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Optically Active β-Methyl-δ-Valerolactone: Biosynthesis and Polymerization

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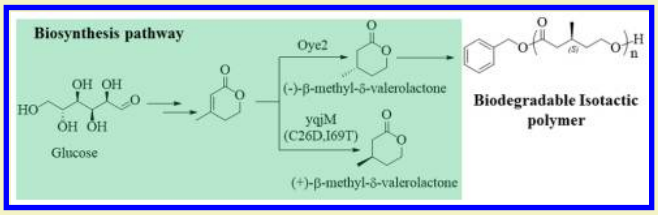
Supporting Information

ABSTRACT: Chemo-enzymatic pathways were developed to prepare optically enriched (+)-β-methyl-δ-valerolactone and (−)-β-methyl-δ-valerolactone. Anhydromevalonolactone, synthesized by the acid-catalyzed dehydration of bioderived mevalonate, was transformed to (+)-β-methyl-δ-valerolactone with 76% ee and 69% conversion using the mutant enoate reductase, YqjM(C26D, I69T). With the same substrate but a different enoate reductase (OYE2), we obtained the other enantiomer ((−)-β-methyl-δ-valerolactone) with higher selectivity and yield (96% ee and a 92% conversion). The enzyme-docking program LibDock was used to help explain the origin of the divergent enatntioselectivity of the two reductases, and complementary *in vitro* experiments were used to determine the turnover number and Michaelis constant for each. Finally, the effect of the enantiopurity of the β-methyl-δ-valerolactone monomer on the properties of the corresponding polyester was investigated. Like atactic poly((±)-β-methyl-δ-valerolactone), the isotactic polymer was determined to be amorphous with a low softening temperature (−52 °C).



KEYWORDS: *Biosynthesis, Chemo-enzymatic pathways, Enatntioselectivity, Isotactic polymer, Thermal properties*

INTRODUCTION

Medium-sized lactones are a common structural motif in biologically active molecules including plant growth inhibitors, insect antifeedants, and antitumor agents.[1−3] In particular, the potential value of optically active δ-lactones as precursors for the synthesis of complex natural products has made the efficient asymmetric synthetic of these molecules an important and long-standing research challenge for chemists and biochemists alike.[4,5] More recently, these versatile molecules have also found additional use as monomers for the preparation of biodegradable aliphatic polyesters.[6−8] Importantly, it has been demonstrated that with many polyesters altering stereochemical composition can be used to tune key physical properties including degradation rate, thermal, and mechanical properties.[6,7]

Myriad synthetic strategies have been used for the synthesis of optically active δ-lactones, however only a few biocatalytic methods have been developed.[4,9] Of these, the lipase-based resolution of racemic lactones has been most widely explored.[10] Notably, optically active polymers have also been prepared from the lipase-catalyzed polymerization of racemic ε-lactones. However; kinetic resolution strategies are problematic because, to achive high optical purity, the reaction must be stopped at low conversion.[7] Asymmetric synthesis methods, such as lactonization of diols using dehydrogenases,[11−13] oxidation of cyclic ketones using monooxygenase enzymes,[14,15] and reduction of unsaturated lactone precursors,[3,16−21] do not suffer from this limitation.

We recently developed a biosynthetic route for the production of β-methyl-δ-valerolactone (MVL) from glucose.[22] The final step in our pathway utilizes an enoate reductase of the old yellow enzyme family (OYE2 or YqjM(C26D, I69T)) to reduce anhydromevalonolactone (AMVL). Because the titers of MVL prepared using biosynthetic route were low, we also developed a semisynthetic route whereby bioderived mevalonate is chemically dehydrated to AML and hydrogenated to prepare MVL.[22] Since the hydrogenation catalyst used is not steroselective, the semisynthetic route yields a racemic mixture of MVL. While we envisioned that our total biosynthetic routes could result in optically active products,[13,23−25] the stereoselectivities of reductases (YqjM(C26D, I69T) and OYE2) were not determined. We also hypothesized that the tacticity of PMVL might effect its thermal properties but were unable to

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test this hypothesis without optically pure (+)-MVL or (−)-MVL.

Here, AMVL prepared from the acid-catalyzed dehydration of bioderived mevalonate is transformed into optically active MVL by either YqjM(C26D, I69T) or OYE2. After 72 h, OYE2 transformed anhydromevalonate to (−)-MVL with good conversion (91.5%) and stereoselectivity (96% ee). Fermentation with YqjM(C26D, I69T) resulted in the production of (+)-MVL, albeit with slightly lower selectivity and conversion (76% ee and 68.9%, respectively). We discuss the mechanistic origins of selectivity of YqjM(C26D, I69T) and OYE2 and compare these enzymes to asymmetric hydrogenation catalysts previous used to prepare optically enriched MVL.[26,27] Finally, we investigated the effect of polymer tacticity on the thermal properties of poly((−)-β-methyl-δ-valerolactone).

METHODS AND MATERIALS

Materials. Benzyl alcohol (Sigma-Aldrich) was purchased and used as received. Diphenyl phosphate (Sigma-Aldrich) was purchased and dried in a vacuum oven at room temperature for 96 h. Both benzyl alcohol and diphenyl phosphate were handled and stored in a glovebox with a nitrogen atmosphere. Reagent grade chloroform and methanol were purchased from Fisher Scientific and used as received. Reagents used in fermentation and culture processes were purchased from Sigma-Aldrich unless otherwise specified. FastDigest restriction enzymes were purchased from Thermo Scientific. Quick ligation, and Phusion high-fidelity PCR kits were supplied by New England Biolabs. Oligonucleotides were ordered from Eurofins MWG Operon.

Characterization. Fermentation products were analyzed using an Agilent 1260 Infinity HPLC equipped with an Aminex HPX-87H column and a refractive index detector. The mobile phase was 5 mM aqueous H_2SO_4 at a flow rate of 0.6 mL/min. The column and detector temperatures were 35 and 50 °C, respectively. The purity of anhydromevalonate precursor and β-methyl-δ-valerolactone monomer was verified using tandem gas chromatography/low resolution mass spectrometry (GC/MS) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR). GC/MS was performed on a mass selective detector that used electron impact (EI) ionization. Samples were prepared in ethyl acetate with a concentration of ∼0.1 mg/mL.

Optical rotations of (+)-β-methyl-δ-valerolactone and (−)-β-methyl-δ-valerolactone were identified using a digital polarimeter with a 100 mm cell. Samples were prepared in chloroform, and data were collected at room temperature. A reference optical rotation of $[\alpha^{23}]_D$ = −27.55 was used to calculate ee.[28] The identity of each previously characterized compound used in this work was established by ^{1}H NMR spectroscopy.^{1}H NMR spectra were collected from $CDCl_3$ solution on a Varian INOVA-500 spectrometer operating at 500 MHz. ^{1}H NMR spectra are reported as the average of at least 24 scans and were acquired using a 5 s acquisition time and a 10 s delay; chemical shifts are referenced to the protic solvent peak at 7.26 ppm.

Polymer mass-average molar mass (M_w) and dispersity ($Đ$) were determined using a size exclusion chromatography instrument with THF as the mobile phase at 25 °C and a flow rate of 1 mL min^{-1}. Size exclusion was performed with three successive Phenomenex Phenogel-5 columns. The mass average molar mass and dispersity were identified using the known concentration of the sample in THF and the assumption of 100% mass recovery to calculate dn/dc from the RI signal. Differential scanning calorimetry was conducted on a TA Instruments Q-1000 DSC. Samples were analyzed in hermetically sealed aluminum pans. The samples were equilibrated at −80 °C, heated to 100 °C at 10 °C min^{-1}, cooled to −80 °C at 5 °C min^{-1}, and then reheated to 100 °C at the same rate. In a separate experiment, a polymer sample was heated to 100 °C at 5 °C min^{-1} and cooled to 20 °C at 5 °C min^{-1} and then annealed at room temperature (20 ± 4 °C) for 90 days. The sample was then heated to 100 °C at a rate of 5 °C min^{-1}.

Bacterial Strain and Plasmid Construction. Bacterial strains and plasmids used in this study are described in Table S1. *E. coli* strain XL10-Gold was used in plasmid construction processes. *E. coli* strain BW25113 was used in fermentation experiments. *E. coli* strain BL21 was used in protein expression and purification experiments. Enoate reductases OYE2 from *S. cerevisiae* and YqjM from *Bacillus subtilis* were used for biosynthesis of enantiopure MVL. The OYE2 fragment was first amplified from *S. cerevisiae* genomic DNA with primers OYE2-*Eco*RI-F (cacacagaattcattaaagaggagaaattaactATGCCATTTGT-TAAGGACTTTAAGCC) and OYE2-XbaI-R (gctgcttctaga-TTAATTTTTGTCCCAACCGAGTTTTA), and then cloned into a pZE vector at *Eco*RI and XbaI sites to form the plasmid pZE-OYE2. YqjM(C26D, I69T) was PCR amplified with primers YqjM-*Eco*RI-F (gctgctgaattcattaaagaggagaaaggtaccatgGCCAGAAAATT-ATTTACACCTAT) and YqjM-XbaI-R (gctgcttctagagctagc-TTACCAGCCTCTTTCGTATTGAACAG) using pMVL-4 as the template, and then cloned into a pZE vector at *Eco*RI and XbaI sites to form the plasmid pZE-yqjM(C26D, I69T).

Vectors (pZE-his6-OYE2 and pZE-his6-yqjM(C26D, I69T)) containing an *n*-terminal Hexa-Histidine (6His) affinity tag were constructed for enzyme purification and characterization. The OYE2 gene was amplified by PCR with primers hisOYE2-*Bam*HI−F (ggatcgcatcaccatcaccatcacggatccATGCCATTTGTTAA-GGACTTTAAGCCACAg) and hisOYE2-XbaI-R (ttttatttgatgcctctaga-TTAATTTTTGTCCCAACCGA) and then inserted into a pZE vector to form pZE-his6-OYE2. YqjM(C26D, I69T) was amplified by PCR with primers hisYqjM-*Bam*HI−F (ggatcgcatcaccatcacc-atcacggatccatgGCCAGAAAATTATTTACACCTATTACA) and hi-sYqjM-XbaI-R (ttttatttgatgcctctagattaCCAGCCTCTTTCGTATT) using pMVL-4 as the template, and then cloned into a pZE vector to form the plasmid pZE-his6-yqjM(C26D, I69T)

Fermentation Methods. *Batch Fermentations.* Anhydromevalonate, used as a substrate for all fermentation reactions and enzyme assays, was produced using the methods previously described.[22] For shake flask fermentation, 125 mL conical flasks containing 0.5 g of $CaCO_3$ were autoclaved and dried. Then 5.0 mL of M9 medium supplemented with yeast extract (5 g L^{-1}), glucose (to a final concentration of 40 g L^{-1}), IPTG (0.1 mM), anhydromevalonate (1 g L^{-1}), and ampicillin (100 mg L^{-1}) were added to the sterilized shake flask. The additional glucose was supplied to support cell growth and to provide the reducing power for reductases OYE2 or YqjM(C26D, I69T). The shaker flasks were inoculated with overnight cultures of the appropriate recombinant strain in 2XYT medium (200 μL) and then incubated in a 30 °C shaker for 72 h with a shaker speed of 250 rpm.

Semibatch Fermentations. Fed-batch fermentations were conducted in 1.3-L benchtop bioreactor (New Brunswick Bioflo 115). The primary culture media used for fed batch fermentations was composed of glucose (10 g L^{-1}), K_2HPO_4 (7.5 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (2 g L^{-1}), citric acid monohydrate (2 g L^{-1}), ferric ammonium citrate (0.3 g L^{-1}), yeast extract (20 g L^{-1}), sulfuric acid (98%, 0.8 mL L^{-1}), ampicillin (0.1 g L^{-1}), 1000X modified trace metal solution (1 mL L^{-1}), and vitamin solution (1 mL L^{-1}). The 1000X modified trace metal solution contained NaCl (10 g L^{-1}), citric acid (40 g L^{-1}), $ZnSO_4{\cdot}7H_2O$ (1.0 g L^{-1}), $MnSO_4{\cdot}H_2O$ (30 g L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.1 g L^{-1}), H_3BO_3 (0.1 g L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.1 g L^{-1}), $FeSO_4{\cdot}7H_2O$ (1 g L^{-1}), and $CoCl_2{\cdot}6H_2O$ (1 g L^{-1}). The vitamin solution was composed of thiamine hydrochloride (1 g L^{-1}), (+)-biotin (1 g L^{-1}), nicotinic acid (1 g L^{-1}), pyridoxine hydrochloride (4 g L^{-1}). Inoculum was prepared by adding 1% overnight preculture to a sterile shake flask containing 50 mL LB medium and 0.1 g/L ampicillin. This flask was incubated at 37 °C until the contents reached an optical density (OD_{600}) of 1.0. The cell solution was then transferred to the benchtop bioreactor, and culture media was added to bring the initial volume to 0.5 L. The reactor was incubated in batch mode at 37 °C until the OD_{600} reached 6.0. Then the temperature was decreased to 30 °C, and IPTG (to a concentration of 0.3 mM) was added. Following induction with IPTG, a substrate solution containing AMVL (100 g L^{-1}) in water was fed into the bioreactor to reach a concentration of 1 g L^{-1}. A second solution composed of glucose (600 g L^{-1}), K_2HPO_4 (7.4 g L^{-1}), ampicillin (0.1 g L^{-1}), and IPTG (0.3 mM), modified trace metal solution (1 mL L^{-1}), and vitamin solution (8 mL L^{-1}) was fed at a rate determined by the dissolved oxygen (DO) level. Throughout the

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fermentation, the pH was maintained (at ~6.8) via addition of a 26 wt % aqueous NH_4OH solution. The DO level was maintained above 10% by adjusting agitation rate (from 300 to 800 rpm). The airflow rate was maintained at 0.5 vvm in order to provide oxygen for cells. Throughout the fermentation, concentrations of starting materials and of metabolites were monitored using HPLC.

Protein Expression and Purification. The BL21 *E. coli* strain was transformed with the His-tagged plasmids pZE-his6-OYE2 or pZE-his6-yqjM(C26D, I69T). The recombinant cells were inoculated from an overnight preculture at 1/100 dilution and grown in 500 mL of LB medium containing ampicillin (100 mg L^{-1}) at 30 °C. After the OD_{600} reached 0.6, IPTG (0.5 mM) was added. Following induction, the cells were incubated at 30 °C overnight. Then the cells were harvested by centrifugation. All the subsequent steps were carried out at 4 °C to prevent protein degradation. To lyse the cells, the pellets were resuspended in ~15 mL lysis buffer (composed of 50 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole, 5% glycerol, 1 mM DTT, pH 7.6) and sonicated using a Heat Systems Ultrasonics W-225 sonicator. The extracts were clarified by centrifuging at 10,733 rcf for 15 min. The supernatants were passed through a gravity column containing 4 mL of HisPur Ni-NTA resin (Thermo Scientific). The resin was washed twice with 10 mL of wash buffer (50 mM Tris-HCl, 100 mM NaCl, and 25 mM imidazole, pH 7.6). The bound protein was eluted with 15 mL of elution buffer (50 mM Tris-HCl, 250 mM NaCl, and 250 mM imidazole, pH 8.0). The final protein sample was then purified using buffer-exchanged with Amicon Ultra centrifugal filters (Millipore) and a storage buffer (50 M Tris-HCl, 2 mM $MgSO_4$, 20% glycerol, pH 8.0). The purified protein concentration was determined by a Quick Start Bradford protein assay kit (Bio-Rad Laboratories).

Enzymatic Activity Assays. The reductase activities of OYE2 and YqjM(C26D, I69T) were assessed using NADPH initial consumption rates as determined using a coupled assay. The assay solution was composed of 1 mM NADPH and 0.1−100 mM AMVL in 100 μL of 100 mM Tris-HCl buffer (pH 7.5) with 5 mM $MgSO_4$. Purified enzyme (100 nM of either OYE2 or YqjM(C26D, I69T)) was added to start the reaction, and the NADPH consumption rate was monitored spectroscopically. For each enzyme, three replicate assays were conducted. These data were used to fit the parameters of the Michaelis−Menten kinetic model, k_{cat} and K_m, by nonlinear least-squares regression using the intrinsic *nlinfit* function of the Matlab software program.

Enzyme Docking. All molecular and enzyme docking was performed with Libdock utilizing Discovery Studio 4.1 software. Both PDB files of OYE2 (PDB ID: 4GXM) and YqjM (PDB ID: 1Z42) were downloaded from the Protein Data Bank.[29] The active sites denoted in crystal structure were defined as the binding site for Libdock. Since the crystal structure of YqjM(C26D, I69T) has not been reported, we used sequence alignment and homology modeling in Discovery Studio 4.1 software to build the structure of this mutant YqjM. Subsequently, Libdock was used to analyze the interaction between OYE2 or YqjM(C26D, I69T) and anhydromevalonolactone.

Synthetic Methods. *Anhydromevalonolactone.* The pathway for enzymatic synthesis of mevalonate from glucose has previously been reported.[22] The mevalonate fermentation broth (containing 28 g L^{-1} mevalonate as determined by HPLC) was filtered through activated charcoal to remove cell debris and colored impurities. Then, concentrated H_2SO_4 (200 mL) was slowly charged into the filtered fermentation broth (2 L) under vigorous stirring. The acid solution was refluxed for 15 h, then cooled and extracted with chloroform. The chloroform was removed under reduced pressure, and the crude product was distilled to yield pure AMVL (39 g, 82% gravimetric yield).

Purification of (−)-β-Methyl-δ-valerolactone. To the fermentation broth (1L), chloroform (200 mL) was added to extract residual AMVL. Among numerous potential green solvents, ethyl acetate or isobutanol may be viable, less toxic alternatives to chloroform. The aqueous layer was carefully acidified with H_2SO_4 (to pH ~ 2) and then extracted using chloroform (3X 200 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated under reduced pressure to obtain crude (−)-MVL (0.5 g). Prior to polymerization, this monomer was dried over calcium hydride and distilled. ^{1}H NMR: 4.47−4.36 ppm (m, 1H CH_2CH_2OR) 4.47−4.36 ppm (m, 1H CH_2CH_2OR) 2.76−2.60 ppm (m, 1H $CO_2CH_2CHCH_3$), 2.180−2.05 (m, 1H $CO_2CH_2CHCH_3CH_2$), 1.97−1.87 ppm (m, 1H $CO_2CH_2CHCH_3$), 1.60−1.44 ppm (m, 1H $CH_2CHCH_3CH_2$), 1.1 ppm (d, 3H $CO_2CH_2CHCH_3$), $[\alpha^{23}]_D$ = −25.7 ($CHCl_3$).

(+)-β-Methyl-δ-valerolactone. (+)-MVL was obtained using the method described above, except the crude MVL was purified by column chromatography (silica gel, 30:70 ethyl acetate:hexanes), dried over $MgSO_4$, and filtered. ^{1}H NMR: 4.47−4.36 ppm (m, 1H CH_2CH_2OR) 4.47−4.36 ppm (m, 1H CH_2CH_2OR) 2.76−2.60 ppm (m, 1H $CO_2CH_2CHCH_3$), 2.180−2.05 (m, 1H $CO_2CH_2CHCH_3CH_2$), 1.97−1.87 ppm (m, 1H $CO_2CH_2CHCH_3$), 1.60−1.44 ppm (m, 1H CH_2CH1CH_2), 1.1 ppm (d, 3H $CO_2CH_2CHCH_3$), $[\alpha^{23}]_D$ = +20.9 ($CHCl_3$).

Synthesis of Poly((−)-β-methyl-δ-valerolactone). Benzyl alcohol (2.7 mg) was mixed with (−)-MVL (0.5345 g) in a glass vial with magnetic stirring. When fully dissolved diphenyl phosphate (1.7 mg) was added to initiate the polymerization. The reaction was stirred for 8 h at room temperature. The obtained polymer was purified by precipitation in methanol from dichloromethane/chloroform and subsequently dried. ^{1}H NMR: kg mol^{-1}; 7.37−7.33 ppm (m, 5H Ar-H), 5.14 ppm (s, 2H Ar−CH_2O), 4.19−4.05 ppm (170 H, CH_2OR), 3.73−3.62 ppm (3.3 H, CH_2OH), 2.36−2.30 ppm (m, 80H CO_2CH_2CH), 2.20−2.15 ppm (m, 80H $CO_2CH_2CH_2$), 2.14−2.03 ppm (m, 95H, $CH_2CHCH_3CH_2$), 1.74−1.65 ppm (m, 87H $CH_2CHCH_3CH_2$), 1.57−1.48 ppm (m, 87H $CH_2CHCH_3CH_2$), 1.03 ppm (d, 259H $CH_2CHCH_3CH_2$); MALLS-SEC: M_n = 7.8 kg mol^{-1}, $Đ$ = 1.03; $[\alpha^{23}]_D$ = −7.5 ($CHCl_3$); DSC: T_g = −52.5 °C.

RESULTS

Stereoselective Reduction of AMVL Using Enoate Reductases. Previously, the dehydration of mevalonate to AMVL was found to be the rate-limiting step in the biosynthesis of MVL.[22] Here, we performed the dehydration chemically by adding sulfuric acid directly to the mevalonate fermentation broth. After refluxing for 15 h, the solution was extracted with chloroform to obtain AMVL in high yields (typically, 80%−90% based on the starting concentration of mevalonate as monitored by GC). *E. coli* strains overexpressing an enoate reductase were used to convert AMVL to optically enriched β-methyl-δ-valerolactone. As described in Scheme 1, (−)-β-methyl-δ-valerolactone and (+)-β-methyl-δ-valerolactone were produced by strains expressing OYE2 and YqjM-(C26D, I69T), respectively.

During the fermentation process, glucose (40 g L^{-1}) was supplied to support cell growth and provide cofactors/reducing power for the reduction of AMVL. However, since only the reductase was overexpressed, no glucose was directly converted to MVL. For the OYE2-overexpressing strain (Figure 1a), the concentration of glucose gradually decreased (from 40 to 9.7 g L^{-1} after 72 h). AMVL (1.1 g L^{-1}) was added to the fermentation broth as the substrate. After an initial lag period, MVL increased monotonically (to approximately 1.1 g L^{-1}). The overall conversion of anhydromevalonate reached 92% at the end of fermentation.

Fermentation with the YqjM(C26D, I69T)-overexpressing strain (Figure 1b) was qualitatively similar. Again, no MVL was detected in the first 12 h, while about 0.2 g L^{-1} AMVL was consumed in this period of time. The final concentration of MVL reached 0.8 g L^{-1}. After 72 h, the overall conversion was 69%, slightly lower than that of the fermentation with the OYE2-overexpressing strain. Comparatively, less glucose was consumed and more acetate accumulated (8.1 g L^{-1} was consumed after 72 h).

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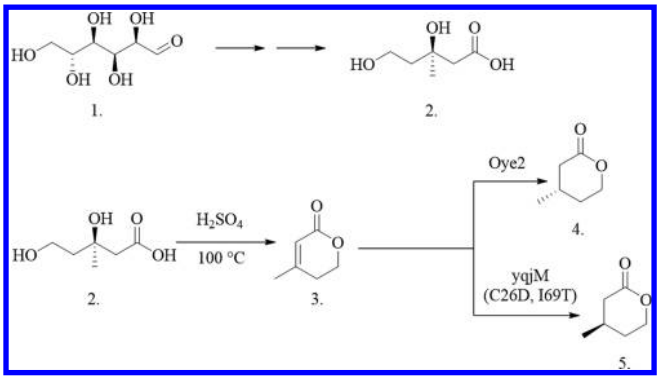
Scheme 1. Chemo-Enzymatic Synthesis of Enantiopure β-Methyl-δ-valerolactone[a]



[a]Glucose (1) is transformed biosynthetically to mevalonate (2). Subsequent chemical dehydration yields AMVL (3). Enzymatic reduction with OYE2 or YqjM(C26D, I69T) yields (−)-β-methyl-δ-valerolactone or (+)-β-methyl-δ-valerolactone, respectively.

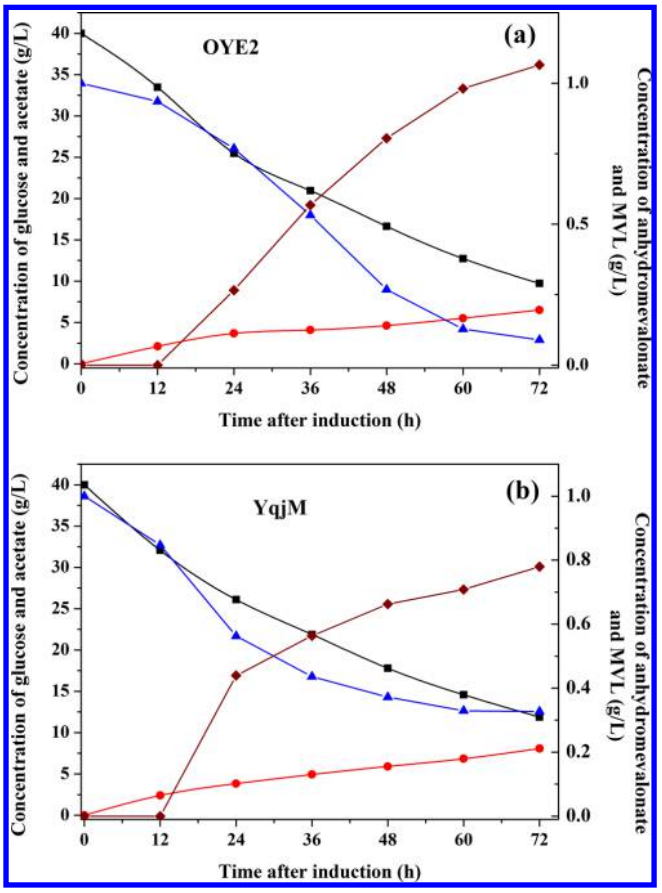


Figure 1. Shake flask fermentation results with (a) BW25113 transformed with pZE-OYE2 and (b) BW25113 transformed with pZE-YqjM(C26D, I69T) showing concentration of glucose (black square), acetate (red circle), anhydromevalonolactone (blue triangle), and β-methyl-δ-valerolactone (burgundy diamond).

We then compared the *in vivo* activity of the respective enoate reductases to their *in vitro* activity. As shown in Figure S1, we first used SDS-PAGE to verify that OYE2 and YqjM were overexpressed by the respective recombinant cells. OYE2 and YqjM(C26D, I69T) were then extracted from the cells and purified; the activity of each enzyme was studied with a coupled assay. Using AMVL as the substrate, we determined the kinetic parameters of OYE2 (K_M = 90 ± 5 mM and k_{cat} = 0.04 ± 0.02 s^{-1}) by monitoring the initial consumption rates of NADPH. Purified YqjM(C26D, I69T) has a faster turnover rate and a lower Michaelis constant (K_M as 61 ± 6 mM and k_{cat} = 0.3 ± 0.05 s^{-1}). Although YqjM(C26D, I69T) showed better *in vitro* activity than OYE2, we found that OYE2 had a higher conversion in fermentation experiments.

Purification and Characterization of β-Methyl-δ-valerolactone. To isolate MVL, the fermentation broth was first washed with chloroform to remove residual AMVL. The solution was acidified with H_2SO_4 and then washed with chloroform to extract MVL. The product was then concentrated and distilled. ^{1}H NMR spectroscopy and GC-MS were used to verify the purity of the product. The optical rotation of the isolated products was used to estimate the enantioselectivity of OYE and YqjM(C26D, I69T). Whereas fermentation with the strain transformed with pZE-OYE2 yielded (−)-MVL (96% ee), the strain transformed with pZE-yqjM(C26D, I69T) was selective for (+)-MVL (76% ee).

Scale-up Synthesis of (−)-β-Methyl-δ-valerolactone. Because the yield and selectivity of fermentation with the strain expressing pZE-OYE2 were higher than those of the strain transformed with pZE-yqjM(C26D, I69T), we chose to use WT strain transformed with pZE-OYE2 for a fed-batch biosynthesis. Scale-up synthesis of (−)-MVL was conducted in a 1.3 L bioreactor. The fed-batch fermentation resulted in a final concentration of 1.0 g L^{-1} (−)-MVL after 48 h. The conversion of (−)-MVL was greater than 93%. The (−)-MVL thus obtained was purified and described previously and polymerized to examine the effect of optical purity on polymer properties.

Synthesis of Poly((−)-β-methyl-δ-valerolactone). To examine the impact of monomer optical purity on polymer properties, we prepared poly((−)-β-methyl-δ-valerolactone) from optically enriched (−)-MVL. Due to the low ceiling temperature of this monomer, the reaction was conducted at room temperature to ensure a high monomer conversion. Benzyl alcohol and diphenyl phosphate were used as an initiator and catalyst, respectively. Using SEC, the molar mass of the polymer was estimated as 7.8 kg mol^{-1}, while the molar mass based on ^{1}H NMR end-group analysis was about 9.9 kg mol^{-1}. The molar mass of the isolated polymer was lower than the theoretical molar mass (calculated from the ratio of MVL monomer to added initiator, e.g., $M_{N\ (target)}$ = $[MVL]_0/[BnOH]_0$ ×114.1 = 21.4 kg mol^{-1}). As the polymer exhibits a narrow molecular weight distribution, we believe this discrepancy is most likely due to the presence of adventitious initiators in the monomer.

The thermal transitions of synthesized polyester were evaluated using differential scanning calorimetry. Initial experiments indicated that, like poly(±)-β-methyl-δ-valerolactone, poly(−)−β-methyl-δ-valerolactone is amorphous with a low glass transition temperature (T_g = −52.5 °C) (Figure 2). To verify these results, the sample was annealed 90 days at room temperature then retested; again no melting endotherm was observed on the initial heating ramp.

DISCUSSION

Enzymes in the Old Yellow Enzyme (OYE) family derive their yellow color from noncovalently bound flavin mononucleotide (FMN) cofactor. Both OYE2 and YqjM catalyze alkene reduction reactions by the net trans-addition of H_2. Since the relative positions of FMN and the tyrosine residue are fixed in

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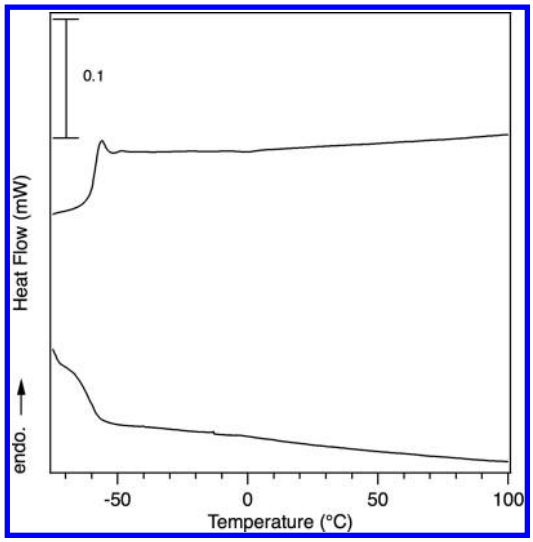

Figure 2. DSC thermogram (endotherm up) of poly(−)-β-methyl-δ-valerolactone. To ensure consistent thermal histories all samples were heated to 100 °C (rate of 5 °C min^{-1}) and cooled at the same rate. The two lines shown are the heating and cooling on the second cycle (ramp rate of 5 °C min^{-1}). To verify that the sample was amorphous, the sample was annealed 90 days at room temperature and heated again to 100 °C (rate of 5 °C min-1); no melting endotherm was observed on the initial heating ramp [data not shown].

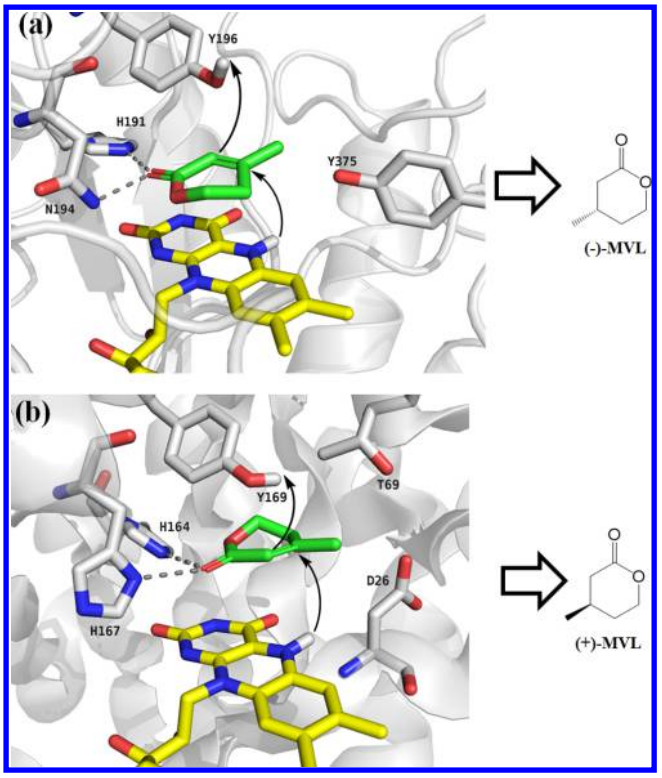

Figure 3. (a) Binding orientation AMVL in enzyme OYE2 and the resulting product, (−)-MVL. N5 of the FMN cofactor adds a hydride to the β-carbon from bottom, and Y196 adds another hydride to the α-carbon from top. PDB ID: 3TX9. (b) The binding orientation AMVL in enzyme YqjM(C26D, I69T) and the resulting product, (+)-MVL. N5 of the FMN cofactor adds a hydride to the β-carbon from bottom and Y196 adds another hydride to the α-carbon from top.

the active site, the orientation of the substrate in the binding pocket determines the stereoselectivity of the enzyme.

Enzyme OYE2 binds different substrates with different orientations mainly because of the steric effects caused by amino acid residue Y375 or W116. In this study, we found that AMVL was reduced steroselectively by OYE2 to (−)-MVL (96% ee); this observation suggests that OYE2 binds AMVL in the orientation shown in Figure 3 (a). Amino acid residues H191 and N194 hydrogen bonded with the carbonyl group and helped fix the substrate. Because AMVL does not have a bulky side chain, this result suggests the internal oxygen atom may play an important role in determining the binding orientation.

YqjM, another member of the old yellow enzyme family, was first isolated from *Bacillus subtilis* where it plays a role in the oxidative stress response.[30] Our experiment results show that YqjM(C26D, I69T) can transform anhydromevalonate into (+)-MVL with 76% ee, which suggests AMVL may prefer a flipped binding mode in YqjM mutant compared with that in OYE2 as shown in Figure 3 (b). Docking experiments, performed to gain insight on the stereoselectivity of YqjM-(C26D, I69T), confirm this idea. Indeed, a flipped binding mode for AMVL was identified in YqjM(C26D, I69T).

Previously, the asymmetric conjugate reduction of AMVL has been studied using copper/BIPHEMP catalysts with poly-methylhydrosiloxane as a stoichiometric reductant. Using this method, (−)-MVL has been prepared from in moderate yield and enantioselectivity (70% and 82%, respectively).[26] Additionally the asymmetric hydrogenation of a number of heterocyclic alkenes has been investigated using homogeneous iridium complexes. In the hydrogenation of AMVL, the authors report moderate selectivity (79%) for (−)-MVL.[27] Like OYE2 and YqjM, the enantioselectivities of these organometallic catalysts are determined by the preferred binding orientation of the substrate to an active coordination site. Because the coordination of a trisubstituted alkene is most favorable when the alkene hydrogen is oriented closest to the most sterically encumbered position, the absolute configuration of the product depends primarily on configuration and position of the chiral ligand. Like OYE2, the iridium and copper catalysts described in previous works are selective for (−)-MVL;[26,27] however, OYE2 shows improved selectivity. Although the YqjM studied in this work only gives modest enatioselectivity, it is a notable method to produce (+)−MVL from anhydromevalonate; heretofore, no transition metal hydrogenation catalysts have been reported to be selective for this enantiomer.

With an optically active monomer in hand, we investigated properties of optically enriched poly(MVL). Although we have previously demonstrated good molar mass control for the polymerization of racemic MVL, in this work, we obtained a lower molar mass polymer than expected. Because the dispersity was relatively narrow, we believe this discrepancy is probably due to the presence of adventitious initiators in the monomer. It is probable that use of higher purity monomer could result in better control; however, this was not practical at the current scale. Like poly(±)-β-methyl-δ-valerolactone, poly(−)−β-methyl-δ-valerolactone is fully amorphous. Although this behavior is similar to that previously reported for poly((−)-4-methyl caprolactone),[7] it is still somewhat surprising because it has been reported that the constitutional isomer, poly((+)−γ-methyl-δ-valerolactone is semicrystalline.[31] We are actively exploring the interesting effects optical purity and monomer structure have on polymer thermal properties.

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CONCLUSIONS

In this work, we use a biocatalytic strategy to prepare both enantiomers of MVL from AMVL, an unsaturated precursor that can be produced efficiently on a large scale via the dehydration of bioderived mevalonate. Specifically, reduction with OYE2 yields (−)-β-methyl-δ-valerolactone (96% e.e. and 91.5% conversion), and reduction with YqjM(C26D, I69T) yields (+)-β-methyl-δ-valerolactone (76% e.e. and 68.9% yield). Reduction of AMVL with OYE2 results in higher yield and enatioselectivity than reductions with transition metal catalysts.[26,27] Interestingly, polymerization of optically active (−)-β-methyl-δ-valerolactone yielded an isotactic polymer that was, like its atactic analog, fully amorphous. This result is surprising, because it is known that isotactic poly((+)-γ-methyl-δ-valerolactone), which is a constitutional isomer, rapidly crystallizes.[31] It is evident that substituent position can dramatically impact the physical properties of optically active aliphatic polyesters, a subject we intend to explore in future publications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.6b00992.

Information mentioned in the text. (PDF)

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Notes

The authors declare no competing financial interest.

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There was an error in the presentation of the names of the authors in the version published ASAP July 19, 2016; the corrected version was published ASAP July 22, 2016.

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