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Fluorogenic substrates for the screening assay of transketolase through beta-elimination of umbelliferone—Development, scope and limitations

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ABSTRACT

5-*O*-Coumarinyl-D-xylulose was studied as a fluorogenic substrate for the stereospecific assay of transketolase enzyme. Enzymatic C2–C3 cleavage released an α-hydroxyl, β-coumarinyl substituted aldehyde. Although the subsequent β-elimination step was rate limiting under chemical or enzymatic catalysis, we detected a TK activity as low as 0.7 mIU. To improve the fluorescence signal release, kinetic and product distribution analyses of this reaction were performed by LC/UV/MS coupling.

1. Introduction

In the course of work on the evolution of a transketolase (TK, E.C.2.2.1.1) to generate mutants able to access D-threo aldoses or L-erythro ketoses, we first focused on existing available TK assays that met the conditions such as sensitivity, selectivity and ease of handling required for the high-throughput screening (HTS) of large libraries of clones. To date, TK activity has been monitored using various spectrophotometric assays based on a TK-catalysed reaction with D-xylulose-5-phosphate (Paoletti, 1983), D-xylulose (Lee et al., 2008), D-fructose-6-phosphate (Naula et al., 2008), or β-hydroxypyruvic acid (HPA) (Hobbs et al., 1993; Hecquet et al., 1994) as donor substrates, coupled with NADH-dependent auxiliary enzymes. An HPLC assay for erythrulose product formation and HPA depletion, together with preparative-scale product isolation has also been described (Mitra et al., 1998). However, although these methods are well suited for the detection of *in vitro* TK activity from its natural substrates, they are hampered by low sensitivities or low throughput. Recently a tetrazolium red-based colorimetric assay was devised to screen for transketolase activity (Smith et al., 2006a,b). This assay offers the advantage of low cost and rapidity, but it is restricted to the non-α-hydroxylated aldehydes as acceptors for TK, which precludes its use in new HTS assays for D-threo aldoses or L-erythro ketoses. Fluorescence spectroscopy has made a great and growing contribution to the detection of many enzymes in recent years. Accordingly, we examined some new fluorogenic substrates for a stereospecific assay of wild-type or altered TK.

In the field of fluorogenic enzyme assays, to avoid the non-specific hydrolysis that occurs for acid-sensitive umbelliferyl esters, some enzyme substrates have been designed to promote an indirect fluorescence release through a β-elimination reaction (Klein and Reymond, 1998). The product of the primary enzyme reaction underwent a fast secondary β-elimination, either spontaneously, or catalysed by bovine serum albumin (BSA), which caused the fluorescent reporter molecule release. The β-elimination approach has been extensively used for various enzyme assays when the primary enzyme reaction product is directly a carbonyl compound or a precursor of a carbonyl compound.

Following this general strategy, new assays were described for lipases and esterases (Klein and Reymond, 1999; Badalassi et al., 2000; Nyfeler et al., 2003; Leroy et al., 2003) (including epoxide hydrolases EH, Badalassi et al., 2000), acylases (Badalassi et al., 2000), phosphatases (Badalassi et al., 2000; Gonzalez-Garcia et al., 2003a), proteases (Badalassi et al., 2002) and Baeyer–Villigerase (Gutiérrez et al., 2003; Sicard et al., 2005).

Enzymes catalysing stereoselective C–C bond formation were also targeted. After a first set of substrates designed to monitor aldolase-like catalytic activities (Jourdain et al., 1998; Pérez Carlòn

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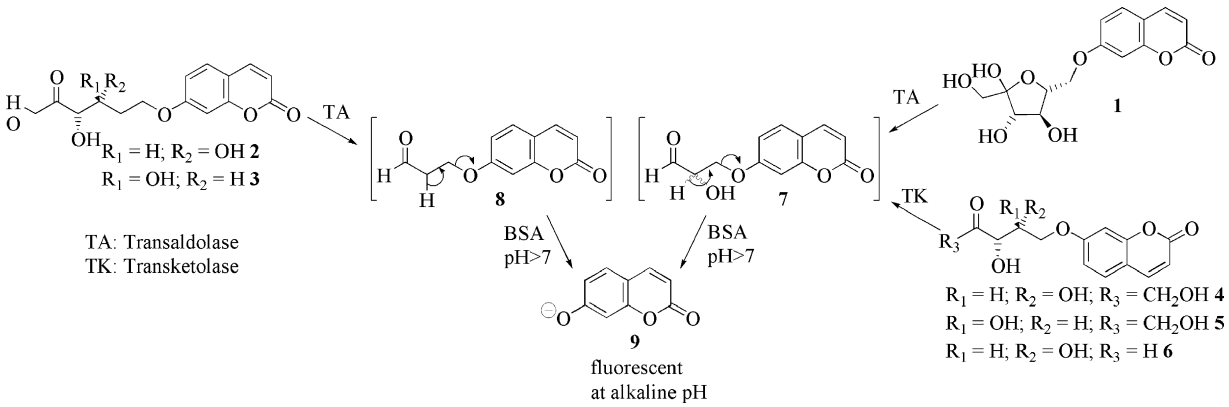

Fig. 1. Stereospecific assays for transaldolase and transketolase.

et al., 2000), the first stereospecific assay for transaldolases (TA; E.C.2.2.1.2) was described in 2003 (Gonzalez-Garcia et al., 2003b). TA was assayed through retro-aldolisation. These authors proposed the synthesis of three different potential fluorescent probes for TA; 6-*O*-coumarinyl-fructose **1**, 6-*O*-coumarinyl-5-deoxy derivative **2**, and its diastereoisomer in the C-4 position **3** (Fig. 1). As expected, on TA and BSA addition, fluorogenic probes **1** and **2** produced a fluorescence signal release, presumably through the formation of compounds **7** and **8**, respectively, which could undergo a β-elimination reaction in the presence of BSA. Compound **3** did not give any fluorescence release: TA, being highly stereoselective, was able to discriminate between a pair of diastereoisomers (**2** and **3**).

In the course of work on transketolase (TK, E.C.2.2.1.1) engineering, we designed some fluorogenic substrates for a stereospecific assay of TK by analogy with the assay proposed for TA (Fig. 1). We have already reported on the stereoselective syntheses of fluorogenic substrates **4**–**6** as probes for measuring wild-type TK activity (compound **4**; Sevestre et al., 2003) or altered TK activity (compounds **5** and **6**; Sevestre et al., 2006).

Here we report the results obtained in a study to optimise a fluorescence stereospecific assay for TK on the basis of the intrinsic reactivity of the initially synthesised fluorogenic probe **4**. Reactions were studied by LC/UV/MS analyses or fluorescence experiments. For the four different buffers tested, we determined variables such as the minimal detectable TK activity, signal-to-blank (S–B) ratios and *Z* values (Zhang et al., 1999), which showed this fluorogenic assay to be very well suited for the HTS of TK mutants.

2. Materials and methods

Chemicals and solvents were purchased from Aldrich and Acros. Q-sepharose resin was purchased from Amersham. BSA was obtained from Sigma. All ^{1}H, and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer: δ values are given in ppm and *J* values in hertz. MS and HRMS were recorded on a Micromass Q-Tof spectrometer fitted with an electrospray ionisation source. All solvents for analysis were LC/MS grade (Riedel de Haen).

2.1. Synthesis

2.1.1. Ring opening of rac-glycidaldehyde diethylacetal 13: general procedure

NaH (0.25 g; 10.5 mmol; 5 equiv.) was added to a 1 M solution of 7-hydroxycoumarin **9** (or 7-hydroxy-4-methylcoumarin **11**) in anhydrous DMF (6.3 mmol; 3 equiv.); *rac*-glycidaldehyde diethylacetal **13** (300 mg; 2.05 mmol; 1 equiv.) dissolved in DMF (5 mL) was then added. The resulting mixture was heated at 60 °C for 48 h. After extraction with AcOEt (three times 75 mL), the organic phases were washed with brine (three times 70 mL), dried with $MgSO_4$, and evaporated to dryness. The residue was purified by flash chromatography on silica gel eluted with CH/AcOEt 6/4 to 4/6.

2.1.2. 7-(3′,3′-Diethoxy-2′-hydroxypropoxy)-2H-chromen-2-one 14

^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.21 (t, 3H, *J* = 7 Hz, $CH_{3acetal}$); 1.25 (t, 3H, *J* = 7 Hz, $CH_{3acetal}$); 2.49 (d, 1H, *J* = 5 Hz, OH); 3.61 (m, 2H, $CH_{2acetal}$); 3.80 (m, 2H, $CH_{2acetal}$); 3.99 (m, 1H, $H_{2'}$); 4.11 (dd, 1H, *J* = 6, 10 Hz, $H_{1a'}$); 4.22 (dd, 1H, *J* = 3, 10 Hz, $H_{1b'}$); 4.63 (d, 1H, *J* = 6 Hz, $H_{3'}$); 6.24 (d, 1H, *J* = 9 Hz, H_{Ar}); 6.85 (s, 1H, H_{Ar}); 6.88 (dd, 1H, *J* = 2, 8 Hz, H_{Ar}); 7.36 (d, 1H, *J* = 8 Hz, H_{Ar}); 7.62 (d, 1H, *J* = 9 Hz, H_{Ar}). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.4 ($CH_{3acetal}$); 15.4 ($CH_{3acetal}$); 63.9 ($CH_{2acetal}$); 64.3 ($CH_{2acetal}$); 69.0 ($C_{1'}$); 70.5 ($C_{2'}$); 101.8 (CH_{Ar} or $C_{3'}$); 102.3 (CH_{Ar} or $C_{3'}$); 112.7 (C_{Ar}); 112.8 (CH_{Ar}); 113.3 (CH_{Ar}); 128.8 (CH_{Ar}); 143.5 (CH_{Ar}); 155.8 (C_{Ar}); 161.2 (C_{Ar}); 162.0 (C_2). HRMS: [M+H$^+$]; calculated for 309.1338; found 309.1335. Yield: 66% from **9**.

2.1.3. 7-(3′,3′-Diethoxy-2′-hydroxypropoxy)-4-methyl-2H-chromen-2-one 15

^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.18 (t, 3H, *J* = 7 Hz, $CH_{3acetal}$); 1.23 (t, 3H, *J* = 7 Hz, $CH_{3acetal}$); 2.35 (d, 3H, *J* = 1 Hz, CH_3Ar); 2.59 (s.wide, OH); 3.59 (m, 2H, $CH_{2acetal}$); 3.77 (m, 2H, $CH_{2acetal}$); 3.97 (m, 1H, $H_{2'}$); 4.08 (dd, 1H, *J* = 6, 10 Hz, $H_{1a'}$); 4.19 (dd, 1H, *J* = 3, 10 Hz, $CH_{1b'}$); 4.59 (d, 1H, *J* = 6 Hz, $H_{3'}$); 6.09 (d, 1H, *J* = 1 Hz, H_{Ar}); 6.81 (d, 1H, *J* = 2 Hz, H_{Ar}); 6.87 (dd, 1H, *J* = 2 Hz and *J* = 9 Hz, H_{Ar}); 7.45 (d, 1H, *J* = 9 Hz, H_{Ar}). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.4 ($CH_{3acetal}$); 15.4 ($CH_{3acetal}$); 18.7 (CH_3Ar); 63.9 ($CH_{2acetal}$); 64.3 ($CH_{2acetal}$); 68.9 ($C_{1'}$); 70.9 ($C_{2'}$); 101.9 (CH_{Ar} or $C_{3'}$); 102.4 (CH_{Ar} or $C_{3'}$); 112.2 (CH_{Ar}); 112.6 (CH_{Ar}); 113.9 (C_{Ar}); 125.6 (CH_{Ar}); 152.6 (C_{Ar}); 155.2 (C_{Ar}); 161.3 (C_{Ar}); 161.9 (C_2). HRMS: [M+H$^+$]; calculated for 323.1495; found 323.1489. Yield: 63% from **11**.

2.1.4. 2-Hydroxy-3-(2′-oxo-2H-chromen-7′-yloxy)-propanal 7

Dowex 50WX8H$^+$ resin (35 mg) was added to **14** (51 mg; 0.165 mmol) in 1 mL of H_2O. The mixture was heated at 40 °C overnight with stirring. After dilution with 0.5 mL of THF, the resin was discarded. The filtrate was evaporated, neutralised with Dowex 1X8 HCO_3^-, and freeze-dried to give **7** as a solid (25 mg; 0.1 mmol) in 61% yield.

HRMS: [M+H$^+$]; calculated for 253.0712 (hydrate); found 253.0679.

2.1.5. 4-Methyl-7-(2′,3′,5′-trihydroxy-4′-oxopentyloxy)-2H-chromen-2-one 10

Dowex 50WX8H$^+$ resin (55 mg) was added to **15** (80 mg; 0.25 mmol) in 1 mL of H_2O. The mixture was heated under stirring