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SYNTHESES OF D,L-1,2,4-BUTANETRIOL
AND ε-CAPROLACTAM FROM D-GLUCOSE-DERIVED
STARTING MATERIALS

presented by

MAPITSO N MOLEFE

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John W. Frost
Major Professor's Signature

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SYNTHESES OF D,L-1,2,4-BUTANETRIOL AND ε-CAPROLACTAM FROM D-GLUCOSE-DERIVED STARTING MATERIALS

By

Mapitso N. Molefe

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ABSTRACT

SYNTHESES OF D,L-1,2,4-BUTANETRIOL AND ε-CAPROLACTAM FROM D-GLUCOSE-DERIVED STARTING MATERIALS

By

Mapitso N. Molefe

Syntheses of D,L-1,2,4-butanetriol and ε-caprolactam, from renewable and abundant starting materials using chemical catalysis and biocatalysis are described. The current syntheses of these chemicals rely on the use of stoichiometric reagents or use of non-renewable starting materials. For the synthesis of D,L-1,2,4-butanetriol, two routes involving catalytic hydrogenation as a common step are described. In the first route, D, L-malic acid as well as L-malic acid were hydrogenated over Ru on carbon to afford D,L-1,2,4-butanetriol in 74% yield as part of product mixture. Variation of hydrogen pressure, reaction temperature, time, concentration, catalysts and catalyst loading were evaluated to find optimum reaction conditions. The major contaminants produced were 1,2-propanediol (12%) and 1,4-butanediol (8%). 1,2-Propanediol was shown to result from C-C bond cleavage of D,L-1,2,4-butanetriol whereas 1,4-butanediol resulted from dehydration of 3,4-dihydroxybutyric acid or the corresponding 3-hydroxy-γ-butyrolactone followed by hydrogenation. Difficulties associated with purifying D,L-1,2,4-butanetriol from contaminating diols led to the development of an alternative route. In the second route, 2-hydroxy-2-buten-4-olide was hydrogenated to D,L-1,2,4-butanetriol in 96% yield, without accumulation of byproducts. This substrate was obtained from

oxidative cleavage of L-ascorbic acid to L-threonate. Dehydration of L-threonate to 4-hydroxy-2-ketobutyrate was catalyzed by dihydroxy-acid dehydratase. Finally, 4-hydroxy-2-ketobutyrate was cyclized to 2-hydroxy-2-buten-4-olide, which was hydrogenated to afford D,L-1,2,4-butanetriol in 53% overall yield.

ε-Caprolactam, a monomer for nylon 6 was synthesized from L-lysine. Several routes for deamination of the α-amino group of L-lysine were explored. The strategy involved cyclization of L-lysine followed by deamination of α-aminocaprolactam. Alternatively, ε-caprolactam was obtained by deamination of L-lysine followed by cyclization of 6-aminocaproic acid. Catalytic deamination over Pt on SiO_2 and Mo-Ni-S on Al_2O_3 catalysts were attempted to deaminate α-aminocaprolactam. Partial deamination was observed when α-aminocaprolactam was subjected to reduction with sodium metal in 2-propanol (20%). Alternatively, deamination of an isomer of L-lysine, L-β-lysine, was attempted. L-β-Lysine is the first intermediate in the biodegradation of L-lysine by some *Clostridium* species under anaerobic conditions. L-Lysine-2,3-aminomutase (LAM) catalyzes the conversion. Intact cells and crude extracts of LAM were explored for the isomerization of L-lysine. The resulting product was cyclized to β-aminocaprolactam and deaminated to ε-caprolactam.

To my family

For their constant love and support.

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LIST OF ABBREVIATIONS

Ac	acetyl
ADP	adenosine diphosphate
ATP	adenosine triphosphate
Ap	ampicillin
Ap^R	ampicillin resistance gene
bp	base pair
BSTFA	bis(trimethylsisly)trifluoroacetamide
BT	1,2,4-butanetriol
BTX	benzene, toluene, xylene
CA	chorismic acid
CIAP	calf intestinal alkaline phosphatase
COMT	catechol-*O*-methyltransferase
DAHP	3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate
DCU	digital control unit
DEAE	diethylaminoethyl
DERA	2-deoxyribose 5-phosphate aldolase
DHAD	dihydroxy-acid dehydratase
DHQ	3-dehydroquinic acid
DHS	3-dehydroshikimic acid
D.O.	dissolved oxygen
DTT	dithiothreitol
E4P	D-erythrose 4-phosphate
EPSP	5-enolpyruvoylshikimate 3-phosphate
FBR	feedback resistant

GA	gallic acid
h	hour
HPLC	high pressure liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
Kan	kanamycin
kb	kilobase
kg	kilogram
LAM	lysine aminomutase
LPA	lysophosphatidic acid
M9	minimal salts
min	minute
mL	milliliter
μL	microliter
mM	millimolar
μM	micromolar
OD	optical density
PBT	polybutyleneterephthalate
PCA	protocatechuic acid
PEG	polyethylene glycol
PEP	phosphoenolpyruvic acid
PHB	*p*-hydroxybenzoic acid
PID	proportional-integral-derivative
PCR	polymerase chain reaction
Phe	L-phenylalanine
PLP	pyrodoxal-5-phosphate
psi	pounds per square inch
PTS	phosphotransferase system

PTT	polytrimethylene terephthalate
PET	polyethylene terephthalate
PVC	polyvinyl chloride
Tyr	L-tyrosine
Trp	L-tryptophan
QA	quinic acid
rpm	rotations per minute
SA	shikimic acid
SAM	*S*-adenosylmethionine
SDS	sodium dodecyl sulfate
S3P	shikimate 3-phosphate
Tc	tetracycline
TSP	sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$
TsOH	*p*-toluenesulfonic acid
UV	ultraviolet

CHAPTER 1

INTRODUCTION

Petroleum oil and natural gas are the raw material from which most organic chemicals are derived.[1] The current geopolitical instabilities, high demand and declining availability have resulted in steadily increasing crude oil prices.[2] Furthermore, these non-renewable resources are slowly dwindling with year 2040 projected to be the end of cheap crude oil.[3] For the chemical industry to continue to thrive, integration of renewable feedstock into their manufacturing process will be critical. Nature provides terrestrial biomass such as sugars, amino acids, lipids, biopolymers such as cellulose, hemicellulose, chitin, starch, lignin and proteins in abundance. Carbohydrates constitute two-thirds of the annually renewable biomass even though this feedstock is under-utilized by the chemical industry.[4] Systematic development of appropriate methodologies to convert carbohydrates into industrially useful organic building blocks that compete with petroleum-derived building blocks is important. Particularly, the use of biotechnology in which microbial transformation, fermentation and enzymatic conversion of renewable feedstock into commodity chemicals, fine chemicals, pharmaceuticals agents, or enantiopure organic building blocks is gaining popularity.[4]

For the completion of this dissertation, two chemicals currently derived from petroleum were synthesized from renewable feedstock by incorporating chemical and chemoenzymatic syntheses. Chapter 2 describes catalytic hydrogenation of petroleum-derived D,L-malic acid and biomass-derived L-malic acid over Ru on C, which afforded D,L-1,2,4-butanetriol in 74% yields as part of a product mixture. The effects of hydrogenation time, pressure, and temperature along with substrate concentration,

catalyst loading and catalyst composition were examined. Complications associated with separating product from the reaction mixture by distillation resulted a 28% yield of D,L-1,2,4-butanetriol with significant contamination from 1,4-butanediol. An alternate chemoenzymatic route to D,L-1,2,4-butanetriol employed D-glucose-derived L-ascorbic acid as the starting material. Chemical oxidation of L-ascorbic acid gave L-threonate, which was dehydrated using dihydroxy-acid dehydratase. Cyclization of the resulting 4-hydroxy-2-ketobutyrate to 2-hydroxy-2-buten-4-olide was followed by hydrogenation over Ru on C to afford D,L-1,2,4-butanetriol as the only product in 53% overall yield from L-ascorbic acid.

Chapter 3 of the thesis explores the use of L-lysine as the starting material for the synthesis of ε-caprolactam. L-Lysine is microbially synthesized from D-glucose. Denitrification using catalytic hydrogenation of L-lysine over Pt/C and Mo-based catalysts was evaluated along with single electron reduction of three substrates: α-aminocaprolactam, the methyl ester of L-lysine, and methyl pipecolinate. To setup removal of the α-amino group of L-lysine, intact *Clostridium subterminale* cells were utilized to isomerize L-lysine to L-β-lysine. Elimination of the β-amino group and subsequent hydrogenation to afford the ε-caprolactam was evaluated over Ru on C.

Biomass-derived building blocks

Carbohydrate starting materials are heavily functionalized with hydroxyl groups and they possess a high density of stereochemistry while petroleum-derived hydrocarbons are bare of functional groups and stereocenters. By exploiting the structural backbone of carbohydrate starting materials, building blocks such as D,L-1,2,4-butanetriol and ε-caprolactam, which have industrial utility, were accessed (Figure 1).

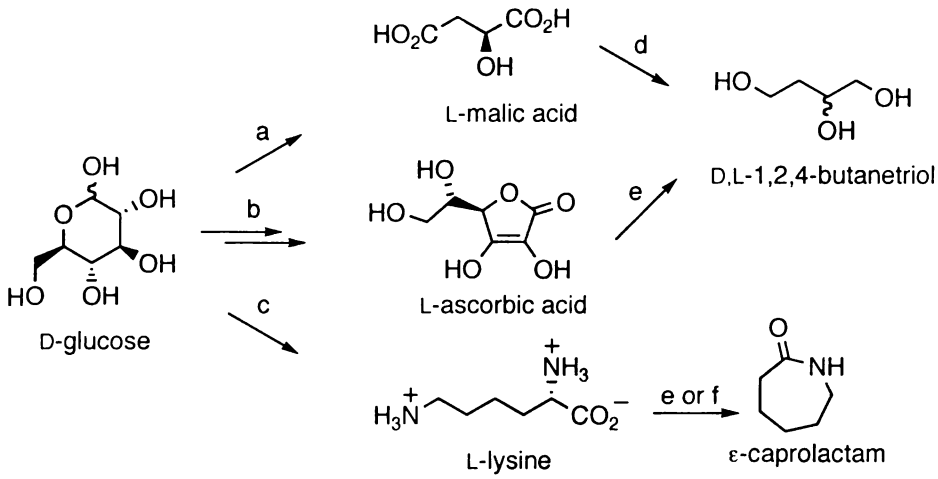

Figure 1. D-Glucose-derived D,L-1,2,4-butanetriol and ε-caprolactam. Key: (a) *Aspergillus flavus*; (b) Reichstein-Grüssner Process; (c) *Corynebacterium glutamicum*; (d) Catalytic hydrogenation; (e) Chemoenzymatic synthesis; (f) Reductive deamination.

For the biomass-based chemical industry to be competitive, replacement of low molecular weight building blocks from petroleum needs to be addressed. The technology for the conversion of petroleum into primary building blocks for the chemical industry such as ethylene, propylene, benzene, toluene, xylene (BTX) and butadiene is very well developed while production of building blocks from biopolymers is still under development.[5] D-Glucose is the most abundant monosaccharide industrially produced in pure form and is obtained from enzymatic hydrolysis of starch.[6] Cellulose is likely to serve as a cost-efficient source of D-glucose in the future and would avoid competition between food and chemical use of starch-derived D-glucose.[7] The cellulose ethanol technology recently developed by Iogen in which 85 gallons of ethanol was obtained from a ton of wheat straws has opened new avenues for the utilization of cellulosic biomass as a source of carbohydrates.[8]

Twelve value-added chemicals from biomass have recently been identified as potential basic building blocks for the chemical industry (Figure 2).[8a,9] These building

blocks along with L-ascorbic acid, L-malic acid and L-lysine discussed in the next chapters, are synthesized from D-glucose.

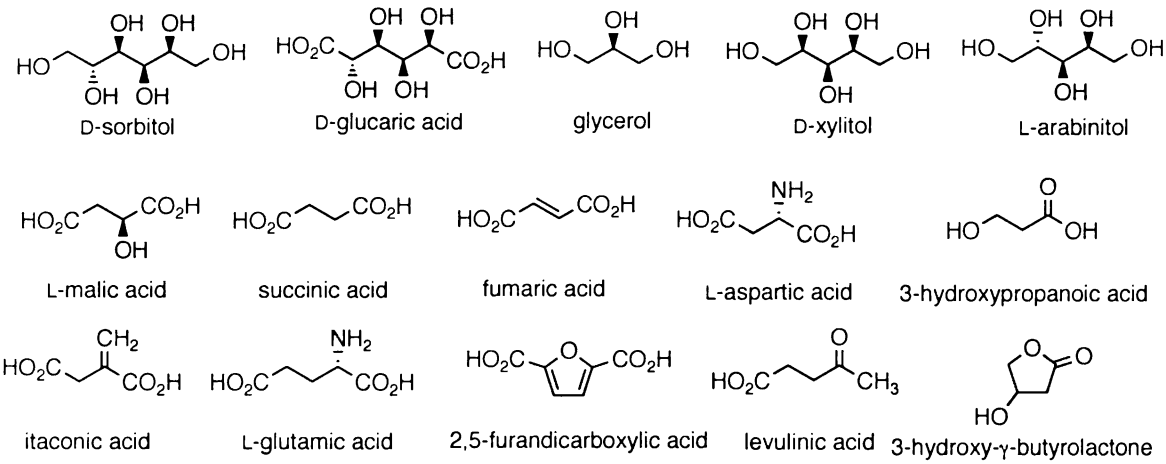

Figure 2. Chemical building blocks derived from biomass.

These molecules include glycerol, 3-hydroxypropanoic acid, 3-hydroxy-γ-butyrolactone, aspartic acid, itaconic acid, levulinic acid, glutamic acid, D-xylitol and L-arabitol, glucaric acid, 2,5-furandicarboxylic acid, D-sorbitol and 1,4-dicarboxylic acids such as succinic acid, L-malic acid and fumaric acid. Because these building blocks have multiple functional groups, they can serve as platform chemicals from which a variety of products could be derived. Synthesis of selected building blocks and their derivatization is examined from two perspectives. The first part involves transformation of sugars into the basic building blocks while the second part entails derivatization of the basic building block to various secondary building blocks. Biotransformation is an important feature of routes from plant feedstock into sugars through to basic building blocks. Chemical transformation on the other hand predominates in converting basic building blocks into secondary building blocks. A brief overview of challenges associated with conversion of sugar into the selected basic building blocks and subsequent conversion into secondary building blocks is given.

3-Hydroxypropionic acid

3-Hydroxypropanoic acid or β-lactic acid has a potential application as a monomer for (co)-polymerization similar to lactic acid, which affords a biodegradable thermoplastic polylactide polymer.[10] Furthermore, it can be hydrogenated to 1,3-propanediol, which along with terephthalic acid, is polymerized to polytrimethylene terephthalate (PTT).[11] PTT is a polymer with desirable properties such as good resilience, stain resistance and low static generation relative to its competitors PET (polyethylene terephthalate) and nylon in fiber and textile applications. Dehydration of β-lactic acid can also result in a family of acrylates such as acrylic acid, methyl acrylate and acrylamide (Figure 3).

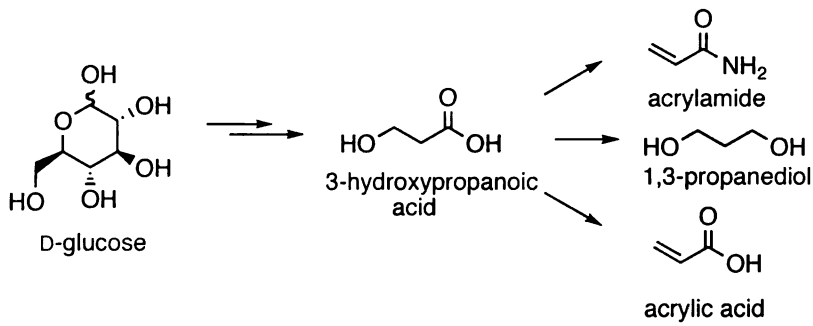

Figure 3. Synthesis of 3-hydroxypropanoic acid and potential derivatives.

Acrylic acid is in high demand (1.2 x 10^9 kg/year in the US) and is currently derived from oxidation of propylene via the intermediacy of acrolein.[12] Acrylates are used primarily to prepare emulsion and solution polymers, which have found application in textile, paint, adhesives and paper industry.[12]

The high price of 3-hydroxypropanoic acid limits its application. Microbial synthesis of 3-hydroxypropanoic acid has not yet been commercialized while synthesis of L-lactic acid from corn starch has been commercialized in a joint venture between Cargill

and Dow Chemical with the annual capacity of 140 × 10^6 kg.[13] In 2001, genetically engineered *E. coli* expressing glycerol dehydratase from *Klebsiella pneumoniae* and nonspecific aldehyde dehydrogenase from *S. cerevisiae* was reported to produce 0.2 g/L of 3-hydroxypropanoic acid from glycerol.[14] Since then, five possible biosynthetic routes from D–glucose have been proposed in the patent literature.[15,16]

The microbial synthesis of 3-hydroxypropanoic acid can only be economically competitive if a minimum concentration of 2.5 g/L is achieved in 1 h.[8a,9] Proposed derivatives from 3-hydroxypropanoic acid such as acrylates, which are currently produced from petroleum, must also be accessible via a low cost and high-yielding route.

2,5-Furandicarboxylic acid

Acid-catalyzed dehydration of biomass derived pentoses and hexoses affords two furanic compounds, furfural and 5-hydroxymethylfurfural **1** (Figure 4).[17] These two compounds and their derivatives represent a class of compounds that are suitable for use as monomers for the preparation of non-petroleum derived polymeric materials. 5-Hydroxymethylfurfural **1** has reportedly been used in the manufacture of phenolic resin.[18] 5-Hydroxymethylfurfural **1** is readily accessible from acid-catalyzed elimination of 3 mol of water from fructose or inulin hydrolysates.[19] This six-carbon commodity chemical is a key intermediate because of various industrially significant chemicals that can be derived from it (Figure 4).

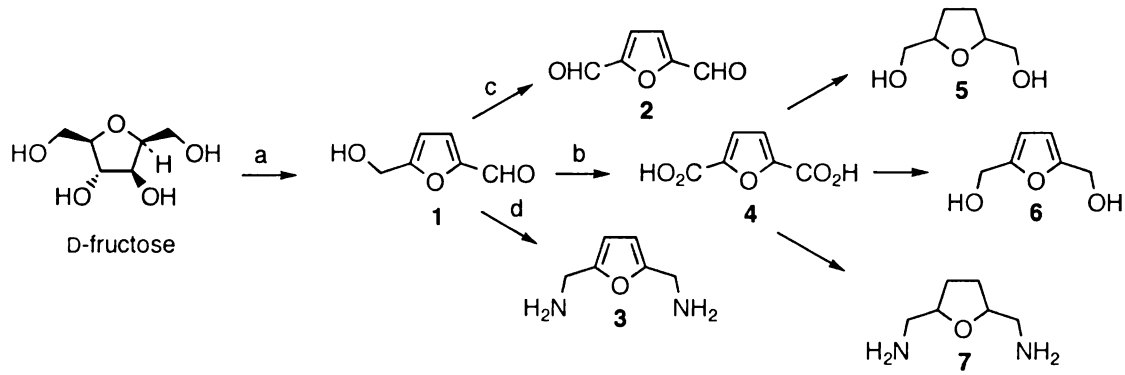

Figure 4. Dehydration of D-fructose to versatile derivatives of 2,5-disubstituted furan. Key: a) H_3O^+; b) Pt/Pd, C/ O_2; c) $BaMnO_4$; d) i. NH_2OH, ii. Ni/ H_2.

These include oxidation to 2,5-furandicarboxaldehyde **2**, reduction of aldehyde to 2,5-bis(hydroxymethyl)-furan **6**, oxidation to 2,5-furandicarboxylic acid **4** or reductive amination to 2,5-bis(aminomethyl)-furan **3**.[20] These monomers have been exploited for the preparation of furanoic polymers. In particular, copolymerization of **3** and **4** to furanoic polyamide has a potential of replacing petroleum-based polyamides.[8a,9,,21] Copolymerization of 2,5-furandicarboxylic acid **4** with aliphatic or aromatic diamines has resulted in an analogue of Kelvar®.[22] This polyamide was found to have a promising glass transition temperature and thermal stability compared to all furanic polyamide. Unlike furfural, which is produced industrially at 200 000 tons/yr capacity from dehydration of pentoses, 5-hydroxymethylfurfural **1** has only been produced on a pilot plant scale. Hydroxymethylation of furfural in excess formaldehyde has been evaluated for the synthesis of 5-hydroxymethylfurfural **1**.[21] Due to the deactivating effect of the aldehyde group, the conversion of furfural was only 50%. Reversal of polarity by protecting the aldehyde as 1,3-dithiolane however, resulted in the formation of 5-hydroxymethylfurfural **1** in 90% yield. 2,5-Furandicarboxylic acid **4** has a potential of replacing terephthalic acid, which is widely used in polymers such as polyethylene terephthate (PET) and polybutyleneterephthalate (PBT).[23] There is an annual demand of

5 billion lb for these polymers.[8a,9] The increasing price of benzene, from which the aromatic monomers are derived, fueled by high demand and limited availability would favor furanic compounds as alternative building blocks.[2]

Levulinic acid

Levulinic acid has great potential as an inexpensive feedstock for producing a wide variety of industrially important products (Figure 5).[24] Currently, the worldwide market for levulinic acid is ~ 1 million lb/year at a price of $4-6/lb. Levulinic esters and salts are used in the food industry as preservatives, stabilizers and as flavoring agents.[25a] These esters increase thermal stability of poly(vinylchloride) when used as additives. δ-Amino levulinic acid is a nontoxic, biodegradable broad-spectrum herbicide, which is triggered by light to kill weeds.[25b]

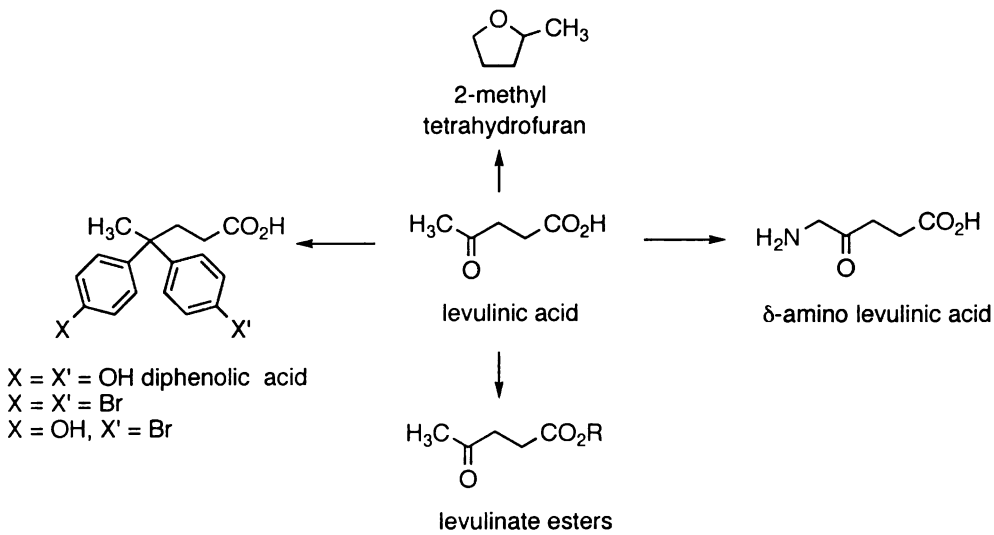

Figure 5. Levulunic acid derivatives with industrial applications.

Diphenolic acid has a potential of replacing bisphenol-A as a monomer in the production of polycarbonate resins. Brominated diphenolic acid on the other hand could serve as an environmentally acceptable marine coating while dibrominated diphenolic acid may find

use a fire retardant. The U. S. Department of Energy has approved the use of methyl tetrahydrofuran as a fuel additive, which increases oxygenate levels in gasoline.[25c-d] This molecule is derived from catalytic hydrogenation of levulinic acid. The process developed by Biofine Inc. for the synthesis of methyl tetrahydrofuran, together with the high demand for δ-amino levulinic acid, may potentially expand the demand of levulininc acid to between 200 million and 400 million lb/year thus decreasing the price to $0.04-$0.10 per pound.

The process for the synthesis of levulinic acid relies on feedstocks such as cellulose-containing waste materials from paper mill sludge, waste wood, paper waste, and agricultural residues. Hydrolysis of cellulosic biomass results in hexoses, which are enolized to enediol **8** under acidic conditions (Figure 6).[26] The first dehydration affords an enol form of 3-deoxyhexosulose **9**, which undergoes further dehydration to 3,4-dideoxyglycosulosene-3 **10**. The latter is readily converted into dienediol **11**, which after cyclization results in **12**. Subsequent dehydration affords 5-hydroxymethyl furfural **1**. Addition of a molecule of water across C2-C3 of 5-hydroxymethylfurfural results in the ring opening of the furfural into an unstable tricarbonyl intermediate **13**. The latter decomposes into levulinic acid and formic acid.

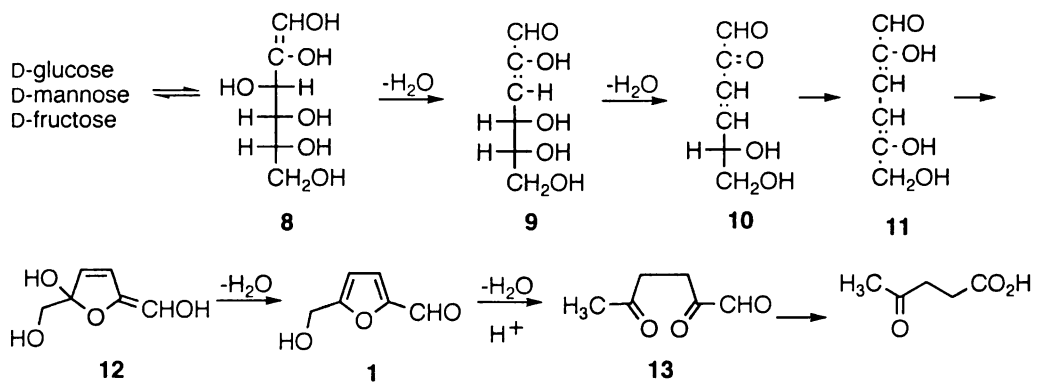

Figure 6. Acid-catalyzed dehydration of hexoses to levulinic acid.

Alternatively, heating of furfuryl alcohol in the presence of HCl has been reported to yield up to 80% of levulinic acid. Both furfuryl alcohol and 5- hydroxymethyl furfural are derived from wood pulp processing.[26] Side products consisting of humic compounds reduce the yield of levulinic acid from cellulosic biomass. Development of a more selective dehydration process is the key to the use of levulinic acid as a building block.[8a,9]

D-Sorbitol

Batch-hydrogenation of D-glucose to D-sorbitol over Raney nickel is an established route for the synthesis of this sugar alcohol (Figure 7).[27] As an alternative to the widely used batch process, a continuous process based on Ru affords an almost quantitative yield of D-sorbitol with a high hourly space velocity.[9] Almost all of the 650,000 tons of sorbitol produced annually is used as a food additive.

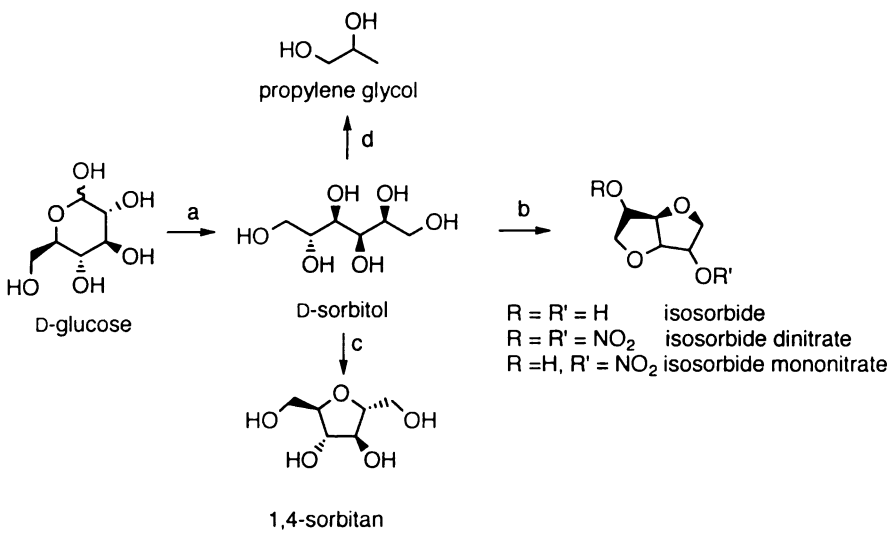

Figure 7. Conversion of glucose to sorbitol and its derivatives. Key: a) Raney Ni 122 atm H_2, 140 °C; b) H_3O^+; c) H_3O^+; d) Ru/C H_2.

D-Sorbitol is also an intermediate in Reichstein's synthesis of L-ascorbic acid from D-glucose.[28] This large-volume sugar alcohol can potentially be the source of dehydration sugars such as isosorbide or 1,4-sorbitan (Figure 7). Isosorbide mono- and

dinitrates are used therapeutically as vasodilators to treat angina pectoris, congestive heart failure and dysphasia.[29] Another commercially viable derivative of sorbitol, sorbitan monoesters (SME) obtained from esterification of 1,4-sorbitan are used commercially as non-ionic surfactants, solubilizers and emulsifiers in cosmetics and various other formulations.[30]

Recent studies have shown that sorbitol is an effective stabilizer of polyvinyl chloride (PVC). This occurs by dehydration of sorbitol to 1,4-anhydrosorbitol. The water formed during the intramolecular dehydration then binds free hydrogen chloride in PVC via hydrogen bonding.[31] Isosorbide has also been reported to improve properties of polyethyleneterephthalate when used as a comonomer.[9] Catalytic hydrogenolysis of sorbitol to 1,2-propanediol is not selective. An alternative synthesis of propylene glycol from hydrogenation of D-glucose-derived lactic acid over Ru on C has been reported.[32] Microbial fermentation of common sugars such as D-glucose and D-xylose have also been shown to afford 1,2-propanediol under anaerobic conditions.[33]

L-Malic Acid

Over 80% of malic acid produced worldwide is used in food and beverages.[34a] Both L- and D, L-malic acid are generally recognized as safe (GRAS) substances for use as flavor enhancers, pH control agents, flavoring agents and adjuvants (Figure 8). D, L-Malic acid is obtained from acid-catalyzed hydration of fumaric acid and hydrolysis of maleic anhydride, which is derived from butane oxidation over $(VO)_2P_2O_7$.[34b]

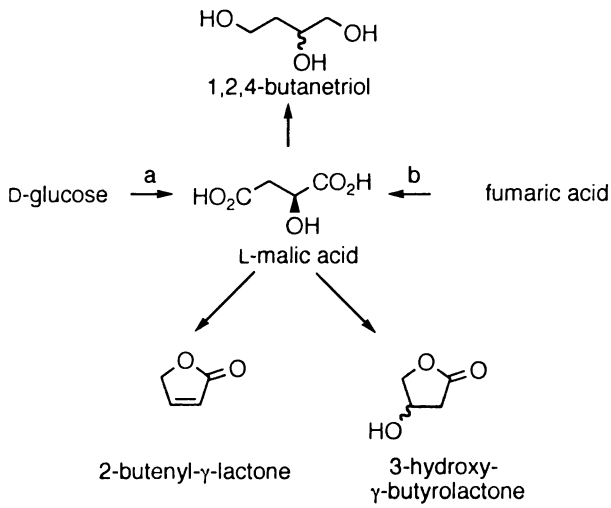

Figure 8. Malic acid and derivatives with industrial applications. Key: (a) *Aspergillus flavus*; (b) *Brevibacterium flavum.*

L-Malic acid is produced by two biocatalytic methods. Enzymatic hydration of fumaric acid is catalyzed by intact cells of *Brevibacterium flavum* or isolated fumarase activity (Figure 8).[35a] The second route involves direct fermentation of D-glucose under aerobic conditions to afford L-malic acid.[35b] A pilot plant aimed at hydrogenating malic anhydride to 3-hydroxy-γ-butyrolactone at 250 atm H_2 was constructed by SK Energy and Chemicals in Daeduk, Korea.[36] It is anticipated that this plant would supply the pharmaceutical industry with 3-hydroxy-γ-butyrolactone, which is currently obtained from oxidation of pentoses and hexoses.[37] Dehydration of 3-hydroxy-γ-butyrolactone to 2-butenyl-γ-lactone, and esterification can yield acrylate lactone, which can potentially find use in the synthesis of new polymers.[9] Complete hydrogenation of malic acid affords 1,2,4-butanetriol, a subject of this thesis. Applications of D,L-1,2,4-butanetriol range from application as a synthetic scaffold in organic chemistry and incorporation into energetic plasticizers to treatment of angina pectoris after nitration to D,L-1,2,4-butanetriol trinitrate.[38]

L-Aspartic acid

L-Aspartic acid is widely used in the food and pharmaceutical industries and is needed for the production of the low-calorie sweetener aspartame (Figure 9).[39] It is obtained by amination of fumaric acid catalyzed by immobilized aspartate ammonia-lyase (aspartase) from *Bacillus* with an annual capacity of 100 ton/yr.[40] The major drawback for the development of aspartic acid as a building block is the lack of a direct fermentation route starting from sugar substrate.[9] Reduction in the cost of fumaric acid production from D-glucose fermentation could potentially reduce the cost of L-aspartic acid.

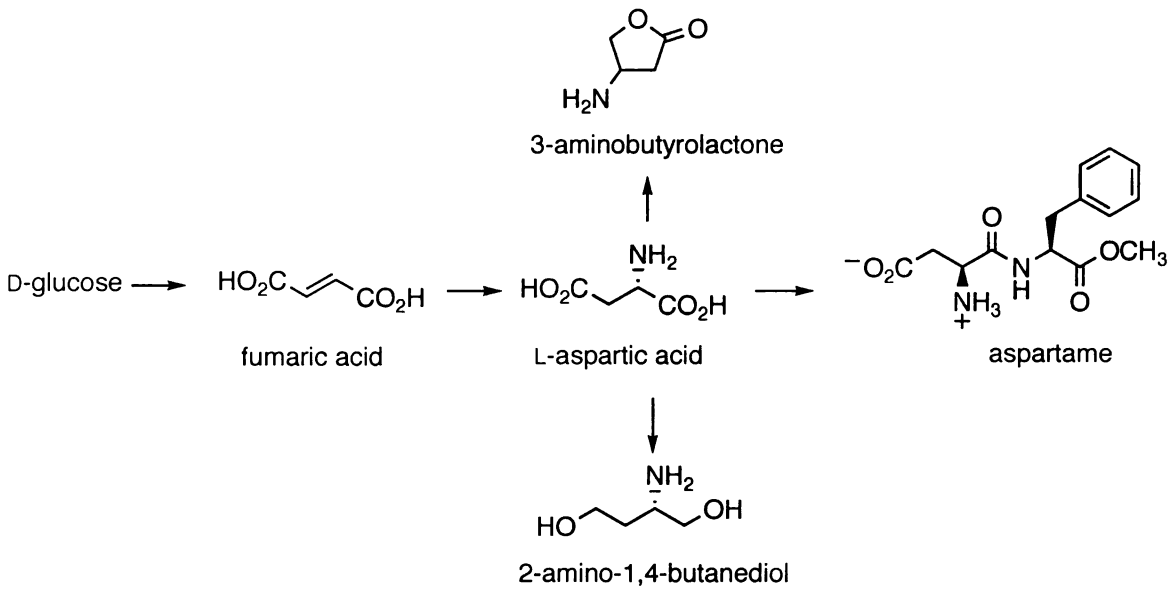

Figure 9. Synthesis of L-aspartic acid and the ensuing derivatives.

Hydrogenation of L-aspartic acid to either 3-aminobutyrolactone or 2-amino-1,4-butanediol would provide amino analogs of 3-hydroxy-γ-butyrolactone and 1,2,4-butanetriol. These derivatives can potentially find use as intermediates for the synthesis of high value pharmaceutical compounds if their proposed derivatization can be accomplished selectively under mild conditions. L-Aspartic acid is an important

monomer for the biodegradable nylon-3 derivative poly(α-isobutyl-L-aspartic acid).[41] This polyamide along with other nylon 3 (poly-β-alanine) and nylon 3-derivatives could serve as substitutes for polyacrylic acid and polycarboxylates.[9]

Use of L-aspartic acid along with derivatives is contingent on the availability of fumaric acid. This unsaturated 1,4-diacid is obtained using byproducts from phthalic anhydride manufacture because the biological production of fumaric acid is too expensive to compete with the petroleum-based manufacture.[42] Although many species produce small amount of fumaric acid as a byproduct metabolite during oxidative metabolism, mycelial fungi are capable of producing significant quantities of fumaric acid from D-glucose and CO_2.[43] Fungal fermentation of *Rhizopus oryzae* has been extensively studied for the production of fumaric acid.[44] When the nitrogen source is limited, the growth of *Rhizopus* is stunted and during this no growth period, fumaric acid is synthesized in a maximum yield of 2 mol/mol of D-glucose.[45] Typically, $CaCO_3$ is added during *Rhizopus oryzae* fermentation for the production of fumaric acid as a neutralizing agent and to aide in the removal of fumarate from the fermentation broth. The process of regenerating free acid from fumarate is complex, tedious and expensive. Several techniques employing adsorbent to bind fumaric acid while it is being produced or use of alternative neutralizing agents are being explored for production of fumaric acid from D-glucose.[44,45]

Aromatic Compounds

Aromatic compounds such as benzene, toluene and xylenes constitute a major part of the primary building blocks from petroleum and they are the basis for industrial

polymers such as nylon, polyester, polystyrene, polyurethane.[1] The recent surge in the price of benzene from $1.40/gal in November 2003 to ~$4.00/gal in November 2004 has resulted in an increase in the price of polymers manufacture from monomer derived from benzene.[46] The high demand for benzene and continued constraints on its availability are predicted to continue to drive the price for benzene upward. This may force the chemical industry to explore other sources for benzene-derived starting materials. Replacing benzene with D-glucose thus becomes an appealing option.

The Shikimate Pathway

Plants, bacteria and fungi utilize the shikimate pathway for the biosynthesis of aromatic amino acids and aromatic vitamins.[47] There are seven enzymatic reactions that convert phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate (E4P) into chorismic acid (Figure 10). The first committed step in the synthesis of aromatic amino acids is the condensation of (PEP) and (E4P) to afford 3-deoxy-D-*arabino*-heptusonic acid 7-phosphate (DAHP) catalyzed by DAHP synthase. Three isozymes of DAHP synthase exist in *E. coli*, and they are sensitive to feedback inhibition by one of the three aromatic amino acids. The genes *aro*F, *aro*G and *aro*H encode for L-tyrosine-sensitive, L-phenylalanine-sensitive, and L-tryptophan-sensitive isozymes of DAHP synthase, respectively.

Conversion of DAHP to 3-dehydroquinic acid (DHQ) is catalyzed by *aro*B-encoded DHQ synthase. The reaction involves an intramolecular oxidation-reduction at C-5 of DAHP with a very tightly bound NAD^+ cofactor, a *syn* elimination of phosphate, and an intramolecular cyclization to afford DHQ.[48] *syn*-Elimination of a molecule of

water from 3-dehydroquinate (DHQ) to 3-dehydroshikimate (DHS) is catalyzed by *aroD*-encoded 3-dehydroquinate dehydratase.[49] NADP-dependent shikimate dehydrogenase, encoded by the *aroE* gene catalyzes the reduction of DHS to shikimic acid (SA).[50] The 3-hydroxyl group of shikimic acid is phosphorylated by *aroL*- and *aroK*-encoded shikimate kinase isozymes to afford shikimate-3-phosphate (S3P), which condenses with the second equivalent of PEP to afford 5-enolpyruvylshikimate-3-phosphate.[51]

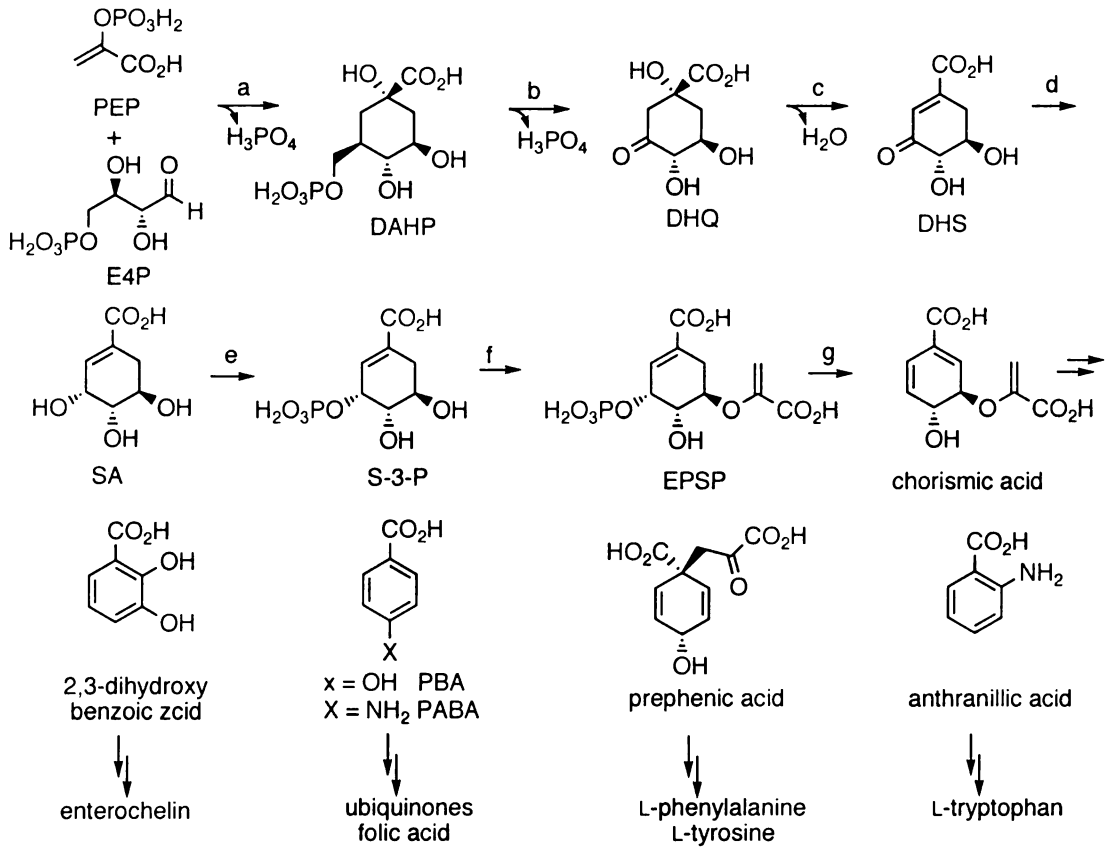

Figure 10. The shikimate pathway for aromatic amino acid biosynthesis.
Key: Abbreviations: PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate, DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimic acid; SA, shikimic acid; S3P, shikimate 3-phosphate; EPSP, 5-enolpyruvylshikimate 3-phosphate; PBA, *p*-hydroxybenzoic acid; PABA, *p*-aminobenzoic acid. Enzymes: a) 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (AroF, AroG, AroH); b) 3-dehydroquinate synthase (AroB); c) 3-dehydroquinate dehydratase (AroD); d) shikimate dehydrogenase (AroE); e) shikimate kinase (AroK, AroL); f) 5-enolpyruvylshikimate 3-phosphate (AroA); g) chorismate synthase (AroC).

This condensation is catalyzed by *aro*A-encoded 5-enolpyruvylshikimate-3-phosphate synthase.[52] As the final step, chorismate synthase catalyzes the concerted 1,4-*trans* elimination of phosphate from 5-enolpyruvylshikimate-3-phosphate to afford chorismic acid, which is the common intermediate from which L-phenylalanine, L-tyrosine and L-tryptophan are derived.[53] Furthermore, biosynthetic pathways leading to ubiquinone, folic acid and enterochelin branch off at chorismic acid (Figure 10). Folic acid-derived coenzymes are frequently involved in the biosynthetic transfer of one-carbon fragments, ubiquinones are involved in electron transport, and enterochelin is an iron chelator responsible for iron uptake in numerous microorganisms.[54]

Phenol and *p*-hydroxybenzoic acid from shikimic acid

Shikimic acid is currently used as the starting material for the synthesis of the neuraminidase inhibitor GS-41014, which is used to treat influenza infection and is marketed as Tamiflu™ by Roche.[55] Before shikimic acid was produced from fermentation, it was obtained from extraction of the fruit of *Illicium* plants, which is commonly known as the anise tree.[56] Production of shikimic acid from D-glucose under fed-batch fermentation conditions have been improved from 52 g/L with *E. coli* SP1.1/pKD12.138 to 87 g/L with SP1.1*pts*/pSC6.090.[57] Shikimic acid and the intermediates in the shikimic acid pathway can be exploited for the synthesis of aromatic compounds. By interfacing biocatalysis with chemical catalysis, value-added aromatic compounds were derived from D-glucose. The next section of this thesis presents a brief overview of benzene-free routes to access some aromatic compounds from D-glucose by exploiting shikimic acid and intermediates in the shikimate pathway. When an aqueous

solution of shikimic acid was heated to 350 °C, phenol was isolated in 45% yield (Figure 11).[58]

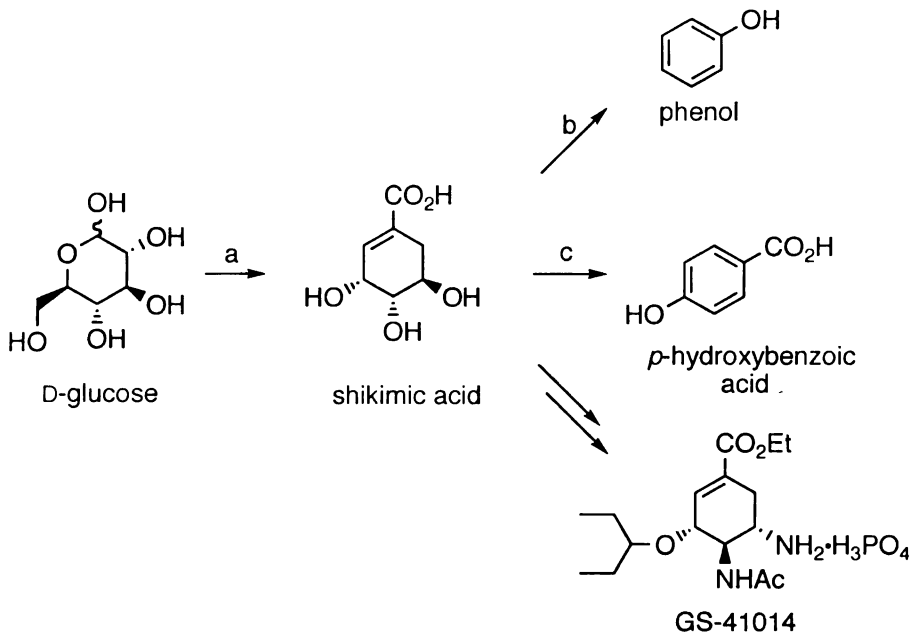

Figure 11. Synthesis of phenol and *p*-hydroxybenzoic acid from shikimic acid. Key: a) *E. coli* SP1.1/pKD12.138; b) H_2O, 350 °C; c) 1 M H_2SO_4 in AcOH.

Phenol is used to make synthetic resins, dyes, pharmaceuticals, pesticides, perfumes, lubricating oils and solvents.[59] The Hock oxidation of benzene-derived cumene is currently the predominant method used in the production of phenol with an annual production of 5×10^9 kg. It is estimated that 20% of the global benzene production is directed to the manufacture of phenol.[59] Benzene, however, is a hazardous organic pollutant whose emission must be reduced as mandated by the Chemical and Manufacturing Rule issued by the U. S. Environmental Protection Agency. Furthermore, exposure to benzene has been linked to acute leukemia and non-Hodgkin's lymphoma.[60] When shikimic acid was heated in the presence of 1 M sulfuric acid and acetic acid at 120 °C under atmospheric pressure, *p*-hydroxybenzoic acid was obtained in 57% yield.[58] *p*-Hydroxybenzoic acid is a component of liquid crystal polymers such as Xydar, which

have attracted considerable attention because of their use in high-performance applications.[61] Esters of *p*-hydroxybenzoic acid are also widely used as food preservatives. *p*-Hydroxybenzoic acid is currently manufactured by Kolbe-Schmitt reaction of dried potassium phenoxide with 20 atm dry CO_2 at 180-250 °C. Product potassium *p*-hydroxybenzoate is converted to its free acid upon addition of mineral acid.

Chemicals derived from 3-Dehydroshikimic acid

Vanillin

Vanillin is a natural flavoring agent, which is isolated from the dried pods of the orchid *Vanillin planifolia.*[62a] It accounts for 20 tons of the 1.2 x 10^5 tons/year world flavor market and is used in food, beverages and perfumes.[62b] Only 0.2% of vanillin is isolated from the natural sources,[62c] the rest is obtained from benzene-derived guaiacol by condensation with glyoxylic acid to afford mandelic acid. Oxidation of mandelic acid followed by decarboxylation affords vanillin (Figure 12).[62a]

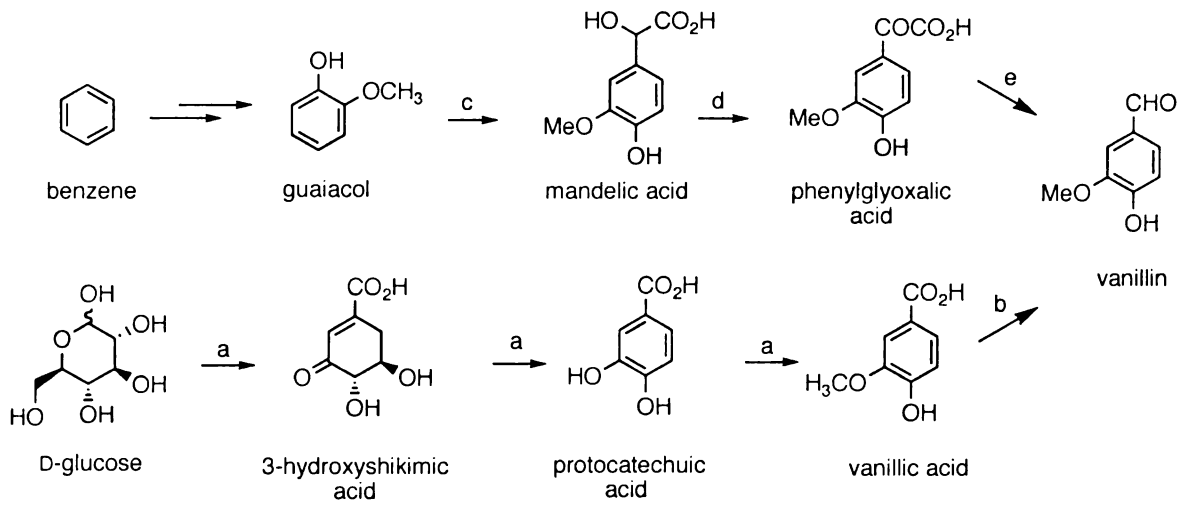

Figure 12. Synthesis of vanillin from benzene and D-glucose. Key: a) KL7/pKL5.26A; b) *N. crassa* aryl aldehyde dehydrogenase; c) $HCOCO_2H$; d) O_2; e) H^+.

Synthetic vanillin sells for $12/kg while natural vanilla flavoring extracted from vanilla bean containing 2% vanillin sells for $30-120/kg.[62c] The high price for natural vanilla flavoring reflects the labor-intensive cultivation, pollination, harvesting and curing of vanilla beans. The demand for natural flavorings has, in turn, prompted the development of biocatalytic routes to vanillin. Biocatalytic conversion of D-glucose to vanillin proceeds via the intermediacy of 3-dehydroshikimic acid. Heterologous expression of the *aroZ* locus in *E. coli aroE* auxotroph KL7 leads to protocatechuic acid.[63] Expression of rat-liver *COMT*-encoded catechol-*O*-methyltransferase in KL7 resulted in 4.9 g/L of vanillic acid by fed-batch fermentation from D-glucose when the construct was supplemented with L-methionine (Figure 12). COMT catalyzes the methylation of protocatechuic acid (PCA) to a mixture of vanillic acid and isovanillic acid. The *in vitro* reduction of vanillic acid to vanillin was carried out by aryl aldehyde dehydrogenase purified from the fungus *Neurospora crassa* in 66% yield.[64] This two-step biocatalytic synthesis of vanillin is the one of two biocatalytic synthesis of vanillin using a carbohydrate as a starting material. Recently, Paolis patented a technology (Gly Link) for the synthesis of vanilla by harnessing yeast fermentation of glucose.[65]

Gallic Acid and pyrogallol

3-Dehydroshikimic acid can also serve as the starting material for 3,4,5-trihydroxybenzoic acid, which is commonly known as gallic acid. This polyhydroxylated aromatic is currently isolated from gall nuts or from seed pods of *Coulteria tinctoria* trees found in Peru.[66] Thermal decarboxylation of gallic acid in copper autoclaves affords pyrogallol.[66] Two biocatalytic routes were developed for the synthesis of DHS to

supplant isolation of gallic acid and pyrogallol from scarce natural resources. In one route, 3-dehydroshikimic acid in acetic acid solution was oxidized by O_2 in the presence of catalytic amounts of Cu^{2+} and Zn^{2+} to afford gallic acid in 67% yield.[67] Alternatively, gallic acid was obtained directly from D-glucose via the intermediacy of protocatechuic acid (Figure 13).[68]

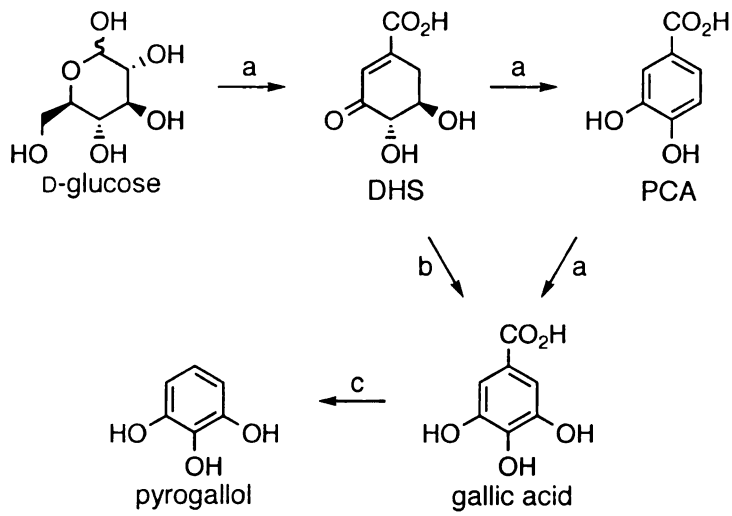

Figure 13. Synthesis of gallic acid and pyrogallol from D-glucose. Keys: a) *E. coli* KL7/pSK6.161; b) O_2, Cu^{2+}, Zn^{2+}, AcOH; (c) *E. coli* RB791*serA*::*aroB*/pSK6.234. Abbreviations: DHS, 3-dehydroshikimic acid; PCA, protocatechuic acid.

E. coli KL7/pSK6.161 expresses a plasmids-localized mutant isozyme of *p*-hydroxybenzoate hydroxylase encoded by plasmid-localized *pobA** and DHS dehydratase encoded by a genomic copy of *aroZ* and feedback-insensitive DAHP synthase encoded by plasmid-localized $aroF^{FBR}$.[69] *E. coli* KL7/pSK6.161 afforded 20 g/L of gallic acid in 12% (mol/mol) yield from D-glucose under fermentor-controlled conditions.[68] Decarboxylation of gallic acid to pyrogallol was effected by *E. coli* RB791*serA*::*aroB*/pSK6.234 expressing *aroY*-encoded PCA decarboxylase. Addition of gallic acid to a batch culture of *E. coli* RB791*serA*::*aroB*/pSK6.234 during its stationary phase of growth afforded pyrogallol in a concentration of 14 g/L in 97% (mol/mol) yield.

The high-yielding biocatalytic decarboxylation of gallic acid to pyrogallol provides an attractive alternative to currently employed chemical decarboxylation process. The toxicity of pyrogallol towards growing *E. coli* cells precluded the direct synthesis of pyrogallol from D-glucose using a single microbial construct.

Catechol and adipic acid

Inclusion of a *catA* encoding catechol 1,2-dioxygenase in a catechol-producing *E. coli* strain resulted in the synthesis *cis,cis*-muconic acid from D-glucose.[70] The *catA*-encoded catechol 1,2-dioxygenase was isolated from *Acinetobacter calcoaceticus*.[71] Catalytic hydrogenation of *cis,cis*-muconic acid under mild conditions afforded adipic acid (Figure 14), a monomer for synthesis of nylon-6,6.[72]

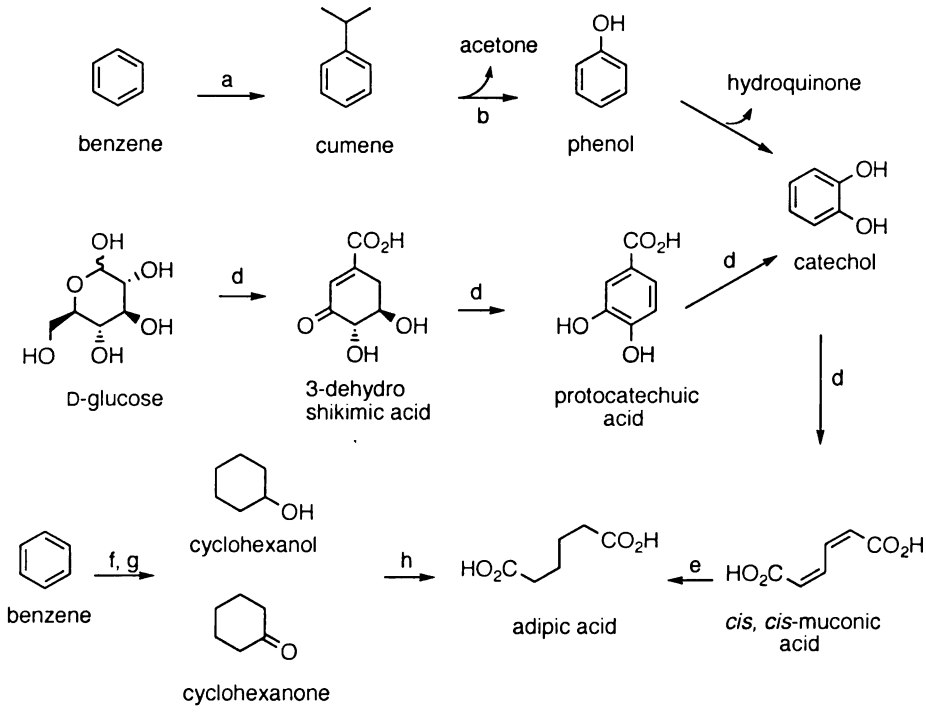

Figure 14. Chemical and biosynthetic route to catechol and adipic acid. (a) propylene, 400-600 psi., solid H_3PO_4 catalyst, 200-260°C; (b) O_2, 80-130 °C, SO_2, 60-100 °C; (c) 70% H_2O_2, EDTA, Fe^{2+} or Co^{2+}, 70-80 °C; (d) *E. coli* WN1/pWN2.248; (e) H_2, 50 psi., 10% Pt/C; (f) Ni-Al_2O_3, H_2, 370-800 psi., 150-250 °C; (g) Co, O_2, 120-140 psi., 150-160 °C; (h) Cu, NH_4VO_3, 60% HNO_3, 60-80 °C.

An improved route to adipic acid via catechol utilized an *aroE* auxotroph *E. coli* WN1 expressing *aroZ*-encoded DHS dehydratase for the conversion of DHS to protocatechuic acid, *aroY*-encoded PCA decarboxylase for the conversion of PCA to catechol, and *catA*-encoded catechol 1,2-dioxygenase for the conversion of catechol to *cis,cis*-muconic acid.[73] The construct *E. coli* WN1/pWN2.248 synthesized 37 g/L of *cis,cis*-muconic acid from D-glucose in 23% yield (mol/mol). Catalytic hydrogenation over Pt/C at 50 psi H_2 afforded adipic acid in 97% yield from *cis,cis*-muconic acid.

Adipic acid is one of the top 50 large-volume chemicals produced in the U. S. at 918,000 tons/year. In 1999, ~84% of the adipic acid produced was used in the synthesis of nylon 6,6, with the rest going to the production of polyurethane and plasticizers.[74] Most of the syntheses rely on benzene-derived cyclohexane as the chemical feedstock. Air oxidation of cyclohexane over cobalt naphthalene or cobalt octanoate affords a mixture of cyclohexanol and cyclohexanone. Cyclohexanol has also been produced from oxidation of cyclohexene. Conversion of cyclohexanol or cyclohexanone to adipic acid is effected by nitric acid oxidation over copper and vanadium catalysts at 92-96% yield. During this process, NO, NO_2 and N_2O are produced as byproduct. Nitrous oxide contributes to the depletion of the ozone layer and global warming.[75] Other routes for adipic acid involved reaction of butadiene and carbon monoxide or carbonylation of 1,4-butanediol. The biocatalytic route starting from D-glucose to *cis, cis*-muconic acid offers a viable alternative to the synthesis of adipic acid monomer for nylon 6,6.

Hydroquinone from Quinic Acid

Microbial synthesis of quinic acid from D-glucose and the subsequent conversion of quinic acid to hydroquinone provide an example of how renewable feedstocks can be

substituted for fossil fuel feedstock in chemical manufacture. Quinic acid is an important chiral synthon isolated from *Chicoma* bark.[76] Hydroquinone is a pseudocommodity chemical used mainly for photographic development with an annual production of approximate 4.5 × 10^7 kg from benzene-derived aniline, phenol and *p*-diisopropylbenzene.[77] Shikimate dehydrogenase, which catalyzes the reduction of 3-dehydroshikimic acid to shikimic acid, also catalyzes in the reduction of 3-dehydroquinic acid to quinic acid. Overexpression of shikimate dehydrogenase in 3-dehydroquinate-synthesizing *E. coli* strains has resulted in a high-yielding microbial synthesis of quinic acid, which after oxidation with household bleach followed by heating at reflux affords hydroquinone in 87% isolated yield.[78] An alternative chlorine-free route was also explored in which oxidation of quinic acid was catalyzed Ag_3PO_4 (10 mol%) in the presence of $K_2S_2O_8$ as a co-oxidant at 50 °C to afford hydroquinone in 85% yield after reflux (Figure 15).[78]

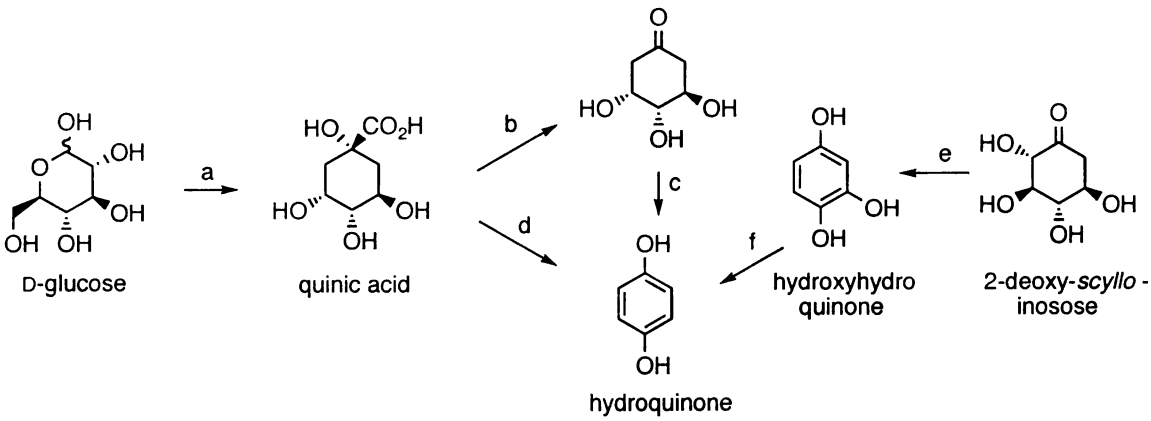

Figure 15. Synthesis of hydroquinone. Key: (a) *E. coli* QP1.1/pKD12.138; (b) i). NaOCl, ii). isopropanol, H^+; (c) reflux; (d) i). Ag_3PO_4 (10 mol%), $K_2S_2O_8$, 50 °C, ii). reflux; (e) 0.5 M H_3PO_4, reflux, 39%; (f) 50 psi H_2, Rh on Al_2O_3, 12 h, 82%

Aromatic Compounds from Non-Shikimate Pathway

Instead of the shikimate pathway, the *myo*-inositol biosynthesis has also been exploited for the synthesis of 1,2,3,4-tetrahydroxybenzene (Figure 16).[79] Polyhydroxy benzenes along with quinones are known to possess biological activity. Specifically, aurantiogliocladin and fumigatin are antibiotics while coenzyme $Q_{n=10}$ is an essential antioxidant in humans.[80]

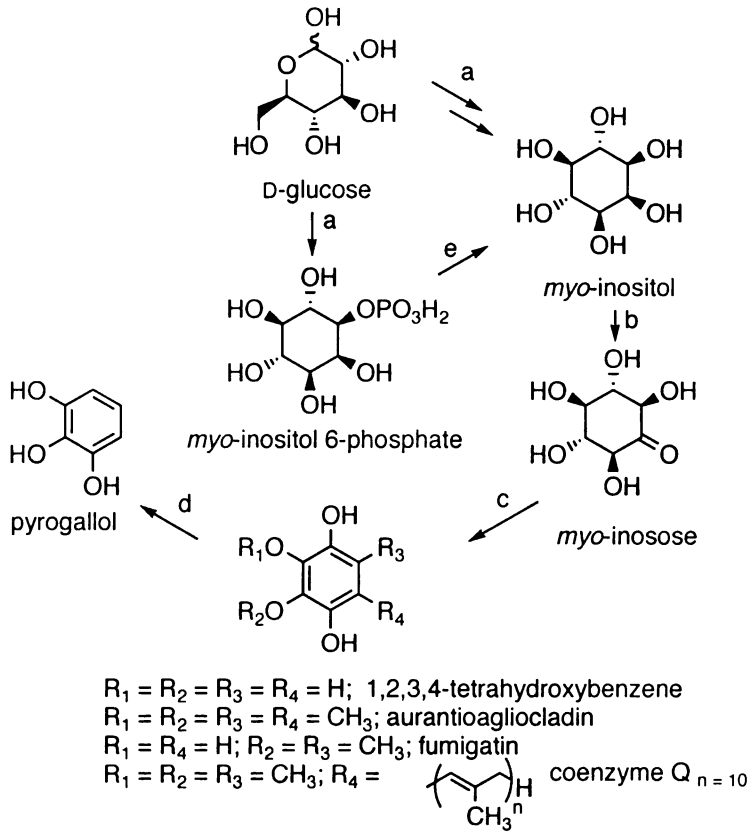

Figure 16. Synthesis of polyhydroxybenzenes from *myo*-inositol. Key: (a) *E. coli* JWF1/pAD1.88A, 11%; (b) *Gluconobacter oxydans*, 95%; (c) H_2SO_4, H_2O, reflux, 66%; (d) 50 psi H_2, Rh on Al_2O_3, 12 h, 44%; (e) phytase.

E. coli JWF1/pAD1.88A synthesized 21 g/L of *myo*-inositol and 4 g/L of *myo*-inositol-1-phosphate in 11% combined yield from D-glucose under fed-batch fermentor conditions.[79] The biocatalyst *E coli* JWF1/pAD1.88A expressed *INO1*-encoded *myo*-inositol-1-phosphate synthase from *Saccharomyces cerevisiae*, which catalyzed

cyclization of D-glucose 6-phosphate into *myo*-inositol-1-phosphate. Hydrolysis of the phosphoester into *myo*-inositol was catalyzed by an unidentified phosphatase within *E. coli* JWF1/pAD1.88A. Oxidation of *myo*-inositol into *myo*-inosose was catalyzed by *Gluconobacter oxydans* (ATCC 621) in 95% isolated yield. Acid-catalyzed dehydration of *myo*-inosose in refluxing in 0.5 M H_2SO_4 for 9 h under Argon afforded 1,2,3,4-tetrahydroxybenzene in 66% isolated yield. Catalytic hydrogenation of 1,2,3,4-tetrahydroxybenzene over Rh on Al_2O_3 afforded pyrogallol in 44% yield.[79] Phloroglucinol, which is a substituent for a variety of natural products, was synthesized by two routes and subsequently deoxygenated to resorcinol (Figure 17).[81]

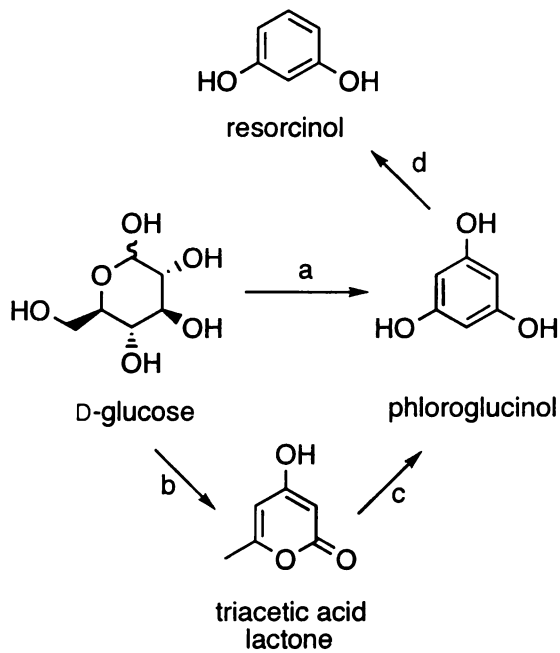

Figure 17. Syntheses of phloroglucinol and resorcinol from D-glucose. Key: (a) *E. coli* JWF1 (DE3)/pJA3.131A; (b) *S. cereviciae*; (c) Na, MeOH, 185 °C; (d) 50 psi H_2, Rh on Al_2O_3, 12 h, 82%.

In the first route, phloroglucinol was synthesized via the intermediacy of triacetic acid lactone by *Saccharomyces cereviciae* harboring fatty acid synthase mutant (Y2226F).[81a] In a different route, heterologous expression of *phlD* from *Pseudomanas*

fluorescens in *E. coli* led to the production of phloroglucinol.[81b] Catalytic hydrogenation of phloroglucinol over Rh on Al_2O_3 afforded resorcinol in 82% yield (Figure 17).[80] Hydroquinone was also synthesized from a non-shikimate pathway (Figure 15).[80,82] D-Glucose-derived 2-deoxy-*scyllo*-inosose was dehydrated under acidic conditions to afford hydroxyhydroquinone. Subsequent hydrogenation of hydroxyhydroquinone over Rh on Al_2O_3 resulted in hydroquinone in 53% yield.

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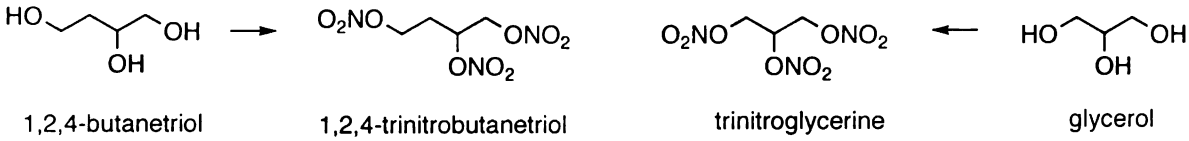
CHAPTER 2

SYNTHESIS OF D,L-1,2,4-BUTANETRIOL

Introduction

1,2,4-Butanetriol is an important industrial precursor and a useful synthetic scaffold in organic syntheses.[1] Nitration of racemic D,L-1,2,4-butanetriol leads in 1,2,4-butanetriol trinitrate, which is the energetic plasticizer used in single stage rockets used in the U.S. military.[2] Compared to trinitroglycerine, 1,2,4-butanetriol trinitrate has reduced sensitivity to impact, enhanced stability, and lower volatility (Figure 18).[3] The heat of explosion is not compromised in substituting trinitroglycerine (1455 kcal/kg) with 1,2,4-butanetriol trinitrate (1440 kcal/kg).[4]



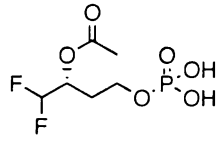
1,2,4-butanetriol | 1,2,4-trinitrobutanetriol | trinitroglycerine | glycerol

Figure 18. 1,2,4-Butanetriol trinitrate and trinitroglycerine.

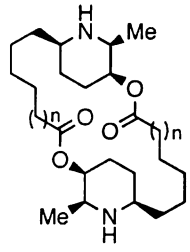
1,2,4-Butanetrioltrinitrate also has potential application as a vasodilator for treatment angina pectoris.[5] Coincidentally, trinitroglycerine is the most prescribed nitrate ester for alleviation of angina.[6] It is assimilated within a minute and offers relief almost instantaneously. However, the relief is short-lived and longterm use requires increasing doses due to nitrate tolerance.[7a] Furthermore, trinitroglycerine can lead to dilation of other vessels including arterioles and veins, which can lead to side effects such as violent headache that can last for several hour.[7b] Studies have shown that the rate of hydrolysis of 1,2,4-butanetriol trinitrate is markedly reduced compared to trinitroglycerine and erythrityl trinitrate.[7c] This slow hydrolysis corresponds to a prolonged effect of the

nitrate ester in assuaging angina.[7c] Replacement of trinitroglycerine, both as an energetic plasticizer and a vasodilator, hinges on the availability of racemic D,L-1,2,4-butanetriol.

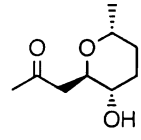
Enantiomerically pure 1,2,4-butanetriol and its derivatives have been employed in the syntheses of important pharmaceutical compounds (Figure 19).[8] Notably, *S*-1,2,4-butanetriol has been used in the synthesis of lysophosphatidic acid (LPA) analogues.[8a] These compounds play a critical role as general growth, survival and pro-angiostenic factor, in the regulation of physiological and pathophysiological processes *in vivo* and *in vitro*.[8b] Abnormalities in LPA metabolism and function in ovarian cancer patients may contribute to the initiation and progression of the disease. Therefore, LPA receptors constitute a potential target for cancer therapy.[8a]



lysophosphatidic acid analogue



(+)-carpaine: n = 3
(+)-azamine: n = 1



decarestrictine L

Figure 19. Biologically active compounds derived from *S*-1,2,4-butanetriol and its derivatives.

(+)-Carpaine, which belongs to a novel class of macrocyclic dilactones containing a 2,3,6-trisubstituted piperidine skeleton, exhibits a range of biological activities including antitumor activities at low concentrations.[8c] These compounds along with (+)-azamine were synthesized from *S*-1,2,4-butanetriol as a single source of chirality.[8d] *S*-3-Hydroxy-γ-butyrolactone, a derivative of *S*-1,2,4-butanetriol, constitutes the core structure of (+)-(2*R*, 3*S*, 6*R*)-decarestrictine L.[8e] The decarestrictine family is a growing

class of natural products with remarkable potential in inhibiting HMG-CoA reductase, the first enzyme involved in the cholesterol biosynthetic pathway.[8f]

Several processes have been developed for the large-scale synthesis of 1,2,4-butanetriol as evident by the patent literature (Figure 20).

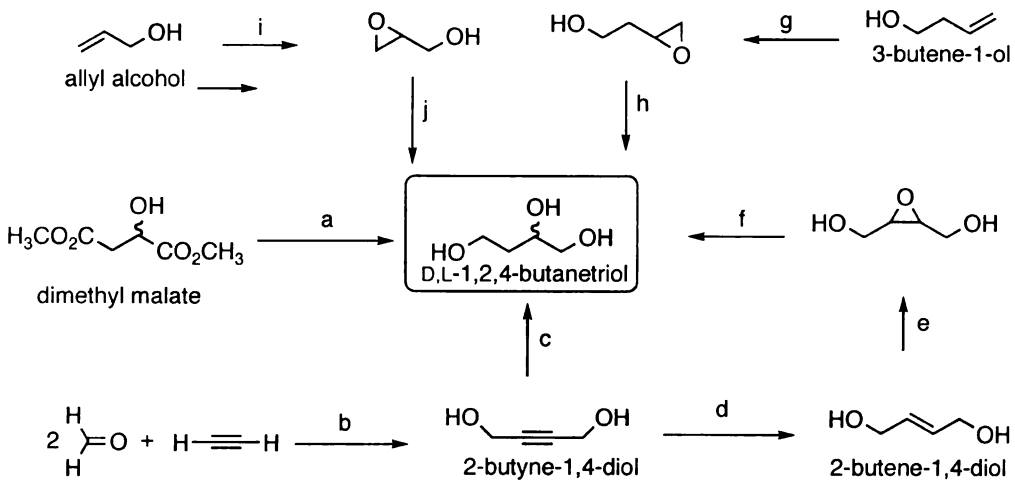


Figure 20. Syntheses of 1,2,4-butanetriol. Key: (a) $NaBH_4$, THF, MeOH; (b) CuC_2, 50 atm; (c) i) $HgSO_4$, H_2SO_4, ii) $2CuO{\cdot}Cr_2O_7$, H_2; (d) Lindlar's catalyst, H_2; (e) H_2O_2, H_2WO_4; (f) Pd/C, H_2; (g) H_2O_2, H_2WO_4; (h) H_2SO_4; (i) i) H_2O_2; (j) $Co_2(CO)_8$, (CO, H_2), ii) $LiAlH_4$.

The current commercial synthesis of D,L-1,2,4-butanetriol relies on stoichiometric reduction of dimethyl D,L-malate.[9] Racemization does not occur during reduction. When dimethyl D- and L-malate is reduced with $NaBH_4$, D- and L-1,2,4-butanetriol is obtained, respectively. 2-Butyn-1,4-diol produced by the reaction of acetylene and formaldehyde (the Reppe process), provides two avenues for D,L-1,2,4-butanetriol production.[10] Oxymercuration of 2-butyn-1,4-diol gives 1,4-dihydroxy-2-butanone, which is subsequently hydrogenated to 1,2,4-butanetriol over $2CuO{\cdot}Cr_2O_7$ catalyst.[10a] The catalyst contained 20% copper and 0.5% chromium on silica.[10b] Alternatively, 2-butyn-1,4-diol can be hydrogenated to 2-buten-1,4-diol, followed by oxidation to the oxirane-(bis)-methanol.[11] Hydrogenation of the epoxide over Pd catalyst yields D,L-1,2,4-butanetriol.[11]

In another route, low yielding dehydration of acetaldehyde-derived 1,3-butanediol leads 3-buten-1-ol.[12a] Oxidation to the corresponding epoxide followed by acid-catalyzed hydrolysis results in D,L-1,2,4-butanetriol.[12b] Hydroformylation of 2,3-epoxy-1-propanol (glycidol) into 3-hydroxy-γ-butyrolactone followed by reduction with $LiAlH_4$ results in D,L-1,2,4-butanetriol in low yield.[13] Glycidol is obtained from epoxidation of allyl alcohol *en route* to synthetic glycerol.[14] This reaction is reminiscent of the Prins reaction in which the condensation of allyl alcohol and formaldehyde in the presence of acid affords 1,2,4-butanetriol.[15]

All the substrates employed thus far for the syntheses of D,L-1,2,4-butanetriol are derived from petroleum. Use of $NaBH_4$ or $LiAlH_4$ in stoichiometric quantities as a reducing agent results in generation of a large amount of salts. Reaction conditions needed to transform 2-butyn-1,4-diol into 1,2,4-butanetriol call for use of mercury and Cr(VI). In addition to being detrimental to the environment, Cr(VI) along with formaldehyde and acetaldehyde are carcinogenic.[16] In light of stricter environmental policies and a long term shift from petroleum-based towards a biomass-derived chemical industry, chemoenzymatic and biocatalytic routes have been explored for the synthesis of enantiomerically pure 1,2,4-butanetriol.

Man Kit Lau in the Frost group has examined a synthesis of D-1,2,4-butanetriol by employing a chemoenzymatic route starting from glycaldehyde and acetaldehyde (Figure 21).[17] 2-Deoxyribose 5-phosphate aldolase catalyzed the condensation of glycaldehyde and acetaldehyde to D-3,4-dihydroxybutanal,[18a] which was hydrogenated over Ru on C to D-1,2,4-butanetriol in 17% overall yield after vacuum distillation.[17]

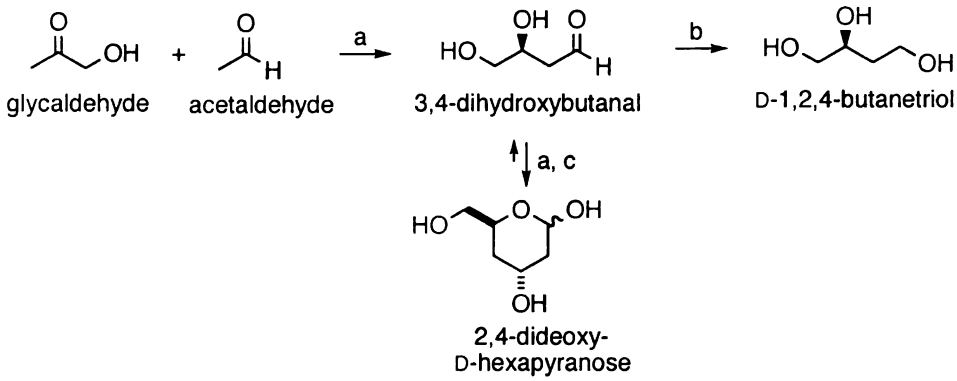


Figure 21. Chemoenzymatic synthesis of D-1,2,4-butanetriol. Key: (a) 2-Deoxyribose- 5-phosphate aldolase (20,000 U), pH 7.6, rt, 35%; (b) 1.0 mol% Ru on C, 14 atm H_2, 30 °C, 5 h, 99%; (c) acetaldehyde.

The double aldol condensation product of one glycaldehyde and two acetaldehyde molecules, 2,4-dideoxy-D-hexapyroanoside, accounted for the majority of the product formed.[18b] Although this route is very concise, it is low yielding and affords only the D-enantiomer of 1,2,4-butanetriol. Moreover, the starting materials are derived from nonrenewable sources. Glycaldehyde is derived from reaction of formaldehyde with syn gas (CO, H_2) over Rh catalyst at 300 atm and 150 °C.[19] Acetaldehyde on the other hand is derived from Wacker oxidation of ethylene over $PdCl_2$ and $CuCl_2$ catalysts at 125-130 °C and 11.3 atm.[20]

Recently, microbes have been constructed that catalyze the synthesis of D-1,2,4-butanetriol and L-1,2,4-butanetriol from D-arabinose and L-xylose, respectively (Figure 22).[21] The first step involves oxidation of L-arabinose and D-xylose to L-arabinoic acid and D-xylonic acid catalyzed by L-arabinose dehydrogenase and D-xylose dehydrogenase, respectively. Dehydration of the L-arabinoic acid and D-xylonic acid to the corresponding pentulosonic acid is catalyzed by L-arabinoate dehydratase and D-xylonate dehydratase, respectively.

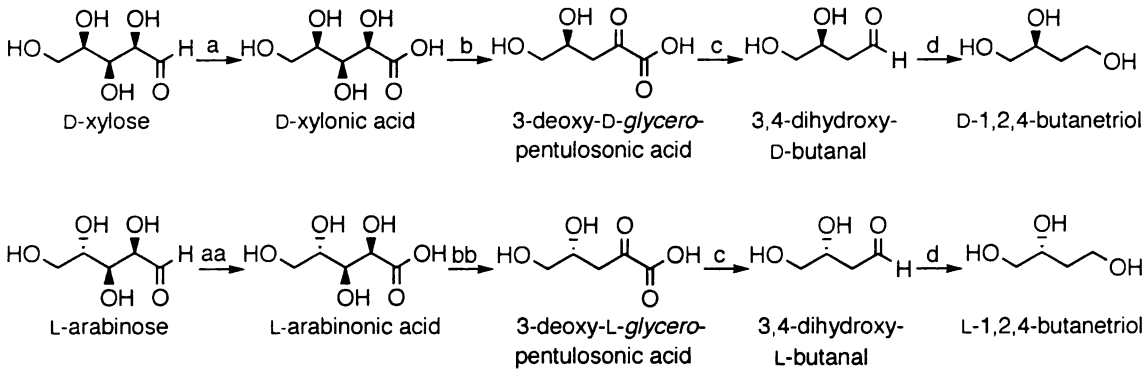


Figure 22. Biosynthetic pathway of D-xylose and L-arabinose to D- and L-1,2,4-butanetriol. Enzymes: (a) D-xylose dehydrogenase; (aa) L-arabinose dehydrogenase; (b) D-xylonate dehydratase; (bb) L-arabinonate dehydratase; (c) 2-keto acid decarboxylase; (d) alcohol dehydrogenase.

Decarboxylation followed by reduction of D- and L-3-deoxy-*glycero*-pentulosonic acid catalyzed by benzylformate decarboxylate and alcohol dehydrogenase afforded L- and D-1,2,4-butanetriol in 19% and 18% overall yield. The starting L-arabinose and D-xylose are derived from abundantly available plant hemicellulose. However, inexpensive, pure streams of these pentoses are not commercially available yet.[22] Catalytic hydrogenation of D, L-malic acid and L-malic acid were explored as alternatives to stoichiometric reduction of methyl D,L-malate. Racemic D,L-1,2,4-butanetriol was obtained as a product from hydrogenation of both substrates. As an alternative route, a chemoenzymatic synthesis of D,L-1,2,4-butanetriol starting from L-ascorbic acid is described. The centerpiece in this route is the formation and catalytic hydrogenation of 2-hydroxy-2-buten-4-olide. Syntheses were examined from the perspective of starting material, the number of steps, reaction conditions, byproducts and product stereochemistry.

Catalytic Hydrogenation of Malic Acid

Based on the successful use of Ru on C in the hydrogenation of lactic acid to 1,2-propanediol in aqueous medium,[23] this catalyst was employed in the hydrogenation of D,L-malic acid **2a,b**. Variation of pressure, temperature, reaction time, concentration of D,L-malic acid, the D,L-malate/Ru mol ratio and catalyst composition were explored (Table 1-2). A typical hydrogenation was conducted by dissolving D, L-malic acid in distilled, deionized water (100 mL) in a glass liner. The catalyst was suspended in malic acid solution. The liner was inserted into a 500 mL Parr 4575 stainless steel high temperature-high pressure reactor, and the vessel was sealed. The temperature and stirring rate were controlled by a Parr 4842 temperature controller. Hydrogen was bubbled through the reaction mixture for 10-15 min to remove air while stirring at 100 rpm. The vessel was then charged with H_2 to a pressure below the desired value. After heating the reaction to desired temperature, the H_2 pressure was adjusted to the desired pressure. The reaction was stirred at 200 rpm for 1-10 h at a constant temperature. When the reaction was complete, the reaction vessel was cooled to rt and the pressure was released. After removal of the catalyst by filtration, the reaction mixture was concentrated to dryness under vacuum to afford a colorless oil, which was derivatized with (*N,O-bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA) and analyzed by gas chromatography.

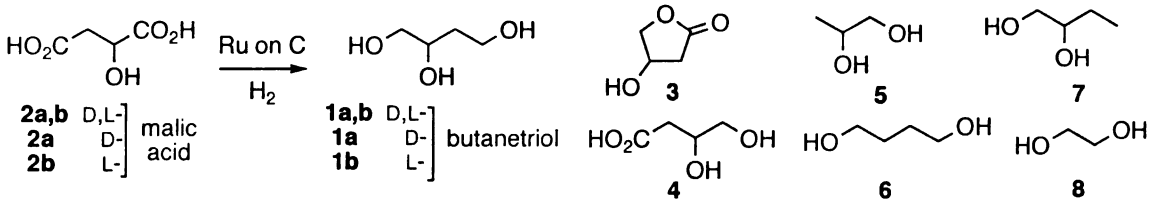


Figure 23. Catalytic hydrogenation of malic acid.

Table 1 a-c. Hydrogenation of D, L-Malic Acid as a Function of H_2 pressure, Temperature, and Reaction Time.

reaction conditions	product, intermediate, byproduct yields (mol %)[1]							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
a. atm	variable: H_2 pressure[2]							
68	24	28	22	17	3	4	0	1
136	67	9	6	7	4	3	0	1
204	70	4	3	2	6	6	0	1
272	71	2	0	1	9	8	0	2
340	73	0	0	0	13	9	0	2
b. °C	variable: temperature[3]							
125	61	14	11	1	4	3	2	0
130	67	1	10	0	10	7	3	2
135	74	0	0	0	12	8	3	3
140	70	0	0	0	15	9	2	2
c. h	variable: time[4]							
1	21	20	46	9	2	5	0	0
5	69	8	5	trace	6	7	2	2
10	72	1	1	1	11	9	2	2
15	71	1	1	trace	13	10	3	2
20	66	0	0	0	14	10	3	2

[1] See Figure **23** for structures. [2] 1 M malate, 135 °C, 5 wt % Ru on C, 1.3 mol % Ru/malate, 10 h. [3] 1 M malate, 340 atm H_2, 5 wt % Ru on C, 1.3 mol % Ru/malate, 10 h. [4] 1 M malate, 135 °C, 340 atm H_2, 5 wt % Ru on C, 1.3 mol % Ru/malate.

At 68 atm H_2 pressure, 3-hydroxy-γ-butyrolactone **3** and 3,4-dihydroxybutyric acid **4** were the dominant products formed along with D,L-1,2,4-butanetriol **1a,b** (Figure 23, Table 1a). Formation of lactone **3** and carboxylate **4** was consistent with a more rapid rate of reduction of the malate C-1 carboxylate with its adjacent α-hydroxyl group relative to the malate C-4 carboxylate with its adjacent methylene group. Formation of 3-

hydroxy-γ-butyrolactone **3** and 3,4-dihydroxybutyric acid **4** declined with increased formation of 1,2,4-butanetriol **1a,b** when the H_2 pressure was increased to 136 atm (Table 1a). Above 136 atm, increasing H_2 pressures led to smaller incremental increases in product D,L-1,2,4-butaentriol **1a,b** and increased formation of byproducts 1,2-propanediol **5**, 1,4-butanediol **6** (Table 1a). At all pressures, temperatures, reaction times, malate concentrations, and Ru/malate ratios examined, at least 90% of the molar decline in D,L-malate **2a,b** starting material could be accounted for by the formation of product D,L-1,2,4-butanetriol **1a,b**, reaction intermediates **3** and **4**, or byproducts **5**, **6**, **7**, and **8** (Figure 23, Table 1a).

Increased temperature had a pronounced impact on product and byproduct formation while the reaction pressure was maintained at 340 atm (Table 1b). The yield of D,L-1,2,4-butanetriol **1 a,b** increased as the temperature was increased from 125 °C to 135 °C. Formation of byproducts 1,2-propanediol **5** and 1,4-butanediol **6** increased across the entire range of examined temperatures. Formation of 1,2-butanediol **7** in addition to ethylene glycol **8** was also observed. The maximum yield of 1,2,4-butanetriol **1 a,b** was obtained at 135 °C and 340 atm H_2 pressure at a reaction time of 10 h (Table 1b). Longer reaction times resulted in reduced yields of D,L-1,2,4-butanetriol **1a,b** and increased yields of byproducts **5** and **6** (Table 1c). A 1 M concentration of D,L-malate **2a,b** led to the highest yield of D,L-1,2,4- butanetriol **1** after reaction for 10 h at 340 atm H_2 and 135 °C (Table 2a). Increasing D,L-malate **2a,b** concentrations beyond 2 M led to a precipitous decline in the yields of product **1** and byproducts **5-8** (Table 2a). The highest yield of 1,2,4-butanetriol **1a,b** was achieved with a Ru/malate mol ratio of 1.3 mol % when 1 M D,L-malate **2a,b** was reacted at 340 atm H_2 and 135 °C.

Table 2 a-c. Hydrogenation of D, L-Malic Acid as a Function of Malate Concentration, Mole Ratio of Ru:Malate and the Mole Ratio of Ru:Re.

reaction conditions	product, intermediate, byproduct yields (mol %)[1]							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
a. M	variable: malate concentration[2]							
1	72	2	1	1	12	9	2	3
2	63	11	1	4	12	6	2	2
5	3	94	0	0	0	0	0	2
10	0	99	trace	trace	0	0	0	2
b. mol%	variable: Ru/malate[3]							
0.66	63	1	6	2	3	12	3	0
1.3	73	0	2	0	10	9	2	2
2.0	69	0	1	0	12	6	2	3
3.3	55	0	1	0	15	3	2	2
c. mol%	variable: mol Ru/mol Re[4]							
10	55	6	0	0	7	5	5	0
25	51	7	2	1	4	5	3	0
45	58	4	2	1	3	6	6	0
65	70	10	trace	1	2	8	3	0
85	34	13	21	6	1	9	1	0

[1] See Figure **23** for structures. [2] 340 atm H_2, 135 °C, 5 wt % Ru on C, 1.3 mol % Ru/malate, 10 h. [3] 1 M malate, 340 atm H_2, 135 °C, 10 h. [4] 1 M malate, 135 °C, 340 atm H_2, 1.3 mol% Ru/Re on C, 10 h.

Increasing the Ru/malate mol ratio beyond 1.3 mol % led to a steady decline in the yield of D,L-1,2,4-butanetriol **1a,b** and had a disproportionate impact on increased formation of 1,2-propanediol **5** relative to 1,4-butanediol **6** formation (Table 2b). Full conversion of D,L-malic acid was observed with intermediates **3** and **4** essentially absent in the solution. This however, did not translate to improved conversion to D,L-1,2,4-butanetriol **1a,b**. Instead, the amount of byproducts increased drastically while the yield of D,L-1,2,4-butanetriol **1a,b** declined. As a final parameter, a mixture of Re and Ru ranging from 10-85 wt % was evaluated for hydrogenation of D, L-malic acid (Table 2c). The overall catalyst loading of Re-Ru on C was maintained at 1.3 mol%. When Ru catalyst was spiked with 10 wt % of Re catalyst, there was over a 90% conversion of D, L-malic acid **2** but only 55% of 1,2,4-butanetriol was observed (Table 2c). The catalyst

composition of 25 wt % Re relative to Ru resulted in a slight decrease both in D.L-1,2,4-butanetriol **1a,b** and 1,2-propanediol **5**. At the optimum catalyst composition of 65 wt % Re/Ru, the amount of 1,2-propanediol **5** diminished to only 2% while 1,4-butanediol **6** increased to 8%. At the highest composition of Ru relative to Re, 1,2-propanediol **5** vanished almost completely while 1,4-butanediol **6** formation was at its highest levels (9%). The amount of D,L-1,2,4-butanetriol **1a,b** also declined significantly from 70% when the catalyst composition of 65 wt % Re/Ru was used down to 34% when the catalyst composition of 85 wt % Re/Ru was used (Table 2c). Hydrogenation over 1.3 mol% of Re on carbon relative to D, L-malic acid **2** resulted in the recovery of unreacted starting material (results not shown). Other catalysts such as Pt, Pd and Rh were examined for the hydrogenation of D, L-malic acid **2**. Unreacted starting material was recovered with 1.3 mol% Pt on C and 1.3 mol% Pd on C while only 12% of D,L–1,2,4-butanetriol **1a,b** and 3-hydroxy-γ-butyrolactone **3** (12%) was obtained with the 1.3 mol% Rh on C.

Catalytic hydrogenation at the optimized conditions of 340 atm H_2 and 135 °C of a 1 M aqueous solution of L-malic acid **2b** over 5% R on C using a 1.3% Ru/malate ratio and a reaction time of 10 h was also examined. Formation of Mosher esters[24] of the product and analysis by HPLC indicated a 74% yield of racemic D,L-1,2,4-butanetriol **1a,b**. Apparently, Ru-catalyzed racemization of the stereogenic center occurred during the course of the catalytic hydrogenation.[25] Hydrogenation of a 1 M aqueous solution of D,L-1,2,4-butanetriol **1a,b** at 340 atm H_2 and 135 °C for 10 h over 5% Ru on C using a 1.3 mol % Ru/ D,L-1,2,4-butanetriol **1a,b** mol ratio was also examined to determine if byproducts were formed as a consequence of product reactivity. In addition to a 70%

recovery of unreacted D,L-1,2,4-butanetriol **1a,b**, formation of 1,2-propanediol **5** (18%), 1,4-butanediol **6** (3%), 1,3-propanediol (2%), 1,2-butanediol **7** (2%), 1,3-butanediol (2%) and ethylene glycol **8** (3%) was observed.

Purification of product D,L-1,2,4-butanetriol **1a,b** was not straightforward. Short path distillation in vacuo of crude product afforded a 28% overall yield from starting D,L-malic acid **2a,b** of a 95:5 mol/mol mixture of D,L-1,2,4-butanetriol **1a,b** and 1,4-butanediol **6**. Trace amounts of other diols could also be detected in the distilled product. The loss of more than half of the product during distillation resulted from decomposition of product D,L-1,2,4-butanetriol **1a,b**. Contamination may reflect azeotropic distillation of the diols with the desired D,L-1,2,4-butanetriol **1a,b** during purification.

Alternative Substrates for Catalytic Hydrogenation

Byproduct diol formation during catalytic hydrogenation of D, L-malic acid **2** is not readily removed by simple distillation. Intermediates **3** and **4** are poised to undergo dehydration via β-elimination to afford 1,4-butanediol **6** after reduction. The remaining diols are presumably derived from C-C and C-O cleavage of product **1a,b** as a result of forcing reaction conditions required for hydrogenation of the unactivated carboxylate of malate. Ideally, substrates that could be hydrogenated to D,L-1,2,4-butanetriol **1a,b** under conditions that do not promote C-C and C-O cleavage are desired (Figure 24). The presence of an α-hydroxy moiety adjacent to the carboxylic acid reduces the energy barrier required to hydrogenate the carboxylic acid. If intermediates are formed during hydrogenation, they should not undergo secondary reactions such as elimination reactions to afford byproducts. 2,4-Dihydroxybutyric acid **9** or the corresponding 2-hydroxy-γ-

butyrolactone **10** are would be ideal substrates for catalytic hydrogeantion to afford D,L-1,2,4-butanetriol.

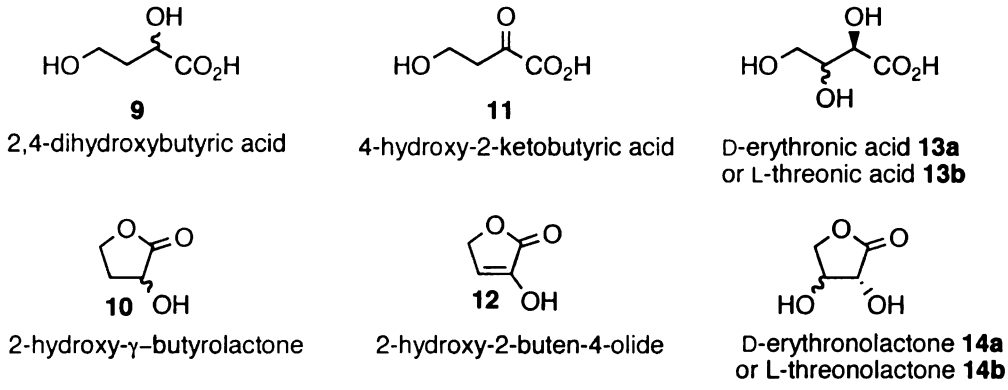


Figure 24. Alternative substrates for catalytic hydrogenation.

They could both be hydrogenated directly to D,L-1,2,4-butanetriol **1a,b**. Under milder reaction conditions, hydrogenolysis of D,L-1,2,4-butanetriol **1a,b** to 1,2-propanediol **5** is not expected to lower the yield of product. Dehydration of the α-hydroxyl group is not expected to occur because of its proximity to the carboxylate. Another substrate that could be hydrogenated to D,L-1,2,4-butanetriol **1a,b** via the intermediacy of 2,4-dihydroxybutyric acid **9** or 2-hydroxy-γ-butyrolactone**10** is 4-hydroxy-2-ketobutyric acid **11** or the corresponding lactone, 2-hydroxy-2-buten-4-olide **12**. D-Erythronic acid **13a** or L-threonic acid **13b** and the corresponding D-erythronolactone or L-threonolactone **14a,b** were also evaluated as possible substrate for catalytic hydrogenation to D,L-1,2,4-butanetriol **1a,b** (Figure 24).

2-Hydroxy-γ-butyrolactone **10**

Two procedures, which involve reduction of the unactivated carboxylic acid group of malic acid **2a,b** have been described (Figure 25).[26] In the first route, the α-

hydroxyl group along with the carboxylate are simultaneously protected as a 2-*t*-butyl-5-substituted 1,3-dioxolanone **15** using pivaldehyde in the presence of acid catalyst.[25a]

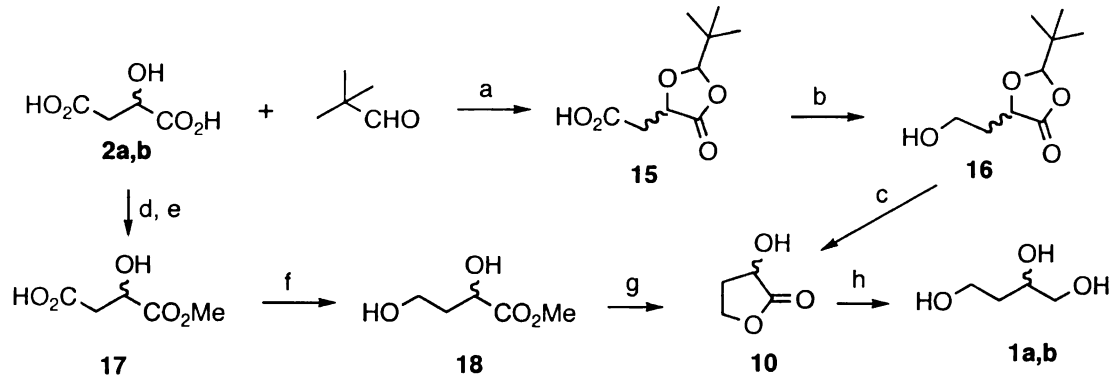


Figure 25. Syntheses of 2-hydroxy-γ-butyrolactone. Key: a) *p*TSA, pentane, Dean-Stark, 79%; b) BH_3-SMe_3, $B(OCH_3)_3$, THF, 97%; c) HCl; d) Trifluoroacetic anhydride; e) Methanol; f) BH_3-SMe_3, $B(OCH_3)_3$, THF, 81% g) Na_2CO_3, Dowex-50 (H^+); (h) 170 atm H_2, 125 °C, 1.0 mol % Ru/C, 10 h, 100%.

The resulting unprotected carboxylic acid was reduced to the corresponding alcohol **16** using 1.3 equivalents of borane-dimethylsulfide.[25b] Alcohol **16** residue was redissolved in MeOH and concentrated several times to remove contaminating methyl borate byproduct. Acid deprotection of alcohol **16** afforded 2-hydroxy-γ-butyrolactone **10** after removal of solvent. In the second procedure, trifluoroacetic anhydride was suspended in D,L-malic acid **2a,b** to afford the anhydride.[25c] Methanolysis of the anhydride resulted in methyl malate **17** as a sole product. Reduction of the C-4 carboxylic acid required 2 equivalents of borane-dimethyl sulfide.[25b] The resulting product was a mixture of 2,4-dihydroxy-methyl-butyrate **18** and 2-hydroxy-γ-butyrolactone **10**. Base hydrolysis of the mixture followed by a cation exchange column resulted in 2-hydroxy-γ-butyrolactone **10** after removal of water. Hydrogenation of the 0.5 M aqueous solution of 2-hydroxy-γ-butyrolactone **10** at 340 atm H_2 for 10 h over 1.3

mol % Ru/C at 135 °C was conducted. D,L-1,2,4-Butanetriol **1a,b** was obtained in 82% yield in addition to formation of 1,2-propanediol **5** in 13% yield. D, L-Malic acid **2** hydrogenation studies (Table 1 and 2) that were conducted established that H_2 pressures between 136 and 204 atm H_2 were sufficient to hydrogenate the carboxylic acid adjacent to the hydroxyl group. To avoid hydrogenolysis of 1,2,4-butanetriol **1a,b** to 1,2-propanediol **5**, it was necessary to reduce temperature and catalyst loading. Dehydration of 3,4-dihydroxybutyric acid **4** and subsequent hydrogenation to 1,4-butanediol **6** was shown to occur during catalytic hydrogenation. With the current substrate, 2-hydroxy-γ-butyrolactone **10**, this type of dehydration is not possible. As predicted, quantitative conversion of 2-hydroxy-γ-butyrolactone **10** to D,L-1,2,4-butanetriol **1a,b** was observed under milder reaction conditions (170 atm H_2, 125 C, and 1.0 mol% Ru on °C) relative to those employed (340 at H_2, 135 °C, and 1.3 mol% Ru on C) for D,L- and L-malic acid (Figure 25).

4-Hydroxy-2-ketobutyric acid **11**

In mammals, 2-keto-4-hydroxybutyrate **11** is an intermediate in L-homoserine degradation (Figure 26).[27a] This degradation is catalyzed by glutamate-aspartate transaminase, where oxaloacetate or 2-ketoglutarate serve as amino group acceptor.

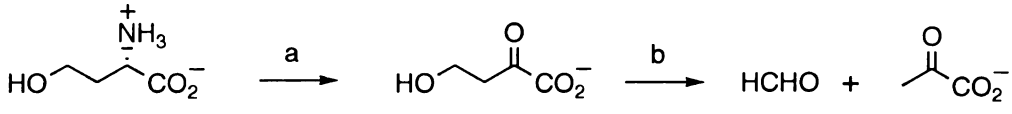


Figure 26. Degradation of L-homoserine in mammals. Key: a) glutamate-aspartate or glutamine-alanine transaminase; b) 2-keto-4-hydroxyglutarate aldolase.

Glutamine-alanine transaminase also catalyzes transamination of L-homoserine to 2-keto-4-hydroxybutyrate **11** and either pyruvate or 2-ketoglutarate serve as the amino group acceptor.[26b] The α-Keto acid that results is further cleaved to pyruvate and formaldehyde by 2-keto-4-hydroxyglutarate aldolase (Figure 26).[26c-f] In bacteria and fungi, L-homoserine is an intermediate in the biosynthesis of L-threonate **13b** from L-aspartate.[28] For the synthesis of 2-keto-4-hydroxybutyrate **11**, L-aspartic acid was used as the starting material (Figure 27). Methanolysis of the aspartic anhydride afforded a mixture of C-1 and C-4 methyl aspartates.[25c] These isomers were separated by trituration with diethyl ether and petroleum ether (2:1). The unprotected carboxylic acid of the desired C-1 methyl aspartate **19** was reduced with two equivalents of borane to afford a methyl ester of homoserine **20**.[25b] Subsequent base hydrolysis of ester **20** afforded the sodium salt of L-homoserine.

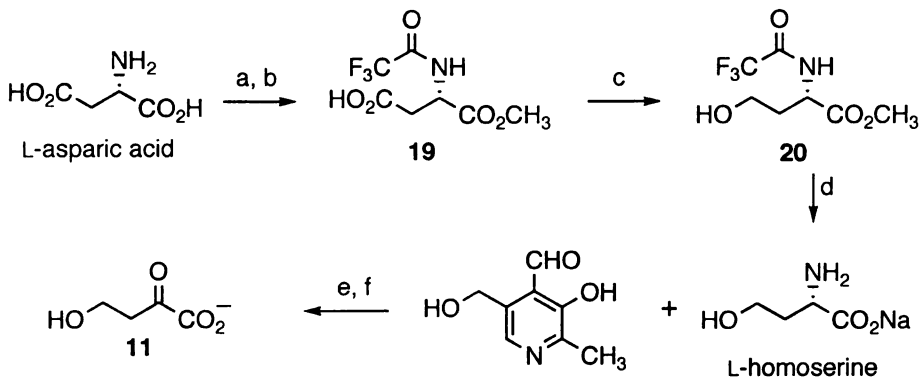


Figure 27. Synthesis of 2-keto-4-hydroxybutyrate from L-aspartic acid.
Key: (a) Trifluoroacetic anhydride; b) Methanol; c) BH_3.THF; d) 2 eq. NaOH; e) $CuCl_2.2H_2O$, acetate buffer, pH 5; f) Dowex-50 (H^+), pH 7.

Transamination of L-homoserine with pyridoxal-HCl was catalyzed by copper chloride to afford 2-keto-4-hydroxybutyrate **11** in disappointing yield 10-15%.[29] The product was contaminated with acetate used in the buffer. This synthesis of 2-keto-4-

hydroxybutyrate **11** along with the two syntheses of 2,4-dihydroxybutyrate **9** are not amenable to scale up due to the stoichiometric use of borane reagent. Furthermore, the methyl borate esters generated as by-products are difficult to separate from product.

D-Erythronolactone **14a** and L-threonolactone **14b**

Oxidative cleavage of D-isoascorbic acid and L-ascorbic acid in the presence of basic hydrogen peroxide solution afforded D-erythronate **13a** and L-threonate **13b**, respectively in addition to sodium oxalate (Figure 28).[30] Acidification of the product mixture to pH 2.5 with HCl resulted in formation of D-erythronolactone **14a** and L-threonolactone **14b**. Following D-erythronolactone **14a** and **14b** D-erythronolactone **14a** and **14b**.

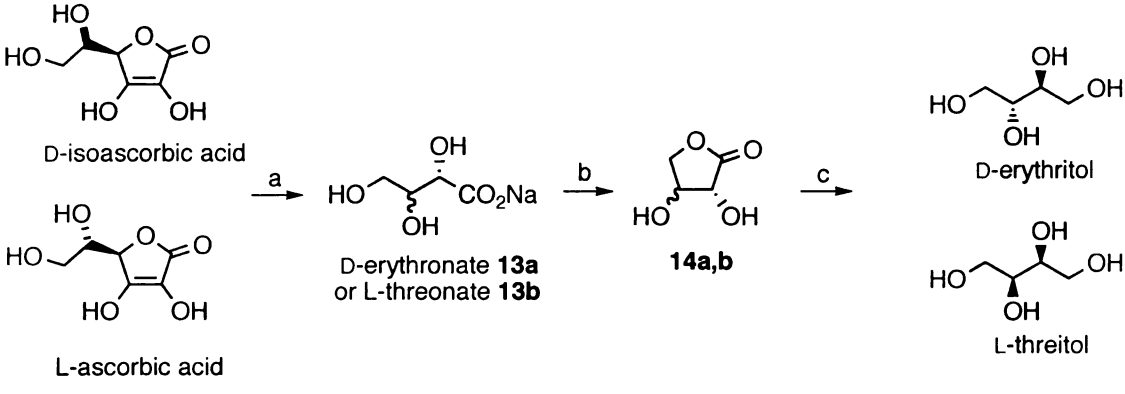


Figure 28. Syntheses of D-erythronolactone and L-threonolactone . Key: (a) $NaCO_3$, H_2O_2; (b) 6 M HCl, pH 2.5, 94%; (c) 340 atm H_2, 135 C, 1.3 mol% Ru on C, 10 h, 100%.

The propensity for elimination of water to occur in D-erythronolactone **14a** under hydrogenation conditions was evaluated. This avenue was pursued because of the previous observation that intermediates 3-hydroxy-γ-butyrolactone **3** and 3,4-dihydroxybutyrate **4** presumably underwent dehydration-hydrogenation sequence to afford 1,4-butanediol **6** during malate hydrogenation (Figure 23). An aqueous solution of

D-erythronolactone **14a** was subjected to hydrogenation conditions at 340 atm H_2 and 135 °C over 1.3 mol% Ru on C, yielding a quantitative amount of D-erythritol (Figure 28). No 1,2,4-butanetriol **1a,b**, the product of dehydration-hydrogenation, was observed. The hydrogenolysis of L-threonolactone also led to of L-threitol as the sole product.

2-Hydroxy-2-buten-4-olide **12** from D-erythronolactone **14a**

With D-erythronolactone **14a** in hand, synthesis of 2-hydroxy-2-buten-4-olide **12** was attempted with hopes to ultimately reduce the butenolide **12** to D,L-1,2,4-butanetriol **1a,b** (Figure 29). The D-erythronolactone **14a** was benzoylated or acetylated and subsequent β-elimination with a base afforded 2-*O*-acetyl-D-erythronolactone or 2-*O*-benzoly-D-erythronolactone, respectively.[31,32] Deprotection of the acetyl or benzoyl group using *in situ* generated lithium hydroxide resulted in the degradation of the product and 2-hydroxy-2-buten-4-olide was not isolated (Figure 29).[30]

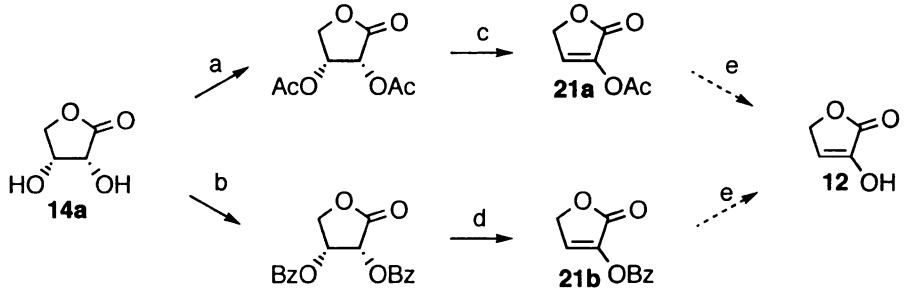


Figure 29. Attempted synthesis of 2-hydroxy-2-buten-4-olide. Key: a) Acetic anhydride; b) pyridine, benzoyl chloride; c) pyridine; d) triethylamine; e) LiOH.

Successful elimination was achieved with trifluoroacetic anhydride in the presence of pyridine as both a solvent and base. 2-Hydroxy-2-buten-4-olide was obtained in quantitative yields after hydrolysis. However, large-scale purification of the

butenolide **12** from pyridine and pyridinium trifluoroacetate using multiple cation exchange columns was tedious. This route was ultimately abandoned for the synthesis of butenolide **12**. An alternative synthesis of this substrate starting from L-ascorbic acid via the intermediacy of L-threonate **13b** and 4-hydroxy-2-ketobutyrate **11** was pursued (Figure 30).

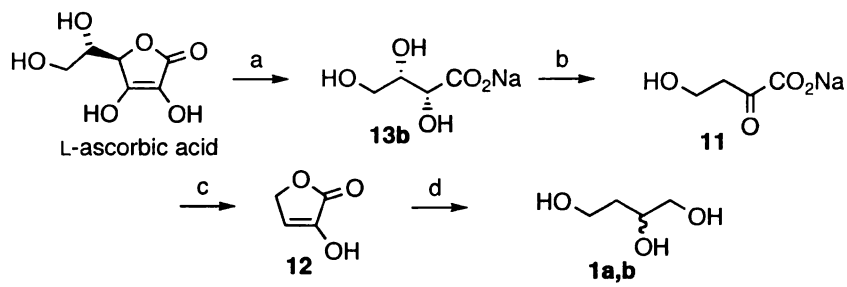


Figure 30. Chemoenzymatic synthesis of D, L-1,2,4-butanetriol from L-ascorbic acid. Key: (a) Na_2CO_3, 30% H_2O_2, 74%; (b) JWF1/pON1.118B, pH 8.2, 37 °C, N_2, 80%; (c) HCl, pH 1.5, 93%; (d) 1.0 mol% Ru on C, 170 atm H_2, 125 °C, 96%.

Chemoenzymatic Synthesis of D,L-1,2,4-Butanetriol

To obtain large quantities of L-threonate **13a**, a literature procedure was modified.[29a] Instead of a 0.2 M solution of L-ascorbic acid, a 1 M solution was used. Because of its elevated concentration, byproduct sodium oxalate precipitated out of solution when the reaction mixture was allowed to stand at 4 °C. This eliminated the need to concentrate large volumes of water along with the multi step procedure (acidification, EtOAc extraction, concentration and base hydrolysis) previously required to obtain L-threonate **13b**. Reaction of L-ascorbate at 1 M concentration with H_2O_2 afforded L-threonate **13b** in 74% yield (Figure 30).

In a chemoenzymatic synthetic study of 2-keto-3-deoxy-aldonic acid starting from aldonic acids, dihydroxy-acid dehydratase was shown to accept different substrates.[30]

This enzyme catalyzes the dehydration of 2,3-dihydroxyisovalerate and 2,3-dihydroxy-3-methylvalerate during the biosyntheses of L-leucine and L-isoleucine the dehydration, respectively.[33] Spinach and *Escherichia coli* were explored as sources for this enzyme. The spinach dihydroxy-acid dehydratase with its active site [2Fe-2S] cluster was stable to air but overexpression was not an option since the encoding gene had not yet been identified.[34] Dihydroxy-acid dehydratase from *E. coli* with its active site [4Fe-4S] cluster was unstable when exposed to air.[35] However, the encoding *ilvD* gene was known and thus was amenable to overexpression. Dihydroxy-acid dehydratase was partially purified by a modified literature procedure from a homogenate prepared from spinach leaves using protamine sulfate precipitation followed by DEAE-cellulose anion exchange chromatography (Table 3).[30]

Table 3. Purification of Dihydroxy-acid Dehydratase from Spinach Leaves.

Step	Protein (mg)	Specific Activity (U/mg)	Total Activity (U)	Yield (%)	Purification (fold)
Crude lysate	6044	0.89	538	100	1
Protamine sulfate	3198	0.13	494	92	1.4
DEAE cellulose	98	3.4	332	60	38

U/mg = 1 μmol/min/mg.

This short purification scheme afforded an enzyme preparation with a specific activity of 3.4 U/mg, which constituted a 38-fold increase in specific activity and a 60% yield in total units relative to the dihydroxy-acid dehydratase activity assayed in crude spinach homogenate. Conversion of L-threonate **13b** (152 mM) to 4-hydroxy-2-ketobutyrate **11** using the spinach dihydroxy-acid dehydratase (630 units) was carried out at 37 °C and pH 8.0 under N_2 (Figure 31). The spinach dihydroxy-acid dehydratase activity was relatively stable (~3.3 U/mg) during the 48 h reaction time but only a 22% yield of 4-hydroxy-2-ketobutyrate **11** was obtained.

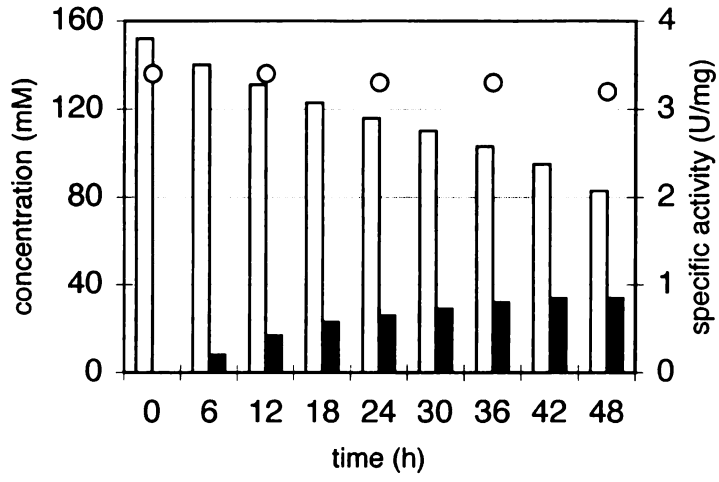


Figure 31. Conversion of L-threonate to 4-hydroxy-2-ketobutyrate catalyzed by dihydroxy-acid dehydratase partially purified from spinach leaves. Legends: L-threonate (white bar), 2-keto-4-hydroxybutyrate (black bar), specific activity (open circle).

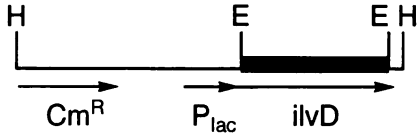
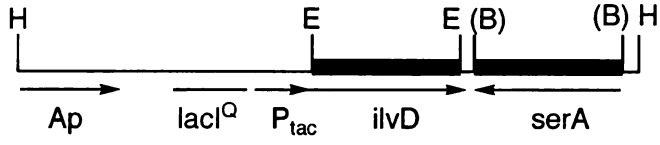
Due to the lack of chromosomal DNA sequence for spinach dihydroxy-acid dehydratase gene, attention was focused on overexpressing the *ilv*D gene locus in *Escherichia coli.*

Construct Design and Culturing Conditions

E. coli JWF1, which was used as the host strain, was derived from *E. coli* RB791 (W3110 $lacI^Q$). Homologous recombination was used to replace the *serA* gene from the RB791 genome with the non-functional *serA* gene.[36a] Consequently, *E. coli* JWF1 encoded catalytically inactive D-3-phosphoglycerate dehydrogenase, which is an enzyme required for L-serine biosynthesis. Dihydroxy-acid dehydratase was encoded by *ilvD* in plasmid pWN3.196A and transcribed from a P_{tac} promoter while in plasmid pON1.118B *ilvD* was transcribed from a P_{tac} promoter (Table 4). Both plasmids used in this study contained the *serA* gene, which encodes D-3-phosphoglycerate dehydrogenase (Table 4). This provided the basis for plasmid maintenance when *E. coli* JWF1 was grown in minimum salts medium L-serine supplementation.[36b,c] The additional plasmid-localized

lacI^{Q} gene in pON1.118B allowed *ilvD* transcription to be controlled by the concentration of isopropyl β-D-thioglactopyranoside (IPTG) in the growth medium.

Table 4. Plasmid Restriction Maps.

Plasmid (size)	Plasmid Map[a]
pWN3.196A (6.0-kb)	
pON1.118B (9.0-kb)	

[a] Restriction enzyme sites are abbreviated as follows: H = *Hind*III, E = *Eco*RI, B = *Bam*HI. Parentheses indicate that the designated enzyme site has been eliminated. Lightface line indicates vector DNA; boldface line indicates insert DNA.

Both *E. coli* JWF1/pWN.196A and JWF1/pON1.118B were cultured under fed-batch fermentor conditions at 33 °C and pH 7.0. Dissolved O_2 levels were maintained at a set point of 20% air saturation. D-Glucose addition was controlled by dissolved O_2 concentration with the rate of D-glucose addition dictated by a proportional-integral-derivative (PID) control loop. When dissolved O_2 level exceeded the set point value indicating decreased microbial metabolism, the rate of D-glucose addition was increased. Conversely, the rate of D-glucose addition was decreased when the dissolved O_2 level declined below the set point value indicating increased microbial metabolism. Transcription of dihydroxy-acid dehydratase in *E. coli* JWF1/pON1.118B was induced by addition of IPTG to a final concentration of 218 mg/L when both the impeller speed and airflow had reached the maximum settings of 1100 rpm and 1.0 L/min, respectively. The respective *E. coli* were cultured for 36 h and the cells were harvested by centrifugation of the fermentation broth.

After *E. coli* JWF1/pWN3.196A cells were harvested, they were resuspended phosphate buffer previously purged with N_2 and lysed by passage through French press. After centrifugation, the resulting crude lysate containing 65,000 U of dihydroxy-acid dehydratase was combined with L-threonate (155 mM) inside a fermentor. A fermentor was used to maintain a reaction temperature of 37 °C, the solution at pH 8.2, and a constant N_2 atmosphere during the enzyme-catalyzed dehydration.

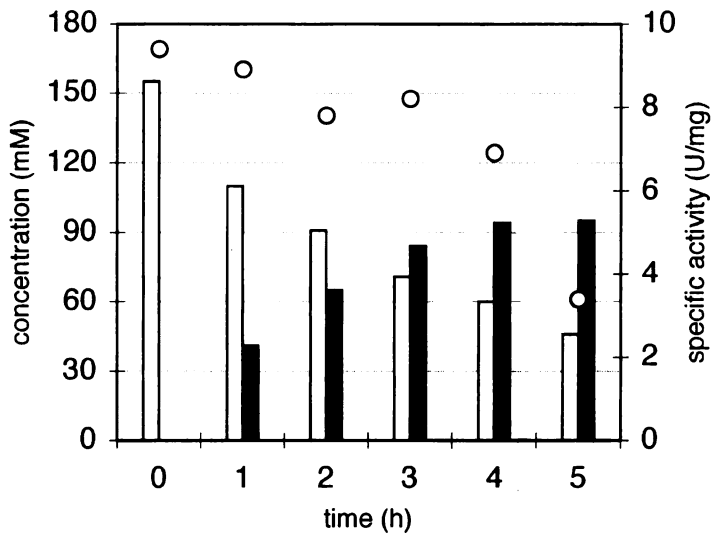


Figure 32. Conversion of L-threonate to 4-hydroxy-2-ketobutyrate and associated specific activity for dihydroxy-acid dehydratase from *E. coli* JWF1/pWN3.196A. Legends: L-threonate (white bar), 2-keto-4-hydroxybutyrate (black bar), specific activity (open circle).

Incubation with crude lysate of *E. coli* JWF1/pWN3.196A resulted in the conversion of 60% of L-threonate to 2-keto-4-hydroxybutyrate in 5 h (Figure 32). About 70% of the initial activity of *E. coli* dihydroxy-acid dehydratase activity was lost after 5 h of bioconversion. Comparison of dihydroxy-acid dehydratase specific activity from spinach leaves and *E. coli* JWF1/pWN196A for the dehydration of native substrate and L-threonate is shown in Table 5.

Table 5. Substrate Specificity Comparison.

Substrate	Specific activity[1]	
	Dihydroxy acid dehydratase of JWF1/pWN3.196A[2]	Spinach dihydroxy acid dehydratase[3]
2,3-dihydroxy isovalerate	9.04	3.39
L-threonate	0.17	0.49
specific activity ratio	53:1	7:1

[1] μmol/min/mg. [2] crude cell lysate. [3] partially purified dihydroxy-acid dehydratase.

The spinach dihydroxy-acid dehydratase had a 1/7 ratio of specific activities for dehydration of L-threonate **13b**/dehydration 2,3-dihydroxy isovalerate (native substrate). *E. coli* dihydroxy-acid dehydratase had a substantially lower 1/53 ratio of specific activities for dehydration of L-threonate **13b**/dehydration of 2,3-dihydroxy isovalerate (native substrate).

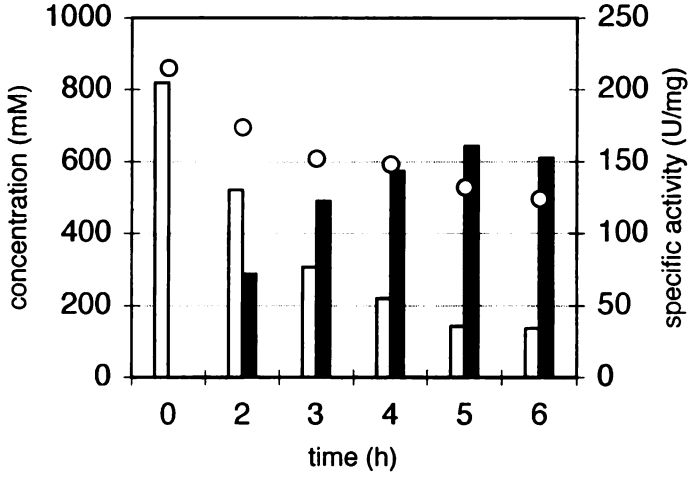


Figure 33. Conversion of L-threonate to 4-hydroxy-2-ketobutyrate and associated specific activity for dihydroxy-acid dehydratase from *E. coli* JWF1/pON1.118B. Legends: L-threonate (white bar), 2-keto-4-hydroxybutyrate (black bar), specific activity (open circle).

The ability to overexpress plasmid-localized *ilvD* from a P_{tac} promoter in *E. coli* JWF1/pON1.118B more than compensated for the instability of *E. coli* dihydroxy-acid dehydratase in air and its lower activity towards L-threonate **13b** relative to spinach dihydroxy-acid dehydratase and expressed by *E. coli* JWF1/pWN3.196A, which

transcribed *ilvD* from a P_{lac} promoter. Harvested *E. coli* JWF1/pON1.118B cells were resuspended in the concentrated solution of L-threonate **13b** after precipitation/removal of sodium oxalate, addition of phosphate buffer, and purging of the reaction solution with N_2. After lysis of the cells and centrifugation, the reaction solution contained 2.4 x 10^6 U of dihydroxy-acid dehydratase. A fermentor was used to maintain a reaction temperature of 37 °C, the solution at pH 8.2, and a constant N_2 atmosphere during the enzyme-catalyzed dehydration. This led to the conversion of L-threonate **13** into 4-hydroxy-2-ketobutyrate **11** in 80% yield after 5 h (Figure 33). Approximately half of the IlvD specific activity was lost during the enzyme-catalyzed dehydration. Allowing the enzyme-catalyzed dehydration to continue beyond 5 h did not lead to conversion of the residual amount of L-threonate **13b** into additional concentrations of 4-hydroxy-2-ketobutyrate **11**.

The crude reaction mixture was acidified to pH 1.5 and the precipitated protein removed by centrifugation. Lactonization of 2-keto-4-hydroxybutyric acid and selective extraction away from L-threonolactone **14b** using liquid-liquid extraction[37] with EtOAc afforded a 93% yield of 2-hydroxy-2-buten-4-olide **12** that was free of contamination by L-threonolactone. Subsequent hydrogenation at 170 atm H_2 and 125 °C of a 0.5 M aqueous solution of 2-hydroxy-2-buten-4-olide **12** over 5 wt % Ru on C at a 1.0 mol% Ru/2-hydroxy-2-buten-4-olide mol ratio for 10 h afforded D,L-1,2,4-butanetriol **1a,b** in 96% yield. The overall yield of D,L-1,2,4-butanetriol from L-ascorbic acid was 53%. No byproducts such as those resulting from C-C and C-O bond cleavage during hydrogenation of D,L-malic acid **2a,b** could be detected. As a consequence, purification of product D,L-1,2,4-butanetriol **1a,b** by distillation was not required.[17]

DISCUSSION

The current commercial manufacture of D,L-1,2,4-butanetriol **1a,b** relies on a stoichiometric $NaBH_4$ reduction of dimethyl D,L-malate (Figure 20).[9] Liquefiable petroleum gas, which is produced from petroleum and natural gas, is the source of the carbon atoms in D,L-malic acid (**2a,b**) and the derived dimethyl D,L-malate (Figure 20).[38] Petroleum-derived acetylene is the starting material for 2-butyne-1,4-diol and 2-buten-1,4-diol used for the synthesis of D,L-1,2,4-butanetriol **1a,b**. Allyl alcohol and acetaldehyde were required for the syntheses of glycidol and 3-buten-1-ol. By contrast, the syntheses described in this chapter employed starting materials derived from abundant and inexpensive D-glucose derived from renewable feedstocks. The source of carbon atoms is an important consideration given the increasing prices of petroleum and natural gas and the projected decline of domestic reserves of fossil fuels.[39] L-Malic acid (**2b**, Figure 23) can be microbially synthesized from D-glucose using *Aspergillus flavus*.[40] L-Ascorbic acid (Figure 30) is manufactured from D-glucose-derived D-sorbitol via the Reichstein-Grussner process.[41]

Stereochemical considerations are also important. 1,2,4-Butanetriol trinitrate that has been used as an energetic material has been a racemic D,L-mixture, which reflects the use of methyl D,L-malate (Figure 20) as the starting material for commercial synthesis of precursor D,L-1,2,4-butantriol **1a,b**. The difference in the melting point of the pure enantiomers relative to the different melting point for the racemic mixture of enantiomers is an important consideration in the utilization of energetic materials. Biocatalytic routes typically lead to either the D- or L-enantiomer of 1,2,4-butanetriol. For example, D-1,2,4-butanetriol **1a** has been microbially synthesized from D-xylose while a different microbe

was used to synthesize L-1,2,4-butanetriol **1b** from L-arabinose.[21] Condensation of glycolaldehyde and acetaldehyde catalyzed by 2-deoxyribose-5-phosphate aldolase gave D-1,2,4-butanetriol **1a** (Figure 21).[17] No enzyme is currently known that is capable of catalyzing the condensation of glycolaldehyde and acetaldehyde to afford L-1,2,4-butanetriol **1b**. By contrast, L-malic acid **2a** (Figure 23) underwent racemization during its catalytic hydrogenation to afford D,L-1,2,4-butanetriol **1a,b**. Racemic D,L-1,2,4-butanetriol **1a,b** was the product of the chemoenzymatic synthesis using L-ascorbic acid as the starting material (Figure 30).[17]

Catalytic hydrogenation of D,L-malic acid **2a,b** and L-malic acid **2b** was the shortest route for synthesis of D,L-1,2,4-butanetriol **1a,b**. Optimized hydrogenation of a 1 M aqueous solution of D,L-malic acid **2a,b** for 10 h at 340 atm H_2 and 135 °C over 5% Ru on C using a 1.3% Ru/malate ratio led to a 74% yield of D,L-1,2,4-butanetriol **1a,b** along with a complex mixture of byproducts. Distillation of this crude product mixture afforded only a 28% yield of impure D,L-1,2,4-butanetriol **1a,b**. Notably, the 340 atm H_2 used in these hydrogenations was at the maximum pressure rating for the reactor used for these experiments. Such elevated H_2 pressures and temperatures are typical for catalytic hydrogenation of unactivated carboxylates.[42] Adkins first reported the synthesis of 1,2,4-butanetriol from hydrogenation of neat dimethyl malate over $2CuO{\cdot}Cr_2O_3$.[43] High catalyst loading relative to substrate, 150 °C reaction temperatures, and H_2 pressures of 340 atm were required to obtain a 67% yield of 1,2,4-butanetriol and a 20% yield of 1,4-butanediol. Anton reported hydrogenation of aqueous malic acid at 200 atm H_2 and 60 °C over Ru-Re to afford D,L-1,2,4-butanetriol **1a,b** in 80% yield.[44]

Hydrogenation of malic acid (Figure 23) was the obvious catalytic alternative to stoichiometric reduction of D,L-malic acid **2a,b** using $NaBH_4$. However, the elevated H_2 pressures and temperatures required for catalytic hydrogenation of D,L-malic acid **2a,b** and the separation of D,L-1,2,4-butanetriol **1a,b** from byproducts compromise the synthetic directness of this route. Malic acid possesses two chemically different carboxylates. One carboxylate is inductively activated by an α-hydroxyl group while the second carboxylate remains unactivated. The first step in malic acid **2a,b** hydrogenation proceeds under relatively mild conditions to afford 3,4-dihydroxybutyric acid **4** and the corresponding 3-hydroxy-γ-butyrolactone **3** (Figure 34). This is presumably due to the presence of an electron-withdrawing hydroxyl group at the alpha position, which eases the hydrogenation barrier.[45] Hydrogenation of the remaining unactivated carboxylate results in D,L-1,2,4-butanetriol **1a,b**.

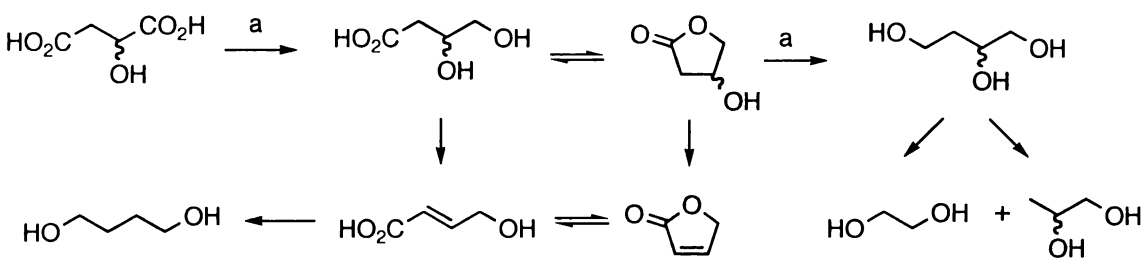


Figure 34. Catalytic hydrogenation of malic acid. Key: 340 atm H_2, 1.3 mol% Ru/malate, 135 °C, 10 h.

Byproduct formation was shown to result mainly from C-C and C-O bond cleavage of D,L-1,2,4-butanetriol **1a,b**. The major byproduct, 1,2-propanediol is derived from C-C bond cleavage of D,L-1,2,4-butanetriol **1a,b**. Ru-catalyzed dehydrogenation of 1,2,4-butanetriol leads to 1,4-dihydroxy-2-butanone or 3,4-dihydroxybutanal, which ultimately undergo retro-aldol followed by hydrogenation to afford 1,2-propanediol and ethylene glycol, respectively (Figure 35).[46]

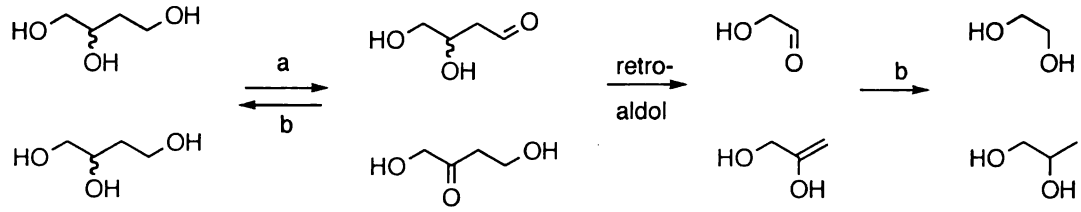


Figure 35. Formation of 1,2-propanediol and 1,2-ethanediol.

1,4-Butanediol, which accounted for 32% of the byproduct formed, did not originate from 1,2,4-butanetriol. Presumably, it was formed from dehydration of the partially reduced intermediates 3,4-dihydroxybutyric acid **4** and 3-hydroxy-γ-butyrolactone **3** followed by hydrogenation (Figure 34).[47] Both 3,4-dihydroxybutyric acid **4** and 3-hydroxy-γ-butyrolactone **3** have a high propensity to undergo β-elimination. An interesting observation was the complete racemization of the L-malic acid **2b** to D,L-1,2,4-butanetriol **1a,b** during the course of hydrogenation. This can be attributed to the high temperatures and length of reaction time employed in this study. Racemization was an advantage because it allowed D,L-1,2,4-butanetriol **1a,b** to be synthesized from D-glucose-derived L-malic acid instead of petroleum-derived sources. However, difficulties associated with purifying product from contaminating byproducts significantly reduced the yield of D,L-1,2,4-butanetriol **1a,b**. In order to make this route viable as a source of D,L-1,2,4-butanetriol **1a,b**, a practical method for the removal of contaminants is needed. Acetalization of the mixture of polyols in the presence of acetaldehyde has been used to separate ethylene glycol from propylene glycol.[48a-d] The resulting 1,3-dioxolanes of ethylene glycol and propylene glycol are more volatile than the corresponding polyols and can be easily separated from each other by fractional distillation. This methodology might be employable for the purification of D,L-1,2,4-butanetriol **1a,b** from contaminating 1,2-glycols such as 1,2-propanediol **5**, 1,2-butanediol **7** and ethylene

glycol **8**. The 1,2-*O*-ethylidenebutane-1,2,4-triol could then be hydrolyzed to afford pure racemic 1,2,4-butanetriol and acetaldehyde, which would need to be recycled.[37]

Catalytic hydrogenation of L-malic acid to D,L-1,2,4-butanetriol **1a,b** route is concise, employs a starting material derived from renewable feedstock and affords the desired racemic mixture. The forcing reaction conditions result in the formation of undesired byproducts, which reduce the yield from 74% to 28% after distillation. Furthermore, the intermediates formed during the hydrogenation process are poised to undergo β-elimination, giving rise to undesired 1,4-butanediol. To circumvent these forcing reaction conditions and reactive intermediates, new substrates for catalytic hydrogenation were explored (Figure 24). The key consideration in choosing the starting material was the carbon source. Carbohydrate-derived starting materials, which afford the desired stereochemistry of product were ideal. Syntheses of three substrates, 2-hydroxy-γ-butyrolactone **10**, 2-hydroxy-2-buten-4-olide **12** and D-erythronolactone **14a,** were undertaken. Because catalytic hydrogenation was to be used, the presence of an activating hydroxyl group at the α-position was imperative. The absence of β-hydroxyl group eliminated the possibility of forming 1,4-butanediol.

Although the synthesis of 2-hydroxy-γ-butyrolactone **10** starts from D,L-malic acid, it was necessary to protect the unactivated carboxylic acid as an ester **17** or 1,3-dioxolanone **15** (Figure 25). Stoichiometric reduction of the unactivated carboxylic acid with borane results in the alcohol (**16** or **18**), which was subsequently deprotected to afford the desired 2-hydroxy-γ-butyrolactone **10**. The protection/deprotection sequence increases the length of the synthesis and requires the use of volatile and toxic reagents. Borane reduction was almost quantitative, but 1 to 1.3 equivalents of borane were

required to transform the acid to the alcohol. Subsequently, methyl borate by-product is generated as with the current commercial synthesis of 1,2,4-butanetriol (Figure 20). Purification of products from methyl borate is tedious. This synthesis cannot be scaled-up but it was useful to generate enough substrate to evaluate hydrogenation to 1,2,4-butanetriol **1a,b**. As predicted, hydrogenation of 2-hydroxy-γ-butyrolactone **10** afforded 1,2,4-butanetriol **1a,b** in quantitative yields (Figure 25). This was because an α-hydroxy carboxylic acid group requires milder hydrogenation conditions as was established during hydrogenation studies of malic acid (Figure 23). No intermediates or diol by-products were observed during the hydrogenation under mild conditions.

Having established that hydrogenation of 2-hydroxy-γ-butyrolactone **10** is more efficient than malic acid in affording 1,2,4-butanetriol, a more practical synthetic route was investigated. Several procedures for making 4-hydroxy-2-ketobutyrate **11** have been reported starting from L-aspartic acid, L-ascorbic acid or D-isoascorbic acid. In the L-aspartic acid route, protection and borane reduction sequence similar to 2-hydroxy-γ-butyrolactone **10** synthetic route was carried out to obtain L-homoserine (Figure 27). The transamination of homoserine to 4-hydroxy-2-ketobutyric acid proceeded in an acetate buffer in the presence of pyridoxal and cuprate. However, low yields, use of stoichiometric borane and tedious purification processes limited application of this process. In an alternative route, intermediates to 4-hydroxy-2-ketobutyrate **11** or the corresponding lactone **12** were obtained from L-ascorbic acid. A synthetic route for D-erythronate **13a** and L-threonate **13b** was reported by Isbel and Frush,[30] which took advantage of the known product of vitamin C oxidation. Hydrogen peroxide was consumed by L-ascorbic acid or D-isoascorbic acid to afford an inert α,β-diketone adduct,

which under basic conditions was cleaved to L-threonate **13b** or D-erythronate **13a** and oxalic acid (Figure 28).[30] Acid-catalyzed lactonization of D-erythronate **13a** afforded the corresponding D-erythronolactone **14a**. Having obtained both L-threonolactone **14a** and D-erythronolactone **14a**, attempts were made to dehydrate and hydrogenate these intermediates to obtain 1,2,4-butanetriol (Figure 28). Although the presence of the β-hydroxyl makes these substrates potentially susceptible to β-elimination, quantitative yields of L-threitol or D-erythritol were obtained even under reaction conditions employing high pressures and temperatures. This suggested that hydrogenation of the carboxylate is faster than the dehydration process. L-Erythritol or D -threitol is not poised to dehydrate under hydrogenation conditions used.

By taking advantage of the *trans*-relationship of the α-hydrogen and the β-hydroxyl group in D-erythronolactone, β-elimination of the benzoyloxy-, acetyloxy- or trifloroacetyloxy- was effected under in the presence of triethylamine or pyridine (Figure 29). Subsequent deprotection of the benzoyloxy and acetyloxy groups was problematic under basic conditions. This was likely due to the degradation of product 2-hydroxy-2-buten-4-olide under basic conditions.[31] When trifluoroacetic anhydride was used to activate the hydroxyl group, excess pyridine was used. Quantitative conversion of D-erythronolactone **14a** to 2-hydroxy-2-buten-4-olide **12** was observed by ^{1}H NMR. However, removal of pyridine was unsuccessful, which compromised this route for the synthesis of 2-hydroxy-2-buten-4-olide **12**.

In the last and final route, L-threonate **13b** was dehydrated to 4-hydroxy-2-keto-butyrate **11** in 80% yield at ambient conditions (Figure 30). Dihydroxy-acid dehydratase obtained from overexpression of the *E. coli ilvD* locus catalyzed the dehydration.

Dihydroxy-acid dehydratase catalyzes the third step of branched-chain amino acid biosynthesis where it catalyzes the dehydration and tautormerization of two naturally occurring 2*R*, 3*R*-dihydroxycarboxylic acids to the corresponding 2-keto acids. When nonnative substrates were used, the *threo*-dihydroxy acid isomer was dehydrated more rapidly relative to the *erythreo*-dihydroxy isomer.[31] Dihydroxy-acid dehydratase is utilizes the Fe-S cluster. Mechanistically related enzymes include aconitase, which catalyzes the reversible dehydration of citrate to *cis*-aconitase, and L-serine dehydratase, which catalyzes the irreversible deamination of serine to pyruvate.[49,50] All of these enzymes along with dihydroxy-acid dehydratase from *E. coli* possess a [4Fe-4S], which in the presence of oxygen is oxidized and partially or completely degraded. Dihydroxy-acid dehydratase from spinach leaves on the other hand, is stable to O_2 and possesses a [2Fe-2S] cluster at its active site.

By expanding its coordination sphere, the iron of the cluster is able to accommodate the 3*R*-hydroxy of the substrate as a ligand.[50] This allows the Fe-S cluster to function as a Lewis acid and activates the 3-hydroxyl leaving group. Removal of the proton *trans* to the hydroxyl furnishes the enol intermediate, which tautormerizes to the 2-keto acid.[31] The catalytic turnover of the spinach dihydroxy-acid dehydratase for L-threonate is much higher relative to *E. coli* dihydroxy-acid dehydratase.[17] Spinach dihydroxy-acid dehydratase stable over 2 days (Figure 31) of reaction while the specific activity of *E. coli* dihydroxy-acid dehydratase drops by 60-70% in 5 h (Figure 32,33). Utilization of the stronger P_{tac} promoter improved expression of *E. coli* dihydroxy-acid dehydratase, which facilitated the conversion of L-threonate **11** to 4-hydroxy-2-ketobutyrate **11** (Figure 30). 4-Hydroxy-2-ketobutyrate **11** was not isolated due to

previous reports of its instability. Instead, acid-catalyzed lactonization furnished 2-hydroxy-2-buten-4-olide **12**, which is stable. Direct hydrogenation via the intermediacy of 2-hydroxy-γ-butyrolactone afforded D,L-1,2,4-butanetriol **1a,b** without concomitant formation of 1,2-propanediol or 1,4-butanediol.

This last route provides an alternative to the current commercial synthesis of D,L-1,2,4-butanetriol **1a,b**. Glucose-derived ascorbic acid is used as the starting material to afford D,L-1,2,4-butanetriol **1a,b** in 53% overall yield. Byproduct formation is completely avoided by hydrogenating under mild conditions. Unlike the current commercial synthesis of D,L-1,2,4-butanetriol **1a,b**, this proceeds in water while deriving its carbon source from renewable and abundant starting materials. On the other hand, the L-ascorbic acid route requires more synthetic steps relative hydrogenation of malic acid.

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CHAPTER 3

CONVERSION OF L-LYSINE TO ε-CAPROLACTAM

Introduction

ε-Caprolactam is the monomer for the synthesis of Nylon 6.[1] Over 90% of the annually produced ε-caprolactam (4 billion kg/year) is polymerized to nylon 6, which along with nylon 6,6 account for 98% of nylon fibers produced worldwide.[2] Most commercial processes for the manufacture of ε-caprolactam start from either toluene or benzene.[3] Oxidation of benzene followed by catalytic hydrogenation of the resulting phenol results in cyclohexanone (Figure 36).

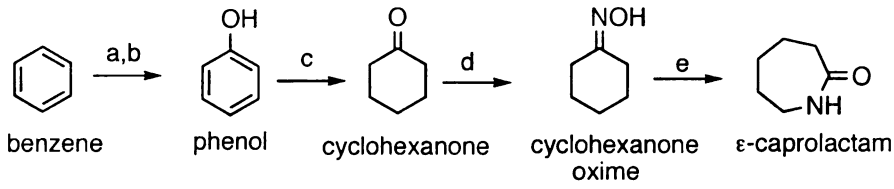


Figure 36. Manufacture of ε-caprolactam from benzene. Key: (a) 2-propene, HZSM-12; (b) O_2, H_2SO_4; (c) Pd, H_2; (d) $(NH_2OH)_2H_2SO_4$, NH_3; (e) $H_2SO_4 \cdot SO_3$, NH_3.

Coupling of the cyclohexanone with hydroxylamine sulfate affords cyclohexanone oxime, along with ammonium sulfate byproduct. Several processes have been developed to minimize formation of ammonium sulfate, which is also used in the manufacture of fertilizers. These include NO reduction and use of phosphate oxime processes licensed and operated by DSM.[4] Alternatively, cyclohexanone oxime is produced by reaction with NH_3 and H_2O_2 in the presence of titanosilicate catalysts. This so-called ammoxidation reaction commercialized by Sumimoto completely eliminates production of ammonium sulfate.[5] Alternatively, cyclohexane is converted into cyclohexanone oxime in the presence of nitrous oxide and HCl. Both cyclohexanone and

hydroxylamine are eliminated in this process.[6] Beckman rearrangement of the cyclohexanone oxime in the presence of oleum results in ε-caprolactam (Figure 36).[7] During this step, formation of ammonium sulfate byproduct results from neutralization of oleum with ammonia. Sumimoto has successfully commercialized an oleum-free Beckman rearrangement process. The process uses a fluid-bed reactor operated at 350 °C in the presence of methanol to effect rearrangement of cyclohexanone oxime to ε-caprolactam.[5] Other than the Sumimoto process in which no ammonium sulfate is produced, other commercial processes produce 1.4-4.3 tons of ammonium sulfate/ton of ε-caprolactam.[3]

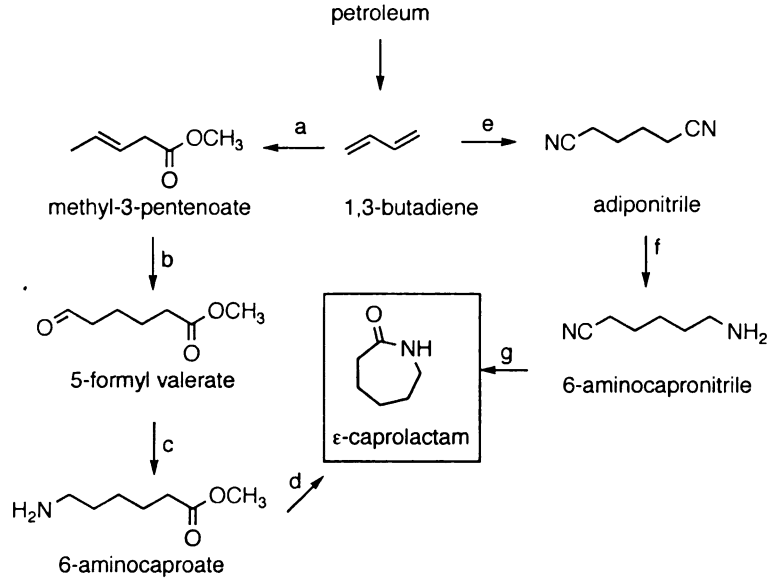


Figure 37. Syntheses of ε-caprolactam from 1,3-butadiene. Key: (a) CO, MeOH, Ni-zeolites; (b) CO, H_2, Co, Rh; (c) NH_3/H_2, Ru; (d) 250 °C; (e) HCN; (f) Ni/Co, H_2; (g) TiO_2, H_2O.

Non-aromatic feedstocks have been used for the syntheses of ε-caprolactam (Figure 37). The DSM-DuPont process employed 1,3-butadiene as the starting material.[8] Carbonylation over Ni-based zeolites or Pd borosilicates catalyst in the presence of

methanol affords pentenoate ester (Figure 37). Hydroformylation followed by reductive amination of the valerate ester results in 6-aminocaproate ester, which is ultimately cyclized to ε-caprolactam at 250 °C. BASF on the other hand converted adiponitrile, which could also be derived from 1,3-butadiene, to 6-aminocapronitrile over Ni-Co catalysts (Figure 37). Hydrolysis of 6-aminocapronitrile over TiO_2 at 240 °C results in ε-caprolactam (Figure 37).[9]

With the synthesis of adipic acid, one of the monomers required for the synthesis of nylon 6,6 from D-glucose delineated, a complementary synthesis of ε-caprolactam starting material derived from renewable feedstocks sought.[10] This chapter explores D-glucose-derived L-lysine as the starting material for the synthesis of ε-caprolactam. After masking the ε-amino group of L-lysine as an amide by cyclization, several strategies were employed to remove the α-amino group. The first approach employed catalytic hydrodenitrogenation employing Mo-based catalysts used in the denitrification of petroleum. A single-electron reduction-deamination sequence was also explored to remove the α-amino group from the methyl ester of L-lysine, methyl pipecolinate and α-amino ε-caprolactam. With limited success in removing the α-amino group, attention was focused on isomerizing L-lysine to β-L-lysine in order to make the amino group susceptible to β-elimination. Although β-L-lysine can be chemically synthesized from L-ornithine by homologation using an Arndt-Eistert reaction, it is not commercially available. Efforts were directed at optimizing the bioconversion of L-lysine to β-L-lysine using *Clostridium subterminale*.

Background

L-Lysine is an ideal precursor for the synthesis of ε-caprolactam because of its carbon skeleton. It is trails behind L-glutamic acid as the second highest produced amino acid at 7.5 x 10^8 kg/year.[11] The increased demand for L-lysine and other amino acids stems from their utilization in food additives, feed supplements, therapeutic agents and precursors for the syntheses of peptides or agrochemicals.[12] Specifically, L-lysine is required as a feed additive for poultry and swine production.[13] The microbial production of L-lysine by *Corynebacterium glutamicum* was introduced in 1957 and has since been improved to afford titer of 170 g/L from D-glucose (Figure 38).[14]

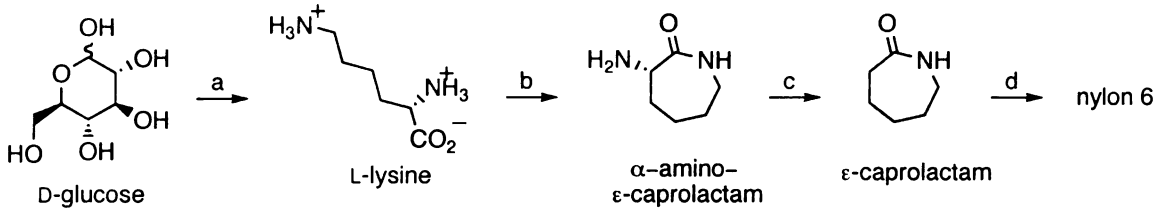


Figure 38. Synthetic route of ε-caprolactam from D-glucose. Key: (a) *Corynebacterium glutamicum*; (b) refluxing 1,2-propanediol, 2 h, 96%; (c) NH_2OSO_3H, KOH, 75%; H^+, 250 °C.

L-Lysine is an ideal precursor for the synthesis of ε-caprolactam because it possesses the C-1 carboxylate and the ε-amine necessary for the amide linkage. Amide formation, which consumes these two functionalities, avoids the need for derivatization. Selective removal of the secondary α-amine group is then required to afford the desired product. To mask the ε-amino group, L-lysine was cyclized in refluxing 1,2-propanediol to afford α-amino-ε-caprolactam (Figure 38). This cyclization was optimized in the Frost group to afford 96% of the desired 7-membered ring lactam.[15] 1,2-Propanediol is derived from lactic acid hydrogenation, which in turn is derived from microbial fermentation of D-glucose. Reported literature procedures for the cyclization of L-lysine employed

petroleum-derived toluene as a solvent for its ability to remove water. Reaction of α-amino-ε-caprolactam with hydroxylamine-*O*-sulfonic acid under basic conditions resulted in the deamination to afford ε-caprolactam in 75% yield.[16] Ironically, traditional synthesis of ε-caprolactam from cyclohexanone employs hydroxylamine-*O*-sulfonic acid (Figure 36). This route constitutes a fundamental departure from reported syntheses of ε-caprolactam, which rely on petroleum-derived benzene or 1,3-butadiene as carbon sources. However, similar to the current synthesis, preparation of the inorganic reagent required to effect the deamination results in the generation of a byproduct salt stream. Ideally, a catalytic deamination process would be more desirable to afford ε-caprolactam from α-amino-ε-caprolactam. To this end, catalytic deamination and catalytic hydrogen transfer were explored along with reductive deamination with single electron transfer reagents.

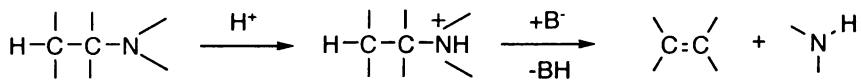
Catalytic Hydrodenitrogenation

Removal of sulfur, nitrogen and metals is one of the most important processes during the refining of petroleum.[17] This process reduces emission of sulfur and nitrogen oxides which are formed when these component of oil are combusted in car and truck engines. This process is referred to as hydrotreating, where crude oil is treated with hydrogen in the presence of a catalyst at high temperature without reducing the boiling point of the oil fractions. Hydrotreating is the largest application in industry. Consequently, hydrotreating catalysts rank third after exhaust gas catalysts and fluid cracking catalysts on the basis of the amount of catalysts sold per year.[17]

The industrial catalysts used to remove sulfur (hydrodesulfurization) from crude oil contain molybdenum and cobalt supported on γ-alumina whereas for removal of nitrogen (hydrodenitrification) a combination of molybdenum and nickel is used.[18] The formation of H_2S, which immediately sulfidates the metal or metal oxide catalyst, is inevitable during hydrotreatment. Catalyst suflidation under controlled conditions prior to the hydrotreating process is therefore necessary. Consequently, molybdenum sulfide is considered the actual catalyst. Nickel and cobalt function as promoters, which increase the activity of molybdenum for the removal of nitrogen, sulfur and oxygen.

The hydrodenitrogenation catalysts were prepared by a two-step pore volume impregnation procedure.[19] In the first step, γ-alumina was impregnated with an aqueous solution of ammonium molybdenate followed by drying and heating (calcine) at 400 °C. In a second step, the resulting Mo-alumina mixture was impregnated with nickel (II) nitrate, dried and calcined. Finally, the Mo-Ni/Al_2O_3 catalyst was activated by treatment with a mixture of H_2S (10%) and H_2 (90%) at 400 °C and 34 atm for 4 h to afford Ni-Mo-S/Al_2O_3. Two mechanisms have been proposed for catalytic hydrodenitrogenation of aliphatic amines (Figure 39). In Hoffmann elimination, the acidic group protonates the amine making it a good leaving and the basic sulfur abstracts the β-hydrogen simultaneously resulting in *trans*-elimination of the amine (Figure 39).[17]

Hoffmann Elimination



Nucleophilic Substitution

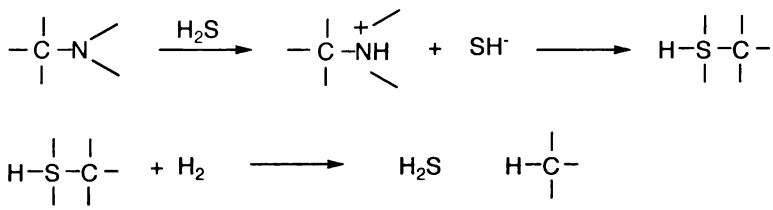


Figure 39. Mechanistic hydrodenitrogenation catalyzed by Mo-based catalyst.

Amines, which have no β-hydrogens are denitrogenated by nucleophilic substitution of the amine group by an SH group followed by hydrogenolysis of the weak C-S bond (Figure 39). As an alternative, a Pt/SiO_2 catalyst was also been explored for the deamination of α-amino-ε-caprolactam.[20] The catalyst was prepared by impregnating SiO_2 with chloroplatinic acid followed by activation at 350 °C and 1 atm H_2. The activated catalyst contained 40% Pt and 60% silica.[20]

Cyclohexylamine was used as a model substrate for catalytic demamination using Mo-Ni and Pt catalysts. Catalytic hydrodenitrogenation was conducted in a flow apparatus consisting of three U-tubes. Cyclohexylamine was places in the first U-tube, which was connected to a second U-tube containing reduced Pt/SiO_2. The last U-tube served as a product chamber and was maintained at -78 °C with dry ice-acetone cooling. Substrate and catalyst U-tubes were heated to 200 °C. H_2 was allowed to flow through the three U-tubes. The vaporized cyclohexylamine was swept through the catalyst and the resulting cyclohexane was trapped in the third U-tube. After all the cyclohexylamine

was evaporated, the sand bath heating was removed. Batch hydrodenitrogenation was also employed with Mo-based catalysts inside a 500 mL 4575 Parr reaction vessel. All catalysts were active in deaminating the cyclohexylamine to cyclohexane. Mo-based catalysts on Al_2O_3, SiO_2 and C along with 40% Pt on SiO_2 were first explored for the deamination of cyclohexylamine (Figure 40, Table 6).

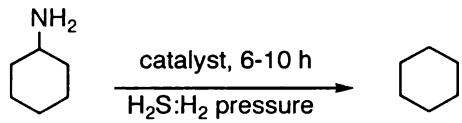


Figure 40. Deamination of cyclohexylamine.

Table 6. Hydrodenitrogenation of cyclohexylamine to cyclohexane.

Entry	Catalyst	Temp (°C)	Pressure (atm)	Yield[1] (%)
1	Pt/SiO_2	200	1	90
2	$Ni\text{-}Mo/SiO_2$	360	68	86
3	$Ni\text{-}Mo/Al_2O_3$	350	68	93
4	Ni-Mo/C	350	68	90

[1] Yield are based on response factor obtained on the GC

Higher temperatures and pressures were necessary with Ni-Mo catalysts to deaminate cyclohexylamine compared to Pt/SiO_2. These reactions with cyclohexylamine were conducted to establish that the catalysts were active. Attempted catalytic denitrogenation of α-amino-ε-caprolactam over Pt/SiO_2 in a U-tube resulted in the recovery of unreacted starting material. The low vapor pressure of the lactam prevented the use of the flow apparatus for deamination.

Batch hydrodenitrogenation was then evaluated with Mo-based catalysts and Pt/SiO_2. α-Amino-ε-caprolactam·HCl, α-amino-ε-caprolactam, L-lysine and methyl L-

lysine in glyme and diglyme were hydrodenitrogenated over Ni-Mo-S (20-40 mol%) under varying conditions of pressures (68-204 atm H_2) and temperatures (100-350 °C).

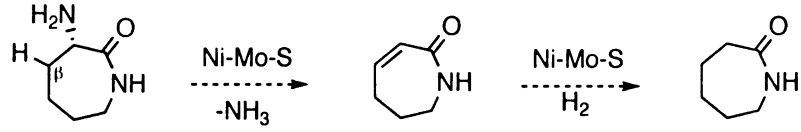


Figure 41. Hydrodenitrogenation of α-amino-ε-caprolactam.

The desired deamination to form ε-caprolactam was not observed. Instead, unreacted starting material was recovered. Oligomerization of α-amino-ε-caprolactam was observed when the temperature was increased beyond 160 °C even with thorough drying of α-amino-ε-caprolactam prior to deamination. A drying pistol was used to dry α-amino-ε-caprolactam with refluxing EtOAc.

Catalytic hydrogen transfer

Hydrogenolysis of the C-N bond has previously been achieved with catalytic hydrogen transfer. This method offers a viable alternative to selective hydrogenation in the presence of sensitive functionalities without the use of special apparatus. Several compounds such as ammonium formate, formic acid, phosphoric acid and cyclohexadiene may serve as a hydrogen source in the presence of metal catalysts like Pd or Ni.[21] Extension of this procedure using ammonium formate as the hydrogen source in methanol over Pd/C was conducted in an attempt to deaminate α-amino-ε-caprolactam (Figure 42).

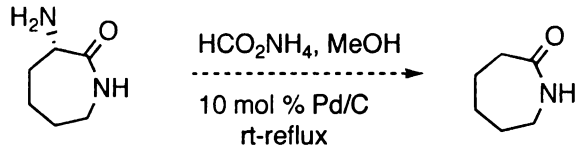


Figure 42. Catalytic hydrogen transfer.

The temperature was varied from rt to refluxing MeOH with an excess of hydrogen source. In all cases, unreacted starting material was recovered. Methylation of the amine in α-amino-ε-caprolactam with formaldehyde and hydrogen and subsequent hydrogenolysis did not yield the desired ε-caprolactam.[22]

Reductive Deamination

Samarium (II) iodine is widely used in organic chemistry as a single-electron reductant. This reagent was first introduced by Kagan and has been used extensively in reductive cleavage of α-heterosubtituted carbonyl substrates.[23] For instance, an important subgroup of compounds possessing a small ring adjacent to the carbonyl such as epoxy ketones and esters and cyclopropyl ketones have been reductively cleaved to afford ring-opened product.[24] Reductive cleavage of 2-acylaziridines has also been observed (Figure 43).[25]

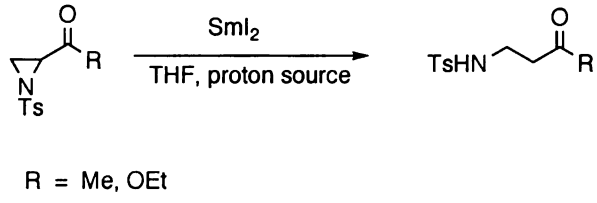


Figure 43. Reductive cleavage of acylaziridine with SmI_2.

This work was extended to the reductive deamination of a variety of amino acids with primary, secondary and tertiary amines.[26] Typically, a stoichiometric amount of SmI_2 and HMPA is required to effect the deamination. A proton source such as methanol, pivalic acid or *N,N*-dimethylaminoethanol (DMAE) is also present during the reaction. Since two electrons are required for the deamination, an excess of SmI_2 as well as hexamethylphosphamide are typically used. This reagent is generated *in situ* from the reaction of Sm powder and CH_2I_2.[27] Following a literature procedure, exploratory reductive deamination of methyl lysine ester with SmI_2 resulted in deamination to 6-amino-methyl-caproate in only 20% yields (Figure 44).

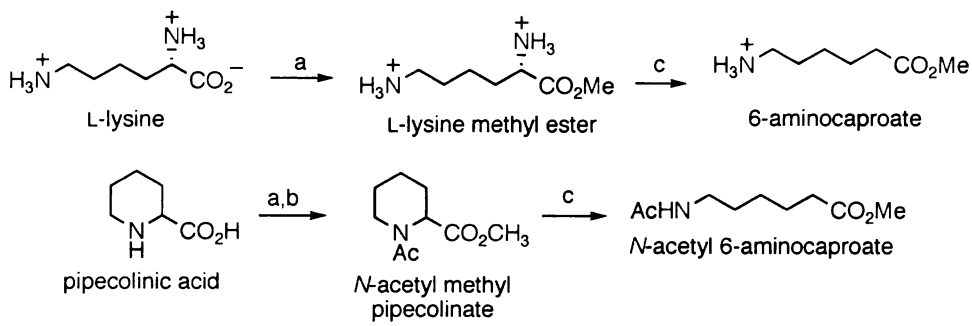


Figure 44. Reductive deamination of L-lysine derivatives with SmI_2. Key: (a) 2,2-dimethoxypropane, refluxing MeOH, HCl; (b) pyridine, Ac_2O; (c) 10 eq SmI_2-HMPA, MeOH, THF, rt.

Excess SmI_2 and a reaction time of 6 h was necessary to effect the deamination. If HMPA was not used, the reaction time was prolonged. This was contrary to the reported procedure in which *N*-acetyl methyl pipecolinate was reductively deaminated to *N*-acetyl methyl 6-aminocaproate in moderate yields (63%). Pipecolinic acid (65%) can be derived from L-lysine by oxidative deamination followed by hydrogenation in the presence of 5 mol% Ru on C at 100 psi H_2 and 200 °C.[15] Methylation of the carboxylate followed by acetylation of the amine resulted in *N*-acetyl methyl pipecolinate. Reductive

deamination of which in THF and MeOH required 10 equivalent of SmI_2. The limited solubility of L-lysine ester·HCl in MeOH was the source of limited deamination of this substrate. Obviating protection-deprotection of the L-lysine starting material is ideal if this process is to be used industrially for the synthesis of caprolactam. Since single electron transfer reaction with SmI_2 showed promising results, other inexpensive reductants were explored for the deamination of L-lysine, methyl L-lysine ester and aminocaprolactam (Figure 45, Table 7). Single-electron reducing agents, such as Li/NH_3, Na, K, Zn powder and activated Mg were investigated for deamination in MeOH, EtOH and *i*-PrOH (Figure 45).[28]

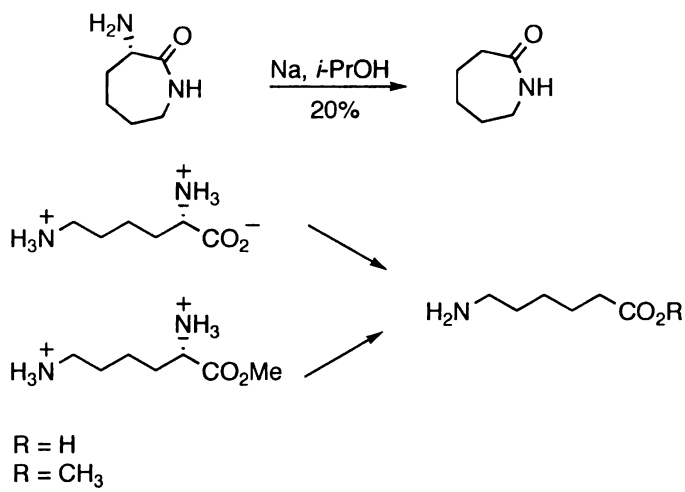


Figure 45. Reductive deamination with alkali metals.

Table 7. Reductive Deamination of L-Lysine and its Derivatives.

Reductant	Proton source	Temp (°C)
Na	*i*-PrOH	rt
Na	*i*-PrOH, EtOH	rt
K	*i*-PrOH	-10
Zn	*i*-PrOH, NaOH	reflux
Mg	*i*-PrOH	reflux
Li	NH_3	-78

Typically, small pieces of the reductant metal were added to a stirring solution of the substrate in alcoholic solvents. When MeOH and EtOH were used as solvents, respective alkoxides were formed and reductive deamination was not observed. Due to the lack of solubility of L-lysine and L-lysine methyl esters in alcoholic solvents, attention was focused on reductive deamination of α-amino ε-caprolactam in *i*-PrOH. Unreacted starting material was recovered when Li/NH_3 or refluxing *i*-PrOH with Zn or Mg were used. Addition of K metal to a solution of α-amino ε-caprolactam in *i*-PrOH was rather violent and resulted in degradation of α-amino ε-caprolactam to unknown products. Reductive deamination was observed with Na metal in *i*-PrOH to afford the desired α-amino ε-caprolactam in 20% as observed by ^1H NMR. When the reaction was allowed to stir long after addition of Na, the mixture thickened. The reaction was quenched by addition of MeOH with 2.5% HCl. Half of the unreacted α-amino ε-caprolactam was hydrolyzed to L-lysine and L-lysine ester.

Isomerization of L-lysine to β-L-lysine

With limited success in deaminating the α-amino group of L-lysine, a new strategy in which the amine group was made more susceptible to elimination by isomerization of L-lysine to β-L-lysine was targeted. In this approach, the synthesis of ε-caprolactam from L-lysine exploited an enzymatic reaction exploited by *Clostridium* species during fermenation of L-lysine.[29] In the first step of L-lysine fermentation, the α-amino group is isomerized to the β-position by L-lysine-2,3-aminomutase (Figure 46).[30]

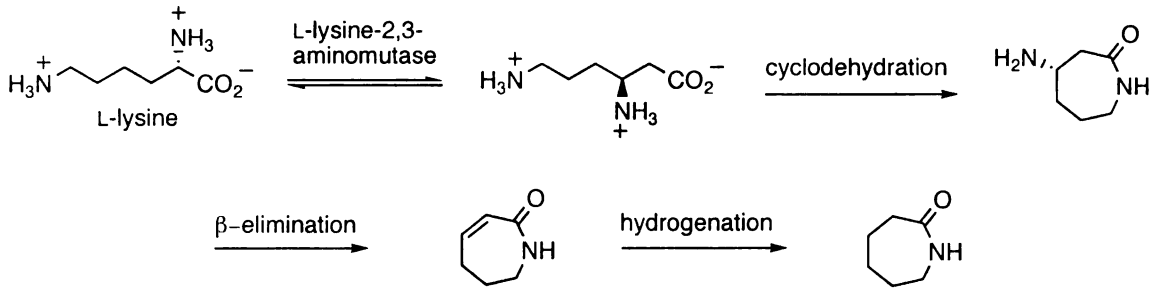


Figure 46. Synthesis of caprolactam from L-lysine via L-β-lysine.

In order to deaminate the secondary amine, the ε-amino group was masked as an amide by cyclization to β-amino ε-caprolactam. It was anticipated that the resulting L-β-lysine would be deaminated to α,β-unsaturated moiety with relative ease compared to L-lysine, which would then afford the desired ε-caprolactam after hydrogenation.

Fermentation of L-lysine

Amino acids can serve as major energy sources for selected species under anaerobic conditions.[31] The fermentation always involves simultaneous oxidation and reduction of a single amino acids or different amino acids. Oxidation reactions in anaerobic bacteria (oxidative deamination, transamination and α-keto acid oxidation) are analogous to the reactions that occur in aerobic bacteria, except for the absence of molecular oxygen and oxidants with high potential.[31,32] The reduction step however, is unique since suitable electron acceptor(s) of appropriate potential such as α- and β-keto acids, α,β-unsaturated acids or their CoA thioesters and protons must be generated by the anaerobe. Reduction of these substrates afford short fatty acid chains, succinic acid, δ-aminovaleric acid and molecular hydrogen.[31]

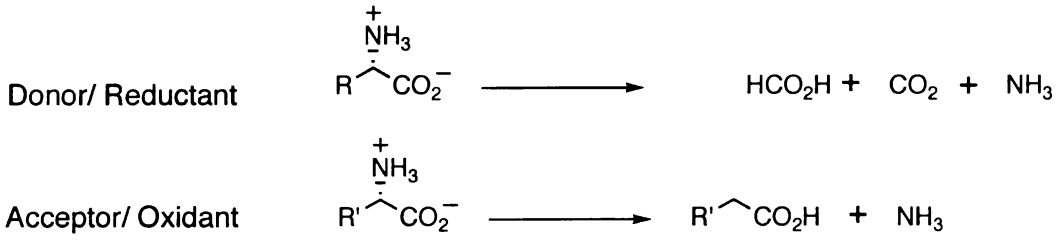


Figure 47. Strickland reaction of amino acids.

In cases where a single amino acid cannot serve as both the oxidant and reductant, certain pairs of amino acids are coupled in a redox reaction to allow for ATP generation (Strickland reaction) in *Clostridium* species (Figure 47).[29] Not all amino acids can serve as donor or acceptor in a Strickland reaction. Branched amino acids typically serve as donors while aromatic amino acids and glycine can function as both electron acceptor and donor.[31] In *Clostridium sporogenes*, ornithine serves as an electron donor and forms 5-aminovalerate via D-proline. In this organism, proline is preferred as an electron acceptor over glycine whereas glycine inhibits proline reduction in *Clostridium sticklandii*.[33] L-Lysine, its ornithine homologue, is metabolized by completely different pathways. In *C. sticklandii* and *C. subterminale* L-lysine is degraded to acetate, butyrate and ammonia via two pathways.

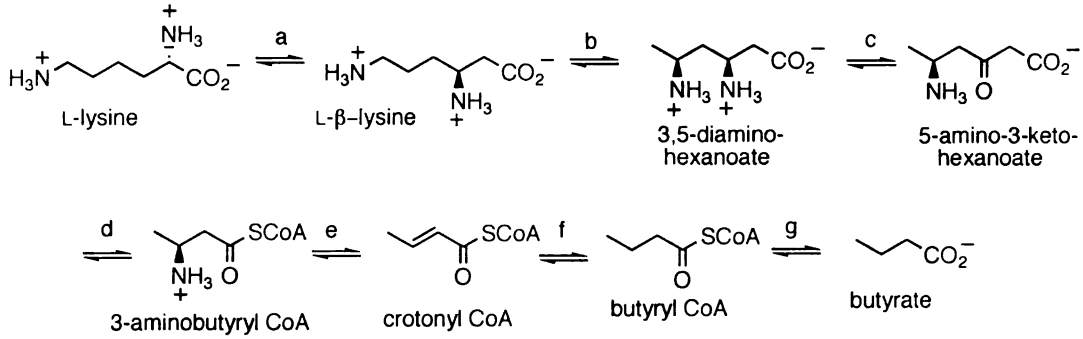


Figure 48. Degradation of L-lysine in *Clostridium subterminale*. Key: (a) L-lysine-2,3-aminomutase; (b) β-L-lysine-5,6-aminomutase; (c) oxidative deamination; (d) 3-keto-5-aminohexanoate cleavage enzyme; (e) 3-aminobutyrl CoA deaminase; (f) reductase; (g) hydrolysis.

Degradation of L-lysine is an example of a single amino acid serving as both an oxidant and reductant with concomitant generation of ATP (Figure 48). In *C. subterminale*,[31] L-lysine is first isomerized to L-β-lysine by pyridoxal 5-phosphate dependent L-lysine-2,3-aminomutase (Figure 48).[34] This is followed by migration of the terminal amino group to position 5 catalyzed by cobalamide coenzyme-dependent L-β-lysine-5,6-aminomutase.[35] The resulting intermediate, 3,5-diaminohexanoate, is poised to undergo oxidative deamination at position 3 to afford 5-amino-3-ketohexanoate. This oxidation is catalyzed by L-*erythro*-3,5-diaminohexanoate dehydrogenase.[36] By forming the 5-amino-3-ketohexanoate intermediate, spontaneous cyclization is avoided. Subsequent cleavage with acetyl CoA catalyzed by 3-keto-5-aminohexanoate cleavage enzyme results in L-3-aminobutyryl-CoA (C3-C6 of lysine) and acetoacetate formation from acetyl CoA. This reaction is said to occur by a concerted mechanism since neither CoA nor intermediates could be detected by several group exchange reactions.

L-3-Aminobutyryl-CoA is reversibly deaminated to crotonyl-CoA and ammonia by 3-aminobutyrl CoA deaminase.[37] Reduction of crotonyl CoA to butyryl CoA, which then transfer its CoA moiety to acetoacetate to afford butyrate and acetoacetate CoA is catalyzed by CoA transferase. Finally, thiolase-catalyzed cleavage of acetoacetate CoA results in two moles of acetyl CoA. One molecule of acetyl CoA participates in the 5-amino-3-ketohexanoate-cleavage reaction while the second one is used to form ATP and acetate via the phosphate acetyltransferase and acetate kinase reactions. Alternatively, crotonyl CoA is converted to 3-hydroxybutyryl CoA by crotonase, which is oxidized to acetoacetate CoA by action of 3-hydroxybutyryl CoA dehydrogenase.[31] This pathway provides additional NADH and ATP that may be needed for biosynthetic reactions.

The first step in the degradation of L-lysine in *C. sticklandii* is initiated by a racemase, which converts L-lysine to D-lysine. Migration of ε-amino group to C-5 is catalyzed by a B_{12}-dependent D-lysine-5,6-aminomutase. Subsequent degradation of the resulting 2,5-diaminohexanoic acid results in butyrate derived from C1-C4 of D-lysine and acetate.[29]

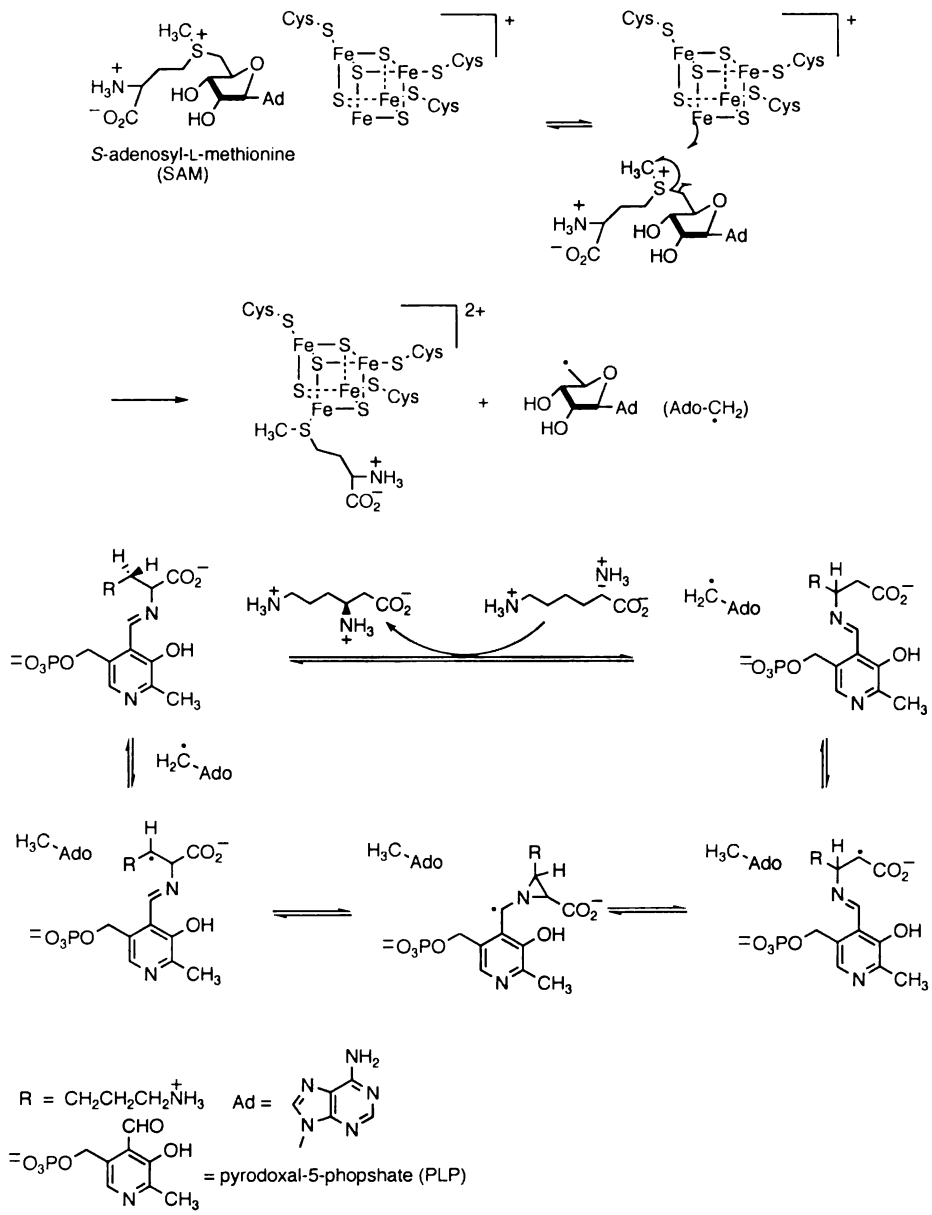


Figure 49. Isomerization of L-lysine to L-β-lysine catalyzed by L-lysine-2,3-aminomutase.

L-Lysine-2,3-aminomutase, which catalyzes the first step in lysine catabolism in *C. subterminale* is a well-characterized enzyme. It possesses an iron-sulfur cluster, [4Fe-4S], and is *S*-adenosylmethionine (SAM) and pyridoxal 5-phosphate (PLP) dependent.[38] The isomerization is initiated by the adenosyl radical, which is generated by homolytic cleavage of *S*-adenosylmethionine by the iron-sulfur cluster (Figure 49).[39] The resulting 5'-deoxynosine-5'-yl abstracts a proton from carbon 3 of the imine formed between PLP and L-lysine. The resulting substrate-related radical imine undergoes a 1,2-imino shift via an azacyclopropylcarbinyl radical intermediate to afford a product-related radical imine. Finally, the product-related radical imine abstracts hydrogen from 5'-deoxyadenosine to produce L-β-lysine and regenerate 5'-deoxyadenosyl radical (Figure 49).[40]

The L-lysine-2,3-aminomutase gene locus (*kamA*) from C. *subterminale* SB4 has previously been overexpressed in *E. coli*.[40] The construct *E. coli* BL21(DE3)/pAF-80/*kam*A was cultured under anaerobic conditions following literature procedure.[41] Cells were harvested by centrifugation, lysed by passage through a French press (12,000 psi). After removal of cellular debris, the extract was kept under N_2 and sodium dithionite was added to scrub O_2. Pyridoxal 5-phosphate and glutathione were added to activate L-lysine-2,3-aminomutase prior to addition of *S*-adenosylmethionine and dithionite to initiate the isomerization of L-lysine to β-L-lysine (Figure 50).[41]

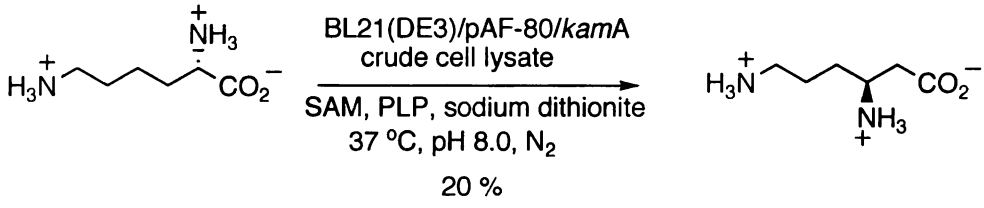


Figure 50. Bioconversion of L-lysine to β-L-lysine catalyzed by *E. coli* BL21(DE3)/pAF-80/*kam*A.

The enzyme was unstable as measured by the specific activity, which dropped from 0.02 U/mg to undetectable levels within 1 h. The isomerization of L-lysine ceased after a 20% conversion of starting material. Crude extracts of L-lysine-2,3-aminomutase from *C. subterminale* was explored in search of better conversion.[30,41]

Clostridium SB4 was obtained from American Type Culture Collection (ATCC 29748). It was grown in a medium containing L-lysine, yeast extract, sodium dithionite, K_2CO_3 and phosphate buffer previously purged with N_2. The cells were cultured in 5 L Pyrex bottle at 37 °C for 3-5 h (A_{600} ~ 0.5) for experiments with cell crude extracts and 12–18 h (A_{600} ~ 2.0-3.2) for experiments with intact cells. Manipulations were conducted in a Coy chamber to avoid exposure of cells to O_2. Cells were harvested by centrifugation and used without washing. The yield of wet, packed-cells was 0.4-0.7 g/L from a 5 h cultures and approximately 2-4 g/L from 18 h cultures.

Bioconversion with crude extracts of L-lysine-2,3-aminomutase

Cell extracts were prepared by suspending the cell pellet in phosphate buffer (0.1 M, pH 7.5) and treated with a 60-watt ultrasonic homogenizer for 10 minutes or by passage through a French press (12,000 psi). After removal of cell debris, the protein concentration was between 8-15 mg/mL. A 10 mL reaction mixture contained 60 mM L-lysine, 25 mM tris-HCl (pH 7.5), 5 mM potassium phosphate (pH 7.5), 10 mM α-ketoglutarate, 10 mg pyridoxal-5-phosphate, 0.2 mM CoA, 0.2 mM *S*-adenosylmethionine, 5-20 mM glutathione, and 10 mg of protein.

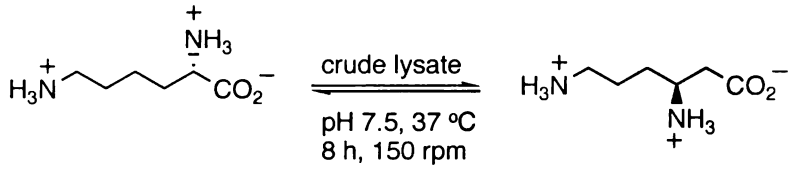


Figure 51. Bioconversion with crude extracts of L-lysine-2,3-aminomutase.

Table 8. Bioconversion of L-lysine to β-L-lysine Using Crude Lysate.

Entry	Additives	L-lysine (%)	L-β-lysine (%)
1	none	100	0
2	α-ketoglutarate, CoA, glutathione	11	77
3	PLP, CoA, glutathione	15	75
4	PLP, SAM, glutathione	17	68
5	α-ketoglutarate, SAM, glutathione	24	64
6	PLP, glutathione	67	21
7	α-ketoglutarate, glutathione	61	28

[1] Yields were determined by ^{1}H NMR.

The reaction mixture was incubated at 37 °C and reached equilibrium after 8 h, at which point it was acidified to pH 3 to precipitate the protein. The supernatant was dried and submitted for ^{1}H NMR (Figure 51, Table 8). Contrary to the enzyme heterologously expressed in *E. coli*, which only gave 20% of the L-β-lysine, crude extracts resulted in moderate isomerization of L-lysine. PLP or ketoglutarate could be interchanged without decreasing the yield (Entry 2-6). However, in the absence of CoA or SAM the yield of L-β-lysine was decreased (1, 6 and 7). Although crude extracts have reportedly been used for the preparative synthesis of L-β-lysine, this route has several disadvantages. When experiments employing crude lysates were conducted with cells harvested after 18 h, isomerization of L-lysine to L-β-lysine was greatly reduced. Because the [4Fe-4S] cluster is deactivated during preparation of crude extracts, activation of the enzyme prior to bioconversion is required. The need to replenish the crude extracts with PLP, SAM and glutathione would require large quantities of these expensive reagents to be added.

Furthermore, it was also necessary to freeze the crude extracts for 2-14 days prior to use. Presumably, heat shock also deactivates L-β-lysine-5.6-aminomuatse. These factors forced us to pursue use of intact of *Clostridium* SB4 cells for the preparative synthesis of L-β-lysine from L-lysine.

Bioconversion with intact cells of *Clostridium subterminale*

In order to accumulate L-β-lysine with intact cells in solution, L-β-lysine-5,6-aminomutase had to be deactivated (Figure 48b). Unlike L-lysine-2,3-aminomutase, L-β-lysine-5,6-aminomutase is not a [4Fe-4S] enzyme. It is a cobalamine dependent enzyme.[35] Therefore it could be selectively deactivated without affecting L-lysine-2,3-aminomutase. *C. subterminale* cells were cultured and harvested after 18 h. After cells were harvested by centrifugation, they were resuspended in a reaction mixture containing 50 mM L-lysine, 80 mM Tris-HCl (pH 7.5), 5 mM potassium phosphate (pH 7.5), and $FeSO_4$ (3 mM). The bioconversion mixture contained 1.4-2.1 g of cells in 100 mL. The reactions were incubated at 37 °C in 250 mL Pyrex bottles, which were flushed with N_2 and sealed with screw caps. Variation in the amount of $FeSO_4$ was adjusted to optimize the yield of L-β-lysine. The Pyrex bottles were wrapped with aluminum foil to prevent exposing cells to light. Alternatively, cells were exposed to intense light from a tungsten lamp irradiation in a Pyrex reaction bottle for 12 h.

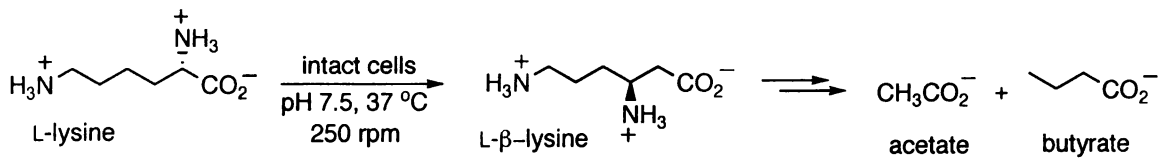


Figure 52. Bioconversion of L-lysine to L-β-lysine using intact *C. subterminale*.

Table 9. Bioconversion of L-lysine β-L-lysine using intact *C. subterminale.*

Entry	$FeSO_4$ (mM)	light	L-lysine (%)[1]	L-β-lysine (%)[1]	Acetate & butyrate (%)[1]
1	0	no	20	0	80
2	3	no	trace	trace	90
3[2]	3	yes	25	60	0
4	3	yes	50	33	15
5	10	yes	>99	0	0
6	3	yes[3]	trace	>95	0

[1] Yields were estimated by ^1H NMR integrations. [2] glutathione was used instead of sodium dithionite. [3] Exposure to light for 24 h.

In the absence of light and $FeSO_4$, 80% of L-lysine was converted to acetate and butyrate (Entry 1). However, addition of $FeSO_4$ in the absence of light resulted in an approximately 100% conversion of L-lysine to acetate and butyrate without the accumulation of L-β-lysine (Entry 2). When cells were exposed to intense light for 12 h, accumulation of L-β-lysine increased at the optimum $FeSO_4$ concentration (Entry 3). Glutathione was effective in keeping the reduced atmosphere and resulted in the higher yield of L-β-lysine than when dithionite was used (Entry 4). A high concentration of $FeSO_4$ inhibited the bioconversion of L-lysine completely (Entry 5). Allowing the reaction to proceed for 24 h resulted in the complete conversion of L-lysine to L-β-lysine (Entry 6). Acetate and butyrate were not observed but there was a doublet centered at 1.2 ppm, which presumably belongs to 3,5-diaminobutyrate (Figure 48). The bioconversion has successfully been scaled-up such that cells harvested from 10 L cultures can convert L-lysine (200 mM) to L-β-lysine (180 mM) within 24 h in 500 mL. L-β-Lysine has been purified from unreacted L-lysine using Dowex 50 (H^+). Sodium formate buffer (0.2 M, pH 2.75) containing NaCl (0.35 M) was used to differentially elute L-lysine and L-β-lysine, with the former eluting first followed by L-β-lysine. L-β-Lysine was then loaded on the second Dowex 50 (H^+) column, washed with water to remove sodium formate. L-

β-Lysine was eluted with 1 N NH_4OH and the eluant was concentrated under vacuum to afford 43% of L-β-lysine based on starting materials. Cyclization of L-β-lysine to β-aminocaprolactam in EtOH at 200 °C was shown to proceed to completion. Preliminary studies showed 13% of caprolactam could be obtained when L-β-lysine in EtOH was heated to 200 °C in the presence of Ru on Al_2O_3 at 1,000 psi H_2. The rest of the starting material was degraded to unidentified products. Detailed evaluations of catalysts and deamination conditions need to be explored.

As a slight departure from chemoenzymatic synthesis of ε-caprolactam from L-lysine, deamination of L-lysine and its derivatives in the presence of mercaptans was also explored. This route was inspired by the mechanism of D-proline reductase, which catalyzes reductive deamination of D-proline to 5-aminovalerate (Figure 53).[42] Presumably, the sulfide anion can replace a hydroxyl group adjacent to the ketone.[43]

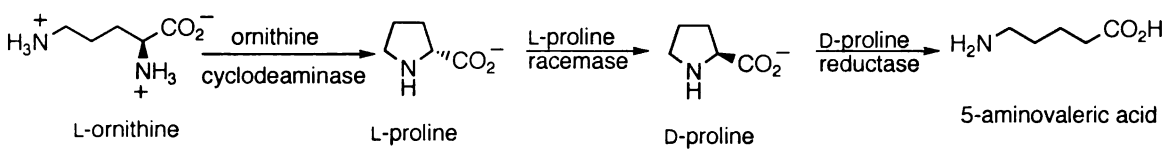


Figure 53. Reductive deamination of L-ornithine to 5-aminovalerate in *Clostridium sticklandii.*

A typical reaction mixture contained methyl L-pipecolinate ester or α-aminocaprolactam in either 1,2-ethylene dithiol or 1,3-propylene dithiol as a solvent in the presence of tetrabutylammonium fluoride (TBAF) or tetraammonium chloride (TBACl).[44] The reaction temperature was increased from rt to refluxing dithiol and resulted in the recovery of unreacted starting materials. Replacement of TBAF with

$ZnCl_2$ or acetylating the amine group of the starting material did not result in any deamination.

DISCUSSION

The recent surge in benzene prices due to high demand and limited supply of benzene had a direct impact on the price of nylon 6 and other polyamides.[7] This development is not surprising because current commercial syntheses of ε-caprolactam utilizes benzene as the main starting material. Synthesis of ε-caprolactam from 1,3-butadiene has also been described. The ultimate source of carbon for all syntheses of ε-caprolactam is petroleum. D-Glucose-derived L-lysine, on the other hand, provides for a renewable carbon source for ε-caprolactam synthesis. L-Lysine is inexpensive and abundant.

Deamination of L-lysine to ε-caprolactam with hydroxylamine-*O*-sulfonic acid under basic conditions is stoichiometric. Catalytic deamination of the α-amino group surveyed with Mo-Ni-S, Pt/SiO_2 and catalytic hydrogen transfer were unsuccessful. Successful deamination of cyclohexylamine with both Mo-Ni-S and Pt/SiO_2 proved that the catalysts employed during those reactions were active. The activity of Ni-Mo-S catalyst was not responsible for the lack of deaminated product. Probably, Hoffmann elimination of the α-amine is not thermodynamically favorable since it requires abstraction of a β-hydrogen, which is not acidic (Figure 39). Similarly, nucleophilic substitution at the α-carbon of α-aminocaprolactam was not favored. It has been reported that C-N bond cleavage starts by insertion of the catalyst between α-C-H to form the σ-complex or the α,β-adsorbed intermediate (Figure 54).[20]

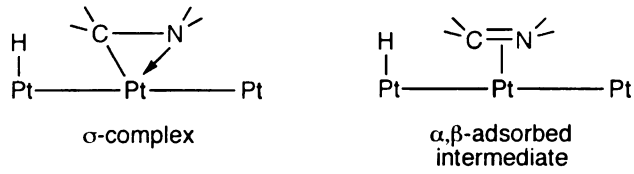


Figure 54. Intermediates formed during C-N hydrogenolysis.

Typically, high temperatures are required to effect C-N hydrogenolysis. Under these forcing conditions on the metal surface, C-C bond cleavage competes with C-N bond cleavage. Presumably, the becomes less basic on the metal surface making chemisorbed N and C to behave similarly, which, in turn, accounts for the lack of selectivity between C-C and C-N hydrogenolysis at high reaction temperatures. The fact that C-C and C-N bond cleavage products were not observed with Mo-Ni and Pt catalysts could mean that α-aminocaprolactam did not form the presumed complexes (Figure 54). High pressures employed during catalytic hydrodenitrogenation may have potentially inhibited deamination of α-aminocaprolactam. Unpublished results in the Frost research groups have shown that catalytic deamination of α-aminocaprolactam over Pt/C at 50 psi H_2 resulted in a 23% caprolactam and the yield was reduced to 12% when H_2 pressure was increased 100 psi H_2. Catalytic transfer hydrogenation did not result in deamination of α-aminocaprolactam.

Single electron reduction with SmI_2 resulted in the deamination of the α-amino-group of L-lysine methyl ester and *N*-acetyl methyl pipecolinate. The low yield observed with the L-lysine methyl ester can be attributed to its limited solubility in THF-MeOH solvent.

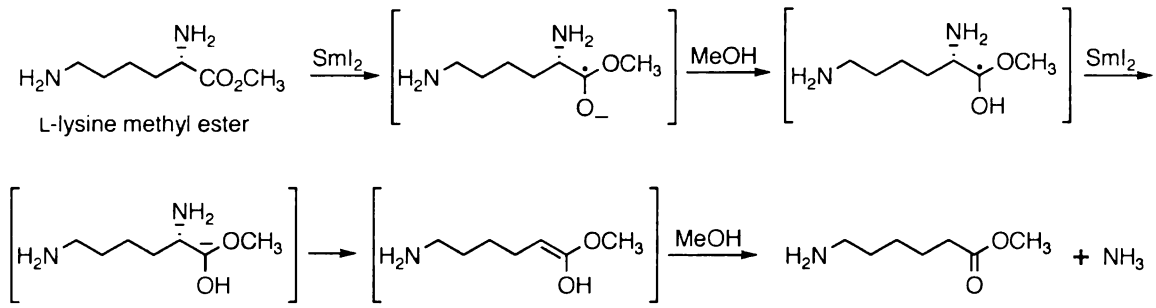


Figure 55. Reductive deamination of L-lysine methyl ester with SmI_2.

Acetylation of the pipecolinate ester improved the yield of the deaminated product by 40%. The expected mechanism for deamination of an amine adjacent to a carboxyl involves the reaction of the carbonyl with SmI_2 to afford a radical anion, which is rapidly protonated MeOH (Figure 55).[45] The second equivalent of SmI_2 reduces the protonated radical to afford a carbanion, which is followed by elimination of the amine. Tautomerization of the intermediate enol then leads to the observed product. The same number of equivalents of HMPA as SmI_2 are required because HMPA serves as a chelator of Lewis acidic Sm(III) species formed during deamination. Although a catalytic reductive deamination with SmI_2 is known in the presence of misch metal, the need to protect the amines is not appealing for large-scale synthesis of ε-caprolactam. Misch metal is an alloy of light lanthanides (La 33%, Ce 50%, Nd 12%, Pr 4%, Sm and other lanthanides 1%) and is available at a low cost.[46] When this alloy is used, catalytic amount of SmI_2 (0.2 eq, 10 mol %) has been effective as a single electron reductant.

Reductive deamination of a 125 mM solution α-aminocaprolactam in *i*-PrOH with Na afforded ε-caprolactam in only 20% yield. Formation of sodium isopropoxide was responsible for consumption of Na. Circumventing protection of the α-amine group

makes this route more viable than reductive deamination with SmI_2. However, the reaction is stoichiometric with respect to Na.

Is the deamination of L-lysine via a ketene a possible route for the synthesis of caprolactam (Figure 56)?[47]

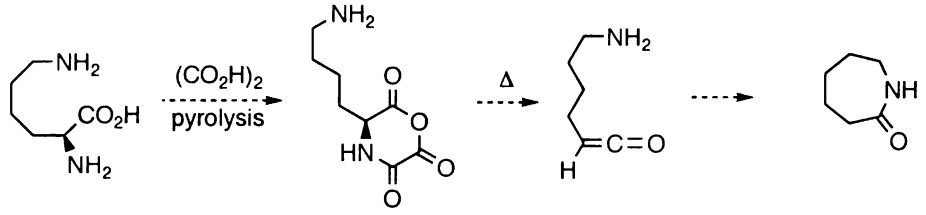


Figure 56. Hypothesized deamination of L-lysine to caprolactam via a ketene.

Hypothetically, the α-amino acid can be simultaneously protected with oxalic acid, which would form an amide and a mixed anhydride with the amine and carboxylic acid, respectively. Decarboxylation would then result in a 6-aminocaproketene along with formamide, which would cyclize to afford caprolactam.

As an alternative strategy to accomplish elimination, L-lysine was isomerized to β-L-lysine. Unlike L-lysine, the α-H in β-L-lysine is acidic and after abstraction would presumably lead to α,β-unsaturated 6-amine hexanoate via α-carbanionic species (Figure 57).

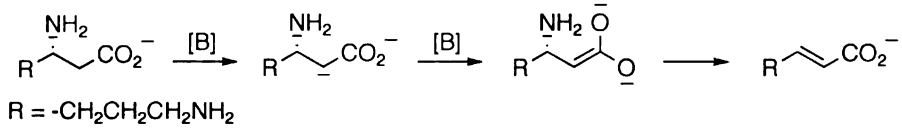


Figure 57. Deamination of β-amino group from β-L-lysine.

The chemical synthesis of β-L- does not utilize L-lysine as the starting material, which is the key goal for the synthesis of caprolactam based on starting materials derived from renewable feedstocks.[48] By recruiting L-lysine-2,3-aminomutase from *Clostridium*

subterminale, an unprecedented preparative synthesis of β-L-lysine was accomplished. Reported syntheses of β-L-lysine employed crude extracts. Approximately, 100 mg of protein is required to convert 100 mg L-lysine to 77 mg of L-β-lysine under optimum conditions. This much protein is obtained from 12 L of cultures incubated for 5 h. Use of crude extracts from stationary phase (12-18 r incubation period) results in a 10-15 % yield of desired L-β-lysine. In addition, additives are imperative for the bioconversion in crude cell lysates. α-Ketoglutarate and PLP are required to make the imine with the lysine whereas SAM is required to generate the 5'-deoxyadenosyl radical necessary to initiate the 1,2-migration reaction of the amino group. Glutathione or mercaptoethanol is required to reduce the iron cluster. This implies that scaling up the isomerization in crude cell lysate would also requires scaling-up of these expensive additives.

Deactivation of the second enzyme in L-lysine biodegradation, L-β-lysine-5,6-aminomutase, was key in accumulating L-β-lysine using intact cells. Fortunately, this enzyme could be differentiated from L-lysine-2,3-aminomutase because it is a cobalamine-dependent enzyme. It has been reported that exposure to intense light inactivate cobalamide-dependent enzyme activity and especially L-β-lysine-5,6-aminomutase.[49] Nitrous oxide, charcoal as well as ornithine, which is a competitive inhibitor, have also been shown to inhibit L-β-lysine-5,6-aminomutase.[50] Intense light in the presence of $FeSO_4$ was effective in blocking L-β-lysine-5,6-aminomutase and resulted in the accumulation of L-β-lysine. The presence of Fe^{2+} is necessary because it is required for the biosynthesis of the [4Fe-4S] cluster of L-lysine-2,3-aminomutase. Irradiation with light (E = 52 kcal/mol for a wavelength of 550 nm) irreversibly

deactivated the L-β-lysine-5,6-aminomutase presumably by cleaving the Co-C, which has been reportedly weak (25-40 kcal/mol).[51]

The advantage of using intact cells instead of crude cell lysate is the elimination of additives such as PLP and SAM. By allowing the bioconversion to proceed for 24 h, a 100% conversion of L-lysine was observed. High concentrations of $FeSO_4$ inhibited the intact cell bioconversion completely. Freezing of cells *Clostridium* SB4 cell at -20 °C overnight improved their ability to catalyze isomerization of L-lysine to L-β-lysine. This heat-shock presumably inactivated the L-β-lysine-5,6-aminomutase. Previous reports showed that the crude cell lysate used for preparative synthesis of L-β-lysine was heated to 55-70 °C to precipitate unwanted proteins.[52] This would suggest that frozen intact cells of *Clostridium* SB4 should be able to catalyze isomerization without irradiation of L-lysine to L-β-lysine. This avenue remains to be pursued along with systematic evaluation of the freezing-thawing cycles of *Clostridium* SB4 at -20 °C or -80 °C. Activity of intact cells to catalyze the isomerization of L-lysine to L-β-lysine with and without irradiation can then be evaluated. Irradiation of intact cells at various wavelengths can also be explored to obtain maximum inactivation of the L-β-lysine-5,6-aminomutase.

When *Clostridium* SB4 cells were cultured in a medium containing more than 18 g/L of L-lysine, the cell mass declined indicating the toxicity of acetate and butyrate to the cells. To overcome this limitation for large-scale production of L-β-lysine from L-lysine, plasmid-localized *kamA* gene encoding L-lysine-2,3-aminomutase could be expressed in *Clostridium subterminale*. The resting cells could then be harvested and used to catalyze the isomerization of L-lysine to L-β-lysine in large quantities.

Six homologous proteins of unknown functions have been revealed to have a sequence identity of 37-72% to L-lysine-2,3-aminomutase from *Clostridium subterminale*. These prokaryotic organisms include *Porphyromonas gingivalus*, *Bacillus subtilis*, *Deinococcus radiodurans*, *Aquiflex aeolicus*, *Treponema pallidum*, *Haemophilus influenza*, and *E. coli*. The *B. subtilis* protein, YodO, is 62% identical to *C. subterminale* L-lysine-2,3-aminomutase. The *yodO* gene has been cloned from the *B. subtilis* and heterologously expressed in *E. coli*.[53] The resulting protein was reported to catalyze the isomerization of L-lysine to L-β-lysine under anaerobic conditions as well. Unlike the *Clostridium* LAM, *Bacillus* was found to be stable in air and was not irreversibly deactivated. Attempts to heterologoulsy express the *yodO* gene in *E. coli* did not result in a viable construct. Intact cells of *Bacillus subtilis* were never explored for the conversion of L-lysine to L-β-lysine during this study. Given the activity of *Clostridium* SB4 to catalyze the isomerization reaction, exploring intact cells of *Bacillus subtilis* would be valuable.

Enzyme-catalyzed β-elimination of L-β-lysine followed by enzymatic reduction is another avenue that can be explored in addition to chemical deamination. Within the degradation pathway of L-lysine in *Clostridium* (Figure 48), L-β-amino-butyryl CoA deaminase catalyzes β-elimination of L-β-amino-butyryl CoA to an enoate CoA. Substrate analogues of L-β-amino-butyryl CoA have never been explored (Figure 58). L-β-Lysine or its cyclic analogue can be evaluated using this L-β-amino-butyryl CoA. This enzyme has previously been purified from *Clostridium subterminale*.

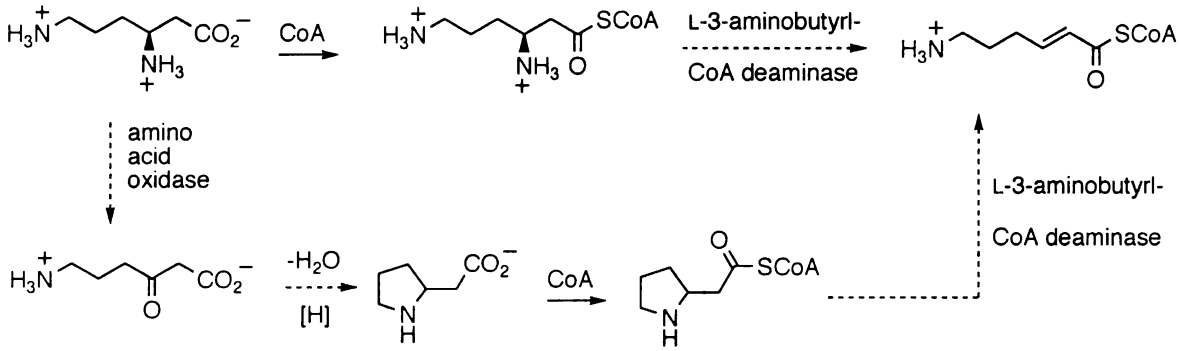


Figure 58. L-β-Amino-butyryl CoA deaminase catalyzes β-elimination.

D-Proline reductase, which is found in *Clostridium sticklandii* catalyzes C-N bond cleavage of D-proline to 5-aminovalerate (Figure 59).

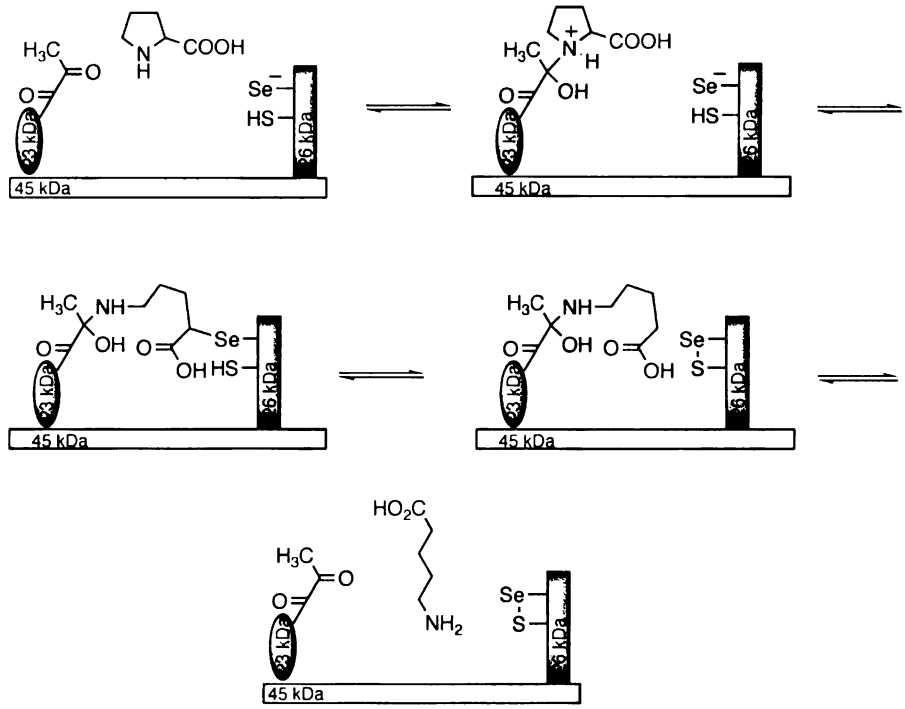


Figure 59. Reductive deamination of D-proline to 5-aminovalerate catalyzed by D-proline reductase.

This enzyme contains three subunits with molecular masses of 23, 26 and 45 kDa.[95] The 23 kDa subunits contains a pyruvoyl moiety which binds the substrate. The 26 kDa subunits houses the selenocysteine moiety (Figure 58).[95c] An enzyme-bound

pyruvoyl group first activates the L-ornithine-derived D-proline substrate. Nucleophilic attack of the α-carbon by the selenol anion of the selenocysteine results in the cleavage C-N bond of proline. Subsequent hydrolysis of the resulting intermediate gives 5-aminovalerate. With the advent of molecular evolution, reductive deamination of L-lysine-derived D-pipecolinate to 6-aminocaproic acid, which can then be cyclized to ε-caprolactam is not a far-fetched idea (Figure 60). L-Pipecolinate reductase is not a known enzyme but L-proline reductase has been purified and characterized.

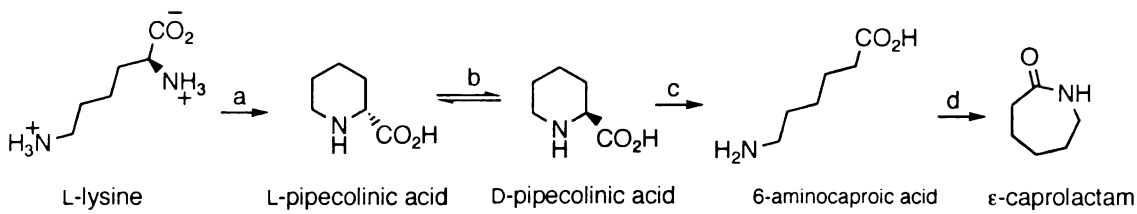


Figure 60. Hypothesized microbial syntheses of 6-aminocaprolactam from L-lysine. Key: (a) L-Lysine cyclase; (b) racemase; (c) L-pipecolinate reductase; (d) EtOH, 200 °C.

Production of L-lysine by *Corynobacterium glutamicum* proceeds under aerobic conditions while its isomerization to L-β-lysine in *Clostridium* proceeds under anaerobic conditions. Given this challenge, a single microbe capable of converting D-glucose to L-lysine may seem unlikely. However, the Frey group has successfully overexpressed L-lysine-2,3-aminomutase in *E. coli*. The resulting construct was cultured under aerobic conditions and expression of L-lysine-2,3-aminomutase was induced by degassing the media to remove O_2. A similar strategy can be adopted for the production of L-β-lysine from D-glucose.

The use of intact cells for the preparative synthesis of L-β-lysine is unmatched. This route relies solely on D-glucose-derived L-lysine as the starting material, without the

need for cofactors. β-Elimination of the β-amino group in the 7-membered ring lactam derived from cyclization of L-β-lysine, followed by hydrogenation hold a promising process for the production of ε-caprolactam from renewable starting materials. L-β-Lysine is not commercially available yet, this route can be a potential source of L-β-lysine.

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CHAPTER 4

EXPERIMENTAL

GENERAL METHODS

General chemistry

All air and moisture sensitive reactions were carried out in oven and/or flame-dried glassware under positive argon pressure. Air or moisture sensitive reagents and solvents were transferred to reaction flasks fitted with rubber septa via syringes or cannula. Solvents were removed using either a Büchi rotary evaporator at water aspirator pressure or under high vacuum. Hydrogenations were performed in the Parr 4575 stainless steel high temperature-high pressure reactor equipped with the Parr 4842 temperature controller, which controlled the temperature and stirring rate.

Reagents and solvents

Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium benzophenone ketyl. Dichloromethane, pyridine, triethylamine and benzene were distilled from calcium hydride under nitrogen. Methanol and hexanol were distilled from sodium metal under argon and stored over Linde 4 Å molecular sieves under argon. DMF, DMSO, hexanes and acetone were dried over activated Linde 4 Å molecular sieves under nitrogen. Water was glass distilled and deionized. Charcoal (Darco® G-60 ~ 100 mesh) was used for discoloration of solutions. All reagents and solvents were used as available from commercial sources or purified according to published procedures. Organic solutions of products were dried over anhydrous $MgSO_4$. All chemicals were

purchased from Aldrich. Ammonium molybdate and nickel nitrate were purchased from Strem. Sodium salt of 3-(trimethylsilyl)-propionic*2,2,3,3-d*$_4$ acid (TSP) was purchased from Lancaster Synthesis Inc. Misch metal was purchased from Alfa Aesar. 3-Hydroxy-γ-butyrolactone, 2-hydroxy-γ-butyrolactone, methyl malate were synthesized from malic acid following a literature procedure.[x] D-Erythronolactone and L-threonolactone were synthesized from D-isoascorbic acid and L-ascorbic acid using a modified procedure. Diazomethane was generated from Diazald® following a literature procedure. *S*-Adenosylmethionine was purified on a (1.2 cm x 6 mL) carboxymethylcellulose (CM) column. The CM column was equilibrated with acetate buffer before 10 mL of 10 mg/mL of *S*-adenosylmethionine iodide was loaded to the column. The column was washed with 6 mL of acetate buffer before eluting with 40 mM HCl. 0.6 mL fractions were collected and those with an absorbance maxima at A_{260} were pooled and the concentration was determined. SAM was frozen at –20 °C.

Chromatography

Radial chromatography was carried out on a Harrison Associates Chromatotron using 1, 2 or 4 mm layers of silica gel 60 PF_{254} containing gypsum (E. Merck). Silica gel 60 (40-63 μm, E. Merck or Spectrum Chemicals) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized pre-coated plates of silica gel K6F 60 Å (0.25 mm, Whatman). TLC plates were visualized by immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% acetic acid and 2.5% anisaldehyde) or phosphomolybdic acid stain (7% 12-molybdophosphoric acid in ethanol, w/v) followed by heating. Amino acids were visualized by staining with ninhydrin stain

prepared by dissolving 15 g of NaOAc in water (40 mL) and adjusting to pH 5-6. Tetramethylene sulfone (40 mL) and ninhydrin (2.0 g) were added followed by hydrindantin (36 mg) after 15 min. The total volume was made up to 100 mL with water.

Diethylaminoethyl cellulose (DEAE) was purchased from Whatman, Dowex 50 (H^+) from Sigma and AG-1X8 from Bio-Rad. Dowex 50 was recycled by first rinsing with 10 column volume of water followed by suspending the resin in 10 N KOH to final pH of 14. The base was decanted and the resin was rinsed with three column volumes of water. Bromine was added to the resin in small portions until the color change stopped. The suspension was allowed to stand for 3 h before bromine before the resin was filtered and washed with 6 N HCl. Finally, the clean Dowex 50 was washed with water to remove all excess acid and was stored 4 °C.

AG-1X8 Cl^- was converted to the hydroxide form by washing with twenty column volumes of 1 N NaOH. The column was then washed with distilled deionized water until all the chloride was displaced as determined by silver nitrate test. Carboxymethylcellulose (CM) column was equilibrated with sodium acetate buffer before use.

Spectroscopic and analytical measurements

^1H NMR and ^{13}C NMR spectra were recorded on a Varian VX-300 FT-NMR spectrometer or a Varian VXR-500 FT-NMR spectrometer. Chemical shifts for ^1H NMR spectra are reported in parts per million (ppm) relative to internal tetramethylsilane (Me_4Si, δ = 0.0 ppm) with $CDCl_3$ as the solvent and to internal sodium 3-(trimethylsilyl) propionate-*2,2,3,3-d*$_4$ (TSP, δ = 0.0 ppm) when D_2O was the solvent. ^{13}C NMR spectra

were recorded at 75 MHz on a Varian VX-300 FT-NMR spectrometer or at 125 MHz on a Varian VXR-500 FT-NMR spectrometer. Chemical shifts for ^{13}C NMR spectra were reported in parts per million (ppm) relative to $CDCl_3$ (δ = 77.0 ppm) or CD_3OD (δ = 49.0 ppm) in D_2O. To determine molar concentrations during L-threonate **10** conversion to 4-hydroxy-2-ketobutyrate **11**, a portion (1.0-3.0 mL) of the bioconversion mixture was taken every h and the protein was precipitated with 10% HCl and spun down in a microfuge. The supernatant was concentrated to dryness, the residue redissolved in 1 mL D_2O, concentrated again to dryness and the residue redissolved in 1 mL D_2O containing 10 mM TSP. The concentrations of L-threonate **10** and 4-hydroxy-2-ketobutyrate **11** were determined by the ratios of the integrated ^1H NMR resonances at δ 3.66 and δ 3.00, respectively, with the integrated resonance corresponding to TSP at δ 0.00. Similarly, the molar concentration during the bioconversion of L-lysine to β-L-lysine was determined as mentioned above. The concentration of β-L-lysine and L-lysine were determined by the ratios of the integrated ^1H NMR resonances at δ 2.8 and δ 2.0, respectively, with the integrated resonance corresponding to TSP at δ 0.00.

Gas chromatography was used to determine the product yields obtained during the hydrogenations of D,L-malic acid **2a,b**, L-malic acid **2b** and 2-hydroxy-2-buten-4-olide **12**. Chromatograms were obtained using an Agilent 6890N equipped with an HP-5 column (30 m x 0.25 mm x 0.25 μm) after samples were derivatized with bis(trimethylsilyl)trifluoroacetamide. Analysis was optimized by using a temperature program in the range from 120 °C to 210 °C at 15 °C/min. GC derivatization was initiated by dissolving the dry sample (~50 mg) in pyridine (1 mL, 12.4 mmol) followed by the addition of dodecane (0.10 mL, 0.44 mmol) and bis(trimethylsilyl)-

trifluoroacetamide (2.0 mL, 7.5 mmol). The reaction was stirred at room temperature for 3 h. Samples were injected (2-5 μL) using an Agilent 7683 series injector. Yields were determined with response factors obtained from authentic samples that were quantified relative to dodecane internal standard.

1,2,4-Butanetriol enantiomers obtained from hydrogenation of L-malic acid were derivatized and the resulting Mosher esters analyzed using Agilent 1100 HPLC interfaced with ChemStation acquisition software (Rev. A.08.03).[15] To 1,2,4-butanetriol (0.003 g, 2.8 x 10^{-5} mol) in pyridine (0.20 mL) was added CH_2Cl_2 (0.30 mL), *p*-dimethylaminopyridine (0.005 g, 4 x 10^{-5} mol) and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (0.0040 g, 17 x 10^{-5} mol). The reaction mixture was allowed to stir at rt for 8 h. The crude product was passed through a silica gel column and eluted with CH_2Cl_2 (3 mL). After removal of CH_2Cl_2 in vacuo, the resulting residue was redissolved in CH_2Cl_2 and washed with 1% $NaHCO_3$ (5 mL) followed by H_2O (2 x 5 mL). The organic layer containing the Mosher esters[1] of 1,2,4-butanetriol enantiomers was concentrated and loaded on the Chiralpak AD column (Daicel Chemical, 4.6 mm x 250 mm) previously equilibrated with 2-propanol:hexanes (2:98, v/v). The column was eluted with a linear gradient of hexanes with 2-15% 2-propanol (v/v) for 28 min. The solvent was eluted at 1.25 mL/min while the eluant was monitored at 260 nm.

Bacterial Strains and plasmids

E. coli JWF1 was prepared by homologous recombination of a non-functional *serA* gene into *E. coli* RB791 (W3110 *lacI*^{q}).[2] Linearization of the 1.9 kb *serA* fragment

obtained from pD2625 into pMAK705 provided pLZ1.68A. Linearization of pLZ1.68A at the unique *Bam*HI site internal to *serA* followed by treatment with Klenow fragment and dNTP's and religation afforded pLZ1.71A.[3] Homologous recombination of the resulting non-functional *serA* locus of the pLZ1.71A into RB791 afforded JWF1. *E. coli* BL21(DE3)/pAF-80-*kam*A + pAlterEX2-argU was obtained from Professor P. Frey at the University of Wisconsin, Madison.[4] Plasmid pET-23(a) was purchased from Novagen. Plasmids pMM4.166B, pMM4.202 and pMM4.257 were constructed following a literature procedure.[5] *Bacillus subtilis*, *Clostridium subterminale* and *Clostridiun sticklandii* were obtained from ATCC.

Storage of microbial strains and plasmids

All bacterial strains were stored at -78 °C in glycerol. Plasmids were transformed into DH5a for long-term storage. Glycerol samples were prepared by adding 0.75 mL of an overnight culture to a sterile vial containing 0.25 mL of 80% (v/v) glycerol. The solution was mixed, left at room temperature for 2 h, and then stored at -78 °C. For *Bacillus subtilis, Clostridium subterminale* and *Clostridium sticklandii*, glycerol was purged with N_2 before the overnight culture was introduced.

Culture medium

All solutions were prepared in distilled, deionized water. LB medium (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). *Bacillus* medium (1 L) was obtained by soaking 50 g of soybean in 1 L of distilled, deionized water overnight. NaOH (2.0 g) was dissolved and neutralized with HCl. The mixture

was steamed for 1 h and filtered through a cheesecloth. The volumes was made up to 1 L and soluble starch (15 g), $(NH_4)_2HPO_3$ (10.0 g) and KCl (0.2 g) were added and the solution adjusted to pH 7 prior to autoclaving. After the solution cooled to rt, a previously autoclaved solution of $MgSO_4$ was added to final concentration of 0.2 g/mL. YT Medium (1 L) contained Bacto-tryptone (16 g), Bacto-yeast extracts (10 g) and NaCl (5 g) in distilled deionized water. TB medium (1 L) contained tryptone (10 g) and NaCl (5 g). After autoclaving and directly before use, $MgSO_4$ (10 mL of 1 M stock) was added to the TB medium. M9 salts (1 L) contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g), and NaCl (0.5 g). M9 medium contained D-glucose (10 g), $MgSO_4$ (0.12 g), and thiamine (0.001 g) in 1 L of M9 salts. CAYE medium (100 mL) contained casamino acids (2 g) and yeast extract (10 g). Minimum salts (1L) contained $(NH_4)_2SO_4$ (0,2 g), KH_2PO_4 (0.6 g), K_2HPO_4 (1.4 g), sodium citrate (0.1 g) and magnesium sulphate (0,02 g). Terrific Broth (1 L) contained Bacto tryptone (12 g), Bacto yeast extract (24 g), glycerol (4 mL) in 900 mL. In a separate flask KH_2PO_4 (2.31 g) and K_2HPO_4 (12.54 g) were dissolved in 90 mL of distilled deionized water. Each was autoclaved separately and the phophates were added to the YTG base after it cooled to 60 °C. SOC medium (1 L) contained Bacto tryptone (20 g), Bacto yeast extract (5 g), NaCl (10 mL, 1 M), KCl (2.5 mL, 1 M), $MgCl_2$ (10 mL, 1 M), $MgSO_4$ (10 mL, 1 M) and glucose (10 mL, 2 M). 2×YT medium (1 L) contained Bacto tryptone (16 g), yeast extract (10 g) and NaCl (5 g). Solutions of inorganic salts, magnesium salts, and carbon sources were autoclaved separately and then mixed at room temperature. Reinforced Clostridium Medium-CM 0149 (1 L) contained yeast extract (3 g), Lablemco powder (10 g), peptone (10 g), glucose (5 g), soluble starch (1 g) NaCl (5 g), sodium acetate (3 g), cysteine-HCl (0.5 g),

agar (0.5 g). Clostridium Medium (1 L) contained L-lysine·HCl (6 g), yeast extracts (6 g), sodium dithionite (30 mg), 1 M phosphate buffer (45 mL) and 1 M potassium carbonate (7.5 mL). X-Gal indicator plates[6] contained glucose (4 g), lactose (4 g), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (1 mL, 3 mg/mL in EtOH:H_2O, 1:1, v/v) in medium E (1 L) with 1.5% (w/v) Difco agar.

Antibiotics were added where appropriate to the following final concentrations unless noted otherwise: ampicillin, 50 μg/mL; kanamycin, 50 μg/mL; tetracycline, 10 μg/mL; and carbernicillin, 20 μg/mL. Stock solutions of antibiotics were prepared in water with the exceptions of tetracycline, which was prepared in 50% aqueous ethanol. Antibiotics, isopropyl β-D-thioglucopyranoside (IPTG), thiamine, and amino acid supplementations were sterilized through 0.22-μm membranes prior to addition to M9 medium. Solid medium was prepared by addition of 1.5% (w/v) Difco agar to the medium.

Fermentation medium (1 L) contained K_2HPO_4 (7.5 g), ammonium iron (III) citrate (0.3 g), citric acid monohydrate (2.1 g and concentrated H_2SO_4 (1.2 mL). The culture medium was adjusted to pH 7.0 by addition of concentrated NH_4OH before autoclaving. The following supplements were added immediately prior to initiation of the fermentation: glucose (19 to 22 g) $MgSO_4$ (0.24 g) and trace minerals $(NH_4)_6(Mo_7O_{24}).4\ H_2O$ (0.0037 g), $ZnSO_4.7\ H_2O$ (0.0029 g), H_3BO_3 (0.0247 g), $CuSO_4.5\ H_2O$ (0.0025 g), and $MnCl_2.4\ H_2O$ (0.0158 g). Solutions of D-glucose and $MgSO_4$ were autoclaved separately. Trace minerals were sterilized through 0.22-*m*m membranes prior to addition to the medium.

Fed-batch fermentation

Fermentations were conducted in a B. Braun M2 culture vessel with a 2 L working capacity. Environmental conditions were supplied by a B. Braun Biostat MD controlled by a DCU-1. Data was acquired on a Dell Optiplex Gs+ 5166M personal computer using B. Braun MFCS/Win software. PID control loops were used to control temperature, pH, and glucose addition. The temperature was maintained at 33 °C or 36 °C as indicated and the pH was maintained at 7.0 by addition of NH_4OH and 2 N H_2SO_4. Glucose was added as a 60% (w/v) solution. Dissolved oxygen (D.O.) was monitored using a Mettler-Toledo 12 mm sterilizable O_2 sensor fitted with an Ingold A-type O_2 permeable membrane. D.O. was maintained at 20% air saturation throughout the course of the fermentations unless otherwise specified. Antifoam (Sigma 204) was manually pumped into the vessel as needed.

Inoculants were prepared by introduction of a single colony into 5 mL of M9 medium. The culture was grown at 37 °C with agitation at 250 rpm until they were turbid (~18-30 h) and subsequently transferred to 100 mL of M9 medium. Cultures were grown at 37 °C for an additional 12 h. The inoculant (OD_{600} = 1.5-3.0) was then transferred into the fermentor vessel and the batch fermentation was initiated (t = 0 h).

Genetic manipulations

Recombinant DNA manipulations generally followed methods described by Sambrook et al.[7] Restriction enzymes were purchased from Invitrogen or New England Biolabs. T4 DNA ligase was obtained from Invitrogen. Fast-Link™ DNA Ligation Kit was obtained from Epicentre. Zymoclean Gel DNA Recovery Kit and DNA Clean &

Concentrator Kit was obtained from Zymo Research Company. Maxi and Midi Plasmid Purification Kits were obtained from Qiagen. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. Agarose (electrophoresis grade) was obtained from Invitrogen. Phenol was prepared by addition of 0.1 % (w/v) 8-hydroxyquinoline to distilled, liquefied phenol. Extraction with an equal volume of 1 M Tris-HCl (pH 8.0) two times was followed by extraction with 0.1 M Tris-HCl (pH 8.0) until the pH of the aqueous layer was greater than 7.6. Phenol was stored at 4 °C under an equal volume of 0.1 M Tris-HCl (pH 8.0). SEVAG was a mixture of chloroform and isoamyl alcohol (24:1, v/v). TE buffer contained 10 mM Tris-HCl (pH 8.0) and 1 mM Na_2EDTA (pH 8.0). TAE buffer contained 40 mM Tris-acetate (pH 8.0) and 2 mM Na_2EDTA. Endostop solution (10× concentration) contained 50% glycerol (v/v), 0.1 M Na_2EDTA, pH 7.5, 1% sodium dodecyl sulfate (SDS) (w/v), 0.1% bromophenol blue (w/v), and 0.1% xylene cyanole FF (w/v) and was stored at 4 °C. Prior to use, 0.12 mL of DNase-free RNase was added to 1 mL of 10X Endostop solution. DNase-free RNase (10 mg mL^{-1}) was prepared by dissolving RNase in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. DNase activity was inactivated by heating the solution at 100 °C for 15 min. Aliquots were stored at -20 °C. PCR amplifications were carried out as described by Sambrook et al. Standard reaction (0.1 mL) contained 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, dATP (0.2 mM), dCTP (0.2 mM), dGTP (0.2 mM), dTTP (0.2 mM), template DNA, 0.5 μM of each primer, and 2 units of the *Taq* polymerase. Template concentration varied from 0.02 μg to 1.0 μg.

Large scale purification of plasmid DNA

Plasmid DNA was purified on a large scale using a modified alkaline lysis method described by Sambrook et al. In a 2 L Erlenmeyer flask, 500 mL of LB containing the appropriate antibiotics was inoculated from a single colony, and the culture was incubated in a gyratory shaker at 37 °C for 14 h with agitation at 250 rpm. Cells were harvested by centrifugation (4 000*g*, 5 min, 4 °C) and then resuspended in 10 mL of cold GETL solution (50 mM glucose, 20 mM Tris-HCl (pH 8.0), 10 mM Na_2EDTA, pH 8.0) into which lysozyme (5 mg/mL) had been added immediately before use. The suspension was stored at room temperature for 5 min. Addition of 20 mL of 1% sodium dodecyl sulfate (w/v) in 0.2 N NaOH was followed by gentle mixing and storage on ice for 15 min. Fifteen milliliters of an ice-cold solution containing 3 M KOAc (prepared by combining 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of H_2O) was added. Vigorous shaking resulted in formation of a white precipitate. After the suspension was stored on ice for 10 min, the cellular debris was removed by centrifugation (48 000*g*, 20 min, 4 °C). The supernatant was transferred to two clean centrifuge bottles and isopropanol (0.6 volumes) was added to precipitate the DNA. After the samples were left at room temperature for 15 min, the DNA was recovered by centrifugation (20 000*g*, 20 min, 4 °C). The DNA pellet was then rinsed with 70% ethanol and dried.

Further purification of the DNA sample involved precipitation with polyethylene glycol (PEG). The isolated DNA was dissolved in TE (3 mL) and transferred to a Corex tube. Cold 5 M LiCl (3 mL) was added and the solution was gently mixed. The sample was then centrifuged (12 000*g*, 10 min, 4 °C) to remove high molecular weight RNA.

The clear supernatant was transferred to a clean tube and isopropanol (6 mL) was added followed by gentle mixing. The precipitated DNA was collected by centrifugation (12 000*g*, 10 min, 4 °C). The DNA was then rinsed with 70% ethanol and dried. After re-dissolving the DNA in 0.5 mL of TE containing 20 μg/mL of RNase, the solution was transferred to a 1.5 mL microcentrifuge tube and stored at room temperature for 30 min. DNA was precipitated from solution upon addition of 500 μL of 1.6 M NaCl containing 13% PEG-8000 (w/v) (Sigma). The solution was mixed and centrifuged (microcentrifuge, 10 min, 4 °C) to recover the precipitated DNA. The supernatant was removed, and the DNA was then re-dissolved in 400 μL of TE. The sample was extracted sequentially with phenol (400 μL), phenol and SEVAG (400 μL each), and finally SEVAG (400 μL). Ammonium acetate (10 M, 100 μL) was added to the aqueous DNA solution. After thorough mixing, 95% ethanol (1 mL) was added to precipitate the DNA. The sample was left at room temperature for 5 min and then centrifuged (microcentrifuge, 5 min, 4 °C). The DNA was rinsed with 70% ethanol, dried, and then redissolved in 200-500 μL of TE.

Alternatively, DNA was purified using a Qiagen Maxi Kit or Midi Kit as described by the manufacturer. The purity of DNA isolated by these kits was adequate for DNA sequencing.

Small scale purification of plasmid DNA

An overnight culture (5 mL) of the plasmid-containing strain was grown in LB containing the appropriate antibiotics. Cells from 3 mL of the culture were collected in a 1.5 mL microcentrifuge tube by centrifugation. The resulting cell pellet was liquefied by vortexing (30 sec) and then resuspended in 0.1 mL of cold GETL solution into which

lysozyme (5 mg/mL) had been added immediately before use. The solution was stored on ice for 10 min. Addition of 0.2 mL of 1% sodium dodecyl sulfate (w/v) in 0.2 N NaOH was followed by gentle mixing and storage on ice for 5-10 min. To the sample was added 0.15 mL of cold KOAc solution. The solution was shaken vigorously and stored on ice for 5 min before centrifugation (15 min, 4 °C). The supernatant was transferred to another microcentrifuge tube and extracted with equal volumes of phenol and SEVAG (0.2 mL). The aqueous phase (approximately 0.5 mL) was transferred to a fresh microfuge tube, and DNA was precipitated by the addition of 95% ethanol (1 mL). The sample was left at room temperature for 5 min before centrifugation (15 min, room temperature) to collect the DNA. The DNA pellet was rinsed with 70% ethanol, dried, and redissolved in 50 to 100 μL TE. DNA isolated using this method was used for restriction enzyme analysis, although the concentration of DNA could not be accurately determined by spectroscopic methods.

Restriction enzyme digestion of DNA

Restriction enzyme digests were performed in buffers provided by Invitrogen or New England Biolabs. A typical restriction enzyme digest contained 0.8 μg of DNA in 8 μL of TE, 2 μL of restriction enzyme buffer (10× concentration), 1 μL of bovine serum albumin (0.1 mg/mL), 1 μL of restriction enzyme and 8 μL TE. Reactions were incubated at 37 °C for 1 h, terminated by addition of 2.2 μL of 10X Endostop solution and analyzed by agarose gel electrophoresis. When DNA was required for cloning experiments, the digest was terminated by addition of 1 μL of 0.5 M Na_2EDTA (pH 8.0)

or by heating at 70 °C for 15 min followed by extraction of the DNA using Zymoclean gel DNA recovery kit.

Determination of DNA concentration

The concentration of DNA in the sample was determined as follows. An aliquot (10 μL) of the DNA was diluted to 1 mL in TE and the absorbance at 260 nm was measured relative to the absorbance of TE. The DNA concentration was calculated based on the fact that the absorbance at 260 nm of 50 μg/mL of double stranded DNA is 1.0.

Agarose gel electrophoresis

Agarose gel typically contained 0.7% agarose (w/v) in TAE buffer. Ethidium bromide (0.5 μg/ml) was added to the agarose to allow visualization of DNA fragments under a UV lamp. Agarose gel was run in the TAE buffer. The size of the DNA fragments were determined using two sets of DNA molecular weight standards: *l* DNA digested with *Hin*dIII (23.1-kb, 9.4-kb, 6.6-kb, 4.4-kb, 2.3-kb, 2.0-kb and 0.6-kb) and *l* DNA digested with *Eco*RI and *Hin*dIII (21.2-kb, 5.1-kb, 5.0-kb, 4.3-kb, 3.5-kb, 2.0-kb, 1.9-kb, 1.6-kb, 1.4-kb, 0.9-kb, 0.8-kb and 0.6-kb).

Isolation of DNA from agarose

The band of agarose containing DNA of interest was excised from the gel while visualized with long wavelength UV light. Two methods were used for isolating DNA from agarose gels. The first method used Zymoclean gel DNA recovery kit to isolate DNA from the agarose gel according to the procedure provided by Zymo Research.

Alternatively, the agarose gel containing DNA was chopped thoroughly with a razor and then transferred to a 0.5 mL microfuge tube packed tightly with glass wool. The tube was centrifuged for 5 min using a Beckman microfuge to extrude the DNA solution from the agarose into a second 1.5 mL microfuge tube. The DNA was precipitated using 3 M NaOAc and 95% ethanol as described previously and subsequently redissolved in TE.

Treatment of DNA with Klenow fragment

DNA fragments with recessed 3' termini were modified to DNA fragments with blunt ends by treatment with the Klenow fragment of *E. coli* DNA polymerase I. After restriction digestion (20 μL) of the DNA (0.8-2 μg) was complete, a solution (1 μL) containing each of the four dNTPs was added to a final concentration of 1 mM for each dNTP. Addition of 1-2 units of Klenow fragment was followed by incubation at room temperature for 20-30 min. Since the Klenow fragment works well in the common restriction enzyme buffers, there was generally no need to purify the DNA after restriction digestion and prior to filling recessed 3' termini. Klenow reactions were quenched by extraction with equal volumes of phenol and SEVAG. DNA was recovered using the Zymoclean gel DNA recovery kit or by precipitation as described previously and subsequently dissolved in TE.

Treatment of vector DNA with calf intestinal alkaline phosphatase

Following restriction enzyme digestion, plasmid vectors were dephosphorylated to prevent self-ligation. Digested vector DNA was dissolved in TE (88 μL). To this sample was added 10 μL of dephosphorylation buffer (10× concentration) and 2 μL of

calf intestinal alkaline phosphatase (2 units). The reaction was incubated at 37 °C for 1-2 h. The phosphatase was inactivated by the addition of 1 *m*L of 0.5 M Na_2EDTA (pH 8.0) followed by heat treatment (70 °C, 15 min). The sample was extracted with phenol and SEVAG (100 μL each) to remove the protein, and the DNA was purified as previously described and subsequently dissolved in TE.

Ligation of DNA

Molar ratios of insert to vector were typically maintained at 3 to 1 for DNA ligations. A typical reaction contained 0.1 μg of vector DNA and 0.05 to 2.0 μg of insert DNA in a total volume of 7 μL. To this was added 2 μL of T4 ligation buffer (5× concentrations) and 1 μL of T4 DNA ligase (2 units). The reaction was incubated at 16 °C for at least 4 h and then used to transform competent cells. Alternatively, Fast-Link™ DNA Ligation Kit (Epicentre, Madison, WI) was used for ligation of insert DNA with cohesive or blunt ends into predigested vectors with compatible ends according to the protocol provided by Epicentre.

Preparation and transformation of competent *E. coli* cells

Competent cells were prepared according to a procedure modified from Sambrook et al. LB medium (5 mL) containing antibiotics where appropriate, was inoculated with a single colony from a LB plate containing antibiotics where appropriate. The culture was grown at 37 °C with shaking at 250 rpm for 10-12 h. An aliquot (1 mL) from the culture (5 mL) was used to inoculate LB (100 mL) containing the appropriate antibiotics. The culture was grown at 37 °C with shaking at 250 rpm in a NBS series 25 incubator shaker

until the optical density at 600 nm was between 0.4 and 0.6. The culture was transferred to a centrifuge bottle that had been sterilized with a 25 % (v/v) bleach solution and rinsed four times with sterile, deionized water. The cells were harvested by centrifugation (4000*g*, 5 min, 4 °C) and the culture medium was decanted. All subsequent manipulations were carried out on ice. The harvested cells were resuspended in ice-cold 0.9 % NaCl (100 mL), and the cells were collected by centrifugation (4000 *g*, 5 min, 4 °C). The 0.9 % NaCl solution was decanted, the cells were resuspended in ice-cold 100 mM $CaCl_2$ (50 mL) and stored on ice for 30 min. After centrifugation (4000 *g*, 5 min, 4 °C), the cells were resuspended in 4 mL of ice-cold 100 mM $CaCl_2$ containing 15% glycerol (v/v). Aliquots (0.25 mL) of competent cells were added to 1.5 mL microfuge tubes, immediately frozen in liquid nitrogen, and stored at -78 °C.

Frozen competent cells were thawed on ice for 5 min before transformation. A small aliquot (1 to 10 *m*L) of plasmid DNA or a ligation reaction was added to the thawed competent cells (0.1 mL). The solution was gently mixed by tapping and stored on ice for 30 min. The cells were then heat shocked at 42 °C for 30 seconds and returned to ice briefly (1 min). LB (0.5 mL, no antibiotics) was added to the cells, and the sample was incubated at 37 °C (no agitation) for 1 h. Cells were collected by centrifugation (30 s) in a microcentrifuge. If the transformation was to be plated onto LB plates, 0.5 mL of the culture supernatant was removed, and the cells were resuspended in the remaining 0.1 mL of LB and subsequently spread onto plates containing the appropriate antibiotics. If the transformation was to be plated onto minimal medium plates, the cells were washed twice with a solution of M9 inorganic salts (0.5 mL). After resuspension in a fresh aliquot of M9 salts (0.1 mL), the cells were spread onto a plate. An aliquot of competent

cells with no DNA added was also carried through the transformation protocol as a control. These cells were used to check the viability of the competent cells and to verify the absence of growth on selective medium.

Transformations were also performed by electroporation using electrocompetent cells. An aliquot (1 mL) from an overnight culture (5 mL) was used to inoculate 500 mL of 2×YT containing the appropriate antibiotics. The cells were cultured at 37 °C with shaking at 250 rpm. Once an absorbance of 0.6-0.8 at 600 nm was observed, the cells were kept on ice for 10 min and harvested (3 000*g*, 5 min, 4 °C). The cells were gently washed three times with sterile, cold water (450 mL once and 250 mL twice) and then resuspended in 100 mL sterile, ice-cold aqueous 10% glycerol (v/v). After centrifugation (3 000*g*, 5 min, 4 °C), the cells were resuspended in 1.5 mL sterile ice-cold aqueous 10% glycerol (v/v). Aliquots (0.1 mL) of electrocompetent cells were dispensed into 1.5 mL microfuge tubes, and immediately frozen in liquid nitrogen and stored at –78 °C.

The electroporation was performed in Bio-Rad Gene Pulser cuvettes with an electrode gap of 0.2 cm. The cuvettes were chilled on ice for 5 min prior to use. Electrocompetent cells were thawed in ice for 5 min, and 40 μL of thawed cells was added to the chilled cuvette. To this was added 1-10 μL of plasmid DNA (1 μg mL^{-1}), and the mixture was gently shaken. The Bio-Rad Gene Pulser was set at 2.5 kvolts, 25 μF and 200 Ohms. The outside surface of the cuvette was wiped clean and it was placed in the sample chamber. A single pulse was applied, the cuvette was removed, and 1 mL of freshly prepared SOC was added into it. The contents of the cuvette were transferred to a 15 mL sterile centrifugation tube. The cells were incubated at 37 °C for 1 h with

shaking at 250 rpm. The transformed cells were plated in the same manner as in the transformation with chemically competent cells.

Purification of *Bacillus subtilis* genomic DNA

Genomic DNA was purified using a method by Doi and McLoughlin. A single colony of a *B. subtilis* was inoculated into a 200 mL of M-1 medium and cultured for 12 h on a gyratory shaker set at 250 rpm (37 °C). The cells were harvested by centrifugation at 4 °C for 10 min on rotor set 10,000 rpm. The cell pellets were gently resuspended in a 20 mL lysis buffer (150 mM NaCl, 100 mM EDTA, pH 8.0, 0.5 mg/mL lysozyme, which was added before use) and were incubated at 37 °C for 60 min with occasional swirling. The following solutions were added in the order written: 1 mL of 10% SDS, 4 mL of 5 M $NaClO_4$ and 40 mL chloroform/octanol (24:1). The mixture was gently shaken (50 rpm) for 15 min at rt and then centrifuged at 18,000*g* for 20 min. The aqueous layer was carefully transferred to a clean 100 mL beaker. Two volumes of cold 96% ethanol were gently added onto the DNA solution. The DNA was spooled out of the solution with a glass rod into a cold 70% solution of ethanol and then was allowed to dry in air for 5 min. The DNA was resuspended in TEN (5 mL). RNase buffer (20 μl) was added and the mixture was incubated at 37 °C for 60 min followed by addition of pronase (50 μl). The mixture was incubated for an additional 60 min. The mixture was gently extracted with 5 mL of phenol/chloroform (1:1) and the upper layer was transferred into a clean tube after centrifugation for 5 min at 10,000*g*. The DNA was recovered by gently adding two volumes of cold ethanol (96%) and was spooled out of the solution with a glass rod. The glass rod was dipped into a cold 70% ethanol solution and allowed to dry in air for 5 min.

The DNA was then resuspended in 2 mL of TE. The purity of DNA was evaluated and stored in solution at 4 °C.

CHAPTER 2

Synthetic Procedures

Hydrogenation of D,L-Malic Acid.[8]

A solution of D,L-malic acid (13.4 g, 0.10 mol) in distilled, deionized water (100 mL) was placed in a glass reaction vessel along with 5 wt % Ru on C (2.68 g, 1.33 mmol). The glass reaction vessel was inserted into a 500 mL Parr 4575 stainless steel high temperature-high pressure reactor and the vessel sealed. The temperature and the stirring rate were controlled by a Parr 4842 temperature controller. Hydrogen was bubbled through the reaction mixture for 10-15 min to remove air while stirring at 100 rpm. The vessel was then charged with 272 atm H_2. After heating the reaction to 135 °C, the H_2 pressure was 340 atm. The reaction was stirred at 200 rpm for 10 h at 135 °C. After removal of the catalyst by filtration through Celite, the reaction solution was concentrated to dryness under vacuum to afford a colorless oil. Distillation of the crude hydrogenation product using a short path distillation apparatus (148-156 °C/1.2 mm Hg) afforded D,L-1,2,4-butanetriol **1a, b** (3.0 g, 28%) and 1,4-butanediol **6** (0.13 g, 1.4%) as determined by GC.

Ru-Re catalyst.[9]

Re_2O_7 (6.3 g, 0.013 mol) and $RuCl_3$ (9.83 g, 0.0473 mol) were dissolved in distilled, deionized H_2O (100 mL) inside a glass reaction vessel, which was then inserted into 500 mL Parr 4575 stainless steel high temperature-high pressure reactor and the vessel sealed. Temperature and stirring rate were contolled by the Parr 4842 temperature controller. H was bubbled through the reaction mixture for 10-15 min to remove air while stirring at 400 rpm. The reactor was charged with 1,500 psi H_2 and the temperature was increased to 120 °C for 1 h. The Parr was cooled to room temperature and the pressure released. The Ru-Re catalyst was filtered off and used in hydrogenation reactions.

2-Hydroxy-γ-butyrolactone 10.[10]

Malic acid (7.7 g, 57 mmol), pivaldehyde (9.7 mL, 89 mmol), *p*-toluenesulfonic acid (1.0 g), a drop of concentrated H_2SO_4 and pentane (60 mL) were refluxed with azeotropic removal of water. The resulting suspension was filtered after cooling. The filter cake was redissolved in CH_2Cl_2 (80 mL) and the filtrate was washed with 8% aqueous phosphoric acid (2x 40 mL). The solution was dried over anhydrous Na_2SO_4 and concentrated to half the volume under reduced pressure. Crystallization at –78 °C afforded 2-(*t*-butyl)-5-(methoxycarbonyl)-1,3-dioxolan-4-one (5.9 g, 51%) as colorless crystals with a melting point of 102-104 °C. The combined mother liquor yielded (2.7 g, 23%). ^{1}H-NMR ($CDCl_3$): δ 10.6 (br s, 1 H), 5.20 (s, 1 H), 4.16 (dd, J_1 and J_2= 4 Hz 1 H), 3.17-2.60 (m, 2H), 1.0 (s, 9 H).

2-(*t*-Butyl)-5-(methoxycarbonyl)-1,3-dioxolan-4-one (173 mg, 0.85 mmol) from above was dissolved in anhydrous THF (1 mL). It was added dropwise to a stirred solution of 2 M BMS.THF complex (0.14 mL, 0.28 mmol) and trimethylborane (0.93 mL, 0.28 mmol) held 0 °C. The solution was stirred at 0 °C for an additional 5-10 min before it was the cooling bath was removed. Stirring was continued at room temperature for 8 h. MeOH (1.2 mL) was added dropwise and the resulting solution was evaporated to dryness. The light yellow residue was further co-evaporated with MeOH (3x 2 mL) to remove residual boron by-product to afford the protected alcohol. 0.156 g, 97%).
The protected alcohol was deacetylated by 2 N HCl (15 mL) at 50 °C overnight to afford 2-hydroxy-4-butyrolactone **10** after removal of water under high vacuum.

Malic acid (13.4 g, 100 mmol) was suspended in trifluoroacetic anhydride (50 mL) and stirred at room temperature until the mixture was homogeneous. Excess trifluoroacetic anhydride was removed under reduced pressure. The remaining white solid, *O*-TFA malate anhydride, was opened by methanolysis to a methyl malate monoester. Excess methanol was removed under vacuum an the monoester was dried and lyphilised. The resulting powder was dissolved in THF (100 mL) and cooled to 0 °C. A 1 M solution of BH_3-Me_3S (200 mL) was added dropwise. After 3 h the reaction was quenched with methanol (50 mL). Solvent was evaporated in vacou and the 1-methyl malate **18** was co-evaporated several times with methanol to remove methyl borate byproducts. The remaining oil, a mixture of 2,4-dihydroxybutanoic acid lactone **10** and the corresponding methyl ester, was treated with aqueous sodium carbonate and evaporation of water gave the sodium salt. The sodium salt 2,4-dihydroxybutanoate was passed through the Dowex-50(H^+) column to yield 2-hydroxy-4-butyrolactone **10** in 86%.

^{1}H-NMR (D_2O, 300 MHz): δ 1.62 (m, 1 H), 1.81 (m, 1 H), 3.50 (t, J = 7.5 Hz, 2 H), 3.96 (q, J = 4.5 Hz, 1 H). ^{13}C-NMR (D_2O, 300 MHz): δ 37.3 (C-3), 59.4 (C-4), 70.6 (C-2), 182.1 (C-1).

Hydrogenation of 2-hydroxy-γ-butyrolactone 10.

The solution of 2-hydroxy-γ-butyrolactone (7.6 g, 75 mmol) in distilled deionized water (75 mL) was placed in a glass reaction vessel along with 5 wt % Ru on C (1.5 g, 0.75 mmol). The glass reaction vessel was inserted into the 500 mL Parr 4575 stainless steel high temperature-high pressure reactor and the vessel sealed. The temperature and stirring rate were controlled by a Parr 4842 temperature controller. Hydrogen was bubbled through the reaction mixture for 10-15 min to remove air while stirring at 100 rpm. The vessel was then charged with 2,000 psi H_2. After heating the reaction to 125 °C, a final pressure of 2,500 psi was reached. The reaction was allowed to stir for 10 h at 125 °C. After removal of the catalyst by filtration through Celite, the reaction solution was concentrated to a light yellow oil, and a portion of the residue was derivatized by bis(trimethylsilyl)trifluoroacetamide followed by GC analysis. D,L-1,2,4-Butanetriol **1a,b** was obtained as a yellow oil (7.8 g, 99%). The ^{1}H NMR and ^{13}C NMR were identical to authentic sample.

2-Keto-4-hydroxybutyrate 11.[11,12]

In a round-bottom flask was dissolved in L-aspartic acid (4.0 g, 30 mmol) in 50 mL trifluoroacetic anhydride for 3 h at room temperature. Excess trifluoroacetatic acid and anhydride were removed under reduced pressure to afford a white solid, which was

redissolved in dry methanol. The mixture was stirred at room temperature for 3 h before excess methanol was removed under vacuum to afford 4-methyl *N*-(trifluoroacetyl)-L-aspartate and 1-methyl *N*-(trifluoroacetyl)-L-aspartate **19** in 1:4 ratio. The former ester was removed with petroleum ether/ether (2:1) and the resulting fine suspension was filtered to remove 4-methyl *N*-(trifluoroacetyl)-L-aspartate. In a separate dry flask was placed borane methyl sulfide (27 mL, 54 mmol) and methyl borate (6 mL, 54 mmol). The flask was held in a ice-acetone bath under argon. 1- Methyl *N*-(trifluoroacetyl)-L-aspartate **19** (4 g, 27 mmol) in THF (100 mL) was introduced slowly via a syringe pump. The reaction was allowed to stir in ice-acetone bath for 2 h before warming up to room temperature for 8 h. Acidic methanol was added before the mixture was concentrated to dryness and co-evaporated with methanol to remove methyl borate. Finally, the resulting homoserine lactone-HCl was hydrolyzed to homoserine (15 mmol) and was mixed with pyridoxal hydrochloride (15 mmol) and $CuCl_2.2H_2O$ (7 mmol) in distilled deionized water (50 mL) to give a deep green solution. The pH was adjustment to pH 5.0 followed by addition of acetate buffer (20 mmol, pH 5). The flask was immersed in boiling and the internal temperature rose to 89 °C in 30-40 min and the mixture turned emerald green. The flask was then poured into ice and neutralized before loading on Dowex 50 (H^+) and eluted with water. The combined eluant were concentrated to 10 mL and quantified by NMR. 4-Hydroxy-2-ketobutyrate **11** was estimated by H NMR integration of a triplet centered at 2.45 ppm relative to TMS peak centered at 0.0 ppm. The yield of 15% was obtained.

D-Erythronolactone 14a.[13]

A 1 L round-bottom flask was charged with D-isoascorbic acid (35.2 g, 200 mmol) and distilled, deionized water (500 mL). The flask was stirred in an ice-water bath while anhydrous sodium carbonate (42.4 g, 400 mmol) was added in small portions over 30 minutes. To the resulting yellow solution was added aqueous hydrogen peroxide (44 ml of 33%, 450 mmol) over an hour. The resulting solution was kept in an ice bath for an additional 30 minutes before it was transferred to a 40 °C water bath, where it was stirred for 30 minutes. Charcoal (8 g) was added in small portions to destroy excess hydrogen peroxide. The temperature was increased to 70 °C and was kept constant until the starch iodide test was negative. The hot mixture was filtered through Celite and washed with hot water (200 mL). The mixture was acidified to pH 1.5 with 6 N HCl and extracted with hot ethyl acetate. The combined extracts were combined and concentrated to D-erythronolactone (22.2 g, 94%). ^{1}H-NMR (D_2O, 500 MHz): δ 4.34 (d, J = 11 Hz, 1H), 4.50 (dd, J_1 = 3 Hz, J_2 = 11 Hz, 1 H), 4.56 (dd, J_1 = 3 Hz, J_2 = 5 Hz, 1 H), 4.69 (d, J = 5 Hz, 1 H). ^{13}C-NMR (D_2O, 500 MHz): δ 61.18 (C-4), 72.67 (C-3), 73.03 (C-2), 177.66 (C-1).

2-Acetyloxy-2-buten-4-olide 21a.

To the solution of D-erythronolactone **14a** (12 g, 100 mmol) in dichloromethane (50 mL) was added pyridine (79 mL) and acetic anhydride (52 mL). The flask was refluxed under argon for 8 h. The dark brown solution that resulted was concentrated and flushed through a silica gel column eluting with ethyl acetate: hexanes (3:7). The eluant was concentrated to give a colorless oil of 2-acetoxy-2-butenolide **21a** (10.1 g, 70%). The

spectra were identical to published spectra. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 2.25 (s, 3 H), 4.9 (d, J = 1.8 Hz, 2 H), 7.28 (d, J = 1.8 Hz, 1 H). ^{13}C-NMR ($CDCl_3$, 300 MHz): δ 22.3 (-CH_3), 68.96 (C-4), 116.68 (C-3), 144.00 (C-2), 172.22 (C-1), 178.07 (C=O).

2-Benzoyloxy-2-buten-4-olide 21a.[14]

To a solution of D-erythronolactone **14a** (4 g, 33 mmol) in anhyd. DMF (40 mL) and dry pyridine (11 mL) was added benzoyl chloride (16 mL, 136 mmol) and the mixture was stirred for 3 h, in which time benzoylation was complete as confirmed by TLC. The mixture was diluted with dichloromethane (60 mL) and poured in to ice water. The organic layer was washed three times with saturated sodium carbonate, dried over sodium sulfate and dried under reduced pressure to afford 2,3-*O*-dibenzyloxy-D-erythronolactone as a light yellow syrup. This was redissolved in dichloromethane (50 mL) and treated with triethylamine (10 mL, 72 mmol) and stirred at room temperature for 4 h. After the solvent was removed, triethylamine was azeotroped off with toluene to afford 2-benzoyloxy-2-buten-4-olide **21b** in quantitative yield (7.1 g). Crystallization from acetonitrile resulted in 89% (6.3 g) yield of light yellow crystals (mp = 106-109 °C). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 4.77 (d, J_1= 2 Hz, 2 H); 7.3 (m, 3 H); 7.45 (m, 3 H); 7.97 (m. 3H). ^{13}C-NMR ($CDCl_3$, 300 MHz): δ 67.5 (C-4); 130.1 (C-3); 128.5, 130 (C-Bz); 133.9 (C-Bz); 137.4 (C-2); 162.3 (C-Bz); 166.8 (C-1).

2-Hydroxy-2-buten-4-olide 12 from D-*erythro*-1,4-lactone 14a.

D-Erythronolactone **14a** (5.9 g, 50 mmol) was dissolved in pyridine (20 mL. 250 mmol) and stirred in an ice-water bath. Trifluoroacetic anhydride (18 mL, 125 mmol)

was introduced dropwise. The color changed from clear to brown during the addition of trifluoroacetic anhydride. The reaction mixture was allowed to stir in an ice-water bath for 2 h before warming to rt. The mixture was quenched with H_2O (12 mL) and 1 mL was concentrated under vacuum. The resulting brown solid was redissolved in water before loading on Dowex 50 (H^+) eluted with water. The resulting eluant was concentrated and decolorized with charcoal to afford 2-hydroxy-2-buten-4-olide **12**. ^1H and ^{13}C NMR were identical to published spectra.[15]

L-Threonate 13b.[16]

A solution of L-ascorbic acid (150 g, 0.75 mol) in deionized water (500 mL) was stirred in an icebath while Na_2CO_3 (30 g, 0.29 mol) was added in small portions. Hydrogen peroxide (165 mL, 1.46 mol) was added over 20 min during which the internal temperature of the flask reached 94 °C with precipitation of sodium oxalate. The reaction mixture was stirred in an icebath until it cooled to rt before the precipitate was filtered off. The resulting yellow solution was refluxed with Darco KB-B activated carbon (30 g) until excess H_2O_2 had decomposed as shown by a negative starch-iodide test. The hot reaction solution was filtered through Celite and after cooling, a solution of 10 M NaOH was added dropwise to pH 8.2. The resulting clear L-threonate **13b** solution was stirred in the cold room to allow precipitation of sodium oxalate, which was removed by filtration to afford 76 g (74%) of sodium threonate. The ^1H NMR and ^{13}C NMR were identical to published spectra.

4-Hydroxy-2-ketobutyrate 11.

To the L-threonate **13b** solution was added $MgCl_2 \cdot 6H_2O$ (1.2 g, 5.9 mmol), Na_2HPO_4 (5.30 g, 37.7 mmol) and KH_2PO_4 (0.27 g, 2.0 mmol). Half of the L-threonate buffer solution was used to resuspend 200 g of harvested *E. coli* JWF1/pON1.118B and the mixture was passed through a French pressure cell 2x (12,000 atm) to lyse the *E. coli* cells. The resulting lysate was combined with the rest of the buffer solution and centrifuged at 9,000*g* for 30 min. The supernatant was transferred to a fermentor and was purged with N_2 until no dissolved O_2 remained in the in the reaction solution. A fermentor was used to maintain the temperature at 37 °C and the stirring rate at 200 rpm. The reaction solution was maintained at pH 8.2 with fermentor-controlled addition of 1 N H_2SO_4 and 0.5 M NaOH, respectively. The bioconversion was monitored by ^1H NMR and after 80% of L-threonate **13b** was converted to 4-hydroxy-2-ketobutyrate **11**, the bioconversion was terminated. A 5 mL sample of the bioconversion mixture was acidified to pH 2.5 with concentrated HCl and the resulting protein precipitate removed by centrifugation. After concentration, the residue was analyzed. ^1H NMR (D_2O): δ 3.01 (t, J = 5.7 Hz, 2 H) 3.88 (t, J = 5.7 Hz, 2 H). ^{13}C NMR (D_2O): δ 40.996, 55.52, 168.997, 203.919.[20]

2-Hydroxy-2-buten-4-olide 12.

The crude reaction solution containing 4-hydroxy-2-ketobutyrate **11** was acidified to pH 2.5 with concentrated HCl. Precipitated protein was removed by centrifugation at 9,000*g* for 20 min. The resulting clear yellow solution was combined with 40 g of Darco KB-B activated carbon and placed on a 37 °C shaker for 3 h. The mixture was then

filtered under reduced pressure through Celite and acidified to pH 1.5 with concentrated HCl. 4-Hydroxy-2-ketobutyric acid **11** and residual L-threonic acid **13b** lactonized to 2-hydroxy-2-bute-4-olide **12** and L-threonolactone **14b**, respectively. Liquid-liquid extraction with EtOAc as a solvent was used to separate 2-hydroxy-2-bute-4-olide **12** from L-threonolactone **14b**.[17] The extraction was conducted over 32 h and was stopped when all the 2-hydroxy-2-bute-4-olide **12** had been extracted from the aqueous solution of L-threonolactone **14b**. The combined EtOAc extracts containing 2-hydroxy-2-bute-4-olide **12** were concentrated to dryness on a rotatory evaporator to afford 2-hydroxy-2-buten-4-olide **12** as a light yellow solid (41 g, 93%). A portion of the crude 2-hydroxy-2-buten-4-olide **12** was sublimed to afford white crystals mp 104-107 °C. ^{1}H and ^{13}C NMR were identical to published spectra.[17,18]

Hydrogenation of 2-hydroxy-2-buten-4-olide 12.

A solution of 2-hydroxy-2-buten-4-olide **12** (5 g, 50 mmol) in distilled, deionized water (100 mL) was placed in a glass reaction vessel along with 5 wt % Ru on C (1.0 g, 0.5 mmol). The glass reaction vessel was inserted into the 500 mL Parr 4575 stainless steel high temperature-high pressure reactor and the vessel sealed. The temperature and the stirring rate were controlled by a Parr 4842 temperature controller. Hydrogen was bubbled through the reaction mixture for 10-15 min to remove air while stirring at 100 rpm. The vessel was heated to 125 °C before it was pressurized with 170 atm H_2 pressure. The reaction was stirred at 200 rpm for 10 h. After removal of the catalyst by filtration through Celite, the reaction mixture was concentrated to dryness under vacuum

to afford 5.1 g (96%) of 1,2,4-butanetriol **1a,b** as a light yellow oil. The overall yield of D,L-1,2,4-butanetriol from L-ascorbic acid was 53%.

Plasmids:

pWN3.196A. This 6.0-kb pSU18-based plasmid encodes *ilvD* and *serA*. Using genomic DNA isolated from *E. coli* K-12 strain W3110 as template, the open reading frame of *ilvD* gene was PCR amplified with its native ribosomal binding site. The 1.8-kb *ilvD* DNA fragment was cloned behind the P_{tac} promoter of pSU18 by insertion into its *Eco*RI site to afford pWN3.172A. A 1.9-kb *serA*-encoding fragment was liberated from pD2625 by digestion with *Eco*RV and *Dra*I. Plasmid pWN3.172A was digested with *Bam*HI and treated with Klenow fragment. Ligation of the two blunt end fragments of DNA yielded pWN3.196A.

pON1.118B. This 9.0-kb pJF118EH-based plasmid encodes *ilvD* and *serA*. Digestion of pWN3.172A with *Eco*RI released a 1.8-kb fragment encoding the *ilvD* gene, which was subsequently cloned behind the P_{tac} promoter of pJF118EH by insertion into its *Eco*RI site to afford pON1.39A. A 1.7-kb *serA*-encoding fragment was liberated from pRC1.55B by digestion with *Sma*I. Plasmid pON1.39A was digested with *Bam*HI and treated with Klenow fragment. Ligation of the two blunt end fragments of DNA yielded pON1.118B. This 9.0-kb pJF118EH-based plasmid encodes *ilvD* and *serA*. Digestion of pWN3.172A with *Eco*RI released a 1.8-kb fragment encoding the *ilvD* gene, which was subsequently cloned behind the P_{tac} promoter of pJF118EH by insertion into its *Eco*RI site to afford pON1.39A. A 1.7-kb *serA*-encoding fragment was liberated from pRC1.55B by digestion with *Sma*I. Plasmid pON1.39A was digested with *Bam*HI and

treated with Klenow fragment. Ligation of the two blunt end fragments of DNA yielded pON1.118B. Transformation of competent of *E. coli* JWF1 with this plasmid afforded JWF1/p1.118B.

CHAPTER 3

α-Amino-caprolactam.[18]

A stirrer mixture of L-lysine·HCl (55 g, 0.3 mol) and NaOH (12 g, 0.3 mol) in 1,2,-propanediol (1.2 L) was heated to reflux for 2 h in the presence of Dean-Stark trap. The solution was then cooled and concentrated under vacuum to afford to α-aminocaprolactam along with byproduct NaCl, which was removed by filtration.
In an alternative method, α-aminocaprolactam was obtained from heating a mixture of L-lysine in EtOH to 200 °C for 2 h inside a 4575 Parr reaction vessel. After removal of EtOH, α-aminocaprolactam was obtained as the only product as confirmed by ^{1}H NMR.

β-L-Lysine.[19]

A solution of L-ornithine·HCl (30.0 g, 0.178 mol) in 5 M NaOH (450 mL) was stirred with icebath cooling while benzyl chloroformate (63.5 mL, 0.440 mol) was added dropwise over 15 min. The resulting mixture was stirred for an additional 6 h in an icebath before it was diluted with H_2O (400 mL) and acidified to pH 5 with concentrated HCl. The solution was extracted 5x with EtOAc (200 mL) and the combined extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to afford a white wax, which was suspended in boiling petroleum ether and filtered to afford N,N'-dibenzyloxycarbonyl L-ornithine (52 g, 80%). To a solution of N,N'-dibenzyloxy-

carbonyl L-ornithine (9.15 g, 0.0250 mol) in EtOAc (250 mL) was added N-methylmorpholine (3.0 mL, 0.0275 mol). The solution was stirred at –10 °C while ethylchloroformate (4.75 mL, 0.050 mol) was introduced dropwise over 20 min. The resulting milky mixture was stirred for an addition 3 h at –10 °C before it was filtered through Celite. The resulting filtrate was combined with a 0.8 M diazomethane in diethyl ether (400 mL) and stirred at 0 °C for 6 h. Excess diazomethane was destroyed by addition of AcOH before the reaction mixture was concentrated under reduced pressure to afford diazoketone as a yellow solid (6.25 g, 85%).

The diazoketone (6.25 g, 0.0214 mol) was dissolved in MeOH (100 mL) and the reaction flask was wrapped with aluminium foil. All subsequent reactions were performed in the dark room. A solution of silver benzoate (1.25 g, 6.55 mmol) in Et_3N (20 mL) was introduced dropwise into the diazoketone solution held at 0 °C. The reaction mixture was stirred for 1 h before an additional solution of silver benzoate was introduced. The resulting muddy mixture was concentrated to dryness and the resulting residue was redissolved in EtOAc (20 mL) and subsequently washed with $NaHCO_3$ (10 mL), brine (2x 10 mL) and 5% HCl (10 mL). After the organic layer was dried and concentrated under reduced pressure, β-L-lysine ester was obtained as an orange solid (4.11 g, 65%).

Pt/SiO_3.[20]

To a solution of $H_2PtCl_6 \cdot H_2$ (2.1 g, 5.1 mmol) in distilled, deionized H_2O (60 mL) was added 1.5 g of silica gel (100-200 mesh). The water was removed under reduced pressure and the catalyst mixture was dried in oven (80 °C) for 4 h. The resulting orange

powder was ground into fine powder with pestle and motar, which was dried in an 80 °C oven for 10 h. The resulting fine powder was placed in a U-tube, which was sealed with glass wool on both ends. The U-tube was heated to 400 °C in a sand bath while passing H_2 through for 1 h, during which the catalyst turned from bright orange to black. The U- was removed from the sand bath and was cooled to 200 °C.

Denitrification of Cyclohexylamine.

Hydrodenitrogenation was conducted in a flow apparatus consisting of three U-tubes. Cyclohexylamine (8.2 g, 80 mmol) was places in the first U-tube, which was connected to a second U-tube containing reduced Pt/SiO_2. The last U-tube served as a product chamber and was maintained at -78 °C with dryice-acetone cooling. Subtrate and catalyst U-tubes were heated to 200 °C while H_2 was allowed to flow from the subtrate through to product. The vaporized cyclohexylamine was swept through the catalyst and the resulting cyclohexane was trapped in the third U-tube. After all the cyclohexylamine was evaporated, the sand bath heating was removed. Cyclohexane (6.0 g, 90%) was obtained as the only product as determined by GC.

Molybdenum Nickel Catalyst.[21]

To the solution of ammonium molybdate (58.8 g, 0.300 mol) in distilled, deionized water (300 mL) was added 70 g of ground δ-Al_2O_3. The water was removed under reduced pressure and the resulting white powder was dried in an oven (80 °C) for 12 h before it was heated to 350 °C for 6 h in a 500 mL Parr 4575 stainless steel high temperature-high pressure reactor. After cooling to rt, a black powder was obtained and

was suspended in nickel nitrate solution (58.2 g, 0.200 mol) in distilled, deionized water (200 mL). Following removal of water in vacuo, the catalyst mixture was dried in an oven for 6 h before it was heated to 400 °C for an addition 6 h in a Parr apparatus. Prior to the reaction, the Mo-Ni/Al_2O_3 catalyst was treated with a mixture of H_2S (10%) and H_2 (90%) at 400 °C and 500 psi for 4 h.

Deamination of Methyl Pipecolinate.[22]

Methyl pipecolinate (0.53 g, 3.7 mmol) and tetrabutylammonium iodide (4.1 g, 11 mmol)were placed in a round-bottom flask. 1,3-Propanedithiol (3.7 mL, 37 mmol), *N*-methylmorpholine (1.2 mL, 11 mmol) and THF (10 mL) were introduced into the flask, which was subsequently stirred at rt 12 h. The mixture was then refluxed for an additional 12 h. After removal of solvent under vacuum, unreacted starting was recovered.

Deamination with SmI_2.[23]

Into a dry two-neck round-bottom flask was placed Sm powder (1.0 g, 6.7 mmol) in THF (12 mL), HMPA (12 mL, 6 7 mmol) L-lysine methyl ester (0.15 g, 1.0 mmol), and MeOH (0.12 mL, 3.0 mmol). The flask was stirred at rt under Ar. Diiodomethane (0.5 mL, 5.8 mmol) was added dropwise over 5 min. Effervescence was observed and the reaction mixture was cooled to –10 °C. The color changed from olive green to olive green after 2 h of stirring. The reaction was quenched with $NaHCO_3$ and MeOH filtered through Celite. The filtrate was concentrated under vacuum to afford a light brown residue. The yield of methyl 6-aminocaproate ester was determined by integration of the

2.5 ppm triplet relative to TSP internal standard centered at 0.0 ppm. The yield of caprolactam was 20 %.

Deamination af aminocaprolactam with Na.[24]

L-2-Aminocaprolactam (0.64 g, 5 mmol) was dissolved in 2-propanol (40 mL) at rt. Na pieces (0.53 g, 23 mmol) were added in small chunks over 1 h. The reaction mixture was stirred at rt until all the Na dissolved. Excess Na was destroyed by addition of acidic methanol. The mixture was concentrated to dryness and ε-caprolactam was quantified by integration of its 2.45 ppm signal on ^{1}H NMR against TSP internal standard (0.0 ppm).

Expression of *yodO* from *B. subtilis.*[25]

Genomic DNA[10] was isolated from *B. subtilis* (ATCC 15818) cells grown in Nutrient Broth (8 g/L, Difco). The *yodO* gene encoding L-lysine-2,3-aminomutase was amplified from *B. subtilis* genomic DNA using the following primer: 5'–TATACATATGAAAAACAAATG GTATAAACCGAAACGGCATTGGAAGG and 5'-TAGACTCGAGTCATGAAGAATCCCCTCCGCATTCAGTCTCTTTCTG. *Nde*I and *Xho*I (underlined) restriction sequences were included to facilitate cloning. Digestion of pMM4.166 with *Xho*I and *Nde*I afforded a 1.4-kb *yodO* fragment, which was subsequently localized in *Xho*I and *Nde*I-digested pET-23a(+) to provide pMM4.197. For expression of *Bacillus subtilis yod*O in *E. coli*, pMM4.197 was transformed into competent BL21(DE3) *E. coli* cells.

Culturing of *Clostridium subterminale*.

Clostridium subterminale SB4 was obtained from the American Type Culture Collection (ATCC 29748). Cells were grown in a medium (1 L) containing yeast extract (6 g), L-lysine·HCl (6 g), 1 M phosphate buffer (45 mL), and 1 M K_2CO_3 (7.5 mL) at pH 7.5. After the medium was autoclaved in a Pyrex bottle, it was purged with N_2 while it was allowed to cool to rt. All subsequent manipulations were performed in a Coy anaerobic chamber. Sodium dithionite (30 mg) was added to the medium inside a Coy anaerobic chamber. Cultures were initiated by inoculating a single colony into 5 mL of semisolid (0.2% agar) clostridium medium. The test tubes were sealed tightly with septa and the inoculants were cultures at 37 °C with agitation at 250 rpm for 12 h. The 5 mL semisolid aliquot was subsequently transferred (inside a Coy chamber) to a 100 mL containing clostridium medium. The cells were grown at 37 °C with agitation at 150 rpm for 12 h and subsequently transferred to 1 L of growth medium, which was cultured for 12-18 h at 37 °C to OD_{600} ~2-3.2. Following centrifugation at 9,000*g* for 10 min, approximately 2-4 g of wet cells were obtained. Precaution was taken not to expose cell to air by conducting all transfers inside the Coy chamber.

Crude Lysate Bioconversion.[26]

Clostridium SB4 culture was harvested after 4 h (OD_{600}~ 0.5) by centrifugation at at 9,000*g* for 10 min to afford approximately 0.4-0.7 g of wet cells/L. All solutions were purged with N_2 before use and only opened inside the Coy chamber. Cell were only opened inside the Coy chamber and the pellet was resuspended in phosphate buffer (0.1 M, pH 7.5) and treated with a 60-watt ultrasonic homogenizer for 10 minutes or by

passage through a French press (12,000 psi). After removal of cell debris by centrifugation at 20,000*g* for 5 min, the protein concentration was between 8-15 mg/mL. The crude extracts were quick frozen in liquid N_2 and stored at -20 °C for 1-14 days. The crude lysate was thawed in an icebath before use. A 10 mL reaction mixture contained 60 mM L-lysine, 25 mM tris-HCl (pH 7.5), 5 mM potassium phosphate (pH 7.5), 10 mM α-ketoglutarate, 10 mg pyridoxal-5-phosphate 0.2 mM CoA, 0.2 mM *S*-adenosylmethionine, 5-20 mM glutathione, and 10 mg of protein. The reaction mixture was incubated in a 37 °C shaker (250 rpm) for 8 h. The reaction was quenched by addition of HCl to pH 2.5. The precipitated protein was removed by centifugation at 20,000*g* for 5 min. The supernatant was concentrated under vacuum to afford a yellow residue, which was redissolved in 1 mL of D_2O and concentrated three times. ^1H NMR was used to identify reaction product, L-β-lysine by a ddd centered at 2.8 ppm. Response factor obtained by integration of this peak relative to TSP (0.0 ppm) was used to calculate the yield of L-β-lysine.

Intact cell bioconversion.

Clostridium SB4 cells harvested after 18 h (OD_{600} ~ 2) from a 4 L medium were resuspended in a reaction mixture, previously purged with N_2, containing 150 mM L-lysine, 80 mM Tris-HCl (pH 7.5), 5 mM potassium phosphate (pH 7.5), and $FeSO_4$ (3 mM). The reaction mixture contained ~20 g of cells/250 mL Pyrex bottle, which was sealed with a screw-cap. The reaction mixture was incubated in a 37 °C shaker (150 rpm) with tungsten lamp (75 watt, 120 V) directly shone at the Pyrex bottles for 24 h. The reaction mixture was centrifuged at 9,000*g* to remove intact cells and the resulting

supernatant was concentrated to 20 mL, which was then acidified to pH 2.5 with HCl. A mL of the product mixture was concentrated to dryness and the residue was redissolved in D_2O (1 mL) and concentrated three times. L-β-Lysine was quantified by integration of its 2.8 ppm peak relative to TSP internal standard.

Purification of β-L-lysine.[27]

The main reaction mixture was acidified to pH 2.5, centrifuged to remove protein, and then loaded on the Dowex 50 (H^+) column (3 x 40 cm) previously washed with 10 mM HCl. The L-lysine and L-β-lysine were differentially eluted with 0.2 M sodium formate buffer, pH 2.75, containing 0.35 M NaCl. Fractions of 10 mL were collected after ~200 mL of eluant was discarded. L-Lysine eluted first in the fractions 22-56 and L-β-lysine in fractions 78-115. The L-β-lysine fraction were pooled and concentrated to 50 mL and loaded on a fresh Dowex 50-column and washed with water. The amino acid was retained on the column and then was eluted with 1 N ammonium hydroxide, which came out as a single 40-mL fraction. The L-β-lysine solution was evaporated under reduced pressure to remove excess ammonia and the resulting yellow solid was redissolved in EtOH and concentrated several times to a to afford a yellow solid in 43% isolated yield based on starting L-lysine. L-β-Lysine obtained was very hygroscopic and was used in the next step.

Deamination of L-β-lysine with Ru on C.

A solution of L-β-lysine (0.4 g, 2.8 mmol) was dissolved in EtOH (80 mL) in a glass reaction vessel. 5 mol % of Ru/Al_2O_3 (0.28 g, 0.14 mmol) was suspended in the

reaction solution. The glass reaction vessel was inserted into the 500 mL Parr 4575 stainless steel high temperature-high pressure reactor and the vessel sealed. The temperature and the stirring rate were controlled by a Parr 4842 temperature controller. Hydrogen was bubbled through the reaction mixture for 10-15 min to remove air while stirring at 100 rpm. The vessel was heated to 200 °C and stirred for 2 h. After 2 h, the reaction vessel before it was pressurized with 1,000 psi H_2 pressure. The reaction was stirred at 400 rpm for another 2 h. After removal of the catalyst by filtration, the reaction mixture was concentrated to dryness under vacuum to afford a brown residue. Quantification was conducted by integration of the caprolactam (13%) peak centered at 2.5 ppm relative to TSP as an internal standard.

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