Glycosidase Antibodies Induced to a Half-Chair Transition-State Analog

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Enzymes stabilize the transition states of chemical reactions to bring about large rate accelerations.[1] Glycosidases, which hydrolyze glycosidic bonds, are no exception: it is believed that glycosidases effect catalysis by stabilizing the developing half-chair conformers along the hydrolysis pathway (Scheme 1).[2-5] The generally accepted mechanism of glycoside hydrolysis involves (i) protonation of the glycosidic *exo*-oxygen of **1** and distortion of the glycoside ring to the half-chair conformer **2**, leading to the rate-determining transition state,[5] (ii) departure of the protonated alkoxy group to afford an oxocarbenium ion **3**, and finally (iii) trapping of **3** with water to eventually yield pyran **4**. As research interest in antibody catalysis continued to increase,[6,7] we became interested in generating glycosidase antibodies[8] and wish to describe herein the successful outcome of our efforts with the use of the hapten **5a**, a *half-chair* transition-state analog (Chart 1).

In the past, enzyme inhibitors have served as a guide for hapten design, and indeed several half-chair analogs of oxocarbenium ion **3** are known to be potent glycosidase inhibitors.[9,10] However, since the rate-determining transition state does involve the departing alkoxy group, it was thought that a preferred hapten for the generation of glycosidase antibodies should accommodate both the half-chair conformation and an appropriately disposed alkoxy group and thus should mimic **2**

Scheme 1

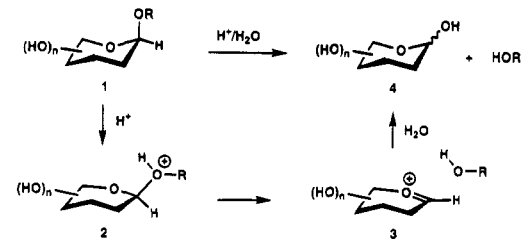
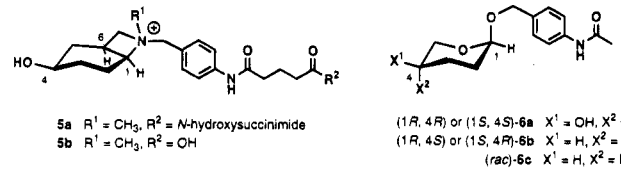


Chart 1



5a $R^1 = CH_3$, R^2 = *N*-hydroxysuccinimide
5b $R^1 = CH_3$, $R^2 = OH$

(1*R*, 4*R*) or (1*S*, 4*S*)-**6a** $X^1 = OH$, $X^2 = H$
(1*R*, 4*S*) or (1*S*, 4*R*)-**6b** $X^1 = H$, $X^2 = OH$
(*rac*)-**6c** $X^1 = H$, $X^2 = H$

rather than **3**.[4,5] The design of hapten **5a** includes the following features: (i) a cyclohexane which takes a half-chair conformation upon fusion with an azetidine, thereby mimicking the structure of **2**, and (ii) a quaternary ammonium ion which mimics the protonated form of the *exo*-oxygen and might generate a stabilizing residue in the antibody combining site. With respect to substrates such as **6a**, the introduction of a 4-hydroxy group on the pyran ring provides an appropriate model for the glycosidic bond hydrolysis[11] and also simplifies the evaluation of catalytic antibodies induced to hapten **5a** in terms of the enantio- and diastereospecificities of the substrates.

The diastereoselective synthesis of hapten **5a** and inhibitor **5b** (both racemic) are outlined in Scheme 2.[12] All four possible stereoisomers of substrates **6a** and **6b** were synthesized according to Scheme 2. The NMR spectra of **6a**, **6b**, and **6c** suggest that all three compounds preferentially exist as their axial anomers.[13]

Hapten **5a** was conjugated with the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Balb/c mice were immunized with the KLH conjugate of **5a**, and hybridomas were prepared and subcloned by standard protocols.[7,14] A total of 15 monoclonal antibodies specific to **5b** were isolated, and these antibodies were initially screened for hydrolytic activity against racemic substrate **6c**. Five monoclonal antibodies accelerated the hydrolysis of **6c** over the uncatalyzed process. Antibody ST-8B1, which showed the most significant activity, was chosen for further study.

The rate of hydrolysis of **6c** catalyzed by antibody ST-8B1, measured as a function of substrate **6c** concentrations, followed Michaelis–Menten kinetics. The apparent values of the Michaelis constant K_m and the catalytic constant k_{cat} were 1.74 mM and 3.6×10^{-2} min^{-1}, respectively, and comparison of k_{cat}/k_{un} affords a rate acceleration of 240 for **6c** (Table 1, entry 1).[15]

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(13) No other anomeric isomers of the substrates were observed in CD_3OD according to their ^1H NMR spectra. The chemical shifts of C-1 protons of **6a**, **6b**, and **6c** were observed at δ 4.72, 4.71, and 4.67 ppm, respectively, and small coupling constants ($J < 2.7$ Hz) of the C-1 protons with the adjacent C-2 protons were observed in all compounds. These results well agree with an axial anomer of pyranoside.

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(15) The uncatalyzed hydrolysis rate constants (k_{un}) of the substrates were determined under conditions identical to those described in Table 1 except in the absence of antibody. The values determined for k_{un} of **6a**, **6b**, and **6c** were 4.0×10^{-6}, 2.0×10^{-5}, and 1.5×10^{-4} min^{-1}, respectively.

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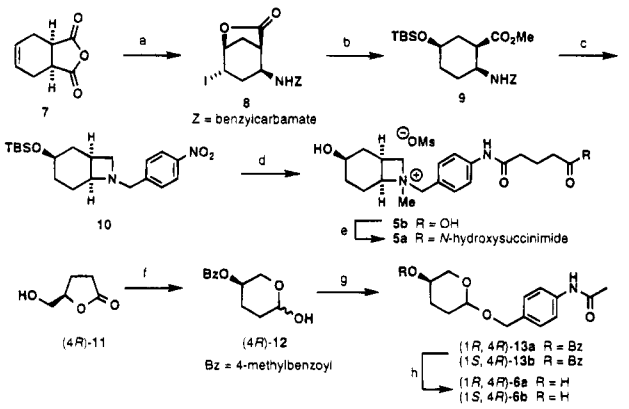
Scheme 2. Synthesis of Hapten **5a**, Inhibitor **5b**, and Substrates **6a** and **6b**[a]



[a] (a) Conditions: (i) MeOH, reflux, (ii) ethyl chlorocarbonate, Et_3N, acetone, 0 °C, then NaN_3, (iii) benzene, reflux, then benzyl alcohol, catalyst *p*-TsOH, reflux, (iv) NaOH, MeOH/H_2O, (v) I_2, KI, $NaHCO_3$, H_2O/CH_2Cl_2, 55% in five steps; (b) (i) Bu_3SnH, catalyst AIBN, benzene, reflux, 87%, (ii) catalyst BF_3OEt_2, MeOH, reflux, 95%, (iii) TBSCl, imidazole, DMF, 98%; (c) (i) NaOH, acetone/H_2O, 89%, (ii) ethyl chlorocarbonate, Et_3N, THF, then $NaBH_4$, 85%, (iii) 10% Pd/C, H_2, MeOH, (iv) *p*-nitrobenzyl bromide, Et_3N, CH_3CN, 50 °C, 89% in two steps, (v) CBr_4, Ph_3P, Et_3N, CH_3CN, 80%; (d) (i) 2.5% HF, CH_3CN/H_2O, 92%, (ii) MeOMs, CH_3CN, 49%, (iii) 5% Pt/C, H_2, MeOH, (iv) glutaric anhydride, CH_3CN, 89% in two steps; (e) *N*-hydroxysuccinimide, DCC, DMF; (f) (i) DHP, catalyst PPTS, CH_2Cl_2, 95%, (ii) LAH, Et_2O, (iii) TBSCl, catalyst DMAP, Et_3N, CH_2Cl_2, then 4-methylbenzoyl chloride, 74% in two steps (iv) Bu_4NF, THF, (v) $(COCl)_2$, DMSO, CH_2Cl_2, then Et_3N, (vi) AcOH, THF/H_2O, 80% in three steps; (g) (i) 4-nitrobenzoyl chloride, Et_3N, CH_2Cl_2, 80%, (ii) 4-acetamidobenzyl alcohol, TMSOTf, CH_2Cl_2, 74%, (iii) recrystallization to separate the diastereomers; (h) K_2CO_3, MeOH, 82%.

Table 1. Michaelis–Menten Parameters of Antibody ST-8B1[a]

entry	substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/k_{un}
1	(*rac*)-**6c**	1.74	3.6×10^{-2}	240
2	(1*R*,4*R*)-**6a**	1.16	1.5×10^{-3}	375
3	(1*R*,4*S*)-**6b**	1.62	2.1×10^{-3}	105

[a] All reactions were carried out in aqueous buffer solution composed of 29 mM bis-tris, 11 mM succinic acid, 10 mM NaCl, and 2% acetonitrile at pH 5.4 and 37 °C. All kinetic assays were performed in duplicate at seven different substrate concentrations (0.5–4.0 mM) in the presence of 18 μM of the antibody. See supplementary material for details.

The catalytic activity of ST-8B1 was competitively inhibited by the addition of **5b**, with an inhibition constant K_i of 8.3 μM, indicating that the catalyzed hydrolysis proceeds in the antibody combining site.[16]

The hydrolysis of enantiomerically and diastereomerically pure substrates **6a** and **6b** was then examined to determine the stereospecificity of antibody ST-8B1. The antibody catalyzed the hydrolysis of the substrates (1*R*,4*R*)-**6a** and (1*R*,4*S*)-**6b** but not the other stereoisomers. This result appears reasonable. The reaction proceeds only with the 1*R* stereoisomers to form a half-chair oxocarbenium ion in the distinctly chiral environment of the antibody combining site. The comparison of apparent kinetic constants (Table 1) reveals that (1*R*,4*R*)-**6a**, giving K_m = 1.16 mM and k_{cat}/k_{un} = 375,[15] is the substrate most efficiently catalyzed by ST-8B1. This suggests that the antibody also discerns the equatorial 4-hydroxy group from the axial stereoisomer as expected from the stereochemistry of hapten **5a**. Thus, antibody ST-8B1 has high stereospecificity[18] with respect to both C-1 and C-4 stereogenic centers of the substrates, indicating that ST-8B1 may have been generated from (1*S*,4*R*,6*S*)-**5a**.

Although the mechanism of the above catalytic reactions is yet to be elucidated, the following observations have been made: (i) pH-dependent kinetics of the antibody-catalyzed hydrolysis of **6c** reveal that k_{cat}/k_{un} and K_m are independent over the pH range from 5.4 to 7.0 (see supplementary material), suggesting no significant participation of an acidic residue (ionizable in this pH region) for the catalysis, and (ii) the observed ratio of k_{cat}/k_{un} is approximately the same as the ratio of K_m/K_i. Thus, it appears that the antibody contributes only to the binding and distortion of the substrate.[19] The lack of specific acid catalysis by ST-8B1 suggests that the quaternary ammonium ion of hapten **5a** might not be appropriate to generate an essential acidic residue *in close proximity* to the *exo*-oxygen of substrate. Further modification on the azetidine moiety might provide a solution to this problem.

We have demonstrated for the first time that a *half-chair* transition-state analog successfully generated catalytic antibodies with glycosidase capability. The antibody ST-8B1 catalyzed the hydrolysis of glycosides via transition-state stabilization in a highly stereospecific manner. Although the catalytic antibodies isolated here are inefficient compared to natural glycosidases, the design of azetidine-containing haptens promises further development of antibody catalysis. These results also imply that structures containing the azetidine ring may be applicable to the design of novel glycosidase inhibitors.[20]

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Supplementary Material Available: Listing of relevant experimental details for synthesis of **5a,b** and **6a–c**, preparation of monoclonal antibodies, kinetic assays, and pH-dependent kinetics profile (12 pages). This material is contained in many libraries on microfilm, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(16) The K_i value (8.3 μM for **6c** and 9.3 μM for (1*R*,4*R*)-**6a**) is close to the dissociation constant (K_d) for **5b** (K_d = 10.0 ± 0.3 μM), determined by a competition enzyme-linked immunosorbent assay (ELISA).[17] Since the competition ELISA can titrate only the combining site of antibody, these results indicated that the catalysis is due only to the antibody catalytic sites, not to contaminants.

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(18) Antibody ST-8B1 also catalyzes the hydrolysis of 4-acetamidophenyl pyranoside. However, the Michaelis–Menten parameters (k_{cat} = 1.5 × 10^{-1} min^{-1}, K_m = 3.3 mM, and k_{cat}/k_{un} = 176) suggest that the phenyl pyranoside is a somewhat poorer substrate than the series of benzylalkoxy substrates **6a–c**. It is noted that catalysis by ST-8B1 in morpholinoethanesulfonic acid buffer is significantly abolished, presumably caused by binding of the morpholine moiety to the antibody active site.

(19) General acid catalysis may be involved in the antibody catalysis, as we observed a modest buffer concentration dependence of the catalytic activity for (1*R*,4*R*)-**6a** (K_m = 1.20 mM, k_{cat} = 1.7 × 10^{-3} min^{-1} at 58 mM bis-tris, 22 mM succinic acid) (cf. Table 1).

(20) The structure of 8-azabicyclo[4.2.0]octane **5a** is somewhat similar to those of natural glycosidase inhibitors such as swainsonine and casternospermine, wherein both structures possess the 1-azabicyclo[4.3.0]-nonane skeleton. Accordingly, an azetidine-containing structure consisting of 1-azabicyclo[4.2.0]octane might be more suitable in the design of glycosidase inhibitors.