A Sensitive and Selective High-Throughput Screening Fluorescence Assay for Lipases and Esterases

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Long-chain fatty acid esters of 7-(3,4-dihydroxybutyloxy)-2*H*-1-benzopyran-2-one (**6**) such as octanoate **2a** are shown to be exceptionally sensitive and selective fluorogenic substrates for lipases and esterases. Umbelliferone (**8**) is released upon hydrolysis of the ester function in **2a** in the presence of bovine serum albumin and sodium periodate. These substrates are at least by one order of magnitude more sensitive to lipases than the commercial fluorogenic substrate 4-methylumbelliferyl heptanoate. Furthermore, they are stable to a broad range of pH-induced- and thermal-hydrolysis conditions and do not react with non-catalytic proteins such as bovine serum albumin (BSA).

Introduction. – Enzyme assays are indispensable tools in enzymology, where they are used to identify enzymes and to evaluate their purity and activity [1]. When an enzyme is discovered, one important question is always the identity of its natural substrate, which is presumably also its best substrate in terms of kinetic behavior. In the context of enzyme assays, the related problem is to find the optimal method for any given enzyme, taking into consideration that assays giving spectroscopic signals are preferred for high-throughput applications, for example, in the context of biodiversity mining and directed evolution [2]. Herein, we report that C_8-C_{14} aliphatic esters of the fluorogenic diol **6** are exceptionally selective and sensitive probes for lipases (*Scheme 1*). These substrates are stable under a variety of conditions, but are rapidly hydrolyzed whenever only traces of an active lipase are present. The assay is applicable for high-throughput screening.

Results and Discussion. – Lipases and esterases are among the most broadly used enzymes. The natural function of most of these enzymes is to hydrolyze triglycerides of fatty acids, which initiates the metabolization of fat. As a consequence, their ideal substrate should be a triglyceride. Indeed, vegetal oil and glycerol tributyrate (tributyrin) are well-known substrates of lipases and esterases, respectively. We recently reported a simple colorimetric enzyme assay with these triglycerides as substrates [3]. However, despite of using optimal substrates, the assay had a detection limit in the usual range of 10–100 μg ml^{-1} of enzyme[2]). We had encountered a similar

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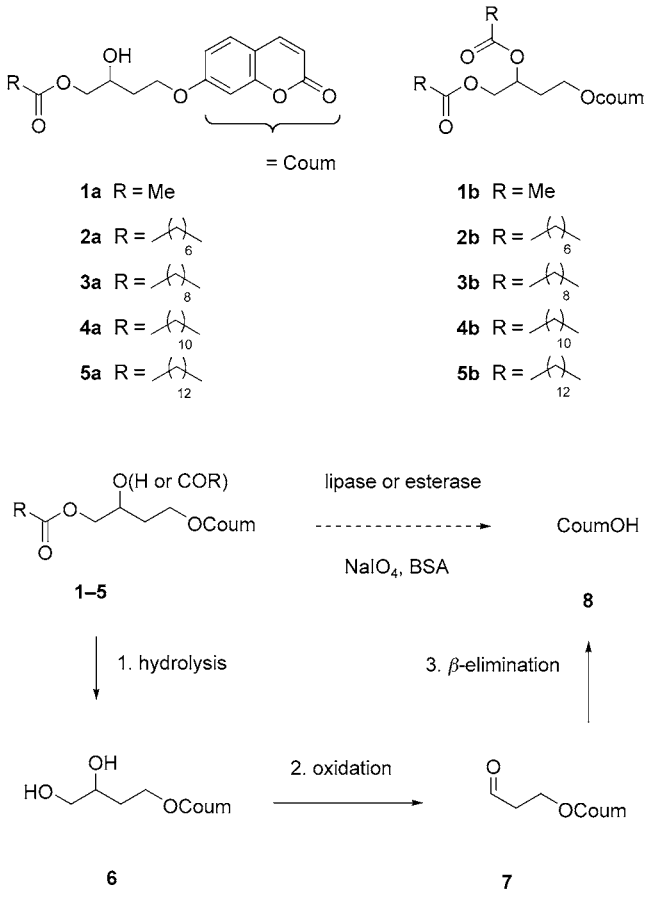
[2]) Commercial enzyme samples were used in all our studies. The activities observed with vegetal oil [3] or with the substrates in the present study did not correlate well with the activity units (U = μmol product min^{-1} mg^{-1}) given by the manufacturer, which are most often defined with 4-nitrophenyl butyrate or palmitate as substrate. This is not surprising considering that enzyme reactivities are generally dependent on substrate structure.

Scheme 1. *Fluorogenic Substrates for Lipases and Esterases, and Detection Principle*

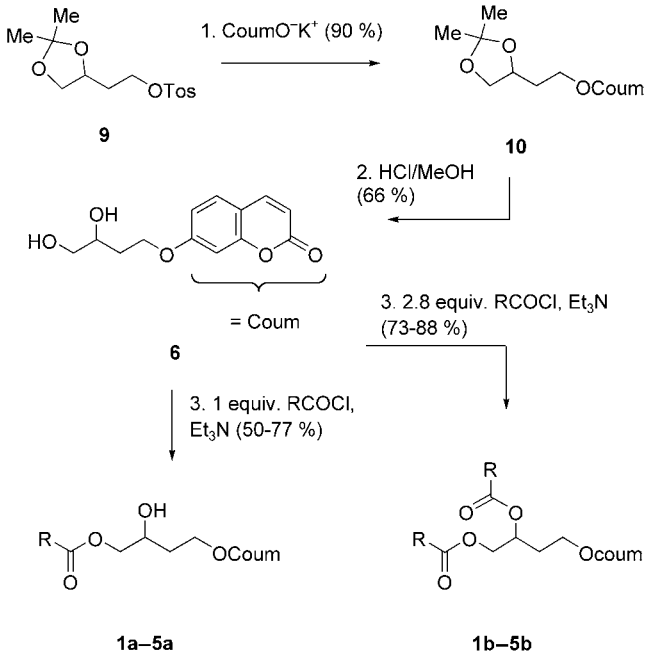


detection limit when using acetates **1a** and **1b** as lipase and esterase substrates (*Scheme 1*) [4]. These substrates release diol **6** upon reaction with the enzymes, and a fluorescence signal is produced in the presence of $NaIO_4$ and bovine serum albumin (BSA) by liberation of umbelliferon (**8**) through an oxidation/β-elimination sequence *via* aldehyde **7** [5]. A first attempt at optimizing the structures of these substrates towards lipases consisted in modifying diol **6** into a branched analog of glycerol to produce diglyceride-like aliphatic ester substrates [6]. However, this strategy did not lead to the expected improvement in sensitivity. We, therefore, went back to the simple diol **6**, which may also be considered as an analog of glycerol, to investigate the effect of substituting long-chain aliphatic acyl groups for the acetyl group in **1a** and **1b**.

Starting with butane-1,2,4-triol, acetonide formation and tosylation gave the known tosylate **9** [7]. Substitution with the sodium salt of umbelliferone gave ether **10**, which was deprotected with acid to give the racemic diol **6** (*Scheme 2*). The four aliphatic acid monoesters **2a**–**5a** and the four aliphatic acid diester derivatives **2b**–**5b** were prepared

by reaction of this diol with the corresponding acyl chloride and Et_3N. These substrates were conditioned as 1 mM stock solutions in MeCN, and tested with a series of lipases and esterases. Assays were initiated by addition of substrate to a prediluted solution of enzyme in a slightly basic buffer (pH 8.8), incorporating $NaIO_4$ as a diol-processing reagent and BSA as an additive. The latter served both as a catalyst for the β-elimination of the intermediate aldehyde and as a non-selective protein to mimic the conditions of high-throughput screening in crude extracts. Fluorescence time profiles were recorded in microtiter plate assay format by using a series of enzymes, and the time curves were used to derive apparent reaction rates with the different enzymes (*Table*).

Scheme 2. *Synthesis of Fluorogenic Lipase Substrates*



All enzymes showed a much higher reactivity with the long-chain aliphatic esters **2**–**5** compared to the acetate derivatives **1a** and **1b**. While the earlier measurements with **1a** and **1b** required 100 $\mu g \cdot ml^{-1}$ enzyme to obtain a good signal, many enzyme samples retained activity upon 10-fold, 100-fold, or even 1000-fold dilution. Analysis by gel electrophoresis (SDS-PAGE) showed that high activity, in fact, correlated with protein purity. The mono-ester derivatives were generally much more reactive than the corresponding diesters, which is not surprising if one considers that the diester requires a double hydrolysis reaction to induce a fluorogenic oxidation sequence. Several esterases also showed reactivities with the long-chain fatty acid esters largely exceeding those observed with the acetate derivatives. This is not necessarily surprising considering the problematic distinction between these two enzyme classes. It should be noted that the acetates were distinct from the long-chain substrates in that the

Table 1. *Reaction Rates Observed with Lipases and Esterases*[a])

Fluka No.	Enzyme	μg ml^{-1}	**1a**[b])	**2a**	**3a**	**4a**	**5a**	**1b**[b])	**2b**	**3b**	**4b**	**5b**
62336	PSBL	0.1	(5.2)	17.0	24.2	23.1	9.7	(21)	16.2	3.4	3.5	7.2
62333	CVL	0.1	(0.2)	11.9	13.7	15.6	17.8	(5.0)	6.7	1.6	1.6	2.9
62321	PFL	0.1	(4.0)	8.6	13.1	11.8	6.4	(14.1)	3.9	2.0	2.1	1.9
62335	PSL	1	(1.9)	30.7	34.4	33.3	25.6	(7.0)	14.4	9.4	9.5	6.8
62285	AOL	1	(0.2)	4.1	8.3	10.5	3.5	(1.3)	3.3	1.5	1.2	1.0
62299	CAL	10	(4.1)	26.6	18.7	9.3	10.8	(1.1)	13.8	9.1	10.9	7.1
62305	RAL	10	n.d.	24.7	14.4	20.3	25.9	n.d.	13.3	1.1	3.2	1.5
62309	PCL	10	(0.4)	13.0	14.5	16.2	8.2	(1.0)	26.0	12.2	7.6	5.0
62294	ANL	10	(0.2)	19.2	16.6	17.1	8.3	(3.1)	15.0	0.2	4.9	2.2
62304	MJL	10	n.d.	16.4	11.5	19.8	12.3	n.d.	9.2	2.4	6.2	1.9
62316	CCL	10	(0.1)	15.4	15.1	9.8	3.7	(0.5)	14.1	2.4	4.1	1.0
62298	MML	10	(0.1)	16.3	11.8	12.2	5.9	(0.7)	7.4	4.2	3.5	1.5
62300	HPL	100	0.3	35.6	28.4	29.6	7.7	2.4	18.0	8.8	6.6	1.8
46069	HLE	100	2.2	38.8	32.0	28.6	15.2	6.0	8.6	3.6	3.4	3.1
62291	RML	100	0.1	26.9	23.6	24.7	11.9	0.6	19.3	5.3	3.6	2.4
46059	MME	100	0.1	31.2	28.3	18.2	7.7	0.3	12.2	8.2	5.1	4.1
46061	TBE	100	0.1	18.5	22.3	21.7	12.7	0.3	3.7	2.1	3.4	2.0
46062	BSE	100	0.1	19.2	18.6	18.8	7.8	0.2	6.1	3.6	4.1	3.1
62310	RNL	100	0.1	17.9	15.7	9.8	10.0	0.4	15.9	3.4	4.2	1.1
46051	BStE	100	1.0	15.5	11.0	10.6	2.3	1.8	2.5	0.3	0.2	0.2
62306	WGL	100	n.d.	6.7	6.3	4.6	1.8	n.d.	1.0	0.9	1.3	1.7
62308	PRL	100	n.d.	4.5	7.3	3.8	3.0	n.d.	0.1	0.1	0.1	0.0
	no E[c])		0.10	0.23	0.28	0.24	0.22	0.04	0.08	0.02	0.06	0.03

[a]) Rate of release of umbelliferone (**8**) in nM s^{-1} from 100 μM substrate, with commercial enzyme samples at the given concentration. Conditions: aq. 20 mM borate buffer, pH 8.8, 5% (*v*/*v*) MeCN, 30°, in the presence of 1 mM $NaIO_4$ and 2 mg · ml^{-1} BSA, 0.1-ml assays were carried out in round-bottom polypropylene *96*-well-plates (*Costar*) and recorded with a *Cytofluor II Fluorescence Plate Reader* (*Perseptive Biosystems*; filters $\lambda_{ex} = 360 \pm 20$, $\lambda_{em} = 460 \pm 20$ nm). Fluorescence was converted to product concentration with a calibration curve with pure **8**, and the steepest linear portion of each curve was used to calculate the reaction rates. [b]) Data for **1a** and **1b** from [4a]. Rates in parentheses are with 100 μg · ml^{-1} enzyme concentration and not the concentration indicated here. [c]) Spontaneous reaction in the absence of enzyme under the assay conditions.

diester derivative **1b** was consistently more enzyme-reactive than the monoacetate **1a**, indicating that the enzymes require a certain level of lipophilicity in the substrate for having a good reactivity.

The reactivity of the octanoate **2a** with *Pseudomonas fluorescens* type *B* lipoprotein lipase (PSBL) was investigated closer. The substrate gave a signal to background ratio $V_{rel} = 185$ with 10 μg ml^{-1} enzyme ($V_{rel} = (V_{enz}/V_{no\,E}) - 1$)[3]). The signal remained very strong upon dilution of the enzyme, and was still at $V_{rel} = 73$ at 0.1 μg ml^{-1} (*Table*). Further dilution down to 0.01 μg ml^{-1} enzyme gave a signal-to-background ratio of $V_{rel} > 10$, which is perfectly sufficient for detection (*Fig. 1*). Both enantiomers (*R*)-**2a** and (*S*)-**2a** showed similar reactivities.

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[3]) At high enzyme concentration, the rate of release of umbelliferone (**8**) is limited by the rate of oxidation/β-elimination of diol **6**, so that the apparent reaction rate is not proportional to enzyme concentration upon dilution of the enzyme.

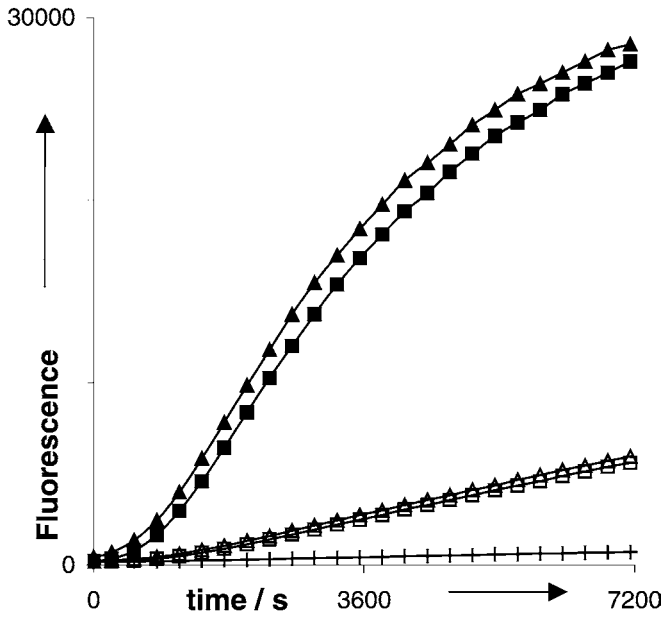


Fig. 1. *Fluorescence signal for the reaction of 100* μM *octanoate* (R)-**2a** *and* (S)-**2a** *in 20 m*M *aq. borate, pH 8.8, 2 mg ml*$^{-1}$ *BSA, 1 m*M $NaIO_4$, *20% (v/v) DMF, 25°.* + : No additive, ■: (*S*)-**2a** with 0.1 μg ml^{-1} BSBL, □: (*S*)-**2a** with 0.01 μg ml^{-1} PSBL, ▲: (*R*)-**2a** with 0.1 μg ml^{-1} PSBL, △: (*R*)-**2a** with 0.01 μg ml^{-1} PSBL.

The high reactivity with lipases could be attributed in part to the aliphatic hydrocarbon chain of the acid. By comparison, we investigated the reactivity of 4-methylumbelliferyl heptanoate (**11**), a commercially available fluorogenic lipase substrate, which is a representative example of aliphatic phenyl ester [8]. 4-Methylumbelliferyl heptanoate (**11**) reacted with PSBL within one order of magnitude of sensitivity of our substrates ($V_{rel} = 4.6$ at 0.1 μg ml^{-1} enzyme; *Fig. 2*). However, the commercial heptanoate had to be used at exactly neutral pH because it spontaneously hydrolyzed at pH 8.8, with or without BSA. In addition, its reaction was not selective for lipases and produced a fluorescence signal in the presence of the noncatalytic protein BSA even at neutral pH. This nonselective reaction can be attributed to an acylation of surface lysine residues. By contrast, octanoate **2a** showed no reaction in the presence or absence of BSA at neutral (pH 7) or slightly basic (pH 8.8) conditions. The reactivity difference between the two ester substrates is a direct consequence of the difference in leaving-group acidity ($pK_a \sim 7$ for the OH group in 4-methylumbelliferone and $pK_a \sim 14$ for diol **6**). The chemical lability problem of 4-methylumbelliferyl heptanoate (**11**) also occurs with the resorufin derivative **12**, a substrate which is insoluble in buffer and must be used with detergents for solubilization. Interestingly, it has been recently shown that substrate **12** undergoes hydrolysis at the activated resorufin ester bond under the action of lipases, while the glycerol ester bond remains stable [9]. Probes **11** and **12** must be stored at −18°, contain several percent of hydrolyzed material when purchased, and must be repurified before use.

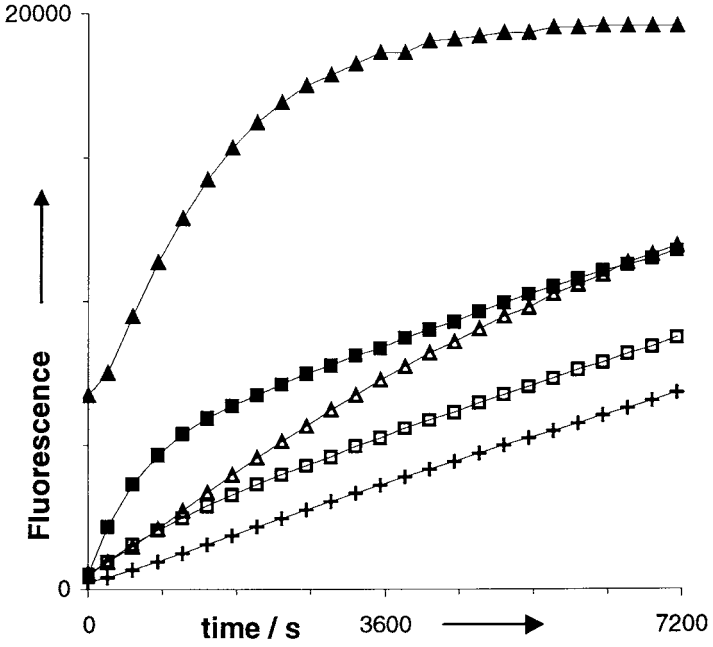
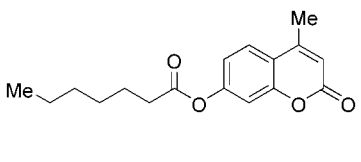


Fig. 2. *Fluorescence signal for the reaction of 100* μM *4-methylumbelliferyl heptanoate in 20 mM aq. phosphate, pH 7.0, 20% (v/v) DMF, 25°.* + : No additive, □: 0.5 mg ml^{-1} BSA, ■: 2 mg ml^{-1} BSA, ▲: 0.1 μg ml^{-1} PSBL, △: 0.01 μg ml^{-1} PSBL. 4-Methylumbelliferyl heptanoate was repurified before use to remove contaminant 4-methylumbelliferone. Stock solutions are unstable upon storage.



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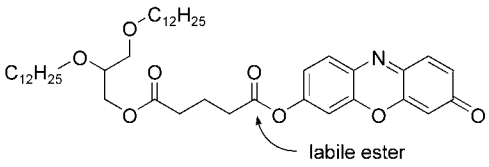

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The resilience of fluorogenic substrate **2a** to nonspecific degradation was remarkable under a variety of conditions such as extreme pH and temperature values. Thus, there was no significant (< 5%) degradation upon incubation for 60 min in aqueous buffers containing BSA at pH values as low as pH 1.0 and as high as pH 11, or by incubation at pH 8.8 at 100° for 60 min. Clearly, the reaction of **2a** with lipases was not only highly sensitive but also highly selective. Esters **3a**–**5a** and diesters **2b**–**5b** showed comparable properties in terms of resistance to nonspecific degradation and sensitivity to lipases and esterases. By contrast to the above mentioned lipase probes, our substrates also proved shelf-stable as solids and could be handled without any problems of nonspecific degradation.

Conclusions. – In summary, long-chain fatty acid esters of the glycerol analog **6** are remarkably sensitive and selective fluorogenic substrates for lipases and esterases. The fluorogenic glyceride analogs reported here are stable under a variety of conditions including extreme pH and temperature values, yet react selectively with only trace amounts of lipases. The substrates are also suitable as fluorogenic substrates for esterases. The coupling of high sensitivity with high selectivity makes these substrates an optimal choice for the detection of esterolytic activities in high-throughput screening. The processing reagents $NaIO_4$ and BSA are necessary but do not affect enzyme activity. These reagents may also be added at the end of the reaction for an endpoint determination of product formation. It must be mentioned that the decomposition of the primary diol product **6** by oxidation and β-elimination leading to the fluorescent signal may become rate-limiting when the enzymatic hydrolysis rate is very high, implying that our detection system is probably not suited for precise kinetic studies. The use of the more hydrophobic substrates, in particular, the diesters of the long-chain esters **3b** – **5b**, in the presence of detergents are under current investigation.

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Experimental Part

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 soln. in 95% EtOH. Silica gel *60* (*Macherey-Nagel*, 230–400 mesh) was used for flash chromatography (FC). M.p.: *Kofler* apparatus or *Büchi 510* apparatus; IR Spectra: *Perkin-Elmer Spectrum One* series FTIR apparatus. ^{1}H- and ^{13}C-NMR spectra: *Bruker AC-300* spectrometer.

*7-[2-(3,3-Dimethyl-1,2-dioxolan-4-yl)ethoxy]-2*H*-1-benzopyran-2-one* (**10**). A suspension of *umbelliferone* (= *7-hydroxy-2*H*-1-benzopyran-2-one* **8**; 270 mg, 1.66 mmol), 18-crown-6 (87 mg, 0.33 mmol), anh. K_2CO_3 (459 mg, 3.32 mmol), and 2-(3,3-dimethyl-1,3-dioxolan-4-yl)ethyl toluene-4-sulfonate (500 mg, 1.66 mmol) in 3 ml of acetone was heated under reflux for 16 h. Evaporation, aq. workup (AcOEt, 0.1N NaOH, then aq. sat. NaCl) and FC (hexane/AcOEt 2:1; R_f 0.23) gave **10** (433 mg, 1.49 mmol, 90%). Solid. M.p. 81°. IR (KBr): 2988*m*, 2956*m*, 2934*m*, 2890*m*, 1726*s*, 1629*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, *J* = 9.6, 1 H); 7.36 (*d*, *J* = 7.7, 1 H); 6.84 (*s*, 1 H); 6.83 (*d*, *J* = 7.7, 1 H); 6.24 (*d*, *J* = 9.6, 1 H); 4.31 (*m*, *J* = 6.3, 6.6, 1 H); 4.13 (*m*, *J* = 6.3, 6.6, 8.1, 3 H); 3.65 (*dd*, *J* = 6.6, 8.1, 1 H); 2.07 (*m*, *J* = 6.3, 1 H); 1.43 (*s*, 3 H); 1.37 (*s*, 3 H). ^{13}C-NMR 75 MHz, $CDCl_3$): 162.7; 144.0; 130.6; 129.5; 128.5; 113.9; 113.5; 113.3; 109.7; 102.2; 73.7; 70.1; 66.0; 34.0; 27.7; 26.4. EI-MS: 290 (M^+), 275, 215, 162, 134, 71, 43.

*7-(3,4-Dihydroxybutyloxy)-2*H*-1-benzopyran-2-one* (**6**). Intermediate **10** (5.47 g, 18.9 mmol) was dissolved in 60 ml MeOH. 0.1N Aq. HCl (50 ml) was added, and the mixture was stirred at 25° for 17 h. Evaporation and co-evaporation with toluene, followed by FC (CH_2Cl_2/acetone 7:3 bis 1:1), gave **6** (3.13 g, 12.5 mmol, 66%). Colorless solid. M.p. 98°. IR (KBr): 3300*s*, 2934*m*, 2876*m*, 1724*s*, 1611*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, *J* = 9.6, 1 H); 7.37 (*d*, *J* = 7.7, 1 H); 6.86 (*d*, *J* = 7.7, 1 H); 6.84 (*d*, *J* = 7.7, 1 H); 6.25 (*d*, *J* = 9.6, 1 H); 4.15 (*m*, 2 H); 3.78 (*m*, 1 H); 3.46 (*m*, 2 H); 2.00 (*m*, 1 H); 1.76 (*m*, 1 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 163.9; 163.3; 157.0; 145.7; 130.3; 114.1; 113.9; 113.2; 102.2; 70.0; 67.4; 66.5; 33.9. Anal. calc. for $C_{13}H_{14}O_5$: C 62.39, H 5.64; found: C 62.33, H 5.60.

*3-Hydroxy-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butyl Octanoate* (**2a**). A soln. of **6** (100 mg, 0.4 mmol) and dry Et_3N (81 mg, 0.8 mmol) in dry CH_2Cl_2 (6 ml) and dry DMF (1 ml) at 0° was treated with octanoyl chloride (69 µl, 0.4 mmol). The mixture was stirred at 0° for 1 h and then at 25° until completion of the reaction (TLC). Aq. workup (CH_2Cl_2/aq. sat. $NaHCO_3$ and brine) and FC (hexane/AcOEt 2:1) gave **2a** (91.7 mg, 0.24 mmol, 59%). Colorless solid. M.p. 41°. TLC (hexane/AcOEt 1:1): R_f 0.53. IR (film): 3466*w* (br.), 3008*w*, 2956*m*, 2930*m*, 2858*m*, 1728*s*, 1613*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, 1 H); 7.37 (*d*, 1 H); 6.86 (*s*, 1 H); 6.84 (*d*, 1 H); 6.25 (*d*, 1 H); 4.04–4.25 (*m*, 5 H); 2.36 (*m*, 2 H); 1.98 (*m*, 2 H); 1.63 (*m*, 2 H); 1.29 (*m*, 8 H); 0.87

(*m*, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.7; 162.6; 161.9; 156.4; 144.1; 129.4; 113.7; 113.5; 113.3; 102.1; 68.9; 67.7; 65.6; 34.8; 33.3; 32.2; 29.7; 29.5; 25.5; 23.2; 14.7. FAB-MS: 377 (M^+), 251, 163.

*Compound (*R*)-***2a***.* The procedure for **2a** was carried out starting with (*R*)-**6** [4d] (96% ee; 85 mg, 0.34 mmol) to give the corresponding ester (*R*)-**2a** (109 mg, 0.29 mmol, 85%). Colorless waxy solid. $[\alpha]_D^{20} = +4.6$ ($c = 1$, $CHCl_3$).

*Compound (*S*)-***2a***.* The procedure for **2a** was carried out starting with (*S*)-**6** [4d] (87% ee; 70 mg, 0.28 mmol) to give the corresponding ester (*R*)-**2a** (108 mg, 0.29 mmol, 81%). Colorless waxy solid. $[\alpha]_D^{20} = -4.0$ ($c = 1$, $CHCl_3$).

*3-Hydroxy-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butyl Decanoate* (**3a**). The procedure for **2a** was applied with decanoyl chloride (83 µl, 0.4 mmol). FC gave **3a** (80 mg, 0.2 mmol, 49%). Colorless solid. M.p. 45°. TLC (hexane/AcOEt 1:1): R_f 0.57. IR (film): 3492*w* (br.), 3013*w*, 2928*m*, 2856*w*, 1728*s*, 1613*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, 1 H); 7.36 (*d*, 1 H); 6.86 (*s*, 1 H); 6.83 (*d*, 1 H); 6.25 (*d*, 1 H); 4.04–4.24 (*m*, 5 H); 2.35 (*m*, 2 H); 1.97 (*m*, 2 H); 1.61 (*m*, 2 H); 1.26 (*m*, 12 H); 0.86 (*m*, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.7; 162.6; 161.8; 156.6; 144.0; 129.5; 113.9; 113.5; 113.4; 102.23; 69.0; 67.9; 65.7; 34.9; 33.3; 32.5; 30.1; 29.9; 29.8; 25.6; 23.3; 14.8. FAB-MS: (M^+), 251, 163, 13. Anal. calc. for $C_{32}H_{32}O_6$: C 68.29, H 7.97; found: C 68.19, H 8.00.

*3-Hydroxy-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butyl Dodecanoate* (**4a**). The procedure for **2a** was applied with dodecanoyl chloride (96 µl, 0.4 mmol). FC gave **4a** (130 mg, 0.3 mmol, 75%). Colorless solid. M.p. 53°. TLC (hexane/AcOEt 1:1): R_f 0.69. IR (film): 3466*w* (br.), 3009*w*, 2927*s*, 2855*m*, 1728*s*, 1613*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, 1 H); 7.37 (*d*, 1 H); 6.86 (*s*, 1 H); 6.84 (*d*, 1 H); 6.25 (*d*, 1 H); 4.01–4.28 (*m*, 5 H); 2.34 (*m*, 2 H); 1.97 (*m*, 2 H); 1.52 (*m*, 2 H); 1.25 (*m*, 16 H); 0.87 (*m*, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.6; 162.6; 161.8; 156.5; 144.0; 129.4; 113.8; 113.5; 113.3; 102.2; 69.0; 67.7; 65.7; 34.8; 33.3; 32.5; 30.2; 30.1; 29.9; 29.9; 29.8; 25.6; 23.3; 14.7. FAB-MS: 433 (M^+), 251, 228, 163. HR-ESI-MS: 455.2425 ($[M + Na^+]$, $C_{25}H_{36}O_6^+$; calc. 455.2409).

*3-Hydroxy-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butyl Tetradecanoate* (**5a**). The procedure for **2a** was applied with tetradecanoyl chloride (108 µl, 0.4 mmol). FC gave **5a** (142 mg, 0.31 mmol, 77%). Colorless solid. M.p. 60°. TLC (hexane/AcOEt 1:1): R_f 0.67. IR (film): 3469*w* (br.), 3011*w*, 2927*s*, 2855*m*, 1728*s*, 1613*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, 1 H); 7.37 (*d*, 1 H); 6.86 (*s*, 1 H); 6.84 (*d*, 1 H); 6.25 (*d*, 1 H); 4.04–4.28 (*m*, 5 H); 2.36 (*m*, 2 H); 1.98 (*m*, 2 H); 1.62 (*m*, 2 H); 1.25 (*m*, 20 H); 0.88 (*m*, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.7; 162.6; 161.9; 156.6; 144.1; 129.5; 113.9; 113.5; 113.4; 102.2; 69.1; 67.9; 65.7; 34.9; 33.3; 32.6; 30.4; 30.3; 30.3; 30.1; 30.0; 29.9; 29.8; 25.6; 23.4; 14.8. FAB-MS: 461 (M^+), 256, 251, 163. HR-ESI-MS: 461.2914 ($[M + H]^+$; $C_{27}H_{40}O_6^+$; calc. 461.2903).

*4-[(2-Oxo-2*H*-1-benzopyran-7-yl)oxy]butane-1,2-diyl Dioctanoate* (**2b**). A soln. of diol **6** (100 mg, 0.4 mmol) and 4-(dimethylamino)pyridine (134 mg, 1.1 mmol) in dry CH_2Cl_2 (7 ml) was treated with octanoyl chloride (171 µl, 1.1 mmol) and stirred at 25° until completion of the reaction (18 h, TLC). Aq. workup (CH_2Cl_2/aq. sat. $NaHCO_3$ sat. and brine) and FC (hexane/AcOEt 5:1) gave **2b** (60 mg, 0.12 mmol, 30%). Colorless solid. M.p. 38°. TLC (hexane/AcOEt 2:1): R_f 0.56. IR (film): 2955*m*, 2928*m*, 2857*m*, 1738*s*, 1614*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, 1 H); 7.35 (*d*, 1 H); 6.84 (*s*, 1 H); 6.80 (*d*, 1 H); 6.25 (*d*, 1 H); 5.33 (*m*, 1 H); 4.35 (*m*, 1 H); 4.04–4.16 (*m*, 3 H); 2.31 (*m*, 4 H); 2.13 (*m*, 2 H); 1.59 (*m*, 4 H); 1.27 (*m*, 16 H); 0.86 (*m*, 6 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.1; 173.83; 162.4; 159.4; 158.8; 144.0; 129.5; 114.0; 113.7; 113.4; 102.0; 69.2; 65.4; 65.1; 35.0; 34.8; 32.3; 31.2; 29.7; 29.6; 25.7; 25.6; 23.3; 14.7. FAB-MS: 503 (M^+), 377, 341, 251, 233, 215, 127. HR-ESI-MS: 525.2827 ($[M + H]$, $C_{29}H_{42}O_7^+$; calc. 525.2828).

*4-[(2-Oxo-2*H*-1-benzopyran-7-yl)oxy]butane-1,2-diyl Didecanoate* (**3b**). The procedure for **2b** was applied with decanoyl chloride (204 µl, 1.1 mmol). FC gave **3b** (166 mg, 0.29 mmol, 73%). Colorless solid. M.p. 55°. TLC (hexane/AcOEt 2:1): R_f 0.67. IR (film): 3024*w*, 2955*m*, 2927*s*, 2855*m*, 1740*s*, 1613*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, 1 H); 7.36 (*d*, 1 H); 6.84 (*s*, 1 H); 6.80 (*d*, 1 H); 6.26 (*d*, 1 H); 5.32 (*m*, 1 H); 4.34 (*m*, 1 H); 4.04–4.16 (*m*, 3 H); 2.31 (*m*, 4 H); 2.14 (*m*, 2 H); 1.57 (*m*, 4 H); 1.24 (*m*, 24 H); 0.86 (*m*, 6 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.1; 173.8; 166.6; 162.44; 161.7; 144.0; 129.5; 114.0; 113.7; 113.4; 102.0; 69.2; 65.4; 65.1; 35.1; 34.8; 32.5; 31.2; 30.1; 30.0; 29.8; 25.7; 25.6; 23.4; 14.8. FAB-MS: 559 (M^+), 405, 251, 233, 215, 155. HR-ESI-MS: 559.3640 ($[M + H]^+$, $C_{33}H_{50}O_7^+$; calc. 559.3635).

*4-[(2-Oxo-2*H*-1-benzopyran-7-yl)oxy]butane-1,2-diyl Didodecanoate* (**4b**). The procedure for **2b** was applied with dodecanoyl chloride (237 µl, 1.1 mmol). FC gave **4b** (184 mg, 0.3 mmol, 75%). Colorless solid. M.p. 64°. TLC (hexane/AcOEt 2:1): R_f 0.58. IR (film): 3020*m*, 2925*s*, 2854*s*, 1735*s*, 1612*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, 1 H); 7.36 (*d*, 1 H); 6.84 (*s*, 1 H); 6.80 (*d*, 1 H); 6.26 (*d*, 1 H); 5.34 (*m*, 1 H); 4.34 (*m*, 1 H); 4.04–4.16 (*m*, 3 H); 2.30 (*m*, 4 H); 2.13 (*m*, 2 H); 1.58 (*m*, 4 H); 1.24 (*m*, 32 H); 0.87 (*m*, 6 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.1; 173.8; 162.4; 161.7; 156.6; 144.0; 129.5; 114.0; 113.7; 113.4; 102.0; 69.2; 65.4; 65.1; 35.0;

34.8; 32.6; 31.2; 30.3; 30.2; 30.1; 30.0; 29.8; 29.7; 25.6; 25.6; 23.4; 14.8. FAB-MS: 615 (M^+), 433, 251, 233, 215. HR-ESI-MS: 615.4242 ($[M+H]^+$, $C_{37}H_{58}O_7^+$; calc. 615.4261).

*4-[(2-Oxo-2*H*-1-benzopyran-7-yl)oxy]butane-1,2-diyl Ditetradecanoate* (**5b**). The procedure for **2b** was applied with tetradecanoyl chloride (297 µl, 1.1 mmol). FC gave **5b** (261 mg, 0.39 mmol, 88%). Colorless solid. M.p. 72°. TLC (hexane/AcOEt 2:1): R_f 0.73. IR (film): 3022*w*, 2927*s*, 2855*m*, 1732*s*, 1614*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.63 (*d*, 1 H); 7.36 (*d*, 1 H); 6.84 (*s*, 1 H); 6.80 (*d*, 1 H); 6.26 (*d*, 1 H); 5.34 (*m*, 1 H); 4.34 (*m*, 1 H); 4.04–4.16 (*m*, 3 H); 2.31 (*m*, 4 H); 2.13 (*m*, 2 H); 1.58 (*m*, 4 H); 1.25 (*m*, 40 H); 0.87 (*m*, 6 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.1; 173.8; 162.4; 161.8; 156.5; 144.0; 129.5; 113.9; 113.7; 113.4; 101.9; 69.2; 65.3; 65.1; 35.0; 34.8; 32.6; 31.1; 30.33; 30.30; 30.27; 30.14; 30.12; 30.03; 29.9; 29.79; 29.76; 25.6; 25.5; 23.3; 14.8. FAB-MS: 671 (M^+), 461, 443, 251, 233, 215.

Kinetic Measurements. All substrates were diluted from stock solns. in 50% aq. MeCN (for **1a**–**5a**) or pure MeCN (for **1b**–**5b**), and stored at −20°. *4-Methylumbelliferyl heptanoate* (**11**) was purchased from *Fluka* (No. 75230), purified by flash chromatography (AcOEt/hexane 1:3) and recrystallization from MeOH, and used as a freshly prepared 10 mM stock soln. in MeCN/H_2O 1:1. Enzymes were diluted from 1 mg ml^{-1} stock solns. 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*) with 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 *Table* are derived from the linear portion in each curve.

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