CHAPTER 4: FUCOSE-GALACTOSE IN LEARNING AND MEMORY

4.1 Introduction

4.1.1 Carbohydrates in neurological processes

Carbohydrates play important roles in numerous biological processes and recently, interest in identifying the biological functions of this class of biomolecule has intensified. Carbohydrates are ubiquitous in nature and structurally diverse, allowing for a broad range of biological functions. They facilitate proper protein localization as well as protein folding and stability.[1] Additionally they are critical to cell-cell communication events such as immune response,[2] microbial virulence,[3] and inflammation.[4] The surface of a cell is decorated with a variety of motifs that play a crucial role in mediating many biological processes—among these motifs are glycosylated lipids and proteins. These glycoconjugates serve as receptors and ligands, providing a structural link between a cell and its environment. The high complexity and heterogeneity of the adorning carbohydrates encode information, allowing cells to communicate.

Not surprisingly, extensive glycosylation has been found on the surface of nerve cells.[5, 6] Carbohydrates present at the synapse have various functions ranging from cell adhesion and axon pathfinding during brain development, to neurotransmitter reception and memory formation processes in mature synapses.[7] In particular, it has been shown that transmembrane glycoproteins containing sialyl or fucosyl epitopes play a role in neuronal processes.[6, 8] A well-studied example is neural cell adhesion molecule (NCAM), a member of the immunoglobulin superfamily which is glycosylated with polysialic acid (PSA) residues. NCAM is crucial in development and regeneration of the nervous system and is also involved in synaptic plasticity associated with learning and memory.[5, 8, 9] Interestingly, the PSA residues on NCAM greatly affect these neuronal processes. Enzymatic removal of

PSA on NCAM using endoneuraminidase (endo N), which selectively degrades PSA, perturbs neuron migration and axon outgrowth during development.[7, 10] Furthermore, injection of endo N into the hippocampus causes significant impairment of spatial memory and completely prevents induction of long term potentiation (LTP), a measure of learning and memory.[10]

4.1.2 Implications for fucosylation in learning and memory

Similar to sialyl epitopes, fucosyl epitopes have also been heavily implicated in neuronal processes such as development and learning and memory. Fucosylated glycoproteins are enriched in the central nervous system and account for as much as 85% of the protein-bound sugars in synaptic plasma membranes.[11] Altered fucosylation of brain glycoconjugates have been shown to play a role in the molecular mechanisms underlying long term memory formation and information processing. Studies have shown an increase in [^{3}H]-fucose incorporation into brain glycoproteins in rats during passive avoidance training tasks.[6, 12, 13] Increased fucose uptake into hippocampal and cortical glycoproteins has also been demonstrated in rats trained in a brightness discrimination task.[14, 15] Furthermore, increased fucosylation was associated with increased activity of fucokinase, an enzyme involved in fucose activation prior to the fucosylation step in a variety of species, including both rats and chicks.[16] Not only has an increase in fucosylation been seen during particular training tasks, but treatment with fucose has been shown to cause an increase in LTP, which is correlated with synaptic strength.[17, 18]

If fucosylation is necessary for the process of memory formation, then inhibition of fucosylation might be expected to disrupt this process. Indeed this is the case. Fucose plays

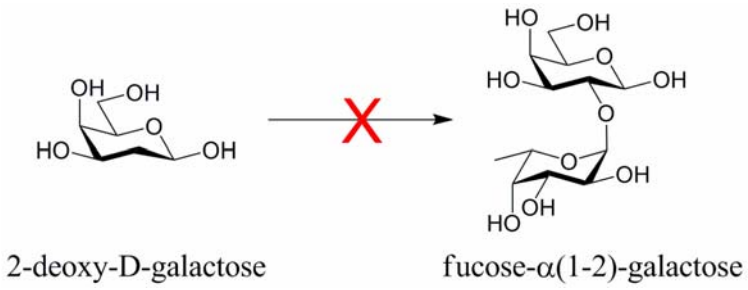

Figure 4.1. 2-Deoxy-D-galactose inhibits fucosylation by competition with galactose for incorporation into oligosaccharaides. The lack of a C-2 hydroxyl group prevents the formation of the α(1-2) glycosidic linkage.

a major role as a terminal sugar in glycoproteins and is often covalently linked to galactose by a 1-2 glycosidic linkage. If galactose is replaced by 2-deoxy-D-galactose, the 1-2 linkage cannot be formed, due to the lack of a C-2 hydroxyl, and fucosylation is inhibited (Figure 4.1). Intracerebral injection of 2-deoxy-D-galactose into the brains of rats and chicks exerts amnesic effects during passive avoidance training tasks—further implicating fucose in learning and memory.[19-22] 2-Deoxy-D-galactose has also been shown to decrease levels of [^{3}H]-fucose incorporation into neuronal glycoproteins.[22] In addition, blocking the fucose-α(1-2)-galactose epitope using an antibody against fucose-α(1-2)-galactose also results in loss of memory in rats.[23]

Taken together, evidence overwhelmingly implicates fucose in memory formation. Despite the evidence, the exact molecular role of fucose is not known and neuronal glycoproteins bearing this epitope have not been identified. Carbohydrate binding proteins (i.e. lectins) to fucose-α(1-2)-galactose epitopes in the brain also have not been identified. In summary, the molecular mechanisms surrounding the role of fucose have not been determined.

4.1.3 Challenges and chemical methodologies in carbohydrate research

Despite the overwhelming evidence demonstrating the involvement of carbohydrates in a vast number of biological systems and processes, elucidation of the molecular basis of their function has been slow relative to studies of proteins and nucleic acids. The study of carbohydrates in biological processes poses numerous challenges due to the high complexity and heterogeneity of carbohydrates found in biological systems. Furthermore, the biosynthesis of carbohydrates is not template driven nor under direct translational control, unlike other biopolymers; therefore, carbohydrates can be highly heterogeneous making it difficult to perform structure-function studies using conventional genetic manipulations. Adding to this complexity is the ability for carbohydrates to undergo further modifications such as sulfation. These modifications are also not directly encoded genetically. Consequently, it has proven to be very difficult to delineate the effects of these modifications on the overall biological function of the carbohydrate. Evidence suggests that minor changes in how the carbohydrate is presented (i.e., type of carbohydrate and sulfation pattern) on the protein have significant consequences to the biological activity of the glycosylated protein so it of great interest to develop methods for the structure-function studies of carbohydrates.

Another challenge in the study of carbohydrates arises from the fact that glycosyltransferases, enzymes that catalyze the transfer of a carbohydrate monomer unit to a protein or another carbohydrate molecule, are fairly promiscuous. The ability for glycosyltransferases to recognize multiple substrates further complicates their study since the removal of a glycosyltransferase by genetic knock out methods can result in lethality or deleterious effects to other biological processes within a system.

Despite the challenges in carbohydrate research, some progress has been made toward

novel methodologies that have allowed for the study of carbohydrates and their role in biological processes. While enzymatic and genetic methods have contributed considerably to functional studies of carbohydrates, chemical tools have proven to be invaluable to glycobiology. For biochemical studies, homogeneous populations of carbohydrates have been obtained using chemical and bioenzymatic synthesis.[24-28] While the chemical synthesis of carbohydrates, especially of larger oligosaccharides, is generally seen as extremely formidable, it affords greater flexibility than enzymatic means. It also allows one to obtain homogeneous carbohydrate samples of well-defined structure and modification.

Chemical methods have also been used in the development of other tools in glycobiology. For example, chemical strategies have been used to inhibit the synthesis or function of specific oligosaccharides and glycoconjugates.[29-34] The inhibition of the synthesis of specific glycoconjugates allows one to ascertain its biological role. Additionally, chemical methods have been used generate probes and scaffolds aimed at studying lectins that recognize glycoconjugates.[35-38] They have also been used in the development of unnatural metabolic substrates that allow for the biosynthetic engineering of cell surface glycoconjugates, termed metabolic oligosaccharide engineering. This particular method has been used to disrupt carbohydrate synthesis, probe metabolic pathways, and to identify lectins/glycoproteins.

Clearly, chemical tools have played and will continue to play a critical role in glycobiology. The coupling of developing chemical strategies with advancing biochemical and genetic methodologies will provide invaluable tools to address challenges in carbohydrate research.

4.2 Experimental design

4.2.1 Proposed models for fucose involvement in memory formation

We propose several models as to how fucose may be involved in memory formation. It is possible that fucose-α(1-2)-galactose serves to mediate protein-protein interactions between a fucose-α(1-2)-galactose lectin and the cognate glycoprotein at the cell surface (figure 4.2, mechanism A). Alternatively, the fucose-α(1-2)-galactose epitope may be acting to target proteins to specific subcellular compartments (figure 4.2, mechanism B). These two mechanisms could also be working in concert as a feedback loop to recruit more fucosyl glycoproteins to the cell surface. Finally, it is also possible that fucosyl lectins and/or glycoproteins act intracellularly in signaling pathways responsible for memory formation (figure 4.2, mechanism C).

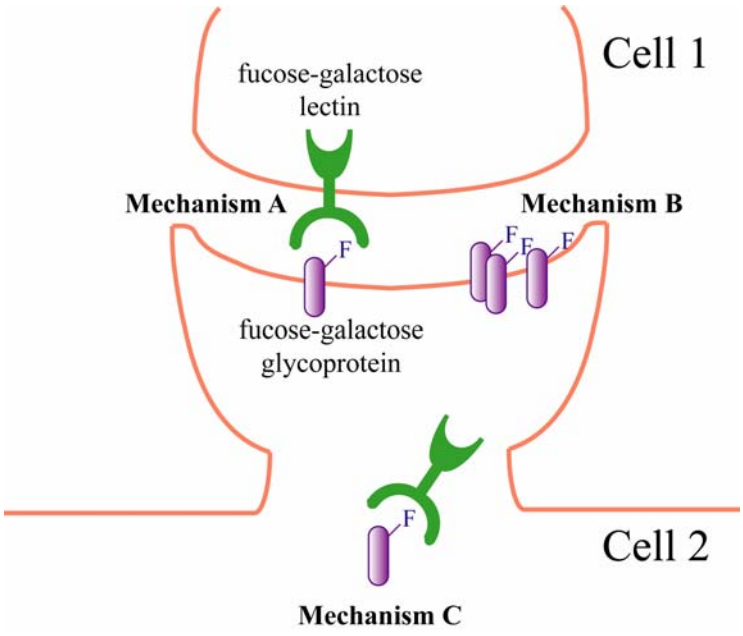

Figure 4.2. Model of how fucosyl saccharides may play a role in learning and memory. Mechanism A) Fucose-galactose glycoproteins are presented at the surface and mediate interactions through binding to a lectin. Mechanism B) Fucose-galactose glycoproteins act as a targeting element to recruit more fucosyl glycoproteins to the surface. Mechanism C) Fucose-galactose glycoproteins and their binding partners act intracellularly during memory formation.

Identification of the fucose-α(1-2)-galactose bearing glycoproteins as well as their binding counterpart proteins may provide new insights into the molecular mechanisms underlying memory formation. Furthermore, identification of these proteins would allow for more detailed study of the role of fucose in learning processes. This research describes efforts to isolate and identify fucose-α(1-2)-galactose bearing glycoproteins as well as fucose-α(1-2)-galactose binding proteins. Molecular techniques such as immunoprecipitation will be employed to isolate fucose-α(1-2)-galactose bearing glycoproteins, while chemical probes will be used to isolate fucose-α(1-2)-galactose binding proteins.

4.2.2 Identification of fucose-α(1-2)-galactose binding proteins with chemical probes

Chemical probes have emerged as a powerful tool to study protein-substrate interactions.[39] The modification of substrates to incorporate "tagging" groups allows for the visualization, tracking, or isolation of enzymes/proteins of interest. We have designed two chemical probes for the purpose of studying and identifying fucose-α(1-2)-galactose binding

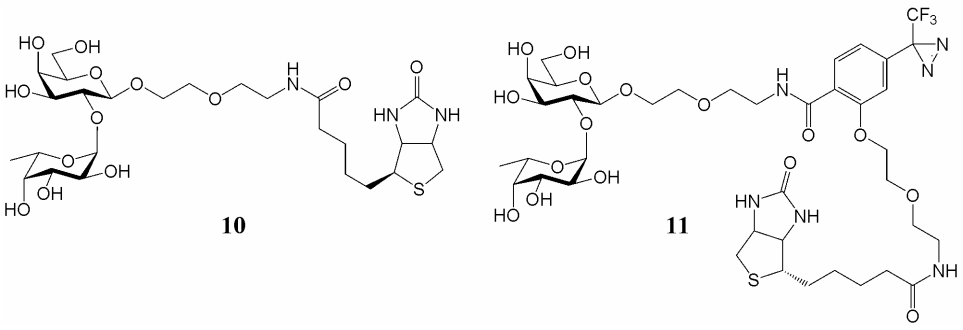

Figure 4.3. Probe **10** contains a fucose-α(1-2)-galactose recognition element and biotin moiety. Photoactivatible probe **11** also contains a photoreactive diazirine group for chemical crosslinking to target proteins.

proteins in the brain (figure 4.3). Probe **10** was synthesized to establish whether fucose-α(1-2)-galactose binding proteins exist in the brain. Probe **11** was synthesized to isolate fucose-α(1-2)-galactose binding proteins.

*4.2.2.1 Design of probe **10***

The design of the probe **10** incorporated two main features: (1) a fucose-α(1-2)-galactose epitope for protein binding, and (2) a biotin moiety for neuron imaging using dye-conjugated streptavidin (figure 4.3A). The two elements are connected via a hydrophilic linker to increase the solubility of the molecule under physiological conditions. The fucose-α(1-2)-galactose epitope was chosen as the recognition element over the monosaccharide and trisaccharide because evidence has strongly implicated the disaccharide and has not conclusively implicated the trisaccharide. The design of probe **10** is modular such that different linkers and reporter groups can be incorporated without major revisions to the overall synthesis.

*4.2.2.2 Design of photoreactive crosslinking probe **11***

Probe **11** was designed for the purpose of isolating fucose-α(1-2)-galactose binding proteins present in the brain (figure 4.3B). In addition to the fucose-α(1-2)-galactose epitope and biotin moiety found in **10**, probe **11** contains a 3-trifluoromethyl-3-phenyldiazirine (TPD) moiety, allowing for chemical crosslinking to capture the target protein(s). By forming a covalent linkage to the proteins of interest, the protein-probe complex can withstand rigorous washing in the purification process. Similar to probe **10**, the synthetic design is very modular such that different analogs can be readily made, if needed. For

example, the linker arm length or crosslinking moiety could easily be changed without the necessity of redesigning the overall synthesis. This convergent synthesis combines the power of photoaffinity crosslinking with the advantages of a biotin-avidin system.

Photoaffinity labeling is a well-established technique to elucidate ligand-biomolecule interactions. This technique has been used to successfully label enzymes, membranes, protein structures, neural receptors and RNA/DNA structures.[40-46] Typically, the substrate is modified to bear the photoreactive element, and radiolabels are incorporated to allow for the identification of the binding site. The covalently labeled enzyme/protein can also be visualized with a variety of other techniques such as spectroscopic analysis and fluorophore methods.[40]

Combining the power of photoaffinity labeling with the advantages of biotinylating substrates creates a chemical probe that allows for a covalent bond to be formed between the enzyme and the substrate, as well as a handle to isolate the complex with the use of immobilized avidin. The photoreactive element is anticipated to overcome weak binding affinities between the protein and substrate, which has been reported for a variety of lectins.[47] The biotin moiety also offers the advantage of sensitive, non-radioactive detection of labeled protein using streptavidin-conjugated-horseradish peroxidase (HRP). A variety of biotinylated photoreactive probes have been synthesized and successfully used to study and isolate protein-substrates. Probes ranging from biotinylated photoactivated γ-secretase[48] inhibitors to bis-mannose photolabels to study glucose transporter isoform 4 (GLUT4)[49] have been synthesized.

For the photoreactive crosslinking moiety, we selected the diazirine group over benzophenone, phenylazides and other photoaffinity labels for several reasons. First, unlike

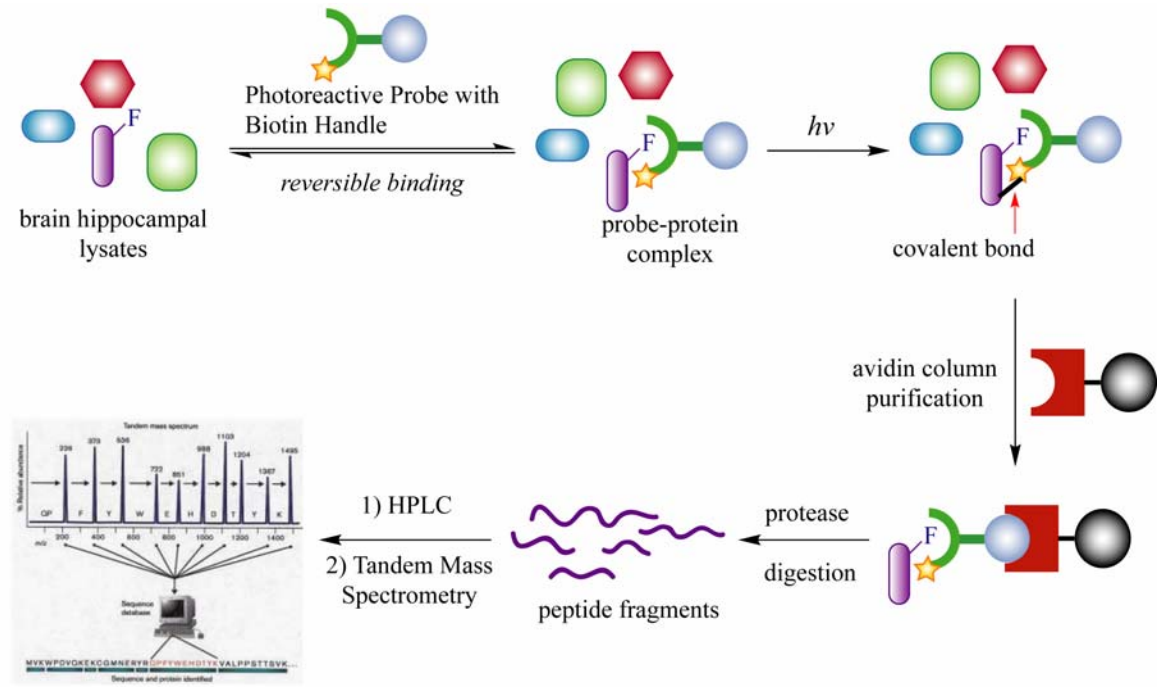

Figure 4.4. Schematic of how compound **11** can be used to isolate and identify fucose-α(1-2)-galactose binding proteins. Brain lysates would be incubated with photoreactive probe **11**. Photo-induced crosslinking results in the formation of a covalent bond between fucose-α(1-2)-galactose binding proteins and **11**. The biotin handle of **11** will allow for the purification of the crosslinked complexes using avidin-agarose. The recovered fucose-α(1-2)-galactose binding proteins can then be identified using tandem mass spectrometry.

phenyl azides, the activation of TPD occurs at 353 nm, which is out of range for protein damage. Photoactivation of diazirines at 353 nm generates a highly reactive triplet carbene species that has been shown to undergo efficient O-H and C-H insertion. The carbene intermediate of the TPD is also more reactive than the nitrene intermediate of phenyl azides. Second, while the activation of benzophenone is above 300 nm, the TPD is small and nonbulky. Furthermore, TPD has relative thermal stability as well as stability to moderately acidic (1N HCl) conditions and moderately basic conditions (1 N NaOH).[50] It has also been shown to be inert to reducing agents, whereas phenylazides are rapidly reduced by reducing agents such as DTT. Given the stability of TPD, it is expected to be stable to conditions

anticipated in biochemical labeling studies.[50]

The strategy for the isolation and identification of fucose-α(1-2)-galactose binding proteins, using photoreactive probe **11** is depicted in Figure 4.4. Rat hippocampal lysates would be incubated with **11** and irradiated with 353 nm light to activate the diazirine moiety for crosslinking to bound proteins. The crosslinked complex would then be isolated using immobilized streptavidin, followed by digestion with proteases and sequencing by tandem mass spectrometry.

4.2.3 Identification of fucose-α(1-2)-galactose bearing proteins with immunoprecipitation

The second portion of the described research was aimed at identifying glycoproteins bearing fucose-α(1-2)-galactose epitopes in neurons. The availability of antibodies specific towards fucose-α(1-2)-galactose carbohydrates allowed for the use of standard molecular biology techniques to detect fucose-α(1-2)-galactose glycoproteins in a variety of studies. The research herein describes preliminary efforts to study fucose-α(1-2)-galactose glycoproteins in neurons using antibodies.

4.3 Results

*4.3.1 Synthesis of Probe **10***

Synthesis of probe **10** began with construction of the disaccharide moiety (figure 4.5). Synthesis of the disaccharide was performed using methods reported by Wegmann, et al.,[51] with some modifications. The synthesis started with treatment of commercially available L-fucose **1** with acidic ion-exchange resin in refluxing methanol to form the methyl glycoside.[52] Benzylation[53, 54] followed by hydrolysis[55] gave **2**, which was then transformed

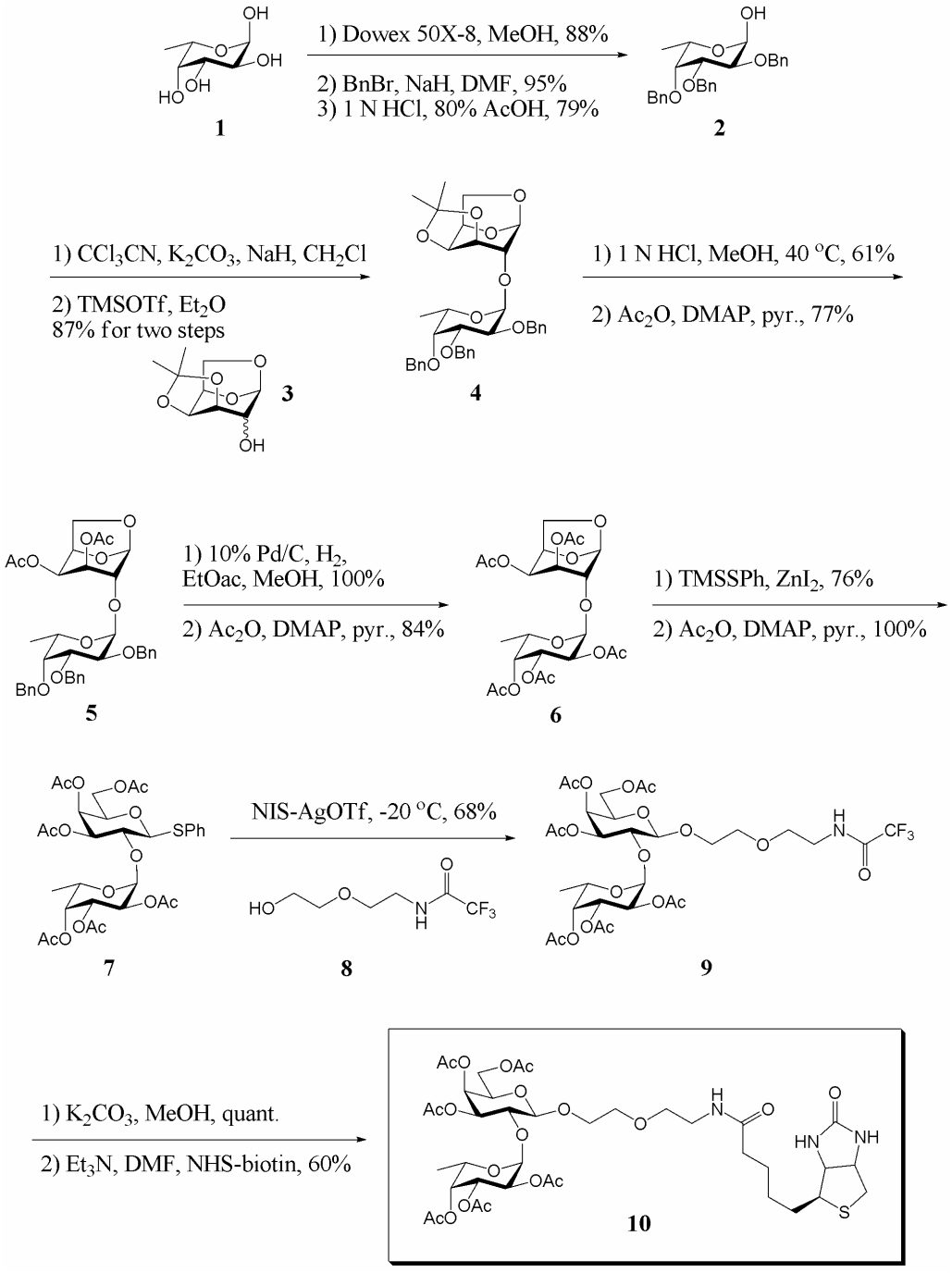

Figure 4.5. Synthesis of probe **10.**

to the corresponding imidate using sodium hydride and trichloroacetonitrile.[56] Attempts to isolate the imidate were unsuccessful due to its susceptibility to hydrolysis. Therefore, the crude product was used in subsequent reactions without further purification. Treatment of

the crude α-imidate and 1,6-anhydro-3,4-isopropylidene-β-D-galactose **3** with trimethylsilyl trifluoromethanesulfonate (TMSOTf) in diethyl ether afforded the disaccharide with the desired α(1-2) glycosidic linkage in 87% yield. At this stage, attempts to deprotect the acetonide group and debenzylate in one step, using standard hydrogenolysis conditions doped with palladium (II) chloride were unsuccessful. Consequently, **4** was treated with 1 N hydrochloric acid and gentle heating to remove the acetonide, and acetylated using acetic anhydride to yield **5**. Debenzylation of **5** followed by acetylation gave **6,** which was then transformed to the β-thioglycoside using the conditions of Motawia et al.[57] This involved treatment of **6** with (phenylthio)trimethylsilane and zinc iodide to open the anhydro ring to form the thioglycoside, followed by acetylation to afford **7**. With the peracetylated thioglycoside in hand, **9** was readily obtained by coupling **7** and **8** using *N*-iodosuccidimide (NIS) and silver triflate.[58] Complete deprotection of **9** using potassium carbonate[59] followed by addition of *N*-hydroxysuccinimide (NHS) biotin under basic conditions yielded the desired product **10**.

4.3.2 Biological studies using probe 10: Imaging hippocampal neurons

With probe **10** in hand, we began the first round of biological experiments to determine whether fucose-α(1-2)-galactose binding proteins were present in the brain. Probe **10** was used to image neurons to examine the subcellular localization of fucose-α(1-2)-galactose binding proteins. The biotin moiety allowed for fluorescence microscopy using streptavidin-dye conjugates (Molecular Probes). Fluorescence microscopy, done by Cristal Gama (Hsieh-Wilson Lab, California Institute of Technology, Pasadena, CA), of cultured neurons incubated with 10 mM solutions of **10** revealed fucose-α(1-2)-galactose binding

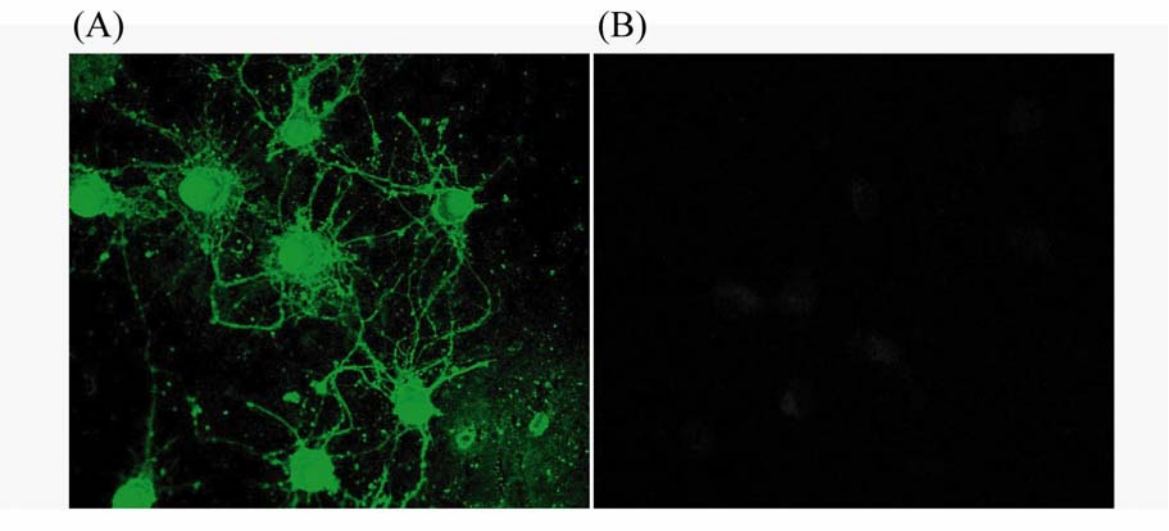

Figure 4.6. Probe **10** binds to cultured hippocampal neurons. A) Cultured embryonic rat hippocampal neurons incubated with probe **10** and treated with streptavidin-dye conjugate and imaged using confocal fluorescence microscopy. B) Cultured embryonic rat hippocampal neurons incubated with streptavidin-dye conjugate as a negative control. (Cristal Gama, Hsieh-Wilson Laboratory).

proteins on the surface of neurons (figure 4.6A). Neurons incubated with biotin and imaged with streptavidin dye-conjugate showed little to no staining, indicating that the binding of the probe was due to the fucose-α(1-2)-galactose epitope (figure 4.6B). These fluorescence microscopy images therefore strongly suggest the presence of fucose-α(1-2)-galactose binding proteins in the brain.

4.3.3 Synthesis of capture probe 11

Synthesis of the capture probe **11** was achieved using methods described by Hatanaka *et al* (figure 4.7).[60, 61] 3'-Bromoanisole was converted to the corresponding Grignard reagent and reacted with *N*-(trifluoroacetyl)piperidine[62] to afford **12**. Treatment of the trifluoroacetophenone with hydroxylamine hydrochloride yielded the oxime **13**, which was then transformed to the diaziridine with *p*-toluenesulfonyl chloride followed by treatment

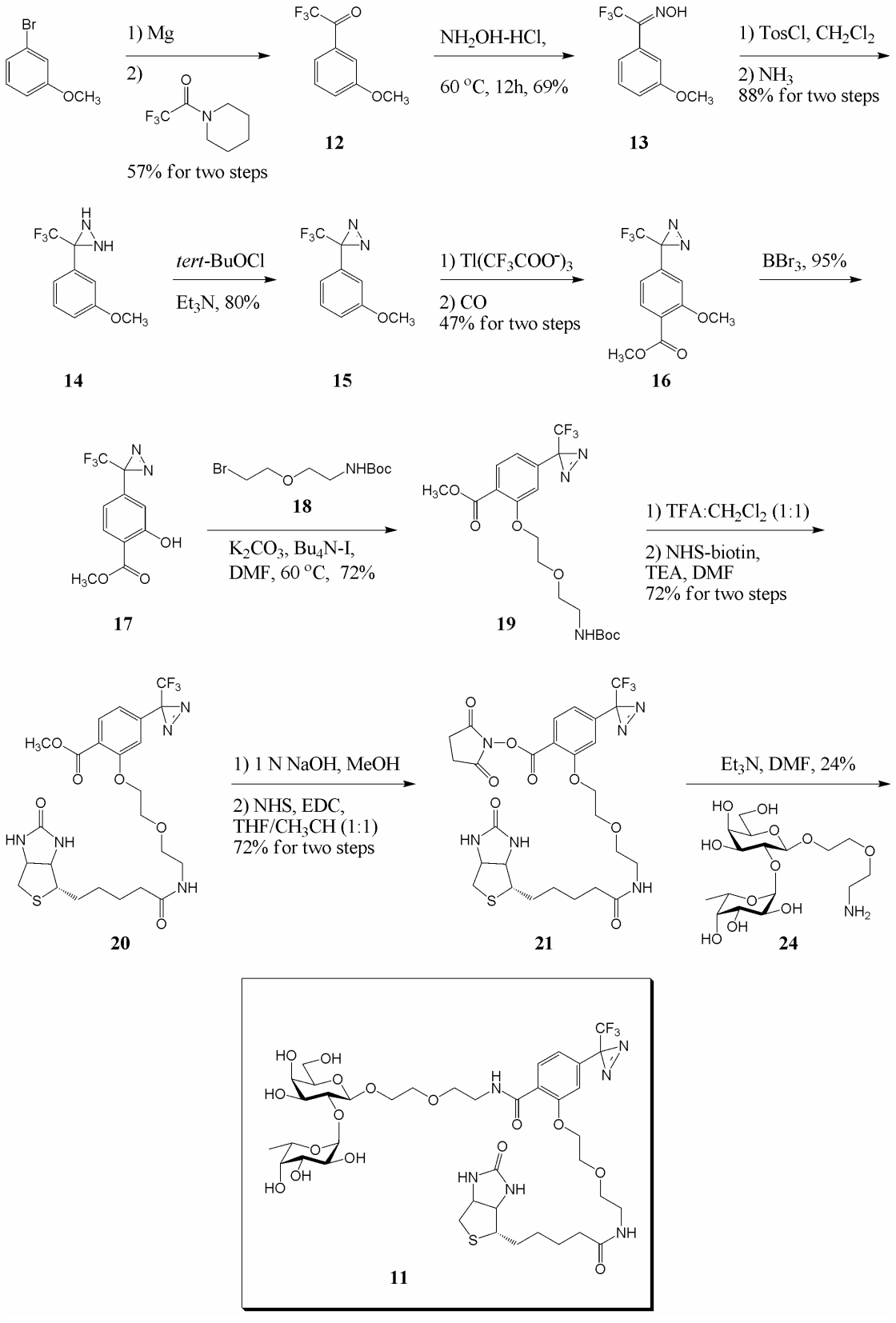

Figure 4.7. Synthesis of photoreactive probe **11.**

with liquid ammonia. The diazirine **15** was obtained with 80% yield via oxidation of **14** with *tert*-butyl hypochlorite.[63] UV spectrophotometry was used to monitor formation of the diazirine structure, which shows an absorption band at approximately 350 nm (whereas the diazo isomer absorbs at around 450 nm). Carboxylation of **15** was performed using conventional thallation conditions, using thallium (III) trifluoroacetate, followed by palladium-catalyzed carboxylation to afford **16**. Because thallium compounds are known to be very toxic, thallations were performed in a glove box with all necessary safety precautions to prevent exposure and contamination. Carboxylations with carbon monoxide were performed in a well-ventilated hood. Demethylation of **16** with boron tribromide afforded **17** in 95% yield. With **17** in hand, all the necessary components of the full probe were ready for the final couplings, using procedures reported by Hatanaka et al.[61]

Alkylation of **17** with linker arm, **18**, was performed using tetrabutylammonium iodide and potassium carbonate to afford **19**. The synthesis of **18** was adapted from previously reported procedures (figure 4.8).[61] Boc-deprotection of **19** with trifluoroacetic acid followed by amide bond coupling to NHS-biotin afforded **20**, which was readily converted to the NHS ester **21** via saponification and reaction with NHS and EDC. The desired capture probe was obtained using conventional amide bond coupling conditions between **21** and **24**. **24** was obtained from complete deprotection of **9** using potassium

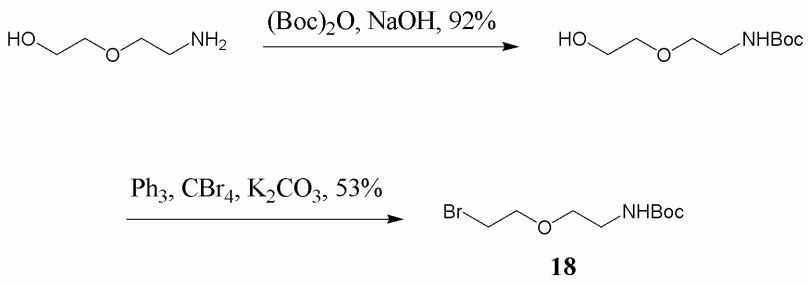

Figure 4.8. Synthesis of PEG linker **18**.

carbonate.

*4.3.4 Biological studies using capture probe **11***

4.3.4.1 General

Once probe **11** was synthesized, a series of experiments were performed to establish the potential of **11** as a suitable means to isolate fucose-α(1-2)-galactose binding proteins. Initial experiments were aimed at examining the binding potential of **11** to fucose-α(1-2)-galactose binding proteins in embryonic rat hippocampal neurons and adult rat hippocampal neurons. Subsequently, a series of experiments were performed to assess the ability for **11** to be used in the isolation of fucose-α(1-2)-galactose binding proteins from lysates. Lastly, competition experiments were performed to establish the binding specificity of **11** via its fucose-α(1-2)-galactose epitope.

*4.3.4.2 Binding potential of probe **11** to fucose-α(1-2)-galactose binding proteins in cultured embryonic rat hippocampal neurons*

Initial experiments using **11** were aimed at determining the potential of **11** as a probe for fucose-α(1-2)-galactose binding proteins before using larger amounts to isolate target proteins. Neurons cultured on coverslips (provided by Cristal Gama, Hsieh-Wilson laboratory) were incubated overnight with a 10 mM solution of **11**. The media was removed and the cells were washed with PBS and then irradiated on ice in PBS, using a handheld 18 W long wavelength UV lamp (365 nm). As a control sample, photolysis of **11** in PBS was monitored by UV. After 2 h of irradiation, a decrease in the absorbance at 350 nm indicated a complete photoactivation of the diazirine moiety (figure 4.9). The cells were removed from

the coverslips and lysed in denaturing sample buffer. Cell lysates were resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was then incubated with strepavidin conjugated to HRP followed by chemiluminescent detection of the photolabeled proteins. Results indicated a potential fucose-α(1-2)-galactose binding protein at approximately 68 kDa (figure 4.9). As a control, cultured hippocampal neurons on coverslips were also incubated with methyl ester **20** (figure 4.7) to determine nonspecific binding of proteins to the probe (figure 4.9, lane 2). Two bands appear at a molecular weight slightly less than that seen with probe **11** (figure 4.9, lane 1). No endogenous streptavidin-

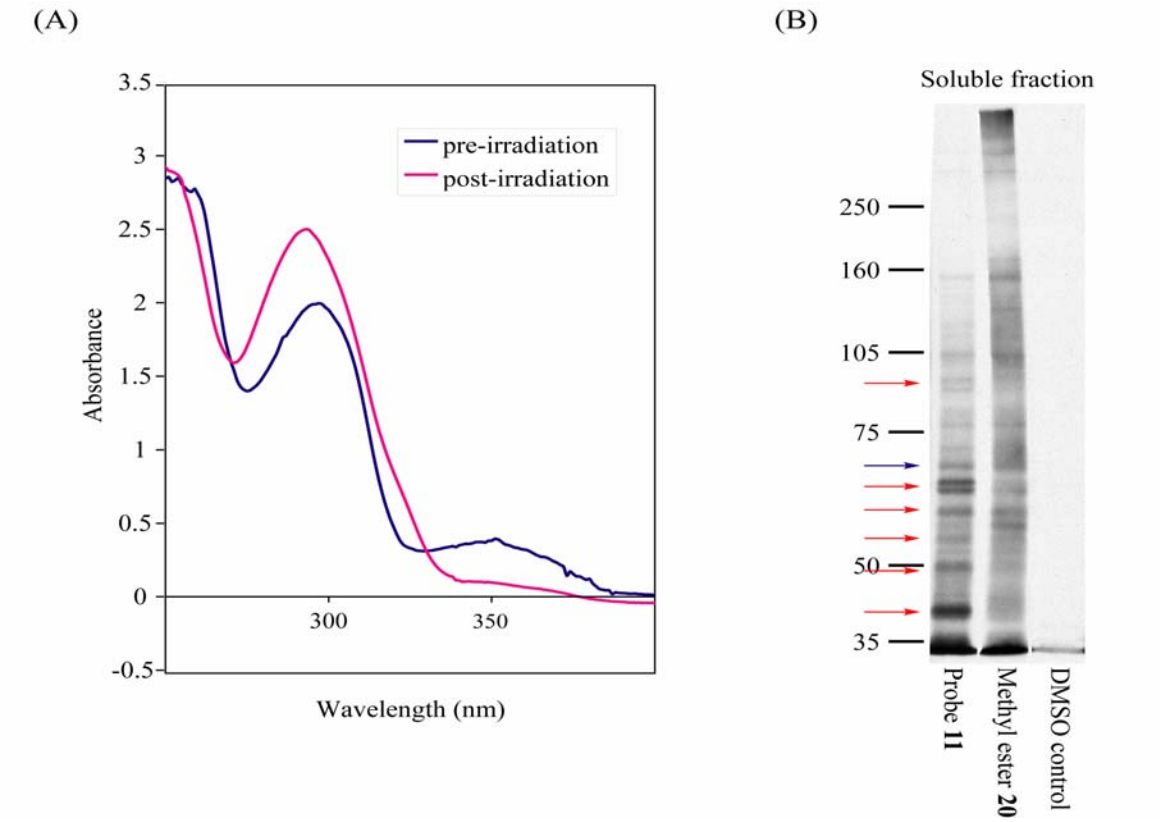

Figure 4.10. A) UV-vis spectrum of probe **11** prior to irradiation and after 4 hours of irradiation. A decrease in absorbance at 350 nm indicates photolyzed product. B) Western blot detection of crosslinked proteins with probe **11** in the soluble fraction of adult rat hippocampal lysates. The blue arrow indicates protein band that was also detected in cultured embryonic rat hippocampal neurons. Note: the exposure time of the methyl ester **20** control lane was 15 s whereas the probe **11** lane and the DMSO control lane have an exposure time of 15 min.

binding proteins were detected from neurons incubated with DMSO (figure 4.9, lane 3). Probing neuons with **11** appears to be sufficient to selectively crosslink to potential proteins of interest with a bit of nonspecific binding as seen probing with compound **20**.

*4.3.4.3 Binding potential of probe **11** to fucose-α(1-2)-galactose binding proteins in adult rat hippocampal neurons*

We also examined whether probe **11** could capture proteins from adult rat hippocampal lysates. Using adult rat hippocampal lysates offers the advantage of a greater amount of total protein over cultured rat embryonic neurons. Briefly, the soluble (S2) protein fraction derived from adult rat hippocampal lysates was incubated with 1 mM of **11** and then irradiated on ice with UV light (365 nm). Again, the photolysis of **11** was monitored by UV, which indicated complete photoactivation after 4 h of irradiation. The samples were then resolved by SDS-PAGE, transferred to PVDF membrane, and probed with streptavidin-HRP. Similar controls were done with methyl ester **20** and DMSO to detect nonspecific binding proteins. Results showed a similar molecular weight band at ~68 kDa to that seen with cultured embryonic hippocampal neurons (figure 4.10, indicated by blue arrow). Additionally, several other protein bands at ~38 kDa, 50 kDa, 58 kDa, 63 kDa, 65 kDa, and 85 kDa (figure 4.10, red arrows) were detected. Comparison with the methyl ester **20** shows that the capture probe has captured proteins of molecular weights that are not seen with the methyl ester. As previously observed, the DMSO control had no detectable levels of endogenous streptavidin-binding proteins. As compared to probing experiments done with cultured neurons on coverslips, there were a greater number of detected proteins in the rat hippocampal lysates. Interestingly, the methyl ester also detected/crosslinked more proteins

in the adult rat hippocampal lysates than in the case of cultured embryonic hippocampal neurons.

*4.3.4.4 Isolation of fucose-α(1-2)-galactose binding proteins in adult rat hippocampal lysates using Probe **11***

Following initial crosslinking experiments, we attempted to isolate the probe-protein complexes using streptavidin-agarose (UltraLink, Pierce, Rockford, IL). As in previous experiments, the soluble protein fraction from adult rat hippocampal lysates was incubated with probe **11** and then irradiated for 4 h. The irradiated lysates were incubated with streptavidin-agarose beads and then the beads were washed to remove unbound proteins. The bound proteins were then eluted by boiling under denaturing conditions, resolved by SDS-PAGE, and subjected to Western blot analysis. Several attempts to remove proteins from the streptavidin-agarose were unsuccessful (data not shown). It is likely that boiling under denaturing conditions was not sufficient to disrupt the high binding affinity between biotin and streptavidin ($K_a \cong 10^{15}$ M). Therefore, attempts to pull down "captured" proteins were carried out using avidin-agarose. The release of biotinylated substrates from avidin-agarose (Sigma, St. Louis, MO) by boiling under denaturing conditions has been well precedented in the literature.[48] Furthermore, UltraLink strepavidin-agarose contains twice the number of biotin-binding sites which may result in a stronger binding affinity due to multivalency. Elution conditions were also modified such that the bound proteins were eluted by boiling under denaturing conditions in the presence of 1000-fold excess of free biotin. If high temperature is not sufficent to completely disrupt biotin-avidin through denaturation, the free biotin would compete for avidin-binding with the photolabeled

proteins—thus, fewer photolysed proteins would return to a bound state on the avidin-agarose.

The soluble protein fraction from adult rat hippocampal lysates was incubated with probe **11**, irradiated for 4 h, and then incubated with avidin-agarose. The beads were then washed to remove unbound proteins. Subsequently, bound proteins were eluted using the modified eluction conditions, resolved by SDS-PAGE, and subjected to Western blot analysis. Western blot analysis of the eluted proteins indicated a faint band at around 58 kDa, which is also seen in the irradiated samples that were not isolated by avidin-agarose purification (figure 4.11, Lane 3, 7). A control with DMSO detected few endogenous avidin-binding proteins (figure 4.11, Lane 8). To ensure the elution conditions were capable of eluting bound proteins from the avidin-agarose, the pull-down experiment was also done with biotinylated bovine serum albumin (BSA). Similar to the irradiated lysates, biotinylated BSA was incubated with avidin-agarose. The beads were washed and bound proteins were eluted using the modified elution conditions, resolved by SDS-PAGE, followed by Western blot analysis. Results from the pull down of biotinylated BSA indicated that the elution conditions were sufficient to disrupt the biotin-avidin affinity and remove bound proteins (figure 4.11, Lane 5, 6).

In addition to pull-down experiments using probe **11**, boiled lysates were also incubated with probe **11** to compare them to unboiled lysates incubated with **11**. Lysates were boiled for 10 min to denature the proteins and then cooled back to room temperature. Subsequently, the boiled proteins were incubated with probe **11**, irradiated for 4 h, and then subjected to Western blot analysis. Similarly, unboiled lysates were treated with probe **11.** DMSO controls were also done with boiled and unboiled lysates. Unboiled lysates incubated

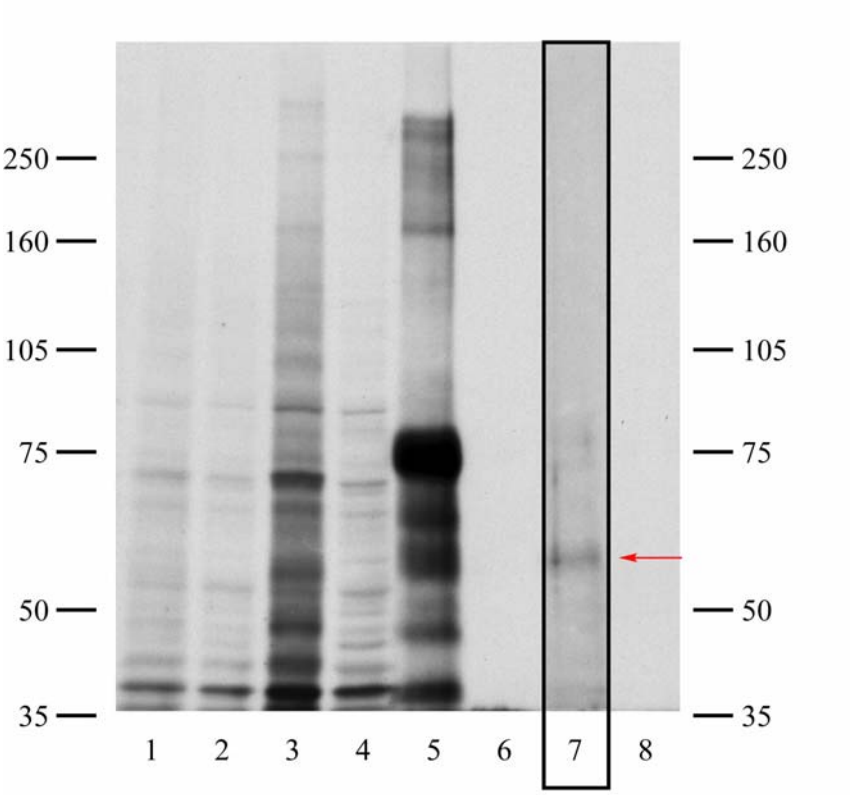

Figure 4.11. Western blot analysis of crosslinked proteins using probe **11** from adult rat hippocampal lysates. Lane 1: 75 μg of boiled adult rat hippocampal lysates with probe **11**; Lane 2: 75 μg of boiled adult rat hippocampal lysates with DMSO; Lane 3: 75 μg of unboiled adult rat hippocampal lysates with probe **11**; Lane 4: 75 μg of unboiled adult rat hippocampal lysates with DMSO; Lane 5: isolated biotinylated BSA using avidin-agarose; Lane 6: avidin-agarose in absence of biotinylated BSA; Lane 7: crosslinked protein complexes with probe **11** from adult rat hippocampal lysates isolated using avidin-agarose; Lane 8: adult rat hippocampal lysates incubated with avidin-agarose. Note: the exposure time for Lane 7 is 12 h while Lanes 1-6, 8 have an exposure time of 1 h.

with probe **11** resulted in bands at similar molecular weights seen in previous experiments (figure 4.11, Lane 3). The boiled lysates treated with probe **11** showed very few bands and are similar to both DMSO controls (figure 4.12, Lane 1, 2, 4). It is likely that the boiling process denatured the proteins, thereby causing loss of function. The observation that binding ability to the fucose-α(1-2)-galactose epitope in probe **11** is lost upon denturing the proteins strongly suggests that the binding to fucose-α(1-2)-galactose is specific. It is

expected then that the results of the boiled lysates incubated with probe **11** would be similar to the DMSO controls. These results were exciting because it demonstrated a loss of function of fucose-galactose binding proteins under denaturing conditions. The presence of bands in the DMSO controls suggests that avidin-agarose is less efficient at removing endogenous streptavidin/avidin-binding proteins, than streptavidin-agarose (which was used to preclear lysates in previous experiments). As a result, future experiments were done using lysates precleared with streptavidin-agarose.

*4.3.4.5 Competition experiments of probe **11** with O-ethyl-fucose-α(1-2)-galactose in adult rat hippocampal lysates*

To further confirm that binding of proteins to probe **11** was due to specificity with the disaccharide moiety, the *O*-ethyl-fucose-α(1-2)-galactose **23** was synthesized. Competition experiments using 1000-fold excess of **23** compared to probe **11** should allow us to determine which proteins are specifically binding to the fucose-α(1-2)-galactose moiety on the probe. Compound **23** was synthesized from the peracetylated thioglycoside **22** (figure 4.12) by treatment of **7** with *N*-iodosuccinimide and silver triflate.[57] Deacetylation of **22** using sodium methoxide in methanol afforded the desired compound **23** in a 1:2 (α:β) ratio. Only the

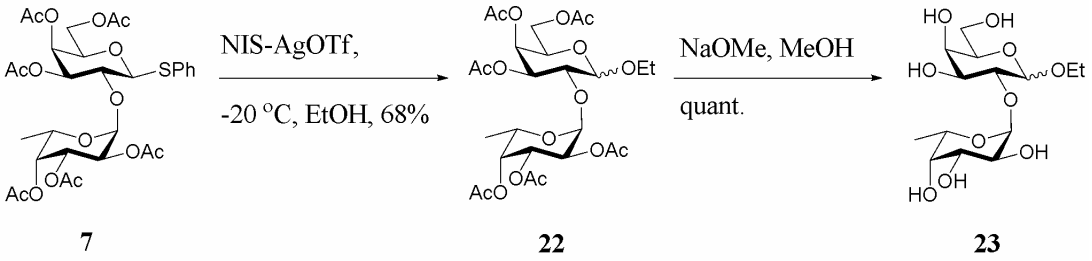

Figure 4.12. Synthesis of *O*-ethyl fucose-α(1-2)-galactose.

β–product was used for the competition experiments. With **23** in hand, the preliminary competition experiments were performed. The soluble protein fraction from adult rat hippocampal lysates was preincubated for 4 h with 1000-fold excess (compared to amount of **11** added later) of **23**. Probe **11** was then added to the mixture and incubated for 8 h, and irradiated. The lysates were then resolved by SDS-PAGE and subjected to Western blot analysis. The same amount of total lysate used in the competition experiment was also incubated with probe **11** to allow for a direct comparison of the intensities for each protein band. A DMSO control was also done with the same amount of total lysate. Results from a preliminary competition experiment showed a decrease in signal upon incubation with the competitor compound **23** (figure 4.13). Specifically, a decrease in intensity was seen with proteins of an approximate molecular weight of 38 kDa, 50 kDa, 65 kDa, and 68 kDa. This suggested that binding of probe **11** to the proteins of interest was due to the fucose-α(1-2)-galactose moiety.

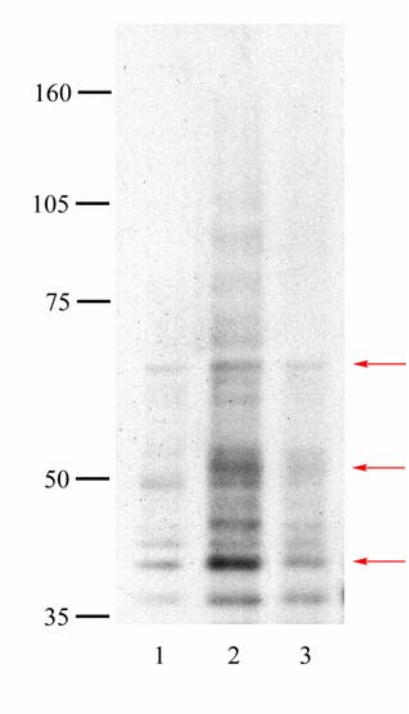

Figure 4.13. Western blot analysis of competition experiments using 1000-fold excess of **23** using the soluble fraction of adult rat hippocampal lysates. Lane 1: adult rat hippocampal lysates with DMSO; Lane 2: adult rat hippocampal lysates with probe **11**; Lane 3: adult rat hippocampal lysates with probe **11** and 1000-fold excess of **23**.

4.3.5 Efforts toward Identification of fucose-α(1-2)-galactose Glycoproteins

In addition to studying fucose-α(1-2)-galactose lectins, we validated the existence of fucose-α(1-2)-galactosyl glycoproteins in the brain using molecular techniques. These

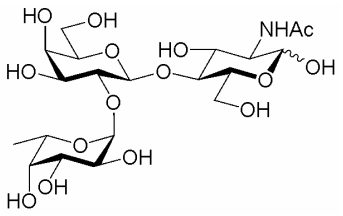

Figure 4.14. Structure of the blood group antigen H type 2, fucose-α(1-2)-galactose-β(1-4)-GlcNAc, which is recognized by the A46-B/B10 antibody.

proteins could be detected by Western blotting with an antibody (A46-B/B10) specific to the blood group antigen H type 2**,** Fucα(1-2)Galβ(1-4)GlcNAc (figure 4.14).[23] Hybridomas were obtained through the generous gift of Dr. Uwe Karsten (Max-Delbruck Centre for Molecular Medicine). Although A46-B/B10 was developed against the trisaccharide, binding studies demonstrated that stringency for selectivity was in the fucose-α(1-2)-galactose moiety, and less so for the trisaccharide.[23] Antibodies were generated (Cocalico) and purified across an IgM antibody purification kit (Pierce, Rockford, IL). Western blotting of adult rat hippocampal lysates indicated the presence of fucose-α(1-2)-galactose bearing proteins of 70 kDa and 80 kDa. Immunoprecipitation of these proteins using immobilized A46-B/B10 antibody was attempted, but was unsuccessful (data not shown). Work towards isolating and identifying the fucose-α(1-2)-galactosyl expressing proteins in the brain is now being continued by Monica Luo (Hsieh-Wilson lab).

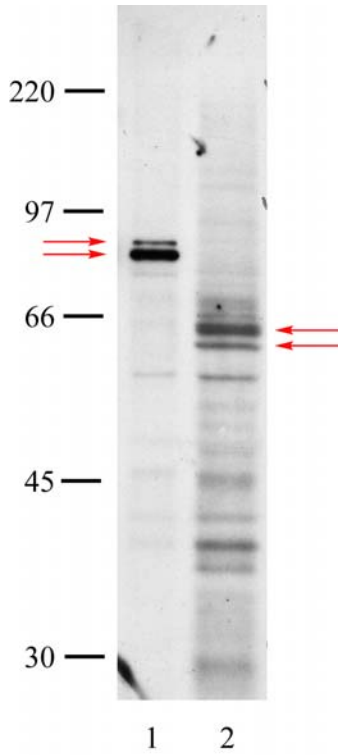

Figure 4.15. Western blot analysis of adult rat hippocampal lysates and cultured embryonic rat hippocampal lysates using A46-B/B-10 antibody. Lane 1: adult rat hippocampal lysates; Lane 2: cultured embryonic (E18) rat hippocampal lysates.

Because carbohydrates are often expressed at different levels during various stages of development, the presence of fucose-α(1-2)-galactose glycoproteins in cultured embryonic hippocampal cells were examined.

Interestingly, Western blotting of cultured embryonic hippocampal cell lysates using the A46-B/B10 antibody detected glycoproteins of different molecular weights than that of adult rat hippocampal tissue (figure 4.15). Probing of adult rat hippocampal lysates revealed fucosylated proteins of 80 kDa and 70 kDa, whereas embryonic lysates have fucosylated proteins of 63 kDa and 61 kDa**.** These results suggest that expression of fucose-α(1-2)-galactose glycoproteins is developmentally regulated. Further studies directed toward identifying the fucose-α(1-2)-galactose glycoproteins, their subcellular localization, and their roles in development are being carried out by Cristal Gama (Hsieh-Wilson laboratory).

4.4 Discussion

4.4.1 General

A growing body of evidence suggests a critical role for carbohydrates in a vast number of neurological processes such as learning and memory. Previous work had strongly suggested a role for fucose-α(1-2)-galactose in learning and memory; however, at the time when this research was undertaken, no fucose-α(1-2)-galactose binding proteins had been identified, nor had there been identification of any fucose-α(1-2)-galactose bearing glycoproteins. Therefore, the herein research was aimed at identifying these proteins through chemical and molecular means. We aimed to use chemical probes to study proteins that bound to fucose-α(1-2)-galactose epitodes. The availability of antibodies specific to fucose-α(1-2)-galactose epitopes afforded us the ability to use molecular means to study fucose-α(1-2)-galactose bearing glycoproteins.

4.4.2 Study of fucose-α(1-2)-galactose binding proteins with chemical probes

Probe **10** was designed and synthesized as an initial probe to examine the presence of fucose-α(1-2)-galactose binding proteins in the brain. It contains a fucose-α(1-2)-galactose epitode tethered to a biotin moiety via a PEG linker. Incubation of **10** with cultured embryonic rat hippocampal neurons followed by imaging using confocal microscopy strongly suggested the presence of fucose-α(1-2)-galactose binding proteins. Incubation of the neurons with biotin attached to a PEG linker, with no fucose-α(1-2)-galactose epitope, resulted in no binding as determined by confocal microscopy. This established the specificity of the binding was via fucose-α(1-2)-galactose epitopes. Furthermore, the fluorescence imaging of the neurons using **10** validated the design of probe **10**—establishing that the disaccharide is sufficient for binding and that the length of the linker arm is compatible with protein binding.

Probe **11** was designed and synthesized to isolate via photoreactive crosslinking fucose-α(1-2)-galactose binding proteins in neurons with lower binding affinities that often hamper "pull-down" experiments. Probe **11** was synthesized in a relatively straightforward fashion with a modular design such that modifications, such as linker length, could be made with relative ease. Initial experiments using **11** were aimed at establishing the ability of **11** to selectively crosslink to proteins of interest in both cultured embryonic rat hippocampal neurons as well as adult rat hippocampal lysates.

Neurons or lysates were incubated with probe **11** or with the corresponding methyl ester **20**. Compound **20** was used as a control to validate the specificity of binding to the fucose-α(1-2)-galactose epitope and not other moieties of **11**. Crosslinking experiments with the cultured embryonic rat hippocampal and adult rat hippocampal lysates both showed

distinct proteins bands, although crosslinking experiments probing the cultured neurons detected fewer proteins than that of the adult lysates (figure 4.9A, 10A). Nonetheless, the results of these experiments were very promising because they suggested that probe **11** is able to selectively capture potential proteins.

In experiments probing cultured embryonic rat hippocampal neurons, protein bands were detected in the negative control using methyl ester **20** (figure 4.9B). The two protein bands seen in the control with the methyl ester **20** may be due to a variety of reasons. It is possible that the increased hydrophobicity of **20** compared to capture probe **11** causes nonspecific hydrophobic interactions between proteins and **20**. It is also possible that some proteins are binding specifically to the aryl ester moiety on **20**. Both possibilities lead to the capture of proteins that may not be specific to the fucose-α(1-2)-galactose epitope.

Interestingly, probing adult rat hippocampal lysates yielded more detected proteins than those found in cultured neurons. These results were very exciting because they indicated that there were potentially more fucose-α(1-2)-galactose binding proteins in the brain than just the single protein initially observed from the cultured neurons experiments. The difference in the proteins seen may be a result of different expression levels of fucose-α(1-2)-galactose binding proteins during different stages of development. These results would support a hypothesis that expression of fucose-α(1-2)-galactose binding proteins is developmentally regulated. Similar to the capture probe, the methyl ester also captures more proteins in the adult rat hippocampal lysates than in the case of cultured embryonic hippocampal neurons. This potentially could be due to nonspecific hydrophobic interactions between the protein and the methyl ester. If this is the case, it is possible that the methyl ester is capturing the most abundant proteins in the lysates. Again, it should be noted that the

protein expression levels are likely to be varied during development, resulting in the capture of different proteins from embryonic hippocampal neurons to adult hippocampal neurons.

Once the binding of compound **11** was established we utilized the biotin handle of **11** to determine if **11** could be used to isolate proteins captured during crosslinking, using avidin-agarose or streptavidin-agarose (figure 4.11). The results of the pull-down experiments were promising because they suggested that fucose-α(1-2)-galactose binding proteins can be isolated through crosslinking with probe **11**, followed by incubation with avidin-agarose. While the results of the pull down experiments using biotinylated BSA indicate that the elution conditions are sufficient for recovery of the bound proteins, the faint appearance of the 58 kDa band in the lysates suggests that the efficiency of pull down experiments needs to be optimized. Future experiments with probe **11** will be aimed at improving the pull-down conditions, possibly through increasing the amount of avidin-agarose used coupled with increasing the incubation time.

We further validated the specificity of the probe towards fucose-α(1-2)-galactose binding proteins with competition experiments using *O*-ethyl-fucose-α(1-2)-galactose **23**. Incubation with 1000-fold excess of **23** over **11** resulted in decreased crosslinking of **11** to fucose-α(1-2)-galactose binding proteins, presumably due to competitive binding of **23** to these proteins (figure 4.13). These results indicated that binding of probe **11** to the proteins of interest was due to the fucose-α(1-2)-galactose moiety. Future experiments aimed at the isolation and identification of fucose-α(1-2)-galactose binding proteins will include additional competition experiments to confirm preliminary results, as well as optimization of pull-down conditions using avidin-agarose.

4.4.3 Study of fucose-α(1-2)-galactose bearing glycoproteins using molecular techniques

To study fucose-α(1-2)-galactose bearing glycoproteins, we utilized the A46-B/B10 antibody that recognizes fucose-α(1-2)-galactose to probe cultured embryonic rat hippocampal neurons and adult hippocampal lysates (Figure 4.15). Our preliminary results demonstrated the presence of fucose-α(1-2)-galactose glycoproteins in both sources of neurons. Interestingly, the fucose-α(1-2)-galactose glycoproteins detected in the cultured embryonic neurons differed in molecular weight from those in the adult lysates. These results suggest that expression of fucose-α(1-2)-galactose glycoproteins is developmentally regulated. Further studies directed toward identifying the fucose-α(1-2)-galactose glycoproteins, their subcellular localization, and their roles in development are being carried out by Cristal Y. Gama (Hsieh-Wilson laboratory).

4.5 Conclusions

In summary, the data strongly suggest the presence of fucose-α(1-2)-galactose binding proteins and fucose-α(1-2)-galactose glycoproteins in the brain. Chemical probes **10** and **11** were synthesized and used to study fucose-α(1-2)-galactose binding proteins. Using probe **10** and confocal fluorescence microscopy, we demonstrated the existence of fucose-α(1-2)-galactose binding proteins in hippocampal neurons. Probe **11** was synthesized to capture fucose-α(1-2)-galactose binding proteins by means of chemical crosslinking. Initial results from experiments with probe **11** were very exciting because they suggest that the design of our probe is sufficient to isolate fucose-α(1-2)-galactose binding proteins from the brain. Furthermore, we were able to use antibodies specific to fucose-α(1-2)-galactose epitopes to examine fucose-α(1-2)-galactose bearing glycoproteins in the brain. Overall, the

described research further demonstrates the power of chemical probes in biological studies. Additionally, the data from both studies utilizing chemical probes and molecular probes strongly suggest that the modifications of proteins with fucose-α(1-2)-galactose epitopes and the expression of fucose-α(1-2)-galactose binding proteins are developmentally regulated. Future studies will be directed at isolating fucose-α(1-2)-galactose binding proteins and identifying these proteins using MALDI-MS. Identification of fucose-α(1-2)-galactose binding proteins and their binding partners at the synapse should provide a greater understanding of the molecular underpinnings of memory formation, through a detailed study of their molecular interactions.

4.6 Experimental methods and materials

4.6.1 General synthetic information.

Unless otherwise stated, all reactions were performed in flame-dried glassware under an atmosphere of nitrogen gas or argon gas. Reagents were obtained from commercial sources and used as received. Thin-layer chromatography (TLC) was performed using E. Merck silica gel 60 F254 precoated plates (0.25 mm). Visualization of the developed chromatogram was performed by fluorescence quenching, cerium ammonium molybdate stain, ninhydrin stain, or *p*-dimethylaminocinnamaldehyde stain (for detection of biotin). Chromatography (flash) was performed using ICN Silica gel (0.032-0.063 mm) or Fluka alumina oxide type 507 C neutral (0.05-0.15 mm). ^{1}H-NMR and ^{13}C-NMR were recorded on a Varian Mercury 300 (300 MHz for ^{1}H-NMR and 74.5 MHz for ^{13}C-NMR). Infrared (IR) spectra were obtained using a Perkin Elmer Paragon spectrometer and are reported in terms of frequency of absorption (cm^{-1}). UV (vis) spectra were recorded on a Unikon Spectrophotometer 930 and are reported in terms of wavelength of maximum absorbance (λ_{max}). Mass spectra were obtained from the Protein/Peptide MicroAnalytical Laboratory at the California Institute of Technology, Pasadena, California.

4.6.2.1 Synthesis of methyl α-L-fucopyranoside

L-fucose (50 g, 0.304 mol) and the acid catalyst Dowex 50W-X8 (Acros) were combined in methanol (500 mL) and refluxed for 48 h. The reaction mixture was cooled, filtered and concentrated under vacuum. The residue solidified and was crystallized from EtOAc. The filtrate, after crystallization, was concentrated and methanol (500 mL) added and the reflux and isolation process was repeated twice to yield methyl α-L-fucopyranoside (45.5 g, 84 %):

^{1}H-NMR (300 MHz, D_2O) δ 1.21 (d, 3H, *J* = 6.6Hz, C*H*$_3$), 3.38 (s, 3H, OC*H*$_3$), 3.77 to 3.90 (m, 3H, H-2, H-3, H-4), 4.04 (m, 1H, *J* = 6.6Hz, H-5**)**, 4.76 (d, 1H, *J* = 3.3Hz, H-1) MS (EI^+) *m/z* 201.0 $[M+Na]^+$.

4.6.2.2 Synthesis of Methyl–2, 3, 4-tri-O-benzyl-α-L-fucopyranoside.

Benzylation of **1** was done as described by Zhang *et al*[53]. To a solution of **1** (25 g, 0.140 mol) in DMF (1.22 L) was added NaH (57%-63% oil dispersion, 11.79 g, 0.280 mol) in portions. After the mixture was stirred for 15 min, benzyl bromide (47.89 g, 0.280 mol) was then added dropwise through an addition funnel at 0 °C. The reaction was stirred for 8 h at room temperature. Methanol (209.48 mL) was then added to the mixture. The reaction mixture was poured to ice water and extracted with EtOAc. The organic layer extract was dried over Na_2SO_4 and filtered. The filtrate was concentrated *in vacuo* to afford an oily residue. Column chromatography on silica gel afforded a colorless oil (59.53 g, 94.8%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.12 (d, 3H, *J* = 6.6Hz, C*H*$_3$), 3.37 (s, 3H, OC*H*$_3$), 3.65 (dd, 1H, *J* < 1, H-4), 3.84 (m, 1H, *J* = 6.6Hz, H-5), 3.94 (dd, 1H, *J* = 10.2Hz, *J* = 3.3Hz, *J* = 2.7Hz, H-3), 4.05 (dd, 1H, *J* = 9.9Hz, *J* = 3.9Hz, H-2), 4.65-5.02 (m, 6H, C*H*$_2$Ph), 4.66 (d, 1H, *J* = 3.9Hz, H-1), 7.26-7.41 (m, 15H, *Ph*); ^{13}C-NMR (74.5MHz, $CDCl_3$) δ17.02, 55.64, 66.37, 73.67, 73.79, 75.10, 76.60, 78.09, 79.71, 99.05, 127.70, 127.79, 127.87, 128.31, 128.39, 128.53, 128.59, 128.65, 138.74, 138.78, 139.14; MS (EI^+) *m/z* 471.2 [M+Na]+, 487.2 [M+K]+; MS (EI^-) *m/z* 483.4 [M+Cl']-.

*4.6.2.3 Synthesis of 2, 3, 4-tri-O-benzyl-α-L-fucopyranoside (**2**)*

Synthesis of 2, 3, 4-tri-O-benzyl-α-L-fucopyranoside was done according to Dejter-Juszynski, M. and Flowers, H. M..[55] A solution of methyl–2, 3, 4-tri-O-benzyl-α-L-fucopyranoside (5.0 g, 11.15 mmol) in 80% acetic acid (80 mL) and 1 N HCl (25 mL) was heated between 95-100 °C for 2 h. After cooling, the reaction mixture was extracted with chloroform. The chloroform solution was washed with a cold saturated solution of $NaHCO_3$, followed by a wash with water. The organic layer was then dried with calcium chloride, filtered, and concentrated *in vacuo*. The solid was crystallized from ether-hexane to yield **2** (3.84 g, 79.2%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.14 (d, 3H, *J* = 6.6Hz, C*H*$_3$), 3.89 (dd, 1H, *J* = 2.7, *J* = 9.9), 3.67 (d, 1H, *J* = 1.8, H-4), 3.89 (dd, 1H, *J* = 2.7, *J* = 9.9, H-3), 4.05 (dd, 1H, *J* = 3.9, *J* = 9.9, H-2), 4.10 (q, 1H, *J* = 6.6Hz, H-5), 4.65-5.00 (m, 6H, C*H*$_2$), 5.27 (s, 1H, H-1); MS (EI$^+$) *m/z* 457.2 [M+Na]+, 473.2 [M+K]+.

*4.6.2.4 Synthesis of 1, 6-Anhydro-3, 4-O-isopropylidene-2-O-(2, 3, 4-tri-O-benzyl-α-L-fucopyranosyl)- β-D-galactopyranose (**4**)*

To a solution of **2** (2.5 g, 5.75 mmol) in CH_2Cl_2 (39.68 mL) was added potassium carbonate (3.04 g, 21.99 mmol) and freshly distilled trichloroacetonitrile (3.17 mL, 4.56 g, 31.61 mmol). The reaction mixture was stirred vigorously for 1 h under argon. Sodium hydride (261.07 g of 57%-63% oil dispersion, 6.2 mmol) was added portion-wise and the reaction mixture was allowed to stir for 5 h at room temperature. The reaction mixed was then cooled on ice and poured into ice-cold saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2 (x 3). The organic layer was dried with Na_2SO_4, filtered and concentrated *in vacuo* to afford a dark orange oil. Attempts to purify the crude material across silica gel were unsuccessful so the O-(2, 3, 4-tri-O-benzyl-α-L-fucopyranoside)trichloroacetimidate was

taken directly to the next step with no purification. The crude imidate was lyophilized for at least 5 h to remove any residual moisture before the synthesis of **4**. To a solution of crude imidate and **3** in dry ether (500 mL), was added trimethylsilyl triflate (5.99 mmol) at room temperature. The reaction was stirred at room temperature until thin layer chromatography (1:1 pet ether: EtOAc) showed one major product. $NaHCO_3$ (39.94 g) was added and the mixture was stirred for 15 min., filtered, and extracted with ether. The extract was washed with saturated aqueous NaCl, dried over $MgSO_4$. Column chromatography (1:1 pet ether: EtOAc) of the residue gave **4** (3.10 g, 87.25% yield overall for both steps): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.12 (d, 3H, *J* = 6.6Hz, C*H*$_3$), 1.31 (s, 3H, C*H*$_3$), 1.53 (s, 3H, C*H*$_3$), 3.56 (dd, 1H, *J* = 7.2Hz, *J* = 5.4Hz, H-5), 3.70 (s, 2H, H-2, H-4′), 3.93 (dd, 1H, *J* = 9.9Hz, *J* = 2.7Hz, H-3′), 3.98 (q, 1H, H-5′), 4.04 (dd, 1H, *J* = 9.6Hz, *J* = 3.9Hz, H-2′), 4.09 (d, 1H, *J* = 7.2Hz, H-6), 4.24 (d, 1H, *J* = 7.2 Hz, H-3), 4.40-4.50 (m, 2H, H-4,6), 4.60-5.00 (m, 7H, 3PhC*H*$_2$, and including at 4.91, H-1′, d, *J* = 3.6Hz), 5.42 (s, 1H, H-1), 7.25-7.45 (m, 15H, 3Ph); ^{13}C-NMR (74.5 MHz, $CDCl_3$) δ 17.0, 24.8, 26.2, 63.5, 67.2, 69.4, 72.5, 73.4, 73.8, 74.8, 75.2, 76.5, 77.6, 79.5, 99.3, 100.6, 108.8, 127.6, 127.7, 127.8, 127.9, 128.0, 128.4, 128.6, 128.6, 138.6, 138.8, 139.0; MS (EI^+) *m/z* 641.4 [M+Na]+, 657.2 [M+K]+.

4.6.2.5 Synthesis of 1, 6-Anhydro-2-O-(2, 3, 4-tri-O-benzyl-α-L-fucopyranosyl)- β-D-galactopyranose

Compound **4** (8 g, 12.93 mmol) was treated with 1 N HCl (18.3 mL) in methanol (91.3 mL) at 40 °C overnight. The reaction was quenched by addition of $NaHCO_3$ at 4 °C (ice bath) until pH was established at approximately 7. The mixture was then filtered through Celite® and concentrated *in vacuo*. Column chromatography on silica gel (1:1 Pet

ether: EtOAc) gave the desired product (4.56 g, 61% yield): ^{1}H-NMR (300 MHz, $CDCl_3$) δ1.10 (d, 1H, *J* = 6.3Hz, C*H*$_3$), 3.58-3.62 (m, 2H), 3.92 (dd, 1H, *J* = 2.7Hz, *J* = 10.2Hz), 3.92 (m, 1H), 3.98-4.05 (m, 3H), 4.20 (d, 1H, *J* = 7.8Hz, H-6), 4.42 (t, 1H, *J* = 3.6Hz), 4.59-4.99 (m, 6H), 4.86 (d, 1H, *J* = 3.00Hz, H-1'), 5.45 (s, 1H, H-1), 7.28-7.38 (m, 15H); ^{13}C-NMR (74.5 MHz, $CDCl_3$) δ 17.0, 30.1, 64.0, 64.6, 67.2, 69.7, 73.4, 73.9, 75.1, 75.2, 76.7.78.5, 79.3, 99.4, 101.2, 127.6, 127.7, 127.8, 127.9, 128.1, 128.4, 128.6, 126.62, 128.64, 138.6, 138.8, 139.0; MS (EI$^+$) *m/z* 601.2 [M+Na]+, 617.4 [M+K]+; MS (EI$^-$) *m/z* 613.4 [M+Cl']-; IR 3446cm^{-1}.

*4.6.2.6 Synthesis of 3,4-Di-O-acetyl-1,6-anhydro-2-O-(2, 3, 4-tri-O-benzyl-α-L-fucopyranosyl)- β-D-galactopyranose (**5**)*

To a solution of 1, 6-Anhydro-2-O-(2, 3, 4-tri-O-benzyl-α-L-fucopyranosyl)- β-D-galactopyranose (4 g, 6.91 mmol) in dry pyridine (88.89 mL) was added 0.2 equivalents of DMAP and the reaction was allowed to stir at room temperature for 10 min. Dry acetic anhydride (44.44 mL) was then added slowly and the reaction was stirred until starting material was consumed as monitored by thin layer chromatography. The mixture was concentrated and repeatedly evaporated with toluene to remove residual pyridine to afford an orange oil. Column chromatography in (2:1 pet ether: EtOAc) yielded **5** in 77% yield; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.10 (d, 3H, *J* = 6.6Hz), 2.05 (s, 3H), 2.12 (s, 3H), 3.56 (s, 1H, H-2), 3.67-3.71 (m, 2H, H-4'. H-6), 3.94 (dd, 1H *J* = 3.0Hz, *J* = 10.2Hz, H-3'), 3.98 (m, 1H, H-5'), 4.03 (dd, 1H, *J* = 3.3Hz, *J* = 10.5Hz, H-2'), 4.31 (d, 1H, *J* = 7.5Hz, H-6), 4.47 (m, 1H, *J* = 3.9Hz, *J* = 4.2Hz, H-5), 4.62-5.00 (m, 6H, C*H*$_2$), 4.94 (d, 1H, *J* = 3.9Hz, H-1'), 5.27 (dd, 1H, *J* < 1Hz, *J* = 5.0Hz, H-4), 5.30 (dd, 1H, *J* < 1Hz, *J* = 5.0Hz, H-3), 5.42 (s, 1H, H-1),

7.26-7.40 (m, 15H); MS (EI^+) *m/z* 685.2 [M+Na]+, 701.2 [M+K]+; MS (EI^-) *m/z* 697.6 [M+Cl']-.

*4.6.2.7 Synthesis of 3,4-Di-O-acetyl-1,6-anhydro-2-O-(2,3,4-tri-O-acetyl-α-L-fucopyranosyl)- β-D-galactopyranose (**6**)*

To a solution of **5** (3 g, 4.53 mmol) in degased EtOAc (62.2 mL) and dry methanol (62.2 mL) was added 10% Pd/C (0.78 g). The reaction mixture was pressurized to 1.3 atm of H_2 gas and allowed to stir for 6 h. Reaction was determined to have proceeded to completion by H-NMR. Solvents were evaporated, and the debenzylated compound was stirred with dry pyridine (77.72 mL) and 0.2 equivalents of DMAP for ten minutes. Dry acetic anhydride (31.09 mL) was added slowly, and the mixture was allowed to stir at room temperature overnight. Solvents were evaporated, and the remaining pyridine was removed by repeated evaporation with toluene. Column chromatography (1:2 = EtOAc:hexanes) afforded **6** (1.97 g, 84 % yield) : ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.13 (d, 3H, *J* = 6.6Hz), 1.99 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.13 (s, 3H), 2.15 (s, 3H), 3.61 (s, 1H, H-2), 3.72 (dd, 1H, *J* = 5.2Hz, *J* = 6.8Hz, H-6), 4.28 (q, 1H, *J* = 6.6Hz, H-5'), 4.31 (d, 1H, *J* = 7.8Hz, H-6), 4.46 (t, 1H, *J* = 4.5Hz, H-5), 5.08 (dd, 1H, *J* = 1.2Hz, *J* = 5.4Hz, H-4), 5.14 (dd, 1H, *J* = 1.2Hz, *J* = 5.4Hz, H-3), 5.19 (t, 1H, *J* = 4.5Hz), 5.24 (d, 1H, *J* = 3.9Hz, H-1'), 5.30-5.34 (m, 2H), 5.42 (s, 1H, H-1); ^{13}C-NMR (74.5 MHz, $CDCl_3$ w/TMS) δ 16.2, 20.9, 20.97, 20.98, 21.0, 21.2, 64.7, 65.4, 65.4, 67.7, 68.2, 68.3, 71.1, 72.3, 77.0, 97.3, 100.5, 169.2, 169.8, 170.1, 170.6, 170.7; MS (EI^+) *m/z* 541.2 [M+Na]+; MS (EI^-) *m/z* 553.2 [M+Cl']-.

4.6.2.8 Synthesis of 5-(acetyl-4,5-diacetyloxy-6-methylperhydro-2H-pyran-2-yloxy)-4-acetyloxy-2-(hydroxymethyl)-6-phenylthioperhydro-2H-pyran-3-yl acetate

A mixture of **6** (1.75 g, 3.38 mmol), (phenylthio)trimethylsilane (1.96 mL, 10.37 mmol), and zinc iodide (3.33 g, 10.44 mmol) in CH_2Cl_2 (19.6 mL) was stirred at room temperature for 21 h. The mixture was diluted with EtOAc (120.23 mL) and washed successively with sat. aq. $NaHCO_3$ (150.15 mL), water (3 x 56.15 mL), and brine (18.77 mL) and dried with sodium sulfate. The organic layer was filtered to remove the sodium sulfate, and then concentrated *in vacuo*. The residue was dissolved in dry THF (15.02 mL) and to that was added 1 M tetrabutylammonium fluoride (TBAF) in THF (6.62 mL). The reaction mixture was allowed to stir for an additional 20 min. The solvent was evaporated and the residue was dissolved in EtOAc (60.03 mL) and washed with water (3 x 30.1 mL), sat. aq. $NaHCO_3$ (30.1 mL), and brine (30.1 mL) and dried over sodium sulfate, filtered and concentrated to afford an oil. Column chromatography (2.5:1= hexanes:EtOAc) afforded the β-thioglycoside (1.61 g, 76%): In future synthesis, this material is taken directly onto next step with no purification.

4.6.2.9 Synthesis of 5-(acetyl-4,5-diacetyloxy-6-methylperhydro-2H-pyran-2-yloxy)-4-acetyloxy-2-(acetyloxymethyl)-6-phenylthioperhydro-2H-pyran-3-yl acetate (7)

To a solution of 5-(acetyl-4,5-diacetyloxy-6-methylperhydro-2H-pyran-2-yloxy)-4-acetyloxy-2-(hydroxymethyl)-6-phenylthioperhydro-2H-pyran-3-yl acetate (312.7 mg, 0.499 mmol) in dry pyridine (6.42 mL) was added acetic anhydride (3.21 mL). The reaction was allowed to stir at room temperature overnight. The solvent was evaporated and residual pyridine was removed by repeated evaporation with toluene. Column chromatography (2.5:1

hexanes:EtOAc) on silica gel afforded **7** (333.69 mg) in quantitative yield: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.97 (d, 1H, *J* = 6.6Hz), 1.92 (s, 3H), 1.96 (s, 6H), 2.00 (s, 3H), 2.10 (s, 3H), 2.11 (s, 3H), 4.03 (d, 2H, *J* = 6.3Hz, H-6), 4.25 (dd, 1H, *J* = 6Hz, *J* = 10.5Hz, H-2), 4.33 (q, 1H, *J* = 6.6Hz, H-5'), 4.70 (t, 1H, *J* = 6.3Hz, H-5), 4.99 (dd, 1H, *J* = 3.7, *J* = 10.4Hz, H-2'), 5.18 (dd, 1H, *J* = 3.3, *J* = 10.2Hz, H-3), 5.27-5.31 (m, 3H, H-1', H-4', H-3), 5.41 (dd, 1H, , *J* = 1.2, *J* = 3.3Hz, H-4), 5.66 (d, 1H, *J* = 5.4Hz, H-1), 7.24-7.5 (m, 5H); MS (EI^+) *m/z* 693.2 [M+Na]+, 709.2 [M+K]+.

*4.6.2.10 Synthesis of 5-(acetyl-4,5-diacetyloxy-6-methylperhydro-2H-pyran-2-yloxy)-4-acetyloxy-2-(acetyloxymethyl)-6-{2-[2-)2,2,2-trifluoroacetylamino)ethoxy]ethoxy}perhydro-2H-pyran-3-yl acetate (**9**)*

Compound **7** (793 mg, 1.19 mmol) and **8** (225 mg, 1.12 mmol) were evaporated repeatly with toluene to remove excess water and then placed under argon gas. **7** and **8** were dissolved in CH_2Cl_2 (8.4 mL) and mol sieves (4 Å). *N*-iodosuccinimide (NIS) (289 mg, 1.28 mmol) was added and the mixture was cooled to -20 °C. Silver triflate (316 mg, 1.23 mmol) was added portionwise at -20 °C. The reaction occurred almost instantly upon addition of silver triflate. The reaction was diluted with CH_2Cl_2 and filtered. The organic layer was washed with 10% aq. sodium thiosulfate, followed by sat. sodium bicarbonate and then brine. The organic layer was dried with sodium sulfate, filtered and concentrated to afford a yellow oil. Column chromatography (2:1 hexanes:EtOAc) on silica gel obtained **9** (810 mg, 95% yield): ^{1}H-NMR (300 MHz, $CDCl_3$) δ1.10 (d, 3H, *J* = 6.0Hz), 1.99 (s, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 2.04 (s, 3H), 2.13 (s, 3H), 2.15 (s, 3H), 3.40 (q, 1H, *J* = 6.6Hz), 3.55-3.78 (m, 6H), 3.88 (t, 1H, *J* = 6.5Hz), 3.97 (dd, 1H, *J* = 7.8Hz, *J* = 10.2Hz), 4.06 (dd, 1H, *J* = 6.6Hz, *J* =

9.3Hz), 4.14 (dd, 1H, *J* = 6.6Hz, *J* = 14.4Hz), 4.187 (m, 1H), 4.50 (d, 1H, *J* = 7.8Hz, H-1**β**, confirmed by decoupling experiments), 4.76 (q, 1H, *J* = 6.6Hz), 5.00 (dd, 1H, *J* = 3.3Hz, *J* = 10.2Hz), 5.02 (dd, 1H, *J* = 3.3Hz, *J* = 10.2Hz), 5.25-5.31 (m, 3H), 5.42 (d, 1H, *J* = 3.6Hz), 7.49 (bs, 1H, N-H); MS (EI^+) *m/z* 784.6 [M+Na]+, 800.4 [M+K]+

4.6.2.11 Synthesis of 5-((2S)-6,8-diazo-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl)-N-(2-{2-[4,5-dihydroxy-6-(hydroxymethyl)-3-(3,4,5-trihydroxy-6-methylperhydro-2H-pyran-2yloxy) perhydro-2H-pyran-2-yloxy]ethoxy}ethyl)pentanamide (10)

Compound **9** (361.2 mg, 0.474 mmol) was dissolved in 50.07 mL of 7% potassium carbonate in methanol/water (2:5) and allowed to stir until complete deprotection has occurred as detected by drop in R_f in thin layer chromatography and appearance of amine as seen by ninhydrin staining. The solvent was removed *in vacuo* and the deprotected product was extracted from the residue with methanol. The extraction was concentrated and the resulting residue was dissolved in DMF (11.73mL). The pH of the mixture was adjusted to 10 using triethylamine (TEA). NHS-biotin is added and the reaction is allowed to stir overnight. The reaction mixture is concentrated to afford an off-white syrup. Purification by HPLC yields **(10)** in 60% yield: ^{1}H-NMR (300 MHz, D_2O) δ 1.05 (d, 3H, *J* = 6.6Hz), 1.23-1.31 (m, 2H), 1.41-1.60 (m, 4H), 2.12 (t, 2H, *J* = 7.0Hz), 2.62 (d, 1H, *J* = 13.2Hz), 2.84 (dd, 1H, *J* = 4.8Hz, *J* = 12.6Hz), 3.17 (dd, 1H, *J* = 4.8Hz, *J* = 9.3Hz), 3.23 (dd, 1H, *J* = 4.8Hz, *J* = 8.7Hz), 3.42-3.75 (m, 11H), 3.90 (dt, 1H, *J* = 4.2Hz, *J* = 11.7Hz), 4.19 (q, 1H, *J* = 6.6Hz), 4.27 (dd, 1H, *J* = 4.7Hz, *J* = 7.8Hz), 4.35 (d, 1H, *J* = 8.4, H-1**β**), 4.46 (dd, 1H, *J* = 4.8Hz, *J* = 8.1Hz), 5.08 (d, 1H, *J* = 6Hz); MS (EI^+) *m/z* 662.2 [M+Na]+, 678.2 [M+K]+.

4.6.2.12 Synthesis of NHS-biotin

Biotin (2.0 g, 8.19 mmol) was dissolved in DMF (24.8 mL) with gentle heating (40 °C). To the reaction mixture was added *N*-hydroxysuccinimide (1.23 g, 10.65 mmol) and dicyclohexylcarbodiimide (DCC) (1.69 g, 8.19 mmol). The reaction was allowed to stir for 24 hours at room temperature. The reaction was then filtered to remove the dicyclohexylurea (DCU) and the filtrate was partially concentrated. The desired product was precipitated from diethyl ether and washed with diethyl ether followed by a wash with isopropanol to afford a white solid (2.43 g, 87%).

4.6.2.13 Synthesis of N-trifluoroacetylpiperidine

Trifluoroacetic anhydride (59.2 g, 0.282 mol) was slowly added to piperidine (20.0 g, 0.235 mol) at 0 °C with vigorous stirring. The reaction was allowed to stir overnight at room temperature. The reaction was concentrated *in vacuo* to remove any trifluoroacetic acid, which afforded the product (42.0 g, 99 %): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.58-1.76 (m, 6H), 3.57 (dt, 4H); MS (EI^+) *m/z* 182.2 $[M+H]^+$.

4.6.2.14 Synthesis of tert-butyl hypochlorite

In a 500 mL flask was placed commercial household bleach (250mL, Clorox®). The flask was placed in an ice bath and vigorously stirred until the temperature dropped below 10 °C. At this point the lights in the vicinity were turned off to protect the reaction from light. A solution of *tert*-butanol (18.5 mL, 0.195 mol) and glacial acetic acid (12.25 mL, 0.215 mol) was added in a single portion to the bleach solution with rapid stirring. The reaction was stirred for about 3 minutes. After stirring, the entire reaction was poured into a

separatory funnel and the lower aqueous layer was discarded while the oily organic layer was washed with 10 % aqueous $NaHCO_3$ (25 mL) and then water (25 mL). The product was dried over $CaCl_2$ (0.5 g) and filtered. The product was used as is in the subsequent reactions and stored over $CaCl_2$.

4.6.2.15 Synthesis of (tert-butoxy)-N-[2-(2-hydroxyethoxy)ethyl]carboxamide

2-(2-aminoethoxy)ethanol (10.0 g, 95.11 mmol) was dissolved in CH_3CN (634 mL). Boc-anhydride (24.3 g, 95.11 mmol) and 1 N NaOH (79.3 mL) was added to the solution and the reaction mixture was stirred for 3 h at room temperature. The reaction mixture was evaporated to dryness and the residue was dissolved in EtOAc. This solution was dried over $MgSO_4$ and filtered. The solvent was removed *in vacuo* and chromatography afforded a yellow oil (17.96 g, 92%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ1.33 (s, 9H), 3.34 (dd, 2H, *J* = 5.4Hz, *J* = 5.7Hz, *J* = 10.4Hz), 3.56 (t, 2H, *J* = 6.1Hz), 3.58 (t, 2H, *J* = 5.1Hz, *J* = 5.4Hz), 3.75 (t, 2H, *J* = 6.0Hz); MS (EI^+) *m/z* 206.3 [M+H]+ 228.3 [M+Na]+.

*4.6.2.16 Synthesis of (tert-butoxy)-N-[2-(2-bromoethoxy)ethyl]carboxamide. **(18)***

(tert-butoxy)-N-[2-(2-hydroxyethoxy)ethyl]carboxamide (5.0 g, 24.36 mmol), carbon tetrabromide (10.11 g, 30.45 mmol), K_2CO_3 (5.12 g, 36.54 mmol) and CH_2Cl_2 (60.9 mL) were placed in a flask. To the mixture was added triphenylphosphine (9.62 g, 36.54 mmol) in CH_2Cl_2 (60.9 mL) over 15 min at room temperature. After stirring for 13 h at room temperature, the reaction mixture is filtered and the filtrate is concentrated. The residue was treated with a mixture of EtOAc:hexanes (1:1, 121.8 mL) by filtration and the filtrate was concentrated. Chromatography of the residue on silica gel afforded the bromide (3.46 g, 53

%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ1.33 (s, 9H), 3.20 (dd, 2H, *J* = 5.4Hz, *J* = 5.7Hz, *J* = 10.4Hz), 3.36 (t, 2H, *J* = 6.0Hz), 3.45 (t, 2H, *J* = 5.1Hz, *J* = 5.4Hz), 3.67 (t, 2H, *J* = 6.0Hz), 5.02 (bs, 1H); ^{13}C-NMR (74.5MHz, $CDCl_3$ w/TMS) δ 28.4, 30.5, 40.3, 60.3, 70.0, 70.7, 155.8; MS (EI^+) *m/z* 291.2 [M+Na]+.

4.6.2.17 Synthesis of 2,2,2-Trifluoro-1-(3-methyoxyphenyl)ethanone ***(12)***

Magnesium turnings (3.22 g), 3-bromoanisole (18.7 g, 100 mmol), and anhydrous THF (100 mL) were placed in a round bottom flask fitted with a condenser. The mixture was cautiously heated with a heating mantle until a vigorous reaction took place. The reaction was removed from heat and the reaction was allowed to proceed until almost all the Mg turnings were dissolved. The reaction was cooled in an ice bath. A solution of *N*-trifluoroacetylpiperidine (14.6 g, 80 mmol) in anhydrous THF (20 mL) was added to the Grignard reagent dropwise with stirring at 0 °C. After the addition, the reaction was stirred at room temperature for 2 h, then quenched with the addition of saturated aqueous NH_4Cl (10 mL). The precipitates were removed by filtration and the filtrate dried over $MgSO_4$. The filtrate was concentrated to afford a dark amber oil. Vacuum distillation of the residual oil afforded a colorless oil (9.34 g, 57%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.80 (s, 3H), 7.16-7.72 (m, 4H); MS (EI^+) *m/z* 205.2 [M+H]+, 227.2 [M+Na]+.

4.6.2.18 Synthesis of 2,2,2-Trifluoro-1-(3-methoxyphenyl)ethanoneOxime ***(13)***

A solution of **12** (10.0 g, 48.98 mmol) and hydroxylamine hydrochloride (3.51 g, 50.45 mmol) in absolute ethanol (27 mL) and dry pyridine (49 mL) was heated at 60 °C for 12 h. After evaporation of the solvents, the residue was partitioned between water and

diethyl ether. The organic layer was washed with 1 N HCl and dried with $MgSO_4$. After evaporation of the solvent, the crude oxime was purified by column chromatography on silica gel (CH_2Cl_2) to afford **13** (10 g, 93%): ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS) δ 3.83 (s, 3H), 7.00-7.43 (m, 4H), 8.67 and 8.85 (each bs, total 1H); ^{13}C-NMR (74.5 MHz, $CDCl_3$ w/TMS) δ 55.6, 114.0, 114.4, 116.3, 116.5, 120.9, 129.8, 129.9, 159.5; MS (EI^+) *m/z* 220.2 $[M+H]^+$; MS (EI^-) *m/z* 218 [M-H]-.

*4.6.2.19 Synthesis of 3-(3-methoxyphenyl)-3-(trifluoromethyl)diaziridine (**14**)*

To a solution of oxime **13** (1.32 g, 6.02 mmol), triethylamine (1.54 g, 15.23 mmol), and (*N*, *N*-dimethylamino)pyridine (36.8 mg, 0.301 mmol) in CH_2Cl_2 (10.8 mL) at 0 °C was added *p*-toluenesulfonyl chloride (1.30 g, 6.80 mmol) portionwise with stirring. After addition, the reaction was allowed to stir at room temperature. After 45 minutes of stirring, the reaction was washed with water, and the organic layer is dried with magnesium sulfate and filtered. The solvent was removed to afford a white solid. The crude oxime was then dissolved in CH_2Cl_2 (3.2 mL) and brought to -78 °C (dry ice and acetone bath). Ammonia gas was condensed into the reaction flask with the use of a cold finger until approximately 3-4 mL of liquid ammonia was present. The reaction was allowed to stir for 12 h at room temperature under NH_3 with a cold finger attached to the reaction flask. After 12 h, the excess ammonia was allowed to evaporate off at room temperature. The reaction was partitioned between water and CH_2Cl_2. The organic layer was washed with water, dried over $MgSO_4$, filtered and concentrated. Column chromatography on alumina (diethyl ether) of the crude oil afforded **14** (1.15 g, 89%): ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS) δ 2.24 (d, 1H, J = 8.1Hz), 2.78 (d, 1H, J = 9Hz), 3.83 (s, 3H), 6.98 (ddd, 1H, J = 1.2Hz, J = 2.2Hz, J = 8.3Hz),

7.15-7.25 (m, 2H), 7.33 (t, 1H, *J* = 7.8Hz, *J* = 8.1Hz); ^{13}C-NMR (74.5 MHz, $CDCl_3$ w/TMS) δ 55.6, 113.7, 115.9, 120.4, 121.7, 125.4, 130.0, 133.1, 159.7; MS (EI^+) *m/z* 219.2 $[M+H]^+$.

Note: The following reaction and reactions thereafter were done amberized glass to protect the product and starting material from light due to its photosensitivity. All precautions were taken to protect the compounds from light sources.

*4.6.2.20 Synthesis of 3-(3-methoxyphenyl)-3-(trifluoromethyl)-3H-diazirine (**15**)*

To a solution of **14** (1.25 g, 5.73 mmol) and triethylamine (2.13 g, 21.03 mmol) in *tert*-butanol (2.88 mL) and absolute ethanol (2.88 mL) at 0 °C, was cautiously added a solution of *tert*-butyl hypochlorite in *tert*-butanol (1.72 mL). The reaction was stirred at room temperature for 2 h. After 2 h of stirring, the reaction was quenched by the addition of 10% aqueous sodium metabisulfite. The reaction mixture was extracted with hexane and the organic layer was dried over $MgSO_4$, filtered and concentrated to afford a crude oil. Column chromatography on silica gel (hexane:CH_2Cl_2 = 2:1) afforded a colorless oil **15** (0.995 g, 80%): ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS) δ 3.80 (s, 3H), 6.69 (s, 1H), 6.78 (d, 1H, *J* = 7.8Hz), 6.94 (dd, 1H, *J* = 2.1Hz, *J* = 2.7Hz, *J* = 8.4Hz), 7.31 (t, 1H, *J* = 8.1Hz, *J* = 8.4Hz); ^{13}C-NMR (74.5 MHz, $CDCl_3$ w/TMS) δ55.5, 112.4, 115.3, 118.8, 120.4, 124.0, 130.1, 130.6, 159.8; MS (EI^+) *m/z* 517.0 $[M+H]^+$; UV(hex) λ_{max} 365 nm

*4.6.2.21 Thallation of **15** in preparation for the synthesis of **16***

Thallium compounds are known to be very toxic. As a result, thallations were performed in a well-ventilated glove box and all safety precautions were taken to prevent exposure and

contamination. Any glassware and tools that came into direct contact with thallium were disposed of properly. Double gloving and rubber gloves were used.

Diazirine **15** (2.0 g, 9.25 mmol) in trifluoroacetic acid (9.25 mL) was treated with 1.1 equiv of thallium(III) trifluoroacetate (5.50 g, 10.18 mmol) for 24 h at room temperature. The resulting arylthallium ditrifluoroacetates were used in the next step without further purification.

*4.6.2.22 Synthesis of 2-Methoxy-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoic Acid Methyl Ester (**16**)*

After the thallation of **15** (2.0 g, 9.25 mmol) as described above, the solvent trifluoroacetic acid was evaporated *in vacuo*. The residual arylthallium compound was dissolved in dry methanol (59.2 mL) and then added to a solution of palladium(II)chloride (210.39 mg, 1.19 mmol), lithium chloride (1.01 g, 23.84 mmol), and magnesium oxide (953.6 mg, 23.66 mmol) in dry methanol (59.2 mL) under an atmosphere of CO. The reaction mixture was allowed to stir at room temperature under an atmosphere of CO for 48 h. *Reactions involving CO were carried out in a well-ventilated hood*. After stirring the reaction mixture was brought to 0 °C and the pH was adjusted to 2 with 1 N HCl. The reaction mixture was partitioned between diethyl ether and water. The organic layer was washed with 1 N HCl and then dried over $MgSO_4$ and filtered. The filtrate was concentrated and column chromatography of the residue on silica gel (hexane:CH_2Cl_2 = 1:1) afforded a colorless oil (1.57 mg, 62%): ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS) δ 3.89 (s, 3H), 3.90 (s, 3H), 6.68 (s, 1H), 6.82 (d, 1H, *J* = 8.3Hz), 7.81 (d, 1H, *J* = 7.8Hz); ^{13}C-NMR (74.5 MHz, $CDCl_3$ w/TMS) δ 52.3, 56.1, 109.7, 118.0, 120.0, 121.3, 123.7, 132.1, 134.3, 159.0, 165.6;

MS (EI^+) *m/z* 297.2 $[M+Na]^+$; IR (neat) ν 1735 (CO) cm^{-1}; UV(EtOH) λ_{max} 349 nm; IR 1733 cm^{-1}.

*4.6.2.23 Synthesis of 2-Hydroxy-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoic Acid Methyl Ester (**17**)*

Compound **16** (1.00 g, 3.65 mmol) was dissolved in dry CH_2Cl_2 (7.25 mL) and cooled to 0 °C. To that was slowly added a 1 M solution of boron tribromide in CH_2Cl_2 (6.21 mL, 6.21 mmol). The reaction was allowed to stir at 0 °C for 30 min. Water was added to quench the reaction. The reaction mixture was extracted with diethyl ether, dried over $MgSO_4$, filtered and concentrated to afford a brown residue. The crude residue was used without further purification: crude ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS) δ 3.97 (s, 3H), 6.65 (d, 1H, *J* = 8.9Hz), 7.84 (d, 1H, *J* = 8.1Hz), 10.83 (s, 1H); MS (EI^+) *m/z* 283.2 [M+Na]+.

*4.6.2.24 Synthesis of **19***

The diazirine **17** (710 mg, 2.73 mmol) was dissolved in DMF (5.0 mL) and to that was added K_2CO_3 (377.3 mg, 2.73 mmol) at 0 °C. **18** (807.1 mg, 3.01 mmol) and tetrabutylammonium iodide (100.8 mg, 0.273 mmol) were added at room temperature. The reaction was refluxed at 60 °C for 19 h with stirring. The reaction was then filtered to remove insoluble material. The solvent was removed *in vacuo* and residue was partitioned between benzene and water. The organic layer was dried with $MgSO_4$, filtered and concentrated under reduced pressure. Chromatography of the residue on silica (hexane:diethyl ether = 1:1) afforded **22** (874.4 mg, 72%): ^{1}H-NMR (300 MHz, $CDCl_3$

w/TMS) δ 1.42 (s, 9H), 3.34 (dd, 2H, *J* = 5.4Hz, *J* = 10.5Hz), 3.64 (t, 2H, *J* = 4.8Hz, *J* = 5.4Hz), 3.87 (dd, 2H, *J* = 4.5Hz, *J* = 9.3Hz), 3.89 (s, 3H), 4.18 (t, 2H, *J* = 4.5Hz, *J* = 5.1Hz), 5.12 (bs, 1H), 6.72 (s, 1H), 6.82 (d, 1H, *J* = 8.7Hz), 7.82 (d, 1H, *J* = 8.4Hz); ^{13}C-NMR (74.5 MHz, $CDCl_3$ w/TMS) δ 28.5, 40.5, 52.4, 53.6, 69.0, 69.1, 70.6, 79.2, 111.5, 118.5, 120.0, 121.8, 123.7, 132.2, 134.3, 158.4, 165.5; MS (EI^+) *m/z* 470.2 $[M+Na]^+$, 486.2 $[M+K]^+$, 442.2 $[M+H]^+$; UV(EtOH) λ_{max} 355 nm.

*4.6.2.25 Synthesis of methyl 2-(2-{2-[5-((2S)-6,8-diaza-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl)pentanoylamino]ethoxy}ethoxy)-4-[3-(trifluoromethyl)(1,2-diazirin-3—yl)]benzoate (**20**)*

Boc-deprotection was performed by treatment of **19** (700 mg, 1.57 mmol) with 50% trifluoroacetic acid in CH_2Cl_2 (3.14 mL) for 3 h at 0 °C with stirring. After 3 h of stirring, the reaction mixture was concentrated. The residue was dissolved in DMF (1.57 mL) and brought to 0 °C. To that, was added triethylamine (544 μL) followed by a solution of NHS-biotin (535.97 mg, 1.57 mmol) in DMF (9.42 mL). The reaction mixture was allowed to stir overnight at room temperature. The solvent was removed *in vacuo* and the residue was partitioned between CH_2Cl_2 and water. The organic layer was dried with $MgSO_4$ and concentrated. Chromatography on silica ($CHCl_3$:ethanol = 6:1) afforded **20** (649 mg, 72%): ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS) δ 1.41 (m, 2H), 1.66 (m, 4H), 2.23 (t, 1H, *J* = 7.2Hz, *J* = 7.8Hz), 2.71 (d, 1H, *J* = 13.2Hz), 2.88 (dd, 1H, *J* = 4.8Hz, *J* =5.1Hz, *J* = 12.8Hz,), 3.12 (m=dd, 1H, *J* = 7.2Hz, *J* = 7.8Hz, *J* = 11.7Hz), 3.47 (dd, 2H, *J* = 5.1Hz, *J* = 5.7Hz, *J* = 10.2Hz), 3.87 (t, 1H, *J* =4.8Hz, *J* = 5.1Hz), 3.87 (t, 2H, *J* = 5.1Hz), 3.88 (s, 3H), 4.18 (t, 2H, *J* =4.5Hz), 4.28 (dd, 1H, *J* = 4.5Hz, *J* = 7.8Hz), 4.47 (dd, 1H, *J* = 4.8Hz, *J* = 7.2Hz), 5.36 (bs,

1H), 6.15 (bs, 1H), 6.70 (s, 1H), 6.72 (bs, 1H), 7.83 (d, 1H, J = 8.1Hz); MS (EI^+) *m/z* 574.2 $[M+H]^+$, 596.2 $[M+Na]^+$, 612.2 $[M+K]^+$; UV(EtOH) λ_{max} 364 nm.

4.6.2.26 Synthesis of 2-(2-{2-[5-((2S)-6,8-diaza-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl)pentanoylamino]ethoxy}ethoxy)-4-[3-(trifluoromethyl)(1,2-diazirin-3—yl)]benzoic acid

Compound **20** (9.4 mg, 0.0164 mmol) was dissolved in methanol (307 μL) and cooled to 0 °C. 1 N aqueous NaOH (82 μL) was added at 0 °C and the reaction was allowed to stir overnight at room temperature. After evaporation of the methanol, the residue was dissolved in water and acidified with 1 N aqueous HCl. The crude acid was purified by reprecipitation in chloroform-hexane to yield the desired product (7.5 mg, 82%): The product was used as is in next step; MS (EI^+) *m/z* 582.0 [M+Na]+, 560.2 [M+H]+.

*4.6.2.27 Synthesis of 2,5-dioxopyrrolidiny 2-(2-{2-[5-((2S)-6,8-diaza-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl)pentanoylamino]ethoxy}ethoxy)-4-[3-(trifluoromethyl)(1,2-diazirin-3—yl)]benzoate. (**21**)*

Compound 2-(2-{2-[5-((2S)-6,8-diaza-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl)pentanoylamino]ethoxy}ethoxy)-4-[3-(trifluoromethyl)(1,2-diazirin-3—yl)]benzoic acid (100 mg, 0.179 mmol) was dissolved THF/CH_3CN (1:1, 4 mL) and *N*-hydroxysuccinimide (21.41 mg, 0.186 mmol) was added. To the reaction mixture was added a solution of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (31.36 mg, 0.202 mmol) in THF (0.8 mL). Triethylamine (20.44 mg, 0.202 mmol) was added to the reaction mixture and the reaction is allowed to stir at room temperature overnight. The reaction was then concentrated and the residue was partitioned between CH_2Cl_2 and water. The organic layer was dried with

$MgSO_4$, filtered and concentrated to afford **21** (85.2 mg, 73%): ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS): δ 1.34 (m, 2H), 1.61 (m, 4H), 2.14 (t, 2H, *J* = 7.2Hz, *J* = 7.8Hz), 2.85 (m, 1H), 2.91 (s, 4H), 3.10 (dd, 1H, *J* = 7.2Hz, *J* = 7.8Hz, *J* = 9.8Hz), 3.40 (dd, 2H, *J* = 4.8Hz, *J* = 5.4Hz, *J* = 10.2Hz), 3.61 (t, 2H, *J* = 4.8Hz, *J* = 5.1Hz), 3.85 (dd, 2H, *J* = 2.7Hz, *J* = 3.6Hz, *J* = 5.0Hz), 4.20 (t, 2H, *J* = 3.9Hz, *J* = 4.8Hz), 4.26 (dd, 1H, *J* = 4.8Hz, *J* = 5.7Hz, *J* = 7.4Hz), 4.45 (dd, 1H, *J* = 4.8Hz, *J* = 5.1Hz, *J* = 7.7Hz), 5.49 (s, 1H), 6.31 (s, 1H), 6.52 (bs, 1H), 6.72 (s, 1H), 6.88 (d, 1H, *J* = 8.1Hz), 8.03 (d, 1H, *J* = 8.1Hz); ^{13}C-NMR (74.5 MHz, $CDCl_3$ w/TMS) δ 25.7, 25.8, 25.9, 28.2, 28.4, 35.9, 39.3, 40.7, 55.8, 60.3, 61.8, 68.8, 69.1, 70.2, 77.4, 111.1, 115.9, 118.6, 119.8, 123.5, 133.1, 136.8, 159.4, 159.8, 164.1, 169.5, 173.4; MS (EI^+) *m/z* 657.2 $[M+H]^+$, 679.2 $[M+Na]^+$, 695.2 $[M+K]^+$, UV(H_2O) λ_{max} 364 nm.

*4.6.2.28 Synthesis of Probe 5-((2S)-6,8-diazo-7-oxo-3-thiabicylco[3.3.0]oct-2-yl)-N-[2-(2-{2-[N-2-{2-[4,5-dihydroxy-6-(hydroxymethyl)-3-(3,4,5-trihydroxy-6-methylperhydro-2H-pyran-2-yloxy)perhydro-2H-pyran-2-yloxy]ethoxyl}ethyl)carbamoyl]-5[3-(trifluoromethyl)(1,2-diazirin-3-yl)phenoxy}ethoxy)ethyl]pentanamide (**11**)*

8 (62.84 mg, 0.152 mmol) was dissolved in the minimal amount of DMF (15 mL) and the pH was adjusted to approximately 9 using TEA. To that was added **21** (100 mg, 0.152 mmol) and the reaction was allowed to stir at room temperature for 10 h. After 10 h of stirring another 0.2 equiv. of **21** was added and the reaction was stirred for another 5 h. Another 0.2 equiv. of **21** was added every 5 h until staining by ninhydrin showed the disappearance of **8**. The reaction was then concentrated and the crude was purified by HPLC (22% CH_3CN/H_2O on C18 resin) to afford **11** (34.9 mg, 24%): ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS) δ1.18 (d, 3H, *J* = 6.6Hz), 1.36-1.46 (m, 2H), 1.51-1.77 (m, 4H), 2.19 (d, 2H, *J* =

7.5Hz), 2.69 (d, 1H, *J* = 12.9Hz), 2.91 (dd, 1H, *J* = 4.8Hz, *J* = 12.6Hz), 3.17 (dt, 2H, *J* = 3.6Hz, *J* = 5.7Hz), 3.41 (t, 2H, *J* = 5.5Hz), 3.54-3.65 (m, 8H), 3.67-3.75 (m, 7H), 3.80 (m, 2H), 3.86-3.93 (m, 4H), 4.16 (q, 1H, *J* = 6.6Hz), 4.28 (dd, 2H, *J* = 4.5Hz, *J* = 8.1Hz), 4.33 (d, 1H, *J* = 8.4, H-1**β**), 4.48 (dd, 1H, *J* = 4.5Hz, *J* = 8.1Hz), 5.00 (d, 1H, *J* = 3.9, H-1'**α**), 6.86 (s, 1H), 7.04 (d, 1H, *J* = 8.1Hz), 8.01 (d, 1H, *J* = 8.1Hz); MS (EI^+) *m/z* 955.4 $[M+H]^+$, 977.4 $[M+Na]^+$, 993.4 $[M+K]^+$.

*4.6.2.29 Synthesis of 4,5-diacetyloxy-2-[4,5-diacetyloxy-6-(acetyloxymethyl)-2-ethoxyperhydro-2H-pyran-3-yloxyl]-6-methylperhydro-2H-pyran-3-yl acetate (**22**)*

7 (143.4 mg, 0.214 mmol) is repeatedly evaporated with toluene to remove excess water. **7** is brought up in CH_2Cl_2 (1.53 mL) and dry EtOH (9.88 mg, 0.214 mmol) and mol. sieves (4 Å) are added. The mixture stirred for 10min and then cooled to -20 °C. To that is added NIS (58.5 mg, 0.247 mmol), followed by addition of solver triflate (60.64 mg, 0.236 mmol). The reaction was allowed to warn to room temperature and proceeds almost instantaneously upon addition of silver triflate. After it had gone to completion, the reaction was diluted with CH_2Cl_2 and filtered. The filtrate is washed with sat. aq. sodium thiosulfate, sat. aq. sodium bicarbonate, and brine. The organic layer is dried with sodium sulfate, filtered and concentrated. Column chromatography (3:1 hexanes: EtOAc) affords **22** (84.9 mg, 70%, α:β = 1:3): only β is used: ^{1}H-NMR (300 MHz, $CDCl_3$ w/TMS) δ1.10 (d, 3H, *J* = 6.6Hz), 1.24 (t, 3H, *J* = 7.2Hz), 1.96 (s, 3H), 1.97 (s, 3H), 1.98 (s, 3H), 2.02 (s, 3H), 2.11 (s, 3H), 2.13 (s, 3H), 3.59 (quin., 1H, *J* = 7.2Hz), 3.85 (t, 1H, *J* = 6.6Hz), 3.92 (dd, 1H, *J* = 7.8Hz, *J* = 9.9Hz), 4.00 (dd, 1H, *J* = 6.9Hz, *J* = 9.3Hz), 4.08 (dd, 1H, *J* = 6.6Hz, *J* = 11.7Hz), 4.16 (dd, 1H, *J* = 6.6Hz, *J* = 11.1Hz), 4.46 (d, 1H, *J* = 7.8Hz, H-1**β**), 4.59 (q, 1H, *J* = 6.6Hz),

4.98 (dd, 1H, *J* = 4.5Hz, *J* = 10.2Hz), 5.00 (dd, 1H, *J* = 3.3Hz, *J* = 9.9Hz), 5.27 (m, 2H), 5.33 (dd, 2H, *J* = 3.6Hz, *J* = 8.4Hz); MS (EI^+) *mz* 629.4 $[M+Na]^+$, 645.4 $[M+K]^+$.

4.6.2 Molecular Biology: General

Unless otherwise stated, chemicals and molecular biology reagents were purchased from Fisher (Fairlawn, NJ) and used as is. Protease inhibitors were purchased from Aldrich Chemicals (St. Louis, MO) and Alexis Biochemicals (San Diego, CA).

4.6.2.1 Periodic Acid Treatment of Bovine Serum Albumin (BSA)

Bovine serum albumin (4 g) was dissolved in 0.1 M sodium acetate, pH 4.5 (90 mL). Periodic acid (227.9 mg, 1 mmol) was dissolved in 0.1 M sodium acetate, pH 4.5 (3 mL). The periodic acid solution was added to the solution of BSA and the mixture was stirred for 6 h or until the absorbance at 265 nm exhibited no change. The reaction was quenched by the addition of glycerol (73 uL, 1 mmol). The reaction was dialyzed against 2 changes of phosphate buffered saline (PBS, pH 7.4) and then diluted to a final concentration of 3 g in 100 mL with PBS. HIO_4 treated BSA was reused and stored at 4 °C.

4.6.2.2 Recovery of adult rat hippocampal tissue

100 g male Sprague-Dawley rats were purchased from Charles River Laboratories (Kingston, Mass) and housed at the California Institute of Technology Laboratory Animal Facilities. Rats were anesthetized with carbon dioxide for 2 min and immediately euthanized by decapitation with a guillotine (Kent Scientific Co.) The brain was promptly removed and

placed on ice. The required portion of the brain dissected on ice and placed a glass dounce homogenizer tube for subsequent treatment.

4.6.2.3 Preparation and A46-B/B10 Western Blotting of Adult Rat Hippocampus Brain and Cultured Rat Enbryonic Hippocampus Neurons

The hippocampus from one adult Sprague-Dawley rat (100 g) was removed and to that was added 4 volumes of boiling 1 % SDS supplemented with protease inhibitors (aprotinin, 20 μg/mL; leupeptin, 20 μg/mL; antipain, 20 μg/mL; pepstatin, 5 μg/mL; chymstatin, 5 μg/mL; PMSF, 1 mmol) The tissue was homogenized with 5 strokes of a teflon-glass homogenizer followed by 5 passes through a 25-gauge syringe needle to clarify the tissue solution. The solution was transferred to an eppendorf tube and boiled for ten minutes. The solution was allowed to cool and the protein concentration of the solution was determined using BCA Assay (Pierce). Cultured E18 hippocampus neurons (provided by Cristal I. Gama) were removed from the culture plate and centrifuged at 1000 x g for 5 min. The supernatant was removed and the pellet was washed with PBS. The pellet was resuspended in 200 μL of boiling 1 % SDS supplemented with protease inhibitors and sonicated for 2 times 5 s on ice (10 s interval in between). The sample was then boiled for 10 min and the concentration determined by BCA Assay. Loading dye was added to each sample of desired protein content and each sample was boiled for 5 min and the cooled to room temperature. The samples were then loaded on to a 10% polyacrylamide stacking gel and resolved at 200 V. The proteins were then transferred to a nitrocellulose membrane at 150 mA. The membrane was blocked using periodic acid treated BSA for 1 h at room temperature. The membrane was then washed with (2 x 10 min) followed by an incubation

with A46-B/B10 antibody overnight at 4 °C. The membrane was then washed with TBST (2 x 20 min) and incubated with goat-anti-mouse antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. The membrane was then washed successively with TBST (1 x 10 min, 2 x 30 min, 1 x 20 min) and visualized by enhanced chemiluminescence (Amersham, Pittsburgh, PA) and developed on to film (Kodak, X-OMAT AR).

4.6.2.4 Representative Protocol for detection of fucosyl-galactose binding proteins using Probe 11

Preparation of lysates was done using procedures adapted from previously reported procedures.[48] The hippocampi of six 100 g Sprague-Dawley rats was removed and homogenized in 5 volumes of Buffer A supplemented with protease inhibitors with ten strokes of a Teflon tissue homogenizer. The lysates was passed through a 26 gauge needle five times to clarify, followed by sonication on ice (2 x 5 s). Lysates were then centrifuged at 800 x g for 10 min to remove cell debris and nuclei. The supernatant was removed and centrifuged at 100,000 x g (Beckman Ultima centrifuge) for 1 h. The supernatant, which was considered the soluble protein fraction (S2), was removed and stored at 4 °C. The pellet was then resuspended in Buffer A plus protease inhibitors and centrifuged at 100,000 x g for 1 h. The supernatant was removed and the pellet was resuspended in the minimal amount of Buffer A plus 1 % CHAPSO supplemented with protease inhibitors. Brief sonication on ice was used to facilitate resuspension. The lysate was diluted to 10 mg/mL with Buffer A plus 1 % CHAPSO and incubated with rocking for 1 h at 4 °C. The lysates was then centrifuged at 100,000 x g for 1 h. The supernatant was removed and considered the membrane fraction. The soluble fraction and the membrane fraction were then diluted 10 fold, using Buffer B (50

mM PIPES plus protease inhibitors, to a final total protein concentration of approximately 0.5 μg/mL. The soluble fraction and the membrane fraction were each precleared to remove any endogenous streptavidin-agarose by incubating the protein fractions with streptavidin-agarose (30 μL of slurry/100 μg of protein) with rocking for 1 h at 4 °C. *The next step was done in the dark to prevent the photoactivation of* **11**. Each fraction of protein (membrane and soluble) was incubated in the dark with 1 mM of **11** with rocking for 8 h at 4 °C. After 8 h of incubation, each sample was irradiated with 365 nm light (handheld 18 W UV lamp) at a 2 cm working distance for 4 h at 4 °C. Photolysis of **11** was monitored by UV-vis spectrophotometry. The samples were treated with denaturing sample loading dye (Pierce), boiled for 5 min and allowed to cool. The samples were resolved using electrophoresis under denaturing conditions on a 7% polyacrylamide gel. Proteins were transferred to PVDF membrane and the blot was blocked with HIO_4 treated BSA for 1 h, rinsed quickly with TBST, and incubated with streptavidin conjugated with horseradish peroxidase (streptavidin-HRP) in TBST for 1 h. The blot was then washed with TBST (1 x 10 min, 3 x 20 min) and visualized using enhanced chemiluminescence (Amersham).

4.7 References

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