Low background FRET-substrates for lipases and esterases suitable for high-throughput screening under basic (pH 11) conditions†

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FRET-based fluorogenic substrates for lipases and esterases were prepared in four steps from commercially available building blocks. The substrates are pyrenebutyric acid monoesters of aliphatic 1,2-diols bearing a dinitrophenylamino group as a quencher. The most enzyme-reactive substrate is ester **2a**. The substrates do not show any measurable background reaction in the absence of enzyme even at pH 11, but react fast and specifically with lipases and esterases. These substrates offer an unprecedented and practical solution to the long-standing problem of a simple yet efficient high-throughput screening tool for lipase activities under basic conditions.

Introduction

High-throughput screening (HTS) enzyme assays are essential tools in biotechnology, in particular for enzyme discovery and engineering.[1] Many enzyme assays are based on fluorogenic and chromogenic substrates. Although such assays may not be desirable for directed evolution experiments, they are particularly useful and popular as reporter assays for routine work and discovery because they are generally adaptable to a range of conditions and provide an operationally simple yet reliable test for enzyme activity. Such assays ideally rely on substrates that are chemically stable and react only with the target enzyme. Substrate stability against non-specific degradation is particularly problematic in assays for lipases and esterases, which is one of the most important enzyme classes.[2] The standard assays use aliphatic esters of nitrophenol and umbelliferone, which show a high level of non-specific degradation due to the strong leaving group ability of the acidic phenol,[3,4] although the problem is suppressed when these substrates are adsorbed onto silicagel prior to the assay.[5] The resistance to non-specific degradation can be improved by using acyloxymethyl ethers of umbelliferone,[6] or acylated cyanohydrins,[7] but these fluorogenic esters are still relatively unstable. HTS assays are also possible with the much less reactive esters of aliphatic alcohols such as tributyrin using indirect assays based on pH-indicators,[8] pH-titration,[9] back-titration of released glycerol,[10] or coupled enzymes,[11] however the requirement for auxiliary reagents renders the procedure inherently more complicated and less flexible.

We have recently reported fluorogenic lipase substrates based on monoesters of 1,2-diols in which the fluorescence signal was released by a selective oxidation of the 1,2-diol product with sodium periodate,[12] a strategy which is also suitable for releasing volatile perfumes from stable precursors.[13] Such substrates are aliphatic esters, and as such they are remarkably resistant towards non-specific degradation. These monoacylated 1,2-diols proved remarkably reactive towards lipases or esterases, but the requirement for an oxidative step based on sodium periodate proved problematic for many applications, in particular for testing in media containing oxidation sensitive compounds such as carbohydrates or amino alcohol buffers.

Herein we report fluorogenic substrates **1a–15a** for lipases and esterases having a similar 1,2-diol core, but which are based on fluorescence resonance energy transfer (FRET) as a signalling mechanism (Scheme 1). The substrates are highly reactive with lipases and esterases, yet show essentially no background reaction in the absence of enzyme. Substrates **1a–15a** are prepared in four steps from commercially available building blocks, which makes them more readily available than the structurally more complex FRET-type lipase substrates reported previously.[14] In particular substrate **2a** shows a particularly high reactivity across a broad range of enzymes while being essentially stable even at pH 11 in the absence of an enzyme.

Results and discussion

Substrate design

FRET-signalling is possible in principle for any bond cleaving enzyme. The method requires a double labeling of the substrate, but leaves the reactivity of the enzyme-labile bond unchanged and therefore allows the use of non-activated functional groups. FRET-based enzyme activity detection has been used in doubly labeled peptides for protease assays.[15] Several FRET-substrates have also been reported for lipases and phospholipases, all of which are close structural analogs of triglycerides.[14] However, an exact structural match to naturally occurring substrates should not be necessary to obtain lipase/esterase reactive FRET-substrates. Indeed many highly reactive lipase probes are not close analogues of natural substrates, with many changes possible on the alcohol side of the ester. Nevertheless, the presence of an aliphatic acid such as octanoic acid is generally necessary to obtain high lipase reactivity, while esterases prefer butyrate esters.

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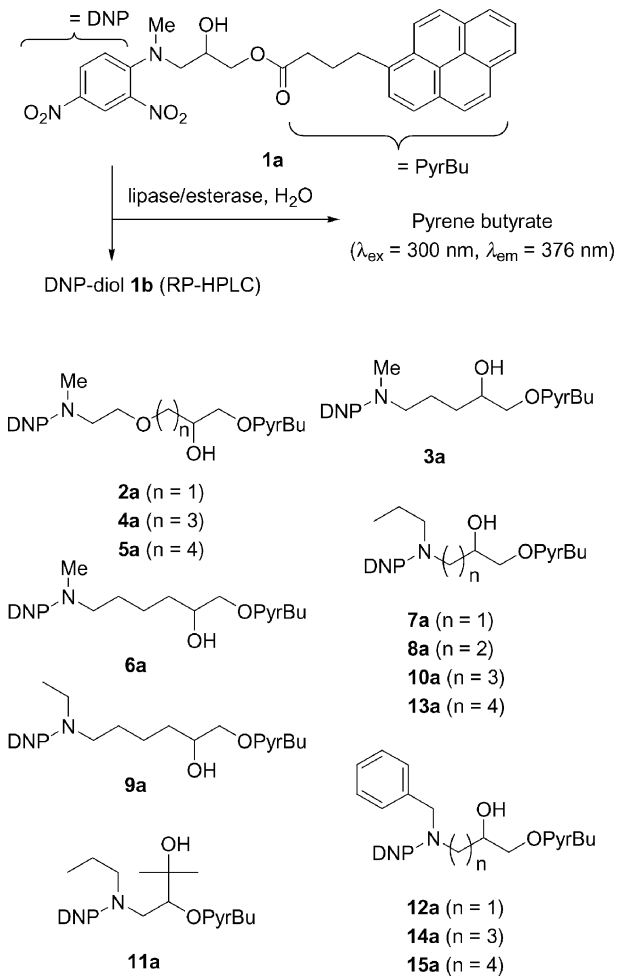
† Electronic supplementary information (ESI) available: Rate data for all enzyme–substrate pairs tested; cocktail assay rate and HPLC traces; NMR and MS spectra of all compounds. See DOI: 10.1039/b601151a



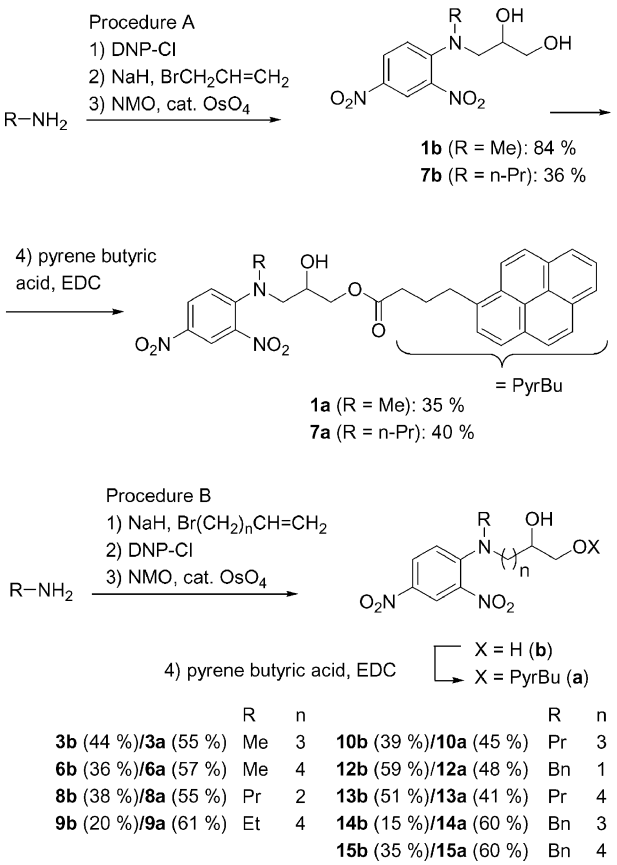
Scheme 1 FRET-fluorogenic substrates for lipases and esterases.

We set out to prepare FRET-signalling lipase/esterase substrates using pyrenebutyric acid as a fluorescent label. This label would be attached to a dinitrophenylamino group as a quencher by mono-esterification to an enzyme reactive 1,2-diol. Variations of the spacer in terms of branching, hydrophobicity and length were envisioned to optimize the substrates towards enzyme reactivity.

Synthesis

Aliphatic 1,2-diols are readily prepared by OsO_4-catalyzed dihydroxylation of the corresponding alkenes, a reaction which is compatible with the presence of chromophores and suitable for the preparation of enzyme substrates.[16] The preparation of DNP-labeled aliphatic alkenes was carried out by the derivatization of aliphatic primary amines with DNP chloride, followed by a second alkylation at the nitrogen with allyl bromide. The crude alkenes were then directly submitted to OsO_4-catalyzed dihydroxylation to yield the dinitrophenylamino-labeled aliphatic 1,2-diols, which were purified by column chromatography. This procedure A was used for the preparation of DNP-labeled alkenes **1b** (from methylamine), and **7b** (from propylamine) (Scheme 2).

The *N*-alkylation of the DNP-labeled amine intermediate used with allyl bromide did not proceed with other less reactive alkenyl bromides, in particular 4-bromobutene, 5-bromopentene and 6-bromohexene. In these cases the desired 1,2-diol products



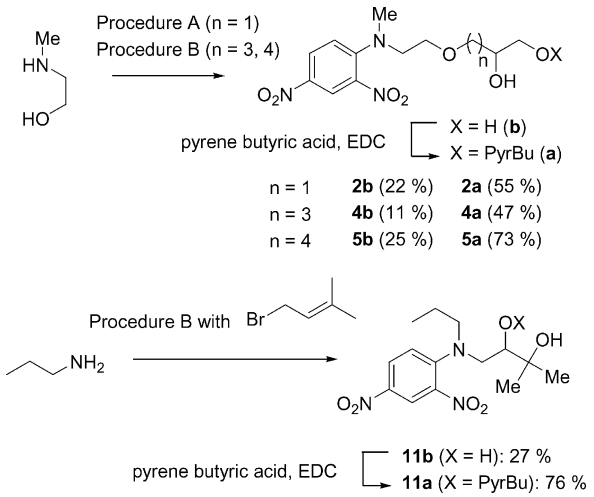
	R	n		R	n
3b (44 %)/**3a** (55 %)	Me	3	**10b** (39 %)/**10a** (45 %)	Pr	3
6b (36 %)/**6a** (57 %)	Me	4	**12b** (59 %)/**12a** (48 %)	Bn	1
8b (38 %)/**8a** (55 %)	Pr	2	**13b** (51 %)/**13a** (41 %)	Pr	4
9b (20 %)/**9a** (61 %)	Et	4	**14b** (15 %)/**14a** (60 %)	Bn	3
			15b (35 %)/**15a** (60 %)	Bn	4

Scheme 2 Synthesis of fluorogenic FRET substrates.

were obtained through protocol B in which the aliphatic amine (methyl-, ethyl-, propyl-, or benzylamine) was first reacted with 0.5 equivalents of the alkenyl bromide in DMF in the presence of sodium hydride, followed by the addition of DNP–Cl. After aqueous work-up the crude products were then subjected to catalytic dihydroxylation to give the 1,2-diols, which were separated from the byproducts by column chromatography and isolated in low to moderate yields. This procedure was used in various combinations to give 1,2-diols **3b**, **6b**, **8b**, **9b**, **10b**, **12b**, **13b**, **14b**, and **15b**.

Additional 1,2-diols incorporating an ethylene glycol unit as a spacer were prepared starting with *N*-methylethanolamine. In this case alkylation with the alkyl bromide involved ether bond formation at the free hydroxyl group, yielding diols **2b**, **4b** and **5b** with allyl bromide, 5-bromopentene and 6-bromohexene, respectively, under procedure B (Scheme 3). Finally, the sterically more demanding secondary alcohol **11b** was prepared starting with 1-bromo-3-methyl-2-butene and propylamine using protocol B.

The 15 different 1,2-diols **1b–15b** were then converted to the corresponding mono-esters by coupling with pyrenebutyric acid (Scheme 2 and 3). A previously reported procedure for the monoesterification of 1,2-diols involving the reaction with the acyl halide in dichloromethane in the presence of pyridine or collidine did not work in this case.[10] We therefore turned to diimide-promoted coupling. Esterification to the mono-esters was carried out by the reaction of pyrenebutyric acid with *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) in dichloromethane



Scheme 3 Synthesis of FRET-fluorogenic lipase substrates.

and pyridine as the solvent. The reaction produced the monoesters **1a–15a** in moderate yields as the only products beside unreacted 1,2-diols.

Enzyme assays

Substrates **1a–15a** were conditioned as 2 mM stock solutions in DMF. A series of 36 commercially available lipases and esterases were prepared as 1 mg mL^{-1} stock solutions. The assays were conducted at 25 °C in aqueous PBS buffer pH 7.4 containing 30% v/v DMSO to ensure complete solubility of the substrates, using 50 μg mL^{-1} enzyme and 10 μM of each substrate. The assays were followed by fluorescence (λ_{ex} = 300 nm, λ_{em} = 376 nm) in a microtiter plate reader instrument (Fig. 1). While the background reaction in the absence of any enzyme was not measurable with any of the substrates, almost all substrates reacted well with the different enzymes, with the exception of the secondary ester **11b**, which only reacted with three enzymes.

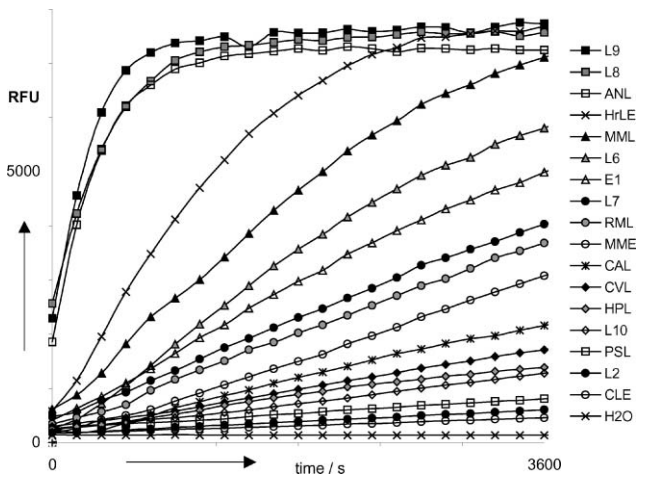


Fig. 1 Fluorescence assay of selected lipases and esterases with substrate **12a**. Conditions: 10 μM **12a** in aq. PBS pH 7.4 with 30% v/v DMSO, 50 μg ml^{-1} enzyme, 25 °C. 100 μL assays were run in 96-well polystyrene plates. Relative fluorescence units (RFU) are reported with λ_{em} = 376 nm (λ_{ex} = 300 nm), and are linearly proportional to the concentration of pyrenebutyric acid in the concentration rage 0–10 μM.

The initial apparent reaction rates were determined from the initial slope of the fluorescence–time plots for each of the 540 substrate–enzyme pairs (Table S1†). The fastest reactions were observed with *Mucor miehei* lipase (Roche Chiralzyme L8), *Thermocyces lanuginose* lipase (Roche Chiralzyme L9) and *Aspergillus niger* lipase (ANL, Fluka 62294). However seven enzyme samples did not show any reaction with the substrates. The absence of a reaction with these seven enzymes is probably caused by the presence of a pyrenebutyric acid group because the same enzymes reacted with fluorogenic lipase probes consisting of similar 1,2-diols esterified to octanoic acid.[17]

Cocktail assays

We have recently shown that cocktail assays based on HPLC-analysis of reaction products give highly reproducible activity fingerprints for enzymes,[17,18] which can be used for functional classification.[19] Although substrates **1a–15a** indeed reacted as a cocktail and could be analyzed by HPLC, the reactivity patterns resulting from this set of substrates were very similar between the different enzymes, probably because the pyrenebutyric ester group governs most of the enzyme–substrate interactions. Furthermore, the same seven enzymes that did not react with individual substrates also failed to react with the cocktail. Under these unfavourable circumstances, the use of the cocktail for functional classification of the enzymes was not investigated further.

Substrate ranking

The substrates were ranked according to their highest reactivities to determine the best substrates of the series. For each enzyme, the relative apparent reactivity of each substrate to the fastest reacting substrate for the enzyme was determined, and the resulting percentage values were summed across all enzymes tested. The data was computed for the initial reaction rates under single substrate assay conditions, as well as for the relative product percentages as determined by HPLC analysis of the cocktail reaction (primary data for all enzymes in Table S1†). Substrate **2a** stood out as the most reactive substrate in both analyses (Fig. 2). The difference was particularly strong in terms of the initial reaction rates, for which **2a** was 40% more reactive than the second most reactive substrate **5a**. Interestingly, both of these substrates contain an oxygen atom in the spacer, which renders the substrates somewhat less hydrophobic. The particularly strong reactivity of **2a** might reflect the fact this substrate is directly derived from glycerol itself, which renders it structurally very similar to natural triglycerides.

The reactivity of the most reactive substrate **2a** against lipases and esterases was investigated closer in the case of the most reactive enzymes L8, L9 and ANL. The apparent reaction rate was found to be independent of the substrate concentration above 10 μM in all cases, implying that the K_M values are much lower than 10 μM. Exact kinetics could not be carried out due to the poor fluorescence signal at very low substrate concentrations. Tight substrate binding is probably due to the strongly hydrophobic nature of the substrates.

Variation of the pH towards basic conditions showed that substrate **2a** was completely stable towards non-specific hydrolysis, with no apparent reaction even at pH 11 (Fig. 3). The substrate was

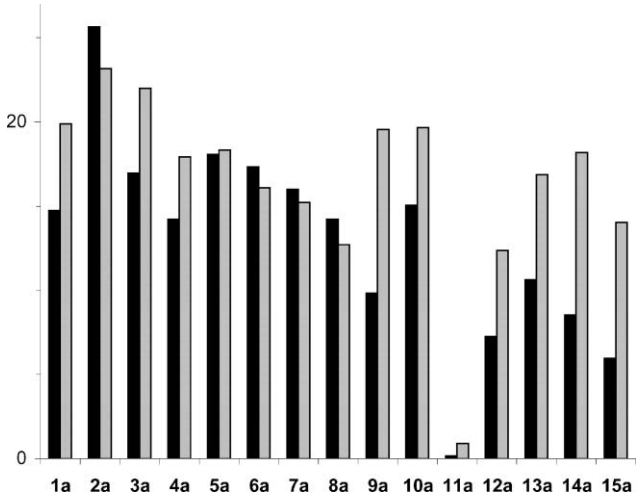


Fig. 2 Cumulative relative reactivities of fluorogenic lipase substrates against 29 enzymes. Black bars: sum of the relative initial rate values for 29 enzyme measurements each under the conditions of Fig. 1. Grey bars: sum of the relative product abundance values determined by HPLC after 2 h of reaction (conditions see Fig. S1 and S2†). In each series of 15 measurements with substrates **1a–15a** and one enzyme, the substrate giving the fastest initial rate or the most abundant product receives a value of 1, and the other substrates receive the ratio of their initial rates or product abundance to those for the fastest substrate. The rate and product percentages for each enzyme–substrate pair are given in Table S1†.

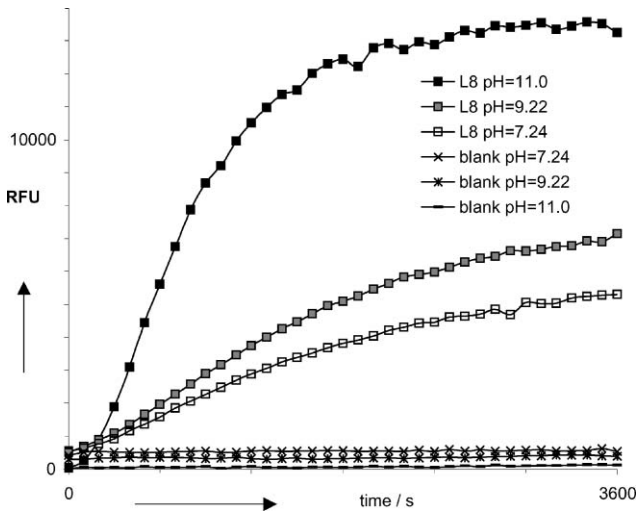


Fig. 3 Fluorescence assay of lipase L8 (Roche *Thermocyces lanuginose* lipase) with pyrene ester **2a** at different pH values. Conditions: 10 μM **2a**, 10 μg ml^{-1} lipase in 20 mM PBS buffer pH = 7.24, 20 mM borate buffer pH = 9.22 and 11.0 with 30% v/v DMSO, 25 °C, see also the conditions in the Experimental section.

therefore used to screen the activity of the different enzymes under these strongly alkaline conditions. Nine of the 36 enzymes tested indeed showed good reactivity with substrate **2a** at pH 11 (Table 1). Enzymes that are inactive are probably denatured at that pH.

Conclusion

FRET-substrates **1a–15a** for lipases and esterases were prepared featuring a dinitrophenylamino group as a quencher linked to an aliphatic 1,2-diol monoacylated with pyrenebutyric acid as a fluorophore. The substrates showed negligible background reactivity and a strong and specific reaction with lipases and esterases. Several enzymes did not show any reaction with these substrates, preventing the use of this substrate series as a general functional classification tool. Substrate **2a** exhibited the strongest reactivity towards most enzymes and was also stable at alkaline pH. Although the assay does not respond with some of the enzymes and is therefore not completely general, substrate **2a** and its analogs offer an unprecedented and practical solution to the long-standing problem of an efficient reporter assay for lipase activity under basic conditions suitable for high-throughput screening.

Table 1 Screening of enzyme activities at pH 11. All enzymes from Table 1 were tested for the reaction with **2a** (10 μM) in aq. 20 mM borate buffer pH 11 containing 30% v/v DMSO, 25 °C. The reaction was followed by fluorescence. Enzymes codes: L8: Roche *Thermocyces lanuginose* lipase; CVL: *Chromobacterium visc.* lipoprotein lipase 2832 U mg^{-1} (Fluka 62333); MJL: *Mucor javanicus* lipase 4.9 U mg^{-1} (Fluka 62304); PSL: *Pseudomonas sp.* lipoprotein lipase 1560 U mg^{-1} (Fluka 62335); E1: Roche pig liver esterase; MML: *Mucor miehei* lipase 1.3 U mg^{-1} (Fluka 62298); CLL: *Candida lipolytica* lipase 1.0 U g^{-1} (Fluka 62303); E2: Roche pig liver esterase; HrLE: horse liver esterase 250 U mg^{-1} (Fluka 46058)

Enzyme	Concentration/μg ml^{-1}	% conversion of **2a** after 30 min
L8	10	75
CVL	10	61
MJL	1	41
PSL	1	41
E1	10	36
MML	10	34
CLL	1	33
E2	10	30
HrLE	10	29

Experimental

All reagents were either purchased from Aldrich or Fluka or synthesized following literature procedures. Chromatography (flash) was performed with Merck silicagel 60 (0.040–0.063 mm). DMF and THF were dried, solvents were distilled from technical solvents. All reactions were followed by TLC on Alugram SIL UV_{254} silica gel sheets (Macherey-Nagel) with detection by UV. NMR spectra were recorded on a BRUKER AC 300 for ^{1}H (300 MHz) or ^{13}C (75 MHz) measurements. Mass spectra were provided by the "Service of Mass Spectrometry" of the Department of Chemistry and Biochemistry.

Preparation of *N*-alkyl-2,4-dinitroanilines

2,4-Dinitrophenylchloride (2.3 g, 11.4 mmol) was added to a solution of the amine in MeOH (2 ml, 1 eq.) and *N*,*N*-diisopropylethylamine (2 ml, 1.2 g, 1 eq.) in CH_2Cl_2 (20 mL) and the reaction was stirred for 4 h at 25 °C. The precipitate was filtered off and washed with CH_2Cl_2, and the filtrate was concentrated to yield *N*-methyl-2,4-dinitroaniline (1.58 g, 70%, 8 mmol) or *N*-propyl-2,4-dinitroaniline (1.7 g, 66%, 7.5 mmol).

3-[*N*-Methyl-*N*-(2,4-dinitrophenyl)amino]propane-1,2-diol (1b). *N*-Methyl-2,4-dinitroaniline (395 mg, 2 mmol) was dissolved in 7 mL DMF and treated with sodium hydride (55% suspension in oil, 167 mg, 4 mmol) and allyl bromide (484 mg, 0.35 mL, 4 mmol)

and the reaction was stirred at 70 °C for 14 h. The reaction was diluted with ethyl acetate (50 mL), washed with water (2 × 30 mL) and brine (30 mL), dried over $MgSO_4$, filtered and evaporated. The residue was purified by FC (eluent: hexane–AcOEt 2 : 1). The main fraction ($R_f = 0.48$) was concentrated to yield *N*-methyl-*N*-allyl-2,4-dinitroaniline (412 mg, 1.73 mmol, 87%) as a dark yellow oil. This product was dissolved in acetone (10 mL) and water (4 mL) and treated with *N*-methylmorpholine oxide monohydrate (282 mg, 2.1 mmol) and catalytic OsO_4 (0.1 mL of a 2.5% solution in *tert*-butanol). After completion of the reaction (12 h at 25 °C), the reaction was diluted with ethyl acetate. Aqueous work-up as above and purification by FC (CH_2Cl_2 + 5% MeOH, $R_f = 0.44$) gave diol **1b** (454 mg, 1.67 mmol, 84%) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.69$ (d, 1H, $J = 2.64$ Hz), 8.22 (dd, 1H, $J = 9.42$, 2.64 Hz), 7.29 (d, 1H, $J = 1.14$ Hz), 4.08 (m, 1H), 3.79 (dd, 1H, $J = 11.1$, 3.57 Hz), 3.51 (m, 3H), 3.03 (s, 3H), 2.23 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 149.5$, 146.4, 127.7, 123.9, 123.5, 119.0, 69.1, 63.8, 56.2, 41.8; ESI-MS: calc. for $C_{10}H_{13}N_3O_6$ $[M + H]^+$: 272.0882, found: 272.0880.

3-[2-(*N*-Methyl-2,4-dinitrophenyl)amino]ethoxypropane-1,2-diol (2b). *N*-Methylethanolamine (188 mg, 2.5 mmol), allyl bromide (302 mg, 2.5 mmol) and 2,4-dinitrophenylchloride (505 mg, 2.5 mmol) were stirred in DMF (5 mL) with NaH (55% suspension in oil, 230 mg, 5 mmol) for 8 hours at 25 °C. Aqueous work-up and FC gave 506 mg of a crude product which was oxidized as above and purified by FC (CH_2CH_2–MeOH 9 : 1, $R_f = 0.38$) to yield diol **2b** (152 mg, 0.55 mmol, 22.4%) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.64$ (d, 1H, $J = 2.64$ Hz), 8.17 (dd, 1H, $J = 9.60$, 2.82 Hz), 7.14 (d, 1H, $J = 9.42$ Hz), 3.73 (m, 3H), 3.59 (q, 3H, $J = 9.99$, 5.28 Hz), 3.49 (m, 3H), 2.99 (s, 3H), 2.47 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 149.0$, 134.2, 127.5, 123.9, 118.0, 72.6, 70.4, 68.2, 63.6, 53.7, 40.6; ESI-MS: calc. for $C_{12}H_{17}N_3O_7$ $[M + H]^+$: 316.1144, found: 316.1056.

5-[*N*-Methyl-*N*-(2,4-dinitrophenyl)amino]pentane-1,2-diol (3b). Methylamine hydrochloride (67.5 mg, 4 mmol) was suspended in 7 mL DMF and treated with 5-bromopentene (298 mg, 0.24 mL, 2 mmol) and sodium hydride (55% suspension in oil, 334 mg, 8 mmol). After 24 h at 25 °C, 2,4-dinitrophenylchloride (808 mg, 8 mmol) was added and the reaction was stirred for an additional 8 h at 25 °C. The reaction was diluted with 50 mL ethyl acetate, washed with water (2 × 30 mL) and brine (30 mL), dried (Na_2SO_4), and concentrated. The residue was dissolved in acetone (10 mL) and water (4 mL) and treated with *N*-methylmorpholine oxide monohydrate (282 mg, 2.1 mmol) and catalytic OsO_4 (0.1 mL of a 2.5% solution in *tert*-butanol). After completion of the reaction (12 h at 25 °C), the reaction was diluted with ethyl acetate and worked up as above. Purification of the residue by FC (CH_2Cl_2–MeOH 95 : 5) gave diol **3b** (263 mg, 0.88 mmol, 44%) as a yellow oil, $R_f = 0.49$ (CH_2CH_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.63$ (dd, 1H, $J = 2.64$, 1.68 Hz), 8.14 (ddd, 1H, $J = 9.60$, 2.82, 1.32 Hz), 7.04 (d, 1H, $J = 9.60$ Hz), 3.61 (m, 2H), 3.39 (q, 3H, $J = 10.71$, 7.32 Hz), 2.92 (s, 3H), 2.42 (bs, 2H), 1.75 (m, 2H), 1.40 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 148.8$, 136.4, 127.6, 124.1, 117.3, 71.5, 66.6, 53.9, 40.4, 29.6, 23.1; ESI-MS: calc. for $C_{12}H_{17}N_3O_6$ $[M + H]^+$: 300.1195, found: 300.1206.

5-[2-(*N*-Methyl-2,4-dinitrophenyl)amino]ethoxypentane-1,2-diol (4b). The procedure for **3b** was applied starting with *N*-methylethanolamine (300 mg, 4 mmol), 5-bromopent-1-ene (0.47 mL, 596 mg, 4 mmol), NaH (55% suspension in oil, 335 mg, 8 mmol) and dinitrophenyl chloride (808 mg, 8 mmol), to yield diol **4b** (148 mg, 0.43 mmol, 11%) as a yellow oil, $R_f = 0.47$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.64$ (d, 1H, $J = 2.64$ Hz), 8.16 (dd, 1H, $J = 9.42$, 2.64 Hz), 7.16 (d, 1H, $J = 9.42$ Hz), 3.56 (m, 6H), 3.37 (m, 3H), 2.99 (s, 3H), 2.20 (s, 2H), 1.53 (m, 2H), 1.34 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 160.6$, 138.7, 127.5, 123.9, 118.3, 71.5 ($J = 26.05$ Hz), 68.9 ($J = 45.26$ Hz), 67.7, 66.7, 54.0, 40.5, 30.0, 25.8; ESI-MS: calc. for $C_{14}H_{21}N_3O_7$ $[M + H]^+$: 344.1457, found: 344.1461.

6-[2-(*N*-Methyl-2,4-dinitrophenyl)amino]ethoxyhexane-1,2-diol (5b). The procedure for **4b** was applied using 6-bromohexene (0.53 mL, 652 mg, 4 mmol) to yield diol **5b** (361 mg, 1.01 mmol, 25%) as a yellow oil, $R_f = 0.48$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.63$ (d, 1H, $J = 2.82$ Hz), 8.15 (dd, 1H, $J = 9.42$, 2.64 Hz), 7.17 (d, 1H, $J = 9.60$ Hz), 3.65 (m, 3H), 3.55 (m, 3H), 3.39 (m, 3H), 2.98 (s, 3H), 2.20 (s, 2H), 1.47 (m, 2H), 1.29 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 149.2$, 127.4, 126.2, 123.9, 118.3, 71.2 ($J = 57.67$ Hz), 66.7 ($J = 66.98$ Hz), 60.3, 54.0, 40.4, 32.7, 29.5, 22.1, 14.1; ESI-MS: calc. for $C_{15}H_{23}N_3O_7$ $[M + H]^+$: 358.1614, found: 358.1621.

6-[2-(*N*-Methyl-2,4-dinitrophenyl)amino]hexane-1,2-diol (6b). The procedure for **3b** was applied using 6-bromohexene (0.27 mL, 326 mg, 2 mmol) to yield diol **6b** (226 mg, 0.72 mmol, 36%) as a yellow waxy solid, $R_f = 0.47$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.63$ (d, 1H, $J = 2.82$ Hz), 8.14 (dd, 1H, $J = 9.42$, 2.64 Hz), 7.02 (d, 1H, $J = 9.60$ Hz), 3.67 (d, 1H, $J = 6.99$ Hz), 3.60 (t, 1H, $J = 3.0$ Hz), 3.37 (q, 3H, $J = 11.7$, 7.35 Hz), 2.92 (s, 3H), 2.51 (s, 2H), 1.67 (ddd, 2H, $J = 19.38$, 12.24, 7.71 Hz), 1.37 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 148.8$, 136.1, 136.0, 127.6, 124.2, 117.2, 71.8, 66.6, 54.0, 40.5, 32.4, 26.8, 22.6; ESI-MS: calc. for $C_{13}H_{19}N_3O_6$ $[M + H]^+$: 314.1352, found: 314.1345.

3-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino]propane-1,2-diol (7b). The procedure for **3b** was applied starting with propylamine (0.5 mL, 354 mg, 6 mmol) and allyl bromide (0.26 mL, 363 mg, 3 mmol), and using *t*-BuOK (672 mg, 6 mmol) in 10 mL dichloromethane, to yield diol **7b** (321 mg, 1.07 mmol, 36%) as a yellow oil, $R_f = 0.56$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.65$ (d, 1H, $J = 2.64$ Hz), 8.21 (dd, 1H, $J = 9.21$, 2.64 Hz), 7.24 (d, 1H, $J = 9.24$ Hz), 3.95 (m, 1H), 3.73 (dd, 1H, $J = 11.28$, 3.57 Hz), 3.44 (m, 3H), 3.08 (m, 2H), 2.33 (s, 2H), 1.56 (ddd, 2H, $J = 21.69$, 14.55, 7.2 Hz), 0.81 (t, 3H, $J = 7.35$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 149.6$, 140.0, 139.1, 127.9, 123.6, 121.0, 68.9, 63.8, 55.5, 53.4, 20.9, 11.1; ESI-MS: calc. for $C_{12}H_{17}N_3O_6$ $[M + H]^+$: 300.1184, found: 300.1188.

4-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino)butane-1,2-diol (8b). The procedure for **3b** was applied starting with propylamine (0.14 mL, 94 mg, 1.6 mmol) and 4-bromobutene (0.08 mL, 108 mg, 0.8 mmol), to yield diol **8b** (96 mg, 0.31 mmol, 38%) as a yellow oil, $R_f = 0.54$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.59$ (d, 1H, $J = 2.8$ Hz), 8.15 (dd, 1H, $J = 9.4$, 2.8 Hz), 7.12 (d, 1H, $J = 9.4$ Hz), 3.66 (dddd, 1H, $J = 12.2$, 10.7, 7.0, 3.4 Hz), 3.57 (dd, 1H, $J = 11.1$, 3.2 Hz), 3.37 (m, 3H), 3.15 (dddd, 2H, $J = 17.3$, 14.1, 9.6, 7.4 Hz), 2.67 (s, 2H), 1.56 (m, 4H), 0.83 (t, 3H, $J = 7.5$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 148.7$, 137.9, 137.2,

127.7, 123.9, 119.2, 69.6, 66.7, 54.6, 48.5, 30.3, 20.7, 11.2; ESI-MS: calc. for $C_{13}H_{19}N_3O_6$ $[M + H]^+$: 314.1352, found: 314.1340.

6-[*N*-Ethyl-*N*-(2,4-dinitrophenyl)amino]hexane-1,2-diol (9b). The procedure for **3b** was applied starting with ethylamine hydrochloride (246 mg, 3 mmol), and 6-bromohexene (489 mg, 3 mmol), to yield diol **9b** (199 mg, 0.61 mmol, 20%) as a yellow oil, $R_f = 0.38$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.61$ (d, 1H, $J = 2.85$ Hz), 8.16 (dd, 1H, $J = 9.42$, 2.64 Hz), 7.04 (d, 1H, $J = 9.60$ Hz), 3.61 (m, 2H), 3.26 (m, 5H), 2.13 (s, 2H), 1.60 (m, 2H), 1.33 (m, 4H), 1.19 (t, 3H, $J = 7.17$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 148.2$, 134.3, 127.5, 123.8, 118.7, 111.4, 71.8, 66.6, 51.1, 47.2, 32.4, 27.1, 22.7, 12.5; ESI-MS: calc. for $C_{14}H_{21}N_3O_6$ $[M + H]^+$: 328.1508, found: 328.1518.

5-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino]pentane-1,2-diol (10b). The procedure for **3b** was applied starting with propylamine (0.33 mL, 236 mg, 4 mmol) and 5-bromopentene (0.24 mL, 298 mg, 2 mmol) to yield diol **10b** (256 mg, 0.78 mmol, 39%) as a yellow oil, $R_f = 0.48$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.62$ (d, 1H, $J = 2.82$ Hz), 8.17 (dd, 1H, $J = 9.60$, 2.82 Hz), 7.07 (d, 1H, $J = 9.60$ Hz), 3.60 (m, 2H), 3.38 (dd, 1H, $J = 10.74$, 7.35 Hz), 3.30 (t, 2H, $J = 7.35$ Hz), 3.20 (t, 2H, $J = 7.35$ Hz), 2.08 (s, 2H), 1.57 (m, 4H), 1.32 (m, 2H), 0.83 (t, 3H, $J = 7.35$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 148.7$, 140.4, 127.6, 123.9, 119.0, 71.5, 66.7, 54.0, 51.9, 29.8, 23.5, 20.7, 11.2; ESI-MS: calc. for $C_{14}H_{21}N_3O_6$ $[M + H]^+$: 328.1508, found: 328.1511.

1-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino]-3-methylbutane-2,3-diol (11b). The procedure for **1b** was applied starting with *N*-propyl-2,4-dinitroaniline (460 mg, 2 mmol) and 1-bromo-3-methyl-2-butene (0.4 mL, 596 mg, 4 mmol) to yield diol **11b** (184 mg, 0.56 mmol, 27%) as a yellow liquid, $R_f = 0.64$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.65$ (d, 1H, $J = 2.64$ Hz), 8.22 (dd, 1H, $J = 9.42$, 2.82 Hz), 7.23 (d, 1H, $J = 9.39$ Hz), 3.63 (dddd, 2H, $J = 10.74$, 6.60, 4.14, 2.46 Hz), 3.34 (dd, 1H, $J = 14.49$, 10.74 Hz), 3.04 (m, 2H), 1.55 (ddd, 2H, $J = 21.63$, 14.49, 7.14 Hz), 1.28 (s, 3H), 1.21 (s, 3H), 0.81 (t, 3H, $J = 7.35$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 149.7$, 138.8, 138.0, 127.7, 123.5, 121.1, 74.1, 71.5, 55.3, 52.5, 26.3, 24.4, 20.8, 11.0; ESI-MS: calc. for $C_{14}H_{21}N_3O_6$ $[M + H]^+$: 328.1508, found: 328.1501.

3-[*N*-Benzyl-*N*-(2,4-dinitrophenyl)amino]propane-1,2-diol (12b). The procedure for **3b** was applied starting with benzylamine (0.273 mL, 268 mg, 2.5 mmol) and allyl bromide (0.254 mL, 338 mg, 2.5 mmol) to yield diol **12b** (511 mg, 1.47 mmol, 59%) as a yellow oil, $R_f = 0.45$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.64$ (d, 1H, $J = 2.82$ Hz), 8.18 (dd, 1H, $J = 9.42$, 2.64 Hz), 7.18 (m, 4H), 7.15 (dd, 2H, $J = 5.28$, 1.71 Hz), 4.36 (dd, 2H, $J = 19.02$, 15.24 Hz), 3.66 (m, 1H), 3.61 (dd, 1H, $J = 11.28$, 3.39 Hz), 3.41 (dd, 1H, $J = 11.49$, 5.46 Hz), 3.36 (d, 2H, $J = 6.42$ Hz), 2.82 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 149.6$, 138.8, 138.3, 135.2, 128.9, 128.2, 127.8, 127.8, 123.5, 121.2, 69.2, 63.7, 57.4, 53.7; ESI-MS: calc. for $C_{16}H_{17}N_3O_6Na$: 370.1015, found: 370.1005.

6-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino]hexane-1,2-diol (13b). The procedure with **3b** was applied starting with propylamine (0.33 mL, 236 mg, 4 mmol) and 6-bromohexene (0.3 mL, 362 mg, 2 mmol) to yield diol **13b** (345 mg, 1.01 mmol, 51%) as a yellow oil, $R_f = 0.47$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.62$ (d, 1H, $J = 2.82$ Hz), 8.16 (dd, 1H, $J = 9.42$, 2.64 Hz), 7.05 (d, 1H, $J = 9.60$ Hz), 3.64 (m, 1H), 3.60 (d, 1H, $J = 3.03$ Hz), 3.38 (dd, 1H, $J = 10.92$, 7.35 Hz), 3.20 (ddd, 4H, $J = 19.59$, 14.52, 7.35 Hz), 2.04 (s, 2H), 1.59 (m, 4H), 1.29 (m, 4H), 0.85 (t, 3H, $J = 7.53$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 148.7$, 137.0, 127.7, 124.0, 119.0, 71.9, 66.7, 54.1, 52.0, 32.6, 27.3, 22.8, 20.8, 11.2; ESI-MS: calc. for $C_{15}H_{23}N_3O_6$ $[M + H]^+$: 342.1665, found: 342.1671.

5-[*N*-Benzyl-*N*-(2,4-dinitrophenyl)amino]pentane-1,2-diol (14b). The procedure for **3b** was applied starting with benzylamine (0.44 mL, 429 mg, 4 mmol), 5-bromopentene (0.24 mL, 298 mg, 2 mmol) to yield diol **14b** (111 mg, 0.30 mmol, 15%) as a yellow oil, $R_f = 0.42$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.66$ (d, 1H, $J = 2.85$ Hz), 8.16 (dd, 1H, $J = 9.42$, 2.82 Hz), 7.28 (m, 3H), 7.18 (q, 2H, $J = 3.78$, 1.50 Hz), 7.09 (d, 1H, $J = 9.42$ Hz), 4.47 (s, 2H), 3.59 (m, 2H), 3.36 (dd, 1H, $J = 10.74$, 7.35 Hz), 3.27 (t, 2H, $J = 7.35$ Hz), 1.98 (s, 2H), 1.65 (m, 2H), 1.35 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 139.4$, 135.4, 128.9, 128.1, 127.7, 127.5, 123.7, 119.6, 71.5, 66.6, 56.2, 52.3, 29.7, 23.4; ESI-MS: calc. for $C_{18}H_{21}N_3O_6$ $[M + H]^+$: 376.1508, found: 376.1523.

6-[*N*-Benzyl-*N*-(2,4-dinitrophenyl)amino]hexane-1,2-diol (15b). The procedure for **3b** was applied starting with benzylamine (0.19 mL, 184 mg, 1.72 mmol) and 6-bromohexene (0.10 mL, 128 mg, 0.86 mmol) to yield diol **15b** (115 mg, 0.30 mmol, 35%) as a yellow oil, $R_f = 0.46$ (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.61$ (d, 1H, $J = 2.82$ Hz), 8.12 (dd, 1H, $J = 9.39$, 2.61 Hz), 7.23 (m, 3H), 7.18 (dd, 2H, $J = 6.21$, 4.14 Hz), 7.07 (d, 1H, $J = 9.60$ Hz), 4.46 (s, 2H), 3.60 (q, 1H, $J = 5.10$, 2.82 Hz), 3.54 (dd, 1H, $J = 11.13$, 3.03 Hz), 3.32 (dd, 1H, $J = 10.92$, 7.53 Hz), 3.22 (t, 2H, $J = 7.35$ Hz), 2.86 (s, 2H), 1.59 (m, 2H), 1.24 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 148.7$, 137.8, 137.7, 135.3, 128.8, 127.9, 127.5, 127.4, 123.6, 119.5, 71.7, 66.5, 56.1, 52.4, 32.2, 27.0, 22.5; ESI-MS: calc. for $C_{19}H_{23}N_3O_6$ $[M + H]^+$: 390.1665, found: 390.1672.

3-[*N*-Methyl-*N*-(2,4-dinitrophenyl)amino]-2-hydroxypropyl-4-(pyren-1-yl)butyrate (1a). A solution of diol **1b** (100 mg, 0.37 mmol) and pyrenebutyric acid (112 mg, 0.39 mmol) in CH_2Cl_2 (10 mL) and pyridine (1 mL) was treated with *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) (115 mg, 0.74 mmol) and 4-dimethylaminopyridine (90 mg, 0.74 mmol) and stirred at room temperature for 15 h. The reaction was diluted with ethyl acetate (150 mL), washed with aq. 1 N HCl (2 × 50 mL), sat. aq. $NaHCO_3$ (50 mL), and brine (50 mL). The organic phase was concentrated and the residue was purified by FC (AcOEt–hexane 1 : 2) to yield ester **1a** (68 mg, 0.13 mmol, 35%) as a yellow oil, $R_f = 0.31$ (hexane–AcOEt 1 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.54$ (d, 1H, $J = 3.42$ Hz), 8.24 (d, 1H, $J = 20.79$ Hz), 8.15 (s, 1H), 8.12 (s, 1H), 8.10 (s, 1H), 8.09 (d, 1H, $J = 2.85$ Hz), 8.06 (d, 1H, $J = 4.14$ Hz), 8.00 (s, 2H), 7.94 (q, 2H, $J = 7.14$, 5.64 Hz), 7.82 (d, 1H, $J = 7.89$ Hz), 6.92 (d, 1H, $J = 9.60$ Hz), 3.95 (m, 3H), 3.33 (dd, 2H, $J = 14.49$, 6.78 Hz), 3.30 (t, 2H, $J = 2.28$ Hz), 2.84 (s, 3H), 2.46 (t, 2H, $J = 6.96$ Hz), 2.04 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.4$, 149.1, 137.3, 135.2, 131.3, 130.8, 130.0, 128.7, 127.4, 127.4, 127.3, 126.8, 125.9, 125.0, 124.9, 124.8, 123.6, 123.1, 118.6, 67.5, 65.6, 56.3, 41.5, 33.5, 32.5, 26.5; ESI-MS: calc. for $C_{30}H_{27}N_3O_7$ M^+: 541.184901, found: 541.1838.

3-[*N*-Methyl-*N*-(2,4-dinitrophenyl)amino]ethoxyl-2-hydroxypropyl-4-(pyren-1-yl)butyrate (2a). The procedure for **2a** was applied starting with diol **2b** (63 mg, 0.2 mmol) and pyrenebutyric acid (60 mg, 0.21 mmol) to yield ester **2a** (65 mg, 0.11 mmol, 55%) as a yellow oil, $R_f = 0.24$ (hexane–AcOEt 1 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.59$ (d, 1H, $J = 2.64$ Hz), 8.26 (d, 1H, $J = 9.21$ Hz), 8.17 (s, 1H), 8.14 (s, 1H), 8.11 (s, 1H), 8.06 (m, 2H), 8.01 (s, 2H), 7.95 (t, 1H, $J = 7.74$ Hz), 7.83 (d, 1H, $J = 7.92$ Hz), 6.97 (d, 1H, $J = 9.60$ Hz), 3.98 (m, 3H), 3.84 (m, 1H), 3.60 (dd, 2H, $J = 9.78$, 4.89 Hz), 3.35 (m, 4H), 2.89 (s, 3H), 2.44 (t, 2H, $J = 7.14$ Hz), 2.04 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.4$, 148.9, 135.4, 131.3, 130.8, 129.9, 128.7, 127.4, 127.4, 127.3, 126.7, 125.8, 125.0, 124.9, 124.9, 124.8, 123.8, 123.2, 117.9, 72.0, 68.7, 68.2, 65.2, 53.6, 40.5, 33.6, 32.6, 26.6; ESI-MS: calc. for $C_{32}H_{31}N_3O_8$ M^+: 586.2189, found: 586.2202.

5-[*N*-Methyl-*N*-(2,4-dinitrophenyl)amino]-2-hydroxypentyl-4-(pyren-1-yl)butyrate (3a). The procedure for **1a** was applied starting with **3b** (90 mg, 0.3 mmol) and pyrenebutyric acid (91 mg, 0.32 mmol) to yield ester **3a** (96 mg, 0.17 mmol, 55%) as a yellow oil, $R_f = 0.18$ (hexane–AcOEt 1 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.60$ (d, 1H, $J = 2.82$ Hz), 8.26 (d, 1H, $J = 9.42$ Hz), 8.16 (s, 1H), 8.14 (d, 1H, $J = 1.14$ Hz), 8.11 (s, 1H), 8.07 (q, 2H, $J = 4.32$, 2.82 Hz), 8.04 (s, 2H), 8.00 (t, 1H, $J = 3.00$ Hz), 7.95 (d, 1H, $J = 7.53$ Hz), 7.83 (d, 1H, $J = 8.61$ Hz), 6.88 (d, 1H, $J = 9.63$ Hz), 4.05 (dd, 1H, $J = 11.1$, 3.21 Hz), 3.89 (dd, 1H, $J = 11.46$, 7.14 Hz), 3.70 (m, 1H), 3.36 (t, 2H, $J = 7.74$ Hz), 3.26 (t, 2H, $J = 7.32$ Hz), 2.82 (s, 3H), 2.46 (t, 2H, $J = 7.14$ Hz), 2.16 (q, 2H, $J = 14.1$, 6.96 Hz), 1.63 (m, 2H), 1.35 (dddd, 2H, $J = 12.06$, 8.67, 3.21 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.5$, 148.7, 135.3, 131.3, 130.8, 130.0, 128.7, 127.5, 127.4, 127.4, 127.3, 126.7, 125.9, 125.0, 125.0, 124.9, 124.8, 124.0, 123.2, 117.2, 69.4, 68.4, 53.7, 40.4, 33.6, 32.6, 29.8, 26.6, 22.8; ESI-MS: calc. for $C_{32}H_{31}N_3O_7Na$ $[M + Na]^+$: 592.2059, found: 592.2262.

5-[*N*-Methyl-*N*-(2,4-dinitrophenyl)amino]ethoxyl-2-hydroxypentyl-4-(pyren-1-yl)butyrate (4a). The procedure for **1a** was applied starting with **4b** (69 mg, 0.2 mmol) and pyrenebutyric acid (60 mg, 0.21 mmol) to yield ester **4a** (58 mg, 0.10 mmol, 55%) as a yellow oil, $R_f = 0.30$ (hexane–AcOEt 1 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.58$ (d, 1H, $J = 3.75$ Hz), 8.28 (d, 1H, $J = 9.21$ Hz), 8.17 (t, 1H, $J = 1.11$ Hz), 8.14 (d, 1H, $J = 1.68$ Hz), 8.12 (s, 1H), 8.09 (s, 1H), 8.06 (t, 1H, $J = 2.64$ Hz), 8.02 (d, 2H, $J = 1.53$ Hz), 7.96 (t, 1H, $J = 7.71$ Hz), 7.85 (d, 1H, $J = 7.74$ Hz), 4.11 (m, 2H), 3.92 (dd, 1H, $J = 11.28$, 6.96 Hz), 3.72 (m, 1H), 3.59 (t, 2H, $J = 5.10$ Hz), 3.43 (dd, 2H, $J = 12.45$, 7.35 Hz), 3.38 (t, 4H, $J = 2.61$ Hz), 2.90 (s, 3H), 2.45 (dd, 2H, $J = 13.38$, 6.21 Hz), 2.17 (q, 2H, $J = 13.74$, 6.39 Hz), 1.40 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.6$, 149.0, 136.7, 135.6, 131.4, 130.9, 130.0, 128.7, 127.5, 127.4, 127.4, 127.4, 126.8, 125.9, 125.1, 125.0, 124.9, 124.8, 124.8, 123.8, 123.3, 118.0, 71.2, 69.6, 68.5, 67.6, 53.9, 40.2, 33.7, 32.7, 30.2, 26.8, 25.6; ESI-MS: calc. for $C_{34}H_{35}N_3O_8Na$ $[M + Na]^+$: 614.2502, found: 614.2514.

6-[*N*-Methyl-*N*-(2,4-dinitrophenyl)amino]ethoxyl-2-hydroxyhexyl-4-(pyren-1-yl)butyrate (5a). The procedure for **1a** was applied starting with **5a** (71 mg, 0.2 mmol) and pyrenebutyric acid (60 mg, 0.21 mmol) to yield ester **5a** (91 mg, 0.15 mmol, 73%) as a yellow oil, $R_f = 0.38$ (hexane–AcOEt 1 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.60$ (d, 1H, $J = 2.82$ Hz), 8.27 (d, 1H, $J = 9.21$ Hz), 8.17 (t, 1H, $J = 1.14$ Hz), 8.14 (t, 1H, $J = 1.71$ Hz), 8.11 (d, 1H, $J = 0.75$ Hz), 8.08 (dd, 2H, $J = 2.82$, 1.50 Hz), 8.02 (s, 2H), 7.96 (t, 1H, $J = 7.89$ Hz), 7.84 (d, 1H, $J = 7.71$ Hz), 7.05 (d, 1H, $J = 9.42$ Hz), 4.18 (m, 1H), 4.08 (dd, 1H, $J = 3.96$, 0.96 Hz), 3.90 (dd, 1H, $J = 11.31$, 7.35 Hz), 3.74 (dd, 2H, $J = 6.96$, 2.82 Hz), 3.56 (q, 2H, $J = 6.21$, 1.68 Hz), 3.46 (q, 2H, $J = 9.42$, 4.92 Hz), 3.32 (m, 4H), 2.91 (s, 3H), 2.47 (t, 2H, $J = 7.14$ Hz), 2.16 (dd, 2H, $J = 14.88$, 7.35 Hz), 1.94 (bs, 1H), 1.38 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.5$, 149.0, 135.5, 131.3, 130.8, 129.9, 128.7, 127.4, 127.3, 127.3, 127.3, 126.7, 125.8, 124.8, 124.7, 124.7, 123.8, 123.2, 118.0, 71.1, 69.7, 68.5, 67.5, 53.9, 40.3, 33.6, 32.8, 32.6, 29.4, 26.7, 21.9; ESI-MS: calc. for $C_{35}H_{37}N_3O_8Na$ $[M + Na]^+$: 650.2478, found: 650.2492.

6-[*N*-Methyl-*N*-(2,4-dinitrophenyl)amino]-2-hydroxyhexyl-4-(pyren-1-yl)butyrate (6a). The procedure for **1a** was applied starting with **6b** (94 mg, 0.3 mmol) and pyrenebutyric acid (91 mg, 0.32 mM) to yield ester **6a** (101 mg, 0.17 mmol, 57%) as a yellow oil, $R_f = 0.40$ (hexane–AcOEt 1 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.57$ (d, 1H, $J = 2.82$ Hz), 8.24 (d, 1H, $J = 9.21$ Hz), 8.15 (s, 1H), 8.12 (s, 1H), 8.09 (s, 1H), 8.06 (d, 1H, $J = 1.32$ Hz), 8.00 (s, 2H), 7.94 (dd, 1H, $J = 8.10$, 7.35 Hz), 7.81 (d, 1H, $J = 7.92$ Hz), 4.07 (dddd, 1H, $J = 12.99$, 12.31, 6.15, 3.21 Hz), 3.90 (dd, 1H, $J = 8.49$, 7.17 Hz), 3.70 (m, 1H), 3.35 (t, 2H, $J = 7.08$ Hz), 3.19 (t, 2H, $J = 7.35$ Hz), 2.77 (s, 3H), 2.46 (t, 2H, $J = 7.35$ Hz), 2.16 (q, 2H, $J = 12.99$, 5.73 Hz), 2.05 (bs, 1H), 1.58 (t, 2H, $J = 3.18$ Hz), 1.38 (t, 2H, $J = 5.41$ Hz), 1.23 (t, 2H, $J = 7.14$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.5$, 148.5, 136.2, 135.4, 131.3, 130.8, 129.9, 128.6, 127.4, 127.4, 127.3, 126.7, 125.8, 125.0, 124.9, 124.8, 124.7, 124.0, 123.1, 117.0, 69.5, 68.4, 53.8, 40.3, 33.6, 32.6, 32.6, 26.6, 26.6, 22.4; ESI-MS: calc. for $C_{33}H_{33}N_3O_7$ $[M + H]^+$: 584.2396, found: 584.2404.

3-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino]-2-hydroxypropyl-4-(pyren-1-yl)butyrate (7a). The procedure for **1a** was applied starting with **7b** (90 mg, 0.3 mmol) and pyrenebutyric acid (91 mg, 0.32 mmol) to yield ester **7a** (68 mg, 0.12 mmol, 40%) as a yellow oil, $R_f = 0.50$ (hexane–AcOEt 1 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.56$ (d, 1H, $J = 2.64$ Hz), 8.24 (d, 1H, $J = 9.21$ Hz), 8.16 (s, 1H), 8.14 (s, 1H), 8.11 (d, 1H, $J = 1.32$ Hz), 8.05 (ddd, 2H, $J = 9.42$, 8.49, 2.82 Hz), 8.02 (s, 2H), 7.96 (dd, 1H, $J = 8.28$, 7.14 Hz), 7.82 (d, 1H, $J = 7.71$ Hz), 7.02 (d, 1H, $J = 9.42$ Hz), 4.20 (m, 1H), 4.13 (dd, 1H, $J = 7.14$, 3.75 Hz), 3.97 (dd, 2H, $J = 13.95$, 5.46 Hz), 3.24 (m, 4H), 3.02 (m, 2H), 2.68 (m, 1H), 2.46 (t, 2H, $J = 7.14$ Hz), 2.18 (t, 2H, $J = 7.35$ Hz), 1.49 (dd, 2H, $J = 14.31$, 7.14 Hz), 0.75 (t, 3H, $J = 7.53$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.5$, 149.2, 139.0, 138.3, 135.3, 133.6, 131.4, 130.8, 130.0, 128.7, 127.7, 127.5, 127.3, 126.8, 125.9, 125.1, 125.0, 124.9, 124.8, 123.4, 123.2, 120.8, 67.4, 65.6, 55.1, 53.7, 33.6, 32.5, 26.6, 20.8, 11.0; ESI-MS: calc. for $C_{32}H_{31}N_3O_7$ M^+: 569.216201, found: 569.2167.

4-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino)-2-hydroxybutyl-4-(pyren-1-yl)butyrate (8a). The procedure for **1a** was applied starting with **8b** (63 mg, 0.2 mmol) and pyrenebutyric acid (61 mg, 0.21 mmol) to yield ester **8a** (66 mg, 0.11 mmol, 55%) as a yellow oil, $R_f = 0.50$ (hexane–AcOEt 1 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.56$ (d, 1H, $J = 2.82$ Hz), 8.24 (d, 1H, $J = 9.21$ Hz), 8.16 (d, 1H, $J = 1.29$ Hz), 8.14 (d, 1H, $J = 0.75$ Hz), 8.10 (s, 1H), 8.05 (q, 2H, $J = 8.28$, 2.82 Hz), 8.01 (s, 2H), 7.96 (t, 1H, $J = 7.71$ Hz), 7.81 (d, 1H, $J = 7.71$ Hz), 6.94 (d, 1H, $J = 9.30$ Hz), 4.03 (q, 1H, $J = 11.31$, 3.39 Hz), 3.90 (dd, 1H, $J = 11.49$, 6.78 Hz), 3.77 (s,

1H), 3.34 (dddd, 4H, $J = 17.34, 14.49, 9.99, 2.28$ Hz), 3.06 (ddd, 2H, $J = 9.24, 6.78, 1.53$ Hz), 2.44 (t, 2H, $J = 7.17$ Hz), 2.32 (bs, 1H), 2.14 (m, 2H), 1.24 (m, 4H), 0.78 (t, 3H, $J = 7.35$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.5, 148.4, 137.9, 137.1, 135.3, 131.3, 130.8, 129.9, 128.7, 127.5, 127.4, 127.4, 127.3, 126.7, 125.9, 124.9, 124.7, 123.7, 123.1, 118.9, 68.3, 67.3, 54.5, 47.8, 33.5, 32.5, 30.5, 26.5, 20.5, 11.0$; ESI-MS: calc. for $C_{33}H_{33}N_3O_7$ $[M + H]^+$: 584.2396, found: 584.2414.

6-[*N*-Ethyl-*N*-(2,4-dinitrophenyl)amino]-2-hydroxyhexyl-4-(pyren-1-yl)butyrate (9a). The procedure with **1a** was applied starting with **9b** (66 mg, 0.2 mmol) and pyrenebutyric acid (61 mg, 0.21 mmol) to yield ester **9a** (73 mg, 0.12 mmol, 61%) as a yellow oil, $R_f = 0.50$ (hexane–AcOEt 1 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.55$ (d, 1H, $J = 2.82$ Hz), 8.25 (d, 1H, $J = 9.21$ Hz), 8.15 (s, 1H), 8.12 (s, 1H), 8.09 (s, 1H), 8.07 (s, 1H), 8.02 (dd, 1H, $J = 9.60$, 2.82 Hz), 8.00 (s, 2H), 7.94 (t, 1H, $J = 7.35$ Hz), 7.81 (d, 1H, $J = 7.71$ Hz), 6.83 (d, 1H, $J = 9.60$ Hz), 4.12 (m, 1H), 3.85 (dd, 1H, $J = 11.49$, 7.14 Hz), 3.75 (m, 1H), 3.34 (t, 2H, $J = 7.92$ Hz), 3.12 (m, 4H), 2.45 (t, 2H, $J = 7.17$), 2.19 (t, 2H, $J = 7.71$ Hz), 2.11 (bs, 1H), 1.49 (dd, 2H, $J = 12.06$, 8.67 Hz), 1.40 (m, 3H), 1.21 (m, 1H), 1.07 (t, 3H, $J = 7.14$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.5, 148.1, 137.5, 136.7, 135.4, 131.3, 130.8, 129.9, 128.6, 127.4, 127.4, 127.3, 126.7, 125.8, 125.0, 124.9, 124.9, 124.7, 124.7, 123.7, 123.2, 118.5, 69.5, 68.4, 51.1, 47.0, 33.6, 32.6, 32.6, 26.9, 26.6, 22.5, 12.4$; ESI-MS: calc. for $C_{34}H_{35}N_3O_7$ $[M + H]^+$: 598.2553, found: 598.2543.

5-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino]-2-hydroxypentyl-4-(pyren-1-yl)butyrate (10a). The procedure for **1a** was applied starting with **10b** (66 mg, 0.2 mmol) and pyrenebutyric acid (61 mg, 0.21 mM) to yield ester **10a** (54 mg, 0.09 mmol, 45%) as a yellow oil, $R_f = 0.56$ (hexane–AcOEt 1 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.58$ (d, 1H, $J = 1.74$ Hz), 8.26 (d, 1H, $J = 9.24$ Hz), 8.16 (t, 1H, $J = 1.32$ Hz), 8.14 (d, 1H, $J = 1.32$ Hz), 8.11 (s, 1H), 8.06 (dd, 2H, $J = 9.42$, 2.64 Hz), 8.02 (s, 2H), 7.95 (dd, 1H, $J = 7.71$, 7.36 Hz), 7.83 (d, 1H, $J = 7.71$ Hz), 6.91 (d, 1H, $J = 9.39$ Hz), 4.03 (dd, 1H, $J = 8.58$, 3.21 Hz), 3.88 (dd, 1H, $J = 11.25$, 7.14 Hz), 3.67 (dddd, 1H, $J = 12.81, 10.47, 7.35, 3.39$ Hz), 3.36 (t, 2H, $J = 7.71$ Hz), 3.19 (t, 2H, $J = 7.32$ Hz), 3.10 (t, 2H, $J = 7.35$ Hz), 2.42 (dd, 2H, $J = 15.45$, 8.10 Hz), 2.15 (m, 2H), 2.02 (bs, 1H), 1.49 (m, 4H), 1.32 (ddd, 2H, $J = 11.85$, 8.64, 3.00 Hz), 0.80 (t, 3H, $J = 7.35$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.5, 148.5, 137.7, 136.9, 135.4, 131.3, 130.8, 129.9, 128.7, 127.5, 127.4, 127.4, 127.3, 126.7, 125.8, 125.0, 124.9, 124.9, 124.7, 123.8, 123.1, 118.8, 69.4, 68.4, 53.9, 51.6, 33.6, 32.6, 30.0, 26.6, 23.2, 20.5, 11.1$; ESI-MS: calc. for $C_{34}H_{35}N_3O_7$ $[M + H]^+$: 598.2556, found: 598.2552.

1-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino]-3-hydroxy-3-methylbutyl-4-(pyren-1-yl)butyrate (11a). The procedure for **1a** was applied starting with **11b** (82 mg, 0.25 mmol) and pyrenebutyric acid (144 mg, 0.50 mmol) to yield ester **11a** (115 mg, 0.19 mmol, 76%) as a yellow oil, $R_f = 0.77$ (hexane–AcOEt 1 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.60$ (d, 1H, $J = 2.64$ Hz), 8.20 (d, 1H, $J = 6.42$ Hz), 8.17 (dd, 1H, $J = 4.14$, 1.32 Hz), 8.14 (t, 1H, $J = 1.11$ Hz), 8.10 (d, 1H, $J = 4.14$ Hz), 8.04 (m, 2H), 8.02 (s, 2H), 7.96 (t, 1H, $J = 7.53$ Hz), 7.76 (d, 1H, $J = 7.71$ Hz), 6.97 (d, 1H, $J = 9.42$ Hz), 5.11 (d, 1H, $J = 2.46$ Hz), 5.07 (d, 1H, $J = 2.43$ Hz), 3.12 (m, 5H), 2.00 (m, 4H), 1.82 (bs, 1H), 1.51 (ddd, 2H, $J = 21.84$, 14.49, 7.17 Hz), 1.22 (s, 3H), 1.18 (s, 3H), 0.82 (t, 3H, $J = 7.53$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 172.6, 148.8, 138.3, 137.6, 135.1, 131.3, 130.7, 129.9, 128.6, 127.4, 127.1, 126.7, 125.8, 125.0, 124.9, 124.8, 124.7, 123.6, 123.0, 119.6, 74.7, 71.4, 54.8, 51.2, 33.5, 32.5, 26.3, 26.1, 25.9, 20.6, 11.0$; ESI-MS: calc. for $C_{34}H_{35}N_3O_7$ $[M + H]^+$: 598.2553, found: 598.2545.

3-[*N*-Benzyl-*N*-(2,4-dinitrophenyl)amino]-2-hydroxypropyl-4-(pyren-1-yl)butyrate (12a). The procedure for **1a** was applied starting with **12b** (70 mg, 0.2 mmol) and pyrenebutyric acid (61 mg, 0.21 mmol) to yield ester **12a** (59 mg, 0.10 mmol, 48%) as a yellow oil, $R_f = 0.32$ (hexane–AcOEt 1 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.60$ (d, 1H, $J = 2.82$ Hz), 8.24 (d, 1H, $J = 9.42$ Hz), 8.18 (s, 1H), 8.15 (s, 1H), 8.11 (d, 1H, $J = 2.55$ Hz), 8.09 (d, 1H, $J = 3.03$ Hz), 8.06 (t, 1H, $J = 2.64$ Hz), 8.03 (s, 2H), 7.97 (dd, 1H, $J = 8.10$, 7.14 Hz), 7.82 (d, 1H, $J = 7.92$ Hz), 7.24 (dd, 3H, $J = 3.57$, 1.50 Hz), 7.08 (ddd, 3H, $J = 9.24, 6.78, 5.10$ Hz), 4.34 (d, 2H, $J = 3.03$ Hz), 4.04 (m, 2H), 3.94 (q, 1H, $J = 12.81, 6.78$ Hz), 3.34 (t, 1H, $J = 7.71$ Hz), 3.26 (t, 2H, $J = 6.96$ Hz), 2.42 (t, 2H, $J = 7.14$ Hz), 2.12 (q, 2H, $J = 14.70, 7.35$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.4, 149.4, 139.0, 138.6, 135.3, 135.2, 131.3, 130.8, 128.9, 128.2, 127.8, 127.7, 127.4, 127.4, 127.3, 126.7, 125.9, 125.0, 124.8, 123.4, 123.1, 121.2, 67.5, 65.5, 57.3, 53.9, 33.5, 32.5, 26.5$; ESI-MS: calc. for $C_{36}H_{31}N_3O_7$ $[M + H]^+$: 618.2240, found: 618.2246.

6-[*N*-(2,4-Dinitrophenyl)-*N*-propylamino)-2-hydroxyhexyl-4-(pyren-1-yl)butyrate (13a). The procedure for **1a** was applied starting with **13b** (102 mg, 0.3 mmol) and pyrenebutyric acid (91 mg, 0.21 mmol) to yield ester **13a** (75 mg, 0.12 mmol, 41%) as a yellow oil, $R_f = 0.15$ (hexane–AcOEt 1 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.59$ (d, 1H, $J = 2.64$ Hz), 8.26 (d, 1H, $J = 9.21$ Hz), 8.17 (s, 1H), 8.14 (d, 1H, $J = 0.75$ Hz), 8.11 (t, 2H, $J = 0.93$ Hz), 8.08 (1H, $J = 2.61$ Hz), 8.02 (s, 2H), 7.96 (t, 1H, $J = 7.92$ Hz), 7.83 (d, 1H, $J = 7.74$ Hz), 6.92 (d, 1H, $J = 9.40$ Hz), 4.06 (dd, 1H, $J = 14.61$, 3.21 Hz), 3.89 (dd, 1H, $J = 11.49$, 6.87 Hz), 3.69 (ddd, 1H, $J = 9.81, 6.78, 3.39$ Hz), 3.36 (t, 2H, $J = 7.92$ Hz), 3.12 (q, 4H, $J = 15.06$, 7.35 Hz), 2.46 (t, 2H, $J = 7.14$ Hz), 2.16 (ddd, 2H, $J = 14.67, 7.35, 1.47$ Hz), 1.51 (ddd, 4H, $J = 21.66, 14.58, 4.87$ Hz), 1.26 (m, 4H), 0.81 (t, 3H, $J = 7.35$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.5, 148.5, 140.0, 136.8, 135.4, 131.3, 130.8, 130.0, 128.9, 127.5, 127.4, 127.4, 127.3, 126.7, 125.8, 125.0, 124.9, 124.9, 124.8, 123.8, 123.2, 118.8, 69.6, 68.5, 53.9, 51.9, 33.6, 32.6, 32.6, 27.1, 26.6, 22.5, 20.6, 11.1$; ESI-MS: calc. for $C_{35}H_{37}N_3O_7$ $[M + H]^+$: 612.2709, found: 612.2713.

5-[*N*-Benzyl-*N*-(2,4-dinitrophenyl)amino]-2-hydroxypentyl-4-(pyren-1-yl)butyrate (14a). The procedure for **1a** was applied starting with **14b** (75 mg, 0.2 mmol) and pyrenebutyric acid (61 mg, 0.21 mmol) to yield ester **14a** (77 mg, 0.12 mmol, 60%) as a yellow oil, $R_f = 0.44$ (hexane–AcOEt 1 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.63$ (d, 1H, $J = 2.82$ Hz), 8.27 (d, 1H, $J = 9.42$ Hz), 8.17 (d, 1H, $J = 1.32$ Hz), 8.14 (d, 1H, $J = 0.75$ Hz), 8.12 (s, 1H), 8.07 (m, 2H), 8.02 (s, 2H), 7.96 (dd, 1H, $J = 7.92$, 7.35 Hz), 7.84 (d, 1H, $J = 7.71$ Hz), 7.26 (ddd, 3H, $J = 8.85, 6.78, 4.89$ Hz), 7.13 (q, 2H, $J = 8.28$, 1.31 Hz), 6.97 (d, 1H, $J = 9.39$ Hz), 4.38 (s, 2H), 4.03 (dd, 1H, $J = 11.49$, 3.39 Hz), 3.87 (dd, 1H, $J = 11.46$, 7.14 Hz), 3.71 (s, 1H), 3.37 (t, 2H, $J = 7.71$ Hz), 3.19 (t, 2H, $J = 7.32$ Hz), 2.45 (t, 2H, $J = 7.14$ Hz), 2.16 (ddd, 2H, $J = 14.85, 6.72, 3.57$ Hz), 1.61 (m, 2H), 1.34 (dd, 2H, $J = 6.99$, 3.03 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.6, 148.8, 135.4, 135.4, 131.4, 130.9, 130.0, 128.9, 128.8, 128.1, 127.6, 127.6, 127.5, 127.4, 126.8, 125.9, 125.0, 125.0, 124.8, 123.7, 123.2, 119.6, 69.5, 68.4, 56.2, 52.2, 33.7, 32.7, 30.0,$

26.6, 23.2; ESI-MS: calc. for $C_{38}H_{35}N_3O_7$ $[M + H]^+$: 646.2553, found: 646.2567.

6-[*N*-Benzyl-*N*-(2,4-dinitrophenyl)amino]-2-hydroxyhexyl-4-(pyren-1-yl)butyrate (15a). The procedure for **1a** was applied starting with **15b** (78 mg, 0.2 mmol) and pyrenebutyric acid (61 mg, 0.21 mmol) to yield ester **15a** (80 mg, 0.12 mmol, 60%) as a yellow oil, $R_f = 0.44$ (hexane–AcOEt 1 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.62$ (d, 1H, $J = 2.82$ Hz), 8.27 (d, 1H, $J = 9.24$ Hz), 8.16 (d, 1H, $J = 1.32$ Hz), 8.14 (d, 1H, $J = 1.32$ Hz), 8.11 (d, 1H, $J = 0.75$ Hz), 8.07 (dd, 2H, $J = 5.46$, 2.82 Hz), 8.02 (s, 2H), 7.96 (t, 1H, $J = 7.53$ Hz), 7.83 (d, 1H, $J = 7.92$ Hz), 7.28 (q, 3H, $J = 3.84$, 2.07 Hz), 7.14 (dd, 2H, $J = 7.35$, 1.50 Hz), 6.96 (d, 1H, $J = 9.39$ Hz), 4.40 (s, 2H), 4.04 (dd, 1H, $J = 8.49$, 6.95 Hz), 3.87 (dd, 1H, $J = 11.49$, 6.95 Hz), 3.69 (t, 2H, $J = 2.82$ Hz), 3.36 (t, 2H, $J = 7.89$ Hz), 3.15 (t, 2H, $J = 7.35$ Hz), 2.46 (t, 2H, $J = 6.84$ Hz), 2.16 (q, 2H, $J = 15.06$, 7.71 Hz), 1.58 (d, 2H, $J = 6.42$ Hz), 1.31 (dd, 4H, $J = 14.70$, 9.24 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta =$ 173.5, 148.7, 137.5, 135.4, 131.3, 130.8, 130.0, 128.9, 128.7, 128.0, 127.5, 127.5, 127.4, 127.3, 127.1, 126.7, 125.8, 125.0, 124.9, 124.9, 124.8, 123.6, 123.2, 119.5, 69.5, 68.5, 60.3, 56.1, 52.4, 33.6, 32.6, 32.6, 27.0, 26.6, 22.5; ESI-MS: calc. for $C_{39}H_{37}N_3O_7$ $[M + H]^+$: 660.2709, found: 660.2698.

Enzyme assays

Fluorescence assay. Substrates **1a–15a** were conditioned as 2 mM stock solutions in DMF by dissolving 1–2 mg waxy solid into the appropriate volume. The fluorescence assay of the cocktail uses the same conditions as with the HPLC assay. For each substrate 5 μL of the 2 mM stock solution were diluted in 945 μL PBS (20 mM phosphate, 160 mM NaCl, pH 7.4) containing 30% v/v DMSO. The solution was distributed in 95 mL aliquots into the wells of a 96 well polystyrene plate and each assay was started by adding 5 μL of a freshly prepared 1 mg mL^{-1} stock solution of the lipase in PBS buffer. The assay concentration under these conditions was 10 μM of each substrate and 50 μg mL^{-1} of the lipase; the assay was conducted for 2 h at room temperature. The reactions were followed using a SPECTRAMax fluorescence detector with wavelength setting $\lambda_{ex} = 300$ nm, $\lambda_{em} = 376$ nm.

HPLC assay

The cocktail was prepared by mixing 5 μL of each substrate stock solution and 5 μL of a 2 mM stock solution of the internal standard *N*-benzyl-2,4-dinitrobenzenamine in 20 μL DMF to make 100 μM of each substrate solution. The stock solution was diluted to the appropriate volume using PBS containing 30% v/v DMSO to a total cocktail concentration of 50 μM or 30 μM. The assay was started by adding 5 μL of a freshly prepared 1 mg mL^{-1} stock solution of the lipase in PBS buffer to 95 μL of the cocktail. The assay concentration under these conditions was 50 μM or 30 μM for the cocktail and 50 μg mL^{-1} for the enzyme. After 2 h at room temperature, the reaction was analyzed by RP-C18 HPLC using a Vydac 218TP54 column, 0.4 × 22 cm, detection by UV at 385 nm. Eluents: A = 0.1% CF_3CO_2H in water, B = 1 : 1 acetonitrile–water. Flow rate: 1.5 mL min^{-1}. Linear gradient: $t = 0$: A : B = 55 : 45, $t = 20$ min: A : B = 40 : 60, $t = 30$ min: A : B = 15 : 85, $t =$ 35 min: A : B = 0 : 100. The column was then washed for 20 min with 100% B and reequilibrated to the initial conditions for 10 min.

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References

1 (*a*) *Enzyme Assays: A Practical Approach*, ed. R. Eisenthal and M. Danson, Oxford University Press, Oxford, 2002; (*b*) *Enzyme Assays: Essential Data*, ed. S. Gul, S. K. Sreedharan and K. Brocklehurst, John Wiley & Sons, New York, 1998; (*c*) M. T. Reetz, *Angew. Chem.*, 2001, **113**, 292, (*Angew. Chem., Int. Ed.*, 2001, **40**, 284); (*d*) D. Wahler and J.-L. Reymond, *Curr. Opin. Chem. Biol.*, 2001, **5**, 152; (*e*) D. Wahler and J.-L. Reymond, *Curr. Opin. Biotechnol.*, 2001, **12**, 535; (*f*) H. Lin and V. W. Cornish, *Angew. Chem., Int. Ed.*, 2002, **41**, 4402; (*g*) F. H. Arnold and G. Georgiou, *Methods Mol. Biol.*, 2003, **230**, 213; (*h*) J.-P. Goddard and J.-L. Reymond, *Trends Biotechnol.*, 2004, **22**, 363; (*i*) J.-P. Goddard and J.-L. Reymond, *Curr. Opin. Biotechnol.*, 2004, **15**, 314; (*j*) *Enzyme Assays*, ed. J.-L. Reymond, Wiley-VCH, Weinheim, Germany, 2006.

2 (*a*) R. D. Schmidt and R. Verger, *Angew. Chem., Int. Ed.*, 1998, **37**, 1608; (*b*) M. T. Reetz, *Curr. Opin. Chem. Biol.*, 2002, **6**, 145.

3 G. G. Guibault and J. Hieserman, *Anal. Chem.*, 1969, **41**, 2006.

4 (*a*) M. Schmidt and U. T. Bornscheuer, *Biomol. Eng.*, 2005, **22**, 51; (*b*) D. Gilham and R. Lehner, *Methods*, 2005, **36**, 139.

5 P. Babiak and J.-L. Reymond, *Anal. Chem.*, 2005, **77**, 373.

6 (*a*) E. Leroy, N. Bensel and J.-L. Reymond, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2105; (*b*) R. Sicard, L. S. Chen, A. J. Marsaioli and J.-L. Reymond, *Adv. Synth. Catal.*, 2005, **347**, 1041; (*c*) Y. Yongzheng, P. Babiak and J.-L. Reymond, *Helv. Chim. Acta*, 2006, **89**, 404–415.

7 E. Leroy, N. Bensel and J.-L. Reymond, *Adv. Synth. Catal.*, 2003, **345**, 859.

8 (*a*) L. E. Janes and R. J. Kazlauskas, *J. Org. Chem.*, 1997, **62**, 4560; (*b*) L. E. Janes, A. C. Löwendahl and R. J. Kazlauskas, *Chem.–Eur. J.*, 1998, **4**, 2324.

9 F. Beisson, A. Tiss, C. Rivière and R. Verger, *Eur. J. Lipid Sci. Technol.*, 2000, 133.

10 (*a*) D. Wahler and J.-L. Reymond, *Angew. Chem., Int. Ed.*, 2002, **41**, 1229; (*b*) D. Wahler, O. Boujard, F. Lefèvre and J.-L. Reymond, *Tetrahedron*, 2004, **60**, 703.

11 (*a*) M. Baumann, R. Stürmer and U. T. Bornscheuer, *Angew. Chem., Int. Ed.*, 2001, **40**, 4201–4204; (*b*) G. Klein and J.-L. Reymond, *Helv. Chim. Acta*, 1999, **82**, 400.

12 (*a*) F. Badalassi, D. Wahler, G. Klein, P. Crotti and J.-L. Reymond, *Angew. Chem., Int. Ed.*, 2000, **39**, 4067; (*b*) D. Lagarde, H.-K. Nguyen, G. Ravot, D. Wahler, J.-L. Reymond, G. Hills, T. Veit and F. Lefevre, *Org. Process Res. Dev.*, 2002, **6**, 441; (*c*) E. M. Gonzalez-Garcia, J. Grognux, D. Wahler and J.-L. Reymond, *Helv. Chim. Acta*, 2003, **86**, 2458; (*d*) E. Nyfeler, J. Grognux, D. Wahler and J.-L. Reymond, *Helv. Chim. Acta*, 2003, **86**, 2919; (*e*) F. Badalassi, G. Klein, P. Crotti and J.-L. Reymond, *Eur. J. Org. Chem.*, 2004, 2557; (*f*) J. Grognux, D. Wahler, E. Nyfeler and J.-L. Reymond, *Tetrahedron: Asymmetry*, 2004, **15**, 2981.

13 Y. Yongzheng, D. Wahler and J.-L. Reymond, *Helv. Chim. Acta*, 2003, **86**, 2928.

14 (*a*) G. Zandonella, L. Haalck, F. Spener, K. Faber, F. Paltauf and A. Hermetter, *Chirality*, 1996, **8**, 481; (*b*) M. Duque, M. Graupner, H. Stutz, I. Wicher, R. Zechner, F. Paltauf and A. Hermetter, *J. Lipid Res.*, 1996, **37**, 868; (*c*) A. Hermetter, *Methods Mol. Biol.*, 1999, **109**, 19.

15 E. D. Matayoshi, G. T. Wang, G. A. Krafft and J. Erickson, *Science*, 1990, **247**, 954.

16 D. Wahler, F. Badalassi, P. Crotti and J.-L. Reymond, *Chem.–Eur. J.*, 2002, **8**, 3211–3228.

17 J.-P. Goddard and J.-L. Reymond, *J. Am. Chem. Soc.*, 2004, **126**, 11116–11117.

18 (*a*) R. Sicard, J.-P. Goddard, M. Mazel, C. Audiffrin, L. Fourage, G. Ravot, D. Wahler, F. Lefèvre and J.-L. Reymond, *Adv. Synth. Catal.*, 2005, **347**, 987–996; (*b*) Y. Yongzheng and J.-L. Reymond, *Mol. BioSyst.*, 2005, **1**, 57–63.

19 (*a*) J.-L. Reymond and D. Wahler, *ChemBioChem*, 2002, **3**, 701–708; (*b*) J. Grognux and J.-L. Reymond, *ChemBioChem*, 2004, **5**, 826–831.