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ESTABLISHING A TWO-COLOR FLUORESCENCE PROBE ASSAY FOR THE SIMULTANEOUS SCREENING OF GLUT5 AND GLUT2 FRUCTOSE TRANSPORTERS IN LIVE CELLS

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By

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DEDICATION

This report is dedicated to myself for the many years of studying, crying and celebrating.

To my parents for their endless sacrifices and unwavering belief in my potential. Your guidance and encouragement have shaped my journey, and I am eternally grateful for your love and support.

To my siblings, for their constant encouragement and for always being there to share in my joys and challenges. Your support has meant the world to me.

To my friends and colleagues, whose companionship and encouragement have made this journey more enjoyable and fulfilling.

Lastly, to my advisor, Dr. Marina Tanasova, whose mentorship and guidance have been invaluable in the completion of this work.

This dedication is a small token of my gratitude for all the love, support, and encouragement you all have provided. Thank you for being my pillars of strength.

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This report is a culmination of the efforts and support of many individuals, and I am sincerely grateful to each and every one of you.

Thank you.

ABSTRACT

The mammalian facilitative glucose transporter (GLUT) family comprises 14 members that mediate the transport of hexoses across cell membranes. GLUT5 is the only member specific to fructose, and together with GLUT2, which transports fructose in addition to glucose, they make up the primary fructose transporters in humans. This study introduces a novel two-color fluorescence assay designed to simultaneously monitor the activity of GLUT5 and GLUT2 in live cells. 2,5-anhydro-D-mannitol (2,5-AM) a GLUT5 targeting compound has facilitated the development of various fructose-mimicking probes with a wide range of properties, such as radioactive imaging, fluorescent, photoactive, and drug delivery capabilities. Effective and accurate screening of fructose transporters GLUT5 and GLUT2 is essential for understanding their unique roles in cellular metabolism and their implications in metabolic disorders. This assay employed two fluorescent probes: P2, a mimicked conformation and configuration variation of fructose, and NBDM, a glucose analog that serves as a GLUT2 substrate. By leveraging their distinct fluorescence properties, P2 and NBDM facilitate the simultaneous visualization of GLUT5 and GLUT2 activities and this assay was validated with triple-negative breast cancer (TNBC) (MDA MB 231, 453,), hepatocellular carcinomas cell line (HEP-G2) proving its ability to differentiate fructose uptake by GLUT5 and GLUT2. This dual-color fluorescence assay marks significant progress in transporter research and has the potential for accelerating drug discovery and elucidating the molecular mechanisms underlying related metabolic diseases.

1 INTRODUCTION

FRUCTOSE METABOLISM IN CANCER

Fructose, a simple sugar that is naturally found in many fruits, vegetables, and honey has recently gained significant attention in cancer research due to its role in cellular metabolism, implications in tumor development and progression, and its significant impact on health particularly in the context of metabolic disorders such as Obesity, Cancer, Diabetes, and High Blood Pressure. Unlike glucose, which is primarily metabolized through glycolysis and the citric acid cycle, fructose follows distinct metabolic routes that can contribute to the metabolic reprogramming observed in cancer cells. This reprogramming, often referred to as the Warburg effect, enables cancer cells to meet their increased energy demands and biosynthetic needs, promoting rapid proliferation and survival under various conditions (Figure 1).

Cancer cells exhibit altered metabolic pathways to support rapid growth and proliferation, often characterized by increased glucose and fructose uptake[1]. The facilitative glucose transporters, GLUT5 and GLUT2, play pivotal roles in fructose transport across cell membranes. GLUT5, the fructose-specific transporter is expressed in various tissues including the small intestine, kidney, and adipose tissue. Notably, GLUT5 is overexpressed in several cancer types such as breast, prostate, glioblastoma, colon, liver, and lung carcinomas [2]. This overexpression is believed to contribute to the increased fructose uptake and utilization observed in cancer cells, providing an alternative energy source to support rapid proliferation and tumor growth.

On the other hand, GLUT2 is a low-affinity transporter that facilitates the bidirectional transport of both glucose and fructose. GLUT2 is primarily expressed in hepatocytes, pancreatic β-cells, and certain cancer cells[2]. Its role in fructose metabolism has been implicated in the development of metabolic disorders, including non-alcoholic fatty liver disease and type 2 diabetes.

In lung adenocarcinoma, an up-regulation of GLUT5 is linked to a poor prognosis, as its depletion attenuates cell proliferation, invasion, and enhances apoptosis, while its overexpression promotes cell proliferation, migration, invasion, and tumorigenesis[3]. Similarly, in acute myeloid leukemia, increased fructose utilization through GLUT5 is associated with higher proliferation, colony growth, migration, invasion, and worsened leukemic phenotypes [4]. Understanding the precise functions and regulation of

these transporters is essential for elucidating the metabolic pathways of fructose and its implications in metabolic disorders.

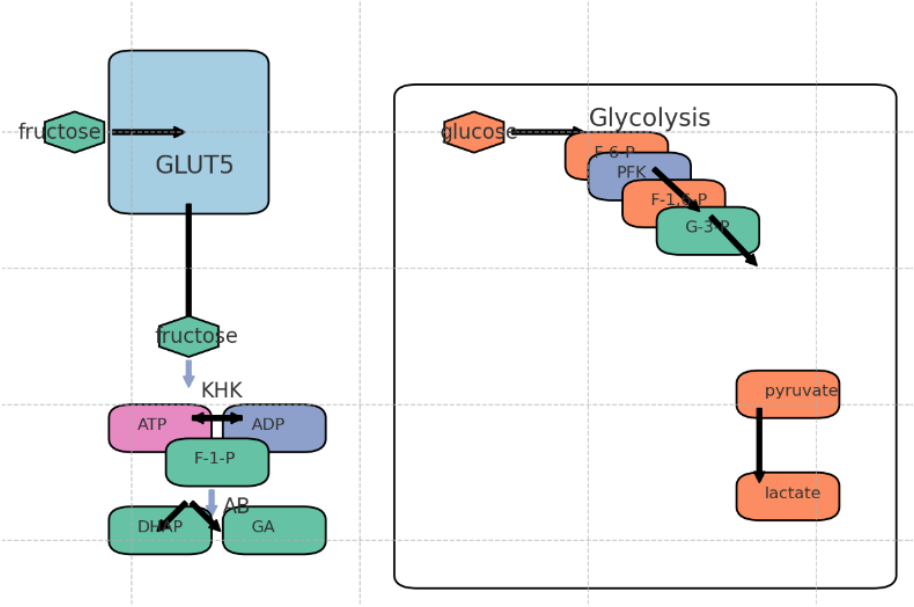


Figure 1. Fructose metabolism highlights the roles of GLUT5 and glycolysis, along with the Warburg effect.

- GLUT5 transporter facilitates the entry of fructose into the cell.
- Once inside, fructose is metabolized by KHK (ketohexokinase) to form F-1-P (fructose-1-phosphate).
- F-1-P is then split by aldolase B (AB) into DHAP (dihydroxyacetone phosphate) and GA (glyceraldehyde).
- DHAP and GA enter the glycolysis pathway, eventually leading to the production of pyruvate.
- Pyruvate is converted to lactate under the Warburg effect, typical in cancer cells.

The reliance of cancer cells on fructose metabolism is complex and multifactorial, involving various enzymes and metabolic pathways. Once inside the cell, fructose is quickly phosphorylated by ketohexokinase (KHK) to form fructose-1-phosphate. This enzyme has been implicated in promoting fructose metabolism in certain cancers, such as colorectal cancer[7]. Additionally, KHK exhibits nuclear kinase activity and influences tumor progression in various cancers. It facilitates tumor growth in glioma and non-small cell lung cancer, activates the MAP kinases pathway to promote pancreatic

cancer growth, contributes to hepatocellular carcinoma formation by triggering a cMyc-induced splicing switch leading to the KHK-A isoform, and enhances fructose-induced metastasis in breast cancer[8]. Unlike glucose metabolism, fructose metabolism bypasses the critical regulatory step involving phosphofructokinase-1 (PFK-1), this diversion results in an augmented flow of metabolites into alternative metabolic pathways[9]

Fructose metabolism in cancer contributes to several key aspects of tumor biology. The enhanced utilization of metabolites derived from fructose, such as dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP), within the glycolytic pathway provides cancer cells with a rapid energy source and building blocks for nucleotide and lipid synthesis. Additionally, fructose metabolism can lead to the accumulation of uric acid, a byproduct of fructose breakdown. High levels of uric acid are associated with oxidative stress and inflammation, promoting a favorable microenvironment for tumor progression and metastasis. Furthermore, uric acid stimulates multiple signaling pathways that enhance cancer cell survival and proliferation[10].

Other factors, such as the availability of fructose in the tumor microenvironment and the overall metabolic profile of the cancer cells, also contribute to their dependence on fructose. Studies on mice have demonstrated that even small amounts of fructose (~3% of the total daily caloric intake) can stimulate tumor growth in intestinal cancer and contribute to liver metastasis in colon cancer[10].

The dysregulation of fructose metabolism in cancer presents a promising target for therapeutic interventions[11]. Fluorescence-based assays offer a promising alternative for studying transporter activity in live cells. By utilizing fluorescent probes, these assays provide real-time monitoring of transporter function and allow for the simultaneous detection of multiple targets. In this context, a two-color fluorescence assay presents a powerful tool for the concurrent screening of GLUT5 and GLUT2 transporters in live cells. This assay leverages distinct fluorescent probes that selectively interact with GLUT5 and GLUT2, enabling the visualization and quantification of their activity within the same cellular environment. Furthermore, it will facilitate high-throughput screening of potential therapeutic compounds targeting these transporters, contributing to the development of novel strategies for managing metabolic diseases associated with fructose dysregulation.

THE ROLE OF FRUCTOSE GLUTS IN HUMAN HEALTH AND DISEASE

GLUTs play a crucial role in regulating glucose homeostasis and facilitating energy metabolism in diverse cells and tissues. GLUTs exhibit unique structural characteristics, regulatory mechanisms, tissue distribution patterns, and notable relevance in both physiological well-being and pathological conditions.

GLUT proteins facilitate the transport of various sugars as well as other substrates, including glucose, fructose, mannose, glucosamine, galactose, mannose, dehydroascorbic acid (DHA), urate, and myoinositol, exhibiting varying affinities for each. Fourteen GLUT proteins are expressed in humans and are categorized into three classes based on their structural, substrate, and amino acid sequence similarities[12,13a,13b].

These isoforms are encoded by different genes and exhibit unique tissue distribution patterns and substrate specificities which are designated GLUT1 through GLUT14. While it is commonly understood that most of these isoforms facilitate the transport of D-glucose, the reason for the presence of multiple isoforms with seemingly comparable substrate preferences and kinetics remains unclear. Each isoform exhibits distinct kinetic properties and tissue-specific expression patterns. The increased uptake of sugars and heightened metabolic activity in cells have been linked to various metabolic disorders, including cancer,[14,15] type 2 diabetes mellitus,[16] non-alcoholic fatty liver disease,[17] obesity,[18] and Alzheimer's disease.

In the case of cancer, the uncontrolled proliferation of cells relies on enhanced sugar uptake to fuel energy production.[19,20] Consequently, changes in the expression and composition of glucose transporters (GLUTs) occur to satisfy the high metabolic demands for uncontrolled cell growth and proliferation.[21,22] Numerous studies have documented elevated levels of glucose transporters, including GLUT1, GLUT3, and GLUT5 in many cancer types.[23-31] These deregulated expressions of GLUTs, each with varying affinities for different sugars, provide cancer cells with the means to optimize their energy supply, ultimately conferring a significant advantage for their growth.

Amongst GLUTs, GLUTs 1-4 and GLUT5 have drawn considerable attention owing to their strong association with cancer development and progression. The upregulation of these GLUT isoforms with enhanced activity has been consistently linked to various types of cancer, underscoring the complexity of their involvement in malignancy.[32]

GLUT1, as the predominant transporter of glucose stands out for its ubiquitous overexpression across a range of cancer types.[27] Notable examples include brain,[33] liver,[34] breast,[35] cervix, colon,[36] lungs,[29] ovary,[37] thyroid,[38] prostate,[39] pancreas,[40] and skin cancers.[41] Beyond the diverse range of cancer types, the augmented expression of GLUT1 has been associated with the invasive and metastatic potential of these tumors.[42] This suggests that GLUT1 plays a crucial role in cancer cell survival and progression. Concurrently, GLUT2 functions as a pivotal transporter in various organs, aiding in the uptake of glucose and fructose in the intestine, transporting fructose to the liver, promoting glucose reabsorption in the kidney, facilitating glucose-stimulated hormone secretion in the pancreas, and contributing to glucose sensing in neurons and blood vessels.[43] Recently, the involvement of GLUT2 in the transport of *Flammulina velutipes* polysaccharide-iron complex (FVP1-Fe(III)) has been shown in the human intestinal epithelial Caco-2 cell line. It showcases the importance of GLUT2 in iron deficiency anemia as FVPI-Fe (III) is a potential iron supplement for treating this disease[44] .

Mutations in the SLC2A2 gene, which encodes GLUT2, can lead to various metabolic disorders, including glycosuria, abnormal feeding behavior, preference for sugar feeding, and type 2 diabetes.[39] One of the genetic diseases associated with GLUT2 mutations is Fanconi-Bickel syndrome. The syndrome is linked to mutations in GLUT2, resulting in either impaired transporters or malfunctioning proteins that cannot effectively reach the plasma membrane. It manifests with glycosuria due to impaired glucose reabsorption in the kidney's basolateral membrane where GLUT2 is expressed.[39]

A growing body of research has provided evidence linking GLUT2 to the development and progression of certain cancer types.[38] In hepatocellular carcinoma, GLUT2 mRNA is expressed at higher levels than other GLUT isoforms and is positively correlated with advanced clinical stage. Ductal invasive breast carcinomas and colorectal cancer tissues also show overexpression of GLUT2 compared to healthy counterparts. Interestingly, GLUT2 is mainly localized in the cytoplasm of malignant cells. This distinct localization and expression may be linked to specific energy requirements and environments in these tissues. Furthermore, GLUT2 appears to play a more significant role in fructose metabolism in certain tumor-bearing mice, potentially compensating for weaker GLUT5 expression in breast cancer cell lines.[38]

Among these transporters, GLUT5 is exclusively specific to fructose transport. However, several other GLUT proteins, including GLUT2, GLUT7, GLUT8, GLUT9, and GLUT12, also possess the ability to transport fructose, despite it not being their primary substrate. The broad range of

dysfunctions linked to GLUT proteins, particularly those involved in fructose transport, underscores the urgent need to gain a deeper understanding of their role in the development of various diseases.

GLUT5 expression and activity levels are closely linked to fructose absorption and metabolism with dysregulation implicated in the development of metabolic disorders and certain cancers. GLUT5 plays a crucial role in facilitating fructose uptake into cells, where it can be metabolized and utilized for energy production or other cellular processes. The primary function of GLUT5 is the transport of fructose from the lumen of the small intestine into enterocytes, facilitating its absorption into the bloodstream. Once in the bloodstream, fructose can be taken up by the liver and other tissues for metabolism. This process is crucial for maintaining energy homeostasis, especially when dietary intake of fructose is high.

A deficiency or dysfunction in GLUT5 can lead to fructose malabsorption, a condition where fructose is not adequately absorbed in the small intestine. This can result in gastrointestinal symptoms such as bloating, diarrhea, and abdominal pain.

Overconsumption of fructose, especially from high-fructose corn syrup and sugary beverages, has been linked to metabolic disorders such as obesity, insulin resistance, non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes. The increased occurrence of inflammatory bowel diseases in modern Western diets is associated with the higher consumption of fructose. This link is supported by a study in which mice with reduced expression of GLUT5 exhibited aggravated experimental colitis when fed a fructose-rich diet. However, the colitis symptoms improved when the mice received antibiotic treatment, indicating a potential connection between GLUT5 expression, dietary fructose intake, and the composition of gut microbiota in colitis risk.[37]

GLUT5 being a major transporter of fructose plays a role in these conditions by regulating fructose uptake and metabolism. Studies have demonstrated that GLUT5 plays a crucial role in facilitating fructose uptake and utilization in acute myeloid leukemia (AML) cells, allowing fructose to serve as an alternative energy source under conditions of glucose restriction.[37] Notably, inhibiting fructose uptake using a fructose analog has shown synergistic efficacy in AML mouse models, indicating a potential advantage over conventional chemotherapy approaches alone.

Some cancer cells exploit fructose metabolism to support their energy needs and rapid proliferation as increased expression of GLUT5 has been observed in certain cancers suggesting that these cells

may rely on fructose as an energy source. This makes GLUT5 a potential target for therapeutic interventions aimed at disrupting cancer cell metabolism.

Due to the non-essential role of fructose in normal cell growth and survival, GLUT5 inhibition holds promise as a more selective approach with potentially fewer adverse effects compared to GLUT1 inhibition. This makes GLUT5 an appealing target for advancing cancer treatment, as it could potentially minimize collateral damage and contribute to improved therapeutic outcomes.[1]

DEVELOPMENT OF GLUTS TARGETED PROBES

The recognition that malignant cells have higher energy requirements for a long time [36] has led to the development of metabolic-based cancer detection methods. The differences in GLUT expression between different cells as well as the large amount of sugar consumption in cancer cells make GLUTs important therapeutic targets. Kinetic analysis of glucose and fructose uptake provides the structural basis for the development of fluorinated hexose derivatives for positron emission tomography (PET) imaging of malignant cells [37]. 18F-labelled 2-fluoro-2-deoxy-D-glucose (2-FDG) is the earliest and most commonly used radiotracer for PET [38]. 2-FDG is not completely metabolized after uptake by malignant cells, which gives rise to its continuous accumulation in malignant cells, and is widely used in clinical practice to observe glucose uptake, tumorigenesis and invasion [39,40]. However, since 2-FDG mainly targets GLUT1, which is ubiquitous in tissue cells, abundant false positive results show the insufficient ability of tracers targeting the glucose transporter GLUT1 to distinguish malignant cells [41]. In particular, in breast cancer, there is no significant difference in glucose uptake between malignant and normal cells, which limits the methods of detecting targeted glucose transport[42]. Since GLUT5 expression upregulation has a significant promoting effect on cancer, people have begun to pay attention to the development of targeted probes for fructose transport.

Fructose phosphorylation can occur at position C_1 (KHK) or C_6 (HK), so targeted probes designed for fructose transport mainly focus on these two sites. The first targeted GLUT5 tracer tested in tumors was the fluorinated fructose derivative 1-[18F]fluoro-1-deoxy-D-fructose (1-FDF), designed by Haradahira and colleagues [43], which showed rapid washout of 1-FDF by the kidney and liver *in vivo*. Triple-negative breast cancer cells and tissues express higher levels of GLUT5 mRNA and protein than estrogen receptor-positive breast cancer cells and tissues, and the growth and progression

of breast cancer are highly dependent on fructose . Thus, the 1-FDF analogue 6-[18F]fluoro-6-deoxy-D-fructose (6-FDF) was tested for PET imaging in murine EMT-6 and human MCF-7 breast cancer cells expressing GLUT5[44] . 6-FDF has been shown to be the substrate of human KHK and is rapidly metabolized *in vivo*. In addition, PET imaging tests of 3-[18F]fluoro-3-deoxy-D-fructose (3-FDF) in EMT-6 and MCF-7 cells demonstrated that GLUT5 can transport both furanose and pyranose forms of fructose [46].

Fluorophore labelling with 7-nitro-1,2,3-benzadiazole (NBD) at the fructose C_1 position (1-NBDF) was able to target breast cancer cells GLUT5 well. The absorption of 1-NBDF probes was studied in three breast cancer cell lines: MCF 7, MDA-MB-435, and MDA-MB-231. 1-NBDF showed very good absorption in all cell lines tested, with uptake levels comparable to the corresponding glucose analogue 2-NBDG. Significant uptake of 1-NBDF was not observed in cells lacking GLUT5, while GLUT5-specific accumulation was detected in cells expressing GLUT5[47].

2,5-AM aryl conjugates with high affinity and specificity for GLUT5 have emerged as a new generation of radiotracer probes. 2,5-AM is a symmetric molecule that exists only as a furanose ring structure and cannot be opened to form isomers [48]. The 1-amino-2,5-anhydro-d-mannitol-NBD conjugate (NBDM) synthesized on the basis of the 2,5-AM ring combines well with GLUT5 in Chinese hamster ovary (CHO) cells and can be used as a fluorescent probe targeting GLUT5 [49,50]. However, it should be noted that the accumulation of NBDM probes in cells is limited, resulting in inadequate fluorescence reporting. Recently, a novel fluorescent glycoconjugate was reported as a GLUT5 probe[51]. This fluorescent glycoconjugate is constructed with 2,5-AM as the fixed fructofuranose ring and various coumarins (Cou) as the fluorescent fraction (Man-Cou probe), which can target GLUT5 in malignant cells for viable cell metabolic analysis, and the positive response does not appear in normal cells. Compared to previously developed probes, the improved Man-Cou probe can process samples in only 10 minutes, which can be used for rapid on-site high-throughput diagnosis[52].

Although many glycoconjugates have been synthesized for cancer research, diagnosis and treatment, GLUT5-mediated uptake is often limited by many factors that produce uncontrollable losses. It must be emphasized that the molecular structural size and hydrophilicity of the conjugate are important factors affecting the efficiency of GLUT5-mediated drug delivery, and these two factors should be prioritized in the synthetic design of novel bioactive or imaging agents [53].

FLUORESCENT GLUT PROBES

Fluorescent GLUT probes are specialized molecular tools designed to study the activity, localization, and function of glucose transporters (GLUTs) within cells. These probes utilize fluorescence to provide real-time, visual information about GLUT proteins, offering insights into their role in cellular metabolism and disease. These probes are designed to target specific GLUT isoforms, ensuring that they bind only to the intended transporter. This specificity is achieved through the molecular structure of the probe, which complements the binding site of the GLUT protein. Once bound to the GLUT protein, the fluorescent probe either remains attached, allowing for visualization of the transporter's location, or it may be transported along with the substrate (e.g., glucose), enabling the study of transporter activity.

This approach offers numerous benefits, including non-invasiveness, swift response, high sensitivity, cost-effectiveness, portability, real-time monitoring, and the crucial absence of ionizing radiation. Targeting GLUTs specifically to detect changes in GLUT levels and activity within the membrane has led to the development of many fluorescent molecular probes. By designing fluorescent probes that target GLUTs, it becomes possible to conduct *in vitro* and i*n vivo* imaging of cancer cells and other diseased cells. Several studies have explored fructose and glucose analog-fluorophore combinations in the development of these specialized probes for glucose and fructose transporting GLUTs. Among those, coumarin as fluorophores are used to access multicolor fluorescent probes compatible with GLUT-mediated uptake. The choice was based on their small size and the potential to tune fluorescence color through coumarin core functionalization. The uptake of a 7-aminocoumarin as a conjugate of GLUT5-targeting 2,5-anhydro-D-mannose was investigated through reductive amination to produce the first blue-fluorescent D-mannitol–coumarin conjugate ManCou. ManCous, GLUT5-specific probes were designed for the direct analysis of GLUT5 activity in cells and signal reporting over a broad range of the fluorescence spectrum. These probes show GLUT5 and fructose dependency evident from the lack of probe uptake in GLUT5-deficient liver carcinoma HepG2 cells. The specific targeting of GLUT5 enables a significant differentiation in the imaging of GLUT5-expressing cells vs. GLUT5-deficient cells, a crucial feature in certain cancers through fluorescence-based analysis with ManCous.

The goal of this study carried out in Chapter 2 is to establish a two-color fluorescence assay for simultaneous screening of GLUT5 and GLUT2 fructose transporters in live cells. To achieve this

goal, our aim is to design, synthesize and characterize coumarin-based fructose probes, testing different cell lines for GLUT5 and GLUT 2 distribution, establishing analysis by confocal microscopy flow cytometry and immunocytochemistry.

Chapter 2

ESTABLISHING A TWO-COLOR FLUORESCENCE ASSAY FOR SIMULTANEOUS SCREENING OF GLUT5 AND GLUT 2 FRUCTOSE TRANSPORTERS IN LIVE CELLS

INTRODUCTION

Metabolic deregulations in cells induce alterations in the cellular GLUT composition, resulting in overexpression of intrinsic or expression of extrinsic GLUTs[32]. Among GLUTs, glucose-transporting GLUT1 attracted attention for half a century as a target for cancer therapy and diagnostics, and other GLUTs started to gather much interest recently because of their direct relationship with cancer[54]. Deregulation in the non-specific glucose/fructose transporter GLUT2 is linked to obesity, diabetes and is a prognostic factor for liver cancers[55]

Particular attention is drawn to fructose-transporting GLUTs and the fructose-specific transporter GLUT5 due to the links between fructose uptake and cancer development, progression, and metastasis[56]. Consequently, the fructose transport-targeting probes are of interest as biochemical and biomedical tools.

Between fructose-transporting GLUTs, only GLUT5 has been established as fructose-specific (Km ~ 11–16 mM).[57,58] GLUT2 is a non-specific low- affinity and high-capacity transporter that can transport glucose (Km ~ 17 mM), fructose (~76 mM), galactose (~92 mM), and glucosamine (0.8 mM). Interestingly, despite the low sensitivity to fructose, the high-capacity GLUT2 is a primary fructose transporter in the liver (where GLUT5 is absent). While the need for fructose transporters is primarily justified by its nutritional properties, the impact of fructose appears to be much broader. As a nutrient, fructose directly participates in glycolysis, increases cell proliferation, migration.[59] and also acts as a signaling molecule in cell proliferation. Among these processes, fructose uptake has

shown considerable importance in cancer, with the activity of the fructose-specific transporter GLUT5 being particularly associated with cancer development, progression, and metastasis [56].

Targeting GLUT5 for drug delivery has faced challenges due to the stringent substrate specificity of the transporter[60]. However, some success has been observed in biomedical applications using the GLUT5 inhibition approach. Specifically, a GLUT5-specific inhibitor has been shown to enhance the effectiveness of conventional anticancer drugs.[61] The significant role of GLUT5 in various metabolic disorders underscores the necessity for effective activity reporters. Progress has been made in targeting GLUT5 using analytical probes that enable fluorescence-based activity analysis in cell cultures. Current molecular probes designed to analyze GLUT activity include small fluorophores, such as NBD and coumarins, which are delivered through GLUT transporters in the form of glycoconjugates[62]. For instance, specific targeting of the fructose transporter GLUT5 has been successfully accomplished using 2,5-anhydro-D-mannitol as a cargo carrier.[63]

In this report, we explored the two-color fluorescence probe assay for simultaneous screening of Glut 5 and Glut 2 Fructose transporter in live cells. A major difference between the two major fructose transporters – GLUT5 and GLUT2 – is their substrate specificity (GLUT5) or their lack thereof (GLUT2). Hence, we hypothesis that the combination of two probes (P2 and NBDM) to profile cell for both GLUT 5 and GLUT 2 activity. Consequently, we show that restricting the conformational flexibility of fructose by removing the anomeric hydroxyl group and controlling its positioning within the GLUT5 binding site through derivatization enhances substrate discrimination. When utilized as a mixture that mimics the nutritionally diverse and configurationally complex fructose environment, the corresponding coumarin derivatives facilitate the distribution of the probe between specific and non-specific fructose GLUTs, enabling us to delineate the activity of GLUT2.

The findings offer valuable insights into how substrates are distributed among GLUTs during fructose uptake in mammalian cells, laying the groundwork for developing GLUT2-specific analytical probes or activity modulators using configurational isomers of fructopyranose. Additionally, the results establish experimental conditions for simultaneously screening fluorescence activity for both specific and non-specific fructose GLUTs in live cells.

RESULTS AND DISCUSSION

PROBE DESIGN, SYNTHESIS, AND PROPERTIES

Within mammalian cells, the fructose uptake by GLUT5 has been extensively studied and research indicates that GLUT5 can transport fructose in both furanose and pyranose conformations with similar efficiency. However, the uptake of the furanose conformation appears to be specific to GLUT5. Given the specific uptake of furanose by GLUT5 and the predominance of pyranose configuration in hexose transport by non-specific transporters (e.g, glucose, galactose), we hypothesized that configurational restrictions might play a crucial role in directing fructose through non-specific versus specific GLUTs. This understanding could offer a strategy for targeting non-specific GLUTs.

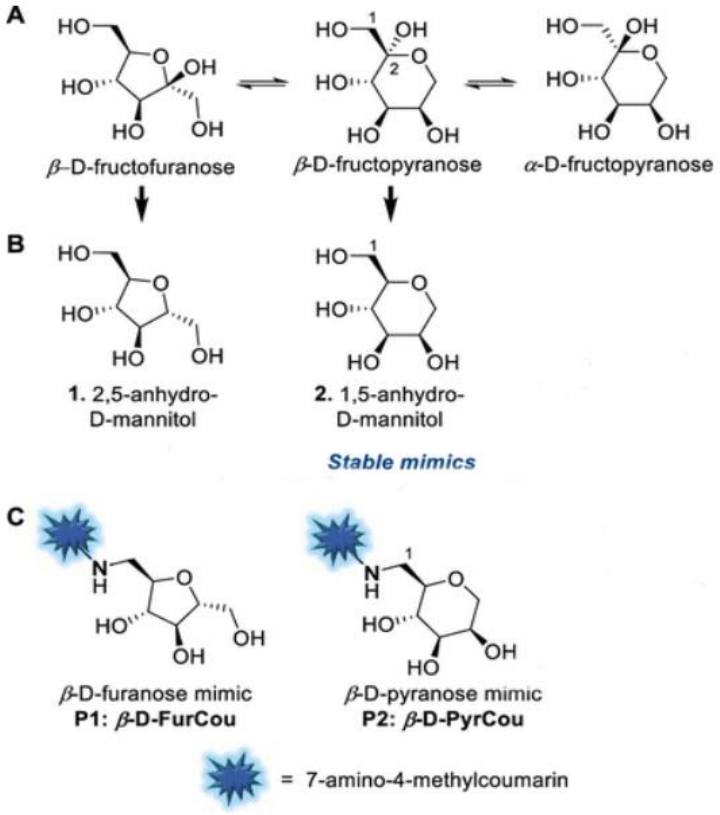
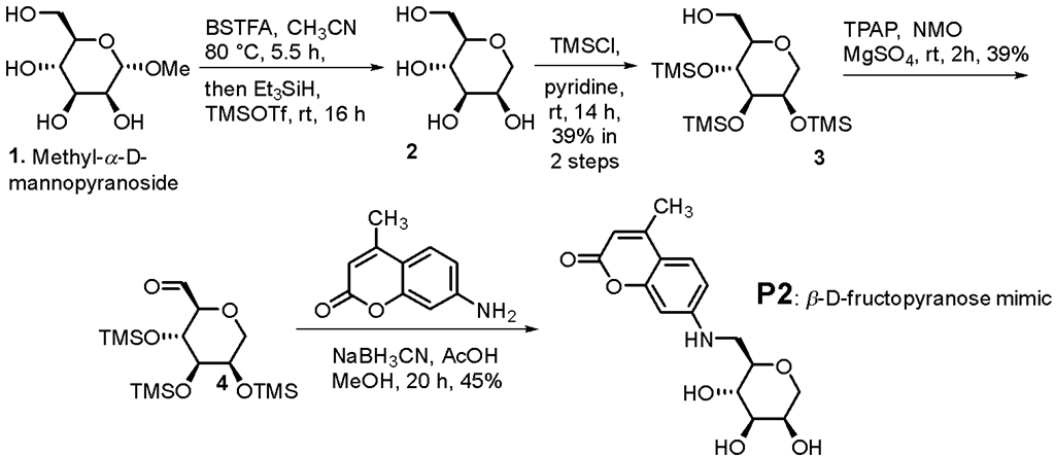


Figure 2. Fluorescently labeled, stable furanose and pyranose mimetics of fructose

β-Fructopyranose is a structural form of fructose characterized by a six-membered ring (pyranose) with a hydroxyl group (-OH) attached to the anomeric carbon in the beta (β) configuration. In aqueous solutions, fructose can cyclize to form either five-membered (furanose) or six-membered (pyranose) rings through the formation of a hemiacetal bond. The position of the hydroxyl group on the anomeric carbon determines the α and β form. In β-fructopyranose, the hydroxyl group is equatorial, which is more stable due to less steric hindrance. β-Fructopyranose is the predominant form of fructose in aqueous solutions, making up about 70% of the fructose population. Its stability is attributed to internal hydrogen bonding and the chair conformation, which reduces steric strain. Fructose exists in equilibrium between various forms, but β-fructopyranose is the most stable and abundant due to its favorable internal hydrogen bonding. The different forms of fructose, including β-fructopyranose, play crucial roles in metabolism. They are substrates for various enzymes involved in glycolysis and fructolysis, contributing to energy production and other metabolic pathways. The specific form of fructose taken up by transporters such as GLUT5 can influence the efficiency and specificity of fructose transport into cells.



Scheme 1. Synthetic Routes to **P2** . Reagents and conditions: (**A**) BSTFA, Ar, 80°C, 5.5 h (step 1), Et3SiH, TMSOTf, Ar, rt, 16–21 h (step 2); (**B**) pyridine, TMSCl, rt, 14 h; (**C**) NMO, MgSO4, TPAP, CH2Cl2, rt, 1–2 h; and (**D**) 7-amino-4-methylcoumarin, NaBH3CN, MeOH, AcOH, pH < 6, rt, 20 h. See the Supporting Information for experimental details.

To establish the simultaneous screening of fructose transporters, we designed the corresponding configurationally locked mimetic (Scheme 1). The probe design considered the non-essential role of the anomeric hydroxyl group in fructose uptake and aimed to create substrates with locked configurations by removing this group. This approach is exemplified by the GLUT5-targeting furanose mimic, 2,5-anhydro-D-mannitol (Figure 2B, **1**). Furthermore, the primary hydroxyl group offers a suitable site for attaching a fluorophore to create the desired fluorescently labeled sugar mimetics. Consequently, we employed this strategy with pyranose isomers, designing configurationally locked pyranose mimetics (Figure 2B, compound **2**) by removing the anomeric hydroxyls and adding a fluorescent label at the C1 position. Configurationally locked pyranose mimetic of β-D-fructopyranose (Figure 2.1C, **P2**) was synthesized from α-Methyl D-Mannopyranoside (**4**) (Scheme 1, see the Supporting Information for synthesis details). Initially, the α-methoxy group of the corresponding α- methyl glucoside (**4**) was cleaved off through a sequential silylation/reduction in the presence of *N*,*O*- Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and Triethylsilane (Et3SiH)/TMSOTf accordingly. The subsequent silylation of **6** allowed for selective protection of secondary hydroxyls (**8**) due to the selective recovery of the primary hydroxyl during compound purification on the silica gel. The primary hydroxyl was then oxidized using Tetrapropylammonium perruthenate TPAP/NMO (**10**), and the resulting pyranose mimetic was conjugated with 7-amino-4-methylcoumarin through reductive amination. The mild acidic conditions of reductive amination also ensured the deprotection of all hydroxyls, yielding fluorescently labeled probe (**P2**). The presence of the fluorescent tracer on each of the synthesized probes allowed us to monitor and assess their uptake directly in live cells.

In the preliminary evaluation of probes separately and in the mixture, the differences in their GLUT preferences have been established. For example, the 5-membered-ring conjugate (**P1**, Figure 2) was shown to be taken preferentially through GLUT5 even in the presence of GLUT2. The six-membered ring conjugate **P2** was also taken up through GLUT5, albeit with 6-fold lesser efficiency. When presented as a mixture, the probes reflected the competition for GLUT5, with P2 also passive through alternative GLUTs, presumably GLUT2. The ability of P2 to change the uptake path when GLUT5 is occupied, provided the feasibility to use P2 uptake as a measure of GLUT2 activity – a task thus far remaining a challenge. Respectively, proof-of-concept analysis of GLUT5 vs. GLUT2 activity in MCF7 cells was carried out, reflecting the 3:1 ratio in the activities of the two transporters

(GLUT5:GLUT2). While the proof of concert supported the feasibility of such analysis, the development of a functional assay requires broader range of data in multiple cell lines.

This analysis focused on establishing the ability of the probes to pass into live cells hence, uptake of these probes were assessed in the following mammalian cell lines:

- **MDA-MB-231**: Highly aggressive, triple-negative breast cancer cells expressing GLUT1, GLUT3, GLUT4, and GLUT5. This cell line is often used to study metastatic breast cancer and tumor progression.
- **MDA-MB-453**: Androgen receptor-positive and HER2-positive breast cancer cells expressing GLUT1, GLUT4, GLUT12, and variably GLUT5. These cells are relevant for research into androgen signaling and HER2-targeted therapies.
- **HEP-G2**: Liver cancer cells expressing GLUT1, GLUT2, GLUT5, and GLUT9. These transporters reflect their liver origin and function in glucose and fructose metabolism, making Hep-G2 cells valuable for research in liver cancer and related metabolic studies.

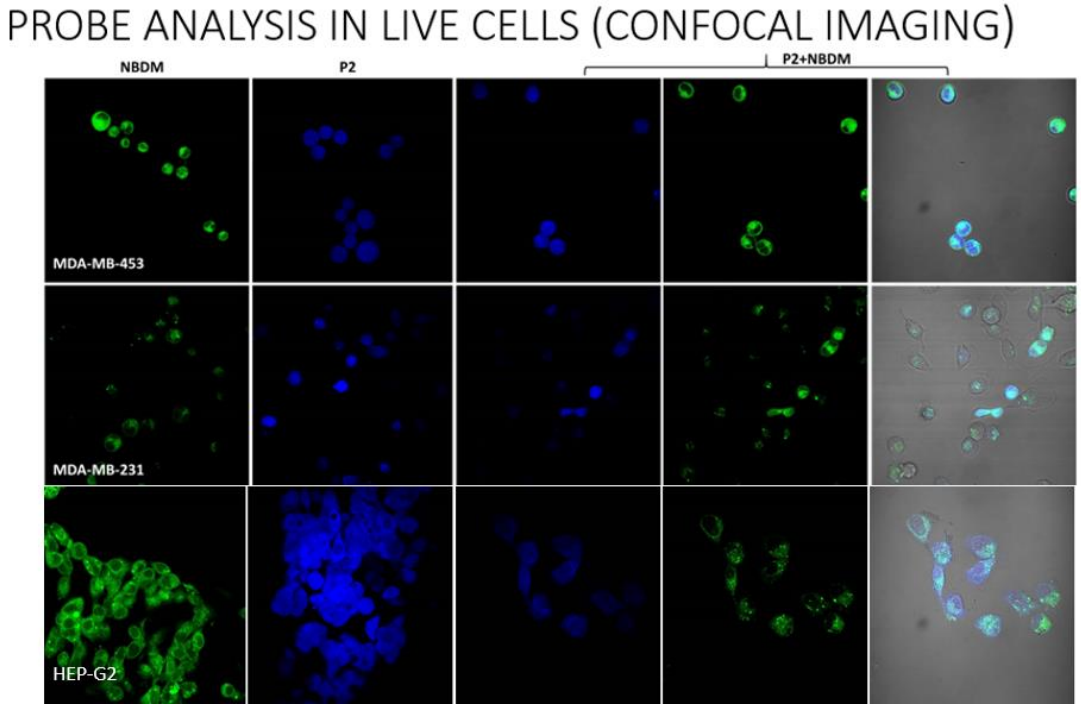
METHODS

Cell Culture: MDA-MB-231, MDA-MB-453, MCF-7 and HEP-G2 cell lines were cultured using complete RPMI-1640 medium, which includes basal RPMI-1640, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin-glutamine. The cells were maintained in 10 cm cell culture dishes (VWR) at 37 °C, with 5% CO2 and 65% relative humidity. Once the cells reached approximately 80% confluence, they were harvested using 2 mL of 0.25% trypsin-EDTA. The trypsin activity was neutralized by adding 3 mL of culture media, and the cells were collected by centrifugation at 1600 rpm for 5 minutes. After centrifugation, the cells were resuspended in 3 mL of complete culture media and seeded onto 35 mm glass-bottom confocal dishes (MatTek) at a density of 100,000 to 150,000 cells per dish. The volume in each dish was adjusted to 2 mL with the corresponding media as needed.

Probe uptake analysis: The evaluation of uptake is dependent on the accumulation of fluorescence signal within the MDA-MB-231, HEP G2 and MDA-MB-453 cells following the application of the probe. Solutions of the probes (P2, NBDM) were prepared at 25 μM in 2 mL of complete cell culture media. These solutions were made using 5 mM stock solutions of the probes in DMSO. For the treatment, cells cultured in glass-bottom confocal dishes were utilized. The cell media were removed,

and the probe solutions (2 mL) of each probe and an addition of both P2 and NBDM were added. The cells were then incubated with probes at 37 °C for 15 minutes. Afterward, the probe solutions were removed, and the cells were washed with complete culture media (2 × 1 mL) to eliminate any remaining probe. Finally, the cells were replenished with 2 mL of complete culture media for the purpose of imaging using confocal microscopy. Fluorescence was captured using a confocal microscope and quantified with ImageJ software. The relative fluorescence was calculated as CTCF/area, where CTCF (corrected total cell fluorescence) is defined as the integrated density minus the product of the area of the selected cell and the mean background fluorescence. The detected blue and green fluorescence signals were observable at concentrations as low as 25 μM.

RESULTS AND DISCUSSION



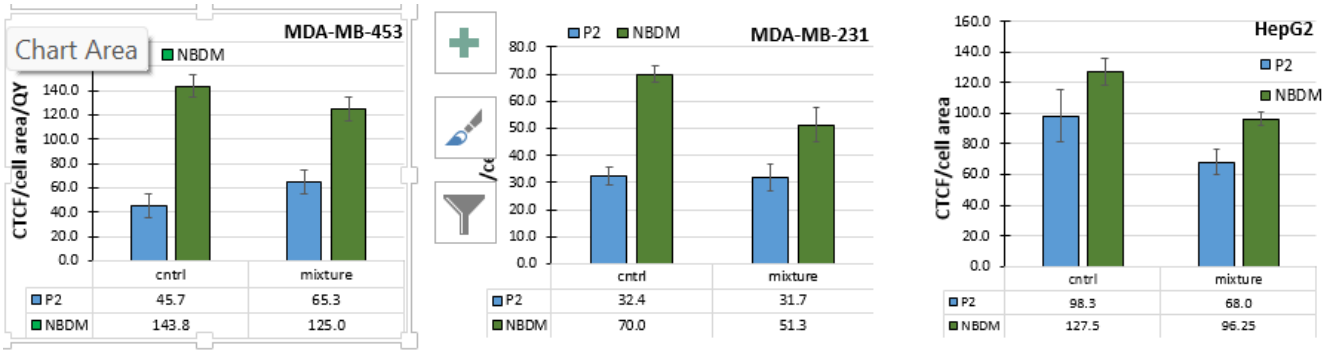


Figure 3: Confocal Images of cell treated with NBDM, P2 and NBDM+P2. Confocal fluorescence images (60X objective) were obtained after treating cells with 25 μM P2 and 25 μM NBDM for 15 min. P2 was images using DAPI filter (405exc nm /461em nm), NBDM was imaged using eGFP filter (488exc nm/525em nm). All images were acquired at the same laser intensity and exposure time.

For the analysis, the cell lines were treated with probes at equimolar concentrations, and fluorescence readouts were obtained using ImageJ. The cumulative data presented below (Figure 2) shows significant differences in probe uptake, as well as relative differences between NBDM and P2.

Overall, we observed that the differences between cell lines are reflected in the relative uptake efficiencies of NBDM versus P2, as well as differences in probe competition. For instance, in MDA-MB-453 cells, NBDM uptake was approximately 30% higher than that of P2. As indicated by the published proof of concept by Gora, GLUT5 differentially recognizes the two probes. Individual probe uptake is assumed to represent GLUT5-mediated translocation, given the low Km of GLUT5 (10 mM). Uptake of probes in the equimolar mixture is assumed to indicate the GLUT5 contribution to NBDM uptake (due to the GLUT5 specificity of a 5-membered fructofuranose) and the mixed uptake for P2.

In MDA-MB-453 cells, the presence of NBDM increased P2 uptake by a factor of six compared to the control. Considering that in the mixture, NBDM predominantly passes through GLUT5 (occupying the transporter), the increased P2 uptake reflects a more efficient alternative uptake pathway for fructose, i.e., GLUT2 (Km 76 mM). Therefore, we can delineate the ratio of GLUT5- versus GLUT2-mediated uptake of P2 as a measure of fructose transport activity. Specifically, the

sixfold increase in P2 uptake represents 43% of the total P2 uptake, indicating the contribution from GLUT2. Thus, for MDA-MB-453 cells, the GLUT5 ratio is approximately 3.3:1.

In MDA-MB-231 cells, we observed only competition between the probes when the mixture was introduced. The lack of enhancement in P2 uptake suggests an absence of GLUT2 activity. It is important to note that no genetic or protein information is available regarding the expression of GLUTs of interest in these cell lines, except for GLUT5. Therefore, further validation of the ratio through immunofluorescence or protein analysis is necessary.

CONCLUSION

In conclusion, our experimental findings with the novel two-color fluorescent probes demonstrate a distinct difference in uptake when the probes are used individually compared to when they are used in combination. The observed lower affinity of GLUT5 for P2, and the competition effects seen in the mixture, suggest an alternative uptake pathway for P2. Further investigations into the cellular GLUT environment, specifically the expression levels of GLUT5 versus GLUT2, are necessary to provide additional support for these findings.

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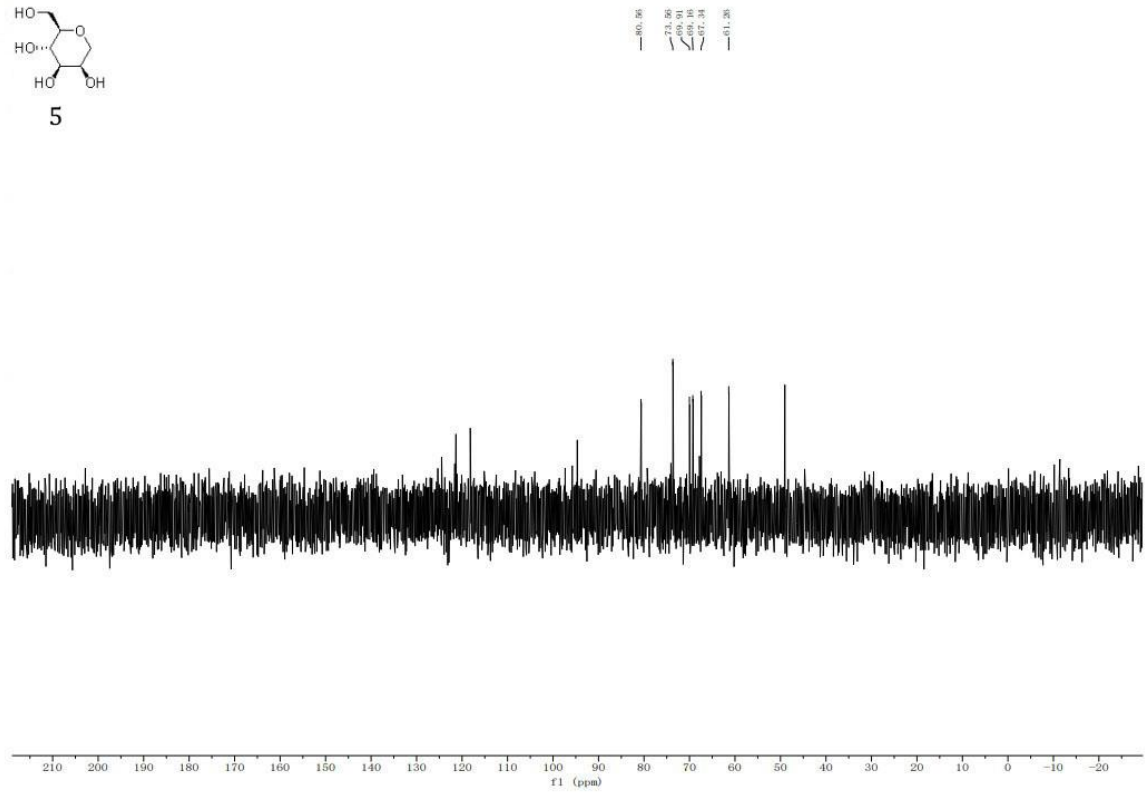

Figure 4. ^{13}C- NMR of crude 5 (100 MHz, D_2O)

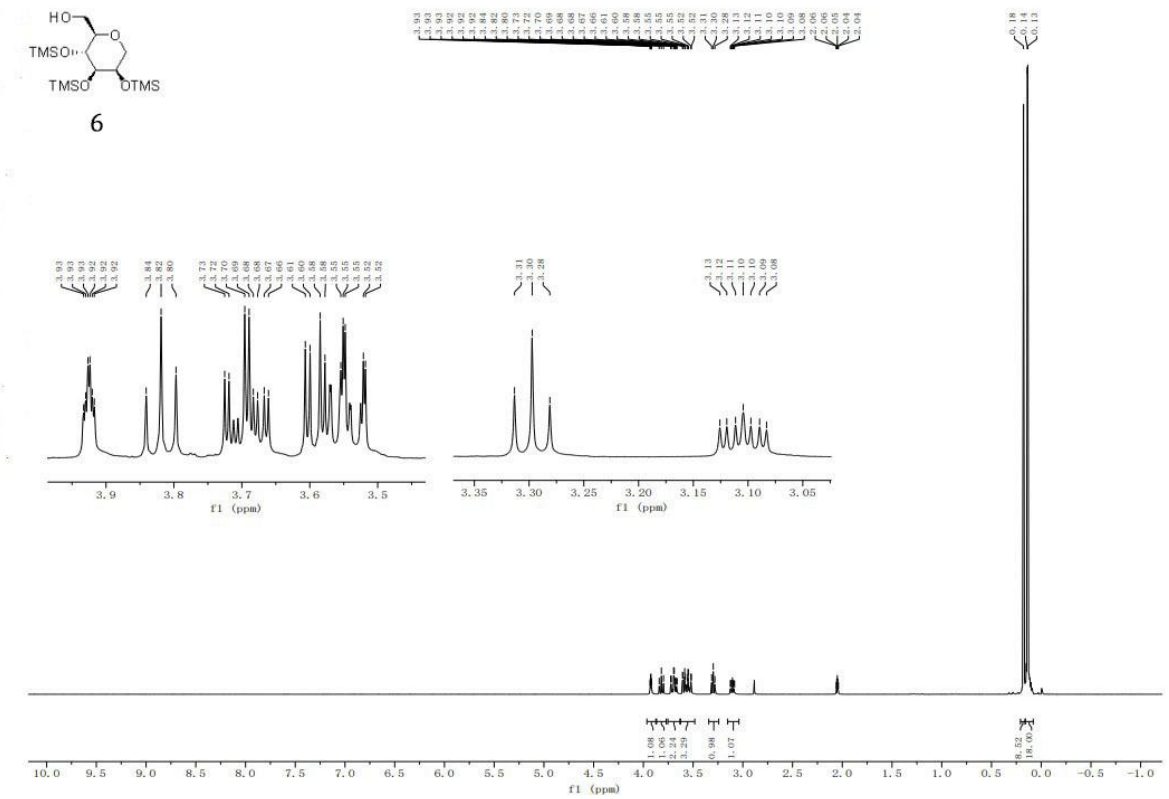

Figure 5. ^{1}H NMR of 6 (400 MHz, acetone – d_6)

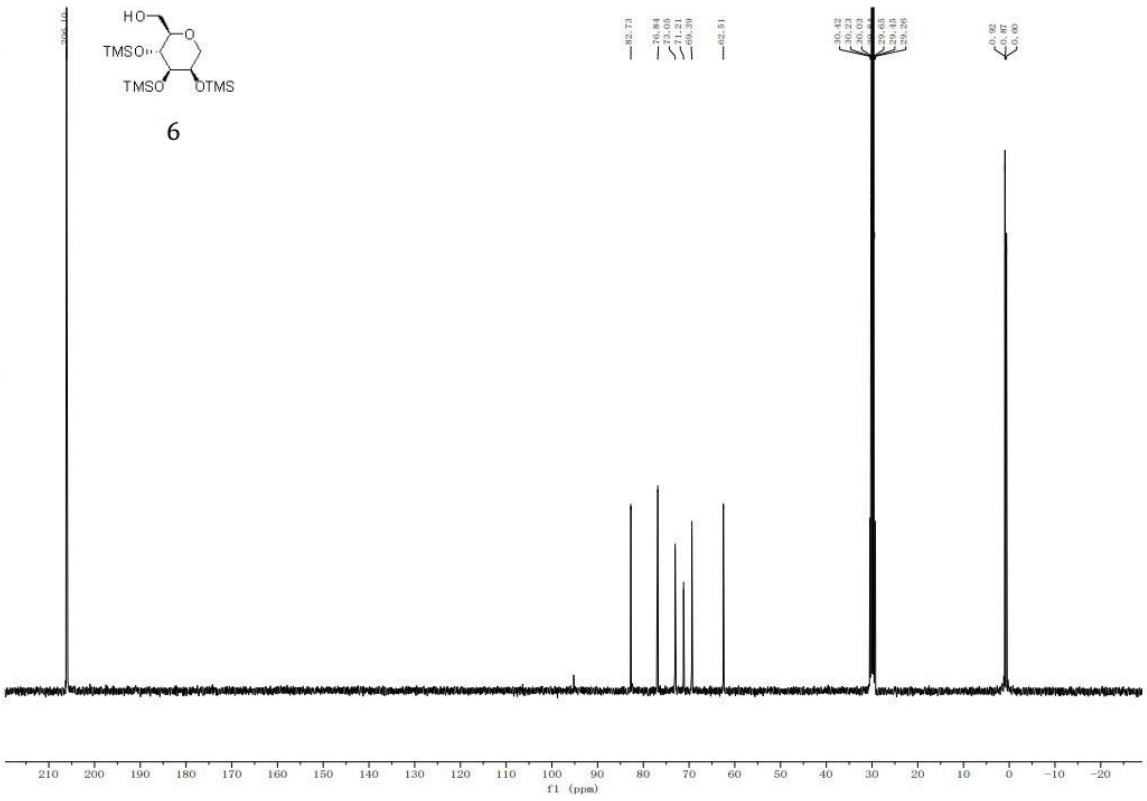

Figure 6. ^{13}C- NMR of crude 6 (100 MHz, acetone – d_6)

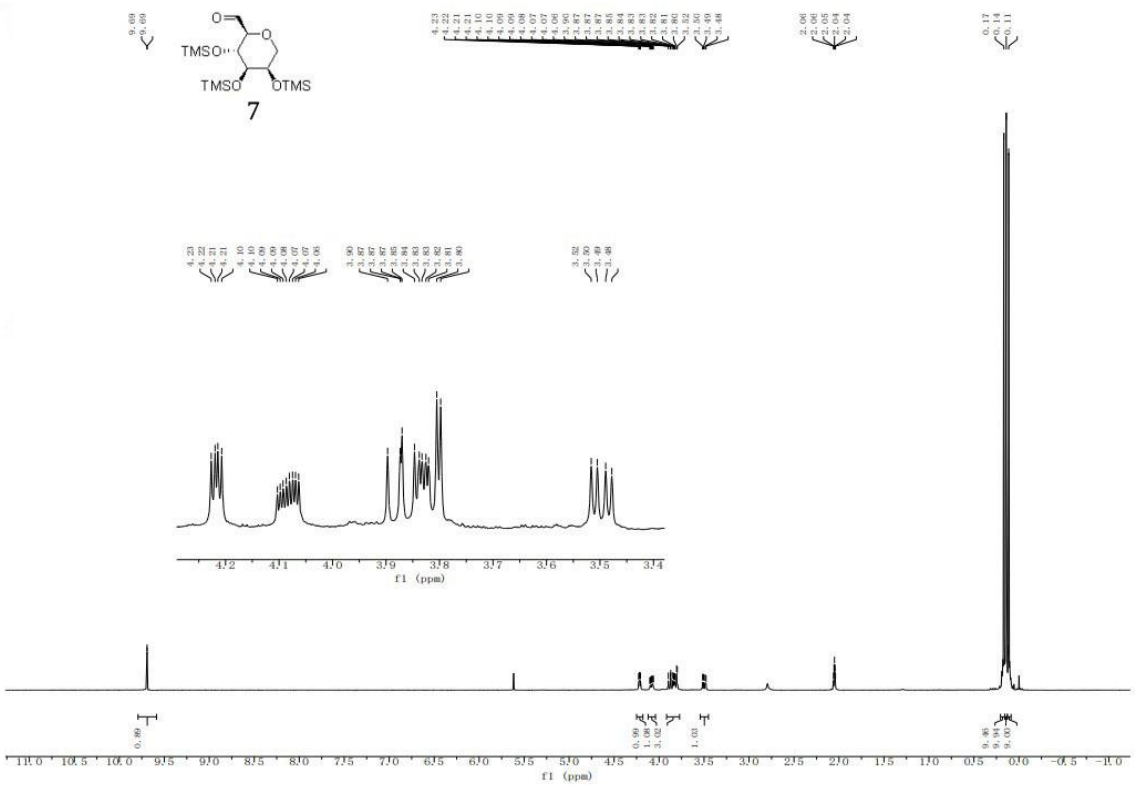

Figure 7. ^{1}H NMR of 7 (400 MHz, acetone – d_6)

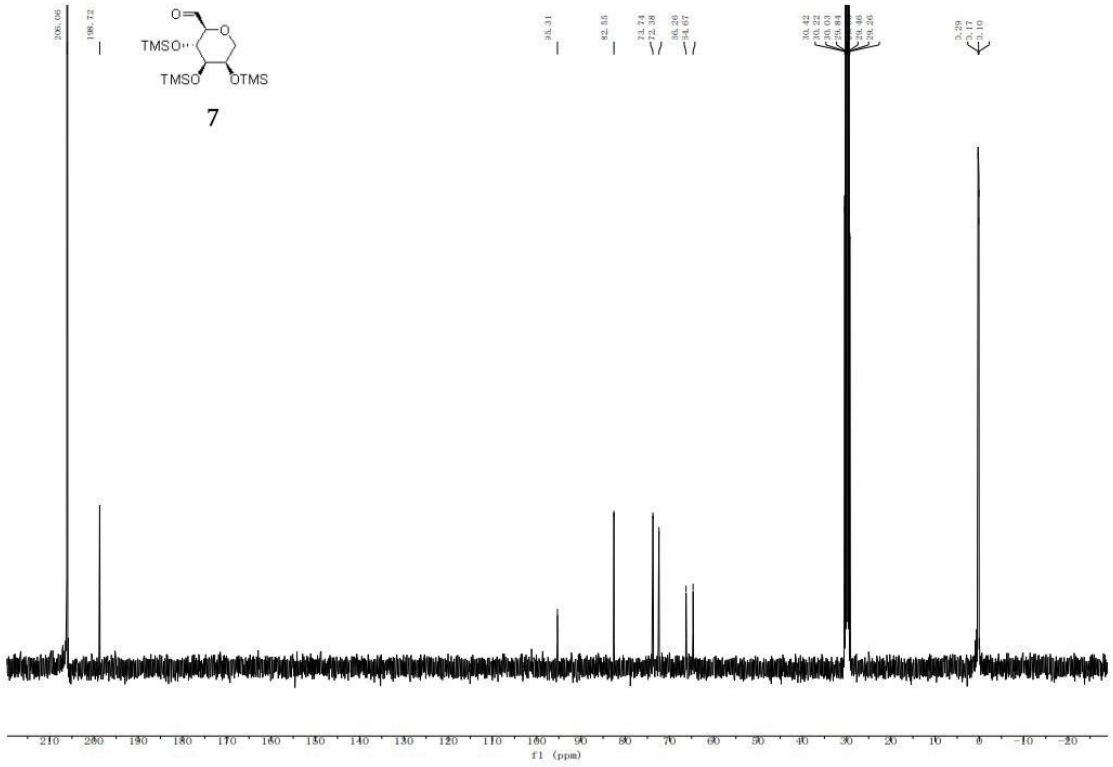

Figure 8. ^{13}C- NMR of crude 7 (100 MHz, acetone – d_6)

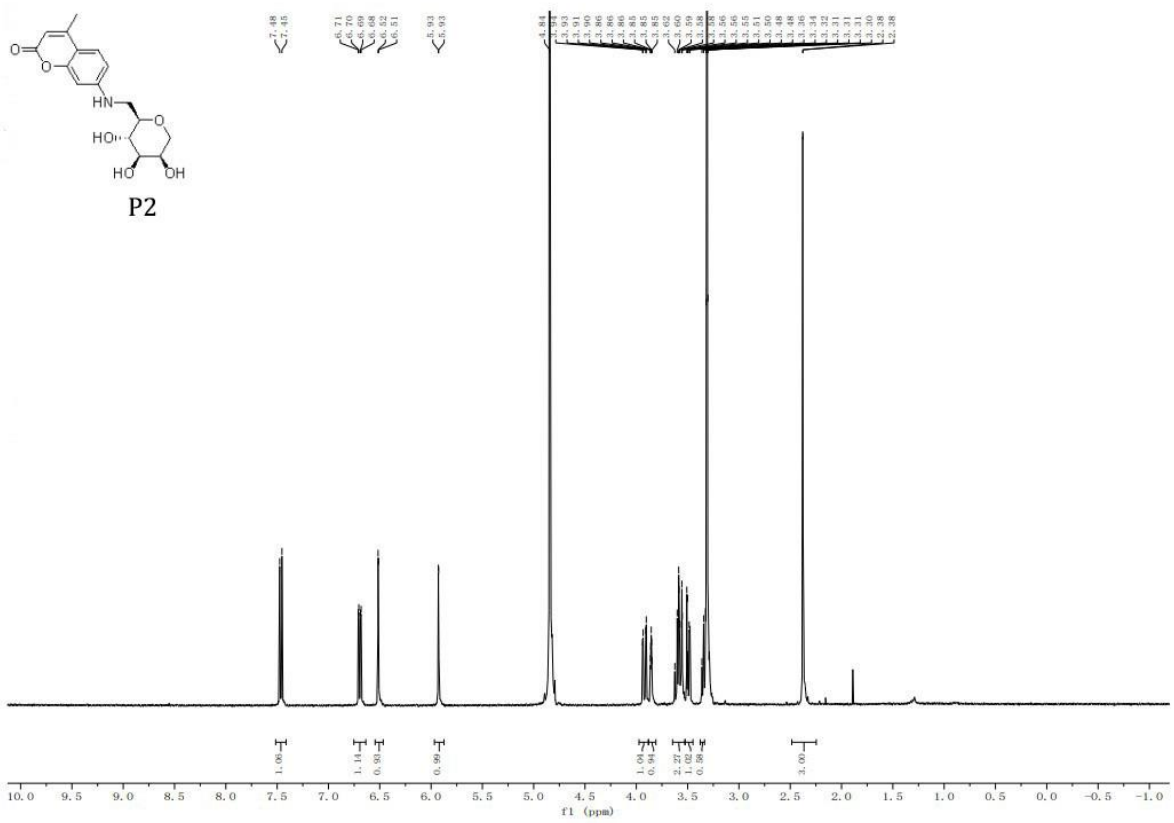

Figure 9. ^{1}H NMR of **P2** (400 MHz, CD_3OD)

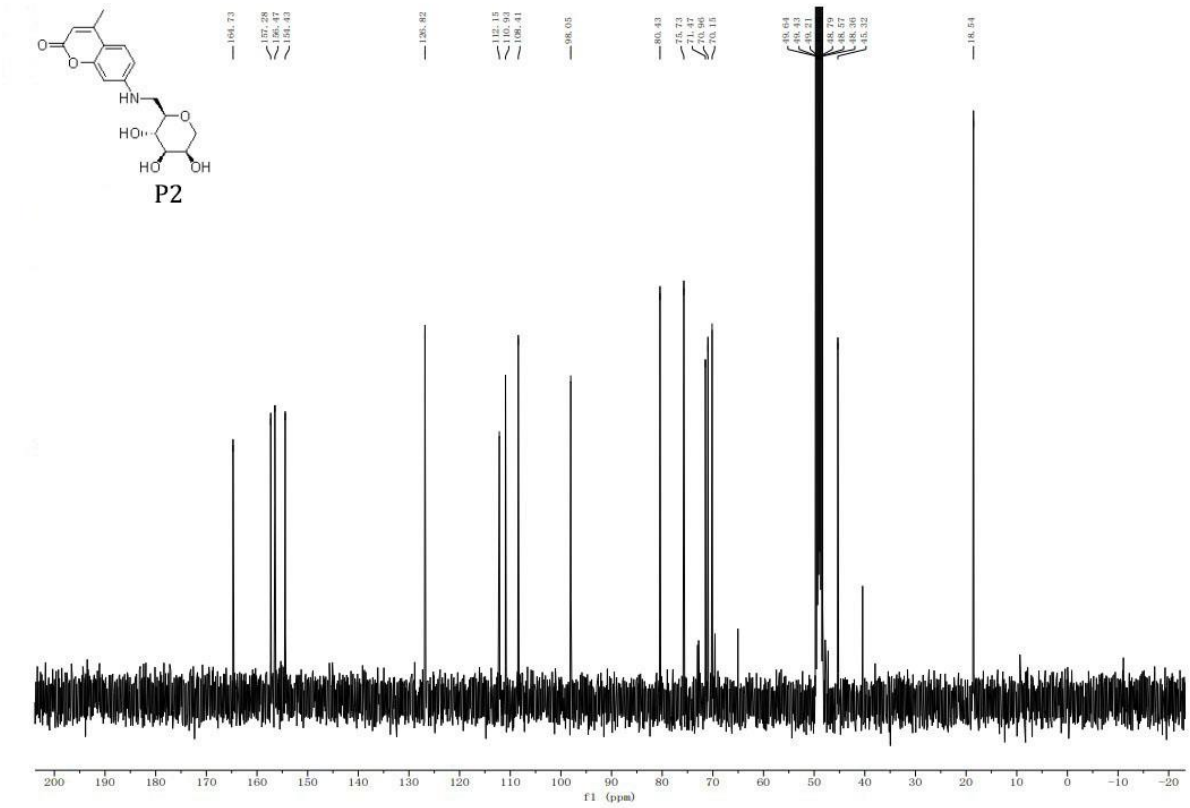

Figure 10. ^{13}C- NMR of **P2** (100 MHz, CD_3OD)