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Universal chromogenic substrates for lipases and esterases

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Abstract—Chromogenic mono- and diacyl-glycerol analogues were prepared and tested as substrates against lipases and esterases in a microtiter plate setup. Thirty-three enzyme samples were analyzed for activity at 100, 10, and 1 μg mL^{-1}. The reaction rates observed were visualized by RGB-color coding of activities, and analyzed by cluster analysis. The C8-, C10-, and C12-monoesters of the nitrophenol-derived diol **13** reacted very strongly across all enzyme samples tested, and appear as universal substrates for these enzymes. Such universal chromogenic probes may be used to detect even dilute lipases and esterases in crude cultures.

1. Introduction

By virtue of their molecular structure, enzymes are complex catalysts and their detailed reactivity is very difficult to predict. 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 (e.g., Scheme 2). This assay methodology practically suppresses any background reactivity of the substrates in the absence of enzyme, and allows the detection of 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] epoxide hydrolases,[5,7] transaldolases,[8] transketolases,[9] and Bayer-Villigerases.[10] These assays may also be applied in parallel using arrays of structurally diverse substrates to produce enzyme-specific activity fingerprints.[11]

Many high-throughput enzyme assays are applied as tools to visualize a particular enzyme class. A number of assays have been described for the particular case of lipases and esterases, which are the main class of enzymes in industrial applications.[12] Lipases can be assayed using fluorescence substrates, which operate by fluorescence resonance energy transfer (FRET).[13] However these substrates are structurally complex since they require two chromophores, and are only suited for lipases. In practice lipase and esterase assays are mostly carried out using the classical aliphatic esters of nitrophenol, umbelliferone, resorufin, and fluorescein (as diester). Unfortunately these substrates are all esters of acidic phenols, and as such they show high spontaneous and nonspecific hydrolysis in aqueous buffer. In addition these substrates do not react very well across all lipases and esterases. In an attempt to reduce spontaneous reactivity, we have recently investigated acyloxymethyl ethers[14] and 1-acyloxy-1-cyano-3-propyl ethers[15] of umbelliferone as fluorogenic substrates for lipases and esterases. These substrates show an approximately 10-fold reduced chemical sensitivity toward spontaneous hydrolysis. We also developed a chromogenic assay for lipases and estereases using the adrenaline test for enzymes.[16] This setup allowed to use triglycerides or 1,2-diol and carbohydrate acetates as substrates.[17] While the use of such aliphatic esters resulted in a practically negligible level of nonspecific hydrolysis, the assay was not suitable to detect trace amounts of lipases.

In our search for optimal probes for lipases and esterases, we later found that long chain aliphatic monoesters of 7-[3,4-dihydroxybut-1-yloxy]coumarin provide optimal fluorogenic probes for lipases and esterases.[18] These substrates liberate 7-hydroxycoumarin (= umbelliferone) as blue fluorescent product by the

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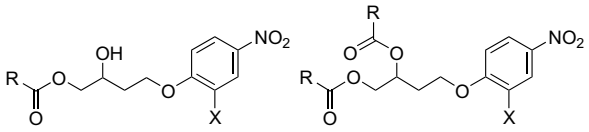
indirect mechanism discussed above using sodium periodate and bovine serum albumin as secondary reagents, and show both minimal spontaneous hydrolysis even under harsh conditions and very high reactivity against lipases and esterases. An array of such fluorogenic substrates allowed to classify these enzyme according to their catalytic functionality.[19]

Herein we show that the related esters of 4-(nitrophenoxy)-1,2-butanediol and 4-(dinitrophenoxy)-1,2-butanediol provide excellent chromogenic probes for lipases and esterases. The chromogenic mono- and diacyl-glycerol analogues **1**–**12b** were synthesized and analyzed for their reactivity against a series of lipases and esterases (Scheme 1). Cluster analysis of the reactivity patterns observed indicate that monoesters of the nitrophenol-derived diol **13** with C8-, C10-, and C12-aliphatic acids behave as highly reactive, universal chromogenic probes for lipases and esterases.



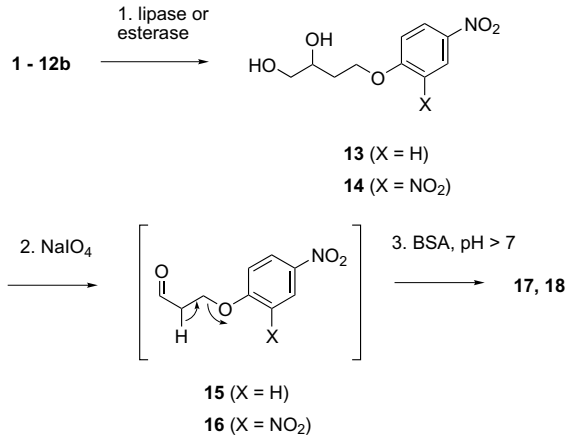
R=		
Me	**1a** (X = H)	**1b** (X = H)
Pr	**2** (X = H)	
C_7H_{15}	**3** (X = H), **4a** (X = NO_2)	**4b** (X = NO_2)
C_9H_{19}	**5a** (X = H)	**5b** (X = H)
$C_{11}H_{23}$	**6** (X = H), **7a** (X = NO_2)	**7b** (X = NO_2)
$C_{13}H_{27}$	**8** (X = H), **9a** (X = NO_2)	**9b** (X = NO_2)
$C_{15}H_{31}$	**10a** (X = H)	**10b** (X = H)
$C_{17}H_{35}$	**11a** (X = H)	**11b** (X = H)
	12a (X = H)	**12b** (X = H)

Scheme 1. Chromogenic substrates for lipases and esterases.

2. Results and discussion

Chromogenic substrates are generally more convenient than fluorogenic substrates. Firstly, their reactions can be detected visually, implying that they are suitable for routine activity control without instruments. Secondly, UV-spectrometers are more broadly available than fluorometers. We therefore decided to investigate chromogenic derivatives of our previously developed fluorogenic substrates as probes for lipases and esterases.

The mono- and diacyl-glycerol analogs esters **1**–**12b** of the glycerol related diols **13** and **14** with aliphatic esters of varying chain length were prepared by mono- or diesterification with the acyl chlorides. The chromogenic reaction followed the oxidation/β-elimination sequence used in our fluorogenic substrate arrays via the intermediate aldehydes **15/16** to produce the colored nitrophenolate **17** and dinitrophenolate **18** as detectable products (Scheme 2). By contrast to the highly reactive esters of nitrophenol, which tend to react non-specifically with proteins,[12] substrates **1**–**12b** were triggered by cleavage of relatively unreactive aliphatic ester bonds, ensuring that only enzyme-specific activities would be revealed.



Scheme 2. Principle of chromogenic periodate coupled assay.

Thirty-three enzymes were tested, including lipases, esterases, one acylase, and one epoxide hydrolase. Reactions were carried out in 96-well microtiter plates, recording all substrates (at concentration of 100 μM each) with each enzyme concentration of 100, 10, and 1 μg mL^{-1}. The results were represented as 4 × 5 color-coded grids for each enzyme, with monoesters of increasing chain length arranged in the first three rows, and diesters of increasing chain length arranged in the two lower rows (Fig. 1). The color was coded using each of the three color channels red (R), green (G), and blue (B) with the activities observed with 100, 10, and 1 μg mL^{-1} enzyme sample. In this display one expects to see blue tones only since the reaction rates should increase with increasing enzyme concentration, even within the limitations imposed by the fact that the secondary oxidation/β-elimination sequence (Scheme 2, steps 2 and 3) becomes rate limiting at high enzyme concentration for very active enzymes, as discussed previously.[5] In practice several enzyme samples, in particular weakly active preparations, exhibited their highest reactivities at the lower enzyme concentrations, probably due to the presence of impurities, or by an enzyme inhibition effect caused by aggregation.

The highest lipase reactivities were observed with monoacyl substrates of intermediate chain length (C8, C10, and C12). In fact these substrates detect lipase activity down to 0.01 μg mL^{-1} for the most active samples, which in our hands is a 100-fold more sensitive than standard substrates such as nitrophenyl octanoate. The acetate substrates **1a/b** showed their highest reactivity with acetyl choline esterase (AchE), in agreement with the known specificity of this enzyme. The C18-derivatives **11a/b** did not show any significant reactivity with any of the enzymes, probably due to limited solubility. By contrast the oleate derivatives **12a/b** did show the highest reactivities of the long chain esters. The most active enzyme samples were those with highest reported specific activ-

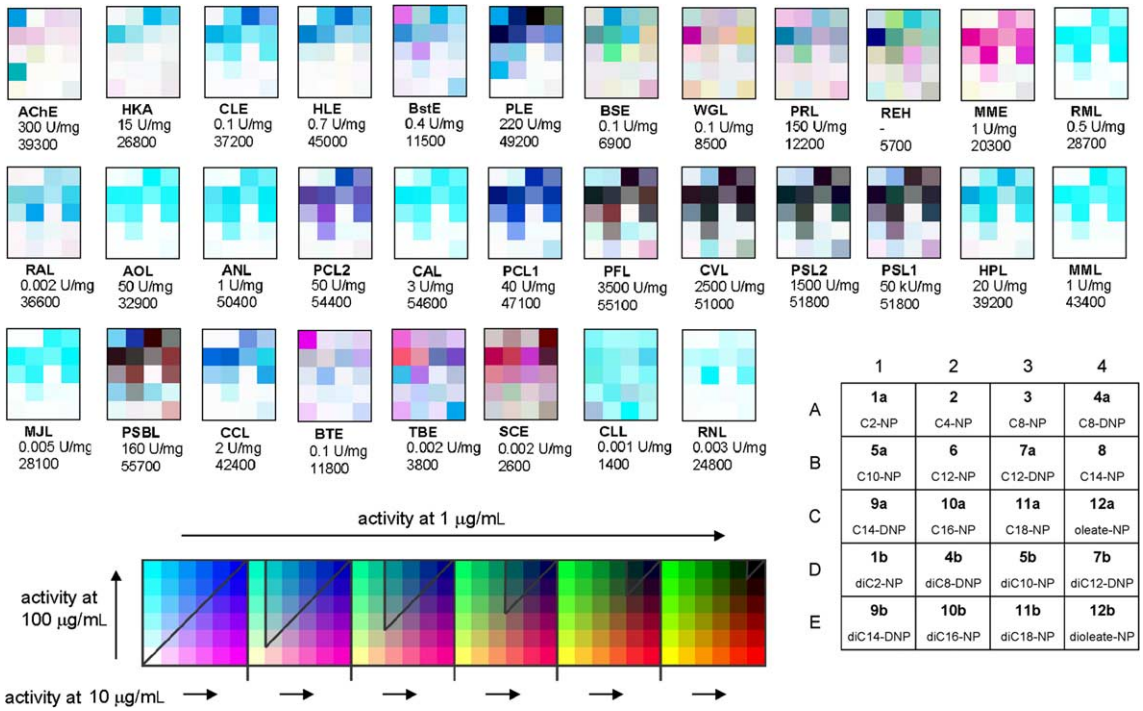


Figure 1. Enzyme activity patterns against chromogenic lipases substrates. Reaction conditions: 100 µM substrate, 5% v/v DMF in aq 20 mM borate buffer pH 8.8, 1 mM $NaIO_4$, 2 mg mL^{-1} BSA, 26 °C. Reactions (0.1 mL each) were followed spectrophotometrically at 405 nm over 1 h in 96-well polystyrene half-area cell-culture plates (Corning–Costar) using a Spectramax 250 microtiter plate reader (Molecular Devices, Inc.). The steepest linear portion of each time curve was used to calculate the apparent reaction rate according to a calibration curve with pure *p*-nitrophenol or 2,4-dinitrophenol. Enzymes are arranged according to clustering (Fig. 2 and text). The background reaction rates in the absence of enzyme was negligible (<100 pM s^{-1}) for all substrates. The display color for each square in each array is defined by setting the intensities of fundamental colors red (R), green (G) and blue (B) in proportion to the relative rates observed at 100, 10, and 1 µg mL^{-1} with the given enzyme. Below each array: enzyme code, activity from manufacturer's data, and reaction rate observed for the fastest reaction in the array in pM s^{-1}. The key at bottom left represents six incremental steps in color intensity. Actual colors are generated proportionally to actual rates, and span the entire color intensity spectrum. Intensity coefficients F of fundamental colors are calculated from relative rates (V_{rel}) as $F_R = 255(1 - V_{rel}\,(100\,\mu g\,mL^{-1}))$, $F_G = 255(1 - V_{rel}\,(10\,\mu g\,mL^{-1}))$, $F_B = 255(1 - V_{rel}\,(1\,\mu g\,mL^{-1}))$. Upper left triangles in each 6 × 6 color grid indicate colors generated from the data for 'well-behaved' systems in which $V(100\,\mu g\,mL^{-1}) > V(10\,\mu g\,mL^{-1}) > V(1\,\mu g\,mL^{-1})$. PSBL = *Pseudomonas* sp. type B lipoprotein lipase (F62336), PFL = *Pseudomonas fluorescens* lipase (F62321), CAL = *Candida antarctica* lipase (F62299), PCL2 = *Pseudomonas cepacia* lipase (F62309), PSL1 = *Pseudomonas* sp. lipoprotein lipase (SL-9656), PSL2 = *Pseudomonas* sp. lipoprotein lipase (F62335), CVL = *Chromobacterium visc*. lipoprotein lipase (F62333), ANL = *Aspergillus niger* lipase (A39,043-7), PCL1 = *Pseudomonas cepacia* lipase (F62312), MML = *Mucor miehei* lipase (F62298), CCL = *Candida cylindracea* lipase (F62316), HPL = hog pancreatic lipase (F62300), RAL = *Rhizopus arrhizus* lipase (F62305), AOL = *Aspergillus oryzae* lipase (F62285), RML = *Rhizomucor miehei* lipase (F62291), MJL = *Mucor javanicus* lipase (F62304), RNL = *Rhizopus niveus* lipase (F62310), PRL = *Penicillium roqueforti* lipase (F62308), WGL = wheat germ lipase (F62306), CLL = *Candida lipolytica* lipase (F62303), PLE = pig liver esterase (F46058), HLE = horse liver esterase (F46069), CLE = *Candida lipolytica* esterase (F46056), MME = *Mucor miehei* esterase (F46059), BTE = *Bacillus thermoglucosidasius* esterase (F46054), BstE = *Bacillus stearothermophilus* esterase, (F46051), BSE = *Bacillus* sp. esterase (F46062), TBE = *Thermoanaerobium brockii* esterase (F46061), SCE = *Saccharomyces cerevisiae* esterase (F46071), AChE = *Electrophorus electricus* acetyl choline esterase (F01023), HKA = hog kidney acylase I (F01821), REH = *Rhodotorula glutinis* epoxide hydrolase.

ity (U/mg). These samples also showed a clear protein band on SDS-PAGE analysis. Octanoate **3** (position A3) and decanoate **5a** (position B1) appeared as the fastest substrates with lipases. This high reactivity also meant that the activities observed in crude samples of non-lipase enzymes such as acylase (HKA) and epoxide hydrolase (REH) most likely indicated the presence of lipase or esterase impurities.

Cluster analysis was performed to gain a systematic insight into the measured reactivity profiles. In order to circumvent the observed problem of enzyme concentration dependent enzyme activities, each enzyme/substrate pair was assigned a total reactivity corresponding to the sum of reaction rates observed with the substrate at the three different enzyme concentrations measured. Each enzyme activity fingerprint was then decomposed in a series of relative activities for each substrate, such that the total reactivity for each enzyme would total 100% across all substrates measured. The analysis of relative rather than absolute reaction rates circumvents the problem of determining the absolute amount of active enzyme in the sample, as well as that of finding a substrate that reacts with all enzymes to assign absolute activity units to each enzyme.[19]

The dataset was processed using the multivariate analysis softwares WINIDAMS and VISTA.[20] Clustering was carried out using Ward's method on the basis of standardized euclidean distances. The 20 substrates were defined as variables and the 32 enzymes tested as observations. Cluster analysis is based on algorithms,

which group observations on the basis of their spatial proximity in an *N*-dimensional space defined by *N* variables.[21] The enzyme series thus ordered was represented as a color-coded representation of the symmetrical dissimilarity matrix of relative distances (Fig. 2).

Analysis of the clustered enzyme set according to the dissimilarity matrix showed a relatively large cluster of 14 similar enzymes at the center (AOL to CCL in Fig. 2). The reactivity pattern in this cluster corresponded to that of the four most reactive lipase samples in the series (PFL, CVL, and two samples of PSL), which was characterized by a very strong reactivity with the 4-nitrophenoxy-derived monoesters **3** (C8), **5a** (C10), **6** (C12), **8** (C14), **10a** (C16), and **12a** (oleate). Two additional clusters appeared at upper left (HKA to RgEH) and at lower right (BTE to CLL) containing mostly esterases. The first cluster was characterized by a very strong reactivity with the nitrophenyl derived C8- and C10-monoesters **3** and **5a** over the other substrates, while the second cluster contained enzymes with generally very low and distributed reactivities across most substrates. The other enzymes appeared singular in their reactivity pattern, including acetyl choline esterase (AchE) reacting more strongly with the mono- and diacetate esters **1a** and **1b**, and the weakly active RNL sample showing an enhanced reactivity with the C16- and oleic acid monoesters **10a** and **12a**.

3. Conclusion

The experiments above demonstrate that aliphatic esters of the periodate-activated chromogenic diols **13** and **14** are sensitive probes for lipase and esterase activities releasing nitrophenol and dinitrophenol, respectively. The mono- and diacetate derivatives exhibit a similarly selective reactivity for acetyl choline esterase (AchE). The monoesters with longer chain aliphatic acids show remarkable reactivities with both lipases and esterases. The most useful lipase probes are the C8-, C10-, and C12-monoesters of the nitrophenol-derived diol **13**. The corresponding dinitrophenol derived esters of diol **14** are generally much less reactive against lipases, which is probably caused by steric hindrance limiting access to the enzyme active sites. These reactivity differences are largely conserved across all enzymes tested.

The nitrophenol-derived substrates **3** (C8), **5a** (C10), and **6** (C12) are optimal to detect lipase and esterase activities with high sensitivity in unknown samples, and it is reasonable to assume that these substrates will react

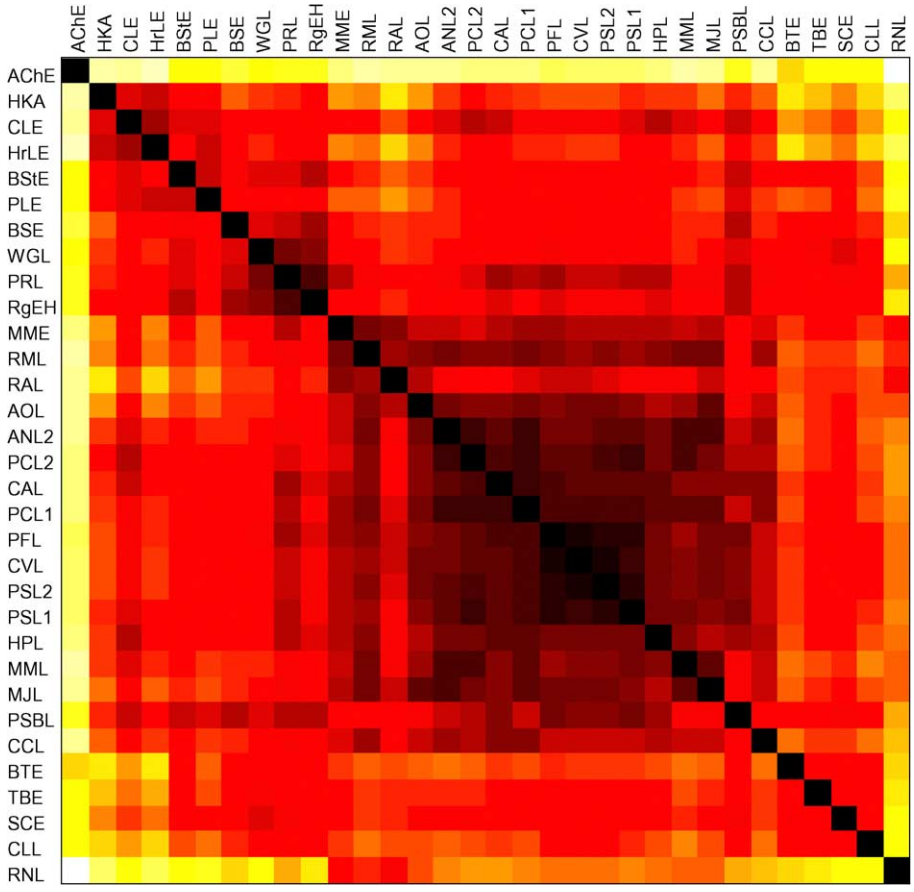


Figure 2. Color-coded representation of the dissimilarity matrix of the enzyme series according to their relative activity fingerprints across the substrates series **1a**–**12b**. Black = identity; white = maximum distance. The actual highest level (dissimilarity) is 0.376, and the agglomerative coefficient is 0.75.

with any lipase or esterase. Such a universal chromogenic lipase and esterase assay can be extremely useful for enzyme discovery during screening. For example, we have used the C10-ester **5a** to discover microbial thermophilic lipases by direct screening.[22] Evidently, it must then be coupled with a more detailed assay protocol to test the enzyme for reactivity and selectivity, including stereo- and enantioselectivity, against particular substrates of synthetic interest.

4. Experimental

4.1. General

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

4.2. (±)-1,2-Isopropylidene-4-(4-nitrophenyloxy)-1,2-butanediol 19

A solution of (±)-1,2-isopropylidene-4-(4-toluene-sulfonate)-1,2,4-butanetriol[23] (5 g, 16.6 mmol), 4-nitrophenol **17** (2.780 g, 20 mmol), K_2CO_3 (4.600 g, 33.2 mmol), and 18-C-6 (0.300 g, 1.1 mmol) in 100 mL acetone is stirred under reflux for 18 h. Acetone is then removed in vacuo, and the residue taken in EtOAc and water. The organic phase is washed with 0.1 N NaOH and brine, dried over $MgSO_4$ and concentrated to dryness. Crude **19** (5.440 g) is used without further purification for the next step. TLC (Hex/EtOAc 75/25, UV 254 nm): R_f 0.49; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.20 (d, J = 9.2 Hz, 2H), 6.95 (d, J = 9.2 Hz, 2H), 4.35–4.27 (m, 1H), 4.22–4.11 (m, 3H), 3.68–3.63 (m, 1H), 2.11–2.04 (m, 2H), 1.43 (s, 3H), 1.36 (s, 3H).

4.3. (±)-4-(4-Nitrophenyloxy)-1,2-butanediol 13

A solution of **19** (5.440 g) in 60 mL MeOH is degassed with nitrogen for 1 h. A solution of 0.1 N HCl (45 mL) is added, and the mixture is further degassed for 30 min. After 18 h stirring at rt under N_2, the solvent is removed in vacuo and the residue taken in EtOAc, washed with satd aq $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated. The crude residue is recrystallized in EtOAc/Hex to give 3.480 g (15.3 mmol, 92% from **19**) of (±)-4-(4-nitrophenoxy)-1,2-butanediol **13** as a yellow solid, mp 98–100 °C; TLC (EtOAc/Hex, 65/35, UV 254 nm): R_f 0.16; ^{1}H NMR (200 MHz, CD_3OD) δ (ppm): 8.2 (d, J = 9.3 Hz, 2H), 7.1 (d, J = 9.3 Hz, 2H), 4.37–4.14 (m, 2H), 3.93–3.76 (m, 1H), 3.53 (d, J = 5.9 Hz, 2H), 2.19–1.97 (m, 1H), 1.93–1.72 (m, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ (ppm): 165.7, 142.7, 126.8, 115.8, 69.9, 67.4, 66.8, 33.9; IR (KBr) ν (cm^{-1}): 3380 (s), 3275 (s), 3081 (w), 2964 (w), 2926 (w), 2880 (w), 1610 (s), 1597 (s), 1513 (s), 1499 (s), 1389 (m), 1333 (s), 1303 (m), 1265 (s), 1243 (m), 1176 (m), 1124 (m), 1105 (m), 1067 (m), 1046 (m) ; Elemental analysis: calcd for $C_{10}H_{13}NO_5$: C, 52.86%, H, 5.77%, N, 6.16%. Found: C, 52.55%, H, 5.91%, N, 5.90%

4.4. (±)-1,2-*O*-Isopropylidene-4-(2,4-dinitrophenyloxy)-1,2-butanediol 20

1,2-*O*-isopropylidene-1,2,4-butanetriol (0.5 g, 3.4 mmol) was treated with 2,4-dinitro fluorobenzene (636 mg, 3.42 mmol) and triethylamine (0.53 mL, 3.76 mmol). The dark brown solution was stirred for 6 h at 25 °C. Aqueous workup (diethyl ether/satd aq $NaHCO_3$, then brine) and FC (hexane/AcOEt 3/1–1/1) gave **20** (0.66 g, 2.00 mmol, 58%) as yellow oil; TLC (Hex/EtOAc 1/1) R_f 0.61; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.74 (d, 1H), 8.43 (dd, 1H), 7.24 (d, 1H), 4.36 (m, 3H), 4.14 (m, 1H), 3.67 (m, 1H), 2.12 (m, 2H), 1.41 (s, 3H), 1.35 (s, 3H). MS (EI) m/z: 313 (M^++1), 297, 167.

4.5. (±)-4-(2,4-Dinitrophenyloxy)-1,2-butanediol 14

The procedure for **13** was applied with **19** (0.6 g, 1.92 mmol) using MeOH (6 mL) and 0.1 N aq HCl (6 mL). FC (acetone/hexane 2/1, R_f 0.68) gave **14** (520 mg, 1.91 mmol, 99%) as pale yellow solid, mp 74 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.78 (d, 1H, J = 2.9 Hz), 8.44 (dd, 1H, J = 2.6 and 9.2 Hz), 7.26 (d, 1H, J = 9.5 Hz), 4.45 (m, 2H), 4.05 (m, 1H), 3.76 (dd, 1H, J = 11.0 and 3.3 Hz), 3.57 (dd, 1H, J = 11.0 and 6.6 Hz), 2.05 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 157.91, 141.32, 140.24, 130.15, 122.35, 116.22, 69.62, 68.80, 67.32, 33.61; IR (KBr) cm^{-1}: 3384 (b, s), 3116 (m), 2932 (m), 2889 (m), 1610 (s), 1529 (s), 1348 (s), 1289 (s); MS (EI) m/z: 241, 184, 168, 154.

4.6. (±)-(4-(*p*-Nitrophenyloxy-2-hydroxybut-1-yl)-butyrate 2

A solution of diol **13** (100 mg, 0.44 mmol) and dry triethylamine (89.0 mg, 0.88 mmol) in dry CH_2Cl_2 (6 mL) and dry DMF (1 mL) at 0 °C was treated with the butyroyl chloride (46 μL, 0.44 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at 25 °C until completion of the reaction (TLC). Aqueous workup (CH_2Cl_2/satd aq $NaHCO_3$ and brine), and FC (hexane/AcOEt 3/1–1/1) gave **2** (73 mg, 0.25 mmol, 56%) as pale yellow oil; TLC (Hex/EtOAc 1/1) R_f 0.65; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.20 (d, 2H), 6.96 (d, 2H), 4.04–4.29 (m, 5H), 2.34 (m, 2H), 1.99 (m, 2H), 1.68 (m, 2H), 0.97 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.50, 164.43, 142.25, 126.57, 115.12, 68.97, 67.69, 65.83, 36.67, 33.30, 19.06, 14.28; IR (film) cm^{-1}: 3525 (b, w), 2957 (s), 2927 (s), 2851 (m), 1732 (m), 1593 (m), 1510 (s), 1342 (s), 1263 (s); MS (FAB) m/z: 298 (M^+), 281, 210, 155, 119.

4.7. (±)-(4-(4-*p*-Nitrophenyloxy-2-hydroxybut-1-yl)-octanoate 3

The procedure for **2** was applied using octanoyl chloride (76 μL, 0.44 mmol). FC gave **3** (97 mg, 0.27 mmol, 62%)

as pale yellow solid, mp 38 °C; TLC (Hex/EtOAc 1/1) R_f 0.68; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.20 (d, 2H), 6.96 (d, 2H), 4.04–4.32 (m, 5H), 2.36 (m, 2H), 1.89–2.08 (m, 2H), 1.64 (m, 2H), 1.28 (m, 8H), 0.88 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): 174.67, 164.43, 142.23, 126.54, 115.10, 68.97, 67.65, 65.83, 34.80, 33.30, 32.26, 29.71, 29.52, 25.56, 23.20, 14.66; IR (film) cm^{-1}: 3505 (b, w), 2957 (m), 2930 (m), 2858 (m), 1731 (m), 1594 (s), 1512 (s), 1342 (s), 1264 (s); MS (FAB) *m/z*: 354 (M^+), 337, 215, 210, 127.

4.8. (±)-(4-(2,4-Dinitrophenyloxy)-2-hydroxybut-1-yl)-octanoate 4a and (±)-(4-(2,4-dinitrophenyloxy)-2-octanoyloxybut-1-yl)-octanoate 4b

A solution of diol **14** (100 mg, 0.37 mmol), triethylamine (153 μL, 1.1 mmol) and 4-dimethylamino-pyridine (2.5 mg, 0.02 mmol) in CH_2Cl_2 (6 mL) was treated at 0 °C with octanoyl chloride (95 μL, 0.55 mmol). The reaction mixture was stirred for 1 h at 0 °C, then at 25 °C until completion of the reaction (5 h, TLC). Aqueous workup (CH_2Cl_2/satd aq $NaHCO_3$, then brine) and separation by FC (hexane/AcOEt 3/1–1/1) gave first **4b** (36 mg, 0.07 mmol, 18%) as pale yellow oil, then **4a** (74 mg, 0.19 mmol, 50%) as pale yellow oil. Compound **4a**: TLC (Hex/EtOAc 1/1) R_f 0.50; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.77 (d, 1H), 8.44 (dd, 1H), 7.25 (d, 1H), 4.06–4.52 (m, 5H), 2.36 (m, 2H), 2.06 (m, 2H), 1.64 (m, 2H), 1.28 (m, 8H), 0.87 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.81, 157.29, 140.80, 139.35, 129.94, 122.69, 115.07, 68.85, 67.99, 67.37, 34.79, 32.95, 32.28, 29.73, 29.55, 25.55, 23.23, 14.70; IR (film) cm^{-1}: 3565 (b, m), 3094 (w), 2956 (s), 2930 (s), 2858 (s), 1732 (s), 1610 (s), 1538 (s), 1344 (s), 1288 (s); MS (FAB) *m/z*: 399 (M^++1), 381, 369, 215, 127.

Compound **4b**: TLC (Hex/EtOAc 1/1) R_f 0.83; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.76 (d, 1H, $H_{3'}$), 8.42 (dd, 1H), 7.17 (d, 1H), 5.30 (m, 1H), 4.08–4.36 (m, 4H), 2.30 (m, 4H), 2.20 (m, 2H), 1.60 (m, 4H), 1.26 (m, 16H), 0.87 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) ppm: 174.04, 173.86, 157.03, 141.01, 139.65, 129.74, 122.69, 114.93, 69.14, 67.74, 65.17, 34.95, 34.77, 32.33, 31.22, 29.74, 29.58, 25.61, 25.56, 23.26, 14.71; IR (film) cm^{-1}: 3031 (w), 2957 (m), 2930 (s), 2858 (m), 1736 (s), 1609 (s), 1534 (s), 1345 (s), 1288 (s); MS (FAB) *m/z*: 525 (M^+), 495, 381, 341, 127.

4.9. (±)-(4-(4-Nitrophenyloxy)-2-hydroxybut-1-yl)-decanoate 5a and (±)-(4-(4-nitrophenyloxy)-2-decanoyloxybut-1-yl)-decanoate 5b

Triethylamine (1.4 mL, 9.5 mmol) is added under N_2 to a solution of (±)-4-(4-nitrophenoxy)-1,2-butanediol **13** (1.080 g, 4.75 mmol) in dry DCM (40 mL) and DMF (10 mL), and the mixture is cooled to 0 °C. Capric acid chloride (1.200 g, 6.3 mmol) is then added, and the mixture stirred at rt for 6 h. After concentration to dryness the crude product is taken in EtOAc and water, washed with satd aq $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated. The residue is finally purified by flash chromatography on silica gel (Hex/EtOAc 75/25–50/50) to give compounds **5a** (1.503 g, 83%) and **5b** (0.117 g, 5%).

Compound **5a**: yellow solid, mp 41 °C; TLC (Hex/EtOAc 50/50, UV 254 nm): R_f 0.80; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.19 (d, J = 8.5 Hz, 2H), 6.95 (d, J = 8.8 Hz, 2H), 4.31–4.04 (m, 5H), 2.38–2.33 (m, 2H), 2.09–1.89 (m, 2H), 1.66–1.61 (m, 2H), 1.28–1.27 (m, 12H), 0.89–0.85 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.7, 164.4, 142.3, 126.6, 115.1, 69.0, 67.7, 65.8, 34.8, 33.3, 32.5, 30.1, 29.9, 25.6, 23.3, 14.8; IR (neat) ν (cm^{-1}): 3479 (w), 2928 (s), 2856 (s), 1737 (s), 1609 (m), 1595 (s), 1515 (s), 1499 (s), 1468 (m), 1343 (s), 1299 (m), 1264 (s), 1174 (s), 1112 (s); HR-ESI-MS: calcd for $C_{10}H_{32}NO_6^+$: 382.2229. Found: 382.2221.

Compound **5b**: yellow oil; TLC (Hex/EtOAc 90/10, UV 254 nm): R_f 0.30; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.19 (d, J = 7.0 Hz, 2H), 6.93 (d, J = 7.3 Hz, 2H), 5.32 (m, 1H), 4.36–4.31 (m, 1H), 4.17–4.08 (m, 3H), 2.35–2.27 (m, 4H), 2.15–2.13 (m, 2H), 1.64–1.56 (m, 6H), 1.26–1.24 (m, 24H), 0.89–0.85 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.1, 173.8, 164.2, 142.4, 126.6, 115.1, 69.2, 65.4, 65.3, 35.0, 34.8, 32.5, 31.2, 30.1, 29.9, 29.8, 25.6, 23.3, 14.7; IR (neat) ν (cm^{-1}): 2928 (s), 2856 (s), 1741 (s), 1610 (m), 1595 (s), 1518 (s), 1499 (m), 1468 (m), 1343 (s), 1299 (m), 1263 (s), 1173 (s), 1112 (s); HR-EI-MS: calcd for $C_{30}H_{49}NO_7$: 535.3509. Found: 535.3526.

4.10. (±)-(4-(4-Nitrophenyloxy-2-hydroxybut-1-yl)-dodecanoate 6

The procedure for **2** was applied using dodecanoyl chloride (104 μL, 0.44 mmol). FC gave **6** (105 mg, 0.26 mmol, 58%) as pale yellow solid, mp 48 °C; TLC (Hex/EtOAc 1/1) R_f 0.70; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.20 (d, 2H), 6.96 (d, 2H), 4.04–4.30 (m, 5H), 2.34 (m, 2H), 1.99 (m, 2H), 1.64 (m, 2H), 1.25 (m, 16H), 0.85 (t, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.72, 164.41, 142.26, 126.60, 115.11, 69.01, 67.72, 65.81, 34.83, 33.28, 32.56, 30.25, 30.10, 29.98, 29.91, 29.80, 25.59, 23.34, 14.77; IR (film) cm^{-1}: 3505 (b, w), 3028 (w), 2927 (s), 2855 (m), 1731 (s), 1594 (s), 1512 (s), 1342 (s), 1263 (s); MS (FAB) *m/z*: 410 (M^+), 393, 271, 210, 183, 119.

4.11. (±)-(4-(2,4-Dinitrophenyl)-2-hydroxybut-1-yl)-dodecanoate 7a and (±)-(4-(2,4-dinitrophenyl)-2-dodecanoyloxy-but-1-yl)-dodecanoate 7b

The procedure for **5a/b** was applied using dodecanoyl chloride (133 μL, 0.56 mmol). FC gave first **7b** (70.3 mg, 0.11 mmol, 30%) as yellow solid, mp 35 °C, then **7a** (91 mg, 0.2 mmol, 54%) as yellow solid, mp 42 °C.

Compound **7a**: TLC (Hex/EtOAc 1/1) R_f 0.44; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.78 (d, 1H), 8.44 (dd, 1H), 7.24 (d, 1H), 4.06–4.52 (m, 5H), 2.36 (m, 2H), 2.05 (m, 2H), 1.62 (m, 2H), 1.25 (m, 16H), 0.86 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) ppm: 174.78, 157.28, 140.86, 139.44, 129.87, 122.66, 115.09, 66.85, 68.03, 67.42,

34.81, 33.01, 32.54, 30.24, 30.09, 29.96, 29.89, 29.79, 25.57, 23.32, 14.74; IR (film) cm^{-1}: 3566 (b, w), 2927 (s), 2855 (m), 1734 (m), 1610 (s), 1538 (s), 1344 (s), 1288 (s); MS (FAB) *m/z*: 455 (M^+), 437, 425, 271, 183, 109.

Compound **7b**: TLC (Hex/EtOAc 1/1) R_f 0.88; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.76 (d, 1H), 8.42 (dd, 1H), 7.17 (d, 1H), 5.30 (m, 1H), 4.12–4.36 (m, 4H), 2.31 (m, 4H), 2.21 (m, 2H), 1.59 (m, 4H), 1.24 (m, 32H), 0.87 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.02, 173.84, 157.02, 140.99, 139.41, 129.72, 122.66, 114.94, 69.14, 67.73, 65.15, 34.95, 34.87, 34.76, 32.57, 31.20, 30.28, 30.15, 30.00, 29.94, 29.89, 29.81, 29.79, 25.61, 25.55, 23.34, 14.76; IR (film) cm^{-1}: 3094 (w), 2927 (s), 2855 (s), 1736 (s), 1609 (s), 1534 (s), 1345 (s), 1287 (s); MS (FAB) *m/z*: 636 (M^+), 618, 606, 453, 183.

4.12. (±)-(4-(4-Nitrophenyloxy-2-hydroxybut-1-yl)-tetradecanoate 8

The procedure for **2** was applied using tetradecanoyl chloride (119 μL, 0.44 mmol). FC gave **8** (126 mg, 0.29 mmol, 65%) as pale yellow solid, mp 81 °C; TLC (Hex/EtOAc 1/1) R_f 0.77; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.20 (d, 2H), 6.96 (d, 2H), 4.04–4.29 (m, 5H), 2.36 (t, 2H), 1.98 (m, 2H), 1.64 (m, 2H), 1.25 (m, 20H), 0.88 (t, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) ppm: 174.70, 164.42, 142.33, 126.61, 115.13, 69.03, 67.79, 65.85, 34.85, 33.32, 32.58, 30.31, 30.13, 30.01, 29.92, 29.82, 25.61, 23.35, 14.77; IR (film) cm^{-1}: 3506 (b, w), 2926 (s), 2854 (m), 1735 (m), 1594 (m), 1516 (s), 1342 (s), 1263 (s); MS (FAB) *m/z*: 438 (M^+), 421, 299, 284, 211, 124.

4.13. (±)-(4-(2,4-Dinitrophenyl)-2-hydroxybut-1-yl)-tetradecanoate 9a and (±)-(4-(2,4-dinitrophenyl)-2-tetradecanoyloxybut-1-yl)-tetradecanoate 9b

The procedure for **5a/b** was applied using diol **14** (60 mg, 0.24 mmol), triethylamine (100 μL, 0.72 mmol), DMAP (1.5 mg, 0.01 mmol) in CH_2Cl_2 (4 mL). FC gave first **9b** (46 mg, 0.07 mmol, 27%) as yellow solid, mp 44 °C, then **9a** (49 mg, 0.1 mmol, 42%) as yellow solid, mp 53 °C.

Compound **9a**: TLC (Hex/EtOAc 1/3) R_f 0.82; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.78 (d, 1H), 8.45 (dd, 1H), 7.24 (d, 1H), 4.06–4.52 (m, 5H), 2.36 (m, 2H), 2.05 (m, 2H), 1.63 (m, 2H), 1.26 (m, 20H), 0.87 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) ppm: 174.80, 157.29, 140.91, 136.74, 129.88, 122.69, 115.08, 68.89, 68.03, 67.46, 34.82, 33.03, 32.58, 30.34, 30.30, 30.26, 30.11, 30.01, 29.92, 29.82, 25.59, 23.35, 14.76; IR (film) cm^{-1}: 3467 (b, w), 3090 (w), 3026 (w), 2926 (s), 2854 (s), 1732 (s), 1610 (s), 1538 (s), 1345 (s), 1289 (s); MS (FAB) *m/z*: 465(M^++1−OH), 453, 299, 211, 167, 149, 113.

Compound **9b**: TLC (Hex/EtOAc 1/3) R_f 0.97; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.76 (d, 1H), 8.42 (dd, 1H), 7.17 (d, 1H), 5.30 (m, 1H), 4.08–4.36 (m, 4H), 2.31 (m, 4H), 2.19 (m, 2H), 1.58 (m, 4H), 1.25 (m, 40H), 0.88 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.05, 173.87, 157.03, 140.44, 139.67, 129.74, 122.70, 114.93, 69.16, 67.75, 65.17, 34.98, 34.78, 32.61, 31.23, 30.34, 30.18, 30.04, 29.97, 29.82, 25.64, 25.57, 23.37, 14.79; IR (film) cm^{-1}: 3031 (w), 2925 (s), 2854 (s), 1737 (s), 1609 (s), 1534 (s), 1344 (s), 1288 (s); MS (FAB) *m/z*: 693 (M^+), 663, 607, 509, 465, 284.

4.14. (±)-(4-(4-Nitrophenyloxy)-2-hydroxybut-1-yl)-hexadecanoate 10a and (±)-(4-(4-nitrophenyloxy)-2-hexadecanoyloxybut-1-yl)-hexadecanoate 10b

Triethylamine (0.7 mL, 4.5 mmol) and DMAP (0.0025 g, 0.02 mmol) are added under N_2 to a solution of (±)-4-(4-nitrophenoxy)-1,2-butanediol **13** (0.500 g, 2.2 mmol) in dry DCM (20 mL) and DMF (5 mL), and the mixture is cooled to 0 °C. Palmitoyl chloride (0.910 g, 3.3 mmol) is then added, and the mixture stirred at rt for 18 h. After concentration to dryness the crude product is taken in EtOAc and water, washed with satd aq $NaHCO_3$, and brine, dried over $MgSO_4$ and concentrated. The residue is finally purified by flash chromatography on silica gel (Hex/EtOAc 100/0–50/50) to give compounds **10a** (0.808 g, 79%) and **10b** (0.032 g, 2%).

Compound **10a**: yellow oil; TLC (Hex/EtOAc 50/50, UV 254 nm): R_f 0.80; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.18 (d, J = 9.2 Hz, 2H), 6.95 (d, J = 9.2 Hz, 2H), 4.28–4.03 (m, 5H), 2.37–2.32 (t, J = 7.5 Hz, 2H), 2.03–1.93 (m, 2H), 1.65–1.61 (m, 2H), 1.25–1.23 (m, 24H), 0.89–0.85 (t, J = 6.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.7, 164.4, 142.3, 126.6, 115.1, 69.0, 67.7, 65.8, 34.8, 33.3, 32.6, 30.3, 30.2, 30.2, 30.0, 29.9, 29.8, 25.6, 23.3, 14.8; IR (neat) ν (cm^{-1}): 3479 (m), 2926 (s), 2855 (s), 1736 (s), 1609 (m), 1595 (s), 1515 (s), 1499 (s), 1468 (m), 1343 (s), 1264 (s), 1174 (s), 1112 (s).

Compound **10b**: yellow solid, mp 50–51 °C; TLC (Hex/EtOAc 90/10, UV 254 nm): R_f 0.38; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.19 (d, J = 9.5 Hz, 2H), 6.93 (d, J = 9.5 Hz, 2H), 5.36–5.31 (m, 1H), 4.32 (dd, J = 3.7, 12.1 Hz, 1H), 4.16–4.08 (m, 3H), 2.33–2.27 (m, 4H), 2.17–2.10 (m, 2H), 1.63–1.57 (m, 4H), 1.25–1.23 (m, 48H), 0.90–0.85 (t, J = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 173.8, 164.2, 145.6, 126.6, 115.1, 69.2, 65.4, 65.3, 35.0, 34.8, 32.6, 31.2, 30.4, 30.2, 30.0, 29.8, 25.7, 25.6, 23.4, 14.8; IR (Chl) ν (cm^{-1}): 3025 (w), 2926 (s), 2855 (s), 1741 (s), 1610 (m), 1595 (m), 1518 (m), 1499 (m), 1468 (m), 1343 (s), 1263 (s), 1173 (m), 1112 (m) ; EI-MS: 704 $[M]^+$.

4.15. (±)-(4-(4-Nitrophenyloxy)-2-hydroxybut-1-yl)-octadecanoate 11a and (±)-(4-(4-nitrophenyloxy)-2-octadecanoyloxybut-1-yl)-octadecanoate 11b

Triethylamine (1.4 mL, 9.5 mmol) and DMAP (0.0025 g, 0.02 mmol) are added under N_2 to a solution of (±)-4-(4-nitrophenoxy)-1,2-butanediol **13** (0.500 g, 2.2 mmol) in dry DCM (20 mL) and DMF (5 mL), and the mixture is cooled to 0 °C. Stearoyl chloride (1 g, 3.3 mmol) is then added, and the mixture stirred at rt for 18 h. After concentration to dryness the crude product is taken in EtOAc and water, washed with satd aq $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated. The residue is finally purified by flash chromatography on silica gel

(Hex/EtOAc 90/10–50/50) to give compounds **11a** (0.680 g, 63%) and **11b** (0.206 g, 12%).

Compound **11a**: yellow oil; TLC (Hex/EtOAc 50/50, UV 254 nm): R_f 0.80; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.17 (d, J = 9.2 Hz, 2H), 6.94 (d, J = 9.2 Hz, 2H), 4.27–4.05 (m, 5H), 2.36–2.31 (m, 2H), 2.00 (m, 2H) 1.64–1.59 (m, 2H), 1.25–1.21 (m, 28H), 0.87–0.83 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.7, 164.4, 126.6, 115.1, 69.0, 67.8, 65.8, 65.3, 35.1, 34.9, 33.3, 32.6, 30.9, 30.4, 30.3, 30.1, 30.0, 29.9, 29.8, 25.7, 25.6, 23.4, 14.8; IR (neat) ν (cm^{-1}): 3492 (w), 3022 (w), 2927 (s), 2855 (s), 1736 (s), 1609 (m), 1595 (s), 1515 (s), 1499 (s), 1468 (m), 1343 (s), 1264 (s), 1174 (s), 1113 (s); EI-MS: 493 $[M]^+$; HR-EI-MS: calcd for $C_{28}H_{47}NO_6$: 493.3403. Found: 493.3403.

Compound **11b**: yellow solid, mp 58–59 °C; TLC (Hex/EtOAc 90/10, UV 254 nm): R_f 0.41; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.19 (d, J = 9.2 Hz, 2H), 6.93 (d, J = 9.2 Hz, 2H), 5.35–5.30 (m, 1H), 4.36–4.31 (m, 1H), 4.16–4.08 (m, 3H), 2.53–2.46 (m, 2H), 2.34–2.27 (m, 2H), 2.15–2.13 (m, 2H), 1.63–1.56 (m, 6H), 1.26–1.24 (m, 56H), 0.90–0.85 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.0, 170.0, 164.1, 126.6, 115.2, 70.3, 70.1, 65.3, 65.2, 65.1, 60.1, 59.6, 42.8, 42.6, 34.8, 32.6, 31.1, 30.4, 30.2, 30.1, 30.0, 29.9, 29.8, 29.0, 28.3, 25.5, 24.2, 23.4, 14.8; IR ($CHCl_3$) ν (cm^{-1}): 3025 (w), 2926 (s), 2855 (s), 1739 (s), 1610 (m), 1595 (s), 1518 (s), 1499 (m), 1468 (m), 1344 (s), 1263 (s), 1216 (s), 1173 (s), 1113 (s).

4.16. (±)-(4-(4-Nitrophenyloxy)-2-hydroxybut-1-yl)-*cis*-9-octadecenoate 12a and (±)-(4-(4-nitrophenyloxy)-2-(*cis*-9-octadecenoyl)oxybut-1-yl)-*cis*-9-octadecenoate 12b

Triethylamine (0.7 mL, 4.5 mmol) and DMAP (0.0025 g, 0.02 mmol) are added under N_2 to a solution of (±)-4-(4-nitrophenoxy)-1,2-butanediol **13** (0.500 g, 2.2 mmol) in dry DCM (20 mL) and DMF (5 mL), and the mixture is cooled to 0 °C. Oleoyl chloride (1 g, 3.3 mmol) is then added, and the mixture stirred at rt for 18 h. After concentration to dryness the crude product is taken in EtOAc and water, washed with satd aq $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated. The residue is finally purified by flash chromatography on silica gel (Hex/EtOAc 100/0–50/50) to give compounds **12a** (0.644 g, 60%) and **12b** (0.025 g, 2%).

Compound **12a**: yellow oil; TLC (Hex/EtOAc 50/50, UV 254 nm): R_f 0.80; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.19 (d, J = 9.2 Hz, 2H), 6.96 (d, J = 9.2 Hz, 2H), 5.36–5.32 (m, 2H), 4.32–4.04 (m, 5H), 2.38–2.33 (m, 2H), 2.05–1.95 (m, 6H), 1.64–1.61 (m, 2H), 1.30–1.26 (m, 20H), 0.88–0.85 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.7, 164.4, 142.2, 130.6, 130.3, 126.5, 115.1, 72.7, 68.9, 67.6, 65.8, 65.6, 65.1, 35.0, 34.8, 33.2, 32.5, 30.8, 30.4, 30.3, 30.1, 29.9, 29.8, 29.7, 27.8, 25.5, 23.3, 14.7; IR (neat) ν (cm^{-1}): 3481 (m), 3006 (m), 2927 (s), 2856 (s), 1739 (s), 1609 (s), 1595 (s), 1516 (s), 1499 (s), 1467 (s), 1343 (s), 1264 (s), 1174 (s), 1112 (s); EI-MS: 491 $[M]^+$; HR-ESI-MS: calcd for $C_{28}H_{46}NO_6^+$: 492.3325. Found: 492.3321.

Compound **12b**: yellow oil; TLC (Hex/EtOAc 90/10, UV 254 nm): R_f 0.38; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.19 (d, J = 9.2 Hz, 2H), 6.93 (d, J = 9.2 Hz, 2H), 5.37–5.32 (m, 5H), 4.33 (dd, J = 3.7, 12.1 Hz, 1H), 4.17–4.08 (m, 3H), 2.34–2.27 (m, 4H), 2.17–2.10 (m, 2H), 2.01–1.99 (m, 8H), 1.61–1.57 (m, 4H), 1.30–1.26 (m, 40H), 0.90–0.85 (t, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 173.8, 164.2, 142.1, 130.8, 130.4, 130.3, 126.6, 115.1, 69.2, 65.3, 35.0, 34.8, 32.6, 30.4, 30.2, 30.0, 29.9, 29.8, 27.9, 25.6, 23.4, 14.8; IR (neat) ν (cm^{-1}): 2928 (s), 2857 (m), 1737 (m), 1646 (s), 1518 (w), 1499 (w), 1467 (w), 1343 (w), 1263 (w), 1216 (w), 1174 (w), 1112 (w).

5. Enzyme measurements

All substrates were diluted from 1 mM stock solutions in 50% aq DMF. The solutions could be stored over months at +4 °C without noticeable decomposition of the substrates. Commercial enzyme samples were freshly prepared by weighting the solid sample and diluting it to a stock solution of 1 mg/mL in PBS (aq 10 mM phosphate, 160 mM NaCl, pH 7.4). BSA (bovine serum albumin) was diluted from a 40 mg/mL stock solution in 20 mM aqueous borate buffer pH 8.8. Sodium periodate ($NaIO_4$) was freshly prepared as a 10 mM stock solution in water. Measurements were carried out in 96-well half-area clear-bottom polystyrene microtiter plates. Substrate solutions (10 μL) were first placed in the wells. A prediluted solution of 2.5 mL containing the enzyme at the indicated concentration, BSA (2 mg/mL) and sodium periodate (1 mM) in aq 20 mM borate buffer pH 8.8 was prepared, and 90 μL were added to each substrate-containing well. The microtiter plates were then placed in a Spectramax 250 Microplate Spectrophotometer (Molecular Devices) and the reaction followed at 405 nm over 1 h. The primary OD-versus-time data were first converted to product-versus-time using a calibration curve with pure nitrophenol or dinitrophenol under the same conditions. The steepest linear portion of each curve was then used to calculate the reaction rate in each well. There was no significant reaction with buffer, $NaIO_4$, and BSA under the reaction conditions in the absence of enzyme.

6. Data treatment

All data were processed in MS-Excel. A table with four columns and five rows containing the observed reaction rates with the different substrates following the layout in Figure 1 was assembled. For each enzyme, a second table was then assembled with 12 columns and 5 rows. Starting at the first row and the first column, each group of three entries on the same rows was filled with the rate data measured with the enzyme at 0.1, 0.01, and 0.001 mg/mL, again following the layout of substrates. All rate data in the table were then divided by the maximum rate in the table to give relative reaction rates V_{rel}. The color intensity factors F were then computed according to the formula $F = 255(1 - V_{rel})$. The resulting numbers were rounded to 0 decimal, and saved in the 'comma separated value' file format. The file was then

re-opened in a text editor and the following four lines were inserted at the top:

line 1: P3
line 2: # (optional line with identification)
line 3: 4 5
line 4: 255

The file was then saved in the 'portable pixel map' format by adding '.ppm' to the file name. This new file is then opened in a graphical program such as COREL-DRAW, PHOTOSHOP, or PAINTSHOP, and resized to approximately 100 pixel broad for visualization.

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