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An Enantioselective Fluorimetric Assay for Alcohol Dehydrogenases Using Albumin-Catalyzed β-Elimination of Umbelliferone

Gérard Klein and Jean-Louis Reymond*

Department of Chemistry & Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland

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Abstract: *3-hydroxybutyl umbelliferyl ethers (R)-1 and (S)-1 are fluorogenic substrates for alcohol dehydrogenases. Their oxidation forms ketone 2, which undergoes β–elimination of umbelliferone under catalysis by bovine serum albumin, leading to a >20-fold fluorescence increase at* $\lambda_{em} = 460 \pm 20$ *nm (*$\lambda_{ex} = 360 \pm 20$ *nm). Enantioselectivity is determined in two separate tests with each enantiomeric substrate.*

An enormous variety of new catalytic activities are discovered by screening libraries of potential catalysts for a desired reaction.[1] Discovering catalysis by high throughput screening is only possible if the reaction under study can be measured simply, sensitively and reliably.[2] Fluorogenic substrates, which are themselves non-fluorescent but release a fluorescent product upon reaction, offer one of the best solutions to that problem. Typically a fluorescent phenol is released by enzymatic cleavage of the corresponding non-fluorescent ester or ether, or a bond breaking or conformational change upon reaction separates a fluorophore from a nearby quencher.[3] Herein we report a new type of fluorogenic substrate for which reaction simply modifies its chemical reactivity such that a fast secondary reaction is made possible that releases a fluorescent product. This strategy opens a range of normally non-fluorogenic reactions to direct fluorescence measurement in solution.

In conjunction with our interest in alcohol dehydrogenase catalytic antibodies,[4] we needed a simple fluorimetric assay for detecting enantioselective alcohol dehydrogenase activity. Although fluorescence and UV absorbance changes between NAD(P)$^+$ and NAD(P)H can be used to follow alcohol dehydrogenase activity,[5] we found this assay to be unpracticable in cell culture media due to the weak fluorescence and UV absorbance of these cofactors and the sensitivity of NAD(P)H to non-specific oxidation. Substituted naphthaldehydes were recently reported as fluorogenic substrates for aldehyde dehydrogenase.[6] However no fluorogenic substrates were known for alcohol dehydrogenases.

Enantioselective detection of alcohol dehydrogenase activity should be possible using the oxidation of a pair of enantiomeric alcohol to the corresponding ketone in two separate test reactions. Indeed the principle of microscopic reversibility guarantees that a catalyst's enantio- or diastereo-selectivity is identical in forward and

* e-mail: jean-louis.reymond@ioc.unibe.ch; Fax: ++41 31 631 80 57

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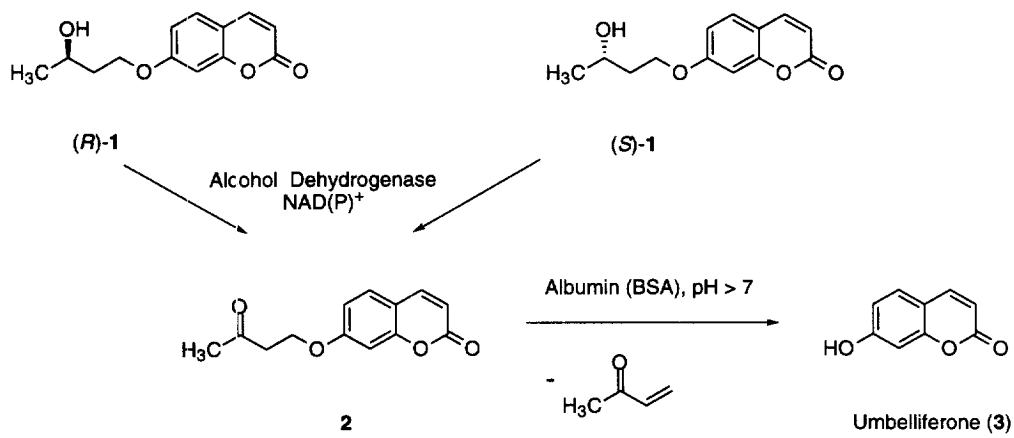
reverse directions.[7] We reasoned that the oxidation reaction could be linked to a fluorescent signal indirectly by placing an umbelliferyl ether in the β-position to the newly formed carbonyl. β-aryloxy carbonyl compounds are unstable and undergo a rapid β-elimination in basic medium.[8] Thus oxidation of umbelliferyl ether (*R*)-**1** or (*S*)-**1** by an alcohol dehydrogenase would lead to ketone **2**, which would in turn release fluorescent umbelliferone[9] (**3**) by β-elimination.



Umbelliferyl ethers (*R*)-**1** or (*S*)-**1** were prepared from (*R*)- and (*S*)-1,3-butanediol by selective tosylation of the primary alcohol[10] followed by displacement of the tosylate with the sodium salt of umbelliferone in DMF (70 %).[11] Ketone **2** was prepared by Swern oxidation of racemic **1**. Calibrations by HPLC and UV showed that **1**, **2** and **3** were completely soluble in various aqueous buffers up to 500 μM. As expected, ketone **2** decomposed in aqueous buffer to release umbelliferone **3**. However this β-elimination was relatively slow ($t_{1/2}$ = 10 h in 20 mM borate, pH 8.8) and would become rate-limiting when coupled to the dehydrogenase reaction. The reaction was accelerated by weak bases such as ethanolamine, morpholine or Tris (tris(hydroxymethyl) aminomethane), but the effect was not sufficient at concentrations compatible with an enzyme assay. To our delight, we found that β-elimination of **3** from **2** was efficiently catalyzed by bovine serum albumin, a protein present in most cell culture media. Catalysis followed Michaelis-Menten kinetics with respect to **2**, with k_{cat} = 0.0078 s^{-1}, K_M = 150 μM and k_{cat}/k_{uncat}= 390 at pH 8.8 (Figure 1). The specificity constant $(k_{cat}/K_M)/k_{uncat}$ = 2.6 x 10^6 M^{-1} was comparable with the value $(k_{cat}/K_M)/k_{uncat}$ = 3.6 x 10^6 M^{-1} reported for the BSA-catalyzed deprotonation of isoxazole at pH 9.[12] In practice, the half-life of **2** was brought down to $t_{1/2}$ = 4 min. in the presence of 2 mg/mL BSA at pH 8.8 (Figure 2).

We then turned to the coupled fluorimetric assay for the enzymatic oxidation of alcohols (*R*)-**1** and (*S*)-**1**. Each alcohol (100 μM) was incubated with each of four different dehydrogenases and the corresponding cofactor NAD^+ or $NADP^+$. A time-dependent fluorescence increase was observed for each enzyme in each of six different buffers with either (*R*)-**1** or (*S*)-**1**. Addition of 2 mg/mL BSA strongly accelerated the fluorescence increase (Figure 2). Under these conditions, the apparent rate was proportional to enzyme concentration, suggesting that β-elimination was not rate-limiting in the overall process. Indeed ketone **2** accumulated in solution in the enzymatic oxidation without BSA, but was not detectable in the presence of 2 mg/mL BSA, as shown by HPLC.

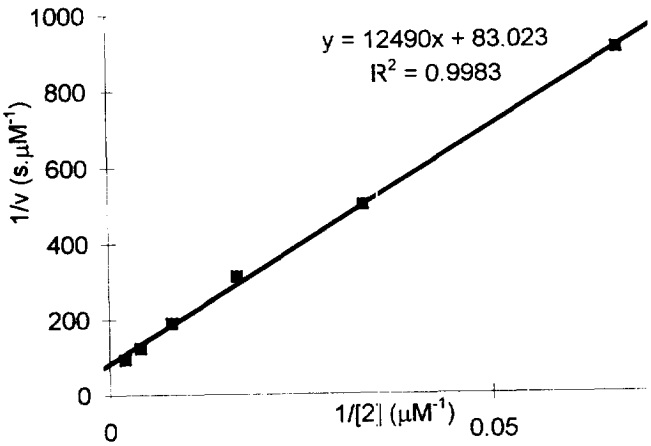


Figure 1. Double reciprocal plot for Albumin (BSA) catalyzed β-elimination of umbelliferone **3** from ketone **2**. Measured by fluorescence with 0.1 mg/mL BSA in 20 mM aqueous borate, pH 8.8.(see also legend of Table 1). Rate constants were calculated from V_{max} assuming one catalytic site per BSA molecule (M.W. 67'000).

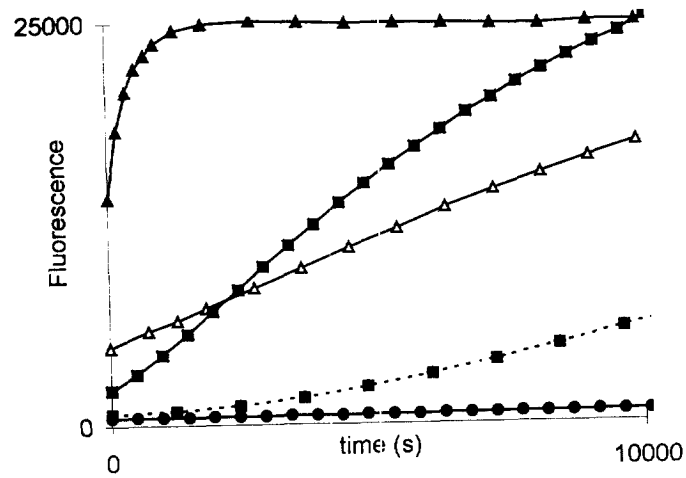


Figure 2. Fluorescence signals (arbitrary units) observed in 20 mM aq. borate, pH 8.8 using λ_{ex} = 360 ± 20 nm, λ_{em} = 460 ± 20 nm for: (△) 30 μM **2** → **3** ; (▲) 30 μM **2** → **3** with 2 mg/mL BSA; (■) 100 μM (*S*)-**1** → **3** with 10 μg/mL *Thermo anaerobium brockii* ADH (---) and with 2 mg/mL BSA added (—); (•) 100 μM (*R*)-**1** → **3** with TBADH and 2 mg/mL BSA. The fluorescence reading at t = 0 reflects fluorescence from **1**, which was completely free of **3** as assessed by HPLC.

The enantioselectivity of the four enzymes was then determined for the oxidation of (*R*)-**1** and (*S*)-**1** in the coupled assay (Table 1). Control experiments showed that there was no measurable release of umbelliferone from the substrates in the absence of either enzyme or cofactor $NAD(P)^+$. Thus even the very low reaction rates measured with the non-reactive enantiomer of **1** can be unambiguously assigned to actual oxidation, which leads to a precise measurement of enantioselectivities up to very high values. The enantiomeric excesses (*ee*, %) given are those predicted for the reduction of ketone **2**. Results are in agreement with the known reactivity of these dehydrogenases for the reduction of methyl ketones.[13] The assay was also practical in cell culture media at pH 7.4. Under these conditions, the detection limit of the assay for alcohol dehydrogenase activity was lowest for the enzyme from *Thermoanaerobium brockii* , with approximately 1.5 μg/mL. At that concentration it was still unambiguously possible to assign the enantioselectivity of the enzyme, illustrating the effectiveness of the assay for testing simultaneously catalytic activity and enantioselectivity.

Table 1. Enantioselectivity of alcohol dehydrogenases determined by oxidation of (*R*)-**1** and (*S*)-**1**.

Alcohol Dehydrogenase	μg/mL	V((*S*)-**1**), μM.s^{-1}[c]	V((*R*)-**1**), μM.s^{-1}[c]	Ratio		*ee* , %
horse liver[a]	10	2.46 x 10^{-3}	1.40 x 10^{-4}	17.6	S	89.2
Yeast[a]	100	5.99 x 10^{-4}	3.31 x 10^{-6}	181	S	98.9[d]
Lactobacillus kefir [b]	50	2.67 x 10^{-5}	7.73 x 10^{-4}	29	R	93.3
Thermoanaerobium brockii [b]	5	1.32 x 10^{-3}	6.61 x 10^{-6}	200	S	99.0[d]

Conditions: 20 mM aq. borate pH 8.8, 26 °C, 100 μM (*R*)-**1** or (*S*)-**1**, 2 mg/mL BSA, enzyme, and [a] 1 mM NAD^+ or [b] 1 mM $NADP^+$. 200 μL assays were followed in individual wells of round-bottom polypropylene 96-well plates (Costar) using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems, filters λ_{ex} = 360 ± 20 nm, λ_{em} = 460 ± 20 nm). Fluorescence was converted to umbelliferone concentration according to a calibration curve with pure **3** in the same buffer containing BSA. [c] Initial rate of release of **3**. [d] may reflect optical purity of 1,3-butanediol used for synthesis. All enzymes and reagents were purchased from Fluka.

In summary a non-fluorogenic reaction, the oxidation of a secondary alcohol, was made fluorogenic by coupling it to a secondary albumin-catalyzed β-elimination of umbelliferone. Reaction with (*R*)-**1** and (*S*)-**1** provides a simple fluorimetric assay for enantioselective alcohol dehydrogenase activity. The assay is compatible with cell culture media. The same principles should be readily applicable to a range of different chiral alcohols containing the 3-hydroxyalkyl umbelliferyl ether moiety. We are currently extending this strategy to other reactions releasing a free carbonyl group, such as the reverse aldolization[7] and the hydrolysis of enol ethers.[14]

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