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Selective Lipase-Catalysed Hydrolysis of a 1,2-Diester in the Development of a New Route to AZD2563 DSP

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Abstract:

During the development of a new route to AZD2563 DSP (DSP = disodium phosphate), a selective enzyme-catalysed hydrolysis of a 1,2-diester moiety to produce the secondary monoester was developed. Apart from two esters, the target molecule also contained three further functional groups prone to hydrolysis. A major challenge to the chosen approach was the very facile rearrangement of the desired secondary monoester product to the undesired primary monoester. This rearrangement was found to be catalysed by a wide range of chemicals and inorganic materials usually considered as inert. The unique selectivity and mild operating conditions of biocatalysis allowed the desired reaction to be developed and successfully scaled up.

Introduction

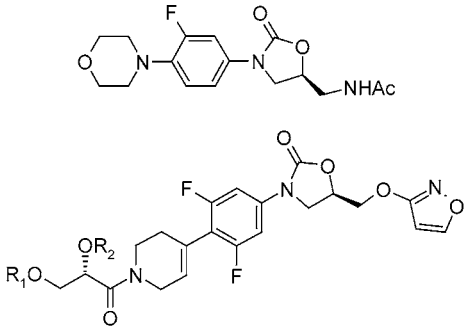
Increasing antibiotic resistance in Gram-positive bacteria typified by the surge in methicillin-resistant *Staphylococcus aureus* (MRSA) has highlighted the urgent need for new and efficient antimicrobial agents. One of the most successful new classes of drug are the oxazolidinones, Linezolid (Zyvox) being the first in the class (Scheme 1).[1]

A member of the oxazolidinone class of new antimicrobials being developed by AstraZeneca, AZD2563, is shown in Scheme 1. AZD2563 was being developed as an oral candidate, and the primary monophosphate disodium salt, AZD2563 DSP, as a water-soluble intravenous (*i.v.*) candidate.[2,3]

At the start of the development programme, the oral and *i.v.* candidates had different synthetic routes branching at the construction of the piperidinyl amide.[4] The diol was derived from *S*-2,2-[1,3]-dioxolane-4-carboxylic acid and the phosphate from *O*-*tert*-butyl serine. After diazotisation, hydrolysis, and amide coupling, the secondary alcohol was phosphitylated with Et_2NP (*O*-*t*-Bu)$_2$ and oxidised with H_2O_2; then the phosphorus and primary alcohol *t*-Bu protecting groups were removed, and the phosphate was migrated from the secondary to the primary alcohol group followed by salt formation (Scheme 2). There existed a number of safety, health, environmental, and scale-up issues with the route to AZD2563 DSP via *O*-*tert*-butyl serine. This was mainly the high cost of *tert*-butyl serine, low yields, and issues with the diazotisation chemistry on-scale. Hence, a more convergent synthetic strategy between oral and *i.v.* dosage forms was sought.[5]

The major application of enzymes in organic synthesis is directed towards the preparation of chiral molecules.[6] In addition to enantioselectivity, many hydrolytic enzymes display a high degree of regioselectivity towards polyfunctional molecules, and we sought to utilize this selectivity in the manipulation of AZD2563 esters. We report the results of our successful study into the application of biocatalysis

Scheme 1. **Oxazolidinone antimicrobials**



Drug candidates

R_1, R_2 = H, AZD2563 – oral candidate

R_1 = $PO(ONa)_2$, R_2= H AZD2563 DSP – *i.v.* candidate

Ester Intermediates

$R_1 = R_2 = CH_3CO$ diacetate = AZD2563 DA

$R_1 = CH_3CO$, R_2 = H primary mono acetate = AZD2563 PMA

R_1 = H, $R_2 = CH_3CO$ secondary mono acetate = AZD2563 SMA

$R_1 = R_2 = C_3H_7CO$ dibutyrate = AZD2563 DB

$R_1 = C_3H_7CO$, R_2 = H primary mono butyrate = AZD2563 PMB

R_1 = H, R_2 C_3H_7CO= secondary mono butyrate = AZD2563 SMB

$R_1 = R_2 = (CH_3)_2CHCO$ di-i-butyrate = AZD2563 DiB

$R_1 = (CH_3)_2CHCO$, R_2 = H primary mono i-butyrate = AZD2563 PMiB

R_1 = H, R_2 = $(CH_3)_2CHCO$ secondary mono i-butyrate = AZD2563 SMiB

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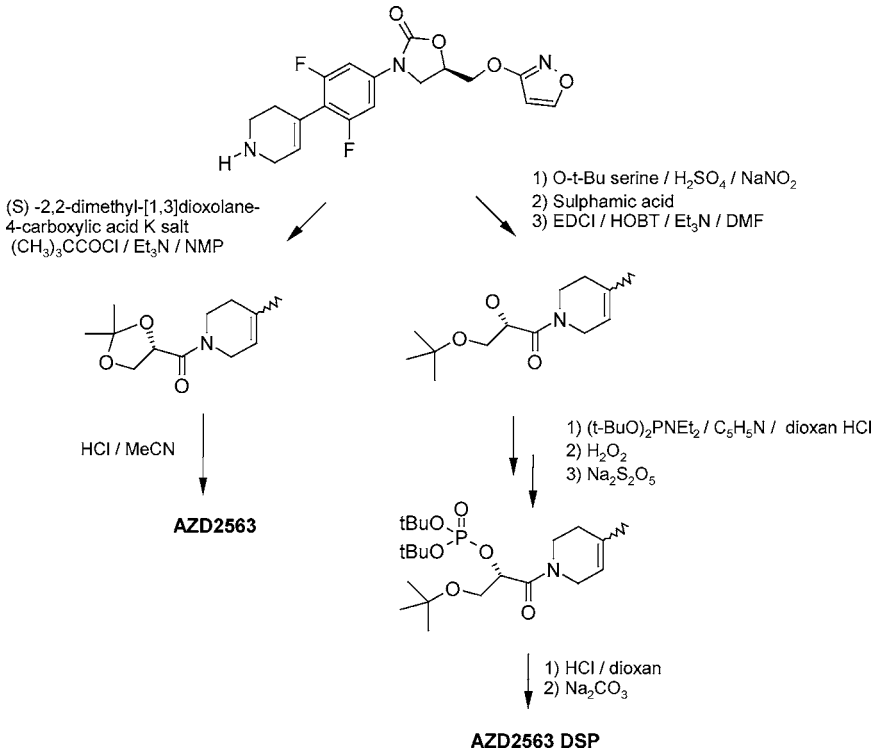
10.1021/op060003h CCC: $33.50

Scheme 2. **Early Synthesis of AZD2563 and AZD2563 DSP**



to convert AZD2563 into AZD2563 DSP via selective manipulation of ester groups on the 1,2-diol portion of the molecule.

Synthesis of Primary Monoesters. The structures of the ester derivatives of AZD2563 are shown in Scheme 1.

Our first approach was to selectively protect the primary alcohol of AZD2563, phosphorylate, remove the primary ester, and then migrate the phosphate group. This would mirror the current synthesis but allow AZD2563 to be used as the starting material for the phosphate pro-drug. Lipases are known to be highly selective catalysts for differentiating primary *vs* secondary and *tert*-alcohol functions in acylation reactions.[7−9] Indeed, this was found to be the case with AZD2563. A number of lipases (*Candida antarctica* B, *Candida antarctica* A, *Candida rugosa*, *Mucor miehei*, *Pseudomonas stutzei*, *Pseudomonas fluorescens*, *Alcaligenes*, *Achrombacter*, *Pseudomonas cepacia*, and *Thermomyces lanuginosus)* were identified that were highly selective for the monoacylation of the primary alcohol group. A variety of acyl donors were effective, e.g., 2,2,2-trifluoroethyl acetate, 2,2,2-trifluoroethyl butyrate, vinyl acetate and butyrate, isoprenyl acetate, and trifluoroisoprenyl acetate. In all cases, no secondary monoesters were seen, the only by-products being very small traces of 1,2-diesters. Some 1,2-diols can be diacylated by lipases.[7,8] The (*S*) configuration of the secondary alcohol in AZD2563 is not favored by lipases; hence, very little diacylation was found. Whilst very selective, the reaction with lipase powders was generally very slow. To obtain reasonable reaction rates the lipases had to be supported on Celite, ceramic particles, or macroporus resins or used as highly pure, cross-linked enzyme crystals (CLECs).[10] Initially, we selected *Candida rugosa* and vinyl acetate from the screening phase, but when scaled-up, the reaction became very slow and difficult to push to completion. This was attributed to inhibition of the enzyme by liberated acetaldehyde.[11]

The better combination identified was *Pseudomonas cepacia* lipase on ceramic particles, Amano PS-C1, and isoprenyl acetate or vinyl butyrate in 1,4-dioxan as solvent.[12] This methodology gave AZD2563 PMA or AZD2563 PMB in ≥95% yield on a multigram scale, but this option was not further developed due to various problems associated with clean and efficient conversion at scale of AZD2563 PMA or PMB into the desired AZD2563 DSP, the chief reason being premature and undesired hydrolysis of the primary ester during the phosphitylation step, leading to complex mixtures.We then chose to look at an alternative strategy via a secondary monoester.

Synthesis of Secondary Monoesters. A variety of diesters could be synthesized from AZD2563 in a straightforward and high-yielding manner by reaction with acid anhydrides in the presence of 4-(dimethylamino)pyridine (DMAP). We expected to be able to find a hydrolytic enzyme that would preferentially remove the primary ester group.[13] We felt that the mild conditions offered by enzymic

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Table 1. **Product distribution in the *C. rugosa* hydrolysis of AZD2563 DA**

composition by HPLC	screening conditions %	scale-up conditions %
AZD2563 DA starting material	1−3	1−3
AZD2563 SMA product	∼93	∼30
AZD2563 PMA by-product	1−2	∼30
AZD2563 diol by-product	2−3	∼30

hydrolysis would be highly suitable for this transformation since other chemistries had demonstrated that the amide, carbamate, and oxazolidine ether linker bond were prone to hydrolysis. The pH stability window for AZD2563 diesters was found to be only between pH 4 and pH 8. It should be noted that AZD2563 also has an epimerisable chiral center to contend with.

We commenced by screening the diacetate, AZD2563 DA. Due to very poor solubility in aqueous buffers, the hydrolysis was run in a 9:1 v/v mixture of *t*-BuOH−water. The screening concentration was very dilute (5−10 mg mL^{-1}).

Esterases were found to be inactivated under these low-water conditions, and proteases that showed reactivity were fairly unselective. Initial results were very encouraging with a lipase, *Candida rugosa*. The results are shown in Table 1. The reaction was then scaled up. The concentration of the substrate was increased to 10 w/v to improve productivity. Whilst maintaining selectivity, rate, and conversion were poor, this was attributed to the fall in pH as butyric acid was liberated, inhibiting the *Candida rugosa* lipase. Thus, for the more concentrated reactions, the pH was maintained at 7.00 by the use of phosphate buffer, or more efficiently, by the automated controlled addition of dilute NH_3 or NaOH via a pH stat. The results obtained now were quite different and were totally unselective (see Table 1). The experiments were rechecked and identical data obtained—excellent selectivity in screening with no additives, very poor under scale-up conditions whilst trying to maintain pH. Scaling up the very dilute screening conditions to several-litre scale worked and gave a few grams of pure AZD2563 SMA for further study.

An examination of the effect of pH was carried out under the scale-up conditions. The selectivity did not improve, but the distribution of reaction products was pH dependent. At pH 8.00 mainly AZD2563 was produced, and at pH 6 mixtures of AZD2563 SMA and AZD2563 PMA were seen.

A study of the various processes occurring during the enzyme hydrolysis of AZD2563 DB was undertaken. A surprisingly complex system was unraveled, summarized in Scheme 3.

Hydrolysis of the diester proceeds to give the desired product, AZD2563 SMB. Some enzymes can further hydrolyse this product to ADZ2563. The major issue was product stability towards rearrangement by migration of the ester group from the secondary to primary alcohol to give AZD2563 PMB. Similar rearrangements have been noted by others working with triglycerides and related materials.[14−18] During the course of the study it became apparent that a large number of reagents and solids would efficiently catalyse the rearrangement of AZD2563 SMB to AZD2563 PMB. These included bases such as pyridine, Et_3N, DMAP, Na_2CO_3, carboxylate salts and even "inert" materials such as polar solvents, NaCl, K_2SO_4, Celite, phosphates, and pH 7.00 buffer solutions and some lipases.[14,17] This rearrangement was thus identified as the loss in selectivity when the hydrolysis was scaled up with neutralization of the carboxylic acid by-product. Some typical results for the rearrangement catalysed by inorganic solids are presented in Table 2. These were obtained by stirring pure AZD2563 SMB with the solids under process conditions, and monitoring the conversion by HPLC analysis. In the presence of a base or nuclophile, the rearrangement can be attributed to a general base or nucleophile-catalysed mechanism. What is happening in the presence of materials such as Celite or Na_2SO_4 is unclear. It has been reported that secondary monoglycerides can undergo similar rearrangements catalysed by charged surfaces.[14,16]

This primary monoester is a better substrate for the enzyme than the diester and, once formed in the presence of lipase, is rapidly hydrolysed to AZD2563. To further complicate the reaction, the butyric acid by-product acted as a competitive inhibitor and lowered the reaction rate. It was also noted that under certain conditions the product AZD2563 SMB could be slowly reacylated back to dibutyrate starting material, the enzyme using butyric acid as acyl donor source.[19]

Choice of Diester A number of secondary monoesters were made to study the tendency of the secondary ester to migrate. The acetate, AZD2563 SMA, was most prone, followed by the butyrate, AZD2563 SMB, the isobutyrate, AZD2563 SMiB, being the most stable. The rate of rearrangement of the primary ester being ∼2 times that of the secondary ester. However, the rate of enzyme hydrolysis of the diisobutyrate ester was unacceptably slow. With *Pseudomonas cepacia* catalyst, at ∼24 h the dibutyrate reaction would be >95% complete. At a comparable time scale, the diisobutyrate reaction would only have gone to 3−5% completion. The use of *Candida rugosa* with the diisobutyrate increased conversions to ∼25%, but the productivity was still deemed too slow for production. Hence, the dibutrate was selected for scale-up, being a good compromise between reactivity and product stability.

Choice of Biocatalyst. For a high yielding reaction, the enzyme has to show high regioselectivity for cleavage of the primary ester, not catalyse the AZD2563 SMB to AZD2563 PMB rearrangement, and be able to work at a pH

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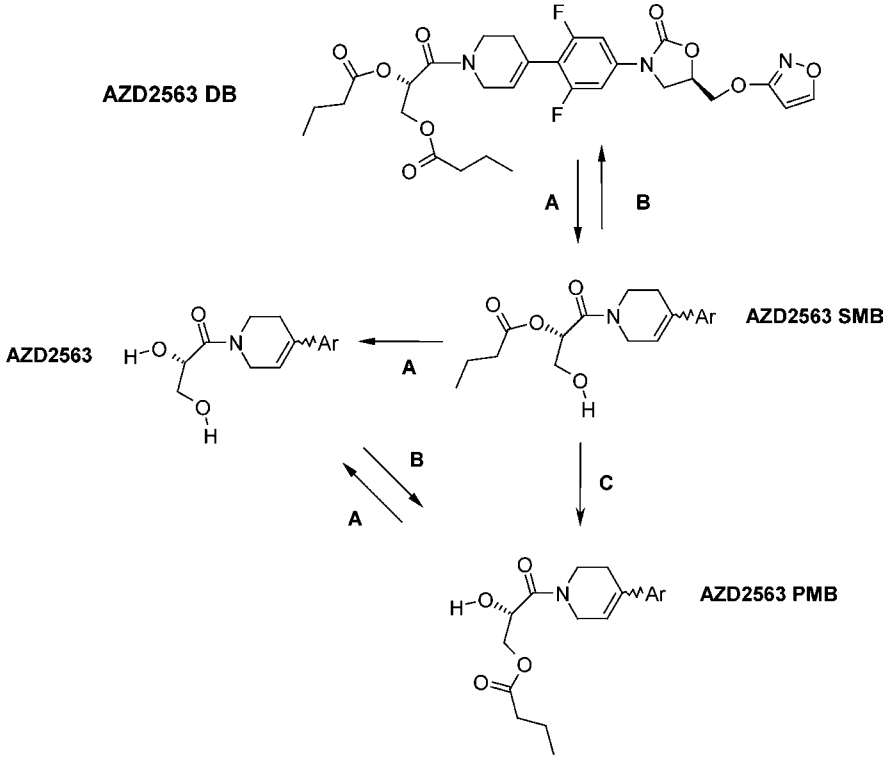
Scheme 3. **Mechanism of the enzyme-catalysed hydrolysis of AZD2563 DB[a]**



[a] **A** = enzyme-catalysed hydrolysis; **B** = enzyme-catalysed acylation; **C** = ester migration. Butyric acid by-product is a reversible competitive inhibitor.

Table 2. **Rearrangement of AZD2563 SMB to AZD2563 PMB with inorganic solids[a]**

	% primary ester formed at 24 h					
solid	Celite	Na_2SO_4	K_2SO_4	NaCl	KCl	$ZnCl_2$
	90	80	75	10	15	100

[a] 5 w/v AZD2563 SMB in 90% v/v *t*-BuOH−water, 40 °C, 5 wt % solid.

of ∼5 since neutralization of the acid liberated during the course of the reaction was not possible. Any atempt to do so via the addition of organic/inorganic bases or buffers resulted in some rearangement of AZD2563 SMB to AZD2563 PMB, thus lowering the observed selectivity of the hydrolysis.Also, any carrier used to support the enzyme had to be an inactive catalyst in the secondary to primary ester rearrangement. *Pseudomonas cepacia* lipase was identified as the best available enzyme from those screened. Various supported *Pseudomonas cepacia* biocatalysts were available, Celite - Amano PS-D, ceramic particles - Amano PS-C1, K_2S0_4 protein-coated microcrystals,[20] CLECs,[21] and macroporus resin - Eupergit.[22] Some care needed to be taken in choice of carrier, since the secondary to primary ester migration is known to be promoted by certain solids used as enzyme supports.[14,16] For use in a pilot plant, we considered Eupergit C resin to be a good choice. However, the hydrolysis was very slow with Eupergit C-based catalysts, probably due to poor diffusion of the hydrophopic substrate into the catalyst beads. This could be improved somewhat by making the beads more hydrophobic. This was done by pretreating the Eupergit C with thiols such as octanethiol prior to attaching the enzyme. Despite achieving ∼10 times the reaction rate with treated Eupergit catalysts, the reaction rate never matched that provided by the lipase supported on powders. Since we had noted the potential of Celite and K_2-SO_4 as catalysts for the rearrangement, these were excluded. *Pseudomonas cepacia* CLEC performed well, but the cost was prohibitive. *Pseudomonas cepacia* lipase on ceramic particles, Amano PS-C1, was found to fulfill all the criteria. At a 10 w/v charge of AZD2563 DB, and 10 w/w PS-C1, the reaction would go to completion with solutions yields of AZD2563 SMB of ∼97%. The final pH of the reaction mixture was 5.2, the optimum for chemical stability of AZD2563 esters.

So now we had a robust reaction; however, the rearrangement of AZD2563 SMB to AZD2563 PMB resurfaced during product isolation as the process was scaled up.

Transesterification. The hydrolysis reaction was then scaled up to 100-g scale. Whilst the selectivity stayed high and solution yields of the desired AZD2564 SMB were ≥95%, problems arose in isolating the product.

The key issue was the butyric acid by-product of the hydrolysis. Butyric acid severely inhibited the crystallisation of AZD2563 SMB. On very small scale, this could be overcome by washing with dilute $NaHCO_3$ solution prior to isolation. However, as the scale increased and contact time with the base increased, significant material (30−40%) was lost via rearrangement to AZD2563 PMB. It became clear that only a process involving direct crystallisation and isolation of AZD2563 SMB would be successful on any

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useful scale, and the production of butyric acid would have to be avoided.

This was solved by switching the reaction to transesterification mode using *n*-butanol as the nucleophile to remove the primary ester as *n*-butyl butyrate.[23] Initial screening demonstrated this to be feasible with the *Pseudomonas cepacia* PS-C1 catalyst. The reaction progressed in a wide range of *n*-butanol mixtures with other solvents, but a key parameter was that, after completion and removal of the biocatalyst by filtration, AZD2563 SMB would have to be directly crystallised in high yield by just cooling or addition of an antisolvent. A mixture of 1:1 methyl-*tert*-butyl ether and *n*-butanol worked well for both reaction and isolation, although to achieve a reasonable and reproducible reaction rate, 3% water had to be added to maintain water activity and effective hydration of the enzyme.[24] Some analysis of reaction mixtures by gas liquid chromatography showed that negligible butyric acid was formed; therefore, the reaction was still predominantly a transesterification, and the very small amounts of butyric acid produced did not hinder product isolation. To obtain a comparable reaction rate to hydrolysis, the charge of PS-C1 was increased from 10% to 15 wt/wt%. The catalyst could be recovered and reused at least 3 times with no loss of rate or selectivity. At the end of the hydrolysis reaction, the mixture was cooled to ambient and filtered to remove the catalyst. The solution was then slowly cooled to −28 °C to crystallise out the AZD2563 SMB. The product could be isolated in ∼80% yield with only ∼0.2% AZD2563 PMB as a by-product.

AZD2563 SMB could be successfully used as a precursor to AZD2563 DSP by phosphorylation of the primary alcohol followed by base-catalysed removal of the ester.[25] No erosion of stereochemical integrity was detected during the conversion of AZD2563 to AZD2563 DSP (Scheme 4), and the transesterification of AZD2563 DB was successfully scaled up in the pilot plant prior to termination of the project.

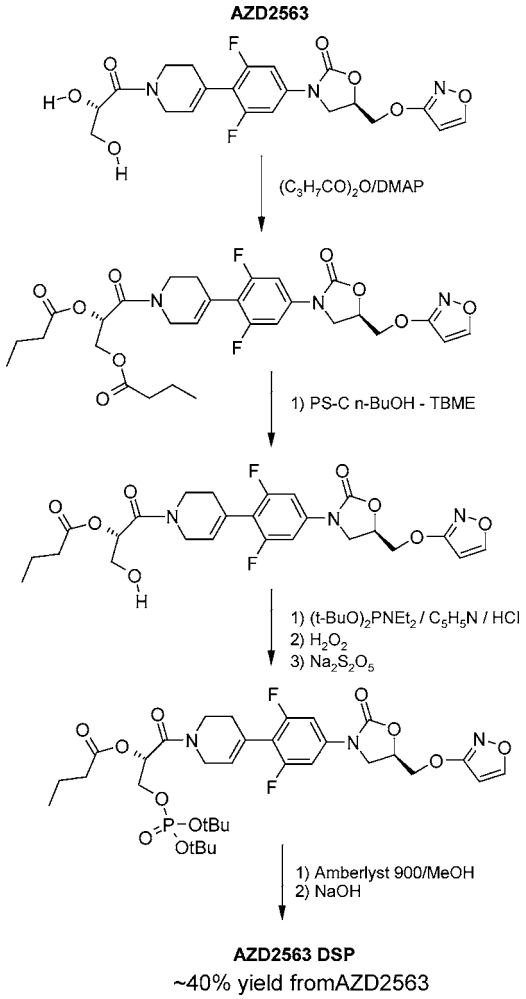
AZD2563 diesters have five hydrolysable groups and an epimerisable chiral centre, and the secondary monoesters are highly sensitive to rearrangement. Only the unique selectivity and mild operating conditions of biocatalysis allowed us to make and handle secondary monoesters such as AZD2563 SMB on scale.

Experimental Details

Synthesis of AZD2563 diol has been published elsewhere.[4] Reagents and solvents were general reagent grade. *Pseudomonas cepacia* lipase on ceramic particles was obtained from Amano Enzymes Inc.

Reaction monitoring was performed by reverse-phase HPLC on a 12.5 cm Genesis lightning C18-130 3 μ HPLC column; temperature 45 °C; flow 1 mL min^{-1}; aqueous eluent 10% MeCN in water plus 0.1% v/v trifluoroacetic acid; organic eluent 10% water in MeCN; gradient 10% organic at start then to 90% organic over 15 min, isocratic at 90% organic for 3 min, then back to 10% organic; detection UV at 254 nM.

Scheme 4. **New route to AZD2563 DSP**



Retention times: AZD2563, 4.60 min; AZD2563 PMB primary monobutyrate ester, 7.5 min; AZD2563 SMB secondary monobutyrate ester, 8.3 min; AZD2563 DB dibutyrate ester, 11.3 min.

Analytical data (^{1}H NMR, ^{13}C NMR, IR, and MS) is reproduced in the Supporting Information

Synthesis of AZD2563 PMA. AZD2563 (15 g, 0.033 mol) was dissolved in 1,4-dioxan (70 mL) and isoprenyl acetate (30 mL) at 70 °C. The resulting solution was cooled to 40 °C, and *Pseudomonas cepacia* lipase on ceramic particles was added (5 g, Amano PS-C1). The reaction was stirred for 20 h at 40 °C. HPLC analysis showed 92% primary monoacetate product and 8% diol. A further 1 g of PS-C1 catalyst was added, and stirring continued for 5 h. HPLC analysis showed greater than 95% product. The reaction was cooled and the catalyst filtered off. The residue was dissolved in ethyl acetate (500 mL) and evaporated. This was repeated with dichloromethane (200 mL). Yield: 17 g quantitative yield (NMR showed 3−4% residual solvent). HPLC of the solid showed 97% AZD2563 PMA and 3% AZD2563.

Synthesis of Dibutyrate Ester (AZD2563 DB). AZD2563 (6.42 kg, 13.8 mol) was charged to a clean, dry, reaction vessel with 4-dimethylamino pyridine (4-DMAP, 336 g, 2.76

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mol). Methyl-*tert*-butyl ether (MTBE, 51 L, 8 vol), and tetrahydrofuran (THF, 6.4 L, 1 vol) were added, and the reaction was stirred to give a slurry which was heated to reflux. Butyric anhydride (4.8 kg, 30.36 mol) was added slowly to the reaction at reflux over 2.15 h, and then the batch was allowed to stir at reflux for a further 2 h. The reaction was judged to be complete following an end-of-reaction analysis by HPLC. The batch was cooled to 43 °C and some seed material added. The batch was allowed to cool to 38 °C over 1 h, by which time crystallisation was well established. The batch was allowed to come to ambient temperature and was stirred overnight. The batch was then cooled to 5 °C and held for 1 h prior to a successful loss-to-liquors analysis. The batch was isolated by filtration on a nutsche. The cake was washed with MTBE (2 × 12 L). The product was dried under vacuum in an oven at 50 °C for 48 h. Yield: AZD2563 DB 7.67 kg, 92% yield.

Synthesis of AZD2563 SMB. AZD2563 DB (7.23 kg, 11.95 mol) was added to *n*-butanol (*n*-BuOH 37 L, 5 vol), MTBE (37 L, 5 vol), and deionised water (2.17 L, 0.3 vol) and heated at 46 °C to dissolve all the solids. The reaction was then cooled to 40 °C. *Pseudomonas cepacia* PS-C1 ex-Amano (1.08 kg) was charged as a solid. The reaction was stirred overnight (17 h) at 40 °C, when HPLC analysis confirmed completion of the transesterification. The enzyme was filtered off through a pad of MTBE-wetted Celite on a nutsche under vacuum and washed with MTBE. The liquors were then screened through a 1.2 μm in-line polishing filter. The liquors were cooled to 14 °C and held at 14 °C for another 30 min, when the product had started to crystallise out. It was then cooled to 12 °C and held at that temperature for 18 h. The batch was then cooled to −28 °C over 1.5 h and then held at −28 °C for another 1.5 h. The product, AZD2563 SMB, was then filtered off under vacuum and the cake washed once with cold MTBE (11 L, 1.5 vol). The batch was dried in the vacuum oven at 40 °C to constant weight.Yield: AZD2563 SMB 4.99 kg, 78%. HPLC analysis showed a further 19% AZD2563 SMB remaining in the filtration liquors.

Acknowledgment

We thank Dr. Bob Osborne and Mr. Tom Vavasour for assistance with the preparation of catalysts and reaction screening.

Supporting Information Available

Analytical data (^{1}H NMR, ^{13}C NMR, IR, and MS). This material is available free of charge via the Internet at http://pubs.acs.org.

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