The Magic Bullet:
Cost-Effective Next Generation Cubicin

*Cheaper production of Cubicin

Directed evolution to produce 6X His-tagged daptomycin *in vivo*

*Oral administration of Cubicin

Chitosan encapsulation of daptomycin to bypass enzymatic liability in GI tract

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Executive Summary

Daptomycin (trade name: Cubicin), marketed by Cubist Pharmaceuticals, is a novel lipoprotein antibiotic specific to gram-positive bacteria. It is effective in treating vancomycin- and methicillin-resistant *Staphylococcus aureus* (VRSA/MRSA), and other antibiotic resistant pathogenic bacteria. However, improvement is required in both the production process and method of administration of Cubicin. The currently employed industrial purification process represents a bottleneck in production. Not only is it complex, it utilizes methods that degrade daptomycin, which requires additional purification steps with decreased yield. A viable solution to this problem is to utilize directed evolution to create a variant of the non-ribosomal peptide synthetase (NRPS), which produces daptomycin in *Streptomyces roseosporus*. The modified NRPS machinery will incorporate 6X His-tag into daptomycin. This thereby allows isolation via nickel column chromatography, decreasing the number of steps that are required for purification. In addition, Cubicin is currently only available for administration by intravenous injection due to enzymatic degradation of the drug if taken orally. A viable solution to this problem is to prepare daptomycin for oral delivery via encapsulation in chitosan. This is a biocompatible and biodegradable polymer with an outer layer of hydroxypropyl methylcellulose phthalate (HPMCP), which will preserve the drug's antibiotic properties as it passes through the gastrointestinal (GI) tract. This unique oral drug delivery platform will facilitate absorption of daptomycin through the intestinal mucosal membrane. In addition, solubility of chitosan can be modified by the degree of crosslinking, acetylation, composition and acidification, which allows for control of the time and anatomical location of daptomycin release in the patient. Cubist Pharmaceuticals should invest in implementing this proposal, which provides a high value proposition: increased output and convenient oral administration of Cubicin. The projected investment for start-up of production is $75,933.31 for capital goods and equipment plus an uncertain additional cost for labor and space for the requisite combinatorial chemistry experiments; the total start-up cost is not projected to exceed approximately $2.02 billion (the maximum amount of money to be recovered within five years of first sale given the following conditions). At the current market price of approximately $165 per dose ($330 per gram), the projected recurring cost of production of $52.81 per dose ($105.62 per gram), and assuming the existence of a market for the sale of approximately 3.6 million doses per year (consistent with sales in the United States alone in 2010), the original investment should very likely be recovered within five years of the first sale (Cubist Pharmaceuticals, Ontario Ministry of Health and Long-Term Care). The actual cost at which daptomycin should be sold to maximize profits can be adjusted once the actual cost of the combinatorial chemistry experiments has been determined following their completion.

Scientific Problems

Cubicin Discovery and Development

Daptomycin was discovered in the late 1980s by researchers at Eli Lilly and Company (Drug Information Portal). It remained with Eli Lilly and Company until 1997, when the rights to the drug changed hands and moved to Cubist Pharmaceuticals. Daptomycin was marketed under the name Cubicin, which was approved by the FDA in 2003. It is currently marketed in multiple countries by companies such as Novartis.

The chemical formula for Cubicin is $C_{72}H_{101}N_{17}O_{26}$. Its chemical name is *N*-decanoyl-L-tryptophyl-D-asparaginyl-L-aspartyl-L-threonylglycyl-L-ornithyl-L-aspartyl-D-alanyl-L-aspartylglycyl-D-seryl-*threo*-3-methyl-L-glutamyl-3-anthraniloyl-L-alanine ε_1-lactone (Tally 2000). When Cubicin was first produced it was classified as part of a new class of antibiotics called cyclic lipopeptides (Baltz 2010).

This antibiotic is naturally made by *S.roseosporus* through the non-ribosomal peptide synthetase (NRPS) machinery. NRPS can synthesize peptides independent of the mRNA and can often incorporate non-proteinogenic amino acids such as D-amino acids (Robbel 2010). In addition, the rate of synthesis can be enhanced by supplying decanoic acid to *S.roseosporus*. Scientists also found that calcium, when bound to daptomycin, causes a conformational change that increases the amphiphilicity of the drug. This in turn will cause it to bind further within the plasma membrane of the cell and consequently cause potassium to flow from the intracellular to the extracellular compartment.

Cubicin demonstrated promising results during preclinical trials. Researchers found that Cubicin was effective against multiple gram-positive bacteria such as *Staphylococcus aureus* endocarditis and *Clostridium difficile* colitis (Bambeke). However, Eli Lilly and Company was not satisfied with its overall performance during Phase II clinical trials due to occurring side-effects such as forearm weakness, myalgia and elevated creatine kinase. Once acquired by Cubist Pharmaceuticals, the drug was modified such that it exhibited lower toxicity and improved activity (Bambeke).

As of 2005, the estimated number of people developing MRSA infection was 94,360, of which 85% were associated with healthcare (Klevens 2007). Currently there are many infections caused by gram-positive bacteria, which are resistant to vancomycin, a very strong antibiotic medicine. Cubicin is the last resort to combat these infections as these bacteria which are resistant to vancomycin and other antibiotics are not resistant to Cubicin. As of today there are no bacterial strains resistant to Cubicin. Although Cubicin presents a possible cure for these invasive infections, it also presents an expensive and an inconvenient treatment option. In this proposal, we demonstrate a new method of production and delivery of Cubicin which is more cost-effective and lucrative (Charles 2004).

Mechanism of Action and Administration

The exact mechanism of daptomycin and its antimicrobial activity has yet to be determined, although several aspects of the mechanism have been elucidated. It is known that the presence of calcium ions (Ca^{2+}) is important for antibiotic activity. Studies, including some involving the determination of nuclear magnetic resonance (NMR) structures, have been conducted to determine the structure and behavior of daptomycin in the presence of Ca^{2+}, in the presence of other divalent ions (e.g. Mg^{2+}) and in the absence of divalent ions. Daptomycin was found to exhibit antibiotic properties in the presence divalent ions other than Ca^{2+}, but the minimum inhibitory concentration (MIC) was increased by a factor of at least 32 in such cases (Robbel 2010).

Early studies into the mechanism of action of daptomycin led to the proposal of mechanisms via micelle formation dependent upon a change in the structure of daptomycin in the presence of Ca^{2+}, which was observed via NMR experiments, fluorescence spectroscopy, and circular dichroism experiments. However, more recent studies have been published which contradict this, by demonstrating that, while daptomycin does not undergo a significant conformational change in the presence of Mg^{2+} ions, these ions nonetheless promote the formation of daptomycin micelles (as shown by equilibrium sedimentation experiments), which is still believed to be a vital factor in daptomycin's antimicrobial activity. These more recent studies indicate that the presence of Ca^{2+} in a 1:1 ratio leads to the formation of micelles of fourteen to sixteen daptomycin molecules, and have led to speculation that the Ca^{2+} cations counteract the negative charge associated with the acidic residues of daptomycin, allowing micelle formation through either favorable interaction of the hydrophobic tails of daptomycin or π-stacking interactions between the aromatic residues of daptomycin (Robbel 2010).

Such recent experiments into the nature of the mechanism of action of daptomycin have thus led to the proposal of a new model for the mechanism of antibacterial activity. In this particular model, daptomycin (in the presence of Ca^{2+}) forms micelles, which is delivered to the cell membranes of gram-positive bacteria in sufficient concentration and appropriate conformation for antimicrobial activity. As daptomycin micelles approach the cell membrane, they dissociate to allow for the insertion of the daptomycin molecules into the membrane, which is facilitated by their nonpolar lipid tails. However, it is still unclear whether daptomycin oligomerizes within the cell membrane or not. Although it is speculated that daptomycin causes potassium efflux and membrane depolarization, it is uncertain whether it is the primary cause of bacterial cell death. It is possible that daptomycin promotes cell death through interference with other cellular processes, such as cellular division (Robbel 2010).

Daptomycin is currently administered through either IV injections over two minutes or IV infusion over thirty minutes. Each method of administration requires that daptomycin is reconstituted in 0.9% sodium chloride to create a 50mg/mL solution. These treatment procedures must be repeated every 24-48 hours, over 7-14 days if the infection is skin-related and over 2-6 weeks if the infection is blood-related (Cubist Pharmaceuticals). The number of injections is unfavorable to patients, thus necessitating an improvement in the administration process such as enabling oral administration. However, daptomycin is a peptide drug, which is susceptible to enzymatic degradation in the stomach by pepsin. In addition, the low pH of the stomach may denature or degrade the drug.

Cubicin Synthesis

The current industrial method for synthesizing Cubicin utilizes *Streptomyces roseosporus*, the species from which the compound was first isolated. *S.roseosporus* utilizes NRPS to synthesize daptomycin, which is then excreted from the cell. However, the synthetase is not specific to daptomycin and many variants of the compound are synthesized with varying tail lengths, of zero to fourteen carbons (C_0-C_{14}), but by feeding *S.roseosporus* n-decanoic acid, the lipoprotein with a carbohydrate chain length of 10 carbons is favored (Kelleher 2004).

The standard industrial method is then continued with a separation of the water-soluble daptomycin from the extracellular fluid by filtration of the fermentation broth, followed by a butanol buffer extraction of daptomycin. Daptomycin is further purified via a series of anion-exchange and hydrophobic interaction columns. The anion-exchange columns utilize a co-polymer of divinylbenzene and styrene (Kelleher 2004). Through a series of washes of varying salt concentrations, daptomycin can be eluted separately from other solution components and degradation products. The anion-exchange columns can be reused after applying a series of regeneration buffers. The hydrophobic interaction column variably binds and elutes compounds of differing hydrophobic properties to, for example, remove the various stereoisomer degradation byproducts, including the beta-isomer of daptomycin and anhydro-daptomycin (Kelleher 2004). The impurities are eluted by varying the concentration of the modified buffer of urea from 2M to 6M and varying the pH from 6 to 7. The sample is then re-applied to the anion-exchange column for further purification. This is followed by ultrafiltration or size exclusion chromatography is performed in which a 10,000 or 30,000 nominal molecular weight membrane is utilized in order to remove low molecular weight compounds from the sample, such as salts and sugars, while retaining daptomycin. The purity of daptomycin in the final product is verified by high pressure liquid chromatography (HPLC), and isolation of daptomycin is performed by micelle formation and extraction (Kelleher 2004).

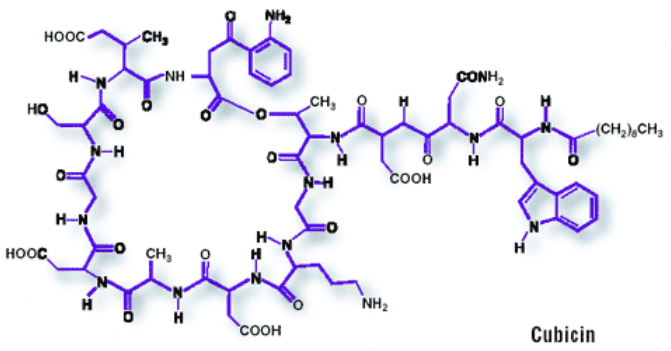

FIGURE 1: Structure of Cubicin
Image courtesy of Team Project 1-Reading from Blackboard

Current Bottleneck in Synthesis

The bottleneck in the method of synthesis currently employed by Cubist is purification; the large number of steps required to purify the drug for administration requires a large investment of time and raw materials. There are at least three columns required for purification, and each requires a series of washes with differing ion concentration, hydrophilicity, and pH; the number of columns and washes necessary results in a low yield of daptomycin. Furthermore, the buffers used affect the purity of the final product due to the conversion of daptomycin into degradation products, only some of which have been identified, including the beta-isomer of daptomycin and anhydro-daptomycin (Kelleher 2004). In an effort to decrease the number of degradation products formed during purification, stringent control of temperature and pH have been instituted, thus further increasing the cost and amount of equipment required for daptomycin production.

Breaking the Bottleneck

Improvement is required in the both the administration and synthesis/purification of daptomycin. In order to improve the purification process, the number of purification steps will be decreased to reduce costs and the number of elution buffer variations will be optimized to prevent the creation of degradation products typical of Cubist's purification methods. Both of these goals can be realized by utilizing poly-histidine tag chromatography, which will accomplish the bulk of the purification in a single step. The transition from intravenous to oral delivery will be performed by administration via chitosan capsules/caplets that will protect the peptide drug from degradation in the harsh conditions of the stomach and allow for delivery of daptomycin in the small intestine.

Research Design: Reduce Cost of Cubicin Production

6X His-Tagged Daptomycin Production in vivo

Daptomycin biosynthesis in *S.roseosporus* is mediated by non-ribosomal peptide synthetase (NRPS): DptA, DptBC, DptD as well as *trans*-acting enzymes. This machinery is organized into modules and domains, which acts in "thiotemplate-directed manner" to synthesize daptomycin (Robbel 2010). Each module recognizes, activates and covalently links the building block onto the peptide chain. The linear lipopeptide is then cyclized by thioesterase enzyme, which aids the nucleophilic attack from the side chain of L-threonine to the electrophilic center of L-kynurenine (Robbel 2010). The natural synthesis is illustrated in Figure 2, which shows the daptomycin biosynthetic gene cluster (A21987C_{1-3}):

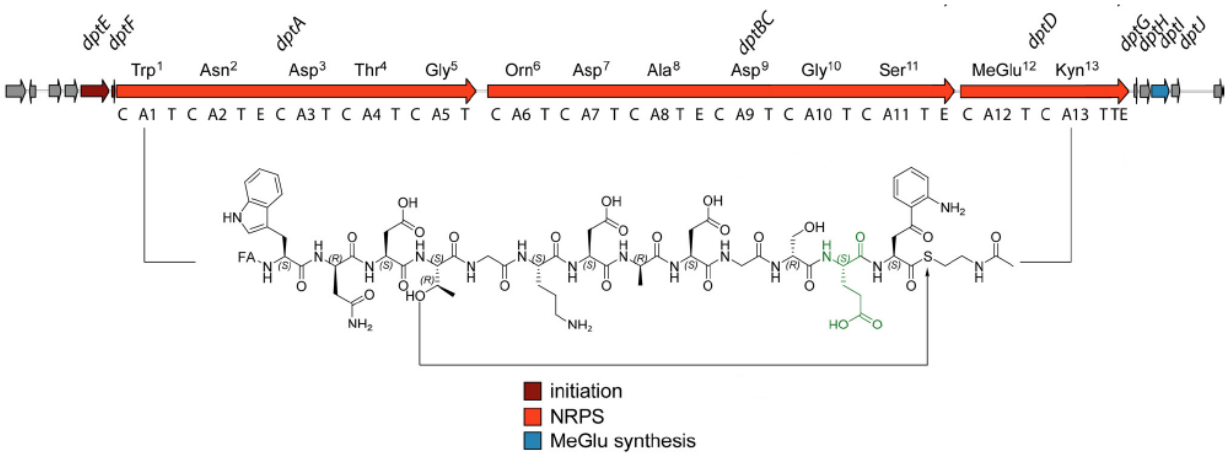

FIGURE 2: Daptomycin Biosynthetic Gene Cluster, A21987C_{1-3}
Image courtesy of Robbel 2010

Each module has three domains for the following reactions: C (condensation), A (adenylation) and T (thiolation). These processes allow the activated substrate to tether to the growing peptide and for the subsequent reactions to occur. We will exploit this daptomycin biosynthetic gene cluster in order to incorporate the 6X His-tag. The overall strategy is to tag Cubicin by the N-terminus L-tryptophan residue *in vivo*. This can be done by modifying one of the components in DptA. As shown in Figure 3, the first step in NRPS is n-decanoic acid linkage to L-tryptophan. This involves modification and condensation of n-decanoic acid by DptE and DptF, respectively:

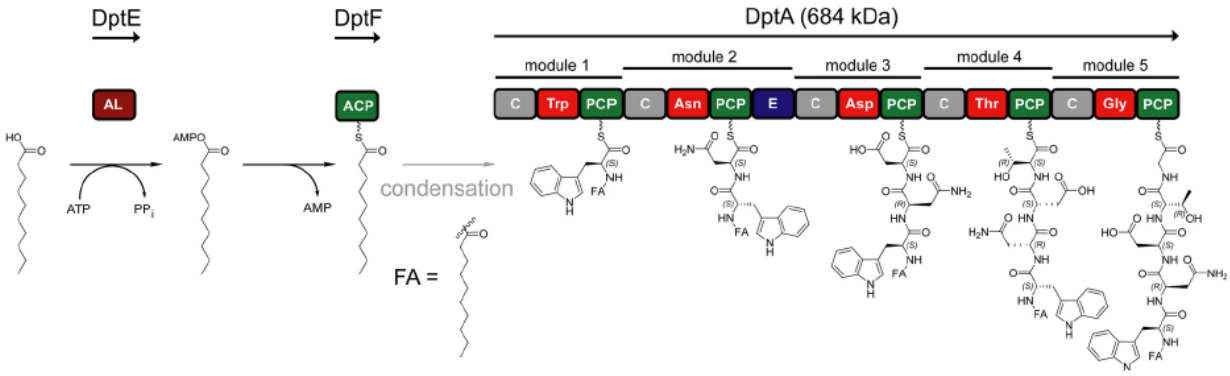

FIGURE 3: NRPS Biosynthetic Enzymatic Machinery (only DptE, F and A shown)
Image courtesy of Robbel 2010

We will use directed evolution so that module 1 of DptA can accept 6X His-tag instead of the n-decanoic acid moiety. This will involve a radical change but directed evolution with a stringent selection scheme can bypass this problem. It also helps that NRPS has already been cloned into bacterial artificial chromosome (BAC) vector in *E.coli* (Miao 2005). We will first subclone module 1 of dptA gene from the BAC vector into a plasmid. We will then generate a large library by inducing random mutations via recombination and error-prone polymerase chain reaction (PCR). These mutations will be analogous to reproduction and random somatic mutations respectively. We will generate a library of variants of dptA module 1 by PCR without primers. This will allow the PCR amplimers to prime off of each other to create a contiguous dptA gene. Figure 4 shows the strategy for generating a library of dptA module 1.

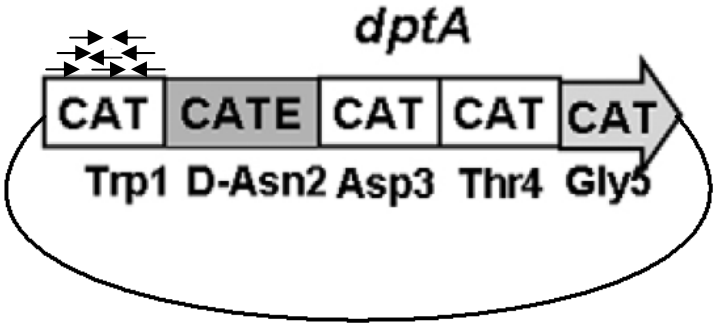

FIGURE 4: PCR Random Mutagenesis for dptA Module 1
The arrow indicates the orientation of the primer from 5' to 3' direction (left to right). Although not shown, the vector has all the other necessary components such as a promoter, an origin of replication and a selectable marker.
Image adapted from Baltz 2010

After *in vitro* recombination and somatic mutations, we will use PCR with primers to amplify the length of dptA module 1. These primers will either have restriction enzyme sites or flanking sequences that facilitate cloning back into the plasmid via ligation or recombination. The plasmid libraries can then be shuttled into *E.coli* to allow recombination to take place between the plasmid and the BAC vector. If these steps are successful, then mutations generated from error-prone PCR will be transferred to the BAC vector with the rest of the gene clusters, dptBC and dptD. This is hereinafter denoted as BAC*.

In *S.roseosporus*, natural daptomycin biosynthesis is initiated by incorporation of n-decanoic acid by DptE and DptF. In our strategy, the goal is to initiate daptomycin synthesis with 6X His-tag. In order to do so, we will transform the mutagenized dptA construct into *E.coli* with knocked out dptE and dptF genes. We are making an assumption that dptE and dptF genes are not essential for cell viability. Our selection is whether the product of NRPS contains 6X His-tag or not. First, we need to make sure that there will be no false positive results. We will express the 6X His-tag in a plasmid in wildtype *E.coli* to ensure that there is no random incorporation of the tag in the proteome. The same can be done by using BLAST to verify that the genome contains no tandem repeats of histidine residues. After this control is done, we will select for those that have incorporated the 6X His-tag in daptomycin.

At this time, the transformed *E.coli* cells will have two selectable markers from BAC* and 6X His-tag. These transformants will be plated on LB under two selectable markers. The resulting colonies will then undergo bulk screening for 6X His-tagged daptomycin. This is outlined in Figure 5 below:

Since directed evolution is a low probability event, we will bulk screen by collective lysis. Although genetically impure, multiple transformants will be grown in one culture tube, which will later undergo cell lysis. Furthermore, we do not need to worry about whether the 6X His-tagged daptomycin will be secreted or not. The cell lysate will then be subjected to nickel column chromatography, 6X His-tag cleavage and n-decanoic acid acylation (discussed later). This reconstituted daptomycin will be used to spot test on a confluent lawn of gram-positive bacteria. Since daptomycin is an effective drug against gram-positive bacteria, it will clear the area around gram-positive bacterial growth.

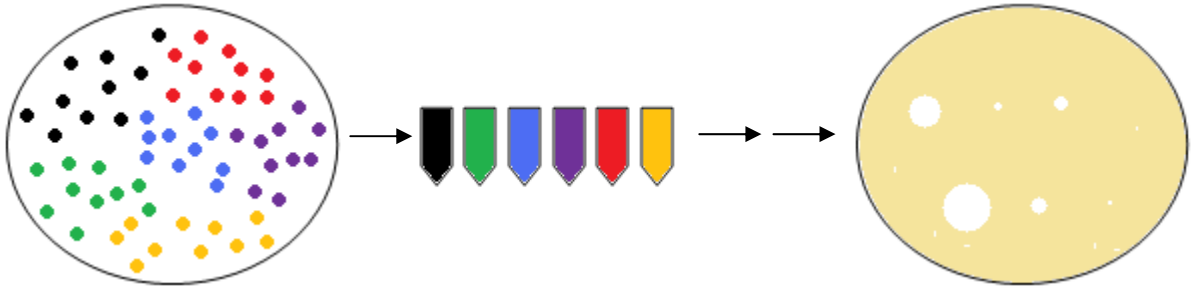

FIGURE 5: Bulk Screening for 6X His-tagged Daptomycin.

A second round of screening can be applied to determine which colony was responsible for production of 6X His-tagged daptomycin. That colony will be selected as the "winner" of directed evolution and grown in culture. A schematic summary of directed evolution is outlined below in Figure 6:

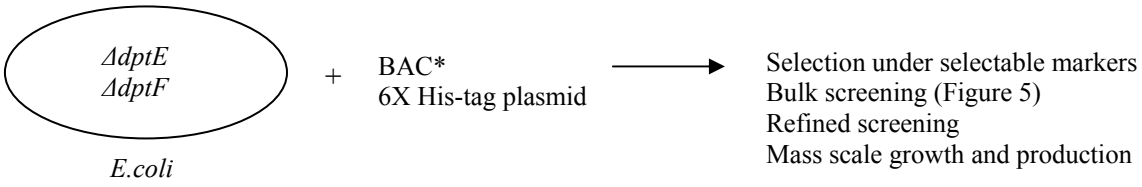

FIGURE 6: Schematic Summary of Directed Evolution

We are assuming that the stereoisomer that may arise from our synthesis is not a major concern. This is because many of the degradative products in Cubicin have not yet been identified and characterized by Cubist Pharmaceuticals (Kelleher 2004).

Purification of 6X His-Tagged Daptomycin in vitro

After verifying *in vivo* synthesis of 6X His-tagged daptomycin, that *E.coli* transformant will be grown in culture to confluence. Then, three-quarters of the batch will be removed for the purification of daptomycin, while the remainder of the culture will be allowed to regenerate. The removed cells will be lysed in preparation for a histidine-tag purification column, which will employ 100mL of High Density Nickel Agarose resin purchased from Jena Bioscience. The histidine residues of the poly-histidine tag will

interact with the nickel ions (Ni^{2+}) of the resin, forming coordination complexes with two histidine residues for every nickel ion.

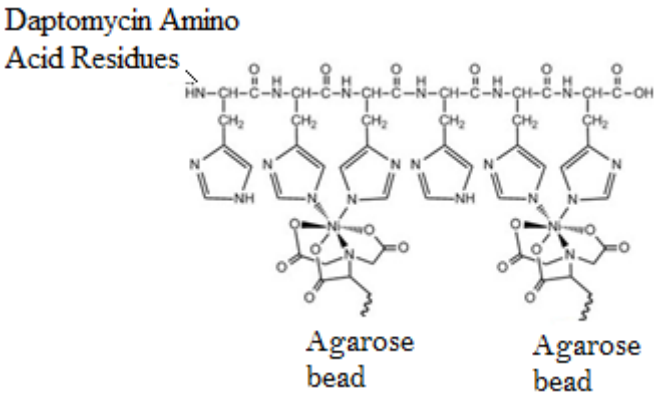

FIGURE 7: Coordination Complex of Histidine and Nickel
Image adapted from http://www.kpl.com/catalog/categories.cfm?Catalog_ID=17&Category_ID=401

The column will be washed, following which daptomycin will be eluted using a 250mM imidazole solution for competitive binding with Ni^{2+}. After elution of the histidine-tagged daptomycin the column will be washed and re-equilibrated or stored. After several cycles of daptomycin purification, the binding capacity of the column may decrease. However, if a significant reduction of daptomycin content in the eluate is observed, the resin can be regenerated by stripping it of (Ni^{2+}) with 20mM sodium phosphate, 0.5M NaCl and 50mM EDTA and applying 500mL of 0.1M nickel chloride. Regeneration in this manner may be performed up to ten times (Jena Bioscience).

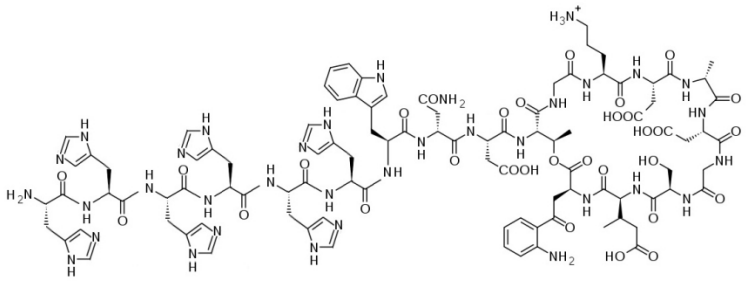

FIGURE 8: Poly-Histidine Tagged Daptomycin
Image adapted from http://en.wikipedia.org/wiki/Daptomycin

After 6X His-tagged daptomycin is purified, the tag must be removed for acylation reaction for the n-decanoic acid tail. There is a commercially available enzyme, TAGZyme DAPase, that removes the 6X His tag but it is limited to specific templates. This is a recombinant exopeptidase that carries a 6X His tag at its C-terminus and removes His tags at the N-terminus (Qiagen). The enzyme sequentially cleaves dipeptides from N-terminus until it reaches an amino acid motif that is not recognized as a substrate (DAPase stop points). These TAGZyme DAPase stop points are listed below:

Amino acid	DAPase stop point (↓) sequence*
Lysine (Lys, K)	Xaa-Xaa...Xaa-Xaa ↓ Lys-Xaa ...
Arginine (Arg, R)	Xaa-Xaa...Xaa-Xaa ↓ Arg-Xaa ...
Proline (Pro, P)	Xaa-Xaa...Xaa-Xaa ↓ Xaa-Xaa Pro-Xaa...
Proline (Pro, P)	Xaa-Xaa...Xaa-Xaa ↓ Xaa-Pro Xaa-Xaa...
Glutamine (Gln, Q)†	Xaa-Xaa...Xaa-Xaa ↓ Gln-Xaa...

FIGURE 9: TAGZyme DAPase Stop Points
Image courtesy of Qiagen

Since our strategy calls for 6X His-tag by L-tryptophan, TAGZyme DAPase from Qiagen will cleave more residues than 6X histidine residues. Furthermore, we will design another round of directed evolution so that this enzyme cannot cleave L-tryptophan. This may be difficult to implement due to commercial patent but similar strategy can be implemented from Figure 4. A large library of TAGZyme DAPase will be generated via error-prone PCR mutagenesis. The vector will be transformed into *E.coli* with amp^r selectable marker. After expressing this enzyme under a promoter, it can be purified by nickel column chromatography because the enzyme itself is 6X His-tagged at the C-terminus. The effect of mutagenesis can be tested by incubating 6X His-tagged daptomycin for 30 minutes at 37°C (Qiagen). This reaction can be spot tested on gram-positive bacteria on LB plate. A confluent plate would indicate that TAGZyme DAPase cleaved more than 6X His residues, which confers non-functional daptomycin. However, if clear spots appear on the plate, then it indicates that the directed evolution was a success. The transformant with the "winner" will be used as the 6X His cleaving enzyme.

Acylation of N-Decanoic Acid Tail of Daptomycin

The eluate containing the poly-histidine tagged daptomycin will be subjected to Fmoc protection, which will protect the primary amine groups to prevent reaction with decanoic acid following cleavage of the poly-histidine tag. The modified daptomycin will next be subjected to treatment with the modified TAGZyme DAPase enzyme, thus cleaving the N-terminal histidine residues from part of the molecule consistent with the original structure of daptomycin (leaving the following tryptophan residue and the following residues unmodified).

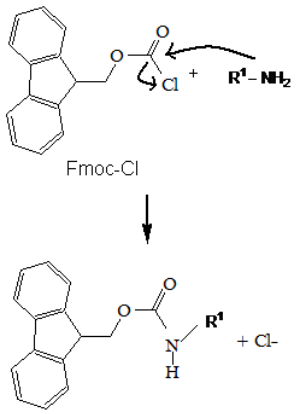

FIGURE 10: Fmoc Protection of Primary Amines
Image adapted from http://www.peptideguide.com/protecting-groups-spps.html

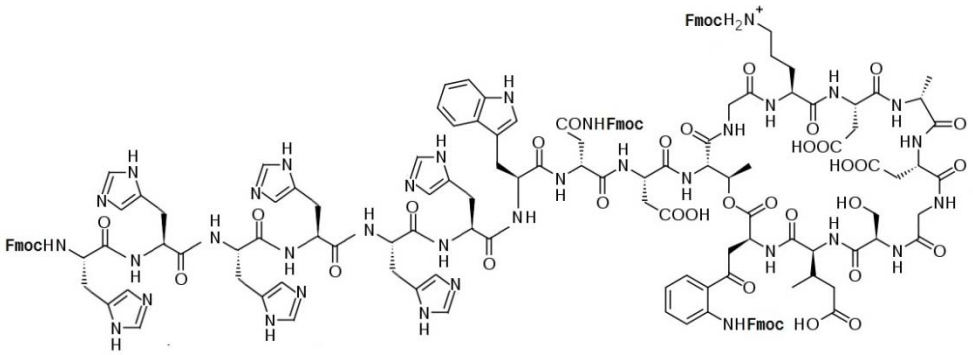

FIGURE 11: Fmoc Protected, Poly-Histidine Tagged Daptomycin
Image adapted from http://en.wikipedia.org/wiki/Daptomycin

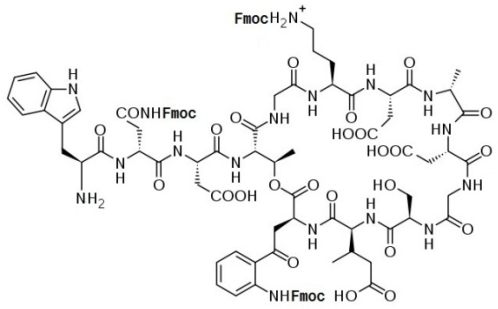

FIGURE 12: Partially Fmoc Protected Daptomycin Without Decanoyl Tail
Image adapted from http://en.wikipedia.org/wiki/Daptomycin

At this stage, the product will be in the form of untagged daptomycin with a primary amine in the place of the decanoic acid tail, and with all other primary amines protected. The eluate will then be subjected to solution phase synthesis to add the decanoic acid tail. DCC and N-hydroxysuccinimide (NHS) will be used to improve the reaction efficiency. The primary amine groups of L-ornithine, D-asparagine and L-kynurinine will be protected, with the only free amine located at the terminal primary amine of the tryptophan residue. Thus, the n-decanoic acid will react at the N-terminus of the tryptophan to complete the daptomycin synthesis. Once significant time has been allowed for all of the daptomycin to react with the decanoic acid, the amino acid side chains will be deprotected by the addition of piperidine.

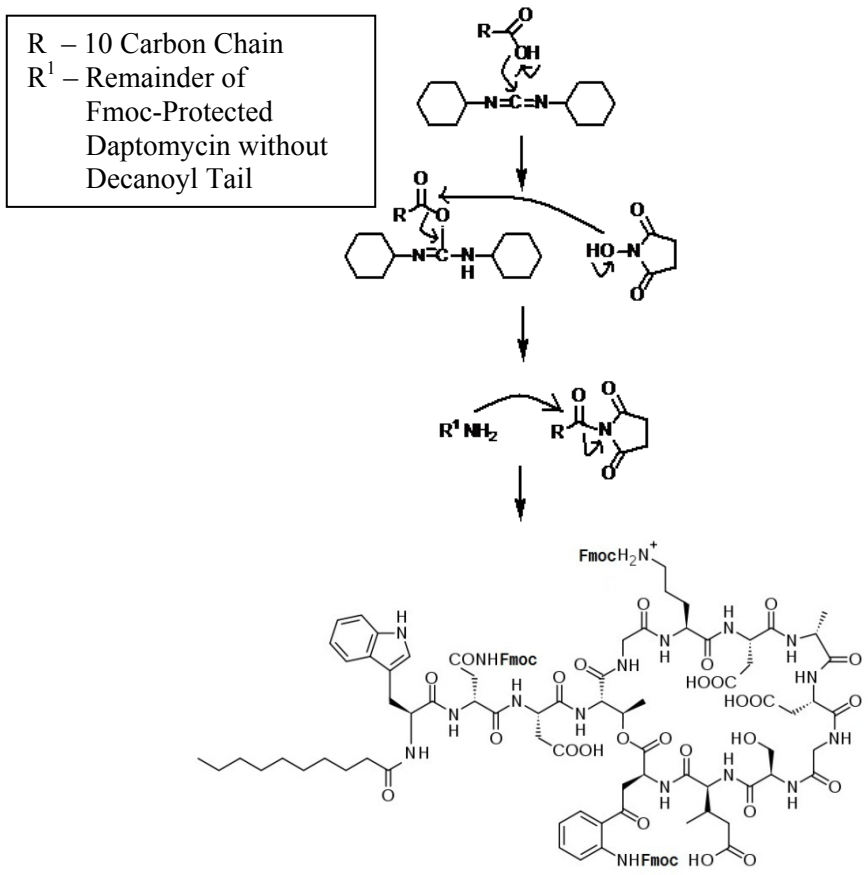

FIGURE 13: Acylation of Daptomycin with n-Decanoic Acid
Image adapted from http://en.wikipedia.org/wiki/Daptomycin

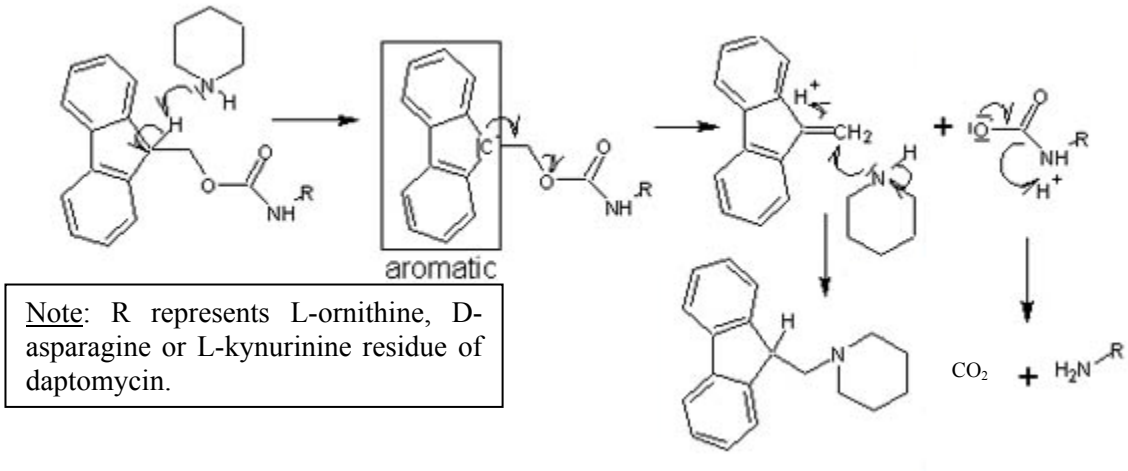

FIGURE 14: Fmoc Deprotection of Daptomycin
Image adapted from http://www1.pacific.edu/~bsztaray/research/undergraduates/

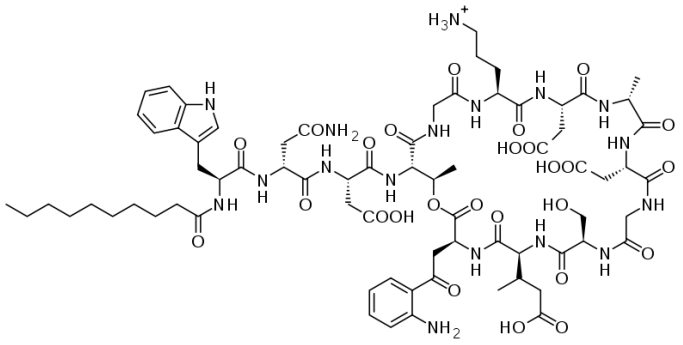

FIGURE 15: Daptomycin
Image from http://en.wikipedia.org/wiki/Daptomycin

The solution containing TAGZyme DAPase enzyme, daptomycin (which has a mass of approximately 1600 Daltons) and any remaining reactants or byproducts will then be subjected to filtration via an ultrafilter with pores sized to exclude masses over 3000 Daltons, which will allow daptomycin to pass through the membrane while preventing the TAGZyme DAPase enzyme to pass through, purifying the enzyme from the drug. A second round of ultrafiltration with a 1000 Dalton filter will be used to remove ions, free amino acids and decanoic acid. This filter will then be removed and dried. The filter will be washed to isolate the daptomycin, which will then be dried in preparation for the creation of the final product for oral delivery.

The proposed method of synthesis confers several advantages to daptomycin production as compared to the currently utilized production method. There is no need for the use of multiple columns, thus leading to a decreased need for equipment maintenance, quantity of resins, buffers, and other solutions, and operator time. Furthermore, unlike the currently employed method of synthesis, this method of synthesis does not result in the product of daptomycin isomers, resulting in decreased yield and purity. Lastly, while *S.roseosporus* requires a 96 hour culture period and a 140 hour period for production of daptomycin, *E.coli* cultures are able to double in number every 40 to 65 minutes under optimal conditions (Miao 2005 and Plank 1979). As *E.coli* is gram-negative, it is thus not adversely affected by the presence of daptomycin (Huber 1988).

Cost Analysis

TABLE 1: Recurring Production Costs- Based on the Production of 1g of Daptomycin

Item	**Product Number**	**Company**	**Unit Price**	**Amount Needed**	**Need Based Cost**
2.5% EDTA	EW-86979-29	Cole-Parmer	$32.00 / 1L	100mL	$3.20
99% Imidazole	EW-88120-00	Cole-Parmer	$14.70 / 5gal	100mL	$0.07
Fmoc	EW-88257-58	Cole-Parmer	$30.80 / 1g	0.747g (0.0024 mol)	$23.01
DCC	36650	Cole-Parmer	$373.50 / 2.5kg	10g	$1.50
n-Decanoic Acid	EW-88177-25	Cole-Parmer	$106.30 / 500g	0.1g (0.0006 mol)	$0.02
TAGZyme pQE Vector Set	32932	Qiagen	$364.00 / 25µg	1µg	$14.56

NHS	130672	Sigma-Aldrich	$80.00 / 100g	10g	$8.00
Piperidine	571261	Sigma-Aldrich	$56.90 / 200mL	50mL (0.0024 mol)	$14.23
Ultrafiltration Membrane Disc Filters (1000kD, 3000kD)	OM 001025	Pall Corporation	$109.10 / 12	2 per 1g of daptomycin	$18.18
Sodium Phosphate	EW-88064-11	Cole-Parmer	$73.50 / 1kg	90mM	$1.00
Distilled Water	15230-001	Invitrogen	$101 / 6L	1L	$16.83
NaCl	7647-14-5	ScienceCompany	$8.95 / 500g	29.22g (0.5 mol)	$0.52
Nickel Chloride	CHEMALDNN 0140-10g	Online Science Mall	$4.50 / 10g	10g	$4.50

Total Recurring Cost for Each Gram of Daptomycin Produced: $105.62
(Assuming the cost of labor and building costs to be relatively small in comparison to raw materials)

Research Design: Develop Oral Delivery of Cubicin

Chitosan-Mediated Oral Drug Delivery Platform

The oral delivery of daptomycin is complicated because daptomycin must pass through the stomach. The stomach has a low pH of 2 due to the presence of hydrochloric acid. It also contains the proteolytic enzymes, which cleave peptide bonds indiscriminately. The highly acidic environment and the presence of proteolytic enzymes (such as pepsin) in the stomach increase the rate of degradation. This poses a problem because daptomycin cannot reach the intestine intact for absorption, hindering all possible therapeutic effects. Therefore, we propose the use of two coats in order to protect Cubicin from the stomach environment and to facilitate daptomycin absorption in the small intestine. These two coats are easily absorbed so the daptomycin delivery is efficient. Also, there have been no unintended pharmacological effects associated with these two coats as they are fully degraded within the gastrointestinal system.

The inner coat, encasing daptomycin, will be a chitosan capsule which promotes the uptake of daptomycin in the small intestine. The outer coat, encasing the daptomycin-chitosan complex, will be a polymer called hydroxypropyl methylcellulose phthalate (HPMCP). HPMCP is designed to be degraded by the stomach environment. In the small intestine, chitosan will be degraded allowing release of daptomycin. The intestine has a pH of 7-8, which will not degrade daptomycin as strongly as with the pH conditions of the stomach. In addition, there are very few proteolytic enzymes in the small intestine, so daptomycin will not be degraded upon release from the chitosan capsule.

Chitosan is a "noninvasive mucoadhesive polysaccharide capable of opening the tight junctions between epithelial cells." (Amidi 2008) It is an absorption enhancer for hydrophilic drugs through mucosal membranes (Singla 2010). Chitosan is able to promote absorption by interacting with the opening mechanism of tight junctions in causing a decrease of ZO-1 proteins and change of cytoskeleton f-actin to g-actin. Chitosan is comprised of copolymers glucosamine and N-acetylglucosamine and is derived from chitin in crustacean shells. It is also naturally present in fungi such as yeast, making it a very easy to grow and harvest.

Chitosan refers to a series of polymers of varying molecular weights (50-2000 kDa), viscosity, and degree of deacetylation (48%-90%). It is insoluble at neutral and alkaline pH values and can form salts with various acids such as glutamic acid, hydrochloric acid, and acetic acid, by which dissolution

occurs allowing for drug release (Illum 1998). Chitosan is nontoxic, and its biocompatibility and biodegradability make it an ideal candidate for oral drug delivery (Hejazi 2003). Figure 16 shows the structure of chitosan and Figure 17 shows how chitosan is derived from the chitin in crustacean shells.

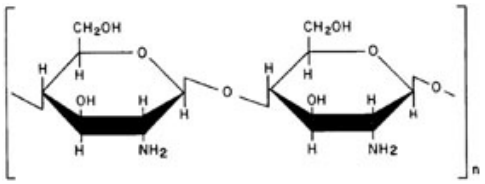

FIGURE 16: Structure of chitosan [(1- 4) 2-amino-2-deoxy-b-D-glucan]

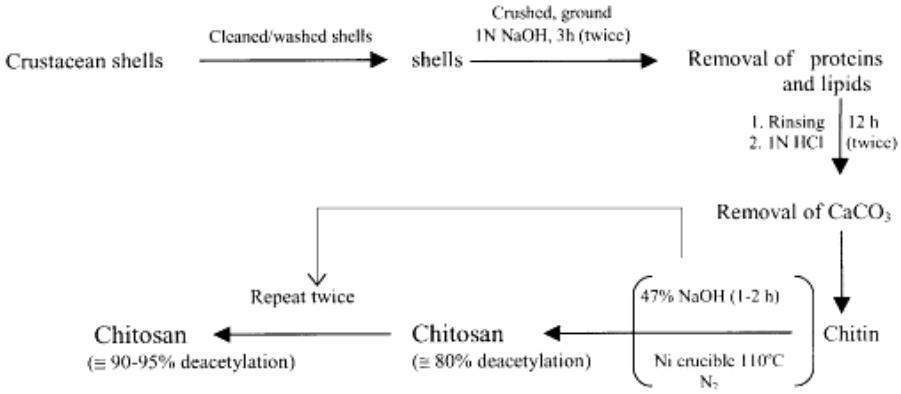

FIGURE 17: Synthesis of chitosan

The solubility of chitosan can depend on many different factors, which can be adjusted depending on the desired time, location, and duration of drug release. One of these factors is degree of deacetylation. Chitosan with a low degree of deacetylation is soluble up to a pH of 9; chitosan with a high degree of deacetylation is soluble up to a pH of 6.5. The solubility also depends on the weight fraction of chitosan in the tablet. It was found that chitosan needed to be at least fifty percent of the tablet's weight for the tablet to be minimally effective as a time release vessel. Solubility can also be adjusted by crosslinking of chitosan polymers with glutaraldehyde, which changes the swelling and gel forming properties of the polymer, allowing for sustained release at intestinal pH (Hejazi 2003).

Our target site of drug release is the small intestine, so we propose adding an enteric coating to the chitosan-drug complex to protect it from the stomach environment. The enteric coating consists of hydroxypropyl methylcellulose phthalate (HPMCP) and also contains additional enzyme inhibitors. HPMCP will be integrated with the chitosan shell through ionic cross-linking, creating a pH sensitive polymer (Makhlof 2010). Figure 18 below shows the structure of HPMCP.

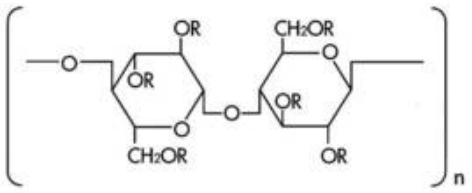

FIGURE 18: Structure of HPMCP [hydroxypropyl methylcellulose phthalate]

When the chitosan-drug-HPMCP complex passes through the stomach, HPMCP disintegrates in the stomach leaving the chitosan-drug complex intact to go onto the small intestine. Note that degradation of HPMCP in the stomach is over a sustained period of time, due to enzyme inhibitors that help preserve the integrity of HPMCP as it passes through the stomach. In the small intestine, chitosan binds and interacts with the tight junctions of the mucosal membrane, allowing for the daptomycin to be released into tissue (Illum 1998).

Since the both chitosan and HPMCP coats are degraded in the digestive tract, we do not have to worry about possible interactions/side effects of these coats, making this the ideal oral delivery system for daptomycin. Indeed, the biggest advantage to using HPMCP and chitosan coats for drug delivery is that its toxicity is negligible. There have been no macroscopic or microscopic effects found in any organs or tissues (Illum 1998).

Cost Analysis

Chitosan capsules can be prepared by obtaining Aicello Chemical Company Capsules. These capsules contain 1.0 mg CF or insulin with 14 mg of bacitracin, 4.0 mg aprotinin, and 3.0 mg of STI as protease inhibitors (Tozaki 1997). The mean diameter of the capsule is 3.5 mm x 1.6 mm. The capsules were coated with hydroxypropyl methylcellulose phthalate (HPMCP) by dissolving HPMCP in a w/w 1:1 solution of acetone:ethanol (Tozaki 1997). The drugs reached the small intestine in two to six hours and the large intestine in six to twelve hours (Tozaki 1997).

We intend to encase the daptomycin in a chitosan shell as proposed by Tozaki *et al*. Chitosan is $13 for 500 mg, but the capsules may be bought from the Aicello Chemical Company. HPMCP is 500g for $104.50 and available through the Cole Parmer company.

TABLE 2: Start-Up Cost of Acquiring Capital Goods and Equipment Necessary for Our Proposal

Product	**Catalog Number**	**Company**	**Unit Price**	**Quantity Needed**	**Cost**
High Density Nickel Agarose Resin	AC-303-500	Jena Bioscience	$18000 / 500mL	100mL	$3600
PBS (10X)	EW-88067-22	Cole-Parmer	$63.30 / 1L	1	$63.30
Ethanol (200 Proof)	EW-89000-60	Cole-Parmer	$75.00 / 4L	1	$75.00
AccuLab V1 Series Scale	V1-10kg	Lab Supply Outlaws.com	$260.00	1	$260.00
Ampicillin (100mg / mL)	*A5354*	Sigma-Aldrich	$64.90 / bottle	2	$129.80

BAXTER Megafuge Tabletop Centrifuge	460522	DOTmed	$1,900.00	1	$1,900.00
Microwave (Used)	N/A	Amazon.com	$58.09	1	$58.09
Sterile Plastic Petri Dishes	B001S4D36A	Amazon.com	$7.50 / 20 plates	5	$37.50
xTractor Buffer-Cell Lysis Buffer	635625	Clontech	$276.00 / 500mL	2	$552.00
Tris Buffer (2M)	EW-88058-14	Cole-Parmer	$54.10 / 100mL	2	$108.20
Hybaid Omn-e Thermal Cycler PCR Machine	431957	LabX	$950.00	1	$950.00
Taq DNA Polymerase	10342-020	Invitrogen	$269 / 500 Units	1	$269.00
dNTPs (10mM)	18342-013	Invitrogen	$75 / 100µL	1	$75.00
Primers	N/A	Invitrogen	$11.40 / primer	4	$45.60
E.coli	BLB1016	Science Stuff	$10 / 0.5 lb	1	$10.00
Distilled Water	15230-001	Invitrogen	$101 / 6L	2	$202.00
PCR Tubes	AM12230	Invitrogen	$143 / 1000 tubes	1	$143.00
Eppendorf Tubes	EW-02560-34	Cole-Parmer	$56 / 500 tubes	2	$112.00
15mL conical screw-cap tubes	1475-0501	USA Scientific	$77.00 / 500 tubes	2	$154.00
UNICO Powerspin Vx Centrifuge (Microcentrifuge)	UI-CE-C816	LabPlanet.com	$249.64	1	$249.64
0.5-10µL Pipette	UPVV-10	Online Science Mall	$144.95 / each	4	$579.80
5-50µL Pipette	UPVV-50	Online Science Mall	$144.95 / each	4	$579.80
10-100µL Pipette	UPVV-100	Online Science Mall	$144.95 / each	4	$579.80
20-200µL Pipette	UPVV-200	Online Science Mall	$144.95 / each	4	$579.80
100-1000µL Pipette	UPVV-1000	Online Science Mall	$144.95 / each	4	$579.80
25mL Pipettes	CLS4489	Sigma-Aldrich	$227 / 200	2	$454.00
Unifit Zero-Fluid-Retention Tips 10µL	UN0010-BP	Pipette.com	$16.00 / 1000	2	$32.00
Unifit Zero-Fluid Retention Tips 200 µL	UN020G-BP	Pipette.com	$18.00 / 1000	2	$36.00
Graduated Cylinder Set (10, 25, 50, 100, 250mL) with	N/A	Amazon.com	$68.99	2	$137.98

Graduated Beaker Set (50, 100, 250, 600, 1000mL) with Erlenmeyer Flask Set (50, 125, 250, 500, 1000mL)					
1L Graduated Cylinder	B0017UE9GS	Amazon.com	$9.15	1	$9.15
1L Erlenmeyer Flask	B001BLOHVK	Amazon.com	$21.95 / each	2	$43.90
UltraSpec-Agarose Powder	WL54470-10	Sargent-Welch	$31.90 / 20g	2	$63.80
Model Horizon 58 Agarose Gel Electrophoresis Apparatus	N/A	LABREPCO	$402.00	2	$804.00
Gel Electrophoresis Powerpack	GT-250 Power Pack	G. BioSciences	$496.00 / each	2	$992.00
Ethidium Bromide	WU-88056-85	Cole-Parmer	$53.70 / 10mL	1	$53.70
LB Broth	0102	Athena Enzyme Systems	$45.00 / 500g	3	$135.00
25x450 mm epoxy coated Chromatography Column	5795-40	Ace Glass Inc.	$71.46 / each	8	$571.68
Pyrex KIMAX Flask Set (200 mL, 500 mL, 1000 mL)	N/A	RecycledGoods.com	$50.15	2	$100.30
Microflex Laboratory Gloves Diamond Grip Plus Latex Gloves	N/A	Pro Safety Supplies	$9.85 / box	10	$98.50
Safety Glasses	174130	Lab Safety Supply	$5.65 / each	8	$45.20
Qiagen Plasmid Mini Kit (100)	12125	Qiagen	$561.00	1	$561.00
Marvel Scientific ADA-Compliant Ice Maker	107067	Lab Safety Supply	$2129.00	1	$2,129.00
Polyurethane Foam Ice Bucket	EW-06274-21	Cole-Parmer	$88.00 /each	2	$176.00
Eppendorf Tube Holders	B0017YAPI0	Amazon.com	$26.81 / 4	2	$53.62
Lab Line 2001 Mult Blok Heater Heat Block	N/A	Ebay	$292.81 / each	1	$292.81
PRECISION Water Bath	66562	DOTmed	$450.00 / each	1	$450.00
VWR Thermo	SB70P	LabPlanet.com	$645.25 /	1	$645.00

Fisher Scientific Symphony pH Meters Benchtop Meter			each		
pH Indicator Paper (1-14)	33811	Indigo.com	$8.70 / 50 strips	2	$17.40
Model SI4 Benchtop Shaking Incubator	1570	ShelLab	$7029.00	1	$7029.00
12x77mm polypropylene culture tubes	1450-0810	USA Scientific	$77.25 / 500 tubes	2	$154.50
Synthware Round-Bottom Flasks, Single-Neck	60002-298	VWR.com	$14.82 / each	4	$59.28
Mettler Sonicator	ME740X	American 3B Scientific	$1695.00	1	$1695.00
Pyrex Funnel	92613	American Science and Surplus	$5.50 / each	4	$22.00
C.B.S. Scientific 4 Plc Conical Tube Rack	RA-415	LabPlanet.com	$97.24 / 4 pieces	2	$194.48
Lab Coats	AD-805	Just Lab Coats	$14.95 / each	10	$149.50
Aegis Refrigerator 4°C	1-RG-25-BB 25	Summit Surgical Technologies	$10,989.60 / each	1	$10,989.60
Thermo Scientific Revco Value Series Ultra-Low Temperature Freezer	ULT1386-E-D	Cooler Direct	$10,008.38	1	$10,008.38
High Density Nickel Agarose Resin	AC-303-500	Jena Bioscience	$18000/500mL	1	$18000
2.5% EDTA	EW-86979-29	Cole-Parmer	$32.00 / 1L	1	$32.00
99% Imidazole	EW-88120-00	Cole -Parmer	$14.70 / 5gal	1	$14.70
Fmoc	EW-88257-58	Cole-Parmer	$30.80 / 1g	2	$61.60
n-Decanoic Acid	EW-88177-25	Cole-Parmer	$106.30 / 500g	1	$106.30
TAGZyme pQE Vector Set	32932	Qiagen	$364.00 / 25 ug of each component	1	$364.00
DCC	36650	Cole-Parmer	$373.50 / 2.5kg	1	$373.50
NHS	130672	Sigma-Aldrich	$80.00 / 100g	1	$80.00

Piperidine	571261	Sigma-Aldrich	$56.90 / 200mL	2	$113.80
Ultrafiltration Membrane Disc Filters (1000kD, 3000kD)	OM 001025	Pall Corporation	$109.10 / 12	2	$218.20
Sodium Phosphate	EW-88064-11	Cole-Parmer	$73.50 / 1kg	1	$73.50
Distilled Water	15230-001	Invitrogen	$101 / 6L	1	$101.00
NaCl	7647-14-5	ScienceCompany	$8.95 / 500g	2	$17.90
Nickel Chloride	CHEMALDNN0140-10g	Online Science Mall	$4.50 / 10g	2	$9.00
Hydroxylpropyl methylcellulose phthalate 55	EW-88349-30	Cole-Parmer	$104.50 / 500g	1	$104.50
Chitosan	N/A	Aicello Chemical Company	$13/500mg	5	$65.00

Cost of Capital Goods Required for Start-Up: $75,933.31

Recommendations

Cubist Pharmaceuticals should attempt to identify fast, easy and cost effective ways to produce Cubicin. The low availability and high cost of Cubicin are major contributors to the spreading of infections due to vancomycin resistant bacteria. Additionally, Cubicin can only be administered through IV which is uncomfortable for the patient and requires hospitalization. Our method introduces a way to administer Cubicin orally which will not only alleviate discomfort, but also lower hospital costs for patients. Increased availability and lower costs will make this Cubicin variant a more attractive option for physicians to prescribe which will increase profits. By using our new mass production method, we can take even better advantage of economies of scale. Since Cubicin is the last line of defense for vancomycin resistant bacterial infections, lowering costs and increasing availability will be considered a humanitarian effort and increase public perception of the company, likely benefiting our shareholders. Making Cubicin production more viable will essentially introduce a new tool for society to use against not only vancomycin resistant bacterial infections, but other gram-positive bacterial infections as well. This will also be more accessible to those with limited health care resources. Cubicin production is essential to the survival of our society and our methods of synthesis and delivery will certainly aide pharmaceuticals and society alike.

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