Antibody-Catalyzed Decarboxylative Oxidation of Vanillylmandelic Acid

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Abstract: The most important industrial process for the synthesis of vanillin is performed in two steps involving an electrophilic aromatic substitution of glyoxylic acid on guaiacol followed by an oxidative decarboxylation of the intermediary α-hydroxy acids formed, thereby producing not only vanillin, but also byproducts which have to be eliminated. In the present study, we took advantage of the high specificity of catalytic antibodies to improve the synthesis of vanillin. Among 11 monoclonal antibodies elicited against the quaternary ammonium hapten **H3**, antibody H3-12 was found to catalyze the oxidative decarboxylation of vanillylmandelic acid (VMA), the precursor of vanillin, in the presence of sodium metaperiodate. The kinetic data of the antibody-catalyzed reaction are consistent with an ordered binding mechanism. At pH 9.0, H3-12 catalyzed the transformation of VMA into vanillin with a k_{cat} of 2.70 min^{-1}, a Michaelis−Menten constant K_a for the binary complex of 260 μM, and a K_b for the ternary complex of 2100 μM. The catalyzed reaction was fully inhibited by a hapten analogue with a K_i of 10 μM. The fine specificity of anti-**H3** monoclonal antibodies was determined using **H3**-related compounds with a competitive enzyme immunoassay. Controls demonstrating that catalytic activity is actually related to antibody binding, and mechanistic studies, are also presented.

Introduction

Among the tools used to catalyze organic reactions, enzymes are playing an increasingly important role due to their high efficiency and selectivity. In addition, when compared to conventional catalytic processes, enzymatic catalysis represents an environmentally compatible and inexpensive approach likely to replace chemical catalysis. However, the main drawback of enzymes is the limited number of accessible reactions and substrates.

To evaluate the potential of catalytic antibodies[1−5] to mediate transformations of a type that are of interest industrially, we examined the concrete case of the industrial synthesis of vanillin and sought to improve this process by the use of catalytic antibodies. Vanillin, the well-known flavor, is a widely used additive in the food and beverage industry. Although it can be obtained from natural sources, the main supply (8000 tons per year) comes from synthetic material. The industrial synthesis of vanillin is currently realized from guaiacol through a two-step procedure developed by Rhône-Poulenc[6] as displayed in Scheme 1.

In the first step of this synthesis, the regioselectivity of the electrophilic substitution of glyoxylic acid on guaiacol is not fully controlled and two byproducts, 2-hydroxy-3-methoxy-mandelic acid (*o*-VMA) and 4-hydroxy 5-methoxy-1,3-dimandelic acid (di-VMA), are generated in a total 15% yield. The oxidative decarboxylation, occurring in the second step of this synthesis, leads to the formation of three aldehydes. To obtain pure vanillin, a time-consuming separation of closely related products must be achieved.

We focused our efforts on the second step of vanillin synthesis, with a view to selective oxidative decarboxylation of vanillylmandelic acid (VMA). So far, only two carbon−carbon bond cleavage reactions catalyzed by abzymes have been described, namely, retroaldolization[7,28] and decarboxylation

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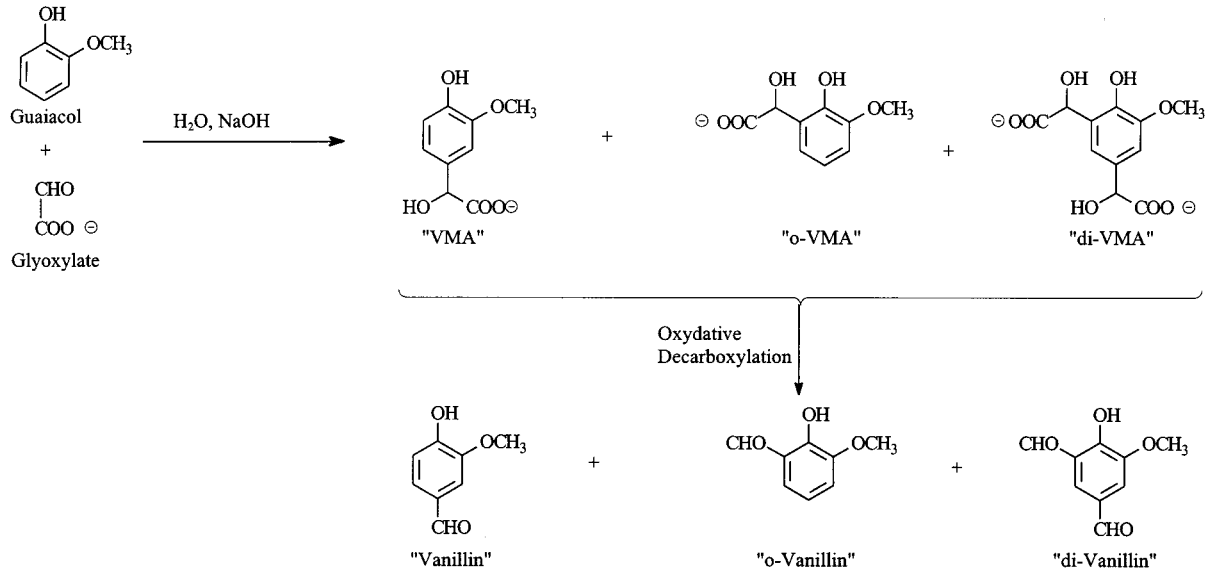
Scheme 1. Rhône-Poulenc Process for the Industrial Synthesis of Vanillin



reactions,[8,9] for which enzymes are known to be efficient. To our knowledge, this is not the case for the decarboxylative oxidation we are considering.

Such an oxidation can be performed with easily available periodinate $NaIO_4$. The aqueous oxidative decarboxylation of VMA leading to vanillin has been widely used[10−12] to measure VMA concentrations in biological media by measuring the UV absorbance of vanillin ($\epsilon^{348}_M = 19\,000$; pH 9). In addition, $NaIO_4$ does not alter the antibodies' recognition activity, since the oxidation of the sugar moiety of monoclonal antibodies is one of the commonest methods of preparing antibody−enzyme conjugates.[13] Furthermore, a catalytic antibody has already been described which achieves $NaIO_4$-mediated sulfur oxidation into sulfoxide.[14]

Results and Discussion

Determination of the Reaction Mechanism. Although the action of $NaIO_4$ on VMA is well-known, its precise mechanism remains unclear. Study of the uncatalyzed reaction mechanism was thus necessary before production of catalytic antibodies. Two mechanisms can be proposed (Scheme 2), based on the well-known action of $NaIO_4$ on α-hydroxy acid,[15−17] glyoxylic acid,[18] and phenols.[19]

In both cases, the oxidative decarboxylation would proceed through a concerted mechanism, after the formation of a reaction intermediate in which the hypervalent iodine atom is covalently attached to one (I_A) or two (I_B) oxygen atoms.

Several experimental arguments allowed us to favor mechanism A. In a first series of experiments, we studied the kinetics of the oxidation of different VMA-related α-hydroxy acids by $NaIO_4$. The phenol function plays a predominant role in this oxidation, since the rate of the reaction is significantly decreased in its absence or when it is substituted by a methoxy group (Table 1).

The velocity of this reaction with VMA increases as the pH rises as a function of the proportion of phenoxide ions, which are very reactive toward $NaIO_4$ ($k_{uncat} = 0.352 \pm 0.004 \times 10^{-5}$ μM^{-1} min^{-1} at pH 7.4; $k_{uncat} = 1.029 \pm 0.010 \times 10^{-5}$ μM^{-1} min^{-1} at pH 9.0).

These observations led us to postulate that mechanism A prevails in this reaction in neutral or alkaline media.

Hapten Design and Synthesis. The key point in the generation of catalytic antibodies is the design of the hapten used to elicit antibodies. According to the previously described mechanisms of the oxidation of VMA by $NaIO_4$, two approaches are conceptually possible: either trigger mechanism B through activation of the α-hydroxy acid moiety, with mimicry of the cyclic intermediate, or enhance the favored mechanism A. To avoid the oxidation of the byproducts also bearing α-hydroxy acid, we chose the second strategy.

We thus chose the hapten **H3** (see Scheme 2) which presents the following required characteristics:

(1) **H3** keeps the global structural features of VMA, with the α-hydroxy acid moiety in the para position toward the phenol, to discriminate between the regioisomers (o-VMA and di-VMA).

(2) The partially positively charged oxygen atom in the transition state is mimicked by the positive charge on the tetrasubstituted nitrogen atom.

(3) In addition, this positively charged nitrogen atom could induce, in the antibody combining site, a general base that would assist the deprotonation to generate the more reactive phenate form of VMA.

Hapten **H3** was synthesized in eight steps with a 33% overall yield from commercially available 4-amino-3-methoxybenzoic acid as depicted in Scheme 3.

The pivotal point in the synthesis of **H3** was to discriminate the acidic functions. Therefore, we decided to perform a one-step introduction of an already functionalized linker bearing an *N*-hydroxysuccinimide-activated acid moiety. Compound **3C** was easily obtained via classical chemical transformations. The unprecedented one-step coupling of a functionalized primary alkyl iodide on a polysubstituted aromatic amine was then efficiently performed using silver trifluoromethanesulfonate, yielding essentially pure **H3** in 65% yield after one single precipitation in diethyl ether.

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Scheme 2. Possible Mechanisms of Oxidation of VMA

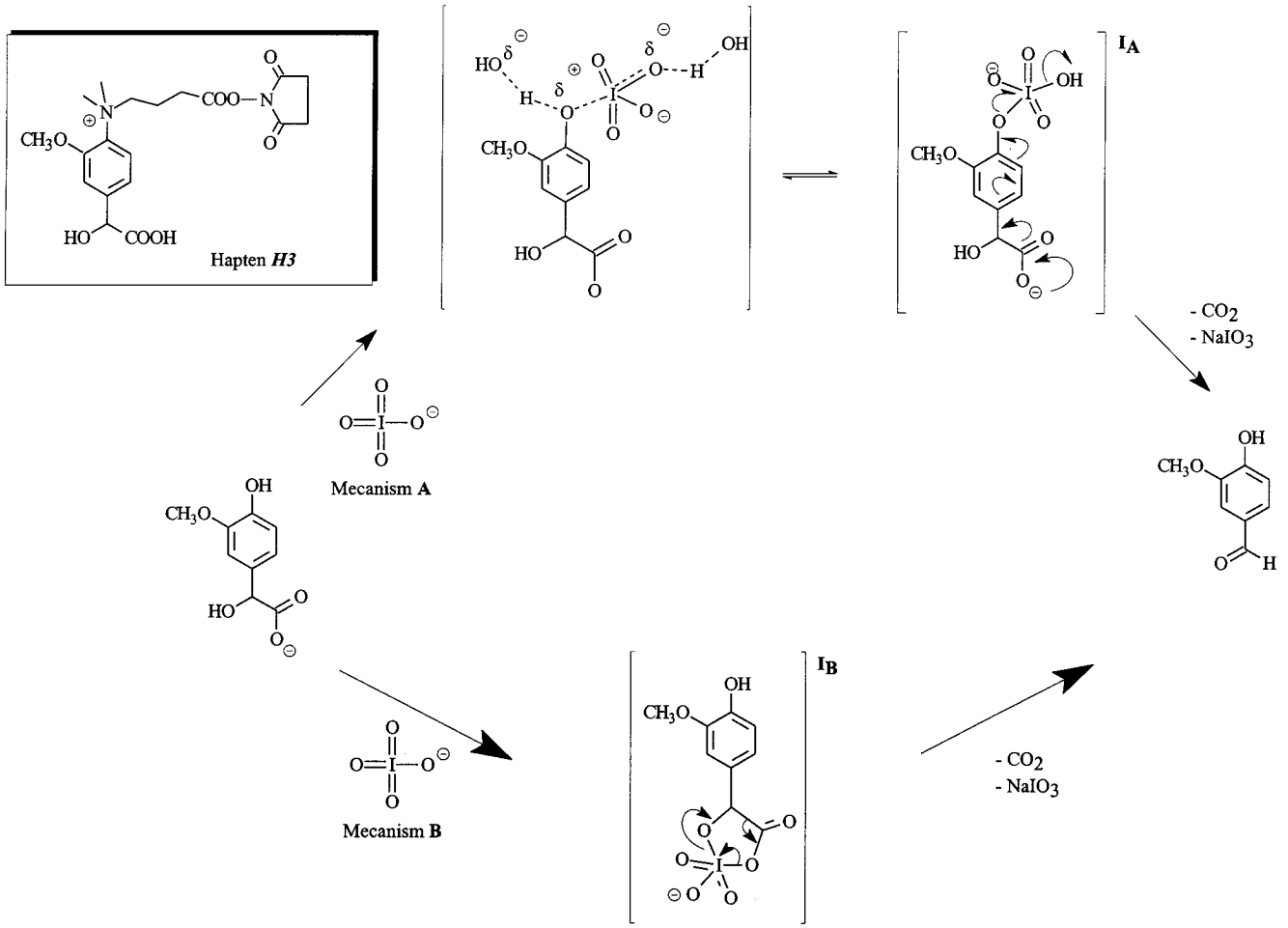
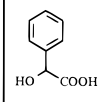
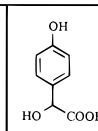
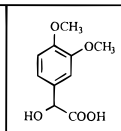
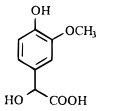


Table 1. Initial Velocities (V_i) of the Oxidation of Various α-Hydroxy Acids (70 μM) with $NaIO_4$ (1220 μM)[a]

Substrate				
Product				
V_i (μM.min^{-1})	0.175 ± 0.008	**2.126 ± 0.005**	0.756 ± 0.006	**7.758 ± 0.002**

[a] The reactions were carried out for 15 min at pH 9.0 and 25 °C in 80 mM potassium carbonate, 50 mM potassium phosphate buffer. The reaction products (aldehydes) were determined by absorbance measurements using a UV−vis spectrophotometer.

Hapten **H3** was covalently coupled to KLH (keyhole limpet hemocyanin, for immunization) or AChE (acetylcholinesterase, to be used as enzymatic tracer in enzyme immunoassays -EIA-experiments) as described in the Experimental Section.[20,21] Three Biozzi mice were immunized, and monoclonal antibodies were produced using conventional procedures.[22,23] Hybridoma supernatants were screened by testing their capacity to bind H3−AChE conjugate as previously described for other haptens.[24] Finally, 11 hybridoma cell lines were stabilized and expended as ascitic fluids.

Monoclonal Antibodies Characterization. To characterize in detail the corresponding monoclonal antibodies, their binding specificity was studied using a competitive enzyme immunoassay (see the Experimental Section), by testing series of compounds structurally related to **H3**. The synthesis of these analogues is described in the Experimental Section. Standard curves were established with each of these compounds for each monoclonal antibody, and the corresponding 50% B/Bo values were compared to that of **H3**, to evaluate the relative affinity of the analogues (see the Experimental Section). It is worth noting that this is only a rough estimation of relative affinities, and that only 10-fold differences can be unambiguously interpreted. The results of these experiments are given in Table 2.

These results showed that the aliphatic linker was poorly involved in hapten−antibody binding for most of monoclonal antibodies excepting monoclonal antibodies H3-12 and H3−15, since compound **I3-1** was recognized with equivalent or even better affinity than **H3**. On the other hand, removal of the methoxy group (compound **I3-2**) was highly detrimental for all monoclonal antibodies (excepting monoclonal antibody H3-6). The presence of the α-hydroxy acid moiety appeared still more important, since compounds **I3-3** and **I3-4** were almost unrecognized by all antibodies. The importance of the ammonium group was demonstrated by the very low cross-reactivities observed with 3-methoxymandelic acid (Table 2). Taken together, these results demonstrated that the binding sites of most of the antibodies cover the entire surface of the haptenic molecule, excepting the aliphatic linker. This is not surprising, taking into account the small size of the molecule. The critical contribution of the α-hydroxy acid group is a direct consequence

Scheme 3. Synthesis of Hapten **H3**

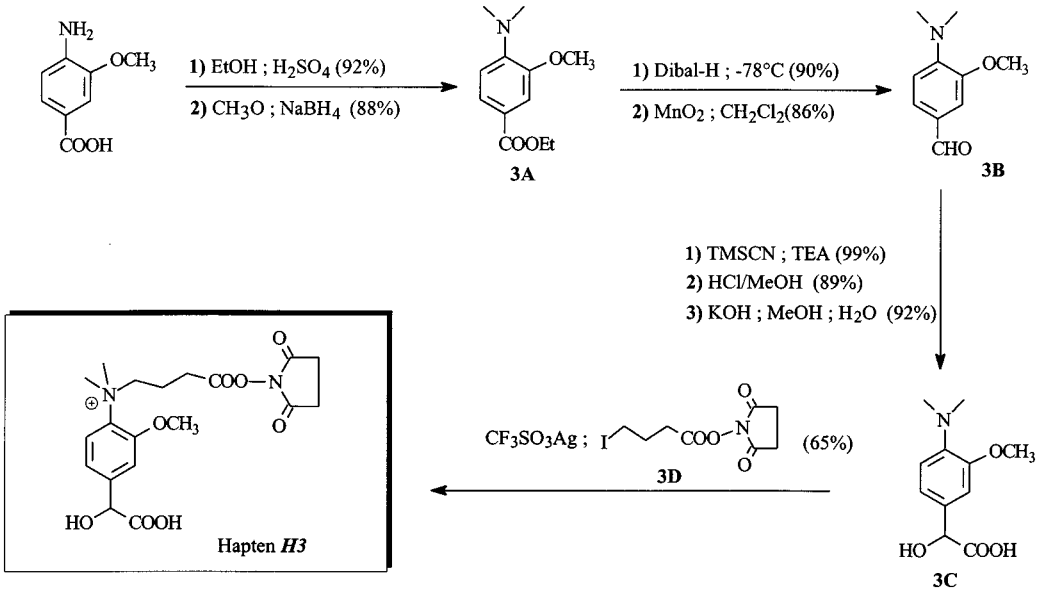
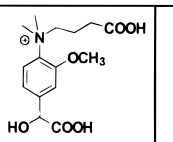
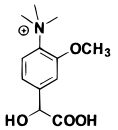
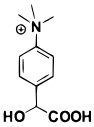
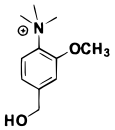
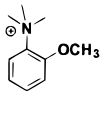
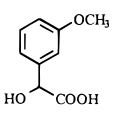


Table 2. Relative Affinities of the Different Monoclonal Antibodies with Regard to Series of **H3** Analogs

ANTIBODY						
	H3 B/Bo 50% (μM)	**I3-1** B/Bo 50% (μM)	**I3-2** B/Bo 50% (μM)	**I3-3** B/Bo 50% (μM)	**I3-4** B/Bo 50% (μM)	3-OMe mandelic acid B/Bo 50% (μM)
2	0.04	0.02	1.75	4340	15530	1290
6	0.03	0.01	0.07	8900	14120	574
8	0.09	0.2	10.2	10600	31800	6.8
10	0.2	0.34	916	960	17400	64700
12	0.5	4.6	5320	$>10^5$	$>10^5$	1280
13	0.025	0.02	1.7	5650	17390	2.5
15	0.27	1.6	200	10^5	68000	1188
16	0.07	0.08	2.8	704	9000	79200
25	0.06	0.02	0.87	4420	16000	1030
32	0.05	0.02	0.4	2560	9090	1.4
36	0.07	0.02	1.6	10900	26400	17

of choosing the quaternary ammonium as coupling point for the preparation of the immunogen.

After having thoroughly studied the specificity of these antibodies, we checked their affinities for the product (vanillin) and the substrates (VMA and $NaIO_4$) of the reaction. Most of the monoclonal antibodies (excepting antibody H3-16) have an ideal specificity profile, affinity for hapten > affinity for substrate > affinity for product, which is compatible with a catalytic activity and even at high concentrations (10 mM) $NaIO_4$ was unable to bind any of the monoclonal antibodies. Indeed, the catalytic act itself (linked to anti-hapten activity) has to be preceded by the formation of an antibody−substrate complex, while vanillin formed should be released thus preventing any product inhibition.

This, however, does not guarantee the actual catalytic activity of the antibodies, since the catalytic process is not an obligatory consequence of the binding of the substrate **H3**.

Catalytic Activity. The next step was to check the capacity of all monoclonal antibodies to catalyze the oxidation of VMA by $NaIO_4$ at pH 7.4 (phosphate buffer) and pH 9.0 (phosphate/carbonate buffer). The reaction was monitored by measuring the formation of vanillin by means of UV absorbance measurements ($\epsilon^{348}_M = 17\,000$; pH 7.4; $\epsilon^{348}_M = 19000$; pH 9.0). No catalytic activity could be detected at pH 7.4 for any of the

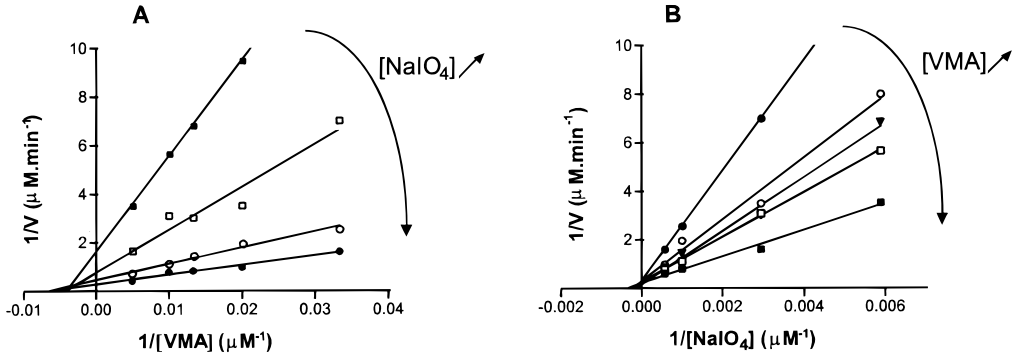


Figure 1. Lineweaver−Burk plots for vanillin formation: (A) as a function of VMA concentration at various concentrations of $NaIO_4$ (170, 340, 1000, and 1760 mM) and (B) as a function of $NaIO_4$ concentration at various concentrations of VMA (30, 50, 75, 100, and 200 μM). Assays were conducted in 80 mM potassium carbonate, 50 mM potassium phosphate buffer, pH 9.0 at 25 °C. The antibody concentration was 3 μM. The reaction was monitored at 348 nm with a UV−vis spectrophotometer.

monoclonal antibodies, but monoclonal antibody H3-12 significantly enhanced the rate of formation of vanillin at pH 9.0.

We therefore selected this monoclonal antibody H3-12 for further studies. Catalysis obeyed Michaelis−Menten kinetics (Figure 1), with a k_{cat} of 2.70 min^{-1} and catalytic efficiencies $(k_{cat}/K_a)/k_{uncat} = 815$ and $(k_{cat}/K_b)/k_{uncat} = 100$.

Changes in the Lineweaver−Burk plots as a function of VMA and $NaIO_4$ concentrations corresponded to an ordered substrate association.[25] First, antibody H3-12 binds VMA to form a binary complex ($K_a = 260 \pm 15$ μM). It is only from this complex that an $NaIO_4$ molecule can be recognized as a second substrate. This step leads to the formation of a ternary complex ($K_b = 2100 \pm 380$ μM) which results in the formation of the products. This catalytic mechanism perfectly corresponds to the specificity of H3-12, which has a marked affinity for VMA but none for $NaIO_4$.

The K_a value (260 ± 15 μM) obtained from the Lineweaver−Burk plots is in close agreement with the dissociation constant of the antibody−VMA complex as estimated by competitive EIA. This correlates with the catalytic mechanism mentioned above, and suggests that the catalytic reactions take place in the combining site of the antibody.

Further experiments were performed to confirm that the observed catalytic activity was actually related to the presence and the binding capacity of the monoclonal antibody H3-12.

(1) Monoclonal antibody H3-12 from various batches of ascitic fluids was affinity purified one to three times on a protein A column and successively purified on a protein A column followed by size exclusion chromatography with ACA 44 gel. Whatever the preparation, activity was recovered together with the antibody protein yielding solutions with equivalent specific activities (results not shown). The purity of these preparations was attested by SDS-PAGE analysis in reducing conditions (results not shown).

(2) When kinetic measurements were performed without one of the two substrates, no detectable vanillin was formed.

(3) None of the control reactions performed with other anti-**H3** monoclonal antibodies and with irrelevant monoclonal antibodies purified under the same conditions as H3-12 displayed any catalytic activity.

(4) The initial rate for the catalyzed reaction is proportional to the antibody concentration (in the 1−10 μM range, results not shown).

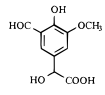
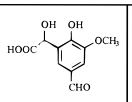
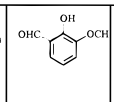
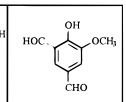
(5) Catalysis was totally inhibited by compound **I3-1,** which binds monoclonal antibody H3-12 with micromolar affinity but is not subject to $NaIO_4$ oxidation in the reaction period used. Lineweaver−Burk plots recorded with variable concentrations of **I3-1** allowed us to calculate an inhibition constant ($K_i = 10 \pm 13$ μM). This inhibition constant is consistent with the apparent affinity determined in EIA experiments (50% B/Bo = 4.6 μM). Nevertheless, it is worth noting that in these Lineweaver−Burk experiments the K_i measured is limited by the amount of antibody used (3 μM here), and thus the apparent inhibition constant calculated here has to be carefully interpreted, taking into account the active sites concentration.

(6) Both the catalytic activity and the binding properties of monoclonal antibody H3-12 were unchanged after prolonged treatment with excess $NaIO_4$ (results not shown).

Further investigations were performed in order to characterize the specificity of the monoclonal antibody H3-12-catalyzed reaction. Oxidation was carried out with various substrates including *o*-VMA, di-VMA and mandelic acid derivatives and was monitored by HPLC detection of the aldehydes formed. As expected, only oxidation of VMA[26] and, to a lesser extent, of 4-hydroxymandelic acid (both accepted as substrates) was significantly accelerated by monoclonal antibody H3-12. Because of the structural differences between di-VMA, *o*-VMA, and hapten **H3**, antibody H3-12 does not bind to these molecules. As a consequence, these two byproducts of the first step of vanillin synthesis are not accepted as a substrate by H3-12 and their oxidation was not catalyzed. On the other hand, mandelic acid, 3-methoxymandelic acid and 3,4-dimethoxymandelic acid display a marked affinity for H3-12 (even higher than VMA), but their oxidation is not catalyzed by antibody H3-12. It is noteworthy that for these compounds, oxidative decarboxylation can only occur via $NaIO_4$ addition on the α-hydroxy acid moiety (mechanism B). These data support the hypothesis that the monoclonal antibody H3-12 catalytic oxidation process occurs through mechanism A. These results, and the pH-dependence of the catalysis (as far as VMA is concerned, no catalytic activity was detected at pH 7.4, and a slight, but noticeable activity was detected at pH 8.0; the optimum activity occurred between pH 8.7 and 9.0), are consistent with the presence of an anionic group in the binding site of the antibody close to the phenol, as expected, due to the presence of the charged nitrogen atom on hapten **H3**.

We took advantage of the selectivity of monoclonal antibody H3-12-induced catalysis to perform the decarboxylative oxidation of the crude reaction mixtures from industrial batches. Reaction progress was monitored by HPLC. In every case, formation of *p*-vanillin was significantly enhanced compared to *o*-vanillin and other byproducts. A typical experiment performed with Rhône-Poulenc crude condensation mixture is shown in Table 3. The results showed that the rate of formation

Table 3. Improvement of *p*-Vanillin Formation in the Presence of Monoclonal Antibody H3-12[a]

Aldehyde					
Relative Amount (cat/ncat)	0.23	0.44	**2.31**	0.52	0.27

[a] The amounts of the various aldehydes formed in the presence (cat) or absence (ncat) of antibody H3-12 were determined after oxidation by 1500 μM $NaIO_4$ of a 100 μM crude reaction mixture of the condensation of glyoxylic acid on guaiacol obtained from Rhône-Poulenc industrial batches. Assays were conducted in 80 mM potassium carbonate, 50 mM potassium phosphate buffer, pH 9.0 at 25 °C. The antibody concentration was 6 μM, the reaction was stopped after 45 s by addition of a 10-fold excess of ethylene glycol, and the amounts of aldehydes formed were determined after HPLC separation and UV absorbance measurements (see the Experimental Section). Results are expressed as the ratios of the amount formed in the presence or absence of monoclonal antibody H3-12.

of *p*-vanillin increased more than 2-fold while the production of all other aldehydes was significantly reduced (up to 4-fold).

Conclusion

We have shown that the monoclonal antibody H3-12 elicited against hapten **H3** significantly increases the rate of oxidative decarboxylation of VMA in the presence of $NaIO_4$. This reaction was chosen as a typical example of an industrial process which could be improved by the use of catalytic antibodies.

The catalytic activity of this antibody remains relatively low (2.70 min^{-1}), so that it cannot be seriously envisaged for use in an actual production process. However, it displayed several particularly interesting and original features, as a remarkable substrate specificity which enabled the antibody to favor VMA oxidation with regard to the other regioisomers *o*-VMA and di-VMA formed during the first step of the industrial synthesis of vanillin.

Moreover, we have shown that simple immunological methods (here a competitive enzyme immunoassay) are well suited to precise determination of the binding properties of the different monoclonal antibodies, using series of relevant **H3** analogues. Even if these properties are not directly related to the catalytic potency of the monoclonal antibodies, a good knowledge of their fine specificity may help in understanding the catalytic process and/or the reaction mechanism.

In addition, the fact that H3-12 is the only monoclonal antibody (with H3-15) exhibiting significant recognition of the linker (Table 2) may be related to the catalytic capacity, assuming that the part of the antibody binding site in contact with the linker could be involved in $NaIO_4$ binding to form the tertiary complex, a step required for catalytic activity.

It is also noteworthy that monoclonal antibody H3-12, the only anti-**H3** antibody with catalytic activity, has the lowest affinity for the hapten (0.5 μM) of all 11 monoclonals tested. Clearly, the conventional procedure, which consists of selecting antibodies according to their binding affinity for the hapten, would not have proven felicitous in this case. This illustrates the importance of an early detection of the catalytic activity of an antibody, a question which has been widely discussed in previous papers. Finally, the results observed with monoclonal antibody H3-12 also provide information on the uncatalyzed reaction mechanism. Indeed, the fact that an antibody produced against hapten **H3** can accelerate the oxidative decarboxylation of VMA, with an abzymatic mechanism that favors mechanism A constitutes, in our minds, an additional indication in favor of the predominance of this mechanism A in the uncatalyzed reaction. It is, to our knowledge, the first time that such a fundamental issue has been addressed by the use of a catalytic antibody.

Experimental Section

A. Synthesis. General Remarks. Reagents were from Aldrich. All chromatography (flash) was performed with Merck Silicagel 60 (0.02−0.04 mm). TLC was performed with fluorescent Merck F254 glass plates. NMR spectra were recorded on a Brucker AC-300 MHz. Chemical shifts (δ) are given in ppm, and the coupling constant *J* is expressed in hertz. IR spectra were recorded in a Perkin-Elmer spectrophotometer 2000-FT/IR, and MS were obtained with a Finnigan-Mat 4600 quadrupole system. Elemental analysis was done by the "Institut des Substances Naturelles" in Gif sur Yvette.

Hapten H3 was prepared from commercial 4-amino-3-methoxybenzoic acid in an 8-step sequence as outlined in Scheme 4.

4-(Dimethylamino)-3-methoxyethyl Benzoate (3A). 4-amino-3-methoxybenzoic acid (4.54 g, 23.28 mmol) was dissolved in 100 mL of methanol. Concentrated sulfuric acid (11 mL, 232.8 mmol) was slowly added. The mixture was then refluxed for 6 h, cooled to room temperature, neutralized with a saturated K_2CO_3 aqueous solution, and extracted three times with Et_2O. Organic phases were then dried over Na_2SO_4, filtered, and concentrated. The resultant orange oil was chromatographed on silica gel (hexane/AcOEt, 8/2). The white solid was further purified by three successive crystallizations, yielding 4.18 g (92%) of 4-amino-3-methoxyethyl benzoate (mp 77 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.37 (t, J = 7 Hz, 3H), 3.9 (s, 3H), 4.31 (q J = 7 Hz, 2H), 6.66 (d, J = 9 Hz, 1H), 7.46 (d, J = 1.5 Hz, 1H) 7.56 (dd, J = 1.5 and 9 Hz, 1H). ^{13}C NMR: δ 15.4, 56.5, 61.4, 112.0, 114.0, 120.7, 124.9, 142.0, 147.0. IR (neat): 3366, 3010, 1685, 1522, 1295. MS (CI, NH_3): 196 ($[M + H]^+$), 213 ($[M + NH_4]^+$). Anal. Calcd for $C_{10}H_{13}NO_3$: C, 61.53; H, 6.71; N, 7.17. Found: C, 61.57; H, 6.71; N,7.17.

A mixture of 4-amino-3-methoxyethyl benzoate (1.8 g, 9.23 mmol) and $NaBH_4$ (2 g, 56.5 mmol), suspended in 30 mL of THF, was added dropwise to a 37% aqueous formaldehyde solution (4.5 mL, 48.4 mmol) and 3 M sulfuric acid (3.3 mL, 8.07 mmol) in 50 mL of THF. When half of the suspension had been added, an extra 3.3 mL of 3 M sulfuric acid was added, and the mixture was vigorously stirred for 1 h, neutralized with a saturated aqueous solution of K_2CO_3, and extracted three times with Et_2O. The organic phases were dried over Na_2SO_4, filtered, and concentrated. Chromatography on silica gel (hexane/AcOEt, 9/1) gave 1.82 g (88%) of dimethylamine **3A** as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.24 (t, J = 8 Hz, 3H), 2.73 (s, 6H), 3.78 (s, 3H), 4.21 (q, J = 8 Hz, 2H), 6.7 (d, J = 8 Hz, 1H), 7.51 (d, J = 2 Hz, 1H), 7.56 (dd, J = 2 and 8 Hz, 1H). ^{13}C NMR: δ 15.3, 43.6 (2C), 56.4, 61.5, 112.85, 117.6, 124.0, 124.2, 147.5, 152.0. IR (neat): 2950, 1710, 1514, 1293, 1124. MS (CI, NH_3): 224 ($[M + H]^+$). Anal. Calcd for $C_{12}H_{17}NO_3$: C, 64.55; H, 7.67; N, 6.27. Found: C, 64.58; H, 7.57; N, 6.41.

4-(Dimethylamino)-3-methoxybenzaldehyde (3B). Dimethylamine **3A** (1.7 g, 7.62 mmol) was dissolved in 100 mL of hexane. The solution is cooled to −78 °C, and 1 M DiBAl-H (17 mL, 16.75 mmol) in toluene was added dropwise. The solution was then stirred at −78 °C for 30 min, and excess hydride was destroyed with successive additions of methanol and water. The resultant suspension was filtered on Celite and extracted four times with AcOEt, dried, and concentrated. The viscous oil is chromatographed on silica gel (hexane/AcOEt, 2/8)

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yielding 1.24 g (90%) of (4-(dimethylamino)-3-methoxyphenyl)-methanol as a pale yellow oil. ^{1}H NMR ($CDCl_3$): δ 2.72 (s, 6H), 3.78 (s, 3H), 3.82 (s, 3H), 4.54 (s, 2H), 6.84 (s, 3H). ^{13}C NMR: δ 44.3 (2C), 56.2, 65.95, 111.1, 118.8, 120.25, 136.5, 142.7, 153.4. IR (neat): 3386, 2950, 1513, 1258, 1121. MS (CI, NH_3): 182 ([M + H]$^+$).

(4-(Dimethylamino)-3-methoxyphenyl)methanol (1.06 g, 5.85 mmol) was dissolved in 100 mL of freshly distilled CH_2Cl_2. MnO_2 (10 g, 115 mmol) was added, and the black suspension was stirred at room temperature for 16 h. The suspension was then filtered over Celite, concentrated, and chromatographed on silica gel (hexane/AcOEt, 8/2), yielding 0.9 g (86%) of aldehyde **3B** as a pale yellow oil. ^{1}H NMR ($CDCl_3$): δ 2.9 (s, 6H), 3.93 (s, 3H), 6.9 (d, J = 8 Hz, 1H), 7.30 (dd, J = 2 and 8 Hz, 1H), 7.36 (d, J = 2 Hz, 1H), 9.8 (s, 1H). ^{13}C NMR: δ 43.5 (2C), 56.5, 110.1, 117.3, 127.7, 130.7, 149.2, 152.5, 191. IR (neat): 2950, 1678, 1515, 1255, 1124. MS (CI, NH_3): 180 ([M + H]$^+$). Anal. Calcd for $C_{12}H_{17}NO_3$: C, 67.02; H, 7.31; N, 7.81. Found: C, 66.89; H, 7.28; N, 7.77.

1-(4-(Dimethylamino)-3-methoxyphenyl)hydroxyacetic Acid (3C). Aldehyde **3B** (850 mg, 4.75 mmol) was dissolved in 150 mL of anhydrous CH_2Cl_2. The solution was cooled to 0 °C, and TMSCN (1.3 mL, 9.5 mmol) and Et_3N (0.1 mL, 0.95 mmol) were added dropwise. The solution was stirred for 8 h at 0 °C and then brought to room temperature overnight. Evaporation of the solvent yielded 1.32 g (99%) of (4-(dimethylamino)-3-methoxyphenyl)trimethylsilanoxy)-acetonitrile as a colorless oil, which could be used without further purification. ^{1}H NMR ($CDCl_3$): δ 0.23 (s, 9H), 2.80 (s, 6H), 3.93 (s, 3H), 5.44 (s, 1H), 6.92 (d, J = 8.5 Hz, 1H), 6.96 (d, J = 3 Hz, 1H), 6.97 (dd, J = 3 and 8.5 Hz, 1H). ^{13}C NMR: δ 0.8 (3C), 44.1 (2C), 56.4, 64.6, 110.05, 118.9, 120.1, 120.3, 130.8, 144.5, 153.5. IR (neat): 2956, 1515, 1458, 1253, 1126. MS (CI, NH_3): 279 ([M + H]$^+$).

Cyanhydrine (1.32 g, 4.75 mmol) was placed in 120 mL of 1 N HCl solution. The solution was refluxed for 7 h, cooled, and then lyophilized. The resultant yellow paste was dissolved in 100 mL of methanol, 0.05 mL of concentrated sulfuric acid was added, and the reaction mixture was stirred at room temperature for 14 h. The solution was then neutralized with saturated aqueous $NaHCO_3$ solution and extracted three times with AcOEt. The organic phase was dried over Na_2SO_4 and concentrated. The resultant yellow oil is chromatographed on silica gel (hexane/AcOEt, 1/1) to yield (4-(dimethylamino)-3-methoxyphenyl)hydroxymethyl acetate (1.01 g, 89%) as a white powder (mp: 82 °C). ^{1}H NMR ($CDCl_3$): δ 2.73 (s, 6H), 3.71 (s, 3H), 3.84 (s, 3H), 5.08 (s, 1H) 6.87 (s, 3H). ^{13}C NMR: δ 44.0 (2C), 53.8, 56.3, 73.8, 110.1, 118.9, 120.1, 133.3, 143.5, 153.3, 175.1. IR (neat): 3431, 2951, 1742, 1512, 1460, 1252, 1123. MS (CI, NH_3): 240 ([M + H]$^+$). Anal. Calcd for $C_{12}H_{17}NO_4$: C, 60.23; H, 7.16; N, 5.85. Found: C, 59.99; H, 6.94; N, 5.81.

(4-(Dimethylamino)-3-methoxyphenyl)hydroxymethyl acetate (335 mg, 1.4 mmol) was dissolved in 5 mL of methanol, and 3 M aqueous KOH solution (0.5 mL, 1.4 mmol) was added. The solution was stirred for 1 h at room temperature and then neutralized with dilute HCl. Water was evaporated, and the crude material was chromatographed on silica gel (CH_2Cl_2/MeOH, 1/1) to yield α-hydroxy acid **3C** (0.29 g, 92%) as a white powder (mp = 183 °C). ^{1}H NMR (CD_3OD): δ 2.71 (s, 6H), 3.88 (s, 3H), 4.82 (s, 1H), 6.92 (d, J = 12 Hz, 1H), 6.96 (d, J = 3 Hz, 1H), 7.02 (dd, J = 3 and 12 Hz, 1H). ^{13}C NMR: δ 44.5 (2C), 55.8, 76.7, 111.7, 118.9, 120.9, 138.35, 142.3, 153.5, 180.4.

4-Iodo-[2,5-dioxopyrrolidin-1-yl] Butyrate (3D). 4-Bromobutyric acid (5 g, 29.94 mmol) and sodium iodide (13.35 g, 86.72 mmol) were dissolved in 100 mL of anhydrous acetone. The solution was stirred for 16 h under argon, and 400 mL of water were added. The aqueous phase was then extracted three times with 200 mL of Et_2O, and the organic phase was washed with 400 mL of 10% aqueous $Na_2S_2O_3$ solution, dried over $MgSO_4$, and concentrated. The crude material was then chromatographed on silica gel (CH_2Cl_2/EtOH, 98/2) yielding 5.45 g (85%) of 4-iodobutyric acid as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 2.12 (quint, J = 6.5 Hz, 2H), 2.51 (t, J = 6.5 Hz, 2H), 3.84 (t, J = 6.5 Hz, 2H). ^{13}C NMR: δ 5.3, 27.9, 34.3, 178.4. MS (CI, NH_3): 232 ([M + NH_4]$^+$).

4-Iodobutyric acid (1 g, 4.67 mmol), *N*-hydroxysuccinimide (0.54 mg, 4.69 mmol), and dicyclohexylcarbodiimide (0.96 g, 4.66 mmol) were dissolved in 10 mL of AcOEt. The reaction mixture was stirred at room temperature for 16 h. The dicyclohexyl urea formed was discarded by filtration over Celite, and the crude material was concentrated and chromatographed on silica gel (petroleum ether/AcOEt, 1/1). The resultant yellow powder was recrystallized from petroleum ether/EtOH (9/1) yielding activated ester **3D** (1.38 g, 95%) as white crystals (mp = 86 °C). ^{1}H NMR ($CDCl_3$): δ 2.24 (quint, J = 6.5 Hz, 2H), 2.76 (t, J = 6.5 Hz, 2H), 2.83 (s, 4H), 3.28 (t, J = 6.5 Hz, 2H). ^{13}C NMR: δ 3.5, 25.3 (2C), 28.0, 31.6, 168.7 (3C). IR (neat) 1803, 1734, 1201. MS (CI, NH_3): 329 ([M + NH_4]$^+$). Anal. Calcd for $C_8H_{10}INO_4$: C, 30.89; H, 3.24; N, 4.5. Found: C, 30.66; H, 3.17; N, 4.36.

[4-(1-Carboxy-1-hydroxymethyl)-2-methoxyphenyl]-[3-((2,5-dioxopyrrolidin-1-yloxy)carbonyl)propyl]dimethylammonium Trifluoromethanesulfonate (H3). α-Hydroxy acid **3C** (0.040 g, 0.18 mmol), activated ester **3D** (0.055 g, 0.18 mmol), and silver trifluoromethanesulfonate (0.046 g, 0.18 mmol) were dissolved in 2 mL of anhydrous acetone. The solution was stirred for 16 h at room temperature. The silver iodide formed was discarded by filtration over Celite, and the solvent was evaporated. The resultant orange oil was dissolved in 1 mL of MeOH, and hapten **H3** was precipitated by addition of 10 mL of Et_2O. The white powder (53 mg, 54% yield) was collected and used without further purification. ^{1}H NMR (DMSO-d_6): δ 2.58 (quint, J = 9 Hz, 2H), 2.74 (t, J = 9 Hz, 2H), 2.87 (s, 4H), 3.89 (s, 6H), 4.09 (s, 3H), 4.21 (t, J = 9 Hz, 2H), 5.44 (s, 1H), 7.33 (dd, J = 2 and 10.5 Hz, 1H), 7.56 (d, J = 2 Hz, 1H), 7.86 (d, J = 10.5 Hz, 1H). ^{13}C NMR: δ 24.35, 25.5 (2C), 27.7, 55, 57.2 (2C), 64.4, 72.8, 113.5, 118.5, 120.2, 123.8, 124.9, 130.9, 168.8; 170.5 (2C), 172.6. IR (neat): 3448, 2955, 1815, 1783, 1731, 1418, 1267, 1166. MS (EI): 408 (*m*/*z*).

[4-(1-carboxy-1-hydroxymethyl)-2-methoxyphenyl]trimethylammonium Iodide (I3-1). (4-(Dimethylamino)-3-methoxyphenyl)hydroxymethyl acetate (0.254 g, 1.06 mmol) and methyl iodide (0.2 mL, 3.2 mmol) are dissolved in 10 mL of acetone. The solution was refluxed for 16 h. After cooling, the ammonium salt was precipitated by addition of Et_2O. The white solid was washed with Et_2O, yielding 0.36 g (89%) of [4-(1-carbxy-1-hydroxymethyl)-2-methoxyphenyl]-trimethylammonium iodide as a white powder (mp = 142 °C). ^{1}H NMR (CD_3OD): δ 3.71 (s, 3H), 3.76 (s, 9H), 4.11 (s, 3H), 5.35 (s, 1H), 7.25 (dd, J = 2 and 12 Hz, 1H), 7.5 (d, J = 2 Hz, 1H), 7.81 (d, J = 12 Hz, 1H). ^{13}C NMR: δ 53, 56.65 (3C), 57.2, 73.1, 114, 120.7, 122.55, 134.55, 145.2, 153.2, 173. IR (neat): 3310, 3028, 1748, 1419, 1084. Anal. Calcd for $C_{13}H_{20}INO_4$: C, 40.96; H, 5.29; N, 3.67. Found: C, 40.79; H, 5.14; N, 3.63.

This ammonium salt (0.17 g, 0.45 mmol), K_2CO_3 (0.06 g, 0.44 mmol), and water (0.04 mL) were dissolved in 3 mL of THF. The solution was stirred for 18 h at room temperature and concentrated. The crude material was dissolved in 5 mL of methanol, and the ammonium salt was precipitated with Et_2O; 165 mg (99%) **I3-1** was then collected as a white powder (mp = 155 °C). ^{1}H NMR (D_2O): δ 3.73 (s, 9H), 4.15 (s, 3H), 4.81 (s, 1H), 6.84 (d, J = 12 Hz, 1H), 7.14 (s, 1H), 7.48 (d, J = 12 Hz, 1H). ^{13}C NMR: δ 56.45 (3C), 56.9, 75.2, 113.9, 120.5, 121.6, 133.6, 148.8, 152.8, 177.9. IR (neat): 3421, 1608, 1384, 1088.

(2-Methoxyphenyl)trimethylammonium iodide (I3-3). 2-Aminophenol (2 g, 18.3 mmol) was dissolved in 50 mL of anhydrous THF under nitrogen. Sodium hydride (1.65 g, 80% dispersion in mineral oil, 55 mmol) was added in one portion. The solution was stirred for 15 min at room temperature, and methyl iodide (5.7 mL, 91.5 mmol) was added. The solution was refluxed for 16 h, cooled, and filtered. The yellow powder was then washed with Et_2O, yielding 3.55 g (70%) of ammonium salt **I3-3**. ^{1}H NMR (D_2O): δ 3.57 (s, 9H), 3.91 (s, 3H), 7.02 (t, J = 8.5 Hz, 1H), 7.21 (d, J = 8.5 Hz, 1H), 7.46 (t, J = 8.5 Hz, 1H) 7.49 (d, J = 8.5 Hz, 1H).

(4-(Hydroxymethyl)-2-methoxyphenyl)trimethylammonium Iodide (I3-4). (4-(Dimethylamino)-3-methoxyphenyl)methanol (0.17 mg, 0.94 mmol) and methyl iodide (0.3 mL, 4.82 mmol) were dissolved in 10 mL of anhydrous acetone. The solution was refluxed for 16 h and cooled, and the quaternarized amine was precipitated with 100 mL of

Et_2O. The white solid was washed with Et_2O, yielding 243 mg (80%) of **I3-4** (mp = 145 °C). ^{1}H NMR (D_2O): δ 3.52 (s, 9H), 3.87 (s, 3H), 4.52 (s, 2H), 6.94 (d, J = 8.5 Hz, 1H), 7.14 (s, 1H), 7.51 (d, J = 8.5 Hz, 1H). ^{13}C NMR: δ 59.1 (3C), 59.6, 66.15, 116.6, 122.6, 124.4, 136, 148.4, 155.4, 173.. IR (neat): 3341, 3041, 1426, 1086. Anal. Calcd for $C_{11}H_{18}INO_2$: C, 40.88; H, 5.61; N, 4.33. Found: C, 40.44; H, 5.39; N, 4.53.

B. Antibody Assays. General Remarks. Immunogen Preparation. The conjugate H3−KLH was prepared by adding 30 μmol of hapten **H3** in 100 μL of dimethylformamide (DMF) to 5 mg of KLH in 10 mL of 0.1 M borate buffer pH 9.0. After 1 h of stirring at room temperature, the mixture was dialyzed against 0.1 M phosphate buffer pH 7.4 at 4 °C. Immunogen H3−KLH was stored at −20 °C until use.

Enzymatic Tracer Preparation. Enzymatic tracer was prepared by covalent linkage of hapten **H3** to the G4 form of AChE as follows: 30 μL (10 nmol) of hapten **H3** in DMF were added to 500 μL of 0.1 M pH 9.0 borate buffer containing 100 μg (0.32 nmol) of AChE. The reaction was allowed to proceed for 30 min at room temperature and was then stopped by addition of 500 μL of EIA buffer (0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 10^{-3} M EDTA, 0.1% bovine serum albumin, and 0.01% sodium azide). Enzymatic tracer was purified and stored as described elsewhere.[33]

Monoclonal Antibodies. Generation and Purification. Three mice each received a subcutaneous injection of 100 μL of 0.5 mg/mL H3−KLH conjugate emulsified in complete Freund's adjuvant. Boosters were given at two-week intervals. After receiving a final intravenous injection of 100 μg of H3−KLH, the spleen cells of the mouse presenting the highest antiserum titer for H3−AChE conjugate were fused with NS1 myeloma cells as described previously.[20] Hybridoma supernatants were screened for their capacity to bind H3−AChE conjugate using EIA as described previously.[21] After selected hybridoma cells were cloned, monoclonal antibodies were expanded as ascitic fluids in mice. Monoclonal antibodies were purified with standard protein A chromatography: to a 5 mL sample of ascitic fluid was added 20 mL of binding buffer (aqueous 0.1 M borate, 0.1 M NaCl, pH 9.0) and 1.0 g of silica gel immobilized protein A. This solution was applied to a column and washed with binding buffer until the absorbance of the eluant was <0.05 absorbance units (at 280 nm). Elution buffer (0.1 M citrate buffer, pH 3.5) was applied to the column, and 2 mL fractions were collected. IgG-containing fractions were pooled and dialyzed against 0.1 M phosphate buffer. Purified monoclonal antibody solutions were stored at −20 °C until use.

Competitive EIA Procedure. Antibody Affinity Measurement. Competitive EIA was performed as previously described[34] using 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 10^{-3} M EDTA, 0.1% bovine serum albumin, and 0.01% sodium azide (EIA buffer). Briefly, 96-well microtiter plates coated with affinity-purified goat polyclonal anti-mouse IgGs were used to ensure separation between bound and free moieties of the tracer. The reagents were dispensed in the following order: 50 μL of standard or buffer, 50 μL of enzymatic tracer, and 50 μL of diluted selected antibody. The plates were reacted for 18 h at 4 °C and washed before 200 μL of Ellman's reagent were dispensed into each well. After 2 h of enzymatic reaction, the absorbance at 414 nm of each well was measured. Results were expressed in terms of B/Bo (%), where B and Bo represents the amount of solid phase-bound tracer in the presence or absence of competitor, respectively. 50% B/Bo values correspond to the concentration of competitor for which the solid phase-bound tracer activity is half of Bo. These values can be considered as a good approximation to the K_D value of the desired product toward the antibody when K_D > [Ab] > [tracer].

UV-Monitored Kinetic Assays. The rates were determined by measuring the initial change in absorbance (<5% substrate depletion) at 348 nm reflecting vanillin release. The background rate of oxidation of VMA was also measured each time and subtracted from the velocities noted in the presence of antibody H3-12.

HPLC-Monitored Kinetics Assays. HPLC separation is achieved on a C18 reverse-phase nucleosil 5 μm column. Flow rate: 1 mL/min Column temperature: 40 °C. Elution conditions: 0−10 min, water/AcOH 98/2 (solvent A); 10−30 min, gradient up to 100% solvent B (water/MeOH/AcOH 69/29/2); 30−50 min 100%, solvent B. Aldehydes were quantified by UV absorbance measurement at 310 and 255 nm.

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