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Nitrilase-Catalysed Desymmetrisation of 3-Hydroxyglutaronitrile: Preparation of a Statin Side-Chain Intermediate

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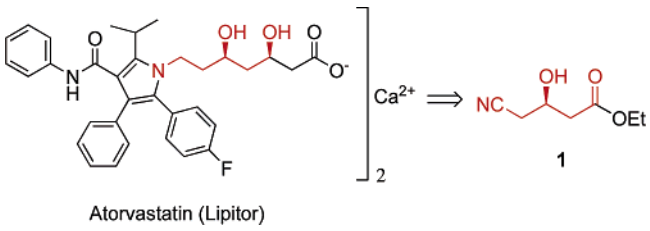
Abstract:

An efficient, scaleable synthesis of ethyl (*R*)-4-cyano-3-hydroxybutyrate, a potential intermediate in the synthesis of Atorvastatin (Lipitor), has been developed. The three-stage process starts with reaction of low-cost epichlorohydrin with cyanide to give 3-hydroxyglutaronitrile (3-HGN). The second stage utilises a nitrilase-catalysed desymmetrisation of 3-HGN. The nitrilase reaction has been optimized to work at 3 M (330 g/L) substrate concentration, pH 7.5, 27 °C. Under these conditions, with an enzyme loading of 6 wt %, 100% conversion and 99% ee product is obtained in 16 h. This material is then esterified to give the target compound, ethyl (*R*)-4-cyano-3-hydroxybutyrate. The cost-effectiveness of the process is determined by three factors: use of a low-cost starting material, the introduction of the chiral centre by desymmetrisation as opposed to kinetic resolution, and the use of *Pf*ēnex Expression Technology to allow a lower-cost supply of biocatalyst.

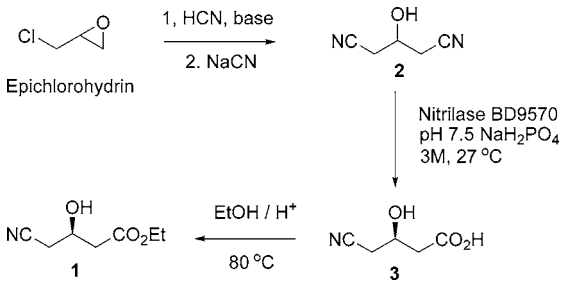
Introduction

Statins are HMG-CoA reductase inhibitors that are used for the treatment of hypocholesterolemia and atherosclerosis. Atorvastatin (Lipitor),[1] a statin launched in 1997, is now the world's largest-grossing drug with 2004 sales of $12 billion. A number of different routes to statin side chains have been reported using a biocatalytic step to introduce one of the two chiral centres.[2] Recently, scientists at Codexis described another process whereby ethyl-4-chloroacetoacetate is reduced to ethyl (*S*)-4-chloro-3-hydroxybutyrate by a ketoreductase, followed by an enzymatic cyanation to give ethyl (*R*)-4-cyano-3-hydroxybutyrate (**1**), a useful intermediate in statin side-chain synthesis (Scheme 1).[3] In this paper we will describe development of an alternative efficient route to **1**.

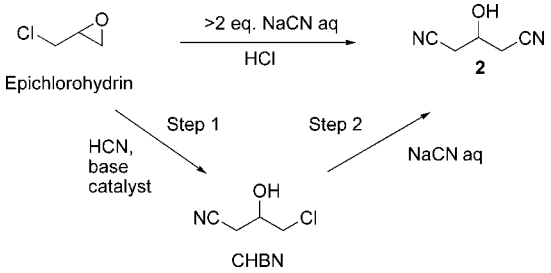
Scheme 1



Scheme 2



Scheme 3



The key step employs a highly volume-efficient nitrilase[4]-catalysed desymmetrisation of the *meso*-dinitrile species 3-HGN (**2**), allowing the product **1**, to be obtained in three steps from epichlorohydrin, an inexpensive starting material (Scheme 2).

Results and Discussion

Preparation of 3-Hydroxyglutaronitrile (2). The synthesis of **2** was investigated by looking at two main routes (Scheme 3). For the direct, one-pot route, it was discovered that precise pH control was necessary for the reaction to work efficiently. Since the pH control required would be difficult

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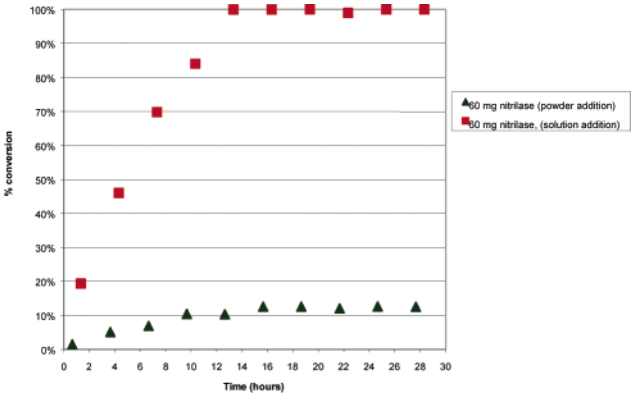
at plant scale, this route was quickly eliminated. Therefore, a two-step process was developed.

Step 1. The synthesis of 4-chloro-3-hydroxybutyronitrile (CHBN) was optimised to give yields of 93%. The major impurity found in the CHBN was the target molecule, **2**. The main consideration in this step is the highly reactive nature of the reaction substrates, epichlorohydrin and hydrogen cyanide. It was considered unsafe to charge the reactor with epichlorohydrin.[5] Accordingly, the reactants were co-fed into an aqueous solution containing base catalyst. The reaction is initially two phase since epichlorohydrin is not water soluble. As the reaction proceeds and the water-soluble CHBN concentration increases, the system becomes homogeneous which can lead to a significantly increased and uncontrollable rate of reaction. It was found that the addition of phase transfer catalyst increases the rate of reaction in the early stages and avoids any sudden uncontrollable exothermic activity.

Step 2. The highest yield achieved for the displacement of the chloride of CHBN by cyanide was 68%. DOE was used to broadly identify important parameters in this stage of the process. Stoichiometry, temperature, reaction volume, and pH were investigated in a series of screening reactions. The temperature range 40−55 °C was found to be optimum; below 40 °C the reaction becomes prohibitively slow, and above 55 °C side reactions become significant. Dilute conditions were found to reduce impurities arising from intermolecular interactions, e.g., dimers and polyols. At pH 10 the reaction yield is highest, although polymerisation of cyanide may be increased. In all cases a yield maximum (typically 68%) was achieved with further reaction time only resulting in more byproduct formation. Thus, a yield of 68% is accompanied with a significant residual concentration of CHBN; typically 15%. Isolation of **2** proved problematic. Downstream processing required that the HGN stream should have low ionic strength and be as clean as possible. Attempts to distill **2** from aqueous salt solution resulted in significant tar formation and low recovery. Azeotropic removal of water, as a strategy to remove inorganic salts, was attempted. This approach resulted in a tarry phase that was impossible to filter. Solvent extraction was found to be the only practical solution. Following a brief series of trials, 2-methyl-propan-1-ol was selected as the extraction solvent since it was efficient, inert to the process, easy to recover, and unlikely to have a detrimental effect in the downstream biotransformation. Due to the hydrophilic polar nature of **2**, it was found that multiple extractions were necessary. Further purification of **2** was necessary to remove volatile impurities which inhibit the nitrilase enzyme in the next stage. Small-scale batch distillations gave poor yields of good-quality material, but which performed well in the biotransformation. However, thermal screening analysis (using the Thermal Screening Unit[6] instrument supplied by Hazard Evaluation Laboratories) showed the crude **2** to decompose exothermically at temperatures approaching 150 °C; as a result, batch distillation was excluded as an option for large-scale purification on safety grounds. A scaleable solution was to perform a single pass through a wiped film evaporator to remove the troublesome volatile impurities. This was achieved at an acceptable temperature and pressure with reasonable recovery (96 °C, 1.4 mbar, 67% recovery). Material treated in this way was shown to perform well in the nitrilase desymmetrisation. GC−MS identified the principal impurities removed in the distillate as 4-hydroxy-but-3-enenitrile and CHBN. The economics for the overall process are important, and the modest yield for the preparation of **2** will be further addressed in future work. However, on the positive side, the raw materials are inexpensive when compared to the product and processing costs.

Chart 1. **Effect of the method of nitrilase addition on reaction progress**



Preparation of (*R*)-4-Cyano-3-hydroxybutyric acid (3). A nitrilase enzyme (BD9570, Diversa) was used in the desymmetrisation of the prochiral substrate **2** to afford **3**. A series of small-scale experiments were performed to optimize the nitrilase-catalysed step. First, the method of nitrilase addition to the reaction mixture was investigated. The nitrilase (Diversa BD9570, 0.1 unit mg^{-1} of lyophilised powder) was either added directly to the reaction mixture as a lyophilised powder or first rehydrated with buffer (sodium phosphate, 100 mM, pH 7.2) and then added to the reaction mixture containing **2**. Addition of lyophilized nitrilase directly to a solution consisting of **2** and buffer resulted in the enzymatic reaction stalling at 13% conversion after 10 h (Chart 1). However, addition of a solution consisting of nitrilase and buffer to **2** resulted in complete conversion within 14 h (Chart 1). Therefore, it is critical to rehydrate the lyophilised nitrilase prior to the addition of substrate to preserve the nitrilase activity.

Next, optimisation of the loading of the nitrilase enzyme (20, 30, 40, and 60 mg of lyophilized nitrilase, 0.1 unit mg^{-1} of lyophilised powder) with 1 g of 3-HGN (3 M) was

(5) Epichlorohydrin is unstable in the presence of acid or base. In this reaction, base catalyst and water are charged to the reaction vessel. Hydrolysis of the epichlorohydrin is possible, but in the worst case, autopolymerisation may occur. For these reasons the inventory of epichlorohydrin present is minimized by batch feeding. HCN is batch fed in all processes. When acid stabilised, HCN may be considered inert. However, in the presence of catalytic quantities of any base, cyanide ion is generated which initiates polymerisation of HCN. In the worst case, explosive polymerisation may occur. For this reason the inventory of HCN is minimized by batch feeding. The enthalpy of reaction (for the required reaction) was estimated at ~100 kJ/mol. The reaction diluent, water, provides a good heat sink, but the reaction power is controlled by feed rate and efficient cooling.

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Chart 2. **Effect of nitrilase loading on reaction progress**

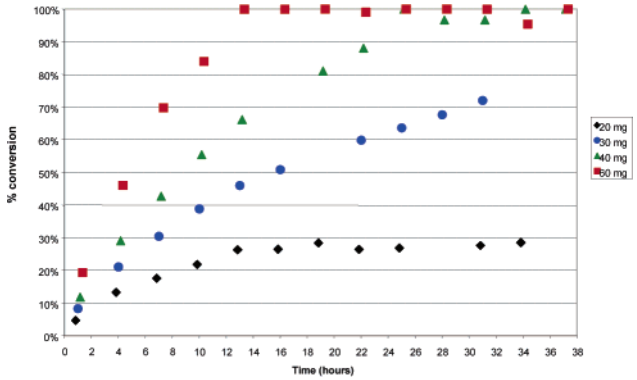


Chart 3. **Effect of pH on the nitrilase-catalysed reaction**

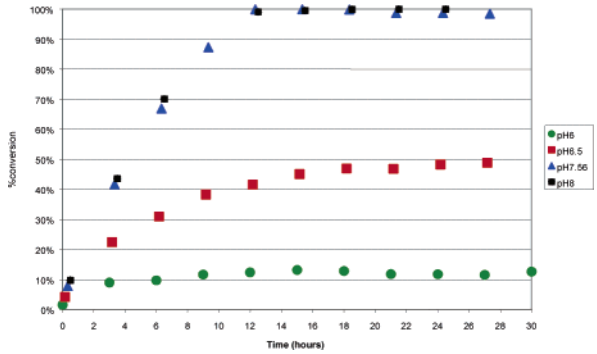
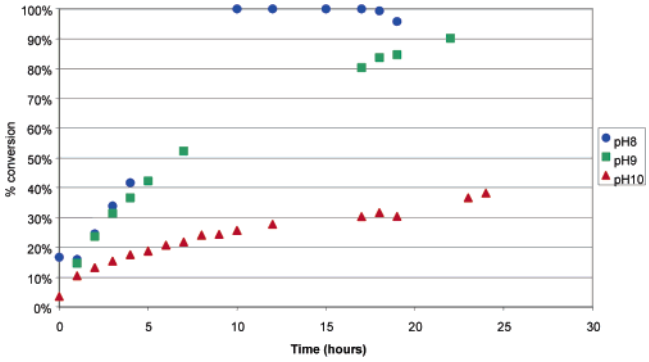


Chart 4. **Effect of alkaline pH on the nitrilase-catalysed reaction**



investigated at pH 7.2. In each case the nitrilase enzyme was rehydrated prior to addition to the reaction mixture. At a nitrilase loading of 20 mg the reaction stalled at 29% conversion (Chart 2). Whereas, with a nitrilase loading of 30 mg the reaction proceeded to 71% conversion within 31 h (Chart 2). At nitrilase loadings of 40 and 60 mg the reactions proceeded to completion within 34 and 13 h, respectively (Chart 2). The nitrilase loading of 60 mg was chosen for all subsequent optimisation experiments with 3-HGN (1 g, 3 M).

The effect of pH on nitrilase activity was also investigated (Charts 3 and 4). Under acidic conditions of pH 6.0 and pH 6.5, the reaction stalled at <50% conversion, whereas, at pH 7.5 and pH 8.0 the reaction proceeded to completion within 12.5 h. Under more alkaline conditions a slowing in the rate of conversion was observed. Thus, an optimum pH range for this reaction is pH 7.5−8.

As a result, under the optimized conditions of the biotransformation using rehydrated nitrilase BD9570 (6 wt %), the 3-HGN (3 M) substrate was shown to be converted to the hydroxy acid (**3**) in ∼99% conversion within 16 h at pH 7.5. These conditions were used for scale-up.

The isolation of **3** from the biotransformation was challenging due to its water solubility and the presence of cell debris and salts in the reaction mixture. Filtration of the acidified mixture, or centrifugation, followed by continuous liquid−liquid extraction with MTBE was the best way to achieve high yield in the laboratory (ca. 89%). However, long extraction times (>20 h) meant that it was necessary to investigate alternative isolation procedures. In some cases the calcium salt could be formed by treatment of the reaction mixture directly with calcium hydroxide. This resulted in a poorly filterable solid, albeit with a slight upgrade in enantiomeric excess (98.6% to 99.4%). The calcium salt could be used directly in the next esterification step, if sufficient acid was added, but was also accompanied by a slow filtration during product isolation. In other cases the biotransformation reaction mixture was telescoped into the esterification step by azeotropic removal of water by co-distillation with ethanol or methyl ethyl ketone followed by treatment with sulphuric acid−ethanol at reflux. For reasons of volume efficiency and throughput, the workup of choice involved acidification and filtration with Celite filter aid, followed by conventional batch extraction with methyl ethyl ketone. This gave good yields (81%), albeit with the principal byproduct of the nitrilase hydrolysis, 3-hydroxypentanedioc acid, still present.

Preparation of Ethyl (*R*)-4-Cyano-3-hydroxybutyrate (1). The esterification of **3** was accomplished by treatment with ethanol and sulphuric acid catalyst at reflux, and was generally complete within 2 h. However, it was noted that larger-scale laboratory experiments occasionally stalled at ca. 90% conversion. In these cases complete conversion was achieved by evaporation of ethanol and resubmitting the partially converted substrate to the reaction conditions. On larger-scale this would be a somewhat cumbersome and inefficient approach. Thus, care is needed in the biotransformation workup to remove as much water as possible from the intermediate acid. On plant scale this esterification would be performed by concomitant distillation from, and resparging of, ethanol into the reaction vessel. Final purification of **1** was achieved by two passes through a wiped film evaporator. This was the purification method of choice since small-scale fractional vacuum distillation had resulted in significant yield loss due to product decomposition.

Biocatalyst Production. A key aspect of process economics is the cost of the catalyst. With this in mind, the nitrilase was expressed in a strain of *Pseudomonas fluorescens* (*Pf*ēnex Expression Technology), a robust gene expression host developed by The Dow Chemical Company.[7] The organism has proved to have broad application. It grows to very high cell densities, and target proteins are commonly

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obtained in very high yield and soluble, active form. When applied to the nitrilase used in this process, excellent results were obtained. The enzyme was obtained solely as a soluble, active multimer in excess of 25 g L^{-1} of fermentation, a quantity that represented >50% of the total cell protein. An added advantage of such a high level of expression of protein is the greatly simplified downstream processing of the enzyme, a further contributing factor to the enzyme cost.

Conclusion

An efficient three-stage synthesis of ethyl (*R*)-4-cyano-3-hydroxybutyrate (**1**) from low-cost epichlorohydrin in 23% overall yield, 98.8% ee, and 97% purity has been developed. Further scale-up will allow additional process improvements to produce the optimum process. The key step is the introduction of the chiral centre using a nitrilase-catalysed desymmetrisation of 3-HGN. The nitrilase reaction has been optimized to work at 3 M (330 g/L) substrate concentration, pH 7.5, 27 °C. Under these conditions, with an enzyme loading of 6 wt %, 100% conversion and 99% ee product is obtained in 16 h. The use of *Pf*ēnex Expression Technology to produce the enzyme for this transformation allows a lower-cost supply of biocatalyst. All of these factors allow a cost-efficient synthesis of a key chiral building block.

Experimental Section

General Experimental Procedures. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz for proton and 100 MHz for carbon. Chemical shifts are reported in ppm using Me_4Si or residual nondeuterated solvent as reference. GC−MS data were obtained using a HP 5890 series 2 GC fitted with a HP 5972 series Mass Selective Detector using electron impact ionization.

Preparation of 3-Hydroxyglutaronitrile, (2). Caution! Free HCN may be present at all stages of the following process description. All work must be carried out in an efficient fume cupboard. Waste streams should be rendered cyanide free by treatment with base and formaldehyde or hypochlorite. During step 1, exothermic activity should be detected soon after the co-feeds have been initiated. Absence of any exotherm indicates an accumulation of unreacted materials which may lead to a subsequent uncontrollable exotherm.

Step 1: 4-Chloro-3-hydroxybutyronitrile. Water (540 g, 2.5 mol), triethanolamine (44.7 g, 0.025 mol), and tetrabutylammonium bromide (19.3 g, 0.005 mol) are premixed and charged to a reaction vessel fitted with a reflux condenser and efficient stirring and temperature controls. Hydrogen cyanide (356.4 g, 1.10 mol) and epichlorohydrin (1110 g, 1 mol) are co-fed from separate feeds to the reaction vessel over 4 h, maintaining the temperature at 45−50 °C. The reaction is then allowed to stir out at this temperature for up to 8 h. The progress of the reaction is monitored by GC.[8] The resultant product (2070 g, 1680 mL) is a yellow solution of typically 63.7 w/w% CHBN (92% yield) and 2.4 w/w% HGN (3.5% yield). No attempt is made to remove residual cyanide since this can be part of the charge in step 2.

Step 2: 3-Hydroxyglutaronitrile, 2. Water (855 g, 19.0 mol) and 30% aq sodium cyanide (408 g, 1.0 mol) are charged to a reaction vessel having heating and cooling control as previously described. Hydrochloric acid (35%) is added (typically 15.6 g, 0.06 mol) to adjust to pH 10. This solution is heated to and maintained at 50−55 °C whilst charging the prepared CHBN solution (431 g, 1 mol epichlorohydrin basis) over 4 h. The extent of reaction is monitored by GC.[9] The reaction is stirred out at 50−55 °C, typically 6 h, until the yield maximum is achieved. Hydrochloric acid, (35% , 6.5 g, ∼0.025 mol) is added to adjust to pH neutral. The dark reaction mixture, (1716 g, 1687 mL) so obtained, contains ∼11.1% w/w **2**; 68% yield based on epichlorohydrin input.

Purification of 2. Aqueous reaction liquor (4.39 kg) was extracted with 2-methylpropan-1-ol (4 × 1.6 L). The combined organic layers were evaporated in vacuo (45 °C, 10 mmHg) to yield crude **2** as a brown liquid (390 g). Purification was achieved by passing the liquid through a wiped film evaporator and retaining the residue (jacket temperature = 96 °C; vacuum = 1.4 mbar; throughput = 46 g h^{-1}; jacket surface area = 0.02 m^2). Yield of residue was 263 g (67% recovery) containing **2** at 91.2% purity (GC); ^{1}H NMR (400 MHz, D_2O) δ 4.25−4.33 (m, 1H), 2.65−2.80 (m, 4H).

Preparation of (*R*)-4-Cyano-3-hydroxybutyric Acid (3). To an aqueous solution of 100 mM NaH_2PO_4 at pH 7.5 (510 mL; adjusted to pH 7.5 by addition of 1 N sodium hydroxide solution) was added lyophilized nitrilase enzyme powder (15.15 g). The mixture was stirred at 27 °C (to rehydrate the lyophilised enzyme powder) for 40 min. 3-Hydroxyglutaronitrile (252.5 g, 2.29 mol) was then charged over ∼10 min. The mixture was stirred at 27 °C for 16 h and then cooled to 2 °C prior to acidification by controlled addition of concentrated sulphuric acid (∼56 mL) to give pH 2 (exotherm is observed). Celite was charged (25 g) and the slurry filtered. The filtrate was extracted with methyl ethyl ketone (4 × 400 mL). The combined methyl ethyl ketone extracts were evaporated in vacuo (15 mbar, 40 °C) to yield the product **3** as a brown liquid (240.8 g, 81% yield; ee 98.8%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.25−4.34 (m, 1H), 2.50−2.77 (m, 4H).

Preparation of Ethyl (*R*)-4-Cyano-3-hydroxybutyrate (1). 3 (239.5 g, 1.855 mol) was dissolved in absolute ethanol (480 mL). Concentrated sulphuric acid (3.3 mL) was dosed in (exotherm observed). The mixture was heated at reflux (80 °C) for 1 h, after which time the reaction was found to have stalled at ca. 90% conversion. The ethanol was

(8) GC assay: J&W-DB17 column; injector temperature, 250 °C; detector temperature, 275 °C; carrier gas, He @ 0.95 kg/cm^2; oven program: 75 °C (hold 5 min), heat to 275 °C @ 10 °C/min, hold for 5 min; CHBN elutes at 10.7 min.

(9) GC assay: J&W-DB17 column; injector temperature, 250 °C; detector temperature, 275 °C; carrier gas, He @ 0.95 kg/cm^2; oven program: 75 °C (hold 5 min), heat to 275 °C @ 10 °C/min, hold for 5 min; CHBN elutes at 10.7 min, HGN elutes at 14.5 min.

Table 1

	1st pass	2nd pass
input ester/g	290	275
jacket temp/°C	51	113
vacuum/mbar	1.4	1.4
condensor temp/°C	6	6
throughput/g h^{-1}	62	57
distillate yield/%	5	66
residue yield/%	95	34

subsequently evaporated in vacuo. Ethanol (400 mL) and concentrated sulphuric acid (3.3 mL) were recharged, and heating resumed for 1.25 h, after which time conversion was complete. Ethanol was evaporated in vacuo to yield the crude product **1** (290 g, yield 99%; ee 98.7%). The crude product was purified by two passes through a wiped film evaporator. The residue from the first pass acted as input for the second as shown in Table 1.

The final product **1** was obtained as a colourless liquid (181.5 g, 62% yield); purity (GC peak area[10]) 97%; ee 98.8%;[11] ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.31−4.37 (m, 1H), 4.23 (q, 2H), 2.60−2.67 (m, 4H), 1.27 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.9, 117.4, 64.5, 61.6, 40.4, 25.4, 14.5; $[\alpha]^{25}_D$ −26.2° (c = 1, $CHCl_3$).

Acknowledgment

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(10) GC assay: DB-1701 column; injector temperature, 250 °C; detector temperature, 300 °C; carrier gas, He @ 15 psi; oven program: 60 °C (hold 5 min), heat to 220 °C @ 5 °C/min; **1** elutes at 19.6 min.

(11) GC assay: Chiraldex G-TA column; injector temperature, 180 °C; detector temperature, 180 °C; carrier gas, He @ 14 psi; oven program: 150 °C (hold for 10 min), heat to 180 °C @ 10 °C/min, hold for 10 min; (*R*)-enantiomer elutes at 9.66 min; (*S*)-enantiomer elutes at 9.44 min.