

Fluorogenic Substrates for Assessing Cytochrome P450 Isozyme Activity

Introduction

Metabolic oxidation of chemical compounds, including many pollutants, is the function of the cytochrome-mediated monooxygenase or mixed-function oxidase system. Several enzymes are involved, including cytochrome P-448 monooxygenase (aryl hydrocarbon hydroxylase), which is induced by carcinogenic polyaromatic hydrocarbons. Cytochrome P450 is a useful marker of endoplasmic reticulum membranes.¹ The low turnover rate of these enzymes can be followed using various fluorogenic alkyl ether derivatives of coumarin² and resorufin.³

Resorufin-Based Cytochrome P450 Substrates

The four resorufin ether substrates (R351, R352, R441, R1147), which all yield fluorescent resorufin (excitation/emission maxima = 571/585 nm), have been extensively used to differentiate isozymes of cytochrome P450.^{3–9} A fluorescence microplate assay has been developed for the simultaneous determination of ethoxyresorufin *O*-de-ethylase and total protein concentration using ethoxyresorufin (R352) and fluorescamine¹⁰ (F2332). Because the cytochrome isozymes exhibit differential activity on the various resorufin ethers, we offer the Alkoxyresorufin Sampler Kit (R6564), which contains 1 mg samples of our four resorufin ethers, 10 mg of resorufin (R363), and an extensive bibliography on these derivatives.

Coumarin-Based Cytochrome P450 Substrates

Possibly the most extensively used substrate for detecting cytochrome P450 has been 7-ethoxycoumarin (E186), which upon dealkylation yields a blue fluorescent product.^{11,12} Fluorescence detection of the de-ethylation of 3-cyano-7-ethoxycoumarin (C684) is reported to be 50–100 times more sensitive than that of resorufin ethyl ether (ethoxyresorufin), primarily because of the faster turnover rate of 3-cyano-7-ethoxycoumarin;^{13,14} however, ethoxyresorufin exhibits lower fluorescence background due to its more favorable spectral shifts. The de-ethylase product of 3-cyano-7-ethoxycoumarin (3-cyano-7-hydroxycoumarin; C183) has a lower pK_a than that of 7-ethoxycoumarin,^{11,12} allowing continuous measurements of enzyme activity at pH 7.

The cytochrome P450 substrate 7-ethoxy-4-trifluoromethylcoumarin (E2882) yields a product with a fluorescence emission that is distinct from that of the substrate and of NADPH, making this substrate useful for the direct measurement of enzymatic activity.^{15,16} Researchers have shown that this substrate is cleaved by at least the 1A2, 2E1, and 2B1 isozymes of cytochrome P450.^{15,17}

New Cytochrome P450 Substrates

The fluorescent products of most cytochrome P450 substrates rapidly leak from live cells, making them ineffective for measuring intracellular enzymatic activity by imaging or flow cytometric analysis. Using techniques for product retention that proved

successful for our patented DetectaGene™ and ImaGene™ glycosidase substrates, we have developed unique new substrates that can potentially be used to detect cytochrome activity in single cells.

Like the DetectaGene™ β -galactosidase substrates, the mildly thiol-reactive chloromethyl moiety of 4-chloromethyl-6,8-difluoro-7-ethoxycoumarin (C12202) and 5-chloromethylfluorescein diethyl ether (C6533) should react with glutathione or other intracellular thiols to produce products that are retained in cells through cell division. The de-ethylase product of the glutathione adduct of 4-chloromethyl-6,8-difluoro-7-ethoxycoumarin has a much lower pK_a than that of 4-chloromethyl-7-ethoxycoumarin and is much more fluorescent in cells. Coumarin and fluorescein ethers are known cytochrome P450 substrates.¹⁴ Adding a chloromethyl moiety to our glycosidase and peptidase substrates has enabled researchers to identify cells with enzymatic activity 24 hours after loading the substrate.¹⁸

Handling and Storage

These products are provided as lyophilized solids and should be stored desiccated at room temperature, 2–6°C, or $\leq -20^\circ\text{C}$ until use. The molecular weight is indicated on the product's label. Allow products to warm to room temperature before opening. A 1–20 mM stock solution may be prepared in high-quality, anhydrous dimethylsulfoxide (DMSO) or dimethylformamide (DMF). This solution can be stored desiccated at 2–6°C or below for at least six months. PROTECT FROM LIGHT.

Applications

Methods for assaying enzyme activity using these substrates should be obtained from the references cited in this document. Substrate working concentrations given in the literature range widely from about 1 to 50 μM . In most cases, the substrate stock solution in DMSO or DMF is diluted into the assay buffer just prior to initiating the assay by addition of the enzyme preparation, microsomes, or cells. Samples are generally incubated in substrate-containing buffers for periods ranging from several minutes to several hours at 37°C. Quantitative studies universally employ high purity reference standards (see Table 1) for assay standardization.

Some of the products described in this document, in particular those designed for enhanced cellular retention, have not yet been characterized as substrates for de-ethylases, either in solution or in cells. Methods for using these products must therefore be developed by the researcher using published protocols for similar substrates as a starting point.

Table 1. Properties of reference standards for cytochrome P450 substrates.

Catalog Number	Reference Standard	Abs *	Em *	pK _a	ε × 10 ⁻³ †	Standard for Substrate
D6566	6,8-difluoro-7-hydroxy-4-methyl-coumarin ‡	365	460	4.9	18	C12202, D12203
T659	7-hydroxy-4-trifluoromethyl-coumarin ‡	385	502	7.3	16	E2882
C183	3-cyano-7-hydroxy-coumarin ‡	408	450	~7.5	43	C684
C1904	5-(and-6)-carboxyfluorescein §	492	517	6.4	78	C6533
R363	Resorufin **	571	585	<7	54	R351, R352, R441, R1147

* Absorption and fluorescence emission maxima, in nm. Spectral maxima listed here are for an aqueous suspension. † Molar extinction coefficient determined at the wavelength in the Abs column. Listed values should be multiplied by 1000 to convert to the conventional units of cm⁻¹M⁻¹. ‡ The spectra of these hydroxycoumarins are pH dependent. Below the pK_a, the absorbance peak shifts to shorter wavelengths (325–340 nm) and the fluorescence intensity decreases. § The intensity of the fluorescence signal is pH dependent, becoming very weak below the pK_a. ** The absorption and fluorescence of resorufin are pH dependent. Below the pKa absorbance shifts to ~480 nm and both ε and fluorescence quantum yield are markedly reduced. The compound is also unstable in the presence of thiols such as dithiothreitol (DTT) and β-mercaptoethanol.

References

1. J Biol Chem 270, 24327 (1995);
2. Anal Biochem 191, 354 (1990);
3. Biochem J 240, 27 (1986);
4. Biochem Pharmacol 47, 893 (1994);
5. Biochem Pharmacol 46, 933 (1993);
6. Anal Biochem 188, 317 (1990);
7. Biochem Pharmacol 40, 2145 (1990);
8. Eur J Immunol 16, 829 (1986);
9. Biochem Pharmacol 34, 3337 (1985);
10. Anal Biochem 222, 217 (1994);
11. Anal Biochem 115, 177 (1981);
12. Hoppe Seyler Z Physiol Chem 353, 1171 (1972);
13. Anal Biochem 172, 304 (1988);
14. Biochem J 247, 23 (1987);
15. Biochem Pharmacol 46, 1577 (1993);
16. Biochem Pharmacol 37, 1731 (1988);
17. Arch Biochem Biophys 323, 303 (1995);
18. J Neurosci 15, 1025 (1995).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
C1904	5-(and-6)-carboxyfluorescein (5(6)-FAM) *high purity* *mixed isomers*	100 mg
C12202	4-chloromethyl-6,8-difluoro-7-ethoxycoumarin (CMDiFUET)	5 mg
C6533	5-(and-6)-chloromethylfluorescein diethyl ether *mixed isomers*	5 mg
C684	3-cyano-7-ethoxycoumarin	10 mg
C183	3-cyano-7-hydroxycoumarin	100 mg
D12203	6,8-difluoro-7-ethoxy-4-methylcoumarin (DiFMUET)	5 mg
D6566	6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) *reference standard*	10 mg
E2882	7-ethoxy-4-trifluoromethylcoumarin	25 mg
R441	benzyloxyresorufin (resorufin benzyl ether)	10 mg
R6564	Alkoxyresorufin Sampler Kit	1 kit
R352	ethoxyresorufin (resorufin ethyl ether)	5 mg
R351	methoxyresorufin (resorufin methyl ether)	5 mg
R1147	pentoxyresorufin (resorufin pentyl ether)	5 mg
R363	resorufin, sodium salt *reference standard*	100 mg
T659	β-trifluoromethylumbelliferone (7-hydroxy-4-trifluoromethylcoumarin) *reference standard*	100 mg

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

Please visit our website — probes.invitrogen.com — for the most up-to-date information.

Molecular Probes, Inc.

29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 • Fax: (541) 335-0504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)

Phone: (541) 335-0338 • Fax: (541) 335-0305 • probesorder@invitrogen.com

Toll-Free Ordering for USA:

Order Phone: (800) 438-2209 • Order Fax: (800) 438-0228

Technical Service: 8:00 am to 4:00 pm (Pacific Time)

Phone: (541) 335-0353 • Toll-Free (800) 438-2209
Fax: (541) 335-0238 • probestech@invitrogen.com

Invitrogen European Headquarters

Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100 • Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Limited Use Label License

For research use only. Not intended for any animal or human therapeutic or diagnostic use. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541) 465-8300. Fax: (541) 335-0504.

Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2005, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.