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Adrenaline profiling of lipases and esterases with 1,2-diol and carbohydrate acetates

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Abstract—The adrenaline test for enzymes is a general back-titration procedure to detect 1,2-diols, 1,2-aminoalcohols and α-hydroxyketones reaction products of enzyme catalysis by colorimetry. The method was used to profile a series of esterases and lipases for their esterolytic activity on a series of carbohydrate and polyol acetates. Substrates were prepared by peracetylation and used for parallel microtiter-plate analysis of enzyme activities. This method can be used to achieve a rapid and automated characterization of a set of enzymes during HTS screening.

1. Introduction

Enzyme assays suitable for high-throughput screening are essential for the exploration of biological diversity in search for novel enzymes,[1] and the use of biocatalysis in industrial processes is rapidly growing.[2] The vast majority of processes are based on hydrolytic enzymes, in particular lipases and esterases, which are essential tools in fine chemical synthesis. Several thousand different lipases and esterases have been described, yet there is almost no information available in the literature that allows comparing these different enzymes in terms of their reactivities and selectivities. We have described recently a general procedure for generating activity fingerprints of hydrolytic enzymes using arrays of fluorogenic and chromogenic substrates.[3] The method was based on an indirect release strategy including an oxidation step with $NaIO_4$, followed by a β-elimination leading to a fluorescent product, such that various enzyme classes could be assayed including alcohol dehydrogenases,[4] aldolases,[5] proteases,[6] and lipases.[7] The diversity of reactivity patterns observed even between closely related enzymes suggested that a large body of differential reactivity information can indeed be established by such procedures.[8] Nevertheless each of the substrates used in this study had to be individually synthesized by asymmetric multistep synthesis, and was available only in limited quantities. We wanted to explore the possibility of using more readily available substrates such that the method would become more broadly available even for laboratories not familiar with organic synthesis. Herein we report that acetate derivatives of commercially available carbohydrates and diols provide a readily available substrate family for profiling lipases and esterases based on the chromogenic adrenaline test for enzymes.

2. Results and discussion

The adrenaline test is a back-titration procedure which measures the concentration of periodate-sensitive reaction products formed from periodate-resistant substrates by an enzymatic transformation.[9] The test solution is treated with a measured amount of sodium periodate, which rapidly reacts with oxidizable functional groups presents, in particular 1,2-diols, 1,2-aminoalcohols or α-hydroxyketones released from the hydrolysis of ester, amide, phosphate or epoxide precursors used as substrates for the corresponding enzymes. The unreacted periodate reagent is then revealed by addition of adrenaline **1**, which undergoes an instantaneous oxidation with periodate to give adrenochrome **2**, a cationic orthoquinone dye with a red absorption maximum in the visible spectrum (Scheme 1). We had demonstrated that this colorimetric procedure provided off-the-shelf endpoint assays for lipases using vegetal oils as substrates, phytases using phytic acid as substrate, and epoxide hydrolases using epoxides as substrates.

In the perspective of fine chemical synthesis, kinetic resolution of chiral alcohols by enantioselective lipases is one of the most popular and reliable methods for producing enantiomerically pure products. The preferred reactions are the formation or hydrolysis of acetate esters of these

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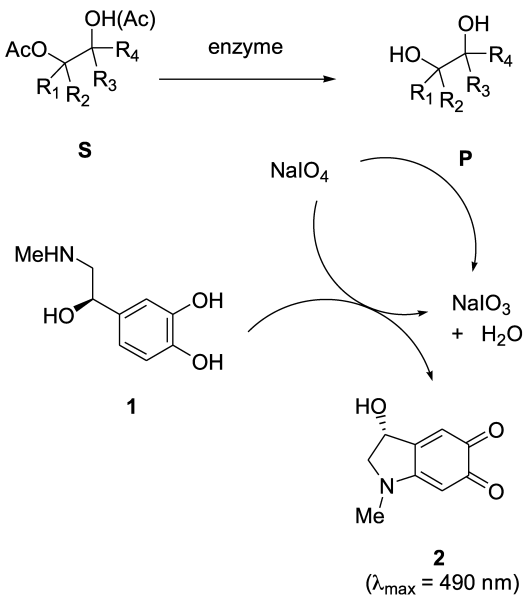

Scheme 1. Principle of the adrenaline test for esterolysis of 1,2-diol acetates.

alcohols. However many enzymes show only very poor reactivities towards acetates, and a preliminary screening against such substrates is a necessary first step for assessing the value of any enzyme collection in view of such applications. It was shown recently that a multi-enzyme detection kit for acetic acid can be used to test such reactivities.[10]

We envisioned that our adrenaline test for enzymes would provide an interesting opportunity for profiling such reactivities with an array of 1,2-diol and carbohydrate acetates. Indeed these substrates could be easily prepared by peracetylation of the parent polyols. The resulting substrate family was structurally diverse, such that the resulting reactivity profiles would be useful for selecting enzymes on the basis of their reactivity. Peracetate derivatives were prepared by exhaustive acetylation with acetic anhydride in pyridine. The products were purified simply by liquid extraction and their structure confirmed by NMR-analysis. The series was extended with a few commercially available di- and triacetates to give a total of 35 different acetates (Fig. 1).

The polyol substrates and their acetate derivatives were conditioned as 10 mM stock solutions in water/acetonitrile mixtures for the assay procedure. First, the reactivity of the compounds against periodate in the adrenaline assay was calibrated by testing serial dilutions of the products against periodate (1 mM) followed by addition of **1** (1.5 mM). The ester derivatives showed either full signal or only a weak decrease of adrenochrome absorption with the adrenaline test, in agreement with the absence of any free 1,2-diol functionality in these derivatives. By contrast, the corresponding polyols showed the expected reactivity, and the results are shown in Table 1. The adrenaline procedure efficiently detected most polyol in the expected range of $100\ \mu M < EC_{50} < 750\ \mu M$ based on the expected stoichiometry. Free hexitols and hexoses were detected with the highest sensitivity in agreement with the fact that these

Table 1. Detection of free polyols by back-titration of sodium periodate with adrenaline. EC_{50} is the product concentration that inhibits 50% of the color reaction. Data calculated from OD decrease at 490 nm by increasing polyol molar concentration, in the presence of 1 mM $NaIO_4$ in 50 mM aqueous borate buffer pH 8.0 for 1 h at 37 °C, followed by reaction with 1.5 mM of **1** for 5 min at 26 °C

Alcohol	Acetates	EC_{50} (mM)
D-Mannitol	**37**	0.09
D-Glucose	**28**	0.10
D-Lactose	**31**	0.10
D-Sorbitol	**32**	0.11
D-Mannose	**24**	0.12
D-Maltose	**36**	0.12
D-Galactose	**34**	0.13
D-Fructose	**29**	0.13
D-Xylose	**25**	0.14
D-Fucose	**34**	0.15
L-Arabinose	**35**	0.15
D-Arabinose	**30**	0.16
D-Ribose	**23**	0.18
D,L-Glyceraldehyde	**13**	0.26
2,3-Butanediol	**10**	0.52
Glycerol	**8, 9**	0.55
1,2-Dodecanediol	**7**	0.65
Ethyleneglycol	**3**	0.74
1,2-Propanediol	**4**	0.74
1,2-Butanediol	**5**	0.74
Cis-1,2-cyclopentanediol	**18**	0.74
Cis-1,2-cyclohexanediol	**19**	0.78
1,2-Octanediol	**6**	0.79
(1*R*,2*R*)-*trans*-1,2-cyclohexanediol	**21**	0.81
3,3-Dimethyl-1,2-butanediol	**11**	1.00
(*S*)-(−)-1,1-Diphenyl-1,2-propanediol	**22**	1.35
Pinacol	**12**	2.24
D-Sucrose	**26**	2.24
D-Trehalose	**27**	2.24

carbohydrates undergo fivefold oxidation with periodate. Sucrose, trehalose, and pinacol gave EC_{50} values in the 2 mM range, probably because their 1,2-diol functional groups only react very slowly with periodate. This calibration showed that all substrates were in principle suited to generate a colorimetric signal upon complete deacetylation. In general however, any partial deacetylation liberating at least one 1,2-diol functionality would lead to a decrease in the colorimetric signal in the adrenaline test.

A series of 33 lipases and esterases was assayed with the peracetate substrate array shown in Figure 1, comprising 21 commercial lipases and 12 proprietary enzymes derived from culture collections at Protéus (Table 2). These proprietary enzymes have been obtained during HTS screening programs dedicated to the discovery of thermophilic esterases and lipases that can be used in the industrial synthesis of fine chemical compounds at high temperature. These enzymes, originating from strains isolated in either deep sea hydrothermal vents, oil fields or hot terrestrial soil, were characterized for their acyl chain length specificity (ranging from C2 to C18).

The enzymes were incubated under a standard microtiter-plate assay procedure consisting in incubating each of the 35 substrates at 1 mM in 100 mL of 50 mM aqueous borate buffer pH 8.0, 5% DMF cosolvent, with 1 mg/mL enzyme and 1 mM $NaIO_4$ at 37 °C for 1 h, followed by treatment with **1** for 5 min. The red color was quantitated at 490 nm with a microtiter-plate reader. The relative signal intensity

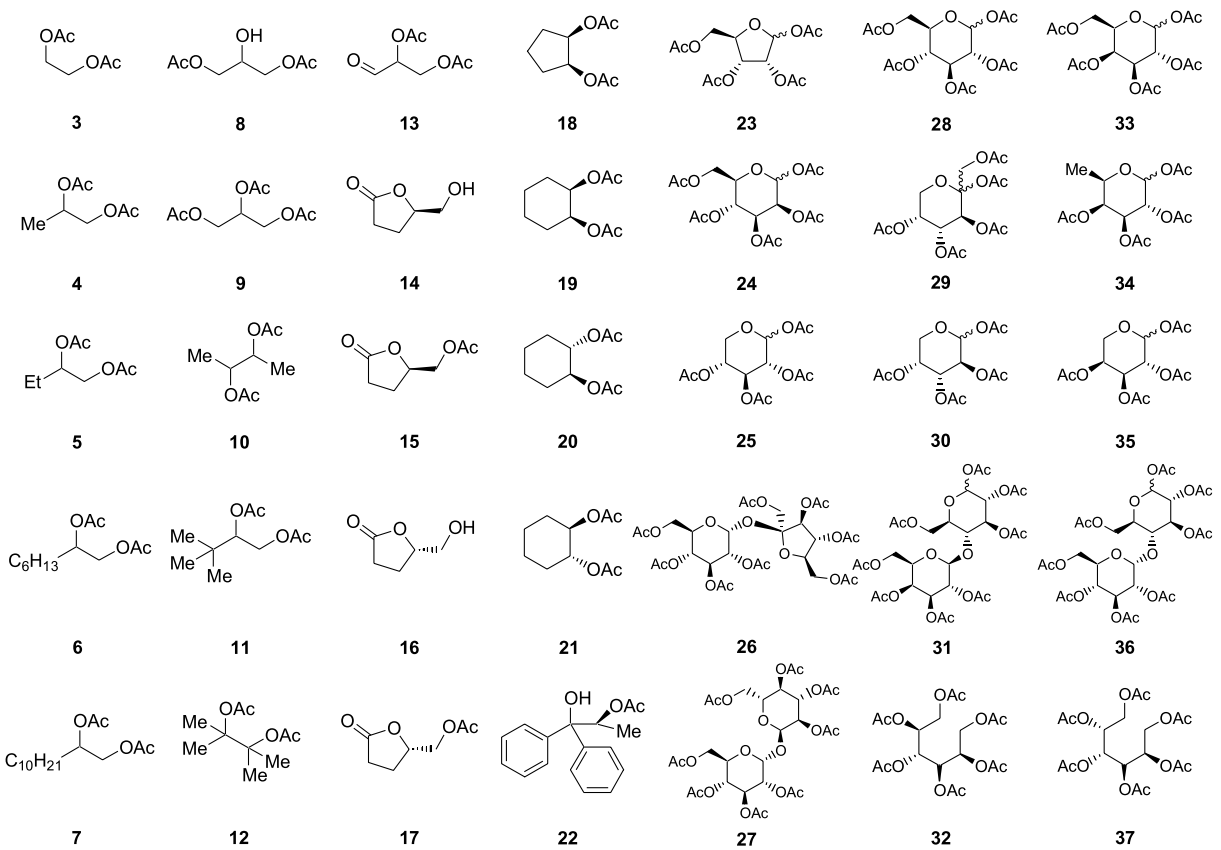

Figure 1. Array of polyol acetates used for adrenaline profiling.

was then corrected by subtracting the blank measurement, which concerned mainly the acetylated fructose derivative **29**. The net signal thus obtained was then used to generate activity patterns in grayscale, which is a convenient display form for data analysis. The results are shown in Figure 2.

The reactivity patterns obtained were strongly dependent on the enzyme used. The reactivities observed should be compared with the blank data observed without enzyme, as well as with the values generated by reaction in the presence of bovine serum albumin, which may be considered as a measure of the non-specific reactivity of the substrates. These blank values show an unusually high reactivity of peracetylated fructose **29** and mannitol hexaacetate **37** and may be associated with a spontaneous partial deacetylation or intrinsic chemical unstability of such substrates. The intrinsic reactivity of the substrates was tested by treatment at higher temperatures under conditions where complete solubility of all substrates was ensured (Fig. 3). Most carbohydrate derivatives were labile under these conditions, while simple aliphatic diols not containing further oxygenated substituents were clearly the most stable. The higher reactivity of carbohydrate acetates compared to less substituted alcohol esters might be related to their higher polarity as well as to the lower pK_a of their hydroxyl groups, which are acidified by inductive effects of the neighbouring oxygen atoms. Indeed the simple effect of stoichiometry (Table 1) is not sufficient to explain the reactivity difference between the two ester classes.

Remarkably, the reactivity with the enzymes did not parallel that of the blank data, showing that the specific enzyme reactivities reflected selective interactions with the enzymes rather than non-specific acceleration of the chemical hydrolysis by the enzymes. Most enzymes showed the strongest signal with acetylated carbohydrates in agreement with the fact that these derivatives release the highest number of periodate-sensitive 1,2-diols. All enzymes tested showed individually differentiated reactivity patterns. These patterns were reproducible and could thus be considered as showing the intrinsic selectivity pattern of each enzyme. The broad array of patterns observed illustrates the enormous diversity in reactivities available in esterolytic enzymes, despite of the fact that all of them are mostly naturally selected to hydrolyze triglycerides. There was no detectable association of the reactivity pattern observed with the association of the enzyme to a lipase or esterase type reactivity, in agreement with the fact that this classification is problematic and sometimes grounded in historical reasons.

As for the proprietary enzymes, PE6 clearly resembled the famous CAL-B enzyme often used for kinetic resolutions. Remarkably, PE3 and PE4 displayed a high reactivity on the hindered secondary ester **22** which was not found in any of the other enzymes tested. PE7 was highlighted by a strong and highly enantioselective reaction with the (*R*,*R*)-enantiomer of *trans*-cyclohexanediol **21**. This selectivity was clearly more pronounced than that of ACE and PLE2 on the **20/21** enantiomeric pair. These interesting reactivities are under further investigation.

Table 2. Enzymes tested using the acetylated substrates array

Code	Enzyme[a]	Specific activity[b] (U/mg)	Specific periodate activity on 23[c] (mU/mg)	Specific periodate activity on 9[d] (mU/mg)
PE1	Proteus esterase number 1	–	5.5	<0.1
PE2	Proteus esterase number 2	–	3.4	0.3
PE3	Proteus esterase number 3	–	31.5	8.2
PE4	Proteus esterase number 4	–	6.8	1.0
PE5	Proteus esterase number 5	–	5.3	5.2
PE6	Proteus esterase number 6	–	5.5	5.4
PE7	Proteus esterase number 7	–	3.7	0.4
PE8	Proteus esterase number 8	–	9.8	0.7
PE9	Proteus esterase number 9	–	0.1	0.1
PE10	Proteus esterase number 10	–	0.1	0.7
PE11	Proteus esterase number 11	–	2.4	<0.1
PE12	Proteus esterase number 12	–	5.5	0.1
ACE	*Electrophorus electricus* AChE (F-01022)	850[e]	14.2	14.2
ASL	*Aspergillus* lipase (F-84205)	0.5[f]	14.4	1.4
BAE	*Bacillus* sp. esterase (F-46062)	0.1[g]	0.7	3.2
BSE	*Bacillus stearothermophilus* esterase (F-46051)	0.4[h]	10.5	2.1
BTE	*Bacillus thermoglucosidasius* esterase (F-46054)	0.1[g]	10.3	2.4
CAL	*Candida antarctica* lipase (F-62299)	3[i]	8.0	<0.1
CAL B	*Candida antarctica* lipase B		5.6	14.7
CCL 1	*Candida cylindracea* lipase (F-62316)	2[i]	13.1	4.8
CCL 2	*Candida cylindracea* lipase (F-62302)	20[i]	12.1	3.7
CLE	*Candida lipolytica* esterase (F-46056)	0.1[j]	13.8	3.5
HKA	Hog kidney acylase I (F-01821)	16.5[k]	14.7	3.5
HLE	Horse liver esterase (F-46069)	0.7[l]	2.8	1.8
HPL	Hog pancreatic lipase (F-62300)	25[m]	14.7	<0.1
MML	*Mucor miehei* lipase (F-62298)	1[i]	1.8	1.4
PCL	*Pseudomonas cepacia* lipase (F-62309)	50[i]	9.0	1.1
PFL	*Pseudomonas fluorescens* lipase (F-62312)	36[i]	10.8	4.8
PLE 1	Pig liver esterase (S-E3019)	15[m]	13.9	<0.1
PLE 2	Pig liver esterase (F-46058)	130[g]	14.8	14.8
RAL	*Rhizopus arrhizus* lipase (F-62305)	10[n]	7.9	0.9
RNL	*Rhizopus niveus* lipase (F-62310)	1.5[o]	13.9	2.6
WGL	Wheat germ lipase (S-L3001)	10[p]	13.1	0.8

[a] Product reference from Fluka (F) or Sigma (S).
[b] Activity given by the provider in units per milligram of powder; definition given in footnote.
[c] Activity given in milliunits per milligram of powder of commercial samples, or per milligram of protein of proprietary enzyme samples; 1 U corresponds to the amount of enzyme which liberates 1 μmol diol per minute at pH 8.0 and 37 °C, peracetylated D-ribose **23** as substrate.
[d] Activity given in milliunits per milligram of powder of commercial samples, or per milligram of protein of proprietary enzyme samples; 1 U corresponds to the amount of enzyme which liberates 1 μmol diol per minute at pH 8.0 and 37 °C, triacetin **9** as substrate.
[e] 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol acetylcholine per minute at pH 8.0 and 37 °C.
[f] 1 U corresponds to the amount of enzyme which liberates 1 μmol acetic acid per minute at pH 7.4 and 40 °C (triacetin as substrate).
[g] 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol of ethyl valerate per minute at pH 8.0 and 25 °C.
[h] 1 U corresponds to the amount of enzyme which releases 1 μmol 4-nitrophenol per minute at pH 7.0 and 65 °C (4-nitrophenyl-*n*-caproate as substrate).
[i] 1 U corresponds to the amount of enzyme which liberates 1 μmol oleic acid per minute at pH 8.0 and 40 °C (triolein as substrate).
[j] 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol of α-methyl-DL-phenylalanine-*O*-methylester per minute at pH 7.5 and 25 °C.
[k] 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol of *N*-acetyl-L-methionine per minute at pH 7.0 and 25 °C.
[l] 1 U will hydrolyze 1 μmol of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 and 25 °C.
[m] 1 U corresponds to the amount of enzyme which liberates 1 μmol fatty acid from triglycerides per minute at pH 8.0 and 37 °C (olive oil as substrate).
[n] 1 U corresponds to the amount of enzyme which liberates 1 μmol butyric acid per minute at pH 8.0 and 40 °C (tributyrin as substrate).
[o] 1 U corresponds to the amount of enzyme which liberates 1 μmol fatty acid from a triglyceride per minute at pH 7.7 and 40 °C (olive oil as substrate).
[p] 1 U will hydrolyze 1 μequiv. of fatty acid from a triglyceride in 1 h at pH 7.7 and 37 °C.

3. Conclusion

Esterases and lipases are involved in industrial processes for the synthesis of regioisomers of polyfunctional compounds, resolutions of secondary alcohols and asymmetric synthesis from prochiral or *meso*-compounds. The commercially available enzymes are not robust and efficient enough to investigate the feasibility of carrying out industrially interesting syntheses, leading to increasing requests from industry for novel enzymes. During the discovery processes of such enzymes (which include microbial screening, HTS gene isolation, and recombinant expression), the key point is to be compatible with the timelines of the industry. To fit with these timelines, robust and versatile HTS screening tests that mimic the final industrial substrates and conditions, while lowering the level of false positive must be used.[11] At the end of the discovery process, novel enzymes are validated for their intrinsic biochemical properties. This step is generally cumbersome and time consuming.

The experiments above address this issue and demonstrate that enzyme reactivity patterns can be readily obtained using the adrenaline test and acetate esters of commercially available diols and carbohydrates. Such reactivity profiling can be readily established in any laboratory since the synthetic chemistry involved is very limited and the measurement method only uses inexpensive and simple reagents. This biochemical fingerprint can be automated using usual liquid handling systems. Comparison of reactivity patterns of novel enzymes should help early

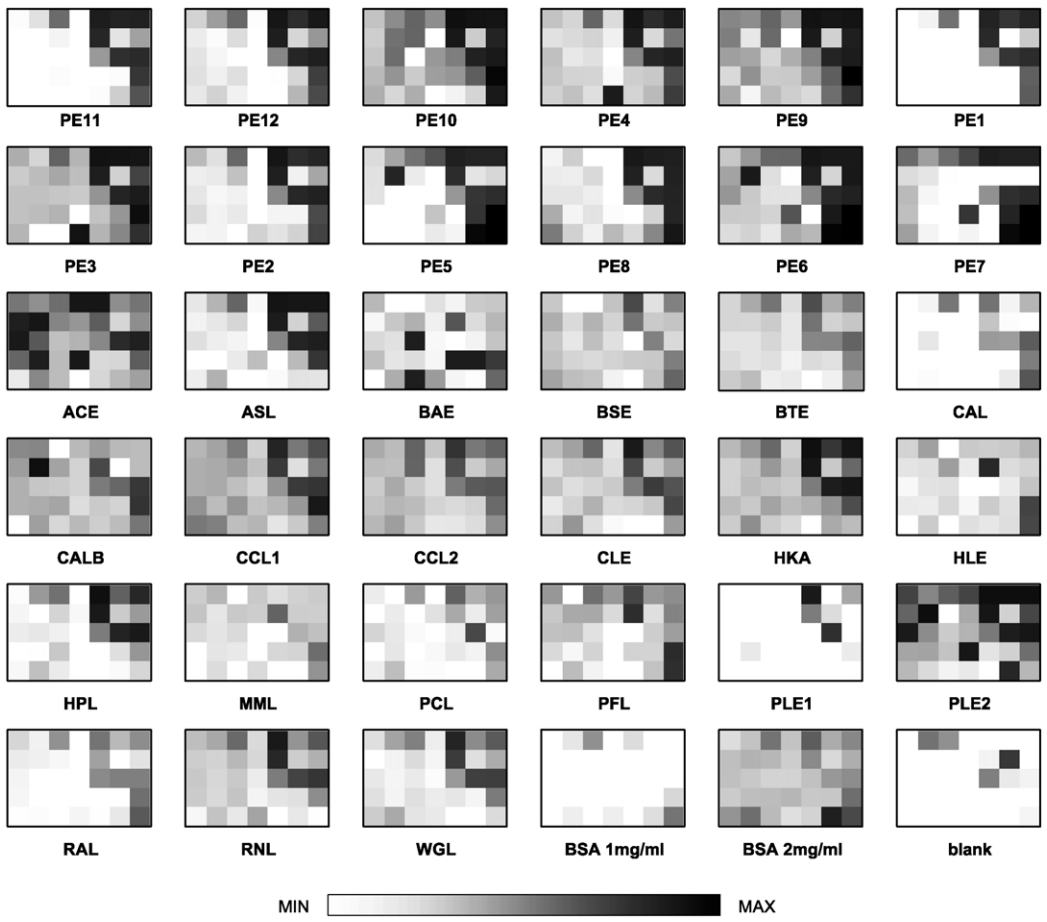

Figure 2. Activity of esterases and lipases towards polyacetates **3**–**37** as determined by the adrenaline test. Each grayscale square corresponds to one ester substrate according to the layout in Figure 1, and shows the percent reduction of OD_{490} from white (0%, no activity) to black (100%, maximum activity), after correction from blank values. Conditions: (1) Enzyme in 50 mM aq. borate pH 8.0, 1 mM $NaIO_4$, 1 mM substrate, 60 min at 37 °C; (2) 1.5 mM **1**, 5 min, 26 °C. Key for enzyme samples are given in Table 2. Commercial enzyme samples were tested at 1 mg/ml, proprietary esterases and lipases samples were used at one tenth of crude enzyme extracts filtered through size-exclusion chromatography columns.

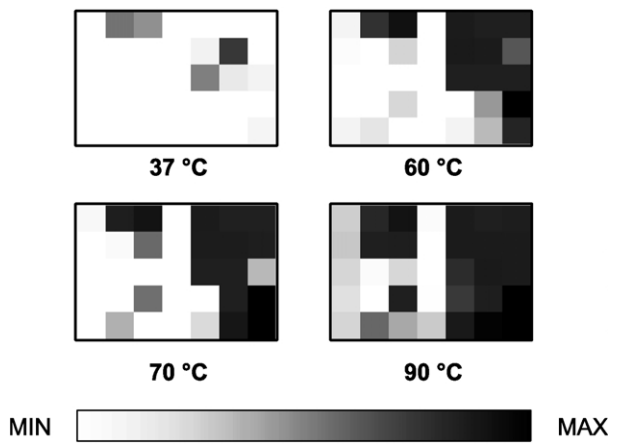

Figure 3. Intrinsic hydrolytic lability of acetate substrates without enzyme. Each grayscale square corresponds to one ester substrate according to the layout in Figure 1, and shows the percent reduction of OD_{490} from white (0%, no degradation) to black (100%, total consumption of sodium periodate). Conditions: (1) 1 mM substrate in 50 mM aq. borate pH 8.0, 1 mM $NaIO_4$, 60 min at the indicated temperature; (2) 1.5 mM adrenaline, 5 min, 26 °C.

validation of discoveries and guide research efforts towards those new enzymes displaying the most interesting and novel reactivity patterns. In that respect, the fact that the proprietary enzymes tested here all display patterns that differ strongly from standard esterases and lipases highlights the validity of pursuing biodiversity mining as a source for new enzymes with novel selectivity properties.

4. Experimental

4.1. General

NMR spectra were recorded on a Bruker Avance 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz) with a Bruker auto-sampler B-ACS 120. Deuterated chloroform with the chemical shifts ^{1}H: 7.26 ppm, ^{13}C: 77.7 ppm, or deuterated methanol with the chemical shifts ^{1}H: 3.31, 4.79 ppm, ^{13}C: 49.0 ppm were used as solvents. All chemical shifts were expressed in ppm according to the reference solvent peaks. Coupling constants (J) are given in Hertz (Hz). Atoms of first acetate residue were quoted with $'$, atoms of the second acetate residue with $''$, etc. The reactions were monitored by thin layer chromatography (TLC) using plastic sheets with silica

gel 60 F_{254} (Merck). The melting points were determined with a Büchi Type S melting point apparatus and are uncorrected.

Pyridine as the solvent for synthesis was purified and dried before use. Polyols were obtained from Sigma-Aldrich-Fluka or Lancaster. Diacetin, triacetin and ethyleneglycol diacetate, as commercial polyacetate substrates, were obtained from Fluka.

4.2. General procedure for the preparation of compounds 4–7, 10–11, 13, 15, 17–21, 24–25, 28, 30, and 32–35

To an ice-bath cooled, stirred mixture of polyol in dry pyridine were added 2 molar equiv. of acetic anhydride per alcohol function. The reaction mixture was stirred overnight, the temperature coming back slowly to room temperature. The reaction was stopped by dilution with water and ethylacetate, and the mixture washed with water and brine. After separation and drying over magnesium sulfate, the organic phase was concentrated to dryness. Coevaporation under vacuum with pyridine and toluene of the residue afforded pure polyacetate. Products were characterized and validated by NMR analysis (proton and carbon), and their homogeneity verified by TLC. Yields obtained ranged from 83 to 99%.

4.3. General procedure for the preparation of compounds 12, 22 and 29

To an ice-bath cooled, stirred mixture of polyol in dry pyridine were added 2 molar equiv. of acetic anhydride and 0.1 molar equiv. of 4-dimethylamino-pyridine per alcohol function. The reaction mixture was stirred overnight, the temperature coming back slowly to room temperature. The reaction was stopped by dilution with water and ethylacetate, and the mixture washed with water and brine. After separation and drying over magnesium sulfate, the organic phase was concentrated to dryness. Coevaporation under vacuum with pyridine and toluene of the residue afforded pure polyacetate. Products were characterized and validated by NMR analysis (proton and carbon), and their homogeneity verified by TLC. Yields obtained ranged from 54 to 78%.

4.3.1. 1,2-Propanediol diacetate 4.[12] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.23 (d, 3H, J=6.4 Hz, C3H_3), 2.04 (s, 3H, C2′H_3), 2.05 (s, 3H, C2″H_3), 4.02 (dd, 1H, J=11.8, 6.6 Hz, C1HH), 4.15 (dd, 1H, J=11.8, 3.6 Hz, C1HH), 5.11 (m, 1H, C2H_1). ^{13}C NMR (75 MHz): δ=17.1 (C3), 21.4 (C2′), 21.8 (C2″), 66.7 (C1), 68.9 (C2), 171.1 (C=O′), 171.4 (C=O″).

4.3.2. 1,2-Butanediol diacetate 5.[13] Yellow syrup. ^{1}H NMR (300 MHz): δ=0.91 (t, 3H, J=7.4 Hz, C4H_3), 1.60 (m, 2H, C3H_2), 2.04 (s, 3H, C2′H_3), 2.05 (s, 3H, C2″H_3), 4.03 (dd, 1H, J=11.8, 6.6 Hz, C1HH), 4.21 (dd, 1H, J=11.8, 3.4 Hz, C1HH), 4.99 (m, 1H, C2H_1). ^{13}C NMR (75 MHz): δ=10.1 (C4), 21.4 (C2′), 21.7 (C2″), 24.4 (C3), 65.4 (C1), 73.4 (C2), 171.2 (C=O′), 171.4 (C=O″).

4.3.3. 1,2-Octanediol diacetate 6.[14] Yellow syrup. ^{1}H NMR (300 MHz): δ=0.86 (t, 3H, J=6.6 Hz, C8H_3), 1.22 (m, 8H, C4H_2, C5H_2, C6H_2, C7H_2), 1.54 (m, 2H, C3H_2), 2.04 (s, 3H, C2′H_3), 2.05 (s, 3H, C2″H_3), 4.01 (dd, 1H, J=11.8, 6.6 Hz, C1HH), 4.21 (dd, 1H, J=12.0, 3.0 Hz, C1HH), 5.05 (m, 1H, C2H_1). ^{13}C NMR (75 MHz): δ=14.7 (C8), 21.4 (C2′), 21.7 (C2″), 23.2 (C7), 25.7 (C6), 29.7 (C5), 31.3 (C4), 32.3 (C3), 65.8 (C1), 72.2 (C2), 171.3 (C=O′), 171.4 (C=O″).

4.3.4. 1,2-Dodecanediol diacetate 7.[15] Yellow syrup. ^{1}H NMR (300 MHz): δ=0.83 (t, 3H, J=6.4 Hz, C12H_3), 1.20 (m, 16H, C4H_2, C5H_2, C6H_2, C7H_2, C8H_2, C9H_2, C10H_2, C11H_2), 1.51 (m, 2H, C3H_2), 2.01 (s, 3H, C2′H_3), 2.02 (s, 3H, C2″H_3), 3.97 (dd, 1H, J=11.8, 6.6 Hz, C1HH), 4.17 (dd, 1H, J=11.9, 3.2 Hz, C1HH), 5.01 (m, 1H, C2H_1). ^{13}C NMR (75 MHz): δ=14.7 (C12), 21.3 (C2′), 21.6 (C2″), 23.3 (C11), 25.7 (C10), 29.8 (C9), 29.9 (C8), 30.0 (C7), 30.1 (C6), 30.2 (C5), 31.3 (C4), 32.5 (C3), 65.8 (C1), 72.3 (C2), 171.6 (C=O′), 171.7 (C=O″).

4.3.5. 2,3-Butanediol diacetate 10.[16] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.19 (m, 3H, C1H_3), 1.23 (m, 3H, C4H_3), 2.04 (s, 3H, C2′H_3), 2.06 (s, 3H, C2″H_3), 4.98 (m, 2H, C2H_1, C3H_1). ^{13}C NMR (75 MHz): δ=8.3 (C1), 9.3 (C4), 14.4 (C2′), 14.5 (C2″), 64.6 (C2), 64.7 (C3), 163.7 (C=O′, C=O″).

4.3.6. 3,3-Dimethyl-1,2-butanediol diacetate 11. Yellow syrup. ^{1}H NMR (300 MHz): δ=0.94 (m, 9H, C4H_3, C5H_3, C6H_3), 2.01 (s, 3H, C2′H_3), 2.07 (s, 3H, C2″H_3), 3.98 (dd, 1H, J=11.7, 8.9 Hz, C1HH), 4.37 (dd, 1H, J=11.7, 2.4 Hz, C1HH), 4.93 (dd, 1H, J=8.9, 2.4 Hz, C2H_1). ^{13}C NMR (75 MHz): δ=21.5 (C2′), 21.6 (C2″), 26.6 (C4, C5, C6), 34.2 (C3), 64.2 (C1), 67.4 (C2), 171.3 (C=O′), 171.6 (C=O″).

4.3.7. Pinacol diacetate 12.[17] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.20 (m, 12H, C1H_3, C4H_3, C5H_3, C6H_3,), 2.05 (m, 6H, C2′, C2″H_3). ^{13}C NMR (75 MHz): δ=22.4 (C1, C4, C5, C6), 25.6 (C2′, C2″), 75.3 (C2, C3), 172.1 (C=O′, C=O″).

4.3.8. D,L-Glyceraldehyde diacetate 13.[18] White solid. Mp 142–144 °C. ^{1}H NMR (300 MHz): δ=2.08 (s, 3H, C2′H_3), 2.14 (s, 3H, C2″H_3), 4.15 (dd, 1H, J=12.4, 3.0 Hz, C3HH), 4.27 (dd, 1H, J=12.5, 4.7 Hz, C3HH), 5.76 (d, 1H, J=7.9 Hz, C2H_1). ^{13}C NMR (75 MHz): δ=21.4 (C2′), 21.6 (C2″), 62.3 (C3), 75.0 (C2), 169.3 (C=O′), 171.2 (C=O″).

4.3.9. (*R*)-(−)-Dihydro-5-(hydroxymethyl)-2(3*H*)-furanone acetate 15.[19] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.10 (s, 3H, C2′H_3), 2.35 (m, 2H, C3H_2), 2.58 (m, 2H, C2H_2), 4.14 (dd, 1H, J=12.2, 5.6 Hz, C5HH), 4.30 (dd, 1H, J=12.2, 3.2 Hz, C5HH), 4.73 (m, 1H, C4H_1). ^{13}C NMR (75 MHz): δ=21.4 (C2′), 24.6 (C3), 28.8 (C2), 66.0 (C5), 77.9 (C4), 171.2 (C=O′), 177.1 (C=O).

4.3.10. (*S*)-(+)-Dihydro-5-(hydroxymethyl)-2(3*H*)-furanone acetate 17.[19] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.09 (s, 3H, C2′H_3), 2.34 (m, 2H, C3H_2), 2.59 (m, 2H, C2H_2), 4.13 (dd, 1H, J=12.2, 5.6 Hz, C5HH), 4.30 (dd, 1H, J=12.2, 3.2 Hz, C5HH), 4.73 (m, 1H, C4H_1). ^{13}C NMR

(75 MHz): δ=21.4 (C2′), 24.6 (C3), 28.8 (C2), 66.0 (C5), 77.9 (C4), 171.2 (C=O′), 177.1 (C=O).

4.3.11. *cis*-1,2-Cyclopentanediol diacetate 18.[20] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.80 (m, 12H, C3H$_2$, C4H$_2$, C5H$_2$, C2′, C2″H$_3$), 5.12 (m, 2H, C1H$_1$, C2H$_1$). ^{13}C NMR (75 MHz): δ=19.8 (C4), 21.6 (C2′, C2″), 28.8 (C3, C5), 74.8 (C1, C2), 171.1 (C=O′, C=O″).

4.3.12. *cis*-1,2-Cyclohexanediol diacetate 19.[21] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.70 (m, 14H, C3H$_2$, C4H$_2$, C5H$_2$, C6H$_2$, C2′H$_3$, C2″H$_3$,), 5.00 (m, 2H, C1H$_1$, C2H$_1$). ^{13}C NMR (75 MHz): δ=21.8 (C2′, C2″), 22.3 (C4, C5), 28.3 (C3, C6), 71.6 (C1, C2), 171.1 (C=O′, C=O″).

4.3.13. (1*S*,2*S*)-*trans*-1,2-Cyclohexanediol diacetate 20.[22] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.70 (m, 14H, C3H$_2$, C4H$_2$, C5H$_2$, C6H$_2$, C2′H$_3$, C2″H$_3$,), 4.78 (m, 2H, C1H$_1$, C2H$_1$). ^{13}C NMR (75 MHz): δ=21.8 (C2′, C2″), 24.1 (C4, C5), 30.8 (C3, C6), 74.4 (C1, C2), 171.1 (C=O′, C=O″).

4.3.14. (1*R*,2*R*)-*trans*-1,2-Cyclohexanediol diacetate 21.[23] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.70 (m, 14H, C3H$_2$, C4H$_2$, C5H$_2$, C6H$_2$, C2′H$_3$, C2″H$_3$,), 4.78 (m, 2H, C1H$_1$, C2H$_1$). ^{13}C NMR (75 MHz): δ=21.8 (C2′, C2″), 24.0 (C4, C5), 30.8 (C3, C6), 74.4 (C1, C2), 171.3 (C=O′, C=O″).

4.3.15. (S)-(−)-1,1-Diphenyl-1,2-propanediol 2-acetate 22.[24] White solid. Mp 137–139 °C. ^{1}H NMR (300 MHz): δ=1.09 (d, 3H, *J*=6.4 Hz, C3H$_3$), 1.87 (s, 3H, C2′H$_3$), 5.91 (q, 1H, *J*=6.4 Hz, C2H$_1$), 7.28 (m, 10H, C$_{Arom}$). ^{13}C NMR (75 MHz): δ=15.1 (C3), 21.8 (C2′), 74.5 (C2), 80.2 (C1), 126.2, 126.5, 127.7, 128.9, 129.0, 143.8, 146.1 (C$_{Arom}$), 170.8 (C=O′).

4.3.16. D-Mannose pentaacetate 24.[25] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.99 (s, 3H, C2′H$_3$), 2.03 (s, 3H, C2″H$_3$), 2.07 (s, 3H, C2‴H$_3$), 2.15 (s, 3H, C2⁗H$_3$), 2.16 (s, 3H, C2⁗′H$_3$), 4.09 (m, 2H, C5H$_1$, C6*H*H), 4.26 (dd, 1H, *J*=12.2, 4.7 Hz, C6*H*H), 5.28 (m, 3H, C2H$_1$, C3H$_1$, C4H$_1$), 6.06 (m, 1H, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C2′, C2″, C2‴, C2⁗, C2⁗′), 62.7 (C6), 66.2 (C4), 69.0 (C2), 69.4 (C3), 71.2 (C5), 91.2 (C1), 168.7 (C=O′), 170.1 (C=O″), 170.3 (C=O‴), 170.6 (C=O⁗), 171.2 (C=O⁗′).

4.3.17. D-Xylose tetraacetate 25.[26] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.02 (s, 3H, C2′H$_3$), 2.04 (s, 3H, C2″H$_3$), 2.05 (s, 3H, C2‴H$_3$), 2.17 (s, 3H, C2⁗H$_3$), 3.71 (dd, 1H, *J*=11.0 Hz, C5*H*H), 3.93 (dd, 1H, *J*=11.0, 5.9 Hz, C5*H*H), 5.02 (m, 2H, C2H$_1$, C4H$_1$), 5.46 (dd, 1H, *J*=9.5 Hz, C3H$_1$), 5.47 (m, 1H, C3H$_1$), 6.25 (d, 1H, *J*=3.7 Hz, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.2 (C2′), 21.3 (C2″), 21.4 (C2‴), 21.5 (C2⁗), 61.3 (C5), 69.3 (C2, C4), 70.0 (C3), 89.9 (C1), 169.7 (C=O′), 170.4 (C=O″), 170.5 (C=O‴), 170.8 (C=O⁗).

4.3.18. D-Glucose pentaacetate 28.[27] White solid. Mp 100–102 °C. ^{1}H NMR (300 MHz): δ=2.01 (s, 3H, C2′H$_3$), 2.04 (s, 3H, C2″H$_3$), 2.05 (s, 3H, C2‴H$_3$), 2.06 (s, 3H, C2⁗H$_3$), 2.09 (s, 3H, C2⁗′H$_3$), 4.09 (m, 2H, C5H$_1$, C6*H*H), 4.26 (dd, 1H, *J*=12.5, 4.1 Hz, C6*H*H), 5.11 (m, 2H, C2H$_1$, C4H$_1$), 5.41 (m, 1H, C3H$_1$), 6.32 (d, 1H, *J*=3.7 Hz, C1H$_1$).

4.3.19. D-Fructose pentaacetate 29.[28] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.04 (s, 3H, C2′H$_3$), 2.06 (s, 3H, C2″H$_3$), 2.08 (s, 3H, C2‴H$_3$), 2.09 (s, 3H, C2⁗H$_3$), 2.10 (s, 3H, C2⁗′H$_3$), 4.16 (m, 2H, C5H$_2$,), 4.75 (m, 2H, C6H$_2$), 5.38 (m, 3H, C2H$_1$, C3H$_1$, C4H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C2′, C2″, C2‴, C2⁗, C2⁗′), 62.3 (C5), 63.6 (C2), 64.1 (C3), 67.2 (C4), 68.8 (C6), 103.2 (C1), 170.2 (C=O′, C=O″, C=O‴, C=O⁗, C=O⁗′).

4.3.20. D-Arabinose tetraacetate 30.[26] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.02 (s, 3H, C2′H$_3$), 2.06 (s, 3H, C2″H$_3$), 2.12 (s, 3H, C2‴H$_3$), 2.15 (s, 3H, C2⁗H$_3$), 4.10 (m, 2H, C5H$_2$), 5.34 (m, 3H, C2H$_1$, C3H$_1$, C4H$_1$), 6.34 (d, 1H, *J*=2.9 Hz, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.2 (C2′), 21.4 (C2″), 21.6 (C2‴), 21.7 (C2⁗), 63.4 (C5), 67.3 (C2), 67.7 (C3), 69.1 (C4), 90.9 (C1), 169.8 (C=O′), 170.6 (C=O″), 170.8 (C=O‴), 171.0 (C=O⁗′).

4.3.21. D-Sorbitol hexaacetate 32.[29] White solid. Mp 87–89 °C. ^{1}H NMR (300 MHz): δ=2.05 (s, 3H, C2′H$_3$), 2.06 (s, 3H, C2″H$_3$), 2.07 (s, 3H, C2‴H$_3$), 2.08 (s, 3H, C2⁗H$_3$), 2.09 (s, 3H, C2⁗′H$_3$), 2.13 (s, 3H, C2⁗″H$_3$), 4.01 (dd, 1H, *J*=12.1, 6.2 Hz, C1*H*H), 4.12 (dd, 1H, *J*=12.1, 5.2 Hz, C6*H*H), 4.24 (dd, 1H, *J*=12.1, 3.7 Hz, C1*H*H), 4.36 (dd, 1H, *J*=12.1, 4.3 Hz, C6*H*H), 5.05 (m, 1H, C2H$_1$), 5.24 (m, 1H, C5H$_1$), 5.42 (m, 2H, C3H$_1$, C4H$_1$). ^{13}C NMR (75 MHz): δ=21.2 (C2′, C2″, C2‴, C2⁗, C2⁗′, C2⁗″), 62.1 (C1), 62.5 (C6), 69.0 (C2), 69.2 (C5), 69.3 (C3), 70.0 (C4), 170.4 (C=O′), 170.5 (C=O″), 170.6 (C=O‴), 170.7 (C=O⁗), 171.1 (C=O⁗′), 171.2 (C=O⁗″).

4.3.22. D-Galactose pentaacetate 33.[30] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.98 (s, 3H, C2′H$_3$), 1.99 (s, 3H, C2″H$_3$), 2.02 (s, 3H, C2‴H$_3$), 2.03 (s, 3H, C2⁗H$_3$), 2.14 (s, 3H, C2⁗′H$_3$), 4.07 (m, 2H, C5H$_1$, C6*H*H), 4.32 (dd, 1H, *J*=12.1, 5.6 Hz, C6*H*H), 5.31 (m, 2H, C2H$_1$, C4H$_1$), 5.48 (m, 1H, C3H$_1$), 6.35 (m, 1H, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C2′, C2″, C2‴, C2⁗, C2⁗′), 61.9 (C6), 67.1 (C4), 68.0 (C2), 68.1 (C3), 69.4 (C5), 90.3 (C1), 169.5 (C=O′), 170.0 (C=O″), 170.5 (C=O‴), 170.8 (C=O⁗), 171.0 (C=O⁗′).

4.3.23. D-Fucose tetraacetate 34.[31] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.13 (d, 3H, *J*=6.4 Hz, C6H$_3$), 1.98 (s, 3H, C2′H$_3$), 1.99 (s, 3H, C2″H$_3$), 2.16 (s, 3H, C2‴H$_3$), 2.17 (s, 3H, C2⁗H$_3$), 4.25 (m, 1H, C3H$_1$), 5.31 (m, 3H, C2H$_1$, C4H$_1$, C5H$_1$), 6.31 (d, 1H, *J*=2.8 Hz, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C6), 21.5 (C2′, C2″, C2‴, C2⁗), 63.4 (C5), 66.8 (C2), 66.9 (C3), 68.0 (C4), 91.6 (C1), 169.4 (C=O′), 170.1 (C=O″), 170.4 (C=O‴), 170.6 (C=O⁗).

4.3.24. L-Arabinose tetraacetate 35.[32] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.01 (s, 3H, C2′H$_3$), 2.02 (s, 3H, C2″H$_3$), 2.12 (s, 3H, C2‴H$_3$), 2.14 (s, 3H, C2⁗H$_3$), 4.05 (m, 2H, C5H$_2$), 5.30 (m, 3H, C2H$_1$, C3H$_1$, C4H$_1$), 6.32 (d, 1H, *J*=2.6 Hz, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C2′, C2″, C2‴, C2⁗), 63.4 (C5), 67.3 (C2), 67.7 (C3), 69.1 (C4), 90.8 (C1), 169.8 (C=O′), 170.5 (C=O″), 170.8 (C=O‴), 170.9 (C=O⁗).

4.4. Enzyme measurements

Substrates were diluted from 10 mM stock solutions in

water/acetonitrile mixtures. Enzymes were diluted from 10 mg mL^{-1} stock solutions in aqueous borate buffer (50 mM, pH 8.0). $NaIO_4$ was added as a freshly prepared 10 mM stock solution in water. Adrenaline (as. HCl salt) was added as a 15 mM stock solution in water. Assays (0.1 mL) were conducted in individual wells of 96-well flat-bottom half-area polystyrene microtiter-plates (Costar) as described in the figure legends. The OD was recorded using a Spectramax 190 Microplate Spectrophotometer (Molecular Devices, λ=490 nm).

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