A Fluorescence-Based Assay for Baeyer–Villiger Monooxygenases, Hydroxylases and Lactonases

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Abstract: Alkylation of umbelliferone and nitrophenol with chloroacetone, 3-chlorobutanone, 2-chlorocyclopentanone and 2-chlorocyclohexanone gave the corresponding 2-coumaryloxy and 2-nitrophenoxy ketones. The 2-coumaryloxy ketones were used as fluorogenic substrates to detect Baeyer–Villiger monooxygenases activities of microbial cultures in high-throughput using microtiter plates. The 2-coumaryloxy ketones were oxidized by microorganisms producing Baeyer–Villiger monooxygenases (BVMO), releasing umbelliferone as a fluorescent signal. The substrates were also biotransformed by a microbial monooxygenase (Trichosporon cutaneum). Chemical Baeyer–Villiger oxidation of 2-coumaryloxy ketones using meta-chloroperbenzoic acid proceeded regioselectively to the corresponding acyloxyalkyl derivatives of umbelliferone and nitrophenol. These chiral lactones underwent a fluorogenic and chromogenic reaction upon hydrolysis by esterases, in particular pig liver esterase. Enantioselectivity of the ester hydrolysis reaction was determined by chiral-phase analysis of the unreacted lactones.

Keywords: biotransformations; enzyme catalysis; fluorescent probes; lactones; oxidation

Introduction

Most enzymes of industrial interest are currently isolated from microbial sources or from genetic libraries.[1] In the search for novel enzymes, it is necessary to apply functional tests for high-throughput screening that are capable of detecting enzyme activities with high selectivity and sensitivity.[2] Recently we developed enzyme substrates that release either umbelliferone as a blue-fluorescent product or nitrophenol as a yellow colored product by β-elimination from an intermediate carbonyl product formed after the enzyme-catalyzed step. This assay methodology detects various enzyme types with high selectivity, including alcohol dehydrogenases,[3] aldolase catalytic antibodies,[4] acylases and phosphatases,[5] proteases,[6] lipases and esterases,[3,5,7] epoxide hydrolases,[5,8] and transaldolases.[9] These assays may also be applied in parallel using arrays of structurally diverse substrates to produce enzyme-specific activity fingerprints.[10,11] The same fluorescence release principle has also been used by other laboratories in fluorescence assays for transketolases[12] and Baeyer–Villiger monooxygenases.[13] In the latter case Baeyer–Villiger monooxygenase activity was detected with umbelliferone 4-oxopentyl ether and an auxiliary enzyme, an alcohol dehydrogenase.

In all of these assays a sequence of secondary reaction steps converts the primary product of the enzyme-catalyzed reaction to a fluorescent product. While this feature provides highly selective assays for reactions that often cannot be rendered fluorogenic otherwise, it also complicates the reaction set-up and the kinetic analysis. Recently we reported that acyloxymethyl ethers of umbelliferone such as **3**, which we originally used to discover catalytic antibodies by screening cell culture supernatants,[14] are useful fluorogenic probes for lipases and esterases (Scheme 1).[15] In this case the primary hydroxymethyl ether product is very unstable and immediately releases umbelliferone **4** without any kinetic delay or auxiliary reagent.

Herein we report the preparation and evaluation of cyclic and acyclic 2-coumaryloxy-ketones **1** and **5**–**7** as fluorogenic substrates for detecting Baeyer–Villiger monooxygenase activities using the same principle (Scheme 1). The Baeyer–Villiger oxidation, the oxidative transformation of a ketone to the corresponding ester or lactone, is a useful transformation in organic synthesis. An asymmetric version of this reaction is possible applying biocatalytic methods with BVMO allowing the

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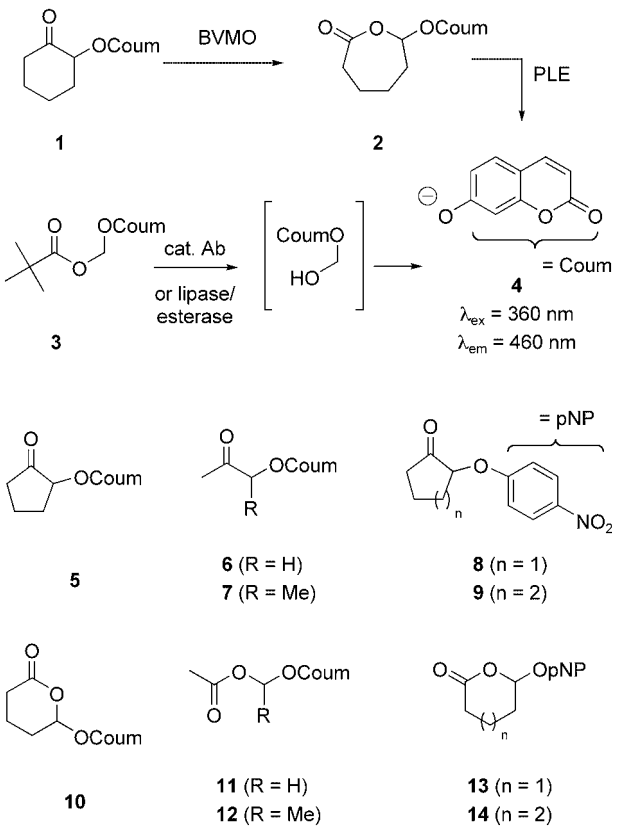


Scheme 1. Substrates and principle of BVMO and esterase fluorescence assay.

preparation of chiral lactones from achiral ketones.[16,17] Up to now only two methods have been reported to assay BVMO in high-throughput,[13,18] which is a prerequisite for microbial screening or directed evolution efforts in this area. The use of our fluorogenic BV-substrates, e.g., **1**, is demonstrated by the direct detection of BV-activity in microbial cell cultures. In addition, the intermediate lactones, e.g., **2**, provide chiral fluorogenic and chromogenic probes for esterases.

Results and Discussion

BV-Assay Design

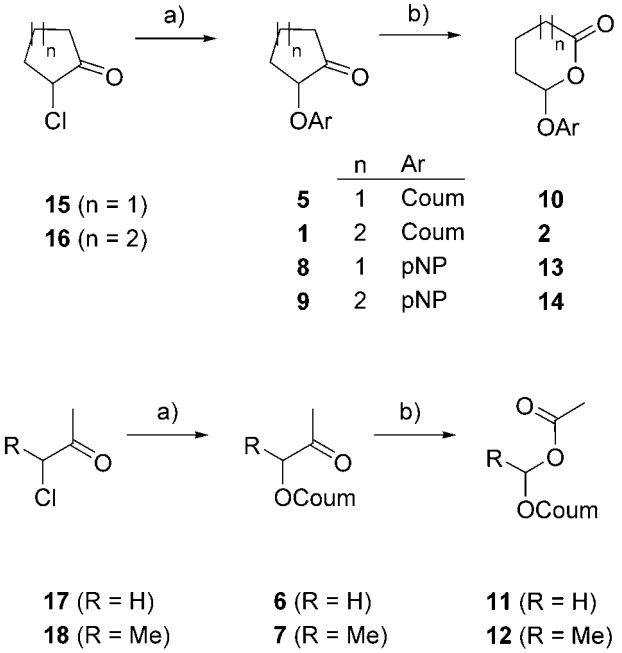
BVMO are mostly membrane-bound enzymes that require a cofactor and a cofactor regeneration system. As a consequence these enzymes are assayed and used in whole cell system. A Baeyer–Villiger oxidation reaction would therefore become fluorogenic if the primary oxidation product could release a fluorescent product under whole-cell reaction conditions. We envisioned that 2-coumaryloxy ketones such as **1** should be preferentially oxidized to the acyloxymethyl ether of umbelliferone **2** due to the stronger group migration ability of the secondary ether carbon center (Scheme 1). Based on our previous experiments with such esters,[15] this product would be relatively unstable under whole-cell conditions and would readily hydrolyze to form umbelliferone **4** as a fluorescent product. This scheme should be applicable for a variety of 2-coumaryloxy ketones featuring aliphatic substituents around the reacting carbonyl groups, which are usually the reactive substrate class for BVMO.

Substrate Synthesis

The synthesis of the target ketones was accomplished in one step by alkylation of 2-chloro ketones **15**–**18** with either umbelliferone or *p*-nitrophenol as sodium salts in DMF (Scheme 2). BV oxidation of the ketones with *m*-CPBA proceeded regioselectively to afford the corresponding ester and lactones as reference products. An attempted synthesis of 1,3-bis-umbelliferyloxy-2-propanone from 1,3-dichloro-2-propanone did not give any product. Considering that the lactones were worthy of investigation as chiral probes for esterases and lipases, the nitrophenol-derived lactones **13** and **14** were also prepared by the same synthetic route.

BV-Detection in Microbial Culture

The BV enzymatic activity was investigated in a series of 15 microbial strains, some of which were known to con-



Scheme 2. Synthesis of BVMO and esterase substrates. Conditions: a) umbelliferone or *p*-nitrophenol, NaI, K_2CO_3, acetone, 25 °C, 5 h (70–97%); b) *m*-CPBA, $NaHCO_3$, CH_2Cl_2, 0 °C to 25 °C, 15 h (70–87%).

tain a monooxygenase activity.[19] The pure cultures were acquired from the CCT collection (CCT – Coleção de Culturas Tropical, Fundação Tropical de Pesquisas André Tosello) and tested for a fluorogenic reaction using ketones **1** and **5**–**7** as fluorescent probes in a microtiter-plate set-up. A relatively slow but steady fluorogenic reaction was observed upon continuous monitoring of the reaction at 30 °C over a 24-h incubation time in half of the 60 microorganism/substrate combinations tested (Figure 1, Table 1). When the fluorescence did not develop, revealing an absence of BVMO, a second set of experiments was performed incubating the experiment for 24 h, after which BSA was added and the fluorescence was measured for 24 h. The preincubation period was introduced to detect the inducible BVMO which was present in 5 microorganisms (Table 1, entries 6, 9, 11, 12 and 14).

The fluorescence assays were compared with GC/MS monitoring of the BVMO activity on 3-hexyl-cyclobutanone **19** and 4-methyl-cyclohexanone **20** (Scheme 3).[19] A Baeyer–Villiger oxidation of **19** to **19a** (Scheme 3 , Table 2) was observed with *Cunnighamela echinulata* CCT 4259, *Curvularia lunata* CCT 5629, *Curvularia eragrostidis* CCT 5634, *Curvularia pallescence* CCT 5654 and *Aspergillus niger* CCT 5559, in agreement with the fluorescence assay (Table 1). However 4-methylcyclohexanone **20** was converted to **20a** (Scheme 3, Table 2) by *Aspergillus oryzae* CCT 0975 *and Geotrichum candidum* CCT 1205, but these microorganisms did not convert our fluorogenic substrates (*A. oryzae*) or convert very little (*G. candidum*). This might be assigned to: 1) unfavorable reaction conditions in the microtiter plate format (24 h standing *vs.* 72–90 h in shaker in the classical BVMO assay); 2) an oxidation with migration of the less substituted carbon leading to the formation of the chemically disfavored regioisomer, which is not fluorogenic; 3) the particular substrate selectivity of the BVMO produced by these microorganisms; 4) a competing reduction of the ketone **1**, **5**–**7** to the corresponding alcohols taking place preferentially to the BV oxidation. Reduction of ketone **6** to the alcohol was indeed observed by HPLC/UV in the microbial reaction with *Emericela nidulans* CCT 3119 and *C. eragrostidis* CCT 5634 (data no shown). In the course of these microbial reactions **19** and **20** were also in part reduced to secondary alcohols, **19b** and **20b** (Scheme 3, Table 2). The alcohols were the only products formed with microbial culture of *Aspergillus niger* CCT 4648 and *Aspergillus terreus* CCT 3320,[19] for which also no fluorescence reaction was observed with probes **1** and **5**–**7**.

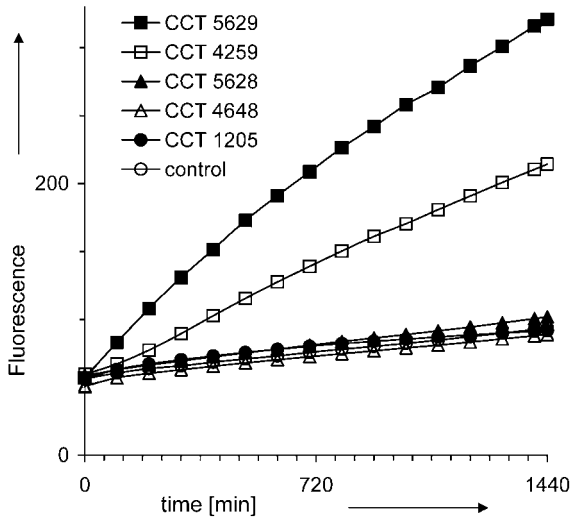


Figure 1. Whole-cell fluorescence screening of monooxygenase activity with 2-couramyloxycyclopentanone **5**. Conditions: 100 μM **5**, 0.5 mg mL^{-1} cells, 2 mg mL^{-1} BSA, 20 mM aqueous borate buffer pH 8.8, 30 °C, λ_{ex} = 365 nm, λ_{em} = 460 nm. See also data in Table 1.

Table 1. Fluorescence microtiter-plate assay in whole cells using 2-coumaryloxy ketones **1** and **5**–**7** as substrates.[a]

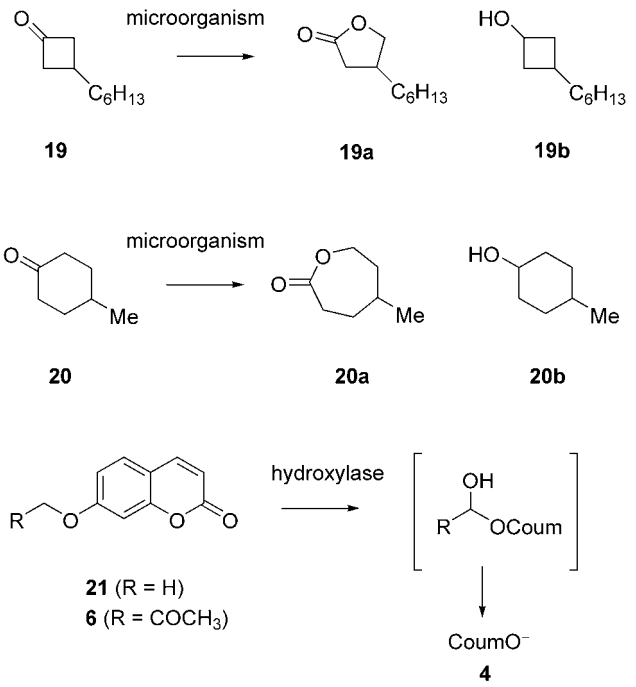
Entry	Microorganisms	**1**	**5**	**6**	**7**
1	*Aspergillus niger* CCT 4648	–	–	–	–
2	*Aspergillus niger* CCT 5559	13	40	–	–
3	*Aspergillus oryzae* CCT 0975	–	–	–	–
4	*Aspergillus terreus* CCT 3320	–	–	–	–
5	*Cunnighamela echinulata* CCT 4259	13	120	90	70
6	*Curvularia eragrostidis* CCT 5634[b]	3	110	16	–
7	*Curvularia lunata* CCT 5628	–	–	–	–
8	*Curvularia lunata* CCT 5629	7	230	–	–
9	*Curvularia pallescens* CCT 5654[b]	4	110	9	–
10	*Geotrichum candidum* CCT 1205	5	–	–	–
11	Fungi CCT 5560[b]	17	29	–	–
12	Fungi CCT 5632[b]	30	–	10	–
13	Fungi CCT 5661	5	260	–	12
14	*Rhizopus oryzae* CCT 4964[b]	7	65	35	27
15	*Trichosporon cutaneum* CCT 1903	16	230	350	290

[a] The fluorescence intensity difference (λ_{ex} = 365 nm, λ_{em} = 460 nm) between each microorganism reaction and the reference (substrate + buffer + BSA) after 24 h incubation is given in arbitrary units. There was no detectable fluorescence of the microorganism cultures. Conditions: 100 μM substrate (diluted from a 2 mM stock solution in 1 : 1 acetonitrile/water), 0.5 mg mL^{-1} of microbial cells, 20 mM borate pH 8.8, 2 mg mL^{-1} BSA, 30 °C. The above listed results are the mean values of duplicate and triplicate reactions.

[b] With preincubation with substrate at 28 °C for 24 h.

Monooxygenase Activity

The strongest fluorescence signal with ketones **1** and **5**–**7** was produced by *Trichosporon cutaneum* CCT 1903, which was consistent with previous observations.[20] Monitoring of the reaction over 24 h by HPLC/UV indicated the direct formation of umbelliferone from these substrates without any detectable intermediates. Al-



Scheme 3. Substrates used in detecting monooxygenases activity.

Table 2. Biocatalytic products using 3-hexylcyclobutanone **19** and 4-methylcyclohexanone **20**.[a]

Microorganisms	19a	19b	20a	20b
Aspergillus niger CCT 4648	–	–	–	25
Aspergillus niger CCT 5559	80	–	1	23
Aspergillus oryzae CCT 0975	–	–	54	23
Aspergillus terreus CCT 3320	–	–	–	93
Cunnighamela echinulata CCT 4259	80	–	–	81
Curvularia eragrostidis CCT 5634	20	10	2	75
Curvularia lunata CCT 5628	44	26	–	51
Curvularia lunata CCT 5629	80	–	3	94
Curvularia pallescens CCT 5654	54	–	1	97
Geotrichum candidum CCT 1205	–	–	100	–

[a] Biocatalytic reactions: ketones **19–20** (approximately 20 mg each) and wet fungal biomass (2.0 g) were mixed in Erlenmeyer flasks (125 mL) containing 30 mL of buffer phosphate solution pH 7.0. The mixture was stirred on a rotary shaker (28 °C, 140 rpm) and monitored by GC/MS (Ref.[19]).

though the intermediate lactones of the putative Baeyer–Villiger reaction might be too unstable in the medium to be detectable, the presence of a possible BVMO was not be confirmed by traditional biocatalytic reactions with several ketones. In most bioreactions the ketones were reduced.

The key to this apparent enigma came from a biocatalytic experiment in which *T. cutaneum* was responsible for the epoxidation of the *cis*-jasmone double bond indicating the presence of monooxygenases.[19] In nature these enzymes are responsible for double bond epoxidation and hydroxylation of activated methine or methylene groups similar to those present in the florescent probes **1** and **5**–**7**. The presence of a monooxygenase with hydroxylating properties in *T. cutaneum* was established by converting camphor to 6-hydroxycamphor which was confirmed by GC/MS and NMR spectroscopy.[21] The fluorogenic reaction observed with *T. cutaneum* and ketones **1** and **5**–**7** is therefore best ascribed to a direct hydroxylation of the ether methylene group to form an unstable hemiacetal which then gives umbelliferone **4** (Scheme 3). The hydroxylation of the fluorogenic substrates such as the methyl ether of umbelliferone **21** is an expected hydroxylase biocatalytic reaction at an activated methylene carbon.

Esterase Assay with Fluorogenic and Chromogenic Lactone Substrates

The use of lactones **2**, **10**, **13**, **14** as spectrophotometric probes for lipases and esterase was investigated next. A spectrophotometric lactonase assay has been described based on 3,4-dihydrocoumarin hydrolysis (UV 270 nm),[22] but no fluorogenic or chromogenic lactone substrates have been described thus far. The lactones were conditioned as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and assayed for their reactivity in aqueous buffer in the presence of 10% v/v DMSO as cosolvent. The spontaneous hydrolysis with these lactone substrates was much higher than previously observed with the corresponding acyclic acyloxymethyl ethers. The seven-membered ring lactones were significantly more stable than the six-membered rings, whereas the nitrophenol derived lactone **13** was particularly unstable.

Assays were conducted under neutral conditions in phosphate buffer saline pH 7.4 to keep the background hydrolysis at a low level. Under these conditions, a positive catalysis signal was obtained in the presence of several lipases and esterases, with pig liver esterase samples showing the strongest reactivity (Figure 2, Table 3) which is in agreement of Rousseau's study.[23] The seven-membered ring umbelliferone-derived lactone **2** gave a useful signal with all enzymes, as indicated by the value of V_{rel}, which measures the apparent signal over background. This high specific reactivity was due to the very low background hydrolysis with this substrate compared to the other lactone substrates tested.

Enantioselectivity in the pig liver esterase-catalyzed reaction was investigated by analyzing the reaction by chiral phase HPLC, taking advantage of the strong aromatic chromophore for detection. An excellent chiral separation was obtained for each of the four lactones on a Chiralcel-OD-H (Figure 3). Application of the kinetic resolution equation of Sih to product formation al-

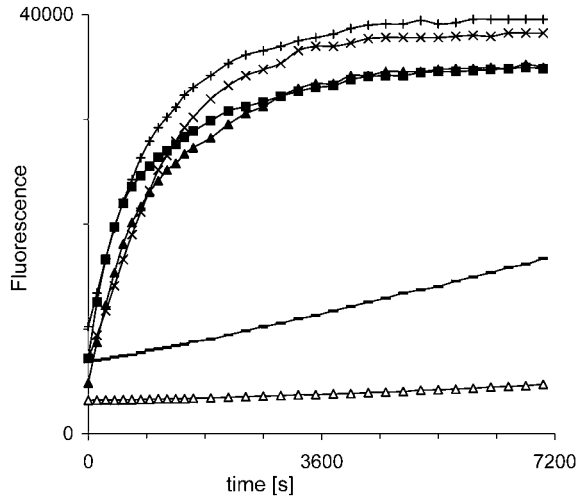


Figure 2. Esterase fluorescence assay with fluorogenic lactones **2** and **10**. (△) 100 μM **2** in aqueous PBS, 10% v/v DMSO, 35 °C; (▲) 100 μM **2** with 10 μg mL^{-1} chirazyme E1; (■) 100 μM **2** with 10 μg mL^{-1} chirazyme E2; (–) 100 μM **10** in aqueous PBS, 10% v/v DMSO, 35 °C; (+) 100 μM **10** with 10 μg mL^{-1} chirazyme E1; (x) 100 μM **10** with 10 μg mL^{-1} chirazyme E2.

lowed the determination of enantioselectivity E in each case.[24] Both samples of pig liver esterases gave enantioselectivities in the range between 2.3 > E > 6.6 (Table 4).

Conclusion

2-Coumaryloxy ketones **1** and **5**–**7** were prepared in one step from the corresponding commercially available 2-chloro ketones. These easily accessible substrates serve as useful fluorogenic probes for monooxygenases under direct culture conditions. The probes were validated against cultures of 15 microorganisms and the results were confirmed by GC/MS assays with 3-hexylcyclobutanone **19** and 4-methylcyclohexanone **20** as substrates. Our results indicate the above mentioned probes **1** and **5**–**7** are excellent for screening monooxygenases, either hydroxylases or BVMO. Some fungi (entries 11–14, Table 1) isolated in our laboratory[25] display interesting monooxygenase activities and will be further investigated. The lactones **2** and **10** produced by Baeyer–Villiger oxidation of **1** and **5**, respectively, and the corresponding nitrophenoxy-substituted lactones **13** and **14** serve as spectroscopic chiral probes for esterases, with a particularly strong reactivity with pig liver esterase samples. The seven-membered ring coumarin-derived lactone **2** is particularly useful due to a very low background hydrolysis. Enantioselectivity can be estimated by chiral-phase HPLC of the unreacted substrates.

Experimental Section

Reagents were purchased from Fluka, Aldrich, Sigma or Acros. Solvents were distilled from technical solvents. All reactions were followed by TLC on Alugram SIL G/UV$_{254}$ silica

Table 3. Rates of hydrolysis of lactone substrates with different enzymes.

Name of enzymes	V_{obs}, nM s^{-1} (V_{rel})			
	10	**2**	**13**	**14**
No enzyme	1.9	0.1	8.0	0.9
Pseudomonas sp. Lipase[a]	7.5	1.6 (15)	13	2.4
Pig liver esterase 1[b]	35 (17)	53 (530)	29	40 (44)
Candida antarctica lipase[c]	7.1	1.6 (15)	11	5.4 (5)
Thermomyces lanuginosa lipase[d]	7.3	1.9 (18)	8.8	1.8
Pig pancreas lipase[e]	6.2	1.5 (14)	11	1.9
Pig liver esterase 2[f]	35 (17)	51 (510)	32	49 (54)
Mucor miehei lipase[g]	7.9	1.9 (18)	13	2.0
Mucor miehei esterase[h]	6.9	1.7 (16)	12	2.0
Saccharomyces cerevisiae esterase[i)]	7.2	1.6 (16)	12	1.8

Conditions: 100 μM substrate in aqueous PBS pH 7.4, 10% v/v DMSO, 35 °C, 10 μg mL^{-1} enzyme. $V_{rel} = (V_{obs}/V_{noE}) - 1$ is indicated in brackets when $V_{rel} > 5$.
Enzyme sources:
[a] Roche Chirazyme L6.
[b] Chirazyme E1.
[c] Chirazyme L5.
[d] Chirazyme L8.
[e] Chirazyme L7.
[f] Chirazyme E2.
[g] Chirazyme L9.
[h] Fluka F46059.
[i] Fluka F46069.

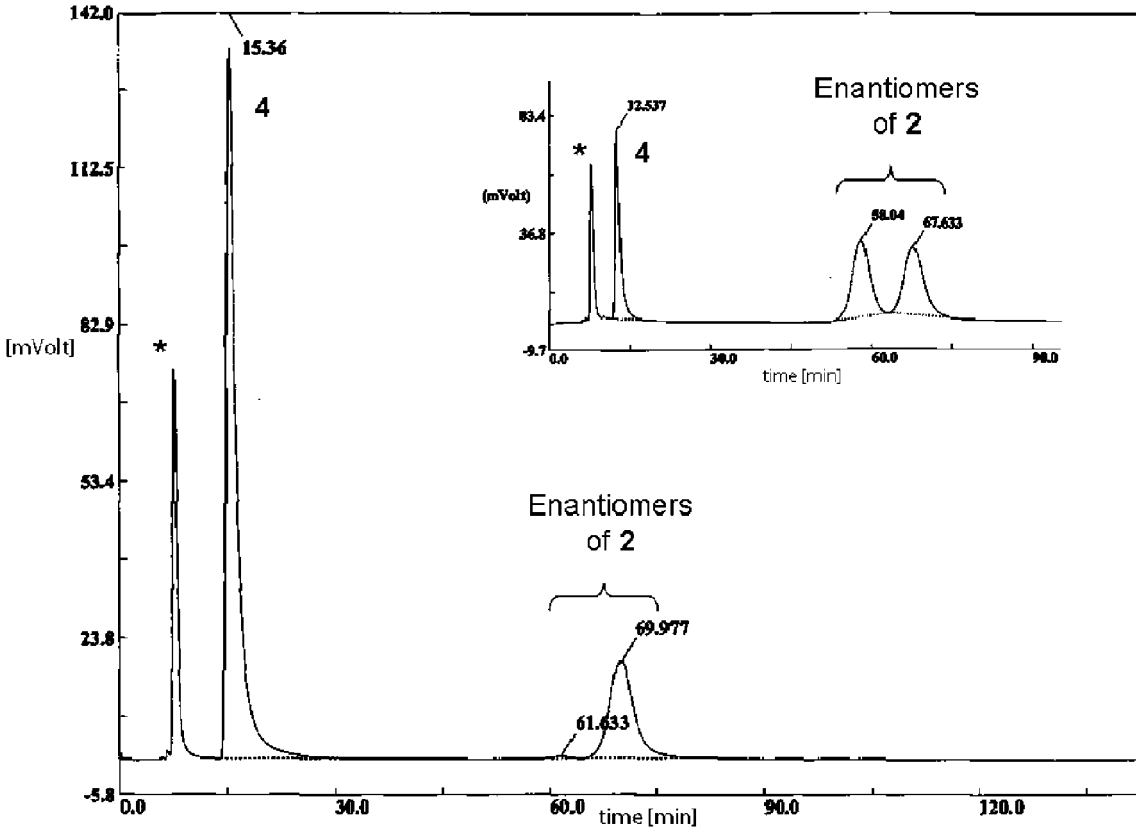


Figure 3. Chiral-phase HPLC analysis of the reaction of lactone **2** with pig liver esterase (chirazyme E1). Main plot: HPLC-trace after 25 min and 75% of conversion. reaction. Insert: control without enzyme after 25 min. Conditions: Conditions: 100 μM substrate in aqueous PBS pH 7.4, 10% v/v DMSO, 10 μg mL^{-1} enzyme, 25 °C. HPLC analysis was performed after extraction with AcOEt using a Chiracell OD-H 0.4 × 22 cm column, flow rate 0.5 mL min^{-1} 2-propanol/hexane 15/85 (* solvent peak).

Table 4. Enantioselectivities of pig liver esterase samples determined by chiral-phase HPLC analysis of the fluorogenic and chromogenic reactions of lactone substrates.

Substrates	t_R1 [a] [min]	t_R2 [b] [min]	*E* (chirazyme E1) [c]	E(*Chirazyme E2*) [c]
10	88.9	107.2	2.3 ± 0.5	2.3 ± 0.5
2	60.4	68.4	6.6 ± 0.2	5.6 ± 1.1
13	39.2	60.8	4.0 ± 1.5	2.7 ± 0.7
14	31.4	42.5	3.5 ± 1.0	4.5 ± 1.3

Conditions: 100 μM substrate in aqueous PBS pH 7.4, 10% v/v DMSO, 10 μg mL^{-1} enzyme, 25 °C. Aliquots were extracted with AcOEt at fixed time intervals and analyzed by HPLC. Each datapoint is the average of at least three different HPLC-analyses.

[a] Retention time of first lactone enantiomer.

[b] Retention time of second lactone enantiomer. HPLC conditions see Figure 3.

[c] Enantioselectivity E was calculated in each case according to the equation: $E = \ln[(1-c)(1-ee)]/\ln[(1-c)(1+ee)]$, where c = conversion of substrate to product and ee is the enantiomeric purity of unreacted substrate.

gel sheets (Macherey-Nagel) with detection by UV (245 and 365 nm) or with reagents like 10% ninhydrin solution in 90% EtOH. All chromatographies (flash) were performed with Fluka Silica gel 60 (0.04–0.063 mm, 230–400 mesh ASTM). Melting points were determined on a Büchi 510 apparatus and are not corrected. NMR spectra were recorded on a Bruker AC 300 for ^1H (300 MHz) or C^{13} (75 MHz) measurements or on a Bruker AC 400 for ^1H (400 MHz) or C^{13} (100 MHz) measurements. Chemical shifts δ are given in ppm and coupling constants *J* in Hertz. Mass spectra and accurate mass were provided by the "Service of Mass Spectrometry" of the Department of Chemistry and Biochemistry.

7-(2-Oxocyclohexyloxy)-*2H*-chromen-2-one (1)

Umbelliferone (0.65 g, 4 mmol) was dissolved in acetone (15 mL). After addition of 2-chlorocyclohexanone (1.32 g, 10 mmol, 2.5 equivs.), K_2CO_3 (0.94 g, 6 mmol, 1.5 equivs.) and NaI (0.9 g, 6 mmol, 1.5 equivs.) at 0 °C, the mixture was stirred at 60 °C overnight. After the reaction was complete, the mixture was filtered and the filtrate was evaporated. The residue was purified by column chromatography (EtOAc/hexane, 1:1) to afford **1** as a colorless solid; yield: 0.6 g (2.3 mmol, 58%); mp 167–169 °C; R_f = 0.4; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.63 (d, J = 9.5 Hz, 1 H), 7.37 (d, J = 8.6 Hz, 1 H), 6.83 (dd, J = 2.4 Hz and 8.6 Hz, 1 H), 6.70 (d, J = 2.4 Hz, 1 H), 6.25 (d, J = 9.6 Hz, 1 H), 4.75 (dd, J = 10.1 Hz and 5.4 Hz, 1 H), 2.62 (m, 1 H), 2.43 (m, 2 H), 2.07 (m, 3 H),1.81 (m, 2 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 207.1, 161.7, 161.4, 156.3, 143.9, 129.5, 114.1, 113.8, 113.7, 103.1, 81.4, 41.3, 34.9, 28.3, 23.7; EI-MS: m/z = 258 $[M]^+$; HR-MS-EI: calcd. for $C_{15}H_{14}O_4$: 258.0892; m/z = 258.0892.

7-(7-Oxooxepan-2-yloxy)-*2H*-chromen-2-one (2)

Ketone **1** (150 mg, 0.58 mmol) was dissolved in dichloromethane (2 mL). After addition of *m*-CPBA (186 mg, 0.76 mmol, 1.3 equivs.) and $NaHCO_3$ (64 mg, 0.76 mmol, 1.3 equivs.) at 0 °C, the mixture was stirred at 25 °C during 3 hours. Aqueous work-up (CH_2Cl_2/water/brine), drying ($MgSO_4$), evaporation of the solvent and purification by column chromatography (hexane/ethyl acetate, 6:4, R_f = 0.2) gave **2** as colorless solid; yield: 110 mg (0.40 mmol, 69%); mp 116–118 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.64 (d, J = 9.6 Hz, 1 H), 7.41 (d, J = 9.3 Hz, 1 H), 7.01 (m, 2 H), 6.29 (d, J = 9.6 Hz, 1 H), 5.92 (d, J = 5.4 Hz, 1 H), 2.75 (m, 2 H), 1.74–2.34 (m, 5 H); ^{13}C NMR ($CDCl_3$, 75 MHz): 173.4, 161.3, 159.3, 156.1, 143.7, 129.8, 115.3, 115.1, 113.9, 105.1, 98.9, 37.4, 34.4, 23.7, 23.5; EI-MS: m/z = 274 $[M]^+$; HR-MS-EI: calcd. for $C_{15}H_{14}O_5$: 274.0841; m/z = 274.0839.

7-(2-Oxocyclopentyloxy)-*2H*-chromen-2-one (5)

Umbelliferone (0.65 g, 4 mmol) was dissolved in acetone (15 mL). After addition of 2-chlorocyclopentanone (1 mL, 10 mmol, 2.5 equiv.), K_2CO_3 (0.94 g, 6 mmol, 1.5 equivs.) and NaI (0.9 g, 6 mmol, 1.5 equivs.) at 0 °C, the mixture was stirred at 60 °C overnight. After the reaction was complete, the mixture was filtered and the filtrate was evaporated. The residue was purified by column chromatography (EtOAc/hexane, 4:6) to afford **5** as a colorless solid; yield: 0.6 g (2.5 mmol, 62%); mp 125–127 °C; R_f = 0.3; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.64 (d, J = 9.5 Hz, 1 H), 7.38 (d, J = 8.3 Hz, 1 H), , 6.94 (dd, J = 2.6 Hz and 8.3 Hz, 1 H), 6.90 (d, J = 2.4 Hz, 1 H), 6.28 (d, J = 9.4 Hz, 1 H), 4.68 (m, 1 H), 2.43 (m, 1 H), 2.40 (m, 2 H), 2.07 (m, 1 H), 2.05 (m, 2 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 213.5, 161.7, 161.7, 156.3, 144.0, 129.5, 114.3, 114.1, 113.9, 103.4, 80.2, 34.9, 30.0, 17.9; EI-MS: m/z = 244 $[M]^+$; HR-MS-EI: calcd. for $C_{14}H_{12}O_4$: 244.0735, m/z = 244.0733.

7-(2-Oxopropoxy)-chromen-2-one (6)

Umbelliferone (0.65 g, 4 mmol) was dissolved in chloroacetone (6 mL). After addition of potassium carbonate (0.94 g, 6 mmol, 1.5 equivs.) and sodium iodide (0.9 g, 6 mmol, 1.5 equivs.) at 0 °C, the mixture was stirred at 25 °C for 4 hours. After the reaction was complete water (20 mL) was added. Aqueous work-up (AcOEt/brine), drying ($MgSO_4$), evaporation of the solvent, and recrystallization (acetone/hexane) gave **6** as a colorless solid; yield: 0.605 mg (2.7 mmol, 70%); mp 107–109 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.64 (d, J = 9.6 Hz, 1 H), 7.41 (d, J = 8.7 Hz, 1 H), 6.86 (dd, J = 2.7 Hz and 8.7 Hz, 1 H), 6.75 (d, J = 2.7 Hz, 1 H), 6.27 (d, J = 9.6 Hz, 1 H), 4.64 (s, 2 H),2.30 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 204.2, 161.3, 160.2, 156.4, 143.8, 129.8, 114.6, 114.0, 113.3, 102.5, 73.6, 27.3; EI-MS: m/z = 218 $[M]^+$; HR-MS-EI: calcd. for $C_{13}H_{12}O_4$: 218.0579; m/z = 218.0578.

7-(1-Methyl-2-oxopropoxy)-chromen-2-one (7)

Umbelliferone (0.65 g, 4 mmol) was dissolved in acetone (10 mL). After addition of 3-chloro-2-butanone (1 mL, 10 mmol, 2.5 equivs.), K_2CO_3 (0.94 g, 6 mmol, 1.5 equivs.) and NaI (0.9 g, 6 mmol, 1.5 equivs.) at 0 °C, the mixture was stirred at 60 °C overnight. After the reaction was complete, the mixture was filtered and the filtrate was evaporated to afford **7** as a colorless solid; yield: 0.9 g (3.88 mmol, 98%); mp 86–88 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.63 (d, J = 9.4 Hz, 1 H), 7.38 (d, J = 8.7 Hz, 1 H), 6.81 (dd, J = 2.4 Hz and 8.7 Hz, 1 H), 6.73 (d, J = 2.4 Hz, 1 H), 6.27 (d, J = 9.6 Hz, 1 H), 4.70 (q, J = 7.8 Hz, 1 H), 2.20 (s, 3 H),1.55 (d, J = 7.8 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 208.9, 161.5, 161.0, 156.4, 143.8, 129.8, 114.4, 113.9, 113.2, 103.0, 80.1, 25.5, 18.0; EI-MS: m/z = 232 $[M]^+$; HR-MS-EI: calcd. for $C_{13}H_{12}O_4$: 232.0735; m/z = 232.0735.

2-(4-Nitrophenoxy)-cyclopentanone (8)

4-Nitrophenol (0.46 g, 3.3 mmol) was dissolved in acetone (15 mL). After addition of 2-chlorocyclopentanone (1 mL, 10 mmol, 3 equivs.), potassium carbonate (1.03 g, 6.6 mmol, 2 equivs.) and sodium iodide (0.99 g, 6.6 mmol, 2 equivs.) at 0 °C, the mixture was stirred at 60 °C overnight. After the reaction was complete, the mixture was filtered and the filtrate was evaporated. Aqueous work-up (AcOEt/NH_4Cl, then water, then brine), evaporation of the organic phase and column chromatography (EtOAc/hexane, 2:8, R_f = 0.3) gave **8** as a dark yellow solid; yield: 0.45 g (3 mmol, 69%); mp 70–73 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.11 (d, J = 9.2 Hz, 2 H), 6.98 (d, J = 9.2 Hz, 2 H), 4.74 (m, 1 H), 2.50 (m, 1 H), 2.36 (m, 2 H), 2.13 (m, 1 H),1.95 (m, 2 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 213.3, 163.6, 126.5, 116.1, 80.1, 35.9, 30.1, 17.7; EI-MS: m/z = 221 $[M]^+$; HR-MS-EI: calcd. for $C_{11}H_{11}NO_4$: 221.0688; m/z = 221.0688.

2-(4-Nitrophenoxy)-cyclohexanone (9)

4-Nitrophenol (0.46 g, 3.3 mmol) was dissolved in acetone (15 mL). After addition of 2-chlorocyclohexanone (1.14 mL, 10 mmol, 3 equivs.), K_2CO_3 (1.03 g, 6.6 mmol, 2 equivs.) and

NaI (0.99 g, 6.6 mmol, 2 equivs.) at 0 °C, the mixture was stirred at 60 °C overnight. After the reaction was complete, the mixture was filtered and the filtrate was evaporated. Aqueous work-up (AcOEt/NH_4Cl, then water, then brine), evaporation of the organic phase and recrystallization (acetone/hexane) gave **9** as a colorless solid; yield: 0.71 g (3 mmol, 92%); mp 90–92 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.15 (d, *J* = 9.2 Hz, 2 H), 6.87 (d, *J* = 9.2 Hz, 2 H), 4.77 (m, 1 H), 2.5 (m, 1 H), 2.41 (m, 2 H), 2.07 (m, 3 H), 1.80 (m, 2 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 206.9, 163.4, 126.5, 115.8, 81.4, 41.3, 34.9, 28.2, 23.7; EI-MS: *m/z* = 235 $[M]^+$; HR-MS-EI: calcd. for $C_{12}H_{13}NO_4$: 235.0845; *m/z* = 235.0844.

7-(Tetrahydro-6-oxo-*2H*-pyran-2-yloxy)-*2H*-chromen-2-one (10)

Ketone **5** (50 mg, 0.205 mmol) was dissolved in dichloromethane (1 mL). After addition of *m*-CPBA (65.7 mg, 0.266 mmol, 1.3 equivs.) and $NaHCO_3$ (22 mg, 0.266 mmol, 1.3 equivs.) at 0 °C, the mixture was stirred at 25 °C during 3 hours. Aqueous work-up (CH_2Cl_2/water/brine), drying ($MgSO_4$), evaporation of the solvent and purification by column chromatography (hexane/ethyl acetate, 1:1, R_f = 0.4) gave **10** as colorless solid; yield: 46 mg (0.177 mmol, 86%); mp 151–153 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.64 (d, *J* = 9.6 Hz, 1 H), 7.41 (d, *J* = 9.3 Hz, 1 H), 7.01 (m, 2 H), 6.29 (d, *J* = 9.5 Hz, 1 H), 6.05 (dd, *J* = 3.2 Hz and 3.7, 1 H), 2.72 (m, 2 H), 1.91–2.32 (m, 4 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 169.5, 161.4, 159.6, 156.1, 143.7, 129.7, 115.1, 115.0, 114.1, 105.2, 98.6, 30.5, 27.4, 15.9; EI-MS: *m/z* = 260 $[M]^+$; HR-MS-EI: calcd. for $C_{14}H_{12}O_5$: 260.0684; *m/z* = 260.0683.

Acetic Acid 1-(2-Oxo-*2H*-chromen-7-yloxy)-methyl Ester (11) by Oxidation of Ketone 1

Ketone **6** (300 mg, 1.37 mmol) was dissolved in dichloromethane (20 mL). After addition of *m*-CPBA(714 mg, 2.06 mmol, 1.5 equivs.) and $NaHCO_3$ (150 mg, 1.78 mmol, 1.3 equivs.) at 0 °C, the mixture was stirred at 25 °C overnight. Aqueous work-up (CH_2Cl_2/aqueous NH_4Cl/brine), drying ($MgSO_4$), evaporation of the solvent and recrystallization of the residue (acetone/hexane) gave **11** as a colorless solid; yield: 260 mg (1.11 mmol, 81%); mp 161–165 °C; R_f = 0.3 (hexane/ethyl acetate, 1:1); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.64 (d, *J* = 9.6 Hz, 1 H), 7.42 (d, *J* = 8.4 Hz, 1 H), 7.02 (d, *J* = 2.4 Hz, 1 H), 6.95 (dd, *J* = 2.4 Hz and 8.4 Hz, 1 H), 6.32 (d, *J* = 9.6 Hz, 1 H), 5.80 (s, 2 H), 2.14 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 170.3, 161.5, 160.3, 156.2, 143.8, 129.7, 115.0, 114.7, 114.2, 104.1, 85.4, 21.7; ESI-MS: *m/z* = 235 $[M+H]^+$; calcd. for $C_{12}H_{10}O_5$: 235.0606; *m/z* = 235.0611.

Acetic Acid 1-(2-Oxo-*2H*-chromen-7-yloxy)-ethyl Ester (12)

Ketone **7** (200 mg, 0.86 mmol) was dissolved in dichloromethane (5 mL). After addition of *m*-CPBA (223 mg, 1.3 mmol, 1.5 equivs.) and $NaHCO_3$ (74 mg, 1.1 mmol, 1.3 equivs.) at 0 °C, the mixture was stirred at 25 °C during 10 hours. Aqueous work-up (CH_2Cl_2/water/brine), drying ($MgSO_4$), evaporation of the solvent and purification by column chromatography (hexane/ethyl acetate, 6:4, R_f = 0.3) gave **12** as a colorless solid; yield: 170 mg (0.69 mmol, 80%); mp 80–83 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.62 (d, *J* = 9.6 Hz, 1 H), 7.37 (d, *J* = 8.5 Hz, 1 H), 6.84 (dd, *J* = 2.4 Hz and 8.5 Hz, 1 H), 6.82 (d, *J* = 2.4 Hz, 1 H), 6.56 (q, *J* = 5.1 Hz, 1 H), 6.24 (d, *J* = 9.4 Hz, 1 H), 2.04 (s, 3 H),1.60 (d, *J* = 5.1 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 170.4, 161.5, 159.5, 156.2, 143.8, 129.7, 115.0, 114.7, 114.1, 104.8, 93.7, 21.7, 21.1; EI-MS: *m/z* = 248 $[M]^+$; HR-MS-EI: calcd. for $C_{13}H_{12}O_5$: 248.0685; *m/z* = 248.0688.

6-(4-Nitrophenoxy)-tetrahydropyran-2-one (13)

Ketone **8** (0.2 g, 0.9 mmol) was dissolved in dichloromethane (5 mL). After addition of *m*-CPBA (0.29 g, 1.18 mmol, 1.3 equivs.) and $NaHCO_3$ (0.1 mg, 1.18 mmol, 1.3 equivs.) at 0 °C, the mixture was stirred at 25 °C during 3 hours. Aqueous work-up (CH_2Cl_2/aqueous NH_4Cl/brine), drying ($MgSO_4$), evaporation of the solvent and recrystallization (dichloromethane/hexane) gave **13** as a colorless solid; yield: 120 mg (0.45 mmol, 50%); mp 85–90 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.21 (d, *J* = 7.2 Hz, 2 H), 7.17 (d, *J* = 7.2 Hz, 2 H), 6.07 (m, 1 H), 2.67 (m, 2 H), 2.29 (m, 1 H), 2.15 (m, 2 H), 1.96 (m, 1 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 169.9, 161.5, 143.8, 126.6, 117.2, 99.3, 30.5, 28.0, 15.8; ESI-MS: *m/z* = 238 $[M+H]^+$; HR-MS: calcd. for $C_{12}H_{13}NO_5$: 238.0715; *m/z* = 238.0715.

7-(4-Nitrophenoxy)oxepan-2-one (14)

Ketone **9** (0.5 g, 2.13 mmol) was dissolved in dichloromethane (15 mL). After addition of *m*-CPBA (0.68 g, 2.76 mmol, 1.3 equivs.) and $NaHCO_3$ (0.23 mg, 2.76 mmol, 1.3 equivs.) at 0 °C, the mixture was stirred at 25 °C during 3 hours. Aqueous work-up (CH_2Cl_2/aqueous NH_4Cl/brine), drying ($MgSO_4$), evaporation of the solvent and recrystallization (acetone/hexane) gave **14** as a colorless solid; yield: 360 mg (1.5 mmol, 67%); mp 112–117 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.22 (d, *J* = 7.1 Hz, 2 H), 7.17 (d, *J* = 7.1 Hz, 2 H), 5.93 (d, *J* = 4.7 Hz, 1 H), 2.73 (m, 2 H), 2.36 (m, 1 H), 2.16 (m, 2 H), 1.59–1.96 (m, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 173.2, 161.1, 143.9, 126.6, 117.1, 98.6, 37.4, 34.3, 23.5, 23.4; EI-MS: *m/z* = 251 $[M]^+$; HR-MS-EI: calcd. for $C_{12}H_{13}NO_5$: 251.0796; *m/z* = 251.0794.

Monooxygenase Assays

Microorganisms: Yeast and fungi were cultured for 3–4 days, at 28 °C, in test tubes (h = 18 cm; ø = 1.5 cm) containing *ca.* 10 mL of yeast-malt-agar (YMA) medium (3 g L^{-1} yeast extract *Merck*, 3 g L^{-1} malt extract *Merck*, 5 g L^{-1} peptone *Difco*, 10 g L^{-1} glucose, 20 g L^{-1} agar *Merck*, H_2O) and 10 mL of malt agar (MA) medium (20 g L^{-1} malt extract Merck, 20 g L^{-1} agar Merck, H_2O), respectively. Just prior to the tests the grown colonies were weighed into 1.5 mL Eppendorf tubes and suspended in borate buffer in order to obtain 1 mg of wet cells per mL.

Fluorescence measurements without preincubation: All buffers and solutions were prepared using deionized H_2O. Microbial cells were diluted from 1 mg mL^{-1} suspensions in 20 mmol

L^{-1} borate buffer (pH 8.8; 100 μL). Substrates were diluted from 2 mmol L^{-1} stock solutions in 50% aqueous MeCN, (10 μL). BSA was diluted from a 4.4 mg mL^{-1} stock solution in 20 mmol L^{-1} borate (pH 8.8; 90 μL). The 200 μL assays were followed in individual wells of flat-bottom polypropylene 96-well microtiter plates (*Costar*) with a *Fluoroskan Ascent* fluorescence plate reader (*Labsystems*, filters λ_{ex} 365 ± 20 nm, λ_{em} 460 ± 20 nm) (Table 1, entries 1–5, 7, 8, 10, 13, 15)

Fluorescence measurements with preincubation: These experiments were performed following the above described protocol without BSA and incubating the microorganisms in the microtiter plate at 28 °C for 24 h.. Fluorescence was monitored for 24 h after BSA addition. (Table 1, entries 6, 9, 11, 12, 14).

Esterase Assays

Kinetic measurements: All buffers and solutions were prepared using MilliQ deionized water, the pH being adjusted with aqueous NaOH and aqueous HCl solutions. Enzymes were used as 1 mg mL^{-1} stock solutions in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) and preserved at 4 °C. Stock solutions of substrates (10 mM and 1 mM) were prepared in DMSO and stored at −20 °C. Assays (0.2 mL for fluorogenic substrates and 0.1 mL for chromogenic substrates) were followed in individual wells of round-bottom polypropylene 96-well-plates (*Costar*) with *Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems,* filters $\lambda_{ex} = 360 \pm 20$, $\lambda_{em} = 460 \pm 20$ nm) for fluorogenic substrates and in individual wells of round-bottom polyprostyrene *96*-well-plates (*Costar*) with *Spectramax 250 Microplate Spectrophotometer (Molecular Devices)* for chromogenic substrates. The rates indicated in Table 2 are derived from the steepest linear portion of each curve. Conditions of assay: to a solution (0.1 or 0.05 mL) of enzyme at 0.02 mg mL^{-1} (0.01 mg mL^{-1} final concentration), is added 0.1 or 0.05 mL of a substrates solution at 0.2 mmol L^{-1} (0.1 mM final concentration) containing 20% of DMSO (10% final concentration).

Determination of enantioselectivity: Reactions were followed in Eppendorf tubes (1 mL). For measurement set, a stock solution of substrates at 1 mM in DMSO were prepared and mixed to the stock solution of enzyme. Then an extraction using EtOAc was done just before the measurement by HPLC. Reaction conditions: 100 μM substrate, 10% DMSO, incubation with enzyme (10 μg mL^{-1}) at room temperature with different reaction time (5, 10 and 15 min). Experiments were done on column Chiralcel OD-H (0.46 × 25 cm), detection by UV 320 nm, eluent: hexane/2-propanol, 85/10; flow: 0.5 mL min^{-1}, UV 320 nm.

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References

[1] a) B. Marrs, S. Delagrave, D. Murphy, *Curr. Opin. Microbiol.* **1999**, *2*, 241–245; b) W. P. C. Stemmer, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 10747–10751; c) W. P. C. Stemmer, *Nature* **1994**, *370*, 389–391; d) L. You, F. H. Arnold, *Protein. Eng.* **1996**, *9*, 77–83; e) M. T. Reetz, K.-E. Jaeger, *Chem. Eur. J.* **2000**, *6*, 407–412; f) S. V. Taylor, P. Kast, D. Hilvert, *Angew. Chem.* **2001**, *113*, 3408–3436; *Angew. Chem. Int. Ed.* **2001**, *40*, 3310–3335.

[2] a) *Enzyme Assays: A Practical Approach*. (Eds.: R. Eisenthal, M. Danson), Oxford University Press, Oxford, **2002**; b) *Enzyme Assays: Essential Data*. (Eds.: S. Gul, S. K. Sreedharan, K. Brocklehurst), John Wiley & Sons, Chicester, **1998**; c) M. T. Reetz, *Angew. Chem.* **2001**, *113*, 292–320; *Angew. Chem. Int. Ed.* **2001**, *40*, 284–310; d) D. Wahler, J.-L. Reymond, *Curr. Opin. Chem. Biol.* **2001**, *5*, 152–158; e) D. Wahler, J.-L. Reymond, *Curr. Opin. Biotechnol.* **2001**, *12*, 535–544; f) J.-P. Goddard, J.-L. Reymond, *Trends Biotechnol.* **2004**, *22*, 363–370; g) J.-P. Goddard, J.-L. Reymond, *Curr. Opin. Biotechnol.* **2004**, *15*, 314–322.

[3] a) G. Klein, J.-L. Reymond, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1113; b) G. Klein, J.-L. Reymond, *Helv. Chim. Acta* **1999**, *82*, 400.

[4] a) N. Jourdain, R. Perez-Carlon, J.-L. Reymond, *Tetrahedron Lett.* **1998**, *39*, 9415; b) R. Pérez Carlòn, N. Jourdain, J.-L. Reymond, *Chem. Eur. J.* **2000**, *6*, 4154.

[5] a) F. Badalassi, D. Wahler, G. Klein, P. Crotti, J.-L. Reymond, *Angew. Chem. Int. Ed.* **2000**, *39*, 4067–4070; b) E. M. Gonzalez-Garcia, J. Grognux, D. Wahler, J.-L. Reymond, *Helv. Chim. Acta* **2003**, *86*, 2458–2470; c) E. Nyfeler, J. Grognux, D. Wahler, J.-L. Reymond, *Helv. Chim. Acta* **2003**, *86*, 2919–2927.

[6] F. Badalassi, P. Crotti, J.-L. Reymond, *Helv. Chim. Acta* **2002**, *82*, 3090.

[7] E. Leroy, N. Bensel, J.-L. Reymond, *Adv. Syn. Catal.* **2003**, *345*, 859–865.

[8] F. Badalassi, G. Klein, P. Crotti, J.-L. Reymond, *Eur. J. Org. Chem.* **2004**, 2557–2566.

[9] E. M. Gonzalez-Garcia, V. Helaine, G. Klein, M. Schuermann, G. Sprenger, W.-D. Fessner, J.-L. Reymond, *Chem. Eur. J.* **2003**, *9*, 893.

[10] a) D. Wahler, F. Badalassi, P. Crotti, J.-L. Reymond, *Angew. Chem. Int. Ed.* **2001**, *40*, 4457–4460; c) D. Wahler, F. Badalassi, P. Crotti, J.-L. Reymond, *Chem. Eur. J.* **2002**, *8*, 3211–3228; d) J.-L. Reymond, D. Wahler, F. Badalasssi, H. K. Nguyen, *Patent* WO0192563, 2001-12-07; e) J.-L. Reymond, D. Wahler *ChemBioChem* **2002**, *3*, 701–708; f) J. Grognux, J.-L. Reymond, *ChemBioChem* **2004**, *5*, 826–831; g) J.-P. Goddard, J.-L. Reymond, *J. Am. Chem. Soc.* **2004**, *126*, 11116–11117.

[11] a) D. Wahler, O. Boujard, F. Lefèvre, J.-L. Reymond, *Tetrahedron* **2004**, *60*, 703–710; b) J. Grognux, D. Wahler, E. Nyfeler, J.-L. Reymond, *Tetrahedron: Asymmetry* **2004**, *15*, 2981–2989.

[12] A. Sevestre, V. Hélaine, G. Guyot, C. Martin, L. Hecquet, *Tetrahedron Lett.* **2003**, *44,* 827–830.

[13] M. C. Gutierrez, A. Sleegers, H. D. Simpson, V. Alphand, R. Furstoss, *Org. Biomol. Chem.* **2003**, *1*, 3500–3506.
[14] N. Bensel, M. T. Reymond, J.-L. Reymond, *Chem. Eur. J.* **2001**, *7*, 4604.
[15] E. Leroy, N. Bensel and J.-L. Reymond, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2105–2108.
[16] M. D. Mihovilovic, B. Müller, P. Stanetty, *Eur. J. Org. Chem.* **2002**, 3711–3730.
[17] C. T. Walsh, Y.-C. J. Chen, *Angew. Chem.* **1988**, *100*, 342–352.
[18] A. B. Watts, J. Whitcher, C. S. Whitcher, J. A. Littlechild, *Biocatal. Biotrans.* **2002**, *20*, 209–214.
[19] R. A. C. Gonçalves, A. L. M. Porto, L. Pinheiro, J. R. Cagnon, G. P. Manfio, A. J. Marsaioli, *Food Technol. Biotechnol*. **2004**, 42, 355–361.
[20] B. Bicalho, L. S. Chen, J. Grognux, J-L. Reymond, A. J. Marsaioli, *J. Braz. Chem. Soc.* **2004**, *15*, 911–916.
[21] A. L. M. Porto. *PhD thesis*, **2002**, Chemistry Institute, State University of Campinas.
[22] M. Kataoka, K. Honda, S. Shimizu, *Eur. J. Biochem.* **2000**, *267*, 3–10.
[23] L. Blanco, E. Guibé-Jampel, G. Rousseau, *Tetrahedron Lett.* **1988**, *29,* 1915–1918.
[24] a) C-S. Chen, Y. Fujimoto, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1982**, *104*, 7294–7298.; b) H. B. Kagan, J. C. Fiaud, in: *Topics in Stereochemistry*; John Wiley & Sons, New York, **1988**, Vol. 18, p. 249.8.
[25] B. Bicalho, R. A. C. Gonçalves, A. P. M. Zibordi, G. P. Manfio, A. J. Marsaioli, *Z. Naturforsch.* **2003**, 58c, 746–751.

