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

Hui Fei, Gang Xu, Jian-Ping Wu*, Li-Rong Yang

Institute of Biological Engineering, Department of Chemical and Biological Engineering, Zhejiang University, 310027 Hangzhou, China

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),

* Corresponding author.
E-mail address: 10928041@zju.edu.cn (J.-P. Wu).

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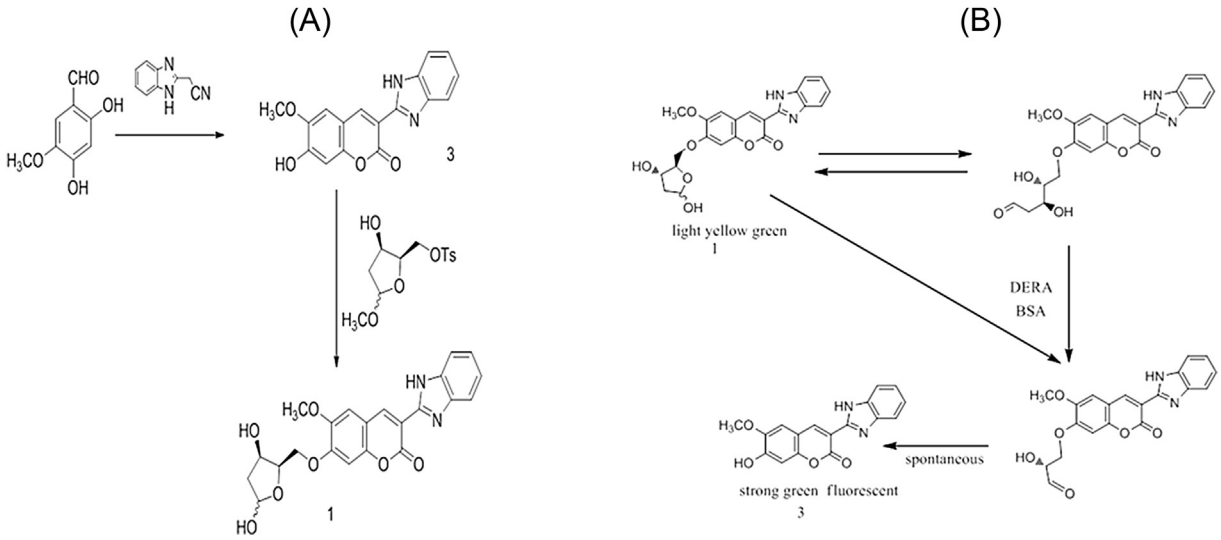

Fig. 1. (A) The chemical synthesis of deoxyriboside-substituted coumarin; (B)The retro-aldol reaction catalyzed by 2-deoxyribose aldolase.

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).