Fluorogenic Cyanohydrin Esters as Chiral Probes for Esterase and Lipase Activity

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Abstract: Fluorogenic cyanohydrin esters were prepared that release the fluorescent product umbelliferone by secondary decomposition of the primary cyanohydrin reaction product by cyanide elimination to the aldehyde and subsequent β-elimination. Whereas butyrate **1b** and octanoate **1d** show the highest reaction rates with enzymes, the highest relative rates above the non-catalyzed background reaction are achieved with pivalate **1c** and benzoate **1e**. Enantioselective reactions are detected when the conversion stabilizes at 50% of the maximum fluorescence release, and enantioselectivity is confirmed by chiral-phase HPLC analysis of the unreacted cyanohydrin ester substrate.

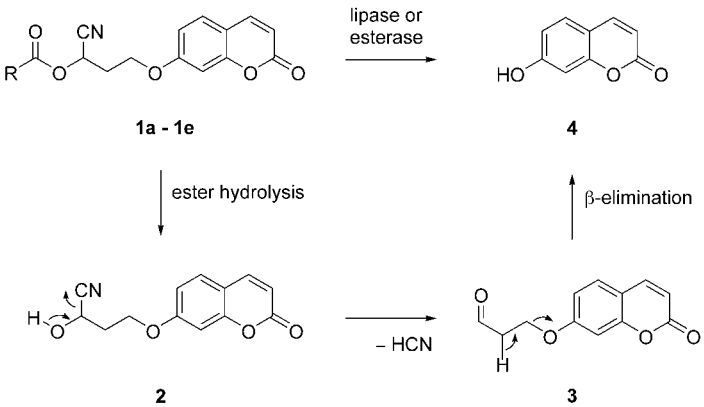
Keywords: enantioselectivity; enzyme assay; fluorescence; high-throughput screening; lipases

Introduction

Lipases and esterases are the most widely used enzyme type, serving various needs ranging from detergent components to chiral catalysts for fine chemical synthesis. Numerous variants of these enzymes are available from a plethora of sources such as prokaryotes (e.g., *Pseudomonas* species), eukaryotes (e.g., yeast), plants (e.g., wheat germs), vertebrates (e.g., electric eel) or mammalian organs (e.g., pig liver).[1] Their success as reagents is due to their generally excellent operational stability under a variety of conditions, particularly in the presence of organic solvents. Yet the desirable activities, enantio- or stereoselectivities for a particular application are often still not available off-the-shelf in the existing collections, and large efforts continue to be invested in the discovery of further esterase and lipase variants by biodiversity mining, mutagenesis or directed evolution.[2] High-throughput screening (HTS) assays are an essential component of this effort. Colorimetric and fluorimetric assays amenable to microtiter-plate format without the need for special instrumentation are very popular because they only require a particular set of reagents. These include among others chromogenic and fluorogenic ester substrates,[3] pH-indicators,[4] and enzyme-coupled assays.[5] Chiral fluorogenic alcohols providing chemically stable esters suitable for screening under a variety of conditions would be desirable in an optimal fluorogenic assay for enantioselective ester hydrolysis. Herein we report the use of chiral fluorogenic cyanohydrin esters **1a – e** as probes for enantioselective esterolytic activities. Cyanohydrin **2** produced by ester hydrolysis undergoes spontaneous elimination of HCN to aldehyde **3**, followed by β-elimination to give the strongly fluorescent product umbelliferone (**4**). These cyanohydrin esters represent a promising substrate class for screening enantioselective esterolytic activities on chiral secondary esters.

Results and Discussion

We recently reported enantioselective high-throughput screening assays for lipases and esterases using esters of enantiomeric fluorogenic alcohols. While the alcohols produced were not fluorescent by themselves, they were



Scheme 1. Principle of fluorogenic cyanohydrin esters.

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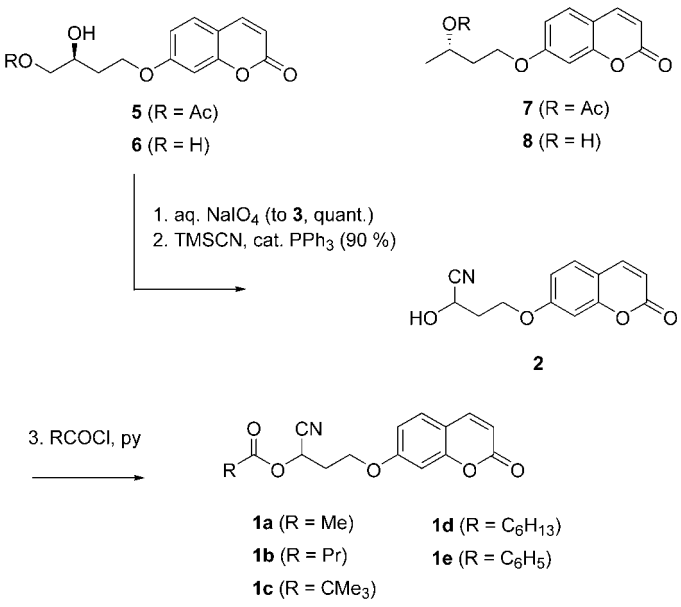
processed *in situ* by a sequence of oxidation and β-elimination to produce umbelliferone as fluorescent signature. Thus, aldehyde **3** and related 3-oxopropyl ethers of acidic phenols (e.g., nitrophenol or umbelliferone) undergo rapid β-elimination in aqueous buffer under catalysis by bovine serum albumin (BSA), an inexpensive protein additive, so that the half-life of these compounds is less than 2 minutes under these conditions. We have used this reaction to design enzyme assays in which aldehyde **3** is the primary reaction product. These include an assay for alcohol dehydrogenases,[6] as well as assays for retroaldolase catalytic antibodies and transaldolases.[7] The assay was also adapted to assay hydrolytic enzymes, in which case the hydrolases produced an alcohol precursor of **3** as primary product. The assay was conducted in the presence of an oxidizing agent, either sodium periodate when assaying the hydrolysis of precursors of diol **6** such as ester **5**,[8] or an alcohol dehydrogenase when assaying the hydrolysis of ester **7** to alcohol **8**.[9] These substrates proved particularly useful due to the absence of non-specific degradation, in contrast to assays based on simple esters of umbelliferone **4** or nitrophenol, which also react non-specifically with non-catalytic lipase fragments. This property has been used successfully to isolate thermophilic enzymes under extreme conditions.[10] A variety of structural variants of diol **6** were also readily prepared using asymmetric dihydroxylation of various olefinic precursors, leading to a broad structural family of fluorogenic and chromogenic enzyme substrate suitable for fingerprinting enzyme activities.[11]

While the low reactivity of screening substrates such as **5** or **7** are highly desirable, assaying microbial enzymes in crude cultures would be simplified if the use of a secondary oxidant could be avoided altogether. The key to the low reactivity was clearly the high pKa of the aliphatic alcohol involved in the enzyme-labile bond ($pK_a > 14$) as compared to that of the final phenol leaving group ($pK_a \sim 7$). We reasoned that cyanohydrin esters such as **1a – e** might combine the advantage of low reactivity with that of spontaneous fluorescence release, considering that the cyanohydrin product **2**, which could be expected to dissociate spontaneously in dilute aqueous environment to the labile aldehyde **3**, possessed a hydroxy group with intermediate acidity.

The synthesis of the target cyanohydrin esters was accomplished *via* aldehyde **3**. Indeed, aldehyde **3** is only unstable in aqueous environment in the presence of BSA, yet can be prepared and used in organic solvent without decomposition. Although aldehyde **3** could be prepared by Dess–Martin oxidation of the parent alcohol, the reaction proved sluggish and required in-house synthesis of the oxidant since the commercially available periodinane is both expensive and of very poor quality. We found it much more convenient to prepare the aldehyde by periodate oxidation of diol **6** in aqueous acetone. The crude aldehyde was then converted to the silylated cyanohydrin by treatment with TMSCN in anhydrous dichloromethane under catalysis by triphenylphosphine. Aqueous work-up and chromatography led to the free cyanohydrin **2**. Esterification with various acyl chlorides in the presence of pyridine then led to the corresponding esters **1a – e** (Scheme 2).

All substrates were diluted from 10 mM stock solution in 50% aqueous solvent. The solutions could be stored at low temperature without decomposition for several weeks. We first investigated the rate of decomposition of cyanohydrin **2** in the presence or absence of bovine serum albumin (BSA) as additive to catalyze the β-elimination of the intermediate aldehyde **3**. We chose either neutral (PBS) or slightly basic (pH 8.8) conditions since these are known to be optimal for most lipases and esterases, and are also necessary to obtain a strong fluorescence signal of the anionic form of umbelliferone **4**, which is strongly fluorescent and exists above pH 7 [pK_a(**4**) ~ 7]. Cyanohydrin **2** released umbelliferone as expected from the spontaneous equilibration to the aldehyde followed by β-elimination under all conditions tested. The reaction was relatively slow, with an apparent half-life of $t_{1/2} = 15$ min in PBS with BSA. The rate of decomposition was thus clearly slower than that of aldehyde **2** ($t_{1/2} = 2$ min), suggesting that cyanide elimination was the rate-limiting step. Indeed addition of formaldehyde had no effect on the reaction rate, showing that there was no pre-equilibrium formation of aldehyde **2** under the reaction conditions.

We next investigated the reactivity of the cyanohydrin esters **1a – e** in the presence of BSA in PBS pH 7.4, under which conditions the spontaneous reaction of the more



Scheme 2. Fluorogenic substrates and synthesis of target cyanohydrin esters.

reactive straight-chain aliphatic substrates **1a, 1b** and **1d** was reasonably slow. The cyanohydrin esters of more bulky acids such as benzoate **1e** or pivalate **1c** were quite resistant to non-specific degradation even under more basic conditions. It should be noted that the useful range of the planned enzyme assay would be limited by the rate of decomposition of the intermediate cyanohydrin **2**. The reactivity of the different cyanohydrin esters against enzymes was investigated by assaying a series of commercially available lipases and esterases.

As for the reactions without enzyme, the time-profiles of fluorescence increase were very well behaved and easy to measure due to the relatively low reaction rates. Indeed, very labile substrates giving extremely high reaction rates are very inconvenient in the practice of high-throughput measurements where a typical 96-well plate requires at least several minutes for filling, even using robotic instruments, and where recording a fluorescence data-point over the entire plate requires at least 10 to 30 seconds. The raw fluorescence data were acquired over two hours in each experiment, which was sufficient for completion of the reaction with the more reactive lipase samples. Cyanohydrin **2** was used in each assay as a reference for the maximum recordable rate. There was no significant effect of the enzymes on the rate of decomposition of the cyanohydrin, showing that the rate effect observed reflected esterolytic activity only. The data were recorded in series of 10 experiments with six substrates each (five esters and one cyanohydrin), grouping nine enzymes with one control without enzyme.

The fluorescence data were converted to product concentration using a calibration curve with pure umbelliferone **4** under the same conditions (PBS, 2 mg mL^{-1} BSA). The fluorescence of **4** was linear up to 50 μM concentration, above which a slight curvature due to autoquenching was observable. The linear portion of each curve was used to calculate the absolute reaction rates shown in Table 1. The two bottom lanes of the Table show two measurements of the non-catalyzed reaction in the absence of enzyme. An average of both data-sets was then used to determine the relative rate ratios, calculated as $r_{enz} = (V_{enz}/V_{noE}) - 1$ (Table 2). The relative rate data are extremely useful to judge the signal

Table 1. Fluorescence assay of lipases and esterases using fluorogenic cyanohydrins. Absolute reaction rates in nM s^{-1} in aqueous PBS pH 7.4, 30 °C, 200 μM substrate, 50 μg mL^{-1} enzyme, 2 mg mL^{-1} BSA. 0.1 mL assays were run in 96-well polypropylene round-bottom microtiter plates. Fluorescence at $\lambda_{em} = 440$ nm ($\lambda_{ex} = 360$ nm) was recorded using a Cytofluor II fluorescence microtiter plate reader and converted to umbelliferone **4** concentration using a calibration curve. For enzyme codes see ref.[9b] The gray-scale grid on the right was generated from the Table data. The maximum possible rate is given by the rate of decomposition of the cyanohydrin **2** under the assay conditions, which was measured as 40 nM s^{-1}. Error margin in all data is 10% and is caused by pipetting accuracy. Enzymes are sorted in descending order according to the maximum reactivity, given by the sum of observed reaction rates across all five substrates.

Absolute rates		Ac	Bu	Piv	Oct	Bz
Enzyme	tot. act.	**1a**	**1b**	**1c**	**1d**	**1e**
PSBL	113	21	37	26	23	6.4
CVL	94	16	32	19	24	2.5
PSL	83	23	29	1.7	28	1.1
PSL	72	21	26	1.5	23	1.1
TBE	55	12	21	3.3	17	1.8
CCL	48	2.6	16	0.6	18	11
BTE	44	5.8	17	7.4	12	1.0
BStE	31	4.5	12	0.7	12	0.63
BSE	30	5.1	11	5.2	8.2	0.79
AOL	28	1.3	3.9	0.15	22	0.06
PCL	26	6.1	6.5	0.50	12	0.28
ANL	24	2.3	5.1	0.61	16	0.41
PRL	21	1.8	6.8	0.25	12	0.20
RAL	16	2.3	5.1	0.38	8.0	0.14
MJL	15	1.9	3.8	0.36	9.0	0.41
CLL	15	2.0	5.7	0.27	7.0	0.19
MME	14	2.2	3.1	0.87	7.3	0.43
RML	13	1.3	1.7	0.17	9.4	0.13
MML	11	1.1	2.4	0.22	7.1	0.28
RNL	7.3	1.5	2.2	0.21	3.3	0.14
CLE	5.2	1.3	1.7	0.38	1.7	0.08
SCE	4.1	1.3	0.89	0.17	1.7	0.03
noE	2.6	1.0	0.45	0.10	1.0	0.05
noE	2.2	0.87	0.61	0.13	0.53	0.05

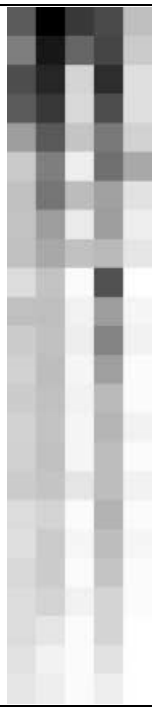
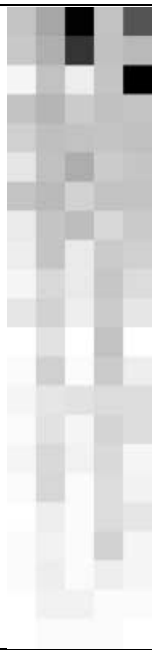


Table 2. Fluorescence assay of lipases and esterases using fluorogenic cyanohydrins. Relative reaction rates were tabulated from the data in Table 1 using $r_{enz} = (V_{enz}/V_{noE}) - 1$, V_{noE} is the spontaneous reaction rate under the reaction conditions in the absence of enzyme. For enzyme codes see ref.[9b] The gray-scale grid on the right was generated from the table data. Enzymes are sorted in descending order according to the maximum relative reactivity r_{enz}, given by the sum of r_{enz} across all five substrates.

Relative rates		Ac	Bu	Piv	Oct	Bz	
Enzyme	tot. act.	**1a**	**1b**	**1c**	**1d**	**1e**	
PSBL	472	22	68	221	29	132	
CVL	320	17	60	163	30	50	
CCL	281	2	29	4	22	224	
PSL	149	24	54	14	35	22	
TBE	136	13	39	27	20	37	
BTE	134	5	32	62	14	20	
PSL	132	22	48	12	28	23	
BSE	92	5	19	43	9	15	
BStE	58	4	22	5	15	12	
ANL	41	1	9	4	19	7	
PCL	40	6	11	3	15	5	
AOL	35	0	6	0	27	0	
PRL	32	1	12	1	15	3	
MME	29	1	5	6	8	8	
MJL	27	1	6	2	11	7	
RAL	23	1	9	2	9	2	
CLL	23	1	10	1	8	3	
MML	18	0	4	1	8	5	
RML	16	0	2	0	11	2	
RNL	10	1	3	1	3	2	
CLE	7	0	2	2	1	1	
SCE	2	0	1	0	1	0	

quality in terms of catalysis signal over background, which is the most significant parameter for detecting catalysis. For both Tables, a gray scale rendering of the numerical data is shown on the right to facilitate data inspection.

As can be seen from the absolute rate data in Table 1, the most reactive substrate across all enzymes is butyrate **1b**, followed by octanoate **1d**. This behavior is in agreement with the known reactivity of lipases and esterases. Butyrates and octanoates of various chromogenic or fluorogenic phenols are indeed standard substrates that are used as "esterase specific" and "lipase specific" substrates, respectively. It must be noted, however, that butyrate **1b** and octanoate **1d** are also the most reactive in the non-catalyzed reaction in the presence of BSA only. This high reactivity reflects not only chemical instability (the acetate **1a** is the most chemically labile ester in the series), but also the tendency towards non-specific interaction with proteins, here with BSA as a bulk additive.

The most relevant data for estimating assay sensitivity are the relative rate over background V_{rel} reported in Table 2. Indeed the relative rate is more significant than the absolute rate observed with fluorogenic reactions because fluorescence can be detected at very low levels. In Table 2, pivalate **1c** appears to be the best substrate, giving a signal to background ratio of over two hundred with *Pseudomonas fluorescens* type B lipoprotein lipase (PSBL). Interestingly, benzoate **1e** also fares excellently and appears with a similarly high signal to background ratio for the reaction with *Candida cylindracea* lipase (CCL). These very high values of catalysis over background are made possible by the almost negligible background rate with **1c** and **1e**. A detection limit for catalytic activity may generally be set at $V_{rel} > 5$, which is sufficient to distinguish catalysis from background. That condition is fulfilled by almost all samples tested, and one must take into account that some of the samples are crude extracts that contain only traces of the desired enzyme.

In principle, an enantioselective enzyme should hydrolyze only half of a chiral substrate, which would be easily detectable at the fluorescence intensity by the end of the reaction. We found one example of such a behavior in our series with the reaction of benzoate **1e** with *Candida cylindracea* lipase (CCL). As can be seen from the fluorescence profile (Figure 2), approximately 50% of this ester was consumed, after which the reaction stopped. The enantioselectivity of the reaction was confirmed by chiral-phase HPLC analysis of the unreacted ester **1e**, which we were able to separate into its enantiomers on a Chiralcel-OD-H column (eluent:

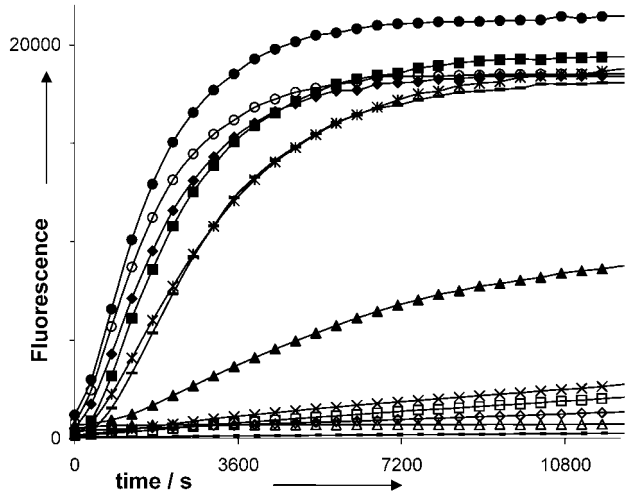


Figure 1. Release of umbelliferone **4** from fluorogenic cyanohydrins. Measured at 30 °C with $\lambda_{em} = 460$ nm ($\lambda_{ex} = 360$ nm) in PBS (160 mM NaCl, 10 mM phosphate, pH 7.4) in the presence of BSA (2 mg mL^{-1}, bovine serum albumin) with 200 μM substrate, with or without 0.05 mg mL^{-1} of *Pseudomonas* sp. type B lipoprotein lipase (PSBL). Cyanohydrin **2** (o), + PSBL (●); acetate **1a** (□), + PSBL(■); butyrate **1b** (◇), + PSBL (◆); pivalate **1c** (−), + PSBL (−); octanoate **1d** (x), + PSBL (**x**); benzoate **1e** (△), + PSBL (▲).The reactions (0.1 mL each) were conducted in wells of 96-well polypropylene microtiter plates and initiated by addition of substrate. Fluorescence data were converted to product concentration using a calibration curve with **4**. The linear portion of each curve was used to generate the rate data in Table 1.

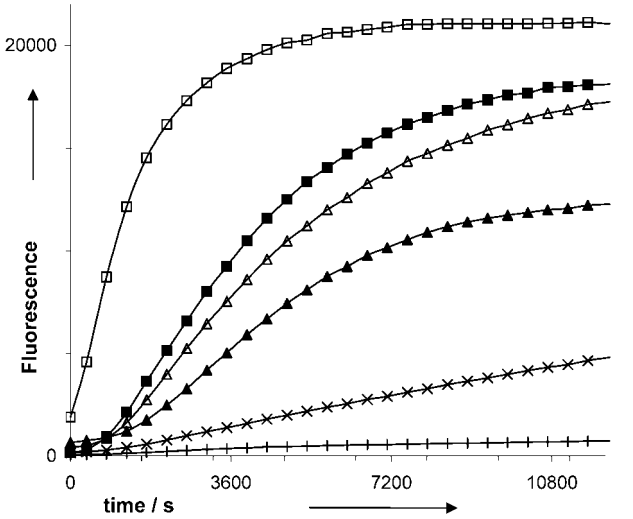


Figure 2. Reaction of cyanohydrin esters with *Candida cylindracea* lipase. Substrates were incubated at 200 μM in PBS at 30 °C with 2 mg mL^{-1} BSA and 50 μg mL^{-1} lipase. Cyanohydrin **2** (□); acetate **1a** (x); butyrate **1b** (△); pivalate **1c** (+); octanoate **1d** (■); benzoate **1e** (▲). See also legend of Figure 1 and Experimental Section for details.

1 mL min^{-1} hexane/2-propanol 1:1, $t_R = 13.37$ and 15.47 min). Thus, while enantioselectivity was not detectable as a fluorescent signal directly in solution, the observation of a 50% conversion after completion of the reaction provides a simple hint towards samples with possible enantioselective reaction. Chiral-phase HPLC analysis is facilitated by the umbelliferone chromophore, which allows us to detect minute amounts of product such as those at the screening concentration. In the present study we did not have chiral columns available capable of separating the other four substrates **1a – d**. Nevertheless, the principle of this measurement combining fluorescence detection with HPLC-separation is not only simple, but allows for the analysis of kinetic resolution with racemic substrate, thus reflecting true kinetic resolution potential by the lipase.

Conclusion

Esters of fluorogenic cyanohydrins are simple fluorogenic probes for fluorescence screening of enzyme activity on chiral secondary esters. Pivalate **1c** and benzoate **1e** were found to show a very low rate of spontaneous hydrolysis, which may be advantageous if testing has to be done under harsh conditions. In the case of benzoate ester **1e**, chiral phase analysis of the unreacted ester allows us to confirm whether an observed partial activity with 50% conversion of substrate, detected at the fluorescence signal of the released umbelliferone, reflects true enantioselectivity, as exemplified by the reaction with *Candida cylindracea* lipase. These substrates represent practical and highly relevant probes for screening lipases for synthetic applications. First, they are chiral secondary alcohols, which is the most important class of substrates used in kinetic lipase resolution. Second, they present a very low spontaneous reactivity, which avoids non-specific catalysis, as well as greatly facilitates handling and set-up of high-throughput experiments, where pipetting operations typically require several minutes and are thus not practically realizable with highly reactive enzyme substrates. A fluorescence assay based on the fluorogenic cyanohydrin esters **1a – e** can be applied readily in a microtiter plate format of any degree of miniaturization, provided that a suitable pipetting and fluorescence recording instrument is available. The overall throughput will be limited by the pipetting operations, and by the fact that the reaction should be allowed to proceed for at least thirty minutes in each sample to allow for the secondary decomposition of the primary cyanohydrin hydrolysis product **2** to the fluorescent umbelliferone **4**.

Experimental Section

General

All reactions were followed by TLC on Alugram SIL G/UV$_{254}$ silica gel sheets (Macherey-Nagel) with detection by UV or with 0.5% phosphomolybdic acid solution in 95% EtOH. Silica gel 60 (Macherey-Nagel 230–400 mesh) was used for flash chromatography (FC). Melting points were determined with a Büchi 510 apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with Bruker AC-300 spectrometer. Infra-red spectroscopy was performed on a Perkin-Elmer *Spectrum One* series FTIR apparatus.

7-[(3-Cyano-3-acetoxy-prop-1-yl)oxy]-*2H*-1-benzopyran-2-one (1a)

Cyanohydrin **2** (100 mg, 0.045 mmol) was treated at 0 °C with acetic anhydride (1 mL) and pyridine (0.05 mL). After 16 h at 20 °C, the reagents were removed by coevaporation with toluene, and the crude product was purified by flash chromatography (hexane/ethyl acetate 7:3) to give acetate **1a** as a colorless solid; yield: 104 mg (0.36 mmol, 95%); mp 81 °C; R_f = 0.25 (hexane/EtOAc, 7/3); IR ($CHCl_3$): ν = 3414, 2359, 1716, 1616, 1384, 1210, 829 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.66 (d, 1H, J = 9.6 Hz), 7.46 (d, 1H, J = 8.8 Hz), 6.85 (dd, 1H, J = 8.8, 2.2 Hz), 6.79 (d, 1H, J = 2.2 Hz), 6.28 (d, 1H, J = 9.6 Hz), 5.59 (t, 1H, J = 6.6 Hz), 4.25 (m, 2H), 2.51 (m, 2H), 2.20 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 168.9, 161.1, 160.8, 155.7, 143.2, 128.9, 116.3, 113.6, 113.1, 112.8, 101.4, 62.9, 58.2, 32.0, 20.2; MS (EI): m/z = 287 (M^+), 162, 134, 126, 105, 43; HRMS: calcd. for $C_{15}H_{13}NO_5$: 287.07937; found: 287.07937; anal. calcd. (%) for $C_{15}H_{13}NO_5$: C 62.72, H 4.56; found: C 62.67, H 4.54.

7-[(3-(Butyryloxy)-3-cyanoprop-1-yl)oxy]-*2H*-1-benzopyran-2-one (1b)

To a solution of cyanohydrin **2** (100 mg, 0.45 mmol) in dry dichloromethane (4 mL), were added butanoyl chloride (141 μL, 3 equivalents) and pyridine (108 μL, 3 equivalents) at 0 °C. The reaction mixture was stirred overnight, extracted with EtOAc (2 × 10 mL), dried on Na_2SO_4 and evaporated. Flash chromatography (hexane/EtOAc = 6/4, R_f = 0.31) gave ester **1b** as a colorless oil; yield: 105 mg (74%); R_f = 0.31 (hexane/EtOAc, 6/4); IR ($CHCl_3$): ν = 2973, 2881, 2401, 1732, 1615, 1216, 838 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.65 (d, 1H, H_4, J = 9.5 Hz), 7.40 (d, 1H, J = 8.4 Hz), 6.85 (dd, 1H, J = 8.4 Hz, J = 2.2 Hz), 6.80 (d, 1H, J = 2.2 Hz), 6.27 (d, 1H, J = 9.6 Hz), 5.62 (t, 1H, J = 7.0 Hz), 4.20 (m, 2H), 2.47 (m, 2H), 2.38 (t, 2H, J = 7.4 Hz), 1.69 (qt, 2H, J = 7.4, 7.4 Hz), 0.95 (t, 3H, J = 7.4 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 171.6, 161.1, 161.0, 155.7, 143.2, 128.9, 113.6, 113.0, 112.8, 101.3, 62.9, 57.9, 35.4, 32.0, 18.1, 13.4; MS (EI): m/z = 315 (M^+), 162, 154; HRMS: calcd. for $C_{17}H_{17}NO_5$: 315.110530; found: 315.110673; anal. calcd. (%) for $C_{17}H_{17}NO_5$: C 64.75, H 5.43; found C 64.71, H 5.37.

7-[(3-Cyano-3-(octanoyloxy)prop-1-yl)oxy]-*2H*-1-benzopyran-2-one (1d)

To a solution of cyanohydrin **2** (120 mg, 0.49 mmol) in dry dichloromethane (4 mL) were added octanoyl chloride (252 μL, 3 equivalents) and dry pyridine (120 μL) at 0 °C. The reaction mixture was stirred overnight, extracted with EtOAc (3 × 10 mL), dried on Na_2SO_4 and evaporated. Flash chromatography (hexane/EtOAc = 6/4, R_f = 0.40) gave ester **1d** as a colorless oil; yield: 92 mg (51%); IR ($CHCl_3$): ν = 2933, 2860, 2401, 1732, 1615, 1216, 838 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.65 (d, 1H, J = 9.2 Hz), 7.40 (d, 1H, J = 8.7 Hz), 6.85 (dd, 1H, J = 8.8 Hz, J = 2.2 Hz), 6.81 (d, 1H, J = 2.2 Hz), 6.28 (d, 1H, J = 9.5 Hz), 5.62 (dd, 1H, J = 6.6 Hz), 4.18 (m, 2H), 2.42 (m, 4H), 1.64 (m, 2H), 1.26 (m, 8H), 0.87 (t, 3H, J = 6.6 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 171.8, 161.1, 161.0, 155.7, 143.2, 128.9, 116.4, 113.6, 113.1, 112.8, 101.3, 62.9, 57.9, 33.6, 32.0, 31.5, 28.9, 28.8, 22.5, 24.6, 14.0; MS (EI): m/z = 371 (M^+), 210, 162; HRMS: calcd. for $C_{21}H_{25}NO_5$: 371.17327; found: 371.17337; anal. calcd. (%) for $C_{21}H_{25}NO_5$: C 67.91, H 6.78; found C 67.99, H 6.84.

7-[(3-Cyano-3-(pivaloyloxy)prop-1-yl)oxy]-*2H*-1-benzopyran-2-one (1c)

To a solution of cyanohydrin **2** (11 mg, 0.045 mmol) in dry dichloromethane (4 mL) were added pyvaloyl chloride (11 μL, 2 equivalents) and pyridine (7 μL, 2 equivalents) at 0 °C. The reaction mixture was stirred overnight, extracted with EtOAc (2 × 10 mL), dried on Na_2SO_4 and evaporated. After a chromatography column (hexane/EtOAc, 6/4, R_f = 0.56), ester **1d** was obtained as a colorless oil; yield: 13.7 mg (94%); IR ($CHCl_3$): ν = 2978, 1749, 1616, 1230 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.65 (d, 1H, J = 9.2 Hz), 7.40 (d, 1H, J = 8.5 Hz), 6.86 (dd, 1H, J = 8.5, 2.6 Hz), 6.81 (d, 1H, J = 2.6 Hz), 6.29 (d, 1H, J = 9.2 Hz), 5.61 (t, 1H, J = 7.0 Hz), 4.20 (m, 2H), 2.49 (m, 2H), 1.24 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 177.1, 161.8, 161.6, 156.5, 143.9, 129.7, 117.2, 114.4, 113.8, 113.5, 102.1, 63.7, 58.8, 39.6, 32.7, 27.6; MS (EI): m/z = 329 (M^+), 168, 162; HRMS: calcd. for $C_{18}H_{19}NO_5$: 329.12632; found: 329.12619; anal. calcd. (%) for $C_{18}H_{19}NO_5$: C 65.64, H 5.81; found C 65.37, H 5.79.

7-[(3-Cyano-3-benzoyloxyprop-1-yl)oxy]-*2H*-1-benzopyran-2-one (1e)

A solution of cyanohydrin **2** (100 mg, 0.045 mmol) in 4 mL anhydrous CH_2Cl_2 at 0 °C was treated with pyridine (0.05 mL, 0.9 mmol) and benzoyl chloride (0.095 mL, 115 mg, 1.2 mmol). The reaction mixture was stirred for 17 h at 20 °C. Aqueous work-up (AcOEt) and flash chromatography (hexane/EtOAc, 6:4) of the organic residue gave benzoate ester **1d** as a colorless solid; yield: 125 mg (0.36 mmol, 88%); mp 137 °C; R_f = 0.5 (hexane/EtOAc, 6/4); IR ($CHCl_3$): ν = 3414, 1732, 1613, 1259, 1124, 849 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.05 (m, 2H), 7.65 (d, 1H, J = 9.6 Hz); 7.62 (m, 1H), 7.50 (m. 2H), 7.38 (d, 1H, J = 8.4 Hz), 6.85 (dd, 1H, J = 2.6, 8.8 Hz), 6.79 (d, 1H, J = 2.6 Hz), 6.24 (d, 1H, J = 9.6 Hz), 5.86 (t, 1H, J = 6.6 Hz), 4.29 (m, 2H), 2.31 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 165.3, 161.8, 161.6, 156.4, 143.9, 134.9, 130.6, 129.6, 129.4, 128.6, 117.1, 114.3, 113.8, 113.5, 102.1, 63.8, 59.5, 32.9; HRMS: calcd for

$C_{20}H_{15}NO_5$: 349.09502; found: 349.095023; anal. calcd. (%) for $C_{20}H_{15}NO_5$: C 68.76, H 4.33; found C 68.72, H 4.33.

7-[(3-Cyano-3-hydroxyprop-1-yl)oxy]-*2H*-1-benzopyran-2-one (2)

To a solution of diol **6** (140 mg, 0.56 mmol) in water (2 mL) and acetone (2 mL) was added sodium periodate (240 mg, 2 equivalents) at 0 °C. The reaction mixture was stirred for 1 hour at 0 °C and extracted with CH_2Cl_2 (2×50 mL). After evaporation of the solvent, the white solid residue of aldehyde **3** was dissolved in CH_3CN (5 mL) and treated with triphenylphosphine (18 mg, 0.15 equivalents) and trimethylsilyl cyanide (57 μL, 1 equivalent) at room temperature. The reaction mixture was stirred 2 hours and evaporated. Flash chromatography of the residue (hexane/EtOAc, 3/1) gave cyanohydrin **2** as a colorless solid; yield: 108 mg (79%); mp 134 °C; IR ($CHCl_3$): $\nu = 3312, 2930, 1686, 1682, 1624, 1556, 1512, 1483, 1456, 1433, 1404, 1330, 1294, 1243, 1202, 1146$ cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.65$ (d, 1H, $J = 9.6$ Hz), 7.40 (d, 1H, $J = 8.8$ Hz), 6.86 (dd, 1H, $J = 8.8$ Hz, 2.2 Hz), 6.85 (br s, 1H), 6.29 (d, 1H, $J = 9.6$ Hz), 4.86 (dd, 1H, $J = 12.5, 6.2$ Hz), 4.30 (m, 2H), 2.93 (d, 1H, $J = 6.2$ Hz), 2.40 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 144.0, 129.7, 114.4, 113.3, 102.3, 64.4, 59.3, 35.0$; EIMS: $m/z = 245$ [M]$^+$, 220, 218, 190, 175, 162, 134, 105, 89, 78; HRMS: calcd. for $C_{13}H_{11}NO_4$: 245.0688; found: 245.0692.

Kinetic Measurements

All substrates were diluted from stock solutions in 50% aqueous acetonitrile, and stored at +4 °C. Enzymes were diluted from 1 mg mL^{-1} stock solutions of the supplied solid in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4). Assays (0.1 mL) were followed in individual wells of round-bottom polypropylene 96-well plates (Costar) using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems, filters $\lambda_{ex} = 360 \pm 20$, $\lambda_{em} = 460 \pm 20$ nm). Fluorescence data were converted to umbelliferone concentration by means of a calibration curve. The rates indicated in the Tables are derived from the linear portion in each curve. Commercial enzyme preparations were purchased from Fluka, Aldrich, Sigma, Boehringer Mannheim or Serva.

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