Chemical Selection for Catalysis in Combinatorial Antibody Libraries

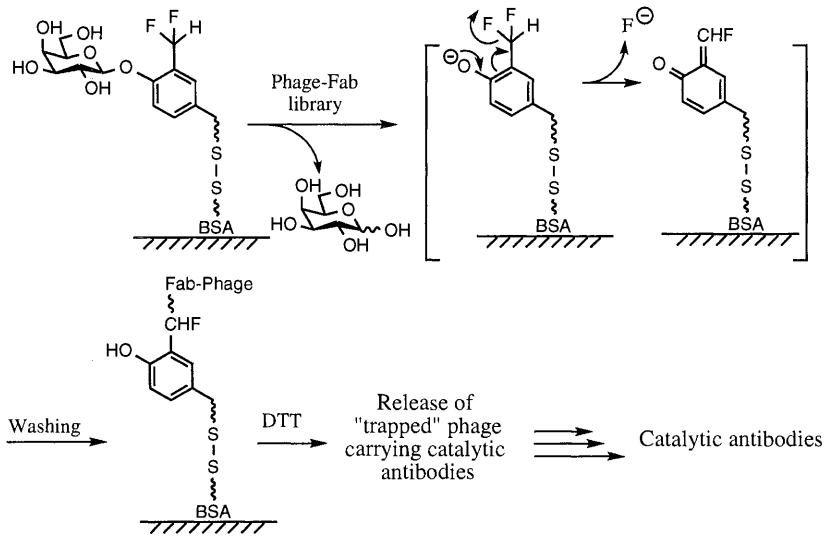
Kim D. Janda*, Lee-Chiang Lo, Chih-Hung L. Lo, Mui-Mui Sim, Ruo Wang, Chi-Huey Wong,* and Richard A. Lerner*

For the past decade the immune system has been exploited as a rich source of de novo catalysts. Catalytic antibodies have been shown to have chemoselectivity, enantioselectivity, large rate accelerations, and even an ability to reroute chemical reactions. In many instances catalysts have been made for reactions for which there are no known natural or man-made enzymes. Yet, the full power of this combinatorial system can only be exploited if there was a system that allows for the direct selection of a particular function. A method that allows for the direct chemical selection for catalysis from antibody libraries was so devised, whereby the positive aspects of hybridoma technology were preserved and re-formatted in the filamentous phage system to allow direct selection of catalysis. This methodology is based on a purely chemical selection process, making it more general than biologically based selection systems because it is not limited to reaction products that perturb cellular machinery.

The science of antibody catalysis has taught much about the use of libraries of molecules to do chemistry (*1*). It can be argued that the immune system appears to be the most powerful combinatorial system known for achieving a diverse set of binding molecules. This power is attributable to its diversity, which can be programmed experimentally, and to its selectivity, based on binding energy. The success of any combinatorial chemical system in obtaining a particular function depends on the size of the library and the ability to access its members. Usually access of function is a more serious obstacle than the size of the library. In nature, this problem is solved by the process of selection. However, most combinatorial chemistry in use at present involves simple screening and only rarely can the desired functionality be coupled to a selectable marker (*2*). This is also true for antibody catalysis where a library can be made of binding proteins of an almost unlimited size, but the low concentration of individual members necessitates that the initial screen be for binding rather than catalysis (*1*). Thus, most often the antibodies that are made in an animal against a hapten that mimics the transition state of a reaction are first screened for binding to the hapten and then screened again for catalytic activity (*1*).

A major advance was achieved when antibody libraries were made in phage systems because the size and composition of the library was no longer limited to the natural system (*3*). The usefulness of the phage system is that the essence of the natural antibody system can be duplicated in phage by linking recognition and replication. But, for two reasons selection for catalysis is not optimal. First, a diversity-generating function has not yet been built into the system. Second, even though recognition and replication are linked, the system is scored for simple binding rather than catalysis. The lack of a diversity-generating system may not be of major consequence because the number of library members can probably be made large enough to overcome this problem. However, the selection for function is more complicated and, until a selection program is available, any access procedure will remain limited. For example, although it is possible to envision various chemical (*4*) or immunological methods (*5*) that might detect product formation, none appears sufficiently sensitive to detect catalysis in single phage particles and thus the essential linkage between the chemical event and a replication system is broken. Here, we describe a method (Scheme 1) that allows direct selection for catalysis from antibody libraries. The method preserves the positive aspects of hybridoma technology but the system is re-formatted in phage so as to allow for the direct selection of catalysis (*6*), thereby linking chemistry and replication.

Hapten design and antibody library generation. We studied antibody catalysis of glycosidic bond cleavage to test our method for direct selection of catalysis (*7*). This reaction was chosen because, despite obvious difficulties, much is understood about the mechanism of this reaction (Scheme 2) (*8*) and the means by which it can be inhibited (*9*). Furthermore, there are a number of bacterial systems that require complementation with glycosidases for survival and therefore have the advantage of an additional test of enzyme function in a phage particle system (*10*). As to mechanism, glycosidic bond cleavage is thought to proceed through a flattened half-chair (or twist-boat) conformation transition state with substantial sp^2 character at the anomeric position. Hapten 1 was designed and synthesized (Fig. 1) to induce antibodies that accommodate a number of features of this transition state. Hapten 1 contains a glycon site, which is a five-membered iminocyclitol, and an aglycon site, which is an aromatic ring. The nitrogen atom in the iminocyclitol is near



Scheme 1. The mechanism-based panning procedure for the selection of antibodies that can catalyze the hydrolysis of a galactopyranoside substrate.

The authors are at The Scripps Research Institute, Department of Chemistry and the Skaggs Institute for Chemical Biology, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

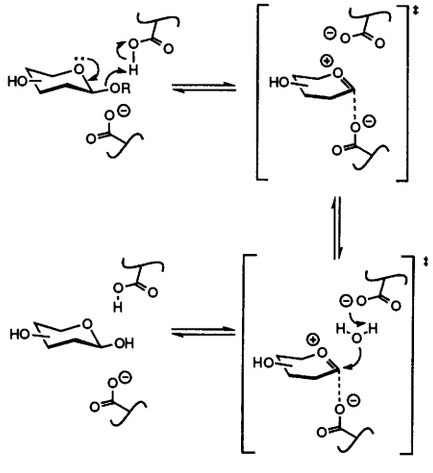
*To whom correspondence should be addressed.

the anomeric position and was anticipated to mimic the electronic character developing in the transition state. In addition, this charged moiety has the potential to elicit complementary charged carboxylate residues in the antibody-combining site which are intimately involved in the catalysis reaction observed with natural glycocidases (*11*). The five-membered iminocyclitol also provides a topography that favorably mimics not only the steric constraints imposed by the flattened chair conformation that occurs during glycosidic bond cleavage but also the hydroxyl distribution seen with galactopyranosides (*12*). Although the iminocyclitol seems a more appropriate mimic for the transition state of β-glucosidic cleavage, the five-membered ring appears to be flexible enough to also mimic β-galactosidic cleavage because it can be a potent inhibitor of a variety of glycosidases (*12*).

The methodology described in Scheme 1 could be used for any library. We elected to use an "enriched library" for several reasons. First, clones responding to the carrier protein, keyhole limpet hemocyanin (KLH), were eliminated at an early stage thereby greatly increasing the population of clones against the hapten of interest. Second, combinatorial library construction was accompanied by chain shuffling which expands the diversity of the library (*13*). Third, by immunization and selection with a transition-state analog, the potential for transition state stabilization was built into the antibody library. Approximately 100 clones that bound hapten 1 [enzyme-linked immunosorbent assay (ELISA)], were generated by established hybridoma methodology (*14*). However, at this early point the usual procedure was interrupted, and all cell lines that produced binding proteins were pooled and RNA was isolated from the pooled cells. Complementary DNA was made for the construction of a combinatorial phage-Fab library by polymerase chain reaction (PCR) amplification of both heavy and light chains.

A set of primers was designed to amplify immunoglobin G1 (IgG1), IgG2a, IgG2b, and IgG3 of heavy Fd fragments and κ light chains (*15*). The libraries were not designed to include λ light chains based on the restriction sites used for cloning. Since λ chains constitute 10 percent of the light chain population in the murine repertoire (*16*), we estimate our phage-Fab combinatorial library contains approximately nine times 10^3 clones (90 light chains × 100 heavy chains). Both chains were subcloned into the pComb3H vector, where the light chain was expressed in a soluble form with an OmpA leader sequence. The heavy chain–truncated gene III fusion protein was expressed with the use of a pelB leader sequence (Fig. 2). In this way, both chains were transported into the periplasmic space of *Escherichia coli* where they were anchored to the membrane to await packaging onto the surface of filamentous phage (*17*).

Direct selection for antibody catalysis. Unlike biological systems in which a selected event improves survival, in chemistry, a selected event should allow discrimination between library members that accomplish a chemical feat and those that do not. In phage systems this reduces to either simple binding or, in the system studied here, the more stringent requirement of a physical change in those library members that accomplish catalysis. The simplest way to accomplish this is to design a reaction that, if accomplished, leads to a covalent interaction between phage and an insoluble matrix. Both ortho- and para-(difluoromethyl)aryl-β-D-glucosides have been shown to be mechanism-based inhibitors of β-glucosidases (*18*). On enzymatic cleavage, the difluoromethylphenol moiety generates a reactive quinone methide species at or near the active site, thereby alkylating any nucleophile. On this basis, we designed a multifunctional linker that contained three essential functions. It allowed (i) coupling of substrate to various protein and polymer matrices, (ii) covalent trapping of catalytic clones, and (iii) recovery of immobilized phage with retention of replication compe-



Scheme 2. A plausible mechanism for glycosidic bond cleavage by a glycosidase.

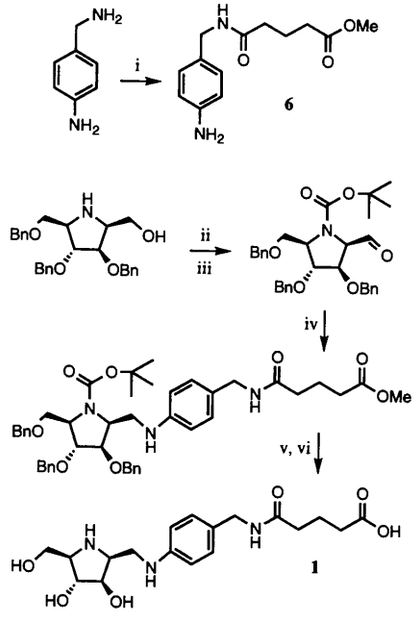


Fig. 1. Synthesis of hapten **1**. Conditions: (i) $MeOOC(CH_2)_3COCl$, −78°C, 61 percent; (ii) Boc_2O, TEA, CH_2Cl_2, 82 percent; (iii) Dess-Martin, 84 percent; (iv) **6**, $NaCNBH_3$, MeOH, 64 percent; (v) BCl_3, CH_2Cl_2, −40°C to −4°C, 92 percent; and (vi) LiOH, MeOH-H_2O, 44 percent.

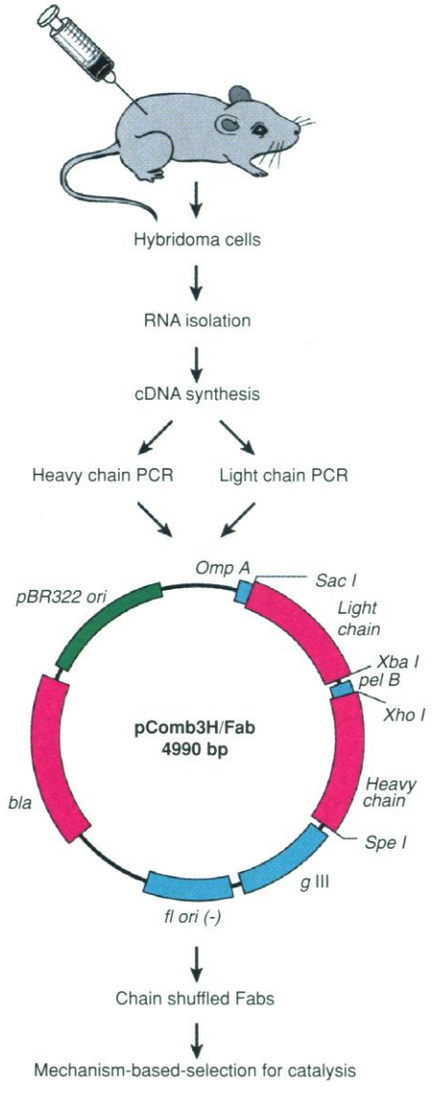


Fig. 2. Construction of a chain-shuffled Fab combinatorial library.

tence. The mechanism-based panning reagent **2** consists of a "head" group containing a galactose recognition unit with a latent quinone methide species similar to other glucosidase inhibitors (as above mentioned) and a "tail" protected as a 2-pyridinyldithiol group. The protected tail functionality allows coupling of the substrate to bovine serum albumin (BSA) which was first treated with Traut's reagent to convert surface lysine residues into free thiol groups, through a disulfide exchange reaction (*19*). This disulfide bridge may be cleaved by the reducing agent dithiothreitol (DTT) for subsequent recovery of catalytic phage clones.

To demonstrate the applicability of this covalent trapping device, compound **3**, a closely related synthetic cognate of **2** was prepared (Fig. 3) and examined as a substrate for β-galactosidase. The biotinylated derivative **3** (Fig. 3), was found to act as a substrate-alkylating agent of this enzyme in an ELISA formatted assay wherein a mixture of **3** and β-galactosidase were reacted and later applied to a streptavidin-coated ELISA plate to trap modified enzyme. Although the enzyme was biotinylated, it still retained its full catalytic activity. Therefore reagent **3** acted as a nondestructive covalent trapping device. The preservation of enzyme activity in the presence of a mechanism-based inactivator is known (*20*). This feature is highly desirable in that it provides the potential to select clones with an ability to undergo multiple turnovers because it allows trapping of catalysts that release product rapidly.

According to Scheme 1, only clones that catalyzed hydrolysis of the glycosidic bond could lead to quinone methide formation and thus capture. The Fab-phage library was applied to a microtiter plate coated with compound **2**–BSA. Nonbinders and phage that did not bind covalently were removed by washing with buffer and acid, respectively. These trapped Fab-phage were eluted with DTT and amplified through infection of *E. coli*. After four rounds of panning, the DNA of gene III was removed by digestion with Spe I and Nhe I from the pComb3H-Fab and was self-ligated. This process allowed selected soluble Fabs to be expressed in β-galactosidase–deficient XL1-Blue cells (*21*). The second selection for identification of catalysts was accomplished by plating the cells onto Luria-Bertani (LB) agar plates containing 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, compound **4**, Fig. 4). The cleavage of **4** gives an indoxyl product that rapidly oxidizes to an insoluble indigo dye in aqueous solution (in contrast to **5**, which on hydrolysis gives *p*-nitrophenol, which in turn rapidly diffuses through the medium) and accumulates as a blue precipitate at the site of reaction, affording a high sensitivity to detection (Fig. 4) (*22*). The plates were incubated for 3 days at 37°C from which a blue-white selection could be performed to confirm the activity of selected Fabs. Active clones show dark-blue color resulting from glycosidic bond cleavage of **4**. Several blue-colored clones were observed, and one clone termed Fab 1B (Fig. 4A) was chosen for further study.

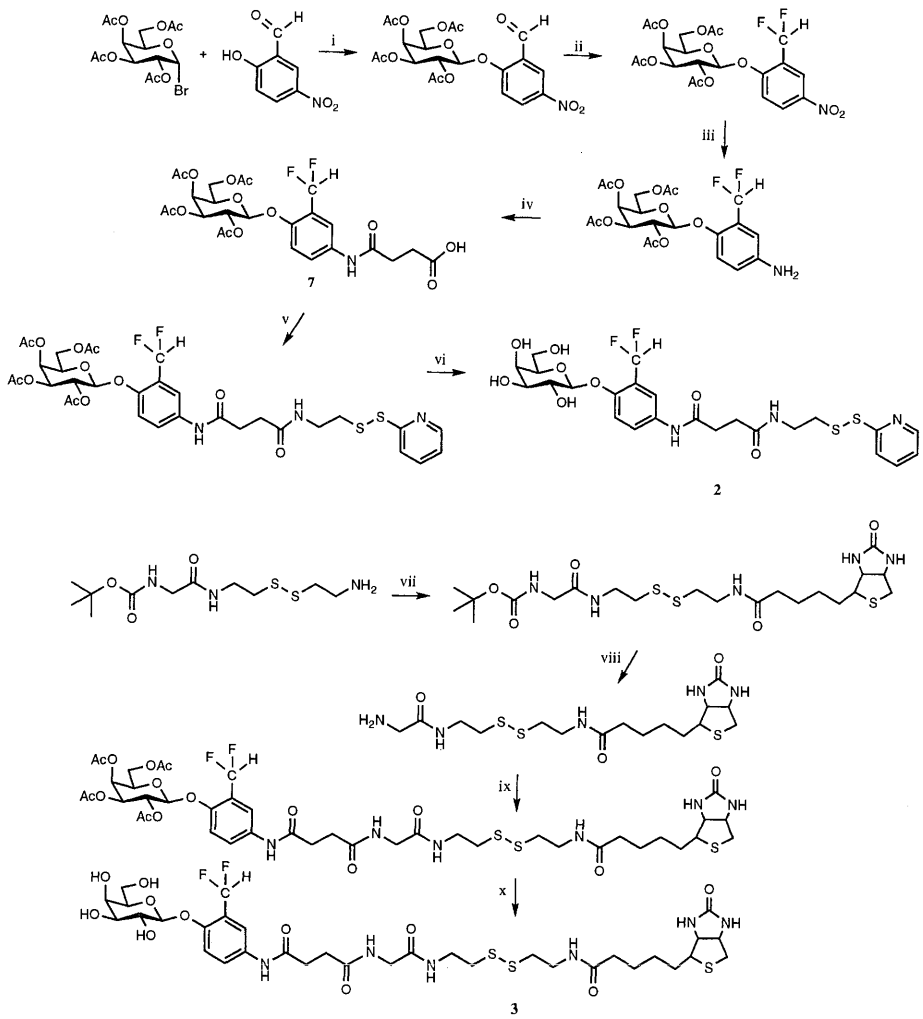


Fig. 3. Synthesis of mechanism-based panning reagent **2** and its biotinylated derivative **3**. Conditions: (i) NaOH, Bu_4NBr, H_2O-CH_2Cl_2, 55 percent; (ii) DAST, CH_2Cl_2, 96 percent; (iii) 10 percent Pd/C, H_2, EtOAc, 98 percent; (iv) succinic anhydride, TEA, CH_2Cl_2, 95 percent; (v) 2-pyridyldithioethylamine hydrochloride, DIEA, EDC, HOBt, CH_2Cl_2, 84 percent; (vi) Na_2CO_3, MeOH, 77 percent; (vii) (+)-biotin 4-nitrophenyl ester, TEA, MeCN, 87 percent; (viii) TFA; IRA-400 (OH^-), Amberlite ion-exchange resin, 99 percent; (ix) **7**, EDC, HOBt, DMF, 53 percent; and (x) Na_2CO_3, MeOH, 78 percent.

Kinetic analysis. Fab 1B was overexpressed in a β-galactosidase–deficient *E. coli* (*21*) and purified by affinity chromatography. At pH 7.8, Fab 1B catalyzed the hydrolysis of *p*-nitrophenyl-β-galactopyranoside (compound **5**, Fig. 4) and demonstrated turnover. The Michaelis-Menten constants were determined at $k_{cat} = 0.007$ min^{-1} and $K_M = 530$ μM, corresponding to a rate enhancement (k_{cat}/k_{uncat}) of 7×10^4. The hydrolysis of substrate **5** by Fab 1B could be competitively inhibited by hapten **1** with a K_i of 15 μM. The question of whether **2** acts as a nondestructive or suicide inactivator of antibody activity was also investigated. As expected, neither panning reagent **2** or **3** altered catalysis; but **3** was able to biontinylate Fab 1B. These results are consistent with our findings when β-galactosidase was used as the enzyme and indicate that Fab 1B was modified by panning reagent **2** at a site distant from the essential catalytic machinery.

It is also important to compare Fab 1B with antibodies obtained from simple hybridoma screening. A total of 22 monoclonal antibodies were obtained from hybridoma protocol when **1** was used as the antibody-eliciting hapten. The best catalyst (1F4) among these 22 clones for hydrolyzing *p*-ni-

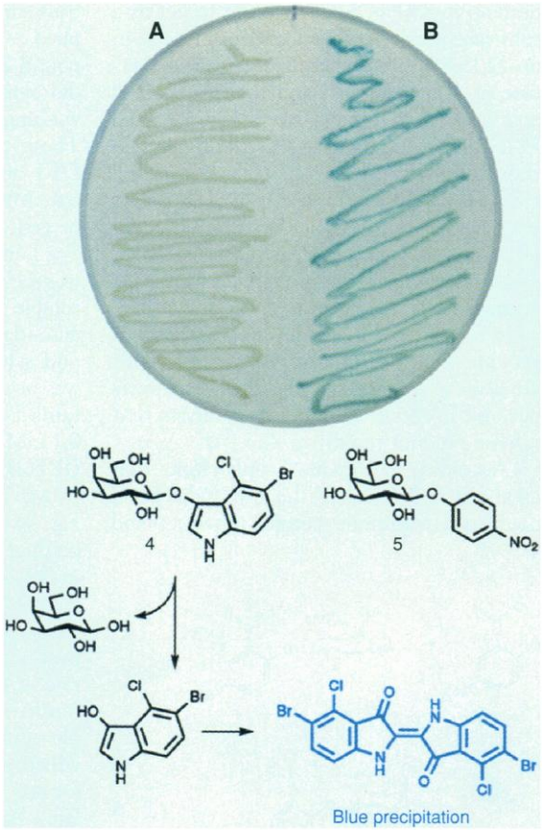


Fig. 4. Detection of catalytic antibody activity with a substrate-precipitating chromogenic assay: The DNA of Fab 1B was subcloned into the pWPY501 vector where protein expression is under the control of the T7 promoter. A positive selection for catalysis was confirmed by transformation of the pWPY501-1B plasmid into NoveBlue(DE3) cells, which are β-galacosidase deficient. The cells were plated onto Luria-Bertani (LB) agar plates containing 0.1 mM IPTG and 2 percent X-gal. (**A**) An unrelated mouse Fab elicited to a boronic acid hapten was cloned into pWPY501 and transformed into NoveBlue(DE3) cells (negative control). (**B**) The selected Fab-1B catalyst in NoveBlue(DE3) cells. Both cells containing the Fab genes (negative control and selected catalyst) were streaked onto a LB–IPTG–X-gal plate from a single clone. The photograph was taken after incubation of the plate for 72 hours at 37°C. The compound **4** corresponds to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal); the compound **5** corresponds to *p*-nitrophenyl-β-D-galactopyranoside.

trophenyl-β-galactopyranoside **5** provided the following kinetic parameters, $k_{cat} = 10^{-5}$ min^{-1}, $K_M = 330$ μM and a rate enhancement of only 10^2. These data support our contention that better catalysts may be isolated through a direct catalytic screen rather than simple binding, probably because the method combines the power of induction with the power of selection. Induction gives a protein binding pocket with defined binding specificities, whereas selection tests which of the solutions to binding give catalysis.

The method demonstrated above is for glycosidic bond cleavage, it should be applicable to any replicating combinatorial system in which a reactive species is formed during a chemical transformation. Many such systems have been described concerning mechanism-based inhibitors and suicide substrates (*23*). Some of these systems are quite sophisticated and depend on the presence of more than one functionality in proximity to the substrate. The ability to achieve complex mechanisms in antibody catalysis by selection on reactive species has already been shown (*24*). The use of such "reactive haptens" in conjunction with the catalyst screening format described above could allow selection for complex catalytic machinery such as the catalytic triad of the serine proteases.

A feature of the design of our experimental work is that we constructed a trapping reagent that did not interfere with turnover, thereby allowing selection for catalysis rather than stoichiometric reactivity. In view of the potential generality of our method, we should ask whether this chemical approach obviates the need for the construction of genetic selection systems in which survival of an organism such as an *E. coli* or yeast cell depends on the generation of a function that gives a growth advantage (*25*). Although these powerful genetic systems remain an option (especially for selection of variants with improved function), the improving methodology to increase the size and diversity of combinatorial libraries coupled with the ability to directly screen for chemical reactivity may obviate the need in many cases for biologically based selection systems. The advantage of purely chemical systems is one of generality in that many desired reactions do not yield intermediates or products that perturb cellular machinery, and thus biologically based selection systems cannot be used.

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