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THE UNIVERSITY OF ALBERTA
ASSAYS AND INHIBITORS FOR FUCOSYLTRANSFERASES AND AMINO ACID DECARBOXYLASES

BY
LOUIS DENNIS HEERZE

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN FOOD CHEMISTRY

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA
SPRING 1990

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NAME OF AUTHOR: Louis Dennis Heerze
TITLE OF THESIS: Ass and Inhibitors for Fucosyltransferases and Amino Acid Decarboxylases
DEGREE: Doctor of Philosophy
YEAR GRANTED: Spring 1990

(Signed) Louis D. Heerze

Permanent Address:
1245 13 Ave. N.
Lethbridge, Alberta
CANADA T1H 1S8

Date: Feb 1, 1990

THE UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ASSAYS AND INHIBITORS FOR FUCOSYLTRANSFERASES AND AMINO ACID DECARBOXYLASES submitted by LOUIS DENNIS HEERZE in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in food chemistry.

Marcia Palcic
(Supervisor)

(External Examiner)

DATE: January 29, 1990

FOR MOM, DAD, AND MANDY

Abstract

Porcine submaxillary gland α(1→2)-fucosyltransferase catalyzes the transfer of the sugar L-fucose from guanosine 5'-diphosphofucose (GDP-fucose) to the 2-hydroxyl group of β-D-galactopyranoside acceptors. A general method is described to assay fucosyltransferase activity which utilizes synthetic hydrophobic acceptor substrates. The radiolabeled products formed during reaction with ^{14}C-labeled GDP-fucose are adsorbed onto C-18 reverse phase Sep Pak cartridges. After washing with water to remove unreacted nucleotide donor, radiolabeled products can be eluted with methanol and quantitated by scintillation counting.

A bisubstrate analog (**3-1**) of the postulated transition-state of the α(1→2)-fucosyltransferase reaction which encompasses portions of both donor and acceptor was found to be a good competitive inhibitor with respect to both GDP-fucose and phenyl-β-D-galactopyranoside with K_i's ranging from 2.3-16 μM. A 2'-deoxy analog of βGal(1→3)βGlcNAcO$(CH_2)_8$COOMe was also found to be a good competitive inhibitor for α(1→2)-fucosyltransferase with a K_i of 800 μM.

L-Aspartate-α-decarboxylase, isolated from *E. coli*, catalyzes the conversion of L-aspartate to β-alanine which is subsequently used in pantothenic acid biosynthesis. Analogs of the substrate aspartic acid, can serve as mechanism-based inhibitors of the decarboxylation reaction. Analogs of aspartic acid which contain substitution at the amino nitrogen were found to be slow irreversible inhibitors of aspartate-α-decarboxylase (K_i's 34 to 106 μM). Several β substituted analogs of aspartic acid were found to be moderate competitive inhibitors of the enzyme (K_i's 2.7 to 4.2 mM). All inhibitors exhibit an unusual D-specificity, with D isomers being more inhibitory than the corresponding L-isomer.

A general radiochemical method for estimating amino acid decarboxylase activity which utilizes ion exchange cartridges to separate product amine from substrate is reported. Acidic, basic, and neutral amino acid decarboxylases can be assayed by using the appropriate choice of ion exchange cartridge. The assay is simple, rapid and sensitive if uniformly ^{14}C labeled substrates are used. This method was applied to three different decarboxylase enzymes.

ACKNOWLEDGEMENTS

I would like to thank Dr. M. Palcic for her guidance and assistance during the course of my studies.

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My appreciation goes out to my fiance Mandy who helped me so much when times were bad.

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LIST OF ABBREVIATIONS

ER endoplasmic reticulum
TGN trans golgi network
GDP-fucose guanosine 5'-diphospho-L-fucose
GlcNAc *N*-acetylglucosamine
GalNAc *N*-acetylgalactosamine
HPLC high performance liquid chromatography
GMP guanosine 5'-monophosphate
TLC thin layer chromatography
UDP-gal uridine 5'-diphospho-D-galactose
UDP-GlcNAc uridine 5'-diphospho-D-*N*-acetylglucosamine
β-NADH β-nicotinamide adenine dinucleotide (reduced form)
ATP adenosine 5'-triphosphate
$MnCl_2$ manganese chloride
KCl potassium chloride
cpm counts per minute
NaN_3 sodium azide
NaCl sodium chloride
BSA bovine serum albumin
dpm disintegrations per minute
Mn manganese
GDP guanosine 5'-diphosphate
Phenyl Gal Phenyl β-D-galactopyranoside
GlcNAc T-V *N*-acetylglucosaminyltransferase V
UDP uridine 5'-diphosphate
NeuAc *N*-acetylneuraminic acid (sialic acid)
ADC L-aspartate-α-decarboxylase
CO_2 carbon dioxide
UV ultraviolet spectroscopy
EDTA ethylene diamine tetraacetic acid tetra sodium salt

ATCC American Type Culture Collection

BnBr benzyl bromide

Tf_2O triflic anhydride

Pd palladium

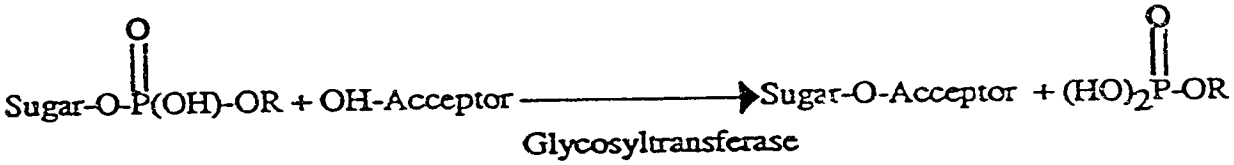
QMA quaternary methyl ammonium

CM carboxymethyl

1. Introduction to Glycosyltransferases

Enzyme catalyzed glycosyl transfer reactions are the key biosynthetic processes in the assembly of the large variety of sugar containing compounds in biological systems. It is well recognized that carbohydrates serve as energy storage compounds (starch and glycogen) as well as structural components in cells (cellulose and chitin). During the last ten years, however, scientists have come to realize the crucial roles played by carbohydrates in intercellular functioning. Complex carbohydrates containing one or more chains covalently attached to peptides (glycoproteins) or lipids (glycolipids) play a role in the cell's ability to recognize and bind selectively to other cells or to soluble molecules such as hormones.

Glycosyltransferases are a class of enzymes which catalyze the specific transfer of a single sugar residue from a sugar donor (usually a sugar nucleotide) to the hydroxyl group of an acceptor sugar molecule which is part of a glycoprotein, glycolipid, or polysaccharide with the release of a nucleoside phosphate as is shown below.



The first nucleotide sugar discovered was uridine diphosphoglucose (1) which was found to participate in the biosynthesis of glycogen (2). Since this discovery, the synthesis of almost all glycosidic bonds in mammals has been found to utilize sugar nucleotide substrates. Uridine diphospho sugars serve as donor substrates for the formation of glycosides of galactose, glucose, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), xylose, glucuronic acid, and *N*-acetylglucosamine phosphate while guanosine diphosphate sugars of fucose and mannose are used in the formation of fucosides and mannosides. In contrast, the transfer of sialic acid is achieved by the use of cytidine monophosphosialic acid. The discovery that sugar nucleotides are utilized as glycosyl donors in glycosyltransferase reactions, has lead to a vast amount of literature describing the occurence, properties, and regulation of glycosyltransferase enzymes. Several reviews have been written describing the research that has been done in the thirty years (3-5). Glycosyltransferases are classified according to the type of sugar transfered from the donor substrate, and possess strict substrate specificity for the acceptor substrate with at least one

enzyme required to synthesize each of the glycosidic linkages observed in nature. The term "one enzyme-one linkage" has been adopted to describe this substrate specificity (6). In nature there exist many complex and diverse carbohydrate structures contained in glycoproteins and glycolipids, therefore there must be many glycosyltransferases present to be able to synthesize these complex structures.

A few exceptions have been noted, the first example being lactose synthetase from milk. This enzyme catalyzes the transfer of galactose from uridine diphosphogalactose to the 4 position of glucose (7). Further research on this enzyme has demonstrated that another protein is closely associated with this galactosyltransferase enzyme (8). The other protein was found to be α-lactalbumin and was required for the synthesis of lactose. If α-lactalbumin was not present with the galactosyltransferase, the enzyme would only transfer galactose to the 4 position of *N*-acetylglucosamine forming *N*-acetyllactosamine (8). The function of α-lactalbumin, found only in mammary glands, was to inhibit the formation of *N*-acetyllactosamine and this is the only case described so far in which another protein modulates the functioning of a glycosyltranferase enzyme activity. Another exception to the concept exists in the case of the Lewis B blood group (1→3)-galactosyltransferase enyme. This enzyme has the additional capacity to transfer not only galactose but also *N*-acetylgalactosamine forming the Lewis blood group A antigenic determinant (9,10). The last anomaly to the "one enzyme-one linkage" concept is observed with an enzyme involved in the Lewis blood group system. This enzyme found in human milk transfers the sugar L-fucose either to the 3 or 4 position of the *N*-acetylglucosamine residue of *N*-acetyllactosamine forming either the Le^a or the Le^b blood group determinants (11,12). This is the only case known where a glycosyltransferase can transfer a sugar to more than one position on an acceptor substrate.

In addition to the strict substrate specificity of glycosyltransferase enzymes, there must be other mechanisms to explain the large diversity observed in the structures of complex carbohydrates found in mammals. Synthesis of complex carbohydrates proceeds along a preferred pathway where the product of one glycosyltransferase reaction serves as a substrate for the next glycosyltransferase enzyme (3,13). The enzyme which operates next in the synthesis is governed by the relative specificities of the glycosyltransferases present towards the product formed in the initial reaction, as well as the distribution of transferases within the endoplasmic reticulum and golgi. The second product that is synthesized will be the result of the transferase that is most successful in the competition for the initial product. The overall concentrations and distribution of both acceptor and donor substrates also influence the distribution of products formed. Addition of specific

sugars such as fucose and sialic acid can signal termination of the synthesis of the complex carbohydrate molecule.

Complex carbohydrates are found attached to proteins by two linkages. They can either be *N*-linked through an asparagine or *O*-linked through a serine or threonine residue to the protein molecule. Carbohydrates can also be *O*-linked to lipids to form a glycolipid. While much information is available for *O*-linked glycoproteins, has the complete biosynthesis of *N*-linked glycoproteins been elucidated (3-5,14,15). The biosynthesis of all *N*-linked glycoproteins begins in the lumen of endoplasmic reticulum of cells with the synthesis of a common seven sugar core structure as shown in Figure 1-1. This process occurs in the lumen of the endoplasmic reticulum. Synthesis of the core structure begins by the addition of *N*-acetylglucosamine to a lipid carrier dolichol pyrophosphate. Sequential addition of 1 GlcNAc, 9 mannose, and 3 glucose sugars produces a 14 sugar glycolipid carrier molecule. The oligosaccharide portion of this glycolipid carrier is then transferred to an asparagine residue on a growing polypeptide chain with the release of the lipid carrier. Subsequent trimming of the 14 sugar oligosaccharide by glycosidase enzymes located in the later part of the endoplasmic reticulum, results in a 7 sugar core structure that is transferred to the golgi apparatus. At this point more saccharides (*N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, fucose, and sialic acid) can be sequentially added by individual transferase enzymes to produce a final complex carbohydrate structure that may be multibranched. The overall biosynthesis of *O*-linked glycolipids is not as well understood, but it is thought to proceed through a different biosynthetic pathway.

A complete understanding of glycosyltransferases and their unique ability to synthesize complex carbohydrate structures can be accomplished by a thorough study of each enzyme's substrate specificities. This is achieved by the use of a purified glycosyltransferase enzyme free of competing activities. Until recently, it was difficult to obtain enough purified glycosyltransferases for thorough enzyme characterization without going to large scale isolation procedures. This can be attributed to relatively low concentrations of these enzymes in tissues. Most transferases are membrane bound and require detergents to solubilize the enzyme activity. This often results in enzyme activities that are prone to denaturation. Glycosyltransferases are also sensitive to dilution and surface inactivation; some even require the addition of detergent to prevent aggregation, but their susceptibility to denaturation varies from enzyme to enzyme. The advent of several affinity purification techniques has provided a means by which many transferases can be isolated. The advantage of using affinity purification is that the transferase enzyme can be selectively removed from large amounts of contaminating protein by adsorption onto an

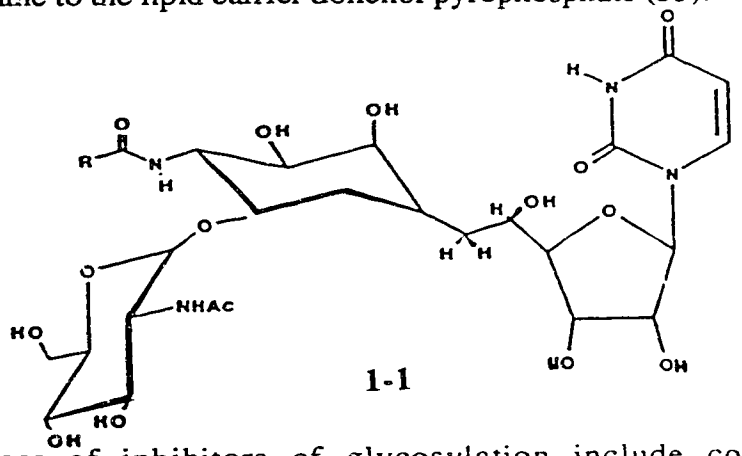
affinity matrix under optimal conditions for maintenance of enzyme stability. Another important consideration is that a concentrated enzyme is obtained as a result of affinity purification. The affinity matrices that have been developed make use of analogs of either the acceptor or donor substrates or the products covalently attached to a support through a six carbon spacer arm (16-18). More recently, advances transferase enzymes have been isolated using an affinity matrix of a covalently attatched dye (19,20).

In order to study the substrate specificity of a transferase, one also requires a reliable enzyme assay. The most common method for estimating enzyme activity is with a radiochemical assay in which sugar nucleotide donor, radiolabeled in the sugar, is incubated with enzyme and an appropriate acceptor. Upon reaction, the resulting radiolabeled product is isolated from reactants by high voltage electrophoresis, thin layer chromatography, gel filtration, ion exchange, or HPLC and then quantitated by scintillation counting (17,21-23). The acceptor substrates used in these assays are usually complex oligosaccharides isolated in small amounts from natural sources. Several problems arose when natural substrates were used in transferase assays. The natural acceptors were usually substrates for more than one transferase enzyme, or the extract containing the transferase activity contained hydrolase enzymes which partially degraded the natural substrate making it difficult to use for studies on enzyme specificity. It is difficult to obtain sufficient amounts of the appropriate acceptor in pure form free of any other potential acceptor substrates. Many of the above difficulties can be overcome by using synthetic acceptor substrates. Many transferase activities can be measured by the use of simple carbohydrate acceptors, and in one case a simple monosaccharide acceptor (24) can be utilized which could be obtained commercially or chemically synthesized by published methods. Other assay methods include a coupled enzyme system to measure nucleotide diphosphate product spectrophotometrically (25). Several enzyme linked immunosorbant assays (ELISA) which utilize specific antibodies directed against the product formed during reaction have also been developed (26,27).

A better understanding of the enzymes that are responsible for the biosynthesis of the complex carbohydrates contained in proteins and lipids on the surface of cells may provide some insight in terms of their absolute function. As mentioned previously, cell surface carbohydrates have been implicated in such intercellular functions as recognition, adhesion, and differentiation as well as the binding of antibodies, lectins, or bacterial toxins to other cells (28,29). Many changes in the distribution of structures of cell surface glycoproteins and glycolipids occur during normal cellular development, during embryogenesis and cellular differentiation, and abnormal cellular development during tumor

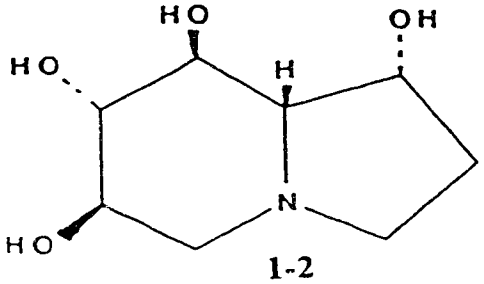
progression (30,31). The altered distribution of carbohydrate structures that appear on cell surfaces during both normal and abnormal cellular development is the direct result of an altered carbohydrate biosynthesis and may be caused by irregularities within the cell that may prevent normal carbohydrate expression. Differing levels of certain glycosyltransferase enzymes are responsible for the altered distribution of carbohydrate structures. The structures that appear on tumor cells have been referred to as "tumor associated antigens" (30,31) and has been directly correlated with an increase in the levels of a specific glycosyltransferase activity in at least one instance (21).

Inhibition of the biosynthesis of complex carbohydrates may provide important information about the overall biosynthesis as well as the biological role of cell surface carbohydrates. Inhibitors of *N*-linked glycosylation fall into two classes: either they inhibit protein glycosylation completely or they inhibit the glycosidase enzymes responsible for the trimming of the 14 sugar mannose containing glycoprotein in the biosynthesis of *N*-linked glycoproteins. The first class of inhibitors includes compounds such as tunicamycin (**1-1**). This compound inhibits the enzyme UDP-GlcNAc:dolichol phosphate GlcNAc-phosphotransferase (E.C. 2.7.8.15) which is responsible for the addition of *N*-acetylglucosamine to the lipid carrier dolichol pyrophosphate (33).



1-1

The second class of inhibitors of glycosylation include compounds such as castanospermine (**1-2**) and swainsonine (**1-3**) which inhibit the trimming reactions of the mannose containing oligosaccharide intermediates (33).



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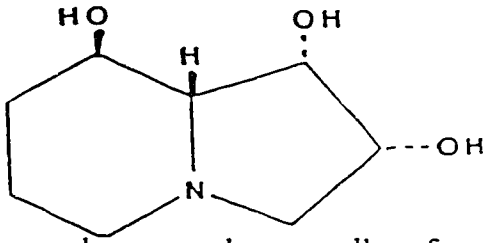
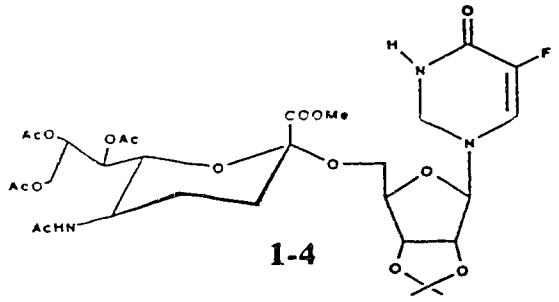


1-3

Both types of inhibitor have been shown to change cell surface carbohydrate structures causing altered cell functioning (33-35). Both classes of inhibitors only affect the early stages in *N* -linked glycoprotein biosynthesis and they do not alter the synthesis of either *O*-linked oligosaccharides or glycolipids which are synthesized by a different route.

Inhibitors of the glycosyltransferases responsible for the later stages of glycoprotein biosynthesis would be beneficial in understanding cell functioning and glycoprotein biosynthesis. Inhibitors of the tranferase enzymes which are at elevated levels in tumor cells exhibiting altered carbohydrate cell surface structures may provide a means for controlling the expression of these altered structures. Several unreactive sugar nucleotide substrate analogs have been synthesized and found to be inhibitory towards individual glycosyltransferases. Two analogs (**1-4**,**1-5**) of cytidine monophosphosialic acid, the sugar nucleotide donor of sialyltranferases, have been found to be weak competitive inhibitors, of a mouse lymphocyte sialyltransferase (36).



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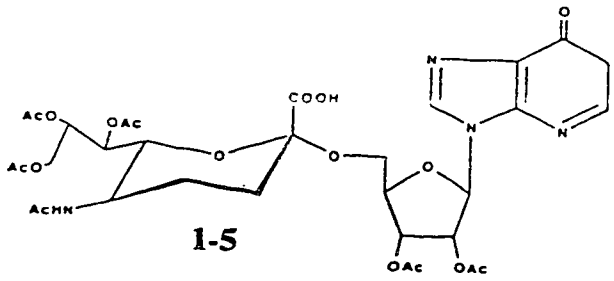
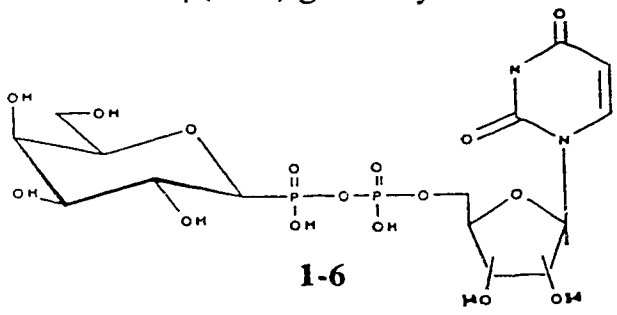


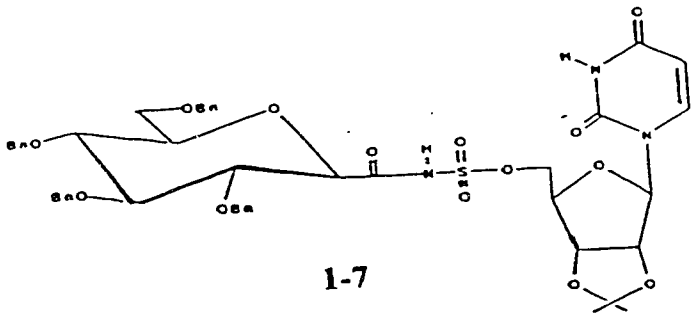
1-5

An analog (**1-6**) of the donor substrate uridine diphosphogalactose was found to be a modest competitive inhibitor of a β(1→4)-galactosyltransferase from human ascites fluid (37).



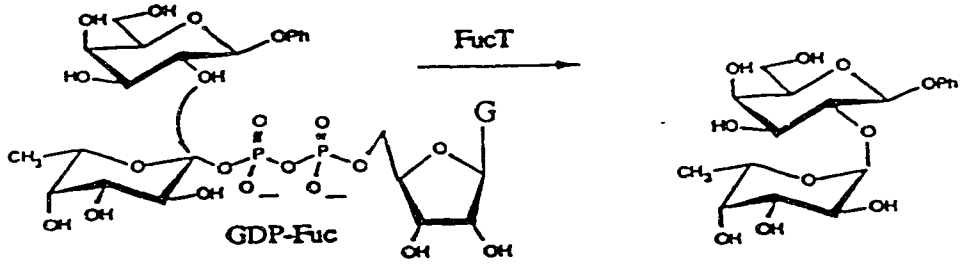
1-6

A derivative of the donor substrate (**1-7**) for a glucosyltransferase was found to inhibit glycoprotein synthesis and posessed anti-viral activity against herpes simplex virus 1 (38).



1-7

The present research deals with fucosyltransferases which transfer L-fucose (6-deoxy-L-galactose) from guanosine diphosphofucose, which is synthesized from guanosine diphosphomannose, to suitable acceptor substrates with the release of product and guanosine diphosphate as is shown below for (1→2)-fucosyltransferase.



The sugar L-fucose is widely distributed in glycoproteins, glycolipids, and oligo-saccharides and is found both in mammals and in plants (4). Fucose is found exclusively α-linked to the non anomeric position of galactose, *N*-acetylglucosamine, or glucose. Fucose can be *O*-linked through either the 2, 3 or 6 hydroxyl group of galactose, the 3, 4 or 6 position of *N*-acetylglucosamine, or the 3 or 4 hydroxyl of glucose. Although all these linkages have been found for fucose on natural structures, the only fucosyltransferase activities demonstrated *in vitro* are those responsible for the addition of fucose to the 3, 4, or 6 hydroxyl of *N*-acetylglucosamine and the 2 position of galactose. The major interest in fucose is that it plays a key role in the formation of the ABH and the Lewis blood group antigenic determinants (39). Several fucosylated structures have been implicated as "tumor

associated antigens" (40) and increased levels of (1→3)-fucosyltransferase activity have been demonstrated in human leukemic cells (41), human lung cancer cells (42), and in blood serum (43).

The specific fucosyltransferase described in this study is the enzyme which transfers fucose to the 2 hydroxyl of a terminal galactose residue, an α(1→2)-fucosyltransferase. This enzyme is responsible for the formation of the H blood group antigenic determinant (α-L-fucose-(1→2)β-D-galactose) which is also a common precursor for the A and B antigens in the ABO blood group system (39). Two genetically distinct forms of this enzyme, differing only in their substrate specificities, are present in humans: the blood group H fucosyltransferase, found in hematopoietic tissues and plasma, and the secretor (Se) fucosyltransferase which is present in secretory tissues and fluids. The human H fucosyltransferase has been recently cloned and expressed in a mouse cell line (44,45). The α(1→2)-fucosyltransferase is also present in several animal tissues which include hog gastric mucosa, rat small intestinal mucosa, and bovine spleen (4). The α(1→2)-fucosyltransferase best studied is one that has been purified to homogeneity from porcine submaxillary glands (46,47), and this same enzyme was used in the present investigations.

The fucosyltransferase from porcine submaxillary glands has two forms with molecular weights of 55,000 and 60,000 respectively and shows a broad pH optimum between pH 6 and 7. Addition of Mn^{2+} or Mg^{2+} stimulates enzyme activity 2 to 6 fold, but remains active even in the absence of divalent cations. The porcine submaxillary fucosyltransferase has a broad acceptor substrate specificity (47) and will act on a variety of glycoprotein and glycolipid acceptors terminating in β-galactose as well as on simple monosaccharide glycosides such as phenyl-β-D-galactopyranoside (24). The best acceptor substrates are those compounds which terminate in the structure β-Gal(1→3)βGalNAc-R. Fucosyltransferase activity is most commonly assayed by using ^{14}C labeled guanosine diphosphofucose (GDP-fucose) and an appropriate acceptor substrate. Upon reaction the product is removed from reactants either by gel filtration or by ion exchange. Few inhibitors exist for this fucosyltransferase; guanosine monophosphate (GMP) was found to be a competitive inhibitor of the donor substrate GDP-fucose (K_i 22 μM), and there are no known inhibitors of the acceptor substrate (47).

This thesis is written in manuscript format, and thus each of the following three chapters, and subsequent chapters on aspartate-α-decarboxylase, are presented as manuscripts that have been submitted for publication or are in press at this time. For this reason many of the introductions may appear to be similar. The topics that will be

discussed below are:

1. The development of a rapid and general radiochemical assay for glycosyltransferases which utilizes hydrophobic acceptor substrates that can be adsorbed onto reverse phase Sep Pak C 18 cartridges.
2. The kinetic evaluation of a bisubstrate analog mechanism-based inhibitor of the porcine submaxillary gland $\alpha(1\rightarrow2)$-fucosyltransferase.
3. The kinetic evaluation of a deoxy analog of an acceptor substrate which was found to be a specific inhibitor of the porcine submaxillary gland fucosyltransferase. For completeness, the results obtained by collaborators for other deoxy analogs will also be included and discussed.

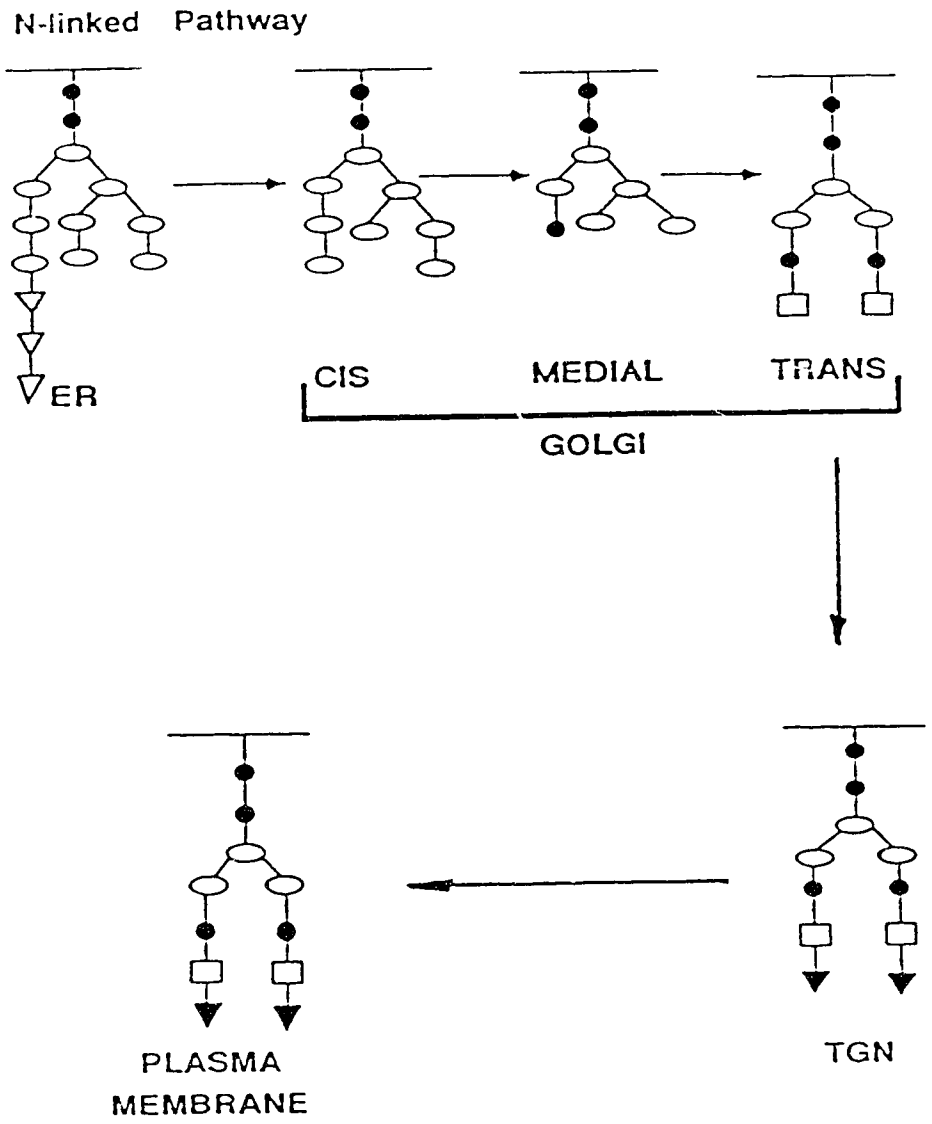


Figure 1-1. Biosynthesis of *N*-linked glycoproteins. The following symbols represent the monosacccharides: *N*-acetylglucosamine (●), mannose (○), glucose (△), galactose (□), and sialic acid (▲) respectively (48).

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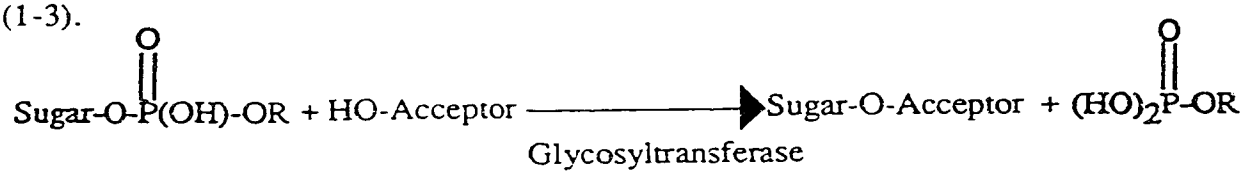
2. The Use Of Hydrophobic Synthetic Glycosides as Acceptors in Glycosyltransferase Assays*

Introduction

Glycosyltransferases are a class of enzymes that catalyze the transfer of a glycosyl residue, usually from a sugar nucleotide donor, to the hydroxyl group of an acceptor saccharide which generally forms part of a glycoprotein, glycolipid or polysaccharide (1-3).



The activity of glycosyltransferases is most commonly assayed by incubating the sugar nucleotide, radiolabeled in the sugar moiety, with a source of enzyme and an appropriate acceptor substrate. Labeled products are then quantitated after separation from the unreacted donor and its degradation products: the sugar phosphate and the free sugar. Separation is carried out in a variety of ways. These include high voltage paper electrophoresis, TLC, gel filtration or HPLC, which are tedious, and time consuming; or by ion exchange chromatography, which is prone to interference by detergents (such as Triton X-100) frequently used to solubilize the enzymes (3-6). These assays are further complicated by the fact that the acceptors for many of these enzymes are complex oligosaccharides which are very difficult to purify, even in milligram amounts, from natural sources.

In this chapter, we report a general method to assay glycosyltransferases which makes use of synthetic glycoside acceptors attached to hydrophobic aglycones. By virtue of their hydrophobicities, the labeled products from an enzyme incubation are readily separated from unreacted sugar nucleotide and by products by adsorption onto a reverse phase C 18 cartridge, from which they are eluted by methanol after aqueous washing.

* Palcic, M.M., Heerze, L.D., Pierce, M., and Hindsgaul, O. (1988)*Glycoconjugate J.* 5, 49-63.
Dr. M. Palcic Department of Food Science, University of Alberta carried out the β(1→4)-galactosyltransferase work.
Dr. O. Hindsgaul, Department of Chemistry, University of Alberta was responsible for the acceptor synthesis.

The assay which we refer to here as the Sep Pak assay is rapid, requiring less than one minute processing time per sample, has excellent sensitivity due to the low background achieved and requires no specialized equipment. Triton X-100 does not interfere and the assay works well for both crude cell homogenates and purified enzymes. We demonstrate here the suitability of this method for the assay of *N*-Acetylglucosaminide β(1→4)-galactosyltransferase, β-galactoside α(1→2)-fucosyltransferase. Some of this data have already been presented in preliminary form (7).

Experimental Procedures

Materials

Bovine milk UDP-Gal β-D-N-Acetylglucosaminide β(1→4)-galactosyltransferase (EC 2.4.1.90), specific activity 4.3 units/mg protein, UDP-Gal, UDP-GlcNAc, β-NADH, ATP phosphoenolpyruvate, rabbit muscle L-lactic dehydrogenase (EC 1.1.1.27) Type II, specific activity 920 units/mg, rabbit muscle pyruvate kinase (EC 2.7.1.40) Type III, specific activity 500 units/mg, 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D- glucopyranoside (**2-2**), phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2-3**), p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2-4**), phenyl β-D-galactopyranoside (**2-5**), o-nitrophenyl β-D-galactopyranoside (**2-8**), and n-octyl β-D-glucopyranoside were from Sigma (St. Louis,MO, USA). 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2-1**) (8), 8-methoxycarbonyloctyl β-D-galactopyranoside (**2-6**) (9), 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (**2-7**) (8) were prepared by chemical synthesis. Uridine diphospho-D-[6-^{3}H]galactose (17.3 Ci/mmol), guanosine diphospho-L-[U-^{14}C]fucose (129 mCi/mmol) and ACS liquid scintillation cocktail were from Amersham (Oakville, Ont. Canada). Uridine diphospho-*N*-Acetyl-D-[1-^{14}C]glucosamine (9.5 mCi/mol) was from New England Nuclear (Lachine, Que., Canada). Sep Pak C 18 reverse phase cartridges were obtained from Waters (Mississauga, Ont., Canada), and were conditioned before use by washing with 10 mL of methanol and 20 mL of water. GDP-fucose was synthesized by Dr. O. Hindsgaul's group, according to the method of Nunez *et al* (10). Porcine submaxillary glands were obtained from Pel-Freeze Biologicals (Rogers, Ark., USA). A crude extract of the soluble form of GDP-Fucose β-galactoside α(1→2)-fucosyltransferase was prepared from porcine submaxillary glands (11). Protein concentrations were estimated using the Bio Rad protein assay kit which is based on the method described by Bradford

(12) using bovine serum albumin as a standard.

Methods

Acceptor Evaluations

400 nmol of **2-1** or **2-2**, or 900 nmol of **2-3** or **2-4** were incubated with 100 nmol UDP-Gal, 0.37 μCi of UDP-[6-^{3}H]Gal, 7 milliunits of galactosyltransferase, 107 μmol sodium cacodylate, pH 7.5, and 10 μmol $MnCl_2$ in a total volume of 1.07 mL. Following incubation at 37°C for 3 hr, each reaction mixture was diluted to 5 mL with water and applied to a conditioned Sep Pak C 18 cartridge attached to a 5 mL syringe. The unreacted UDP-Gal and its hydrolysis products were eluted from the cartridge with 20-25 mLwater or until constant counts near background were reached. Radiolabeled reaction products were eluted in 15 mL methanol, evaporated to dryness, dissolved in 5 mL of water and reapplied to another Sep Pak cartridge. The cartridge was washed with 5 x 5 mL fractions of water, which were collected directly in scintillation vials, then with 5 mL fractions of methanol. After addition of ACS liquid scintillation cocktail, the samples were counted on a Beckman LS 1801 scintillation counter. The data were obtained as dpm, by using a series of standards to generate quench curves relating the counting efficiency to H number for the isotopes used, as specified by the manufacturer. This compensates for the different counting efficiencies of the aqueous and methanol eluents (25 and 18% respectively) for tritium.

α(1→2)-Fucosyltransferase incubations contained in a volume of 0.69 mL: 5.9 μmol of **2-5**, 2.2 μmol of **2-6**, or 0.7 μmol of **2-7**, 0.12 μCi GDP-[U-^{14}C]fucose, 30 μmol $MnCl_2$, 0.2 μmol ATP, 12.5 μmol sodium cacodylate buffer pH 6.0, and an extract of fucosyltransferase which contained 3.4 mg protein. After incubation, at 37°C for 6 to 20 hr, the reaction mixture was quenched with 5 mL of water and applied to a conditioned Sep Pak cartridge. The cartridge was washed with 5 x 5 mL of water, then 15 mL of methanol to elute radiolabeled reaction products. The methanol fraction was evaporated to dryness, reapplied to a Sep Pak cartridge and washed with 5 mL fractions of water which were collected in scintillation vials. The cartridges were then washed with 5 mL fractions of methanol, which were also collected in vials and counted as described above. The counting efficiencies for the aqueous and methanol eluents were 89 and 86% respectively.

β(1→4)-Galactosyltranferase Assays

Bovine milk β(1→4)-galactosyltransferase activity was estimated spectrophotometrically, using the method of Fitzgerald *et al.*(13). Cuvettes containing 107 μmol sodium cacodylate buffer pH 7.5, 10 μmol $MnCl_2$, 100 nmol UDP-Gal, 50 μmol KCl, 1.07 μmol phosphenolpyruvate, 200 nmol NADH, 50 units pyruvate kinase, 50 units lactate dehydrogenase and 20-308 nmol of **2-1** or 17-250 nmol **2-2** were equilibrated to 37°C in a Beckman DU-8 spectrophotometer. β(1→4)-Galactosyl- transferase (0.7-1.7 μg) was added such that the total volume was 1.07 mL, and the decrease in absorbance monitored at 340 nm. Initial rates of product formation were estimated using a millimolar extinction coefficient of 6.2 mM^{-1} cm^{-1} for NADH absorbance. The kinetic parameters V_{max} and K_m were evaluated from the inital rate data using a computer program based on the statistical method of Wilkinson (14).

The radiochemical assay was done in the same manner as the spectrophotometric assay, except the incubations were carried out in plastic liquid scintillation vials and contained 0.3-0.6 μCi of UDP-[6-^{3}H]Gal in addition to the other components. Following incubation at 37°C, 5 mL of water was added to the vials and the samples applied immmediately to a Sep Pak cartridge. The cartridges were washed with water until background levels were achieved and then the sample was eluted in 2 x 5 mL fractions of methanol and counted in 10 mL of ACS cocktail.

α(1→2)-Fucosyltransferase Assays

These assays were done in plastic scintillation vials which contained 0.01 μCi of GDP-[U-^{14}C]fucose, 9 nmol GDP-fucose, 30 μmol $MnCl_2$, 0.2 μmol ATP, 0.39-7.4 μmol of **2-5**, 50-360 nmol of **2-6**, 20-360 nmol of **2-7** or 0.14-1.4 μmol of **2-8** as acceptors. An extract of fucosyltransferase containing 14 mg of protein was added such that the total volume was 0.62 mL. The mixtures were incubated at 37°C for 1 hr, 5 mL of water was added and the samples applied to a Sep Pak cartridge. The cartridge was washed until eluent reached 50-70 cpm, then radiolabeled products were eluted with 5 mL of methanol and counted in 10 mL ACS cocktail.

Routine Sep Pak Assay

After incubation of an enzyme extract and an appropriate donor and acceptor, the reaction mixture was diluted to 5 mL with water, then applied to a freshly conditioned Sep Pak cartridge. The cartridge was washed with 20 mL of water. An additional 5 mL water wash was collected in a scintillation vial and counted to ensure that elution of labeled donor was complete. Radiochemical products were then eluted with 2 x 5 mL methanol and counted separately. The second 5 mL fraction rarely contains significant radioactivity except when large amounts of enzyme are present. The time required for aqueous washing and methanol elution was under one minute. Control assays which contain all the components except acceptor were used to establish blanks. We have not observed interference by endogenous acceptor substrates in our crude fractions; however, for such samples a boiled enzyme solution would serve as a appropriate control. For routine assays cpm rather than dpm can be used for data analysis. Sep Paks can be reused after regeneration according to the manufacturer's recommendations.

Results and Discussion

In principle, any structure that is an acceptor for a glycosyltransferase is useful in the Sep Pak assay, provided that the glycosylated product is partially adsorbed onto the reverse phase matrix under conditions where the labeled donor and degradation products are eluted. In order to obtain meaningful and reproducible kinetic parameters however, we operationally define a "suitable acceptor" as one where over 90% of the product remains adsorbed to the Sep Pak (and is later eluted with methanol) during a water wash sufficient to achieve an eluate radioactivity indistinguishable from background, usually 15 to 25 mL.

To assess the suitability of a potential acceptor, a "preparative scale" synthesis of the product is first carried out using an available source of enzyme where the product is isolated using the routine Sep Pak procedure. After evaporation of the methanol from the eluate, the labeled product is redissolved in water and reisolated on a Sep Pak cartridge to determine whether it satisfies the suitability criteria noted above. The results of such evaluation for the four hydrophobic 2-acetamido-2-deoxy-ß-D-glucopyranosides **2-1**-**2-4** as acceptors for β(1→4)-galactosyltransferase are summarized in Figure 2-1 and Table 2-1. The conclusion from these data is that only the 8-methoxycarbonyloctyl and 4-methylumbelliferyl, acceptors **2-1** and **2-2**, are satisfactory, while the phenyl and p-nitrophenyl glycosides, **2-3** and **2-4**, are not suitable as acceptors for the quantitation

of enzyme activity. These results contrast with the similar evaluation of the phenyl and 8-methoxylcarbonyloctyl galactopyranosides (**2-5** and **2-6**) and o-nitrophenyl-β-D-galactopyranoside (**2-8**) (data not shown), as acceptors for a crude α(1→2)-fucosyltransferase extract from porcine submaxillary glands, where both products are sufficiently hydrophobic, Figure 2-2 and Table 2-2. In this instance, the addition of the fucose residue appears to substantially increase the hydrophobicity of phenyl-β-D-galactopyranoside and o-nitrophenyl-β-D-galactopyranoside which are only poorly adsorbed during the Sep Pak procedure, when the water wash is monitered spectrophotometrically or by the phenol sulfuric acid assay (15).The disaccharide Galβ(1→3)GlcNAc-O(CH_2)COOMe, **2-7** was also evaluated and, as expected, found to be satisfactory as an acceptor (Figure 2-2 and Table 2-2).

The suitablity of acceptors **2-1** and **2-2** for the estimation of standard kinetic parameters for purified bovine milk β(1→4)-galactosyltransferase, using the Sep Pak assay is demonstrated in Figure 2-3 and 2-4. The formation of product is linear with both enzyme concentration and time (Figure 2-3 and 2-4 insets). The kinetic parameters V_{max} and K_m obtained with the Wilkinson method (14) for these substrates are 7.5 ± 0.5 μmol/min/mg and 134 ± 17 μM for **2-1**, and 4.0 ± 0.6 μmol/min/mg and 168 ± 24 μM for **2-2**. Substrates **2-1** and **2-2** were evaluated independently using the spectrophotometric method of Fitzgerald *et al.* (13). The V_{max} and K_m values obtained for these substrates are 6.8 ± 0.5 μmol/min/mg and 118 ± 20 μM for **2-1** and 6.3 ± 0.8 μmol/min/mg and 163 ± 32 μM, respectively. The kinetic parameters obtained by these two independent assays agree reasonably well and the K_m for **2-2** agrees with 220 μM reported previously (16).

The suitability of the Sep Pak assay for detecting fucosyltransferase activity present in crude extracts from pig submaxillary glands is demonstrated in Figures 2-5 to 2-8, where V_{max} and K_m are determined for compounds **2-5** to **2-8**. The enzyme activities detected with these substrates was again linear for increasing protein concentration and product formation increased linearly with time (data not shown). The products were not structurally characterized but since porcine submaxillary glands are a well known source of the α(1→2)-fucosyltransferase, this is undoubtedly the activity being detected with these synthetic substrates. Both phenyl-β-D-galactopyranoside **2-5** and the disaccharide

sequence Galβ1→3GlcNAc **2-7** are known to be substrates for this enzyme (3).

In the forgoing examples we have demonstrated that hydrophobic glycosides of appropriate carbohydrate structure are ideal as acceptors in the assay of glycosyltranferases by virtue of their unique chromatographic behaviour on reverse phase C 18 supports. The hydrophobic aglycones confer glycolipid like properties to the acceptors and products. Sep Pak C 18 cartridges have been used previously in assaying glycosyltransferases which act on isolated glycolipids (17). The examples presented here demonstrate that eight carbons in the aglycon appear to be suffficient to allow a quantitative enzyme assay. The 4-methylumbelliferyl aglycon is acceptable in the Sep Pak assay in the case of β(1→4)-galactosyltransferase, while a simple phenyl group is borderline, being sufficient in some instances but not in others. The success of the type of Sep Pak assay presented here requires each new acceptor to be evaluated with respect to the hydrophobicity of the radiolabeled product, as we have describe above. The increasing commericial availability of synthetic oligosaccharides, many of which are already attached to hydrophobic aglycones, places the scope of the Sep Pak assays beyond the synthetic chemistry laboratories. Subsequent to the publication of this paper, this method has been used to estimate the activity of many other glycosyltransferase enzymes as shown in Table 2-3, indicating that this method is a general assay.

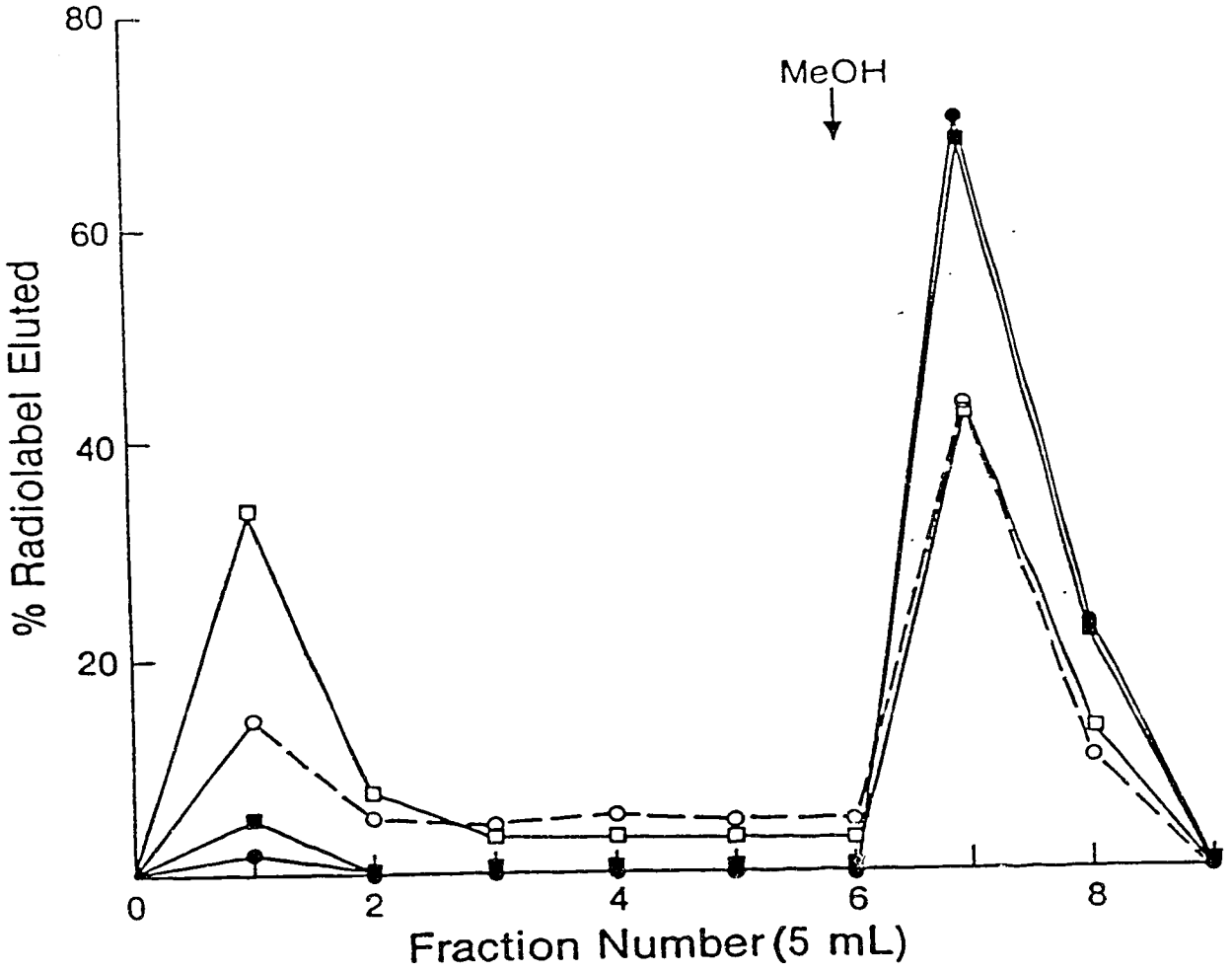
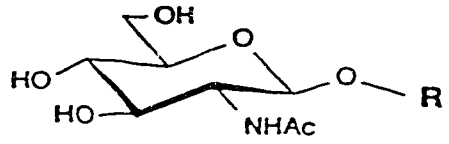


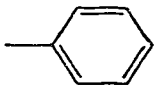
Figure 2-1. Elution profiles of radiolabeled reaction products of β(1-4)-galactosyltransferase on Sep Pak C 18 cartridge. The methanol eluants from Sep Pak eluents of preparative incubations of **2-1** (●), **2-2** (■), **2-3** (○) and **2-4** (□), as described for acceptor evaluations in the experimental sections were evaporated to dryness, dissolved in 5 mL of H_2O, and reapplied to Sep Pak cartridges.. The cartridges were washed with water, then methanol; 5 mL fractions were collected and counted. Recovery is expressed as a percentage of the amount loaded on the cartridge.

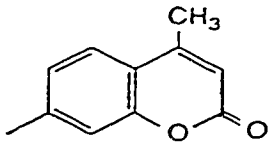
Table 2-1. Evaluation of the Suitability of Hydrophobic Glycosides in the Sep Pak Assay for β(1→4)-Galactosyltransferase.[a]

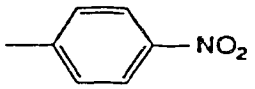
Acceptor	DPM Product Loaded	DPM H_2O Wash	DPM Methanol Eluate	% Recovery
2-1	743,235	20,100 (2.7%)	705,550 (94.9%)	97.6
2-2	531,570	41,050 (7.7%)	490,280 (92.2%)	99.9
2-3	323,740	121,840 (37.6%)	176,320 (54.5%)	92.1
2-4	352,020	188,920 (53.7%)	197,750 (56.2%)	109.9



2-1 : R= $-(CH_2)_8COOMe$

2-3 : R= 

2-2 : R= 

2-4 R= 

[a] Incubations were carried out for 3 hr as described for acceptor evaluations in the experimental procedures, and the products were isolated on Sep Pak cartridges, evaporated, dissolved in 5 mL water, and loaded onto a Sep Pak cartridge.

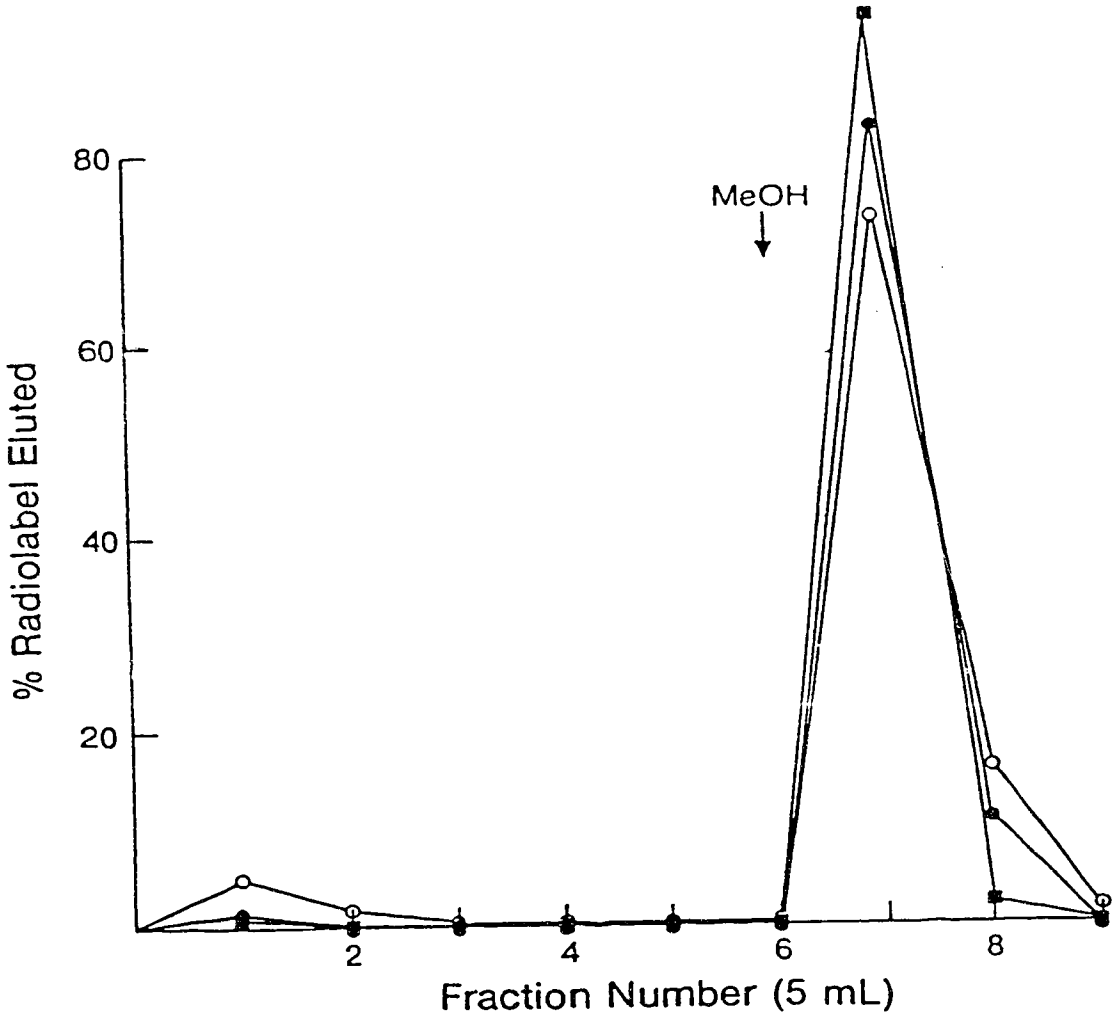
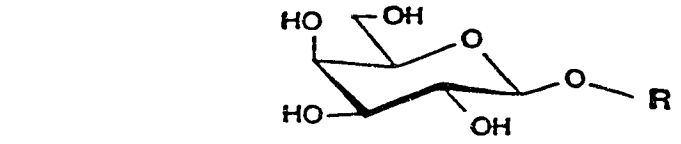
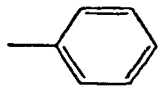


Figure 2-2. Elution profiles of radiolabeled reaction products of α(1→2)-fucosyltransferase on Sep Pak C 18 cartridge. The methanol eluants from Sep Paks of preparative incubations of **2-5** (■), **2-6** (●) and **2-7** (○)were evaporated to dryness, dissolved in 5 mL of H_2O, and reapplied to Sep Pak cartridges. The cartridges were washed with water, then methanol; 5 mL fractions were collected and counted. Recovery is expressed as a percentage of the amount loaded on the cartridge.

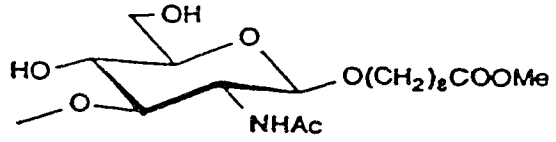
Table 2-2. Evaluation of the Suitablility of Hydrophobic Glycosides as Acceptors in the Sep Pak Assay of α(1→2)-Fucosyltransferase[a].

Acceptor	DPM Product Loaded	DPM H_2O Wash	DPM Methanol Eluate	% Recovery
2-5	21,030	570 (2.7%)	20,430 (96.4%)	99.7
2-6	13,460	500 (3.7%)	12,980 (96.4%)	100.1
2-7	36,320	3,250 (8.96%)	33,010 (90.9%)	99.9



2-5 : R= 

2-6 R= $-(CH_2)_8COOMe$

2-7 : R= 

[a] Incubations were carried out for 6 to 20 hr as described for acceptor evaluations in the experimental procedures, and the products were isolated on Sep Pak cartridges, evaporated, dissolved in 5 mL water, and loaded onto a Sep Pak cartridge.

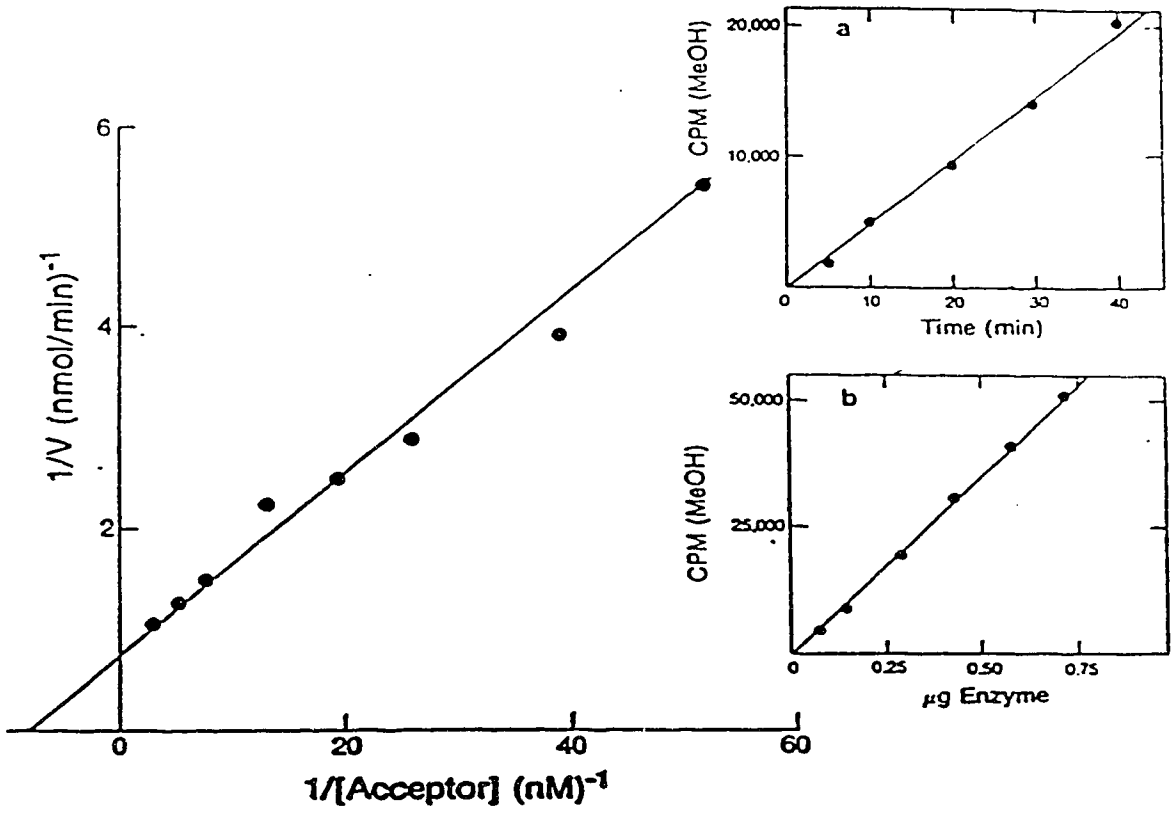


Figure 2-3. Double reciprocal plot for **2-1** as a substrate for β(1→4)-galactosyltransferase. Incubations contained 20-308 nmol **2-1**, 0.18 µg of enzyme, 100 nmol UDP-Gal, 0.4 µCi UDP-[6-^{3}H]Gal, and coupling components in a total volume of 1.07 mL, as described in the methods section. Reaction rates were determined by measuring radiolabeled product formation for 14 min at 37°C. Each point represents an average of duplicates, and the kinetic parameters, V_{max} and K_m, obtained using the Wilkinson method (14) were 1.38±0.089 nmol/min and 134±17 µM respectively.

A. Effect of incubation time on β(1→4)-galactosyltransferase activity. Incubations contained 206 nmol **2-1**, 0.12 µg of enzyme, 100 nmol UDP-Gal, 0.37 µCi UDP -[6-^{3}H] Gal, and coupling components in a total volume of 1.07 mL. The products from the reactions for the indicated period of time were isolated on Sep Pak cartridges, eluted with methanol and counted.

B. Effect of enzyme protein concentration on β(1→4)galactosyltransferase activity. Incubations contained 206 nmol **2-1**,100 nmol UDP-Gal, 0.5 µCi UDP -[6-^{3}H]Gal, coupling components, and enzyme in a total volume of 1.07 mL. The products from incubation at 37°C for 10 min for the indicated concentration of enzyme were isolated on Sep Pak cartridges, eluted with methanol, and counted.

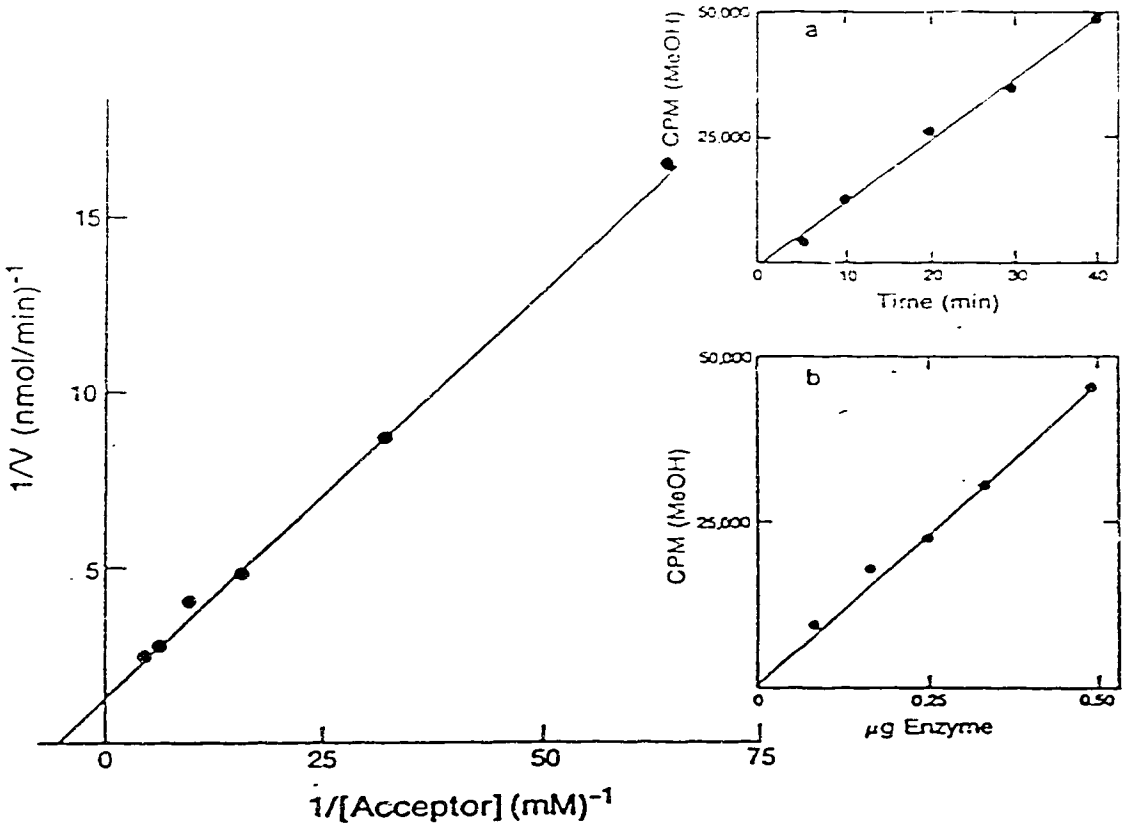


Figure 2-4. Double reciprocal plot for **2-2** as a substrate for β(1→4)-galactosyltransferase. Incubations contained 17-250 nmol **2-2**, 0.21 μg of enzyme, 100 nmol UDP-Gal, 0.56μCi UDP -[6-^{3}H]Gal and coupling components in a total volume of 1.07 mL, as described in the methods section. Reaction rates were determined by measuring radiolabeled product formation for 14 min at 37°C. Each point represents an average of duplicates, and the kinetic parameters, V_{max} and K_m, were obtained using the Wilkinson method (14) were 0.83±0.12 nmol/min and 168±24 μM respectively.

A. Effect of incubation time on β(1→4)-galactosyltransferase activity. Incubations contained 239 nmol **2-2**, 0.21 μg of enzyme, 100 nmol UDP-Gal, 0.57 μCi UDP -[6-^{3}H] Gal, and coupling components in a total volume of 1.07 mL. The products from the reactions for the indicated period of time were isolated on Sep Pak cartridges, eluted with methanol, and counted.

B. Effect of enzyme protein concentration on β(1→4)-galactosyltransferase activity. Incubations contained 239 nmol **2-2**,100 nmol UDP-Gal, 0.57 μCi UDP -[6-^{3}H]Gal, coupling components, and enzyme in a total volume of 1.07 mL. The products from incubation at 37°C for 10 min for the indicated concentration of enzyme were isolated on Sep Pak cartridges, eluted with methanol, and counted.

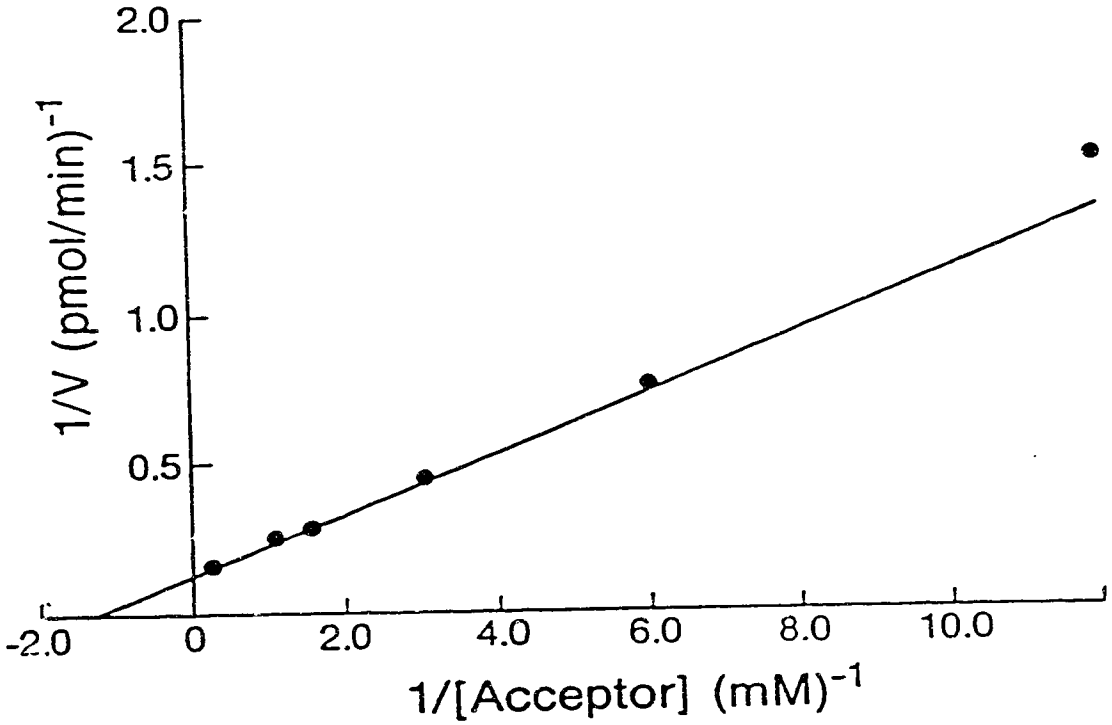


Figure 2-5. Double reciprocal plot of **2-5** as an acceptor for α(1→2)-fucosyltransferase. Incubations contained in 0.62 mL: 0.39-7.4 μmol of **2-5**, 9 nmol GDP-fucose, 0.01 μCi GDP-[U-^{14}C]fucose, an extract of fucosyltransferase containing 14 mg of protein and buffer components as described in the methods section. Reaction rates were determined by measuring product formation for 1 hr at 37°C. The kinetic parameters V_{max} and K_m obtained using the Wilkinson method (14) were 2.6±0.1 pmol/min and 4.4±0.4 mM respectively.

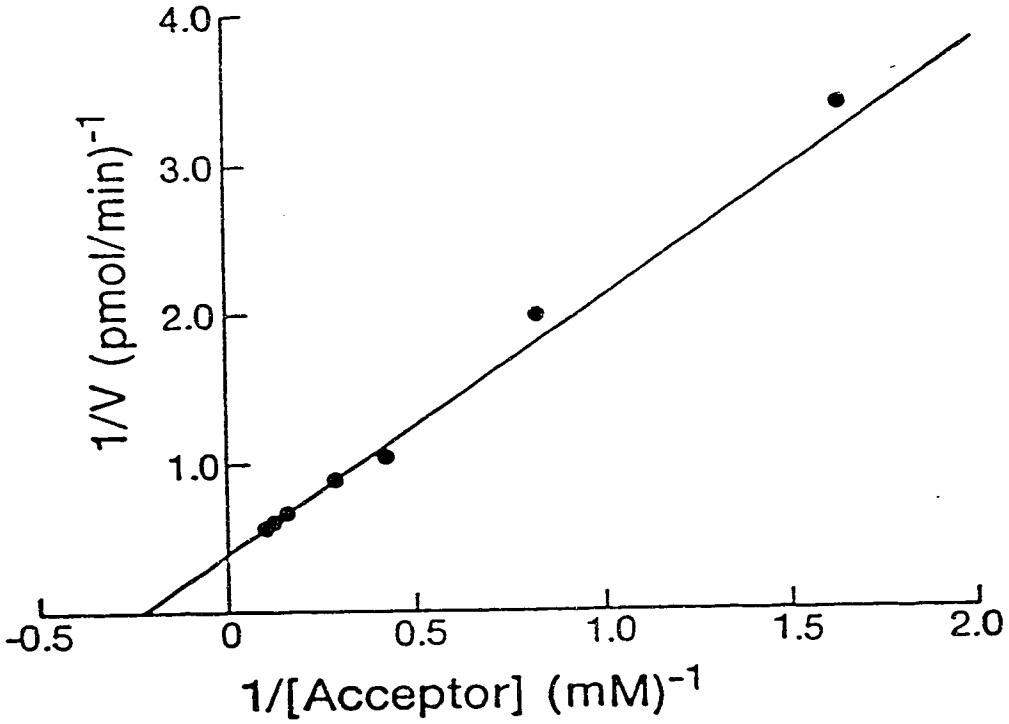


Figure 2-6. Double reciprocal plot of **2-6** as an acceptor for α(1→2)-fucosyltransferase. Incubations contained in 0.62 mL: 50-360 nmol of **2-6**, 9 nmol GDP-fucose, 0.01 μCi GDP-[U-^{14}C]fucose, an extract of fucosyltransferase containing 14 mg of protein and buffer components as described in the methods section. Reaction rates were determined by measuring product formation for 1 hr at 37°C. The kinetic parameters V_{max} and K_m obtained using the Wilkinson method (14) were 8.0±0.1 pmol/min and 0.82±0.03 mM respectively.

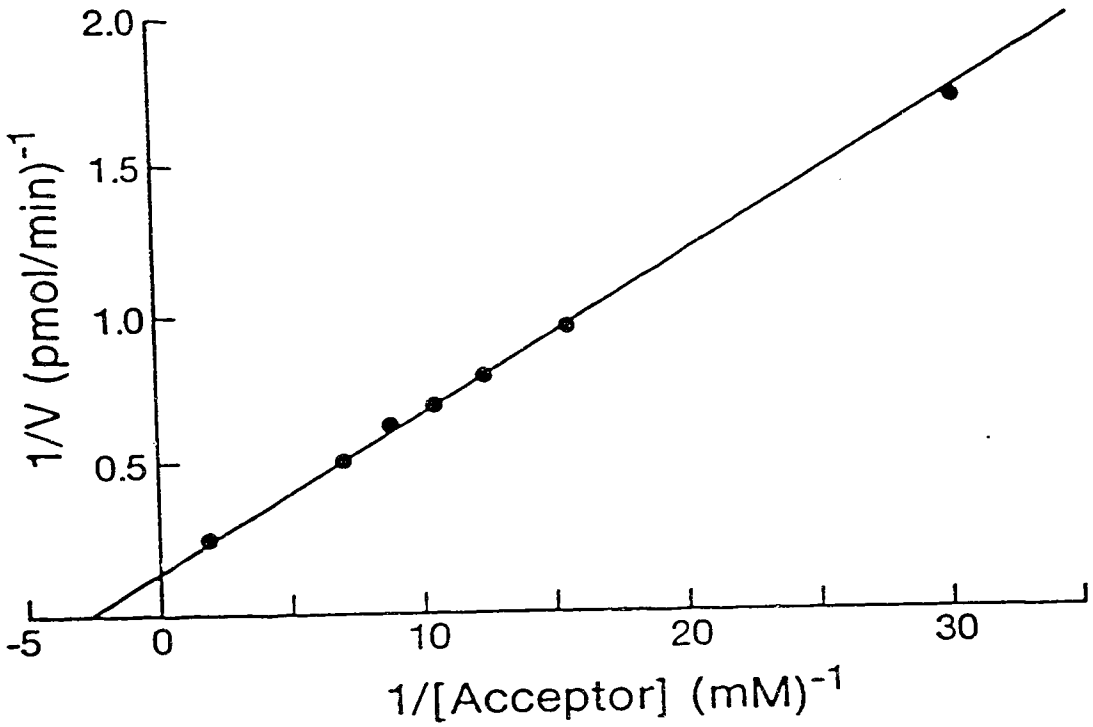


Figure 2-7. Double reciprocal plot of **2-7** as an acceptor for α(1→2)-fucosyltransferase. Incubations contained in 0.62 mL: 20-360 nmol of **2-7**, 9 nmol GDP-fucose, 0.01 μCi GDP-[U-^{14}C]fucose, an extract of fucosyltransferase containing 14 mg of protein and buffer components as described in the methods section. Reaction rates were determined by measuring product formation for 1 hr at 37°C. The kinetic parameters V_{max} and K_m obtained using the Wilkinson method (14) were 7.2±0.1 pmol/min and 0.509±0.008 mM respectively.

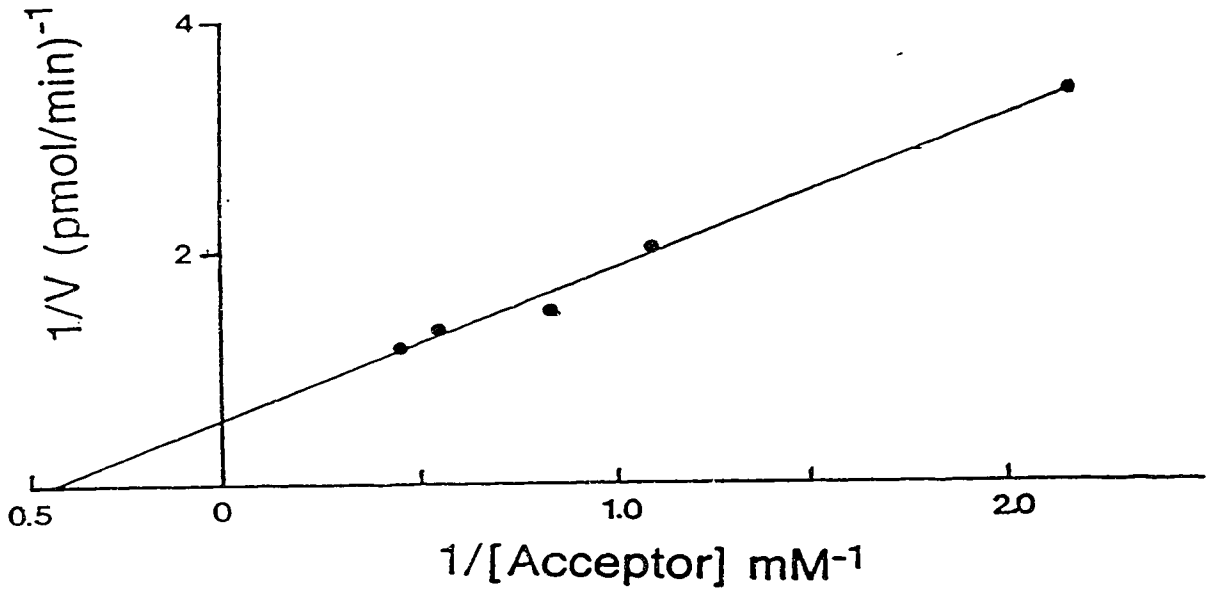


Figure 2-8. Double reciprocal plot of **2-8** as an acceptor for α(1→2)-fucosyltransferase. Incubations contained in 0.62 mL: 0.14-1.4 μmol of **2-8**, 9 nmol GDP-fucose, 0.01 μCi GDP-[U-^{14}C]fucose, an extract of fucosyltransferase containing 14 mg of protein and buffer components as described in the methods section. Reaction rates were determined by measuring product formation for 1 hr at 37°C. The kinetic parameters V_{max} and K_m obtained using the Wilkinson method (14) were 1.6±.07 pmol/min and 2.2±.2 mM respectively.

Table 2-3: Other Transferases Assayed by the Sep Pak Method

Transferase Enzyme	Source	Reference
α(1→3/4)-fucosyltransferase	human milk	18
α(1→4)-fucosyltransferase	mung beans	19
α(1→2)-fucosyltransferase	human serum	20
α(1→3)-fucosyltransferase	human serum	20
α(2→3)-sialyltransferase	porcine submaxillary glands	18
N -acetylglucosaminyl-transferase I	rabbit liver	21
N -acetylglucosaminyl-transferase V	baby hamster kidneys	21-23

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3. A Bisubstrate Analog Inhibitor for α(1→2)-Fucosyltransferase*

It is now well recognized that the complex carbohydrates of mammalian cell surface glycoproteins and glycolipids can play important roles in a variety of physiological processes critical to either the maintenance of health or the establishment of disease. Important examples of oligosaccharides as acceptors in cell adhesion are found in neuronal development (1,2), sperm egg binding and fertilization (3,4), and in the homing of lymphocytes (5,6). Cell surface carbohydrates have been found to modulate growth factor receptor activity (7,8) as well as to provide sites of attachment necessary for both bacterial (9,10) and viral infection (11,12). Alterations in the structures of cell surface carbohydrates are known to accompany normal cellular development during embryogenesis and cell differentiation, as well as abnormal development in the course of tumor progression (13-16). The new structures that appear at the cell surface during these processes have been referred to as developmental, stage specific and tumor associated antigens. Their role in cellular development is under intense investigation but remains poorly understood.

Inhibitors of glycosylation have been invaluable tools both in unravelling the biosynthesis of *N*-linked oligosaccharides and in studying the biological function of these molecules (17,18). The most commonly used *N*-linked glycosylation inhibitors described are of two classes: 1) tunicamycin (and related compounds) which inhibit the UDP-GlcNAc:dolichol phosphate GlcNAc-phosphotransferase (E.C. 2.7.8.15) essential for all *N*-linked glycosylation and 2) a series of glycosidase inhibitors, including the alkaloids swainsonine and castanospermine, which interfere with the so called "trimming" reactions of high-mannose oligosaccharides (18). Both classes of inhibitors have been shown to cause changes in the structures of cell surface *N*-linked oligosaccharides resulting in modified cell social behaviour including metastatic potential (18-20). These inhibitors do not, however, directly affect the biosynthesis of either *O*-linked oligosaccharides or glycolipids since neither the dolichol pathway nor glycosidases appear to be involved in the biosynthesis of these latter classes of structures.

* Palcic, M.M., Heerze, L.D., Srivastava, O.P., and Hindsgaul, O. (1989) *J. Biol. Chem.* **264**, 17174-17181.
Dr. O.Hindsgaul's group from the Department of Chemistry, University of Alberta was responsible for the synthesis of the bisubstrate analog.

The biosynthesis of complex carbohydrates requires the concerted action of a large number of glycosyltransferases which transfer single sugar residues from their activated glycosyl phosphate derivatives (usually sugar nucleotides) to specific acceptor oligosaccharides (21). Clearly, specific inhibitors of glycosyltransferases, of which there are no examples, could be of great value in the study of both biosynthesis and function of complex carbohydrate chains. Recent efforts at producing such inhibitors have concentrated on the preparation of unreactive sugar nucleotide analogs (21-25). While such analogs will indeed prove valuable in the study of the mechanism of action of glycosyltransferases, it is improbable that this approach can provide specific glycosyltransferase inhibitors since any given sugar nucleotide can be utilized by many, often competing, glycosyltransferases (21,26). It seems likely that the origin of specificity of glycosyltransferases arises from their ability to recognize the oligosaccharide acceptor in addition to the sugar nucleotide donor. In order to be specific, a glycosyltransferase inhibitor should therefore reflect some molecular structure in common with the natural acceptor oligosaccharide.

We report here the potential of a mechanism based approach to the preparation of specific glycosyltransferase inhibitors. This approach involves the covalent attachment of an acceptor specific for a glycosyltransferase to the nucleotide portion of the donor sugar nucleotide through the acceptor hydroxyl group to which the enzyme normally transfers a glycosyl residue. The resulting "bisubstrate analog" (27) then contains structural elements of both the donor and the acceptor components of the reaction and may therefore show the corresponding specificity as a glycosyltransferase inhibitor. The enzyme chosen to evaluate this inhibition strategy was a β-galactoside α(1→2)-fucosyltransferase which has been purified from porcine submaxillary glands (28). This particular glycosyltransferase was selected because it accepts the simple phenyl β-D-galactopyranoside as a substrate, a molecule that could be readily chemically manipulated.

Experimental Procedures

Materials

Porcine submaxillary glands were from Pel-Freeze Biologicals, Inc. Sep Pak C 18 reverse phase cartridges were from Waters Asociates. SP-Sephadex C-50, Sepharose 4B, and PD-10 columns were from Pharmacia. Guanosine diphospho-L-[U-^{14}C] fucose (248 mCi/mmol) was from New England Nuclear and unlabeled GDP-fucose was

synthesized by Dr. O. Hindsgaul's group as previously described (37). Aqueous scintillation cocktail was from Amersham. Triton X-100, phenyl-β-D-galactopyranoside, GMP-morpholidate, and N-iodosuccinamide were from Sigma Chemical CO. 8-Methoxycarbonyloctyl-2-acetamido-2-deoxy-3-O-β-D-glucopyranoside (38) was a generous gift from Chembiomed Ltd. Solvents and all buffer components were reagent grade. A kit for determining protein concentrations, based on the method of Bradford (39), was obtained from Bio Rad Laboratories.

Enzyme Preparation

The soluble and membrane bound forms of β-galactoside α(1→2)-fucosyltransferase were purified from porcine submaxillary glands using slight modifications of methods described previously (28). The soluble form of α(1→2)-fucosyltransferase was obtained by repeated homogenization of the ground submaxillary glands in 20 mM Tris buffer at pH 7.0 containing 2 mM NaN_3 (Buffer A). The crude extract was passed trough SP-Sephadex C-50 that had been equilibrated in Buffer A. The enzyme containing fractions were affinity purified using GDP-hexanolamine Sepharose (4 μmol ligand/ mL gel) (40) by eluting the enzyme activity with Buffer A containing 5 mM GMP and 10% glycerol. The eluate was immediatly brought to 0.17 mg/mL in BSA to stabilize the enzyme activity, and desalted on PD-10 columns equlibriated in buffer A containing 10% glycerol and concentrated by ultrafiltration. The soluble form of α(1→2)-fucosyltransferase was purified 49,000 fold and had a specific activity of 44 munits/mg protein.

The membrane bound form of α(1→2)-fucosyltransferase was isolated from a Triton X-100 extract as previously reported (28). The Triton extract was obtained by stirring the crude porcine submaxillary gland homogenate in 1% (w/v) Triton X-100 in Buffer A. This was immediately passed through SP-Sephadex and purified on GDP-hexanolamine by non specific desorption using NaCl elution and by specific desorption using GMP. The membrane bound form of α(1→2)-fucosyltransferase was purified 45,000 fold and had a specific activity of 66.4 munits/mg protein. Enzyme activity was determined as described below and protein was measured by the Bradford assay using BSA as a standard. One unit is defined as 1 μmol product formed/ min at 37°C.

Fucosyltransferase Assays

During purification - For routine assays during purification, reaction mixtures contained in 65μL, 3.57 mM phenyl-β-D-galactopyranoside, 25 μM GDP-fucose, 7000 dpm GDP-[U-^{14}C]fucose, 2.85 mM $MnCl_2$, 2.85 mM ATP and enzyme in Buffer A.

After incubation at 37°C for 1-2 hrs incubation mixtures were diluted with 1 mL of H_2O and immediately placed at 4°C. Reaction mixtures were loaded onto preconditioned Sep Pak cartridges (41) and unreacted GDP-fucose was eluted until background counts were obtained by washing with H_2O (30 mL). Radiolabeled product was eluted with methanol (2 x 5 mL) and quantitated as dpm in 10 mL of scintillation cocktail in a Beckman LS 1801 scintillation counter.

Inhibition Kinetics

Initial rate data were obtained in the presence and absence of inhibitor using a fixed and saturating level of one substrate while varying the concentration of the second substrate at 5 different levels. Two to three concentrations of inhibitor were used and each point represents an average of at least two determinations. Under the conditions described above, no more than 15% turnover occurred to ensure initial rate conditions. The kinetic parameters, V_{max} and K_m for the primary data were estimated using a computer program based on the method of Wilkinson (42). The true kinetic and inhibition constants were obtained by plotting the slope, intercepts, and K_m(app) versus the inhibitor concentration. The data for the secondary plots were fit to the Line program of Cleland (43).

Inhibition kinetics at saturating concentrations of GDP-fucose- Incubation mixtures in 65 µL contained 64 µM GDP-fucose, 30,000 dpm GDP-[U-^{14}C]fucose, 1.54-6.15 mM phenyl-β-D-galactopyranoside or 0.174-0.625 mM βGal(1→3)βGlcNAcO$(CH_2)_8$COOMe, 2.85 mM $MnCl_2$, 2.85 mM ATP, and fucosyltransferase (11µunits) in Buffer A. After incubation at 37°C for 1 hr incubation mixtures were diluted with 1 mL of H_2O and immediately placed at 4°C. No more than 10 assays were done at one time. Radiolabeled reaction products were separated on Sep Pak cartridges and quantitated as described above (41).

Inhibition kinetics at saturating concentrations of acceptor - Incubations were carried out in an analogous manner, but contained varying concentrations of GDP-fucose 5.11- 25.54 µM, 30,000 (membrane bound) and 60,000 (soluble form) dpm of GDP-[U-^{14}C]fucose. The concentation of acceptor was held constant at 25 (for membrane form) and 80 (for soluble form) mM, respectively, and 3.1 µunits of fucosyltranferase was used. After reaction for 1 hour at 37°C, the reaction product was isolated as before.

Chemical Synthesis of 3-1 and 3-2 - Compounds **3-1** and **3-2** were synthesized by Dr. O.P. Srivastava of Dr. O. Hindsgaul's research group as outlined in Scheme 3-1 and described in the original manuscript.

Results and Discussion

The porcine submaxillary α(1→2)-fucosyltransferase transfers a fucosyl residue from GDP-fucose to the 2-OH group of non reducing terminal β-Gal residues (28,29). The best disaccharide acceptor reported for this enzyme is βGal(1→3)GalNAc (K_m = 0.28 mM) but both βGal(1→3)GlcNAc (K_m = 0.63 mM) and βGal(1→4)GlcNAc (K_m = 2.11 mM), as well as more complex oligosaccharides, are substrates (29). The ability of the porcine submaxillary α(1→2)-fucosyltransferase to utilize the simple substrate phenyl β-D-galactopyranside as an acceptor, as is also the case for other α(1→2)-fucosyltransferases (30), made this enzyme an ideal target for the evaluation of a bisubstrate analog inhibition strategy for glycosyltransferases. This was because the synthetic chemistry envisaged for the preparation of a bisubstrate analog had little precedent and might have been substantially more difficult if an acceptor of the complexity of a disaccharide or polysaccharide had been required.

Very little is known about the detailed mechanism of glycosyl transfer by the α(1→2)-fucosyltransferase. Glycosylation occurs with inversion of configuration at the anomeric carbon of the fucosyl residue, which is in the β configuration in the sugar nucleotide, to produce the α–Fuc(1→2)βGal terminating product. The enzyme requires Mn^{2+} for full activity and evidence has been presented that the reaction may proceed by a random mechanism, such that either GDP-fucose or acceptor can bind first to the enzyme (29). There is no evidence for any covalent fucosyl enzyme intermediate nor for multiple inversions. These considerations led us to the view that the glycosyl transfer might occur by a simple ion pair mechanism as shown in Scheme 3-2, such a mechanism having strong analogy in the solution chemistry of glycosylating agents (31). While there is no direct experimental evidence for this mechanism, a key feature of Scheme 3-2 is that O-2 of the acceptor galactosyl-residue and the terminal phosphorous atom of the GDP-fucose would be separated by only about 3 covalent bond lengths in the transition state of such a concerted reaction. Both the galactosyl residue and the GDP moiety would be bound at the same time, although their relative orientations could not be predicted from this model. The stable bisubstrate analog **3-1** was therefore chemically synthesized and kinetically

evaluated as a potential inhibitor of the α(1→2)-fucosyltransferase. Compound **3-1** attaches the terminal GDP phosphorus to O-2 of the galactosyl residue through a flexible 3 bond ethylene bridge which, it was hoped, might then allow the proper orientation of both moieties in the enzyme combining site.

The behaviour of inhibitor **3-1** was first examined using the membrane bound form of the porcine submaxillary α(1→2)-fucosyltransferase which was purified 45,000 fold to a specific activity of 66 munit/mg according to reported procedures (28). The apparent K_m's of this enzyme for phenyl β-D-galactopyranoside and GDP-fucose were determined to be 2.6 mM (Figure 3-1) and 7.3 μM (Figure 3-2), respectively. Figure 3-1 summarizes the effect of inhibitor concentration of the rate of enzymatic fucosylation in the presence of saturating GDP-fucose, and Figure 3-2 in the presence of saturating phenyl β-D-galactopyranoside. The mode of inhibiton is clearly seen to be competitive with respect to both GDP-fucose and the acceptor with K_i's of 16 μM and 2.3 μM, respectively. These data provide firm evidence that the inhibitor **3-1** can occupy both the GDP and the phenyl β-D-galactopyranoside binding sites, as required for a true bisubstrate analog (27). Furthermore, the mechanism of the enzyme reaction is thereby established to be random order addition of either donor or acceptor, since these dual competitive inhibition patterns are consistent only with a random kinetic mechanism (32). Since **3-1** binds to the enzyme at both of the substrate combining sites the measured K_i need not be the true dissociation constant for the enzyme bisubstrate complex (33). This may explain the differences in the inhibition constants obtained at a single saturating concentration of one substrate while varying the other. Alternatively, the high acceptor concentrations (25 mM) used in the experiments summarized in Figure 3-1a may result in partial substrate inhibtion, evidenced by the decreased values of V_{max} obtained from these plots. Regardless of the reasons for the diffence between the K_i values obtained, the kinetic behaviour of inhibitor **3-1** shows it to be a classical bisubstrate analog reflecting both the acceptor and donor recognition properties of the fucosyltransferase.

Further experiments were performed in order to evaluate the contributions to inhibition of the individual portions of the bisubstrate analog **3-1**. Since the K_i for GDP is 8.7 μM (data not shown), and that for GMP is 22 μM (29), the binding energy of the GDP portion appears to be fully realized in the bisubstrate analog. The synthetic

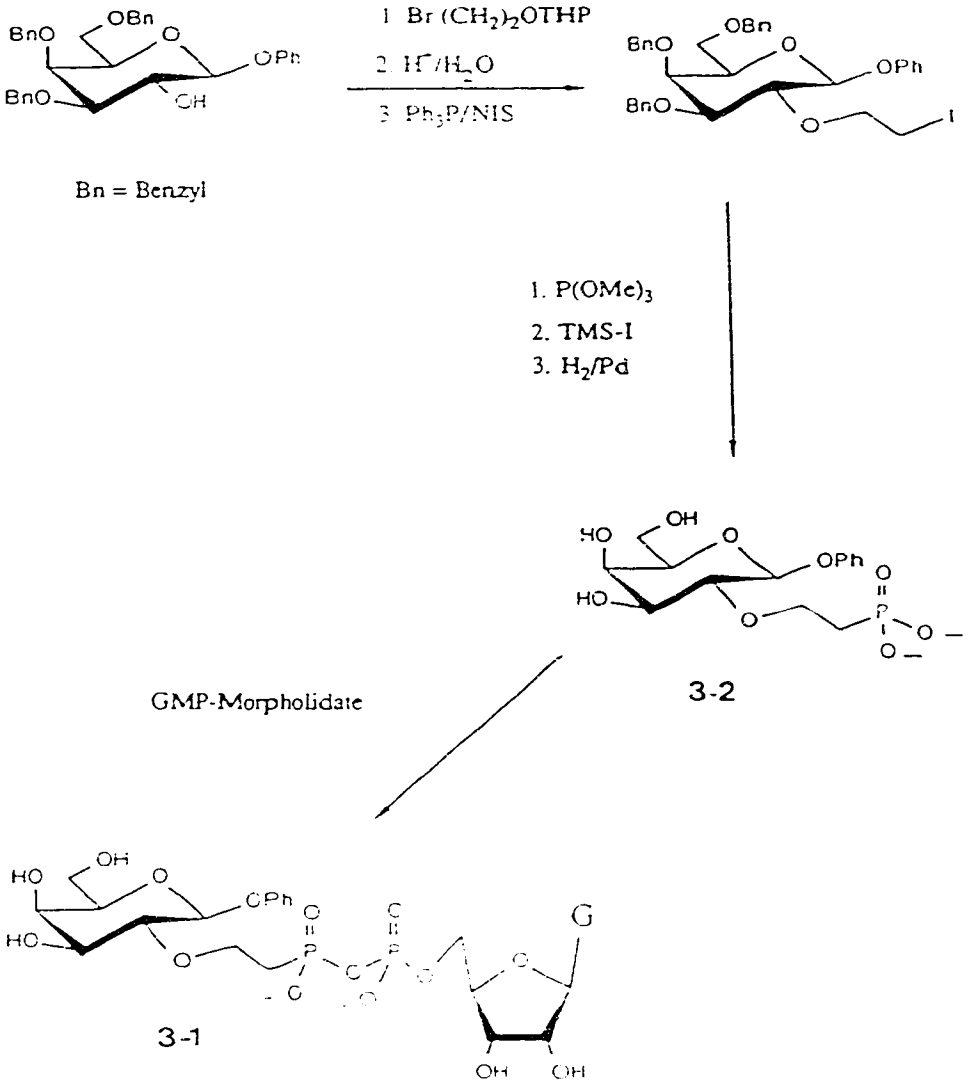
2-*O*-(2-phosphonoethyl)-β-D-galactopyranoside **3-2**, which lacks the guanosine phosphate moiety of **3-1**, was found to be a competitive inhibitor with respect to the acceptor (K_i = 133 μM, Figure 3-3) and a mixed inhibitor with respect to GDP-fucose (K_i= 760 μM, Figure 3-4). The binding properties of the phenyl β-D-galactopyranoside portion of **3-1** are therefore concluded to be strongly dependent on recognition of the GDP moiety by the enzyme since the K_i of **3-2** was increased over 50 fold compared to **3-1**. The mixed inhibition observed against GDP-fucose is expected for a random binding mechanism (34).

The possibility that the hydrophobic phenyl group of **3-1** was causing an artifact in the above experiments by binding to the enzyme in a site distinct from that of the natural oligosaccharide acceptors was investigated by examining the ability of **3-1** to inhibit the fucosylation of the Type 1 structure βGal(1→3)βGlcNAcO$(CH_2)_8$COOMe (**3-3**). The K_m for this acceptor was determined to be 200 μM (Figure 3-5a). Compound **3-1** was found to be a competitive inhibitor of the fucosylation of **3-3** with a K_i of 4.4 μM (Figure 3-5c). The phenyl β-D-galactopyranoside portion of **3-1** is thereby concluded to bind to the normal acceptor oligosaccharide domain of the α(1→2)-fucosyltransferase.

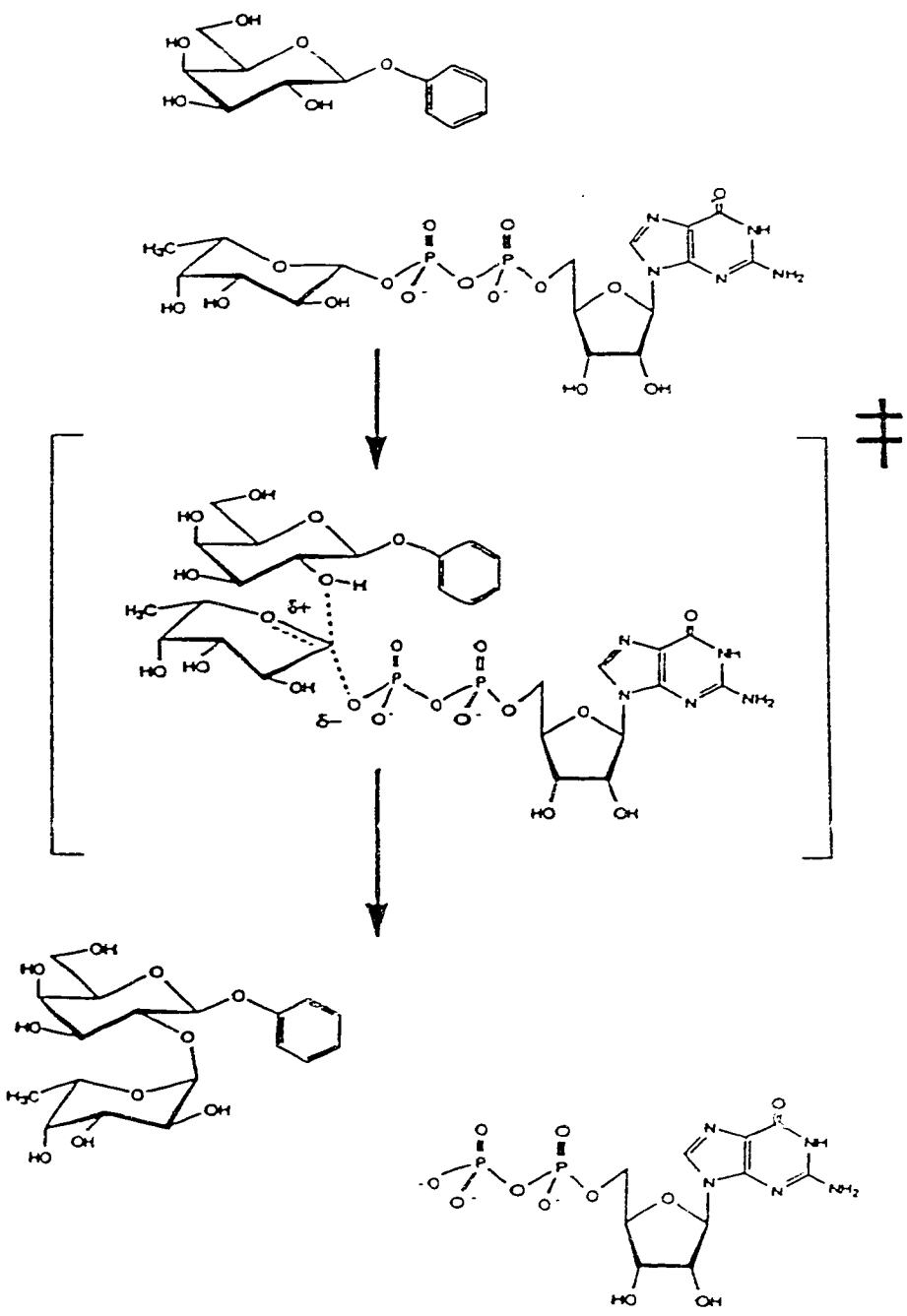
The bisubstrate analog was also found to be an effective inhibitor of the soluble form of α(1→2)-fucosyltransferase isolated from the same porcine submaxillary glands (28). Inhibition was competitive with respect to both GDP-fucose and phenyl β-D-galactopyranoside (Figure 3-6,3-7) with K_i's of 15 μM and 4 μM, respectively. The K_m's for GDP-fucose and phenyl β-D-galactopyranoside were 7 μM and 3 mM respectively for the soluble enzyme (Table 3-1). The similarity in these kinetic constants for the membrane bound and soluble α(1→2)-fucosyltransferase suggest a close relationship for the different forms.

The key kinetic parameters obtained from the experimental data presented in Figures 3-1 to 3-7 are summarized in Table 3-1. The kinetic behaviour of **3-1** shows it to exhibit the characteristics of a classic bisubstrate analog, with a K_i in the micromolar range. Compound **3-1** is the first example of a mechanism based glycosyltransferase inhibitor, and it utilizes both the acceptor and donor recognition potential of the enzyme active site. The possibility of producing specific glycosyltransferase inhibitors by way of a bisubstrate

analog strategy is therefore clearly indicated. The activity of compound **3-1** against fucosyltransferases with acceptor specificities different from that of the α(1→2) enzyme described above is currently under study. Bisubstrate analogs are under preparation for the α(1→3) (35,36) and α(1→3/4) Lewis fucosyltransferases (35) as well as for the *N*-acetylglucosaminyltransferases which control the branching of *N*-linked oligosaccharides (26).



Scheme 3-1. Chemical Synthesis of Compounds 3-1 and 3-2



Scheme 3-2. Postulated transition state for the fucosylation of phenyl β-D-galacto pyranoside catalyzed by the α(1→2)-fucosyltransferase.

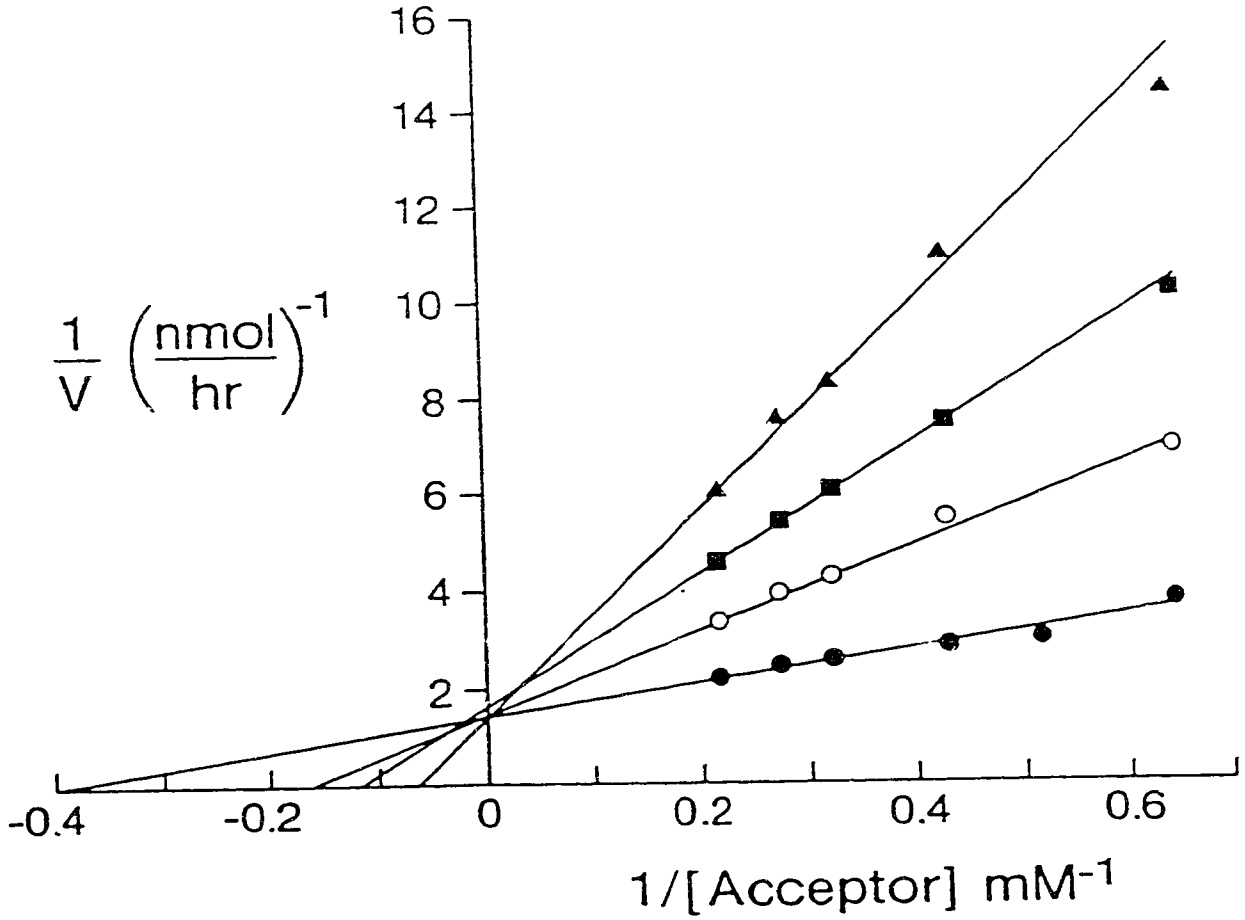


Figure 3-1. Competitive inhibition of membrane bound α(1→2)-fucosyltransferase using phenyl β-D-galactopyranoside as the variable substrate with increasing concentrations of the bisubstrate analog **3-1**. The concentration of GDP-fucose was 64 μM. The concentrations of inhibitor **3-1** were 0 μM (●), 2.7 μM (○), 5.3 μM (■) and 10.7 μM (▲). The points in the reciprocal plots are the experimental values, while the lines are calculated from the fit of the data using the Wilkinson method (42).

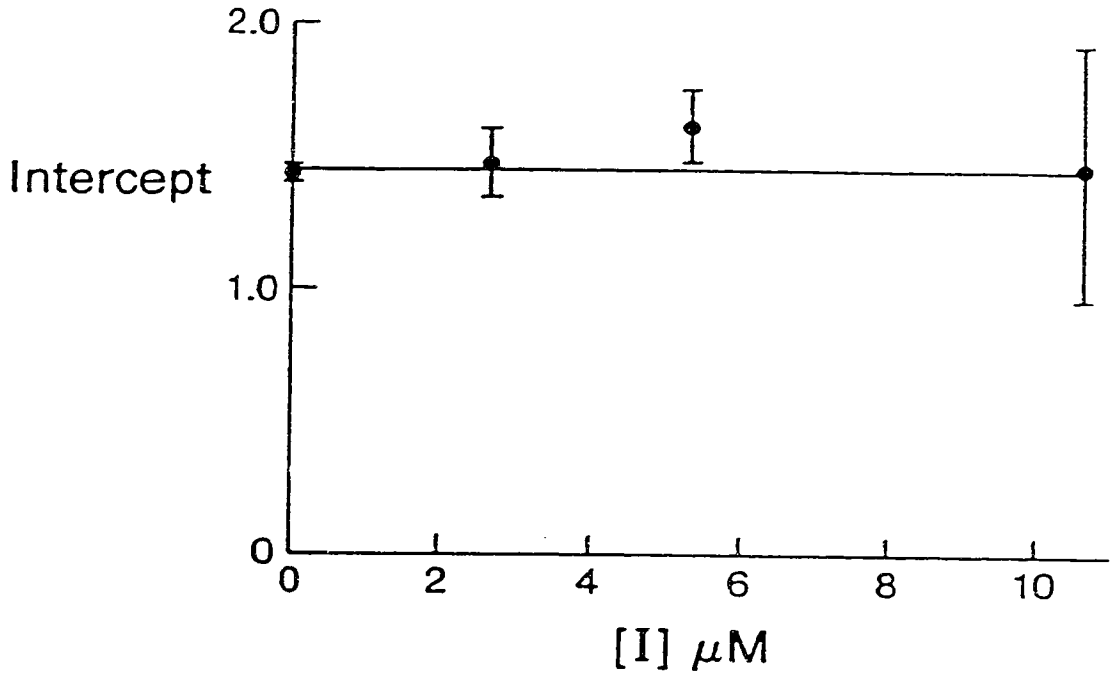


Figure 3-1a. Replot of the intercepts obtained from the data of Figure 3-1. The invariance (within experimental error) is consistent with a competitive inhibitor.

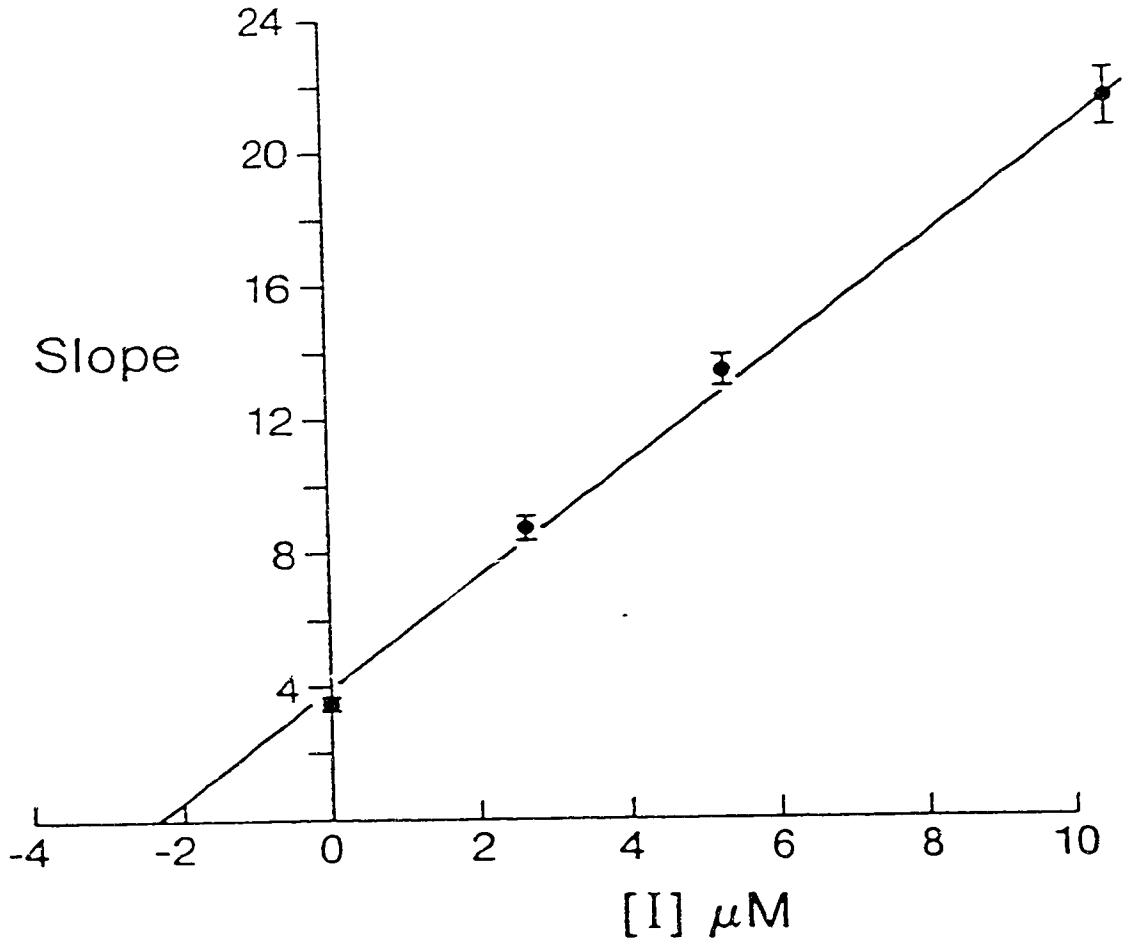


Figure 3-1b. Replot of the slopes (arbitrary units) obtained by analysis of the data in Figure 3-1. A K_i of 2.4±0.3μM was obtained from fits of the data using the LINE program of Cleland (43). Analysis of K_m(app) vs [3-1] gave a K_i of 2.2± 0.2μM (plot not shown).

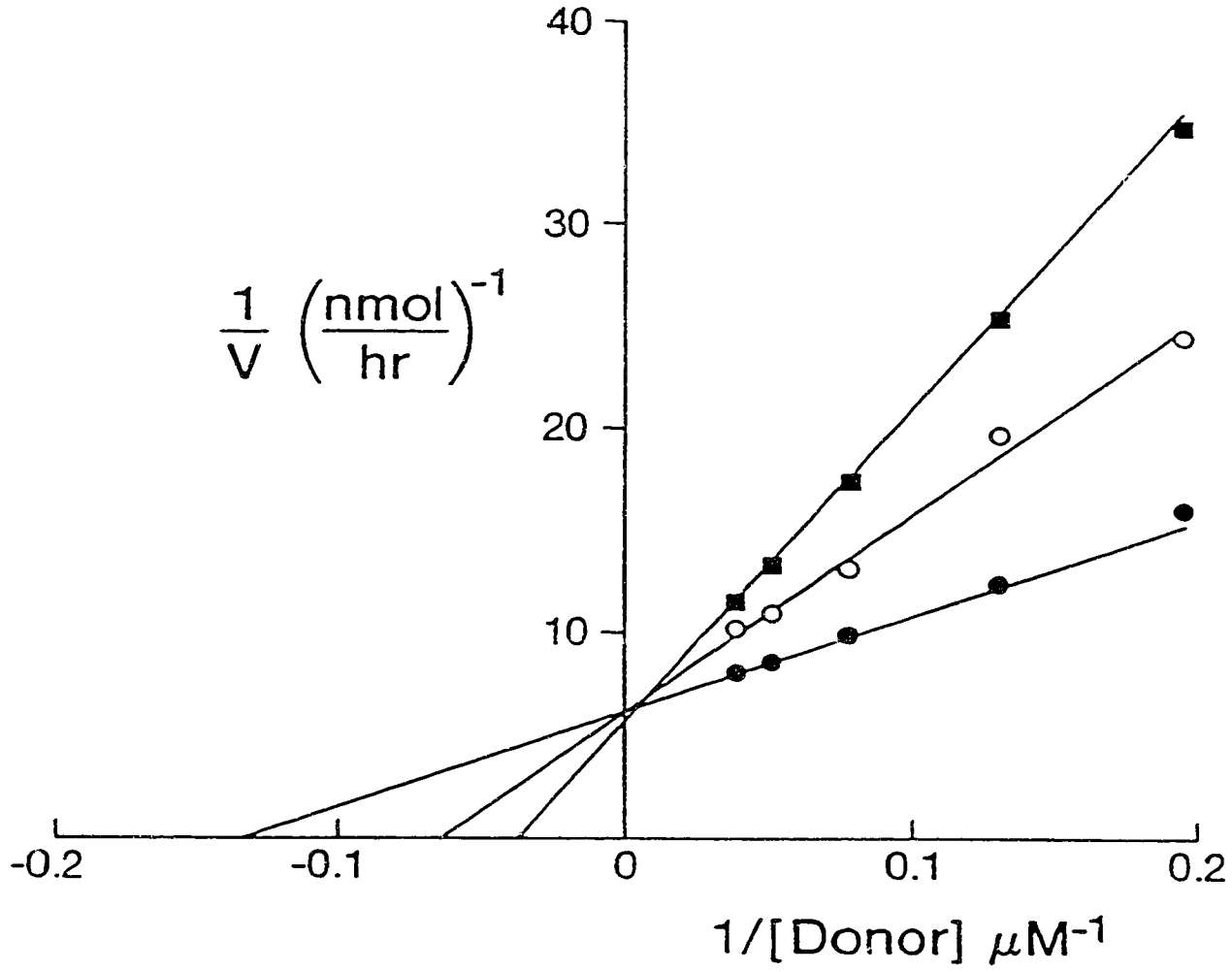


Fig 3-2. Competitive inhibition of membrane bound α(1→2)-fucosyltransferase using GDP-fucose as the variable substrate with increasing concentrations of the bisubstrate analog **3-1**. The concentration of phenyl β-D-galactopyranoside was 25 mM. The concentrations of inhibitor **3-1** were 0 μM (●), 21.3 μM (○) and 42.6 μM (■). The points in the reciprocal plots are the experimental values, while the lines are calculated from the fit of the data using the Wilkinson method (42).

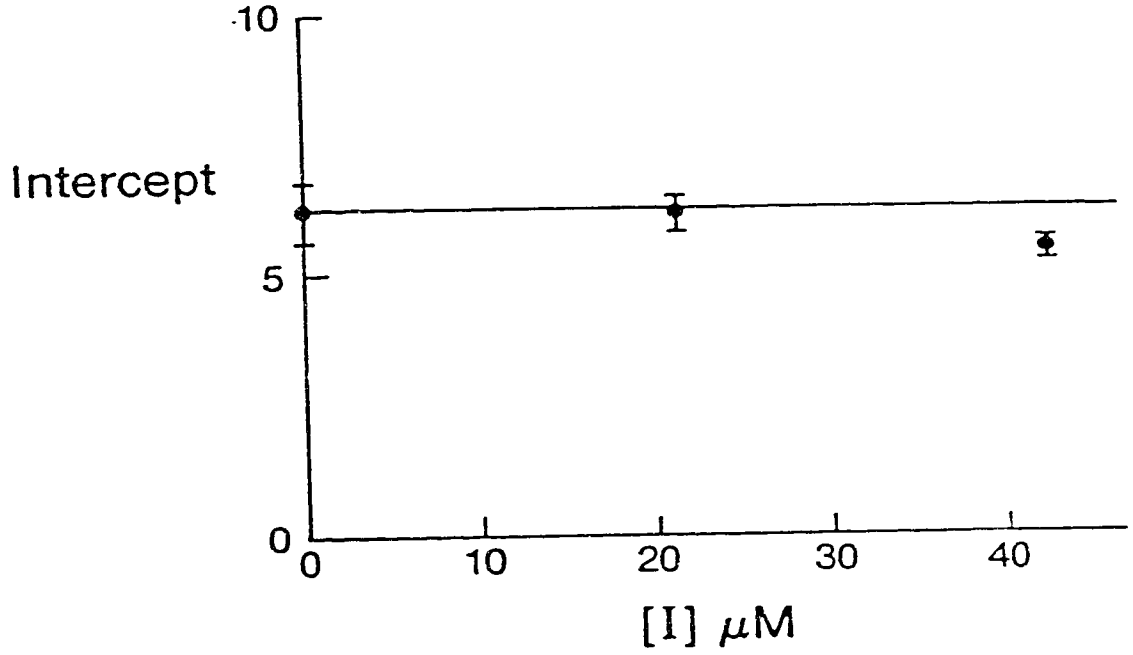


Figure 3-2a. Replot of the intercepts obtained from the data of Figure 3-2. The invariance is consistent with a competitive inhibitor.

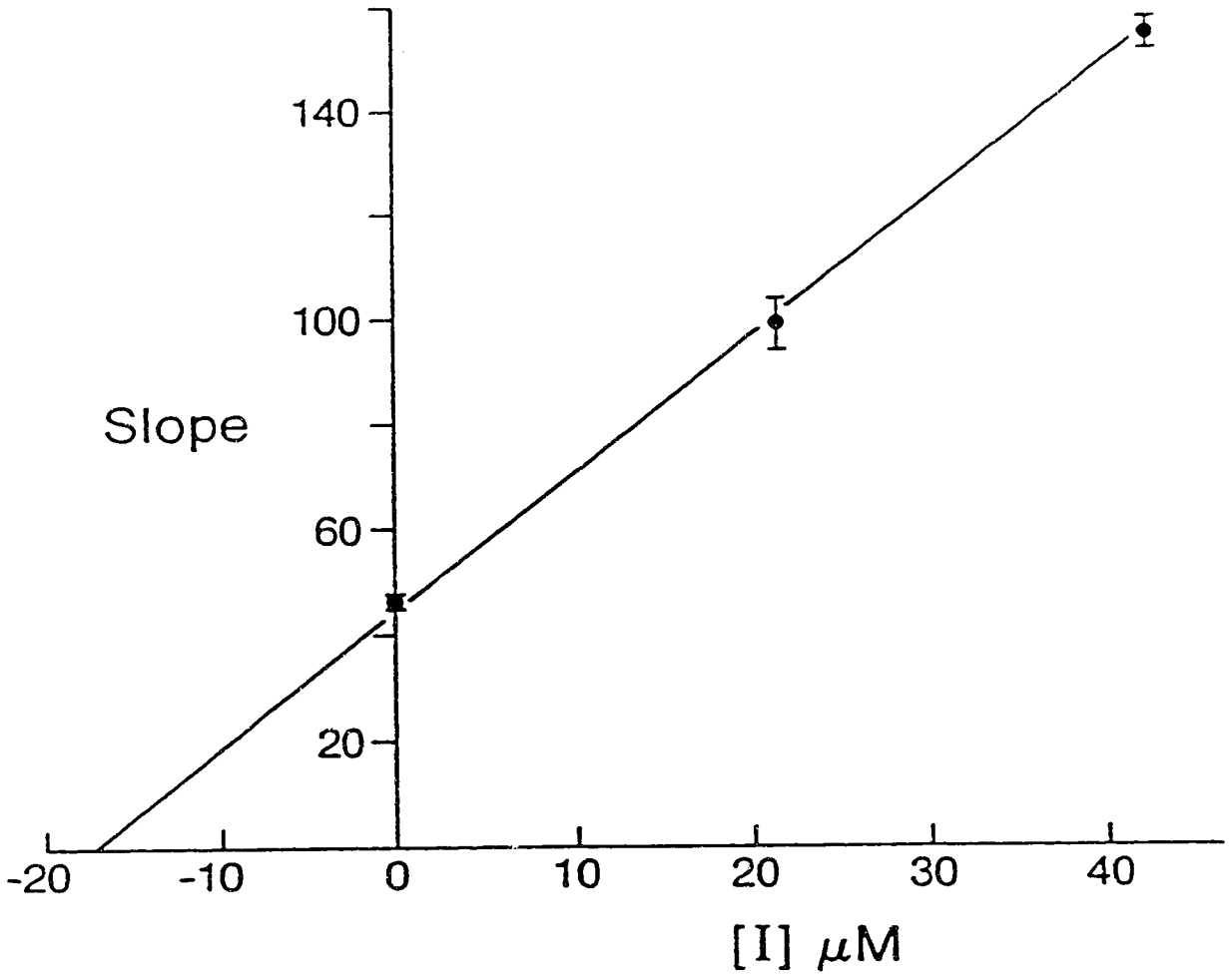


Figure 3-2b. Replot of the slopes (arbitrary units) obtained from Figure 3-2. A K_i of 18 ± 1 μM was obtained using the LINE program of Cleland (43). Analysis of K_m (app) vs [3-1] gave a K_i of 14 ± 4 μM (plot not shown).

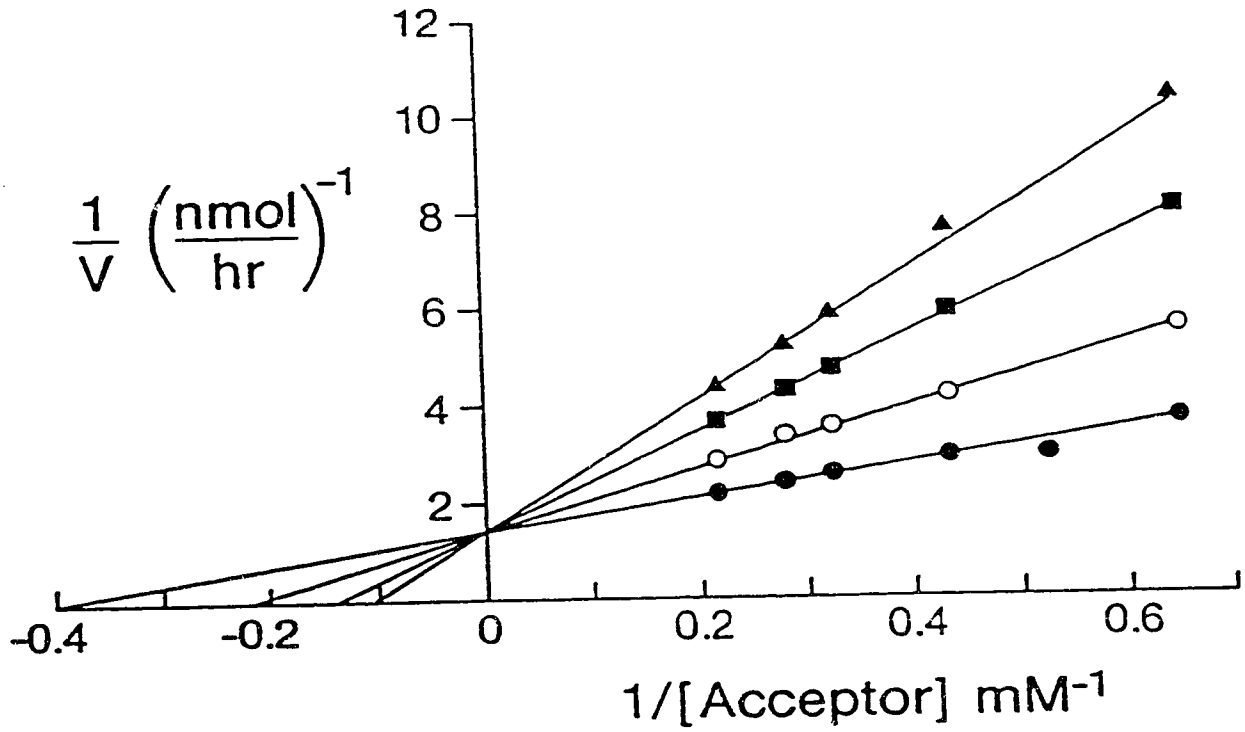


Figure 3-3. Competitive inhibition of membrane bouund α(1→2)-fucosyltranferase using phenyl β-D-galactopyranoside as the variable substrate with increasing concentrations of the phosphonate analog 3-2. The concentration of GDP-fucose was 64 μM. The concentrations of inhibitor 3-2 were 0 μM (●), 132 μM (○), 264 μM (■) and 395 μM (▲). The points in the reciprocal plots are the experimental values, while the lines are calculated from the fit of the data using the Wilkinson method (42).

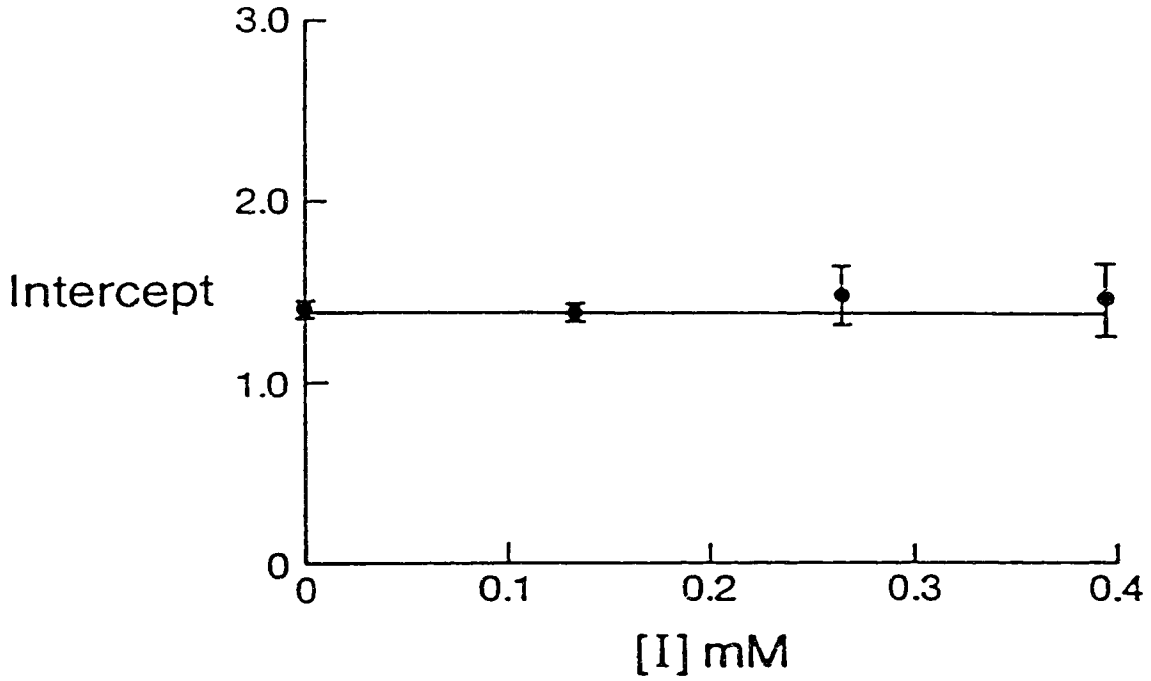


Figure 3-3a. Replot of the intercepts obtained from the data of Figure 3-3. The invariance is consistent with a competitive inhibitor.

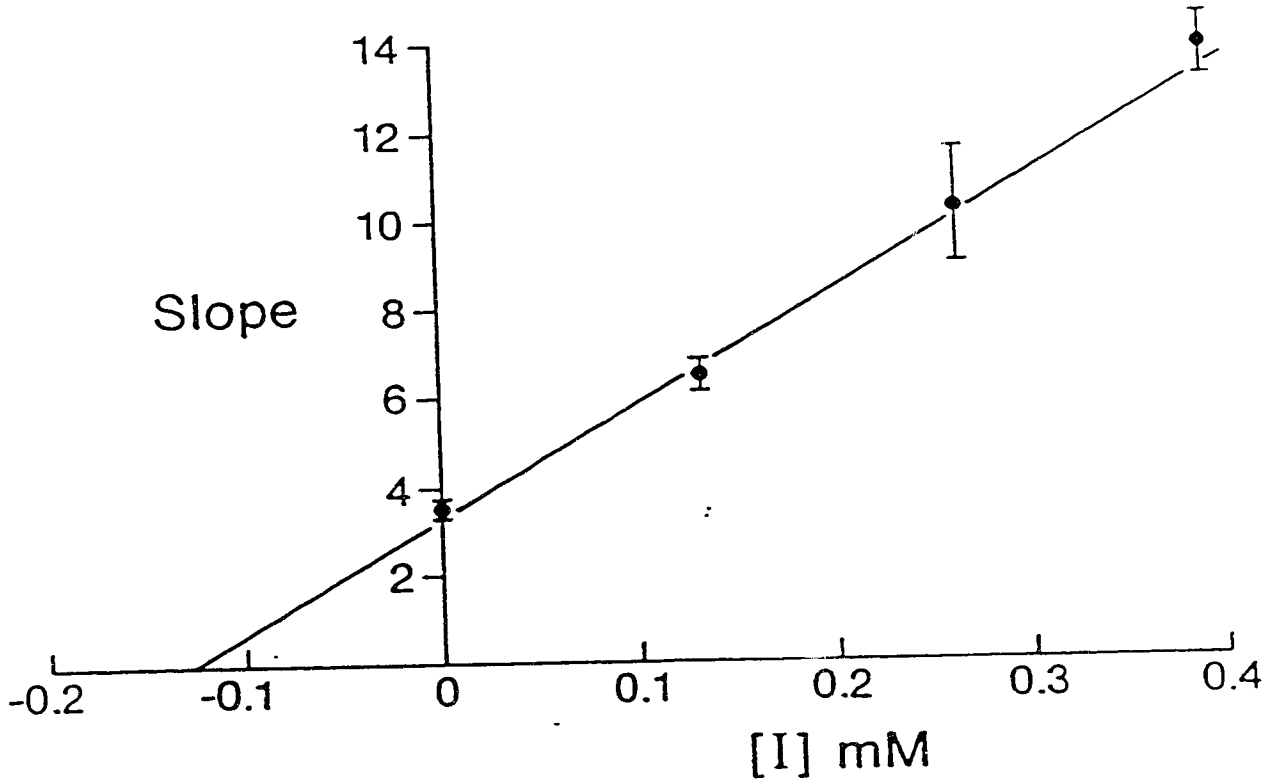


Figure 3-3b. Replot of the slopes (arbitrary units) obtained from Figure 3-3. A K_i of 125±14 μM was obtained using the LINE program of Cleland (43). Analyis of K_m (app) vs [3-2] gave a K_i of 140± 5 μM (plot not shown).

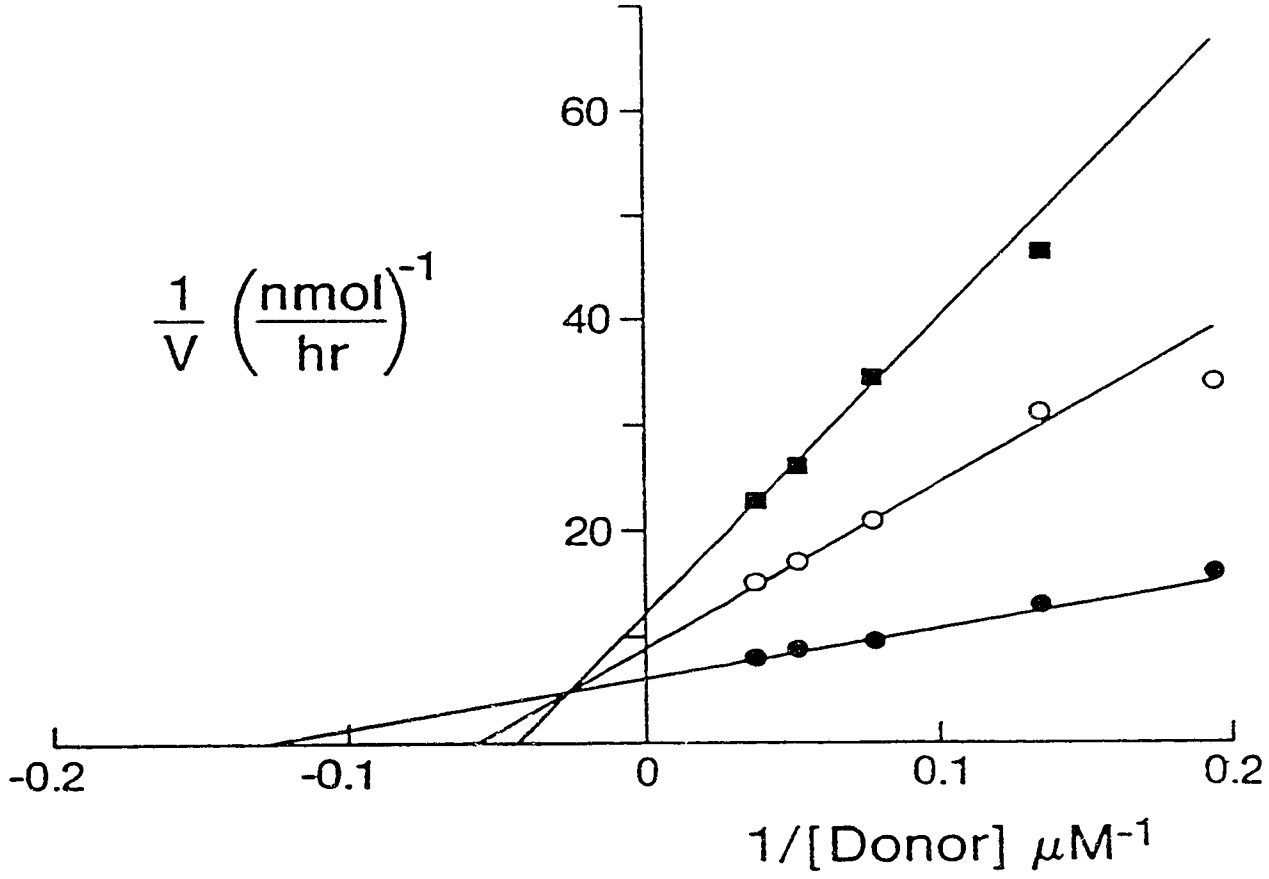


Figure 3-4. Mixed inhibition of membrane bound α(1→2)-fucosyltransferase using GDP-fucose as the variable substrate with increasing concentrations of the phosphonate analog **3-2**. The concentration of phenyl β-D-galactopyranoside was 25 mM, and the concentrations of inhibitor **3-2** were 0 mM (●), 1.05 mM (○) and 1.58 mM (■).

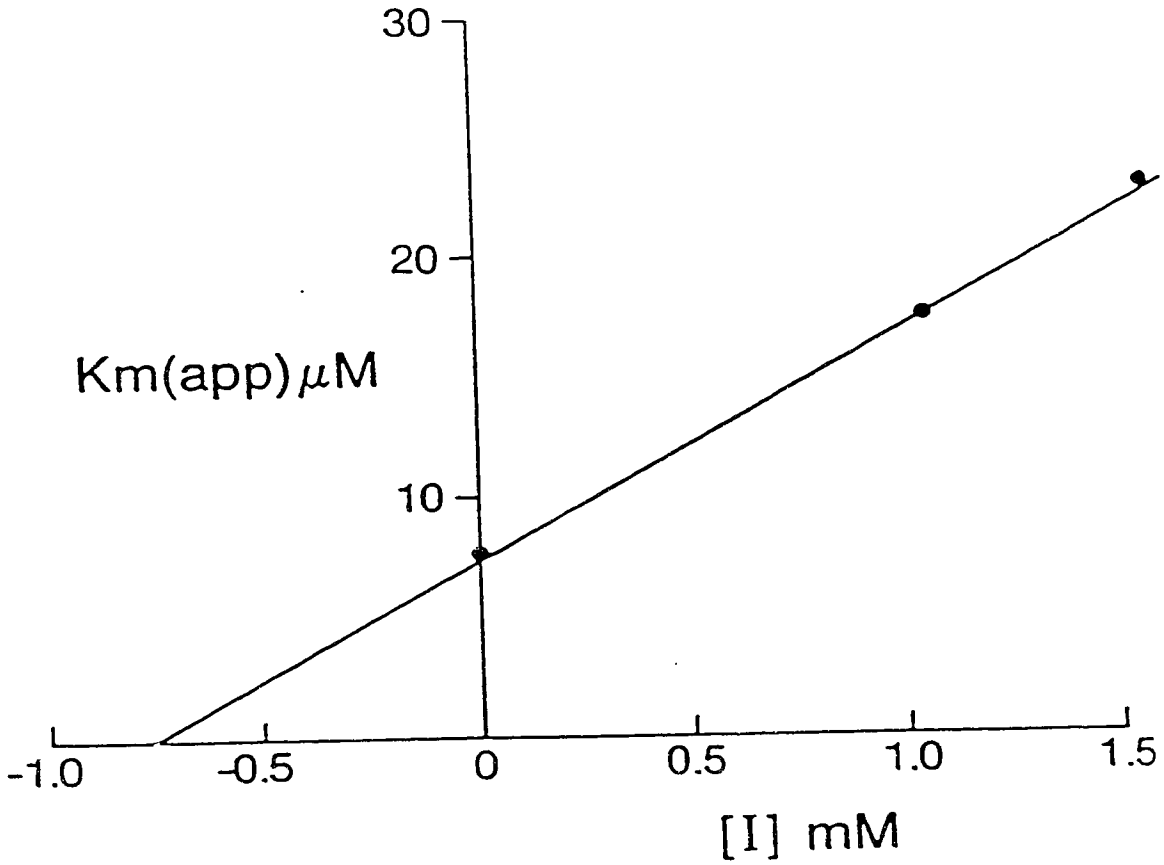


Figure 3-4a. Replot of K_m(app) obtained from Figure 3-4 vs [3-2]. A K_i of 760±37 μM was obtained using the LINE program of Cleland (43).

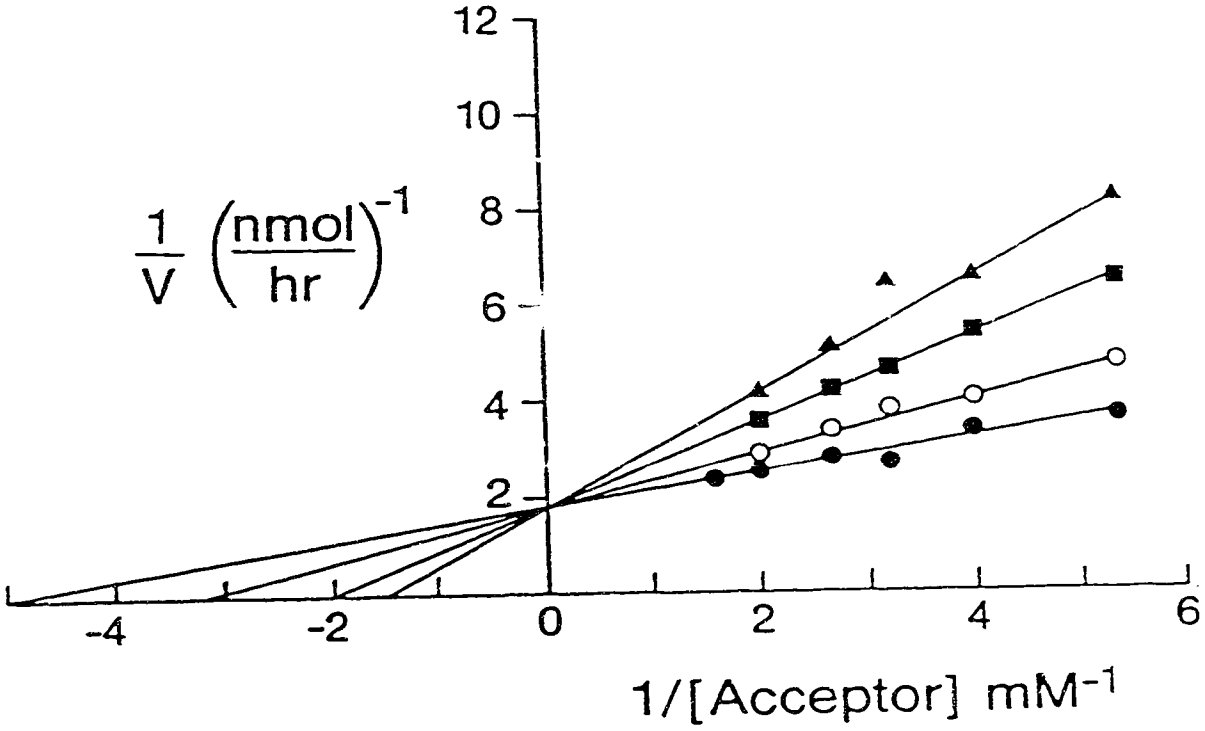


Figure 3-5. Competitive inhibition of membrane bound α(1→2)-fucosyltransferase using βGal(1→3)βGlcNAcO$(CH_2)_8$COOMe (Type 1 acceptor) as the variable substrate with increasing concentrations of the bisubstrate analog **3-1**.The concentration of GDP-fucose was 63.4 μM. The concentrations of inhibitor **3-1** were 0 μM (●), 2.7 μM (○), 5.3 μM (■) and 10.7 μM (▲).

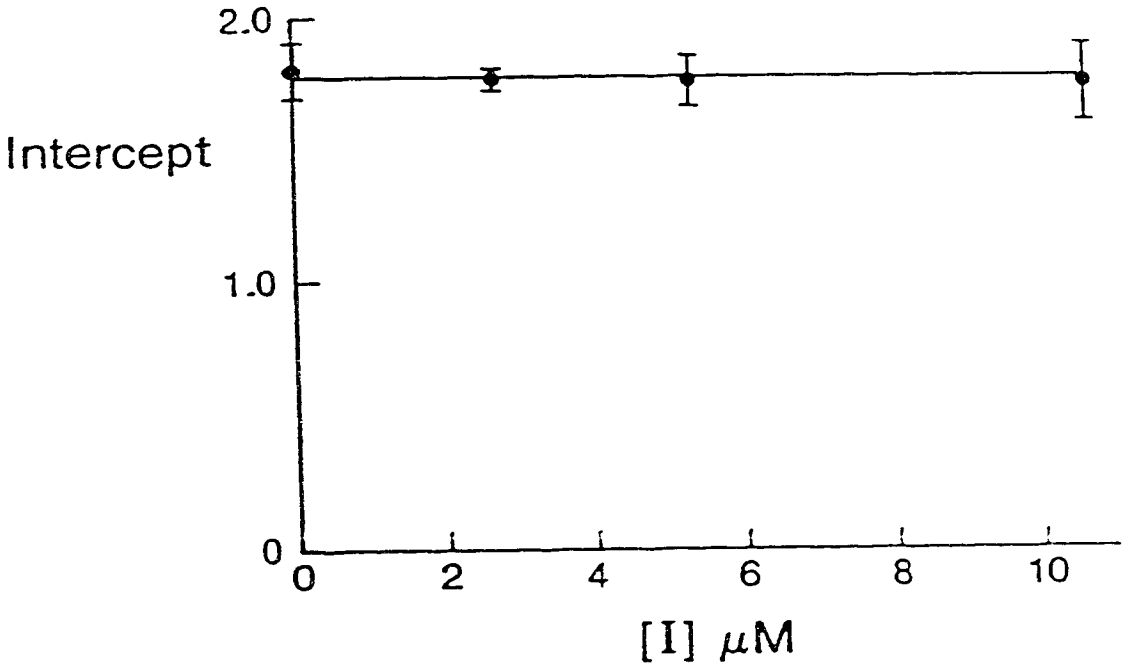


Figure 3-5a. Replots of the intercepts obtained by analysis of the data of Figure 3-5. The invariance is consistent with a competitive inhibitor.

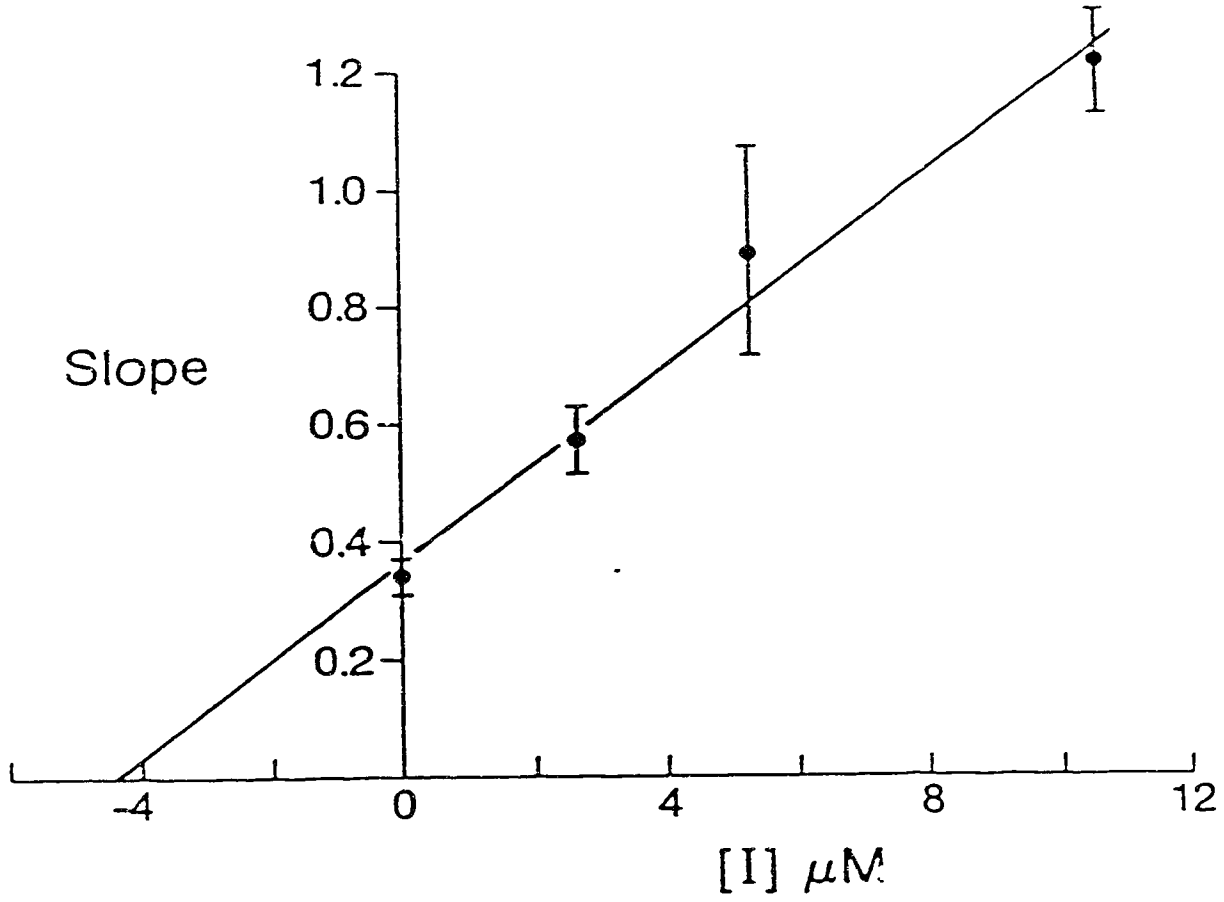


Figure 3-5b. Re[illegible]he slopes of the data in Figure 3-5. A K_i of 4.4±1.1 μM was obtained using th[illegible] program of Cleland (43). Analysis of K_m (app) vs [**3-1**] gave a K_i of 4.3±1.1 μM (plot not shown).

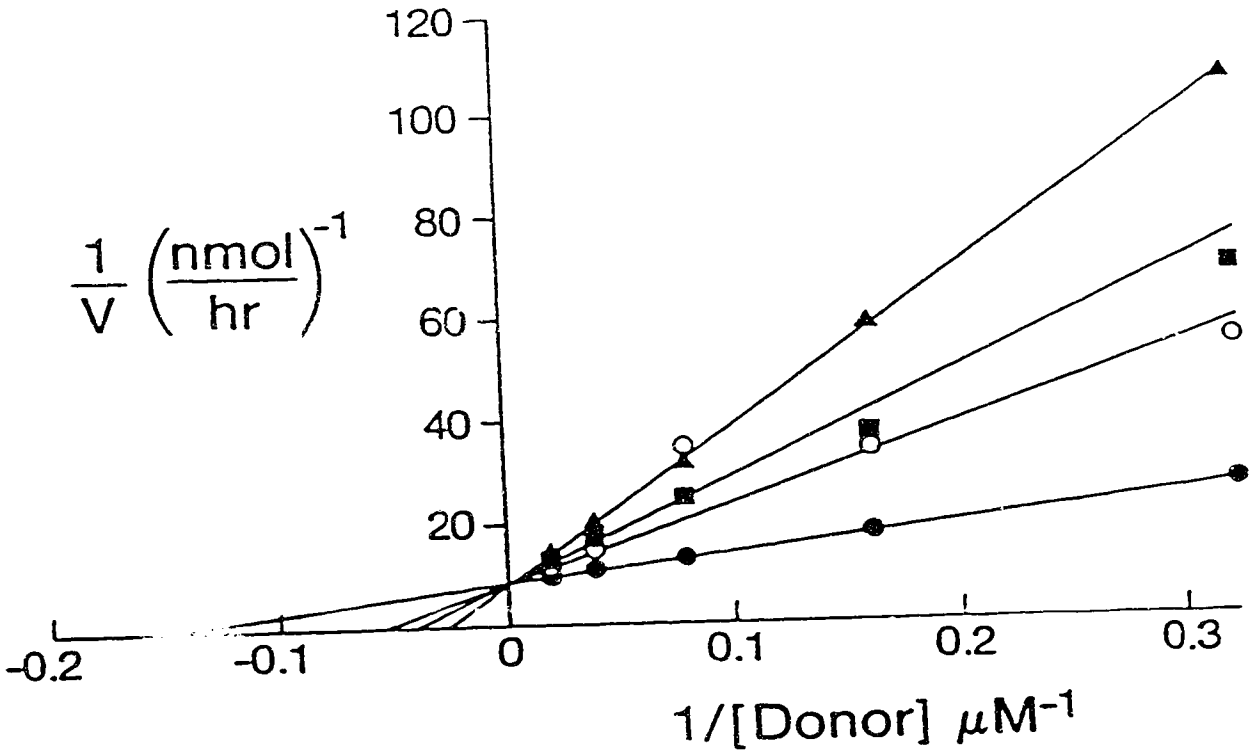


Figure 3-6. Competitive inhibition of the soluble form of α(1→2) fucosyltransferase using GDP-fucose as the variable substrate with increasing concentrations of the bisubstrate analog **3-1**. The concentration of phenyl β-D-galactopyranoside was 80 mM. The concentrations of inhibitor were 0 μM (●), 20.1 μM (○), 40.2 μM (■), and 60.4 μM (▲). The points on the reciprocal plots are the experimental values while the lines are calculated from the fit of the data using the Wilkinson method (42).

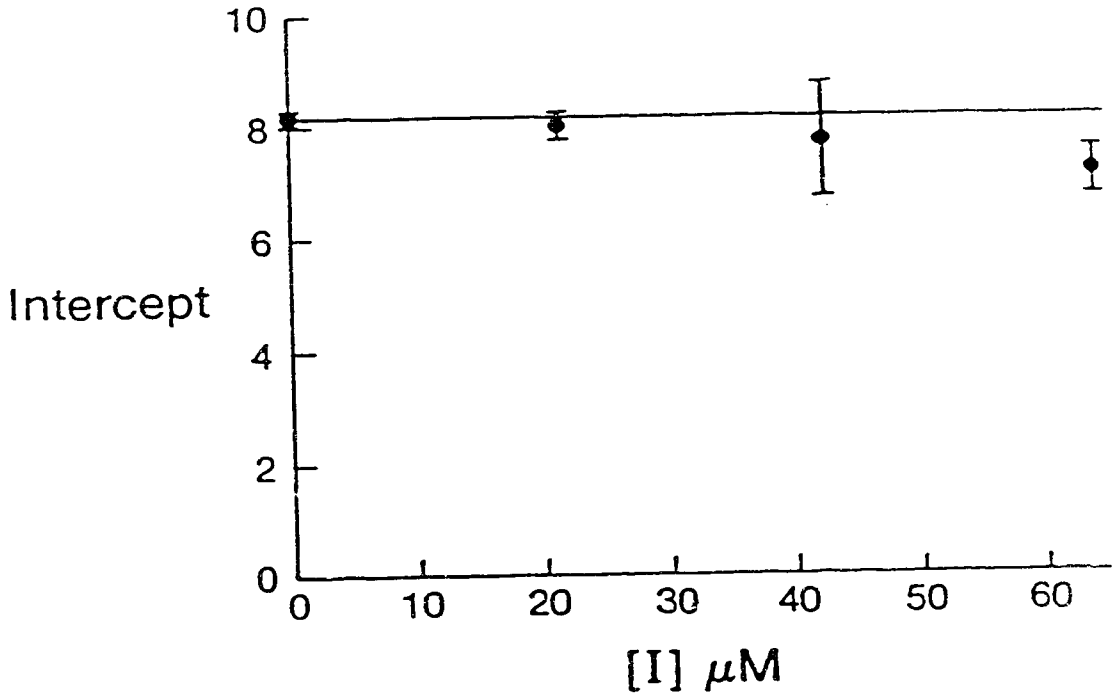


Figure 3-6a. Replot of the intercepts obtained from the data of Figure 3-6. The invariance is consistent with a competitive inhibitor.

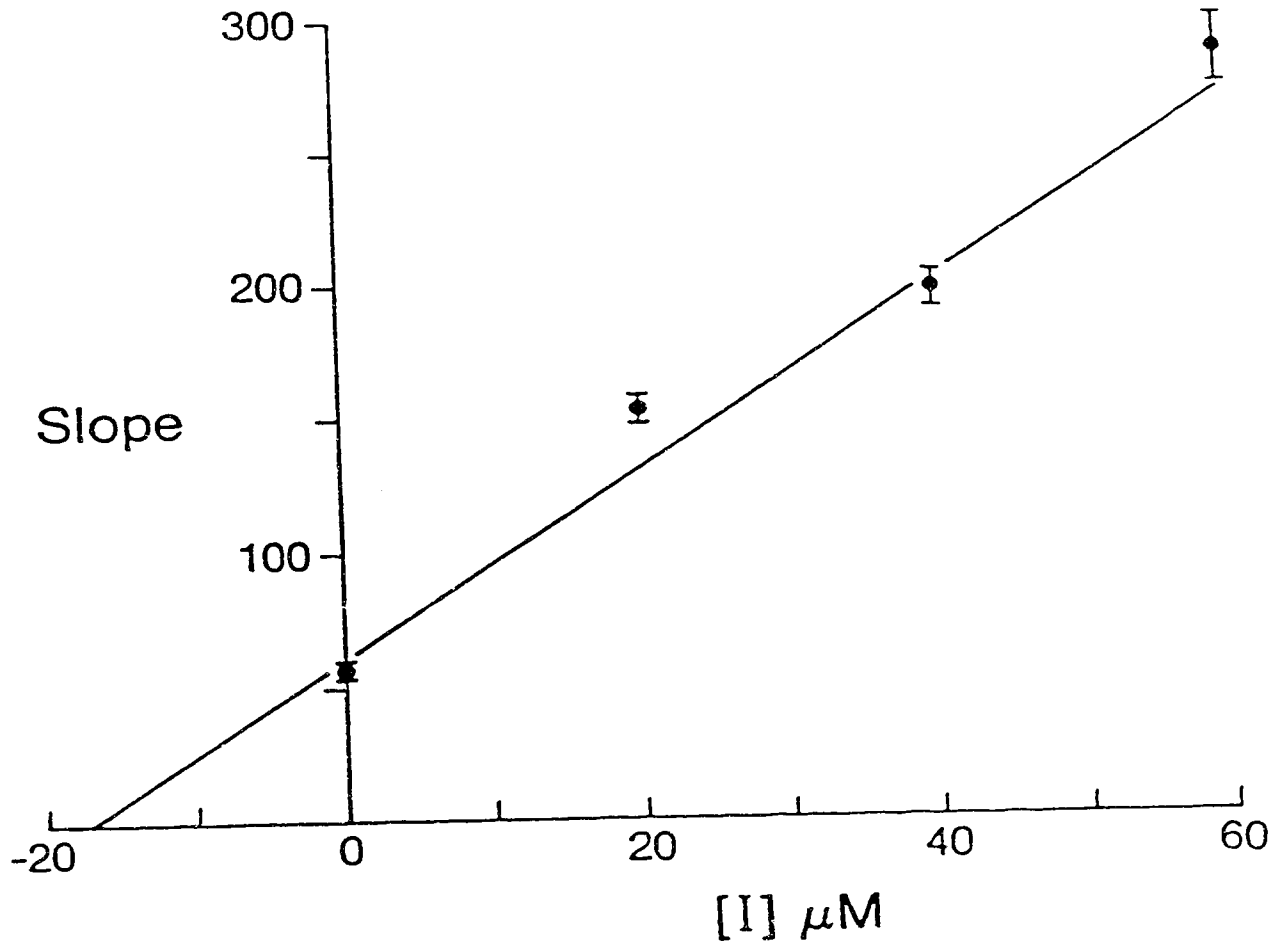


Figure 3-6b. Replot of slopes (arbitrary units) obtained from Figure 3-6. A K_i of 17±5 μM was obtained using the LINE program of Cleland (43). Analysis of K_m (app) vs [3-1] gave a K_i of 13±1 μM (plot not shown).

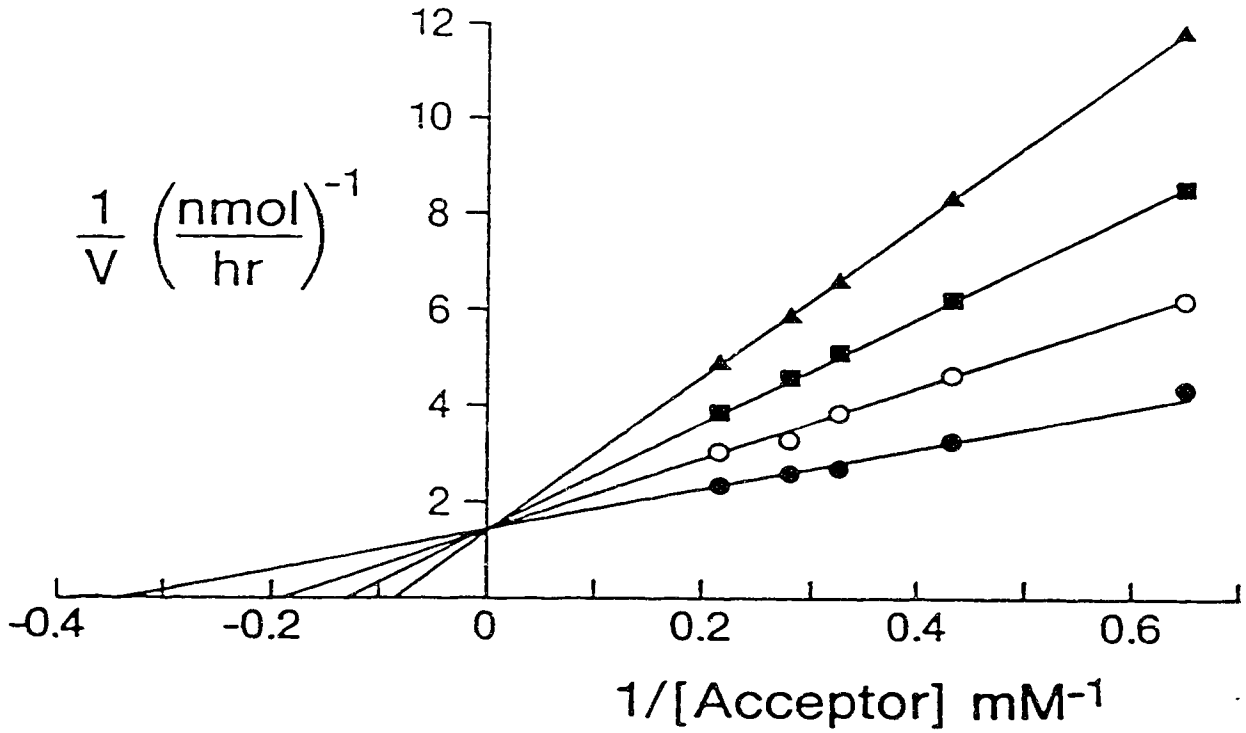


Figure 3-7. Competitive inhibiton of the soluble form of α(1→2)-fucosyltransferase using phenyl β-D-galactopyranoside as the variable substrate with increasing concentrations of bisubstrate analog **3-1**. The concentrations of GDP-fucose was 64 μM. The concentrations of inhibitor **3-1** were 0 μM (●), 2.7 μM (○), 5.3 μM (■) and 10.7 μM (▲). The points in the reciprocal plots are the experimental values, whicle the lines are calculated from the fit of the data using the Wilkinson method (42).

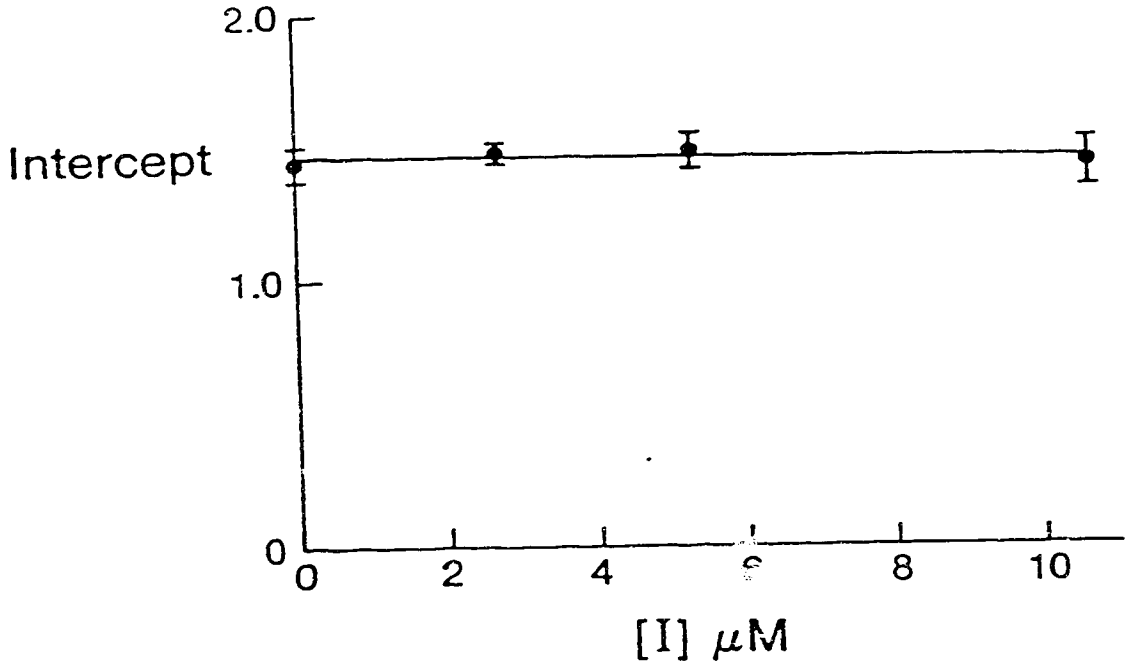


Figure 3-7a. Replot of the intercepts obtained from Figue 3-7. The invariance is consistent with a competitive inhibitor.

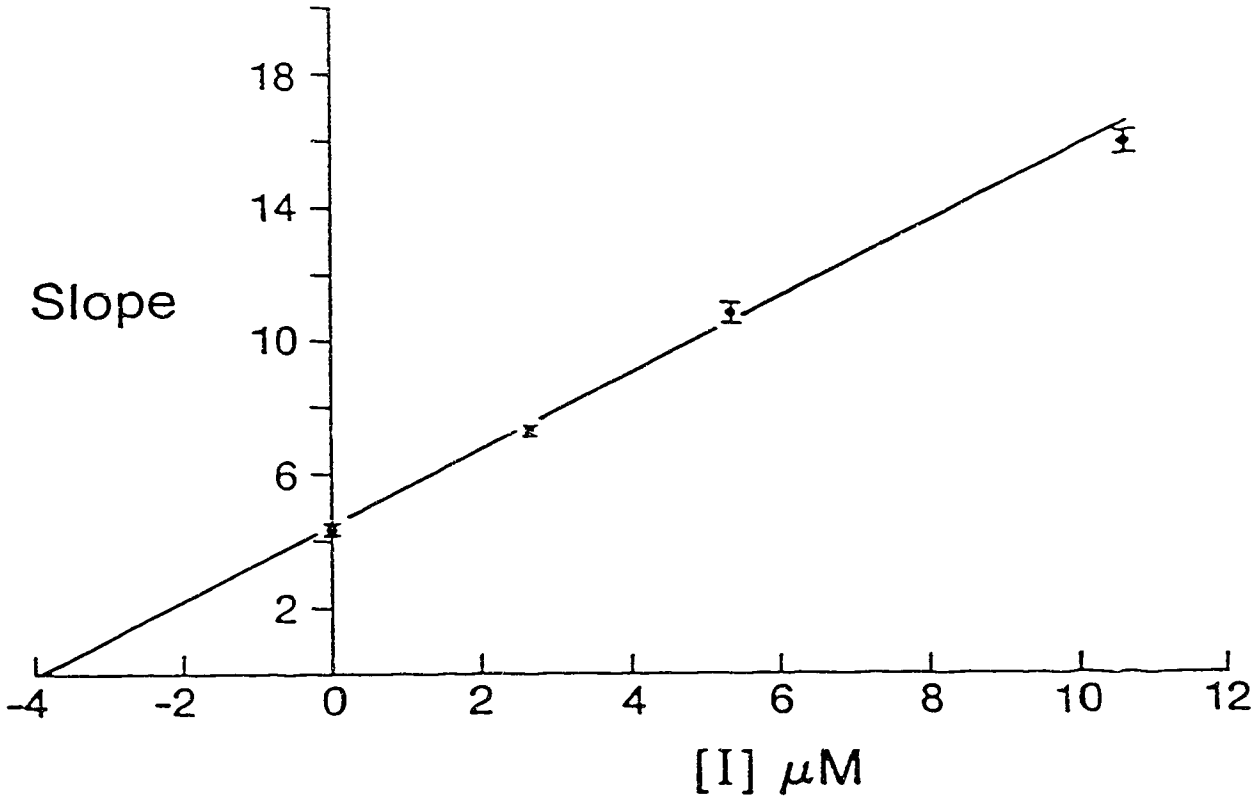


Figure 3-7b. Replot of the slopes (arbitrary units) obtained from Figure 3-7. A K_i of 4± 0.5 μM was obtained using the LINE program of Cleland (43). Analysis of K_m (app) vs [**3-1**] gave a K_i of 4±0.3 μM (plot not shown).

Table 3-1. Kinetic Constants for Inhibition of α(1→2)-Fucosyltransferases*

Enzyme Type	inhibitor	Substrate	K_m	K_i	(Inhibition Type)
Membrane	**3-1** (bisubstrate)	PhenylGal	2600	2.3	(competitive)
Membrane	**3-1** (bisubstrate)	GDP-Fucose	7.3	16	(competitive)
Membrane	**3-2** (phosphonate)	PhenylGal	2600	133	(competitive)
Membrane	**3-2** (phosphonate)	GDP-Fucose	7.3	760	(mixed)
Membrane	**3-1** (bisubstrate)	βGal(1→3) βGlcNAcOR	200	4.4	(competitive)
Soluble	**3-1** (bisubstrate)	PhenylGal	3000	4.0	(competitive)
Soluble	**3-1** (bisubstrate)	GDP-Fucose	7.3	15	(competitive)

*Kinetic constants are the average values obtained from analysis of slope and K_m (app) data with the LINE program, (see figure legends).

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4. Deoxy Acceptor Analogs as Specific Inhibitors of Glycosyltransferases*

Mammalian cell surface carbohydrates have been strongly implicated in several molecular recognition events such as neuronal development (1,2), fertilization (3), tumor progression (4-7), and the specific binding of bacteria and viruses (8,9). Selective inhibition of glycosylation reactions can provide valuable tools for elucidating the biosynthetic pathways responsible for the production of cell surface oligosaccharides, and also provide information as to the importance of these structures as cell recognition markers.

The most frequently used inhibitors of glycosylation are of two types, tunicamycin which inhibits the enyme UDP GlcNAc:dolichol phosphate GlcNAc-phosphotransferase (E.C. 2.7.8.15). The second class of inhibitors includes several alkaloids: castanospermine, swainsonine, and nojirimycin. These are glycosidase inhibitors which hinder the trimming reactions of high mannose containing oligosaccharides (10). Both types of inhibitors cause changes in the oligosaccharide structures on cell surfaces resulting in modified cell behaviour. These inhibitors, however, do not affect the overall biosynthesis of *O*-linked glycolipids which are thought to be biosynthesized by a different pathway.

The biosynthesis of complex oligosaccharide structures requires the sequential action of many different glycosyltransferase enzymes which facilitate the transfer of a sugar from a sugar nucleotide to the hydroxyl group of an acceptor saccharide substrate. These enzymes provide suitable targets for the development of specific inhibitors which have the potential to modify the structures of complex cell surface carbohydrates. Very few inhibitors of glycosyltranferases have been found and most research efforts have centered around the development of unreactive sugar nucleotide analogs (11-15).

Sugar nucleotide analogs, however, do not provide specific inhibitors for glycosyltranferases since a single sugar nucleotide donor can serve as substrates for more than one transferase enzyme. A good example is found with the *N*-acetylglucosaminyltransferases for which there are at least six different enzymes known,

*This work will be submitted to *J. Biol. Chem.*
Dr. M. Palcic Department of Food Science, University of Alberta was responsible of the *N*-acetylglucosaminyltransferase V inhibitor evaluation.
Dr. O. Hindsgaul, Department of Chemistry, University of Alberta was responsible for the chemical synthesis of the deoxyacceptor analogs.

each forming a specific linkage to the core mannose containing structure. All these *N*-acetylglucosaminyltransferases utilize UDP-GlcNAc as the sugar donor; thus an unreactive sugar nucleotide donor would not be able to inhibit a specific *N*-acetylglucosaminyltransferase enzyme. Specific inhibition of a glycosyltransferase activity which utilizes a common donor substrate will only arise by the development of an analog of the acceptor substrate which incorporates structural components that can be recognized by the target enzyme but is unable to participate in the desired reaction. To explore this theory we synthesized and kinetically evaluated acceptor analogs that were deoxygenated at the position of the reactive hydroxyl group which included all the required structural recognition components for the enzyme.The target enzyme systems that we chose to inhibit were the α(1→2)-fucosyltransferase from porcine submaxillary glands, the α(1→3/4)-fucosyltransferase from human milk, and two "tumor associated" transferase enzymes, the α(1→3)-fucosyltransferase from human serum and the *N*-acetylglucosaminyltransferase V (GlcNAc T-V) from crude microsomal extracts of baby hamster kidney cells as shown in Scheme 4-1.

Experimental

Materials - Porcine submaxillary gland α(1→2)-fucosyltransferase was available from previous work (16) and had a specific activity of 66.4 munits/ mg protein. One unit is defined as the amount of enzyme catalizing the formation of 1 μmol of product formed per minute at 37°C. Human milk α(1→3/4)-fucosyltransferase was also available from previous work (17) and had a specific activity of 107 munits/ mg protein. GlcNAc T-V was from a crude microsomal extract of baby hamster kidney cells (18). α(1→3)-Fucosyltransferase was isolated from human serum and had a specific activity of 1.6 munits/ mg protein (19). Sep Pak C18 reverse phase cartridges were from Waters Associates. Guanosine diphospho-L-[U-^{14}C] fucose (248 mCi / mmol) was from New England Nuclear and unlabeled GDP-fucose was synthesized by Dr. O. Hindsgaul's group as previously described elsewhere (20). βGal(1→3)βGlcNAc-O-$(CH_2)_8$COOMe and βGal(1→4)βGlcNAc-O-$(CH_2)_8$COOMe was a generous gift from Chembiomed Ltd. UDP-GlcNAc, ATP, $MnCl_2$, and UDP were from Sigma Chemical Co. UDP-[^{3}H] GlcNAc (448 mCi / mmol) was synthesized as described elsewhere (21). Deoxy analogs **4-1** to **4-3** (22) and **4-4** (23) were chemically synthesized. ACS aqueous scintillation

cocktail was from Amersham.

Inhibition Kinetics

a) For α(1→2)-fucosyltransferase - Incubation mixtures in 65 µL contained 64 µM GDP-fucose, 30,000 dpm GDP-[U-^{14}C]fucose, 0.174-0.625 mM βGal(1→3)βGlcNAc -O-$(CH_2)_8$COOMe, 2.85 mM $MnCl_2$, 2.85 mM ATP, fucosyltransferase (11 µunits), and acceptor analog **4-1** in 20 mM Tris buffer at pH 7.0 containing 2mM sodium azide. After incubation at 37°C for 1 hr incubation mixtures were diluted with 1 mL of H_2O and immediately placed at 4°C. Reaction mixtures were loaded onto preconditioned Sep Pak (30) cartridges and unreacted GDP-fucose was eluted until background counts were obtained by washing with H_2O (30 mL). Radiolabeled product was eluted with methanol (2 x 5 mL) and quantitated as dpm in 10 mL of scintillation cocktail in a Beckman LS 1801 scintillation counter. No more than 10 assays were done at one time. Under the conditions described above, no more than 15% turnover occurred to ensure initial rate conditions. Initial rate data were obtained in the presence and absence of inhibitor using a fixed and saturating level of GDP-fucose while varying the acceptor concentration at 5 different levels. Two concentrations of inhibitor were used and each point represents an average of at least two determinations. The kinetic parameters, V_{max} and K_m for the primary data were estimated using a computer program based on the method of Wilkinson (31). The true kinetic and inhibition constants were obtained by plotting the slope, intercepts, and K_m(app) versus the inhibitor concentration. The data for the secondary plots were fit to the Line program of Cleland (32).

b) *For N-acetylglucosaminyltransferase V* - The following assay components were dried under reduced pressure in 1.5 mL microfuge tubes: 0.72 µCi UDP-[^{3}H]GlcNAc, 25 nmol N-acetyl-β-D-glucopyranosylamine (a hexosaminidase inhibitor), 0.208-10 nmol of βGlcNAc(1→2)αMan(1→6)βMan-O-$(CH_2)_8$COOMe, and analog **4-4**. No unlabeled UDP-GlcNAc was added to enhance the sensitivity of the assay to maximize the amount of radioactivity transferred. The final concentration of UDP-GlcNAc was 160 µM. Ten µL of a microsomal extract of baby hamster kidney cells was added to the dried assay components, briefly shaken on a vortex mixer, and incubated at 37°C for 3-6 hours. After incubation, reaction mixtures were diluted with water (1mL) and applied to a preequilibrated Sep Pak C 18 cartridges. Unreacted UDP-GlcNAc was removed by washing with water until background counts were obtained. Products were eluted with methanol (2x5

mL) and quantitated in 10 mL of scintillation cocktail. The kinetic parameters, V_{max} and K_m and the inhibition constant K_i were determined as described above.

Results

Deoxygenation of the reactive hydroxyl group on the acceptor substrate provides an attractive approach for inhibiting glycosyltransferase activities. A deoxy analog of an acceptor substrate, being less sterically demanding should recognize and bind to the corresponding transferase enzyme in a similar fashion as the substrate unless the hydroxyl group is required for binding to the enzyme.

The porcine submaxillary gland α(1→2)-fucosyltransferase transfers a fucose group from GDP-fucose to the 2 hydroxyl of non reducing terminal β-galactose units (33,34). The effect of 2'-deoxygenated βGal(1→3)βGlcNAc-O-$(CH_2)_8$COOMe (**4-1**) on the membrane bound α(1→2)-fucosyltransferase at saturating concentrations of GDP-fucose is shown in Figure 4-1. From the kinetic plots obtained, one can see that the analog **4-1** is a competitive inhibitor with respect to the acceptor substrate βGal(1→3)βGlcNAc -O-$(CH_2)_8$COOMe with a K_i of 800 μM. The 4'-deoxy βGal(1→3)βGlcNAc -O-$(CH_2)_8$COOMe (**4-2**) and the 3'-deoxy βGal(1→4)βGlcNAc-O-$(CH_2)_8$COOMe (**4-3**) analogs were completely inactive as inhibitors for the human milk α(1→4)-fucosyltransferase and the human serum α(1→3)-fucosyltransferase respectively which transfers fucose to the 3 and 4 positions of a GlcNAc residue, even at ten times the K_m of the corresponding acceptor substrate.

N-acetylglucosaminyltransferase V is an enzyme which is responsible for forming the β(1→6) linkage of GlcNAc to a mannose sugar in complex oligosaccharide structures. Earlier work had demonstrated that small oligosaccharide structures could serve as suitable acceptors for this enzyme (30,35). Competitive inhibition (K_i 70 μM) was achieved with a deoxygenated analog (**4-4**) of a specific GlcNAc T-V acceptor βGlcNAc(1→2)αMan(1→6) βMan-O-$(CH_2)_8$COOMe where the 6'-hydroxyl of the corresponding acceptor had been removed at a concentration of UDP-GlcNAc of 160 μM for a microsomal preparation of GlcNAc T-V from baby hamster kidney cells (Figure 4-2).

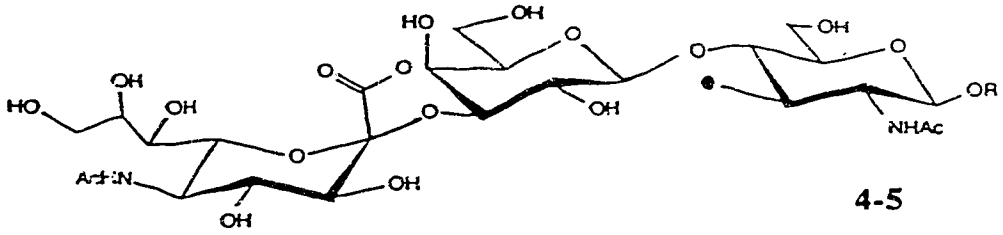
The lack of inhibition for the 3'-deoxy and 4'-deoxy acceptor analogs (**4-2** and **4-3**) for the corresponding fucosyltransferase was unexpected since moderate competitive

inhibition was observed with the 2'-deoxy analog (**4-1**). In principle all three acceptor analogs, being structurally similar, should have provided the minimum structural requirements for proper binding of the analog to the active site.

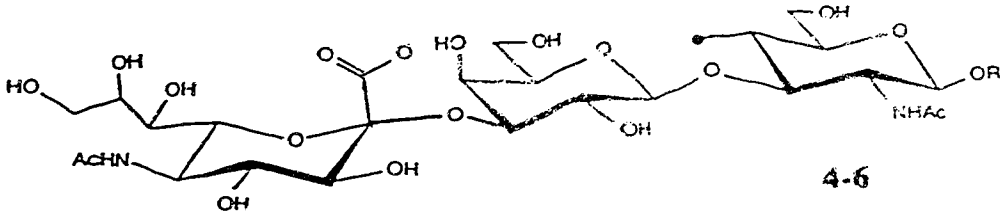
Extensive research has provided data demonstrating that critical hydroxyl groups may be important for the binding of oligosaccharides to lectin I of *Ulex europaeus* and lectin IV of *Griffonia simplicifolia*, and to antibodies directed against several blood group structures (22,36). Polar and hydrophobic interactions between complimentary surfaces on the oligosaccharide and the protein are thought to control the binding between them. Several deoxy analogs of blood group determinants were inactive in inhibiting the binding of the lectin or antibody to the parent blood group structure. The inability of the deoxy analog to prevent binding was attributed to the removal of a hydroxyl group that is involved in the binding reaction. The interaction of an acceptor with the corresponding glycosyltransferase enzyme should involve similar types of polar and hydrophobic interactions. The removal of the hydroxyl group in deoxy analogs **4-2** and **4-3** may have eliminated a critical group which is required for binding of the acceptor analog to the active site of the transferase enzyme resulting in a completely inactive analog.

One approach to improving the performance of analogs **4-2** and **4-3** may be to add another sugar. This may provide additional structural components which would enable the acceptor analog to bind more effectively to the transferase enzyme. Addition of a sialic acid residue to the 3 hydroxyl of βGal(1→4)βGlcNAc to form αNeuAc(2→3)βGal(1→4)βGlcNAc was found to decrease the K_m seven fold over the disaccharide βGal(1→4)βGlcNAc (K_m 1.4 mM) for the human serum α(1→3)fucosyltransferase (37). A three fold decrease in K_m has been observed with the addition of sialic acid to the 3 position of βGal(1→4)βGlcNAc-O-$(CH_2)_8$COOMe (K_m 45 μM) for the human milk α(1→3/4)-fucosyltransferase (17). In these two cases addition of sialic acid to form a trisaccharide structure increased the affinity of the acceptor structure for the corresponding enzyme. Addition of sialic acid to the deoxy analogs **4-2** and **4-3** may provide additional structural components which will increase the affinity of the deoxytrisaccharides to an extent where they can compete with the acceptor substrate efficiently to produce inhibition. Enhanced inhibition cannot be observed with the sialylated 2'-deoxy βGal(1→3)βGlcNAc-O-$(CH_2)_8$COOMe since the structure αNeuAc(2→3)βGal(1→3)βGlcNAc is not a substrate for the porcine submaxillary gland α(1→2)fucosyltransferase. Research is

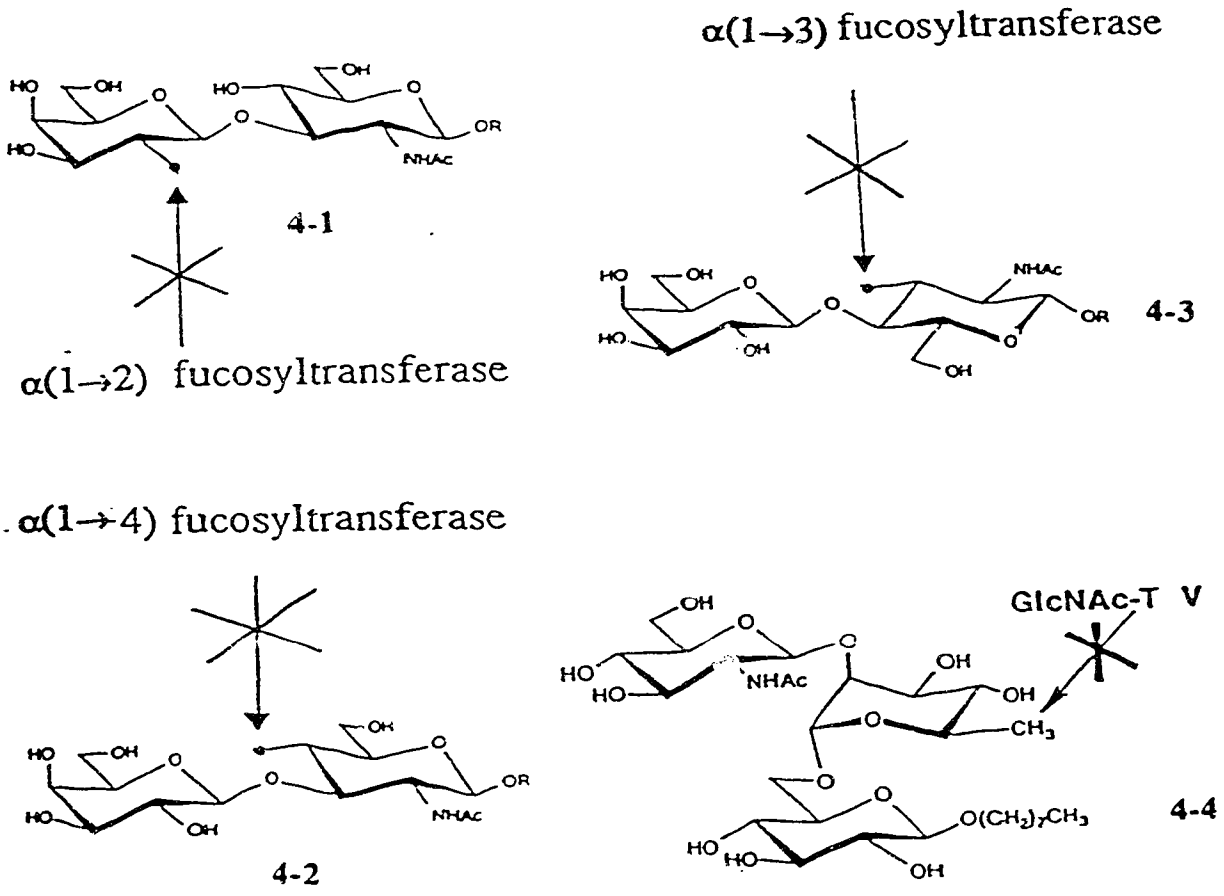
presently underway in our laboratory to enzymatically synthesize the 3'-deoxy αNeuAc(2→3)βGal(1→4)βGlcNAc-O-$(CH_2)_8$COOMe (**4-5**) and 4'-deoxy αNeuAc(2→3) βGal(1→3)βGlcNAc-O-$(CH_2)_8$COOMe (**4-6**) using an α(2→3)-sialyltransferase (17). The resulting deoxy trisaccharides **4-5** and **4-6** will be reevaluated as inhibitors for the α(1→3)-fucosyltransferase and the α(1→3/4)-fucosyltransferase enzymes.



4-5



4-6



Scheme 4-1. Strategy for Inhibition by Deoxy Acceptor Analogs

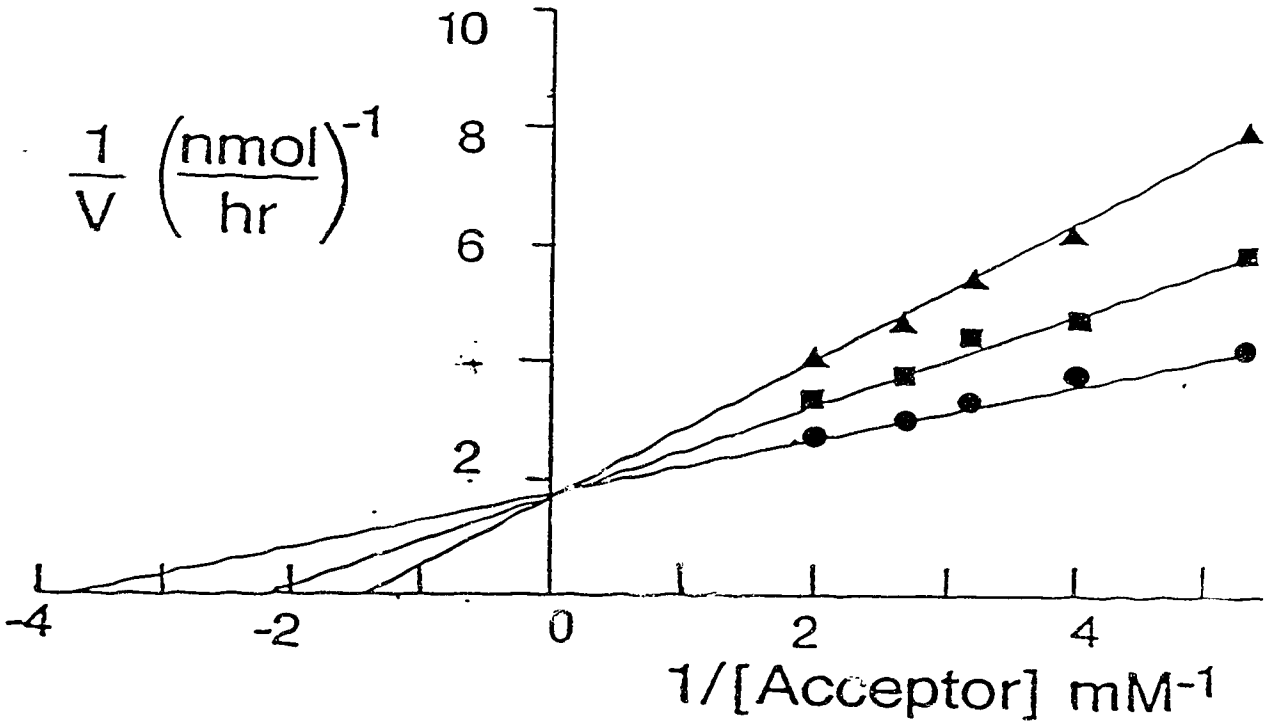


Figure 4-1. Competitive inhibition of membrane bound α(1→2)-fucosyltransferase using βGal(1→3)βGlcNAcO$(CH_2)_8$COOMe (Type 1 acceptor) as the variable substrate with increasing concentrations of the acceptor analog **4-1**. The concentration of GDP-fucose was 64 μM. The concentrations of inhibitor **4-1** were 0 mM (●), 0.373 mM (■) and 1.12 mM (▲).

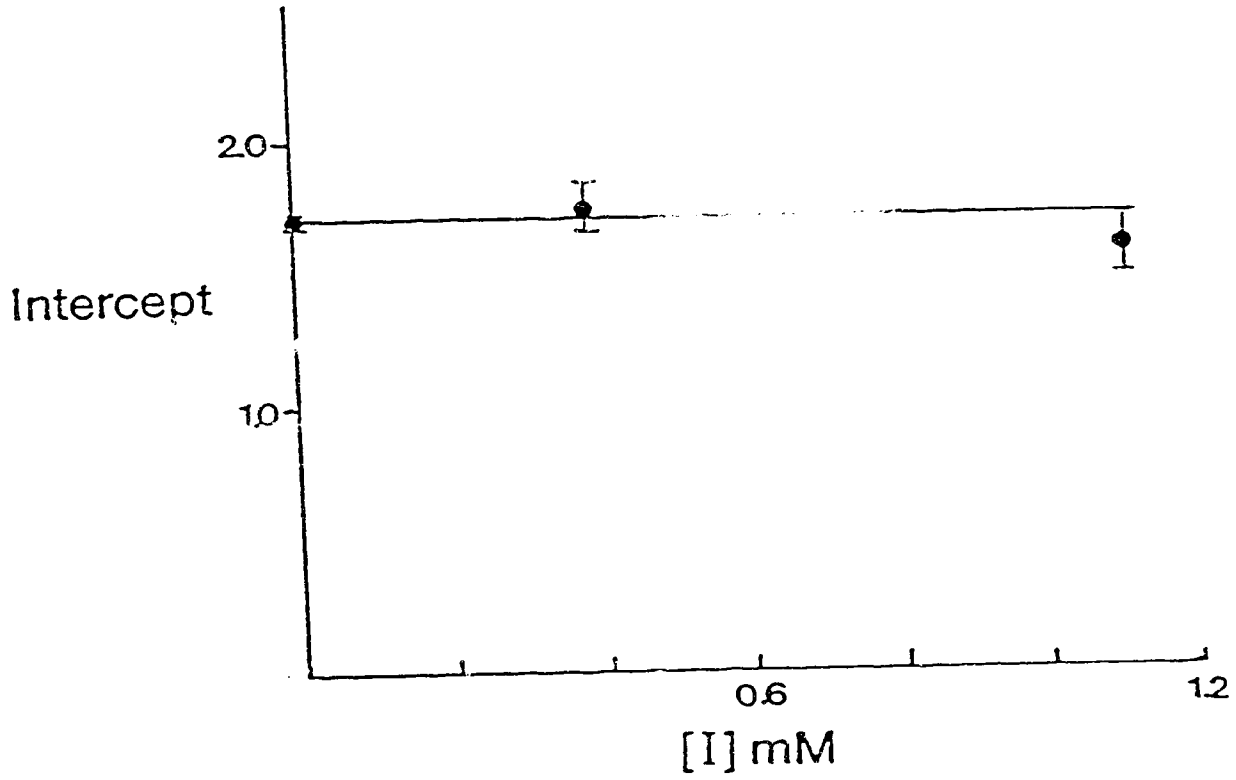


Figure 4-1a. Replots of the intercepts obtained by analysis of the data of Figure 4-1. The invariance is consistent with a competitive inhibitor.

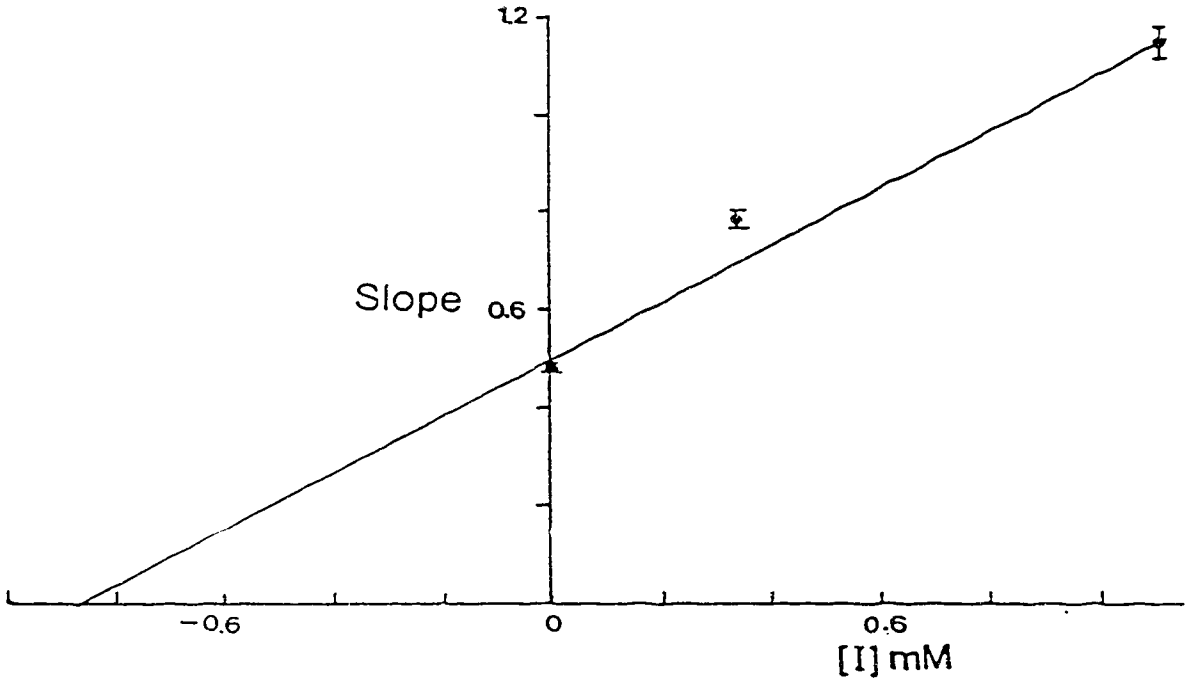


Figure 4-1b. Replot of the slopes of the data in Figure 4-1. A K_i of 0.864±0.036 mM was obtained using the LINE program of Cleland (32). Analysis of K_m (app) vs [**4-1**] gave a K_i of 0.727± 0.054 mM (plot not shown).

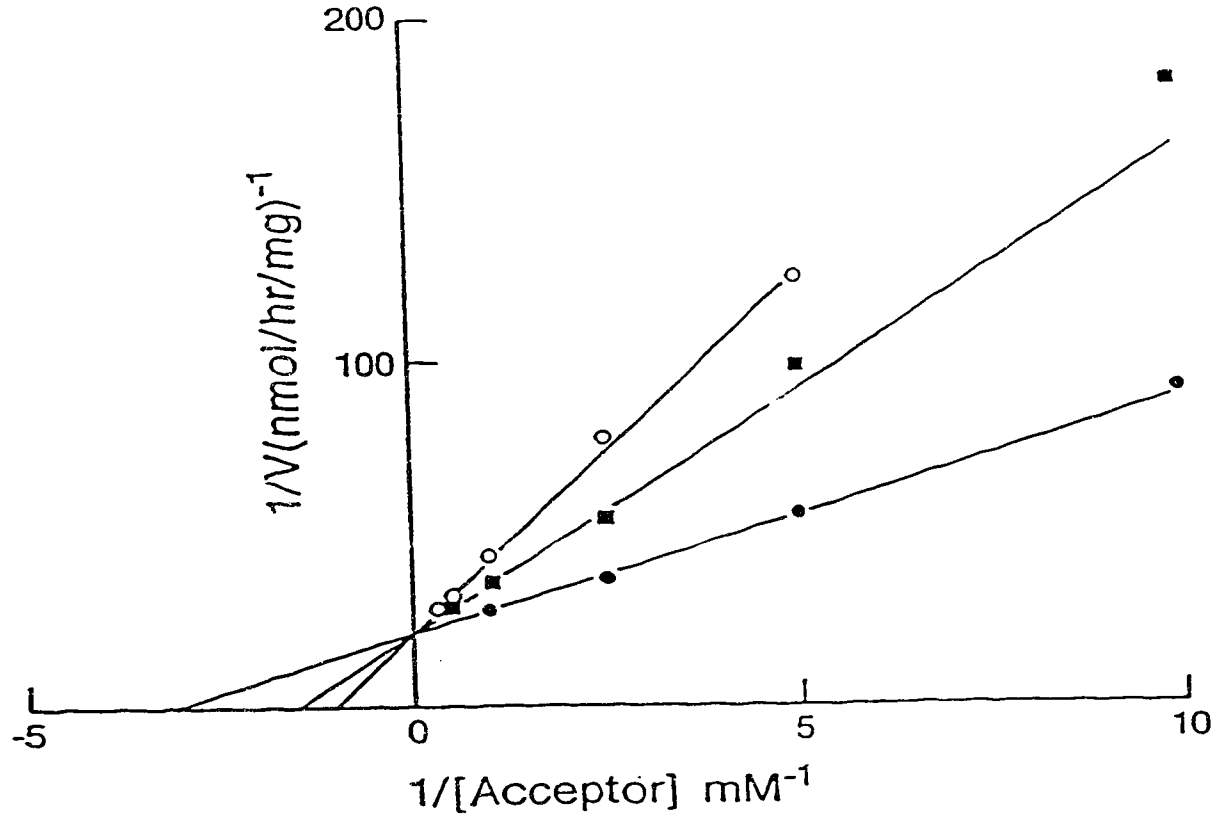


Figure 4-2. Competitive inhibition of the BHK GlcNAc T-V reaction with increasing concentrations of the deoxy analog **4-4**. The concentration of UDP-GlcNAc was 160 μM. The concentration of inhibitor **4-4** were 0 (●), 80 μM (■) and 160 μM (○).

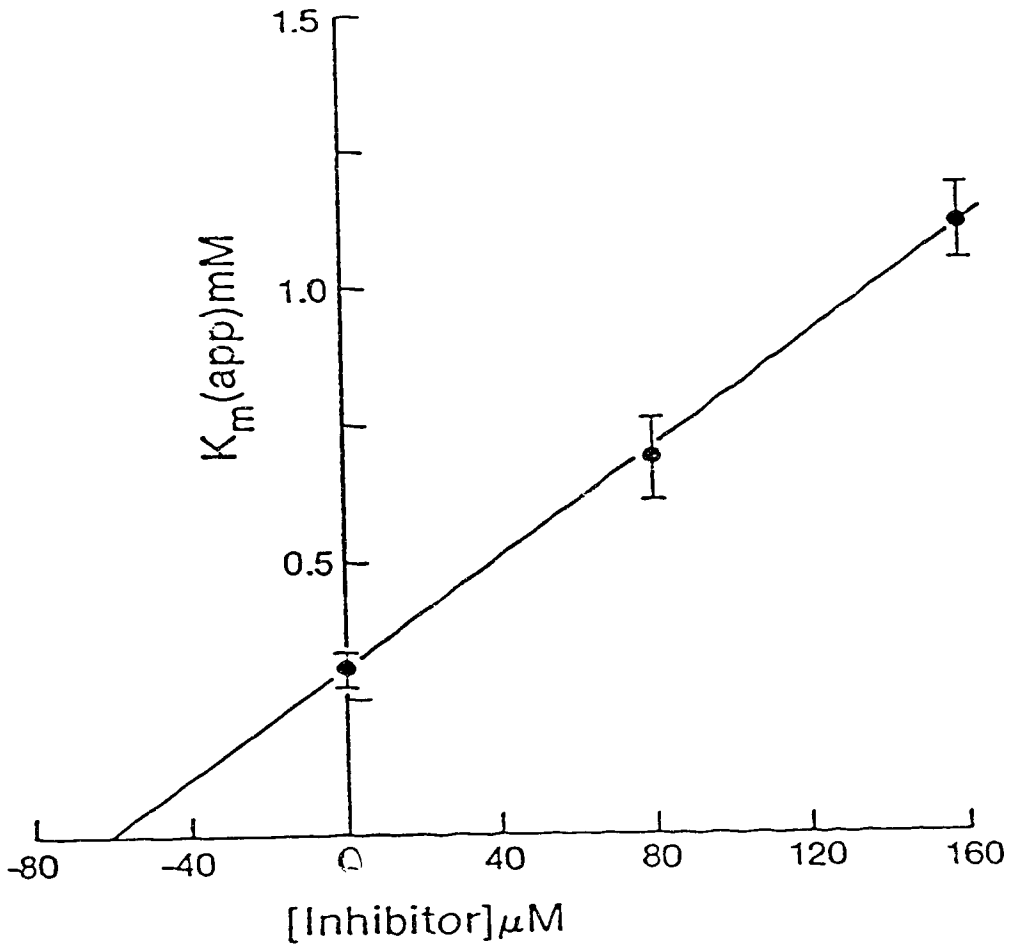


Figure 4-2a. Secondary plot of K_m (app) versus [**4-4**] obtained for the data in Fig. 4-2. A K_i of 60 μM was obtained with the line program of Cleland (32).

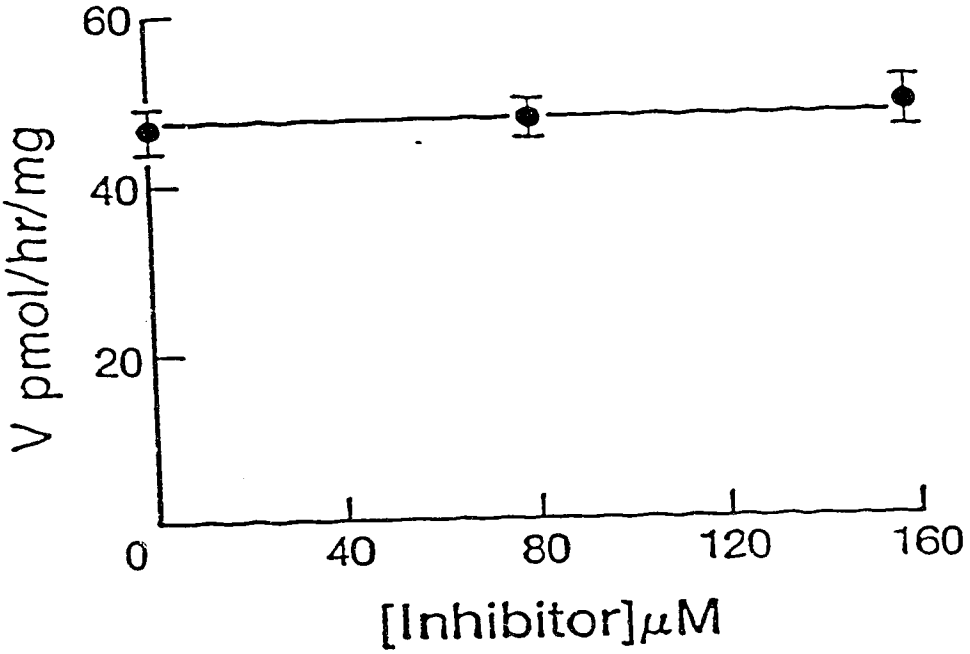


Figure 4-2b. Secondary plot of V_{max} versus [**4-4**] obtained for the data in Figure 4-2. The invariance of V_{max} as predicted for a competitive inhibitor.

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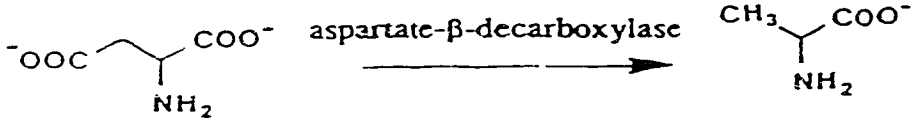
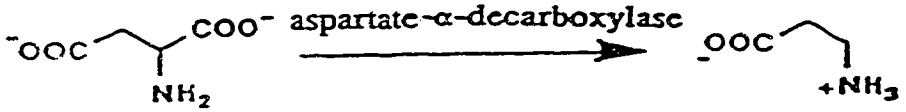
5. Introduction to Aspartate-α-Decarboxylase

Decarboxylase enzymes which catalyze the formation of amines from amino acids are widespread in nature and occur in animals, plants, and microorganisms. Two different enzymes are known to act on the amino acid aspartic acid, aspartate-β-decarboxylase (E.C.4.1.1.12), and aspartate-α-decarboxylase (E.C.4.1.1.11). Aspartate-β-decarboxylase catalyses the formation of L-aspartate to L-alanine as shown in equation 1.



(1)

Aspartate-β-decarboxylase contains pyridoxal phosphate as a cofactor, similar to the majority of the other amino acid decarboxylase enzymes (1). The enzyme has been purified to homogeneity and has been studied extensively (2). Aspartate-α-decarboxylase (E.C.4.1.1.11), the enzyme examined in the present work catalyzes the conversion of aspartic acid to β-alanine as shown in equation 2.



(2)

The function of β-alanine in microorganisms is to provide a substrate for the biosynthesis of pantothenic acid as shown in Scheme 5-1. Pantothenic acid is further converted enzymatically into it's metabolically active form, coenzyme A, which is an essential cofactor in fatty acid biosynthesis.

Aspartate-α-decarboxylase (ADC) was first isolated from *Escherichia coli* in 1979 (3,4). Recent efforts in cloning the same enzyme have had resonable success. An overproducing strain of *E. coli* was found to have levels of the enzyme 50 times greater than the wild type strain (5). The only other attempt in characterizing ADC has involved a chemically induced enzyme in a strain of *E. coli* (6). ADC was found to be pyruvoyl-dependent utilizing a covalently bound pyruvate prosthetic group as an essential cofactor rather than pyridoxal phosphate. Only three other decarboxylase enzymes are found to utilize this unusual cofactor (7). They include histidine decarboxylase from *Lactobacillus* 30a (8), S-adenosylmethionine decarboxylase from *E. coli.* (9), yeast (10), and rat liver (11,12) and phosphatidylserine decarboxylase from *E. coli* (13). The pyruvate cofactor in principle should function in an analogous manner to pyridoxal phosphate, that is by functioning as an electron sink for temporarily storing the electrons of a carbanionic intermediate for later use in bond formation (Scheme 5-2) (1). Histidine

decarboxylase from *Lactobacillus* 30a, the best studied pyruvoyl dependent decarboxylase has been shown to proceed by a similar mechanism (14) and has provided some insight as to the origin of the covalently bound pyruvate. The pyruvate cofactor has been found at the amino terminal of the enzyme. Histidine decarboxylase initially exists as a proenzyme which undergoes proteolytic cleavage exposing a terminal serine residue which is then converted to pyruvate, producing the active form of the enzyme (15). Recent findings with the major form of purified ADC indicate that the origin of the covalently bound pyruvate residue may be the result of proteolytic cleavage of a larger proenzyme as has been described for histidine decarboxylase (5). The purified enzyme had a molecular weight of 58000 which consisted of three subunits of 11800, 9800, and 6400 daltons respectively. The 9800 subunit was found to contain the covalently bound pyruvate cofactor. The enzyme had a pH optimum of 6.8-7.5, a temperature optimum of 55°C, and an isoelectric point of 4.67. The K_m for the substrate aspartic acid is 0.16 mM (3).

Mechanistic information about ADC has been obtained through inhibition studies using β substituted substrate anologs which can serve as mechanism based inhibitors (3,16). The general strategy employed was the use of amino acid analogs which contain a leaving group at the β position that would undergo enzyme mediated elimination (Scheme 5-3). The first step in the overall inhibition is the initial formation of a carbanionic species either by deprotonation or decarboxylation of the imine intermediate. The next step in the process is the elimination of the leaving group at the β position yielding a conjugated olefin system that would be set up for Michael addition by a nucleophile leading to alkylation and inactivation of the enzyme. Earlier work had shown that ADC was irreversibly inactivated by β-chloro-D-alanine, β-trifluoro-D-alanine, β-trifluoro-D,L-alanine, β-fluoro-D-alanine, *O*-carbamoyl-D-serine, and *O*-acetyl-D-serine (16).The L isomers of β-fluoro-alanine, β-chloro-alanine, *O*-carbamoyl-serine, and *O*-acetyl-serine were inactive. Both the D and L isomers of serine and D-cysteine were also found to be time dependent inactivators of ADC.

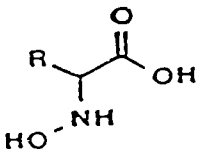
The course of the inhibition was found to proceed through two stages. Normal time dependent inhibition was observed until moderate levels of enzyme activity remained. The inhibition then entered a second stage where the inactvation occurred at a much reduced rate or ceased altogether. Addition of another aliquot of inhibitor resulted in no further inhibition indicating that depletion of inhibitor was not the cause of incomplete inactivation. The activity could not be restored upon exhaustive dialysis indicating that the inactivation was totally irreversible (16).

The results indicated that the initial step in the overall inhibition was deprotonation rather than decarboxylation. The orientation of the inhibitor in the active site was found to play a major role in allowing inhibition by isomers of the opposite configuration to the substrate. The orientation of the inhibitor in the active site allows for the labilization of the proton which is orientated perpendicular to the plane of the π system of the pyruvate group. The resulting carbanionic species formed after deprotonation is stabilized by an extended π system which is similar to the functioning of pyridoxal phosphate in pyridoxal dependent decarboxylases (Scheme 5-4). The labilized bond also provides an additional point of attatchment which prevents any alternate configuration at the active site that would result in differential bond labilization by isomers of the opposite configuration (1).

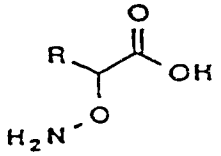
ADC was found to be inactivated in a linear time dependent manner by its substrate L-aspartate. Inactivation has been attributed to a decarboxylation transamination side reaction that takes place between the substrate and the pyruvate cofactor in the active site during normal catalysis (Scheme 5-5) (16). D-aspartate was found to be a non competitive inhibitor of the substrate (K_i 2.2 mM) (16). Several competitive inhibitors (3) were also found (K_i,mM): L-glutamate (0.76), succinate (0.73), oxaloacetate (0.81), L-cysteic acid (0.08), and β-hydroxy-D,L-aspartic acid (0.13). No inhibition was observed for metabolic end products of pantothenic acid biosynthesis indicating that ADC activity was not regulated by feedback inhibition (3).

The design of mechanism based inhibitors for ADC is not only limited to amino acid analogs with leaving groups at the β position. Analogs of aspartic acid which contain substitution at the amino nitrogen have the potential of being mechanism based inhibitors of ADC.

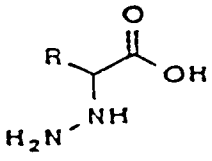
Evidence in the literature suggest compounds such as *N*-hydroxyamino acids (**5-1**), oxyamino acids (**5-2**), and hydazino acids (**5-3**) are good inhibitors of enzymes which utilize either pyridoxal phosphate or pyruvate as a cofactor.



5-1



5-2



5-3

Several hydrazino analogs of the amino acids histidine, diaminopimelate, ornithine, dopa, and phenylalanine have been found to be good competitive or irreversible inhibitors of histidine decarboxylase (17-19), diaminopimelate decarboxylase (20), ornithine decarboxylase (21-24), dopa decarboxylase (25), and aromatic acid decarboxylase (26)

which are all pyridoxal dependent enzymes. *N*-hydroxyamino acids of glutamic acid and diaminopimelic acid were also found to be an irreversible inhibitor of glutamic acid decarboxylase (27) and a competitive inhibitor of diaminopimelate decarboxylase (20).

ADC would be inhibited by the above amino substituted amino acid analogs in the following manner. The initial step in the inibition would be the formation of an imine between the amino acid analog and the pyruvate cofactor. Because of the substituent on the amino nitrogen the substrate analog would not undergo normal enzymatic reaction and is much more stable towards hydrolysis than normal substrate amines. This would result in a covalently bound inhibitor that irreversibly inactivates ADC. The mechanism of inhibition is shown in Scheme 5-6.

Many methods are available to estimate amino acid decarboxylase activity. The most commonly applied method is the use of radiolabeled amino acid substrates which upon reaction with the corresponding amino acid decarboxylase enzyme, liberate $^{14}CO_2$ which can be trapped in base and quantitated by scintillation counting. There are several drawbacks to this method which include the lack of availability of the radiolabeled substrate and the inability in obtaining continuous enzyme data (28,29). Another method for measuring decarboxylase reactions is by measuring CO_2 evolution manometrically (30,31). The manometric method allows for continuous data collection, but the procedure is insensitive, tedious, and time consuming. Two other methods for monitoring CO_2 evolution include spectrophotometric assays which measure CO_2 production continuously with a coupled enzyme assay system (32,33) and the use of a CO_2 sensitive electrode (34). The spectrophotometric assays suffer from the drawback that the coupled assay system can only function under a narrow pH range and the CO_2 sensitive electrode involves an elaborate calibration procedure before useful data can be obtained.

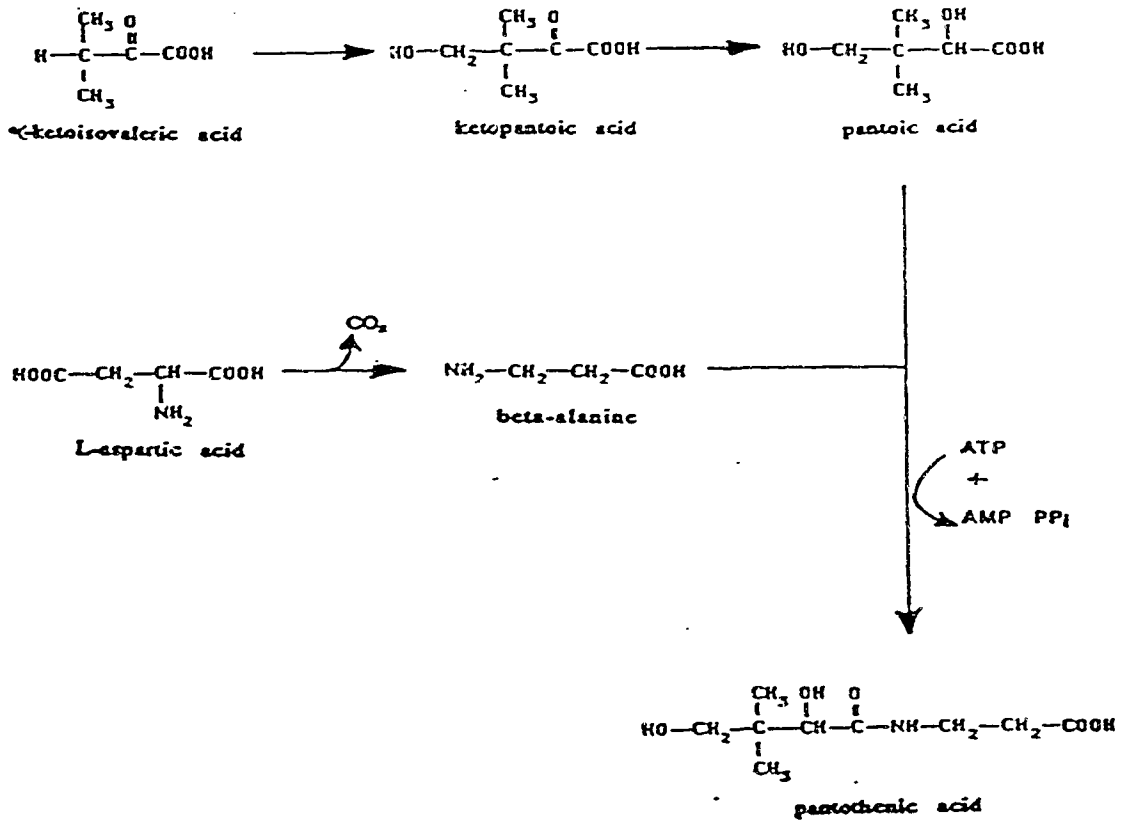
The amine products resulting from an amino acid decarboxylase reaction can also be isolated and quantitated to estimate enzyme activity. The most common methods of isolation of product amine are by HPLC (35), paper chromatography, electrophoresis (36) and ion exchange chromatography (38). Quantitation of the amine product is commonly achieved by derivatization of the amine with a molecule that can be measured either by UV (37), fluorescence (38,39), or by electrochemical means (40). These techniques are all highly sensitive but do not provide continuous enzyme data. Quantitation of the amine has also been achieved by coupling of the amine product to a secondary enzyme such as an amine oxidase (41), but this is not a general method since the amine product must be a

substrate for the amine oxidase. The final method for estimating decarboxylase activity involves the use of acid base indicators to measure the change in pH during reaction (42). This method works for decarboxylase enzymes which function in the acidic pH range.

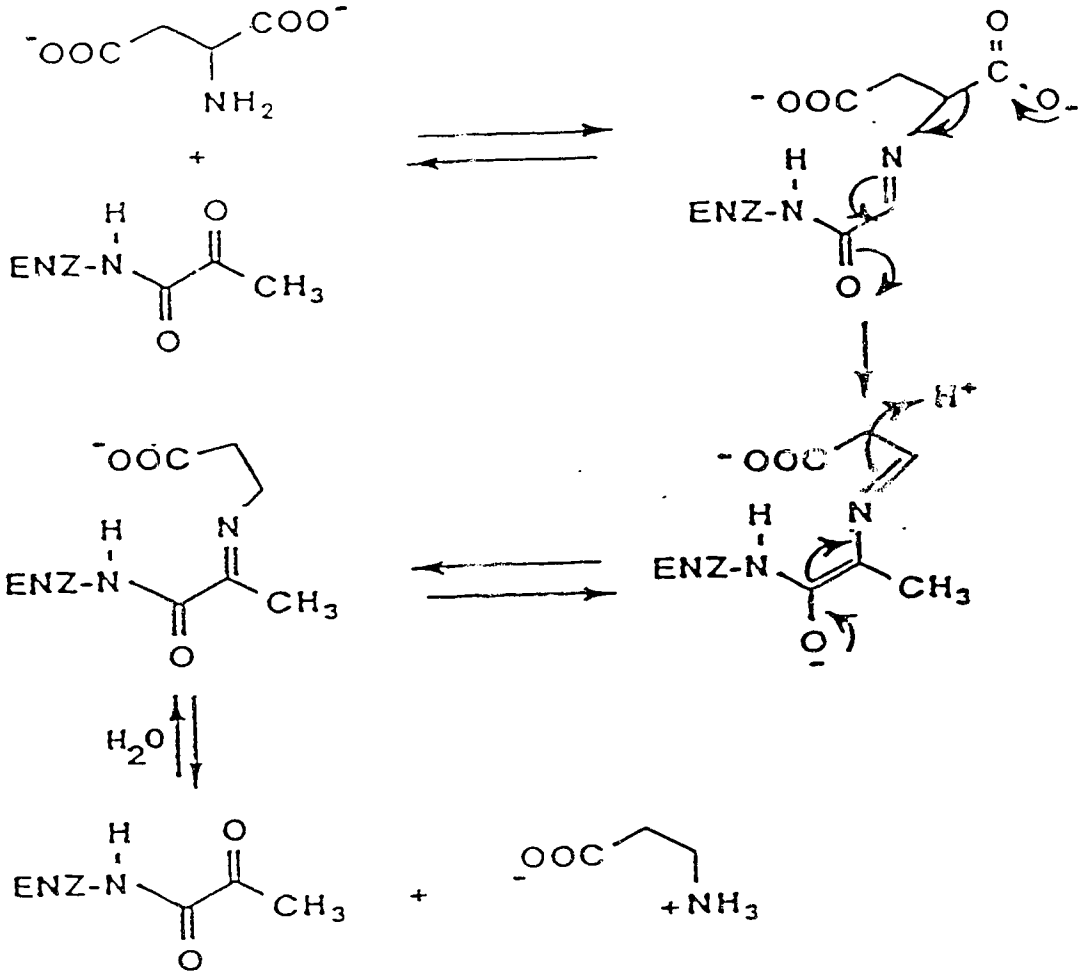
The research that will be discussed in the following two chapters is:

1. The results of a kinetic evaluation of amino acid analogs which include leaving groups at the β position, substitution at the amino nitrogen and derivatization at the substrate carboxy groups that can serve as potential mechanism based inhibitors for purified aspartate-α-decarboxylase. An unusual D specificity was observed in the inhibitor results.

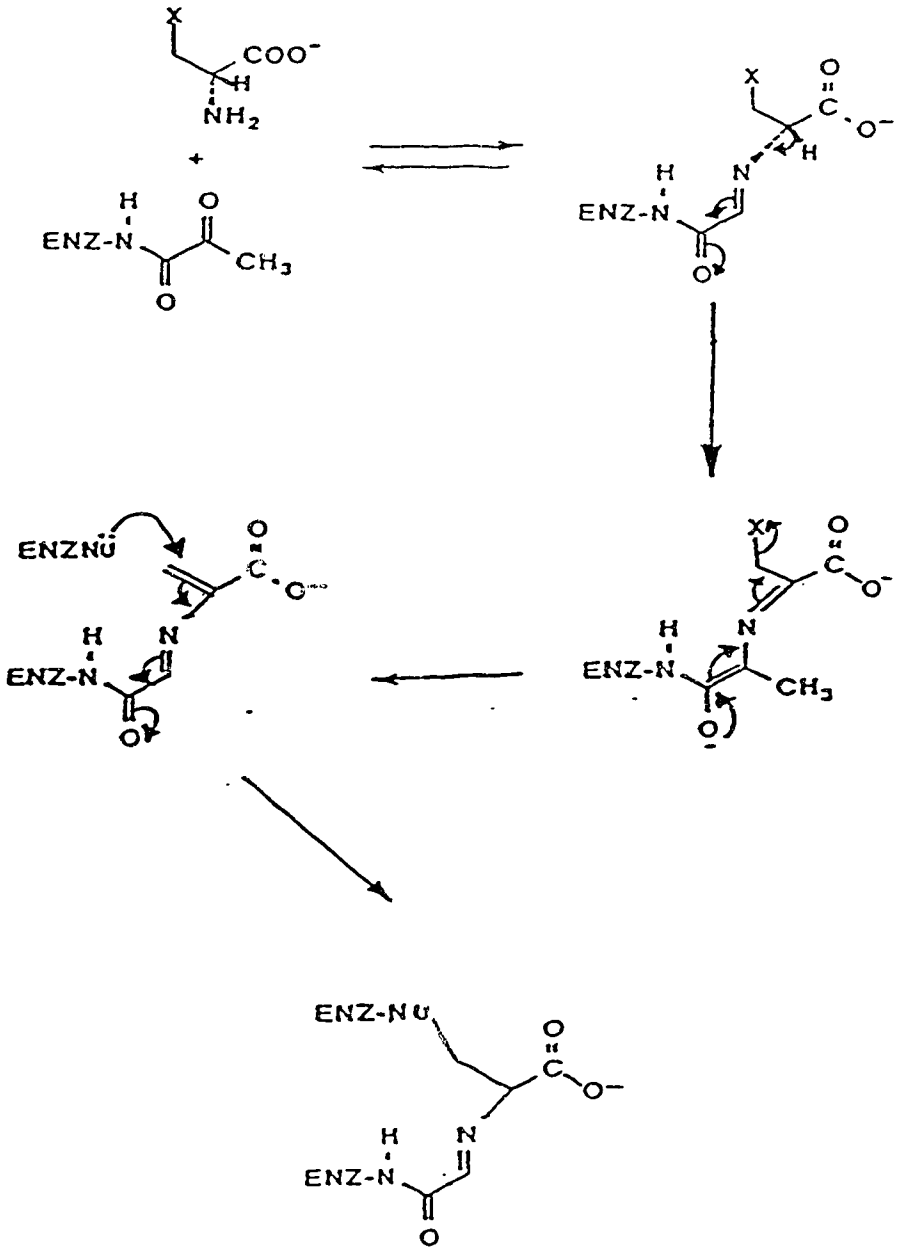
2. A new and rapid radiochemical assay for aspartate-α-decarboxylase, tyrosine decarboxylase and lysine decarboxylase was developed which utilizes ion exchange cartridges to separate product amine from amino acid substrate. If uniformly ^{14}C amino acid substrates are used, enhanced sensitivity is obtained. Further results indicate that this method can be applied to a wide variety of amino acid decarboxylase enzymes.



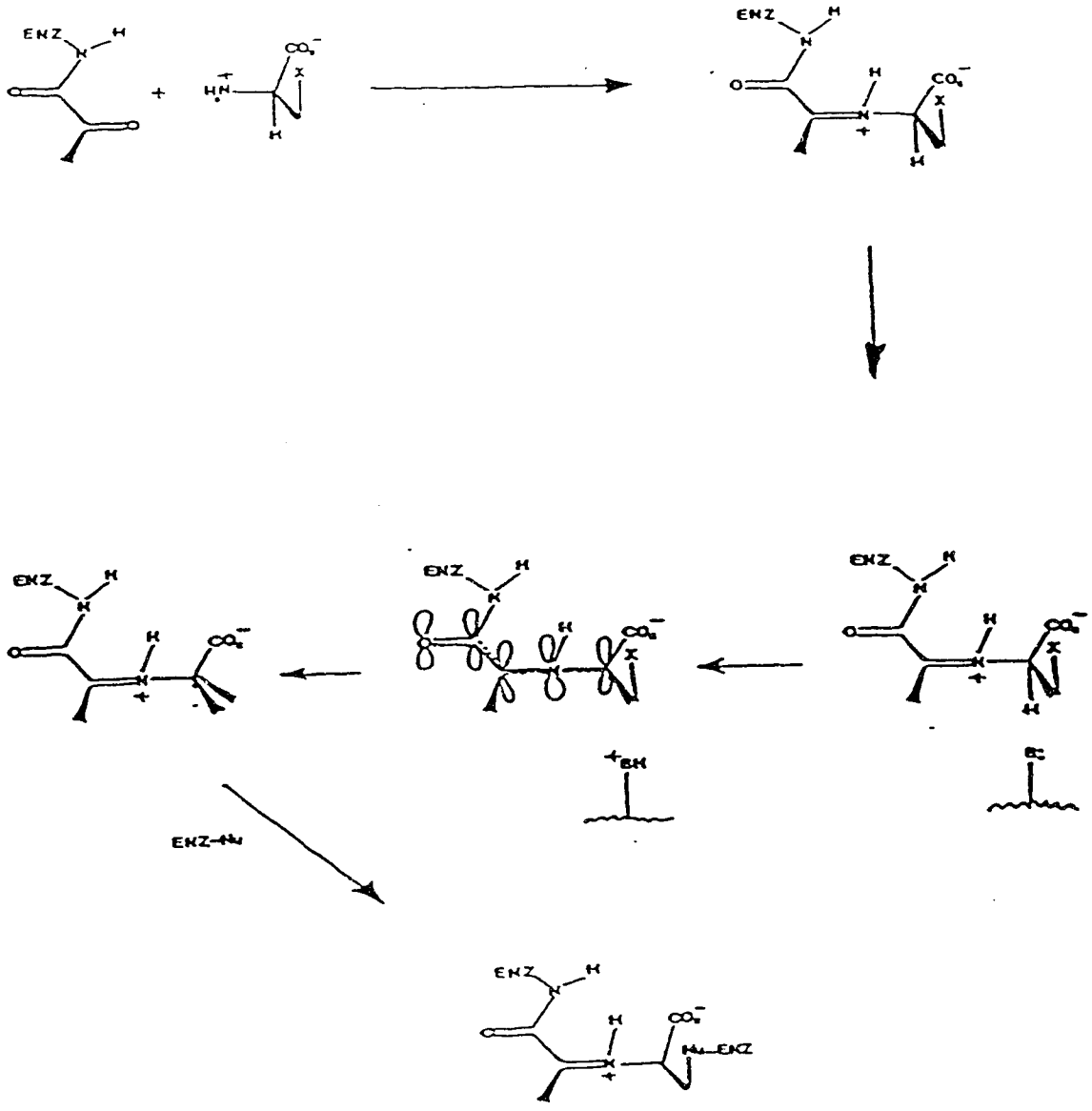
Scheme 5-1. Pantothenic acid Biosynthesis (5).



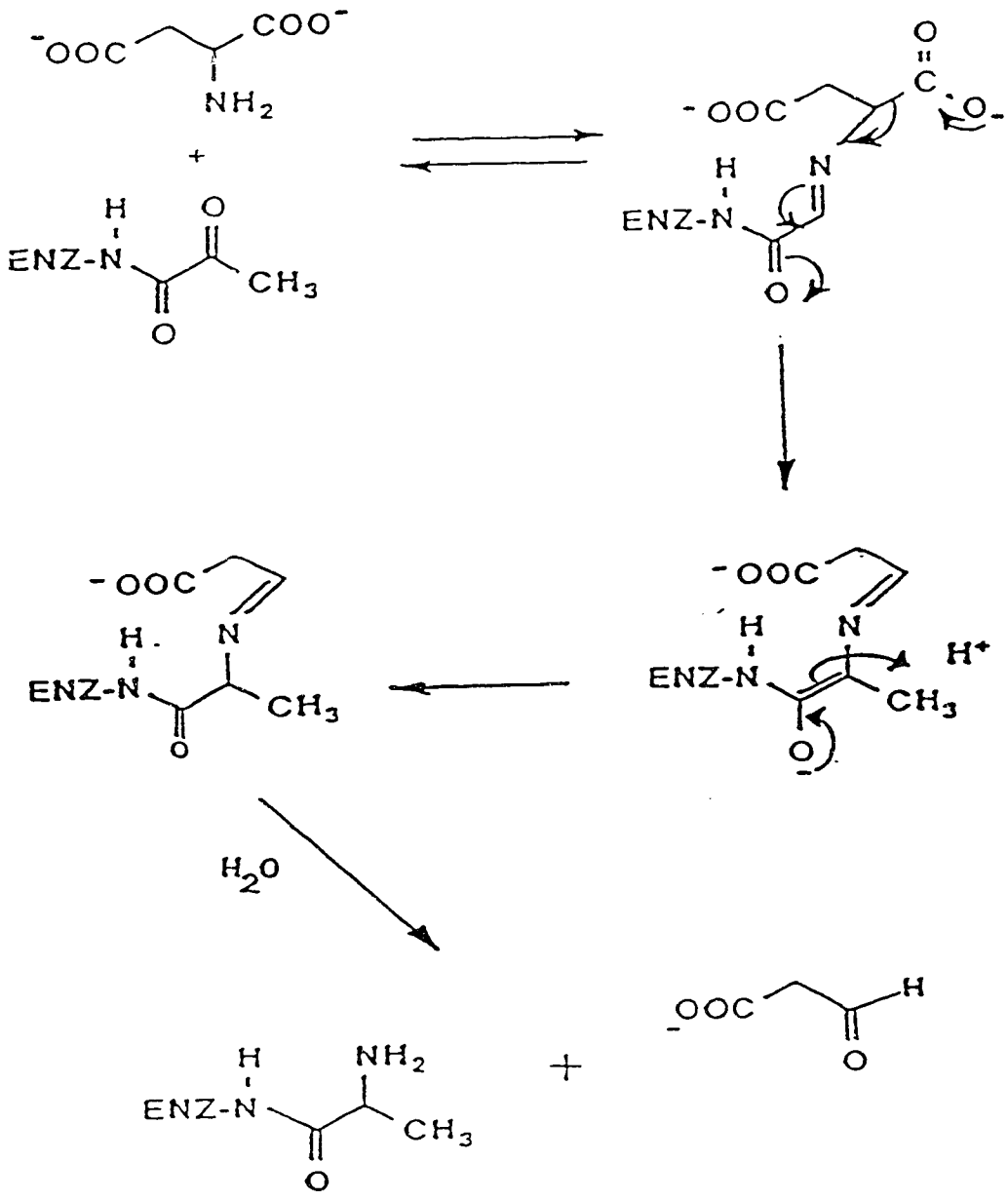
Scheme 5-2. Proposed mechanism for the decarboxylation of aspartate by L-aspartate-α-decarboxylase (43).



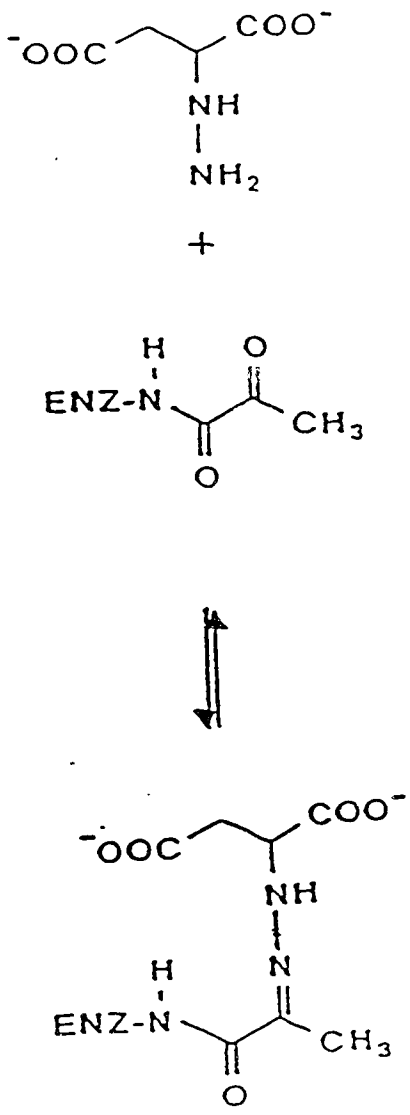
Scheme 5-3. Enzyme mediated β elimination of substrate analogs that contain leaving groups at the β position.



Scheme 5-4. Orientation of inhibitor in the active site which allows for labilization of the hydrogen.



Scheme 5-5. Substrate inactivation of L-aspartate-α-decarboxylase.



Scheme 5-6 Proposed mechanism of inactivation by *N*-substituted aspartic acid analogs.

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43. Figure 5-2 is taken from M. Gore (1988) Ph. D. thesis, Department of Chemistry, University of Alberta.

6. Analogs of Aspartic Acid as Inhibitors of Aspartate-α-Decarboxylase*

L-Aspartate-α-decarboxylase (E.C.4.1.1.11) catalyzes the conversion of L-aspartate to β-alanine. This enzyme provides a source of β-alanine that is used for the conversion of pantoic acid into pantothenic acid which is the major component of coenzyme A, an essential cofactor in fatty acid biosynthesis. The biosynthesis of pantothenic acid from β-alanine has been found to take place in plants and in microorganisms, but not in mammals which have to obtain pantothenic acid from dietary sources. Selective inhibition of pantothenic acid biosynthesis in plants and microorganisms may provide the basis for the development of antibacterial and herbicidal agents which exhibit low toxicity in mammals due to differences in metabolism.

L-Aspartate-α-decarboxylase was purified to homogeneity from *Escherishia coli W* (1). This enzyme is unusual in that the essential cofactor required for enzyme activity is a covalently bound pyruvate group rather than pyridoxal phosphate which is the cofactor most commonly used in amino acid decarboxylase enzymes (2). Only a small number of enzymes are known to contain pyruvoyl groups. These include histidine decarboxylase from several Gram postive bacteria (3), S-adenosylmethionine decarboxylase from *E. coli.* (4), phosphatidylserine decarboxylase from *E. coli* (5), and proline reductase from *Clostridium striklandi* (6).

Mechanistically, the covalently bound pyruvate should function in an analogous manner as pyridoxal phosphate (1), by initially forming an imine intermediate with the substrate and then functioning as an electron sink facilitating the decarboxylation reaction. Recently this has been shown to be the case for histidine decarboxylase, the best studied pyruvoyl dependent decarboxylase enzyme (7).

L-Aspartate-α-decarboxylase has been shown to be inactivated in a time dependent manner by its substrate L-aspartate while D-aspartate was also found to be a non competitive inhibitor (8). The mechanism of inactivation for L-aspartate appears to be a transamination reaction between aspartate and pyruvate, similar to what is observed for pyridoxal dependent decarboxylases. Inhibition of L-aspartate-α-decarboxylase has been

* Dr. J. Vederas, Department of Chemistry, University of Alberta was responsible for the chemical synthesis of aspartate analogs **6-1-6-4**.
Dr. M.Pickard, Department of Microbiology University of Alberta, was responsible for the antimicrobial testing and the growth of *E. coli.* for enzyme isolation.

achieved with amino acid analogs which contain good leaving groups at the β positions that undergo enzyme mediated β elimination reactions which can lead to enzyme inactivation (8,9). The D-isomers of β-chloro, β-fluoro and β-trifluoro alanine were found to be weak irreversible inhibitors of L-aspartate-α-decarboxylase while the L-isomers were inactive. Several derivatives of D-serine and D-cysteine were also shown to be irreversible inhibitors of the enzyme. L-Serine was also found to be a slow irreversible inhibitor of the decarboxylase enzyme. The irreversible inhibition was found to proceed through a two stage process, initially a first order time dependent inactivation occurs until intermediate levels of enzyme activity remain. During the second stage of inactivation, inhibition occurs at a much slower rate or ceases altogether. Addition of more inhibitor had no effect on further decreasing enzyme activity indicating that inhibition was not due to inhibitor depletion. Several competitive inhibitors of L-aspartate-α-decarboxylase have also been observed (1), they include: L-glutamate, β-hydroxy-D,L-aspartic acid, oxaloacetate, succinate and L-cysteic acid. Metabolic end products such as pantothenic acid and coenzyme A were not found to be inhibitors of L-aspartate-α-decarboxylase indicating that regulation of enzyme activity was not the result of feedback inhibition by metabolic end products.

Analogs of aspartic acid which contain derivatization at the amino nitrogen have the potential to be more effective inhibitors of L-aspartate-α-decarboxylase than β substituted amino acid analogs. *N*-hydroxyglutamic acid was found to be a potent inactivator of several pyridoxal dependent glutamic acid metabolizing enzymes including glutamic acid decarboxylase(10), while *N*-hydroxy-diaminopimelic acid was found to be a good competitive inhibitor of diaminopimelate decarboxylase (11). N-Amino derivatives (hydrazino) analogs of several amino acids have also been found to be potent competitive inhibitors of several pyridoxal dependent enzymes (11-13). Potent irreversible inhibition has been observed with the methyl and ethyl esters for the bacterial pyruvoyl dependent histidine decarboxylase (14). The mechanism of inactivation is not known, but one explanation that has been proposed (14) is that histidine methyl ester acylates a nucleophilic group at the active site forming a covalently bound enzyme. In the present work we describe the evaluation of several amino substituted and β substituted aspartic acid analogs, as well as several esters of aspartate, as inhibitors for purified L-aspartate-α-decarboxylase.

Experimental Procedures

Materials

L-aspartate, D-alaninol, L-alaninol, *N*-acetyl-L-aspartic acid, *N*-acetyl-D,L-aspartic acid, *N*-acetyl-D-alanine, *N*-acetyl-L-alanine, D-aspartate dimethyl ester, L-aspartate β-benzyl ester, D,L-malic acid, and 1M hyamine hydroxide were from Sigma Chemical Co. L-[U-^{14}C] aspartic acid (216mCi /mmol) and ACS scintillation cocktail were from Amersham. DEAE-Sephadex A-50 and Ultrogel AcA 44 were from Pharmacia LKB Biotechnology Inc. Hydroxyapatite (Bio-Gel HTP) was from Bio Rad. Ultrafiltration membranes (PM 10) were from Amicon. D and L-*N*-amino succinates (**6-3,6-4**) and D and L-oxyamino succinates (**6-1,6-3**) were chemically synthesized as described in detail elsewhere (15). All other reagents including buffer components were of reagent grade. Protein concentration was estimated with a protein assay kit from Bio Rad which is based on the method of Bradford (16) using bovine serum albumin as a standard.

Enzyme Purification

L-aspartate-α-decarboxylase was purified from 400g (wet weight) of *E.coli* (ATCC 9637) by slight modifications of the method of Williamson and Brown (1). All purification steps except for those mentioned were done at 4°C. Freshly grown cells were harvested, suspended in Buffer A (50 mM potassium phosphate pH 7.0, 5 mM EDTA, 50 μM dithiothreitol), and sonicated two times. The resulting cell sonicate was treated with DNase type 1 (25mg) for 20 min at room temperature and then centrifuged at 24,000xg to remove cellular debris. The supernatant solution was divided into 200 mL aliquots, heated to 55°C, and maintained at that temperature for 3 min. Each portion was immediately placed on ice and rapidly cooled to 4°C. The heat treated suspension was centrifuged as described above and the resulting pellets were discarded. Solid ammonium sulfate was added to the heat treated supernatant solution to 40% saturation (22), stirred overnight at 4°C, and centrifuged at 10,000xg for 30 min to remove precipitated protein. An additional amount of solid ammonium sulfate was added to the supernatant to make the solution 60% saturated in ammonium sulfate and stirred for an additional 10 hrs at 4°C. The resulting solution was centrifuged again at 10,000xg for 30 min and the pellet was dissolved in the minimum amount of Buffer A. The dissolved pellet solution was dialyzed against 3- 4 L changes of Buffer A and concentrated by ultrafiltration. The concentrated enzyme solution was loaded onto a DEAE-Sephadex A-50 column (5 x 38 cm) that was equilibrated in Buffer A in two

portions. The column was developed with a step wise salt gradient by washing the column with Buffer A containing increasing amounts of KCl ranging from 0.1 to 0.3 M, and fractions (25 mL) were collected at a flow rate of 150 mL/hr. A portion of enzyme activity was eluted in Buffer A containing no KCl, but the majority of the enzyme activity was eluted with 0.2 M KCl. Enzyme containing fractions were combined, concentrated by ultrafiltration, and dialyzed against 2-4 L changes of Buffer A. The concentrated decarboxylase enzyme was loaded onto Ultrogel AcA 44 (2.5 x 90 cm) at a flow rate of 60 mL/hr and the column was washed with Buffer A. Decarboxylase containing fractions were pooled, concentrated, and dialyzed overnight against Buffer B (5mM potassium phoshate, 5mM EDTA, 50 μM dithiothreitol). The final purification step was performed on hydroxyapatite (2.5 x 25 cm) that was equilibriated with Buffer B. The column was developed with the same buffer at a flow rate of 60 mL/hr and fractions were collected. A purification of 100 fold was obtained in a yield of 20% (700 units of L-aspartate-α-decarboxylase were obtained from 400g of freshly grown cells). The purified enzyme had a specific activity of 10.2 units/ mg where 1 unit is defined as the amount of enzyme producing 1 nmole of CO_2 per min at 37°C under standard assay conditions.

Enzyme Assays

a) *During Purification* - L-Aspartate-α-decarboxylase activity was assayed by measuring $^{14}CO_2$ evolved from L-[U-^{14}C] aspartic acid. Assays were carried out in plastic mini-scintillation vials. Assay buffer contained 50mM potassium phosphate, pH 7.0, 0.2mM aspartate, 65,000 dpm L-[U-^{14}C] aspartic acid, 5mM EDTA, 50 μM dithiothreitol and crude enzyme in a total volume of 125 μL. After incubation for 1 hr at 37°C, reaction mixtures were quenched with 100 μL of 10% trichloroacetic acid. Radiolabeled CO_2 evolved during reaction was trapped on a 1 x 1 cm piece of filter paper impregnated with 1M hyamine hydroxide that was attached to the cap of the vial. To ensure complete evolution of $^{14}CO_2$, reaction mixtures were shaken for 45 min after quenching. The filter papers were removed and counted in 10 mL of ACS scintillation cocktail.

b) *K_m Determination and Competitive Inhibition Studies* - Assays were carried out in an analagous manner in 50mM potassium phosphate buffer that contained 0.08-1.25mM aspartic acid,200,000 dpm L-[U-^{14}C] aspartic acid, 5mM EDTA, 50 μM dithiothreitol, (0.3 unit) enzyme and inhibitor in a total volume of 125 μL. Upon incubation for 1 hr at

37°C reaction mixtures were quenched with 100 μL of 10% trichloroacetic acid and processed as described above.

c) *Time Dependent Inhibition Studies* - The time dependence of inhibition was examined by incubating inhibitor and enzyme at 25°C and withdrawing 210 μL aliquots at 0, 10, 20, 40, 60, or 90 minute time intervals for assay by the above mentioned procedure. The substrate concentration was 0.2mM and 200,000 dpm L-[U-^{14}C] aspartic acid contained in a total volume of 250 μL. Assays were incubated for 1 hr and $^{14}CO_2$ evolution was estimated as before. Control experiments without inhibitor were carried out simultaneously.

Inhibition Kinetics

All substrate analogs were initially analyzed for time dependent inhibition by the procedure described above. Those that did not exhibit any time dependent inhibition were analyzed for competitive inhibition.

a) *Competitive Inhibitors* - Initial rate data were obtained in the presence and absence of inhibitor at five or six concentrations of substrate. Two concentrations of competitive inhibitor were used. Under the conditions previously described, no more than 15% turnover of substrate occurred to ensure linearity. Each point represents an average of duplicate determinations. The kinetic parameters, V_{max} and $K_{m,}$ data were estimated from initial rate using a computer program based on the method of Wilkinson (17). The true kinetic parameters and inhibition constants were obtained by plotting the slopes, intercepts and K_m(app) versus the concentration of inhibitor. Data obtained from the secondary plots were fit to the LINE program of Cleland (18).

b) *Time Dependent Inhibitors* - Irreversible inhibitors were analyzed according to the method of Kitz and Wilson (19). The primary data were plotted as natural log of the % activity versus time for each concentration of inhibitor. A secondary plot of the reciprocal of k_{cat} (obtained from the slope of the primary plots) versus the reciprocal of the inhibitor concentration provided a value for the inhibition constant (K_i) and a value for K_{cat}.

Antibacterial Testing

All bacteria used were from the American Type Culture Collection (ATCC, Rockville, MD) and identified by the following accession numbers: *Arthrobacter simplex* 6946, *Bacillus subtilis* 6051, *B. cereus* 27348, *B. megaterium* 15374, *Escherichia coli* 9637, *Micrococcus roseus* 186, *M.luteus (lysodeikticus)* 4698, *Pseudomonas aeruginosa* 10145, *Propionibacterium theoni* 4874, *Staphylococcus aureus* 6538P,

Streptomyces antibioticus 8663. All organisms grew well at optimal growth temperatures in the media recommended in the ATCC catalogue. Some studies were carried with bacteria grown in defined media. *B. megaterium* and *B. cereus* were grown in modified medium W as described by White (20) while *E.coli* and *P. aeruginosa* were both grown in the media of Davis and Mingioli (21). *P. theoni* was grown in an anaerobic jar using a BBL Gas Pac (Becton Dickinson , Cockeysville, MD), all other bacteria were grown aerobically with shaking.

Inhibition of growth in liquid culture was performed in roller tubes with 10 mL of medium, sterilized by autoclaving for 20 min at 121°C, and inoculated with 1% of an overnight culture. Analogs were added as filter sterilized aqueous solutions at a concentration of 5, 20, or 100 μg/mL. Growth was for 18 hrs or until tubes with no analog showed adequate turbidity (OD 600nm >0.2). Experimental values were recorded relative to controls. All tests were done in duplicate.

Results

Synthesis of D and L Oxyamino- and N-Aminosuccinates

D-Oxyaminosuccinate (**6-1**) and D-*N*-aminosuccinate (**6-3**) were synthesized by the route shown in Scheme 6-1. L-Malic acid furnished the starting material for the overall synthesis, and was converted into the dibenzyl esters which were further treated with triflic anhydride to furnish the corresponding triflates. D-Oxyaminosuccinate (**6-1**) was prepared by treating the triflate derived from L-malic acid with the lithium salt of tert-butyl-*N*-hydroxycarbamate to afford *N*-Boc protected derivative of **6-1** which was deprotected by hydrogenolysis and acid treatment to give the desired compound. D-*N*-Aminosuccinate (**6-3**) was obtained by treating a similar triflate intermediate with benzylcarbazate to afford **6-3** after deprotection. L-Oxyaminosuccinate (**6-2**) and L-*N*-aminosuccinate (**6-4**) were obtained by a similar route starting with D-malic acid as shown in scheme 6-2.The chemical synthesis was done by Dr. M. Gore in Dr. J. Vederas's group in the Department of Chemistry.

Interaction of Substrate Analogs with L-Aspartate-α-Decarboxylase

A survey was conducted to examine which substrate analogs inhibited the decarboxylation reaction catalyzed by L-aspartate-α-decarboxylase. The results of the inhibition studies are shown in Tables 6-1 and 6-2. Inhibitor results indicated that

compounds **6-1** to **6-4** were good slow acting irreversible inhibitors of aspartate-α-decarboxylase with K_i's of 70 μM for D-oxyaminosuccinate **6-1** (Figure 6-1), 106 μM for L-oxyaminosuccinate **6-2** (Figure 6-2), 34 μM for D-*N*-aminosuccinate **6-3** (Figure 6-3), and 76 μM for L-*N*-aminosuccinate **6-4** (Figure 6-4) respectively. Inactivation proceded by a two stage mechanism; slow linear inactivation initially occured until intermediate levels of enzyme activity remained, followed by a second stage where no further inactivation occurred. Inhibition was irreversible since activity could not be restored upon exhaustive dialysis. Several moderate competitive inhibitors were found for L-aspartate-α-decarboxylase; D and L alaninol had K_i's of 2.7 and 4.2 mM respectively (Figure 6-5 and 6-6). All other substrate analogs tested exhibited no inhibition at the concentrations indicated in Table 6-2.

Antibacterial Activity

The results of the antibacterial testing indicated that D and L-oxyamino and D and L-*N*-amino succinates (**6-1-6-4**) inhibited the growth of *E.coli* under defined conditions. No inhibition was observed with other organisms examined. Analogs **6-1**, **6-2**, and **6-4** inhibited *E.coli* growth at a concentration of 5 μg/ mL by 46, 43, and 65% respectively. Analog **6-3** exhibited somewhat weaker inhibition, 14% inhibition at an analog concentration of 20 μg/ mL.

Discussion

If one examines the overall decarboxylation mechanism of L-aspartate-α-decarboxylase (See Scheme 5-2), analogs of aspartic acid which contain derivatization at the substrate nitrogen might serve as mechanism based inhibitors. Several *N*-amino acid analogs have been shown to be potent inhibitors of mammalian histidine decarboxylase, aspartate aminotranferase, and diaminopimelate decarboxylase (11-13). Inhibition was thought to occur by the formation of a stable hydrazone intermediate that would not be susceptible to decarboxylation nor imine hydrolysis resulting in a covalently bound intermediate (See Scheme 5-6). The oxyamino succinates should function in an analogous manner. Both the D and L enantiomers of *N*-amino and oxyaminosuccinates exhibited a slow two stage irreversible inhibition of the decarboxylase activity that indicated an unusual D specificity. The best inhibitor was D-*N*-aminosuccinate **6-1**(K_i 34 μM) while the L enantiomer **6-2** was slightly weaker (K_i 76 μM). D-oxyaminosuccinate **6-3** (K_i 70

μM) was also found to be a better inhibitor than the corresponding L-enantiomer **6-4** (K_i 106μM). These results are somewhat unusual, but not unexpected, since Williamson and Brown (1) and Smith (8) observed the unusual D specificity with β substituted amino acid analog inhibitors for L-aspartate-α-decarboxylase. Preferential inhibition with D isomers of *N*-amino and oxyaminosuccinates may be due to the D isomer bearing a greater structural resemblance to the natural substrate than the corresponding L isomer when bound to the enzyme.

The two stage inhibition that was observed is similar to the results of Smith (8) with the D-isomers of β-chloro, β-fluoro, and β-trifluoro alanine which irreversibly inhibited L-aspartate-α-decarboxylase only to intermediate levels. Highly purified L-aspartate-α-decarboxylase has been shown to exist as a mixed enzyme species as shown by electrophoresis by non denaturing gels and staining for activity (9). The mixed enzyme species may contain a form which is resistant to inactivation by aspartic acid analogs which contain substitution at the amino nitrogen or at the β position, resulting in the observed partial inactivation.

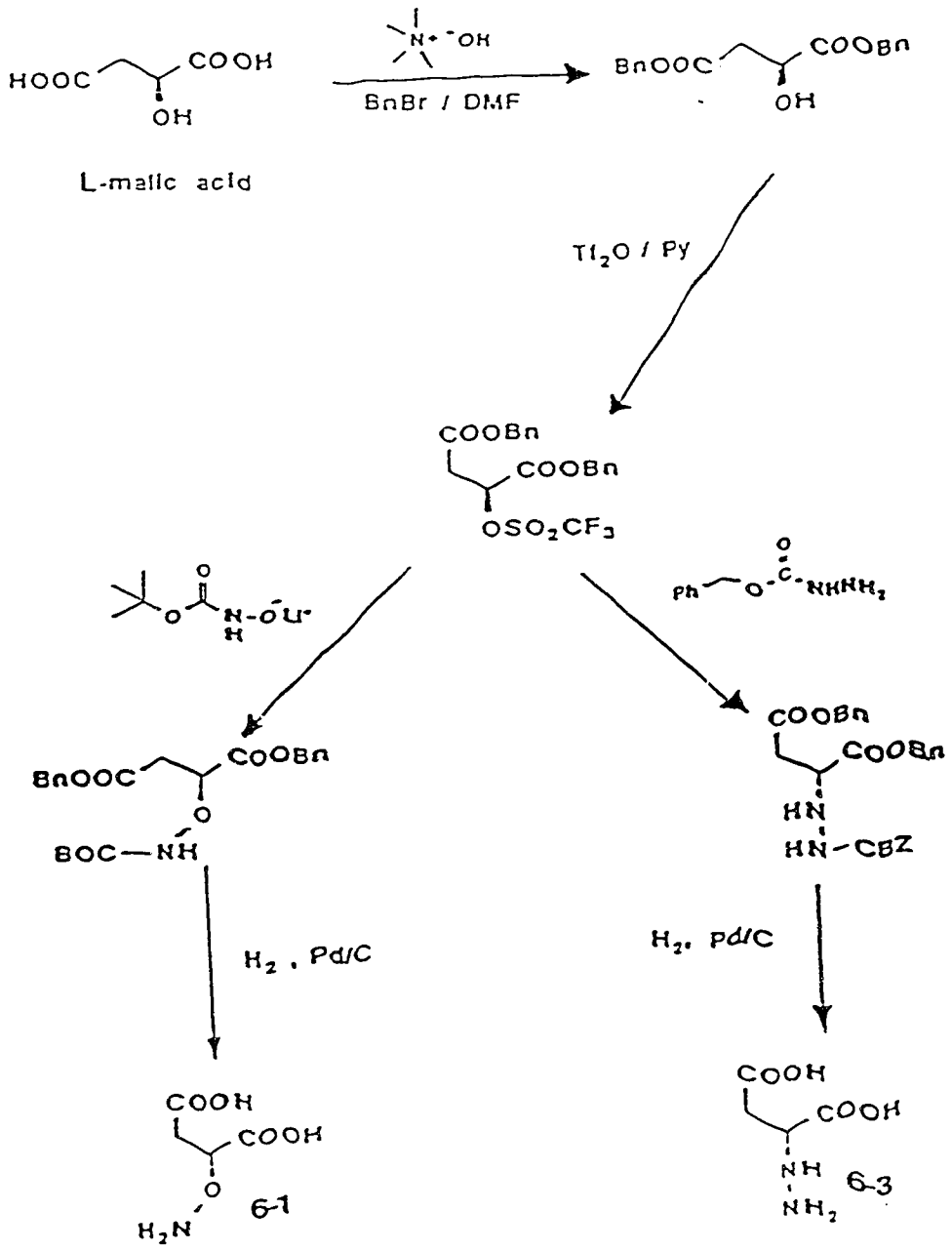
Inhibition of L-aspartate-α-decarboxylase by amino acid analogs which contain leaving groups at the β position can be achieved by undergoing enzyme mediated β elimination, then nucleophilic attack with the formation of a covalently bound inactive enzyme (See Scheme 5-3). The initial step in the overall inactivation is the labilization of the proton on the α carbon which is orientated perpendicular to the π system of the pyruvate cofactor which stabilizes the resulting carbanionic intermediate. The leaving group at the β position must also be orientated perpendicular to the π system to facilitate the elimination reaction. The elimination reaction generates an intermediate that is prone to nucleophilic attack which may lead to the production of a covalently modified enzyme active site. D and L-Alaninol in principle should inhibit L-aspartate-α-decarboxylase by a similar mechanism, thus resulting in irreversible inhibition. D and L-Alaninol, however, were found to be moderate competitive inhibitors with K_i's of 2.7 and 4.2 mM respectively. The failure of alaninols to exhibit time dependent inactivation may be due to the poor leaving group ability of the hydroxyl group which is found in D and L-alaninol. Alternatively, orientation of the hydroxyl group at the β position, such that elimination cannot occur, would also account for the lack of irreversible inhibition.

Esters of aspartate provided no inhibition even at millimolar concentrations. These results are similar to those observed for the methyl esters of L-tyrosine, L-phenylalanine, and L-arginine with their corresponding decarboxylase enzymes (14). The lack of

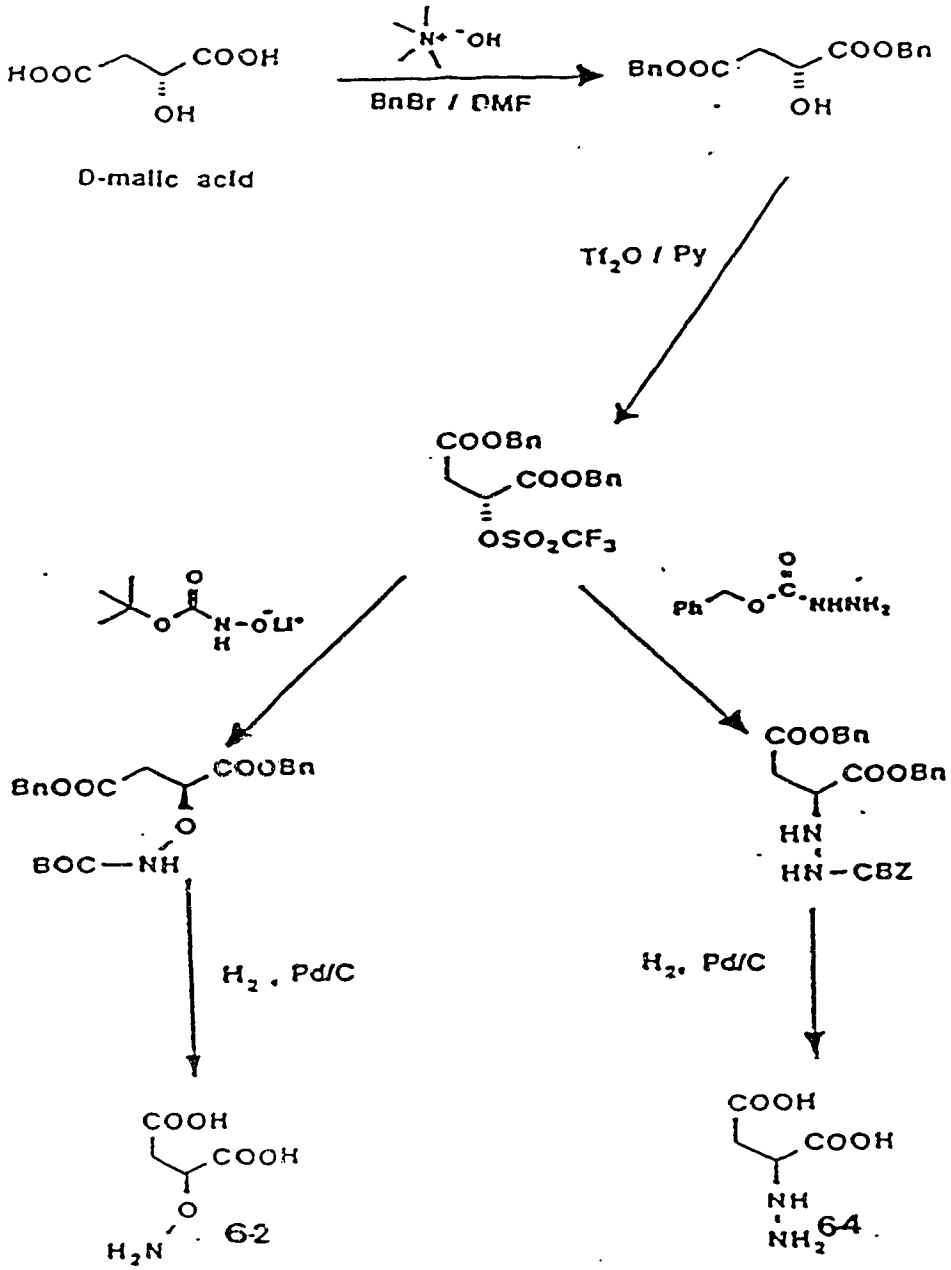
inhibition by aspartate esters may be due to the inability of the analogs to enter the active site due to steric constraints.

Similarly, the lack of inhibition by the *N*-acetyl analogs of aspartic acid and alanine may also be due to the inability of these analogs to bind to the enzyme. Lack of inhibition with *N*-acetyl analogs along with the inability of D,L-malic acid to inhibit the decarboxylase activity suggests that the substrate nitrogen is an important component in determining whether an analog can enter the enzyme's active site.

Antimicrobial susceptibility tests indicated that analogs **6-1**, **6-2**, and **6-4** showed good antibacterial activity only against *E.coli*.. These results suggest that aspartic acid metabolism plays only a minor role in the vital functioning of the other microorganisms tested. The inability of the analogs to inhibit the growth of the other organisms examined may also be due to ineffective transport of the analogs into the cell. The best inhibitor of L-aspartate-α-decarboxylase, D-*N*-aminosuccinate **6-3** was found only to weakly inhibit the growth of *E.coli* indicating that another aspartate metabolizing enzyme may be inhibited by these aspartate analogs as well, thus producing the observed antibacterial results.



Scheme 6-1. Stereospecific synthesis of D-oxyaminosuccinate **6-1** and D-*N*-amino-succinate **6-3**.



Scheme 6-2. Stereospecific synthesis of L-oxyaminosuccinate **6-2** and L-*N*-aminosuccinate **6-4**.

Table 6-1: Inhibitors of L-Aspartate-α-decarboxylase*

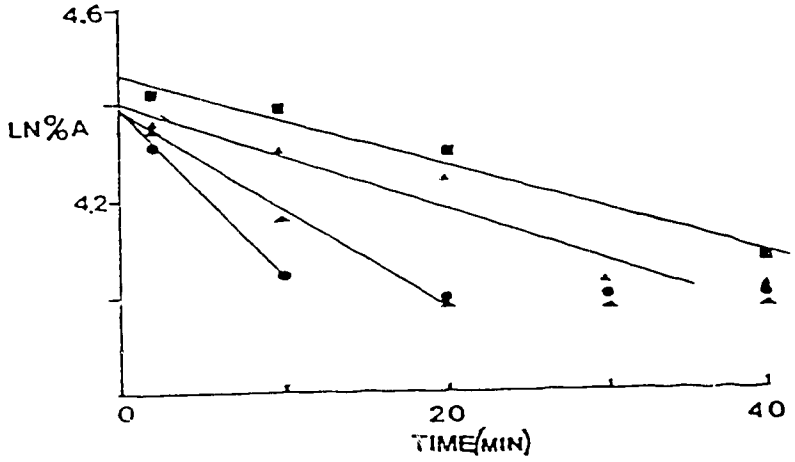
Analog	Structure R_1	R_2	R_3	K_i	k_{cat}
D-*N*-aminosuccinate (**6-1**)	NNH_2	COOH	COOH	34 μM	$2.6X10^{-2}min^{-1}$
L-*N*-aminosuccinate (**6-2**)	NNH_2	COOH	COOH	76 μM	$8.5X10^{-3}min^{-1}$
D-oxyaminosuccinate (**6-3**)	ONH_2	COOH	COOH	70 μM	$8.8X10^{-3}min^{-1}$
L-oxyaminosuccinate (**6-4**)	ONH_2	COOH	COOH	106 μM	$1.9X10^{-2}min^{-1}$
D-alaninol	NH_2	OH	CH_3	2.7 mM	
L-alaninol	NH_2	OH	CH_3	4.2 mM	

* Assay conditions and kinetic evaluations described in experimental procedures.

Table 6-2: Noninhibitors of L-Aspartate-α-decarboxylase +

Analog	R_1	R_2	R_3	[I]mM
D-aspartate-dimethyl ester	NH_2	COOMe	COOMe	16
L-aspartate-β-benzyl ester	NH_2	CH_2Ph	COOH	3.2
N-acetyl-L-aspartate	NHAc	COOH	COOH	16
N-acetyl-D,L-aspartate	NHAc	COOH	COOH	8
N-acetyl-D-alanine	NHAc	H	COOH	5.4
N-acetyl-L-alanine	NHAc	H	COOH	5.4
D,L-malic acid	OH	COOH	COOH	14

+ not inhibitory at the concentration indicated. Aspartate concentration was 0.2 mM



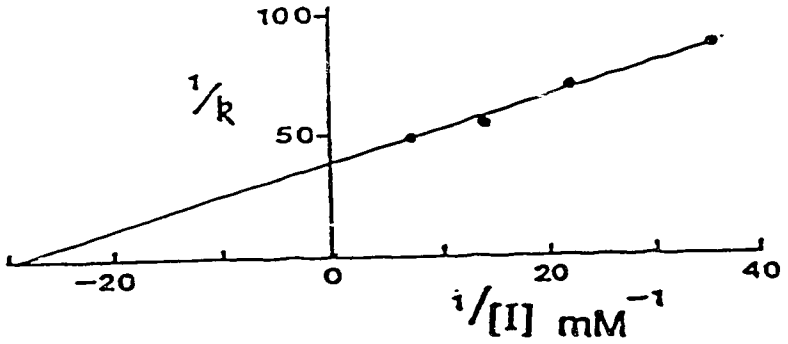
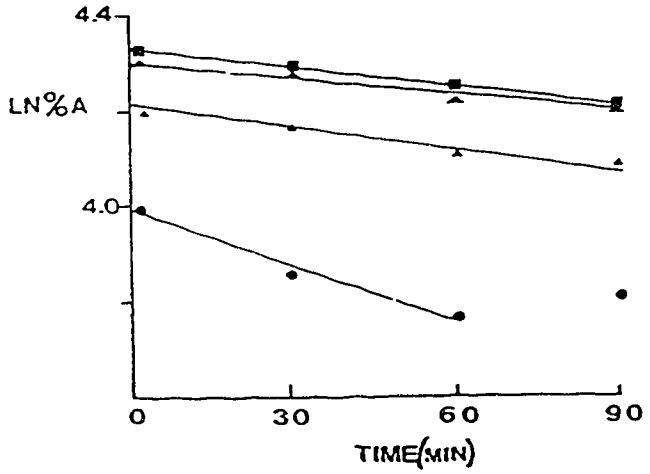


Figure 6-1. Time dependent inactivation of L-aspartate-α-decarboxylase by substrate analog **6-1**. Concentrations of **6-1** were: 0.06 mM (■), 0.12 mM (▲), 0.18 mM (●), and 0.24 mM (▲). Enzyme and **6-1** were incubated at 25°C for the indicated time intervals and 210 μL aliquots were removed and assayed under the conditions described in the experimental section. Controls in the absence of inhibitor were done simultaneously. Data were plotted as the natural log of the percent activity versus time. The linear portions of Figure 6-1 were fit by linear regression and the slope of this line yielded the pseudo-first order rate constant for the inactivation (k_{cat}) at the given concentration of **6-1**.

Figure 6-1a. Plot of inverse of k_{cat} against 1/[**6-1**]. The resulting intercepts yielded values for k_{cat} and K_i respectively.



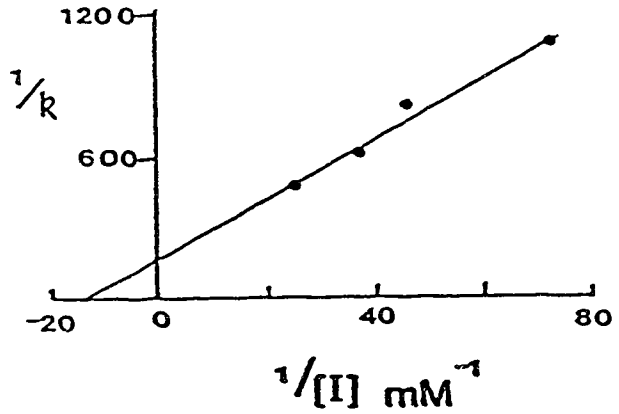
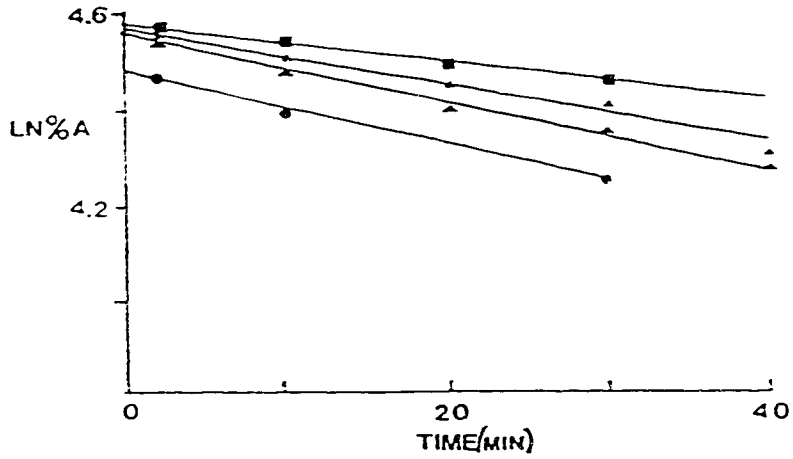


Figure 6-2. Time dependent inactivation of L-aspartate-α-decarboxylase by substrate analog **6-2**. Concentrations of **6-2** were: 0.0115 mM (■), 0.0184 mM (▲), 0.023 mM (▲), and 0.046 mM (●). Enzyme and **6-2** were incubated at 25°C for the indicated time intervals and 210 μL aliquots were removed and assayed under the conditions described in the experimental section. Controls in the absence of inhibitor were done simultaneously. Data were plotted as the natural log of the percent activity versus time. The linear portions of Figure 6-2 were fit by linear regression and the slope of this line yielded the pseudo-first order rate constant for the inactivation (k_{cat}) at the given concentration of **6-2**.

Figure 6-2a. Plot of inverse of k_{cat} against 1/[**6-2**]. The resulting intercepts yielded values for k_{cat} and K_i respectively.



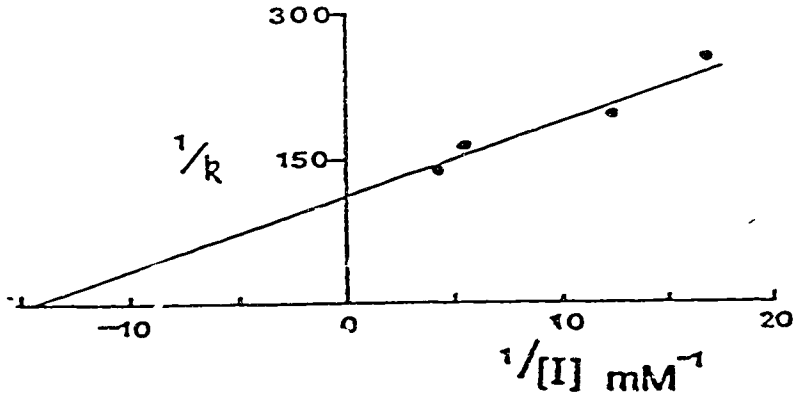
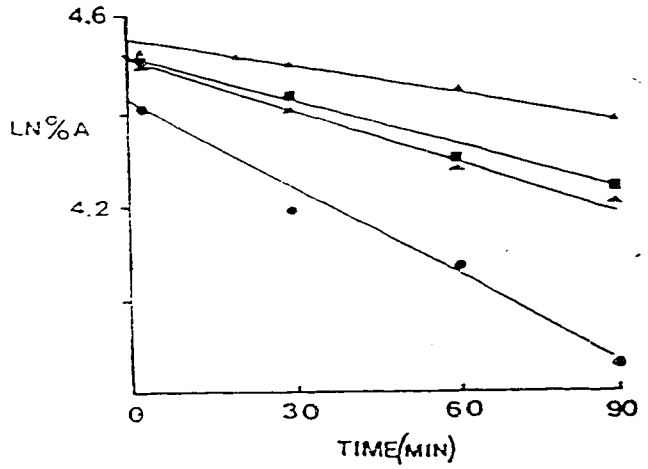


Figure 6-3. Time dependent inactivation of L-aspartate-α-decarboxylase by substrate analog **6-3**. Concentrations of **6-3** were: 0.0278mM (■), 0.044 mM (▲), 0.083 mM (▲), and 0.139 mM (●). Enzyme and **6-3** were incubated at 25°C for the indicated time intervals and 210 μL aliquots were removed and assayed under the conditions described in the experimental section. Controls in the absence of inhibitor were done simultaneously. Data were plotted as the natural log of the percent activity versus time. The linear portions of Figure 6-3 were fit by linear regression and the slope of this line yielded the pseudo-first order rate constant for the inactivation (k_{cat}) at the given concentration of **6-3**.

Figure 6-3a. Plot of inverse of k_{cat} against 1/[**6-3**]. The resulting intercepts yielded values for k_{cat} and K_i respectively.



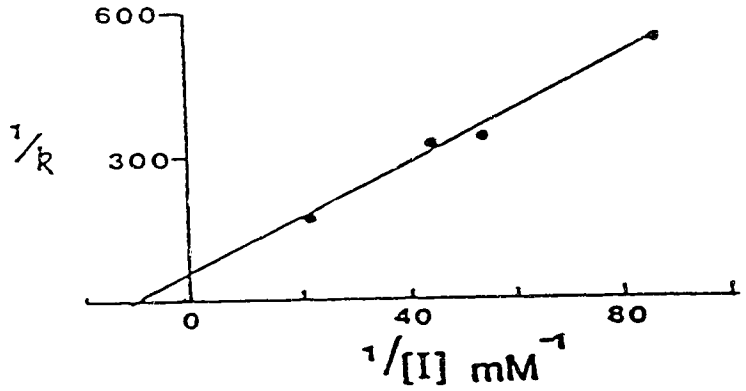


Figure 6-4. Time dependent inactivation of L-aspartate-α-decarboxylase by substrate analog **6-4**. Concentrations of **6-4** were: 0.0138mM (▲), 0.022 mM (■), 0.0275 mM (▲), and 0.0423 mM (●). Enzyme and **6-4** were incubated at 25°C for the indicated time intervals and 210 μL aliquots were removed and assayed under the conditions described in the experimental section. Controls in the absence of inhibitor were done simultaneously. Data were plotted as the natural log of the percent activity versus time. The linear portions of Figure 6-4 were fit by linear regression and the slope of this line yielded the pseudo-first order rate constant for the inactivation (k_{cat}) at the given concentration of **6-4**.

Figure 6-4a. Plot of inverse of k_{cat} verses 1/[**6-4**]. The resulting intercepts yielded values for k_{cat} and K_i respectively.

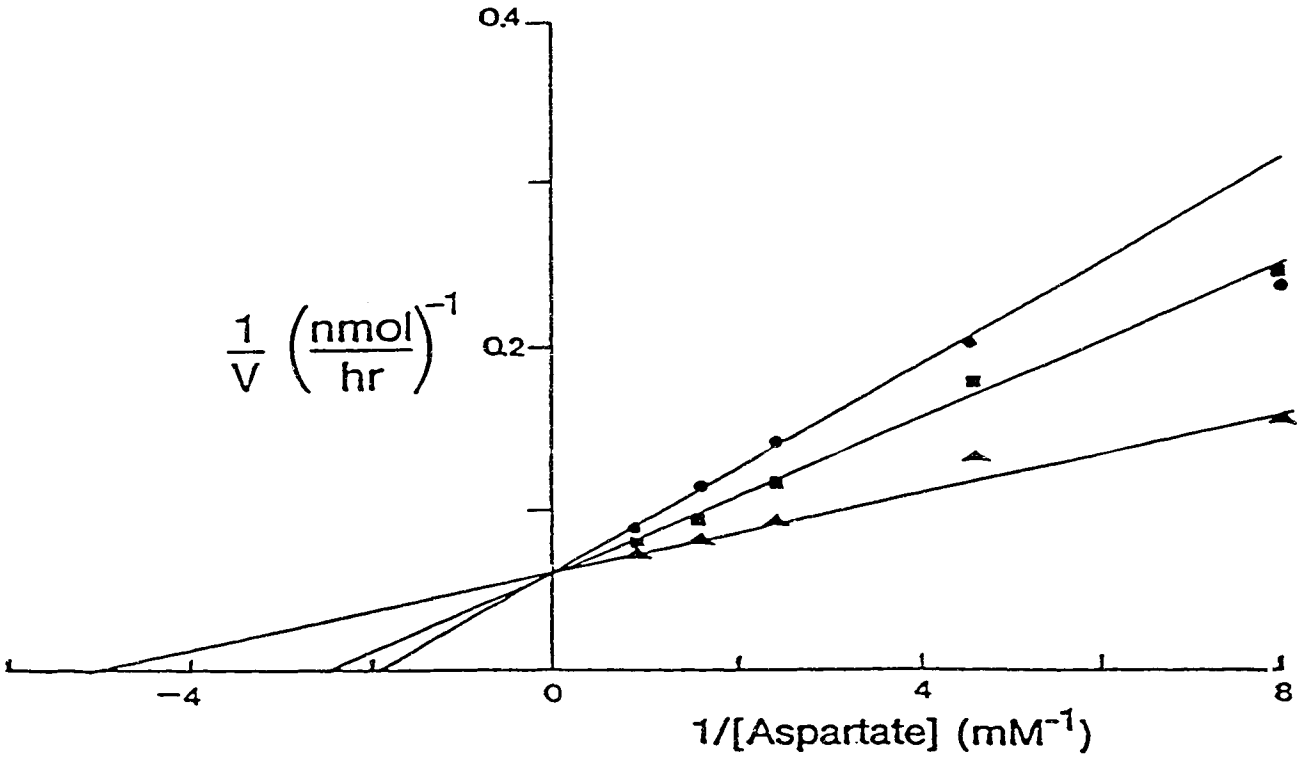


Figure 6-5. Competitive inhibition of the aspartate-α-decarboxylase reaction by D-alaninol. Reactions were done in the presence and absence of inhibitor for 1 hr at 37°C and pH 7.0 using 0.08-1.25 mM aspartate, 130,000 dpm L-[U-^{14}C] aspartic acid and enzyme (0.3 units) in a total volume of 125 µL. Concentrations of D-alaninol were 0 mM (▲), 2.24 mM (■) and 3.73 mM (●).

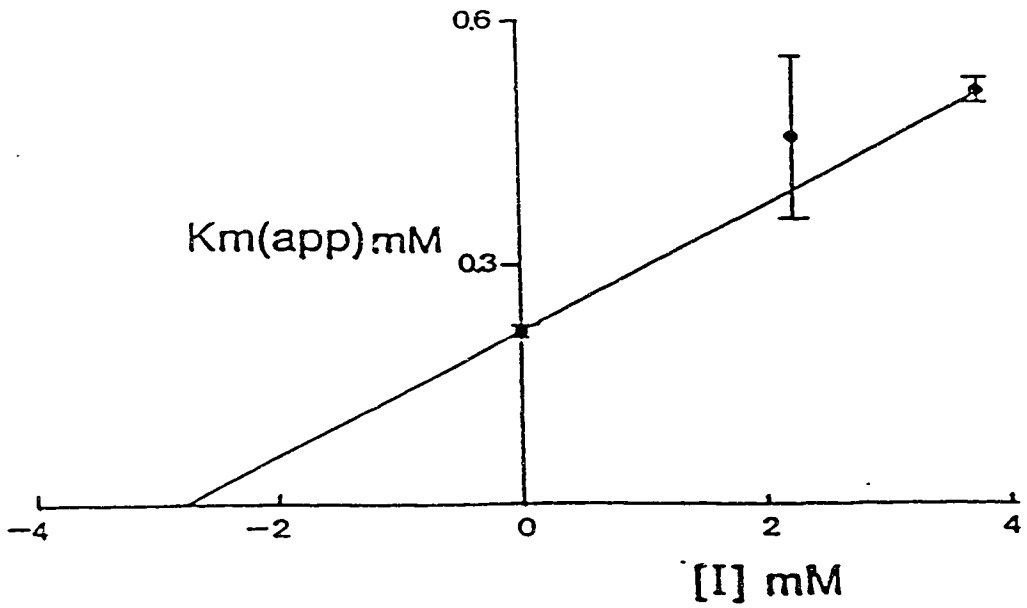


Figure 6-5a. Secondary plot of K_m(app) vs D-alaninol obtained for the data in Figure 6-5. A K_i of 2.76 mM was obtained with the Line program of Cleland (18).

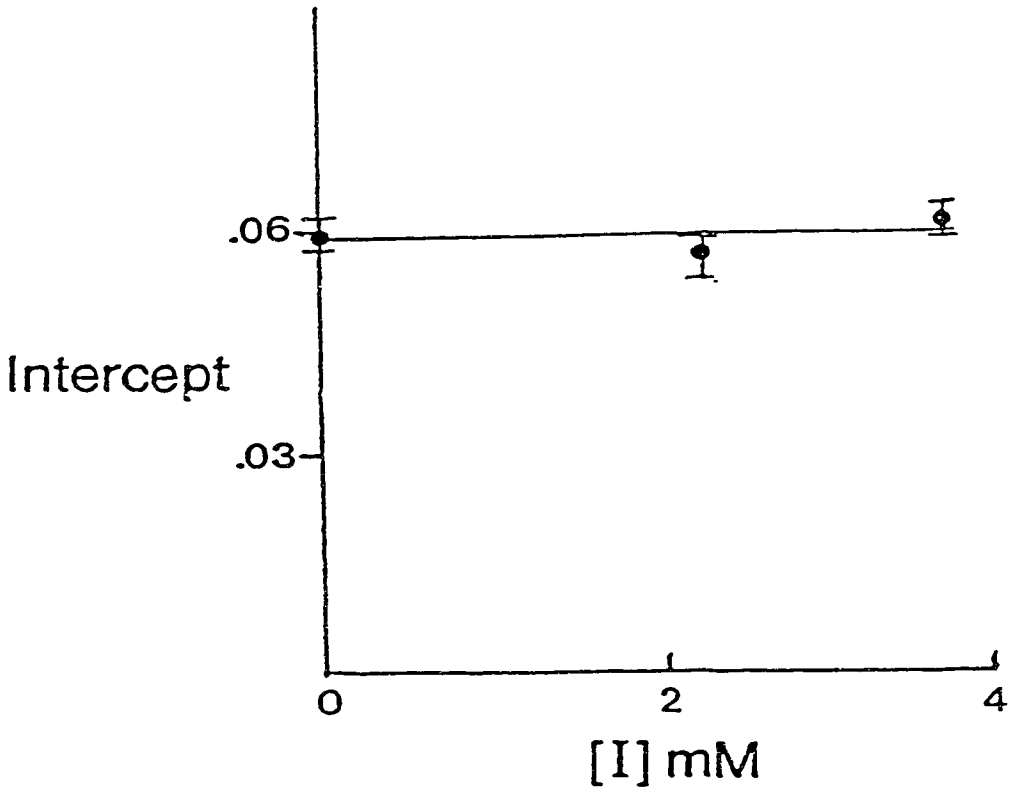


Figure 6-5b. Plot of the intercept vs the concentration of D-alaninol. The invariance of the intercept as predicted for a competitive inhibitor.

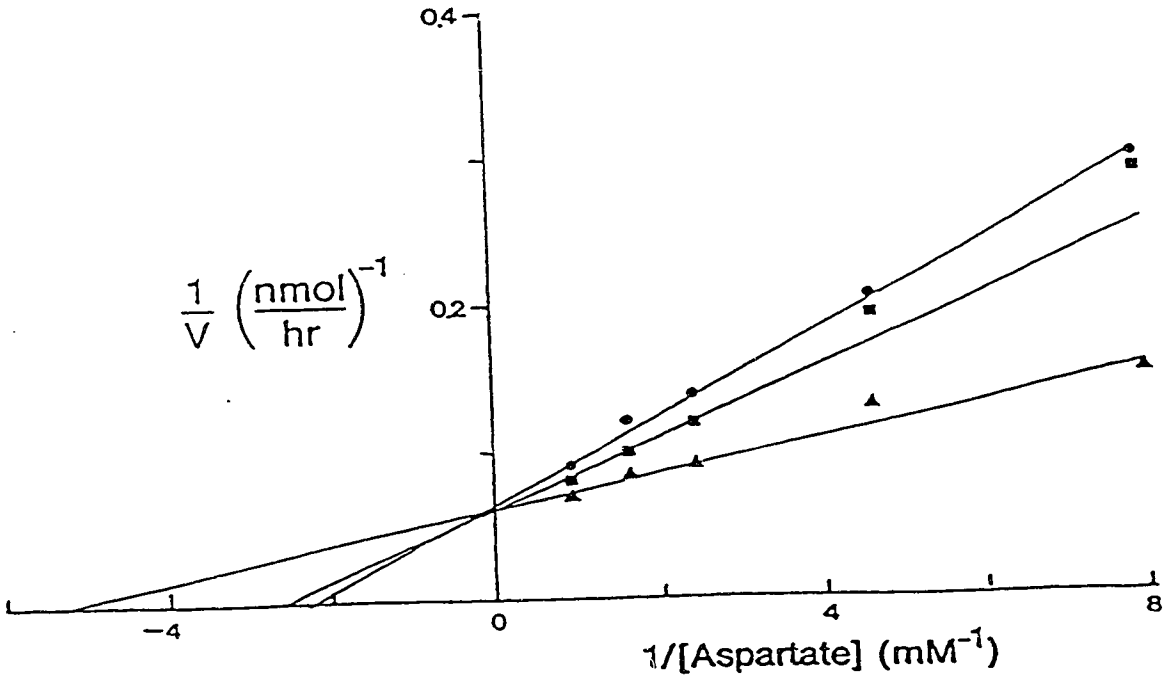


Figure 6-6. Competitive inhibition of the aspartate-α-decarboxylase reaction by L-alaninol. Reactions were done in the presence and absence of inhibitor for 1 hr at 37°C and pH 7.0 using 0.08-1.25 mM aspartate, 130,000 dpm L-[U-^{14}C] aspartic acid and enzyme (0.3 units) in a total volume of 125 µL. Concentrations of L-alaninol were 0 mM (▲), 3.73 mM (■) and 5.63 mM (●).

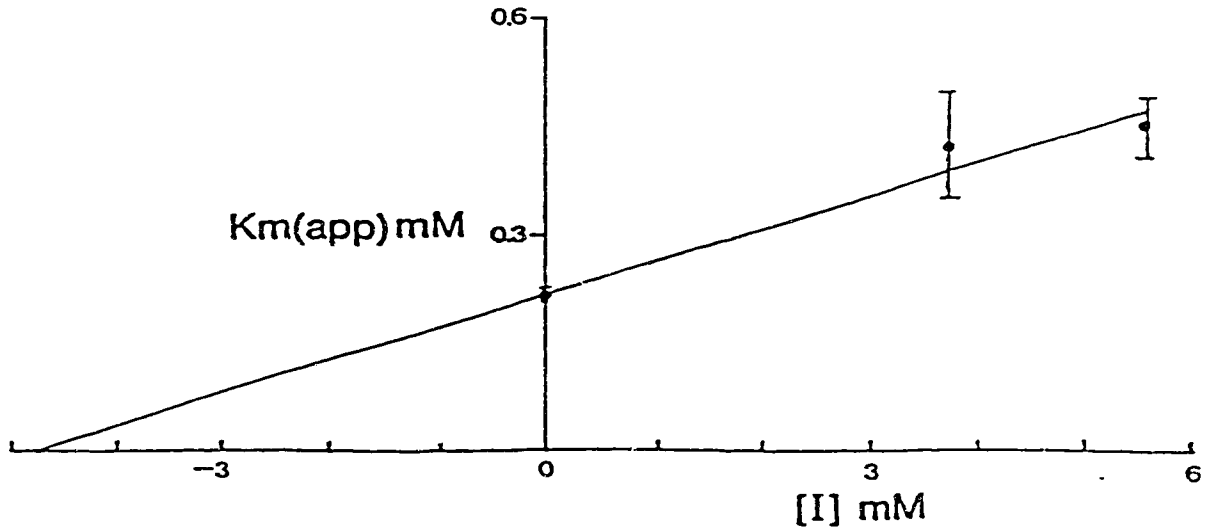


Figure 6-6a. Secondary plot of K_m(app) vs L-alaninol obtained for the data in Figure 6-6. A K_i of 4.2 mM was obtained with the Line program of Cleland (18).

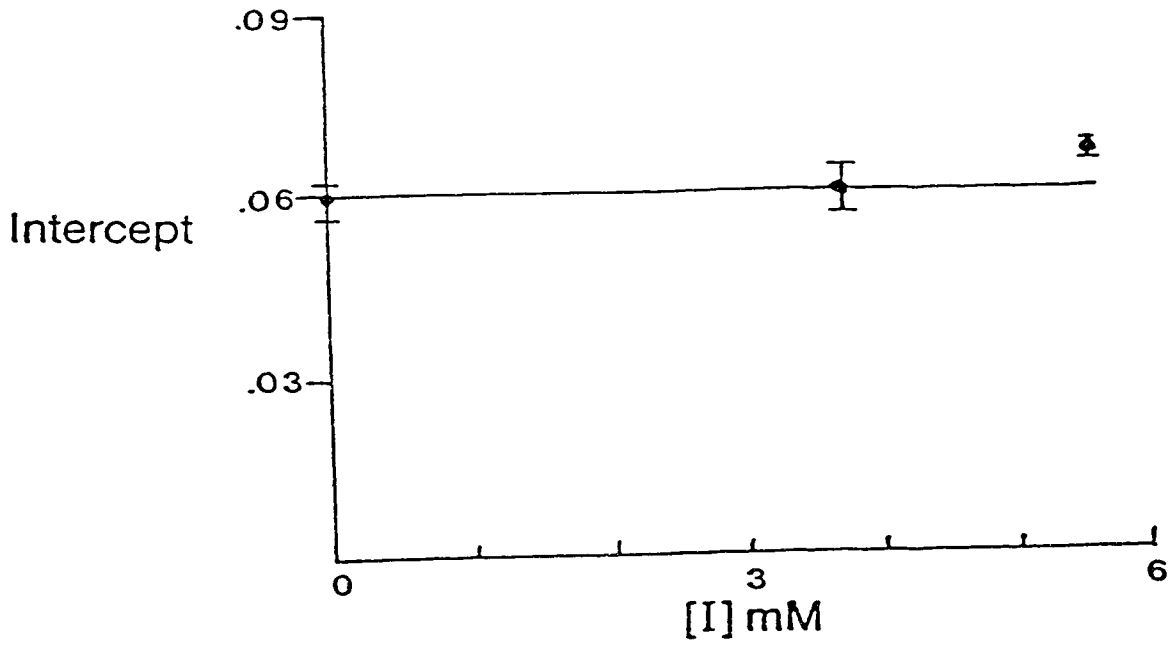


Figure 6-6b. Plot of the intercept vs the concentration of L-alaninol. The invariance of the intercept as predicted for a competitive inhibitor.

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22. Amounts of ammonium sulfate used for percent saturation were determined from a table provided from P.L. Biochemicals, Inc.

7. Assays for Amino Acid Decarboxylase Enzymes using Ion Exchange Cartridges*

Many methods are available to estimate amino acid decarboxylase activity. The most frequently employed procedures monitor CO_2 evolution either manometrically (1,2) or by trapping and counting $^{14}CO_2$ released from appropriately labeled amino acid substrates (3,4). Alternate methods for CO_2 quantitation include spectrophotometric assays which utilize a coupled enzyme system to measure CO_2 production (5,6) and the use of a CO_2 sensitive electrode (7). A variety of procedures based on quantitation of the amines produced in the decarboxylase reactions have been reported including coupled spectrophotometric methods for individual decarboxylases (8,9) as well as isolations using HPLC (10-13), paper chromatography, electrophoresis (14) and ion exchange chromatography (15).

In this paper we report a radiochemical method for assaying amino acid decarboxylases in which labeled product amines are readily separated from unreacted amino acids on ion exchange cartridges by virtue of their different pI's. The assay is most sensitive when uniformly labeled amino acid substrates are used, as well as rapid, with a two to three minute processing time per sample. We report on the use of this method to assay aspartate-α-decarboxylase, tyrosine decarboxylase and lysine decarboxylase.

Materials and Methods

Materials

L-aspartic acid, L-lysine, L-tyrosine, tyrosine decarboxylase (*Streptococcus faecaelis*, 0.66 units/mg), lysine decarboxylase (Type VIII, *Bacterium cadaveris*, 22 units/mg) L-[U-^{14}C] lysine (278 mCi/mmol) hyamine hydroxide and pyridoxal 5'-phosphate were from Sigma Chemical Co. L-[U-^{14}C] aspartic acid (216 mCi/mmol) and ACS scintillation cocktail were from Amersham. L-[U-^{14}C] tyrosine (450mCi/mmol) was from ICN Radiochemicals. Sep Pak Accell QMA and CM ion exchange cartridges were from Waters Associates and Baker SPE sulfonic acid disposable extraction columns were from J. T. Baker Chemical Co. Polygram Ionex 25 cation exchange thin layer chromatography plates were from Machery Nagel and Co. All other reagents including

* Heerze, L.D., Kang, Y.J., and Palcic, M.M.(1990) *Anal. Biochem.* in press. Ms. Young Kang was responsible for the lysine decarboxylase assays.

buffer components were of reagent grade.

The major form of aspartate-α-decarboxylase was isolated from *Escherichia coli* ATCC 9637 by the method of Williamson and Brown (16) and had a specific activity of 10.2 units/ mg when assayed using the $^{14}CO_2$ trapping method described below with 1.25 mM aspartic acid in 50 mM potassium phosphate buffer pH 7.0. A unit of aspartate-α-decarboxylase is defined as the amount of enzyme catalyzing the formation of 1 nmol of product per min at 37°C, while a unit of lysine decarboxylase and tyrosine decarboxylase is given as μmol of product per min. Protein was estimated with a protein assay kit from Bio-Rad which is based on the method of Bradford (17), using bovine serum albumin as a protein standard.

Trial Separation of Substrates and Products of Amino Acid Decarboxylases

The cation (CM) and anion (QMA) exchange Sep Pak cartridges were preconditioned before use by washing with 0.5 M HCl (20 mL) and then water (40 mL). The Baker SPE sulfonic acid columns were preconditioned with water (30 mL) before use. Pairs of amino acid substrate and decarboxylated amine product (2 mg each) were dissolved in 1 mL of buffer at the pH optimum for the corresponding enzyme and were loaded onto a conditioned ion exchange cartridge. Acidic amino acid/amine mixtures were loaded onto anion exchange cartridges, and washed with water or assay buffer to remove uncharged amine products, collecting 5 mL fractions. Aromatic and aliphatic amino acids and their corresponding amines were loaded onto weak cation exchange cartridges. Cartridges were washed first with water or buffer to remove the amino acid, then with 0.5 M HCl to elute the amines. Basic amino acid/amine pairs were loaded onto strong cation exchange cartridges. Selective elution was effected by first washing the cartridge with low ionic strength buffer, pH 6-7, to remove amino acid substrate then with a high ionic strength basic buffer (>pH 8) to elute amines. The efficiency of separation was monitored by spotting 1.5 μL of each 5 mL fraction onto Ionex 25 strong cation exchange thin layer chromatography plates.Plates were developed with 70 mM sodium citrate buffer containing 0.44 M NaCl at pH 3.3 for acidic amino acid/amine pairs, pH 5.1 for basic amino acid pairs and 6.9 for aliphatic and aromatic amino acid pairs. After development, product amines and amino acids were visualized with ninhydrin.

Ion Exchange Cartridge Assays

Aspartate-α-decarboxylase assays were carried out in a total volume of 125 μL which contained 0.08-1.25 mM aspartic acid, 130,000 dpm L-[U-^{14}C] aspartic acid, 5 mM EDTA and 50 μM dithiothreitol and enzyme (0.3 unit), in 50 mM potassium phosphate buffer pH 7.0. After reaction at 37°C for 1 hr, incubation mixtures were

quenched with 1 mL of water and placed at 4°C. To prevent further product formation after quenching, no more than 10 assays were done at one time. Quenched reaction mixtures were immediately loaded onto Accell QMA anion exchange cartridges equilibrated with water. Radiolabeled β-alanine products which eluted with water (2 x 5 mL) were quantitated as dpm in 10 mL of ACS liquid scintillation cocktail on a Beckman LS 1801 scintillation counter. Cartridges were regenerated by washing with 20 mL 0.5 M HCl, then 30 mL H_2O.

Tyrosine decarboxylase was assayed in 100 mM sodium acetate buffer, pH 5.7 containing 0.25 mM pyridoxal phosphate, 0.304-2.13 mM tyrosine, 200,000 dpm L-[U-^{14}C] tyrosine and enzyme (1 munit) in a total volume of 200 µL. Upon incubation at 37°C for 20 minutes, the reaction mixture was quenched with 20 mM potassium phosphate buffer pH 7.2 and loaded onto a preconditioned Accell CM cation exchange Sep Pak cartridge. Unreacted tyrosine was removed by washing with water (25 mL), then radiolabeled tyramine product which eluted with 0.5 M HCl (2 x 5 mL) was quantitated as before. Cartridges were regenerated by washing with 20 mL 0.5 M HCl and 30 mL H_2O.

Lysine decarboxylase assays were carried out in a total volume of 300 µL containing 100 mM sodium phosphate buffer pH 6.0 with 1 µM pyridoxal phosphate, 0.5-20 mM lysine, 200,000 dpm L-[U-^{14}C] lysine and enzyme (4 munit). After incubation at 37°C for 20 min, reaction mixtures were quenched with 1 mL of water and placed at 4°C. Samples were loaded onto preconditioned sulfonic acid cartridges, washed with 0.03 M sodium citrate buffer pH 6.9 (20 mL) to remove unreacted lysine, then 4 mL of H_2O. Labeled cadaverine product was eluted with 1 M potassium phosphate buffer pH 8.0 (12 mL) which was quantitated as before. The cartridges were regenerated by washing with water (40 mL), before reusing.

Standard Radiochemical $^{14}CO_2$ Trapping Assay

Aspartate-α-decarboxylase activity was assayed by measuring $^{14}CO_2$ evolved from L-[U-^{14}C] aspartic acid. Assays were carried out in mini scintillation vials and contained in 50 mM potassium phosphate, pH 7.0, 0.08-1.25 mM aspartic acid, 200,000 dpm L-[U-^{14}C] aspartic acid, 5 mM EDTA, 50 µM dithiothreitol and 0.3 unit enzyme in a total volume of 125 µL . After incubation at 37°C for 1 hr, mixtures were quenched with 100 µL of 10% trichloroacetic acid. Radiolabeled CO_2 evolved during the reaction was trapped on a 1 x 1 cm piece of filter paper impregnated with 1 M hyamine hydroxide that had been secured in the cap of the vial. To ensure complete evolution of $^{14}CO_2$, reaction mixtures

were shaken for 45 min at 37°C after quenching. The filter papers were removed and counted in 10 mL of ACS scintillation cocktail. Assays were standardized and substrates were checked for uniformity of label with an excess of enzyme to completely release and trap $^{14}CO_2$ from substrate.

Tyrosine decarboxylase assays were carried out in an analogous manner in 100 mM sodium acetate buffer pH 5.7. Reaction mixtures contained in a total volume of 200 µL, 0.304-2.13 mM tyrosine, 200,000 dpm L-[U- ^{14}C] tyrosine and 1 munit enzyme. Upon incubation for 1 hr at 37°C, reactions were quenched with 10% trichloroacetic acid and processed as described for the aspartate-α-decarboxylase assays.

Lysine decarboxylase was assayed as before in 100 mM sodium phosphate buffer, pH 6.0, containing 1 µM pyridoxal phosphate, lysine (0.5-20 mM), 200,000 dpm L-[U-^{14}C] lysine and enzyme (4 munit) in a total volume of 300 µL. After incubation for 20 min at 37°C, reaction mixtures were acid quenched and treated as described above.

Results and Discussion

The choice of a suitable ion exchange cartridge is governed by the type of amino acid decarboxylase being investigated and by the ability of the amino acid substrate or the amine product to be quantitatively adsorbed onto the ion exchange cartridge. In principle, acidic amino acids which are negatively charged at the operational pH of a decarboxylase enzyme should be adsorbed onto an anion exchange cartridge. The corresponding product amine is uncharged and thus can be readily removed from the substrate by washing the cartridge with water. Aromatic and aliphatic amino acids yield positively charged amines as decarboxylation products. These will be adsorbed onto weak cation exchange cartridges at neutral pH. The uncharged amino acid is removed by washing the cartridge with water, after which amines can be eluted with (0.5 M HCl) and quantitated. Basic amino acid reactions are best adsorbed onto strong cation exchangers at neutral pH. Selective removal of the amino acid can best be achieved by washing the cartridge with low ionic strength buffer, pH 6-7. The products, which are more positively charged can then be eluted with high ionic strength buffers at basic pH's.

Aspartate-α-decarboxylase was chosen as an example of a decarboxylase acting on an acidic substrate. The reaction product β-alanine is not retained on a weak anion exchange cartridge, and thus can be completely removed from adsorbed unreacted aspartic acid. The ion exchange cartridge assay was found to be linear with time up to 1 hr, and with the concentration of enzyme as shown in Figure 7-1 (correlation coefficients are 0.988 and 0.999 respectively). The suitability of the cartridge assay for estimating the

kinetic paramaters V_{max} and K_m are shown in the reciprocal plot (Figure 7-2). Each point represents the average of duplicate samples and data were analyzed using a computer program based on the statistical method of Wilkinson (18). The major form of aspartate-α-decarboxylase gave a V_{max} of 0.25±0.01 nmol/min with a K_m of 0.18±0.01 mM. When the $^{14}CO_2$ trapping assay was employed under identical assay conditions values of 0.28±0.01 nmol/min and 0.20±0.03 mM were obtained for V_{max} and K_m, respectively. The values obtained for K_m are comparable to 0.16 mM reported by Williamson and Brown (16) for the major form of aspartate-α-decarboxylase. In addition, the sensitivity of the ion exchange assay is three times greater than the $^{14}CO_2$ trapping method when uniformly labeled aspartic acid is used as a substrate. Trial separation experiments also indicate that an identical isolation scheme can be implimented for assaying both aspartate-β-decarboxylase and glutamate decarboxylase.

Tyrosine decarboxylase serves as an example for aliphatic and aromatic decarboxylases. In this case tyramine is adsorbed onto a weak cation exchanger and unreacted tyrosine is removed by washing with water. Tyramine is subsequently eluted with dilute HCl. The tyrosine decarboxylase assay was carried out at pH 5.7 and was found to be proportional to time up to 1 hr and to protein concentration, with correlation coefficients of 0.997 and 0.996, respectively (Figure 7-3). The effect of tyrosine concentration on reaction velocity at pH 5.7 is shown as a reciprocal plot in Figure 7-4. Analysis of the data using the method of Wilkinson provided a V_{max} of 1.34±0.05 nmol/min and a K_m of 0.79±0.08 mM. This is in good agreement with the values of 1.45±0.11 nmol/min and 0.97±0.15 mM obtained with the $^{14}CO_2$ trapping method. A variety of literature values for K_m have been reported for tyrosine decarboxylase, ranging from 0.6 mM (19) to 1.6 mM (20). For uniformly labeled tyrosine, the assay is eight times as sensitive as the trapping method. This isolation scheme can also be used to isolate the products of the phenylalanine decarboxylase, L-DOPA decarboxylase, and valine decarboxylase reactions.

Lysine decarboxylase catalyzes the conversion of a basic amino acid to cadaverine. A strong cation exchanger was employed to adsorb both the amino acid and amine product. Selective desorption of the amino acid was achieved by washing with pH 6.9 buffer of low ionic strength (0.03 M), after which cadaverine was eluted with (1M) phosphate at pH 8, and quantitated. When lysine decarboxylase was assayed using the ion exchange cartridge

method at pH 6.0, a V_{max} of 4.9±0.1 nmol/min and K_m of 1.7±0.2 mM were obtained. The corresponding values from the CO_2 trapping assay are 4.3±0.4 nmol/min and 1.2±0.4 mM for V_{max} and K_m, respectively (data not shown). Both values are in good agreement with those previously reported (21). There is a five fold increase in sensitvity over the trapping method. Other enzymes amenable to analogous exchange assay are arginine decarboxylase, histidine decarboxylase, and ornithine decarboxylase.

This work describes a new method for assaying amino acid decarboxylases. The assay employs ion exchange cartridges from which amine products can readily be separated from unreacted amino acid substrate. The method is can be adopted for the assay of acidic, basic, as well as aliphatic and aromatic amino acid decarboxylases with the use of appropriate ion exchange cartridges. There is an inherent increase in sensivity, since a multilabeled amine product is quantitated rather than the single label in standard $^{14}CO_2$ trapping methods. The enhanced sensitivity will be determined by the molecular weight of the starting amino acid, however, this will range from three to eight fold increases.

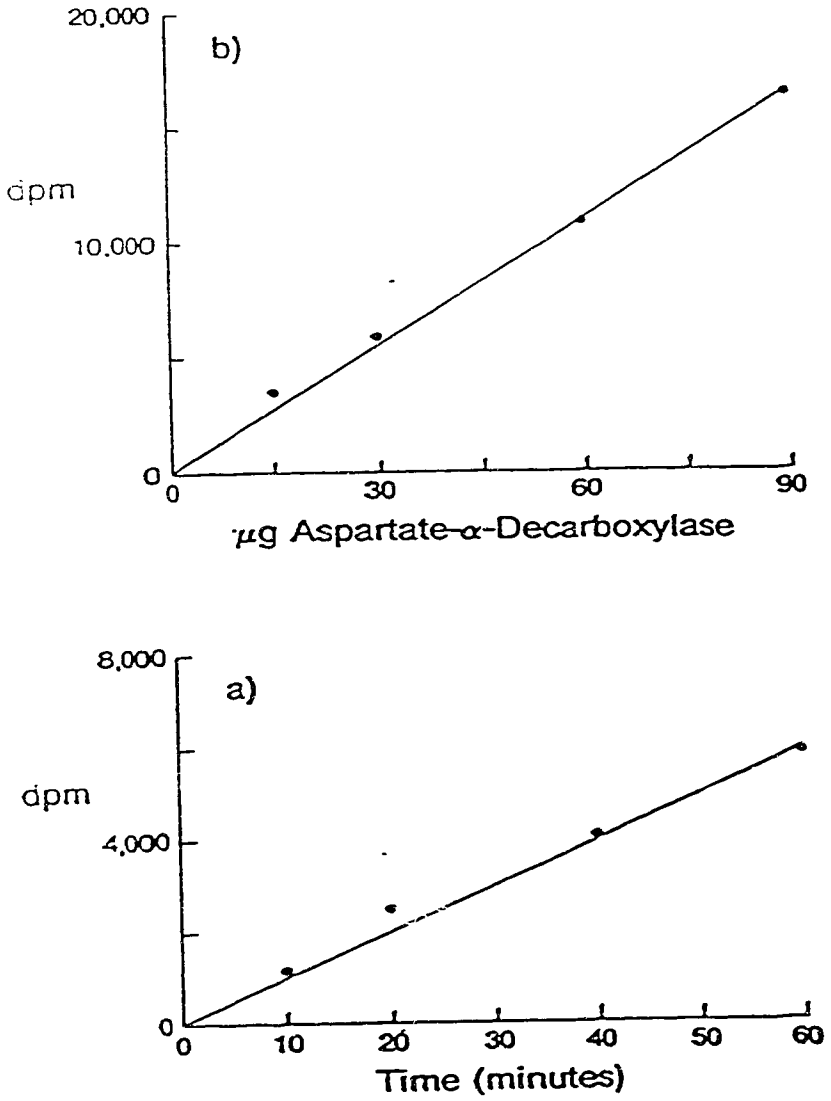

Figure 7-1: The effect of time on the production of β-alanine by aspartate decarboxylase. Assay mixtures contained in potassium phosphate buffer pH 7.0; 0.2 mM aspartate, 130,000 dpm L-[U-^{14}C] aspartic acid, and enzyme (0.3 unit) in a total volume of 125 μL. After incubation at 37°C for the times indicated, mixtures were diluted with 1 mL of water and applied to Accell QMA anion exchange cartridges. β-alanine which eluted with water (2 x 5 mL) was quantitated as dpm by liquid scintillation counting.

Figure 7-1a: The effect of aspartate α-decarboxylase concentration on β-alanine formation. Incubations contained 0.2 mM substrate, 130,000 dpm L-[U-^{14}C] aspartic acid, and enzyme (0.15-0.9 units) in a total volume of 125 μL. The products formed in 60 min incubations at 37°C were isolated on Accell QMA anion exchange cartridges and quantitated as described in Materials and Methods.

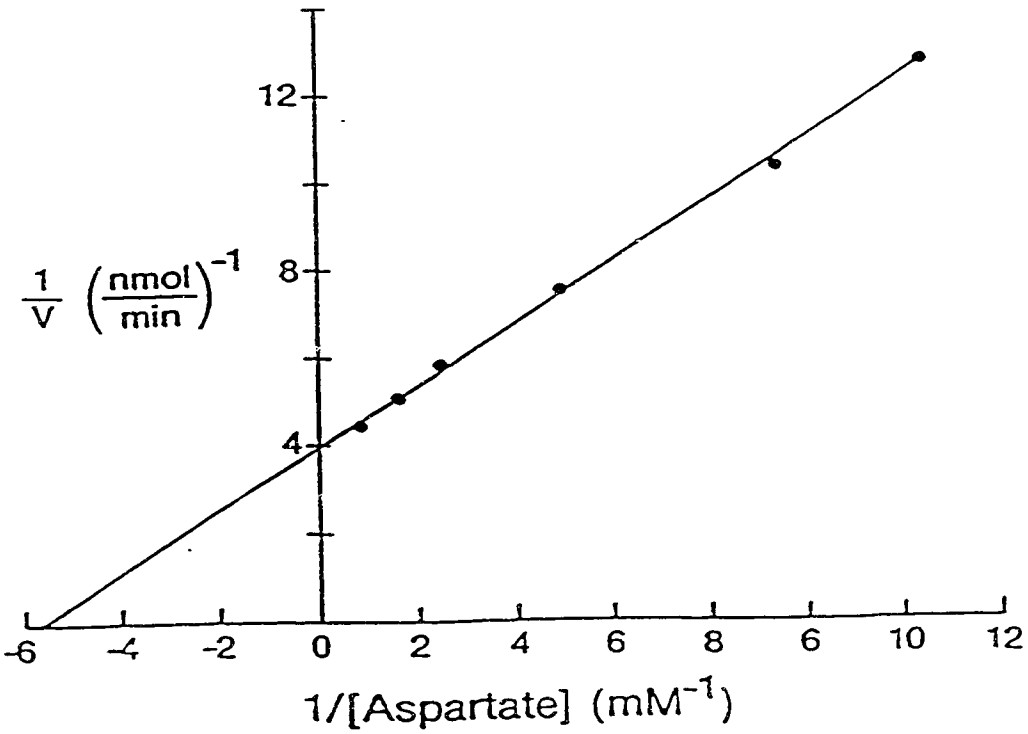

Figure 7-2: The effect of aspartic acid concentration on the rate of β-alanine production. Activity was estimated at 37°C and pH 7.0 using 0.08-1.25 mM aspartate, 130,000 dpm L-[U-^{14}C] aspartic acid and enzyme (0.3 units) in a total volume of 125 µL. After incubation for 60 min products were isolated on Acell QMA cartridges and quantitated as described in Materials and Methods.

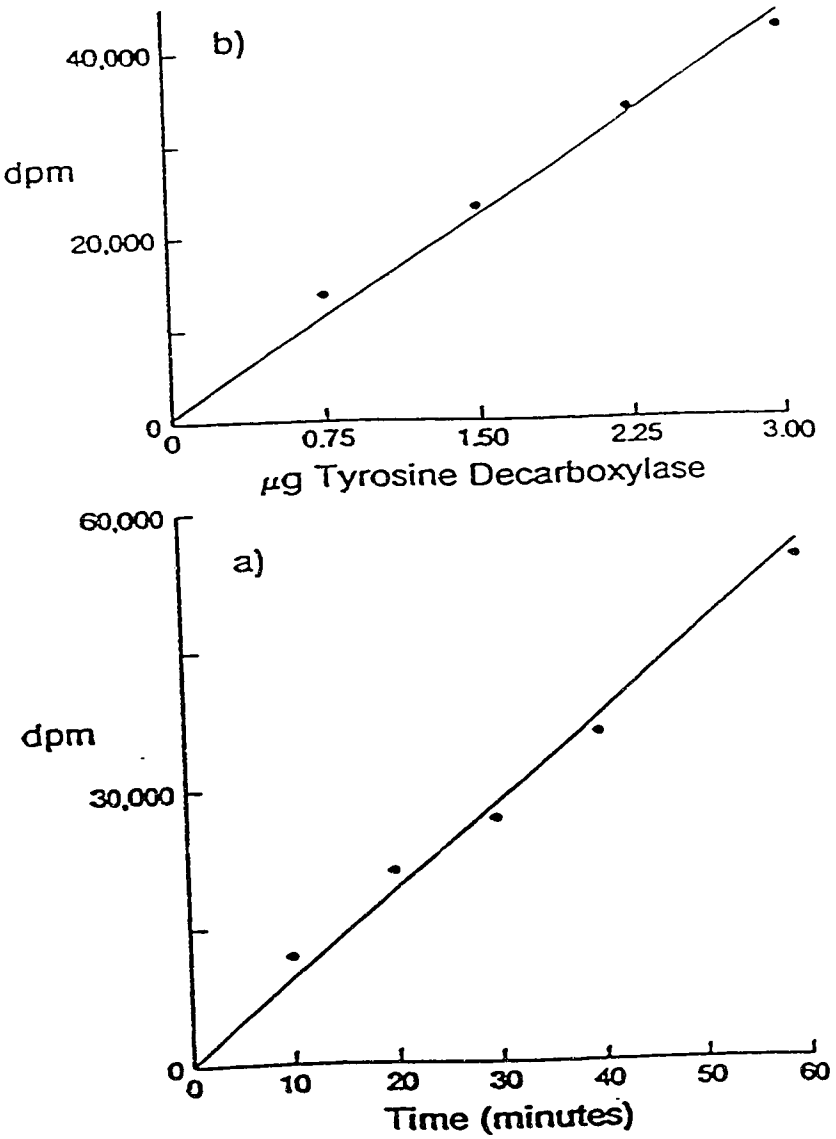

Figure 7-3: The effect of time on the production of tyramine by tyrosine decarboxylase. Reaction mixtures contained in a total volume of 200 μL sodium acetate buffer pH 5.7: 0.61 mM tyrosine, 200,000 dpm L-[U-^{14}C] tyrosine,0.25 mM pyridoxal phosphate and 1milliunit enzyme. After incubation at 37°C for the times indicated, samples were diluted with buffer and applied to Accell CM cation exchange cartridges. Unreacted tyrosine was removed by washing with water, tyramine was eluted in 2 x 5 mL fractions with 0.5 M HCl and quantitated as dpm by scintillation counting as described in Materials and Methods.

Figure 7-3a: The effect of tyrosine decarboxylase concentration on the production of tyramine. Incubations were carried out at 37°C for 20 min at pH 5.7 and contained 0.61 mM substrate, 200,000 dpm L-[U-^{14}C] tyrosine and enzyme. Tyramine was separated from unreacted tyrosine on cation exchange cartridges as described in Materials and Methods.

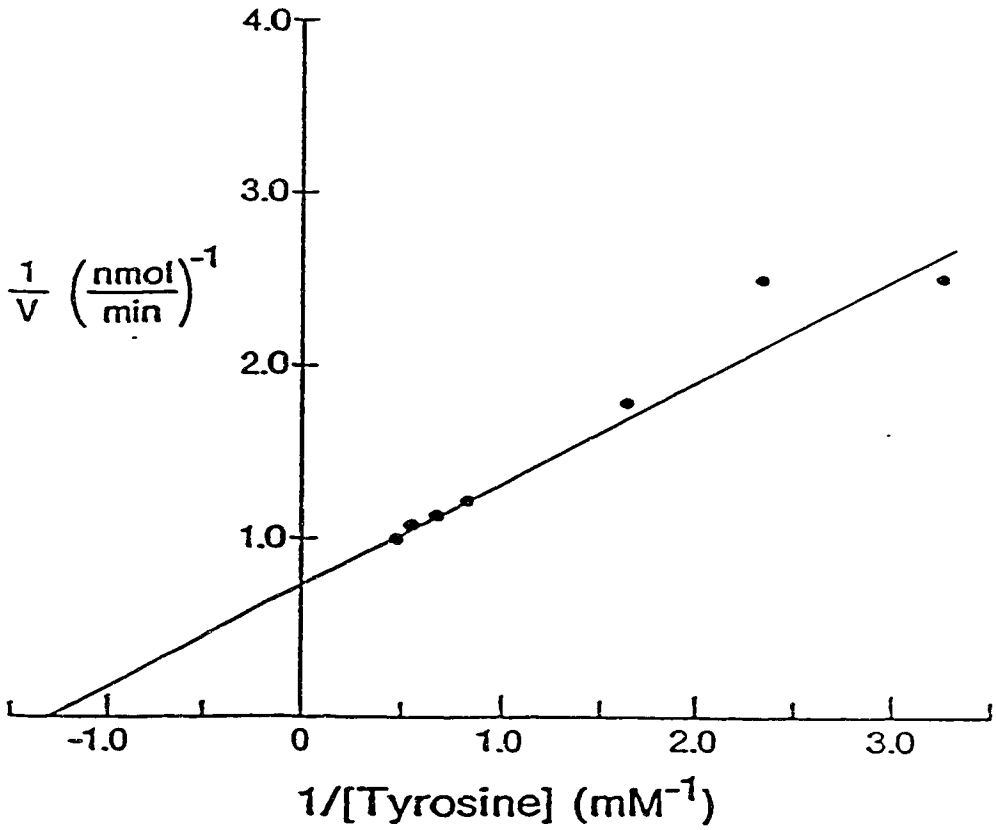

Figure 7-4: The effect of substrate concentration on the rate of tyramine production by tyrosine decarboxylase. Incubations were carried out for 20 min at 37°C and contained in 200 µL sodium acetate buffer pH 5.7, 0.304-2.13 mM tyrosine, 200,000 dpm L-[U-^{14}C] tyrosine and 1 milliunit of tyrosine decarboxylase. Reaction mixtures were diluted,applied to cation exchange cartrides, then tyramine isolated and quantitated as described in Materials and Methods.

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Conclusion

Two different enzymes were investigated in this work. The enzyme systems studied were two forms of β-galactoside α(1→2)-fucosyltransferase from porcine submaxillary glands and L-aspartate-α-decarboxylase from *E. coli*. The research done on α(1→2)-fucosyltransferase was:

1. The development of a new rapid assay for estimating α(1→2)-fucosyltransferase activity which utilizes Sep Pak reverse phase cartridges. My research was combined with those of my collaborators in a paper which describes the use of this assay for 4 different glycosyltransferase enzymes. Subsequent to publishing this research, we have demonstrated that this new assay can be used for at least 14 different glycosyltransferases.

2. The inhibitor evaluation of a bisubstrate analog for the α(1→2)-fucosyltransferase enzyme. The bisubstrate analog was a good inhibitor (K_i's in the micromolar range) for both forms of the enzyme. This was the first mechanism based inhibitor known for glycosyltransferase enzymes. The same strategy that was employed for this analog is being used to develop additional bisubstrate analogs for other transferase enzymes.

3. The inhibitor evaluation of deoxy acceptor analog **1** for the α(1→2)-fucosyltransferase. The deoxy analog was a moderate competitive inhibitor with a K_i of 800 μM.The results that were obtained will be combined with results obtained by my collaborators, in describing the first known inhibitors of the acceptor substrates of glycosyltransferase enzymes.

The research done with L-aspartate-α-decarboxylase was:

1.The inhibitor evaluation of a series of analogs of aspartate which have the potential of being mechanism based inhibitors. The analogs include substitution at the amino nitrogen, the substrate carboxyl groups and at the β positions. The results that were obtained indicate that slow irreversible inhibition was obtained with analogs that had substitution at the amino nitrogen.Two competitive inhibitors were also found in the survey.The inhibitors evaluated show an unuual D specificity in that the D-isomers were better inhibitors than the corresponding L-isomers.

2. A new radiochemical assay was also developed for amino acid decarboxylase enzymes.

Product amines are separated from amino acid substrates by virtue of their different pI's on either cation or anion exchange cartridges. The assay is rapid and more sensitive than standard radiochemical assays if uniformly ^{14}C labeled substrates are used. This assay can be used for a wide spectrum of amino acid decarboxylase enzymes as demonstrated with L-aspartate-α-decarboxylase, tyrosine decarboxylase, and lysine decarboxylase.

Appendix

Isolation of α(1→2)-Fucosyltransferase from Porcine Submaxillary Glands

Fucosyltransferase Assays

During purification - For routine assays during purification, reaction mixtures contained in 65μl, 3.57 mM phenyl-β-D-galactopyranoside, 25 μM GDP-fucose, 7000 dpm GDP-[U-^{14}C] fucose, 2.85 mM $MnCl_2$, 2.85 mM ATP and enzyme in Buffer A. After incubation at 37°C for 1-2 hrs incubation mixtures were diluted with 1 mL of H_2O and immediatly placed at 4°C. Reaction mixtures were loaded onto preconditioned a Sep Pak cartridges (1) and unreacted GDP-fucose was eluted until background counts were obtained by washing with H_2O (30 mL). Radiolabeled product was eluted with methanol (2 x 5 mL) and quantitated as dpm in 10 mL of scintillation cocktail in a Beckman LS 1801 scintillation counter. One unit of enzyme activity is defined as 1 μmol of product formed per minute at 37 °C. Protein concentration was determined by the method of Bradford (2) using BSA as standard.

The α(1→2)-fucosyltransferase was purified by using slight modifications of the method of Beyer et al.(3,4). α(1→2)-Fucosyltransferase is prone to surface denaturation, thus all glass containers including chromatography columns and glass wool were siliconized before use. Column fractions were collected in plastic test tubes and plastic containers were used whenever possible. GDP-hexanolamine Sepharose is regenerated before use by washing the column with 10 volumes of guanidine hydrochloride; 10 volumes of water; and 30 volumes of Buffer A containing 10% glycerol. All steps were done at 4°C unless otherwise stated.

a) *Soluble form of α(1→2)-fucosyltransferase* - The soluble form of the α(1→2)-fucosyltransferase was obtained by repeated homogenization of the submaxillary gland homogenate as described below. 230g of frozen glands were thawed under running tap water and placed at 4°C. The thawed glands were trimmed of any fat or connective tissue and coarsely ground (30 sec) in a Cuisinart food processor. The coarsely ground glands were homogenized with 900 mL of ice cold water (3-30 sec pulses separated by 30 sec rest intervals) and the homogenate was then centrifuged at 7500xg for 45 min. The mucin rich supernatant solution was discarded and the resulting pellet was homogenized in a Cuisinart in 900 mL of Buffer A (20mM Tris.HCl and 2 mM NaN_3), 3-1 min pulses with 30 sec rest intervals). The pellet homogenate was centrifuged at 7500xg for 30min.

The resulting enzyme containing supernatant solution (900 mL) was loaded onto SP-Sephadex column (4.5x30cm) that had been equilibrated in Buffer A in 3-300 mL portions at a flow rate of 90 mL/hr. The column was washed with Buffer A to remove inert protein and the fucosyltransferase activity was eluted with Buffer A containing 0.3M NaCl. Enzyme containing fractions were pooled, made 10% in glycerol, and immediatly loaded at a flow rate of 60 mL /hr onto 5 mL of GDP-hexanolamine sepharose (4 μmol of ligand bound/ mL gel) that had been equilibrated in Buffer A containing 10% glycerol. The column was then washed with Buffer A containing 0.4 M NaCl and 10% glycerol until no protein could be detected in the eluant. α(1-2)-Fucosyltransferase activity was specifically eluted by washing with Buffer A containing 5 mM GMP and 10% glycerol. The eluant was immediatly brought to 0.17 mg/ mL in BSA to stabilize the enzyme activity, and desalted on PD-10 columns equilibrated in Buffer A containing 10% glycerol. Desalted samples were dialyzed overnight against Buffer A containing 10% glycerol and concentrated by ultrafiltration.

The soluble form of α(1→2)-fucosyltransferase was purified 49,000 fold as shown in Table 8-1 and had a specific activity of 44 munits/ mg protein.

b)*Membrane Bound Form of α(1→2)-Fucosyltransferase* - The membrane bound fucosyltransferase is rapidly inactivated on exposure to Triton X-100 or SP-Sephadex, thus the first two steps in the isolation are done as quickly as possible.

300 g of frozen porcine submaxillary glands were thawed, trimmed of fat and connective tissue, and coursely ground in a Cuisinart food procesor. The coarsely ground glands were combined with H_2O (700 mL), homogenized (3-30 sec bursts with a 1 min rest interval) and centrifuged at 7500xg for 45 min. The mucin rich supernatant solution was discarded and the pellets were homogenized in 450 mL of Buffer A (3-30sec pulses followed by a 1 minute rest interval). The tissue homogenate was made 20 mM in $MnCl_2$ by the dropwise addition of 14 mL of 1M $MnCl_2$ and stirred for 15 min. The resulting manganese containing solution was centrifuged at 7500xg for 30 min. This procedure was repeated one more time to yield washed pellets. The washed pellets were combined with 450 mL of Buffer A and homogenized as before. The suspension was made 20 mM in $MnCl_2$ and 1% in Triton X-100 by the addition of 14 mL of 1M $MnCl_2$ and 75 mL of 10% Triton X-100, and stirred for 50 min at 4°C. The Triton-containing solution was centrifuged at 7500xg for 30 min and the supernatant solution was filtered through several layers of cheesecloth to yield Triton extract 1. The extracted pellets were reextracted by the above method to yield a second Triton extract.

The detergent extracts were combined and preeqilibrated SP-Sephadex (20g) was added and slowly stirred for 30 min. The resulting suspension was filtered on a course sinter glass funnel and washed with two volumes of Buffer A. 10g of fresh SP-Sephadex was added to the filtrate, stirred for 30 min and filtered as above. The SP-Sephadex from two batch adsorptions were combined, loaded into a column, and and washed with Buffer A containing 0.3 M NaCl. Enzyme containing fractions were combined, diluted with an equal volume of Buffer, and made 10% with glycerol.

The diluted enzyme solution was loaded onto 4 mLs of GDP-hexanolamine Sepharose (4 μmol of ligand bound/ mL gel) at a flow rate of 100 mL/hr and fucosyltransferase-containing fractions were obtained by non specific desorption using Buffer A which contained 0.4 M NaCl and 10% glycerol. The combined enzyme containing fractions were diluted with an equal volume of Buffer A and loaded onto GDP-hexanolamine Sepharose at a flow rate of 50 mL/hr. The column was washed with Buffer A containing 0.2 M NaCl until no protein was detected in the eluant, and then washed with Buffer A containing 0.2 M NaCl, 10% glycerol, and 5mM GMP. The eluate was made 0.1? mg/mL in BSA to stabilize the enzyme activity, and desalted on PD-10 columns that had been preequilibrated in Buffer A containing 10% glycerol. Desalted enzyme fractions were dialyzed overnight in Buffer A containing 10% glycerol.

The membrane bound form of α(1→2)-fucosyltransferase was purified 45,000 fold as shown in Table 8-2 and had a specific activity of 66.4 munits/ mg protein.

Due to the extreme lability of the porcine submaxillary gland α(1→2)-fucosyltransferase towards denaturation upon exposure to Triton, other detergents were examined as a possible means of extracting the enzyme activity without denaturation. The detergents Tween 20, Nonidet P40, Chaps, and Triton CF-54 at various concentrations (0.1%, 0.5%, and 1%) did not provide any enhancement in the amount of enzyme extracted from the submaxillary glands. A possible means for extracting more activity may lie in the use of a proteolytic enzyme which hydrolyzes the membrane anchor which releases the enzyme from the membrane producing a soluble form of the enzyme. A recent report in the literature had used the enzyme Cathepsin D to solubilize α(2→6)-sialyl-transferase activity from rat liver golgi membranes (5). This method eliminates the need of detergent to solubilize the transferase activity.

Table 8-1 Purification of α(1→2)-Fucosyltransferase

Purification Step	Protein (mg)	Total Activity (munits)	Specific Activity (munits/mg)	Yield (%)	Purification Fold
Crude extract	72072	65.32	0.0009	100.0	1.0
SP Sephadex	6300	42.35	0.0067	64.0	7.4
GDP-hexanolamine	0.04	2.22	44.4	3.4	48990.0

1 unit= 1 μmol of product / minute at 37°C

Table 8-2 Purification of Membrane Bound α(1→2)-Fucosyltransferase

Purification Step	Protein (mg)	Total Activity (munits)	Specific Activity (munits/mg)	Yield (%)	Purification Fold
Crude extract	52000	77.7	0.0015	100.0	1.0
SP Sephadex	320	15.4	0.048	20.0	32.0
GDP-hexanolamine	5	8.32	1.66	11.0	1107.0
GDP-hexanolamine 2	0.05	3.32	66.4	4.0	44438.0

1 unit = 1μmol product/ minute at 37°C.

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