New Monofunctionalized Fluorescein Derivatives for the Efficient High-Throughput Screening of Lipases and Esterases in Aqueous Media

by **Yongzheng Yang**, **Peter Babiak**, and **Jean-Louis Reymond***

Department of Chemistry & Biochemistry, University of Berne, Freiestrasse 3, CH-3012 Berne
(fax: +41-31-631-8057; e-mail: jean-louis.reymond@ioc.unibe.ch)

Monoalkylation or acylation of fluorescein (**1**) with various acyloxymethyl or acyl halides afforded, respectively, a series of ether- (**2**) and ester-functionalized (**3**) fluorogenic probes. The highly reactive and water-soluble substrates release fluorescein (**1**) upon reaction with lipases and esterases within seconds or minutes, both under fully aqueous conditions or in the presence of DMSO (20%) as a co-solvent. The most-reactive substrates in the two series were the octanoic acid derivatives **2f** (=2-{6-[(octanoyloxy)methoxy]-3-oxo-3*H*-xanthen-9-yl}benzoic acid) and **3a** (=2-[6-(octanoyloxy)-3-oxo-3*H*-xanthen-9-yl]benzoic acid). Esterases were found to generally react faster under aqueous conditions, while lipases were more reactive in the presence of DMSO as a co-solvent.

Introduction. – High-throughput-screening (HTS) enzyme assays are essential tools for enzyme discovery and engineering [1]. In particular, assays for lipases and esterases are in great demand due to the importance of this enzyme class in industrial biotechnology [2], and for their role as disease markers [3].

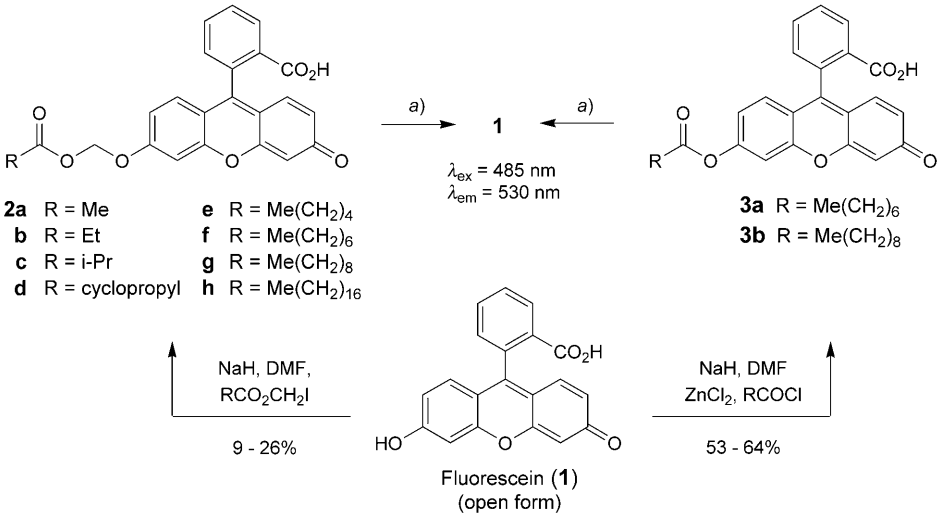
Lipases can be assayed by following the hydrolysis of triglycerides, which are the presumed natural substrates of these enzymes, or with the aid of titration [4], surface-tension balance [4], pH indicators [5], or back-titration of released diols [6]. However, these assays require either specific instrumentation not suitable for HTS, or multiple reagents that complicate the assay. The most-popular HTS assays are based on simple chromogenic/fluorogenic esters of colored/fluorescent phenols such as nitrophenol, umbelliferone (=7-hydroxy-2*H*-1-benzopyran-2-one), or resorufin [7][8]. Activity units for most lipases are, in fact, defined in relation to such substrates.

Unfortunately all of these known substrates are far from ideal because *1*) they are insoluble in aqueous media, requiring co-solvents for reaction, and *2*) they show a high level of non-specific hydrolysis with non-catalytic proteins [4]. HTS Assays with improved performances on both points would, thus, be highly desirable. The problem of non-specific hydrolysis can be solved with FRET-type substrates [9], or esters of aliphatic alcohols releasing a colored or fluorescent phenol *via* a secondary reaction sequence such as the cleavage of a 1,2-diol [10] or a cyanohydrin [11] followed by β-elimination [12]. Simple esters of umbelliferone can also be used in a solid-supported assay by adsorption on silica gel, which suppresses background hydrolysis [13]. However, these modified substrates still show very limited aqueous solubility, and require addition of co-solvents for a reliable reaction.

Herein we show that monoalkylation and monoacylation of fluorescein (**1**) yields highly reactive fluorogenic substrates of type **2** and **3** suitable for lipase and esterase

assays under fully aqueous conditions, with the octanoates **2f** and **3a** being the most-reactive reagents within this series (*Scheme*). Due to their simple synthesis and high reactivity, these substrates are particularly attractive for rapid screening of lipase and esterase activities.

Scheme. *Synthesis and Application of Monofunctionalized Fluorescein Derivatives as Lipase Substrates*



a) Lipase or esterase, H_2O (pH 7.4) or H_2O/DMSO 4:1, 25°.

Results and Discussion. – 1. *General Considerations.* We recently reported that acyloxymethyl ethers of umbelliferone (**4a**) such as **4b** react rapidly with lipases and esterases (> 50% conversion after 10 min), and are more specific towards these enzymes than the corresponding esters, for example **5** (*Fig. 1*) [14]. In particular, the ether **4b** showed a reduced reactivity towards non-catalytic proteins such as bovine serum albumin (BSA) compared to its ester analog **5**. This reactivity difference was attributed to the lower acidity of the primary (aryloxy)methyl alcohol leaving group, leading to a reduced chemical reactivity compared to umbelliferone itself, and its small steric demand allowing better access of the enzyme to the ester function. The corresponding lactones **6a**,**b** also showed high reactivity towards esterases (> 50% conversion after 10 min) [15].

With the aim of establishing a reliable HTS assay for lipases and esterases in fully aqueous media, we set out to prepare acyloxymethyl ethers from fluorogenic phenols bearing water-soluble groups. We first investigated the known 8-octanoyloxypyrene-1,3,6-trisulfonate **7**, which is commercially available [16]. Although this substrate is fully water-soluble and shows a green fluorogenic reaction useful in the context of enzyme model studies such as synthetic channels [17] and dendrimers [18], it reacted relatively slowly with lipases and esterases (less than 5% conversion after 10 min; see legend to *Fig. 3* below). In addition, various attempts of alkylating the OH group with acyloxymethyl halides failed.

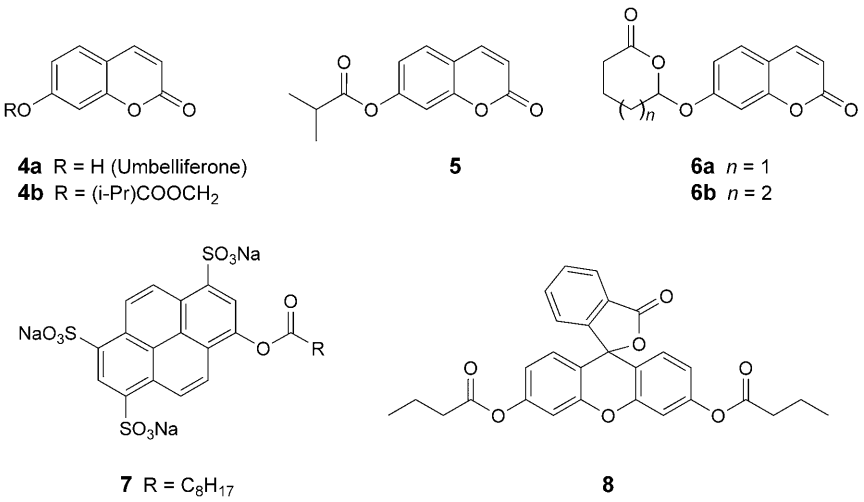


Fig. 1. *Selected, previously reported fluorogenic lipase and esterase substrates*

We next turned to fluorescein (**1**), which is an ideal fluorophore for assembling fluorescent sensors for aqueous media, for example chemosensors for amino acids [19], and sensors for Zn^{2+} [20]. Fluorescein (**1**) is water-soluble due to its polar functional groups (C=O, OH, COOH), gives rise to a green fluorescence ($\lambda_{ex}=485$ nm, $\lambda_{em}=530$ nm), and avoids autofluorescence from biological samples. This chromophore is also inexpensive and, therefore, well-suited for the synthesis of fluorogenic substrates. Esterification of fluorescein with acyl chlorides in the presence of pyridine yields non-fluorescent diesters such as **8**, which are useful as fluorogenic lipase substrates [21]. In these types of compounds, the fluorophore is inactivated by deconjugation through lactonization. These substrates are commercially available as lipase substrates, but are insoluble in aqueous media due to the formation of the cyclic lactone. The dibutyrate **8**, indeed, reacted with several lipases and esterases in the presence of DMSO as a co-solvent (up to 50% conversion after 5 min, see *Fig. 3*), but there was no detectable reaction in neat H_2O.

Considering that the fluorescein chromophore is isoelectronic with resorufin (=7-hydroxy-3*H*-phenoxazin-3-one), and by blocking only one of the two phenolic OH groups by ether- or ester-bond formation, one should obtain a non-fluorescent precursor still retaining its solubilizing COOH group. We had shown earlier in the context of a catalytic-antibody study [22] that fluorescein (**1**) can be monoalkylated with iodomethyl pivalate by reaction of the corresponding Na salt in DMF solution to yield a fluorogenic substrate. We, thus, set out to prepare a series of such (alkanoyloxy)methyl ethers of fluorescein as possible water-soluble fluorogenic substrates for lipases and esterases.

2. *Synthesis.* Chloromethyl esters were prepared by reaction of acyl chlorides with paraformaldehyde in the presence of $ZnCl_2$, as described previously [22]. The chlorides were treated with NaI in MeCN to form the more-reactive iodides, which were directly used in the next step. Treatment of fluorescein (**1**) with excess NaH and the iodomethyl

esters in DMF gave the corresponding monoalkylated fluorescein derivatives **2** as the only products, besides unreacted starting material (see the *Scheme*), and were readily separated by conventional column chromatography. There were no traces of dialkylated products. The modest yields reflect the poor conversion of **1**, which could not be improved by prolonged heating or at higher temperatures. Despite this drawback, the synthetic procedure could be successfully implemented to prepare a series of compounds (**2a**–**h**), including both short-chain esters (expected to react faster with esterases) and longer-chain acyl groups tailored for lipase reactivity. In addition, the monoesters **3a**,**b** were also prepared by reaction of **1** with the appropriate acyl chlorides in the presence of NaH and $ZnCl_2$ in DMF. Under these conditions, only the monoacylated products were formed, which could be readily isolated by column chromatography. The absence of side products in these reactions is probably due to the formation of the Na carboxylate salt of **1**, which does not readily lead to cyclization. This procedure contrasts with the double-acylation method mentioned above to form the known diester **8** from acyl halides in pyridine [21].

3. *Enzyme Assays.* All substrates were used as 4 mM stock solutions in MeCN/H_2O 1:1, and diluted to 0.4 mM in the same solvent mixture for addition to lipase solution. All enzymes were diluted from a stock solution (1 mg/ml) in aqueous phosphate-buffered saline (*PBS* buffer). Assays were initiated by addition of 5 μl of the 0.4 mM substrate solution to 95 μl of a solution of the enzyme ($\leq$50 μg/ml) in *PBS* buffer under rapid mixing. The reactions were then followed with a microtiter plate reader over the next few minutes.

The stabilities of the substrates were first evaluated in aqueous buffer in the presence or absence of BSA, using compounds **2f**,**g** and their acyl analogs **3a**,**b**. The substrates **2f**,**g** showed generally good stability at neutral pH. Addition of BSA increased the background hydrolysis rate of **2f** 11-fold, but not that of **2g** (*Table 1*). The simple monoesters **3a**,**b** showed a *ca.* 5-fold lower background hydrolysis in neat buffer compared to **2f**,**g**, but gave rise to a much faster reaction in the presence of BSA (**2f**: 15-fold, **2g**: 55-fold), in agreement with our previous observations with the corresponding umbelliferyl esters, and suggesting acylation of surface lysine residues in this case [10d]. The presence of specific interactions with the albumine was further evidenced by the observation that addition of BSA strongly reduced the reactivity of the substrates **2** and **3** towards lipases in buffer or in the presence of 20% (*v*/*v*) DMSO. Therefore, BSA was not used as an additive in the enzyme assay. The specific effects of BSA on the reactivity of the substrates might be attributed to binding interactions of the fluorescein chromophore with the known affinity site of BSA for fluorescein (**1**) [23].

Table 1. *Background Hydrolysis Rates* (apparent velocities *v*) *of Selected Fluorophores in the Presence and Absence of Bovine Serum Albumin* (BSA) *as Determined by Fluorescence Spectroscopy.* Conditions: 20 μM substrate and 2 mg/ml BSA in *PBS* buffer (pH 7.4); total volume, 100 μl; temperature, 30°. For details, see *Exper. Part.*

v [nM s^{-1}]	**2f**	**2g**	**3a**	**3b**
BSA	2.5	0.41	1.2	2.1
Control (H_2O)	0.23	0.35	0.082	0.038

Next, the substrates were tested for their activity against lipases and esterases. Although the substrates were reasonably easy to handle as stock solutions, and showed acceptable background-reaction rates, the reactions were extremely rapid, taking place within less than 1 min with certain enzymes. This contrasts sharply with our previous experience with fluorogenic lipase substrates often requiring at least 5 min to reach a significant conversion. In practice, it was not possible to fill a complete 96-well plate with individual assays since the mixing time had to be kept within several seconds only. Therefore, the activity of each enzyme was determined separately for the substrate series, such that only ten assays were conducted in parallel (*Fig. 2*). The experimental method was optimized with pig liver esterase (PLE), and then applied to a series of commercially available lipase and esterase samples. The assay series was performed either in fully aqueous *PBS* buffer, or in buffer containing 20% (*v*/*v*) DMSO, conditions suitable for reactions of water-insoluble substrates. An error rate of 20–30% had to be assumed for any enzyme-substrate pair due to the rapid manual mixing used to start the reactions. In addition, it should be noted that the activities of the enzyme preparations generally decreased over time when stored as stock solutions.

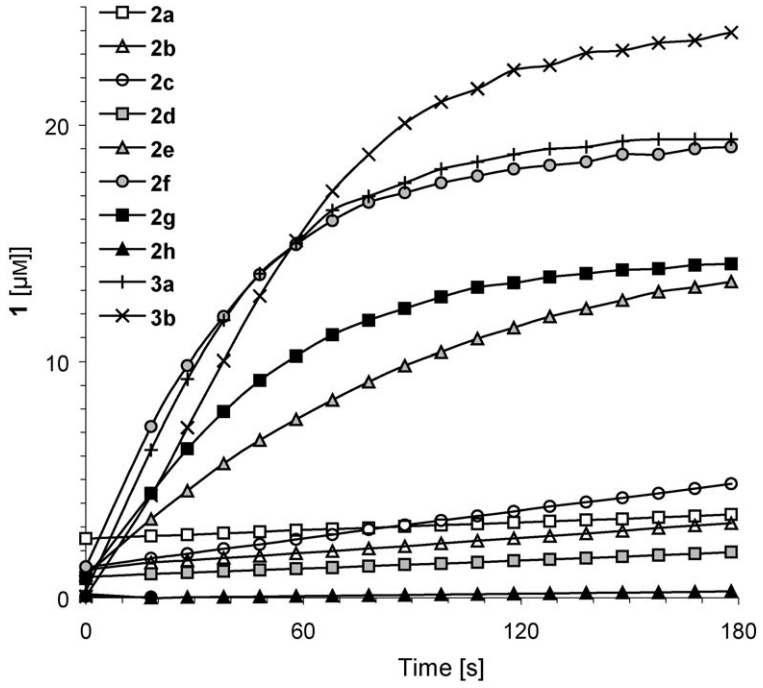


Fig. 2. *Hydrolysis of substrates* **2** *and* **3** *by pig-liver esterase* (PLE). Conditions: substrate, 20 μM; PLE, 5 μg ml^{-1} in *PBS* buffer (pH 7.4); temperature, 30°. For details, see *Exper. Part*.

4. *Data Analysis.* The apparent initial-rate data obtained under the two assay conditions are reported in *Tables 2* and *3*, respectively. The tested enzymes are ordered according to their increasing total reactivity, defined for each enzyme as the sum of all reaction rates observed with the different substrates. The reactivity depends on

Table 2. *Initial Reaction Rates* (in nM s^{-1}) *of the Fluorogenic Substrates* **2** *and* **3** *in Aqueous* PBS *Buffer in the Presence of Different Lipases and Esterases.* Conditions: substrate, 20 μM; enzyme, 50 μg ml^{-1} in aq. *PBS* buffer (pH 7.4); temperature, 25°. Relative error: ±20%. The tested enzymes are ordered according to increasing total reactivity (sum of all observed reaction rates).

Enzyme[a])	**2a**	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**3a**	**3b**
Bckg	0.13	0.17	1.3	0.34	0.58	0.23	0.35	0.06	0.082	0.038
SCE	0.24	0.022	2.1	0.016	1.6	4.1	1.6	1.7	0.015	0.17
WGL	2.1	1.2	0.44	0.28	2	1.6	1.8	0.055	1.7	0.75
CLL	0.23	0.25	0.4	0.12	1.2	1.8	1.8	0.14	1.6	0.98
BTE	0.38	0.49	0.54	0.43	2.1	3.9	4.1	0.31	3.2	2.3
BSE	1.1	1.5	3.6	1.2	2.7	2.7	2.7	0.012	1.7	0.98
PRL	0.37	0.46	0.4	0.31	2.4	2.9	3.3	1.5	2.2	1.6
RML	0.23	0.35	0.78	0.37	1.6	3.9	4	1.1	0.41	0.29
RNL	0.39	0.38	0.59	0.01	2.1	2.9	3.9	0.08	2.7	1.8
HrLE	2.5	1.8	1.5	0.16	12	4.4	4.7	14	4.5	2.7
L9	0.9	1.6	5.7	0.9	37	53	32	13	3.5	3.5
MJL	0.44	0.5	0.76	0.63	2.9	3.1	5.8	0.75	2.5	1.1
TBE	3.1	2.5	0.96	0.28	56	130	98	0.12	1.9	2.3
PLE05	1.2	3.2	14	1.8	59	82	49	0.2	38	10
CLE	1.9	3.9	5.4	1.5	12	26	7.1	0.7	25	8.3
PSBL	1.5	1.6	3.2	2.8	49	36	23	2.1	42	36
ANL	0.66	0.83	1.5	0.71	15	83	58	3.8	2.1	2.2
CAL	0.78	1.3	1.2	0.57	14	40	39	1.1	15	14
HPL	0.66	0.74	0.81	0.3	3.6	7.1	6.1	0.42	530	4.2
MML	0.37	0.21	0.99	0.4	2.9	10	4.7	0.32	1	0.59
CCL	0.69	0.94	2.2	0.38	9.8	76	51	1.1	62	38
L6	5.8	8.1	13	1.5	150	170	140	4.9	8.4	7
AOL	0.68	1	1.2	0.23	68	190	150	47	5.7	4
PFL	22.8	13	26	7.7	84	110	76	55	15	5.6
CVL	1.6	5	3.9	0.8	150	170	140	50	20	12
PLE5	24.2	61	190	51	410	470	180	4	380	220

[a]) Abbreviations: ANL, *Aspergillus niger* lipase (F 62294); AOL, *Aspergillus oryzae* lipase (F 73416); Bckg, background in buffer without enzyme (control); BSE, *Bacillus* sp. esterase (F 46062); BTE, *Bacillus thermoglucosidasius* esterase (F 46054); CAL, *Candida antarctica* lipase (F 62299); CCL, *Candida cylindraceae* lipase (F 62316); CLE, *Candida lipolytica* esterase (F 46056); CLL, *Candida lipolytica* lipase (F 62303); CVL, *Chromobacterium viscosum* lipoprotein lipase (F 62333); HPL, hog-pancreatic lipase (F 62300); HrLe, horse-liver esterase F46069; L6: *Pseudomonas* sp. lipase (*Chirazyme*; *Roche*); L9, *Mucor miehei* lipase (*Chirazyme*; *Roche*); MJL, *Mucor javanicus* lipase (F 62304); MML, *Mucor miehei* lipase (F 62298); PFL, *Pseudomonas fluorescens* lipase (F 62321); PLE, pig-liver esterase (F 46058); PLE05, pig-liver esterase at 0.5 μg ml^{-1}; PLE5, pig-liver esterase at 5 μg ml^{-1}; PRL, *Penicillium roqueforti* lipase (F 62308); PSBL, *Pseudomonas* sp. *B* lipoprotein lipase (F 62336); RML, *Rhizomucor miehei* lipase (F 62291); RNL, *Rhizopus niveus* lipase (F 62310); SCE, *Saccharomyces cerevisiae* esterase (F 46071); TBE, *Thermoanaerobium brockii* esterase (F 46061); WGL, wheat-germ lipase (F 62306).

the particular preference of each enzyme for a given substrate type, but is also critically influenced by the amount of active enzyme per milligram protein, which varies in the commercial samples used.

Table 3. *Initial Reaction Rates* (in nM s^{-1}) *of the Fluorogenic Substrates* **2** *and* **3** *in Aqueous* PBS *Buffer Containing 20% of DMSO as a Co-Solvent in the Presence of Different Lipases and Esterases.* Conditions: substrate, 20 μM; enzyme, 50 μg ml^{-1} in aq. *PBS* buffer (pH 7.4); temperature, 25°. Relative error: ±20%. The tested enzymes are ordered according to increasing total reactivity (sum of all observed reaction rates).

Enzyme[a])	**2a**	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**3a**	**3b**
Bckg	0.2	0.014	0	0	0	0.25	0.35	0.15	0.15	0.1
SCE	0.2	0	0	0	0.076	0.078	0.15	0.0067	0.2	0.21
WGL	4.2	0.95	0.055	0	0.4	1.1	1.3	0.11	1.1	0.69
CLL	0.077	0	0	0	0.17	8.6	4.7	1.1	0.26	0.1
BTE	0.22	0.088	0.04	0	0.41	2.1	2.6	0.45	2.9	1.7
BSE	0.21	0.068	0.0012	0	0.83	2.2	2.3	0.93	2.2	1.7
PRL	0.13	0.011	0	0	1.4	4	5.5	1.6	4.3	4.8
RML	0.16	0.24	0.36	0	3.6	21	30	19	0.55	0.58
RNL	0.71	0.11	0.74	0	5.8	38	25	29	0.92	1.4
HrLE	1.7	0.76	0.64	0.13	9.4	9.1	13	0.78	27	18
L9	0.25	0.07	0.049	0	1.6	34	46	22	0.21	0.31
MJL	0.18	0.2	0.42	0	11	120	60	87	2.6	1.3
TBE	0.036	0	0	0	0.11	2	3.4	1.8	0.22	0.23
PLE05	0.5	0.37	0.72	0.17	3.7	9.7	8.1	0.1	13	9
CLE	0.76	0.91	1.6	0.33	14	45	44	4	65	45
PSBL	0.6	0.76	0.21	0	7	54	81	52	31	25
ANL	0.31	0.34	0.47	0	11	110	160	98	0.91	1.6
CAL	1.3	1.6	1.3	0.098	28	110	130	37	86	37
HPL	0.42	0.34	0.042	0.076	5.1	13	19	12	4.1	1.6
MML	0.67	0.68	0.66	0.017	25	230	250	12	1.4	3.4
CCL	0.79	0.76	0.65	0	3.2	89	145	39	150	63
L6	1.9	1.8	0.9	0.13	14	93	70	55	1.9	2.4
AOL	0.24	0.53	0.35	0	17	58	170	83	1	2
PFL	3.7	3.2	1.2	0.23	20	120	220	170	2	2.9
CVL	1.6	3.1	1.5	0.2	62	200	400	53	20	28
PLE5	6.4	7.4	21	4.7	130	310	200	1.9	320	250

[a]) For abbreviations, see *Table 2* (footnote).

To facilitate analysis, the data set is also represented in a comparative two-color-coded form (*Fig. 3*) [24]. Here, each substrate–enzyme pair is associated with a colored square. The purple-color intensity represents the relative reaction rate in aqueous buffer compared to the fastest reaction rate observed with the enzyme. Similarly, the green-color intensity reports the relative rate observed in the presence of 20% DMSO as a co-solvent. Thus, the purple shades represent substrates reacting faster in H_2O, the green-shaded ones stand for those reacting faster in the presence of DMSO, and the grey scale refers to substrates reacting at equal rates in both solvents. Thus, *Fig. 3* allows a rapid overview of the different enzyme–substrate pairs as a function of reaction conditions.

The ether-functionalized substrates **2e**–**g** showed a consistently strong reactivity towards all but one enzyme. The simple acyl esters **3a**,**b** showed comparable reactivities to their ether analogs **2e**,**f**, but reacted only with half of the enzymes tested. The substrates **2a**–**d** with short acyl chains exhibited only weak enzyme reactivities under aque-

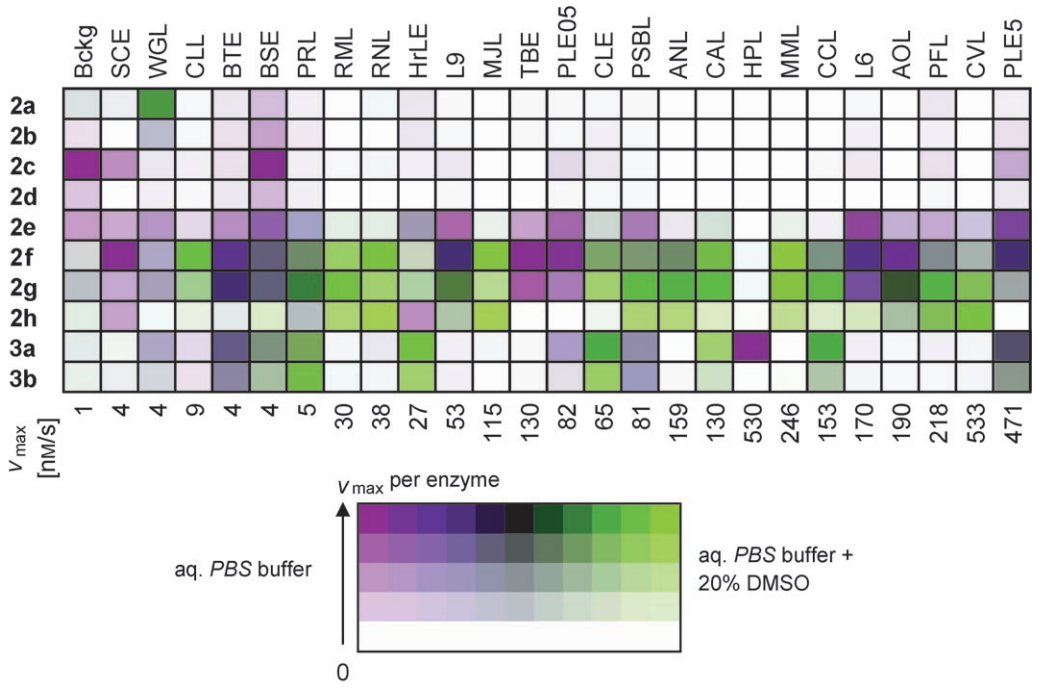


Fig. 3. *Color-coded array of apparent reaction rates* (v) *of the fluorescein derivatives* **2** *and* **3** *in the presence of different lipases and esterases* (visualized data from *Tables 2* and *3*). The value v_{max} below each column indicates the apparent reaction rate for the most-reactive substrate for each enzyme (column). The purple color scale represents the relative reaction rate for the substrate in aqueous *PBS* (*Table 2*) relative to the fastest-reacting substrate for each enzyme. The green color scale refers to reactions in the presence of 20% DMSO (*Table 3*). Grey colors indicate identical reaction rates for a given enzyme–substrate pair in both media; purple means that the reaction is faster in pure H_2O (*PBS* buffer), and green means that the reaction is faster in the binary solvent mixture (H_2O (*PBS*)/DMSO 80:20). For enzyme abbreviations, see *Table 2* (footnote). All enzymes were also tested with the known substrates **7** and **8** under the same conditions, giving apparent rates of <10 nM s^{-1} for **7** with or without DMSO; and <1 nM s^{-1} for **8** in H_2O, and <50 nM s^{-1} in the presence of 20% DMSO.

ous conditions (purple shades in *Fig. 3*), and did not show any reactivity in the presence of DMSO as a co-solvent. It should be noted that these substrates did not show a particularly strong reactivity with esterases, which all preferred the longer-chain substrates. In fact, esterases showed a rather general tendency to react much faster under purely aqueous conditions, including the highly reactive PLE tested at 5 μg ml^{-1} (PLE5) or at 0.5 μg ml^{-1} (PLE05). In contrast, most lipases reacted preferentially in the presence of DMSO (green shades in *Fig. 3*), with several lipases reacting *only* under these conditions. This reactivity difference between lipases and esterases is consistent with the fact that many lipases require interfacial or non-aqueous conditions for full activity due to the presence of a lid covering the hydrophobic active site [2].

Conclusions. – Our experiments have shown that monofunctionalization of fluorescein (**1**) yields fluorogenic substrates suitable for lipase and esterase assays under fully aqueous conditions. The substrates are readily obtained by reaction of **1** with iodomethyl esters or acyl chlorides in DMF. The ether **2f** turned out to be the best fluorescent probe in the series. The structural ester congener **3a**, which is easier to synthesize, also showed good reactivity, but failed to react with about half of the enzymes tested. This highlights the role of the oxymethyl ether function in facilitating enzyme recognition. In general, these substrates react within seconds or minutes with the enzymes, allowing a particularly rapid detection of enzyme activity compared to previously reported fluorogenic substrates for lipases. Several esterases reacted preferentially and, sometimes, only under fully aqueous conditions, but not in the presence of DMSO as a co-solvent. In turn, several lipases were more active (or only active) in the presence of DMSO. Our water-soluble probes thus open up new possibilities in the rapid high-throughput screening of lipases and esterases showing activities in fully aqueous media.

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Experimental Part

General. All reagents were either purchased from *Aldrich* or *Fluka*, or synthesized according to literature procedures. Flash column chromatography (FC) was performed on silica gel *60* (0.040–0.063 mm; *Merck*). DMF and THF were dried, and all solvents were distilled prior to use. Reactions were followed by TLC on *Alugram SIL* UV_{254} silica-gel sheets (*Macherey-Nagel*), with detection under UV light. UV/VIS Spectra were recorded on a *SpectraMax Gemini XS* apparatus; λ_{max} (ε) in nm. NMR Spectra were recorded on a *Bruker AC-300* apparatus at 300 (^{1}H) or 75 MHz (^{13}C). Mass spectra were provided by the mass-spectrometry service of the Department of Chemistry and Biochemistry, University Berne; in *m*/*z*.

Preparation of Chloromethyl Esters. According to a previously reported procedure [22], AcCl (20 g, 268 mmol) was added dropwise to a mixture of paraformaldehyde (8 g, 268 mmol, 1 equiv.) and $ZnCl_2$ (0.73 g, 5.4 mmol, 0.02 equiv.) at 0°, and the mixture was stirred for 1 h at this temp. Then, the cooling bath was removed, and the mixture was stirred for 6 h. Distillation at 75°/1.5 mbar afforded chloromethyl acetate (16.4 g, 150 mmol, 57%) as a colorless liquid. Similarly, propanoyl chloride (26.5 g, 0.289 mol) afforded chloromethyl propanoate (20 g, 58%), isobutyryl chloride (40 g, 0.38 mol) gave chloromethyl isobutyrate (18 g, 35%), cyclopropanoyl chloride (8.6 g, 80 mmol) provided chloromethyl cyclopropanoate (8.2 g, 78%), hexanoyl chloride (9.7 g, 74 mmol) afforded chloromethyl hexanoate (10 g, 83%), octanoyl chloride (9.5 g, 58 mmol) gave chloromethyl octanoate (9.4 g, 84%), decanoyl chloride (18.6 g, 97 mmol) gave chloromethyl decanoate (8 g, 37%), and stearoyl chloride (18.2 g, 60 mmol) provided chloromethyl stearate (14.3 g, 71%).

Preparation of Iodomethyl Esters. Chloromethyl acetate (16.4 g, 150 mmol) was treated with a soln. of NaI (9 g, 150 mmol, 1 equiv.) in MeCN (100 ml). After 12 h of stirring in the dark, the precipitate (NaCl) was filtered off, and the filtrate was concentrated under reduced pressure to yield crude iodomethyl acetate, which was used directly in the next step. The same procedure was used for preparing iodomethyl propanoate, isobutyrate, cyclopropanoate, hexanoate, octanoate, decanoate, and stearate from the corresponding chloromethyl esters.

*2-{6-[(Acetoxy)methoxy]-3-oxo-3*H*-xanthen-9-yl}benzoic Acid* (**2a**). A soln. of fluorescein (**1**) (0.20 g, 0.60 mmol) and iodomethyl acetate (0.36 g, 1.8 mmol) in anh. DMF (4 ml) and anh. THF (6 ml) was treated with NaH (52 mg, 1.2 mmol; 55% suspension in oil) at 0°. After 10 min, the mixture was stirred at r.t. After completion of the reaction (*ca*. 2 h; TLC control), the mixture was poured into aq.

1N HCl (50 ml), and extracted with AcOEt (2×40 ml). The org. phase was washed with brine (2×40 ml), dried (Na_2SO_4), and evaporated. The resulting residue was purified by FC (SiO_2) to afford **2a** (25 mg, 10.3%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.48. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.01 (*d*, *J*=6.6, 1 H); 7.70–7.55 (*m*, 2 H); 7.14 (*d*, *J*=7.4, 1 H); 6.92 (*t*, *J*=1.5, 1 H); 6.80–6.65 (*m*, 3 H); 6.59 (*d*, *J*=8.8, 1 H); 6.51 (*dd*, *J*=8.5, 6.2, 1 H); 5.75 (*s*, 2 H); 2.11 (*s*, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 179.3; 158.9; 158.5; 135.8; 130.5; 130.0; 129.7; 125.7; 124.6; 113.2; 113.0; 104.1; 103.7; 85.5; 21.6. EI-MS: 405 ($[M+H]^+$). HR-EI-MS: 405.0962 ($[M+H]^+$, $C_{23}H_{17}O_7^+$; calc. 405.0974).

*2-(3-Oxo-6-{[(propanoyl)oxy]methoxy}-3*H*-xanthen-9-yl)benzoic Acid* (**2b**). Prepared in analogy to **2a**, but starting from iodomethyl propanoate (0.52 mg, 2.4 mmol). Conditions: 3 h at 0°, purification by FC (SiO_2). Yield: 29 mg (11.5%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.62. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.00 (*dd*, *J*=6.6, 5.5, 1 H); 7.70–7.55 (*m*, 2 H); 7.14 (*d*, *J*=7.4, 1 H); 6.93 (*t*, *J*=1.5, 1 H); 6.80–6.65 (*m*, 3 H); 6.61–6.50 (*m*, 2 H); 5.78 (*s*, 2 H); 2.37 (*dd*, *J*=15.1, 7.7, 2 H); 1.13 (*t*, *J*=7.7, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.1; 170.6; 159.0; 158.7; 153.7; 153.1; 153.0; 135.9; 130.5; 130.0; 127.3; 124.7; 113.9; 113.2; 113.2; 111.6; 104.2; 103.8; 85.6; 28.2; 9.5. EI-MS: 419 ($[M+H]^+$). HR-EI-MS: 419.1131 ($[M+H]^+$, $C_{24}H_{19}O_7^+$; calc. 419.1130).

*2-(6-{[(2-Methylpropanoyl)oxy]methoxy}-3-oxo-3*H*-xanthen-9-yl)benzoic Acid* (**2c**). Prepared in analogy to **2a**, but starting from iodomethyl isobutyrate (0.52 mg, 2.0 mmol). Conditions: 2 h at 0°, 1 h at 25°, purification by FC (SiO_2). Yield: 52 mg (20%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.53. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.03 (*m*, 1 H); 7.70–7.55 (*m*, 2 H); 7.18–7.12 (*m*, 1 H); 6.93 (*t*, *J*=1.5, 1 H); 6.75–6.65 (*m*, 3 H); 6.54 (*dd*, *J*=8.5, 6.3, 2 H); 5.78 (*s*, 2 H); 2.70–2.50 (*m*, 1 H); 1.17 (*d*, *J*=7.0, 6 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 176.8; 159.1; 158.6; 153.7; 153.0; 135.9; 130.5; 130.0; 127.3; 125.8; 124.7; 113.9; 113.2; 113.1; 111.6; 104.3; 103.8; 85.7; 34.7; 19.4. EI-MS: 433 ($[M+H]^+$). HR-EI-MS: 433.1280 ($[M+H]^+$, $C_{25}H_{21}O_7^+$; calc. 433.1287).

*2-(6-{[(Cyclopropylcarbonyl)oxy]methoxy}-3-oxo-3*H*-xanthen-9-yl)benzoic Acid* (**2d**). Prepared in analogy to **2a**, but starting from iodomethyl cyclopropanecarboxylate (0.41 g, 1.8 mmol). Conditions: 4 h at 0°, purification by FC (SiO_2). Yield: 73 mg (26%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.54. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.01 (*dd*, *J*=6 .3, 1.1, 1 H); 7.72–7.55 (*m*, 2 H); 7.15 (*d*, *J*=7.4, 1 H); 6.95 (*t*, *J*=1.5, 1 H); 6.71 (*dd*, *J*=11.8, 1.5, 3 H); 6.58 (*d*, *J*=8.8, 1 H); 6.51 (*dd*, *J*=8.8, 2.2, 1 H); 5.77 (*s*, 2 H); 1.70–1.60 (*m*, 1 H); 1.15–1.05 (*m*, 2 H); 1.0–0.85 (*m*, 2 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.7; 170.6; 159.1; 158.7; 153.8; 153.1; 153.1; 135.9; 130.5; 130.0; 130.0; 127.3; 125.8; 124.7; 113.9; 113.2; 111.6; 104.3; 103.9; 85.6; 13.6; 10.0. EI-MS: 431 ($[M+H]^+$). HR-EI-MS: 431.1122 ($[M+H]^+$, $C_{25}H_{19}O_7^+$; calc. 431.1130).

*2-(6-{[(Hexanoyl)oxy]methoxy}-3-oxo-3*H*-xanthen-9-yl)benzoic Acid* (**2e**). Prepared in analogy to **2a**, but starting from iodomethyl hexanoate (0.62 g, 2.4 mmol). Conditions: 22 h at 0°, purification by FC (SiO_2). Yield: 25 mg (9.0%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.64. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.00 (*t*, *J*=7.0, 1 H); 7.70–7.55 (*m*, 2 H); 7.14 (*d*, *J*=7.4, 1 H); 6.92 (*t*, *J*=1.5, 1 H); 6.70 (*t*, *J*=13.3, 3 H); 6.57 (*d*, *J*=9.0, 1 H); 6.51 (*dd*, *J*=8.8, 2.6, 1 H); 5.77 (*s*, 2 H); 2.32 (*dd*, *J*=14.3, 7.0, 2 H); 1.60 (*t*, *J*=7.0, 2 H); 1.40–1.10 (*m*, 4 H); 0.90–0.70 (*m*, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.6; 170.7; 159.0; 158.9; 153.8; 153.1; 153.1; 135.9; 130.6; 130.1; 130.0; 127.4; 125.8; 124.7; 113.9; 113.2; 111.5; 104.2; 103.9; 85.5; 34.8; 31.9; 25.1; 23.0; 14.6. FAB-MS: 461 ($[M+H]^+$). HR-FAB-MS: 461.1596 ($[M+H]^+$). $C_{27}H_{25}O_7^+$; calc. 461.1600).

*2-(6-{[(Octanoyl)oxy]methoxy}-3-oxo-3*H*-xanthen-9-yl)benzoic Acid* (**2f**). Prepared in analogy to **2a**, but starting from iodomethyl octanoate (0.51 g, 1.8 mmol). Conditions: 22 h at 0°, then 4 h at 25°, purification by FC (SiO_2). Yield: 58 mg (20%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.64. UV (MeOH): 222 (149000), 277 (6500). ^{1}H-NMR (300 MHz, $CDCl_3$): 8.05–7.96 (*m*, 1 H); 7.75–7.55 (*m*, 2 H); 7.15 (*d*, *J*=5.5, 1 H); 6.93 (*t*, *J*=1.5, 1 H); 6.80–6.70 (*m*, 3 H); 6.58 (*d*, *J*=8.4, 1 H); 6.52 (*dd*, *J*=8.8, 2.6, 1 H); 5.78 (*s*, 2 H); 2.35 (*t*, *J*=7.7, 2 H); 1.60 (*dd*, *J*=14.7, 7.4, 2 H); 1.35–1.15 (*m*, 8 H); 0.82 (*dd*, *J*=10.7, 7.0, 2 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.5; 159.0; 158.6; 153.7; 153.0; 135.9; 130.5; 130.0; 130.0; 127.3; 125.8; 124.6; 113.8; 113.1; 113.1; 111.6; 104.2; 103.8; 85.4; 34.8; 32.2; 29.6; 29.5; 25.3; 23.2; 14.7. EI-MS: 488 (M^+). HR-EI-MS: 488.1810 (M^+, $C_{29}H_{28}O_7^+$; calc. 488.1835).

*2-(6-{[(Decanoyl)oxy]methoxy}-3-oxo-3*H*-xanthen-9-yl)benzoic Acid* (**2g**). Prepared in analogy to **2a**, but starting from iodomethyl decanoate (0.77 g, 2.4 mmol). Conditions: 9 h at 0°, then 9 h at 25°, purification by FC (SiO_2). Yield: 60 mg (19%). Yellow solid. TLC (hexane/AcOEt 2:1): R_f 0.45. UV

(MeOH): 222 (149000), 277 (6700). ^{1}H-NMR (300 MHz, $CDCl_3$): 8.05–7.95 (*m*, 1 H); 7.70–7.55 (*m*, 2 H); 7.20–7.10 (*m*, 1 H); 6.93 (*t*, *J* = 1.5, 1 H); 6.80–6.70 (*m*, 3 H); 6.60 (*d*, *J* = 8.8, 1 H); 6.51 (*dd*, *J* = 8.8, 2.6, 1 H); 5.78 (*s*, 2 H); 2.35 (*t*, *J* = 7.7, 2 H); 1.60 (*t*, *J* = 7.4, 2 H); 1.35–1.18 (*m*, 12 H); 0.83 (*t*, *J* = 7.0, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.4; 170.4; 169.3; 158.9; 158.3; 153.6; 153.0; 152.9; 135.7; 130.4; 130.0; 129.9; 129.6; 127.2; 127.0; 125.7; 124.5; 113.8; 113.1; 112.9; 111.7; 110.9; 104.1; 103.7; 85.4; 34.8; 32.4; 30.1; 29.8; 29.8; 29.6; 25.3; 23.3; 14.7. EI-MS: 517 ($[M+H]^+$). HR-EI-MS: 517.2210 ($[M+H]^+$, $C_{31}H_{33}O_7^+$; calc. 517.2226).

*2-(3-Oxo-6-{[(stearoyl)oxy]methoxy}-3*H*-xanthen-9-yl)benzoic Acid* (**2h**). Prepared in analogy to **2a**, but starting from iodomethyl stearate (1.0 g, 2.4 mmol). Conditions: 12 h at 25°, purification by FC (SiO_2). Yield: 41 mg (11%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.76. UV (MeOH): 222 (145000), 277 (4400). ^{1}H-NMR (300 MHz, $CDCl_3$): 8.01 (*d*, *J* = 7.4, 1 H); 7.70–7.60 (*m*, 2 H); 7.14 (*d*, *J* = 7.4, 1 H); 6.93 (*s*, 1 H); 6.71 (*dd*, *J* = 11.8, 1.5, 3 H); 6.59 (*d*, *J* = 8.5, 1 H); 6.51 (*dd*, *J* = 8.8, 2.2, 1 H); 5.78 (*s*, 2 H); 2.45–2.27 (*m*, 2 H); 1.65–1.55 (*m*, 2 H); 1.35–1.18 (*m*, 28 H); 0.85 (*t*, *J* = 6.6, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.4; 159.0; 158.6; 153.8; 153.1; 153.0; 135.8; 130.5; 130.0; 127.4; 125.8; 124.6; 113.9; 113.2; 113.1; 104.2; 103.8; 85.5; 34.8; 32.6; 30.4; 30.4; 30.3; 30.3; 30.3; 30.1; 30.0; 29.9; 29.7; 25.3; 23.4; 14.8. FAB-MS: 629 (M^+). HR-FAB-MS: 629.3481 (M^+, $C_{39}H_4O_7^+$; calc. 629.3478).

*2-{6-[(Octanoyl)oxy]-3-oxo-3*H*-xanthen-9-yl}benzoic Acid* (**3a**). Octanoyl chloride (98 mg, 0.60 mmol) was added dropwise to a mixture of **1** (0.20 g, 0.60 mmol) and $ZnCl_2$ (20 mg, 0.15 mmol) in anh. DMF (10 ml). After 12 h at 25°, the soln. was poured into AcOEt (70 ml), washed with brine (3 × 40 ml), dried (Na_2SO_4), and evaporated. The residue was purified by FC (SiO_2). Yield: 146 mg (53%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.65. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.05–7.95 (*m*, 1 H); 7.70–7.50 (*m*, 2 H); 7.10–7.02 (*m*, 2 H); 6.80–6.70 (*m*, 2 H); 6.66 (*d*, *J* = 2.6, 1 H); 6.58 (*d*, *J* = 8.5, 1 H); 6.49 (*dd*, *J* = 8.5, 2.6, 1 H); 6.13 (*s*, 1 H); 2.55 (*t*, *J* = 7.4, 2 H); 1.80–1.65 (*m*, 2 H); 1.40–1.10 (*m*, 8 H); 0.86 (*t*, *J* = 7.0, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.0; 170.5; 158.6; 153.7; 152.8; 152.6; 152.5; 135.9; 130.5; 129.9; 129.8; 127.0; 125.7; 124.7; 118.0; 117.2; 113.2; 111.3; 111.1; 103.7; 35.0; 32.3; 29.7; 29.5; 25.5; 23.2; 14.7. ESI-MS: 459 ($[M+H]^+$). HR-ESI-MS: 458.1713 (M^+, $C_{28}H_{26}O_6^+$; 458.1729).

*2-{6-[(Decanoyl)oxy]-3-oxo-3*H*-xanthen-9-yl}benzoic Acid* (**3b**). Prepared in analogy to **3a**, but from decanoyl chloride (115 mg, 0.6 mmol). Conditions: 5 h at 25°, purification by FC (SiO_2). Yield: 185 mg (64%). Yellow solid. TLC (hexane/AcOEt 1:1): R_f 0.71. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.00 (*d*, *J* = 6.6, 1 H); 7.70–7.55 (*m*, 2 H); 7.12–7.02 (*m*, 2 H); 6.80–6.70 (*m*, 2 H); 6.65 (*d*, *J* = 2.2, 1 H); 6.57 (*d*, *J* = 8.8, 1 H); 6.48 (*dd*, *J* = 8.8, 2.6, 1 H); 6.29 (*s*, 1 H); 2.55 (*t*, *J* = 7.7, 2 H); 1.80–1.60 (*m*, 2 H); 1.45–1.25 (*m*, 12 H); 0.86 (*t*, *J* = 6.6, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 172.9; 158.6; 153.7; 152.8; 152.6; 152.5; 135.9; 130.5; 129.8; 129.7; 127.0; 125.7; 124.7; 118.0; 117.1; 113.2; 111.3; 111.0; 103.7; 35.0; 32.5; 30.0; 29.8; 29.8; 29.7; 25.5; 23.3; 14.7. ESI-MS: 487 ($[M+H]^+$). HR-ESI-MS: 486.2043 (M^+, $C_{30}H_{30}O_6^+$; calc. 486.2042).

Fluorescence Assays. All enzymes tested were purchased from *Fluka*. All substrates and products were prepared as 4-mM stock solns. in MeCN/H_2O 1:1, and diluted to a concentration of 0.4 mM. All reagents and buffers were prepared in de-ionized H_2O (*milliQ*). Enzymes stock solns. were prepared at 1 mg/ml in phosphate-buffered saline (*PBS*) made from 160 mM NaCl and 10 mM phosphate buffer (pH 7.4), or diluted to 0.1 or 0.01 mg/ml from with *PBS* buffer. BSA stock soln. (40 mg/ml) was prepared in *PBS* buffer. Assays were carried out in round-bottom polypropylene 96-well plates (*Costar*), and monitored with a *Cytofluor II* fluorescence plate reader (*PerSeptive Biosystems*); filters: $\lambda_{ex} = 450 \pm 25$ nm, $\lambda_{em} = 530 \pm 25$ nm.

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