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Enantioselective Hydrolysis of Naproxen Ethyl Ester Catalyzed by Monoclonal Antibodies

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Abstract—This report described that a hapten of racemic phosphonate **3** designed as the mimic of the transition state of hydrolysis of naproxen ethyl ester was successfully synthesized from easily available 2-acetyl-6-methoxy-naphthalene **5**. Then BALB/C mice were immunized and one of the monoclonal catalytic antibodies, N116-27, which enantioselectively accelerated the hydrolysis of the *R*-(−)-naproxen ethyl ester was given. The Michaelis–Menton parameter for the catalyzed reaction was $K_M = 6.67\,mM$ and $k_{cat}/k_{uncat} = 5.8\times10^4$. This enantioselective result was explained by the fact that the *R*-isomer of rac-hapten was more immunogenic than the *S*-isomer.

Introduction

From the hypothesis of Pauling[1] that the binding forces utilized by both enzymes and antibodies are fundamentally identical to the two monumental reports[2,3] appearing on an antibody catalysis, the catalytic antibody opened a new field between chemistry and immunology. The most common strategy for producing catalytic antibodies needs designing and synthesizing for suitable transition state analogues as haptens. Then, after conjugation to carrier proteins to form antigens, the desired monoclonal antibodies were afforded by immunizing the animals with antigens and screening through hybridoma technology. Up to now, catalytic antibodies have shown the ability to catalyze a variety of organic reactions, such as hydrolytic, concerted, carbon–carbon bond forming and redox. Catalytic antibodies can even reverse kinetic control reactions, including ring formation, ring cycloaddition, cationic cyclization and elimination.[4–7] Moreover, they have other applications.[8–10] Therefore, more research on catalytic antibodies will promote deeper understanding of the reaction mechanism and molecular recognition and broaden their applications in organic, biotechnology and medicine.

Naproxen is an important non-steriod anti-flammatory drug, of which the *S*-isomer is 28 times more effective than *R*-isomer.[11] Chemical synthesis of the *S*-isomer involving diastereomeric crystallization from a racemic acid mixture has been exploited.[12] Considerable efforts have been made in improving the methods of its asymmetric synthesis.[13–16]

Recently, we reported a polyclonal antibody that catalyzed the hydrolysis of naproxen ethyl ester **1a**–**c** to naproxen **2a**–**c**, but the value of rate enhancement (k_{cat}/k_{uncat}) was rather too low.[17,18] Somewhat later, Janda et al. reported the hydrolysis of naproxen *p*-methylsulfonylphenyl ester by antibody prepared by the reactive immunization.[19] In continuing our program, herein, we report an enantioselective hydrolysis of naproxen ethyl ester catalyzed by monoclonal antibody with the satisfied rate enhancement. This result also demonstrates that antibodies generated by racemic phosphonate hapten **3** could be used for the kinetic resolution of naproxen ethyl ester.

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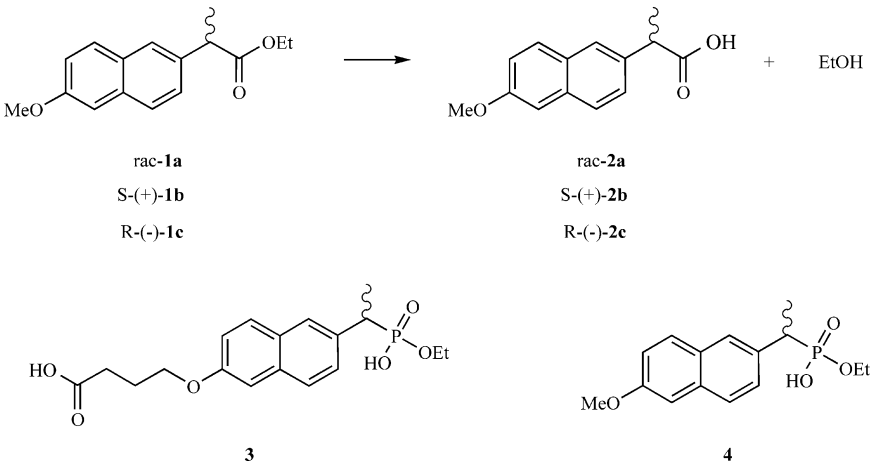
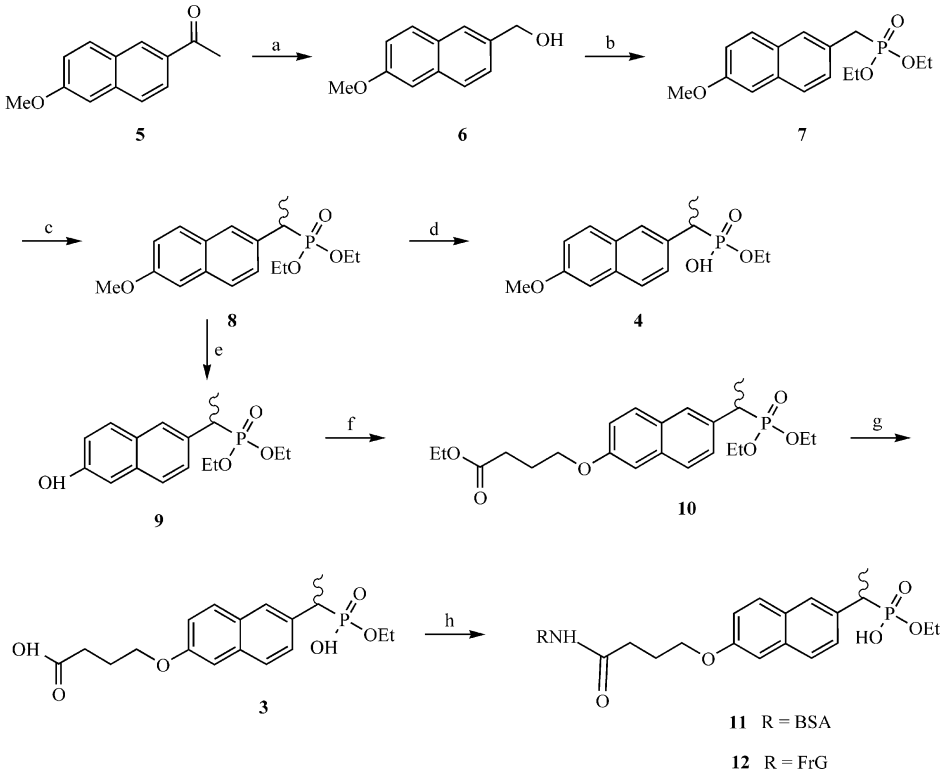


Scheme 1. Substrate **1a**–**c**, product **2a**–**c**, hapten **3**, inhibitor **4** used for monoclonal antibodies catalyzed reactions.



Scheme 2. Reagents and conditions: (a) NaClO, 1,4-dioxane, NaOH, H_2O, 90 °C, 48 h, 83%; $LiAlH_4$, THF, rt, 8 h, 96%; (b) PBr_3, THF, −40 °C, 2 h; $P(OEt)_3$, 120 °C, 10 h; 89%; (c) *n*-BuLi, MeI, THF, −78 °C, 2 h, 87%; (d) NaOH, EtOH, reflux, 16 h, 82%; (e) 1N BBr_3•CH_2Cl_2, CH_2Cl_2, −78 °C, 2 h, 88%; (f) $Br(CH_2)_3CO_2Et$, K_2CO_3, acetone, reflux, 40 h, 82%; (g) NaOH, EtOH, reflux, 24 h, 85%; (h) EDCI, DMF, 10 mmol/L PBS buffer (pH 7.2), rt, 5 h.

Results and Discussion

Preparation of hapten, inhibitor and immunogen

A tetrahedral structure of phosphonates in compounds **3** and **4**, which mimicked the transition state of the hydrolysis of naproxen ethyl ester **1a**–**c**, was designed as a hapten and inhibitor (Scheme 1). They were synthesized from easily available material, 2-acetyl-6-methoxy-naphthalene **5** (Scheme 2).[20] After oxidation and reduction from compound **5**, the 6-methoxy-2-naphthyl-methanol **6** was brominated by phosphorus tribromide followed by Arbuzov reaction in the presence of triethyl phosphite to afford compound **7** in 89% yield. Phosphonate **7** was methylated with iodomethane under *n*-BuLi to produce compound **8** in 87% yield. Then, the intermediate **8** was allowed to synthesize inhibitor **4** and hapten **3** via two different routes. Inhibitor **4** was prepared by selective hydrolysis of the phosphonate ester **8** under sodium hydroxide in only one step in 82% yield. For hapten **3**, at first, the intermediate **8** was demethylated on the methyl ether group under 1 N BBr_3•CH_2Cl_2 solution at −78 °C followed by tethering the spacer with the reagents of

ethyl γ-bromobutyrate and potassium carbonate to give compound **10** in 82% yield. Subsequently, the hapten **3** was prepared by the chemically selective hydrolysis of the triester **10** with sodium hydroxide in 85% yield.

The monoethyl phosphonate **3** (hapten) was coupled to the carrier proteins, bovine serum albumin (BSA) or fowl γ-globulin (FγG) with 1-ethyl-3-(3-dimethylaminopropyl)- carbodiimide (EDCI) as condensation agent to afford the immunogens **11** and **12**.

Production of monoclonal antibodies capable of catalytic power

Among the 10 hybridomas antibodies elicited from immunogens **11** and **12**, seven of them were found to be able to catalyze the hydrolysis of rac-naproxen ethyl ester. By comparison, three of them were allowed to be the best catalysis which were amplified by making ascitic fluid. Based on the establishment of the hybridoma clones against the transition state analogue of hydrolysis of naproxen ethyl ester, the hybridoma cells were injected into the peritoneal cavity of BALB/C mice to induce ascites. The monoclonal antibodies were purified from the ascite as described.[21]

Kinetic evaluation

Our experiment showed that all of the subsequent five monoclonal antibodies could catalyze the hydrolysis of *R*-naproxen ethyl ester. Among them, the most efficient monoclonal catalytic antibody, N116–27, greatly accelerated the hydrolysis of rac-naproxen ethyl ester and the *R*-isomer ester, but not the *S*-isomer ester (Fig. 1).

From Figure 1, the ester of *R*-isomer was 20% consumed during 1 h while the ester of *S*-isomer and background accounted for less than 1% of the total hydrolysis. The rates of the reactions were measured by following the decrease in absorbance of the *R*-isomer ester **1c** at 240 nm by HPLC. The obtained data were corrected for the background reaction and employed to construct a Lineweaver–Burke plot, from which all the kinetic parameters were given (Fig. 2).

The results were observed to follow the Michaelis–Menton kinetic equation of pseudo-first-order reaction. k_M/ν_{max} is determined as the slope of the line and $1/\nu_{max}$ is also determined as the intercept of the vertical axis in Figure 2. The values of the kinetic parameters are: $k_M = 6.67$ mM, $\nu_{max} = 32.3$ μM, $k_{cat} = \nu_{max}/[Ig] = 10.1\ min^{-1}$. Comparison the k_{cat} for the hydrolysis of the *R*-isomer ester with k_{uncat} (1.27×10^{-4} min) for the control experiments gave the rate enhancement of 5.8×10^4-fold. This enhancement was considered to be high for the acceleration rate of hydrolysis of the inactive ester. The catalytic hydrolysis of the naproxen ethyl ester exhibited competitive inhibition by phosphonate **4**, which revealed that catalysis took place at the antibody binding sites.

By exploiting the known enantiomeric discrimination inherent with the immune system, the monoclonal antibody induced from the racemic hapten phosphonate

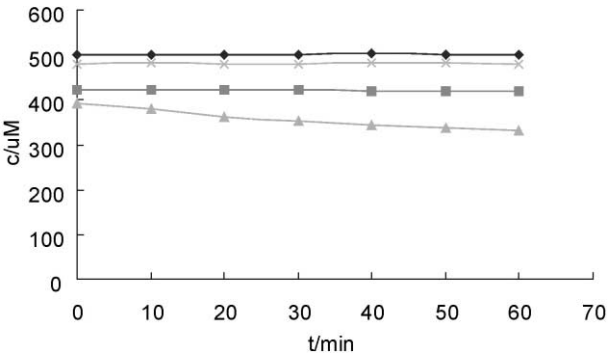


Figure 1. Plot of the concentration of substrates versus the time of hydrolysis. ◆, ×, Control experiment of **1b** and **1c** with non-specific antibodies; ■, △, hydrolysis of **1b** and **1c** by monoclonal antibody N116–27.

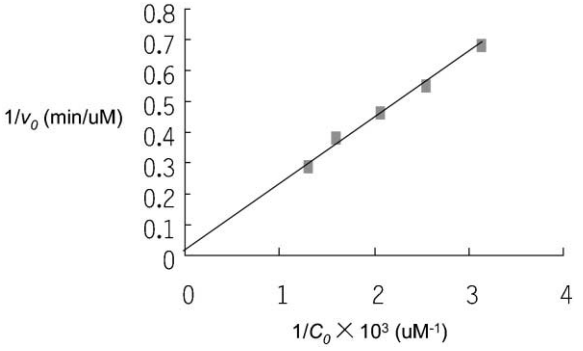


Figure 2. Lineweaver–Burke plot for the reaction of hydrolysis of *R*-(−)-**1c** by N116–27.

could highly enantioselectively catalyze the hydrolysis of *R*-naproxen ethyl ester. This result was contrary to that of the commercial and semipurified lipase of *Candida cyclindracea*, which enantioselectively catalyzed the hydrolysis of *S*-naproxen ethyl ester. These phenomena could be explained that the *R*-isomer of the rac-hapten was more immunogenic than the *S*-isomer, just as the D-amino acids of the hapten were more immunogenic than the L-amino acid. Of course, our results could be further tested by comparing the catalytic activity of antibodies elicited from the *R*-isomer phosphonate of the hapten with that from the *S*-isomer.

Conclusion

We have demonstrated that the racemic hapten **3** and inhibitor **4** were designed and successfully synthesized. Then, the racemic hapten conjugates were immunized and monoclonal antibodies were generated under the standard protocol. The acquired monoclonal antibody N116–27 enantioselectivly catalyzed the hydrolysis of *R*-naproxen ethyl ester with high rate enhancement. Moreover, our experiment affords an example that it should be possible to employ monoclonal antibodies generated from the racemic hapten to effect enantioselectively catalytic results in a variety of reactions, such as acyl-transfer, formation and cleavage of carbon–carbon bonds.

Experimental

General

IR spectra were recorded on a FT-IR instrument. ^{1}H NMR spectra were recorded on Bruker AM-300 (300 MHz) and assigned in ppm (δ) downfield relative to TMS as internal standard. MS spectra were conducted on a HP-5989A and VG QUATTRO mass spectrometers. Microanalyses were preformed in Microanalytical Laboratory at the Shanghai Institute of Organic Chemistry. Flash column chromatography were preformed on silica gel (10–40 μ) using a mixture of petroleum ether–ethyl acetate or dichloromethane–methanol as the eluent. Solvents and reagents were purified and dried by standard methods prior to use.

Diethyl (6-methoxy-2-naphthyl)-methylene phosphonate 7. A 200 mL flask was charged with a solution of **6**[22] (5.56 g, 29.6 mmol) in anhydrous THF (150 mL) under argon. The flask was cooled to −40 °C in dry ice/acetone bath, then PBr_3 (3.4 mL) in anhydrous THF (30 mL) was added dropwise for 30 min and stirring continued for an additional 2 h. The mixture was warmed to room temperature, and satd $NaHCO_3$ was added to neutralize the mixture. The aqueous layer was extracted with EtOAc (150 mL×3) and the combined organic solvent was dried over Na_2SO_4, concentrated in vacuo to give the crude product (7.27 g), which was dissolved in $P(OEt)_3$ (10 mL) and the mixture was stirred at 120 °C for 10 h. EtOAc (150 mL) was added to the cooled mixture and the solution was washed with brine, dried over Na_2SO_4. The solvent was removed in vacuo and the residue was purified by flash chromatography (ethyl acetate/petroleum ether, 2:3) to give **7** (8.13 g, 89%) as a yellow oil. IR (film, cm^{-1}): 2985, 1635, 1508, 1485; MS (EI) *m*/*z* 308 $(M)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69–7.10 (6H, m, Ar), 4.02–3.97 (4H, m, both OCH_2CH_3), 3.90 (3H, s, OCH_3), 3.27 (2H, d, J = 21.4 Hz, CH_2Ar), 1.22 (6H, t, J = 7.2 Hz, both OCH_2CH_3). Anal. calcd for $C_{16}H_{21}O_4P$: C, 62.33; H, 6.87. Found: C, 61.89; H, 7.02.

Diethyl 1-(6-methoxy-2-naphthyl)-ethylidene phosphonate 8. To a solution of **7** (3.550 g, 11.53 mmol) in anhydrous THF (20 mL) was added dropwise *n*-BuLi (6.2 mL, 11.53 mmol) at −78 °C under argon. The mixture was stirred for 45 min and a solution of CH_3I (1.8 mL) in THF (8 mL) was added dropwise. Then the mixture was stirred overnight. Water was added to quench the reaction and 0.1 N HCl was added to neutralize the mixture. The aqueous layer was extracted with EtOAc (100 mL×3) and the combined organic layer was washed with brine, dried over Na_2SO_4. The solvent was removed in vacuo and the residue was purified by flash chromatography (ethyl acetate/petroleum ether 1:2) to give **8** (3.222 g, 87%) as a yellow oil. IR (film, cm^{-1}): 2981, 1634, 1392; MS (EI) *m*/*z* 322 $(M)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.68–7.07 (6H, m, Ar), 4.03–3.73 (4H, m, both OCH_2CH_3), 3.84 (3H, s, OCH_3), 3.27 (1H, m, CHAr), 1.62 (3H, dd, J = 18.4 Hz, J = 7.4 Hz, CH_3), 1.24 (3H, t, J = 7.1 Hz, OCH_2CH_3), 1.08 (3H, t, J = 7.0 Hz, OCH_2CH_3). Anal. calcd for $C_{17}H_{23}O_4P$: C, 63.35; H, 7.14. Found: C, 63.24; H, 7.36.

Diethyl 1-(6-hydroxy-2-naphthyl)-ethylidene phosphonate 9. To a stirred solution of **8** (1.420 g, 4.41 mmol) in 30 mL of CH_2Cl_2 was added dropwise BBr_3·CH_2Cl_2 solution (30 mL, 30 mmol) at −78 °C under argon. The mixture was stirred for 2 h and fragments of ice and water were added. The aqueous layer was extracted with EtOAc (150 mL×3) and the combined organic layer was washed with brine, dried over Na_2SO_4. The solvent was removed in vacuo and the residue was purified by flash chromatography (ethyl acetate/petroleum ether 2:1) to give **9** (1.212 g, 88%) as a yellow oil. MS (EI) *m*/*z* 308 $(M)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58–6.81 (6H, m, Ar), 4.16–3.93 (4H, m, both OCH_2CH_3), 3.36 (1H, m, CHAr), 1.68 (3H, m, CH_3), 1.34 (3H, t, J = 6.8 Hz, OCH_2CH_3), 1.26 (3H, t, J = 7.2 Hz, OCH_2CH_3). Anal. calcd for $C_{16}H_{21}O_4P$: C, 62.33; H, 6.87. Found: C, 62.36; H, 6.97.

Diethyl 1-[6-(3-ethoxycarbopropoxy)-2-naphthyl]-ethylidene phosphonate 10. To a solution of **9** (1.428 g, 4.64 mmol) in 200 mL of acetone was added ethyl 4-bromobutyrate (1.05 mL) and K_2CO_3 (4.50 g). The stirred mixture was refluxed for 40 h followed by evaporated in vacuo. Ice-water was added and the mixture was extracted with EtOAc (100 mL×3). The organic layer was washed with brine, dried over Na_2SO_4. The solvent was removed in vacuo and the residue was purified by flash chromatography (ethyl acetate/petroleum ether 1:1). Compound **10** (1.608 g, 82%) was given as a yellow oil. MS (EI) *m*/*z* 422 $(M)^+$; ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 7.79–7.14 (6H, m, Ar), 4.17–4.03 (6H, m, three OCH_2CH_3), 4.00–3.88 (2H, m, OCH_2CH_2), 3.37 (1H, m, CHAr), 2.53 (2H, t, J = 7.3 Hz, CH_2CO), 2.07 (2H, m, CH_2CH_2CO), 1.59 (3H, dd, J = 9.5 Hz, J = 18.0 Hz, CH_3), 1.26–1.17 (6H, m, both OCH_2CH_3), 1.26 (3H, t, J = 7.2 Hz, OCH_2CH_3). HRMS (EI): $(M)^+$, found 422.1858. $C_{22}H_{31}PO_6$ requires 422.1862.

Ethyl 1-[6-(3-carboxypropoxy)-2-naphthyl]-ethylidene phosphonate, monoethyl ester 3. To a solution of **10** (181 mg, 0.429 mmol) in ethanol (27 mL) was added NaOH (725 mg) and the stirred mixture was refluxed for 24 h. Then, the mixture was acidified to pH 5 and concentrated in vacuo. The residue was purified by flash chromatography (CH_2Cl_2/MeOH 4:1) to afford **3** (133 mg, 85%) as a yellow solid. MS (EI) *m*/*z* 366 $(M)^+$; ESI (CH_3OH) 389 $(M^+ + Na)$; ^{1}H NMR (300 MHz, CD_3OD) δ 7.89–7.23 (6H, m, Ar), 4.28 (2H, t, J = 6.2 Hz, OCH_2), 3.85 (2H, q, J = 7.0 Hz, OCH_2CH_3), 3.32 (1H, m, CHAr), 2.61 (2H, t, J = 5.8 Hz, CH_2CO), 2.16 (2H, m, CH_2CH_2CO), 1.62 (3H, dd, J = 7.4 Hz, J = 17.3 Hz, CH_3), 1.17 (3H, t, J = 7.0 Hz, OCH_2CH_3). HRMS (EI): $(M)^+$, found 366.1232. $C_{18}H_{23}PO_6$ requires 366.1228.

Ethyl (6-methoxy-2-naphthyl)-ethylidene phosphonate, monoethyl ester 4. To a solution of **8** (210 mg, 0.65 mmol) in ethanol (8 mL) was added NaOH (170 mg) and the stirred mixture was refluxed for 16 h. Then the mixture was acidified to pH 5 and concentrated in vacuo. The residue was purified by flash chromatography (EtOAc/EtOH 1:1) to afford **4** (156 mg, 82%) as a yellow solid. IR (KBr) ν_{max} 2980,

1663, 1488; MS (EI) m/z 294 $(M)^+$; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ 7.85–7.13 (6H, m), 4.05 (2H, m, OCH_2CH_3), 3.90 (3H, s, OCH_3), 3.34 (1H, m, CHAr), 1.61 (3H, m, CH_3), 1.17 (3H, m, OCH_2CH_3). HRMS (EI): $(M)^+$, found: 294.1021. $C_{15}H_{19}PO_4$ requires 294.1024.

Synthesis of immunogens 11 and 12

To a mixture of bovine serum albumin (BSA) in 4.0 mL of sodium phosphate buffer (PBS, pH = 7.2, 10 mmol/L) was added a solution of hapten **3** (10 mg) in 2.2 mL of DMF and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI, 15 mg). The mixture was stirred at room temperature for 5 h and the reaction mixture was dialyzed three times in 3 days with 10 mmol/L PBS buffer (pH = 7.2). After that, the immunogen solution was aliquoted, frozen, and stored at −20 °C. Another conjugate of FγG was prepared as described above.

Establishment of mouse lymphocyte hybridoma clones secreting antibodies

The lymphocytes from spleen cells of BALB/C mouse immunized with immunogen **12** were fused with cells of mouse myeloma cell lines Sp2/0 or NS-1 to construct hybridoma. Five hybridoma clones secreting anti-hydrolysis transition state antibodies, named N121–6, N10–16, N116–27, S35–6 and S1–14, were obtained by screening through competitive inhibition assay (CIA) with the inhibitor **4** and indirect enzyme linked immunoabsorbent assay (ELISA) with the immunizing conjugate **11** as coating antigen and cloning by method of limiting dilution. Their immunoglobulin (Ig) subclasses were IgG1, IgG2a, IgG2b, IgG2b and IgG2b, respectively.

Kinetic measurements

Screening for catalytic antibodies: Purified monoclonal antibodies were dialyzed against 10 mmol/L PBS buffer (pH 7.2) three times. Their protein concentrations were determined by the measurement of the absorbance at 260 and 280 nm. The screening assays of hydrolysis were performed under 0.4 mmol/L rac-Naproxen ethyl ester and 10 μmol/L antibody at 37 °C in a pH 8.2 buffer made from 50 mmol/L Tris–HCl and 136 mmol/L NaCl with 5% cosolvent acetonitrile. The reaction was monitored by the decrease absorbance of the ester at 240 nm using a C-18 LiChrosorb RP-18 column with a mobile phase (55% water, 45% acetonitrile at 1 mL/min). An aliquot of reaction mixture (1 mL) was removed (50 μL) and quenched into acetonitrile (50 μL). The background hydrolysis of the substrate in the absence of catalytic antibody was also measured at the same conditions. An internal standard of 6-methoxy-2-naphathyl-methanol was used to calibrate the ester concentrations.

The buffer system and HPLC assay were the same as above. To obtain the kinetic parameters, substrate including rac-naproxen ethyl ester, *S*-(+)-naproxen ethyl ester and *R*-(−)-naproxen ethyl ester over a range of concentrations were assayed. Initial linear rate were measure at less than 5% hydrolysis of the total substrate. For antibody N116–27, the catalyzed hydrolysis rates with five different substrate concentrations from 0.3 to 0.7 mmol/L were measured in the presence of the antibody concentration (6.0 μmol/L). The analysis of v_0 and C_0 by the standard Michaelis–Menton equation gave Michaelis–Menton parameters (K_M, V_{max} and k_{cat}).

Acknowledgements

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