Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 2105–2108

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A Low Background High-Throughput Screening (HTS) Fluorescence Assay for Lipases and Esterases Using Acyloxymethylethers of Umbelliferone

Emmanuel Leroy, Nicolas Bensel and Jean-Louis Reymond*

Department of Chemistry & Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland

Received 23 January 2003; revised 10 April 2003; accepted 12 April 2003

Abstract—Esters and acyloxymethyl ethers of umbelliferone were evaluated as fluorogenic substrates for lipases and esterases with respect to stability and resistance to non-specific hydrolysis. Isobutyryloxymethyl ether **1c** and pivaloxymethyl ether **1d** were found to be optimal substrates for enzyme assays, particularly with respect to HTS applications.

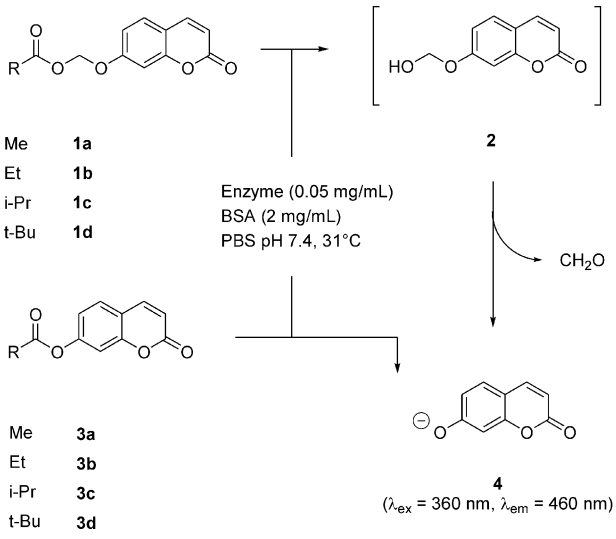
Lipases and esterases are the most broadly used enzyme type, serving various needs ranging from detergent components to chiral catalysts for fine chemical synthesis.[1] Although many such enzymes are known, the desirable activities or selectivities are often not available in existing collections, and large efforts continue to be invested in the discovery of further esterase and lipase variants by biodiversity mining, mutagenesis or directed evolution.[2] High-throughput screening (HTS) assays are an essential component of this effort.[3] Herein, we report the evaluation of acyloxymethyl ethers of umbelliferone as enzyme substrates and demonstrate their use as highly convenient fluorescent probes for lipases and esterases. These substrates combine high specific reactivity with the enzymes tested with excellent stability against non-specific degradation.

The standard, microtiter-plate compatible, high-throughput screening assay for lipases and esterases is based on esters of chromogenic or fluorogenic phenols, for example acetates, butyrates, octanoates, oleates and palmitates of nitrophenol or 4-methylumbelliferone. However, all of these substrates share the serious drawbacks of (1) a high rate of spontaneous hydrolysis in the absence of enzyme, and (2) a tendency to react with non-catalytic proteins, for example BSA (bovine serum albumin) or denatured or non-catalytic lipase fragments. This high and undesirable non-specific reactivity has a clear chemical origin, which is the very low pK_a of the phenol leaving group (pK_a ~7) compared with aliphatic alcohols (pK_a >14), such that these phenyl esters are closer in reactivity to anhydrides (with leaving group pK_a of 4.5 for the carboxylate) than to esters. Thus, nitrophenyl and umbelliferyl esters, for example biotin nitrophenyl ester, are suitable for use as protein acylating agents. Considering that the chromogenic/fluorogenic nature of these aryl esters arises from the strong acidity of the phenol moiety (the color/fluorescence is due to the spectral properties of the phenolate anion), the reactivity problem cannot be solved by altering the phenolic pK_a. The only alternative would be to effect an indirect release of the phenol triggered by ester hydrolysis of a less acidic alcohol function. We have reported an enzyme coupled[4] as well as a sodium periodate coupled[5] solution to this problem, however the utilization of a secondary reaction limits the conditions under which these assays can be used.

A straightforward opportunity to solve the problem of the leaving group pK_a using indirect release is offered by acyloxymethyl ethers of the phenols, such as ethers **1a–d**. These substrates liberate the hydroxymethylether **2** as the primary hydrolysis product (Scheme 1). Hydroxymethyl ether **2** is much less acidic that umbelliferone itself, yet spontaneously collapses in aqueous conditions to give formaldehyde and the fluorescent umbelliferone

*Corresponding author. Tel.: +41-31-631-4325; fax: +41-31-631-8057; e-mail: jean-louis.reymond@ioc.unibe.ch

0960-894X/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00377-9



Scheme 1. Fluorogenic reaction of acyloxymethyl ethers **1a–d** and esters **3a–d**.

4, so that its release is equivalent to that of umbelliferone itself. Clearly noticing the favorable properties of these oxymethyl ethers as fluorogenic substrate in connection with high-throughput screening of catalytic antibodies,[6] we decided to investigate in detail their possible use as fluorescent probes for lipases and esterases.

We first investigated the non-specific reactivity of oxymethyl ethers **1a–d** and of the corresponding esters **3a–d** in aqueous phosphate buffer pH 7.4 in the presence or absence of BSA as an additive to measure the extent of non-specific protein reactivity (Table 1). This non-specific reactivity, which mostly reflects acylation of surface lysine residues in BSA, was quite an important reaction pathway for umbelliferyl acetate **3a**, propionate **3b** and isobutyrate **3c**. Surprisingly, the acetoxymethyl ether **1a** was less prone to reaction with BSA than its propanoyl analogue **1b** despite of its higher chemical reactivity. This may be explained by the higher lipophilicity of the propanoxymethyl ether **1b**, which probably shows a tighter binding tendency with BSA. Umbelliferyl pivalate **3d** was quite unreactive and was less prone to either chemical or BSA-promoted hydrolysis than all other substrates with the exception of oxymethyl ether analogue **1d**, showing the strong influence of steric bulk on reactivity.

Table 1. Hydrolysis rates of umbelliferyl acyloxymethyl ethers **1a–d** and esters **3a–d** in aqueous conditions

	Compd	no BSA[a]	BSA[b]	BSA/no BSA[c]
Acetoxymethyl	**1a**	0.9	2.9	3
Propanoxymethyl	**1b**	0.5	6.3	14
Isobutoxymethyl	**1c**	0.1	1.4	10
Pivaloxymethyl	**1d**	0.02	0.1	6
Acetyl	**3a**	1.2	24.8	21
Propanoyl	**3b**	0.8	35.4	45
Isobutyryl	**3c**	0.5	31.4	69
Pivalyl	**3d**	0.1	0.8	11

[a]Hydrolysis rate in aq PBS pH 7.4, 31 °C, in nM s^{-1} for a 200 μM solution of substrate.
[b]Hydrolysis rate in aq PBS pH 7.4, 31 °C, containing 2 mg mL^{-1} BSA, in nM s^{-1} for a 200 μM solution of substrate. This rate was used as background rate V_{noE} to calculate the net rates and relative rates in Table 2.

Oxymethyl ether substrates **1a–d** and umbelliferyl pivalate **3d**, which all showed an acceptably low level of non-specific reactivity with BSA, were examined as lipase and esterase substrates. A series of commercial enzymes were assayed in the presence of BSA to mimic high-throughput screening conditions where the protein being tested contains not only the active catalyst (Fig. 1). The rate data was tabulated either as net reaction rate above the background reaction with BSA ($V_{net} = V_{obs} - V_{noE}$, Table 2a), or as relative reaction rate to this background reaction ($V_{rel} = V_{net}/V_{noE}$, Table 2b). All enzymes showed satisfactory levels of reaction with at least one of the substrates. The fastest reaction rates were observed with propionyloxymethyl ether **1b**. However this substrate also hydrolyzed quickly in the background with BSA only. The relative rates above background (Table 2b) showed that **1b** was in fact quite poor in terms of specific enzyme acceleration over background. Under these terms, isobutyryloxymethyl ether **1c** and pivaloyloxymethyl ether **1d** appeared clearly as the best substrates, with **1c** combining fast reaction and a highly specific acceleration above background with most enzymes.

The above experiments clearly show that lipases and esterases react readily with esters of relatively hindered carboxylic acids such as isobutyric or pivalic acid. The

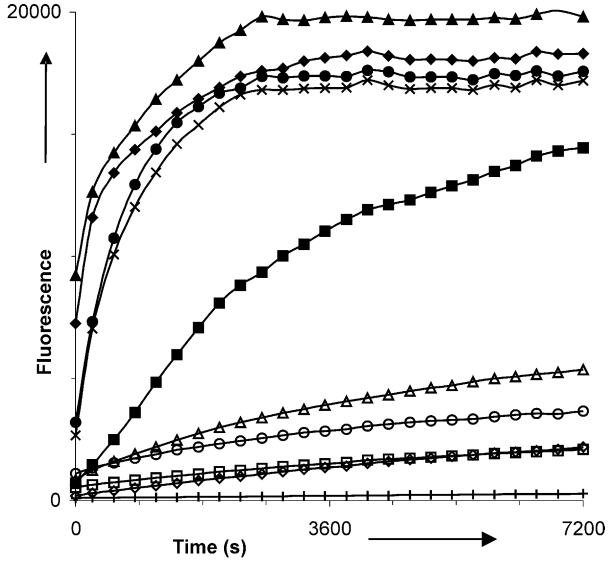


Figure 1. Fluorescence assay with substrates **1a–d** and **3d** and *Pseudomonas sp. type B* lipase (PSBL) in aqueous PBS (160 mM NaCl, 10 mM phosphate) pH 7.4, 2 mg mL^{-1} BSA, 200 μM substrate, 50 μg mL^{-1} enzyme, 31 °C. With enzyme: (●) **1a**, (▲)**1b**, (◆) **1c**, (x) **1d**, (■) **3d**. Without enzyme: (○) **1a**, (△) **1b**, (◇) **1c**, (+) **1d**, (□) **3d**. 0.1 mL assays were carried out in round-bottom polypropylene 96-well-plates (Costar) and recorded using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems, filters $\lambda_{ex} = 360 \pm 20$, $\lambda_{em} = 460 \pm 20$ nm). Fluorescence was converted to product concentration using a calibration curve with pure **4**, and the steepest linear portion of each curve was used to calculate the reaction rates.

Table 2. (a) Rate of hydrolysis rate of substrates **1a–d** and **3d** with different enzymes. Net rates above background $V_{net} = V_{obs} - V_{noE}$ in nM s^{-1} (conditions: 200 μM substrate in aq PBS pH 7.4, 30 °C, 2 mg mL^{-1} BSA, 50 μg mL^{-1} enzyme; see also legend of Figure 1)

Fluka no.	Enzyme	V_{net} (nM s^{-1})					Grayscale
		1a	**1b**	**1c**	**1d**	**3d**	
62336	PSBL	128	92	113	122	33	
46056	CLE	54	84	100	114	75	
46069	HLE	24	97	91	71	57	
62335	PSL	107	135	91	39	7	
62333	CVL	33	56	52	18	5	
46059	MME	12	26	50	7	1	
46062	BSE	8	43	39	7	6	
62309	PCL	58	104	37	1	1	
62310	RNL	19	50	34	6	2	
46062	BSE	8	31	32	6	5	
46051	BStE	13	61	29	4	1	
62300	HPL	13	50	39	2	0	
62305	RAL	11	51	28	2	1	
62308	PRL	10	41	32	1	1	
62285	AOL	7	34	28	2	1	
62316	CCL	5	32	30	2	1	
62298	MML	8	35	19	2	0	
62300	HPL	7	39	27	1	0	
62303	CLL	9	37	18	2	1	
46071	SCE	8	20	25	1	0	
62298	MML	7	40	17	2	1	
62303	CLL	7	32	17	1	1	
62299	CAL	5	34	24	1	1	
62294	ANL	6	25	28	0	0	
62285	AOL	4	35	16	1	0	

(b) Relative rates of reaction of fluorogenic substrates **1a–d** and **3d** with enzymes, expressed as $V_{rel} = V_{net}/V_{noE}$, calculated from the data in Tables 1 and 2 (a)

Fluka no.	Enzyme	$V_{rel} = V_{net}/V_{noE}$					Grayscale
		1a	**1b**	**1c**	**1d**	**3d**	
62336	PSBL	32	6	33	307	22	
46056	CLE	14	6	30	286	50	
46069	HLE	6	7	27	178	38	
62335	PSL	27	9	27	98	5	
62333	CVL	8	4	15	44	3	
46059	MME	3	2	15	18	0	
46062	BSE	2	3	11	17	4	
62309	PCL	15	7	11	3	0	
62310	RNL	5	3	10	16	1	
46062	BSE	2	2	9	14	3	
46051	BStE	3	4	9	11	1	
62300	HPL	3	3	11	4	0	
62305	RAL	3	3	8	5	1	
62308	PRL	3	3	10	4	1	
62285	AOL	2	2	8	6	0	
62316	CCL	1	2	9	5	0	
62298	MML	2	2	6	5	0	
62300	HPL	2	3	8	2	0	
62303	CLL	2	3	5	4	0	
46071	SCE	2	1	7	3	0	
62298	MML	2	3	5	4	0	
62303	CLL	2	2	5	4	1	
62299	CAL	1	2	7	2	0	
62294	ANL	1	2	8	1	0	
62285	AOL	1	2	5	3	0	

The grayscale grid at right was generated from the table data.

specific enzyme acceleration over background is generally higher than for more reactive esters, which translates into a better signal to noise ratio for detection of catalysis. The least reactive substrate is pivaloxymethyl ether (POM) **1d**, which can be readily prepared from umbelliferone and the commercially available chloromethyl pivalate.[6] The corresponding isobutyrate **1c** seems to react with almost all enzymes tested, although its background rate is significantly higher. POM derivative **1d** is particularly convenient for HTS experiments due to its low level of reactivity in the absence of enzyme. Indeed, HTS experiments usually involve reactions in 96-well plates running in parallel, and the pipetting operations imply that several minutes pass

between the reaction initiation and the recording of the fluorescence signal. In addition, **1c** and **1d** are crystalline solids that can be kept for long periods without noticeable degradation. Stock solutions in 50% aq DMF are stable at +4 °C for at least several weeks. The solubility of these substrates also appears to be excellent under the assay conditions, which allows to follow the reaction over several hours without any problems of precipitation. These practical advantages over the notoriously unstable nitrophenyl and umbelliferyl esters makes umbelliferyl acyloxymethyl ethers **1c** and **1d** a very recommendable choice for high-throughput screening of esterolytic enzymes, as well as for routine quality control of enzyme samples. The corresponding nitrophenyl ethers show similar properties and can be used in a chromogenic version of the assay.

Acknowledgements

This work was supported by the Swiss National Science Foundation, the Swiss Office Fédéral de l'Education et de la Science, and Protéus SA, Nîmes, France.

References and Notes

1. (a) Schmidt, R. D.; Verger, R. *Angew. Chem. Int. Ed.* **1998**, *37*, 1608. (b) Reetz, M. T. *Curr. Opin. Chem. Biol.* **2002**, *6*, 145.
2. (a) Marrs, B.; Delagrave, S.; Murphy, D. *Curr. Opin. Microbiol.* **1999**, *2*, 241. (b) Stemmer, W. P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10747. (c) Stemmer, W. P. C. *Nature* **1994**, *370*, 389. (d) You, L.; Arnold, F. H. *Protein. Eng.* **1996**, *9*, 77. (e) Reetz, M. T.; Jaeger, K.-E. *Chem. Eur. J.* **2000**, *6*, 407. (f) Taylor, S. V.; Kast, P.; Hilvert, D. *Angew. Chem.* **2001**, *113*, 3408; *Angew. Chem. Int. Ed.* **2001**, *40*, 3310.
3. (a) Reetz, M. T. *Angew. Chem.* **2001**, *113*, 292; *Angew. Chem. Int. Ed.* **2001**, *40*, 284. (b) Wahler, D.; Reymond, J.-L. *Curr. Opin. Chem. Biol.* **2001**, *5*, 152. (c) Beisson, F.; Tiss, A.; Rivière, C.; Verger, R. *Eur. J. Lipid Sci. Technol.* **2000**, 133. (d) Wahler, D.; Reymond, J.-L. *Curr. Opin. Biotechnol.* **2001**, *12*, 535.
4. Klein, G.; Reymond, J.-L. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1113. (b) Klein, G.; Reymond, J.-L. *Helv. Chim. Acta* **1999**, *82*, 400.
5. (a) Badalassi, F.; Wahler, D.; Klein, G.; Crotti, P.; Reymond, J.-L. *Angew. Chem. Int. Ed.* **2000**, *39*, 4067. (b) Wahler, D.; Badalassi, F.; Crotti, P.; Reymond, J.-L. *Angew. Chem. Int. Ed.* **2001**, *40*, 4457. (c) Wahler, D.; Badalassi, F.; Crotti, P.; Reymond, J.-L. *Chem. Eur. J.* **2002**, *8*, 3211. (d) Wahler, D.; Reymond, J.-L. *Angew. Chem. Int. Ed.* **2002**, *41*, 1229.
6. Bensel, N.; Reymond, M. T.; Reymond, J.-L. *Chem. Eur. J.* **2001**, *7*, 4604.