NMR to follow proton transfer is not clear. Such studies are rather scarce (*12–15*), and none of them have been conclusive for the proton transfer dynamics.

The symmetric double minimum potential for the asymmetric hydrogen bond between peptide units shows that such potentials can occur even for hydrogen bonds that are not formally symmetric (A···H···A). However, this picture has been derived within the Born-Oppenheimer approximation applied at two distinct levels: (i) the electrons follow the atomic nuclei adiabatically, and (ii) the light protons follow the heavy atoms adiabatically. Use of INS reveals that this framework is not adequate to describe the dynamics in hydrogen bonds.

The impact of INS spectroscopy is bound to extend far beyond the study of proton transfer along hydrogen bonds. Many fields of research concerned with proton mobility in the solid state will benefit from recent and future developments of INS techniques.

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RESEARCH ARTICLE

Antibody Catalyzed Cationic Cyclization

Tingyu Li, Kim D. Janda,* Jon A. Ashley, Richard A. Lerner*

Two major goals for the design of new catalysts are the facilitation of chemical transformations and control of product outcome. An antibody has been induced that efficiently catalyzes a cationic cyclization in which an acyclic olefinic sulfonate ester substrate is converted almost exclusively (98 percent) to a cyclic alcohol. The key to the catalysis of the reaction and the restriction of the product complexity is the use of antibody binding energy to rigidly enforce a concerted mechanism in accord with the design of the hapten. Thus, the ability to direct binding energy allows the experimenter to dictate a reaction mechanism which is an otherwise difficult task in chemistry. New catalysts for cationic cyclization may be of general use in the formation of carbon-carbon and carbon-heteroatom bonds leading to multiring molecules including steroids and heterocyclic compounds.

The study of carbocations has added to the understanding of reaction mechanisms in organic chemistry (Fig. 1) (*1*). Nevertheless, controlling the reaction pathways of this highly reactive species is not easy. Among the many transformations where carbocations appear on the reaction pathway, cationic cyclization is one of the most important carbon-carbon bond forming processes in chemistry and biochemistry (*2–17*). Cationic processes are central to many synthetic strategies as well as the polyene cyclization cascade that leads to the formation of steroids. Some of the earliest synthetic work on cationic cyclization reactions was that of Johnson and his colleagues, who studied the formation of six-membered rings from acyclic unsaturated compounds (*2*). Their work led to the recognition that the process must be initiated in a way that generates a cationic center on carbon without affecting the olefinic bonds.

Typically, a cationic cyclization reaction is initiated by the formation of a carbocation, either by electrophilic addition to a double bond or by ionization at a sp^3 hybridized carbon. The reaction is thought to proceed via a transition state in which the reactants adopt a quasi-chairlike conformation, thereby allowing participation of the olefinic bond in what is essentially a concerted transformation.

To catalyze cationic cyclization requires control of the initial generation and stabilization of the carbocation as well as the attendant entropic and stereoelectronic parameters intrinsic to the cyclization reaction. Antibodies should, in principle, be ideal catalysts for initiation and control of this process in that they have been shown to be capable of catalyzing complex reactions in which it was necessary to simultaneously neutralize point charges, overcome entropic barriers, and provide a chiral binding pocket for stereoselectivity (*18*). In essence, the problem reduces to that of generation of the carbocation in an environment that fosters and controls the cyclization reaction. We now describe the implementation of these concepts to achieve antibody catalyzed cationic cyclization.

We studied the classical system of Johnson in which the initiating carbocation is formed by the solvolysis of sulfonate esters; we used **1** as a substrate (Fig. 2) (*2*). At acidic pH several zwitterionic or cationic species or both (Fig. 2) were developed in the transition state, of which **2** is representative. Although such systems can undergo solvolysis-cyclization reactions with electron-rich olefins, they are not always useful because the yield is poor and complex mixtures of products are produced (*19*). However, antibody catalysis permits selective control of the mechanism of the solvolysis of **1**, thereby reducing the complexity of the reaction and improving the yield of desired products.

The reaction and hapten design. To catalyze the cationic cyclization of **1**, we designed a hapten that induced an antibody that simultaneously facilitates the cleavage of the sulfonate and controls the conformation of the substrate in the transition state such that the olefin is properly aligned to participate in the reaction. The cyclic *N*-oxide **3** would seem to be an ideal hapten to induce antibodies capable of catalyzing release of the sulfonate from **1**. The anionic oxygen should elicit a functionality in the antibody capable of operating by way of a process that we have termed "bait and switch" catalysis to stabilize the developing negative charge on the departing sulfonate

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(*20*). Such interaction with the leaving group was necessary because our sulfonate ester was not as activated as the nosylate used in the original Johnson system (*19*). Likewise, the cationic nitrogen can be expected to induce an anionic functionality in the antibody combining site, which should stabilize the developing carbocation so that it is not prohibitively high in energy (*18*).

We envisioned that the reactants would adopt a quasi-chairlike conformation in the transition state with the leaving group in the pseudo-equatorial position and the olefinic bond aligned to participate in what is essentially a concerted transformation (Fig. 2). The expected chair conformation of the cyclic *N*-oxide hapten should induce an antibody which favors a similar conformation of the reactants in the transition-state, thereby facilitating an energetically favorable route for the cyclization process (Fig. 2). Proper alignment of the olefin is further ensured by conjugation with the equatorial carbon-silicon bond, which correctly positions the pi orbital for backside attack on the carbon atom.

Once the cyclization takes place, the newly formed carbocation can be captured, for example, by elimination or attack by an internal or external nucleophile (*16*). The dimethylphenyl silyl group (silane) was included in the molecule to give mechanistic information by offering yet another alternative for capture of the carbocation via loss of the silane and formation of the olefin, a process that occurs readily in organic solvents. In addition, the silane might be expected to participate in the cyclization by resonance stabilization of the incipient carbocation β to the silicon (β effect) (*21*). Both formation of the olefin via loss of the silane and stabilization of the carbocation were expected to be contingent on the ability of the molecule to adopt a conformation in the antibody binding site compatible with the stereoelectronic requirements of these processes.

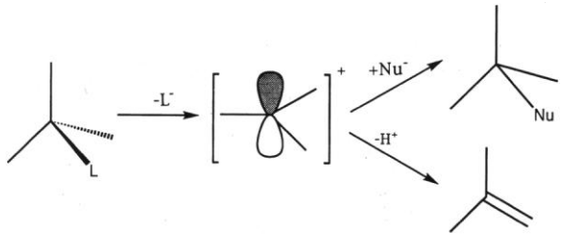


Fig. 1. Generic reaction pathway involving a carbocation. The leaving group (L) in the starting material departs from a tetrahedral carbon to generate a planar carbocation in which the empty *p*-orbital is orthogonal to the plane containing the remaining three substituents. The reaction terminates by nucleophilic addition or loss of a proton.

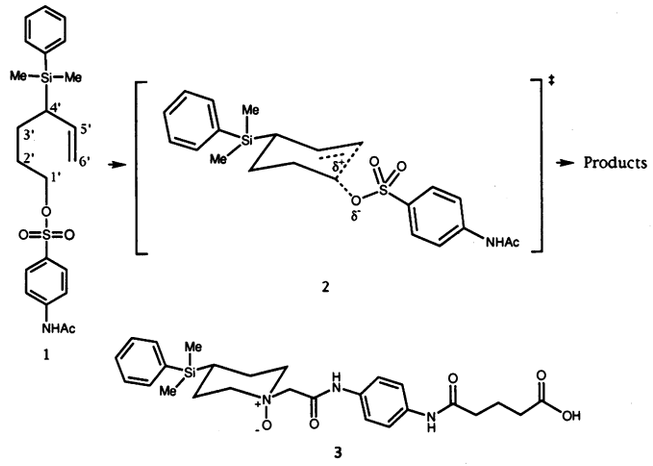


Fig. 2. Reaction mechanism and hapten design for cationic cyclization.

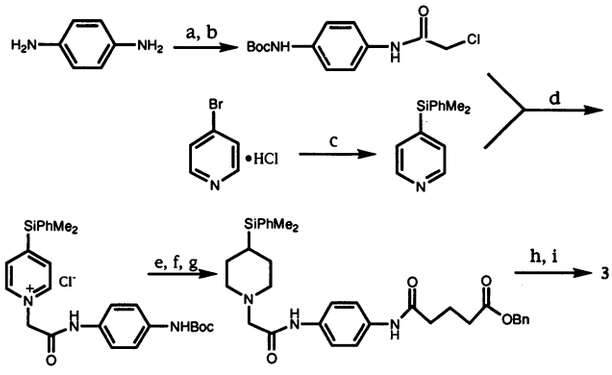


Fig. 3. Synthesis of hapten **3**. Conditions: (a) Boc_2O, di-*tert*-butyl dicarbonate; 64 percent yield. (b) Chloroacetylchloride, 94 percent. (c) *n*-BuLi, *n*-butyllithium; chlorodimethylphenylsilane; 30 percent. (d) NaI (cat), sodium iodide (cat); 70 percent. (e) H_2/PtO_2, hydrogen–platinum (IV) oxide; 80 percent. (f) HCl/dioxane, hydrogen chloride/dioxane; 100 percent. (g) Benzylglutaryl chloride; 82 percent. (h) MCPBA, 3-chloroperoxybenzoic acid; 100 percent. (i) NaOH, sodium hydroxide; 50 percent.

The hapten **3**, which includes a five-carbon spacer, was prepared from 4-bromopyridine hydrochloride and 1,4-phenylenediamine (Fig. 3). Because the overall shape of the hapten was important to this study, we used nuclear magnetic resonance (NMR) to study the conformation of **3** in solution. The silicon appendage and the phenylenediamide moiety were unambiguously assigned to equatorial positions by nuclear Overhauser effect (NOE) measurements (*22*). This result is consistent with previous findings that place the oxygen atom on *N*-oxides in the axial orientation (*23*). While the axial orientation of the oxygen atom might not be considered optimal for induction of a functionality to stabilize a pseudo-equatorial leaving group, the distances between a pseudo-equatorial and an axial atom are close enough to expect stabilization by the induced complementary functionalities in at least some antibodies.

Substrates. In addition to **1**, six other possible substrates (**4**, **5**, **6**, **7**, **8**, **9**) are illustrated in Fig. 4 and the synthesis of all substrates in racemic form is shown in Fig. 5. Sulfonate ester **1** is expected to be the best substrate for antibodies elicited to hapten **3**. The other substrates were synthesized to probe the course of the reaction mechanism. Substrate **4** is analogous to **1**, but a methyl group was introduced into the 1′ position to determine whether a primary or secondary carbocation is preferred at the initiating carbon center. Substrates **5** and **6** were designed to investigate whether the silicon heteroatom influences the reaction sequence. Compounds **7** and **8** were prepared to explore whether the double bond participates via anchimeric assistance (neighboring-group participation) in the solvolysis of the sulfonate. The epoxide-containing compound **9**, while obviously less similar to the hapten than any of the other substrates, was used to investigate the possibility of effecting a cationic ring closure with an initiating event that more closely mimics what occurs in nature.

Antibody induction and catalysis. *N*-Oxide **3** was coupled to keyhole limpet hemocyanin (KLH) and the conjugate was used to immunize 129 $G1X^+$ mice for pro-

duction of monoclonal antibodies (*24*). Twenty-nine monoclonal antibodies were shown by an enzyme-linked immunosorbent assay (ELISA) (*25*) to bind **3** conjugated to bovine serum albumin (BSA) (*26*). Each of the cloned 29 cell lines was injected into mice for production of ascites fluid. Antibody from each sample of ascites fluid was purified by salt precipitation, anion exchange, and affinity chromatography (*27*).

To analyze the set of 29 monoclonal antibodies for catalytic activity we used several assays of which the first was based on high-performance liquid chromatography (HPLC). This assay consisted of incubating the antibodies in a biphasic reaction mixture (83 percent pentane, 2 percent chloroform, 15 percent 50 mM bis-tris, pH 7.0), and following the reaction for the release of 4-acetamidobenzenesulfonic acid (*28*). We have previously shown that for some antibodies catalysis under biphasic conditions has no detrimental effects on either the structural integrity of the antibody or on its kinetic parameters (*29*). From this study, four antibodies (4C6, 16B5, 1C9, and 6H5) were identified as potential "initiating-catalysts" on the basis of their ability to catalyze the cleavage of the sulfonate ester bond in **1**. To ascertain the nature of the terminated products, we used gas chromatography (GC) (*30*). Analytical GC was used for one of these four antibodies (4C6), which was chosen on the basis of its rate of sulfonate ester cleavage. The products observed from the 4C6 antibody-catalyzed reaction were cyclohexene **11** (2 percent) and *trans*-2-dimethylphenylsilylcyclohexanol **12** (98 percent) (Fig. 6). Other potential products (**13**–**15**) were not observed. Only the trans isomer of **12** was observed but the absolute configuration has not yet been determined. The mass balance of the products matched the production of 4-acetamidobenzenesulfonic acid (*31*). Such a narrow distribution and accountability of products is important because typically a plethora of cyclized and uncyclized compounds can be expected from cationic cyclization reactions conducted under solvolysis conditions (Fig. 6). We attribute the almost singular production of the cyclized product, **12**, to both the antibody's ability to enforce a pseudo-cyclic transition state and its capacity to trigger the reaction under conditions so mild that there is no detectable background reaction.

Finally, antibody 4C6 is more than a simple initiating catalyst because not all antibodies that accelerated sulfonate ester cleavage led to the formation of cyclization products. For example, antibody 16B5 displayed an initial rate enhancement for the release of 4-acetamidobenzenesulfonic acid from **1**, but this antibody-accelerated process furnished no straight-chain or cyclic products. We ascribe this lack of productivity to an alkylation event within the antibody's combining site, either as the result of an irreversible S_N2 type process, or the trapping of the carbocation by the antibody after release of the sulfonate. Two pieces of evidence suggest that, unlike 4C6, antibody 16B5 is alkylated. First, the reaction accelerated by antibody 16B5 never proceeded beyond one turnover. Second, this single turnover event destroyed the ability of the immunoglobulin to bind to the hapten **3** (*32*).

Kinetic analysis and mechanism. The most active antibody, 4C6, was studied in more detail using our biphasic solvent conditions at pH 7.0. The initial rate of cleavage, measured as a function of substrate **1** concentrations, followed Michaelis-Menten kinetics (k_{cat} = 0.02 min^{-1}, K_m = 230 μM, for sulfonate release). Multiple turnovers could be seen and no product inhibition was observed even when the reaction was allowed to proceed to more than 70 percent of reacted starting material, a finding likely attributable to the favorable partitioning of products into the organic layer. The efficiency of the antibody-catalyzed reaction is underscored by the fact that even after several days no solvolysis of sulfonate ester **1** was observed without 4C6 present (detection limits < 0.1 μM), thereby precluding determination of k_{uncat}. In

Fig. 4. Additional substrates to probe the reaction mechanism.

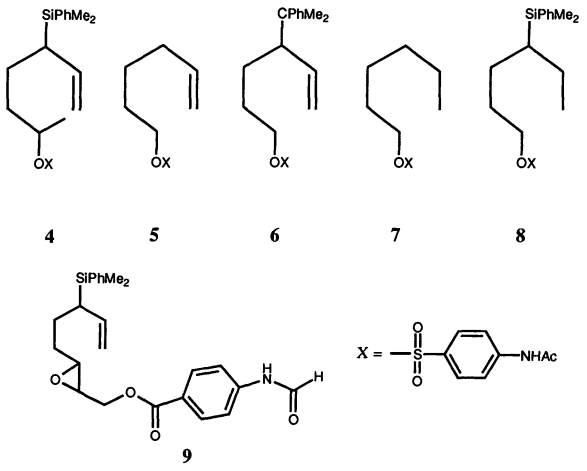
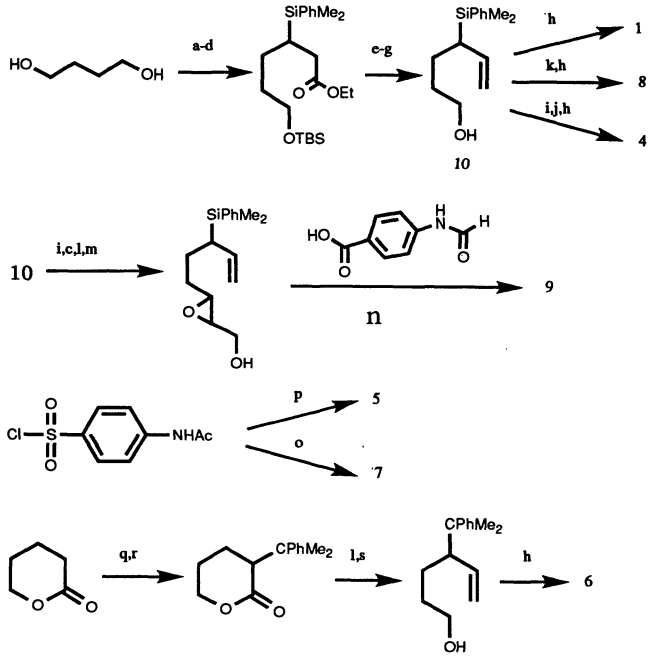


Fig. 5. Synthesis of substrates. (a) NaH, sodium hydride; TBSCl, *tert*-butyldimethylsilyl chloride, 50 percent yield. (b) Swern, 99 percent. (c) (Carbethoxymethylene)-triphenylphosphorane, 62 percent. (d) $(PhSiMe_2)_2CuLi$, lithium bis(phenyldimethylsilyl) cuprate; 88 percent. (e) LAH, lithium aluminium hydride; 96 percent. (f) *n*-Bu_3P, tributylphosphine; 2-nitrophenyl selenocyanate; hydrogen peroxide; 88 percent. (g) HF, hydrofluoric acid; 95 percent. (h) *N*-acetylsulfanilyl chloride; DMAP, 4-dimethylaminopyridine; 50 percent. (i) PCC, pyridinium chlorochromate; 80 percent. (j) MeLi, methyllithium; 90 percent. (k) H_2-Pd, hydrogen-palladium on activate carbon; 95 percent. (l) DIBAL-H, diisobutylaluminum hydride; 80 percent. (m) *Tert*-BuOOH, *tert*-butyl hydroperoxide; $Vo(AcAc)_2$, vanadium (III) acetylacetonate; 20 percent. (n) DCC, 1,3-dicylohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; 65 percent. (o) 1-hexanol; 60 percent. (p) 5-hexen-1-ol; 60 percent. (q) LDA, lithium diisopropylamide; TMSCl, chlorotrimethylsilane; 80 percent. (r) 2-phenyl-2-propylacetate; $ZnCl_2$, zinc chloride; 85 percent. (s) Methyltriphenylphosphonium bromide, *n*BuLi, *n*-butyllithium; 60 percent.



addition, **3** was a potent inhibitor of the antibody catalyzed reaction (K_i = 1.0 μM).

To explore the mechanism of the cyclization reaction, we examined compounds **4** to **9** as potential substrates. The most interesting comparison was between compounds **1** and **8**, which differ only by the presence of an olefin. Only **1** is a substrate for the reaction, thereby giving evidence that the olefin assists in the departure of the sulfonate ester via anchimeric assistance, and the entire process proceeds by a concerted pi route. The failure of the silane to depart also provides mechanistic information about the antibody-catalyzed process. Termination of a fully enforced concerted process with the silane departing from the equatorial position would require formation of the highly unfavorable *trans*-cyclohexene. Thus, for termination to proceed concertedly, nucleophilic trapping of the incipient carbocation must occur, which in this case simply involves the addition of water.

Sulfonate **4**, the closest homolog of **1**, was found to be a substrate for 4C6 (k_{cat} = 0.3 min^{-1}, K_m = 1.8 mM, k_{cat}/k_{uncat} = 12,000, for sulfonate release), but clean cyclization was not observed. Instead, antibody-catalyzed reaction of **4** yielded a complex mixture of products, which were not analyzed further. The differences in products for substrates **1** and **4** may simply reflect the variances in reaction pathways open to the reactants. In the catalytic transformation of **1**, the obligatory anchimeric assistance by the olefin necessarily leads to a restriction in product complexity. Such constraints do not pertain to **4** where a more stable secondary carbocation appears on the pathway. It is well known for many reactions that, when the possibility of alternative stabilization of the carbocation exists, the reaction may proceed by a route that no longer involves anchimeric assistance (*33*).

Compound **6**, which differs from **1** only by substitution of a carbon for a silicon atom, is not only a poor substrate (k_{cat} = 7.8 × 10^{-4} min^{-1}, K_m = 330 μM, for sulfonate release) but also yielded a complex mixture of products. This suggests that the silicon atom plays an important role in the reaction by activation of the olefin, or participation in the overall stabilization of the cationic process, or both. Furthermore, the silane may be conjugated to the olefin, thereby ensuring its proper alignment in the transition state. The ability to simultaneously initiate cationic cyclization by ionization and retain the silane is unprecedented and is allowed by the mild conditions of antibody catalysis. Under the usual solvolysis conditions (formic acid, 80°C) (*2*, *19*), we observed loss of the silane from substrate **1** in a matter of minutes (*34*).

Compounds **5**, **7**, and **8** were ineffective substrates for antibody 4C6, and **8** is, in fact, an inhibitor of the reaction (K_i = 200 μM). These findings suggest that the dimethylphenyl functionality plays an important role in antibody-substrate recognition, but this recognition event by itself does not provide enough binding energy to initiate cleavage of the sulfonate ester. Thus, the effectiveness of our catalyst appears to be governed by several factors including stabilization of point charges, provision of sufficient binding to overcome entropic barriers, and the more complicated stereoelectronic effects which can be provided by precise control of the conformation of the reactants. Finally, the epoxide-containing compound, **9**, was not a substrate for the antibody catalyst 4C6. Presumably, an overall lack of congruency with hapten **3** precludes compound **9** from being a substrate for antibody catalysis.

In summary, we have elicited antibodies that catalyze the process of cationic cyclization. This reaction is interesting for several reasons, not least of which is that carbon-carbon bond formation has been catalyzed. These results again illustrate the power of merging the understanding of a reaction mechanism with the ability of the immune system to yield binding proteins programmed to interact in highly specific ways as the substrate traverses the reaction coordinate. The ability to dictate the reaction mechanism by design allows unprecedented control of the product outcome. But a particular feature of our study is the fact that the cyclization that we have catalyzed can be considered an initiator that is capable of starting the cascade of contingent events implicit in chemical processes involving carbocations. As such, our work may be considered a first step toward the generation of catalytic antibodies that mimic the natural enzymes that achieve polyene cyclization. If these goals can be accomplished, catalytic antibodies may allow the generation of carbon-heteroatom bonds, complex steroid molecules, and other compounds produced by cationic cyclization and not easily attainable by organic synthesis. Apart from discovering new carbon-carbon bond-forming reactions, further studies should provide better understanding of the critical enzymological factors involved that function in polyene cyclization reactions.

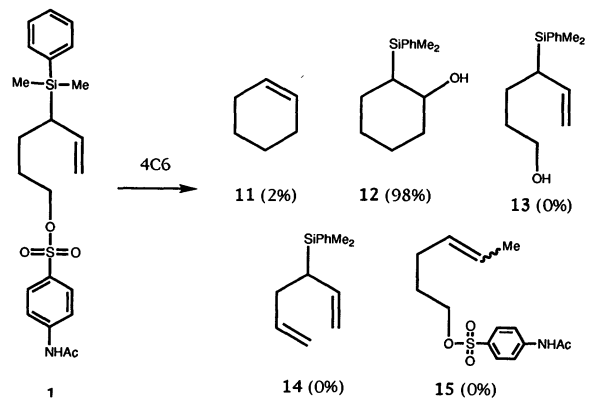


Fig. 6. Product distribution from antibody 4C6-catalyzed reaction.

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22. The NMR coupling constants observed support a chair conformation for piperidine *N*-oxide **3** with the silicon appendage occupying an equatorial position. An NOE was observed between the methylene protons of the phenylenediamide linker and the α (to the *N*-oxide) methylene protons of the piperidine *N*-oxide unit. An NOE effect was not detected between the β (to the *N*-oxide) methylene protons of the piperidine *N*-oxide moiety and the methylene protons of the phenylenediamide appendage. These latter observations provide strong support that the phenylenediamide moiety occupies an equatorial position in space.
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26. After 2 weeks, the antibodies produced by wells containing macroscopic colonies were assayed by ELISA for binding to **3**. Colonies that initially produced antibodies that bound **3** were subcloned twice, after which 29 remained active. The subtype distribution of the 29 monoclonal antibodies was as follows: 15 were immunoglobulin G1 (IgG1), 9 were $IgG2_a$, 3 were $IgG2_b$, and 2 were IgG3. All 29 monoclonal antibodies were injected into pristine-primed 129G1X$^+$ × BALB/c mice to generate ascitic fluid.
27. The γ-globulin–containing fractions from ascitic fluid were precipitated by dropwise addition of saturated ammonium sulfate at 4°C, pH 7.2, until a final concentration of 45 percent was achieved. The ammonium sulfate was removed by dialysis against 10 mM tris, pH 8. The concentrated antibodies were then purified by anion exchange chromatography on DEAE-Sephacel and eluted with a stepwise salt gradient (50 to 500 mM NaCl). The antibodies that eluted in the 100 mM NaCl fraction were concentrated by ultrafiltration before affinity purification on a protein G–Sepharose column. The antibody was placed on the column, and nonadherent material was removed by extensive washing (20 to 30 column volumes). The column was eluted with 0.05 M citric acid, pH 3.0, and fractions were immediately neutralized by collecting into 1 M tris, pH 9.0. All antibodies were then concentrated and dialyzed into 50 mM Bis-tris, pH 7.0, and assayed by HPLC or GC.
28. The biphasic reactions were performed in Polyallomer Eppendorf tubes at 23°C on an IKA-VIBRAX-VXR vortexer shaking at 1200 to 1400 rev/min. Typical reactions were 1 ml in total volume and all contained final concentrations (v/v) of 83 percent pentane, 2 percent chloroform, and 15 percent bis-tris buffer (50 mM, pH 7.0). Catalyzed reactions were performed in the presence of 2 μM monoclonal antibody and were initiated by the addition of various amounts of substrate from a chloroform stock solution. The antibody was stored in 50 mM, pH 7.0 bis-tris buffer; the concentration was determined from an ultraviolet absorbance assay [$\epsilon_{280} = 1.35$ (mg/ml)$^{-1}$ cm^{-1}] and an assumed molecular mass of 150,000 for the immunoglobulin G. Initial velocities and other kinetic parameters for the sulfonate ester cleavage reaction were obtained with HPLC on an analytical reversed-phase C-18 column (VYDAC 218TP54) eluting with an isocratic mobile phase of water (0.1 percent trifluoroacetic acid). Formation of the sulfonate product (calibrated by both peak height and area) was followed at 254 nm by taking 50-μl (aqueous) samples from individual reactions removed from the mechanical shaker at appropriate times.
29. J. A. Ashley and K. D. Janda, *J. Org. Chem.* **57**, 6691 (1992); C. G. Shevlin, S. A. Hilton, K. D. Janda, *Bioorg. Med. Chem. Lett.* **4**, 297 (1994).
30. While the initial sulfonate product of these biphasic reactions was soluble only in the aqueous phase, the resulting straight chain and cyclized products were soluble and detectable only in the organic phase. Formation of these products was followed by injecting small portions from the pentane layer into a Hewlett-Packard 5890A Gas Chromatograph with an Alltech Econo-cap column (30 m by 0.25 mm). Cyclohexene formation was followed at a column temperature of 40°C, and injector temperature of 100°C. All other products were followed at column and injector temperatures of 200° and 250°C, respectively. The amount of product was determined by comparison to calibrated peak areas of known product concentrations.
31. As one example, a reaction was set up containing 6 μM 4C6 antibody and 200 μM substrate **1** under the same conditions and with the same procedures as described (*28*). The reaction was allowed to shake for 24 hours and was then halted by separation of the two phases. The aqueous phase was assayed by the same HPLC as described (*28*) and was found to contain 64 ± 4 μM 4-acetamidobenzenesulfonic acid product. At the same time, GC assays (*30*) were used to observe 1.5 ± 0.2 μM cyclohexene product **11** and 68 ± 7 μM cyclohexanol product **12** in the organic phase. The product concentrations reported from each phase were calibrated on the basis of the total (1 ml) volume of the reaction.
32. A similar reaction assay (*28*) was used with antibody 16B5. Three experiments were performed: (i) 16B5 (10 μM), buffer, and no substrate (antibody control); (ii) 16B5 (10 μM), biphasic conditions, and no substrate (antibody-biphasic control); and (iii) 16B5 (10 μM), biphasic conditions, and substrate **1** (200 μM). Reaction assays (ii) and (iii) were shaken for 24 hours; reactions (ii) and (iii) were halted by the removal of the aqueous layer containing the antibody. Individual ELISA's were performed on the three antibody experiments (as shown below) to determine the binding competency of 16B5. The antigen **3**-BSA was coated on a 96-well ELISA plate (0.12 μg per well), and antibody solutions were diluted in serial twofold dilutions starting at 1:1000. The titers of the reaction assays (i) to (iii) were: (i) 64,000, (ii) 64,000, and (iii) 2,000. These findings demonstrate that substrate **1** reduces the binding ability of 16B5 by more than 95 percent.
33. J. March, *Advanced Organic Chemistry* (Wiley, New York, ed. 3, 1985), pp. 312–326; P. G. Gassman, J. Zeller, J. T. Lumb, *J. Chem. Soc. Chem. Commun.* **1968**, 69 (1968).
34. Sulfonate **1** (3.8 mg, 8.8 μmol) was stirred with formic acid (1 ml) and sodium formate (3.0 mg) at 80°C. After 5 minutes, all the starting material was converted to the desilylation product **15** as indicated by thin-layer chromatography (50 percent ethylacetate in hexanes).
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