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Identification of novel esterases for the synthesis of sterically demanding chiral alcohols by sequence-structure guided genome mining

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ABSTRACT

Six esterases isolated from sequence and structure-guided genome mining approaches were evaluated for the kinetic resolution of secondary and tertiary alcohols that find application in the fine chemical and pharmaceutical industries. Activity and enantioselectivity with *E*-values of up to 24 were determined towards a range of sterically demanding tertiary alcohol esters. Excellent enantioselectivity ($E > 100$) was also achieved in the hydrolysis of a less challenging secondary alcohol ester, menthyl acetate. These results highlight that these approaches can be used for the identification of novel esterases applicable to the preparation of commercially desirable alcohols.

1. Introduction

Tertiary alcohols (TAs) have become interesting targets for organic synthesis either in their own right, as flavour fragrance compounds, or as building blocks for valuable pharmaceutical compounds [1,2]. In the first instance, linalool is widely used in fragrance products such as perfumes, soaps and detergents, whilst also being important as an odour-active component in the hopping of beer [3]. Gossonorol, originally isolated from the cotton plant (*Gossypium*), possesses a floral scent, and is often contained within oils extracted from medicinal plants [4]. It has also attracted interest as a precursor in the synthesis of other compounds used in biologically active remedies [5,6]. In terms of pharmaceutical precursors, tertiary cyanohydrins are versatile precursors for the syntheses of α-hydroxy acids, β-amino alcohols and β-hydroxy amides [5]. Pyridine-derived tertiary alcohols have been used as building blocks for the synthesis of A_{2A} receptor antagonists, promising compounds for the therapy of Parkinson's disease [6]. Ekegren et al. [7] have also described a new class of HIV-1 protease inhibitors containing a tertiary alcohol in the transition-state mimicking scaffold. In the search for a therapy for Alzheimer's disease, a new class of compounds based on BACE-1 inhibitors containing the tertiary alcohol motif has also been investigated [8].

Biocatalytic routes employing enantioselective hydrolases offer promise in the preparation of enantiopure TAs, but have thus far failed to achieve their potential owing to the sterically demanding nature of these substrates. For example, there have been reports of the application of both whole-cell preparations of bacteria or cell-extracts to the production of enantio-enriched linalool by hydrolysis of racemic linalyl acetate [9–11]. Whole cells of *Rhodococcus ruber* DSM 43338 hydrolysed linalyl acetate to give (*S*)-linalool with a conversion of 25%, an enantiomeric excess (*ee*) of 56%, and a selectivity (*E*) of 4.2 [9]. An isolated enzyme EstA from *Rhodococcus* sp. was applied for the enantioselective hydrolysis of linalyl acetate [12], however the enantioselectivity was again modest ($ee_P = 45\%$ at 20% conversion, and $E = 3$) and suggests there may be considerable scope for improvement. In targeting therapeutically valuable pyridine-derived TAs, a recent study showed that some recombinant esterases have excellent enantioselectivity against the nitrogen-bearing TA esters [13], however the substrate range of the individual enzymes was quite limited. Recently [13] we developed a straightforward chemoenzymatic route to the chiral tertiary alcohol (*S*)-2-hydroxy-2-methylbutyric acid, which occurs naturally in clerodendrin-A [14], and has also been used as a precursor for the synthesis of a cyclooxygenase inhibitor [15]. Two

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enzymes, each displaying moderate enantioselectivity, were identified from that study; a thermostable esterase A from *Pyrubaculus calidifontis* and the metagenome-derived esterase Est8. Interestingly, both belong to the family of hormone-sensitive-lipase-like enzymes and share a high sequence similarity.

In this paper, we report the further investigation of hydrolytic enzymes for the hydrolysis of TAs, using two complementary approaches. First, the genome sequence of the actinomycete *N. farcinica* IFM10152 [16], was screened for the presence of esterase genes encoding enzymes containing the GGG(A)X motif, which is known to confer activity of these enzymes towards TAEs [16]. The cloning, expression and characterisation of activity and enantioselectivity, with particular focus on chiral tertiary alcohols, for two of these esterases (EstA1 and EstA2), is described herein.

Second, we used the new α/β-Hydrolase Fold Enzyme Family 3DM Database (ABHDB), which is a structure-based classification of 12,431 available sequences of α/β-hydrolase fold enzymes, to search for enzymes with high similarity to esterase A from *P. calidifontis* and the metagenome-derived esterase Est8, and which may therefore show potential for improved enantioselectivity in the kinetic resolution of TAEs. The ABHDB facilitates a deeper analysis of structure–function relationships within this diverse class of enzymes [17] and was recently applied as a tool for the prediction of key residues for the engineering of the enantioselectivity of an esterase [18] and for the guidance in library generation for protein engineering [19,20]. These complementary approaches have proved to be successful in unearthing new esterase activities towards sterically demanding TA esters of relevance to both flavour/fragrance and pharmaceutical chemistry.

2. Experimental

2.1. General

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany), unless stated otherwise. NMR spectroscopy experiments were performed on an ARX300 (300.13 MHz for ^{1}H and 75.5 MHz for ^{13}C, Bruker, Karlsruhe, Germany), using the δ scale (ppm) for chemical shifts; ^{13}C-spectra were edited using DEPT techniques. *J* values are given in Hz. Mass spectra were recorded on a QP2010 GC–MS device (electron impact, 70 eV, Shimadzu, Japan). The 3DM database [17] is accessible under http://fungen.wur.nl/ABHDB. *Escherichia coli* NovaBlue singles and BL21 (DE3) cells were obtained from Novagen. PCR primers designed towards the *Nocardia farcinica* esterase were ordered from MWG-Eurofins (Germany). Synthetic genes were obtained from GenScript USA Inc. (New Jersey, USA) and transformed into competent *E. coli* BL21 cells. KOD hot start DNA polymerase was obtained from Novagen. The pET-YSBLIC3C vector was obtained from the Structural Biology Laboratory (University of York). Restriction enzymes were purchased from New England Bio Labs. PCR clean-up and mini-prep kits were purchased from Qiagen, and a gel extraction kit from Sigma Aldrich. Isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Melford Labs. The homology models of EstA4, EstA5, EstA6 were created using the PHYRE web server (http://www.sbg.bio.ic.ac.uk/~phyre/). The hit was discovered based on the template of the protein with the PDB code c2c7bA.

2.2. Synthesis of tertiary alcohol esters

(*R*,*S*)-2-(Pyridin-2-yl)but-3-yn-2-yl acetate (**1d**), (*R*,*S*)-2-(pyridin-4-yl)but-3-yn-2-yl acetate (**1b**) and (*R*,*S*)-2-(tert-butylcarbamoyl)-1,1,1-trifluorobut-2-yl acetate (**1e**) were prepared as described [13,15]. 2-Hydroxy-2-methyl-1-phenylbutane-1-one (**2f**) was prepared from 2-hydroxy-2-methylbutanoic acid by formation of an intermediate pyrrolidinyl amide and subsequent arylation (818 mg, 62%) [21]. The spectroscopic data matched literature data. Gossonorol (**2g**) was prepared by addition of toluyl-magnesium bromide to 6-methyl-5-heptene-2-one as described [4]. The spectroscopic data matched literature data [4]. Preparation of 2-acetoxy-2-methyl-1-phenylbutane-1-one (**1f**) and gossonoryl acetate (**1g**) was made according to Bäckvall et al. [22].

2.2.1. Acetoxy-2-methyl-1-phenylbutane-1-one (*1f*)

(650 mg, 82% yield). ^{1}H NMR: δ = 0.99 (t, 3H, *J* = 7.5 Hz, CH_3), 1.69 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 2.00–2.3 (m, 2H, CH_2), 7.4 (m, 3H, H–Ar), 8.03 (d, 2H, H–Ar). ^{13}C NMR: δ = 7.6, 21.2, 21.3, 30.6, 87.0, 128.29, 128.33, 132.33, 134.9, 179.0, 199.1. MS (EI): 177 (M+-43), 159, 145, 132, 115, 105, 73, 43.

2.2.2. Gossonoryl acetate (*1g*)

^{1}H NMR: δ = 1.51 (s, 3H, C(CH_3)2x1), 1.64 (s, 3H, C(CH3)2x1), 1.82 (s, 3H, CCH_3), 1.85–2.02 (4H, m, CH_2CH_2), 2.05 (s, 3H, $COCH_3$), 2.3 (s, 3H, Ar–CH_3), 5.04 (t, 1H, CCH), 7.2 (m, 5H, H–Ar). ^{13}C NMR: δ = 17.49, 20.95, 22.23, 22.51, 24.91, 25.61, 42.22, 83.86, 123.7 124.47, 128.83 131.75 137.27, 141.96, 169.67. MS (EI): 201, 91, 77, 69, 43, 41.

2.3. Cloning of GGG(A)X motif esterases from N. farcinica

From the genomic DNA of *N. farcinica*, the GGG(A)X motif esterases EstA1, EstA2, and EstA3 (accession numbers: Q5YP18, Q5YQP8, Q5YPM0 respectively) were amplified using the following oligonucleotide primers with ligation independent cloning (LIC) specific sites EstA1: 5′-CCAGGGACCAGCAATGGACAACGTGGTCG-AAGCGCCCTCG-3′ and 5′-GAGGAGAAGGCGTTATGCACGGCAAGCT-GTCGAGGGGACT-3′; EstA2: 5′-CCAGGGACCAGCAATGACCATCCG-ATACGACACCACCGTC-3′ and 5′-GAGGAGAAGGCGTTAGCTGGTCC-GCCAGCCGAAGTCGACT-3′; EstA3: 5′-CCAGGGACCAGCAATGGT-GGCAACGATCGACATCACGACC-3′ and 5′-GAGGAGAAGGCGT-TAGCAGTCCCACGGCTGGGACTGGACT-3′ in a standard reaction mixture (0.4 μM of each primer, 0.2 mM dNTPs, 1 unit of KOD hot start DNA polymerase, 1 mM $MgSO_4$, 10× buffer, 50 ng template DNA, 10% (v/v) DMSO, and water to 50 μL) using a temperature cycling program of: 4 min at 94 °C, followed by 35 cycles of: 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C, with a final extension step for 3 min at 72 °C. The band from agarose gel electrophoresis was excised and the DNA extracted (gel extraction kit – Sigma-Aldrich) before determining the concentration using a spectrophotometer. The purified PCR product was cloned into the cleavable N-terminally his-tagged pET-YSBLIC3C vector using a ligation independent cloning protocol as described previously [23,24]. Transformation of the plasmid into chemocompetent *E. coli* NovaBlue cells, was carried out by incubating on ice for 5 min, followed by a heat-shock at 42 °C for 30 s, and then incubation on ice for a further 5 min before the addition of 0.5 mL of LB medium and incubation at 37 °C for 1 h. Transformed cells were plated onto LB agar plates containing kanamycin (100 μg mL^{-1}) and incubated at 37 °C overnight. Several colonies were picked and the plasmids isolated. Sanger sequencing (Technology Facility, Department of Biology, University of York) of cloned inserts was used to verify that genes contained no mutations.

2.4. Expression and purification of recombinant esterases

500 mL cultures of *E. coli* BL21 (DE3) cells containing the transformed plasmid were grown in LB media containing appropriate antibiotics (EstA1: kanamycin 100 μg mL^{-1}, EstA4 and EstA5 ampicillin 100 μg mL^{-1} and chloramphenicol 50 μg mL^{-1}) at 37 °C until an $OD_{600\,nm}$ 0.5, before inducing with IPTG (0.1 mM) and growing