Enzyme Fingerprints by Fluorogenic and Chromogenic Substrate Arrays**

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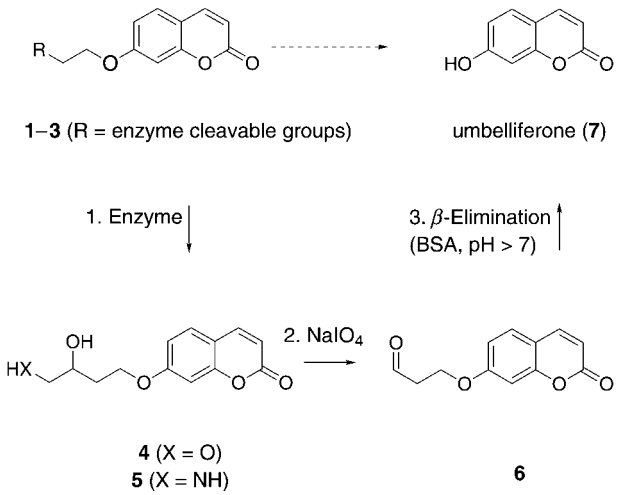
Enzymes are finding numerous uses as mild, environmentally benign, and selective catalysts in a variety of settings, such as consumer products, diagnostics, laboratory kits, and in the production of fine chemicals.[1] This broad variety of uses is made possible by the tremendous diversity of enzymes available either from the biosphere or by in vitro evolution of known enzymes.[2] Besides enzyme discovery, the problem arises of being able to differentiate between similar enzymes—either to better identify interesting new enzymes or for quality control purposes. An enzyme sample can be checked for purity by electrophoresis and for identity by mass spectrometry.[3] Its actual catalytic function, however, can only be assessed by testing a proper chemical reaction for catalysis. While active-site-specific reagents have been used for cysteine proteases,[4] serine hydrolases,[5] or the proteasome,[6] the most convenient and direct method consists of measuring the reaction of a chromogenic or fluorogenic substrate,[7] sometimes in combination with an electrophoretic separation.[8] The information can be refined using several fluorogenic or chromogenic substrates simultaneously. Microorganisms can be unequivocally identified using a series of 19 chromogenic substrates known as the API ZYM system, in which each substrate reacts selectively with one type of enzyme.[9] Libraries of fluorogenic or chromogenic peptide substrates have been used to characterize the cleavage specificity of proteases.[10] Herein we report arrays of chromogenic and fluorogenic substrates tailored for hydrolytic enzymes, such as esterases, lipases, amidases, and epoxide hydrolases. These arrays allow closely related enzymes to be differentiated and relate to the enzyme's catalytic potential for hydrolyzing synthetically relevant functional groups in a variety of steric and stereochemical environments.

We recently reported a versatile fluorogenic assay for hydrolytic enzymes based on a new type of fluorogenic substrates, such as **1**–**3**.[11] Enzymic reactions on these substrates release a vicinal diol **4** or aminoalcohol **5** as a primary reaction product, which is then processed in situ to the strongly fluorescent product umbelliferone (**7**). The reaction proceeds via the intermediate aldehyde **6** following an oxidation/β-elimination sequence (Scheme 1).[12] Since the



Scheme 1. Principle of periodate-coupled fluorescence assay (see Scheme 2 for R groups).

enzyme-labile functional groups in these substrates are chemically nonactivated, their spontaneous hydrolysis rate is negligible in the absence of a specific catalyst. We reasoned that it might therefore be possible to reliably profile the specific activity of a given enzyme by using an array of such substrates without dealing with nonspecific effects. The general applicability of the oxidation/β-elimination scheme enabled us to readily obtain structural variations close to the reaction center of the substrates. We hoped that the identity of a given hydrolytic enzyme would reveal itself not only by its reaction with a particular functional group, but also by its selectivity pattern among a family of structurally diverse, but functionally related, substrates.

Various amides, epoxides, esters, and carbonates related to **1**–**3** were prepared as components of our substrate arrays. Such functional groups would react with the large family of hydrolase enzymes, many of which share an Asp-His-Ser[13] or Asp-His-Asp[14] catalytic triad. The substrates were assembled in arrays layed out in wells of microtiter plates (Scheme 2). Our fluorogenic substrate array consisted of 40 samples distributed as a 5×8 grid. This array also included substrates releasing fluorescent 6-methoxynaphthaldehyde by oxidation of diol **8** as the primary enzyme reaction product.[15] A chromogenic 4×3 array was prepared with analogues of some of the umbelliferone-derived substrates with *p*-nitrophenol as the chromogenic leaving group. Each array included two diol or aminoalcohol reaction products as positive controls.[16]

We used our arrays to measure a series of hydrolytic enzymes. Reactions were initiated by adding a prediluted solution of the enzyme in buffer containing $NaIO_4$ and bovine serum albumin (BSA) to each substrate stock solution. One array was measured without enzyme to provide the reference

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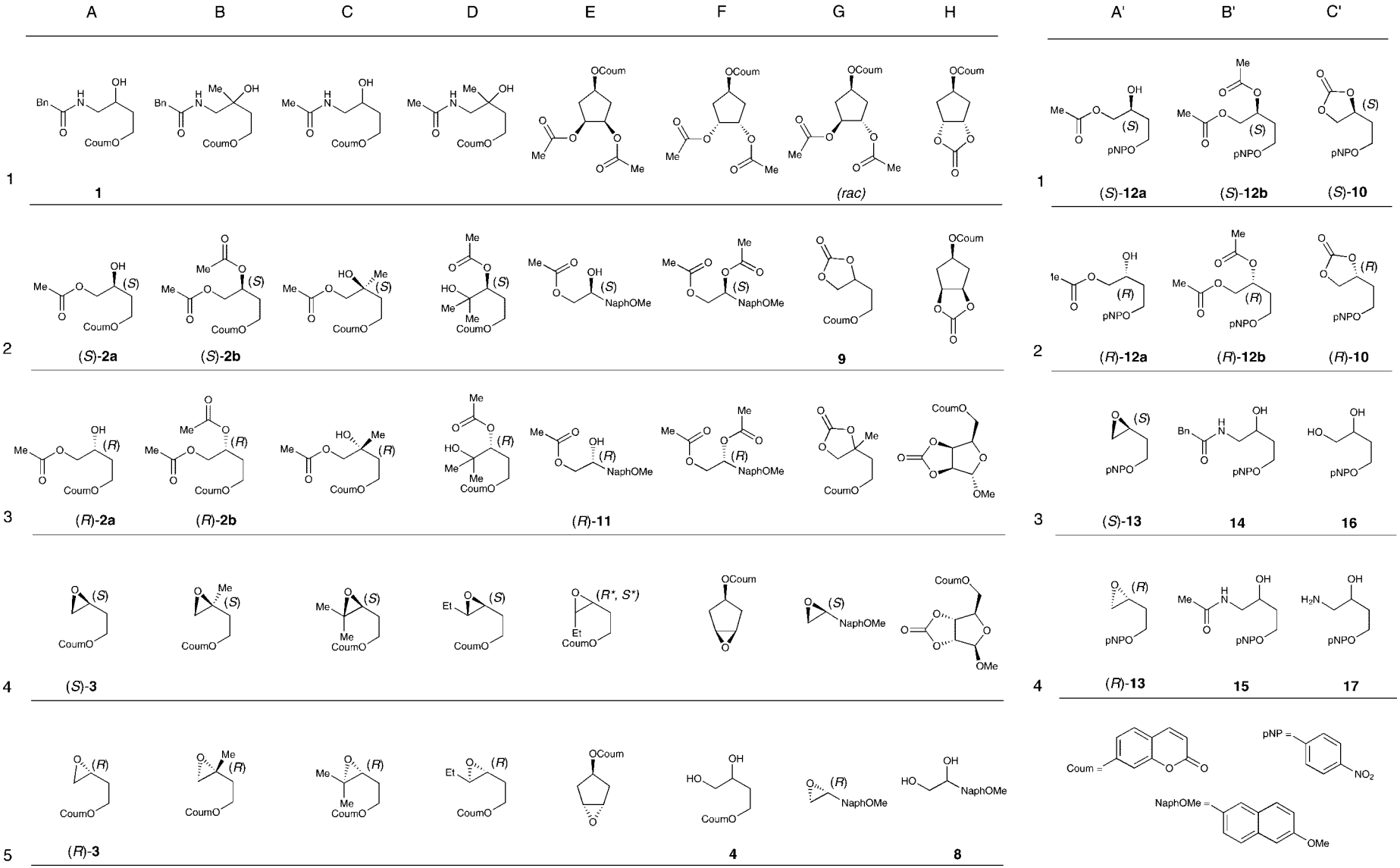

Scheme 2. Arrays of fluorogenic and chromogenic substrates for hydrolytic enzymes (for clarity, only the compounds cited directly in the text and figure have been numbered).

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rates for the very slow spontaneous hydrolysis of the substrates (negative controls) and for the fast oxidation of reference products (positive controls). All reactions were followed over two hours by using the corresponding microtiter plate reader instrument. The recorded signals (Figure 1) were converted into product concentrations by using calibration curves, and the steepest linear portion of each reaction profile was used to calculate the apparent reaction rate in each well. For each enzyme array, the rates measured in the reference plate without enzyme were substracted from the apparent rates, and the resulting net rates plotted in gray-scale relative to the maximum net rate observed in the array, which was set as black. The gray-scale rendering allowed the reactivity pattern of each enzyme to be displayed in an easily identifiable, visual manner. A selection of representative fingerprints is shown in Figure 2.

The analysis for both the fluorogenic and the chromogenic substrate arrays results in activity patterns which clearly differentiate between the different enzymes tested (Figure 2). Enzyme classes, such as esterases and lipases, amidases, and epoxide hydrolases, stand out by their preferential reactivity with the corresponding substrates. Furthermore, the array analysis allows the differentiation between enzymes within one class, with each enzyme displaying a unique pattern. The preferential reactivity of *Rhizopus niveus* lipase with the carbonates **9** and (*S*)-**10**, and the apparent reactivity of elastase with acetate (*R*)-**11** are noteworthy.

The patterns observed are reproducible within less than 10% in the apparent reaction rate at the array positions. These patterns therefore may be considered as fingerprints for the enzyme samples. In each case the question arises as to whether the fingerprint reveals the catalytic action of a single enzyme or whether it combines activities stemming from enzyme impurities not related to the listed main component. In the present set, the fingerprint recorded with *Aspergillus niger* epoxide hydrolase (AEH) is probably enzyme specific since the sample, although not highly purified, only reacts with epoxide substrates, which is an unusual type of reactivity. By contrast, the chemoselectivity observed for lipases and esterases towards esters and carbonates should not be interpreted as a purity indicator because of the ubiquity of this reactivity type. In the general case, the fingerprints could well be specific to the particular preparation or even to a particular enzyme batch.

The substrate arrays shown here are small compared to the peptide libraries developed for proteases.[10] Nevertheless,

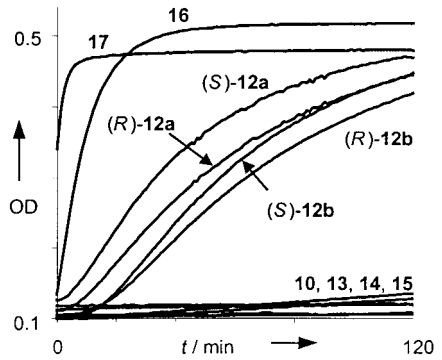

Figure 1. Time course of optical density (OD, at $\lambda = 405$ nm) observed for the reaction of *Pseudomonas fluorescens* lipase with the chromogenic substrate array. Conditions: 0.1 mg mL^{-1} enzyme, 100 μM substrate, 20 mM aq borate pH 8.8, 2.5% v/v DMF, 26 °C, 2 mg mL^{-1} BSA, 1 mM $NaIO_4$. The rates derived from these curves for the different substrates are: **17**: 209300 pM s^{-1}, **16**: 130800 pM s^{-1}, (*S*)-**12a**: 31800 pM s^{-1}, (*S*)-**12b**: 25500 pM s^{-1}, (*R*)-**12a**: 25000 pM s^{-1}, (*R*)-**12b**: 22000 pM s^{-1}, **15**: 4000 pM s^{-1}, (*R*)-**10**: 2600 pM s^{-1}, (*S*)-**10**: 2300 pM s^{-1}, **14**: 1600 pM s^{-1}, (*S*)-**13**: 1400 pM s^{-1}, (*R*)-**13**: 1300 pM s^{-1}. See text and Experimental Section for more information.

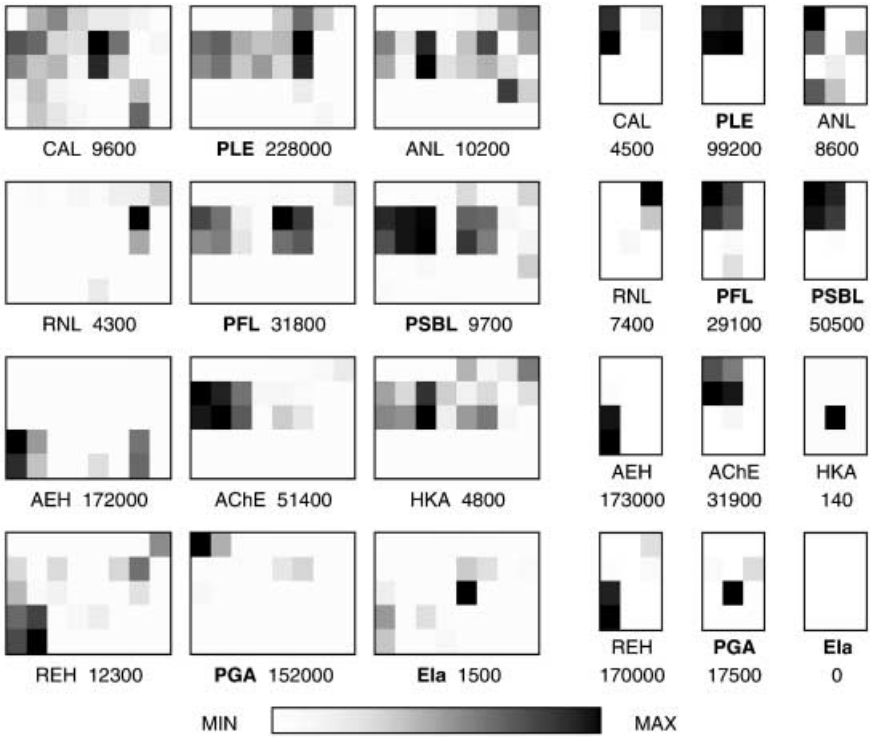

Figure 2. Enzyme fingerprints with fluorogenic and chromogenic substrate arrays. Below each array are given enzyme code and the apparent maximum rate in pM s^{-1}, which is set as black in each array. Enzymes marked in bold appear homogeneous (>90%) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Substrates are positioned as shown in Scheme 2. Conditions: 0.1 mg mL^{-1} enzyme, 100 μM substrate, 20 mM aq. borate pH 8.8 with 2.5% v/v DMF, 26 °C, 2 mg mL^{-1} BSA, 1 mM $NaIO_4$. See text for data treatment. Codes for enzymes: CAL = *Candida antarctica* lipase F62299, PLE = pig liver esterase F46058, ANL = *Aspergillus niger* lipase A39,043-7, RNL = *Rhizopus niveus* lipase F62310, PFL = *Pseudomonas fluorescens* lipase F62321, PSBL = *Pseudomonas* sp. type B lipoprotein lipase F62336, AEH = *Aspergillus niger* epoxide hydrolase, AChE = *Electrophorus electricus* acetylcholine esterase F01023, HKA = hog kidney acylase I F01821, REH = *Rhodotorula glutinis* epoxide hydrolase, PGA = *Escherichia coli* penicillin G acylase F76427, Ela = porcine pancreatic elastase Se20929. Suppliers: F = Fluka, A = Aldrich, Se = Serva. Fingerprints were recorded for a total of 45 different enzymes: 17 lipases, 4 lipoprotein lipases, 10 esterases, 12 amidases (proteases and acylases), and 2 epoxide hydrolases. The following enzymes did not show significant reactivity with any of the substrates: subtilisin, papain, trypsin, chymotrypsin, clostripain, and pepsin.

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our arrays provide fingerprints that differentiate between closely related hydrolytic enzymes. In theory, the amount of information recorded in an array with only a few tens of data points should be sufficient to encode for thousands of different enzymes. The practical realization of a differentiating array depends mainly on the choice of the palette of substrates with which different enzymes react differently. The excellent differentiation observed here might depend on the fact that the structural variations between the different substrates are very close to their enzyme-reactive group.

The concept of enzyme fingerprinting as shown here should be quite general. Arrays could include substrates for other enzyme classes and should also be easily realizable using other high-throughput screening (HTS) assays,[7] provided that these produce simple photometric signals that can be recorded in a highly parallel manner, for example by pH[17] or pM[18] indicators. Fingerprint analyses with substrate arrays might prove useful in quality control, batch identification procedures, as well as in enzyme discovery programs by helping to point out novel enzymes with unusual reactivities.[19]

Experimental Section

Synthesis: All substrates and reference products were prepared by functionalization of the parent olefins starting either with epoxidation or with dihydroxylation. Optically pure compounds were obtained from diols prepared by using Sharpless asymmetric dihydroxylation as described before.[11] Substrates in array positions H3 and H4 were derived from D-lyxose and D-ribose, respectively. All new compounds gave satisfactory spectral data and elemental analysis or HR-MS data. Optical purities were checked by chiral-phase HPLC and had $>95\,\%$ *ee* values in all cases.

Measurements: Substrates and reference products were diluted from 2 mM stock solutions in 50 % aqueous *N*,*N*-dimethylformamide (DMF). These solutions were stored at $+4\,^{\circ}C$. Fluorogenic reactions were carried out in round-bottom polypropylene 96-well plates (Costar) and monitored using a Cytofluor II fluorescence plate reader (Perseptive Biosystems, filters $\lambda_{ex} = 360 \pm 20$, $\lambda_{em} = 460 \pm 20$ nm). Chromogenic reactions were carried out in half-area flat-bottom polystyrene 96-well cell culture plates (Costar) and monitored using a Spectramax 250 microplate spectrophotometer (Molecular Devices, $\lambda = 405$ nm). Each well of the array was filled with 5 μL of a substrate stock solution following the layout shown in Scheme 2. The reactions were initiated by adding 95 μL of a freshly prepared solution containing enzyme (0.1 mg mL^{-1}, weighed in from the supplied solid), BSA (2 mg mL^{-1}), and $NaIO_4$ (1 mM) in 20 mM aqueous borate at pH 8.8 to each well of the array. The reactions were recorded over a period of 2 h. See text and figure legend for data treatment.

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[16] The alcohols and aminoalcohols that are the primary enzyme reaction products were found to release their colored or fluorescent product rapidly ($t_{1/2} < 15$ min) in aqueous buffer in the presence of sodium periodate and bovine serum albumin. Only the D-lyxose- and D-ribose-derived diols, which are released by substrates in positions H3 and H4, respectively, have an unusually slow rate of fluorescence release as a result of the resistance of the intermediate aldehyde to β-elimination ($t_{1/2} > 2$ h). The signal is nevertheless still strong enough in these cases to give good data points.

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[19] Reactivity with a particular substrate can be assayed reliably when testing individual substrates separately as shown here in the arrays. Chemo-, enantio-, or stereoselectivity, by contrast, might be masked if a crude enzyme extract containing several enzymes with complementary selectivites is tested. Nevertheless, many well-known enzyme mixtures give useful selective transformations, for example, pig liver esterase; see C.-H. Wong, G. M. Whitesides, *Enzymes in Synthetic Organic Chemistry*, Pergamon, Elnsford, NY, **1994**, pp. 66 – 68.

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