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Mapping the substrate selectivity and enantioselectivity of esterases from thermophiles

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Abstract—To identify potential applications of nineteen esterases from thermophiles, we mapped their substrate selectivity and enantioselectivity using a library of 50 esters. We measured the selectivities colorimetrically using Quick *E*, which uses pH indicators to detect hydrolysis and a chromogenic reference compound as an internal control. The substrate selectivity mapping revealed one esterase, E018b, with a strong preference for acetyl esters (14- to 25-fold over hexanoate). The enantioselectivity mapping revealed a number of cases of high enantioselectivity. Thirteen of the 19 esterases showed moderate or better enantioselectivity (>19) toward 1-phenethyl butyrate favoring the (*R*)-enantiomer and two esterases (E008, E013) showed moderate or better enantioselectivity (>20) toward methyl 2-chloropropionate favoring the (*S*)-enantiomer. Three esterases (E001, E004, E005) showed high (>46) enantioselectivity toward menthyl acetate favoring the (*R*)-enantiomer. This rapid mapping of the selectivity simplifies the characterization of new enzymes.

1. Introduction

Enzymes from thermophiles (microorganisms that grow in unusual environments such as hot springs) may be useful for chemical synthesis.[1] For example, they allow using higher temperatures where reactions are faster and substrates are more soluble.[2] In addition, these enzymes may also tolerate other unusual conditions such as high concentrations of organic solvents. Although thermophiles and other extremophiles are difficult to grow in the laboratory, advances in molecular biology permit researchers to produce extremophile enzymes in microorganisms such as *E. coli* that grow more easily in the laboratory.[3]

To apply these new enzymes in organic synthesis, one needs some idea of their substrate range and selectivity. Unfortunately, substrate mapping of enzymes is still a slow and tedious process. Recently we developed a fast colorimetric method for measuring the selectivity of hydrolases.[4] Herein, we apply this fast colorimetric method to map the selectivity of nineteen esterases from ThermoGen.[5] These esterases come from thermophiles and are stable at high temperatures. Dimirjian et al. have already mapped some of their substrate selectivity.[6]

2. Results

2.1. Screening method

Preliminary screening measured the initial rate of hydrolysis of different esters. The rate of reaction was measured by monitoring the release of protons using a pH indicator, 4-nitrophenol (Fig. 1a). The yellow color of the phenoxide fades as the reaction proceeds and reveals quantitatively the amount of ester hydrolyzed as described previously.[2]

The second screening measured selectivity. Selectivity measurements require a competition between substrates.[7] We used a reference compound, usually resorufin acetate, as the competitive substrate (Fig. 1b). Hydrolysis of resorufin acetate yields the pink resorufin. We previously used this method to measure enantioselectivity (Quick *E*).[2] In this paper we adapt this method to also measure substrate selectivity (Quick *S*).

Accurate measurement of the selectivities requires that the substrate and the reference compound react at

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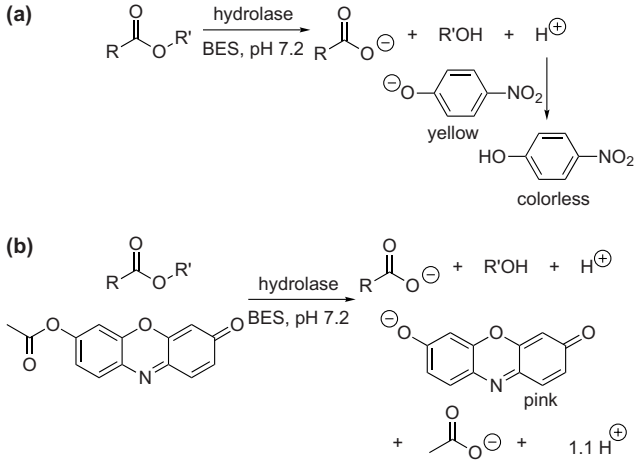


Figure 1. Measuring selectivity of hydrolases. (a) Estimated selectivities compared the initial rates of hydrolysis of esters measured colorimetrically using 4-nitrophenol as a pH indicator. (b) Quantitative measure of selectivity used resorufin esters as internal standards.

comparable rates to measure both reaction rates. When the substrate hydrolysis was much slower than resorufin acetate hydrolysis, we replaced resorufin acetate with the slower reacting resorufin pivaloate or resorufin isobutyrate as reference compounds and extended the measurement time. However, even after this change, some substrates reacted too slowly for an accurate measure of selectivity using this Quick *E*/*S* method.

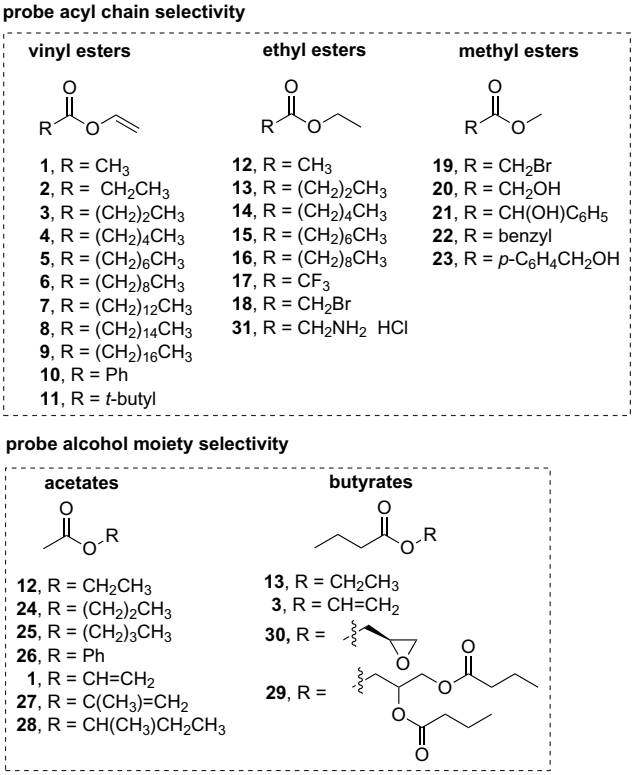
2.2. Survey of substrate selectivity

To survey the substrate selectivity of the thermophile esterases, we first measured their ability to hydrolyze 31 different commercially available esters (Scheme 1, Table 1). Good substrates showed a specific activity >1 μmol ester hydrolyzed/mg protein/min, while very good substrates showed a specific activity >10. (The specific activities listed in the Tables are multiplied by 100 for convenience.) The best substrates were activated esters, which are also chemically the most reactive in the library: vinyl esters **1**–**11**, ethyl trifluoroacetate **17**, and phenyl acetate **26**. Among the vinyl and ethyl esters with different chain lengths, most of the esterases appear to favor the hexanoate or octanoate, while E018b appears to favor acetate esters. The sterically hindered vinyl pivaloate **11** was a poor substrate for all the esterases, as was vinyl benzoate **10**. Polar esters, for example, **20**, **21**, **30** were usually poor substrates. Ethyl butyrate **13** reacted much slower that the more hydrophobic tributyrin **29**. This preference for hydrophobic substrates suggests that these esterases may be related to lipases.

Three pairs of esterases (E010/E011, E013/E014, E019/E020) showed similar reaction rates with all substrates and therefore might be very similar or even identical enzymes.

2.3. Acyl chain length selectivity

We determined the true selectivity of the thermophile esterases toward different acyl chain length using a competitive experiment. We used resorufin esters as the competitive substrate as explained above and normalized the results to hexanoate, Table 2. Details for selected experiments are given in Table 3 and Figure 2 shows a gray-scale array of the selectivities toward vinyl esters. The trends for the true selectivities were similar to the estimated selectivities. Among the vinyl esters, the hexanoate or octanoate was the best substrate for all except E018b, where the acetate was the best substrate. E018b favored vinyl acetate 18-fold over vinyl hexanoate (1800/100, Table 2). The acetate was a very poor substrate for many esterases (e.g., E006, E008, E011, E014). For example, E006 favored vinyl hexanoate >700-fold over vinyl acetate (100/0.13, Table 2).[8]



Scheme 1. Esters used to survey the substrate selectivity of esterases. Some substrates are listed more then once because they fit into more than one category.

The hexanoate versus acetate selectivity varied for other esters, but did not reverse selectivity. For the ethyl esters, E018b favored ethyl acetate 21-fold over ethyl hexanoate (1400/100, Table 2), while E002 favored ethyl hexanoate >1000-fold over ethyl acetate (100/<0.48, Table 2). For the phenyl esters, E018b favored phenyl acetate 25-fold over the hexanoate (2500/100, Table 2). E011 favored phenyl hexanoate 47-fold over phenyl acetate (100/2.1, Table 2). In comparison, this enzyme showed much higher chain length selectivity for the vinyl esters: it favored vinyl hexanoate 1100-fold over vinyl acetate (100/0.089, Table 2). For the glycerol esters E018b favored triacetin 19-fold over trihexanoin (hexanoic acid triglyceride, 2800/150, Table 2). Confirming the selectivity of E018b for acetyl groups, E018b only catalyzed the hydrolysis of acetyl esters, but not

Table 1. Specific activities of thermophile esterases toward esters **1**–**31**[a]

Substrate	Esterase																		
	E001	E002	E003	E004	E005	E006	E007	E008	E009	E010	E011	E012	E013	E014	E015	E016	E018b	E019	E020
1	**150**	**160**	63	60	**140**	**120**	2.3	12.0	**340**	5.5	5.1	3.1	3.0	1.2	89	63	**150**	**120**	**110**
2	**940**	**730**	**220**	**210**	**780**	**260**	**130**	**270**	**1400**	**140**	**160**	11	**140**	**170**	**350**	**220**	20	**400**	**370**
3	**860**	**1000**	**300**	**790**	**550**	**410**	**76**	**320**	**2000**	**190**	**220**	37	**240**	**180**	**280**	**230**	1.1	**490**	**550**
4	**4900**	**3000**	**1400**	**760**	**4100**	**1700**	**510**	**980**	**3800**	**630**	**680**	48	**660**	**540**	**1100**	**1300**	9.3	**2400**	**3000**
5	**2100**	**1700**	**1600**	**960**	**1200**	**960**	**160**	**300**	**1800**	**220**	**260**	50	**6700**	**4600**	**320**	**1200**	2.0	**880**	**1200**
6	**1800**	**1600**	<5	150	**1500**	0.55	10	2.8	**1600**	**240**	**280**	27	**190**	**170**	**330**	15	4.8	**600**	**590**
7	2.6	0.53	16	13	1.1	0.61	0.43	0.88	2.1	0.93	0.45	7.6	0.44	0.81	3.0	21	5.6	0.68	1.0
8	0.19	0.06	<5	3.0	0.13	0.13	<0.1	<0.2	<0.2	<0.2	<0.1	<2	0.10	<0.1	0.16	2.2	1.3	<0.2	<0.2
10	**110**	**140**	44	58	**120**	5.7	1.4	6.5	**230**	2.6	2.7	22	1.7	1.0	5.9	50	19	59	14
11	1.9	2.1	8.2	44	1.6	0.52	0.02	3.2	27	1.2	0.80	16	0.42	0.40	4.0	20	1.1	0.58	1.2
12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
13	4.1	3.3	8.8	26	2.8	1.1	0.19	2.4	**120**	1.1	0.71	<2	0.63	0.40	2.0	3.5	<1	1.5	3.0
14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15	**280**	**240**	96	51	**250**	**160**	3.7	**120**	**570**	**590**	**1200**	19	6.7	5.1	**120**	83	19	1.2	2.6
16	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
17	8.2	**320**	**1600**	**1400**	4.4	1.7	1.9	8.8	**750**	4.1	2.7	**1200**	2.3	1.4	**190**	**1500**	**1100**	1.2	0.35
18	19	75	25	54	66	4.8	1.0	15	**410**	6.6	5.5	5.9	**920**	**660**	**100**	16	19	2.5	3.1
19	8.8	4.9	9.9	9.3	4.1	1.9	0.27	4.3	97	1.8	1.6	2.4	1.3	0.73	2.6	7.6	7.8	1.9	1.6
20	0.25	0.15	<5	13	0.15	0.11	<0.1	<0.2	0.52	0.30	<0.1	<2	<0.1	<0.1	<0.1	<2	1.8	<0.2	<0.2
21	0.12	<0.05	<5	<2	<0.1	<0.1	<0.1	<0.2	<0.2	<0.2	0.04	<2	<0.1	<0.1	<0.1	<2	<1	<0.2	<0.2
22	0.51	0.30	<5	24	0.27	0.16	<0.1	0.21	0.83	0.26	<0.1	<2	<0.1	<0.1	0.14	<2	<1	0.21	0.28
23	0.73	0.50	<5	<2	0.45	0.27	0.19	0.22	0.63	<0.2	<0.1	<2	<0.1	<0.1	0.13	<2	<1	0.24	0.47
24	1.3	0.82	<5	<2	0.69	0.31	<0.1	0.22	4.4	0.48	0.25	2.8	<0.1	<0.1	0.26	<2	**230**	0.49	0.85
25	1.9	1.6	6.1	2.8	1.5	0.54	<0.1	0.85	20	<0.2	0.5	<2	0.35	0.21	1.0	13	48	1.6	2.0
26	**520**	**490**	**350**	**290**	**570**	**300**	87	**340**	**1900**	**210**	**270**	30	**210**	**150**	**320**	**190**	**150**	**250**	**390**
27	**110**	**100**	38	56	**150**	69	0.92	5.2	**250**	**320**	**590**	2.4	2.0	0.73	**100**	49	75	3.1	3.3
28	0.66	0.52	<5	<2	0.38	0.16	<0.1	0.5	4.3	0.37	0.19	<2	0.19	<0.1	0.29	<2	12	0.51	2.1
29	**720**	**570**	**190**	**290**	**640**	**370**	88	**600**	**3000**	**430**	**450**	37	**330**	**280**	**550**	**130**	3.4	**1400**	**1600**
30	14	58	22	9.9	65	3.5	0.65	3.5	94	1.4	1.2	3.4	1.0	0.53	3.2	14	1.6	1.2	2.0
31	<0.1	<0.05	14	12	<0.1	<0.1	<0.1	<0.2	<0.2	<0.2	<0.1	6.0	<0.1	<0.1	<0.1	13	**250**	0.9	1.4

[a] In μmol ester hydrolyzed/min/mg protein × 100. Bold numbers highlight all values >100. ND = not determined.

Table 2. Acyl chain length selectivity of thermophile and several acetyl selective esterases toward vinyl, phenyl, glyceryl, and ethyl esters (hexanoate = 100)[a]

Esterase	Vinyl esters						Phenyl esters				Glycerol esters			Ethyl esters		
	C2, 1	C3, 2	C4, 3	C6, 4	C8, 5	C10, 6	C2, 26	C4	C6	C8	C2	C4, 29	C6	C2, 12	C6, 14	C10, 16
E001	0.45[b]	3.4	12	100	49	11[c]	9.5	47	100	160	<0.053[b]	100	72[d]	ND	ND	ND
E002	19[d]	71	260	100	650	300[c]	5.9	30	100	32	<0.075[b]	100	120[d]	<0.48[b]	100[d]	230[d]
E003	0.97[d]	7.3	19	100	46	22[c]	6.0	173	100	180	<1.23[b]	100	33[d]	<0.053[b]	100[d]	55[d]
E004	0.18[d]	0.70	7.9	100	8.0	2.1[d]	3.3	147	100	110	<0.015[b]	100	6.6[d]	ND	ND	ND
E005	6.23[d]	11	29	100	96	48[c]	3.0	16	100	45	<0.050[b]	100	89[d]	ND	ND	ND
E006	0.13[c]	26	82	100	330	70[c]	5.2	40	100	39	<0.20[b]	100	40[d]	ND	ND	ND
E007	2.0[d]	4.3	16	100	64	13[c]	12	141	100	240	<2.0[b]	100	16[d]	ND	ND	ND
E008	0.56[d]	5.3	5.3	100	7.3	7.8[d]	3.1	39	100	24	<0.0020[b]	100	77[d]	<0.20[b]	100[d]	23[d]
E009	0.14[d]	0.86	8.3	100	10	1.8[d]	2.3	140	100	68	0.0010[d]	100	5.9[c]	ND	ND	ND
E010	0.38[d]	2.7	9.8	100	14	8.5[d]	2.0	65	100	18	<0.016[b]	100	2.1[c]	ND	ND	ND
E011	0.089[d]	0.08	13	100	18	1.4[d]	2.1	60	100	27	<0.0045[b]	100	2.4[c]	<0.76[b]	100[d]	15[d]
E012	0.11[c]	2.0	7.8	100	26	4.1[c]	6.7	74	100	110	0.17[d]	100	6.4[c]	ND	ND	ND
E013	3.0[d]	19	13	100	18	40[d]	3.3	77	100	56	<0.0011[b]	100	42[c]	ND	ND	ND
E014	0.010[d]	1.2	13	100	18	4.6[d]	1.8	98	100	73	<0.15[b]	100	21[d]	ND	ND	ND
E015	0.46[d]	3.3	13	100	18	12[d]	3.2	95	100	44	<0.015[b]	100	1.0[c]	ND	ND	ND
E016	24[d]	25	77	100	192	30[c]	6.5	74	100	81	<0.065[b]	100	9.8[c]	ND	ND	ND
E018b	1800[c]	680[c]	74[c]	100[c]	220[c]	110[c]	2500[c]	310[c]	100[c]	48[c]	2800[c]	100[d]	150[d]	1400[c,e]	100[d]	420[c]
E019	1.7[c]	13	53	100	230	36[c]	3.4	54	100	23	<0.067[b]	100	5.1[c]	ND	ND	ND
E020	1.5[d]	12	41	100	220	34[c]	3.2	50	100	29	0.010[d]	100	5.4[c]	ND	ND	ND
AcCE[f]	6.2[c]	6.9[c]	5.4[c]	100[c]	23[c]	25[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AcE[f]	850[c]	370[c]	140[c]	100[c]	150[c]	160[c]	3500[c]	122[c]	100[d]	67[d]	730[c]	100[d]	87[d]	ND	ND	ND

[a] True selectivities (estimated error limits are ±10–30%) measured using resorufin acetate as the reference compound unless otherwise noted. ND = not determined. Measurements using different reference compounds are scaled so that relative values for one enzyme and a substrate series (e.g., vinyl esters) can be compared. Figure 2 comes from the data for vinyl esters in this table, scaled by the vinyl hexanoate activities listed in Table 1.

[b] Substrate reacted much slower than all available resorufin esters. The value listed is an estimated upper limit.

[c] Resorufin isobutyrate as the reference compound.

[d] Resorufin pivaloate as the reference compound.

[e] Estimated error limits are larger, ±50%.

[f] AcCE = acetylcholine esterase; AcE = acetyl esterase from orange peel.

Table 3. Representative experimental data for Quick *S* and Quick *E* measurements in Tables 2 and 9[a]

	Ester	Substrate		Reference[b]		Selectivity[e]	Quick *E*/*S*[f]
		Obsvd rate[c]	Specific activity[d]	Obsvd rate[c]	Specific activity[d]		
AcE	**1**	−16	0.67	20	0.22	3.0	8.5 (**1**)
	4	−15	0.091	23	0.26	0.35	
E005	(*R*)-**45**	−6	3.2	3	0.48	6.7	4.9 (*S*)
	(*S*)-**45**	−24	15	2	0.47	33	
E011	(*R*)-**45**	−34	0.86	33	0.33	2.6	5.1 (*S*)
	(*S*)-**45**	−69	2.4	17	0.18	13	
E013	(*R*)-**45**	−38	0.58	22	0.11	5.3	2.8 (*S*)
	(*S*)-**45**	−82	1.5	20	0.10	15	
E014	(*R*)-**45**	−24	0.47	18	0.13	3.6	2.7 (*S*)
	(*S*)-**45**	−54	1.3	17	0.13	9.9	

[a] AcE is acetyl esterase from orange peel.

[b] Quick *E* measurements used resorufin acetate, while Quick *S* measurements resorufin pivaloate as reference compound.

[c] Observed rate is in mOD/min and is an average of three or four readings, typical errors are 10–20%. Negative values reflect the fading yellow color of the pH indicator during the reaction, while positive values reflect the increase in the pink color of resorufin.

[d] In U/mg protein; reactions contained differing amounts of protein.

[e] Selectivity for the substrate versus the reference compound.

[f] The favored substrate or enantiomer is in parentheses.

butanoyl or other esters, in the enantiomer pair library (see below). For the vinyl, phenyl, glyceryl, and ethyl esters E018b favored acetyl esters 14- to 25-fold over hexanoyl esters.

To compare E018b to other acetyl-selective enzymes, we measured the chain length selectivity of acetyl esterase from orange peel (AcE) and acetylcholine esterase from electric eel (AcCE). AcE has been used several times in organic synthesis for removal of acetyl groups.[10] For the vinyl esters, AcE favored an acetyl group 8.5-fold over a hexanoyl group (850/100, Table 2), approximately a 2-fold lower selectivity than E018b, 18-fold, Table 2. For the glycerol esters, AcE preferred the ace-

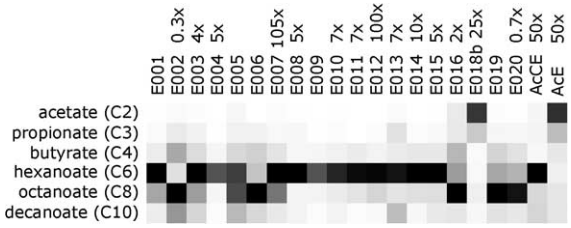


Figure 2. Acyl length selectivity of thermophile esterases toward vinyl esters. Darker squares correspond to higher activity. Most esterases favor either vinyl hexanoate or octanoate, but E018b and AcE (acetyl esterase from orange peel) favor vinyl acetate. To show selectivity clearly, the absolute enzyme activities were scaled as indicated. This grayscale array representation was created from the data in Tables 1 and 2 as described by Reymond et al.[9] AcCE = acetylcholine esterase.

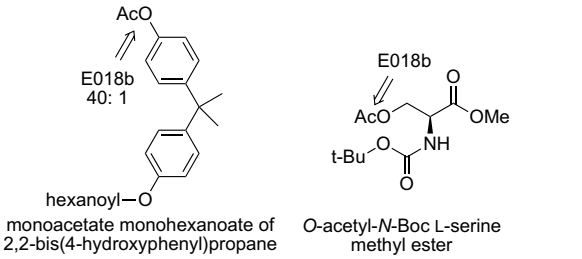


Figure 3. Chemoselective hydrolysis of acetyl esters with E018b. (a) Selective removal of an acetate ester with E018b and a hexanoate ester with E015 in a mixed diester of a bisphenol. (b) Selective removal of the acetyl group in a serine derivative with no detectable hydrolysis of the methyl ester or the *tert*-butoxycarbonyl group.

tate over the hexanoate by 8.4-fold (730/87, Table 2), approximately 2-fold less then E018b, 19-fold. For the phenyl esters, AcE favored an acetyl group 35-fold over a hexanoyl group (3500/100, Table 2), slightly higher than the selectivity of E018b, 25-fold. AcCE did not show the expected acetyl preference with vinyl esters, but favored vinyl hexanoate 16-fold over the acetate (100/6.2, Table 2). Thus, E018b shows comparable or higher acetyl selectivity than other acetyl-selective enzymes.

A competition between vinyl butyrate and acetate monitored by ^{1}H NMR confirmed the high acetyl selectivity of E018b. The resonances of both substrate vinyl esters, and the products, butyric, and acetic acid, were monitored over time and revealed a 17-fold acetyl preference, Table 10 below. In comparison the Quick *S* measurements showed a 24-fold acetyl preference (1800/74 in Table 2). For comparision, acetyl esterase from orange peel showed only a 4-fold preference for acetyl in this experiment. This value is similar to the value of 5.9 (840/144 from Table 2) predicted by the Quick *S* measurements. As a complementary enzyme, we tested E015, which showed an 18-fold preference for butyryl over acetyl, as compared to the 51-fold preference in Quick *S* measurements (3.7/0.072 from Table 2). Small differences in the experimental conditions, such as added detergent Triton X-100 for the Quick *S* measurements, but not the NMR measurements, may cause these differences in selectivity.

We also demonstrated the selective removal of an acetyl group in a mixed diester of bisphenol A using E018b (Fig. 3). HPLC analysis showed selective removal of the acetyl group and a 40:1 ratio of the monohexanoate over the mono acetate. This selectivity was slightly higher than that measured for the phenyl esters: phenyl acetate, **26**, versus phenyl hexanoate was 25:1, likely as a result of the slightly different substrates. E018b also selectively removed the acetyl ester in *O*-acetyl-*N*-Boc L-serine methyl ester.

2.4. Estimated enantioselectivity

To evaluate the potential of the thermophile esterases for enantioselective reactions, we measured the initial rates of hydrolysis of the esterases using 21 pairs of enantiomers (Scheme 2, Tables 4–7). The ratio of the initial rates of the enantiomers is an estimated enantioselectivity.

Substrates with the chirality in the alcohol portion (Tables 4, 5) usually reacted faster than substrates where the chirality is in the carboxylic acid portion (Tables 6–8). The best substrates—**35**, **36**, and **38**—were nonpolar molecules with the stereocenter in the alcohol portion. The poorest substrates—**39**, **40**, **44**, **46**, **47**, **50**, **51**, and **52**—were polar molecules with the stereocenter in the carboxylic acid portion. This observation is consistent with the screening above which also showed that polar esters were poor substrates.

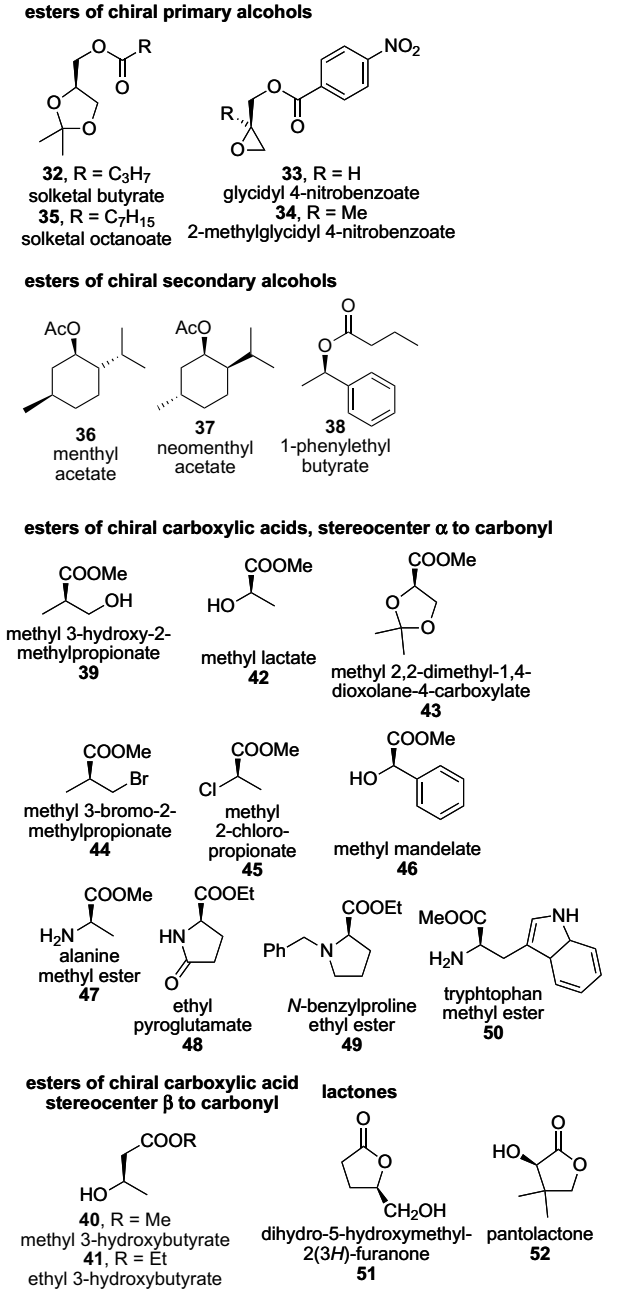
The estimated enantioselectivities were below two in most cases, but two substrates (**38** and **45**) showed higher estimated enantioselectivities with most thermophile esterases. In addition, seven other substrates (**34**, **36**, **42**–**44**, **47**, **50**) showed estimated enantioselectivities above two with several esterases. These esters were screened further using the more accurate Quick *E* method.

2.5. True enantioselectivity

We measured the true enantioselectivity for esters **34**, **36**, **38**, **43**, and **45** using a resorufin ester as a reference compound as described above. When the slow enantiomer reacted much slower than the reference compound, we could only set a lower limit on the enantioselectivity. For several other substrates (**42**, **44**, **47**, and **50**), both enantiomers reacted very slowly compared to the resorufin esters used as reference compounds, so we could not accurately measure enantioselectivity using Quick *E*. Results are summarized in Table 9, detailed data for selected measurements is in Table 3 above.

None of the esterases showed enantioselectivities above five for **34**, 2-methyl glycidyl 4-nitrobenzoate, an ester of a primary alcohol. This is consistent with the screening using estimated *E* (Table 4), where none of the esterases showed an estimated enantioselectivity of above three.

For menthyl acetate, **36**, an acetate of a secondary alcohol, the estimated enantioselectivities (Table 5) were also



Scheme 2. Enantiomer pairs used to survey the enantioselectivity of esterases. Only one enantiomer is shown.

below three. However, Quick *E* identified one esterase, E004, with high enantioselectivity (*E* >81), favoring the (*R*) enantiomer. In addition, two other esterases, E001 and E002, may also be highly enantioselective, but our measured lower limits for the enantioselectivity were only 3.2 and 8.9, respectively. The favored enantiomer was (*R*), as previously seen for esterases and lipases.

For 1-phenylethyl butyrate, **38**, another ester of a secondary alcohol, most of the esterases showed high enantioselectivity (Table 9). Esterases E001, E002, E004, E005, E008, E009, E010, E011, E013, E014, E015, E019, and E020 showed enantioselectivities >19 and E006 showed a lower limit of 16. Like other lipases and esterases, the thermophile esterases favored the (*R*)-enantiomer of **38**. These true enantioselectivities measured by Quick *E* were usually higher than the estimated *E* values in Table 5 above.

Quick *E* measurements with methyl 2,2-dimethyl-1,4-dioxolane-4-carboxylate, **43**, were difficult because the reactions were slow. Quick *E* measurement only set lower limits of >2.2 to >5.5 on the enantioselectivity for five esterases. By comparison, estimated enantioselectivity values for these five esterases ranged from 4.1 to 8.9 (Table 7).

Most of these esterases showed moderate or better enantioselectivity toward methyl 2-chloropropionate, **45**. Esterase E008 showed the highest enantioselectivities, 21, and the lower limits on the enantioselectivity of esterases E010 and E011 were >6.2 and >15. As noted above, these two esterases are very similar and may be identical. All esterases favored the (*S*)-enantiomer.

To confirm these Quick *E* measurements, we also measured the enantioselectivity of several reactions using scale-up reaction.[11] In most cases, enantioselectivities measured by Quick *E* and scale-up reactions agreed with one another (Table 10).

For substrate **36**, the enantioselectivities measured by Quick *E* and by the endpoint (scale-up) method agreed. The enantioselectivity of E004 was high using either method—>81 using Quick *E* and >86 using the endpoint method. Due to low reactivity, Quick *E* could only set lower limit of enantioselectivity for esterases E001 (>3.2) and E005 (>2.0), but the endpoint method revealed that the enantioselectivity was very high, >46 and >500, respectively (Table 10).

For substrate **38**, the enantioselectivities measured by Quick *E* and the endpoint method agreed in seven out of nine cases. The two exceptions were E002 and E013, where the lower limits on the enantioselectivity measured by Quick *E* were slightly higher than the end point values. In the first case, the Quick *E* value was >55, while the end point value was 46. In the second case, the Quick *E* value was >38, while the end point value was 25.

3. Discussion

This pH-indicator based colorimetric screening allowed the rapid testing of 19 esterases with more than 50 substrates, representing more than 1000 selectivity measurements. Researchers previously measured the chain length selectivity of hydrolases by mixing substrates and measuring the relative amounts of hydrolysis by TLC or GC.[12] Similarly, researchers have developed GC, MS, and electrophoresis-based methods for measuring enantioselectivity.[13] The main advantages of the pH-indicator based methods are simplicity and speed. They use unmodified ester substrates, so that adding more substrates to refine screening is easy. They do

Table 4. Specific activities and estimated enantioselectivities of thermophile esterases toward esters of chiral primary alcohols[a]

	(*R*)-**32**	(*S*)-**32**	Est. *E*[b]	(*R*)-**35**	(*S*)-**35**	Est. *E*[b]	(*R*)-**33**	(*S*)-**33**	Est. *E*[b]	(*R*)-**34**	(*S*)-**34**	Est. *E*[b]
E001	9.4	7.1	1.3	**200**	**140**	1.4	12	12.5	1.0	16	40	2.6
E002	7.3	5.6	1.3	**250**	**160**	1.6	7.8	8.5	1.1	36	85	2.4
E003	19	15	1.3	**140**	92	1.5	18	13	1.4	16	27	1.7
E004	27	20	1.4	**180**	**160**	1.1	34	34	1.0	68	53	1.3
E005	5.8	4.4	1.3	**300**	**170**	1.8	6.9	7.2	1.0	44	96	2.2
E006	2.4	1.8	1.3	**100**	61	1.7	2.5	2.9	1.2	3.0	7.9	2.7
E007	0.44	0.32	1.4	4.0	2.5	1.6	0.44	0.46	1.0	0.43	1.3	2.9
E008	6.0	4.2	1.4	93	**150**	1.6	6.4	8.9	1.4	17	16	1.0
E009	**150**	**110**	1.4	**300**	**490**	1.6	**150**	**190**	1.2	**360**	**320**	1.2
E010	2.8	2.7	1.0	81	**110**	1.4	2.9	3.6	1.3	6.4	6.0	1.1
E011	2.2	2.2	1.0	58	94	1.6	2.6	3.0	1.2	6.1	5.3	1.1
E012	3.2	3.0	1.1	19	18	1.0	5.1	4.3	1.2	14	9.6	1.5
E013	1.5	1.1	1.3	94	84	1.1	1.4	2.0	1.4	3.0	3.0	1.0
E014	1.4	1.2	1.2	83	76	1.1	1.2	1.4	1.2	3.3	3.4	1.0
E015	7.2	6.3	1.2	**230**	**190**	1.2	7.6	9.2	1.2	18	18	1.0
E016	14	9.3	1.5	93	57	1.6	13	10	1.3	11	26	2.3
E018b	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]
E019	3.1	2.9	1.1	**120**	76	1.6	3.8	3.1	1.3	2.7	4.8	1.8
E020	4.5	4.5	1.0	**120**	85	1.5	6.1	4.8	1.3	3.9	8.9	2.3

[a] Specific activity in μmol ester hydrolyzed/mg protein/min × 100. Rates ⩾100 are bolded for emphasis.
[b] Estimated enantioselectivity is the ratio of the initial rates of hydrolysis of the two enantiomers (rate for fast enantiomer/rate for slow enantiomer).
[c] Could not be measured because the substrate did not react.

Table 5. Specific activities and estimated enantioselectivities of thermophile esterases toward esters of chiral secondary alcohols[a]

	(*R*)-**36**	(*S*)-**36**	Est. *E*[b]	(*R*)-**37**	(*S*)-**37**	Est. *E*[b]	(*R*)-**38**	(*S*)-**38**	Est. *E*[b]
E001	0.82	1.8	2.2	0.52	0.40	1.3	24	2.0	11
E002	0.54	1.4	2.6	0.08	0.08	1.1	**110**	21	5.1
E003	4.2	7.0	1.6	3.5	3.2	1.1	37	5.6	6.6
E004	62	**120**	2.0	3.8	4.9	1.3	**160**	28	5.7
E005	0.53	1.3	2.4	0.21	0.19	1.1	4.0	0.31	13
E006	0.45	0.54	1.2	0.27	0.25	1.1	5.0	0.27	18
E007	0.22	0.26	1.2	0.14	0.14	1.0	1.2	0.23	5.2
E008	150	**200**	1.0	0.32	0.41	1.3	**260**	57	4.6
E009	**930**	**930**	1.0	0.65	1.3	2.0	**900**	**120**	6.7
E010	**150**	**130**	1.1	0.28	0.32	1.1	16	1.4	11
E011	**180**	**150**	1.2	0.14	0.19	1.4	**130**	15	8.6
E012	16	19	1.2	8.1	7.8	1.1	12	<2	>9.3
E013	76	70	1.5	0.20	0.23	1.2	5.3	0.46	12
E014	34	47	1.4	0.13	0.14	1.1	5.0	0.32	15
E015	93	**150**	1.7	0.25	0.38	1.5	24	1.5	16
E016	1.5	1.4	1.1	5.2	5.3	1.0	23	3.5	6.4
E018b	11	6.2	1.7	6.9	6.1	1.1	1.3	1.3	1.0
E019	0.44	0.68	1.6	0.20	0.19	1.0	7.5	0.45	17
E020	0.64	1.1	1.7	0.31	0.34	1.1	9.2	0.63	15

[a] Specific activity in μmol ester hydrolyzed/mg protein/min × 100. Rates ⩾100 are bolded for emphasis.
[b] Estimated enantioselectivity is the ratio of the initial rates of hydrolysis of the two enantiomers (rate for fast enantiomer/rate for slow enantiomer).

not require finding GC or other separation conditions for each new substrate.

This screening yielded three main conclusions. First, esterase pairs E010/E011, E013/E014, and E019/E020 are likely identical. Second, esterase E018b is highly selective for acetyl esters, while the other esterases favor hexanoyl or octanoyl acyl groups. Third, at least one of the esterases shows high enantioselectivity toward substrates, **36**, **38**, and **45**.

Acetyl selectivity can be useful to protect or deprotect substituents, and the mild conditions of an enzymatic reaction avoid reaction in other parts of the molecule.[14] This is particularly important in sugars, which contain many difficult-to-distinguish alcohol residues. Previously enzymes have been discovered, which chemoselectively hydrolyze phenylacetate,[15] phenylalanine ester,[16] or acetate[14] esters. Waldmann's group[17] used the acetyl selectivity of acetyl esterase (AcE) to distinguish between acetyl groups in acetyl-protected carbohydrates and nucleosides.[18] Several other applications, for example, the synthesis of a lipopeptide, relied on the selectivity of AcE for acetyl groups.[19] For example, AcE removed an acetyl protective group while leaving a C-terminal peptide allyl ester and a palmitoyl thioester

Table 6. Specific activities and estimated enantioselectivities of thermophile esterases toward esters of chiral carboxylic acids (stereocenter at the α-position)[a]

	(*R*)-**39**	(*S*)-**39**	Est. *E*[b]	(*R*)-**42**	(*S*)-**42**	Est. *E*[b]	(*R*)-**44**	(*S*)-**44**	Est. *E*[b]	(*R*)-**45**	(*S*)-**45**	Est. *E*[b]	(*R*)-**47**	(*S*)-**47**	Est. *E*[b]	(*R*)-**50**	(*S*)-**50**	Est. *E*[b]
E001	<0.1	<0.1	[c]	0.60	0.51	1.2	0.38	0.18	2.1	2.3	20	8.5	<0.1	<0.1	[c]	<0.1	0.22	>2.2
E002	<0.05	<0.05	[c]	0.45	0.30	1.6	0.19	0.053	2.1	50	**100**	3.4	<0.05	<0.05	[c]	0.02	0.17	8.5
E003	6.7	5.4	1.2	15	15	1.0	<2.5	<2.5	[c]	11	38	3.4	<2.5	<2.5	[c]	4.3	7.2	1.7
E004	27	29	1.1	46	39	1.2	3.0	<2	>2.3	13	31	2.4	<2	<2	[c]	<2	<2	[c]
E005	<0.1	<0.1	[c]	0.37	0.16	2.4	0.035	0.015	2.1	56	**120**	2.2	<0.1	<0.1	[c]	<0.1	0.73	>7.3
E006	<0.1	<0.1	[c]	0.20	0.12	1.6	<0.1	<0.1	[c]	0.81	4.9	6.1	<0.1	<0.1	[c]	<0.1	0.28	>2.8
E007	<0.06	<0.06	[c]	<0.06	<0.06	[c]	<0.06	<0.06	[c]	0.21	1.0	5.1	<0.06	<0.06	[c]	<0.06	<0.06	[c]
E008	<0.2	<0.2	[c]	0.42	0.46	1.1	0.63	0.21	3.0	3.1	7.3	2.3	0.28	0.66	2.4	<0.2	<0.2	[c]
E009	<0.2	<0.2	[c]	1.0	1.9	1.8	3.1	0.79	4.0	56	**180**	3.2	<0.2	<0.2	[c]	<0.2	0.33	>1.6
E010	<0.1	<0.1	[c]	0.19	0.21	1.1	<0.1	<0.1	[c]	1.1	2.5	2.3	<0.1	<0.1	[c]	<0.1	<0.1	[c]
E011	<0.05	<0.05	[c]	0.050	0.080	1.5	<0.05	<0.05	[c]	28	45	1.6	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E012	29	28	1.1	34	37	1.1	<2	<2	[c]	<2	<2	[c]	<2	<2	[c]	<2	<2	[c]
E013	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	0.58	1.5	2.6	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E014	<0.05	<0.05	[c]	<0.05	<0.05	[c]	0.023	0.015	1.5	0.35	0.97	2.8	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E015	<0.05	<0.05	[c]	0.095	0.25	2.6	0.24	0.06	4.0	2.9	7.5	2.5	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E016	27	29	1.1	37	34	1.1	1.2	1.2	1.0	9.6	28	2.9	<0.5	<0.5	[c]	<0.5	1.7	>3.4
E018b	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]
E019	0.10	0.16	1.6	0.25	0.16	1.6	<0.1	<0.1	[c]	0.74	5.4	7.3	<0.1	<0.1	[c]	<0.1	<0.1	[c]
E020	0.64	0.68	1.1	0.41	0.29	1.6	<0.2	<0.2	[c]	1.7	7.5	4.5	<0.2	<0.2	[c]	<0.2	<0.2	[c]

[a] Specific activity in μmol ester hydrolyzed/mg protein/min × 100. Rates $\geqslant$ 100 are bolded for emphasis.
[b] Estimated enantioselectivity is the ratio of the initial rates of hydrolysis of the two enantiomers (rate for fast enantiomer/rate for slow enantiomer).
[c] Could not be measured because the substrate did not react.

Table 7. Specific activities and estimated enantioselectivities of thermophile esterases toward esters of chiral carboxylic acids (stereocenter at the α-position)[a]

	(*R*)-**43**	(*S*)-**43**	Est. *E*[b]	(*R*)-**46**	(*S*)-**46**	Est. *E*[b]	(*R*)-**48**	(*S*)-**48**	Est. *E*[b]	(*R*)-**49**	(*S*)-**49**	Est. *E*[b]
E001	3.2	0.4	8.1	0.13	0.14	1.1	0.13	0.14	1.0	0.34	0.42	1.2
E002	50	43	1.2	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E003	8.2	5.6	1.5	<2.5	<2.5	[c]	<2.5	<2.5	[c]	<2.5	<2.5	[c]
E004	<2	<2	1.2	<2	<2	[c]	<2	<2	[c]	<2	<2	[c]
E005	61	52	1.2	<0.1	<0.1	[c]	<0.1	<0.1	[c]	<0.1	<0.1	[c]
E006	0.75	0.11	6.6	<0.1	<0.1	[c]	<0.1	<0.1	[c]	0.15	0.14	1.1
E007	<0.06	<0.06	[c]	<0.06	<0.06	[c]	<0.06	<0.06	[c]	0.10	0.17	1.7
E008	0.26	0.22	1.2	<0.2	<0.2	[c]	0.70	1.1	1.5	0.81	0.90	1.1
E009	0.63	0.37	1.7	0.33	0.26	1.3	0.80	0.85	1.1	0.69	0.91	1.3
E010	<0.1	<0.1	[c]	<0.1	<0.1	[c]	0.17	0.25	1.4	0.17	0.25	1.4
E011	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	0.12	0.10	1.3
E012	<2	<2	[c]	<2	<2	[c]	<2	<2	[c]	<2	<2	[c]
E013	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	0.11	0.13	1.3
E014	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E015	0.13	0.060	2.1	<0.05	<0.05	[c]	<0.05	<0.05	[c]	0.20	0.18	1.1
E016	5.3	1.3	4.1	1.2	1.5	1.2	0.90	0.75	1.2	6.7	8.4	1.2
E018b	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]
E019	0.90	0.10	8.9	<0.1	<0.1	[c]	<0.1	<0.1	[c]	0.23	0.21	1.1
E020	1.3	0.25	5.4	<0.2	<0.2	[c]	<0.2	<0.2	[c]	<0.2	<0.2	[c]

[a] Specific activity in μmol ester hydrolyzed/mg protein/min × 100.
[b] Estimated enantioselectivity is the ratio of the initial rates of hydrolysis of the two enantiomers (rate for fast enantiomer/rate for slow enantiomer).
[c] Could not be measured because the substrate did not react.

Table 8. Specific activities and estimated enantioselectivities of thermophile esterases toward esters of chiral carboxylic acids (stereocenter at the β-position) and lactones[a]

	(*R*)-**40**	(*S*)-**40**	Est. *E*[b]	(*R*)-**41**	(*S*)-**41**	Est. *E*[b]	(*R*)-**51**	(*S*)-**51**	Est. *E*[b]	(*R*)-**52**	(*S*)-**52**	Est. *E*[b]
E001	<0.1	<0.1	[c]	0.16	0.15	1.1	<0.1	<0.1	[c]	<0.1	<0.1	[c]
E002	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E003	<0.25	<0.25	[c]	11	9.4	1.2	10	11	1.1	7.7	11	1.4
E004	41	39	1.1	24	33	1.4	<2	<2	[c]	<2	<2	[c]
E005	<0.1	<0.1	[c]	0.15	0.22	1.4	<0.1	<0.1	[c]	<0.1	<0.1	[c]
E006	<0.1	<0.1	[c]	<0.1	<0.1	[c]	<0.1	<0.1	[c]	<0.1	<0.1	[c]
E007	<0.06	<0.06	[c]	<0.06	<0.06	[c]	<0.06	<0.06	[c]	<0.06	<0.06	[c]
E008	<0.2	<0.2	[c]	0.27	0.25	1.1	<0.2	<0.2	[c]	<0.2	<0.2	[c]
E009	0.30	0.35	1.2	0.34	0.47	1.4	<0.2	<0.2	[c]	<0.2	<0.2	[c]
E010	0.24	0.24	1.0	<0.1	<0.1	[c]	<0.1	<0.1	[c]	<0.1	<0.1	[c]
E011	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E012	29	31	1.1	21	28	1.3	<2	<2	[c]	<2	<2	[c]
E013	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E014	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E015	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]	<0.05	<0.05	[c]
E016	27	28	1.0	24	32	1.3	1.0	1.1	1.0	1.0	1.0	1.0
E018b	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]	<1.5	<1.5	[c]
E019	<0.1	<0.1	[c]	<0.1	<0.1	[c]	<0.1	<0.1	[c]	<0.1	<0.1	[c]
E020	<0.2	<0.2	[c]	<0.2	<0.2	[c]	<0.2	<0.2	[c]	<0.2	<0.2	[c]

[a] Specific activity in μmol ester hydrolyzed/mg protein/min × 100.
[b] Estimated enantioselectivity is the ratio of the initial rates of hydrolysis of the two enantiomers (rate for fast enantiomer/rate for slow enantiomer).
[c] Could not be measured because the substrate did not react.

untouched.[20] However, AcE was not sufficiently acetyl selective in the synthesis of *O*-sialyl-Lewis-X peptides because AcE removed both the acetyl groups and the C-terminal ester.[21] Esterase E018b is often more selective than AcE for acetyl groups and may solve these synthetic problems. Additionally, more than 10 thermophile hydrolases favor hexanoate or octanoate esters over their shorter acyl chain analogues. This selectivity provides a method for selectively hydrolysis of one type of ester in the presence of the other.

Our enantioselectivity results are similar, but not identical, to those reported by ThermoGen.[6] For example, in the hydrolysis of 1-phenethyl acetate, Dimirjian et al. reported enantioselectivities >50 for E002, E003, E005, E009, and E020, but moderate enantioselectivities (E = 8–10) for E004, E008, and E013. In agreement with the ThermoGen group, we measured enantioselectivities >50 for all five esterases, but found slightly higher results for E008, and E013 (E = 25–30). There are two likely reasons for these differences. We tested the butyrate ester,

Table 9. Enantioselectivity of thermophile esterases toward selected substrates[a]

	34	**36**	**38**	**43**	**45**
E001	3.4 (*S*)	>3.2 (1*R*)	>22 (*R*)	>4.7 (*R*)	9.7 (*S*)
E002	3.6 (*S*)	>8.9 (1*R*)	>55 (*R*)	ND	7.0 (*S*)
E003	ND	ND	>19 (*R*)	ND	6.4 (*S*)
E004	ND	>81 (1*R*)	>3.4 (*R*)	NR	7.2 (*S*)
E005	3.9 (*S*)	>2.0 (1*R*)	>31(*R*)	ND	4.9 (*S*)
E006	3.7 (*S*)	ND	>16 (*R*)	>2.0 (*R*)	10 (*S*)
E007	2.4 (*S*)	ND	>2.1 (*R*)	NR	4.2 (*S*)
E008	ND	ND	>33 (*R*)	ND	21 (*S*)
E009	ND	ND	110 (*R*)	ND	6.9 (*S*)
E010	ND	ND	>33 (*R*)	NR	>6.2 (*S*)
E011	ND	ND	>66 (*R*)	NR	5.1 (*S*)
E012	ND	ND	2.8 (*R*)	NR	NR
E013	ND	ND	>38 (*R*)	NR	2.8 (*S*)
E014	ND	ND	>28 (*R*)	NR	2.7 (*S*)
E015	ND	ND	>150 (*R*)	ND	10 (*S*)
E016	3.3 (*S*)	ND	12 (*R*)	>5.5 (*R*)	5.4 (*S*)
E018b	NR	ND	ND	NR	NR
E019	ND	ND	>24 (*R*)	>2.2 (*R*)	13 (*S*)
E020	3.1 (*S*)	ND	>50 (*R*)	>2.3 (*R*)	11 (*S*)

[a] NR = not determined because the substrate did not react. ND = not determined because the estimated enantioselectivity was <2. Unless otherwise noted, the true enantioselectivities were measured using resorufin esters as a reference compound and have an estimated error of ±10–30%. When the rate of the slow enantiomer was too slow to measure accurately, a lower limit for the enantioselectivity is given.

Table 10. Selectivity measurement using scale up reactions to confirm Quick *E*/*S* measurements[a]

Substrate (s)	Enzyme	Estimated *E*/*S*	Quick *E*/*S*	Ee_s	Ee_p	End-point	Lit.[b]
36	E001	2.2 (*R*)	>3.2 (*R*)	0.029	>0.96	>46 (*R*)	NR
36	E004	2.0 (*R*)	>81 (*R*)	0.13	>0.86	>86 (*R*)	NR
36	E005	2.4 (*R*)	>2.0 (*R*)	0.80	>0.99	>500 (*R*)	NR
38	E002	5.1 (*R*)	>55 (*R*)	0.91	0.68	46 (*R*)	103 (*R*)
38	E003	6.6 (*R*)	>19 (*R*)	0.73	0.98	200 (*R*)	98 (*R*)
38	E004	5.7 (*R*)	>3.9 (*R*)	0.71	0.76	16 (*R*)	9 (*R*)
38	E005	13 (*R*)	>31 (*R*)	0.23	>0.95	>52 (*R*)	101 (*R*)
38	E007	5.2 (*R*)	>2.1 (*R*)	0.10	0.19	1.6 (*R*)	20 (*R*)
38	E008	4.6 (*R*)	>33 (*R*)	0.29	>0.92	32 (*R*)	7.7 (*R*)
38	E009	6.7 (*R*)	110 (*R*)	0.092	>0.97	>80 (*R*)	88 (*R*)
38	E013	12 (*R*)	>38 (*R*)	0.023	0.88	25 (*R*)	10 (*R*)
38	E020	15 (*R*)	>50 (*R*)	0.36	>0.85	>170 (*R*)	57 (*R*)
1, **3**	E018b	136 (**1**)	24 (**1**)	0.37[c]	0.92[c]	17 (**1**)	NR
1, **3**	AcE	ND	5.9 (**1**)	0.69[c]	0.35[c]	4.0 (**1**)	NR
1, **3**	E015	3.1 (**3**)	28 (**3**)	0.82[c]	0.56[c]	18 (**3**)	NR

[a] ND = not determined. Ee_s = enantiomeric excess of the substrate at the end of the reaction. Ee_p = enantiomeric excess of the product at the end of the reaction.
[b] Enantioselectivity measured using 1-phenethyl acetate instead of 1-phenethyl butyrate, **38**, from Ref. 6. NR = not reported. Based on NMR data.
[c] Excess in starting materials and products respectively at the end of the reaction.

while the ThermoGen group tested the acetate ester. Hydrolysis of butyrates often shows higher selectivity than hydrolysis of acetates, consistent with our generally higher enantioselectivies.[22] Second, all our reactions included 7% acetonitrile while Dimirjian et al. did not and organic co-solvents can change enantioselectivity.[23]

Using qualitative screening the ThermoGen group also identified esterases E002, E003, E005, E006, E007, and E012 as highly selective for the (*R*)-enantiomer of dioxolane **43**. We found very slow reaction for this substrate and thus could not confirm these results. Esterases E007 and E012 did not react, and it was only possible to estimate a lower limit for the enantioselectivity for five esterases.

In many methods, the solution to measuring slow substrates is to extend the reaction time or to add more enzyme. This solution does not work for Quick *E* methods because the key measurement is the relative rate of hydrolysis of the substrate and the reference compound. An accurate relative rate for a slow substrate requires a slow reference compound. The slowest-reacting resorufin compounds we used were the isobutyrate and pivaloate esters. For example, we could only place a lower limit of 2 on the enantioselectivity of E005 toward menthyl acetate, **36**. However, a scale-up reaction revealed that the enantioselectivity was very high, $E > 100$. Our current research has discovered other slow reacting reference compounds that promise to minimize this limitation in the future.

The thermophile esterases resemble lipases. Like lipases, they favor hydrophobic substrates over polar substrates. They also tolerate a wide range of alcohol moieties in the ester, but favor straight chain acyl groups similar to lipases. The high selectivity of all the Thermophilic esterases, but E018b, for hexanoyl or octanoyl esters suggests that future screening with these enzymes should use hexanoyl or octanoyl esters, not acetyl or other esters. However, unlike lipases, there is no evidence that thermophile esterases show interfacial activation.

4. Experimental

4.1. General

Unless otherwise noted, esters, reagents, and solvents were purchased from commercial sources. Enzyme solutions were prepared by dissolving the powder in BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 5.0 mM, pH 7.20) buffer at a concentration of 0.5–2.0 mg solid/mL. The solid was 2–60% protein as determined by the BioRad dye binding assay. Solutions were stored at −20 °C. Solketal butyrate and resorufin acetate were prepared as described previously.[4] Solketal octanoate, 1-phenethyl butyrate, and other resorufin esters were prepared similarly. ^{1}H NMR spectra were run in deuteriochloroform at 200 MHz. Low resolution MS spectra were obtained by direct inlet using electron ionization.

4.2. Phenyl butyrate, 26

Butanoyl chloride (4.8 mL, 42 mmol) was added drop wise to a flame-dried, round-bottomed flask containing a solution of phenol (2.0 g, 21 mmol), and pyridine (2.5 mL, 32 mmol) in dry ether (30 mL) under nitrogen and cooled in an ice bath. After stirring the resulting suspension for 2 h, it was washed with water (2 × 30 mL), saturated sodium bicarbonate (2 × 40 mL), HCl (2 × 30 mL, 0.2 N), and saturated NaCl (2 × 30 mL). The organic phase was dried over magnesium sulfate, concentrated by rotary evaporation, and purified by column chromatography on silica gel (4:1:2 hexanes–ethyl acetate–chloroform, $R_f = 0.67$) yielding a colorless oil: 1.7 g (49%). ^{1}H NMR δ 1.0–1.1 (t, 3H, CH_3) 1.8–1.9 (m, 2H, CH_2), 2.5–2.6 (t, 2H, CH_2), 7.1–7.4 (m, 5H, aromatic). MS *m*/*z*: 164 ($M^{\cdot+}$, 27); 94 (100); 77 (5.4); 71 (46); 43 (27).

4.3. Phenyl hexanoate

Phenyl hexanoate was prepared as above for the butyrate, colorless oil: 3.2 g (79%). $R_f = 0.71$; ^{1}H NMR δ 0.9–1.0 (t, 3H, CH_3) 1.4–1.8 (m, 6H, CH_2), 2.5–2.6 (t, 2H, CH_2), 7.1–7.4 (m, 5H, aromatic). MS *m*/*z*: 192 ($M^{\cdot+}$, 28); 99 (77); 94 (100); 71 (38); 43 (47).

4.4. Phenyl octanoate

Phenyl octanoate was prepared as above for the butyrate, colorless oil: 4.7 g (98%). $R_f = 0.68$; ^{1}H NMR δ 1.0 (s, 3H, CH_3) 1.4–1.9 (m, 8H, CH_2), 2.4–2.5 (m, 2H, CH_2), 2.6–2.7 (t, 2H, octanoyl CH_2), 7.1–7.4 (m, 5H, aromatic). MS *m*/*z*: 220 ($M^{\cdot+}$, 25); 127 (92); 101 (9); 94 (89); 60 (37); 57 (100); 29 (7.5).

4.5. Monohexanoate of 2,2-bis(4-hydroxyphenyl)propane

Monohexanoate of 2,2-bis(4-hydroxyphenyl)propane was prepared as above for phenyl butyrate, yellowish oil: 3.7 g (77%). $R_f = 0.18$ (9:1 hexane/ethyl acetate); ^{1}H NMR δ 0.9 (t, 3H, hexanoyl CH_3) 1.3 (apparent s, 6H, CH_2), 1.6 (s, 6H, propane CH_3), 2.0 (t, 2H, CH_2), 6.7–7.2 (m, 8H, aromatic). MS *m*/*z*: 327 ($M^{\cdot+}$, 31); 326 (32); 213 (100); 135 (17); 119 (20); 99 (6.8); 91 (6.7), 71 (9.2), 43 (18).

4.6. Monoacetate of 2,2-bis(4-hydroxyphenyl)propane

Monoacetate of 2,2-bis(4-hydroxyphenyl)propane was prepared as *N*,*N*-dimethylaminopyridine and acetic anhydride and worked up as above for phenyl butyrate, colorless oil: 0.58 g (24%). $R_f = 0.26$ (9:1 hexane/ethyl acetate); ^{1}H NMR δ 1.6 (s, 6H, propane CH_3), 2.3 (s, 3H, acetyl CH_3), 6.7–7.2 (m, 8H, aromatic). MS *m*/*z*: 271 ($M^{\cdot+}$, 4.3); 255 (6.9); 228 (925); 213 (100); 135 (15); 119 (16); 91 (6.0); 74 (67); 59 (47); 45 (26); 31 (31).

4.7. Monohexanoate monoacetate of 2,2-bis(4-hydroxyphenyl)propane

Monohexanoate monoacetate of 2,2-bis(4-hydroxyphenyl)propane was prepared from the monohexanoate as above for the monoacetate, colorless oil, 1.3 g (95%). ^{1}H NMR δ 0.9 (t, 3H, hexanoyl CH_3) 1.4 (apparent s, 4H, hexanoyl CH_2), 1.7 (s, 6H, propane CH_3), 1.8 (t, 2H, hexanoyl CH_2), 2.3 (s, 3H, acetyl CH_3), 2.6 (t, 2H, hexanoyl CH_2), 7.0–7.2 (m, 8H, aromatic). MS *m*/*z*: 368 ($M^{\cdot+}$, 26); 270 (100); 255 (38); 228 (100); 213 (100); 135 (19); 119 (21); 99 (10); 91 (7.6); 71 (18); 55 (9.2); 43 (40).

4.8. Initial screening (estimated selectivity)

To each well of a 96-well polystyrene microplate was added substrate solution (7.0 μL of a 14.3 mM solution in acetonitrile), 4-nitrophenol solution (23 μL of a 1.9 mM in BES buffer (1.0 mM, pH 7.20) and BES buffer (65 μL, 1.0 mM, pH 7.20). This mixture yielded final concentrations of 1.0 mM substrate, 0.437 mM 4-nitrophenol, 1.13 mM BES, and 7.0 vol% acetonitrile. Esterase solution (5 μL in 5 mM BES, typically 1 mg solid/mL) was added to each well. This mixture yielded final concentrations of 1.0 mM substrate, 0.437 mM 4-nitrophenol, 1.13 mM BES, and 7.0 vol% acetonitrile. The microplate was placed in the microplate reader, shaken for 5 s and the decrease in absorbance at 404 nm was measured as often as permitted by the microplate reader, typically every 10 s. Data was collected for 20 min, at 25 °C, in triplicate and was averaged. For slow reactions, a more concentrated esterase solution was used so that the absorbance change was >2 mA/min, while for fast reactions a less concentrated esterase solution was used so that the absorbance change was <200 mA/min. The reaction rates were calculated using Eq. 1 below.

$$\text{rate}(\mu\text{mol/min}) = \frac{dA_{404}/dt}{\Delta\varepsilon_{404} \times l} \times \left(\frac{[\text{buffer}]}{[\text{indicator}]} + 1\right) \times V \times 10^6 \quad (1)$$

In this equation, dA_{404}/dt is the absorbance decrease at 404 nm per minute, $\Delta\varepsilon_{404}$ is the difference in extinction co-efficients for the protonated and unprotonated forms of the indicator (17,300 $M^{-1}cm^{-1}$), l is the path length (0.2919 cm for a 100 μL reaction volume; in microplates, the path length varies with reaction volume), and V is the reaction volume in liters. For the reaction conditions above, this equation simplifies to Eq. 1a.

$$\text{rate}(\mu\text{mol/min}) = \frac{dA_{404}}{dt} \times 0.07101 \quad (1a)$$

The observed rates were divided by the protein amount in the well to give the values in the Tables. Blanks contained either enzyme, but no substrate or substrate, but no enzyme.

4.9. True selectivity and enantioselectivity

A buffer/indicator solution was prepared by mixing 4-nitrophenol solution (1.2 mL of a 1.8 mM solution in 1.0 mM BES containing 0.33 mM Triton X-100, pH 7.2), BES buffer (3.3 mL of a 1.0 mM solution containing 0.33 mM Triton X-100, pH 7.20), and acetonitrile (65 μL). Substrate solution (e.g., 35 μL of a 150 mM vinyl pivaloate in acetonitrile) and resorufin ester solution (e.g., 260 μL of 2.0 mM resorufin pivaloate in acetonitrile) were added dropwise with continuous vortexing to form a clear emulsion that was stable for at least several hours. Final concentrations in the well were 1.03 mM substrate (vinyl pivaloate), 0.102 mM resorufin pivaloate, 0.423 mM pNP, 1.16 mM BES, 0.29 mM Triton X-100, and 7.05 vol% acetonitrile. This solution was pipetted into a 96-well polystyrene microplate (100 μL/well). Esterase solution (5 μL in 5 mM BES, typically 1 mg solid/mL) was added to each well and the microplate was placed in the microplate reader, shaken for 5 s and the decrease in absorbance at 404 nm and the increase in absorbance at 574 nm were measured as often as permitted by the microplate reader, typically every 11 s. Data was collected for 20 min, at 25 °C, in triplicate and was averaged. The rate of hydrolysis of the reference compound and the substrate were calculated using Eqs. 3 and 4 below using the initial, linear parts of the curve.

$$\text{rate}_{\text{ref}}(\mu\text{mol/min}) = \frac{dA_{574}/dt}{\Delta\varepsilon_{574} \times l} \times V \times 10^6 \quad (2)$$

$$\text{rate}_{\text{sub}}(\mu\text{mol/min}) = \left[\frac{dA_{404}/dt}{\Delta\varepsilon_{404} \times l} \times \left(\frac{[\text{buffer}]}{[\text{indicator}]} + 1\right) \times V \times 10^6\right] - 1.1[\text{rate}_{\text{ref}}] \quad (3)$$

The symbols are the same as those defined for Eqs. 1 and 2 above, while $\Delta\varepsilon_{574}$ is the difference in extinction co-efficients at 574 nm for resorufin and the resorufin ester (15,100 $M^{-1}cm^{-1}$).

For the reaction conditions above, these equations simplify to Eqs. 2a and 3a.

$$\text{rate}_{\text{ref}}(\mu\text{mol/min}) = \frac{dA_{574}}{dt} \times 0.02269 \quad (2a)$$

$$\text{rate}_{\text{sub}}(\mu\text{mol/min}) = \left[\frac{dA_{404}}{dt} \times 0.07411\right] - 1.1[\text{rate}_{\text{ref}}] \quad (3a)$$

The enantioselectivity was calculated from two measurements (one for each enantiomers) and adjusted for the concentration of substrate and reference compound in each measurement as shown below in Eq. 4.

$$\text{Quick } E = \frac{\text{rate}_R}{\text{rate}_{\text{ref }R}} \times \frac{[\text{ref}_R]}{[R]} \times \frac{\text{rate}_{\text{ref }S}}{\text{rate}_S} \times \frac{[S]}{[\text{ref}_S]} \quad (4)$$

In this equation, rate_R represent the rate of hydrolysis of the (*R*)-enantiomer, $\text{rate}_{\text{ref }R}$ is the rate of hydrolysis of the reference compound in the presence of the (*R*)-enantiomer, $[\text{ref}_R]$ is the concentration of the reference compound during the measurement for the (*R*)-enantiomer, and $[R]$ is the concentration of the (*R*)-enantiomer.

4.10. Use of different reference compounds

In some cases we used a faster reacting reference compound (e.g., resorufin acetate) with the faster reacting enantiomer and a slower reacting reference compound (e.g., resorufin pivaloate) with the slower reacting enantiomer. Such measurements were corrected for the selectivity of the enzyme for the two reference compounds, $S_{\text{res 1/res 2}}$. This selectivity was measured using a substrate of intermediate reaction rate. In this case the substrate serves as the reference compound. The selectivity was calculated using Eq. 5 below where $\text{rate}_{\text{res 1}}$ is the observed rate of hydrolysis of resorufin ester **1**, $\text{rate}_{\text{sub res 1}}$ is the observed rate of hydrolysis of the substrate when measured with resorufin ester **1** and the brackets indicate concentrations.

$$S_{\frac{\text{res 1}}{\text{res 2}}} = \frac{\text{rate}_{\text{res 1}}}{\text{rate}_{\text{sub res 1}}} \times \frac{[\text{sub}]}{[\text{res 1}]} \times \frac{\text{rate}_{\text{res 2}}}{\text{rate}_{\text{sub res 2}}} \times \frac{[\text{sub}]}{[\text{res 2}]} \quad (5)$$

4.11. Acyl chain length selectivity using the endpoint method

The selectivity of E018b for vinyl acetate versus vinyl butyrate was also measured by ^{1}H NMR. Vinyl acetate (0.92 μL, 10 μmol), vinyl butyrate (1.3 μL, 10 μmol) were dissolved D_2O (1.0 mL containing BES (1.0 mM, pH 7.20) and 7 vol% CD_3CN). Enzyme solution (10 μL) was added and the resonances corresponding to vinyl acetate (CH_3, δ 2.0), vinyl butyrate (CH_2, δ 2.3), acetic acid (CH_3, δ 1.9), and butyric acid (CH_2, δ 2.2) were monitored by ^{1}H NMR (500 MHz). Data in Table 10.

4.12. Selective hydrolysis of acetyl ester in monohexanoate monoacetate of 2,2-bis(4-hydroxyphenyl)propane

Esterase E018b (1 mL) was added to a solution of monohexanoate monoacetate of 2,2-bis(4-hydroxyphenyl)pro-

pane (0.24 g, 0.67 mmol) in BES (8.3 mL of a 1.0 mM solution) and acetonitrile (700 μL). The pH of the solution was maintained at 7.20 with a pHstat, which controlled the addition of NaOH (0.1 N). When the consumption of base indicated ~40% conversion, the reaction mixture was extracted with ether (2 × 10 mL). The organic phase was concentrated by rotary evaporation, the residue was dissolved in acetonitrile (1 mL), and analyzed by high performance liquid chromatography on a reversed phase column (Zorbax C8, 4.6 mm × 25 mm) eluted with 0.60 mL/min using 50/50 water/methanol at 25 °C. The detector was set at 254 nm. $k_{Ac} = 9.6$ for the monoacetate of 2,2-bis(4-hydroxyphenyl)propane; $k_{Hx} = 15.3$ for the monohexanoate of 2,2-bis(4-hydroxyphenyl)propane. The molar extinction co-efficients for the two monoesters were assumed to be equal.

4.13. True enantioselectivity using the endpoint method

Hydrolase solution (1 mL) was added to a solution of racemic ester (e.g., 110 μL, 51 μmol of menthyl acetate) in BES (8.1 mL of a 1.0 mM solution, pH 7.20) containing 7 vol% acetonitrile (700 μL). The pH of the solution was maintained at 7.20 with a pHstat, which controlled the addition of NaOH (0.1 N). When the consumption of base indicated ~40% conversion, the reaction mixture was extracted with ether (2 × 10 mL). The organic phase was concentrated by rotary evaporation, the residue was dissolved in ethyl acetate (1 mL), and analyzed by gas chromatography on a Chirasil-DEX CB capillary column (Chrompack, Raritan, NJ) at 120 °C: $k_R = 7.15$, $k_S = 6.95$, and $\alpha = 1.03$ for menthyl acetate, **42**, $k_R = 12.1$, $k_S = 11.2$, and $\alpha = 1.08$ for 1-phenethyl butyrate, **41**. Data are in Table 9.

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8. An alternate explanation for the long chain preference is the formation of micelles and interfacial activation, as observed for lipases. This hypothesis is unlikely for two reasons. First, not all of the longer chain esters react faster; for example vinyl decanoate reacts slowly. Second, all reactions contain the surfactant Triton X-100, which causes micelle formation regardless of the substrate added.
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