Stereoselective ketone reduction by a carbonyl reductase from *Sporobolomyces salmonicolor*. Substrate specificity, enantioselectivity and enzyme-substrate docking studies†

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Received 27th April 2006, Accepted 30th May 2006
First published as an Advance Article on the web 15th June 2006
DOI: 10.1039/b606001c

In our effort to search for effective carbonyl reductases, the activity and enantioselectivity of a carbonyl reductase from *Sporobolomyces salmonicolor* have been evaluated toward the reduction of a variety of ketones. This carbonyl reductase (SSCR) reduces a broad spectrum of ketones including aliphatic and aromatic ketones, as well as α- and β-ketoesters. Among these substrates, SSCR shows highest activity for the reduction of α-ketoesters. Aromatic α-ketoesters are reduced to (*S*)-α-hydroxy esters, while (*R*)-enantiomers are obtained from the reduction of aliphatic counterparts. This interesting observation is consistent with enzyme-substrate docking studies, which show that hydride transfer occurs at the different faces of carbonyl group for aromatic and aliphatic α-ketoesters. It is worthy to note that sterically bulky ketone substrates, such as 2′-methoxyacetophenone, 1-adamantyl methyl ketone, ethyl 4,4-dimethyl-3-oxopentanoate and ethyl 3,3-dimethyl-2-oxobutanoate, are reduced to the corresponding alcohols with excellent optical purity. Thus, SSCR possesses an unusually broad substrate specificity and is especially useful for the reduction of ketones with sterically bulky substituents.

Introduction

Asymmetric reduction of ketones is a pivotal transformation in organic synthesis because chiral carbinols are useful target bioactive compounds or their precursors. Therefore, many methodologies have been developed to effect this important transformation. These methods include metal hydride reduction,[1,2] catalytic hydrogenation[3,4] and hydrogen transfer reactions.[5–8] However, hazardous materials such as metal hydrides and hydrogen often result in difficulties and safety concerns in the process operation. In addition, residual metals in the products originating from the metal catalysts present another challenge because of ever more stringent regulatory restrictions on the level of metals allowed in pharmaceutical products.[9] Because of environmentally benign reaction conditions and unparalleled chemo-, regio- and stereoselectivities, biocatalytic protocol has attracted more and more attention from organic chemists.[10–13] Biocatalytic ketone reductions can be realized using isolated enzymes[14–20] or whole cell systems.[21–24] However, the use of isolated enzymes is advantageous because undesirable enantiomer formation mediated by contaminating ketoreductases is minimized,[25] the enzymes can be stored and used as normal chemical reagents and no microbiological knowledge is required, providing more convenience for organic chemists. Although the cofactors required for such ketoreductases are expensive, the efficient cofactor recycling systems are available. Until recently the application of isolated carbonyl reductases to ketone reduction has been hampered by their limited availability.[25]

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† Electronic supplementary information (ESI) available: PDB files of ethyl phenylglyoxylate and ethyl 3,3-dimethyl-2-oxobutyrate docked into SSCR (1Y1P) active site. See DOI: 10.1039/b606001c

In this regard, we have been interested in searching for carbonyl reductase enzymes and assessing their substrate specificity and stereoselectivity.[26,27] Recently, a carbonyl reductase gene from *Sporobolomyces salmonicolor* AKU 4429 has been cloned and over-expressed in *E. coli*. The X-ray structures of the encoded protein (SSCR) and its complex with a coenzyme, NADPH, have been determined.[28,29] SSCR belongs to the short-chain dehydrogenase/reductase family. The structure of the NADPH-binding domain and the interaction between the enzyme and NADPH are very similar to those found in other enzymes of same family, while the structure of substrate-binding domain is unique. This unique substrate-binding domain suggests that SSCR might possess an unusual substrate specificity and stereoselectivity. Although it has been reported that the carbonyl reductase from *Sporobolomyces salmonicolor* reduced ethyl 4-chloro-3-oxo-butyrate to ethyl (*S*)-4-chloro-3-hydroxy-butyrate and was active to a few aldehydes and β-ketoesters,[30] no details about the enantioselectivity of β-ketoester reduction were provided. The substrate specificity and enantioselectivity of this interesting carbonyl reductase have not been fully evaluated. Therefore, we studied the activity and enantioselectivity of SSCR toward the reduction of a variety of ketones with diverse structures and found that SSCR showed a very broad substrate range including aliphatic and aromatic ketones, as well as α- and β-ketoesters. Herein we present these results together with the enzyme-substrate docking studies.

Results and discussion

The carbonyl reductase gene from *Sporobolomyces salmonicolor* was over-expressed in *E. coli* and the encoded protein was purified and lyophilized by following the literature procedure.[28] This lyophilized enzyme was stable for months at 4 °C without significant loss of activity. The specific activity of the carbonyl

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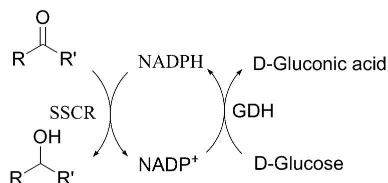
reductase from *Sporobolomyces salmonicolor* (SSCR) toward the reduction of ketones was determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm at room temperature. The enantioselectivity of SSCR toward the reduction of ketones was studied using a NADPH regeneration system consisting of D-glucose dehydrogenase (GDH) and D-glucose as shown in Scheme 1.



Scheme 1 Reduction of ketones by SSCR with NADPH regeneration system.

The enantiomeric excess (ee) values of the product alcohols were determined by chiral GC or HPLC analysis. The absolute configurations of product alcohols were assigned by comparing the retention times with those of standard samples. The standard samples of (*S*)- or (*R*)-enantiomer of the product alcohols were purchased from Aldrich or prepared in our previous studies.[26,27,31] The specific activities and enantioselectivities for different ketone substrates are presented in Tables 1–4.

As shown in Table 1, the carbonyl reductase from *Sporobolomyces salmonicolor* effectively catalyzed the reduction of various substituted acetophenones. The substituent at the phenyl ring exerted effect on the activity, although no general trend was observed. The position of the substituent at the phenyl ring also affected the enzyme activity, with activity increasing from $4' < 2' < 3'$ for Cl and CH_3 groups. The substituent and its position at the phenyl ring also affected the enantioselectivity of SSCR for the aryl ketone reductions. The acetophenone derivatives with a substituent at the 2′- or 3′-position showed higher enantioselectivity than those with a 4′-substituent. It is worth while noting that a methoxy group at the 2′-position greatly enhanced the enantioselectivity to 99% ee, while the ee value was 57% with 4′-OCH_3 substituent. For the substrate with two CF_3 substituents at 3′,5′-positions, the enzyme activity increased to 4 times of that for unsubstituted acetophenone, and (*S*)-1-(3′,5′-ditrifluoromethylphenyl)ethanol was obtained in optically pure form (99% ee). SSCR also efficiently reduced α-chloroacetophenone to (*S*)-2-chloro-1-phenylethanol in 98% ee. Aromatic cyclic ketones such as tetralone and 6-methyl-4-chromanone were also good substrates for this carbonyl reductase. These bicyclic ketones were reduced to the corresponding (*R*)-chiral alcohols in excellent enantiomeric purity.

Table 1 Reduction of aryl ketones by SSCR

X	R	Specific activity[a]	Ee (%)
H	4′-H	28	42 (*R*)
H	4′-F	14	46 (*R*)
H	4′-Cl	20	14 (*R*)
H	4′-Br	13	42 (*R*)
H	4′-CH_3	11	59 (*R*)
H	4′-OCH_3	20	57 (*R*)
H	4′-CF_3	92	17 (*S*)
H	2′-Cl	78	15 (*R*)
H	3′-Cl	280	66 (*R*)
H	2′-CH_3	14	70 (*R*)
H	3′-CH_3	64	92 (*R*)
H	2′-OCH_3	24	99 (*R*)
H	3′,5′-$(CF_3)_2$	120	99 (*R*)
Cl	4′-H	370	98 (*S*)
1-Tetralone		379	94 (*R*)
6-Methyl-4-chromanone		—[b]	99 (*R*)

[a] The specific activity was defined as nmol min^{-1} mg^{-1}. [b] Absorbance at 340 nm was too high, preventing the measurement of specific activity.

Table 2 presents the specific activity and enantioselectivity of the carbonyl reductase from *Sporobolomyces salmonicolor* for the aliphatic ketone reduction. It can be seen that SSCR was active for the reduction of various aliphatic ketones. The activity and enantioselectivity (even stereopreference) were dependent upon the structures of both R and R′ groups. For example, reduction of *n*-pentyl methyl ketone gave (*S*)-enantiomer as the major product, while the (*R*)-alcohol was obtained when methyl was changed to ethyl group. It is worthy to note that SSCR efficiently catalyzed the reduction of bulky 1-adamantyl methyl ketone to (*S*)-1-(1-adamantyl)ethanol in 99% ee.

From Table 3 it can be seen that SSCR was very active for the reduction of both aromatic and aliphatic α-ketoesters. SSCR efficiently catalyzed the enantiospecific reduction of ethyl phenylglyoxylate to ethyl (*S*)-2-hydroxy-2-phenylacetate. Both activity and enantioselectivity of SSCR were affected by the substituents on the phenyl ring of aromatic α-ketoesters with

Table 2 Reduction of aliphatic ketones by SSCR

R	R′	Specific activity[a]	Ee (%)
n-Pentyl	Methyl	50	30 (*S*)
n-Hexyl	Methyl	210	44 (*S*)
n-Heptyl	Methyl	40	4 (*R*)
1-Adamantyl	Methyl	140	>99 (*S*)
n-Pentyl	Ethyl	40	72 (*R*)

[a] The specific activity was defined as nmol min^{-1} mg^{-1}.

Table 3 Reduction of α-ketoesters by SSCR

R	Specific activity[a]	Ee (%)
Phenyl	6640	99 (*S*)
4-Cyanophenyl	2280	82 (*S*)
4-Fluorophenyl	5070	74 (*S*)
4-Chlorophenyl	6360	63 (*S*)
4-Bromophenyl	1400	56 (*S*)
4-Methylphenyl	1200	88 (*S*)
3,5-Difluorophenyl	5730	43 (*S*)
Isopropyl	17540	99 (*R*)
tert-Butyl	5560	99 (*R*)

[a] The specific activity was defined as nmol min^{-1} mg^{-1}.

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ethyl phenylglyoxylate being the most active and enantioselective substrate. The aliphatic α-ketoesters were effectively reduced to the (*R*)-enantiomer of the corresponding α-hydroxyesters in optically pure form with ethyl 3-methyl-2-oxobutyrate being the most active substrate among all the tested ketones. It is astounding that SSCR catalyzed the reduction of aromatic α-ketoesters to afford (*S*)-enantiomers as the major products, while the reduction of aliphatic counterparts gave (*R*)-enantiomers with excellent ee values, indicating that the hydride transfer from NADPH to the carbonyl group occurred from different faces for aromatic and aliphatic α-ketoesters. To gain insight on how the R group affected the faces of hydride transfer, enzyme–substrate docking was performed with ethyl phenylglyoxylate and ethyl 3,3-dimethyl-2-oxobutyrate. Computer-assisted docking was thus performed on SSCR protein structure (PDB file 1Y1P) using the FlexX program. Three residues (Ser133, Try177 and Lys181) were proposed as the catalytic triad in this short-chain dehydrogenase/reductase enzyme. The carbonyl oxygen atom of a substrate forms hydrogen bonds with both Tyr177 and Ser133 residues, and it is protonated from the Tyr177 residue, followed by the attacking of a hydride from C4 atom of NADPH to the carbonyl carbon atom of the substrate.[29,32] Therefore, care was taken to orient the hydrogens of the hydroxy groups of Ser133 and Tyr177 toward the water molecule (HOH175), which is coincident with the carbonyl oxygen of the prospective substrate. The lowest-energy docked conformations of these two substrates into the enzyme active site are deposited as supplementary information† and the active site details are shown in Fig. 1. In both lowest-energy conformational ensembles the carbonyl carbon is in a position proximal to the C4 atom of the nicotinamide ring of NADPH cofactor (3.49 and 3.87 Å for ethyl phenylglyoxylate and ethyl 3,3-dimethyl-2-oxobutyrate, respectively). The ensembles are distinguished by the orientations of the substrates and consistent with the experimental observations. As shown in Fig. 1, the NMN (nicotinamide mononucleotide) ring of the cofactor is located at the *Re*-face of ethyl phenylglyoxylate (A), but at the *Si*-face of ethyl 3,3-dimethyl-2-oxobutyrate (B). Thus these ketones are reduced to ethyl (*S*)-2-hydroxy-2-phenylacetate and ethyl (*R*)-3,3-dimethyl-2-hydroxybutyrate, respectively.

The reductions of β-ketoesters catalyzed by SSCR were also examined (Table 4). The results showed that the carbonyl reductase from *Sporobolomyces salmonicolor* also effectively catalyzed the enantioselective reduction of β-ketoesters. The R group exerted effect on both the enzyme activity and enantioselectivity. Although ethyl 3-oxopentanoate was reduced to ethyl (*R*)-3-hydroxypentanoate in moderate optical purity, when R is the bulky isopropyl or *tert*-butyl group the β-ketoesters were reduced to the (*S*)-enantiomer of the β-hydroxyesters in 99% ee. Among the tested β-ketoesters, SSCR showed highest activity for the reduction of ethyl 4-chloro-3-oxobutyrate, affording ethyl (*S*)-4-chloro-3-hydroxybutyrate (97% ee), an important pharmaceutical intermediate. This carbonyl reductase also catalyzed the reductions of ethyl 3-oxo-3-phenylpronionate and ethyl 3,3,3-trifluoro-3-oxobutyrate to the corresponding (*S*)-alcohol in moderate to high ee values.

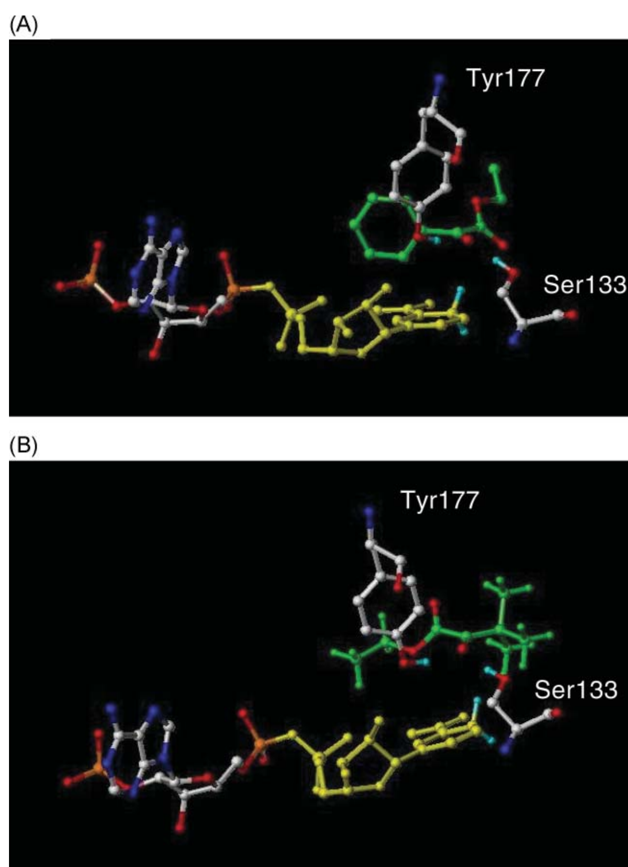

Fig. 1 The substrate ethyl phenylglyoxylate (A) and ethyl 3,3-dimethyl-2-oxobutyrate (B) docked into the enzyme (SSCR) active site. NMN is shown in yellow and the substrate is in green.

Table 4 Reduction of β-ketoesters by SSCR

R	Specific activity[a]	Ee (%)
Chloromethyl	1400	97 (*S*)
Ethyl	195	61 (*R*)
Isopropyl	800	99 (*S*)
tert-Butyl	180	99 (*S*)
Trifluoromethyl	120	90 (*S*)
Phenyl	100	56 (*S*)

[a] The specific activity was defined as nmol min^{-1} mg^{-1}.

As shown above, this carbonyl reductase (SSCR) catalyzes the reduction of a broad spectrum of ketones including aliphatic, aromatic ketones, α- and β-ketoesters. Among these substrates, SSCR shows highest activity for the reduction of α-ketoesters. SSCR belongs to short-chain dehydrogenase/reductase family, and the observation is similar to that for 7α-hydroxysteroid dehydrogenase (7α-HSDH) from *Bacteroides fragilis* ATCC 25285 which is also a member of short-chain dehydrogenase/reductase family.[33,34] Although 7α-HSDH is an effective catalyst for the reduction of α-ketoesters, it shows extremely low activity for the other ketones including aliphatic, aromatic ketones, and β-ketoesters. The enzyme activity of SSCR is higher for every category of ketones than 7α-HSDH. Therefore, SSCR is an efficient catalyst for the reduction of all the tested ketones, indicating this carbonyl reductase possesses unusually broad substrate specificity.

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Conclusions

In conclusion, the carbonyl reductase from *Sporobolomyces salmonicolor* (SSCR) possesses an unusual substrate range by showing activity toward the reduction of diverse ketones. Among the four types of tested carbonyl compounds, this carbonyl reductase shows highest activity for aromatic and aliphatic α-ketoesters. Both experimental and enzyme-substrate docking studies show that the hydride transfer from NADPH to the carbonyl group occurs at different faces for aromatic and aliphatic α-ketoesters, leading to different enantiomers of chiral α-hydroxyesters. It is more important that SSCR efficiently catalyzed the reductions of several bulky ketones, such as 2′-methoxyacetophenone, 3′,5′-ditrifluoromethylacetophenone, 1-adamantyl methyl ketone, ethyl 4,4-dimethyl-3-oxopentanoate, ethyl 4-methyl-3-oxopentanoate, ethyl 3,3-dimethyl-2-oxobutanoate and ethyl 3-methyl-2-oxobutanoate, to the corresponding chiral alcohols in enantiomerically pure form, indicating this carbonyl reductase is a useful enzyme catalyst for the reduction of sterically bulky ketones.

Experimental

Chiral GC analysis was performed on a Hewlett Packard 5890 series II plus gas chromatograph equipped with autosampler, EPC, split/splitless injector, FID detector and CP-Chirasil-Dex CB chiral capillary column (25 m × 0.25 mm). Chiral HPLC analysis was performed on an Agilent 1100 series high-performance liquid chromatography system with (*S*,*S*)-Whelk-O 1 column (25 cm × 4.6 mm, Regis Technologies Inc.). The enzyme activities toward the reduction of ketones were assayed using a SpectraMax M2 microplate reader (Molecular Devices). All the ketones and β-ketoesters were purchased from Aldrich or Acros Organics. Ethyl phenylglyoxylate, ethyl (4-cyanophenyl)glyoxylate, ethyl (3,5-difluorophenyl)-glyoxylate, and ethyl 3-methyl-2-oxobutyrate were purchased from Aldrich or Acros. All the other α-ketoesters were prepared by Friedel–Crafts acylation of substituted benzene with ethyl oxalyl chloride in the presence of anhydrous $AlCl_3$,[35] or reaction of diethyl oxalate with the corresponding Grignard reagents.[36] The racemic alcohol standards were prepared by reduction of ketones with sodium borohydride. (*S*)- or (*R*)-Enantiomers of the product alcohols were purchased from Aldrich or prepared in our previous studies.[26,27,31]

Gene expression and purification of the carbonyl reductase from *Sporobolomyces salmonicolor* (SSCR)

The carbonyl reductase gene from *Sporobolomyces salmonicolor* was cloned by gene assembly techniques.[37] Twelve oligonucleotides ranging from 100 to 120 nucleotides were designed on the basis of nucleotide sequence of *Sporobolomyces salmonicolor* carbonyl reductase gene,[28] the open reading frame of which is composed of 1032 nucleotides (344 amino acid residues). The *Nco* I and *Bam* HI sites were franked to the open reading frame for easy cloning into expression vector pET15b (Novagen). Plasmid DNA containing SSCR gene was transformed into *E. coli* BL21(DE3) strain. Overnight culture was diluted into fresh LB medium containing ampicillin (100 μg ml^{-1}) and incubated at 37 °C until optical density reached 0.6 at 595 nm. The expression was induced by addition of IPTG to 0.1 mM and the culture was incubated at 30 °C for another 6 h. Cells were harvested by centrifugation at 4100 rpm at 4 °C for 30 min. The cell pellet was resuspended in potassium phosphate buffer (100 mM, pH 7.4) and the cells were disrupted by EmulsiFlex-C5 Homogenizer. The cell-free extract was mixed with an equal volume of PEI solution (0.25% polyethyleneimine MW 40 K–60 K, 6% NaCl, 100 mM Borax, pH 7.4) to remove lipids.[38] The supernatant was precipitated with 50% ammonium sulfate. The resulting precipitate was collected after centrifugation and dissolved in potassium phosphate buffer (10 mM, pH 7.4) containing 0.1 mM dithiothreitol. The lysate was desalted by gel filtration into potassium phosphate buffer (10 mM, pH 7.4, 0.1 mM dithiothreitol), and lyophilized to afford the SSCR enzyme as a white powder with a protein content of 83% measured with the Bradford assay.

Activity assay of the carbonyl reductase from *Sporobolomyces salmonicolor* (SSCR)

The activities of the carbonyl reductase from *Sporobolomyces salmonicolor* toward the reductions of ketones were determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) in the presence of an excess amount of ketones. The activities were measured at room temperature in a 96-well plate, in which each well contained ketone (6.25 mm) and NADPH (0.25 mm) in potassium phosphate buffer (100 mM, pH 6.5, 180 μl). The reactions were initiated by the addition of the carbonyl reductase (20 μl solution containing 2–40 μg of enzyme). The specific activity was defined as the number of nmol of NADPH converted in one minute by 1 mg of enzyme (nmol min^{-1} mg^{-1}).

Enantioselectivity of ketone reduction catalyzed by the carbonyl reductase from *Sporobolomyces salmonicolor* (SSCR)

The enantioselectivity of the enzymatic reduction of ketones was studied using an NADPH recycle system. The general procedure was as follows: D-glucose (4 mg), D-glucose dehydrogenase (0.5 mg), NADPH (0.5 mg), the carbonyl reductase (SSCR, 0.5 mg) and ketone solution in DMSO (50 μl, 0.25 M) were mixed in a potassium phosphate buffer (1 ml, 100 mM, pH 6.5) and the mixture was shaken overnight at room temperature. The mixture was extracted with methyl *tert*-butyl ether (1 ml). The organic extract was dried over anhydrous sodium sulfate and was subjected to chiral GC and HPLC analysis to determine the enantiomeric excess.[39] The absolute configurations of product alcohols were identified by comparing the chiral GC data with the standard samples.[26,27,31] The details of chiral GC and HPLC analysis are summarized in Table 5.

Enzyme-substrate docking

The crystal structure of the SSCR–NADPH (PDB code: 1Y1P) was used. In the crystal structure, there were two protein molecules (Mol-1 and Mol-2) and they are almost identical. The docking was performed on Mol-1 where an NADPH was tightly bound. Since the carbonyl oxygen atom of a substrate is proposed to form hydrogen bonds with Ser133 and Tyr177, the torsion angles of Ser133 and Tyr177 were adjusted, so that the hydrogens of the hydroxy groups of Ser133 and Tyr177 were oriented toward the water molecule (HOH175) in the crystal structure, which is

Table 5 Details of chiral HPLC and GC analysis

Alcohols	Method[a]	Retention time/min	
		t_R	t_S
1-Phenylethanol	A	5.04	5.43
1-(4′-Fluorophenyl)ethanol	A	5.97	6.66
1-(4′-Chlorophenyl)ethanol	A	13.61	15.07
1-(4′-Bromophenyl)ethanol	A	19.45	21.10
1-(4′-Methylphenyl)ethanol	A	6.86	7.63
1-(4′-Methoxyphenyl)ethanol	A	13.74	14.54
1-(4′-Trifluoromethylphenyl)ethanol	A	7.27	8.37
1-(2′-Chlorophenyl)ethanol	A	12.96	15.88
1-(3′-Chlorophenyl)ethanol	A	13.49	14.65
1-(2′-Methylphenyl)ethanol	A	8.98	10.70
1-(3′-Methylphenyl)ethanol	A	7.46	7.95
1-(2′-Methoxyphenyl)ethanol	A	13.33	12.48
1-[3′,5′-Bis(trifluoromethyl)phenyl]ethanol	A	4.50	3.98
2-Chloro-1-phenylethanol	A	14.83	15.88
1,2,3,4-Tetrahydro-1-naphthalenol	A	17.59	17.18
6-Methyl-4-chromanol	A	23.16	23.63
2-Heptanol[b]	B	8.73	6.36
2-Octanol[b]	B	14.87	11.32
2-Nonanol[b]	B	22.56	18.28
1-(1-Adamantyl)ethanol[b]	A	23.67	22.51
3-Octanol[b]	B	11.40	9.60
Ethyl 2-hydroxy-2-phenylacetate	C	9.8	10.7
Ethyl 2-hydroxy-2-(4-fluorophenyl)acetate	C	8.4	8.9
Ethyl 2-hydroxy-2-(4-chlorophenyl)acetate	C	8.9	9.5
Ethyl 2-hydroxy-2-(4-bromophenyl)acetate	C	9.4	10.0
Ethyl 2-hydroxy-2-(4-methylphenyl)acetate	C	11.6	12.3
Ethyl 2-hydroxy-2-(4-cyanophenyl)acetate	C	21.6	22.6
Ethyl 2-hydroxy-2-(3,5-diflurophenyl)acetate	D	9.2	9.7
Ethyl 2-hydroxy-3-methylbutyrate	E	22.0	22.3
Ethyl 2-hydroxy-3,3-dimethylbutyrate	B	11.2	11.8
Ethyl 4-chloro-3-hydroxybutyrate[b]	A	7.69	7.94
Ethyl 3-hydroxybutyrate	F	5.37	5.23
Ethyl 3-hydroxypentanoate	F	8.69	8.37
Ethyl 3-hydroxyhexanoate	F	14.19	13.57
Ethyl 3-hydroxy-4-methylpentanoate	F	11.64	11.85
Ethyl 3-hydroxy-4,4-dimethylpentanoate[c]	G	10.05	10.54
Ethyl 3-hydroxy-4,4,4-trifluorobutyrate[c]	G	5.87	6.47
Ethyl 3-hydroxy-3-phenylpropionate	H	18.00	18.24

[a] A) GC, 120 °C for 2 min, 1 °C min^{-1} to 150 °C, 150 °C for 5 min; B) GC, 80 °C for 2 min, 1 °C min^{-1} to 110 °C, 110 °C for 5 min; C) HPLC, flow rate 1.0 ml min^{-1}, hexane–isopropanol (0.1% HOAc) = 95 : 5; D) HPLC, flow rate 1.0 ml min^{-1}, hexane–isopropanol (0.1% HOAc) = 99 : 1; E) GC, 60 °C for 2 min, 1 °C min^{-1} to 90 °C, 90 °C for 5 min; F) GC, 90 °C for 2 min, 1 °C min^{-1} to 120 °C, 120 °C for 5 min; G) GC, 100 °C for 2 min, 1 °C min^{-1} to 130 °C, 130 °C for 5 min; H) GC, 130 °C for 2 min, 1 °C min^{-1} to 160 °C, 160 °C for 5 min. [b] Hydroxyl group was acylated as acetate. [c] Hydroxyl group was acylated as trifluoroacetate.

coincident with the carbonyl oxygen of the docked substrate. All docking calculations were performed with FlexX program in the SYBYL7.1 modeling package. A docking algorithm that took account of ligand flexibility but kept the protein rigid was employed. Docking runs were carried out using the standard parameters of the program for interactive growing and subsequent scoring.

Acknowledgements

We acknowledge the support from the Robert A. Welch Foundation (Grant N-0871) for JDB and the start-up funds from the Southern Methodist University for LH.

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