Small Molecule Probes for Studying Cellular Receptors and Enzymes

by

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ABSTRACT

Small molecules have proven to be very important tools for exploration of biological systems including diagnosis and treatment of lethal diseases like cancer. Fluorescent probes have been extensively used to further amplify the utilization of small molecules. The manipulation of naturally occurring biological targets with the help of synthetic compounds is the focus of the work described in this thesis.

Bleomycins (BLMs) are a class of water soluble, glycopeptide-derived antitumor antibiotics consisting of a structurally complicated unnatural hexapeptide and a disaccharide, clinically used as an anticancer chemotherapeutic agent at an exceptionally low therapeutic dose. The efficiency of BLM is likely achieved both by selective localization within tumor cells and selective binding to DNA followed by efficient double-strand cleavage. The disaccharide moiety is responsible for the tumor cell targeting properties of BLM. A recent study showed that both BLM and its disaccharide, conjugated to the cyanine dye Cy5**, bound selectively to cancer cells. Thus, the disaccharide moiety alone recapitulates the tumor cell targeting properties of BLM. Work presented here describes the synthesis of the fluorescent carbohydrate conjugates. A number of dye-labeled modified disaccharides and monosaccharides were synthesized to study the nature of the participation of the carbamoyl moiety in the mechanism of tumor cell recognition and uptake by BLM saccharides. It was demonstrated that the carbamoylmannose moiety of BLM is the smallest structural entity capable for the cellular targeting and internalization, and the carbamoyl functionality is indispensible for tumor cell targeting. It was also confirmed that BLM is a modular molecule, composed of

a tumor cell targeting moiety (the saccharide) attached to a cytotoxic DNA cleaving domain (the BLM aglycone). These finding encouraged us to further synthesize carbohydrate probes for PET imaging and to conjugate the saccharide moiety with cytotoxins for targeted delivery to tumor cells.

The misacylated suppressor tRNA technique has enabled the site-specific incorporation of noncanonical amino acids into proteins. The focus of the present work was the synthesis of unnatural lysine analogues with nucleophilic properties for incorporation at position 72 of the lyase domain of human DNA polymerase beta, a multifunctional enzyme with dRP lyase and polymerase activity.

To Maa and Bapi…….

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AcOH	Acetic Acid
Ac_2O	Acetic Anhydride
APCI	Atmospheric Pressure Chemical Ionization
anh	Anhydrous
aq	Aqueous
atm	Atmosphere
BH_3	Borane
BLM	Bleomycin
BLEDTA	Bleomycin–ethylenedinitrilotetraacetic Acid Conjugate
Bn	Benzyl
BnBr	Benzyl Bromide
Boc	t-Butoxycarbonyl
br s	Broad Singlet
Bu_2SnO	Dibutyltin Oxide
^oC	Degrees Celsius
$^{13}CNMR$	Carbon Nuclear Magnetic Resonance Spectroscopy
cat	Catalytic
CBr_4	Tetrabromomethane
CBz	Carboxybenzyl
$CDCl_3$	Deuterated Chloroform
cm	Centimeter

conc.	Concentrated
$CoCl_2$	Coballt(II) Chloride
CSA	Camphorsulfonic Acid
$CuCl_2$	Copper(II) Chloride
CuO	Copper(II) Oxide
Cy5	Cyanine 5 (Lumiprobe Co.)
$Cy5^{GE}$	Cyanine 5 (General Electric Co.)
Cy5**	Cyanine 5** (General Electric Co.)
$Cy5.5^{GE}$	Cyanine 5 (General Electric Co.)
Cy7	Cyanine 7 (Lumiprobe Co.)
δ	Chemical Shift (ppm)
d	Doublet
dd	Doublet of Doublet
ddd	Doublet of Doublet of Doublet
dRP	Deoxyribosephosphate
DAPI	4′,6-Diamino-2-phenylindole
DBU	1,8-Diazabiocyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DIBAL-H	Diisobutylaluminium Hydride
DIC	Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DMAP	Dimethylaminopyridine

DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenedinitrilotetraacetic Acid
EtOAc	Ethyl Acetate
Et_3N	Triethylamine
EtSH	Ethanethiol
ESI	Electrospray Ionization
FAB	Fast Atom Bombardment
FDG	2-Fluoro-2-deoxy-D-glucose
Fmoc	9-Fluorenylmethoxycarbonyl
g	Gram(s)
h	Hours
H_2	Hydrogen Gas
H_2O	Water
H_2SO_4	Sulfuric Acid
HCl	Hydrochloric Acid
$HgCl_2$	Mercury(II) Chloride
HgO	Mercury(II) Oxide
^{1}HNMR	Proton Nuclear Magnetic Resonance Spectroscopy
HPLC	High-performance Liquid Chromatography
Hz	Hertz

J	Coupling Constant
L	Liter
LiOH	Lithium Hydroxide
m	Multiplet
M	Molar
M^+	Molecular Ion
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MeOH	Methanol
MeOTf	Methyl Triflate
mg	Milligram(s)
μm	Microgram(s)
$MgSO_4$	Magnesium Sulfate (Anhydrous)
MHz	Mega Hertz
min	Minutes
mL	Milliliter
mM	Millimolar
mmol	Millimole(s)
μmol	Micromole(s)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
N	Normal
N_2	Nitrogen Gas
$NaBH_4$	Sodium Borohydride

$NaHCO_3$	Sodium Bicarbonate
Na_2CO_3	Sodium Carbonate
Na_2SO_4	Sodium Sulfate (Anhydrous)
NaOMe	Sodium Methoxide
NH_3	Ammonia
NH_4OAc	Ammonium Acetate
NHS	*N*-Hydroxysuccinimide
nm	Nanometer
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffer Saline
*p*CN-Phe	*p*-Cyanophenylalanine
p-$NO_2C_6H_4$.OCOCl	*p*-Nitrophenyl Chloroformate
p-TsOH	*p*-Toluenesulfonic Acid
Pd	Palladium
Pd/C	Palladium on Carbon
$Pd(OH)_2$	Palladium Hydroxide on Carbon (Pearlman's catalyst)
pdCpA	5″-*O*-Phosphoryl-2″-deoxycytidylyl(3″→5″)adenosine
$(PhO)_2P(O)Cl$	Diphenyl Phosphoryl Chloride
$PhCH(OMe)_2$	Benzaldehyde Dimethyl Acetal
ppm	Parts Per Million
PPh_3	Triphenylphosphine
PTC	Peptidyltransferase Center

q	Quartet
quin	Quintet
R_f	Ratio of Fronts
RNA	Ribonucleic Acid
s	Singlet
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sulphoCy5	Sulphocyanine 5 (Lumiprobe Co.)
t	Triplet
TBA	Tetrabutylammonium
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
tRNA	Transfer RNA
TMSOTf	Trimethylsilyl Triflate
TSTU	*O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate
UV	Ultraviolet
v	Volume

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CHAPTER 1

INTRODUCTION

1.1. Cancer

The term "cancer" was derived from the Greek word *carcinos,* which means crab. It was used by Hippocrates to describe the ulcerous diseases causing finger-spreading projections.[1] The disruption of the balance between cell division and cell death results in the transition of a normal cell to a cancerous cell. This results in faster multiplication of the cells leading to augmented uncontrolled proliferation and loss of cellular differentiation, producing a growing mass of tissue called a 'tumor' or 'malignancy' (Figure 1.1). Biologically, cancer is a disease which involves a cycle of dynamic genetic and epigenetic changes in a normal cell.[2] For several decades, studies have been ongoing to discover the specific alterations that result in a malignancy.

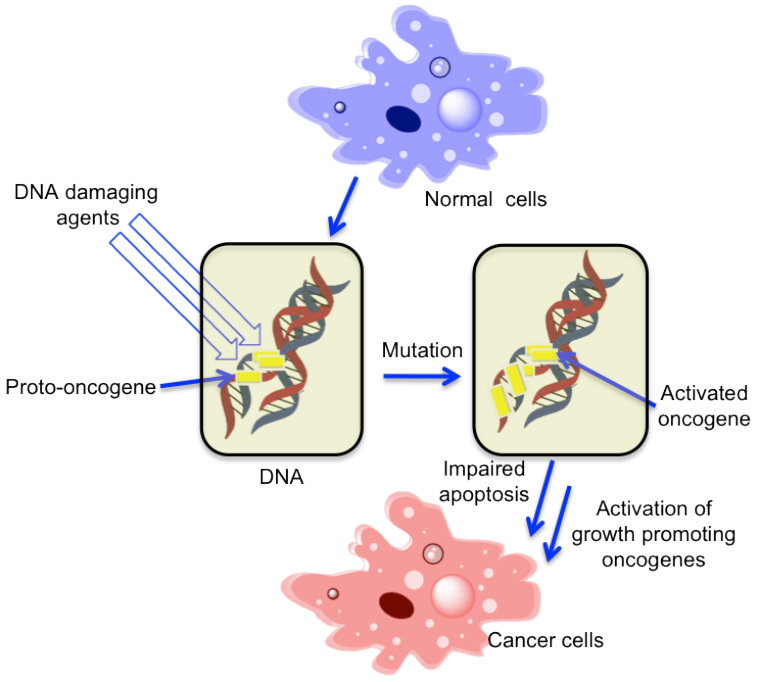


Figure 1.1. Overview of Oncogenesis.

There are more than 100 clinically different types of cancer, which are recognized by their unique properties and symptoms, and require specific treatment strategies.[3] However, they still can be categorized into four major subdivisions: leukemias, lymphomas, sarcomas and carcinomas. A leukemia involves an abnormality in white blood cells or leukocytes, which are produced by the bone marrow. A lymphoma results from an abnormality in lymphocytes, which are produced by spleen and lymph nodes; these are considered similar to a leukemia. A sarcoma is solid tumor of mesodermal origin such as connective tissues, bone, and muscles. Finally, a carcinoma is a solid tumor of epithelial origin and constitutes the major form of cancer, accounting for about of all malignant neoplasms.[3] The hallmarks of cancer mainly involves six biological traits acquired during the various stages of cancer development.[4] They include hyperplasia (uncontrolled proliferation), growth factor abnormalities, suppression of programmed cell death or apoptosis, replication of immortality in the cells, angiogenesis (expansion of blood vessels), and metastasis (the ability of cells to dissociate from a tumor mass and create a new tumor at an anatomically distant site).[4]

1.2. Bleomycins

Bleomycins (BLMs) are a class of water soluble, glycopeptide-derived antitumor antibiotics consisting of a structurally complicated unnatural hexapeptide and a disaccharide (Figure 1.2).[5] It was first isolated from a culture broth of a bacterium, *Streptomyces verticillus*, as a Cu(II) chelate by Umezawa and co-workers in 1966.[6] Clinically, bleomycin is used for the treatment of various kinds of tumors of soft tissues,

especially squamous cell carcinomas and malignant lymphomas.[7] In the United States, it is sold under the tradename Blenoxane as a mixture consisting mainly bleomycin A_2 and B_2, whereas bleomycin A_5 is used clinically in Japan, Russia and China.[8] Bleomycin mediates oxidative cleavage of DNA selectively at 5′-GPy-3′ sequences in the presence of a metal co-factor and oxygen.[5] The exceptionally low therapeutic dose of BLM (~5 μmol) implies extremely high therapeutic efficiency in comparison to other drugs. Such efficiency of BLM is likely achieved both by selective localization within tumor cells[9,10] and selective binding to DNA followed by efficient double-strand cleavage.[11]

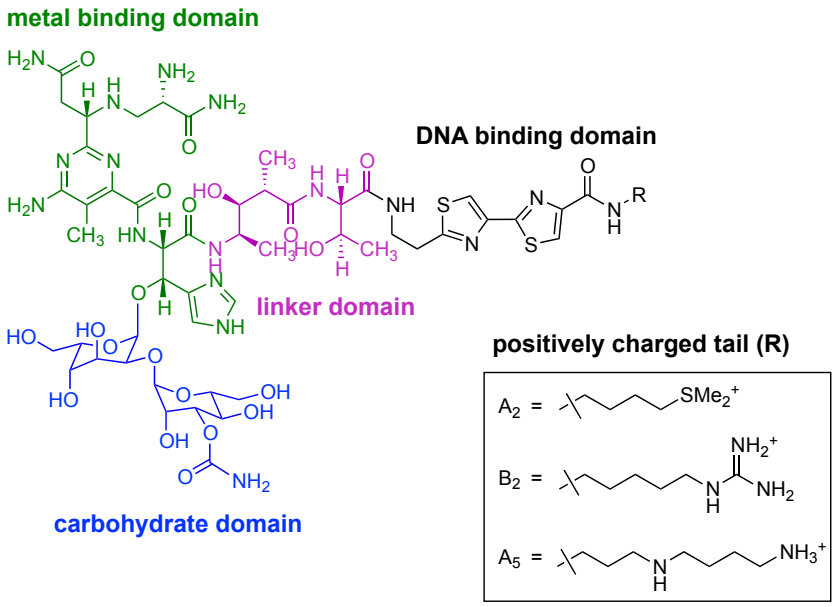


Figure 1.2. Core Structure of BLM, Illustrating its Various Domains.

The efficiency of DNA cleavage by BLM depends on its complex structure which can be dissected into different structural domains: the C-terminal DNA binding domain comprising of a bithiazole attached to a positively charged alkyl substituent, the linker domain with threonine and methylvalerate constituents, the metal binding domain

containing the pyrimidoblamic acid and β-hydroxyhistidine moieties, and the disaccharide moiety. The metal binding domain is the primary determinant of the sequence selectivity of DNA, which is responsible for chelation to metal and oxygen required for the activation of BLM.[12-15] The DNA binding domain binds to DNA through specific interactions. The linker domain maintains the essential compact folded structure required for the cleavage.[16,17] The disaccharide sugar moiety is essential for cancer cell targeting.[10,18] The first total synthesis of bleomycin was accomplished by the Umezawa[19] and Hecht[20] laboratories in 1982. In 1994, Boger and co-workers also reported a total synthesis of BLM A_2.[21]

Though bleomycin is not an enzyme, the interactions of BLM with oxygen possess some similarities to the activation mediated by the metalloenzyme cytochrome P450.[22-24] There are two notable pathways for DNA strand cleavage both of which require activated Fe(II)•BLM (Figure 1.3). The specific mechanism involved in the degradation depends on the availability of oxygen (Figure 1.4). The frank strand scission pathway is favored by a high concentration of oxygen, whereas the mechanism shifts toward the alkali labile lesion pathway in a low oxygen environment.[25-27] As the ratio of products depends on concentration of oxygen, both the pathways are believed to involve the common radical intermediate[28,29] produced by abstraction of the C-4′ hydrogen atom from the deoxyribose of the susceptible nucleotide (Figure 1.4).

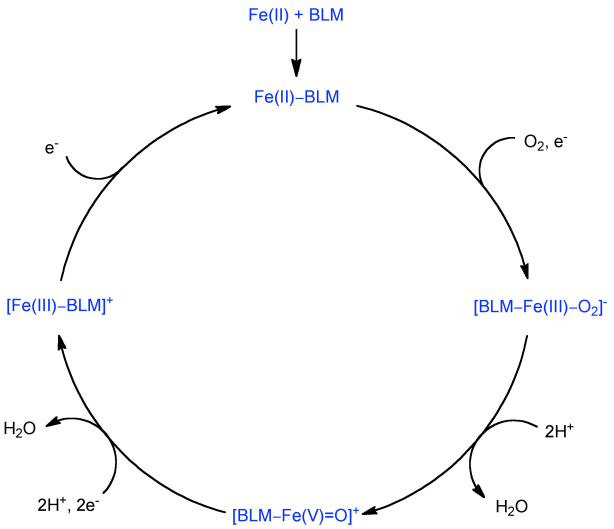


Figure 1.3. Proposed Catalytic Cycle for Activation of Bleomycin.[30]

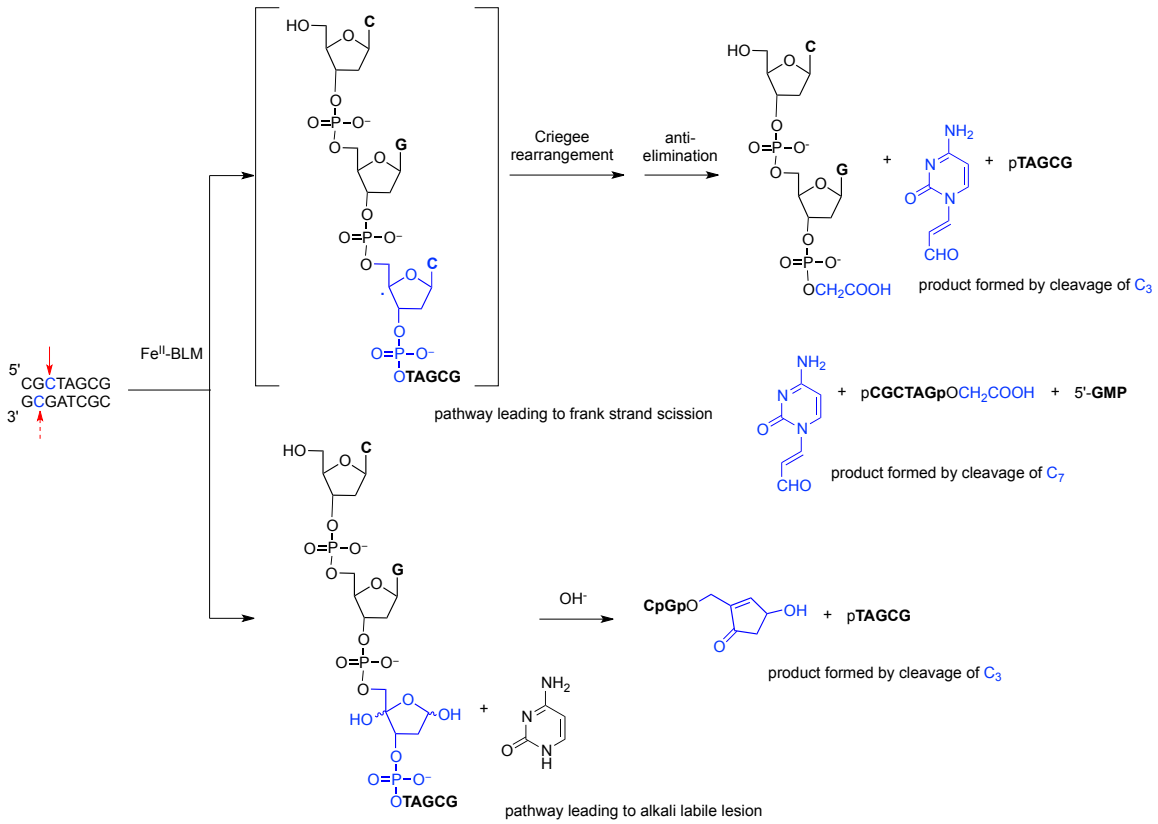


Figure 1.4. Bleomycin-mediated DNA Degradation by the Frank Strand Scission and Alkali-labile Lesion Pathways.[5]

The ability of BLM to mediate double-strand cleavage of DNA depends on the binding interactions between DNA and BLM. Double-strand cleavage of DNA by bleomycin was first studied systematically by the Povirk laboratory.[31-34] Accordingly, a single molecule of BLM effects double-strand cleavage of DNA. After the first lesion generated on the DNA, the BLM is reactivated and the specificity of the DNA gets altered in the complementary strand which results in the second break at one of the two nucleotide positions opposite to the primary site of cleavage.[35] However, recently Hecht and co-workers reported a new mechanism of double-strand cleavage not previously described, which showed that many of the double-strand cleavages associated with the strongly bound hairpin DNAs results from two independent events rather than the coupled event (Figure 1.5).[11]

The high valence iron complex is regenerated from sequestered hydroxyl radical analogous to the propagation in lipid peroxidation. Double-strand cleavages are refractory to repair by DNA repair enzymes and, hence, is highly cytotoxic, thereby making bleomycin selective and cytotoxic.[35]

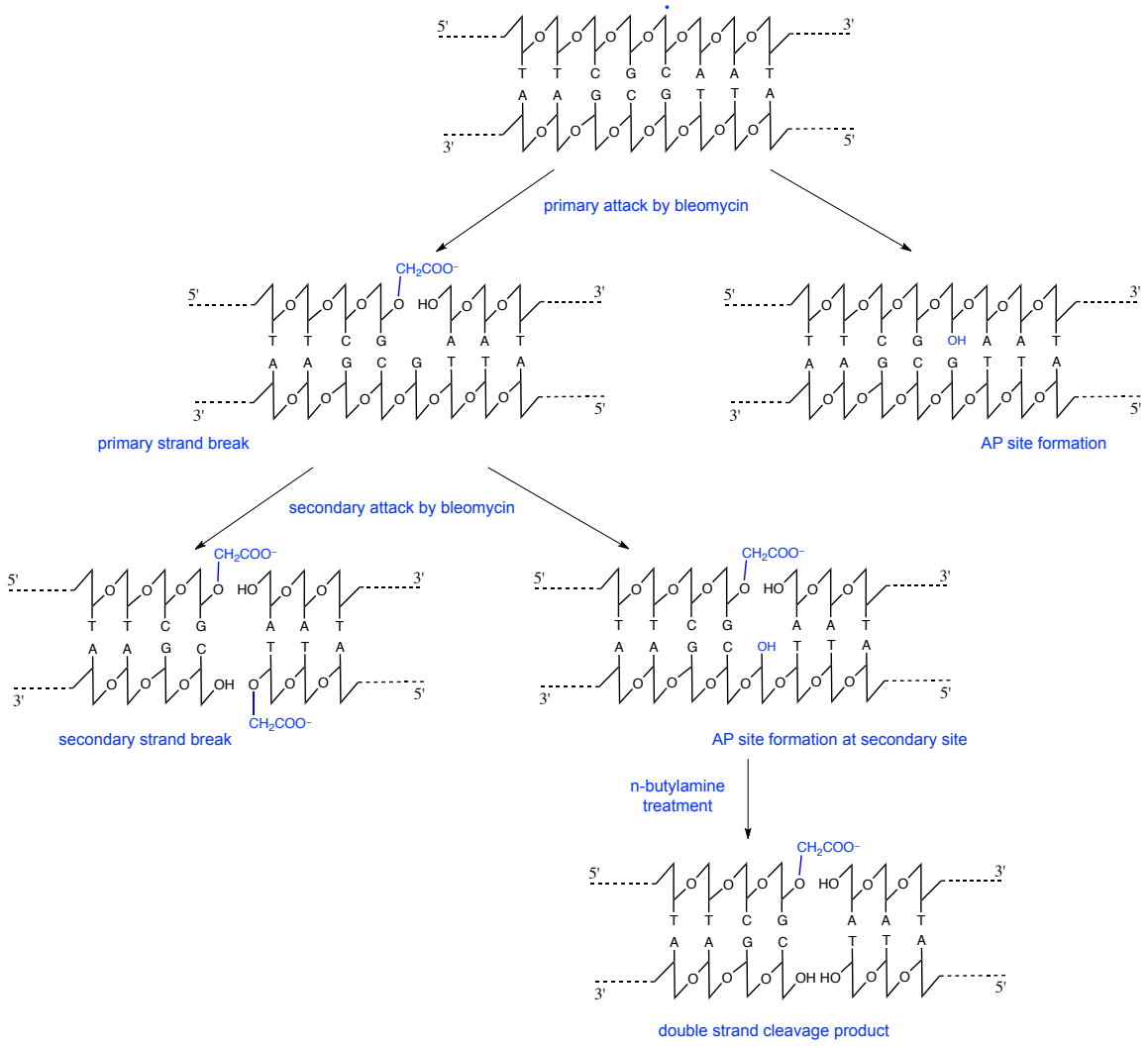


Figure 1.5. Proposed Mechanism for Double-strand Cleavage of DNA.[11,35]

In spite of its therapeutic efficiency, a part of the drug undergoes catabolism to deamido bleomycin by cysteine proteinase bleomycin hydrolase[36-39] (Figure 1.6). Though in earlier reports, deamido bleomycin was asserted to possess no antitumor activity, it was later found to retain one-half of its ability to mediate single strand scission and one-eighth of its ability to mediate double-strand scission.[40]

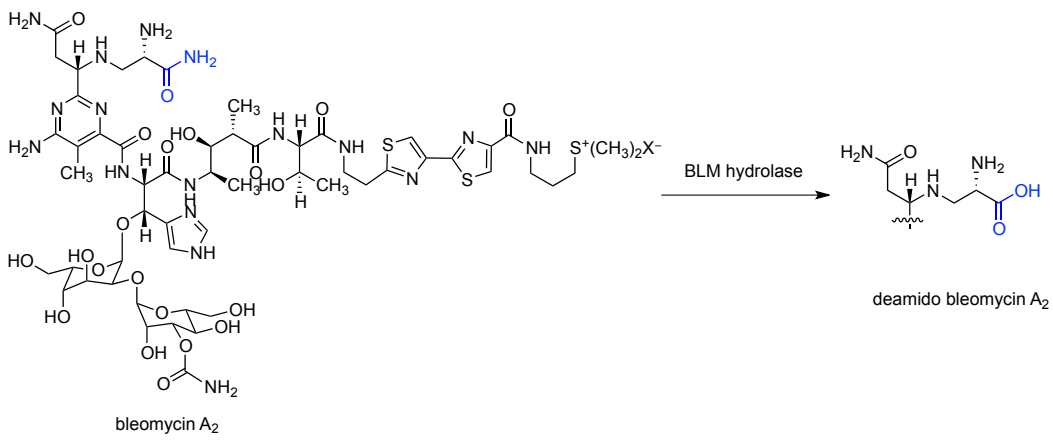


Figure 1.6. Catabolism of Bleomycin to Deamido Bleomycin.[40]

Though bleomycin has proved to be an excellent therapeutic agent for cancer chemotherapy, a significant barrier to its widespread clinical use is a side effect involving lung fibrosis.[41] This raises the question of the requirement for a greater understanding of the chemistry associated with BLM.

1.3. Carbohydrates

Carbohydrates are a class of oxygen containing biomolecules, which play numerous crucial roles in living systems. In principle, they are hydrates of carbon and can be classified as monosaccharides, disaccharides, oligosaccharides, and polysaccharides. The various roles of carbohydrates in the biological processes, apart from being a primary source of energy (starch and glycogen), were not discovered until recently due to the presence of a relatively constrained number of monomers constituting the biologically important polymers.[42] With time, the detailed and closer examination of cellular functions and structure revealed the complexities associated with carbohydrates.

The cell surface is covered by a carbohydrate-rich matrix consisting of glycoproteins, glycolipids and proteoglycans, known as glycocalyx.[43] The glycoproteins are generally large proteins present on the cell surface coupled with sugar residues (Figure 1.7). The majority of glycoproteins are formed by post-translational modification of the protein through glycosylation on the amide nitrogen of the side chain of asparagine (*N*-glycosylation) or the hydroxy group containing side chain of hydroproline, serine, threonine or hydrolysine (*O*-glycosylation).[44] Glycolipids are also found on the cell surface and are composed of hydrophilic saccharides anchored to the hydrophobic lipid cell membrane.[45,46] However, proteoglycans are the major components of extracellular matrix, consisting of the core proteins carrying large polysaccharide units (more than 100 monosaccharides) dangling outside the cell membrane.[47]

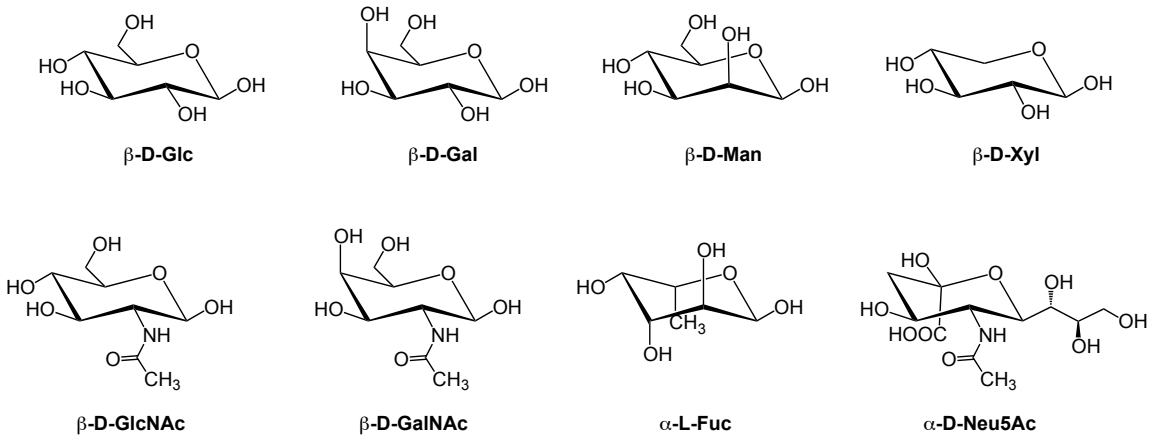


Figure 1.7. The Major Monomeric Monosaccharide Constituents Found in Glycoconjugates: Glycoproteins, Glycolipids and Proteoglycans.

Extracellular communication is critical for the manifestation of the various cellular processes contributing to growth, reproduction, migration, immune response, and signaling events.[48,49] This kind of communication is triggered by the cellular recognition

that involves the interaction of the receptors on the cell surface with their ligands, which was first described as "lock-and-key" hypothesis by Emil Fischer in 1894.[50] Receptor–ligand interaction is effected by the organization and activity of the receptor, which is influenced by the bulky constituents of the glycocalyx. In addition, the larger glycoproteins can influence protein sorting, endocytosis, and neurological and immunological developments.[51-55]

Due to the diverse biological functions associated with the glycan, a comprehensive understanding of protein–carbohydrate interactions is vital in order to study the biological processes.[56,57] In nature, there are several carbohydrate-binding proteins which are mainly classified as lectins and antibodies. The lectins have high affinity towards carbohydrates and bind them reversibly through their carbohydrate recognition domains. They have evolved to recognize the specific carbohydrate at the surface of the cell to mediate cell–cell adhesion, trafficking, triggering immune responses and cell signaling.[58-61] Antibodies are glycoproteins secreted by the plasma cells, which are used by the immune system and are directed towards cell surface glycans. Different glycan signatures are expressed by different cells and have a vital role clinically.[62] One such example for antibody–carbohydrate interaction is represented by the blood group ABO antigen–antibody interaction. The different structure of glycan chains is responsible for the generation of different antigens which forms the ABO blood group system (Figure 1.8) and failure of unmatched blood transfusions.[63,64]

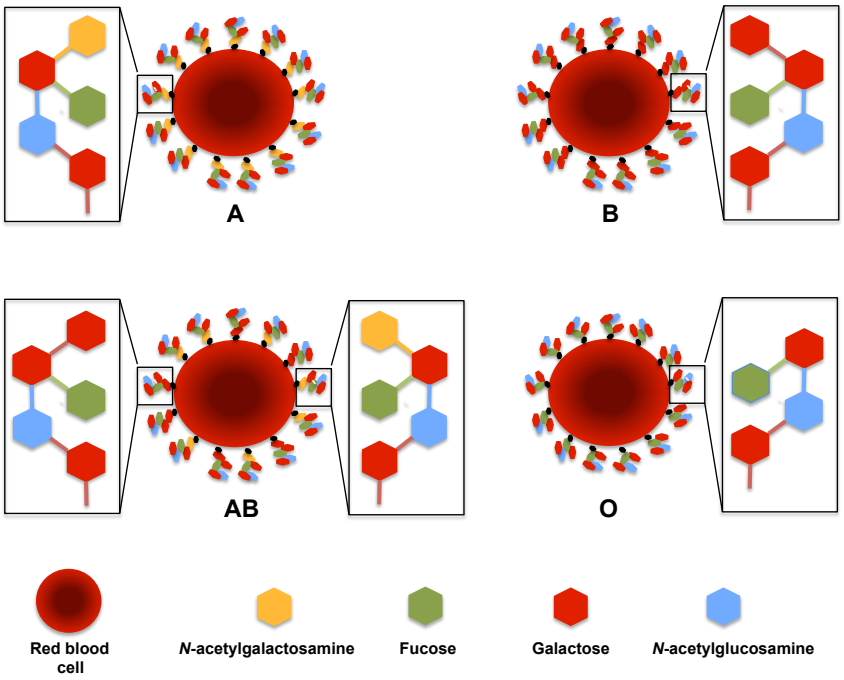


Figure 1.8. ABO Blood Group System Showing the Glycan Chains That Determine the Blood Type.[63]

Many proteins bind to the glycan ligands through polyvalent interactions and possess multiple binding sites.[65] The binding efficiency in the case of polyvalent interaction increases with an increase in the concentration of the monovalent ligands near the site of binding. On the basis of these interactions, many scaffolds were developed for drug delivery, diagnosis and other therapeutic purposes such as cyclodextrins, chitins, chitosans and calixerenes.[66,67]

O-linked-β-*N*-acetylglucosamine (*O*-GlcNAc), a post-translational modification of the glycoproteins, plays a crucial regulatory role in cellular processes and is termed a nutritional sensor.[68] UDP-GlcNAc, the end product in the hexosamine biosynthetic pathway (HBP), is the donor substrate for the enzymatic addition of *O*-GlcNAc used by *O*-GlcNAc transferase (OGT). The removal of *O*-GlcNAc from the proteins is catalyzed

by *O*-GlcNAcase (OGA). The cycling of *O*-GlcNAc in proteins depends on the balance of activity of OGT and OGA, and plays a crucial role in control of cellular processes.[69-73] Excessive *O*-GlcNAc substitution is a general feature associated with cancer.[74-78] As a result of cancer, the energy metabolism of the cell shifts from oxidative phosphorylation to a less efficient energy pathway involving glycolysis; this shift is known as the Warburg effect.[79-81] This results in an increase in the demand for glucose in the cell and eventually increases the uptake of glucose and glucosamine. Further, the levels of the glucose transporters and glycolytic enzymes can be upregulated by Kras oncogene. As a result of the increase in HBP flux, the level of UDP-GlcNAc increases and results in hyper-*O*-GlcNAcylation.[82] The reduction of hyper-*O*-GlcNAcylation, however, inhibits cellular proliferation and results in apoptotic cell death.[73]

Recently, it has been reported that the surface of the glycocalyx in the cancer cells are covered with bulky glycoproteins. These bulky substituents help to facilitate the metastatic spread of cancer cells and to regulate cell survival.[55,83]

1.4. Protein Biosynthesis

Proteins are the biochemical polymeric macromolecules made up of amino acid residues that play a crucial role in a vast array of cellular processes including DNA replication, catalyzing metabolic reactions and the internalization of molecules in the cell. Each protein contains a signature sequence of amino acid residues that arranges itself into a specific three-dimensional structure and thereby contributes to the structure and activity of the protein. In nature, the genetic information encoded in a messenger RNA (mRNA)

is translated into the protein by the cellular organelle called a ribosome. The ribosome is a large nucleoprotein complex made up of a complex array of ribosomal RNAs (rRNAs) and proteins. The ribosome is comprised of two subunits, denoted as 30S and 50S in bacteria.[84,85] Each subunit in the ribosome has three sites for binding transfer RNA (tRNA), namely the A-site (aminoacyl-tRNA binding site), P-site (peptidyl-tRNA transfer site) and E-site (exit site for deacylated tRNA). Each tRNA decodes the genetic information by recognizing a set of three nucleotides known as a codon (triplet sequence), which is complementary to tRNA anticodon, and linking the nucleotide sequence with amino acid residues found at the opposite end of the tRNA. The high fidelity of protein synthesis is due to accurate codon–anticodon base pairing among the different RNAs during the decoding process.[86]

The translation of mRNA proceeds through three distinct steps, namely initiation, elongation and termination. The process begins with activation of tRNA with its cognate amino acid by its aminoacyl-tRNA synthetase. Initiation of protein translation involves the binding of the 30S ribosomal subunit with the mRNA and aminoacylated tRNA with the rRNA having the base pairs complementary to the Shine–Dalgarno sequence of the bound mRNA.[84] This event is followed by binding of the 50S ribosomal subunit to the 30S subunit, affording the 70S ribosomal complex. The formation of initiation complex requires initiation factors (IF1, IF2 and IF3). Generally, the initiation codon translated is AUG, which codes for methionine. Only the activated form *N*-formylmethionine is able to form the initiation complex and occupies the P-site of ribosomal subunit. After initiation, the empty A-site of the ribosomal subunit is charged with aminoacyl-tRNA

having anticodon corresponding to the next codon on mRNA. This event is conducted with the help of GTP and elongation factor EF-Tu, the latter of which is recharged by another elongation factor EF-Ts. The α-amino group of the aminoacyl-tRNA present in the A-site mediates a nucleophilic attack on the carbonyl carbon of the adjacent nascent peptide in P-site and results in transfer of the amino group to the tRNA in the A site thereby elongating the peptide chain (Figure 1.9). Subsequently, the tRNAs in the A-site and P-site are translocated to the P-site and E-site, respectively, with the help of elongation factor EF-G.

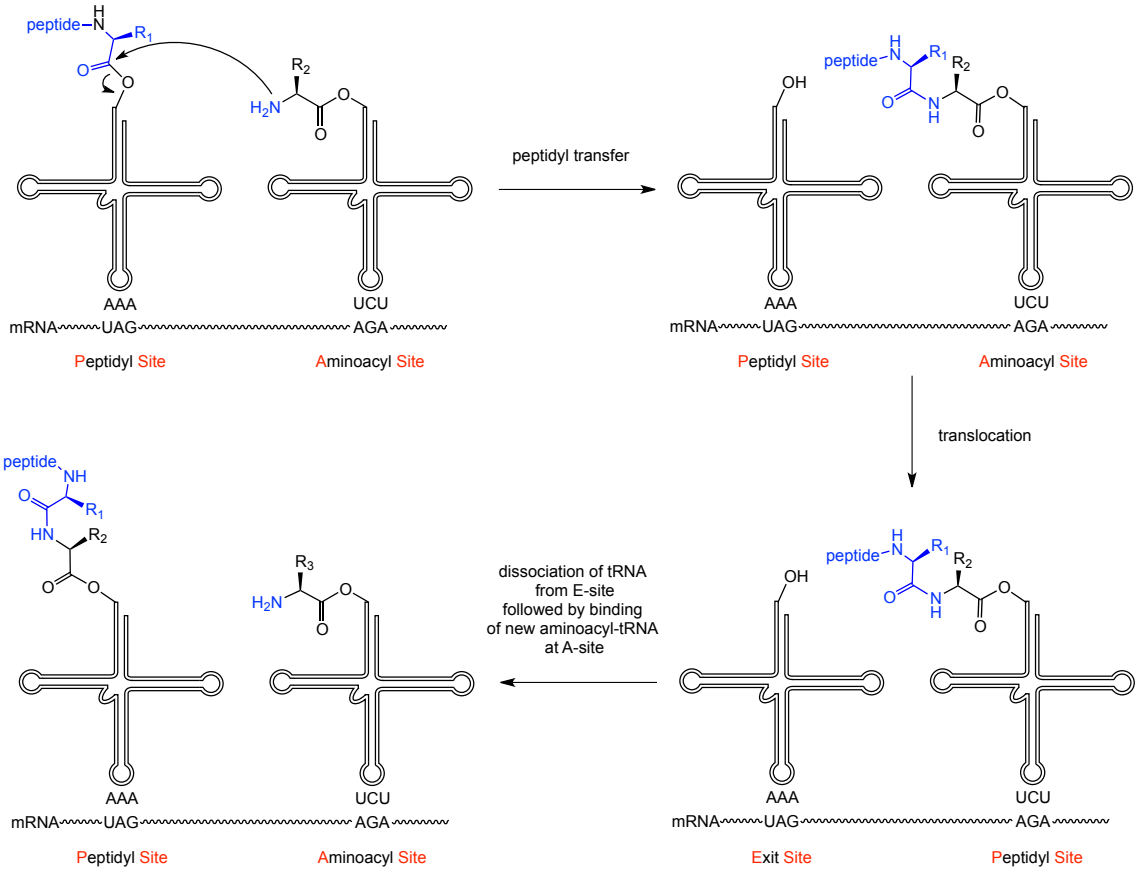


Figure 1.9. Peptide Bond Formation During Protein Translation (R_1, R_2 and R_3 are the side chain substituents in the amino acids).[84]

This event is followed by release of the tRNA from E-site and binding of the next aminoacylated-tRNA, having anticodon corresponding to the next codon on mRNA, to the vacant A-site.[84,87] The process of elongation continues until the termination is signaled by the presence of one of the stop codons (UAG, UAA or UGA) on mRNA. The presence of the stop codon in the A-site results in the binding of the release factors (RF1, RF2 and RF3) and eventually triggers the release of the nascent polypeptide and dissociation of ribosomal complex into 50S and 30S subunits.[87]

1.5. Site-specific Incorporation of Unnatural Amino Acids into Proteins

In nature, there are 20 proteinogenic amino acids and the different order in arrangement of these resides in proteins gives rise to an array of different proteins with a variety of biological roles. To study protein structure, function, dynamics and intermolecular interactions, unnatural amino acids can be introduced into the proteins using misacylated suppressor tRNAs.[88-91] This technique has developed into a very powerful tool and involves site-directed mutagenesis of DNA to replace the codon for a specific amino acid of interest with a nonsense codon (Figure 1.10).

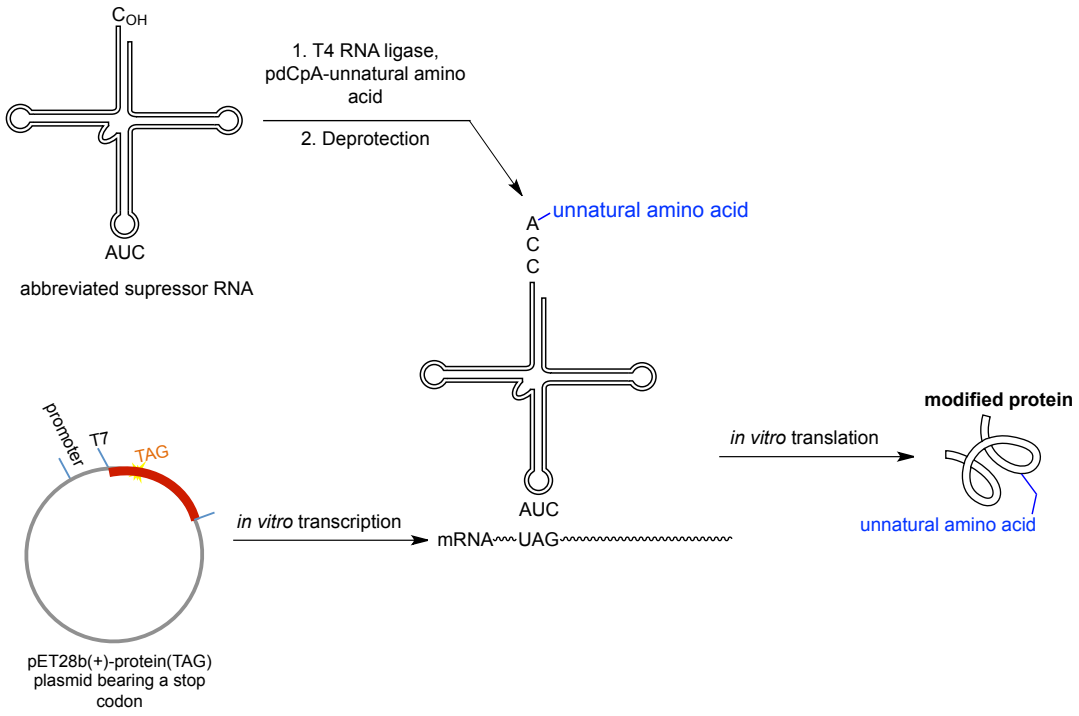


Figure 1.10. Strategy for the Site-specific Incorporation of Unnatural Amino Acids Into Proteins *In Vitro*.[84]

The translation of a stop codon by circumventing the effects of the release factors responsible for the termination of protein synthesis is referred to as suppression event. The nonsense codons used for this purpose are mainly UAG (amber)[88], UAA (opal)[92] and UGA (ochre).[93] Hecht and co-workers first developed the general technique for misacylation of the suppressor tRNA comprising the anticodon complementary to the nonsense codon by ligating a tRNA, lacking the 3′-terminal cytidine and adenosine moieties, to the aminoacylated dinucleotide pCpA with the help of T4 RNA ligase.[94-96] Later, Schultz and co-workers modified the method by replacing the aminoacylated dinucleotide pCpA with dinucleotide pdCpA that is now extensively used for misacylation of tRNA.[97,98] This technology has proven revolutionary in the field of

protein engineering and helped in the incorporation of a wide variety of unnatural amino acids in proteins with novel properties. Further, Hecht and co-workers demonstrated that the modification of the 23S rRNA in bacteria can dramatically changes the ribosomal architecture and permit the incorporation of D-amino acids and β-L-amino acids.[99-102]

CHAPTER 2

SYNTHESIS OF BLEOMYCIN SACCHARIDE CONJUGATES FOR IMAGING STUDIES

2.1. Introduction

2.1.1. Synthesis of Bleomycin Saccharide–dye Conjugates for Selective Targeting of Cancer Cells

For many years the role of the disaccharide moiety in bleomycin was a matter of debate. It was postulated that the C-3 carbamoyl group attached to the mannose is involved in metal binding as the sixth ligand (Figure 2.1) and that the sterically hindered disaccharide forms a protective pocket to shield the reactive radical intermediates.[103-106]

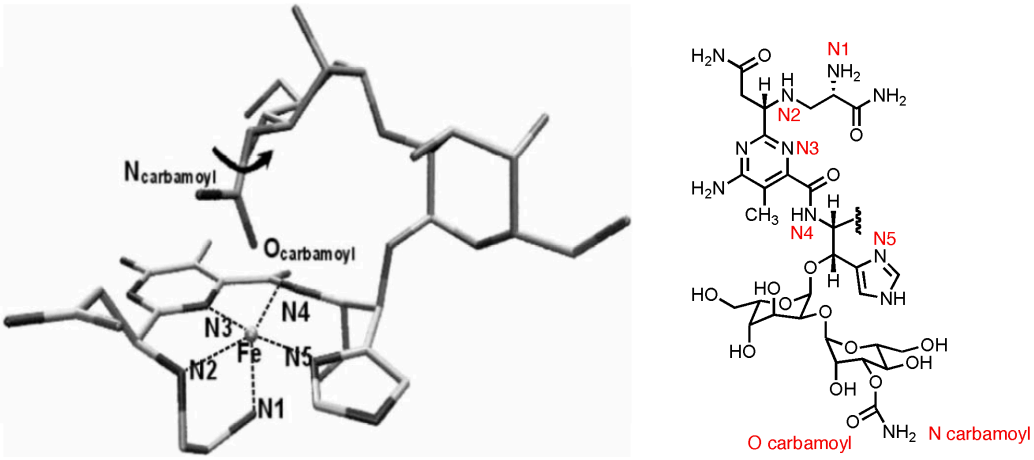


Figure 2.1. Proposed Model for Metal Coordination of Fe(II)•BLM.[104]

However, the documented DNA cleavage activity of deglycoBLM in a cell free system rendered the involvement of the sugar in metal binding moot since deglycoBLM has no sugar.[107,108] In parallel, extensive *in vivo* imaging studies were carried out with ^{57}Co-BLM and ^{111}In-BLM complexes.[109,110] Later, a new modified imaging agent

BLEDTA, containing an ethylenedinitrilotetraacetic acid (EDTA) moiety attached to the C-terminal end of BLM A_2 was developed and ^{111}In-BLEDTA was used successfully to image several carcinomas *in vivo*. These studies failed to show comparably effective imaging for ^{111}In-deglycoBLEDTA.[111-113] Thus, these results strongly implied that the carbohydrate moiety in bleomycin could possibly play a role in cellular recognition and internalization of the drug. In order to further study the role of the carbohydrate moiety, our laboratory designed a targeting experiment in which cancer cells were treated with microbubbles that had been derivatized with bleomycin.[10]

Microbubbles are lipid membranes made up of albumin or other materials, and enclosing a gaseous core such as air, nitrogen or a perfluorocarbon (Figure 2.2). They are used as contrast agents in ultrasonography.[114] The surface of microbubbles that has been modified with streptavidin was used to bind biotin–BLM A_5 and biotin–deglycoBLM A_5 (Figure 2.3) through a biotin–streptavidin specific interaction. The derivatized microbubbles were then incubated with cultured MCF-7 breast cancer cells and MCF-10A normal breast cells and imaged using a Zeiss Axiovert 200M microscope.[10]

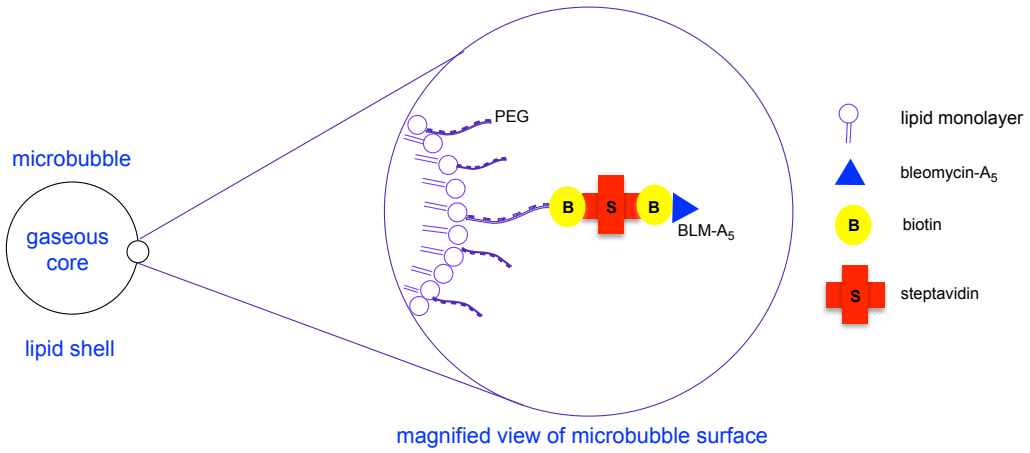


Figure 2.2. Magnified Picture of the Surface of Microbubble Derivatized BLM A_5.[10]

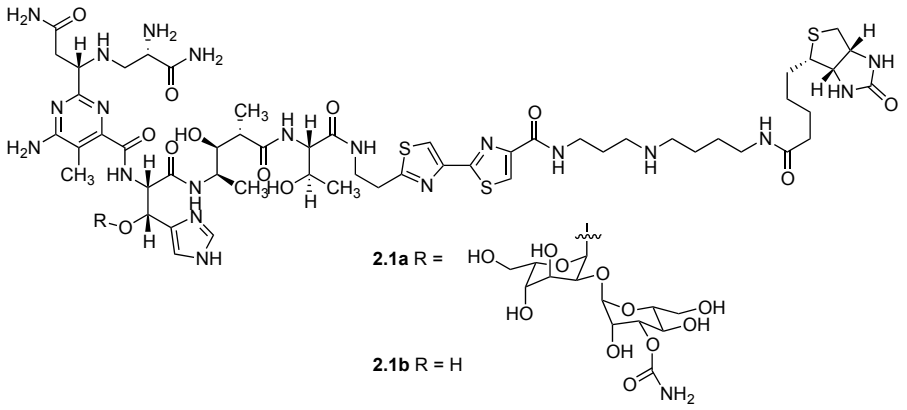


Figure 2.3. Structures of Biotin Derivatized Bleomycin A_5 and Deglycobleomycin A_5.[10]

The BLM–microbubble conjugate was observed to adhere selectively to MCF-7 cells, but the deglycoBLM conjugate lacking the sugar moiety failed to show any cell attachment (Figures 2.4a and 2.4d). Lack of cell attachment was observed for MCF-10A cells incubated with BLM conjugated microbubbles and MCF-7 cells incubated with underivatized microbubbles (Figures 2.4b and 2.4c). The inability of deglycoBLM to bind to cancer cells in comparison to BLM established the requirement of the carbohydrate moiety for targeting cancer cells. As the BLM–microbubble conjugate was derivatized with multiple copies of BLM, the interaction observed by the binding experiment might have been facilitated by the polyvalent nature of the interaction. However, the possible mechanism of cellular uptake of bleomycin and whether the BLM disaccharide moiety alone was sufficient to mediate internalization remained unresolved. To address these issues, fluorescently labeled BLMs and carbohydrates were synthesized and internalization was studied microscopically.[18]

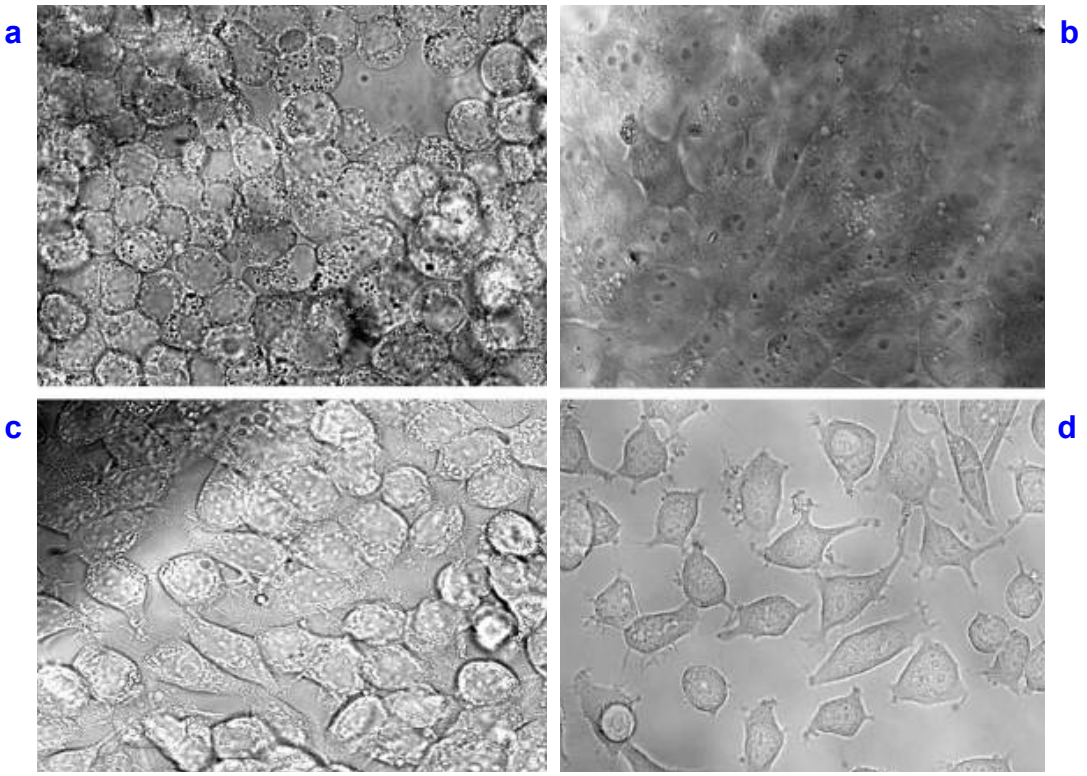


Figure 2.4. Microscopic Images of Microbubble Experiment: (a) Microscopic image of MCF-7 breast cancer cells treated with BLM A_5 derivatized microbubbles. (b) Microscopic image showing MCF-10A normal breast cells treated with BLM A_5 derivatized microbubbles. (c) Microscopic image of MCF-7 breast cancer cells treated with underivatized microbubbles. (d) Microscopic image showing MCF-7 breast cancer cells treated deglycoBLM A_5 derivatized microbubbles.[10]

Classically, in a fluorescence microscopy experiment, the fluorescent tag or label is attached chemically to the molecule of interest to enable its detection following incubation with the target cell lines. Fluorescence microscopy is then used to image the chemical tag localized in the cell. In order to choose the correct reporter, the fluorescent dye should not bind to the cell on its own. Also, it should have sufficient fluorescence signal strength to be detectable above the autofluorescence of the cells studied. Cy5** was chosen as the reporter after many trials[115] and was attached to BLM A_5, deglycoBLM A_5 and BLM disaccharide (Figure 2.5).[18] The conjugates were incubated

with breast, prostate, colon and lung cell lines, as well as their matched normal cell lines, and were imaged using a Zeiss Axiovert microscope. Both BLM–Cy5** (**2.2a**) and BLM disaccharide–Cy5** (**2.3**) showed selective uptake in the MCF-7 breast cancer cells relative to matched normal cells (Figure 2.6 and Figure 2.7). Similar results were also obtained in the case of DU-145 prostate cells and matched normal prostate cells (Figure 2.8 and Figure 2.9).[18]

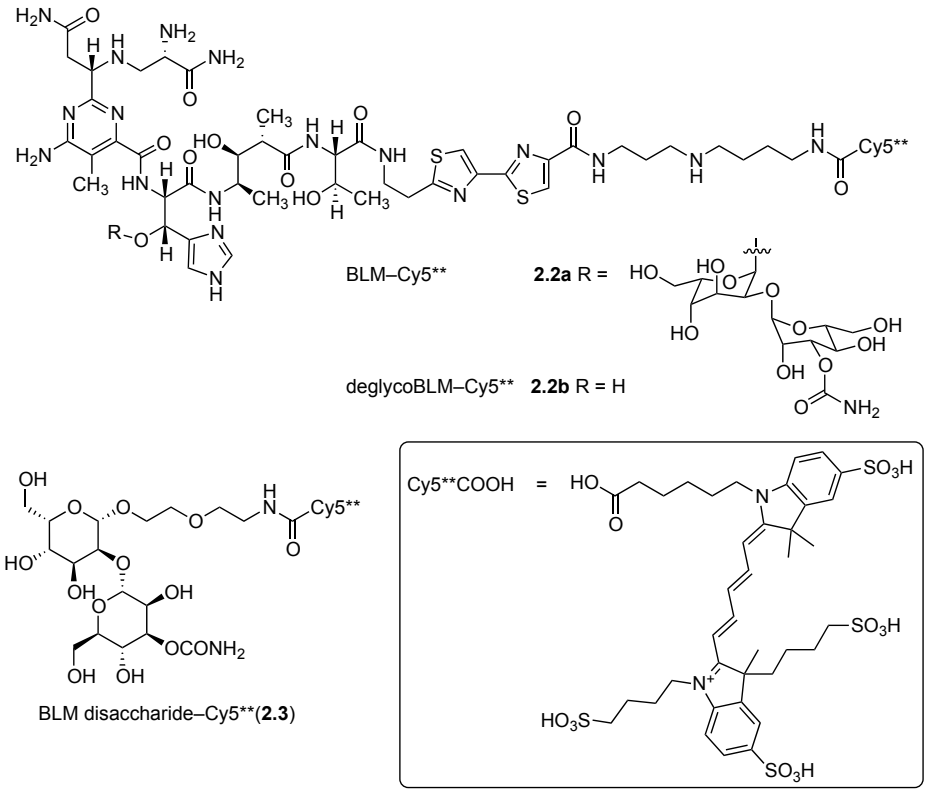


Figure 2.5. Structures of BLM–Cy5** (**2.2a**), DeglycoBLM–Cy5** (**2.2b**) and BLM Disaccharide–Cy5** (**2.3**).[18]

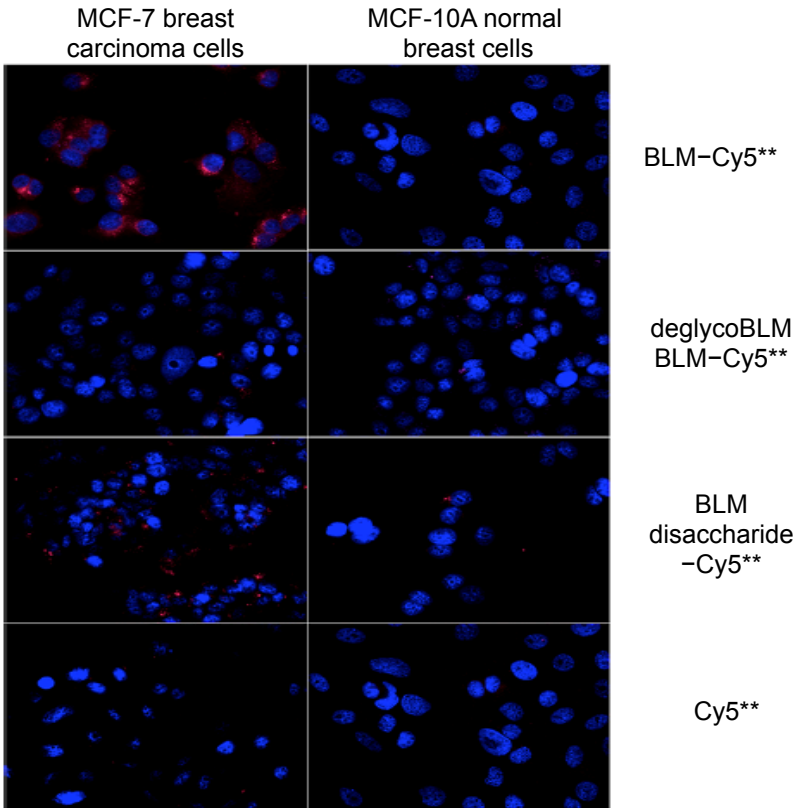


Figure 2.6. Comparison of the Binding/Uptake of BLM–Cy5** (**2.2a**), DeglycoBLM–Cy5** (**2.2b**) and BLM Disaccharide–Cy5** (**2.3**) in MCF-7 Breast Cancer Cells and Matched Normal Breast Cells.[18]

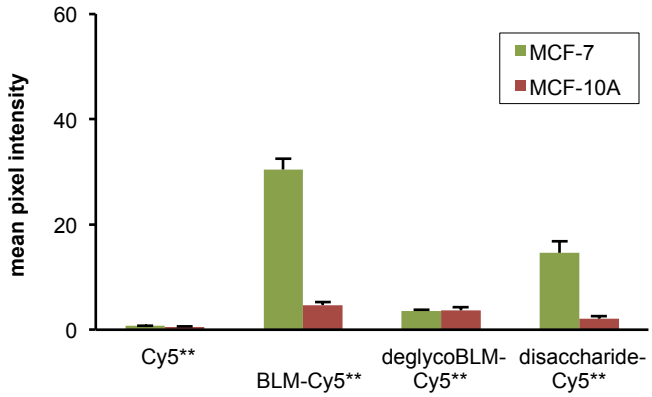


Figure 2.7. Quantification of the Binding/Uptake of BLM–Cy5** (**2.2a**), DeglycoBLM–Cy5** (**2.2b**) and BLM Disaccharide–Cy5** (**2.3**) in MCF-7 Breast Cancer Cells and Matched Normal Breast Cells.[18]

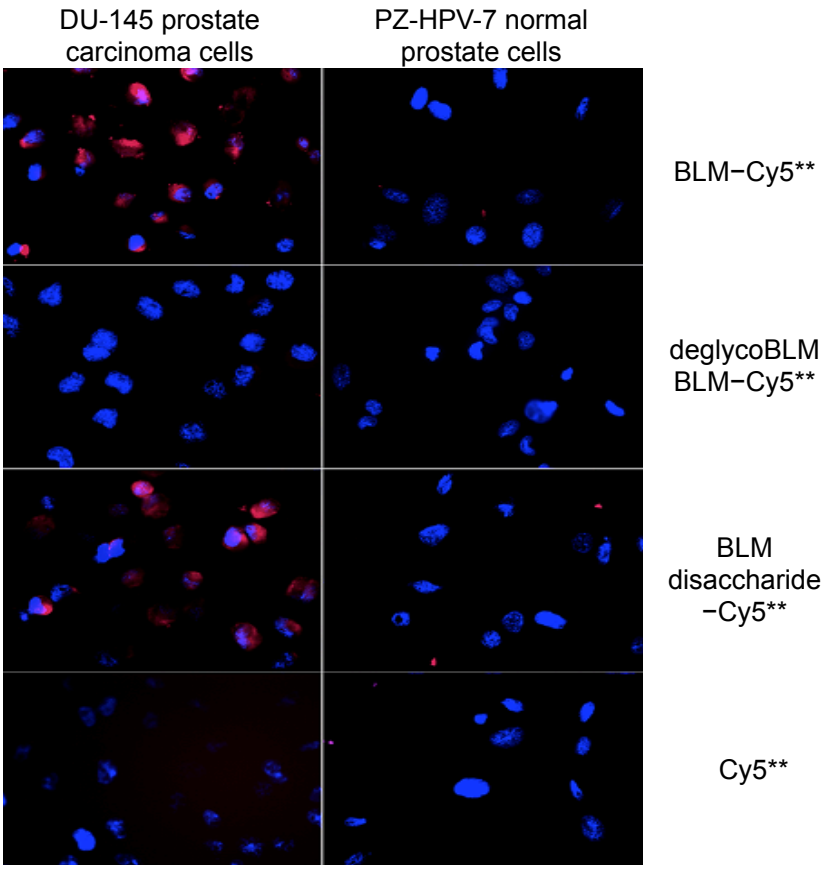


Figure 2.8. Comparison of the Binding/Uptake of BLM–Cy5** (**2.2a**), DeglycoBLM–Cy5** (**2.2b**) and BLM Disaccharide–Cy5** (**2.3**) in DU-145 Prostate Cancer Cells and Matched Normal Prostate Cells.[18]

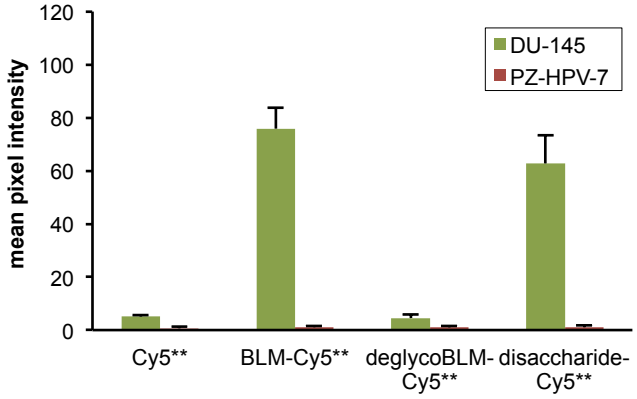


Figure 2.9. Quantification of the Binding/Uptake of BLM–Cy5** (**2.2a**), DeglycoBLM–Cy5** (**2.2b**) and BLM Disaccharide–Cy5** (**2.3**) in DU-145 Prostate Cancer Cells and Matched Normal Prostate Cells.[18]

DeglycoBLM–Cy5** (**2.2b**), lacking the disaccharide moiety showed no uptake in any cell line, which was consistent with the results obtained from the microbubble experiment.[18] Hence, the targeting ability of the carbohydrate domain of BLM along with the sufficiency of the sugar to internalize the reporter molecule can be potentially utilized to develop a better therapeutic agent or drug delivery vehicle for chemotherapy. To further understand the importance of the carbohydrate domain of BLM and explore the participation of the carbamoyl moiety, a library of disaccharide–Cy5** conjugates was synthesized (Figure 2.10).

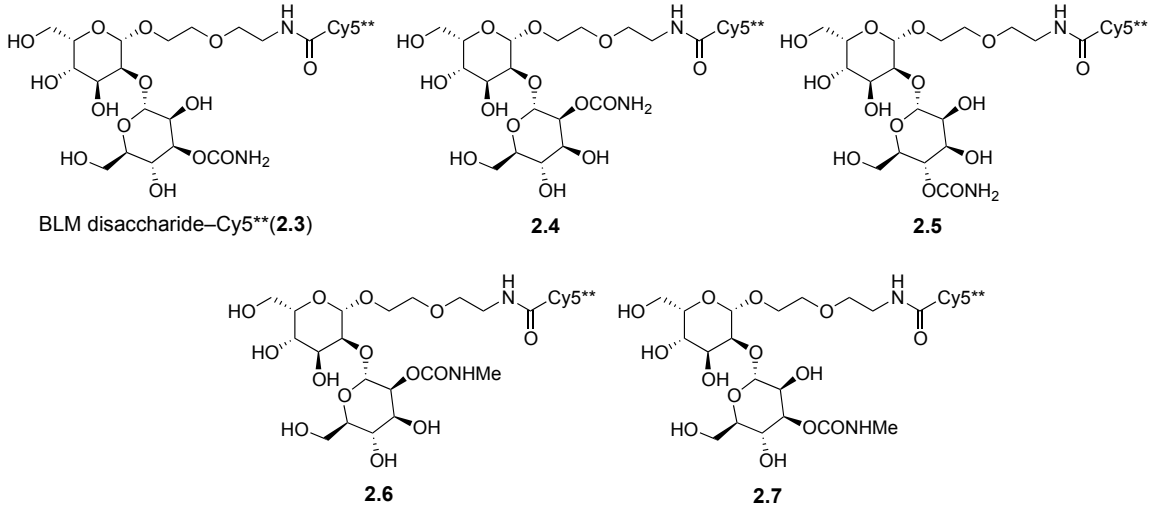
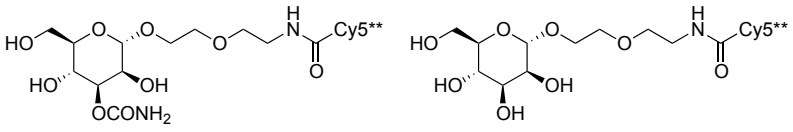


Figure 2.10. Library of Disaccharide–dye Conjugates **2.3**–**2.7** Synthesized and Evaluated. (The synthesis of **2.6** and **2.7** were carried out by Dr. Manikandadas Mathilakathu Madathil).[116]

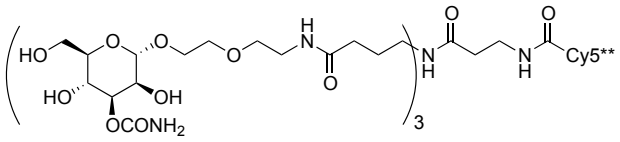
To address the issue of whether the BLM disaccharide, while small and uncomplicated relative to the natural product itself, actually represents the simplest structural entity capable of selective tumor cell targeting, C3-carbamoyl mannose (BLM monosaccharide) conjugated to Cy5** (**2.8)** was also synthesized. Further, to

demonstrate the importance of carbamoyl moiety in selective tumor cell targeting and the effect of trimeric carbohydrate cluster of BLM monosaccharide on cell targeting, mannose (decarbamoyl BLM monosaccharide) conjugated to Cy5** (**2.9**) and a trimer cluster of BLM monosaccharide conjugated to Cy5** (**2.10**) were synthesized (Figure 2.11) and evaluated for their tumor cell targeting ability.



BLM monosaccharide–Cy5**(**2.8**)

2.9



2.10

Figure 2.11. Structures of BLM Monosaccharide–Cy5** (**2.8)**, Decarbamoyl BLM Monosaccharide–Cy5** (**2.9)** and BLM Monosaccharide–Cy5** Trimer (**2.10)** Synthesized and Evaluated.[117]

To further explore the importance of the carbamoyl moiety and its positioning in the monosaccharide subunit, efforts were then extended to synthesize a library of monosaccharides with modifications of the carbamoyl group and alteration of its position on the mannose ring (Figure 2.12).

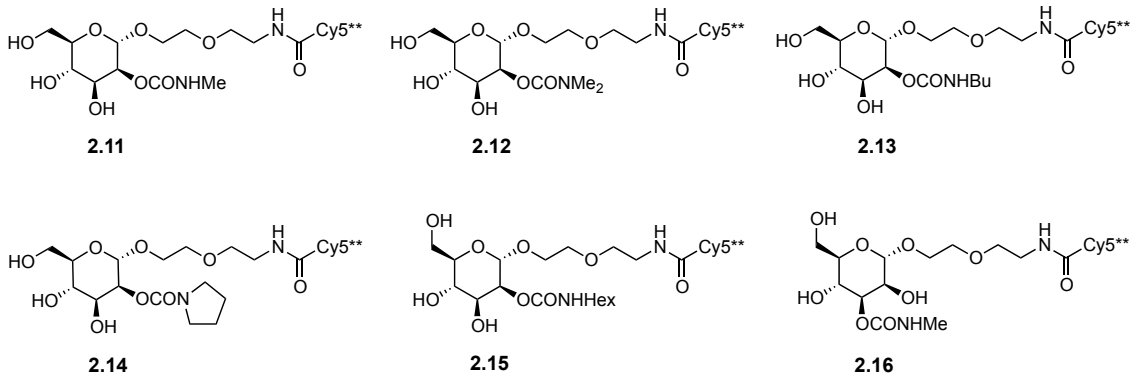


Figure 2.12. Library of Monosaccharide–dye Conjugates **2.11**–**2.16** Synthesized and Evaluated.

Though the reporter dye Cy5** was affording excellent results, the lack of availability of the dye resulted in a search for a replacement dye with similar properties. Hence, a number of commercially available dyes with similar extinction coefficients, and absorption and emission spectra to Cy5** were selected and conjugated with BLM monosaccharide (Figure 2.13) and later with other saccharides (Figure 2.14).

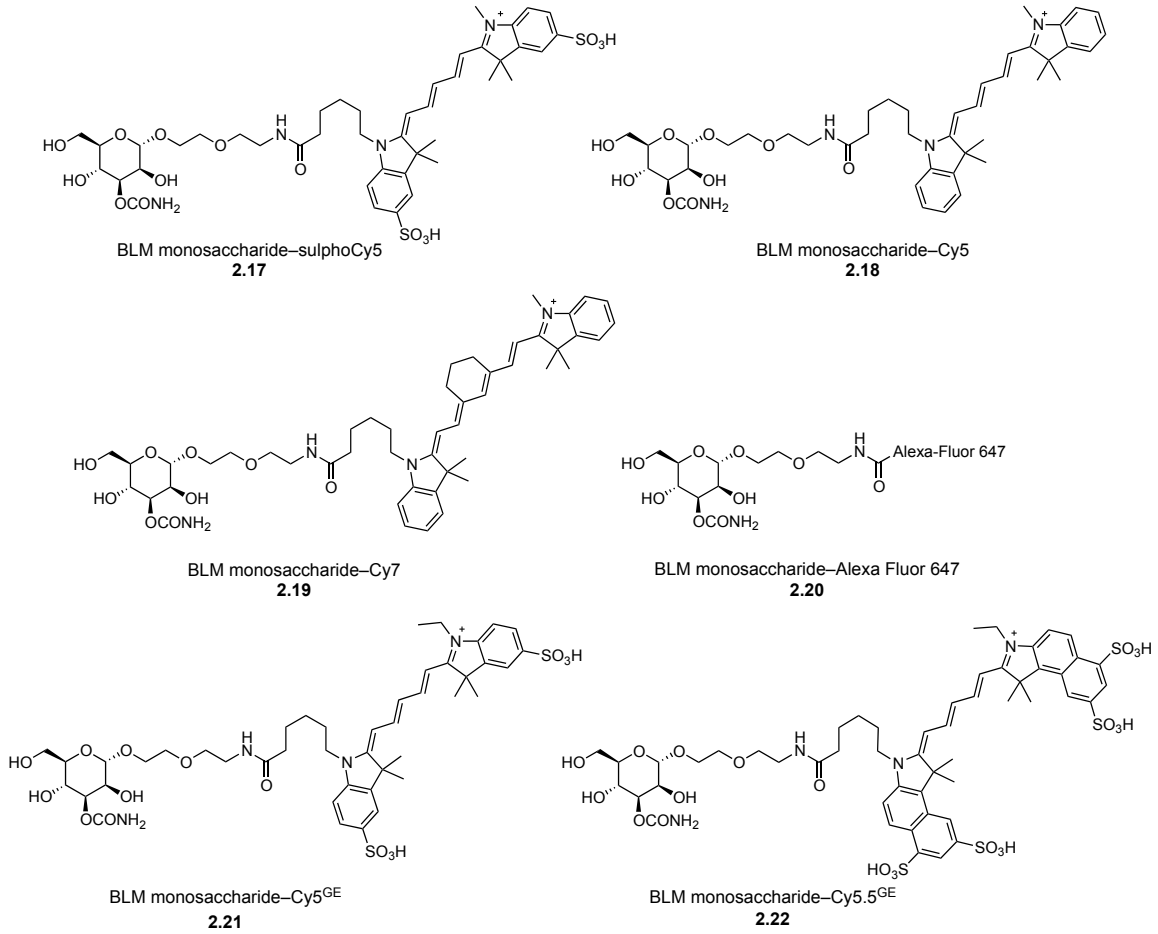


Figure 2.13. Structures of Different Monosaccharide–dye Conjugates **2.17**–**2.22** Synthesized for Selection of a Dye to Replace Cy5**.

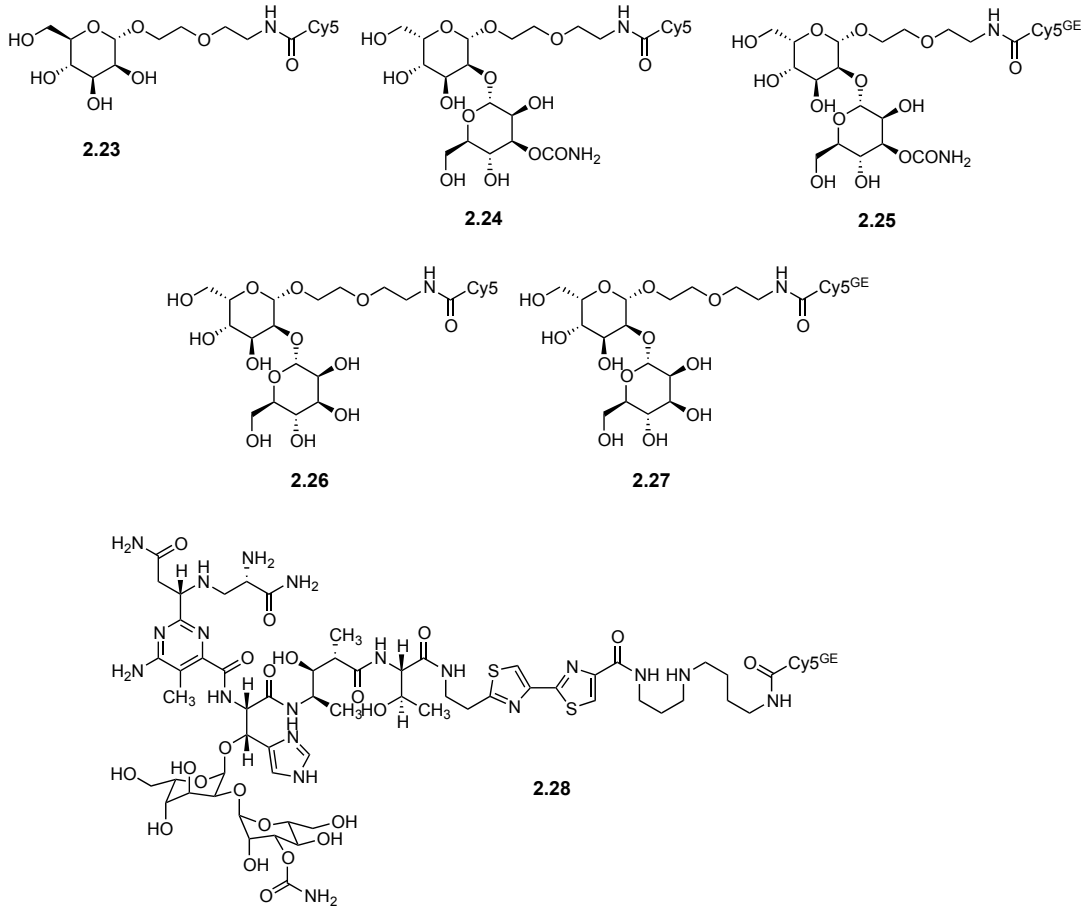


Figure 2.14. Series of Additional Dye Conjugates **2.23**–**2.28** Synthesized and Evaluated.

To explore the modular nature of BLM suggested by diminished cytotoxicity of deglycoBLM in comparison to BLM, different BLM analogues were prepared in which the disaccharide moiety was attached to novel positions of deglycobleomycin, mainly via the C-terminal substituent (**2.29**)[118] and the threonine moiety (**2.31** and **2.32**). Compounds **2.31** and **2.32** were synthesized by Dr. Benjamin R. Schroeder.[119] Later, to study the cellular uptake of the modified BLMs, their dye conjugates **2.30**, **2.33** and **2.34** were synthesized and evaluated (Figures 2.15 and 2.16).

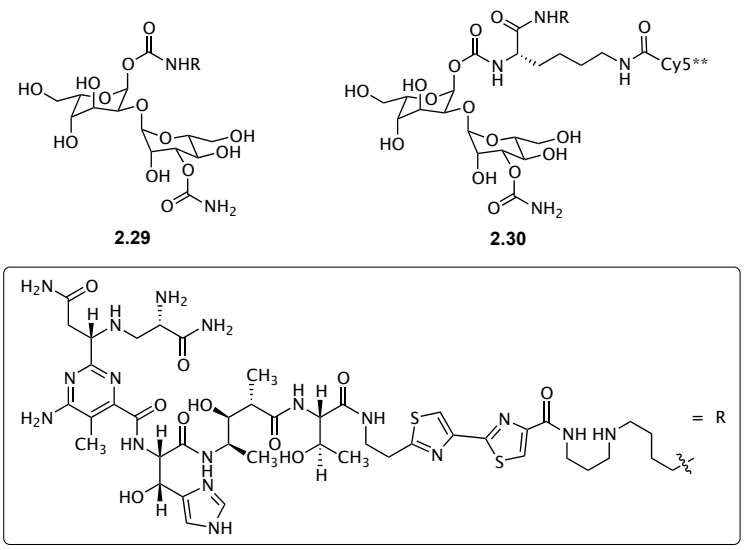


Figure 2.15. Structures of a Modified BLM With a C-terminal Disaccharide **2.29** and its Conjugate With Cy5** **2.30** Synthesized and Evaluated.

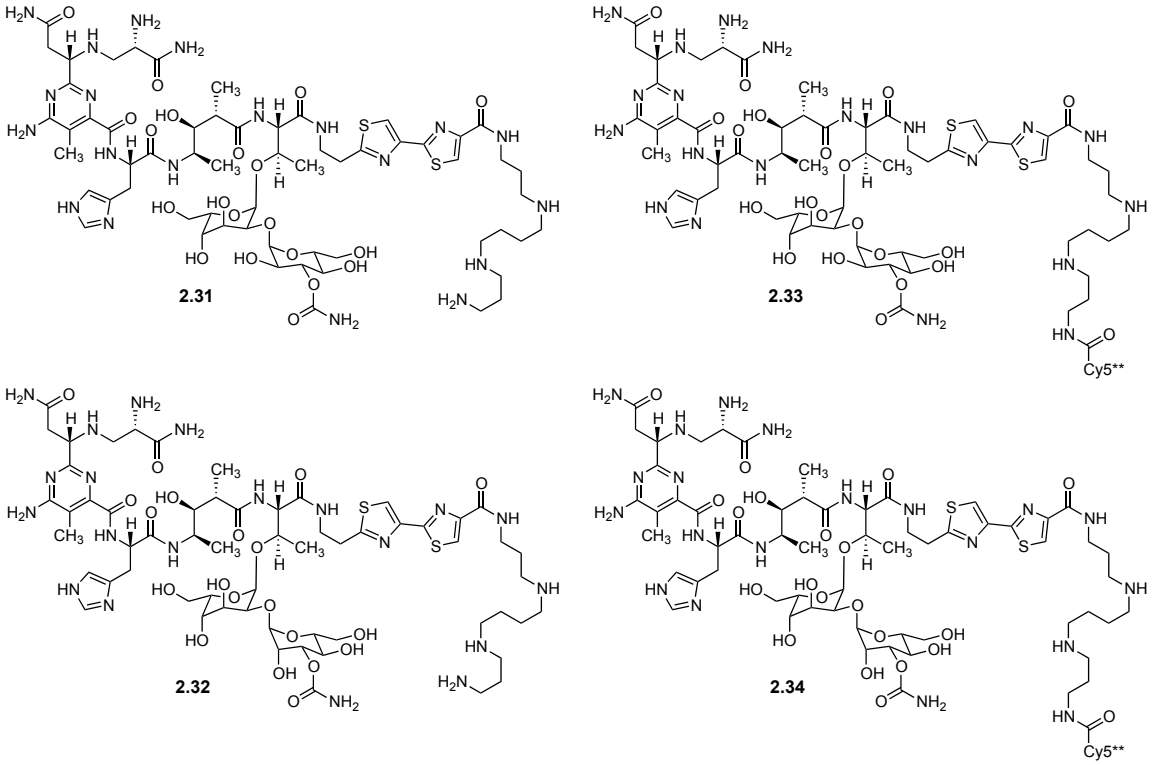


Figure 2.16. Structures of Modified BLMs With the Disaccharide Attached to Threonine Moiety **2.31** and **2.32** (synthesized by Dr. Benjamin R. Schroeder),[119] and Their Fluorescent Dye Conjugates **2.33** and **2.34** Synthesized and Evaluated.

2.1.2. Synthesis of Fluorinated Saccharides for Imaging Studies

In the field of nuclear medicine, the radioisotopically labeled drugs are used for diagnosis of many diseases like cancer.[120] The molecular imaging technique in which compounds labeled with positron emitting radioisotopes are used to produce three-dimensional images of the functional processes in the body is known as PET. The radiolabeled molecules are retained in the tissues and the tomographic images of the biodistribution within the body are generated by detection of the gamma rays.[121] Clinically, ^{18}F-FDG (2-[^{18}F]-fluoro-2-deoxy-D-glucose) has been extensively used for various PET studies. ^{18}F-FDG is a glucose analogue in which C-2 carbon has a fluoride atom in place of the hydroxyl group present in glucose. It undergoes receptor mediated transport and phosphorylation but cannot undergo further metabolism like glucose.[122] The higher uptake in tumor tissues than in normal tissues because of increased glycolysis helps in strong signal intensity in PET imaging (Figure 2.17).[123]

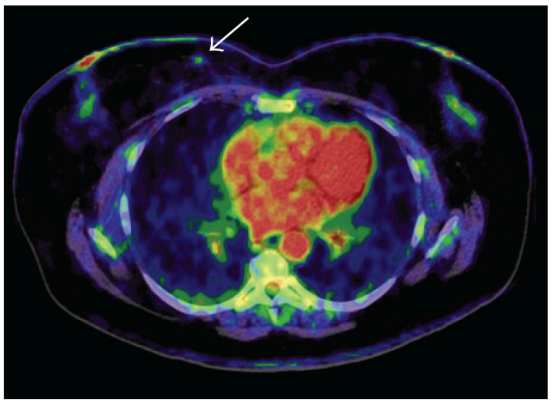


Figure 2.17. PET-CT Image of Breast Ductal Carcinoma Treated With ^{18}F-FDG.[123]

As described earlier, due to the unique ability of BLM disaccharide to target tumors,[18] it represents a potentially attractive PET biomarker enabling possible cancer localization, their biodistribution, *in vivo* quantification of metastatic cells and eventual elimination. Hence, the non-radioactive fluorinated disaccharides were synthesized as non-radioactive reference compounds for disaccharide PET biomarkers (Figure 2.18).

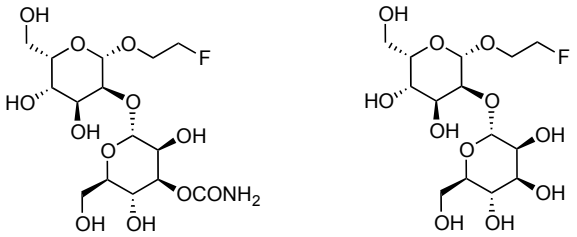


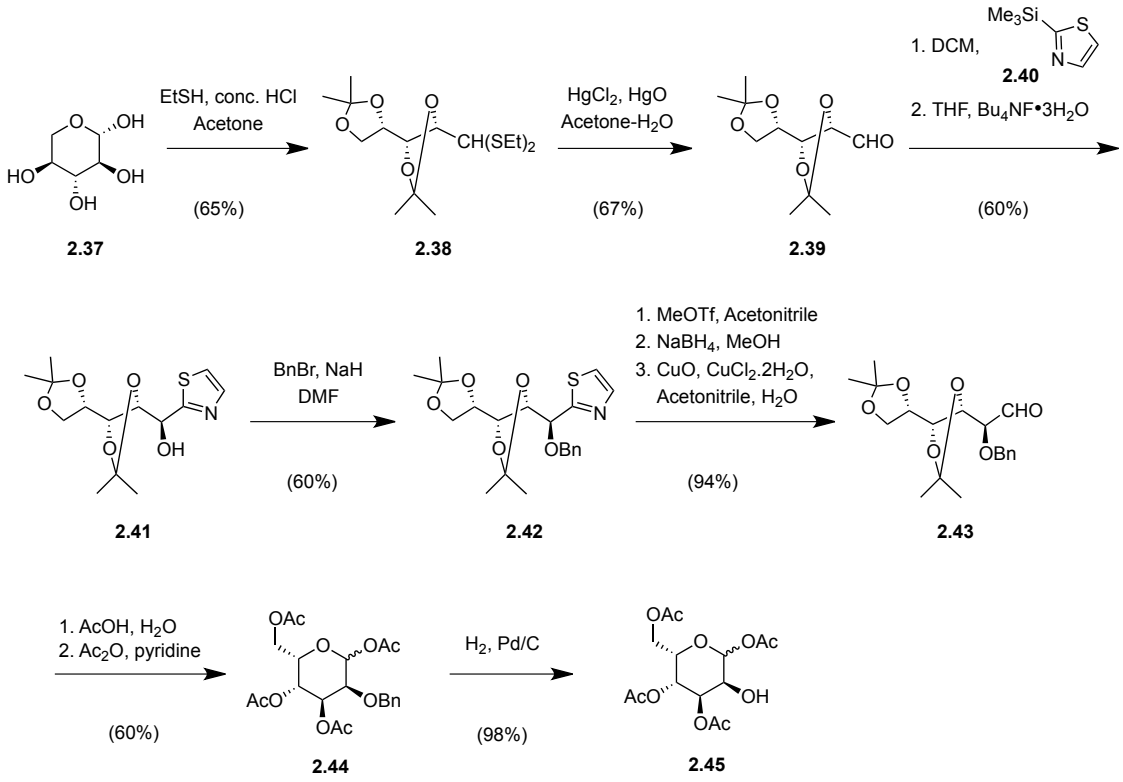
Figure 2.18. Structures of Fluorinated BLM Disaccharide **2.35** and Fluorinated Decarbamoyl BLM Disaccharide **2.36** Synthesized.

2.2. Results

2.2.1. Synthesis of Fluorescent Dye Conjugates

The fluorescent BLM disaccharide–Cy5** conjugate was synthesized from the natural BLM disaccharide according to a reported procedure.[18] The preparation of different disaccharide–dye conjugates began with the synthesis of the corresponding disaccharides attached to a protected amine linker. The syntheses of dye conjugates **2.3**, **2.4** and **2.5** were effected by the coupling of gulose glycosyl acceptor with corresponding mannose donors. The synthesis of gulose glycosyl acceptor was achieved by homologation of commercially available L-xylose following Dondoni's method (Scheme 2.1).[124] L-xylose (**2.37**) was fully protected as dithioacetal **2.38** in 65% yield, the latter of which was selectively hydrolyzed to afford aldehyde **2.39** in the presence of Hg(II) in

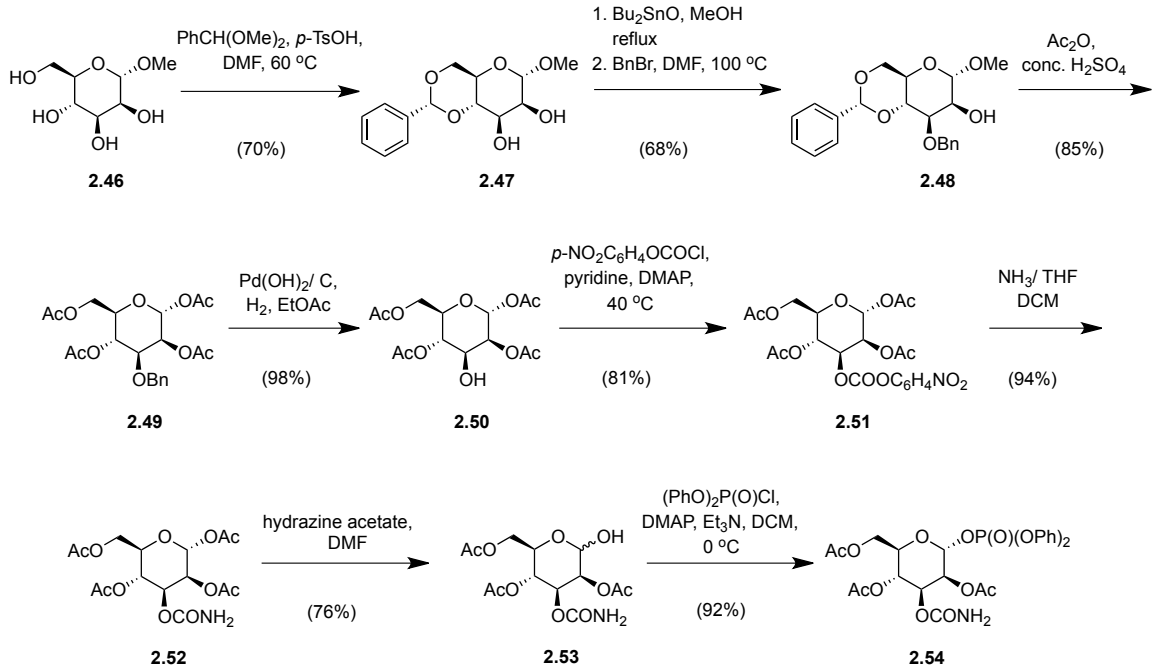
67% yield. Aldehyde **2.39** was immediately coupled with 2-(trimethylsilyl)thiazole (**2.40**)[125] followed by desilylation to afford the alcohol **2.41** in 60% yield. The free hydroxyl group of **2.41** was protected as benzyl ether **2.42** in 60% yield. The thiazole ring of **2.42** was then converted into aldehyde **2.43** in 94% yield through a series of transformations. Acetal deprotection followed by exhaustive acetylation of aldehyde **2.43** afforded tetra-*O*-acetyl-2-*O*-benzyl-L-gulopyranoside (**2.44**) in 60% yield. Reductive debenzylation of gulopyranoside **2.44** gave the gulose acceptor **2.45** in 98% yield.



Scheme 2.1. Synthesis of the Gulose Glycosyl Acceptor **2.45**.

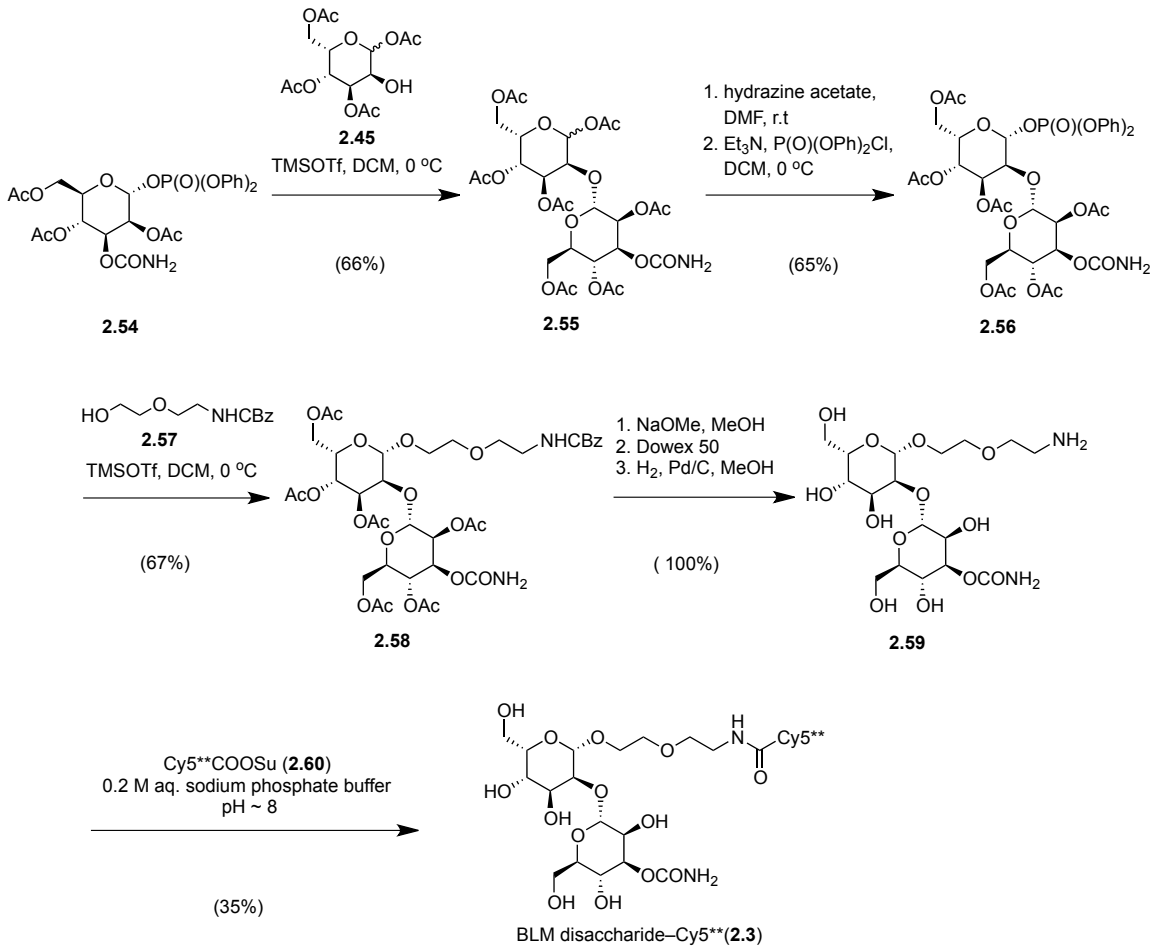
Mannose donor **2.54** was prepared from commercially available α-D-methylmannopyranoside (**2.46**) following reported procedures with slight modifications (Scheme 2.2).[21] The synthesis began with protection of α-D-methylmannopyranoside

(**2.46**) as the benzylidene acetal **2.47** in 70% yield. Selective benzylation of acetal **2.47** at the C-3 position using Bu_2SnO afforded alcohol **2.48** in 68% yield. Deprotection of the acetal and exhaustive acetylation of alcohol **2.48** gave peracetylated mannose **2.49** in 85% yield. Debenzylation using $Pd(OH)_2/C$ (Pearlman's catalyst) yielded **2.50** in 98% yield. C-3 alcohol **2.50** was then activated as the *p*-nitrophenyl carbonate (**2.51**) in 81% yield, the latter of which was subjected to aminolysis with ammonia, yielding carbamate **2.52** in 94% yield. The anomeric acetate of carbamate **2.52** was removed selectively using hydrazine acetate to afford alcohol **2.53** in 76% yield. Alcohol **2.53** was subsequently activated as a diphenyl phosphate ester in presence of DMAP and dry triethylamine to obtain the activated mannose phosphate **2.54** in 92% yield.[126]



Scheme 2.2. Synthesis of the Activated C-3-carbamoylmannose Glycosyl Donor **2.54**.

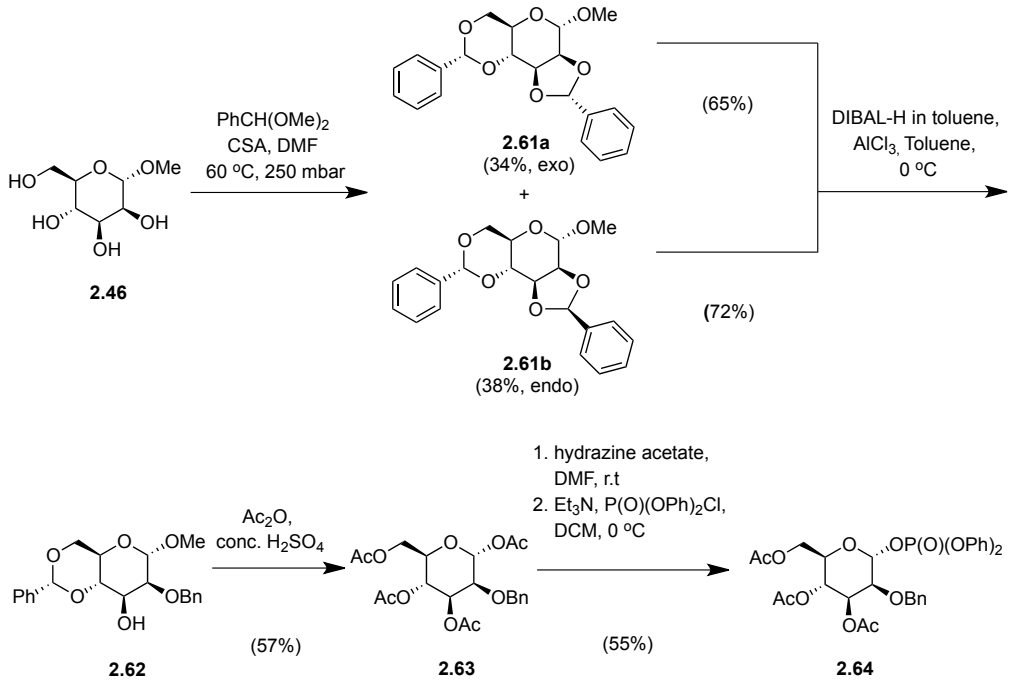
Activated mannose donor **2.54** was coupled with the gulose acceptor **2.45** in the presence of TMSOTf to afford peracetylated BLM disaccharide **2.55** in 66% yield (Scheme 2.3).[21] The anomeric acetate of disaccharide **2.55** was selectively cleaved using hydrazine acetate to afford the corresponding alcohol, the latter of which was activated as a diphenyl phosphate ester **2.56** in presence of DMAP and dry triethylamine in 65% yield over two steps. Activated phosphate ester **2.56** was then coupled to a commercially available linker that had been protected as the benzyloxycarbonyl (CBz) derivative **2.57** to afford BLM disaccharide–linker **2.58** in 67% yield. Deacetylation of the acetates followed by debenzylation afforded the primary amine **2.59** (quantitative yield), the latter of which was conjugated with the *N*-hydroxysuccinimide (NHS) ester of Cy5** (**2.60**)[127] to provide the BLM disaccharide–Cy5** conjugate (**2.3**) in 35% yield.[18]



Scheme 2.3. Synthesis of the BLM Disaccharide–Cy5** (**2.3**).

Utilization of a similar coupling strategy of activated C-2 carbamoylmannose with gulose acceptor **2.45** resulted in unwanted side products. This was due to the presence of the carbamoyl group in close proximity to the anomeric carbon. To address this issue, the carbamoyl group was introduced after formation of the disaccharide. The route employed for the synthesis of C-2 carbamoylmannose modified disaccharide–Cy5** **2.4** is illustrated in Scheme 2.4 and Scheme 2.5. Accordingly, the synthesis of C-2 modified mannose donor **2.64** began with formation of dibenzylidene acetal **2.61** in two isomeric forms, exo and endo, in 34% and 38% yields, respectively (Scheme 2.4).[128] Selective

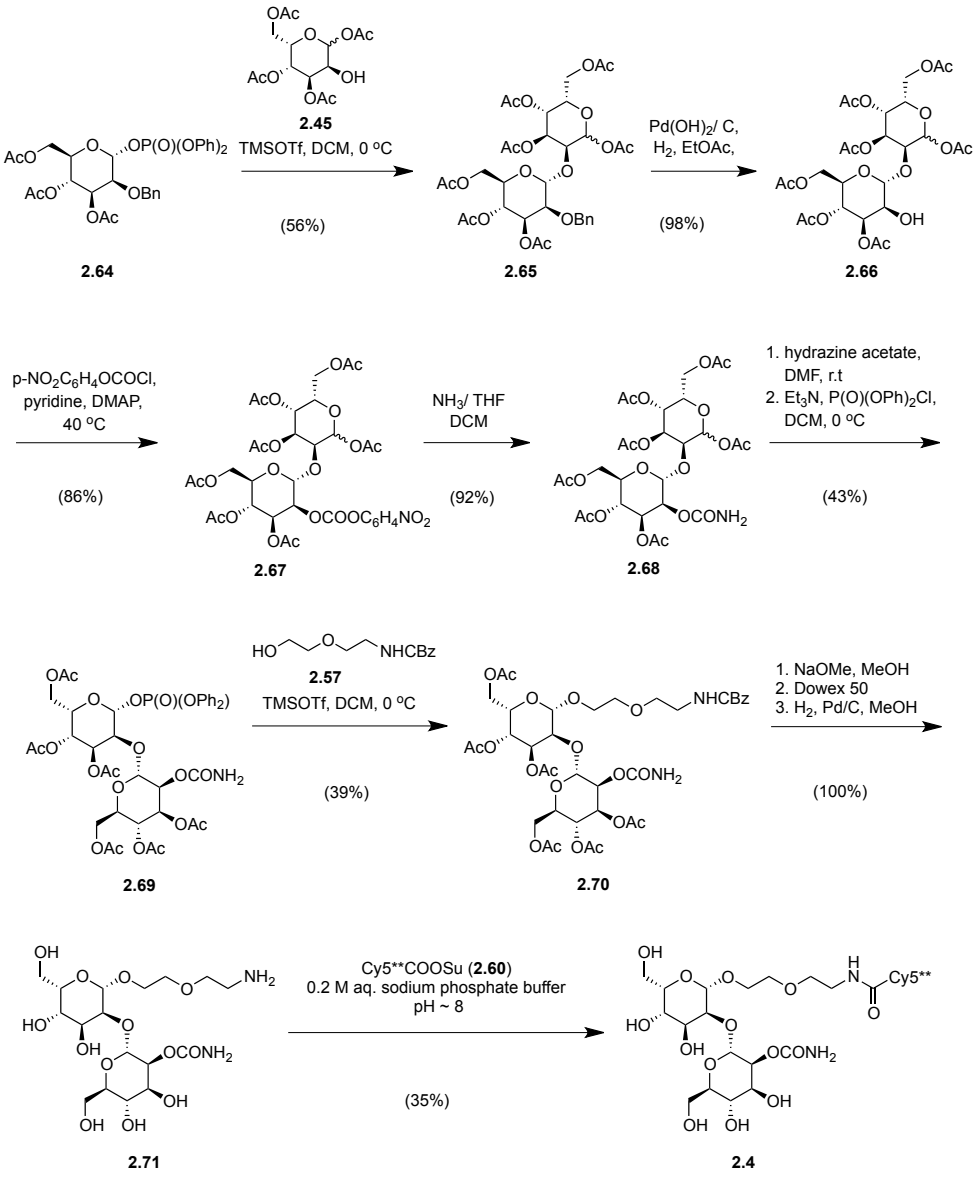
reduction of the acetal at the C-2 and C-3 positions of both exo and endo isomers of acetal **2.61** with DIBAL-H in presence of 5% aluminum chloride afforded alcohol **2.62** in 65% and 72% yield, respectively.[129] Deprotection of the acetal followed by exhaustive acetylation of alcohol **2.62** gave peracetylated mannose **2.63** in 57% yield. The anomeric acetate of mannose **2.63** was selectively cleaved by hydrazine acetate to afford the alcohol, the latter of which was subsequently activated as a diphenyl phosphate ester in the presence of DMAP and dry triethylamine to obtain the activated mannose phosphate **2.64** in 55% yield over two steps.



Scheme 2.4. Synthesis of the Activated C-2-*O*-benzylmannose Glycosyl Donor **2.64**.

Coupling of activated mannose donor **2.64** with the gulose acceptor **2.45** in the presence of TMSOTf afforded disaccharide **2.65** in 56% yield (Scheme 2.5). Debenzylation using $Pd(OH)_2/C$ (Pearlman's catalyst) yielded alcohol **2.66** in 98% yield.

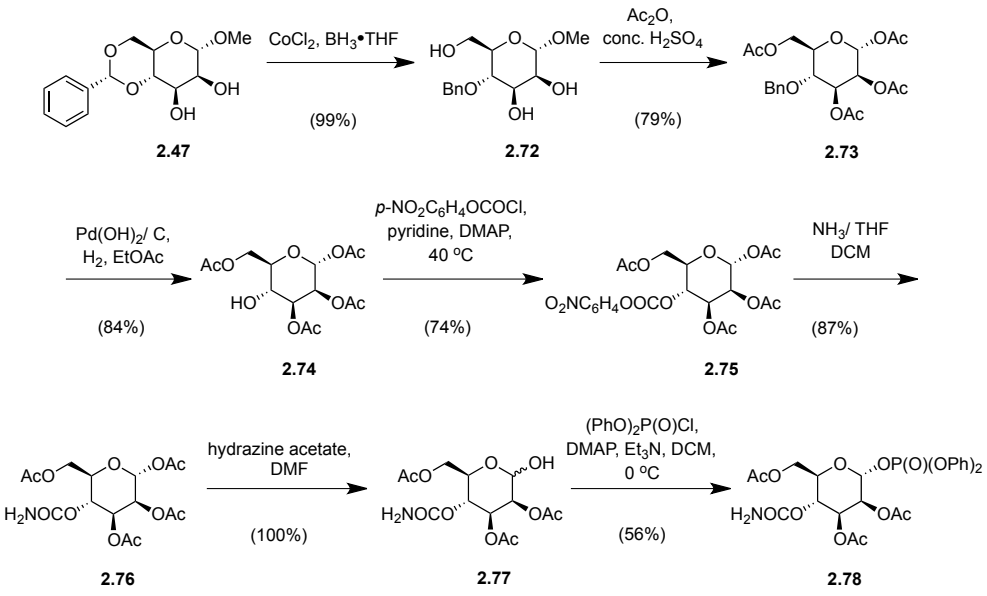
C-2 alcohol **2.66** was then activated as the *p*-nitrophenyl carbonate (**2.67**) in 86% yield, the latter of which was subjected to aminolysis with ammonia yielding carbamate **2.68** with 92% yield. The anomeric acetate of disaccharide **2.68** was selectively cleaved by hydrazine acetate to afford the corresponding alcohol, the latter of which was activated as a diphenyl phosphate ester **2.69** in the presence of DMAP and dry triethylamine in 43% yield over two steps. Activated phosphate ester **2.69** was then coupled to the linker protected as the benzyloxycarbonyl (CBz) derivative **2.57**[18] to afford BLM disaccharide–linker **2.70** in 39% yield. Deacetylation of the acetates followed by debenzylation gave the primary amine **2.71** (quantitative yield), the latter of which was treated with the NHS ester of Cy5** (**2.60**)[127] to provide the modified disaccharide–Cy5** conjugate **2.4** in 35% yield.



Scheme 2.5. Synthesis of the C-2-carbamoylmannose Modified Disaccharide–Cy5** **2.4**.

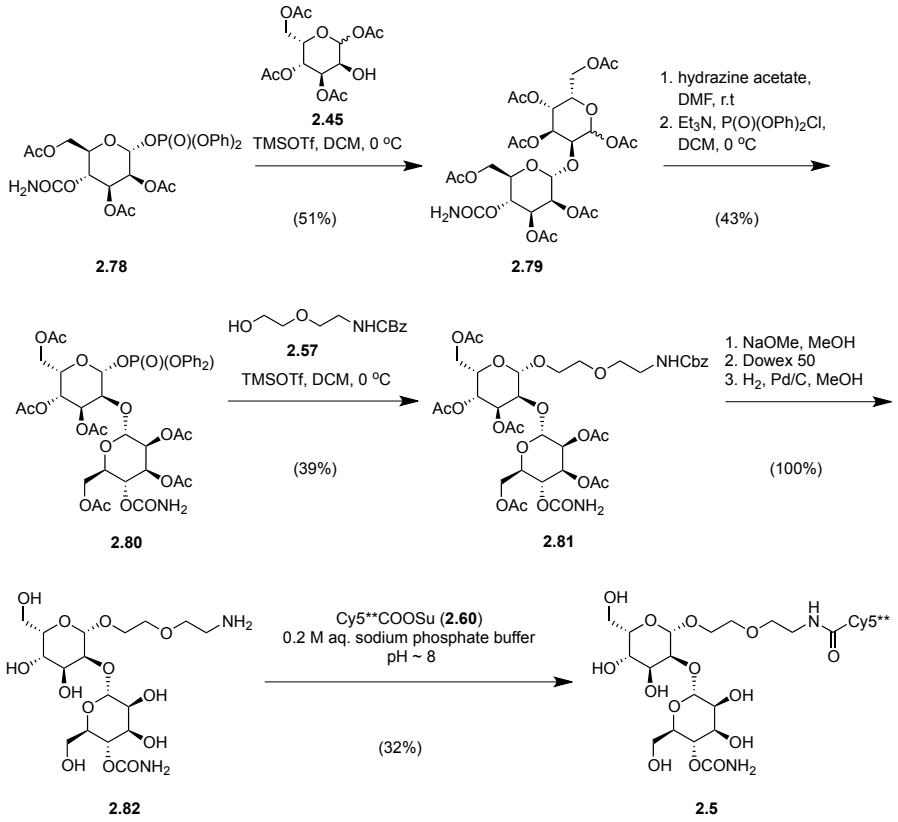
The route employed for the synthesis of C-4 carbamoylmannose modified disaccharide–Cy5** **2.5** was similar to that used for the natural disaccharide–Cy5** conjugate. The synthesis of C-4 carbamoylmannose modified disaccharide–Cy5** was achieved by coupling of activated C-4 carbamoylmannose donor **2.78** with gulose

acceptor **2.45**. The synthesis began with regioselective reductive ring opening of acetal **2.47** in presence of $CoCl_2$ and BH_3•THF to afford alcohol **2.72** in 99% yield (Scheme 2.6).[130] Exhaustive acetylation of alcohol **2.72** in acidic medium gave peracetylated mannose **2.73** in 79% yield. Debenzylation using $Pd(OH)_2/C$ (Pearlman's catalyst) yielded alcohol **2.74** in 84% yield. C-4 alcohol **2.74** was then activated as the *p*-nitrophenyl carbonate (**2.75**) in 74% yield, the latter of which was subjected to aminolysis with ammonia yielding carbamate **2.76** in 87% yield. The anomeric acetate of carbamate **2.76** was removed selectively using hydrazine acetate to afford alcohol **2.77** in 100% yield, the latter of which was subsequently activated as a diphenyl phosphate ester in the presence of DMAP and dry triethylamine to obtain the activated mannose phosphate **2.78** in 56% yield.



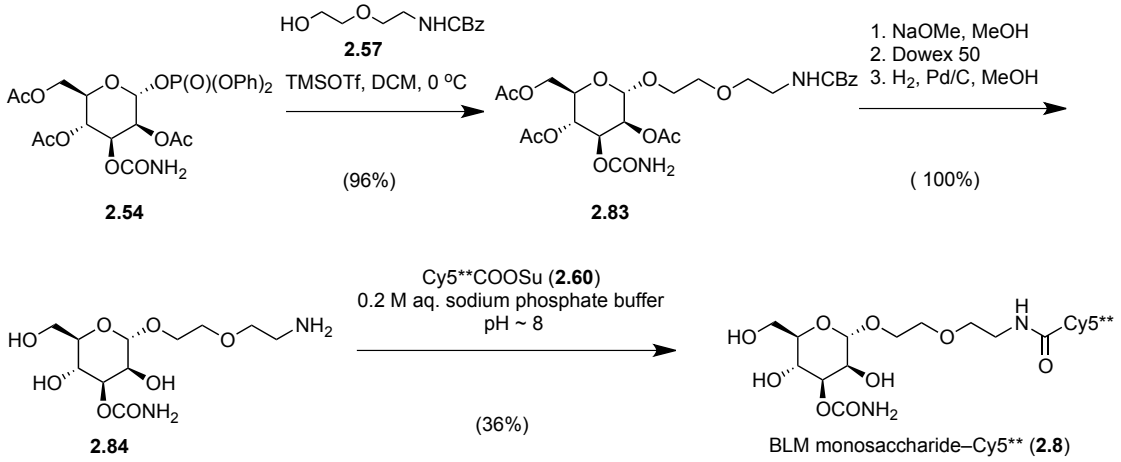
Scheme 2.6. Synthesis of the Activated C-4-carbamoylmannose Glycosyl Donor **2.78**.

Activated mannose donor **2.78** was coupled with gulose acceptor **2.45** in the presence of TMSOTf to afford disaccharide **2.79** in 51% yield (Scheme 2.7). Selective removal of the anomeric acetate of disaccharide **2.79** followed by activation as a diphenyl phosphate ester in the presence of DMAP and dry triethylamine afforded glycosyl donor **2.80** in 43% yield over two steps. Activated glycosyl phosphate **2.80** was then coupled to the linker protected as a benzyloxycarbonyl (CBz) derivative **2.57**[18] to afford disaccharide–linker **2.81** in 39% yield. Deacetylation of the acetates followed by debenzylation gave primary amine **2.82** (quantitative yield), the latter of which was subsequently conjugated with the NHS ester of Cy5** (**2.60**)[127] to afford the modified disaccharide–Cy5** conjugate **2.5** in 32% yield.



Scheme 2.7. Synthesis of the C-4-carbamoylmannose Modified Disaccharide–Cy5** **2.5**.

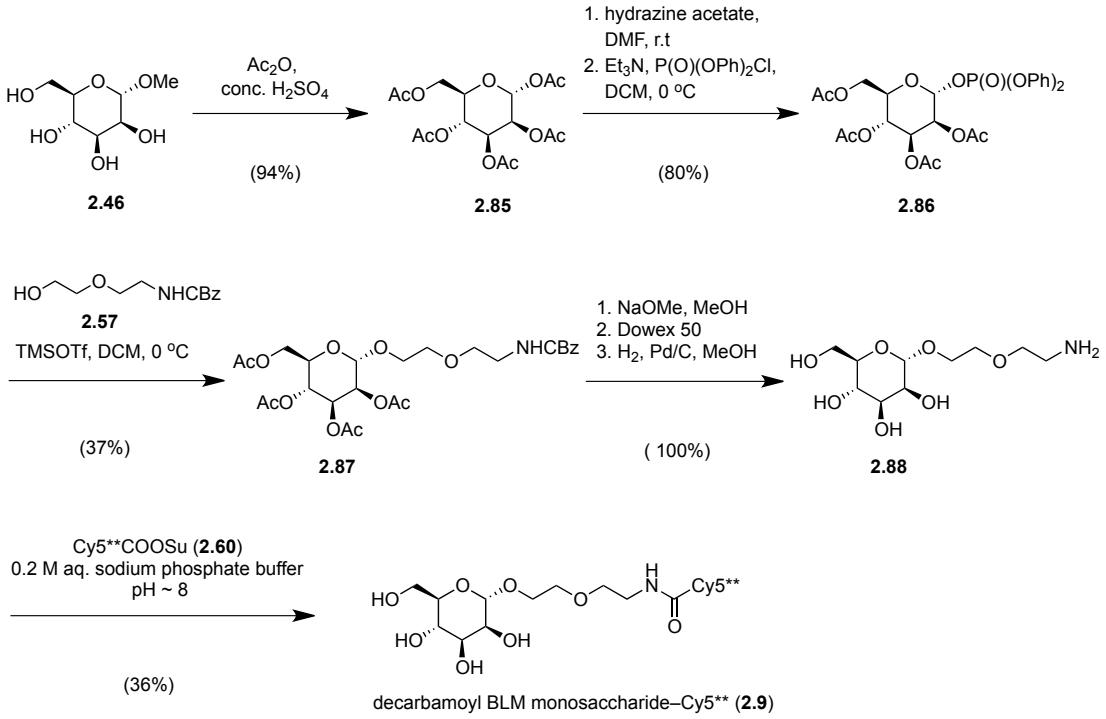
As a part of our ongoing investigation of whether the BLM disaccharide was actually the simplest structural entity conferring the tumor targeting ability of BLM, BLM monosaccharide–Cy5** (**2.8**) was synthesized. Mannose glycosyl donor **2.54** was coupled to the linker protected as the benzyloxycarbonyl (CBz) derivative **2.57**[18] to afford BLM monosaccharide–linker **2.83** in 96% yield (Scheme 2.8). Deacetylation of the acetates followed by debenzylation of the primary amine of **2.83** gave amine **2.84** (quantitative yield), the latter of which was treated with the NHS ester of Cy5** (**2.60**)[127] to give the BLM monosaccharide–Cy5** (**2.8**) in 36% yield.



Scheme 2.8. Synthesis of the BLM Monosaccharide–Cy5** (**2.8**).

To demonstrate that the carbamoyl moiety is absolutely necessary for selective tumor cell targeting, the decarbamoyl BLM monosaccharide–Cy5** (**2.9**) lacking the carbamoyl group at C-3 of the mannose residue was synthesized. Peracetylation of α-D-methylmannopyranoside (**2.46**) gave mannose derivative **2.85** in 94% yield (Scheme 2.9).[131] The anomeric acetate of mannose **2.85** was selectively cleaved by treatment with hydrazine acetate to afford the corresponding alcohol, the latter of which was

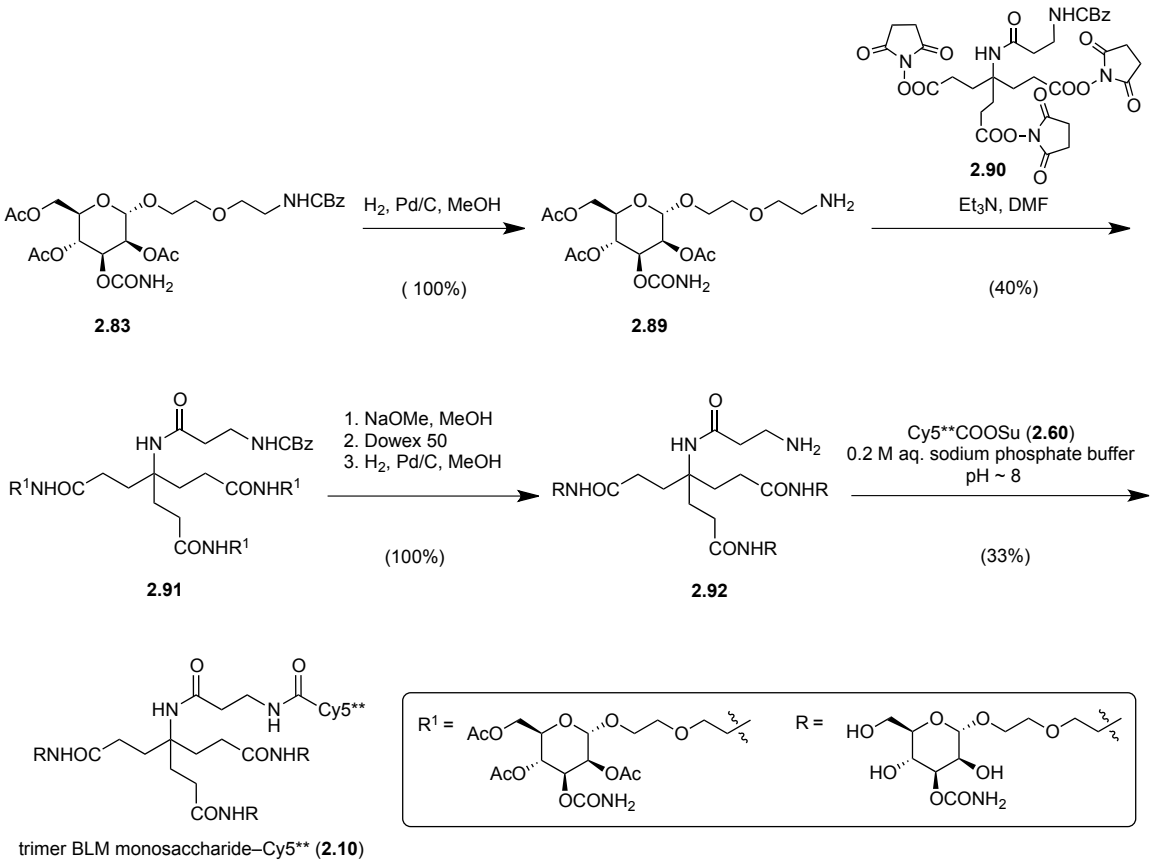
subsequently activated as a diphenyl phosphate ester in the presence of DMAP and dry triethylamine to obtain the activated mannose phosphate **2.86** in 80% yield over two steps.[132] Mannose glycosyl donor **2.86** was coupled with the linker protected as the benzyloxycarbonyl (CBz) derivative **2.57**[18] to afford decarbamoyl BLM monosaccharide–linker **2.87** in 37% yield. Deacetylation followed by debenzylation of the primary amine of **2.87** gave amine **2.88** (quantitative yield), the latter of which was then conjugated with the *N*-hydroxysuccinimide (NHS) ester of Cy5** (**2.60**)[127] to give decarbamoyl BLM monosaccharide–Cy5** (**2.9**) in 36% yield.



Scheme 2.9. Synthesis of Decarbamoyl BLM Monosaccharide–Cy5** (**2.9**).

To study the nature of the receptor that might be responsible for the process of internalization, the dye-labeled trimeric carbohydrate cluster **2.10** was prepared.

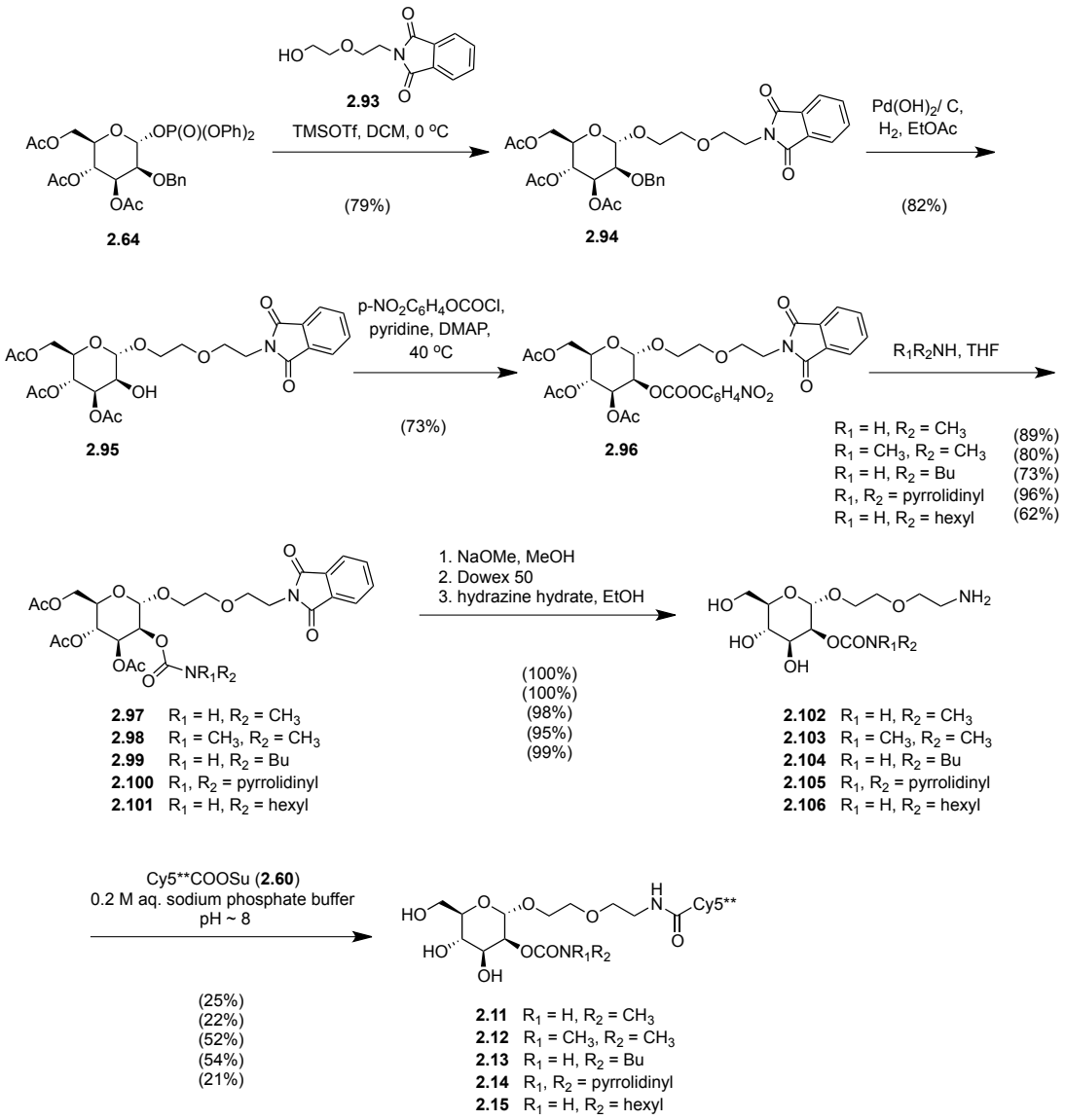
The synthesis commenced with debenzylation of the primary amine of **2.83** to give amine **2.89** in 100% yield. Compound **2.89** was conjugated to the NHS ester of protected trimer–linker **2.90**[118] to afford BLM monosaccharide trimer linker **2.91** in 40% yield (Scheme 2.10). Complete deacetylation followed by debenzylation of the primary amine of **2.91** gave trimer cluster linker amine **2.92** quantitatively. Coupling of trimer linker amine **2.92** with the NHS ester of Cy5** (**2.60**)[127] provided the BLM monosaccharide–Cy5** trimer conjugate **2.10** in 33% yield.



Scheme 2.10. Synthesis of the BLM Monosaccharide–Cy5** Trimer (**2.10**).

In an effort to improve the targeting ability of the natural monosaccharide moiety of the bleomycin, a number of modified monosaccharide–Cy5** conjugates were

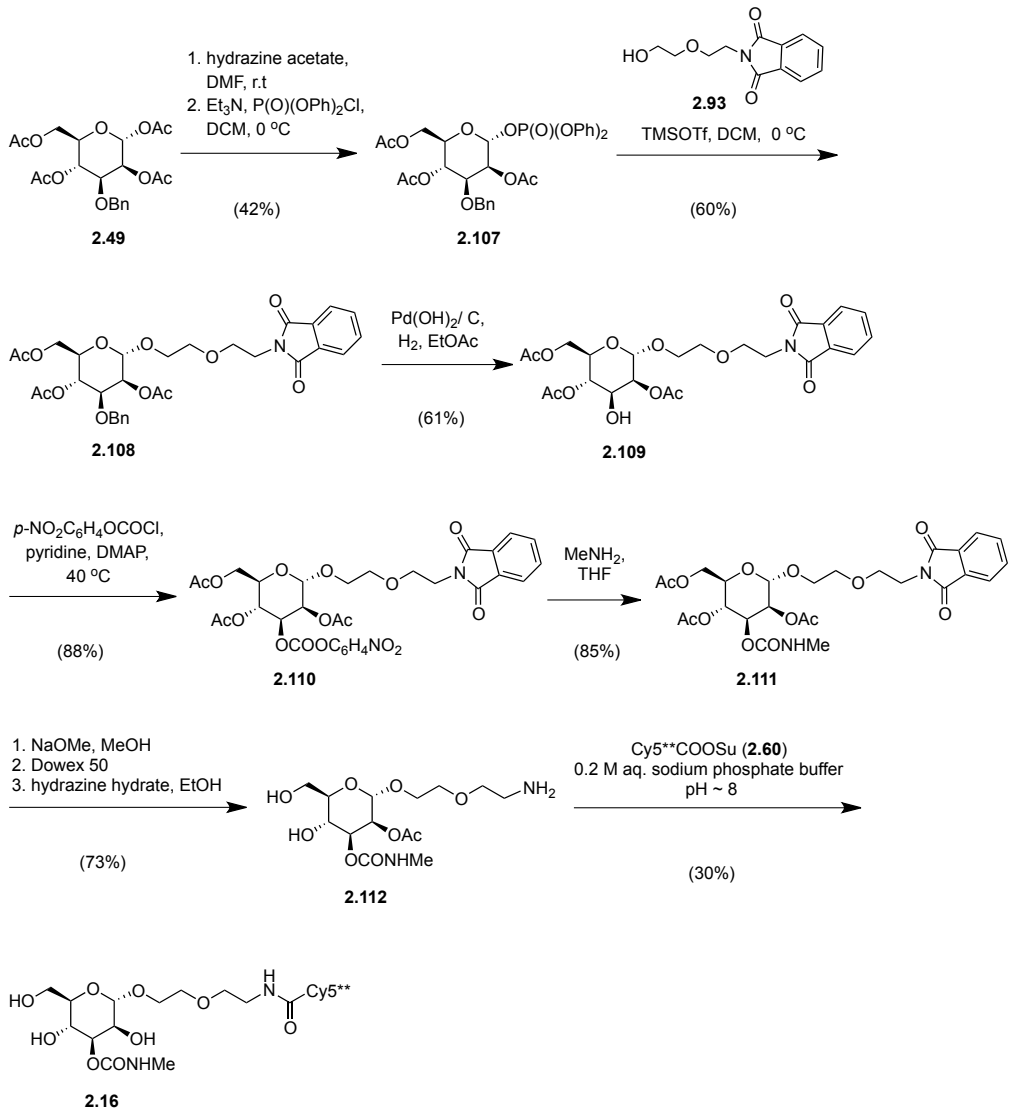
synthesized. The synthesis of C-2 modified monosaccharide–Cy5** began with the coupling of mannose donor **2.64** with the linker protected as the phthalimide derivative **2.93**[133] to afford monosaccharide–linker **2.94** in 79% yield (Scheme 2.11). Debenzylation using $Pd(OH)_2/C$ (Pearlman's catalyst) yielded alcohol **2.95** in 82% yield. C-2 alcohol **2.95** was then activated as the *p*-nitrophenyl carbonate (**2.96**) in 73% yield. Subsequently, carbonate **2.96** was subjected to aminolysis with methylamine, dimethylamine, butylamine, pyrrolidine and hexylamine to afford C-2 methylcarbamate **2.97** in 89% yield, C-2 dimethylcarbamate **2.98** in 80% yield, C-2 butylcarbamate **2.99** in 73% yield, C-2 pyrrolidinylcarbamate **2.100** in 96% yield and C-2 hexylcarbamate **2.101** in 62% yield. Deacetylation of the acetates followed by deprotection of the phthalimide group using hydrazine hydrate afforded amine–linkers **2.102**, **2.103**, **2.104**, **2.105** and **2.106** in 100%, 100%, 98%, 95% and 99% yields, respectively. Amines **2.102**, **2.103**, **2.104**, **2.105** and **2.106** were conjugated with NHS ester of Cy5** (**2.60**)[127] to give modified monosaccharide–Cy5** conjugates **2.11**, **2.12**, **2.13**, **2.14** and **2.15** in 25%, 22%, 52%, 54% and 21% yields, respectively.



Scheme 2.11. Synthesis of the C-2-carbamoyl Modified Monosaccharide–Cy5** **2.11**–**2.15**.

The synthesis of C-3 modified monosaccharide–Cy5** started with the selective removal of the anomeric acetate of mannose **2.49** using hydrazine acetate to afford the corresponding alcohol, the latter of which was subsequently activated as a diphenyl phosphate ester in the presence of DMAP and dry triethylamine to obtain the activated

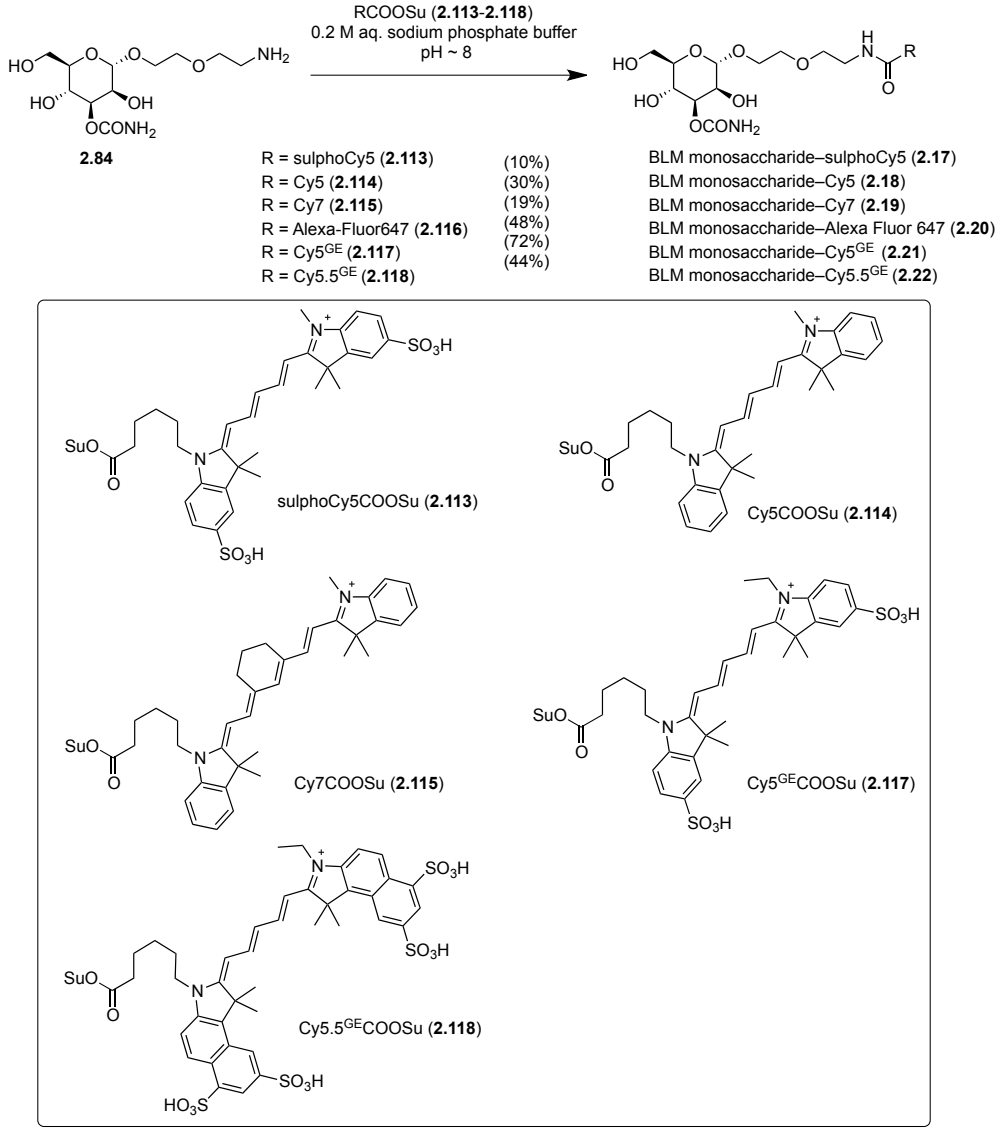
mannose phosphate **2.107** in 42% yield over two steps. Mannose donor **2.107** was then coupled with the linker protected as the phthalimide derivative **2.93**[133] to afford monosaccharide–linker **2.108** in 60% yield (Scheme 2.12). Debenzylation using $Pd(OH)_2/C$ (Pearlman's catalyst) yielded the alcohol **2.109** in 61% conversion, the latter of which was activated as the *p*-nitrophenyl carbonate (**2.110**) in 88% yield. Carbonate **2.110** was subjected to aminolysis with methylamine to afford C-2 methylcarbamate **2.111** in 85% yield. Deacetylation followed by deprotection of the phthalimide group using hydrazine hydrate afforded amine–linker **2.112** in 73% yield, the latter of which was conjugated with the NHS ester of Cy5** (**2.60**)[127] to give modified monosaccharide–Cy5** conjugate **2.16** in 30% yield.



Scheme 2.12. Synthesis of the C-3-methylcarbamoyl Modified Monosaccharide–Cy5** **2.16**.

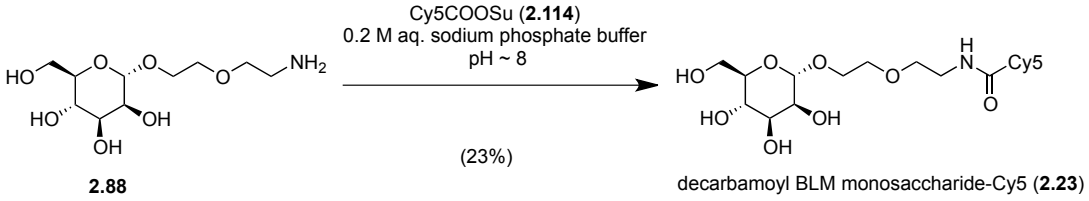
In search of a new dye to replace Cy5** due to its restricted availability, several dyes with similar spectral properties were selected and coupled with the free amine of BLM monosaccharide–linker **2.84** (Scheme 2.13). Conjugation of primary amine **2.84** to the NHS ester of sulphoCy5 (**2.113**), the NHS ester of Cy5 (**2.114**), the NHS ester of Cy7 (**2.115**), the NHS ester of Alexa-Fluor 647 (**2.116**), the NHS ester of $Cy5^{GE}$ (**2.117**) and

the NHS ester of Cy5.5^{GE} (**2.118**) gave BLM monosaccharide–sulphoCy5 conjugate (**2.17**), BLM monosaccharide–Cy5 conjugate (**2.18**), BLM monosaccharide–Cy7 conjugate (**2.19**), BLM monosaccharide–AF647 conjugate (**2.20**), BLM monosaccharide–Cy5^{GE} conjugate (**2.21**) and BLM monosaccharide–Cy5.5^{GE} conjugate (**2.22**) in 10%, 30%, 19%, 48%, 72% and 44% yields, respectively.

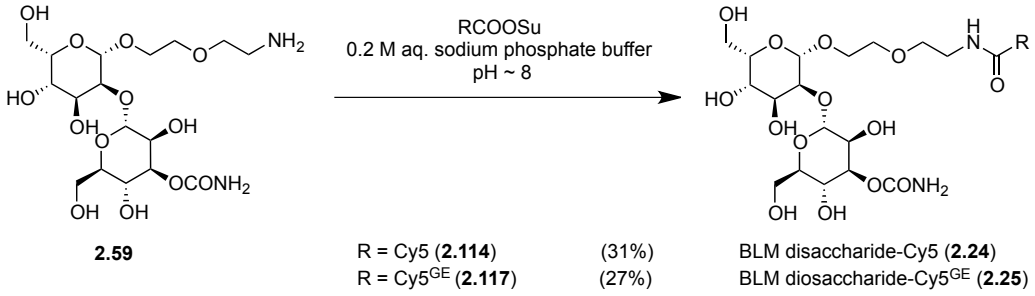


Scheme 2.13. Synthesis of BLM Monosaccharide–dye Conjugates **2.17**–**2.22**.

The primary amine of decarbamoyl BLM monosaccharide–linker **2.88** was also coupled with the NHS ester of Cy5 (**2.114**) to give decarbamoyl BLM monosaccharide–Cy5 conjugate (**2.23**) in 23% yield (Scheme 2.14). Similarly, the primary amine of BLM disaccharide–linker **2.59** was coupled with the NHS ester of Cy5 (**2.114**) and the NHS ester of $Cy5^{GE}$ (**2.117**) to give BLM disaccharide–Cy5 conjugate (**2.24**) and BLM disaccharide–$Cy5^{GE}$ conjugate (**2.25**)[115] in 31% and 27% yields, respectively (Scheme 2.15).



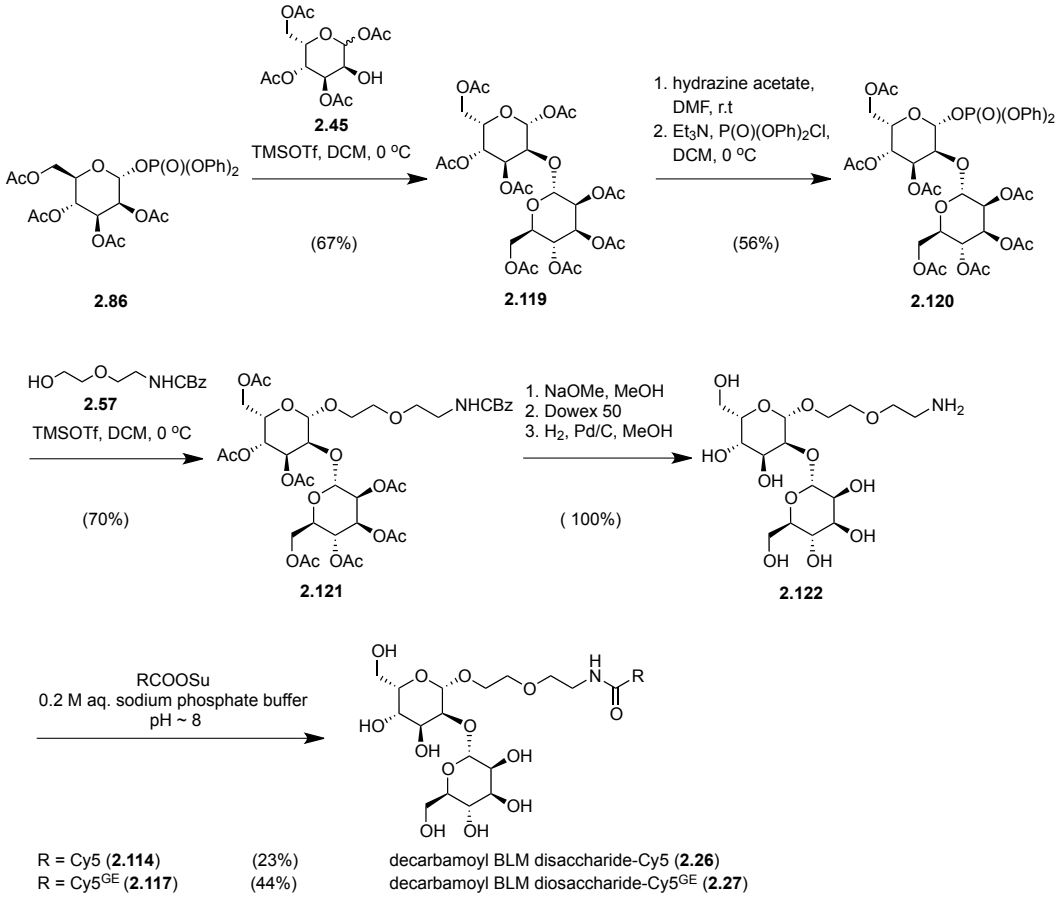
Scheme 2.14. Synthesis of Decarbamoyl BLM Monosaccharide–Cy5 (**2.23**).



Scheme 2.15. Synthesis of BLM Disaccharide–dye Conjugates **2.24** and **2.25**.

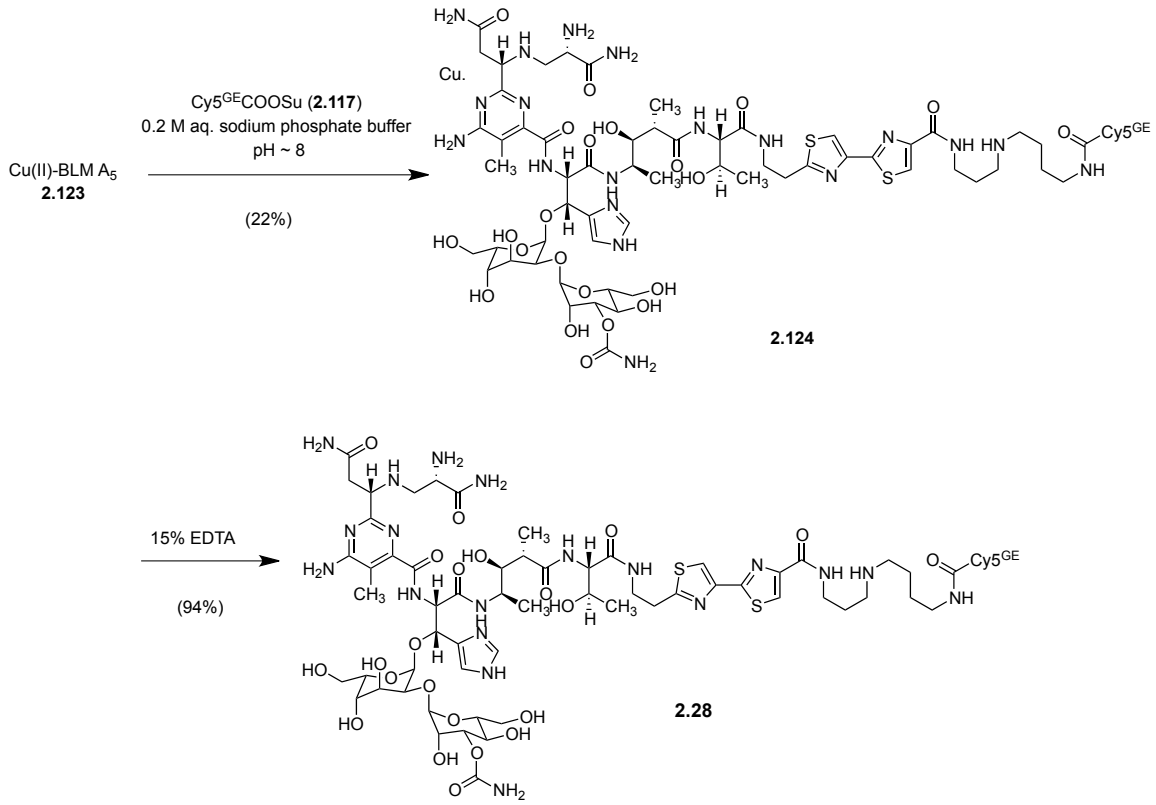
Activated mannose donor **2.86** was coupled with the gulose acceptor **2.45** in the presence of TMSOTf to afford peracetylated disaccharide **2.119** in 67% yield (Scheme 2.16).[118] The anomeric acetate of disaccharide **2.119** was selectively cleaved by hydrazine acetate to afford the corresponding alcohol, the latter of which was activated as diphenyl phosphate ester **2.120** in 56% yield over two steps. Activated glycosyl

phosphate **2.120** was then coupled with the linker protected as the benzyloxycarbonyl (CBz) derivative **2.57**[18] to afford decarbamoyl BLM disaccharide–linker **2.121** in 70% yield.[118] Deacetylation of the acetates followed by debenzylation of the primary amine of **2.121** gave amine **2.122**, the latter of which was coupled with the NHS ester of Cy5 (**2.114**) and the NHS ester of Cy5^{GE} (**2.117**) to give decarbamoyl BLM disaccharide–Cy5 conjugate (**2.26**) and decarbamoyl BLM disaccharide–Cy5^{GE} conjugate (**2.27**) in 23% and 44% yields, respectively.



Scheme 2.16. Synthesis of Decarbamoyl BLM Disaccharide–dye Conjugates **2.26** and **2.27**.

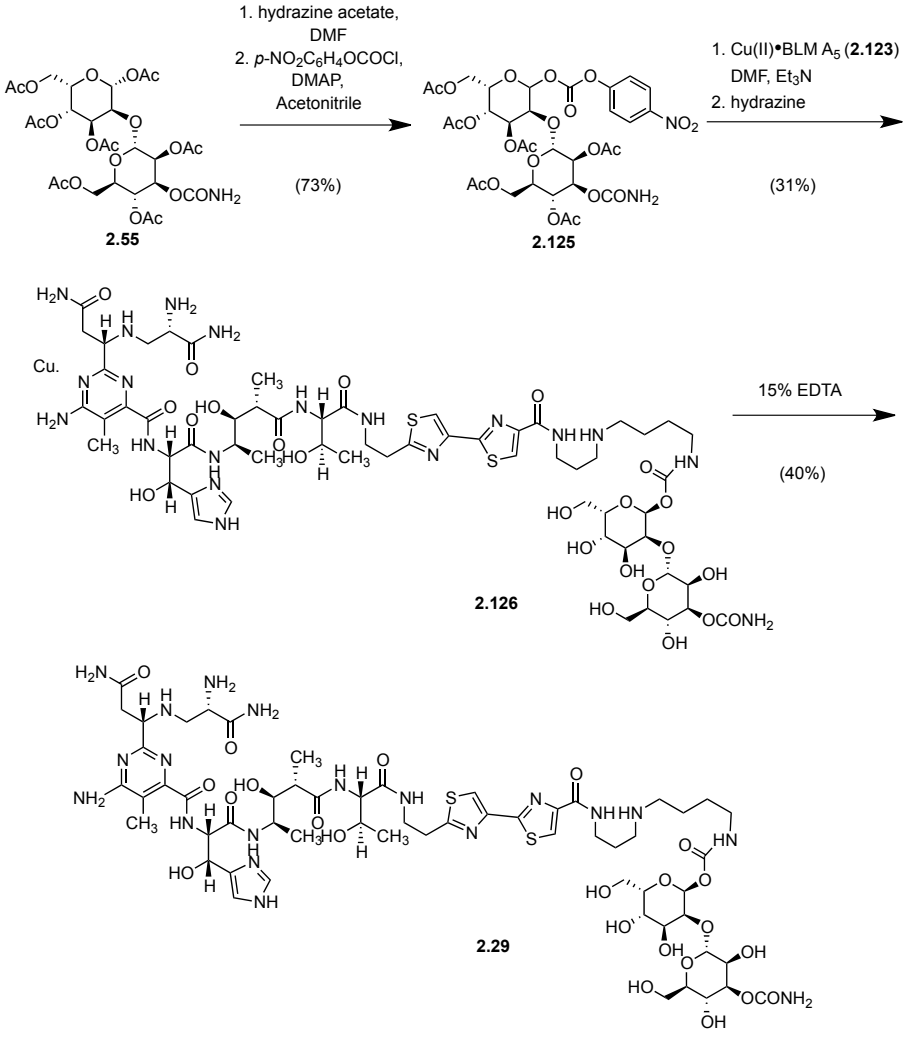
The primary amine in the C-substituent of Cu(II)•BLM A_5 (**2.123**) was coupled with the NHS ester of $Cy5^{GE}$ (**2.117**) to give Cu(II)•BLM–$Cy5^{GE}$ conjugate **2.124** in 22% yield (Scheme 2.17), the latter of which was demetallated using 15% EDTA to afford BLM–$Cy5^{GE}$ (**2.28**) in 94% yield.[115]



Scheme 2.17. Synthesis of BLM–$Cy5^{GE}$ (**2.28**).

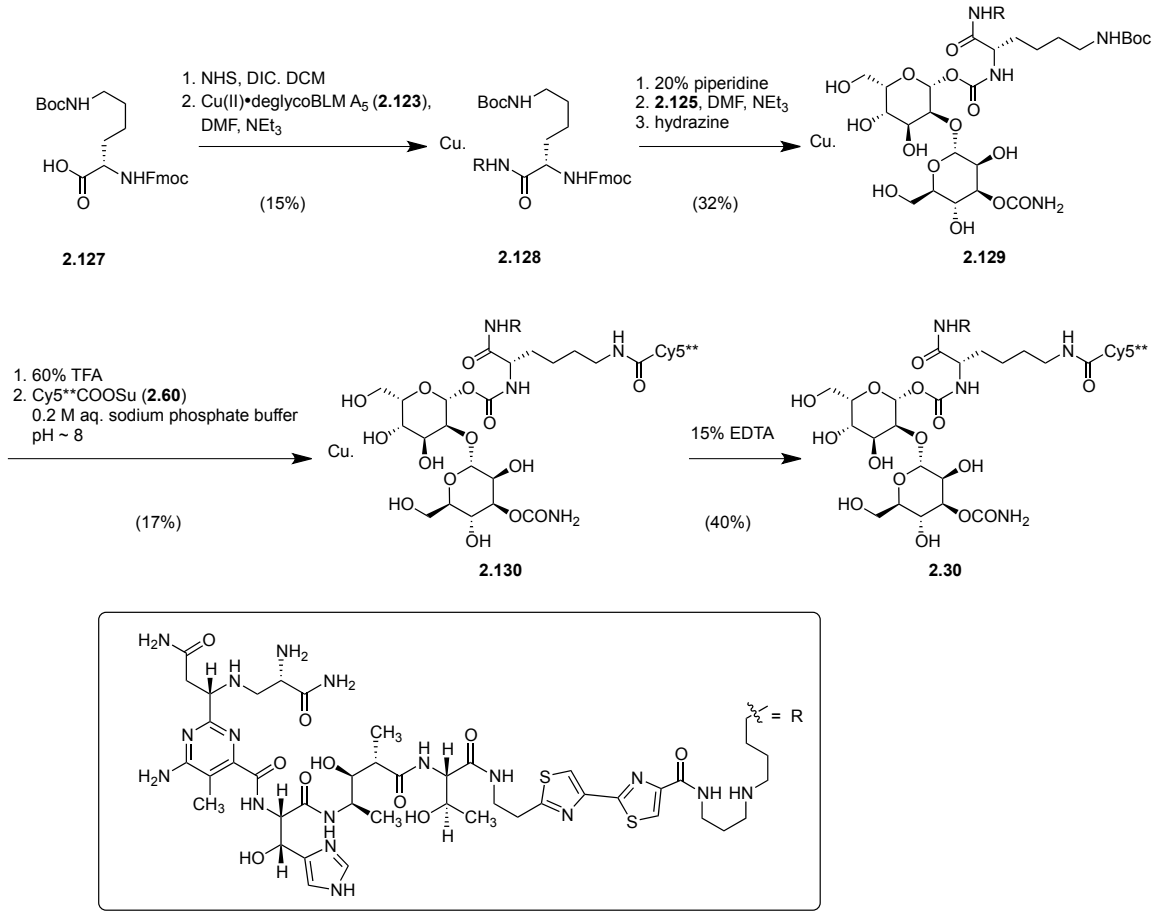
To gain improved insight into the nature of BLM and the role of the BLM disaccharide moiety in cell targeting, different BLM analogues (**2.29**, **2.31** and **2.32**) were prepared as probes. BLM analogues **2.31** and **2.32** were synthesized by Dr. Benjamin R. Schroeder.[119] The bleomycin analogue **2.29** was prepared following previously reported procedures (Scheme 2.18).[118] The synthesis began with the selective deacetylation of the anomeric acetate of disaccharide **2.55** using hydrazine acetate followed by activation as

the *p*-nitrophenyl carbonate to give carbamate **2.125** in 73% yield. The carbonate was then coupled with Cu(II)•deglycoBLM (**2.123**). The resulting intermediate was deprotected by the use of hydrazine to afford modified Cu(II)•BLM **2.126** in 31% yield.[118] The latter was then subjected to demetallation with 15% EDTA to afford modified BLM **2.29** in 40% yield.



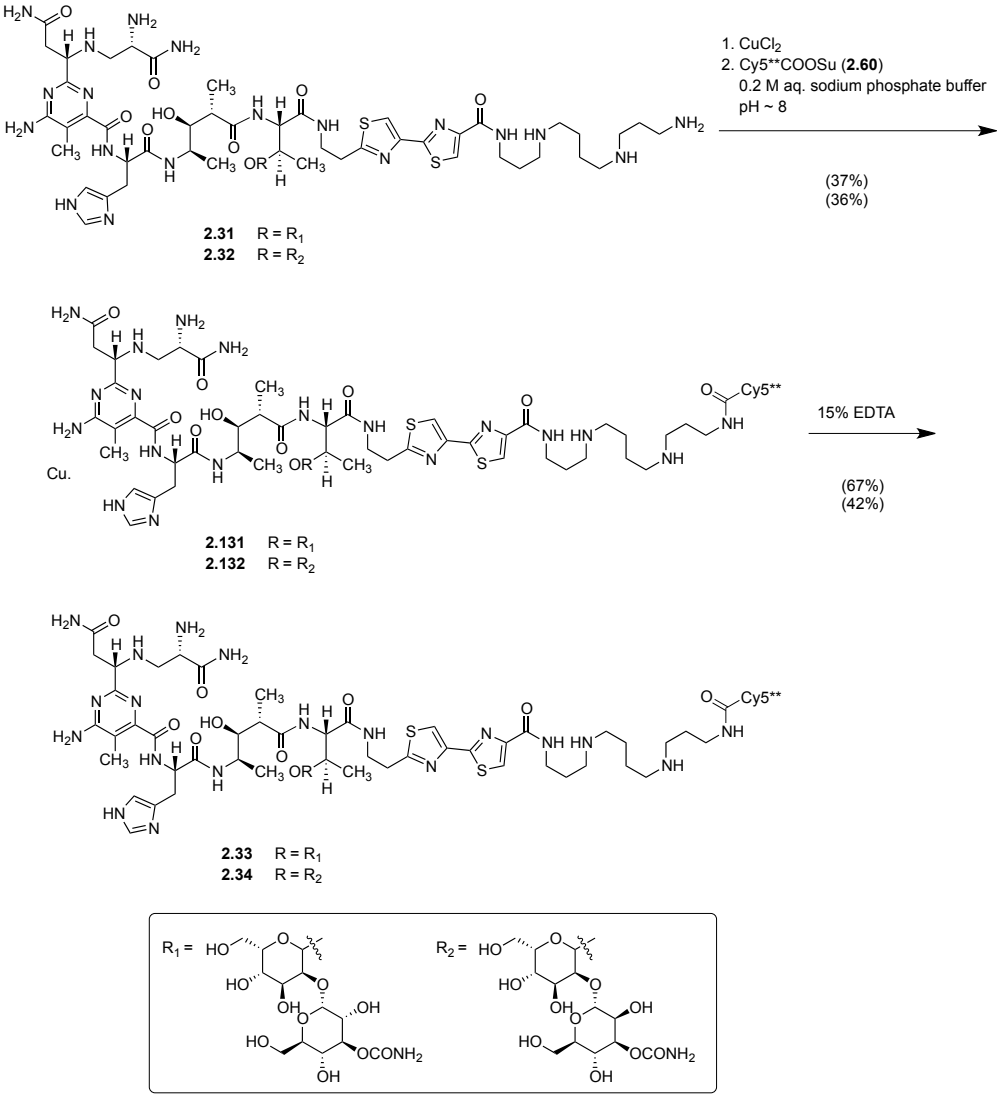
Scheme 2.18. Synthesis of Modified BLM **2.29**

To study the cellular uptake of modified BLMs, the dye-labeled analogues were prepared. The synthesis of dye-labeled analogue **2.30** began with the coupling of the activated NHS ester of acid **2.127** with Cu(II)•deglycoBLM (**2.123**) to afford modified linker BLM **2.128** in 15% yield over two steps (Scheme 2.19). The Fmoc group of linker BLM **2.128** was removed using 20% piperidine in DMF followed by coupling with carbonate **2.125** to provide modified linker BLM–disaccharide **2.129** in 32% yield over two steps. The deprotection of Boc group followed by coupling with the NHS ester of Cy5** (**2.60**)[127] gave conjugate **2.130** in 17% yield over two steps. The demetallation of conjugate **2.130** with 15% EDTA afforded modified BLM–Cy5** **2.29** in 40% yield.



Scheme 2.19. Synthesis of Modified BLM–Cy5** Conjugate **2.30**.

The syntheses of dye labeled analogues of modified BLMs **2.31**[119] and **2.32**[119] started by metallation of each with $CuCl_2$, followed by coupling with the NHS ester of Cy5** (**2.60**),[127] the latter of which afforded conjugates **2.131** and **2.132** in 37% and 36% yields, respectively (Scheme 2.20). Successive demetallation of **2.131** and **2.132** using 15% EDTA afforded modified BLM–Cy5** conjugates **2.33** and **2.34** in 67% and 42% yields, respectively.



Scheme 2.20. Synthesis of Modified BLM–Cy5** Conjugates **2.33** and **2.34**.

2.2.2. Biological Evaluation of Fluorescent Carbohydrate Conjugates

The natural disaccharide moiety in BLM is comprised of D-mannose and L-gulose monosaccharide subunits. The importance of the disaccharide moiety of the bleomycin in the tumor cell selectivity has been well documented by the microbubble[10] and fluorescent microscopy experiments.[18] To better study the importance of the carbamoyl moiety, a library of disaccharide–dye conjugates was synthesized (Figure 2.10). Cy5** was used as the fluorescent probe; it was the perfect dye candidate for the study as it has negligible cell surface affinity and is soluble in aqueous media due to the presence of four sulfonate groups. The cell binding/uptake of BLM disaccharide–Cy5** conjugate (**2.3**), and the newly synthesized disaccharide–Cy5** conjugates **2.4**–**2.7** (Figure 2.10) by A549 lung carcinoma cells, DU-145 prostate carcinoma cells, A498 kidney carcinoma cells, and BxPC-3 pancreatic carcinoma cells were quantified by fluorescence imaging.

Disaccharide conjugate **2.4**, having a unmodified carbamoyl group at the C-2 position of D-mannose (Figure 2.10) showed comparable binding/uptake relative to the natural disaccharide conjugate **2.3** in all the cancer cell lines tested except BxPC-3 pancreatic cancer cells (Figure 2.19). Disaccharide conjugate **2.6** having an *N*-methylated carbamoyl group at the C-2 position of D-mannose exhibited best binding/uptake in all tested cancer cell lines, especially in BxPC-3 pancreatic cancer cells. Disaccharide conjugate **2.7** having an *N*-methylated carbamoyl group at the C-3 position of D-mannose also showed excellent binding/uptake characteristics, almost comparable to disaccharide conjugate **2.6** in A498 kidney cancer cells and BxPC-3 pancreatic cancer cells, but much

lower in A549 lung cancer cells and DU-145 prostate cancer cells. In comparison, disaccharide conjugate **2.5** having an unmodified carbamoyl group at the C-4 position of D-mannose exhibited relatively poor binding/uptake in all of the tumor cell lines tested, underscoring the importance of the position and orientation of the carbamoyl group in the BLM disaccharide. This was especially true for the BxPC-3 pancreatic cancer and A498 kidney cancer cell lines. All of the disaccharide–dye conjugates exhibited negligible binding/uptake in normal cells (data not shown), highlighting the role of the disaccharide and carbamoyl moiety in the cancer cell selectivity of BLM.

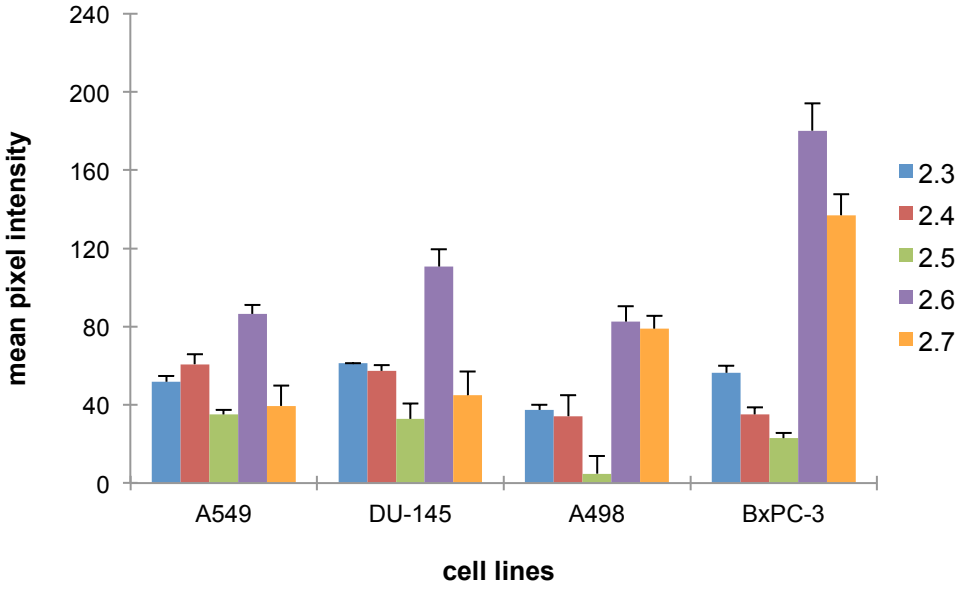


Figure 2.19. Comparison of the Binding/Uptake of BLM Disaccharide–Cy5** Conjugate (**2.3**) and Modified Disaccharide–Cy5** Library Conjugates **2.4**–**2.7** in Four Cancer Cell Lines. (The cells were treated with 25 μM of BLM disaccharide–Cy5** conjugate (**2.3**) or disaccharide–Cy5** conjugates **2.4**–**2.7** at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 2 s exposure time. The experiment was carried out by Dr. Zhiqiang Yu).

Carbamoylmannose conjugated to Cy5** was synthesized (Figure 2.11) to evaluate its effect on cellular internalization. Six cancer cell lines were cultured on 16-well glass chamber slides for 48 h and the cell binding/uptake of BLM monosaccharide–Cy5** (**2.8**) was compared to BLM disaccharide–Cy5** (**2.3**) conjugate (Figure 2.10). The cells were stained with DAPI to permit evaluation of localization of the conjugates relative to the cell nuclei. It was clear that both BLM monosaccharide–Cy5** (**2.8**) and BLM disaccharide–Cy5** (**2.3**) conjugates underwent significant binding and uptake in all six cell lines (Figure 2.20). The binding/uptake was found to be specific for the cancer cell lines, as compared with matched normal controls (data not shown). Quantification of the data revealed that the binding/uptake of the BLM monosaccharide–Cy5** (**2.8**) was much (~2-fold) greater than that of BLM disaccharide–Cy5** (**2.3**) in A549 lung cancer cells, A498 kidney cancer cells, and MCF-7 breast carcinoma cells. The binding/uptake was almost identical for BT-474 breast ductal carcinoma cells and BxPC-3 pancreas cells. In comparison, the uptake exhibited by the BLM disaccharide–Cy5** (**2.3**) was much greater than that of monosaccharide **2.8** in DU-145 prostate cancer cells.

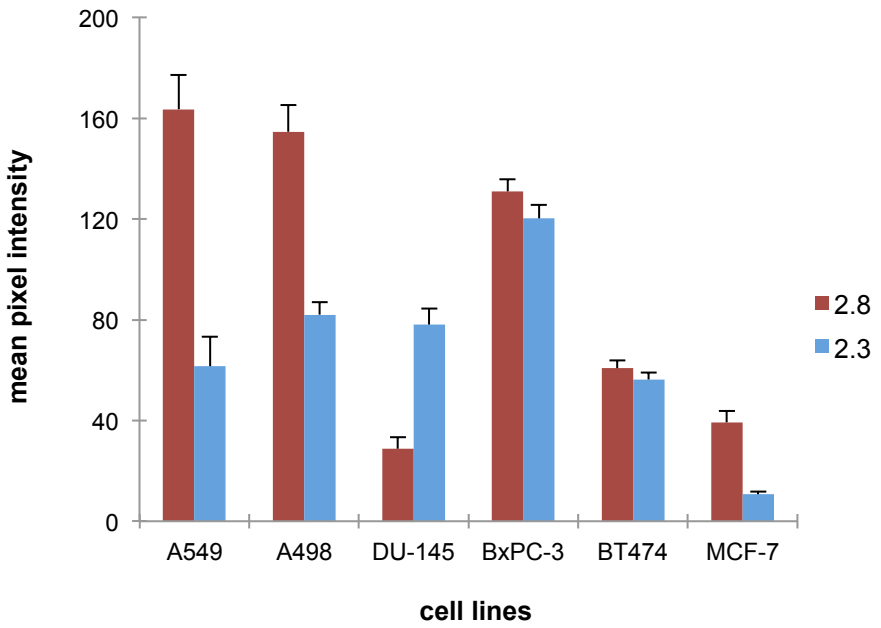


Figure 2.20. Comparison of the Binding/Uptake of BLM Monosaccharide–Cy5** (**2.8**) and BLM Disaccharide–Cy5** (**2.3**) Conjugates in Six Cancer Cell Lines. (The cells were treated with 25 μM BLM monosaccharide–Cy5** (**2.8**) or BLM disaccharide–Cy5** (**2.3**) at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 3 s exposure time. The experiment was carried out by Dr. Zhiqiang Yu).

To better define the importance of the carbamoyl group in cellular recognition, binding, and internalization, an experiment was conducted in which BLM monosaccharide–Cy5** (**2.8**) and decarbamoyl BLM monosaccharide–Cy5** (**2.9**) conjugates (Figure 2.11) were treated with four cancer cell lines and evaluated for cellular localization using fluorescent microscopy. None of the four cancer cell lines bound decarbamoyl BLM monosaccharide–Cy5** (**2.9**) to a significant extent, indicating that the carbamoyl group is essential for cell surface receptor interaction and uptake in these tumor cell lines (Figure 2.21).

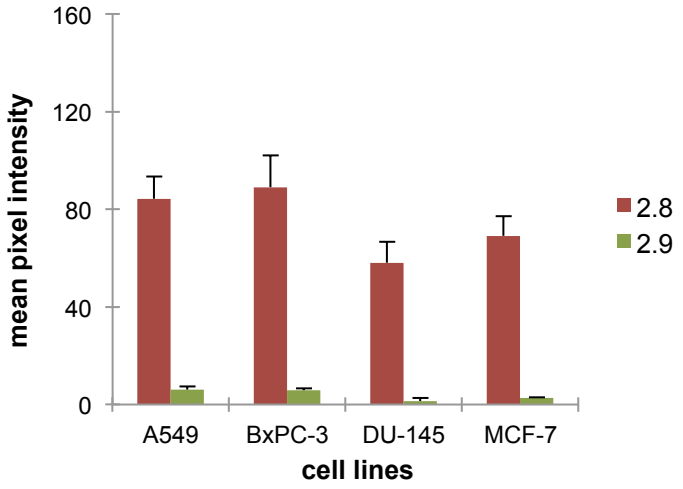


Figure 2.21. Comparison of the Binding/Uptake of Decarbamoyl BLM Monosaccharide–Cy5** (**2.9**) and BLM Monosaccharide–Cy5** (**2.8**) Conjugates in Four Cancer Cell Lines. (The cells were treated with 25 μM decarbamoyl BLM monosaccharide–Cy5** (**2.9**) or BLM monosaccharide–Cy5** (**2.8**) at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 3 s exposure time. The experiment was carried out by Dr. Zhiqiang Yu).

Cellular receptors responsible for carbohydrate binding frequently exhibit multivalency. Accordingly, the cellular targeting and uptake of the Cy5** conjugate containing a cluster of three carbamoylmannose molecules (**2.10**) was studied. The BLM monosaccharide–Cy5** conjugate (**2.8**) and the BLM monosaccharide–Cy5** trimer (**2.10**) (Figure 2.11) were studied in six cancer cell lines. The quantified data reflect the (1.6–2.3-fold) greater binding/uptake of the BLM monosaccharide–Cy5** trimer (**2.10**) compared to the BLM monosaccharide–Cy5** conjugate (**2.8**) in all six cancer cell lines, consistent with multivalent targeting of the putative cell surface carbohydrate receptors (Figure 2.22).

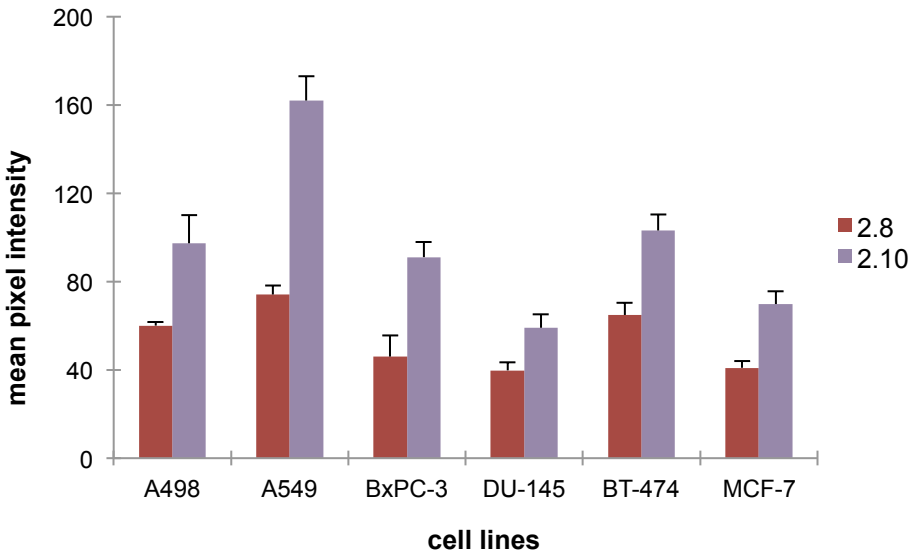


Figure 2.22. Comparison of the Binding/Uptake of BLM Monosaccharide–Cy5** (**2.8**) and BLM Monosaccharide–Cy5** Trimer (**2.10**) Conjugates in Six Cancer Cell Lines. (The cells were treated with 25 μM BLM monosaccharide–Cy5** (**2.8**) or BLM monosaccharide–Cy5** trimer (**2.10**) at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 3 s exposure time. The experiment was carried out by Dr. Zhiqiang Yu).

One interesting aspect of the cellular interaction of the BLM disaccharide–Cy5** conjugate (**2.3**) with cancer cells was found to be its temperature-dependent binding/uptake by cultured MCF-7 breast cancer cells.[18] In order to test the mechanism of uptake of the BLM monosaccharide–Cy5** conjugate (**2.8**), the uptake was measured in seven cell lines at both 4 °C and 37 °C. The uptake measured at 4 °C after 1 h was similar to that observed at 37 °C (Figure 2.23), suggesting that the mechanism of internalization of the BLM monosaccharide–Cy5** conjugate (**2.8**) apparently differs from that of the BLM disaccharide–Cy5** conjugate (**2.3**), at least in this regard.

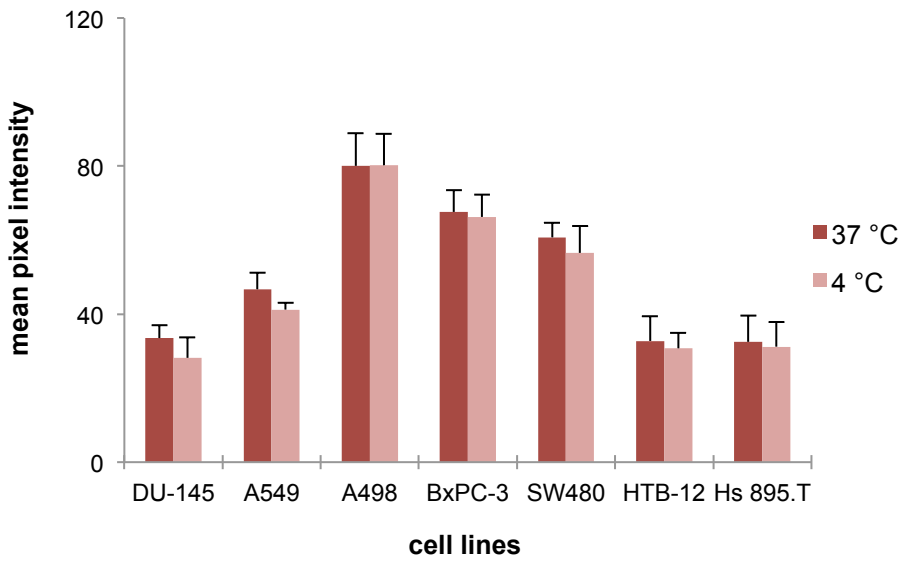


Figure 2.23. Effect of the Incubation Temperature on the Internalization of BLM Monosaccharide–Cy5** Conjugate (**2.8**) in Seven Cancer Cell Lines. (The cells were treated with 25 μM BLM monosaccharide–Cy5** (**2.8**) at 4 °C or 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 2 s exposure time. The experiment was carried out by Dr. Zhiqiang Yu).

The present study was undertaken to further explore the effects of modifications of the carbamoyl moiety in alternative locations within the carbamoylmannose moiety of BLM. The cell binding/uptake of BLM monosaccharide–Cy5** conjugate (**2.8**) (Figure 2.11), and the modified monosaccharide–Cy5** conjugates **2.11**–**2.16** (Figure 2.12) were studied in four cancer cell lines. It was clear that monosaccharide conjugates **2.11** and **2.13** having an *N*-methylated carbamoyl group and an *N*-butylated carbamoyl group at the C-2 position of D-mannose, respectively, both underwent significantly high binding/uptake relative to BLM monosaccharide–Cy5** (**2.8**) in all four cancer cell lines (Figure 2.24). The quantification data also revealed that monosaccharide conjugates **2.12** and **2.14** having *N*,*N*-dimethylated carbamoyl group and an *N*,*N*-pyrrolidinyl carbamoyl

group at the C-2 position of D-mannose, respectively, exhibited relatively poor binding/uptake in all of the tumor cell lines tested, underscoring the importance of the substitution and electron density on the cell binding/uptake. However, monosaccharide conjugate **2.15**, having an *N*-hexylated carbamoyl group at the C-2 position of D-mannose showed high binding/uptake in BxPC-3 pancreatic cancer lines and much lower binding/uptake in A549 lung cancer cells and DU-145 prostate cancer cells. In the case of MCF-7 breast cancer cells, it showed moderate binding/uptake. Monosaccharide conjugate **2.16**, having an *N*-methylated carbamoyl group at the C-3 of D-mannose exhibited considerably lower binding/uptake relative to BLM monosaccharide–Cy5** conjugate **2.8** and monosaccharide conjugate **2.11** in A549 lung cancer cells, DU-145 prostate cancer cells and MCF-7 breast cancer cells which is consistent with the results obtained in case of the disaccharide conjugates **2.6** and **2.7** (Figure 2.12) having an *N*-methylated carbamoyl group at the C-2 and C-3 positions of D-mannose subunit, respectively. The binding/uptake was almost identical to the uptake exhibited by monosaccharide conjugate **2.11** in BxPC-3 pancreatic cancer cells.

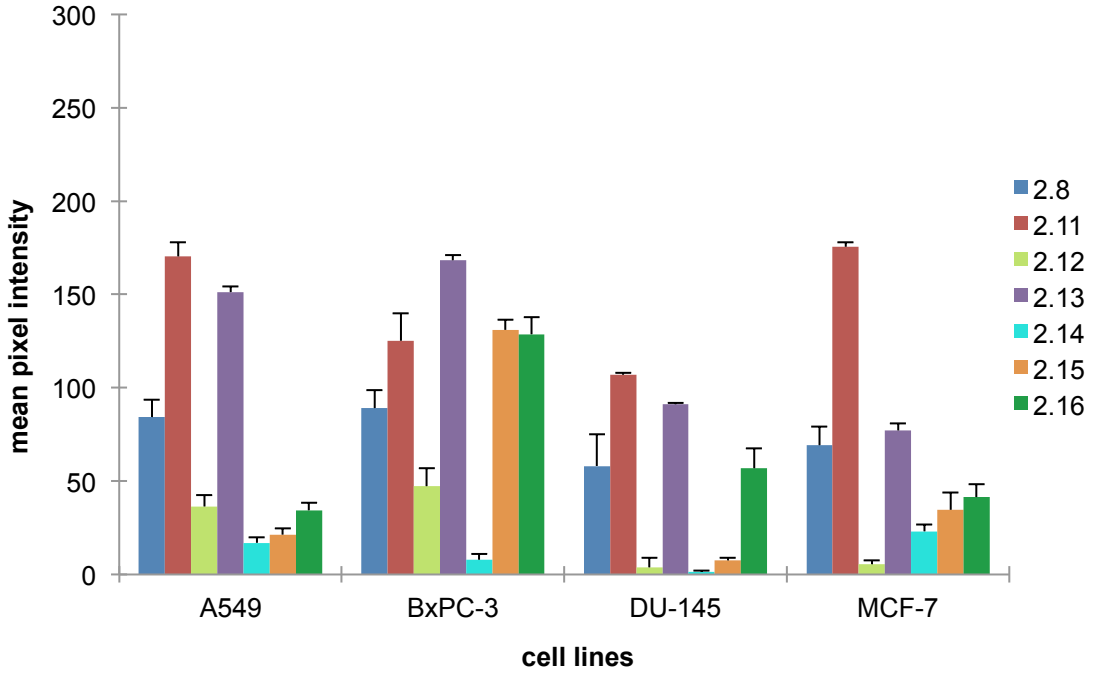


Figure 2.24. Comparison of the Binding/Uptake of Modified Monosaccharide–Cy5** Library Conjugates **2.11**–**2.16** in Four Cancer Cell Lines. (The cells were treated with 25 μM of monosaccharide–Cy5** conjugates **2.11**–**2.16** at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 3 s exposure time. The experiment was carried out by Dr. Zhiqiang Yu).

Cyanine dye Cy5** produced excellent reproducible results, but due to the lack of availability of Cy5** dye several commercially available dyes with similar extinction coefficients, absorption and emission spectra to Cy5** were coupled with the BLM monosaccharide and tested for binding/uptake in the cancer cells. BLM monosaccharide–Cy5 (**2.18**) and BLM monosaccharide–Cy5^{GE} (**2.21**) conjugates (Figure 2.13) showed significant intensity of binding/uptake in all of the tested cancer cell lines (Figure 2.25). The binding/uptake observed was very low for BLM monosaccharide–sulphoCy5 (**2.17**), BLM monosaccharide–Cy7 (**2.19**) and BLM monosaccharide–Cy5.5^{GE} (**2.22**) in all of the

tested cancer cell lines. However, the binding/uptake of the BLM monosaccharide–AF647 (**2.20**) was high in A549 lung cancer cells and A498 kidney cancer cells, but the uptake was non evenly distributed throughout the cells. Hence, Cy5 and Cy5^{GE} were selected as promising candidates for the future experiments.

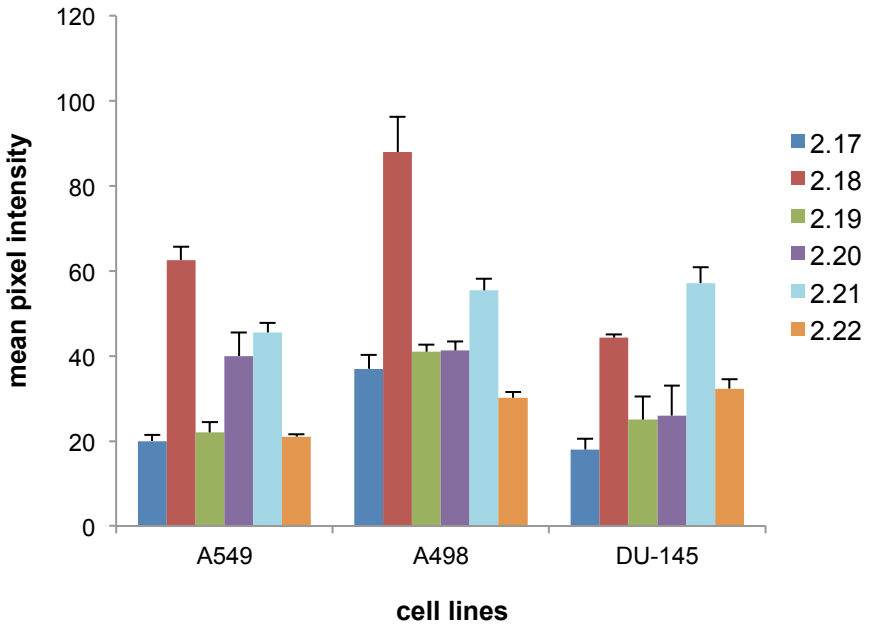


Figure 2.25. Comparison of the Binding/Uptake of BLM Monosaccharide–dye Conjugates **2.17**–**2.22** in Three Cancer Cell Lines. (The cells were treated with 25 μM BLM monosaccharide–dye conjugates **2.17**–**2.22** at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 3 s exposure time. The experiment was carried out by Justin Kaye).

Due to significantly higher uptake of the BLM monosaccharide–Cy5 conjugate **2.18** observed in comparison to the other conjugates, the cell binding/uptake of BLM monosaccharide–Cy5 conjugate (**2.18**) and free dye Cy5, used as a control, were studied in A549 lung cancer cells, A498 kidney cancer cells and DU-145 prostate cancer cells. The free dye Cy5 showed significant background binding/uptake in all of the cell lines

(Figure 2.26). The uptake of Cy5 dye was almost half that of the binding of BLM monosaccharide–Cy5 conjugate (**2.18**) in DU-145 prostate cancer cells and was permeable to the cell membrane on its own to some extent.

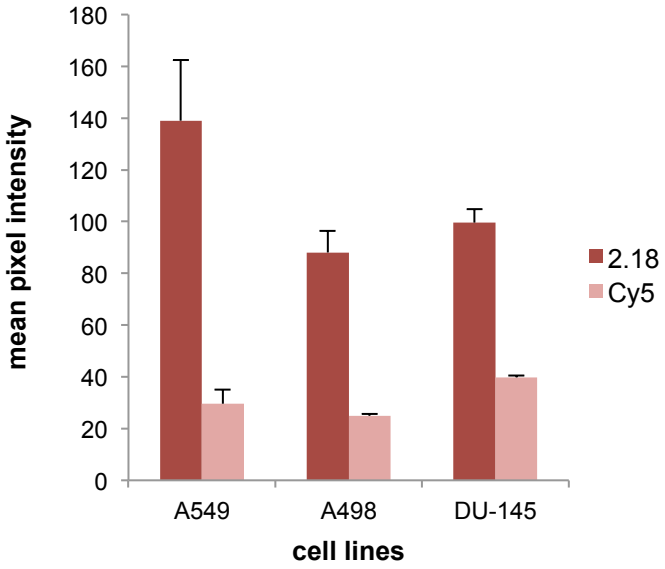


Figure 2.26. Comparison of the Binding/Uptake of BLM Monosaccharide–Cy5 (**2.18**) and the Free Dye Cy5 in A549 Lung Cancer Cells, A498 Kidney Cancer Cells and DU-145 Prostate Cells. (The cells were treated with 25 μM BLM monosaccharide–Cy5 (**2.18**) or Cy5 dye at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 3 s exposure time. The experiment was carried out by Justin Kaye).

As the free dye Cy5 was significantly permeable to the cell membrane on its own, which likely reflected the absence of any polar negatively charged sulfonate group, $Cy5^{GE}$ was next chosen. The cell binding/uptake of BLM monosaccharide–$Cy5^{GE}$ (**2.21**) (Figure 2.13) was compared to BLM disaccharide–$Cy5^{GE}$ (**2.25**)[115] (Figure 2.14) in A549 lung cancer cells and BxPC-3 prostate cancer cells and their matched normal cells. BLM monosaccharide–$Cy5^{GE}$ (**2.21**) and BLM disaccharide–$Cy5^{GE}$ (**2.25**) underwent significant binding and uptake in both cancer cell lines (Figure 2.27). There was no

binding/uptake observed for the matched normal controls cells. The binding/uptake of the BLM monosaccharide–Cy5^{GE} (**2.21**) was much (~2-fold) greater in A498 kidney cancer cells and lower than BLM disaccharide–Cy5^{GE} (**2.25**) conjugate in case of DU-145 prostate cancer cells which was consistent with the results obtained in case of the Cy5** conjugates of BLM monosaccharide (**2.8**) (Figure 2.11) and BLM disaccharide (**2.3**) (Figure 2.10).

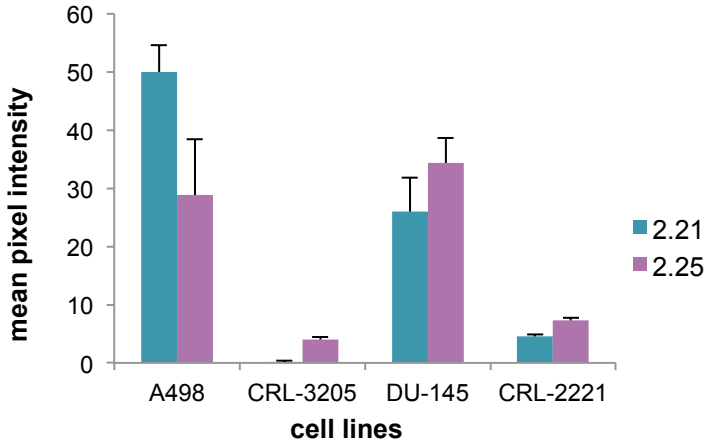


Figure 2.27. Comparison of the Binding/Uptake of BLM Monosaccharide–Cy5^{GE} (**2.21**) and BLM Disaccharide–Cy5^{GE} (**2.25**) Conjugates in A498 Kidney Cancer Cells and BxPC-3 Pancreatic Cancer Cells and Their Matched Normal Cells. (The cells were treated with 25 μM BLM monosaccharide–Cy5^{GE} (**2.21**) or BLM disaccharide–Cy5^{GE} (**2.25**) at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 3 s exposure time. The experiment was carried out by Justin Kaye).

To further confirm the impermeable nature of the Cy5^{GE} conjugates to the cell membrane on its own, the decarbamoyl BLM monosaccharide–Cy5^{GE} (**2.27**) conjugate (Figure 2.14) was used to three cancer cell lines and was compared with BLM monosaccharide–Cy5^{GE} (**2.21**) and BLM disaccharide–Cy5^{GE} (**2.25**). None of the three cell lines showed any binding/uptake in case of decarbamoyl BLM monosaccharide–

Cy5^{GE} (**2.27**), while both BLM monosaccharide–Cy5^{GE} (**2.21**) and BLM disaccharide–Cy5^{GE} (**2.25**) conjugates showed significant binding/uptake in all tested cancer cell lines (Figure 2.28). These results are consistent with the results obtained in case of the respective Cy5** conjugates of BLM monosaccharide (**2.8**), BLM disaccharide (**2.3**) and decarbamoyl BLM disaccharide (**2.9**).

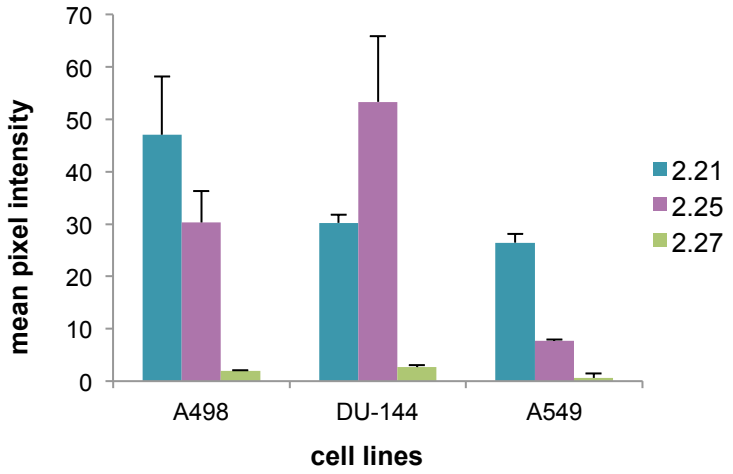


Figure 2.28. Comparison of the Binding/Uptake of BLM Monosaccharide–Cy5^{GE} (**2.21**), BLM Disaccharide–Cy5^{GE} (**2.25**) and Decarbamoyl BLM Disaccharide–Cy5^{GE} (**2.27**) Conjugates in A498 Kidney Cancer Cells, DU-145 Prostate Cancer Cells and A549 Lung Cells. (The cells were treated with 25 μM BLM monosaccharide–Cy5^{GE} (**2.21**) or BLM disaccharide–Cy5^{GE} (**2.25**) or decarbamoyl BLM disaccharide–Cy5^{GE} (**2.27**) at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out with a 3 s exposure time. The experiment was carried out by Justin Kaye).

To better define the possible modular nature of bleomycin as an antitumor agent, derivatives of deglycoBLM in which the BLM disaccharide is attached to the C-terminal substituent (**2.29**)[118] (Figure 2.15) or to the threonine moiety of deglycoBLM (**2.32**)[119] were prepared (Figure 2.16). The cytotoxicity of the BLM analogues was determined by

use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a vital dye in DU-145 prostate cancer cells. It was clear from the assay that BLM A_5 was the most potent of the derivatives, whereas BLM analogue **2.29**, containing the BLM disaccharide attached to the C-terminal substituent, was less cytotoxic than BLM A_5 but significantly more cytotoxic than deglycoBLM or BLM analogue **2.32**, containing the BLM disaccharide attached to the threonine moiety (Figure 2.29). DeglycoBLM A_5, lacking the tumor-targeting disaccharide, was cytotoxic to DU-145 cells but much less than BLM A_5 or BLM analogue **2.32**.

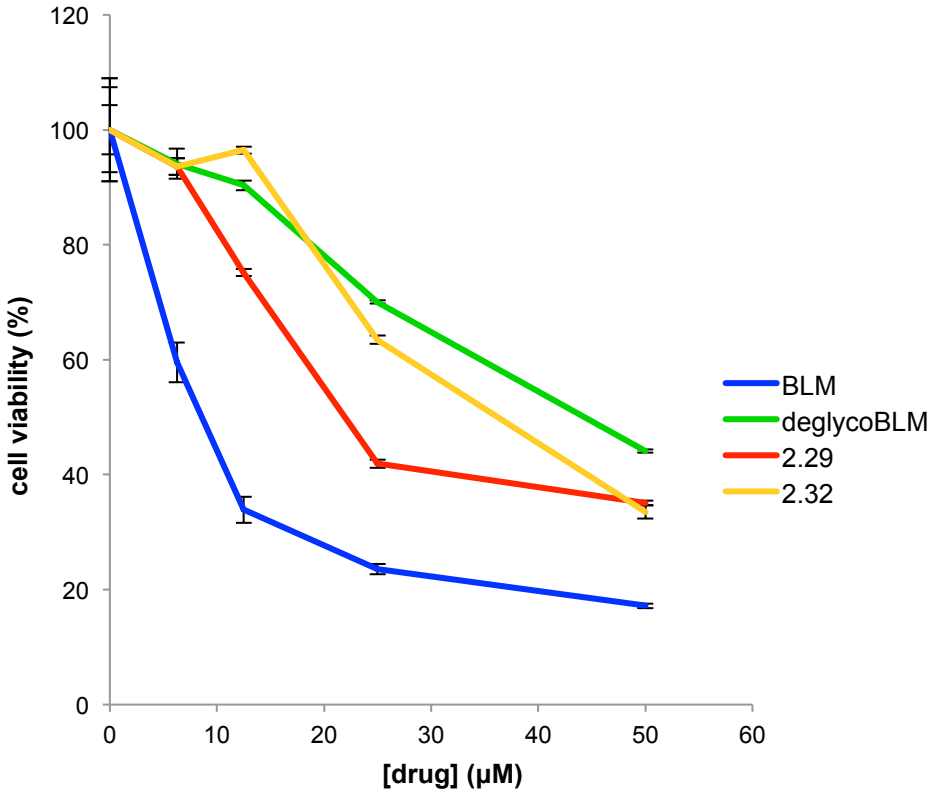


Figure 2.29. Dose-dependent Effects of Modified BLM Analogues **2.29**, **2.31** and **2.32** on the Viability of Cultured DU-145 Prostate Cancer Cells. (The experiment was carried out by Imran Ghare).

To provide additional evidence in support of the basis for expression of cytotoxicity by glycosylated deglycoBLMs, the cytotoxic derivatives **2.29** and **2.32** were conjugated to the cyanine dye Cy5**. The modified BLM–Cy5** conjugates **2.30** (Figure 2.15) and **2.34** (Figure 2.16) were studied in DU-145 prostate cancer cells and PZ-HPV-7 normal prostate cells. It was clear from the quantification data that BLM–Cy5** conjugate (**2.2a**) was internalized most efficiently in DU-145 prostate cancer cells, while uptake of deglycoBLM–Cy5** (**2.2b**) was the least efficient (Figure 2.30). The modified BLM–Cy5** conjugate **2.30** was internalized more efficiently than the modified BLM–Cy5** conjugate **2.34** but less efficiently than that of BLM–Cy5** (**2.2a**). None of the conjugates showed any binding/uptake in PZ-HPV-7 normal prostate cells. Thus, the rank order of uptake of these conjugates was precisely the same as the rank order of observed cytotoxicities toward DU-145 prostate cancer cells.

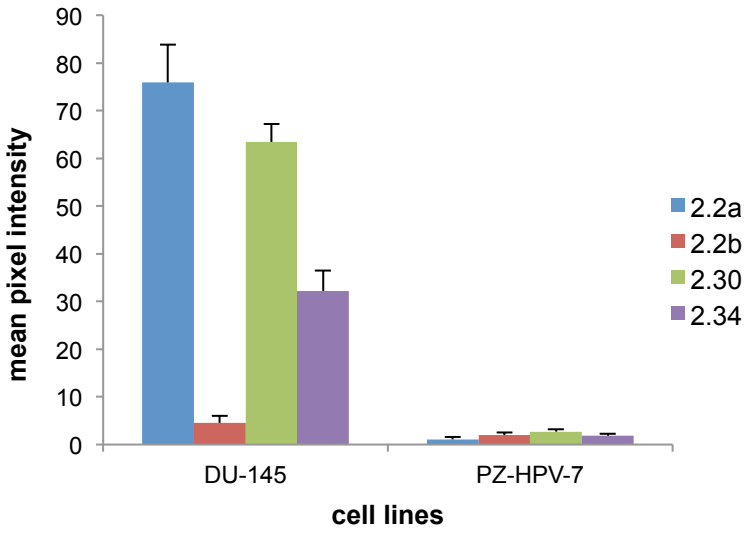
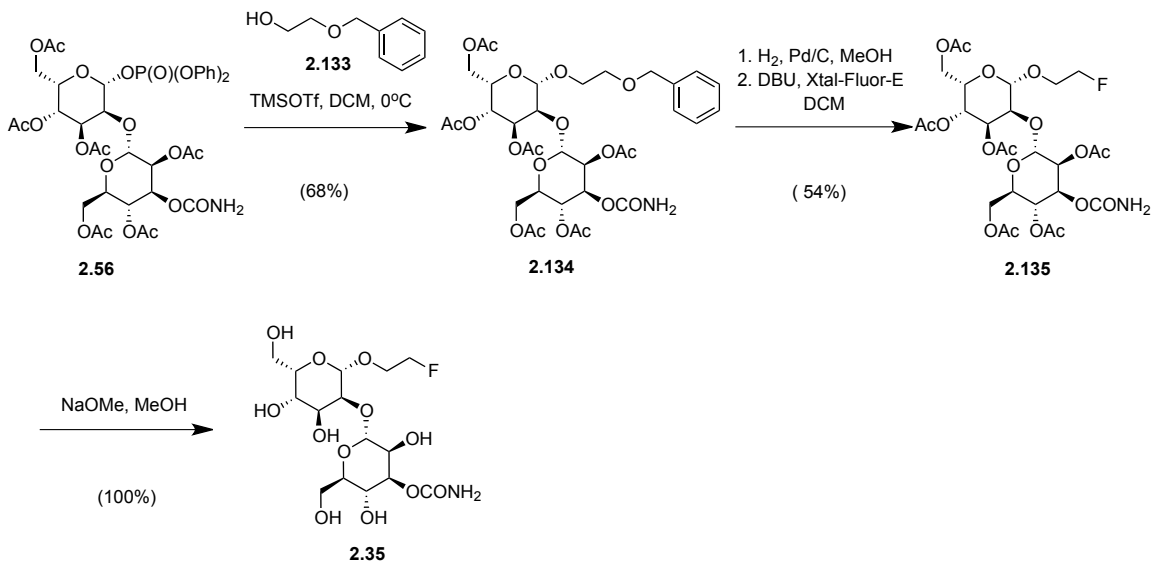


Figure 2.30. Comparison of the Binding/Uptake of BLM–Cy5** (**2.2a**), DeglycoBLM–Cy5** (**2.2b**), and Modified BLM–Cy5** Conjugates **2.30** and **2.34** in DU-145 Prostate Cancer Cells and PZ-HPV-7 Normal Prostate Cells. (The cells were treated with 25 μM Cy5** conjugates (**2.2a**, **2.2b**, **2.30** and **2.34)** at 37 °C for 1 h, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out after a 3 s exposure. The experiment was carried out by Dr. Zhiqiang Yu).

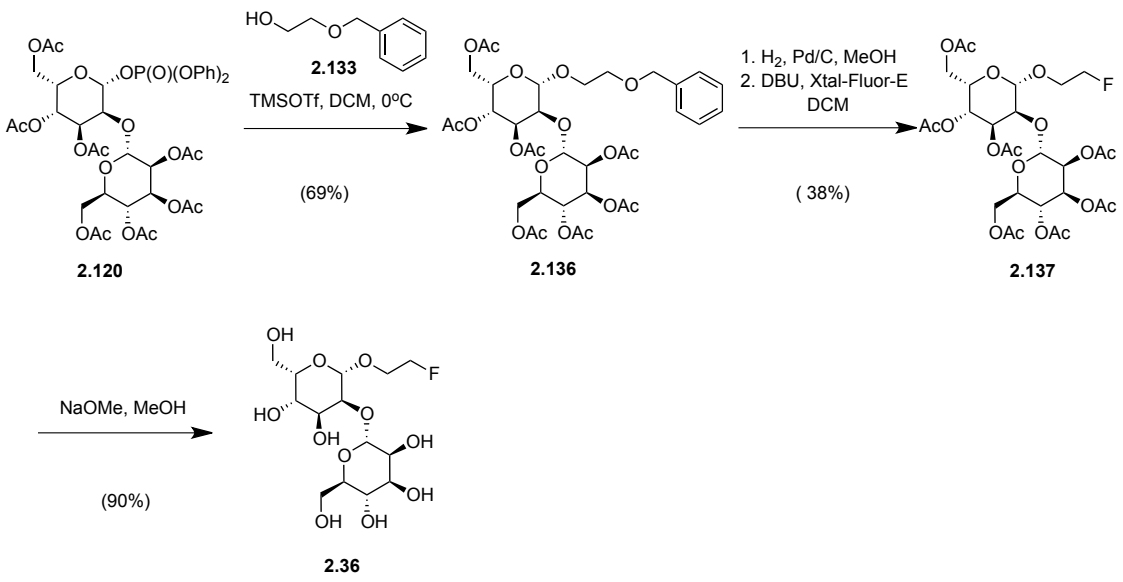
2.2.3. Synthesis of Fluorinated Saccharides

To extend the application of tumor targeting by BLM saccharides, the fluorinated disaccharides were synthesized as references for the preparation of the radiolabeled ^{18}F-saccharide PET biomarkers for tumor diagnostic studies. The synthesis of fluorinated BLM disaccharide started with the coupling of activated disaccharide phosphate ester **2.56** with the commercially available linker **2.133** to afford BLM disaccharide–linker **2.134** in 68% yield (Scheme 2.21). Debenzylation of disaccharide **2.134** gave the free alcohol, the latter of which was fluorinated with XtalFluor-E[134] to give fluorinated disaccharide **2.135** in 54% yield over two steps. Complete deacetylation with sodium methoxide in methanol afforded fluorinated BLM disaccharide **2.35** in quantitative yield.



Scheme 2.21. Synthesis of Fluorinated BLM Disaccharide **2.35**.

Similarly, the fluorinated decarbamoyl BLM disaccharide was synthesized by coupling phosphate ester **2.120** with the commercially available linker **2.133** to afford decarbamoyl BLM disaccharide–linker **2.136** in 69% yield (Scheme 2.22). Debenzylation of disaccharide **2.136** followed by fluorination with XtalFluor-E[134] afforded fluorinated disaccharide **2.137** in 38% yield over two steps. Complete deacetylation with sodium methoxide in methanol afforded fluorinated decarbamoyl BLM disaccharide **2.36** in 90% yield.



Scheme 2.22. Synthesis of Fluorinated Decarbamoyl BLM Disaccharide **2.36**.

2.3. Discussion

The present study was designed to investigate the contributions of the carbohydrate moiety of bleomycin to the cytotoxic and DNA cleaving actions of this clinically utilized antitumor antibiotic. DeglycoBLM, the analogue of bleomycin lacking the carbohydrate moiety, has been fairly extensively exploited and demonstrated to cleave duplex DNA using the same chemistry as BLM and with analogous sequence selectivity.[12,107,108] However, the supercoiled DNA relaxation efficiency of deglycoBLM is (2–3-fold) less than for BLM, and much less double-strand cleavage is relevantly observed for deglycoBLM.[107,108,135-139] Two studies in which the disaccharide moiety of BLM was replaced with a single sugar resulted in BLM analogues with diminished potency of DNA cleavage[140,141] as well as a lesser ability to cleave RNA.[141] Thus, the disaccharide moiety attached to BLM via β-hydroxyhistidine significantly potentiates DNA cleavage.

It was initially uncertain whether the diminished cytotoxic potential of deglycoBLM[107,108,135-139] was due to diminished potency of DNA cleavage, lack of significant double-strand cleavage, or lack of tumor cell targeting and uptake. The recent study from our laboratory documenting tumor targeting and uptake by the disaccharide moiety itself[18] highlights the ambiguity intrinsic to defining the role(s) of the BLM disaccharide. In order to gain insights into possible roles of the disaccharide, a library of disaccharide–Cy5** conjugates was synthesized.

The synthesis of different disaccharide–dye conjugates started with the coupling of the gulose glycosyl acceptor with the corresponding mannose donor. The gulose glycosyl acceptor was obtained from L-xylose following Dondoni's method (Scheme 2.1).[124] At first, L-xylose (**2.37**) was fully protected as dithioacetal **2.38**, the latter of which was selectively converted into aldehyde **2.39** in presence of Hg(II). Due to the unstable nature of aldehyde **2.39**, it was immediately coupled with 2-(trimethylsilyl)thiazole (**2.40**)[125] and desilylated to afford alcohol **2.41**. The protection of the free hydroxyl group as benzyl ether **2.42** and subsequent cleavage of the thiazole ring to provide a formyl group gave aldehyde **2.43**. Acetal deprotection followed by peracetylation of aldehyde **2.43** gave pyranoside **2.44**, the latter of which was debenzylated to give gulose acceptor **2.45**.

The synthesis of mannose donor **2.54** was achieved following reported procedures with slight modifications (Scheme 2.2).[21] The synthesis began with protection of commercially available α-D-methylmannopyranoside (**2.46**) as its benzylidene acetal **2.47** followed by selective benzylation at C-3 position using Bu_2SnO to give alcohol

2.48. Acetal deprotection of alcohol **2.48** followed by exhaustive acetylation gave peracetylated mannose **2.49**, the latter of which was debenzylated using $Pd(OH)_2/C$ (Pearlman's catalyst) to yield C-3 alcohol **2.50**. Activation of the alcohol as its *p*-nitrophenyl carbonate (**2.51**) followed by aminolysis with ammonia gave carbamate **2.52**. Selective deacetylation at the anomeric position of carbamate **2.52** with hydrazine acetate afforded alcohol **2.53**. Subsequent activation of alcohol **2.53** as a diphenyl phosphate ester in the presence of DMAP and dry triethylamine gave the activated mannose phosphate **2.54**,[126] the latter of which was then coupled with gulose acceptor **2.45** in the presence of TMSOTf to afford peracetylated BLM disaccharide **2.55** (Scheme 2.3).[21] Selective deacetylation at anomeric position of disaccharide **2.55** with hydrazine acetate, followed by activation as a diphenyl phosphate ester in presence of DMAP and dry triethylamine, gave activated ester **2.56**. Activated phosphate ester **2.56** was then conjugated with the benzyloxycarbonyl (CBz) protected commercially available linker derivative **2.57** to afford BLM disaccharide–linker **2.58**. Complete deacetylation of the acetates followed by debenzylation afforded primary amine **2.59**, the latter of which was conjugated with the NHS ester of Cy5** (**2.60**)[127] to provide the BLM disaccharide−Cy5** conjugate (**2.3**).[18]

During the synthesis of C-2 carbamoylmannose modified disaccharide–Cy5**, a similar coupling strategy of activated C-2 carbamoylmannose with gulose acceptor **2.45** was tried. Several attempts were made to isolate the coupled C-2 disaccharide but each time a cyclic carbamate by-product was formed due to attack of the C-2 carbamoyl amine on the activated C-1 carbon. Finally, a new strategy was developed, involving the

introduction of the carbamoyl group later in synthesis after formation of the disaccharide. The synthesis of C-2 modified mannose donor **2.64** began with protection of α-D-methylmannopyranoside (**2.46**) as dibenzylidene acetal **2.61** in two isomeric forms, exo and endo, the latter of which were easily separable by crystallization (Scheme 2.4).[128] Selective reduction of the acetal at the C-2 and C-3 positions of both the isomers of **2.61** with DIBAL-H in the presence of aluminium chloride afforded the C-3 alcohol **2.62**,[129] the latter of which was acetylated in presence of concentrated H_2SO_4 to gave peracetylated mannose **2.63**. The selective cleavage of anomeric acetate of **2.63** with hydrazine acetate followed by activation as a diphenyl phosphate ester in presence of DMAP and dry triethylamine gave the activated mannose phosphate **2.64**. Coupling of activated mannose donor **2.64** with gulose acceptor **2.45** in the presence of TMSOTf afforded disaccharide **2.65** (Scheme 2.5). The latter was debenzylated using $Pd(OH)_2/C$ (Pearlman's catalyst) to give alcohol **2.66**. C-2 alcohol **2.66** was then activated as the *p*-nitrophenyl carbonate (**2.67**) and subjected to aminolysis with ammonia to give carbamate **2.68**. The selective removal of the anomeric acetate of disaccharide **2.68**, followed by activation as a diphenyl phosphate ester in presence of DMAP and dry triethylamine, gave activated ester **2.69**. Compound **2.69** was coupled to benzyloxycarbonyl (CBz) protected linker **2.57**[18] to afford BLM disaccharide–linker **2.70**. Complete deacetylation of the acetates followed by debenzylation gave primary amine **2.71**, the latter of which was treated with the NHS ester of Cy5** (**2.60**)[127] to provide the modified disaccharide–Cy5** conjugate **2.4**.

The synthesis of C-4 carbamoylmannose modified disaccharide–Cy5** was

achieved using the same strategy as for the natural disaccharide. The synthesis started with regioselective reductive ring opening of acetal **2.47** in presence of $CoCl_2$ and BH_3•THF to afford alcohol **2.72** (Scheme 2.6).[130] Exhaustive acetylation of alcohol **2.72** in acidic medium gave peracetylated mannose **2.73**, the latter of which was debenzylated using $Pd(OH)_2/C$ (Pearlman's catalyst) to give the alcohol **2.74**. C-4 alcohol **2.74** was then activated as the *p*-nitrophenyl carbonate (**2.75**) and subjected to aminolysis with ammonia to form the carbamate **2.76**. Selective deprotection of the anomeric acetate with hydrazine acetate followed by activation as a diphenyl phosphate ester in presence of DMAP and dry triethylamine gave the activated mannose phosphate **2.78**. This activated mannose donor was then coupled with gulose acceptor **2.45** in the presence of TMSOTf to afford disaccharide **2.79** (Scheme 2.7). Selective removal of the anomeric acetate of disaccharide **2.79**, followed by activation as a diphenyl phosphate ester, afforded glycosyl donor **2.80**, the latter of which was coupled to the benzyloxycarbonyl (CBz) protected linker **2.57**[18] to afford disaccharide–linker **2.81**. Complete deacetylation of the acetates followed by debenzylation gave primary amine **2.82**, the latter of which was subsequently conjugated with the *N*-hydroxysuccinimide (NHS) ester of Cy5** (**2.60**)[127] to afford the modified disaccharide–Cy5** conjugate **2.5**.

In order to find the simplest structural entity capable of mediating tumor cell targeting, the BLM monosaccharide–Cy5** (**2.8**) was synthesized. Mannose glycosyl donor **2.54** was coupled to the benzyloxycarbonyl (CBz) protected linker **2.57**[18] to afford BLM monosaccharide–linker **2.83** (Scheme 2.8). Complete deacetylation of the acetates followed by debenzylation of primary amine of **2.83** gave amine **2.84**, the latter of which

was treated with the NHS ester of Cy5** (**2.60**)[127] to generate the BLM monosaccharide–Cy5** conjugate (**2.8**).

To confirm the necessity of the carbamoyl moiety for selective tumor cell targeting, the decarbamoyl BLM monosaccharide–Cy5** (**2.9**) lacking the carbamoyl group at C-3 of the mannose residue was also synthesized. Exhaustive acetylation of α-D-methylmannopyranoside (**2.46**) gave peracetylated mannose **2.85**[131] (Scheme 2.9), the latter of which was converted into activated mannosyl phosphate **2.86** by selective removal of anomeric acetate and subsequent activation as a diphenyl phosphate ester in presence of DMAP and dry triethylamine.[132] This mannose glycosyl donor **2.86** was coupled with the benzyloxycarbonyl (CBz) protected linker **2.57**[18] to afford decarbamoyl BLM monosaccharide–linker **2.87**. Complete deacetylation of the acetates followed by debenzylation of the primary amine of **2.87** gave amine **2.88**, the latter of which was conjugated to the NHS ester of Cy5** (**2.60**)[127] to afford decarbamoyl BLM monosaccharide–Cy5** conjugate (**2.9**).

To study the nature of the cell receptor–saccharide interaction, the fluorescently labeled trimeric carbohydrate cluster **2.10** was prepared. The synthesis commenced with debenzylation of the primary amine of **2.83** to give amine **2.89**. Coupling of amine **2.89** with the NHS ester of protected trimer–linker **2.90**[118] was very challenging. Several attempts were made to isolate the trimeric sugar cluster but very low yields were obtained. Finally, the reaction was optimized using three equivalents of amine **2.89** relative to NHS ester of protected trimer–linker **2.90** affording BLM monosaccharide trimer linker **2.91** in better yield (Scheme 2.10). Complete deacetylation followed by

debenzylation of the primary amine in **2.91** gave trimer cluster linker amine **2.92**. Coupling of trimer linker amine **2.92** with the NHS ester of Cy5** (**2.60**)[127] provided the BLM monosaccharide–Cy5** trimer conjugate (**2.10**).

In order to further explore the possible roles of the carbamoyl moiety of BLM in tumor cell targeting and modify the properties of the carbamoylmannose moiety, a library of monosaccharide–Cy5** conjugates was synthesized. The synthesis of the C-2 modified monosaccharide conjugates started with the coupling of mannose donor **2.64** with the phthalimide protected linker **2.93**[133] to afford monosaccharide–linker **2.94** (Scheme 2.11). Debenzylation using $Pd(OH)_2/C$ (Pearlman's catalyst) yielded the alcohol **2.95**, the latter of which was then activated as the *p*-nitrophenyl carbonate (**2.96**). Carbonate **2.96** was subjected to aminolysis with methylamine, dimethylamine, butylamine, pyrrolidine and hexylamine to afford C-2 methylcarbamate **2.97**, C-2 dimethylcarbamate **2.98**, C-2 butylcarbamate **2.99**, C-2 pyrrolidinylcarbamate **2.100** and C-2 hexylcarbamate **2.101**, respectively. Complete deacetylation of the acetates followed by deprotection of the phthalimide group using hydrazine hydrate afforded amine–linkers **2.102**, **2.103**, **2.104**, **2.105** and **2.106**, respectively, the latter of which were conjugated to the NHS ester of Cy5** (**2.60**)[127] to give modified monosaccharide–Cy5** conjugates **2.11**-**2.15**, respectively.

The synthesis of the C-3 modified monosaccharide conjugate **2.16** started with the selective deacetylation of the anomeric acetate of mannose **2.49** followed by activation as a diphenyl phosphate ester in presence of DMAP and dry triethylamine, giving the activated mannose phosphate **2.107** (Scheme 2.12). Mannose donor **2.107** was then

coupled to the phthalimide protected linker **2.93**[133] to afford monosaccharide–linker **2.108**. Debenzylation of **2.108** using $Pd(OH)_2/C$ (Pearlman's catalyst) yielded alcohol **2.109**, the latter of which was then activated as the *p*-nitrophenyl carbonate (**2.110**). Aminolysis with methylamine then afforded C-2 methylcarbamate **2.111**. Complete deacetylation followed by deprotection of the phthalimide group using hydrazine hydrate afforded amine–linker **2.112**, the latter of which was conjugated with the NHS ester of Cy5** (**2.60**)[127] to give modified monosaccharide–Cy5** conjugate **2.16**.

In order to find a new dye with properties similar to Cy5**, several dyes were coupled to the free amine of BLM monosaccharide–linker **2.84** (Scheme 2.13). Conjugation of the primary amine in **2.84** to the NHS ester of sulphoCy5 (**2.113**), the NHS ester of Cy5 (**2.114**), the NHS ester of Cy7 (**2.115**), the NHS ester of Alexa Fluor 647 (**2.116**), the NHS ester of $Cy5^{GE}$ (**2.117**) and the NHS ester of $Cy5.5^{GE}$ (**2.118**) gave BLM monosaccharide–sulphoCy5 conjugate (**2.17**), BLM monosaccharide–Cy5 conjugate (**2.18**), BLM monosaccharide–Cy7 conjugate (**2.19**), BLM monosaccharide–Alexa Fluor 647 conjugate (**2.20**), BLM monosaccharide–$Cy5^{GE}$ conjugate (**2.21**) and BLM monosaccharide–$Cy5.5^{GE}$ conjugate (**2.22**), respectively. Later, additional conjugates of the selected dyes (Cy5 and $Cy5^{GE}$) were also synthesized. The primary amine of decarbamoyl BLM monosaccharide–linker **2.88** was also coupled with the NHS ester of Cy5 (**2.114**) to give decarbamoyl BLM monosaccharide–Cy5 conjugate (**2.23**) (Scheme 2.14). Similarly, the primary amine of BLM disaccharide–linker **2.59** was coupled with the NHS ester of Cy5 (**2.114**) and the NHS ester of $Cy5^{GE}$ (**2.117**) to give

BLM disaccharide–Cy5 conjugate (**2.24**) and BLM disaccharide–Cy5^{GE} conjugate (**2.25**), [115] respectively (Scheme 2.15).

In order to obtain controls lacking uptake in any of the cancer cells, decarbamoyl BLM disaccharide conjugates were synthesized.[130] Activated mannosyl donor **2.86** was coupled with gulose acceptor **2.45** in the presence of TMSOTf to afford peracetylated disaccharide **2.119** (Scheme 2.16).[115] Selective deacetylation of the anomeric acetate of disaccharide **2.119** with hydrazine acetate afforded the corresponding alcohol, the latter of which was activated as a diphenyl phosphate ester **2.120** in presence of DMAP and dry triethylamine. Activated glycosyl phosphate **2.120** was then coupled with the benzyloxycarbonyl (CBz) protected linker **2.57**[18] to afford protected decarbamoyl BLM disaccharide–linker **2.121**.[118] Complete deacetylation followed by debenzylation of the primary amine of **2.121** gave amine **2.122**, the latter of which was coupled with the NHS ester of Cy5 (**2.114**) and the NHS ester of Cy5^{GE} (**2.117**) to give decarbamoyl BLM disaccharide–Cy5 conjugate (**2.26**) and decarbamoyl BLM disaccharide–Cy5^{GE} conjugate (**2.27**), respectively. The primary amine of Cu•BLM A_5 (**2.123**) was coupled with the NHS ester of Cy5^{GE} (**2.117**) to give Cu•BLM–Cy5^{GE} conjugate **2.124** (Scheme 2.17). This intermediate was demetallated using 15% EDTA to afford BLM–Cy5^{GE} (**2.28**).[115]

To test the hypothesis that BLM may be modular in nature, composed of a carbohydrate moiety responsible for tumor targeting and an aglycone that mediates DNA strand scission, different BLM analogues **2.29**,[118] **2.31** and **2.32** were prepared containing BLM disaccharide at altered positions.[119] The BLM analogues **2.31** and **2.32** were synthesized by Dr. Benjamin R. Schroeder.[119] The C-terminal disaccharide substituted

bleomycin **2.29** was prepared following previously reported procedures (Scheme 2.18).[118] The synthesis began with the selective deacetylation of the anomeric acetate of disaccharide **2.55** using hydrazine acetate, followed by activation as the *p*-nitrophenyl carbonate (**2.125**). Carbonate **2.125** was then coupled with Cu(II)•deglycoBLMA$_5$ (**2.123**) and was deprotected by the use of hydrazine to afford C-terminal modified Cu(II)•BLM **2.126**,[118] the latter of which was then subjected to demetallation with 15% EDTA to afford modified BLM **2.29**. The synthesis of the dye labeled analogue of C-terminal disaccharide substituted bleomycin **2.30** started with the coupling of the activated NHS ester of acid **2.127** with Cu(II)•deglycoBLMA$_5$ (**2.123**) to afford modified linker BLM **2.128** (Scheme 2.19). The Fmoc group of linker BLM **2.128** was removed using 20% piperidine in DMF, followed by coupling with activated carbonate **2.125** to provide modified linker BLM–disaccharide **2.129**. The butyloxycarbonyl group of disaccharide **2.129** was removed using TFA and then coupled with the NHS ester of Cy5** (**2.60**)[127] to give conjugate **2.130**. Demetallation of conjugate **2.130** with 15% EDTA afforded modified BLM–Cy5** **2.30**. The synthesis of dye labeled analogues of modified BLMs **2.31**[119] and **2.32**[119] started with metallation with $CuCl_2$ followed by coupling with the NHS ester of Cy5** (**2.60**),[127] which afforded conjugates **2.131** and **2.132** (Scheme 2.20). Successive demetallation of **2.131** and **2.132** using 15% EDTA afforded modified BLM–Cy5** conjugates **2.33** and **2.34**, respectively.

The cell binding/uptake of the various dye–conjugates was analyzed using a Zeiss Axiovert 200M inverted microscope, having a 40× oil objective. The binding/cellular uptake of BLM disaccharide–Cy5** conjugate (**2.3**), and disaccharide–Cy5** conjugates

2.4–**2.7** (Figure 2.10) in four cancer cell lines were quantified by fluorescence imaging. It was observed that disaccharide conjugate **2.6** having an *N*-carbamoyl group at the C-2 position of D-mannose exhibited the best binding/uptake profile in all four cancer cell lines studied (Figure 2.19). The binding/uptake of disaccharide conjugate **2.4** having an unmodified carbamoyl group at the C-2 position of D-mannose was found to be comparable to the binding/uptake of natural disaccharide conjugate **2.3** in all of the cancer cell lines studied (Figure 2.19), but disaccharide conjugate **2.7** having an *N*-carbamoyl group at the C-3 position of D-mannose showed significant binding in A498 kidney cancer cells and BxPC-3 pancreatic cancer cells and rather poor binding in A549 lung cancer cells and DU-145 prostate cancer cells. The binding/uptake of disaccharide conjugate **2.5** having an unmodified carbamoyl group at the C-4 position of D-mannose gave very poor results. It is obvious from the results that the position and orientation of the carbamoyl group in the BLM disaccharide is crucial for cell targeting. In common with the results of earlier studies,[18] little dye was observed to be associated with the cell surface of any of the tumor cell lines studied. The lack of cell surface fluorescence is consistent with the interpretation that internalization/uptake of the disaccharide–dye conjugate is rapid relative to cell surface binding. This interpretation is supported by the observation that microbubbles whose surface had been derivatized covalently with multiple BLM or BLM disaccharide molecules bound selectively to cultured cancer cells but could not be internalized due to their large sizes. Hence, only cell surface binding with no uptake was observed in case of the microbubbles derivatized BLM or BLM disaccharide.[18]

The cell binding/uptake of BLM monosaccharide–Cy5** (**2.8**) (Figure 2.11) was compared to the BLM disaccharide–Cy5** (**2.3**) conjugate (Figure 2.10). The binding/uptake of BLM monosaccharide–Cy5** (**2.8**) was much (~2-fold) greater in A549 lung cancer cells, A498 kidney cancer cells and MCF-7 breast carcinoma cells, essentially identical for BT-474 breast ductal carcinoma cells and BxPC-3 pancreas cells and much lower in DU-145 prostate cancer cells (Figure 2.20). The cell binding/uptake of decarbamoyl BLM monosaccharide−Cy5** conjugate (**2.9**) revealed that there was no uptake in any of the four cell lines tested. This confirmed that the carbamoyl group is indispensible for cell surface receptor interaction, and for facilitating selective cell uptake in tumor cells (Figure 2.21).

The cellular targeting and uptake of the Cy5** conjugate containing a cluster of three carbamoylmannose molecules (**2.10**) (Figure 2.11) indicated that the binding/uptake of the BLM monosaccharide–Cy5** trimer (**2.10**) was (1.6–2.3-fold) greater than that of the BLM monosaccharide–Cy5** conjugate (**2.8**) in each of six cancer cell lines tested (Figure 2.22), suggesting the involvement of a multivalent cell surface carbohydrate receptor. As for the BLM disaccharide–Cy5** conjugate (**2.3**), the BLM monosaccharide–Cy5** conjugate (**2.8**) presumably first binds to a cell surface receptor followed by internalization. This model is supported by the results obtained with the BLM monosaccharide–Cy5** trimer (**2.10**), which showed enhanced uptake in each of six cell lines. The majority of fluorescence within the cells was found to colocalize with DAPI, suggesting that the conjugates were present within the nucleus. The increase in binding/uptake observed for the BLM monosaccharide−Cy5** trimer provides evidence

of the nature of the receptor, and a tool for further studying and exploiting the tumor targeting properties of 3-*O*-carbamoylmannose.

The cell binding/uptake study of BLM monosaccharide–Cy5** (**2.8**) at different incubation temperatures (4 and 37 °C) was found to be quite similar (Figure 2.23), suggesting that the mechanism of cellular internalization of the BLM disaccharide−Cy5** conjugate (**2.3**), which exhibits temperature-dependent binding/uptake, may be different from the mechanism of cellular internalization of BLM monosaccharide−Cy5** conjugate (**2.8**), at least in this regard.

The cell binding/uptake of BLM monosaccharide–Cy5** conjugate (**2.8**), and the modified monosaccharide–Cy5** conjugates **2.11**–**2.16** (Figure 2.12) showed that the monosaccharide conjugates **2.11** and **2.13** having *N*-methylated and *N*-butylated carbamoyl groups at the C-2 position of D-mannose exhibited best binding/uptake profiles in all six cancer cell lines (Figure 2.24). It was also evident that monosaccharide conjugates **2.12** and **2.14** having an *N,N*-dimethylated and *N*,*N*-pyrrolidinylated carbamoyl groups at the C-2 position of D-mannose, respectively, exhibitted almost no binding/uptake in any of the tumor cell lines tested. This result clearly establishes the involvement of steric factors in the cell surface binding interaction and internalization. Binding/uptake exhibited by the sterically hindered monosaccharide conjugate **2.15** having an *N*-hexylated carbamoyl group at the C-2 position of D-mannose was very low in A549 lung cancer cells, DU-145 prostate cancer and MCF-7 breast cancer cells, but comparable to BLM monosaccharide–Cy5** (**2.8**) in the BxPC-3 pancreatic cancer line. The monosaccharide conjugate **2.16**, having an *N*-methylated carbamoyl group at the C-3

position of D-mannose, exhibited considerably lower uptake than either the BLM monosaccharide–Cy5** conjugate (**2.8**) or monosaccharide conjugates **2.11** having an *N*-methylated carbamoyl group at the C-2 position of D-mannose in A549 lung cancer cells and MCF-7 breast cancer cells. This was consistent with the results obtained for disaccharide conjugates **2.6** and **2.7** (Figure 2.10) having an *N*-methylated carbamoyl group at the C-2 and C-3 positions of D-mannose, respectively. The efficient cellular uptake of monosaccharide conjugates **2.11** and **2.13** make them potentially good candidates for targeted drug delivery or as *in vivo* imaging agents.

Further, to choose an optimal fluorophore in lieu of Cy5**, several dye–monosaccharide conjugates were tested for binding/uptake in cancer cells. It was found that BLM monosaccharide–Cy5 (**2.18**) and BLM monosaccharide−$Cy5^{GE}$ (**2.21**) (Figure 2.13) conjugates showed the highest efficiency of binding and uptake in all the cancer cell lines tested (Figure 2.25). In case of the other dye–monosaccharide conjugates, the binding/uptake was either very low or not uniformly efficient for all cell lines studied. However, the free dye Cy5 itself showed significant background binding/uptake in representative cell lines (Figure 2.26). Hence, $Cy5^{GE}$ was chosen as the fluorophore to be used in future experiments. Subsequently, the cell binding/uptake of BLM monosaccharide–$Cy5^{GE}$ (**2.21**) (Figure 2.13) was compared to BLM disaccharide–$Cy5^{GE}$ (**2.25**) conjugate (Figure 2.14) in A549 lung cancer cells and BxPC-3 pancreatic cancer cell lines and their matched normal cells. The binding/uptake was observed to be significantly higher in both cancer cell lines relative to the binding/uptake observed for the matched normal controls cells (Figure 2.27). The binding/uptake of the BLM

monosaccharide–Cy5^{GE} (**2.21**) was much (~2-fold) greater in A549 lung cancer cells than the disaccharide **2.25** and lower than BLM disaccharide–Cy5^{GE} conjugate (**2.25**) in DU-145 prostate cancer cells, consistent with the results obtained in the case of the Cy5** conjugates of BLM monosaccharide (**2.8**) (Figure 2.11) and BLM disaccharide (**2.3**) (Figure 2.10). These findings were further confirmed by the observation that the decarbamoyl BLM monosaccharide–Cy5^{GE} conjugate (**2.27**) failed to show any binding/uptake in any of three cancer cell lines (Figure 2.28).

It was evident from the MTT assay of the modified BLMs in DU-145 prostate cancer cells that BLM analogue **2.29**[118] (Figure 2.15) was less cytotoxic than BLM A_5 but significantly more cytotoxic than deglycoBLM or BLM analogue **2.32**[119] (Figure 2.16); deglycoBLM A_5, lacking the tumor-targeting disaccharide, was the least toxic (Figure 2.29). To provide additional evidence in support of the basis for expression of cytotoxicity by glycosylated deglycoBLMs, the modified BLM–Cy5** conjugates **2.30** and **2.34** were studied in four cancer cell lines. The result from the quantification data showed that the binding/uptake of modified BLM–Cy5** conjugate **2.30** (Figure 2.15) was higher than both deglycoBLM–Cy5** (**2.2b**) and the modified BLM–Cy5** conjugate **2.34** (Figure 2.16), but lower than that of BLM–Cy5** (**2.2a**) (Figure 2.30).

Hence, the effect of inserting the BLM disaccharide to different positions of deglycoBLM was investigated. Attachment of BLM disaccharide to the β-hydroxyhistidine moiety, as in the natural product, significantly amplified cytotoxicity toward cultured DU-145 prostate cancer cells (Figure 2.29). The relationship between cytotoxicity and BLM sugar-mediated uptake was confirmed by measuring the actual

extent of internalization of cytotoxic BLM analogues conjugation to the cyanine dye (Figure 2.30). These findings establish that BLM is a modular molecule, comprised of a tumor cell targeting moiety (the saccharide) attached to a cytotoxic DNA cleaving domain (the BLM aglycone) and further proves that BLM disaccharide plays a key role in the targeted cell surface binding followed by internalization of the drug.

From the viewpoint of drug discovery, the current findings are of great interest. It was demonstrated that 3-*O*-carbamoyl-D-mannose, one of the two sugars present in the BLM disaccharide, is sufficient to mediate selective tumor targeting and internalization with absolute requirement of the carbamoyl moiety. The development of bleomycin analogues containing disaccharide such as **2.6** or monosaccharides such as **2.11** and **2.13** or trivalent cluster of monosaccharide such as **2.10** in lieu of the normal BLM disaccharide **2.3** may show superior antitumor properties to those of BLM itself. Additionally, development of multivalent clusters of the disaccharide such as **2.6** or monosaccharides such as **2.11** and **2.13** with improved tumor targeting actions would provide more efficient delivery of attached cargoes similar to the promising strategy developed using antibodies in antibody–drug conjugates.[142-144]

Another possibility for the utilization of optimized BLM saccharides might be to serve as PET biomarkers for tumor diagnostic studies. For this purpose, fluorinated BLM disaccharide was synthesized as a reference standard. The synthesis of fluorinated BLM disaccharide started by the coupling of activated disaccharide phosphate ester **2.56** with the benzyl protected linker **2.133** to afford BLM disaccharide–linker **2.134**. Debenzylation of primary amine of **2.134** followed by fluorination with XtalFluor-E[134]

gave fluorinated disaccharide **2.135**. Complete deacetylation with sodium methoxide in methanol afforded fluorinated BLM disaccharide **2.35**. Similarly, the fluorinated decarbamoyl BLM disaccharide was synthesized by coupling phosphate ester **2.120** with the benzyl protected linker **2.133** to afford decarbamoyl BLM disaccharide–linker **2.136**. Debenzylation of primary amine of **2.136** followed by fluorination with XtalFluor-E[134] afforded fluorinated disaccharide **2.137**, the latter of which was deacetylated with sodium methoxide in methanol to afford fluorinated decarbamoyl BLM disaccharide **2.36**. These analogues were sent to our collaborators for use as authentic standard.

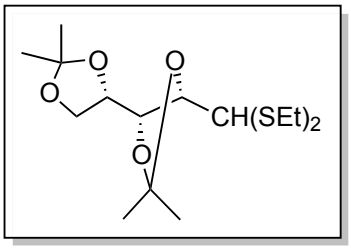
2.4. Experimental Procedures

Materials. The chemicals used were purchased from Aldrich Chemical Co., Sigma Chemical Co. or Combi-Blocks and were used without further purification. Anhydrous methanol, DMF, acetonitrile and toluene were used as purchased. Tetrahydrofuran and dichloromethane were distilled from sodium/benzophenone and calcium hydride, respectively. The cyanine dye, Cy5** was obtained from our collaborator Dr. Michael Rishel at General Electric Co. SulphoCy5, Cy5 and Cy7 dyes were purchased from Lumiprobe Co., and Alexa Fluor 647 was purchased from Life Technologies Co. The cyanine dyes, $Cy5^{GE}$ and $Cy5.5^{GE}$ were purchased from General Electric Co.

General Experimental Procedures. Reactions were carried out under an argon atmosphere in flame-dried glassware. Flash column chromatography was carried out using silica gel (Silicycle R10030B, 60 particle size, 230-400 mesh), applying a low-pressure stream of nitrogen or dry air. Analytical thin layer chromatographic separations

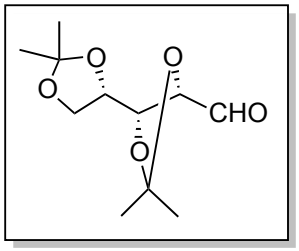
were carried out on glass plates coated with silica gel (60 particle size F254, Silicycle TLG-R10011B-323) and were developed by immersing the plates in a solution of ceric ammonium molybdate (CAM) stain followed by heating with a heat gun.

Instrumentation. HPLC was performed using an Agilent 1100 series instrument. ^{1}H and ^{13}C NMR spectra were recorded on Varian Inova 400 MHz and 100 MHz, respectively, using $CDCl_3$ as solvent and internal standard. ^{1}H NMR and ^{13}C NMR chemical shifts were reported relative to a residual $CHCl_3$ at 7.26 ppm and 77.16 ppm, respectively. Splitting patterns are designated as s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet; q, quartet and quint, quintet. High resolution mass spectrometric data were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State Mass Spectrometry Facility.



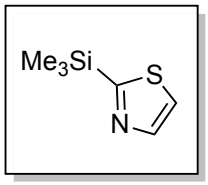
2,3,4,5-Di-*O*-isopropylidene-L-xylose Diethyl Dithioacetal (2.38).[124] To a vigorously stirred suspension of 10.0 g (66.6 mmol) of L-xylose (**2.37**) in 4.0 mL of conc HCl was added 14.8 mL (12.4 g, 200 mmol) of ethanethiol. The reaction mixture was stirred at room temperature until the two-layer mixture gave a homogenous solution (usually after 15-20 min). The reaction was then diluted with 200 mL of acetone and stirred for 5 h, at which time the solution was neutralized with saturated aq NH_4OH solution and co-

evaporated with six 20-mL portions of toluene to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (25 × 8 cm). Elution with 1:1 hexanes–ethyl acetate gave **2.38** as a colorless syrup: yield 14.5 g (65%); $[\alpha]_D$ + 57.2 (*c* 1.8, C_6H_6), lit.[145] $[\alpha]_D$ + 51.3 (*c* 1.8, C_6H_6); silica gel TLC R_f 0.59 (3:1 ethyl acetate–hexanes); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.26-1.28 (m, 6H), 1.37 (s, 3H), 1.42 (2s, 6H), 1.46 (s, 3H), 2.74 (m, 4H), 3.92 (m, 2H), 4.04 (m, 1H), 4.13 (m, 1H) and 4.32 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.26, 14.34, 24.9, 25.3, 25.6, 26.1, 27.1, 27.3, 53.0, 65.9, 75.2, 78.7, 80.1, 109.5 and 110.0.



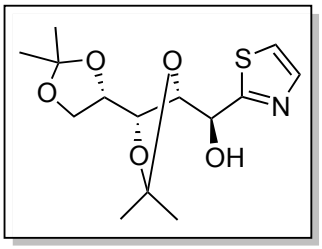
2,3,4,5-Di-*O*-isopropylidene-aldehydo-L-xylose (2.39).[124] To a stirred solution containing 6.37 g (18.9 mmol) of thioacetal **2.38** in 64 mL of acetone diluted with 6.4 mL of water was added 9.44 g (43.6 mmol) of yellow mercury(II) oxide and 9.27 g (34.1 mmol) of mercury(II) chloride. The reaction mixture was stirred at 55 °C for 2 h and then allowed to cool to room temperature. The solvent was filtered through a pad of Celite 545® and concentrated under diminished pressure to afford a crude residue. The residue was suspended in three 100-mL portions of dichloromethane and filtered through a pad of Celite 545®. The organic layer was washed with 100 mL of 1 M aq KI, dried ($MgSO_4$), filtered and then concentrated under diminished pressure to afford the aldehyde **2.39** as

oil: yield 2.91 g (67%); silica gel TLC R_f 0.24 (1:1 hexanes–ethyl acetate). The aldehyde was used for the next reaction immediately.



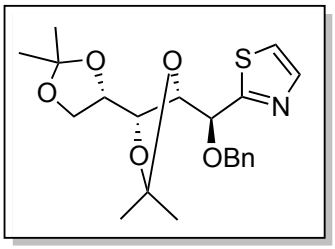
2-(Trimethylsilyl)thiazole (2.40).[125] A 500-mL four-necked round-bottomed flask was equipped with two 100-mL pressure-equalizing dropping funnels and a low temperature thermometer. The anhydrous apparatus was kept under argon and cooled to −78 ºC. The flask was charged with 98 mL of freshly distilled diethyl ether and 33 mL (83.0 mmol) of a 2.5 M solution of *n*-BuLi in hexane. One of the two dropping funnels was charged with 6.80 mL (12.4 g, 75.0 mmol) of 2-bromothiazole in 25 mL of diethyl ether and the other with 9.60 mL (8.26 g, 75.0 mmol) of chlorotrimethylsilane in 25 mL of diethyl ether. While the solution in the flask was stirred, 2-bromothiazole was added dropwise at −78 ºC over a period of 1 h. After 20 min of additional stirring, chlorotrimethylsilane was added dropwise at −78 ºC over a period of 30 min and the stirring was continued for 1 h at −78 ºC. The resulting mixture was then allowed to warm to room temperature, quenched with 100 mL saturated aq $NaHCO_3$ and extracted with three 200-mL portions of diethyl ether. The combined organic layer was dried (Na_2SO_4), filtered, and concentrated under diminished pressure (temperature not exceeding 40 °C). The residue was distilled from a 100-mL flask at diminished pressure in a Claisen apparatus at 45 °C. The pure product **2.40** was isolated as a clear solution: yield 9.91 g (83%); ^{1}H NMR

($CDCl_3$, 400 MHz) δ 0.39 (s, 12H), 7.50 (d, 1H, J = 3.0 Hz) and 8.09 (d, 1H, J = 2.9 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 1.0, 127.3, 145.6 and 174.2.



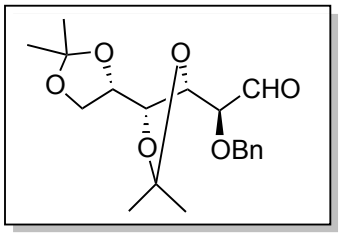
1,2,3,4-Bis-*O*-(1-methylethylidene)-5-*C*-2-thiazolyl-(5*S*)-D-xylitol (2.41).[124] To a stirred solution containing 2.91 g (12.6 mmol) of aldehyde **2.39** in 50 mL of anh dichloromethane cooled to −42 °C was added 2.60 mL (16.4 mmol) of 2-(trimethylsilyl)thiazole (**2.40**) dropwise over a period of 15 min. The solution was stirred at 0 °C for 1 h and then concentrated under diminished pressure to afford a crude residue. The residue was dissolved in 50 mL of anh THF and treated with 3.99 g (12.6 mmol) of *n*-$Bu_4NF•3H_2O$ at 20 °C for 30 min and then concentrated under diminished pressure. The residue was diluted by the addition of 250 mL of dichloromethane and washed with three 50-mL portions of water, dried (Na_2SO_4), filtered and then concentrated under diminished pressure to yield compound **2.41** as a crude residue. Recrystallization of the residue from cyclohexane afforded alcohol **2.41** as a colorless solid: yield 2.39 g (60%); $[\alpha]_D$ + 18.2 (*c* 1.1, $CHCl_3$), lit.[124] $[\alpha]_D$ + 18.5 (*c* 1.1, $CHCl_3$); silica gel TLC R_f 0.24 (1:1 hexanes–ethyl acetate); ^1H NMR ($CDCl_3$, 400 MHz) δ 1.28 (s, 3H), 1.36 (s, 6H), 1.40 (s, 3H), 3.67 (t, 1H, J = 6.6 Hz), 3.82 (m, 2H), 4.12 (dd, 1H, J = 7.2 and 3.6 Hz), 4.32 (m, 1H), 4.56 (br s, 1H), 5.10 (d, 1H, J = 5.5 Hz), 7.30 (d, 1H, J = 3.2 Hz) and 7.71 (d, 1H, J

= 3.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 25.6, 26.1, 27.07, 27.13, 65.7, 71.7, 75.5, 77.4, 79.8, 109.5, 110.2, 119.7, 142.1 and 170.9.



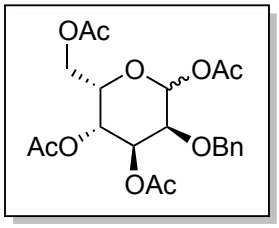
1,2,3,4-Bis-*O*-(1-methylethylidene)-5-*O*-(phenylmethyl)-5-*C*-2-thiazolyl-(5*S*)-D-xylitol (2.42).[124] To a solution containing 2.11 g (6.69 mmol) of alcohol **2.41** in anh DMF cooled to 0 °C was added 0.54 g (60% dispersion in oil, 13.4 mmol) of NaH portionwise and the reaction mixture was stirred at 0 °C for 30 min. To this solution was added 1.20 mL (1.73 g, 9.51 mmol) of benzyl bromide and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was quenched by the addition of 1.2 mL of methanol, then stirred for 10 min and diluted with 40 mL of distilled water. The aqueous layer was extracted with three 100-mL portions of ether. The combined organic layer was dried ($MgSO_4$), filtered and concentrated under diminished pressure to afford a crude residue, which was purified by flash chromatography on a silica gel column (25 × 4 cm). Elution with 6:1 hexanes–ethyl acetate gave ether **2.42** as a colorless solid: yield 1.40 g (60%); $[\alpha]_D$ −32.2 (*c* 1.1, $CHCl_3$), lit.[124] $[\alpha]_D$ −32.3 (*c* 1.1, $CHCl_3$); silica gel TLC R_f 0.56 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20 (s, 3H), 1.25 (s, 3H), 1.29 (s, 3H), 1.33(s, 3H), 3.65 (m, 1H), 3.78 (m, 1H), 3.91 (m, 1H), 3.98 (m, 1H), 4.35 (dd, 1H, *J* = 7.3 and 2.5 Hz), 4.44 (d, 1H, *J* = 12.1 Hz), 4.63 (d, 1H, *J* = 12.1 Hz), 4.80 (d, 1H, *J* = 4.8 Hz), 7.21-7.28 (m, 5H), 7.32 (d, 1H, *J* = 3.2 Hz) and 7.78 (d, 1H, *J* = 3.2 Hz);

^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.0, 25.5, 26.03, 26.05, 26.7, 27.0, 65.5, 72.2, 75.5, 77.7, 78.5, 79.4, 109.4, 110.3, 120.1, 127.9, 128.1, 128.3, 136.8, 142.4 and 168.9.

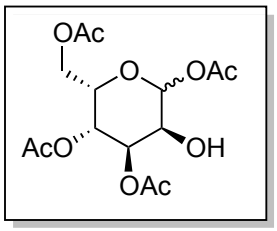


2-*O*-Benzyl-3,4,5,6-di-*O*-isopropylidene-aldehydo-L-gulose (2.43). [124] A solution containing 4.18 g (10.3 mmol) of *O*-benzyl ether **2.42** and 20.0 g of activated 4Å molecular sieves dissolved in 100 mL of anh acetonitrile was stirred at 20 °C for 10 min and then 1.52 mL (2.20 g, 13.4 mmol) of methyl triflate was added dropwise. The suspension was stirred at room temperature for 15 min and then concentrated under diminished pressure to afford the crude *N*-methylthiazolium salt. To a stirred solution of the crude *N*-methylthiazolium salt in 100 mL of anh methanol cooled to 0 °C was added 0.86 g (22.7 mmol) of sodium borohydride. The reaction mixture was stirred at room temperature for 5 min and diluted with 10 mL of acetone. The solvent was filtered through a pad of Celite 545® and concentrated under diminished pressure to afford a crude mixture of thiazolidines. The residue was dissolved in 88 mL of acetonitrile and 8 mL of water and treated with 6.56 g (82.5 mmol) of CuO and 1.76 g (10.3 mmol) of $CuCl_2 \cdot 2H_2O$ under vigorous stirring. The reaction mixture was stirred at 20 °C for 15 min, filtered through a pad of Celite 545® and then concentrated under diminished pressure to remove acetonitrile and most of the water (bath temperature not exceeding 40 °C) to afford a crude residue. The brown residue was triturated with four 100-mL portions of ether and the liquid phase was pipetted and filtered through a pad of Florisil®

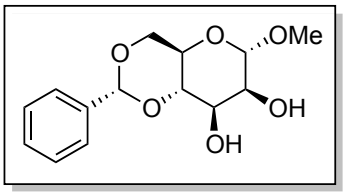
(60–100 mesh) to afford a colorless solution. After a further washing of Florisil® with 100 mL of ethyl acetate, the combined organic layer was concentrated under diminished pressure to yield the aldehyde **2.43** as a brown syrup; yield 3.40 g (94%); silica gel TLC R_f 0.47 (2:1 hexanes–ethyl acetate). The aldehyde was used for the next reaction immediately.



1,3,4,6-Tetra-*O*-acetyl-2-*O*-benzyl-L-gulopyranose (2.44).[124] To 3.40 g (9.70 mmol) of aldehyde **2.43** was added 67.4 mL of 4:1 acetic acid-water and stirred at 100 °C for 40 min. The reaction was cooled and co-evaporation with three 20-mL portions of toluene to afford the crude 2-*O*-benzyl-L-gulose as a mixture of β-pyranose, α-pyranose, and furanose forms. To the solution of crude residue in 25 mL of dry pyridine were added 1.18 g (4.02 mmol) of DMAP and 25 mL of acetic anhydride. The reaction was stirred overnight at room temperature. The mixture was concentrated under diminished pressure and purified by flash chromatography on a silica gel column (30 × 4 cm). Elution with 3:1 hexanes–ethyl acetate afforded benzyl ether **2.44** as a yellow oil: yield 2.54 g (60%); silica gel TLC R_f 0.44 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.01 (s, 3H), 2.05 (s, 3H), 2.08 (s, 3H), 2.11 (s, 3H), 3.64 (dd, 1H, J = 8.3 and 4.9 Hz), 4.01 (m, 2H), 4.28 (m, 1H), 4.49 (d, 1H, J = 11.9 Hz), 4.63 (d, 1H, J = 11.9 Hz), 4.95 (dd, 1H, J = 3.9 and 2.5 Hz), 5.44 (m, 1H), 5.89 (d, 1H, J =8.3 Hz) and 7.23-7.34 (m, 5H).

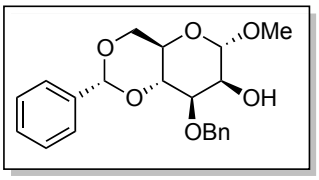


1,3,4,6-Tetra-*O*-acetyl-L-gulopyranose (2.45).[124] To a solution containing 2.15 g (4.90 mmol) of **2.44** in 30 mL of ethyl acetate was added 1.06 g of 10% Pd/C and the reaction mixture was stirred overnight under 1 atm of H_2 (g). The solvent was filtered through a pad of Celite 545® and concentrated under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (15 × 4 cm). Elution with 1:1 hexanes–ethyl acetate afforded **2.45** as a colorless oil: yield 1.67 g (98%); silica gel TLC R_f 0.16 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.91 (s, 3H), 2.00 (s, 3H), 2.03 (s, 6H), 3.22-3.52 (br s, 1H), 3.80 (dd, 1H, J = 8.4 and 3.5 Hz), 3.94 (m, 1H), 4.02 (m, 1H), 4.16 (m, 1H), 4.85 (m, 1H), 5.19 (t, 1H, J = 3.6 Hz) and 5.70 (d, 1H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.4, 20.5, 20.6, 20.8, 61.6, 66.2, 67.5, 69.5, 70.9, 92.1, 169.4, 169.6, 169.7 and 170.5.



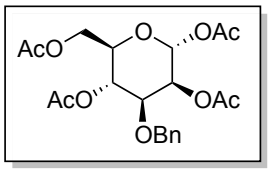
Methyl-4,6-*O*-benzylidene-α-D-mannopyranoside (2.47).[21] To a solution containing 7.00 g (36.0 mmol) of methyl-α-D-mannopyranoside (**2.46**) in 85 mL of anh DMF was added 5.60 mL (5.68 g, 37.3 mmol) of benzaldehyde dimethyl acetal and a catalytic amount of *p*-TsOH. The reaction mixture was stirred at 60 °C under diminished pressure

for 1 h, allowed to cool to room temperature and then poured into a stirring mixture of 120 mL of ethyl acetate and 100 mL saturated aq $NaHCO_3$. The organic layer was washed with three 50-mL portions of brine, dried ($MgSO_4$) and filtered. The solvent was concentrated under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (30 × 5 cm). Elution with 4:1 hexanes–ethyl acetate afforded acetal **2.47** as a colorless solid: yield 7.13 g (70%); silica gel TLC R_f 0.31 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.38 (s, 3H), 3.78 (m, 2H), 3.87 (m, 1H), 3.98 (m, 2H), 4.25 (m, 1H), 4.72 (m, 1H), 5.55 (s, 1H), 7.36 (m, 3H) and 7.47 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.2, 63.3, 68.8, 69.0, 71.1, 79.0, 101.6, 102.4, 126.5, 128.6, 129.5 and 137.4.



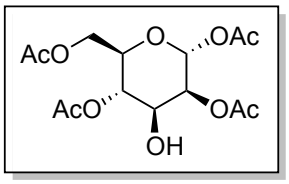
Methyl 4,6-*O*-Benzylidene-3-*O*-benzyl-α-D-mannopyranoside (2.48).[21] To a solution of 2.50 g (8.86 mmol) of acetal **2.47** in 76 mL of anh methanol was added 2.42 g (9.71 mmol) of Bu_2SnO and the solution was heated at reflux for 1.5 h to afford a clear solution. The solvent was removed under diminished pressure and the resulting solid was dried under vacuum overnight to remove traces of methanol. The white residue was dissolved in 76 mL of anh DMF, treated with 2.10 mL (17.7 mmol) of BnBr, and warmed to 100 °C for 30 min. The cooled reaction mixture was poured into a stirring solution of 125 mL of ethyl acetate and 75 mL of saturated aq $NaHCO_3$ and the white precipitate of tin oxide was filtered off. The aqueous layer was extracted with two 50-mL portions of

ethyl acetate. The combined organic extract was washed with 50 mL of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 4 cm). Elution with 5:1→3:1 hexanes–ethyl acetate afforded alcohol **2.48** as a colorless oil: yield 2.24 g (68%); silica gel TLC R_f 0.30 (3:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.38 (s, 3H), 3.77 (m, 3H), 4.05 (m, 2H), 4.27 (m, 1H), 4.70 (m, 2H), 4.84 (m, 1H), 5.62 (s, 1H) and 7.28-7.52 (m, 10H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 54.9, 63.3, 68.8, 69.8, 73.0, 75.7, 78.8, 101.2, 101.6, 126.1, 127.8, 128.2, 128.4, 128.4, 128.9, 137.6 and 138.0.

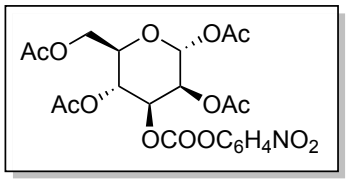


1,2,4,6-Tetra-*O*-acetyl-3-*O*-benzyl-α-D-mannopyranose (2.49).[21] To a solution of 1.60 g (4.30 mmol) of alcohol **2.48** in 26 mL of acetic anhydride was added 6 drops of concentrated H_2SO_4 and stirred overnight at room temperature. The solution was poured into a stirring solution of 100 mL of ethyl acetate and 70 mL of saturated aq $NaHCO_3$. The organic layer was washed with 50 mL of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 4 cm). Elution with 4:1→2:1 hexanes–ethyl acetate afforded peracetylated benzyl ether **2.49** as a yellow oil: yield 1.60 g (85%); silica gel TLC R_f 0.34 (3:1 hexanes–ethyl acetate) and 0.5 (1:1 in hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.99 (s, 3H), 2.04 (s, 3H), 2.08 (s, 3H), 2.12 (s, 3H), 3.83 (dd, 1H, J = 9.7 and 3.4 Hz), 3.90 (m, 1H), 4.04 (m, 1H), 4.19 (m, 1H), 4.41 (m, 1H), 4.64 (m, 1H), 5.25 (m, 1H), 5.34 (dd, 1H, J = 3.4 and 2.1), 6.06 (d, 1H, J = 2.0 Hz) and 7.21-7.33

(m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.79, 20.84, 20.89, 20.94, 62.5, 66.9, 67.1, 70.9, 71.6, 74.2, 91.1, 127.8, 128.0, 128.5, 137.5, 168.1, 169.6, 170.0 and 170.8.

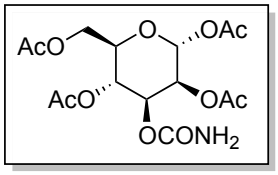


1,2,4,6-Tetra-*O*-acetyl-α-D-mannopyranose (2.50).[21] To a solution of 1.10 g (2.51 mmol) of benzyl ether **2.49** in 30 mL of ethyl acetate was added a catalytic amount of $Pd(OH)_2/C$ and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3.5 cm). Elution with 2:1→1:1 hexanes–ethyl acetate afforded alcohol **2.50** as a colorless oil: yield 856 mg (98%); silica gel TLC R_f 0.13 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.07 (s, 3H), 2.11 (s, 3H), 2.12 (s, 3H), 2.17 (s, 3H), 2.48 (d, 1H, J = 8 Hz), 3.91 (m, 1H), 4.18 (m, 2H), 4.19 (dd, 1H, J = 12.3 and 4.8 Hz), 5.07 (m, 1H), 5.13 (m, 1H) and 6.10 (d, 1H, J = 1.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.8, 20.9, 20.96, 20.98, 62.3, 68.5, 68.9, 70.4, 71.2, 90.5, 168.2, 170.3, 170.8 and 171.2.



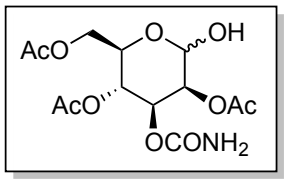
1,2,4,6-Tetra-*O*-acetyl-3-*O*-((*p*-nitrophenyl)carbamoyl)-α-D-mannopyranose (2.51).[21] To a solution of 660 mg (1.89 mmol) of alcohol **2.50** in 7.0 mL of dry pyridine was added 926 mg (7.58 mmol) of DMAP and 1.53 g (7.58 mmol) of *p*-nitrophenyl

chloroformate. The reaction was stirred at 40 °C for 2 h at which time it was poured into a mixture of 40 mL ethyl acetate and 10 mL of water. The organic layer was washed with three 10-mL portions of 1N HCl, 10 mL of saturated aq $NaHCO_3$ and 10 mL of brine. The solution was dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (30 × 3.5 cm). Elution with 4:1→1:2 hexanes–ethyl acetate afforded carbonate **2.51** as a light yellow oil: yield 788 mg (81%); silica gel TLC R_f 0.53 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.11 (s, 3H), 2.12 (s, 3H), 2.17 (s, 3H), 2.21 (s, 3H), 4.08 (m, 2H), 4.25 (m, 1H), 5.15 (m, 1H), 5.42 (m, 2H), 6.11 (s, 1H), 7.34 (d, 2H, J = 9.2 Hz) and 8.23 (d, 2H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.8, 20.86, 20.89, 20.92, 62.1, 65.2, 67.7, 70.8, 74.4, 90.8, 122.0, 125.5, 125.7, 151.9, 155.4, 168.0, 169.5, 170.1 and 170.7.

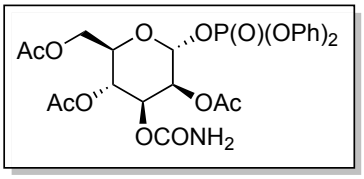


1,2,4,6-Tetra-*O*-acetyl-3-*O*-carbamoyl-α-D-mannopyranose (4.52).[21] To a solution of 630 mg (1.22 mmol) of carbonate **4.51** in 33 mL anh dichloromethane was added 16 mL of anh THF saturated with NH_3 (g). The reaction was stirred at room temperature for 2.5 h at which time the TLC indicated that the reaction was complete. The solution was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (24 × 3.5 cm). Elution with 2:1→1:2 hexanes–ethyl acetate afforded carbamate **4.52** as a colorless oil: yield 450 mg (94%); silica gel TLC R_f 0.13 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.03 (s, 3H),

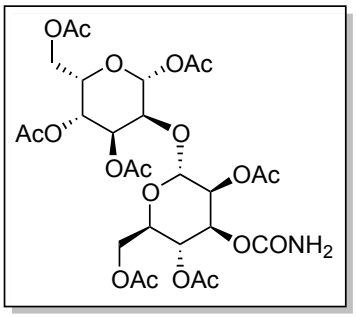
2.04 (s, 3H), 2.12 (s, 3H), 2.13 (s, 3H), 4.00-4.10 (m, 2H), 4.24 (dd, 1H, J = 12.6 and 5.0), 5.00 (br s, 2H), 5.18-5.30 (m, 3H) and 6.04 (d, 1H, J = 1.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7, 20.78, 20.81, 20.9, 62.1, 65.7, 68.8, 69.7, 70.6, 90.6, 155.4, 168.2, 169.76, 169.84 and 170.7.



2,4,6-Tri-*O*-acetyl-3-*O* carbamoyl-α,β-D-mannopyranose (2.53).[21] To a solution of 369 mg (0.93 mmol) of carbamate **2.52** in 5.7 mL of DMF was added 120 mg (1.32 mmol) of hydrazine acetate. The solution was stirred at room temperature for 2 h. The reaction was diluted with 60 mL of ethyl acetate and washed with 30 mL of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 1:1→1:3 hexanes–ethyl acetate afforded pyranoside **2.53** as a colorless oil: yield 249 mg (76%); silica gel TLC R_f 0.32 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.07 (s, 3H), 2.11 (s, 3H), 2.16 (s, 3H), 4.15 (m, 1H), 4.23 (m, 2H), 4.83 (s, 2H) and 5.25 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.9, 21.1, 62.7, 66.4, 68.8, 69.8, 70.5, 77.4, 92.4, 155.3, 170.16, 170.25 and 170.9.

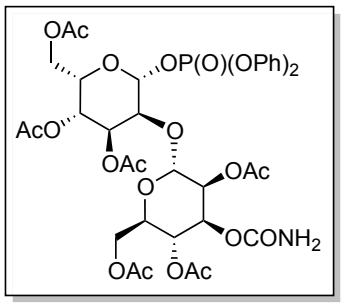


2,4,6-Tri-*O*-acetyl-3-*O*-carbamoyl-α-D-mannopyranosyl Diphenyl Phosphate (2.54).[21,126] To a solution of 189 mg (0.54 mmol) of pyranoside **2.53**, 84.0 mg (0.69 mmol) of DMAP and 0.75 mL (5.40 mmol) of dry triethylamine in 2.6 mL of dry dichloromethane at 0 °C was added 1.11 mL (5.28 mmol) of diphenyl chlorophosphate dropwise. The solution was stirred at 0 °C for 1.5 h and was poured into a mixture of 30 mL of ethyl acetate and 15 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 10-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (18 × 3 cm). Elution with 2:1→1:3 hexanes–ethyl acetate afforded the phosphate ester **2.54** as a colorless oil: yield 288 mg (92%); silica gel TLC R_f 0.51 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.98 (s, 3H), 2.06 (s, 3H), 2.16 (s, 3H), 3.92 (dd, 1H, J = 12.4 and 1.6 Hz), 4.08 (m, 1H), 4.19 (dd, 1H, J = 12.0 and 4.8 Hz), 4.70 (br s, 2H), 5.32 (m, 3H), 5.88 (dd, 1H, J = 6.5 and 1.6 Hz), 7.20-7.27 (m, 6H) and 7.34-7.38 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7, 20.8, 20.9, 61.8, 65.4, 69.2, 69.3, 70.8, 96.2, 120.18, 120.24, 120.3, 120.4, 125.9, 126.0, 130.08, 130.13, 155.0, 169.6, 169.9 and 170.7.



1,3,4,6-Tetra-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl-α-D-mannopyranosyl)-α-L-gulopyranoside (2.55).[21] To a solution of 147 mg (0.25 mmol) of phosphate ester **2.54**

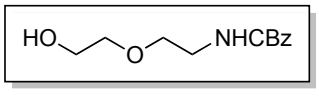
and 70.0 mg (0.20 mmol) of gulose alcohol **(2.45)** in 4.3 mL of anh dichloromethane was added 82.0 μL (102 mg, 0.46 mmol) of TMSOTf at 0 °C. The reaction mixture was stirred at 0 °C for 20 min, at which time it was poured into a mixture of 50 mL of ethyl acetate and 20 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 20-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 3 cm). Elution with 1:1→1:3 hexanes–ethyl acetate afforded disaccharide **2.55** as a colorless oil: yield 90.0 mg (66%); silica gel TLC R_f 0.27 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.04 (2s, 6H), 2.11(s, 3H), 2.12 (2s, 6H), 2.14 (s, 3H), 2.18 (s, 3H), 3.98 (dd, 1H, J = 8.4 and 3.6 Hz), 4.11 (m, 3H), 4.23 (m, 2H), 4.35 (m, 1H), 4.69 (br s, 2H), 4.99 (m, 2H), 5.06 (m, 1H), 5.10 (m, 1H), 5.24 (t, 1H, J = 3.6 Hz), 5.43 (t, 1H, J = 3.6 Hz) and 5.87 (d, 1H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.8, 20.81, 20.9, 20.94, 61.5, 62.2, 65.6, 66.0, 67.80, 69.20, 69.3, 69.8, 69.9, 71.5, 90.8, 95.1, 155.1, 168.8, 169.37, 169.39, 169.5, 169.9, 170.6 and 170.7.



3,4,6-Tri-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl-α-D-mannopyranosyl)-α-L-gulopyranosyl Diphenyl Phosphate (2.56).[21] To a solution of 90.0 mg (0.13 mmol) of disaccharide **2.55** in 1.4 mL of anh DMF was added 17.0 mg (0.18 mmol) of hydrazine acetate. The solution was stirred at room temperature for 2.5 h and diluted with 15 mL of

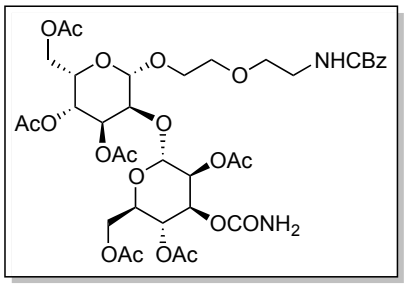
ethyl acetate. The organic solution was washed with 15 mL of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure to afford the product as a colorless oil: yield 72.0 mg; silica gel TLC R_f 0.25 (1:3 hexanes–ethyl acetate).

To a solution of above residue, 18.0 mg (0.14 mmol) of DMAP and 0.16 mL (1.13 mmol) of dry triethylamine in 2.9 mL of anh dichloromethane at 0 °C was added 0.22 mL (1.08 mmol) of diphenyl chlorophosphate dropwise. The solution was stirred at 0 °C for 1.5 h and was poured into a mixture of 20 mL of ethyl acetate and saturated 10 mL of aq $NaHCO_3$. The organic layer was washed with two 10-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (22 × 3cm). Elution with 2:1→1:3 hexanes–ethyl acetate afforded the phosphate ester **2.56** as a colorless oil: yield 75.0 mg (65% over two steps); silica gel TLC R_f 0.51 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.70 (s, 3H), 1.97 (s, 3H), 2.05 (s, 3H), 2.11 (s, 3H), 2.13 (s, 3H), 2.19 (s, 3H), 4.13 (m, 5H), 4.31 (m, 2H), 4.76 (s, 2H), 4.96 (m, 1H), 4.98 (m, 1H), 5.18 (m, 3H), 5.43 (m, 1H), 5.69 (m, 1H) and 7.25 (m, 10H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.2, 20.6, 20.7, 61.1, 61.7, 65.3, 65.4, 67.3, 69.0, 69.8, 71.5, 95.3, 96.1, 120.1, 120.2, 125.5, 129.6, 129.8, 129.9, 155.0, 169.2, 169.3, 169.7, 170.3 and 170.5.



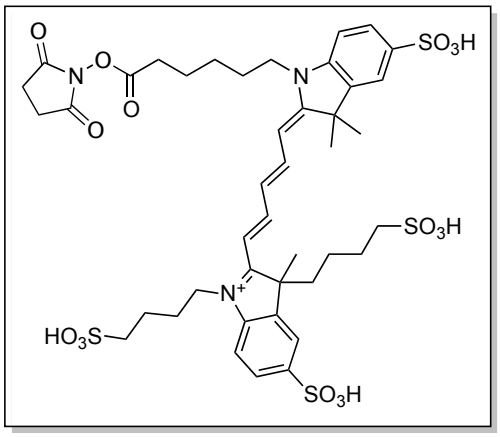
Benzyl 2-(2-Hydroxyethoxy)ethylcarbamate (2.57).[18] To a solution containing 1.01 g (9.61 mmol) of 2-(2-aminoethoxy)ethanol in 100 mL of anh THF at room temperature were added 1.34 mL (9.61 mmol) of dry triethylamine and 1.49 mL (1.78 g, 10.6 mmol) of CBzCl. The reaction mixture was stirred for 1 h and was then diluted with 100 mL of

ethyl acetate. The organic layer was washed with two 50-mL portions of water, 50 mL of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (30 × 4 cm). Elution with 9:1 hexanes–ethyl acetate afforded alcohol **2.57** as a colorless oil: yield 2.21 g (96%); silica gel TLC R_f 0.30 (9:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) 3.30 (m, 2H), 3.45 (m, 4H), 3.52 (s, 1H), 3.62 (m, 2H), 5.03 (s, 2H), 5.86 (m, 1H) and 7.27 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) 40.5, 61.1, 66.3, 69.7, 72.0, 127.72, 127.75, 128.1, 136.3 and 156.5.

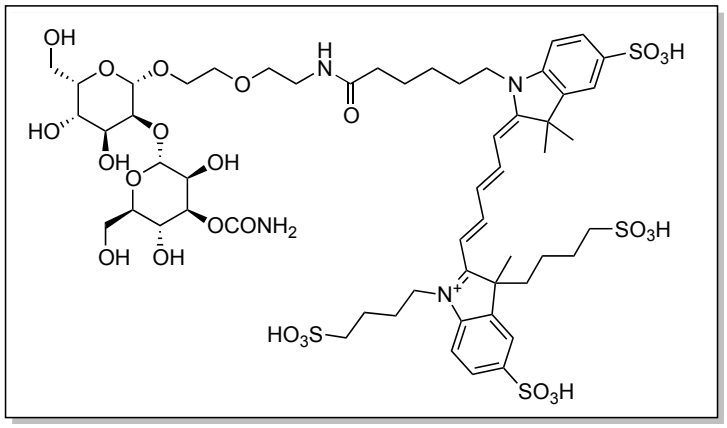


3,4,6-Tri-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl-α-D-mannopyranosyl)-α-L-gulopyranosyl Benzyl 2-(2-Ethoxy)ethylcarbamate (2.58).[18] To a solution of 40.0 mg (0.05 mmol) of phosphate ester **2.56** and 10.0 mg (0.04 mmol) of alcohol **2.57** in 1.1 mL of anh dichloromethane was added 15.0 μL (17.8 mg, 0.08 mmol) of TMSOTf at 0 °C. The reaction mixture was stirred at 0 °C for 20 min at which time it was poured into a mixture of 20 mL of ethyl acetate and 10 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 5-mL portions of brine, ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 1.5 cm). Elution with 1:16:16→1:12:24 methanol–hexanes–ethyl acetate afforded disaccharide–linker conjugate **2.58** as a colorless oil: yield 26.0 mg (67%); silica

gel TLC R_f 0.30 (1:4 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.03 (s, 6H), 2.07 (s, 3H), 2.09 (s, 3H), 2.11 (s, 3H), 2.12 (s, 3H), 3.54 (m, 8H), 3.83 (m, 1H), 3.96 (m, 1H), 4.05 (m, 4H), 4.25 (m, 1H), 4.46 (m, 1H), 4.69 (s, 1H), 4.91 (m, 1H), 5.12 (m, 8H), 5.61 (m, 1H) and 7.34 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.6, 20.7, 20.8, 29.6, 40.9, 62.1, 62.5, 63.7, 65.5, 66.1, 66.6, 67.6, 68.5, 69.1, 69.6, 69.7, 70.0, 70.3, 70.6, 97.0, 97.1, 128.1, 128.2, 128.4, 136.5, 156.5, 169.3, 169.5, 169.8 and 170.5; mass spectrum (MALDI-TOF), *m/z* 859.30 $(M + H)^+$, 881.30 $(M + Na)^+$ (theoretical *m/z* 858.29).



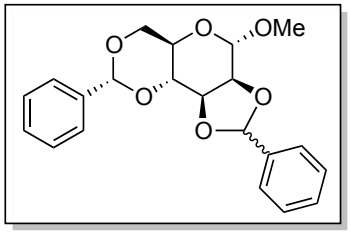
Cy5**succinimidyl ester (2.60).[127] To a vial containing 0.50 mg (0.6 μmol) of Cy5**COOH were added 5.00 mg (16.0 μmol) of TSTU dissolved in 100 μL of anh DMF, followed by 7.50 μL (5.60 mg, 43.0 μmol) of anh DIPEA dissolved in 75 μL of anh DMF. The reaction mixture was stirred at room temperature for 3 h and then diluted with 3 mL of ethyl acetate. The solution was then centrifuged at 15000 rpm for 15 min. The supernatant solution was discarded and the residue was washed with 1 mL of ethyl acetate. The residue was then dried under vacuum in the dark for 30 min to afford the NHS ester **2.60** as a dark blue solid: yield 0.55 μg (100%); mass spectrum (MALDI-TOF), *m/z* 984.55 $(M + H)^+$ (theoretical *m/z* 984.24).



BLM Disaccharide–Cy5 (2.3).**[18] To a solution of 8.00 mg (9.30 μmol) of disaccharide–linker **2.58** in 2 mL of anh methanol was added 0.2 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF) *m/z* 629.40 $(M + Na)^+$ (theoretical *m/z* 629.23). To the solution of the crude product in 5 mL of anh methanol was added Pd/C and H_2 gas was bubbled through for 45 min. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered through Celite 545® and then concentrated under diminished pressure to afford amine **2.59** as a colorless oil: yield 4.78 mg (100%), which was used directly for the next reaction; mass spectrum (MALDI-TOF), *m/z* 473.35 $(M + H)^+$ and 495.32$(M + Na)^+$ (theoretical *m/z* 473.19 and 495.19).

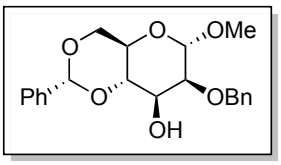
To 175 μg (0.37 μmol) of amine **2.59** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu (**2.60**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction

mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 3 mL/min. The fractions containing the desired product eluted at 18.5 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give BLM disaccharide–Cy5** (**2.3**) as a blue solid: yield 63.0 μg (35% over two steps); mass spectrum (MALDI-TOF), *m/z* 1363.50 $(M - H + Na)^+$, 1385.50 $(M - 2H + 2Na)^+$ and 1407.44 $(M - 3H + 3Na)^+$ (theoretical *m/z* 1363.38, 1385.36 and 1407.35).



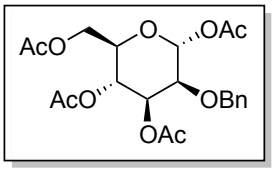
Methyl (*S*),(*R*)-2,3,4,6-di-*O*-benzylidene-α-D-mannopyranoside (2.61a) and Methyl(*R*),(*R*)-2,3,4,6-di-*O*-benzylidene-α-D-mannopyranoside (2.61b).[128] To a solution of 8.00 g (41.2 mmol) of methyl-α-D-mannopyranoside (**2.46**) in 88 mL of DMF were added 16.0 mL (106 mmol) of benzaldehyde dimethylacetal and 120 mg (0.52 mmol) of camphor sulfonic acid. The reaction was heated at 45 °C under reduced pressure in rotatory evaporator for 3 h. To the reaction mixture were then added 8.0 mL (53.2 mmol) of benzaldehyde dimethyl acetal and 60.0 mg (0.26 mmol) of camphor sulfonic acid and heated at 45 °C for additional 2 h. The solvent was concentrated and the residue was co-evaporated with three 20-mL portions of toluene. The residue was dissolved in 120 mL of dichloromethane and washed with 60 mL of saturated aq

$NaHCO_3$, 60 mL of brine (60 mL), dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The resulting residue was purified by recrystallization (ethyl acetate−methanol) to give the exo-dibenzylidene isomer **2.61a** as a white solid: yield 5.20 g (34%); silica gel TLC R_f 0.80 (3:1 hexanes–ethyl acetate); mp 170-172 °C, lit.[145] mp 176-180 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.41 (s, 3H), 3.84 (m, 2H), 3.90 (m, 1H), 4.15 (d, 1H, J = 5.4 Hz), 4.37 (m, 1H), 4.64 (dd, 1H, J = 7.8 and 5.4 Hz), 5.03 (s, 1H), 5.65 (s, 1H), 6.30 (s, 1H), 7.33-7.43 (m, 6H), 7.44-7.50 (m, 2H) and 7.51-7.57 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.2, 60.3, 68.9, 75.3, 75.5, 77.5, 98.8, 102.0, 103.0, 126.3, 128.2, 128.3, 129.1, 137.1 and 138.6. The mother liquor was concentrated and recrystallization in ethanol to give the endo-dibenzylidene isomer **2.61b** as a white solid: yield 5.86 g (38%); silica gel TLC R_f 0.80 (3:1 hexanes–ethyl acetate); mp 80-85 °C, lit.[145] mp 95-97 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.44 (s, 3H), 3.79 (m, 3H), 4.31 (m, 2H), 4.48 (t, 1H, J = 6.7 Hz), 5.09 (s, 1H), 5.54 (s, 1H), 5.98 (s, 1H), 7.34-7.39 (m, 4H), 7.40-7.46 (m, 2H), 7.49 -7.52 (m, 2H) and 7.53-7.59 (m, 2H).



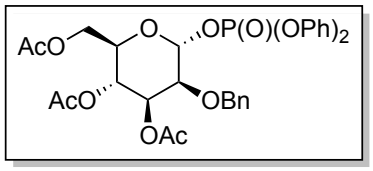
Methyl 4,6-*O*-benzylidene-2-*O*-benzyl-α-D-mannopyranoside (2.62).[129] To a stirred suspension of 4.22 g endo-dibenzylidene isomer **2.61b** (7.69 mmol) in 68 mL of anh toluene were added 105 mg of 5 mol% of aluminium chloride and 34.6 mL (34.6 mmol) of 1M solution of DIBAL-H in toluene dropwise at 0 °C. The reaction was stirred at 0 °C for 1 h and quenched by the addition of 27 mL of methanol. The solution was diluted with 300 mL diethyl ether, washed with 120 mL 10% potassium tartarate. The aqueous

layer was extracted with 400 mL diethyl ether and the combined extract was washed with 120 mL brine, dried ($MgSO_4$), and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 6 cm) with 4:1 hexanes–ethyl acetate to give alcohol **2.62** as colorless oil: yield 4.22 g (72%); silica gel TLC R_f 0.52 (2:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.36 (s, 3H), 3.72-3.96 (m, 4H), 4.08 (dd, 1H, J = 9.8 and 3.7 Hz), 4.26 (dd, 1H, J = 9.5 and 4.1 Hz), 4.72 (m, 3H), 5.58 (s, 1H), 7.27-7.42 (m, 7H) and 7.44-7.53 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.0, 63.3, 68.7, 68.8, 73.7, 78.4, 79.5, 99.4, 102.13, 102.13, 126.3, 127.9, 128.2, 129.1 and 137.3; similarly exo-dibenzylidene isomer **2.61a** gave alcohol **2.62** in 65% yield.



1,3,4,6-Tetra-*O*-acetyl-2-*O*-benzyl-α-D-mannopyranoside (2.63).[147] To a solution of 2.16 g (11.2 mmol) of alcohol **2.62** in 40 mL of acetic anhydride was added a drop of concentrated H_2SO_4 at 0 °C and stirred overnight at room temperature. The solution was poured into a mixture of 200 mL of ethyl acetate and 100 mL of saturated aq $NaHCO_3$. The organic layer was washed with 30 mL of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (22 × 5 cm). Elution with 4:1→2:1 hexanes–ethyl acetate afforded benzyl ether **2.63** as a colorless oil: yield 1.46 g (57%); silica gel TLC R_f 0.44 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.99 (s, 3H). 2.04 (s, 3H), 2.09 (s, 3H), 2.13 (s, 3H), 3.82 (dd, 1H, J = 3.4 and 2.0 Hz), 3.99-4.03 (m, 1H), 4.12

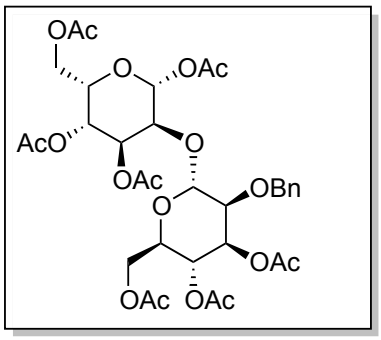
(dd, 1H, J = 12.4 and 2.4 Hz), 4.26 (dd, 1H, J = 12.3 and 4.9 Hz), 4.58-4.75 (m, 2H), 5.19 (dd, 1H, J = 10.1 and 3.3 Hz), 5.48 (t, 1H, J = 10.1 Hz), 6.19 (d, 1H, J = 1.9 Hz) and 7.28-7.36 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.6, 20.65, 20.69, 20.9, 62.2, 65.8, 70.5, 70.9, 72.8, 73.8, 91.0, 127.9, 128.0, 128.2, 128.3, 128.4, 137.2, 168.6, 169.4, 170.1 and 170.6.



3,4,6-Tri-*O*-acetyl-2-*O*-benzyl-α-D-mannopyranosyl Diphenyl Phosphate (2.64). To a solution of 920 mg (2.10 mmol) of benzyl ether **2.63** in 14.3 mL of anh DMF was added 271 mg (2.94 mmol) of hydrazine acetate. The solution was stirred at room temperature for 2.5 h and diluted with 100 mL of ethyl acetate. The organic solution was washed with 50 mL of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure to afford the alcohol as a light yellow oil: crude yield 873 mg (100%), which was used for the next reaction; silica gel TLC R_f 0.42 (1:2 hexanes–ethyl acetate).

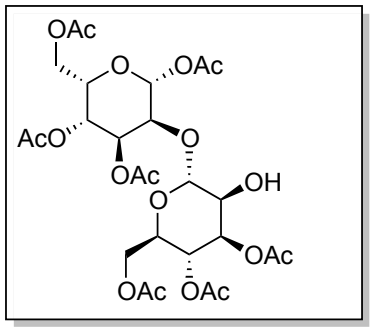
To a solution of above residue in 50 mL of anh dichloromethane at 0 °C were added 336 mg (2.75 mmol) of DMAP, 3.30 mL (2.42 g, 23.9 mmol) of dry triethylamine, and 4.40 mL (5.71 g, 21.2 mmol) of diphenyl phosphoryl chloride. The solution was stirred at room temperature for 1.5 h and poured into a mixture of 70 mL of ethyl acetate and 35 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 35-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 4 cm). Elution with 2:1 hexanes–ethyl acetate afforded phosphate ester **2.64** as a white foam:

yield 730 mg (55% over two steps); silica gel TLC R_f 0.49 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.98 (s, 3H), 1.99 (s, 3H), 2.03 (s, 3H), 3.86 (m, 1H), 3.93 (dd, 1H, *J* = 12.3 and 2.3 Hz), 4.00 (ddd, 1H, *J* = 10.2, 4.7 and 2.2 Hz), 4.17 (dd, 1H, *J* = 12.4 and 4.7 Hz), 4.61 (m, 2H), 5.23 (dd, 1H, *J* = 10.2 and 3.3 Hz), 5.46 (t, 1H, *J* = 10.1 Hz), 5.91 (dd, 1H, *J* = 6.5 and 2.0 Hz), 7.19-7.25 (m, 6H) and 7.28-7.39 (m, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.6, 20.7, 61.8, 65.5, 70.0, 70.8, 73.3, 74.6, 74.7, 96.5, 96.6, 120.1, 120.2, 120.3, 125.7, 125.7, 128.0, 128.1, 128.5, 129.89, 129.92, 136.9, 150.0, 150.3, 169.4, 170.0 and 170.7; mass spectrum (FAB), *m*/*z* 629.1795 $(M + H)^+$ ($C_{31}H_{34}O_{12}P$ requires *m*/*z* 629.1788).



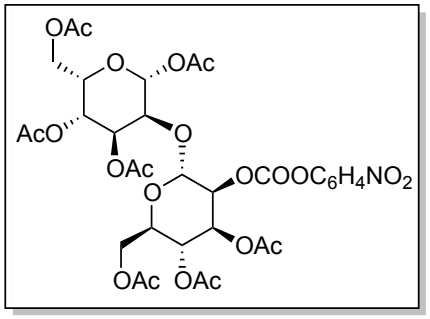
1,3,4,6-Tetra-*O*-acetyl-2-*O*-(3,4,6-tri-*O*-acetyl-2-*O*-benzyl-α-D-mannopyranosyl)-β-L-gulopyranose (2.65). To a solution of 263 mg (0.418 mmol) of phosphate ester **2.64** and 124 mg (0.36 mmol) of gulose alcohol **2.45** in 5.6 mL of dichloromethane was added 153 μL (167 mg, 0.75 mmol) of TMSOTf at 0 °C. The reaction was stirred for 15 min at which time it was poured into a mixture of 20 mL of ethyl acetate and 10 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 10-mL portions of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 3.5 cm). Elution with 1:1→1:2

hexanes–ethyl acetate afforded disaccharide **2.65** as a white foam: yield 170 mg (56%); silica gel TLC R_f 0.28 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.90 (s, 3H), 2.01 (s, 3H), 2.06 (s, 3H), 2.12 (s, 3H), 2.14 (s, 3H), 2.16 (s, 6H), 3.61 (m, 1H), 3.98-4.24 (m, 5H), 4.36 (m, 1H), 4.53 (m, 1H), 4.70 (m, 2H), 4.97 (dd, 1H, J = 10.2 and 3.4 Hz), 4.97 (dd, 1H, J = 10.2 and 3.4 Hz), 5.12 (d, 1H, J = 1.6 Hz), 5.39 (m, 1H), 5.48 (m, 1H), 5.84 (d, 1H, J = 8.8 Hz), 7.28-7.35 (m, 3H) and 7.39-7.42 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.65, 20.69, 20.7, 20.8, 61.3, 62.2, 65.4, 65.4, 65.8, 66.1, 67.7, 68.1, 69.2, 70.5, 71.3, 72.3, 74.0, 77.2, 90.6, 94.3, 127.8, 128.16, 128.19, 137.6, 168.7, 169.4, 170.1, 170.4 and 170.7; mass spectrum (APCI), m/z 727.2455 $(M + H)^+$ ($C_{33}H_{43}O_{18}$ requires m/z 727.2450).



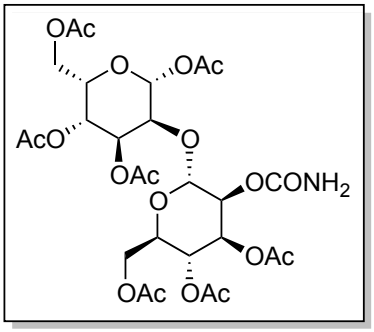
1,3,4,6-Tetra-*O*-acetyl-2-*O*-(3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-β-L-gulopyranose (2.66). To a solution of 236 mg (0.32 mmol) of disaccharide **2.65** in 4.7 mL of ethyl acetate was added 117 mg of $Pd(OH)_2/C$ and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and concentrated under diminished pressure affording alcohol **2.66** as a white foam: yield 203 mg (98%); silica gel TLC R_f 0.13 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.01 (s, 3H), 2.06 (2s, 6H), 2.11 (s, 3H), 2.15 (s, 6H), 2.16 (s, 3H),

3.85 (m, 1H), 4.01 (dd, 1H, J = 8.5 and 3.3 Hz), 4.09 (m, 2H), 4.16 (m, 1H), 4.20 (m, 1H), 4.27 (m, 1H), 4.36 (ddd, 1H, J = 7.4, 6.0 and 1.5 Hz), 4.99 (dd, 1H, J = 3.8 and 1.4 Hz), 5.03 (d, 1H, J = 1.8 Hz), 5.07 (dd, 1H, J = 9.8 and 3.2 Hz), 5.35 (t, 1H, J = 10.0 Hz), 5.45 (t, 1H, J = 3.5 Hz) and 5.87 (d, 1H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.6, 20.7, 20.8, 20.9, 61.3, 61.9, 65.7, 65.8, 67.7, 68.5, 69.9, 69.5, 71.2, 71.3, 77.2, 90.6, 90.7, 168.7, 169.4, 168.5, 168.6, 169.8, 170.4 and 170.9; mass spectrum (APCI), m/z 637.1985 $(M + H)^+$ ($C_{26}H_{37}O_{18}$ requires m/z 637.1980).



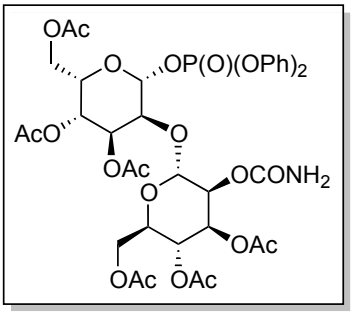
1,3,4,6-Tetra-*O*-acetyl-2-*O*-(3,4,6-tri-*O*-acetyl-2-*O*-((*p*-nitrophenyl)carbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranose (2.67). To a solution of 203 mg (0.319 mmol) of alcohol **2.66** in 2.1 mL of dry pyridine was added 156 mg (1.276 mmol) of DMAP and 257 mg (1.28 mmol) of *p*-nitrophenyl chloroformate. The reaction was stirred overnight at 40 °C at which time it was poured into a mixture of 6 mL ethyl acetate and 2.2 mL of water. The organic layer was washed with three 3 mL portions of 1N HCl, 3 mL of saturated aq $NaHCO_3$ and 3-mL of brine. The solution was dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 3 cm). Elution with 1:1 hexanes–ethyl acetate affording carbonate **2.67** as a white foam: yield 220 g (86%); silica gel TLC R_f

0.46 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.01 (s, 3H), 2.06 (2s, 6H), 2.13 (s, 3H), 2.15 (s, 3H), 2.17 (s, 3H), 2.20 (s, 3H), 4.03 (dd, 1H, *J* = 8.5 and 3.3 Hz), 4.08-4.20 (m, 3H), 4.26 (m, 2H), 4.38 (m, 1H), 5.02 (m, 2H), 5.17(m, 1H), 5.23 (dd, 1H, *J* = 10.1 and 3.3 Hz), 5.35 (m, 1H), 5.46 (t, 1H, *J* = 3.5 Hz), 5.90 (d, 1H, *J* = 8.5 Hz), 7.41-7.44 (m, 2H) and 8.28-8.32 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.6, 20.7, 20.8, 61.3, 61.8, 62.0, 65.4, 65.6, 65.9, 67.7, 68.7, 69.3, 70.0, 71.3, 73.4, 89.2, 90.5, 94.4, 121.6, 125.3, 145.5, 151.6, 155.2, 168.6, 169.3, 169.5, 169.6, 169.7, 170.4 and 170.5; mass spectrum (APCI), *m*/*z* 802.2033 $(M + H)^+$ ($C_{33}H_{40}O_{22}$ requires *m*/*z* 802.2042).



1,3,4,6-Tetra-*O*-acetyl-2-*O*-(3,4,6-tri-*O*-acetyl-2-*O*-(carbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranose (2.68). To a solution containing 94.0 mg (0.12 mmol) of carbonate **2.67** in 5.0 mL of dichloromethane was added 2.2 mL of THF saturated with NH_3 (g). The reaction mixture was stirred at room temperature for 3 h. The solvent was concentrated under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (15 × 2.5 cm). Elution with 1:3 hexanes–ethyl acetate afforded carbamate **2.68** as a white foam: yield 73.0 mg (92%); silica gel TLC R_f 0.13 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.98 (s, 3H), 2.02 (s, 3H), 2.04 (s, 3H), 2.11 (s, 3H), 2.13 (s, 3H), 2.14 (s,

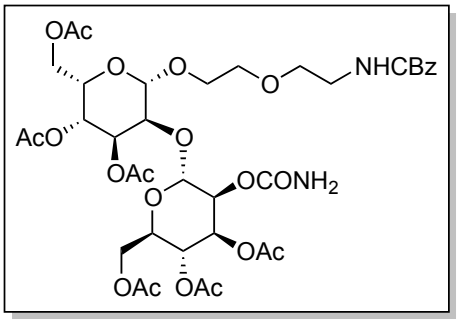
3H), 2.18 (s, 3H), 3.98 (dd, 1H, J = 8.4 and 3.3 Hz), 4.08 (m, 2H), 4.15 (m, 1H), 4.18 (m, 1H), 4.25 (m, 1H), 4.35 (m, 1H), 4.85 (br s, 2H), 4.96 (m, 1H), 5.01 (m, 2H), 5.11 (m, 1H), 5.25 (m, 1H), 5.44 (t, 1H, J = 3.6 Hz) and 5.89 (d, 1H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.62, 20.65, 20.68, 20.71, 20.8, 61.4, 62.1, 65.6, 65.7, 67.6, 68.9, 69.1, 69.4, 69.8, 71.3, 90.7, 95.3, 154.9, 168.7, 169.2, 169.6, 169.9, 170.4 and 170.5; mass spectrum (APCI), m/z 680.2026 $(M + H)^+$ ($C_{27}H_{38}O_{19}$ requires m/z 680.2038).



3,4,6-Tri-*O*-acetyl-2-*O*-(3,4,6-tri-*O*-acetyl-2-*O*-(carbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranosyl Diphenyl Phosphate (2.69). To a solution containing 66.0 mg (0.10 mmol) of disaccharide **2.68** in 1.0 mL of anh DMF was added 13.0 mg (0.14 mmol) of hydrazine acetate. The reaction mixture was stirred at room temperature for 3 h and diluted by the addition of 20 mL of ethyl acetate. The organic solution was washed with 10 mL of water, 10 mL of saturated aq $NaHCO_3$, 10 mL of brine. The solvent was dried ($MgSO_4$), filtered and concentrated under diminished pressure to afford the crude product as a light yellow oil: yield 56.0 mg (90%); silica gel TLC R_f 0.23 (1:4 hexanes–ethyl acetate). MALDI, m/z 660.18 for $(M + Na)^+$ (theoretical m/z 660.18); the residue was used for next reaction.

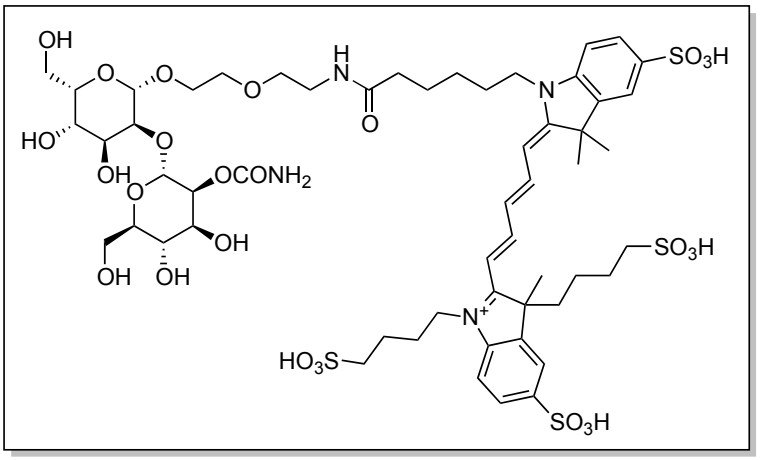
To a stirred solution containing 56.0 mg (0.09 mmol) of the crude residue in 3.3 mL of anh dichloromethane were added 13.0 mg (0.11 mmol) of DMAP, 133 μL (96.0

mg, 0.95 mmol) of dry triethylamine and 176 μL (229 mg, 0.85 mmol) of diphenyl chlorophosphate. The reaction mixture was stirred at 0 °C for 2 h and poured into a mixture of 5 mL of ethyl acetate and 5 mL of saturated aq $NaHCO_3$. The organic layer was washed with 10 mL of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 1:2 hexanes–ethyl acetate afforded phosphate ester **2.69** as a colorless oil: yield 36.0 mg (47% over two steps); silica gel TLC R_f 0.18 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.77 (s, 3H), 2.03 (s, 3H), 2.05 (s, 3H), 2.13 (s, 3H), 2.21 (s, 3H), 2.27 (s, 3H), 4.07 (m, 2H), 4.18 (m, 2H), 4.26 (m, 1H), 4.39 (m, 2H), 4.87 (br s, 2H), 5.08 (m, 3H), 5.26 (m, 1H), 5.32 (m, 1H), 5.52 (m, 1H), 5.77 (m, 1H), 7.22-7.28 (m, 2H) and 7.32-7.43 (m, 8H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.3, 20.63, 20.64, 20.68, 20.71, 61.2, 61.8, 65.3, 65.6, 67.4, 69.0, 69.1, 69.5, 71.1, 71.2, 71.6, 95.6, 96.17, 96.22, 120.19, 120.24, 125.6, 125.70, 125.71, 129.6, 129.9, 150.0, 150.1, 154.9, 169.2, 169.6, 169.7, 170.4 and 170.6; mass spectrum (APCI), *m*/*z* 870.2224 $(M + H)^+$ ($C_{37}H_{45}NO_{21}P$ requires *m*/*z* 870.2222).



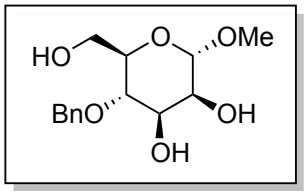
3,4,6-Tri-*O*-acetyl-2-*O*-(3,4,6-tri-*O*-acetyl-2-*O*-(carbamoyl)-α-D-mannopyranosyl)-α,β-L-gulopyranosyl Benzyl 2-(2-Ethoxy)ethylcarbamate (2.70). To a stirred solution

containing 31.0 mg (0.04 mmol) of phosphate ester **2.69** in 3.9 mL of anh dichloromethane was added a solution of 9.40 mg (0.04 mmol) of CBz linker **2.57** in 4.5 mL of anh dichloromethane at 0 °C. To the cooled reaction mixture was added 41.0 μL (51.0 mg, 0.23 mmol) of TMSOTf. The reaction mixture was stirred at 0 °C for 15 min at which time it was poured into a mixture of 20 mL of ethyl acetate and 10 mL of saturated aq $NaHCO_3$. The organic layer was washed with 10mL of brine, and dried ($MgSO_4$) and filtered. The solvent was concentrated under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (12 × 2 cm). Elution with 1:3 hexanes–ethyl acetate afforded disaccharide–linker conjugate **2.70** as a colorless oil: yield 12.0 mg (39%); silica gel TLC R_f 0.12 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.99 (s, 3H), 2.02 (s, 2H), 2.04 (s, 3H), 2.07 (s, 3H), 2.09 (s, 3H), 2.12 (s, 3H), 3.37-3.42 (m, 2H), 3.55-3.65 (m, 3H), 3.67-3.69 (m, 2H), 3.83-3.88 (m, 1H), 3.97 (t, 1H, J = 3.9 Hz), 4.03-4.09 (m, 2H), 4.10-4.15 (m, 1H), 4.28 (dd, 1H, J = 11.9 and 5.2 Hz), 4.46 (t, 1H, J = 6.6 Hz), 4.77-4.90 (br s, 2H), 4.93 (d, 1H, J = 3.9 Hz), 5.03-5.06 (m, 3H), 5.09 (s, 2H), 5.23-5.29 (m, 3H), 5.46-5.48 (m, 1H) and 7.28-7.37 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.62, 20.65, 20.72, 20.76, 40.9, 62.1, 62.5, 63.8, 65.8, 66.1, 66.6, 67.6, 68.6, 68.7, 69.0, 70.1, 70.2, 70.3, 71.0, 77.2, 97.0, 97.6, 128.1, 128.2, 128.5, 136.5, 155.0, 169.3, 169.72, 169.73, 170.0, 170.56 and 170.59; mass spectrum (APCI), m/z 859.2987 $(M + H)^+$ ($C_{37}H_{51}N_2O_{21}$ requires m/z 859.2984).



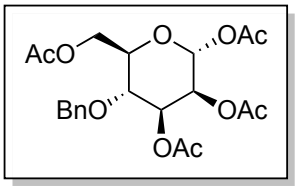
Disaccharide−Cy5 Conjugate 2.4.** To a solution of 2.20 mg (2.60 μmol) of disaccharide−linker **2.70** in 1 mL of anh methanol was added 0.1 mL of a freshly prepared 25% w/w solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 3 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 629.64 $(M + Na)^+$ (theoretical *m/z* 629.23). To the solution of the crude product in 5 mL of anh methanol was added Pd/C and H_2 gas was bubbled through for 1 h. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered through Celite 545® and the filtrate was concentrated under diminished pressure to afford amine **2.71** as a colorless oil: yield 1.32 mg (100%), which was used directly for the next reaction; mass spectrum (MALDI-TOF), *m/z* 495.44 $(M + Na)^+$; mass spectrum (APCI), *m/z* 473.1986 $(M + H)^+$ ($C_{17}H_{33}N_2O_{13}$ requires *m/z* 473.1983).

To 101 μg (0.21 μmol) of amine **2.71** was added a solution of 106 μg (0.11 μmol) of Cy5**COOSu (**2.60**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Alltech Alltima C_{18} reversed phase semi-preparative (250 × 10 mm, 5 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 35 min at a flow rate of 4 mL/min. The fractions containing the desired product eluted at 23.5 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give disaccharide–Cy5** conjugate **2.4** as a blue solid: yield 48.0 μg (35% over two steps); mass spectrum (APCI), *m/z* 669.1883 $(M - K - 2H)^{2-}$ ($C_{55}H_{78}N_4O_{26}S_4^{2-}$ requires *m/z* 669.1899).

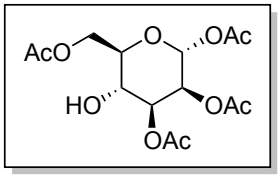


4-*O*-benzyl-α-D-mannopyranoside (2.72).[130] To 2.49 g (8.82 mmol) of acetal **2.47** in 50 mL of anh THF were added 26.5 mL (26.5 mmol) of a 1 M solution of BH_3 in THF and 3.44 g (26.5 mmol) of anh $CoCl_2$ at 0 °C. The reaction mixture was stirred for 3.5 h at room temperature and diluted by the addition of 100 mL of ethyl acetate. The solution was filtered and the filtrate was treated with 200 mg (5.29 mmol) of $NaBH_4$ in 30 mL of water. The solution was again filtered and washed successively with 50 mL of saturated aq $NaHCO_3$, 50 mL of brine, and dried ($MgSO_4$) and filtered. The solution was concentrated under diminished pressure to afford benzyl ether **2.72** as a colorless oil: yield 2.48 g (99%); silica gel TLC R_f 0.27 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$,

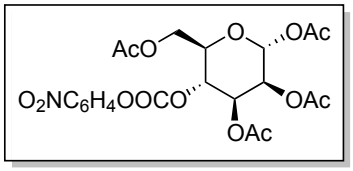
400 MHz) δ 3.27 (s, 3H), 3.34 (m, 1H), 3.52 (m, 2H), 3.61 (br s, 1H), 3.78 (m, 2H), 3.87 (br s, 1H), 3.92-3.95 (m, 1H), 4.45 (br s, 1H), 4.67-4.84 (m, 3H) and 7.22-7.33 (m, 5H).



1,2,3,6-Tetra-*O*-acetyl-4-*O*-benzyl-α-D-mannopyranoside (2.73).[148] To a solution containing 2.49 g (8.44 mmol) of benzyl ether **2.72** in 58 mL of acetic anhydride was added a catalytic amount of concentrated H_2SO_4 at 0 °C. The solution was stirred at room temperature for 12 h. The reaction mixture was quenched by the addition of 120 mL of ethyl acetate and 80 mL of saturated aq $NaHCO_3$. The organic layer was washed with 50 mL of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (30 × 5 cm). Elution with 3:1→1:1 hexanes–ethyl acetate afforded peracetylated benzyl ether **2.73** as a colorless oil: yield 2.91 g (79%); silica gel TLC R_f 0.5 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.00 (s, 3H), 2.08 (s, 3H), 2.13 (s, 3H), 2.15 (s, 3H), 3.87 (t, 1H, J = 9.7 Hz), 3.99 (dt, 1H, J = 9.9 and 3.4 Hz), 4.32 (d, 2H, J = 3.5 Hz), 4.59 (d, 1H, J = 11.2 Hz), 4.70 (d, 1H, J = 10.8 Hz), 5.26 (dd, 1H, J = 3.3 and 2.1 Hz), 5.37 (dd, 1H, J = 9.5 and 3.4 Hz), 6.04 (t, 1H, J = 6.1 Hz) and 7.24-7.38 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.92, 20.97, 20.99, 21.04, 62.9, 68.9, 71.6, 71.8, 72.6, 75.2, 90.8, 127.9, 128.3, 128.7, 137.5, 168.4, 169.8, 169.9 and 170.8.

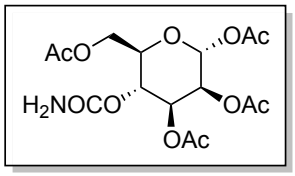


1,2,3,6-Tetra-*O*-acetyl-α-D-mannopyranoside (2.74).[149] To a solution of 2.90 g (6.61 mmol) of benzyl ether **2.73** in 50 mL of ethyl acetate was added 550 mg of $Pd(OH)_2/C$ and the reaction was stirred under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and the filtrate was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 5 cm). Elution with 2:1 hexanes–ethyl acetate afforded alcohol **2.74** as a colorless oil: yield 1.93 g (84%); silica gel TLC R_f 0.25 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.06 (s, 3H), 2.12 (s, 6H), 2.14 (s, 3H), 2.94 (br s, 1H), 3.88 (m, 2H), 4.26 (m, 1H), 4.52 (m, 1H), 5.21 (m, 2H) and 6.04 (d, 1H, J = 1.6 Hz).



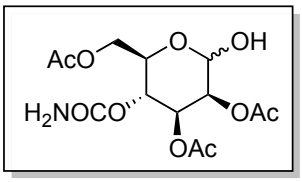
1,2,3,6-Tetra-*O*-acetyl-4-*O*-(*p*-nitrophenyloxy)carbonyloxy-α-D-mannopyranoside (2.75). To a solution of 1.74 g (5.00 mmol) of alcohol **2.74** in 17.8 mL of dry pyridine was added 2.44 g (20.0 mmol) of DMAP and 4.03 g (20.0 mmol) of *p*-nitrophenyl chloroformate. The reaction was stirred at 40 °C for 2.5 h at which time it was poured into a mixture of 50 mL of ethyl acetate and 19 mL of water. The organic layer was washed successively with three 25-mL portions of 1 N HCl, 25 mL of saturated aq $NaHCO_3$ and 25 mL of brine. The solution was dried ($MgSO_4$), filtered and concentrated

under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (28 × 5 cm). Elution with 2:1 hexanes–ethyl acetate afforded carbonate **2.75** as a colorless foam: yield 1.91 g (74%); silica gel TLC R_f 0.21 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.05 (s, 3H), 2.11 (s, 3H), 2.18 (s, 3H), 2.19 (s, 3H), 4.18 (m, 2H), 4.56 (m, 1H), 5.23 (t, 1H, J = 9.9 Hz), 5.33 (m, 1H), 5.45 (dd, 1H, J = 10.1 and 3.5 Hz), 6.12 (d, 1H, J = 1.9 Hz), 7.38 (d, 2H, J = 9.2 Hz) and 8.29 (d, 2H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.66, 20.69, 20.8, 61.6, 68.3, 68.6, 70.1 70.9, 90.4, 121.6, 125.4, 145.7, 151.7, 155.1, 167.9, 169.5, 169.8 and 170.6; mass spectrum (ESI), m/z 531.1472 $(M + NH_4)^+$ ($C_{21}H_{27}N_2O_{14}$ requires m/z 531.1462).

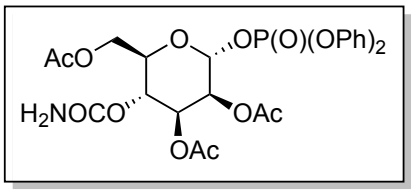


1,2,3,6-Tetra-*O*-acetyl-4-*O*-(carbamoyloxy)-α-D-mannopyranoside (2.76). To a solution of 1.90 g (3.70 mmol) of carbonate **2.75** in 100 mL of dichloromethane was added a solution of 35 mL of anh THF that had been saturated with NH_3 at 0 °C. The reaction mixture was allowed to warm to room temperature and then stirred at room temperature overnight. The solution was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 1:1 hexanes–ethyl acetate afforded carbamate **2.76** as a colorless foam: yield 1.25 g (87%); silica gel TLC R_f 0.12 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.03 (s, 3H), 2.09 (s, 3H), 2.16 (s, 3H), 2.17 (s, 3H), 4.03 (m, 1H), 4.17 (m, 1H), 4.29 (m,

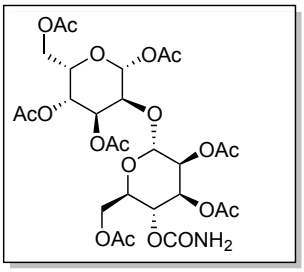
1H), 4.73 (br s, 2H), 5.19 (t, 1H, J = 10.1 Hz), 5.24-5.25 (m, 1H), 5.34-5.37 (m, 1H) and 6.07 (d, 1H, J = 1.9 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.68, 20.72, 20.76, 20.85, 62.3, 66.7, 68.4, 68.6, 70.7, 90.6, 154.9, 168.0, 169.8, 170.1 and 170.7; mass spectrum (APCI), m/z 392.1203 $(M + H)^+$ ($C_{15}H_{22}NO_{11}$ requires m/z 392.1193).



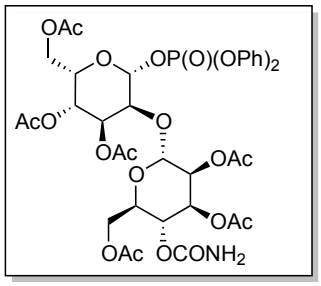
2,3,6-Tri-*O*-acetyl-4-*O*-(carbamoyloxy)-α-D-mannopyranoside (2.77). To a solution containing 553 mg (1.41 mmol) of carbamate **2.76** in 9.2 mL of anh DMF was added 182 mg (1.98 mmol) of hydrazine acetate. The solution was stirred at 25 °C for 2 h and then treated with 120 mL of ethyl acetate. The organic solution was washed successively with 120 mL of water, 120 mL of saturated aq $NaHCO_3$, 120 mL of brine, dried ($MgSO_4$) and filtered. The solvent was concentrated under diminished pressure and co-evaporated with three 10-mL portions of toluene to afford pyranoside **2.77** as a colorless foam: yield 501 mg (100%); silica gel TLC R_f 0.28 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.00 (s, 3H), 2.08 (s, 3H), 2.14 (s, 3H), 4.17-4.24 (m, 3H), 4.58-4.64 (br s, 1H), 5.10 (t, 1H, J = 9.6 Hz), 5.07-5.15 (br s, 2H), 5.20-5.22 (m, 2H) and 5.37-5.41 (m, 1H); mass spectrum (ESI), m/z 348.0932 $(M - H)^-$ ($C_{13}H_{18}NO_{10}$ requires m/z 348.0931).



2,3,6-Tri-*O*-acetyl-4-*O*-(carbamoyloxy)-α-D-mannopyranosyl Diphenyl Phosphate (2.78). To a solution of 496 mg (1.42 mmol) of pyranoside **2.77** in 29 mL of dichloromethane at 0 °C were added 217 mg (1.78 mmol) of DMAP, 2.10 mL (15.0 mmol) of dry triethylamine and 2.80 mL (13.6 mmol) of diphenylphosphoryl chloride. The reaction mixture was stirred for 1.5 h and the solution was poured into a mixture of 40 mL of ethyl acetate and 20 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 10-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (20 × 3 cm). Elution with 2:3 hexanes–ethyl acetate afforded phosphate ester **2.78** as a colorless oil: yield 460 mg (56%); silica gel TLC R_f 0.33 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.06 (s, 3H), 2.12 (s, 3H), 2.24 (s, 3H), 4.15-4.19 (m, 1H), 4.28-4.32 (m, 1H), 4.37-4.41 (m, 1H), 4.80 (s, 1H), 4.82-4.90 (br s, 2H), 5.21-5.30 (m, 1H), 5.41-5.50 (m, 1H), 5.95-5.97 (m, 1H), 7.24-7.36 (m, 6H) and 7.44-7.48 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.56, 20.62, 20.7, 61.9, 66.3, 68.0, 68.7, 68.8, 70.8, 96.0, 120.01, 120.05, 120.16, 120.21, 125.7, 125.85, 125.86, 129.93, 129.99, 169.6, 169.9 and 170.6; mass spectrum (APCI), *m*/*z* 582.1387 $(M + H)^+$ ($C_{25}H_{29}NO_{13}P$ requires *m*/*z* 582.1377).



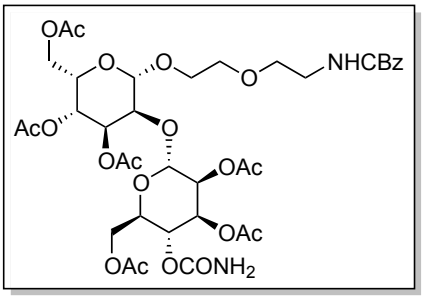
1,3,4,6-Tetra-*O*-acetyl-2-*O*-(2,3,6-tri-*O*-acetyl-4-*O*-(carbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranose (2.79). To a solution of 460 mg (0.79 mmol) of phosphate ester **2.78** in 5.1 mL of dichloromethane containing 4Å molecular sieves was added 191 mg (0.33 mmol) of gulose alcohol **2.45** in 4.8 mL of dichloromethane. The solution was cooled to 0 °C and was then treated with 220 μL (1.22 mmol) of TMSOTf. The reaction mixture was stirred for 20 min at which time it was poured into a mixture of 70 mL of ethyl acetate and 40 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 50-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (25 × 3 cm). Elution with 1:3 hexanes–ethyl acetate afforded **2.79** as a colorless oil. The product was isolated as a (5:1) mixture of anomers: yield 275 mg (51%); silica gel TLC R_f 0.26 (3:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) (major anomer) δ 1.99 (s, 3H), 2.05 (s, 3H), 2.12 (s, 3H), 2.13 (s, 6H), 2.14 (s, 3H), 2.18 (s, 3H), 3.97-4.00 (m, 1H), 4.03-4.16 (m, 2H), 4.26-4.28 (m, 1H), 4.33-4.37 (m, 1H), 4.73 (br s, 2H), 4.94-4.97 (m, 1H), 4.99-5.01 (m, 1H), 5.06-5.09 (m, 2H), 5.13-5.15 (m, 2H), 5.14-5.15 (m, 1H), 5.43 (t, 1H, J = 3.6 Hz) and 5.88 (d, 1H, J = 8.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) (mixture of anomers) δ 20.56, 20.63, 20.68, 20.69, 20.7, 20.8, 20.9, 61.3, 62.2, 65.5, 65.89, 66.9, 67.0, 67.57, 67.60, 68.66, 68.71, 69.4, 69.8, 71.3, 90.6, 95.1, 155.0, 155.2, 168.7, 169.24, 169.26, 169.5, 170.0, 170.4 and 170.6; mass spectrum (FAB), m/z 680.2045 $(M + H)^+$ ($C_{27}H_{38}NO_{19}$ requires m/z 680.2038).



3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,6-tri-*O*-acetyl-4-*O*-(carbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranosyl Diphenyl Phosphate (2.80). To a solution containing 62.0 mg (0.09 mmol) of disaccharide **2.79** in 1.5 mL of anh DMF was added 12.0 mg (0.13 mmol) of hydrazine acetate. The reaction mixture was stirred at room temperature for 2.5 h and diluted by the addition of 15 mL of ethyl acetate. The organic solution was washed with 10 mL of brine, dried ($MgSO_4$) and filtered. The solvent was concentrated under diminished pressure to afford the product as a yellow oil: yield 51 mg (88%); silica gel TLC R_f 0.1 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.00 (s, 3H), 2.07 (s, 3H), 2.12 (s, 3H), 2.13 (s, 3H), 2.14 (s, 3H), 2.17 (s, 3H), 3.74-3.77 (m, 1H), 4.11-4.19 (m, 2H), 4.23-4.26 (m, 2H), 4.33-4.38 (m, 1H), 4.53-4.56 (br s, 2H), 4.94-4.95 (m, 1H), 4.97-5.01 (m, 2H), 5.09-5.15 (m, 3H), 5.26-5.30 (m, 1H) and 5.39 (t, 1H, J = 3.6 Hz). The crude residue was used directly in the next reaction.

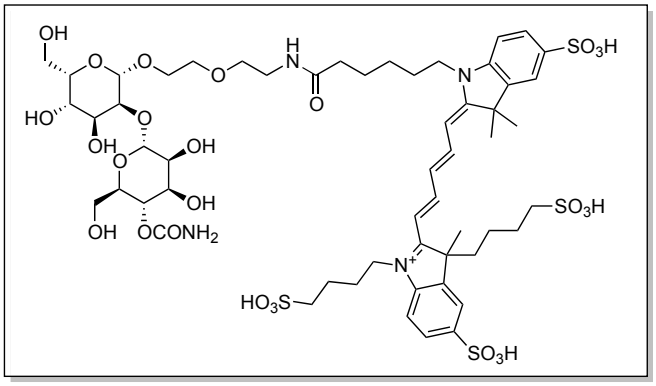
To a stirred solution containing 51.0 mg (0.10 mmol) of the above residue in 3 mL of anh dichloromethane was added 15.0 mg (0.12 mmol) of DMAP, 147 μL (106 mg, 1.04 mmol) of dry triethylamine and 194 μL (252 mg, 0.94 mmol) of diphenylphosphoryl chloride. The reaction mixture was stirred at 0 °C for 2 h and was then poured into a mixture of 40 mL of ethyl acetate and 20 mL of saturated aq $NaHCO_3$. The organic layer was washed successively with 10 mL brine, dried ($MgSO_4$), filtered and concentrated

under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 1:2 hexanes–ethyl acetate afforded the phosphate ester **2.80** as a colorless oil: yield 41.0 mg (52% over two steps); silica gel TLC R_f 0.23 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.95 (s, 3H), 1.97 (s, 3H), 2.07 (s, 3H), 2.11 (s, 3H), 2.13 (s, 3H), 2.20 (s, 3H), 4.00 (m, 2H), 4.12 (m, 1H), 4.19 (m, 2H), 4.30 (m, 2H), 4.97 (m, 2H), 5.09 (m, 3H), 5.22 (m, 1H), 5.40-5.45 (br s, 2H), 5.70 (t, 1H, J = 8.0 Hz), 7.15-7.21 (m, 4H) and 7.28-7.38 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.6, 20.7, 20.8, 61.1, 62.0, 65.4, 66.4, 67.4, 68.6, 68.9, 69.2, 71.6, 95.1, 96.2, 120.18, 120.23, 120.4, 120.5, 125.6, 125.7, 129.7, 129.9, 154.9, 169.3, 169.5, 169.8, 170.4 and 170.6; mass spectrum (APCI), m/z 870.2230 $(M + H)^+$ ($C_{37}H_{45}NO_{21}P$ requires m/z 870.2222).



3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,6-tri-*O*-acetyl-4-*O*-(carbamoyl)-α-D-mannopyranosyl)-α,β-L-gulopyranosyl Benzyl 2-(2-Ethoxy)ethylcarbamate (2.81). To a stirred solution containing 27.0 mg (0.03 mmol) of phosphate ester **2.80** in 3.9 mL of anh dichloromethane was added a solution of 8.20 mg (0.03 mmol) of CBz-protected linker **2.57** in 3.9 mL of anh dichloromethane at 0 °C. To the cooled solution was added 8.20 μL (10.1 mg, 0.04 mmol) of TMSOTf. The reaction mixture was stirred at 0 °C for 15 min and then poured into a mixture of 20 mL of ethyl acetate and 4 mL of saturated aq

$NaHCO_3$. The organic layer was washed with 10 mL of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (12 × 2 cm). Elution with 1:3 hexanes−ethyl acetate afforded disaccharide–linker conjugate **2.81** as a colorless oil. The product was isolated as a mixture of anomers: yield 7.00 mg (26%); silica gel TLC R_f 0.11 (1:4 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) (major anomer) δ 1.99 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.10 (s, 3H), 2.12 (s, 3H), 2.13 (s, 3H), 3.33-3.45 (br s, 2H), 3.56-3.65 (m, 2H), 3.67-3.73 (m, 2H), 3.82-3.88 (m, 1H), 3.96 (t, 1H, J = 4.0 Hz), 4.03-4.11 (m, 3H), 4.12-4.19 (m, 2H), 4.30 (dd, 1H, J = 12.0 and 5.7 Hz), 4.42 (t, 1H, J = 6.5 Hz), 4.93-4.98 (m, 3H), 5.00-5.03 (m, 1H), 5.07 (s, 2H), 5.12-5.17 (m, 2H), 5.24-5.30 (m, 3H) and 7.30-7.36 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.81, 20.89, 20.90, 20.93, 21.0, 29.8, 41.2, 62.3, 62.9, 63.8, 65.7, 67.0, 67.1, 68.1, 68.69, 68.72, 69.6, 70.1, 71.1, 77.5, 97.2, 97.6, 128.32, 128.38, 128.7, 136.5, 155.5, 156.9, 169.5, 169.8, 169.9, 170.2, 170.7 and 170.8; mass spectrum (APCI), *m*/*z* 859.2975 $(M + H)^+$ ($C_{37}H_{51}N_2O_{21}$ requires *m*/*z* 859.2984).

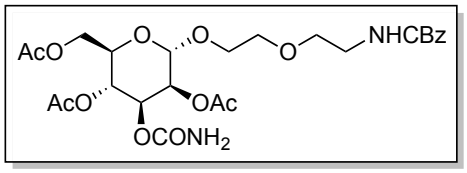


Disaccharide–Cy5 Conjugate 2.5.** To a solution containing 2.20 mg (2.56 mmol) of disaccharide–linker **2.81** in 1 mL of anh methanol was added 0.1 mL of a freshly

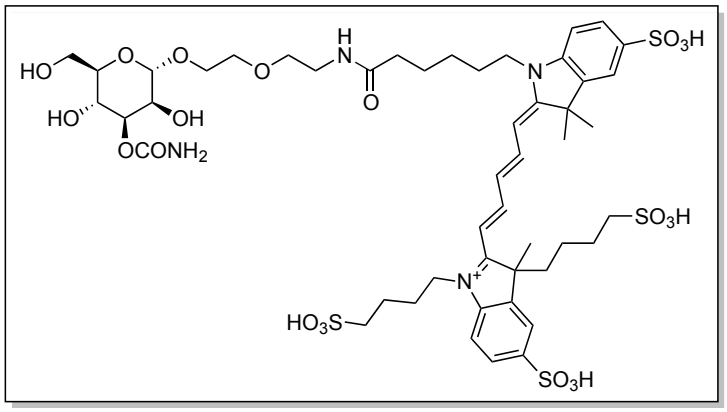
prepared 25% w/w solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 3 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF) *m/z* 629.30 $(M + Na)^+$ (theoretical *m/z* 629.23). To the solution of the crude product in 5 mL of anh methanol was then added a catalytic amount of Pd/C and H_2 gas was bubbled through for 1 h. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered through Celite 545® and the filtrate was concentrated under diminished pressure to afford amine **2.82** as a colorless oil: yield 1.32 mg (100%), which was used directly in the next reaction; mass spectrum (MALDI-TOF) *m/z* 495.44 $(M + Na)^+$; mass spectrum (APCI), *m/z* 473.1972 $(M + H)^+$ ($C_{17}H_{33}N_2O_{13}$ requires *m/z* 473.1983).

To 101 μg (0.21 μmol) of amine **2.82** was added a solution of 106 μg (0.11 μmol) of Cy5**COOSu (**2.60**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Alltech Alltima C_{18} reversed phase semi-preparative (250 × 10 mm, 5 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 35 min at a flow rate of 4 mL/min. The fractions containing the desired product eluted at 23.5 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give disaccharide–

Cy5** conjugate **2.5** as a blue solid: yield 44.0 μg (32% over two steps); mass spectrum (APCI), *m/z* 669.1880 $(M - K - 2H)^{2-}$ ($C_{55}H_{78}N_4O_{26}S_4^{2-}$ requires *m/z* 669.1899).

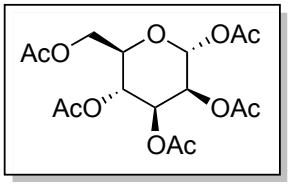


2,4,6-Tri-*O*-acetyl-3-*O*-(carbamoyl)-α-D-mannopyranosyl Benzyl 2-(Ethoxy)ethylcarbamate (2.83). To a solution of 1.87 g (3.22 mmol) of phosphate ester **2.54** and 692 mg (2.89 mmol) of CBz-protected linker **2.57** in 50 mL of anh dichloromethane was added 1.10 mL (1.29 g, 5.79 mmol) of TMSOTf at 0 °C. The reaction mixture was stirred at 0 °C for 20 min at which time it was poured into a mixture of 70 mL of ethyl acetate and 28 mL of saturated aq $NaHCO_3$.The organic layer was washed with two 30-mL portions of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 5 cm). Elution with 1:1→1:2→1:3 hexanes–ethyl acetate afforded linker conjugate **2.83** as a colorless oil: yield 1.58 g (96%); silica gel TLC R_f 0.26 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.02 (s, 3H), 2.08 (s, 3H), 2.12 (s, 3H), 3.39 (m, 2H), 3.54 (m, 2H), 3.64 (m, 3H), 3.79 (m, 1H), 4.08 (m, 2H), 4.26 (m, 1H), 4.71 (br s, 2H), 4.91 (s, 1H), 5.10 (s, 2H), 5.25 (m, 3H), 5.37 (br s, 1H) and 7.35 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.8, 20.9, 21.0, 41.1, 62.7, 66.5, 66.8, 67.3, 68.5, 70.0, 70.1, 70.3, 70.4, 77.4, 97.6, 128.1, 128.2, 128.6, 136.8, 155.3, 170.1, 170.2 and 170.8; mass spectrum (APCI), *m/z* 571.2141 $(M + H)^+$ ($C_{25}H_{35}N_2O_{13}$ requires *m/z* 571.2139).



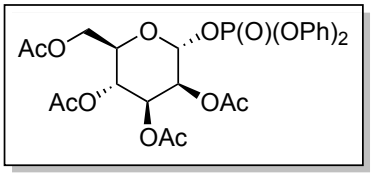
BLM Monosaccharide–Cy5 (2.8).** To a solution of 4.60 mg (8.06 μmol) of monosaccharide–linker **2.83** in 2 mL of anh methanol was added 0.20 mL of a freshly prepared 25% w/w solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 467.27 $(M + Na)^+$; mass spectrum (APCI), *m/z* 445.1815 $(M + H)^+$ ($C_{19}H_{29}N_2O_{10}$ requires *m/z* 445.1822). To the solution of the crude product in 5 mL of anh methanol was added Pd/C and H_2 gas was bubbled through for 45 min. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered through Celite 545® and the filtrate was concentrated under diminished pressure to afford **2.84** as a colorless oil: yield 2.50 mg (100%), which was used directly for the next reaction; mass spectrum (MALDI-TOF), *m/z* 333.22 $(M + Na)^+$; mass spectrum (APCI), *m/z* 311.1455 $(M + H)^+$ ($C_{11}H_{23}N_2O_8$ requires *m/z* 311.1454).

To 122 μg (0.39 μmol) of **2.84** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu (**2.60**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 35 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 22.5 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give BLM monosaccharide–Cy5** (**2.8**) as a blue solid: yield 50.0 μg (36% over two steps); mass spectrum (MALDI-TOF), *m/z* 1201.47 $(M - H + Na)^+$, 1223.47 $(M - 2H + 2Na)^+$, 1245.45 $(M - 3H + 3Na)^+$ and 1267.43 $(M - 4H + 4Na)^+$; mass spectrum (ESI), *m/z* 588.1614 $(M - 3H)^{2-}$ ($C_{49}H_{68}N_4O_{21}S_4^{2-}$ requires *m/z* 588.1629).



Penta-*O*-acetyl-α-D-mannopyranose (2.85).[131] To a solution containing 1.00 g (5.15 mmol) of methyl-α-D-mannopyranose (**2.46**) in 19.0 mL of acetic anhydride was added a catalytic amount of concentrated H_2SO_4, and the solution was stirred at room temperature for 12 h. The reaction mixture was poured into a stirred mixture of 150 mL of ethyl acetate and 80 mL of saturated aq $NaHCO_3$. The organic layer was washed with 40 mL of saturated aq $NaHCO_3$, 30 mL of brine, then dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel

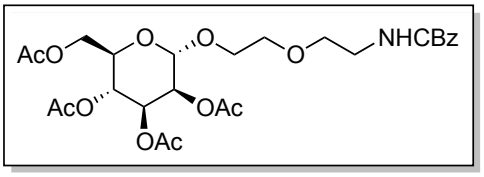
column (18 × 5 cm). Elution with 5:1→3:1 hexanes–ethyl acetate afforded mannose **2.85** as a colorless oil: yield 1.97 g (98%); silica gel TLC R_f 0.60 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.86 (s, 3H), 1.91 (s, 3H), 1.95 (m, 3H), 2.04 (m, 6H), 3.94 (m, 2H), 4.13 (m, 1H), 5.12 (s, 1H), 5.20 (m, 2H) and 5.94 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.40, 20.43, 20.47, 20.53, 20.6, 61.9, 65.3, 68.1, 68.6, 70.4, 76.8, 77.2, 77.5, 167.8, 169.3, 169.5, 169.7 and 170.3.



2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl Diphenyl Phosphate (2.86).[132] To a solution of 525 mg (1.34 mmol) of mannose **2.85** in 8.1 mL of dry DMF was added 170 mg (1.88 mmol) of hydrazine acetate. The reaction was stirred at room temperature for 2 h until analysis by silica gel TLC indicated it was complete. The reaction mixture was diluted with 50 mL of ethyl acetate and washed with three 20-mL portions of brine. The aqueous layer was re-extracted with three 30-mL portions of ethyl acetate. The combined organic layer was dried (Na_2SO_4), filtered and concentrated under diminished pressure to afford the pyranoside as a colorless oil; silica gel TLC R_f 0.39 (3:1 hexanes–ethyl acetate).

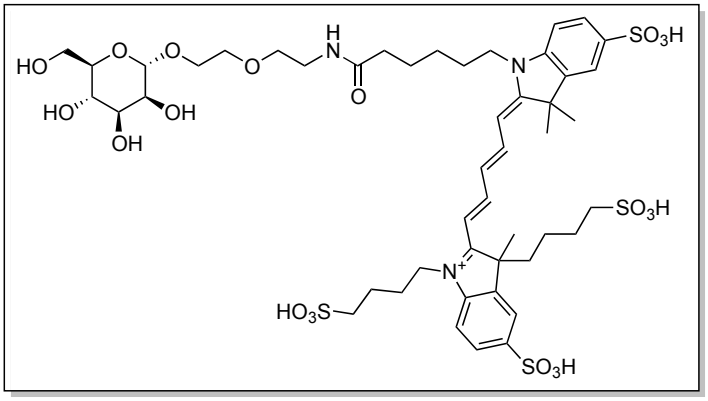
To the solution of 397 mg (1.14 mmol) of the above residue alcohol in 16.5 mL of dry dichloromethane was added 180 mg (1.47 mmol) of DMAP and 1.6 mL (11.4 mmol) of dry triethylamine. The reaction mixture was stirred for 10 min, followed by the addition of 2.30 mL (10.9 mmol) of diphenyl chlorophosphate dropwise at 0 °C. The

solution was stirred at 0 °C for 1.5 h and was poured into a mixture of 100 mL of ethyl acetate and 50 mL of saturated aq $NaHCO_3$ soln. The organic layer was washed with two 30-mL portions of brine, dried (Na_2SO_4), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (18 × 5 cm). Elution with 3:1→2:1 hexanes–ethyl acetate afforded phosphate ester **2.86** as a colorless oil: yield 424 mg (54% over two steps); silica gel TLC R_f 0.54 (3:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.04 (s, 3H), 2.06 (s, 3H), 2.10 (s, 3H), 2.22 (s, 3H), 3.98 (dd, 1H, J = 12.4 and 2.0 Hz), 4.14 (m, 1H), 4.25 (dd, 1H, J = 12.4 and 4.8 Hz), 5.40 (m, 3H), 5.92 (dd, 1H, J = 6.8 and 1.6 Hz), 7.28-7.33 (m, 6H) and 7.40-7.45 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.70, 20.72, 20.78, 20.84, 61.8, 65.2, 68.3, 68.7, 68.8, 70.9, 96.17, 96.22, 120.18, 120.22, 120.3, 120.4, 125.90, 125.91, 126.0, 130.1, 130.2, 169.6, 169.9 and 170.7.



2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl Benzyl 2-(2-Ethoxy)ethylcarbamate (2.87). To a solution of 300 mg (0.52 mmol) of phosphate ester **2.86** and 111 mg (0.46 mmol) of the CBz-protected linker **2.57** in 5.5 mL of anhydrous dichloromethane was added 168 μL (207 mg, 0.93 mmol) of TMSOTf at 0 °C. The reaction was stirred at 0 °C for 18 min and was then poured into a mixture of 100 mL of ethyl acetate and 40 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 40-mL portions of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was

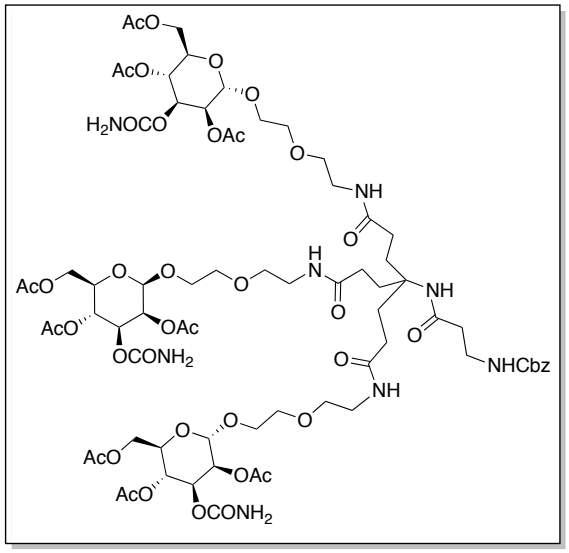
purified by flash chromatography on a silica gel column (25 × 2.5 cm). Elution with 2:1→1:2 hexanes–ethyl acetate afforded linker conjugate **2.87** as a colorless oil: yield 110 mg (37%); silica gel TLC R_f 0.35 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.98 (s, 3H), 2.00 (s, 3H), 2.08 (s, 3H), 2.13 (s, 3H), 3.40 (m, 2H), 3.53 (m, 2H), 3.62 (m, 3H), 3.77 (m, 1H), 4.06 (m, 1H), 4.11 (m, 1H), 4.24 (m, 1H), 4.87 (br s, 1H), 5.08 (s, 2H), 5.24 (m, 2H), 5.35 (m, 2H) and 7.26-7.34 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.80, 20.82, 20.9, 21.0, 39.4, 41.1, 62.7, 66.4, 66.8, 67.2, 68.6, 69.1, 69.8, 70.1, 70.4, 97.7, 128.2, 128.6, 136.8, 169.9, 170.0, 170.3, 170.8 and 170.9; mass spectrum (MALDI-TOF), *m/z* 592.34 $(M + Na)^+$; mass spectrum (APCI), *m/z* 570.2182 $(M + H)^+$ ($C_{26}H_{36}NO_{13}$ requires *m/z* 570.2187).



Decarbamoyl BLM Monosaccharide–Cy5 (2.9).** To a solution of 8.90 mg (15.6 μmol) of linker **2.87** in 2.00 mL of anh methanol was added 0.20 mL of a freshly prepared 25% w/w solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min,

filtered and concentrated; mass spectrum (MALDI-TOF) *m/z* 424.24 $(M + Na)^+$; mass spectrum (APCI), *m/z* 402.1759 $(M + H)^+$ ($C_{18}H_{28}NO_9$ requires *m/z* 402.1764). To a solution of the crude product in 5 mL of anh methanol was added Pd/C and H_2 gas was bubbled through for 45 min. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered through Celite 545® and then concentrated under diminished pressure to afford amine **2.88** as a colorless oil: yield 4.17 mg (100%), which was used for the next reaction; mass spectrum (MALDI-TOF), *m/z* 268.25 $(M + H)^+$ and 290.25 $(M + Na)^+$; mass spectrum (APCI), *m/z* 268.1391 $(M + H)^+$ ($C_{10}H_{22}NO_7$ requires *m/z* 268.1396).

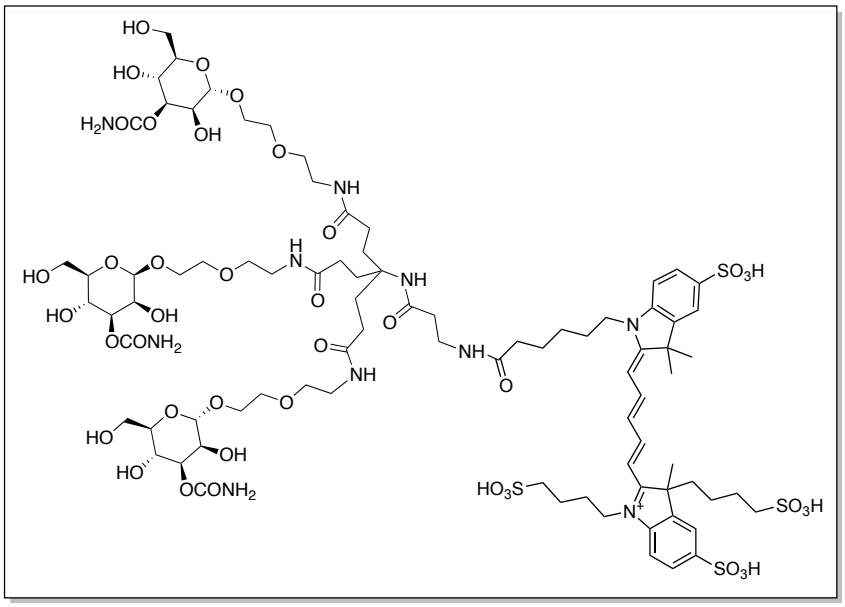
To 152 μg (0.57 μmol) of amine **2.88** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu (**2.60**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 35 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 19.5 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give decarbamoyl BLM monosaccharide–Cy5** (**2.9**) as a blue solid: yield 39.0 μg (30% over two steps); mass spectrum (MALDI-TOF), *m/z* 1158.40 $(M - H + Na)^+$ and 1180.47 $(M - 2H + 2Na)^+$; mass spectrum (ESI), *m/z* 1136.3409 $(M)^+$ ($C_{48}H_{70}N_3O_{20}S_4$ requires *m/z* 1136.3435).



BLM Monosaccharide Trimer (2.91). H_2 gas was bubbled through a mixture containing 36.0 mg (21.0 μmol) of monosaccharide–linker **2.83** and a catalytic amount of Pd/C in 6 mL of dry THF for 45 min. The reaction mixture was filtered through a pad of Celite 545® and the filtrate was concentrated under diminished pressure to obtain amine **2.89** as a colorless oil, which was used immediately in the next step: crude yield 27.0 mg (99%); silica gel TLC R_f 0.29 (1:3 hexanes–ethyl acetate); mass spectrum (MALDI-TOF), *m/z* 459.26 $(M + Na)^+$; mass spectrum (APCI), *m/z* 437.1768 $(M + H)^+$ ($C_{17}H_{29}N_2O_{11}$ requires *m/z* 437.1772).

To a solution containing 27.0 mg (61.8 μmol) of amine **2.89** in 0.53 mL of dry DMF were added 13.0 μL (0.09 mmol) of dry triethylamine and 15.2 mg (20.4 μmol) of NHS ester of the trimeric linker **2.90**[130] and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 1.5 cm). Elution with 16:12:1→11:12:1 chloroform–acetone–methanol afforded BLM

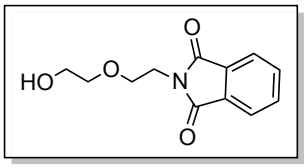
monosaccharide trimer−linker conjugate **2.91** as a colorless oil: yield 15.0 mg (43%); silica gel TLC R_f 0.56 (4:4:1 chloroform–acetone–methanol); mass spectrum (MALDI-TOF), *m/z* 1730.76 $(M + Na)^+$; mass spectrum (ESI), *m/z* 854.3351 $(M + 2H)^{2+}$ ($C_{72}H_{108}N_8O_{39}$ requires *m/z* 854.3357).



BLM Monosaccharide–Cy5 Trimer (2.10).** To a solution of 4.20 mg (2.46 μmol) of linker **2.91** in 2 mL of anh methanol was added 200 μL of a freshly prepared 25% w/w solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 1351.40 $(M + Na)^+$ (theoretical m/z 1328.56). To the solution of the crude product in 5 mL of anh methanol was added Pd/C and H_2 gas was bubbled through for 45 min. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture

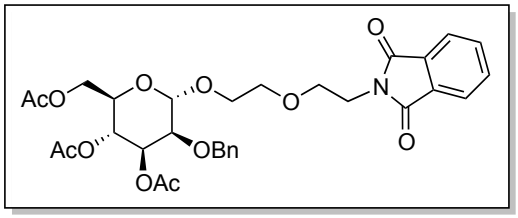
was filtered through Celite 545® and then concentrated under diminished pressure to afford amine **2.92** as a colorless oil: yield 2.90 mg (100%), which was used directly for the next reaction; mass spectrum (MALDI-TOF), *m/z* 1217.62 $(M + Na)^+$; mass spectrum (ESI), *m/z* 1229.4961 $(M + Cl)^-$ ($C_{46}H_{82}N_8O_{28}Cl$ requires *m/z* 1229.4927).

To 540 μg (0.448 μmol) of amine **2.92** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu (**2.60**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 21.0 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give BLM monosaccharide–Cy5** trimer **2.10** as a blue solid: yield 77.0 μg (33%); mass spectrum (MALDI-TOF), *m/z* 2085.85 $(M + Na - H)^+$ and 2107.85 $(M + 2Na - 2H)^+$; mass spectrum (ESI), *m/z* 686.6584 $(M - 4H)^{3-}$ ($C_{84}H_{127}N_{10}O_{41}S_4$ requires *m/z* 686.5679).



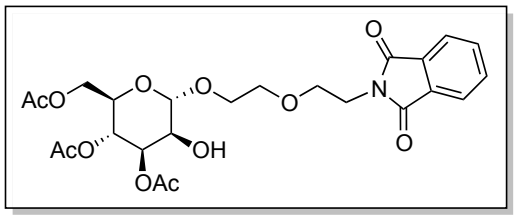
2-(2-(2-Hydroxyethoxy)ethyl)isoindoline-1,3-dione (2.93).[133] To a solution of 2.10 g (19.9 mmol) of 2-(2-aminoethyl)-ethanol in 90 mL dry toluene was added 2.95 g (19.9 mmol) of phthalic anhydride. The resulting solution was then heated under reflux for 6 h with Dean-Stark apparatus. The reaction mixture was allowed to cool, dried ($MgSO_4$), filtered, and concentrated under diminished pressure to afford protected linker **2.93** as a

white solid: yield 4.67 g (99%); silica gel TLC R_f 0.35 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.61 (m, 2H), 3.69 (m, 2H), 3.75 (t, 2H, J = 5.3 Hz), 3.91 (t, 2H, J = 5.3 Hz), 7.73 (m, 2H) and 7.85 (m, 2H).

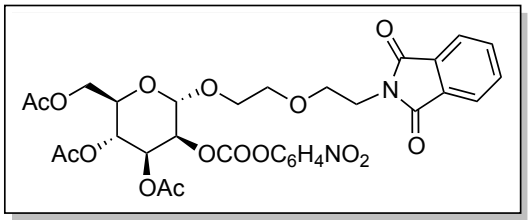


3,4,6-Tri-*O*-acetyl-2-*O*-benzyl-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.94). To a solution of 1.11 g (1.77 mmol) of phosphate ester **2.64** and 374 mg (1.59 mmol) of the alcohol **2.93** in 30 mL of anh dichloromethane was added 0.58 mL (707 mg, 3.18 mmol) of TMSOTf at 0 °C. The reaction was stirred at 0 °C for 20 min and was then poured into a mixture of 150 mL of ethyl acetate and 60 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 20-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (22 × 5 cm). Elution with 4:1→1:1 hexanes–ethyl acetate afforded monosaccharide **2.94** as a colorless oil: yield 860 mg (79%); silica gel TLC R_f 0.41 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.91 (s, 3H), 1.96 (s, 3H), 2.00 (s, 3H), 3.54 (m, 3H), 3.67 (m, 3H), 3.78 (m, 1H), 3.82 (m, 1H), 3.88 (m, 1H), 4.00 (m, 1H), 4.16 (m, 1H), 4.57 (q, 2H, J = 12.0 Hz), 4.82 (d, 1H, J = 1.2 Hz), 5.16 (m, 1H), 5.32 (t, 1H, J = 10.0 Hz), 7.22-7.30 (m, 5H), 7.63 (m, 2H) and 7.76 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.83, 20.85, 20.9, 37.3, 62.8, 66.7, 67.0, 68.1, 68.6, 69.6, 71.3, 73.1, 75.5, 98.0, 123.3, 127.9, 127.9, 128.4, 132.2, 133.0,

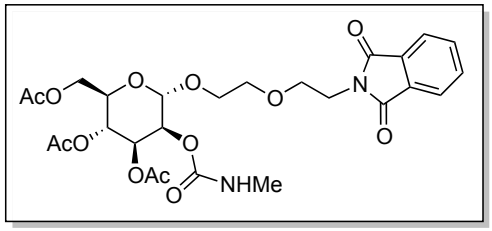
137.9, 168.3, 169.8, 170.2 and 170.9; mass spectrum (ESI), *m/z* 636.2040 $(M + Na)^+$ ($C_{31}H_{35}NO_{12}Na$ requires *m/z* 636.2057).



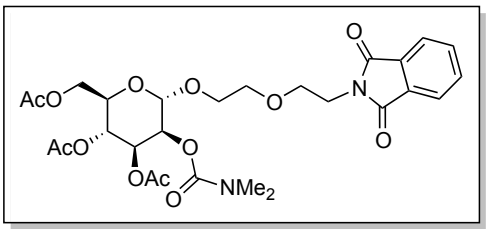
3,4,6-Tri-*O*-acetyl-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.95). To a solution of 860 mg (1.40 mmol) of monosaccharide **2.94** in 23 mL of ethyl acetate was added 420 mg of $Pd(OH)_2/C$ and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (18 × 3.5 cm). Elution with 3:1→1:1 hexanes–ethyl acetate afforded alcohol **2.95** as a colorless foam: yield 601 mg (82%); silica gel TLC R_f 0.13 (1:1 hexanes–ethyl acetate). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.97 (s, 3H), 2.01 (2s, 6H), 2.94 (br s, 1H), 3.60 (m, 3H), 3.69 (m, 3H), 3.84 (m, 2H), 3.94 (m, 1H), 4.00 (m, 2H), 4.20 (m, 1H), 4.84 (d, 1H, J = 2.0 Hz), 5.17 (m, 1H), 5.28 (t, 1H, J = 10.0 Hz), 7.67 (m, 2H) and 7.80 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.73, 20.77, 20.9, 37.4, 62.6, 66.3, 67.0, 68.1, 68.3, 69.1, 69.7, 71.6, 99.9, 123.3, 132.0, 134.0, 168.3, 169.9, 170.0 and 170.9; mass spectrum (ESI), *m/z* 546.1596 $(M + Na)^+$ ($C_{24}H_{29}NO_{12}Na$ requires *m/z* 546.1588).



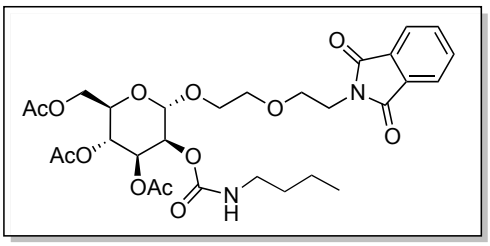
3,4,6-Tri-*O*-acetyl-2-*O*-((*p*-nitrophenyl)carbamoyl)-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.96). To a solution of 461 mg (0.88 mmol) of alcohol **2.95** in 4.8 mL of dry pyridine was added 430 mg (3.52 mmol) of DMAP and 710 mg (3.52 mmol) of *p*-nitrophenyl chloroformate. The reaction was stirred at 40 °C overnight until the analysis by silica gel TLC indicated it was complete. The solution was cooled and poured into a mixture of 20 mL ethyl acetate and 5 mL of water. The organic layer was washed successively with three 10-mL portions of 1N HCl, 5 mL of saturated aq $NaHCO_3$ and 5 mL of brine. The solution was dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (30 × 2.5 cm). Elution with 4:1→1:1 hexanes–ethyl acetate afforded carbonate **2.96** as a white foam: yield 443 mg (73%); silica gel TLC R_f 0.55 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.02 (s, 3H), 2.05 (s, 3H), 2.07 (s, 3H), 3.66 (m, 2H), 3.72 (m, 3H), 3.79 (m, 1H), 3.89 (m, 2H), 4.06 (m, 2H), 4.17 (m, 1H), 5.08 (m, 1H), 5.18 (m, 1H), 5.30 (t, 1H, J = 10.0 Hz), 5.41 (m, 1H), 7.44 (d, 2H, J = 9.2 Hz), 7.70 (m, 2H) 7.84 (m, 2H) and 8.29 (d, 2H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.83, 20.85, 20.86, 37.5, 62.4, 66.0, 67.3, 68.4, 68.6, 69.1, 69.8, 74.5, 97.2, 121.9, 123.4, 125.5, 132.2, 134.1, 145.7, 152.1, 155.5, 168.4, 169.8 and 170.8; mass spectrum (APCI), *m/z* 689.1817 $(M + H)^+$ ($C_{31}H_{33}N_2O_{16}$ requires *m/z* 689.1830).



3,4,6-Tri-*O*-acetyl-2-*O*-(methylcarbamoyl)-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.97). To a solution containing 166 mg (0.24 mmol) of carbamate **2.96** in 7.6 mL of anh THF was added 0.12 mL (0.24 mmol) of 2 M solution of methylamine in THF dropwise at 0 °C. The reaction mixture was allowed to warm to room temperature and then stirred overnight at room temperature. The solvent was concentrated under diminished pressure and was purified by flash chromatography on a silica gel column (18 × 2.5 cm). Elution with 3:1→1:2 hexanes–ethyl acetate afforded monosaccharide **2.97** as a colorless foam: yield 125 mg (89%); silica gel TLC R_f 0.11 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.96 (s, 3H), 2.01 (s, 3H), 2.04 (s, 3H), 2.77 (d, 3H, J = 4.8 Hz), 3.62 (m, 3H), 3.71 (m, 3H), 3.89 (m, 2H), 4.01 (m, 2H), 4.27 (m, 1H), 4.85 (s, 1H), 4.89 (q, 1H, J = 4.8 Hz), 5.11 (m, 1H), 5.21 (m, 1H), 5.28 (m, 1H), 7.68 (m, 2H) and 7.82 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.80, 20.85, 27.6, 37.3, 62.6, 64.4, 66.2, 67.2, 68.1, 68.3, 69.4, 70.1, 77.4, 98.1, 123.4, 132.2, 134.0, 155.8, 168.2, 169.9, 169.9 and 170.8; mass spectrum (ESI), *m/z* 603.1797 (M + Na)$^+$ ($C_{26}H_{32}N_2O_{13}Na$ requires *m/z* 603.1802).

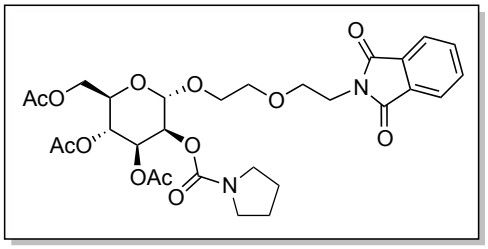


3,4,6-Tri-*O*-acetyl-2-*O*-(dimethylcarbamoyl)-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.98). To a solution containing 180 mg (0.26 mmol) of carbamate **2.96** in 8.2 mL of anh THF was added 0.13 mL (0.26 mmol) of 2 M solution of dimethylamine in THF dropwise at 0 °C. The reaction mixture was allowed to warm to room temperature and then stirred overnight at room temperature. The solvent was concentrated under diminished pressure and was purified by flash chromatography on a silica gel column (18 × 2.5 cm). Elution with 3:1→1:2 hexanes–ethyl acetate afforded monosaccharide **2.98** as a colorless foam: yield 125 mg (80%); silica gel TLC R_f 0.15 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.95 (s, 3H), 2.01 (2s, 6H), 2.88 (s, 3H), 2.96 (s, 3H), 3.61 (m, 3H), 3.72 (m, 3H), 3.86 (m, 2H), 4.02 (m, 2H), 4.25 (m, 1H), 4.85 (d, 1H, J = 1.6 Hz), 5.08 (m, 1H), 5.28 (m, 2H), 7.68 (m, 2H) and 7.81 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7, 20.80, 20.84, 36.0, 36.6, 37.3, 62.5, 66.2, 67.3, 68.1, 68.4, 69.39, 69.41, 70.6, 97.9, 123.3, 132.2, 134.0, 155.2, 168.2, 169.87, 169.92 and 170.6; mass spectrum (ESI), *m/z* 617.1963 $(M + Na)^+$ ($C_{27}H_{34}N_2O_{13}Na$ requires *m/z* 617.1959).



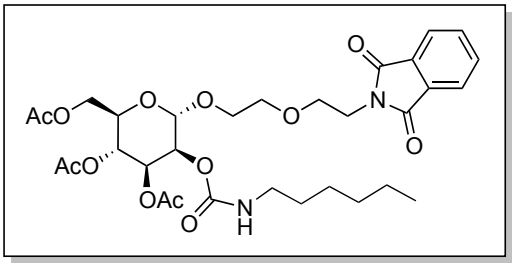
3,4,6-Tri-*O*-acetyl-2-*O*-(butylcarbamoyl)-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.99). To a solution containing 41.0 mg (0.06 mmol) of carbamate **2.96** in 1.9 mL of anh THF was added 30.0 μL (0.06 mmol) of 2 M solution of butylamine in THF dropwise at 0 °C. The reaction mixture was allowed to

warm to room temperature and then stirred overnight at room temperature. The solvent was concentrated under diminished pressure and was purified by flash chromatography on a silica gel column (18 × 2 cm). Elution with 3:1→1:2 hexanes–ethyl acetate afforded monosaccharide **2.99** as a colorless foam: yield 27.0 mg (73%); silica gel TLC R_f 0.17 (1:1 hexanes–ethyl acetate); ^{1}H ($CDCl_3$, 400 MHz) δ 0.91 (t, 3H, J = 7.2 Hz), 1.35 (m, 2H), 1.48 (m, 2H), 1.98 (s, 3H), 2.03 (s, 3H), 2.06 (s, 3H), 3.16 (q, 2H, J = 6.8 Hz), 3.63 (m, 3H), 3.75 (m, 3H), 3.89 (m, 2H), 4.04 (m, 2H), 4.30 (m, 1H), 4.87 (s, 2H), 5.13 (br s, 1H), 5.27 (m, 2H), 7.70 (m, 2H) and 7.84 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.8, 20.0, 20.8, 20.87, 20.92, 29.8, 32.0, 37.3, 41.0, 62.7, 66.4, 67.3, 68.2, 68.4, 69.4, 69.5, 70.0, 98.2, 123.4, 132.2, 134.0, 155.3, 168.3, 170.0 and 170.8; mass spectrum (ESI), *m/z* 645.2278 $(M + Na)^+$ ($C_{29}H_{38}N_2O_{13}Na$ requires *m/z* 645.2272).



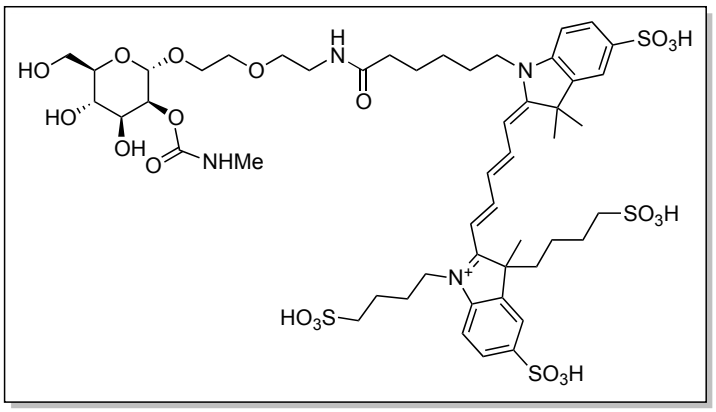
3,4,6-Tri-*O*-acetyl-2-*O*-(pyrrolidinylcarbamoyl)-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.100). To a solution containing 80.0 mg (0.12 mmol) of carbamate **2.96** in 3.7 mL of anh THF was added 10.0 μL (0.12 mmol) of pyrrolidine at 0 °C. The reaction mixture was allowed to warm to room temperature and then stirred overnight at room temperature. The solvent was concentrated under diminished pressure and was purified by flash chromatography on a silica gel column (18 × 2.5 cm). Elution with 3:1→1:2 hexanes–ethyl acetate afforded monosaccharide **2.100**

as a colorless foam: yield 69.0 mg (96%); silica gel TLC R_f 0.19 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.88 (m, 4H), 1.96 (s, 3H), 2.01 (s, 3H), 2.03 (s, 3H), 3.35 (m, 2H), 3.45 (s, 2H), 3.62 (m, 3H), 3.71 (m, 3H), 3.89 (m, 2H), 4.04 (m, 2H), 4.26 (m, 1H), 4.87 (s, 1H), 5.11 (m, 1H), 5.31 (m, 2H), 7.68 (m, 2H) and 7.82 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.75, 20.83, 20.9, 25.0, 25.7, 37.3, 46.0, 46.3, 62.6, 66.3, 67.3, 68.1, 68.4, 69.41, 69.45, 70.2, 98.0, 123.4, 132.2, 134.0, 153.6, 168.3, 169.95, 169.98 and 170.7; mass spectrum (ESI), *m/z* 643.2123 $(M + Na)^+$ ($C_{29}H_{36}N_2O_{13}Na$ requires *m/z* 643.2115).



3,4,6-Tri-*O*-acetyl-2-*O*-(hexylcarbamoyl)-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.101). To a solution containing 43.0 mg (0.06 mmol) of carbamate **2.96** in 2 mL of anh THF was added 31.0 μL (0.06 mmol) of 2 M solution of hexylamine in THF dropwise at 0 °C. The reaction mixture was allowed to warm to room temperature and then stirred overnight at room temperature. The solvent was concentrated under diminished pressure and was purified by flash chromatography on a silica gel column (18 × 2 cm). Elution with 3:1→1:2 hexanes–ethyl acetate afforded monosaccharide **2.101** as a colorless oil: yield 25.0 mg (62%); silica gel TLC R_f 0.23 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (m, 3H), 1.24-1.35 (m, 7H), 1.99 (s, 3H), 2.04 (s, 3H), 2.07 (s, 3H), 2.39 (m, 1H), 3.15 (m, 2H), 3.64 (m, 3H), 3.73

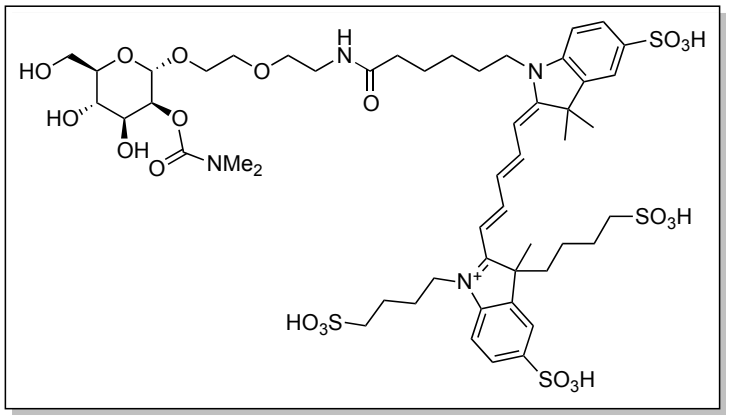
(m, 3H), 3.90 (m, 2H), 4.05 (m, 2H), 4.30 (m, 1H), 4.88 (m, 2H), 5.14 (s, 1H), 5.27 (m, 2H), 7.71 (m, 2H) and 7.85 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 20.88, 20.89, 20.93, 22.7, 26.5, 29.9, 31.6, 37.35, 37.40, 41.4, 62.7, 66.4, 67.3, 68.2, 68.3, 68.4, 69.46, 69.51, 69.6, 70.0, 98.2, 123.4, 132.3, 134.1, 155.28, 168.3, 170.0 and 170.9; mass spectrum (APCI), *m/z* 651.2771 $(M + H)^+$ ($C_{31}H_{43}N_2O_{13}$ requires *m/z* 651.2765).



Monosaccharide–Cy5 Conjugate 2.11.** To a solution of 9.00 mg (15.5 μmol) of compound **2.97** in 2 mL of anh methanol was added 0.2 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 477.33 $(M + Na)^+$; mass spectrum (APCI), *m/z* 445.1661 $(M + H)^+$ ($C_{20}H_{27}N_2O_{10}$ requires *m/z* 445.1665). To the solution of the crude product in 0.2 mL of ethanol was added 0.48 μL (15.5 μmol) of hydrazine hydrate and stirred at 60 °C overnight. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered

and concentrated under diminished pressure to afford **2.102** as a colorless oil: yield 4.90 mg (100%), which was used for the next reaction; mass spectrum (MALDI-TOF), *m/z* 347.12 $(M + Na)^+$.

To 191 μg (0.59 μmol) of **2.102** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu **(2.60)** in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 19.4 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give monosaccharide–Cy5** conjugate **2.11** as a blue solid: yield 34.0 μg (25%); mass spectrum (MALDI), *m/z* 1193.34 $(M)^+$ and 1215.33 $(M - H + Na)^+$; mass spectrum (ESI), *m/z* 396.4448 $(M - 3H)^{3-}$ ($C_{50}H_{72}N_4O_{21}S_4^{3-}$ requires *m/z* 396.4446).

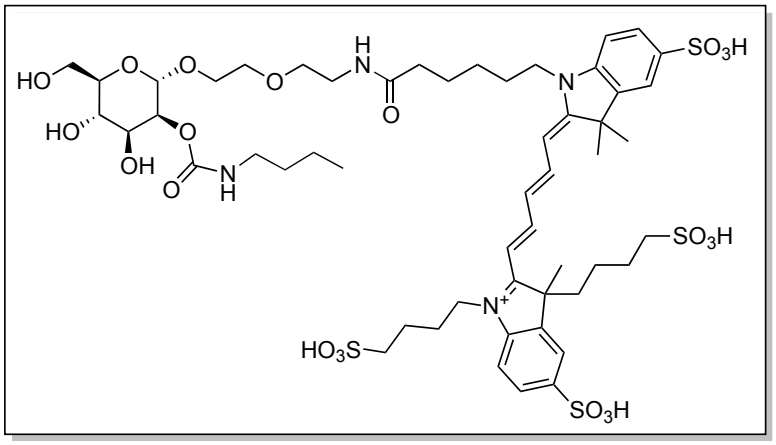


Monosaccharide–Cy5 Conjugate 2.12.** To a solution of 9.00 mg (15.1 μmol) of compound **2.98** in 2 mL of anh methanol was added 0.2 mL of 25% w/w freshly prepared

solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 491.32 $(M + Na)^+$; mass spectrum (APCI), *m/z* 491.1639 $(M + Na)^+$ ($C_{21}H_{28}N_2O_{10}Na$ requires *m/z* 491.1642). To the solution of the crude product in 0.2 mL of ethanol was added 0.48 μL (15.1 μmol) of hydrazine hydrate and stirred at 60 °C overnight. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered and concentrated under diminished pressure to afford **2.103** as a colorless oil: yield 5.00 mg (100%), which was used for the next reaction; mass spectrum (MALDI-TOF), *m/z* 339.22 $(M + H)^+$; mass spectrum (APCI), *m/z* 339.1773 $(M + H)^+$ ($C_{13}H_{27}N_2O_8$ requires *m/z* 339.1767).

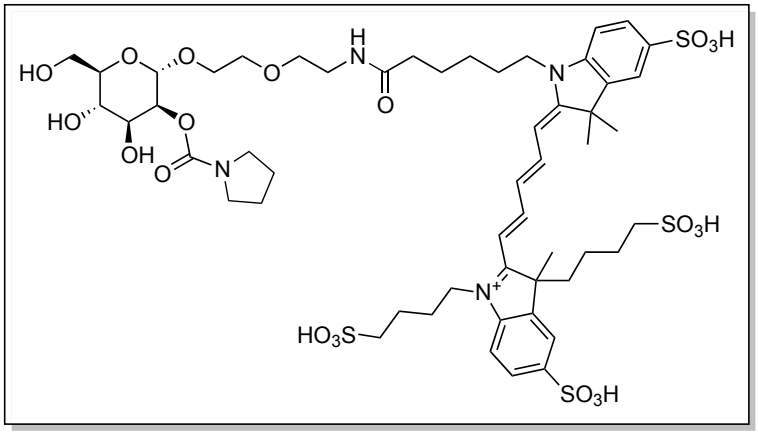
To 192 μg (0.57 μmol) of **2.103** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu (**2.60**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 21.0 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give monosaccharide–Cy5** conjugate **2.12** as a blue solid: yield 30.0 μg (22%); mass

spectrum (MALDI), *m/z* 1208.36 $(M)^+$ and 1246.34 $(M - H + K)^+$; mass spectrum (ESI), *m/z* 401.1162 $(M - 3H)^{3-}$ ($C_{51}H_{74}N_4O_{21}S_4^{3-}$ requires *m/z* 401.1165).



Monosaccharide–Cy5 Conjugate 2.13.** To a solution of 10.0 mg (16.1 μmol) of compound **2.99** in 2 mL of anh methanol was added 0.2 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 519.40 $(M + Na)^+$; mass spectrum (ESI), *m/z* 519.1958 $(M + Na)^+$ ($C_{23}H_{32}N_2O_{10}Na$ requires *m/z* 519.1954). To the solution of the crude product in 0.5 mL of ethanol was added 0.50 μL (16.0 μmol) of hydrazine hydrate and stirred at 60 °C overnight. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered and concentrated under diminished pressure to afford **2.104** as a colorless oil: yield 5.10 mg (98%), which was used for the next reaction; mass spectrum (APCI), *m/z* 367.2078 $(M + H)^+$ ($C_{15}H_{31}N_2O_8$ requires *m/z* 367.2080).

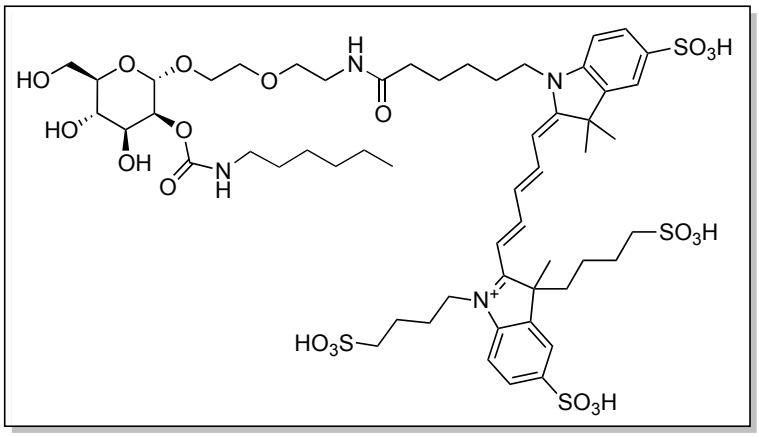
To 221 μg (0.60 μmol) of **2.104** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu **(2.60)** in 100 μL of 0.2 M phosphate buffer and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 19.3 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give monosaccharide–Cy5** **2.13** as a blue solid: yield 73.0 μg (52%); mass spectrum (MALDI-TOF), *m/z* 1235.45 $(M)^+$; mass spectrum (ESI), *m/z* 1279.3722 $(M - 2H + 2Na)^+$ ($C_{53}H_{77}N_4O_{21}S_4Na_2$ requires *m/z* 1279.3753).



Monosaccharide–Cy5 Conjugate 2.14.** To a solution of 18.0 mg (29.0 μmol) of compound **2.100** in 4 mL of anh methanol was added 0.4 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then

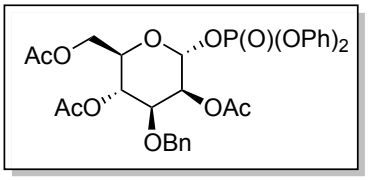
quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (ESI), *m/z* 517.1808 $(M + Na)^+$ ($C_{23}H_{30}N_2O_{10}Na$ requires *m/z* 517.1798). To the solution of the crude product in 0.4 mL of ethanol was added 0.90 μL (29.0 μmol) of hydrazine hydrate and stirred at 60 °C overnight. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered and concentrated under diminished pressure to afford **2.105** as a colorless oil: yield 10.0 mg (95%), which was used for the next reaction; mass spectrum (APCI), *m/z* 365.1926 $(M + H)^+$ ($C_{15}H_{29}N_2O_8$ requires *m/z* 365.1924).

To 220 μg (0.60 μmol) of **2.105** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu **(2.60)** in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 35 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 20.7 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give **2.14** as a blue solid: yield 75.0 μg (54%); mass spectrum (MALDI-TOF), *m/z* 1255.47 $(M - H + Na)^+$, 1277.55 $(M - 2H + 2Na)^+$ and 1299.49 $(M - 3H + 3Na)^+$; mass spectrum (ESI), *m/z* 1233.4021 $(M)^+$ ($C_{53}H_{77}N_4O_{21}S_4$ requires *m/z* 1233.3958).



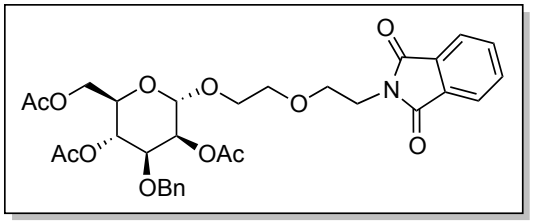
Monosaccharide–Cy5 Conjugate 2.15.** To a solution of 3.70 mg (5.69 μmol) of compound **2.101** in 1 mL of anh methanol was added 0.1 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 547.57 $(M + Na)^+$; mass spectrum (APCI), *m/z* 525.2447 $(M + H)^+$ ($C_{25}H_{37}N_2O_{10}$ requires *m/z* 525.2448). To the solution of the crude product in 0.2 mL of ethanol was added 0.18 μL (5.70 μmol) of hydrazine hydrate and stirred at 60 °C overnight. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered and concentrated under diminished pressure to afford **2.106** as a colorless oil: yield 2.24 mg (99%), which was used for the next reaction; mass spectrum (MALDI-TOF), *m/z* 395.30 $(M + H)^+$; mass spectrum (APCI), *m/z* 395.2402 $(M + H)^+$ ($C_{17}H_{35}N_2O_8$ requires *m/z* 395.2393).

To 220 μg (0.57 μmol) of **2.106** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu **(2.60)** in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 21.0 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give monosaccharide–Cy5** conjugate **2.15** as a blue solid: yield 30.0 μg (21%); mass spectrum (MALDI-TOF), *m/z* 1263.55 $(M)^+$ and 1285.43 $(M - H + Na)^+$ (theoretical *m/z* 1263.44 and 1307.44).



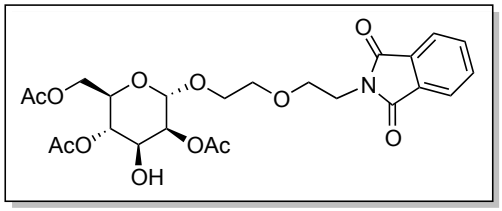
2,4,6-Tri-*O*-acetyl-3-*O*-benzyl-α-D-mannopyranosyl Diphenyl Phosphate (2.107).[114] To a solution of 910 mg (2.07 mmol) of benzyl ether **2.49** in 14 mL of anh DMF was added 262 mg (2.91 mmol) of hydrazine acetate. The solution was stirred at room temperature for 2.5 h and diluted with 100 mL of ethyl acetate. The organic solution was washed with 50 mL of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure to afford the alcohol as a light yellow oil: crude yield 820 mg (100%), which was used for the next reaction; silica gel TLC R_f 0.40 (1:1 hexanes–ethyl acetate).

To a solution of above residue in 32 mL of anh dichloromethane at 0 °C were added 326 mg (2.67 mmol) of DMAP, 2.90 mL (2.01 g, 20.7 mmol) of dry triethylamine, and 4.10 mL (5.32 g, 19.8 mmol) of diphenyl phosphoryl chloride. The solution was stirred at room temperature for 1.5 h and poured into a mixture of 70 mL of ethyl acetate and 35 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 35-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 4 cm). Elution with 2:1 hexanes–ethyl acetate afforded phosphate ester **2.107** as a colorless oil: yield 550 mg (42% over two steps); silica gel TLC R_f 0.38 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.01 (s, 3H), 2.08 (s, 3H), 2.15 (s, 3H), 3.94 (dd, 1H, J = 6.0 and 3.6 Hz), 4.11 (m, 1H), 4.14 (m, 1H), 4.19 (m, 1H), 4.55 (ABq, 2H, J = 12.4 Hz), 5.26 (m, 2H), 5.37 (m, 1H), 7.14-7.18 (m, 5H) and 7.26-7.36 (m, 10H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.9, 21.0, 21.2, 63.0, 67.6, 68.6, 69.0, 71.6, 74.1, 92.7, 120.3, 120.4, 125.5, 127.9, 128.0, 128.5, 128.8, 129.9, 137.8, 150.6, 169.9, 170.6 and 171.0.



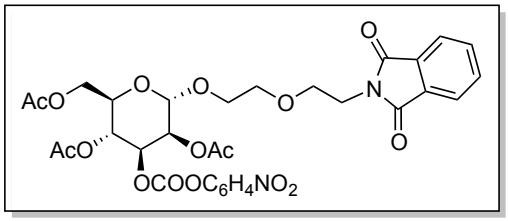
2,4,6-Tri-*O*-acetyl-3-*O*-benzyl-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.108). To a solution of 550 mg (0.87 mmol) of phosphate ester **2.107** and 185 mg (0.79 mmol) of the alcohol **2.57** in 15 mL of anh dichloromethane was added 0.29 mL (350 mg, 1.58 mmol) of TMSOTf at 0 °C. The reaction was stirred at 0 °C for 20 min and was then poured into a mixture of 75 mL of ethyl acetate and 30 mL of saturated aq

$NaHCO_3$. The organic layer was washed with two 20-mL portions of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (22 × 4 cm). Elution with 4:1→1:1 hexanes–ethyl acetate afforded monosaccharide **2.108** as a colorless oil: yield 290 mg (60%); silica gel TLC R_f 0.40 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.00 (s, 3H), 2.06 (s, 3H), 2.13 (s, 3H), 3.64 (m, 3H), 3.73 (m, 3H), 3.84-3.94 (m, 4H), 4.05 (m, 1H), 4.23 (m, 1H), 4.52 (ABq, 2H, J = 12.0 Hz), 4.86 (s, 1H), 5.22 (t, 1H, J = 10.0 Hz), 5.37 (d, 1H, J = 0.8 Hz), 7.22-7.34 (m, 5H), 7.70 (m, 2H) and 7.84 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7, 20.8, 21.0, 37.2, 62.7, 67.0, 67.4, 68.0, 68.2, 68.6, 69.3, 71.4, 74.7, 97.8, 123.3, 127.6, 127.7, 128.3, 132.0, 134.0, 137.9, 168.2, 169.7, 170.2 and 170.7; mass spectrum (APCI), *m/z* 614.2237 $(M + H)^+$ ($C_{31}H_{36}NO_{12}$ requires *m/z* 614.2238).



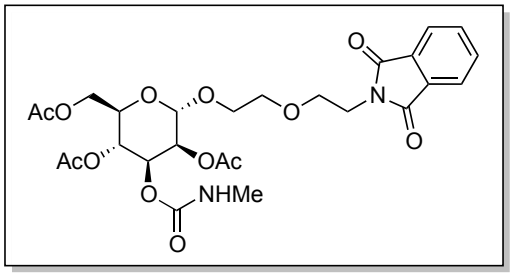
2,4,6-Tri-*O*-acetyl-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.109). To a solution of 275 mg (0.45 mmol) of monosaccharide **2.108** in 10 mL of ethyl acetate was added 135 mg of $Pd(OH)_2/C$ and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (18 × 3 cm). Elution with 3:1→1:1 hexanes–ethyl acetate afforded alcohol **2.109** as a colorless foam: yield 142 mg (61%); silica gel TLC R_f

0.21 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.07 (s, 3H), 2.12 (s, 3H), 2.15 (s, 3H), 2.22 (m, 1H), 3.59 (m, 1H), 3.65 (m, 2H), 3.75 (m, 3H), 3.89 (m, 2H), 3.95 (m, 1H), 4.07 (m, 2H), 4.29 (m, 1H), 4.88 (d, 1H, J = 1.2 Hz), 5.05 (m, 2H) 7.71 (m, 2H) and 7.85 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7, 20.9, 21.0, 37.1, 62.5, 67.1, 68.0, 68.1, 68.5, 69.2, 69.3, 72.3, 97.3, 123.3, 132.0, 134.0, 168.2, 170.4, 170.7 and 171.2; mass spectrum (APCI), *m/z* 524.1764 $(M + H)^+$ ($C_{24}H_{30}NO_{12}$ requires *m/z* 524.1768).



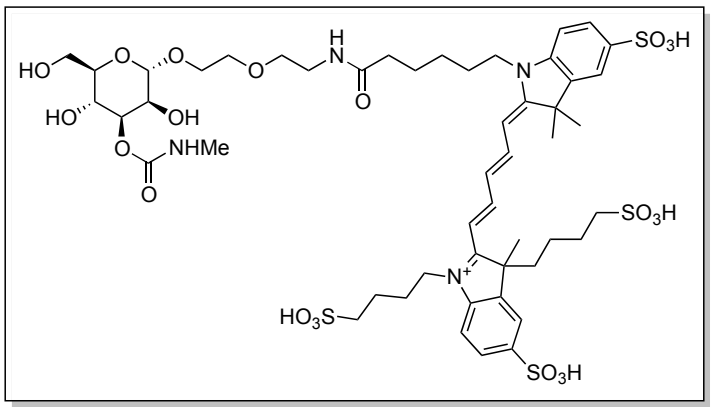
2,4,6-Tri-*O*-acetyl-2-*O*-((*p*-nitrophenyl)carbamoyl)-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.110). To a solution of 39.0 mg (0.07 mmol) of **2.109** in 0.42 mL of dry pyridine were added 3.00 mg (0.30 mmol) of DMAP and 60.0 mg (0.30 mmol) of *p*-nitrophenyl chloroformate. The reaction was stirred at 40 °C overnight until the analysis by silica gel TLC indicated it was complete. The solution was cooled and poured into a mixture of 15 mL ethyl acetate and 5 mL of water. The organic layer was washed successively with three 5-mL portions of 1 N HCl, 5 mL of saturated aq $NaHCO_3$ and 5 mL of brine. The solution was dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (30 × 1.5 cm). Elution with 4:1→1:1 hexanes–ethyl acetate afforded carbonate **2.110** as a white foam: yield 45.0 mg (88%); silica gel TLC R_f 0.55 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.08 (s, 3H),

2.10 (s, 3H), 2.17 (s, 3H), 3.64 (m, 3H), 3.72 (m, 2H), 3.77 (m, 1H), 3.89 (m, 2H), 4.08 (m, 2H), 4.30 (m, 1H), 4.92 (s, 1H), 5.21 (s, 1H), 5.36 (t, 1H, J = 10.0 Hz), 5.47 (m, 1H), 7.38 (d, 2H, J = 9.2 Hz), 7.69 (m, 2H), 7.83 (m, 2H) and 8.26 (d, 2H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.8, 20.9, 21.0, 37.3, 62.4, 65.8, 67.3, 68.3, 68.5, 68.8, 69.6, 74.8, 97.7, 122.0, 123.4, 125.4, 132.2, 134.1, 145.7, 151.7, 155.5, 168.3, 169.8, 170.3 and 170.8; mass spectrum (APCI), *m/z* 689.1839 $(M + H)^+$ ($C_{31}H_{33}N_2O_{16}$ requires *m/z* 689.1830).



2,4,6-Tri-*O*-acetyl-2-*O*-(methylcarbamoyl)-α-D-mannopyranosyl 2-(2-(Ethoxy)ethyl)isoindoline-1,3-dione (2.111). To a solution containing 21.0 mg (0.03 mmol) of carbamate **2.110** in 1 mL of anh THF was added 15.0 μL (0.03 mmol) of 2 M solution of methylamine in THF dropwise at 0 °C. The reaction mixture was allowed to warm to room temperature and then stirred overnight at room temperature. The solvent was concentrated under diminished pressure and was purified by flash chromatography on a silica gel column (18 × 1.5 cm). Elution with 3:1→1:2 hexanes–ethyl acetate afforded monosaccharide **2.111** as a colorless foam: yield 15.0 mg (85%); silica gel TLC R_f 0.21 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.04 (s, 3H), 2.07 (s, 3H), 2.13 (s, 3H), 2.76 (d, 3H, J = 4.8 Hz), 3.63 (m, 3H), 3.72 (m, 3H), 3.88 (m, 2H), 4.04 (m, 2H), 4.28 (m, 1H), 4.61 (d, 1H, J = 4.8 Hz), 4.85 (s, 1H), 5.25 (m, 3H), 7.70 (m,

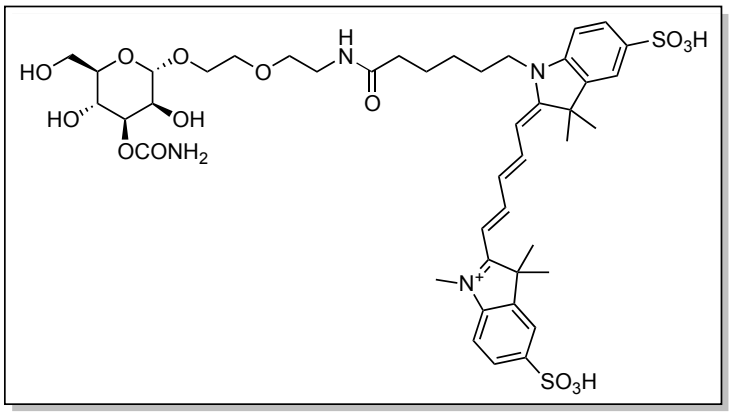
2H) and 7.85 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.89, 20.94, 21.1, 27.7, 29.8, 37.4, 62.7, 66.5, 67.3, 68.3, 68.5, 69.6, 69.9, 70.3, 97.8, 123.4, 132.3, 134.0, 155.7, 168.4, 170.1 and 170.8; mass spectrum (APCI), *m/z* 581.1983 $(M + H)^+$ ($C_{26}H_{33}O_{13}N_2$ requires *m/z* 581.1983).



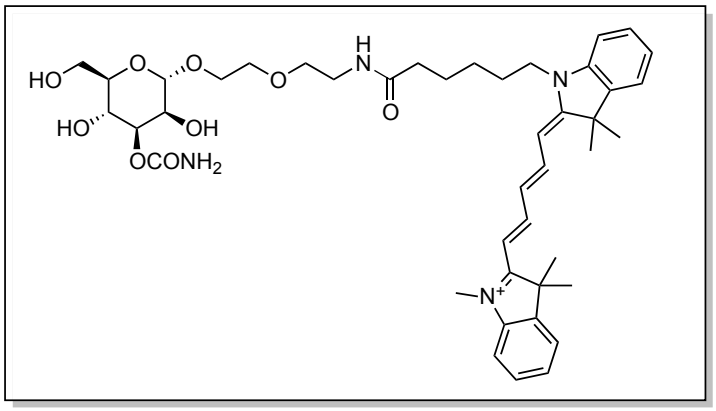
Monosaccharide–Cy5 Conjugate 2.16.** To a solution of 3.70 mg (6.37 μmol) of compound **2.111** in 1 mL of anh methanol was added 0.1 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 477.46 $(M + Na)^+$; mass spectrum (ESI), *m/z* 445.1484 $(M + Na)^+$ ($C_{20}H_{26}N_2O_{10}Na$ requires *m/z* 445.1485). To the solution of the crude product in 0.2 mL of ethanol was added 0.48 μL (15.5 μmol) of hydrazine hydrate and stirred at 60 °C overnight. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered and concentrated under diminished pressure to afford **2.112** as a colorless oil: yield 1.50

mg (73%), which was used for the next reaction; mass spectrum (MALDI-TOF), *m/z* 347.36 $(M + Na)^+$; mass spectrum (APCI), *m/z* 325.1612 $(M + H)^+$ ($C_{12}H_{25}N_2O_8$ requires *m/z* 325.1611).

To 185 μg (0.57 μmol) of **2.112** was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu **(2.60)** in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 19.4 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give monosaccharide–Cy5** conjugate **2.16** as a blue solid: yield 40.0 μg (30%); mass spectrum (MALDI-TOF), *m/z* 1215.71 $(M - H + Na)^+$, 1237.73 $(M - 2H + 2Na)^+$ and 1259.74 $(M - 3H + 3Na)^+$; mass spectrum (ESI), *m/z* 396.4445 $(M - 3H)^{3-}$ ($C_{50}H_{72}N_4O_{21}S_4^{3-}$ requires *m/z* 396.4446).

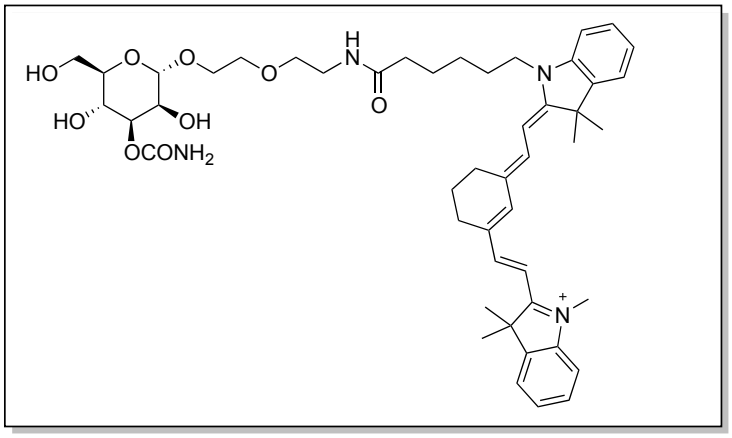


BLM Monosaccharide–sulphoCy5 (2.17). To 251 μg (0.81 μmol) of amine **2.84** was added a solution of 114 μg (0.15 μmol) of sulphoCy5COOSu (**2.113**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 25.3 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give BLM monosaccharide–sulphoCy5 conjugate (**2.17**) as a blue solid: yield 14.0 μg (10%); mass spectrum (MALDI-TOF), *m/z* 957.62 $(M + Na - H)^+$ and 979.59 $(M + 2Na - 2H)^+$; mass spectrum (ESI), *m/z* 933.3231 $(M)^+$ ($C_{43}H_{57}N_4O_{15}S_2^+$ requires *m/z* 933.3262).



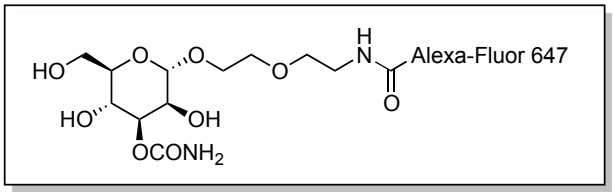
BLM Monosaccharide–Cy5 (2.18). To 1.40 mg (4.38 μmol) of amine **2.84** was added a solution of 500 μg (0.81 μmol) of Cy5COOSu (**2.114**) in 500 μL of 0.2 M phosphate buffer (pH 8.0) and 100 μL of DMSO. The reaction mixture was stirred overnight in the dark and purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10

μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile→ 0:100 0.1% aq TFA–acetonitrile) over a period of 10 min and 5 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 15.6 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give BLM monosaccharide–Cy5 conjugate (**2.18**) as a blue solid: yield 185 μg (30%); mass spectrum (MALDI-TOF), *m/z* 775.57 $(M)^+$ and 776.67 $(M + H)^+$; mass spectrum (ESI), *m/z* 775.4278 $(M)^+$ ($C_{43}H_{59}N_4O_9$ requires *m/z* 775.4277).

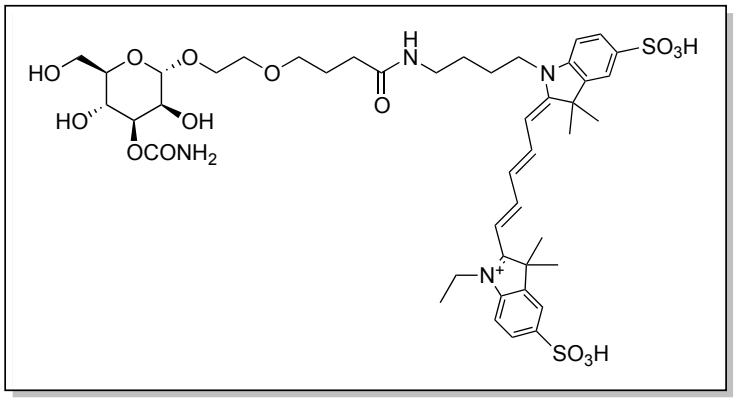


BLM Monosaccharide–Cy7 (2.19). To 284 μg (0.92 μmol) of amine **2.84** was added a solution of 116 μg (0.17 μmol) of Cy7COOSu (**2.115**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and 20 μL of DMSO. The reaction mixture was stirred overnight in the dark and purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile→ 0:100 0.1% aq TFA–acetonitrile) over a period of 12 min and 13 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 16.0 min

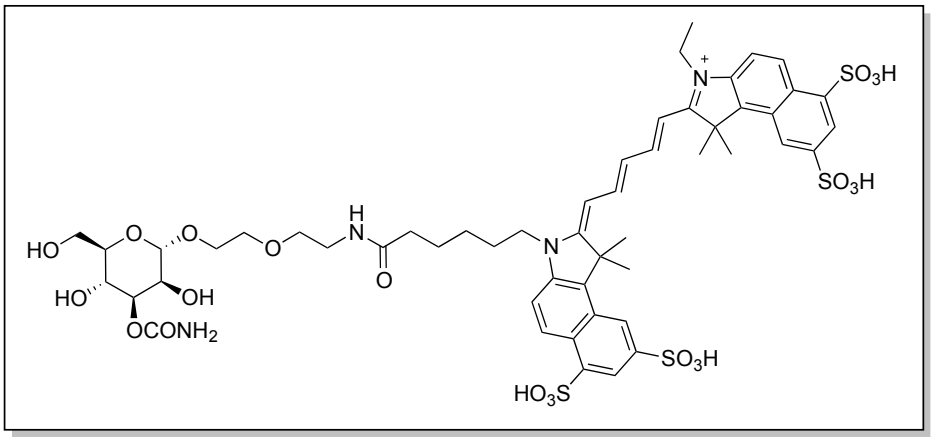
(monitoring at 750 nm) and were collected, frozen and lyophilized to give BLM monosaccharide–Cy7 conjugate (**2.19**) as a blue solid: yield 27.0 μg (19%); mass spectrum (MALDI-TOF), *m/z* 842.73 $(M)^+$; mass spectrum (ESI), *m/z* 841.4730 $(M)^+$ ($C_{48}H_{65}N_4O_9^+$ requires *m/z* 841.4746).



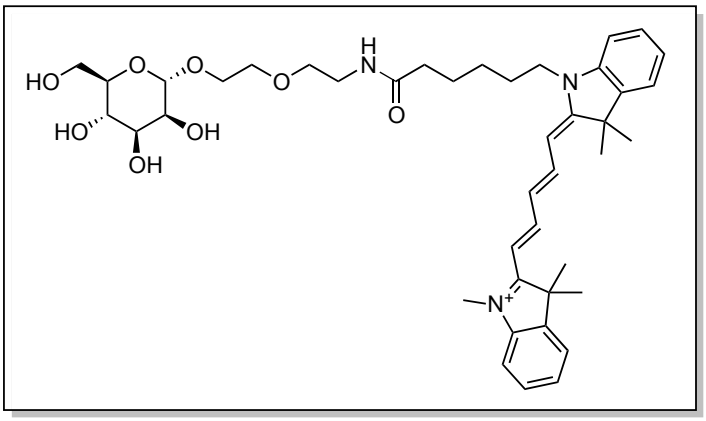
BLM Monosaccharide–Alexa Fluor 647 (2.20). To 134 μg (0.43 μmol) of amine **2.84** was added a solution of 100 μg (0.08 μmol) of Alexa-Fluor647COOSu (**2.116**) in 100 μL of 0.2 M phosphate buffer and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile) over a period of 25 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 17.4 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give BLM monosaccharide–Alexa Fluor 647 conjugate (**2.20**) as a blue solid: yield 56.0 μg (48%); mass spectrum (MALDI-TOF), *m/z* 1369.32 $(M)^+$, (theoretical *m/z* 1369; the structure of Alexa Fluor 647 fluorophore has not been reported).



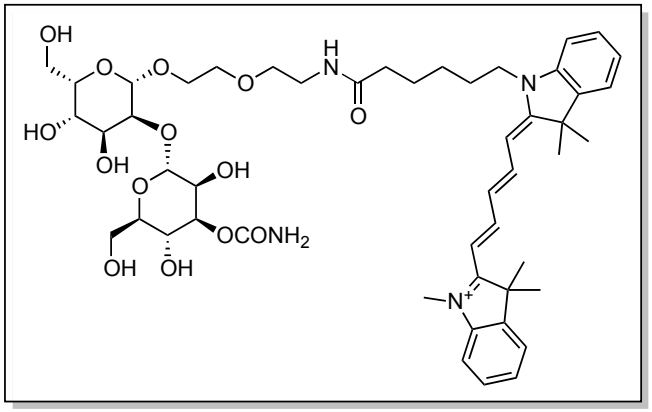
BLM Monosaccharide–Cy5^{GE} (2.21). To 211 μg (0.68 μmol) of amine **2.84** was added a solution of 100 μg (0.13 μmol) of Cy5^{GE}COOSu (**2.117**) in 100 μL of 0.2 M phosphate buffer and 20 μL DMSO. The reaction mixture was stirred overnight in the dark and was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 12 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 6.9 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give BLM monosaccharide–Cy5^{GE} conjugate (**2.21**) as a blue solid: yield 86.0 μg (72%); mass spectrum (MALDI-TOF), *m/z* 949.48 $(M)^+$ and 971.49 $(M + Na)^+$; mass spectrum (ESI), *m/z* 949.3538 $(M)^+$ ($C_{44}H_{61}N_4O_{15}S_2^+$ requires *m/z* 949.3575).



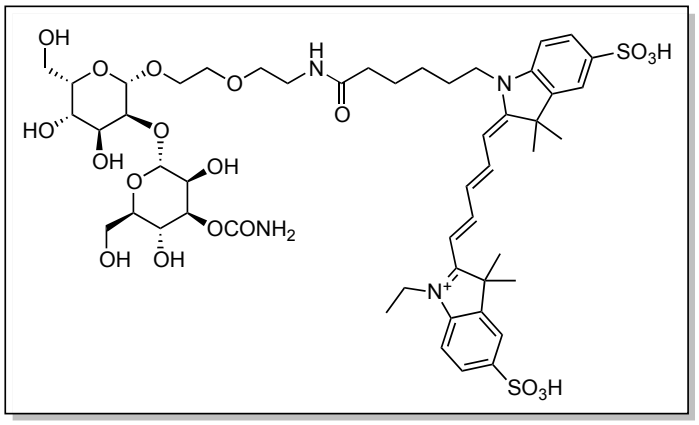
BLM Monosaccharide–Cy5.5^{GE} (2.22). To 148 μg (0.48 μmol) of amine **2.84** was added a solution of 100 μg (0.09 μmol) of Cy5.5^{GE}COOSu (**2.118**) in 100 μL of 0.2 M phosphate buffer and 20 μL DMSO. The reaction mixture was stirred overnight in the dark and was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→70:30 0.1% aq TFA–acetonitrile) over a period of 18 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 16.5 min (monitoring at 675 nm) and were collected, frozen and lyophilized to give BLM monosaccharide–Cy5.5^{GE} (**2.22**) as a blue solid: yield 47.0 μg (44%); mass spectrum (MALDI-TOF), *m/z* 1231.55 $(M - H + Na)^+$, 1247.57 $(M - H + K)^+$, 1253.55 $(M - 2H + 2Na)^+$, 1269.57 $(M - 2H + Na + K)^+$ and 1275.55 $(M - 3H + 3Na)^+$ (theoretical *m/z* 1231.38, 1247.26, 1253.27, 1269.24 and 1275.25).



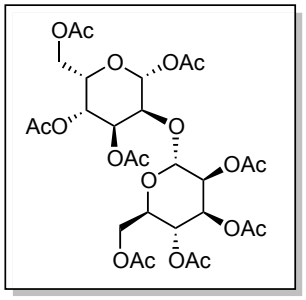
Decarbamoyl BLM Monosaccharide–Cy5 (2.23). To 1.80 mg (4.38 μmol) of amine **2.88** was added a solution of 500 μg (0.81 μmol) of Cy5COOSu (**2.114**) in 500 μL of 0.2 M phosphate buffer and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 10 and 5 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 15.7 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give decarbamoyl BLM monosaccharide–Cy5 (**2.23**) as a blue solid: yield 169 μg (23%); mass spectrum (ESI), *m/z* 732.4219 $(M)^+$ ($C_{42}H_{58}N_3O_8^+$ requires *m/z* 732.4218).



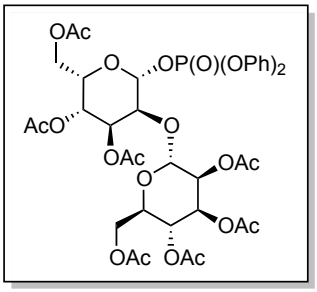
BLM Disaccharide–Cy5 (2.24). To 1.90 mg (4.02 μmol) of amine **2.59** was added a solution of 500 μg (0.81 μmol) of Cy5COOSu (**2.114**) in 500 μL of 0.2 M phosphate buffer and 100 μL of DMSO. The reaction mixture was stirred overnight in the dark and purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 10 min and 5 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 15.0 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give BLM disaccharide–Cy5 (**2.24**) as a blue solid: yield 235 μg (31%); mass spectrum (MALDI-TOF), *m/z* 937.65 $(M)^+$; mass spectrum (ESI), *m/z* 937.4803 $(M)^+$ ($C_{49}H_{69}N_4O_{14}^+$ requires *m/z* 937.4805).



BLM Disaccharide–Cy5^{GE} (2.25).[115] To 322 μg (0.68 μmol) of amine **2.59** was added a solution of 100 μg (0.13 μmol) of Cy5^{GE}COOSu (**2.114**) in 100 μL of 0.2 M phosphate buffer and 20 μL DMSO. The reaction mixture was stirred overnight in the dark and was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 12 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 6.7 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give BLM disaccharide–Cy5^{GE} (**2.25**) as a blue solid: yield 38.0 μg (27%); mass spectrum (MALDI-TOF), *m/z* 1111.41 $(M)^+$ (theoretical *m/z* 1111.41).



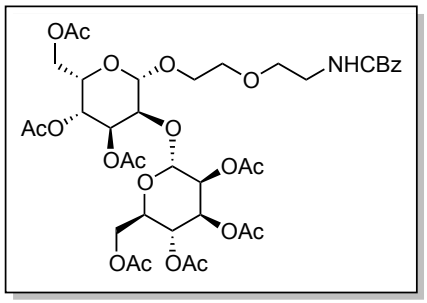
1,3,4,6-Tetra-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-L-gulopyranoside (2.119).[150] To a solution of 300 mg (0.52 mmol) of phosphate ester **2.86** and 144 mg (0.41 mmol) of gulose alcohol **2.45** in 8.6 mL of anh dichloromethane at 0 °C was added 0.17 mL (207 mg, 0.93 mmol) of TMSOTf dropwise. The reaction mixture was stirred at 0 °C for 18 min at which time it was poured into a mixture of 60 mL of ethyl acetate and 30 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 20-mL portions of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (22 × 3 cm). Elution with 3:1→1:1 hexanes–ethyl acetate afforded disaccharide **2.119** as a colorless oil: yield 150 mg (53%); silica gel TLC R_f 0.36 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.96 (s, 3H), 2.02 (s, 3H), 2.05 (s, 3H), 2.12 (s, 3H), 2.13 (2s, 6H), 2.15 (s, 3H), 2.18 (s, 3H), 3.98 (dd, 1H, J = 4.8 and 3.6 Hz), 4.05-4.18 (m, 4H), 4.22 (m, 2H), 4.36 (m, 1H), 4.97 (d, 1H, J = 1.6 Hz), 5.00 (m, 1H), 5.09 (m, 1H), 5.14 (m, 1H), 5.26 (m, 1H), 5.43 (t, 1H, J = 3.6 Hz) and 5.89 (d, 1H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.78, 20.81, 20.88, 20.94, 61.5, 62.2, 65.6, 65.9, 67.8, 68.7, 68.8, 68.9, 69.3, 69.8, 71.5, 90.8, 95.1, 168.8, 169.38, 169.42, 169.6, 169.7, 170.0, 170.6 and 170.7.



3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-L-gulopyranosyl Diphenyl Phosphate (2.120).[118] To a solution of 150 mg (0.22 mmol) of disaccharide **2.119** in 1.9 mL of anh DMF was added 28.0 mg (0.31 mmol) of hydrazine acetate salt and the reaction mixture was stirred for 2.5 h at room temperature. The solution was diluted with 50 mL of ethyl acetate and washed with three 20-mL portions of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was co-evaporated with toluene to give the product as a colorless oil: yield 140 mg (99%); silica gel TLC R_f 0.25 (1:2 hexanes–ethyl acetate).

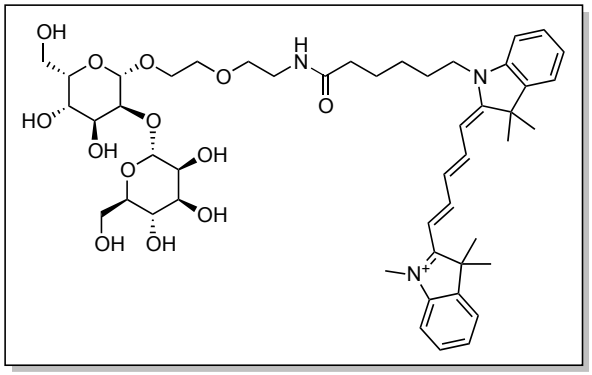
To the solution of 140 mg of the residue in 5.5 mL of anh dichloromethane were added 35.0 mg (0.28 mmol) of DMAP, 0.31 mL (222 mg, 2.20 mmol) of dry triethylamine and 0.44 mL (564 mg, 2.10 mmol) of diphenyl chlorophosphate at 0 °C. The solution was stirred at 0 °C for 1.5 h and was then poured into a mixture of 50 mL of ethyl acetate and 20 mL of saturated aq $NaHCO_3$. The organic layer was washed with 15 mL of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 2.5 cm). Elution with 2:1→1:1 hexanes–ethyl acetate afforded phosphate ester **2.120** as a colorless oil: yield 108 mg (56% over two steps); silica gel TLC R_f 0.28 (1:2 hexanes–ethyl acetate). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.70 (s, 3H), 1.94 (s, 3H), 1.99 (s, 3H), 2.08 (s, 3H), 2.13 (s, 3H), 2.15 (s, 3H), 2.21 (s, 3H), 4.00 (m, 3H), 4.13 (m, 2H), 4.33 (dd, 2H, *J* = 15.5 and 8.8 Hz), 4.99 (m, 2H), 5.14 (m, 1H), 5.24 (m, 2H), 5.44 (s, 1H), 5.71 (t, 1H, *J* = 7.3 Hz) and 7.28 (m, 10H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.2, 20.60, 20.61, 20.65, 20.7, 61.1, 61.8, 65.3, 65.5, 67.4, 68.6, 69.0, 69.1, 71.0, 71.6, 95.4, 96.1, 120.2, 120.2,

125.6, 125.7, 129.6, 129.9, 150.0, 150.3, 169.2, 169.3, 169.4, 169.5, 169.6, 170.3 and 170.5.



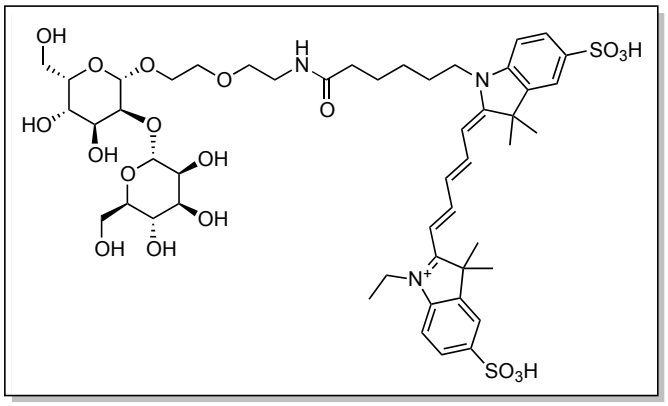
3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α,β-L-gulopyranosyl Benzyl 2-(2-ethoxy)ethylcarbamate (2.121).[118] To a solution of 26.0 mg (30.0 μmol) of phosphate ester **2.120** and 6.50 mg (27.0 μmol) of CBz-protected linker **2.57** in 1.2 mL of anh dichloromethane was added 10.0 μL (12.3 mg, 54.0 μmol) of TMSOTf at 0 °C. The reaction was stirred at 0 °C for 17 min and was then poured into a mixture of 25 mL of ethyl acetate and 10 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 5-mL portions of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 2 cm). Elution with 32:16:1→12:12:1 hexanes–ethyl acetate–methanol afforded compound **2.121** as a colorless oil: yield 18.0 mg (70%); silica gel TLC R_f 0.80 (12:12:1 hexanes–ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.98 (s, 3H), 2.02 (s, 3H), 2.04 (s, 3H), 2.08 (s, 3H), 2.10 (s, 3H), 2.12 (s, 3H), 2.14 (s, 3H), 3.40 (m, 2H), 3.61 (m, 3H), 3.68 (m, 2H), 3.85 (m, 1H), 3.97 (m, 1H), 4.18 (m, 5H), 4.27 (m, 4H), 4.47 (t, 1H, J = 6.4 Hz), 4.92 (d, 1H, J = 3.7 Hz), 5.01 (m, 2H), 5.09 (m, 2H), 5.15 (s, 1H), 5.45 (s, 1H) and 7.33 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.61, 20.62, 20.70, 20.75, 20.8, 29.7, 40.9, 62.1, 62.5, 63.7, 65.5, 66.6, 68.61, 68.62, 69.1, 69.2, 70.1, 70.3,

70.6, 97.0, 97.1, 128.05, 128.15, 128.5, 136.5, 156.4, 169.3, 169.5, 169.6, 169.8, 169.9, 170.52 and 170.53.



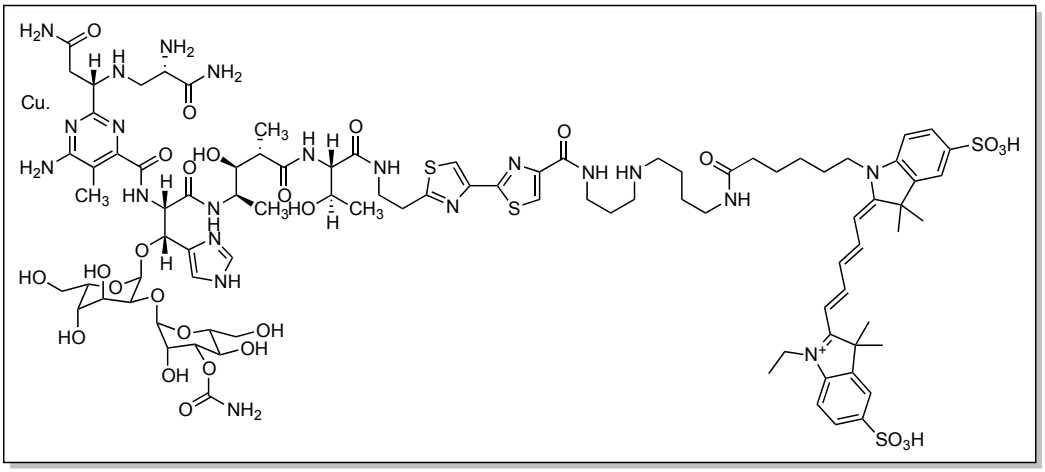
Decarbamoyl BLM Disaccharide–Cy5 (2.26). To a solution of 12.0 mg (14.0 mmol) of decarbamoyl disaccharide CBz protected linker **2.121** in 3 mL of anh methanol was added 0.3 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered and concentrated; mass spectrum (MALDI-TOF), *m/z* 586.34 $(M + Na)^+$ (theoretical *m/z* 563.22). To the solution of the crude product in 5 mL of anh methanol was added Pd/C and H_2 gas was bubbled through for 45 min. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered through Celite 545® and then concentrated under diminished pressure to afford amine **2.122** as a colorless oil: yield 6.00 mg (100%), which was used for the next reaction; mass spectrum (MALDI-TOF), *m/z* 430.32 $(M + Na)^+$.

To 1.80 mg (4.38 μmol) of amine **2.122** was added a solution of 500 μg (0.81 μmol) of Cy5COOSu (**2.114**) in 500 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→69:31 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 10 min and 5 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 14.9 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give decarbamoyl BLM disaccharide–Cy5 **2.26** as a blue solid: yield 169 μg (23%); mass spectrum (MALDI-TOF), *m/z* 894.53 $(M)^+$; mass spectrum (ESI), *m/z* 894.4737 $(M)^+$ ($C_{48}H_{68}N_3O_{13}^+$ requires *m/z* 894.4747).



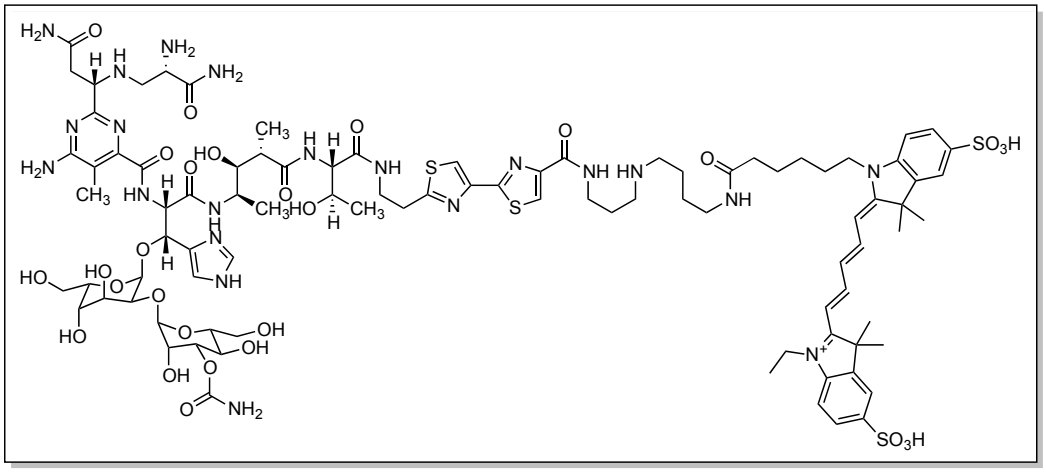
Decarbamoyl BLM Disaccharide–Cy5^{GE} (2.27). To 293 μg (0.68 μmol) of amine **2.122** was added a solution of 100 μg (0.13 μmol) of Cy5^{GE}COOSu (**2.117**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and 20 μL DMSO. The reaction mixture was stirred overnight in the dark and was purified on an Econosil C_{18} reversed phase semi-preparative (250 ×

10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 12 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 6.6 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give decarbamoyl BLM disaccharide–Cy5^{GE} (**2.27**) as a blue solid: yield 59.0 μg (44%); mass spectrum (ESI), *m/z* 1066.3878 $(M - 2H)^-$ ($C_{49}H_{68}N_3O_{19}S_2$ requires *m/z* 1066.3888).

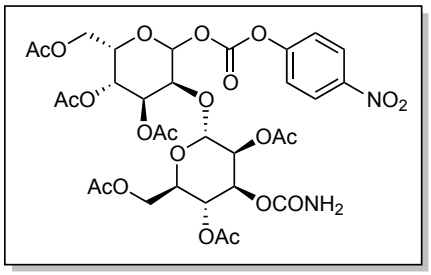


Cu(II)•Bleomycin–Cy5^{GE} (2.124).[115] To 912 μg (0.61 μmol) of Cu(II)•deglycobleomycin A_5 (**2.123**) was added a solution of 200 μg (0.25 μmol) of Cy5^{GE}COOSu (**2.117**) in 200 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 ×10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 12 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 6.9 min

(monitoring at 650 nm) and were collected, frozen and lyophilized to give Cu(II)•bleomycin–Cy5^{GE} (**2.124**) as a blue solid: yield 120 μg (22%); mass spectrum (MALDI-TOF), *m/z* 2079.55 $(M + H)^+$ (theoretical *m/z* 2079.82).



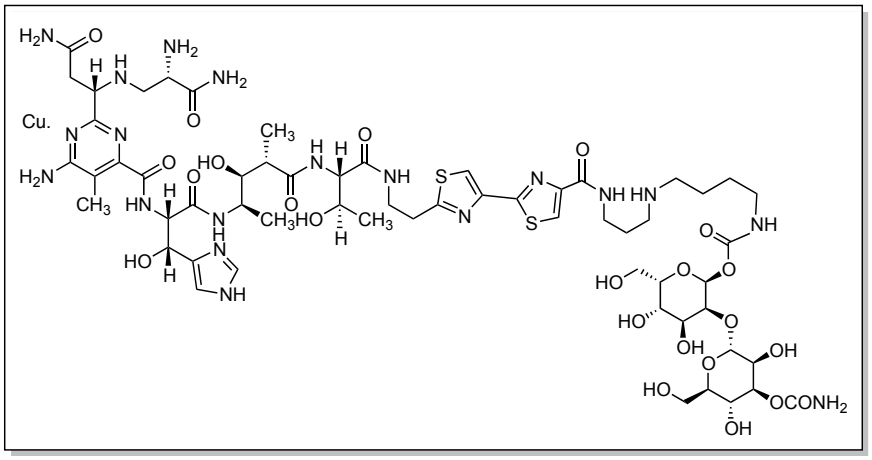
Bleomycin–Cy5^{GE} (2.28).[115] To 120 μg (0.06 μmol) of **2.124** was added 150 μL of 15% EDTA and 20 μL of DMSO. The reaction mixture was stirred overnight in the dark and was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 12 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 6.9 min (monitoring at 650 nm) and were collected, frozen and lyophilized to give bleomycin–Cy5^{GE} (**2.28**) as a blue solid: yield 110 μg (94%); mass spectrum (MALDI-TOF), *m/z* 2079.55 $(M + H)^+$ (theoretical *m/z* 2079.82).



1-*O*-[(*p*-Nitrophenyl)oxycarbonyl]-3,4,6-tri-*O*-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl-α-D-mannopyranosyl)-α,β-L-gulopyranoside (2.125).[118] To a solution of 7.00 mg (0.01 mmol) of disaccharide **2.55** in 0.2 mL of anh DMF was added 1.30 mg (0.01 mmol) of hydrazine acetate. The solution was stirred at room temperature for 2.5 h and diluted with 15 mL of ethyl acetate. The organic solution was washed with 5 mL of brine, dried ($MgSO_4$), filtered, and concentrated under diminished pressure to afford the product as a colorless oil: yield 6.50 mg; silica gel TLC R_f 0.25 (1:3 hexanes–ethyl acetate).

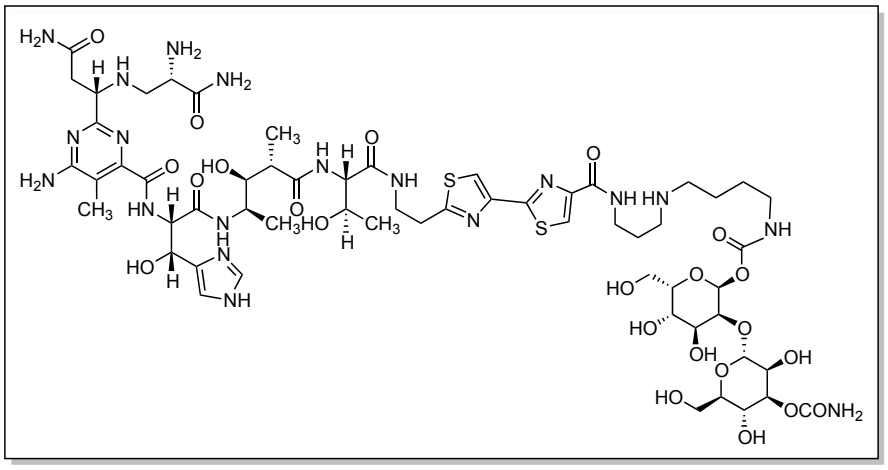
To a solution of 6.50 mg (0.01 mmol) of the above residue in 1.1 mL of dry acetonitrile were added 1.90 mg (15.3 μmol) of DMAP and a solution containing 3.10 mg (15.3 μmol) of *p*-nitrophenyl chloroformate in 100 μL of acetonitrile. The reaction mixture was stirred at room temperature for 2 h, at which time silica gel TLC analysis showed complete consumption of the starting material. The reaction mixture was concentrated under diminished pressure, dissolved in 10 mL of ethyl acetate, and then washed successively with 5 mL of water, 5 mL of 1 N aqueous HCl and 5 mL of brine. The solution was dried (Na_2SO_4), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 1.5 cm). Elution with 1:1→1:2 hexanes–ethyl acetate afforded the carbonate **2.125** as a colorless

oil: yield 6.00 mg (73%); silica gel TLC R_f 0.38 (2:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.94 (s, 3H), 2.06 (s, 3H), 2.10 (s, 3H), 2.15 (s, 3H), 2.16 (s, 3H), 2.21 (s, 3H), 4.11 (m, 6H), 4.41 (m, 1H), 4.69 (br s, 2H), 5.04 (m, 2H), 5.16 (m, 2H), 5.29 (m, 1H), 5.48 (m, 1H) 5.83 (d, 1H, J = 8.4 Hz), 7.47 (dd, 1H, J = 6.8 and 2 Hz) and 8.31 (dd, 1H, J = 6.8 and 2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.78, 20.84, 20.95, 61.4, 62.0, 65.6, 65.9, 67.5, 69.2, 69.7, 69.8, 70.0, 72.0, 95.3, 95.5, 122.1, 125.6, 146.0, 151.1, 155.1, 155.2, 169.3, 169.3, 169.6, 169.8, 170.5 and 170.7.



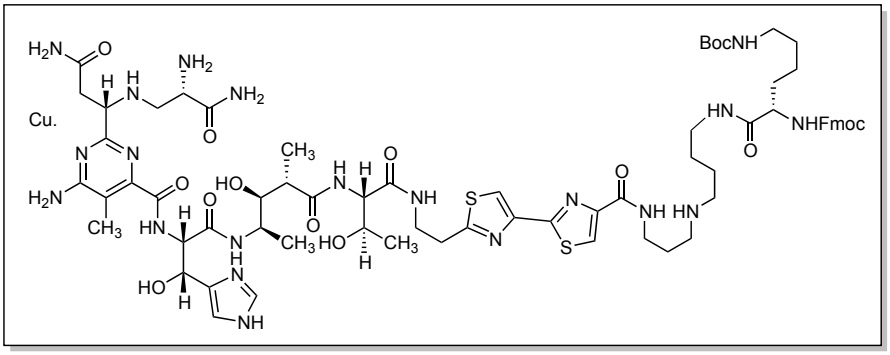
Cu(II)•Deglycobleomycin–Disaccharide Conjugate 2.126.[118] To a solution containing 2.00 mg (2.40 μmol) of carbamate **2.125** and 2.00 mg (1.80 μmol) of Cu(II)•deglycobleomycin A_5 (**2.123**) in 1 mL of anhydrous DMF was added 10.0 μL (7.40 mg; 72.0 μmol) of dry triethylamine and shaken at room temperature for 3 h. The reaction mixture was treated with 100 μL (102 mg; 3.20 mmol) of hydrazine and was shaken at room temperature for additional 1 h. The reaction mixture was then concentrated under diminished pressure and purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–

acetonitrile→50:50 0.1% aq TFA–acetonitrile) over a period of 28 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 11.0 min (monitoring at 292 nm) and were collected, frozen and lyophilized to give conjugate **2.126** as a light blue solid: yield 843 μg (31%); mass spectrum (MALDI-TOF), *m/z* 1546.8 $(M)^+$ (theoretical *m/z* 1546.5); mass spectrum (ESI), *m/z* 773.2547 $(M + 2H)^{2+}$ ($C_{58}H_{87}N_{19}O_{23}S_2Cu$ requires *m/z* 773.2558).



Deglycobleomycin–Disaccharide Conjugate 2.29. To 0.83 mg (0.54 μmol) of Cu(II)•deglycoBLM–disaccharide **2.126** was added 0.8 mL of 15% EDTA solution and the reaction mixture was shaken at room temperature for overnight. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→50:50 0.1% aq TFA–acetonitrile) over a period of 18 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 3.2 min (monitoring at 292 nm) and were collected, frozen, and lyophilized to give conjugate **2.29** as white solid: yield 0.31 mg (40%); mass spectrum (MALDI-TOF), *m/z* 1484.9 $(M + H)^+$ (theoretical *m/z* 1483.6); mass spectrum (ESI), *m/z*

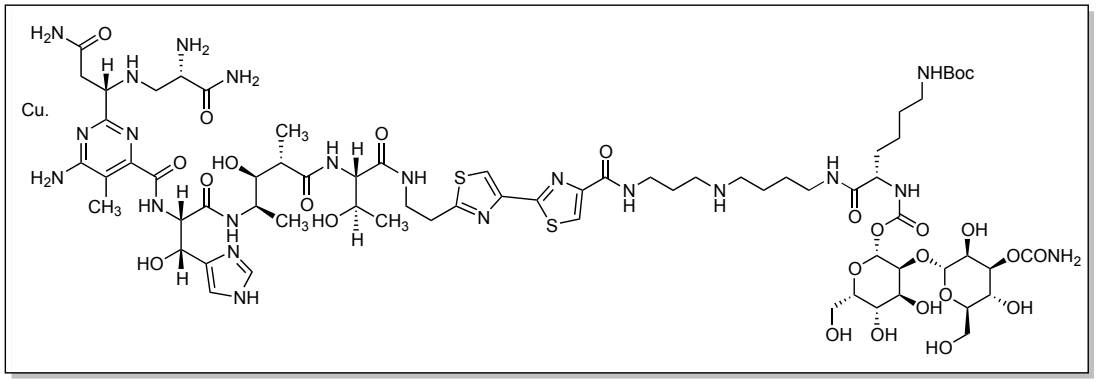
742.7975 $(M + 2H)^{2+}$ ($C_{58}H_{89}N_{19}O_{23}S_2$ requires *m/z* 742.7988).



Cu(II)•Deglycobleomycin–Linker Conjugate 2.128. To a solution containing 4.00 mg (8.50 μmol) of lysine linker **2.127** in 1.1 mL anh dichloromethane were added 1.20 mg (10.0 μmol) of *N*-hydroxysuccinimide and 3.10 μL (2.52 mg, 20 μmol) of *N,N′*-diisopropylcarbodiimide. The reaction mixture was stirred overnight at room temperature. The reaction mixture was concentrated to give the activated acid as a colorless solid: yield 4.80 mg (99%); mass spectrum (MALDI-TOF), *m/z* 588.31 $(M + Na)^+$ (theoretical *m/z* 588.23).

To a solution containing 2.40 mg (4.20 μmol) of compound the activated acid and 5.00 mg (4.40 μmol) of Cu(II)•deglycobleomycin A_5 (**2.123**) in 0.9 mL of anhydrous DMF was added 6.00 μL (4.40 mg, 40.0 μmol) of dry triethylamine and the reaction mixture was shaken overnight at room temperature. The reaction mixture was concentrated under diminished pressure and purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→50:50 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 16 min and 4 min, respectively, at a flow rate of 4.5 mL/min. The fractions

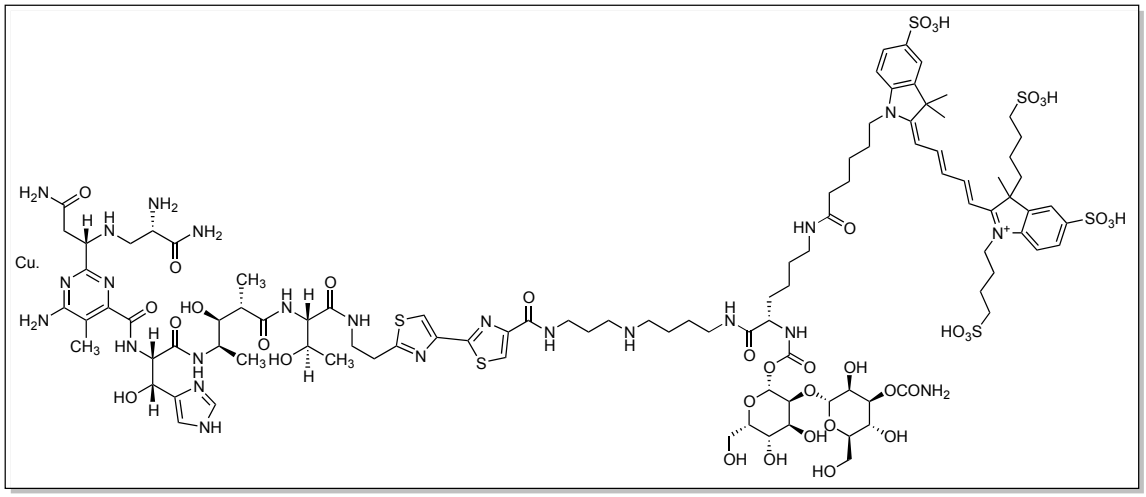
containing the desired product eluted at 22.8 min (monitoring at 292 nm) and were collected, frozen, and lyophilized to give linker **2.128** as a light blue solid: yield 1.04 mg (15% over two steps); mass spectrum (MALDI-TOF), *m/z* 1545.79 $(M - Cu + Na)^+$ (theoretical *m/z* 1545.69).



Cu(II)•Deglycobleomycin–Linker–Disaccharide Conjugate 2.129. To 1.03 mg (0.65 μmol) of linker **2.128** was added 0.24 mL of 20% piperidine in DMF and the reaction mixture was stirred for 1.5 h. The reaction mixture was frozen and lyophilized to give the crude amine as a white solid: yield 844 μg (95%); mass spectrum (MALDI-TOF), *m/z* 1300.81 (M − Cu)+ (theoretical *m/z* 1300.63).

To a solution containing 0.84 mg (0.62 μmol) of the crude amine and 0.84 mg (0.86 μmol) of carbonate **2.125** in 0.5 mL of anh DMF was added 10.0 μL (7.40 mg; 72.0 μmol) of dry triethylamine and the reaction mixture was shaken overnight at room temperature. Fifty μL (51.0 μg; 1.60 mmol) of hydrazine was added and the reaction mixture was shaken at room temperature for 1 h and then concentrated under diminished pressure. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile

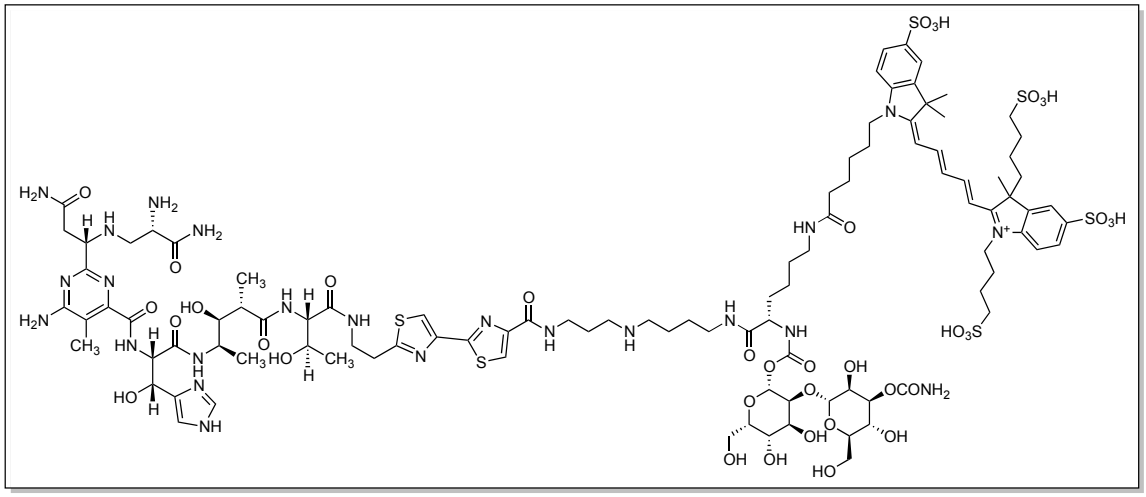
mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→50:50 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 16 min and 4 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted 10.4 min (monitoring at 292 nm) and were collected, frozen, and lyophilized to give Cu(II)•deglycoBLM–linker–disaccharide **2.129** as a light blue solid: yield 367 μg (32%); mass spectrum (MALDI-TOF), *m/z* 1712.52 $(M - Cu + H)^+$ (theoretical *m/z* 1712.74).



Cu(II)•Deglycobleomycin–Linker–Disaccharide–Cy5 Conjugate 2.130.** To 363 μg (0.20 μmol) of linker **2.129** was added 0.3 mL of 60% TFA and the reaction mixture was stirred for 2 h. The reaction mixture was frozen and lyophilized to give the free amine as a light blue solid: yield 342 μg (100%); mass spectrum (MALDI-TOF), *m/z* 1613.94 $(M - Cu + 2H)^+$ (theoretical *m/z* 1613.69).

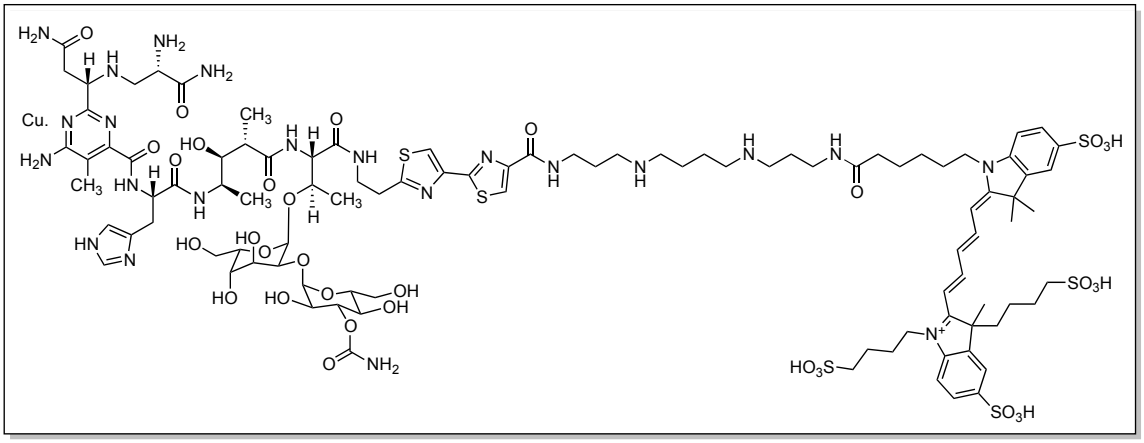
To 341 μg (0.20 μmol) of the free amine was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu (**2.60**) in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on

an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→50:50 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 16 min and 4 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 10.1 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give Cy5** conjugate **2.130** as a blue solid: yield 47.0 μg (17%); mass spectrum (MALDI-TOF), *m/z* 2480.66 $(M - Cu)^+$ and 2585.02 $(M + 3Na + K - 3H)^+$, (theoretical *m/z* 2480.89 and 2585.80).



Deglycobleomycin–Linker–Disaccharide–Cy5 Conjugate 2.30.** To 46.0 μg (0.02 μmol) of Cy5** conjugate **2.130** was added 100 μL of 15% EDTA and the reaction mixture was stirred at room temperature overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→50:50 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 16 min and 4 min, respectively, at a flow rate

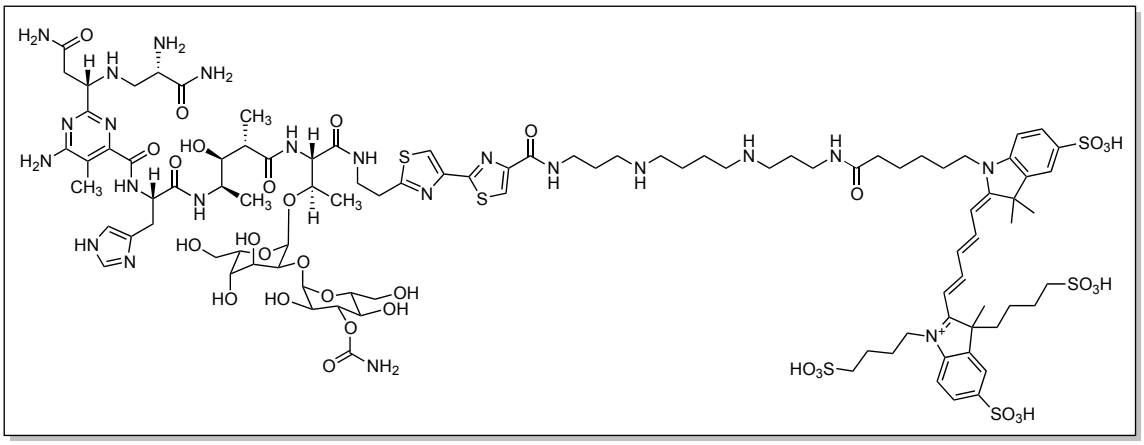
of 4.5 mL/min. The fractions containing the desired product eluted at 10.1 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give Cy5** conjugate **2.30** as a blue solid: yield 18.0 μg (40%); mass spectrum (MALDI-TOF), *m/z* 2480.66 $(M)^+$, 2556.46 $(M - 2H + 2K)^+$, 2564.08 $(M + 2Na + K - H)^+$ and 2586.87 $(M + 3Na + K - 2H)^+$, (theoretical *m/z* 2480.89, 2556.80, 2564.82 and 2586.81).



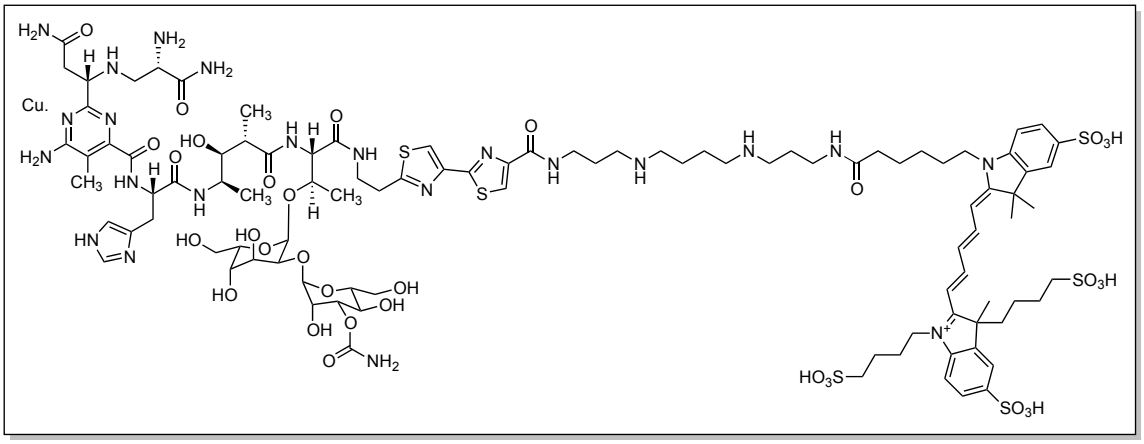
Cu(II)•Deglycobleomycin–Cy5 Conjugate 2.131.** To 400 μg (0.27 μmol) of modified BLM **2.31** was added 200 μL of a 2.94 mM aq $CuCl_2$ solution and the reaction mixture was stirred overnight in the dark. The reaction mixture was frozen and lyophilized to give the metallated BLM as a light blue solid: yield 417 μg (100%); mass spectrum (MALDI-TOF), *m/z* 1543.45 $(M)^+$ and 1481.53 $(M - Cu + H)^+$, (theoretical *m/z* 1543.58 and 1481.66).

To 417 μg (0.27 μmol) of the metallated BLM was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 ×10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1

0.1% aq TFA–acetonitrile→75:25 0.1% aq TFA–acetonitrile) over a period of 23 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 21.1 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give Cy5** conjugate **2.131** as a blue solid: yield 100 μg (37%); mass spectrum (MALDI-TOF), *m/z* 2388.36 $(M - Cu + K)^+$ (theoretical *m/z* 2388.83).

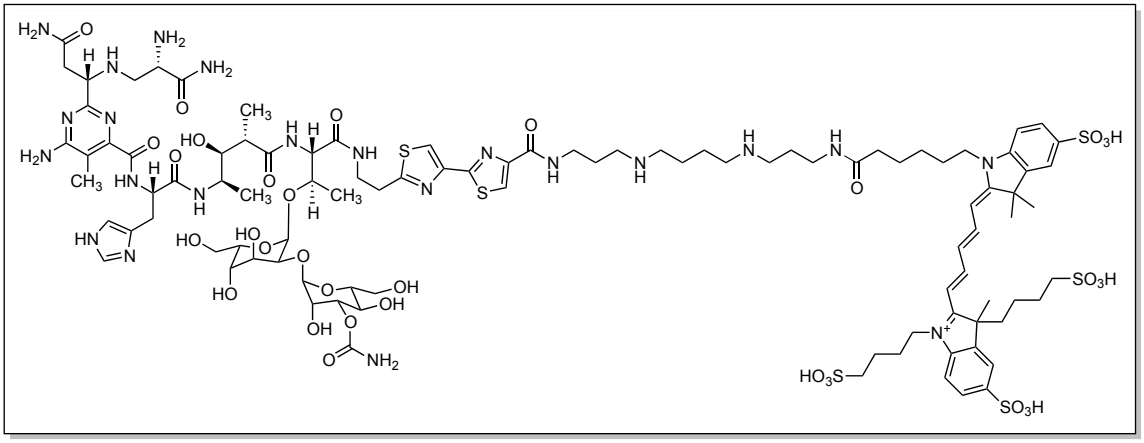


Deglycobleomycin–Cy5 Conjugate 2.33.** To 98.0 μg (0.04 μmol) of Cy5** conjugate **2.131** was added 100 μL of 15% EDTA and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 15 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 6.6 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give Cy5** conjugate **2.33** as a blue solid: yield 64.0 μg (67%); mass spectrum (MALDI-TOF), *m/z* 2388.51 (M + K)+ (theoretical *m/z* 2388.83).

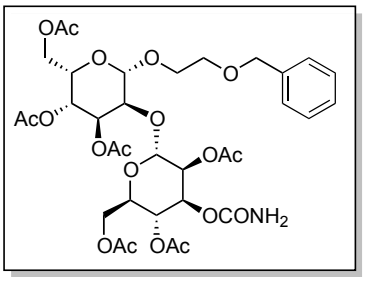


Cu(II)•Deglycobleomycin–Cy5 Conjugate 2.132.** To 200 μg (0.14 μmol) of modified BLM **2.32** was added 150 μL of a 2.94 mM aq $CuCl_2$ solution and the reaction mixture was stirred overnight in the dark. The reaction mixture was frozen and lyophilized to give the metallated BLM as a light blue solid: yield 208 μg (100%); mass spectrum (MALDI-TOF), *m/z* 1543.53 $(M)^+$, 1481.61 $(M - Cu + H)^+$, (theoretical *m/z* 1543.58, 1481.66).

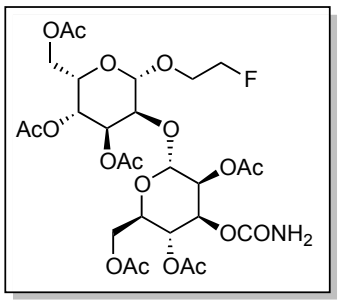
To 208 μg (0.13 μmol) of the metallated BLM was added a solution of 110 μg (0.11 μmol) of Cy5**COOSu in 100 μL of 0.2 M phosphate buffer (pH 8.0) and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 ×10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→75:25 0.1% aq TFA–acetonitrile) over a period of 23 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 21.2 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give Cy5** conjugate **2.132** as a blue solid: yield 97.0 μg (36%); mass spectrum (MALDI-TOF), *m/z* 2388.11 $(M - Cu + K)^+$ (theoretical *m/z* 2388.83).



Deglycobleomycin–Cy5 Conjugate 2.34.** To 95.0 μg (0.04 μmol) of Cy5** conjugate **2.132** was added 100 μL of 15% EDTA and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→0:100 0.1% aq TFA–acetonitrile) over a period of 15 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 6.6 min (monitoring at 651 nm) and were collected, frozen and lyophilized to give Cy5** conjugate **2.34** as a blue solid: yield 39.0 μg (42%); mass spectrum (MALDI-TOF), *m/z* 2388.01 (M + K)+, (theoretical *m/z* 2388.83).



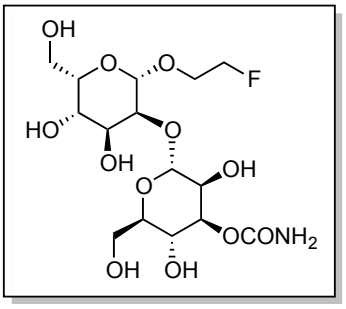
3,4,6-Tri-acetyl-2-O-(2,4,6-tri-O-acetyl-3-O-carbamoyl-α-D-mannopyranosyl)-α-L-gulopyranosyl 2-(Benzyloxy)ethane (2.134). To a solution of 63.0 mg (0.07 mmol) of phosphate ester **2.56** and 9.20 μL (9.13 mg, 0.06 mmol) of 2-(benzyloxy)ethanol **(2.133)** in 1.8 mL of anh dichloromethane was added 24.0 μL (29.0 mg, 0.13 mmol) of TMSOTf at 0 °C. The reaction mixture was stirred at 0 °C for 18 min at which time it was poured into a mixture of 30 mL of ethyl acetate and 15 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 10-mL portions of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 1.5 cm). Elution with 1:1→1:3 hexanes–ethyl acetate afforded disaccharide **2.134** as a colorless oil: yield 34.0 mg (68%); silica gel TLC R_f 0.41 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.98-2.14 (m, 18H), 3.69 (m, 3H), 3.81 (m, 2H), 4.19 (m, 4H), 4.26 (m, 2H), 4.57 (m, 3H), 4.75 (m, 1H), 4.91 (m, 2H), 5.35 (m, 3H) and 7.27-7.31 (m, 5H); mass spectrum (APCI), *m/z* 772.2665 $(M + H)^+$ ($C_{34}H_{46}NO_{19}$ requires *m/z* 772.2664).



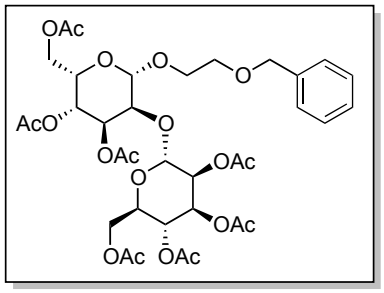
3,4,6-Tri-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl-α-D-mannopyranosyl)-α-L-gulopyranosyl 2-(Fluoro)ethane (2.135).[134] To a solution of 20.0 mg (0.03 mmol) of disaccharide **2.134** in 3 mL of anh methanol was added a catalytic amount of Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by

filtration through a pad of Celite 545® and concentrated under diminished pressure to give the alcohol as a colorless oil: yield 19.0 mg (100%); silica gel TLC R_f 0.08 (1:3 hexanes–ethyl acetate).

To the solution of 19.0 mg (0.3 mmol) of the alcohol in 0.3 mL of anh dichloromethane at −78 °C were added 6.2 μL (6.09 mg, 0.04 mmol) of DBU and 10.0 mg (0.04 mmol) of XtalFluor-E and stirred for 30 min. The reaction mixture was then allowed to warm to room temperature and stirred for 24 h at room temperature. The solution was poured into a mixture of 10 mL of ethyl acetate and 5 mL of saturated aq $NaHCO_3$. The organic layer was washed with 5 mL portions of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 1.5 cm). Elution with 1:1→1:3 hexanes–ethyl acetate afforded compound peracetylated fluoro BLM disaccharide (**2.135**) as a colorless oil: yield 9.00 mg (54% over two steps); silica gel TLC R_f 0.34 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.99-2.17 (m, 18H), 3.41 (m, 3H), 4.17 (m, 3H), 4.28 (m, 2H), 4.52 (m, 2H), 4.64 (br s, 2H), 4.71 (m, 2H), 4.91 (m, 1H), 5.08 (m, 2H) and 5.29 (m, 3H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ −223.1 (m), −223.9 (m); *m/z* 722.43 $(M + K)^+$; mass spectrum (APCI), *m/z* 684.2151 $(M + H)^+$ ($C_{27}H_{39}FNaO_{18}$ requires *m/z* 684.2151).

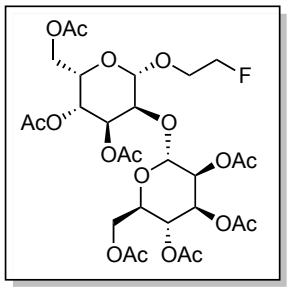


Fluoro BLM Disaccharide (3.35). To a solution of 1.00 mg (1.5 μmol) of peracetylated fluoro BLM disaccharide (**2.135**) in 1.1 mL of anh methanol was added 0.11 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, filtered, concentrated and lyophilized to afford fluoro BLM disaccharide (**2.35**) as a white solid: yield 0.70 mg (100%); mass spectrum (MALDI-TOF), *m/z* 454.33 $(M + Na)^+$ (theoretical *m/z* 454.13).



3,4,6-Tri-acetyl-2-*O*-(2,3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-α-L-gulopyranosyl 2-(Benzyloxy)ethane (2.136). To a solution of 108 mg (0.12 mmol) of phosphate ester **2.120** and 16.0 μL (18.3 mg, 0.12 mmol) of 2-(benzyloxy)ethanol **(2.133)** in 3.5 mL of anh dichloromethane was added 40.0 μL (48.9 mg, 0.22 mmol) of TMSOTf at 0 °C. The reaction mixture was stirred at 0 °C for 20 min at which time it was poured into a mixture of 30 mL of ethyl acetate and 15 mL of saturated aq $NaHCO_3$. The organic layer was washed with two 15-mL portions of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 2.5 cm). Elution with 3:1→1:1 hexanes–ethyl acetate afforded

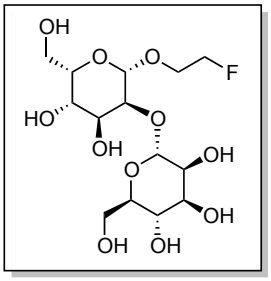
disaccharide **2.136** as a colorless oil: yield 60.0 mg (69%); silica gel TLC R_f 0.51 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.97-2.15 (m, 21H), 3.67 (m, 3H), 3.84 (m, 2H), 4.09 (m, 4H), 4.25 (m, 2H), 4.55 (m, 3H), 4.76 (m, 1H), 4.95 (m, 2H), 5.32 (m, 3H) and 7.33 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.77, 20.83, 20.90, 20.92, 21.0, 21.1, 62.6, 66.2, 66.4, 67.5, 68.0, 68.5, 68.9, 69.0, 69.3, 69.4, 70.2, 70.2, 71.5, 73.36, 73.40, 97.8, 127.6, 127.81, 127.84, 128.6, 138.2, 155.3, 169.8, 170.1 and 170.8; mass spectrum (ESI), *m/z* 793.2529 $(M + Na)^+$ ($C_{35}H_{46}O_{19}Na$ requires *m/z* 793.2531).



3,4,6-Tri-acetyl-2-*O*-(2,3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-α-L-gulopyranosyl 2-(Fluoro)ethane (2.137).[134] To a solution of 60.0 mg (0.08 mmol) of disaccharide **2.136** in 8.2 mL of anh methanol was added a catalytic amount of Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and concentrated under diminished pressure to give the alcohol as a colorless oil: yield 45.0 mg (85%); silica gel TLC R_f 0.08 (1:1 hexanes–ethyl acetate).

To the solution of 45.0 mg (0.06 mmol) of the alcohol in 0.3 mL of anh dichloromethane at −78 °C were added 15.0 μL (15.2 mg, 0.10 mmol) of DBU and 23 mg (0.10 mmol) of XtalFluor-E were added and stirred for 30 min. The reaction mixture was then allowed to warm to room temperature and stirred for 24 h at room temperature. The solution was poured into a mixture of 15 mL of ethyl acetate and 7.5 mL of saturated aq

$NaHCO_3$. The organic layer was washed with 7.5 mL of brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 1.5 cm). Elution with 3:1→1:2 hexanes–ethyl acetate afforded compound peracetylated fluoro decarbamoyl BLM disaccharide (**2.137**) as a colorless oil: yield 20.0 mg (38% over two steps); silica gel TLC R_f 0.37 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.98-2.18 (m, 21H), 3.40 (m, 1H), 4.07 (m, 2H), 4.14 (m, 3H), 4.27 (m, 2H), 4.52 (m, 1H), 4.64 (m, 1H), 4.71 (m, 2H), 4.89 (m, 1H), 5.01 (m, 2H) and 5.29 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.2, 20.77, 20.82, 20.88, 20.91, 20.96, 20.98, 21.1, 62.6, 62.7, 66.2, 66.3, 67.4, 67.6, 68.7, 68.8, 68.8, 70.0, 70.1, 70.1, 81.5, 83.2, 97.9, 98.7, 155.3, 170.1 and 170.8; ^{19}F NMR ($CDCl_3$, 376 MHz) δ -224.43 (m); mass spectrum (MALDI-TOF), *m/z* 705.46 $(M + Na)^+$; mass spectrum (ESI), *m/z* 705.2027 $(M + Na)^+$ ($C_{28}H_{39}FNaO_{18}$ requires *m/z* 705.2018).



Decarbamoyl Fluoro BLM Disaccharide (3.36). To a solution of 7.00 mg (10.2 mmol) of peracetylated fluoro decarbamoyl BLM disaccharide (**2.137**) in 2.2 mL of anh methanol was added 0.22 mL of 25% w/w freshly prepared solution of sodium methoxide in methanol. The reaction mixture was allowed to stir at room temperature for 2.5 h and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of

Dowex 50x resin, shaken for 15 min, filtered, concentrated, and lyophilized to afford fluoro decarbamoyl BLM disaccharide (**2.36)** as a light yellowish solid: yield 3.60 mg (90%); mass spectrum (MALDI-TOF), *m/z* 411.31 $(M + Na)^+$; mass spectrum (ESI), *m/z* 411.1280 $(M + Na)^+$ ($C_{14}H_{25}FNaO_{11}$ requires *m/z* 411.1279).

Cell Growth Conditions.

MCF-7 cells (ATCC HBT-22), A498 cells (ATCC HTB-41), A549 cells (ATCC CCL-185) and BT474 cells (ATCC HTB-20) were grown in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) and 1% penicillin–streptomycin mix antibiotic supplement (Cellgro, Manassas, VA). MCF-10A cells (ATCC CRL-10317) were grown in MEGM (Invitrogen, Grand Island, NY) supplemented with 100 ng/mL cholera toxin (Sigma-Aldrich) and 1% penicillin-streptomycin mix antibiotic supplement. DU-145 (ATCC HTB-81) prostate cells, BxPC-3 (ATCC CRL-1687) pancreas cells, SVR A221a (CRL-2386) pancreatic normal cells, PZ-HPV-7 (ATCC CRL-2221) prostate normal cells, WI-38 (ATCC CCL-75) lung normal cells and CCD-1105 KIDTr (CRL-2305) kidney normal cells were grown in MEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin–streptomycin mix antibiotic supplement. Cell lines were maintained at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air.

Fluorescence microscopy.

The fluorescent images were obtained using a Zeiss Axiovert 200M inverted fluorescence microscope fitted with an AxioCam MRm camera equipped with a 300-w xenon lamp (Sutter, Novato, CA) and a Cy5 cyanine filter (Chroma, Bellows Falls, VT). The cells were grown on 16-well Lab-Tek glass chamber slides at a cell density of 5000 cells/well (Thermo Scientific, Waltham, MA) at 37 °C for 48 h. When the cell confluency reached about 70%, the cells were rinsed twice with phosphate buffered saline (PBS), and the old medium was replaced with RPMI 1640 (no phenol red). Subsequently, the dye-labeled conjugates were added to the final desired concentrations and incubation of the cells was carried out at 37 °C for 1 h. Thereafter, the cells were washed with PBS, and fixed with 4% paraformaldehyde at 37 °C for 5 min. Finally, the slide was mounted with Prolong Antifade Gold reagent with DAPI (Invitrogen). The cells were imaged using a Zeiss EC Plan Neofluor 40x/1.3 DIC M27 oil objective and the target cells were counted for quantification. For comparative studies, the exposure time and source intensity were kept identical for accurate measurements. Three different viewing fields each containing at least 10 individual cells were analyzed from each experiment. The mean pixel intensities [per unit area of the cells] in the viewing fields were measured to give the normalized fluorescence and quantification values were generated by using AxioVision 4 v 4.7.1.0 software in conjunction with the interactive measurement tool.

CHAPTER 3

SYNTHESIS OF SACCHARIDE–DRUG CONJUGATES FOR TARGETED DELIVERY TO CANCER CELLS

3.1. Introduction

A major problem associated with conventional chemotherapy is the lack of specificity of the drug for the cancer cells, resulting in high cytotoxicity towards normal cells. This type of treatment results in side effects, disease relapse and poor quality of life after treatment for many patients. However, more recently chemotherapy has focused not only in finding the correct drug molecule with optimized pharmacokinetic properties but also in finding a way to improve its selectivity towards cancer cells. One such approach is the identification of therapeutic targets with over-expressed receptors or antigens.

The conjugation of drugs to the monoclonal antibodies that can precisely deliver drugs to cancer cells with overexpressed antigens has been a very promising area of development.[151] The linkers that attach the cytotoxic agent to the antibody are designed in such a way that they are stable in the circulatory system and release the cytotoxic drug inside the targeted cells (Figure 3.1).[142,152,153]

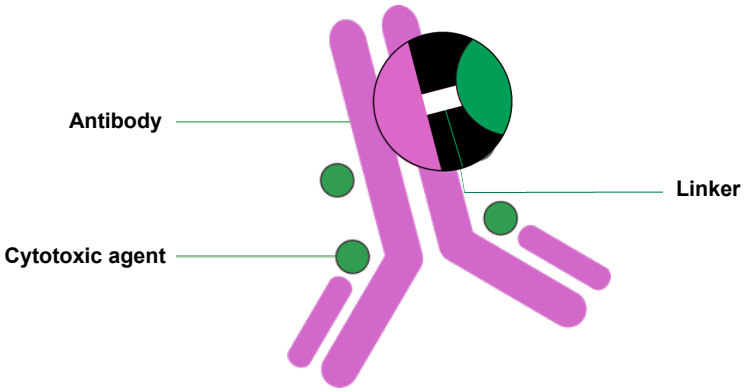


Figure 3.1. Elements of an Antibody–Drug Conjugate (ADC).[142]

The use of antibody drug conjugates (ADC) in cancer therapy has led to increased therapeutic efficacy for many drugs (Figure 3.2). However, the development of ADCs requires the optimization of several parameters, including immunogenicity associated with the conjugate, stability of the conjugate, pharmacokinetics, retention of immugenic response and proper release of the drug at the target site.[154,155] The proposed mechanism of internalization of ADCs is illustrated in Figure 3.3.

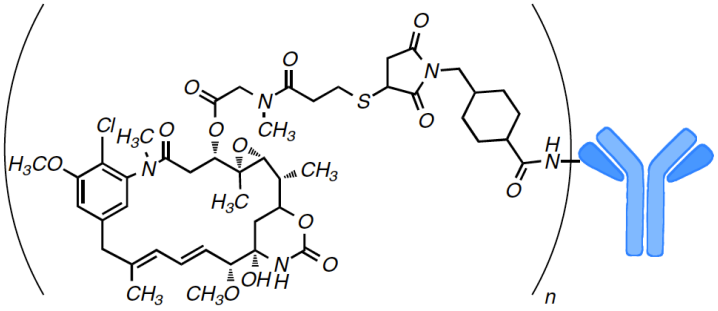


Figure 3.2. Structure of Antibody–Drug Conjugate: Trastuzumab-DM1 (recently approved by FDA).

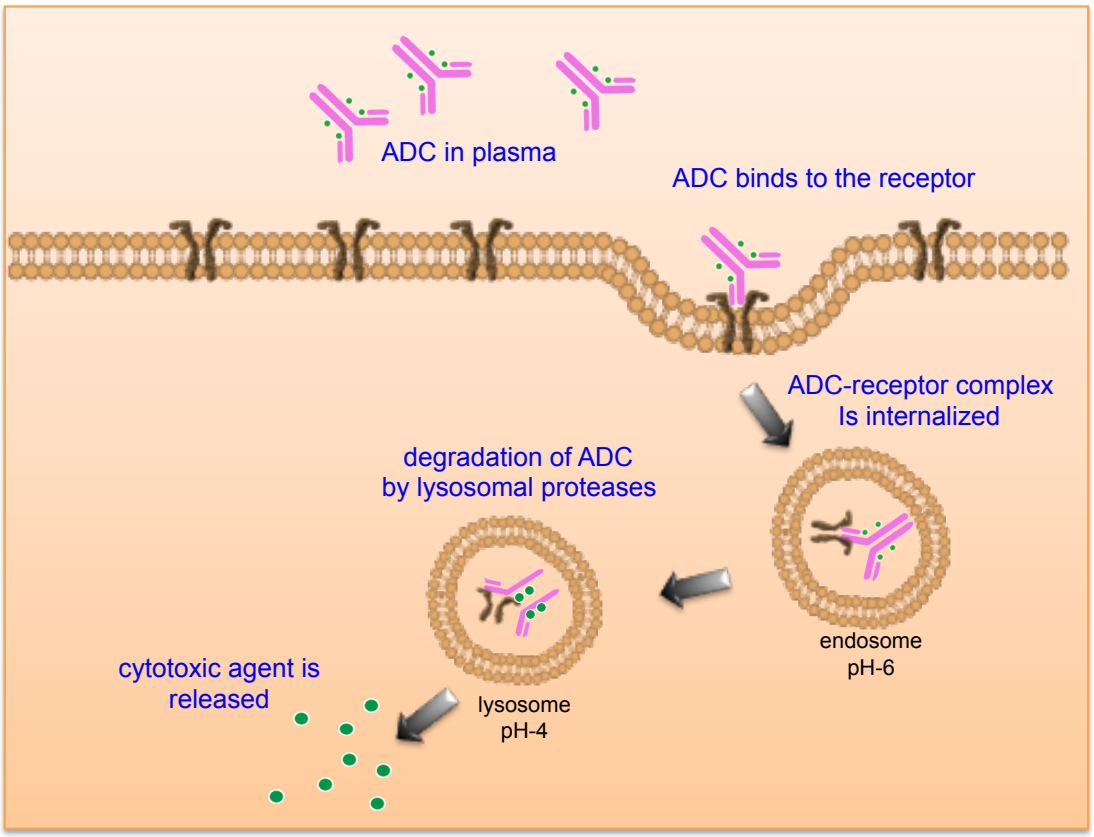


Figure 3.3. Proposed Mechanism of Targeted Delivery of a Cytotoxic Agent by Antibody–Drug Conjugates (ADCs) (adapted from ref. 142).

Another approach has involved utilization of an overexpressed receptor on the cancerous cell to guide the toxin to tumor cells. As described earlier, the BLM saccharide is responsible for tumor targeting ability possessed by BLM[18] and hence, it seems possible that BLM saccharides might be able to serve the role of delivering the toxins selectively to the cancer cells.

Camptothecin (CPT) is a naturally occurring pentacyclic alkaloid isolated from *Camptotheca acuminate* exhibiting antitumor activity towards solid tumors.[156] It inhibits the action of DNA topoisomerase I (type I inhibitor) and causes programmed cell death.[157] Due to high cell toxicity, it has been a very interesting lead compound for

cancer chemotherapy for decades. But the clinical utility of CPT was restricted due to poor solubility in water, which was addressed by utilization of the sodium salt of the ring-opened compound in the earliest clinical trials, the latter of which proved to be inactive. Later, the limitation associated with water solubility was resolved by the discovery of its more water soluble congeners topotecan and irinotecan (Figure 3.4), which are now used clinically for the treatment of ovarian and colon cancers.[158-160]

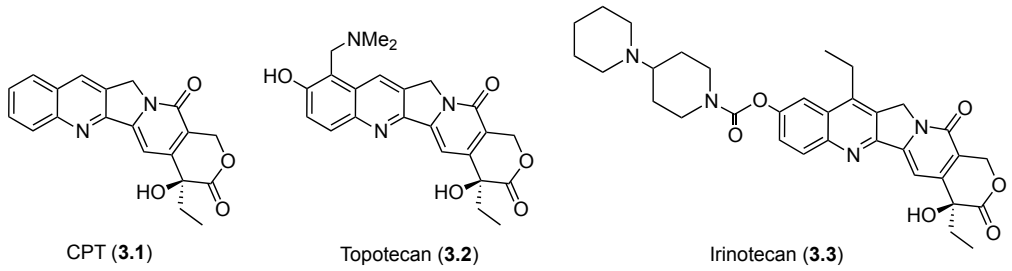


Figure 3.4. Structures of CPT Congeners: Topotecan and Irinotecan.

Base treatment of **3.1** opens the lactone ring to form the inactive water-soluble carboxylate **3.4** (Figure 3.5). This occurs to some extent under physiological conditions in human blood circulation. Hence, the prodrug approach by esterification of 20-OH may be useful in the development of a better therapeutic form of CPT.[161] In an effort to design and develop a more selective CPT pro-drug, a series of CPT–saccharide carbamates were synthesized and their cytoxicities were evaluated (Figure 3.6).

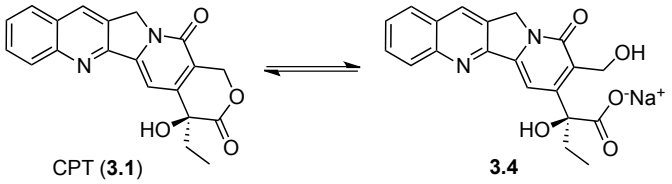


Figure 3.5. Equilibrium Between the Closed and Open Lactone Ring of CPT.

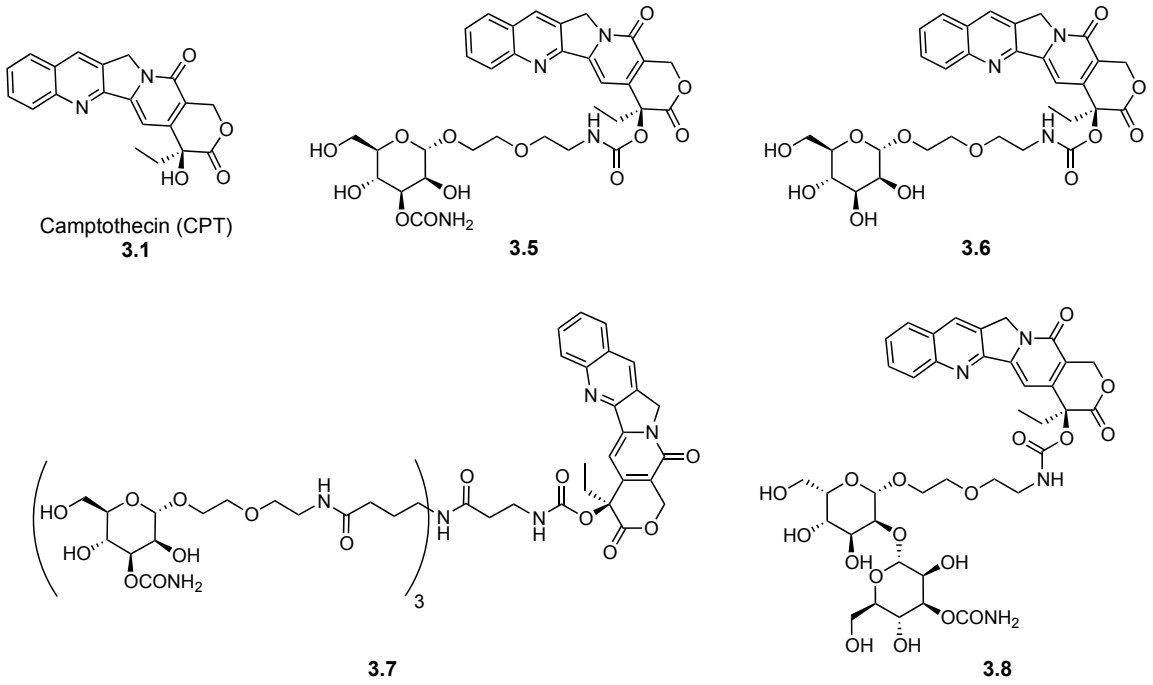


Figure 3.6. Series of CPT–saccharide Carbamates Synthesized and Evaluated.

Later, a series of CPT–saccharide esters and CPT–bis-saccharide esters were also synthesized to study the effect of the increase in the number of conjugated sugars to the cytoxicity of the drug (Figures 3.7 and 3.8).

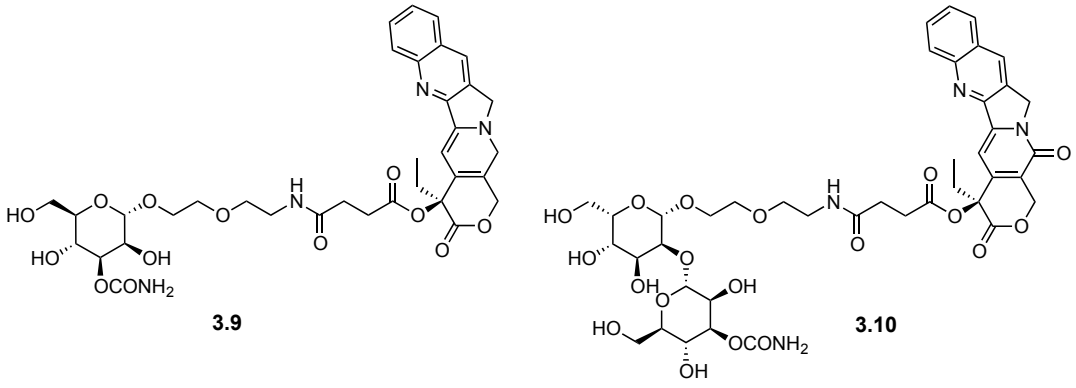


Figure 3.7. CPT–saccharide Esters Synthesized and Evaluated.

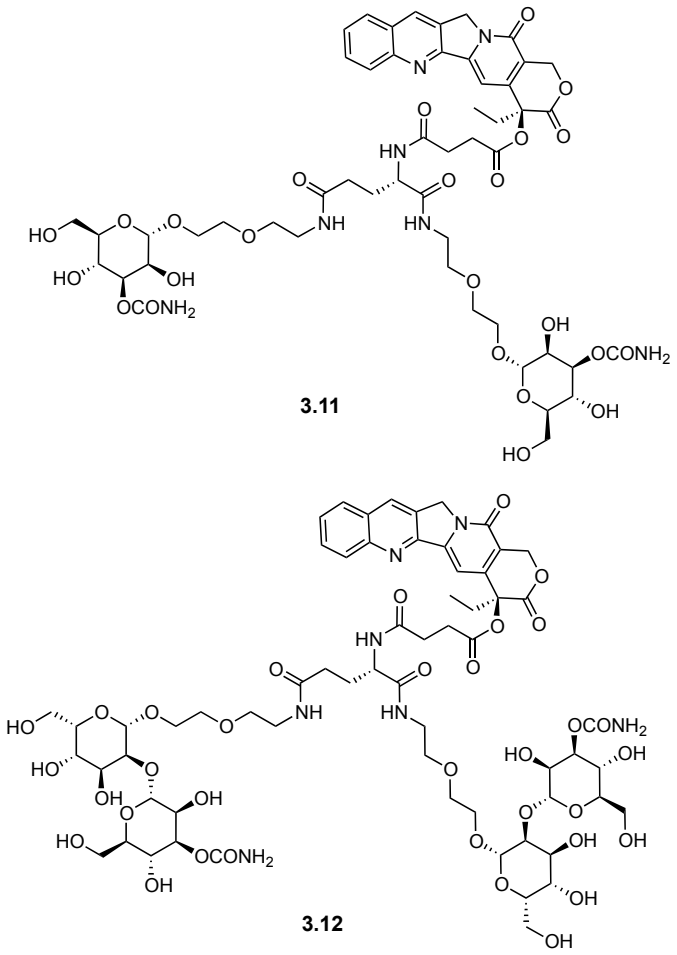
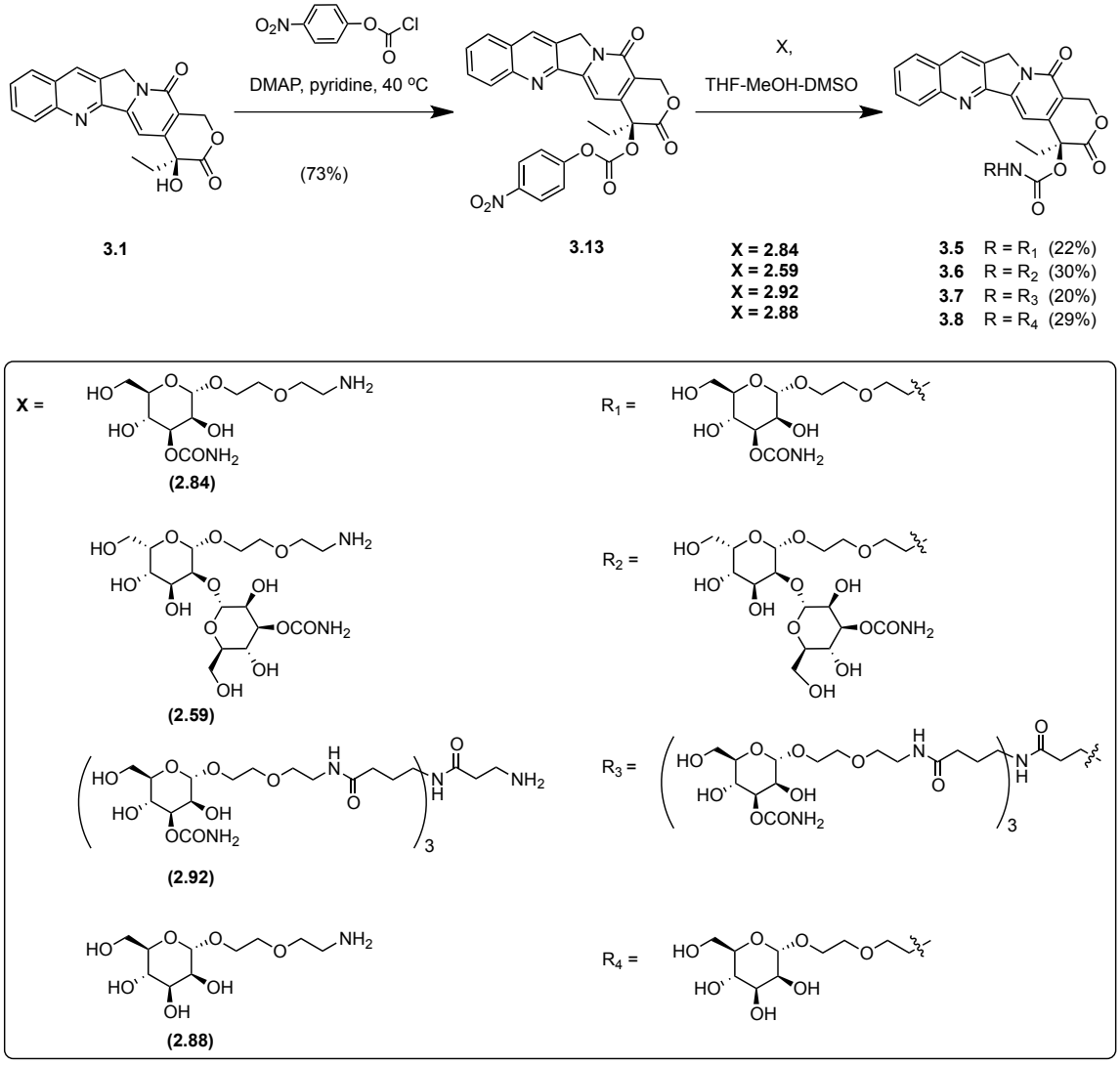


Figure 3.8. CPT–bis-saccharide Esters Synthesized and Evaluated.

3.2. Results

The synthesis of the CPT–saccharide carbamate conjugates started with the activation of the 20-OH group of CPT as the *p*-nitrophenyl carbonate (**3.13**) in 73% yield (Scheme 3.1).[161] Carbonate **3.13** was coupled with BLM monosaccharide linker **2.84**, BLM disaccharide linker **2.59**, BLM monosaccharide trimer linker **2.92** and decarbamoyl BLM monosaccharide linker **2.88** to give CPT–BLM monosaccharide carbamate **3.5**, CPT–BLM disaccharide carbamate **3.6**, CPT–BLM monosaccharide trimer carbamate **3.7**

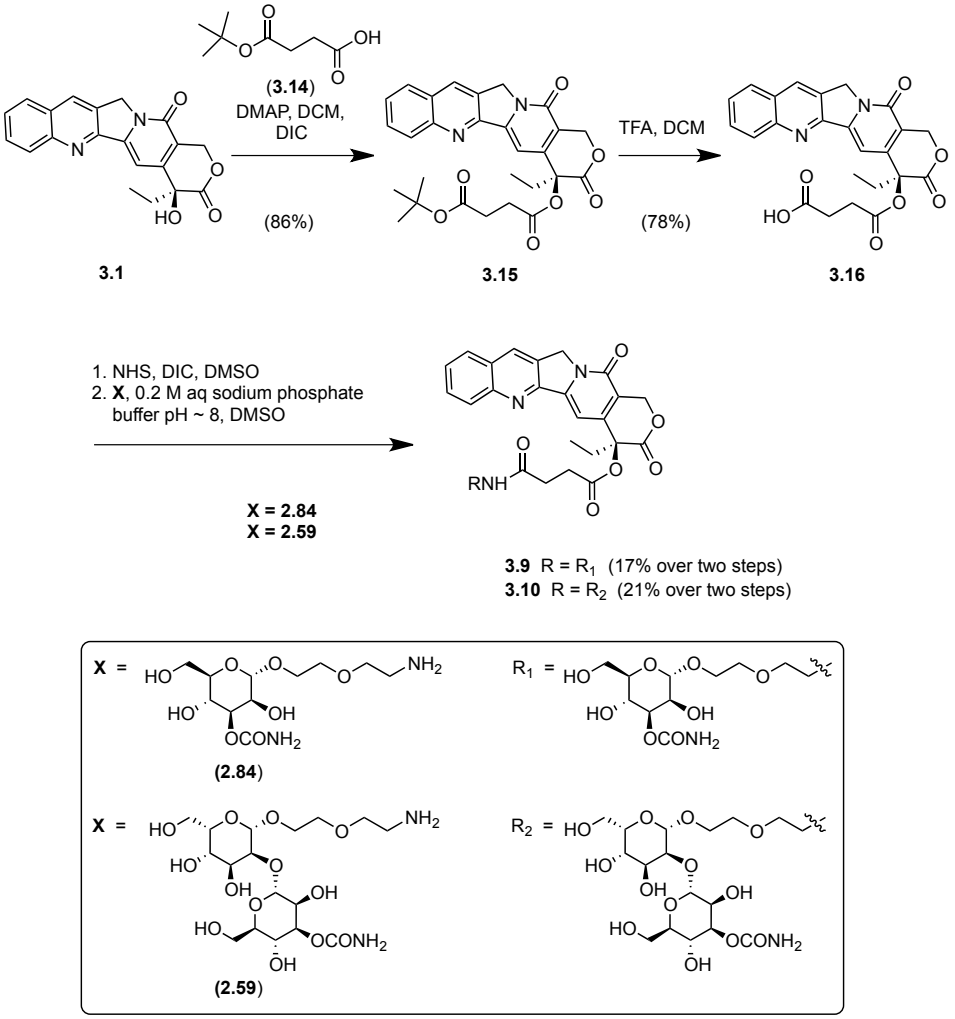
and CPT–decarbamoyl BLM monosaccharide carbamate **3.8** in 22%, 30%, 20% and 29% yields, respectively.



Scheme 3.1. Synthesis of CPT–saccharide Carbamate Conjugates **3.5**–**3.8**.

CPT–saccharide ester conjugates were synthesized to afford easily hydrolyzable saccharide prodrugs of CPT. The synthesis of CPT–saccharide esters began with the esterification of 20-OH group of CPT with mono protected succinic acid ester (**3.14**) to form CPT ester linker **3.15** in 86% yield (Scheme 3.2).[162] The *tert*-butyl ester of

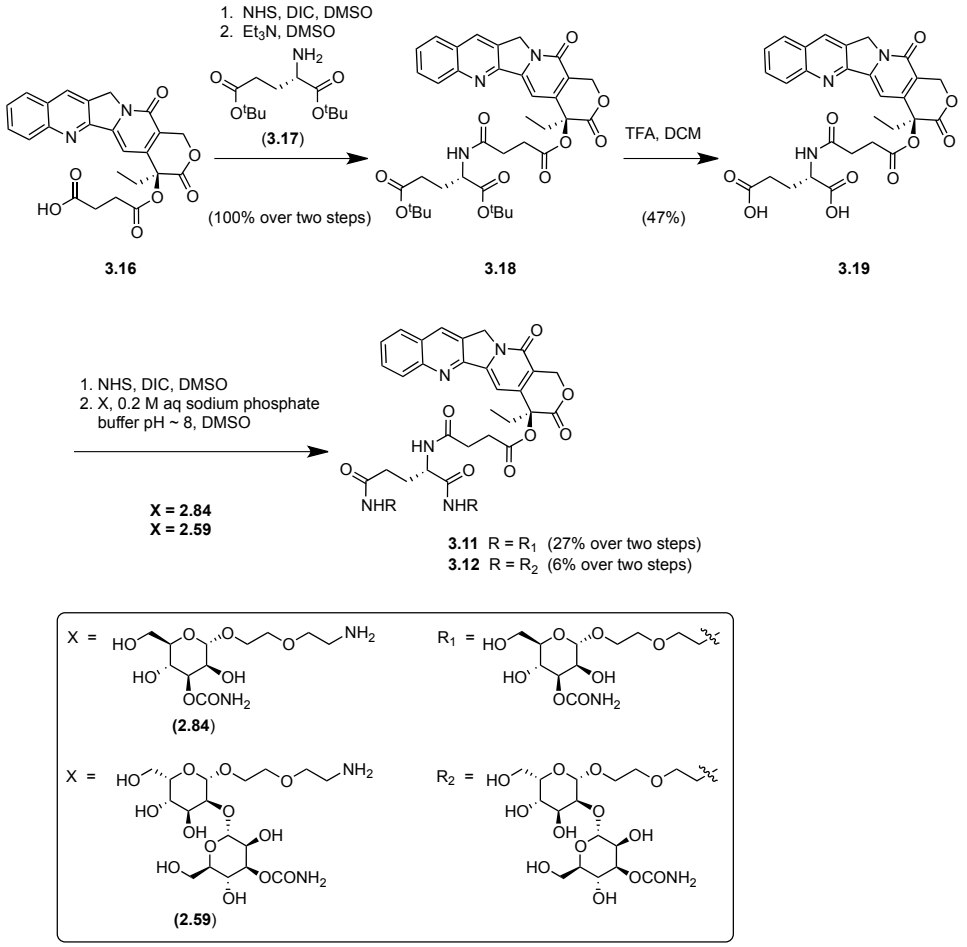
compound **3.15** was cleaved with TFA to afford CPT acid linker **3.16** in 78% yield. CPT acid linker **3.16** was activated as the NHS ester and coupled with BLM monosaccharide linker **2.84** and BLM disaccharide linker **2.59** to afford CPT–BLM monosaccharide ester conjugate **3.9** and CPT–BLM disaccharide ester conjugate **3.10** in 17% and 21% yields, respectively, over two steps.



Scheme 3.2. Synthesis of CPT–saccharide Ester Conjugates **3.9** and **3.10**.

To further study the effect of increasing the number of sugar moieties attached to the prodrug on cytotoxicity, CPT–bis-saccharide ester conjugates were synthesized. The

synthesis of bis-saccharide conjugates started with the coupling of the NHS ester of CPT acid linker acid **3.16** with the amine of di-*tert*-butyl ester of glutamic acid (**3.17**) to form CPT bis-ester linker **3.18** in quantitative yield (Scheme 3.3). The *tert*-butyl ester of compound **3.18** was cleaved with TFA to give CPT bis-acid linker **3.19** in 47% yield. Linker **3.19** was then activated as the bis-NHS ester and coupled with BLM monosaccharide linker **2.84** and BLM disaccharide linker **2.59** to afford CPT–bis-monosaccharide ester **3.11** and CPT ester–bis-disaccharide ester **3.12** in 27% and 6% yields, respectively, over two steps.



Scheme 3.3. Synthesis of CPT–bis-saccharide Ester Conjugates **3.11** and **3.12**.

3.3. Discussion

The cytotoxic drug camptothecin (CPT) was modified with different BLM saccharides as 20-OH prodrugs to promote targeted delivery to tumor cells. The 20-OH *p*-nitrophenyl carbonate of CPT (**3.13**) was synthesized from CPT (**3.1**) by following reported procedures (Scheme 3.1).[21,161] Carbonate prodrugs **3.5**–**3.8** were then prepared by coupling the free amine of BLM monosaccharide linker **2.84**, BLM disaccharide linker **2.59**, trimer BLM monosaccharide linker **2.92** and decarbamoyl BLM monosaccharide linker **2.88**, respectively with carbonate **3.13**.

The easily hydrolyzable ester prodrugs of CPT were also prepared to ensure complete release of the drug after saccharide–mediated internalization. The 20-OH CPT ester **3.15** was synthesized from CPT (**3.1**) by following reported procedures (Scheme 3.2).[162] The *tert*-butyl ester of **3.15** was cleaved using TFA and the resulting free acid of CPT linker **3.16** activated as the NHS ester, was condensed with the free amine of BLM monosaccharide linker **2.84** and BLM disaccharide linker **2.59** to afford CPT esters **3.9** and **3.10**.

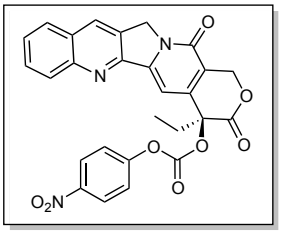
Later, the bis-sugar ester prodrugs of CPT were synthesized to facilitate polyvalent interaction with the cell surface receptor. CPT free acid **3.16**, activated as the NHS ester, was coupled with linker amine **3.17** to give the *tert*-butyl ester **3.18** (Scheme 3.3). Hydrolysis of the *tert*-butyl ester, followed by subsequent coupling of the NHS ester of the bis-acid **3.19** with BLM monosaccharide linker **2.84** and BLM disaccharide linker **2.59** afforded CPT esters **3.11** and **3.12**. The biological evaluation of these analogues is in progress.

3.4. Experimental Procedures

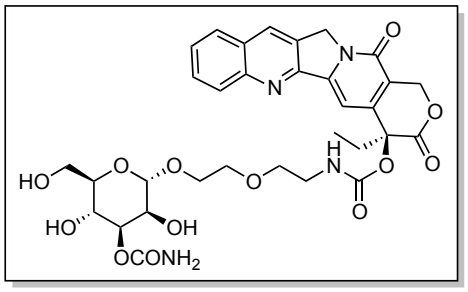
Materials. The chemicals used were purchased from Aldrich Chemical Co., Sigma Chemical Co. or Combi-Blocks and were used without further purification. Anhydrous methanol, DMSO and pyridine were used as purchased. Dichloromethane was distilled from calcium hydride.

General Experimental Procedures. Flash column chromatography was carried out using silica gel (Silicycle R10030B, 60 particle size, 230-400 mesh), applying a low-pressure stream of nitrogen or dry air. Analytical thin layer chromatographic separations were carried out on glass plates coated with silica gel (60 particle size F254, Silicycle TLG-R10011B-323) and the developed chromatogram was observed under UV light.

Instrumentation. HPLC was performed using an Agilent 1100 series instrument. High resolution mass spectrometric data were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State Mass Spectrometry Facility. ^{1}H and ^{13}C NMR spectra were recorded on Varian Inova 400 MHz and 100 MHz, respectively, using $CDCl_3$ as solvent and internal standard with a residual peak at 7.26 ppm and 77.16 ppm, respectively. Splitting patterns are designated as s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet and q, quartet.

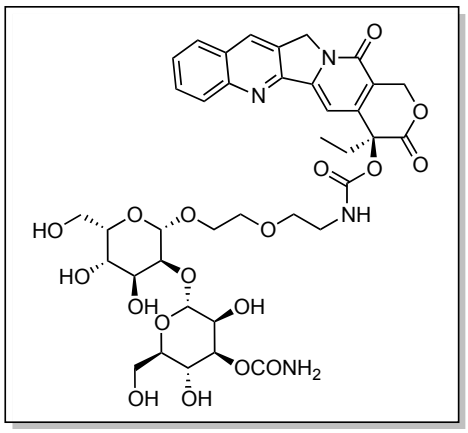


CPT 20-(*p*-nitrophenyl)carbonate (3.13).[21,161] To a solution of 40.0 mg (0.11 mmol) of CPT (**3.1**) in 0.42 mL of dry pyridine were added 56.0 mg (0.46 mmol) of DMAP and 92.0 mg (0.46 mmol) of *p*-nitrophenyl chloroformate and was stirred at 40 °C overnight. The solution was cooled and poured into a mixture of 20 mL ethyl acetate and 5 mL of water. The organic layer was washed with three 5-mL portions of 1N HCl, 5 mL of saturated aq $NaHCO_3$ and 5 mL of brine. The solution was dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 2.5 cm). Elution with 15:1→8:1 chloroform–methanol afforded carbonate **3.13** as a light yellow solid: yield 43.0 mg (73%); silica gel TLC R_f 0.30 (7:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.07 (m, 3H), 2.25 (m, 1H), 2.37 (m, 1H), 5.31 (m, 2H), 5.42 (d, 1H, J = 16.5 Hz), 5.72 (d, 1H, J = 17.0 Hz), 7.40 (m, 2H), 7.70 (m, 1H), 7.86 (m, 1H), 7.96 (d, 1H, J = 7.5 Hz), 8.23 (m, 3H) and 8.42 (s, 1H).



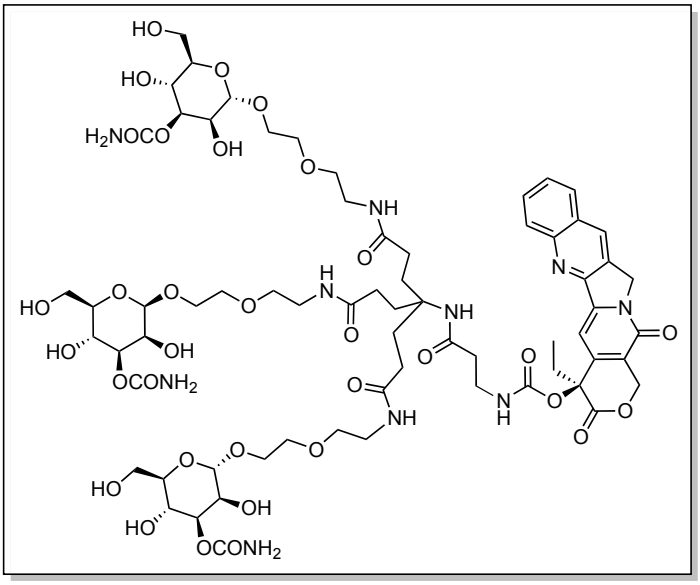
CPT 20-(BLM Monosaccharide)carbamoyl Conjugate (3.5) To 6.00 mg (19.5 μmol) of amine **2.84** in 1.75 mL of 2:4:1 THF–methanol–DMSO was added 5.00 mg (9.74 μmol) of carbamate **3.13** and the reaction mixture was stirred overnight. The crude reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A

linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→60:40 0.1% aq TFA–acetonitrile) over a period of 18 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 15.9 min (monitoring at 364 nm) and were collected, frozen and lyophilized to give CPT carbamate conjugate **3.5** as a light yellow solid: yield 1.50 mg (22%); mass spectrum (MALDI-TOF), *m/z* 685.72 $(M + H)^+$; mass spectrum (ESI), *m/z* 683.2197 $(M - H)^-$ ($C_{32}H_{35}N_4O_{13}$ requires *m/z* 683.2201).

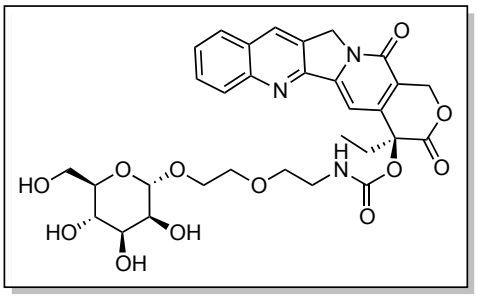


CPT 20-(BLM Disaccharide)carbamoyl Conjugate (3.6). To 6.60 mg (14.0 μmol) of amine **2.59** in 1.8 mL of 2:1 THF–methanol was added 3.60 mg (7.01 μmol) of carbamate **3.13** and the reaction mixture was stirred overnight. The crude reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→60:40 0.1% aq TFA–acetonitrile) over a period of 18 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 13.8 min (monitoring at 364 nm) and were collected, frozen and lyophilized to give CPT carbamate conjugate **3.6** as a white solid:

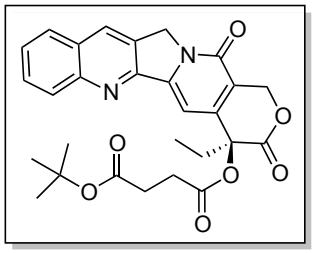
yield 1.80 mg (30%); mass spectrum (MALDI-TOF), *m/z* 869.43 $(M + Na)^+$; mass spectrum (ESI), *m/z* 869.2706 $(M + Na)^+$ ($C_{38}H_{46}N_4O_{18}Na$ requires *m/z* 869.2705).



CPT 20-(BLM Monosaccharide Trimer)carbamoyl Conjugate (3.7). To 9.30 mg (7.78 μmol) amine **2.92** in 1.5 mL of 2:4:1 THF–methanol–DMSO was added 2.00 mg (3.90 μmol) of carbamate **3.13** and the reaction mixture was stirred overnight. The crude reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→60:40 0.1% aq TFA–acetonitrile) over a period of 18 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 16.5 min (monitoring at 364 nm) and were collected, frozen and lyophilized to give CPT carbamate conjugate **3.7** as a light yellow solid: yield 1.20 mg (20%); mass spectrum (MALDI-TOF), *m/z* 1591.89 $(M + Na)^+$ and 1607.61 $(M + K)^+$ (theoretical *m/z* 1591.61 and 1607.61).

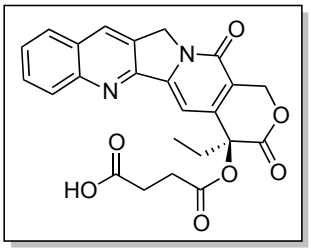


CPT 20-(Decarbamoyl BLM Monosaccharide)carbamoyl Conjugate (3.8). To 5.20 mg (19.5 μmol) of amine **2.88** in 1.5 mL of 2:1 THF–MeOH was added 5.00 mg (9.74 μmol) of carbamate **3.13** and the reaction mixture was stirred overnight. The crude reaction mixture was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→60:40 0.1% aq TFA–acetonitrile) over a period of 18 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 15.2 min (monitoring at 364 nm) and were collected, frozen and lyophilized to give CPT carbamate conjugate **3.8** as a white solid: yield 1.80 mg (29%); mass spectrum (APCI), *m/z* 642.2280 $(M + H)^+$ ($C_{31}H_{36}N_3O_{12}$ requires *m/z* 642.2299).



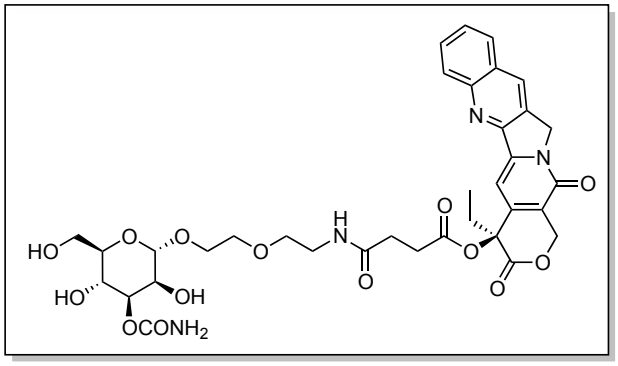
***tert*-Butyl CPT 20-Succinate Ester (3.15).**[162] To a solution of 198 mg (1.13 mmol) of acid **3.14** in 12 mL of dry dichloromethane were added 93.0 mg (0.76 mmol) of DMAP, 0.18 mL (145 mg, 1.15 mmol) of diisopropylcarbodiimide (DIC) and 200 mg (0.57

mmol) of CPT (**3.1**). The reaction was stirred at room temperature overnight and diluted with 10 mL dichloromethane. The solution was washed with 3 mL of 0.1 N HCl, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was crystallized from methanol, filtered and dried to afford **3.15** as a yellow solid: yield 170 mg (56%); silica gel TLC R_f 0.30 (12:1 chloroform–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.99 (t, 3H, J = 7.2 Hz), 1.36 (s, 9H), 2.15 (m, 1H), 2.26 (m, 1H), 2.56 (m, 2H), 2.78 (m, 2H), 5.26 (d, 2H, J = 3.2 Hz), 5.38 (d, 1H, J = 17.2 Hz), 5.68 (d, 1H, J = 17.2 Hz), 7.31 (s, 1H), 7.66 (m, 1H), 7.82 (m, 1H), 7.92 (d, 1H, J = 8.4 Hz), 8.22 (d, 1H, J = 8.4 Hz) and 8.37 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 7.8, 28.1, 29.3, 30.2, 31.9, 50.0, 67.2, 76.3, 81.1, 96.6, 120.2, 128.1, 128.3, 128.3, 128.6, 129.9, 130.7, 131.2, 146.2, 146.3, 149.0, 152.6, 157.5, 167.6, 171.2 and 171.6.

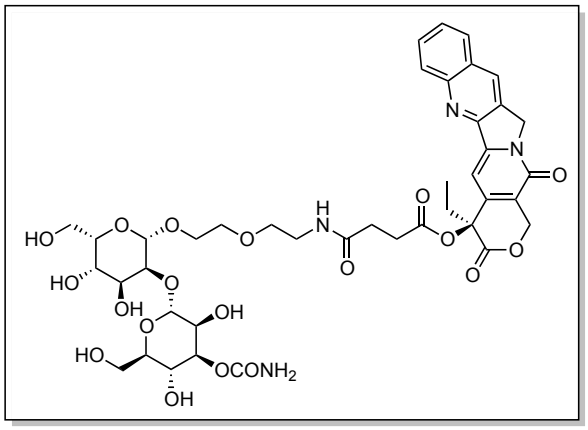


CPT 20-Succinate Acid (3.16).[162] To 134 mg (0.27 mmol) of ester **3.15** in 1.5 mL of dichloromethane was added 0.6 mL of TFA and the reaction mixture was stirred for 5 h at room temperature. The reaction mixture was concentrated under diminished pressure, crystallized from methanol and dried to afford acid **3.16** as a pale yellow solid: yield 98.0 mg (82%); silica gel TLC R_f 0.29 (12:1 chloroform–methanol); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.05 (m, 3H), 2.29 (m, 2H), 2.61 (m, 2H), 2.90 (m, 2H), 5.41 (d, 2H, J = 1.2 Hz), 5.62 (s, 2H), 7.26 (s, 1H), 7.84 (t, 1H, J = 7.2 Hz), 8.00 (m, 1H), 8.25 (d, 1H, J = 8.0

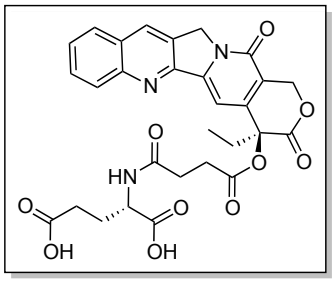
Hz), 8.30 (d, 1H, J = 8.4 Hz) and 8.80 (s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 7.5, 28.4, 28.6, 30.4, 50.2, 66.3, 75.9, 95.1, 118.9, 127.7, 128.0, 128.5, 129.0, 129.8, 130.4, 131.5, 145.2, 145.9, 145.9, 152.4, 156.5, 167.2, 171.3 and 173.0.



BLM Monosaccharide CPT 20-Succinate Ester Conjugate (3.9). To 5.00 mg (11.0 μmol) of acid **3.16** in 0.15 mL of DMSO were added 1.50 mg (13.0 μmol) of *N*-hydroxysuccinimide and 1.70 μL (1.40 mg, 11 μmol) of diisopropylcarbodiimide and the reaction mixture was stirred at room temperature for 24 h. To the activated acid solution was added 3.50 mg (11.0 μmol) of amine **2.84** in 0.5 mL of 1:1 0.2 M aq sodium phosphate buffer (pH 8.0)–DMSO. The reaction mixture was stirred overnight at room temperature and purified on an Econosil C_{18} reversed phase semi-preparative HPLC column (250 × 10 mm, 10 μm) using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→60:40 0.1% aq TFA–acetonitrile) over a period of 18 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 18.8 min (monitoring at 364 nm) and were collected, frozen and lyophilized to give CPT ester conjugate **3.9** as a light yellow solid: yield 1.40 mg (17% over two steps); mass spectrum (MALDI-TOF), *m/z* 779.68 (M + K)$^+$ (theoretical requires *m/z* 779.22).



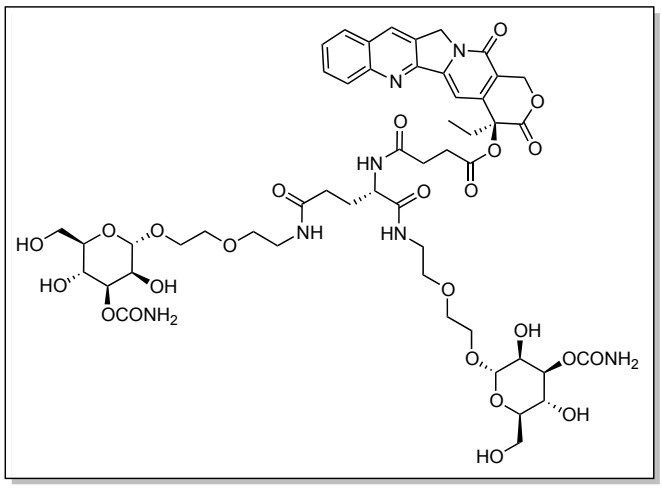
BLM Disaccharide CPT 20-Succinate Ester Conjugate (3.10). To 2.00 mg (4.50 μmol) of acid **3.16** in 0.15 mL of DMSO were added 1.00 mg (8.90 μmol) *N*-hydroxysuccinimide and 1.40 μL (1.10 mg, 8.90 μmol) of diisopropylcarbodiimide and the reaction mixture was stirred at room temperature for 24 h. To the activated acid solution was added 3.20 mg (6.77 μmol) of amine **2.59** in 0.4 mL of 1:1 0.2 M aq sodium phosphate buffer (pH 8.0)–DMSO. The reaction mixture was stirred overnight at room temperature and purified on an Econosil C_{18} reversed phase semi-preparative HPLC column (250 × 10 mm, 10 μm) using aq 0.1% TFA and CH_3CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→60:40 0.1% aq TFA–acetonitrile) over a period of 18 min at a flow rate of 4.5 mL/min. The fractions containing the desired product eluted at 17.9 min (monitoring at 364 nm) and were collected, frozen and lyophilized to give CPT ester conjugate **3.10** as a light yellow solid: yield 2.08 mg (21% over two steps); mass spectrum (MALDI-TOF), *m/z* 925.22 $(M + Na)^+$; mass spectrum (ESI), *m/z* 903.3157 $(M + H)^+$ ($C_{41}H_{51}N_4O_{19}$ requires *m/z* 903.3148).



L-Glutamic Acid CPT 20-Succinate (3.19). To a solution of 174 mg (0.39 mmol) of acid **3.16** in 5 mL of dry DMSO were added 89.0 mg (0.78 mmol) *N*-hydroxysuccinimide and 0.12 mL (98.0 mg, 0.78 mmol) of diisopropylcarbodiimide (DIC) and the reaction mixture was stirred at room temperature for 24 h. To the activated acid solution were added 92.0 mg (0.31 mmol) of amine **3.17** and 43.0 μL of dry triethylamine (31.3 mg, 0.31 mmol) in 3 mL of dry DMSO and stirred at room temperature overnight. The mixture was then diluted with 20 mL dichloromethane and washed with 5 mL of 0.1 N HCl, then dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was crystallized from methanol, filtered and dried to afford bis-ester **3.18** as a yellow solid: yield 280 mg (100%); silica gel TLC R_f 0.35 (12:1 chloroform–methanol).

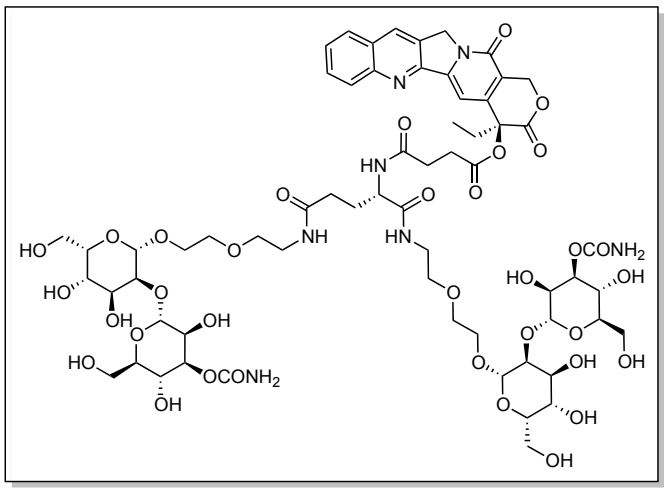
To 280 mg (0.41 mmol) bis-ester **3.18** in 3.1 mL of dichloromethane was added 1.3 mL of TFA and the reaction mixture was stirred for 5 h. The reaction mixture was concentrated under diminished pressure and crystallized from methanol, filtered and dried to afford bis-acid **3.19** as a pale yellow solid: yield 110 mg (47%); silica gel TLC R_f 0.25 (12:1 chloroform–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.93 (t, 3H, J = 7.6 Hz), 1.79 (m, 1H), 1.99 (m, 1H), 2.13 (m, 2H), 2.29 (m, 2H), 2.41 (m, 1H), 2.56 (m, 1H), 2.73 (m, 2H), 4.40 (m, 1H), 5.26 (s, 2H), 5.48 (s, 2H), 7.12 (s, 1H), 7.70 (t, 1H, J = 7.2 Hz), 7.84 (t, 1H, J = 7.4 Hz), 8.11 (d, 1H, J = 8.0 Hz), 8.22 (dd, 2H, J = 8.4 and 4.8 Hz) and

8.66 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 7.6, 26.5, 29.0, 29.4, 30.1, 30.3, 50.2, 51.1, 66.2, 75.8, 95.3, 118.7, 127.7, 127.9, 128.5, 129.0, 129.7, 130.3, 131.5, 145.5, 145.9, 147.9, 152.4, 156.5, 167.2, 170.5, 171.2, 173.3 and 173.7; mass spectrum (APCI), *m/z* 578.1774 $(M + H)^+$ ($C_{29}H_{28}N_3O_{10}$ requires *m/z* 578.1774).



(Bis-BLM Monosaccharide)-L-Glutamate CPT 20-Succinate Ester Conjugate (3.11). To 3.00 mg (5.20 μmol) of bis-acid **3.19** in 0.2 mL of DMSO were added 2.20 mg (18.7 μmol) *N*-hydroxysuccinimide and 2.40 μL (2.0 mg, 15.6 μmol) of diisopropylcarbodiimide (DIC) and the reaction mixture was stirred at room temperature for 24 h. To the activated acid solution was added 3.30 mg (10.5 μmol) of amine **2.84** in 0.3 mL of 0.2 M sodium phosphate buffer (pH 8.0). The reaction mixture was stirred overnight at room temperature and purified on an Econosil C_{18} reversed phase semi-preparative HPLC column (250 × 10 mm, 10 μm) using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→70:30 0.1% aq TFA–acetonitrile→50:50 0.1% aq TFA acetonitrile) over a period of 8 min and 12 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired

product eluted at 11.7 min (monitoring at 364 nm) and were collected, frozen and lyophilized to give CPT ester conjugate **3.11** as a light yellow solid: yield 1.60 mg (27% over two steps); mass spectrum (ESI), *m/z* 1162.4308 $(M)^+$ ($C_{51}H_{68}N_7O_{24}$ requires *m/z* 1162.4316).



(Bis-BLM Monosaccharide)-L-Glutamate CPT 20-Succinate Ester Conjugate (3.12). To 3.00 mg (5.20 μmol) of bis-acid **3.19** in 0.2 mL of DMSO were added 2.20 mg (18.7 μmol) *N*-hydroxysuccinimide and 2.40 μL (2.00 mg, 15.6 μmol) of diisopropylcarbodiimide and the reaction mixture was stirred at room temperature for 24 h. To the activated acid solution was added 5.00 mg (10.5 μmol) of amine **2.59** in 0.3 mL of 0.2 M sodium phosphate buffer (pH 8.0). The reaction mixture was stirred overnight at room temperature and purified on an Econosil C_{18} reversed phase semi-preparative HPLC column (250 × 10 mm, 10 μm) using aq 0.1% TFA and acetonitrile mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–acetonitrile→70:30 0.1% aq TFA–acetonitrile→50:50 0.1% aq TFA–acetonitrile) over a period of 8 min and 12 min, respectively, at a flow rate of 4.5 mL/min. The fractions containing the desired product

eluted at 10.8 min (monitoring at 364 nm) and were collected, frozen and lyophilized to give CPT ester conjugate **3.12** as a light yellow solid: yield 0.44 mg (6% over two steps); mass spectrum (MALDI-TOF), *m/z* 1508.43 $(M + Na)^+$; mass spectrum (ESI), *m/z* 1508.5216 $(M + Na)^+$ ($C_{63}H_{87}N_7O_{34}Na$ requires *m/z* 1508.5192).

CHAPTER 4

SYNTHESIS OF LYSINE ANALOGUES FOR MODIFICATION OF HUMAN DNA POLYMERASE BETA

4.1. Introduction

A variety of chemical, biochemical and physical events can lead to alteration or deletion of nucleotide bases in cellular genomic DNA. Such damage in DNA repair genes or cell cycle regulation genes may prove lethal. Hence, the cell maintains genomic stability by using a set of DNA repair pathways, which include base excision repair (BER), DNA mismatch repair (MMR) and nucleotide excision repair (NER).[163,164] Base excision repair (BER) is the major pathway among these and protects the cell mainly against single base damage. BER is a sequential multistep process involving a cascade of events including (i) recognition and removal of the altered base, resulting in the generation of an apurinic/apyrimidinic (AP) site by DNA glycosylase, (ii) cleavage of the phosphodiester bond 5′- to the lesion by AP endonuclease, (iii) removal of the remaining sugar by polymerase beta, (iv) addition of the missing nucleotide by DNA polymerase beta, and (v) sealing of the nick by DNA ligase.[165] DNA polymerase beta is very important for BER as it is involved in both removal of the sugar residue and addition of the missing nucleotide.

DNA polymerase beta is a multifunctional enzyme comprised of two domains including a 31 kDa C-terminal polymerase and an 8 kDa N-terminal dRP lyase separated by a protease sensitive linker.[166] In solution it exists as a monomeric polypeptide of 335

amino acids[167] (Figure 4.1) and has the lowest fidelity of all DNA polymerases on the order of 1error/1500 bases.[168]

```
1   MSKRKAPQET LNGGITDMLT ELANFEKNVS QAIHKYNAYR KAASVIAKYP HKIKSGAEAK
61  KLPGVGTKIA EKIDEFLATG KLRKLEKIRQ DDTSSSINFL TRVSGIGPSA ARKFVDEGIK
121 TLEDLRKNED KLNHHQRIGL KYFGDFEKRI PREEMLQMQD IVLNEVKKVD SEYIATVCGS
181 FRRGAESSGD MDVLLTHPSF TSESTKQPKL LHQVVEQLQK VHFITDTLSK GETKFMGVCQ
241 LPSKNDEKEY PHRRIDIRLI PKDQYYCGVL YFTGSDIFNK NMRAHALEKG FTINEYTIRP
301 LGVTGVAGEP LPVDSEKDIF DYIQWKYREP KDRSE
```

Figure 4.1. Amino Acid Sequence of Human DNA Polymerase Beta, Highlighted Red Letters Include the Residues Present in the Active Site of dRP Lyase Domain.

Polymerase beta contains a 'helix-hairpin-helix' (HhH) motif in both the domains which is similar to those found in other DNA repair enzymes.[169,170] The dRP lyase domain of polymerase mediates excision of the dRP group of the AP lesion by elimination, as suggested by Matsumoto and Kim.[171] Later, Pierson et al. demostrated that β-elimination involves the formation of a Schiff base between the enzyme and dRP-containing DNA (Figure 4.2).[172] Although the active site of dRP domain is Lys-rich (Figure 4.3), it was shown by site-directed mutagenesis that N^{ϵ} of Lys72 is the atom involved in Schiff base formation.[173,174]

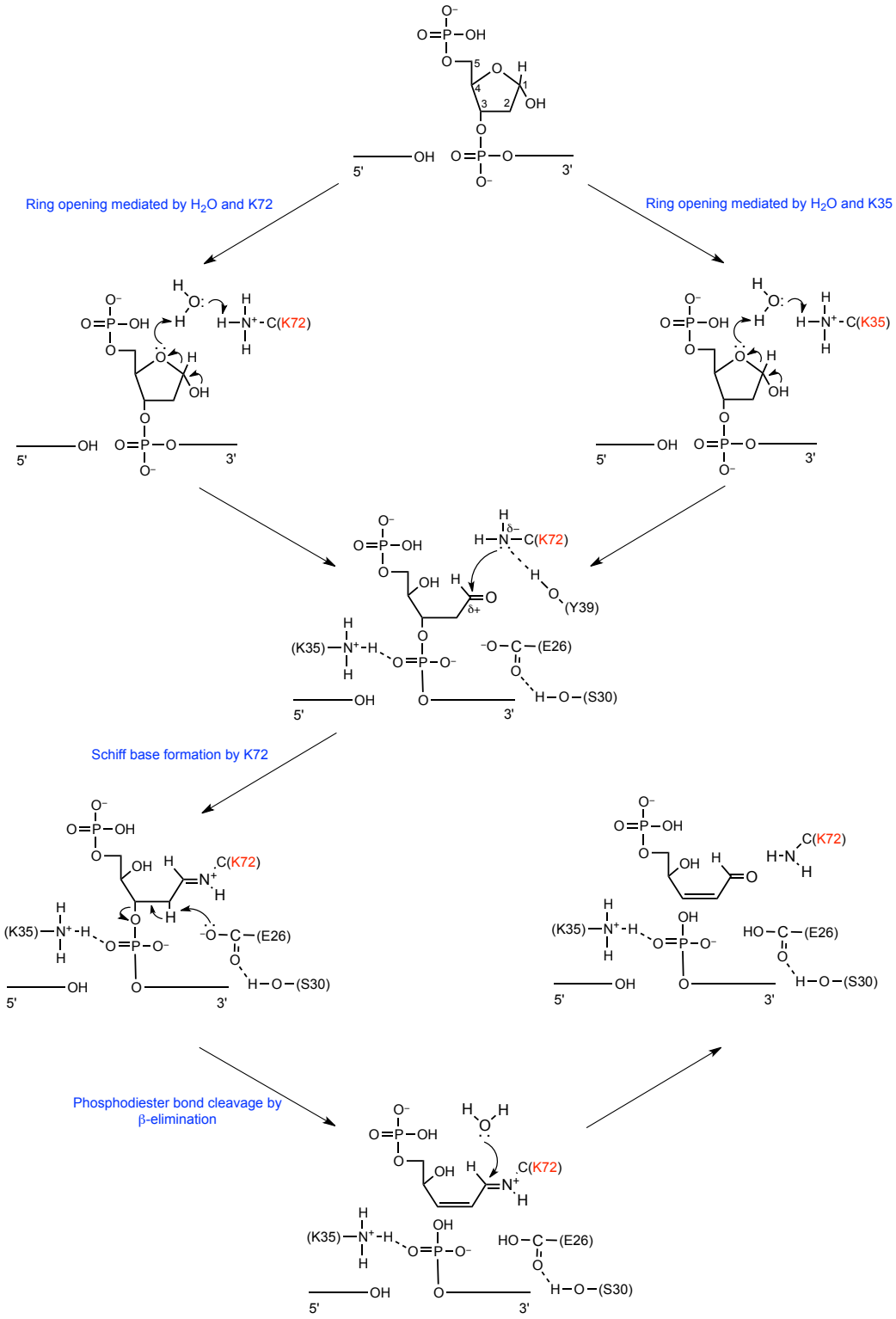


Figure 4.2. Proposed Mechanism of dRP Excision.[175]

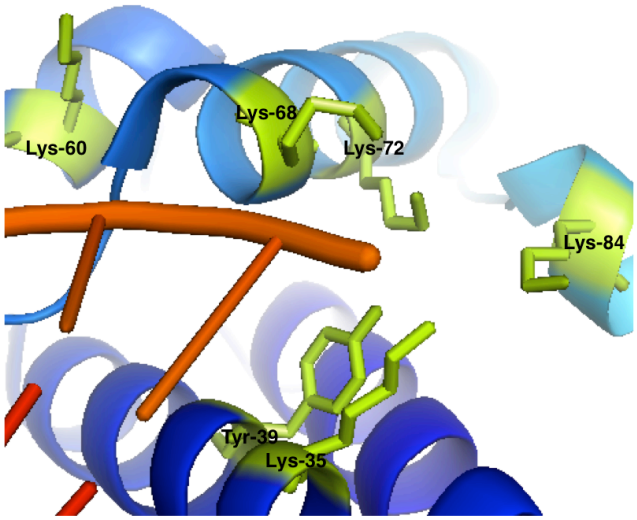


Figure 4.3. Active Site of dRP Lyase Domain of Human DNA Polymerase Beta Including Lys35, Tyr39, Lys60, Lys68, Lys72 And Lys84. (PBD ID of human DNA polymerase beta used is 3C2K).

To gain a better understanding of the mechanism involved in the excision of dRP, our laboratory decided to modify position 72 of rat DNA polymerase beta with a number of unnatural synthesized amino acids by site directed mutagenesis (Fig 4.4) and studied their effect on the dRP lyase activity of the enzyme.[176]

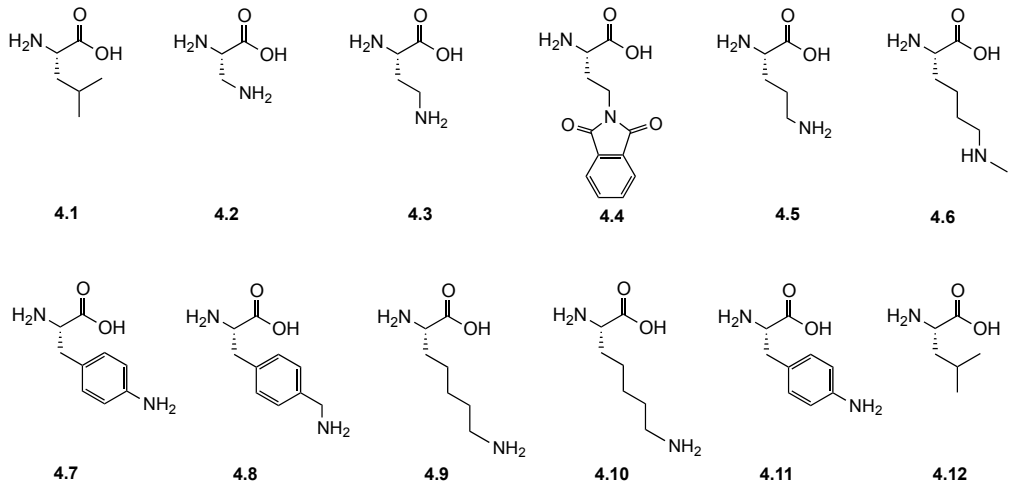


Figure 4.4. Amino Acids Used for Modification of Lys72 of Rat DNA Polymerase Beta.[176]

It was concluded from the study of dRP excision activity of the modified proteins that nature has already optimized the required nucleophile for the excision reaction. The modified protein obtained by site-directed mutagenesis of Lys 72 with N^{ε}-methyllysine (**4.6**) retained 86% of the activity of wild type (Figure 4.5), and is thought to stabilize the Schiff base compared to wild type making the adjacent C-2 proton of the sugar less acidic (Figure 4.2). The lower activity of modified protein obtained by site-directed mutagenesis of Lys 72 with analogue **4.9** implied that the distance between the N^{ε} and α-C of amino acid is critical as it helps in proper positioning of the nucleophile to attack the dRP DNA bound in the catalytic pocket.[176]

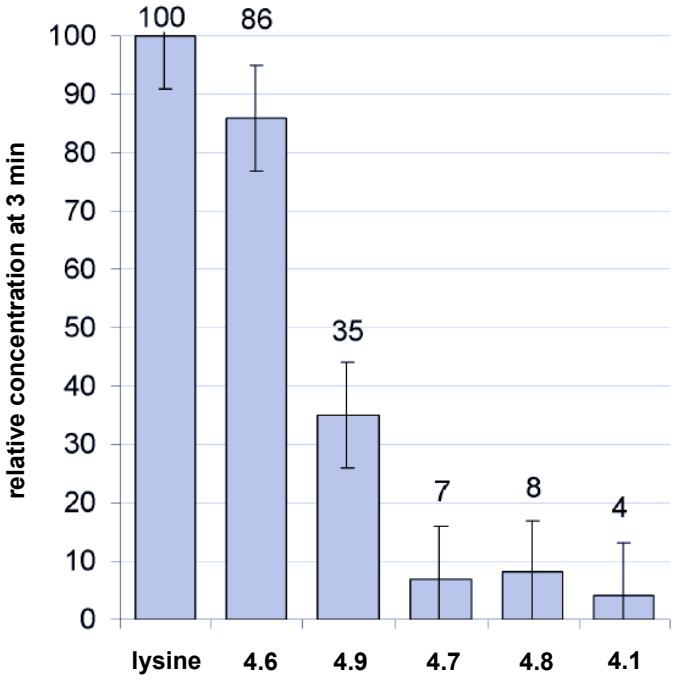


Figure 4.5. Summary of dRP Excision Activity of Rat Polymerases Beta Modified at Position 72 With Lysine Analogues.[176]

As the rat and human polymerase beta have 95.5% sequence homology (Figure 4.6), the conclusions drawn from the modification of rat polymerase beta can presumably

be reasonably extended to human polymerase beta. Hence, the ideal amino acid required to substitute at position 72 should be an amino acid with nucleophile at the ε–position of the side chain and will be able to stabilize the formed Schiff base without making the adjacent C-2 proton more acidic. Thus, in the quest for exploring a better nucleophile for DNA excision of human polymerase beta, a series of unnatural aminoacyl–pdCpA derivatives were designed and synthesized for site-specific incorporation in human DNA polymerase beta (Figure 4.7).

```
1   MSKRKAPQET LNGGITDMLV ELANFEKNVS QAIHKYNAYR KAASVIAKYP HKIKSGAEAK
61  KLPGVGTKIA EKIDEFLATG KLRKLEKIRQ DDTSSSINFL TRVTGIGPSA ARKLVDEGIK
121 TLEDLRKNED KLNHHQRIGL KYFEDFEKRI PREEMLQMQD IVLNEVKKLD PEYIATVCGS
181 FRRGAESSGD MDVLLTHPNF TSESSKQPKL LHRVVEQLQK VRFITDTRSK GETKFMGVCQ
241 LPSENDENEY PHRRIDIRLI PKDQYYCGVL YFTGSDIFNK NMRAHALEKG FTINEYTIRP
301 LGVTGVAGEP LPVDSEQDIF DYIQWRYREP KDRSE
```

Figure 4.6 Amino Acid Sequence of Rat DNA Polymerase Beta. (The modification at 15 positions of rat enzyme sequence will result into the human enzyme sequence; V20T, T104S, L114F, E144G, L169V, P171S, N119S, S205T, R113Q, R222H, R228L, E244K, N248K, Q317K, and R326K).

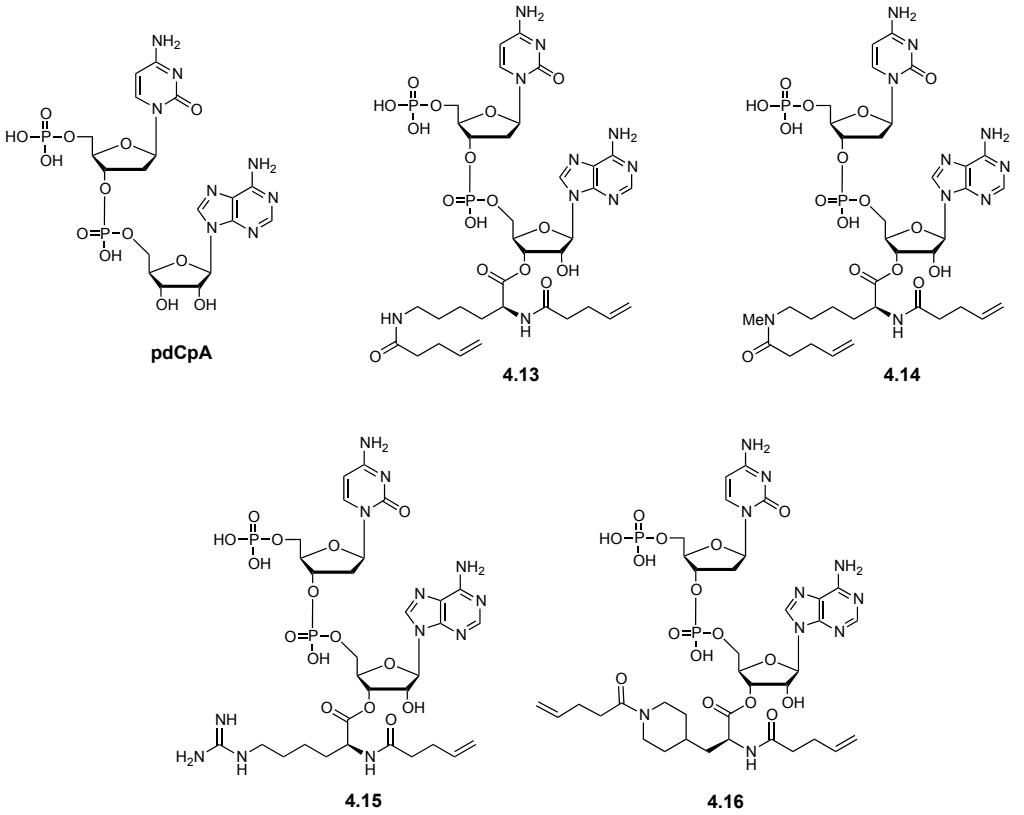
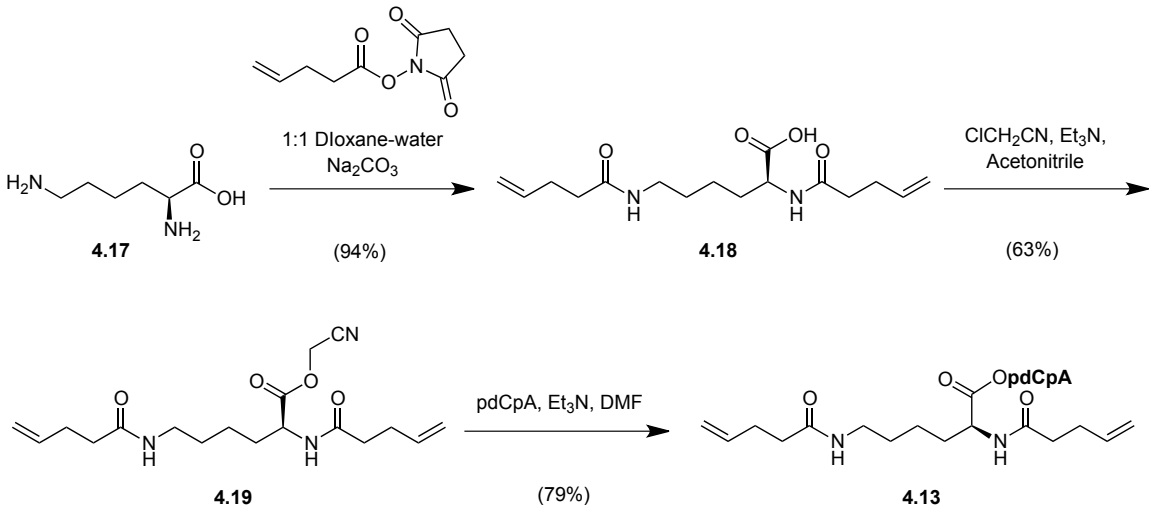


Figure 4.7. Series of Aminoacylated pdCpA Derivatives Synthesized for Site Directed Incorporation at Position 72 of Human DNA Polymerase Beta.

4.2. Results

In recent years, misacylated tRNAs have been used extensively to facilitate the introduction of unnatural amino acids into predetermined positions in proteins of interest.[88,95,96,177-179] The same strategy was utilized for the incorporation of unnatural lysine analogues into position 72 of human DNA polymerase beta. In order to synthesize aminoacylated tRNA, the pdCpA derivatives of lysine and lysine analogues were generated from their respective amino acids. The key intermediate cyanomethyl ester of the *N*-protected amino acid was coupled with pdCpA to give a mixture of 2′ and 3′ dinucleotide esters. Pentenoyl protected lysine **4.18** was prepared from lysine (**4.17**) by

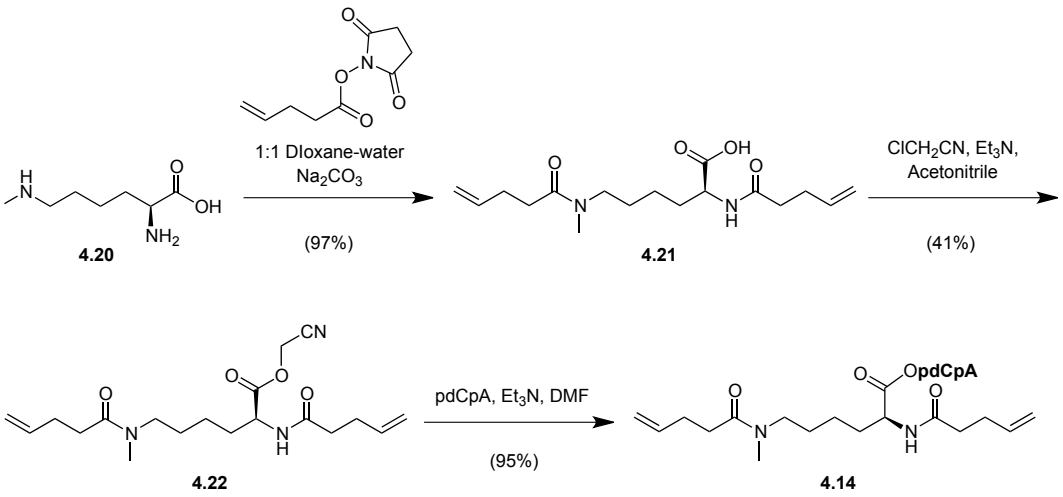
treatment with 4-pentenoic acid succinimide ester in the presence of Na_2CO_3 in 94% yield (Scheme 4.1).[180] The pentenoyl protection was chosen as it can be readily deprotected by treatment with iodine without any undesirable side reaction at the tRNA level.[181] Subsequently, dipentenoyl amide **4.18** was activated as cyanomethyl ester[180] **4.19** by treatment with chloroacetonitrile in 63% yield. The treatment of cyanomethyl ester **4.19** by treatment with tris(tetrabutylammonium) salt of pdCpA[97] in anh DMF afforded the lysine–pdCpA ester **4.13** in 79% yield (Scheme 4.1).



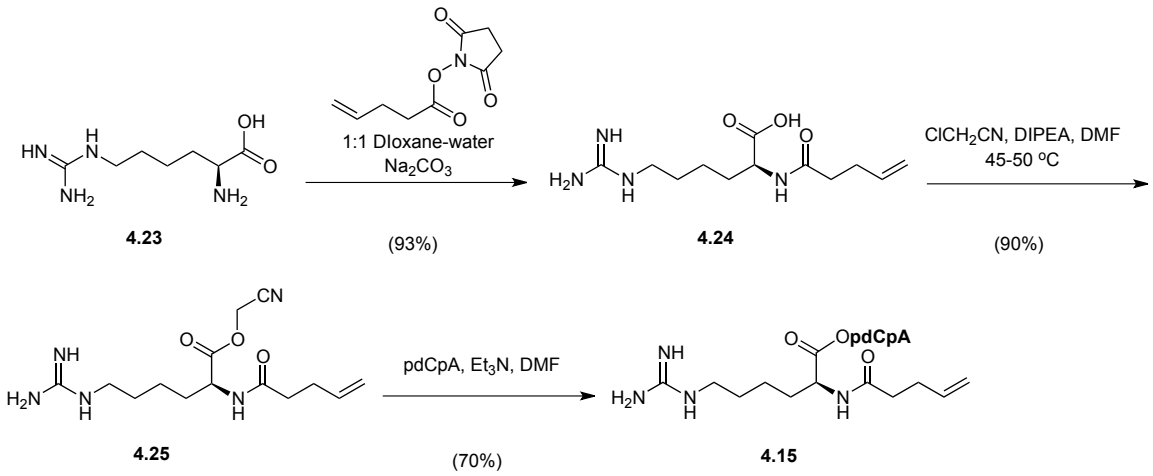
Scheme 4.1. Synthesis of Lysyl–pdCpA **4.13**.

The syntheses of the pdCpA ester of pentenoyl protected *N*-methyllysine **4.14** and pentenoyl protected homoarginine **4.15** were also carried out in similar fashion from *N*-methyllysine (**4.20**) and homoarginine (**4.23**), respectively (Schemes 4.2 and 4.3). The free amine of *N*-methyllysine (**4.20**) was protected using 4-pentenoic acid succinimide ester in the presence of Na_2CO_3 affording dipentenoyl amide **4.21** in 97% yield, the latter of which was activation as cyanomethyl ester[180] **4.22** by treatment with chloroacetonitrile in 41% yield (Scheme 4.2). Cyanomethyl ester **4.22** was then coupled with

tris(tetrabutylammonium) salt of pdCpA[97] in anh DMF to afford the *N*-methyllysine–pdCpA ester **4.14** in 95% yield. Similarly, homoarginine (**4.23**) was converted into *N*-pentenoyl homoarginine (**4.24**) in 98% yield, followed by activation as the respective cyanomethyl ester **4.25** in 90% yield (Scheme 4.3). The pdCpA derivative of homoarginine **4.15** was prepared from cyanomethyl ester **4.25** in 70% yield.[182]

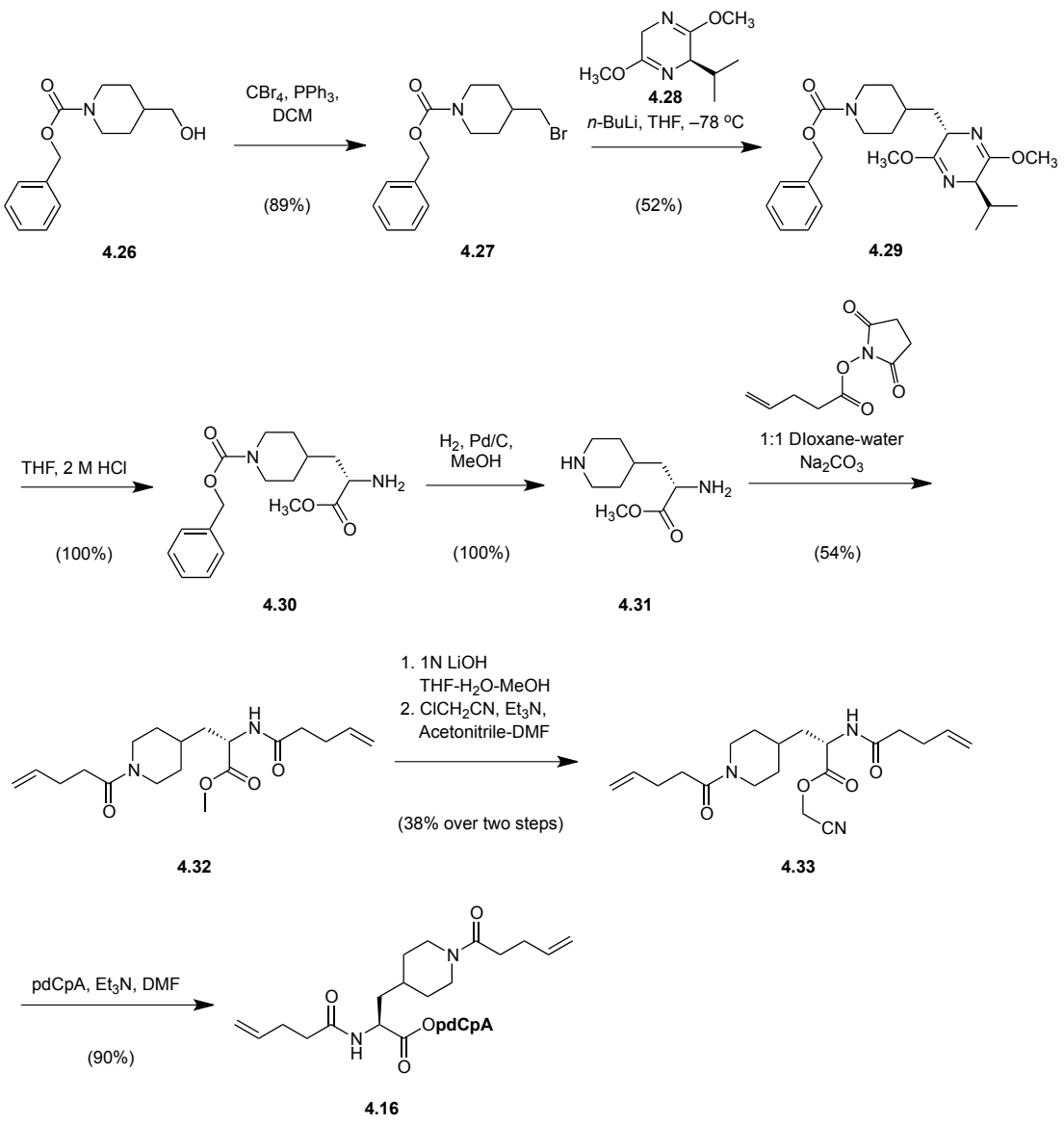


Scheme 4.2. Synthesis of *N*-methyllysyl–pdCpA **4.14**.



Scheme 4.3. Synthesis of Homoargininyl–pdCpA **4.15**.

The synthesis of pdCpA derivative of piperidylalanine commenced with the asymmetric synthesis of the amino acid precursor. To realize the asymmetric synthesis of piperidylalanine, a stereoselective strategy was employed using the Schöllkopf chiral reagent,[183,184] which enabled the successful synthesis of a single unnatural S-isomer of the amino acid. The synthesis began with bromination of CBz protected hydroxymethylpiperidine (**4.26**) to give bromide **4.27** in 89% yield (Scheme 4.4).[185] Regioselective lithiation of Schöllkopf chiral auxiliary (**4.28**) with *n*-BuLi followed by treatment with bromide **4.27** at −78 °C afforded the adduct **4.29** as a single diastereomer in 52% yield. Mild hydrolysis with 2 M HCl[184] afforded the α-substituted amino acid methyl ester **4.30** in quantitative yield. The CBz protecting group was removed from of the piperidyl side chain by hydrogenation over Pd/C to afford piperidylalanine **4.31** quantitatively. The methyl ester of piperidylalanine (**4.31**) was protected as the dipentenoyl amide **4.32** in 54% yield over three steps. *N*-protected methyl ester **4.32** was subsequently hydrolyzed to afford the free acid, the latter of which was treated with chloroacetonitrile to afford the desired cyanomethyl ester[180] **4.33** in 38% yield over two steps. Treatment of cyanomethyl ester **4.33** with the tris(tetrabutylammonium) salt of pdCpA[97] in anh DMF afforded pdCpA ester **4.16** in 90% yield.



Scheme 4.4. Synthesis of Piperidylalanyl–pdCpA **4.16**.

The strategy employed for incorporation of lysine analogues into human DNA polymerase beta is illustrated in Figure 4.8. The suppressor tRNA-C_{OH}, lacking the terminal cytidine and adenosine moieties at the 3′ end, was successfully transcribed from *FokI*-digested pYRNA8 plasmid DNA and was ligated with the pentenoyl protected aminoacylated pdCpA derivatives **4.13**-**4.16** via a T4 RNA ligase.[97] The ligation

efficiencies were evaluated via denaturing acidic PAGE analysis as shown in Figure 4.9.[186] The *N*-pentenoyl protected misacylated tRNAs were deprotected by treatment with aqueous iodine to afford the activated tRNAs having lysine analogues with free α-amines as described previously.[181,187]

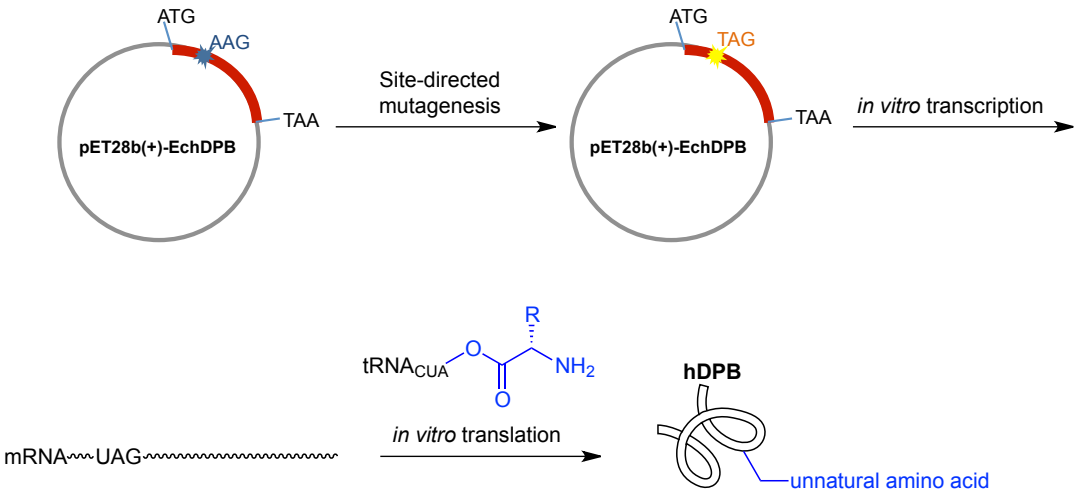


Figure 4.8. Strategy Employed for Incorporation of Lysine Analogues Into Position 72 of Human DNA Polymerase Beta (EchDPB), the Gene for Which had Been Optimized for Expression in *E. coli*.

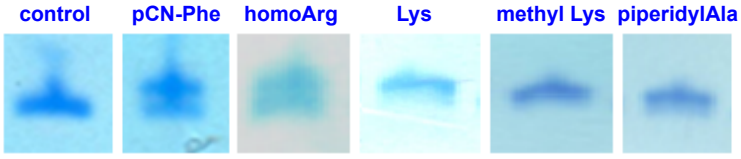


Figure 4.9. Monitoring the Ligation Between the Suppressor tRNA-C_{OH} and Aminoacylated pdCpA Derivatives **4.13-4.16** After Pentenoyl Deprotection by Acidic Polyacrylamide Gel Electrophoresis and Methylene Blue Staining. (The experiment was performed by Dr. Sasha Daskalova).

The AAG lysine codon at position 72 was replaced with amber (TAG) stop codon and *E. coli* codon-optimized human DNA polymerase beta (hDPB) gene was generated. The misacylated tRNA derivatives were incorporated at position 72 of *E. coli* codon-

optimized hDPB. The incorporation of the various lysine analogues was evaluated via denaturing PAGE analysis.[185] *In vitro* translation of modified protein with homoarginine at position 72 from *E. coli* codon-optimized template gave 69.2% incorporation relative to *in vitro* translation of wild type protein from *E. coli* codon-optimized (EchDPB) template, summarized in Figure 4.10(I). Homoarginine (69.2%) and methyl lysine (21.3%) were incorporated in much better yields than lysine (16%), while piperidylalanine incorporation was only 5.8% compared to homoarginine incorporation, illustrated in Figure 4.10(II). This work was performed by Dr. Sasha Daskalova.

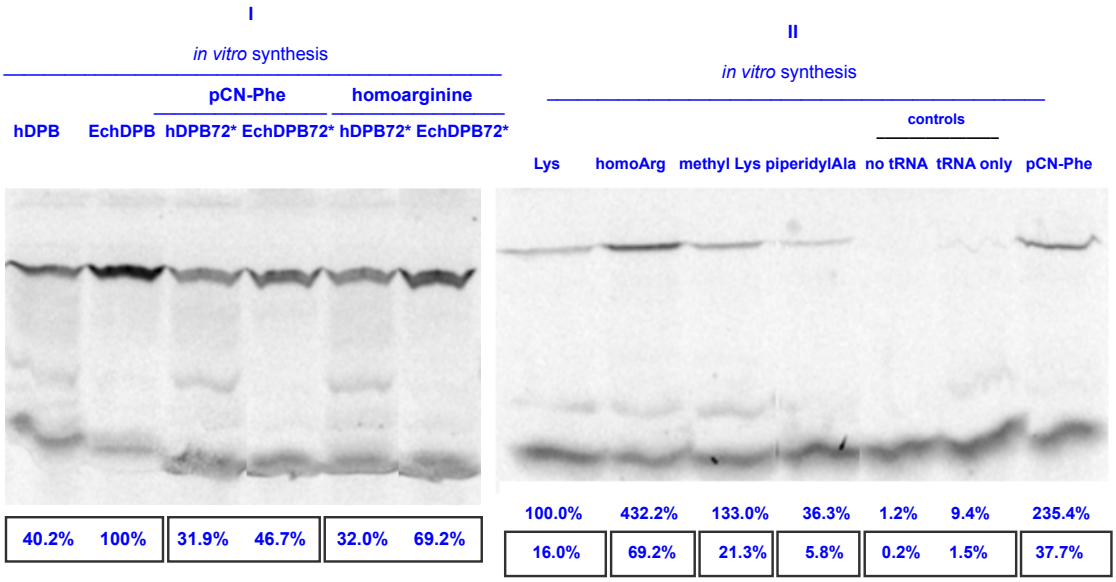


Fig. 4.10. (I) *In Vitro* Translation of Wild Type Protein From *E. coli* Codon-optimized (EchDPB) and Non-optimized (hDPB) Template and *In Vitro* Translation of Modified Proteins With Incorporated *p*CN-Phe or Homoarginine at Position 72 From *E. coli* Codon-optimized Template or Non-optimized template Having an Amber Stop Codon at Position 72 (EchDPB72* and hDPB72*, respectively; monitored by SDS-PAGE and autoradiography); (II) *In vitro* Translation of Modified Proteins With Incorporated Lysine or Lysine Analogues at Position 72 From *E. coli* Codon-optimized Template Having an Amber Stop Codon at Position 72 (monitored by SDS-PAGE and autoradiography; relative incorporation compared to lysine incorporation (100%) is shown on the first row, relative yield compared to the yield of *in vitro* synthesized wild type hDPB from *E. coli* codon-optimized template is shown on the second row; experiment was performed by Dr. Sasha Daskalova).

4.3. Discussion

In the pursuit of a better understanding of the mechanism involved in the excision of dRP by lyase domain of human DNA polymerase beta, a series of modified proteins were synthesized from their respective misacylated tRNAs. Early studies demonstrated that the suppressor tRNA-C_{OH}, lacking the terminal cytidine and adenosine moieties at the 3′-end, could be ligated with the pentenoyl protected amino acid to form the misacylated tRNA.[94-97] A similar strategy was utilized in case of the protein of interest. The pdCpA derivatives **4.13**–**4.15** were prepared by first protecting the free amine as the 4-pentenoyl derivative, followed by activation of the acid as the cyanomethyl ester and coupling with the tris(tetrabutylammonium) salt of dinucleotide pdCpA.[97,180,181] However, the synthesis of pdCpA derivative of piperidylalanine **4.16** was very challenging and started with the asymmetric synthesis of the piperidylalanine. Several attempts were made to alkylate the lithiated Schöllkopf chiral auxiliary with CBz protected chloromethylpiperidine but none of them resulted in successful asymmetric synthesis. Finally, CBz protected bromomethylpiperidine (**4.27**) was synthesized from CBz protected hydroxymethylpiperidine (**4.26**)[184] and coupled with the lithiated Schöllkopf chiral auxiliary affording the adduct[182,183] **4.29** as a single enantiomer. Mild acid hydrolysis followed by hydrogenation with Pd/C afforded piperidylalanine **4.31**, the latter of which was protected as the dipentenoyl methyl ester **4.32**. Subsequently, the ester hydrolysis of the methyl ester **4.32** gave the free acid, the latter of which was treated with chloroacetonitrile to afford the activated cyanomethyl ester **4.33**. The obtained cyanomethyl ester **4.33** was unstable, such that several purification attempts failed.

Finally, the partially purified cyanomethyl ester **4.33**, obtained by extractive work-up, was used to ligate the dinucleotide pdCpA to afford the pdCpA derivatives **4.16** after HPLC purification.

Misacylated tRNAs were generated by ligating abbreviated tRNA, lacking the terminal cytidine and adenosine moieties at the 3′-end, with the pdCpA derivatives **4.13**–**4.16** using T4 tRNA ligase.[97] The ligase is an ATP dependent enzyme that catalyzes 3′→5′ phosphodiester bond formation in RNA. Treatment with aqueous iodine then afforded the tRNAs activated with unprotected amino acids, the latter of which were added to an *in vitro* transcription/translation system. The system contained the modified plasmid having an amber (TAG) stop codon at position 72 of the hDPB gene. The *in vitro* translation gave the modified proteins with unnatural amino acid residues at position 72 of hDPB.

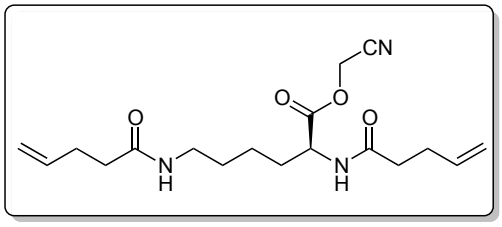
4.4. Experimental Procedures

Materials. The chemicals used were purchased from Aldrich Chemical Co., Sigma Chemical Co. or Combi-Blocks and were used without further purification. Anhydrous methanol, DMF and acetonitrile were used as purchased. Tetrahydrofuran and dichloromethane were distilled from sodium/benzophenone and calcium hydride, respectively. The tris-(tetrabutylammonium) salt of pdCpA was prepared by passing pdCpA through the activated TBA form of Dowex 50W×8 (200-400 mesh).

General Experimental Procedures. Reactions were carried out under an argon atmosphere in flame-dried glassware. Flash column chromatography was carried out

using silica gel (Silicycle R10030B, 60 particle size, 230-400 mesh), applying a low-pressure stream of nitrogen or dry air. Analytical thin layer chromatographic separations were carried out on glass plates coated with silica gel (60 particle size F254, Silicycle TLG-R10011B-323) and were developed by immersing the plates in a solution of potassium permanganate stain or ceric ammonium molybdate (CAM) stain followed by heating with a heat gun.

Instrumentation. HPLC was performed using a Waters 600 pump coupled with a Varian ProStar 340 detector. ^{1}H and ^{13}C NMR spectra were recorded on Varian Inova 400 MHz and 100 MHz, respectively, using $CDCl_3$ as solvent and internal standard. ^{1}H NMR and ^{13}C NMR chemical shifts were reported relative to a residual $CHCl_3$ at 7.26 ppm and 77.16 ppm, respectively. Splitting patterns are designated as s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet; q, quartet and quint, quintet. High resolution mass spectrometric data were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State Mass Spectrometry Facility.

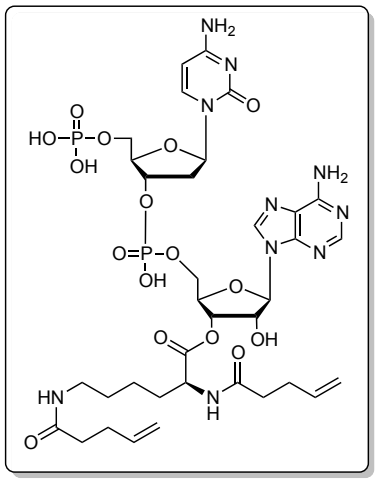


***N,N*-Dipentenoyl-L-lysine Cyanomethyl Ester (4.19).** To 150 mg (1.03 mmol) of lysine **4.17** were added 435 mg (4.10 mmol) of Na_2CO_3 in 5 mL water and 486 mg (2.46 mmol) of 4-pentenoic acid succinimidyl ester in 5 mL of 1,4-dioxane. The heterogeneous reaction mixture was stirred at room temperature overnight. The mixture was diluted with

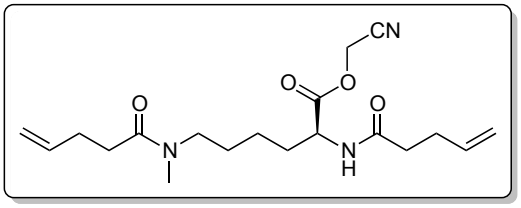
5 mL of water, acidified to pH 2-3 with 1 N $NaHSO_4$ and extracted with three 30-mL portions of ethyl acetate. The organic layer was washed with 30 mL of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The crude residue was quickly passed through a silica gel column (18 × 2 cm). Elution with 15:1→10:1 chloroform–methanol afforded *N,N*-dipentenoyl-L-lysine (**4.18**) as a colorless oil: yield 300 mg (94%); silica gel TLC R_f 0.13 (10:1 chloroform–methanol); mass spectrum (MALDI-TOF), *m/z* 311.37 $(M + H)^+$, 333.38 $(M + Na)^+$. This material was used directly in the next step without further purification.

To 300 mg (0.32 mmol) of dipentenoyl lysine (**4.18**) in 15 mL of dry acetonitrile was added 0.67 mL (489 mg, 4.83 mmol) of dry triethylamine followed by 0.18 mL (219 mg, 2.90 mmol) of chloroacetonitrile. The reaction mixture was stirred under nitrogen at room temperature for 18 h. The reaction mixture was diluted with 30 mL of ethyl acetate, washed successively with 10 mL of 1 N $NaHSO_4$ and 10 mL of brine. The solution was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (18 × 2 cm). Elution with 1:1→1:3 hexanes–ethyl acetate afforded cyanomethyl ester **4.19** as a colorless oil: yield 212 mg (63%); silica gel TLC R_f 0.16 (1:3 hexanes–ethyl acetate); ^1H NMR ($CDCl_3$, 400 MHz) δ 1.34 (m, 2H), 1.47 (m, 2H), 1.79 (m, 2H), 2.23 (m, 2H), 2.29-2.35 (m, 6H), 3.20 (m, 2H), 4.46 (m, 1H), 4.74 (ABq, 2H, *J* = 16.0 Hz), 4.97 (d, 2H, *J* = 12.0 Hz), 5.00 (m, 1H), 5.05 (m, 1H), 5.78 (m, 2H), 6.10 (t, 1H, *J* = 5.6 Hz) and 6.89 (d, 1H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 22.1, 29.1, 29.4, 29.6, 30.5, 35.1, 35.8, 38.2, 48.9,

51.9, 114.3, 115.57, 115.61, 136.9, 137.0, 171.1, 173.06 and 173.13; mass spectrum (MALDI-TOF), *m/z* 350.37 $(M + H)^+$, 372.38 $(M + Na)^+$.



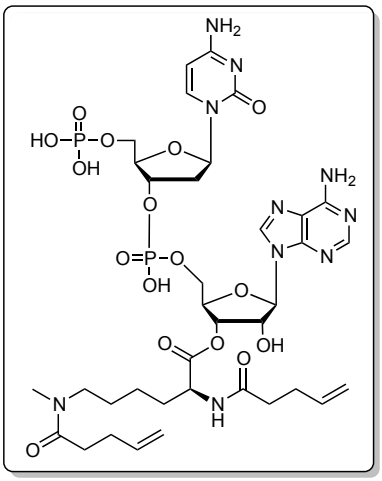
***N,N*-Dipentenoyl-L-lysine pdCpA Ester (4.13).** To 7.40 mg (5.40 μmol) of the tris-(tetrabutylammonium) salt of pdCpA was added a solution of 9.50 mg (27.2 μmol) of cyanomethyl ester **4.19** in 150 μL of anh DMF followed by 15.0 μL of triethylamine. The reaction mixture was sonicated at room temperature for 24 h and was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 5 μm) using acetonitrile and 50 mM aq NH_4OAc (pH 4.5) mobile phases. A linear gradient was employed of (1:99 acetonitrile–50 mM aq NH_4OAc buffer→65:35 acetonitrile–50 mM aq NH_4OAc buffer) over a period of 45 min at a flow rate of 3.5 mL/min. The fractions containing the desired product eluted at 15.6 min (monitoring at 260 nm) and were collected, frozen and lyophilized to give lysine–pdCpA ester **4.13** as a colorless solid: yield 4.00 mg (79%); mass spectrum (MALDI-TOF), *m/z* 929.35 $(M + H)^+$; mass spectrum (ESI), *m/z* 927.2798 $(M - H)^-$ ($C_{35}H_{49}N_{10}O_{16}P_2$ requires *m/z* 927.2803).



***N,N*-Dipentenoyl-L-*N*-methyllysine Cyanomethyl Ester (4.22).** To 130 mg (0.66 mmol) of methyllysine (**4.20**) were added 420 mg (3.97 mmol) of Na_2CO_3 in 5 mL water and 365 mg (1.85 mmol) 4-pentenoic acid succinimidyl ester in 5 mL of 1,4-dioxane. The heterogeneous reaction mixture was stirred at room temperature overnight. The mixture was diluted with 5 mL of water, acidified to pH 2-3 with 1 N $NaHSO_4$ and extracted with three 30-mL portions of ethyl acetate. The organic layer was washed with 30 mL of brine, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The crude residue was quickly passed through silica gel column (18 × 2 cm). Elution with 15:1→10:1 chloroform–methanol afforded dipentenoyl methyllysine (**4.21**) as a colorless solid: yield 208 mg (94%); silica gel TLC R_f 0.4 (10:1 chloroform–methanol); mass spectrum (APCI), *m/z* 325.2130 $(M + H)^+$ ($C_{17}H_{29}N_2O_4$ requires *m/z* 325.2127). This material was used directly in the next step without further purification.

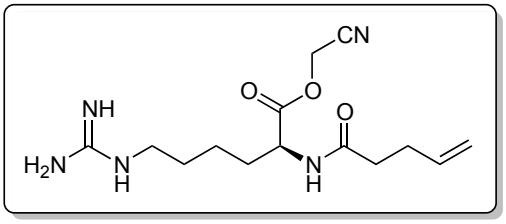
To 208 mg (0.64 mmol) of dipentenoyl methyllysine (**4.21**) in 15 mL of dry acetonitrile was added 0.45 mL (324 mg, 3.21 mmol) of dry triethylamine followed by 0.18 mL (145 mg, 1.92 mmol) of chloroacetonitrile. The reaction mixture was stirred under nitrogen at room temperature for 18 h. The reaction mixture was diluted with 30 mL of ethyl acetate, washed successively with 10 mL of 1 N $NaHSO_4$ and 10 mL of brine. The solution was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The crude residue was purified by flash chromatography on a silica gel column

(18 × 2 cm). Elution with 1:1→1:3 hexanes–ethyl acetate afforded cyanomethyl ester **4.22** as a colorless oil: yield 45.0 mg (41%); silica gel TLC R_f 0.16 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.31 (m, 2H), 1.55 (m, 2H), 1.87 (m, 2H), 2.34-2.45 (m, 8H), 2.99 (s, 3H), 3.36 (m, 1H), 3.44 (m, 1H), 4.44 (ABq, 1H, J = 5.6 Hz), 4.76 (ABq, 2H, J = 15.6 Hz), 4.99-5.11 (m, 4H), 5.84 (m, 2H) and 6.98 (d, 1H, J = 6.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 26.3, 28.6, 29.2, 29.5, 29.9, 33.0, 35.2, 35.4, 46.1, 48.9, 52.3, 114.3, 115.5, 137.1, 137.3, 171.1, 173.3 and 173.4; mass spectrum (APCI), *m/z* 364.2233 $(M + H)^+$ ($C_{19}H_{30}N_3O_4$ requires *m/z* 364.2236).



***N,N*-Dipentenoyl-L-*N*-methyllysine pdCpA Ester (4.14).** To 9.20 mg (6.76 μmol) of the tris-(tetrabutylammonium) salt of pdCpA was added a solution of 12.3 mg (33.8 μmol) of cyanomethyl ester **4.22** in 150 μL of anh DMF followed by 15.0 μL of triethylamine. The reaction mixture was sonicated at room temperature for 18 h and was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 5 μm) using acetonitrile and 50 mM aq NH_4OAc (pH 4.5) mobile phases. A linear gradient was employed of (1:99 acetonitrile–50 mM aq NH_4OAc buffer→65:35 acetonitrile–50 mM aq

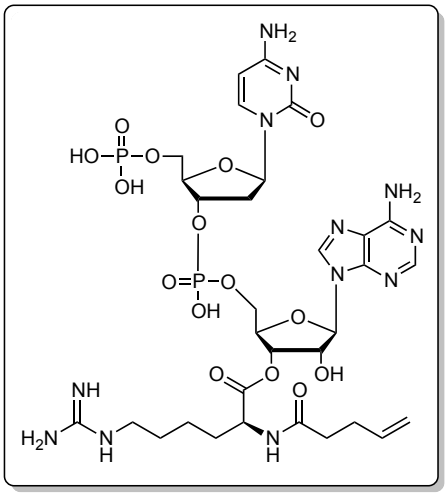
NH_4OAc buffer) over a period of 45 min at a flow rate of 3.5 mL/min. The fractions containing the desired product eluted at 16.5 min (monitoring at 260 nm) and were collected, frozen and lyophilized to give *N*-methyllysine–pdCpA ester **4.14** as a colorless solid: yield 6.00 mg (94%); mass spectrum (MALDI-TOF), *m/z* 943.50 $(M + H)^+$; mass spectrum (ESI), *m/z* 941.2940 $(M - H)^-$ ($C_{36}H_{51}N_{10}O_{16}P_2$ requires *m/z* 941.2960).



***N*-(4-Pentenoyl)-L-homoarginine Cyanomethyl Ester (4.25).**[182] To 150 mg (0.80 mmol) of homoarginine (**4.23**) were added 127 mg (1.20 mmol) of Na_2CO_3 in 5 mL water and 189 mg (0.96 mmol) 4-pentenoic acid succinimidyl ester in 5 mL of 1,4-dioxane. The heterogeneous reaction mixture was stirred at room temperature overnight. The mixture was diluted with 5 mL of water, acidified to pH 4 with 1 N $NaHSO_4$ and extracted with three 20-mL portions of ethyl acetate. The aqueous part was concentrated under diminished pressure, dissolved in methanol and the white precipitate was filtered off. The filtrate was concentrated to afford pentenoyl homoarginine (**4.24**) as a colorless oil: yield 211 mg (98%); silica gel TLC R_f 0.45 (3:1 ethyl acetate–MeOH); mass spectrum (MALDI-TOF), *m/z* 271.35 $(M + H)^+$. This material was used directly in the next step without further purification.

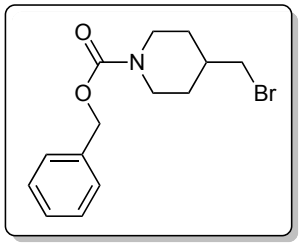
To 210 mg (0.78 mmol) of pentenoyl homoarginine (**4.24**) in 6 mL of dry DMF was added 0.54 mL (489 mg, 3.11 mmol) of dry *N*,*N*-diisopropylethylamine followed by 0.18 mL (219 mg, 2.90 mmol) of chloroacetonitrile. The reaction mixture was heated at

45-50 °C under nitrogen for 48 h. The reaction mixture was concentrated under diminished pressure and purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 3:1→1:1 ethyl acetate–methanol afforded cyanomethyl ester **4.25** as a colorless oil: yield 212 mg (88%); silica gel TLC R_f 0.58 (1:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.30-2.37 (m, 13H), 4.20 (m, 2H), 4.68 (m, 2H) and 5.74 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.4, 19.5, 24.2, 29.5, 30.9, 31.7, 36.0, 44.0, 50.3, 53.6, 56.0, 116.1 and 138.4.

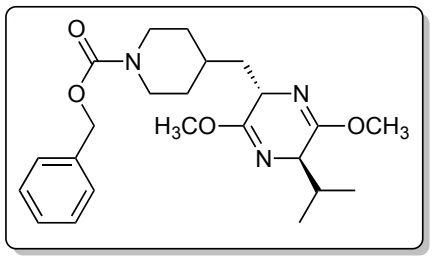


***N*-(4-Pentenoyl)-L-homoarginine pdCpA Ester (4.15).**[182] To 8.70 mg (5.4 μmol) of the tris-(tetrabutylammonium) salt of pdCpA was added a solution of 10.0 mg (27.2 μmol) of cyanomethyl ester **4.25** in 150 μL of anh DMF followed by 15.0 μL of dry triethylamine. The reaction mixture was sonicated at room temperature for 48 h and was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 5 μm) using acetonitrile and 50 mM aq NH_4OAc (pH 4.5) mobile phases. A linear gradient was employed of (1:99 acetonitrile–50 mM aq NH_4OAc buffer→65:35 acetonitrile–50 mM aq NH_4OAc buffer)

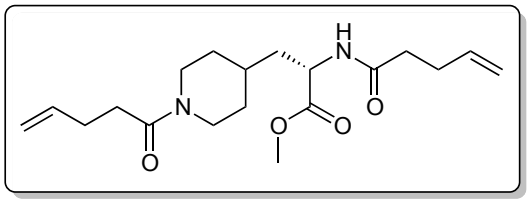
over a period of 45 min at a flow rate of 3.5 mL/min. The fractions containing the desired product eluted at 15.4 min (monitoring at 260 nm) and were collected, frozen and lyophilized to give homoarginine–pdCpA ester **4.15** as a colorless solid: yield 4.00 mg (70%); mass spectrum (MALDI-TOF), *m/z* 889.42 $(M + H)^+$.



Benzyl 4-(Bromomethyl)piperidine-1-carboxylate (4.27).[185] To a stirred solution of 600 mg (2.41 mmol) of alcohol **4.26** in 6.2 mL of anh dichloromethane were added 960 mg (2.89 mmol) of carbontetrabromide and 758 mg (2.89 mmol) of triphenylphosphine. The reaction mixture was stirred at room temperature for 5 h. The reaction mixture was concentrated under diminished pressure and purified by flash chromatography on a silica gel column (18 × 3.5 cm). Elution with 6:1→4:1 hexanes–ethyl acetate afforded bromide **4.27** as a colorless oil: yield 670 mg (89%); silica gel TLC R_f 0.4 (3:1 hexanes–ethyl acetate). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.22 (m, 2H), 1.81 (m, 3H), 2.77 (m, 2H), 3.28 (d, 2H, J = 6.0 Hz), 4.22 (m, 2H), 5.13 (s, 2H) and 7.29-7.38 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 30.8, 38.5, 38.7, 43.8, 67.1, 127.9, 128.0, 128.6, 136.9 and 155.2.



Benzyl 4-(((2*S*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)piperidine-1-carboxylate (4.29). To a stirred solution containing 90.0 μL (92.4 mg, 0.50 mmol) of Schöllkopf's reagent (**4.28**) in 4.1 mL of anhydrous THF at −78 °C was added 0.25 mL (0.63 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at −78 °C for 30 min under argon and then a solution containing 130 mg (0.42 mmol) of bromide **4.27** in 4.1 mL of anh THF was added. The reaction mixture was left to warm slowly to room temperature and stirred for 1 h under argon. The reaction mixture was diluted with 25 mL of saturated aq NH_4Cl, extracted with two 30-mL portions of ethyl acetate, dried ($MgSO_4$), filtered and concentrated under diminished pressure. The crude residue was purified by flash chromatography on a silica gel column (18 × 2 cm). Elution with 8:1→5:1→4:1 hexanes–ethyl acetate afforded adduct **4.29** as a colorless oil: yield 91.0 mg (52%); silica gel TLC R_f 0.4 (3:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.69 (d, 3H, J = 6.8 Hz), 1.05 (d, 3H, J = 6.8 Hz), 1.18 (m, 2H), 1.50 (t, 1H, J = 8.4 Hz), 1.64 (d, 1H, J = 13.2 Hz), 1.76 (m, 3H), 2.27 (m, 1H), 2.78 (m, 2H), 3.67 (s, 3H), 3.68 (s, 3H), 3.93 (t, 1H, J = 3.6 Hz), 4.01 (m, 1H), 4.13 (br s, 2H), 5.12 (s, 2H) and 7.28-7.37 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.6, 19.0, 31.7, 32.2, 32.5, 40.9, 44.15, 44.21, 52.4, 53.3, 60.6, 66.9, 127.8, 127.9, 128.4, 137.0, 155.3, 163.3 and 164.3; mass spectrum (MALDI-TOF), *m/z* 416.48 $(M + H)^+$; mass spectrum (APCI), *m/z* 416.2542 $(M + H)^+$ ($C_{23}H_{34}N_3O_4$ requires *m/z* 416.2549).

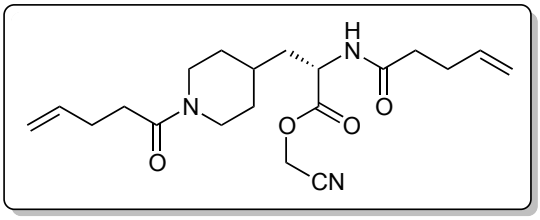


***N,N*-Dipentenoyl-L-piperidylalanine methyl Ester (4.32).** To a stirred solution containing 231 mg (0.56 mmol) of adduct **4.29** in 12 mL of THF at 0 °C was added 8.4 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then slowly poured into 50 mL of saturated aq $NaHCO_3$, extracted with two 50-mL portions of ethyl acetate, dried ($MgSO_4$) and filtered. The filtrate was concentrated under diminished pressure to afford protected amino acid **4.30** as a colorless oil: yield 180 mg (100%); silica gel TLC R_f 0.32 (1:1 ethyl acetate–methanol); mass spectrum (APCI), *m/z* 321.1809 $(M + H)^+$ ($C_{17}H_{25}N_2O_4$ requires *m/z* 321.1814). This material was used directly in the next step without further purification.

To the solution of 180 mg (0.56 mmol) of protected amino acid **4.30** in anh methanol was added catalytic amount of Pd/C and H_2 gas was bubbled through for 45 min. The reaction mixture was stirred at room temperature overnight. After the complete consumption of starting material, the reaction mixture was filtered through Celite 545® and concentrated under diminished pressure to afford L-piperidylalanine methyl ester (**4.31**) as a colorless oil: yield 105 mg (100%); silica gel TLC R_f 0.37 (1:4 ethyl acetate–methanol); mass spectrum (APCI), *m/z* 187.1448 $(M + H)^+$ ($C_9H_{19}N_2O_2$ requires *m/z* 187.1447). This material was used directly in the next step without further purification.

To 105 mg (0.56 mmol) of L-piperidylalanine methyl ester (**4.31**) were added 358 mg (3.38 mmol) of Na_2CO_3 in 7.5 mL water and 311 mg (1.58 mmol) 4-pentenoic acid succinimidyl ester in 7.5 mL of 1,4-dioxane. The heterogeneous reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with 10 mL of brine and extracted with three 30-mL of portions of ethyl acetate. The organic layer was

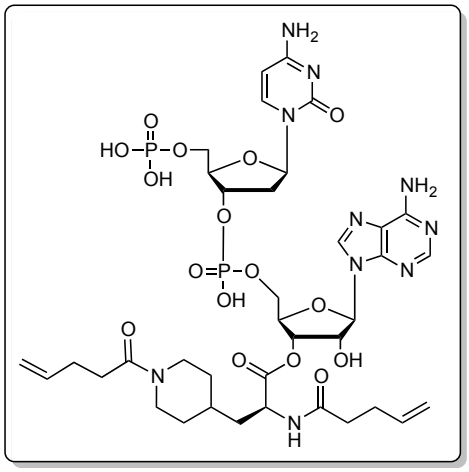
dried ($MgSO_4$), filtered and concentrated under diminished pressure. The crude residue was purified by flash chromatography on a silica gel column (18 × 2.5 cm). Elution with 1:1→1:3 hexanes–ethyl acetate afforded methyl ester **4.32** as a colorless oil: yield 106 mg (54% over three steps); silica gel TLC R_f 0.18 (1:3 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.10 (m, 2H), 1.55 (m, 2H), 1.71 (m, 2H), 1.88 (m, 1H), 2.30-2.44 (m, 8H), 2.51 (m, 1H), 2.96 (m, 1H), 3.73 (s, 3H), 3.83 (d, 1H, J = 12.8 Hz), 4.56 (d, 1H, J = 12.0 Hz), 4.68 (m, 1H), 5.02 (m, 4H), 5.82 (m, 2H) and 6.12 (d, 1H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 25.5, 29.50, 29.54, 32.7, 32.8, 35.7, 39.6, 42.2, 46.0, 49.7, 52.7, 77.4, 115.5, 116.0, 136.9, 137.4, 172.1 and 173.4; mass spectrum (MALDI-TOF), *m/z* 351.32 $(M + H)^+$; mass spectrum (APCI), *m/z* 351.2292 $(M + H)^+$ ($C_{19}H_{31}N_2O_4$ requires *m/z* 351.2284).



***N,N*-Dipentenoyl-L-piperidylalanine Cyanomethyl Ester (4.33).** To a stirred solution containing 74.0 mg (0.21 mmol) of methyl ester **4.32** in 3.8 mL of 1:1:3 THF–water–methanol was added 0.42 mL (0.42 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with acidified to pH 2-3 with 1 N $NaHSO_4$ and extracted with three 15-mL portions of ethyl acetate. The organic layer was washed with 10 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure to gave dipentenoyl piperidylalanine as a colorless oil: yield 42.0 mg (59%); silica gel TLC R_f 0.4 (10:1 chloroform–methanol); mass spectrum (APCI), *m/z*

337.2124 $(M + H)^+$ ($C_{18}H_{29}N_2O_4$ requires *m/z* 337.2127). This material was used directly in the next step without further purification.

To 22.0 mg (0.06 mmol) of dipentenoyl piperidylalanine in 1.8 mL of dry acetonitrile was added 46.0 μL (33.3 mg, 0.33 mmol) of dry triethylamine followed by 13.0 μL (15.1 mg, 0.20 mmol) of chloroacetonitrile. The reaction mixture was stirred under nitrogen at room temperature for 18 h. The reaction mixture was diluted with 10 mL of ethyl acetate, washed with 10 mL of brine, dried ($MgSO_4$) and filtered. The solution was concentrated under diminished pressure to afford cyanomethyl ester **4.33** as a colorless oil: yield 12.0 mg (49%); silica gel TLC R_f 0.21 (1:3 hexanes–ethyl acetate); mass spectrum (MALDI-TOF), *m/z* 376.38 $(M + H)^+$; mass spectrum (APCI), *m/z* 376.2240 $(M + H)^+$ ($C_{20}H_{30}N_3O_4$ requires *m/z* 376.2236). The compound was used directly in the next step without further purification due to unstable nature.



***N,N*-Dipentenoyl-L-piperidylalanine pdCpA Ester (4.16).** To 7.90 mg (5.81 μmol) of the tris-(tetrabutylammonium) salt of pdCpA was added a solution of 11.0 mg (29.0 μmol) of cyanomethyl ester **4.33** in 150 μL of anh DMF followed by 15.0 μL of

triethylamine. The reaction mixture was sonicated at room temperature for 9 h and was purified on an Econosil C_{18} reversed phase semi-preparative (250 × 10 mm, 5 μm) using acetonitrile and 50 mM aq NH_4OAc (pH 4.5) mobile phases. A linear gradient was employed of (1:99 acetonitrile–50 mM aq NH_4OAc buffer→65:35 acetonitrile–50 mM aq NH_4OAc buffer) over a period of 45 min at a flow rate of 3.5 mL/min. The fractions containing the desired product eluted at 17.7 min (monitoring at 260 nm) and were collected, frozen and lyophilized to give piperidylalanine–pdCpA ester **4.16** as a colorless solid: yield 5.00 mg (91%); mass spectrum (MALDI-TOF), *m/z* 955.65 $(M + H)^+$; mass spectrum (ESI), *m/z* 953.2948 $(M - H)^-$ ($C_{37}H_{51}N_{10}O_{16}P_2$ requires *m/z* 953.2960).

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