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Visualizing Antibody-Catalyzed Retro-Aldol-Retro-Michael Reactions

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Abstract—We developed a visible detection system for antibody-catalyzed retro-aldol-retro-Michael reactions. Aldolase antibody 38C2 catalyzed the reaction of substrate **1** to provide 6-bromo-2-napthol that forms a visible colored azo dye with diazonium salts. This system has potential for the screening of novel catalysts.

Antibody-catalyzed prodrug activation has considerable potential as a therapeutic approach towards cancer. Recently we described a generic drug masking/activation strategy based on a tandem retro-aldol-retro-Michael reaction catalyzed by the broad scope antibody 38C2.[1a] This approach has now been validated in an animal model of cancer.[1b] Antibody 38C2 and related catalysts have been obtained by immunization with 1,3-diketone containing haptens designed to covalently trap catalysts possessing an enamine/imine based catalytic mechanism.[2,3] Although in vitro selection strategies using 1,3-diketones can be used to access aldolase catalysts,[3b] direct selection of catalysts based on the retro-aldol-retro-Michael reaction might result in improved catalysts for prodrug activation since catalysis of both reactions would be accounted for in the screen. Here we report a visible detection system for the screening of catalysts that catalyze retro-aldol-retro-Michael reactions. Visual detection of catalytic activity is an efficient approach for catalysts screening.[4] While UV-observable and fluorogenic substrates for the retro-aldol reaction and retro-aldol-retro-Michael reactions have been reported[3,5] and these substrates are useful for catalytic assays in solution with spectrophotometers, substrates for the direct visual detection of catalytic activity based on retro-aldol-retro-Michael reaction have not been reported.

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Substrate **1** was designed to detect catalysis of tandem retro-aldol-retro-Michael reactions. Since napthol derivatives are utilized for the detection of alkaline phosphatase and β-galactosidase enzyme activity with diazonium salts in agar plate assays with bacteria and in the immunoblots on membranes,[6] we took advantage of this napthol-based strategy to address the problem of detecting retro-aldol-retro-Michael catalysis.

In our approach, retro-aldol reaction of **1** generates **2** that is a substrate for a retro-Michael reaction yielding 6-bromo-2-naphthol (**3**). Compound **3** can form a visible azo dye precipitate following reaction with diazonium salts in aqueous media (Scheme 1).

The synthesis of substrate **1** is shown in Scheme 2. Alkylation of the phenolic group of **3** with 4-bromo-1-butene followed by oxidation of the olefin provided ketone **2.** Further elaboration by aldol reaction of **2** with an acetone enolate provided **1**.[7]

To detect **3**, a set of commercially available diazonium salts[8] was tested in PBS (phosphate buffered saline, pH 7.4) since the product azo dye color and the background color from the decomposition of diazonium salts are not systematically reported. Some diazonium salts give significant colored precipitates in PBS in the absence of **3**. For the detection system, it is desired that the product color is different from the color generated from the diazonium salt itself. Five diazonium salts were found to be suitable for the detection of **3** and the product azo dye colors observed with these diazonium salts are shown in

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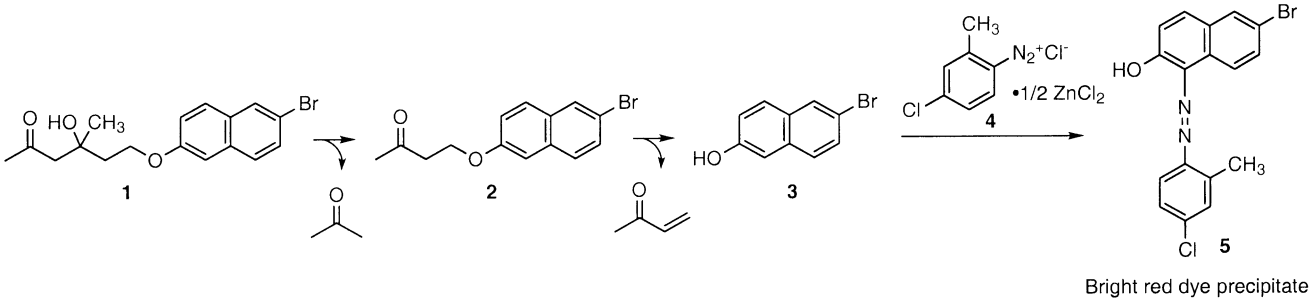
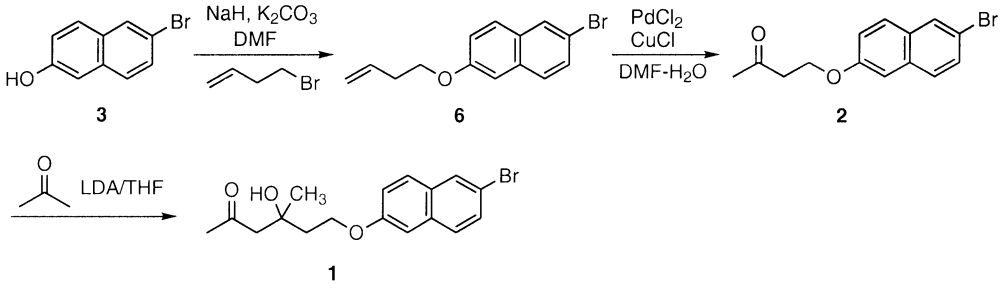


Scheme 1.



Scheme 2.

Table 1. The best results were observed when Fast Red TR salt (**4**) was used: a bright red colored azo coupling product was generated. Therefore, **4** was employed in the further experiments.

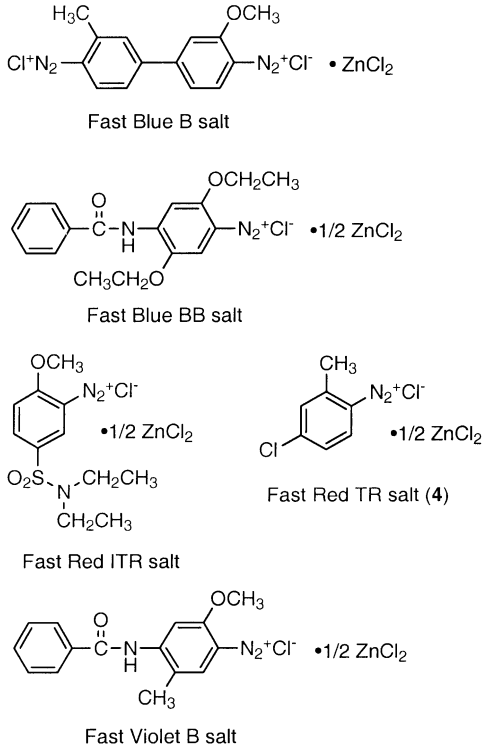
The detection limit of **3** was determined using typical small scale of antibody assay conditions. To 60 μL of a solution of **3** (50, 25, 10, 5, 2.5, 1.25, 0.5, and 0 μM) in 0.5% DMSO/PBS (pH 7.4) at room temperature was added 3 μL of a solution of **4** (10 mg/mL) in PBS to give **5**. Concentrations of 1.25 μM or greater of **3** (75 pmol in 60 μL) were visually detected 10 min after addition of **4** in this experiment.[9] Higher concentrations of **3** gave stronger coloration with **4**. When hybridoma medium (RPMI1640 without phenol red, GIBCO) was used instead of PBS in this experiment, concentrations of 5 μM or greater of **3** were detected visually. Addition of higher concentrations of **4** in these experiments did not change the detection limit.

This detection system was examined in the aldolase antibody 38C2-catalyzed reaction. Reactions were initiated by adding 2.5 μL of substrate **1** (10 mM in DMSO) to 47.5 μL of antibody 38C2 in PBS at 24 °C: The reaction mixtures contained **1** (500 μM) and antibody 38C2 (active site concentration 20, 10, 5, 2.5, 1.25, and 0 μM) in 5% DMSO/PBS (pH 7.4) in the total volume of 50 μL. After 3.5 h, 5 μL of **4** (10 mg/mL in PBS) was added. Reaction mixtures containing 5 μM or more (active sites) of antibody 38C2 gave a visible red precipitate. Higher concentrations of 38C2 gave stronger coloration. In addition, the amount of time required for visible detection was studied. Antibody 38C2-catalyzed reactions were performed in the following conditions:

Table 1. The color of azo coupling product with 6-bromo 2-naphthol (**3**)[a]

Diazonium salt	Color
Fast Blue B salt	Dark violet
Fast Blue BB salt	Violet
Fast Red ITR salt	Bright red
Fast Red TR salt (**4**)	Bright red
Fast Violet B salt	Violet

[a]The color was observed in PBS (pH 7.4).



[antibody 38C2] 10 μM (active site) and [**1**] 100 μM in 1% DMSO/PBS (pH 7.4) (total volume 50 μL). Six identical reactions were used to study the time course of the reaction. After 2 min, 30 min, 1, 3, 5, and 7 h at 24 °C, **4** (10 mg/mL in PBS, 5 μL) was added into the separate tube. With reaction times of 2 and 30 min no visible red color was observed, while reaction times of 1 h or longer provided a red colored precipitate that was detectable by eye. Longer reaction times gave stronger coloration.

The applicability of substrate **1** to screen growing hybridoma cultures was also examined. Antibody 38C2 hybridoma cell[10] culture (1 mL) in hybridoma medium was mixed with **1** (1 mM in 10% DMSO/PBS, 50 μL)[11] (final concentration: [**1**] 50 μM, 0.5% DMSO/hybridoma medium). The cell culture was incubated at 37 °C in a CO_2 incubator for 17 h and then **4** (10 mg/mL in PBS, 50 μL) was added. As a control, hybridoma cells producing a non-catalytic monoclonal antibody binding to a phosphonate hapten were used in the same experiment. The 38C2 hybridoma cell culture was colored red compared to the control. The color difference between 38C2 and the control was more clearly recognized when the culture was centrifuged in a clear tube. Since higher concentrations of substrate **1** were toxic to the cells, 50 μM of **1** provided optimal results in terms of both detection and cell viability.

In summary we have developed an assay that allows for visual screening of antibody-catalyzed retro-aldol-retro-Michael reactions and demonstrated the usefulness of the visible detection system in an antibody 38C2-catalyzed reaction. Since this visible detection system has utility in agar plate screening assays with bacteria as well as in hybridoma screening, this system is promising for the identification of novel or improved prodrug activation catalysts based on retro-aldol-retro-Michael reactions.

Acknowledgements

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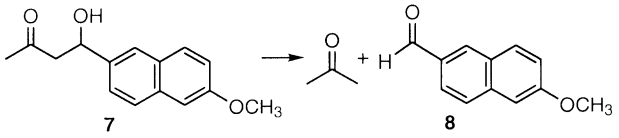
7. **Compound 6**. To a solution of 6-bromo-2-napthol (2.2316 g, 10.0 mmol) in DMF (20 mL) at 0 °C, NaH (60% in mineral oil) (445.3 mg, 11.1 mmol) was added in several portions. After 5 min, the mixture was warmed to room temperature and 4-bromo-1-butene (1.2 mL, 11.8 mmol) was added. After 7 h, K_2CO_3 (842 mg, 6.09 mmol) and 4-bromo-1-butene (0.6 mL, 5.9 mmol) was added. After 5 days, the mixture was added to saturated aq NH_4Cl and extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, evaporated, and flash chromatographed (EtOAc/hexane, 1:20) to give **6** (836.4 mg, 30%). ^{1}H NMR ($CDCl_3$) δ 7.90 (d, J = 1.9 Hz, 1H), 7.63 (d, J = 9.0 Hz, 1H), 7.58 (d, J = 8.7 Hz, 1H), 7.48 (dd, J = 8.7 Hz, 1.9 Hz, 1H), 7.15 (dd, J = 9.0 Hz, 2.4 Hz, 1H), 7.08 (d, J = 2.4 Hz, 1H), 5.94 (m, 1H), 5.20 (dd, J = 17.2 Hz, 1.4 Hz, 1H), 5.13 (d, J = 10.2 Hz, 1H), 4.11 (t, J = 6.7 Hz, 2H), 2.60 (q, J = 6.7 Hz, 2H).

Compound 2. A mixture of CuCl (450.6 mg, 4.55 mmol) and $PdCl_2$ (63.2 mg, 0.356 mmol) in water (0.5 mL) was stirred at room temperature under O_2. After 20 min, a solution of **6** (836.4 mg, 3.02 mmol) in DMF (5.0 mL) was added and the mixture was stirred for 19 h. After Celite filtration, the filtrate was added to 0.2 N HCl and extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, evaporated, and flash chromatographed (EtOAc/hexane, 1:5) to give **2** (674.7 mg, 76%). ^{1}H NMR ($CDCl_3$) δ 7.89 (d, J = 1.9 Hz, 1H), 7.63–7.56 (m, 2H), 7.48 (dd, J = 8.7 Hz, 1.9 Hz, 1H), 7.13–7.09 (m, 2H), 4.31 (t, J = 6.3 Hz, 2H), 2.96 (t, J = 6.3 Hz, 2H), 2.25 (s, 3H). FABMS: m/z 293, 295 (MH^+), 315, 317 (MNa^+). HR-FABMS: calcd for $C_{14}H_{13}O_2BrNa$ (MNa^+) 314.9997, found 314.9994.

Substrate 1. To a solution of LDA at −78 °C (2 M in heptane/THF/ethylbenzene) (3.10 mL, 6.20 mmol) was added acetone (416 μL, 5.67 mmol). After 30 min, a solution of **2** (553.7 mg, 1.89 mmol) in THF (5.0 mL) was added and the solution was stirred for 1.5 h. The mixture was added to a mixture of ice, saturated aq NH_4Cl, and 10% citric acid, and extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, evaporated, and flash chromatographed (EtOAc/hexane, 1:2) to give **1** (534.4 mg, 81%). ^{1}H NMR ($CDCl_3$) δ 7.91 (d, J = 1.9 Hz, 1H), 7.65–7.57 (m, 2H), 7.49 (dd, J = 8.8 Hz, 1.9 Hz, 1H), 7.13–7.10 (m, 2H), 4.30–4.14 (m, 2H), 4.14 (s, 1H), 2.83 (d, J = 17.4 Hz, 1H), 2.68 (d, J = 17.4 Hz, 1H), 2.20 (s, 3H), 2.09 (t, J = 6.2 Hz, 2H), 1.32 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 210.7, 156.8, 133.0, 130.0, 129.6 (x2), 128.5, 128.4, 119.7, 117.1, 106.7, 70.9, 64.3, 52.5, 40.4, 31.8, 27.4. FABMS: m/z 351, 353 (MH^+), 373, 375 (MNa^+). HR-FABMS: calcd for $C_{17}H_{19}O_3BrNa$ (MNa^+) 373.0415, found 373.0427.
8. The following diazonium salts were tested: Fast Black K salt, Fast Blue B salt, Fast Blue BB salt, Fast Blue RR salt, Fast Garnet GBC salt, Fast Blue RR salt, Fast Garnet GBC salt, Fast Red AL salt, Fast Red B salt, Fast Red ITR salt, Fast Red PDC salt, Fast Red TR salt, Fast Red Violet LB salt, Fast Violet B salt.

9. The detection limit of **3** was compared with that of the aldehyde product of fluorogenic substrate **7**.[5b] A solution of 6-methoxy-2-naphthaldehyde (**8**) (50, 25, 10, 5, 2.5, 1.25, 0.5, and 0 μM) in 0.5% CH_3CN/PBS (pH 7.4) (60 μL) in microtiter plate and in clear tube was examined with a standard long wavelength UV lamp (365 nm). Concentrations of 2.5 μM or greater of **8** were visually detected in the dark and concentrations of 5 μM or greater of **8** were visually detected in a typical bright room. A lower concentration of **8** can be detected with a spectrometer.



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10. The hybridoma cell line producing monoclonal antibody 38C2 was originally prepared by the fusion of mouse spleen cells with X63/Ag8.653 myeloma cells. See ref 2.

11. Predilution of substrate **1** into PBS is essential in experiments using living hybridoma cells since they are sensitive to high concentrations of DMSO. Direct addition of a stock solution of **1** in DMSO into the cell culture significantly reduced the viability of the hybridoma cells.