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Immobilization of Cholesterol Esterase for Use in Multiple Batch Biotransformations to Prepare (−)- FTC (Emtricitabine)

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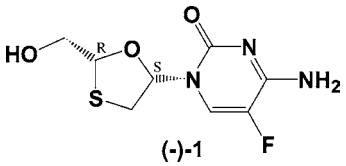
Abstract:

2′,3′-Dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC], the active ingredient in the antiviral drug emtricitabine (Emtriva), was prepared by a bioresolution process using cholesterol esterase immobilized on Accurel PP to resolve optical isomers of racemic FTC butyrate. Cholesterol esterase was immobilized at 1.9-kg scale. Recycling studies were carried out with racemic FTC butyrate that indicated a high degree of immobilized cholesterol esterase stability resulting in 15 successive cycles of use (14 recycles). Racemic FTC butyrate (∼8 kg, 200 g/L) was resolved with immobilized cholesterol esterase using a 1-pentanol/ potassium dihydrogen phosphate buffer−solvent system to give (−)-FTC·HCl (98% ee, 2.17 kg, 31% molar yield based on racemic FTC butyrate).

2′,3′-Dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC, (−)-**1**] is the active ingredient in the antiviral drug Emtriva, a nucleoside reverse transcriptase inhibitor targeted for the treatment of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections.[1] (−)-FTC [(−)-**1**] is an optically active drug, the active form being the (−), or [1′-S,4′-R] (numbering as a ribose analogue) form.



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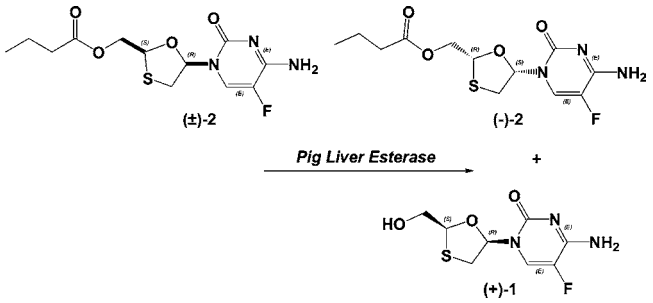
A process for preparing (−)-FTC employing a late-stage resolution of (±)-FTC butyrate using pig liver esterase (PLE) (Scheme 1) has been described by Liotta et al.[2] in which the desired enantiomer is isolated as an unreacted ester (−)-**2**, which is then chemically hydrolyzed to give (−)-FTC. However, the use of an animal-derived enzyme in processing an active pharmaceutical ingredient raises concerns about the security of supply and the potential for PLE to introduce viruses into the process stream and thereby potentially to contaminate the product and equipment in which the process was carried out.[3] In this contribution we describe the development of an alternative transformation using an immobilized enzyme from a microbial source.

Scheme 1. **Bioresolution of (±)-FTC butyrate [(±)-2] using pig liver esterase**



Our route (Scheme 2) for preparing (−)-FTC directly generates (−)-**1** as the HCl salt, racemic butyrate ester (±)-**2** (200 g/L) is treated with an immobilized cholesterol esterase,[4] which results in the cleavage of the required isomer to the corresponding alcohol (−)-**1**, which when isolated as the hydrochloride gave 31% yield (98% ee) based on racemic FTC butyrate.

Cholesterol esterase was chosen from a panel of enzymes to demonstrate the enantioselective hydrolysis of (±)-FTC butyrate to access (−)-FTC directly. Immobilisation and recycle of the cholesterol esterase was a preferred option for the benefit of future process scale economics. A robust method for enzyme immobilization was found to be precipitation of cholesterol esterase onto the Accurel PP support using acetone, followed by cross-linking with glutaraldehyde (0.25% v/v).[5] Optimisation of the bioresolution with (±)-FTC butyrate (200 g/L) was then performed with a SK 233 (Gilson) automated workstation. Various solvent systems were tested with effort focused on bioresolution systems consisting of 50% v/v aqueous buffer in either 1-butanol or 1-pentanol and 20% v/v water in 2-propanol. Further experiments were then performed to test the thermal stability and solvent tolerance of the immobilized cholesterol esterase.

Thermal and Solvent-Stable Immobilization of Cholesterol Esterase. Immobilized cholesterol esterase (10 g) was washed five times with a 50:50 solvent/ buffer mixture. The washings and the washed immobilized enzyme were then

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(3) Gaede, B. J.; Nardelli, C. A. *Org. Process Res. Dev.* **2005**, *9* (1), 23.

(4) Cholesterol esterase from *Candida cyclindracea* was purchased from Boehringer Mannheim.

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Scheme 2. **Bioresolution of (±)-FTC butyrate [(±)-2] using immobilized cholesterol esterase**

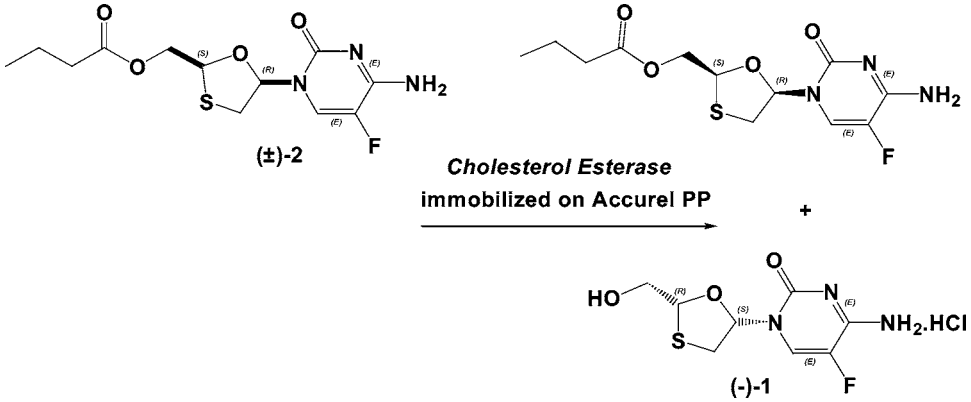
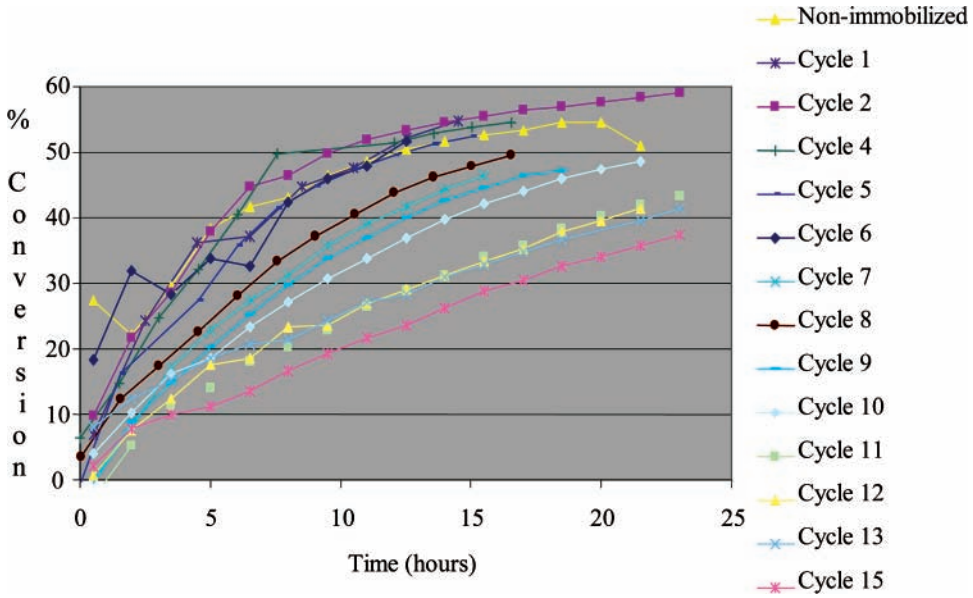


Chart 1. **Successive recycles of immobilized cholesterol esterase with (±)-FTC butyrate bioresolutions**[a]



[a] Reaction conditions: (±)-FTC butyrate (200 g/L), 2-propanol/water mixture (80:20), 30 °C

tested for cholesterol esterase activity. Washing did not appear to cause loss of activity from the immobilized enzyme into the solvent−buffer mixture. The immobilized enzyme was stored at 4 and 25 °C, and cholesterol esterase activity was measured in duplicate after 1, 5, 7, 9, 15, and 27 days. There was no discernible loss of activity compared to a control of lyophilised cholesterol esterase (nonimmobilized).

Recycles of Immobilized Cholesterol Esterase. Immobilized cholesterol esterase was recycled over several (±)-FTC butyrate resolutions under standard operation conditions with a final reaction mixture volume of 10 mL. This work was carried out using a SK 233 automated workstation. Immobilized cholesterol esterase was shown to be robust and able to undergo at least 15 recycles, each cycle comparable to the lyophilized enzyme. There were apparent drops in activity between cycles 6 to 7, 10 to 11, and 13 to 15. The other cycles, however, show a high level of consistency (Chart 1). The apparent decrease in activity over the 14 successive recycles was attributed to mechanical loss at small scale and, as previously shown, was not likely to be due to thermal denaturation or desorption of the enzyme from the support. The excellent recycling properties of the immobilized cholesterol esterase indicate the possibility of a much reduced cost contribution of the enzyme to the desired process.

Scale-Up of the (±)-FTC Butyrate Bioresolution Process. (±)-FTC butyrate (7.85 kg) was resolved with immobilized cholesterol esterase (738 g) using a 1-pentanol/potassium dihydrogen phosphate (5 mM, pH 7.0) buffer−solvent system, to give material of 91.2% ee at 52% conversion. No problems were encountered in the scale-up, and material of excellent enantiomeric excess and chemical purity was isolated through the formation of the hydrochloride, which improves enantiomeric excess at the expense of yield; (−)-FTC·HCl (2.17 kg) was isolated as an off-white crystalline solid (98% ee, 31% molar yield based on (±)-FTC butyrate). (−)-FTC·HCl could be dissociated using Amberlite LA2 liquid ion-exchange resin to afford (−)-FTC in excellent recovery and enantiomeric excess (>99%).

In conclusion robust chemical processes for the preparation of immobilized cholesterol esterase and its use in the bioresolution of (±)-FTC butyrate were developed which could be performed in standard chemical manufacturing equipment. The immobilised cholesterol esterase can be recycled at least 14 times, allowing the cost contribution of

the enzyme to the biotransformation process to be significantly reduced.

Experimental Section

General. Reagents and anhydrous solvents were purchased from Aldrich and used as received**.** ^{1}H NMR spectra were acquired on a Brucker Advance NMR spectrometer at 400 MHz.

For the investigation of the recycling of immobilized cholesterol esterase, all experiments were performed at the 10-mL scale; (±)-FTC-butyrate (2 g) was suspended in a mixture of 2-propanol/water (80:20, 10 mL). Immobilized cholesterol esterase was added to the reaction mixture at the equivalence of 2% w/w lyophilized enzyme with respect to (±)-FTC-butyrate. Using an SK 233 automated workstation, samples (50 μL) of the reaction mixture were taken over time diluted with ethanol, and dried over magnesium sulfate. Samples were analyzed by chiral HPLC (column = Chiralpak AS, eluent = 2-propanol/ethanol (1:1), flow rate = 1 mL/ min, detection by UV spectrophotometry at 254 nm). Reaction progress (% conversion) was calculated from the enantiomeric excess of remaining substrate (**2**) and product [(−)-**1**] using the equation; $c = ee_s/(ee_p + ee_s)$.[6] Immobilized cholesterol esterase was recovered and washed using a mixture of 2-propanol/water (80:20), under vacuum using a small glass sinter between recycles.

Immobilization of Cholesterol Esterase at 1.9 kg Scale. Lyophilized cholesterol esterase (Boehringer Mannheim, 410 g, approximately 10% of this is actually protein) was resuspended in potassium phosphate buffer (5 mM, pH 7.0, 4 L). Accurel microporous polypropylene (Accurel PP, particle size 400−1000 μm, 810 g) was wetted with ethanol (7 L). Excess ethanol was removed by vacuum filtration. The resuspended cholesterol esterase was added to prewetted Accurel PP followed by water (3 L) to allow covering of the support. The suspension was stirred for 4 h at ambient temperature, and excess buffer was removed by vacuum filtration. Acetone (4 L) at 0 °C was added to promote precipitation of the protein onto the support. The suspension was stirred for 1 h before the addition of glutaraldehyde (0.25% v/v) followed by 16−18 h of stirring to permit cross-linking. The immobilized enzyme was obtained by filtration and then dried by rotary evaporation. A final yield of 1.98 kg was achieved; the support was assayed for cholesterol esterase activity; 1 g of lyophilized cholesterol esterase was found to be equivalent to 4.725 g of immobilized enzyme, and using this equivalence, (±)-FTC-butyrate (200 g/L) was taken to 49% conversion after 16.5 h. This was found to be the same rate as lyophilized cholesterol esterase (nonimmobilized). This suggested that immobilization of cholesterol esterase had been achieved with near complete retention of activity.

Resolution of (±)-FTC Butyrate on ~8 kg Scale. A 200-L jacketed vessel was heated to 30 °C and charged with (±)-FTC butyrate (Pharm-Eco Labs Inc, U.S.A., 7.85 kg, 24.7 mol), 1-pentanol (15.7 kg, 17.8 mol), and potassium dihydrogen orthophosphate solution (pH 7.0, 5 mM, 20.15 kg, 14.8 mol). The pH of the mixture was adjusted to pH 7.0 with potassium hydroxide (50 g, 1.1 mol) and stirred at 300 rpm. When the internal temperature of the vessel reached 30 °C, cholesterol esterase immobilized on Accurel PP (738 g) was charged to the vessel. The reaction was monitored by removing an aliquot and analyzing by chiral HPLC (column = Chiralpak AS, eluent = 1:1 2-propanol/ethanol, flow rate = 1 mL/ min, detection by UV spectrophotometry at 254 nm, retention times: (−)-FTC = 4.4 min, (+)-FTC = 6.4 min). When the conversion had reached 49−50% (48 h at 30 °C), toluene (17.3 kg, 18.7 mol) was added, the mixture was stirred for 10 min and then filtered. The bulk filtrate was transferred to a clean jacketed vessel, which was heated to 50 °C and stirred at 300 rpm. When the internal jacketed temperature had reached 50 °C, the stirring was turned off, and the mixture was allowed to settle. The lower aqueous phase was separated, water (8 kg) was charged to the vessel containing the organic layer, and mixture was stirred and separated at 50 °C. This was repeated, and the aqueous layers were combined to yield a solution of (−)-FTC in water. To the solution of (−)-FTC was charged 1-pentanol (16 kg, 26.6 mol). The combined aqueous and 1-pentanol layers were transferred to a vessel set up for vacuum Dean−Stark distillation. The water was removed under best vacuum via azeotropic distillation; the azeotrope was approximately 1:1 at 30−50 mBar. The water was distilled until it was essentially replaced with 1-pentanol. The alcohol was recycled. Ethanol (10.45 kg) was cooled and added to the jacketed reactor. The jacket temperature was set to 50 °C. The reaction mixture was stirred for 45 min at 50 °C, then filtered and washed with ethanol. Hydrochloric acid (36%, 1.65 kg, 45.2 mol) was charged to the vessel. Crystallisation of the (−)-FTC·HCl salt occurred at 50 °C. The mixture was stirred and slowly cooled to 20 °C before being allowed to stir for 16 h. The (−)-FTC·HCl salt was collected by filtration, the filter cake was washed with 2-propanol (4 kg), and the product was dried under vacuum at 40 °C to give (−)-FTC·HCl (2.17 kg) as an off-white crystalline solid (98% ee, 31% molar yield based on (±)-FTC butyrate). A sample of (−)-FTC·HCl (100 g) was dissociated using Amberlite LA2 liquid ion-exchange resin to afford 78.5 g of (−)-FTC (90% recovery, >99% ee). 30 g of this material was recrystallised to provide 25 g of pure (−)-FTC of >99% ee.

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Supporting Information Available

^{1}H NMR and chiral HPLC spectra of FTC and (±)-FTC butyrate. This material is available free of charge via the Internet at http://pubs.acs.org.

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