

Probing Receptors and Enzymes with Synthetic Small Molecules

by

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## ABSTRACT

Manipulation of biological targets using synthetic or naturally occurring organic compounds has been the focal point of medicinal chemistry. The work described herein centers on the synthesis of organic small molecules that are targeted either to cell surface receptors, to the ribosomal catalytic center or to human immunodeficiency virus reverse transcriptase.

Bleomycins (BLMs) are a family of naturally occurring glycopeptidic antitumor agents with an inherent selectivity towards cancer cells. DeglycoBLM, which lacks the sugar moiety of bleomycin, has much lower cytotoxicity in cellular assays. A recent study using microbubble conjugates of BLM and deglycoBLM showed that BLM was able to selectively bind to breast cancer cells, whereas the deglyco analogue was unable to target either the cancer or normal cells. This prompted us to further investigate the role of the carbohydrate moiety in bleomycin. Fluorescent conjugates of BLM, deglycoBLM and the BLM carbohydrate were studied for their ability to target cancer cells. Work presented here describes the synthesis of the fluorescent carbohydrate conjugate. Cell culture assays showed that the sugar moiety was able to selectively target various cancer cells. A second conjugate was prepared to study the importance of the C-3 carbamoyl group present on the mannose residue of the carbohydrate. Three additional fluorescent probes were prepared to improve the uptake of this carbohydrate moiety into cancer cells. Encouraged by the results from the fluorescence experiments, the sugar moiety was conjugated to a cytotoxic molecule to selectively deliver this drug into cancer cells.

The nonsense codon suppression technique has enabled researchers to site specifically incorporate noncanonical amino acids into proteins. The amino acids successfully incorporated this way are mostly  $\alpha$ -L-amino acids. The non- $\alpha$ -L-amino acids are not utilized as substrates by ribosome catalytic center. Hoping that mutations near the ribosome peptidyltransferase site might alleviate its bias towards  $\alpha$ -L-amino acids, a library of modified ribosomes was generated. Analogues of the naturally occurring antibiotic puromycin were used to select promising candidates that would allow incorporation of non- $\alpha$ -L-amino acids into proteins. Syntheses of three different puromycin analogues are described here.

The reverse transcriptase enzyme from HIV-1 (HIV-1 RT) has been a popular target of HIV therapeutic agents due to its crucial role in viral replication. The 4-chlorophenyl hydrazone of mesoxalic acid (CPHM) was identified in a screen designed to find inhibitors of strand transfer reactions catalyzed by HIV-1 RT. Our collaborators designed several analogues of CPHM with different substituents on the aromatic ring using molecular docking simulations. Work presented here describes the synthesis of eight different analogues of CPHM.

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## LIST OF ABBREVIATIONS

ACN	acetonitrile
APA	4-amino-4-deoxy-10- <i>N</i> -methylpropanoic acid
aq	aqueous
BLM	bleomycin
Bn	benzyl
Boc	<i>t</i> -butoxycarbonyl
Boc <sub>2</sub> O	<i>t</i> -butoxycarbonyl anhydride
°C	degrees Celsius
cat	catalytic
cm	centimeter
δ	chemical shift (ppm)
d	doublet
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCC	<i>N,N'</i> -dicyclohexylurea
dec	decomposition
dd	double doublet
ddd	doublet of doublet of doublet
DIPEA	diisopropylethylamine
DMAP	dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid

EDTA	ethylenedinitrilotetraacetic acid
ESI	electrospray ionization
EtOAc	ethyl acetate
Fmoc	<i>N</i> -(9-fluorenylmethoxy-carbonyloxy)
FmocOSu	<i>N</i> -(9-fluorenylmethoxy-carbonyloxy)succinimide
g	grams
h	hours
<sup>1</sup> HNMR	proton nuclear magnetic resonance spectroscopy
HATU	2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	<i>O</i> -benzotriazole- <i>N,N,N',N'</i> -tetramethyl-uronium-hexafluoro- phosphate
HPLC	high pressure liquid chromatography
Hz	Hertz
<i>J</i>	coupling constant
L	liter
M	molar
m	multiplet
MALDI-TOF	matrix assisted laser desorption ionization - time of flight
MBL	mannose-binding lectin
mCPBA	metachloroperbenzoic acid
MHz	mega Hertz
min	minutes



mL	milliliter
mM	millimolar
mmol	millimole(s)
μmol	micromole(s)
NHS	<i>N</i> -hydroxysuccinimide
nm	nanometer
NMR	nuclear magnetic resonance
proton sponge	1,8-Bis(dimethylamino)naphthalene
PTC	peptidyl transferase center
PANS	puromycin aminonucleoside
$R_f$	ratio of fronts
RNA	ribonucleic acid
s	singlet
satd	saturated
soln	solution
t	triplet
TBA	tetrabutylammonium
TCEP	tris(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer Chromatography
TMSOTf	trimethylsilyl triflate
TSTU	<i>O</i> -( <i>N</i> -succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate

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## CHAPTER 1

### INTRODUCTION

#### 1.1. CARBOHYDRATES

Carbohydrates are a class of biopolymers that play an essential role in a range of crucial biological processes. They can be classified into four categories, namely monosaccharides, disaccharides, oligosaccharides and polysaccharides. Saccharides usually occur in nature as glycoconjugates like glycoproteins, proteoglycans, glycolipids, etc. Detailed determination of the functional aspects of these saccharides can be difficult for a number of reasons including the lack of facile carbohydrate sequencing techniques and efficient amplification methods.

The glycoconjugates that are present on cell surfaces are believed to participate in extracellular communication.<sup>1</sup> Glycolipids on the cell surface are composed of a lipid membrane anchor that has a hydrophilic saccharide at the extracellular end.<sup>2</sup> A significant number of proteins are modified post-translationally through glycosylation.<sup>3</sup> Glycoproteins usually have oligosaccharides comprising less than 15-20 monosaccharides. Proteoglycans, on the other hand, carry large polysaccharides (more than 100 monomers).<sup>4</sup> In both cases, the sugar moiety is usually presented outside of the cell while the protein spans the membrane.<sup>5</sup> Interactions of these surface carbohydrates with extracellular carbohydrates or receptor proteins are implicated in immune recognition, cell adhesion, cell migration, cellular recognition, and signaling events.<sup>6</sup> Many diseases such as bacterial and viral infection, inflammation and

cancer have been associated with an abnormal expression of cell surface carbohydrates.<sup>7</sup>

Due to their crucial role in a range of biological processes, considerable effort has been dedicated to understanding protein–carbohydrate interactions.<sup>8</sup> Proteins that function by binding to carbohydrates can be broadly classified into three categories such as enzymes (glycosidases and glycosyltransferases), carbohydrate binding antibodies and lectins. Lectins recognize specific carbohydrate ligands to mediate biological phenomena like cell attachment, migration and invasion.<sup>9</sup> Along with other receptors, lectins are an integral part of the innate immune system.<sup>10</sup> Lectins utilize specific sugar motifs displayed by microorganisms to identify the pathogens and trigger an immune response.<sup>11</sup> The carbohydrate ligands they interact with can be polysaccharides, oligosaccharides or even simple monosaccharides.<sup>12</sup> For example, mannose-binding lectin (MBL) plays a crucial role in immune response by binding to specific oligosaccharides and monosaccharides present on the cell surface of many bacteria.<sup>13</sup>

Frequently, these proteins bind to multiple carbohydrate ligands simultaneously to achieve the desired affinity and specificity. Mechanistically, there are two pathways that can lead to enhancement of binding affinity due to polyvalency.<sup>14</sup> If the protein has multiple binding sites, a multivalent ligand can potentially act as a chelating agent and give rise to stronger binding compared to a monomeric ligand. The structure of the spacer that carries all the ligands can be very important for efficient chelation. Another mechanism is called statistical rebinding.<sup>15</sup> A multivalent ligand can increase the effective concentration of the

monovalent ligand near the binding site. This causes a decrease in the off-rate of protein–ligand interaction. Plant lectins such as concanavalin A (con A) and wheat germ agglutinin (WGA) are examples of carbohydrate binding proteins that bind to multiple mannose and GlcNAc residues, respectively.<sup>16</sup> The cholera toxin is a member of the AB<sub>5</sub> family of toxins that binds to multiple sugars simultaneously.<sup>17</sup> Bacterial adhesion lectins such as FimH (from *E. coli*), LecB (from *P. aeruginosa*) also involve in polyvalent interactions with host cell-carbohydrates.<sup>18</sup> Many multivalent carbohydrate-based ligands have been developed targeting these proteins for diagnostic or therapeutic purposes. Popular scaffolds used to produce multivalent systems have included cyclodextrins, calixerenes and dendritic spacers.<sup>19</sup> More recently, gold nanoparticles, quantum dots and carbon nanotubes have been prepared that were equipped with several carbohydrates.<sup>20</sup>

Protein–carbohydrate interactions play an important role during cellular transformations. The state of glycosylation of glycoproteins and glycolipids on the cell-surface is regulated by glycosyltransferases and glycosidases. The level of expression of these enzymes can change dramatically depending on cell metabolism, activation and malignant transformation.<sup>21</sup> Tumor cells express an altered distribution and composition of glycan epitopes (called tumor antigens) on their cell surface.<sup>22</sup> This helps cancer cells escape recognition by immune cells during tumor development and metastasis.<sup>23</sup> Glycoproteins such as carcinoembryonic antigen (CEA) and MUC1 have been used as biomarkers for certain types of cancer.<sup>24</sup> Cancer cells also produce unusual levels of several

carbohydrate binding proteins such as galectins and selectins. These lectins have been implicated in cancer progression, metastasis, apoptotic resistance and angiogenesis.<sup>9</sup>

Altered expression of cell surface glycans on tumor cells is a biomarker for disease diagnosis. Plant lectins that recognize cancer-specific glycan changes in tumor biopsies have been utilized to assess cancer prognoses and metastasis.<sup>25</sup> The lectins *Helix pomatia* agglutinin, *Ulex europaeus* agglutinin I and leucocyte-agglutinating phytohaemagglutinin have been used for the diagnosis of breast and colorectal cancers, respectively.<sup>26</sup> On the other hand, carbohydrate based ligands have been developed to inhibit carbohydrate-binding proteins that are involved in tumor growth, metastasis and angiogenesis.<sup>9</sup>

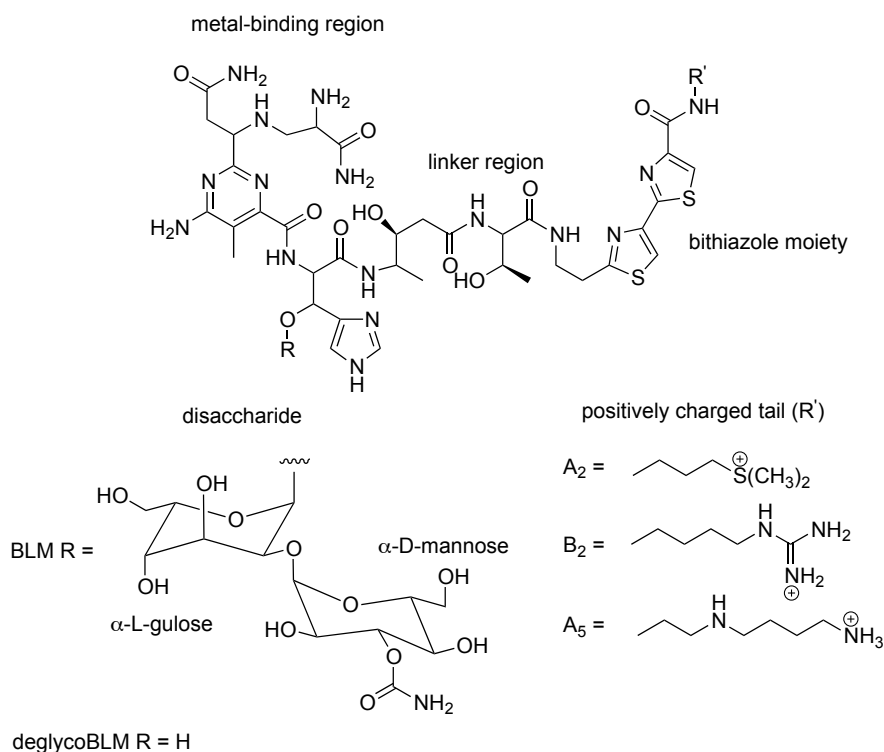
## 1.2. BLEOMYCINS

There are several anti-cancer drugs that have a sugar moiety attached to the cytotoxic core.<sup>27</sup> It has been shown that the presence of sugar moieties causes a significant increase in cytotoxicity.<sup>28</sup> DNA binding molecules such as epirubicin, esperamicin A<sub>1</sub>, and chromomycin A<sub>3</sub> belong to this class.<sup>27</sup>

Bleomycins are a family of glycopeptidic antitumor agents that are used clinically in combination chemotherapy against malignant lymphomas and squamous cell carcinomas. Umezawa and coworkers first isolated bleomycin from *Streptomyces verticillus* in 1966.<sup>29</sup> Since its discovery bleomycin (BLM) quickly gained attention because of its ability to selectively target various cancer cells.<sup>30</sup> As shown in Figure 1.1, the BLMs have four distinct structural domains, including an



N-terminal metal binding domain, linker domain, C-terminal bithiazole domain and the carbohydrate domain, the last consisting of D-mannose and L-gulose. The clinically used bleomycin mixture, called blenoxane has BLM A<sub>2</sub> and BLM B<sub>2</sub> as its major constituents.<sup>31</sup> BLM targets 5'-GC-3' and 5'-GT-3' sequences in DNA and causes oxidative DNA damage. *In vitro* experiments have shown it can also damage RNA and RNA–DNA heteroduplexes.<sup>32</sup>



**Figure 1.1.** Structural domains of bleomycin.<sup>33</sup>

### 1.3. INCORPORATION OF UNNATURAL AMINO ACIDS INTO PROTEINS

Techniques that allow the manipulation of protein structure are invaluable for understanding the correlation between protein structure and function, and for

generating proteins with novel properties. Site directed mutagenesis of DNA has been used to replace a specific residue of a protein with another desired amino acid. Recently site-specific incorporation of noncanonical amino acids has drawn significant attention because of its potential applications in studies of protein stability and folding, protein–ligand and protein–protein interactions. This strategy enables researchers to introduce a range of new functionalities other than those found in 20 proteinogenic amino acids.

The translation of a protein requires the sequential condensation of specific amino acids according to the nucleotide sequence of the respective mRNA. There are 64 codons and 61 of them code for the natural amino acids. These leave three that are called nonsense codons or stop codons. The amino acids are assigned to a particular codon by the use of tRNAs which have an anticodon loop. The enzymes that activate a particular tRNA with its cognate amino acid are called aminoacyl-tRNA synthetases (aa-RSs).<sup>34</sup> Although the genetic code is nearly universal, aa-RSs from one species generally do not readily aminoacylate tRNAs from other species.<sup>35</sup> The process of peptide bond formation is catalyzed by the ribosome and includes four steps: initiation, elongation, termination and recycling. Ribosome, mRNA, initiator tRNA, initiation factors and guanosine triphosphate form the initiation complex. Then the next aminoacyl-tRNA (aa-tRNA) is introduced into the ribosome catalytic center with the help of elongation factors. The new aminoacyl-tRNA pairs with the next codon in the mRNA and the amino acid from the initiator tRNA is transferred to it at the peptidyltransferase center. Then the next tRNA is introduced and the elongation

cycle is repeated until the ribosome encounters one of the three stop codons. The stop codon causes release of the peptide from the last aminoacyl-tRNA and the ribosomal apparatus with the help of release factors. Finally, the ribosome is recycled for another round of protein production.

Incorporation of unnatural amino acids has been attempted using both chemical and biosynthetic methods. Initial efforts involved chemical modification of certain amino acids within a biochemically expressed protein. This method often lacked the desired specificity and selectivity.<sup>36</sup> Solid phase peptide synthesis, in combination with native chemical ligation, can be used to incorporate almost any noncanonical amino acid. This strategy, however, is limited to smaller proteins (~200 residues) and restricted by the requirement for amino acid residues that can participate in the native ligation.<sup>37</sup> On the other hand, first of the biosynthetic strategies involved growing bacteria in the presence of noncanonical  $\alpha$ -amino acids. The unnatural amino acids incorporated were close structural analogues of the natural ones that the bacteria were unable to synthesize.<sup>38</sup>

Incorporation of unnatural amino acids into proteins at predetermined positions was achieved by the use of suppressor tRNA technology.<sup>39</sup> At first, a stop codon such as TAG (the amber stop codon) was introduced at the desired position of the gene of interest. Then a suppressor tRNA, having CUA as the anticodon, was misacylated with the amino acid of interest.<sup>40</sup> The misacylated suppressor tRNA recognized the stop codon in the mRNA and mediated the incorporation of the amino acid using an *in vitro* protein synthesizing system.

Both chemical and biosynthetic approaches have been utilized for the misacylation of tRNA.<sup>41</sup>

Hecht and coworkers first achieved tRNA chemical misacylation by ligating the aminoacylated dinucleotide pCpA to a tRNA lacking the last two nucleotides at its 3' end, using T4 RNA ligase.<sup>42</sup> Schultz and colleagues later used aminoacylated pdCpA, containing a deoxycytidine, to overcome the synthetic difficulties for aminoacylation of pCpA.<sup>43</sup> Aminoacyl-pdCpAs are now used routinely to produce tRNAs misacylated with unnatural amino acids. As the chemically misacylated tRNAs cannot be regenerated in the cell, a stoichiometric amount is consumed during protein synthesis. An alternative strategy for misacylating tRNAs involves the evolution of an orthogonal tRNA:aa-RS pair. Pairs from different organisms have been used for this purpose.<sup>44</sup> In addition, directed evolution has allowed the isolation of specific tRNA:aa-RS pairs selected for aminoacylation of various noncanonical amino acids.<sup>44</sup>

#### **1.4. ANTI-HIV THERAPIES**

Human immunodeficiency virus (HIV) induces acquired immune deficiency syndrome (AIDS) in humans by gradually damaging the host immune system.<sup>45</sup> Since its discovery, substantial effort has been made to develop anti-HIV therapies. The drugs currently approved by the Food and Drug Administration (FDA) for anti-HIV treatment can be classified into six categories including non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs), fusion

inhibitors (FIs), CCR5 antagonists, and integrase inhibitors (INIs). Drugs belonging to each class block a crucial step of the viral life cycle.<sup>46</sup>

The life cycle of HIV starts by its recognition of the CD4 receptors on the host cell surface.<sup>47</sup> Following interaction with the chemokine co-receptor CXCR4 or CCR5 the viral envelope is fused with the cell membrane, causing the release of the viral RNA into the cytoplasm of the host cell. The enzyme reverse transcriptase then synthesizes double stranded viral DNA from the viral RNA templates. The double stranded DNA is incorporated into the host genome with the help of integrase. The cellular transcription and translation machinery then produces viral proteins and viral RNA. Finally the virion is assembled and it buds off from the host cell as a Gag-Pol polyprotein. This polyprotein is subsequently cleaved by viral protease to generate mature virion particles.

The nucleoside reverse transcriptase inhibitors (NRTIs) are nucleoside analogues that lack 2' and 3' OH groups. Inside the cell, these compounds are converted to their triphosphate forms and participate in the reverse transcription process, resulting in chain termination.<sup>48</sup> The first of these three phosphorylations is believed to be the slowest. NRTIs already equipped with a phosphoramidate group are being developed as pro-drugs to bypass the first phosphorylation step.<sup>49</sup> The incorporated NRTIs can also be cleaved from the 3'-end of the growing DNA in the drug resistant HIV strains. To reduce the occurrence of drug resistance, phosphonate groups have been attached at the 5'-end of the nucleoside inhibitors. Once incorporated, phosphonate esters are harder to repair as compared to

phosphodiester linkages.<sup>50</sup> At present there are seven NRTIs approved by the FDA for clinical use.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind to an allosteric pocket of HIV RT near its polymerase active site.<sup>51</sup> Binding of these inhibitors causes conformational changes in the architecture of the active site, resulting in diminished polymerase activity. Rapid emergence of drug resistance has plagued the clinical use of NNRTIs targeted against HIV.<sup>52</sup> Various new drugs belonging to this class are being developed for use against the drug-resistant strains. Five FDA-approved NNRTIs are used currently for the treatment of HIV-infected patients.

The FDA has approved as many as eight protease inhibitors (PIs) for clinical use against HIV. During replication, the viral proteins are initially expressed as polymeric precursors gag and gag-pol. HIV protease then cleaves the polyproteins to form mature virions.<sup>53</sup> This enzyme, having two aspartic acid residues at the catalytic center, behaves like an aspartic acid protease.<sup>54</sup> Most of the protease inhibitors are non-hydrolyzable transition state analogues of the peptide hydrolysis reaction. Appearance of drug resistance and significant toxicity has limited the use of PIs. New inhibitors are being designed to overcome these problems.

During the anchoring of the virion on the host cell surface, glycoprotein gp120 interacts with host cell surface receptors and releases the fusion peptide gp41. This peptide catalyzes the fusion of the host cell membrane and virion envelope.<sup>55</sup> The fusion inhibitors (FIs) mimic the gp41 peptide and block the

fusion process. Currently enfuvirtide is the only FDA-approved FI.<sup>56</sup> The cost of production and emergence of resistance are the major obstacles for new fusion inhibitors to overcome in clinical trials.

The cellular targeting process of HIV commences with the interaction between CD4 receptors on the cell surface and gp120 viral protein. This binding causes conformational changes in gp120 exposing a new site for interaction with cellular chemokine co-receptors such as CXCR4 and CCR5.<sup>57</sup> Antagonists of these co-receptors can be used to block the HIV infection process. Maraviroc, a CCR5 antagonist, is the only drug approved for clinical use from this class.

Integrase inhibitors (INIs) block the process of integration of proviral DNA into the host cell chromosome. This step is catalyzed by the viral enzyme integrase. Integrase is also responsible for the processing of the newly reverse-transcribed viral cDNA.<sup>58</sup> There is only one integrase inhibitor, raltegravir, which is currently approved by the FDA.

Most of these drugs are used in combinations. Although these drugs have significantly improved the state of morbidity and mortality in HIV-infected patients, rapid emergence of drug resistance and cytotoxicities have been their dose limiting factors. The discovery of new candidates with low toxicity, high bioavailability and new modes of action are always desirable.

## CHAPTER 2

### SYNTHESIS OF BLEOMYCIN-DISACCHARIDE CONJUGATES FOR SELECTIVE TARGETING OF CANCER CELL LINES

#### 2.1. INTRODUCTION

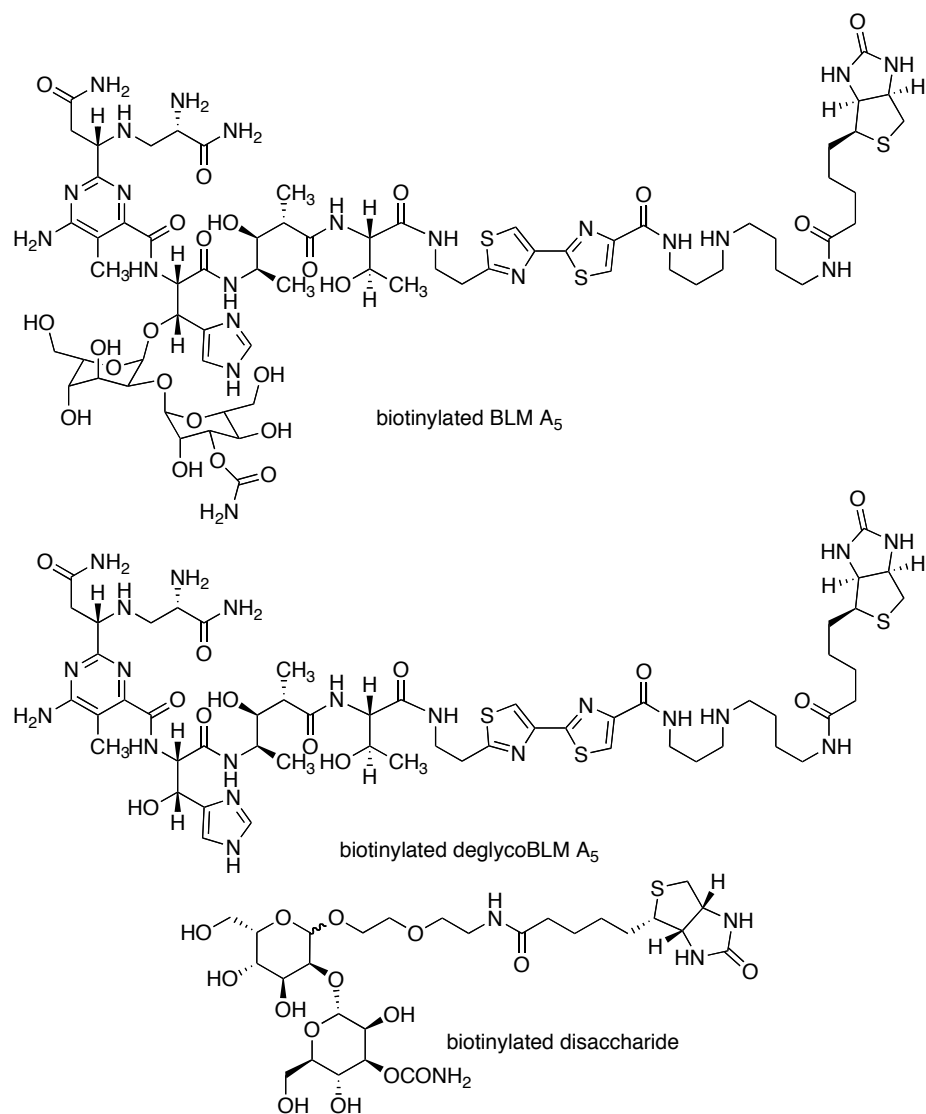
The exact roles of the carbohydrate moiety in the cytotoxic activity of bleomycins (BLMs) still require definition. Reports in the literature indicate stronger cytotoxicity of bleomycin relative to deglycoBLM in cellular and *in vivo* assays.<sup>59</sup> The carbamoyl group present on the D-mannose of the disaccharide has been implicated as the sixth metal binding site of BLM.<sup>60</sup> However, this hypothesis cannot explain the fact that in cell free systems deglycoBLM retains significant DNA cleaving activity. In addition, in order to induce DNA damage, the metal ion chelated by BLM binds molecular oxygen, which necessitates a free binding site on the metal ion.<sup>61</sup> DeglycoBLM analogues have even been utilized previously as synthetically expedient models to determine the DNA damage profiles of the corresponding BLM analogues.<sup>62</sup>

Due to their inherent selectivity towards tumor cells,<sup>63</sup> BLM-metal complexes such as <sup>57</sup>Co-BLM and <sup>111</sup>In-BLM have been used as *in vivo* radioactive tumor-imaging agents.<sup>64</sup> The Co-BLM complex, however, was not usable clinically due to the extended half-life (270 days) of <sup>57</sup>Co and the rapid excretion rate of the complex. <sup>111</sup>In-BLM complex was too weak to be useful *in vivo*.<sup>64</sup> A new imaging agent, BLEDTA, was developed in which an ethylenedinitrilotetraacetic acid (EDTA) moiety was attached to the C-terminal end of BLM A<sub>2</sub>. <sup>111</sup>In-BLEDTA successfully imaged various carcinomas in



patients. Notably,  $^{111}\text{In}$ -deglycoBLEDTA was ineffective in similar imaging experiments.<sup>65</sup> This result hints at the carbohydrate domain being involved in the cellular recognition and uptake of BLM.

The above results prompted our lab to further investigate the role played by the carbohydrate domain in the tumor cell recognition process. A detailed understanding of tumor cell recognition and uptake might lead to identification of particular cell-surface receptors and the participating structural domains in bleomycin. The structural domain(s) of BLM directly responsible for tumor targeting could be further exploited to develop selective tumor imaging agents/targeted drug delivery vehicles. The tumor targeting ability of BLM and deglycoBLM were studied through imaging experiments involving cells treated with microbubbles conjugated to bleomycins.<sup>66</sup> Originally used as contrasting agents in ultrasonography, microbubbles are lipid membranes encapsulating a gaseous core containing some inert gas such as air, nitrogen, or a perfluorocarbon.<sup>67</sup> The microbubble surface was modified with streptavidin. BLM A<sub>5</sub>, deglycoBLM A<sub>5</sub> and BLM-disaccharide were conjugated to biotin (Figure 2.1) and were conjugated to the microbubbles through streptavidin–biotin binding. The targeted microbubbles were then incubated with cultured MCF-7 breast carcinoma cells and MCF-10A non-cancerous breast cells and visualized through a Zeiss Axiovert 200M microscope. BLM and BLM-disaccharide-conjugated microbubbles adhered selectively to the cancer cells but not to the “normal” cells.<sup>66</sup>



**Figure 2.1.** Biotin conjugates of bleomycin A<sub>5</sub>, deglycobleomycin A<sub>5</sub> and BLM-disaccharide.

DeglycoBLM conjugated microbubbles, on the other hand, did not adhere either to the cancer cells or the normal cells under the same incubation conditions. The inability of deglycoBLM to target tumor cells suggested the importance of the sugar moiety in cancer cell surface recognition. The ability of the BLM-

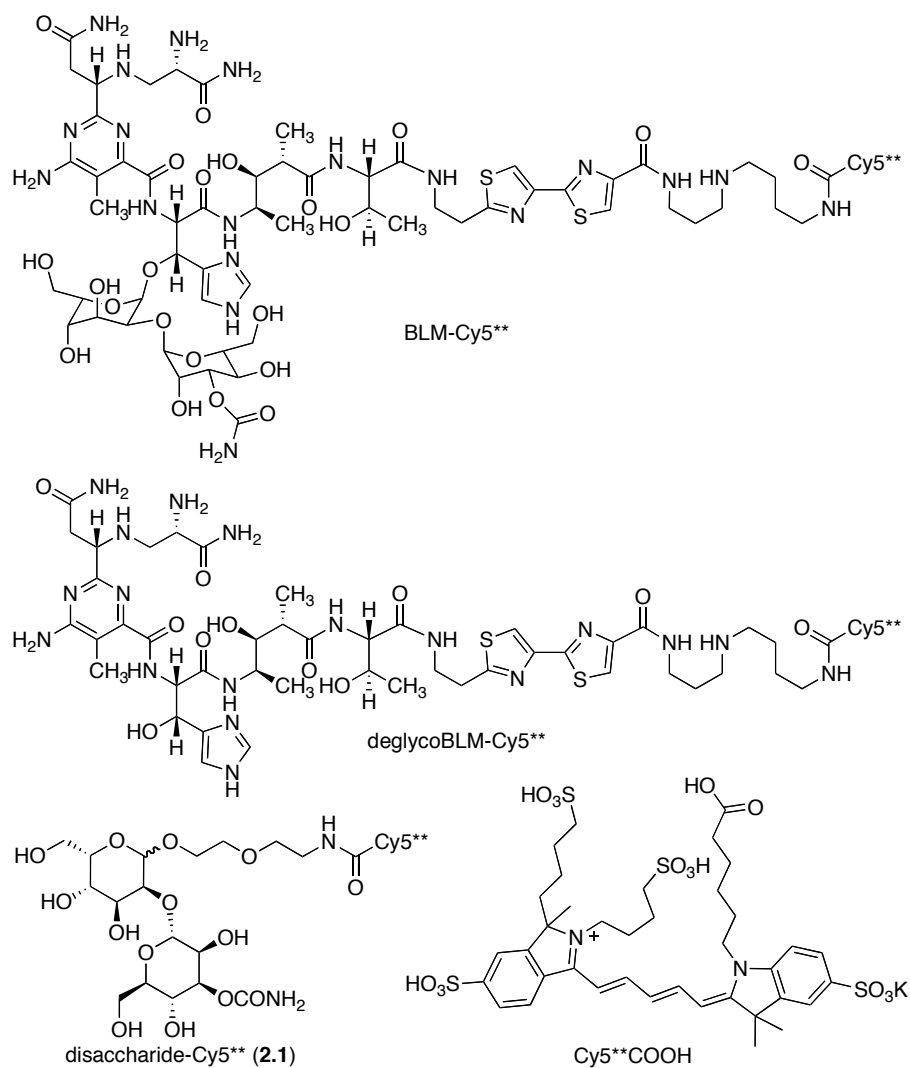
disaccharide itself to adhere to the tumor cells established that this domain is sufficient for tumor targeting. As the microbubbles were modified with multiple BLMs or sugars the observed interaction with the cell surface receptors might have been facilitated by polyvalency.

The microbubble experiment established the role of the disaccharide domain in tumor cell surface binding by BLM but this technique is limited to cell surface interactions. The possible mechanism of cell uptake of bleomycin, whether by passive diffusion or active transport, cannot be resolved using this technique. Accordingly, we employed fluorescently labeled BLMs and analogues, in conjunction with fluorescence microscopy, to address these issues. Typically, fluorescence microscopy experiments require fluorescent reporter molecules (e.g., dye) which are tagged to analytes, and the resulting fluorescent conjugates are incubated with the target cell lines *in vitro*. A fluorescence microscope is then used to visualize the fluorescence signal from the probe, permitting its location to be determined.

There are several factors to be considered while choosing the fluorescent probe. First, the conjugation reaction should lead to a product that has a known number of reporter molecules attached to the analyte in a structurally defined fashion so that meaningful quantitative data can be extracted from the imaging experiments. Fluorescent dyes can bind nonspecifically to cells and can also possess their own cell-uptake mechanisms. Ideally the fluorescent reporter molecule should not be able to bind to or enter the cell on its own. This can be easily tested using a control probe without the analyte. Usually the dye and the

analyte molecule are connected through a linker of sufficient length so that the interaction of the analyte with the cell surface is unperturbed by the dye. Another significant issue associated with fluorescent microscopy in cellular systems is the occurrence of autofluorescence, which results in very high background signals.<sup>68</sup> Autofluorescence can be avoided either by using a reporter with very high signal strength or by employing a dye that emits outside of the range of cellular autofluorescence wavelengths.

After much trial and error, we focused on Cy5\*\* as the reporter of choice. This dye was conjugated with BLM A<sub>5</sub> and deglycoBLM A<sub>5</sub> (Figure 2.2) and these probes were incubated with breast, prostate, colon and lung cell lines (both cancer and non-cancerous). Cy5\*\*COOH, used as the control, showed no binding/uptake by any of these cell lines. BLM-Cy5\*\* showed selective association/uptake by all of the cancer cell lines mentioned above. DeglycoBLM-Cy5\*\* had minimal association with the cells. The work described herein further explored the role of the disaccharide domain in the binding/uptake of bleomycins, with the goal of utilizing the acquired knowledge for application in targeted tumor imaging and drug delivery.



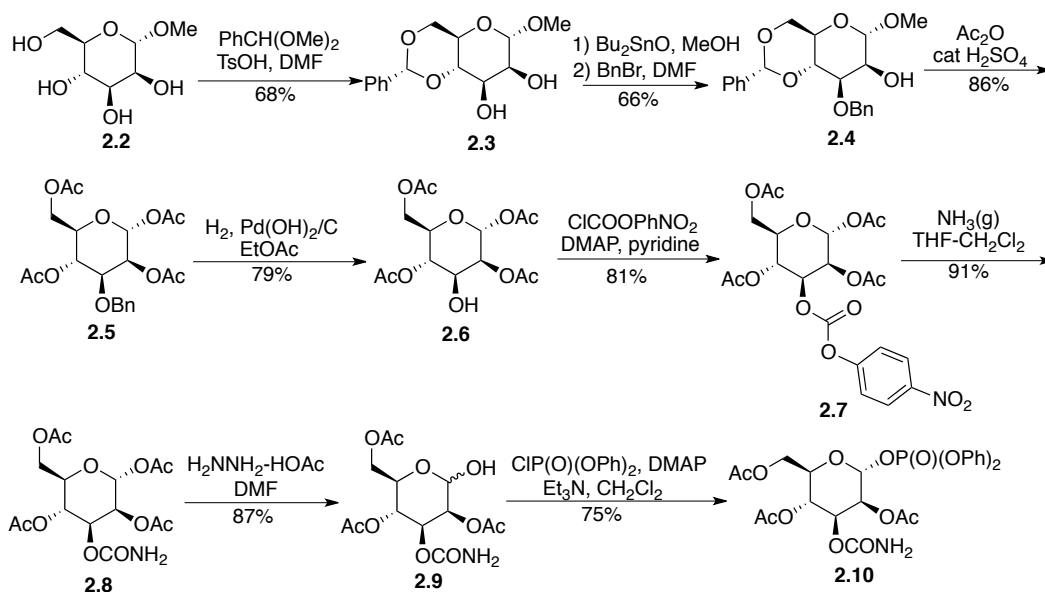
**Figure 2.2.** Structure of Cy5\*\*-conjugates with BLM, deglycoBLM and the BLM-disaccharide.

## 2.2. RESULTS

### 2.2.1. Synthesis of fluorescent conjugates

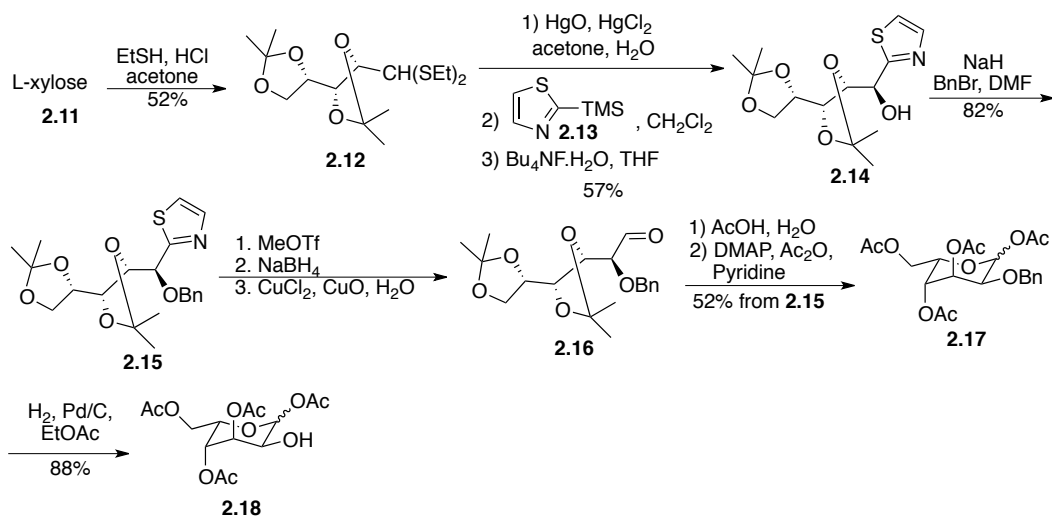
BLM-disaccharide-Cy5\*\* was synthesized from the natural disaccharide moiety in bleomycin, which was prepared following reported procedures with slight modifications.<sup>69</sup> Synthesis of the mannose residue (**2.10**) of the disaccharide

is described in Scheme 2.1. The C-4 and C-6 OH groups of methyl  $\alpha$ -D-mannopyranoside were protected as a benzylidene acetal. Selective protection at C-2 with dibutyl tin oxide followed by alkylation at C-3 with benzyl bromide gave compound **2.4**. Treatment with acetic anhydride in presence of catalytic  $\text{H}_2\text{SO}_4$  resulted in the acetylated product **2.5** in 86% yield. Reductive debenzylation followed by activation as the *p*-nitrophenyl carbonate provided compound **2.7**. Ammonolysis of the carbonate gave 3-*O*-carbamoylmannose intermediate **2.8** in 91% yield. The anomeric acetate was selectively removed with the acetate salt of hydrazine. The resulting free hydroxyl group was subsequently activated as a diphenyl phosphate ester using DMAP and chlorodiphenyl phosphate to obtain the activated mannose monosaccharide **2.10** in 75% yield.



**Scheme 2.1.** Synthesis of the activated mannose monosaccharide (**2.10**).<sup>69</sup>

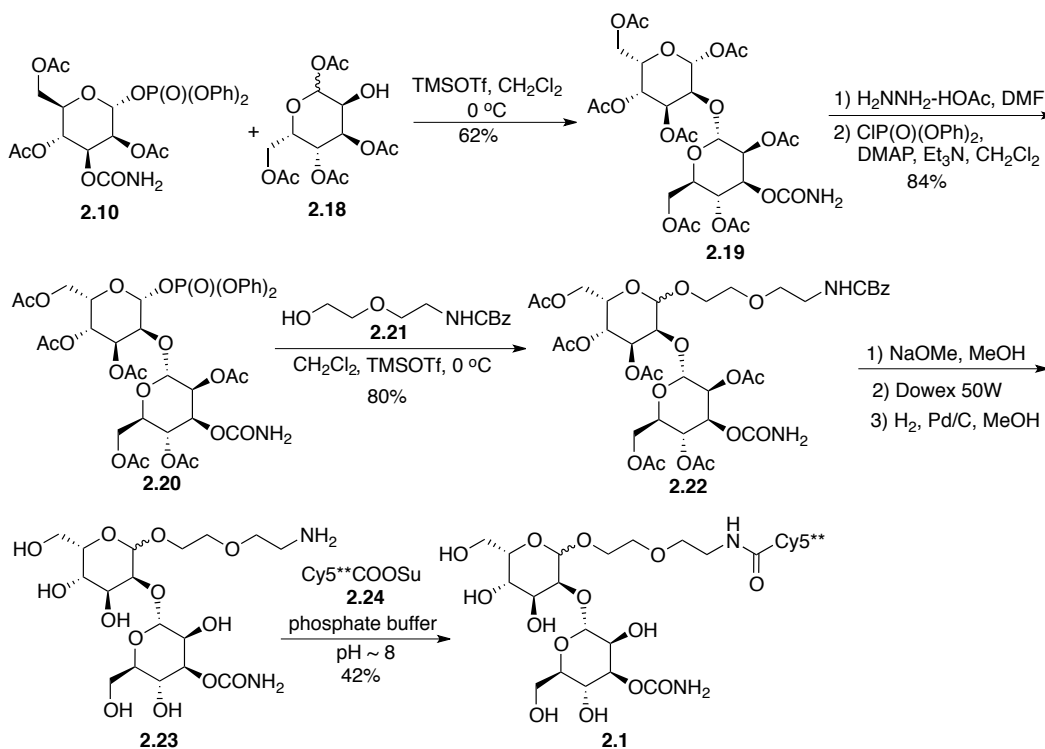
Synthesis of the requisite gulose moiety (Scheme 2.2) was achieved following Dondoni's method.<sup>69</sup> L-Xylose (**2.11**) was protected as diethylthioacetal **2.12**. The aldehyde was regenerated from **2.12** by treatment with HgO/HgCl<sub>2</sub> and immediately treated with 2-(trimethylsilyl)thiazole (**2.13**) to avoid decomposition. Desilylation of the addition product with TBAF provided intermediate **2.14** in 57% yield from compound **2.12**. The free hydroxyl group was then benzylated to obtain compound **2.15**. The thiazole moiety was converted to the aldehyde. Intermediate **2.16** was immediately treated with aq AcOH to permit cyclization and the free OH groups were acetylated with acetic anhydride to afford compound **2.17** in 52% yield. Debenzylation with Pd/C gave the appropriately protected acceptor **2.18** in 88% yield.



**Scheme 2.2.** Synthesis of the gulose acceptor (**2.18**).<sup>69</sup>

The activated phosphate ester **2.10** and compound **2.18** were coupled in the presence of TMSOTf in anhydrous CH<sub>2</sub>Cl<sub>2</sub> to obtain the acetylated BLM-

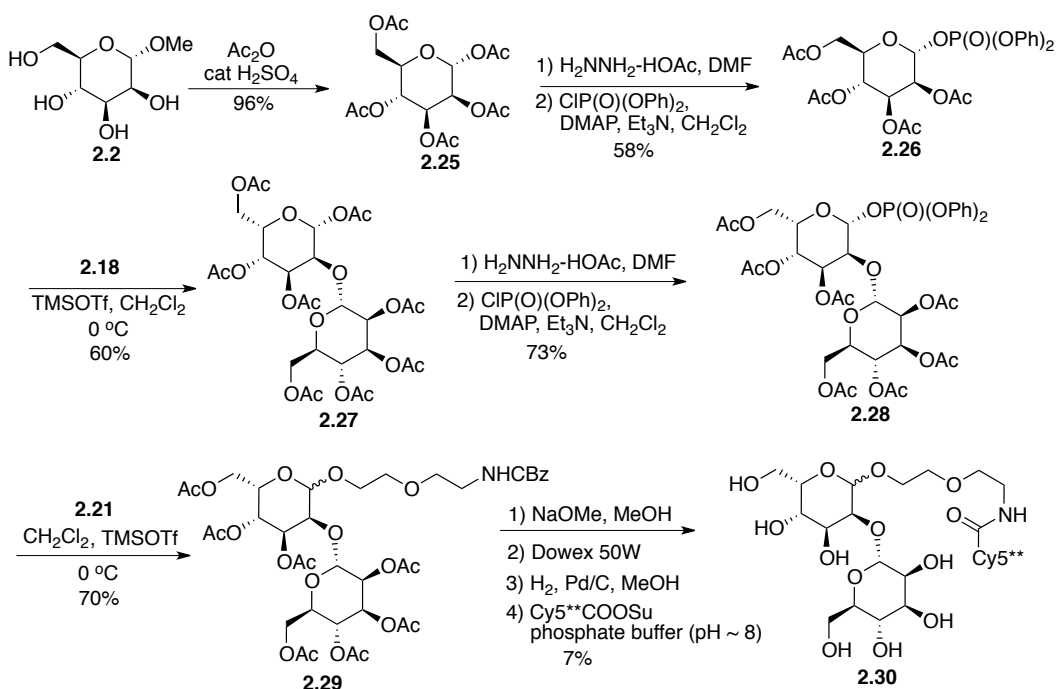
disaccharide **2.19** in 62% yield (Scheme 2.3). Selective deprotection of the anomeric OH group followed by activation as the diphenyl phosphate ester **2.20** proceeded in 84% yield. The glycosyl phosphate was subjected to TMSOTf mediated coupling with linker **2.21** to give compound **2.22** in 80% yield. The linker was synthesized from 2-(2-aminoethoxy)ethanol by treatment with CBzCl and triethylamine. Complete deacetylation with NaOMe-MeOH, followed by reductive removal of the CBz group, afforded the BLM-disaccharide linker **2.23**. Finally, compound **2.23** was condensed with the succinimidyl ester **2.24** (prepared from the corresponding carboxylic acid) to obtain the BLM-disaccharide-Cy5\*\* **2.1** in 42% yield.



**Scheme 2.3.** Synthesis of BLM-disaccharide-Cy5\*\* (**2.1**).<sup>69</sup>



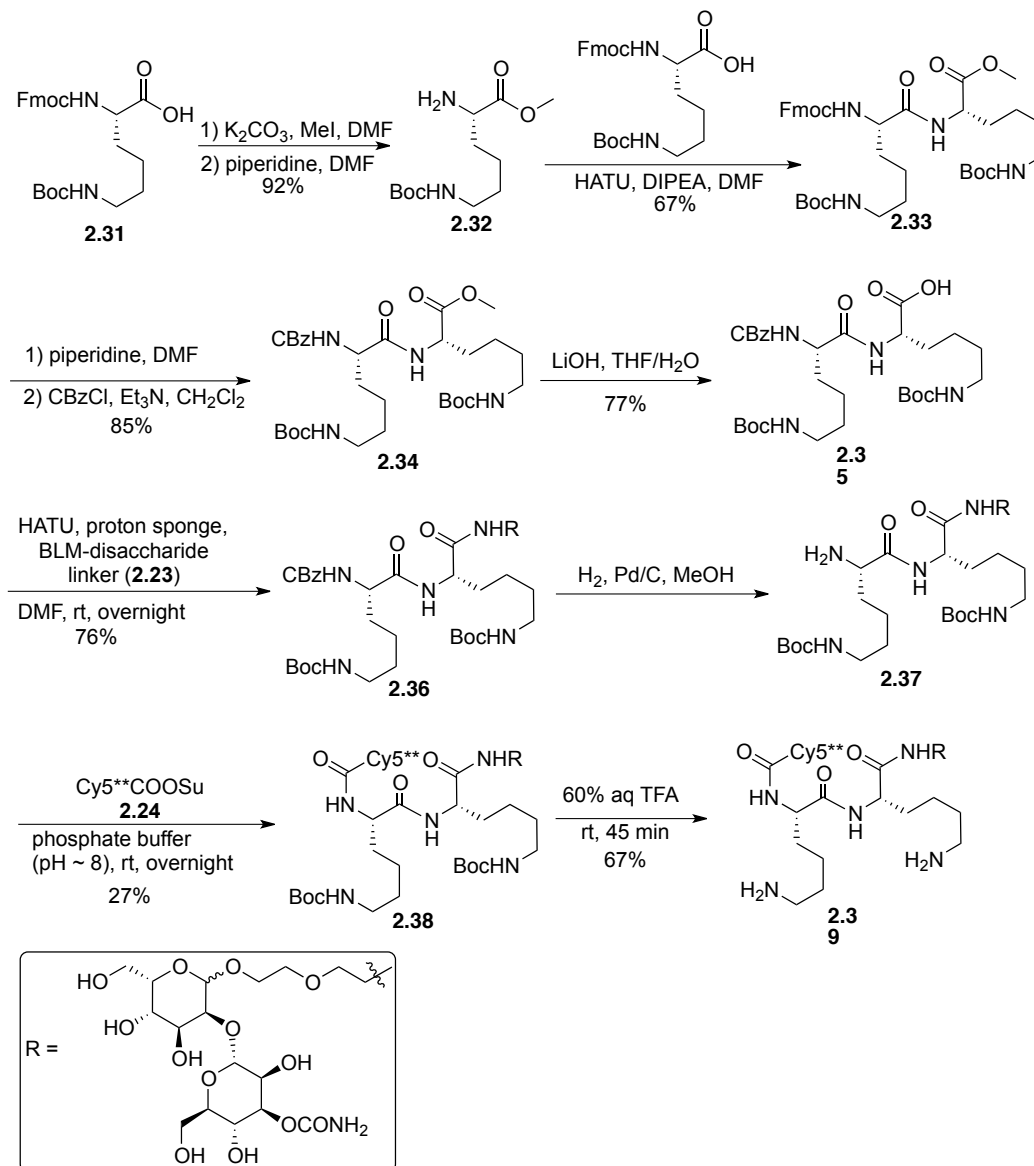
DecarbamoylBLM-disaccharide-Cy5\*\* **2.30** lacked the carbamoyl group at C-3 of the mannose residue. The purpose of synthesizing this fluorescent conjugate was to test whether the C-3 carbamoyl group played any role in tumor targeting. It was synthesized from disaccharide **2.27** (Scheme 2.4).<sup>70</sup> Synthesis of compound **2.27** commenced with peracetylation of  $\alpha$ -D-mannopyranoside. Selective deacetylation of the anomeric OH group and subsequent activation with chlorodiphenyl phosphate in the presence of DMAP and triethylamine afforded the glycosyl donor **2.26**. Coupling of the activated ester and glycosyl acceptor **2.18** was promoted by TMSOTf and afforded the disaccharide **2.27** in 60% yield. The disaccharide was subjected to selective deprotection at the anomeric position followed by activation as diphenyl phosphate ester **2.28**. Coupling of **2.28** with linker **2.21** proceeded in 70% yield. Compound **2.29** was subjected to complete deacetylation followed by CBz deprotection. The intermediate was treated with Cy5\*\*COOSu to afford the decarbamoylBLM-disaccharide-Cy5\*\* **2.30**.



**Scheme 2.4.** Synthesis of decarbamoylBLM-disaccharide-Cy5\*\* (**2.30**).<sup>70</sup>

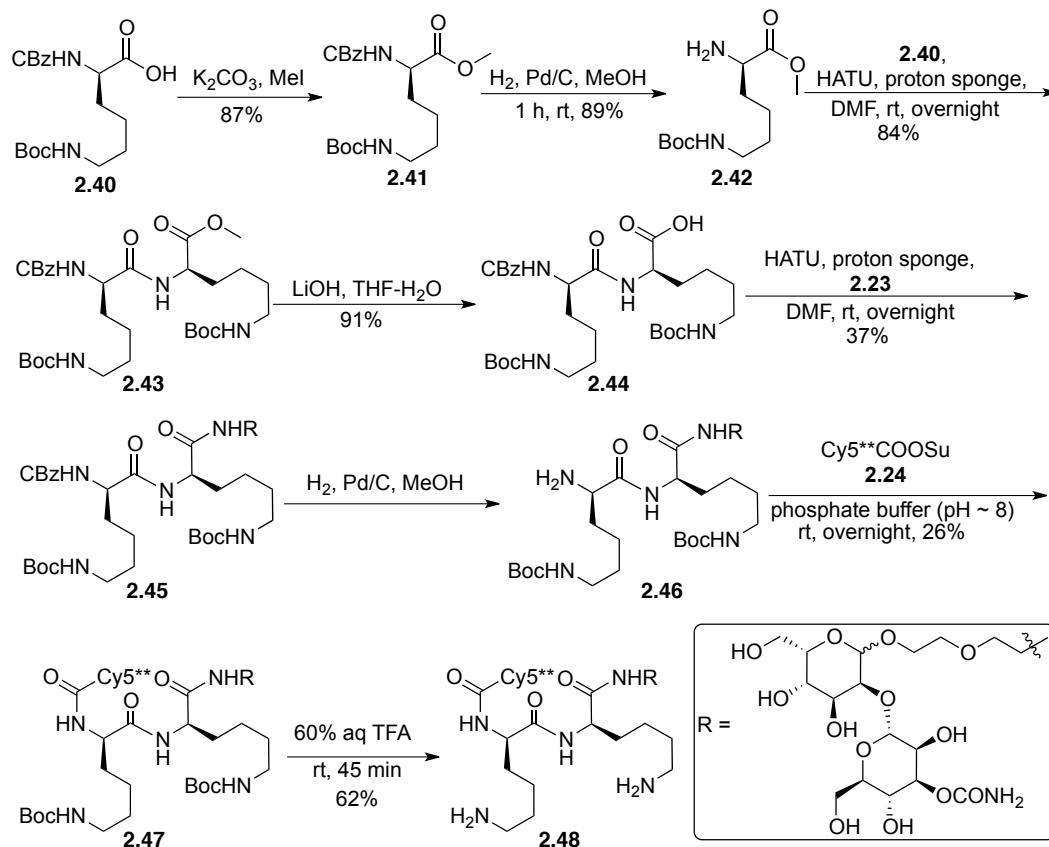
The fluorescent BLM-disaccharide conjugate **2.39** was prepared to introduce positive charge into the linker (Scheme 2.5). Synthesis of the fully protected linker **2.35** began with methylation of *N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-Boc-L-lysine (**2.31**) using iodomethane in the presence of K<sub>2</sub>CO<sub>3</sub>. The crude intermediate was treated with 20% piperidine in DMF to afford the free amine **2.32** in 92% yield over two steps. The intermediate was subsequently condensed with *N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-Boc-L-lysine (**2.31**) to afford the Fmoc-protected dipeptide methyl ester **2.33** in 67% yield. The Fmoc protecting group was replaced with a CBz group to provide the CBz-protected dipeptide methyl ester **2.34**. Saponification of the methyl ester afforded the dipeptide linker **2.35** in 77% yield. Compound **2.23** was then conjugated with this dipeptide to afford *N*<sup>α</sup>-CBz-*N*<sup>ε</sup>-Boc-L-lysyl-*N*<sup>ε</sup>-Boc-L-lysyl-

disaccharide (**2.36**) in 76% yield. Reductive deprotection of the CBz group followed by condensation with succinimidyl ester **2.24** provided the Boc protected fluorescent conjugate **2.38**. The Boc groups were removed by treatment with 60% aq CF<sub>3</sub>COOH to afford the fluorescent conjugate **2.39**, having two primary amino groups, in 67% yield.



**Scheme 2.5.** Synthesis of Cy5<sup>\*\*</sup>-L-lysyl-L-lysyl-BLM-disaccharide (**2.39**).

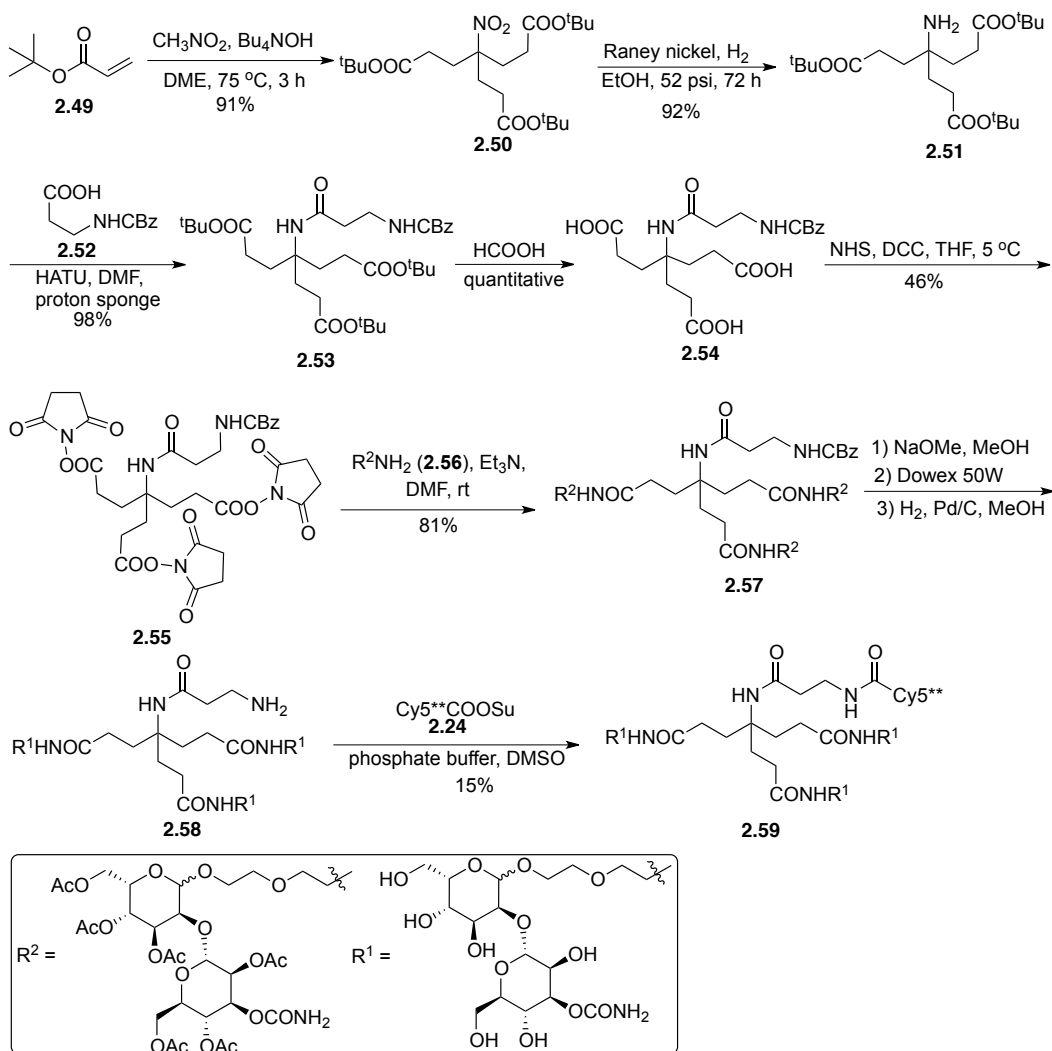
The dipeptide in the conjugate **2.39**, containing two L-lysines, might be susceptible to peptidases present inside the cells or in the cell culture media. Conjugate **2.48** was synthesized to overcome this problem by replacing the L-lysines with their D-enantiomers (Scheme 2.6). Methylation of the commercially available *N*<sup>α</sup>-CBz-*N*<sup>ε</sup>-Boc-D-lysine (**2.40**) proceeded in 87% yield. The CBz group was removed reductively to give compound **2.42** having a free amine. Coupling of this free amine with the protected lysine **2.40** was promoted by HATU affording the protected dipeptide ester **2.43** in 84% yield. The methyl ester was cleaved using LiOH in aq THF to obtain the dipeptide **2.44**. BLM-disaccharide linker **2.23** was then condensed with this dipeptide linker in the presence of HATU. Compound **2.45** was then subjected to reductive deprotection of the CBz group and the product was condensed with succinimidyl ester **2.24** to afford compound **2.47** in 26% yield. Removal of the Boc groups with 60% aq TFA generated the fluorescent probe **2.48** in 62% yield.



**Scheme 2.6.** Synthesis of Cy5\*\*-D-lysyl-D-lysyl-BLM-disaccharide (**2.48**).

Suspecting that the interaction between the BLM-disaccharide and the cellular receptors might involve polyvalency, the fluorescent probe **2.59** was prepared by conjugating three BLM-disaccharide moieties to Cy5\*\*COOH through a branched linker (Scheme 2.7). The synthesis of the linker commenced with the exhaustive alkylation of nitromethane with *tert*-butyl acrylate in the presence of tetrabutylammonium hydroxide to obtain compound **2.50**. Hydrogenation of the nitro group was catalyzed by Raney nickel in ethanol to afford amine **2.51** in 92% yield. The amine was condensed with CBz protected  $\beta$ -alanine to provide the fully protected linker **2.53** in 98% yield. Treatment with

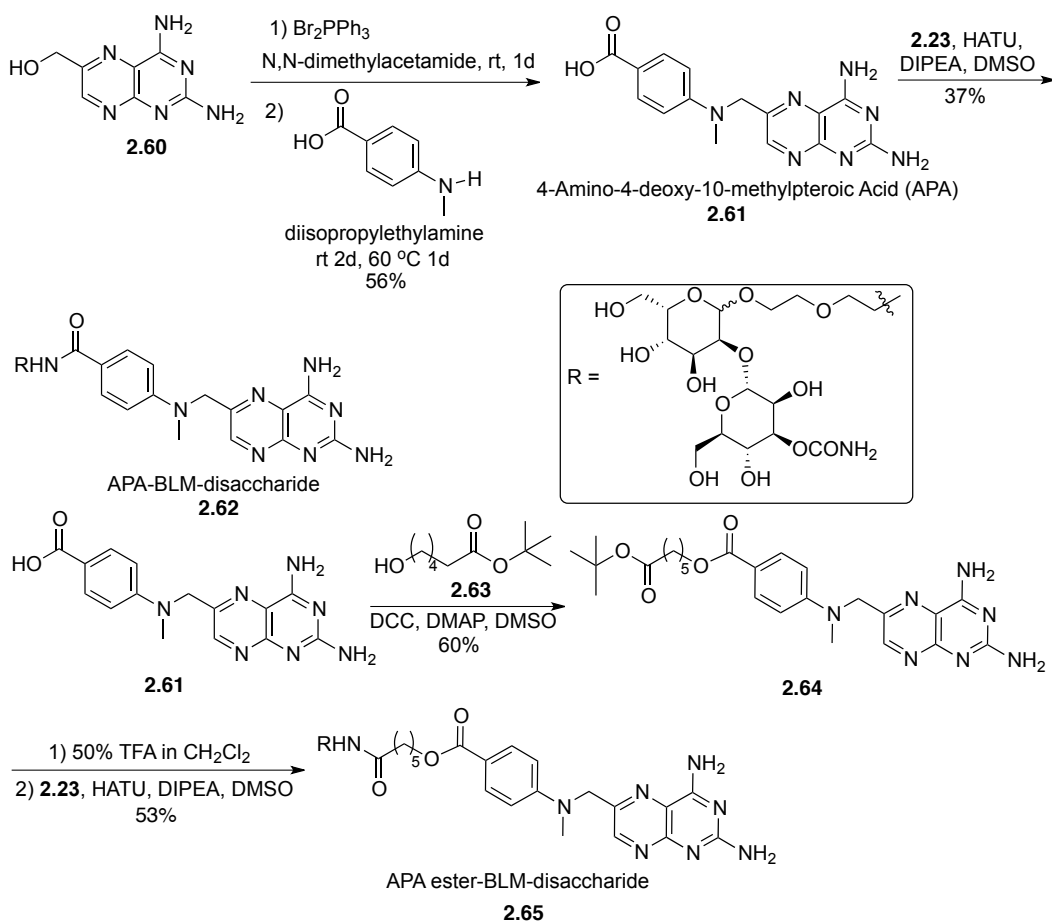
CF<sub>3</sub>COOH in CH<sub>2</sub>Cl<sub>2</sub> proceeded in quantitative yield to produce tri-acid **2.54**. The tri-succinimidyl ester **2.55** was prepared by the use of *N*-hydroxysuccinimide and DCC in THF. Compound **2.22** (Scheme 2.3) was subjected to hydrogenolysis of the CBz and the free amine intermediate **2.56** was immediately coupled with the activated tri-ester **2.55** to produce the trimerBLM-disaccharide **2.57** in 81% yield. Deprotection of acetyl and CBz groups was followed by condensation with Cy5\*\*COOSu (**2.24**) to obtain the trimerBLM-disaccharide-Cy5\*\* **2.59** in 15% yield.



**Scheme 2.7.** Synthesis of trimerBLM-disaccharide-Cy5\*\* (**2.59**).

In order to permit a study of the efficacy of the BLM-disaccharide as a tumor targeting vehicle for cytotoxic agents, APA (4-amino-4-deoxy-10-*N*-methylpteroic acid) was conjugated to the BLM-disaccharide (Scheme 2.8). 2,4-Diamino-6-(hydroxymethyl)pteridine hydrochloride (**2.60**) was treated with triphenylphosphine dibromide to generate the alkyl bromide *in situ*; this was coupled with 4-*N*-methylaminobenzoic acid to obtain APA (**2.61**) in 56% yield.<sup>71</sup>

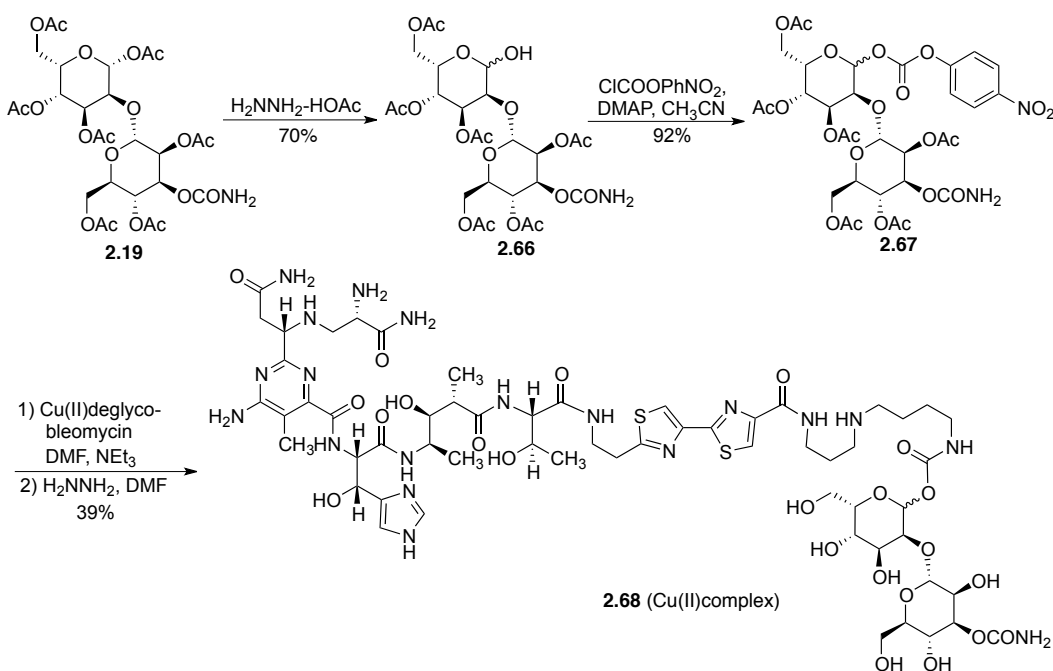
Compound **2.23** was condensed with APA to provide the APA-BLM-disaccharide conjugate **2.62** in 37% yield. Conjugate **2.65** was designed to be a tumor selective drug delivery vehicle. Once inside the cell, the cellular esterases should release the cytotoxic drug, in this case APA, from the conjugate. The synthesis started with the esterification of APA with *tert*-butyl 6-hydroxyhexanoate (**2.63**) to obtain compound **2.64** in 60% yield. The *tert*-butyl group was removed by the use of CF<sub>3</sub>COOH in dichloromethane and the resulting acid was condensed with compound **2.23** to afford the conjugate **2.65** in 53% yield.



**Scheme 2.8.** Synthesis of APA-BLM-disaccharide conjugates **2.62** and **2.65**.



Scheme 2.9 describes the synthesis of a bleomycin A<sub>5</sub> analogue **2.68** in which the disaccharide is attached to deglycoBLM via a polyamine linker. The acetylated disaccharide **2.19** was activated as the *p*-nitrophenyl carbonate (**2.67**) by selective deacetylation of the anomeric OH with acetate salt of hydrazine followed by treatment with *p*-nitrophenyl chloroformate in the presence of DMAP. The carbonate was then coupled with deglycoBLM-Cu(II) complex. The resulting intermediate was deprotected by the use of hydrazine to afford compound **2.68** in 39% yield.



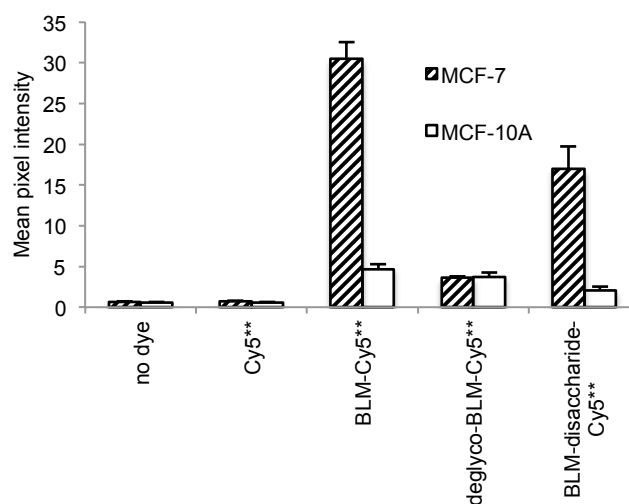
**Scheme 2.9.** Synthesis of bleomycin A<sub>5</sub> analogue **2.68**.

### 3.2.4 Biological Evaluation of Fluorescent Carbohydrate Conjugates

The emission maxima ( $\lambda_{\text{max}}$ ) of Cy5\*\*, the fluorescent dye used in this study, is at 651 nm. Emission at such long wavelengths allows for complete

elimination of the interference arising from cellular autofluorescence. The presence of four sulfonates in the dye molecule (Figure 2.2) makes it highly soluble in aqueous media and also impermeable to the cell membrane on its own. All these characteristics made Cy5\*\* a perfect dye candidate for our study. The cells were cultured on 16-well glass chamber slides until the cell confluence was about 70%. The fluorescent conjugates were then incubated with the cells at 37 °C for 24 h. The cells were washed with PBS and fixed with 4% paraformaldehyde at 37 °C for 5 min. After drying for 24 h the slides were imaged using a Zeiss Axiovert 200M inverted microscope equipped with a 300-w xenon lamp (Shutter), ET-CY5 cyanine filter (Chroma) and an AxioCam MRm camera.

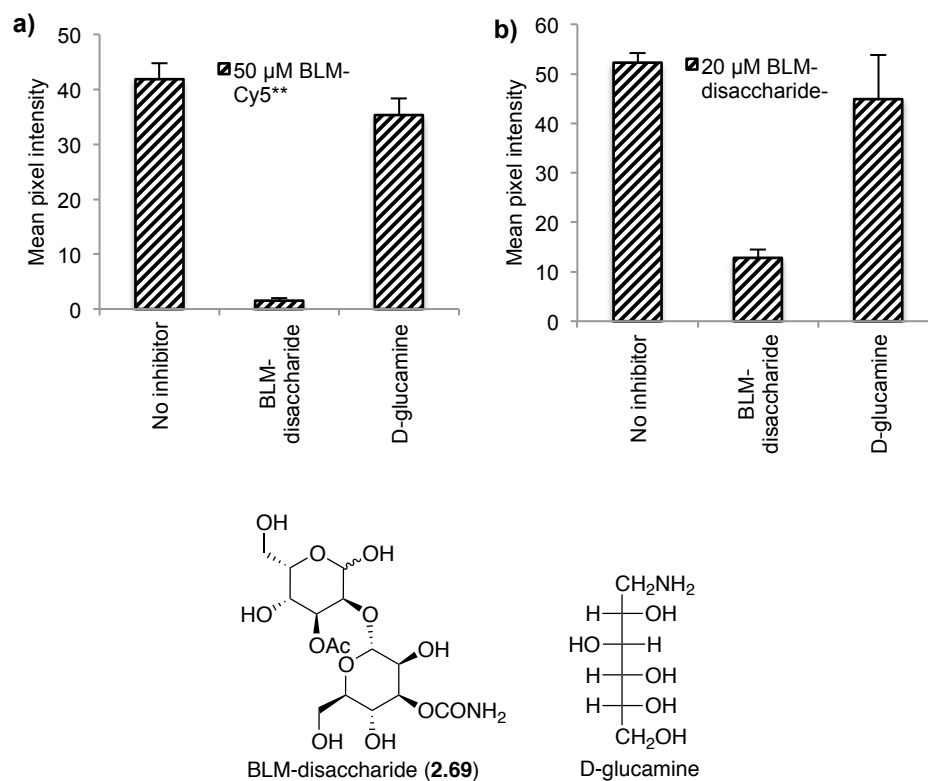
The microbubble experiment had shown that both BLM and the BLM-disaccharide adhered selectively to MCF-7 human breast cancer cells compared to MCF-10A normal breast cells.<sup>66</sup> On the other hand deglycoBLM did not adhere to either of the two cell lines. Similar results were obtained when we treated the same two cell lines with the fluorescent conjugates BLM-Cy5\*\*, deglycoBLM-Cy5\*\* and BLM-disaccharide-Cy5\*\* (Figure 2.3). BLM bound to the MCF-7 cells but not to noncancerous MCF-10A cells. DeglycoBLM bound minimally to either cell lines. The BLM-disaccharide also targeted the cancer cells selectively although the uptake was much lower than BLM itself. The free dye Cy5\*\*, used as a control, showed no binding/uptake in any of the cell lines.



**Figure 2.3.** Relative uptake of Cy5\*\* dye conjugates in human breast cancer cells (MCF-7) and human breast cells (MCF-10A). Cultured cells were incubated with 50  $\mu$ M of each of the fluorescent conjugates at 37  $^{\circ}$ C for 1 h. The cells were washed with PBS and were fixed with 4% paraformaldehyde at 37  $^{\circ}$ C for 5 min. The slide was dried for 1 h and imaged with a Zeiss Axiovert 200M inverted microscope. Dr. Alan Yu carried out this experiment.

Similar profiles of cell uptake were observed for these fluorescent conjugates in prostate, lung, colon cancer cells and the respective non-cancerous cells (data not shown). The above results suggested that the disaccharide moiety in BLM is absolutely essential for its selective uptake into cancer cells. Further support came from the uptake-inhibition experiments described in Figure 2.4. The binding of BLM-Cy5\*\* was reduced in MCF-7 cells when the cells were pre-incubated with 50 mM BLM-disaccharide (**2.69**)<sup>72</sup> (Figure 2.4) in comparison with its binding to cells with no pre-incubation (panel a, Figure 2.4). Not

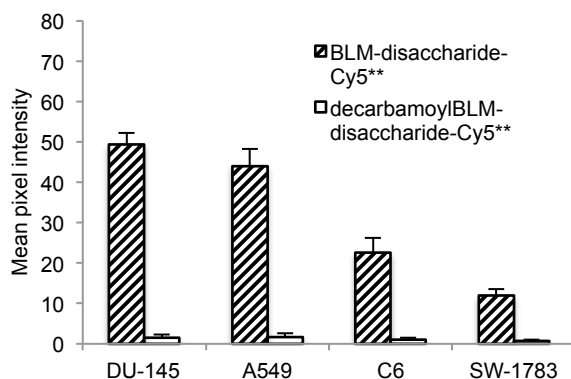
surprisingly, the BLM-disaccharide was also able to inhibit the binding of BLM-disaccharide-Cy5\*\* (**2.1**) to DU-145 prostate cancer cells (panel b, Figure 2.4).



**Figure 2.4.** Inhibition of binding of BLM-Cy5\*\* by MCF-7 cells and binding of BLM-disaccharide-Cy5\*\* by DU-145 cells by BLM-disaccharide. Cultured cells were incubated with a 50 mM solution of either BLM-disaccharide (**2.69**) or D-glucamine at 37 °C for 2 h. (a) The MCF-7 cells were subsequently treated with a 50 μM solution of BLM-Cy5\*\* at 37 °C for 1 h and (b) the DU-145 cells were subsequently treated with a 20 μM solution of BLM-disaccharide-Cy5\*\* (**2.1**) at 37 °C for 1 h. The cells were washed with PBS and fixed with 4% paraformaldehyde at 37 °C for 5 min. The slide was dried for 1 h and imaged with

a Zeiss Axiovert 200M inverted microscope. Dr. Alan Yu carried out this experiment.

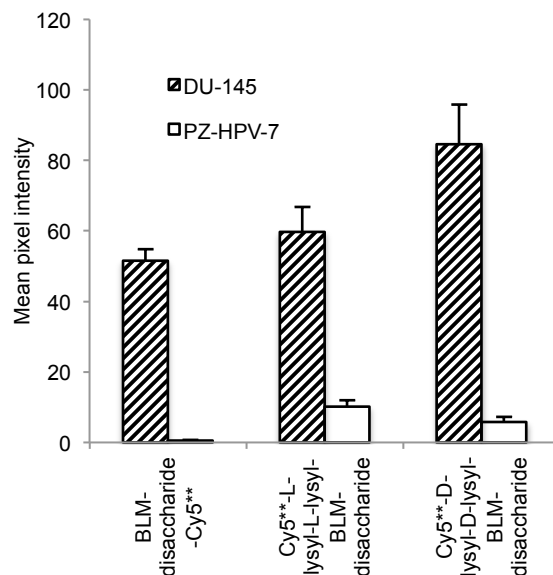
The carbohydrate domain in BLM contains a carbamoyl group at C-3 in the mannose residue. In the next experiment four different types of cancer cells such as prostate, lung, glioma and astrocytoma cells were treated with either BLM-disaccharide-Cy5\*\* (2.1) or decarbamoylBLM-disaccharide-Cy5\*\* (2.30). As shown in Figure 2.5, the decarbamoylBLM-disaccharide-Cy5\*\* had very low binding to each of the four cancer cell lines compared to the BLM-disaccharide. Clearly, the carbamoyl group plays an important role in the binding of the BLM-disaccharide (and possibly bleomycin) to various cancer cell lines.



**Figure 2.5.** Relative binding of BLM-disaccharide-Cy5\*\* (2.1) and decarbamoylBLM-disaccharide-Cy5\*\* (2.30) to human prostate cancer (DU-145), lung cancer (A549), glioma (C6, ATCC CCL-107), astrocytoma (SW-1783, ATCC HTB-13) cell lines. Cultured cells were incubated with 25  $\mu$ M of each of the two fluorescent conjugates at 37  $^{\circ}$ C for 1 h. The cells were washed with PBS

and were fixed with 4% paraformaldehyde at 37 °C for 5 min. The slide was dried for 1 h and imaged with a Zeiss Axiovert 200M inverted microscope. Dr. Alan Yu carried out this experiment.

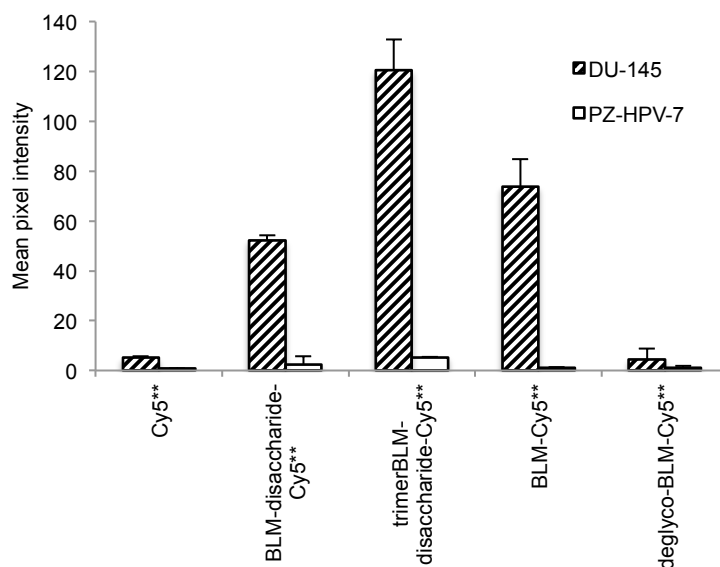
Although BLM-disaccharide can target the cancer cells selectively on its own, cancer cell association was relatively low. Our next objective was to improve its cellular association so that it can be developed as a tumor selective imaging agent or as a targeted delivery vehicle for cytotoxic drugs. The cell membrane impermeability of the Cy5\*\* molecule might also hinder the uptake of the BLM-disaccharide-Cy5\*\*. Bleomycin A<sub>5</sub>, on the other hand, has several amines that could become protonated in the cellular media. This would certainly reduce the overall net negative charge on the BLM-Cy5\*\* molecule and facilitate its uptake. To introduce positive charge into the disaccharide conjugate **2.1**, probe **2.39**, having a L-lysyl-L-lysine dipeptide was synthesized. Incubating DU-145 prostate cancer cells with **2.39**, however, did not afford any significant increase in cell binding/uptake (Figure 2.6). Small peptides are known to be prone to degradation mediated by peptidases present in cellular media. To avoid degradation, we sought to replace the L-lysyl-L-lysine dipeptide in **2.39** with D-lysyl-D-lysine. The resulting fluorescent conjugate **2.48** showed improved uptake in DU-145 cells compared to BLM-disaccharide-Cy5\*\* (Figure 2.6).



**Figure 2.6.** Relative binding of Cy5\*\*-L-lysyl-L-lysyl-BLM-disaccharide (**2.39**) and Cy5\*\*-D-lysyl-D-lysyl-BLM-disaccharide (**2.48**) in human prostate cancer (DU-145), and normal (PZ-HPV-7) cells. Cultured cells were incubated with a 25  $\mu$ M solution of each of the fluorescent conjugates at 37  $^{\circ}$ C for 1 h. The cells were washed with PBS and were fixed with 4% paraformaldehyde at 37  $^{\circ}$ C for 5 min. The slide was dried for 1 h and imaged with a Zeiss Axiovert 200M inverted microscope. This experiment was carried out by Dr. Alan Yu.

Another strategy to boost the binding of the BLM-disaccharide involved the use of a multivalent linker. This prompted us to synthesize the trimerBLM-disaccharide-Cy5\*\* (**2.59**) having three BLM-disaccharides connected to Cy5\*\* through a branched linker. Binding of this conjugate was studied in DU-145 cells and was compared with the binding of BLM-disaccharide-Cy5\*\* and BLM-Cy5\*\*. It is clear from Figure 2.7 that introduction of polyvalency greatly

facilitated the binding of the fluorescent conjugate **2.59** by prostate cancer cells. Notably, the binding of this conjugate by noncancerous prostate cells was still very low.



**Figure 2.7.** Uptake of trimerBLM-disaccharide-Cy5\*\* (**2.59**) in human prostate cancer (DU-145), and normal (PZ-HPV-7) cells. Cultured cells were incubated with a 25  $\mu$ M solution of each of the fluorescent conjugates at 37 °C for 1 h. The cells were washed with PBS and were fixed with 4% paraformaldehyde at 37 °C for 5 min. The slide was dried for 1 h and imaged with a Zeiss Axiovert 200M inverted microscope. Dr. Alan Yu carried out this experiment.

## 2.3 DISCUSSION

### 2.3.1. Chemistry

The synthesis of the disaccharide **2.19** was achieved using published procedures with slight modifications.<sup>69</sup> Commercially available mannose



derivative **2.2** was protected as benzylidene acetal **2.3** (Scheme 2.1). To selectively alkylate at C-3, compound **2.3** was first protected with dibutyltin oxide and then treated with benzyl bromide to afford compound **2.4**. The acetylated intermediate **2.5** was prepared by the use of acetic anhydride in the presence of a catalytic amount of H<sub>2</sub>SO<sub>4</sub>. The benzyl protecting group was removed under reductive conditions and the hydroxyl intermediate **2.6** was activated with *p*-nitrophenyl chloroformate to afford carbonate **2.7**. The carbamoyl group was introduced at the C-3 position by treatment with ammonia. This reaction was complete in 1.5 h and started to generate side products when continued longer than 3 h. Acetylated carbamoyl mannose **2.8** was then treated with the acetate salt of hydrazine for deprotection of the C-1 OH group. Once the starting material was consumed, it was necessary to quench this reaction by the addition of ethyl acetate to avoid formation of undesired by-products. A DMAP–triethylamine mixture was employed for the activation of compound **2.9** to avoid the frequently observed deprotonation of the carbamoyl group when nBuLi is used.

Synthesis of the gulose derivative **2.18** was completed following reported procedures starting from L-xylose (Scheme 2.2). L-Xylose was protected as the thioacetal (**2.12**). The aldehyde functional group was regenerated by the use of HgO and HgCl<sub>2</sub> and immediately treated with 2-trimethylsilylthiazole (**2.13**) to afford the hydroxylated intermediate **2.14** having the desired stereochemistry. The OH group was benzylated and thiazole **2.15** was converted to the benzylated gulose residue **2.17** via formation of the aldehyde **2.16**. Reductive debenzylation

of **2.17** afforded compound **2.18** which was used in crude form without any chromatographic purification.

Compound **2.18** was coupled with compound **2.10** in the presence of TMSOTf (Scheme 2.3). The reaction mixture was stirred at 0 °C for 17 min to obtain the best yield of product **2.19**. Disaccharide **2.19** was then deprotected at the anomeric position using conditions similar to those used for the preparation of **2.9**. In this case, however, the crude intermediate was used directly in the next step to avoid loss of material during silica gel column chromatography. The activation of the anomeric OH group afforded much higher yields of the desired product **2.20** when DMAP–triethylamine was used instead of nBuLi. The phosphate ester was coupled with the linker alcohol **2.21** in the presence of TMSOTf to afford compound **2.22** as mixture of anomers. Treatment of **2.22** with NaOMe affected complete deacetylation. The acidic ion exchange resin Dowex 50W was used to quench the remaining NaOMe. The deacetylated intermediate was subjected directly to catalytic hydrogenation which removed the CBz group in 30 min. The disaccharide linker **2.23** was condensed with the succinimidyl ester of the dye (**2.24**) to obtain BLM-disaccharide-Cy5\*\* (**2.1**). The succinimidyl ester was prepared from the corresponding dye carboxylic acid following a reported procedure.<sup>76</sup>

The decarbamoyl analogue **2.30** was prepared following a similar synthetic strategy (Scheme 2.4). Methyl  $\alpha$ -D-mannopyranoside was acetylated in the presence of acetic anhydride and a catalytic amount of H<sub>2</sub>SO<sub>4</sub>. Compound **2.25** was then subjected to anomeric deprotection followed by activation as the

diphenyl phosphate **2.26**. The phosphate ester was coupled with gulose derivative **2.18** to obtain the decarbamoyl disaccharide **2.27**. This disaccharide was then coupled with the linker alcohol **2.21** via anomeric deacetylation and diphenyl phosphate activation. Compound **2.29** was then subjected to complete deacetylation, reductive removal of the CBz protecting group and condensation with succinimidyl ester **2.24** to afford decarbamoylBLM-disaccharide-Cy5\*\* (**2.30**).

The synthesis of Cy5\*\*-L-lysyl-L-lysyl-BLM-disaccharide (**2.39**) involved attaching the linker disaccharide **2.23** at the C-terminus of lysyllysine followed by coupling of the dye at the N-terminus (Scheme 2.5). Synthesis of the dipeptide commenced with methylation and subsequent Fmoc deprotection of Fmoc-L-lys(Boc)-OH to afford compound **2.32**. Compound **2.32** was then condensed with a second molecule of Fmoc-L-lys(Boc)-OH. The Fmoc group was exchanged for the CBz group and the methyl ester **2.34** was saponified to provide compound **2.35**. Compound **2.35** was then condensed with the disaccharide linker **2.23**; the product was purified by reversed phase HPLC. The CBz group was subsequently removed and crude intermediate **2.37** was pure enough to be condensed with the activated ester of Cy5\*\* (**2.24**). Finally the Boc groups were removed by the use of aq CF<sub>3</sub>COOH. Cy5\*\*-D-lysyl-D-lysyl-BLM-disaccharide was synthesized following a similar synthetic route (Scheme 2.6). CBz-D-lys(Boc)-OH was protected as the methyl ester (**2.41**). The CBz group was deprotected and compound **2.42** was coupled to another molecule of CBz-D-lys(Boc)-OH. The resulting methyl ester (**2.43**) was subjected to saponification

and condensation with the disaccharide linker **2.23** to afford compound **2.45**. The CBz group was subsequently removed and the crude intermediate **2.46** was condensed with activated ester **2.24**. Removal of the Boc groups afforded dye conjugate **2.48**.

Synthesis of the branched linker **2.55** started with Michael addition of nitromethane to *tert*-butyl acrylate (Scheme 2.7). The branched nitro compound **2.50** was converted to the respective amine (**2.51**) and subsequently coupled with protected  $\beta$ -alanine **2.52**. The *tert*-butyl esters were removed by the use of HCOOH. Triacid **2.54** was activated as tri-succinimidyl ester **2.55** in the presence of *N*-hydroxysuccinimide and DCC. This compound was purified by recrystallization from ethanol because purification attempts by silica gel column chromatography lead to degradation of the desired product. Preparation of compound **2.56** by hydrogenation of compound **2.22** gave inconsistent results, most probably due to the extreme sensitivity of this reaction toward any catalyst poison. The condensation of **2.56** with compound **2.55** proceeded in reasonable yield. Compound **2.57** was then subjected successively to complete deacetylation and catalytic hydrogenation to obtain intermediate **2.58**. Coupling of **2.58** with compound **2.24** produced trace amounts of trimerBLM-disaccharide-Cy5\*\* (**2.59**) when aq phosphate buffer was used as the solvent. Addition of a small amount of DMSO to the reaction gave better yields of the conjugate **2.59**, possibly due to the greater solubility of compound **2.58** in aq DMSO.

The cytotoxic core of the anticancer drug methotrexate, 4-amino-4-deoxy-10-*N*-methylpropanoic acid (APA) (**2.61**), was synthesized by following reported

procedures from compound **2.60** (Scheme 2.8).<sup>71</sup> The disaccharide linker **2.23** was coupled with compound **2.61** to afford the APA-BLM-disaccharide conjugate **2.62**. Synthesis of APA ester-BLM-disaccharide (**2.65**) commenced with esterification of APA with alcohol **2.63**. The *tert*-butyl ester was cleaved by the use of CF<sub>3</sub>COOH and the resulting intermediate was condensed with the disaccharide linker **2.23** to give conjugate **2.65**.

To attach the BLM-disaccharide at the C-terminus of deglycoBLM, disaccharide **2.19** was first deacetylated at C-1 by the use of acetate salt of hydrazine (Scheme 2.9). Compound **2.66** was then activated as *p*-nitrophenyl carbonate **2.67**. The carbonate was then treated with deglycoBLM, which had been protected as the Cu(II) complex. The coupled product was deacetylated by the use of hydrazine to afford the BLM A<sub>5</sub> analogue **2.68**.

### **2.3.2. Biological Evaluation**

The obvious difference in cytotoxicities of BLM and deglycoBLM in cellular or *in vivo* assays strongly suggest that the carbohydrate moiety in BLM plays a key role in cellular targeting or uptake. The study involving microbubbles conjugates of BLM, deglycoBLM and BLM-disaccharide clearly showed that the BLM-disaccharide moiety was necessary for cell surface binding. The issue of cellular uptake, however, required further investigation.

Fluorescent conjugates of BLM, deglycoBLM and BLM-disaccharide were prepared to compare their cell binding/uptake profiles in different cell lines. The conjugates were first tested in breast cells where the microbubble study had

shown a selective binding of BLM and BLM-disaccharide to MCF-7 breast cancer cells and not to normal MCF-10A breast cells. As expected, BLM-Cy5\*\* and BLM-disaccharide-Cy5\*\* were selectively bound by MCF-7 cells and not by MCF-10A normal breast cells (Figure 2.3). The dye conjugate deglyco BLM-Cy5\*\*, which lacked the carbohydrate moiety, did not bind to or enter the cells from either of the two cell lines. The dye Cy5\*\*COOH, used as a negative control, also did not show any binding/uptake. Similar pattern of cellular binding/uptake of these fluorescent conjugates was observed in several other cell lines (data not shown). These results confirmed that the disaccharide moiety in BLM is essential for cancer cell targeting and uptake.

Although BLM-disaccharide was able to selectively target the cancer cells, its binding/uptake was lower than that of the BLM conjugate. One possible reason for the observed difference in uptake between BLM and BLM-disaccharide could be the difference in formal charges on these fluorescent conjugates in the cellular media (pH ~ 7). BLM-disaccharide-Cy5\*\* would have four net negative charges arising from the four sulfonate groups. This would certainly cause a significant repulsion from the negatively charged cell membrane, thus hindering cell surface interactions and uptake. The bleomycin molecule, on the other hand, contains several amine groups that should be at least partially protonated at pH 7, thus reducing the overall negative charge on BLM-Cy5\*\*. In fact, the polyamine tail in BLM A<sub>5</sub> has been implicated in its very high uptake in certain cancer cells that overexpress polyamine receptors.<sup>73</sup>

The inhibition experiments described in Figure 2.4 were performed to investigate whether the selective uptake of BLM and BLM-disaccharide in cancer cells is mediated by cell surface receptor that recognizes the BLM-disaccharide moiety. The cells were preincubated with high concentrations (50 mM) of disaccharide **2.69** to effectively saturate any cell surface receptor. The uptake of BLM-Cy5\*\* and BLM-disaccharide-Cy5\*\* was then studied in these preincubated cells. Pretreatment of the cancer cells with **2.69** blocked the uptake of both of the dye conjugates, suggesting that the carbohydrate moiety of BLM might be involved in the cellular recognition process. When the cells were preincubated with D-glucamine there was no inhibition of uptake of the Cy5\*\* conjugates. Further studies are required to identify this potential receptor that preferentially interacts with the BLM-disaccharide.

The carbamoyl moiety present on the mannose residue of BLM-disaccharide is quite uncommon among naturally occurring carbohydrates. The fluorescent conjugate decarbamoylBLM-disaccharide-Cy5\*\* was prepared to define the importance of the carbamoyl group in the cancer cell recognition process. The uptake of this conjugate was compared with that of BLM-disaccharide-Cy5\*\* in four different cancer cell lines. It is obvious from Figure 2.5 that the carbamoyl group is indispensable for uptake of the fluorescent conjugate into cancer cells. Presumably, this group participates actively in the interaction of the BLM-disaccharide with potential cell surface receptors.

Having clearly established the ability of the BLM-disaccharide to target cancer cells, it was of interest to employ this sugar as a tumor imaging agent or a

drug delivery vehicle. The BLM-disaccharide itself was not suitable for these applications due to its relatively low uptake. This prompted us to manipulate the linker region of the BLM-disaccharide-Cy5\*\* conjugate to allow optimal uptake of the disaccharide. Two different strategies were employed. The first approach was directed towards reducing the formal net negative charge on the dye conjugate by introducing lysine residues in the linker. Another strategy involved the use of polyvalency by employing a branched linker.

The dye conjugate Cy5\*\*-L-lysyl-L-lysyl-BLM-disaccharide (**2.39**) has a L-lysyl-L-lysine dipeptide inserted between the disaccharide and the dye. The side chain amine groups should be protonated at pH ~ 7. This should help reduce the electrostatic repulsion between the dye conjugate and the negatively charged cell membrane and improve its uptake. Figure 2.6 shows that introduction of the dipeptide did not result in any significant increase in uptake in DU-145 prostate cancer cells. The reason for the low uptake could be the hydrolytic degradation of the dipeptide by the peptidases present in the cell culture media. Cy5\*\*-D-lysyl-D-lysyl-BLM-disaccharide (**2.48**) was synthesized to overcome this problem. The dipeptide present in this molecule consists of two D-lysines and should be resistant to peptidase activities. Figure 2.6 shows that introduction of two D-lysines caused almost a twofold increase in binding/uptake. Once again, none of the conjugates showed any significant binding to normal cell lines, suggesting that their selectivity for the cancer cells was still intact.

The trimer BLM-disaccharide-Cy5\*\* (**2.59**) connects three sugars to a single Cy5\*\* molecule through a branched linker. The binding/uptake profile of

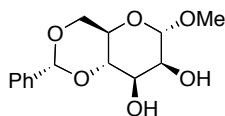


this conjugate was examined in DU-145 prostate cancer cells and was found to be almost 2.5 times that of BLM-disaccharide-Cy5\*\*. This result suggests that the potential receptor on the cell surface probably interacts with multiple sugar residues simultaneously. The conjugate **2.59** probably increases the local concentration of the BLM-disaccharide around the dye molecule, thereby facilitating a polyvalent interaction with the cell surface receptor. High selectivity towards cancer cells makes this conjugate a potential candidate for further development as an *in vivo* tumor imaging agent or as a targeted drug delivery vehicle.

#### **2.4. EXPERIMENTAL**

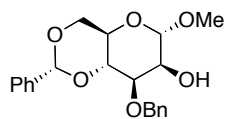
All the chemicals were purchased from Aldrich Chemical Co., Sigma Chemical Co or TCI America and used without further purification. Dichloromethane was dried by distillation from calcium hydride and tetrahydrofuran was dried by distillation from sodium. All reactions involving air or moisture sensitive reagents or intermediates were performed under an argon atmosphere. Flash chromatography was carried out using Silicycle 200–400 mesh silica gel. Analytical thin layer chromatography (TLC) was carried out using 0.25 mm EM Silica Gel 60 F<sub>250</sub> plates that were visualized by irradiation (254 nm) or by staining with Hanessian's stain (cerium molybdate). <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained using an Inova 400 MHz Varian instrument. Chemical shifts were reported in parts per million (ppm,  $\delta$ ) referenced to the residual <sup>1</sup>H resonance of the solvent (CDCl<sub>3</sub>, 7.26 ppm). <sup>13</sup>C spectra were referenced to the residual <sup>13</sup>C

resonance of the solvent (CDCl<sub>3</sub>, 77.16 ppm). Splitting patterns were designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublet; dt doublet of triplet; m, multiplet. HPLC was performed using an Agilent 1100 series instrument. High resolution mass spectra were obtained at the Arizona State University High Resolution Mass Spectrometry Laboratory or at the Michigan State University Mass Spectrometry Facility.



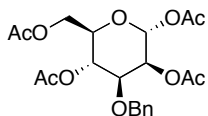
**Methyl 4,6-*O*-Benzylidene- $\alpha$ -D-mannopyranoside (2.3).**<sup>69</sup>

To a solution containing 5.08 g (26.2 mmol) of methyl- $\alpha$ -D-mannopyranoside in 62 mL of DMF were added 4.10 mL (27.3 mmol) of benzaldehyde dimethyl acetal and a catalytic amount of *p*-TsOH. The reaction mixture was swirled at 60 °C for 1 h under diminished pressure and was then poured into a stirred mixture containing 120 mL of ethyl acetate and 100 mL of satd aq NaHCO<sub>3</sub>. The organic layer was washed with three 50-mL portions of brine, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure to afford a crude, colorless oil. The residue was purified by flash chromatography on a silica gel column (25 × 4 cm). Elution with 2:1 hexanes–ethyl acetate afforded compound **2.3** as a colorless solid: yield 5.05 g (68%); silica gel TLC *R*<sub>f</sub> 0.31 (1:1 ethyl acetate–hexanes); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  2.90 (s, 2H), 3.38 (s, 3H), 3.78 (m, 2H), 3.87 (m, 1H), 3.98 (m, 2H), 4.25 (m, 1H), 4.72 (d, 1H), 5.55 (s, 1H), 7.36 (m, 3H) and 7.47 (m, 2H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  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.



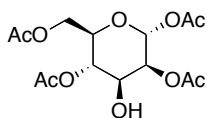
**Methyl 4,6-*O*-Benzylidene-3-*O*-benzyl- $\alpha$ -D-mannopyranoside (2.4).**<sup>69</sup>

To a solution containing 2.40 g (8.52 mmol) of **2.3** in 71 mL of MeOH was added 2.34 g (9.35 mmol) of Bu<sub>2</sub>SnO and the reaction mixture was heated to reflux for 1.5 h to afford a clear solution. The solvent was concentrated under diminished pressure and the resulting solid was dried under vacuum overnight. The white residue was dissolved in 71 mL of DMF, treated with 2.03 mL (1.4 g, 17.0 mmol) of BnBr, and heated to 100 °C for 45 min. The reaction mixture was cooled to room temperature and poured into a stirred mixture of 100 mL of ethyl acetate and 70 mL of satd aq NaHCO<sub>3</sub>. The organic phase was separated and washed with 60 mL of brine, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 4 cm). Elution with 30% ethyl acetate in hexanes afforded compound **2.4** as a colorless oil: yield 2.10 g (66%); silica gel TLC *R*<sub>f</sub> 0.30 (30% ethyl acetate in hexanes); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  3.06 (s, 1H), 3.36 (s, 3H), 3.79 (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); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  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- $\alpha$ -D-mannopyranose (2.5).**<sup>69</sup>

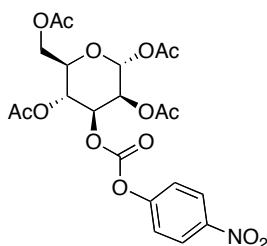
To a solution containing 0.80 g (4.40 mmol) of intermediate **2.4** in 13 mL of Ac<sub>2</sub>O was added a catalytic amount of H<sub>2</sub>SO<sub>4</sub> and the solution was stirred at room temperature for 1 h. The reaction mixture was poured into a stirred mixture of 100 mL of ethyl acetate and 60 mL of satd aq NaHCO<sub>3</sub>. The organic phase was separated and washed with 30 mL of brine, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (24 × 3 cm). Elution with 2:1 hexanes–ethyl acetate afforded the mannose derivative **2.5** as a yellow oil: yield 0.81 g (86%); silica gel TLC *R<sub>f</sub>* 0.34 (30% ethyl acetate in hexanes); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.02 (s, 3H), 2.07 (s, 3H), 2.11 (s, 3H), 2.15 (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), 5.24 (m, 1H), 5.34 (dd, 1H, *J* = 3.4 and 2.1 Hz), 6.09 (d, 1H, *J* = 2.0 Hz) and 7.24–7.37 (m, 5H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 14.4, 21.0, 21.1, 21.1, 62.6, 67.1, 67.2, 71.9, 71.7, 74.3, 91.2, 128.0, 128.2, 128.6, 137.6, 168.3, 169.8, 170.2 and 171.0.



**1,2,4,6-Tetra-*O*-acetyl- $\alpha$ -D-mannopyranose (**2.6**).**<sup>69</sup>

To a solution containing 0.88 g (2.00 mmol) of compound **2.5** in 24 mL of ethyl acetate was added a catalytic amount of Pd(OH)<sub>2</sub>/C and the reaction was maintained under 1 atm of H<sub>2</sub>(g) overnight. The catalyst was removed by filtration through a pad of Celite and the filtrate was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 3 cm). Elution with 75% ethyl acetate in hexanes afforded

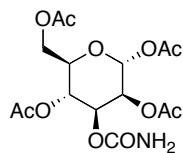
compound **2.6** as a colorless oil: yield 550 mg (79%); silica gel TLC  $R_f$  0.11 (1:1 ethyl acetate–hexanes);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.04 (s, 3H), 2.09 (s, 3H), 2.13 (s, 3H), 2.15 (s, 3H), 2.97 (s, 1H), 3.95 (m, 1H), 4.04 (m, 1H), 4.09 (m, 1H), 4.19 (dd, 1H,  $J = 12.3$  and 4.8 Hz), 5.07 (m, 1H), 5.13 (m, 1H) and 5.99 (m, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  20.7, 20.8, 62.3, 68.0, 68.6, 70.3, 70.9, 90.4, 168.2, 170.3, 170.8 and 170.9.



**1,2,4,6-Tetra-*O*-acetyl-3-*O*-((*p*-nitrophenyl)carbamoyl)- $\alpha$ -D-mannopyranose (2.7).**<sup>69</sup>

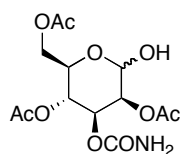
To a solution containing 0.55 g (1.60 mmol) of **2.6** in 5.6 mL of pyridine were added 0.77 g (6.30 mmol) of DMAP and 1.30 g (6.30 mmol) of *p*-nitrophenyl chloroformate. The reaction mixture was stirred at 40 °C for 2 h at which time it was poured into a two-phase solution of 40 mL of ethyl acetate and 10 mL of  $\text{H}_2\text{O}$ . The organic layer was washed successively with three 10-mL portions of 1 N HCl, 10 mL of satd aq  $\text{NaHCO}_3$  and 10 mL of brine. The solution was dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20  $\times$  3 cm). Elution with 50% ethyl acetate in hexanes afforded compound **2.7** as a yellow oil: yield 0.66 g (81%); silica gel TLC  $R_f$  0.58 (1:1 ethyl acetate–hexanes);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.07 (s, 3H), 2.09 (s, 3H), 2.14 (s, 3H), 2.17 (s, 3H), 4.08 (m, 2H), 4.25 (m, 1H),

5.15 (dd, 1H), 5.41 (m, 2H), 6.11 (s, 1H), 7.34 (d, 2H) and 8.23 (d, 2H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  20.6, 20.9, 61.8, 64.9, 67.4, 70.5, 74.1, 90.5, 121.8, 125.2, 145.5, 151.6, 155.1, 167.8, 169.3, 169.9 and 170.5.



**1,2,4,6-Tetra-*O*-acetyl-3-*O*-carbamoyl- $\alpha$ -D-mannopyranose (2.8).**<sup>69</sup>

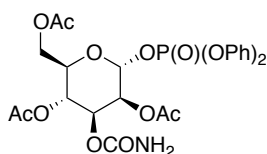
To a solution of 0.51 g (1.31 mmol) of carbonate **2.7** in 27 mL of anhydrous  $\text{CH}_2\text{Cl}_2$  was added 15 mL of THF that had been saturated with  $\text{NH}_3$  (g). The solution was stirred at room temperature for 1.5 h (at which time silica gel TLC analysis indicated that the reaction was complete). The solution was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (14  $\times$  3 cm). Elution with 3:1  $\rightarrow$  1:2 hexanes–ethyl acetate afforded compound **2.8** as a colorless oil: yield 355 mg (91%); silica gel TLC  $R_f$  0.10 (1:1 hexanes–ethyl acetate).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.02 (s, 3H), 2.04 (s, 3H), 2.11 (s, 3H), 2.12 (s, 3H), 4.04 (m, 2H), 4.22 (dd, 1H,  $J = 12.6$  and 5.0 Hz), 5.03 (br s, 2H), 5.24 (m, 3H) and 6.03 (d, 1H,  $J = 1.7$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  20.6, 20.6, 20.7, 61.9, 65.4, 68.6, 69.4, 70.4, 90.3, 155.2, 168.0, 169.6, 169.6 and 170.5.



**2,4,6-Tri-*O*-carbamoyl- $\alpha,\beta$ -D-mannopyranose (2.9).**<sup>69</sup>

To a solution of 365 mg (0.93 mmol) of compound **2.8** in 10.5 mL of dry DMF was added 120 mg (1.31 mmol) of acetate salt of hydrazine. The reaction mixture

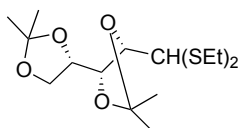
was stirred at room temperature for 1 h (at which time silica gel TLC analysis indicated that **2.8** had been consumed) and diluted with 80 mL of ethyl acetate. The solution was washed with three 25-mL portions of brine and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 1:1 hexanes–ethyl acetate afforded compound **2.9** as a colorless oil: yield 285 mg (87%); silica gel TLC  $R_f$  0.24 (1:1 hexanes–ethyl acetate).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.06 (s, 3H), 2.09 (s, 3H), 2.15 (s, 3H), 4.15 (m, 1H), 4.23 (m, 2H), 4.83 (s, 2H) and 5.25 (m, 4H).



**2,4,6-Tri-*O*-acetyl-3-*O*-carbamoyl- $\alpha$ -D-mannopyranosyl Diphenyl Phosphate (**2.10**).**<sup>69</sup>

To a solution of 160 mg (0.46 mmol) of intermediate **2.9**, 64.0 mg (0.57 mmol) of DMAP and 640  $\mu\text{L}$  (468 mg; 4.63 mmol) of  $\text{Et}_3\text{N}$  in 12 mL of  $\text{CH}_2\text{Cl}_2$  at 0 °C was dropwise added 0.95 mL (1.23 g; 4.6 mmol) of diphenyl chlorophosphate. The solution was stirred at 0 °C for 1.5 h and was poured into a two-phase solution of EtOAc (100 mL) and saturated aq  $\text{NaHCO}_3$  (40 mL). The organic layer was washed with two 30-mL portions of brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 2:1  $\rightarrow$  1:2 hexanes–ethyl acetate afforded the phosphate ester **2.10** as a colorless oil: yield 201 mg (75%); silica gel TLC  $R_f$  0.41 (2:3 hexanes–ethyl acetate).  $^1\text{H}$  NMR

(CDCl<sub>3</sub>)  $\delta$  1.95 (s, 3H), 2.03 (s, 3H), 2.12 (s, 3H), 3.91 (d, 1H,  $J$  = 12.4 and 2.2 Hz), 4.08 (m, 1H), 4.17 (dd, 1H,  $J$  = 12.4 and 4.7 Hz), 4.66 (br s, 2H), 5.30 (m, 3H), 5.87 (dd, 1H,  $J$  = 6.5 and 1.6 Hz) and 7.28 (m, 10H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  20.6, 20.7, 20.7, 61.7, 65.3, 69.0, 69.1, 69.2, 70.7, 96.0, 96.1, 120.1, 120.1, 120.2, 120.3, 125.8, 125.9, 130.0, 129.0, 150.0, 150.1, 150.2, 150.3, 155.2, 169.5, 169.8 and 170.6.

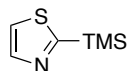


**2,3,4,5-Di-O-isopropylidene-L-xylose Diethylthioacetal (2.12).<sup>69</sup>**

To a suspension of 5.0 g (33 mmol) of L-xylose (**2.11**) in 3.2 mL of conc HCl was added with vigorous magnetic stirring, 7.4 mL (6.38 g; 0.10 mol) of ethanethiol. Stirring was continued at room temperature until the two-layer mixture gave a homogenous solution (usually after 15-20 min) which was then diluted with 120 mL of acetone. After stirring for 5 h, the solution was neutralized with satd aq NH<sub>4</sub>OH solution and co-evaporated with six 20-mL portions of toluene several times to remove water and most of acetone-diethyl dithioacetal to afford a crude residue. The crude residue was applied to a silica gel column (28 × 5 cm). Elution with 1:1 ethyl acetate–hexanes gave compound **2.12** as a colorless syrup: yield 5.23 g (52 %); silica gel TLC  $R_f$  0.59 (3:1 ethyl acetate–hexanes); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.23-1.28 (m, 6H), 1.36 (s, 3H), 1.41 (s, 6H), 1.45 (s, 3H), 2.72 (m, 4H), 3.91 (dd, 2H,  $J$  = 9.8 and 4.5 Hz), 4.04 (m, 1H), 4.13 (dd, 1H,  $J$  = 5.3 and



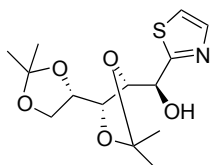
2.1 Hz) and 4.32 (m, 2H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.26, 14.34, 24.9, 25.3, 25.6, 26.1, 27.1, 27.3, 53.0, 65.9, 75.2, 78.7, 80.1, 109.5 and 110.0.



**2-(Trimethylsilyl)thiazole (2.13).**<sup>74</sup>

A 500-mL, four-necked round bottomed flask, containing a magnetic stirring bar, was equipped with two 100 mL, pressure-equalizing dropping funnels and a low-temperature thermometer. The anhydrous apparatus was filled with argon and kept under a slightly positive pressure of this gas during the entire reaction. The flask was charged with 80 mL of freshly distilled  $\text{Et}_2\text{O}$  and 42 mL (67.1 mmol) of a 1.6 M solution of *n*-BuLi in hexane. One of the two dropping funnels is charged with 5.5 mL (10 g, 61 mmol) of 2-bromothiazole in 20 mL of  $\text{Et}_2\text{O}$  and the other with 7.7 mL (6.6 g; 61 mmol) of chlorotrimethylsilane in 20 mL of  $\text{Et}_2\text{O}$ . The reaction flask was cooled to  $-78\text{ }^\circ\text{C}$  in an anhydrous acetone bath. While the solution in the flask was stirred, 2-bromothiazole was added dropwise over a period of 1 h. After 20 min of additional stirring, chlorotrimethylsilane was added dropwise over 30 min and the stirring was continued for 1 h at  $-78\text{ }^\circ\text{C}$ . The resulting mixture was then allowed to warm to room temperature. A satd aq  $\text{NaHCO}_3$  solution was added and the mixture was transferred into a 1-L separatory funnel. After the mixture was shaken, the organic layer is recovered and the aqueous layer was extracted with two 200-mL portions of  $\text{Et}_2\text{O}$ . The combined organic layer was dried ( $\text{Na}_2\text{SO}_4$ ), filtered, and concentrated under diminished pressure with the external bath temperature not exceeding  $40\text{ }^\circ\text{C}$ . The residue was

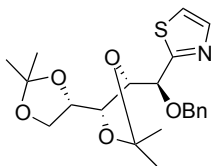
distilled from a 100 mL flask at reduced pressure in a Claisen apparatus. The distillation was carried out under diminished pressure at 45 °C after a forerun at 25 °C consisting mainly of bromobutane was collected. The pure product **2.13** was isolated as a colorless oil: yield 6.5 g (67%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.39 (s, 12H), 7.50 (1H, d, *J* = 3.0 Hz) and 8.09 (1H, d, *J* = 2.9 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 1.03, 127.3, 145.6 and 174.2.



**(1S)-((4R,4'S)-2,2,2',2'-Tetramethyl-[4,4'-bi(1,3-dioxolan)]-5-yl)(thiazol-2-yl)methanol (*anti*-6) (2.14).**<sup>69</sup>

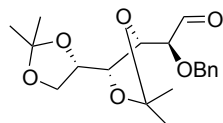
To a stirred solution containing 5.01 g (14.8 mmol) of thioacetal **2.12** in 50 mL of acetone diluted with 5 mL of water were added 7.6 g (35 mmol) of yellow mercury(II) oxide and 7.6 g (28 mmol) of mercuric(II) chloride. The reaction mixture was stirred at 55 °C for 2 h and then allowed to cool to room temperature. The solvent was filtered through a pad of Celite and the filtrate was concentrated under diminished pressure to afford a crude residue. The crude residue was suspended in three 60-mL portions of dichloromethane and filtered through a pad of Celite. The filtrate was washed with 40 mL of 1 M aq KI, dried (Na<sub>2</sub>SO<sub>4</sub>) and then concentrated under diminished pressure to afford 2,3,4,5-di-*O*-isopropylidene-*aldehydo*-L-xylose: crude yield 2.8 g. The crude aldehyde was used for the next reaction immediately without further purification.

To a stirred solution containing 2.8 g (12.2 mmol) of crude aldehyde in 38 mL of anhydrous dichloromethane cooled to  $-20\text{ }^{\circ}\text{C}$  was added 2.5 mL (2.46 g; 15.7 mmol) of 2-(trimethylsilyl)thiazole (**2.13**) dropwise over a period of 15 min. The solution was stirred at  $0\text{ }^{\circ}\text{C}$  for 1 h and then concentrated under diminished pressure to afford a crude residue. The crude residue was dissolved in 50 mL of anhydrous THF and treated with 3.8 g (12 mmol) of *n*-Bu<sub>4</sub>NF•3H<sub>2</sub>O at room temperature for 30 min, then concentrated under diminished pressure. The residue was dissolved in 300 mL of dichloromethane. The solution was washed with three 80-mL portions of water and dried (Na<sub>2</sub>SO<sub>4</sub>) and then concentrated under diminished pressure to yield **2.14** as a crude residue. Repeated recrystallization of the crude residue from cyclohexane afforded intermediate **2.14** as a colorless crystals: yield 2.7g (57% over two steps); silica gel TLC *R*<sub>f</sub> 0.49 (1:1 ethyl acetate–hexanes); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.28 (s, 3H), 1.36 (s, 6H), 1.40 (s, 3H), 3.67 (t, 1H, *J* = 6.6 Hz), 3.81 (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); <sup>13</sup>C NMR (CDCl<sub>3</sub>) 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.



**2-((1S)-(Benzyloxy))((4R,4'S)-2,2,2',2'-tetramethyl-[4,4'-bi(1,3-dioxolan)]-5-yl)methylthiazole (2.15).**<sup>69</sup>

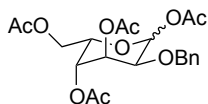
To a solution containing 2.65 g (8.40 mmol) of compound **2.14** in anhydrous DMF, cooled to 0 °C, was added 0.67 g (60% dispersion in oil, 17 mmol) of NaH portionwise and the reaction mixture was stirred for 0.5 h. To this solution was added 1.50 mL (2.16 g, 12.6 mmol) of benzyl bromide and the reaction mixture stirred at room temperature for 0.5 h. The reaction mixture was quenched by the addition of 1.5 mL methanol, stirred for 10 min and then diluted with 50 mL of distilled water. The aqueous layer was extracted with three 140-mL portions of ether. The combined organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure to afford a crude residue. The crude residue was purified by flash chromatography on a silica gel column (25 × 4 cm). Elution with 6:1 ethyl acetate–hexanes gave **2.15** as colorless solid: yield 2.8 g (82%); silica gel TLC *R<sub>f</sub>* 0.36 (9:1 toluene–methanol); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.20 (s, 3H), 1.25 (s, 3H), 1.29 (s, 3H), 1.33(s, 3H), 3.65(m, 1H), 3.77 (m, 1H), 3.91 (m, 1H), 3.97 (m, 1H), 4.35 (dd, 1H, *J* = 7.3 and 2.5 Hz), 4.47 (m, 2H), 4.80 (d, 1H, *J* = 4.8 Hz), 7.24 (m, 5H), 7.32 (d, 1H, *J* = 3.2 Hz) and 7.78 (d, 1H, *J* = 3.2 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>) 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.



**3,4:5,6-Di-*O*-isopropylidene-2-*O*-(benzyl)-aldehyde-L-gulose (**2.16**).**<sup>69</sup>

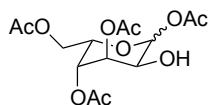
A solution containing 3.80 g (9.30 mmol) of *O*-benzyl ether **2.15** and 17.4 g of activated 4Å Molecular Sieves dissolved in 93 mL of anhydrous acetonitrile was

stirred at room temperature for 10 min and then 1.37 mL (2.05 g; 12.1 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 93 mL of methanol cooled to 0 °C was added 0.75 g (20.0 mmol) of sodium borohydride. The reaction mixture was stirred at room temperature for 5 min and then diluted with 31 mL of acetone. The solvent was filtered through a pad of Celite and concentrated under diminished pressure to afford a crude mixture of thiazolidines. The crude mixture obtained was dissolved in 87 mL of acetonitrile and 8.7 mL of water and treated under vigorous stirring with 5.98 g (74.8 mmol) of CuO and 1.62 g (9.34 mmol) of CuCl<sub>2</sub>•2H<sub>2</sub>O. The reaction mixture was stirred at 20 °C for 15 min and filtered through a pad of Celite 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 310-mL portions of ether and the liquid phase separated and filtered through a pad of Florisil (60–100 mesh) to afford a colorless solution. After a further washing of the Florisil with 310 mL of ethyl acetate, the combined organic layer was concentrated under diminished pressure to yield the crude aldehyde **2.16** as a brown syrup which was used immediately for the next reaction without further purification: crude yield 2.23 g.



**1,3,4,6-Tetra-*O*-acetyl-2-*O*-benzyl-*L*-gulopyranose (2.17).**<sup>69</sup>

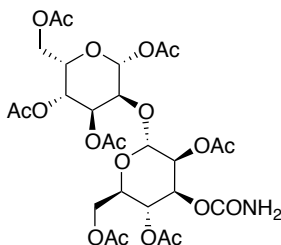
A solution containing 2.23 g (6.36 mmol) of crude aldehyde **2.16** was dissolved in 35 mL of glacial acetic acid and 9 mL of distilled water and stirred at 100 °C for 40 min. The cooled reaction mixture was then concentrated by co-evaporation three times with portions of toluene to afford the crude 2-*O*-benzyl-*L*-gulose as a mixture of  $\beta$ -pyranose,  $\alpha$ -pyranose and furanose forms. A solution of the crude residue and 0.76 g (6.36 mmol) of 4-(*N,N*-dimethylamino)pyridine in 16 mL of pyridine and 16 mL of acetic anhydride was stirred at room temperature for 12 h and concentrated under diminished pressure to yield a brown syrup. The crude residue was purified by flash chromatography on a silica gel column (30 × 4 cm). Elution with 3:1 ethyl acetate–hexanes gave **2.17** as a yellow oil: yield 2.14 g (52% from compound **2.15**); silica gel TLC  $R_f$  0.44 (1:1 ethyl acetate–hexanes);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  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), 4.04 (m, 2H), 4.28 (m, 1H), 4.55 (m, 2H), 4.95 (dd, 1H,  $J = 3.9$  and 2.5 Hz), 5.44 (m, 1H), 5.89 (d, 1H,  $J = 8.3$  Hz) and 7.28 (m, 5H).



**1,3,4,6-Tetra-*O*-acetyl-*L*-gulopyranose (**2.18**).**<sup>69</sup>

To a solution containing 1.73 g (3.95 mmol) of **2.17** in 27 mL of ethyl acetate was added a catalytic amount of 10% Pd/C and the reaction mixture was stirred at room temperature overnight under 1 atm of  $\text{H}_2$  (g). The solvent was filtered through a pad of Celite and the filtrate concentrated under diminished pressure to afford **2.18** as a mixture of  $\beta$ -pyranose,  $\alpha$ -pyranose and furanose forms which was used in the next reaction without further purification: yield 1.21 g (88%); silica

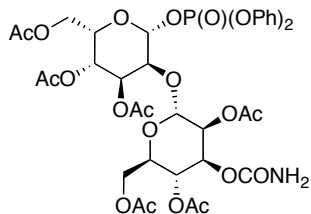
gel TLC  $R_f$  0.52 (ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.91 (s, 3H), 2.00 (s, 3H), 2.03 (s, 6H), 3.32 (br s, 1H), 3.80 (dd, 1H,  $J = 8.4$  and 3.5 Hz), 3.94 (m, 1H), 4.02 (m, 1H), 4.16 (m, 1H), 4.85 (m, 1H), 5.19 (t, 1H,  $J = 3.6$  Hz) and 5.70 (d, 1H,  $J = 8.4$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  20.4, 20.5, 20.6, 20.8, 61.6, 66.2, 67.5, 69.5, 70.9, 92.1, 169.4, 169.5, 169.7 and 170.5.



**1,3,4,6-Tetra-*O*-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl- $\alpha$ -D-mannopyranosyl)- $\alpha$ -L-gulopyranoside (**2.19**).**<sup>69</sup>

To a round bottom flask containing 200 mg (0.34 mmol) of **2.10** was added a solution of 95.0 mg (0.27 mmol) of **2.18** in 3.8 mL of anhydrous  $\text{CH}_2\text{Cl}_2$ . The solution was cooled to 0 °C and to it was added 98.0  $\mu\text{L}$  (120 mg; 0.55 mmol) of TMSOTf dropwise and the reaction mixture was stirred at 0 °C for 17 min at which time it was poured into a two-phase solution of EtOAc (60 mL) and saturated aq  $\text{NaHCO}_3$  (25 mL). The organic layer was washed with two 20-mL portions of brine, dried ( $\text{Na}_2\text{SO}_4$ ), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25  $\times$  2 cm). Elution with 3:2  $\rightarrow$  1:3 hexanes–ethyl acetate afforded the disaccharide **2.19** as a colorless oil: yield 115 mg (62%); silica gel TLC  $R_f$  0.38 (1:4 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.05 (s, 3H), 2.06 (s, 3H), 2.07

(s, 3H), 2.14 (s, 6H), 2.16 (s, 3H), 2.20 (s, 3H), 3.98 (dd, 1H,  $J = 8.4$  and  $3.3$  Hz), 4.19 (m, 2H), 4.38 (m, 1H), 4.85 (s, 2H), 5.13 (m, 7H), 5.45 (m, 1H) and 5.88 (d, 1H,  $J = 8.4$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.4, 20.9, 21.0, 21.0, 21.2, 21.3, 60.6, 61.6, 62.3, 65.7, 66.1, 67.9, 69.3, 69.4, 69.9, 71.5, 90.8, 95.2, 155.4, 168.9, 169.5, 169.6, 170.0, 170.7 and 170.8.



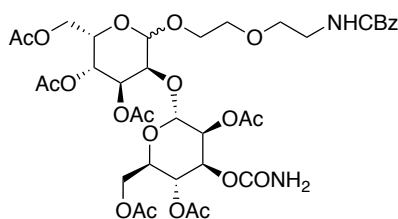
**3,4,6-Tri-*O*-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl- $\alpha$ -D-mannopyranosyl)- $\alpha$ -L-gulopyranosyl Diphenyl Phosphate (2.20).<sup>69</sup>**

To a solution containing 112 mg (0.165 mmol) of **2.19** in 0.8 mL of anhydrous DMF was added 21 mg (0.23 mmol) of acetate salt of hydrazine. The reaction mixture was stirred at room temperature for 1 h and quenched by the addition of 60 mL of ethyl acetate. The organic layer was washed with three 10-mL portions of brine and dried ( $\text{Na}_2\text{SO}_4$ ). The solvent was filtered and then concentrated under diminished pressure to afford the deacetylated intermediate as a crude residue which was used for next reaction without further purification.

To a solution of 115 mg of the crude residue, 26 mg (0.21 mmol) of DMAP and 242  $\mu\text{L}$  (177 mg, 1.75 mmol) of  $\text{Et}_3\text{N}$  in 16.5 mL of anhydrous  $\text{CH}_2\text{Cl}_2$  at  $0^\circ\text{C}$  was added 0.33 mL (428 mg, 1.59 mmol) of diphenyl chlorophosphate dropwise. The solution was stirred at  $0^\circ\text{C}$  for 1.5 h and was then poured into a two-phase solution of EtOAc (80 mL) and saturated aq  $\text{NaHCO}_3$



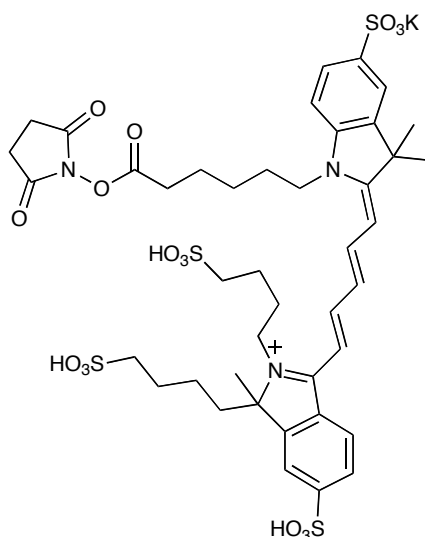
soln (30 mL). The organic layer was washed with three 25-mL portions of H<sub>2</sub>O, two 25-mL portions of brine, then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (22 × 2 cm). Elution with 1:1 → 1:3 hexanes–ethyl acetate afforded compound **2.20** a colorless oil: yield 121 mg (84%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 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); <sup>13</sup>C NMR (CDCl<sub>3</sub>) 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.



**3,4,6-Tri-*O*-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl- $\alpha$ -D-mannopyranosyl)- $\alpha$ -L-gulopyranosyl benzyl 2-(2-ethoxy)ethylcarbamate (2.22).**<sup>75</sup>

To a solution of 78 mg (91  $\mu$ mol) of **2.20** and 19 mg (79  $\mu$ mol) of **2.21** in 2.4 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> was added 28  $\mu$ L (34 mg, 0.16 mmol) of TMSOTf at 0 °C. The reaction mixture was stirred at 0 °C for 17 min, at which time it was poured into a two-phase solution of EtOAc (50 mL) and saturated aq NaHCO<sub>3</sub> (20 mL). The organic layer was washed with two 20-mL portions of brine, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated under diminished pressure. The residue was purified by

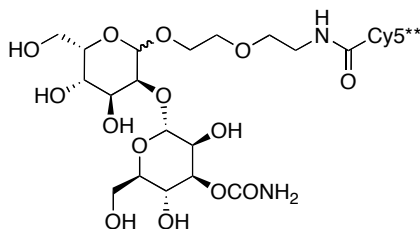
flash chromatography on a silica gel column (25 × 2 cm). Elution with 15:32:1 → 11:36:1 hexanes–ethyl acetate–methanol afforded compound **2.22** as a colorless oil: yield 62 mg (80%); silica gel TLC  $R_f$  0.30 (1:4 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.03 (s, 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);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  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.



**Cy5\*\* Carboxylic Acid Succinimidyl Ester (2.24).**<sup>76</sup>

To a 1.5 mL vial containing 0.50 mg (57 nmol) of Cy5\*\*COOH were added a solution of 5 mg (16.6  $\mu\text{mol}$ ) of TSTU in 100  $\mu\text{L}$  of DMF and a solution of 6  $\mu\text{L}$  (34.4  $\mu\text{mol}$ ) of DIPEA in 60  $\mu\text{L}$  of DMF. The mixture was shaken at room temperature for 2.5 h, diluted with ethyl acetate and centrifuged. The supernatant was discarded, the pellet was washed with ethyl acetate and dried under

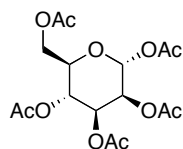
diminished pressure to afford Cy5\*\* carboxylic acid succinimidyl ester (Cy5\*\*COOSu) (**2.24**) as dark blue solid: yield 0.55 mg (100%); mass spectrum (MALDI) 984.55 (M)<sup>+</sup> (C<sub>42</sub>H<sub>54</sub>N<sub>3</sub>O<sub>16</sub>S<sub>4</sub><sup>+</sup> requires 984.24).



### BLM-disaccharide-Cy5\*\* (**2.1**).<sup>77</sup>

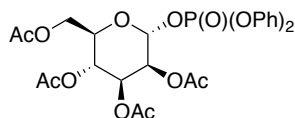
To a solution of 8.0 mg (9.3  $\mu$ mol) of **2.22** in 2 mL of dry MeOH was added 0.2 mL of a 25% w/w solution of NaOMe in MeOH and the mixture was shaken at room temperature for 2 h. Fifty mg of Dowex 50W resin was added and the mixture was shaken at room temperature for 30 min. The mixture was filtered, diluted to 5 mL with methanol and a catalytic amount of 10% Pd/C was added. H<sub>2</sub> gas was bubbled through the solution for 30 min, the mixture was filtered and concentrated to obtain compound **2.23** as colorless foam: yield 3.5 mg (80%). Compound **2.23** was dissolved in 2 mL of 0.2 M aq sodium phosphate buffer solution (pH ~ 8). One hundred  $\mu$ L (175  $\mu$ g; 0.37  $\mu$ mol) of this solution was added to a vial containing 110  $\mu$ g (0.112  $\mu$ mol) of Cy5\*\* carboxylic acid succinimidyl ester **2.24** and the mixture was shaken at room temperature overnight in the dark. The crude reaction mixture was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250  $\times$  10 mm, 10  $\mu$ m) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (1  $\rightarrow$  55% CH<sub>3</sub>CN in 0.1% aq TFA) over a period of 30 min at a flow rate of 3 mL/min.

Fractions containing the desired product eluted at 16.1 min (monitoring at 651 nm) and were collected, frozen, and lyophilized to give BLM-disaccharide-Cy5\*\* (2.1) as a blue solid: yield 62  $\mu\text{g}$  (42%); mass spectrum (MALDI),  $m/z$  1379.59 ( $M$ )<sup>+</sup> (theoretical  $m/z$  1379.36).



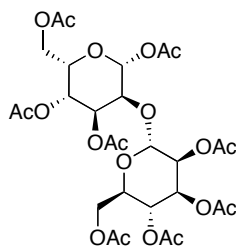
**Penta-*O*-acetyl- $\alpha$ -D-mannopyranose (2.25).**<sup>78</sup>

To a solution containing 1.09 g (5.60 mmol) of 1-*O*-methyl- $\alpha$ -D-mannopyranose in 18 mL of  $\text{Ac}_2\text{O}$  was added a catalytic amount of  $\text{H}_2\text{SO}_4$ , 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 satd aq  $\text{NaHCO}_3$ . The organic phase was separated and washed with 40 mL of satd aq  $\text{NaHCO}_3$ , 30 mL of brine, then dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (16  $\times$  3 cm). Elution with 4:1 hexanes–ethyl acetate afforded **2.25** as a light yellow oil: yield 2.10 g (96%); silica gel TLC  $R_f$  0.64 (30% ethyl acetate in hexanes);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  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);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  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.



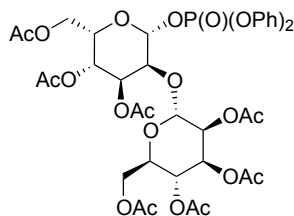
**2,3,4,6-Tetra-*O*-acetyl- $\alpha$ -D-mannopyranose (2.26).**<sup>79</sup>

To a solution of 300 mg (0.77 mmol) **2.25** in 10 mL of dry DMF was added 99.0 mg (1.07 mmol) of acetate salt of hydrazine. The reaction was stirred at room temperature for 1 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 aq layer was re-extracted with three 30-mL portions of ethyl acetate. The combined organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure. The residue was co-evaporated with portions of toluene several times, then dried and dissolved in 40 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. To the solution were added 117 mg (0.96 mmol) of DMAP and 1.14 mL (0.834 g; 8.19 mmol) of Et<sub>3</sub>N. The reaction mixture was stirred for 10 min, followed by the addition of 1.49 mL (1.93 g; 7.19 mmol) of diphenyl chlorophosphate dropwise at 0 °C. The solution was stirred at 0 °C for 1 h and was poured into a two-phase solution of EtOAc (200 mL) and saturated aq NaHCO<sub>3</sub> soln (80 mL). The organic layer was washed with two 50-mL portions of brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 3:1 → 2:1 hexanes–ethyl acetate afforded compound **2.26** as a colorless oil: yield 258 mg (58% over two steps); silica gel TLC *R*<sub>f</sub> 0.41 (3:1 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.96 (s, 3H), 1.99 (s, 3H), 2.03 (s, 3H), 2.15 (s, 3H), 3.91 (dd, 1H, *J* = 2.04 and 12.43 Hz), 4.07 (m, 1H), 4.17 (m, 1H), 5.32 (m, 3H), 5.87 (m, 1H), 7.23, (m, 6H) and 7.36 (m, 4H).



**1,3,4,6-Tetra-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl- $\alpha$ -D-mannopyranosyl)- $\alpha$ -L-gulopyranoside (**2.27**).**<sup>70</sup>

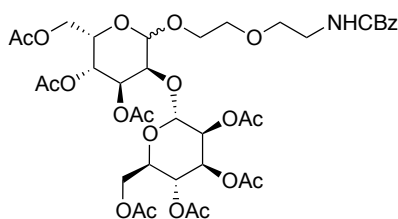
To a solution of 51 mg (0.15 mmol) of **2.18** 100 mg (0.17 mmol) of **2.26** in 1.3 mL of dry CH<sub>2</sub>Cl<sub>2</sub> at 0 °C was added 53.0  $\mu$ L (65.2 mg; 0.29 mmol) of TMSOTf dropwise and the reaction mixture was stirred at 0 °C for 17 min at which time it was poured into a two-phase solution of EtOAc (60 mL) and saturated aq NaHCO<sub>3</sub> (30 mL). The organic layer was washed with two 20-mL portions of brine, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25  $\times$  2 cm). Elution with 3:1  $\rightarrow$  1:1 hexanes–ethyl acetate afforded the disaccharide **2.27** as a colorless oil: yield 60 mg (60%); silica gel TLC *R*<sub>f</sub> 0.36 (1:2 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.93 (s, 3H), 1.99 (s, 3H), 2.01 (s, 3H), 2.10 (m, 12H), 2.15 (s, 3H), 3.95 (dd, 1H, *J* = 8.4, 3.0 Hz), 4.09 (m, 4H), 4.18 (t, 2H, *J* = 3.8 Hz), 4.32 (t, 1H, *J* = 6.5 Hz), 4.94 (s, 1H), 4.97 (d, 1H, *J* = 3.7 Hz), 5.05 (m, 1H), 5.11 (dd, 1H, *J* = 10.0 and 3.3 Hz), 5.23 (t, 1H, *J* = 10.0 Hz), 5.40 (t, 1H, *J* = 3.3 Hz) and 5.85 (d, 1H, *J* = 8.4 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  20.7, 20.78, 20.84, 20.9, 61.4, 62.2, 65.6, 65.8, 67.7, 68.7, 68.9, 69.3, 69.7, 71.4, 90.7, 95.1, 168.7, 169.32, 169.35, 169.5, 169.6, 169.9, 170.5 and 170.6.



**3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl- $\alpha$ -D-mannopyranosyl)- $\alpha$ -L-gulopyranosyl Diphenyl Phosphate (2.28).<sup>77</sup>**

To a solution of 30 mg (44  $\mu$ mol) of compound **2.27** in 1 mL of dry DMF was added 5.5 mg (60  $\mu$ mol) of acetate salt of hydrazine and the reaction mixture was stirred for 1 h at which time analysis by silica gel TLC indicated complete consumption of the disaccharide. The solution was diluted with 40 mL of EtOAc and washed with three 15-mL portions of brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure. The residue was co-evaporated with toluene, dried under diminished pressure and dissolved in 5 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. To the solution were added 6.5 mg (54  $\mu$ mol) of DMAP and 65  $\mu$ L (47 mg; 0.5 mmol) of Et<sub>3</sub>N; the mixture was stirred for 10 min, followed by addition of 86.0  $\mu$ L (111 mg; 0.41 mmol) of diphenyl chlorophosphate dropwise at 0 °C. The solution was stirred at 0 °C for 1 h and was then poured into a two-phase solution of EtOAc (50 mL) and saturated aq NaHCO<sub>3</sub> soln (20 mL). The organic layer was washed with two 15-mL portions of brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (22  $\times$  2 cm). Elution with 2:1  $\rightarrow$  1:1 hexanes–ethyl acetate afforded the activated ester **2.28** as a colorless oil: yield 28 mg (73% over two steps); silica gel TLC *R*<sub>f</sub> 0.28 (1:2 hexanes–ethyl acetate); <sup>1</sup>H

NMR (CDCl<sub>3</sub>) 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); <sup>13</sup>C NMR (CDCl<sub>3</sub>) 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; mass spectrum (ESI), *m/z* 869.2253 (M + H)<sup>+</sup> (C<sub>38</sub>H<sub>46</sub>O<sub>21</sub>P requires *m/z* 869.2264)

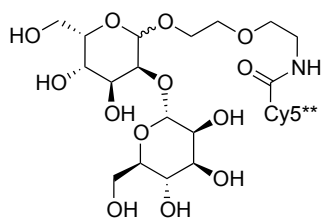


**3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl- $\alpha$ -D-mannopyranosyl)- $\alpha,\beta$ -L-gulopyranosyl benzyl 2-(2-ethoxy)ethylcarbamate (**2.29**).<sup>77</sup>**

To a solution of 26 mg (30  $\mu$ mol) of phosphate ester **2.28** and 6.5 mg (27  $\mu$ mol) of the alcohol **2.21** in 1.0 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> was added 10  $\mu$ L (12.3 mg; 54  $\mu$ mol) of TMSOTf at 0 °C. The reaction was stirred at 0 °C for 17 min and was then poured into a two-phase solution of EtOAc (50 mL) and saturated aq NaHCO<sub>3</sub> (20 mL). The organic layer was washed with two 20-mL portions of brine, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25  $\times$  2 cm). Elution with 32:16:1  $\rightarrow$  12:12:1 hexanes–ethyl acetate–methanol afforded compound **2.29** a colorless oil: yield 18 mg (70%); silica gel TLC *R*<sub>f</sub> 0.80



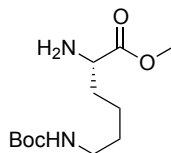
(12:12:1 hexanes–ethyl acetate–methanol);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.97 (s, 3H), 2.02 (s, 3H), 2.04 (s, 3H), 2.08 (s, 3H), 2.10 (s, 3H), 2.12 (s, 3H), 2.14 (s, 3H), 3.40 (m, 2H), 3.61 (m, 3H), 3.68 (m, 2H), 3.85 (m, 1H), 3.97 (m, 1H), 4.18 (m, 5H), 4.27 (m, 4H), 4.47 (t, 1H,  $J = 6.4$  and  $6.4$  Hz), 4.92 (d, 1H,  $J = 3.7$  Hz), 5.01 (m, 2H), 5.09 (s, 2H), 5.15 (s, 1H), 5.28 (m, 3H), 5.45 (s, 1H) and 7.33 (m, 5H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  20.61, 20.62, 20.70, 20.75, 20.8, 29.7, 40.9, 62.1, 62.5, 63.7, 65.5, 66.0, 66.6, 68.6, 68.6, 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; mass spectrum (ESI),  $m/z$  858.3036 ( $\text{M} + \text{H}^+$ ) ( $\text{C}_{38}\text{H}_{52}\text{NO}_{21}$  requires  $m/z$  858.3026)



### Decarbamoyl BLM-disaccharide-Cy5\*\* (2.30).

To a solution of 5.0 mg (5.8  $\mu\text{mol}$ ) of compound **2.29** in 2 mL of dry MeOH was added 0.2 mL of a 25% w/w solution of NaOMe in MeOH and the mixture was shaken at room temperature for 2 h. Fifty mg of Dowex 50W resin was added and the mixture was shaken at room temperature for 30 min. The mixture was filtered, diluted to 5 mL with methanol and catalytic amount of Pd/C was added.  $\text{H}_2$  gas was bubbled through the solution for 30 min and the mixture was filtered through a pad of Celite. The filtrate was concentrated to obtain the free amine as a colorless oil: crude yield 2 mg (73%). Two (2.0) mg of this compound was dissolved in 1 mL of 0.2 M aq sodium phosphate buffer solution (pH  $\sim$  8). One

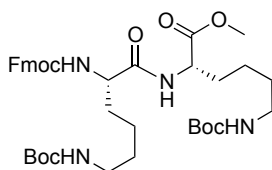
hundred  $\mu\text{L}$  of this solution was added to a vial containing 110  $\mu\text{g}$  of Cy5\*\* carboxylic acid succinimidyl ester (**2.24**) and the mixture was shaken at room temperature overnight in the dark. The crude reaction mixture was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250  $\times$  10 mm, 10  $\mu\text{m}$ ) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. The column was washed with 1  $\rightarrow$  55% CH<sub>3</sub>CN in 0.1% aq TFA over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 16.05 min (monitoring at 651 nm) and were collected, frozen, and lyophilized to give the dye conjugate **2.30** as a blue solid: yield 34  $\mu\text{g}$  (7%); mass spectrum (MALDI),  $m/z$  1336.99 (M)<sup>+</sup> (theoretical 1336.35); mass spectrum (ESI),  $m/z$  647.6858 (M – K – 2H)<sup>2-</sup> (C<sub>54</sub>H<sub>77</sub>N<sub>3</sub>O<sub>25</sub>S<sub>4</sub> requires  $m/z$  647.6870).



***N*<sup>ε</sup>-Boc-L-Lysine Methyl Ester (2.32).**<sup>80</sup>

To a mixture of 160 mg (0.34 mmol) of *N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-Boc-L-lysine (**2.31**) and 94 mg (0.68 mmol) of K<sub>2</sub>CO<sub>3</sub> in 4 mL of anhydrous DMF was added 64.0  $\mu\text{L}$  (145 mg; 1.0 mmol) of CH<sub>3</sub>I at room temperature. The reaction mixture was stirred at 50 °C for 2 h. The cooled reaction mixture was diluted with 50 mL of diethyl ether. The organic layer was washed with 20 mL of H<sub>2</sub>O, 20 mL of 0.1 N aq HCl and 15 mL of brine. The ether layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure. The crude *N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-Boc-L-lysine methyl ester was then dissolved in 3 mL of a solution of 20% piperidine in anhydrous DMF and the

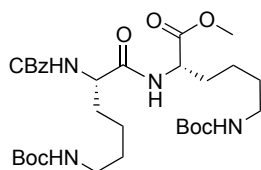
reaction mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (8 × 3 cm). Elution with 25:1 → 15:1 chloroform–methanol afforded compound **2.32** as a colorless oil: yield 82 mg (92%); silica gel TLC  $R_f$  0.46 (20:1 chloroform–methanol);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.32–1.77 (m, 15H), 3.10 (d, 2H,  $J = 6.1$  Hz), 3.43 (dd, 1H,  $J = 9.6$  and 3.7 Hz), 3.70 (s, 3H) and 4.58 (s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  22.8, 28.4, 29.8, 34.4, 40.3, 51.9, 54.2, 79.0, 155.9 and 176.4.



**$N^\alpha$ -Fmoc- $N^\epsilon$ -Boc-L-lysyl- $N^\epsilon$ -Boc-L-lysine Methyl Ester (2.33).**

To a solution of 60.0 mg (0.23 mmol) of compound **2.32**, 108 mg (0.23 mmol) of compound **2.31** and 99.0 mg (0.46 mmol) of 1,8-bis(dimethylamino)naphthalene (proton sponge) in 4 mL of anhydrous DMF was added 131 mg (0.35 mmol) of HATU. The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated under diminished pressure and the residue was diluted in 50 mL of diethyl ether. The ether layer was washed with three 15-mL portions of 1 N aq HCl, 20 mL of satd aq  $\text{NaHCO}_3$  soln and 20 mL of brine. The organic layer was then dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 2:1 → 1:1 hexanes–ethyl acetate afforded compound **2.33** as a colorless oil: yield 110 mg (67%); silica gel TLC  $R_f$  0.42 (1:1

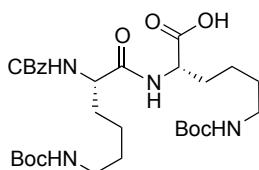
hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.41 (m, 26H), 1.66 (m, 2H), 1.84 (m, 2H), 3.06 (m, 4H), 3.66 (s, 3H), 4.40 (m, 4H), 4.54 (dd, 1H,  $J = 12.4$  and  $7.4$  Hz), 4.82 (s, 2H), 5.81 (s, 1H), 6.92 (d, 1H,  $J = 6.1$  Hz), 7.27 (m, 2H), 7.37 (t, 2H,  $J = 7.5$  Hz), 7.58 (d, 2H,  $J = 7.3$  Hz) and 7.73 (d, 2H,  $J = 7.5$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  22.3, 22.5, 28.47, 28.48, 29.4, 29.6, 31.5, 32.3, 39.9, 40.0, 47.1, 52.2, 52.4, 54.6, 67.2, 79.1, 119.98, 120.01, 125.16, 125.21, 127.1, 127.7, 141.28, 141.29, 143.8, 143.9, 156.20, 156.24, 156.3, 171.9 and 172.6; mass spectrum (ESI),  $m/z$  711.3976 ( $\text{M} + \text{H}$ ) $^+$  ( $\text{C}_{38}\text{H}_{55}\text{N}_4\text{O}_9$  requires  $m/z$  711.3964).



**$N^\alpha$ -CBz- $N^\epsilon$ -Boc-L-lysyl- $N^\epsilon$ -Boc-L-lysine Methyl Ester (2.34).**<sup>81</sup>

To a solution of 110 mg (0.15 mmol) of compound **2.33** in 2 mL of anhydrous DMF was added 0.4 mL of piperidine and the solution was stirred at room temperature for 1 h. The reaction mixture was concentrated under diminished pressure and the residue was co-evaporated with three 10-mL portions of toluene. To a solution of the residue in 3 mL of dry THF were added 0.22 mL (0.16 mg; 1.60 mmol) of anhydrous triethylamine and 0.11 mL (0.13 g, 0.77 mmol) of benzyl chloroformate. The reaction mixture was stirred at room temperature for 5 h. The reaction mixture was diluted with 60 mL of diethyl ether, washed with two 20-mL portions of water and 20 mL of brine. The ether layer was dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15  $\times$  3 cm). Elution with 2:1  $\rightarrow$  1:2

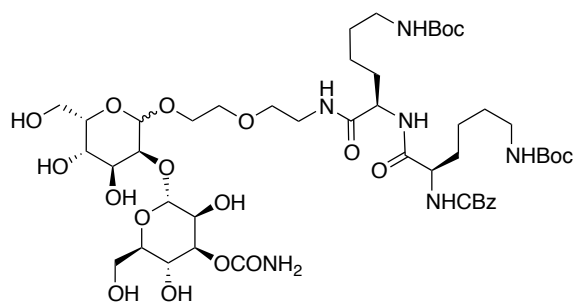
hexanes–ethyl acetate afforded the methyl ester **2.34** as a colorless oil: yield 82 mg (85%); silica gel TLC  $R_f$  0.50 (1:1 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.30–1.53 (m, 26H), 1.67 (dd, 3H,  $J = 13.7$  and  $7.1$  Hz), 1.83 (m, 2H), 3.06 (s, 4H), 3.72 (s, 3H), 4.21 (d, 1H,  $J = 5.8$  Hz), 4.67 (m, 3H), 5.10 (s, 2H), 5.64 (s, 1H), 6.75 (d, 1H,  $J = 6.9$  Hz) and 7.32 (m, 5H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  22.4, 22.5, 27.8, 28.5, 29.3, 29.5, 29.6, 31.7, 39.9, 40.1, 52.1, 54.8, 67.1, 79.2, 128.2, 128.3, 128.6, 136.3, 156.2, 156.3, 156.4, 171.9 and 172.7.



**$N^\alpha$ -CBz- $N^\epsilon$ -Boc-L-lysyl- $N^\epsilon$ -Boc-L-lysine (2.35).**<sup>82</sup>

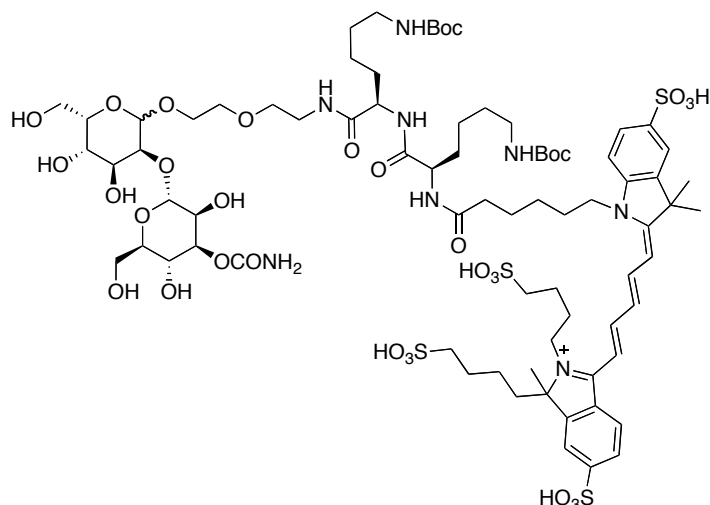
To a solution containing 82.0 mg (0.13 mmol) of compound **2.34** in 1.2 mL of THF was added a solution of 13 mg of LiOH (0.53 mmol) in 0.6 mL of water. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with 10 mL of  $\text{Et}_2\text{O}$  and 25 mL of water and the phases were separated. The cooled aqueous phase (ice bath) was acidified to pH  $\sim$  3 with 5% aq  $\text{NaHSO}_4$  soln and extracted with three 20-mL portions of ethyl acetate. The combined organic layer was then washed with 10 mL of brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under diminished pressure to afford the acid **2.35** as a colorless oil: yield 62 mg (77%); silica gel TLC  $R_f$  0.27 (1:3 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  1.44 (s, 26H), 1.68 (m, 2H), 1.86 (m, 2H), 3.01 (m, 4H), 4.14 (m, 1H), 4.38 (m, 1H), 5.09 (s, 2H) and 7.33 (m, 5H);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  24.0, 24.1, 28.8, 30.4, 30.5, 32.3, 32.9, 41.0, 41.1, 53.4,

53.5, 56.2, 67.6, 79.8, 128.8, 129.0, 129.4, 138.2, 158.4, 158.49, 158.53, 174.9 and 175.1.



***N*<sup>α</sup>-CBz-*N*<sup>ε</sup>-Boc-L-lysyl-*N*<sup>ε</sup>-Boc-L-lysyl-BLM-disaccharide (2.36).**

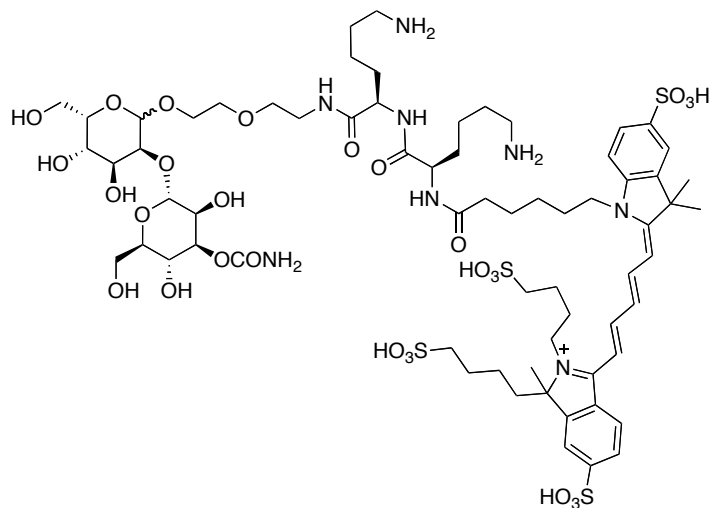
To a solution of 5.0 mg (10 μmol) of compound **2.23**, 4.5 mg (7.4 μmol) of the dipeptide **2.35** and 6.4 mg (30 μmol) of 1,8-bis(dimethylamino)naphthalene (proton sponge) in 0.2 mL of anhydrous DMF was added 5.6 mg (15 μmol) of HATU. The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. The column was washed with 1 → 55% CH<sub>3</sub>CN in 0.1% aq TFA over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 29.8 min (monitoring at 220 nm) and were collected, frozen, and lyophilized to give compound **2.36** as a colorless solid: yield 6 mg (76%); mass spectrum (ESI), *m/z* 1063.5287 (M + H)<sup>+</sup> (C<sub>47</sub>H<sub>79</sub>N<sub>6</sub>O<sub>21</sub> requires *m/z* 1063.5293).



***N*<sup>α</sup>-Cy5<sup>\*\*</sup>-*N*<sup>ε</sup>-Boc-L-lysyl-*N*<sup>ε</sup>-Boc-L-lysyl-BLM-disaccharide (**2.38**).**

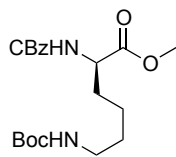
H<sub>2</sub> (g) was bubbled through a solution of 4.5 mg (4.2 μmol) of compound **2.36** in 2 mL of methanol for 10 min, and the reaction mixture was stirred for 30 min under a hydrogen atmosphere. The reaction mixture was filtered and the filtrate was concentrated under diminished pressure to obtain compound **2.37** as colorless solid: crude yield 4.2 mg. Two (2.0) mg of the amine **2.37** was dissolved in 0.8 mL of 0.2 M aq sodium phosphate buffer solution (pH ~ 8). Two hundred μL (0.5 mg; 0.54 μmol) of this solution was added to a vial containing 100 μg (0.10 μmol) of Cy5<sup>\*\*</sup> carboxylic acid succinimidyl ester (**2.24**) and the mixture was shaken at room temperature overnight in the dark. The crude reaction mixture was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–CH<sub>3</sub>CN → 45:55 0.1% aq TFA–CH<sub>3</sub>CN) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 20.2 min (monitoring at 651 nm) and were collected, frozen, and

lyophilized to give the dye conjugate **2.38** as a blue solid: yield 50  $\mu\text{g}$  (27%); mass spectrum (ESI),  $m/z$  897.3375 ( $M - 3H$ )<sup>2-</sup> ( $C_{77}H_{118}N_8O_{32}S_4$  requires  $m/z$  897.3373).



***N* <sup>$\alpha$</sup> -Cy5\*\*-L-lysyl-L-lysyl-BLM-disaccharide (2.39).**

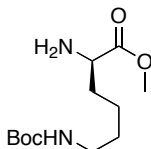
A solution of 32  $\mu\text{g}$  (18 nmol) of **2.38** in 200  $\mu\text{L}$  of 60% aq TFA was shaken at room temperature for 40 min and was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250  $\times$  10 mm, 10  $\mu\text{m}$ ) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–CH<sub>3</sub>CN  $\rightarrow$  45:55 0.1% aq TFA–CH<sub>3</sub>CN) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 16.1 min (monitoring at 651 nm) and were collected, frozen, and lyophilized to afford the dye conjugate **2.39** as a blue solid: yield 19  $\mu\text{g}$  (67%); mass spectrum (ESI),  $m/z$  797.2850 ( $M - 3H$ )<sup>2-</sup> ( $C_{67}H_{102}N_8O_{28}S_4$  requires  $m/z$  797.2850).





***N*<sup>α</sup>-CBz-*N*<sup>ε</sup>-Boc-D-lysine Methyl Ester (2.41).**<sup>83</sup>

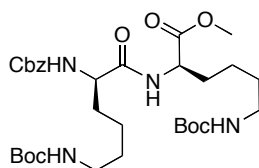
To a mixture of 149 mg (0.39 mmol) of *N*<sup>α</sup>-CBz-*N*<sup>ε</sup>-Boc-D-lysine (**2.40**) and 108 mg (0.78 mmol) of K<sub>2</sub>CO<sub>3</sub> in 4 mL of anhydrous DMF was added 73.0 μL (0.17 g; 1.2 mmol) of CH<sub>3</sub>I at room temperature. The reaction mixture was stirred at 50 °C for 2 h. The cooled reaction mixture was diluted with 50 mL of diethyl ether. The organic layer was washed with 20 mL of H<sub>2</sub>O, 20 mL of 0.1 N aq HCl and 15 mL of brine. The ether layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (12 × 3 cm). Elution with 3:1 → 1:1 hexanes–ethyl acetate afforded compound **2.41** as a colorless oil: yield 134 mg (87%); silica gel TLC *R*<sub>f</sub> 0.31 (3:1 hexanes–ethyl acetate); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 1.31–1.67 (m, 15H), 2.87 (m, 2H), 3.62 (m, 1H), 3.99 (s, 3H), 5.03 (s, 2H), 6.78 (m, 1H), 7.71 (m, 1H) and 7.36 (m, 5H).



***N*<sup>ε</sup>-Boc-D-lysine Methyl Ester (2.42).**<sup>83</sup>

H<sub>2</sub> (g) was bubbled through a mixture of 134 mg (0.34 mmol) of compound **2.41** and a catalytic amount of Pd/C in 8 mL of methanol for 10 min. The reaction mixture was stirred under an atmosphere of H<sub>2</sub> for 30 min and filtered through a pad of Celite. The filtrate was concentrated under diminished pressure to obtain compound **2.42** as a colorless oil: yield 79 mg (89%); silica gel TLC *R*<sub>f</sub> 0.46 (20:1 chloroform–methanol); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.32–1.77 (m, 15H), 3.10 (d, 2H, *J* =

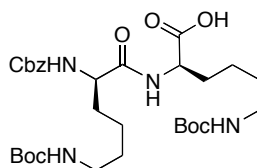
6.1 Hz), 3.43 (dd, 1H,  $J = 9.6$  and  $3.7$  Hz), 3.70 (s, 3H) and 4.58 (s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  22.8, 28.4, 29.8, 34.4, 40.3, 51.9, 54.2, 79.0, 155.9 and 176.4.



***N*<sup>α</sup>-CBz-*N*<sup>ε</sup>-Boc-D-lysyl-*N*<sup>ε</sup>-Boc-D-lysine Methyl Ester (2.43).**

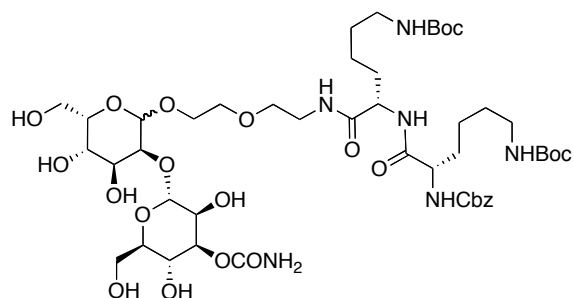
To a solution of 79 mg (0.30 mmol) of compound **2.42**, 109 mg (0.29 mmol) of compound **2.40** and 123 mg (0.57 mmol) of 1,8-bis(dimethylamino)naphthalene (proton sponge) in 4 mL of anhydrous DMF was added 163 mg (0.43 mmol) of HATU. The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated under diminished pressure and the residue was diluted in 80 mL of diethyl ether. The ether layer was washed with three 25-mL portions of 1 N aq HCl, 30 mL of satd aq NaHCO<sub>3</sub> soln and 20 mL of brine. The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 2:1 → 1:1 hexanes–ethyl acetate afforded the methyl ester **2.43** as a colorless oil: yield 150 mg (84%); silica gel TLC  $R_f$  0.50 (1:1 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.30–1.53 (m, 26H), 1.67 (dd, 3H,  $J = 13.7$  and  $7.1$  Hz), 1.83 (m, 2H), 3.06 (s, 4H), 3.72 (s, 3H), 4.21 (d, 1H,  $J = 5.8$  Hz), 4.67 (m, 3H), 5.10 (s, 2H), 5.64 (s, 1H), 6.75 (d, 1H,  $J = 6.9$  Hz) and 7.32 (m, 5H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  22.4, 22.5, 27.8, 28.5, 29.3, 29.5, 29.6, 31.7, 39.9, 40.1, 52.1, 54.8, 67.1, 79.2, 128.2, 128.3, 128.6, 136.3, 156.2, 156.3, 156.4, 171.9 and 172.7;

mass spectrum (ESI),  $m/z$  623.3652 ( $M + H$ )<sup>+</sup> ( $C_{31}H_{51}N_4O_9$  requires  $m/z$  623.3650).



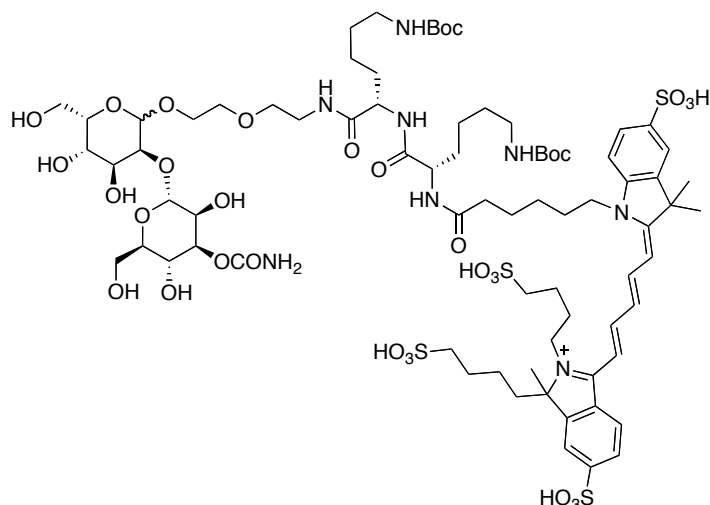
***N*<sup>α</sup>-CBz-*N*<sup>ε</sup>-Boc-D-lysyl-*N*<sup>δ</sup>-Boc-D-lysine (2.44).**

To a solution containing 145 mg (0.37 mmol) of the methyl ester **2.34** in 3.2 mL of THF was added a solution of 35 mg of LiOH (1.47 mmol) in 1.6 mL of water. The reaction mixture was stirred at room temperature for 2 h. The mixture was diluted with 20 mL of Et<sub>2</sub>O and 35 mL of water and the phases were separated. The cooled aqueous phase (ice bath) was acidified to pH ~ 3 with 5% aq NaHSO<sub>4</sub> and extracted with three 30-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure to afford the free acid **2.44** as a colorless oil: yield 129 mg (91%); silica gel TLC  $R_f$  0.27 (1:3 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.24–1.96 (m, 30H), 3.04 (d, 4H,  $J = 5.1$  Hz), 4.25 (m, 2H), 4.89 (s, 1H), 5.06 (d, 2H,  $J = 12.7$  Hz), 6.05 (m, 1H), 7.25 (m, 5H) and 10.55 (br s, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 22.5, 22.6, 28.5, 29.5, 31.5, 32.4, 40.2, 52.3, 54.8, 67.0, 79.4, 128.1, 128.2, 128.6, 136.4, 156.4, 156.5, 158.0, 172.4 and 174.9; mass spectrum (ESI),  $m/z$  609.3495 ( $M + H$ )<sup>+</sup> ( $C_{30}H_{49}N_4O_9$  requires  $m/z$  609.3494).



***N*<sup>α</sup>-CBz-*N*<sup>ε</sup>-Boc-D-lysyl-*N*<sup>ε</sup>-Boc-D-lysyl-BLM-disaccharide (2.45).**

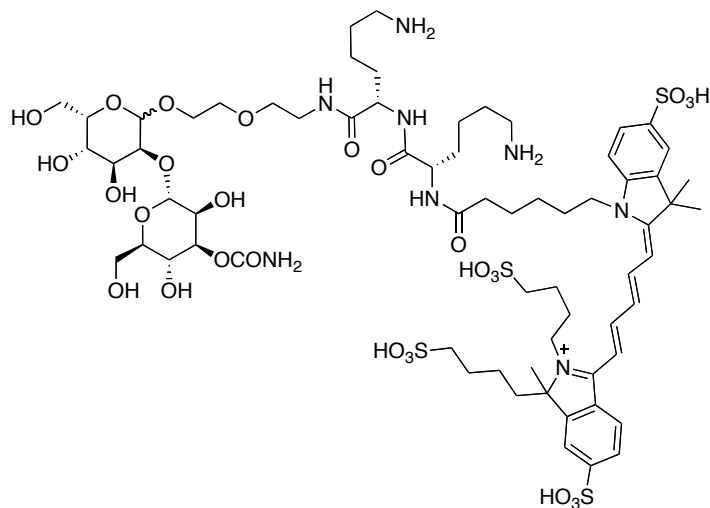
To a solution of 3.0 mg (6.3 μmol) of compound **2.23**, 3.8 mg (6.2 μmol) of compound **2.44** and 4.5 μL (3.2 mg; 25 μmol) of DIPEA in 0.15 mL of anhydrous DMF was added 4.8 mg (13 μmol) of HATU. The reaction mixture was stirred at room temperature for 16 h. The reaction was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–CH<sub>3</sub>CN → 45:55 0.1% aq TFA–CH<sub>3</sub>CN) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 29.8 min (monitoring at 220 nm) and were collected, frozen, and lyophilized to give **2.45** as a colorless solid: yield 2.5 mg (37%); mass spectrum (ESI), *m/z* 1063.5280 (M + H)<sup>+</sup> (C<sub>47</sub>H<sub>79</sub>N<sub>6</sub>O<sub>21</sub> requires *m/z* 1063.5293).



***N*<sup>α</sup>-Cy5\*\*-*N*<sup>ε</sup>-Boc-D-lysyl-*N*<sup>ε</sup>-Boc-D-lysyl-BLM-disaccharide (2.47).**

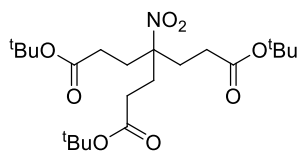
H<sub>2</sub> gas was bubbled through a solution of 1.5 mg (4.2 μmol) of compound **2.45** in 2 mL of methanol for 10 min, and the reaction mixture was stirred for 30 min under a hydrogen atmosphere. The reaction mixture was filtered and the filtrate was concentrated under diminished pressure to afford compound **2.46** as a colorless solid: yield 1.1 mg. To 1.1 mg (1.2 μmol) of **2.46** was added 0.44 mL of 0.2 M aq sodium phosphate buffer solution (pH ~ 8). Two hundred μL (0.5 mg; 0.54 μmol) of this solution was added to a vial containing 110 μg (0.11 μmol) of Cy5\*\* carboxylic acid succinimidyl ester (**2.24**) and the mixture was shaken at room temperature overnight in the dark. The crude reaction mixture was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250 × 10 mm, 10 μm) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA-CH<sub>3</sub>CN → 45:55 0.1% aq TFA-CH<sub>3</sub>CN) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 20.1 min (monitoring at 651 nm) and were collected, frozen, and

lyophilized to give the dye conjugate **2.47** as a light blue solid: yield 52  $\mu\text{g}$  (26%); mass spectrum (ESI),  $m/z$  897.3370 ( $M - 3H$ )<sup>2-</sup> ( $C_{77}H_{118}N_8O_{32}S_4$  requires  $m/z$  897.3373).



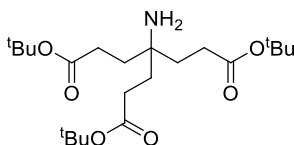
***N* <sup>$\alpha$</sup> -Cy5<sup>\*\*</sup>-D-lysyl-D-lysyl-BLM-disaccharide (**2.48**).**

A solution of 45  $\mu\text{g}$  (25 nmol) of **2.47** in 200  $\mu\text{L}$  of 60% aq TFA was shaken at room temperature for 40 min and was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250  $\times$  10 mm, 10  $\mu\text{m}$ ) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–CH<sub>3</sub>CN  $\rightarrow$  45:55 0.1% aq TFA–CH<sub>3</sub>CN) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 16.2 min (monitoring at 651 nm) and were collected, frozen, and lyophilized to give **2.48** as a blue solid: yield 25  $\mu\text{g}$  (62%); mass spectrum (ESI),  $m/z$  797.2845 ( $M - 3H$ )<sup>2-</sup> ( $C_{67}H_{102}N_8O_{28}S_4$  requires  $m/z$  797.2850).



**4-(2-*tert*-Butoxycarbonyl-ethyl)-4-nitro-heptanedioic Acid Di-*tert*-butyl Ester**  
**(2.50).**<sup>84</sup>

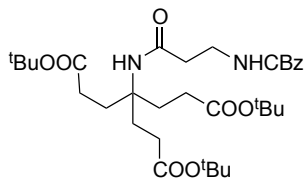
To a solution of 2.14 mL (2.43 g; 39.8 mmol) of nitromethane in 10 mL of dimethoxyethane at 65 °C was added 0.4 mL of 40% aq tetrabutylammonium hydroxide soln and the reaction mixture was heated to 75 °C. To the reaction mixture was added dropwise 18.2 mL (125 mmol) of *tert*-butyl acrylate (**2.49**). To this mixture was added 0.8 mL of 40% aq tetrabutylammonium hydroxide soln in portions over a period of 1 h. The reaction mixture was stirred at 75 °C for 2 h. The reaction mixture was concentrated under diminished pressure and the residue was diluted in 100 mL of diethyl ether. The ether layer was washed with two 30-mL portions of 10% aq citric acid soln, two 30-mL portions of sat aq NaHCO<sub>3</sub> soln, 20 mL of brine, then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure. The residue was recrystallized from absolute ethanol to afford compound **2.50** as colorless needles: yield 16.1 g (91%); mp 92-94 °C, lit<sup>84</sup> mp 98-100 °C <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.43 (s, 27H) and 2.19 (m, 12H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 28.2, 29.9, 30.5, 81.3, 92.3 and 171.2.



**4-Amino-4-(2-*tert*-butoxycarbonylethyl)heptanedioic Acid Di-*tert*-butyl Ester**  
**(2.51).**<sup>84</sup>

A mixture of 1.02 g (2.29 mmol) of compound **2.50**, ~6 mL of T1-Raney Ni (suspension in ethanol) and 18 mL of absolute ethanol was shaken in a Parr

shaker at room temperature and 52 psi H<sub>2</sub> for 72 h. The reaction mixture was filtered through a pad of Celite and the filtrate was concentrated under diminished pressure to afford the amine **2.51** as a waxy solid which was used directly in the next step: yield 0.88 g (92%); silica gel TLC *R<sub>f</sub>* 0.14 (1:3 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.42 (s, 27H), 1.58 (t, 6H, *J* = 8.4 Hz) and 2.22 (t, 6H, *J* = 8.4 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 28.0, 29.9, 34.4, 52.3, 80.3 and 173.0.

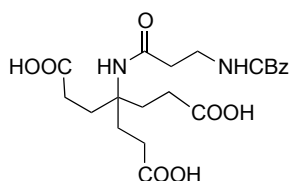


**4-(3-Benzyloxycarbonylamino)propionylamino)-4-(2-*tert*-butoxycarbonyl-ethyl)-heptanedioic Acid Di-*tert*-butyl Ester (2.53).**

To a solution of 0.84 g (2.02 mmol) of compound **2.51** and 0.43 g (1.91 mmol) of CBz-β-alanine (**2.52**) in 15 mL of dry DMF were added 0.74 g (1.95 mmol) of HATU and 0.82 g (3.82 mmol) of proton sponge. The resulting yellow mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated under diminished pressure and the residue was dissolved in 80 mL of ethyl acetate. The ethyl acetate layer was washed with two 40-mL portions of 2 M aq HCl, two 30-mL portions of H<sub>2</sub>O, and 20 mL of brine, then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (12 × 3 cm). Elution with 1:1 hexanes–ethyl acetate gave compound **2.53** as a colorless solid: yield 1.17 g (98%); silica gel TLC *R<sub>f</sub>* 0.40 (1:1 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.42 (s, 27H), 1.94 (t, 6H, *J* = 8.0 Hz), 2.19 (t, 6H, *J* = 8.4 Hz), 2.34 (m, 2H), 3.44

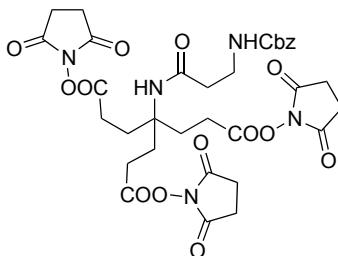


(m, 2H), 5.09 (s, 2H), 5.57 (brs, 1H), 5.99 (brs, 1H) and 7.32 (m, 5H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  28.1, 29.8, 30.0, 36.8, 37.3, 57.8, 66.6, 80.8, 128.0, 128.5, 136.7, 156.6, 170.9 and 172.9; mass spectrum (ESI),  $m/z$  621.3753 ( $\text{M} + \text{H}$ ) $^+$  ( $\text{C}_{33}\text{H}_{53}\text{N}_2\text{O}_9$  requires  $m/z$  621.3746).



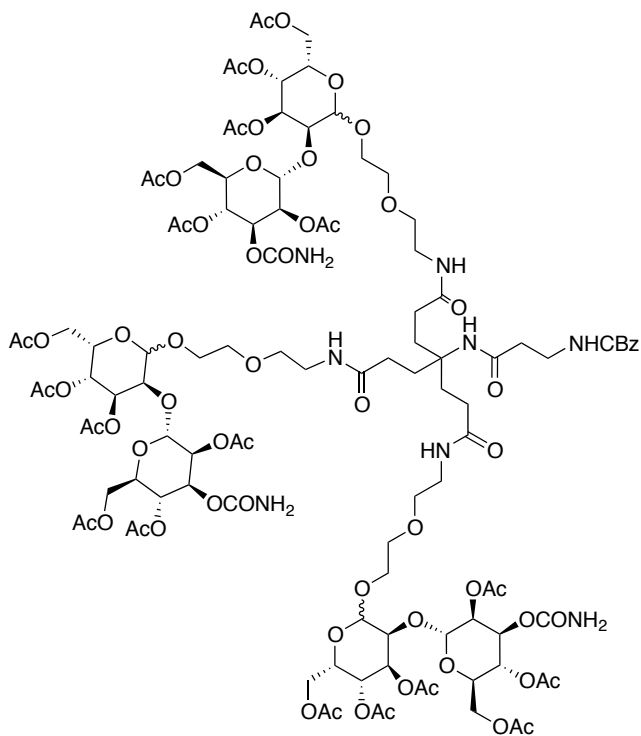
**4-(3-Benzyloxycarbonylamino)propionylamino)-4-(2-carboxy-ethyl)-heptanedioic Acid (2.54).**

A solution of 1.21 g (1.93 mmol) of **2.53** in 25 mL of formic acid was stirred at room temperature for 12 h. The reaction mixture was concentrated under diminished pressure. The residue was co-evaporated with six 10-mL portions of toluene to afford the tri-acid **2.54** as colorless oil: yield 0.91 g (100%);  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  2.01 (m, 6H), 2.26 (m, 6H), 2.40 (m, 2H), 3.36 (m, 2H), 5.07 (s, 2H) and 7.31 (m, 5H);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  28.1, 29.0, 36.2, 37.3, 56.4, 65.2, 127.71, 127.75, 137.2, 156.0, 170.0 and 174.5; mass spectrum (ESI),  $m/z$  453.1886 ( $\text{M} + \text{H}$ ) $^+$  ( $\text{C}_{21}\text{H}_{29}\text{N}_2\text{O}_9$  requires  $m/z$  453.1868).



**4-(3-Benzoyloxycarbonylaminopropionylamino)-4-[2-(2,5-dioxo-pyrrolidin-1-  
yloxycarbonyl)-ethyl]-heptanedioic Acid Bis-(*N*-hydroxysuccinimide) Ester  
(**2.55**).**

To a solution of 0.48 g (1.06 mmol) of compound **2.54** and 0.44 g (3.82 mmol) of *N*-hydroxysuccinimide in 9 mL dry THF at 0 °C was added dropwise a solution of 0.83 g (4.03 mmol) of DCC in 2 mL of dry THF. The resulting reaction mixture was stirred at 5 °C for 16 h. The reaction mixture was concentrated under diminished pressure and the residue was suspended in 10 mL of acetonitrile. The suspension was filtered and the filtrate was concentrated under diminished pressure. The residue was purified by crystallization from absolute ethanol to afford **2.55** as a colorless crystals: yield 366 mg (46%); <sup>1</sup>H NMR (CD<sub>3</sub>CN) δ 2.08 (m, 6H), 2.31 (m, 2H), 2.58 (m, 6H), 2.74 (s, 12H), 3.28 (m, 2H), 5.02 (s, 2H), 5.73 (brs, 1H), 6.10 (brs, 1H) and 7.32 (m, 5H); <sup>13</sup>C NMR (CD<sub>3</sub>CN) δ 25.9, 26.3, 29.5, 37.0, 37.9, 58.0, 66.7, 128.6, 128.7, 129.3, 138.3, 157.2, 169.8, 171.0 and 172.1; mass spectrum (ESI), *m/z* 744.2342 (M + H)<sup>+</sup> (C<sub>33</sub>H<sub>38</sub>N<sub>5</sub>O<sub>15</sub> requires *m/z* 744.2359).

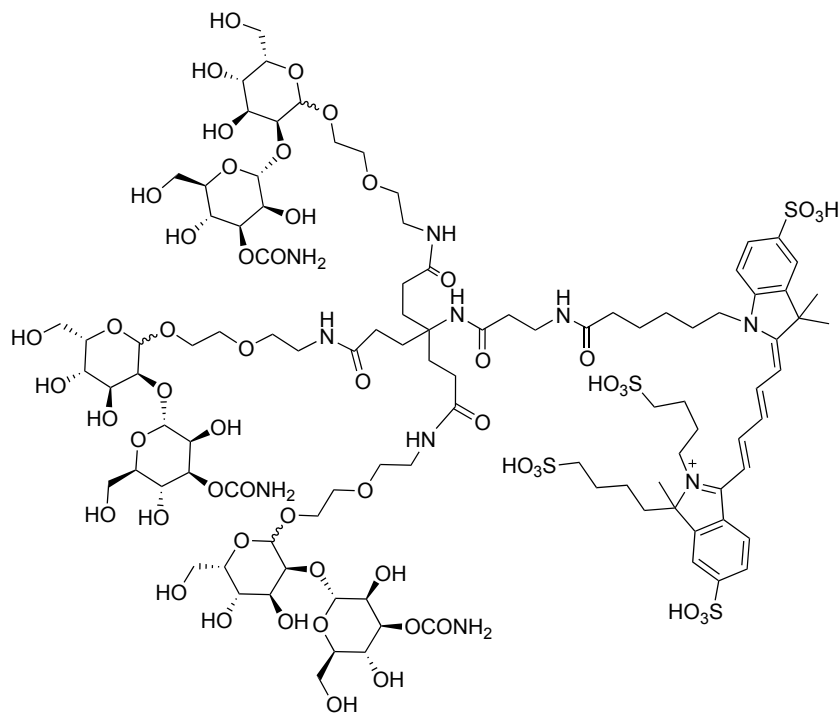


**TrimerBLM-disaccharide (2.57).**

H<sub>2</sub> gas was bubbled through a mixture containing 18 mg (21 μmol) of **2.22** and a catalytic amount of Pd/C in 5 mL of dry THF for 45 min. The reaction mixture was filtered through a pad of Celite and the filtrate was concentrated under diminished pressure to obtain crude **2.56** as a colorless oil, which was used immediately in the next step: crude yield 14 mg; mass spectrum (MALDI) *m/z* 725.28 (M + H)<sup>+</sup> (theoretical *m/z* 725.26).

To a solution containing 14 mg (19 μmol) of **2.56** and 20 μL (15 mg, 0.14 mmol) of triethylamine in 1.5 mL of dry DMF was added 1.6 mg (2.2 μmol) of **2.55** and the mixture was stirred at room temperature for 20 h. The reaction mixture was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (14 × 1 cm). Elution with 32:15:1 →

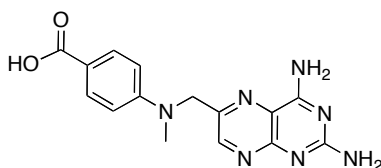
11:10:1 chloroform–acetone–methanol afforded trimerBLM-disaccharide **2.57** as a colorless oil: yield 4.5 mg (81%); silica gel TLC  $R_f$  0.60 (4:4:1 chloroform–acetone–methanol); mass spectrum (MALDI),  $m/z$  2595.11 ( $M + Na$ )<sup>+</sup> (theoretical  $m/z$  2594.90); mass spectrum (ESI),  $m/z$  1297.4575 ( $M + H + Na$ )<sup>2+</sup> ( $C_{108}H_{155}N_8O_{63}Na$  requires  $m/z$  1297.4529).



### TrimerBLM-disaccharide-Cy5\*\* (**2.59**).

To a solution of 5.0 mg (1.94  $\mu$ mol) of **2.57** in 2 mL of dry MeOH was added 0.3 mL of a 25% w/w solution of NaOMe in MeOH. The reaction mixture was shaken at room temperature for 2 h. One hundred mg of Dowex 50W resin was added and the mixture was shaken at room temperature for 30 min. The mixture was filtered, diluted to 5 mL with methanol and a catalytic amount of Pd/C was added. H<sub>2</sub> gas was bubbled through the mixture for 30 min and the mixture was

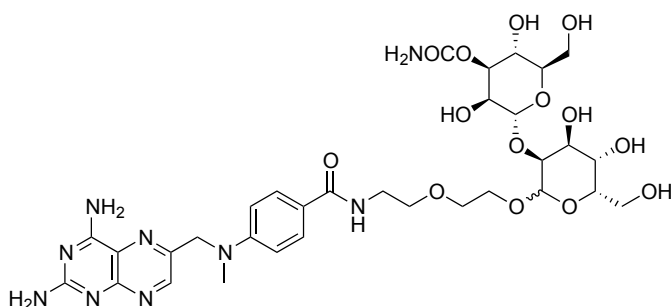
filtered. The filtrate was concentrated to obtain compound **2.58** as colorless solid: crude yield 2.6 mg (80%). To 110  $\mu\text{g}$  (0.11  $\mu\text{mol}$ ) of Cy5\*\*COOSu (**2.24**) was added 100  $\mu\text{L}$  of 0.2 M aq sodium phosphate buffer (pH  $\sim$  8). This solution was added to a vial containing 0.56 mg (0.34  $\mu\text{mol}$ ) of **2.58** in 40  $\mu\text{L}$  of DMSO and the mixture was shaken at room temperature overnight in the dark. The crude reaction mixture was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250  $\times$  10 mm, 10  $\mu\text{m}$ ) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–CH<sub>3</sub>CN  $\rightarrow$  45:55 0.1% aq TFA–CH<sub>3</sub>CN) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 14.8 min (monitoring at 651 nm) and were collected, frozen, and lyophilized to give the trimerBLM-disaccharide-Cy5\*\* **2.59** as a blue solid: yield 43  $\mu\text{g}$  (15%); mass spectrum (MALDI),  $m/z$  2588.4 (M)<sup>+</sup> (theoretical  $m/z$  2587.8); mass spectrum (ESI),  $m/z$  848.6223 (M – 4H)<sup>3-</sup> (C<sub>102</sub>H<sub>167</sub>N<sub>10</sub>O<sub>56</sub>S<sub>4</sub> requires  $m/z$  848.6215).



#### 4-Amino-4-deoxy-10-N-methylpteridic Acid (APA, **2.61**).<sup>71</sup>

A mixture of 249 mg (0.59 mmol) dibromotriphenylphosphorane and 45.0 mg (0.20 mmol) 2,4-diamino-6-(hydroxymethyl)pteridine hydrochloride (**2.60**) in 1.5 mL of anhydrous dimethylacetamide was stirred at room temperature for 24 h under an argon atmosphere. To the reaction mixture were added 41 mg (0.27 mmol) of 4-(methylamino)benzoic acid and 0.16 mL (116 mg, 0.90 mmol) of

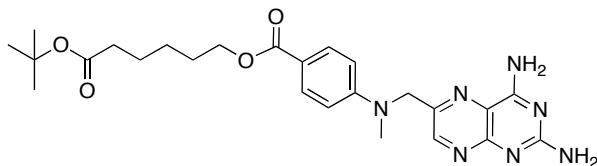
DIPEA and the reaction mixture was stirred at room temperature for 48 h, and then at 60 °C for 24 h. The cooled reaction mixture was poured into 25 mL of 0.33 M aq NaOH and the precipitate was filtered. The filtrate was adjusted to pH 5.5 with 10% acetic acid and the resulting precipitate was collected through filtration, washed with water and dried under diminished pressure at 80 °C overnight to obtain **2.61** as an orange solid: yield 42 mg (56%); silica gel TLC  $R_f$  0.47 (5:4:1 chloroform–methanol–water);  $^1\text{H NMR}$  (DMSO- $d_6$ )  $\delta$  3.19 (s, 3H), 4.76 (s, 2H), 6.61 (s, 2H), 6.78 (d, 2H,  $J = 8.9$  Hz), 7.72 (d, 2H,  $J = 8.7$  Hz) and 8.56 (s, 1H).



#### APA-BLM-disaccharide (**2.62**).

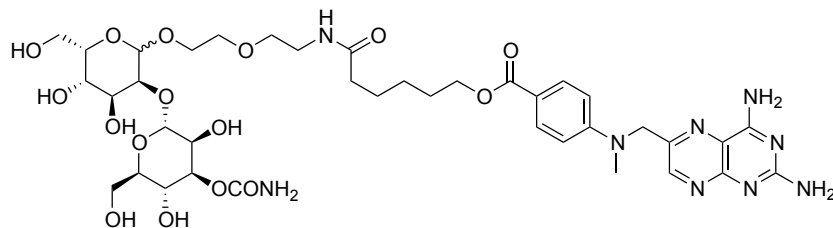
To a solution containing 3.5 mg (7.4  $\mu\text{mol}$ ) of **2.23**, 2.5 mg (7.6  $\mu\text{mol}$ ) of **2.61** and 3.0  $\mu\text{L}$  (2.2 mg; 17  $\mu\text{mol}$ ) of DIPEA in 0.12 mL of anhydrous DMSO was added 4.3 mg (11  $\mu\text{mol}$ ) of HATU. The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250  $\times$  10 mm, 10  $\mu\text{m}$ ) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–CH<sub>3</sub>CN  $\rightarrow$  45:55 0.1% aq TFA–CH<sub>3</sub>CN) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 26.5 min

(monitoring at 292 nm) and were collected, frozen, and lyophilized to give APA-BLM-disaccharide conjugate **2.62** as a yellow solid: yield 2.2 mg (37%); mass spectrum (ESI),  $m/z$  780.3168 ( $M + H$ )<sup>+</sup> ( $C_{32}H_{46}N_9O_{14}$  requires  $m/z$  780.3159).



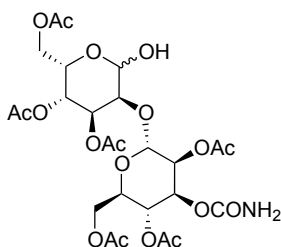
**4-Amino-4-deoxy-10-N-methylpteroic Acid 6-(*tert*-Butoxy)-6-oxohexyl Ester (2.64).**

To a solution of 36 mg (0.2 mmol) of **2.63**, 12 mg (37  $\mu$ mol) of **2.61** and 45 mg (0.4 mmol) of DMAP in 1 mL of anhydrous DMSO was added 39 mg (0.2 mmol) of DCC and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was filtered and the filtrate was purified on a  $C_{18}$  reversed phase semi-preparative (250  $\times$  10 mm, 10  $\mu$ m) HPLC column using 0.1% aq TFA and  $CH_3CN$  mobile phases. A linear gradient was employed (99:1 0.1% aq TFA– $CH_3CN$   $\rightarrow$  1:99 0.1% aq TFA– $CH_3CN$ ) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 25.8 min (monitoring at 292 nm) and were collected, frozen, and lyophilized to give **2.64** as a yellow solid: yield 11 mg (60%);  $^1H$  NMR ( $CD_3CN$ )  $\delta$  1.40 (m, 9H), 1.58 (m, 2H), 1.70 (m, 2H), 2.19 (t, 2H,  $J = 7.3$  Hz), 3.24 (s, 3H), 4.19 (t, 2H,  $J = 6.4$  Hz), 4.84 (s, 2H), 5.45 (s, 2H), 6.79 (m, 2H), 7.26 (s, 1H), 7.72 (s, 1H), 7.8 (m, 2H) and 8.73 (s, 1H); mass spectrum (ESI),  $m/z$  496.2676 ( $M + H$ )<sup>+</sup> ( $C_{25}H_{34}N_7O_4$  requires  $m/z$  496.2667).



**APA ester-BLM-disaccharide (2.65).**

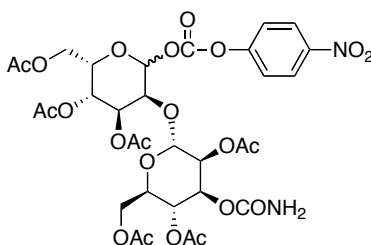
A solution containing 2.8 mg (5.6  $\mu\text{mol}$ ) of compound **2.64** in 1:1 TFA–CH<sub>2</sub>Cl<sub>2</sub> was shaken at room temperature for 1 h and concentrated under diminished pressure. The residue was co-evaporated with five 2-mL portions of toluene and dissolved in 0.1 mL of anhydrous DMSO. To this solution were added 2.9 mg (6.2  $\mu\text{mol}$ ) of **2.23**, 2.0  $\mu\text{L}$  (1.5 mg; 12  $\mu\text{mol}$ ) of DIPEA and 3.5 mg (9.3  $\mu\text{mol}$ ) of HATU. The reaction mixture was stirred at room temperature for 16 h. The reaction was purified on an Econosil C<sub>18</sub> reversed phase semi-preparative (250  $\times$  10 mm, 10  $\mu\text{m}$ ) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–CH<sub>3</sub>CN  $\rightarrow$  45:55 0.1% aq TFA–CH<sub>3</sub>CN) over a period of 30 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 20.7 min (monitoring at 292 nm) and were collected, frozen, and lyophilized to give APA-BLM-disaccharide conjugate **2.65** as a yellow solid: yield 2.2 mg (53%); mass spectrum (ESI),  $m/z$  894.3853 ( $M + H$ )<sup>+</sup> (C<sub>32</sub>H<sub>46</sub>N<sub>9</sub>O<sub>14</sub> requires  $m/z$  894.3840).





**3,4,6-Tri-*O*-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl- $\alpha$ -D-mannopyranosyl)- $\alpha,\beta$ -L-gulopyranoside (2.66).<sup>69</sup>**

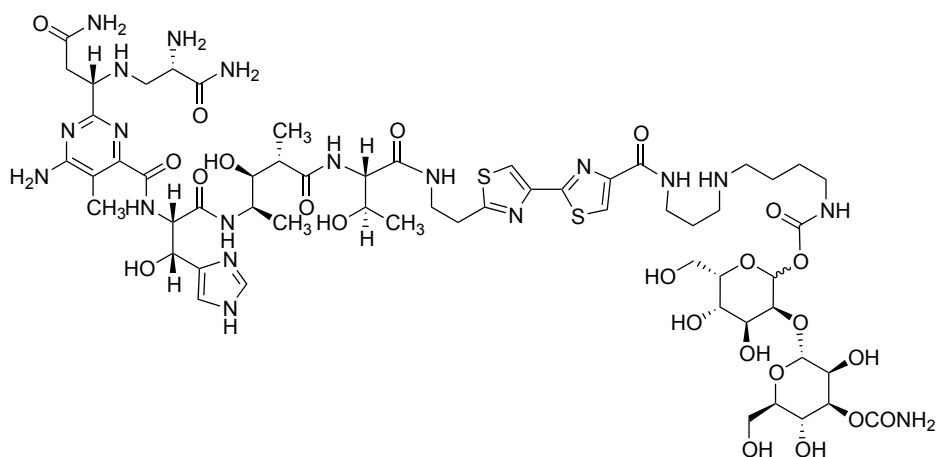
To a solution of 83.0 mg (0.12 mmol) of **2.19** in 2 mL of dry DMF was added 15.0 mg (0.16 mmol) of acetate salt of hydrazine at 0 °C. The solution was warmed to room temperature and stirred for 1 h at which time analysis by silica gel TLC indicated that the starting material had been completely consumed. The reaction mixture was diluted with 50 mL of EtOAc and washed with three 15-mL portions of brine, then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 1:3 hexanes–ethyl acetate afforded compound **2.66** as a colorless oil: yield 55 mg (70%); silica gel TLC *R*<sub>f</sub> 0.30 (1:2 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  2.02 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.10 (s, 3H), 2.12 (s, 3H), 2.15 (s, 3H), 3.73 (dd, 1H, *J* = 8.0 and 3.4 Hz), 4.12 (m, 3H), 4.34 (m, 1H), 4.95 (m, 5H), 5.10 (m, 1H), 5.16 (d, 1H, *J* = 3.3 Hz), 5.25 (m, 1H) and 5.37 (m, 1H).



**1-*O*-((*p*-Nitrophenyl)carbamoyl)- 3,4,6-tri-*O*-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl- $\alpha$ -D-mannopyranosyl)- $\alpha,\beta$ -L-gulopyranoside (2.67).**

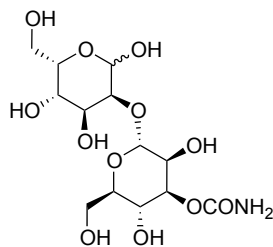
To a solution of 25 mg (39  $\mu$ mol) of compound **2.66** in 1 mL of dry acetonitrile were added 7.0 mg (58  $\mu$ mol) of DMAP and a solution of 12 mg (58  $\mu$ mol) of *p*-

nitrophenyl chloroformate in 125  $\mu$ L of acetonitrile. The mixture was stirred at room temperature for 1 h at which time silica gel TLC analysis showed complete consumption of the starting material. The reaction mixture was concentrated under diminished pressure, diluted in 30 mL ethyl acetate, and then washed with 10 mL of H<sub>2</sub>O, 10 mL of 1 N aq HCl and 10 mL of brine. The ethyl acetate layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15  $\times$  2 cm). Elution with 1:1  $\rightarrow$  1:2 hexanes–ethyl acetate afforded the carbonate **2.67** as a colorless oil: yield 28 mg (92%); silica gel TLC  $R_f$  0.38 (2:3 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  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); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  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, 122.09, 125.6, 146.0, 151.1, 155.1, 155.2, 169.3, 169.3, 169.6, 169.8, 170.5 and 170.7; mass spectrum (ESI),  $m/z$  825.1821 (M + Na)<sup>+</sup> (C<sub>32</sub>H<sub>38</sub>N<sub>2</sub>O<sub>22</sub>Na requires  $m/z$  825.1808).



### Cu(II)•deglycoBLM-disaccharide **2.68**.

To a solution containing 1.0 mg (1.2  $\mu\text{mol}$ ) of compound **2.67** and 1.0 mg (0.9  $\mu\text{mol}$ ) of Cu(II)•deglycobleomycin in 0.5 mL of anhydrous DMF was added 5.0  $\mu\text{L}$  (3.7 mg; 36  $\mu\text{mol}$ ) of dry triethylamine and the mixture was shaken at room temperature for 3 h. Fifty  $\mu\text{L}$  (51 mg; 1.59 mmol) of hydrazine was added and the reaction mixture was shaken at room temperature for 1 h and then concentrated under diminished pressure. The mixture was diluted with 0.2 mL of 1:1 0.1% aq TFA–CH<sub>3</sub>CN and purified on an Alltech Alltima C<sub>18</sub> reversed phase semi-preparative (250 × 10 mm, 5  $\mu\text{m}$ ) HPLC column using 0.1% aq TFA and CH<sub>3</sub>CN mobile phases. A linear gradient was employed (99:1 0.1% aq TFA–CH<sub>3</sub>CN → 50:50 0.1% aq TFA–CH<sub>3</sub>CN) over a period of 35 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 17.0 min and were collected, frozen, and lyophilized to give **2.68** as a colorless solid: yield 0.53 mg (39%); mass spectrum (MALDI-TOF),  $m/z$  1484.9 (M – Cu + H)<sup>+</sup> (theoretical  $m/z$  1484.6); mass spectrum (ESI),  $m/z$  742.7995 (M – Cu + 2H)<sup>2+</sup> (C<sub>58</sub>H<sub>91</sub>N<sub>19</sub>O<sub>23</sub>S<sub>2</sub> requires  $m/z$  742.7983).



### **Bleomycin-disaccharide (2.69).**<sup>72</sup>

To a solution of 20 mg (29  $\mu\text{mol}$ ) of compound **2.19** in 10 mL of dry MeOH was added 0.5 mL of a 25% w/w solution of NaOMe in MeOH. The mixture was shaken at room temperature for 2 h. One hundred mg of Dowex 50W resin was added and the mixture was shaken at room temperature for 30 min. The mixture was filtered and concentrated to obtain compound **2.69** as colorless syrup: 10 mg (88%); mass spectrum (MALDI),  $m/z$  408.31 ( $M + \text{Na}$ )<sup>+</sup> (theoretical  $m/z$  408.11).

### **Cell Growth Conditions.**

MCF-7 cells (ATCC HBT-22) 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) and PZ-HPV-7 (ATCC CRL-2221) prostate 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<sub>2</sub> and 95% air. Dr. Alan Yu carried out this experiment.

### **Fluorescence Microscopy.**

Fluorescence images were obtained using a Zeiss Axiovert 200M inverted microscope fitted with an AxioCam MRm camera equipped with a 300-w xenon lamp (Sutter, Novato, CA), Cy5 and Cy7 cyanine filter (Chroma, Bellows Falls, VT). Adherent cancer 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. Cells were rinsed twice with phosphate buffered saline (PBS) when the cell confluence was about 70%, then the medium was replaced with RPMI 1640 (no phenol red). The dye-labeled conjugates were subsequently added to the final desired concentrations. The cells were incubated at 37 °C for 1 h, washed with PBS, then fixed with 4% paraformaldehyde at 37 °C for 5 min. Finally the slide was mounted with Prolong Antifade Gold reagent with DAPI (Invitrogen), then covered with a glass coverslip and dried for 24 h before fluorescence microscope imaging analysis. All images were recorded and target cells counted using a 40× oil objective. For comparative studies, the exposure time and laser intensity were kept identical for accurate intensity measurements. Pixel intensity was quantified using AxioVision Release 4.7 version software, and the mean pixel intensity was generated as gray level. Dr. Alan Yu carried out this experiment.

## CHAPTER 3

### SYNTHESIS OF PUROMYCIN ANALOGUES

#### 3.1. INTRODUCTION

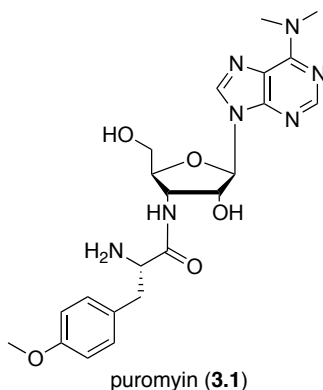
Site-specific incorporation of unnatural amino acids into proteins has been facilitated by the use of suppressor tRNAs misacylated with these amino acids.<sup>39</sup> Several  $\alpha$ -L-analogues of natural amino acids, containing various side chains, were successfully incorporated into proteins albeit with varying yields of the modified protein. Studies have been conducted to better understand the structural constraints placed upon amino acid residues that can be utilized by the ribosome.<sup>85</sup> Incorporation efficiencies of  $\alpha$ -L-amino acids with large side chains, as well as  $\alpha$ -hydroxy acids,<sup>86</sup>  $\alpha$ -hydrazino amino acids<sup>87</sup> and  $\alpha$ -aminooxy acids<sup>88</sup> were quite low. This suggests that the peptidyl transferase center (PTC) is at least partially selective in accepting individual amino acids during protein synthesis.

Using the same technique, incorporation of  $\alpha$ -D-amino acids was not successful as well.<sup>89</sup> Naturally occurring peptides containing D-amino acids are produced either by nonribosomal synthesis or by post-translational modifications.<sup>90</sup> To facilitate the incorporation of D-amino acids into proteins, Dedkova et al. re-engineered regions of 23S rRNA in an effort to make the PTC better able to utilize D-aminoacyl-tRNAs.<sup>91</sup> Mutated 23S rRNA genes were introduced into bacteria and the colonies expressing modified ribosomes were examined for their ability to incorporate D-amino acids. A number of colonies containing modified ribosomes were then used to successfully incorporate the D-amino acids into firefly luciferase and dihydrofolate reductase (DHFR). Notably,

these clones retained the ability to express functional wild-type proteins *in vitro* when unmodified mRNAs were used. The utility of this technique is limited, however, due to its laborious and low-throughput nature.

A detailed study of high-resolution structure of the PTC, conducted by Yonath and coworkers, revealed some possible mechanisms by which ribosomes could exclude D-amino acids during protein production, and the nucleotides in the 23S rRNA putatively involved.<sup>92</sup> When the crystal structure of the PTC in complex with a substrate analogue mimicking a D-aminoacyl-tRNA was studied, the amino group (of the amino acid) was not present within a distance and in a spatial orientation suitable for a peptide bond-forming reaction. Instead, the amino group was within a hydrogen bonding distance that would lock the group in an unproductive conformation. This may well be the reason that substantial structural changes in the PTC were required to accommodate tRNAs activated with  $\alpha$ -D-amino acids.<sup>91</sup>

Puromycin is an antibiotic produced by *Streptomyces alboniger* (Figure 3.1). Owing to its structural resemblance to the 3'-end of aminoacyl-tRNAs, puromycin can bind to the A-site of ribosomes and cause premature release of polypeptides by accepting the growing peptide chain from peptidyl-tRNAs in the ribosomal P-site.<sup>93</sup> It was hypothesized that puromycin analogues containing non- $\alpha$ -L-amino acids, rather than the normal substituent with an  $\alpha$ -L-tyrosine moiety, might bind selectively to modified ribosomes capable of utilizing the corresponding aminoacylated suppressor tRNAs.<sup>94</sup>



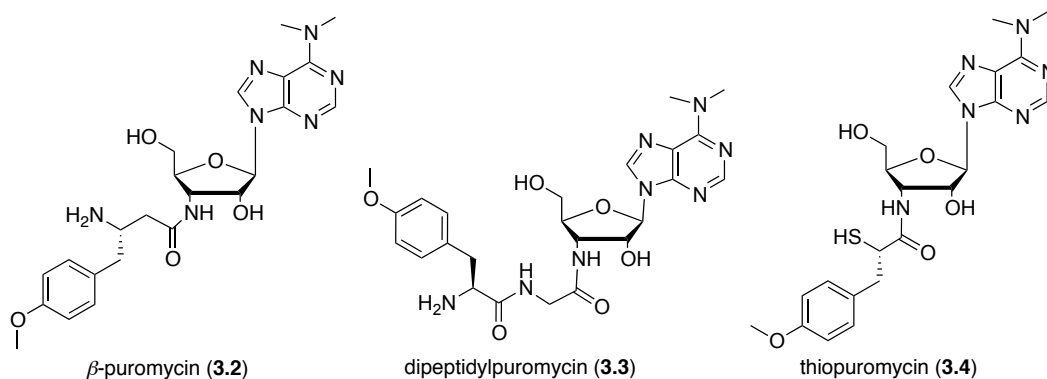
**Figure 3.1.** Structure of the antibiotic puromycin.

The architecture of the PTC of the selected ribosomes would have to accommodate the geometry of the aminoacyl-tRNAs charged with non- $\alpha$ -L-amino acids, including placing the nucleophilic N atom at the required distance and in a favorable orientation for successful “peptide bond” formation. Such puromycin analogues could then be used for identifying ribosomes having an enhanced ability to incorporate the desired noncanonical amino acids from a library of bacteria containing re-engineered ribosomes.

*In vitro* experiments have shown that L- $\beta$ -(4-Me)-Phe-PANS (puromycin aminonucleoside) and L- $\beta$ -Ala-PANS was able to disrupt normal translation.<sup>95</sup> This suggests that  $\beta$ -amino acids could bind to the A-site of the PTC but that the distance between the activated carbonyl of the peptidyl-tRNA and the amino group of the aminoacyl-tRNA might not be favorable for peptide bond formation. Accordingly, we generated a library of ribosomes with alterations in two regions of the PTC.<sup>94</sup>  $\beta$ -Puromycin (Figure 3.2) was chosen as the initial puromycin analogue to be used for selection of modified ribosomes capable of incorporating



$\beta$ -amino acids into proteins while maintaining the fidelity of protein synthesis for the incorporated natural  $\alpha$ -amino acids. Two additional puromycin analogues were prepared. Dipeptidylpuromycin (**3.3**) was synthesized to enable the selection of ribosomes that could incorporate two amino acids per mRNA codon. Thiopuromycin (**3.4**) was prepared to select modified ribosomes that would accept  $\alpha$ -thiocarboxylic acids during protein synthesis.



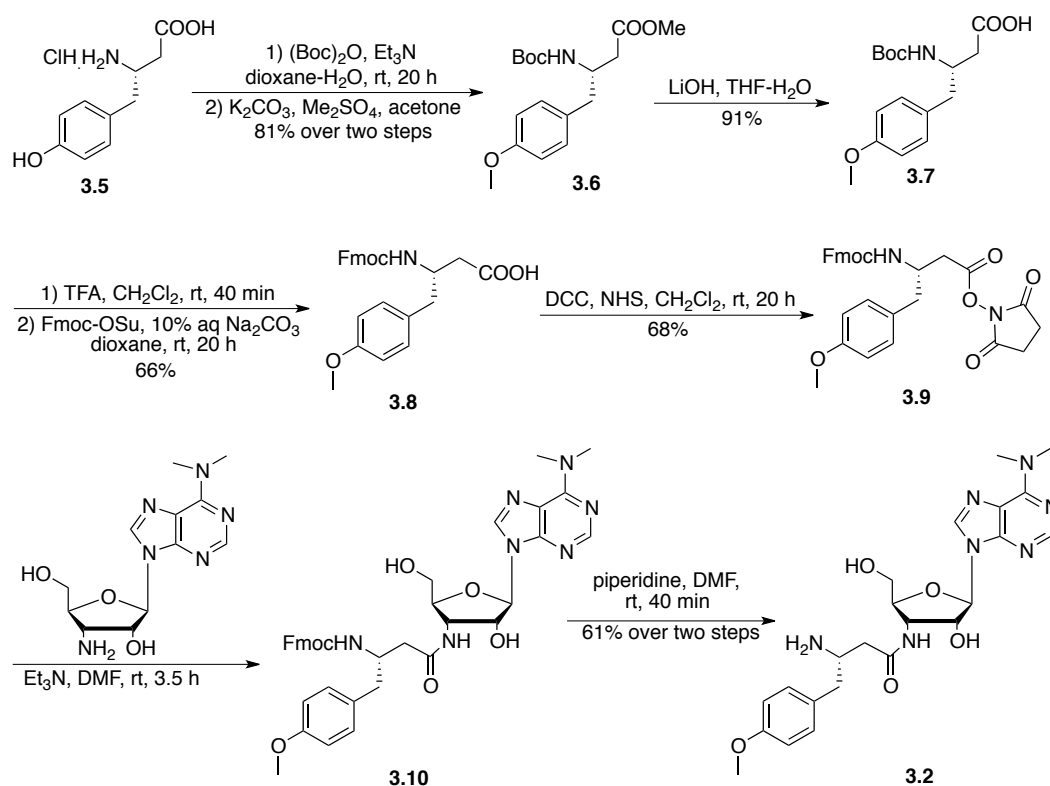
**Figure 3.2.** Structure of puromycin analogues prepared.

## 3.2. RESULTS

### 3.2.1. Synthesis of $\beta$ -Puromycin (**3.2**)

The synthesis of  $\beta$ -puromycin was achieved by acylating the amine functionality of puromycin aminonucleoside (PANS) with *N*-Fmoc-(*S*)-3-amino-4-(*p*-methoxyphenyl)butyric acid (**3.8**) (Scheme 3.1). The primary amine of *L*- $\beta$ -homotyrosine hydrochloride (**3.5**) was protected as the *tert*-butyl carbamate and the intermediate was subjected to exhaustive methylation using dimethyl sulfate and  $K_2CO_3$  to afford compound **3.6** in 81% yield. Methylation permitted the protected amino acid to be isolated and purified readily. The methyl ester was

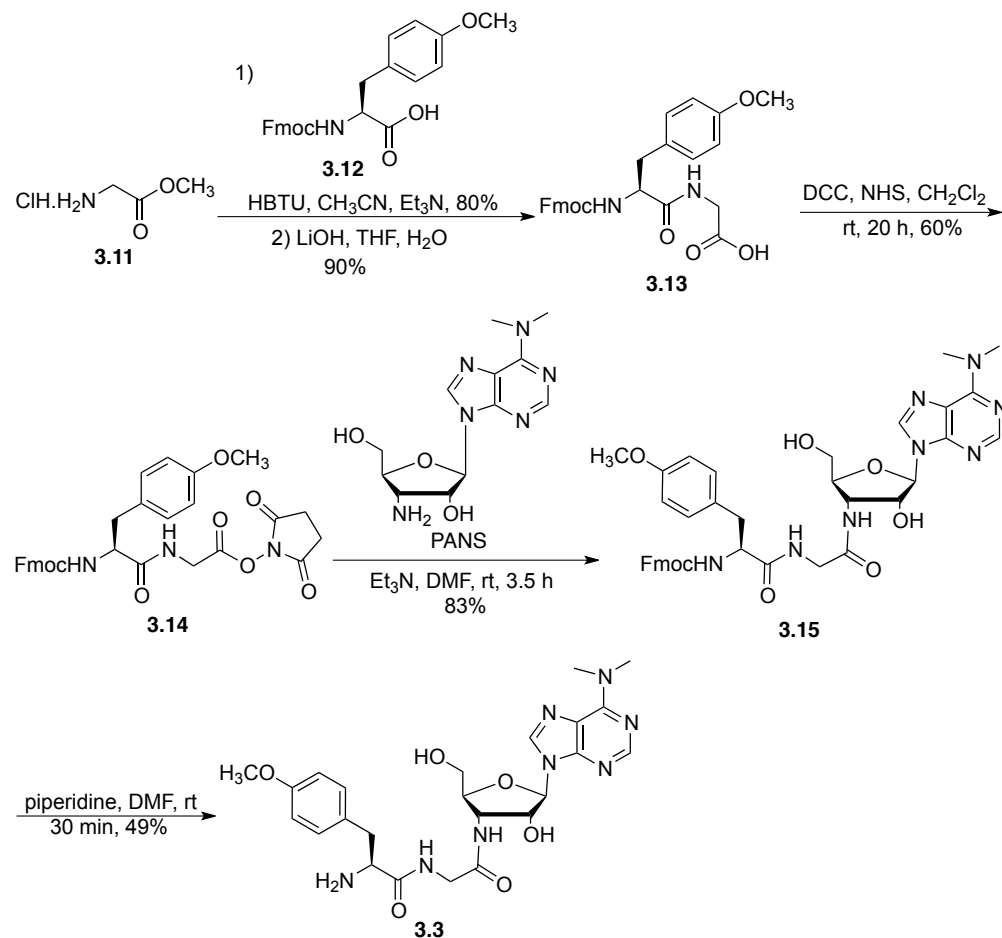
saponified with LiOH in aqueous THF to obtain compound **3.7** in 91% yield. The Boc group was removed by treatment with CF<sub>3</sub>COOH and the resulting free amine was re-protected with a Fmoc group. Carboxylic acid **3.8** was activated as the *N*-hydroxysuccinimidyl ester **3.9**, which was subsequently condensed with PANS in the presence of triethylamine to provide Fmoc-protected  $\beta$ -puomycin **3.10**. Removal of the Fmoc group with piperidine in DMF afforded  $\beta$ -puomycin (**3.2**) in 61% yield for the last two steps.



**Scheme 3.1.** Synthesis of  $\beta$ -puomycin (**3.2**).<sup>94</sup>

### 3.2.2. Synthesis of Dipeptidylpuomycin (**3.3**)

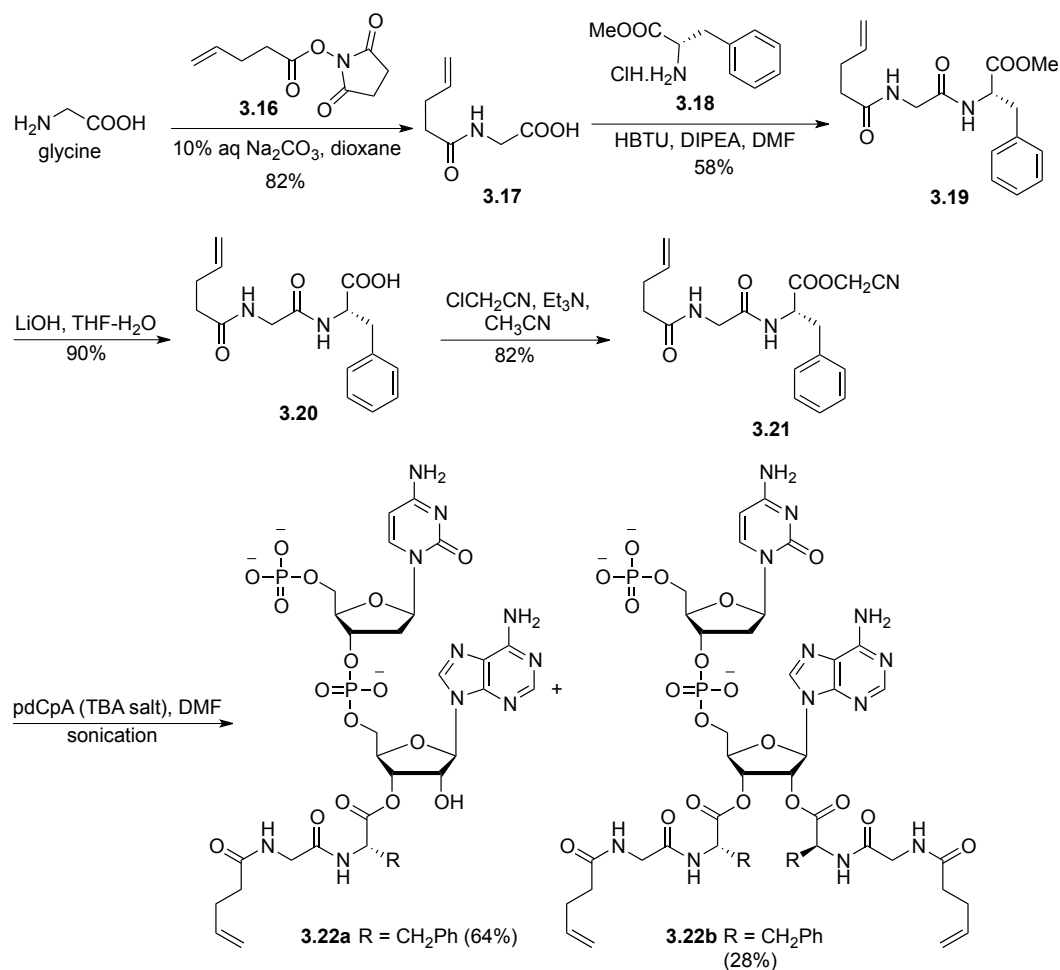
Sriloy Dey synthesized compound **3.14** starting from methyl glycinate and *N*-Fmoc-*O*-methyl-L-tyrosine (Scheme 3.2). The *N*-hydroxysuccinimide ester **3.14** was condensed with puromycin aminonucleoside to afford Fmoc-protected dipeptidylpuromycin **3.15** (Scheme 3.2). Dipeptidylpuromycin (**3.3**) was then prepared in 49% yield from **3.15** by the use of 20% piperidine in DMF.



**Scheme 3.2.** Synthesis of dipeptidylpuromycin (**3.3**).

### 3.2.3. Synthesis of Dipeptidyl-pdCpAs **3.22a** and **3.22b**

Synthesis of the requisite dipeptidyl-pdCpA (Scheme 3.3) required the synthesis of dipeptide **3.20**. The dipeptide synthesis commenced with the protection of glycine as *N*-pentenoylglycine **3.17**. This protected glycine was subsequently condensed with phenylalanine methyl ester (**3.18**) in the presence of HBTU and DIPEA to afford the *N*-protected dipeptide methyl ester **3.19** in 58% yield. The methyl ester was subjected to saponification in the presence of aqueous LiOH to produce the *N*-pentenoyl dipeptide **3.20** in 90% yield. The dipeptide was then converted to the corresponding cyanomethyl ester **3.21** in 82% yield. The cyanomethyl ester was used for the acylation of the dinucleotide pdCpA.<sup>96</sup> The acylation reaction was promoted by the use of sonication and provided both mono- and bisacyl-pdCpA derivatives, **3.22a** and **3.22b**, respectively.

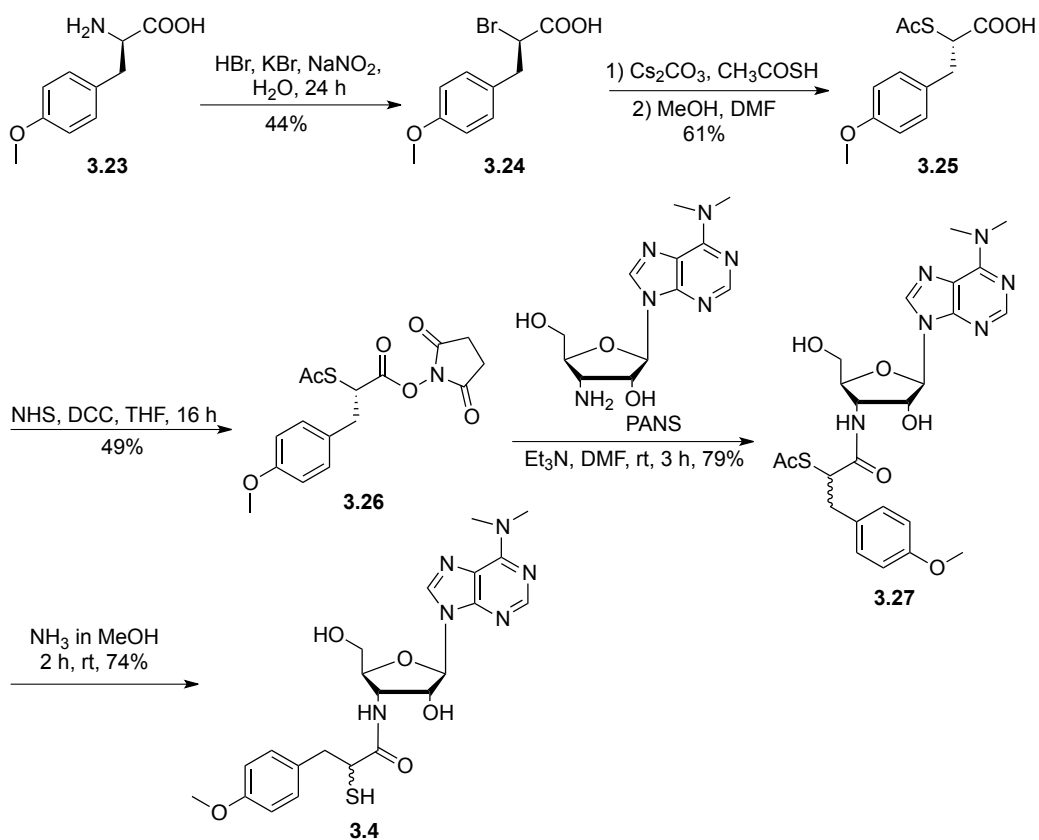


**Scheme 3.3.** Synthesis of mono-2'(3')-O-(N-4-pentenoylglycylphenylalanyl)-pdCpA (**3.22a**) and bis-2',3'-O-(N-4-pentenoylglycylphenylalanyl)-pdCpA (**3.22a**).

### 3.2.4. Synthesis of Thiopuromycin (3.4)

The synthesis of thiopuromycin (**3.4**) started from 4-O-methyl-D-tyrosine **3.23** (Scheme 3.4). The amino acid was subjected to diazotization in the presence of KBr to obtain the bromide **3.24** as a crude intermediate. Cesium thioacetate was generated *in situ* from cesium carbonate and thioacetic acid, and was

combined with the bromide to afford compound **3.25** in 61% yield. The carboxylate was activated as *N*-hydroxysuccinimidyl ester **3.26** by the use of DCC and *N*-hydroxysuccinimide in THF. The activated ester was then condensed with PANS to afford *S*-acetylthiopuromycin **3.27** as mixture of epimers. The acetyl group was removed by the treatment with ammonia in MeOH to generate thiopuromycin **3.4** in 74% yield.



**Scheme 3.4.** Synthesis of thiopuromycin (**3.4**).

### 3.2.5. $\beta$ -Puromycin (**3.2**) Selection of Erythromycin Resistant Clones of 23S

#### rRNA<sup>94</sup>

The 23S rRNA gene was subjected to systemic alteration in three different regions in order to derive modified ribosomes exhibiting greater tolerance towards

$\beta$ -amino acids. An iterative selection process was employed to identify modified ribosomes having the ability to incorporate both  $\alpha$ - and  $\beta$ -amino acids into proteins while maintaining the fidelity of translation. The antibiotic erythromycin is known to bind close to the PTC.<sup>97</sup> Thus modified ribosomes with improved resistance against this antibiotic compared to wild type are likely to have altered PTC architectures.

Initially, the region 2057–2063 in the 23S rRNA gene was altered to obtain a library of *E. coli* clones. These clones were grown in the presence of 3.5  $\mu\text{g/mL}$  erythromycin and the minimum inhibitory concentration (MIC) of this antibiotic was determined for each clone. Eight clones were chosen based on their MIC values. Plasmids from these clones were isolated, sequenced and transformed into freshly grown *E. coli* cells. The  $\beta$ -puromycin sensitivities of these clones were determined (Table 3.1). The modified clones with reduced sensitivity to erythromycin did show increased cell growth inhibition by  $\beta$ -puromycin (100  $\mu\text{g/mL}$ ) compared to the wild-type strain.

**Table 3.1:** Characterization of Erythromycin-Resistant Clones Harboring Plasmids with Modified *rrnB* Operons.

name/no. of clones	sequence in the 2057-2063 region of 23SrRNA gene	inhibition by	
		erythromycin (MIC, $\mu\text{g/mL}$ )	$\beta$ -puromycin (% cell growth inhibition)
01/05	TGCGTGG	12.5 – 6.25	26 $\pm$ 10
02/02	TTGGTCG	6.25 – 3.12	15 $\pm$ 6
03/01	ATGGTTG	6.25 – 3.12	20 $\pm$ 7
04/02	AGCGTGA	6.25 – 3.12	28 $\pm$ 13
05/02	TCGTCCA	12.5 – 6.25	16 $\pm$ 7
06/01	AGGGACA	12.5 – 6.25	27 $\pm$ 11
07/01	ATTCCGG	6.25 – 3.12	16 $\pm$ 7
08/01	AGTGAGA	25 – 12.5	23 $\pm$ 9
wt	GAAAGAC	1.56 – 0.78	<1

Dr. Larisa Dedkova carried out this experiment.

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The next round of mutagenesis involved alterations of the eight 23S rRNA variants in the regions 2582–2588, 2496–2501 or 2502–2507. Approximately  $6 \times 10^3$  clones were generated and were screened for their erythromycin resistance and  $\beta$ -puromycin sensitivity. Isolated plasmids from the selected clones were transformed into freshly grown *E. coli* cells to verify their sensitivity to both the antibiotics. Table 3.2 summarizes the detailed characterization of 17 clones of interest.



**Table 3.2:** Characterization of Selected  $\beta$ -Puromycin-Sensitive and Erythromycin-Resistant Clones.

name of plasmid	sequence in regions of mutagenesis		sensitivity to	
	first region	second region	$\beta$ -puromycin (IC <sub>50</sub> , $\mu$ g/mL)	erythromycin (MIC, $\mu$ g/mL)
wt	2057GAAAGAC2063	2496CACCTC2501 2502GATGTC2507 2582GGTTTAG2588	>1000	3.12 – 1.56
010102	2057TGCGTGG2063	2582TTCAAGA2588	390 $\pm$ 32	6.25 – 3.12
030116	2057ATGGTTG2063	2582TCAACTC2588	320 $\pm$ 90	6.25 – 3.12
030130	2057ATGGTTG2063	2582TCAGGGC2588	314 $\pm$ 20	6.25 – 3.12
030185	2057ATGGTTG2063	2582CCCGATT2588	320 $\pm$ 90	12.5 – 6.32
080110	2057AGTGCGG2063	2582TCAGATC2588	250 $\pm$ 50	12.5 – 6.32
080118	2057AGTGCGG2063	2582ATGGGCT2588	450 $\pm$ 90	12.5 – 6.32
020250	2057TTGGTCG2063	2496TCAGCG2501	260 $\pm$ 80	6.25 – 3.12
020252	2057TTGGTCG2063	2496TCGAGA2501	230 $\pm$ 40	6.25 – 3.12
030201	2057ATGGTTG2063	2496AGGTCT2501	290 $\pm$ 100	6.25 – 3.12
040217	2057AGCGTGA2063	2496ATAGAA2501	250 $\pm$ 30	12.5 – 6.32
060203	2057AGGGACA2063	2496ATAAAT2501	170 $\pm$ 45	6.25 – 3.12
060233	2057AGGGACA2063	2496ACAAAT2501	220 $\pm$ 97	12.5 – 6.32
020322	2057TTGGTCG2063	2502ACGAAG2507	145 $\pm$ 70	6.25 – 3.12
020328	2057TTGGTCC2063	2502ACGAAG2507	130 $\pm$ 90	6.25 – 3.12
040321	2057AGCGTGA2063	2502AGATAA2507	430 $\pm$ 85	12.5 – 6.32
040329	2057AGCGTGA2063	2502TGGCAG2507	320 $\pm$ 98	12.5 – 6.32
080302	2057AGTGCGG2063	2502TACAAC2507	210 $\pm$ 60	12.5 – 6.32

Dr. Larisa Dedkova carried out this experiment.

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The selected mutants were then checked for their ability to support non-specific readthrough of mRNAs by the use of a  $\beta$ -galactosidase assay. The clones having mutations in the region 2582-2588 were excluded from subsequent experiments due to their lesser fidelity during protein translation. Finally, cells transformed by plasmids bearing the selected 23S rRNA genes were grown in the

presence of erythromycin and their sensitivity towards  $\beta$ -puromycin was measured (Table 3.3).

**Table 3.3:** Characterization of Rate of Growth and  $\beta$ -Puromycin Sensitivity of Clones Having Modified Ribosomes, in the Presence of Erythromycin.

clones	doubling time, min	$\beta$ -puromycin inhibition (IC <sub>50</sub> , $\mu$ g/mL)
020322	137 $\pm$ 32	66 $\pm$ 20
020328	270 $\pm$ 25	41 $\pm$ 6
030201	540 $\pm$ 120	63 $\pm$ 10
040217	160 $\pm$ 80	46 $\pm$ 2
040321	220 $\pm$ 90	208 $\pm$ 130
040329	140 $\pm$ 25	53 $\pm$ 14
0403x4 <sup>a</sup>	120 $\pm$ 40	76 $\pm$ 15
060203	102 $\pm$ 7	58 $\pm$ 4
080302	88 $\pm$ 20	53 $\pm$ 6
wt	no growth	
wt (no Ery)	38 $\pm$ 4	>1000

<sup>a</sup>Derived in analogy with 040329, but in a parallel set of experiments. Dr. Larisa Dedkova carried out this experiment.

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Five clones with acceptable growth rates in the presence of erythromycin and sufficient sensitivity to  $\beta$ -puromycin were utilized to prepare S-30 extracts. Each of the five S-30 preparations was used for the *in vitro* translation of proteins. The *E. coli* DHFR gene was modified to incorporate a TAG codon corresponding to position 10 of DHFR. The ability of the S-30 systems to suppress this stop codon in the presence of  $\beta$ -alanyl-tRNA<sub>CUA</sub> was measured and compared with their suppression efficiency in the presence of L- $\alpha$ -valyl-tRNA<sub>CUA</sub> (Table 3.4).

**Table 3.4:** Characterization of DHFR Synthesis Using S-30 Systems Prepared from Clones with Modified Ribosomes.<sup>a</sup>

S-30 name	modified ribosome		suppression efficiency (%)	
	sequence	abundance (%)	tRNA <sub>CUA</sub>	$\beta$ -alanyl-tRNA <sub>CUA</sub>
B1 (wt)		no	1.0 ± 0.4	4.0 ± 1.3
020322	2057UUGGUCG2063 2502ACGAAG2507	22	1.3 ± 0.3	7.5 ± 2.3
040329	2507AGCGUGA2063 2502UGGCAG2507	66	3.3 ± 0.7	12.3 ± 2.2
0403x4	2507AGCGUGA2063 2502AGCCAG2507	58	3.2 ± 0.2	9.7 ± 3.6
040217	2507AGCGUGA2063 2496AUAGAA2501	50	0.9 ± 0.4	4.8 ± 2.0
060203	2507AGCGUGA2063 2496AUAAAU2501	53	0.8 ± 0.5	3.8 ± 1.5

<sup>a</sup>The mRNA utilized for translation had a UAG codon corresponding to position 10 of DHFR. The suppression efficiency reported is relative to that obtained using L- $\alpha$ -valyl-tRNA<sub>CUA</sub>. Dr. Larisa Dedkova carried out this experiment.

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### 3.2.6. Dipeptidylpuromycin (3.3) Selection of Erythromycin Resistant Clones of 23S rRNA

A solution of 100  $\mu$ g/mL of dipeptidylpuromycin (**3.3**) was used for selection of 415 clones having mutated ribosomes. Clones with increased sensitivity (>50% inhibition of cell growth) to dipeptidylpuromycin were identified and the plasmids from these clones were sequenced (Table 3.5). These

modified ribosomes with increased sensitivity to **3.3** are expected to accept dipeptides from dipeptidyl-tRNAs during protein synthesis.

**Table 3.5.** Characterization of Dipeptidylpuromycin-Sensitive Clones.

clones	cell-growth inhibition (%)	sequence in mutagenized regions	
		first region	second region
010120	65.4	2057TGCGTGG2063	2582CCGAATC2588
080118	66.5	2057AGTGAGA2063	2582ATGGGCT2588
020213	54	2057TTGGTCC2063	2496CACCTC2501
020309	63.2	2057TTGGTCC2063	2502CTACGG2507
020310	52.9	2057TTGGTCC2063	2502CGCAAT2507
020322	77	2057TTGGTCC2063	2502ACGGAA2507
020326	50.4	2057TTGGTCC2063	2502CTACAG2507
020328	77	2057TTGGTCC2063	2502ACGAAG2507
030240	56	2057TTGGTCC2063	2496AGTGGT 2501
030247	64	2057AGCGTGA2063	2496CACCTC2501
030248	61	2057AGCGTGA2063	2496CACCTC2501
040228	51	2057AGCGTGA2063	2496CACCTC2501
040322	54	2057AGCGTGA2063	2502CTGCTT2507
040329	54	2057AGCGTGA2063	2502TGGCAG2507
040338	56	2057AGCGTGA2063	2502ATCAGG2507
070307	77	2057ATTCCGG2063	2502GATGTC2507
080337	51	2057AGTGAGA2063	2502ATCCGA2507

Dr. Larisa Dedkova carried out this experiment.

### **3.2.7. Thiopuromycin (3.4) Selection of Erythromycin Resistant Clones of 23S rRNA**

A library of 264 clones was selected for their sensitivity to 100 µg/mL of thiopuromycin (**3.4**). The solution of thiopuromycin used for this assay contained 0.3 M of 3-mercaptpropionic acid (3-MPA) to prevent disulfide formation during the selection experiment. Clones that exhibited >50% inhibition of cell growth in the presence of thiopuromycin were chosen for further characterization of the plasmids (Table 3.6). It was presumed that the modified ribosomes selected

against thiopuromycin would show enhanced ability to incorporate  $\alpha$ -thiocarboxylic acids into proteins.

**Table 3.6.** Characterization of Thiopuromycin-Sensitive Clones.

clones	cell-growth inhibition (%)	sequence in the regions of mutagenesis	
		first region	second region
010342	56.1	2057TGCGTGG2063	2502GATGTC2507
020213	84	2057TTGGTCC2063	2496CACCTC2501
020219	51.1	2057TTGGTCC2063	2496CACCTC2501
020220	91.8	2057TTGGTCC2063	2496CACCTC2501
020309	83	2057TTGGTCC2063	2502CTACGG2507
020310	61	2057TTGGTCC2063	2502CGCAAT2507
020312	59	2057TTGGTCC2063	2502CTCCAG2507
020322	59.4	2057TTGGTCC2063	2502ACGGAA2507
020326	65	2057TTGGTCC2063	2502CTACAG2507
020328	58.6	2057TTGGTCC2063	2502ACGAAG2507
030304	57.1	2057TTGGTCC2063	2502GATGTC2507
030307	75.1	2057TTGGTCC2063	2502GATGTC2507
030310	57.8	2057TTGGTCC2063	2502ACGCGG2507
030322	72	2057TTGGTCC2063	2502GATGTC2507
030326	64.6	2057TTGGTCC2063	2502GATGTC2507
030332	51.0	2057TTGGTCC2063	2502GATGTC2507
030342	55.3	2057TTGGTCC2063	2502ATCTAT2507
030343	61.5	2057TTGGTCC2063	2502GATGTC2507
040217	54.2	2057AGCGTGA2063	2496ATAGTT2501
040329	52	2057AGCGTGA2063	2502TGGCAG2507
060325	52	2057AGGGACA2063	2502 TGAAGT2507
070307	67	2057ATTCCGG2063	2502GATGTC2507

Dr. Larisa Dedkova carried out this experiment.

### 3.3. DISCUSSION

#### 3.3.1. Chemistry

Boc-protection of the L- $\beta$ -homotyrosine followed by methylation of the phenolic OH with dimethyl sulfate afforded the methyl ester **3.6**. The acid was regenerated by the treatment of aq LiOH. The Boc protecting group was removed in the presence of CF<sub>3</sub>COOH. The crude residue had to be dried thoroughly by co-evaporation with toluene to obtain reasonable yields of the Fmoc-protected compound **3.8**. Purification of the succinimidyl ester **3.9** by column

chromatography on silica gel was difficult due to contamination from dicyclohexylurea (DCU). Re-suspending the crude residue in acetonitrile followed by filtration removed most of the DCU. Condensation of the ester **3.9** with puromycin aminonucleoside (PANS) and subsequent Fmoc deprotection was achieved using 20% piperidine in DMF (Scheme 3.1).

The dipeptidylpuromycin **3.3** contains a glycine residue which connects the tyrosine and the aminonucleoside. Modified ribosomes selected using dipeptidylpuromycin should be promising candidates for accepting a dipeptide from dipeptidyl-tRNA during protein synthesis. The succinimidyl ester **3.14** was prepared by Sriloy Dey. The synthesis of analogue **3.3** from the activated ester **3.14** was achieved in 41% yield over two steps (Scheme 3.2).

Chemical misacylation of a suppressor tRNA with a dipeptide required the synthesis of dipeptidyl-pdCpA. The dipeptide consisted of glycine and phenylalanine. Glycine was protected as *N*-(4-pentenoyl)glycine (**3.17**) and condensed with phenylalanine methyl ester (Scheme 3.3). The methyl ester **3.19** was converted to the cyanomethyl ester **3.21** via the formation of the acid **3.20**. The cyanomethyl ester then was utilized to form pdCpA esters **3.22a** and **3.22b**.

The bromide **3.24** was prepared from the amino acid **3.23** by diazotization in the presence of KBr (Scheme 3.4). Exclusion of aerial oxygen and ambient light during the reaction afforded better yields of the bromide. After extractive work-up the bromide was sufficiently pure to be used in the next reaction. Careful exclusion of oxygen from the reaction mixture was required to avoid formation of side products during the substitution reaction with CsSAc. The thioacetate (**3.25**)

was activated as the succinimidyl ester (**3.26**) which was then condensed with PANS. The coupling reaction afforded the product **3.27** as a mixture of epimers. Epimerization was unavoidable even without the presence of any base (such as triethylamine) in the reaction mixture. Deacetylation with methanolic ammonia was complete in 2 h. TCEP (tris(2-carboxyethyl)phosphine) was added to the reaction mixture before HPLC purification to prevent thiol oxidation. After lyophilization, the peaks collected from HPLC showed partial disulfide formation in MALDI analysis.

### **3.3.2. Biological Evaluation**

A dual selection strategy was employed to identify modified ribosomes having an A-site architecture that would allow incorporation of  $\beta$ -amino acids during protein production. The ribosomes were initially modified in the region 2057–2063. Ribosomes that had altered erythromycin resistance as compared to wild-type ribosomes were selected in the first round. These were judged more likely to have alterations close the PTC where erythromycin binds. Moreover, the erythromycin resistance allowed the modified ribosomes to be studied independently without inference from the wild-type ribosomes that were encoded by chromosomal genes in *E. coli*. The clones that showed improved resistance to erythromycin were extra-sensitive to  $\beta$ -puromycin in comparison with the wild-type strain, thus validating the selection strategy.

The next round of mutagenesis performed on these selected clones then afforded a large library of clones containing modified ribosomes. All of the clones were checked for their erythromycin resistance and sensitivity to  $\beta$ -puromycin.

Seventeen clones with the best activity profile were chosen for further characterization and subsequent rounds of selections. The enhanced sensitivity of these clones to  $\beta$ -puromycin (in the presence of erythromycin) suggested that they were heavily dependent on the modified ribosomes for survival, probably due to increased production of the modified ribosomes.

To express full-length proteins with meaningful activity containing a  $\beta$ -amino acid at a certain position, the altered ribosomes must retain their fidelity of protein synthesis involving natural (i.e.  $\alpha$ -L) amino acids. Accordingly, next round of selection involved exclusion of the modified ribosomes that support nonspecific readthrough of a nonsense codon. The selected clones were then tested for their ability to express wild-type proteins in their active form from their wild-type mRNAs. S-30 preparations from the selected clones were then checked for their  $\beta$ -puromycin sensitivity during protein expression. Interestingly, S-30 extract from one of the clones did not show significant  $\beta$ -puromycin inhibition of protein synthesis.

Finally four S-30 preparation were utilized for suppressing an amber codon in the presence of a suppressor tRNA that was chemically misacylated with  $\beta$ -alanine. The DHFR mRNA was modified with a UAG codon at the place corresponding to position 10 (valine) of DHFR and was translated using the modified ribosomes in the presence of  $\beta$ -alanyl-tRNA<sub>CUA</sub>. Enhanced suppression of the nonsense codon in the presence of  $\beta$ -alanyl-tRNA<sub>CUA</sub> suggested incorporation of the noncanonical amino acid  $\beta$ -alanine into DHFR. The unmodified aminoacyl-tRNA, L- $\alpha$ -valyl-tRNA<sub>CUA</sub>, was used as the positive



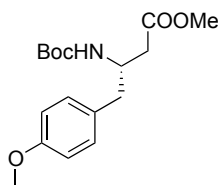
control and tRNA<sub>CUA</sub>, bearing no amino acid was used as the negative control. Four of the S-30 preparations showed enhanced expression of full length protein in the presence of  $\beta$ -alanyl-tRNA<sub>CUA</sub> compared to the wild-type S-30 system.

A similar selection strategy was adopted for the other two analogues. Erythromycin resistant clones were selected for their sensitivity to dipeptidylpuromycin (**3.3**) and thiopuromycin (**3.4**). The clones with most favorable results were sequenced. Verification of the fidelity of protein synthesis and subsequent expression of proteins containing noncanonical residues using these modified ribosomes has recently been achieved.

### 3.4. EXPERIMENTAL

Reagents and solvents for chemical synthesis were purchased from Aldrich Chemical Co. or Sigma Chemical Co. and were used without further purification. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under argon. Flash chromatography was performed using Silicycle silica gel (40–60 mesh). Analytical TLC was performed using EM silica gel 60 F<sub>254</sub> plates (0.25 mm) and was visualized by UV irradiation (254 nm). <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained using a 400 MHz Varian NMR instrument. Chemical shifts are reported in parts per million (ppm,  $\delta$ ) referenced to the residual <sup>1</sup>H resonance of the solvent (CDCl<sub>3</sub>,  $\delta$  7.26; CD<sub>3</sub>OD,  $\delta$  3.31; DMSO-*d*<sub>6</sub>,  $\delta$  2.50). <sup>13</sup>C NMR spectra were referenced to the residual <sup>13</sup>C resonance of the solvent (CDCl<sub>3</sub>,  $\delta$  77.16; CD<sub>3</sub>OD,  $\delta$  49.00; DMSO-*d*<sub>6</sub>,  $\delta$  39.52). Splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublet of doublet; dt, doublet of triplet; t, triplet; q,

quartet; m, multiplet; br, broad. High resolution mass spectra were obtained at the Michigan State University High Resolution Mass Spectrometry Laboratory.

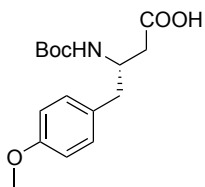


***N*-Boc-(*S*)-3-amino-4-(4-methoxyphenyl)butyric Acid Methyl Ester (3.6).<sup>94</sup>**

To a cooled (0 °C) solution containing 161 mg (0.68 mmol) of  $\beta$ -L-homotyrosine hydrochloride (3.5) in 2.6 mL of 1:1 dioxane–water was added 0.24 mL (176 mg; 1.7 mmol) of triethylamine, and the reaction mixture was stirred at 0 °C for 5 min. Di-*tert*-butyl dicarbonate (237 mg, 1.09 mmol) was added, and the reaction mixture was stirred at 0 °C for 30 min, and then at 25 °C for 20 h. The reaction mixture was concentrated under diminished pressure, and the residue was diluted with 20 mL of water and washed with two 8-mL portions of Et<sub>2</sub>O. The cooled aqueous phase (ice bath) was acidified to pH ~3 with 5% aq NaHSO<sub>4</sub> and extracted with three 20-mL portions of ethyl acetate. The combined organic layer was washed with 10 mL of brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure to afford *N*-Boc-(*S*)-3-amino-4-(4-methoxyphenyl)butyric acid as a light yellow oil: crude yield 228 mg; silica gel TLC *R*<sub>f</sub> 0.20 (9:1 chloroform–methanol). This compound was used directly in the next step without further purification.

To a suspension containing 228 mg of the crude carboxylic acid and 470 mg (3.41 mmol) of oven-dried K<sub>2</sub>CO<sub>3</sub> in 4 mL of anh acetone was added dropwise 0.20 mL (0.27 mg; 2.0 mmol) of dimethyl sulfate, and the reaction

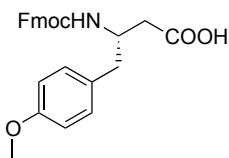
mixture was stirred at reflux for 36 h. The cooled reaction mixture was filtered and washed with ethyl acetate, and the combined filtrate was concentrated under diminished pressure. The light yellow residue was purified on a silica gel column (12 × 3 cm), which was eluted with 4:1 hexanes–ethyl acetate. Compound **3.6** was obtained as a colorless oil: yield 179 mg (81% over 2 steps); silica gel TLC  $R_f$  0.30 (2:1 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.38 (s, 9H), 2.46 (dq, 2H,  $J = 15.8$  and  $5.7$  Hz), 2.80 (m, 2H), 3.64 (s, 3H), 3.74 (s, 3H), 4.10 (m, 1H), 5.01 (br, 1H), 6.80 (d, 2H,  $J = 8.4$  Hz) and 7.06 (d, 2H,  $J = 8.4$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 28.4, 37.5, 39.5, 49.0, 51.6, 55.2, 79.3, 113.9, 129.7, 130.3, 155.1, 158.4 and 172.1; mass spectrum (ESI),  $m/z$  324.1800 ( $\text{M} + \text{H}^+$ ) ( $\text{C}_{17}\text{H}_{26}\text{NO}_5$  requires  $m/z$  324.1811).



***N*-Boc-(*S*)-3-amino-4-(4-methoxyphenyl)butyric Acid (**3.7**).**<sup>94</sup>

To a solution containing 76 mg (0.27 mmol) of compound **3.6** in 2.5 mL of THF was added a solution of 27 mg (1.1 mmol) of LiOH in 1.25 mL of water, and the reaction mixture was stirred at 25 °C for 20 h. The reaction mixture was diluted with 10 mL of  $\text{Et}_2\text{O}$  and 25 mL of water, and the phases were separated. The cooled aqueous phase (ice bath) was acidified to pH ~ 3 with 5% aq  $\text{NaHSO}_4$  and extracted with three 20-mL portions of ethyl acetate. The combined organic layer was washed with 10 mL of brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated under diminished pressure to afford **3.7** as a colorless solid: yield 65

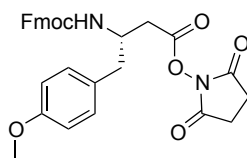
mg (91%); silica gel TLC  $R_f$  0.17 (1:1 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$ : 1.37 (s, 9H), 2.40, (m, 2H), 2.72 (m, 2H), 3.75 (s, 3H), 4.07 (m, 1H), 6.82(d, 2H,  $J = 8.4$  Hz) and 7.12 (d, 2H,  $J = 8.4$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  28.7, 39.5, 40.8, 50.7, 55.6, 79.9, 114.8, 131.3, 131.5, 157.5, 159.7 and 175.0.



***N*-Fmoc-(*S*)-3-amino-4-(4-methoxyphenyl)butyric Acid (3.8).**<sup>94</sup>

A solution containing 220 mg (0.71 mmol) of **3.7** in 4.2 mL of 4:1  $\text{CH}_2\text{Cl}_2$ – $\text{CF}_3\text{COOH}$  was stirred at 25 °C for 40 min. The reaction mixture was concentrated under diminished pressure and the residual  $\text{CF}_3\text{COOH}$  was removed by coevaporation with five 4-mL portions of toluene. The solid residue was dissolved in 4.2 mL of 10% aq  $\text{Na}_2\text{CO}_3$  and a solution containing 290 mg (0.86 mmol) of Fmoc-OSu in 4.2 mL of dioxane was added dropwise. The resulting mixture was stirred at 25 °C for 24 h. Water (25 mL) was added and the aqueous layer was washed with two 10-mL portions of ether. The cooled aqueous phase (ice bath) was acidified to pH ~ 3 with 5% aq  $\text{NaHSO}_4$  and extracted with three 30-mL portions of ethyl acetate. The combined organic layer was washed with 15 mL of brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under diminished pressure. Compound **3.8** was purified by precipitation from hexanes–ethyl acetate and was obtained as a colorless solid: yield 202 mg (66%); silica gel TLC  $R_f$  0.12 (1:1 hexanes–ethyl acetate); mp 178 °C;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  2.37 (dd, 2H,  $J = 6.8$  and 2.2 Hz), 2.66 (d, 2H,  $J = 6.8$  Hz), 3.67 (s, 3H), 3.95 (dd, 1H,

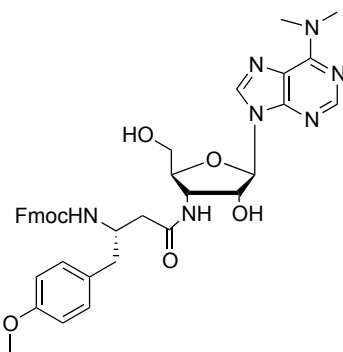
$J = 14.9$  and  $7.0$  Hz),  $4.16$  (dd,  $1\text{H}$ ,  $J = 12.9$  and  $6.0$  Hz),  $4.22$  (dd,  $2\text{H}$ ,  $J = 7.0$  and  $2.9$  Hz),  $6.80$  (d,  $2\text{H}$ ,  $J = 8.6$  Hz),  $7.08$  (d,  $2\text{H}$ ,  $J = 8.6$  Hz),  $7.32$  (q,  $2\text{H}$ ,  $J = 6.3$  Hz),  $7.41$  (t,  $2\text{H}$ ,  $J = 7.4$  Hz),  $7.65$  (d,  $2\text{H}$ ,  $J = 7.5$  Hz) and  $7.88$  (d,  $2\text{H}$ ,  $J = 7.5$  Hz);  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$   $46.7$ ,  $49.8$ ,  $54.9$ ,  $65.2$ ,  $113.6$ ,  $120.1$ ,  $125.2$ ,  $127.0$ ,  $127.6$ ,  $130.2$ ,  $130.4$ ,  $140.7$ ,  $143.8$ ,  $143.9$ ,  $155.3$ ,  $157.7$  and  $172.46$ ; mass spectrum (ESI),  $m/z$   $432.1820$  ( $\text{M} + \text{H}$ ) $^+$  ( $\text{C}_{26}\text{H}_{26}\text{NO}_5$  requires  $m/z$   $432.1811$ ).



***N*-Fmoc-(*S*)-3-amino-4-(4-methoxyphenyl)butyric Acid Succinimidyl Ester (3.9).**<sup>94</sup>

To a suspension containing  $46$  mg ( $0.11$  mmol) of **3.8** and  $19.0$  mg ( $0.16$  mmol) of *N*-hydroxysuccinimide in  $3$  mL of anhydrous  $\text{CH}_2\text{Cl}_2$  was added  $33.0$  mg ( $0.16$  mmol) of *N,N'*-dicyclohexylcarbodiimide and the reaction mixture was stirred at  $25$  °C for  $24$  h. The insoluble precipitate was filtered and the filtrate was concentrated under diminished pressure. The residue was suspended in  $3$  mL of ethyl acetate, filtered and the filtrate was concentrated under diminished pressure. The crude product was purified on a silica gel column ( $12 \times 2$  cm); elution was with  $2:1$  hexanes–ethyl acetate. Compound **3.9** was obtained as a colorless solid: yield  $38$  mg ( $68\%$ ); mp  $172$  °C; silica gel TLC  $R_f$   $0.75$  ( $9:1$  chloroform–methanol);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$   $2.70$ – $3.01$  (m,  $8\text{H}$ ),  $3.78$  (s,  $3\text{H}$ ),  $4.28$  (dt,  $4\text{H}$ ,  $J = 14.1$  and  $6.7$  Hz),  $5.35$  (d,  $1\text{H}$ ,  $J = 8.8$  Hz),  $6.85$  (d,  $2\text{H}$ ,  $J = 8.3$  Hz),  $7.15$  (d,  $2\text{H}$ ,  $J = 8.0$  Hz),  $7.30$  (t,  $2\text{H}$ ,  $J = 7.4$  Hz),  $7.40$  (t,  $2\text{H}$ ,  $J = 7.4$  Hz),  $7.56$  (dd,  $2\text{H}$ ,  $J = 7.4$

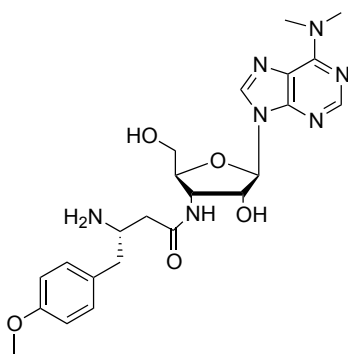
and 3.7 Hz) and 7.76 (d, 2H,  $J = 7.5$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  25.7, 34.7, 38.7, 47.3, 49.6, 55.4, 66.9, 114.3, 120.0, 125.3, 127.1, 127.8, 129.0, 130.5, 141.4, 143.98, 144.02, 155.7, 158.6, 166.7 and 169.1; mass spectrum (ESI),  $m/z$  529.1956 ( $\text{M} + \text{H}^+$ ) ( $\text{C}_{30}\text{H}_{29}\text{N}_2\text{O}_7$  requires  $m/z$  529.1975).



**9-[3'-Deoxy-3'-(*N*-Fmoc-(*S*)-3-amino-4-(4-methoxyphenyl)butyramido)- $\beta$ -D-ribofuranosyl]-6-(*N,N'*-dimethylamino)purine (3.10).<sup>94</sup>**

To a solution containing 14 mg (26  $\mu\text{mol}$ ) of **3.9** and 3.0  $\mu\text{L}$  (2.2 mg; 22  $\mu\text{mol}$ ) of  $\text{Et}_3\text{N}$  in 0.6 mL of anhydrous DMF was added 5.0 mg (17  $\mu\text{mol}$ ) of puromycin aminonucleoside (PANS). The reaction mixture was stirred at 25  $^\circ\text{C}$  for 3.5 h (at which time silica gel TLC analysis showed complete consumption of **3.9**). The solvent was concentrated under diminished pressure and the residue was purified on a silica gel column (14  $\times$  2 cm); elution was with 25:1 chloroform–methanol. Compound **3.10** was obtained as a colorless solid: yield 12 mg (the product was slightly impure and was used directly in the next step); silica gel TLC  $R_f$  0.52 (9:1 chloroform–methanol);  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  2.31 (d, 2H,  $J = 6.7$  Hz), 2.55-2.82 (m, 2H), 3.50 (s, 6H), 3.68 (m, 5H), 3.97 (s, 2H), 4.20 (m, 3H), 4.45 (dd, 2H,  $J = 16.1$  and 8.5 Hz), 5.17 (s, 1H), 5.97 (s, 2H), 6.80 (d, 2H,  $J = 8.1$  Hz), 7.06 (d,

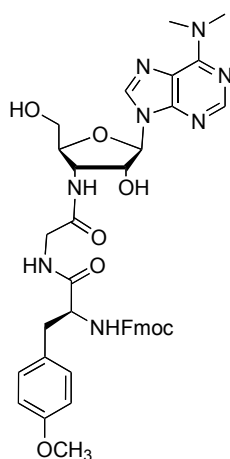
2H,  $J = 8.0$  Hz), 7.18 (d, 1H,  $J = 8.3$  Hz), 7.36 (m, 4H), 7.63 (t, 2H,  $J = 7.1$  Hz), 7.87 (d, 2H,  $J = 8.0$  Hz), 7.91 (d, 1H,  $J = 8.0$  Hz), 8.20 (s, 1H) and 8.44 (s, 1H);  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  24.9, 33.8, 47.1, 50.5, 50.6, 55.3, 60.9, 65.6, 73.6, 83.3, 89.7, 113.9, 120.0, 120.5, 125.6, 127.4, 128.0, 130.7, 138.1, 141.1, 144.2, 144.4, 150.0, 152.2, 154.7, 155.8, 158.0 and 170.8; mass spectrum (MALDI)  $m/z$  708.2 (M + H) $^+$  (theoretical 708.3) and 730.1 (M + Na) $^+$  (theoretical 730.3); mass spectrum (ESI),  $m/z$  708.3163 (M + H) $^+$  (C<sub>38</sub>H<sub>42</sub>N<sub>7</sub>O<sub>7</sub> requires  $m/z$  708.3146).



**9-[3'-Deoxy-3'-((*S*)-3-amino-4-(4-methoxyphenyl)butyramido)- $\beta$ -D-ribofuranosyl]-6-(*N,N'*-dimethylamino)purine ( $\beta$ -Puromycin) (**3.2**).<sup>94</sup>**

A solution containing 12 mg (17 mmol) of **3.10** in 3 mL of 4:1 DMF–piperidine was stirred at 25 °C for 40 min. The solvent was concentrated under diminished pressure and the residue was purified by chromatography on a silica gel column (5  $\times$  2 cm). Elution with 9:1 chloroform–methanol afforded **3.2** as a colorless solid: yield 5 mg (61% over two steps); silica gel TLC  $R_f$  0.12 (9:1 dichloromethane–methanol);  $^1\text{H}$  NMR (CD<sub>3</sub>OD)  $\delta$  2.42 (dd, 1H,  $J = 15.9$  and 8.4 Hz), 2.60 (dd, 1H,  $J = 15.9$  and 4.2 Hz), 2.82 (m, 2H), 3.51 (s, 6H), 3.59 (m, 1H), 3.73 (dd, 1H,  $J = 12.6$  and 2.9 Hz), 3.77 (s, 3H), 3.94 (dd, 1H,  $J = 12.5$  and 2.0

Hz), 4.17 (d, 1H,  $J = 7.3$  Hz), 4.62 (m, 2H), 6.01 (d, 1H,  $J = 3.0$  Hz), 6.90 (d, 2H,  $J = 8.5$  Hz), 7.17 (d, 2H,  $J = 8.5$  Hz), 8.21 (s, 1H) and 8.35 (s, 1H);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  38.8, 39.0, 40.5, 51.5, 52.0, 55.7, 62.2, 75.0, 84.8, 91.9, 115.3, 121.6, 129.6, 131.4, 139.1, 150.6, 153.0, 156.2, 160.4 and 173.3; mass spectrum (MALDI)  $m/z$  486.2 ( $\text{M} + \text{H}$ ) $^+$  (theoretical 486.2) and 508.2 ( $\text{M} + \text{Na}$ ) $^+$  (theoretical 508.2); mass spectrum (ESI),  $m/z$  486.2482 ( $\text{M} + \text{H}$ ) $^+$  ( $\text{C}_{23}\text{H}_{32}\text{N}_7\text{O}_5$  requires  $m/z$  486.2465).

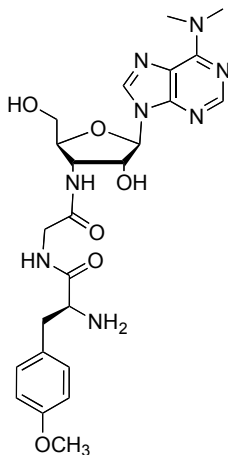


**9-[3'-Deoxy-3'-(*N*-Fmoc-4-*O*-methyl-(*S*)-tyrosylglycyl)- $\beta$ -D-ribofuranosyl]-6-(*N,N'*-dimethylamino)purine (3.15).**

To a solution of 14 mg (24  $\mu\text{mol}$ ) of **3.14** and 4.7 mg (16  $\mu\text{mol}$ ) of puromycin aminonucleoside in 0.8 mL of dry DMF was added 3  $\mu\text{L}$  (2.4 mg; 24  $\mu\text{mol}$ ) of  $\text{Et}_3\text{N}$ . The reaction mixture was stirred at 25  $^\circ\text{C}$  for 3.5 h and then concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15  $\times$  2 cm). Elution with 1:15 methanol–chloroform afforded **3.15** as a colorless solid: yield 10 mg (83%);  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  2.73 (m, 1H), 2.97 (dd, 1H,  $J = 13.7$  and 3.7 Hz), 3.47 (br s, 6H), 3.68 (s, 3H),



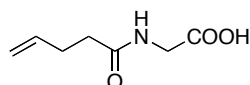
3.74, (m, 1H), 3.82 (d, 2H,  $J = 5.6$  Hz), 4.04 (m, 1H), 4.17 (m, 4H), 4.50 (m, 2H), 6.01 (d, 1H,  $J = 2.4$  Hz), 6.80 (d, 2H,  $J = 8.4$  Hz), 7.21 (m, 2H), 7.29 (m, 2H), 7.39 (d, 2H,  $J = 7.8$  Hz), 7.63 (d, 3H,  $J = 7.8$  Hz), 7.87 (d, 2H,  $J = 7.5$  Hz), 7.96 (d, 1H,  $J = 7.6$  Hz), 8.25 (s, 1H) and 8.49 (s, 1H);  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  29.0, 36.5, 42.0, 46.5, 48.6, 50.3, 54.9, 56.5, 65.7, 73.2, 83.1, 89.4, 113.5, 119.6, 120.0, 125.30, 125.31, 127.0, 127.6, 130.0, 130.2, 138.1, 140.6, 140.6, 143.7, 143.8, 149.3, 151.1, 153.6, 155.9, 157.7, 169.0 and 171.9; mass spectrum (MALDI)  $m/z$  751.5 (M + H) $^+$  (theoretical  $m/z$  751.3),  $m/z$  773.5 (M + Na) $^+$  (theoretical  $m/z$  773.3); mass spectrum (APCI),  $m/z$  751.3201 (M + H) $^+$  ( $\text{C}_{39}\text{H}_{43}\text{N}_8\text{O}_8$  requires  $m/z$  751.3204).



**9-[3'-Deoxy-3'-(*O*-methyl-(*S*)-tyrosylglycyl)- $\beta$ -D-ribofuranosyl]-6-(*N,N'*-dimethylamino)purine (dipeptidylpuromycin) (3.3).**

A solution of 10 mg (13  $\mu\text{mol}$ ) of **3.15** in 1 mL of 5:1 DMF–piperidine was stirred at 25  $^{\circ}\text{C}$  for 40 min and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (5  $\times$  2 cm). Elution with 1:9 methanol–chloroform afforded **3.3** as a colorless solid: yield 3.1

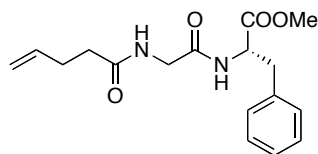
mg (49%);  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  2.85 (m, 1H), 3.07 (m, 1H), 3.51 (s, 6H), 3.76 (m, 4H) 3.93 (m, 3H), 4.18 (m, 1H), 4.63 (m, 2H), 6.03 (d, 1H,  $J = 3.0$  Hz), 6.87 (d, 2H,  $J = 8.5$  Hz), 7.17 (d, 2H,  $J = 8.6$  Hz), 8.21 (s, 1H) and 8.36 (s, 1H);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  39.0, 39.3, 43.3, 52.1, 55.7, 62.3, 68.6, 75.1, 85.0, 92.0, 106.4, 115.2, 121.6, 131.4, 139.2, 150.6, 153.0, 156.2, 160.4 and 171.6; mass spectrum (MALDI)  $m/z$  529.3 ( $\text{M} + \text{H}$ ) $^+$  (theoretical  $m/z$  529.2), 551.3 ( $\text{M} + \text{Na}$ ) $^+$  (theoretical  $m/z$  551.2); mass spectrum (APCI),  $m/z$  529.2530 ( $\text{M} + \text{H}$ ) $^+$  ( $\text{C}_{24}\text{H}_{33}\text{N}_8\text{O}_6$  requires  $m/z$  529.2523).



***N*-(4-pentenyl)glycine (3.17).**

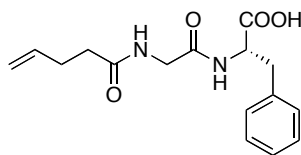
To a solution of 250 mg (3.33 mmol) of glycine in 10 mL of 10% aq  $\text{Na}_2\text{CO}_3$  was added a solution of 1.31 g (6.66 mmol) of pentenoylsuccinimide (**3.16**) in 10 mL of dioxane. The reaction mixture was stirred at 25 °C for 12 h. The mixture was acidified with 1 N aq HCl and the aq layer was extracted with three 50-mL portions of ethyl acetate. The combined organic extract was dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (16  $\times$  3 cm). Elution with 1:5:50 acetic acid–methanol–dichloromethane afforded **3.17** as a colorless oil: yield 0.430 g (82%); silica gel TLC  $R_f$  0.52 (1:10:50 acetic acid–methanol–dichloromethane);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.37 (m, 4H), 4.05 (d, 2H,  $J = 5.1$  Hz), 5.04 (m, 2H), 5.80 (m, 1H), 6.58 (m, 1H) and 10.63 (br s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  29.3, 35.3, 41.5,

115.9, 136.4, 172.8 and 174.0. mass spectrum (APCI),  $m/z$  158.0815 ( $M + H$ )<sup>+</sup>  
( $C_7H_{12}NO_3$  requires  $m/z$  158.0817).



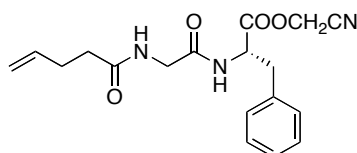
**(*N*-(4-Pentenyl)glycyl)phenylalanine Methyl Ester (3.19).**

To a solution of 0.41 g (2.60 mmol) of **3.17**, 673 mg (3.13 mmol) of **3.18** and 0.55 mL (3.95 mmol) of triethylamine in 20 mL of dry DMF was added 1.48 g (3.91 mmol) of HBTU. The reaction mixture was stirred at 25 °C for 5 h. The mixture was concentrated under diminished pressure and the residue was diluted in 80 mL of ethyl acetate. The organic layer was washed with two 40-mL portions of 1 N aq HCl, 40 mL of water and 20 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 (20 × 3 cm). Elution with 1:25 methanol–dichloromethane afforded **3.19** as a colorless oil: yield 0.49 g (58%); silica gel TLC  $R_f$  0.59 (9:1 chloroform–methanol);  $^1H$  NMR ( $CDCl_3$ )  $\delta$  2.28 (m, 4H), 3.02 (m, 2H), 3.64 (s, 3H), 3.86 (m, 2H), 4.78 (m, 1H), 4.97 (m, 2H), 5.76 (m, 1H), 7.01 (t, 1H,  $J = 5.1$  Hz), 7.09 (m, 2H), 7.19 (m, 3H) and 7.36 (d, 1H,  $J = 8.0$  Hz);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  29.3, 35.1, 37.7, 42.9, 52.2, 53.4, 115.4, 126.9, 128.4, 129.1, 135.9, 136.8, 169.1, 171.7 and 173.0; mass spectrum (ESI),  $m/z$  319.1655 ( $M + H$ )<sup>+</sup>  
( $C_{17}H_{23}N_2O_4$  requires  $m/z$  319.1652).



**(N-(4-Pentenoyl)glycyl)phenylalanine (3.20).**

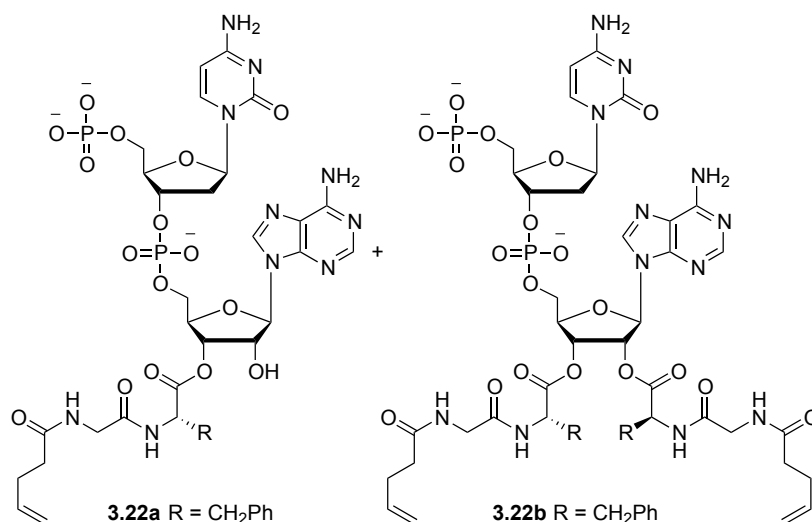
To a solution of 0.35 g (1.10 mmol) of **3.19** in 10 mL of THF was added dropwise a solution of 105 mg (4.39 mmol) of LiOH in 5 mL of water at 0 °C. The reaction mixture was stirred at 25 °C for 12 h, diluted with 30 mL of water and washed with two 15-mL portions of Et<sub>2</sub>O. The aqueous layer was acidified with 1 N aq HCl to pH ~ 2 and extracted with three 40-mL portions of ethyl acetate. The combined organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure to obtain **3.20** as a colorless solid: yield 301 mg (90%); silica gel TLC *R<sub>f</sub>* 0.25 (9:1 chloroform–methanol); <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 2.32 (s, 4H), 3.01 (dd, 1H, *J* = 13.9 and 8.0 Hz), 3.19 (dd, 1H, *J* = 13.9 and 5.2 Hz), 3.82 (q, 2H, *J* = 16.7 Hz), 4.68 (dd, 1H, *J* = 7.6 and 5.4 Hz), 4.96 (m, 1H), 5.04 (d, 1H, *J* = 17.0 Hz), 5.83 (m, 1H) and 7.24 (m, 5H); <sup>13</sup>C NMR (CD<sub>3</sub>OD) δ 29.2, 34.7, 36.9, 41.8, 52.5, 114.4, 126.4, 128.0, 128.9, 136.7, 136.8, 169.9, 172.9 and 174.3; mass spectrum (APCI), *m/z* 305.1503 (M + H)<sup>+</sup> (C<sub>17</sub>H<sub>23</sub>N<sub>2</sub>O<sub>4</sub> requires *m/z* 305.1501).



**(N-(4-pentenoyl)glycyl)phenylalanine Cyanomethyl Ester (3.21).**

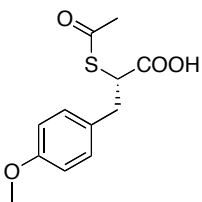
To a solution of 301 mg (0.99 mmol) of compound **3.20** in 18 mL anhydrous acetonitrile was added 0.70 mL (0.51 g; 5.06 mmol) of Et<sub>3</sub>N followed by 0.63 mL

(0.53 g; 9.9 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 16 h and then concentrated under diminished pressure. The residue was diluted in 80 mL of ether. The ether layer was washed successively with 40 mL of water, 40 mL of 1 N HCl and 30 mL of brine. The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 1:3 hexanes–ethyl acetate afforded the ester **3.21** as a colorless oil: yield 0.28 g (82%); silica gel TLC *R<sub>f</sub>* 0.67 (9:1 chloroform–methanol); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.31 (m, 4H), 3.09 (m, 2H), 3.89 (m, 2H), 4.67 (m, 2H), 4.81 (m, 1H), 5.01 (m, 2H), 5.76 (m, 1H), 6.79 (m, 1H), 7.14 (d, 2H, *J* = 7.7 Hz), 7.26 (m, 3H) and 7.44 (d, 1H, *J* = 7.6 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 29.3, 35.2, 37.5, 43.1, 49.0, 53.4, 114.0, 115.7, 127.4, 128.8, 129.2, 135.2, 136.8, 169.4, 170.2 and 173.3; mass spectrum (APCI), *m/z* 344.1607 (M + H)<sup>+</sup> (C<sub>18</sub>H<sub>22</sub>N<sub>2</sub>O<sub>4</sub> requires *m/z* 344.1610).



**Mono-2' (3')-O-(N-(4-pentenoyl)glycyl)phenylalanyl-pdCpA (3.22a) and Bis-2',3'-O-(N-(4-pentenoyl)glycyl)phenylalanyl-pdCpA (3.22a).**

A solution containing 35.0 mg (0.10 mmol) of cyanomethyl ester **3.21** and 8.0 mg (5.9  $\mu\text{mol}$ ) of the tris(tetrabutylammonium) salt of pdCpA<sup>96</sup> in 100  $\mu\text{L}$  of DMF was subjected to sonication at room temperature for 1 h. The reaction mixture was diluted with 2:1  $\text{CH}_3\text{CN}$ –50 mM  $\text{NH}_4\text{OAc}$ , pH 4.5, to a total volume of 800  $\mu\text{L}$  and purified using a semipreparative  $\text{C}_{18}$  reversed phase column (250  $\times$  10 mm). The column was washed with 1  $\rightarrow$  63%  $\text{CH}_3\text{CN}$  in 50 mM  $\text{NH}_4\text{OAc}$ , pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). After lyophilization of the appropriate fractions two compounds were obtained as colorless solids: mono-2'(3')-*O*-(*N*-(4-pentenoyl)glycyl)phenylalanyl-pdCpA (**3.22a**) (retention times 18.4 and 18.8 min, for the two positional (2',3') isomers): yield 3.5 mg (64%). mass spectrum (ESI),  $m/z$  921.2365 ( $\text{M} - \text{H}$ )<sup>-</sup> ( $\text{C}_{35}\text{H}_{43}\text{N}_{10}\text{O}_{16}\text{P}_2$  requires  $m/z$  921.2339); bis-2',3'-*O*-(*N*-(4-pentenoyl)glycyl)phenylalanyl-pdCpA (**3.22a**): yield 2.0 mg (28%). mass spectrum (ESI),  $m/z$  603.1809 ( $\text{M} - 2\text{H}$ )<sup>2-</sup> ( $\text{C}_{51}\text{H}_{60}\text{N}_{12}\text{O}_{19}\text{P}_2$ <sup>2-</sup> requires  $m/z$  603.1792).



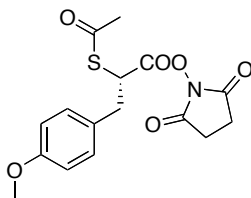
**(*S*)-2-(Acetylthio)-3-(4-methoxyphenyl)propanoic Acid (3.25).**<sup>98</sup>

A solution of 400 mg (2.05 mmol) of (*R*)-4-*O*-methyltyrosine, 829 mg (6.97 mmol) of KBr, and 0.51 mL of 48% HBr in 2 mL of water was cooled to  $-13$   $^{\circ}\text{C}$  in an ice–salt water bath with argon bubbling through the solution.  $\text{NaNO}_2$  (176

mg; 2.54 mmol) was added portionwise over a period of 45 min and the reaction mixture was allowed to warm to room temperature. The argon bubbling was ceased and the reaction mixture was stirred at room temperature for 20 h. The solution was bubbled with argon for 1 h and then extracted with three 20-mL portions of diethyl ether. The combined organic extract was dried ( $\text{Na}_2\text{SO}_4$ ), filtered and concentrated under diminished pressure to give the bromo acid **3.24** as a yellow oil which was used in the next step without further purification: yield 230 mg (44%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.20 (dd, 1H,  $J = 14.4$  and 7.2 Hz), 3.41 (dd, 1H,  $J = 14.4$  and 8.0 Hz), 3.80 (s, 3H), 4.38 (dd, 1H,  $J = 8.0$  and 7.2 Hz); 6.58 (d, 2H,  $J = 8.4$  Hz) and 7.50 (d, 2H,  $J = 8.4$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  39.9, 45.0, 55.2, 114.1, 128.4, 130.2, 158.9 and 175.2.

A suspension of 464 mg (1.42 mmol)  $\text{Cs}_2\text{CO}_3$  in 3.5 mL of dry MeOH was bubbled with argon for 10 min and cooled to 0 °C. To this was added dropwise 0.1 mL (92 mg; 1.5 mmol) of thioacetic acid and the mixture was stirred for 1 h while warming to room temperature. The solvent was concentrated under diminished pressure and the residue was dissolved in 1 mL of dry DMF. Bromo-acid **3.24** (230 mg, 0.89 mmol) in 1.2 mL of DMF was added dropwise and the resulting solution was stirred at room temperature for 20 h. The solution was poured over 10 mL of 1 N HCl and extracted with three 15-mL portions of EtOAc. The combined organic extract was dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (14 × 3 cm). Elution with 1:1:50 acetic acid–methanol–chloroform afforded **3.25** as a colorless oil: yield 0.14 g (61%); silica gel TLC  $R_f$  0.31 (9:1

chloroform–methanol);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.32 (s, 3H), 2.97 (dd, 1H,  $J = 14.0$  and 7.6 Hz), 3.22 (dd, 1H,  $J = 14.2$  and 7.6 Hz), 3.77 (s, 3H), 4.40 (m, 1H); 6.82 (d, 2H,  $J = 8.0$  Hz), 7.14 (d, 2H,  $J = 7.9$  Hz) and 10.45 (br s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  30.3, 36.7, 47.5, 55.3, 114.0, 129.0, 130.3, 158.8, 176.6 and 193.9.

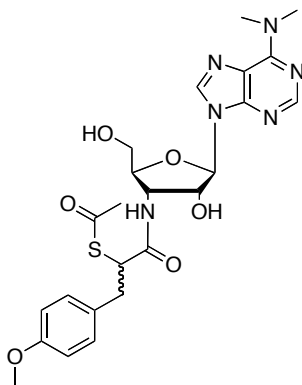


**(*S*)-2-(Acetylthio)-3-(4-methoxyphenyl)propanoic Acid Succinimidyl Ester (3.26).**

To a cooled (0 °C) mixture of 110 mg (0.43 mmol) of **3.25** and 74.0 mg (0.65 mmol) *N*-hydroxysuccinimide in 1.5 mL of dry THF was added dropwise a solution of 165 mg (0.80 mmol) of DCC in 1.5 mL of dry THF. The reaction mixture was stirred at room temperature overnight and then concentrated under diminished pressure. The residue was suspended in 4 mL of acetonitrile, filtered and the filtrate was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (18 × 2 cm). Elution with 2:1 hexanes–ethyl acetate afforded **3.26** as a colorless solid: yield 75 mg (49%); silica gel TLC  $R_f$  0.42 (15:1 chloroform–methanol);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.32 (s, 3H), 2.76 (s, 4H), 3.09 (dd, 1H,  $J = 11.6$  and 6.0 Hz), 3.32 (dd, 1H,  $J = 11.2$  and 7.6 Hz), 3.76 (s, 3H), 4.63 (m, 1H); 6.86 (d, 2H,  $J = 7.2$  Hz) and 7.23 (d, 2H,  $J = 7.2$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  26.36, 26.39, 30.4, 36.8, 45.7, 55.9, 114.8, 129.3,



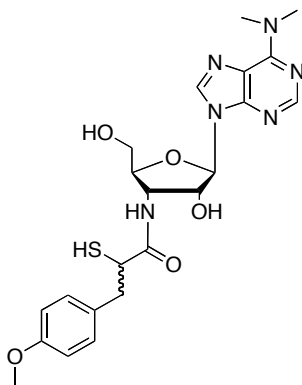
131.5, 159.9, 168.3, 170.7 and 193.7; mass spectrum (APCI),  $m/z$  352.0863 ( $M + H$ )<sup>+</sup> ( $C_{16}H_{18}NO_6S$  requires  $m/z$  352.0855).



**9-[3'-Deoxy-3'-(2-(acetylthio)-3-(4-methoxyphenyl)propanamido)-β-D-ribofuranosyl]-6-(N,N'-dimethylamino)purine (3.27).**

To a solution of 20 mg (57 μmol) of compound **3.26** and 7.0 mg (24 μmol) of puromycin aminonucleoside in 1.0 mL of dry DMF was added 3 μL (2.4 mg; 24 μmol) of Et<sub>3</sub>N. The reaction mixture was stirred at room temperature for 3.5 h and then concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 1:15 methanol–chloroform afforded **3.27** as a colorless solid (mixture of epimers): yield 10 mg (79%); silica gel TLC  $R_f$  0.36 (9:1 chloroform–methanol); <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 2.30 (s, 3H), 2.91 (dd, 1H,  $J = 13.6$  and 6.0 Hz), 3.08 (dd, 1H,  $J = 13.5$  and 9.9 Hz), 3.46 (m, 8H), 3.74 (s, 3H), 3.83 (ddd, 1H,  $J = 6.7, 5.3$  and 2.2 Hz), 4.37 (dd, 1H,  $J = 9.9$  and 6.1 Hz), 4.51 (m, 3H); 5.86 (d, 1H,  $J = 3.1$  Hz), 6.84 (d, 2H,  $J = 8.6$  Hz), 7.16 (d, 2H,  $J = 8.5$  Hz), 8.17 (s, 1H) and 8.27 (s, 1H); <sup>13</sup>C NMR (CD<sub>3</sub>OD) δ 30.2, 38.6, 49.9, 52.1, 55.7, 62.3, 74.7, 85.1, 91.9, 114.9, 121.6,

130.9, 131.4, 139.2, 150.6, 152.9, 156.2, 160.2, 173.1 and 196.2; mass spectrum (ESI),  $m/z$  530.2023 ( $M + H$ )<sup>+</sup> ( $C_{24}H_{31}N_6O_6S$  requires  $m/z$  530.2020).



**9-[3'-Deoxy-3'-(2-thio-3-(4-methoxyphenyl)propanamido)-β-D-ribofuranosyl]-6-(N,N'-dimethylamino)purine (3.4).**

A solution of 7.0 mg (13 μmol) of compound **9** in 1.2 mL of dry MeOH was saturated with NH<sub>3</sub> and the reaction mixture was stirred at room temperature for 2 h. To the reaction mixture were added 0.8 mL of 50 mM aq NH<sub>4</sub>OAc buffer and ~ 5 mg of tris(2-carboxyethyl)phosphine, and the mixture was purified using a semipreparative C<sub>18</sub> reversed phase column (250 × 10 mm). The column was washed with 1 → 63% CH<sub>3</sub>CN in 50 mM NH<sub>4</sub>OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). Fractions containing the desired product eluted at 32.0 min and were collected, frozen, and lyophilized to give **3.4** as a colorless solid: yield 4.8 mg (74%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.75 (m, 4H), 3.08 (m, 3H), 3.54 (m, 4H), 3.71 (s, 3H), 3.71 (s, 3H), 3.74 (m, 3H), 3.99 (m, 1H), 4.35 (br s, 12H), 4.45 (m, 2H), 5.08 (t, 1H, *J* = 5.4 Hz), 5.20 (t, 1H, *J* = 5.4 Hz), 5.94 (d, 1H, *J* = 3.1 Hz), 5.98 (d, 1H, *J* = 2.6 Hz), 6.03 (m, 1H), 6.13 (d, 1H, *J* = 5.0 Hz), 6.82 (d, 2H, *J* = 2.9 Hz), 6.84 (d, 2H, *J* = 2.9 Hz), 7.13 (d, 4H, *J* = 8.6

Hz), 8.09 (d, 1H,  $J = 8.1$  Hz), 8.13 (d, 1H,  $J = 7.4$  Hz), 8.23 (s, 1H), 8.23 (s, 1H), 8.42 (s, 1H) and 8.44 (s, 1H);  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  41.08, 41.09, 42.5, 42.7, 50.3, 50.4, 54.9, 55.0, 60.8, 61.0, 73.01, 73.02, 83.4, 83.5, 89.31, 89.32, 113.50, 113.55, 119.60, 119.61, 130.0, 130.1, 130.47, 130.55, 137.8, 149.6, 151.8, 154.2, 157.8, 157.9, 172.0 and 172.4; mass spectrum (MALDI),  $m/z$  489.3 (M + H) $^+$  (theoretical  $m/z$  489.2); mass spectrum (APCI),  $m/z$  489.1921 (M + H) $^+$  ( $\text{C}_{22}\text{H}_{29}\text{N}_6\text{O}_5\text{S}$  requires  $m/z$  489.1920).

## CHAPTER 4

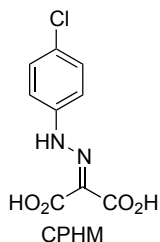
### SYNTHESIS OF MESOXALIC ACID HYDRAZONES

#### 4.1. INTRODUCTION

Due to its central role in the viral replication process the reverse transcriptase enzyme of human immunodeficiency virus (HIV) has been the target of a large number of anti-HIV therapeutic agents. The p66 subunit of HIV-1 reverse transcriptase (HIV-1 RT) has two active sites.<sup>99</sup> The polymerase site catalyzes RNA/DNA-dependent DNA polymerization and the RNase active site is responsible for selective hydrolysis of the RNA strands from RNA–DNA heteroduplexes. There are two kinetically different pathways for RNA hydrolysis, namely polymerase-dependent RNase activity and polymerase-independent RNase activity.<sup>100</sup> RNase activity is very important for the two DNA strand transfer reactions that occur during the reverse transcription process.<sup>100</sup> The strand transfer reactions involve translocation of a nascent DNA strand from one template strand to another.

In an automated screening of a library of compounds, 4-chlorophenylhydrazone of mesoxalic acid (CPHM) was identified as an inhibitor of the DNA strand transfer reactions catalyzed by the reverse transcriptase with an  $IC_{50}$  value of 4.5  $\mu$ M (Figure 4.1).<sup>101</sup> The structure-activity relationship studies revealed that both the carboxylic acid moieties are essential for the inhibitory activity. Initially, it was proposed that CPHM chelates the  $Mg^{2+}$  ion present at the RNase active site and blocks the hydrolysis of RNA from RNA–DNA duplexes. Inhibition of the RNase activity prevents the transfer of the newly synthesized

DNA strand to a new template. CPHM was also shown to be specific for the reverse transcriptase from HIV-1.<sup>102</sup>

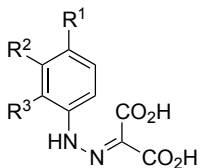


**Figure 4.1.** Structure of 4-chlorophenylhydrazone of mesoxalic acid (CPHM).

In 2002, Shaw-Reid and coworkers studied the mode of action of CPHM using mutant HIV-1 RT enzymes.<sup>103</sup> CPHM inhibited both the polymerase and the RNase activity of wild type HIV-1 RT but it did not show any activity against a polymerase active site mutant (D185N). Interestingly, its activity against an RNase active site mutant (D443N) was similar to that against the wild-type enzyme. CPHM also failed to inhibit the isolated RNase H domain chimera of HIV-1 RT. This data clearly suggested that CPHM binds near the polymerase active site and causes inhibition of RNase activity, possibly through allosteric effects. In cellular assays, however, CPHM did not show any antiviral activity. This was probably due to the inefficient uptake of this drug across the cell membrane.

Development of new inhibitors of HIV-1 RT might be useful in elucidating the mechanism of several steps of the viral reverse transcription process. Discovery of more potent inhibitors might also lead to new anti-viral therapies. We synthesized a series of analogues of CPHM (Figure 4.2) with

various substituents on the aromatic ring. Our collaborators designed these analogues using molecular docking simulations based on their ability to bind to the polymerase active site.

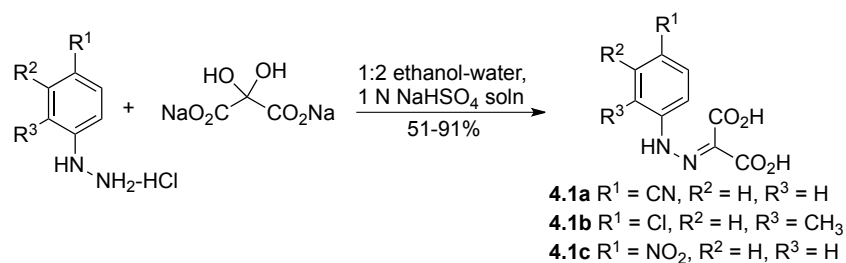


- 4.1a** R<sup>1</sup> = CN, R<sup>2</sup> = H, R<sup>3</sup> = H  
**4.1b** R<sup>1</sup> = Cl, R<sup>2</sup> = H, R<sup>3</sup> = CH<sub>3</sub>  
**4.1c** R<sup>1</sup> = NO<sub>2</sub>, R<sup>2</sup> = H, R<sup>3</sup> = H  
**4.1d** R<sup>1</sup> = NHAc, R<sup>2</sup> = H, R<sup>3</sup> = H  
**4.1e** R<sup>1</sup> = OBn, R<sup>2</sup> = H, R<sup>3</sup> = H  
**4.1f** R<sup>1</sup> = Cl, R<sup>2</sup> = CH<sub>3</sub>, R<sup>3</sup> = H  
**4.1g** R<sup>1</sup> = OMe, R<sup>2</sup> = H, R<sup>3</sup> = H

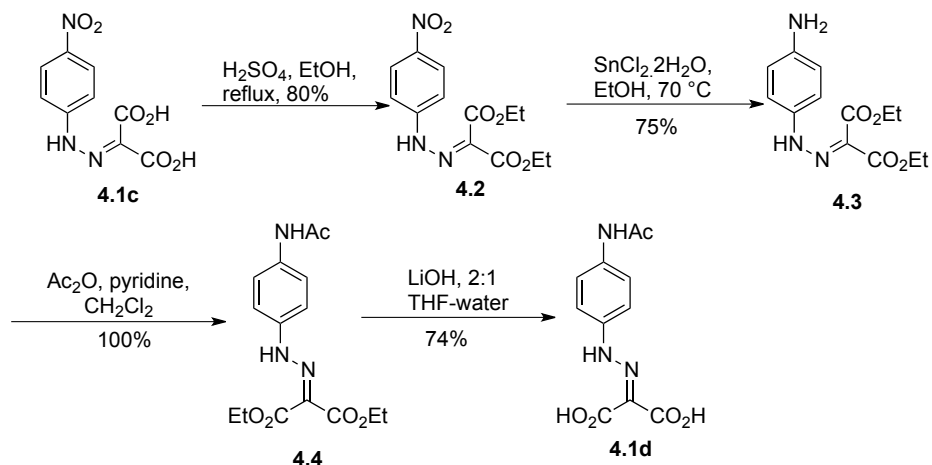
**Figure 4.2.** Structures of CPHM analogues prepared.

## 4.2 RESULTS

Hydrazones **4.1a**, **4.1b** and **4.1c** were prepared in a single step by condensing the corresponding arylhydrazines with disodium mesoxalate in the presence of an acid catalyst (Scheme 4.1). Compound **1c** was subsequently esterified with ethanol and catalytic conc. H<sub>2</sub>SO<sub>4</sub> to afford the diester **4.2** in 80% yield. Reduction of the nitro group with stannous chloride followed by acetylation of the resulting amine gave intermediate **4.4**. Ester hydrolysis of the diester **4.4** using aqueous LiOH afforded the diacid **4.1d** in 74 % yield (Scheme 4.2).<sup>104</sup>

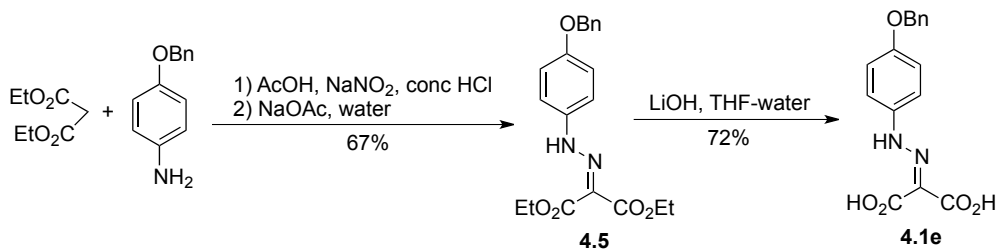


**Scheme 4.1.** Synthesis of CPHM analogues **4.1a**, **4.1b** and **4.1c**.<sup>104</sup>

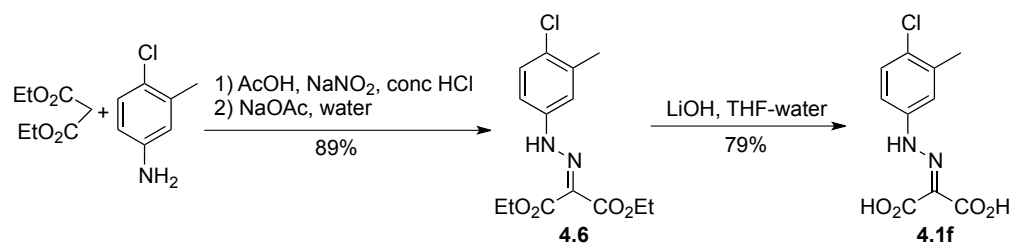


**Scheme 4.2.** Synthesis of 4-*N*-acetyl analogue **4.1d**.<sup>104</sup>

Analogue **4.1e** was synthesized from 4-*O*-benzylaniline hydrochloride by diazonium coupling with diethyl malonate to form intermediate **4.5**, followed by hydrolysis of the diester using LiOH (Scheme 4.3).<sup>104</sup> Similarly, 4-chloro-3-

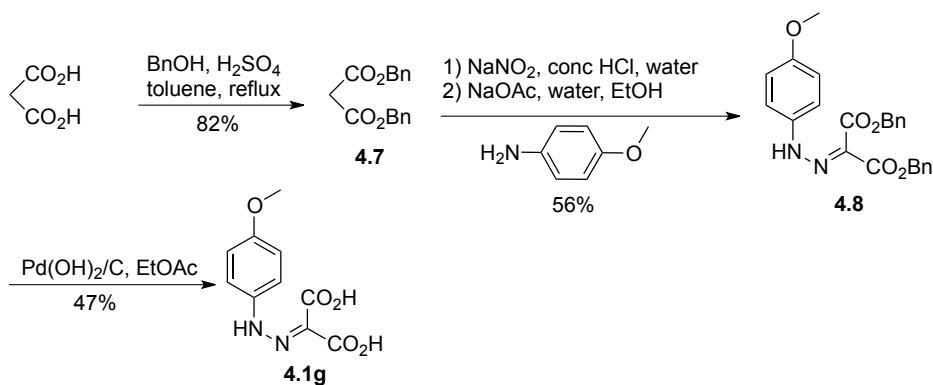


**Scheme 4.3.** Synthesis of 4-*O*-benzyl analogue **4.1e**.<sup>104</sup>



**Scheme 4.4.** Synthesis of 4-chloro-3-methyl analogue **4.1f**.

methylaniline was diazo-coupled with diethyl malonate and the intermediate **4.6** was subjected to ester-hydrolysis to produce diacid **4.1f** in 70% overall yield (Scheme 4.4). The synthesis of the 4-*O*-methyl analogue **4.1g** was achieved using a slightly different route. Diazo coupling of *p*-anisidine with dibenzyl malonate **4.7** (prepared from malonic acid)<sup>105</sup> afforded intermediate **4.8** in 56% yield. Reductive debenzoylation using Pd(OH)<sub>2</sub>/C provided analogue **4.1g** in 47% yield (Scheme 4.5).<sup>104</sup>



**Scheme 4.5.** Synthesis of 4-*O*-methyl analogue **4.1g**.<sup>104</sup>



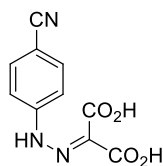
### 4.3. DISCUSSION

Syntheses of the analogues **4.1a–c** were achieved in one step from the corresponding hydrazones, which were commercially available. Reduction of the nitro group to the amine group in Scheme 4.2 was much cleaner when the diacid moiety was protected as the ethyl esters. Analogues **4.1e–g** were prepared by condensing diazotized aniline derivatives with malonate esters and subsequent deprotection of the esters. For the synthesis of 4-*O*-methyl analogue (**4.1g**), dibenzyl malonate was used instead of diethyl malonate. The diethyl analogue of **4.8** never afforded the desired product (**4.1g**) when subjected to saponification conditions, most probably due to oxidative decomposition. Reductive removal of the benzyl groups was employed to circumvent this problem (Scheme 4.5). These analogues were sent to our collaborators for biochemical evaluation.

### 4.4. EXPERIMENTAL

Chemicals and solvents were of reagent grade and were used without further purification. All reactions involving air or moisture sensitive reagents or intermediates were performed under an argon atmosphere. Flash chromatography was carried out using Silicycle 200–400 mesh silica gel. Analytical TLC was carried out using 0.25 mm EM Silica Gel 60 F250 plates that were visualized by irradiation (254 nm) or by staining with *p*-anisaldehyde stain. <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained using an Inova 400 MHz Varian instrument. Chemical shifts were reported in parts per million (ppm, δ) referenced to the residual <sup>1</sup>H resonance of the solvent (CDCl<sub>3</sub>, 7.26 ppm; DMSO-*d*<sub>6</sub>, 2.50 ppm). <sup>13</sup>C spectra

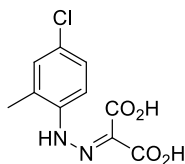
were referenced to the residual  $^{13}\text{C}$  resonance of the solvent ( $\text{CDCl}_3$ , 77 ppm;  $\text{DMSO}-d_6$ , 39.5 ppm). Splitting patterns were designated as follow: s, singlet; br, broad; d, doublet; dd, doublet of doublet; dt, doublet of triplet; m, multiplet. High resolution mass spectra were obtained at the Arizona State University High Resolution Mass Spectrometry Laboratory or at the Ohio State University Mass Spectrometry Facility.



#### **2-(2-(4-Cyanophenyl)hydrazono)malonic Acid (4.1a).**

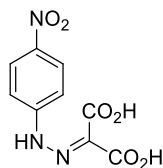
To a suspension containing 180 mg (1.00 mmol) of disodium ketomalonate in 3 mL of 1:2 ethanol–water was added 170 mg (1.00 mmol) of 4-cyanophenylhydrazine hydrochloride with vigorous stirring. The pH of the resulting mixture was adjusted to ~5 by adding 1 N aq  $\text{NaHSO}_4$  soln and the reaction mixture was stirred at 23 °C for 12 h. The mixture was filtered and the precipitate was washed with cold acidic (pH ~4) water. The precipitate was dissolved in 40 mL of ethyl acetate and was extracted with three 20-mL portions of 1 N aq  $\text{NaOH}$  soln. The combined  $\text{NaOH}$  extract was washed with two 10-mL portions of ethyl acetate, cooled in an ice bath and acidified to pH ~3 with 3 N  $\text{HCl}$ . The formed precipitate was filtered, washed with cold acidic (pH ~4) water and air-dried to obtain **4.1a** as a pale yellow solid: yield 182 mg (78%); mp >200 °C; silica gel TLC  $R_f$  0.45 (4:1 chloroform–methanol);  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  7.49 (d, 2H,  $J = 8.8$  Hz), 7.77 (d, 2H,  $J = 8.8$  Hz) and 13.55 (br s, 1H);  $^{13}\text{C}$  NMR

(DMSO- $d_6$ )  $\delta$  104.3, 115.2, 115.2, 119.2, 125.6, 133.8, 133.8, 146.3, 165.0 and 165.3; mass spectrum (APCI),  $m/z$  232.0342 ( $M - H$ )<sup>-</sup> ( $C_{10}H_6N_3O_4$  requires 232.0358).



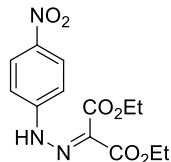
**2-(2-(4-Chloro-2-methylphenyl)hydrazono)malonic Acid (4.1b).**

To a suspension containing 180 mg (1.00 mmol) of disodium ketomalonate in 3 mL of 1:2 ethanol–water was added 198 mg (1.00 mmol) of 4-chloro-2-methylphenylhydrazine with vigorous stirring. The pH of the resulting mixture was adjusted to ~5 by adding 1 N aq NaHSO<sub>4</sub> soln and the reaction mixture was stirred at 23 °C for 12 h. The mixture was filtered and the precipitate was washed with cold acidic (pH ~4) water. The precipitate was dissolved in 40 mL of ethyl acetate and extracted with three 20-mL portions of 1 N aq NaOH soln. The combined NaOH extract was washed with two 10-mL portions of ethyl acetate, cooled in an ice bath and acidified to pH ~3 with 3 N HCl. The formed precipitate was filtered, washed with cold acidic (pH ~4) water and air-dried to obtain **4.1b** as a yellow solid: yield 132 mg (51%); mp >200 °C; silica gel TLC  $R_f$  0.33 (4:1 chloroform–methanol); <sup>1</sup>H NMR (DMSO- $d_6$ )  $\delta$  2.23 (s, 3H), 7.26 (m, 2H) and 7.51 (m, 1H); <sup>13</sup>C NMR (DMSO- $d_6$ )  $\delta$  16.9, 114.7, 125.1, 125.4, 126.0, 127.3, 130.4, 140.4, 165.6 and 169.0; mass spectrum (APCI),  $m/z$  255.0176 ( $M - H$ )<sup>-</sup> ( $C_{10}H_8N_2O_4Cl$  requires 255.0173).



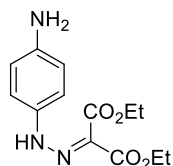
**2-(2-(4-Nitrophenyl)hydrazono)malonic Acid (4.1c).**<sup>104</sup>

To a suspension containing 180 mg (1.00 mmol) of disodium ketomalonate in 3 mL of 1:2 ethanol–water was added 208 mg (1.10 mmol) of 4-nitrophenylhydrazine hydrochloride with vigorous stirring. The pH of the resulting mixture was adjusted to ~5 by adding 1 N aq NaHSO<sub>4</sub> soln and the reaction mixture was stirred at 23 °C for 12 h. The mixture was filtered and the precipitate was washed with cold acidic (pH ~4) water. The precipitate was dissolved in 40 mL of ethyl acetate and extracted with three 20-mL portions of 1 N aq NaOH soln. The combined NaOH extract was washed with two 10-mL portions of ethyl acetate, cooled in an ice bath, acidified to pH ~3 with 3 N HCl and re-extracted with three 40-mL portions of ethyl acetate. The combined organic layer was then washed with 20 mL of brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure to afford **4.1c** as a yellow solid: yield 230 mg (91%); mp 196–198 °C (dec); silica gel TLC *R<sub>f</sub>* 0.40 (4:1 chloroform–methanol); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 7.56 (d, 2H, *J* = 9.2 Hz), 8.23 (d, 2H, *J* = 9.2 Hz) and 12.84 (br s, 1H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) δ 115.0, 115.0, 125.6, 125.6, 126.8, 142.2, 148.1, 163.5 and 165.0; mass spectrum (APCI), *m/z* 252.0266 (M – H)<sup>–</sup> (C<sub>9</sub>H<sub>6</sub>N<sub>3</sub>O<sub>6</sub> requires 252.0257).



**2-(2-(4-Nitrophenyl)hydrazono)malonic Acid Diethyl Ester (4.2).**<sup>104</sup>

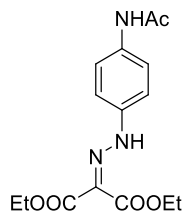
To a solution of 235 mg (0.760 mmol) of **4.1c** in 15 mL of ethanol was added 6–7 drops of H<sub>2</sub>SO<sub>4</sub> and the reaction mixture was heated to reflux for 16 h. The cooled reaction mixture was concentrated under diminished pressure and the residue was diluted in 40 mL of ethyl acetate. The organic layer was washed with two 20-mL portions of sat aq NaHCO<sub>3</sub>, 10 mL of brine, then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure. The resulting residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 50:1 chloroform–methanol gave **4.2** as a light yellow solid: yield 230 mg (80%); mp 64–66 °C; silica gel TLC *R*<sub>f</sub> 0.72 (1:1 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.38 (m, 6H), 4.36 (m, 4H), 7.38 (d, 2H, *J* = 9.2 Hz), 8.24 (d, 2H, *J* = 9.2 Hz) and 12.72 (br s, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 14.0, 14.2, 61.8, 62.1, 115.0, 115.0, 124.3, 125.7, 125.7, 143.7, 143.9, 162.6 and 162.7; mass spectrum (ESI), *m/z* 332.0844 (M + Na)<sup>+</sup> (C<sub>13</sub>H<sub>15</sub>N<sub>3</sub>O<sub>6</sub>Na requires 332.0859).



**2-(2-(4-Aminophenyl)hydrazono)malonic Acid Diethyl Ester (4.3).**

A suspension containing 44.0 mg (0.14 mmol) of **4.2** and 160 mg (0.71 mmol) of SnCl<sub>2</sub>•2H<sub>2</sub>O in 2 mL of absolute ethanol was purged with argon for 15 min. The

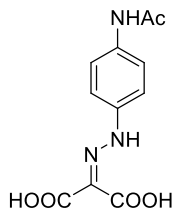
reaction mixture was heated to 70 °C for 12 h under an argon atmosphere. The pH of the cooled reaction mixture was adjusted to ~9 by addition of sat aq NaHCO<sub>3</sub> soln and the resulting mixture was stirred at 23 °C for 1 h. The reaction mixture was then extracted with three 20-mL portions of ethyl acetate. The combined organic layer was washed with three 20-mL portions of brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (12 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave **4.3** as an orange solid: yield 30 mg (75%); mp 91 °C; silica gel TLC *R*<sub>f</sub> 0.44 (1:1 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.36 (m, 6H), 3.68 (br s, 2H), 4.32 (m, 4H), 6.67 (d, 2H, *J* = 8.8 Hz), 7.17 (d, 2H, *J* = 8.8 Hz) and 12.99 (br s, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 14.3, 14.4, 60.9, 61.1, 115.9, 115.9, 117.2, 117.6, 117.6, 134.1, 144.1, 163.8 and 164.2; mass spectrum (APCI), *m/z* 280.1299 (M + H)<sup>+</sup> (C<sub>13</sub>H<sub>18</sub>N<sub>3</sub>O<sub>4</sub> requires 280.1297).



**2-((4-Acetylamino)phenyl)hydrazono)malonic Acid Diethyl Ester (4.4).**<sup>104</sup>

To a solution containing 20.0 mg (72 μmol) of **4.3** and 57.0 μL (56 mg; 0.72 mmol) of pyridine in 2 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> was added 36.0 μL (39 mg; 0.36 mmol) of acetic anhydride and the reaction mixture was stirred at 23 °C for 8 h. The reaction mixture was concentrated under diminished pressure and diluted in 15 mL of ethyl acetate. The organic layer was washed with three 5-mL portions of

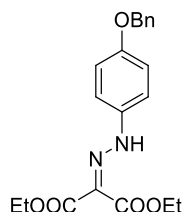
1 N HCl, 5 mL of satd aq NaHCO<sub>3</sub> soln, 5 mL of brine, then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure. The residue was purified by flash column chromatography on a silica gel column (12 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave **4.4** as yellow solid: yield 23 mg (100%); mp 154–156 °C; silica gel TLC *R<sub>f</sub>* 0.28 (1:1 hexanes–ethyl acetate); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.36 (m, 6H), 2.15 (s, 3H), 4.32 (m, 4H), 7.26 (d, 2H, *J* = 8.4 Hz), 7.52 (d, 2H, *J* = 8.4 Hz), 7.71 (br s, 1H) and 12.84 (s, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 14.2, 14.4, 24.6, 61.3, 61.4, 116.1, 116.1, 119.6, 121.1, 121.1, 135.1, 138.2, 163.66, 163.68 and 168.5; mass spectrum (APCI), *m/z* 322.1403 (M + H)<sup>+</sup> (C<sub>15</sub>H<sub>20</sub>N<sub>3</sub>O<sub>5</sub> requires 322.1403).



**2-((4-Acetylamino-phenyl)-hydrazono)malonic Acid (4.1d).**<sup>104</sup>

To a solution containing 23.0 mg (72 μmol) of **4.4** in 1 mL THF was added dropwise a solution containing 8.0 mg (0.3 mmol) of LiOH in 0.5 mL of water. The reaction mixture was stirred at 23 °C for 16 h. The reaction was diluted with 5 mL of Et<sub>2</sub>O and 10 mL of water and the phases were separated. The aqueous layer was washed with two 5-mL portions of ethyl acetate, cooled in an ice bath, acidified to pH ~3 with 1 N HCl and extracted with three 10-mL portions of ethyl acetate. The combined organic layer was then washed with 10 mL of brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under diminished pressure to

afford **4.1d** as a yellow solid: yield 14 mg (74%); mp 176–178 °C (dec); silica gel TLC  $R_f$  0.19 (4:1 chloroform–methanol);  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  2.03 (s, 3H), 7.45 (d, 2H,  $J = 8.8$  Hz), 7.61 (d, 2H,  $J = 8.8$  Hz), 9.99 (s, 1H) and 13.24 (s, 1H);  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  24.0, 116.4, 116.4, 116.9, 119.9, 119.9, 136.6, 136.9, 164.6, 168.2 and 168.6; mass spectrum (APCI),  $m/z$  264.0627 (M–H) $^-$  ( $\text{C}_{11}\text{H}_{10}\text{N}_3\text{O}_5$  requires 264.0620).

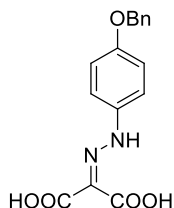


**2-((4-Benzoyloxyphenyl)hydrazono)malonic Acid Diethyl Ester (4.5).**<sup>104</sup>

To a solution containing 2.36 g (10.0 mmol) of 4-*O*-benzylaniline hydrochloride in 51 mL of acetic acid was added 2.5 mL of conc HCl and the reaction mixture was cooled to 0 °C. To the cooled reaction mixture was added a solution of 0.90 g (13.0 mmol) of sodium nitrite in 26 mL water and resulting mixture was stirred at 0 °C for 30 min. The mixture was then added dropwise into a previously prepared mixture of 1.51 mL (1.44 g; 10.0 mmol) of diethyl malonate and 31.0 g (0.38 mol) of sodium acetate in 250 mL water. The reaction mixture was stirred at 23 °C for 24 h and then filtered. The precipitate was washed with water, air dried and purified by flash column chromatography on a silica gel column (15 × 4 cm). Elution with 2:1 hexanes–ethyl acetate afforded **4.5** as yellow solid: yield 2.49 g (67%); mp 70–72 °C; silica gel TLC  $R_f$  0.69 (1:1 hexanes–ethyl acetate);  $^1\text{H}$  NMR (CDCl<sub>3</sub>)  $\delta$  1.26 (m, 6H), 4.23 (m, 4H), 4.93 (m, 2H), 6.86 (m, 2H), 7.23 (m, 7H)

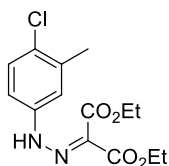


and 12.81 (s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.1, 14.3, 60.9, 61.1, 70.3, 115.8, 115.8, 116.8, 116.8, 118.6, 127.4, 127.4, 128.0, 128.6, 128.6, 136.8, 153.7, 156.2, 163.4 and 163.8; mass spectrum (APCI),  $m/z$  371.1621 ( $\text{M} + \text{H}^+$ ) ( $\text{C}_{20}\text{H}_{23}\text{N}_2\text{O}_5$  requires 371.1607).



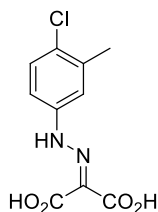
**2-((4-Benzyloxyphenyl)hydrazono)malonic Acid (4.1e).**<sup>104</sup>

To a solution containing 493 mg (1.33 mmol) of **4.5** in 12 mL THF was added dropwise a solution of 160 mg (6.66 mmol) of LiOH in 6 mL of water and the reaction mixture was stirred at 23 °C for 16 h. The reaction was diluted with 20 mL of  $\text{Et}_2\text{O}$  and 25 mL of water and the phases were separated. The aq layer was washed with two 10-mL portions of ethyl acetate, cooled in an ice bath and acidified to pH ~3 with 3 N HCl. The formed precipitate was filtered, washed with cold acidic (pH ~4) water and air dried to obtain **4.1e** as a yellow solid: yield 300 mg (72%); mp 153 °C (dec); silica gel TLC  $R_f$  0.43 (4:1 chloroform–methanol);  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  5.10 (s, 2H), 7.14 (m, 2H), 7.40 (m, 7H) and 13.30 (br s, 1H);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  69.5, 115.7, 115.7, 116.2, 117.5, 117.5, 127.7, 127.7, 127.9, 128.4, 128.4, 135.4, 136.9, 136.9, 156.0, 164.6 and 168.8; mass spectrum (APCI),  $m/z$  313.0827 ( $\text{M} - \text{H}^-$ ) ( $\text{C}_{16}\text{H}_{13}\text{N}_2\text{O}_5$  requires 313.0824).



### 2-(2-(4-Chloro-3-methylphenyl)hydrazono)malonic Acid Diethyl Ester (4.6).

To a solution containing 142 mg (1.00 mmol) of 4-chloro-3-methylaniline in 5.1 mL of acetic acid was added 0.3 mL of conc HCl and the mixture was cooled to 0 °C. To the cold mixture was added a solution of 89.0 mg (1.30 mmol) of sodium nitrite in 2.5 mL water and the reaction mixture was stirred at 0 °C for 30 min. The reaction mixture was then poured into a previously prepared mixture of 0.13 mL (120 mg; 0.86 mmol) of diethyl malonate and 2.54 g (31.0 mmol) of sodium acetate in 20 mL of water. The reaction mixture was stirred at 23 °C for 24 h and filtered. The precipitate was washed with water, air dried and purified by flash chromatography on a silica gel column (14 × 2 cm). Elution with 25:1 chloroform–methanol gave **4.6** as light yellow solid: yield 250 mg (89%); mp 46 °C; silica gel TLC  $R_f$  0.72 (1:1 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.39 (m, 6H), 2.39 (m, 3H), 4.35 (m, 4H), 7.10 (m, 1H), 7.26 (m, 2H) and 12.75 (br s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.1, 14.3, 20.3, 61.3, 61.5, 114.2, 117.7, 120.4, 129.94, 129.93, 137.4, 140.4, 163.3 and 163.5; mass spectrum (APCI),  $m/z$  313.0951 ( $\text{M}+\text{H}^+$ ) ( $\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_4\text{Cl}$  requires 313.0955).



### 2-((4-Chloro-3-methylphenyl)hydrazono)malonic Acid (4.1f).

To a solution of 75.0 mg (0.24 mmol) of **4.6** in 2.5 mL of THF was added dropwise a solution of 24.0 mg (0.96 mmol) of LiOH in 1.25 mL of water and the

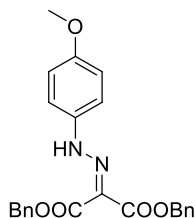
reaction mixture was stirred at 23 °C for 16 h. The reaction mixture was diluted with 10 mL of Et<sub>2</sub>O and 20 mL of water and the phases were separated. The aqueous layer was washed with two 8-mL portions of ethyl acetate, cooled in an ice bath and acidified to pH ~3 with 1 N HCl. The formed precipitate was filtered, washed with cold acidic (pH ~4) water and air dried to obtain **4.1f** as a yellow solid: yield 45 mg (79%); mp 150–152 °C (dec); silica gel TLC *R<sub>f</sub>* 0.38 (4:1 chloroform–methanol); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ and 2.33 (s, 3H), 7.33 (m, 1H), 7.40 (m, 1H), 7.46 (m, 1H) and 12.75 (br s, 1H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) 19.8, 114.8, 118.0, 119.8, 128.4, 129.7, 136.6, 140.8, 164.2 and 167.3; mass spectrum (APCI), *m/z* 255.0175 (M – H)<sup>–</sup> (C<sub>10</sub>H<sub>8</sub>N<sub>2</sub>O<sub>4</sub>Cl requires 255.0173).



**Dibenzyl Malonate (4.7).**<sup>105</sup>

To a solution containing 4.02 g (38.6 mmol) of malonic acid and 9.40 mL (9.00 g; 91 mmol) of benzyl alcohol in 15 mL of toluene was added 5 drops of H<sub>2</sub>SO<sub>4</sub> and the reaction mixture was heated to reflux using a Dean Stark apparatus for 2 h. The cooled reaction mixture was concentrated under diminished pressure and the residue was redissolved in 80 mL of ethyl acetate. The ethyl acetate layer was washed with two 30 mL-portions of sat aq NaHCO<sub>3</sub> soln, one 20-mL portion of brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, 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 hexanes–ethyl acetate gave **4.7** as a colorless oil: yield 9.01 g (82%); silica gel TLC *R<sub>f</sub>* 0.57 (2:1 hexanes–ethyl

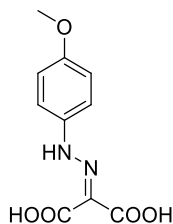
acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.48 (s, 2H), 5.18 (s, 4H) and 7.35 (m, 10H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  41.7, 67.4, 67.4, 128.4, 128.4, 128.4, 128.4, 128.5, 128.5, 128.7, 128.7, 128.7, 128.7, 135.3, 135.3, 166.3 and 166.3.



### 2-((4-Methoxyphenyl)hydrazono)malonic Acid Dibenzyl Ester (**4.8**).

To a mixture containing 0.62 g (5.00 mmol) of *p*-anisidine in 1.25 mL of conc HCl was added 2.5 g of ice and the mixture was cooled to 0 °C. To the cold mixture was added dropwise a solution of 0.35 g (5.11 mmol) of sodium nitrite in 0.85 mL water and the reaction mixture was stirred at 0 °C for 20 min. The resulting mixture was then poured into a previously prepared mixture of 1.40 g (4.90 mmol) of dibenzyl malonate (**4.7**), 0.95 g (11.6 mmol) of sodium acetate and 1.5 mL of water in 10 mL of ethanol. The reaction mixture was stirred at 23 °C for 12 h and then concentrated under diminished pressure. The residue was diluted in 40 mL ethyl acetate. The organic layer was washed with two 15-mL portions of water, 10 mL of brine, then dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 2:1 hexanes–ethyl acetate gave **4.8** as orange oil: yield 1.21 g (56%); silica gel TLC  $R_f$  0.72 (1:1 hexanes–ethyl acetate);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.81 (s, 3H), 5.34 (s, 4H), 6.91 (m, 2H), 7.37 (m, 12H) and 13.00 (br s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  55.6, 66.6, 66.6,

114.8, 114.8, 117.1, 117.1, 118.0, 127.9, 127.9, 128.00, 128.00, 128.05, 128.3, 128.5, 128.5, 128.7, 128.7, 135.5, 135.5, 136.3, 157.4, 163.3 and 163.6; mass spectrum (APCI),  $m/z$  419.1616 ( $M + H$ )<sup>+</sup> ( $C_{24}H_{23}N_2O_5$  requires 419.1607).



**2-((4-Methoxyphenyl)hydrazono)malonic Acid (4.1g).**<sup>104</sup>

Hydrogen gas was bubbled through a stirred suspension containing 298 mg (0.71 mmol) of **4.8** and 15 mg of 20% palladium hydroxide in 15 mL of ethyl acetate for 25 min. The reaction mixture was filtered through a Celite pad and the pad was washed with ethyl acetate. The filtrate was concentrated under diminished pressure to a volume of 30 mL and extracted with three 15-mL portions of 1 N aq NaOH soln. The combined NaOH extract was washed with two 10-mL portions of ethyl acetate, cooled in an ice bath, acidified to pH ~3 with 3 N HCl and re-extracted with three 15-mL portions of ethyl acetate. The combined organic layer was then washed with one 20-mL portion of brine, dried over anhydrous  $Na_2SO_4$ , filtered and concentrated under diminished pressure to afford **4.1g** as a yellow solid: yield 80 mg (47%); mp 128–130 °C (dec); silica gel TLC  $R_f$  0.86 (4:1 chloroform–methanol); <sup>1</sup>H NMR (DMSO- $d_6$ )  $\delta$  3.75 (s, 3H), 6.98 (d, 2H,  $J = 8.4$  Hz), 7.49 (d, 2H,  $J = 8.4$  Hz) and 13.30 (br s, 1H); <sup>13</sup>C NMR (DMSO- $d_6$ )  $\delta$  55.4, 114.7, 114.7, 115.8, 115.8, 117.6, 135.1, 157.1, 164.5 and 169.0; mass spectrum (ESI),  $m/z$  237.0503 ( $M - H$ )<sup>-</sup> ( $C_{10}H_9N_2O_5$  requires 237.0511).

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REFERENCES:

- (1) Brandley, B. K.; Schnaar, R. L. *J. Leukoc. Biol.* **1986**, *40*, 97-111.
- (2) (a) Wickner, W. T.; Lodish, H. F. *Science* **1985**, *230*, 400. (b) Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell*; Garland Science: New York, NY, 2002.
- (3) Apweiler, R.; Hermjakob, H.; Sharon, N. *Biochim. Biophys. Acta* **1999**, *1473*, 4.
- (4) Sznajdman, M. In *Bioorganic Chemistry*; Hecht, S. M., Ed.; Oxford University Press: New York, NY, 1999, p 1-55.
- (5) Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell*; Garland Science: New York, NY, 2002.
- (6) (a) Inatani, M.; Irie, F.; Plump, A. S.; Tessier-Lavigne, M.; Yamaguchi, Y. *Science* **2003**, *302*, 1044. (b) Pilobello, K. T.; Mahal, L. K. *Curr. Opin. Chem. Biol.* **2007**, *11*, 300. (c) Drickamer, K.; Taylor, M. E.; *Trends Biochem. Sci.* **1998**, *23*, 321.
- (7) (a) Sharon, N.; Lis, H. *Glycobiology* **2004**, *14*, R53. (b) Ohyama, C.; Tsuboi, S.; Fukuda, M. *EMBO J.* **1999**, *18*, 1516.
- (8) (a) Paulson, J.C.; Blixt, O.; Collins, B.E. *Nat. Chem. Biol.* **2006**, *2*, 238. (b) Pohl, N. L. *Curr. Opin. Chem. Biol.* **2005**, *9*, 76.
- (9) Nangia-Makker, P.; Conklin, J.; Hogan, V.; Raz, A. *Trends. Mol. Med.* **2002**, *8*, 187.
- (10) (a) Saito, A.; Fujimura, M.; Inoue, T.; Shimizu, H.; Tominaga, T. *Acta Neurochir. (Wien)* **2010**, *152*, 589. (b) Shimizu, T.; Nishitani, C.; Mitsuzawa, H.; Arikawa, S.; Takahashi, M.; Ohtani, K.; Wakamiya, N.; Kuroki, Y. *Biochim. Biophys. Acta* **2009**, *1790*, 1705. (c) Van De Wetering, J. K.; Van Golde, L. M. G.; Batenburg, J. J. *Eur. J. Biochem.* **2004**, *271*, 1229.
- (11) (a) Yu, L.; Chen, S. *Cancer Immunol. Immunother.* **2008**, *57*, 1271. (b) Sharon, N. *FEBS Lett.* **1987**, *217*, 145.
- (12) (a) Goldstein, I. J.; Portez, R. D. In *The Lectins: Properties, Functions, and Applications in Biology and Medicine*; Liener, I. E., Sharon, N., Goldstein, I. J., Eds.; Academic Press, Inc.: Orlando, 1986, p 35. (b) Sharon, N.; Lis, H. *Lectins*; Chapman and Hall: London, UK, 1989.

---

(13) Lee, R. T.; Ichikawa, Y.; Fay, M.; Drickamer, K.; Shao, M. C.; Lee, Y. C. *J. Biol. Chem.* **1991**, *266*, 4810.

(14) Pieters, R. *J. Org. Biomol. Chem.*, **2009**, *7*, 2013.

(15) (a) Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. *Angew. Chem. Int. Ed.* **2006**, *45*, 2348; (b) Gestwicki, J. E.; Cairo, C. W.; Strong, L. E.; Oetjen, K. A.; Kiessling, L. L.; *J. Am. Chem. Soc.* **2002**, *124*, 14922; (c) Wolfenden, M. L.; Cloninger, M. J. *Bioconj. Chem.* **2006**, *17*, 958.

(16) Kanellopoulos, P. N.; Pavlou, K.; Perrakis, A.; Agianian, B.; Vorgias, C. E.; Mavrommatis, C.; Soufi, M.; Tucker, P. A.; Hamodrakas, S. J. *J. Struct. Biol.* **1996**, *116*, 345. (b) Page, D.; Roy, R. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1765.

(17) (a) Fan, E.; Merritt, E. A.; Verlinde, C. L. M. J.; Hol, W. G. J. *Curr. Opin. Struct. Biol.* **2000**, *10*, 680. (b) Merritt, E. A.; Kuhn, P.; Sarfaty, S.; Erbe, J. L.; Holmes, R. K.; Hol, W. G. J. *J. Mol. Biol.* **1998**, *282*, 1043.

(18) (a) Bouckaert, J.; Berglund, J.; Schembri, M.; de Genst, E.; Cools, L.; Wuhrer, M.; Hung, C. S.; Pinkner, J.; Slattegard, R.; Zavialov, A.; Choudhury, D.; Langermann, S.; Hultgren, S. J.; Wyns, L.; Klemm, P.; Oscarson, S.; Knight, S. D.; de Greve, H. *Mol. Microbiol.* **2005**, *55*, 441. (b) Loris, R.; Tielker, D.; Jaeger, K. E.; Wyns, L. *J. Mol. Biol.* **2003**, *331*, 861.

(19) (a) Lee, R. T.; Lee, Y. C. *Glycoconj. J.*, **2000**, *17*, 543. (b) Roy, R. *Trends Glycosci. Glyc.* **2003**, *15*, 291.

(20) (a) Barrientos, A. G.; de la Fuente, J. M.; Rojas, T. C.; Fernandez, A.; Penades, S. *Chem. Eur. J.* **2003**, *9*, 1909. (b) Babu, P.; Sinha, S.; Suroliya, A. *Bioconjugate. Chem.*, **2007**, *18*, 146.

(21) van Kooyk, Y.; Saeland, E. *Biochem. Soc. Trans.* **2011**, *39*, 388.

(22) Ohtsubo, K.; Marth, J.D. *Cell* **2006**, *126*, 855.

(23) Ohshima, C.; Tsuboi, S.; Fukuda, M. *EMBO J.* **1999**, *18*, 1516.

(24) (a) Gorelik, E.; Galili, U.; Raz, A. *Cancer Metastasis Rev.* **2001**, *20*, 245. (b) Patton, S.; Gendler, S.J.; Spicer, A.P. *Biochim. Biophys. Acta* **1995**, *1241*, 407.

(25) Drake, P.M.; Cho, W.; Li, B.; Prakobphol, A.; Johansen, E.; Anderson, N.L.; Regnier, F.E.; Gibson, B.W.; Fisher, S. J. *Clin. Chem.* **2010**, *56*, 223.

- 
- (26) (a) Brooks, S.A.; Leatham, A.J. *Lancet* **1991**, 338, 71. (b) Seelentag, W.K.; Li, W.P.; Schmitz, S.F.; Metzger, U.; Aeberhard, P.; Heitz, P.U.; Roth, J. *Cancer Res.* **1998**, 58, 5559.
- (27) (a) Kahne, D.; Silva, D.; Walker, S. In *Bioorganic Chemistry: Carbohydrates*; Hecht, S. M., Ed.; Oxford University Press: New York, NY, 1999.
- (28) (a) Chaires, J. B. In *DNA Sequence Specific Agents*; Hurley, L. H., Chaires, J. B., Eds.; JAI Press: Greenwich, CT, 1996; Vol. 2, p 141. (b) Drak, J.; Iwasawa, N.; Danishefsky, S.; Crothers, D. M. *Proc. Natl. Acad. Sci. U S A* **1991**, 88, 7464.
- (29) Umezawa, H.; Suhara, Y.; Takita, T.; Maeda, K. *J. Antibiot. (Tokyo)* **1966**, 19, 210.
- (30) (a) Jones, S. E.; Lilien, D. L. ; O'Mara, R. E.; Durie, B. G.; Salmon, S. E. *Med. Pediatr. Oncol.* **1975**, 1, 11. (b) Silverstein, M. J.; Verma, R. C.; Greenfield, L.; Morton, D. L. *Cancer* **1976**, 37, 36. (c) Bekerman, C.; Moran, E. M.; Hoffer, P. B.; Hendrix, R. W.; Gottschalk, A. *Radiology* **1977**, 123, 687. (d) Burton, I. E.; Todd, J. H.; Turner, R. L. *Br. J. Radiol.* **1977**, 50, 508. (e) Goodwin, D. A.; Meares, C. F.; DeRiemer, L. H.; Diamanti, C. I.; Goode, R. L.; Baumert Jr., J. E.; Sartoris, D. J.; Lantieri, R. L.; Fawcett, H. D. *J. Nucl. Med.* **1981**, 22, 787. (f) Stern, P. H.; Helpern, S. E.; Hagan, P. L.; Howell, S. B.; Dabbs, J. E.; Gordon, R. M. *J. Natl. Cancer Inst.* **1981**, 66, 807.
- (31) Hecht, S. M. In *Cancer Chemotherapeutic Agents*; Foye, W. O., Ed.; American Chemical Society: Washington, DC, 1995, p 369-388.
- (32) (a) Holmes, C. E.; Carter, B. J.; Hecht, S. M. *Biochemistry* **1993**, 32, 4293. (b) Kane, S. A.; Hecht, S. M. *Prog. Nucleic Acid Res. Mol. Biol.* **1994**, 49, 313. (c) Claussen, C. A.; Long, E. C. *Chem. Rev.* **1999**, 99, 2797. (d) Hecht, S. M. *J. Nat. Prod.* **2000**, 63, 158. (e) A. T. Abraham, A. T.; Lin, J. J.; Newton, D. L.; Rybak, S.; Hecht, S. M. *Chem. Biol.* **2003**, 10, 45. (f) Chen, J.; Stubbe, J. *Nat. Rev. Cancer* **2005**, 5, 102. (g) Tao, Z. F.; Konishi, K.; Keith, G.; Hecht, S. M. *J. Am. Chem. Soc.* **2006**, 128, 14806.
- (33) Chen, J.; Stubbe, J. *Nat. Rev. Cancer* **2005**, 5, 102.
- (34) Ibba, M.; Söll, D. *EMBO Reports* **2001**, 2, 382.
- (35) Wakasugi, K.; Quinn, C. L.; Tao, N.; Schimmel, P. *EMBO J.* **1998**, 17, 297.
- (36) DeSantis, G.; Jones, J. B. *Curr. Opin. Biotech.* **1999**, 10, 324.



- 
- (37) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.
- (38) Hortin, G.; Boime, I. *Methods Enzymol.* **1983**, *96*, 777.
- (39) Hecht, S. M. Synthesis of modified proteins using misacylated tRNAs, in Protein Engineering (RajBhandary, U. L., and Koehrer, C., Eds.) 2008, pp 251, Springer, Berlin.
- (40) (a) Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R. *J. Am. Chem. Soc.* **1989**, *111*, 8013. (b) Heckler, T. G.; Chang, L.-H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468. (c) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182.
- (41) Strømgaard, A.; Jensen, A. A.; Strømgaard K. *ChemBioChem* **2004**, *5*, 909.
- (42) T. G. Heckler, L.-H. Chang, Y. Zama, T. Naka, M. S. Chorghade, S. M. Hecht, *Biochemistry* **1984**, *23*, 1468.
- (43) Robertson, S. A.; Noren C. J.; Anthony-Cahill S. J.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1989**, *17*, 9649.
- (44) Wang, L.; Xie, L.; Schultz, P. G. *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 225.
- (45) Barré-Sinoussi, F.; Chermann, J. C.; Rey, F.; Nugeyre, M. T.; Chamaret, S.; Gruest, J.; Dauguet, C.; Axler-Blin, C.; Vézinet-Brun, F.; Rouzioux, C.; Rozenbaum, W.; Montagnier, L. *Science* **1983**, *220*, 868.
- (46) DeClercq, E. *Drug Discovery* **2002**, *1*, 13.
- (47) Coffin, J. M.; Hughes, S. H.; Varmus, H. E. (1997). *Retroviruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- (48) Balzarini, J. *Pharm. World Soc.* **1994**, *16*, 113.
- (49) Cahard, D.; McGuigan, C.; Balzarini, J. *Mini-Rev. Med. Chem.* **2004**, *4*, 371.
- (50) De Clercq, E. *Expert Rev. Anti-Infect. Ther.* **2003**, *1*, 21.
- (51) De Clercq, E. *Chem. Biodiversity* **2004**, *1*, 44.
- (52) Domaoal, R. A.; Demeter, L. M. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 1735.
- (53) De Clercq, E. *J. Med. Chem.* **2005**, *48*, 1297.

- 
- (54) Wlodawer, A.; Gustchina, A. *Biochim. Biophys. Acta* **2000**, *1477*, 16.
- (55) Matthews, T.; Salgo, M.; Greenberg, M.; Chung, J.; DeMasi, R.; Bolognesi, D. *Nat. Rev. Drug Discovery* **2004**, *3*, 215.
- (56) www.fda.gov (August, 2012).
- (57) Berger, E. A.; Murphy, P. M.; Farber, J. M. *Annu. Rev. Immunol.* **1999**, *17*, 657.
- (58) Pommier, Y.; Johnson, A. A.; Marchand, C. *Nat. Rev. Drug Discovery* **2005**, *4*, 236.
- (59) (a) Brahim, S.; Abid, K.; Kenani, A. *Cell. Biol. Int.* **2008**, *32*, 171. (b) Brahim, S.; Bettaieb, A.; Kenani, A. *J. Oral. Pathol. Med.* **2008**, *37*, 352.
- (60) (a) Oppenheimer, N. J.; Rodriguez, L. O.; Hecht, S. M. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 5616. (b) Karawajczyk, A.; Buda, F. *Molecular Simulation* **2006**, *32*, 1233. (c) Kane, S. A.; Hecht, S. M. *Prog. Nucleic Acid Res. Mol. Biol.* **1994**, *49*, 313. (d) Akkerman, M. A. J.; Neijman, E. W. J. F.; Wijmenga, S. S.; Hilbers, C. W.; Bermel, W. J. *J. Am. Chem. Soc.* **1990**, *112*, 7462-7474.
- (61) Natrajan, A.; Hecht, S. M. In *Molecular Aspects of Anticancer Drug-DNA Interactions*; Neidle, S., Waring, M. J., Ed.; Macmillan Press: London, 1994, p 197.
- (62) Cai, X.; Zaleski, P. A.; Cagir, A.; Hecht, S. M. *Bioorg. Med. Chem.* **2011**, *19*, 3831.
- (63) (a) Umezawa, H. *Pure Appl. Chem.* **1971**, *28*, 665-680. (b) Grove, R. B.; Eckelman, W. C.; Reba, R. C. *J. Nucl. Med.* **1973**, *14*, 917-919. (c) Thakur, M. L. *Int. J. Appl. Radiat. Isot.* **1973**, *24*, 357-359.
- (64) (a) Grove, R. B.; Reba, R. C.; Eckelman, W. C.; Goodyear, M. *J. Nucl. Med.* **1974**, *15*, 386-390. (b) Lilien, D. L.; Jones, S. E.; O'Mara, R. E.; Salmon, S. E.; Durie, B. G. *Cancer* **1975**, *35*, 1036-1049.
- (65) (a) DeRiemer, L. H.; Meares, C. F.; Goodwin, D. A.; Diamanti, C. I. *J. Med. Chem.* **1979**, *22*, 1019-1023. (b) Goodwin, D. A.; Meares, C. F.; DeRiemer, L. H.; Diamanti, C. I.; Goode, R. L.; Baumert, J. E., Jr.; Sartoris, D. J.; Lantieri, R. L.; Fawcett, H. D. *J. Nucl. Med.* **1981**, *22*, 787-792. (c) Mears C. F.; Hecht S. M. unpublished results.

- 
- (66) Chapuis, J. C.; Schmaltz, R. M.; Tsosie, K. S.; Belohlavek, M.; Hecht, S. M. *J. Am. Chem. Soc.* **2009**, *131*, 2438.
- (67) Lindner, J. R. *Nat. Rev. Drug Discov.* **2004**, *3*, 527.
- (68) Monici, M. *Biotechnol. Annu. Rev.* **2005**, *11*, 227-256.
- (69) (a) Boger, D. L.; Honda, T. *J. Am. Chem. Soc.* **1994**, *116*, 5647. (b) Dondoni, A.; Marra, A.; Massi, A. *J. Org. Chem.* **1997**, *62*, 6261.
- (70) (a) Tsuchiya, T; Miyake, T; Kageyama, S; Umezawa, S; Umezawa, H; Takita, T. *Tet. Lett.* **1981**, *22*, 1413. (b) Schmaltz, R.; Tsosie, K.; Hecht, S., unpublished results.
- (71) Kralovec, J.; Spencer, G.; Blair, A. H.; Mammen, M.; Singh, M.; Ghose, T. *J. Med. Chem.*, **1989**, *32*, 2426.
- (72) Katano, K.; Millar, A.; Pozsgay, V.; Primeau, J. L. Hecht, S. M. *J. Org. Chem.* **1986**, *51*, 2927.
- (73) Aouida, M.; Ramotar, D. *Curr. Drug Metab.* **2010**, *11*, 595.
- (74) Dondoni, A.; Fantin, G.; Fogagnolo, M.; Medici, A.; Pedrini, P. *J. Org. Chem.* **1988**, *53*, 1748.
- (75) Hecht, S.; Schmaltz, R.; Tsosie, K. PCT Int. Appl. 2011, WO 2011019419 A1 20110217.
- (76) West, R., Martin.; Bosworth, N.; Mujumdar, R., B; WIPO, Ed. WO/2005/04493. 2005.
- (77) Schmaltz, R.; Hecht, S. unpublished results.
- (78) Watt, J. A.; Williams, S. J. *J. Org. Biomol. Chem.* **2005**, *3*, 1982.
- (79) Sabesan, S.; Neira, S. *Carbohydrate Res.* **1992**, *223*, 169.
- (80) Manesis, N. J.; Goodman, M. *J. Org. Chem.* **1987**, *52*, 5331.
- (81) Bailly, F.; Bailly, C.; Helbecque, N.; Pommery, N.; Colson, P.; Houssier, C.; Henichart, J. P. *Anti-Cancer Drug Res.* **1992**, *7*, 83.
- (82) Doyle, V. E.; Klimkait, T.; Mahmood, N.; Slater, M.; Hazen, R. J.; Gilbert, I. H. *J. Enzyme Inhib. Med. Ch.* **2002**, *17*, 175.

- 
- (83) Hartwig, S.; Hecht, S. *Macromolecules* **2010**, *43*, 242.
- (84) Cruz-Morales, J. A.; Guadarrama, P. *J. Mol. Struct.* **2005**, *779*, 1.
- (85) (a) Hohsaka, T.; Sato, K.; Sisido, M.; Takai, K.; Yokoyama, S. *FEBS Lett.* **1993**, *355*, 47. (b) Sisido, M.; Hohsaka, T. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 274.
- (86) (a) Fahnstock, S.; Rich, A. *Science* **1971**, *173*, 340. (b) Bain, J. D.; Diala, E. S.; Glabe, C. G.; Wacker, D. A.; Lyttle, M. H.; Dix, T. A.; Chamberlin, A. R. *Biochemistry* **1991**, *30*, 5411.
- (87) Killian, J. A.; Van Cleve, M. D.; Shayo, Y. F.; Hecht, S. M. *J. Am. Chem. Soc.* **1998**, *120*, 3032.
- (88) Eisenhauer, B. M.; Hecht, S. M. *Biochemistry* **2002**, *41*, 11472.
- (89) (a) Roesser, J. R.; Xu, C.; Payne, R. C.; Surratt, C. K.; S. M. Hecht, S. M. *Biochemistry* **1989**, *28*, 5185. (b) Ellman, J. A.; Mendel, D.; Schultz, P. G. *Science* **1992**, *255*, 197. (c) Bain, J. D.; Wacker, D. A.; Kuo, E. E.; Chamberlin, A. R. *Tetrahedron* **1991**, *47*, 2389.
- (90) (a) Kreil, G. *Annu. Rev. Biochem.* **1997**, *66*, 337. (b) Soyea, D.; Toullec, J.-Y.; Ollivaux, C.; Geraund, G. J. *J. Biol. Chem.* **2000**, *275*, 37870.
- (91) (a) Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y.; Hecht, S. M. *J. Am. Chem. Soc.* **2003**, *125*, 6616. (b) Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y. Hecht, S. M. *Biochemistry* **2006**, *45*, 15541.
- (92) Zarivach, R.; Bashan, A.; Berisio, R.; Harms, J.; Auerbach, T.; Schluenzen, F.; Bartels, H.; Baram, D.; Pyetan, E.; Sittner, A.; Amit, M.; Hansen, H. A. S.; Kessler, M.; Liebe, C.; Wolff, A.; Agmon, I.; Yonath, A. *J. Phys. Org. Chem.* **2004**, *17*, 901.
- (93) Nathans, D.; Neidle, A. *Nature* **1963**, *197*, 1076.
- (94) Dedkova, L. M.; Fahmi, N. E.; Paul, R.; del Rosario, M.; Zhang, L.; Chen, S.; Feder, G. and Hecht, S. M. *Biochemistry* **2012**, *51*, 401.
- (95) Starck, S. R.; Qi, X.; Olsen, B. N.; Roberts, R. W. *J. Am. Chem. Soc.* **2003**, *125*, 8090.
- (96) Robertson, S. A.; Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1989**, *17*, 9649.

- 
- (97) (a) Vannuffel, P.; Di Giambattista, M.; Morgan, E. A.; Cocito, C. *J. Biol. Chem.* **1992**, *267*, 8377. (b) Hansen, J. L.; Ippolito, J. A.; Ban, N.; Nissen, P.; Moore, P. B.; Steitz, T. A. *Mol. Cell* **2002**, *10*, 117.
- (98) Fournie-Zaluski, M.; Coric, P.; Thery, V.; Gonzalez, W.; Meudal, H.; Turcaud, S.; Michel, J.; Roques B. P. *J. Med. Chem.* **1996**, *39*, 2594.
- (99) Repaske, R.; Hartley, J. W.; Kavlick, M. F.; O'Neill, R. R.; Austin, J. B. *J. Virol.* **1989**, *63*, 1460.
- (100) (a) Furfine, E. S.; Reardon, J. E. *J. Biol. Chem.* **1991**, *266*, 406. (b) Gopalakrishnan, V.; Peliska, J. A.; Benkovic, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10763. (c) Peliska, J. A.; Benkovic, S. J. *Science* **1992**, *258*, 1112.
- (101) Gabbara, S.; Davis, W. R.; Hupe, L.; Hupe, D.; Peliska, J. A. *Biochemistry* **1999**, *38*, 13070.
- (102) Davis, W. R.; Tomsho, J.; Nikam, S.; Cook, E. M.; Somand, D.; Peliska, J. A. *Biochemistry* **2000**, *39*, 14279.
- (103) Shaw-Reid, C. A.; Munshi, V.; Graham, P.; Wolfe, A.; Witmer, M.; Danzeisen, R.; Danzeisen, R.; Olsen, D. B.; Carroll, S. S.; Embrey, M.; Wai, J. S.; Miller, M. D.; Cole, J. L.; Hazuda D. J. *J. Biol. Chem.* **2003**, *278*, 2777.
- (104) Barber, H. J.; Washbourn, K.; Wragg W. R.; Lunt, E. *J. Chem. Soc.* **1961**, 2828.
- (105) Saul, W.; Ro, S.; Kim, C.; Shi, Z. *Can. J. Chem.* **2001**, *79*, 1238.