in all examined metaphases of SQ-20B. These chromosomes had apparently formed stable markers represented by M3, M11, M16, and M18 (Fig. 3). Human c-*raf*-1 has been localized on chromosome 3 [3p25 (*11*)]. Double-minute chromosomes representative of amplified DNA sequences (*12*) were found in NIH/3T3 cl-1 (Fig. 3) and may account for the amplified *raf* hybridization apparent in this and other transfected clones.

Similar transfection-mediated identifications of the c-*raf* protooncogene have been noted for DNAs obtained from carcinomas of the stomach (*3*) and liver (*5*), as well as from a glioblastoma (*4*). It seems likely that a truncated form of c-*raf* is responsible for transformation of transfected NIH/3T3 cells. In those instances where the recombinational limits can be approximated, removal of at least the first five or six exons has led to activation. Analysis of the predicted amino acid sequence of c-*raf*-1 (*8*) reveals that these exons encode domains that exhibit strong primary and secondary structural homology to the ligand-binding region of the protein kinase C family (*13, 14*). The region of highest homology encompasses the two cysteine "fingers" of protein kinase C and the related sequences of c-*raf*-1 (exons 3, 4, and 5). By analogy to protein kinase C, events associated with ligand binding may result in alterations in protein conformation so as to allow adenosine 5′-triphosphate (ATP) binding and subsequent phosphorylation mediated by the catalytic (kinase) domain. Thus, one might surmise that removal of regions capable of directly or indirectly blocking ATP binding may constitutively activate the kinase. The v-*raf* (*15*) and v-*mil* (*16*) fusion proteins would thus be examples of activated kinases, whose expression in infected cells is also unrestrained.

We believe the c-*raf*-1 product is related to the tumor phenotype for three reasons. First, the frequency of its identification by NIH/3T3 transfection of SQ-20B DNA suggests the presence of some genetic abnormality within the c-*raf*-1 locus, perhaps related to that described by Chang *et al.* (*17*). The nature of this abnormality remains to be elucidated. Second, since we have found that NIH/3T3 cells in which unrestrained normal c-*raf*-1 expression occurs are malignant in nude mice (*18*), similar unregulated expression in another competent cell may also contribute to its transformed phenotype. Finally, by means of the same approaches described in this report, two additional squamous cell carcinomas of head and neck origin (JSQ-3 and SCC-35) have yielded DNAs that produced NIH/3T3 foci in which truncated human c-*raf*-1 loci were frequently found. As with the SQ-20B–derived transformants, the majority of these transfectants were tumorigenic in athymic nude mice (*10*).

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22. We thank W. D. Peterson for karyotype analysis, B. K. Chun and E. Bergoffer for histopathology, W. Lancaster for papilloma virus screening, and S. Hawkins for secretarial assistance. Supported by the Department of Radiation Medicine and NIH grant CA425969 to R.R.W.

3 February 1987; accepted 27 May 1987


# A Stereospecific Cyclization Catalyzed by an Antibody


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**A monoclonal antibody elicited by a transition-state analog that is representative of an intramolecular six-membered ring cyclization reaction acted as a stereospecific, enzyme-like catalyst for the appropriate substrate. Formation of a single enantiomer of a δ-lactone from the corresponding racemic δ-hydroxyester was accelerated by the antibody by about a factor of 170, which permitted isolation of the lactone in an enantiomeric excess of about 94 percent. This finding demonstrates the feasibility of catalytic-antibody generation for chemical transformations that require stereochemical control.**


RECENT EXPERIMENTS HAVE BEGUN to exploit the repertoire of receptor molecules that can be generated by the immune system for chemical transformations through strategies designed to elicit antibodies capable of selective chemical catalysis (*1, 2*). Since antibodies can be produced against virtually any antigen with precise structural specificity (*3*), the possibility exists for a general catalytic method for the synthesis and functionalization of mole-


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**Table 1.** Kinetic parameters for the cyclization of ester **1** by monoclonal antibody 24B11. Velocities were determined spectrophotometrically by measuring the initial linear absorbance change at 271 nm. The antibody (2 μ*M*, from Lowry assay, with a molecular weight of 150,000 for immunoglobulin G) was first incubated at 25°C in 100 m*M* KCl in 25 m*M* phosphate buffer at *p*H 7.0. Reactions were initiated by addition of varying amounts of a stock solution of ester **1** to give a substrate concentration of 20 to 100 μ*M*. Ester **1** was obtained by deprotection of the trimethylsilyl derivative with 5% citric acid in methanol followed by dilution with 25 m*M* phosphate buffer, *p*H 7.0, to give the desired concentration of stock solution. The concentrate was stored frozen at −80°C until immediately prior to use. The first-order rate constant ($k_{uncat}$) for the cyclization in the absence of antibody was measured similarly and used to correct the initial rate data, which were used to obtain kinetic parameters from the Lineweaver-Burk plots shown in Fig. 4.

| $K_M$* (μ*M*) | $K_i$ (μ*M*) | $V_{max}$ (μ*M* min$^{-1}$) | $k_{cat}$ (minute$^{-1}$) | $k_{uncat}$ (minute$^{-1}$) | $k_{cat}/k_{uncat}$ |
|---|---|---|---|---|---|
| 76 | 0.25 | 0.99 | 0.50 | 0.003 | 167 |

*In the absence of binding by the unreactive enantiomer, $K_M \approx 38$ μ*M* for the reactive substrate.

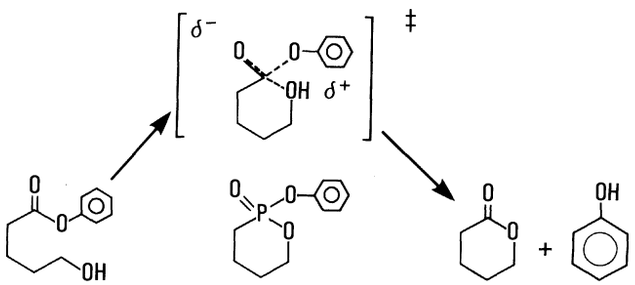


**Fig. 1.** Conversion of a hydroxy ester to a δ-lactone proceeds through a cyclic product-like transition state. A cyclic phosphonate ester is a stable representation for this state.

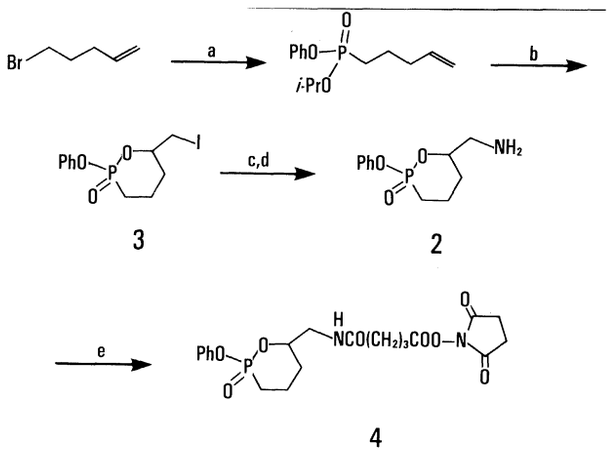


**Fig. 2.** Reagents and conditions (a) $PhOP(O\text{-}i\text{-}Pr)_2$, NaI, 170°C; (b) $I_2$, $CHCl_3$, 10°C, 3 days; (c) $NaN_3$, $Bu_4NBr$, 50% dimethylformamide-benzene, 80°C; (d) $H_2$, 10% Pd-C, 24 hours; (e) NHS glutarylchloride, $NEt_3$, $CH_2Cl_2$. Abbreviations: Ph, phenyl; *i*-PrO, isopropyl.

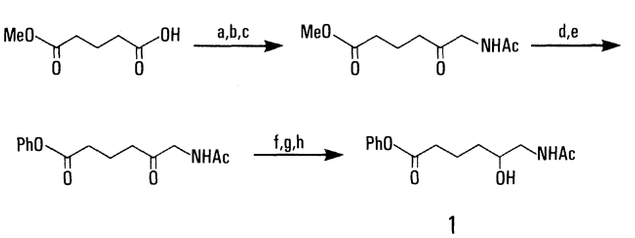


**Fig. 3.** Reagents and conditions: (a) $SOCl_2$; (b) CuCN, $CH_3CN$, 80°C; (c) $H_2$, 5% Pd-C, $Ac_2O$, AcOH; (d) KOH, 95% EtOH, reflux, 5 minutes; (e) PhOH, dicyclohexylcarbodiimide, *N*,*N*-dimethylaminopyridine, $CH_2Cl_2$; (f) $NaBH_3CN$, MeOH, *p*H 3; (g) $Me_3SiCl$, $(Me_3Si)_2NH$, pyridine, 5 minutes; (h) citric acid, MeOH. Abbreviations: Me, methyl; Et, ethyl; Ph, phenyl; Ac, acetyl.

cules by antibodies. Antibody reagents have been used as catalysts for aryl ester (*1*) or carbonate hydrolysis (*2, 4*). The concept of transition-state analogs was used, which is based on the hypothesis of Pauling (*5*) that the free energy difference between the enzyme-bound substrate and its transition state is reduced by specific binding interactions that develop along the reaction coordinate during passage through the transition state (*6*). Since the tetrahedral configuration is the key mechanistic feature of ester hydrolysis, tetrahedral phosphonate mimics were used as haptens to elicit the desired antibodies. The collective results were notable for the rate acceleration of $10^3$ to $10^4$ of the antibody-catalyzed reaction relative to the spontaneous hydrolysis and for their high specificity, that is, a trifluoroacetyl compared to an acetyl substituent served to differentiate active from inactive substrates (*1*).

We examined an intramolecular cyclization reaction, having noted that constraints imposed by the antibody binding pocket should confer a modest degree of rate acceleration dependent on the extent of reduction of rotational entropy (*7*) (Fig. 1). This intramolecular acyl transfer reaction has a transition state that may be mimicked by a cyclic phosphonate ester. The system we selected offered another test of the enzyme-like qualities of antibodies; namely, a choice between two reaction pathways that in the absence of a chiral reagent would be equal in free energy (enantiomeric) but that in the presence of the antibody should be unequal in free energy (diastereomeric) and lead to a stereospecific cyclization. We constructed amide derivatives of the phosphonate ester 2,2-phenoxy-2-oxo-6-(aminomethyl)-1,2-oxaphosphorinane (Fig. 2), which resembles a "product-like" intermediate along the cyclization route. The corresponding ester **1**, with chirality at the carbinol atom, was prepared as the *O*-protected trimethylsilyl derivative (Fig. 3).

The substrate was generated in situ by dissolution in acidic methanol prior to its addition to the aqueous assay buffers. The synthesis of the intermediate **2** was stereospecific and yielded only one diastereomer, as revealed by a single $^{31}P$ nuclear magnetic resonance (NMR) peak at 27.2 ppm (relative to 85% $H_3PO_4$). Confirmation that the phenoxy and aminomethyl substituents are in a 1,3-trans orientation (axial-equatorial) was obtained from the x-ray crystal structure of the iodo derivative **3**. An immunogenic conjugate was prepared by reaction of the phosphonate **4** with a carrier protein (keyhole limpet hemocyanin). Monoclonal antibodies were obtained by standard protocols (*8*) and purified to >90% homogeneity as judged by sodium dodecyl sulfate (SDS) gel electrophoresis (*9*); 24 antibodies were screened for hydrolytic activity (25 m*M* phosphate, *p*H 7.0, 25°C) by monitoring substrate depletion with high-performance liquid chromatography (HPLC). One (24B11) was chosen for further analysis.

The rates of phenol release from the ester **1** (20 to 100 μ*M*) in the presence of 24B11 (2 μ*M*) were determined spectrophotometrically. The initial rates as a function of substrate concentration followed Michaelis-Menten kinetics, which is consistent with Eq. 1:

$$\begin{array}{ccc} 24B11\cdot\mathbf{1} & \xrightarrow{k_{cat}} & 24B11 + \text{products} \\ \Big\uparrow\Big\downarrow K_m & & \\ 24B11 + \mathbf{1} & \xrightarrow{k_{uncat}} & \text{products} \end{array} \qquad (1)$$

Over this concentration range there was no apparent substrate inhibition. The relevant kinetic parameters in the presence and absence of antibody are listed in Table 1 and indicate a 167-fold rate acceleration. To confirm that the catalytic activity is a property of 24B11 and not of a contaminating esterase, we demonstrated that: (i) neither the hydrolytically more labile coumarin ester corresponding to **1** nor the phenyl-5-hydroxypentanoate, which lacks the important acetamidomethyl recognition element, are substrates; (ii) a second antibody (24E12) that bound **4** did not catalyze the liberation of phenol from **1**; and (iii) the reaction of 24B11 with **1** is competitively inhibited linearly by the addition of the transition-state analog, the *N*-acetyl derivative of **2** (Fig. 4), with a $K_i = 0.25$ μ*M*.

A striking characteristic of the time course of the reaction is its cessation at ~50% of the initial ester concentration (Fig. 5). To eliminate the possibility of a coincidental product inhibition, we added another substrate solution to the initial reaction solution which resulted in a second depletion of ~50% of the freshly added substrate. Introduction of fresh antibody was without effect; moreover, the addition of phenol (0.5 equivalents of **1**) revealed the expected absorbance increase. Analysis by HPLC of the completed reaction solution yielded ~50% of **1**. These results are consistent with the stereospecific antibody-catalyzed cyclization of one enantiomer of **1**. There was no evidence for cyclization by the antibody of the remaining enantiomer over the observed time course (about 30 minutes).

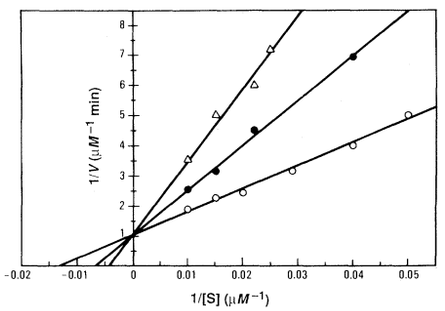



**Fig. 4.** Lineweaver-Burk plot for cyclization of substrate **1** (*S*) by the monoclonal antibody, 24B11. Velocities were determined as described in Table 1. (○) No inhibitor present. (●) Inhibited by 0.25 μ*M* *N*-acetyl derivative of **2**. (△) Inhibited by 0.50 μ*M* *N*-acetyl derivative of **2**.

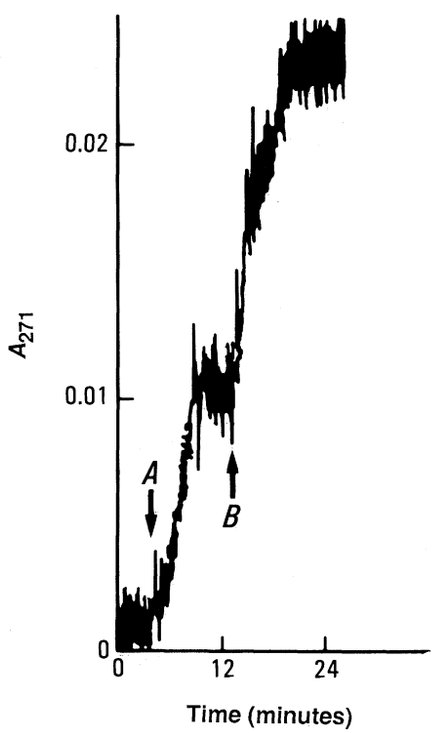
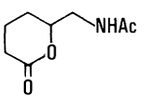



**Fig. 5.** Cyclization of **1** by the monoclonal antibody 24B11. The reaction was followed by monitoring the absorbance at 271 nm ($A_{271}$) due to release of phenol. The antibody (20 μ*M* in 25 m*M* phosphate buffer, *p*H 7.0) was incubated at 25°C. A volume of solution containing **1** (3.34 m*M* in phosphate buffer, *p*H 7.0) that was calculated to give a 40 μ*M* solution was added at points A and B. The average observed absorbance increase was shown to correspond to the consumption of 49 ± 13% of **1** present relative to a phenol standard.

To confirm that the reaction is a stereospecific cyclization of **1** to the lactone **5**, the latter was independently synthesized by treatment of 6-iodomethylvalerolactone (*10*) with sodium azide followed by reduction in the presence of acetic anhydride.




**5**

Extraction and chromatographic purification of the lactone permitted us to examine its enantiomeric purity by $^1$H NMR in the presence of a chiral lanthanide shift reagent, tris[3-(heptafluoropropylhydroxymethylene) - *d* - camphorato]europium(III) [Eu(hfbc)$_3$], in which the acetamido function binds the reagent (*11*). Clear separation of each of the three single $^1$H NMR resonances for the protons (underlined) of the C$\underline{H}_3$CONH substituent and side chain C$\underline{H}_2$ of **5** into two proton signals was achieved for both the antibody-generated and chemically synthesized samples of **5**. A portion of the spectrum is shown in Fig. 6. The equivalence of peak areas for the synthetic sample, as expected for a racemate, validates the analytical method and indicates that the observed enantiomeric excess (the percentage of the major peak minus the percentage of the minor peak) generated by the antibody-catalyzed cyclization is 66 ± 4%. The latter can be corrected for the competing spontaneous cyclization of the substrate under these conditions by a computer simulation of Eq. 1 (*12*) with the rate constants in Table 1, which calculated that 86% of the minor peak arose from the uncatalyzed reaction. Thus the stereospecificity of the antibody-catalyzed cyclization of **1** favors one enantiomer in 94 ± 8% excess, given the limits of our present determinations. Experiments with the separate enantiomers should permit a more precise measure of this enantiomeric differentiation, which probably is nearly absolute.

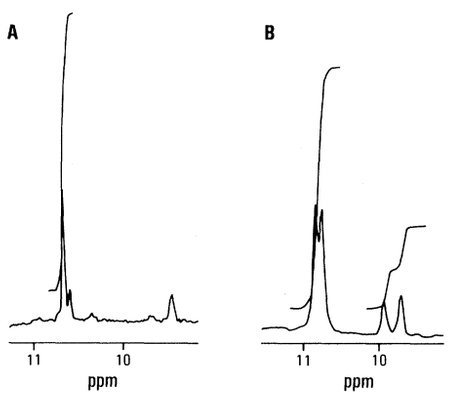



**Fig. 6.** Part of the $^1$H NMR (360 MHz, $CDCl_3$) spectrum for the two enantiomers of **5** in the presence of ~1 equivalent of tris[heptafluoropropylhydroxymethylene) - *d* - camphorato] europium(III). Chemical shifts (ppm) are shown downfield from tetramethylsilane (TMS). Peak assignments and the chemical shift differences between enantiomers (ΔΔδ) are as follows: (**A**) δ 9.45, 9.68 (one of C$\underline{H}_2$NHAc, ΔΔδ = 0.23), 10.60, and 10.67 (NHCOC$\underline{H}_3$, ΔΔδ = 0.07); (**B**) δ 9.71, 9.94 (one of C$\underline{H}_2$NHAc, ΔΔδ = 0.23), 10.74, and 10.82 (NHCOC$\underline{H}_3$, ΔΔδ = 0.08). The lactone **5** was obtained by cyclization of ester **1** for 55 minutes at 25°C in 25 m*M* phosphate buffer, *p*H 7, in the presence (A) or absence (B) of antibody 24B11. The antibody was removed by Centricon filtration; **5** was isolated from the filtrate by $CH_2Cl_2$ extraction followed by column chromatography on silica. The initial ratio of **1** to 24B11 was 9.2 m*M* to 115 μ*M*.

The cyclization reaction described here differs in its potential for molecular assembly from the hydrolytic reactions reported previously (*1, 2, 4*). More significantly, the stereochemical control of a reaction course so typical of enzymes extends to antibodies as well. The vision that general catalysts can be tailor-made from immunological sources for reactions that require stereochemical specificity is a step closer to reality.

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12. It is not necessary to introduce explicitly a term for the spontaneous hydrolysis of **5** since both enantiomers decay at identical rates and thus maintain their relative ratio.


13. This work was supported in part by NIH grants GM 13306 (S.J.B.) and GM 35318 (A.T.) and NSF grant DCB 8607352 (A.T. and R.A.L.). We acknowledge the technical assistance of D. Schloeder and C. Palmer.

9 July 1987; accepted 5 August 1987