Antibody-Catalyzed Benzoin Oxidation as a Mechanistic Probe for Nucleophilic Catalysis by an Active Site Lysine

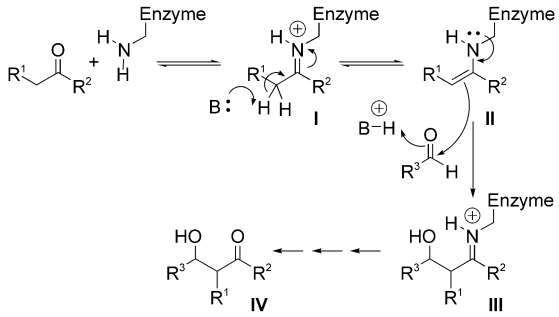
Genia Sklute,[a] Rachel Oizerowich,[a] Hagit Shulman,*[a] and Ehud Keinan*[a, b]

Abstract: Aldolase antibody 24H6, which was obtained by reactive immunization against a 1,3-diketone hapten, is shown to catalyze additional reactions, including H/D exchange and oxidation reactions. Comparison of the H/D exchange reaction at the α-position of a wide range of aldehydes and ketones by 24H6 and by other aldolase antibodies, such as 38C2, pointed at the significantly larger size of the 24H6 active site. This property allowed for the catalysis of the oxidation of substituted benzoins to benzils by potassium ferricyanide. This reaction was used as a mechanistic probe to learn about the initial steps of the 24H6-catalyzed aldol condensation reaction. The Hammett correlation ($\rho = 4.7$) of $\log(k_{cat})$ versus the substituent constant, σ, revealed that the reaction involves rapid formation of a Schiff base intermediate from the ketone and an active site lysine residue. The rate-limiting step in this oxidation reaction is the conversion of the Schiff base to an enamine intermediate. In addition, linear correlation ($\rho = 3.13$) was found between $\log(K_M)$ and σ, indicating that electronic rather than steric factors are dominant in the antibody–substrate binding phenomenon and confirming that the reversible formation of a Schiff base intermediate comprises part of the substrate-binding mechanism.

Keywords: aldol reaction · catalytic antibodies · reaction mechanisms · Schiff bases

Introduction

An active site nucleophilic lysine residue with a highly perturbed pK_a is an essential element of the catalytic machinery available to both the natural, type I aldolase enzymes[1] and the aldolase antibodies that were elicited by reactive immunization with 1,3-diketones.[2] These chemically programmed antibodies, which include 33F12, 38C2,[2,3] 24H6,[4] 93F3, and 84G3,[5] were shown to catalyze aldol reactions with broad substrate scope, remarkable enantioselectivity, and high reaction rates. The crystal structure of 33F12 indeed revealed a lysine residue that is buried in a hydrophobic pocket.[6] This lysine residue can catalyze the aldol reaction by converting the carbonyl donor (ketone or aldehyde) into a protonated Schiff base (**I**; Scheme 1), which can then tautomerize to an enamine intermediate (**II**). The latter electron-rich



Scheme 1. Pathway for the antibody-catalyzed aldol reaction. B = base.

species may participate in variety of carbonyl transformations, such as aldol and retroaldol reactions, alkylation, deuteration, decarboxylation, and so forth. The aldol reaction, for example, involves nucleophilic addition of **II** to a carbonyl acceptor to produce a new Schiff base intermediate (**III**) and, following hydrolysis, an aldol product (**IV**).

Beyond the synthetic significance of catalyzing asymmetric carbonyl transformations by new biocatalysts, it is important to understand the details of their catalytic machinery,

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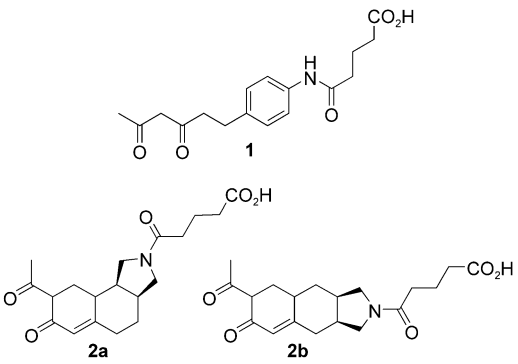
particularly in comparison with the naturally occurring counterparts. Mechanistic information and identification of enzyme–substrate intermediates could be achieved by using various reactions as mechanistic probes. For example, linear free-energy relationship studies,[7] using substituted aromatic substrates, could teach us about charge development in the transition state and point at rate-limiting steps. We have recently carried out such Hammett correlation studies of the 38C2- and 24H6-catalyzed aldol and retroaldol reactions,[4,8] taking advantage of the broad substrate scope of these biocatalysts. We found that although the two antibodies exhibit broad substrate specificities, they utilize slightly different mechanisms. While antibody 38C2 adopts a mechanism that is reminiscent of an acid-catalyzed aldol reaction, antibody 24H6 uses a mechanism that is similar to the base-catalyzed reaction.

In those studies,[4,8] we used acetone with a series of substituted cinnamaldehydes to investigate the substituent effect on the acceptor partner of the aldol/retroaldol reaction. To study the aldol reaction, we employed acetone in large excess, rendering the kinetics pseudo-first order. The use of one of the reactants in large excess was essential not only for the simplification of the kinetics, but, more importantly, for pushing the highly reversible aldol reaction towards the products. A complementary study with a series of substituted donor substrates is highly desirable. Unfortunately, the reversibility of the aldol/retroaldol reactions would require the employment of the acceptor aldehyde in large excess, which would result in total inhibition of the catalyst by quantitatively converting its active-site lysine residue into a Schiff base. In addition, the aldolase antibodies, 38C2, 33F12, 93F3, and 84G3 elicited against small haptens, such as **1**, although quite indiscriminate with respect to the acceptor molecules, do not accept large donors, such as substituted acetophenones, which are required for Hammett studies.

We anticipated that the donor size problem could be solved with antibody 24H6, which was induced by the large dicarbonyl haptens, **2a** and **2b**. To address the problem of inhibition by excess acceptor, we searched for another reaction that shares the first mechanistic steps with the aldol reaction, but that continues irreversibly to products.



The notion of using another reaction as a mechanistic probe for the first steps of the biocatalyzed aldol reaction has been demonstrated by Christen,[9] who investigated the rabbit muscle aldolase-catalyzed oxidation of α-hydroxycarbonyls to α-dicarbonyls. The aldolase-catalyzed oxidation of various substrates, including dihydroxyacetone phosphate, fructose-1-phosphate, and fructose-1,6-diphosphate, with tetranitromethane as well as with other oxidants testified to the formation of Schiff base and enamine intermediates. These oxidation reactions were also used more generally as mechanistic probes to identify other oxidizable substrate–enzyme intermediates with several other enzymes, including type I and type II aldolases, aspartate aminotransferase, pyruvate decarboxylase, and 6-phosphogluconate dehydrogenase.[10] In all cases, the enzyme-catalyzed oxidations were found to be highly specific with respect to the substrate, but rather nonspecific with respect to the oxidant.

Here we focus on our recently reported aldolase antibody 24H6, showing that it indeed accepts large substrate molecules and catalyzes oxidation reactions that are not catalyzed by other aldolase antibodies. More importantly, we report on a Hammett correlation study that sheds light on the initial steps of the catalytic aldol mechanism with this antibody. The specific reaction used as the mechanistic probe was the oxidation of α-ketoalcohols to 1,2-diketones by potassium ferricyanide.

Results and Discussion

Antibody 24H6, which was raised against a mixture of **2a** and **2b**, catalyzes aldol and retroaldol reactions with a broad variety of substrates. In order to learn more about the substrate preferences of this antibody, we studied the relative rates of the H/D exchange reaction at the α-position of a wide range of aldehydes and ketones (Figure 1). As seen in the figure, aldehydes are very reactive substrates with this antibody, and cyclic ketones are generally better substrates than the acyclic ones. This tendency and the general preference of 24H6 for aromatic substrates in the aldol/retroaldol reactions[4] agree with a highly hydrophobic active site that is lined up with many aromatic residues.

Abstract in Hebrew:

הנוגדן 24H6, אשר התקבל כתוצאה מחיסון כנגד 1,3-דיקטון, ידוע בפעילותו כזרז של תגובות אלדול. בעבודה הנוכחית נמצא כי הנוגדן מזרז גם תגובות נוספות, כגון חילוף של H/D בעמדה α לקרבוניל ותגובת חימצון של כהל לקטון. תגובת החילוף של H/D נבדקה עם מגוון גדול של קטונים ואלדהידים, תוך השוואה לפעילותו של אלדולז נוגדני אחר, 38C2. התוצאות מצביעות על כך שהאתר הפעיל בנוגדן 24H6 גדול באופן משמעותי מזה של 38C2. תכונה זאת איפשרה זירוז של תגובת החימצון של בנזואינים מותמרים לבנזילים המתאימים באמצעות אשלגן פריציאניד. חקירתה של תגובת החימצון הזאת שופכת אור על מנגנון זירוז השלבים הראשונים של תגובת אלדול ע״י הנוגדן 24H6. קורלצית Hammett בין $\log(k_{cat})$ לבין ערכי σ בתגובת החימצון מצביעה על כך שבשלב הראשון של התגובה מתרחשת יצירה מהירה של בסיס שיף בין קבוצת ליזין בנוגדן לבין הקטון. לאחר מכן, בשלב קובע המהירות, עובר בסיס השיף איזומריזציה לאן-אמין. בנוסף לממצאים האלו נצפתה קורלציה לינארית בין $\log(K_M)$ לבין ערכי σ. הקורלציה הזאת מוליכה למסקנה כי גורמים אלקטרוניים ולא סטריים פועלים בתהליך הקישור בין הנוגדן לסובסטרט וכי בתהליך הזה נוצר בסיס שיף בין הסובסטרט לבין קבוצת ליזין באתר הקישור של הנוגדן.

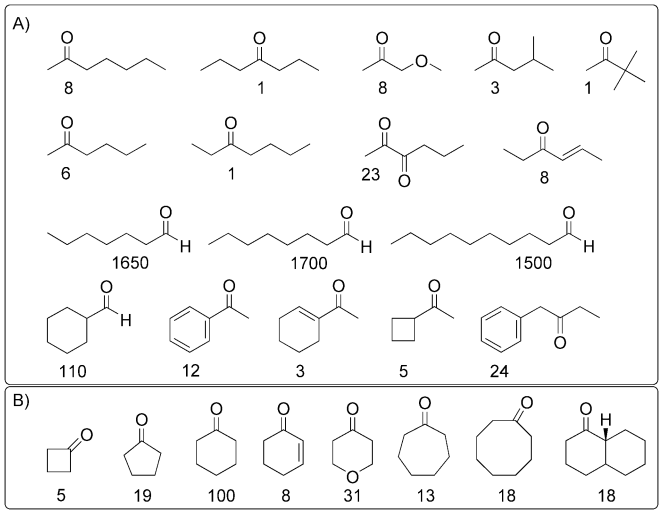


Figure 1. Ketone and aldehyde substrates used for 24H6-catalyzed deuterium exchange reactions at the α-carbon. The numbers represent relative rates. A) Acyclic ketones and aldehydes. B) Cyclic ketones.

Use of the deuteration reaction to compare the substrate profile of 24H6 (Figure 1)[11] with that of 38C2 (Figure 2)[12] indicates that 38C2 is more selective for small substrates,

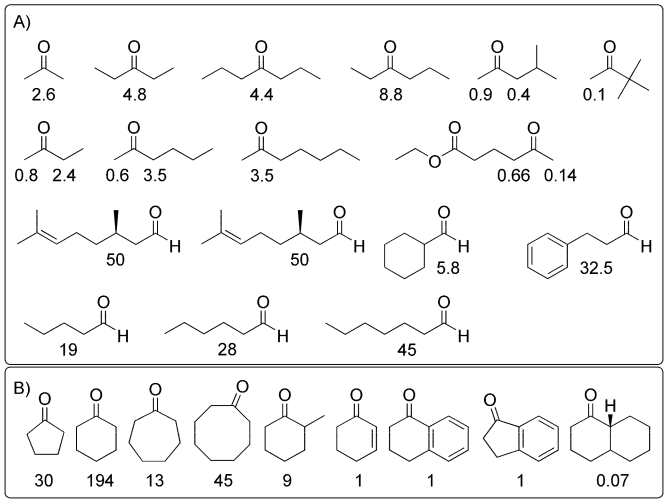
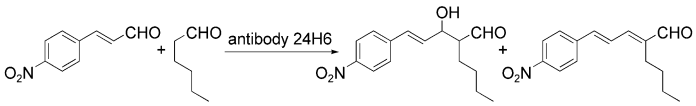


Figure 2. Ketone and aldehyde substrates used for 38C2-catalyzed deuterium exchange reactions at the α-carbon. The numbers, taken from reference [12], are rate constants [min^{-1}]. When two numbers are given for one substrate, they refer to the α- and α′-positions. A) Acyclic ketones and aldehydes. B) Cyclic ketones.

particularly for monocyclic ketones. For example, with 38C2 decalone is 3000 times less reactive than cyclohexanone. By contrast, with 24H6 decalone is only five times less reactive than cyclohexanone, indicating that this antibody is more indiscriminate towards large substrates than 38C2.

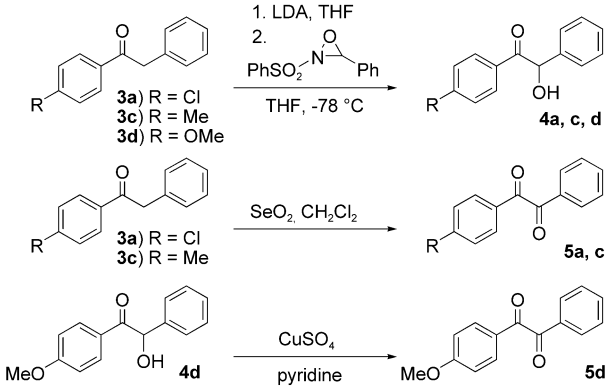
In accordance with these results, which point at a larger active site in 24H6, we also found that 24H6 can catalyze aldol condensation reactions with relatively large aliphatic aldehydes as donors. For example, butanal, valeraldehyde, hexanal, and heptanal were found to be good aldol donors, as exemplified by the cross aldol reaction between hexanal and *p*-nitrocinnamaldehyde (Scheme 2), which proceeded



Scheme 2. Cross aldol reaction between hexanal and *p*-nitrocinnamaldehyde.

with $k_{cat} = 0.002\ min^{-1}$ and $K_M = 40$ mM. By contrast, 38C2 is more limited with respect to the donor size. For example, 38C2 can use propanal and butanal as donors, but not heptanal and higher aldehydes.[11] It appears that despite the broad substrate range, which characterizes antibodies produced through reactive immunization, the general shape and size of the hapten and the positioning of the 1,3-diketone functionality in it play significant roles in determining the dimensions and topography of the resultant antibody active site. Although both 38C2 and 24H6 were elicited against diketone haptens, they show different reactivity and substrate range. The significantly larger size of the 24H6 active site suggests that this antibody could catalyze the above-mentioned oxidation of substituted benzoins to benzils.

For the Hammett correlation studies we synthesized a series of substituted benzoin substrates and benzil products as described in Scheme 3. The substituted desoxybenzoins,



Scheme 3. Syntheses of substrates and products.

3a and **3c** were prepared from the appropriate benzaldehydes by reaction with phenyldiazomethane (formed in situ from the sodium salt of phenyltosylhydrazone in THF[13]). Compound **3d** was prepared from phenylacetic acid and 4-anisaldehyde.[14] Hydroxylation of **3d** with *N*-phenylsulfonyloxaziridine (Davis reagent)[15] afforded **4a**, **4c**, and **4d**. The substituted benzils **5a** and **5c** were obtained by direct oxidation with SeO_2.[16] 4-Methoxybenzil (**5d**) was obtained by oxidation of **3d** with copper sulfate in refluxing pyridine.[17] Benzoin (**4b**) and benzil (**5b**) were commercially available.

Antibody 24H6 was found to catalyze the oxidation of all substituted benzoins (**4a–d**) to the corresponding benzil derivatives (**5a–d**) in the presence of $K_3[Fe(CN)_6]$. Catalysis followed Michaelis–Menten saturation kinetics. Antibody 38C2 failed to catalyze this reaction, thus serving for control experiments that confirmed specific catalysis at the binding

site of 24H6. Apparently, such substituted aromatic ketone substrates are too large to fit within the active site of 38C2. Another control experiment that supported catalysis by the active-site lysine residue was carried out with 24H6 in the presence of 10% acetone as a co-solvent instead of DMSO. Expectedly, since acetone can form a Schiff base with the antibody lysine residue, the reaction was inhibited by approximately 30% under these conditions. The antibody-catalyzed oxidation reactions were carried out at 25°C with a constant concentration of 24H6 (10.7 μM) and $K_3[Fe(CN)_6]$ (2 mM) and variable concentrations of substrate in phosphate buffered saline (PBS, 50 mM phosphate, 100 mM NaCl, pH 9.0) containing 10% DMSO. The progress of the reaction was monitored by HPLC equipped with a UV detector. The kinetic parameters, k_{cat} and K_M (Table 1), were extracted from a Lineweaver–Burk analysis of the kinetic data. Originally, we attempted to increase the range of substrates for the Hammett correlation study and include three more substrates with higher and lower σ values. To that end we synthesized **4e** (R = NMe_2), **4f** (R = CF_3), and **4g** (R = NO_2) using the above-described procedures. However, the 24H6-catalyzed oxidation reaction with **4e** produced the corresponding benzil along with other products. Substrates **4f** and **4g**, which bear electron-withdrawing substituents, were found to be too unstable under the antibody-catalyzed reaction conditions. Fortunately, the quality of the correlation line and the relatively steep slope made a four-point line sufficient for this study.

Table 1. Kinetic parameters of the antibody 24H6-catalyzed oxidation reactions with substrates **4a–d**. The σ values were taken from reference [14].

	R	σ	k_{cat} [min^{-1}]	K_M [mM]	k_{cat}/K_M
4a	Cl	0.24	9.10×10^{-2}	50.05	1.82×10^{-3}
4b	H	0.00	1.96×10^{-3}	7.98	2.50×10^{-4}
4c	Me	−0.16	4.45×10^{-4}	3.76	1.18×10^{-4}
4d	OMe	−0.28	1.20×10^{-4}	0.52	2.30×10^{-4}

Plotting the values of $\log(k_{cat})$ against the values of the Hammett substituent constant, σ (Figure 3),[18] afforded a linear correlation with a positive slope ($\rho = 4.7$). This Hammett correlation coefficient suggests that a positive charge is diminished in the transition state of the rate-limiting step. A similarly large coefficient ($\rho = 5.3$), which has been reported for the alkaline hydrolysis of 2-methoxy tropones, was also interpreted in terms of formation of a significant negative charge in the transition state.[19]

A plausible mechanistic pathway of the 24H6-catalyzed oxidation reaction (Scheme 4) starts with a nucleophilic attack of a low-pK_a amine residue on the substrate carbonyl to form a Schiff base (or a protonated Schiff base) intermediate (**I**). Tautomerization of the latter to an enamine intermediate (**II**) occurs by means of a base-mediated deprotonation at the α-carbon atom. The highly electron-rich double bond in **II** is prone to oxidation, which probably occurs through two consecutive one-electron oxidation steps by Fe^{III} to produce intermediates **V** and **VI**. Finally, hydrolysis of **VI** would release the product, **5**.

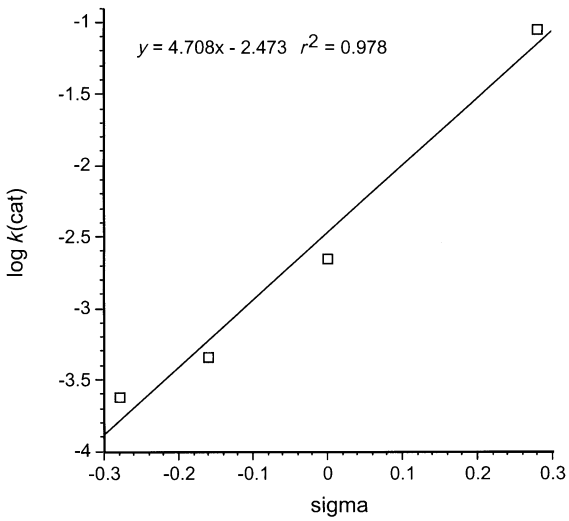
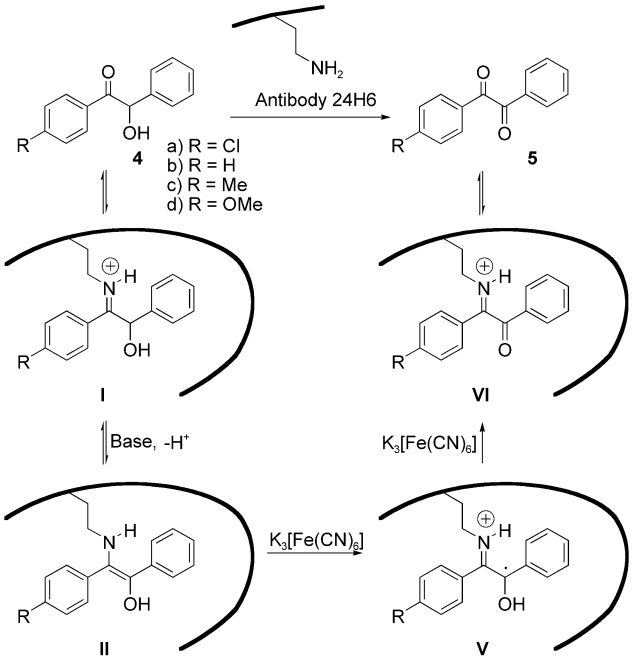


Figure 3. Hammet correlation for the 24H6-catalyzed oxidation reaction.



Scheme 4. Mechanistic pathway of the antibody-catalyzed oxidation reaction.

This mechanistic scheme suggests that the first two steps, formation of intermediates **I** and **II**, involve accumulation of a negative charge in the benzylic position in the transition state, which would be reflected by a positive ρ value. Both oxidation steps involve partial formation of a positive charge in the benzylic position, which would be reflected by a negative ρ value. Thus, based on the large, positive value of ρ in our case, we conclude that both oxidation steps which form intermediates **V** and **VI** are fast. Thus, the rate-limiting step would be either formation of **I** or **II**. Mechanistic studies of Schiff base formation suggest that the rate-determining step depends on the pH.[20] In acidic solution the reversible attack of free amine on the carbonyl carbon to form a tetrahedral intermediate is rate-limiting; this is char-

acterized by Hammett constants ranging between 1.5 and 2.[20] Conversely, in basic solution, the attack of free amine is fast, while the loss of water (or hydroxide ion) becomes the slow step. The much larger value of 4.7 found in our case suggests that formation of **I** is not rate-limiting, pointing at the deprotonation of **I** to produce **II** as the rate-limiting step.

These findings agree with our previously reported 38C2-catalyzed isotope-exchange experiments[12] that supported formation of the Schiff base intermediate in a rapid pre-equilibrium. The antibody-catalyzed $^{16}O/^{18}O$ exchange of the carbonyl oxygen of cycloheptanone in $H_2{}^{18}O$, was found to be very fast ($k_{cat}=418\ min^{-1}$, $K_M=21$ mM). Comparison of these results with the kinetic parameters for the 38C2-catalyzed H/D exchange with the same substrate ($k_{cat}=13\ min^{-1}$, $K_M=16$ mM) indicated that the formation of the enamine intermediate is approximately 32 times slower than the formation of the Schiff base. These studies have also indicated that in the 38C2-catalyzed aldol condensation reaction, the C–C bond-forming step is approximately 10^4 times slower than the formation of the enamine.[12] Accordingly, we believe that this bond-forming step is rate-limiting in the 24H6-catalyzed aldol reaction as well.

It is interesting to compare the Hammett correlation coefficient of the 24H6-catalyzed oxidation of **4** ($\rho=4.7$) with that of the uncatalyzed reaction ($\rho=1.7$, Figure 4), which

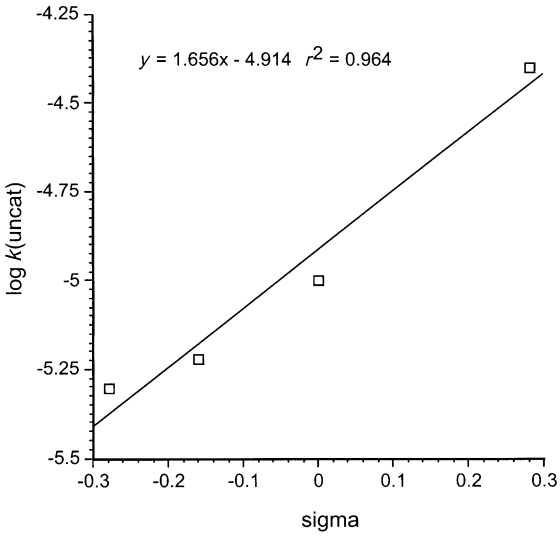
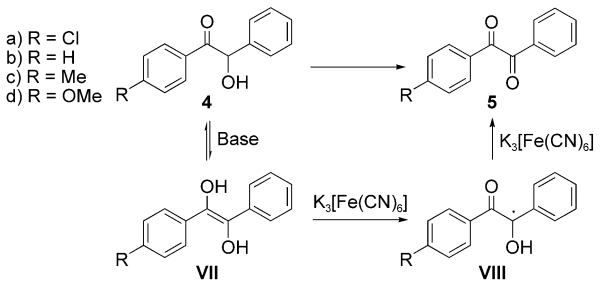


Figure 4. Hammet correlation for the uncatalyzed oxidation reaction.

was performed under the same conditions in the absence of the antibody. The proposed mechanistic pathway of the latter reaction, which is probably general base-catalyzed, is outlined in Scheme 5. Again, as is the case in the antibody-catalyzed reaction, the positive ρ value of the uncatalyzed reaction indicates that the rate-limiting step is the formation of the enol intermediate (**VII**). As in the catalyzed reaction, the two irreversible one-electron oxidation steps that form intermediate **VIII** and the final product (**5**) are much faster



Scheme 5. Mechanistic pathway of the uncatalyzed oxidation reaction.

than the enolization step. The irreversibility of the oxidation steps renders the enolization step practically irreversible as well, thus providing an opportunity of directly measuring the Hammett correlation coefficient of the enolization process.

To examine the substituent effect on the binding constant we plotted the $\log(K_M)$ values against the Hammett σ substituents constants (Figure 5). Interestingly, a nearly linear

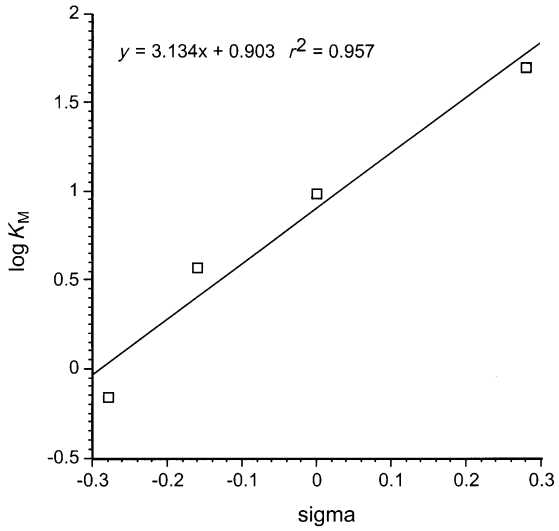


Figure 5. $\text{Log}(K_M)$ versus σ for the oxidation reaction.

correlation with a positive slope ($\rho=3.13$) was found. In our previously reported Hammett correlation studies with aldol reaction catalyzed either by 38C2 or by 24H6 we employed substituted benzaldehyde acceptors rather than substituted donors. Very little effect of the substituent on binding (K_M) was observed in those cases.[4,8] The large value of 3.13, as well as the remarkable difference between the substituent effects on the binding of either donor or acceptor, suggests that electronic rather than steric factors are dominant in binding. These findings support the assumption that a chemical bond is formed between the antibody and the donor molecule during the catalytic event. This notion has been previously suggested for all aldolase antibodies,[6] including 24H6,[4] based on chemical modifications (reductive alkyla-

tion of the antibody lysine residue with acetone and borohydride) and on spectral data (formation of a new UV absorption band at 316–318 nm, which was attributed to the formation of a vinylogous amide upon binding of acetylacetone to the lysine residue).

In conclusion, oxidation of a series of substituted benzoins to benzils by potassium ferricyanide was used as a mechanistic probe to better understand the initial steps of the 24H6-catalyzed aldol condensation reaction. Hammett correlation ($\rho = 4.7$) of $\log(k_{cat})$ versus the σ substituent constant revealed that the rate-limiting step in this oxidation reaction is the conversion of the Schiff base (initially formed from the ketone and an active site lysine residue) to an enamine intermediate. In addition, linear correlation ($\rho = 3.13$) was found between $\log(K_M)$ and the Hammett σ substituents constants. This correlation suggests that electronic rather than steric factors are dominant in the antibody–substrate binding phenomenon, consistent with reversible formation of a Schiff base intermediate.

Experimental Section

General methods: Most ^{1}H NMR spectra were recorded on a Bruker AM200 spectrometer, operating at 200 MHz using $CDCl_3$ as a solvent (unless otherwise specified). Positive ion mass spectra, using the fast atom bombardment (FAB) technique, were obtained on a VG ZAB-VSE double-focusing, high-resolution mass spectrometer equipped with either a cesium or sodium ion gun. EI-MS spectra were measured on a Finnigan MAT-711 spectrometer. CI-MS spectra were measured on a Finnigan TSQ-70 spectrometer. UV/Vis spectra were recorded on a Shimadzu UV-1601 spectrometer. Long period reactions were maintained at 25 °C by using a Friocell incubator. TLC was performed on glass sheets pre-coated with silica gel (Merck, Kieselgel 60, F254, Art. 5715). Column chromatographic separations were performed on silica gel (Merck, Kieselgel 60, 230–400 mesh, Art. 9385) under pressure (flash chromatography). Dry THF (Sure-seal) was purchased from Aldrich. HPLC analyses were carried out with a Merck–Hitachi Lachrom system equipped with an L-7100 pump, an L-7400 UV/Vis detector, and a D-7000 system manager with a Supelco RP LC-18 analytical column. All starting materials and reagents, including benzoin (**4b**) and benzil (**5b**), were purchased from Aldrich.

General procedure for α-hydroxylation: The appropriate ketone (1 mmol) in dry THF (6 mL) was added dropwise to a freshly prepared, cold (−78 °C) solution of LDA (1.1 mmol) in dry THF (2 mL), and the mixture was stirred at the same temperature for 30 min. A solution of phenylsulfonyloxaziridine (1.4 mmol in 6 mL THF) was added dropwise, and the progress of the reaction was monitored by TLC. Upon completion the reaction was quenched with saturated aq NH_4Cl; the mixture was then allowed to warm to room temperature, was extracted with diethyl ether, was washed with saturated aq $Na_2S_2O_3$ (2×15 mL) and brine (2×15 mL), and was dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by flash chromatography.

4-Chlorobenzoin (4a): ^{1}H NMR: $\delta = 7.83$ (d, $J = 8.6$ Hz, 2H) 7.31 (d, $J = 8.6$ Hz, 2H), 7.28 (m, 5H), 5.86 (br d, $J = 7$ Hz, 1H), 4.45 ppm (br d, $J = 7$ Hz, 1H); MS (CI): m/z: 247 $[M+H]^+$.

4-Methylbenzoin (4c): ^{1}H NMR (300 MHz): $\delta = 7.76$ (d, $J = 8.4$ Hz, 2H) 7.25 (m, 5H), 7.22 (d, $J = 8.4$ Hz, 2H), 5.87 (br d, $J = 5$ Hz, 1H) 4.55 (br d, $J = 5$ Hz, 1H) 2.29 ppm (s, 3H); MS (CI): m/z: 227 $[M+H]^+$.

4-Methoxybenzoin (4d): ^{1}H NMR: $\delta = 7.89$ (d, $J = 8.6$ Hz, 2H) 7.29 (m, 5H), 6.84 (d, $J = 8.6$ Hz, 2H), 5.86 (br s, 1H) 4.64 (br s, 1H) 3.79 ppm (s, 3H); ^{13}C NMR: $\delta = 196.5$, 164.0, 139.6, 131.5, 129.0, 128.3, 127.6, 126.5, 113.9, 75.7, 55.4 ppm; MS (CI): m/z: 243 $[M+H]^+$.

General procedure for selenium dioxide oxidation: A solution of either **3a** or **3c** (1 mmol) and selenium dioxide (1.1 mmol) were dissolved in 70% acetic acid (5 mL) and the mixture was heated to 90 °C for 12 h. The solution was then poured into water and extracted with diethyl ether, washed with saturated aqueous K_2CO_3, and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by flash chromatography.

4-Chlorobenzil (5a): ^{1}H NMR: $\delta = 7.93$ (m, 4H), 7.48 ppm (m, 5H); MS (CI): m/z: 244 $[M]^-$.

4-Methylbenzil (5c): ^{1}H NMR: $\delta = 7.95$ (br d, $J = 7.4$ Hz, 2H), 7.85 (d, $J = 8.1$ Hz, 2H), 7.61 (d, $J = 7.4$ Hz, 1H) 7.48 (t, $J = 7.4$ Hz, 2H), 7.29 (d, $J = 8.1$ Hz, 2H), 2.42 ppm (s, 3H); MS (CI): m/z: 244.1 $[M]^-$.

4-Methoxybenzil (5d): 4-Methoxybenzoin (0.2 g, 0.83 mmol) was refluxed for 5 h with a solution of copper sulfate (0.4 g) and pyridine (5 mL) in water (1 mL). Aqueous HCl (2 M, 5 mL) was added to the cooled mixture and the aqueous layer was extracted three times with dichloromethane. The combined organic extracts washed twice with brine and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by flash chromatography. ^{1}H NMR: $\delta = 7.97$ (br d, $J = 8.4$ Hz, 2H), 7.94 (br d, $J = 8.4$ Hz, 2H), 7.61 (d, $J = 7.4$ Hz, 1H), 7.50 (t, $J = 7.4$ Hz, 2H), 6.96 (dd, $J = 8.0$, 1.9 Hz, 2H), 3.87 ppm (s, 3H); MS (CI): m/z: 241 $[M+H]^+$.

Antibody-catalyzed reactions: All antibody-catalyzed reactions were carried out in PBS (50 mM phosphate, 100 mM NaCl, pH 9.0) containing 10% DMSO as organic co-solvent. The progress of the reactions was monitored by HPLC using an RP Supelcosil LC18 column, with the initial rates calculated by regression analysis. Antibody concentration was 10.7 μM for 24H6. The rate of the uncatalyzed reactions was subtracted.

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