SYNTHETIC APPLICATIONS OF HOMOCHIRAL GLYCIDIC ESTERS DERIVED FROM ENZYMATIC REDUCTIONS

By

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by

Brent Derek Feske
This document is dedicated to my loving parents.
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SYNTHETIC APPLICATIONS OF HOMOCHIRAL GLYCIDIC ESTERS DERIVED FROM ENZYMATIC REDUCTIONS

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Chair: Jon D. Stewart
Major Department: Chemistry

A library of eighteen known bakers' yeast reductases has been screened for their ability to reduce several \( \alpha \)-chloro-\( \beta \)-keto esters. By using these enzymes in whole-cell biotransformations an easily scaleable method to synthesize gram quantities of homochiral chlorohydrins can be achieved. A stereoselective, base catalyzed ring closure can be used to transform these compounds into enantiopure glycidic esters, which are useful intermediates to several biologically active molecules.

Using a whole-cell reduction and a Ritter reaction as key steps, we were able to develop a new route to both antipodes of the C-13 Taxol side chain and a formal total synthesis of \((-\)-Bestatin. We synthesized the protected form of the Taxol side chain in four steps with an overall yield of 49%. \((2S, 3R)-3\)-Amino-2-hydroxy-4-phenylbutyric acid was synthesized in six steps with an overall yield of 42%, thus completing a formal synthesis of \((-\)-Bestatin.
Depending on the substrate, we were able to consistently yield 1 to 4.5 g/L of product for our whole-cell biotransformations. Thus, the overexpression of bakers’ yeast reductases in whole-cells proved to be an adequate and scaleable method for the synthesis of homochiral chlorohydrins.
CHAPTER 1
HISTORICAL BACKGROUND OF TAXOL

Discovery of Taxol

In 1960, the National Cancer Institute established a program that purified organic molecules from biological samples and screened the compounds for their pharmaceutical activities. This program was triggered by the early success of Beer et al., who found the antileukemic agents vinblastine 1 and vincristine 2 in periwinkle leaves from Madagascar. \(^1\) Throughout this program’s 22 year tenure, only two compounds were found to show medicinal potential, Taxol 3 (paclitaxel) and camptothecin 4 (Figure 1-1).

![Chemical structures of vinblastine, vincristine, Taxol, and camptothecin](image)

Figure 1-1. Natural products with anticancer activity: Vinblastine 1, Vincristine 2, Taxol 3, Camptothecin 4.

Taxol was isolated from the bark of the Pacific Yew tree (Taxus brevifolia) and shown to have anti-tumor activity in 1962. Even though Taxol showed...
promising anti-cancer activity, further testing slowed because it was highly insoluble in water, which would make it almost impossible to deliver to patients. In addition, as the demand for Taxol increased, the supply of Pacific Yew trees and thus Taxol was diminishing.

In 1979, there was a breakthrough in understanding Taxol’s anti-cancer activity. Susan B. Horwitz, a molecular pharmacologist, along with Schiff et al. found that Taxol’s mode of action was completely different from those of traditional cancer drugs. Many of these drugs destabilize a cell’s ability to make microtubules, which are essential for cell replication. Taxol’s mode of action is actually the opposite: it stabilizes the microtubules during cell replication, preventing their separation (Figure 1-2). Due to the cells’ inability to divide they will eventually grow so large as to trigger their own death.

Figure 1-2. Taxol bound to a tubulin α,β dimer
This newly discovered mode of action accelerated the clinical trials of Taxol. After many years of thorough testing, the Food and Drug Administration (FDA) approved Taxol for the treatment of ovarian and breast cancer in 1992. This newly approved and promising cancer drug quickly developed a strong commercial demand. This led to a problem, however, since the slow growing Pacific Yew tree was considered near the brink of extinction and this precluded an adequate supply of Taxol for the clinical use by isolating the natural product. To solve this problem, chemists were given the task of producing what would become the largest selling cancer drug ever placed on the pharmaceutical market, yielding sales in the billions of dollars.

**Synthetic Strategies for Taxol**

The isolation of Taxol from the Pacific Yew tree has low yields, so auxiliary strategies have been developed to obtain this drug. These include total synthesis, plant tissue cultures, engineering of a Taxol producing fungus, and the coupling of Baccatin III (which contains the ring structure of Taxol) with the Taxol side chain. Due to low yields and the high number of steps, the total synthesis of Taxol is unlikely to become an option for commercial production. Ongoing work on the engineering of plant tissue cultures continues, but many scientists believe that plant cells are too difficult to manipulate for high Taxol yields. As a result, engineering the Taxol producing fungus and semi-synthesis by coupling of Baccatin III to the side-chain are considered the primary strategies for commercial production.
Total Synthesis of Taxol

In 1971, the Wani et al. group published the structure of Taxol 3, which is composed of a complex poly-oxygenated diterpene and a phenylisoserine side-chain.\(^3\) As a result, synthetic chemists were intrigued by the challenge of synthesizing such a complex organic molecule. It took nearly 20 years, but in 1994 the Holton group and Nicolaou group almost simultaneously reported the total synthesis of Taxol. Since that time, four more total syntheses have been completed on Taxol.

The Holton lab approach\(^4,5\) used (-)-camphor 5 as the starting material for a strategy in which the A and B rings of Taxol were created first, then this unit was fused to the C ring (Scheme 1-1). The oxetane (D) ring was subsequently formed through a tosylate intermediate. This synthesis was composed of 41 steps with an overall yield of 2%.

Scheme 1-1. Summary of Holton’s approach

The Nicolaou group\(^6-9\) first constructed the A and C ring systems from hydrazone 8 and bicyclic aldehyde 9, followed by a McMurry cyclization to form the ABC ring system intermediate 11 (Scheme 1-2). The D ring was added through the formation of a triflate silyl ether intermediate and treated under mildly acidic conditions to make the oxetane. This total synthesis of Taxol was completed in fifty-one steps with an unreported overall yield.
Scheme 1-2. Summary of Nicolaou’s approach

The Danishefsky group\textsuperscript{10} used the Wieland - Miescher ketone \textsuperscript{12} and trimethylcyclohexane-1,3-dione \textsuperscript{13} as starting materials for the synthesis of the C and D rings (Scheme 1-3). They then used an intramolecular Heck reaction to fuse the AB rings to the CD ring structure. This synthesis required 47 steps and resulted in an overall yield of less than 0.1%.

Scheme 1-3. Summary of Danishefsky’s approach

The Wender group\textsuperscript{11} utilized \(\alpha\)-pinene \textsuperscript{15} as a matrix to make the A and B rings through a fragmentation technique with an epoxy alcohol (Scheme 1-4). The C ring was added via an aldol condensation and then formation of the D ring following in several steps by direct closure of the diol. This synthesis of Taxol was accomplished in 37 steps with an unreported overall yield.
Scheme 1-4. Summary of Wender’s approach

In 1997, the Mukaiyama group\textsuperscript{12,13} reported the total synthesis of Taxol utilizing L-serine \textsuperscript{18} as the starting material (Scheme 1-5). In their strategy, the BC ring system was synthesized via a pinacol coupling cyclization; then addition of the A ring followed. In subsequent steps they added the D ring and the Taxol side-chain, thereby affording Taxol with an unreported yield.

Scheme 1-5. Summary of Mukaiyama’s approach

The Kuwajima group’s\textsuperscript{14} synthesis started with a Peterson olefination to afford dienol silyl ether \textsuperscript{21} to form the A ring (Scheme 1-6). Next, 2-bromobenzaldehyde dibenzylacetal \textsuperscript{22} was used to synthesize the C-ring fragment. The coupling of the A and C rings and subsequent cyclization to form the B ring resulted in the tricarbocycle. This was converted to Taxol after several additional steps in an unreported overall yield.
Scheme 1-6. Summary of Kuwajima’s approach

**Isolation of Taxol from Plant Tissue Cultures**

Large-scale plant cell cultures have been shown to be useful sources of certain natural products.\(^{15-17}\) This usually requires a cell selection with medium optimization, genetic engineering, elicitation of enzyme systems, precursor feeding, and overall process optimization. Research on the optimization of Taxol producing plant cells is still ongoing; however, due to the difficulty of engineering plant cells it is not expected to be a practical route for the production of Taxol.\(^{18-20}\)

As an example, Chang *et al.* recently collected tissues from *Taxus mairei*, a plant found in Taiwan at an altitude of 2000 m above sea level, and discovered that the amount of Taxol and Taxol derivatives found in this plant were higher than the pacific yew and other Taxus species.\(^{21}\) However, when using callus cells from *Taxus mairei*, an optimized cell line was only able to produce 200 mg/L of Taxol after a six week incubation period.

**Taxol Producing Fungus**

In 1993, Stierle *et al.* found that Taxol is produced by the fungus *Taxomyces andreanae*.\(^{22}\) Unfortunately, Taxol is only produced at
concentrations of 25-50 ng/L in this organism. On the other hand, fungal cells can be engineered more easily than plant cells, so the future of using engineered cells for the production of Taxol is promising.

The Croteau lab has taken on the challenge of fully deciphering the Taxol biosynthetic pathway in *Taxomyces andreanae*. His group has found that there are 19 enzymatic steps from the basic geranylgeranyl diphosphate used as the isoprenoid precursor. The biosynthesis of Taxol begins with the 2-C-methyl-D-erythritol phosphate pathway (MEP) (Scheme 1-7). Isopentenyl diphosphate and dimethylallyl diphosphate are then combined to form geranylgeranyl diphosphate (GGPP) from geranylgeranyl diphosphate synthase. The next step is the cyclization of GGPP to taxadiene by taxadiene synthase. Over the next three steps, taxadiene is decorated with an alcohol and acetate functionality. The order of additional oxygenations beyond this point has not been totally deciphered.
Scheme 1-7. The biosynthetic pathway to Baccatin III

Once Baccatin III 30 has been formed, the C-13 Taxol side chain is added. The side chain begins from α-phenylalanine 31, which is converted to β-phenylalanine 32 by phenylalanine aminomutase PAM (Scheme 1-8). In the next step, 32 is activated as the corresponding CoA thioester followed by an aroyl transfer to Baccatin III resulting in 33. Lastly, the side chain is hydroxylated at the C-2 position and then N-benzoylated to afford the biosynthetic product, Taxol 3.

Scheme 1-8. The biosynthetic pathway of Taxol side chain and its coupling to Baccatin III

The strategy for engineering the Taxol-producing fungus is to locate the bottlenecks in the pathway and overexpress the genes responsible for the slow steps. This plan also includes knocking out competitive pathways that may lead to undesirable products; this can be done by adding elicitors or by gene
knockout. If these two strategies are successful, it would increase the concentration of the final product and also allow for easier purification.

**Semi-synthesis of Taxol: Synthesis of the Taxol Side Chain**

Taxol’s path to becoming a commercial drug was an uphill battle, but in the late 1980’s it became evident that Taxol would soon become FDA approved. Since a commercial source had not yet been found, Pierre Potier *et al.* began to study this problem. They found and extracted a compound from the Pacific Yew bush (*Taxus baccata*), which contains the terpene core of Taxol. This compound, 10-deacetylbaccatin III 34, can be isolated from the leaves of the bush in high yield. In addition, only the leaves are removed from the bush, which can be regenerated by the plant, allowing it to be a renewable source. Upon isolation of 10-deacetylbaccatin III 34, it can be coupled to the protected form of the Taxol side chain 35 and/or 36 yielding the full structure of Taxol 3 (Scheme 1-9). Greene *et al.* developed a side chain synthesis and coupling procedure; however, it resulted in an enantiomeric excess (e.e.) of only 78%. Due to the low enantiomeric excess, this synthesis did not meet the purity standards set by the FDA.
To answer the commercial demand for Taxol, Dr. Robert Holton developed a metal alkoxide process for the Taxol semi-synthesis in the early 1990’s with a 74% overall yield. This patent was licensed by Bristol-Myers Squibb and has been used for the commercial production of Taxol since its approval in 1993. Since then, a number of different approaches to the Taxol side chain have been reported. These approaches are listed within three categories: asymmetric metal catalysis, enzymatic catalysis, and enantiomer separation.

**Asymmetric metal catalysis**

The main challenge for the synthesis of the Taxol side chain is to define the stereochemistries of the C-2 and C-3 positions with high selectivities. Popular solutions to this product involve asymmetric metal catalysts that afford the desired enantiomer in good to high enantiomeric excess.

In 1986, The Greene lab was the first to publish the synthesis of the Taxol side chain using this approach (Scheme 1-10). He applied the Sharpless epoxidation methodology to cis-cinnamyl alcohol 37 to afford chiral epoxy alcohol
38 in 78 % e.e., which was then oxidized and esterified by diazomethane to yield glycidic ester 39. Ring opening of the 39 with azidotrimethylsilane yielded azide 40. Reaction with benzoyl chloride under Schotten-Baumann conditions followed by hydrogenation resulted in the Taxol side chain 41.

Scheme 1-10. Greene’s Strategy to the Taxol side chain

The Jacobsen group synthesis started by a Lindlar reduction of ethyl phenylpropiolate 42 (Scheme 1-11). The key step used (salen) Mn (III) complex 48 as an inorganic asymmetric catalyst resulting in the glycidic ester 44. Ring opening with ammonia and hydrolysis of the amide resulted in α-hydroxy-β-amino acid 46. Addition of benzoyl chloride and treatment with aqueous hydrochloric acid afforded the Taxol side chain 47.
Scheme 1-11. Jacobsen’s strategy to the Taxol side chain

Sharpless et al. used an inorganic catalyst approach to carry out the asymmetric dihydroxylation of trans-methyl cinnamate 49 (Scheme 1-12). The syn-diol 50 was converted to acetoxy bromo ester 51 by reaction with trimethyl orthoacetate in the presence of a catalytic amount of p-TsOH. After reacting 51 with sodium azide, the acetoxyazide ester was hydrogenated giving N-acetyl-3-phenylisoserine 52. The amide ester was then hydrolyzed to afford the acid and benzoylated to the Taxol side chain 47.

Scheme 1-12. The Sharpless strategy to the Taxol side chain

Ham et al. synthesis began with the protection of L-phenylglycine 53, followed by treatment with N,O-dimethylhydroxylamine hydrochloride to afford
Weinreb amide 54 (Scheme 1-13). Reduction of 54 by lithium aluminum hydride followed by a Wittig reaction resulted in ester 55. Treatment of 55 with DIBAL gave alcohol 56, which was acetylated over a three step process resulting in 57. The key step was a palladium catalyzed oxazoline formation yielding only trans-compound 58. This was then oxidized to acid 59 by sodium periodate. The resulting acid 59 was reacted with diazomethane resulting in the protected Taxol side chain 60.

Scheme 1-13. Ham’s strategy to the Taxol side chain

The Barua lab began by reacting benzyl alcohol 61 and epichlorohydrin to afford epoxide 62 (Scheme 1-14). Treatment of 62 with 30% HClO₄ gave the
diol, which was oxidized to the aldehyde $63$ with Pb(OAc)$_4$. The key step of this synthesis was a Shibasaki asymmetric Henry reaction with aldehyde $63$ and phenylnitromethane $64$ yielding alcohol $65$ with a 90% e.e. Simple acetylation of the Henry adduct, followed by a hydrogenation resulted in $67$. After oxidation of the alcohol using CrO$_3$, $68$ was benzylation with benzoyl chloride and then the acetate was hydrolytically removed to the C-13 Taxol side chain $47$.

Scheme 1-14. Barua’s strategy to the Taxol side chain

**Enzyme catalysis**

Another strategy to afford the proper stereochemistry at the C-2 and C-3 positions of the Taxol side chain involves the use of enzymes. Typically, acylases or lipases are the enzymes of choice for organic chemists. These enzymes can be an efficient source for chirality; however, the maximum yield possible from the racemate is fifty percent. Despite these problems, there have been several published syntheses using enzymes as a key step in their synthesis.
The Kim lab’s Taxol side chain synthesis began with a kinetic resolution of racemic diol 69, using the lipase from *Pseudomonas cepacia* (Scheme 1-15).\(^{36}\) Unlike other kinetic resolutions, in which there is at least a fifty percent loss in yield, Kim’s synthesis utilized both lipase products as precursors for the Taxol side chain. After the enzymatic reaction, 70 was tosylated, which allowed for ring closure by the addition of a weak base. Glycidic ester 39 was then reacted with sodium azide to form 40. The other enzymatic product, 72, was brominated by hydrobromic acid and acetic acid to afford 73. Reaction of 73 with sodium azide and then sodium acetate also gave compound 40. These compounds were combined and treated with benzoyle chloride, followed by a palladium catalyzed hydrogenation resulting in the Taxol side chain 41.
Scheme 1-15. Kim’s strategy to the Taxol side chain

In 1999, Kayser and Stewart et al. also used an enzymatic approach to synthesize the Taxol side chain. They developed two syntheses, both utilizing stereoselective ketone reductions by bakers’ yeast.

In the first strategy, racemic glycidic ester 74 was opened with sodium azide, then the resulting alcohol was oxidized by Jones reagent to yield 75 (Scheme 1-16). Incubation of racemic 75 with bakers’ yeast gave the diastereomeric reduction products 76. Syn-Azido alcohol 40 was purified by column chromatography and the Greene strategy (Scheme 1-10) was used to complete the Taxol side chain 41.
Scheme 1-16. Kayser and Stewarts’s first strategy to the Taxol side chain using bakers’ yeast

The second approach began with the LDA-mediated addition of 77 and 78, resulting in racemic β-lactam 79 (Scheme 1-17). The ketal was hydrolyzed with concentrated sulfuric acid, then the ketone was reduced by incubation with bakers’ yeast resulting in three of the four possible diastereomers. syn-Alcohols 81 and 82 were separated from 83 by chromatography to yield a protected form of the Taxol side chain with an enantiomeric excess of 82%.

Scheme 1-17. Kayser and Stewart’s second strategy to the Taxol side chain

The Cardillo group synthesized racemic 88 by reaction of benzaldehyde 85 with malonic acid 86 and ammonium acetate 87, which was subsequently benzoylated with benzoyl chloride (Scheme 1-18). Incubating 89 with penicillin G acylase resulted in formation of homochiral β-amino acid 91. This was benzoylated and reacted with thionyl chloride and methanol, resulting in methyl
ester 92. The addition of LiHMDS produced the lithium dianion, which after reacting with iodine, led to rearrangement product 60. Refluxing oxazoline 60 with weak aqueous hydrochloric acid afforded the Taxol side chain 41.

Scheme 1-18. Cardillo’s strategy to the Taxol side chain

The Hamamoto lab’s synthesis approach began with the Darzens condensation of 85 and 93 to produce α-keto-β-chloro ester 94 (Scheme 1-19). KS-Selectride reduced 94 to give predominately anti-chlorohydrin 95. Lipase then resolved 95 to afford homochiral chlorohydrin 96, which was reacted with sodium azide to afford 40. Reaction of 40 with benzoyl chloride followed by a palladium catalyzed hydrogenation afforded the Taxol side chain 41.
Scheme 1-19. Hamamoto’s strategy to the Taxol side chain

The Mandai group began by reacting phenylacetic acid 97 with LDA to form the lithium dienolate, which was reacted with acrolein, then stirred with 3 N hydrochloric acid to afford acid 98 (Scheme 1-20). In the next step, 98 was esterified with allyl alcohol to give 99, which was reacted with Chirazyme® in 2-propenyl acetate and toluene to give 100 and 101. The latter was transformed into cyclic carbamate 102, via the Curtius rearrangement of the free acid produced by the palladium-catalyzed hydrogenolysis of the allyl ester. Carbamate 102 was then protected with (Boc)₂O and oxidized with ruthenium oxide and sodium periodate to afford acid 103. Treatment with 2 M sodium hydroxide resulted in ring opening, and trifluoroacetic acid was used to remove the Boc protecting group. In the last step, benzoyl chloride was used to afford the Taxol side chain 47.
Scheme 1-20. Mandai’s strategy to the Taxol side chain

The Botta group began by adding the Grignard salt of acetylene to benzaldehyde 85 (Scheme 1-21). Next, the racemic alcohol 104 was subjected to a Ritter reaction with acetonitrile and sulfuric acid. Alkyne 105 was then hydrogenated in the presence of Lindlar catalyst, and deacetylated by aqueous hydrochloric acid to yield 107. The enantioselective acetylation of 107 using Candida antartica lipase resulted in 108 and 109. Amide 109 was deacetylated with aqueous acid to afford 110, which was then benzyolated with benzoyl chloride. The addition of OsO₄ and NMO to 111 oxidized the alkene to a mixture of alcohol diastereomers. After oxidation with Jones reagent, L-Selectride yielded predominately 114. The Deoxo-Flour reagent was added which resulted in the ring closure product, oxazoline 115. Oxidation of 115 with PCC and then acid catalyzed hydrolysis resulted in the Taxol side chain 41.
Resolution of enantiomers

As seen above, popular published methods of the Taxol side chain use either an asymmetric metal catalyst or an enzymatic catalyst to afford the proper stereochemistry. However, some other strategies involve the racemic synthesis of the Taxol side chain, followed by the separation and purification of enantiomers.
The McChesney lab’s approach utilized the Darzen reaction of methyl chloroacetate 116 and benzaldehyde 85 (Scheme 1-22). 42 Reaction of 117 with dry hydrochloric acid opened the epoxide with retention of configuration at C-3 affording chlorohydrin 118. Ring closure using basic Amberlite 400 resin gave cis-glycidic ester 119. Ring opening with ammonia followed by benzoylation afforded 120. Reaction of 120 with the acidic Amberlite resin 120 in methanol resulted in the racemic Taxol side chain. This was then resolved by entrainment to afford 41 with an enantiomeric excess of 95%.

Scheme 1-22. McChesney’s strategy to the Taxol side chain

The Zhou group began with ammonolysis of the glycidic ester 121 to yield isoserineamide 122 (Scheme 1-23). 43 Benzoylation provided 123, and acid catalyzed methanolysis gave the methyl ester 124. The use of thionyl chloride and hydrochloric acid inverted the C-2 hydroxyl to produce 41, which was subsequently hydrolyzed to the racemic acid. This was resolved with R-(+)-α-
methyl benzylamine to provide the Taxol side chain 47 with an unreported enantiomeric excess.

Scheme 1-23. Zhou’s strategy to the Taxol side chain

As seen above, there are numerous approaches to the synthesis of the Taxol side chain. Many of these routes are often limited by the use of unsafe chemicals and/or conditions that are difficult for industrial scale-up. In addition, sometimes the synthetic routes do not yield a product with a high enantiomeric excess (e.e.) or diastereomeric excess (d.e.), which is essential for the commercial sale of pharmaceuticals. Many strategies to the Taxol side chain use reactions with lipase or the resolution of enantiomers, both of which can be very time consuming, thus inhibiting an industrial high-throughput process. With this said, it was our goal to develop an efficient and easily scaleable reaction for the homochiral C-13 Taxol side chain.

We proposed a five step synthesis for the Taxol side chain, which begins with the reaction of ethyl benzoylacetate 125 with sulfuryl chloride to afford α-chloro-β-keto ester 126 (Scheme 1-24). Ester 126 can be added to Escherichia coli (E. coli) that have overexpressed a single bakers’ yeast reductase, to yield
homochiral chlorohydrin 127. Treatment of 127 with a weak base should yield the optically pure cis-glycidic ester 128. Subsequent treatment of the epoxide with benzonitrile and a catalytic Lewis acid can result in trans-oxazoline 129. Oxazoline 129 can then be treated under mildly acidic conditions to form the optically pure Taxol side chain 130. This reaction scheme utilizes mild conditions and reagents that can be used on a large scale.

Scheme 1-24. Our proposed synthesis of the Taxol side chain

As seen in the proposed synthesis of the Taxol side chain we plan to utilize the reduction products of α-chloro-β-keto esters using a single bakers’ yeast reductase. The enzymatic product has four possible diastereomers, given by a dynamic kinetic resolution (Scheme 1-25). This is made possible through the low pKa of the α-proton in β-keto esters. The average pKa for this functionality is 10, which allows the α-carbon to quickly epimerize. Thus, if a reductase exhibits a preference for either substrate enantiomer, the rapid racemization re-establishes the equilibrium. This allows all of the starting material to be converted to a single diastereomer product. Once the substrate has entered the active site, the reductase will transfer a hydride from NADPH to the β-carbon
typically resulting in a highly stereoselective reduction. This characteristic is vital to our strategy, because it inserts two chiral centers in our scheme in a highly stereoselective manner.

Scheme 1-25. Dynamic Kinetic Resolution
CHAPTER 2
HISTORICAL BACKGROUND OF BESTATIN

Discovery of Bestatin

In 1975, The Umezawa group discovered and isolated an antitumor and antimicrobial agent from *Streptomyces olivoreticuli* named Bestatin 131 (ubenimex) (Figure 2-1).\(^{44}\) Bestatin was found while screening cultures of actinomycetes for their ability to inhibit aminopeptidase B. This screening was ignited by the recent findings that exopeptidases have a strong effect on mammalian cell surfaces.\(^{45}\) At that time, the research started as a hypothesis, but after 30 years, several aminopeptidase inhibitors are now used to treat a variety of cancers and antibiotic infections.

![Structure of Bestatin 131 (ubenimex)](image)

Figure 2-1. Structure of Bestatin 131 (ubenimex)\(^{46,47}\)

The function of Aminopeptidase B is to hydrolyze the N-terminal lysyl and arginyl residues from peptide substrates.\(^{44,48}\) Aminopeptidase B is also thought to play a role in processing various peptide signals and precursor enzymes, by binding to membrane macrophages and lymphocytes through membrane aminopeptidases.\(^{49,50}\) This binding induces a cascade of responses like increased cytokines, colony stimulating factors, and cell apoptosis.\(^{50-52}\)
Presently, Bestatin is used as an oral medication for the treatment of cancer and bacterial infection in Japan. In addition, Bestatin is often used in conjunction with other antibiotics and anticancer drugs because it causes the proliferation of T cells thus enhances the immune response.\textsuperscript{50} Bestatin also shows potential as an anti-inflammatory agent and for the treatment of HIV.\textsuperscript{53-59}

In 1976, shortly after its discovery, the Nakamura lab’s published the crystal structure of Bestatin, confirming that the compound was the dipeptide [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine.\textsuperscript{47} Upon verification of the correct configuration, Suda et al. began the first synthesis of this challenging molecule that contains three asymmetric centers. Since this publication, there have been several documented asymmetric syntheses of this popular anticancer and antimicrobial agent, which will be introduced in chronological order.

**Synthetic Approaches to Bestatin**

The Suda lab’s\textsuperscript{60} 1976 strategy began with Boc-protected D-phenylalanine \textsuperscript{132}, which was coupled with pyrazole to form pyrazolide \textsuperscript{133} (Scheme 2-1). This was reacted with 2 equivalents of lithium aluminum hydride to afford aldehyde \textsuperscript{134}. The diastereomeric mixture of bisulfite adducts \textsuperscript{135} was then reacted with sodium cyanide resulting in acyl cyanide \textsuperscript{136}. Hydrolysis of \textsuperscript{136} with 6 M hydrochloric acid gave the diastereomeric acid mixture, which was separated by chromatography to afford the natural occurring amino acid (2S, 3R)-(3-amino-2-hydroxy-4-phenylbutanoic acid (AHPA) \textsuperscript{137}. This was reprotected with Boc-Cl before it was reacted with benzoyl protected L-leucine. A simple hydrogenation of \textsuperscript{139} afforded Bestatin \textsuperscript{131} in an overall yield of 14%. 
Scheme 2-1. Suda’s strategy to Bestatin

The Umezawa lab’s$^{61}$ synthesis of Bestatin commenced by reacting $N$-acyl-$\alpha$-aminoacetophenone 140 and glyoxylic acid 141 to yield 142 (Scheme 2-2). This was then subjected to a palladium catalyzed hydrogenation, which reduced the benzylic carbon to afford 143. The racemate of 143 was resolved with $S$-(−)-$\alpha$-methylbenzylamine to give the optically pure salt 144. Refluxing 144 in aqueous hydrochloric acid gave the free amine 136 that was Boc-protected. The protected peptide 145 was formed by the DCC coupling of benzyl protected L-leucine. A simple hydrogenation resulted in Bestatin 131 in an overall yield of 10%.
Scheme 2-2. Umezawa’s strategy to Bestatin

Pearson and Hines\textsuperscript{62} synthesis began with dioxalanone 146, which underwent an aldol condensation with phenyl acetaldehyde resulting in the mixture of diastereomers 147 (Scheme 2-3). The diastereomers were separated by column chromatography and homochiral 147 was reacted with diphenylphosphoryl azide to form azido compound 148. Refluxing 148 in aqueous hydrochloric acid resulted in ester 149. The ethyl ester was saponified, followed by a DCC coupling of the acid and the benzyl protected L-leucine. The peptide 150 was then deprotected by a palladium catalyzed hydrogenation resulting in Bestatin 131.
Scheme 2-3. Pearson and Hine’s strategy to Bestatin

Norman and Morris’s synthesis began with the diesterification of L-malic acid 151 resulting in 152 (Scheme 2-4). This was benzylated under basic conditions and the adduct was saponified to diacid 153. This was selectively esterified by forming the cyclic anhydride intermediate, which was opened regioselectively by ethanol to form 154. A base catalyzed Curtius rearrangement with diphenylphosphoryl azide resulted in the protected compound 155. Ester 155 was saponified with lithium hydroxide, which was coupled using EDC with L-leucine methyl ester affording 156. This was deprotected with 1 M sodium hydroxide to yield Bestatin 131.
Scheme 2-4. Norman and Moris’s strategy to Bestatin

The Palomo group started their approach by coupling 157 and 158 to form \(\beta\)-lactam 159 (Scheme 2-5). This was followed by a two-step dehydroxylation of the benzyl carbon yielding 160. The \(\beta\)-lactam 160 was protected with \((\text{Boc})_2\text{O}\) and then reacted with L-leucine and sodium azide to form adduct 162. The addition of TFA followed by hydrogenation gave the deprotected compound 131.

Scheme 2-5. Palomo’s strategy to Bestatin

The Koseki group commenced their synthesis of Bestatin from 2,3-isopropylidene-D-ribose 163 (Scheme 2-6). The addition of phenyl magnesium bromide to the sugar gave diastereomeric mixture of Grignard adducts 164. The addition of sodium periodate gave the cyclic product 165, which was purified and
reacted with Jones reagent to form cyclic ester 166. A hydrogenation followed by a DCC coupled addition of benzyl protected L-leucine afforded amide 167. Diol 168 underwent a two-step process mediated by 1-methyl-2-fluoropyridine to afford azide 169, which was hydrogenated to afford Bestatin 131.

Scheme 2-6. Koseki’s strategy to Bestatin

Bergmeier and Stanchina\textsuperscript{66} began with the protection of mannitol 170, which was oxidized to acid 172 (Scheme 2-7). This was reduced by sodium borohydride to form aldehyde 173, which was subjected to a Wittig reaction, and subsequent acid treatment to afford allylic alcohol 175. This was selectively monosilylated with tert-butyldiphenylsilyl chloride, followed by reaction with CDI and sodium azide to form 177, which was then heated in a sealed tube to yield cyclized product 178. Aziridine 178 was reacted sequentially with phenyllithium
and TBAF to form 179, which was oxidized and coupled with protected L-leucine to provide dipeptide 180. The next three steps resulted in Bestatin 131 with an overall yield of 6% in 18 steps.

Scheme 2-7. Bergmeier and Stanchina’s strategy to Bestatin

The Seki lab’s approach began with protected L-aspartic acid 182, which was converted in three steps to oxazolidinone 183 (Scheme 2-8). Treatment of 183 with benzyl bromide afforded 184. The lithium enolate of 184 generated by LiHMDS and subsequent treatment with 3-phenyl-2-(phenylsulfonyl)oxaziridine gave the chiral alcohol 185. Hydrogenation of 185 resulted in amino alcohol 186,
which was then treated with benzyl chloroformate to protect the free amine.

Alcohol 187 was reacted with DEAD and formic acid resulting in formate 188. This 189 was reacted with L-leucine, then deprotected with hydrogenolysis to yield Bestatin 131.

Scheme 2-8. Seki’s strategy to Bestatin

The Semple group\(^6^8\) approached their synthesis by utilizing the coupling of 192 and 194 (Scheme 2-9). The synthesis began with \(N\)-\(\alpha\)-Cbz-\(D\)-Phe-H that was reduced over two steps to form aldehyde 192. Next, benzyl protected L-
leucine 193 was reacted over two steps to form isonitrile 194. After the addition of trifluoroacetic acid, 192 and 194 were reacted to form the diastereomeric mixture of dipeptides 195. After a simple hydrogenation, the diastereomers of 131 were separated by liquid chromatography, resulting in pure Bestatin 131 in a 13% overall yield.

Scheme 2-9. Semple’s strategy to Bestatin

The Park group\textsuperscript{69} synthesis began with an alkyne Grignard reaction on (R)-phenylalaninal resulting in predominately syn-197 (Scheme 2-10). O-Benzylaion of 197 afforded 199, and the alkyne was subsequently oxidized to acid 200. This underwent a DCC coupling to produce 201, which was subjected to a two-step deprotection process that resulted in Bestatin 131.
Scheme 2-10. Park’s strategy to Bestatin

In 2003, the Jurczak lab’s\textsuperscript{70} began their synthesis with aldehyde 202. They utilized a nitro aldol reaction with 1-nitro-2-phenylethane 203 (Scheme 2-11). The aldol product was purified by chromatography to yield homochiral 204. A Nitro group reduction by Raney-Ni hydrogenation, followed by the addition of a Boc protecting group resulted in 205. This was cyclized in the presence of DMP to the protected form of β-amino α-hydroxy acid 206. Addition of L-leucine to 206 resulted in protected dipeptide 207. This was followed by two deprotection steps that yielded optically pure Bestatin 131.
Scheme 2-11. Jurczak’s strategy to Bestatin

The Wasserman group’s synthesis began with N-Boc-D-phenylalanine 132 which was coupled with (cyanomethylene) triphenylphosphorane 208 to afford 209 (Scheme 2-12). This was reacted with ozone and L-Leu-OBn to form the doubly protected dipeptide 210. A stereoselective reduction with zinc borohydride resulted in 145 with a diastereomeric excess of 86%. Refluxing with aqueous hydrochloric acid followed by a palladium catalyzed hydrogenation resulted in Bestatin 131.
Over the past 30 years, there have been several documented strategies for the synthesis of Bestatin. Most of these utilize amino acids or other naturally occurring chiral compounds for their starting material. Other strategies require enantiomer separation by chiral resolution; however, this process can be very difficult and often inefficient. It was our goal to develop a novel synthesis for AHPA and Bestatin through our simple glycidic ester approach.

Our proposed synthesis begins with a literature procedure using meldrum’s acid 212 and phenylacetyl chloride 211 to yield 213 (Scheme 2-13). Reaction of β-keto ester 213 with sulfuryl chloride affords α-chloro-β-keto ester 214. Compound 214 can be added to E. coli that have overexpressed a bakers’ yeast reductase, affording (2R-3S)-chlorohydrin 215 as the homochiral reduction product. Treatment of chlorohydrin 215 with potassium carbonate will result in cis-glycidic ester 216. Epoxide 216 can be treated with boron trifluoride diethyl etherate and benzonitrile to afford the rearrangement product 217. Reflux with 6 M HCl should result in hydrolysis product (2S, 3R)-(3-amino-2-hydroxy-4-phenylbutanoic acid (AHPA) 137. Following the Suda lab’s synthetic strategy, AHPA 137 can be coupled with L-leucine over three steps to yield Bestatin 131.
Scheme 2-13. Our proposed synthesis of AHPA and Bestatin
CHAPTER 3
STEREOSELECTIVE, BIOCATALYTIC REDUCTIONS OF \(\alpha\)-CHLORO-\(\beta\)-KETO ESTERS

Introduction

Homochiral glycidic esters are versatile intermediates that can be converted into a variety of high-value products. Optically active glycidates can be prepared by a number of routes including asymmetric Darzens reactions, chiral alkene oxidation methodologies and by ring closure of homochiral \(\alpha\)-halo-\(\beta\)-hydroxy esters.\(^{72-86}\) We were particularly interested in the last strategy because asymmetric reductions of \(\alpha\)-chloro-\(\beta\)-keto esters might afford each of the four possible glycidate precursors via dynamic kinetic resolution processes from single, inexpensive starting materials (Scheme 3-1). Here, we explore the potential of individual reductase enzymes from baker’s yeast (\textit{Saccharomyces cerevisiae}) as solutions to the problem of obtaining homochiral glycidate precursors.

Reductions of \(\alpha\)-chloro-\(\beta\)-keto esters by whole cells of commercial baker’s yeast generally produce disappointing mixtures of alcohol diastereomers.\(^{87-90}\) Recent work has revealed that the yeast genome encodes a large number of reductases and it seemed likely that their simultaneous participation was mainly responsible for the modest stereoselectivities commonly observed in yeast-mediated ketone reductions.\(^{91-93}\) In response, we have adapted a fusion protein strategy\(^{94}\) that allows the properties of yeast reductases to be assessed.
Scheme 3-1. Four possible reduction products of α-chloro-β-keto esters: (2S-3S)-white, (2R-3S)-black, (2R-3R)-black/white lines, (2S-3R)-black dashes individually, so that enzymes yielding homochiral products can be uncovered.\(^95,96\)

Moreover, after a reductase with the desired properties has been identified, whole *Escherichia coli* cells expressing the same protein can be employed for bioconversions on preparative scales using glucose fed-batch conditions.\(^97\)

Cellular metabolic pathways supply NADPH, and the whole cells display very high stereoselectivities because they overexpress only a single yeast reductase.

**Results and Discussion**

A series of five α-chloro-β-keto esters was used in this study (Scheme 3-1). Eighteen yeast reductases were isolated as fusion proteins with glutathione S-transferase using previously-described methods.\(^96\) The collection of enzymes included members of the aldose reductase, D-hydroxyacid dehydrogenase, medium chain dehydrogenase and short chain dehydrogenase superfamilies.

Each α-chloro-β-keto ester was tested as a substrate for each reductase in the presence of NADPH, which was supplied by a cofactor regeneration system. For comparison, parallel reductions were also carried out with commercial bakers' yeast cells for the two cases where literature data were not available.\(^98\)
Since we were concerned with the enantiomeric and diastereomeric excess values for each reduction, we had to develop analytical techniques to resolve the enantiomers. Our general approach is to generate a mixture of all four possible products by sodium borohydride. The products are then analyzed by chiral gas chromatography (chiral GC) (Scheme 3-2).

![Scheme 3-2. Synthesis of all 4 diastereomers by sodium borohydride, which can be separated by chiral gas chromatography]

This strategy typically works for most alcohols; however, occasionally full separation cannot be achieved. This was found during the attempt to separate chlorohydrin 218. To solve this problem, the alcohol 218 was acetylated using acetic anhydride affording derivatives that can be fully resolved on a chiral gas chromatography column (Scheme 3-3).

Individual stereoisomers were linked to the appropriate GC peak by isolating alcohols from enzymatic conversions that afforded only single products. Where literature data were available, optical rotation values were used to determine absolute stereochemistry; these assignments were consistent with those made by NMR in all cases.
Comparing the outcomes of reactions using whole bakers’ yeast cells with those employing isolated yeast reductases clearly demonstrates the utility of examining individual biocatalysts (Figure 3-1). Not only did the purified yeast reductases deliver higher stereoselectivities in most cases, they also produced diastereomers not observed in reductions employing commercial yeast cells. This may result from low expression of some reductases under the physiological conditions prevailing in commercial bakers’ yeast, and this highlights an important advantage of using isolated reductases, rather than relying on whole yeast cells. Alternative methods to increase expression levels of desirable reductases, such as adding specific enzyme inhibitors, are more difficult to optimize and control.\textsuperscript{93,99,100} It should also be noted that the screening reactions could be carried out rapidly, and a complete data set was typically obtained for each substrate within 48 h.

The smallest substrate, $220$, was accepted by all of the yeast reductases examined, although the stereoselectivities of these reactions were relatively poor except for YOR120w and YGL157w, which afforded ($2S$, $3S$) and ($2R$, $3S$) configuration as the major products, respectively. In all cases, however, only L-
**Figure 3-1.** Biocatalytic reductions of α-chloro-β-keto esters. Yeast enzymes are referred to by their systematic names and grouped by superfamilies. Product compositions from reactions that proceeded to at least 20% conversion within 24 hr are shown in pie charts (2S-3S)-white, (2R-3S)-black, (2R-3R)-black/white lines, (2S-3R)-black dashes.
alcohols were formed. This behavior parallels our earlier observations from reactions in which ethyl acetoacetate was used as a substrate for the same collection of yeast fusion proteins. The behavior of higher homolog provides an interesting contrast. In four cases, D-alcohols were the major products. This is significant because D-alcohols are observed much less commonly in biocatalytic reductions and enzymes that deliver this enantioselectivity are correspondingly important. Six enzymes examined accepted as a substrate: four afforded only (2S, 3R) configuration while the remaining two produced mainly (2R, 3S). Benzyl-substituted β-keto ester was reduced by three enzymes, with very high stereoselectivities in two cases.

Taken together, our results have demonstrated that reductase enzymes uncovered by an analysis of the yeast genome can deliver important chiral building blocks for organic synthesis. At least two of the four possible α-chloro-β-hydroxy ester diastereomers could be produced in high optical purities in most cases. The major deficiency in the present collection is a lack of stereoselective reductases with D-specificities. Biocatalysts with these properties might be identified by including enzymes from additional organisms in our collection of fusion proteins and the increasing pace of genome sequencing project bodes well for expanding the utility of our chemo-enzymatic approach.
CHAPTER 4
SYNTHESIS OF THE C-13 TAXOL SIDE CHAIN

Racemic Synthesis

Our approach to the synthesis of the Taxol side chain began with a racemic synthesis. This was developed to test the feasibility of important chemical steps, and also to optimize the reaction conditions without consuming homochiral starting material.

This synthesis began with a Darzen’s reaction of benzaldehyde 85 and methyl chloroacetate 116 to yield trans-methyl 3-phenylglycidate 117\(^{42}\) (Scheme 4-1). Treatment of trans-117 with dry hydrochloric acid for several hours allowed a highly stereoselective ring opening to afford chlorohydrin 118\(^{42}\), which was reacted with potassium carbonate to yield cis-epoxide 119.\(^{89}\) The racemic epoxide 119 underwent a Ritter reaction with benzonitrile, catalyzed by boron trifluoride etherate to form cyclic product 60.\(^{101,102}\) Treatment of trans-oxazoline 60 with 0.5 M hydrochloric acid yielded the racemic Taxol side chain 41 with an overall yield of 16%.\(^{41,103}\)
Scheme 4-1. Racemic synthesis of the Taxol side chain

Once the racemic synthesis was complete, we began our work on the chiral route. Our first step was the chlorination of ethyl benzoylacetate 125, which can be achieved by reaction with tetrabutylammonium bromide and chlorotrimethylsilane\textsuperscript{104} (Scheme 4-2). However, we found that this chlorination technique gave side products that were difficult to separate from the desired product 126. After several failed attempts, an alternate literature procedure was found using sulfuryl chloride\textsuperscript{105-108}, giving high yields and allowing a simple distillation for purification.

Scheme 4-2. Two chlorination methods for β-keto esters

**Biotransformation Strategy**

As discussed in chapter 3, we recently published the reduction results for several α-chloro-β-keto esters using our library of purified bakers’ yeast
reductases. This strategy of using purified reductases works well for the small scale screening of substrates; however, due to the high cost of NADPH, this method is not practical for gram scale syntheses. A simple and economical reduction method is the use of whole cells, utilizing the cell’s cofactors for the reaction.

Our strategy was to implement a scaleable synthesis of the Taxol side chain using an enzymatic reduction as the key step, thus we needed to utilize whole-cell biotransformations. Adam Walton and Parag Parekh initiated our group’s research on whole-cell catalyzed reactions using *E. coli* with overexpressed GRE2<sup>97</sup>, a known yeast reductase. They established that the cells kept their reducing capabilities longer in a non-growing nitrogen deprived environment, compared to reactions in complete growth media. Using ethyl acetoacetate as their substrate, they were able to reach a product concentration of 250 mM over a 30 hour period (Figure 4-1).

The key step in our route to the Taxol side chain involves the enzymatic reduction of α-chloro-β-keto ester 126, using a similar whole-cell approach as developed by Walton and Parekh. After analyzing the results presented in Chapter 3, we chose two reductases for our synthesis of the Taxol side chain: YDL124w, which affords (2S-3R)-chlorohydrin 127 and YGL039w, which affords
Figure 4-1. Production of (S)-ethyl 3-hydroxybutyrate by engineered *E. coli* cells under non-growing conditions

(2R-3S)-chlorohydrin *ent*-127 (Scheme 4-3). By using these enzymes, we can synthesize both enantiomers of the optically pure Taxol side chain.

Scheme 4-3. The Taxol side chain and its enantiomer can be synthesized by utilizing two different enzymes; YDL124w and YGL039w, respectively

**Optimization of Our Whole-cell System**

A review from Saluta and Bell reports conditions that can potentially effect protein overexpression by a T7 promoter such as glucose concentration, induction optical density, induction temperature, and induction time. Before we
began our biotransformation studies, these conditions were optimized. In their review, these authors recommend certain parameters to be followed for general protein overexpressions. First, it is known that the overexpression plasmid can be 'leaky', thus a catabolite repressor should be used to inhibit the protein expression in the growing phase. They recommend supplementation of the growth media with 2% glucose for this reason. Second, they reported that the most advantageous optical density for inductions in these systems are $A_{600} = 0.5 – 1.0$. The recommended glucose concentration and optical density for induction were used in our system.

It is known that the optimal induction temperature can vary when trying to overexpress a protein in its active form. A common complication in the overexpression of proteins is the formation of insoluble-misfolded peptides called inclusion bodies, which are usually caused by the expression at high temperatures (~37 °C).\textsuperscript{110} This was most likely the case in our early attempts to use YGL039w in our whole-cell biotransformations. SDS-PAGE confirmed the overexpression of our protein of interest; however, reduction attempts were unsuccessful (Figure 4-2).
Figure 4-2. SDS-Page of the overexpression of YGL039w over a 4-hour time period. The arrow marks the expected position of the YGL039w fusion protein.

Whole-cell Assays

To investigate the inclusion body theory, experiments were developed to test the cells’ reducing activity when expressing protein at 37 °C, 30 °C, and 24 °C. Three separate cell batches were grown at 37 °C until they reached an O.D. = 0.6, then these were cooled to the corresponding temperatures. Once the cells reached their final temperature, they were induced with IPTG (0.1 mM final concentration). Aliquots were taken at various time points and the cell suspension was lysed by sonication, followed by centrifugation to remove the cellular debris. The activity of soluble reductases was screened by the addition of NADPH and ethyl acetoacetate (an excellent substrate for YGL039w). This
solution was monitored at 340 nm over 2 minutes to observe the loss of NADPH, which is directly proportional to the reduction of ethyl acetoacetate. The specific activity for each aliquot was calculated as described in Appendix B, and the activities were plotted versus the time after induction (Figure 4-3).

Figure 4-3. Specific activity of the *E. coli* cells overexpressing YGL039w, which have been grown under different induction temperatures (37 °C, 30 °C, and 24 °C)

From this experiment we concluded that overexpression of YGL039w at 24 °C gave the highest activity per cell. In addition, it confirmed our suspicion that YGL039w overexpressed at 37 °C formed inclusion bodies, which explained why it was unable to reduce the ketone substrate. These experiments also answered an additional question. It was first considered important to stop the growth of the induced cells after 4 hours. However, this experiment ran for 24 hours, and we saw no evidence for a significant loss of specific activity from the cells up to this point.
In addition to the induction temperature, we were also curious about the effect of the GST tag on the activity of the protein. Therefore, YGL039w was overexpressed both with and without the GST tag, and the same methods to evaluate reductase activity described above were used to compare the activities (Figure 4-4). The results implied that the GST tag, at least in this case, does not adversely affect the activity of the reductase.

![GST Effect on Specific Activity](image)

**Figure 4-4.** Whole cell activity of an overexpressed YGL039w with a GST tag and YGL039w without a GST tag versus time

**Whole-cell Reduction of 126**

After the optimization of protein expression and activity, the whole-cell reductions of the α-chloro-β-keto esters could begin. The reduction of 126 was run on a 1 liter scale fermentation reaction in a nitrogen-free phosphate buffer. Unfortunately, we encountered a problem while conducting this experiment: 126 was found to decompose while dissolved in water. After analyzing its stability at different pH values, the molecule was found to be reasonably stable at a working
pH under 6.0. Second, we noticed that some α-chloro-β-keto ester will undergo reductive dechlorination, which Bertau and Jorg\cite{90,111,112} suggested was due to a reaction with free glutathione in the cell. In our whole-cell reductions of 126, we found that the amount of dechlorinated product can widely vary for each reaction, and we do not have a strong hypothesis to why there is such a vast inconsistency.

Our last problem observed in the whole-cell reduction of 126 was its high toxicity and inhibitory effect towards the cells. Two actions were taken to minimize these toxicity effects. First, we slowed substrate feeding to keep the starting material concentration at a minimum. Second, a non-polar adsorbing Amberlite XAD-4 resin was added to the fermentation reaction. This resin adsorbs the product from the aqueous phase, thereby lowering its inhibitory effect on the cells. On average, we have seen a 20 – 25 % increase of isolated product for 127 and ent-127 using these tactics.

After optimization of both the growing conditions and the conditions for the whole-cell reduction, we were able to achieve a final product concentration of ~6 mM. These concentrations were calculated by GC using the ratio of product peak area versus internal standard peak area.

The next step, extraction of the product, proved to be difficult because of the formation of an emulsion caused by cellular debris. To avoid the emulsion, we centrifuged the cells and extracted the supernate with an organic phase. However, product was found in the cell pellet, resulting in a significant loss in
yield. We therefore developed a gentle extraction method in which the aqueous phase was slowly circulated through methylene chloride (Figure 4-6).

Figure 4-5. Concentration of the product for the biotransformation using YDL124w and YGL039w

**Product extraction apparatus**

Figure 4-6. Diagram of our gentle extraction technique
After purification by flash chromatography, we were able to achieve a 91% and 85% yield of 127 and ent-127 from the whole-cell reduction with YDL124w and YGL368w, respectively (Scheme 4-4).

Scheme 4-4. Final results for the whole-cell biotransformations after purification

Base Catalyzed Ring Closure

Our next step required a base catalyzed ring closure to form the corresponding glycidic ester (Scheme 4-5). This reaction can be directed to the cis or trans product by adjusting the strength of the base. Reaction of chlorohydrin 223 and 225, with a weak base will yield exclusively the kinetic product for this reaction 224 and 226, respectively. Treatment of the same two chlorohydrins with a strong base will selectively yield trans-epoxide 226.
Scheme 4-5. Results for the ring closure of chlorohydrins: Potassium carbonate results in the kinetic product, whereas sodium ethoxide affords the thermodynamic product.

The Azerad lab reported\(^8^9\) that the reaction involving ethoxide ion preceded by initial epimerization of the chlorohydrin via the formation of the enolate prior to cyclization (Scheme 4-6). Once epimerized to the more thermodynamically favored anti conformation, the chlorohydrin can undergo closure to trans-epoxide \(\text{226}^\).

Scheme 4-6. Mechanism for the sodium ethoxide promoted epoxidation and a Newman projection describing syn versus anti configuration of chlorohydrins.

Epoxide formation using potassium carbonate required some water to afford glycidic ester \(\text{128}^\) with a 99% yield, and the amount of added water proved critical (Scheme 4-7). We found that 1 – 2 equivalents of water would result in a slow reaction, and more than 3 equivalents of water would often lead to by-
products. It was also necessary to use chlorohydrin purified by column chromatography, because the impurities found with the reduction product led to a drastic loss in yield.

![Chemical structure of 127 and 128](image)

Scheme 4-7. Base promoted formation of cis-glycidic ester 128

**Ritter Reaction**

Once the synthesis of glycidic ester 128 was achieved, it underwent a Ritter reaction with benzonitrile to afford predominately trans-oxazoline 129 with a 55% yield (Scheme 4-8). This reaction also yielded a small amount of cis-oxazoline 227 which decreased the yield of the desired product. Fortunately, the cis isomer was separable by flash chromatography allowing pure trans-oxazoline 129 to be isolated.

![Chemical structure of 128, 129, and 227](image)

Scheme 4-8. The Ritter reaction of glycidic ester 128 and benzonitrile

An attempt was made to increase selectivity for the trans oxazoline by varying the temperature, solvent polarity, and strength of the Lewis acid. Different temperatures and solvents were found to have no effect on the reaction selectivity. A variety of Lewis acids were also screened to see if they could
increase the trans: cis ratio (Figure 4-7). These attempts were unsuccessful and the original conditions were found to be optimal.

<table>
<thead>
<tr>
<th>Lewis Acid</th>
<th>Conversion</th>
<th>Ratio (Trans: Cis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium Bromide</td>
<td>0%</td>
<td>---</td>
</tr>
<tr>
<td>Lithium Chloride</td>
<td>0%</td>
<td>---</td>
</tr>
<tr>
<td>Ytterbium Triflate</td>
<td>99%</td>
<td>5:1</td>
</tr>
<tr>
<td>Tin (II) Ethyl Hexanoate</td>
<td>0%</td>
<td>---</td>
</tr>
<tr>
<td>Aluminum Chloride</td>
<td>0%</td>
<td>---</td>
</tr>
<tr>
<td>p-Toluene Sulfonic Acid</td>
<td>99%</td>
<td>5:1</td>
</tr>
<tr>
<td>Triflic Acid</td>
<td>99%</td>
<td>5:1</td>
</tr>
<tr>
<td>BF₃-etherate</td>
<td>99%</td>
<td>5:1</td>
</tr>
</tbody>
</table>

Figure 4-7. Effect of Lewis acids on the Ritter reaction

**Ring Hydrolysis to the Taxol Side Chain**

Oxazoline 129 has been used by Bristol Myers-Squibb as a protected form of the Taxol side chain¹¹³ that can be coupled to the terpene core of Taxol (Scheme 1-9). With this said, we were able to synthesize the protected Taxol side chain in 4 steps with an overall yield of 49%.

For academic reasons, and to compare the optical rotation of our material with that synthesized previously, we treated oxazoline 129 and ent-129 with aqueous acid to afford the Taxol side chain 130 and ent-130 as their ethyl esters. Optical rotations for the Taxol side chain 130, and its enantiomer ent-130 were \([\alpha]_D = -11.6 \text{ (c = 2.0, CHCl}_3\text{)}\) and \([\alpha]_D = +12.3, \text{ (c = 1.0, CHCl}_3\text{)}\), respectively. As expected, the \(^1\text{H NMR spectra of 130 and ent-130 were found to overlap (Appendix B). In addition, to confirm the enantiopurity of the final products 130 and ent-130, were derivatized with (S)-\(\alpha\)-methoxy-\(\alpha\)-phenylacetic (MPA) and the spectra of the two derivatives were compared (Appendix B). The spectra of ent-}
130 was found to be 99% enantiomerically pure. A small amount of enantiomer was found in 130, which can be attributed to a small amount of trans-epoxide that was not separated in the ring closure step. However, since ent-130 was synthesized in a 99% ee, it confirmed that subsequent chemical steps in our reaction scheme did not provoke any racemization.

For the final step, it is essential that the reaction be carried out under mildly acidic conditions. It has been reported that treatment with weak aqueous acid will result in the hydrolysis product from attack at the C-4 position\(^\text{103}\) (Scheme 4-9). However, treatment of oxazoline 129 with 6 M hydrochloric acid will result in amide hydrolysis resulting in compound 228. In addition, we found that storing the oxazoline in the open air at room temperature would result in the hydrolysis product 130 after several weeks.

Scheme 4-9. Hydrolysis of oxazoline 129 under mildly acidic conditions versus strongly acidic conditions
CHAPTER 5
SYNTHESIS OF BESTATIN

Over the past 30 years, there have been several strategies reported for the synthesis of Bestatin \(131\) (Chapter 2). Most of these utilize amino acids or other naturally occurring chiral compounds for their starting material. Other strategies utilize the separation of enantiomers by chiral resolution; however, this process can be very difficult and often inefficient. It was our goal to develop a novel synthesis for AHPA \(137\) and Bestatin \(131\) through our simple homochiral glycidic ester approach.

**Synthesis of β-Keto Ester 213**

Our approach to Bestatin began by synthesizing β-keto ester \(213\) by a literature procedure using Meldrum’s acid \(212\) and phenylacetyl chloride \(21\) (Scheme 5-1). The product of this reaction \(213\) can be easily purified by a simple vacuum distillation.

![Scheme 5-1. Synthesis of β-keto ester 213](image)

**Chlorination of β-Keto Ester 213**

The chlorination of compound \(213\) with sulfuryl chloride was found to be difficult, because performing this reaction with stoichiometric amounts of starting material resulted in a large amount of dichlorinated byproduct \(229\) accompanying
the desired product 214 (Scheme 5-2). These compounds were inseparable by distillation, and difficult to separate by flash chromatography. This problem was solved by lowering the reaction temperature and decreasing the ratio of sulfuryl chloride to β-keto ester, thus simplifying the purification process.

Scheme 5-2. Two different approaches to chlorinate 213 with sulfuryl chloride

**Enzymatic Reduction of 214**

The key step in our approach to AHPA 137 and Bestatin 131 is the whole-cell reduction of compound 214, using an overexpressed bakers’ yeast reductase in *E. coli*. This process allows us to introduce two chiral centers into our reaction scheme, and we hoped to achieve this on a gram scale. We recently published the reduction results for several α-chloro-β-keto esters using our library of bakers' yeast reductases\(^\text{109}\) (Chapter 3). Benzenebutanoic acid, α-chloro-β-oxo-ethyl ester 214 was shown to be a substrate for three bakers’ yeast reductases; YDR368w, YGL157w, and YGL039w (Scheme 5-3).
Scheme 5-3. Three bakers’ yeast reductases that accept 214 as a substrate; YDR368w, YGL039w, and YGL157w

Previously, for compounds 127 and ent-127 (Chapter 4), we were able to use literature data to determine the absolute stereochemistry; unfortunately, no literature data was found for compounds 215 and 250. Comparison of the $^1$H NMR of these compounds confirmed that YDR368w and YGL157w afforded syn and anti chlorohydrins, respectively (Appendix B). However, since literature data did not exist, another method was used to define the absolute stereochemistry for 215 and 250. We first attempted X-ray crystallography; however, we were unable to grow diffraction-quality crystals from these compounds. Our next approach was NMR analysis by derivatizing the chlorohydrins with (R)- and (S)-α-methoxy-α-phenylacetic (MPA) acid. Dr. Ion Ghivirigia analyzed these derivatized compounds for their $^1$H-$^1$H, $^1$H-$^{13}$C one-bond and $^1$H-$^{13}$C long-range couplings to determine the absolute stereochemistry.$^{115-118}$ (Appendix B).

The results from NMR experiments were used to assign the (2R, 3S) configuration to the YDR368w product, and the (2S, 3S) configuration to the
YGL157w and YGL039w products. Fortunately, the (2R-3S)-chlorohydrin 215, is the intermediate needed for our approach to AHPA 137 and Bestatin 131.

**Whole-cell Reduction of 214**

The reduction of 214 was performed in a 1 liter fermentation reaction in a nitrogen-free phosphate buffer. The growth and biotransformation conditions for the reduction of compound 214 followed those optimized for YGL039w in our Taxol side chain synthesis (Chapter 4). As seen before, this whole-cell reduction also yielded dechlorinated product that formed in the beginning of the reaction (Scheme 5-4). Typically, the first five percent of the starting material would suffer this dechlorination reaction. Occasionally there would be vast variations in the extent of dechlorination, and this phenomenon has yet to be fully understood.

![Scheme 5-4. The whole-cell biotransformation resulted in the chlorohydrin 215 and dechlorinated product 231.](image)

Substrate 214, much like compound 126, was highly toxic toward the *E. coli* cells. To lessen this effect, two actions were taken. First, we fed the cells small portions of starting material every hour, thereby keeping the concentration of the toxic starting material at a minimum. Second, a non-polar adsorbing XAD-4 resin was added to the fermentation system. This resin would slowly adsorb the toxic compounds from the reaction system, thus allowing the cells to further reduce substrates and consume glucose and oxygen.
The whole-cell reduction of \(214\) by cells overexpressing the reductase YDR368w yielded a final product concentration of 5 mM. Using the previously described gentle extraction method, we were able to obtain 1.1 g of product after purification by flash chromatography with an overall yield of 82% (Scheme 5-5).

![Chemical structure](image)

Scheme 5-5. Results for our optimized whole-cell reduction of \(214\)

**Base Catalyzed Ring Closure of 215**

Treatment of chlorohydrin \(215\) with potassium carbonate cleanly yielded cis-glycidic ester \(216\)\textsuperscript{89}, with no need for further purification (Scheme 5-6). Since DMF was used as the solvent for this reaction, it was important to wash the organic phase with several small portions of water to remove any residual DMF. In addition, the amount of water was critical to the success of this reaction. We found that 1 – 2 equivalents of water would result in a slow reaction, and that more than 3 equivalents of water would often lead to by-products.

![Chemical structure](image)

Scheme 5-6. Base promoted ring closure for glycidic ester \(216\)

**Ritter Reaction**

Epoxide \(216\) was opened by benzonitrile and boron trifluoride diethyl etherate to afford the protected form of AHPA \(217\)\textsuperscript{101,102} (Scheme 5-7). Unlike
the Ritter reaction in our synthesis of the Taxol side chain (Scheme 4.8), this reaction was completely selective for *trans*-oxazoline 217, with an overall yield of 78%.

\[
\begin{align*}
\text{216} & \quad \text{Benzonitrile / BF}_3\text{OEt}_2 \\
\end{align*}
\]

Scheme 5-7. The Ritter reaction of 216 and benzonitrile only afforded the *trans*-oxazoline 217

**Synthesis of Bestatin from 217: First Generation**

In our first attempt to complete the synthesis of Bestatin, oxazoline 217 was saponified to produce acid 232 (Scheme 5-8). This was subjected to a DCC coupling with L-Leu-OBn in an attempt to form the protected form of Bestatin 233. The final deprotection step was expected to utilize hydrogenolysis of both oxazoline and the benzyl ester, thus yielding Bestatin directly. While the coupling with L-Leu-OBn appeared to have been successful, a number of attempts to hydrogenolyze the oxazoline failed, and we found that the oxazoline could only be deprotected by reflux in the presence of 6 M HCl. Such conditions would also cleave the peptide bond, and this strategy was therefore abandoned.
Scheme 5-8. First attempt for the synthesis of Bestatin

**Synthesis of Bestatin from 217: Second Generation**

Our second attempt to convert oxazoline 217 to Bestatin involved nucleophilic attack on the ring carbon to yield an sp$^3$ center that would be more susceptible to deprotection (Scheme 5-9). We chose a nickel-catalyzed addition of PhMgBr to afford Grignard product $234^{119}$. We attempted a DCC coupling with L-Leu-OtBu and crude 234, but unfortunately the products of the Grignard reaction and the Leucine coupling reaction were very difficult to dissolve in a variety of solvents, thus making these intermediates extremely difficult to characterize. Since Bestatin was not observed at the end of this sequence, this route was also abandoned.
Scheme 5-9. Second attempt for the synthesis of Bestatin

**Synthesis of Bestatin from 217: Third Generation**

Based on the difficulties encountered in converting oxazoline 217 directly to Bestatin 131, a simpler approach was taken. As mentioned in Chapter 2, Bestatin is composed of two main parts, (2S, 3R)-3-Amino-2-hydroxy-4-phenylbutanoic acid 137 (AHPA) and L-leucine. The most difficult challenge for the synthesis of Bestatin 131 is the synthesis of AHPA 137. Our final approach was to synthesize AHPA by simply deprotecting oxazoline ester 217 with 6 M HCl (Scheme 5-10). Since literature methods can be used to convert AHPA 137 to Bestatin 131, this completes a formal total synthesis of this molecule.

Scheme 5-10. Final Strategy to AHPA 137 and Bestatin 131

Hydrolysis of 217 proceeded smoothly and after a simple purification by a Dowex cation exchange resin, we were able to isolate AHPA with a 40% overall yield. As a confirmation, commercially available AHPA was purchased and compared with our synthetic material. The \(^1\)H NMR spectrum of the
commercially available AHPA and our synthesized AHPA were identical
(Appendix B). In addition, the optical rotation for our synthetic AHPA was $[\alpha]_D = 23.2^\circ$ (c = 1.3, 1 M HCl), AHPA (Sigma-Aldrich) $[\alpha]_D = 23.4^\circ$ (c = 1.0, 1 M HCl).
The overlap of spectral data and optical rotation values\textsuperscript{67} confirms that our
previous definition of absolute stereochemistry was correct using NMR.
CHAPTER 6
SYNTHETIC APPROACH TO CHUANGXINMYCIN

Introduction

Chuangxinmycin 286 is a natural product isolated from Actinoplanes tsinanensis (Figure 6-1). Initial studies suggested that it has in vitro antibacterial activity against a variety of gram-negative and gram-positive bacteria. In addition, it has shown antimicrobial activity against Escherichia coli and Shigella dysenteriae in mouse models\textsuperscript{120}. Clinical results have shown successful treatment for septicaemia and for urinary and binary infections cause by E. coli. Presently, Chuangxinmycin’s mode of action is not completely understood; however, it is reported that this drug has an affect on the tryptophan biosynthetic pathway.

![Figure 6-1. Chuangxinmycin 236](image)

The Akita lab’s Approach to Chuangxinmycin

In 1997, the Akita group published synthesis of racemic Chuangxinmycin\textsuperscript{121} by coupling (+/-)-(2,3)-syn-epoxy butanoate 243 and 4’-iodoindole 242 (Scheme 6-1). This synthesis began with 2-amino-6-nitrotoluene 237, which was treated...
with CH(OEt)$_3$ to afford 238. Imidate ester 238 was reacted with potassium ethoxide followed by a palladium catalyzed hydrogenation to yield 4-amino indole derivative 241. Diazotization of 241 with sodium nitrite and subsequent treatment with potassium iodide resulted in 4-iodoindole derivative, which was converted into 4-iodoindole 242 by hydrolysis. Reaction of racemic glycidic ester 243 and 242 in the presence of tin (IV) chloride afforded (+/-)-4'-iodoindolmycenate 244. Treatment of 244 with methanesulfonyl chloride in pyridine gave thioacetoxy ester 245, which was deacetylated under weak alkaline conditions. Treatment of 246 with Pd(PPh$_3$)$_4$ gave methyl ester 247 that was treated with aqueous base to result in Chuangxinmycin 236.
Enzymatic Reduction of 220

This publication\textsuperscript{121} described a method to synthesize racemic Chuangxinmycin through trans-glycidic ester 243. However, to achieve the absolute stereochemistry at the C-4 and C-5 positions, one must develop a chiral synthesis to this ester. We recently disclosed that reductase YOR120w afforded the (2R, 3S) chlorohydrin by reduction of 220 with a >98% e.e and >98% d.e.\textsuperscript{109} Using this chlorohydrin, we proposed a practical, gram-scale synthesis to make (2R, 3S)-epoxy butanoate 243 with a whole-cell biotransformation using an overexpressed bakers’ yeast reductase as the key step (Scheme 6-2).
Whole-cell Reduction of 220

The whole-cell reduction of 2-chloro ethylacetoacetate 220 was carried out in a 1 liter fermentation vessel in a nitrogen deprived phosphate buffer, using conditions similar to those described earlier (Chapter 4). This reduction was able to achieve a much larger final concentration when compared to the whole-cell reductions of 126 and 214. Nonetheless, this reaction fell short in final product concentration when compared to the reduction of ethyl acetoacetate by GRE2 (Figure 4-1). Between these cases, there was about a ten-fold decrease for 2-chloro ethylacetoacetate 220 (Figure 6-2). This is most likely due to the increased toxicity toward the cells from the chlorine functionality on substrate 220. Whole-cell reduction of 2-chloro ethylacetoacetate did yield an approximately four-fold higher final concentration when compared to the whole-cell reduction of 214. We believe this is because 220 is less hydrophobic than 214, and thus has a smaller inhibitory effect on the cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>249</td>
<td>32.2 g/L</td>
</tr>
<tr>
<td>248</td>
<td>4.4 g/L</td>
</tr>
<tr>
<td>215</td>
<td>1.1 g/L</td>
</tr>
</tbody>
</table>

Figure 6-2. Final product concentrations for 249, 248, and 215 by the corresponding engineered E. coli

The reduction of 220 was catalyzed by E. coli cells containing overexpressed reductase YOR120w. To lessen the toxicity to the cells, the substrate was added in small increments over 24 hours, and a one liter reaction afforded 4.4 grams of product with an 89% overall yield. As was also seen in
other reductions of chlorinated β-keto esters, there was a small dechlorination by-product. In this case, however, we found only a small percentage of this by-product.

While separation was simpler due to the small amount of dechlorinated by-product, we did encounter a problem while trying to extract 248 from the reaction mixture. Even using our gentle extraction technique (Chapter 4), we were unable to achieve complete extraction of product from the aqueous phase, even after 5 days of extraction. This can be attributed to the high solubility of the reduction product 248 in water. As a result, the organic layer was replaced with fresh methylene chloride three times to allow an adequate extraction.

**Base Catalyzed Ring Closure of 248**

To synthesize the homochiral glycidic ester, the chlorohydrin 248 was treated with sodium ethoxide to afford predominately *trans*-glycidic ester 243. Unfortunately, this reaction did not follow the trend seen in the literature, which reported the *trans*-epoxide as the only product formed. Our ring closure reported a 5 : 1 (trans : cis) product ratio and we were unable to get adequate separation of these diastereomers (Scheme 6-4).

![Scheme 6-4. Ring closure promoted by sodium ethoxide](image)

This observation can be explained by the size of the γ-carbon chain. The reaction of chlorohydrins with sodium ethoxide is directed by the thermodynamic
stability of the *anti* versus the *syn* conformation (Scheme 4.6). Due to its small size, the methyl group does not adequately direct the steric course of the reaction.

We also investigated ring closure of 248 by treatment with a weak base. Chlorohydrin 248 was reacted with potassium carbonate (3 equiv) and a catalytic amount of water (3 equiv) over a 5 hour period (Scheme 6-5). Gas chromatography and NMR analysis confirmed that this epoxidation followed the general trend, affording only the *cis*-glycidic ester.

![Scheme 6-5. Ring closure of chlorohydrin 248 using potassium carbonate and water.](image)

We were generally unsuccessful in the synthesis of (2R, 3S)-epoxy butanoate 243. A simple solution to this problem is to find a reductase that will result anti-chlorohydrin 251 with a high d.e. and e.e. This will then allow for the epoxidation using potassium carbonate, which should afford only the trans-glycidic ester 243 (Scheme 6-6).

![Scheme 6-6. Proposed synthesis to (2R, 3S)-epoxy butanoate 243.](image)
CHAPTER 7
CONCLUSIONS AND FUTURE WORK

Studying our collection of purified bakers’ yeast reductases has shown that they can be utilized to afford highly synthetically useful intermediates with high stereoselectivities. In addition, these reductions can be carried out with engineered whole-cells to yield products on gram scales. We have also shown that these reduction products can be easily transformed into glycidic esters, which are popular intermediates for a variety of pharmaceutical drugs.

Our reduction library allowed us to make the (2S-3R)-127 and (2R-3S)-chlorohydrins ent-127 needed for synthesizing both Taxol side-chain antipodes with high enantiomeric excess. Using these intermediates, we were able to react these chlorohydrins with a weak base to afford the corresponding glycidic esters. The glycidic esters underwent a Ritter reaction with benzonitrile to form the protected Taxol side chain with an overall yield of 49% and its enantiomer with an overall yield of 38%. This is advantageous because it eliminates any additional steps that are needed to take the Taxol side chain to a form in which it can be coupled to Baccatin III.

Benzenebutanoic acid, α-chloro-β-oxo-, ethyl ester 214 was shown to be a substrate for YDR368w, thus affording (2R, 3S)-chlorohydrin 215 in high enantiopurity. This result allowed us to synthesize oxazoline 217 through our glycidic ester and Ritter reaction route, thus resulting in a protected form of AHPA. Treatment of oxazoline 217 with strong acidic conditions resulted in
AHPA 137 in 6 steps with an overall yield of 42%. This completes a formal total synthesis of Bestatin 131.

The whole-cell reductions of three key α-chloro-β-keto esters resulted in final concentrations of 1.1 g for 215, 1.39 g for 127, and 4.4 g for 248. This trend is a direct result of the product toxicity to the cells. This toxicity is probably due to the hydrophobicity of the compounds, along with the addition of the chlorine moiety. Future work for these reductions would focus on the optimization of these biotransformations. For example, the dechlorination of the starting material may be eliminated by incubating the cells with a chlorinated compound that is commercially available and easily removed. Additional work would focus on engineering at the genomic level, thus making cells more resistant to product toxicity and/or more efficient at reducing substrates.

The two pharmaceutical routes reported in this thesis are only two examples from a wide range of possibilities. If we increase our library of purified bakers' yeast reductases, or expand the library with reductases from other organisms, it may help in expanding our synthetic potential. As seen in Chapter 6, if a reductase is found to yield the (2S, 3S)-chlorohydrin 251, we will be able to synthesize the (2R, 3S)-epoxy butanoate 243, which is an intermediate to Chuangxinmycin 236. Other possible pharmaceutical intermediates that can be formed through homochiral glycidic esters are, but not limited to: Diltiazem 252, KRI-1230 253, Amistatin ent-253, and Indolmycin 254 (Figure 7-1)
Figure 7-1. Other pharmaceutical drugs that can be synthesized from homochiral glycidic ester intermediates: Diltiazem 252, KRI-1230 253, Amistatin ent-253, and Indolmycin 254
APPENDIX A
EXPERIMENTAL

General Methods and Instrumentation

Standard media and techniques for growth and maintenance of *E. coli* were used, and Luria-Bertani (LB) medium contained 1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, and 1% NaCl. Synthetic reactions were carried out under argon atmosphere, with the exception of water containing reactions. Reactions were monitored by TLC (silica, 60 Å) or by GC using a DB-17 column (0.25 mm x 25 m x 0.25 µm thickness) with a flame ionization detector, and for 137 reversed-phase HPLC (4.6 × 250 mm C\textsubscript{18} column) using a water-CH\textsubscript{3}CN solvent system (both solvents containing 0.1% trifluoracetic acid) was used. For chiral separation, GC was used with a Chirasil-Dex CB column (0.25 mm x 25 m x 0.25 µm thickness) or a Chirasil-L-Val (0.25 mm x 25 m x 0.25 µm thickness) with a flame ionization detector. NMR spectra for \textsuperscript{1}H and \textsuperscript{13}C were recorded on Varian 300 MHz instruments. Chemical shifts are reported at 25 °C in ppm relative to TMS. Optical rotations were measured in CHCl\textsubscript{3} at room temperature (Perkin-Elmer 241 digital polarimeter) unless otherwise stated. Elemental analysis was performed by Atlantic Microlab, Inc. in Atlanta, Georgia. Racemic alcohols were prepared from the corresponding ketones by reduction with sodium borohydride.
Whole-cell Activity Assays for Inductions Carried Out at Different Temperatures

A 35 mL solution of LB broth (supplemented with 30 µg/mL kanamycin) was inoculated with a single colony of *E. coli* (BL21(DE3)(pIK6)) and shaken overnight at 37 °C. The preculture (10 mL) was diluted (1: 100) into 1 L of LB (supplemented with 30 µg/mL kanamycin and 4 g/L glucose) in three 2 L baffled flasks. The cultures were grown for 2 hours at 37 °C with shaking at 400 rpm until they reached an O.D.\textsubscript{600} = 0.6. One flask was placed at 24 °C, one at 30 °C and the other was left at 37 °C. The cells were allowed to shake at 400 rpm for 15 minutes and then reductase overproduction was induced with isopropylthio- β-D-galactoside at a final concentration of 0.1 mM. The cells were kept under the same conditions and aliquots (100 mL) were taken at various times. Cells were collected by centrifugation (6000 g for 10 min at 4 °C), resuspended in phosphate buffer (3 mL, 100 mM, pH = 7), then PMSF was added to a final concentration of 1.5 mM. The aliquots were stored at 4 °C and then sonicated for 10 seconds. The cell suspension was centrifuged (6000 g for 10 min at 4 °C) to remove cellular debris, decanted, and the supernate was stored on ice.

A premixed solution of phosphate buffer (10 mM, pH = 7) and ethyl acetoacetate (5 mM) was maintained at 30 °C. The premixed solution (1 mL) was added to a quartz cuvette, followed by 10 µL of NADPH solution (20 mM) and the cellular supernate (volume varied). The cuvette was gently mixed and immediately monitored at 340 nM (120 sec at 20 sec intervals). The slope was calculated and used to find the specific activity (Appendix B).
Procedures and Data

Ethyl benzoylacetate 125 (6.0 g, 28.0 mmol) was added to chloroform (170 mL). The reaction mixture was purged with argon at 50 °C and then sulfuryl chloride (2.5 mL, 28.0 mmol, 1 equiv) was added over 15 minutes. The reaction mixture was stirred at 50 °C for 3 hours and then allowed to cool to room temperature. Water (200 mL) was added to the mixture and the aqueous layer was extracted with methylene chloride (3 x 100 mL). The combined organic layers were dried with magnesium sulfate and concentrated. The resulting residue was purified by vacuum distillation (2 mm Hg, 120 °C) to afford 6.2 g as a colorless oil 126 in 98% yield. ¹H NMR: (CDCl₃) δ: 8.00 (d, 2H), 7.60 (m, 3H), 5.62 (s, 1H), 4.29 (q, 2H, J = 6.9), 1.24 (t, 3H, J = 6.9). ¹³C NMR: (CDCl₃) δ: 188.6, 165.6, 134.7, 133.8, 129.6, 129.3, 63.6, 58.4, 14.3. IR (neat): ν(cm⁻¹): 2984.5, 1763.8, 1691.7, 1268.3, 1182.8.

A 45 mL solution of LB broth (supplemented with 30 µg/mL kanamycin) was inoculated with a single colony of E. coli (BL21(DE3)(pIK8)) and shaken overnight at 37 °C. The preculture (40 mL) was diluted (1: 100) to 4.0 L of LB (supplemented with 30 µg/mL kanamycin and 4 g/L glucose) in a New Brunswick M19 fermenter. The culture was grown for 2 hours at 37 °C with a stir rate of 800
rpm and an air flow of 0.5 vessel volumes per minute (vvm) until it reached
\( O.D_{600} = 0.6 \). The cell suspension was cooled to 28 °C over 15 minutes and
then reductase overexpression was induced with isopropylthio-β-D-galactoside at
a final concentration of 0.1 mM. The cells were kept under the same conditions
for 6 hours and then collected by centrifugation (6000 g for 10 min at 4 °C). Half
of the cells (25 g wet weight) were resuspended in 1 L of 10 mM KP\(_i\) (pH = 5.6)
containing 4 g/L glucose. The bioconversion was carried out in a Braun Biostat B
fermenter at 30 °C with the pH maintained at 5.6 using 3 M NaOH. The
dissolved oxygen was maintained at 75% saturation using a fixed air flow of 0.25
vvm and variable stirring rate. After the addition of the XAD-4 resin (0.5 g),
portions of neat 126 (0.2 mL) were added approximately every hour over a total
of 12 hours to provide a final concentration of 6 mM. Portions of glucose were
added after 3.0 hours and 6.0 hours to maintain the glucose concentration at
approximately 4 g/L. Consumption of 126 and glucose slowed significantly after
8 hours. After 24 hours, the reaction was gently extracted with methylene
cloride (2 x 300 mL) to avoid an emulsion. The combined organic layers were
dried with magnesium sulfate and concentrated under vacuum. The crude oil
was purified by flash chromatography (Cyclohexane: Ether 85:15) to afford 1.33
g of 127 as a colorless oil with a 91% yield after recovered starting material.
\([\alpha]_D = -3 ^\circ \) (c = 0.68, CHCl\(_3\)), Lit.\(^{88}\) \([\alpha]_D = -3 ^\circ \) (c = 1.7, CHCl\(_3\)) ¹H NMR: (CDCl\(_3\))
\( \delta: 7.25 \) (m, 5H), 5.00 (d, 1H, \( J = 6.3 \)), 4.35 (d, 1H, \( J = 6.3 \)), 3.99 (q, 2H, \( J = 6.9 \)),
1.02 (t, 3H, \( J = 6.9 \)). ¹³C NMR: (CDCl\(_3\)) \( \delta: 168.4, 138.7, 129.1, 128.9, 127.2,
77.1, 75.1, 63.4, 62.7, 14.2 \). IR (neat): \( \nu(\text{cm}^{-1})\): 3475.3, 2982.2, 1745.8, 1373.1,
A 45 mL solution of LB broth (supplemented with 30 µg/mL kanamycin) was inoculated with a single colony of *E. coli* (BL21(DE3)(pIK6)) and shaken overnight at 37 °C. The preculture (40 mL) was diluted (1: 100) to 4.0 L of LB (supplemented with 30 µg/mL kanamycin and 4 g/L glucose) in a New Brunswick M19 fermenter. The culture was grown for 2 hours at 37 °C with a stir rate of 800 rpm and an air flow of 0.5 vessel volumes per minute (vvm) until it reached an O.D.₆₀₀ = 0.6. The cell suspension was cooled to 28 °C over 15 minutes and then reductase overexpression was induced with isopropylthio-β-D-galactoside at a final concentration of 0.1 mM. The cells were kept under the same conditions for 6 hours and then collected by centrifugation (6000 g for 10 min at 4 °C). Half of the cells (25 g wet weight) were resuspended in 1 L of 10 mM KPₙ (pH = 5.6) containing 4 g/L glucose. The bioconversion was carried out in a Braun Biostat B fermenter at 30 °C with the pH maintained at 5.6 using 3 M NaOH. The dissolved oxygen was maintained at 75% saturation using a fixed air flow of 0.25 vvm and variable stirring rate. After the addition of the XAD-4 resin (0.5 g), portions of neat 126 (0.2 mL) were added approximately every hour over a total of 12 hours to provide a final concentration of 6 mM. Portions of glucose were added after 3.0 hours and 6.0 hours to maintain the glucose concentration.
at approximately 4 g/L. Consumption of 126 and glucose slowed significantly after 8 hours. After 24 hours, the reaction was gently extracted with methylene chloride (2 x 300 mL) to avoid an emulsion. The combined organic layers were dried with magnesium sulfate and concentrated under vacuum. The crude oil was purified by flash chromatography (Cyclohexane: Ether 85:15) to afford 1.39 g of ent-127 as a colorless oil with an 85% yield after recovered starting material. 

$[\alpha]_D = +4 \degree (c = 0.68, \text{CHCl}_3)$  
$^1$H NMR: (CDCl$_3$) $\delta$: 7.25 (m, 5H), 5.00 (d, 1H, $J$ = 6.3), 4.35 (d, 1H, $J$ = 6.3), 3.99 (q, 2H, $J$ = 6.9), 1.02 (t, 3H, $J$ = 6.9).  
$^{13}$C NMR: (CDCl$_3$) $\delta$: 168.4, 138.7, 129.1, 128.9, 127.2, 77.1, 75.1, 63.4, 62.7, 14.2.  
IR (neat): v(cm$^{-1}$): 3475.3, 2982.2, 1745.8, 1373.1, 1258.9, 1095.7.

Chlorohydrin 127 (1.3 g, 5.7 mmol) was added to DMF (28 mL) and stirred at room temperature. Potassium carbonate (2.2 g, 17.1 mmol, 3 equiv) and water (525 µL) were added to the reaction mixture and stirred for 5 hours. The resulting mixture was diluted with water (75 mL), and then the aqueous layer was extracted with diethyl ether (3 x 75 mL). The organic layer was then washed with water (6 x 5 mL) to remove residual DMF. The combined organic layers were dried with magnesium sulfate and concentrated under reduced pressure to yield 1.08 g of 128 as a colorless oil in a 99% yield.  

$[\alpha]_D = +24 \degree (c = 1.5, \text{CHCl}_3)$ Lit.$^{89}$  
$[\alpha]_D = +25 \degree (c = 1.1, \text{CHCl}_3)$  
$^1$H NMR: (CDCl$_3$) $\delta$: 7.31 (m, 5H), 4.27 (d, 1H, $J$ = 4.8), 4.00 (m, 2H), 3.82 (d, 1H, $J$ = 4.8), 1.01 (t, 3H, $J$ = 6.9).  
$^{13}$C NMR: (CDCl$_3$)
δ: 167.0, 133.3, 129.0, 128.8, 128.4, 127.0, 126.2, 61.6, 57.8, 56.1, 14.3. IR (neat): v(cm⁻¹): 2982.2, 1753.0, 1729.6, 1245.0, 1053.6, 698.7. Anal. Calcd for C₁₁H₁₂O₃: C, 68.74; H, 6.29. Found: C, 68.34; H, 6.57. ent-128 was prepared the same way and spectral data matched that reported above, except for the optical rotation which was [α]D = -28.9 ° (c = 2.9, CHCl₃).

Glycidic ester 128 (0.50 g, 2.6 mmol) was added to benzonitrile (4 mL) and cooled to 0 °C under argon atmosphere. Boron trifluoride diethyl etherate (330 µL, 2.6 mmol, 1 equiv) was slowly added to the reaction mixture over a 10 minute period. The reaction was allowed to stir and warm to room temperature over 3 hours. Saturated sodium bicarbonate (4 mL) was then added to the reaction and stirred for 2 hours. The reaction mixture was diluted with water (20 mL) followed by extraction with methylene chloride (3 x 20 mL). The combined organic layers were separated and dried with magnesium sulfate, followed by purification by flash chromatography (silica, Cyclohexane: Ether 85: 15) affording 0.42 g of 129 as a colorless oil with a 55% yield. [α]D = +11 ° (c = 1.1, CHCl₃) ¹H NMR: (CDCl₃) δ: 8.02 (d, 2H), 7.30 (m, 8H), 5.37 (d, 1H, J = 6.3), 4.81(d, 1H, J = 6.3), 4.25 (m, 2H), 1.26 (t, 3H, J = 7.2). ¹³C NMR: (CDCl₃) δ: 170.3, 141.4, 132.1, 129.1, 128.9, 128.7, 128.6, 128.2, 126.7, 74.9, 62.0, 14.4. IR (neat): v(cm⁻¹):
2982.2, 1753.0, 1729.6, 1245.0, 1053.6, 698.7. \textit{ent-129} was prepared the same way and spectral data matched that reported above, except for the optical rotation which was \([\alpha]_D = -12^\circ \) (c = 1.7, CHCl$_3$).

\begin{center}
\textbf{130}
\end{center}

A solution of compound \textbf{129} (0.32 g, 1.10 mmol) in 0.5 M HCl (2.5 mL) and ethanol (7 mL) was held at reflux for 6 hours. After the solvent was removed under reduced pressure, the residue was dissolved in methylene chloride (10 mL) and washed with water (2 x 7.5 mL). The combined organic layers were dried with magnesium sulfate and concentrated under reduced pressure. The residue was purified by flash chromatography (silica, Hexane: EtOAc 50: 50) to afford 0.3 g of \textbf{130} as a white solid in an 85% yield. Mp = 162 – 163 °C. \([\alpha]_D = -12^\circ \) (c = 2.0, CHCl$_3$) \textsuperscript{1}H NMR: (CDCl$_3$) \(\delta\): 7.76 (d, 2H), 7.30 (m, 8H), 6.98 (d, 1H, \(J = 8.3\)), 5.76 (d, 1H, \(J_1 = 2.1, J_2 = 9.3\)), 4.62 (q, 1H, \(J = 2.1\)), 4.30 (m, 2H), 3.30 (d, 1H, \(J = 4.2\)), 1.30 (t, 3H, \(J = 6.9\)). \textsuperscript{13}C NMR: (CDCl$_3$) \(\delta\): 173.1, 167.1, 138.9, 134.4, 131.9, 128.9, 128.8, 128.1, 127.2, 127.1, 62.9, 60.6, 55.0, 14.4. IR (neat): \(\nu(\text{cm}^{-1})\): 3416.0, 3352.3, 1718.0, 1637.9, 1528.6. \textit{ent-130} was prepared the same way and spectral data matched that reported above, except for the optical rotation, which was \([\alpha]_D = +12^\circ \) (c = 1.0, CHCl$_3$).
Meldrum’s acid 212 (13.2 g, 91.6 mmol) was added to methylene chloride (170 mL) under an argon atmosphere. This solution was cooled to 0 °C, and then phenylacetyl chloride 211 (14.16 g, 91.6 mmol, 1 equiv) was added to the reaction mixture over a 15 minute period. The reaction was allowed to reach room temperature and then stirred overnight. The reaction mixture was followed by a wash with 10% HCl (2 x 25 mL) and water (1 x 25 mL). The combined organic layers were then dried with magnesium sulfate and concentrated. The oily residue was dissolved in 100 mL of ethanol and held at reflux for 3 hours. The solution was concentrated and then distilled under vacuum (80 °C, 0.3 mmHg) to afford 16.2 g of 213 as a colorless oil in an 86% yield. \(^1\)H NMR: (CDCl\(_3\)) \(\delta\): 7.30 (m, 5H), 4.14 (q, 2H, \(J = 6.1\) Hz), 3.79 (s, 2H), 3.43 (s, 2H), 1.23 (t, 3H, \(J = 6.1\) Hz). \(^1^3\)C NMR: (CDCl\(_3\)) \(\delta\): 167.5, 129.9, 129.8, 129.6, 129.3, 129.1, 128.9, 127.8. IR (neat): \(v(\text{cm}^{-1})\): 2984.3, 1742.3, 1730.0, 1315.9, 1030.4.

\[ \beta\text{-Keto ester 213} \] (15.5 g, 75 mmol) was added to chloroform (170 mL) and stirred under argon at 25 °C, then sulfuryl chloride (5.7 mL, 60 mmol, 0.6 equiv) was slowly added over a 15 minute period. The reaction mixture was stirred
under argon for 3 hours or until the reaction was confirmed complete by TLC. Water (100 mL) was added to the mixture and the aqueous layer was extracted with methylene chloride (3 x 50 mL). The combined organic layers were dried with magnesium sulfate and concentrated yielding a light pink oil. The oil was separated by flash chromatography (silica, Hexanes: Toluene 1:1) to yield 13.2 g of 214 as a pink oil in 92% yield with recovered starting material. $^1$H NMR: (CDCl$_3$) $\delta$ 7.32 (m, 5H), 4.87 (s, 1H), 4.22 (q, 2H, $J = 6.3$ Hz), 4.01 (d, 2H, $J = 4.5$ Hz), 1.27 (t, 3H, $J = 6.1$ Hz). $^{13}$C NMR: (CDCl$_3$) $\delta$: 196.5, 165.1, 132.7, 129.9, 129.0, 127.7, 63.4, 60.6, 45.9, 14.1. IR (film): $\nu$ (cm$^{-1}$): 2987, 1764, 1692, 1269, 1185.

![Structure of compound 215](image)

A 45 mL solution of LB broth (supplemented with 30 µg/mL kanamycin) was inoculated with a single colony of *E. coli* (BL21(DE3)(pIK4)) and shaken overnight at 37 °C. The preculture (40 mL) was diluted (1: 100) into 4.0 L of LB (supplemented with 30 µg/mL kanamycin) in a New Brunswick M19 fermenter. The culture was grown for 2 hours at 37 °C with a stir rate of 800 rpm and an air flow of 0.5 vessel volumes per minute (vvm) until it reached O.D.$\_600 = 0.6$. The cell suspension was cooled to 28 °C over 15 minutes and then reductase overexpression was induced with isopropylthio-β-D-galactoside to a final concentration at 0.1 mM. The cells were kept under the same conditions for 6
hours and then collected by centrifugation (6000 g for 10 min at 4 °C). Half of the cells (25 g wet weight) were resuspended in 1 L of 10 mM KP$_i$ (pH = 5.6) containing 4 g/L glucose. The bioconversion was carried out in a Braun Biostat B fermenter at 30 °C with a pH maintained at 5.6 using 3 M NaOH. The dissolved oxygen was maintained at 75% saturation using a fixed air flow of 0.25 vvm and variable stirring rate. After the addition of the XAD-4 resin (0.5 g), portions of neat 214 (0.2 mL) were added approximately every hour over a total of 12 hours to provide a final concentration of 5 mM. Portions of glucose were added after 3.0 hours and 6.0 hours to maintain the glucose concentration at approximately 4 g/L. Consumption of 214 and glucose slowed significantly after 7 hours. After 24 hours, the reaction was gently extracted with methylene chloride (2 x 300 mL) to avoid an emulsion. The combined organic layers were dried with magnesium sulfate and concentrated under vacuum. The crude oil was purified by flash chromatography (Cyclohexane: Ether 85:15) to afford 1.1 g of 215 as a colorless oil with an 82% yield. [α]$_D$ = +23.7° (c = 0.7, CHCl$_3$). $^1$H NMR: (C$_6$D$_6$) δ 7.06 (m, 5H), 4.24 (ddd, 1H, $J_1$ = 3.3 Hz, $J_2$ = 6.6 Hz, $J_3$ = 7.1 Hz), 4.05 (d, 1H, $J_1$ = 3.3 Hz), 3.78 (q, 2H, $J$ = 7.2 Hz), 2.82 (dd, 1H, $J_1$ = 7.1 Hz, $J_2$ = 13.8 Hz), 2.72 (dd, 1H, $J_1$ = 6.6 Hz, $J_2$ = 13.8 Hz), 2.4 (br s, 1H), 0.79 (t, 3H, $J$ = 7.2 Hz). $^{13}$C NMR: (CDCl$_3$) δ 169.1, 137.1, 129.8, 129.2, 127.4, 73.4, 63.0, 61.2, 40.4, 14.4. IR (film): ν (cm$^{-1}$): 3483, 1740, 1300, 1183, 1025. Anal. Calcd for C$_{12}$H$_{15}$ClO$_3$: C, 59.39; H, 6.23. Found: C, 59.75; H, 6.72.
Chlorohydrin 215 (1.0 g, 4.1 mmol) was added to DMF (19 mL) and stirred at room temperature. Potassium carbonate (1.7 g, 12.4 mmol, 3 equiv) and water (390 µL) was added to the reaction mixture and stirred for 5 hours. The resulting mixture was diluted with water (50 mL), and the aqueous layer was extracted with ether (3 x 50 mL). The combined organic layers were then washed with water (6 x 5 mL) to remove residual DMF. The organic layer was dried with magnesium sulfate and purified by flash chromatography (silica, Cyclohexanes: Ether 85: 15) to afford 0.84 g of 216 as a colorless oil in a 99% yield. \([\alpha]_D = +37.1^\circ\) (c = 3.0, CHCl3). \(^1\)H NMR: (CDCl3) δ: 7.10 (m, 5H), 3.87 (dd, 2H, \(J = 2.7\) Hz, \(J = 6.9\)), 3.17 (d, 1H, \(J = 4.2\) Hz), 3.00 (dq, 2H, \(J_1 = 6.3\), \(J_2 = 14.8\)), 0.83 (t, 3H, \(J = 7.2\) Hz) \(^{13}\)C = NMR: (CDCl3) δ: 168.6, 137.0, 129.3, 129.1, 127.2, 62.0, 58.1, 53.3, 34.2, 14.7. IR (neat): ν(cm\(^{-1}\)): 2983.6, 2917.8, 1748.8, 1197.8, 1032.0. Anal. Calcd for C\(_{12}\)H\(_{14}\)O\(_3\): C, 69.89; H, 6.84. Found: C, 70.10; H, 6.91.
Glycidic ester 216 (100 mg, 0.49 mmol) was added to benzonitrile (900 µL) and cooled to 0 °C under an argon atmosphere. Boron trifluoride diethyl etherate (61.5 µL, 0.49 mmol, 1 equiv) was then slowly added to the reaction mixture over 10 minutes. The reaction was allowed to stir and warm up to room temperature over a 3 hour period. Saturated sodium bicarbonate (2 mL) was then added to the reaction and stirred for 2 hours. The reaction mixture was diluted with water (10 mL) followed by extraction with methylene chloride (3 x 15 mL). The organic layers were combined and dried with magnesium sulfate, followed by purification by flash chromatography (silica, Cyclohexane: Ether 9: 1) affording 0.12 g of 217 as a colorless oil with a 78% overall yield. \([\alpha]_D = -57.1^\circ (c = 2.0, \text{CHCl}_3)\).

$^1$H NMR: (C$_6$D$_6$) $\delta$ 8.37 (m, 2H), 7.28 (m, 8H), 4.90 (ddd, 1H, $J_1 = 6.3$ Hz, $J_2 = 6.3$ Hz, $J_3 = 6.9$ Hz), 4.78 (d, 1H, $J = 6.3$ Hz), 3.93 (m, 2H), 3.18 (dd, 1H, $J_1 = 6.3$ Hz, $J_2 = 13.8$ Hz), 2.92 (dd, 1H, $J_1 = 6.9$ Hz, $J_2 = 13.8$ Hz), 0.91 (t, 3H, $J = 7.2$ Hz).

$^{13}$C NMR: (C$_6$D$_6$) $\delta$ 170.2, 163.2, 131.8, (other aromatic signals obscured by solvent), 80.3, 73.7, 61.2, 42.1, 14.0. IR (film): $\nu$ (cm$^{-1}$): 3029, 2982, 1752, 1655, 1206, 1027, 695. Anal. Calcd for C$_{19}$H$_{19}$O$_3$N: C, 73.77; H, 6.19; N, 4.53. Found: C, 73.63; H, 6.14; N, 4.56.

Oxazoline 217 (323 mg, 1.04 mmol) was dissolved in ethanol (8 mL) and water (6 mL) and then concentrated HCl (15 mL, 12 M) was added. This solution
was heated at reflux for 8 hours and then concentrated under reduced pressure. The residue was resuspended in water (3 mL) and ethanol (1.5 mL) and then a Dowex 50WX8-100 resin (5 g) was added and gently swirled by hand for 5 minutes. The suspension was filtered and rinsed with water (15 mL), followed by a rinse with ethanol (15 mL). The beads were then rinsed with 2 M ammonium hydroxide and the filtrate was collected and lyophilized to afford 0.16 g of 137 as a white powder. The solid was dissolved in 6 M HCl (4 mL) and the solution was lyophilized to afford 0.20 g of the HCl salt with an 83% overall yield. \([\alpha]_D = +23.2^\circ (c = 1.3, 1 \text{ M HCl})\). (Sigma-Aldrich) \([\alpha]_D = +23.4^\circ (c = 1.0, 1 \text{ M HCl})\) Lit\(^67\). \([\alpha]_D = +26.8^\circ (c = 0.7, 1 \text{ M HCl})\) \(^1\)H NMR: (D2O) \(\delta:\) 7.39 (m, 5H), 4.37 (d, 1H, \(J = 3.0 \text{ Hz})\), 3.98 (dt, 1H, \(J_1 = 3.0 \text{ Hz, } J_2 = 7.5 \text{ Hz}\)), 3.10 (dq, 2H, \(J_1 = 7.5 \text{ Hz, } J_2 = 15 \text{ Hz}\)). \(^13\)C NMR: (D2O) \(\delta:\) 174.6, 135.3, 129.8, 129.6, 128.1, 68.8, 54.8, 35.5. IR (KBr): \(\nu(\text{cm}^{-1}):\) 3385.1, 2916.9, 2848.6, 1731.5, 1604.6, 1496.8, 1029.4. Melting Point: 201 – 203 °C. Melting Point: (Sigma-Aldrich) 203 – 204 °C.

![Image](https://via.placeholder.com/150)

\(\text{H}_2\text{C} \quad \text{OHO} \quad \text{OEt} \quad \text{Cl} \quad 248\)

A 45 mL solution of LB broth (supplemented with 30 µg/mL kanamycin) was inoculated with a single colony of \(E. \text{coli}\) (BL21(DE3)(pIK30)) and shaken overnight at 37 °C. The preculture (40 mL) was diluted (1: 100) to 4.0 L of LB (supplemented with 30 µg/mL kanamycin) in a New Brunswick M19 fermenter. The culture was grown for 2 hours at 37 °C with a stir rate of 800 rpm and an air
flow of 0.5 vessel volumes per minute (vvm) until it reached O.D.₆₀₀ = 0.6. The cell suspension was cooled to 28 °C over 15 minutes and then reductase overexpression was induced with isopropylthio-β-D-galactoside to a final concentration at 0.1 mM. The cells were kept under the same conditions for 6 hours and then collected by centrifugation (6000 g for 10 min at 4 °C). Half of the cells (25 g wet weight) were resuspended in 1 L of 10 mM KPᵢ (pH = 5.6) containing 4 g/L glucose. The bioconversion was carried out in a Braun Biostat B fermenter at 30 °C with a pH maintained at 5.6 using 3 M NaOH. The dissolved oxygen was maintained at 75% saturation using a fixed air flow of 0.25 vvm and variable stirring rate. Portions of commercially available ethyl 2-chloroacetoacetate 220 (0.25 mL) were added approximately 4 times an hour over a total of 8 hours to provide a final concentration of 24 mM. Portions of glucose were added after 2.0 hours and 5.0 hours to maintain the glucose concentration at approximately 4 g/L. Consumption of 220 and glucose slowed significantly after 6 hours. After 12 hours, the reaction was gently extracted with methylene chloride (4 x 300 mL) to avoid an emulsion. The combined organic layers were dried with magnesium sulfate and concentrated under vacuum. The crude oil was purified by flash chromatography (Cyclohexane: Ether 85:15) to afford 4.4 g of 248 as a colorless oil with an 89% yield. [α]D = +10.8° (c = 1.9, CHCl₃). Lit.¹⁰⁷ [α]D = +12.4° (c = 1.0, CHCl₃). ¹H NMR: (C₆D₆) δ: 4.00 (m, 1H, J = 5.7 Hz), 3.88 (d, 1H J = 4.7 Hz), 3.82 (q, 2H, J = 7.2 Hz), 2.33 (bs, 1H), 1.02 (t, 3H, J = 6.3), 0.84 (t, 3H, J = 7.2). ¹³C NMR: (C₆D₆) δ: 168.6, 68.6, 63.5,
62.3, 19.7, 13.9. IR (neat): v(cm\(^{-1}\)): 3458.3, 2984.3, 1747.1, 1182.6, 944.1, 878.4.

Chlorohydrin 248 (100 mg, 0.6 mmol) was added to DMF (3.2 mL) and stirred at room temperature. Potassium carbonate (249 mg, 1.80 mmol, 3 equiv) and water (62 µL, 3 equiv) was added to the reaction mixture and stirred for 5 hours. The resulting mixture was dissolved in water (30 mL), and the aqueous layer was extracted with diethyl ether (3 x 30 mL). The organic layer was then washed with water (6 x 3 mL) to remove residual DMF. The combined organic layers were dried with magnesium sulfate and purified by flash chromatography (silica, Cyclohexanes: Ether 85: 15) to afford 0.66 g of 250 as a colorless oil in an 85% yield. \([\alpha]_D = -13.8^\circ\) (c = 1.4, CHCl\(_3\)). \(^1\)H NMR: (C\(_6\)D\(_6\)) \(\delta\): 3.91 (q, 2H, \(J = 7.2\) Hz), 3.19 (d, 1H \(J = 4.2\) Hz), 2.73 (m, 1H, \(J = 4.5\) Hz), 1.11 (d, 3H, \(J = 5.4\)), 0.90 (t, 3H, \(J = 7.2\)). \(^13\)C NMR: (C\(_6\)D\(_6\)) \(\delta\): 168.0, 60.9, 53.0, 52.8, 14.2, 12.9. IR (neat): v(cm\(^{-1}\)): 3418.5, 2929.5, 1663.3, 1389.9, 1258.8, 1098.5.

Chlorohydrin 248 (500 mg, 3.01 mmol) was dissolved in ethanol (12 mL) and cooled to 0 °C. A small chunk of excess sodium (213 mg, 9.00 mmol) was slowly added to the reaction mixture and allowed to stir for three hours at 0 °C.
The solution was concentrated under reduced pressure and then 0.5 M HCl (20 mL) added to the residue. The aqueous mixture was extracted with diethyl ether (3 x 20 mL) and the organic layers were combined and dried by magnesium sulfate. The combined organic layers were concentrated under reduced pressure to afford a mixture of cis and trans isomers that could not be fully separated. Spectral data was not obtained because 243 was not obtained in pure form.
APPENDIX B
ADDITIONAL INFORMATION

NMR Analysis for Absolute Configuration of Compound 215 and 230

NMR spectra were recorded on a Varian Inova spectrometer equipped with a 5 mm indirect detection probe, operating at 500 MHz for $^1$H and at 125 MHz for $^{13}$C. Chemical shifts at 25ºC are reported in ppm relative to TMS. In this case benzene-$d_6$ was used as the solvent to analyze compounds 215 and 230 to remove some signal overlap. Complete $^1$H and $^{13}$C chemical shifts assignments for the compound were based on the $^1$H-$^1$H, $^1$H-$^{13}$C one-bond and $^1$H-$^{13}$C long-range couplings seen in the proton and the G-BIRD-HSQMBC spectra.

The relative configuration of the reduction product was determined by NMR through the combined use of $^3$J(H-H) homonuclear and $^n$J(C-H) long range heteronuclear coupling constants$^{115,116}$. Of the six staggered rotamers of the erythro (anti) and threo (syn) configurations, presented in Figure B-3, four can be identified by the pattern of coupling constants. The other two rotamers, A3 and B3, display the same pattern of coupling constants. Fortunately, in none of the pairs of diastereomers that we analyzed was threo in the A3 conformation and the erythro in the B3.

The long range coupling constants presented in Figure B-1 were measured in $f_2$ slices of the G-BIRD-HSQMBC spectra,$^{122}$ with a precision of ± 0.5 Hz. The patterns of coupling constants indicate that the preferred conformation is A1 for the threo configuration and B3 for the erythro, i.e. the conformation in which the
R and the COOEt groups are *anti*. These are the only conformations for 215 and 230.

![Chemical structures of 215, 230, and 230](image)

<table>
<thead>
<tr>
<th></th>
<th>YDR368w</th>
<th>YGL157w</th>
<th>YGL039w</th>
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</thead>
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<td>7.8</td>
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<td>2.5</td>
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<td>3.1</td>
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<tr>
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<td>erythro</td>
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Figure B-1. Long range coupling constants (Hz) for the major enzymatic reduction product of YDR368w, YGL157w, and YGL039w

Absolute configurations were determined based on the differences in chemical shifts between the (R)- and (S)-α-methoxy-α-phenylacetic (MPA) esters. The esterification was carried out in the NMR tube, by adding one equivalent of a mixture consisting of 2 parts (R)-MPA and 1 part (S)-MPA to the alcohol, followed by the addition of DCC (1.5 equiv.) and DMAP (0.5 equiv.). The chemical shifts difference between the R- and S-MPA esters demonstrate that 215 is the 2R,3S ester and 230 is the 2S,3S ester (Figure B-2).
<table>
<thead>
<tr>
<th>Species</th>
<th>Alcohol δH2</th>
<th>Alcohol δH1</th>
<th>Alcohol δH1'</th>
<th>Alcohol δH2'</th>
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<th>R-MPA Ester δH4a</th>
<th>R-MPA Ester δH4b</th>
<th>R-MPA Ester δHortho</th>
<th>S-MPA Ester δH3</th>
<th>S-MPA Ester δH4a</th>
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<td>YDR368w</td>
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Figure B-2. The chemical shifts for the major reduction products of YDR368w, YGL157w, and YGL039w
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<table>
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**Figure B-3.** Coupling constants in the staggered rotamers of threo and erythro diastereomers of reduction products.

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<th>Coupling Constant</th>
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<td>$^3J(H_2-H_3)$</td>
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<td>1 to 4</td>
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<td>$^3J(H_2-C_4)$</td>
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<td>1 to 3</td>
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<tr>
<td>$^3J(H_3-C_1)$</td>
<td>6 to 8</td>
<td>1 to 3</td>
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<tr>
<td>$^2J(H_2-C_3)$</td>
<td>-5 to -7</td>
<td>0 to -2</td>
</tr>
</tbody>
</table>

**Figure B-4.** Range of values for the large and small coupling constants (Hz).
Specific Activity Calculations

\[ A = \varepsilon_{bc} \]
\[ \varepsilon = \text{L / mol-cm} \quad b = \text{cm} \quad c = \text{mol / L} \]

Velocity (mol / L-min) = \[\frac{\Delta A}{\Delta t}\] / \(\varepsilon b = \Delta c / \Delta t\)

Activity (µmol / min-mL) = (moles NADPH consumed / unit) / Volume of protein used

Specific activity (µmol / min-mg) = activity / protein concentration

Protein concentration was calculated by a traditional Bradford assay using BSA, monitored at 595 nm (Figure B-5).

![Figure B-5. Line equation for the Bradford assay](image-url)
Figure B-6. NMR of (+)-AHPA synthesized by our strategy
Figure B-7. NMR spectra of authentic (+)-AHPA
Figure B-8. NMR spectra of the Taxol side chain 130
Figure B-9. NMR spectra of the Taxol side chain enantiomer ent-130
Figure B-10. $^1$H NMR for derivatized 130 and ent-130: Top spectra is the (S)-MPA ester of ent-130; bottom spectra is the (S)-MPA ester of the Taxol side chain 130.
Figure B-11. $^1$H NMR spectra of syn product from YDR368w (top) and anti product from TGL157w (bottom)
LIST OF REFERENCES


(43) Zhou, Z.; Mei, X. *Synthetic Communications* 2003, 33, 723-728.


(67) Seki, M.; Nakao, K. *Bioscience, Biotechnology, and Biochemistry* 1999, 63, 1304-1307.


BIOGRAPHICAL SKETCH

Brent Derek Feske was born in Joliet, Illinois, on June 28th, 1978. He began his initial research working with Dr. John Allen on the synthesis of ion selective electrodes to monitor nitrate levels in local wetlands. He then worked for Dr. Newton Hilliard on the isolation of the proteins involved for the unusual metabolic pathway in *Thiobacillus neapolitanus*. Brent also spent two summers at Albemarle Corporation. His first summer was spent in the analytical department working with HPLC and GC. His second summer was spent in the applications department working with flame retardants in polyurethane foam. In 2000, Brent graduated *cum laude* from Southeastern Louisiana University and then moved his scientific career to the University of Florida. His graduate research was on the synthesis of chiral molecules and pharmaceuticals using *E. coli* engineered to overexpress a single bakers’ yeast reductase. He will further this research, along with teaching undergraduate chemistry, at Armstrong Atlantic State University in Savannah, Georgia.