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Identification of an Esterase Isolated Using Metagenomic Technology which Displays an Unusual Substrate Scope and its Characterisation as an Enantioselective Biocatalyst

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Abstract. Evaluation of an esterase annotated as 26D isolated from a marine metagenomic library is described. Esterase 26D was found to have a unique substrate scope, including synthetic transformations which could not be readily effected in a synthetically useful manner using commercially available enzymes. Esterase 26D was more selective towards substrates which had larger, more sterically demanding substituents (i.e. *iso*-propyl or *tert*-butyl groups) on the β-carbon, which is in contrast to previously tested commercially available enzymes which displayed a preference for substrates with sterically less demanding substituents (i.e. methyl group) at the β-carbon.

Keywords: Esterase; Metagenomic Library; Stereochemistry; Biocatalyst; Enantiopurity

Introduction

Due to the importance of stereochemistry in drug discovery and the associated clinical and regulatory implications, the capability of producing enantiomerically pure synthons and Active Pharmaceutical Ingredients (APIs) in an efficient, highly stereoselective manner has never been more important.[1] Moreover, with increasing focus on "green" synthetic routes, a requirement exists for technologies which operate under milder conditions and minimise waste generation. Biocatalysis has emerged as a very favourable solution to these challenges.[2]

Biocatalysts are inherently stereoselective and many can outperform alternative methods prevalent in asymmetric synthesis, such as transition metal catalysis or chiral auxiliary chemistry.[3] In addition to the aforementioned advantages for enantioselective synthesis, biocatalysts are also obtained from renewable resources, can operate at neutral pH and wide ranges of temperatures, and are safe for both humans and the environment. Hence, there are significant advantages to this enabling technology when compared to "traditional" organic synthesis, which often relies on harsh reaction conditions or heavy metal catalysts.[2a, 4]

Hydrolytic biocatalysts are very important to pharmaceutical, chemical and food industries.[5] These enzymes can be used to effect achiral transformations, however their primary use in synthetic chemistry is in asymmetric synthesis, where they can lead to enantioselective (dynamic) kinetic resolutions of esters and related compounds.[6] Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are sub-groups of hydrolases. Esterases and lipases catalyse the hydrolysis of short chain and long chain glycerol esters, respectively.[6a, 7]

A common strategy employed for the discovery of novel biocatalysts is through functional metagenomic screening to discover proteins with novel sequences.[8]

There exists a requirement for new enzyme discovery, not least from the point of view of investigation of new activities and functionality,[9] as well as from the perspective of novel substrate specificity. The dereplication of functionally active clones is a vital step in the identification of novel biocatalysts. The DNA sequencing of the novel biocatalysts can be searched against large sequence databases such as NCBI, which currently contains over 125 million sequences of different proteins. [10] This step of bioinformatic dereplication of positive clones at an early stage of the process, saves much time and cost on downstream analysis. Furthermore, these newly discovered biocatalysts can act as templates for further evolution, starting from a point at which the enzyme displays some activity for the target transformation.

The marine environment is an unexploited resource for the mining of hydrolases such as esterases and lipases. [11] This niche environment harbours a wide range of enzymes that tolerate varying reaction conditions such as high salt concentration, high pressure and pH tolerance, as well as functioning in extreme temperatures, which enable their use in diverse applications including industrial scale transformation. [12] In the past when using traditional microbiology techniques, only a minority of the bacteria were culturable from a given environment and therefore a vast array of important enzymes could have been overlooked. [13] To combat this challenge, culture-independent techniques have now been successfully employed. The construction and functional screening of large metagenomic libraries have successfully uncovered many novel biocatalysts. [14] Furthermore the screening of these libraries can be adapted to high-throughput (10^3 to 10^5 variants/day) or ultrahigh-throughput (10^6 to 10^9 variants/day) functional screening assays. [15]

Previously, our group [11] and others have had success with enzyme mining in niche environments as a biocatalyst source.[16] The marine environment, which has bestowed upon us many pharmaceutically interesting natural products, has proven to be a rich source of protein diversity.[11, 17]

Exploring the potential of the novel biocatalysts in enantioselective synthesis

Via the culture-independent sampling of the marine environment, a number of enzymes were identified in our laboratory as having hydrolytic activity through use of a tributyrin screen. The hydrolase family of enzymes make up approximately 55% of all enzymes used in biocatalytic processes, with only 8% of these being esterases.[18] Following a screen of target substrates with these biocatalysts, esterase 26D was identified as an active and selective enzyme when tested, showing an altered substrate preference to previously tested commercial hydrolases.[1b, 19] Esterase 26D shows greater selectivity when the substituent on the β-carbon is a *tert*-butyl group (sterically bulky) versus a methyl group.

Results and Discussion

The novel esterase 26D was identified in a functional screen of an *Axinella dissimilis* marine sponge metagenomic library (n = 20,352 clones) constructed in *E. coli* EPI300 cells using the fosmid vector pCCFOS1. Comparative analysis of the gene sequence encoding the 26D esterase revealed sequence identity (71%) with hydrolase WP_108845108.1 from a *Phyllobacteriaceae* bacterium and a hypothetical protein (70%) OUW21162.1 from a *Rhizobiales* bacterium. The clustering tree demonstrated that the 26D sequence branched on the same node as the aforementioned proteins, (WP_108845108.1 (■) and OUW21162.1 (●)) however importantly the 26D esterase was on a distinct branching point to these (Figure 1). The percentage identity of the sequences in the tree, range from 44-71% compared to 26D. We theorized that this novel sequence could give rise to a novel functioning esterase with a unique substrate profile when compared with existing esterase activities.

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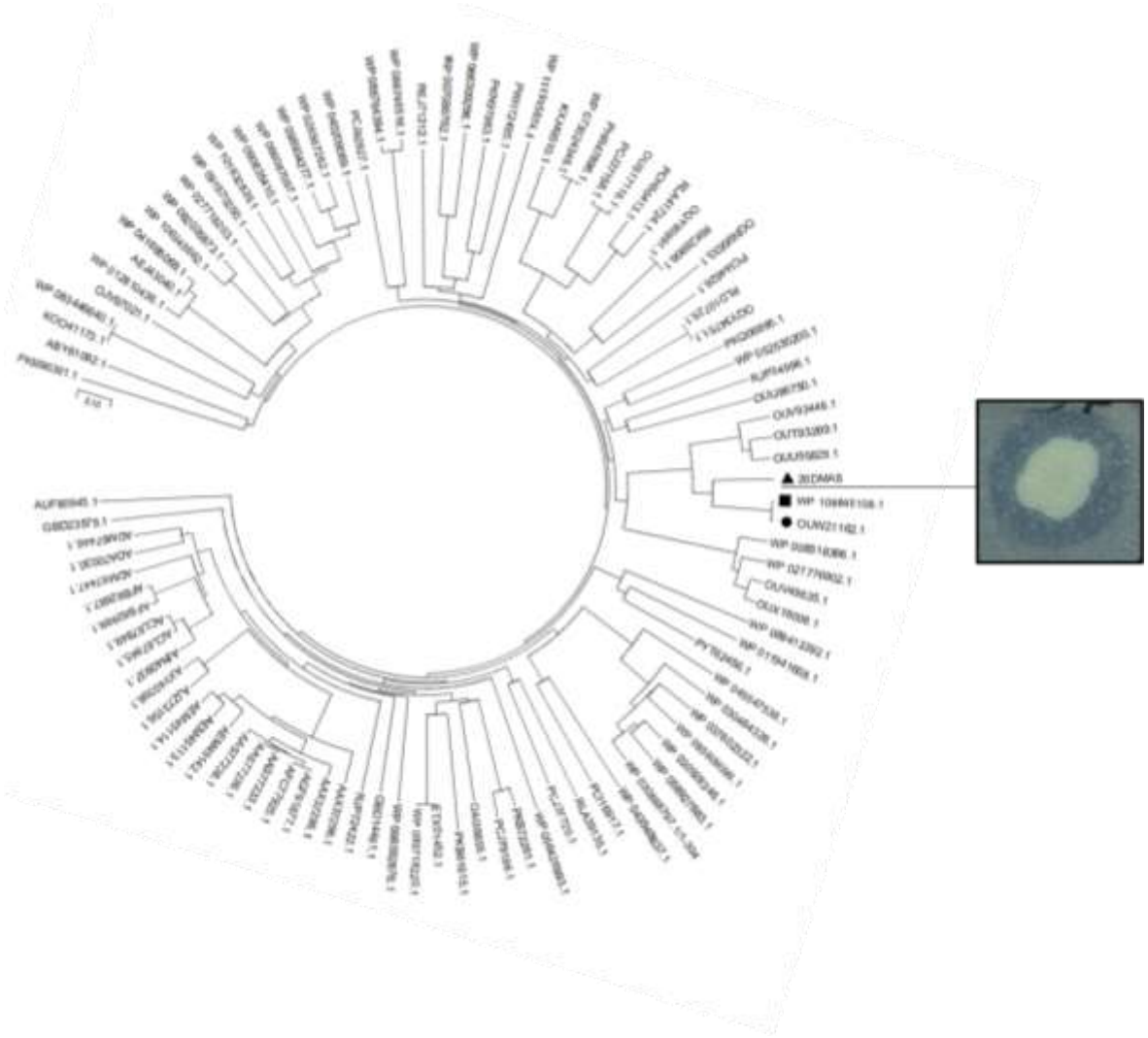
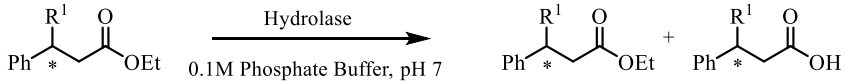


Figure 1. Clustering Tree to define the uniqueness of the esterase positive clone 26D

Table 1. Commercial enzymes used in the kinetic resolution of 3-arylalkanoic acids.[19]



1 R^1 = Me
3 R^1 = Et
4 R^1 = ^{i}Pr
5 R^1 = ^{t}Bu

6 R^1 = Me
8 R^1 = Et
9 R^1 = ^{i}Pr
10 R^1 = ^{t}Bu

Conversion: up to 50%
Acid, ester up to >99% ee

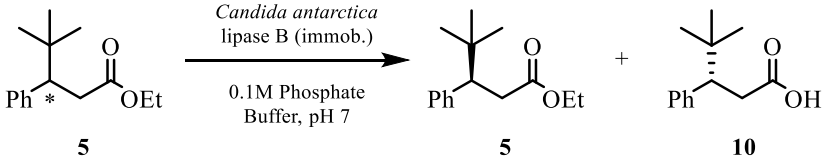
Entry	R	No. of enzymes which gave >10% conversion	No. of enzymes which furnished acid product in 90% *ee*
1	Me	15	7
2	Et	5	1[a]
3	^{i}Pr	5	1
4	^{t}Bu	2[b]	1

(a) Use of co-solvent required (b) Heating to 40 °C required

Previous work in our group investigated the kinetic resolution of 3-arylalkanoic acids. Using a systematic approach with commercial enzymes and by varying the co-solvent, a range of compounds were resolved with excellent enantiopurity (Table 1).[1b, 19] One of the features of these compounds is that the chiral

centre is not adjacent to the reacting site (i.e. at the α-carbon) but at the β-carbon, which brings about significant challenges in terms of selectivity and activity.[1b, 19-20]

Notably, from this previously completed screen the number of enzymes which processed the substrate decreased as the steric bulk of the β-substituent increased, Table 1.[19] Concurrently the number of enzymes exhibiting enantiodiscrimination also decreased. In particular, enzymatic hydrolysis of substrate **5** bearing a *t*-butyl substituent was extremely challenging, with only two of the previously screened commercial hydrolases showing activity. After considerable optimisation of the conditions for the biotransformation (immobilised *Candida Antarctica* Lipase B as the biocatalyst), the optimum outcome achieved led to the acid product in excellent enantiopurity (98% *ee*) with moderate conversion (23%) in 72 hours at 40 °C (Scheme 1). Even under the optimised conditions, the kinetic resolution of **5** is of limited synthetic utility as the enantiopurity of the recovered ester is, of necessity, compromised by the limited extent of reaction.[19] One possible explanation for this given by Müller *et al* is that, given the paucity of natural products featuring the *t*-butyl group, enzyme evolution in nature to accommodate this sterically demanding substituent is less likely.[21]



Standard conditions = Ambient temp and 66 h - 1% conversion, 1% ee_s , ≥99% ee_p

Optimised conditions = 35-45 °C and 72 h - 23%

Scheme 1: Previously screened hydrolysed-mediated kinetic resolution of (±)-ethyl 4,4-dimethyl-3-phenylpentanoate (±)-**5** at variable reaction times and temperature.[19]

In this work identifying an enzyme which would hydrolyse substrate **5**, the challenging substrate, in a synthetically useful manner would be very attractive. Accordingly, wild type esterase 26D was tested for activity against the challenging ester substrate **5**.

Gratifyingly, in the initial screens using whole cells expressing the wild type esterase 26D both activity and excellent enantioselectivity were seen with substrate **5**. Whole cells expressing the esterase furnished the acid product in excellent enantiopurity (98% *ee*) with 21% conversion (E_{calc}) (Table 2, entry 5).

In light of this result, screening this novel enzyme against the series of esters **1**-**4** was undertaken to probe the substrate scope of the enzyme by varying the alkyl substitution on the β-carbon (as summarised in Table 2). Interestingly, esterase 26D, while active across the series, displayed the opposite trend to all other enzymes screened against this similar series, with enantioselectivity increasing with increased steric demand at the β-substituent. Hydrolysis of the esters bearing ethyl (**3**) and isopropyl (**4**) substituents proceeded with good enantioselectivity, while decreased selectivity was seen with the smaller methyl substituent, even though resolution of this derivative was by far the most efficient in our earlier study.[1b, 19]

Interestingly resolution of the trifluoromethyl substituted ester **2** was more efficient than that of the methyl derivative **1** under the same conditions.

While the hydrolysis of ester **5** was promising (Table 2, entry 5), to enhance its synthetic utility, process optimisation was undertaken (Table 2, entry 6) involving repeated additions of the biocatalyst at 0, 12 and 24 hours, which led to recovery of the ester in **73% *ee*** albeit with a reduction in the enantiopurity of the acid (**91% *ee***) under these more forcing conditions. This result demonstrates that with further optimisation, esterase 26D can lead to a synthetically feasible kinetic resolution, whereby both enantiomeric series can be accessed. Use of alternative co-solvents (1,4-dioxane, MTBE, EtOH), variation of pH (6 to 7.8) and extended reaction (72 h) time were explored without significant impact.

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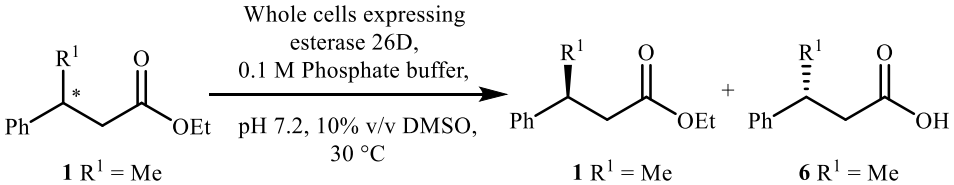
Table 2. Kinetic resolution of 3-arylalkanoic acid ethyl esters **1-5** using esterase 26D



1 R^1 = Me
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5 R^1 = ^{t}Bu

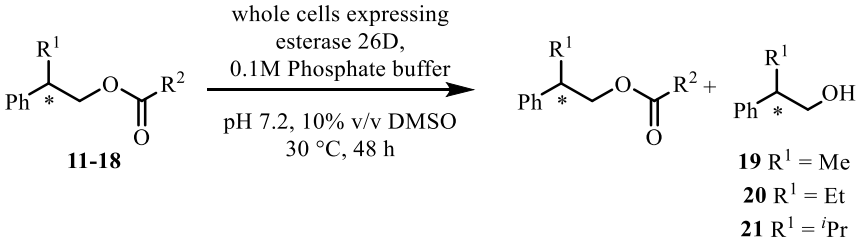
6 R^1 = Me
7 R^1 = CF_3
8 R^1 = Et
9 R^1 = ^{i}Pr
10 R^1 = ^{t}Bu

Entry	R^1	Time (h)	Conversion (%)		% *ee*		*E*[22]
			^{1}H NMR[a]	E_{calc}[b] [22]	Ester	Acid	
1	Me[c]	24	25	22	18 (*S*)	63 (*R*)	5
2	CF_3[c]	24	60	42	64	88	
3	Et[d]	24	30	24	31(*S*)	96 (*R*)	66
4	^{i}Pr[c]	24	-[g]	42	65 (*R*)	88 (*S*)	31
5	^{t}Bu[c]	24	30	21	26 (*R*)	98 (*S*)	127
6	^{t}Bu[e]	48	-[g]	45	73 (*R*)	91(*S*)	40
7	^{t}Bu[f]	48	-[g]	37	55 (*R*)	93 (*S*)	47

(a) Conversion was estimated using ^{1}H NMR spectroscopy by integration of the signal for esters and alcohols; (b) Conversion (E_{calc}) and the enantiomeric ratio (*E*) were calculated from enantiomeric excess of substrate ester (ee_s) and product acid (ee_p); (c) 50 mg/mL of whole cells expressing esterase 26D; (d) 25 mg/mL of whole cells expressing esterase 26D; (e) 50 mg/mL of whole cells expressing esterase 26D was added in three portions, 0h, 12 h and 24 h; (f) 150 mg/mL of whole cells expressing esterase 26D; (g) percentage conversion of ester to acid could not be determined by ^{1}H NMR spectroscopy analysis.

Having established that 26D could be used for a transformation which was not readily achieved with existing biocatalysts, its substrate scope was further investigated. We had previously examined a panel of hydrolase enzymes in the kinetic resolution of 2-phenylalkanols.[23] Excellent enantiopurities were obtained for a number of alcohol products when CAL-B was used as the biocatalyst. In contrast, esterase 26D was a poor biocatalyst for the resolution of 2-phenylalkanols. A range of ester substrates was screened, with the substituent on the β-carbon (R^1) as well as the substituent (R^2) on the carboxylic acid moiety of the ester being varied (Table 3). Product *ee* was poor to moderate, although in some instances hydrolysis was seen for substrates (**14** and **15)** which were not processed by CAL-B [23] (Table 3, entries 4 and 5), further highlighting the different profile in terms of the enzyme's substrate scope.

Table 3. Kinetic resolution of 2-phenylalkanols using esterase 26D



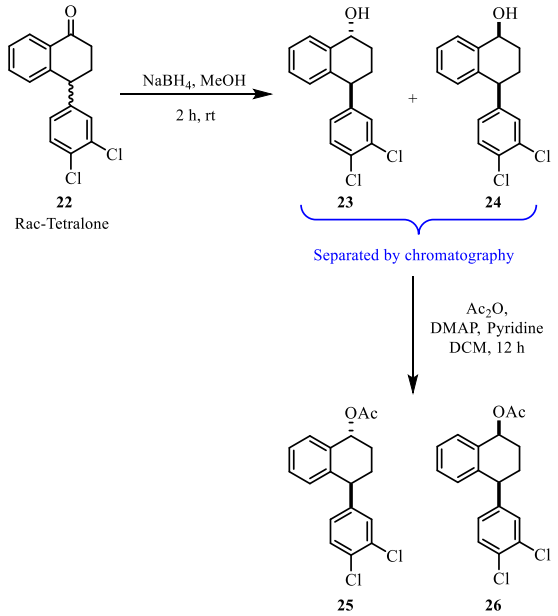
Entry	Compound	R^1	R^2	Conversion (%)		% ee		$E^{[22]}$
				^{1}H NMR [a]	E_{calc} [b] [22]	Ester	Alcohol	
1	**11**	Me	Ph	81	67	36 (*R*)	18 (*S*)	2
2	**12**	Me	^{t}Bu	56	15	3 (*R*)	19 (*S*)	2
3	**13**	Et	Ph	55	48	37	40	3
4	**14**	Et	^{t}Bu	55	34	40	77	11
5	**15**	^{i}Pr	^{i}Pr	55	49	29	30	2
6	**16**	^{i}Pr	Ph	25	19	14	61	5
7	**17**	^{i}Pr	^{t}Bu	-[c]	15	16	91	25
8	**18**	Me	Bn	22	21	10	38	2

(a) Conversion was estimated using ^{1}H NMR spectroscopy by integration of the signal for esters and alcohols; (b) conversion (E_{calc}) and enantiomeric ratio (*E*) were calculated from enantiomeric excess of substrate ester (ee_s) and alcohol product (ee_p); (c) percentage conversion of ester to alcohol could not be determined by ^{1}H NMR spectroscopy analysis

We then turned our attention to applying the atypical substrate acceptance of esterase 26D to a pharmaceutical intermediate, since assimilation of a novel biocatalyst into an industrially relevant synthetic route would underline its value.

This work was initially extended to cyclic substrates **27-29**, as summarised in **Table 4**. Esterase 26D hydrolyses acetates of α- and β-tetralols (**27** & **28**) and 1-indanol (**29**), affording high conversion to the alcohols **31** & **32** but with very poor enantiodiscrimination.

Following on from this work, the novel acetates **25** and **26** were prepared by acetylation of the corresponding tetralols **23** and **24,** which were accessed by reduction of the tetralone **22** (Scheme 2). An intermediate in the synthesis of the Pfizer antidepressant sertraline, racemic tetralone **22** was obtained from the condensation of 1-napthol and 1,2-dichlorobenzene in the presence of a strong Lewis acid. Enantiopure (4*S*)-tetralone **22** was also reduced and acetylated. The tetralin core (α- or β- substituted) is in itself a privileged moiety in drug discovery, being present in a number of drugs and bioactive compounds.[24]

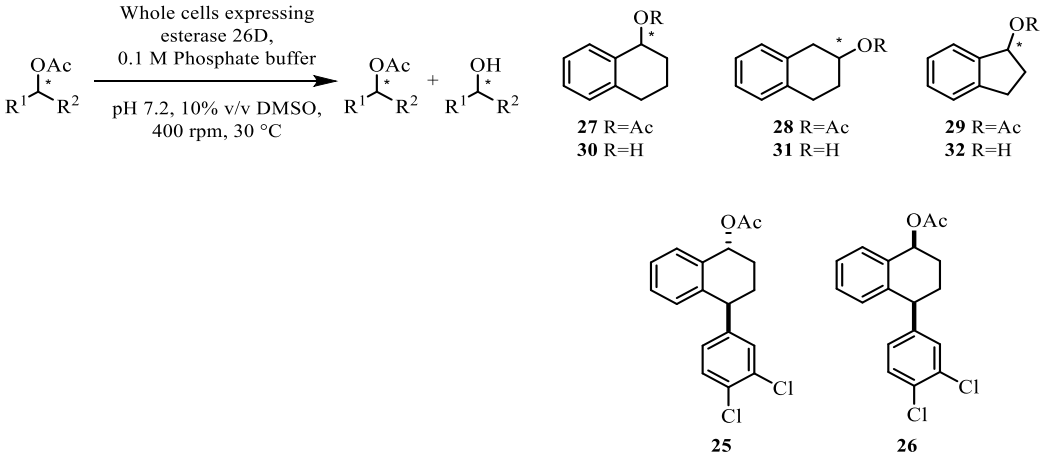


Scheme 2. Synthesis of acetates **25** and **26**

When the *trans*- and *cis*- acetates **25** and **26** were exposed to esterase 26D, hydrolysis of the *trans*-acetate **25** is more rapid than that of the *cis*-acetate **26** (Table 4 entry 4 and 5). Excellent enantioselectivity was observed in the formation of the *cis*-tetralol **24** (93% *ee*). As the *cis*-tetralol **24** product has been used

as a synthetic intermediate for sertraline,[25] this observation is particularly interesting and highlights the potential of the novel biocatalyst to be utilised for the resolution of pharmaceutically related compounds. Absolute stereochemistries of the biotransformation products were deduced by comparing the HPLC traces with that of the (4*S*)-tetralol and the corresponding acetates.

Table 4. Hydrolysis of acetates **25**-**29** using esterase 26D.



Entry	Substrate	Time (h)	Conversion		% ee		*E*[22]
			^{1}H NMR	E_{calc}[a] [22]	Ester	Alcohol	
1	**27**	4	-[b]	5	30	10	2
2	**28**	1	-[b]	86	32	5	1
3	**29**	2	22	50	4	4	1
4	**25**	24	38	36	~31[c]	55 (1*R*,4*S*)	5
5	**26**	24	20	18	21	93 (1*R*,4*R*)	39

(a) Conversion (E_{calc}) and the enantiomeric ratio (*E*) were calculated from enantiomeric excess of substrate ester (ee_s) and product alcohol (ee_p); (b) percentage conversion of ester to alcohol could not be determined by ^{1}H NMR spectroscopy analysis; (c) full resolution of signals in the HPLC was not possible

Conclusion

In conclusion, the novel esterase 26D acts as a synthetically valuable biocatalyst displaying a unique substrate scope, at least within the range of biocatalysts which we have explored, and to date is the optimum biocatalyst for the resolution of the challenging ester substrate **5**, highlighting the importance of metagenomic screening as a valuable approach for the discovery of novel biocatalysts. The success in resolving an ester substrate where the chirality lies in the acid portion (**6**-**10**) relative to the other substrates explored where the chirality lies in the alcohol (e.g. compounds **11**-**18** and **25**-**29**) moiety is unsurprising in an esterase.[26] Furthermore, a potential intermediate in the synthesis of sertraline (compound **26**) was resolved in excellent enantiopurity, suggesting the potential of this biocatalyst to be used in the synthetic route of a pharmaceutical compound.

Experimental Section

General

Solvents were distilled prior to use as follows: dichloromethane was distilled from phosphorus pentoxide, ethyl acetate was distilled from potassium carbonate. Hexane was distilled prior to use. Organic phases were dried over anhydrous magnesium sulfate. Infrared spectra were recorded neat using a Perkin Elmer FTIR UATR2 spectrometer.^{1}H (300 MHz) and ^{13}C (75.5 MHz) NMR spectra were recorded on a

Bruker Avance 300 MHz NMR spectrometer and all spectra were recorded at room temperature (~20 °C) in deuterated chloroform (CDCl3), unless otherwise stated, using tetramethylsilane (TMS) as an internal standard and chemical shifts (δH and δC) are reported in parts per million (ppm) and coupling constants are expressed in Hertz (Hz). Low resolution mass spectra were recorded on a Waters Quattro Micro triple quadrupole spectrometer in electrospray ionisation (ESI) mode using 50% water/acetonitrile containing 0.1% formic acid as eluent. High resolution mass spectra (HRMS) were recorded on a Waters LCT Premier Time of Flight spectrometer in electrospray ionisation mode (ESI) using 50% water/acetonitrile containing 0.1% formic acid as eluent. Wet flash chromatography was performed using Kieselgel Silica Gel 60, 0.040–0.063 mm (Merck). Thin layer chromatography (TLC) was carried out on precoated silica gel plates (Merck 60 PF254). Visualisation was achieved by UV (254 nm) light detection and $KMnO_4$ staining. Candida Antarctica lipase B (immobilised on ImmoBead 150) was purchased from Sigma-Aldrich chemical company. All enzymatic reactions were performed on a VWR Incubating Mini Shaker 4450. All reagents are analytical grade and purchased from Sigma-Aldrich, Acros Organics, Fluka or TCI. Enantiomeric excess values were measured by high performance liquid chromatography (HPLC) using a Chiralcel® OB-H column (5 × 250 mm), Chiralcel® OD-H column (5 × 250 mm), Chiralcel® OJ-H column (5 × 250 mm) and Chiralpak® IB (5 × 250 mm) purchased from Daicel Chemical Industries, Japan. Phenomenex Amylose 1 column (5 × 250 mm), Phenomenex Amylose 2 column (5 × 250 mm), Phenomenex Cellulose 4 column (5 × 250 mm), purchased from Phenomenex Inc., UK. Mobile phase and flow rate are included where appropriate, and the detector wavelength was 209.5 nm. HPLC analysis was performed on a Waters alliance 2690 separations module with a PDA detector.

Expression and purification of lipase/esterase

His-tagged esterase 26D fusion proteins derived from a tributyrin screen of a pCC1FOS™ (Epicentre) metagenomic library transformed in EPI300™ Electrocompetent *E. coli* were prepared for recombinant protein expression. Single colonies were inoculated in LB (Luria-Bertani) medium supplemented with kanamycin (50 μg/ml), chloramphenicol (50 μg/ml) and streptomycin (75 μg/ml) and incubated at 37°C with shaking at 180 rpm overnight. Overnight cultures were transferred (1%) to fresh media with antibiotics and grown to an OD_{600nm} of 0.8-1.0, at which point the medium was supplemented with 0.5 mM IPTG (Melford) to induce recombinant protein expression. After 4 hrs post-induction, the culture was centrifuged at 12,000 rpm for 10 min at 4°C to harvest the cells. The cell pellet was stored at -80°C until ready for expression analysis.

Bioinformatic Dereplication for Novel Activity

The DNA sequences of the positive clone was bioinformatically aligned using Clustal Omega [27] and a consensus sequence was defined using Jalview Version 2 [28]. A Translated BLAST: blastx search was conducted against the NCBI Non-redundant protein sequence database [29]. The cluster relationship of 26D was inferred using the Neighbor-Joining method. The distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 100 amino acid sequences of the top BLAST hits by sequence identity. All ambiguous positions were removed for each sequence pair. This analysis was conducted in MEGA X.

Lipase-catalysed hydrolysis reactions

In a typical experiment, 50 mg of whole cells expressing esterase 26D were added to a 15 mL centrifuge tube (unless otherwise stated) followed by 0.1 M potassium phosphate buffer at pH 7.2 (900 μL/1000 μL). Substrates **1 - 5**, **11 - 18** and **25 - 29** (20 mM) were dissolved in 100 μL DMSO (10% v/v) and then added to the centrifuge tube to make the total volume 1 mL. The reaction mixture was agitated at 400-450 rpm and incubated at 30 °C for 24 h unless otherwise stated. Ethyl acetate (3mL) was added to the centrifuge tube and the reaction mixture was centrifuged at 5000 rpm for 10 minutes. The layers were separated, the aqueous layer was washed with ethyl acetate (2 × 3 mL), the combined organic extracts were filtered through Celite® and concentrated under reduced pressure. The entire reaction sample was analysed by ^{1}H NMR spectroscopy, reconcentrated and dissolved in a mixture of ^{i}PrOH /hexane [10:90 (HPLC grade)] and enantiopurity determined by chiral HPLC analysis.

Compounds **1, 3-5, 7-12**, **15**, **18**, **21, 23-24** and **27-29** were synthesised according to previously reported methods.[19, 23, 25, 30] Details of novel compounds are given in the SI.

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FULL PAPER

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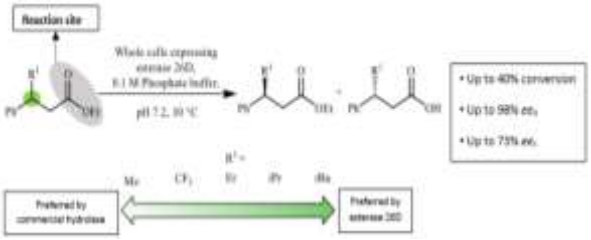

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