Biochemical and Biophysical Research Communications 460 (2015) 826–830

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

An improved flurogenic probe for high-throughput screening of 2-deoxyribose aldolases

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ARTICLE INFO

Article history:
Received 12 March 2015
Available online 28 March 2015

Keywords:
High-throughput screen
Daylight-viewable fluorescence
2-deoxyribose aldolase

ABSTRACT

2-Deoxyribose aldolase-catalyzed C–C bond-forming reactions have become 1more and more important in synthesis of statins and other drug intermediates. Many methods have focused on improving the aldolase properties and harvesting new aldolases, but a good outcome depends on the efficiency of the high-throughput screening system. We have developed a visible green fluorescence probe based on a coumarin derivative, which can be reversibly modulated by a retro-aldol reaction catalyzed by 2-deoxyribose aldolase for selecting aldolase mutants with high activity. This assay system provides a convenient and effective way for high-throughput screening aldolases as the green fluorescence is sensitively detected and daylight-viewable without the need for specialist equipment. We used our probe to successfully harvest aldolase mutants with higher activities than the parent from a random mutagenesis library.

1. Introduction

The aldol reaction is one of the most important C–C bond-forming reactions used in organic synthesis. Therefore, the identification of new organic and bioorganic aldol catalysts is very important. Since Wong [1,2] reported that a one-pot tandem reaction catalyzed by 2-deoxy-D-ribose-5-phosphate aldolase (DERA) could be used to synthesize six membered lactol derivatives, aldolases have attracted the interest of researchers because of their ability to efficiently catalyze the formation of C–C bonds with stereospecific control to yield a single product [3–5]. In recent years, DERA has been used for the synthesis of the side chain of atorvastatin, providing an attractive alternative to traditional chemical methodologies [6].

Although the 2-deoxyribose aldolase-catalyzed reaction can greatly simplify the process of synthesizing the chiral side chain of statins [1], at present there are not many reliable approaches that allow screening of biocatalysts in a short time [8]. Fortunately, non-fluorogenic substrates that afford fluorescent products can be used as sensitive probes for the high-throughput screening of biocatalysts [7–9], and Greenberg reported the use of 4-methylumbelliferone to screen 2-deoxy-D-ribose from environmental DNA libraries [10]. However, this fluorescence can only be detected under ultraviolet excitation. Here we investigate the fluorescence mechanism of coumarin derivatives and describe the design of a daylight-viewable green fluorescent probe which can be seen with the naked eye, and used in the high-throughput screening of 2-deoxyribose aldolases.

2. Materials and methods

2.1. Chemistry

2.1.1. Materials

Reagents were purchased in the highest quality available from Sigma or Aldrich. All solvents used in reactions were obtained from SCRC. Solvents for extractions were distilled from technical quality. Sensitive reactions were carried out under nitrogen. ESI analyses were provided by the mass spectrometry service of the Department of Chemistry, University of Zhejiang, ^{1}H NMR spectra were recorded on Bruker Advanced 2B (400 MHz) instrument. The fluorescence was recorded on a fluorospectrophotometer (Hitachi).

2.1.2. Synthetic route

The synthetic route described below is illustrated in Fig. 1A.

Compound 1:

3-(1H-Benzo[d]imidazol-2-yl)-7-(((2R,3S)-3,5-dihydroxytetrahydrofuran-2-yl)methoxy)-6-methoxy-2H-chromen-2-one **(1):** The known methyl-5-toluenesulfonyl-2-deoxyriboside (3.0 g, 10 mmol) was dissolved in DMF (45 mL),

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http://dx.doi.org/10.1016/j.bbrc.2015.03.116
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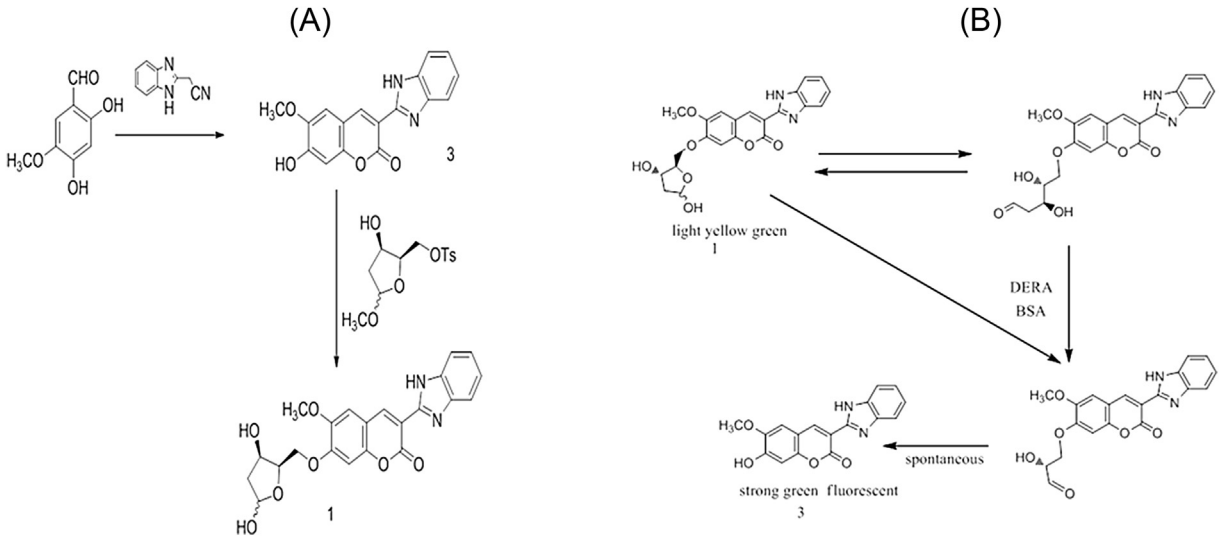

Fig. 1. (A) The chemical synthesis of deoxyriboside-substituted coumarin; (B)The retro-aldol reaction catalyzed by 2-deoxyribose aldolase.

and potassium carbonate (2.0 g, 14.5 mmol) and compound **3** (3.08 g, 10 mmol) were added. The mixture was stirred at 90 °C for 24 h). Water (100 mL) was added, and the mixture was extracted with ethyl acetate (2 × 50 mL). The organic layer was back-extracted with aqueous sodium hydroxide, dried with sodium sulfate and concentrated to give pale yellow oil. This was dissolved in acetonitrile (2.5 mL) and water (10 mL) [10], Dowex 50WX8-100 (0.24 g) was added, and the mixture was stirred at room temperature. After 2 h, the methanol was removed under vacuum and the residue was filtered, concentrated under reduced pressure, and purified by silica gel chromatography (ethyl acetate: hexane, 4:1) to give a pale yellow foam (0.817 g). ^{1}H NMR spectrum ($CDCl_3$) δ, ppm: 7.89 (1H, s, 4-H), 6.92–7.72 (6H, m, 2H coumarin (coum) residue and 4H benzimidazole (bzi) residue), 5.46 (1H, t), 4.55 (1H, m), 4.23–4.38 (2H, d), 3.58 (3H, s, OCH_3), 3.36 (3H, br.s), 1.35–1.78 (2H, m); ^{13}C NMR spectrum ($CDCl_3$) δ 161.18, 155.78, 148.08, 146.92, 146.21, 140.97, 139.10, 139.10, 128.67, 122.55, 122.55, 115.43, 115.43, 114.38, 107.90, 102.85, 95.12, 87.42, 70.91, 66.79, 57.12, 43.51. ESI-MS: *m*/*z* % 425.2 [M+H].

Compound 3:

3-(1H-Benzo[d]imidazol-2-yl)-7-hydroxy-6-methoxy-2H-chromen-2-one **(3)**: 2,4-dihydroxy-5-methoxy benzaldehyde (2.35 g, 13.8 mmol) and 2-(cyanomethyl)benzimidazole (2.0 g, 13.8 mmol) were dissolved in the minimum volume of isopropyl alcohol in a round-bottom flask at 40 °C, then 0.1 mL piperidine was added and the solution was stirred at room temperature for 16 h. After filtration, the precipitate was washed with alcohol and refluxed for 2 h in water (50 mL) containing conc. H_2SO_4 (2 mL). The precipitate formed was filtered off, dried and recrystallized from DMF to give a yellow powder (1.74 g). ^{1}H NMR spectrum (DMSO-d^6) δ, ppm: 7.92 (1H, s, 4-H), 6.82–7.81 (6H, m, 2H coumarin (coum) residue and 4H benzimidazole (bzi) residue), 3.82 (3H, s, OCH_3); ^{13}C NMR spectrum (DMSO-d^6) δ 161.91, 149.47, 146.25, 145.19, 141.53, 138.93, 138.93, 128.81, 123.09, 123.09, 115.40, 115.80, 114.33, 112.33, 110.27, 102.85, 57.23. ESI-MS: *m*/*z* % = 307.0 [M−H], 309.2 [M+H].

2.1.3. Measuring the fluorescence features of compounds 1 and 3

Compounds **1** and **3** were dissolved in 5% DMF/1.0 mL sodium phosphate (50 mM, pH 7.0) respectively, added into two quartz cuvette, the excitation and emission wavelengths were assayed (λ_{ex} and λ_{em}, respectively) on fluorospectrophotometer, and the relationship between fluorescence intensity and concentration of compound **3** was determined.

2.2. Biology

2.2.1. Materials

2-Deoxy-D-ribose-5-phosphate (DRP) and glycerol-3-phosphate dehydrogenase (GPD)/triose-phosphate isomerase (TPI) were obtained commercially from Sigma (3050 Spruce St., St. Louis, USA), and 16 × D/100 mm POROS MC20 μm columns were obtained from Applied Biosystems Co., USA, plasmid pDEST14 was used for overexpression of the protein.

2.2.2. Construction of the E. coli variant DERA$_{gth}$ library by random mutagenesis

Random mutagenesis was carried out using error-prone PCR with primers 5′-GGG GACAAGTTTGTACAAAAAAGCAGGCTTCG AAGGAGATAGAACCATGACGGT GAATATTGCTAAAATGA-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGG GTCTTAATAGTTAGCGCCG CCGG-3′, respectively. The *E. coli* BL21 (pDEST14) was used for the mutated DERA$_{gth}$ gene (cloned from *Geobacillus thermodenitrificans* ACCC 10254) expression for variant DERA enzymes.

2.2.3. Expression of mutated DERA$_{gth}$ gene in deep 96-well plates

Cells were plated on agar plates containing 100 μg/mL ampicillin, the plates were incubated at 37 °C for 24 h, and the bacterial colony was transferred into a 96-well plate (each well contained 200 μL LB medium and 100 μg/mL ampicillin). After culturing at 37 °C for 12 h, 10 μL of the pre-culture was transferred into another 96-well plate (each well contained 500 μL LB medium and 100 μg/mL ampicillin), and was incubated at 37 °C until the OD reached 0.6, 0.5 mM IPTG, then it was incubated at 18 °C for 6 h. Cells were harvested by centrifugation for 15 min, then washed with 50 mM phosphate buffer (pH 7.5) twice, centrifuged to remove the buffer and stored at −80 °C.

2.2.4. High-throughput screening of DERA$_{gth}$ mutations

The obtained cell pellets were lysed in 300 μL lysis buffer at 37 °C for 2 h. Cell debris was removed by centrifugation (5000 rpm, 4 °C, 15 min) and 50 μL cell-free extract from each well was transferred into a new 96-well shallow plate (each well contained 130 μL, 50 mM sodium phosphate buffer (pH 7.5), 10 μL bovine). Compound **1** (5 μM) dissolved in dimethylformamide (5%) was then added to each well using a biopette, the plate was incubated at 37 °C for 3 min (Fig. 1B).

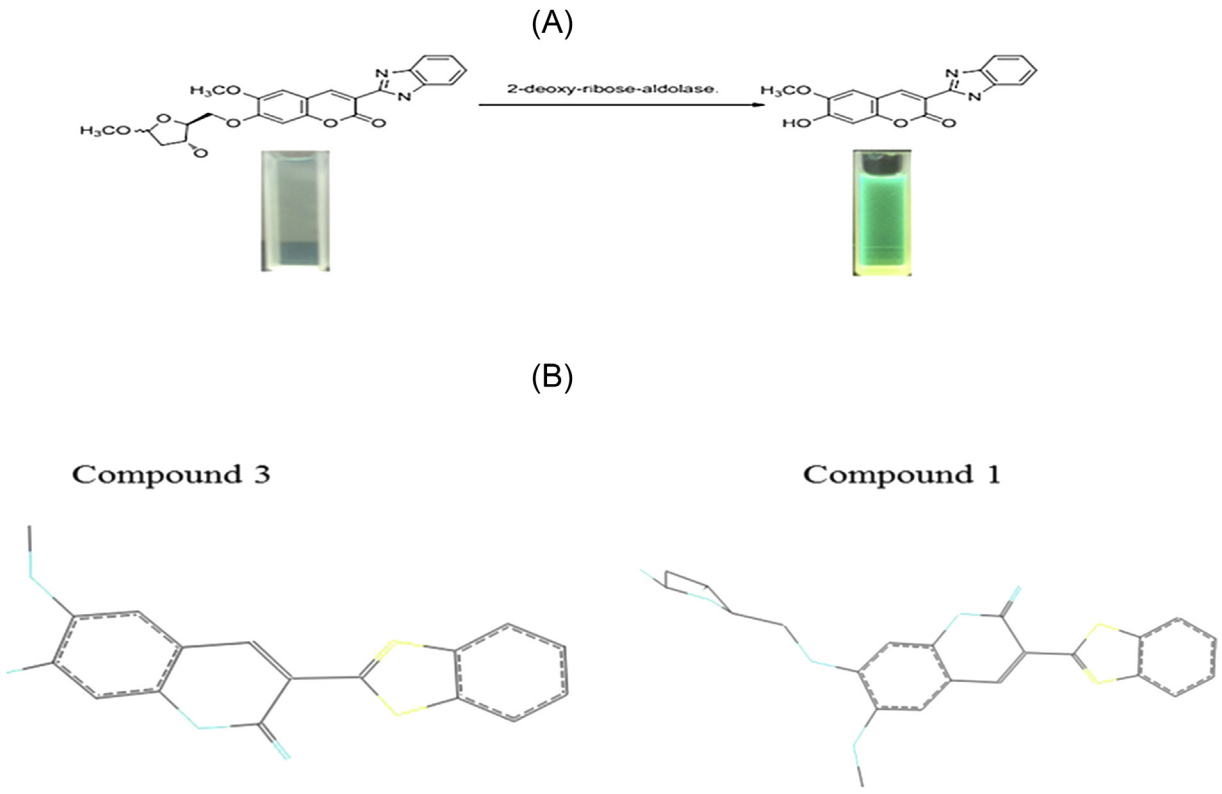

Fig. 2. (A)The fluorescence changes before and after the reaction; (B)The torsion angle between the benzoimidazole group and the coumarin ring.

2.2.5. Purification of recombinant $DERA_{gth}$ proteins

The favorable mutant cells were grown in 200 mL of LB medium and induced by adding 0.5 mM IPTG when the OD reached 0.6. Cells were harvested by centrifugation for 15 min and then washed with 50 mM sodium phosphate buffer (pH 7.5) twice.

The purification procedure was performed on the AKTA™ explorer system (Amersham Biosciences Corp., Uppsala, Sweden) with a 16 × D/100 mm POROS MC20 μm column. The cell pellet was solubilized in 50 mM NaH_2PO_4 buffer with 0.5 mM imidazole (pH 7.5), and the cells were disrupted using ultrasonication (sonicate: 3 s, interval: 7 s, 80 times) in an ice bath. After centrifugation at 4 °C and 12,000 × g for 15 min, the supernatant was filtered through a 0.45 μm filter and loaded onto the column (chelated with Ni^{2+}). Two column volumes (CVs) of 50 mM NaH_2PO_4 buffer containing 50 mM imidazole and 500 mM NaCl were used to wash out any proteins that were bound non-specifically to the column [11]. The recombinant DERA was eluted with a linear gradient of 50–500 mM imidazole in 50 mM sodium phosphate buffer (pH 7.5) containing 500 mM NaCl. The samples were dialyzed overnight against deionized water and then freeze-dried. The purified

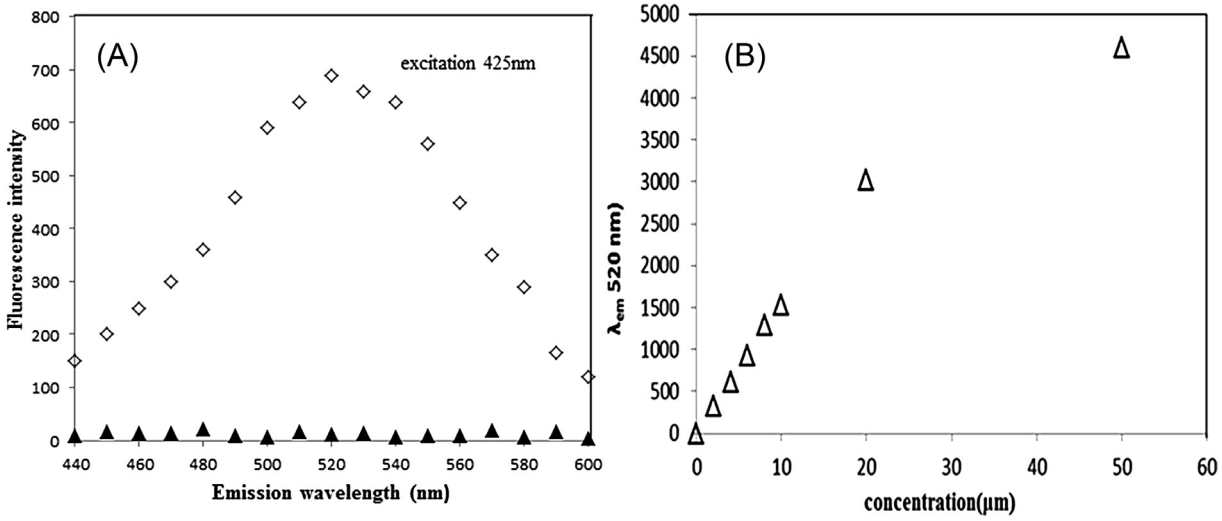

Fig. 3. (A) Selected fluorescence spectra. Conditions: compound **1** and compound **3** were dissolved in 5%DMF/sodium phosphate buffer (50 Mm, pH 7.0). Key: open square, compound **3**; solid triangle, compound **1**; (B) The relationship between fluorescence intensity and concentration of compound **3.**

Table 1
Fluorescence of Compound.1 and Compound.3 in 5% DMF/sodium phosphate buffer (50 Mm, pH 7.0), relative fluorescence intensity after background correction.

Entry	Concentration (μM)	λ_{ex} (nm)	λ_{em} (nm)	Compound.1 fluorescence intensity	Compound.3 fluorescence intensity
1	5	425	520	<10	698
2	2	425	520	<10	301
3	4	425	520	<10	548
4	6	425	520	<10	832
5	8	425	520	<10	1170
6	10	425	520	33	1405
7	20	425	520	61	2576
8	50	425	520	104	4572

recombinant protein was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the protein concentration was measured using the Bradford method [12,13].

2.2.6. Enzyme activity assay of selected mutants

The DRP cleavage activity was determined by measuring the oxidation of NADH using a coupled assay containing TPI and GPD. The mixture contained 100 mM buffer, 0.1 mM NADH, 0.4 mM DRP, 11 units of TPI (rabbit muscle), 4 units of GPD (rabbit muscle), The change in absorbance of NADH was assayed at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) [14].

3. Results and discussion

3.1. The mechanism of the fluorescent probe

Many coumarin derivatives show different fluorescence features when bearing an electron-donating group or an electron-withdrawing group on the coumarin ring. As reported, attaching an electron-donating group onto the 6- or 7-position and an electron-withdrawing group onto the 3- or 4-position of the coumarin ring causes absorption and fluorescence at longer wavelengths than compounds without any substituents [15]. Compound **3**, which emits daylight viewable green fluorescence in aqueous buffers, has a methoxy group, a hydroxy group (electron-donating), and a benzoimidazole group (electron-withdrawing) on the coumarin ring. Compared with compound **3**, where the hydroxy group is substituted by deoxyriboside group, compound **1** is not fluorescent under the same assay conditions as compound **3** (Fig. 2A). We suggest that when the deoxyriboside group is attached to the coumarin ring, this results in an increase in the torsion angle between the benzoimidazole group and coumarin ring (from 0.9° to 1.5°, calculated using SYBYL software), which makes the conjugative effect weaker or even disappear (Fig. 2B).

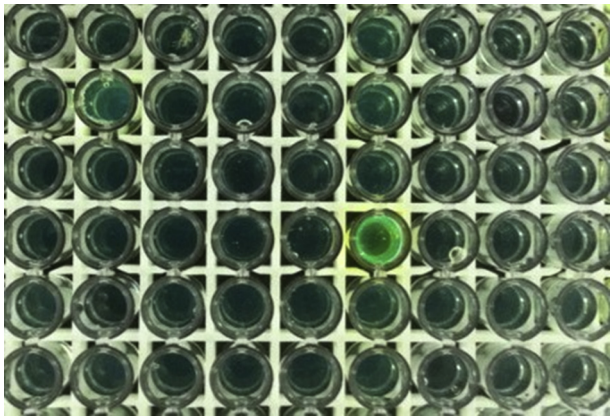

Fig. 4. The outcome of the high-throughput screen of $DERA_{gth}$ mutants, two wells emit an obviously stronger fluorescence than the others.

Table 2
The amino acid changes and specific activity (to catalyze the natural substrate) of 10 $DERA_{gth}$ mutants.

$DERA_{gth}$	Amino acid changes	Specific activity (U/mg)
w.t	/	22.5
Mutants1	T207	31.4
Mutants2	S185A	40.3
Mutants3	F159I, S209G	105.8
Mutants4	L14E	18.7
Mutants5	R189A	39.7
Mutants6	T12s	35.2
Mutants7	K125N	39.3
Mutants8	S185A, L14E	56.2
Mutants9	T157S	40.5
Mutants10	F159I	34.7

3.2. Fluorescence features of compounds 1 and 3

Selected fluorescence spectra are shown in Fig. 3A. At λ_{ex} 425 nm and λ_{em} 520 nm, compound **3** shows much higher fluorescence intensity than compound **1** which is almost non fluorescent at 2, 4, 6, 8, 10, 20, 50 μM (Table 1). There is a linear relationship between fluorescence intensity and concentration of compound **3** at low concentration (Fig. 3B).

3.3. The outcome of high-throughput screen of $DERA_{gth}$ mutations

We assayed approximately 10,000 clones (each plate has a $DERA_{gth}$ control) for improved activities with this high-throughput screening method. Fortunately some wells showed a strong green fluorescence (Fig. 4). According to the fluorescence intensity, we selected 10 mutants which had the strongest fluorescence (including on other plates), and their proteins were purified in His-tagged forms and analyzed for their ability to catalyze the natural substrate.

The DNA sequences of the mutants were confirmed by Invitrogen, and all the mutants had 1 or 2 amino acid changes. Among the 10 mutants, the most outstanding result was obtained with $DERA^{mutant\ 3}$, which gave an almost 4.7-fold increase in catalytic activity compared with parent $DERA_{gth}$ (Table 2).

We have developed a new daylight viewable fluorogenic probe for high-throughput screening of 2-deoxyribose aldolases in a short time, and found a mutant of $DERA_{gth}$ with outstanding activity. Compound **1** was essentially non-fluorescent, and compound **3** showed much stronger fluorescence than the compound **1** in aqueous solutions. The fluorescence assay system will be useful for screening 2-deoxyribose aldolases.

Acknowledgments

The research was supported by the National Basic Research Program of China (973 Program, No. 2011CB710800), the Hi-Tech

Research and Development Program of China (863 Program, 2011AA02A209), and the Zhejiang University Foundation (No. 2013QNA4034).

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.116.

References

[1] C.H. Wong, E. Garcia-Junceda, L. Chen, O. Blanco, H.J. Gijsen, D.H. Steensma, Recombinant 2-deoxyribose-5-phosphate aldolase in organic synthesis: use of sequential two-substrate and three-substrate aldol reactions, J. Am. Chem. Soc. 117 (12) (1995) 3333–3339.
[2] H.J. Gijsen, C.H. Wong, Unprecedented asymmetric aldol reactions with three aldehyde substrates catalyzed by 2-deoxyribose-5-phosphate aldolase, J. Am. Chem. Soc. 116 (18) (1994) 8422–8423.
[3] A. Bolt, A. Berry, A. Nelson, Directed evolution of aldolases for exploitation in synthetic organic chemistry, Arch. Biochem. Biophys. 474 (2008) 318–330.
[4] D.G. Gillingham, P. Stallforth, A. Adibekian, P.H. Seeberger, D. Hilvert, Chemoenzymatic synthesis of differentially protected 3-deoxysugars, Nat. Chem. 2 (2) (2010) 102–105.
[5] M.J. Walters, E.J. Toone, Pyruvate aldolases in chiral carbon–carbon bond formation, Nat. Protoc. 2 (7) (2007) 1825–1830.
[6] J.M. Patel, Biocatalytic synthesis of atorvastatin intermediates, J. Mol. Catal. B: Enzym. 61 (3) (2009) 123–128.
[7] R. Biswas, N. Maillard, J. Kofoed, J.L. Reymond, Comparing dendritic with linear esterase peptides by screening SPOT arrays for catalysis, Chem. Commun. 46 (46) (2009) 8746–8748.
[8] Hai-Ming Guo, Maki Minakawa, Fujie Tanaka, Fluorogenic imines for fluorescent detection of Mannich-type reactions of phenols in water, J. Org. Chem. 73 (10) (2008) 3964–3966.
[9] L. Jiang, E.A. Althoff, F.R. Clemente, L. Doyle, D. Röthlisberger, A. Zanghellini, D. Baker, De novo computational design of retro-aldol enzymes, science 319 (5868) (2008) 1387–1391.
[10] W.A. Greenberg, A. Varvak, S.R. Hanson, K. Wong, H. Huang, P. Chen, M.J. Burk, Development of an efficient, scalable, aldolase-catalyzed process for enantioselective synthesis of statin intermediates, Proc. Natl. Acad. Sci. U. S. A. 101 (16) (2004) 5788–5793.
[11] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (5259) (1970) 680–685.
[12] H. Fei, G. Xu, J.P. Wu, L.R. Yang, Improvement of the thermal stability and aldehyde tolerance of deoxyriboaldolase via immobilization on nano-magnet material, J. Mol. Catal. B: Enzym. 101 (2014) 87–91.
[13] S.J. Lee, H.Y. Kang, Y. Lee, High-throughput screening methods for selecting L-threonine aldolases with improved activity, J. Mol. Catal. B: Enzym. 26 (3) (2003) 265–272.
[14] H.M. Guo, F. Tanaka, A fluorogenic aldehyde bearing a 1, 2, 3-triazole moiety for monitoring the progress of aldol reactions, J. Org. Chem. 74 (6) (2009) 2417–2424.
[15] H. Sakuraba, K. Yoneda, K. Yoshihara, K. Satoh, R. Kawakami, Y. Uto, , et al.T. Ohshima, Sequential aldol condensation catalyzed by hyperthermophilic 2-deoxy-D-ribose-5-phosphate aldolase, Appl. Environ. Microbiol. 73 (22) (2007) 7427–7434.