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รายงานการวิจัย

การสังเคราะห์และการวิเคราะห์โครงสร้าง Dihexulose Dianhydrides และสารอื่นที่เกี่ยวข้อง

(Synthesis and Structural Characterization of Dihexulose Dianhydrides and Related Compounds)

ด้รับทุนอุดหนุนการวิจัยจาก
มหาวิทยาลัยเทคโนโลยีสุรนารี

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คณะผู้วิจัย

หัวหน้าโครงการ

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ผู้ช่วยวิจัย

นางสาวสายฝน จันผกา

ได้รับทุนอุดหนุนการวิจัยจากมหาวิทยาลัยเทคโนโลยีสุรนารี ปีงบประมาณ พ.ศ. 2547

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

กุมภาพันธ์ 2554

บทคัดย่อ

งานวิจัยนี้ได้ทำการทดลองสังเคราะห์สารDixhexulosedianhydridesจากสารละลาย3ชนิดชนิดแรกคือสารละลายเดี่ยวhexuloseketosemonosaccharides(ใช้D-fructoseหรือL-sorboseอย่างใดอย่างหนึ่ง) ชนิดที่สองคือสารละลายผสมhexuloseketosemonosaccharides(สารละลายผสมของD-fructoseและL-sorbose)และชนิดที่สามคือสารละลายเดี่ยวhexuloseketosemonosaccharidesกับhexulose aldose monosaccharides(สารผสมของD-mannose กับD-fructose หรือL-sorboseอย่างใดอย่างหนึ่ง)กรดที่ใช้3ชนิดคือกรดไฮโดรคลอริคกรดซัลฟูริคและกรดไนตริกจากผลการทดลองพบว่ากรดทั้งสามชนิดใช้ในการสังเคราะห์สารDixhexulosedianhydridesได้ดีโดยสภาวะการทดลองที่เหมาะสมคือความเข้มข้นของกรดที่ใช้ 1.25 M อุณหภูมิ75^{o}Cระยะเวลาทำปฏิกิริยา 2ชั่วโมง

สารสังเคราะห์ที่ได้ถูกทำให้บริสุทธิ์ด้วยการหมักกับยีสต์และการตกผลึกผลิตภัณฑ์ที่ได้นำมาศึกษาคุณลักษณะด้วยเครื่องวิเคราะห์^{13}C NMR, Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Fourier-Transform Infrared (FT-IR) spectroscopy และDispersive X-Ray (EDX) spectroscopy จากผลการวิเคราะห์พบว่าผลิตภัณฑ์ที่ได้คือdifructosedianhydridesอย่างไรก็ตามผลึกที่เตรียมได้จาก^{13}C NMR พบว่ามีขนาดเล็กเกินไปที่จะศึกษาโครงสร้างผลึกด้วยเครื่องX-Ray diffraction

Abstract

Dixhexulosedianhydrides have been synthesized from a range of single hexuloseketosemonosaccharides (including D-fructose and L-sorbose, however D-tagatose was also shown to be a potential candidate for production of dianhydrides), from mixtures of hexuloseketosemonosaccharides (mixtures of D-fructose and L-sorbose were used), and also mixtures of hexuloseketosemonosaccharides and hexulose aldose monosaccharides (mixtures of D-glucose with either D-fructose or L-sorbose, and mixtures of D-mannose with either D-fructose or L-sorbose were used). All acids used, including hydrochloric, sulfuric, nitric, and hydrofluoric, were successful in synthesizing the desired dihexulosedianhydride products. Conditions which resulted in near optimum results for both yield and selectivity of the desired products were determined, and these conditions were at approximately 1.25 M acid (which appeared to have small dependence on the type of acid used), 75ºC, and 2 h reaction time. Although there is a broad optimum for synthesis conditions, using acid concentrations in excess of 1.5 M, reaction times in excess of 3 h, and/or reaction temperatures in excess of 90ºC will lead to further degradation of the desired products into lower molecular weight residues; these conditions are not recommended.

Many of the reaction products have been purified using a combination of yeast degradation of residual sugar reagents and crystallization. These products have been characterized by a range of techniques, including ^{13}C NMR, Gas Chromatography (GC) of the trimethylsilyl (TMS) derivatives of the compounds, high performance liquid chromatography (HPLC), Fourier-Transform Infrared (FT-IR) spectroscopy, and Energy Dispersive X-Ray (EDX) spectroscopy. Based on results from previous researchers the structures of several products could be specified, which were mainly difructosedianhydrides. Due to the inaccessibility of a preparative-scale HPLC our ability to separate enough pure product to achieve diffraction-sized single crystals was greatly inhibited, however diffraction was performed on several crystals that were found in product samples. Unfortunately these were found to be crystals of unreacted reagents or crystals of salts precipitating as a result of the reaction between the acid used to cause reaction and the base used later to neutralize the mixture. Crystals shown to be products by ^{13}C NMR were unfortunately too small for single crystal X-Ray diffraction.

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Chapter I
Introduction

Dihexulose dianhydrides are molecules which have a number of uses including animal feed additives andlow calorie sweeteners (since themolecules cannot be metabolized in the body or fermented) among others.They have also been shown to be a preventative agent for tooth decay, assist in absorption of minerals in the body, and are a factor in the production of the *Bifidus* bacteria (Rhee et al., 2005)[1]. Patents for producing them include JP 62275693 (difructosedianhydride I) by Kishimoto and Haraguchi (1987)[2], US2007/0287835 (difructosedianhydride III) by Taizo et al. (2007)[3], and US6841368 (difructose dianhydride IV) by Rhee et al. (2005)[1].

They may in general be formed by dehydration of monosaccharide hexose sugars. As the name dianhydride suggests, it is necessary to remove two molecules of water from the (two) combined monosaccharides in order for the reaction to produce a dihexulose dianhydride. The product molecules have two intramolecular bonds between what were originally hydroxyl groups in the monosaccharide sugars. An example of a dihexulose dianhydride is shown in Figure 1.1.

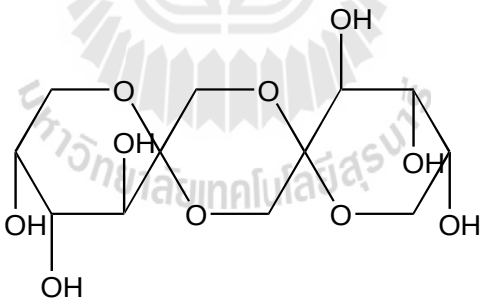


Fig. 1.1 An example of a dihexulose dianhydride, in this case α-D-fructopyranose-1,2':2,1'-β-D-fructopyranose.

The number of possible dianhydrides is clearly very large due to the large numbers of hexulose sugars, the large number of conformations of each sugar (usually 4 ring forms and two straight-chain forms are possible for each hexulose sugar), the number of hydroxyl groups on each sugar molecule, and hence the very large number of possible permutations. However dianhydrides usually only form when at least one of the hexulose sugars is a ketose sugar (fructose, sorbose, tagatose,…), and many of the possible configurations are

sterically impossible. This reduces the number of likely dihexulose dianhydrides to about 100 molecules, around 34 of which have already been characterized (Manley-Harris and Richards, 1997)[4]. The molecules formed are named after the two monosaccharides that formed them. Since a single sugar may take on at least 4 ring forms,2 six-membered ring forms (pyranoses) and 2 five membered ring forms (furanoses), (for instance D-fructose appears in solution in four forms; α-D-fructopyranose, β-D-fructopyranose, α-D-fructofuranose, and β-D-fructofuranose), then the structural forms of the monosaccharide sugars must be taken into account when naming dianhydrides. To form the name of the dianhydride the name of the first monosaccharide is determined by (i) which monosaccharide is first alphabetically (fructopyranose would precede tagatopyranose, while fructofuranose would precede fructopyranose), (ii) the D- sugar should precede the L-sugar, and (iii) the α- anomers should precede the β- anomers. The positions of the intramolecular bonds between the moieties is used between the two component names; this is done by determining the carbon atom on the first molecule (1-6) which is written without a prime, followed by the carbon on the other side of the bond (to the other monosaccharide moiety) which is written with a prime. This must be done twice (there are two such bonds in the dianhydride) and there is a colon between the two bond locations. For example the dianhydride shown in Figure 1.1 is named α-D-fructopyranose-1,2':2,1'-β-D-fructopyranose, or in short form α-D-fru*p*-1,2':2,1'-β-D-fru*p*. The short form is sufficient since no other relevant monosaccharide begins with 'Fru', and pyranose (*p*) and furanose (*f*) are the only likely ring forms for dihexulose dianhydrides.

The great majority of the dihexulose dianhydrides discovered thus far are difructose dianhydrides. Some fructose-glucose dianhydrides and some fructose-sorbose dianhydrides have also been discovered. A table originally appearing in Manley-Harris and Richards is given as Table 1.1: it represents the known dihexulose dianhydrides as of 1997. To the best of the authors knowledge no new dianhydrides have been discovered between that time and the time of the present study, where we have also begun looking a dianhydrides involving tagatose, and both fructose and tagatose.

Table 1.1 Known dihexulose dianhydrides and related compounds and their common names (Manley-Harris and Richards, 1997).

Abbrev. of IUPAC name	Trivial name
α-D-fru*p*-1,2':2,1'-β-D-fru*p*	Diheterolevulosan I
α-D-fru*f*-1,2':2,1'-β-D-fru*p*	Diheterolevulosan II
β-D-fru*f*-1,2':2,1'-β-D-fru*p*	Diheterolevulosan III
β-D-fru*p*-1,2':2,1'-β-D-fru*p*	Diheterolevulosan IV
α-D-fru*f*-1,2':2,1'-α-D-fru*p*	
β-D-fru*f*-1,2':2,1'-α-D-fru*p*	
β-D-fru*f*-2,1':3,2'-α-D-fru*p*	
β-D-fru*f*-2,1':3,2'-β-D-fru*p*	
α-D-fru*f*-1,2':2,1'-α-D-fru*p*	
β-D-fru*f*-1,2':2,1'-β-D-fru*f*	
α-D-fru*f*-1,2':2,1'-β-D-fru*f*	Difructose anhydride I
β-D-fru*f*-1,2':2,3'-β-D-fru*f*	Difructose anhydride II
α-D-fru*f*-1,2':2,3'-β-D-fru*f*	Difructose anhydride III
β-D-fru*f*-2,6':6,2'-β-D-fru*f*	Difructose anhydride IV
α-D-fru*f*-1,2':2,6'-β-D-fru*f*	Difructose anhydride V
?-D-fru*f*-2,6':6,2'-?-D-fru*f*	Alliuminoside
3,6-β-D-fru*f*-1,2':2,1'-α-D-glu*p*-3',6'	2,1':3,6':3',6'-trianhydrosucrose
β-D-fru*f*-1,2':2,1'-α-D-glc*p*-3',6'	2,1': 3,6-dianhydrosucrose
β-D-fru*f*-1,2':2,1'-α-D-glc*p*	2,1'-anhydrosucrose
α-D-fru*f*-1,1':2,2'-α-D-glc*p*	
β-D-fru*f*-1,1':2,2'-α-D-glc*p*	
β-D-fru*p*-1,1':2,2'-α-D-glc*p*	
β-D-fru*f*-2,1':3,2'-α-D-glc*p*	2,3'-anhydrosucrose
β-D-fru*p*-1,2':2,1'-α-D-sor*p*	
β-D-fru*p*-1,2':2,1'-α-L-sor*p*	
α-L-sor*f*-1,2':2,3'-α-L-sor*f*	
α-L-sor*f*-2,1':3,2'-α-L-sor*p*	
α-L-sor*p*-1,2':2,1'-α-L-sor*p*	
α-D-sor*p*-1,2':2,1'-α-L-sor*p*	

Table 1.1 (*cont*.)	
β-L-sor*p*-1,2':2,1'-β-L-sor*p*	
α-L-sor*p*-1,2':2,1'-β-L-sor*p*	Diheterosorbosan I
α-L-sor*f*-1,2':2,1'-α-L-sor*p*	
β-L-sor*f*-1,2':2,1'-α-L-sor*p*	Diheterosorbosan II
α-L-sor*f*-1,2':2,1'- β-L-sor*f*	

Not all of the entries in the table have adequate crystal structures; some confirmations have been deduced from other techniques which makes them somewhat less certain. In some instances (for instance Binkley et al., 1974)[5] assumptions based on N.M.R. results have been later proven incorrect by X-Ray crystallographic studies. The study by Binkley et al. assumed that dihereolevulosan II was the molecule β-D-fru*f*-1,2':2,1'-α-D-fru*p*, whereas later crystal structure determinations (by Kanters et al., 1990)[6] showed that the molecule is in fact α -D-fru*f*-1,2':2,1'-β-D-fru*p*. Thus, X-Ray crystal structure data is by far the most valuable method to obtain the correct structural form of such molecules. Examples of crystal structure determinations of difructose-dianhydrides include α-D-fru*f*-1,2':2,3'-β-D-fru*f* (Taniguchi and Uchiyama; 1982)[7], β-D-fru*f*-1,2':2,3'-β-D-fru*f*(Taniguchi et al.; 1988)[8], and α-D-fru*f*-1,2':2,1'-β-D-fru*f* (Shalby et al; 1994)[9].

A brief summary of structure of the dihexulose dianhydrides which have been synthesized and separated, or have had structural determinations, is given in Table 1.2. Details in the table include the name and molecular structure of the compound, and remarks on what has been achieved with the compound, and citation details of relevant studies. Other significant references are also known.[10-16]

The current study aims to synthesize more such compounds, and to characterize the molecules synthesized with a range of techniques, including structural characterization by single crystal X-Ray structure characterization if it is possible to obtain suitable crystals for the techniques. Originally the project aimed to synthesize difructose dianhydrides, which have special significance in the sugar industry, but since so many of these had been synthesized already the project expanded to other types of dihexulose dianhydrides involving mainly fructose, glucose, sorbose, and tagatose moieties.

Table 1.2 Summary of structural details of known dihexulosedianhydrides.

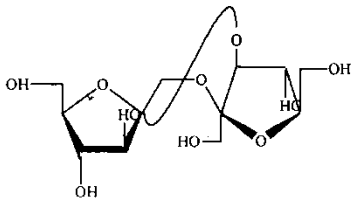
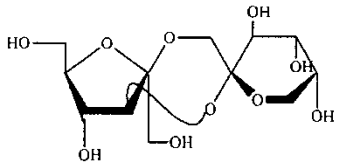
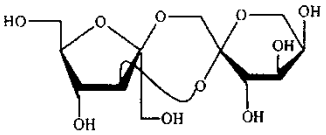
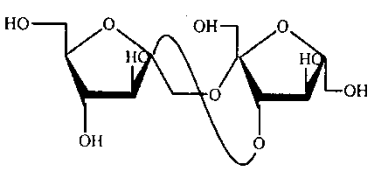
Dihexulose-dianhydrides	Remarks
α-D-Fruf-1,2':2,3'–β-D-Fruf (DFA III) 	**Crystal structure has been published in:** Taniguchi, T.; Uchiyama, T. The crystal structure of di-D-fructose anhydride III, produced by inulin D-fructotransferase. *Carbohydr. Res.* **1982**, *107*, 255-262.
β-D-Fruf-2,1':3,2'- α-D-Frup 	**LC separation** was isolated as a colorless glass, $[\alpha]^{25}$ D180 ° (c 0.5, H_2O); found (for per-O-methylated [M + H]+: 409.2056, calculated for $C_{18}H_{33}O_{10}$: 409.2074. **Methylation analysis** yielded 1,2,6-tri-O-acetyl-(2-deuterio)-3,4,5-tri-O-methylhexitol and 2,3,5-tri-O-acetyl-(2-deuterio)-l,4,6-tri-O-methylhexitol. **References:** M. Manley-Harris, G.N. Richards. *Carbohydr. Res.* **1996**, *287* 183-202.
β-D-Fruf-2,1':3,2'-β-D-Frup 	**LC separation** Colorless glass **Specific rotation** $[\alpha]^{25}D$ -68° (c 0.44 H_2O); lit 58.5° (20°, c 1.03 H_2O) (Defay, Gadelle & Pedersen, 1985) **Methylation analysis** yielded 1,2,6-tri-O- acetyl-(2-deuterio)-3,4,5-tri-O-methylhexitol and 2,3,5-tri-O-acetyl-(2-deuterio)-l,4,6-tri-O-methylhexitol. **References:** M. Manley-Harris, G.N. Richards. *Carbohydr. Res.* **1996**, *287* 183-202. J. Defaye, A. Gadelle, and C. Pedersen, *Carbohydr. Res.*, 136 (1985) 53-65.
β-D-Fruf-1,2':2,3'-β-D-Fruf (DFA II) 	**Crystal structure has been published in** Taniguchi, T.; Sawada, M.; Tanaka, T.; Uchiyama, T. Crystal structure of di-β-D-fructofuranose 2,1':2,3'-dianhydride. *Carbohydr. Res.* **1988**, *177*, 13-20. (DFA II).

Table 1.2 (*cont.*)

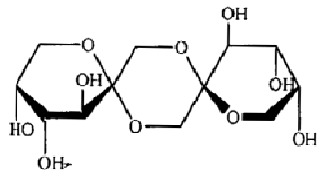
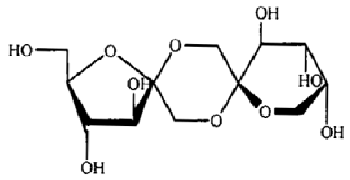
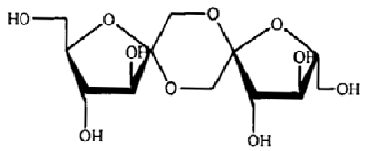
α-D-Frup-1,2':2,1'-β-D-Frup (DHL I) 	could not be isolated in sufficiently pure form for measurement of optical rotation or accurate methylation analysis. However, the per-O-Me3Si ether of 8 co-eluted with that of an authentic sample and had the same mass spectrum. **Remarks:** hygroscopic (unfermented anhydro sugar): Sattler, Serban, Clark, Chu, Albon, Gross & Whalley, 1952 **References:** M. Manley-Harris, G.N. Richards. *Carbohydr. Res.* **1996**, *287* 183-202. L.Sattler, F.W. Serban, G.L.Clark, C.C. Chu, N. Albon, D.Gross, H.C.S. Whalley.; *In. Eng. Chem.* 1952, 44,1127-1135.
β-D-Fruf-1,2':2,1'-α-D-Frup 	
α-D-Fruf-1,2':2,1'-α-D-Fruf 	**LC separation** colorless glasses **Methylation** analyses yielded only 1,2,5-tri-O-acetyl-(2-deuterio)-3,4,6-tri-O-methylhexitol. + 114.8 ° (c 6.0, H_20); lit. (Defay, Gadelle & Pedersen, 1985) +93 ° (H20); found (for per-O-methylated) [M +H]+: 409.2081, calculated for $C_{18}H_{33}0_{10}$: 409.2074 **References:** M. Manley-Harris, G.N. Richards. *Carbohydr. Res.* **1996**, *287* 183-202. J. Defaye, A. Gadelle, and C. Pedersen, *Carbohydr. Res.*, 174 (1988) 323-329.

Table 1.2 (*cont.*)

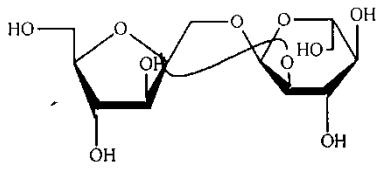
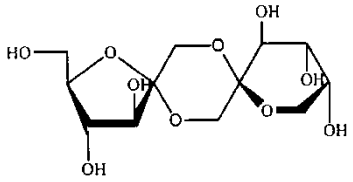
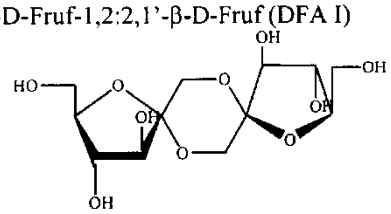
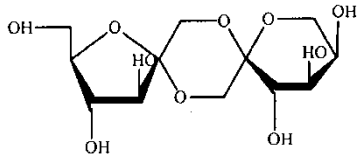
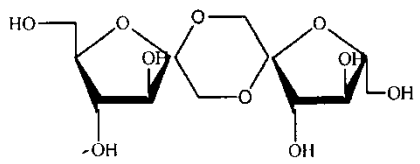
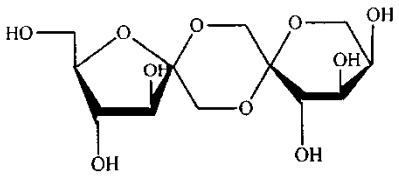
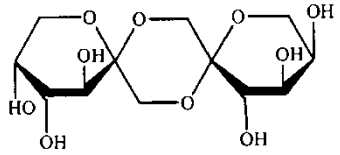
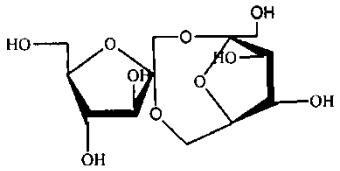
α-D-Fruf-1,1':2,2'α-D-Glup 	**LC separation** colorless glasses [α]25 + 26.8 ° (c 2.98, H_20); found (for per-O-methylated 11) [M + H]+: 409.2075, calculated for C~8H330~0: 409.2074. Methylation analysis 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylhexitol, the position of deuteration **References:** M. Manley-Harris, G.N. Richards. *Carbohydr. Res.* **1996**, *287* 183-202.
α-D-Fruf-1,2':2,1'-β-D-Frup (DHL II) 	**LC separation** colorless glasses [cα] 25 +4.8 ° (c 2.1, H_20); found (for per-O-methylated) [M + H] +: 409.2073, calculated for $C_{18}H_{33}0_{10}$: 409.2074. **Methylation analysis** yielded approximately equal amounts of 1,2,5-tri-O-acetyl-(2-deuterio)-3,4,6-tri-O-meth- ylhexitol and 1,2,6-tri-O-acetyl-(2-deuterio)-3,4,5-tri-O-methylhexitol. **Remarks** hygroscopic (unfermented anhydro sugar): Sattler, Serban, Clark, Chu, Albon, Gross & Whalley.; 1952 **References:** M. Manley-Harris, G.N. Richards. *Carbohydr. Res.* **1996**, *287* 183-202. . Manley-Harris, G.N. Richards. *Carbohydr. Res.* **1996**, *287* 183-202. L.Sattler, F.W. Serban, G.L.Clark, C.C. Chu, N. Albon, D.Gross, H.C.S. Whalley.; In. Eng. Chem. 1952, 44,1127-1135.
α-D-Fruf-1,2:2,1'-β-D-Fruf (DFA I) 	**Crystal structure has been published in:** Shalaby, M.A.; Fronczek, F.R.; Younathan, E.S. The configuration and conformation of di-D-fructose anhydride I. The crystal and molecular structure of 3,4,3',4'-tetra-O-acetyl-6,6'-di (triphenylmethyl)-di-D-fructose anhydride I. *Carbohydr.Res.* **1994**, *265*, 207-214.
α-D-Fruf-1,2':2,1'–α-D-Frup 	**LC separation** colorless glasses +94.1 ° (c 1.6, H_20); found (for per-O-methylated 14) [M + H]+: 409.2088, calculated for $C_{18}H_{32}0_{10}$: 409.2074. **Methylation analysis** yielded approximately equal amounts of 1,2,5-tri-O-acetyl-(2-deuterio)-3,4,6-tri-O- methylhexitol and 1,2,6-tri-O-acetyl-(2-deuterio)-3,4,5-tri-O-methylhexitol **References:** M. Manley-Harris, G.N. Richards / Carbohydrate Research 287 (1996) 183-202

Table 1.2 (*cont.*)

β-D-Fruf-1,2':2,1'-β-D-Fruf 	
β-D-Fruf-1,2':2,1'-β-D-Frup (DHL III) 	**LC separation** colorless glasses **Methylation analysis** yielded approximately equal amounts of 1,2,5-tri-O-acetyl-(2-deuterio)-3,4,6-tri-O-methylhexitol and 1,2,6-tri-O- acetyl-(2-deuterio)-3,4,5-tri-O-methylhexitol. The optical rotation was - 189 ° (c 1.3, H_20); lit. - 179 ° (H_20) [25], - 183 ° (H_20) [26] **References:** M. Manley-Harris, G.N. Richards. *Carbohydr. Res.* **1996**, *287* 183-202.
β-D-Frup-1,2':2,1'-β-D-Frup (DHL IV) 	
α-D-Fruf-1,2':2,6'-β-D-Fruf 	

Chapter II
Materials and Methods

2.1 Introduction

There are a range of materials and methods used in the current study. Materials involved include the monosaccharide sugars used as starting materials for the production of the dianhydrides, acids used for the dehydration reactions, bases for neutralization of solutions after the syntheses, salts for precipitating out ions left over from the reactions or neutralizations, and materials needed for analytical characterization techniques such as solvents for Nuclear Magnetic Resonance (N.M.R.) characterizations and materials needed for chromatography separations of the synthesis products. There were also a range of methods used for syntheses, a range of methods used for separation of products from the syntheses, and also for the characterizations of the reaction products. The materials used, and the methods used, are characterized in the sections below. Because methods used sometimes changed during the course of the research in order to optimize the reactions, the methods given below are typical of those used. When more exact methods are required to understand the results discussed in later sections more exact techniques may be given with the results.

2.2 Materials

2.2.1 Sugars Used in the Chemical Syntheses

Most sugars involved in the research were purchased from Carlo Erba at Analytical Reagent grade. These sugars included D-(-)-fructose, D-(+)-glucose, D-(+)-mannose, and D-sucrose.L-(-)-sorbose was obtained from Acros Chemicals. A sample of food grade D-tagatose (a rare sugar, before commercial production was begun based on a new method) was obtained from Arla Food Ltd. in the United States. Sugar samples were used as received.

2.2.2 Acids Used in theChemical Syntheses

Acids used were purchased from Carlo Erba reagents, and diluted with deionised water to produce stock solutions of known molarities. Hydrochloric acid (37%), nitric acid (70%), sulfuric acid (97%) and hydrofluoric acid (48%) were all diluted significantly with distilled and deionized water before use, since highly concentrated acids dehydrate the sugars to a far larger extent than desired in the current work. The acids used, and dilutions

used for the acids, varied in order to determine the effect of acid strength and type on the yield and composition of the dianhydride reaction products.

2.2.3 Other Solvents and Non-Solvents used in Synthesis and Characterization

A number of other solvents were used in the synthesis, separation and characterization of the dihexulose dianhydrides. Anhydrous ethanol (>99.5%) was purchased from Carlo Erba and was used as a non-solvent to assist in preparing crystal products. D_2O was used as a solvent in experiments to characterize reagents and products by N.M.R.

2.2.4 Other Materials

A number of bases were used to neutralize the solutions after the desired reaction time in acidic solution was complete. In most cases sodium hydroxide (NaOH) was used as a base to neutralize solutions to stop the reaction and prepare the solution for further reactions. Calcium hydroxide solutions, $Ca(OH)_2$ were also used for neutralization in some experiments. Stock solutions of 1 M were prepared for neutralizations.

2.3 Methods

There are a number of different methods which need to be presented, relating to synthesis, separation and characterization of products. These methods are described in the following sections. There were a large number of variations used in the methods, since we were attempting to optimize the synthesis and separation. Typical methods are given below: when a significant change to these methods was used then it will be mentioned in the results section.

2.3.1 Synthesis Methods

Reactions of sugars under acidic conditions and at elevated temperatures were performed in glass vials sealed with plastic caps. In some cases a single monosaccharide ketose sugar (for example D-fructose, L-sorbose, D-tagatose) was used; in some cases mixtures of these monosaccharide ketose sugars were used; and in some cases mixtures of these ketose sugars and monosaccharide aldose sugars (for example, D-glucose, D-xylose, and D-mannose)were used for the preparation of the dianhydrides. If mixtures of only aldose sugars are treated thermally under acidic conditions then the dehydration reactions are usually intra-molecular, producing anhydrides rather than dianhydrides, and hence these reactions were not attempted. A Schott hot plate stirrer was used for mixing the reagents during the reaction synthesis, following which solutions were neutralized with bases (typicallyNaOH) for further treatment prior to characterization. AHeto CBN cooling

bath and Heto HMT200 temperature controller (Heto, Denmark) were used with anInjenieurburo CAT Rotator (Germany) for controlling a post-synthesis yeast fermentation to remove reagent sugars from the mixture.A BUCHI B-480 water bath and a BUCHI B-480 Rotavapor equipped with the BUCHI B-169 vacuum system (BUCHI, Switzerland) were used to partially vacuum distill the solvent from the reaction mixture to concentrate the products.

Syntheses had some variation in methods, which was necessary since both reagents and products had some variation in properties (for instance, some reagents could be fermented away the products after a synthesis, particularly fructose and glucose, whereas others could not, particularly sorbose) and also because syntheses were modified in order to attempt to optimize the production of particular products, but a typical method will be described below.

12 g of D-fructose was weighed into a glass vial sealed with a plastic cap. 2 mLof 1.0 M hydrochloric acid stock solution was added and the mixture was stirred briefly using the magnetic stirrer, until the fructose had completely dissolved into the acid solution. The vial containing the reaction solution was then transferred to a water bath and maintained at a temperature of 80°C for a period of 1 hr, with continuous agitation during this time. After the reaction period the sample was quickly cooled to room temperature, and 10 g of the sample, which had changed to a brown colored solution, was transferred to a 250 mL beaker and adjusted to a pH of 5.0 using a NaOH solution.

Due to difficulties in accessing (or being allowed to use) a preparative-scale HPLC unit at the site of the research it was decided to remove unreacted reagents from the product solution via yeast fermentation, a novel technique in synthesis of these type of compounds formed in the present study, in some experiments. Simple monosaccharide sugars tend to be fermented by yeasts, thus removing them from the sample mixture, while all of the dihexulose-dianhydrides are not fermented to any significant extent. The mixture was thus fermented to remove residual sugar which had not reacted. This is possible since most monosaccharide sugars can be decomposed via baker's yeast, whereas the dihexulose dianhydride products can not be decomposed via this technique. 1.0 g of baker's yeast (*Saccharomyces cerevisiae*) was diluted in warm deionized water and added to the solution producedby the step above, after which warm deionized water was added to increase the volume of the solution to 100 mL. The solution was held in a constant temperature bath at 30.0±1.0°C with continuous stirring for 3 days.

After the fermentation, yeast was removed using a centrifuge followed by filtration through a Whatman No. 42 filter paper (Whatman, England). The syrup, now a yellow color, was dried through the addition of anhydrous ethanol, followed by removal of the solvent via vacuum distillation under reduced pressure (such that the solvent removal could be achieved at slightly above 40°C. After this procedure about 2 g of product remained, and cold ethanol was added to induce crystallization. This solution was held at 5°C for a period of 2 days. A white powder was formed which was hygroscopic.

Note that this method is a characteristic example; many details of this procedure were varied for other experiments, including (i) sugar, or binary sugar mixture used, (ii) acid used, (iii) strength of the acid, (iv) reaction temperature, (v) reaction time, (vi) product separation technique.

In cases involving L-sorbose the separation of the reagents by yeast fermentation was not performed, as it would not have assisted in the removal of reagent material. In these cases chromatography is necessary for separation of the different fractions of the reaction solution in order to purify the products.

Further experiments were performed using a solvothermal technique in a microwave assisted bomb, however the microwave synthesis equipment in the equipment center of SUT was not operating correctly during this period (the temperature could not be held at a correct value for the full synthesis time) and so this line of experiments was discontinued.

2.3.2 Characterization Techniques

(1) ^{13}C Nuclear Magnetic Resonance: ^{13}C N.M.R. was performed on both reagent materials (to help separate reagent peaks from product peaks when reagents and products were both present in a synthetic product solution) and products from syntheses. The spectra were measured for 500 μL of sample mixed with 100 μL D_2O by^{13}CN.M.R. at 300 MHzon a Varian 300MHz spectrometer at the Center for Technological Equipment, Suranaree University of Technology.

(2) Gas Chromatography/Mass Spectometry: Compositions of reaction products were also analyzed using Gas Chromatgraphy/Mass Spectometry (GC/MS). Although neither the reagents nor products of the syntheses were volatile materials it was possible to characterize them both using GC/MS based on a method from Flood et al. (1996)[17]. GC/MS analysis of the trimethlysilyl (TMS) derivatives of the sugar molecules (which *are* volatile) was performed based on the method of Flood et al. In short; derivative mixtures

were prepared by adding 0.1 mL of trimethylsilylimidazole (Sigma, UK) to 0.9 mL of anhydrous pyridine (Ajax, Australia) which had been dried over anhydrous sodium sulfate. These mixtures were prepared in 2.0 mL septum capped vials, and 3 μL of reaction mixture was added to the vial, and the vial shaken vigorously for a period of 1 minute. The reaction is very fast and very selective such that the derivatized products are exactly representative of the compounds that the reaction mixture originally contained.

After derivatization a 5 μL sample of the mixture was injected into a Varian CP-3800 gas chromatograph which contained a 30m × 0.25 mm DF 0.25 VF-5 ms Factor Four capillary column (Varian, Germany). The operating conditions were; helium carrier gas flowrate = 1.2 mL/min; split ratio = 20; column temperature = 200°C; injector temperature = 250°C. The temperature program of the column oven is given in Table 2.1.

The GC was interfaced with a 1200L quadrupole MS/MS Varian mass spectrometer configured in electron impact (EI) mode, with a source temperature of 200°C, a source voltage of 70 eV, and a vacuum of 3.5×10^{-7}Torr. Data were collected in full-scan acquisition mode. Mass spectra were recorded in the range *m/z* 50-600 in positive ion mode. The Varian MS Workstation version 6.3 was used for all data acquisition.

Table 2.1 Temperature profile of the column oven for the GC/MS technique used.

Temperature (°C)	Rate (°C/min)	Hold time (min)	Total (min)
150		5	5
190	20	7	14
240	30	40	55.67

(3) Liquid Chromatography: Preparative liquid chromatography was performed on aqueous samples of approximately 1 g/mL using Waters Delta Pak C_{18} cartridges (25 × 100 mm, 100 Å) using a Guard Pak to protect the column. Elution was at 10.0 mL/min using H_2O.

(4) Single Crystal X-ray Structure Determination: Crystals produced from reaction mixtures were investigated under a microscope to check for quality. Good quality crystals of appropriate size for diffraction were mounted. Reflection intensities were collected using a Bruker-NoniusKappaCCDdiffractometer which used COLLECT software. The source was a fine focus Mo X-Ray source (graphite monochromated Mo *Kα* radiation, λ =

0.71073 Å). Where necessary, data was solved and then refined using SHELXTL (Bruker AXS Inc., 1998).

(5) Fourier Transform Infra-Red (FT-IR) Spectometry: Since it was difficult to crystallize the reaction products, and since it is known that carbohydrates can complex with metal ions to form less soluble products, a series of experiments to form complexes between the reagents (such as fructose) and dihexulose dianhydride products and metal ions (Ca^{2+} and Ba^{2+}) were performed. These complexes were crystallized and analyzed by astandard FT-IT technique using a KBr pellet. Spectra were recorded between 370 and 4,000 cm^{-1}.

(6) Energy Dispersive X-Ray Spectroscopy (EDX): EDX was performed to analyze further the products mentioned in section (5) above. EDX was performed on the solids ina SEM using energies up to 20keV.

Chapter III
Results and Discussion

Dihexulose dianhydrides are usually formed via three potential reactions. The first of these reaction is of a single hexuloseketose sugar reacted under elevated temperature in acidic conditions which causes dehydration reactions to condense two similar molecules into a single dianhydride. The second of these reactions is where two different hexuloseketosemonosaccharide sugars are reacted under elevated temperature and acidic conditions to condense two different monosaccharides into a dianhydride molecule: this reaction may also produce dianhydrides produced from two equivalent moieties, making the selectivity of this reaction likely to be lower, however giving greater potential to create new (unknown) molecules.The thirdtype of reaction is where a monosaccharide hexuloseketose sugar is reacted with a monosaccharide hexulose aldose sugar under elevated temperature and acidic conditions: this may result in dianhydride molecules consisting of a ketose moiety with an aldose moiety, or with dianhydrides consisting of two ketose moieties. Reactions of monosaccharide hexulose aldose sugars tend to yield dehydrated products which are the result of intramolecular dehydration (molecules where a *single* monosaccharide moiety has been dehydrated twice leading to intramolecular bonds) rather than the dianhydride molecules desired from the current study. To the best knowledge of the authors no dianhydride molecule has been created from a reaction mixture containing only aldose sugars. For this reason reaction mixtures using solely aldose sugars were not created. Reaction mixtures were created for single componentmonosaccharide hexuloseketose sugars (D-fructose, L-sorbose, and D-tagatose were all used), for mixtures of monosaccharide hexuloseketose sugars (mixtures of the three components mentioned above), and mixtures of these ketose sugars with monosaccharide hexulose aldose sugars (D-glucose, and D-mannose have been used). It is hoped that this variation in reaction mixtures can create a variety of unknown dihexulose dianhydrides.

3.1 Optimizing Yield and Selectivity and GC-MS Studies of the Trimethylsilyl (TMS) Derivatives of Reagents and Products

In any synthesis it is desirable to obtain a good yield to minimize the amount of unreacted reagents remaining in solution, and good selectivity in order to minimize the

amount of unwanted products, and also to make separation and purification of the products simpler. It was expected that the yield and selectivity of the reaction between the monosaccharide ketose sugars (or mixtures of different monosaccharide ketose sugars, or mixtures of monosaccharide ketose sugars and monosaccharide aldose sugars) and acids would depend strongly on the strength of the acid, the temperature, and the reaction time, and possible also on the type of acid used. If the acid strength is very high, total dehydration of the sugar to carbon will be seen in a highly exothermic reaction, producing a black amorphous material as a product, which is strongly undesired in this case. If the acid strength is moderately large the reaction will produce a wide range of further decomposition products from decomposition of the dianhydride materials; these are likely to include pyrroles, pyrazines, pyridines, imidazoles, and anhydrosugars (which are often monosaccharide derivatives with two waters removed causing intra-molecular bonds to form). This is also strongly undesirable, since these products will reduce the yield of the desired product (the dianhydride sugar)

In order to ascertain reactions at close to optimum conditions a set of screening tests was performed using ranges of conditions and observing product solutions in order to determine appropriate conditions for further reactions. It is known from previous literature that reactions producing the desired dianhydrides create solutions of yellow to light brown color (from solutions of monosaccharides, which are clear solutions). If the solutions remain clear or colorless it is an indication that the reaction has not progressed to sufficient yield. If the solutions become dark brown or black then the reaction has progressed too far and the dianhydrides initially produced have been further degraded into a range of products that have been further dehydrated. The screening tests were performed with an equimolar mixture of D-fructose and L-sorbose, with these mixtures assumed to be representative of other reagent mixtures. The sugar contents of the solutions were set to 400 g/L which is lower than the solubility of the sugars. Acid solutions of various molarity were prepared from 37% hydrochloric acid. Temperature control was achieved by placing the vials containing the solution (25 mL) into a temperature controlled bath (Heto CBN 18-30 and Heto HMT200, Denmark) which could control temperature to within ±0.5°C. Reaction temperatures were controlledbetween 60.0 and 90.0°C; these limits were used because of the need for the reactions to occur at elevated temperatures and to avoid solutions nearing their boiling point. After the desired reaction time, samples were taken and the reaction stopped using solutions of analytical grade sodium hydroxide to increase the pHto between

7 and 8 (at which point the reactions stops progressing). Further analysis was performed on samples of the product, and this will be discussed following data on observations of the product solutions.Preliminary observations of color produced in the solutions created from the screening test are shown in Table 3.1.

Table 3.1 Observations of color produced in the screening tests.

Acid concen-tration (M)	Temperature (°C)	Reaction time (hr)	Observation of product solution
1.00	60.0	1	Yellow solution
1.00	60.0	2	Yellow solution
1.00	60.0	3	Yellow solution
1.00	75.0	1	Light brown solution
1.00	75.0	2	Light brown solution
1.00	75.0	3	Light brown solution
1.00	90.0	1	Dark brown solution, black solid
1.00	90.0	2	Dark brown solution, black solid
1.00	90.0	3	Dark brown solution, black solid
1.25	60.0	1	Yellow solution
1.25	60.0	2	Yellow solution
1.25	60.0	3	Yellow solution
1.25	75.0	1	Light brown solution, black solid
1.25	75.0	2	Light brown solution, black solid
1.25	75.0	3	Brown solution, black solid
1.25	90.0	1	Brown solution, black solid
1.25	90.0	2	Brown solution, black solid
1.25	90.0	3	Dark brown solution, black solid
1.50	60.0	1	Yellow solution
1.50	60.0	2	Yellow solution
1.50	60.0	3	Orange solution
1.50	75.0	1	Light brown solution
1.50	75.0	2	Light brown solution
1.50	75.0	3	Brown solution, brown solid (small amt.)

1.50	90.0	1	Dark brown solution, black solid
Table 3.1 (cont.)			
1.50	90.0	2	Dark brown solution, black solid
1.50	90.0	3	Dark brown solution, black solid

It is clear from the results shown in Table 1 that a combination of high temperature and high acid strength forced the reaction too far and produced a series of further degradation products of dianhydrides. Longer reaction times also promoted the further degradation of the desired products. All solutions displayed some color change, indicating that some products were produced even under mild reaction conditions for short reaction times. However it appears that the optimum reaction conditions are likely to occur using an acid strength of approximately 1.25 M, a temperature of 75°C, and reaction times of 1 – 2 hr.

Further analysis was performed using a number of techniques.The first of these techniques was gas-chromatography with mass spectroscopy (GC-MS) analysis of the trimethylsilyl(TMS) derivatives of the compounds in the reaction mixture. This technique assists in determination of compounds present in the mixture analyzed. The method used was based on an earlier method of Flood et al. (1996)[17]. The method was to prepare a reaction solution of 0.1 mL of trimethylsilylimidazole, TMSI (Sigma, UK) to 0.9 mL of anhydrous pyridine (Ajax, Australia) that had been dried over anhydrous sodium sulfate to ensure a total lack of water in the reaction mixture. (Water content must be minimized since water also reacts with the TMSI). 3 μL of the product sample was added to this reaction mixture in order to produce the TMS derivatives of the product compounds, and also any residual reagents. The mixtures were sealed in 2.0 mL septum-capped vials and shaken vigorously for a period of 1 minute, which is sufficient for the derivatization to be complete. After derivatization, a 5 μL sample from the mixture was injected into a Varian CP-3800 gas chromatograph. Chromatography was performed on a 30 m × 0.25 mm DF 0.25 VF-5 ms Factor Four capilliary column (Varian, Germany). The operating conditions were as follows: helium carrier gas flow of 1.2 mL/min, split ratio equal to 20, column temperature of 200°C, and injector temperature equal to 250°C. The GC was interfaced with a 1200L quadrupole MS/MS Varian Mass Spectrometer configured in electron impact (EI) mode, using a source temperature of 200°C, a source voltage of 70 eV, and a vacuum

of 3.5×10^{7}torr. The data was collected in full-scan acquisition mode. Mass spectra were recorded over the range *m/z* 50-600 in positive ion mode. The Varian Workstation version 6.3 was used for the data acquisition. Results for a small number of selected samples are shown in Figures 3.1 to 3.15. Further results can be obtained by contacting the authors.

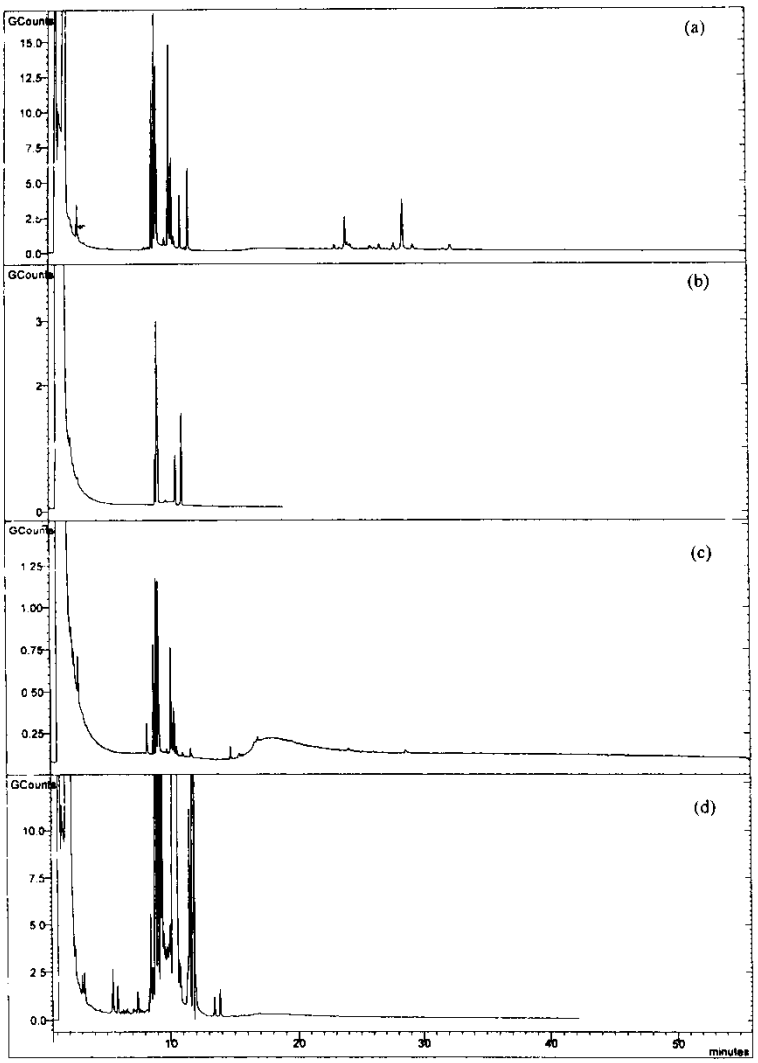


Figure 3.1 Full chromatograms of TMS derivatives of (a) product mixture for reaction at 75°C, HCl at 1.5 M, and a reaction time of 3 hr: (b) fructose reagent: (c) sorbose: (d) product mixture for reaction at 90°C, HCl at 1.0 M, and a reaction time of 2 hr.

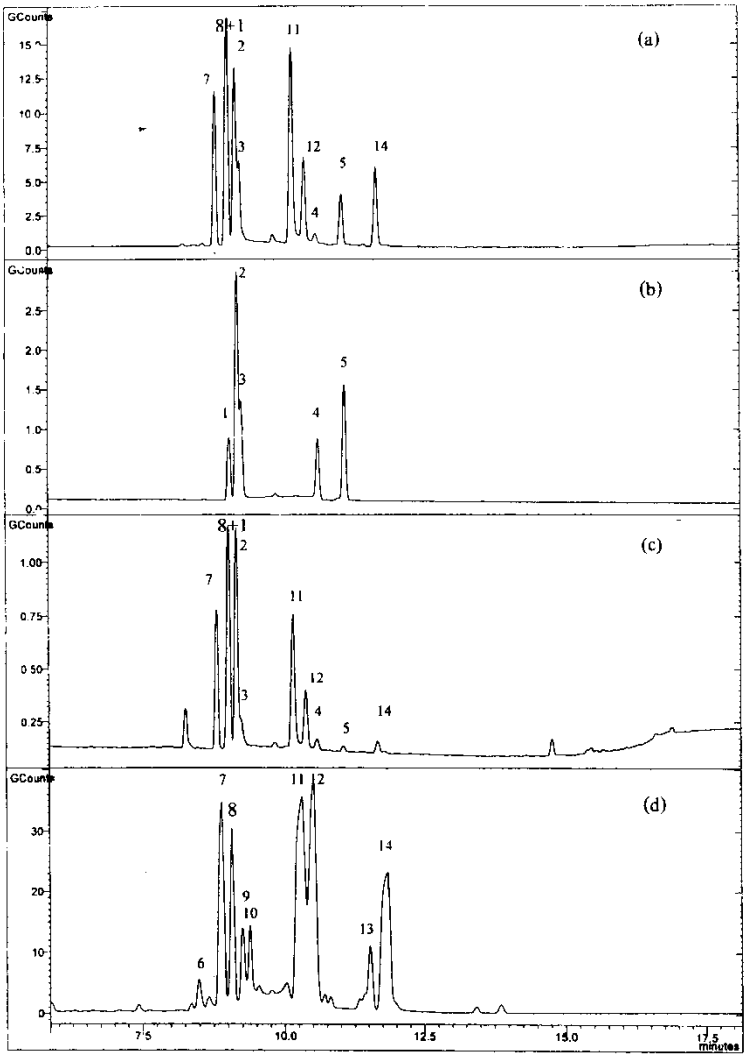


Figure 3.2Chromatograms of TMS derivatives expanded to show the characteristic peaks of the species of (a) product mixture for reaction at 75°C, HCl at 1.5 M, and a reaction time of 3 hr: (b) D-fructose reagent: (c) D-sorbose reagent: (d) product mixture for reaction at 90°C, HCl at 1.0 M, and a reaction time of 2 hr.

The evidence of new peaks in the chromatograms from the reaction products shows that new compounds (almost certainly dihexulosedianhydrides based on the reaction conditions) have been produced. In particular peaks labeled 13 and 14 are likely to be difructosedianhydrides or mixed fructose – sorbosedianhydrides. Further confirmation of this was attempted through characterization of the mass spectra of the material as these peaks were eluted. These mass spectra could be compared to standard libraries of mass spectra to search for possible matches. Mass spectra of particular peaks are given in the following figures.

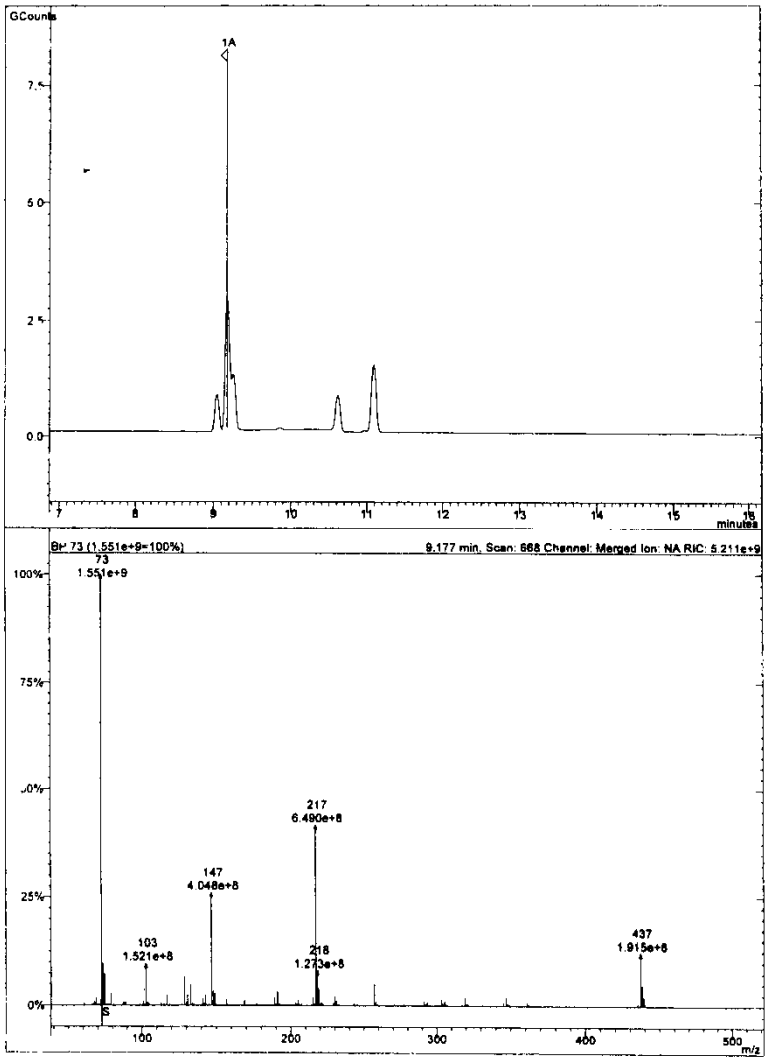


Figure 3.3 Gas chromatogram of TMS derivatives of D-fructose reagent and the characteristic spectra from the peak 1A.

It is important to note that the mass numbers of the fragments detected may be larger than the mass of the products (dihexulosedianhydrides) being considered. This is because of the reaction with the trimethylsilyl group (mass equal to 73.2 g/mol) which increases the mass of the compounds in the GC chromatogram. This group reacts with available hydroxyl groups, of which there are several in a dianhydride compound, thus leading to large mass compounds. This is why (even for sugar reagents) large fragments such as m/z = 437 can be detected. The molecular weight of all the desired products is 324 Da.

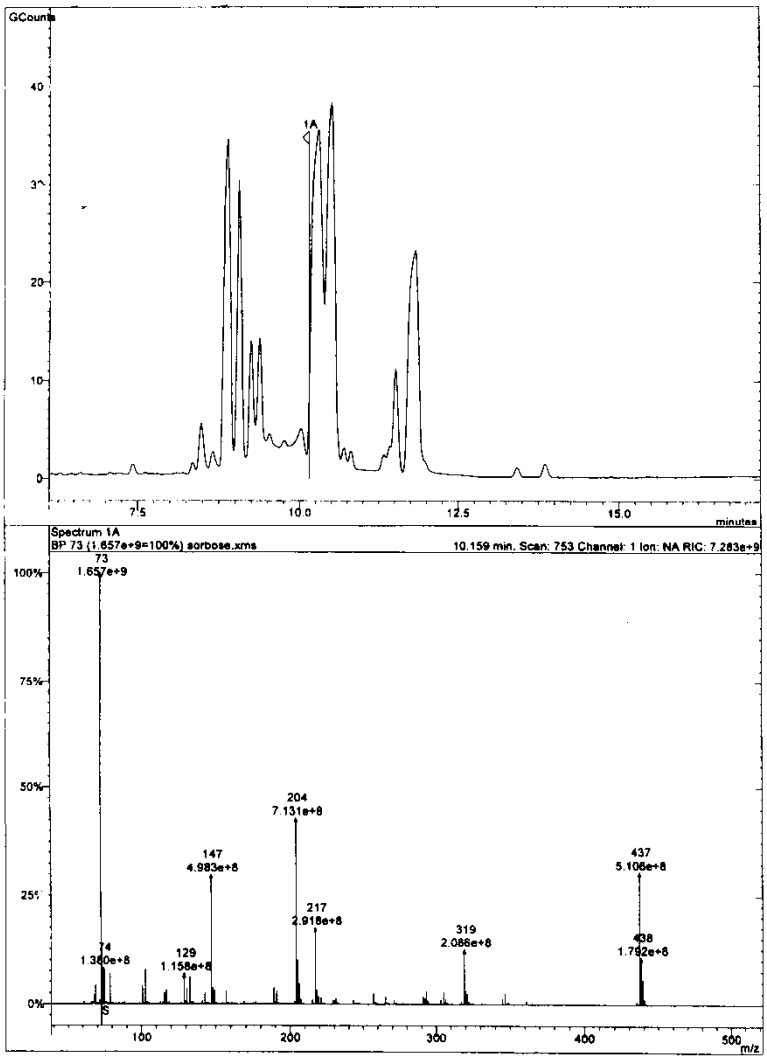


Figure 3.4 Gas chromatogram of TMS derivatives of L-sorbose reagent and the characteristic spectra from the peak 1A.

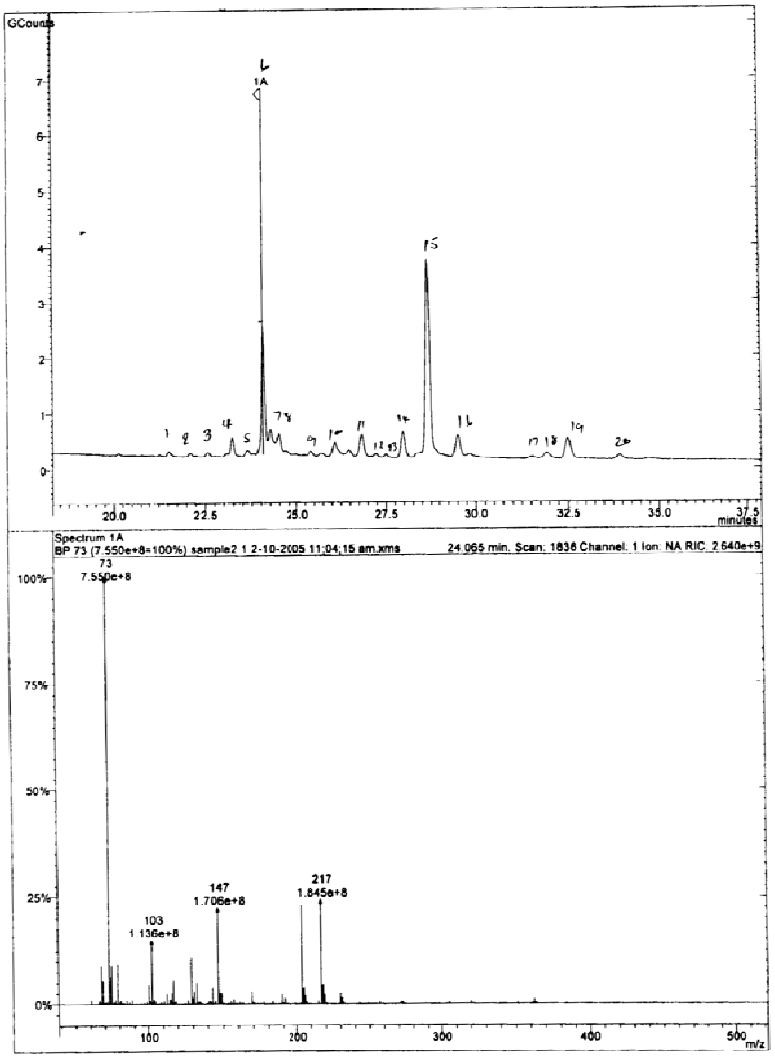


Figure 3.5 Gas chromatogram of TMS derivatives of the reaction solution and the characteristic spectra from the peak 1A. Reactions conditions are: 75ºC; 1.5 M acid concentration; reaction time 3 hr. GC-MS conditions: injection volume, 5 µL; scanning range 40-400 *m*/*z*.

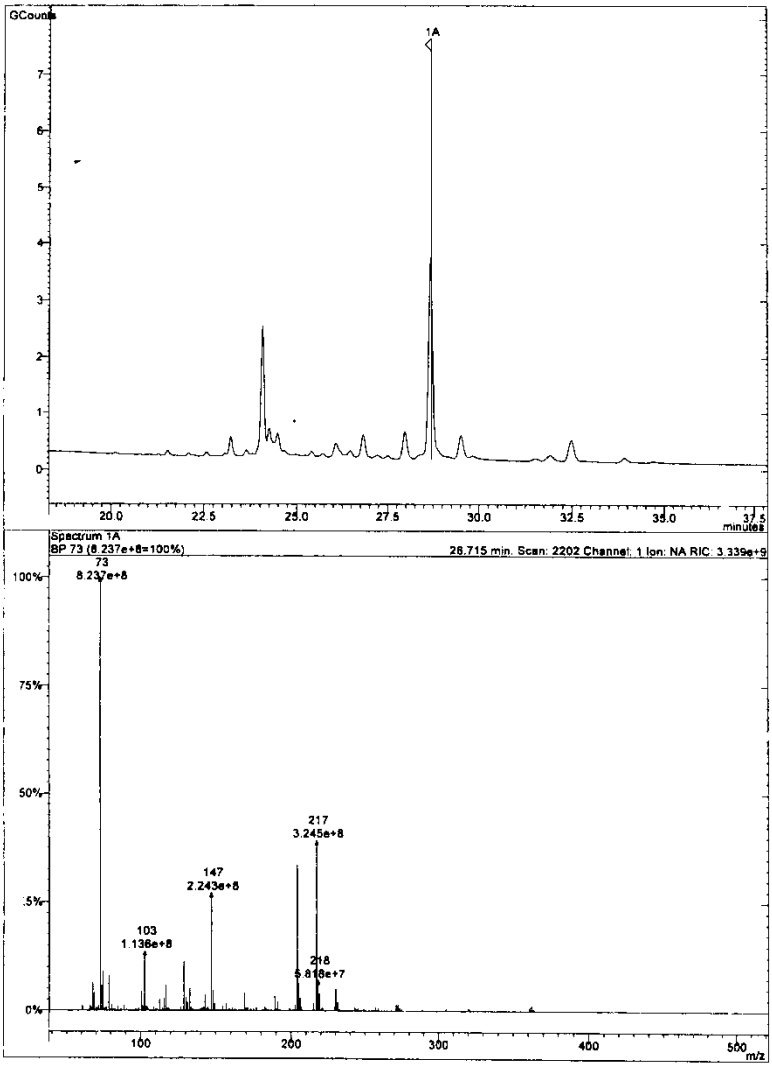


Figure 3.6 Gas chromatogram of TMS derivatives of areaction solution and the characteristic spectra from the peak 1A. Reactions conditions are: 75ºC; 1.5 M acid concentration; reaction time 3 hr. GC-MS conditions: injection volume, 5 µL; scanning range 40-400 *m*/*z*.

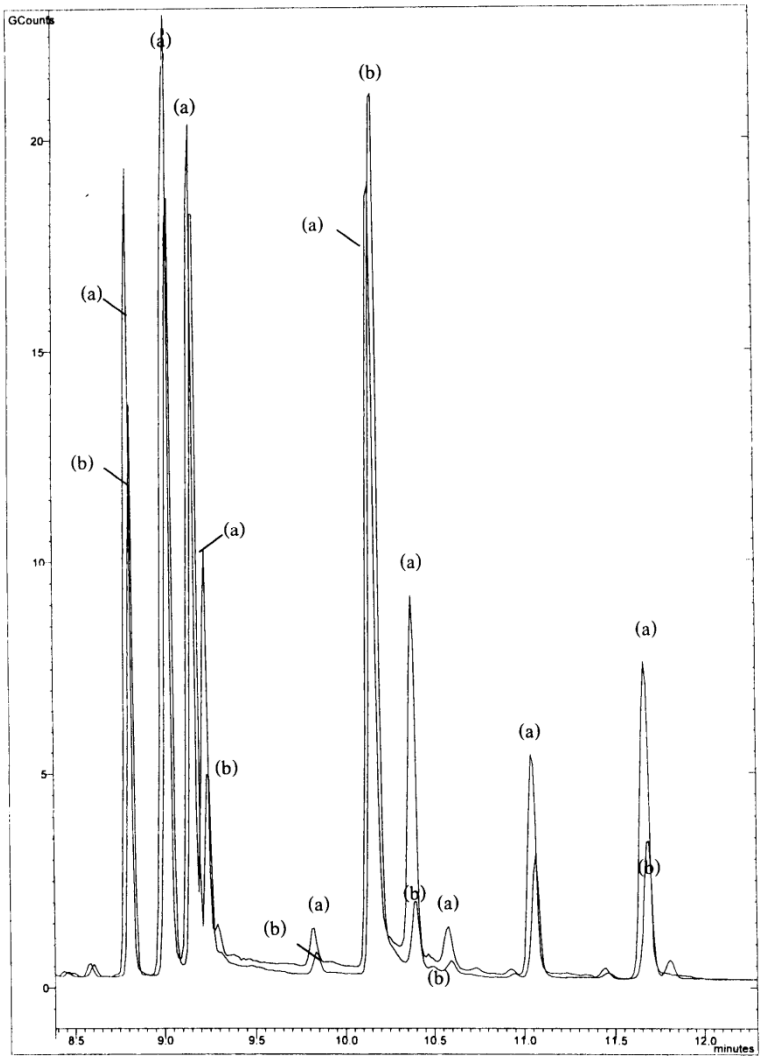


Figure 3.7 Gas chromatograms of TMS derivatives of reaction solutions to compare product traces for different reaction conditions. (a) reaction mixture; 75ºC; acid concentration equal to 1.5 M; reaction time 3 hr. Injection volume 5 µL. (b)reaction mixture; 90ºC; acid concentration equal to 1.0 M; reaction time 2 hr. Injection volume 5 µL. Retention time 8.40 – 12.3 min.

Note that both these conditions resulted in similar products (the retention times of the newly created peaks are identical) but that the yield and the selectivity of the reaction varied according to the reaction conditions and reaction time. The difference is not too great for this example, since although curve (a) has a lower reaction temperature it also has a larger acid strength and reaction time.

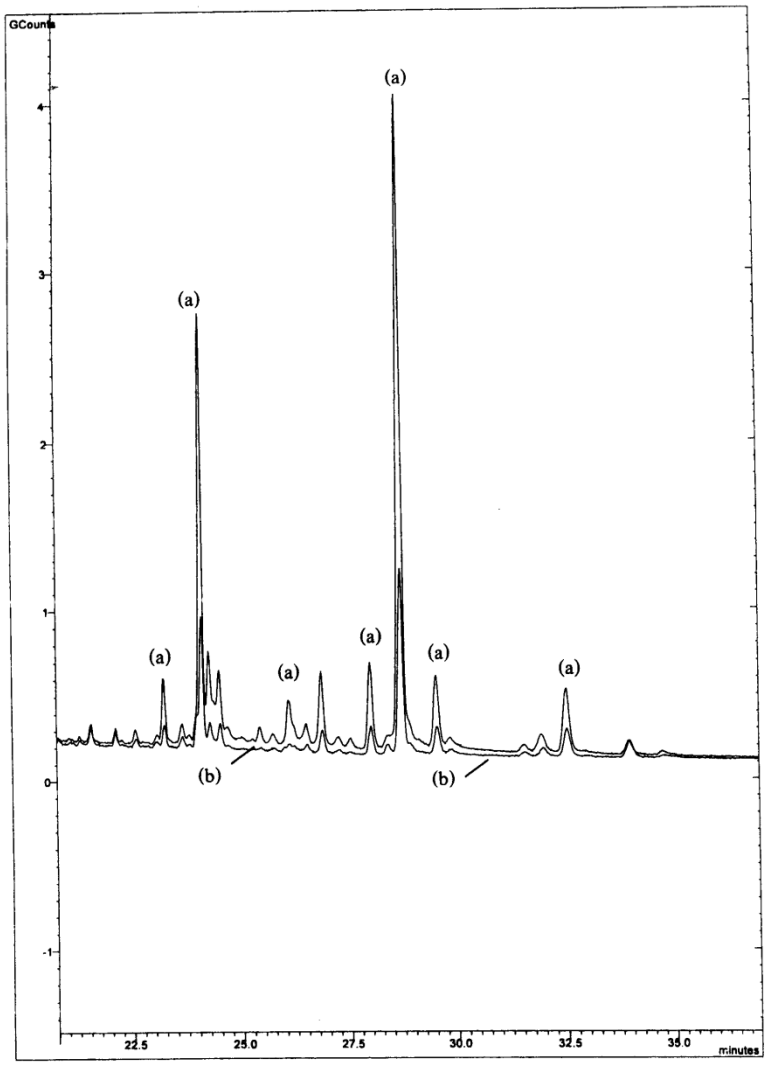


Figure 3.8 Gas chromatograms of TMS derivatives of reaction solutions to compare product traces for different reaction conditions. (a) reaction mixture; 75ºC; acid concentration equal to 1.5 M; reaction time 3 hr. Injection volume 5 µL. (b)reaction mixture; 90ºC; acid concentration equal to 1.0 M; reaction time 2 hr. Injection volume 5 µL. Retention time 20.0 – 37.5 min.

3.2 ^{13}C Nuclear Magnetic Resonance Studies of Reagents and Products

In previous work in this study, dehydrations of monosaccharides were performed in the presence of diluted acids. Reaction progress was monitored and products were characterized by NMR spectroscopy.

The ^{13}C NMR spectra of reaction products in D_2O were obtained using a Varian 300 MHz spectrometer. Four difructosedianhydride compounds were produced at the

example conditions in the presence of 1.0 M acid, at the temperature of80°C, and with a reaction time of 60 min. The structures were assigned by comparison with literature values (Manley-Harris & Richards, 1997)[4]. Four dianhydrides were produced from fructose solutions, and examples of spectraare given in Figures 3.9 to 3.13 (which come from various reaction conditions). They and three forms of unreacted D-fructose were identified in the reaction product solution (fructose occurs as tautomers in solution so there will always be more than one form apparent in a liquid solution). The first compound was assigned to be α-D-fructofuranose-l,2':2,-β-D-fructopyranosedianhydride (α-D-fruf-l,2':2,1'-β-D-frup) which was previously designated asdiheterolevulosan I (DHL I). The ^{13}C NMR of the second compound is identical with thatof the difructosedianhydride, assigned as α-D-fructofuranose-l,2':2,l'-β-D-fructofuranose (α-D-fruf-l,2':2,1'-β-D-fruf) or difructosedianhydride I (DFA I). Thethird and fourth compounds were identified as β-D-fructofuranose-l,2':2,3'-β-D-fructofuranose (β-D-fruf-l,2':2,3'-β-D-fruf) designated as difructosedianhydride II(DFA II) and α-D-fructofuranose-l,2':2,l'-α-D-fructofuranose (α-D-fruf-l,2':2,l'-α-D-fruf). Yeast fermentation was applied for remove the part ofunreacted D-fructose.

Comparison of the ^{13}C NMR spectra, before and after fermentation, showed thataction of yeast enzymes on the reaction mixture containing a mixture of difructosedianhydride products and unreacted fructose in different forms, completely removed theunreacted fructose, but did not affect the difructosedianhydride products. The majordifructosedianhydride product from these preparations with diluted acid isdiheterolevolusan II, as was previously found for preparations utilizing the action ofconcentrated hydrochloric acid (Wolfrom, Hilton &Blinkley, 1952)[18].

From the results, it can be concluded that *Saccharomyces cerevisiae*can beconveniently used to remove the unreacted monosaccharides that are constituents of thedifructosedianhydride preparations. The major product obtained from the crystallization step was diheterolevulosan II. Data on the ^{13}C NMR chemical shifts of products produced by the synthesis are given in Table 3.2.

In order to preparation of the new structure of dihexulosedianhydride compounds, D-glucose was used as the new starting material. The preparation method was started, and followed by the preparation of glucose caramel which was reported on the qualitative and quantitative evaluation of mono- and disaccharides in D-fructose, D-glucose and sucrose caramels by gas-liquid chromatography-mass spectrometry which di-D-fructose

dianhydrides were contained as tracers of caramel authenticity (Ratsimba et al., 1999)[18]. One dianhydride product could be separated, crystallized and an NMR spectrum obtained (see Figure 3.9).

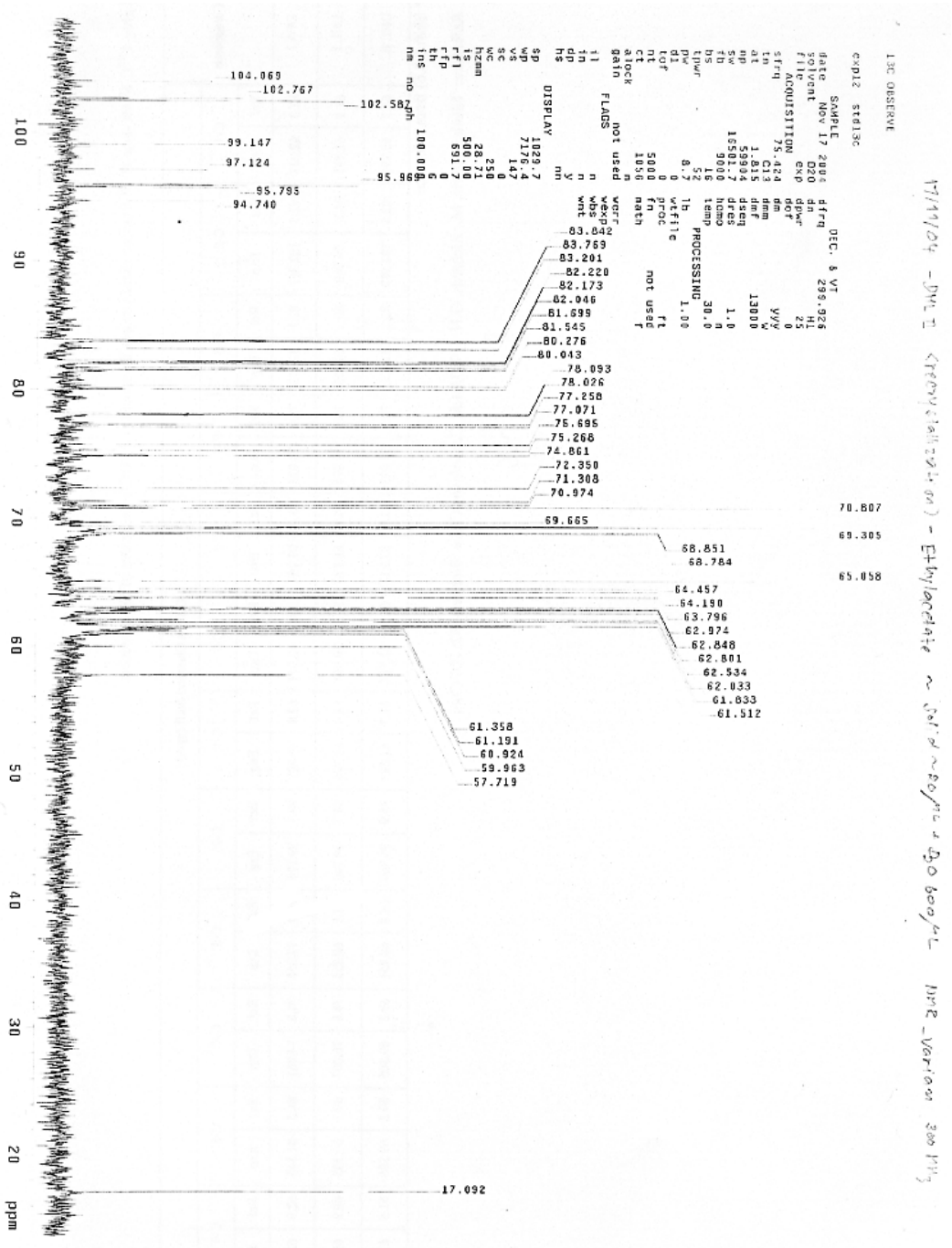


Figure 3.9 ^{13}C NMR spectrum (20-100 ppm) after fermentation of reaction products produced by fructose dehydration, and recrystallization, showing the product DHLII. This compound is further described in the text.

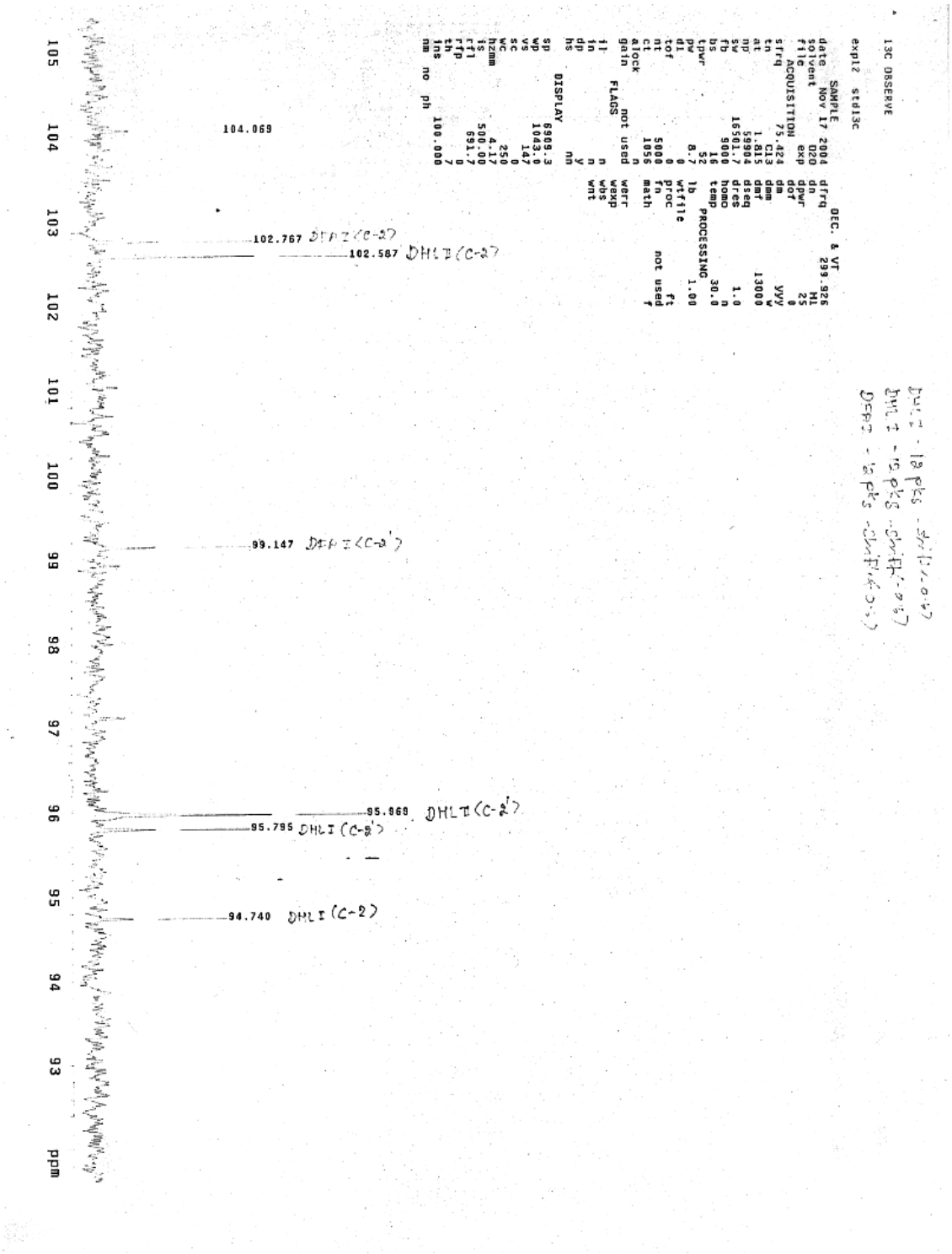


Figure 3.10 ^{13}C NMR spectrum of reaction products (92-105 ppm) produced by fructose dehydration before fermentation: note the complex mixture of reactants and products. These compounds are further described in the text.

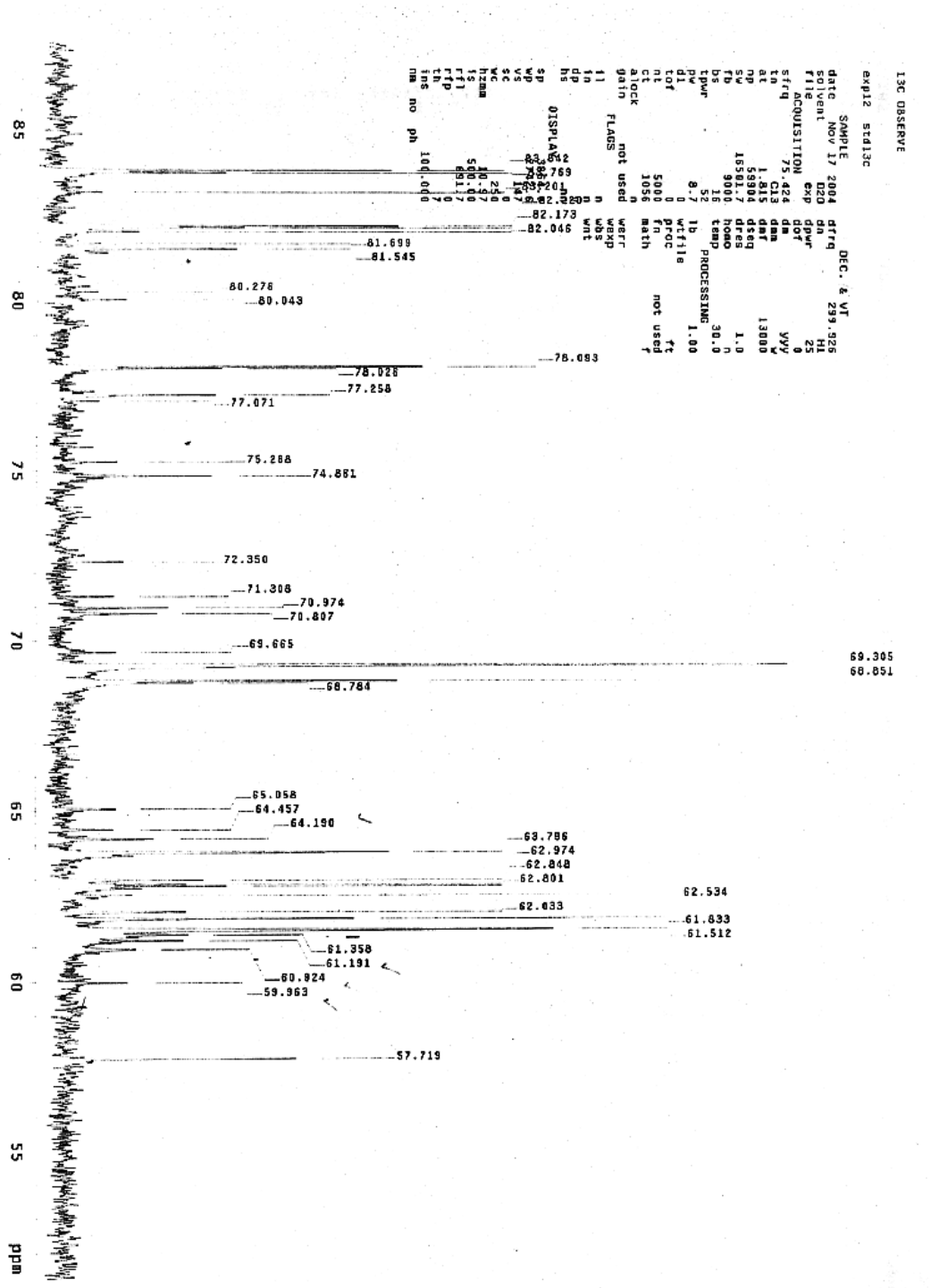


Figure 3.11 ^{13}C NMR spectrum of reaction products (54-86 ppm) produced by fructose dehydration before fermentation: note the complex mixture of reactants and products. These compounds are further described in the text.

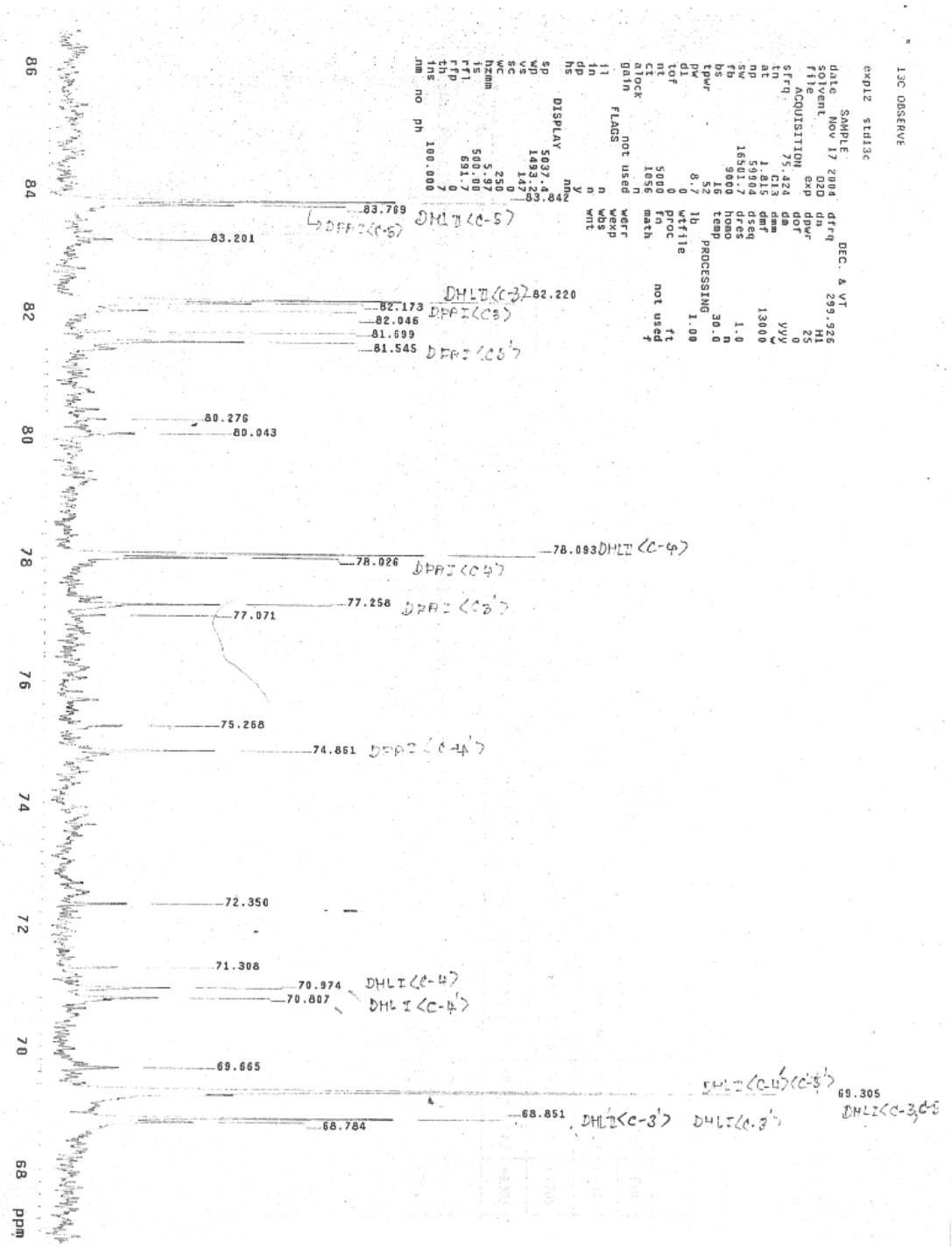


Figure 3.12 ^{13}C NMR spectrum of reaction products (54-86 ppm) produced by fructose dehydration after fermentation: products include DFAI, DHLI, and DHLII. These compounds are further described in the text.

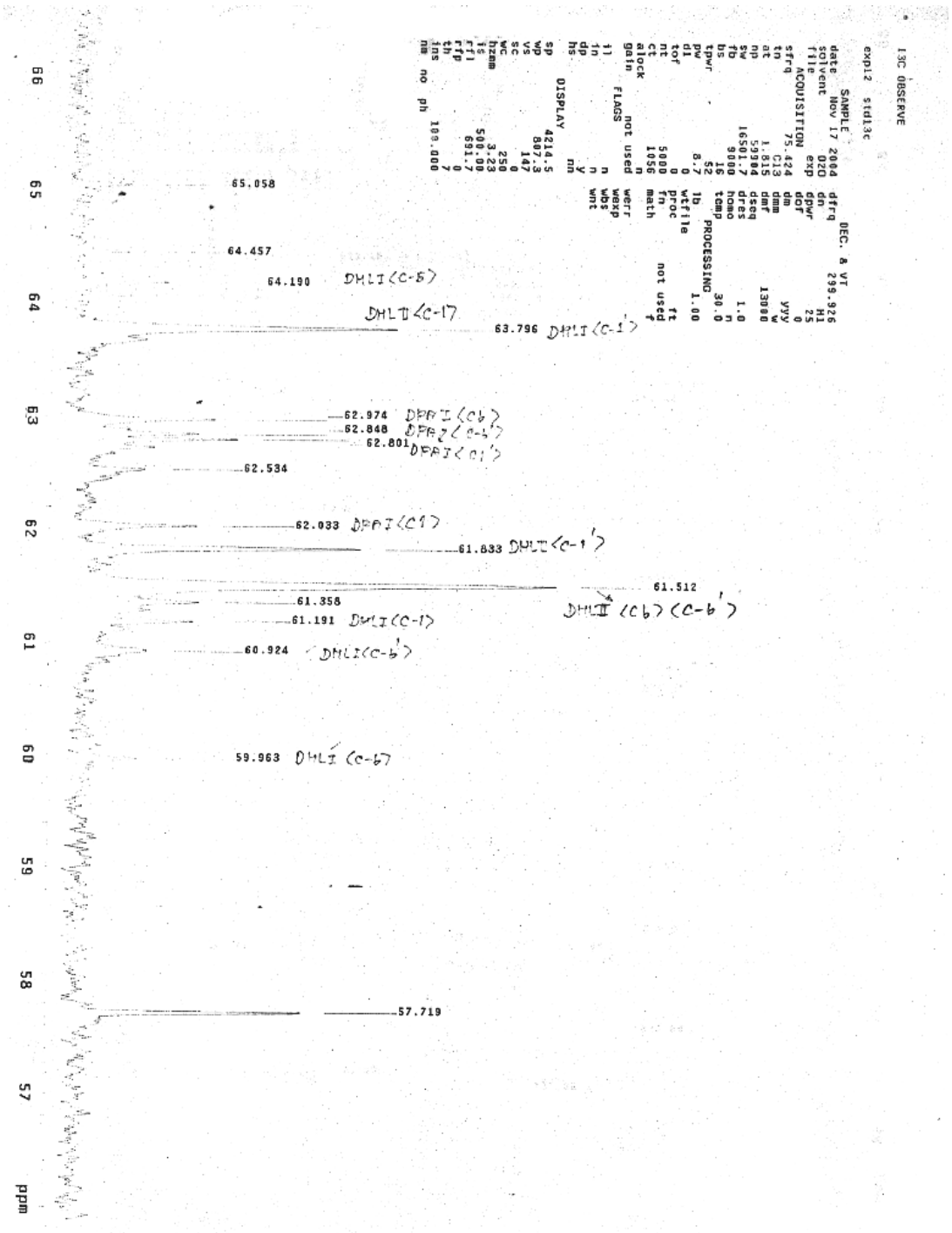


Figure 3.13 ^{13}C NMR spectrum of reaction products (56-66 ppm) produced by fructose dehydration after fermentation: products include DFAI, DHLI, and DHLII. These compounds are further described in the text.

Table 3.2 Comparison of experimental ^{13}C NMR peaks of products from experimental samples (which had been treated by yeast fermentation to remove reactants) with standard peaks for particular difructosedianhydrides recorded in the literature.

Unfortunately the crystalline dianhydride product obtained, while suitable for NMR analysis, was not suitable for single crystal structure determination. Re-crystallizations of the compound were not successful in obtaining a crystal suitable for diffraction studies.

Compounds	Carbon Chemical Shift (ppm)*																							
	C-1		C-2		C-3		C-4		C-5		C-6		C-1'		C-2'		C-3'		C-4'		C-5'		C-6'	
	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp	Ref	Exp
DFA I	62.0	62.033	102.7	102.767	82.1	82.173	78.0	78.026	83.7	83.769	62.9	62.974	62.8	62.801	99.1	99.174	77.2	77.258	74.8	74.861	81.5	81.545	62.9	62.848
DHL I	61.1	61.191	94.7	94.740	69.3	69.305	70.9	70.974	64.2	64.190	59.9	59.963	63.8	63.796	94.7	94.740	68.8	68.851	70.8	70.807	69.3	69.305	60.9	60.924
DHL II	63.7	63.796	102.5	102.587	82.2	82.220	78.0	78.093	83.7	83.842	61.5	61.512	61.7	61.883	95.9	95.969	68.8	68.851	69.3	69.305	69.3	69.305	61.5	61.512

* Shift (-0.6) from reference

Reference: Manley-Harris, M., Richards, G.N. (1997) *Adv. Carbohydr. Chem. Biochem.* **52:** 207-266.

3.3 HPLC of Product Solutions

Although (for reasons unknown to us) we were not allowed access to the preparative scale HPLC equipment in Suranaree University of Technology, we felt it necessary to determine the feasibility of separating dianhydride compounds from each other (and from starting materials) by HPLC. The chromatography setup consisted of a Waters 600 Dual Pump (Waters, USA), and a Rheodyne injector with a 20 μL sample loop. A Waters 2487 dual λ Absorbance Detector, and a Waters 2414 Refractive Index Detector equipped with a computer, and a Millenium 32 data acquisition system was used for the detection/acquisition. Chromatography was accomplished with a Bio-Sil C18 HP 90-55 column, 250 × 4.6 mm, with a particle size of 5 μm. This was coupled to a guard column, with elution of 1.0 mL/min of water. Examples of results for chromatograms of reagents and product solutions are given in Figures 3.14 to 3.17.

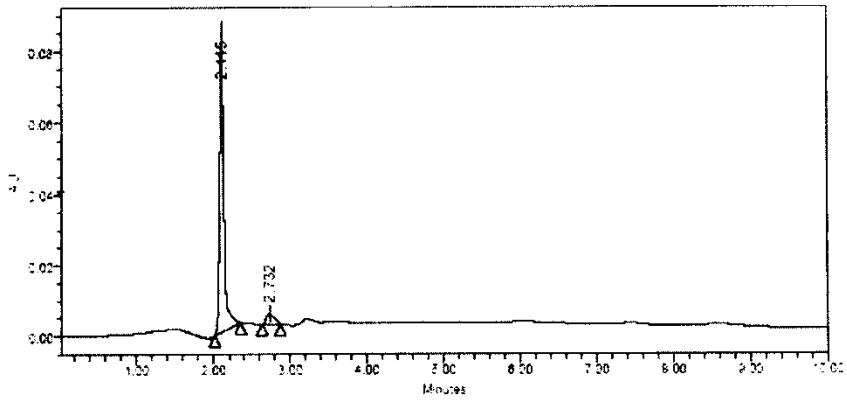


Figure 3.14 Chromatogram of fructose standard (UV-Vis detector, 230 nm).

Retention times of pure reagent compounds (for instance D-fructose and L-sorbose) were determined in order to remove these peaks from the chromatograms of reaction product mixtures. Since (unlike the GC-MS of the TMS derivatives) the method could not distinguish between different tautomers or anomers of the sugars both fructose and glucose show a clearly defined single peak on the chromatogram. Both UV-Vis and RI detectors are suitable to determine retention times of compounds in the mixture.

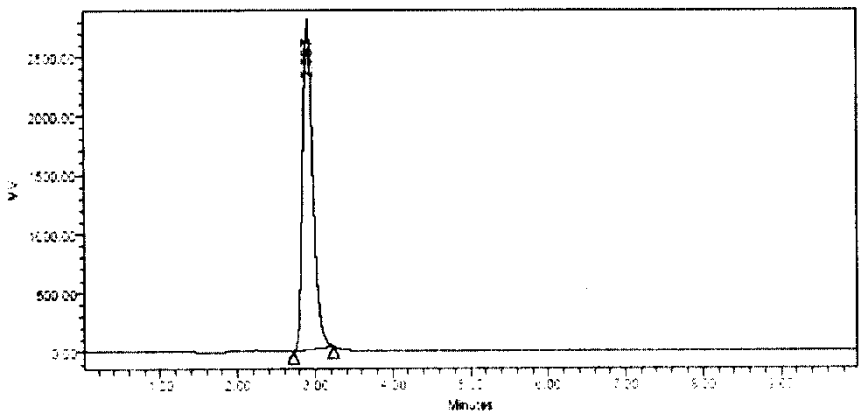


Figure 3.15 Chromatogram of sorbose standard (refractive index detector).

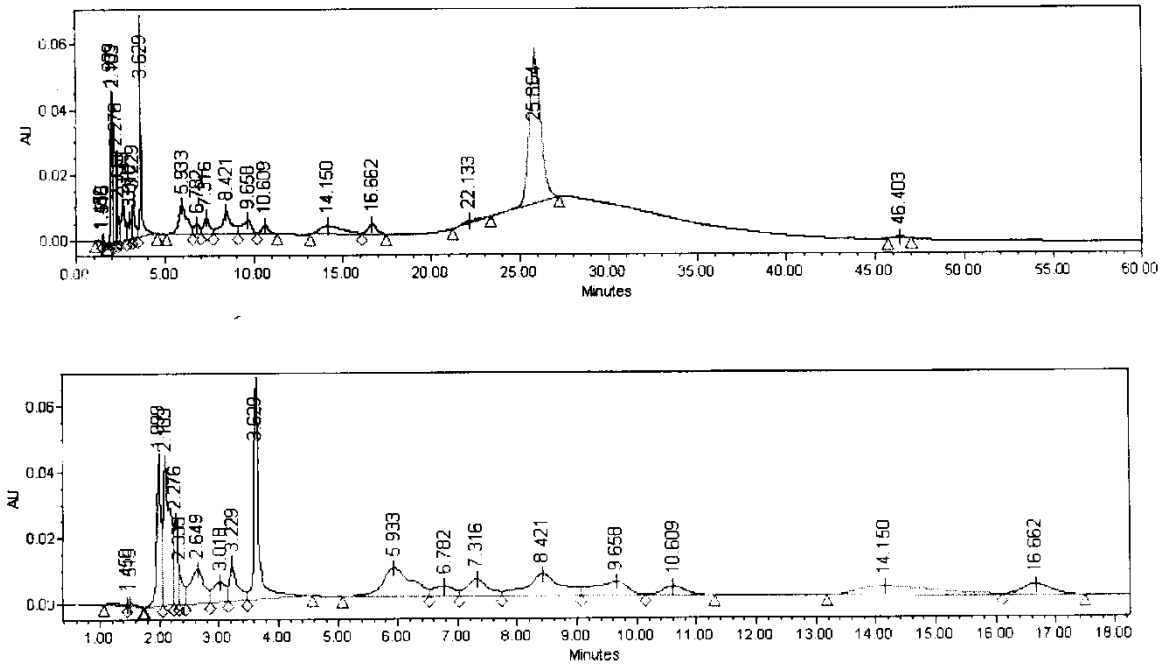


Figure 3.16 Chromatogram of product solution mixture; 75ºC, acid concentration of 1.5 M (HF), and reaction time equal to 3 hr. Taken after yeast fermentation.(UV-Vis detector, 230 nm).

These results show that a wide range of products is produced, but that many of the products occur at low concentrations. It is also evident that preparative-scale HPLC should be able to separate products from reagents, and also separate different products from each other. This will be useful to prepare solutions suitable for crystallization, particularly to

enable sufficient pure product to be created for crystal growth work that is necessary for X-Ray structure determination.

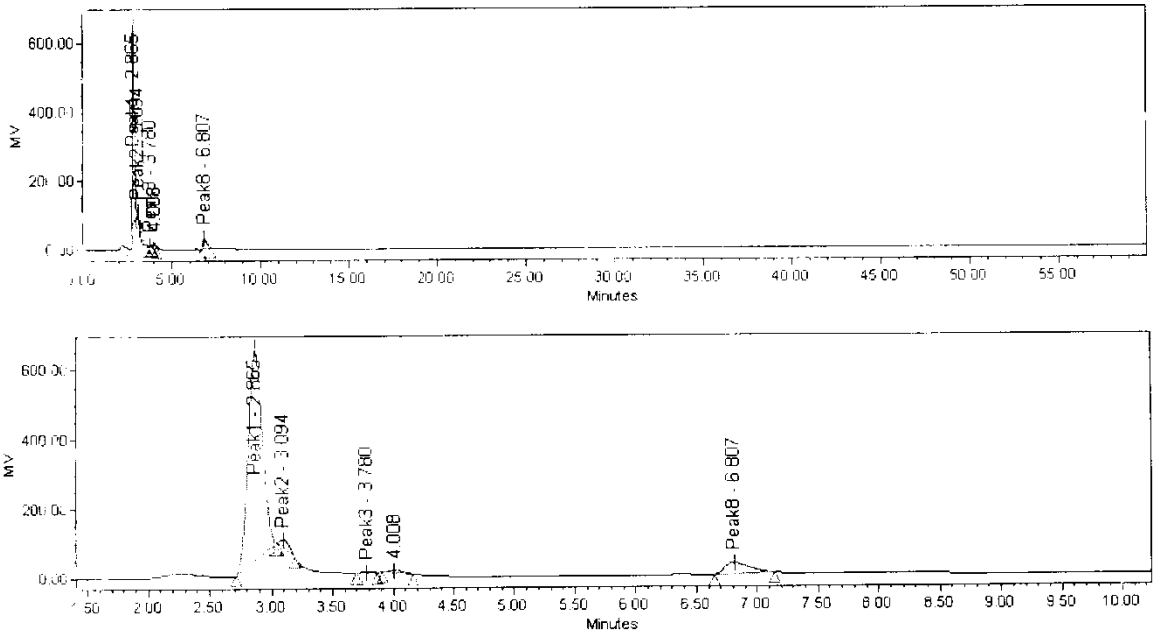


Figure 3.17 Chromatogram of product solution mixture; 75ºC, acid concentration of 1.5 M (HF), and reaction time equal to 3 hr. Taken after yeast fermentation. (UV-Vis detector, 230 nm).

Retention times of major reactant and product peaks are shown in Table 3.3.

Table 3.3 Retention times of standards and reaction product mixtures from chromatographic solution (as described in the text). Results of both refractive index (RI) and UV-Vis (at 230 nm) detectors are shown.

Fructose		Sorbose		50% Fr+Sr/HCl		50% Fr+Sr/HF (nonfermented)		50% Fr+Sr/HF (fermented)	
UV-Vis	RI	UV-Vis	RI	UV-Vis	RI	UV-Vis	RI	UV-Vis	RI
-	-	-	-	-	-	-	-	1.999	-
2.139	-	2.115	-	-	-	2.113	-	2.103	-
-	-	-	-	-	-	2.176	-	-	-
-	-	-	-	-	-	-	-	2.276	-
-	-	-	-	-	-	-	-	2.333	-

Table 3.3 (*Cont.*)

-	-	-	-	-	-	2.450	-	-	-
-	-	-	-	2.652	-	-	-	2.649	-
-	-	2.372	-	-	2.745	2.745	-	-	-
2.802	-	-	2.897	-	-	-	-	-	2.865
-	2.980	-	-	2.904	2.949	-	2.994	-	-
-	-	-	-	-	-	-	-	3.018	3.094
-	-	-	-	3.279	-	-	-	3.229	-
-	-	-	-	-	3.533	-	-	-	-
-	-	-	-	-	-	-	-	3.629	-
-	-	-	-	-	-	-	-	-	3.780
-	-	-	-	-	3.960	-	-	-	-
-	-	-	-	-	-	-	-	-	-
-	-	-	-	5.926	-	-	4.086	5.933	-
-	-	-	-	-	-	6.038	-	-	-
-	-	-	-	-	-	-	-	6.782	-
-	-	-	-	-	-	-	-	-	6.800
-	-	-	-	7.236	-	-	-	-	-
-	-	-	-	-	-	-	-	7.316	-
-	-	-	-	-	-	7.445	-	-	-
-	-	-	-	8.302	-	-	-	-	-
-	-	-	-	-	-	-	-	8.421	-
-	-	-	-	-	-	8.563	-	-	-
-	-	-	-	9.467	-	-	-	-	-
-	-	-	-	-	-	-	-	9.658	-
-	-	-	-	-	-	9.799	-	-	-
-	-	-	-	-	-	-	-	10.609	-
-	-	-	-	13.369	-	-	-	-	-
-	-	-	-	-	-	-	-	14.150	-
-	-	-	-	-	-	-	-	-	-
-	-	-	-	16.288	-	-	-	-	-
-	-	-	-	-	-	16.687	-	16.662	-

Table 3.3 (*Cont.*)									
-	-	-	-	-	-	-	-	22.133	-
-	-	-	-	-	-	25.816	-	25.864	-
-	-	-	-	39.529	-	-	-	-	-
-	-	-	-	-	-	45.623	-	-	-
-	-	-	-	-	-	-	-	46.403	-

3.4 Production of Barium Complexes of Sugars and Dianhydride Products

It was also interesting to determine whether barium complexes of compounds produced in the reaction mixtures were suitable for crystallization. This is also interesting because metal-saccharide complexes have major significance in biological processes. Initially it was decided to work with barium complexes of fructose to determine how convenient it is to crystallize these complexes.

It was found to be reasonable straightforward to produce the fructose-barium complexes and also to crystallize them. D-fructose (0.09 g, 5 mmol) and a stoichiometric amount of barium chloride ($BaCl_2$) were dissolved in 2 mL of distilled water, after which 2 mL of ethanol was added. Clear prismatic crystals with good aspect ratios were produced from this solution at room temperature, approximately 24ºC. The crystals were separated by paper filtration.

The crystals produced were analyzed using EDX in a Scanning Electron Micrscope (SEM), and also with FT-IR. The results of the EDX are shown in Figure 3.18. The crystals show significant amounts of barium, but little chloride, suggesting that these crystals are barium complexes of D-fructose rather than barium chloride. Some chloride may be from solution that evaporated from the surface of the crystals or from a small amount of barium chloride which co-precipitated from the solution mixture.

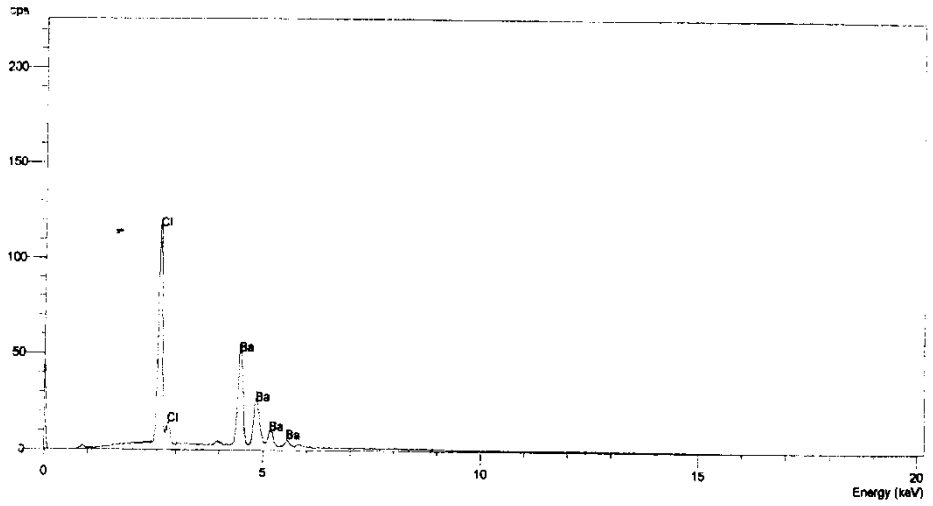


Fig. 3.18 EDX spectra of the Ba-fructose complex produced.

FT-IR spectra of both the Ba-fructose complex and D-fructose were determined. The results are shown in Figure 3.19. Functional group analysis of the FT-IR spectra clearly shows that the complex has been formed. This suggests that it may be possible to use the method to obtain barium complexes of the dianhydrides, and that this is a potential separation method that could be used to produce crystalline material from the reaction products.

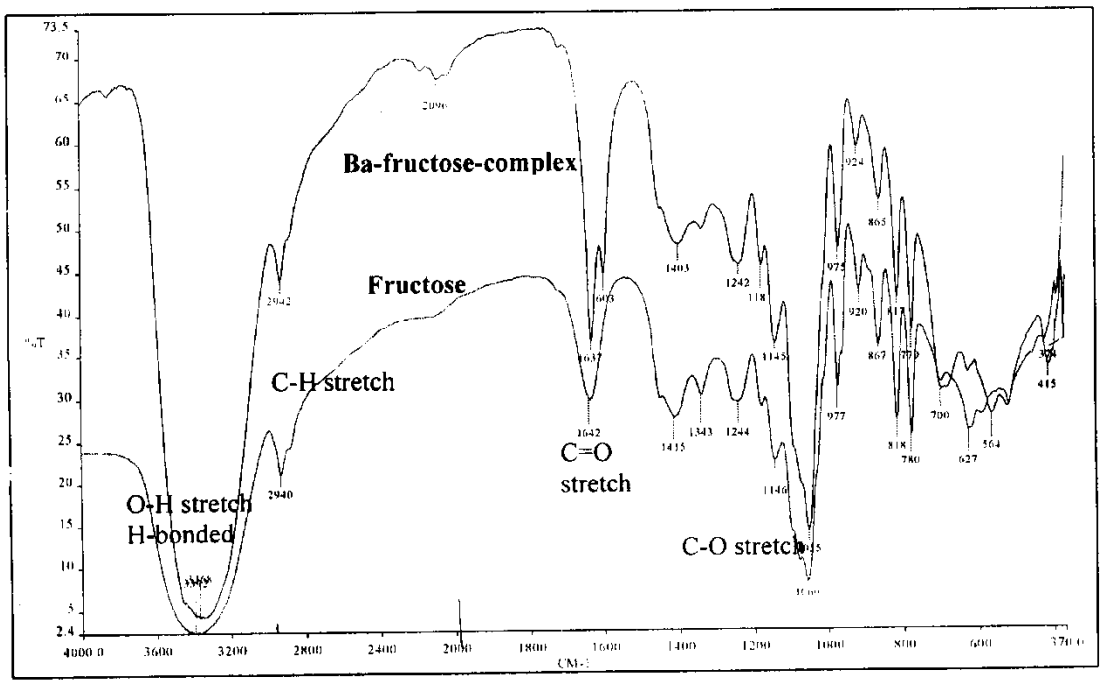


Fig. 3.19 FT-IR spectra of fructose and barium fructose complex: KBr pellet.

The spectrum analysis of the FT-IR data is shown in Table 3.4.

Table 3.4 Spectrum analysis of FT-IR of fructose and barium – fructose complex.

Type of spectrum	Significant frequencies		Inferences
	Fructose	Ba-fructose complex	
FT-IR, KBr pellet	3392 cm^{-1}	3368 cm^{-1}	O-H stretch, H-bond
	2940 cm^{-1}	2942 cm^{-1}	C-H stretch
	1642 cm^{-1}	1637 cm^{-1}	C=O stretch
	1145 cm^{-1}	1146 cm^{-1}	C-O stretch

Summary

The project determined reaction conditions and reagents that produced a range of pre-existing and novel dihexulosedianhydride compounds. The reactions involved starting materials including D-fructose and L-sorbose, D-tagatose, D-glucose and D-mannose. The conditions used varied typically in the range of 60 - 90ºC, 1.0 – 1.5 M acid (using any of hydrochloric, sulfuric, nitric, and hydrofluoric acids), and reaction times between 1 and 3 h. The optimum conditions for yield and selectivity of the desired products were somewhere in the center of this range, and the use of 75ºC at 1.25 M acid for 2 h is likely to produce products at acceptable yield and selectivity.

Products can be separated from the reagent mixture in most cases by yeast fermentation, although this does not work when sorbose is a reagent since it is not fermentable. The fermentation does not affect the yield or selectivity of the dianhydride products since they are not fermentable. This is a good method (and novel for these molecules) to purify reagents away from suitable products, and aids in further separation to obtain individual products, or for chemical characterization of the products.

The many products produced via the reactions have been characterized by a range of techniques, including ^{13}C NMR, Gas Chromatography (GC) of the trimethylsilyl (TMS) derivatives of the compounds, high performance liquid chromatography (HPLC), Fourier-Transform Infrared (FT-IR) spectroscopy, and Energy Dispersive X-Ray (EDX) spectroscopy. Based on results from previous researchers the structures of several products could be specified, and these were mainly difructosedianhydrides. Due to the inaccessibility of a preparative-scale HPLC our ability to separate enough pure product to achieve diffraction-sized single crystals was greatly inhbited, however diffraction was performed on several crystals that were found in product samples. Unfortunately these were found to be crystals of unreacted reagents or crystals of salts precipitating as a result of the reaction between the acid used to cause reaction and the base used later to neutralize the mixture. Crystals shown to be products by ^{13}C NMR were unfortunately too small for single crystal X-Ray diffraction.However the research has produced novel products, characterized a number of products, and has enabled methods to purify them based on chromatography. The only remaining step requires the availability of preparative-scale HPLC for the authors.

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