A General Fluorogenic Assay for Catalysis Using Antibody Sensors

Paul Geymayer, Nicolaus Bahr, and Jean-Louis Reymond*[a]

Abstract: A general assay for monitoring catalysis by fluorescence in real time has been developed by use of an antibody sensor. The sensor consists of a product-specific antibody tightly bound to a product analogue covalently labeled with the fluorescent tag acridone. Acridone fluorescence is quenched in the bound state. The reaction is monitored by following the fluorescence increase caused by displacement of the acridone-labeled product from the antibody combining site by the released product. Fluorescence detection of enzymatic hydrolysis of a β-galactoside and butyrate by β-galactosidase and esterase, respectively, are demonstrated. The assay operates by modulation of fluorescence intensity at 445 nm, a signal compatible with currently available instruments measuring in 96-well or 384-well plastic microtiter plates. The method is potentially general and only limited by binding selectivities of the product versus the substrate that can be encountered in antibodies.

Keywords: acridone · antibodies · enzyme catalysis · fluorescence spectroscopy · sensors

Introduction

An enormous variety of new catalytic activities are found by screening libraries of potential catalysts for a desired reaction.[1] Discovering catalysis in high-throughput screening is only possible if the reaction under study can be measured simply, sensitively, and reliably. Screening for catalysis is generally achieved by measuring product formation. Several methods for detecting product formation in high-throughput format have been reported. With substrates on solid supports, product formation can be measured by means of a product-specific reagent, for example, a product-specific antibody (*cat*-ELISA),[2] or a biotin[3] or DNA[4] tag added to the reaction. We have reported a highly sensitive assay based on analyzing reactions of substrates labeled with the fluorescent tag acridone by thin-layer chromatography.[5]

Although generally applicable, the above-mentioned assays are used in practice as single point measurements of product formation. However the ideal setup for catalysis screening involves continuous monitoring of product increase in solution. Indeed only by looking at the direct kinetics of a reaction can one distinguish real catalysts from product-inhibited catalysts that do not turn over. This can readily be done if a reaction involves a fluorogenic or chromogenic substrate.[6] However such substrates are usually biased in their reactivity or may not be available for a particular reaction. Herein we report a new approach for continuous monitoring of catalysis by fluorescence in solution using antibody sensors. This methodology can be applied generally to reactions of nonchromogenic and nonfluorogenic substrates.

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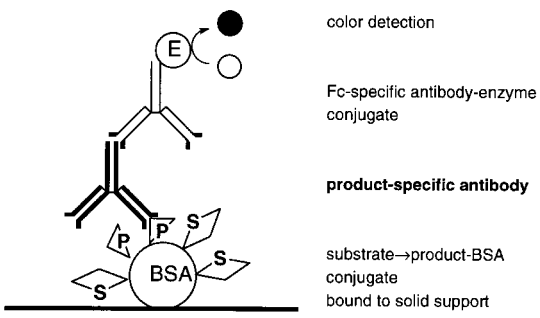
Results

Assay design: The *cat*-ELISA technique developed simultaneously by Green and Hilvert involves detection of product on a solid support. In this assay the solid-support-bound substrate is exposed to a test solution containing a possible catalyst. After some reaction time, the test solution is washed away and the product quantitated by ELISA (enzyme-linked immuno-sorbent assay) with a product-specific polyclonal rabbit antibody (Scheme 1).



Scheme 1. Principle of *cat*-ELISA assay. Step 1: Substrate on solid support is converted to product by catalytic reaction (not shown). Step 2: Product is quantitated by ELISA with a product-specific antibody.

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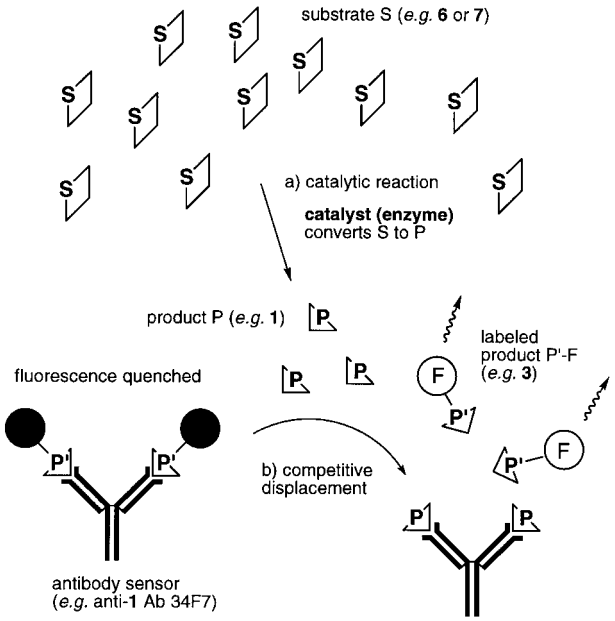
This assay furnishes a single-point measurement of product formation. In principle a similar measurement should be possible in real time with a product-specific sensor present in solution and capable of modulating a signal in real time upon selective product binding. We set out to investigate if such a sensor could be assembled from antibodies in analogy to *cat*-ELISA.

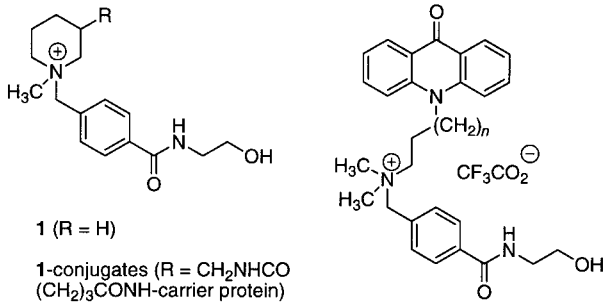
Due to the high intensity of fluorescence signals and their compatibility with biological buffers, we chose to develop a product-selective antibody sensor based on fluorescence.[7] Synthetic or semisynthetic fluorescent sensors[8] are known for inorganic ions,[9–12] carbohydrates,[13] imidazoles,[14] c-AMP[15, 16] and cholic acids.[17] Fluorescent sensors for organic molecules such as the typical organic reaction product are very readily obtained from polyclonal or monoclonal antibodies for use in homogeneous immunoassays.[18] Typical examples are sensors for gentamycin,[19] amphetamine,[20] glucose,[21] and estrone-3-glucuronide.[22]

Fluorescent antibody sensors can be constructed by combining analyte-specific polyclonal or monoclonal antibodies with an analyte analogue covalently labeled with a fluorescent tag.[23, 24] In many cases fluorescence of the tag is quenched or polarized upon binding to the antibody compared with its intensity in solution. The analyte then induces a signal modulation by competitive displacement of the labeled analyte from the antibody-combining site. For following a reaction one would use a product-specific antibody combined with a fluorescently labeled product analogue (Scheme 2).

To develop our catalysis assay we decided to work on monoclonal antibodies derived from hapten **1**.[25] Mechanistic studies with antibody 14D9,[26] an anti-**1** antibody that catalyzes an enantioselective protonation reaction,[27] have shown that anti-**1** antibodies display strong binding specificity for the aromatic benzamide group. For example antibody 14D9 binds hapten **1** approximately 100 times more tightly than its benzoate analogue **2**.[28] Anti-**1** antibodies would, therefore, be most likely to bind **1** selectively over derivatives of **1** modified at the *N*-hydroxyethylamide side chain, for example hydrolytically labile ester and acetal derivatives.



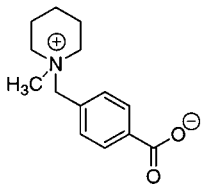
Scheme 2. Real-time catalysis assay with the use of a fluorescent antibody sensor. a) substrate (S) reacts to product (P) under the influence of a catalyst; b) released product (P) displaces quenched labeled product (P′-F) from the combining site of anti-P antibodies (Ab), whereupon fluorescence of the tag returns.



1 (R = H)

1-conjugates (R = $CH_2NHCO(CH_2)_3CONH$-carrier protein)

3 (*n* = 1)
4 (*n* = 4)



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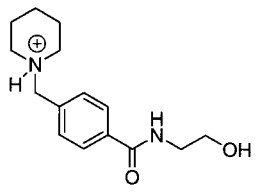

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Abstract in French: *Nous avons developpé une méthode générale pour mesurer la catalyse en temps réel par fluorescence à l'aide d'un anticorps-senseur. Ce senseur consiste en un anticorps spécifique pour le produit de réaction complexé avec un analogue de ce produit marqué par un noyau fluorescent de type acridone. La fluorescence de l'acridone est neutralisée par la complexation avec l'anticorps. On suit la réaction en mesurant l'augmentation de fluorescence produite par le déplacement du produit marqué à l'acridone par le produit formé. Ce principe est illustré pour l'hydrolyse enzymatique d'un β-galactoside et d'un butyrate par une β-galactosidase et une estérase, respectivement. La modulation d'intensité de fluorescence à 445 nm utilisée dans notre mesure est compatible avec les spectromètres pour plaques à 96 ou 384 puits disponibles actuellement. Cette méthode est potentiellement générale et seulement limitée par les discriminations substrat-produit réalisables à l'aide d'anticorps.*

Antibody sensors: Selective fluorescent sensors for hapten **1** can be prepared by combining anti-**1** antibodies with **3** or **4**, two achiral analogues of hapten **1** covalently labeled with the fluorescent tag acridone.[29] Among fourty different monoclonal anti-**1** antibodies, about a quarter of them quench the fluorescence of the acridone label by 80% or more. These quenching interactions take place by selective binding to the combining site of the antibodies, as evidenced by the fact that fluorescence of the labels **2** or **3** returns to full level upon

addition of excess hapten **1**. Thus, complexes of quenching antibodies (Ab) with **3** or **4** function as selective chemosensors for hapten **1** [see Eq. (1)].

$$\{(\mathbf{3}\text{ or }\mathbf{4})^{\text{quenched}}\cdot \text{Ab}\}+\mathbf{1}\rightarrow\{\mathbf{1}\cdot\text{Ab}\}+(\mathbf{3}\text{ or }\mathbf{4})^{\text{fluorescent}} \quad (1)$$

Two of these complexes, {**3**·34F7} and {**3**·83B8}, have been characterized in detail. The systems are well behaved and fluorescence can be cleanly titrated to two equivalents of acridone derivative **3** per antibody molecule (Figure 1).

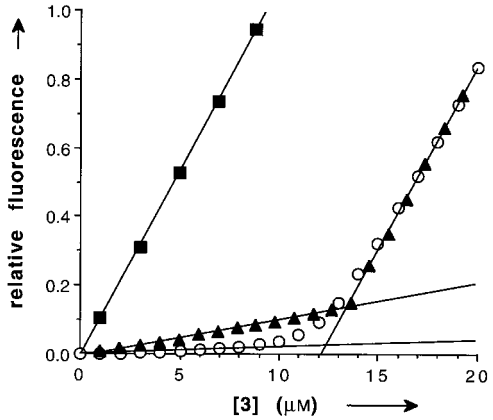


Figure 1. Fluorescence titration of antibodies 34F7 and 83B8 with **3**. Relative fluorescence emission at 445 nm upon excitation at 356 nm as a function of concentration. (■) aqueous buffer 10 mM phosphate, 160 mM NaCl, pH 7.4. (▲) antibody 34F7 at 1.125 mg mL^{-1}. (○) antibody 83B8 at 1.125 mg mL^{-1} in the same buffer. Saturation of antibody binding sites is reached at 13.6 μM **3** for antibody 34F7 and 12.2 μM **3** for antibody 83B8.

Binding studies with **1** and its close analogues **2** and **5** reveal that {**3**·34F7} displays good binding selectivity at the hydroxyethyl side chain, while {**3**·83B8} is highly selective for the *N*-methyl substituent of the piperidine nitrogen atom (Table 1).

Table 1. Binding selectivity of antibody sensors.

EC_{50}[a]	**1**	**2**	**5**
{**3**·34F7}	2 μM	530 μM	40 μM
{**3**·83B8}	8 μM	18 μM	980 μM

[a] EC_{50} is defined as the analyte concentration inducing 50% of the maximum fluorescence increase. Measured at 20 °C in aq. 160 mM NaCl, 10 mM phosphate, pH 7.4 with 1 μM **2** + 0.5 μM antibody 34F7 and/or 0.5 μM antibody 83B8.

In view of developing a real-time assay for catalysis based on these sensors, we investigated closely the kinetics of signal switching. Chemosensor {**3**·83B8} responded within the mixing time upon addition of analyte, and the detailed kinetics could not be measured precisely. For chemosensor {**3**·34F7} signal equilibration required between thirty seconds and three minutes for complete equilibration, depending on product concentration (Figure 2). The apparent first-order rate constant in the experiment with excess hapten **1** (50 μM) provided an estimate for the rate of dissociation of the sensor {34F7·**3**}, k_{off} (**3**) = 0.017 s^{-1}. Since $K_D = k_{off}/k_{on}$ and K_D(**3**) ~5 nM, the rate constant for association is k_{on} (**3**) ~ 3.4 × 10^6 M^{-1}s^{-1}, which is well-below diffusion control. While much

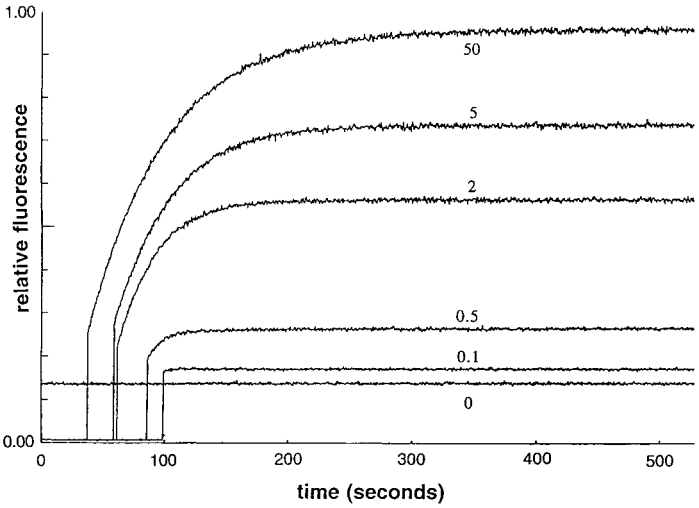
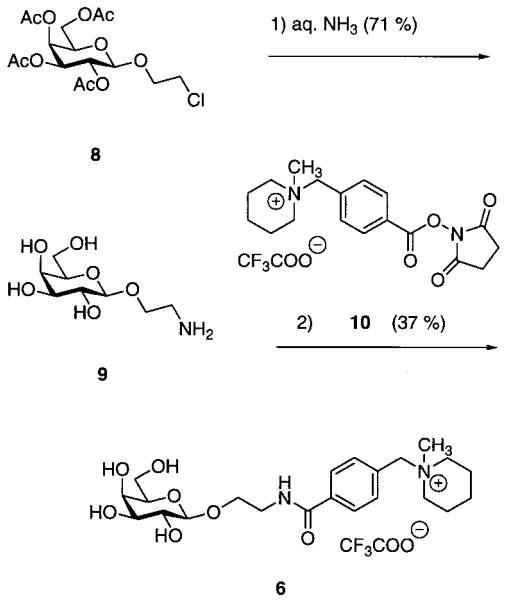


Figure 2. Time-response curves of fluorescent antibody-sensor 34F7·**3** (0.5 μM in 10 mM phosphate, 160 mM NaCl, pH 7.4) at different concentrations of hapten **1** in μM. For each curve the start of the reaction was approximately 5 seconds before the initial vertical line.

longer than for {**3**·83B8}, the equilibration time with {**3**·34F7} was shorter than the reported 5–20 minute equilibration times usually required in antibody-based homogeneous fluorescence assays.

Catalysis assay: Enzymatic hydrolyses of **6** by β-galactosidase and of butyrate **7** by esterases were chosen as test reactions. Compound **8,** obtained from 1-bromo-tetraacetyl galactose and chloroethanol, gave directly aminogalactoside **9** upon treatment with ammonia in water. Coupling with *N*-hydroxysuccinimide ester **10**, obtained by activation of **2**, gave substrate **6** (Scheme 3). Butyrate **7** was obtained by quaternization of *N*-methylpiperidine with **11** (Scheme 4). Both substrate were purified by preparative reverse-phase HPLC and isolated as trifluoroacetate salts.



Scheme 3. Synthesis of β-galactosidase substrate **6**.

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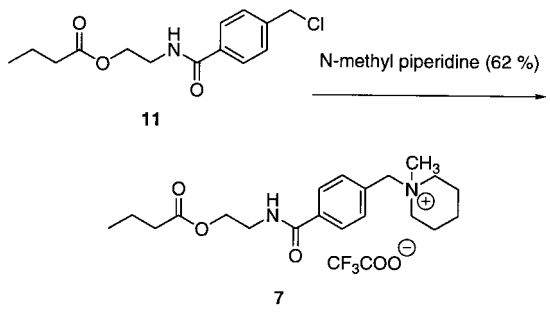

Scheme 4. Synthesis of esterase substrate **7**.

As expected, sensor {**3**·34F7} bound **1** with high selectivity over its galactoside **6** or butyrate **7** (Figure 3). By contrast sensor {**3**·83B8} showed no binding selectivity for **1** against these substrates. Kinetic measurements were therefore carried out with the sensor {**3**·34F7}.

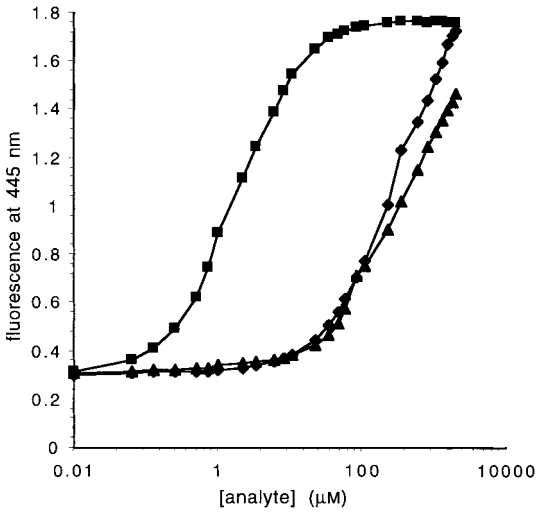

Figure 3. Fluorescence readings of chemosensor {**3**·34F7} at 445 nm as a function of analyte concentration, as a logarithmic plot: (■) product **1**; (▲) galactoside **6**; (◆) butyrate **7**. Measured in 160 mM NaCl, 10 mM phosphate, pH 7.4 with 1 μM sensor. The lines were obtained by interpolation from the experimental points.

First we investigated if nonspecific processes might lead to a signal increase in the absence of our substrates **6** and **7**. Both sensors {**3**·34F7} and {**3**·83B8} were found to be highly stable and photoresistant in the quenched state over several days at 20 °C. However, fluorescence increase was observed upon addition of cosolvents such as dimethyl formamide (10 % v/v), most likely by the weakening of noncovalent interactions between **3** and the antibodies. Thus our fluorescence assay would require an aqueous buffered environment. Under these conditions, however, the sensors were found to be stable in the presence of different enzyme preparations, showing that direct action of enzymes on the sensors would not lead to a fluorescence signal.

We then turned to fluorogenic measurement of enzymatic hydrolysis of **6** and **7** with the sensor {**3**·34F7}. Substrates at either 10 μM, 50 μM, or 100 μM were incubated in the presence of different concentrations of either hog-liver esterase or β-galactosidase and 1 μM sensor {34F7·**3**}. A rapid fluorescence increase was observed within seconds when β-galactosidase was added to **6** or when esterase was added to **7** (Figures 4 and 5). HPLC analysis confirmed that product **1** was being

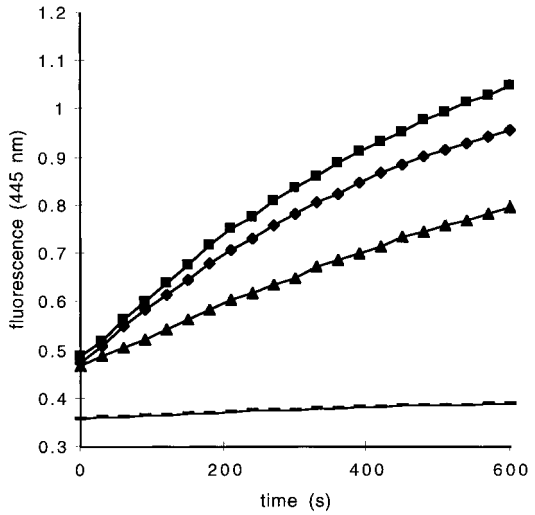

Figure 4. Time course of fluorescence catalysis assay for β-galactosidase with 50 μM **6**, 1 μM sensor {**3**·34F7}, and: (—) no enzyme, (▲) 3.33 μg mL^{-1}, (◆) 6.67 μg mL^{-1}, and (■) 13.3 μg mL^{-1} of a β-galactosidase preparation from *E. coli*. Measured at 20 °C in aq. 160 mM NaCl, 10 mM phosphate, pH 7.4. The lines were obtained by interpolation from the experimental points.

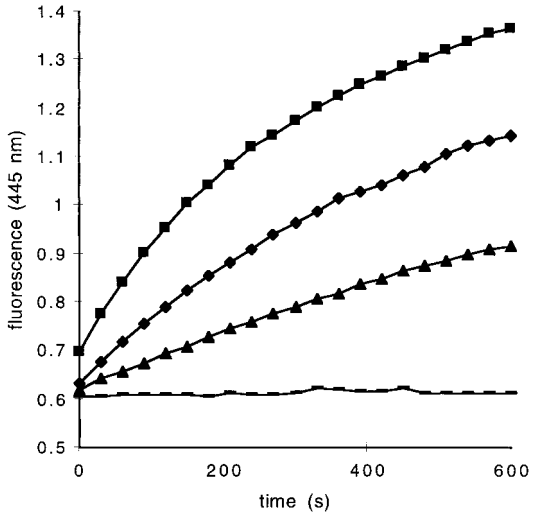

Figure 5. Time course of fluorescence catalysis assay for hog-liver esterase with 50 μM **7**, 1 μM sensor {**3**·34F7}, and: (—) no enzyme, (▲) 1.5 μg mL^{-1}, (◆) 3 μg mL^{-1}, and (■) 6 μg mL^{-1} of hog-liver esterase. Measured at 20 °C in aq. 160 mM NaCl, 10 mM phosphate, pH 7.4. The lines were obtained by interpolation from the experimental points.

released from the substrates under these conditions. By contrast there was no fluorescence increase when galactoside **6** was treated with esterase, or when butyrate **7** was treated with β-galactosidase. Thus sensor {**3**·34F7} allowed unequivocal identification of catalysis by fluorescence.

For each assay, the apparent rate for product release was calculated by converting fluorescence to the concentration of compound **1** from the calibration curve in Figure 3. Apparent rates were linear over the first ten minutes of reaction, corresponding to the release of up to 4 μM of the product (Table 2). Initial velocities were calculated from the first three

Table 2. Initial reaction rates for enzyme-catalyzed release of **1** [$\mu M\,s^{-1} \times 10^5$], at different enzyme concentrations [$\mu g\,mL^{-1}$], as calculated from fluorescence data from the catalysis sensor {**3**·34F7}.[a]

		β-Galactosidase				Hog-liver esterase			
		0	3.3	6.7	13.3	0	1.5	3.0	6.0
galactoside **6**	10 μM	1.2	6.6	24	78	–	–	–	–
galactoside **6**	50 μM	1.3	116	201	224	–	–	–	5.3
galactoside **6**	100 μM	11	125	232	238	–	–	–	–
butyrate **7**	10 μM	–	–	–	–	3.7	29	72	184
butyrate **7**	50 μM	–	–	–	4.0	0.25	98	190	440
butyrate **7**	100 μM	–	–	–	–	6.5	170	410	–[b]

[a] Reactions were carried out in aqueous 160 mM NaCl, 10 mM phosphate, pH 7.4 in the presence of 1 μM sensor {**3**·34F7}. Fluorescence was recorded with $\lambda_{exc} = 356$ nm, $\lambda_{em} = 445$ nm. Fluorescence data was converted to product concentration from the calibration curve derived from Figure 3. [b] Data points were outside the linear range of log[**1**] versus fluorescence.

minutes of the reaction. The results show that the apparent reaction rates were proportional to enzyme concentration at low catalyst concentration. The observed kinetic saturation at high enzyme concentration must reflect the rate-limiting displacement of **3** by product **1** from the binding pocket of antibody 34F7. The measured off-rate for {34F7·**3**} is k_{off} (**3**) = 0.017 s^{-1} (see Figure 2). However, since EC_{50}(**1**) = 2 μM (Table 1), only 0.5 μM of **3** must be exchanged from 1 μM sensor {**3**·34F7} to measure the release of 2 μM product **1**. One could therefore expect the sensor to measure a rate of release up to four times higher than its off-rate, depending on the extent of competing recomplexation of **3**. The maximum rate of product release measured by sensor {**3**·34F7} in the enzyme assays is approximately 0.044 $\mu M\,s^{-1}$ (3 $\mu M\,min^{-1}$), which is well within this limit.

Discussion

The above described sensor allows direct continuous measurement of catalysis for two enzymatic hydrolytic reactions liberating product **1**. In both cases it is clear that the activity of the enzymes used here can also be recorded spectroscopically by means of simple fluorogenic or chromogenic substrates such as β-D-nitrophenylgalactoside or nitrophenyl acetate. However, it must be noted that such substrates are chemically activated for their hydrolysis, a property that is almost always encountered for chromogenic or fluorogenic substrates. While this may not matter for detecting an existing enzyme, this represents a serious limitation when discovery of new catalysts is the goal, since very different chemical properties may be required for cleaving nonactivated bonds. Using our antibody sensor, we have recorded an enzymatic ester and glycoside cleavage of a nonactivated alkyl alcohol with unbiased reactivity.

More generally, the strategy outlined here can be applied to fluorescence sensing of any chemical transformation. Sensor preparation involved the preparation of monoclonal antibodies against a protein conjugate of the reaction product, here compound **1**, and the selection of antibodies that would efficiently quench the fluorescence of a labeled analogue of this product upon binding. Owing to the high proportion (25%) of quenching antibodies found in our library and the ample precedent for fluorescence quenching by antibodies in related systems, one can reasonably assume that similar antibodies could also be found in immunizations against other products.

Finding good binding selectivity of product over substrate is critical to achieve catalysis sensing. Evidently condensation or cleavage reactions such as those used for demonstrating solid-supported *cat*-ELISA and the present assay are readily amenable to high product-binding selectivities that result from large changes in functional groups or charges, which are easily recognized by antibodies. Reactions involving more modest functional changes, such as olefin hydrogenation or epoxidation, could prove more challenging.

Direct monitoring of product increase in our method critically depends on the exchange rate of product against fluorescence-labeled product in the binding pocket of the antibody sensor. The rate of signal exchange is limited by k_{off}, the off-rate for release of labeled product from the antibody binding pocket. Due to the necessity for the antibody sensor to bind both product and labeled product tightly, their dissociation constant K_D must be small. Since $K_D = k_{off}/k_{on}$ and k_{on} is limited by diffusion, k_{off} is bound to be relatively small in a sensitive and selective sensor. Despite of this fundamental limitation, our experiments clearly show that a kinetically practical sensor can be prepared.

Conclusion

A fluorogenic assay for catalysis has been developed by means of a sensor consisting of a product-specific antibody combined with a product analogue covalently labeled with the fluorescent tag acridone. Hydrolysis of alkyl galactoside **6** and butyrate **7** are readily detected by fluorescence. Sensor equilibration becomes rate-limiting at high catalyst or substrate concentration. It should be noted that our catalysis sensor operates by the simple modulation of fluorescence intensity above 400 nm. Unlike wavelength shifts or polarization changes, which are used in many fluorescent sensors, straight intensity modulation is a signal compatible with currently available high-throughput screening instruments measuring in 96-well or 384-well plastic plates. This direct assay for catalysis is potentially general and is only limited by binding selectivities of product versus substrate that can be encountered in antibodies.

Experimental Section

All reagents and enzymes were purchased from Aldrich or Fluka. All chromatographies (flash) were performed with Merck Silicagel 60 (0.040–0.063 mm). Preparative HPLC was done with HPLC grade acetonitrile and MilliQ deionized water with a Waters prepak cartridge 500g installed on a Waters Prep LC 4000 system from Millipore, flow rate 100 $mL\,min^{-1}$, gradient +0.5% min^{-1} CH_3CN, detection by UV at 230 nm. The HPLC conditions ofr substrates and products are given in Table 3. TLC was performed with fluorescent F254 glass plates. MS, HRMS (high resolution mass spectra) were provided by Dr. Thomas Schneeberger (University of Bern). Fluorescence measurements were carried out with an SPF-500C spectrofluorometer from SLM Instruments, a FluoroMax Spectrofluoro-

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meter from SPEX Industries, and a Cytofluor II Plate-Reader from Perseptive Biosystems.

***N*-{[4′-(Hydroxyethyl)carbamido]phenyl}methyl-*N*-[3-(*N*′-acridonyl)-1″-propyl]-*N*,*N*-dimethylpiperidinium trifluoroacetate (3)**: *N*-(3-dimethylamino-1-propyl)-acridone[30] (20 mg, 0.07 mmol) and *N*-hydroxyethyl-4-chloro methyl-benzamide[26] (30 mg, 0.14 mmol) were stirred in DMF (0.2 mL) for two days at 20 °C. Dilution with water and purification by preparative RP-HPLC gave **3** as the trifluoroacetate salt (40 mg, 0.07 mmol, 100 %). Pale yellow crystalline solid, m.p. 206–208 °C (decomp); ^{1}H NMR (300 MHz, D_2O): $\delta = 8.13$ (dd, $^3J = 8.5$, 2 Hz, 2 H), 7.72 (ddd, $^3J = 9$, 7, 2 Hz, 2 H), 7.47 (d, $^3J = 9$ Hz, 2 H), 7.33 (d, $^3J = 8.5$ Hz, 2 H), 7.26 (t, $^3J = 7$ Hz, 2 H), 7.01 (d, $^3J = 8.5$ Hz, 2 H), 4.40 (t, $^3J = 7$ Hz, 2 H), 4.31 (s, 2 H), 3.80, 3.53 (2 t, $^3J = 6$ Hz, 2 × 2 H), 2.96 (s, 8 H), 2.28 (m, 2 H); ^{13}C NMR (100 MHz, $D_2O + 30\%$ CD_3OD): $\delta = 179.3$, 170.1, 141.7, 136.5, 136.1, 133.4, 131.0, 128.5, 127.5, 123.2, 121.6, 116.0, 68.5, 61.0, 51.3, 43.1, 42.1, 21.9; IR (KBr): $\tilde{\nu} = 3412$, 1682, 1652, 1598, 1558, 1500, 1462, 1292, 1182, 1122, 754, 674 cm^{-1}; HRMS (FAB +): $C_{28}H_{32}N_3O_3^+$ calcd 458.2444, found 458.2461.

1-(2-Aminoethyl)-β-D-galactopyranoside (9):[31] The product was obtained by aminolysis of 2-chloroethyl-tetra-*O*-acetyl-β-D-galactopyranoside[32] (1.22 g, 2.97 mmol) in aqueous ammonium hydroxyde (25 %, 20 mL) at 65 °C over 3 days in a pressure vial. After evaporation of solvent, the crude product was dissolved in water (20 mL) and purified on Dowex 50X8 (SO_3H-form, washing with water and water/MeOH 1:1, elution with 5 % and 25 % NH_3 in water). After lyophilization **9** (473 mg, 2.12 mmol, 71 %) was obtained. Spectral data corresponded to published data.

***N*-{[4′-(β-D-Galactosyloxyethyl)carbamido]phenyl}methyl-*N*-methylpiperidinium trifluoroacetate (6)**: 1-(4-carboxy-benzyl)-1-methylpiperidinium chloride **2** (584 mg, 2 mmol) in DMF (6 mL) and water (0.6 mL) was treated overnight with *N*-ethyl-*N*′-diethylaminopropylcarbodiimide hydrochloride (EDC; 766 mg, 4 mmol) and *N*-hydroxysuccinimide (460 mg, 4 mmol) at 20 °C. Purification by preparative HPLC and lyophilization of the product-containing fractions gave **10** (549 mg, 1.23 mmol, 62 %) as a colorless solid. The product contained 15–20 % (according to HPLC analysis) of **2** and was used as such.

Spectral data for **10**: ^{1}H NMR (300 MHz, D_2O): $\delta = 7.86$ (d, $J = 8.5$ Hz, 2 H), 7.45 (d, $J = 8.5$ Hz, 2 H), 4.34 (s, 2 H), 3.15 (m, 4 H), 2.75 (s, 3 H), 2.74 (s, 4 H), 1.68 (m, 4 H), 1.47 (m, 1 H), 1.34 (m, 1 H); ^{13}C NMR (100 MHz, D_2O/CD_3OD): $\delta = 176.7$, 173.3, 169.7, 135.1, 134.8, 131.7, 127.3, 61.1, 47.5, 26.5, 26.3, 21.5, 20.6.

Amine **9** (112 mg, 0.43 mmol), activated ester **10** (266 mg, 0.6 mmol), and $NaHCO_3$ (80 mg, 1 mmol) were stirred overnight. HPLC analysis showed complete consumption of active ester. Preparative HPLC and lyophilization of the product containing fractions gave **6** as colorless sirup (88 mg, 0.16 mmol, 37 %). ^{1}H NMR (300 MHz, D_2O): $\delta = 7.75$, 7.53 (2 d, $J = 8.5$ Hz, 2 × 2 H), 4.44 (s, 2 H), 4.33 (d, $J = 7.7$ Hz, 1 H), 3.98 (m, 1 H), 3.80 (m, 2 H), 3.64–3.49 (m, 6 H), 3.43 (dd, $J = 7.7$, 9.9 Hz, 1 H), 3.30 (m, 4 H), 2.87 (s, 3 H), 1.82 (m, 4 H), 1.62 (m, 1 H), 1.54 (m, 1 H); ^{13}C NMR (100 MHz, D_2O/CD_3OD): $\delta = 171.03$, 136.59, 134.31, 131.45, 128.71, 104.11, 76.14, 73.71, 71.78, 69.58, 69.42, 68.15, 61.93, 61.90, 47.51, 41.04, 21.57, 20.61; HRMS (EI$^+$): $C_{22}H_{35}N_2O_7^+$ [M^+] calcd 439.2435, found 439.24597.

***N*-(Butanoyloxyethyl)-4-chloromethylbenzamide (11)**: Triethylamine (160 mg, 1.6 mmol), catalytic amounts of DMAP and butyroyl chloride (173 mg, 1.6 mmol) were added to a suspension of *N*-(hydroxyethyl)-4-chloromethylbenzamide[26] (213 mg, 1 mmol) in dry toluene (20 mL). The mixture was stirred for 90 min at 20 °C and then diluted with toluene (20 mL), washed (3 × aq. sat. $NaHCO_3$), and dried over Na_2SO_4. Solvent evaporation and chromatography (hexane/EtOAc 1:3) gave **11** (256 mg, 0.9 mmol, 90 %) as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.73$, 7.42 (2 d, $J = 8.5$ Hz, 2 × 2 H), 6.65 (s, 1 H), 4.58 (s, 2 H), 4.28 (t, $J = 5.15$ Hz, 2 H), 3.69 (m, $J = 5.15$, 5.51 Hz, 2 H), 2.30 (t, $J = 7.7$ Hz, 2 H), 1.63 (m, $J = 7.7$, 7.4 Hz, 2 H), 0.91 (t, $J = 7.4$ Hz, 3 H); HRMS (LSIM): $C_{14}H_{18}ClNO_3$ [M^+] calcd. 284.10491 found 284.10516; IR ($CHCl_3$): $\tilde{\nu} = 3018$, 2968, 1730, 1654, 1534, 1504, 1458, 1266, 1182, 1094, 668 cm^{-1}.

***N*-{4′-[(Butanoyloxyethyl)carbamido]phenyl}methyl-*N*-methylpiperidinium trifluoroacetate (7)**: Butyrate **11** (256 mg) in *N*-methylpiperidine (1 mL) and DMF (1 mL) was stirred for 60 min at 40 °C. After solvent evaporation the residue was dissolved in water (30 mL) and purification by preparative RP-HPLC gave substrate **7** (255 mg, 0.554 mmol, 62 %) as colorless solid. ^{1}H NMR (300 MHz, D_2O): $\delta = 7.72$, 7.54 (2 d, $J = 8.1$ Hz, 2x2 H), 4.45 (s, 2 H), 4.21 (t, $J = 5.2$ Hz, 2 H), 3.57 (t, $J = 5.2$ Hz, 2 H), 3.27 (m, 4 H), 2.87 (s, 3 H), 2.23 (t, 7.4 Hz, 2 H), 1.82 (m, 4 H), 1.63 (m, 1 H), 1.51 (m, 1 H), 1.45 (m, 2 H), 0.70 (t, $J = 7.4$ Hz, 3 H); ^{13}C NMR (D_2O/CD_3OD): $\delta = 177.51$, 170.74, 136.68, 134.36, 131.51, 128.63, 68.10, 63.91, 61.99, 47.50, 39.89, 36.68, 21.62, 20.63, 18.96, 13.75; HRMS (LSIM): $C_{20}H_{31}N_2O_3^+$ calcd 347.2327, found 347.23343.

Table 3. HPLC Conditions for substrates and products.[a]

	% A	% B	t_R [min]	HPLC purity
1[b]	90	10	4.99	> 99 %
2[b]	90	10	7.53	> 98 %
3[c]	50	50	3.80	> 99 %
6[b]	90	10	4.73	> 99 %
7[b]	70	30	8.03	> 99 %
10[b]	80	20	11.40	~ 80 %

[a] Isocratic elution at 1.5 mL min^{-1}, detection by UV at 230 nm, A = 0.1 % TFA in H_2O, B = 50:50 CH_3CN/H_2O, analytical column: [b] Vydac 218TP-54 (C_{18}, poresize 300 Å) or [c] Microsorb-MV 86–203-C5 (C_{18}, poresize 300 Å), 0.45 × 22 cm. Preparative HPLC was performed at 100 mL min^{-1} on a Waters prepak cartridge 500 g, gradient +1 % B min^{-1} starting with 10–15 % less B than the isocratic conditions.

Antibodies: Monoclonal antibodies against the KLH conjugate of **1** were produced by standard procedures, and were obtained from ascites fluid grown from the individual hybridoma cell lines.[33] Each antibody was purified to homogeneity by ammonium sulfate precipitation, followed by anion exchange and protein G chromatography, dialyzed into phosphate (10 mM) and NaCl (160 mM) with pH 7.4, and its concentration estimated by UV at 280 nm as c (mg mL^{-1}) = Abs/1.4.

Fluorescence measurements: Fluorescence measurements were carried out at 20 °C in a 1 × 1 cm cuvette with $\lambda_{exc} = 356$ nm, $\lambda_{em} = 445$ nm, set to give maximum fluorescence reading (2.0) at 445 nm for 2 μM free **3**. All measurements were taken at 20 °C in PBS (aqueous 160 mM NaCl, 10 mM phosphate, pH 7.4). Kinetic assays were also carried out in 96-well polypropylene round-bottom plates by use of a Cytofluor II Plate-Reader from Perseptive Biosystems, with emission filter $\lambda_{ex} = 360 \pm 20$ nm, and emission filter $\lambda_{em} = 440 \pm 20$ nm. Results were identical to measurements done in 1 × 1 cm cells.

Equilibrium measurements (data in Table 1 and Figure 1): Aliquots of properly prediluted solutions of analyte **1**, **2**, **5**, **6** and **7** were added to the sensor (2 mL; 1 μM **3** + 0.5 μM antibody 34F7 and/or 0.5 μM antibody 83B8). The solution was then mixed with a 1 mL pipetter and allowed to stand for 2 minutes before recording fluorescence.

Kinetics of exchange (data in Figure 2): The reaction was initiated by adding a prediluted solution of hapten **1** (1 mL) to the sensor solution (1 mL; 1 μM {**3**·34F7} complex) in the fluorometer cell and mixing for 5 seconds.

Enzyme kinetics (data in Figures 4 and 5, Table 2): Assay mixtures were initiated by adding an enzyme stock solution to PBS (2 mL) containing sensor {**3**·34F7} (1 μM) and substrate **6** or **7** at the given concentration in PBS. β-galacotsidase (Fluka 48274) contained 540 U mg^{-1} {1 U (U = unit) hydrolyses 1 μmol min^{-1} 4-nitrophenyl-β-D-galactoside at pH 7.8, 37 °C). Hog-liver esterase (Fluka 46058) contained 240 U mg^{-1} (1 U hydrolyses 1 μmol min^{-1} ethyl valerate at pH 8.0, 25 °C).

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[1] a) P. G. Schultz, R. A. Lerner, *Science* **1995**, *269*, 1835; b) S. Borman, *Chem. Eng. News* **1996**, *74*(45), 37.

[2] a) D. S. Tawfik, B. S. Green, R. Chap, M. Sela, Z. Eshhar, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 373; b) G. MacBeath, D. Hilvert, *J. Am. Chem. Soc.* **1994**, *116*, 6101; c) F. Benedetti, F. Berti, F. Massimiliano, M. Resmini, E. Bastiani, *Anal. Biochem.* **1998**, *256*, 67.

[3] J. W. Lane, X. Hong, A. W. Schwabacher, *J. Am. Chem. Soc.* **1993**, *115*, 2078.
[4] H. Fenniri, K. D. Janda, R. A. Lerner, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2278.
[5] J.-L. Reymond, T. Koch, J. Schröer, E. Tierney, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4251.
[6] a) M. T. Reetz, A. Zonta, K. Schimossek, K. Liebeton, K.-E. Jaeger, *Angew. Chem.* **1997**, *109*, 2961; b) L. E. Janes, R. J. Kazlauskas, *J. Org. Chem.* **1997**, *62*, 4560; c) G. Klein, J.-L. Reymond, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1113; d) N. Jourdain, R. Pérez-Carlón, J.-L. Reymond, *Tetrahedron Lett.* **1998**, *39*, 9415; e) G. Klein, J.-L. Reymond, *Helv. Chim. Acta* **1999**, in press.
[7] R. P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed., Molecular Probes, **1995**, pp. 201 – 244.
[8] a) R. Y. Tsien, *Fluorescent Chemosensors for Ion and Molecule Recognition* (Ed.: A. W. Czarnik), American Chemical Society, Washington, DC, **1993**; b) A. P. de Silva, H. Q. N. Gunaratne, T. Gunnlaugsson, A. J. M. Huxley, C. P. McCoy, J. T. Rademacher, T. E. Rice, *Chem. Rev.* **1997**, *97*, 1515.
[9] a) A. W. Czarnik, *Chem. Biol.* **1995**, *2*, 423; b) R. A. Bissell, A. P. de Silva, H. Q. N. Gunaratne, P. L. M. Lynch, G. E. M. Maguire, C. P. McCoy, K. R. A. S. Sandanayake, *Top. Curr. Chem.* **1993**, *168*, 223; c) R. Krämer, *Angew. Chem.* **1998**, *110*, 804; *Angew. Chem. Int. Ed.* **1998**, *37*, 772.
[10] a) L. Fabbrizzi, M. Licchelli, P. Pallavicini, A. Perotti, D. Sacchi, *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1975; b) L. Fabbrizi, M. Licchelli, P. Pallavicini, A. Perotti, A. Taglietti, D. Sacchi, *Chem. Eur. J.* **1996**, *2*, 75; c) A. P. de Silva, H. Q. N. Gunaratne, C. P. McCoy, *J. Am. Chem. Soc.* **1997**, *119*, 7891; d) B. Ramachandram, A. Samanta, *Chem. Commun.* **1997**, 1037; e) G. E. Collins, L.-S. Choi, *Chem. Commun.* **1997**, 1135; f) L. Fabbrizzi, M. Licchelli, P. Pallavicini, L. Parodi, *Angew. Chem.* **1998**, *110*, 838; *Angew. Chem. Int. Ed.* **1998**, *37*, 800.
[11] a) G. K. Walkup, B. Imperiali, *J. Am. Chem. Soc.* **1996**, *118*, 3053; b) J. A. Godwin, J. M. Berg, *J. Am. Chem. Soc.* **1996**, *118*, 6514; c) A. Torrado, G. K. Walkup, B. Imperiali, *J. Am. Chem. Soc.* **1998**, *120*, 609; d) G. K. Walkup, B. Imperiali, *J. Am. Chem. Soc.* **1997**, *119*, 3443; e) J. Yoon, N. E. Ohler, D. H. Vance, W. D. Aumiller, A. W. Czarnik, *Tetrahedron Lett.* **1997**, *38*, 3845; f) U. Oguz, E. U. Akkaya, *Tetrahedron Lett.* **1997**, *38*, 4509; g) E. Brunet, M. T. Alonso, O. Juanes, R. Sedano, J. C. Rodriguez-Ubis, *Tetrahedron Lett.* **1997**, *38*, 4459; h) J. D. Winkler, C. M. Bowen, V. Michelet, *J. Am. Chem. Soc.* **1998**, *120*, 3237.
[12] A. Miyawaki, J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams, M. Ikura, R. Y. Tsien, *Nature* **1997**, *388*, 882.
[13] a) T. D. James, K. R. A. S. Sandanayake, S. Shinkai, *Angew. Chem.* **1994**, *106*, 2287; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 2207; b)T. D. James, K. R. A. S. Sandanayake, S. Shinkai, *Nature* **1995**, *374*, 345; c) T. D. James, H. Shimmori, S. Shinkai, *Chem. Commun.* **1997**, 71; d) M. Takeuchi, M. Yamamoto, S. Shinkai, *Chem. Commun.* **1997**, 1731; e) C. R. Cooper, T. D. James, *Chem. Commun.* **1997**, 1419; f) J. S. Marvin, H. W. Hellinga, *J. Am. Chem. Soc.* **1998**, *120*, 7.
[14] L. Fabbrizzi, G. Francese, M. Licchelli, A. Perotti, A. Taglietti, *Chem. Commun.* **1997**, 581.
[15] S. R. Adams, A. T. Harootunian, Y. J. Buechler, S. S. Taylor, R. Y. Tsien, *Nature* **1991**, *349*, 694.
[16] M. A. DeBernardi, G. Brooker, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4577.
[17] J. Wang, A. Nakamura, K. Hamasaki, H. Ikeda, T. Ikeda, A. Ueno, *Chem.Lett.* **1996**, 303.
[18] a) I. Hemmilä, *Clin. Chem.* **1985**, *31*, 359; b) J. P. Gosling, *Clin. Chem.* **1990**, *36*, 1408; c) C. L. Morgan, D. J. Newman, C. P. Price, *Clin. Chem.* **1996**, *42*, 193; d) E. Gizeli, C. R. Lowe, *Curr. Opin. Biotechnol.* **1996**, *7*, 66.
[19] E. J. Shaw, R. A. A. Watson, D. S. Smith, *Clin. Chem.* **1979**, *25*, 322.
[20] D. L. Colbert, G. Gallacher, R. W. Mainwaring-Burton, *Clin. Chem.* **1985**, *31*, 1193.
[21] J. S. Schultz, S. Mansouri, I. J. Goldstein, *Diabetes Care* **1982**, *5*, 245.
[22] G. Barnard, F. Kohen, H. Mikola, T. Lövgren, *Clin. Chem.* **1989**, *35*, 555.
[23] For a general introduction on antibody-based sensors: A. M. Campbell, *Monclonal Antibodies and Immunosensor Technology* (Ed.: P. C. van der Vilet), Elsevier, Amsterdam, **1991**, chapter 12, pp. 343 – 372.
[24] For antibodies as fluorescent chemosensors, see: D. S. Smith, M. H. H. Al-Hakiem, J. Landon, *Ann. Clin. Biochem.* **1981**, *18*, 253.
[25] T. Koch, J.-L. Reymond, R. A. Lerner, *J. Am. Chem. Soc.* **1995**, *117*, 9383, and references therein.
[26] a) J.-L. Reymond, G. K. Jahangiri, C. Stoudt, R. A. Lerner, *J. Am. Chem. Soc.* **1993**, *115*, 3909; b) G. K. Jahangiri, J.-L. Reymond, *J. Am. Chem. Soc.* **1994**, *116*, 11 264.
[27] J.-L. Reymond, J.-L. Reber, R. A. Lerner, *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 475.
[28] D. Shabat, S. C. Sinha, J.-L. Reymond, E. Keinan, *Angew. Chem.* **1996**, *108*, 2800; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 2628.
[29] Preliminary communication: N. Bahr, E. Tierney, J.-L. Reymond, *Tetrahedron Lett.* **1997**, *38*, 1489.
[30] S. J. Schmolka, H. Zimmer, *Synthesis* **1984**, *1*, 29.
[31] a) C. K. Chiang, M. McAndrew, R. Barker, *Carbohydr. Res.* **1979**, *70*, 93; b) H. Susaki, K. Suzuki, M. Ikeda, H. Yamada, H. K. Watanabe, *Chem. Pharm. Bull.* **1994**, *32*, 2090.
[32] A. Y. Chernyak, G. V. M. Sharma, L. O. Kononov, P. R. Krishna, A. B. Levinsky, N. K. Kochetkov, *Carbohydr. Res.* **1992**, *223*, 303.
[33] a) G. Köhler, C. Milstein, *Nature* **1975**, *256*, 495; b) E. Enguall, *Methods Enzymol.* **1980**, *70*, 419.

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