DESIGN, SYNTHESIS, AND EVALUATION OF SYNTHETIC MIMICS OF CELL SURFACE RECEPTORS

A Dissertation in
Chemistry
by
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ABSTRACT

The plasma membrane of mammalian cells is composed of a vast array of lipids and proteins arranged in a lipid bilayer. Many of these proteins function as receptors and facilitate or control cell signaling events. In addition, some receptors mediate the uptake of specific ligands from the surrounding environment. These receptors include at least three regions: an extracellular ligand binding motif, a linker region, and a membrane-binding domain. These receptors can also have an internal cytoplasmic region. The extracellular domains can be targeted to deliver drugs or macromolecules to the intracellular environment through receptor-mediated endocytosis.

We constructed artificial cell surface receptors that comprise an outward-projecting ligand binding domain, a linker region, and the membrane anchor \( N \)-alkyl-3\( \beta \)-cholesterylamine. These artificial receptors mimic trafficking and membrane association properties of endogenous cholesterol by inserting into the plasma membranes of living mammalian cells and cycling between the cell surface and endosomal compartments. We report here that calix[4]arene scaffolds linked to a cholesterylamine anchor can be used to effect the intracellular delivery of vascular endothelial growth factor (VEGF), a growth factor that plays key roles in cancer proliferation. We further report the effective delivery of both monomeric and dimeric versions of the drug vancomycin into mammalian cells using the ligand-binding peptide D-Phe-D-Ala, enabling eradication of intracellular bacteria. Synthetic cell surface receptors represent promising tools for drug delivery, novel cellular probes, and innovative modulators of biological systems.
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Chapter 1

Synthetic Mimics of Cell Surface Receptors

1.1 Introduction

The plasma membrane of cells is composed of a vast array of proteins and lipids arranged in a lipid-bilayer. Phospholipids in the membrane are arranged in a “tail to tail” fashion. The hydrophobic tail region is shielded from the surrounding environment, whereas the hydrophilic polar head groups project into the cytosol and extracellular space. Membrane associated proteins, steroids, and carbohydrates are interspersed within the bilayer to facilitate intracellular communication and the uptake of molecules (Figure 1.1). The plasma membrane is selectively permeable, allowing the passage of

Figure 1.1: Cartoon diagram of the lipid-bilayer.
certain molecules by a variety of mechanisms. Small hydrophobic compounds can diffuse across the cell membrane by passive transport without the use of cellular energy. In contrast, active transport mechanisms require cellular energy. Certain ions and small polar molecules can be transported against a concentration gradient through protein pores on the cell surface. Large and polar molecules such as proteins and DNA are taken up by cells through the active transport mechanism of endocytosis.²

1.2 Receptor-Mediated Endocytosis

During endocytosis, mammalian cells take up materials from the extracellular environment by engulfment and entrapment within membrane-sealed compartments. There are three main types of endocytosis: phagocytosis, pinocytosis, and receptor-mediated endocytosis. Phagocytosis is a process by which cells ingest relatively large particles such as bacteria or viruses by folding the membrane around the particle and sealing it in a large vacuole called a phagosome. Pinocytosis is much less specific in the molecules it transports. In this process, the membrane forms invaginations around solutes that are subsequently internalized.

Receptor-mediated endocytosis is very specific; cells internalize molecules that bind specific receptors by the inward budding of the membrane. This process often involves clustering of receptors in clathrin-coated pits. The protein clathrin controls the endocytosis of numerous cell surface receptors such as the low-density lipoprotein receptor.³ Receptor-mediated endocytosis can also involve lipid-raft domains called
caveolae. Lipid-raft-mediated endocytosis is one of the uptake mechanisms thought to be involved in the internalization of cholera toxin mediated by the glycolipid ganglioside GM1.4

1.2.1 The LDLR Receptor

Clathrin-mediated endocytosis occurs in all mammalian cells and is involved in the uptake of numerous cell surface receptors responsible for cellular communication, cellular homeostasis, and the uptake of essential nutrients.2 One of the most extensively characterized cell surface receptors involved in clathrin-mediated endocytosis is the low-density lipoprotein receptor (LDLR).3,5 This protein spans the plasma membrane of cells, and consists of three parts: an extracellular domain, a transmembrane domain, and a cytoplasmic domain (Panel A, Figure 1.2 ). This receptor allows the cellular uptake of exogenous cholesterol in LDL particles for use in the biosynthesis of steroid hormones and cellular membranes.6 The LDL particle is composed of cholesterol, cholesteryl esters, phospholipids, triglycerides, and the protein apolipoprotein B-100 (Panel B, Figure 1.2).7
When an LDL particle binds to the extracellular domain of LDLR, the particle and receptor become clustered on the cell surface in clathrin-coated pits. Clathrin is a three armed protein that forms a structure termed a triskelion that has the ability to self-assemble under non-physiological conditions. In order for clathrin assemblies to form under physiological conditions, specific proteins termed assembly proteins (APs) are required. The adaptor proteins direct the clathrin into curved lattices and bind cytoplasmic tyrosine-based internalization motifs on the LDLR to cause the receptor to concentrate into the coated pits. These pits invaginate and pinch off with the

**Figure 1.2:** The LDLR receptor (Panel A), an LDL particle (Panel B) and receptor-mediated endocytosis of the LDLR-LDL complex (Panel C). Adapted from Peterson, B.R.
assistance of the GTPase protein dynamin to form intracellular endosomes, although the exact role of this protein is unclear. Once the pit is pinched off, an uncoating of the endocytic machinery occurs for use in subsequent invaginations. The pH of the resulting endosome becomes acidic due to the activation of proton pumps, causing the LDLR to dissociate from the LDL. The receptor is then free to recycle back to the cell surface, whereas endosomes containing LDL fuse with lysosomes to liberate the cholesterol in the LDL particles for use by the cell (Panel C, Figure 1.2).

1.2.2 Ganglioside GM1

Ganglioside GM1 is a small cell surface receptor (~1600 Da) consisting of a lipid tail and a pentasaccharide head group (1, Figure 1.3). This glycolipid binds to the bacterial protein cholera toxin (Panel A, Figure 1.3), promoting its cellular uptake. Unlike the membrane-spanning LDLR, ganglioside GM1 is restricted exclusively to the outer leaflet, resulting in a mechanistically different method of receptor-mediated endocytosis. During endocytosis, CT binds to the headgroup of ganglioside GM1 and is internalized by a variety of mechanisms. The complex dissociates in the acidic endosomes and CT undergoes trafficking to the Golgi complex and endoplasmic reticulum. Upon escape into the cytosol, it acts as a catalytic toxin and is responsible for some of the adverse effects associated with cholera infection.
Early studies suggested that the cellular uptake of cholera toxin (CT) mediated by ganglioside GM1 involved a caveolae-/lipid-raft-dependant pathway. Lipid-rafts are membrane sub-domains enriched with cholesterol and sphingolipids, and have been proposed to regulate the activation of signal transduction pathways and control endocytosis of specific receptors. Endocytic mechanisms involving these domains remain largely unknown. Cells that do not express caveolin, such as Caco-2 human epithelial cells, are also sensitive to CT internalization and toxicity. This suggests that other endocytic mechanisms are involved. Recent research has indicated that CT can enter cells by both clathrin-dependent and clathrin-independent mechanisms. In BHK cells, HeLa cells, and hippocampal neurons, it was demonstrated that CT enters the cells by clathrin-mediated mechanisms after binding to ganglioside GM1. This implies that the differing cell membrane compositions may affect the endocytic pathway.
utilized. CT can also be endocytosed into cells by noncaveolar and nonclathrin-mediated pathways.\textsuperscript{26,27} In monkey kidney epithelial (BSC-1) cells, the contributions of different endocytic pathways was examined using co-localization markers for the clathrin-, caveolin-, and Arf6-dependent pathways.\textsuperscript{26} It was found that CT colocalized with all three different markers, indicating that endocytosis occurs through all three pathways. Inhibiting all three pathways simultaneously did not prevent CT toxicity, suggesting that there is alternative unknown mechanism of CT entry.\textsuperscript{26} Studies in both wild-type and caveolin-deficient mouse embryonic fibroblast (MEF) cells demonstrated that the CT uptake mechanism was clathrin-independent but relatively cholesterol-dependent.\textsuperscript{27} Using immuno-electron microscopy, it was determined that the major mediator in CT uptake was unidentifiable tubular/ring-shaped structures that contained GPI-anchored proteins.\textsuperscript{27} These studies suggest that the endocytic uptake of CT by ganglioside GM1 is very complex and cell-type dependent.

\textbf{1.3 Synthetic Receptor Targeting}

Receptor-mediated endocytosis is an efficient method for the delivery of components into membrane-sealed compartments within cells. As a consequence, cell surface receptors have been used to enable cellular uptake of a variety of impermeable molecules. Ligands of surface receptors have been linked to macromolecules, fluorescent probes, and drugs to promote intracellular delivery.\textsuperscript{28} Many natural cell surface receptors
targeted in this way are over-expressed in rapidly proliferating cells and include the glycosylphosphatidylinositol (GPI) lipid-anchored folate receptors, transferrin receptors, and LDL receptors.

The Peterson group has been investigating cholesterol analogues designed to mimic cellular uptake functions of natural cell surface receptors. These small receptors comprise three domains: a cholesterol derivative as a plasma-membrane anchor, a peptide-like linker region, and a head group such as a fluorophore or protein-binding motif. As shown in Figure 1.4, N-alkyl-3β-cholesterylamine has been used as a plasma-membrane anchor for headgroups such as fluorophores (2, 8, and 9), biotin (3), peptides (4, 5, 6), and dinitrophenyl hapten (7). Receptors 2 through 9 incorporate into the plasma membrane of mammalian cells and rapidly recycle between endosomes.
and the cell surface. When treated with the appropriate cell-impermeable ligands, these molecules enable cells to endocytose their ligands. Following endocytosis, ligands become localized in late endosomes and lysosomes.\(^\text{32-35}\) The process was termed “synthetic receptor targeting” (Figure 1.5) because of the similarity of these synthetic systems to the natural cell surface receptors.\(^\text{35}\)

**Figure 1.5:** Synthetic receptor targeting approach for enhancing the cellular uptake of impermeable ligands. Synthetic mimics of cell surface receptors are added to living cells. These cells internalize the ligands such as IgG bound to bacterial protein A (PrA) by receptor-mediated endocytosis.\(^\text{7}\)

### 1.3.1 DNP and NBD Receptors

Working in collaboration with Dr. Sirawutt Boonyarattanakalin, compounds 7, 8, and several derivatives were synthesized and studied during the course of my thesis research.\(^\text{33}\) Cellular uptake mediated by these receptors appears to mimic cellular entry mechanisms involving clathrin-mediated endocytosis and lipid-raft mediated endocytosis that are commonly used by viruses and other cellular toxins.\(^\text{37}\) Synthetic receptor-mediated cellular uptake of fluorescent anti-dinitrophenyl (anti-DNP) IgG ligand was
observed by confocal microscopy and flow cytometric analysis of cells treated with 7. Sucrose density gradient ultracentrifugation experiments revealed that the ligand co-fractionates with cholera toxin in the low-density lipid-raft fraction of the plasma membrane.33

Synthetic receptors derived from cholesterylamine rapidly recycle between the plasma membrane and endosomal compartments in the absence of a ligand. When human Jurkat lymphocyte cells are treated with 7 followed by the addition of fluorescent anti-DNP IgG bound to bacterial Protein A, uptake of this complex is increased by over 200-fold with low toxicity.33 A model of this delivery system is shown in Figure 1.6.

Figure 1.6: A simple model of synthetic receptor-mediated endocytosis. Synthetic receptors embedded in the cellular plasma membrane rapidly cycle between the cell surface and intracellular endosomes. Binding of IgG ligand results in association with lipid-rafts and uptake of the complex by endocytosis. Dissociation in endosomes frees the receptor to return to the cell surface. The protein is sorted to late endosomes and lysosomes.33
The linker region between the \textit{N-alkyl-3\beta-cholesterylamine} and the head group of the synthetic receptor derivatives has a profound effect on cell membrane association, cellular uptake, and intracellular localization. Studies of receptors 7 and 8 and related compounds demonstrated that the insertion of additional \textit{\beta-alanine} units into the linker region substantially increased the number of these receptors on the cell surface compared to the number of receptors located in endosomes.\textsuperscript{33} This modification also resulted in increased uptake of ligand. When \textit{\beta-alanine} units were removed, more compound was located in endosomes than on the plasma membrane surface and the uptake of ligand was decreased.\textsuperscript{33} Profound effects were also seen when amino acids with charged head-groups were inserted into the linker region. The incorporation of multiple positively charged residues such as lysine and arginine increased cell death, whereas negatively charged residues such as glutamic acid led to an increase in cell-surface association in addition to attenuating toxicity.\textsuperscript{38} Reversing the amide bond order in the linker region and inclusion of a urea functional group did not affect receptor-cell surface association or trafficking.\textsuperscript{39}

In addition to the importance of the linker region, it was discovered that the secondary amine of the \textit{N-alkyl-3\beta-cholesterylamine} derivatives is very important for receptor-like properties. At physiological pH, the amine is protonated and presumably stabilizes the association of the membrane anchor with the cell surface. Replacing the amine functionality with an amide has deleterious effects. Amide versions of 7 and 8 are not inserted into the plasma membrane as efficiently as their amine counterparts, and as a result, decrease the uptake of ligand. This effect is also observed with the amide version of the D-Phe-D-Ala vancomycin receptor 6, and is discussed at length in Chapter 3.\textsuperscript{36}
Fluorescent amide analogs with uncharged head groups tend to localize at internal membranes of the Golgi apparatus and nuclear membranes of living cells.\textsuperscript{33} Cholesteryl esters of similar compounds share this type of localization which likely mimics the natural trafficking of these cholesterol derivatives.\textsuperscript{40}

**1.4 Other Related Approaches for the Delivery of Impermeable Compounds**

Numerous other methods have been developed to alter cell surface chemistry or deliver impermeable cargo into cells. These methods include modification of the plasma membrane with PEG derivatives\textsuperscript{41,42} and lipid anchors, chemical modification of membrane proteins by metabolic engineering,\textsuperscript{43,44} and the use of cell-penetrating peptides (CPPs) to promote cellular delivery.\textsuperscript{45,46} These methods and others have been extensively reviewed.\textsuperscript{28,47,48,49}

**1.4.1 Modification of the Plasma Membrane with PEG and Lipid Anchors**

It has been previously established that long chain diacyl-phosphatidyl ethanolamine-polyethylene-glycols (PE-PEGs) can rapidly integrate into lipid bilayers when initially dispersed in aqueous media.\textsuperscript{50,51} These results led to the investigation of artificial lipid-anchored streptavidin conjugates that become incorporated into the plasma membrane of living cells. Biotinylated PE-PEGs were successfully synthesized and
incorporated into the cell membrane of human Jurkat lymphocyte cells. These receptors bound streptavidin on the cell surface. Following the addition of anti-streptavidin antibodies, it was found that an antibody-mediated cross-linking of conjugates occurred, triggering an intracellular signaling event that elevated intracellular calcium levels. Another study anchored proteins (streptavidin, EGFP) directly to the plasma membrane with a single oleyl chain derivative or dioleylphosphatidylethanolamine derivative coupled with a hydrophobic poly(ethylene glycol) (PEG90). The retention time of the conjugated proteins on the cell surface exceeded 4 hours, and was found to be serum dependent. This lipid-anchoring method is conceptually related to the Peterson cholesterol-anchoring approach, but PEG conjugates are typically not efficiently internalized by endocytosis, limiting their utility for promoting the uptake of molecules.

Another strategy that incorporates glycosylphosphatidylinositol (GPI) lipid anchors on proteins added to plasma membranes is termed “cellular painting”. Chemical modification of GPI anchored proteins has been used to study the organization of the plasma membrane, cell signaling, and immune responses to modified cell surfaces. GPI lipids conjugated to green fluorescent proteins (GFPs) and an immunoglobulin Fc receptor have been shown to incorporate into the plasma membrane of living cells and undergo endocytosis. This strategy is also similar to the Peterson approach and offers promise for the delivery of specific cargo into cells.
1.4.2 Chemical Modification of Membrane Proteins by Metabolic Engineering

Carbohydrates on cell surfaces offer an attractive target for cell surface modification. Bertozzi and coworkers have installed bioorthogonal functional groups such as ketones and azides on cell surface carbohydrates using metabolic oligosaccharide engineering.\(^{44,58}\) In this method, cells are incubated with unnatural sugars. By using the cell’s biosynthetic machinery, the unnatural sugar is incorporated into glycoconjugates presented on the cell surface. The added functional groups become available for modification with complementary reactive groups. In this manner, ketones can be reacted with hydrazine derivatives to give hydrazones\(^{58}\) and azides can be reacted with phosphines in a Staudinger ligation\(^{44}\) to immobilize molecules on the cell surface. Bertozzi and co-workers used the azide functionality for glycan incorporation due to its small size and limited reactivity with biological functionalities. The scope of the Staudinger ligation in vivo can be expanded and used with a variety of phosphine analogues to tag cell surfaces with fluorescent probes and proteins.\(^{59}\) An important aspect of metabolic engineering was the ability to utilize this cell surface labeling system in living animals. This novel cell surface modification was used to label incorporated N-azidoacetylmannosamine in living mice without harmful side effects.\(^{60}\) It was additionally demonstrated that N-azidoacetylglucosamine can be metabolically incorporated into nuclear and cytoplasmic proteins in cells,\(^{61}\) and an N-azidoacetylgalactosamine can effectively label glycoproteins in living animals.\(^{62}\) Despite these successes, certain applications are limited by the slow kinetics of incorporation and
phosphine moieties required for the Staudinger ligation reaction. To circumvent these issues, a bioorthogonal reaction was developed based on the Huisgen [3+2] dipolar cycloaddition of alkynes with azides.\textsuperscript{63} Using ring strain of alkynes incorporated into 8-membered rings, azido sugars were successfully modified on cell surfaces faster than the traditional Staudinger ligation reaction and without the use of a toxic copper catalyst.\textsuperscript{63}

1.4.3 Delivery of Cargo into Cells with Cell Penetrating Peptides

Cell penetrating peptides (CPPs) and proteins have been widely used to deliver molecules both \textit{in vivo} and \textit{in vitro} and have been recently reviewed.\textsuperscript{64,65,66,67} CPPs are generally small peptide sequences of less than 30 amino acids derived from HIV Tat protein, polyarginine peptides, and other charged oligomers.\textsuperscript{45,46} These sequences are covalently attached to a variety of non-permeable drugs, proteins, RNA, and DNA to allow cellular internalization and modulation of biological responses.\textsuperscript{65} The mechanism and rate of uptake depends primarily on the CPP, size of the conjugated molecule, and cell type. Delivery may be mediated by endocytosis or by a direct translocation across the lipid bilayer. The use of fluorescent endocytic probes and endocytic inhibitors have revealed information regarding CPP cellular association and endocytic uptake mechanisms.\textsuperscript{66} For example, HIV Tat has been shown to utilize clathrin-mediated endocytosis when conjugated to a fluorophore\textsuperscript{68} and lipid-raft-mediated endocytosis when conjugated to a protein.\textsuperscript{69} Non-endocytic plasma membrane translocation is thought to occur when the CPP peptide compromises the structural integrity of the cell
membrane. Specifically, the CPP TP-10 binds the membrane surface, causing a mass imbalance that perturbs the bilayer, allowing for direct translocation.\textsuperscript{70} Evidence for the CPP penetratin (Antp) suggests that the peptides bind the plasma membrane, accumulate in large numbers, and form a membrane pore.\textsuperscript{71} However, another recent study suggests that Antp enters cells through three simultaneous endocytic routes in HeLa cells.\textsuperscript{72} These studies demonstrate the fact that the specific uptake mechanisms of CPPs are largely debated. Despite this, the conjugation of biologically active molecules and drugs of interest to these peptides is an exciting and promising area of research.

1.5 Conclusions

Derivatives of $N$-alkyl-3$\beta$-cholesterylamine appear to mimic natural glycolipids in both size and cellular uptake mechanisms. Modification of the linker region has proven to be an effective means of altering cellular activity and subcellular localization. In addition, the ligand-binding head group of these molecules may be altered in innumerable ways to deliver proteins and biologically active small molecules into cells through endocytosis. This thesis investigates the delivery of the drug vancomycin and the protein vascular endothelial growth factor (VEGF) by small synthetic cell surface receptors.
1.6 References

1. Villareal, M.R. Unpublished and used with permission.


38. Sun, Q.; Cai, S.; Peterson, B.R. Unpublished results.


Chapter 2

Calix[4]arene-derived Synthetic Receptors that Promote Cellular Uptake of VEGF

2.1 Introduction

Angiogenesis is a complex process leads to the formation of new blood vessels. In adults, angiogenesis is suppressed and occurs transiently during reproduction, development, and wound healing. However, in cancer, angiogenesis is activated to enable tumor growth, invasiveness, and metastasis. Specifically, the progression of cancer involves an imbalance of angiogenic and angiostatic mediators, favoring the expression of angiogenic factors that include transforming growth factor (TGF) beta, angiopoietin-1, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF).

VEGF is an important mediator of breast cancer angiogenesis, promoting both the invasiveness and survival of breast cancer cells. In human breast cancers, several isoforms of VEGF are overexpressed. Specifically, the serum level of VEGF in breast cancer patients is dramatically elevated (median 365.2 pg/mL) compared to serum levels following tumor removal (median 158.7 pg/mL) and healthy controls (median 88.5 pg/mL). These elevated levels of VEGF in human breast cancers are primarily promoted by hypoxia, a deficiency in the amount of oxygen reaching body tissues. Several drugs
are directed at inhibition of VEGF signaling because of its importance in the progression of cancer. These drugs and drug candidates include humanized monoclonal antibodies against VEGF (Bevacizumab), antibodies against the VEGF receptor VEGFR-2, soluble competitive VEGF receptors (SU5416), and small molecules (Suramin) that can inhibit the growth of multiple tumor cell lines \textit{in vivo} \textsuperscript{3}. \textsuperscript{3} It was recently demonstrated by Huh et al. that inhibition of VEGF receptors with a novel small molecule significantly impaired breast cancer growth in transgenic mice. \textsuperscript{8} Therapeutics directed against VEGF may retard tumor growth with potentially high levels of specificity. Therefore, the targeting of VEGF or its receptor by angiogenesis inhibitors is an appealing alternative to traditional cancer treatments due to the potential reduction of adverse side effects and decreased cytotoxicity.

\textbf{2.2 Vascular Endothelial Growth Factor (VEGF)}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{X-ray crystal structure of the disulfide-linked dimeric VEGF protein. Lysine residues on the surface of this basic protein are shown as CPK models.}
\end{figure}
The VEGF family of protein ligands comprises structurally similar dimeric proteins linked by inter-chain disulfide bridges (Figure 2.1). Five VEGF isoforms are generated as a result of alternative splicing of the gene encoding VEGF. The major mediator of breast cancer progression is VEGF-A (also referred to as VEGF), a 165 amino acid protein with a molecular weight of 42,321 daltons. VEGF enhances angiogenesis and vascular permeability in breast cancer by its over-expression and subsequent binding to a single transmembrane cell surface receptor of the tyrosine kinase family. Binding of VEGF to the receptor VEGFR-2 results in activation of signal transduction and concomitant endocytosis of VEGF and VEGFR-2. The VEGF ligand is targeted to late endosomes and lysosomes for degradation, providing a mechanism for downregulation of VEGF by removal of this protein from circulation. Mutational analysis has revealed two symmetrical “hot spots” on opposite ends of the VEGF molecule that bind its receptors. Twelve lysine residues decorate the surface of these basic growth factor proteins, providing numerous potential targets for electrostatic interactions with acidic small molecules and peptides covering large surface areas.

2.3 Design of a Small Molecule that Binds VEGF

The laboratories of Hamilton and Sebti have reported the synthesis of novel calix[4]arenes termed growth factor binders (Figure 2.2). GFB-111 (10) was the first example of a calix[4]arene reported to exhibit high affinity for platelet-derived growth factor (PDGF), a protein similar in structure and function to VEGF. In
addition, this compound was shown to exhibit both anti-angiogenic and anticancer activity against human tumors in nude mice. More recently, compound GFB-204 (11) was reported to bind both VEGF and PDGF with high affinity.\textsuperscript{16} Compared with the lead compound GFB-111, GFB-204 is an easily synthesized and highly potent inhibitor of endothelial cell migration and capillary network formation \textit{in vitro}. Moreover, this compound inhibits angiogenesis by binding VEGF as well as PDGF resulting in blocking of the growth of human tumors \textit{in vivo} in nude mouse models.\textsuperscript{16}

The recognition mechanism of VEGF and PDGF by these compounds has been shown to involve binding of the tetracarboxylic acid functionality of the calix[4]arene to the numerous basic residues on the surface of the protein ligands. This molecular recognition event by GFB-204 is thought to prevent the initiation of angiogenesis by VEGF, block the maintenance of blood vessels by PDGF, and confer potent anticancer activity.\textsuperscript{16}

**Figure 2.2:** Structures of growth factor binders (GFBs) reported by Hamilton and Sebti. The activities of these compounds as inhibitors of VEGF and / or PDGF signaling are also shown (NT = not tested).
2.4 Synthetic Receptors Designed to Bind VEGF

We hypothesized that novel synthetic cell surface receptors composed of a similar calix[4]arene headgroup to GFB-204 and an N-alkyl-3β-cholesterylamine membrane anchor (Figure 2.3) should bind the VEGF protein through interactions between the carboxylic acid moieties displayed on the upper calix[4]arene ring and the numerous lysine residues that are present on the surface of VEGF. This interaction might promote the endocytic destruction of these proteins, removing the mitogenic VEGF ligand from cell culture media.

Starting with a calix[4]arene core, it was postulated that mono-alkylation on the lower rim with a carbon linker containing a protected amine functionality would enable the attachment of the fluorescent linker and plasma-membrane anchor. Modification of the upper rim by installation of carboxylic acids, amines, or iodines would enable further elaboration to install headgroups similar in orientation to the ester headgroups of GFB-

![Diagram of calix[4]arene synthetic receptor](image)

**Figure 2.3:** Design of a calix[4]arene synthetic receptor to bind VEGF.
An important change was made in this headgroup. Replacing the ester functionality in the GFB-204 head group to an amide bond would most likely increase the stability of the final molecule to esterases \textit{in vitro}.

\textbf{2.4.1 Synthesis of Calix[4]arene Scaffolds using Aromatic Formylation}

The formation of amide bonds on the upper rim of calix[4]arene was hypothesized to be an efficient way to introduce carboxylic acid functionalities that would bind to VEGF, similar to the synthesis of the potent VEGF-binding compound GFB-204.\textsuperscript{16} As shown in Figure 2.4, using a modified Zinke-Cornforth procedure, \textit{p}-tert-butyl phenol \textbf{12} was condensed with formaldehyde at 120 °C in the presence of NaOH to form an uncharacterized compound. This compound was pyrolyzed in diphenyl ether at 230 °C to form \textit{p}-tert-butylcalix[4]arene \textbf{13}.\textsuperscript{17} Removal of the tert-butyl groups under Lewis acidic conditions afforded calix[4]arene \textbf{14}.\textsuperscript{18} The calix[4]arene \textbf{14} was then mono-O-alkylated\textsuperscript{19} on the lower rim with mesylate \textbf{21} (prepared in two steps from 3-chloropropanol\textsuperscript{20}) to form azide \textbf{15} in 98% yield. This latter step is selective and high yielding due to the differing acidities of the phenolic groups on calix[4]arene.\textsuperscript{19} (The p\textsubscript{Ka} of the first calix[4]arene phenol to be deprotonated is approximately 4-5 p\textsubscript{Ka} units lower than the other three phenolic units).\textsuperscript{19}
Subsequent reaction of 15 with 1-bromobutane and an excess of NaH afforded the peralkylated product 16. The azide of 16 was reduced to the corresponding amine using lithium aluminum hydride, and a tosyl protecting group was introduced to form 17 which can be deprotected and further elaborated with an N-alkyl 3β-cholesterylamine derivative later in the synthesis. Protected 17 was subjected to aromatic formylation conditions to
afford tetraaldehyde 18. Oxidation of 18 with NaHPO₄, H₂O₂, and NaO₂Cl provided tetracarboxylic acid 19. Before conversion of tetracarboxylic acid 19 to the corresponding acid chloride, amine 24 was synthesized in two steps starting with 5-nitroisophthalic acid monomethylester 22 (Figure 2.5). The acid chloride derived from 22 was treated with N-methylbenzylamine to obtain amide 23. Reduction of the nitro group was achieved with Pd/C and hydrogen to produce amine 24. Unfortunately, attempts to convert 19 to the tetraacid chloride and add amine 24 to form tetraamide 20 were unsuccessful under a variety of conditions due to the degradation of 19, and the subsequent formation of complex mixtures of products.

2.4.2 Synthesis of Calix[4]arene Scaffolds using Nitration

Figure 2.6: Synthesis of calix[4]arenes nitrated on the upper rim.
In light of the inability to generate an acid chloride on the upper rim of calix[4]arene, it was hypothesized that switching the functionality on the upper rim of 19 from carboxylic acid groups to amino groups might be more successful and increase the stability of the substrate. These amines could be derived from nitro groups installed by standard nitration reactions. In addition, we hypothesized that a longer linker on the lower rim of the calix[4]arene would alleviate any puckering on the upper rim due to bulky amine protecting groups on the lower rim that may have affected the reactivity of 19.

Starting with calix[4]arene 14, mono-alkylated 25 was obtained using NaH and an azido mesylate in DMF (Figure 2.6). Treatment of 25 using NaH and subsequent addition of 1-bromobutane in DMF generated the peralkylated product 26. Azide reduction with LAH followed by protection with TsCl afforded the protected calixarene 27. Nitration conditions of NaNO₃ in TFA afforded the tetranitrated 28. Subsequent hydrogenation with Pd/C generated the tetraamine 29. Acylation of 29 with an acid chloride derived from carboxylic acid 34 (Figure 2.7) was attempted under a variety of conditions, but unfortunately the desired product was never detected. It was found that tetraamine 29 was highly unstable and rapidly decomposed upon purification.

Figure 2.7: Synthesis of aromatic carboxylic acid derivatives.
2.4.3 Synthesis of Calix[4]arene Scaffolds Using the Sonogashira Reaction

The inability to acylate the upper rim of calix[4]arene scaffolds led us to investigate the use of carbon-carbon bond coupling reactions. Utilization of the Sonogashira reaction to modify the upper rim of calixarenes has been previously reported.\textsuperscript{24,25,26} Iodination of calixarene 26 with silver TFA and iodine afforded tetraiodo calixarene 35 (Figure 2.8).\textsuperscript{27} Sonogashira reaction of 35 with alkyne 41 (Figure 2.8) afforded the tetrasubstituted alkyne calixarene 36. Alkyne 41 was synthesized in five steps from isophthalate 37 (Figure 2.9). Subsequent azide reduction of 36 to the

![Diagram](image)

**Figure 2.8:** Sonogashira reaction with upper-rim substituted calix[4]arenes.

![Diagram](image)

**Figure 2.9:** Synthesis of the alkyne precursor for the Sonogashira reaction.
corresponding amine using mild reducing agents (triphenylphosphine or SnCl\textsubscript{2}) was not successful (no reduction occurred). The use of harsher reducing agents such as lithium aluminum hydride led to degradation of the starting material. However, modification of the upper rim with the Sonogashira reaction proved to be an efficient and successful means of introducing functionality.

Because of the failure to reduce the azide of \textit{36} to the corresponding amine, we proposed that an alternative functionality was needed that would be stable to the Sonogashira reaction. In addition, we thought that the reactivity of the system during carbon-carbon bond formation might improve if we changed the alkyne group of \textit{41} to an aromatic iodide for reaction with a tetraalkyne calix[4]arene derived from tetrainodo calix[4]arene \textit{35}.

To explore this idea, the azide of tetrainodo calixarene \textit{35} was reduced to the corresponding amine with SnCl\textsubscript{2} and subsequently protected as a Boc carbamate to give \textit{42} (Figure 2.10). Sonogashira reaction of \textit{42} with TMS acetylene in triethylamine afforded the tetra TMS-alkyne calixarene \textit{43}. Removal of TMS with TBAF afforded \textit{44}. The aromatic iodine \textit{52} was synthesized in three steps from the nitro-isophthalate \textit{49} (Figure 2.11) and was subjected to a Sonogashira reaction with tetraalkyne \textit{44} to afford tetrakisubstituted \textit{45}. The Boc protecting group of \textit{45} was removed with 15% TFA in dichloromethane and subsequently acylated with cholesterol derivative \textit{53} (synthesized on solid phase from a valeric acid-derived cholesterol)\textsuperscript{28} to give \textit{46}. We used PyBOP for this transformation to reduce epimerization of lysine.
Figure 2.10: Synthesis of fluorescent calix[4]arene-derived synthetic receptors.
The methyl ester protecting groups on the upper rim of 46 were removed with LiOH in methanol, followed by Boc deprotection of the lysine and secondary steroidal amine using 20% TFA in dichloromethane. For visualization in cells the final fluorescently labeled compounds 47 and 48 were obtained by coupling with Oregon Green-NHS ester or Penn Green-NHS ester.

![Chemical Reaction Diagram](image)

**Figure 2.11**: Synthesis of an aromatic iodide derivative.

### 2.5 Analysis of Calix[4]arene Receptors on Living Cells

Living human Jurkat lymphocytes and Chinese hamster ovary (CHO) cells were treated with compounds 47 and 48 and examined by confocal laser scanning microscopy. The cells were treated for 4 h, centrifuged, washed to remove unincorporated receptors, and imaged to examine cellular fluorescence. Both the Oregon Green (47) and Pennsylvania Green (48) derivatives are internalized by both cell lines and become located primarily in endosomes (Figure 2.12). The Oregon Green version of the calixarene receptor is brighter in both cell lines, and appears to be taken up more readily in Jurkat cells than the Pennsylvania Green receptor. In comparison to Jurkat cells, CHO cells appear to take up both receptors more extensively.
Figure 2.12: Differential interference contrast (DIC) and confocal laser scanning micrographs of human Jurkat lymphocytes and Chinese hamster ovary cells after incubation with calixarene receptors 47 and 48 for 4 h. Laser settings were identical for all micrographs. The cells were treated with 10 μM of receptor 47 or 48 in DMSO for 4 h, washed, and imaged.

Figure 2.13: Whole cell fluorescence of living Jurkat lymphocytes treated with calix[4]arene compounds as measured by flow cytometry. Cells were treated with compounds at the indicated concentrations for 4 h, washed, and analyzed.
Flow cytometry was further used to quantify whole cell fluorescence of Jurkat lymphocytes. Cells were treated with compounds 47 and 48 for 4 h, washed, re-suspended, and subjected to flow cytometry. As shown in Figure 2.13, the receptors were taken up in a dose-dependent fashion. Consistent with the confocal micrographs in Figure 2.11, the Oregon Green receptor is considerably brighter than the Pennsylvania Green receptor at all concentrations. A concentration of 10 μM was chosen for co-localization studies with the VEGF protein.

2.6 Cellular Uptake of VEGF-633

2.6.1 Purification of VEGF

VEGF was obtained from the NCI pre-clinical repository as a solid lyophilized with bovine serum albumin (BSA). The ratio of VEGF to BSA was 1 μg to 50 μg. In order to fluorescently label the growth factor, VEGF was purified by affinity chromatography using heparin high-trap columns from GE biosciences. After concentrating the protein to 1 mg/mL using the centricon filter system, VEGF was labeled with AlexaFluor-633 using a protein labeling kit from Molecular Probes. The degree of labeling was determined to be 1.2 fluorophores per protein by UV/Vis spectroscopy. The final labeled VEGF concentration was determined to be 150 μg/mL by Coomassie protein assay. A reducing SDS-page protein gel was used to examine the
purity and fluorescent labeling of the protein as indicated by a band at 21 kDa (Figure 2.14).

Figure 2.14: Analysis of labeled protein by SDS PAGE. Panel A: Sigma protein ladder (Lane 1), BSA (lane 2), VEGF with residual BSA (lane 3) and VEGF-AlexaFluor633 (lane 4). Panel B: SDS gel irradiated with UV light to examine the fluorescence of labeled VEGF.

2.6.2 Confocal Microscopy Co-localization Studies

For co-localization assays, the OG and PG calixarene receptors (10 μM, 1% DMSO) and VEGF-633 (10 μg/mL) were pre-equilibrated for 1 h at 23 °C. The complex was then added to Jurkat lymphocytes or CHO cells for 4 h. After washing, the cells
were imaged by confocal microscopy. Following simultaneous excitation with the 488 and 633 laser lines, VEGF-633 and the calixarene-derived receptors were found to co-localize (Panel A and B, Figure 2.15). Some VEGF was taken up by cells in the absence of a receptor, as indicated by the observed internal red fluorescence (Panel C, Figure 2.14). However, the red fluorescence was appreciably enhanced by the addition of

![Figure 2.15](image)

**Figure 2.15**: Differential interference contrast (DIC) and confocal laser scanning micrographs of Jurkat cells after incubation for 4 h. Laser settings were identical for all micrographs. Panel A: Cells were treated with Oregon Green calixarene 47 (10 μM) and VEGF-633 (10 μg/mL). Panel B: Cells were treated with Pennsylvania Green calixarene 48 (10 μM) and VEGF-633 (10 μg/mL). Panel C: VEGF-633 alone (10 μg/mL).
the receptors (Panel A and B) compared to the VEGF-633 only control (Panel C). These results indicate that the receptors can increase the cellular uptake of VEGF protein.

The uptake of VEGF mediated by the calixarene-derived receptors was also investigated in CHO cells. Both the OG and PG calixarene-derived receptors were found to enhance VEGF-633 uptake (Figure 2.16).

Figure 2.16: Differential interference contrast (DIC) and confocal laser scanning micrographs of CHO cells after incubation for 4 h. Laser settings were identical for all micrographs. Panel A: Cells were treated with Oregon Green calixarene 47 (10 μM) and VEGF-633 (10 μg/mL). Panel B: Cells were treated with Pennsylvania Green calixarene 48 (10 μM) and VEGF-633 (10 μg/mL). Panel C: VEGF-633 alone (10 μg/mL).
2.6.3 Quantitation of VEGF-633 Uptake by Flow Cytometry

The uptake of the red fluorescent VEGF protein by the green fluorescent calixarene-derived receptors was measured quantitatively by flow cytometry. VEGF-633 (10 μg/mL) and receptors 47 and 48 (1, 5, and 10 μM) were pre-equilibrated for 1 h at 23 °C. The complex then was added to human Jurkat lymphocytes for 4 h at 37 °C. The cells were subsequently washed and analyzed. As a control, Jurkat cells were incubated with receptors and DMSO alone or VEGF-633 and DMSO alone. As shown in Figure 2.17, the cellular uptake of the green fluorescent calixarene-derived receptors by Jurkat cells was not affected by the addition of VEGF-633 (Panel A, blue bars, Figure 2.16) and increased in a dose-dependent manner when compared to the receptor-only DMSO controls (Panel A, purple bars, Figure 2.16). The spectrally orthogonal red fluorescence of VEGF-633 was found to increase in the presence of the receptors (Panel B, Figure 2.16). For example, treatment of Jurkat cells with VEGF-633 in the presence of DMSO provided a fluorescence value of 3.47, while cells treated with only DMSO provided a background fluorescence value of ~ 1.0. However, when VEGF-633 was added to cells combined with receptors 55 and 56, the value of red fluorescence increased in a small but dose-dependent manner (Panel B, red bars, Figure 2.16). Cells treated with the receptor-DMSO control displayed background red fluorescent values less than or equal to a value of 1.0 (Panel B, green bars, Figure 2.16). In the presence of 10 μM receptor 55 and 56, it can be seen that the red fluorescence increases by two-fold to 8.68 and 7.84 respectively, when compared to VEGF-633 alone.
Figure 2.17: Fluorescence of Jurkat lymphocytes treated with calix[4]arene derivatives at the indicated concentrations and VEGF-633 (10 μg/mL) as measured by flow cytometry. Living cells were treated with compounds at the indicated concentrations for 4 h, washed, and analyzed.
2.7 Conclusions

We report the synthesis and biological activity of synthetic receptors derived from fluorescent calix[4]arenes and N-alkyl-3β-cholesterylamine. These compounds become incorporated into membranes of living Jurkat lymphocytes and Chinese hamster ovary cells. In addition, these compounds enhance the uptake of the protein ligand, VEGF. Future work is needed to determine the affinity of our synthetic receptor for the protein and enhance the population of the receptors on the cell surface compared with endosomes. The identification of highly active compounds would lead to studies in models of angiogenesis.

2.8 Experimental Section

2.8.1 General

Chemical reagents were obtained from Acros, Aldrich, Alfa Aesar, or TCI America. Solvents were from EM Science. Media and antibiotics were purchased from Mediatech. Commercial grade reagents were used without further purification unless otherwise noted. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). All reactions were performed under an atmosphere of dry argon or nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica
gel 60 F254 (EM Science). TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of Cerium Molybdenate (Hanessian’s stain). Flash column chromatography employed ICN SiliTech Silica Gel (32-63 µm). Purification by preparative reverse phase HPLC employed an Agilent 1100 preparative pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrenedivinylbenzene) reverse phase column (7 µm particle size, 21.5 mm x 25 cm). The HPLC flow rate was 20 mL/min throughout the run. Melting points were measured with a Thomas Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR, or a Thermo Scientific Nicolet 380 FT-IR Spectrometer equipped with either a Smart Diffuse Reflectance Accessory or a Smart Orbit Diamond ATR accessory. NMR spectra were obtained with Bruker CDPX-300, DPX-300, AMX-360, or DRX-400, instruments with chemical shifts reported in parts per million (ppm, δ) referenced to either CDCl₃ (¹H 7.27 ppm; ¹³C 77.23 ppm), DMSO-d₆ (¹H, 2.50 ppm; ¹³C, 39.51 ppm), or methanol-d₄ (¹H, 3.35, 4.78 ppm; ¹³C, 49.3 ppm). High-resolution mass spectra were obtained from the Pennsylvania State University Mass Spectrometry Facility (ESI and CI). Peaks are reported as m/z.
2.8.2 Synthetic procedures and compound characterization data

28-(3-azidopropoxy)pentacyclo[19.3.1.1^3,7.1^9,13.1^15,19]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaene-25,26,27-triol. Sodium hydride (34 mg, 1.41 mmol) was suspended in 50 mL of dry toluene and cooled to 4 °C in an ice bath. Calix[4]arene (462 mg, 1.09 mmol) was slowly added to the slurry as a solution in dry toluene. The reaction mixture was allowed to warm to 23 °C over 1 h followed by the addition of MsO(CH₂)₃N₃ 21 (2.1 g, 11.8 mmol). After stirring at 23 °C for 2 h, the solution was heated at 70 °C for 12 h. The reaction was quenched by the addition of deionized water (10 mL) and solvent was removed in vacuo. The resulting residue was dissolved in CH₂Cl₂ (100 mL) and washed with saturated aqueous sodium chloride solution (2 x 50 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed in vacuo. Flash chromatography (10% ethyl acetate in hexanes) afforded 15 (553 mg, 98%) as a white solid, mp 144 - 148 °C; ¹H NMR (300 MHz, CDCl₃) δ 9.77 (s, 1H), 9.44 (s, 2H), 7.19 (m, 8H), 6.84 (m, 4H), 4.46 (m, 4H), 4.34 (t, J = 6 Hz, 2H), 4.00 (t, J = 6 Hz, 2H), 3.62 (m, 4H), 2.46 (q, J = 6 Hz,
25-(3-azidopropoxy)-26,27,28-tributoxypentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaene. Mono-substituted calixarene 15 (508 mg, 1 mmol) was dissolved in dry DMF (50 mL) and heated to 70 °C. NaH (192 mg, 8 mmol) was added and the solution was stirred for 10 min at 70 °C. 1-bromobutane (1.01 g, 8 mmol) was added, and the reaction was stirred at 70 °C for an additional hour. Upon cooling to 23 °C, the reaction mixture was poured into ethyl acetate (100 mL) and was washed with deionized water (3 x 50 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed in vacuo. Flash chromatography (3% ethyl acetate in hexanes) afforded 16 (606 mg, 90%) as a clear oil, ^1H NMR (400 MHz, CDCl₃) δ 6.65 (m, 12H), 4.43 (m, 4H), 4.02 (t, J = 8 Hz, 2H), 3.89 (m, 6H), 3.52 (t, J = 8 Hz, 2H), 3.18 (m, 4H), 2.23 (q, J = 8 Hz, 2H), 1.91 (q, J = 8 Hz, 6H), 1.47 (m, 6H), 1.02 (t, J = 8 Hz, 9H); ^13C NMR (75 MHz, CDCl₃) 156.7, 156.5, 156.3, 156.2, 153.5, 153.4, 135.2, 135.1, 134.8, 134.6, 134.3, 128.5, 128.4, 128.2, 128.1, 128.1, 127.9, 122.3,
4-methyl-N-(3-{[26,27,28-tributoxypentacyclo[19.3.1.1^{3}.7.1^{9}.13.1^{15}.19]octacosa-
1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaen-25-
yl]oxy}propyl)benzenesulfonamide. Peralkylated calixarene 16 (300 mg, 0.44 mmol) was dissolved in dry ether (100 mL) and cooled to 4 °C in an ice bath. Lithium aluminum hydride (253 mg, 6.65 mmol) was added in 50 mg increments over 10 min. The reaction was then allowed to warm to 23 °C for 1 h. The reaction was quenched by the slow addition of deionized water (50 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was redissolved in dry CH₂Cl₂ (100 mL). Triethylamine (89 mg, 0.88 mmol) and toluene sulfonyl chloride (110 mg, 0.572 mmol) were added and the reaction was stirred for 2 h. Solvent was removed in vacuo and the remaining residue applied directly to the column. Flash chromatography (20% ethyl acetate in hexanes) afforded 17 (253 mg, 72% over 2 steps) as a clear oil, ¹H NMR (400 MHz, CDCl₃) δ 7.79 (d, J = 10 Hz, 2H), 7.31 (d, J = 10 Hz, 2H), 6.61 (m, 12H), 4.80 (t, J = 8 Hz, 1H), 4.44 (m, 4H), 3.91 (m, 8H), 3.16 (m, 6H), 2.43 (s, 3H), 2.12
4-methyl-N-(3-[(26,27,28-tributoxy-5,11,17,23-tetraformylpentacyclo[19.3.1.13,7.19,13.115,19]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaen-25]-yl]oxy)propyl)benzenesulfonamide. A solution of dichloromethyl methylether (9.26 g, 80.5 mmol) in CHCl₃ (50 mL) was heated to 40 °C in a three-neck round bottom flask. Two separate solutions were added simultaneously to the reaction mixture over 10 min; TiCl₄ (7.67 g, 40.32 mmol) in CHCl₃ (50 mL) and tosyl protected calixarene 17 (1.3 g, 1.61 mmol) in CHCl₃. The reaction was stirred an additional 2 h at 40 °C and was subsequently quenched with 50 mL of deionized water and stirred for 30 min. The organic layer was separated and dried over anhydrous sodium sulfate, and the solvent was removed in vacuo. Column chromatography (30% ethyl acetate in hexanes) afforded 18 (798 mg, 55%) as a pale yellow solid, mp 75 - 79 °C; ¹H NMR (400 MHz, CDCl₃) δ 9.64

(q, J = 6.8 Hz, 2H), 1.91 (m, 6H), 1.46 (m, 6H), 1.01 (t, J = 7.2 Hz, 9H); ¹³C NMR (75 MHz, CDCl₃) 156.9, 156.8, 156.5, 148.8, 137.4, 135.6, 135.5, 135.3, 135.2, 130.2, 128.7, 128.6, 127.5, 122.6, 122.4, 75.3, 72.0, 41.0, 32.7, 31.4, 30.7, 21.9, 19.8, 19.7, 14.5; IR (ATR) ν max 3271, 2924, 1450, 1084; HRMS (ESI) m/z 826.4120 (MH⁺, C₅₀H₆₁NO₆SNa requires 826.4117).
(s, 1H), 9.52 (s, 3H), 9.09 (s, 1H), 7.71, (d, J = 10 Hz), 7.32 (d, J = 10 Hz), 7.13 (m, 6H),
6.56 (m, 2H), 4.44 (m, 4H), 3.89 (m, 8H), 3.51 (m, 2H), 3.27 (m, 4H), 2.42 (s, 3H), 2.14
(m, 2H), 1.83 (m, 6H), 1.41 (m, 6H), 0.96 (m, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$) 191.2,
161.9, 161.1, 155.8, 145.5, 136.3, 135.6, 135.3, 134.8, 134.1, 131.1, 131.0, 130.2, 129.7,
128.6, 127.1, 122.8, 75.2, 72.1, 39.9, 32.1, 30.8, 30.7, 29.1, 21.5, 19.1, 13.8; IR (ATR)
$\nu_{\text{max}}$ 2927, 1687, 1590, 1123; HRMS (ESI) $m/z$ 915.4016 (MH$^+$, C$_{54}$H$_{60}$NO$_{10}$ requires
915.4011).

25,26,27-tributoxy-28-(3-[(4-methylphenyl)sulfonyl]amino)propoxy)
pentacyclo[19.3.1.1$^3$.7$^9$.1$^{13}$.1$^{15}$.1$^{19}$]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),
16,18,21,23-dodecaene-5,11,17,23-tetracarboxylic acid. Tetraaldehyde 18 (540 mg,
0.59 mmol) was dissolved in of an acetonitrile/deionized water mixture (20 mL, 6:1).
NaHPO$_4$ (82 mg, 0.59 mmol) was added, and the reaction mixture was cooled to 4 °C.
Hydrogen peroxide (114 mg, 3.54 mmol) was added followed by NaClO$_2$ (429 mg, 4.72
mmol), and the reaction was warmed to 23 °C for 5 h. Aqueous HCl (1M, 50 mL) was
added, and the resulting precipitate was collected and dried yielding 19 (529 mg, 91%) as
a white solid, mp 196 - 201 °C; $^1$H NMR (400 MHz, d$_6$-MeOH) $\delta$ 9.05 (s, 1H), 7.73 (d, J
= 8 Hz, 2H), 7.53 (m, 3H), 7.46 (d, J = 8 Hz, 2H), 6.95 (m, 2H), 6.22 (m, 3H), 4.30 (m, 4H), 3.91 (m, 4H), 3.80 (m, 2H), 3.69 (m, 2H), 3.56 (m, 2H), 3.17 (m, 4H), 2.31 (s, 3H), 1.97 (m, 2H), 1.75 (m, 6H), 1.44 - 1.24 (m, 6H), 0.91 (m, 9H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) 169.9, 169.8, 162.8, 162.7, 162.7, 161.4, 156.7, 147.1, 137.3, 136.9, 136.6, 135.5, 134.8, 131.8, 131.5, 131.2, 129.2, 128.6, 125.6, 125.3, 76.3, 73.6, 41.2, 33.5, 31.9, 31.8, 30.4, 21.7, 20.6, 20.3, 14.5; IR (ATR) \(\nu_{\text{max}}\) 3100, 2929, 1688, 1592, 1284; HRMS (ESI) \(m/z\) 986.4388 (MH\(^+\), C\(_{54}\)H\(_{68}\)NO\(_{14}\)S requires 986.4361).

3-azidopropyl methanesulfonate. 3-Chloropropanol (40 g, 210 mmol) was added to a suspension of sodium azide (33.2 g, 255 mmol) in dry DMF (300 mL). The reaction was heated at 100 °C for 48 h. After cooling to 23 °C, the reaction was diluted with diethyl ether (1 L), washed with saturated aqueous sodium chloride solution (5 x 500 mL), and dried over anhydrous sodium sulfate. Solvent was removed \(\textit{in vacuo}\) and the resulting residue was dissolved in CH\(_2\)Cl\(_2\) (200 mL) and cooled to 4 °C. \(N,N\)-dimethylaminopyridine (5.13 g, 42 mmol) and triethylamine (85 g, 82 mmol) were added followed by mesyl sulfonylchloride (62.6 g, 273 mmol). The reaction was stirred at 4 °C for 30 min, and allowed to warm to 23 °C over 20 min. The reaction was diluted with CH\(_2\)Cl\(_2\) (200 mL), poured into deionized ice water, washed with aqueous HCl (1M, 2 x 100 mL and saturated aqueous sodium chloride solution (2 x 100 mL), and dried over anhydrous sodium sulfate. Solvent was removed \(\textit{in vacuo}\) and flash chromatography (25% ethyl acetate in hexane) afforded 21 (46.8 g, 63%, 2 steps) as a yellow oil; \(^1\)H NMR
Methyl 3-[[benzyl(methyl)amino]carbonyl]-5-nitrobenzoate. Mono-methyl 5-nitroisophthalate (1 g, 4.44 mmol) was dissolved in thionyl chloride (100 mL) and refluxed at 85 °C for 3 h. Upon cooling to 23 °C, solvent was removed in vacuo. The resulting film was redissolved in dry CH₂Cl₂ (100 mL). N-Methyl benzylamine (404 mg, 3.33 mmol) was added dropwise followed by the addition of diisopropylethylamine (1.1 g, 8.88 mmol) and was stirred for 1 h. Solvent was removed in vacuo and the crude product was applied directly to a column. Flash chromatography (30% ethyl acetate in hexanes) afforded 23 (950 mg, 86%) as a yellow solid, mp 90 – 95 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.71 (s, 1H), 8.39 (s, 1H), 8.34 (s, 1H), 7.23 (m, 5H), 4.68 (br s, 0.67 of 2H), 4.43 (br s, 0.33 of 2H), 3.87 (s, 3H), 2.99 (br s, 0.33 of 3H), 2.84 (br s, 0.67 of 3H); ¹³C NMR (100 MHz, CDCl₃) 168.2, 167.5, 163.8, 147.7, 137.9, 135.8, 135.3, 133.1, 131.8, 128.6, 128.4, 127.9, 127.4, 126.2, 125.6, 124.74, 54.7, 53.3, 52.6, 50.6, 36.5, 33.3; IR (ATR) νmax 1721, 1627, 1295; HRMS (ESI) m/z 329.1127 (MH⁺,C₁₇H₁₆N₂O₅ requires 329.1137).
Methyl 3-amino-5-\{benzyl(methyl)amino\}carbonyl\}benzoate. Compound 23 (950 mg, 2.89 mmol) was dissolved in methanol (100 mL) and was hydrogenated with 10% palladium on carbon under hydrogen atmosphere with a balloon for 2 h. The palladium catalyst removed by filtration over celite and solvent removed in vacuo. Flash chromatography (30% ethyl acetate in hexane) yielded 24 (737 mg, 86%) as a white solid, mp 136 - 138 °C; \(^1\)H NMR (360 MHz, CDCl\(_3\)) \(\delta\) 7.21 (m, 7H), 6.81 (s, 1H), 4.60 (br s, 0.67 of 2H), 4.35 (br s, 0.33 of 2H), 3.72 (s, 3H), 2.86 (br s, 0.33 of 3H), 2.71 (br s, 0.67 of 3H); \(^13\)C NMR (90.5 MHz, CDCl\(_3\)) 172.2, 171.5, 167.1, 147.9, 137.7, 137.2, 136.7, 131.5, 129.1, 128.5, 127.9, 127.3, 117.8, 117.4, 117.1, 55.5, 52.6, 51.1, 37.3, 33.5; IR (ATR) \(\nu_{max}\) 3448, 3331, 1701, 1589, 1255; HRMS (ESI) \(m/z\) 299.1389 (MH\(^+\),C\(_{17}\)H\(_{18}\)N\(_2\)O\(_3\) requires 299.1396).

28-\{(6-azidohexyl)oxy\}pentacyclo[19.3.1.1\(^3\).7\(^1\).1\(^9\).1\(^3\).1\(^5\).1\(^9\)]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaene-25,26,27-triol. Sodium hydride (68 mg, 2.82 mmol) was suspended in 100 mL of dry toluene and cooled to 4 °C
in an ice bath. Calix[4]arene (1 g, 2.35 mmol) was slowly added to the slurry as a solution in dry toluene. The reaction mixture was allowed to warm to 23 °C over 1 h followed by the addition of 3-azidohexyl methanesulfonate (4.152 g, 18.8 mmol). After stirring at 23 °C for 2 h the solution was heated at 70 °C for 12 h. The reaction was quenched by the addition of deionized water (50 mL) and solvent was removed in vacuo. The resulting residue was dissolved in CH₂Cl₂ (100 mL) and washed with saturated aqueous sodium chloride solution (2 x 50 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed in vacuo. Flash chromatography (10% ethyl acetate in hexanes) afforded 25 (955 mg, 75%) as a white solid, mp 128 - 130 °C; ¹H NMR (300 MHz, CDCl₃) δ 9.94 (s, 1H), 9.63 (s, 2H), 7.23 (m, 8H), 7.06 (t, J = 7.5 Hz, 1H), 6.88 (t, J = 7.5 Hz, 3H), 4.53 (m, 4H), 4.35 (t, J = 6.6 Hz, 2H), 3.68 (m, 4H), 3.50 (t, J = 6.6 Hz, 2H), 2.372 (q, J = 6.6 Hz, 2H), 1.90 (m, 4H), 1.77 (m, 2H); ¹³C NMR (75 MHz, CDCl₃) 151.8, 151.3, 149.6, 134.6, 129.9, 129.3, 129.2, 69.3, 51.8, 32.4, 31.9, 30.3, 29.2, 27.1, 26.0; IR (ATR) υ max 3300, 2093, 1453; HRMS (ESI) m/z 548.2542 (MH⁻, C₃₄H₃₄N₃O₄ requires 548.2549).

25-[(6-azidohexyl)oxy]-26,27,28-tributoxypentacyclo[19.3.1.1³,7.1⁹,13.1¹⁵,1⁹]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaene. Monosubstituted calixarene
25 (995 mg, 1.74 mmol) was dissolved in dry DMF (100 mL) and heated to 70 °C. NaH (333 mg, 13.89 mmol) was added and the solution was stirred for 10 min at 70 °C. 1-bromobutane (1.90 g, 13.89 mmol) was added and the reaction was stirred at 70 °C for an additional hour. After cooling to 23 °C, the reaction mixture was poured into ethyl acetate (200 mL) and was washed with deionized water (3 x 100 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed in vacuo. Flash chromatography (3% ethyl acetate in hexanes) afforded 26 (1.12 g, 90%) as a clear oil, 1H NMR (400 MHz, CDCl3) δ 6.49 (m, 12H), 4.35 (m, 4H), 3.79 (m, 8H), 3.16 (t, J = 7 Hz, 2H), 3.06 (m, 2H), 1.81 (m, 8H), 1.54 (m, 2H), 1.36 (m, 10H), 0.93 (t, J = 6.2 Hz, 9H); 13C NMR (75 MHz, CDCl3) 156.4, 135.0, 128.1, 121.8, 74.7, 51.3, 32.2, 30.9, 29.9, 28.8, 26.7, 25.7, 19.3, 14.0; IR (ATR) νmax 2958, 2092, 1454, 1192; HRMS (ESI) m/z 740.4411 (MH+, C43H53N3O4Na requires 740.4403).

4-methyl-N-(6-[[26,27,28-tributoxypentacyclo[19.3.1.13,7.19,13.115,19]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaen-25-yl]oxy]hexyl)benzenesulfonamide. Peralkylated calixarene 26 (306 mg, 0.44 mmol)
was dissolved in dry diethyl ether (100 mL) and was cooled to 4 °C on an ice bath. Lithium aluminum hydride (243 mg, 6.39 mmol) was added in 50 mg increments over 10 min. The reaction was then allowed to warm to 23 °C for 1 h. The reaction was quenched by the slow addition of deionized water (50 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was redissolved in dry CH$_2$Cl$_2$ (100 mL). Triethylamine (87 mg, 0.86 mmol) and toluene sulfonyl chloride (107 mg, 0.56 mmol) were added and the reaction was stirred for 2 h. Solvent was removed in vacuo and the remaining residue applied directly to the column. Flash chromatography (20% ethyl acetate in hexanes) afforded the protected calixarene **27** (350 mg, 96% over 2 steps) as a clear oil, $^1$H NMR (400 MHz, CDCl$_3$) δ 7.65 (d, J = 8 Hz, 2H), 7.16 (d, J = 8 Hz, 2H), 6.48 (m, 12H), 4.78 (t, J = 6 Hz, 1H), 4.33 (m, 4H), 3.76 (m, 8H), 3.04 (m, 4H), 2.82 (q, J = 6.8 Hz, 2H), 2.29 (s, 3H), 1.77 (m, 8H), 1.31 (m, 12H), 0.88 (m, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$) 157.1, 157.0, 156.9, 147.3, 143.7, 142.1, 137.5, 135.7, 135.6, 135.5, 134.4, 130.1, 128.6, 127.6, 122.4, 75.3, 43.7, 32.7, 31.5, 31.2, 30.5, 30.2, 27.2, 26.2, 22.3, 21.9, 19.8, 14.6; IR (ATR) $\nu_{\text{max}}$ 2924, 1452, 1156, 1085; HRMS (ESI) $m/z$ 868.4555 (MH$^+$ requires C$_{53}$H$_{67}$NO$_6$SNa requires 868.4587).
4-methyl-N-(6-[[26,27,28-tributoxy-5,11,17,23-tetranitropentacyclo [19.3.1.1^{3,7}.9.1^{15,19}]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23- dodecaen-25-yl]oxy]hexyl)benzenesulfonamide. Protected calixarene 27 (50 mg, 0.06 mmol) was dissolved in trifluoroacetic acid (10 mL). NaNO₃ (120 mg, 1.41 mmol) was added and the reaction was stirred until the initial bluish black color dissipated. The reaction was subsequently poured into deionized ice water, and extracted with CH₂Cl₂ (2 x 20 mL). The organic phase was washed with deionized water until neutrality of the aqueous phase was achieved and dried over anhydrous sodium sulfate. Flash chromatography (1% methanol in benzene) afforded nitro compound 28 (43 mg, 70%) as a brown solid, mp 88 - 92 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.09 (d, J = 8 Hz, 2H), 7.45 (m, 10H), 5.27 (s, 1H), 4.46 (d, J = 12 Hz, 4H), 4.05 (m, 8H), 3.39 (d, J = 12 Hz, 4H), 2.52 (m, 2H), 2.36 (s, 3H), 1.84 (m, 8H), 1.43 (m, 12H), 1.02 (m, 9H); ¹³C NMR (75 MHz, CDCl₃) 161.9, 161.8, 161.7, 146.6, 143.3, 142.7, 142.6, 142.5, 135.6, 135.5, 133.9, 129.7, 124.1, 75.9, 53.6, 32.5, 32.1, 31.0, 30.5, 29.6, 22.4, 21.6, 19.1, 14.2, 14.0, 13.9; IR (ATR) νmax 2929, 1518, 1339; HRMS (ESI) m/z 1048.4025 (MH⁺, C₅₃H₆₃N₅O₁₄SNa requires 1048.3990).
4-methyl-\(N\)-(6-[[5,11,17,23-tetraamino-26,27,28-tributoxypentacyclo[10.3.1.1^{3,7,1^{9,13},1^{15,19}}]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaen-25-yl]oxy]hexyl)benzenesulfonamide. Nitrated calixarene 28 (275 mg, 0.27 mmol) was dissolved in methanol and ethyl acetate (50 mL, 2:1). 10% Pd on carbon was added (290 mg, 2.7 mmol) and the reaction was placed under a hydrogen atmosphere with a balloon and stirred for 3 h. The reaction was filtered through a pad of celite, and the solvent was removed \textit{in vacuo} to afford amine 29 (220 mg, 91%) as a tan solid, mp 120 - 122 °C; \textsuperscript{1}H NMR (400 MHz, d\textsubscript{4}-Methanol) \(\delta\) 7.57 (d, \(J = 10.8\) Hz, 2H), 7.20 (d, \(J = 10.8\) Hz, 2H), 6.57 (m, 8H), 4.34 (m, 4H), 3.81 (m, 8H), 3.16 (m, 4H), 2.71 (m, 2H), 2.26 (s, 3H), 1.76 (m, 8H), 1.31 (m, 12H), 0.87 (m, 9H); \textsuperscript{13}C NMR (75 MHz, d\textsubscript{4}-Methanol) 158.2, 144.9, 138.2, 131.2, 128.5, 127.3, 124.2, 77.0, 55.2, 44.3, 34.1, 32.2, 31.6, 31.2, 28.2, 27.2, 21.9, 20.8, 21.8, 20.8, 14.9; IR (ATR) \(\nu\)\textsubscript{max} 2931, 1665, 1191; HRMS (ESI) \(m/z\) 906.5243 (MH\textsuperscript{+}, C\textsubscript{53}H\textsubscript{72}N\textsubscript{5}O\textsubscript{6}S requires 906.5203).
Dimethyl 1,3,5-benzene tricarboxylate (32) was prepared as previously described. Tricarboxylate (2.5 g, 9.9 mmol) was dissolved in methanol (200 mL) and aqueous NaOH was added (1M, 8.9 mL) and the solution was stirred for 18 h. The solvent was removed in vacuo. The resulting residue was redissolved in CH$_2$Cl$_2$ (200 mL) and was washed with saturated aqueous NaHCO$_3$ (2 x 100 mL). The aqueous layer containing the product was acidified with concentrated HCl then extracted with ethyl acetate (5 x 100 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo to afford 32 (2.1 g, 87%) as a white solid, mp 115 - 119 °C; $^1$H NMR (400 MHz, d$_4$-Methanol) δ 8.35 (s, 2H), 8.24 (s, 1H), 3.89 (s, 6H); $^{13}$C NMR (75 MHz, d$_4$-Methanol) 167.7, 166.7, 135.5, 135.1, 133.3, 132.4; IR (ATR) $\nu_{\max}$ 2955, 2633, 1630, 1236; HRMS (ESI) m/z 237.0397 (MH$,^+$ C$_{11}$H$_9$O$_6$ requires 237.0399).

Dimethyl 5-[[benzyl(methyl)amino]carbonyl]isophthalate. Monocarboxylic acid 32 (1 g, 4.21 mmol) was dissolved in thionyl chloride (100 mL) and refluxed at 85 °C for 3 h. Upon cooling to 23 °C solvent was removed in vacuo. The resulting film was redissolved in dry CH$_2$Cl$_2$ (100 mL). N-Methyl benzylamine (407 mg, 3.37 mmol) was
added dropwise followed by the addition of diisopropyl ethylamine (1.6 g, 12.8 mmol), and was stirred for 1 h. Solvent was removed in vacuo and the crude product was applied directly to a column. Flash chromatography (30% ethyl acetate in hexane) afforded 33 (1.13 g, 79%) as a clear oil; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.44 (s, 1H), 8.16 (s, 2H), 7.17 (m, 5H), 5.08 (br s, 0.67 of 2H), 4.59 (br s, 0.33 of 2H) 3.71 (s, 6H), 2.89 (br s, 0.33 of 3H), 2.71 (br s, 0.67 of 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) 168.4, 167.7, 163.7, 135.6, 135.0, 134.5, 130.5, 129.8, 129.4, 127.3, 127.1, 126.6, 126.0, 125.1, 53.5, 50.8, 49.2, 35.2, 31.7; IR (ATR) $\nu_{max}$ 1724, 1635, 1236; HRMS (ESI) $m/z$ 342.1334 (MH$^+$, C$_{19}$H$_{20}$NO$_5$ requires 342.1341).

3-[[benzyl(methyl)amino]carbonyl]-5-(methoxycarbonyl)benzoic acid. Methyl ester 33 (1.13 g, 3.31 mmol) was dissolved in methanol (100 mL) and aqueous NaOH was added (1M, 3 mL) and stirred for 18 h. The solvent was removed in vacuo. The resulting residue was redissolved in CH$_2$Cl$_2$ (100 mL) and washed with saturated aqueous NaHCO$_3$ (2 x 50 mL). The aqueous layer containing the product was acidified with concentrated HCl then extracted with ethyl acetate (5 x 50 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo to afford 34 (1.04 g, 96%) as a white solid, mp 52 – 55 °C; $^1$H NMR (400 MHz, d$_4$-Methanol) $\delta$ 8.65 (s, 1H), 8.25 (s, 2H), 7.30 (m, 5H), 4.76 (br s, 0.67 of 2H), 4.51 (br s, 0.33 of 2H), 3.93 (s, 3H), 3.05 (br s, 0.33 of 3H), 2.81 (br s, 0.67 of 3H); $^{13}$C NMR (75 MHz, d$_4$-Methanol) 171.4,
25-[(6-azidohexyl)oxy]-26,27,28-tributoxy-5,11,17,23-tetraiodopentacyclo
[19.3.1.1^3.7.1^9.13.1^15.19]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-
dodecaene. Azido calixarene 26 (900 mg, 1.25 mmol) and silver TFA (1.2 g, 5.52
mmol) were dissolved in CHCl₃ (200 mL) and refluxed at 80 °C for 3 h. The reaction
was subsequently cooled to 50 °C and iodine (1.4 g, 5.52 mmol) was added followed by
stirring at 50 °C for 18 h. After cooling to 23 °C, the precipitated AgI was filtered off.
The filtrate was washed with saturated aqueous Na₂S₂O₅ (5 x 50 mL) and saturated
aqueous sodium chloride solution (2 x 50 mL), then dried over anhydrous sodium sulfate.
Flash chromatography (20% ethyl acetate in hexane) afforded 35 (1.44 g, 94%) as a pale
yellow solid, mp 75 - 80 °C; ¹H NMR (300 MHz, CDCl₃) δ 6.98 (m, 8H), 4.27 (m, 4H),
3.82 (m, 8H), 3.23 (t, J = 6.9 Hz, 2H), 3.05 (m, 4H), 1.85 (m, 8H), 1.42 (m, 12H), 0.97
(m, 9H); ¹³C NMR (75 MHz, CDCl₃) 156.7, 137.5, 137.2, 86.6, 75.6, 51.7, 32.5, 30.8,
30.2, 29.3, 27.2, 26.1, 19.7, 14.5; IR (ATR) vₘₐₓ 2953, 2090, 1452, 1193; HRMS (ESI)
m/z 1244.0297 (MH⁺, C₄₆H₅₅I₄N₃O₄Na requires 1244.0269).
tetramethyl3,3',3'',3'''-{[25-[(6-azidohexyl)oxy]-26,27,28-tributoxypentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaene-5,11,17,23-tetrayl]tetraethyne-2,1-diyl}tetrakis(5-[[benzyl(methyl)amino]carbonyl]benzoate). Iodinated calixarene 35 (210 mg, 0.17 mmol) and alkyne 41 (200 mg, 0.65 mmol) were dissolved in tetrahydrofuran and triethylamine (100 mL, 1:1) under argon. PdCl$_2$(PPh$_3$)$_2$ (10 mg, 0.014 mmol) and CuI (1.3 mg, 0.007) were added. The reaction was refluxed at 65 °C for 18 h. The solvent was removed in vacuo and the resulting residue was redissolved in CH$_2$Cl$_2$ (100 mL), washed with aqueous HCl (1M, 2 x 50 mL) then saturated aqueous sodium chloride solution (2 x 50 mL), and dried over anhydrous sodium sulfate. Flash chromatography afforded 36 (125 mg, 37%) as a yellow solid, mp 100 - 103 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 7.98 (s, 4H), 7.84 (s, 4H), 7.59 (s, 4H), 7.18 (m, 20 H), 7.16 (m, 8H), 4.38 (m 12H), 3.82 (m, 20H), 3.21 (m, 6H), 2.85 (m, 12H), 1.84 (m, 8H), 1.45 (m, 12H), 0.96 (m, 9H); $^{13}$C NMR (75 MHz, CDCl$_3$) 170.7, 170.1, 165.9, 156.1, 136.9, 136.8, 135.4, 135.3, 134.4, 133.6, 132.5, 132.5, 129.4, 129.3, 129.1, 128.8, 128.6, 128.3, 128.0, 127.8, 124.9, 117.1, 92.8, 89.5, 75.7, 55.6, 52.8, 51.7, 51.2, 37.3, 33.8, 32.6, 31.3, 30.4, 30.1, 29.4, 27.3, 26.2, 23.8, 20.6, 19.7, 14.5; IR (ATR)
\( \nu_{\text{max}} \) 2922, 2091, 1722, 1633, 1233; HRMS (ESI) \( m/z \) 1938.8781 (MH\(^+\), \( \text{C}_{122}\text{H}_{120}\text{N}_7\text{O}_{16} \) requires 1938.8792).

**Dimethyl 5-\([(\text{trimethylsilyl})\text{ethynyl}]\text{isophthalate} \).** Dimethyl-5-hydroxy isophthalate (5.0 g, 23.8 mmol) and pyridine (2.82 g, 35.7 mmol) were dissolved in dry \( \text{CH}_2\text{Cl}_2 \) (300 mL) and cooled to 4 °C. Trifluoromethanesulfonic anhydride (7.38 g, 26.2 mmol) was added dropwise over 10 min and the reaction was stirred for 30 min at 4 °C. After quenching with aqueous HCl (1M, 50 mL), the organic layer was washed with deionized water (2 x 100 mL) and saturated aqueous sodium chloride solution (2 x 100 mL), dried over anhydrous sodium sulfate, then concentrated *in vacuo*. The resulting residue was redissolved in dry benzene (60 mL). DBO (2.9 g, 26 mmol) and TMS-acetylene (3 mL, 21.5 mmol) were added, followed by the addition of PdCl\(_2\)(PPh\(_3\))\(_2\) (302 mg, 0.43 mmol) and CuI (41 mg, 0.22 mmol). The reaction was stirred at 23 °C for 18 h, after which the solvent was removed *in vacuo*. After re-dissolving in \( \text{CH}_2\text{Cl}_2 \) (100 mL) the organic layer was washed with aqueous HCl (1M, 2 x 50 mL) and saturated aqueous sodium chloride solution (2 x 50 mL) then dried over anhydrous sodium sulfate. Flash chromatography (10% ethyl acetate in hexane) afforded 38 (2.5 g, 74%, 2 steps) as a white solid, mp 105 - 106 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 8.28 (s, 1H), 7.99 (s, 2H), 3.67 (s, 6H), 0.00 (s, 9H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) 165.7, 137.0, 131.0, 130.4, 124.4, 102.9, 53.1, 52.7,
Dimethyl 5-ethynylisophthalate. TMS alkyne 38 (2.5 g, 8.62 mmol) was dissolved in THF (100 mL) and cooled to 4 °C. Tetrabutylammonium fluoride (10 mL, 10 mmol) was added and the reaction was stirred for 10 min. After the addition of aqueous HCl (1M, 50 mL), the solvent was removed in vacuo. The resulting residue was dissolved in CH₂Cl₂ (100 mL), washed with saturated aqueous sodium chloride solution (2 x 50 mL), dried over anhydrous sodium sulfate, and concentrated in vacuo. Flash chromatography (10% ethyl acetate in hexane) afforded 39 (1.1 g, 60%) as white solid, mp 130 - 132 °C; ¹H NMR (400 MHz, d₆-DMSO) δ 8.29 (s, 1H), 8.05 (s, 2H), 4.42 (s, 1H), 3.86 (s, 6H); ¹³C NMR (75 MHz, d₆-DMSO) 164.8, 136.4, 131.1, 129.7, 123.3, 83.5, 81.4, 53.1; IR (ATR) v max 3245, 1721, 1258; HRMS (ESI) m/z 218.0580 (MH⁺, C₁₂H₁₀O₄ requires 218.0579).

3-ethynyl-5-(methoxycarbonyl)benzoic acid. Dimethylester 39 (550 mg, 2.52 mmol) was dissolved in methanol and THF (20 mL, 3:1) and aqueous LiOH was added (1M, 2.7
mL, 2.7 mmol). The reaction was stirred at 23 °C for 18 h, after which some starting material remained. The solvent was removed in vacuo, and CH₂Cl₂ (50 mL) was added. The organic layer was washed with aqueous LiOH (1M, 2 x 10 mL), resulting in residual starting material in the organic phase and product in the basic aqueous phase. The organic phase was dried over anhydrous sodium sulfate and concentrated in vacuo to afford 200 mg of starting material. The aqueous phase was acidified with concentrated HCl and the resulting precipitate was collected to afford 40 (270 mg, 83% corrected for recovered starting material) as a white solid, mp 248 - 252 °C; ¹H NMR (300 MHz, CDCl₃) δ 8.56 (s, 1H), 8.24 (s, 2H), 3.87 (s, 3H), 3.16 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) 166.1, 164.8, 136.4, 135.9, 129.9, 122.2, 80.6, 78.2, 51.6; IR (ATR) υₘₐₓ 3076, 1702, 1239, 1181; HRMS (ESI) m/z 203.0328 (MH⁻, C₁₁H₇O₄ requires 203.0344).

Methyl 3-[[benzyl(methyl)amino]carbonyl]-5-ethylbenzoate. Monocarboxylic acid 40 (270 mg, 1.33 mmol) was dissolved in dry DMF (50 mL) and cooled to 4 °C. Triethylamine (0.28 mL, 1.99 mmol) and diphenylphosphoryl azide (0.43 mL, 1.99 mmol) were added and the reaction was stirred at 4 °C for 1 h. N-Methyl benzylamine (0.21 mL, 1.6 mmol) was added and the reaction was stirred at 23 °C for 18 h. Ethyl acetate (100 mL) was added and the organic layer was extracted with deionized water (3 x 50 mL) and saturated aqueous sodium chloride solution (3 x 50 mL). The solution was then dried over anhydrous sodium sulfate and concentrated in vacuo. Flash
chromatography (25% ethyl acetate in hexane) afforded 41 (388 mg, 95%) as a yellow solid, mp 72 – 75 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.15 (s, 1H), 8.05 (s, 1H), 7.71 (s, 1H), 7.32 (m, 5H), 4.71 (br s, 0.67 of 2H), 4.44 (br s, 0.33 of 2H), 3.88 (s, 3H), 3.14 (br s, 0.33 of 3H), 3.00 (s, 1H), 2.82 (br s, 0.67 of 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) 168.4, 164.4, 135.9, 133.5, 133.0, 129.7, 127.8, 127.2, 127.1, 126.7, 125.7, 122.1, 80.7, 78.2, 54.2, 51.4, 49.8, 35.8, 32.3; IR (ATR) $\nu_{\text{max}}$ 3219, 1720, 1624, 1248; HRMS (ESI) $m/z$ 308.1276 (MH$^+$, C$_{19}$H$_{18}$NO$_3$ requires 308.1287).

tert-butyl(6-[[26,27,28-tributoxy-5,11,17,23-tetraiodopentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaen-25-yl]oxy]hexyl)carbamate. Azide 26 (1.0 g, 0.82 mmol) was dissolved in ethyl acetate and methanol (200 mL, 1:1). SnCl$_2$ (1.5 g, 6.5 mmol) was added and the reaction was stirred at 23 °C for 18 h. The solvent was removed in vacuo, and ethyl acetate (100 mL) was added. The organic layer was washed with aqueous KOH (2M, 2 x 50 mL), dried over anhydrous sodium sulfate, and concentrated in vacuo. The resulting amine residue was redissolved in dry THF (100 mL) and diisopropylethylamine (0.72 mL, 4.1 mmol) and Boc$_2$O (358 mg, 1.64 mmol) were added. After 2 h, the solvent was
concentrated *in vacuo* and the reaction mixture was applied directly to a column. Flash chromatography (20% ethyl acetate in hexane) afforded 42 (998 mg, 93%, 2 steps) as a pale yellow solid, mp 89 - 92 °C ; $^1$H NMR (400 MHz, CDCl$_3$) δ 6.94 (m, 8H), 4.19 (m, 4H), 3.76 (m, 8H), 3.01 (m, 6H), 1.76 (m, 8H), 1.32 (m, 21H), 0.90 (t, J = 7.2 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$) 156.4, 156.3, 156.2, 155.9, 137.1, 136.9, 136.8, 136.7, 136.6, 86.5, 75.6, 54.1, 32.4, 30.7, 30.3, 28.8, 27.3, 26.2, 19.6, 14.4; IR (ATR) $\nu_{\text{max}}$ 2954, 1700, 1452, 1194; HRMS (ESI) m/z 1318.0864 (MH$^+$, C$_{51}$H$_{65}$I$_4$NO$_6$Na requires 1318.0889).

tert-buty1(6-[26,27,28-tributoxy-5,11,17,23-tetrakis[(trimethylsilyl)ethynyl]pentacyclo[19.3.1.1$^3$.7.1$^9$.13.1$^{15}$.19]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaen-25-yl]oxy}hexyl)carbamate. Tetraiodo calixarene 42 (2.0 g, 1.54 mmol) was dissolved in dry triethylamine (100 mL) under argon. PdCl$_2$(PPh$_3$)$_2$ (108 mg, 0.15 mmol) and Cul (15 mg, 0.08 mmol) were added, followed by the addition of TMS-acetylene (1 mL, 6.48 mmol). The reaction was stirred at 23 °C under argon for 18 h then the solvent was removed *in vacuo*. The residue was redissolved in CH$_2$Cl$_2$ (100 mL), washed with aqueous HCl (1M, 3 x 100 mL), and dried over anhydrous sodium
sulfate. After concentrating the solvent \textit{in vacuo}, flash chromatography (10\% ethyl acetate in hexane) afforded \textbf{43} (1.84 g, 96\%) as a pale brown solid, mp 110 - 112 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 6.93 (m, 8H), 4.50 (s, 1H), 4.33 (m, 4H), 3.85 (m, 8H), 3.11 (m, 4H), 1.84 (m, 8H), 1.38 (m, 21H) 0.97 (t, J = 7.2 Hz, 9H), 0.00 (m, 36H); $^{13}$C NMR (75 MHz, CDCl$_3$) 156.7, 156.6, 156.5, 155.8, 134.1, 134.0, 132.3, 116.8, 105.3, 92.6, 75.0, 40.1, 31.9, 30.6, 28.2, 25.6, 24.5, 19.1, 13.9, 0.00; IR (ATR) $\nu_{max}$ 2956, 1714, 1457, 1245, 837; HRMS (ESI) $m/z$ 1198.611 (MH$, C_{71}H_{101}NO_6Si_4$ requires 1198.6604).

tert-butyl\{6-\{[26,27,28-tributoxy-5,11,17,23-tetraethynylpentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaen-25-yl]oxy\}hexyl\}carbamate. TMS alkyne \textbf{43} (400 mg, 0.34 mmol) was dissolved in THF (50 mL) and cooled to 4 °C. Tetrabutylammoniumfluoride (1M, 1.5 mL, 1.5 mmol) was added and stirred for 10 min. The reaction was poured into deionized water (50 mL) and the aqueous phase was extracted with CH$_2$Cl$_2$ (3 x 50 mL). The organic phase was dried over anhydrous sodium sulfate then concentrated \textit{in vacuo}. Flash chromatography (20\% ethyl acetate in hexane) afforded \textbf{44} (267 mg, 89\%) as a pale
brown solid, mp 76 – 80 °C; ¹H NMR (400 MHz, CDCl₃) δ 6.72 (m, 8H), 4.51 (s, 1H), 4.26 (m, 4H), 3.78 (m, 8H), 2.99 (m, 6H), 2.78 (s, 4H), 1.76 (m, 8H), 1.31 (m, 21H), 0.91 (t, J = 7.2 Hz, 9H); ¹³C NMR (75 MHz, CDCl₃) 157.5, 157.4, 156.3, 135.1, 135.0, 132.6, 116.4, 84.34, 79.3, 76.4, 75.4, 40.1, 32.6, 31.0, 30.6, 30.4, 28.8, 27.3, 26.2, 19.7, 14.5; IR (ATR) νmax 3288, 2956, 2104, 1702, 1457, 1213; HRMS (ESI) m/z 910.5015 (MH⁺, C₇₁H₁₄₁NO₆ requires 910.5023).

Tetramethyl3,3',3'',3'''-[25,26,27-tributoxy-28-((6-[((tert-butoxycarbonyl)amino]hexyl)oxy)pentacyclo[19.3.1.1³,7,1⁹,1³,1⁵,1⁹]octacosa-1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaene-5,11,17,23-tetrayl]tetraethyne-2,1-diyl]tetrakis(5-[[benzyl(methyl)amino]carbonyl]benzoate). Tetraalkyne calixarene 44 (267 mg, 0.30 mmol) and iodo compound 52 (515 mg, 1.26 mmol) were dissolved in diisopropylamine (75 mL) under argon. PdCl₂(PPh₃)₂ (21 mg, 0.03 mmol) and CuI (3 mg, 0.06 mmol) were added, and the reaction was heated at 60 °C for 18 h. The solvent was removed in vacuo, and the residue was redissolved in CH₂Cl₂ (100 mL) and washed with aqueous HCl (1M, 2 x 50 mL). The organic layer was dried over anhydrous sodium sulfate and
concentrated in vacuo. Flash chromatography (50% ethyl acetate in toluene) afforded 45 (419 mg, 69%) as a yellow solid, mp 114 - 118 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.99 (s, 4H), 7.84 (s, 4H), 7.59 (s, 4H), 7.19 (m, 20H), 6.91 (m, 8H), 4.38 (m, 13H), 3.75 (m, 20H), 3.11 (m, 6H), 2.88 (m, 12H), 1.81 (m, 8H), 1.37 (m, 21H), 0.89 (t, $J = 7.2$ Hz, 9H); $^{13}$C NMR (90.5 MHz, CDCl$_3$) 195.1, 170.3, 169.6, 165.6, 157.3, 156.0, 145.3, 136.7, 136.5, 134.9, 133.8, 133.4, 133.2, 132.1, 130.5, 128.7, 128.3, 128.2, 127.6, 126.9, 124.6, 116.4, 91.9, 86.8, 79.0, 75.3, 55.2, 52.6, 52.3, 50.8, 40.5, 36.9, 33.3, 32.2, 30.8, 30.6, 30.3, 30.1, 29.7, 28.7, 28.2, 26.9, 25.8, 19.3, 14.1; IR (thin film) $\upsilon_{\text{max}}$ 2919, 1725, 1637, 1448, 1448, 1243; HRMS (ESI) $m/z$ 2012.9363 (MH$^+$, C$_{127}$H$_{130}$N$_5$O$_{18}$ requires 2012.9411).

$N$-(5-{$(\text{tert-butoxycarbonyl})[3\beta]$-cholest-5-en-3-yl}amino)pentanoyl)-$\beta$-alanyl-$\beta$-alanyl-$N^6$-{$(\text{tert-butoxycarbonyl})$-$N$-(6-{$[5,11,17,23$-tetrakis{$[3$-[benzyl(methyl)amino]carbonyl}-5-(methoxycarbonyl)phenyl]ethynyl$]-26,27,28$-tributoxy$\text{pentacyclo}[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]\text{octacosa-1}(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23$-dodecaen-25-yl}oxy$]hexyl$)$lysamipamide. Calixarene 45 (85 mg, 0.042
mmol) was dissolved in CH$_2$Cl$_2$ containing TFA (20%, 20 mL) and stirred at 23 °C for 3 h. The reaction was then poured into aqueous NaOH (1M, 10 mL) and the organic layer was separated, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was redissolved in CHCl$_3$ (10 mL) and PyBOP (24 mg, 0.046 mmol), cholesterol compound 53 (36 mg, 0.042 mmol), and triethylamine (0.018 mL, 0.105 mmol) were added and the reaction was stirred at 23 °C for 5 h. The solvent was concentrated in vacuo and the residue was applied directly to a column. Flash chromatography (5% methanol in CH$_2$Cl$_2$) afforded 46 (58 mg, 48%) as a pale yellow solid, mp 146 - 150 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.84 (m, 12H), 7.17 (m, 28H), 5.25 (s, 1H), 4.37 (m, 13H), 3.86 (m, 20H), 3.16 – 0.77 (m, 135H), 0.59 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) 173.8, 172.5, 170.7, 170.1, 166.0, 157.7, 156.8, 155.9, 141.7, 137.1, 136.9, 136.6, 136.1, 135.3, 134.7, 134.2, 133.7, 133.6, 132.4, 132.0, 131.1, 131.0, 130.8, 129.1, 128.6, 127.9, 127.5, 127.3, 124.9, 121.7, 116.8, 92.3, 91.9, 87.4, 87.2, 79.6, 77.6, 75.6, 58.4, 57.1, 56.5, 55.6, 54.0, 53.8, 52.7, 51.2, 50.5, 44.8, 42.7, 40.1, 40.0, 39.9, 38.8, 37.3, 37.0, 36.6, 36.2, 33.7, 32.7, 32.6, 32.2, 31.9, 31.3, 30.4, 30.1, 29.9, 29.7, 29.0, 28.8, 28.6, 28.4, 27.5, 27.2, 26.2, 26.0, 24.7, 24.2, 23.7, 23.2, 22.9, 21.4, 19.9, 19.8, 19.7, 19.1, 14.6, 14.5, 12.2; IR (thin film) $\nu_{\text{max}}$ 3295, 2919, 1731, 1642, 1460, 1243; HRMS (ESI) $m/z$ 2872.5571 (MH$^+$, C$_{176}$H$_{212}$N$_{10}$O$_{24}$ requires 2872.5574).
$N\{5\{3\beta\}-cholest-5-en-3-ylamino\}pentanoyl\}-\beta\text{-}alanyl\}-\beta\text{-}alanyl\}-N^6\{3\text{-}carboxy\text{-}4\text{-}(2,7\text{-}difluoro\text{-}6\text{-}hydroxy\text{-}3\text{-}oxo\text{-}3H\text{-}xanthen\text{-}9\text{-}yl)benzoyl\}-N\{6\{5,11,17,23\text{-}tetrakis\{3\{benzyl\text{(methyl)amino}\}carbonyl\text{-}5\text{-}carboxyphenyl\}ethynyl\}-26,27,28\text{-}tributoxypentacyclo\{19.3.1.1^{3,7}.1^{9,13}.1^{15,19}\}\text{octacosa-}1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23\text{-}dodecaen\text{-}25\text{-}yl\}oxy\}$hexyllysaminamide.

Calixarene 46 (29 mg, 0.01 mmol) was dissolved in methanol and THF (10 mL, 3:1) and aqueous LiOH (1M, 0.3 mL, 0.3 mmol) was added. After stirring at 23 °C for 18 h, the reaction was concentrated in vacuo then redissolved in CH$_2$Cl$_2$ (20 mL). The organic layer was washed with aqueous HCl (1M, 2 x 10 mL), dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was dissolved in CH$_2$Cl$_2$ containing TFA (20%, 10 mL) and stirred at 23 °C for 12 h. After removing the solvent, the residue was redissolved in DMF (2 mL) and Oregon Green-NHS ester (5 mg, 0.01 mmol) and triethylamine (0.030 mL, 0.3 mmol) were added. After 12 h, the mixture was directly purified by HPLC (89.9% deionized water, 10% MeCN, 0.1% TFA to 0.9% deionized
water, 99% MeCN, 0.1% TFA over 15 min, hold at 99% MeCN to 25 min; RT = 20 min) to afford labeled calixarene 47 (15 mg, 50%) as an orange solid, mp 145 - 149 °C; HRMS (ESI) m/z 2987.4283 (MH⁺, C₁₈₃H₁₉₆F₂N₁₀O₂₆ requires 2987.4290).

Analytical HPLC profile of the HPLC-purified analogue 47 (absorbance at 215 nm, gradient 9.9% MeCN, 90% H₂O, and 0.1% TFA to 98.9% MeCN, 1% H₂O, and 0.1% TFA over 15 min, hold for 10 min; retention time = 17.9 min).

\[ N\{5\text{-}(3\beta\text{-cholest-5-en-3-ylamino})\text{pentanoyl}\}\beta\text{-alanyl}\beta\text{-alanyl}\text{-N}^6\{4\text{-}(2,7\text{-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl})\text{-3-methylbenzoyl}\}\text{-N}\{6\text{-}[5,11,17,23\text{-tetrakis}[3\{[benzyl(methyl)amino]carbonyl\}\text{-5-carboxyphenyl}]\text{ethynyl}]\text{-26,27,28-tributoxypentacyclo}[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]\text{octacosa-}\]
1(25),3(28),4,6,9(27),10,12,15(26),16,18,21,23-dodecaen-25-yl)oxy}hexyl)lysinamide.

Calixarene 46 (29 mg, 0.01 mmol) was dissolved in methanol and THF (10 mL, 3:1) and aqueous LiOH (1M, 0.3 mL, 0.3 mmol) was added. After stirring at 23 °C for 18 h, the reaction was concentrated in vacuo then redissolved in CH₂Cl₂ (20 mL). The organic layer was washed with aqueous HCl (1M, 2 x 10 mL), dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was dissolved in CH₂Cl₂ containing TFA (20%, 10 mL) and stirred at 23 °C for 12 h. After removing the solvent, the residue was redissolved in DMF (2 mL) and Pennsylvania Green-NHS ester (5.2 mg, 0.01 mmol) and triethylamine (0.030 mL, 0.3 mmol) were added. After 12 h, the mixture was directly purified by HPLC (89.9% deionized water, 10% MeCN, 0.1% TFA to 0.9% deionized water, 99% MeCN, 0.1% TFA over 15 min, hold at 99% MeCN to 25 min; RT = 20 min) to afford labeled calixarene 48 (16 mg, 50%) as an orange solid, mp 142 - 146 °C; HRMS (ESI) m/z 2957.4542 (MH⁺, C₁₈₃H₁₉₈F₂N₁₀O₂₄ requires 2957.4549).

Analytical HPLC profile of the HPLC-purified analogue 48 (absorbance at 215 nm, gradient 9.9% MeCN, 90% H₂O, and 0.1% TFA to 98.9% MeCN, 1% H₂O, and 0.1% TFA over 15 min, hold for 10 min; retention time = 18.1 min).
3-amino-5-(methoxycarbonyl)benzoic acid. 3-Nitro-5-(methoxycarbonyl)benzoic acid (5.0 g, 22.2 mmol) was dissolved in methanol (250 mL) and 10% Pd on carbon was added. The reaction was placed under a hydrogen atmosphere with a balloon and stirred for 3 h. After filtering through a pad of celite, the solvent was concentrated *in vacuo* to afford amine 50 (4.2 g, 98%) as a yellow solid, mp 210 - 212 °C; ¹H NMR (400 MHz, d₄-Methanol) δ 7.93 (s, 1H), 7.54 (s, 1H), 7.51 (s, 1H), 3.89 (s, 3H); ¹³C NMR (75 MHz, d₄-Methanol) 170.1, 168.8, 150.3, 133.6, 132.7, 121.5, 121.1, 120.8, 53.2; IR (ATR) υmax 3460, 3375, 2847, 1712, 1596, 1248; HRMS (ESI) m/z 194.0452 (MH⁻, C₉H₈NO₄ requires 194.0453).

![50](image)

3-iodo-5-(methoxycarbonyl)benzoic acid. Amino compound 50 (1.73 g, 9.01 mmol) was dissolved in aqueous HCl (23%, 50 mL) and cooled to 4 °C. NaNO₂ (687 mg, 9.96 mmol) was added dropwise as a solution in deionized water (2 mL) and the reaction was stirred for 5 min at 4 °C. A solution of KI (1.49 g, 9.96 mmol) in deionized water (3.5 mL) was added and the reaction was heated at 90 °C for 1 hour. After cooling to 23 °C, the resulting precipitate was collected and recrystallized (20% ethanol in deionized water) to afford 51 (2.68 g, 97%) as an orange solid, mp 195 - 200 °C; ¹H NMR (400

![51](image)
Methyl 3-[[benzyl(methyl)amino]carbonyl]-5-iodobenzoate. Carboxylic acid 51 (410 mg, 1.34 mmol) was dissolved in dry DMF (50 mL) and cooled to 4 °C. Triethylamine (0.28 mL, 2.01 mmol) and diphenylphosphoryl azide (0.43 mL, 2.01 mmol) were added and the reaction was stirred at 4 °C for 1 h. N-methyl benzylamine (0.21 mL, 1.6 mmol) was added and the reaction was stirred at 23 °C for 18 h. Ethyl acetate (100 mL) was added, and the organic layer was extracted with deionized water (3 x 50 mL) and saturated aqueous sodium chloride solution (3 x 50 mL) and dried over anhydrous sodium sulfate and concentrated in vacuo. Flash chromatography afforded 52 (530 mg, 96%) as a yellow oil, 1H NMR (400 MHz, CDCl3) δ 8.31 (s, 1H), 7.97 (s, 1H), 7.88 (s, 1H), 7.20 (m, 5H), 4.63 (br s, 0.67 of 2H), 4.38 (br s, 0.33 of 2H), 8.81 (s, 3H), 2.94 (br s, 0.33 of 3H), 2.76 (br s, 0.67 of 3H); 13C NMR (75 MHz, CDCl3) 167.6, 163.8, 138.9, 138.3, 130.9, 127.9, 127.7, 127.2, 126.7, 126.1, 125.7; 92.8, 54.1, 51.5, 49.8, 35.8, 32.4; IR (ATR) v max 2168, 1274, 1634, 1271, 1181; HRMS (ESI) m/z 410.0274 (MH+; C17H17INO3 requires 410.0253).
(3β)N-{5-{(tert-butoxycarbonyl)(cholest-5-en-3-yl)amino}pentanoyl}−β-alanyl−β-alanyl-N⁶-(tert-butoxycarbonyl)lysine. The linker region was synthesized on solid phase using the method described in Chapter 3 with the amino acids Fmoc-Lys(Boc)-OH (1.2 mmol), Fmoc-β-Ala-OH (1.76 mmol), and the coupling reagents DIEA (2.3 mmol), HBTU (1.64 mmol), and HOBT (1.46 mmol). After removal of the Nα-Fmoc group of the amino terminal AA, the amino terminus was capped by addition of DIEA (0.40 mL, 1.75 mmol), HOBT (134 mg, 0.878 mmol), valeric acid-derived cholesterol (570 mg, 0.87 mmol) and PyBOP (459 mg, 0.878 mmol). The product was cleaved with CH₂Cl₂ containing acetic acid (20%, 5 mL) to retain the lysine protecting group. The crude product was purified by flash chromatography (10% MeOH, 0.5% AcOH in dichloromethane) to afford 53 (450 mg, 68%) as a sticky white foam; ¹H NMR (400 MHz, CDCl₃) δ 8.41 (m, 4H), 5.32 (s, 1H), 4.46 (m, 1H), 3.61-3.15 (m, 15H), 2.45-0.85 (m, 72H), 0.65 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 173.9, 172.7, 172.6, 172.5, 156.6, 142.3, 121.6, 79.3, 79.2, 57.1, 56.4, 52.9, 50.4, 46.6, 42.6, 40.5, 40.4, 40.0, 39.8, 38.7, 37.0, 36.2, 36.1, 32.2, 31.5, 29.8, 28.5, 28.4, 28.3, 28.2, 26.7, 24.6, 24.1, 23.3, 23.1, 21.5, 21.3, 19.8, 19.0, 12.2; IR (KBr) νmax 2980, 1731, 1690, 1250; HRMS (ESI) m/z 971.7286 (MH⁺, C₅₅H₉₇N₅O₉ requires 971.7293).
2.8.3 Assays and Protocols

**Purification of VEGF.** Vascular endothelial growth factor was obtained from the NCI BRB Preclinical Repository as a lyophilized solid containing 50 µg of bovine serum albumin per 1 µg of VEGF. HiTrap Heparin HP pre-packed columns (1 mL) were used to purify VEGF from BSA. Sodium phosphate buffer (10 mM, pH 7) was used as the loading and binding buffer. Ten column volumes of binding buffer were used to elute the BSA while retaining the VEGF on the column. The protein was subsequently eluted with aqueous NaCl in aqueous sodium phosphate (1.2 mM NaCl, 10 mM sodium phosphate, pH 7). The protein was concentrated using a Centricon centrifuge concentrator at 4 °C to 1 mg/mL. Final protein concentrations were determined using a Pierce Coomassie Assay. The protein was labeled with an AlexaFluor-633 labeling kit from Invitrogen. After isolation and concentration, the degree of labeling was calculated to be 1.2 moles of dye per mole of protein at a final concentration of 150 µg/mL using UV/Vis spectroscopy. The purity of the protein was observed by performing SDS PAGE using a non-gradient reducing gel in 1x SDS. Proteins were denatured in a DTT containing loading buffer at 90 °C for 5 min, and loaded onto the gel along side a Sigma protein ladder. The electrophoresis apparatus was run at 200 volts until the markers migrated to the bottom of the gel. The gel was irradiated with UV light to visualize the fluorescent VEGF and then stained/destained to visualize the protein ladder. The tris-glycine protein gels were purchased from Jule Inc (part number 15D75HMC10P).
Cell culture. Jurkat lymphocytes (human acute T-cell leukemia, ATCC #TIB-152) were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units per mL), and streptomycin (100 µg per mL). RPMI media used for cell culture and washing steps contained antibiotics and FBS unless otherwise noted. CHO-K1 (Chinese hamster ovary cells, ATCC #CCL-61) were maintained in F-12K media supplemented with fetal bovine serum (FBS, 10%), penicillin (100 units per mL), and streptomycin (100 µg per mL). F-12K media used for cell culture and washing steps contained antibiotics and FBS unless otherwise noted. All cells were propagated in a humidified 5% CO₂ incubator at 37 °C.

Flow cytometry. Analyses were performed with a Beckman-Coulter XL-MCL bench-top flow cytometer or Beckman-Coulter FC-500. Forward scatter (FS) and side-scatter (SSC) dot plots afforded cellular physical properties of size and granularity that allowed gating of live cells. After gating, 10,000 cells were counted. To measure the uptake of Oregon Green calixarene and Pennsylvania Green calixarene by Jurkat lymphocytes, the fluorophores were excited at 488 nm with a 15 mW air-cooled argon-ion laser. The emission was split with a 550 nm dichroic and filtered through a 510 nm long pass filter and 530/30-nm band pass filter using the XL-MCL cytometer. The PMT voltage for this instrument was set to 724 for the detection of Oregon Green and Pennsylvania Green. Calibration with Sphero Rainbow Calibration particles (Spherotech) bearing 330,000 molecules of fluorescein per particle provided a fluorescence of 17.5 at this voltage.
Microscopy. A Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat objective (63X) was employed. Fluorophores were excited with the 488 spectral line of a 25 mW argon ion laser and emitted photons were collected through a 505 nm LP filter. VEGF-AlexaFluor-633 was excited with the 633 spectral line of the HeNe laser and emitted photons were collected through a 650 nm LP filter.

Treatment of Jurkat lymphocytes with protein and synthetic receptors. VEGF-633 (25 µL, 10 µg / mL) and calix[4]arene compounds (final concentration = 10 µM, 1% DMSO) were pre-equilibrated in RPMI media (0.5 mL) for 1 h. This mixture was then added to Jurkat lymphocytes (5 x 10^5) or CHO-K1 cells (5 x 10^5). These cells were incubated at 37 °C for 4 to 24 h to load the protein-calixarene complex into cellular membranes and intracellular endosomes. The cells were then washed with media (0.5 mL) to remove unincorporated protein, receptor, and DMSO. After re-suspension in fresh media the cells were analyzed by confocal microscopy or flow cytometry.

2.9 References


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Chapter 3

Artificial Cell Surface Receptors that Bind Vancomycin

3.1 Introduction

Antibiotic resistance has become increasingly problematic over the past decade as more bacterial strains have become resistant to the effects of penicillin. For example, certain strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) have acquired resistance to virtually all clinically used antibiotics including cephalosporins, tetracyclines, aminoglycosides, erythromycin, and the sulfonamides.\(^1\) This resistance has lead to widespread bacterial infections and death. The broad spectrum antibiotic vancomycin (54, Figure 3.1) is the last line of defense in hospitals.

![Figure 3.1: Structures of vancomycin and derivatives.](image)
against some of these resistant bacteria.\textsuperscript{1} By binding to peptidoglycan precursors that terminate in the peptide sequence L-Lys-D-Ala-D-Ala, vancomycin blocks cross-linking of the bacterial cell wall, providing an effective mode of action against gram positive bacteria.\textsuperscript{2} However, the high polarity and resulting low cell permeability of glycopeptide antibiotics render these drugs ineffective against bacteria capable of replicating either in the cytoplasm of mammalian host cells or in organs such as the brain that are protected by membrane barriers.\textsuperscript{3,4}

3.2 Synthetic Receptors Designed to Bind Vancomycin

We hypothesized that a synthetic receptor capable of promoting the endocytosis of vancomycin might allow this cell-impermeable antibiotic to access the cytoplasm of cells infected with pathogenic bacteria and cross tissue barriers that restrict the delivery of this drug. To test this idea, we synthesized a fluorescent derivative of vancomycin (55, Figure 3.1) and an artificial cell surface receptor (57, Figure 3.2) comprising the vancomycin-binding dipeptide D-Phe-D-Ala\textsuperscript{5} linked to an N-alkyl derivative of 3β-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{synthetic_vancomycin_receptors.png}
\caption{Structures of synthetic vancomycin receptors.}
\end{figure}
cholesterylamine.\(^6\) Compared with the natural D-Ala-D-Ala ligand (K\(_d\) \(~ 1\) μM), the D-Phe-D-Ala substrate has an improved affinity for vancomycin (K\(_d\) \(~ 7\) μM).\(^5\) Control compounds that do not bind vancomycin (L-Phe-L-Ala analogue \(^{58}\)) or exhibit lower affinity for the plasma membrane (amide analogue \(^{59}\)) were synthesized for comparison. Other vancomycin binding motifs linked to lipids have been previously described.\(^{7,8,9,10}\)

### 3.2.1 Synthesis of Artificial Cell Surface Receptors

Compounds \(^{57}\) and \(^{58}\) were synthesized using solid-phase peptide synthesis. These compounds were originally prepared by Dr. Sirawutt Boonyarattanalin and capped with a valeric-acid cholesterol derivative.\(^{11}\) Amide receptor \(^{59}\) was also synthesized on solid-phase, but was capped with a glutaric-acid cholesterol derivative (\(^{65}\), Figure 3.3) that was prepared by reacting cholesterylamine \(^{64}\) with glutaric anhydride. As shown in Figure 3.3, the synthesis of \(^{64}\) incorporated a Swern oxidation of cholesterol (\(^{60}\)) to ketone \(^{61}\). Subsequent reduction to an alcohol with L-selectride inverted the

![Figure 3.3](image)

**Figure 3.3:** Synthesis of a cholesterol derivative used for capping in solid-phase peptide synthesis.
stereochemistry providing epicholesterol 62. A Mitsunobu reaction with HN₃ gave the corresponding azide-derived cholesterol 63, and reduction with lithium aluminum hydride afforded 3β-cholesterylamine 64.⁶

3.3 Biological Evaluation of Receptors for Vancomycin

It was envisioned that addition of the synthetic receptor 57 to healthy (non-infected) mammalian cells would cause this compound to insert into the plasma membrane and project the vancomycin-binding motif from the cell surface. Upon addition of vancomycin, the interaction between the drug and receptor would trigger endocytosis of the complex. Since endosomes are acidic, these conditions would cause the vancomycin and receptor to dissociate, leaving the receptor free to cycle back to the cell surface. The vancomycin would then be degraded in lysosomes. However, in cells infected with an intracellular bacterial pathogen such as *Listeria monocytogenes*,

![Figure 3.4](image)

**Figure 3.4:** The synthetic receptor targeting strategy for endocytic delivery of vancomycin.
endosomes would become disrupted by the secretion of membrane lytic proteins by the bacteria, resulting in delivery of the vancomycin into the cytoplasm (Figure 3.4).

3.3.1 Confocal Imaging of Vancomycin Receptors

Confocal laser scanning microscopy was used to examine the delivery of fluorescent vancomycin (55) into J-774 mouse macrophage cells. Treatment with 55 alone did not appreciably enhance cellular fluorescence, but cells treated with 55 and 57

![Image of confocal laser scanning microscopy](image)

**Figure 3.5:** Confocal laser scanning and differential interference contrast (DIC) microscopy of living J-774 macrophages alone (Panel A) and infected with L. monocytogenes (Panel B). Prior to microscopy, receptor 57 (10 μM) was added to cells for 1 h at 37 °C, cells were washed, and 55 (3.6 μM) was added for 2 h at 37 °C. Scale bar: 10 μm.
internalized this fluorescent probe (Panel A, Figure 3.5). In non-infected cells, this intracellular fluorescence was localized in endosomes and lysosomes. However, in living J-774 cells infected with \textit{L. monocytogenes}, the fluorescence of 55 was observed to be distributed throughout the cytoplasm and nucleus (Panel B, Figure 3.5). This change in localization was presumably due to disruption of endosomes by the pathogen.

### 3.3.2 Quantification by Flow Cytometry

The dose-dependent effectiveness of delivery of 55 into mammalian cells mediated by receptor 57 was examined quantitatively by flow cytometry. Pre-equilibration of 55 with 57 prior to addition to cells was ca. 2-fold more effective for delivery compared with preloading of cellular plasma membranes with 57, washing of cells to remove unincorporated receptor, and subsequent addition of 55 (Panel A and B, Figure 3.6). However, both conditions substantially enhanced ligand uptake. Addition of the soluble competitor AcNH-D-Phe-D-Ala-CO$_2$H blocked these effects. Treatment with the L-Phe-L-Ala-analogue 58 did not enhance cellular fluorescence, demonstrating a requirement for specific recognition. Consistent with previous studies of amide derivatives of 3β-cholesterylamine, analogue 59 was much less active.
3.3.3 Rescue of HeLa Cells from Bacterial Infection

The ability of 57 to deliver vancomycin (55) into HeLa cells infected by *L. monocytogenes* was examined. As shown in Panel A, Figure 3.7, 57 (10 μM) uniquely enabled 55 to eliminate this intracellular microorganism. Control compounds 58 or 59 did not confer antibiotic activity under the same conditions. Moreover, the combination of 57 and 55 rescued HeLa cells from the lethal effects of the pathogen (Panel B, Figure 3.7), whereas neither 58 nor 59 combined with 55 blocked the toxicity of *L. monocytogenes*. 

**Figure 3.6:** Flow cytometric analysis of uptake of vancomycin receptors by J-774 cells (A) and HeLa cells (B). PL = Pre-loading conditions: 65, 66, or 67 was added to cells for 1 h at 37 °C, washed, then OG Van (63) added for 4 h at 37 °C. PE = Pre-equilibration conditions: 65, 66, or 67 was combined with OG Van (63) at 23 °C for 1 h followed by addition to cells for 4 h at 37 °C.
3.3.4 In Vivo Studies on the Uptake of Fluorescent Vancomycin

We examined the potential of synthetic receptor targeting in vivo by injecting fluorescent vancomycin (55) into mice. Balb/c mice were injected intraperitoneally (ip) with 57, premixed solutions of 57 combined with 55, or control compounds (58, 59). Fluorescence of cells isolated from specific tissues was quantified by flow cytometry. As shown in Figure 3.8, 57 uniquely enabled 55 to accumulate in the brain and other tissues with no apparent toxicity. This delivery was highly dependent on molecular structure; altering the stereochemistry of the vancomycin-binding motif (58) or acylation of 3β-
cholesterylamine (59) substantially reduced tissue targeting \textit{in vivo}. This system shows much promise for intracellular drug delivery.

![Delivery of Vancomycin (55) into Tissues of Balb/c Mice](image)

**Figure 3.8:** Delivery of fluorescent vancomycin into tissues of Balb/c mice in vivo. Compounds were injected ip (50 mg/kg), mice were sacrificed after 8 h, and fluorescence of cells from tissues was analyzed by flow cytometry. Error bars reflect the standard deviation (n = 3).

### 3.4 Improvements on the Vancomycin Receptor System

#### 3.4.1 Design and Synthesis of Second Generation Receptors

In an effort to improve the delivery of vancomycin into cells \textit{in vitro} and \textit{in vivo}, three new receptors were synthesized. Based on the crystal structure of vancomycin, it was hypothesized that the peptide L-Lys-D-Phe-D-Ala might exhibit a greater affinity towards the drug due to an additional electrostatic interaction between the lysine and a carboxylic acid on vancomycin.\textsuperscript{12,13,14} A synthetic receptor 66 was designed to
incorporate this amino acid and generate an analogue of 57. In addition, studies of the linker region by Sutang Cai in the Peterson lab established that installation of glutamic acids can enhance uptake of synthetic receptors by cells (unpublished results). Thus, receptors 67 and 68 were designed. We also wanted to investigate the use of Pennsylvania Green as a fluorescent label for studies of vancomycin uptake (Figure 3.1, 56). Receptors 66, 67, and 68 were synthesized on solid phase and capped with a valeric acid-derived cholesteryl amine11 (Figure 3.9).

![Figure 3.9: Structures of analogues of vancomycin synthetic receptor 57.](image)
3.4.2 Confocal Imaging

The uptake of receptors 66 - 68 was examined by confocal microscopy. As shown in Figure 3.10, the addition of two glutamic acid amino acids into the linker region of the receptor (68) did not significantly enhance uptake of the fluorescent vancomycin 55 when compared to the original receptor 57 (Panel A and B, Figure 3.10). However, the addition of a lysine moiety into the vancomycin binding head group (66) demonstrated a substantial increase in the uptake of OG-vancomycin 55, but also an increase in cellular toxicity (Panel C, Figure 3.10). This toxicity was evidenced by the large amount of cell debris and dead cells observed during confocal imaging and flow cytometry analysis.

![Figure 3.10](image)

**Figure 3.10:** Confocal laser scanning and differential interference constrast (DIC) micrographs of Jurkat cells treated with synthetic receptors (10 μM) for 1 h at 37 °C followed by OG-Van (3 μM) for 4 h at 37 °C. Cells were subsequently washed and imaged.
Qi Sun in the Peterson lab previously found that positive charges in the linker region of synthetic receptors can lead to an increase in cell death (unpublished results). However, this toxicity was attenuated by the addition of two glutamic acids into the linker region in receptor 67 (Panel D, Figure 3.10). In addition to an increased uptake of the fluorescent vancomycin 55, Receptor 67 demonstrated greater solubility in aqueous media compared with the original D-Phe-D-Ala receptor 57.

3.4.3 Flow Cytometry analysis

**Figure 3.11:** Uptake of fluorescent vancomycin 55 and 56 mediated by synthetic receptors 57 and 67 in Jurkat lymphocytes. Each receptor was added to cells for 1 h to load the plasma membrane, cells were washed, and 55 or 56 (3 μM) was added for 4 h. Cells were analyzed by flow cytometry.
The uptake of Oregon Green vancomycin (55) and the new Pennsylvania Green vancomycin (56) by 57 and the improved receptor 67 was measured by flow cytometry. Figure 3.11 demonstrates the enhanced uptake of vancomycin by receptor 67 in a dose-dependent fashion. However, the Penn Green vancomycin 56 was not as bright as the Oregon green derivative 55 in vitro.

3.5 Conclusions

We constructed artificial cell-surface receptors composed of a peptide vancomycin binding motif linked to 3-β-cholesterylamine. Receptor 57 was identified as an effective mediator of the endocytic uptake of Oregon Green vancomycin 55. Through dynamic recycling between the plasma membrane and endosomes, this receptor effectively enabled the drug to eradicate an intracellular pathogen. In addition, this receptor 57 was able to deliver vancomycin 55 into tissues of mice in vivo. An improvement on this system was developed by modification of the vancomycin binding domain and linker region in receptor 67. This approach has the potential to provide new tools for intracellular drug delivery.
3.6 Experimental Section

3.6.1 General

Chemical reagents were obtained from Acros, Aldrich, Alfa Aesar, or TCI America. Chemicals for solid phase syntheses were purchased from Novabiochem. Vancomycin hydrochloride hydrate was purchased from Aldrich. Solvents were obtained from EM Science. Dulbecco's Modification of Eagle's Medium (DMEM) and antibiotics were purchased from Gibco BRL. Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) media were purchased from Valley Biomedical. Brain and Heart Infusion (BHI) powder was purchased from Becton, Dickinson and Company. Oregon Green N-hydroxysuccinimidyl ester was purchased from Molecular Probes. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). All solution phase reactions were performed under an atmosphere of dry argon. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254 (EM Science). TLC plates were visualized by UV irradiation (254 nm) or stained with cerium molybdenate (Hannessian’s Stain). Flash column chromatography employed ICN SiliTech Silica Gel (32-63 μm). Purification by preparative reverse phase HPLC employed an Agilent 1100 preparative pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase column (7 μm particle size, 21.5 mm x 25 cm). The HPLC flow rate was maintained at 20 mL/min.
Melting points were measured with a Thomas Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. NMR spectra were obtained with Bruker AMX-360, DRX-400, or AMX-2-500 instruments with chemical shifts reported in parts per million (ppm, δ) which were referenced to either CDCl₃ (¹H 7.27 ppm; ¹³C 77.23 ppm) or DMSO-d₆ (¹H 2.50 ppm; ¹³C 39.51 ppm). High-resolution mass spectra were obtained from the University of Texas at Austin Mass Spectrometry Facility (FAB) or the Penn State University Mass Spectrometry Facility (ESI). Peaks are reported as m/z. Listeria monocytogenes 10403S (L. monocytogenes) was obtained as a gift from Dr. Nancy Freitag (UCLA).

### 3.6.2 Solid-Phase Peptide Synthesis

Coupling of the carboxylic acid of the first amino acid (1.2 mmol) to 2-chlorotrityl chloride resin (250 mg of 1.2 mmol/g, 0.3 mmol) were run in glass reaction vessels with a medium size frit in dry CH₂Cl₂ (4 mL) in the presence of diisopropylethylamine (DIEA, 1.5 mmol). After shaking with a wrist shaker for 4 h, the resin was washed thoroughly with DMF (6 x 3 mL) and CH₂Cl₂ (4 x 1 mL). Peptide synthesis employed standard Nα-Fmoc methodology. The following amino acids were used to construct peptides: Fmoc-D-Ala-OH, Fmoc-L-Ala-OH, Fmoc-D-Phe-OH, Fmoc-Phe-OH, Fmoc-ε-Ahx-OH (aminohexanoic acid), Fmoc-β-Ala-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Glu(OtBu)-OH. Amino acids were coupled in glass reaction vessels with a medium size frit. Removal of Fmoc carbamates from N-termini of amino acids on the
resin was effected by addition of 20% piperidine in DMF (3 x 2 mL for 5 min followed by 2 mL for 15 min). Nα-Fmoc amino acids (AA) were consecutively coupled to the treated resin by sequential addition of the following reagents: AA (1.2 mmol), DIEA (1.5 mmol), HBTU (1.14 mmol), and HOBT (1.14 mmol) in DMF. The resin was shaken at 22 °C for 2 to 4 h. After removal of the Nα-Fmoc group of the amino terminal AA, the amino terminus was capped by addition of DIEA (0.12 mL, 0.9 mmol), HOBT (50 mg, 0.33 mmol), valeric-acid derived cholesterol (193 mg, 0.33 mmol) or glutaric-acid derived cholesterol (65, 185 mg, 0.33 mmol) and PyBOP (173 mg, 0.33 mmol) in 4 mL of DMF. The resin was shaken at 22 °C by the wrist shaker for 24 h followed by washing thoroughly with DMF (8 x 10 mL). Capped peptides were simultaneously cleaved from the resin and globally deprotected by treatment with TFA containing triisopropyl silane (TIS, 2.5%) and deionized H2O (2.5%). After gently shaking for 1 h, the resin was removed by filtration and washed twice with TFA (4 mL). The filtrates were collected and solvent removed in vacuo. The brown crude oily product was dried under high vacuum for 16 h, redissolved in DMSO (4 mL), and purified by preparative reverse-phase HPLC (using gradients of solutions containing MeCN, H2O, and 0.1% TFA). HPLC solvents were removed by lyophilization to afford the desired products as white solids.
3.6.3 Synthetic Procedures and compound characterization data

Oregon Green Vancomycin, aka - 3,1S,2R,18R,19R,22S,25R,28R)-22-(2-amino-2-oxoethyl)-48-{[2-O-(3-D[3-carboxylato-4-(2,7-difluoro-6-oxido-3-oxo-3H-xanthen-9-yl]benzoyl)amino]-2,3,6-trideoxy-3-methylhexopyranosyl)hexopyranosyl]oxy}-5,15-dichloro-2,18,32,35,37-pentahydroxy-19-{[(2R)-4-methyl-2-(methylammonio)pentanoyl]amino}-20,23,26,42,44-pentaoxo-7,13-dioxa-21,24,27,41,43-pentaazaoctacyclo [26.14.2.3^6, 1^8, 12^10, 29^9, 33^9, 34^39, 0^1, 0^10, 25^0]pentaconta-3,5,8(48),9,11,14,16,29(45),30,32,34,36,38,46,49-pentadecaene-40-carboxylate (Originally prepared by Dr. Sirawutt Boonyarattanakalin). A solution of Oregon Green 488 carboxylic acid succinimidyld ester (5 mg, 9.8 µmol), vancomycin hydrochloride hydrate (20.0 mg, 24.0 µmol), and potassium carbonate (10 mg, 72.4 µmol) in DMSO (1.5 mL) was stirred at 22 °C for 12 h. The reaction mixture was directly loaded on a preparative reverse-phase HPLC
column. HPLC purification (gradient: 19.9% MeCN, 80.0% H₂O, and 0.1% TFA to 37.9% MeCN, 62% H₂O, and 0.1% TFA over 18 min; retention time = 13.7 min) afforded 55 (12.3 mg, 68%); FAB+ m/z 1842.4682 (M⁺, C₈₇H₉₄N₉O₃₀F₂Cl₂ requires 1842.4669).

Analytical HPLC profile of the HPLC-purified Oregon Green vancomycin 55 (absorbance wavelength = 215 nm, gradient: 10.0% MeCN, 89.9% H₂O, and 0.1% TFA to 99.9% MeCN, 0% H₂O, and 0.1% TFA over 20.0 min; retention time = 10.0 min).

A solution of Pennsylvania Green-succinimidyld ester (prepared as previously reported)\textsuperscript{15} (10 mg, 0.011 mmol), vancomycin hydrochloride hydrate (62 mg, 0.021 mmol), and triethylamine (7 uL, 0.055 mmol) in 1:1 DMF/DMSO (2 mL) was stirred at 22 °C for 12 h. The reaction mixture was directly loaded on a preparative reverse-phase HPLC column. HPLC purification (gradient: 9.9% MeCN, 90.0% H\textsubscript{2}O, and 0.1% TFA to 98.9% MeCN, 1% H\textsubscript{2}O, and 0.1% TFA over 20 min; retention time = 9.1 min) afforded \textit{56} (8.2 mg, 41%); HRMS (ESI) \textit{m/z} 1812.4955 (M\textsuperscript{+}, C\textsubscript{87}H\textsubscript{86}N\textsubscript{9}O\textsubscript{28}Cl\textsubscript{12}F\textsubscript{2} requires 1812.4927).

Analytical HPLC profile of the HPLC-purified Penn Green vancomycin \textit{56} (absorbance wavelength = 215 nm, gradient: 10.0% MeCN, 89.9% H\textsubscript{2}O, and 0.1% TFA to 99.9% MeCN, 0% H\textsubscript{2}O, and 0.1% TFA over 20.0 min; retention time = 9.1 min).
**alaninamide** (Originally prepared by Dr. Sirawutt Boonyarattanakalin). The crude product from solid-phase synthesis was redissolved in DMSO (4 mL). Purification by preparative reverse-phase HPLC (gradient: 49.9% MeCN, 50% H₂O, and 0.1% TFA to 62.9% MeCN, 37.0% H₂O, and 0.1% TFA over 20 min; retention time = 13.9 min) afforded 57 (84.3 mg, 24%) as a white solid, mp 226 – 228 °C; ¹H NMR (500.1 MHz, DMSO) δ 12.57 (br s, 0.4 H), 8.35 (br s, 2H), 8.30 (d, J = 7.2 Hz, 1H), 7.96 (d, J = 8.6 Hz, 1H), 7.92 – 7.81 (m, 3H), 7.78 (t, J = 5.3 Hz, 1H), 7.28 – 7.21 (m, 3H), 7.19 – 7.14 (m, 1H), 5.40 (br s, 1H), 4.58 – 4.51 (m, 1H), 4.25 – 4.18 (m, 1H), 3.52 – 3.14 (m, 11H), 3.02 (dd, J₁ = 3.7 Hz, J₂ = 13.8 Hz, 1H), 2.99 – 2.83 (m, 5H), 2.70 (dd, J₁ = 10.6 Hz, J₂ = 13.7 Hz, 1H), 2.58 – 2.53 (m, 1H), 2.41 – 2.13 (m, 9H), 2.09 (t, J = 6.8 Hz, 2H), 2.03 – 1.69 (m, 7H), 1.51 – 0.87 (m, 28H), 1.29 (d, J = 7.3 Hz, 3H), 0.95 (s, 3H), 0.89 (d, J = 6.4 Hz, 3H), 0.85 (d, J = 2.3 Hz, 3H), 0.83 (d, J = 2.2 Hz, 3H), 0.65 (s, 3H); ¹³C NMR (125.7 MHz, DMSO) δ 174.0, 171.9, 171.7, 171.4, 170.3, 170.1, 138.6, 138.1, 129.2, 127.9, 126.1, 122.5, 56.6, 56.1, 55.5, 53.4, 49.3, 47.5, 45.7, 43.6, 41.8, 40.5, 40.3, 38.4, 37.6, 36.4, 36.2, 35.6, 35.4, 35.3, 35.2, 35.1, 34.5, 34.4, 34.3, 31.3, 28.8, 27.8, 27.4, 25.9, 25.4, 24.9, 24.4, 23.8, 23.2, 22.7, 22.4, 22.2, 20.5, 18.8, 18.6, 17.1, 11.7 ; FAB+ m/z 1101.7684 (MH⁺, C₆₀H₁₀₁N₈O₉ requires 1101.7691).

![HPLC Graph](image-url)
Analytical HPLC profile of the HPLC-purified synthetic receptor (absorbance wavelength = 215 nm, gradient: 10.0% MeCN, 89.9% H₂O, and 0.1% TFA to 99.9% MeCN, 0% H₂O, and 0.1% TFA over 20.0 min; retention time = 17.3 min).

\[N\{5-[(3\beta)-cholest-5-en-3-ylamino]pentanoyl\}-\beta\text{-alanyl}-\beta\text{-alanyl}-\beta\text{-alanyl}\text{-}N\{6-[(1S)-1-benzyl-2\{[(1S)-1-carboxyethyl]amino\}-2\text{-oxoethyl]amino\}-6\text{-oxohexyl\}-}\beta\text{-alaninamide}\] (Originally prepared by Dr. Sirawutt Boonyarattanakalin). The crude product from solid-phase synthesis was redissolved in DMSO (4 mL). Purification by preparative reverse-phase HPLC (gradient: 49.9% MeCN, 50% H₂O, and 0.1% TFA to 62.9% MeCN, 37.0% H₂O, and 0.1% TFA over 20 min; retention time = 15.9 min) afforded (27.1 mg, 20%) as a white solid, mp 224 – 226 °C; FAB\(^+\) \textit{m/z} 1101.7670 (\textit{MH}^+, C_{62}H_{101}N_{8}O_{9} requires 1101.7692).

Analytical HPLC profile of the HPLC-purified L-Phe-L-Ala analogue (absorbance wavelength = 215 nm, gradient: 10.0% MeCN, 89.9% H₂O, and 0.1% TFA to 99.9% MeCN, 0% H₂O, and 0.1% TFA over 20.0 min; retention time = 17.7 min).
N-{5-[(3β)-cholest-5-en-3-ylamino]-5-oxopentanoyl}-β-alanyl-β-alanyl-β-alanyl-N-{6-[(1R)-1-benzyl-2-[(1R)-1-carboxyethyl]amino]-2-oxoethyl]amino}-6-oxohexyl}-β-alaninamide. The crude product from solid-phase synthesis was redissolved in MeOH (6 mL). Purification by preparative reverse-phase HPLC (gradient: 9.9% MeCN, 90% H2O, and 0.1% TFA to 62.9% MeCN, 37% H2O, and 0.1% TFA over 40 min; retention time = 12.21 min) afforded 59 (5.0 mg, 18%) as an off-white solid, mp 160-162 °C; CI m/z 1113.7325 ((M-H)- C62H97N8O10 requires 1113.7333).

Analytical HPLC profile of the HPLC-purified amide analogue 59 (absorbance at 215 nm, gradient 10% MeCN, 89.9% H2O, and 0.1% TFA to 99.9% MeCN, 0% H2O, and 0.1% TFA over 40 min; retention time = 15.3 min).
Epicholesterol. Dry CH₂Cl₂ (100 mL) was injected into a flame-dried Ar-filled three-neck flask and cooled to −78 °C. Oxalyl chloride (11.60 mL, 2 M in CH₂Cl₂, 22.20 mmol) was added, followed by slow addition of dry dimethyl sulfoxide (1.80 mL, 25.35 mmol). Cholesterol (6.00 g, 15.46 mmol) in dry CH₂Cl₂ (50 mL) was added by addition funnel over 30 minutes to this −78 °C solution. The reaction mixture was warmed to −45 16 °C and stirred for 1.5 h. Triethylamine (11.25 mL, 80.39 mmol) was added at −45 °C followed by warming of the reaction mixture to 23 °C. The reaction was quenched by the addition of deionized H₂O (300 mL). This mixture was extracted with CH₂Cl₂ (1 x 200 mL), the organic layers were combined, washed with saturated aqueous NaCl (2 x 200 mL), and dried over anhydrous Na₂SO₄. The solution was concentrated in vacuo to afford 5-cholestene-3-one (61) as a yellow solid that was used immediately in the next reaction without further purification. A solution of L-selectride (20.00 mL, 1 M in THF, 20.00 mmol) in dry THF (50 mL) was cooled to −78 °C. Crude 5-cholestene-3-one (61) was dissolved in 20 mL of THF, cooled to 4 °C, and added to the L-selectride solution. The reaction mixture was stirred for 2 h at −78 °C, warmed to 23 °C, and stirred for an additional 1 h. The reaction was quenched by the careful addition of aqueous KOH (50 mL, 2 M) followed by methanol (50 mL). Solvents were reduced in vacuo to 20% of the original volume and the mixture was extracted with ether (2 x 100 mL). The ether layers were combined, washed with saturated aqueous NaCl (3 x 100 mL), dried over anhydrous Na₂SO₄, and concentrated in vacuo to afford a yellow-green solid. This crude product
was re-crystallized (10% ether in methanol) to afford epicholesterol 62 (4.98 g, 83%) as white solid, mp 135 – 136 °C; \(^1\)H NMR (300 MHz, CDCl3) \(\delta\) 5.32 (s, 1H), 3.92 (s, 1H), 2.47 (d, \(J = 14.6\) Hz, 1H), 2.02-0.78 (m, 40H), 0.61 (s, 3H); \(^1^3\)C NMR (75.4 MHz, CDCl3) \(\delta\) 138.7, 123.9, 67.1, 56.9, 56.3, 50.4, 42.4, 39.9, 39.7, 37.4, 36.3, 36.0, 33.3, 32.1, 32.0, 29.0, 28.4, 28.1, 24.4, 24.0, 23.0, 22.7, 20.9, 18.9, 18.8, 12.0; IR (film) \(\nu_{\text{max}}\) 3330, 2933, 1467 cm\(^{-1}\); APCI \(m/z\) 387.3614 (MH\(^+\), \(C_{27}H_{47}O\) requires 387.3627).

3β-Azido-5-cholesten. Sodium azide (10.00 g, 153.82 mmol) and deionized H\(_2\)O (10 mL) were combined to form a white paste. Benzene (100 mL) was added, and the mixture was cooled to 4 °C. Concentrated sulfuric acid (6 mL) was added to this flask over 30 min by addition funnel. The reaction mixture was warmed to 23 °C, the benzene layer was separated, and the organic layer was dried over anhydrous Na\(_2\)SO\(_4\). This solution of HN\(_3\) in benzene was immediately added to a mixture of epicholesterol (62, 4.90 g, 12.63 mmol) and PPh\(_3\) (5.30 g, 20.21 mmol) in benzene (200 mL). Diethylazodicarboxylate (9.16 mL, 40% in toluene, 20.21 mmol) in benzene (20 mL) was added, and the reaction was stirred for 2 h at 23 °C to yield a white precipitate. The precipitate was removed by filtration, and the filtrate was concentrated \textit{in vacuo} to yield a crude orange-yellow solid. Flash column chromatography (hexanes) afforded 63 as a white solid (3.30 g, 64%), mp 93 – 94 °C; \(^1\)H NMR (300 MHz, CDCl3) \(\delta\) 5.37 (s, 1H), 3.30 – 3.13 (m, 1H), 2.26 (d, \(J = 7.9\) Hz, 2H), 2.03 – 0.83 (m, 37H), 0.66 (s, 3H); \(^1^3\)C
NMR (75.4 MHz, CDCl3) δ 140.3, 123.0, 61.6, 57.1, 56.6, 50.5, 42.7, 40.1, 39.9, 38.0, 37.8, 36.8, 36.6, 36.2, 32.3, 32.2, 28.6, 28.4, 24.7, 24.3, 23.2, 23.0, 21.2, 19.7, 19.1, 12.3; IR (film) νmax 2100, 1600 cm⁻¹; APCI m/z 411.3607 (M⁺, C₂₇H₄₅N₃ requires 411.3613).

(3β)-cholesta-5-en-3-amine. Azide 70 (6.3 g, 15.3 mmol) was dissolved in dry ether (200 mL) and cooled to 0 °C. Lithium aluminum hydride (2.9 g, 76.4 mmol) was added in small portions to the solution. The reaction was stirred for 2 h, upon which the excess LAH was quenched by the slow addition of water (100 mL). The organic and aqueous layers were separated, and the organic layer was dried over anhydrous sodium sulfate then concentrated in vacuo to give the amine 71 as a white foam (5.1 g, 88%); mp 52 – 58 °C; ¹H NMR (400 MHz, CDCl3) δ 5.31 (s, 1H), 2.59 (m, 1H), 2.16-0.91 (m, 42H), 0.83 (s, 3H); ¹³C NMR (75.4 MHz, CDCl3) δ 142.1, 121.1, 57.2, 56.5, 52.3, 50.6, 43.6, 42.7, 40.2, 39.9, 38.6, 36.9, 36.6, 36.2, 32.9, 32.3, 28.6, 28.4, 24.7, 24.2, 23.2, 22.9, 21.4, 19.9, 19.1, 12.2; IR (film) νmax 3662, 2939 cm⁻¹; HRMS (ESI) m/z 385.5079 (M⁺, C₂₇H₄₇N requires 385.5075).

5-(3β)-cholesta-5-en-3-ylamino]-5-oxopentanoic acid. 3β-Cholesterylamine (0.9 g, 2.3 mmol) was dissolved in distilled dioxane (50 mL). To this solution was added glutaric
anhydride (267 mg, 2.3 mmol). The reaction was heated at 50 °C for 5 h. Solvent was removed in vacuo. The crude product was recrystallized (20:1 EtOH/H₂O) to afford 65 (995.0 mg, 85%) as a white solid, mp 230 °C; ¹H NMR (300 MHz, d₆-DMSO) δ 11.9 (s, 1H), 7.6 (d, 1H), 5.2 (s, 1H), 2.08-0.71 (m, 47H), 0.5 (s, 3H); ¹³C NMR (75.4 MHz, d₆-DMSO) δ 174.3, 170.5, 140.9, 120.7, 56.2, 55.6, 49.6, 41.9, 37.6, 36.1, 35.7, 35.2, 34.6, 33.1, 31.4, 31.3, 28.3, 27.8, 27.4, 23.9, 23.2, 22.70, 22.4, 20.7, 20.5, 19.0, 18.6, 11.9. IR (KBr) vmax 3326, 2933, 1703, 1610 cm⁻¹; Cl m/z 500.4104 (MH⁺, C₃₂H₅₄NO₃ requires 500.4104).

N-{5-[(3β)-cholest-5-en-3-ylamino]pentanoyl}-β-alanyl-β-alanyl-β-alanyl-N-{6-[((1S)-5-ammonio-1-{{[(1R)-1-benzyl-2-{{[(1R)-1-carboxyethyl]amino}-2-oxoethyl]amino}carbonyl}pentyl)amino]-6-oxohexyl}-β-alaninamide. The crude product from solid-phase synthesis was re-dissolved in DMSO (4 mL). Purification by preparative reverse-phase HPLC (gradient 9.9% MeCN, 90% water, 0.1% TFA to 98.9% MeCN, 1% water, 0.1% TFA over 20 min, RT = 15.8 min) afforded 66 (210 mg, 57%) as an off-white solid, mp 188 - 192 °C; ¹H NMR (400 MHz, d₆-DMSO) δ 12.3 (s, 1H), 8.36 (s, 2H), 7.87 (m, 9H), 7.18 (m, 5H), 5.38 (s, 1H), 4.52 (m, 1H), 4.19 (m, 2H), 3.21-2.58 (m, 17H), 2.39-0.85 (m, 72H), 0.63 (s, 3H); HRMS (ESI) m/z 1229.8660 (MH⁺, C₆₈H₁₁₃N₁₀O₁₀ requires 1229.8641).
Analytical HPLC profile of the HPLC-purified analogue 66 (absorbance at 215 nm, gradient 9.9% MeCN, 90% H2O, and 0.1% TFA to 98.9% MeCN, 1% H2O, and 0.1% TFA over 20 min; retention time = 15.8 min).

\[ \text{N-\{5-[(3\beta)-cholest-5-en-3-ylamino\}pentanoyl\}-\beta-alanyl-L-\alpha-glutamyl-L-\alpha-glutamyl-} \]
\[ \text{N-\{6-\{[(1S)-5-amino-1-\{[(1R)-1-benzyl-2-\{[(1R)-1-carboxyethyl]amino\}-2-} \]
\[ \text{oxoethyl]amino\}carbonyl\}pentyl]amino\}-6-oxohexyl\}-\beta-alaninamide.} \]

The crude product from solid-phase synthesis was redissolved in DMSO (4 mL). Purification by preparative reverse-phase HPLC (gradient 9.9% MeCN, 90% water, 0.1% TFA to 98.9% MeCN, 1% water, 0.1% TFA over 20 min, RT = 15.9 min) afforded 67 (251 mg, 62%) as an off-white solid, mp 160 – 165 °C; \(^1\)H NMR (400 MHz, d\(_6\)-DMSO) \(\delta\) 12.32 (s, 3H), 8.39-7.63 (m, 11H), 7.16 (m, 5H), 5.39 (s, 1H), 4.52 (m, 1H), 4.14 (m, 4H), 3.49-2.66 (m, 14H), 2.45-0.85 (m, 74H), 0.64 (s, 3H); (ESI) \(m/z\) 1345.8782 (MH\(^+\), C\(_{72}\)H\(_{117}\)N\(_{10}\)O\(_{14}\) requires 1345.8751).
Analytical HPLC profile of the HPLC-purified analogue 67 (absorbance at 215 nm, gradient 9.9% MeCN, 90% H2O, and 0.1% TFA to 98.9% MeCN, 1% H2O, and 0.1% TFA over 20 min; retention time = 15.9 min).

\[
N\{\text{5-[(3}β\text{-cholest-5-en-3-ylamino]pentanoyl}\}-\beta\text{-alanyl-L-}α\text{-glutamyl-L-}α\text{-glutamyl-}
N\{\text{6-[[}\text{1R}\text{-1-benzyl-2-[(1R)-1-carboxyethyl]amino]-2-oxoethyl]amino]-6-oxohexyl \}
\}-\beta\text{-alaninamide.}\] The crude product from solid-phase synthesis was redissolved in DMSO (4 mL). Purification by preparative reverse-phase HPLC (gradient 9.9% MeCN, 90% water, 0.1% TFA to 98.9% MeCN, 1% water, 0.1% TFA over 30 min, RT = 18.5 min) afforded 77 (mg, %) as an off-white solid, mp 182 - 186 °C; \(^1\)H NMR (400 MHz, \text{d}_6\text{-DMSO}) \text{δ} 12.5 (s, 3H), 8.32 (m, 8H), 7.15 (m, 5H), 5.39 (s, 1H), 4.52 (m, 1H), 4.14 (m, 3H), 3.49-2.66 (m, 15H), 2.45-0.85 (m, 65H), 0.64 (s, 3H); HRMS (ESI) \text{m/z} 1217.7747 (MH\(^+\), C\text{66}H\text{105}N\text{8}O\text{13} \text{requires} 1217.7801).
Analytical HPLC profile of the HPLC-purified analogue 77 (absorbance at 215 nm, gradient 9.9% MeCN, 90% H₂O, and 0.1% TFA to 98.9% MeCN, 1% H₂O, and 0.1% TFA over 30 min; retention time = 18.5 min).

3.6.4 Biological Assays and Protocols

**Cell Culture:** HeLa (ATCC# CCL-2) and J-774 (ATCC #TIB-67) cells were maintained in DMEM supplemented with FBS (10%) without antibiotics in a humidified 37 °C CO₂ incubator. These cells were seeded in a 24-well plate for 12 h prior to experiments. A single colony of *L. monocytogenes* was inoculated in 2 mL of BHI broth (BHI powder (14.8 g) in DDW (400 mL) with streptomycin (200 µg/mL)). The culture was incubated at 37 °C for 18 h with shaking (250 rpm). This saturated *L. monocytogenes* culture (~1 x 10⁷ CFU/mL) was diluted 20-fold in BHI broth and immediately used for infection of mammalian cells. Jurkat T lymphocytes (human acute T-cell leukemia, ATCC #TIB-152) were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units per mL),
and streptomycin (100 µg per mL). RPMI media used for cell culture and washing steps contained antibiotics and FBS unless otherwise noted.

**Confocal Microscopy:** A Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat objective (63X) was employed. Fluorophores were excited with the 488 spectral line of a 25 mW argon ion laser and emitted photons were collected through a 505 nm LP filter.

**Flow Cytometry:** Analyses were performed with a Beckman-Coulter XL-MCL bench-top flow cytometer. Forward-scatter (FS) and side-scatter (SSC) dot plots afforded cellular physical properties of size and granularity that allowed gating of live cells. After gating, 10,000 cells were counted. For studies of uptake of Pennsylvania Green and Oregon Green vancomycin mediated by synthetic receptors, the fluorophores were excited at 488 nm with a 15 mW air-cooled argon-ion laser, the emission was split with a 550 nm dichroic and filtered through a 510 nm long pass filter and 530/30-nm band pass filter using the XL-MCL cytometer. The PMT voltage for this instrument was set to 724 for detection of Oregon Green 488. Calibration with Sphero Rainbow Calibration particles (Spherotech) bearing 330,000 molecules of fluorescein/particle provided a fluorescence of 17.5 at this voltage. For studies of cell viability, live and dead cells were distinguished by visual observation and light scattering.
**Oregon Green Vancomycin Uptake Assays:**

**Preloading Conditions** (Performed by Dr. Sirawutt Boonyarattanakalin):

Receptors in DMSO were diluted in DMEM media to their final working concentrations with DMSO concentration at 1% and added to wells in 24-well plates containing HeLa or J-774 cells (70% confluence). These receptor-treated cells were incubated at 37 °C for 1 h, washed with DMEM media (2 x 0.5 mL) to remove unincorporated receptors and DMSO, and fresh media (300 µL) containing Oregon Green vancomycin (3.6 µM) was added. Cells were typically maintained at 37 °C for 4 h to promote synthetic receptor-mediated endocytosis. Prior to analysis, treated cells were washed with media (2 x 0.5 mL) and DPBS (2 x 0.5 mL). After washing, cells were trypsinized and resuspended in fresh media (0.4 mL) for analysis by confocal microscopy or flow cytometry.

**Premixing Conditions** (Performed by Dr. Sirawutt Boonyarattanakalin):

Receptors in DMSO were pre-equilibrated (preincubated at 22 °C for 1 h) with Oregon Green vancomycin in DPBS. These pre-equilibrated complex solutions were diluted in DMEM media to their final working concentrations (DMSO = 1%) and added to wells in 24-well plates containing HeLa or J-774 cells (60% confluence). These treated cells were typically maintained at 37 °C for 4 h to promote synthetic receptor-mediated endocytosis. Treated cells were washed with media (2 x 0.5 mL) and DPBS (2 x 0.5 mL). After washing, cells were trypsinized and resuspended in fresh media (0.5 mL) for analysis by confocal microscopy or flow cytometry.
**Rescue of HeLa and J-774 Cells from Lethal* L. monocytogenes* (Performed by Dr. Sirawutt Boonyarattanakalin):** HeLa or J-774 cells in 24-well plates (20% confluence) were treated with BHI broth containing *L. monocytogenes* (200 µL of a saturated culture diluted 20-fold in BHI broth). After 30 min of bacterial infection, the excess *L. monocytogenes* was washed away with DPBS (4 x 0.8 mL). The infected mammalian cells were treated with a pre-equilibrated solution (500 µL) of vancomycin (50 µM) and receptor (10 µM) in DMEM media with no other antibiotics. These treated cells were maintained in a humid 37 °C CO₂ incubator for 7 h to promote endocytic delivery of vancomycin by synthetic receptors. After treatment, the drug-complex solution was removed, and cells were washed with DMEM (500 µL) and DPBS (500 µL). The treated, infected mammalian cells were maintained in DMEM media with no antibiotics at 37 °C for 36 h before analysis by microscopy. Prior to flow cytometric analysis, the supernatant from each well was removed and saved. Cells were washed immediately with DPBS (500 µL), trypsinized, combined with the reserved well supernatant, and analyzed by flow cytometry for cell viability.

**Viability of Intracellular* L. monocytogenes* (Performed by Dr. Sirawutt Boonyarattanakalin):** HeLa or J-774 cells were subjected to identical conditions utilized for the rescue of HeLa and J-774 cells from lethal *L. monocytogenes* (described above). After the drug complex solution was removed by washing, the treated, infected mammalian cells were maintained in BHI broth in a 37 °C CO₂ incubator for 24 h. Growth of *L. monocytogenes* was analyzed by the absorbance at 590 nm.
Localization of Oregon Green Vancomycin in Non-Infected and \textit{L. monocytogenes} Infected J-774 cells (Performed by Dr. Sirawutt Boonyarattanakalin): J-774 cells were seeded on cover slides in a 6-well plate 24 h prior to the experiment (70% confluence). These cells were treated with a solution of receptor in DMEM media (0.5 mL, final concentrations of receptor = 10 µM, and DMSO = 1%) at 37 °C for 1 h, washed with DMEM media (2 x 1 mL) to remove unincorporated receptor and DMSO, and fresh media (300 µL) containing Oregon Green vancomycin (3.6 µM) was added. Cells were maintained at 37 °C for 2 h. Prior to bacterial infection, treated cells were washed with media (2 x 1 mL) and DPBS (2 x 1 mL). After washing, cells were treated with \textit{L. monocytogenes} in DMEM media (1 mL*) for 2 h and analyzed by confocal microscopy.

*A single colony of \textit{L. monocytogenes} was inoculated into 5 mL of BHI broth with streptomycin (200 µg/mL). The culture was incubated at 37 °C for 13 h with shaking (250 rpm). This \textit{L. monocytogenes} culture (optical density at 590 nm = 1.20) was centrifuged (4300 rpm) and the bacteria were re-suspended in DMEM media (5 mL).

Injection of Compounds into Mice (performed by Jianfang Hu and Dr. Avery August, Penn State University): Mice (Balb/c background) were divided into 4 groups. In group I, mice received a mixture of receptor (1 mg/mouse), Oregon Green vancomycin (1 mg/mouse), and (2-hydroxypropyl)-β-cyclodextrin (210 mg/mouse) in DPBS. Group II mice received a mixture of Oregon Green vancomycin (1 mg/mouse) and (2-hydroxypropyl)-β-cyclodextrin (210 mg/mouse) in DPBS. Group III mice received a solution of (2-hydroxypropyl)-β-cyclodextrin (210 mg/mouse) only in DPBS. Each mixture was injected into mice intraperitoneally (IP) with the final volume of 300 µL,
and mice were sacrificed 8 h after the injection. Tissues were harvested and single cell suspensions were prepared.

**Preparation of single cell suspensions** (preformed by Jianfang Hu and Dr. Avery August): Mice were sacrificed and the spleen, lymph nodes, thymus, liver, lungs, kidneys, pancreas, and heart were removed. Spleens, lymph nodes and thymus were homogenized by using a dounce homogenizer to generate a single cell suspension. The other tissues were minced using curved scissors and dissociated using collagenase (150 U/mL). All of the cell suspensions were forced through nylon mesh to obtain a clean suspension. Cells were centrifuged (4300 RPM) and re-suspended in PBS (6 mL).

**Additional Uptake Studies of Oregon Green and Pennsylvania Green Vancomycin:** Receptors in DMSO were diluted in RPMI media to their final working concentrations with DMSO concentration at 1% and added to Jurkat cells (5 x 10⁵). These receptor-treated cells were incubated at 37 °C for 1 h, pelleted (4000 RPM, 4 min) and washed with RPMI media (2 x 0.5 mL) to remove unincorporated receptors and DMSO. Fresh media (300 µL) containing Oregon Green vancomycin (3 µM) was added. Cells were typically maintained at 37 °C for 4 h to promote synthetic receptor-mediated endocytosis. Prior to analysis, treated cells were pelleted then washed with media (2 x 0.5 mL) and DPBS (2 x 0.5 mL). After washing, cells were resuspended in fresh media (0.5 mL) for analysis by confocal microscopy or flow cytometry.
3.7 References


11. Sun, Q.; Peterson, B.R. Unpublished data.


Chapter 4

Multivalent Synthetic Receptors

4.1 Introduction

Bacterial resistance to antibiotics is a global health issue. We are interested in studying whether new approaches for the delivery of vancomycin might be useful in combating this problem. Although infections caused by methicillin resistant Staphylococcus aureus (MRSA) can be treated with vancomycin, other Gram positive bacteria have developed resistance to this drug. Termed vancomycin-resistant Enterococci (VRE), these bacteria cause complicated urinary tract infections, blood infection, and death.\(^1\) The transmission of vancomycin resistance to S. aureus (VRSA) is of great concern.\(^2,3\) Resistance can be caused by alteration of the cell-wall binding sequence from L-Lys-D-Ala-D-Ala to L-Lys-D-Ala-D-Lac, leading to an approximate \(10^3\) loss of affinity for vancomycin.\(^4,5\) As a result, much effort has been devoted to developing new antibacterial agents against MRSA and VRE. Multiple approaches have been taken, including the development of novel antibiotics that act against targets other than cell wall biosynthesis\(^6\) and the modification of existing glycopeptide antibiotics.\(^7\) However, one of the most promising approaches uses multivalency to improve the affinity of vancomycin for its peptide ligand.
4.2 The Multivalency Approach

Polyvalent interactions involve the simultaneous binding of multiple ligands on one molecule or surface to multiple receptors on another molecule or surface. These types of interactions are common in biology and can be much stronger than the corresponding monovalent interaction. Important examples of multivalency have been reviewed, and include the multivalent binding of antibodies to antigens, transcription factor binding to DNA, and viral and bacterial adhesion to cell surfaces. Studies of multivalency may provide new drug design strategies.

Whitesides has used polyvalency in designing a high affinity vancomycin derivative. A trivalent vancomycin and ligand system was designed and synthesized (Figure 4.1) with a very high affinity ($K_d \sim 4 \times 10^{-17}$ M), about 25 times tighter than the avidin-biotin interaction, one of the strongest known interactions in biological systems. In comparison, the monomeric interaction of vancomycin with a D-Ala-D-Ala ligand

![Figure 4.1: Structures of the trivalent derivatives of vancomycin (A) and the peptide ligand D-Ala-D-Ala (B).](image-url)
exhibits a binding constant $K_d \sim 1 \times 10^{-6}$ M. This multivalency approach to vancomycin resistance has been pursued by numerous other researchers and include head-to-head, tail-to-tail, and head-to-tail dimeric vancomycins with different lengths and types of linkers, some of which have activity against VRE.

4.3 Design and Synthesis of Multivalent Synthetic Receptor Systems

We hypothesized that a dimeric synthetic receptor might bind a dimeric vancomycin and substantially increase affinity and delivery into mammalian cells through endocytosis. To test this hypothesis, we synthesized a series of dimeric artificial cell surface receptors 69 - 72 based our previously employed D-Phe-D-Ala vancomycin binding motif. In addition, two glutamic acids were installed in the linker region (Figure 4.2). We also synthesized dimeric fluorescent derivatives of vancomycin (73 -

![Diagram of vancomycin binding motif](image)

**Figure 4.2:** Structures of dimeric synthetic receptors for vancomycin and vancomycin dimers.
with varying linkers to determine how linker structures would impact solubility, receptor association, and uptake by endocytosis (Figure 4.3).

![Figure 4.3: Dimeric fluorescent vancomycin derivatives.](image)

### 4.3.1 Synthesis of Dimeric Receptors

Compounds 69 - 72 were synthesized using solution phase peptide synthesis starting with NH$_2$-D-Ala-OtBu (76) and Fmoc-D-Phe-OH. These two amino acids were coupled in chloroform using PyBOP and diisopropylethylamine to give the dipeptide 77. Subsequent Fmoc deprotection and PyBOP coupling with different amino acids provided the corresponding tri-peptides (78 - 81, Figure 4.4).
An aromatic core was synthesized from 5-amino-isophthalate 82 using a previously published synthesis in four steps. Acylation using chloroacetyl chloride afforded 83. Conversion of the chloride group to the amine 84 was effected with aqueous ammonia. The free amine was Fmoc protected with FmocCl and sodium bicarbonate to give the novel aromatic core 85 (Figure 4.5).

Compounds 78 – 81 were Fmoc deprotected and coupled to aromatic core 85 to afford the dimeric compounds 86 – 89. Another Fmoc deprotection, coupling of a solid-phase synthesized cholesterol derivative 93, and global deprotection with TFA gave the corresponding dimeric synthetic receptors 69 – 72 (Figure 4.6).
4.3.2 Synthesis of Dimeric Fluorescent Vancomycins

Dimeric vancomycin derivatives were synthesized by coupling 2 equivalents of vancomycin with a corresponding diamine using HBTU and DIEA in DMF to afford 90-92. These compounds were labeled with Oregon Green-NHS ester to give the final fluorescent dimeric vancomycin derivatives 73-75 (Figure 4.7). The xylene-derived dimeric vancomycin 73 was based on previously reported compound 90, which demonstrated a potent antibiotic effect against VRE.\textsuperscript{16} Compound 74 utilized a PEG-type linker to improve the solubility properties of the dimer. Compound 75 was synthesized as a direct analogue to 74 to compare the effects of a PEG-linker and carbon-linker with the same number of atoms (8 atoms). A related vancomycin dimer with a 6-carbon linker was reported to display potent antibiotic activity.\textsuperscript{16}
To determine if the dimeric nature of 73 – 75 had an impact on fluorescence intensity, emission curves were collected and compared with the monomeric 55. Stock solutions of compound in DMSO were diluted in PBS to a concentration of 10 nM and were normalized by UV/Vis spectroscopy such that the concentration of fluorophores was identical. As shown in Figure 4.8, the fluorescent emission of monomeric Oregon Green-Van 55 was twice that of the xylene-derived 73, suggesting that the dimer undergoes some homo-FRET quenching. The PEG-derived 74 and octyl-derived 75 also displayed a lower fluorescent intensity compared to 55. These results suggest that the more hydrophobic linkers of 73 and 75 experience “hydrophobic collapse” that causes
the fluorophores attached to vancomycin to move closer in proximity. PEG-derived 74 may have an increased fluorescence due to dihedral angles; the lone pairs on the oxygen atoms in the PEG linker may enforce the extended conformation more so than the hydrocarbon linker of 73 or 75.

Figure 4.8: Emission spectra of fluorescent vancomycin derivatives excited at 494 nm. The absorbance of compounds was normalized by UV/Vis spectroscopy prior to data collection. Compounds were diluted from concentrated DMSO stock solutions in PBS to a final concentration of 10 nM.
4.4 Biological Evaluation of the Dimeric Vancomycin Synthetic Receptor System

It was envisioned that dimeric synthetic receptors 69 - 72, when added to cells, would project the dimeric D-Ala-D-Phe binding motif outward from the cell surface. Upon addition of one of the fluorescent dimeric vancomycin compounds (73 - 75), the ligand and receptor would bind more tightly than the corresponding monomeric system explored in the previous chapter and the complex would undergo endocytosis.

4.4.1 Flow Cytometry Analysis of the Dimeric System

The dose-dependent effectiveness of delivery of 73 - 75 and monomeric OG-Van 55 into mammalian cells mediated by receptors 69 - 72 and 57 was examined quantitatively by flow cytometry. The receptors and ligands were pre-equilibrated for 1 h, and then added to human Jurkat lymphocytes for 4 h. As shown in Figure 4.9, the uptake of dimeric vancomycins 73 - 75 proved to be greater in comparison to the uptake of monomeric OG-Van 55 mediated by all of the receptors. Additionally, the uptake of monomeric OG-Van 55 mediated by monomeric receptor 57 proved to be less than the uptake of the dimeric derivatives, suggesting that the dimeric nature of the receptor and ligand substantially impacted affinity.
Cellular uptake of the three dimeric fluorescent vancomycin derivatives (73 - 75) mediated by the dimeric receptors (69 - 72) was very similar and occurred in a dose-dependent manner. However, a correlation was observed between the length of the linker in the receptor and the magnitude of uptake of a dimeric vancomycin derivative. In other words, the shorter the carbon chain is in the tri-peptide region of the receptor, the greater the impact was on cellular uptake. Receptor 69 demonstrated the greatest uptake of 73 - 75.

The cellular uptake of this system was affected by the solubility of the dimeric ligands. Dimeric xylene-linked vancomycin 73 had very poor solubility and precipitated.

![Cellular Uptake of Fluorescent Vancomycin Derivatives](image)

**Figure 4.9:** Flow cytometry data with Jurkat lymphocyte cells. Receptors (57, 69 - 72) were pre-equilibrated with vancomycin ligands (55, 73 - 75) at the indicated concentrations for 1 h at 23 °C. The complex was added to the cells for 4 h at 37 °C. After washing away excess compound the cells were analyzed by flow cytometry.
in media at all concentrations even though it demonstrated an increased uptake when compared to PEG-linked 74 and octyl-linked 75. Dimeric octyl-linked vancomycin 75 was slightly more soluble than the xylene derivative, but uptake by the cells was decreased. The addition of the PEG-linker in 74 greatly increased the solubility properties of the fluorescent dimeric vancomycin and retained cellular association activity.

4.4.2 Confocal Imaging of the Dimeric System

Confocal laser scanning microscopy of Jurkat lymphocytes was used to examine the delivery of the PEG-linked dimeric fluorescent vancomycin 74 by receptors 69 - 72. The receptors (10 μM) and dimeric vancomycin 74 (10 μM) were pre-equilibrated for 1 h before addition to Jurkat cells for 4 h. The cells were subsequently washed and imaged. Receptors 70 -72 displayed a “capping” effect on the cell surface (Panel B, C, and D, Figure 4.10). Receptor 69 (Panel A, Figure 4.10) exhibited the greatest cellular fluorescence and was observed as a bright ring around the cells. Because other PEG derivatives are known to inhibit endocytosis, it was hypothesized that the PEG-linker of dimeric vancomycin 74 might inhibit endocytosis.22,23
To test this hypothesis, receptor 69 was pre-equilibrated with dimeric PEG-linked vancomycin 74, dimeric octyl-linked vancomycin 75, or monomeric OG-Van 55 for 1 h. The complex was added to Jurkat cells for 4 h which were subsequently washed and imaged. The octyl-linked dimeric vancomycin (Panel B, Figure 4.11) and the monomeric OG-Van (Panel C, Figure 4.11) did not display the prominent fluorescent ring around the cell surface observed with the PEG-linked dimeric vancomycin (Panel A, Figure 4.11). The fluorescence was intracellular and concentrated mainly in endosomes in direct contrast to the “ring” seen with the PEG linker. These results indicated that the PEG linker of 74 exhibits a profound inhibition effect on endocytosis.

**Figure 4.10**: Confocal laser scanning and differential interference contrast (DIC) microscopy of living Jurkat lymphocytes. Prior to microscopy, receptors 69 - 72 (10 μM) and PEG-linked 74 (10 μM) were pre-equilibrated at 23 °C for 1 h prior to addition to cells at 37 °C for 4 h. Cells were subsequently washed and imaged. (A) Cells treated with 74 and receptor 69. (B) Cells treated with 74 and 70. (C) Cells treated with 74 and 71. (D) Cells treated with 74 and 72.
Figure 4.11: Confocal laser scanning and differential interference contrast (DIC) microscopy of living Jurkat lymphocytes. Prior to microscopy, receptor 69 (10 μM) and vancomycin derivatives (10 μM) were pre-equilibrated for 1 h at 23 °C, prior to addition to cells for 4 h at 37 °C. Cells were subsequently washed and imaged. (A) Cells treated with dimeric receptor 69 and PEG-linked 74. (B) Cells treated with 69 and octyl-linked 75. (C) Cells treated with 69 and monomeric OG-Van 55.
4.5 Inhibition of Endocytosis

Studies of endocytosis can facilitate elucidation of cellular uptake mechanisms of impermeable molecules. Tools to study endocytosis have been developed and include fluorescent labeling of ligands and internal organelles. Specific pharmacological and chemical compounds have been investigated and reviewed that inhibit different modes of internalization by either clathrin-coated pits or other mechanisms of endocytosis.

Early inhibitors of clathrin-mediated endocytosis include monodansylcadaverine (MDC), potassium depletion, and hypertonic sucrose. Other methods include the use of chlorpromazine, phenylarsine oxide, or cytosolic acidification. Some of these methods block not only clathrin-mediated endocytosis, but also uptake by macropinocytosis. Many of these methods have been utilized in efforts to distinguish between clathrin-dependent and lipid-raft-dependent pathways.

Most inhibitors of caveolae/lipid-raft mediated endocytosis target the cholesterol present that is abundant in these domains. The synthesis of cholesterol is inhibited by statins or cholesterol can be depleted from cell surfaces with methyl-β-cyclodextrin, which in turn has a general and inhibitory effect on both clathrin-dependent and lipid-raft mediated endocytosis. A more specific inhibition of lipid-raft/caveolae endocytosis uses polyene antibiotics such as filipin and nystatin, which create large disruptive cholesterol aggregates in the cell membrane.
The presence of a PEG-linker in compounds that associate with the plasma membrane can also impact endocytosis. Cholesterol derivatives of PEG inhibit clatherin-independent but not clatherin-dependent endocytosis in HT-1080 cells. In a different study, the uptake of fluorescent cholera toxin-B through caveolae was inhibited by a similar cholesterol-PEG compound in A431 cells. The clathrin-dependent uptake of transferrin was not affected until the cholesterol-PEG compound exceeded 20% of the plasma membrane surface area. It has been proposed that PEG influences fluidity and membrane dynamics critical to endocytosis.

We proposed that our fluorescent dimeric PEG-vancomycin (74) might inhibit endocytosis by binding to synthetic receptor through an unknown mechanism. To investigate this, we added a preequilibrated receptor and ligand complex to cells for 1 h to begin the endocytosis inhibition, washed away excess receptor, and then added fluorescent proteins known to be internalized by endocytosis. After 1 h, the cells were washed again, and imaged. To demonstrate that the PEG linker and not the synthetic receptor was affecting endocytosis, fluorescent dimeric vancomycin 75 with an 8-carbon linker was examined as a control.

### 4.5.1 Endocytosis Inhibition Demonstrated by Inhibition of Red Fluorescent Proteins

The mechanism of uptake of cholera toxin mediated by ganglioside GM1 was previously discussed in Chapter 1 and is thought to involve a variety of endocytic mechanisms. To examine the inhibition of uptake of this protein, synthetic receptor 69
(10 μM) was pre-equilibrated with dimeric vancomycin compounds 74 or 75 (10 μM) for 1 h at 23 °C. Jurkat cells and CHO cells were then treated with this complex for 1 h at 37 °C. Excess compound was washed away and the cells were treated with cholera toxin-594 (CT-594, 170 nM) for 1 h (Jurkat cells) or 5 min (CHO cells) at 37 °C. Figure 4.12 (Panels A and B) shows Jurkat and CHO cells treated with CT-594 alone. The protein demonstrated significant uptake in both cell lines and was primarily intracellular. Jurkat cells treated with receptor 69 and the fluorescent octyl-linked dimeric vancomycin 75 (Panel C, Figure 4.12) demonstrated that the red fluorescent CT-594 and the green fluorescent vancomycin were both endocytosed, as evidenced by the internal red-green co-localization. However, when Jurkat cells were treated with the PEG-linked 74 and receptor 69, a red-ring and a green-ring were both co-localized on the plasma membrane of the cells (Panel E, Figure 4.12). Only a very small amount of protein and compound was internalized, indicating that these compounds have an inhibitory effect on the endocytosis of CT. The effect in CHO cells was not as profound and was observed to be less differential with respect to the octyl linked 75 (Panel D, Figure 4.12) and the PEG-linked 74 (Panel F, Figure 4.12). The CT-594 and compounds were primarily endosomal and indicated that the endocytic uptake of cholera toxin is more pronounced in Jurkat cells.
A protein that is exclusively internalized by clathrin-dependent endocytosis is transferrin. To investigate if our system would specifically inhibit clathrin-dependent uptake of transferrin, synthetic receptor 69 (10 μM) was pre-equilibrated with dimeric vancomycin compounds 74 or 75 (10 μM) for 1 h at 23 °C. Jurkat cells and CHO cells were then treated with this complex at 37 °C for 1 h. After washing away excess compound, the cells were treated with red-fluorescent Texas Red Transferrin (TR-Trans,

**Figure 4.12:** Confocal laser scanning and differential interference contrast (DIC) microscopy of living Jurkat lymphocytes and Chinese hamster ovary cells. Prior to microscopy, receptor 69 (10 μM) and vancomycin compounds 74 or 75 (10 μM) were pre-equilibrated at 23 °C for 1 h, then added to cells at 37 °C for 1 h. Excess compound was washed away, and the cells were incubated with cholera toxin-594 (170 nM) at 37 °C for 1 h (Jurkat cells) or 5 min (CHO cells), then washed and imaged. A and B: Cells treated with no compound followed by cholera toxin-594. C and D: Cells treated with 69 and octyl-linked 75 followed by cholera toxin-594. E and F: Cells treated with 69 and PEG-linked 74 followed by cholera toxin-594.
500 nM) for 1 h (Jurkat cells) or 5 min (CHO cells) at 37 °C. Cells treated with TR-Trans alone showed that the protein had significant uptake in both cell lines (Panel A and B, Figure 4.13) and was located mainly in endosomes. Cells treated with receptor 69 and the octyl-linked 75 (Panel C and D, Figure 4.13) demonstrated that the red fluorescent TR-Trans and the green fluorescent vancomycin were both endocytosed, as evidenced by the red-green co-localization inside both cell lines. When Jurkat cells were treated with the PEG-linked 74 and receptor 69 followed by TR-Trans, a bright red fluorescent ring and a green fluorescent ring were exclusively colocalized on the plasma membrane of these cells (Panel E, Figure 4.13). There was no endocytosis of TR-Trans in the presence of the PEG-linked vancomycin 74 and receptor 69. The inhibitory effect of the PEG-linked vancomycin in CHO cells was more profound (Panel F, Figure 4.13) when compared to the same experiments with CT-594 and Dil-LDL. There was a greater amount of co-localization on the cell surface even though the protein and compounds were mostly endosomal.
The mechanism of uptake of LDL by its receptor was previously discussed in Chapter 1 and also involves clathrin-mediated endocytosis. To investigate if our system would also inhibit clathrin-dependent uptake of Dil-LDL, synthetic receptor 69 (10 μM) was pre-equilibrated with dimeric vancomycin compounds 74 or 75 (10 μM) for 1 h at 23 °C. Jurkat cells and CHO cells were treated with this complex at 37 °C for 1 h. After excess compound was washed away, the cells were treated with red-fluorescent Dil-LDL.

Figure 4.13: Confocal laser scanning and differential interference contrast (DIC) microscopy of living Jurkat lymphocytes and Chinese hamster ovary cells. Prior to microscopy, receptor 69 (10 μM) and vancomycin compounds 74 or 75 (10 μM) were pre-equilibrated at 23 °C for 1 h, then added to cells at 37 °C for 1 h. Excess compound was washed away, and the cells were incubated with Texas Red-Transferrin (500 nM) at 37 °C for 1 h (Jurkat cells) or 5 min (CHO cells), then washed and imaged. A and B: Cells treated with no compound followed by Texas Red-Transferrin. C and D: Cells treated with 69 and octyl-linked 75 followed by Texas Red-Transferrin. E and F: Cells treated with 69 and PEG-linked 74 followed by Texas Red-Transferrin.
(20 nM) for 1 h (Jurkat cells) or 5 min (CHO cells) at 37 °C. In cells treated with DiI LDL alone, the protein was taken up by both cell lines and was located mainly in endosomes (Panel A and B, Figure 4.14). Cells treated with receptor 69 and the fluorescent octyl-linked dimeric vancomycin 75 (Panel C and D, Figure 4.14) demonstrated that the red fluorescent DiI-LDL and the green fluorescent vancomycin were both endocytosed, evidenced by the red-green co-localization inside both cell lines. Jurkat cells treated with the PEG-linked 74 and receptor 69 showed both a faint red fluorescent ring indicating DiI-LDL and a green-ring were colocalized on the plasma membrane of these cells (Panel E, Figure 4.14). The uptake of DiI-LDL was greater than the uptake of CT-594 in the presence of the PEG-linked vancomycin and receptor complex, indicating a partial inhibition of clathrin-mediated endocytosis. The inhibitory effect of the PEG-linked vancomycin 74 with DiI-LDL in CHO cells was not as profound (Panel F, Figure 4.14), similar to the CT-594 results. There was a small amount of co-localization on the cell surface, but the protein and compounds were primarily endosomal.

The apparent lack of endocytosis inhibition of DiI-LDL by receptor 69 and PEG-linked 74 in Jurkat cells requires further study because the endocytosis of Texas Red Transferrin that also utilizes clathrin-mediated uptake was completely inhibited under the same conditions. We postulated that because of their similar mechanisms of cellular uptake, the uptake of LDL was being inhibited similar to the inhibition of Transferrin uptake in the presence of our dimeric receptor system. However, in contrast to the covalently attached Texas Red dye in Transferrin, the DiI dye is not covalently attached to the LDL particle. It is possible that the DiI dye is leaching out of the LDL liposome
and entering into the cells by diffusion, leading to the observed results. Further experiments would be needed to confirm this hypothesis.

**Figure 4.14:** Confocal laser scanning and differential interference contrast (DIC) microscopy of living Jurkat lymphocytes and Chinese hamster ovary cells. Prior to microscopy, receptor $69$ (10 μM) and vancomycin compounds $74$ or $75$ (10 μM) were pre-equilibrated at 23 °C for 1 h, then added to cells at 37 °C for 1 h. Excess compound was washed away, and the cells were incubated with Dil-LDL (20 nM) at 37 °C for 1 h (Jurkat cells) or 5 min (CHO cells), then washed and imaged. A and B: Cells treated with no compound followed by Dil-LDL. C and D: Cells treated with $69$ and octyl-linked $75$ followed by Dil-LDL. E and F: Cells treated with $69$ and PEG-linked $74$ followed by Dil-LDL.
4.6 Conclusions

We designed and synthesized a series of dimeric vancomycins and receptors aimed to improve the potency of the monomeric synthetic receptor system. Receptor 69 was identified as an efficient receptor for the delivery of three dimeric vancomycin derivatives 73 - 75. Upon further investigation, it was discovered that PEG-linked dimeric vancomycin 74 bound to receptor 69 was a potent inhibitor of both clathrin-mediated and lipid-raft-mediated endocytosis of red fluorescent proteins. An octyl-linked dimeric vancomycin derivative 75 did not demonstrate endocytic inhibition, suggesting that the PEG linker is involved in the mechanism. Further studies are needed to determine the manner and extent of inhibition.

4.7 Experimental Section

4.7.1 General

Chemical reagents were obtained from Acros, Aldrich, Alfa Aesar, NovaBioChem, or TCI America. Solvents were from EM Science. Media and antibiotics were purchased from Mediatech. Commercial grade reagents were used without further purification unless otherwise noted. Anhydrous solvents were obtained after passage
through a drying column of a solvent purification system from Glass Contour (Laguna Beach, CA). All reactions were performed under an atmosphere of dry argon or nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254 (EM Science). TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of Cerium Molybdenate (Hanessian’s stain). Flash column chromatography employed ICN SiliTech Silica Gel (32-63 µm). Purification by preparative reverse phase HPLC employed an Agilent 1100 preparative pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrenedivinylbenzene) reverse phase column (7 µm particle size, 21.5 mm x 25 cm). The HPLC flow rate was 20 mL/min throughout the run. Melting points were measured with a Thomas Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR or a Thermo Scientific Nicolet 380 FT-IR Spectrometer equipped with either a Smart Diffuse Reflectance Accessory or a Smart Orbit Diamond ATR accessory. NMR spectra were obtained with Bruker CDPX-300, DPX-300, AMX-360, or DRX-400, instruments with chemical shifts reported in parts per million (ppm, δ) referenced to either CDCl3 (1H 7.27 ppm; 13C 77.23 ppm), DMSO-d6 (1H, 2.50 ppm; 13C, 39.51 ppm) or methanol-d4 (1H, 3.35, 4.78 ppm; 13C, 49.3 ppm). High-resolution mass spectra were obtained from either the Pennsylvania State University Mass Spectrometry Facility or the University of Texas Mass Spectrometry Facility at Austin (ESI and CI). Peaks are reported as m/z.
4.7.2 Synthetic procedures and compound characterization data

\[
N-[5-(3\beta)(cholest-5-en-3-ylamino)pentanoyl]-\beta\text{-}\text{alanyl}\text{-}\alpha\text{-}\text{glutamyl}\text{-}\alpha\text{-}\text{glutamyl}\text{-}\beta\text{-}\text{alanyl}\text{-}N\text{-}[3,5\text{-}\text{bis}((3\text{-}\{1\text{-}\text{benzyl}-2\text{-}[(1\text{-}\text{carboxyethyl})\text{amino}]\text{-}2\text{-}\text{oxoethyl})\text{amino}\text{-}3\text{-}\text{oxopropyl})\text{amino})\text{carbonyl})\text{phenyl}]\text{glycinamide}
\]

Compound \(86\) (32 mg, 0.028 mmol) was dissolved in DMF containing piperidine (20\%, 0.5 mL) and stirred at 23 °C for 20 min. Solvent was removed \textit{in vacuo} and the remaining residue was re-dissolved in CHCl\(_3\) (5 mL). PyBOP (16 mg, 0.031 mmol), diisopropylethylamine (0.02 mL, 0.07 mmol), and cholesterol derivative \(93\) (30 mg, 0.028 mmol) were added and the reaction was stirred at 23 °C for 12 h. The solvent was removed \textit{in vacuo} and the residue was dissolved in trifluoroacetic acid, deionized water and triisopropyl silane (2 mL, 95:5:5) for global deprotection. After stirring at 23 °C for 5 h, the solution was directly purified by preparative reverse-phase HPLC (gradient 9.9\% MeCN, 90\% deionized water, 0.1\% TFA to 99.9\% MeCN, 10\% deionized water, 0.1\% TFA over 20 min; retention time = 16.9 min) afforded \(69\) (21 mg, 44\%) as a white solid, mp 176 to 180 °C; \(^1\)H NMR (400 MHz, d\(_6\)-DMSO) \(\delta\) 12.33 (s, 4H), 10.2 (s, 1H), 8.39-7.87 (m, 15H), 7.13 (m, 10H), 5.38 (s, 1H), 4.55 (m, 2H), 4.20 (m, 4H), 3.89 (m, 2H), 3.45-0.89 (m, 83H), 0.69 (s, 3H); HRMS (ESI) m/z 1683.9164 (M\(^+\), C\(_{88}H_{125}N_{13}O_{20}\) requires 1683.9159).
Analytical HPLC profile of the HPLC purified 3-carbon dimeric receptor 69 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 16.9 min).

\[ \text{N-}[5-(3\beta)(\text{cholest-5-en-3-ylamino})\text{pentanoyl}]-\beta\text{-alanyl-}\alpha\text{-glutamyl-}\alpha\text{-glutamyl-}\beta\text{-alanyl-N-[3,5-bis(\{}4\text{-}(1\text{-benzyl-2-[}(1\text{-carboxyethyl)}\text{amino})\text{-2-oxoethyl)}\text{amino})-4-oxobutyl\text{amino}\text{carbonyl}\text{phenyl}]}\text{glycinamide} \]

Compound 87 (121 mg, 0.103 mmol) was dissolved in DMF containing piperidine (20%, 0.5 mL) and stirred at 23 °C for 20 min. Solvent was removed \textit{in vacuo} and the remaining residue was re-dissolved in CHCl₃ (10 mL). PyBOP (60 mg, 0.113 mmol), diisopropylethylamine (0.05 mL, 0.26 mmol), and cholesterol derivative 93 (111 mg, 0.103 mmol) were added and the reaction was stirred at 23 °C for 12 h. The solvent was removed \textit{in vacuo} and the residue was
dissolved in trifluoroacetic acid, deionized water and triisopropyl silane (2 mL, 95:5:5) for global deprotection. After stirring at 23 °C for 5 h, the solution was directly purified by preparative reverse-phase HPLC (gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 16.9 min) afforded 70 (52 mg, 29%) as a white solid; mp 174 to 178 °C; ¹H NMR (400 MHz, d₆-DMSO) δ 12.31 (s, 4H), 10.23 (s, 1H), 8.47-7.88 (m, 15H), 7.14 (m, 10H), 5.39 (s, 1H), 4.53 (m, 2H), 4.14 (m, 6H), 3.89 (m, 2H), 3.44-0.85 (m, 85H), 0.67 (s, 3H); HRMS (ESI) m/z 1712.9561 (MH⁺, C₉₀H₁₃₀N₁₃O₂₀ requires 1712.9555).

Analytical HPLC profile of the HPLC purified 4-carbon dimeric receptor 70 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 16.9 min).
**N-[5-(3β)(cholest-5-en-3-ylamino)pentanoyl]-β-alanyl-α-glutamyl-α-glutamyl-β-alanyl-N-[3,5-bis({[5-({1-benzyl-2-[(1-carboxyethyl)amino]-2-oxoethyl}amino)-5-oxopentyl]amino}carbonyl)phenyl]glycinamide.** Compound 88 (124 mg, 0.103 mmol) was dissolved in DMF containing piperidine (20%, 0.5 mL) and stirred at 23 °C for 20 min. Solvent was removed *in vacuo* and the remaining residue was re-dissolved in CHCl₃ (10 mL). PyBOP (60 mg, 0.113 mmol), diisopropylethylamine (0.05 mL, 0.26 mmol), and cholesterol derivative 93 (111 mg, 0.103 mmol) were added and the reaction was stirred at 23 °C for 12 h. The solvent was removed *in vacuo* and the residue was dissolved in trifluoroacetic acid, deionized water and triisopropyl silane (2 mL, 95:5:5) for global deprotection. After stirring at 23 °C for 5 h, the solution was directly purified by preparative reverse-phase HPLC (gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 16.9 min) afforded 71 (41 mg, 23%) as a white solid, mp 184 to 188 °C. \(^1\)H NMR (400 MHz, d₆-DMSO) δ 12.26 (s, 4H), 10.15 (s, 1H), 8.41-7.78 (m, 15H), 7.04 (m, 10H), 5.31 (s, 1H), 4.47 (m, 2H), 4.11 (m, 8H), 3.81 (m, 2H), 3.39-0.81 (m, 87H), 0.68 (s, 3H); HRMS (ESI) \(m/z\) 1739.9790 (MH⁺, \(C_{92}H_{133}N_{13}O_{20}\) requires 1739.9781).
Analytical HPLC profile of the HPLC purified 5-carbon dimeric receptor 71 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90 % deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 16.8 min).

\[ N-[5-(3\beta)(cholest-5-en-3-ylamino)pentanoyl]-\beta\text{-alanyl-}\alpha\text{-glutamyl-}\alpha\text{-glutamyl-}\beta\text{-alanyl-}N-[3,5\text{-bis(}[6-(\{1\text{-benzyl-2-}[\text{1-carboxyethyl}amino]-2\text{-oxoethyl}amino]-6\text{-oxohexyl}amino}\text{carbonyl}phenyl]\text{glycinamide.} \]

Compound 89 (25 mg, 0.020 mmol) was dissolved in DMF containing piperidine (20%, 0.5 mL) and stirred at 23 °C for 20 min. Solvent was removed \textit{in vacuo} and the remaining residue was re-dissolved in CHCl₃ (5 mL). PyBOP (11 mg, 0.022 mmol), diisopropylethylamine (0.01 mL, 0.05 mmol), and cholesterol derivative 93 (25 mg, 0.022 mmol) were added and the reaction was stirred at 23 °C for 12 h. The solvent was removed \textit{in vacuo} and the residue was dissolved in trifluoroacetic acid, deionized water and triisopropyl silane (2 mL, 95:5:5)
for global deprotection. After stirring at 23 °C for 5 h, the solution was directly purified by preparative reverse-phase HPLC (gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 16.9 min) afforded 72 (19 mg, 56%) as a white solid; mp 143 to 146 °C; \(^1\)H NMR (400 MHz, d6-DMSO) \(\delta\) 12.29 (s, 4H), 10.25 (s, 1H), 8.43-7.79 (m, 15H), 7.19 (m, 10H), 5.33 (s, 1H), 4.46 (m, 2H), 4.15 (m, 10H), 3.80 (m, 2H), 3.39-0.81 (m, 89H), 0.71 (s, 3H), HRMS (ESI) \(m/z\) 1769.0161 (\(M^+\), \(C_{94}H_{138}N_{13}O_{20}\) requires 1769.0181).

[Graphical representation of analytical HPLC profile]

Analytical HPLC profile of the HPLC purified 6-carbon dimeric receptor 72 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 16.9 min).
Dimeric-Oregon Green-Xylene Vancomycin or Bis-[40-([4-(aminomethyl)benzyl]amino)carbonyl]- (1S,2R,18R,19R,22S,25R,28R)-22-(2-amino-2-oxoethyl)-5,15-dichloro-2,18,32,35,37-pentahydroxy-19-[(N-methyl-D-leucyl)amino]-20,23,26,42,44-pentaaxo-7,13-dioxo-21,24,27,41,43-pentaazaoctacyclo[26.14.2.23,6.214,17.18,12.129,33.010,25.034,39]pentaconta-3,5,8(48),9,11,14,16,29(45),30,32,34,36,38,46,49-pentadecaen-48-yl-2-O-(3-{3-carboxy-4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoyl]amino}-2,3,6-trideoxy-3-methylhexopyranosyl)hexopyranoside]. Compound 90 (15 mg, 0.005 mmol) was dissolved in DMF (1.8 mL). Oregon Green-NHS ester (5 mg, 0.0098 mmol) and triethylamine (5 μL, 0.025 mmol) were added, and the reaction was stirred at 23 °C for 24 h. The reaction mixture was directly purified by preparative reverse-phase HPLC (9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 1% deionized water, 0.1% TFA over 20 min, RT = 9.6 min) afforded 73 (14 mg, 78%) as an orange solid, mp = decomposition over 220 °C; HRMS (ESI) m/z 1893.6160 ([M-2H]²⁻, C₁₈₂H₁₇₂Cl₄F₄N₂₀O₅₈²⁻ requires 1893.6169).
Analytical HPLC profile of the HPLC purified fluorescent dimeric xylene vancomycin 73 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 9.6 min).

3,5,8(48),9,11,14,16,29(45),30,32,34,36,38,46,49-pentadecaen-48-yl-2-O-(3-[[3-carboxy-4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoyl]amino]-2,3,6-trideoxy-3-methylhexopyranosyl)hexopyranoside]. Compound 91 (15 mg, 0.005 mmol) was dissolved in DMSO and DMF (1:1, 1.8 mL). Oregon Green-NHS ester (5 mg, 0.0098 mmol) and triethylamine (5 μL, 0.025 mmol) were added, and the reaction was stirred at 23 °C for 24 h. The reaction mixture was directly purified by preparative reverse-phase HPLC (9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 1% deionized water, 0.1% TFA over 20 min, RT = 9.3 min) afforded 74 (10 mg, 55%) as an orange solid, mp = decomposition over 220 °C; HRMS (ESI) m/z 1899.6219 ([M-2H]^{2-}, C_{180}H_{176}Cl_{4}F_{4}N_{2}O_{60}^{2-} requires 1899.6215).

Analytical HPLC profile of the HPLC purified fluorescent dimeric PEG vancomycin 74 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 9.3 min).
Dimeric-Oregon Green Octyl Vancomycin or Bis-[40-\{(aminooctyl)amino\}carbonyl]-1(S,2R,18R,19R,22S,25R,28R)-22-(2-amino-2-oxoethyl)-5,15-dichloro-2,18,32,35,37-pentahydroxy-19-[\((N\text{-methyl-D-leucyl)}amino\]-20,23,26,42,44-pentaaoxo-7,13-dioxo-21,24,27,41,43-pentaazaoctacyclo[26.14.2.23.6.214.17.18.12.19.33.010.25.034.39]pentaconta-3,5,8(48),9,11,14,16,29(45),30,32,34,36,38,46,49-pentadecaen-48-yl-2-O-(3-\{[3-carboxy-4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoyl\}amino\]-2,3,6-trideoxy-3-methylhexopyranosyl)hexopyranoside. Compound 92 (15 mg, 0.005 mmol) was dissolved in DMSO and DMF (1:1, 1.8 mL). Oregon Green-NHS ester (5 mg, 0.0098 mmol) and triethylamine (5 μL, 0.025 mmol) were added, and the reaction was stirred at 23 °C for 24 h. The reaction mixture was directly purified by preparative reverse-phase HPLC (9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 1% deionized water, 0.1% TFA over 20 min, RT = 9.1 min) afforded 75 (8 mg, 44%) as an orange solid, mp = decomposition over 220 °C; HRMS (ESI) m/z 1897.6494 ([M-2H]^{2-}, C_{182}H_{180}Cl_{4}F_{4}N_{20}O_{58}^{2-} requires 1897.6487).
Analytical HPLC profile of the HPLC purified fluorescent dimeric octyl vancomycin 75 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 9.1 min).

tert-butyl N-[(9H-fluoren-9-ylmethoxy)carbonyl]-D-phenylalanyl-D-alaninate. D-Ala-OtBu (182 mg, 1.0 mmol) and Fmoc-D-Phe-OH (426 mg, 1.1 mmol) were dissolved in dry CHCl₃ (50 ml). PyBOP (512 mg, 1.1 mmol) and diisopropylethylamine (0.5 mL, 2.5 mmol) were added and the reaction was stirred at 23 °C for 1 h. After pouring into deionized water (50 mL) the organic layer was washed with aqueous saturated NaCl (2 x 50 ml) and dried over anhydrous sodium sulfate. After concentrating, flash chromatography (50% ethyl acetate in hexane) afforded the 77 (496 mg, 96%) as a white solid, mp 112 - 116 °C; ¹H NMR (300 MHz, CDCl₃) δ 5.54 (d, J = 7.5 Hz, 2H), 7.34 (m,
2H), 7.17 (m, 3H), 7.04 (m, 7H), 6.11 (d, 8.4 Hz, 1H), 4.56 (m, 1H), 4.29 (t, J = 7.3 Hz, 1H), 4.21 (t, J = 8.1 Hz, 1H), 3.96 (m, 3H), 2.97 (m, 2H), 1.28 (s, 9H), 1.13 (d, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, CDCl$_3$) 172.3, 171.7, 156.7, 144.4, 144.3, 141.7, 137.2, 129.9, 128.9, 128.1, 127.5, 127.3, 125.7, 125.6, 120.4, 82.2, 67.6, 56.5, 49.2, 47.5, 39.3, 28.4, 18.7; IR (KBr) $\nu_{\text{max}}$ 3305, 2976, 1726, 1692, 1652, 1530; HRMS (ESI) $m/z$ 515.2547 (MH$^+$, C$_{31}$H$_{35}$N$_2$O$_5$ requires 515.2546).

**tert-butyl-N-[(9H-fluoren-9-ylmethoxy)carbonyl]-β-alanyl-D-phenylalanyl-D-alaninate.** Dipeptide 77 (200 mg, 0.37 mmol) was Fmoc-deprotected in DMF containing piperidine (20%, 0.5 mL) for 30 min, and the solvent was removed *in vacuo*. The resulting residue was redissolved in CHCl$_3$ and Fmoc-β-Ala-OH (115 mg, 0.37 mmol), PyBOP (211 mg, 0.41 mmol), and diisopropylethylamine (0.16 ml, 0.92 mmol) were added. After stirring for 2 h, the solvent was removed. Flash chromatography (5% methanol in CH$_2$Cl$_2$) afforded the tri-peptide 78 (193 mg, 89%) as a white solid, mp 164 - 166 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.77 (d, J = 7.2 Hz, 2H), 7.59 (d, J = 7.2 Hz, 2H), 7.39 (t, J = 7.2 Hz, 2H), 7.30 (m, 8H), 5.81 (s, 1H), 4.69 (m, 1H), 4.36 (m, 3H), 4.19 (m, 1H), 3.37 (m, 3H), 3.11 (m, 4H), 2.37 (s, 1H), 1.93 (s, 2H), 1.45 (m, 12H); $^{13}$C NMR (75 MHz, CDCl$_3$) 171.6, 170.6, 156.6, 143.7, 141.1, 136.3, 129.0, 128.3, 127.5, 126.8, 126.7, 124.9, 119.7, 81.9, 66.5, 54.1, 48.8, 47.0, 38.1, 35.7, 27.7, 25.9, 17.8; IR (ATR) $\nu_{\text{max}}$
tert-butyl-N-(4-[(9H-fluoren-9-ylmethoxy)carbonyl]amino)butanoyl)-D-phenylalanyl-D-alaninate. Dipeptide 77 (190 mg, 0.37 mmol) was Fmoc-deprotected in DMF containing piperidine (20%, 0.5 mL) for 30 min, and the solvent was removed \textit{in vacuo}. The resulting residue was redissolved in CHCl$_3$ and Fmoc-ABa-OH (121 mg, 0.37 mmol), PyBOP (211 mg, 0.41 mmol), and diisopropylethylamine (0.16 mL, 0.92 mmol) were added. After stirring for 2 h, the solvent was removed. Flash chromatography (5% methanol in CH$_2$Cl$_2$) afforded the tri-peptide 79 (213 mg, 96%) as a white solid, mp 156 - 158 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.68 (d, $J = 7.5$ Hz, 2H), 7.43 (d, $J = 7.5$ Hz, 2H), 7.24 (m, 9H), 6.76 (s, 1H), 6.61 (s, 1H), 5.17 (m, 1H), 4.61 (q, $J = 6.6$ Hz, 1H), 4.26 (m, 3H), 4.11 (t, $J = 6.9$ Hz, 1H), 3.59 (m, 2H), 3.06 (m, 4H), 2.09 (t, $J = 6.6$ Hz, 2H), 1.65 (m, 2H), 1.35 (m, 12H); $^{13}$C NMR (75 MHz, CDCl$_3$) 173.2, 172.0, 171.1, 157.3, 144.3, 141.7, 137.0, 129.7, 128.9, 128.1, 127.5, 127.4, 125.5, 120.4, 82.3, 67.0, 54.9, 49.2, 47.6, 40.3, 38.5, 33.5, 28.3, 26.4, 13.1; IR (ATR) $\nu_{\text{max}}$ 3314, 2972, 1693, 1637, 1534, 1261; HRMS (ESI) $m/z$ 600.3040 (MH$^+$, C$_{35}$H$_{42}$N$_3$O$_6$ requires 600.3074).
tert-butyl-N-(5-[[9H-fluoren-9-ylmethoxy]carbonyl]amino)pentanoyl)-D-phenylalanyl-D-alaninate. Dipeptide 77 (232 mg, 0.45 mmol) was Fmoc-deprotected in DMF containing piperidine (20%, 0.5 mL) for 30 min, and the solvent was removed \textit{in vacuo}. The resulting residue was redissolved in CHCl$_3$ and Fmoc-AVa-OH (153 mg, 0.45 mmol), PyBOP (257 mg, 0.49 mmol), and diisopropylethylamine (0.19 ml, 1.12 mmol) were added. After stirring for 2 h, the solvent was removed. Flash chromatography (5% methanol in CH$_2$Cl$_2$) afforded the tri-peptide 80 (255 mg, 92%) as a white solid, mp 149 - 150 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.65 (d, $J = 7.5$ Hz, 2H), 7.49 (d, $J = 7.5$ Hz, 2H), 7.21 (m, 9H), 7.11 (s, 1H), 6.93 (s, 1H), 4.65 (m, 1H), 6.70 (s, 1H), 4.24 (m, 3H), 4.10 (m, 1H), 3.54 (m, 2H), 3.03 (m, 4H), 2.07 (m, 2H), 1.34 (m, 14H); $^{13}$C NMR (75 MHz, CDCl$_3$) 173.1, 171.5, 170.8, 156.5, 143.8, 141.1, 136.6, 129.2, 128.3, 127.5, 126.9, 126.6, 124.9, 119.8, 81.7, 66.4, 54.3, 48.7, 47.0, 40.3, 38.0, 35.5, 28.8, 27.7, 22.5, 12.5; IR (ATR) $\nu_{\text{max}}$ 3292, 2934, 1732, 1648, 1637, 1534; HRMS (ESI) $m/z$ 614.3235 (MH$^+$, C$_{36}$H$_{44}$N$_3$O$_6$ requires 614.3230).
tert-butyl-N-(6-[(9H-fluoren-9-ylmethoxy)carbonyl]amino)hexanoyl)-D-phenylalanyl-D-alaninate. Dipeptide 77 (200 mg, 0.388 mmol) was Fmoc-deprotected in DMF containing piperidine (20%, 0.5 mL) for 30 min, and the solvent was removed in vacuo. The resulting residue was redissolved in CHCl₃ and Fmoc-εAhx-OH (175 mg, 0.388 mmol), PyBOP (225 mg, 0.427 mmol), and diisopropylethylamine (0.2 ml, 0.97 mmol) were added. After stirring for 2 h, the solvent was removed. Flash chromatography (5% methanol in CH₂Cl₂) afforded the tri-peptide 81 (218 mg, 90%) as a white solid mp 118 - 120 °C; ¹H NMR (300 MHz, CDCl₃) δ 7.66 (d, J = 7.2 Hz, 2H), 7.50 (d, J = 7.2 Hz, 2H), 7.29 (t, J = 7.2 Hz, 2H), 7.15 (m, 7H), 6.69 (s, 1H), 6.40 (s, 1H), 4.94 (m, 1H), 4.67 (q, J = 7 Hz, 1H), 4.27 (m, 3H), 4.10 (m, 1H), 2.97 (m, 4H), 2.05 (t, J = 6.9 Hz, 2H), 1.35 (m, 18H); ¹³C NMR (75 MHz, CDCl₃) 173.2, 172.0, 171.0, 156.8, 144.4, 141.7, 136.9, 129.8, 129.6, 129.1, 128.7, 128.2, 127.9, 127.5, 127.3, 125.5, 120.3, 82.3, 66.8, 54.5, 49.2, 41.1, 38.8, 36.5, 29.9, 28.3, 28.2, 26.5, 25.4, 18.7; IR (ATR) vₘₐₓ 3304, 2933, 1732, 1684, 1635, 1534, 1257; HRMS (ESI) m/z 628.3392 (MH⁺, C₃₇H₄₆N₃O₆ requires 628.3387).
5-(N-{(9H-fluoren-9-ylmethoxy)carbonyl}glycyl)amino)isophthalic acid. 5-(2-aminoacetamido)isophthalic acid (600 mg, 2.52 mmol, prepared as described in Org. Lett., 2005, 7, 1761-1764) and NaHCO$_3$ (423 mg, 5.04 mmol) were dissolved in deionized water (20 mL) and cooled to 4 °C. Fmoc-Cl (979 mg, 3.78 mmol) was added as a solution in dioxanes (5 mL). The reaction was stirred at 4 °C for 1 h, then 23 °C for 18 h. Aqueous NaHCO$_3$ (10 mL, 10%) was added, and the reaction was extracted with diethyl ether (2 x 50 mL). The aqueous layer was acidified with concentrated HCl forming a white precipitate that was filtered, washed, and freeze-dried to afford 85 (956 mg, 82%) as a white solid, mp 298 - 301 °C; $^1$H NMR (300 MHz, d$_6$DMSO) $\delta$ 13.27 (s, 2H), 10.37 (s, 1H), 8.44 (s, 2H), 8.15 (s, 1H), 7.88 (d, J = 7.2 Hz, 2H), 7.72 (d, J = 7.2 Hz, 2H), 7.38 (m, 4H), 4.26 (m, 3H), 3.82 (s, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) 168.9, 166.8, 156.9, 144.2, 141.1, 139.8, 132.1, 127.9, 127.4, 125.6, 124.9, 123.8, 120.4, 66.1, 46.9, 44.4; IR (ATR) $\nu_{\text{max}}$ 3200, 1685, 1538, 1251; HRMS (ESI) $m/z$ 461.1349 (MH$^+$, C$_{25}$H$_{21}$N$_2$O$_7$ requires 461.1349).
di-tert-butyl 2,2'-{5-[(9H-fluoren-9-ylmethoxy)carbonyl]amino}acetyl)amino]-1,3-phenylene}bis[carbonylimino(1-oxopropane-3,1-diyl)imino(1-oxo-3-phenylpropane-2,1-diyl)imino]}dipropanoate. Tripeptide 78 (120 mg, 0.21 mmol) was Fmoc-deprotected in DMF containing piperidine (20%, 0.5 mL) for 30 min, and the solvent was removed in vacuo. The resulting residue was redissolved in CHCl₃ and isophthalate 85 (50 mg, 0.10 mmol), PyBOP (112 mg, 0.21 mmol), and diisopropylethylamine (0.09 mL, 0.49 mmol) were added. After stirring for 18 h, the solvent was removed. Flash chromatography (5% methanol in CH₂Cl₂) afforded 86 (92 mg, 82%) as a white solid, mp 106 - 109 °C; ¹H NMR (300 MHz, d₆DMSO) δ 10.1 (s, 1H), 8.27 (m, 4H), 8.01 (m, 4H), 7.77 (m, 3H), 7.60 (m, 3H), 7.12 (m, 14H), 4.42 (m, 2H), 4.10 (m, 5H), 3.66 (s, 2H), 3.19 (m, 4H), 2.90 (m, 2H), 2.61 (m, 2H), 2.18 (m, 4H), 1.24 (s, 18H), 1.13 (d, J = 7.2 Hz, 6H); ¹³C NMR (75 MHz, d₆DMSO) 172.0, 171.6, 170.4, 168.6, 166.1, 144.2, 141.1, 139.2, 138.3, 135.7, 129.5, 128.3, 127.9, 127.4, 126.5, 125.6, 121.0, 120.4, 80.7, 66.1, 53.8, 48.7, 46.9, 38.0, 36.4, 35.3, 27.9, 17.2; IR (ATR) νmax 3278, 2967, 1730, 1635, 1534, 1159; HRMS (ESI) m/z 1151.5529 (MH⁺, C₆₃H₇₅N₈O₁₃ requires 1151.5454).
di-tert-butyl 2,2'-{[5-{{[(9H-fluoren-9-ylmethoxy)carbonyl]amino}acetyl]amino}1,3-phenylene}bis[carbonylimino(1-oxobutane-4,1-diyl)imino(1-oxo-3-phenylpropane-2,1-diyl)imino]}dipropanoate. Tripeptide 79 (220 mg, 0.37 mmol) was Fmoc-deprotected in DMF containing piperidine (20%, 0.5 mL) for 30 min, and the solvent was removed in vacuo. The resulting residue was redissolved in CHCl₃ and isophthalate 85 (81 mg, 0.18 mmol), PyBOP (200 mg, 0.39 mmol), and diisopropylethylamine (0.15 mL, 0.88 mmol) were added. After stirring for 18 h, the solvent was removed. Flash chromatography (5% methanol in CH₂Cl₂) afforded 87 (121 mg, 59%) as a white solid, mp 104 - 106 °C; ¹H NMR (300 MHz, d₆-DMSO) δ 10.27 (s, 1H), 8.45 (m, 4H), 8.12 (m, 4H), 7.91 (m, 4H), 7.69 (m, 4H), 7.31 (m, 11H), 4.57 (m, 2H), 4.35 – 3.84 (m, 8H), 3.09 (m, 8H), 2.09 (m, 4H), 1.64 (m, 4H), 1.39 (m, 24H); ¹³C NMR (75 MHz, d₆-DMSO) 172.1, 171.8, 168.7, 166.3, 156.9, 144.2, 141.1, 139.2, 138.4, 136.0, 129.5, 128.3, 127.9, 127.4, 126.5, 125.6, 124.7, 124.7, 121.0, 120.4, 119.4, 110.0, 80.7, 66.1, 60.1, 53.8, 48.7, 46.9, 38.0, 33.2, 27.9, 25.7, 21.1, 17.2, 14.4; IR (ATR) vₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚportion of the document. Please note that the text above is a direct transcription of the image and may contain some formatting errors. It is intended to provide a clear representation of the content in a plain text format.
di-tert-butyl 2,2'-%{5-[(9H-fluoren-9-y1methoxy)carbonyl]amino}acetyl]amino]-1,3-phenylene}bis[carbonylimino(1-oxopentane-5,1-diyl)imino(1-oxo-3-phenylpropane-2,1-diyl]imino]}dipropanoate. Tripeptide 80 (117 mg, 0.19 mmol) was Fmoc-deprotected in DMF containing piperidine (20%, 0.5 mL) for 30 min, and the solvent was removed in vacuo. The resulting residue was redissolved in CHCl₃ and isophthalate 85 (40 mg, 0.08 mmol), PyBOP (104 mg, 0.20 mmol), and iisopropylethylamine (0.08 mL, 0.44 mmol) were added. After stirring for 18 h, the solvent was removed. Flash chromatography (5% methanol in CH₂Cl₂) afforded 88 (94 mg, 89%) as a white solid, mp 102 - 104 °C; ¹H NMR (300 MHz, d₆DMSO) δ 10.26 (s, 1H), 8.41 (m, 4H), 8.18 (s, 2H), 8.02 (m, 6H), 7.75 (m, 4H), 7.36 (m, 11H), 4.59 (m, 2H), 4.25 (m, 6H), 4.12 (s, 2H), 3.07 (m, 8H), 2.06 (m, 4H), 1.38 (m, 32H); ¹³C NMR (75 MHz, d₆DMSO) 172.2, 172.0, 171.8, 168.6, 166.2, 156.9, 144.2, 141.1, 138.2, 136.1, 129.5, 128.3, 128.2, 127.9, 127.5, 127.4, 126.5, 125.6, 124.7, 120.4, 119.4, 109.4, 80.6, 66.1, 53.7, 48.7, 46.9, 44.4, 42.1, 38.1, 35.2, 28.9, 27.9, 23.0, 17.2; IR (ATR) ʋ max 3282, 2930, 1727, 1689, 1637, 1531, 1145; HRMS (ESI) m/z 1229.5946 (MH⁺, C₆₇H₈₂N₈O₁₃Na requires 1229.5899).
di-<i>tert</i>-butyl 2,2'\{-[5-\{[(9H-fluoren-9-ylmethoxy)carbonyl]amino\}acetyl]amino\}-1,3-phenylene\}bis[carbonylimino(1-oxohexane-6,1-diyl)imino(1-oxo-3-phenylpropane-2,1-diyl)imino]\}dipropanoate. Tripeptide 81 (200 mg, 0.32 mmol) was Fmoc-deprotected in DMF containing piperidine (20%, 0.5 mL) for 30 min, and the solvent was removed <i>in vacuo</i>. The resulting residue was redissolved in CHCl<sub>3</sub> and isophthalate 85 (70 mg, 0.15 mmol), PyBOP (175 mg, 0.33 mmol), and diisopropylethylamine (0.13 mL, 0.76 mmol) were added. After stirring for 18 h, the solvent was removed. Flash chromatography (5% methanol in CH<sub>2</sub>Cl<sub>2</sub>) afforded 89 (150 mg, 79%) as a white solid, mp 98 – 102 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 9.60 (s, 1H), 7.96 (m, 3H), 7.64 (d, J = 6 Hz, 2H), 7.49 (m 4H), 7.18 (m, 18H), 6.54 (s, 1H), 4.80 (m, 2H), 4.27 (m, 4H), 3.99 (m, 3H), 3.02 (m, 8H), 2.00 (m, 4H), 1.27 (m, 36H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) 174.1, 172.1, 169.2, 167.4, 156.2, 144.1, 141.6, 137.2, 136.0, 129.7, 128.7, 128.2, 127.5, 127.1, 125.5, 121.7, 120.4, 82.3, 77.8, 54.8, 49.3, 47.4, 40.0, 38.9, 36.1, 28.4, 28.3, 26.3, 25.2, 18.4; IR (ATR) ν<sub>max</sub> 2948, 1730, 1641, 1535, 1204; HRMS (ESI) <i>m/z</i> 1235.6353 (MH<sup>+</sup> C<sub>69</sub>H<sub>87</sub>N<sub>8</sub>O<sub>13</sub> requires 1235.6393).
Dimeric-Xylene Vancomycin or Bis-[40-N-[4-(aminomethyl)benzyl]-
(1S,2R,18R,19R,22S,25R,28R)-22-(2-amino-2-oxoethyl)-48-{[2-O-(3-amino-2,3,6-
trideoxy-3-methylhexopyranosyl)hexopyranosyl]oxy}-5,15-dichloro-2,18,32,35,37-
pentahydroxy-19-[(N-methyl-D-leucyl)amino]-20,23,26,42,44-pentaaoxo-7,13-dioxa-
3,5,8(48),9,11,14,16,29(45),30,32,34,36,38,46,49-pentadecaene-40-carboxamide].

Compound 90 was synthesized as previously reported. Vancomycin (100 mg, 0.067
mmol) and diisopropylethylamine (11 μL, 0.065 mmol) were dissolved in DMF and
DMSO (1:1, 2 mL). HBTU (27 mg, 0.071 mmol) and p-xylylenediamine (4.1 mg,
0.0301 mmol) were added and the reaction was stirred at 23 °C for 18 h. The mixture was
directly purified by preparative reverse-phase HPLC (0% MeCN, 99.9% deionized water,
0.1% TFA to 29.9% MeCN, 70% deionized water, 0.1% TFA over 20 min, then 29.9%
MeCN, 70% deionized water, 0.1% TFA to 49.9% MeCN, 50% deionized water, 0.1%
TFA until 30 min; retention time = 19 min) to afford 90 (89 mg, 99%) as a white fluffy
solid, HRMS (ESI) m/z 999.3212 (MH3+\textsuperscript{3+}, C\textsubscript{140}H\textsubscript{161}Cl\textsubscript{4}N\textsubscript{20}O\textsubscript{46}\textsuperscript{3+} requires 999.3204).
Dimeric-PEG Vancomycin or Bis-[40-N-{2-[2-(aminoethoxy)ethoxy]ethyl}- (1S,2R,18R,19R,22S,25R,28R)-22-(2-amino-2-oxoethyl)-48-\{2-O-(3-amino-2,3,6- trideoxy-3-methylhexopyranosyl)hexopyranosyl\oxy\}-5,15-dichloro-2,18,32,35,37- pentahydroxy-19-{(N-methyl-D-leucyl)amino}-20,23,26,42,44-pentaaxo-7,13-dioxa- 21,24,27,41,43-pentaazaoctacyclo[26.14.2.2\textsuperscript{3,6}.2\textsuperscript{14,17}.1\textsuperscript{8,12}.1\textsuperscript{29,33}.0\textsuperscript{10,25}.0\textsuperscript{34,39}]pentaconta- 3,5,8(48),9,11,14,16,29(45),30,32,34,36,38,46,49-pentaadecaene-40-carboxamide].

Vancomycin (100 mg, 0.067 mmol) and diisopropylethylamine (11 μL, 0.065 mmol) were dissolved in DMF and DMSO (1:1, 2 mL). HBTU (27 mg, 0.071 mmol) and 1,8-Diamo-3,6-dioxoactane (4.5 mg, 0.0301 mmol) were added and the reaction was stirred at 23 °C for 18 h. The mixture was directly purified by preparative reverse-phase HPLC (0% MeCN, 99.9% deionized water, 0.1% TFA to 29.9% MeCN, 70% deionized water, 0.1% TFA over 20 min, then 29.9% MeCN, 70% deionized water, 0.1% TFA to 49.9% MeCN, 50% deionized water, 0.1% TFA until 30 min; retention time = 19 min) to afford 91 (80 mg, 86%) as a white fluffy solid; mp = decomposition > 200 °C; HRMS (ESI) m/z 752.7474 (MH\textsubscript{4}\textsuperscript{4+}, C\textsubscript{138}H\textsubscript{166}Cl\textsubscript{4}N\textsubscript{20}O\textsubscript{48}\textsuperscript{4+} requires 752.7486).
Analytical HPLC profile of the HPLC purified dimeric PEG vancomycin 91 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 7.0 min).


Vancomycin (200 mg, 0.135 mmol) and diisopropylethylamine (25 μL, 0.32 mmol) were
dissolved in DMF and DMSO (1:1, 2 mL). HBTU (56 mg, 0.147 mmol) and 1,8-Diaminoctane (8.6 mg, 0.0602 mmol) were added and the reaction was stirred at 23 °C for 18 h. The mixture was directly purified by preparative reverse-phase HPLC (0% MeCN, 99.9% deionized water, 0.1% TFA to 29.9% MeCN, 70% deionized water, 0.1% TFA over 20 min, then 29.9% MeCN, 70% deionized water, 0.1% TFA to 49.9% MeCN, 50% deionized water, 0.1% TFA until 30 min; retention time = 18 min) to afford 92 (120 mg, 62%) as a white fluffy solid; mp = decomposition > 200 °C; HRMS (ESI) m/z 1002.0079 (MH33+, C140H169Cl4N20O463+ requires 1002.0091).

Analytical HPLC profile of the HPLC purified dimeric Octyl vancomycin 92 (absorbance wavelength = 215 nm, gradient 9.9% MeCN, 90% deionized water, 0.1% TFA to 99.9% MeCN, 10% deionized water, 0.1% TFA over 20 min; retention time = 7.9 min).
**N-{5-[(tert-butoxycarbonyl)(3β-cholest-5-en-3-yl)amino]pentanoyl}-β-alanyl-α-tert-butyl-glutamyl-α-tert-butyl-glutamyl-β-alanine.** The linker region was synthesized on solid phase using the method described in Chapter 3 with the amino acids Fmoc-β-Ala-OH (1.76 mmol) and Fmoc-Glu(OtBu)-OH (1.76 mmol), and the coupling reagents diisopropylethylamine (2.3 mmol), HBTU (1.64 mmol), and HOBT (1.46 mmol). After removal of the Nα-Fmoc group of the amino terminal AA, the amino terminus was capped by addition of diisopropylethylamine (0.40 mL, 1.75 mmol), HOBT (134 mg, 0.878 mmol), valeric acid-derived cholesterol (570 mg, 0.87 mmol) and PyBOP (459 mg, 0.878 mmol) in 4 mL of DMF. The product was cleaved with CH$_2$Cl$_2$ containing acetic acid (20%, 5 mL) to retain the glutamic acid protecting groups. The crude product was purified by flash chromatography (10% MeOH, 0.5% AcOH in dichloromethane) afforded 93 (465 mg, 73%) as a white fluffy solid; mp = 142 – 144 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 12.3 (s, 1H), 8.03 (s, 4H), 5.24 (s, 1H), 4.66 (m, 2H), 3.44-0.83 (m, 92H), 0.61 (s, 3H); $^{13}$C NMR (75.4 MHz, CDCl$_3$) δ 174.3, 171.8, 171.6, 170.7, 155.1, 140.6, 120.5, 80.1, 79.7, 55.9, 55.3, 52.2, 51.3, 49.3, 45.5, 41.5, 38.9, 38.6, 37.6, 36.2, 35.8, 35.4, 34.9, 33.1, 31.0, 30.8, 30.5, 28.8, 27.7, 27.4, 27.2, 27.1, 26.0, 23.4, 23.0, 22.4, 22.0, 21.8, 20.2, 18.6, 17.9, 11.1; IR (KBr) $\nu_{max}$ 2993, 1721, 1695, 1252; HRMS (ESI) $m/z$ 1098.7662 (MH$^+$, C$_{61}$H$_{104}$N$_5$O$_{12}$ requires 1098.7681).
4.8 Biological Assays and Protocols

**Emission Curves.** Emission spectra for the fluorescent vancomycin derivatives was obtained with a PTi MD-5020 fluorimeter in phosphate-buffered saline, pH = 7.4 with less than 1% DMSO. Compounds were diluted from DMSO stock solutions to a concentration of 10 nM and normalized by UV/Vis spectroscopy. They were then excited at 494 nm and the emission from 515 to 650 nm was collected using a 1 cm path length quartz cuvette.

**Cell Culture.** Jurkat lymphocytes (human acute T-cell leukemia, ATCC #TIB-152) were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units per mL), and streptomycin (100 µg per mL). RPMI media used for cell culture and washing steps contained antibiotics and FBS unless otherwise noted. CHO-K1 (Chinese hamster ovary cells, ATCC #CCL-61) were maintained in F-12K media supplemented with fetal bovine serum (FBS, 10%), penicillin (100 units per mL), and streptomycin (100 µg per mL). F-12K media used for cell culture and washing steps contained antibiotics and FBS unless otherwise noted. All cells were propagated in a humidified 5% CO2 incubator at 37 °C.

**Flow cytometry.** Analyses were performed with a Beckman-Coulter XL-MCL bench-top flow cytometer or Beckman-Coulter FC-500. Forward scatter (FS) and side-scatter (SSC) dot plots afforded cellular physical properties of size and granularity that
allowed gating of live cells. After gating, 10,000 cells were counted. To measure the uptake of Oregon Green compounds by Jurkat lymphocytes, the fluorophores were excited at 488 nm with a 15 mW air-cooled argon-ion laser. The emission was split with a 550 nm dichroic and filtered through a 510 nm long pass filter and 530/30-nm band pass filter using the XL-MCL cytometer. The PMT voltage for this instrument was set to 724 for the detection of Oregon Green. Calibration with Sphero Rainbow Calibration particles (Spherotech) bearing 330,000 molecules of fluorescein per particle provided a fluorescence of 17.5 at this voltage.

**Microscopy.** A Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat objective (63X) was employed. Fluorophores were excited with the 488 spectral line of a 25 mW argon ion laser and emitted photons were collected through a 505 nm LP filter.

**Dimeric-Oregon Green Vancomycin Uptake Assays.** Receptors and dimeric vancomycin compound stock solutions were made in DMSO and were subsequently diluted in RPMI media to their final working concentrations (DMSO concentration 1%). They were pre-equilibrated for 1 h at 23 °C. The complexes in media were then added to Jurkat cells (5 x 10⁵). Cells were typically maintained at 37 °C for 4 h to promote synthetic receptor-mediated endocytosis. Prior to analysis, treated cells were washed with media (2 x 0.5 mL) and DPBS (2 x 0.5 mL). After washing, cells were resuspended in fresh media and analyzed by confocal microscopy or flow cytometry.
**Endocytosis Inhibition Studies.** Receptor 69 (10 μM) and either vancomycin compounds 74 or 75 (10 μM) were pre-equilibrated for 1 h at 23 °C in media, then added to Jurkat cells (5 x 10^5) or CHO cells (5 x 10^5) for 1 h at 37 °C. The treated cells were washed with media (2 x 0.5 mL) then were incubated at 37 °C with either cholera toxin-594 (170 nM), TR-transferrin (500 nM), or DiL-LDL (20 nM) for 1 h (Jurkat cells) or 5 min (CHO cells). Prior to analysis, treated cells were washed with media (2 x 0.5 mL) and DPBS (2 x 0.5 mL), and analyzed by confocal microscopy. Control cells were treated with either cholera toxin-594 (170 nM), TR-transferrin (500 nM), or DiL-LDL (20 nM) for 1 h (Jurkat cells) or 5 min (CHO cells) then washed and imaged.

### References


VITA

Sheryl Ann Rummel

Sheryl A. Rummel was born on December 31, 1980 in Ringwood, N.J. She grew up in Orange County, NY and moved to Sussex County, NJ where she attended High Point Regional High School, graduating as the salutatorian in 1999. She then attended Grove City College in Pennsylvania where she received a B.S. in Biochemistry with honors in 2003. Sheryl completed her graduate work at The Pennsylvania State University in 2008 where she worked under the supervision of Dr. Blake R. Peterson. Upon graduation, Sheryl will be employed by The Pennsylvania State University as the Director of Instrumentation for the Undergraduate Chemistry program in Whitmore.