1,2-ethanediyl)-D-mannitol, was successfully prepared by a cyclo-dialkylation to produce a strained, trans-fused 2,5,8-trioxabicyclo[4.3.0]nonane derivative in three steps from 2,5-anhydro-D-mannitol. The product was studied by X-ray single crystal diffraction. As regards the five-membered ring, it was found to exist in an ideal $^{4}\text{T}_3$ conformation. The product was also studied utilizing n.m.r. spectrometry. Simulations of the two independent parts of the molecule yielded two spectra which when combined reproduced the experimental spectrum. The torsion angles derived from the n.m.r. data and the X-ray results were identical within experimental error. A temperature dependent solution n.m.r. investigation showed no line shape change
over the range from 25°C to 80°C. The only change was a
differential change in relative chemical shifts which
resulted in the signals being better separated at 60°C
than at the other temperatures investigated.
This confirms that the molecule, in solution, is also
locked in the $^{4T_3}$ conformation. Further studies are
planned to use the mono-phosphate of 2,5-anhydro-3,4-\(\alpha\)-
(1,2-ethanediyl)-D-mannitol to verify that
phosphofructokinase does prefer the $^{4T_3}$ conformation of its
physiological substrate.

In the second study, which was directed toward the
exploration of the activator site of phosphofructokinase,
this candidate has investigated in detail the structure of
a recently discovered (1981) potent activator of
phosphofructokinase by n.m.r. techniques. This compound
has the putative structure: D-fructose 2,6-bisphosphate.
It simultaneously activates the glycolytic pathway and
acts as an inhibitor of the reverse pathway which is
gluconeogenesis; thus suppressing futile cycling.
Rigorous proof of its structure and experimental evidence
for its enzymatically active conformer were lacking.
A very detailed $^1$H-, $^{13}$C-, and $^{31}$P-n.m.r. investigation in
conjunction with certain chemical reactions studied by
$^1$H-n.m.r. confirms that the structure of the activator is
in fact D-fructose 2,6-bisphosphate. The data indicate
that it has the $\beta$-anomeric configuration. This conclusion
was only possible by careful analysis of several unexpected chemical shifts and coupling constants and use of molecular mechanics calculations. Taking into consideration all the data, the structure of the activator was finally established to be $\beta$-D-fructose 2,6-bisphosphate. Furthermore, the activator appears to be predominately in the $^4T_3$ conformation in solution based upon the magnitude of the coupling constants obtained. The data suggest that the activator site of phosphofructokinase, like its active site, has evolved to bind preferentially the $^4T_3$ conformer of the activator.

Pertinently, Poorman et al.\textsuperscript{1} postulated that the activator site of phosphofructokinase has evolved from its active site by a process of gene duplication and divergence. The preference of the active site for the $^4T_3$ conformer and the evidence presented in this dissertation that the activator is predominantly in the $^4T_3$ conformation seem to corroborate this postulation.

The data obtained in the course of this investigation and their interpretations will allow further studies on the enzyme-ligand molecular recognition using n.m.r. techniques for not only phosphofructokinase but all the other proteins that bind this important effector e.g. fructose 1,6-bisphosphatase, pyruvate kinase and 6-phosphogluconate dehydrogenase.

In summary, this candidate has provided evidence that both the active site and the regulatory site of phosphofructokinase seem to prefer the $^{4}T_{3}$ conformer of these fructofuranose ligands. Moreover, this study opens the way for further experimentation to verify this hypothesis using techniques described in detail in this dissertation.
APPENDIX

List of Publications Of Ronald J. Voll

1. "The Fructose 6-Phosphate Site of Phosphofructokinase; I. Tautomeric and Anomeric Specificity"
   T. A. W. Koerner, Jr., E. S. Younathan, A. E. Ashour, and R. J. Voll,

2. "The Fructose 6-Phosphate Site of Phosphofructokinase; II. Epimeric Specificity"
   T. A. W. Koerner, Jr., R. J. Voll, A. E. Ashour, and E. S. Younathan,

3. "Tautomeric and Anomeric Specificity of Allosteric Activation of Yeast Pyruvate Kinase by Fructose 1,6-Bisphosphate and its Relevance in D-Glucose Catabolism"
   B. Wurster, B. Hess, T. A. W. Koerner, Jr., R. J. Voll, and E. S. Younathan,
4. "A Proposed Model for the Regulation of Phosphofructokinase and Fructose 1,6-Bisphosphatase Based on Their Reciprocal Anomeric Specificities"
   T. A. W. Koerner, Jr., R. J. Voll, and E. S. Younathan,

5. "Isolation of 2,5-Anhydro-1,3-O-Isopropylidene-6-O-Trityl-D-Glucitol and Conformations of its 4-O-Substituted and Deprotected, Acylated Derivatives. New Derivatives of 2,5-Anhydro-D-Hexitols. Part I"
   T. A. W. Koerner, Jr., R. J. Voll, and E. S. Younathan,

6. "Photoelectron Spectroscopy of Some Biological Molecules"

7. "Reassignment of the Methine Resonances of D-Fructose Based on the Carbon-13 N.M.R. Spectrum of 3-O-Methyl-D-Fructose"
   T. A. W. Koerner, Jr., R. J. Voll, L. W. Cary, and E. S. Younathan,
8. "Synthesis of 2,5-anhydro-\( \text{D} \)-glucitol 6-phosphate. New Derivatives of 2,5-Anhydro-\( \text{D} \)-hexitols. Part II"  
T. A. W. Koerner, Jr., R. J. Voll, and E. S. Younathan,  

T. A. W. Koerner, Jr., R. J. Voll, L. W. Cary, and E. S. Younathan,  
Biochemistry, 19, 2795-2801 (1980).

10. "Anomeric Specificity and Regulation of Phosphofructokinase. Stereochemical Considerations in Carbohydrate Metabolism in Normal and Certain Pathological States"  

11. "Purification of 2,5-Anhydro-\( \text{D} \)-Hexitol Bis(phosphates) and Identification of a Major 1,4,6-Tris(phosphate) Contaminant by \(^{31}\text{P}-\), \(^{13}\text{C}-\), and \(^{1}\text{H}-\text{N.M.R.} \) Spectroscopy"  
R. J. Voll, T. A. W. Koerner, Jr., P. A. Bartlett, N. S. Bhacca, D. C. Lankin, and E. S. Younathan,  
S. F. Watkins, K. A. Abboud, R. J. Voll,
T. A. W. Koerner, Jr., and E. S. Younathan,

13. "Stereospecificity of the Fructose 2,6-Bisphosphate
Site of Muscle 6-Phosphofructo-1-kinase"
E. L. Kelley, R. J. Voll, V. A. Voll,
and E. S. Younathan,
Biochemistry, 25, 1245-1248 (1986).

14. "Carbohydrate Substrate Specificity of Bacterial and
Plant Pyrophosphate-Dependent Phosphofructokinase"
B. L. Bertagnolli, E. S. Younathan, R. J. Voll,
C. E. Pittman, and P. F. Cook,

15. "Kinetic Studies on the Activation of Pyrophosphate-
Dependent Phosphofructokinase from Mung Bean by
Fructose 2,6-Bisphosphate and Related Compounds"
B. L. Bertagnolli, E. S. Younathan, R. J. Voll,
and P. F. Cook,
Biochemistry, 25, 4682-4687 (1986).

16. "The X-Ray Crystal Structure of D,L-(1,3,5,2,4)-
1,2,3,4-Tetra-acetoxy-5-(acetoxy methyl) cyclohexane"
S. F. Watkins, K. A. Abboud, N. P. Nghiem,
R. J. Voll, and E. S. Younathan,
17. "Ab Initio SCF Energy Calculations of The Rotational Conformations of Both Hydroxymethyl Groups of The KetoheXose 6-O-Methyl β-D-Tagatofuranose"
J. A. Darsey, R. J. Voll, E. S. Younathan, and T. A. W. Koerner, Jr.,
18. "The X-Ray Crystal Structure of 2,3:4,5-Di-O-Isopropylidene-1-O-Methyl-β-D-Fructopyranose"
S. F. Watkins, S. K. Kim, F. R. Fronczek, R. J. Voll, and E. S. Younathan,
19. "Two-Dimensional 1H-, 13C- and 31P- Nuclear Magnetic Resonance and Molecular Mechanics Investigation of D-Fructose 2,6-Bisphosphate"
R. J. Voll, S. Ramaprasad, D. Vargas, E. S. Younathan, S. Laban, and T. A. W. Koerner,
20. "Structure of 1,2,3,4,5,6-Hexa-O-Acetyl-myo-Inositol"
K. A. Abboud, S. H. Simonsen, R. J. Voll, and E. S. Younathan,
J. G. Garcia, R. J. Voll, and E. S. Younathan,
VITA

RONALD JOSEPH VOLL

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate:  Ronald J. Voll

Major Field:  Chemistry

Title of Dissertation:  Studies in Fructofuranose Chemistry

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

July 10, 1991