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Catalysis of the Photo-Fries Reaction: Antibody-Mediated Stabilization of High Energy States

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Abstract: A conformationally constrained hapten is presented that is capable of catalyzing the first antibody-mediated photo-Fries rearrangement. In this reaction, absorption of light energy by a diphenyl ether substrate results in homolytic C−O bond cleavage followed by recombination to yield biphenyl-derived products. The most proficient antibody studied converts 4-phenoxyaniline **15** into 2-hydroxy-5-aminobiphenyl **16** under high-intensity irradiation at a rate of 8.6 μM/min. These results support a recent hypothesis stating that immunization with conformationally constrained haptens provides higher titers for the acquisition of simple binding antibodies; however, in this case, conformational constraint does not ensure the development of more efficient catalysts. Using the obtained antibodies, the presence of products resulting from escape of free radicals from the solvent cage can be suppressed, altering the excited state energy surface such that free radicals are funneled into the formation of the desired biphenyl product. However, studies also show the inactivation of the antibodies as a result of photodecay of the biphenyl product. Using an isocyanate scavenging resin, the photodecay product could be removed and the inactivation of the antibody drastically reduced. Furthermore, despite the observed photodecay, turnover of the antibody was present; this represents the first case in which true turnover of a photochemical reaction using a catalytic antibody could be observed.

Introduction

A number of rearrangements of aromatic compounds are known including the Claisen rearrangement,[1] the Fries rearrangement,[2] and the benzidine rearrangement.[3] Each of these reactions can proceed via two distinct and readily differentiated mechanisms, photochemical and acid-catalyzed rearrangement. Although diphenyl ethers such as **1** have been shown to be stable to acids and high temperatures, upon UV irradiation these compounds can be converted into products **2**−**5**, albeit in low quantum yield (Scheme 1). As a result of this low reactivity coupled with limited synthetic utility, this reaction has only been sparingly studied in the greater than fifty years since its discovery.[4] Formally, this reaction is a photo-Claisen rearrangement, albeit distinct from the [3,3] photo-Claisen rearrangement.

It is widely accepted that the photo-Fries reaction proceeds through radical pairs; however little physical evidence has been reported. A report by Haga studied this process in detail, and a number of interesting features were uncovered.[5] Mechanistically, it has been suggested that the reaction occurs by excitation of **1** from the ground state to a singlet state, followed by homolytic cleavage of the C−O bond to give a singlet radical pair. Recombination and aromatization of these radical pairs leads to some of the observed products, while others result from dissociation of the radical pairs to free radicals and hydrogen abstraction from the solvent. Asymmetric ethers having their para position occupied by a substituent were also studied, and it was found that the product distribution was heavily influenced by the electronic character of this substitutent (Scheme 2). Specifically, electron-donating substituents predominantly gave products resulting from path a, while electron-withdrawing substituents gave products exclusively from path b. Amazingly, despite the radical nature of this reaction, the preference of the mechanism for aryloxy−phenyl cleavage with electron-donating substitutent and aryl−phenoxy cleavage with electron-withdrawing substituents correlated with the Hammett σ values.

To effectively exploit the energy potential provided by light, nature has evolved particularly efficient mechanisms to both harness and direct light-based energy. Tantamount among these

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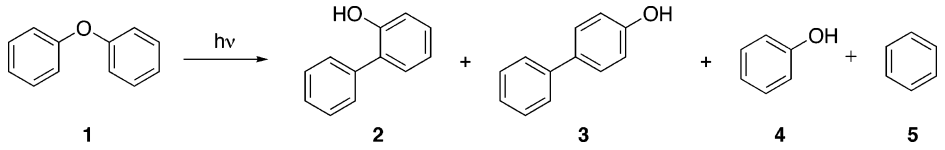
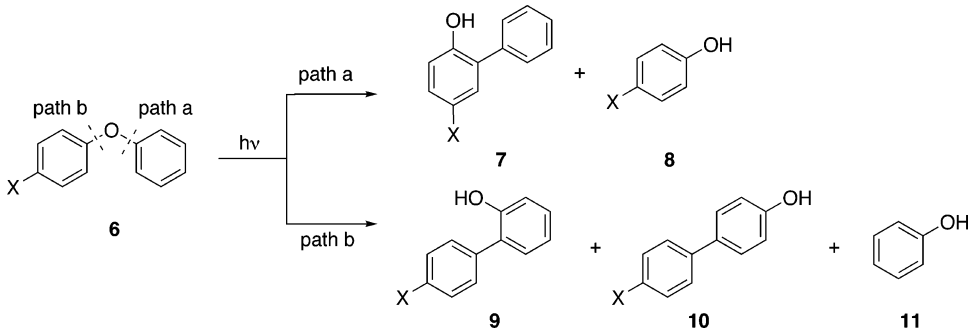
10.1021/ja038016n CCC: $25.00

Scheme 1. Photo-Fries Rearrangement of a Diphenyl Ether and Observed Reaction Products



1 2 3 4 5

Scheme 2. Photo-Fries Rearrangement of Asymmetric Diphenyl Ethers[a]



6 7 8 9 10 11

[a] When X = EDG (e.g., OMe, OH, NH_2, CH_3), path a ≫ path b. When X = EWG (e.g., CO_2Me, CN), path a ≪ path b.

processes are the rhodopsin-activated enzymatic cascade in vision,[6] the electron-transfer events present in photosynthesis,[7] and the fragmentation of thymine dimers in DNA repair.[8] These enzymes have evolved in such a way that high energy states are processed correctly to produce only the desired chemical outcome. Thus, a critical challenge in the production of photochemical catalysts is not only the stabilization of a given transition state, but also rigorous control over the high-energy intermediates produced along the reaction coordinate.

Alternative protein-based entities that can provide mechanistic insight into biochemical processes include catalytic antibodies. The development of the first catalytic antibodies in 1986 helped galvanize new research opportunities into the creation of *de novo* biocatalysts.[9] Quickly following this seminal discovery, it was realized that the much of the true power of catalytic antibodies was in the catalysis of reactions which have no known natural counterpart.[10] In this context, numerous antibody catalysts have since been identified which can catalyze reactions such as Diels−Alder and [1.3]-dipolar cycladditions,[11] anti-Baldwin ring closure reactions,[12] cationic cyclizations,[13] and the Bergman cyclization of enediynes.[14] Furthermore, the recent demonstration that all antibodies can produce reactive oxygen species including H_2O_2, H_2O_3, and ozone from singlet oxygen clearly shows that the antibody structure is surprisingly resilient to a variety of highly reactive chemical species.[15] The vast majority of reported antibody-catalyzed reactions are thermal in nature, and in fact, there are no examples of photochemical reactions demonstrating uninterrupted antibody turnover. However, examples do exist of antibody accelerated/energy altered photochemical reactions including Norrish type II reactions,[16] pyrimidine dimer cleavage,[17] and the production of an antibody-stilbene "exciplex" which productively funnels energy into a long-lived blue fluorescent species.[18] Little is known regarding the chemical structure of photochemical transition states and corresponding stable analogues; thus, in all reported antibody photochemical reactions, haptens have been designed which directly resemble ground-state species.

In this study, we report the development of the first antibodies that are capable of catalyzing the Fries photochemical rearrangement of diphenyl ethers. By not only affecting the energy surface of the ground state but also altering the surface of the excited state, these antibodies accelerate the formation of the desired photochemical product while also drastically reducing the proportion of byproducts produced as a result of escape of radicals from the solvent cage. This study also demonstrates that antibodies can turnover substrates for photochemical reactions under standard conditions used for studying reaction

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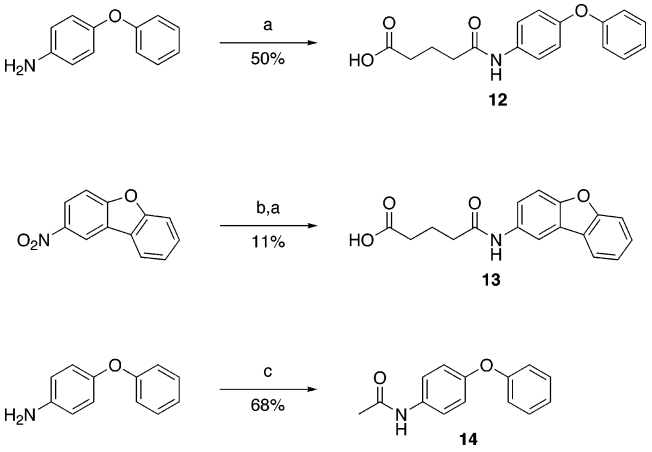
Scheme 3. Synthesis of Haptens Used to Elicit Catalytic Antibodies for Catalysis of the Photo-Fries Rearrangement[a]



[a] (a) glutaric anhydride, diisopropylethylamine, $CHCl_3$, 0 °C. (b) $SnCl_2$, EtOH, reflux. (c) AcCl, DMAP, pyridine.

kinetics. This is critical as while the occurrence of turnover has been claimed, it has not been demonstrated on a practical level.

Results and Discussion

Two haptens were designed to resemble points along differing reaction coordinates. Hapten **12** is a flexible "substrate" and was made in an attempt to let the combinatorial power of the immune repertoire sculpt a *de novo* pocket such that the formed radicals could seek either path a or b (Scheme 3). Using this approach allows for an examination of the interaction of light with a suitable substrate within the confines of an antibody combining site where binding energy could alter the energy surface of the conversion of excited states to the corresponding photochemical products. Additionally, this type of hapten could provide an environment such that escape of free radicals from the solvent cage would be precluded, thereby eliminating the production of **8** or **11** (Scheme 2). A hapten-substrate approach, although atypical in relation to other catalytic antibodies, has proven suitable in the development of antibodies for photochemical reactions as an external source of energy is present in these reactions.[16] In contrast, hapten **13** was designed as a rigid analogue for the reaction shown in Scheme 2; this hapten is a planar structure and therefore should elicit complimentary aromatic residues that could serve as stabilizing moieties upon the generation of radical species. Additionally, the fortuitous positioning of a tryptophan residue in the antibody combining site could also lead to antibody-based sensitization of the photo-Fries reaction.[17b] Based upon our recent studies demonstrating enhanced serum titer with conformationally constrained haptens,[19] we felt **13** should also provide an additional model system for testing this hypothesis in terms of obtaining competent catalysts.

Hapten **12** was synthesized in one step from 4-phenoxyaniline and glutaric anhydride in good yield (50%) (Scheme 3). Reduction of the nitro group of 2-nitrobenzofuran with tin(II) chloride, followed by reaction with glutaric anhydride, gave analogous constrained benzofuran hapten **13** in 11% yield over two steps. Both haptens were conjugated to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) using standard protocols.[10] Two panels of 2 and 14 monoclonal antibodies obtained from immunization with hapten **12** and **13**, respectively, were purified[20] and screened under described conditions (vide supra). The most congruent substrate to haptens **12** and **13** was deemed to be **14**, wherein the linker has been replaced with an acetamide functionality. High-intensity, broad band UV irradiation of substrate **14** under the assay conditions (50 mM phosphate-buffered saline, pH 7.4, 2% acetonitrile) revealed that extended reaction times (>12 h) were necessary in order to achieve significant production of product. These extended reaction times are presumed to be due to the relatively minor effect an acetamido functionality has on the electronic character of the phenyl ring ($\sigma_{para} = -0.01$). In previous experiments, both strongly donating and strongly withdrawing substituents decreased the necessary irradiation times.[5] Unfortunately, although six-members of the antibody panel showed increased rates of product formation over background, the extended irradiation times necessary (>12 h) also caused antibody precipitation and an inability to measure kinetics. We envisioned that the obtained antibodies might also bind the close homologue 4-phenoxyaniline **15**, a compound which is expected to react significantly faster in this reaction.[5] Irradiation of **15** under the assay conditions yielded primarily biphenyl product **16** corresponding to homolytic cleavage C−O from path a (Scheme 4), in agreement with previous studies. From the obtained panels of antibodies, six of the mAbs raised against hapten **13** (MT4-1G11, 3G2, 5A1, 6F11, 7E4, and 8D5) and one mAb raised against hapten **12** (MT2-21C4) showed enhanced formation of **16** relative to uncatalyzed rates. Of these, two mAbs (MT4-3G2 and MT2-21C4) that showed the greatest enhancement were chosen for further study. Interestingly, both haptens led to products resulting from path a, implying that stereoelectronic effects inherent in the substrate take precedence in determining the mode of C−O homolytic cleavage even in the antibody combining site. Furthermore, the observed rate acceleration could be completely inhibited by preincubation of the antibody (20 μM) with 1 equiv of the corresponding hapten (2-acetamidodibenzofuran, 40 μM), highly suggesting that the catalysis occurs in the antibody combining site. As a further control, substrate **15** was also irradiated in the presence of BSA, a protein previously shown to bind small molecules and accelerate some reactions,[21] as well as several nonspecific antibodies; yet no effect or acceleration on product formation was observed.

In accordance with previous observations in our laboratories,[19] immunization with conformationally constrained hapten **13** leads to significantly increased serum titers. Analysis by standard ELISA techniques of the serum from mice immunized with **13** revealed titers of approximately 128 000−256 000, while immunization with hapten **12** gave serum titers of only 8000−64 000. This clearly gives further credence to the hypothesis that conformational constraint leads to an elevated immune response. However, this is not to imply that the use of conformational constraint in hapten design will lead to increased catalytic activity.[22] Indeed, the most proficient antibodies from either panel had approximately equal catalytic activity.

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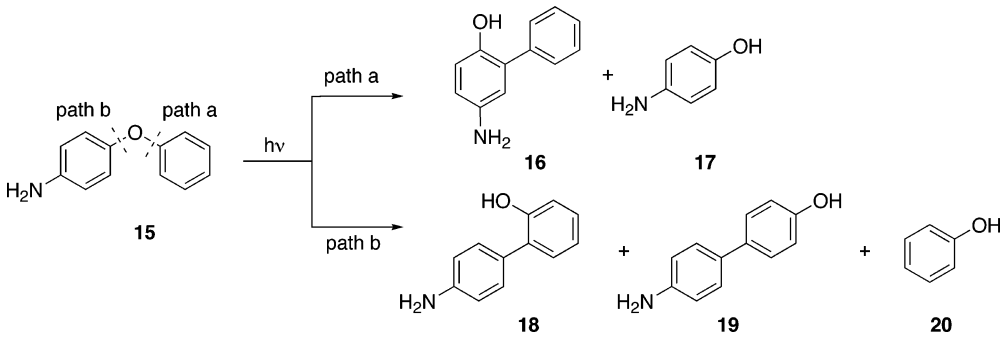
Scheme 4. Photo-Fries Rearrangement of 4-Phenoxyaniline



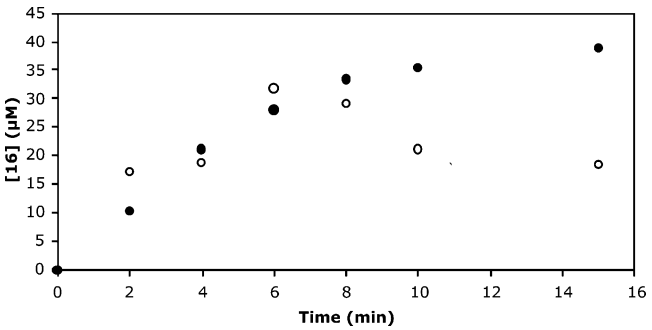


Figure 1. Observed formation of **16** in the presence of antibodies raised against constrained and unconstrained haptens. The data shown have been corrected for the background reaction. (●) MT2-21C4, (○) MT4-3G2.

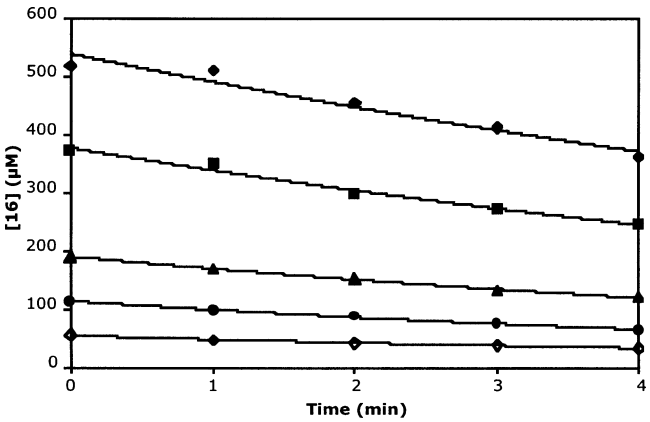


Figure 2. First-order photodecay of photo-Fries biphenyl product **16**. (◆) 500 μM **16**, (■) 400 μM **16**, (▲) 200 μM **16**, (●) 100 μM **16**, (◇) 40 μM **16**.

At low antibody concentrations, the background reaction dominates the kinetic profile. Thus, kinetic measurements under standard Michaelis−Menten conditions (i.e., low catalyst concentrations relative to substrate) were not possible. Furthermore, tight binding of the substrate prevented an accurate determination of K_m. In all studied antibodies, the observe rate showed minimal dependence on the concentration of substrate down to the lowest concentrations at which product formation could be detected (10 μM), indicating a $K_m \ll 10$ μM. Consequently, examinations of the reaction were performed under set conditions (phosphate-buffered saline, pH 7.4, 2% acetonitrile, 100 μM **15**, 20 μM mAb), and initial rates measured. Using an antibody raised against hapten **13**, MT4-3G2, the initial rates were 8.6 μM/min, while an antibody raised against hapten **12**, MT2-21C4, had an initial product rate of 5.2 μM/min. It was also noted, under these conditions, the product **16** also presented itself to be an inactivating photodecay component of the antibody-mediated process. Interestingly, in the presence of MT2-21C4, this photoinactivation decay product was clearly reduced relative to the reaction performed with MT4-3G2 (Figure 1). Presumably, this phenomenon is due to the modification of a critical combining site residue of MT4-3G2 by the photodecay product, while the combining site of MT2-21C4 is more robust and able to withstand exposure to this product.

Based upon our kinetic data with MT4-3G2, the photodecay of **16** was further explored. In aqueous solution, **16** readily decayed under the high-intensity UV light used for the experiments with an apparent first-order rate constant of 0.11 min^{-1} (Figure 2). This finding is not particularly surprising as **16** greatly resembles a 1,4-hydroquinone, a known antioxidant. Furthermore, the reaction of phenolic compounds with singlet oxygen is well-characterized and of significant interest in the field of waste water purification.[23]

Although it has been demonstrated that the antibody structure is surprisingly resilient to UV irradiation,[15] based upon our kinetic results and the observed reduction in catalytic activity (vide supra), the ability of the antibodies to bind the substrate after irradiation was examined. As in previous studies, in the absence of substrate, no significant loss of binding of either MT4-3G2 or MT2-21C4 to its antigen was observed after 2 h of UV irradiation. However, in the presence of substrate, a pronounced loss in the binding capacity of MT4-3G2 is observed, as measured by ELISA (Figure 3). Surprisingly, after only 10 min, a 15% loss in relative binding affinity is observed, with a 50% loss being observed after 1 h of irradiation. This supports the notion that the substrate is, in fact, acting in the combining site of the antibody and that the reaction of interest also occurs at this site.

The observed decrease in reaction rate can be attributed to one of two factors, either the produced radicals are covalently modifying the antibody, thereby serving as mechanism-based inhibitors, or the photodecay of the recombination product yields a reactive intermediate which prevents further reaction in the antibody combining site. Covalent modification of an antibody combining site is not unprecedented and has been previously observed in catalytic antibodies for cationic reactions.[13] To assess this difference, MT4-3G2 was irradiated in the presence of synthetically prepared **16** and then assayed for the ability of the antibody to bind its hapten. Hapten binding was inhibited

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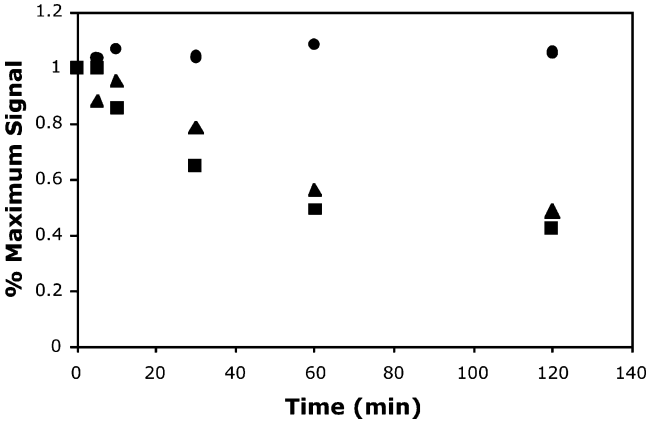

Figure 3. Loss of antibody binding capacity as a result of irradiation in the presence of photo-Fries substrate and product. (●) 3G2 alone, (■) 3G2 exposed to **15** during irradiation, (▲) 3G2 exposed to **16** during irradiation.

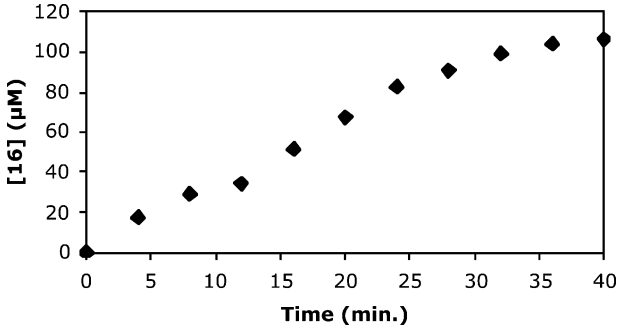

Figure 4. Observed turnover of mAb MT4-3G2. The reaction was performed with 20 μM MT4-3G2 and 200 μM **13** in the presence of an isocyanate scavenging resin. Data shown are corrected for the background photochemical reaction.

to an almost identical extent as exposure to irradiated substrate, implying that photodecay of the reaction product serves as an inhibitor of the reaction. Analysis of MT4-3G2 after irradiation by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) revealed no visible fragmentation of the antibody due to the presence of potentially proteolytic radicals or other reactive species (data not shown).

Previous studies using antibodies for photochemical reactions have focused on observing turnover through a variety of methods including dialysis of the antibody after the reaction of 1 equiv of substrate[16b] and continuously pulsing the reaction with 1 equiv of substrate and driving the reaction to completion.[16a] In our initial studies, turnover could not be directly assessed as detection was limited by the photodecay of reaction product **16** and the subsequent antibody inactivation (vide supra). To suppress the inactivation of the antibody, an isocyanate polystyrene resin was examined to trap **16** out of the reaction mixture. Although the resin did not seem to efficiently trap **15**, fortuitously, the resin reacted with the photodecay product, thereby removing the inhibitory effects of this product. This effect is surprising as the resin used has a polystyrene backbone and thus is not expected to appreciably swell in a polar solvent such as water. Under these conditions, a minimum of 2.5 turnovers could be clearly observed (Figure 4). Presumably, the entirety of the photodecay product is not removed from the reaction, thus eventually leading to antibody inactivation and the observed decrease in rate. Importantly, this represents the first observation of true turnover in an antibody-catalyzed reaction involving biradical intermediates.

Conclusion

Traditionally, the design of haptens for the production of catalytic antibodies has been grounded in transition state theory and the stabilization of this transient species. However, it has recently been appreciated that antibodies are also capable of altering the energy surface of excited states, thereby stabilizing highly reactive species and productively channeling their energy into the appropriate reaction pathways. This study has demonstrated the ability of an antibody to catalyze the photo-Fries rearrangement of a diphenyl ether substrate. In the antibodies studied here, catalysis could be due to multiple factors including conformational control of the biradical intermediates as well as the positioning of residues that serve as potential photosensitizers. Despite the presence of significant photodecay of the product of this reaction and its subsequent inactivation of the antibody, using a polystyrene resin to trap the photodecay product, turnover could be observed without using specialized conditions such as dialysis or "pulsing" the reaction. Furthermore, this photodecay is the limiting factor in the reaction as the antibody structure was extremely resilient to short-term irradiation with high-intensity ultraviolet light. Reiterating the concept of conformational constraint in the elicitation of high antibody titers,[19] a constrained hapten yielded extremely high immunization responses; although this concept is significant in the design of new haptens for a number of scenarios, it does not imply that constraint necessarily leads to higher catalytic efficiency. Clearly, hapten design with regard to transition state mimicry of the desired reaction and the induction of essential catalytic residues remain a challenge in the development of efficient catalytic antibodies. The results reported expand our current understanding of antibody catalysts and their catalytic diversity.

Experimental Section

General Methods. Unless otherwise stated, all reactions were performed under an inert atmosphere with dry reagents and solvents and flame-dried glassware. Analytical thin-layer chromatography (TLC) was performed using 0.25 mm precoated silica gel Kieselgel 60 F_{254} plates. Visualization of the chromatogram was by UV absorbance, iodine, dinitrophenylhydrazine, ceric ammonium molybdate, ninhydrin, or potassium permanganate as appropriate. Preparative and semi-preparative TLC was performed using Merck 1 mm or 0.5 mm coated silica gel Kieselgel 60 F_{254} plates, respectively. Analytical HPLC was performed on a Hitachi L-5000 series HPLC using a Vydac 201TP54 reversed phase C_{18} column. Methylene chloride and chloroform were distilled from calcium hydride. Tetrahydrofuran (THF) was distilled from sodium/benzophenone. Methanol was distilled from magenesium. High-intensity photolysis was performed in a Rayonet photochemical reactor with RPR-3400 bulbs. Analytical HPLC was performed on a Hitachi L5000 series HPLC using a Vydac 201TP54 reversed phase column. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer at 500 and 125 MHz, respectively, and are reported in ppm, unless otherwise noted. Matrix-assisted laser desorption/ionization (MALDI) FTMS experiments are performed on an IonSpec FTMS mass spectrometer. Electrospray ionization (ESI) mass spectrometry experiments were performed on an API 100 Perkin-Elmer SCIEX single quadrupole mass spectrometer.

4-(4-Phenoxyphenylcarbamoyl)butyric Acid (12). In a dry round-bottom flask under argon, a cooled (0 °C) solution of 4-phenxoyaniline (510 mg, 2.8 mmol) in dry $CHCl_3$ (5 mL) was treated successively with diisopropylethylamine (1.9 mL, 11 mmol) and glutaric anhydride (628 mg, 5.5 mmol). After 16 h of stirring at room temperature, the

reaction mixture was then diluted with one volume of $CHCl_3$. Upon washing with 1 N HCl, a white precipitate was formed and filtered off the organic layer. The fluffy solid was dried under high vacuum to give 407 mg (50% after crystallization) of the desired hapten **12**. ^{1}H NMR (DMSO-d_6) 1.80 (q, J = 7.3 Hz, 2H), 2.26 (t, J = 7.3 Hz, 2H), 2.33 (t, J = 7.3 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 9.2 Hz, 2H), 7.07 (m, 1H), 7.34 (dd, J = 8.8 Hz, 7.3 Hz, 2H), 7.59 (d, J = 9.2 Hz, 2H), 9.91 (s, 1H); ^{13}C NMR (DMSO-d_6) 20.5, 33.0, 35.4, 117.8, 119.5, 120.7, 122.9, 130.0, 135.3, 151.4, 157.5, 170.6, 174.2. MALDI-FTMS for $C_{17}H_{18}NO_4$ ($M + H^+$) calculated 300.1230, found 300.1229.

4-(Dibenzofuran-2-ylcarbamoyl)butyric Acid (13). 2-Aminodibenzofuran (23 mg, 0.13 mmol) was dissolved in $CHCl_3$ (2 mL) and cooled to 0 °C. This solution was then treated successively with diisopropylethylamine (88 μL, 0.5 mmol) and glutaric anhydride (29 mg, 0.25 mmol). After 3 h at room temperature, the reaction mixture was then diluted with one volume of $CHCl_3$. Upon washing with 1 N HCl, a white precipitate was formed and filtered off the organic layer. The organic layer was then concentrated under reduced pressure and the residue combined to the solid obtained by filtration. The crude material was purified by successive preparative TLC eluting first with EtOAc/hexane/AcOH (4:4:0.8) followed by toluene/EtOAc/AcOH (7:3:0.5) to give 9 mg (24%) of the desired compound as an off-white solid. ^{1}H NMR (CD_3OD) 2.02 (q, J = 7.3 Hz, 2H), 2.35 (t, J = 7.3 Hz, 2H), 2.46 (t, J = 7.3 Hz, 2H), 7.93 (td, J = 7.8 Hz, 1.5 Hz, 1H), 7.46 (td, J = 7.8 Hz, 1.5 Hz, 1H), 7.52 (m, 2H), 7.56 (d, J = 8.0 Hz, 1H), 7.98 (d, J = 8.5 Hz, 1H), 8.31 (s, 1H); ^{13}C NMR (CD_3OD) 21.3, 33.4, 37.2, 104.8, 111.9, 113.6, 118.2, 121.6, 124.1, 125.7, 128.3, 135.5, 147.1, 155.6, 172.0, 177.4. MALDI-FTMS for $C_{17}H_{16}NO_4$ ($M + H^+$) calculated 298.1074, found 298.1066.

4-Phenoxyacetanilide (14). To a cooled solution (0 °C) of 4-phenoxyaniline (570 mg, 4.05 mmol) in pyridine (4 mL) under argon, acetyl chloride (864 μL, 12 mmol), and *N*,*N*-dimethylaminopyridine (50 mg, 0.4 mmol) were added successively. The reaction was allowed to stir at room temperature for 16 h, after which time EtOAc was added and the organic layer washed several times with aqueous 1 N HCl and brine and then dried over $MgSO_4$. Purification of the crude material by flash chromatography on SiO_2 eluting with hexane/EtOAc (3:2) gave 625 mg (68%) of the desired material as a white solid. ^{1}H NMR ($CDCl_3$) 2.14 (s, 3H), 6.95 (m, 4H), 7.08 (t, J = 7.3 Hz, 1H), 7.31 (dd, J = 8.5 Hz, 7.6 Hz, 2H), 7.45 (d, J = 8.8 Hz, 1H), 8.00 (br s, 1H); ^{13}C NMR ($CDCl_3$) 24.8, 118.3, 119.4, 121.8, 123.0, 129.7, 133.4, 153.4, 157.4, 168.8. MALDI-FTMS for $C_{14}H_{14}NO_2$ ($M + H^+$) calculated 228.1019, found 228.1017.

4-*N*-Acetamido-2′-hydroxybiphenyl. To a solution of 2-iodophenol (220 mg, mmol) in water (10 mL), 4′-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)acetanilide (261 mg, mmol), K_2CO_3 (415 mg, mmol), and 10% palladium on carbon (10 mg) were added. The reaction mixture was stirred for 12 h vigorously and then quenched slowly with 1.5 M HCl (10 mL). The solution was then filtered to isolate a tan solid along with the catalyst. The solid was then triturated with ethyl acetate, and the resulting solution was concentrated to yield 200 mg (88%) of the desired compound. ^{1}H NMR ($CDCl_3$) 2.22 (s, 3H), 6.97 (d, J = 8.1 Hz, 2H), 7.22 (m, 2H), 7.44 (d, J = 8.4 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H); ^{13}C NMR ($CDCl_3$): 24.3, 116.7, 122.5, 129.6, 134.4, 142.7, 151.4, 169.3. MALDI-FTMS for $C_{14}H_{14}NO_2$ ($M + H^+$) calculated 228.1019, found 228.1022.

2-Hydroxy-5-nitrobiphenyl. 2-Hydroxybiphenyl (2 g, 11.8 mmol) was dissolved in glacial acetic acid (50 mL) and cooled to 0 °C. To this solution, concentrated nitric acid (680 μL) in glacial acetic acid (10 mL) was added dropwise via addition funnel. The reaction was then warmed to room temperature and stirred vigorously for 2 h. Saturated aqueous $NaHCO_3$ (300 mL) was then added slowly, and the aqueous layer was extracted with CH_2Cl_2 (3 × 200 mL). The combined organic layers were then dried on $MgSO_4$ and concentrated. Purification on silica with EtOAc/hexane (1:9) yielded the desired compound as an orange powder (475 mg, 19%). ^{1}H NMR ($CDCl_3$) 6.98 (d, J = X Hz, 1H), 7.40 (m, 3H), 7.57 (m, 2H), 8.09 (m, 1H), 8.18 (s, 1H); ^{13}C NMR ($CDCl_3$) 117.2, 123.8, 124.6, 125.9, 127.8, 128.2, 129.9, 135.4, 140.2, 153.9. MALDI-FTMS for $C_{12}H_{10}NO_3$ ($M + H^+$) calculated 216.0655, found 216.0654.

2-Hydroxy-5-aminobiphenyl (16). 2-Hydroxy-5-nitrobiphenyl (25 mg, 0.12 mmol) was dissolved in methanol (30 mL), and Pd/C (5%, 5 mg) was added. The reaction was then shaken on a Parr shaker with hydrogen (40 psi) for 2 h. The crude mixture was then passed through a pad of Celite and concentrated to give the desired compound (21 mg, 98%). ^{1}H NMR ($CDCl_3$) 6.78 (m, 3H), 7.29 (m, 3H), 7.54 (s, 2H); ^{13}C NMR ($CDCl_3$) 116.6, 117.9, 126.2, 128.4, 131.9, 142.6, 153.8. MALDI-FTMS for $C_{12}H_{12}NO$ ($M + H^+$) calculated 186.0913, found 186.0914.

2-Hydroxy-5-acetamidobiphenyl. 2-Hydroxy-5-aminobiphenyl (150 mg, 0.81 mmol) was dissolved in CH_2Cl_2 (10 mL), and DIEA (157 mg, 212 μL, 1.22 mmol) was added. The mixture was cooled to 0 °C, acetyl chloride (70 mg, 63 μL, 0.89 mmol) was slowly added, and the reaction was stirred 12 h at room temperature. The crude diacetyl product was then treated with a solution of 1 M NaOH and methanol (1:3; 10 mL) and stirred for an additional 2 h. The reaction was then concentrated and purified by preparative TLC (5% MeOH/CH_2Cl_2) to yield 114 mg of the desired product (62%). ^{1}H NMR ($CDCl_3$) 2.15 (s, 3H), 6.82 (m, 1H), 7.35 (m, 5H), 7.57 (m, 2H); ^{13}C NMR ($CDCl_3$) 24.1, 114.2, 118.9, 122.2, 125.4, 128.1, 129.5, 134.2, 136.7, 152.1, 168.2. MALDI-FTMS for $C_{14}H_{14}NO_2$ ($M + H^+$) calculated 228.1019, found 228.1015.

4-Amino-4′-hydroxybiphenyl (19). 4-Nitro-4′-hydroxybiphenyl (100 mg, 0.47 mmol) was dissolved in ethyl acetate (40 mL), and palladium on carbon was added (10%, 10 mg). The resulting suspension was then hydrogenated at 36 psi for 18 h at room temperature. The reaction was then filtered through a cake of Celite and concentrated to yield a yellow-brown solid (81 mg, 93%). ^{1}H NMR ($CDCl_3$) 6.68 (m, 4H), 7.28 (m, 4H); ^{13}C NMR ($CDCl_3$) 115.4, 116.2, 126.0, 127.9, 129.6, 139.3, 145.8, 158.5. MALDI-FTMS for $C_{12}H_{12}NO$ ($M + H^+$) calculated 186.0913, found 186.0911.

4-*N*-Acetamido-4′-hydroxybiphenyl. 4-Amino-4′-hydroxybiphenyl (89 mg, 0.48 mmol) was dissolved in THF (50 mL), and DIEA (93 mg, 126 μL, 0.72 mmol) was added. The mixture was cooled to 0 °C, acetyl chloride (42 mg, 38 μL, 0.53 mmol) was slowly added, and the reaction was stirred 12 h at room temperature. The crude diacetyl product was then treated with a solution of 1 M NaOH and methanol (1:3; 10 mL) and stirred for an additional 2 h. The reaction was then concentrated and purified by preparative TLC (5% MeOH/CH_2Cl_2) to yield 63 mg of the desired product (58%). ^{1}H NMR ($CDCl_3$) 2.38 (s, 3H), 7.19 (m, 4H), 7.62 (m, 4H); ^{13}C NMR ($CDCl_3$) 24.5, 116.8, 120.6, 124.3, 125.8, 132.6, 142.0, 145.3, 158.6, 169.2. MALDI-FTMS for $C_{14}H_{14}NO_2$ ($M + H^+$) calculated 228.1019, found 228.1021.

Preparation of Immunoconjugates. Hapten (5 mg) was treated with 1.3 equiv of 3-sulfo-*N*-hydroxysuccinimide and 1.3 equiv of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride for 24 h in DMF. This reaction was then added to solutions (5 mg/mL) of keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) in PBS (50 mM, pH 7.4), respectively, and allowed to react 24 h at 4 °C.

Hybridoma Production. See ref 21.

Kinetic Assays. Antibody stock concentrations were determined by UV−vis spectroscopy using 1 OD = 1.25 mg/mL at 280 nm and 1 mg/L = 6.7 μM. The assay was initiated by the addition of a solution of antibody (20 μM in PBS) to a solution of 4-phenoxyaniline **13** (200 μM) in buffer (10 mM PBS, pH 7.4) with 2% acetonitrile in 4 mL glass vials. High-intensity photolysis was performed for 30 min intervals, and at times throughout the assay, aliquots (20 μL) were removed and directly injected onto a RP-C18 Vydac HPLC column, equipped with a guard column (HPLC conditions, Isocratic mobile phase of acetonitrile/water (20:80) with 0.1% TFA. Solvent flow rate of 1 mL min^{-1} and detection at 254 nm). Biphenyl product **16** (retention

time = 5.2 min) was determined by interpolation of peak height and area values relative to standard curves.

ELISA Binding Experiments. An ELISA plate (CoStar; 96-well) was coated uncovered overnight at 37 °C with 25 μL of the MT4-BSA conjugate diluted to 5 μg/mL. To this, methanol (50 μL/well) was added and allowed to sit 5 min at room temperature. The methanol was then removed from the wells, and the plates were allowed to dry. Wells were then blocked with 50 μL of Blotto (5% skim milk powder in PBS) for 10 min at 37 °C. Typically, 25 μL of a 1:1000 dilution of mAbMT4-3G2 in Blotto were then added and incubated 1 h at 37 °C. After washing, 25 μL of a 1:1000 dilution of a goat-anti-mouse/horseradish peroxidase conjugate in Blotto were added and incubated for 30 min. The plate was developed with the colorimetric reagent ABTS for 30 min, and the absorbance was measured on an ELISA plate reader at 405 nm.

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