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Article

Chemoenzymatic Synthesis of Apremilast: A Study Using Ketoreductases and Lipases

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The key step in the chemoenzymatic synthesis of apremilast was to produce the chiral alcohol (*R*)-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanol, (*R*)-**3**. Two enzymatic approaches were evaluated to obtain (*R*)-**3**, one using ketoreductases and the other lipases. Bioreduction of 1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanone (**2**), using ketoreductase KRED-P2-D12, led to (*R*)-**3** with 48% conversion and 93% enantiomeric excess (*ee*). Kinetic resolution of *rac*-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl acetate (*rac*-**4**), via hydrolysis reaction, with 20% of *n*-butanol, catalyzed by lipase from *Aspergillus niger* yielded (*R*)-**3** with > 99% *ee*, 50% conversion and E-value (enantiomeric ratio) > 200. The reaction between enantiomerically pure (*R*)-**3** and 4-acetylamino-isoindol-1,3-dione (**8**) afforded apremilast in 65% yield and 67% *ee*.

Keywords: apremilast, biocatalysis, ketoreductases, lipases, enzymatic kinetic resolution

Introduction

Apremilast (Figure 1), (*S*)-*N*-(2-(1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl)-1,3-dioxoisoindolin-4-yl)acetamide, is a selective inhibitor of the enzyme phosphodiesterase 4 (PDE-4). It was developed by a pharmaceutical company and was approved by the US FDA (Food and Drug Administration) in 2014 and by the European agency EMA (European Medicines Agency) in 2015 for the treatment of moderate to severe active psoriasis and for psoriatic arthritis.[1,2] More recently, in 2018, the Brazilian agency responsible for sanitary surveillance, ANVISA (National Health Surveillance Agency), approved apremilast for commercialization.[3] Apart from psoriasis, this drug has shown relevant versatility for the treatment of several diseases including acute lung injury,[4] hidradenitis suppurativa,[5] atopic dermatitis,[6] recalcitrant aphthous stomatitis,[7] inflammation in the heart caused by the action of doxorubicin-induced

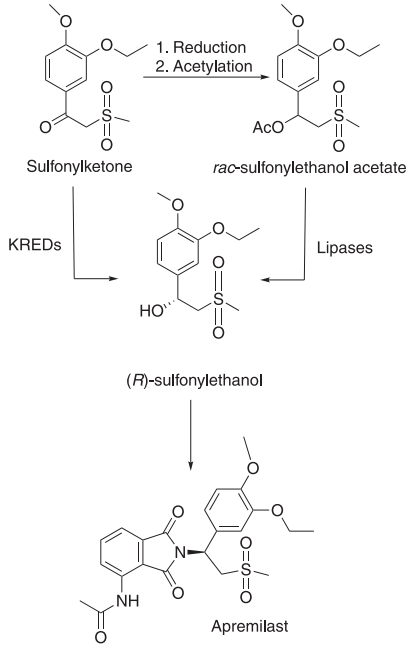

Figure 1. Chemoenzymatic approaches to produce apremilast.

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apoptosis,[8] recalcitrant pyoderma gangrenous[9] and Alzheimer's disease.[10]

Apremilast has a stereogenic center with (*S*)-configuration, being five times more active than the *ent*-apremilast.[11] The key step of the synthesis was the kinetic resolution of β-aminosulfone 1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanamine using *N*-acetyl-L-leucine as resolution agent, leading to the corresponding (*S*)-β-aminosulfone with 98.4% enantiomeric excess (*ee*). Then, the chiral (*S*)-β-aminosulfone reacted with 3-*N*-acetylaminophthalic anhydride to afford apremilast with 75% yield and 99.5% *ee*, this obtained after recrystallization from ethanol.[11]

Subsequently, the same developer[1] reported the synthesis of apremilast via catalytic asymmetric hydrogenation of enamine (*E*)-*N*-(1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)vinyl-2,2,2-trifluoroacetamide in the presence of the rhodium catalyst $Rh(cod)_2OTf$ and the chiral ligand (*R*,*R*)-Me_2-DuPhos, providing, after deprotection of the amine, the chiral β-aminosulfone (*S*)-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl) ethanamine with 68% yield and 96% *ee*.[1] In an alternative protocol, the asymmetric hydrogenation of the enamine was carried out in the presence of the ferrocene catalyst (*S*,*R*)-*t*-Bu-Josiphos, followed by deprotection of the amine and treatment with *N*-acetyl-L-leucine. This protocol resulted the corresponding (*S*)-β-aminosulfone with 80% yield and 99.2% *ee*.[1] In this same paper,[1] a third option was reported for obtaining apremilast via enantioselective reduction of ketone 1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanone (**2**) through Noyori's asymmetric transfer hydrogenation. By using the ruthenium catalyst RuCl(*p*-cymene)[(*S*,*S*)-Ts-DPEN], DPEN: diphenylethylenediamine, the hydroxysulfone (*R*)-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanol (*R*-**3**) was produced with 87% yield and 99.4% *ee*. Then, the (*R*)-alcohol underwent Mitsunobu reaction, in the presence of hydrazoic acid, being converted into the corresponding (*S*)-azide with only 41.7% *ee*. The authors considered that the degree of racemization observed could be attributed to the formation of a carbocation from intermediate (*R*)-**3** (an *ortho*- and *para*-alkoxybenzyl alcohol) via an S_N1-type reaction. Thereafter, the conversion of (*S*)-azide into the corresponding (*S*)-amine, followed by treatment with *N*-Ac-Leu salt and crystallization, produced the corresponding salt of (*S*)-β-aminosulfone with 94.4% *ee*.[1] In all these cases, the reaction between (*S*)-β-aminosulfone and 3-*N*-acetylaminophthalic anhydride led to apremilast in the enantiomerically enriched form.[1]

In 2017,[12] chiral β-acetylamino sulfide was reported to be a potential intermediary in the synthesis of apremilast. The latter was obtained with 99% yield and 96% *ee*, through asymmetric hydrogenation of *N*-(1-(3-ethoxy-4-methoxyphenyl)-2-(methylthio)vinyl)acetamide, in the presence of the rhodium catalyst $Rh(NBD)_4BF_4$ NBD: norbornadiene, and the ligand (S_c,R_p)-DuanPhos. After that, the chiral β-acetylamino sulfide was oxidized, in the presence of 30% H_2O_2 and $TaCl_5$ catalyst, to produce the corresponding β-acetylamino sulfone with 50% yield and 94% *ee*.[12] More recently, a new approach was reported[13] for the synthesis of apremilast, which consisted of the reaction between 4-amino-2-(2-methylsulfonyl)vinyl)isoindoline-1,3-dione and (3-ethoxy-4-methoxyphenyl)boronic acid, using the rhodium catalyst $[RhCl(C_2H_4)_2]_2$ and a chiral ligand, (1*S*,4*S*)-1,7,7-trimethyl-2,5-diphenylbicyclo[2.2.1] hepta-2,5-diene or (1*S*,4*S*)-2,5-diphenylbicyclo[2.2.1] hepta-2,5-diene. After acetylation of the amino group attached to the benzenic ring, apremilast was obtained with 94% yield and 97% *ee*.[13]

Biocatalysis is a powerful alternative tool to mainstream chemical synthesis to produce chiral drugs in enantioriched/enantiopure forms. This methodology has evolved to industrial application due to the ease in complementing conventional methods of synthesis of active pharmaceutical intermediates (APIs). The differential of a biocatalytic processes lies in the fact that an enzyme is obtained from renewable sources and it is biodegradable and non-hazardous, therefore being environmentally friendly.[14] Conversely, the use of conventional synthetic methodologies is faced with the need to remove traces of metal catalysts such as platinum, rhodium, ruthenium, and palladium in the final stages of the process, increasing the cost of producing APIs. Biocatalytic processes can be economically more attractive, as they are generally conducted under mild conditions that include atmospheric pressure, room temperature and aqueous reaction medium, reducing waste generation.[14] Moreover, the advent of direct protein evolution has enabled to tailor an enzyme to accept a specific unnatural substrate, improving catalytic performance, thermostability and stereospecificity.[15] Consequently, biocatalysis is one of the main technologies endorsed by the pharmaceutical industry to produce APIs, a fact that can be proven by the large number of patents that have been granted over the last five years. Currently, approximately 300 biocatalytic processes are operating on an industrial scale.[15-22]

Ketoreductases (KREDs), also called alcohol dehydrogenases (ADHs) or carbonyl reductases (CRs), together with lipases, have been the most used biocatalysts at industrial scale.[17] It is noteworthy that KREDs represent more than a quarter of the commercially available enzymes. This class of enzymes requires a cofactor,

such as nicotinamide adenine dinucleotide (NADH) or its phosphorylated form (NADPH), together with a regeneration system.[23] These enzymes have been widely used to produce chiral secondary alcohols, an important building block in the pharmaceutical industry to produce APIs and in fine chemistry.[24-27]

Lipases stand out among the enzymes used to produce APIs, since they can be used without the need of cofactor addition, besides acting on a wide range of substrates. Both factors, high abundance in nature and ease of genetic manipulation, contributed to the existence of a reasonable set of commercially available lipases. Finally, these enzymes are chemo-, regio- and enantioselective, and are generally active in organic solvent, constituting an advantage for solubilizing the organic substrate to be modified.[28-30]

Herein, we report the study on the chemoenzymatic synthesis of apremilast using as key chiral intermediate a (*R*)-sulfonylethanol, Figure 1. Attempts to produce the intermediate (*R*)-sulfonylethanol involved the use of commercial KREDS and lipases from a sulfonylketone and *rac*-sulfonylethanol acetate, respectively, Figure 1. All investigated approaches focused, especially, on the optimization of the reactional conditions.

Experimental

Enzymes

(*i*) Codex® Ketoreductase (KRED) screening kit was provided by Codexis (California, USA); (*ii*) immobilized lipases: *Candida antarctica* lipase type B immobilized on acrylic resin (CAL-B, Novozym 435, 7300.0 U g^{-1}) was purchased from Novozymes® (Bischheim, France). *Thermomyces lanuginosus* lipase immobilized on immobead-150 (TLL, 250.0 U g^{-1}), Amano lipase PS immobilized on diatomaceous earth (PS-IM, ≥ 500 U g^{-1}), Amano lipase from *Pseudomonas fluorescens* immobilized on immobead 150 (≥ 600 U g^{-1}), Amano lipase from *Pseudomonas fluorescens* immobilized in sol gel AK (≥ 30 U g^{-1}), Amano lipase from *Pseudomonas fluorescens* immobilized in sol gel AK on pumice (≥ 8 U g^{-1}), and Lipozyme RM IM (> 30 U g^{-1}) were acquired from Sigma-Aldrich® (Munich, Germany); (*iii*) crude lipase preparations: *Pseudomonas fluorescens* lipase (AK, 22100.0 U g^{-1}), *Penicillium camemberti* lipase (G, 50.0 U g^{-1}), Amano lipase PS from *B. cepacia* (PS, ≥ 30,000 U g^{-1}), lipase from *Rhizopus niveus* (RNL, ≥ 1.5 U mg^{-1}), Amano lipase from *Mucor javanicus* (MJL, ≥ 10,000 U g^{-1}) and lipase from *Aspergillus niger* (ANL, 200 U g^{-1}) were acquired from Sigma-Aldrich® (Munich, Germany). Porcine pancreas lipase (PPL, 46.0 U g^{-1} solid), and *C. rugosa* lipase (CRL, 1.4 U g^{-1}) were obtained from Sigma® (São Paulo, Brazil).

Chemical materials

Chemical reagents were purchased from different commercial sources and used without further purification. Acetone, hexane, dichloromethane, chloroform, ethyl acetate, methanol and isopropanol were acquired from Biograde (Anápolis, Brazil) acetonitrile was obtained from Tedia (Anápolis, Brazil); toluene, 1,4-dioxane and dimethylsulfoxide (DMSO) were obtained from Synth (Diadema, Brazil); *n*-butanol was purchased from Dinamica (Indaiatuba, Brazil); petroleum ether was from Neon (Suzano, Brazil). High performance liquid chromatography (HPLC) grade isopropanol (IPA) and hexane were purchased from Bio Scie (Anápolis, Brazil); tetrahydrofuran (THF), heptane, cyclohexane, and methyl *tert*-butyl ether (MTBE) were acquired from Sigma-Aldrich (São Paulo, Brazil). Solvents were distilled over an appropriate desiccant under nitrogen. Analytical thin layer chromatography (TLC) analyses were performed on aluminum sheets pre-coated with silica gel 60 F254 (0.2 mm thick) from Merck (Darmstadt, Germany). Flash chromatographies were performed using silica gel 60 (230-240 mesh).

Analysis

Melting points were determined on a Microquimica model APF 301 (Marconi, São Paulo, Brazil) apparatus and are uncorrected. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) were obtained using spectrometer Bruker model Avance DRX-300 (Billerica, USA), operating at frequency of 300 MHz for hydrogen and frequency of 75 MHz for carbon. The chemical shifts are given in delta (δ) values and the absolute values of coupling constants (J) in hertz (Hz). Measurement of the optical rotation was done in a PerkinElmer 341 polarimeter (Shelton, USA). Conversions and enantiomeric excesses were measured by HPLC using a Shimadzu chromatograph model LC solution 20A (Missouri, USA) with a chiral column Chiracel® OD-H (150 m × 4.6 mm I.D.), with oven set at 40 °C for the intermediaries *rac*-**3** and *rac*-**4**. For apremilast and *ent*-apremilast, a chiral column Chiracel® IA (150 m × 4.6 mm I.D.) with oven set at 30 °C was used. The injection volume was 20 μL and flow was 1.0 mL min^{-1} using hexane:IPA (isopropyl alcohol) as eluent and UV detector. The elution conditions for the intermediates *rac*-**3** and *rac*-**4** were as follows: 0-41 min: gradient 95-90% hexane, 41-45 min: gradient 90-97% hexane, 45-50 min: gradient 97-95% hexane, 50-55 min: isocratic 95%

hexane. Retention times were (207 nm): (*R*)-**3** = 31.15 min, (*S*)-**3** = 33.86 min, (*S*)-**4** = 36.32 min; (*R*)-**4** = 41.95 min. The elution conditions for apremilast and *ent*-apremilast were as follows: hexane:IPA (80:20). Retention times were (220 nm): apremilast = 21.88 min, *ent*-apremilast = 25.31 min.

Gas chromatography-mass spectrometry (GC-MS) analyses were obtained on an Agilent 6890 Series GC System (Santa Clara, California, USA) and mass spectra were recorded with a Hewlett-Packard 5973 (Santa Clara, California, USA) mass selective detector (70 eV) using a fused silica capillary column (DB-5MS 5% phenyl 95% dimethylarylene siloxane (30 m × 0.25 mm ID × 0.25 μm film thickness)) and helium as carrier gas (1 mL min^{-1}). The injector temperature was kept at 250 °C and the detector at 280 °C. The column temperature was held at 60 °C for 0.5 min, increased to 290 °C at a rate of 25 °C min^{-1}, and then kept constant for 5 min. Retention times were: ketone **2** = 8.93 min, alcohol (*R*)-**3** = 9.04 min.

Synthesis of 1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanone (**2**)

Dimethylsulfone (926 mg, 9.85 mmol) and 7 mL of dry THF were added into a 25 mL flask, under nitrogen atmosphere and at 0 °C. Then, a solution of *n*-butyllithium in hexane (2.5 M, 3.8 mL, 9.45 mmol) was added to the same flask. The resulting mixture was stirred at 0 °C for 1 h. Subsequently, 3-ethoxy-4-methoxybenzonitrile (**1**) (700 mg, 3.94 mmol) was dissolved in 2.3 mL of dry THF at 0 °C and added dropwise to the reaction mixture, maintaining this temperature for 1 h. After this period, the temperature was raised to room temperature and the reaction system was stirred for 12 h. After, the reaction was quenched with 6 mL of 2.5 M HCl solution and the mixture was stirred for 1 h. Then, the solid formed was filtered and recrystallized from petroleum ether/EtOAc, obtaining a white solid identified as 1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanone (910.5 mg, 85%) with Rf = 0.47 (hexane:EtOAc (1:1)) and mp 144-145 °C.

Synthesis of *rac*-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanol (*rac*-**3**)

In a 20 mL flask, the ketone **2** (300 mg, 1.1 mmol) was dissolved in CH_2Cl_2 and MeOH (1:4, 0.1 M). Then, $NaBH_4$ (50.1 mg, 1.32 mmol) was added to the flask at 0 °C. After 5 min, the temperature was raised to room temperature and the reaction system was stirred for 25 min, followed by the evaporation of solvents under reduced pressure. Then, 10 mL of 1 M HCl solution was added and the product was extracted with EtOAc (3 × 30 mL). The organic phase was treated with anhydrous Na_2SO_4 and, after filtration, the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatographic column with flash silica gel using hexane:EtOAc (1:1) as eluent, to give a white solid identified as *rac*-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethanol, *rac*-**3** (295 mg, 98%), with Rf = 0.33 (hexane:EtOAc (1:1)) and mp 115-117 °C.

Synthesis of *rac*-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl acetate (*rac*-**4**)

In a 10 mL flask, 4-dimethylaminopyridine (DMAP) (55.7 mg, 0.456 mmol) and Ac_2O (259.8 μL, 2.735 mmol) were dissolved in 4 mL of CH_2Cl_2. Then, *rac*-**3** (250 mg, 0.912 mmol) and Et_3N (55 μL, 0.456 mmol) were added to the flask and the reaction mixture was stirred for 1 h. After this time, 2 mL of a saturated solution of $NaHCO_3$ was added and the aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL). The organic phases were combined and treated with anhydrous Na_2SO_4. After filtration, the solvent was evaporated under reduced pressure and the crude product was purified by chromatographic column with flash silica gel using hexane:EtOAc (1:1) as eluent, to give the product 1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl) ethyl acetate, *rac*-**4** (246 mg, 85%), as a white solid with Rf = 0.43 (hexane:EtOAc (1:1)) and mp = 99-100 °C.

Bioreduction of ketone **2** catalyzed by KRED-P2-D12

KRED-P2-D12 (2 mg) and ketone **2** (1.3 mg) were added in a 2 mL Eppendorf. Then, 25 μL of ethanol and 400 μL of a stock solution of KRED Recycle Mix P (composed of 125 mmol L^{-1} of potassium phosphate, 1.25 mmol L^{-1} of $MgSO_4$, 1 mmol L^{-1} of $NADP^+$, 80 mmol L^{-1}, pH 7.0 from 0.15 g of Mix P dispersed in 5 mL of Milli-Q water) were added. Subsequently, 75 μL of IPA was added and the system was stirred at 180 rpm on the orbital shaker at 30 °C for 24 h. The reaction was quenched with the addition of 1 mL of EtOAc, followed by centrifugation for 5 min at 4500 rpm. Soon after, the organic phase was treated with anhydrous Na_2SO_4 and after filtration, the sample was analyzed in GC-MS and HPLC. After analysis, the formation of (*R*)-**3** was confirmed with a conversion of 48% and 93% *ee*.

Synthesis of (*R*)-**3** and (*S*)-**4** via kinetic resolution of *rac*-**4** catalyzed by lipase from *Aspergillus niger*

A solution of acetate *rac*-**4** (72.8 mg, 0.229 mmol) in 2.3 mL of 0.1 M phosphate buffer pH 7/*n*-butanol (8:2) (v/v)

was prepared. Subsequently, lipase from *Aspergillus niger* (3:1 m/m) was added to the solution and the reaction mixture was stirred at 250 rpm in the orbital shaker at 45 °C for 6 h. After this period, 3 mL of EtOAc was added to the reaction mixture, followed by centrifugation for 5 min at 1000 rpm. Then, the organic phase was treated with anhydrous Na_2SO_4. After filtration, the solvent was evaporated under reduced pressure and the crude product was purified by chromatographic column with flash silica gel using CH_2Cl_2:MeOH (9.7:0.3) as eluent, to give 32 mg of (*R*)-**3** ($[\alpha]_D^{20}$ = –22° (*c* 0.65, EtOAc) for > 99% *ee*) and 30.1 mg of (*S*)-**4** ($[\alpha]_D^{20}$ = +55° (*c* 2.33, acetone) for > 99% *ee*). Lit. $[\alpha]_D^{25}$ = –23.19° (*c* 0.42, EtOAc) for (*R*)-**3** (> 99% *ee*).[31]

Synthesis of 4-nitroisoindoline-1,3-dione (**6**)

In a 5 mL flask, 4-nitroisobenzofuran-1,3-dione (**5**) (1.55 mmol, 300 mg) and 540 µL ammonia hydroxide were added. Then, a reflux condenser was adapted to the system (without water flow) and the reaction system was heated to the boiling temperature until all the water had evaporated. Thereafter, the temperature was increased to 230 °C and heating was continued for 2 h. After this time, a yellow solid, identified as 4-nitroisoindoline-1,3-dione (**6**) (278 mg, 93%), was obtained with Rf = 0.61 (hexane: EtOAc (1:1)) and mp 213-215 °C.

Synthesis of 4-aminoisoindoline-1,3-dione (**7**)

In a Schlenk flask, 10% Pt/C catalyst (47.0 mg, 0.24 mmol) and 13.5 mL of ethanol were added. Subsequently, the nitrophthalimide **6** (220 mg, 1.140 mmol) was added to the reaction mixture, under magnetic stirring. The reaction was maintained at an average temperature of 55 °C and in an atmosphere of H_2 at 2 atm for 24 h. After this period, the reaction system was filtered and ethanol was evaporated under reduced pressure, yielding a green colored solid identified as 4-aminoisoindoline-1,3-dione (**7**) (177.5 mg, 96%), with Rf = 0.59 (hexane:EtOAc (1:1)) and mp 265-266 ºC.

Synthesis of 4-acetylamino-isoindol-1,3-dione (**8**)

In a 5 mL flask at room temperature, the aminophthalimide **7** (170 mg, 1.05 mmol) and acetic anhydride (895 µL, 9.5 mmol) were added. The mixture was heated to 80 °C and maintained under stirring for 1 h. Then, acetic anhydride was evaporated under reduced pressure and the solid was washed with cold ethanol, filtered, and dried at room temperature. A white solid was obtained, which was identified as the 4-acetylamino-isoindol-1,3-dione (**8**) (116.6 mg, 54%), with Rf = 0.6 (hexane: EtOAc (1:1)) and mp 236-237 °C.

Synthesis of apremilast

Triphenylphosphine (45.6 mg, 0.174 mmol) and 700 µL dry THF/toluene (1:1) were added into a 10 mL flask, under nitrogen atmosphere, and at –5 °C. Then, diisopropyl azodicarboxylate (27 µL, 0.139 mmol) was added dropwise and the reaction mixture was stirred until the precipitation of a salt. Subsequently, in another flask, the alcohol (*R*)-**3** (32 mg, 0.116 mmol) and the acetylaminophthalimide **8** (23.7 mg, 0.116 mmol) were dissolved in 0.4 mL of dry THF/toluene (1:1) at –5 °C, and under nitrogen atmosphere. This solution was transferred dropwise to the reaction mixture and the system was stirred for 5 h at –5 °C. Then, the temperature of the reaction system was raised to room temperature and stirred for more 7 h. After, the solvent was evaporated under reduced pressure and the crude product was purified on a chromatographic column with flash silica gel using CH_2Cl_2:MeOH (9.8:0.2) as eluent. This procedure gave 34 mg of a white solid identified as (*S*)-*N*-(2-(1-(3-ethoxy-4-methoxyphenyl)-2-methylsulfonyl)ethyl)-1,3-dioxoisoindolin-4-yl) acetamide (apremilast) with 65% yield, Rf = 0.78 (CH_2Cl_2: MeOH (9.5:0.5)) and mp 175-176 °C.

Results and Discussion

Biocatalytic approach to produce the chiral (*R*)-sulfonylethanol intermediate (*R*)-**3**: via KREDs and lipases

Initially, the ketone (**2**) was obtained from the commercially available 3-ethoxy-4-methoxybenzonitrile (**1**) in two steps. The first step consisted of the reaction of **1** with the dimethylsulfone carbanium formed in the presence of *n*-butyllithium, yielding the corresponding enamine. This latter was treated one-pot with a 2.5 M HCl solution to produce the ketone (**2**) in 85% yield (Figure 2). Two alternative routes were investigated to produce (*R*)-**3**, one involving the reduction of ketone **2** catalyzed by KREDs (Figure 2, path A) and other by lipase-mediated hydrolysis of the *rac*-sulfonylethanol acetate (*rac*-**4**), Figure 2, path B.

Reduction of ketone **2** catalyzed by KREDs

Initially, ketone **2** was subjected to chemical reduction in the presence of sodium borohydride in methanol and dichloromethane, leading to hydroxysulfone *rac*-**3** in 98% yield (Figure 2, path B). Then, a method using GC-MS was developed to separate ketone **2** from hydroxysulfone

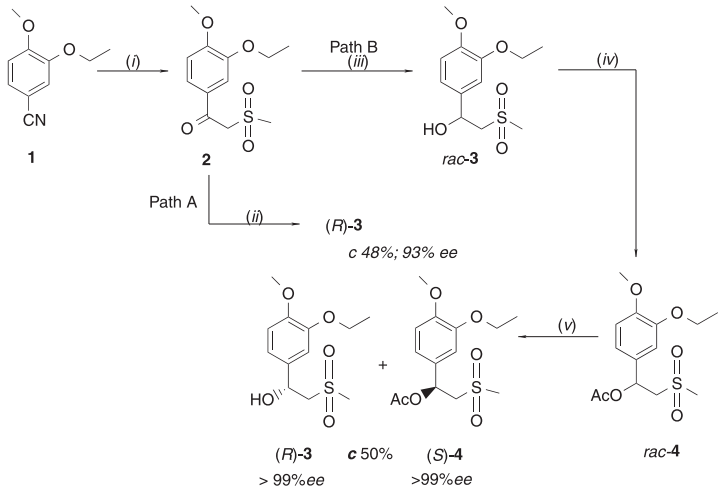

Figure 2. Chemical routes to produce the chiral sulfonylethanol intermediate (*R*)-**3** via reduction of ketone **2** catalyzed by KREDs (path A) or lipase-mediated hydrolysis of *rac*-acetate (path B). Reagents and conditions. Path A: (*ii*) KRED-P2-D12, NAD(P)H, EtOH; IPA, H_2O, $MgSO_4$, K_3PO_4, pH 7, 30 °C, 24 h. Path B: (*i*) Me_2SO_2/*n*-BuLi, THF; 0 °C, then HCl/H_2O; 2.5 M (85%); (*iii*) $NaBH_4$ in CH_2Cl_2/MeOH, 0 ºC to r.t. (98%); (*iv*) Ac_2O, DMAP and Et_3N in CH_2Cl_2, at r.t. (85%); (*v*) lipase from *Aspergillus niger*, phosphate buffer pH 7 0.1 M and *n*-butanol (8:2), 45 °C, 6 h ((*R*)-**3** 44%, > 99% *ee*), ((*S*)-**4** 45%, > 99% *ee*).

rac-**3**, aiming to determine the conversion in the reduction reaction catalyzed by KREDs.

A screening was performed with the 24 ketoreductases from Codexis® (Codex KRED Screening Kit) using previously reported conditions.[32] Ketone **2** was subjected to a reduction reaction at 30 °C, 24 h, 180 rpm in the presence of a KRED with its respective cofactor recycling system and using DMSO as co-solvent (85.7% of KRED Recycle Mix, 9.5% of IPA and 4.8% of DMSO; v:v:v). In this case, DMSO was used to solubilize ketone **2** in the reaction medium. After 24 h of reaction, the conversion value was determined by GC-MS analysis. Among the 24 KREDs assessed, only 8 were active. The results are summarized in Table 1. The table containing all KREDs assessed for ketone **2** reduction is found in Supplementary Information (SI) section.

Among the eight KREDs active in reducing ketone **2**, only two showed *anti*-Prelog selectivity, leading to hydroxysulfone (*R*)-**3** (Table 1, entries 1 and 2). Both KRED-P2-D03 and KRED-P2-D12 produced (*R*)-**3** with values of *ee* > 90%, the latter leading to a higher conversion value (25%). Due to this, KRED-P2-D12 was chosen for further tests with the aim of increasing the conversion of bioreduction reaction of ketone **2**. It is known that one of the advantages of the KRED-P2-D12 is the tolerance to a high amount of IPA used in the recycling of the cofactor, in the same way as the nineteen first KREDs listed in Table S1 (SI section).[33] The reduction of the carbonyl group takes place under thermodynamic control and, generally, requires a large excess of IPA to favor high conversion values.[34] In this way, we decided to evaluate the conversion behavior in the bioreduction of ketone **2**, progressively increasing the concentration of IPA in the reaction medium with a concomitant decrease in the Recycle Mix and keeping the concentration of DMSO in 4.8%. The results are summarized in Table S2 (SI section).

Table 1. Screening of commercial ketoreductases for reduction of ketone **2**

entry	KRED	Cofactor	Cofactor recycling system	c[a] / %	*ee*[b] / %
1	KRED-P2-D12	NADPH	isopropanol	25	91 (*R*)
2	KRED-P2-DO3	NADPH	isopropanol	15	95 (*R*)
3	KRED-P1-B02	NADPH	isopropanol	15	90 (*S*)
4	KRED-P1-C01	NADPH	isopropanol	94	98 (*S*)
5	KRED-P2-B02	NADPH	isopropanol	90	48 (*S*)
6	KRED-P2-C02	NADPH	isopropanol	18	27 (*S*)
7	KRED-P2-D11	NADPH	isopropanol	90	93 (*S*)
8	KRED-P2-G03	NADPH	isopropanol	15	42 (*S*)

[a]Conversion measured by gas chromatography-mass spectrometry (GC-MS); [b]enantiomeric excess determined by HPLC. KRED: ketoreductase; NADPH: nicotinamide adenine dinucleotide in phosphorylated form.

With the increase in IPA up to 14.3%, there was an increase in conversion from 25 to 33%, but concentrations above this value led to a decrease in conversion. This behavior is in line with results published in the literature.[35] In addition to the DMSO, eleven more organic co-solvents were evaluated, which can modify the three-dimensional structure of an enzyme and its catalytic activity.[35,36] In this case, ethanol was the most efficient co-solvent, increasing the conversion from 33 (DMSO) to 48%, acting as a co-solvent, as well as a regenerating cofactor.[37]

Once the conditions for the reduction of ketone **2** in the presence of KRED-P2-D12 were optimized, we turned our attention to the biocatalytic approach consisted of obtaining alcohol (*R*)-**3** via kinetic resolution of acetate *rac*-**4** by hydrolysis reaction catalyzed by lipases (Figure 2, path B).

Kinetic resolution of *rac*-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl acetate (*rac*-**4**) via lipase-mediated hydrolysis

First, the previously prepared hydroxysulfone *rac*-**3** was subjected to acetylation reaction in the presence of acetic anhydride, DMAP and triethylamine in dichloromethane, providing acetate *rac*-**4** in 85% yield. Chiral HPLC methods were developed for both *rac*-**3** and *rac*-**4** to reliably measure the enantiomeric excesses of both the remaining substrate and the final product in the lipase-catalyzed resolution of *rac*-**4**.

Fifteen commercially available lipases (listed in "Enzymes" sub-section) were evaluated in the kinetic resolution of *rac*-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl acetate (*rac*-**4**) via hydrolysis reaction, Figure 2 (path B). The reactions were conducted using conditions previously reported by our research group,[38] such as 1:2 ratio (m:m) of substrate/lipase, phosphate buffer 0.1 M (pH 7.0), acetonitrile as co-solvent (buffer/co-solvent 8:2, v:v), 30 °C, 250 rpm and 24 h. In these conditions, only the lipase from *Aspergillus niger* was active, leading to alcohol (*R*)-**3** with 93% *ee* and acetate (*S*)-**4** with 98% *ee*, E-value (enantiomeric ratio) of 127 and 51% conversion (Figure 2, path B). Although we obtained promising results in the production of (*R*)-**3**, we decided to evaluate the behavior of this kinetic resolution in the absence of a co-solvent and in the presence of several other co-solvents. The results are summarized in Table 2.

Notably, the kinetic resolution of *rac*-**4** in the absence of co-solvent, in just 13 h of reaction, reached the maximum conversion value (ca. 50%) albeit with a low enantioselectivity value (Table 2, entry 1). It is known that water participates directly or indirectly in non-covalent interactions to maintain the conformation of an active enzyme.[39] In a totally aqueous microenvironment, the conformational change of the active site is highly flexible,[40,41] which could provide a favorable adjustment of the two enantiomers of *rac*-**4**, leading to low enantioselectivity. The kinetic resolution of *rac*-**4** in the presence of the most varied co-solvents, polar or nonpolar (with different log P and dielectric constant),

Table 2. Effect of co-solvent, temperature, and enzyme:substrate ratio on the lipase from *Aspergillus niger*-mediated enzymatic kinetic resolution of *rac*-**4**[a]

entry	Co-solvent	Log P	ε	time / h	ee_s[b] / %	ee_p[b] / %	c[c] / %	E[d]
1	none	–	–	13	44	42	51	4
2	acetonitrile	–0.33	38	13	98	93	51	127
3	1,4-dioxane	–0.27	2.21	24	98	87	51	60
4	isopropanol	0.05	18.3	9	72	75	49	15
5	THF	0.46	7.6	11	90	91	50	62
6	*n*-butanol	0.88	18	13	> 99	96	51	> 200
7	*n*-butanol[e]	0.88	18	9	> 99	> 99	50	> 200
8	*n*-butanol[f]	0.88	18	7	3	91	3	21
9	*n*-butanol[g]	0.88	18	9	> 99	98.8	50.3	> 200
10	*n*-butanol[h]	0.88	18	9	> 99	> 99	50	> 200
11	*n*-butanol[i]	0.88	18	6	> 99	> 99	50	> 200
12	*n*-butanol[j]	0.88	18	23	> 99	> 99	50	> 200
13	MTBE	0.94	2.6	13	88	92	49	67

[a]Conditions: 30 °C, 0.1 M phosphate buffer:co-solvent (8:2; v:v), lipase: *rac*-**4** (2:1) at 250 rpm; [b]enantiomeric excess determined by HPLC; [c]conversion, $c = ee_s/(ee_s + ee_p)$; [d]enantiomeric ratio, $E = \ln[1 - c(1 + ee_p)] / \ln[1 - c(1 - ee_p)]$; [e]reaction performed at 45 °C; [f]reaction performed at 50 °C; [g]conditions: 45 °C, 9 h, 0.1 M phosphate buffer:*n*-butanol (9:1; v:v), lipase: *rac*-**4** (2:1); [h]conditions: 45 °C, 9 h, 0.1 M phosphate buffer:*n*-butanol (7:3; v:v), lipase: *rac*-**4** (2:1); [i]conditions: 45 °C, 6 h, 0.1 M phosphate buffer:*n*-butanol (8:2; v:v), lipase: *rac*-**4** (3:1); [j]conditions: 45 °C, 23 h, 0.1 M phosphate buffer:*n*-butanol (8:2; v:v), lipase: *rac*-**4** (1.5:1). log P: 1-octanol-water partition constant; ε: dielectric constant; THF: tetrahydrofuran; MTBE: methyl *tert*-butyl ether.

reached conversion values close to 50% in reaction times that varied from 9 to 24 h with E-values ranging from 15 to > 200 (Table 2). Although there is no general rule that indicates the physical/chemical properties of a co-solvent that act in the alteration of enzyme activity and enantiomeric ratio, the most evaluated are log P and dielectric constant.[42-44] In fact, two distinct enantiomeric ratio values were obtained in co-solvents with log P < 0, since in acetonitrile the E-value was 127 and in 1,4-dioxane it was 60 (Table 2, entries 2 and 3). This difference in enantiomeric ratio could be attributed to the ability of acetonitrile to act in the stabilization of enzyme charges, since it presents a higher dielectric constant value (38) in relation to the 1,4-dioxane dielectric constant (2.21). Among co-solvents with log P > 0, the only one that provided a high E-value (E > 200) was *n*-butanol (Table 2, entry 6). Apparently, *n*-butanol would be the co-solvent that presents an ideal balance between the values of log P (0.88) and dielectric constant (18), enabling a high enantiomeric ratio in the performance of lipase from *A. niger*. Isopropanol has a dielectric constant value (18.3) like *n*-butanol (18), but a much lower log P (0.05) (Table 2, entries 4 and 6, respectively). Although the analysis of log P and dielectric constant are not sufficient to fully explain the behavior of co-solvents in the kinetic resolution of *rac*-**4**, it is evident that both acetonitrile (Table 2, entry 2) and *n*-butanol (Table 2, entry 6) promote a change in the microenvironment of the catalytic site of lipase from *A. niger*, inducing an ideal conformation for high enantiomeric ratio, especially in the case of *n*-butanol which provided an E-value > 200.

In a few more attempts to optimize the kinetic resolution of *rac*-**4**, we decided to increase the temperature from 30 to 45 and 50 °C (Table 2, entries 7 and 8). Actually, at a higher temperature, the resolution of *rac*-**4** occurred in a shorter reaction time and decreased from 13 h at 30 °C to 9 h at 45 °C. Surprisingly, at the same time there was an increase in the *ee* of the product that went from 96% (30 °C) to > 99% (45 °C). However, at 50 °C it was possible to observe a deactivation of lipase from *A. niger*, since in 7 h of reaction the conversion was only 3% (Table 2, entry 8). A similar behavior was observed in the kinetic resolution of *rac*-phenylethylamines catalyzed by lipase from *A. niger*, whose maximum temperature for E-values > 200 was 45 °C.[45] In a glance analysis, it is surprising to see an increase in enzymatic enantioselectivity with increasing temperature, since in most situations the opposite occurs, enantioselectivity decreases with increasing temperature. However, there are several reports in the literature[46-56] in which enzymatic enantioselectivity increases with increasing temperature. In these cases, the enantiomer is favored by entropy and the degree of flexibility at the active site of the enzyme is more evident than electrostatic and stereostatic effects.[47,49,54-56]

Since *n*-butanol showed the best performance as a co-solvent in the kinetic resolution of *rac*-**4**, we decided to investigate the influence of the amount of this co-solvent in the reaction medium, since preliminary studies were carried out in a buffer:*n*-butanol ratio of 8:2 (v:v). In the 9:1 ratio *ee* of (*R*)-**3** had a slight drop (98.8%) and remained at > 99% in a 7:3 ratio, leading to the conclusion that 8:2 is the ideal buffer:*n*-butanol ratio (Table 2, entries 9 and 10, respectively).

In this way, the addition of an ideal amount of *n*-butanol to the aqueous reaction medium and the increase in temperature from 30 to 45 °C provided the achievement of (*R*)-**3** in enantiomerically pure form. It is known that in a totally aqueous medium, lipase exhibits a high flexibility[40,41] and this was probably the dominate factor for the lack of enantioselectivity in the kinetic resolution of *rac*-**4** in the absence of co-solvents (Table 2, entry 1). In the totally aqueous medium, the high flexibility of lipase from *A. niger* was responsible for accommodating the *rac*-**4** enantiomers in a similar way, leading to low enantioselectivity. The addition of a co-solvent to the aqueous reaction medium with a positive log P such as *n*-butanol was responsible for dramatically increasing the enantiomeric ratio (E), which went from a value of 4 to > 200 (Table 2, entries 1 and 6, respectively). The addition of *n*-butanol must have caused a reduction in the flexibility of the lipase,[57] altering the conformation at the active site and providing a more favorable accommodation for the (*R*)-**4** in detriment to its respective enantiomer, leading to a high enantioselectivity. Although the addition of *n*-butanol was responsible for dramatically increasing enantioselectivity, obtaining (*R*)-**3** in enantiomerically pure form was only possible when, at the same time, the temperature was increased from 30 to 45 °C (Table 2, entry 7). In this case, the high enantioselectivity must have been caused by two distinct effects, one related to the decrease in the polarity of *n*-butanol at a higher temperature (ε (30 °C) 17.6 and ε (45 °C) 14.7), which should cause an increase in the conformational rigidity of the enzyme (as observed in preliminary inhouse circular dichroism data, not shown) and the other related to an increase in the conformational flexibility of the enzyme by the temperature increase. These two effects act in the opposite way, but the balance between them is probably responsible for the ideal enantioselectivity observed in the kinetic resolution of *rac*-**4**.

Finally, we investigate the influence of the enzyme loading on the kinetic resolution of *rac*-**4**. With the increase in the enzyme:substrate ratio from 2:1 to 3:1, there was a

decrease in reaction time from 9 to 6 h (Table 2, entry 11). On the other hand, with the decrease in the enzyme:substrate ratio from 2:1 to 1.5:1, there was an increase in reaction time to 23 h (Table 2, entry 12). Therefore, the ideal rate of enzymatic loading enzyme:substrate (m:m) for the kinetic resolution of *rac*-**4** to occur in the shortest possible reaction time is 3:1 (Table 2, entry 11).

Once our objective of obtaining (*R*)-**3** in enantiomerically pure form has been achieved, we focus our attention on the synthesis of apremilast.

Synthesis of apremilast

To perform the synthesis of apremilast, it was necessary to obtain acetylaminophthalimide **8**, starting from commercially available nitro anhydride **5** (Figure 3).

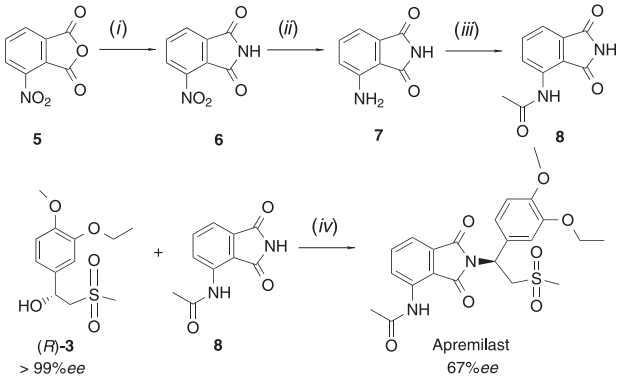

Figure 3. Synthesis of apremilast. Reagents and conditions: (*i*) NH_4OH, 230 °C (93%); (*ii*) H_2/Pt 2 atm, EtOH, 50 °C (96%); (*iii*) Ac_2O, 80 °C (54%); (*iv*) PPh_3, DIAD, THF/toluene, –5 °C to r.t. (65%, 67% *ee*).

The latter was converted to the corresponding phthalimide **6**, in the presence of ammonium hydroxide and heated to 230 °C, with 93% yield. Subsequently, nitrophthalimide **6** was reduced to aminophthalimide **7** in the presence of Pt as a catalyst, with 96% yield. In the last step, aminophthalimide **7** underwent an acetylation reaction, leading to the achievement of acetylaminophthalimide **8** with 54% yield.

Finally, apremilast could be obtained via a Mitsunobu reaction between enantiomerically pure alcohol (*R*)-**3** and acetylaminophthalimide **8**. When the reaction was carried out using THF as solvent at –5 °C, apremilast was obtained with 65% yield and 51% *ee*. In this case, a degree of racemization was observed during the reaction process, suggesting the reaction occurred via an S_N1-type mechanism. Even so, a moderate value of enantiomeric excess was probably assured due to the formation of an intimate ion pair between the phosphonium intermediate with *ortho*- and *para*-alkoxybenzyl alcohol with a carbocation character. To enhance the formation of intimate ion pairs in the reaction medium, leading to an increase in the *ee* of apremilast, the reaction was repeated with the addition of a more non-polar solvent, toluene. Thus, when the Mitsunobu reaction was carried out in the presence of a 1:1 (v:v) mixture of toluene/THF, maintaining the temperature of the reaction medium at –5 °C, apremilast was obtained with a yield of 65% and with 67% *ee* (Figure 3).

Conclusions

In summary, a straightforward chemoenzymatic synthesis from apremilast was developed. Nitrile **1** was an ideal starting material as it provided both intermediates used in the two biocatalytic approaches, ketone **2** and *rac*-**4** acetate, in high yield values. In the first approach studied, among the 24 KREDs evaluated, only two enzymes acted *anti*-Prelog, with emphasis on KRED-P2-D12 which led to alcohol (*R*)-**3** with 48% conversion and 93% *ee*, using ethanol as co-solvent. Between the two biocatalytic approaches for obtaining the alcohol (*R*)-**3**, the one that used lipases stood out compared to the one that used ketoreductases, since in this case the chiral intermediate was obtained at the maximum conversion of 50% and in the enantiomerically pure form. Lipase from *A. niger* has been shown to be an effective enzyme in the key stage of apremilast synthesis. It is worth mentioning that the kinetic resolution of *rac*-**4** was only effective (conversion 50% and E > 200) in the presence of lipase from *A. niger* when the reaction was carried out in phosphate buffer medium containing 20% of *n*-butanol and when the temperature was increased from 30 to 45 °C. A plausible explanation for the increase in enantioselectivity with increasing temperature is that the preferred enantiomer in enzymatic hydrolysis, acetate (*R*)-**4** is favored by entropy, a phenomenon that involves changes in the conformation of the active enzyme site. Thus, a combination and a balance of factors that include an increase in enzymatic stiffness by *n*-butanol and an increase in flexibility due to temperature increase must be responsible for the lipase from *A. niger* to reach an ideal conformation for a high degree of discrimination in relation to (*R*)-**4**.

Finally, apremilast was obtained through the Mitsunobu reaction of alcohol (*R*)-**3**, in the enantiomerically pure form, with acetylaminophthalimide **8**, resulting in 65% yield and 67% *ee*. Although the reaction is expected to undergo S_N1-type mechanism, our data suggests that the use of a mixture of toluene:THF must have ensured a reasonable percentage of intimate ionic pairs in the reaction medium, providing apremilast with a reasonable value of *ee*.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Kimberly B. Vega was responsible for the conceptualization, investigation, data curation, formal analysis and writing original draft; Daniel M. V. Cruz for the investigation; Artur R. T. Oliveira for the investigation; Marcos R. da Silva for the investigation and formal analysis; Telma L. G. de Lemos for the conceptualization and writing original draft; Maria C. F. Oliveira for the conceptualization, funding acquisition and writing original draft; Ricardo D. S. Bernardo for the investigation; Jackson R. de Sousa for the conceptualization and writing original draft; Geancarlo Zanatta for the conceptualization and writing original draft; Fábio D. Nasário for the investigation and formal analysis; Anita J. Marsaioli for the conceptualization, funding acquisition and writing original draft; Marcos C. de Mattos for the conceptualization, funding acquisition, writing original draft and editing.

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