Dimedone Esters as Novel Hydrolase Substrates and their Application in the Colorimetric Detection of Lipase and Esterase Activity

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There is currently considerable interest in the development of novel high-throughput screens for identifying enzymes with specific characteristics (e.g. substrate specificity, enantioselectivity, stability etc.).[1] Such screens can be used to select for variant enzymes from directed-evolution experiments or to probe genomic libraries obtained from environmental DNA samples. In this context, attention has inevitably been directed towards methods for screening for lipases and esterases[2] in view of their importance in the preparation of chiral alcohols and carboxylic acids by hydrolysis, esterification and transesterification reactions.[3] Examples of such high-throughput screens include those based upon changes in the pH,[4] chromogenic[5] and fluorogenic[6] properties of the reaction as well as ESI-MS,[7] IR-thermography,[8] capillary array electrophoresis (CAE)[9] and circular dichroism.[10] Herein we report a simple, inexpensive and easy to use colorimetric screen based upon the use of dimedone esters as substrates.

Although vinyl and *p*-nitrophenyl esters are well documented as lipase substrates,[3] they suffer from the limitation that,

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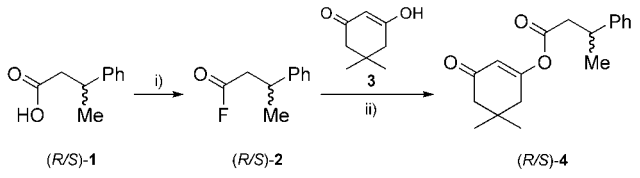
when used to screen for hydrolase activity, they are artificially reactive and hence do not always lead to the selection of highly active enzymes. With this in mind, we considered the use of esters of 1,3-enols, which have not previously been employed as lipase/esterase substrates. Such esters would offer the possibility of developing novel screening methods based upon the liberation and subsequent (colorimetric) detection of the 1,3-diketone/enol. In order to test this idea, the dimedone ester (*R/S*)-**4** of (*R/S*)-3-phenylbutyryl acid was selected as a model substrate based upon the known reactivity of this chiral acyl group towards a range of lipases and esterases.[11] (*R/S*)-**4** was prepared in high yield by treatment of (*R/S*)-**1** with cyanuric fluoride[12] to yield the acyl fluoride (*R/S*)-**2** followed by direct coupling with dimedone **3** in CH_2Cl_2 (DCM)/diisopropylethylamine (DIPEA; Scheme 1).



Scheme 1. *Synthesis of dimedone ester* (R/S)-***4****. Reagents and conditions: i)* $(CNF)_3$*, py, DCM, −20°C, RT, 1 h (93%); ii)* ***3****, DIPEA, DCM, RT, 2 h (90%).*

To establish whether the dimedone-containing ester (*R/S*)-**4** was a suitable substrate for hydrolase-catalyzed reactions, 17 different lipases and esterases were screened for activity (Table 1). The percentage conversion, after 18 h, and the enantiomeric ratio (*E*)[13] of the reaction were determined by using reverse- (C18 column) and normal-phase (chiral-ODH column) HPLC, respectively. High levels of conversion were observed with *Chromobacterium viscosum* lipase (entry 1), *Candida lipolytica* esterase (entry 10) and *Mucor miehei* esterase (entry 12).

The highest selectivity ($E=88$) was observed with CVL, which showed complete hydrolysis of the (*S*)-enol ester **4** after about 3 h (Figure 1 and Scheme 2). Such selectivity is in accord with previous reports[11] and indicates that, as expected, the presence of the dimedone moiety does not alter the enantioselectivity of the hydrolysis. In order to assess the relative reactivity of dimedone esters, the corresponding racemic methyl (**5**) and vinyl (**6**) esters were prepared (Scheme 2).[14] Under identical conditions (*R/S*)-**5** and (*R/S*)-**6** were shown to undergo complete conversion with CVL after 24 h and 15 minutes, respectively. Interestingly, therefore, 1,3-enol esters possess reactivity intermediate between alkyl and vinyl esters. Consideration of leaving-group ability suggests that esters of 1,3-diketones/enols should be more reactive than vinyl esters as a result of stabilization of the departing alcohol. Presumably the presence of the bulky dimedone moiety leads to poorer binding of **4** at the enzyme active site and hence a reduced overall rate of reaction compared to the corresponding vinyl ester **6**.

Dimedone is known to form coloured complexes in aqueous solution in the presence of copper(II) acetate,[15] although the exact structure of the complex is unknown. Treatment of (*R/S*)-**4** with CVL in the presence of a saturated aqueous copper(II) acetate solution led to the formation of a green solution whose intensity of colour was proportional to the concentration of DMSO (Scheme 3). Solutions of the Cu^{II}–dimedone complex were found to have a $\lambda_{max}=417$ nm ($\varepsilon=84\ M^{-1}cm^{-1}$). A

Table 1. *Enzyme-screening reactions with* (R/S)-***4****.*

	Hydrolase	Abbreviation	% Conv.[a]	E[b]
1	*Chromobacterium viscosum* lipase	CVL	>99	88
2	*Pseudomonas cepacia* lipase	PCL	54	6
3	Porcine pancreatic lipase	PPL	69	5
4	*Candida antarctica* lipase B	CAL-B	10	1
5	*Pseudomonas fluorescens* lipase	PFL	51	8
6	*Rhizopus arrhizus* lipase	RAL	60	12
7	*Candida lipolytica* lipase	CLL	50	14
8	*Penicillium roqueforti* lipase	PRL	8.7	1
9	*Mucor javanicus* lipase	MJL	28	1
10	*Candida lipolytica* esterase	CLE	>99	1
11	Acetylcholine esterase	AChE	12	1
12	*Mucor miehei* esterase	MME	>99	4
13	*Bacillus species* esterase	BSpE	18	2
14	*Thermoanaerobium brockii* esterase	TBE	10	1
15	*Saccharomyces cerevisiae* esterase	SCE	7.6	1
16	*Bacillus sterotheromphilus* esterase	BSE	5.6	1
17	*Bacillus thermoglycosidasius* esterase	BTE	8.4	1

Conditions: [a] The ester (*R/S*)-**4** was dissolved in 10% MeCN/0.1 M KP_i buffer solution containing the enzyme (1 mg). Reactions were carried out at RT for 18 h, after which the enzyme was precipitated (0.1% TFA), the mixture was filtered and then analysed by reverse-phase HPLC on a Sphereclone C18 column. [b] *E* values were determined in separate experiments in which the reaction was taken to <50%. Conversions and enantiomeric excesses were determined by HPLC on a Chiracel-ODH column eluting with 10% isopropanol in hexane containing 0.1% TFA.

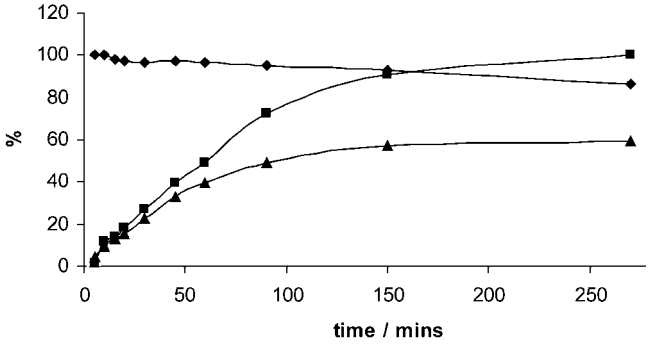
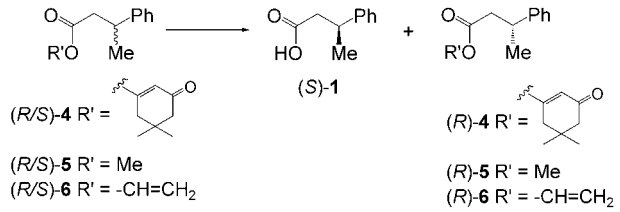
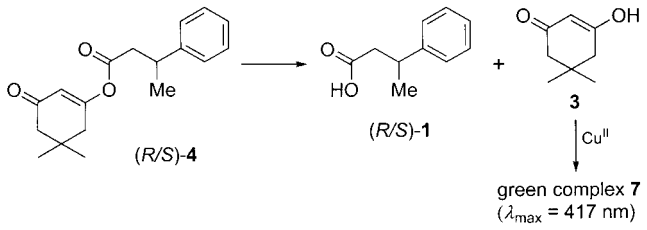


Figure 1. *Plot of* ee *of acid* ***1*** *(◆), ee of enol ester* ***4*** *(■) and % conversion (▲) for the CVL-catalysed hydrolysis of* (R/S)-***4****.*



Scheme 2. *CVL hydrolysis of esters of acid* ***1****. Reagents and conditions: CVL, 10% MeCN/0.1 M* KP_i *buffer (pH 7.0), RT.*



Scheme 3. *Hydrolase screening by detection of the copper–dimedone adduct* ***7****. Reagents and conditions: Substrate (14 μmol per well), hydrolase (1 mg per well), DMSO/H_2O/saturated $Cu(OAc)_{2(aq)}$ (75:15:10, 200 μL per well), 30 °C, 2 h.*

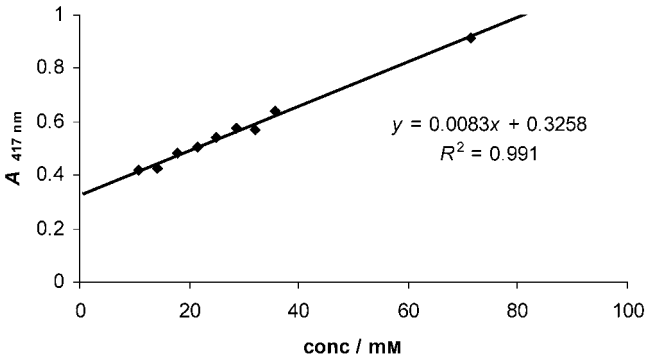


Figure 2. *Calibration curve showing concentration of dimedone plotted against absorbance in a DMSO solution containing copper(II) acetate.*

calibration curve of dimedone concentration plotted vs. absorbance clearly revealed a linear relationship (Figure 2).

To allow for complete dissolution of the substrate and optimal colour formation, the reaction was performed in a 75% DMSO/water mixture. Under these conditions, ^{1}H NMR spectroscopy indicated that dimedone existed mainly (>95%) in the enol form. In order to establish that the absorbance at 417 nm (A_{417}) was proportional to the concentration of CVL and, hence, the rate of hydrolysis, the standard curves shown in Figure 3a and b were generated. Formation of the green copper-dimedone complex is shown in Figure 3c.

Eight lipases and two esterases, each identified as giving high conversion of (*R/S*)-**4** from the HPLC screen, were then chosen to validate the new colorimetric screen. The rates of hydrolysis of the reactions were monitored in a 96-well microtitre plate (MTP) over a period of two hours by using a UV/Vis-plate reader. Figure 4 shows the time course of hydrolysis monitored by detection at $\lambda = 417$ nm. Thus *Chromobacterium viscosum* lipase (CVL), *Rhizopus arrhizus* lipase (RAL), *Candida antarctica* lipase B (CAL-B), *Candida lipolytica* lipase (CLL) and *Mucor miehei* esterase (MME) were all identified as active biocatalysts, and in the same rank order of activity as determined by the HPLC conversions. However, the commercially available preparations of five hydrolases, namely *Pseudomonas cepacia* lipase (PCL), *Pseudomonas fluorescens* lipase (PFL), *Mucor javanicus* lipase (MJL), porcine pancreatic lipase (PPL) and *Candida lipolytica* esterase (CLE) showed unacceptable background absorbance at 417 nm and would therefore need further purification before being suitable for use. Interestingly, although esterases are well-known to be less tolerant to organic solvents, MME retained activity despite the fact that the reaction was performed in a high concentration of DMSO.

Investigations into the rate of the CVL-catalyzed hydrolysis by using individual enantiomers of (*S*)-**4** and (*R*)-**4** showed a preference for (*S*) over (*R*), although, as expected, the difference in rate was low. This lack of selectivity is a consequence of elimination of competitive binding of the two enantiomers at the enzyme active site. Solutions to this problem have been developed by Kazlauskas et al., and exploited in their "Quick *E*" method.[16]

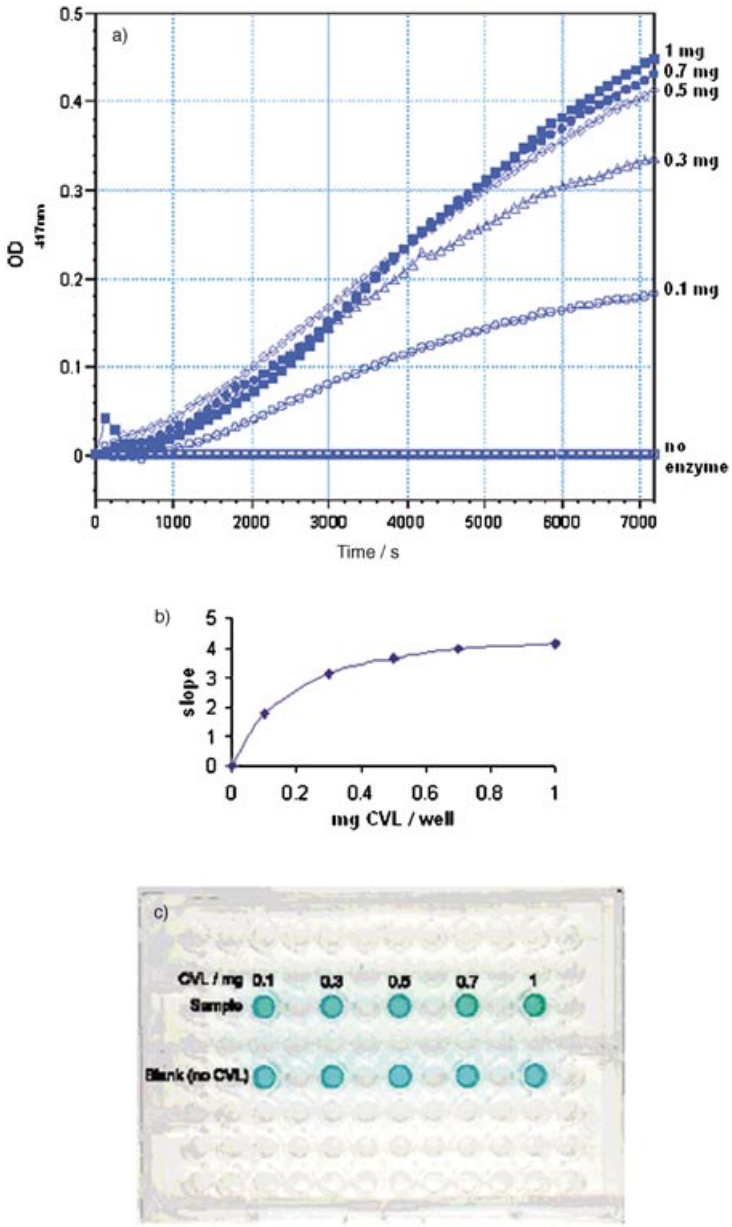


Figure 3. *a) Plot of absorbance against time for CVL-catalyzed (varying concentrations) hydrolysis of dimedone ester (R/S)-***4** *(14 μmol per well) in DMSO/H_2O/saturated $Cu(OAc)_{2(aq)}$ (75:15:10, 200 μL per well). b) Plot of the slope versus the amount of CVL per MTP well; c) 96-well microtitre plate (MTP) showing the green colour of adduct* ***7****.*

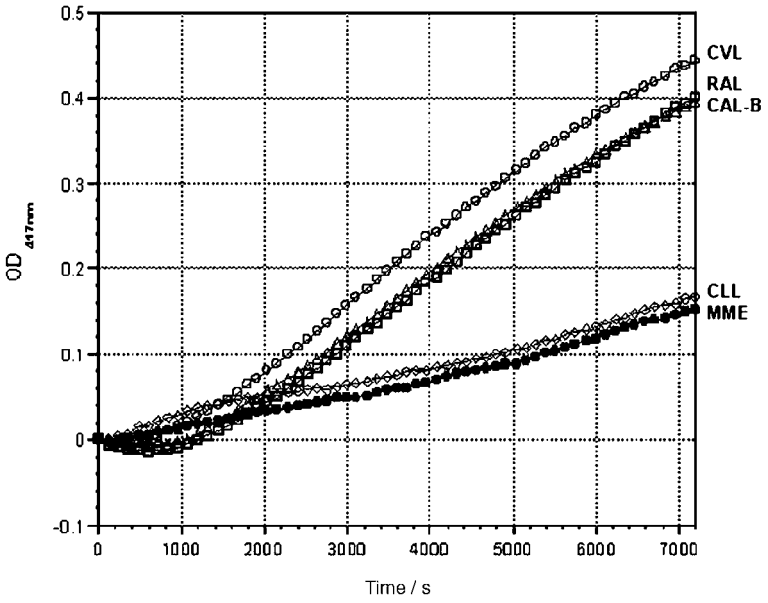


Figure 4. *Hydrolase-catalyzed hydrolysis of 1,3-enol ester (*R/S*)-**4** (14 μmol per well) in DMSO/H_2O/saturated Cu(OAc)$_{2(aq)}$ (75:15:10, 200 μL per well) and in situ formation of the copper–dimedone complex **7**.*

In summary, we have demonstrated that dimedone esters, in the presence of copper(II) acetate, can be used for the colorimetric detection of lipases and esterases in MTP format. The substrates are simple and inexpensive to prepare and, significantly, possess stability and reactivity akin to alkyl esters. Formation of the coloured product upon treatment with the lipase/esterase is instantaneous and does not rely upon a secondary reaction with a chromophore-containing reagent. The use of high concentrations of DMSO enhances the intensity of colour formation and also improves the solubility of the substrate. The latter is important, since screening methods of this type depend upon generating a homogeneous solution in the MTP for accurate measurement of UV absorbance, necessitating that both the substrate and enzyme are fully dissolved.

A further application of the methodology described above derives from our recent report on the synthesis of resin-bound cyclohexane-1,3-diones[17] and their use in lipase-catalysed kinetic resolutions on solid phase (e.g. PEGA) through a novel "capture and release" strategy.[18] Resin-bound reactions offer potential advantages in terms of automation and the synthesis of libraries for screening. In that context, lipases/esterases that have activity on resin-bound substrates could be identified through colorimetric detection of the liberated 1,3-enol by employing the assay described above.

Experimental Section

Analytical reverse-phase HPLC was performed on a Waters 600 controller/pump with a 996 photodiode array detector and equipped with a Phenomenex Sphereclone C18 column; elution: acetonitrile/water (1:1) at 1 mL min^{-1}, analysis at 210 nm. Normal-phase HPLC was performed on a Waters 600 controller/pump with a 486 tunable absorbance detector and equipped with a Chiracel-ODH column; elution: IPA/hexane (1:10) at 0.5 mL min^{-1}, analysis at 210 nm. UV analysis was performed in a Molecular Devices Versamax tunable microplate reader by using Softmax pro software.

Enzymatic HPLC screen: Dimedone ester (*R/S*)-**4** (86 mg, 3 mmol) was dissolved in 10% MeCN/0.1 M KP_i buffer (pH 7.0) and added to the enzymes (1 mg) under study in individual Eppendorf tubes and to one empty tube as a control. The tubes were rotated at room temperature overnight, then 0.1% TFA in MeCN (0.5 mL) was added to each tube to inactivate and precipitate the enzyme. The mixtures were filtered and analyzed by HPLC.

CVL-catalyzed hydrolysis of dimedone ester (*R/S*)-4, methyl ester (*R/S*)-5 and vinyl ester (*R/S*)-6: Fourteen samples of dimedone ester **4** (85.8 μg, 3 μmol), methyl ester **5** (53.4 μg, 3 μmol) or vinyl ester **6** (5.70 μg, 3 μmol) were each dissolved in 10% MeCN/0.1 M KP_i buffer (pH 7.0, 1 mL) containing CVL (0 1 mg) in individual Eppendorf tubes (with one tube containing no CVL as control) and rotated at room temperature for $t = 5$, 10, 15, 20, 30, 45, 60, 90, 150, 270, 480, 960 and 1440 min. After the correct time, the samples were added to ethyl acetate containing 0.1% TFA (1 mL). The ethyl acetate layer was separated and filtered through a plug of anhydrous magnesium sulfate before being concentrated in vacuo for analysis by normal-phase HPLC.

CVL-catalyzed hydrolysis of dimedone ester (*R/S*)-4 at varying enzyme concentrations: Ester (*R/S*)-**4** (4 mg per well, 14 μmol) was dissolved in DMSO (150 μL per well) and saturated aqueous copper(II) acetate solution (20 μL per well). CVL samples (0.1, 0.3, 0.5, 0.7 and 1 mg) were dissolved in unbuffered water (30 μL per well) and added directly to the required reaction well. The UV absorbance at 417 nm was monitored as described above against a background sample containing no enzyme, over 2 h at 30 °C.

Determination of enzymatic activity in MTP: Dimedone ester (*R/S*)-**4** (4 mg per well, 14 μmol) was dissolved in DMSO (150 μL per well) and saturated aqueous copper(II) acetate solution (20 μL per well). The enzyme (1 mg) was dissolved in unbuffered water (30 μL per well) and added directly to the required reaction well. The UV absorbance at 417 nm was monitored as described above against a background sample containing no enzyme, over 2 h at 30 °C.

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Keywords: colorimetric screen · copper · dimedone esters · esterases · hydrolases · lipases

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