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A Stereoselective Fluorogenic Assay for Aldolases: Detection of an *Anti*-Selective Aldolase Catalytic Antibody

Nathalie Jourdain, Raquel Pérez Carlón and Jean-Louis Reymond*

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

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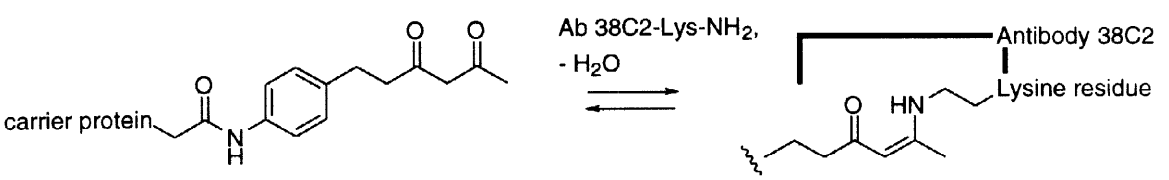
Abstract: *Stereoisomeric aldols* ***6a-d*** *undergo retroaldolization to give 3-oxopropyl umbelliferyl ether* ***5*** *and subsequently umbelliferone* ***4*** *by β-elimination, leading to a fluorescence increase at* λ_{em} *= 460 ± 20 nm (*λ_{ex} *= 360 ± 20 nm). This fluorogenic assay for aldolases operates in cell culture media and can be used to search for new stereoselective aldolase biocatalysts. Aldolase antibody 38C2 (Aldrich no. 47,995-0) catalyzes stereoselectively the retroaldolization of (S)-anti aldol* ***6c****.*

Aldolase catalytic antibodies[1] operating by an enamine mechanism can be obtained by using a primary amine as a key reactive group. We first demonstrated this principle by introducing a primary amine as a cofactor into an antibody combining site.[2] Wagner, Barbas and Lerner have reported that aldolase catalytic antibodies can be prepared using a new principle of reactive immunization with 1,3-diketone **1**, which induces a catalytic lysine side chain in the antibody binding pocket by formation of a vinylogous amide.[3] One of these catalytic antibodies, Ab 38C2, is now commercially available. Despite of the fact that diketone **1** does not contain any stereocenters, Ab 38C2 resulting from immunization against this hapten is found to be highly stereoselective for a variety of substrates.[4] Thus, assuming that there is no bias in the immune response favoring any particular stereochemistry, immunization against an achiral hapten such as **1** might produce highly stereoselective aldolase antibodies with all possible stereoselectivities. Here we report an assay that allows to detect such stereoselective aldolase activities by fluorescence in solution and should facilitate the discovery of aldolase biocatalysts. Using this assay, aldolase antibody 38C2 is found to display an interesting *anti*–diastereoselectivity.



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

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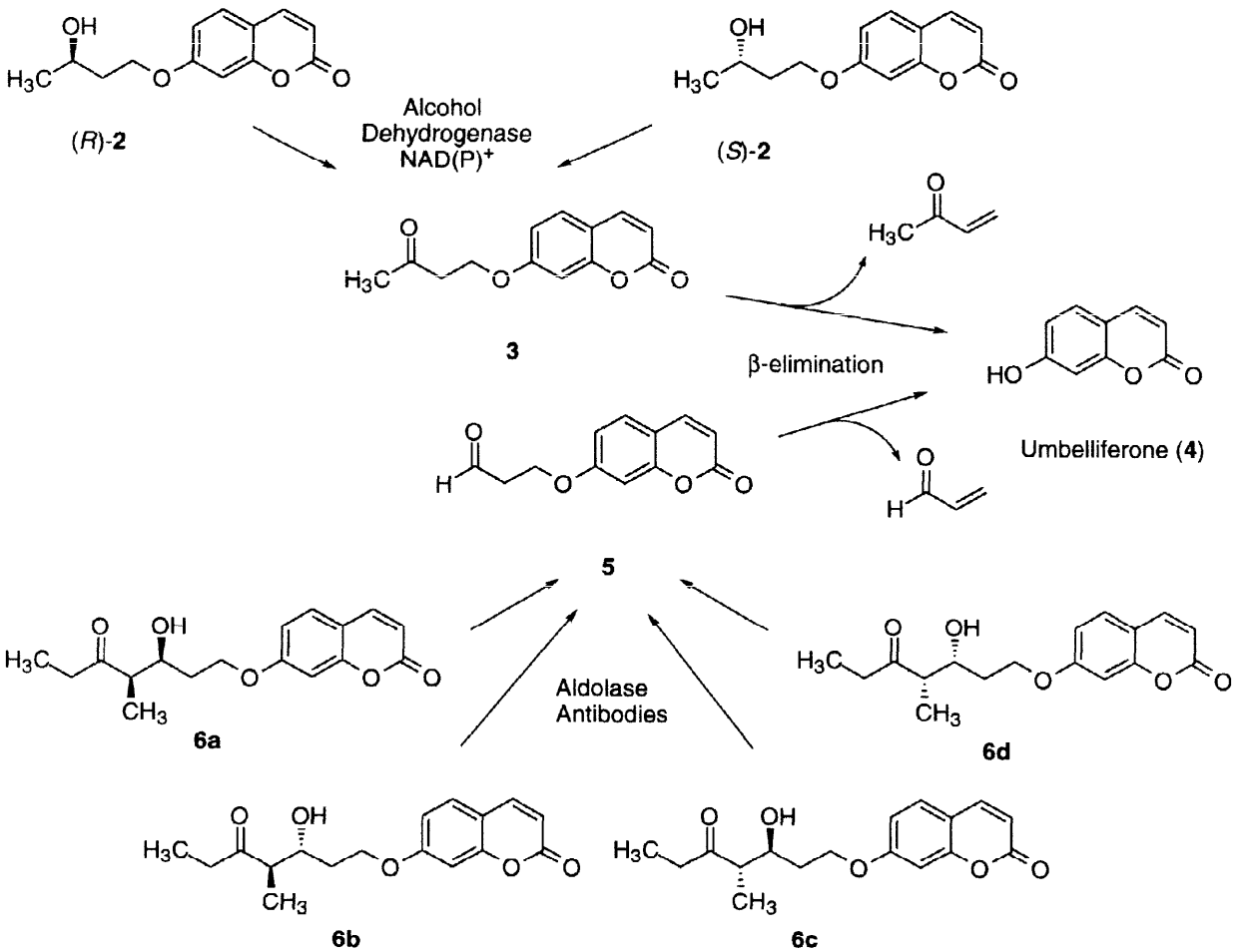
We recently reported an enantioselective fluorogenic assay for alcohol dehydrogenases by using enantiomeric alcohols (*R*)-**2** and (*S*)-**2**.[5] Ketone **3** resulting from their oxidation is unstable and rapidly releases strongly fluorescent umbelliferone **4** by β-elimination. The same principle should allow to design a stereoselective fluorogenic assay for aldolases by measuring the release of aldehyde **5** by retroaldolization. Indeed the aldol reaction is reversible, and aldolase antibodies have identical stereoselectivities for both aldol addition and retroaldolization.[2c] Due to the importance of polypropionate fragments in natural products,[6] we decided to prepare stereoisomeric aldols **6a-d** as fluorogenic substrates.



Aldehyde **5** was prepared from umbelliferone **4** by alkylation with 3-bromo-1-propanol (NaH, DMF, 60°C, 12h, 88 %) followed by oxidation with Dess-Martin periodinane (CH_2Cl_2, 20°C, 10 min, 60 %). The aldehyde was unstable towards β-elimination of umbelliferone but could be purified partially by a rapid flash chromatography. $SnCl_4$-promoted reaction of aldehyde **5** with the TMS enol ether of 3-pentanone gave aldols **6a-d** as a 5 to 1 mixture of *syn*- and *anti*- stereoisomers (-78 °C, THF, 2h, 20 %). Yields were moderate due to competing β-elimination of umbelliferone **4**. Aldols **6a-d** were sensitive on silicagel, but were purified by preparative reverse-phase HPLC (C_{18}-silica, water-acetonitrile gradient). Finally enantiomers were separated using a chiral HPLC column.[7] Stereochemistry was assigned by comparison with literature data of ^{13}C-NMR spectra[8] and optical rotation.[9]

We then turned to fluorescence assays using aldols **6a-d**. The pure stereoisomers were incubated at a concentration of 100 μM either in borate buffer at pH 8.8, in phosphate buffer at pH 7.4 or in cell culture media. The effect of adding BSA (bovine serum albumin) was first tested since we have found it to be an important additive in our assays for catalyzing β-elimination of umbelliferone. This β-elimination,[10] like related deprotonation processes,[11] can be catalyzed by BSA as well as by a number of weak bases and buffers. Adding 2 mg/mL of BSA to substrates **6a-d** resulted in a small time dependent fluorescence increase relative to our reference buffer. The small interference of BSA with our retroaldolization assay is probably due to enamine reactivity of some of its 30 surface lysine residues. Indeed, by contrast with the reactions mentioned above, bimolecular aldolizations and retro-aldolizations in aqueous medium are usually not general acid-base catalyzed and their catalysis follows specific mechanisms such as those found in aldolase enzymes, which include enamine and metal-promoted enolization.[12]

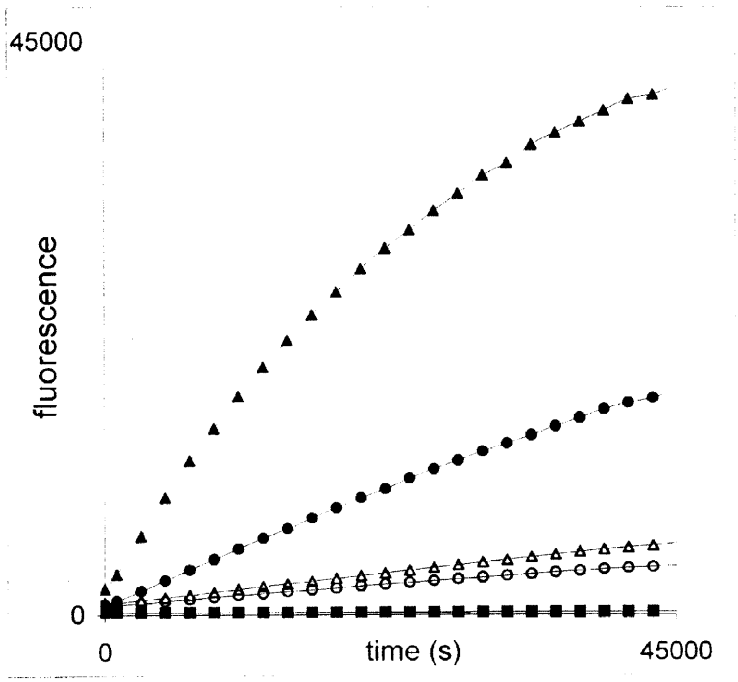


Figure 1. Fluorescence signals (arbitrary units) observed in 20 mM aq. borate, pH 8.8, 2 mg/mL BSA, 1 mg/mL Ab 38C2, using λ_{ex} = 360 ± 20 nm, λ_{em} = 460 ± 20 nm for: (▲) 100 μM **6c** → **4**; (•) 100 μM **6a** → **4**; (△) 100 μM **6b** → **4**; (o) 100 μM **6d** → **4**; (■) control without Ab 38C2: 100 μM **6c** → **4**. The fluorescence reading at t = 0 reflects fluorescence from **6a-d**, which were completely free of **4** as assessed by HPLC.

Table 1. Initial rate of retroaldolization of aldols **6a-d** under catalysis by aldolase antibody 38C2.

Conditions[a]	V_{app} (**6a**) ± 3 x 10^{-5} μM.min^{-1}	V_{app}(**6b**)	V_{app}(**6c**)	V_{app}(**6d**)
A: 20 mM borate, pH 8.8	1.2	3.0	0.4	6.0
B: A + 2 mg/mL BSA	1.2	5.0	1.2	30
C: B + 1 mg/mL Ab 38C2	2440	500	10'400	360

[a] 200 μL assays were run in round-bottom polypropylene 96-well plates (Costar) using a Cytofluor II Plate Reader (Perseptive Biosystems). Fluorescence was converted to umbelliferone concentration according to a calibration curve. Stereoselectivities were calculated from apparent rates (V_{app}) as follows: (*S*)-aldols: (**6a+6c-6b-6d**)/(**6a+6b+6c+6d**) = 87.5 % *ee*, *anti*-aldols: (**6b+6c-6a-6d**)/(**6a+6b+6c+6d**) = 59.1 % *de*.

Aldolase catalytic antibody 38C2 was incubated with substrates **6a-d** in the presence of BSA at pH 8.8. A strong time dependent fluorescence increase was observed (Figure 1 and Table 1). The (*S*)-enantioselectivity for chirality of the secondary alcohol (87.5 % *ee*) is consistent with previous observations with this antibody. More remarkably, the antibody favored (*S*)-*anti*-aldol **6c**. The overall *anti*-diastereoselectivity (59.1 % *de*) for a simple polypropionate fragment is noteworthy since *anti*-aldols are usually difficult to obtain. Similar results were obtained when the reactions were conducted at pH 7.4 or in cell culture media, confirming that our assay was suitable for use in direct activity screening of catalytic antibodies.

In conclusion we have shown that aldols **6a-d** are fluorogenic substrates that can be used to assay the stereoselectivity of aldolases by fluorescence. In contrast to aromatic aldehydes which are often biased in both reactivity and stereoselectivity, the reaction used in our assay involves an aliphatic aldehyde. Therefore there is a good chance that a stereoselective catalyst discovered by this assay will conserve its reactivity and stereoselectivity in reactions with other polypropionate fragments. Antibody 38C2 was shown to display (*S*)-enantioselectivity as well as *anti*-aldol diastereoselectivity, in favor of aldol **6c**. The observation that achiral hapten **1** induces an enantioselective catalytic antibody favoring a normally disfavored *anti*-selective aldolization is encouraging for developing *anti*-selective aldolase catalytic antibodies with even higher stereoselectivities.

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[7] Chiralpak AS (Daicel), 0.45 x 22 cm, 1.0 mL.min^{-1} hexane-isopropanol 1:1; t_R(**6a**) = 16.0 min, t_R(**6b**) = 19.4 min, t_R(**6c**) = 27.3 min, t_R(**6d**) = 35.2 min. *syn*-aldols (**6a+6d**): MS (EI): 304 (M^+). ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.62 (d, *J*=9.4 Hz, 1H), 7.36 (d, *J*=8.5 Hz, 1H), 6.83 (dd, *J*=8.5 and 2.2 Hz, 1H), 6.82 (d, *J*=2.2 Hz, 1H), 6.23 (d, *J*=9.4 Hz, 1H), 4.21 (m, 3H), 2.69-2.43 (m, 4H), 1.87 (m, 2H), 1.20 (d, *J*=7.3 Hz, 3H), 1.09 (t, *J*=7.3 Hz, 3H).^{13}C-NMR ($CDCl_3$, 50 MHz): 216.2, 162.0, 161.1, 155.8, 143.3, 128.7, 113.0, 112.6, 112.5, 101.6, 67.9, 65.6, 49.9, 35.0, 33.3, 10.2, 7.49. *anti*-aldols (**6b+6c**): ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.63 (d, *J*=9.6 Hz, 1H), 7.37 (d, *J*=9.6 Hz, 1H), 6.85 (m, 2H), 6.26 (d, *J*=9.5 Hz, 1H), 4.22 (m, 2H), 3.99 (m, 1H), 2.77-2.43 (m, 3H), 2.05 (m, 1H), 1.88 (m, 1H), 1.70 (bs, 1H), 1.21 (d, *J*=7.0 Hz, 3H), 1.08 (t, *J*=7.1 Hz, 3H).^{13}C-NMR ($CDCl_3$, 50 MHz): 216.6, 162.2, 161.3, 156.1, 143.5, 129.0, 113.4, 112.9, 112.9, 101.9, 71.0, 65.8, 51.2, 36.1, 34.1, 14.4, 7.73.
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