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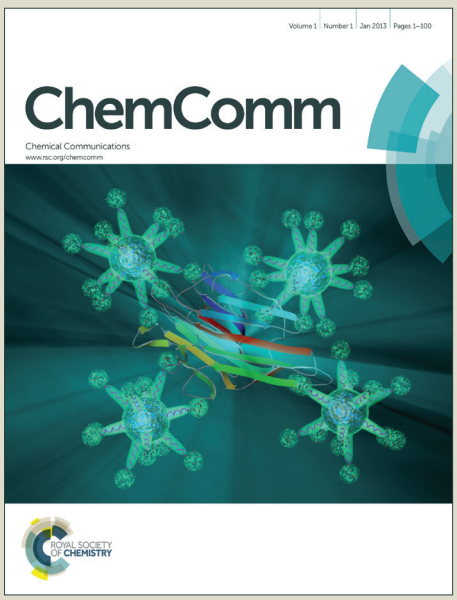

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COMMUNICATION

Asymmetric synthesis of optically active methyl-2-benzamido-methyl-3-hydroxy-butyrate by robust short-chain alcohol dehydrogenases from *Burkholderia gladioli*

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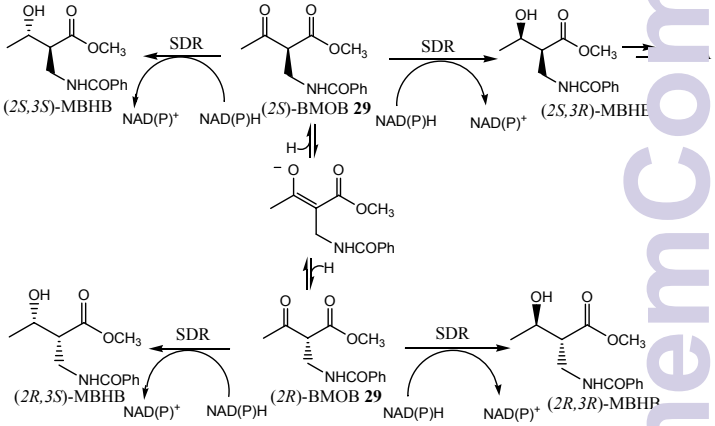
Three short-chain alcohol dehydrogenases from *Burkholderia gladioli* were discovered for their great potential in the dynamic kinetic asymmetric transformation of methyl 2-benzamido-methyl-3-oxobutanoate, and their screening against varied organic solvents and substrates. This is the first report for recombinant enzymes capable of achieving this reaction with the highest enantio- and diastereo-selectivity.

Chiral alcohols are frequently required as important and versatile intermediates for pharmacologically compounds and other fine chemicals. Dynamic kinetic asymmetric transformation (DYKAT) is a powerful tool for accessing single enantiomers with a theoretical yield of 100%.[1] Many short-chain alcohol dehydrogenases/reductases (SDRs) have been exploited and subjected to the DYKAT of racemic ketones.[2] However, upscale DYKAT was hampered due to the limited commercially available enzymes, narrow substrate scope, and poorly soluble substrates.[3] Thus, there is substantial interest in developing robust enzymes and extending the application of DYKAT in stereo-selective synthesis.

The presence of (*2S, 3R*)-methyl-2-benzamido-methyl-3-hydroxy-butyrate (MBHB) in (*3R, 4R*)-4-acetoxy-3-[(*R*)-(*t*-butyl dimethylsilyloxy) ethyl]-2-azetidinone (4-AA), an important building block for carbapenems is well-documented.[4] Achieving this complex compound with excellent enantio- and diastereo-selectivity is normally a difficult task.[5] DYKAT of methyl-2-benzamido-methyl-3-oxobutanoate (BMOB) using a transition metal catalyst has been established, but it requires high-pressure and leaves trace metal contamination in the product.[6] In an attempt to develop a renewable alternative to this target reaction, enzymatic method with ambient pressures and no production of toxic waste is urgent. Several enzymes have been developed to give (*2S, 3R*)-MBHB, but with low to moderate enantio- and distereo-selectivity or low substrate loading (4 mM).[7] Based on the discovery of SDR-producing strain *Burkholderia gladioli* ZJB12126,[8] we envisaged that a highly enantio- and distereo-selective asymmetric synthesis of (*2S, 3R*)-MBHB might be realized by using SDR from ZJB12126 via DYKAT (Scheme 1).

To seek the appropriate enzyme for preparation of (*2S, 3R*)-MBHB, three *bgadh* genes containing distinct sequence motifs similar to the most representative SDR from *Lactobacillus brevis* (GeneBank: CAD66648.1) were identified based on the genome of *B. gladioli* BSR3 (Genbank accession no. NR_102847.1),[9] cloned and overexpressed in *Escherichia coli*. These recombinant enzymes (*Bg*ADH1, *Bg*ADH2, and *Bg*ADH5) displayed 31-34% sequence identities with each other, and owned common "Rossmann-fold" motifs, cofactor-binding domains covered N-terminal TGXXXGXG, NNAG and P[illegible], and N-S-Y-K catalytic tetrads (ESI, † Fig. S3).[10] With the codon optimization of *bgadh2* gene sequence, the expression level of *Bg*ADH2 was significantly improved (ESI, † Fig. S2). After purification, the protein concentration of optimized *Bg*ADH2 (2.6 mg mL^{-1}) was approximately 3-fold enhancement than the counterpart (0.9 mg mL^{-1}). The molecular subunit mass of *Bg*ADHs were around 27 kDa and the quaternary structures of *Bg*ADH1, *Bg*ADH2, and *Bg*ADH5 were tetramers.



Scheme 1 Asymmetric synthesis of enantioenriched MBHB by SDR via dynamic kinetic asymmetric transformation.

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† Electronic Supplementary Information (ESI) available: Table S1-S7, Fig. S1-S38, and detail experimental procedure. See DOI: 10.1039/b000000x/

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*Bg*ADH2 was found to display excellent stereo-selectivity, affording the corresponding (*2S, 3R*)-MBHB with 99% *ee* and 98.5% *de* (Table 1). *Bg*ADH1 and *Bg*ADH5 catalysed this transformation as well yet with low enantio-selectivity or moderate diastereo-selectivity. In comparison, *Bg*ADH2 showed unique and the highest enantio- and distereo-selectivity yielding (*2S, 3R*)-MBHB reported so far. Notably, the K_{mNADPH} values of purified *Bg*ADHs were 3- to 22-fold lower than that against NADH, emphasizing that *Bg*ADHs preferred NADPH over NADH. The overall catalytic efficiency (k_{cat}/K_{mBMOB}) of *Bg*ADH2 (12.3 $s^{-1}\cdot mM^{-1}$) was found to be the highest as compared to *Bg*ADH1(5.83 $s^{-1}\cdot mM^{-1}$), *Bg*ADH5(2.81 $s^{-1}\cdot mM^{-1}$), and *Cp*SCR (10.0 $s^{-1}\cdot mM^{-1}$) from *Candida parapsilosis* (GeneBank: GQ411433.1) (ESI, † Table S6).[11] Although the well-known *Lb*ADH exhibited strong Mg^{2+} dependency,[9a] *Bg*ADHs were typical of non-metal SDRs (ESI, †Table S5). *Bg*ADH2 activity was enhanced 10-fold in response to different temperatures, and the optimal temperature and pH were at 30 °C and 6.5. Half-life more than 48 h was found at 30 °C for *Bg*ADH2, suggesting that it was rather robust and suitable for prolonged incubation. The employment of *Bg*ADH2 also exhibited excellent functions under the "enzyme-coupled" cofactor regeneration using glucose dehydrogenase (GDH) and glucose,[12] avoiding the reversibility and poor thermodynamic driving force in the "substrate-coupled" approach.[13]

The effect of organic solvents, played a crucial role in biocatalysis toward poorly soluble susbtrates,[14] on the activity and stereo-selectivity of *Bg*ADH2 using BMOB as substrate was analyzed in depth. In the presence of water-miscible solvents, insignificant negative effect was observed on the activity of *Bg*ADH2, except for acetone, likely because of the subunit dissociation, cofactor desorption, or the tendency of hydrophilic solvents to strip tightly bound water in the enzyme active site.[15] Similarly, the stereo-selectivity of *Bg*ADH2 was impaired in hydrophilic solvents, except for DMSO and DMF.[16] This might be relieved by immobilization, which can improve enzymatic performance in organic solvents.[17] When the water-immiscible solvents were added, the relative activities were roughly correlated with the log $P_{o/w}$ values of solvents, except for dichloromethane,[18] and remained completely intact in high log $P_{o/w}$ solvents, such as *n*-hexane and *iso*-octane (Fig. 1).[16]

Table 1 DYKAT of BMOB using different recombinant enzymes.[a]

Enzyme	Cf	*ee* (%)	*de* (%)	Cv (%)	Activity[b] (U mg^{-1})
*Bg*ADH1	(*2S,3R*)	1	99	99	3.65
*Bg*ADH2	(*2S,3R*)	99	99	99	5.09
*Bg*ADH5	(*2S,3R*)	99	69	51	2.62
*Cp*SCR	(*2R,3R*)	99	99	99	4.37
LbADH[c]	(*2S,3R*)	99	92	-	-
RDR[c]	(*2S,3R*)	99	89	-	-
FPDH[c]	(*2S,3R*)	94	89	-	-
RAX[c]	(*2S,3R*)	60	49	-	-

[a] Reaction conditions: BMOB (40 mM, DMSO, 5% v/v), $NADP^+$ (0.4 mM), glucose (5%, w/v), purified enzyme (0.1 mg mL^{-1}), GDH (0.1 mg mL^{-1}), pH 6.5, and 30 °C. [b] Activities were measured under the standard assay protocol. Cf, configuration; Cv, conversion. [c] Data from ref. [7c]

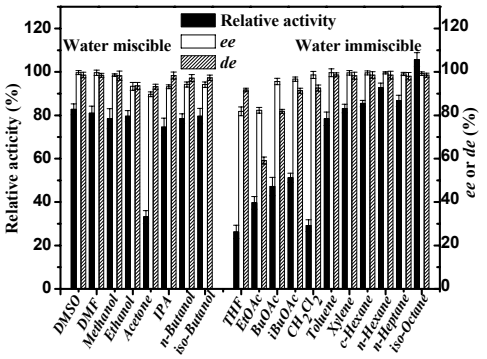

Fig. 1 Effect of organic solvents on the biosynthesis of (*2S, 3R*)-MBHB using purified *Bg*ADH2. The amounts of organic solvents added were 25% (v/v) for water-miscible solvents and 50% (v/v) for water-immiscible solvents. Activities were measured under the standard assay protocol. The activity in the absence of organic solvent was taken as 100%. DMSO, dimethyl sulfoxide; DMF, dimethylformamide; IPA, *iso*-propanol; THF, tetrahydrofuran. EtOAc, ethyl acetate; BuOAc, butyl acetate; iBuOAc, *iso*-butyl acetate; CH_2Cl_2, dichloromethane; c-hexane, cyclohexane.

Importantly, *Bg*ADH2 remained excellent enantio- and diastereo-selectivity as well as high relative activity in the presence of toluene with higher partition efficiencies for BMOB and (*2S, 3R*)-MBHB.

A further study of the developed aqueous-toluene biphasic system for (*2S, 3R*)-MBHB production to increase substrate solubility and avoid product inhibition demonstrated a better productivity than that in monophasic system (Fig. 2a). With a highly optimized process development, full conversion was achieved with 60 mM substrate in short times by the driving force of the acidity of the buffer that faciliated the racemization of (*2R*)-BMOB through the achiral enol.[19] The plateau emerged after only 2 h and the final production of (*2S, 3R*)-MBHB was 55.1 mM (yield 91.1%) in 99% *ee* and 98.5% *de* (Fig. 2b).

To reveal the substrate recognition and catalytic principle of *Bg*ADHs, a set of structurally diverse aldehydes and ketones were subjected to the reduction conditions (Table 2). It was noteworthy that *Bg*ADHs followed the anti-Prelog's rule with a preference and worked well toward varied aryl ketones and ketoesters, except for **20**, **21**, **22**, and **33**. This might be explained by the small hydrophobic active site tunnels in *Bg*ADHs thus the hydrophilic groups or macromolecule were impeded embedding in the hydrophobic cavity.[20] The inverted

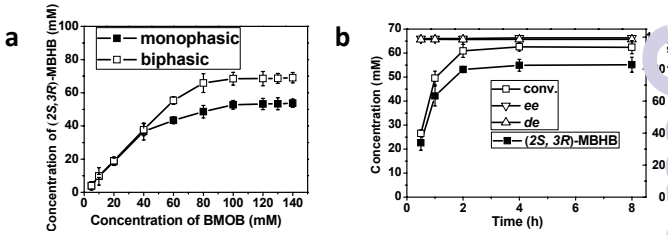

Fig. 2 (a) Effects of substrate concentrations on the (*2S, 3R*)-MBHB production with *Bg*ADH2 in monophasic and biphasic system. (b) Time course of (*2S, 3R*)-MBHB production using *Bg*ADH2. Three independent measurements were taken for each point.

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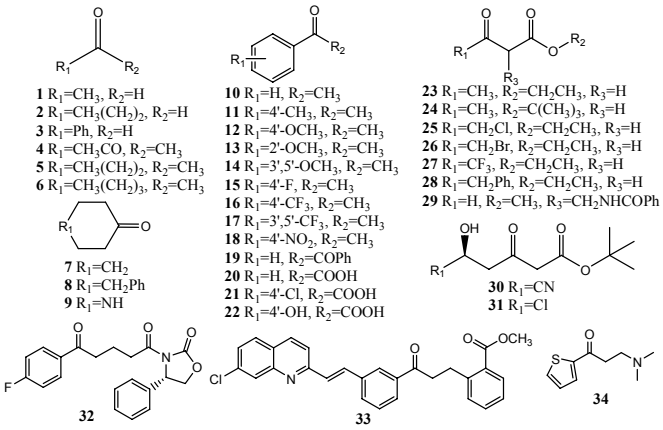
Table 2 Substrate specificities of *Bg*ADHs from *B. gladioli* ZJB12126.



S	*Bg*ADH1		*Bg*ADH2		*Bg*ADH5	
	Activity (U mg^{-1})	Cf *ee*[a] (%)	Activity (U mg^{-1})	Cf *ee*[a] (%)	Activity (U mg^{-1})	Cf *ee*[a] (%)
1	0.32	-	1.33	-	0.32	-
2	0.50	-	1.05	-	0.51	-
3	7.90	-	8.45	-	5.74	-
4	1.15	-	1.15	-	0.82	-
5	1.32	-	1.37	-	1.05	-
6	1.52	-	2.59	-	1.29	-
7	0.74	-	0.85	-	0.85	-
8	1.62	-	1.62	-	1.14	-
9	2.50	-	3.03	-	1.96	-
10	6.13	*R*(99)	6.35	*R*(99)	4.51	*R*(90)
11	5.49	*R*(99)	6.21	*R*(>99)	4.39	*R*(>99)
12	6.29	*R*(99)	6.57	*R*(99)	4.63	*R*(95)
13	4.18	*R*(93)	4.83	*R*(95)	3.64	*R*(90)
14	5.11	*R*(99)	5.19	*S*(99)	4.03	*R*(99)
15	6.28	*R*(99)	6.56	*R*(99)	4.66	*R*(99)
16	6.44	*R*(99)	6.98	*R*(99)	5.35	*R*(99)
17	5.67	*R*(98)	6.13	*R*(99)	4.05	*R*(99)
18	6.22	*R*(87)	6.76	*S*(>99)	4.98	*R*(90)
19	2.43	-	3.52	-	2.32	-
20	-	-	3.35	*R*(41)	-	-
21	-	-	3.64	*R*(98)	-	-
22	-	-	2.02	*R*(90)	-	-
23	4.47	*R*(>99)	5.56	*R*(>99)	4.15	*R*(>99)
24	3.92	*R*(>99)	4.47	*R*(>99)	4.06	*R*(>99)
25	5.14	*S*(95)	5.68	*S*(>99)	4.59	*S*(86)
26	4.71	*S*(99)	5.62	*S*(99)	4.27	*S*(77)
27	3.02	*S*(98)	4.12	*S*(>99)	2.42	*S*(99)
28	3.91	*S*(85)	3.82	*S*(99)	3.37	*S*(15)
29	3.65	*2S,3R* (0)	5.09	*2S,3R* (99)	2.63	*2S,3R* (99)
30	2.55	*3R,5R* (86)	3.11	*3R,5R* (95)	2.55	*3R,5R* (98)
31	3.02	*3R,5S* (88)	3.33	*3R,5S* (96)	3.12	*3R,5S* (97)
32	1.40	*S*(>99)	1.36	*S*(>99)	1.13	*S*(37)
33	-	-	-	-	-	-
34	-	-	1.09	*S*(99)	-	-

[a] The *ee* values of the corresponding products of **10-29** and **34**, or *de* values of the corresponding products of **30**, **31**, and **32**. Cf, configuration.

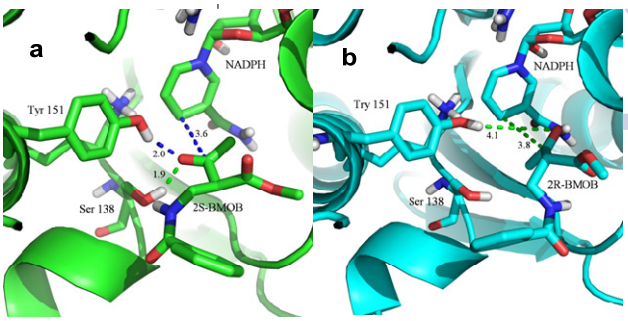


Fig. 3 Binding models of *Bg*ADH2 with BMOB. (a) Detailed view of the interactions between *Bg*ADH2 and (*2S*)-BMOB; (b) Detailed view of the interactions between *Bg*ADH2 and (*2R*)-BMOB.

stereochemical assignment toward **25**-**28**, **32**, and **34** was due to the smaller side of the ketone having a higher Cahn-Ingold-Prelog priority than the large one. The Prelog stereopreference of *Bg*ADH2 against **14** and **18** might be the presence of the hydrogen bond formed between substituents and enzyme, modifying the enzymatic property.[21] Eelectron-withdrawing substituents (**12**, **15**, **16**, **18**, **25**, and **26**) were found to improve the activities, while electron-donating groups (**11**) lead to reduced activities.[11] Substituents close to the carbonyl group (*ortho*-substituted **13**), multi substitution (**14** and **17**), and bulky substituents adjacent to the carbonyl group (**24**, **27**, **28**, **30**, and **31**) have steric hindrance effects on the activities. Remarkably, *Bg*ADH2 was capable of accomplishing the reduction of heterocyclic substrates (**32** and **34**) with high stereo-selectivity and offering a simple access to the corresponding versatile chiral pharmeceutical intermediates.

The molecular basis of *Bg*ADH2 in the binding interaction of BMOB **29** was predicted, wherein C=O oxygen of (*2S*)-BMOB forms hydrogen bonds with both Y151 and S138 (2.0 and 1.9 Å) and it is protonated from Y151-OH, followed by the attack of a hydride from C4-NADPH toward the C=O carbon of (*2S*)-BMOB (3.6 Å) (Fig. 3a), in accordance with the proposed catalytic mechanism of SDR.[3b, 10, 20a] This hypothesis was further supported by the site-directed mutagenesis. Mutation of Y151 or S138 to A resulted in an almost complete (>99%) loss of enzyme activity. Overall, NADPH provides its C4-hydride to attack the *si*-face of (*2S*)-BMOB, giving rise to the (*R*)-product which is consistent with the experimentally observed (*2S, 3R*)-MBHB.[22]

In summary, we have successfully developed a renewable alternative to the asymmetric reduction of BMOB via enzymatic DYKAT, affording an enantio- and distereomerically pure (*2S, 3R*)-MBHB. *Bg*ADH2 from *B. gladioli* ZJB12126 was the first enzyme with excellent enantio- and diastereo-selectivity for (*2S, 3R*)-MBHB production (99% *ee*, 98.5% *de*). An aqueous-toluene biphasic system significantly enhanced the substrate loading (60 mM) and productivity (91.1%). *Bg*ADH2 exhibited also a broad substrate scope followed the anti-Prelog's rule, especially toward aryl ketones and ketoesters. Docking provided insight into the understanding of the molecular basis of *Bg*ADH2, being useful for further engineering of this enzyme for other enantio-selective transformations.

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