[Chem. Pharm. Bull. 35(7)3029—3032(1987)]

Preparation and Evaluation of Fatty Acid Esters of Fluorescent *p*-Substituted Phenols as Substrates for Measurement of Lipase Activity[1)]

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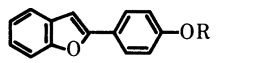
(Received November 6, 1986)

Several kinds of fatty acid esters of fluorescent *p*-substituted phenols (**1a**, **2a**, **3a**, and **4a**) were prepared. Their absorption and fluorescence spectral properties and their usefulness as substrates for the measurement of lipase activity were investigated. Among them, long alkyl chain esters were found to be suitable for fluorescent substrates, because their emission wavelengths were fairly different from those of the corresponding phenols. In particular, 4-(2-benzothiazolyl)phenyl myristate (**3e**), 4-(2-benzothiazolylvinyl)phenyl laurate (**4d**) and 4-(2-benzothiazolylvinyl)phenyl myristate (**4e**) were ascertained to be easily hydrolyzed by porcine pancreatic lipase.

Keywords——lipase; lipase activity; fluorescent *p*-substituted phenol; fluorogenic substrate; fluorometry

In the area of clinical testing, the development of simple, rapid, and sensitive assay methods for lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) activity is important for the diagnosis and follow-up of diseases of the pancreas.[2)] Therefore, many kinds of synthetic substrates for measurement of lipase activity have been reported.[3)] Recently, Orange I-laurate, a synthetic substrate, was found to be most rapidly hydrolyzed in a lipase catalyzed reaction and was applied to the colorimetric assay of lipase activity in blood.[3c)]

In this study, in order to improve in the sensitivity of the assay, various aliphatic carboxylic acid esters [**1**—**4** (R=acyl)] of fluorescent phenols [**1a**—**4a** (R=H)] were newly prepared as potential lipase substrates (Chart 1) and their fluorescence properties were examined. Among the twelve kinds of ester prepared, the laurate (**3d** and **4d**), the myristate (**3e** and **4e**), the palmitate (**3f**), and the stearate (**3g**) were found to be reasonably well hydrolyzed in the lipase-catalyzed reaction.



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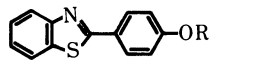

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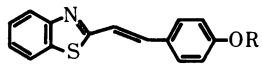

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R	compound			
H	**1a**	**2a**	**3a**	**4a**
$COCH_3$	**1b**		**3b**	**4b**
$CO(CH_2)_2CH_3$			**3c**	
$CO(CH_2)_{10}CH_3$	**1d**	**2d**	**3d**	**4d**
$CO(CH_2)_{12}CH_3$			**3e**	**4e**
$CO(CH_2)_{14}CH_3$			**3f**	
$CO(CH_2)_{16}CH_3$			**3g**	

Chart 1

Experimental

Reagents and Materials——All chemicals were of reagent grade, unless otherwise noted. Porcine pancreatic lipase (100000 U/1.9 mg lyophilized powder) was obtained from Sigma Chemical Co., Ltd., U.S.A. Water was deionized and distilled.

Apparatus——Infrared (IR) spectra were measured in Nujol mull with a JASCO IRA2 grating infrared spectrophotometer, and ultraviolet (UV) and visible absorption spectra and absorbances were obtained with a Hitachi 210 spectrophotometer. Fluorescence spectra were measured with a Hitachi 650 10S fluorescence spectrophotometer and 10 × 10 mm quartz cells. A Toa HM 5A pH-meter was used for pH measurements. Melting points are uncorrected.

Preparation of Substrates[4]——4-(2-Benzofuranyl)phenyl Laurate (**1d**): Acetone (10 ml) solution of lauric anhydride (3.93 g) was added to a solution of 2-(4-hydroxyphenyl)benzofuran (**1a**)[5] (980 mg, 4.7 mmol) in a mixture of pyridine (2 ml) and ether (8 ml). The mixture was refluxed for 5 h and poured into a mixture of ice-water (50 ml). The deposited crystals were filtered off, washed with water, and dried *in vacuo*. Crude **1d** was recrystallized from ethanol; 1.59 g, 87%, mp 103—104 °C. *Anal.* Calcd for $C_{13}H_{32}O_3$: C, 79.59; H, 8.16. Found: C, 79.20; H, 8.15.

4-(2-Benzofuranyl)phenyl Acetate (**1b**): 91%, mp 140—141 °C. *Anal.* Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.80. Found: C, 76.16; H, 4.78.

4-(2-Benzofuranylvinyl)phenyl Laurate (**2d**): **2d** was prepared according to the above procedure (*cf.*, **1d**) in 96% yield from **2a** (mp 201—213 °C), which was obtained by a demethylation of the corresponding methylether (mp 140 °C) with sodium ethylthiolate in *N,N*-dimethylformamide (120 °C, 4 h, 54%); mp 122 °C. *Anal.* Calcd for $C_{28}H_{34}O_3$: C, 80.35; H, 8.19. Found: C, 80.30; H, 8.22.

4-(2-Benzothiazolyl)phenyl Myristate (**3e**): A solution of myristic acid (1.92 g, 8.4 mmol) and *N,N'*-carbodiimidazole (1.43 g, 8.8 mmol) in dry tetrahydrofuran (THF) (10 ml) was refluxed for 1 h in an atmosphere of Ar and then cooled to room temperature. Next, solution of 2-(4-hydroxyphenyl)benzothiazole (**3a**)[6] (2.00 g, 8.8 mmol) in dry THF (9 ml) containing sodium imidazolate (0.66 mmol) was added to the above solution, and the mixture was stirred overnight. The residue obtained by evaporating the solvent *in vacuo* was extracted with benzene (10 ml). The extract was percolated through a short column of alumina (10 g) and concentrated to give colorless crystals (**3e**, 1.72 g, 45%, mp 88—89 °C). *Anal.* Calcd for $C_{27}H_{35}NO_2S$: C, 74.14; H, 8.01; N, 3.20; S, 7.32. Found: C, 74.13; H, 8.18; N, 2.96; S, 7.18.

4-(2-Benzothiazolyl)phenyl Acetate (**3b**): 94%, mp 147 °C. *Anal.* Calcd for $C_{15}H_{11}NO_2S$: C, 66.91; H, 4.09; N, 5.20; S, 11.90. Found: C, 66.79; H, 4.13; N, 5.20; S, 11.90.

4-(2-Benzothiazolyl)phenyl Butyrate (**3c**): 81%, mp 144—145 °C. *Anal.* Calcd for $C_{17}H_{15}NO_2S$: C, 68.69; H, 5.05; N, 4.71; S, 10.77. Found: C, 68.68; H, 5.15; N, 4.11; S, 10.77.

4-(2-Benzothiazolyl)phenyl Laurate (**3d**): 44%, mp 83.5—84 °C. *Anal.* Calcd for $C_{25}H_{31}NO_2S$: C, 73.35; H, 7.58; N, 3.42; S, 7.82. Found: C, 73.60; H, 7.69; N, 3.17; S, 7.81.

4-(2-Benzothiazolyl)phenyl Palmitate (**3f**): 54%, mp 91—92 °C. *Anal.* Calcd for $C_{29}H_{39}NO_2S$: C, 74.84; H, 8.44; N, 2.51; S, 6.68. Found: C, 74.84; H, 8.39; N, 3.01; S, 6.88.

4-(2-Benzothiazolyl)phenyl Stearate (**3g**): 37%, mp 95—96 °C. *Anal.* Calcd for $C_{31}H_{43}NO_2S$: C, 75.41; H, 8.78; N, 2.84; S, 6.49. Found: C, 75.47; H, 8.94; N, 2.73; S, 6.57.

4-(2-Benzothiazolylvinyl)phenyl Acetate (**4b**): The compound was prepared according to the acid anhydride method; 87%, mp 142—143 °C. The starting material, 2-(4-hydroxystyryl)benzothiazole[7] (**4a**), was synthesized by demethylation of the corresponding methylether [**1** (R = Me)].[8]

4-(2-Benzothiazolylvinyl)phenyl Laurate (**4d**): 80%, mp 96—97 °C. *Anal.* Calcd for $C_{27}H_{33}NO_2S$: C, 74.44; H, 7.64; N, 3.33; S, 7.44. Found: C, 74.65; H, 7.67; N, 3.33; S, 7.44.

4-(2-Benzothiazolylvinyl)phenyl Myristate (**4e**): 86%, mp 95—97 °C. *Anal.* Calcd for $C_{29}H_{37}NO_2S$: C, 75.13; H, 8.05; N, 3.02. Found: C, 74.98; H, 8.42; N, 2.93.

Evaluation Procedure for Esters as Lipase Substrates——A solution of a synthetic ester (50 μl) in methyl cellosolve or dioxane was mixed with 40 mM sodium dodecyl sulfate (SDS) in 0.1 M barbital buffer (pH 8.0, 50 μl) and this mixture was further mixed with the same buffer (0.35 ml). After the solution had been sonicated for 1 min, it was mixed with a solution of lipase (1250 U/ml, 50 μl), and incubated at 37 °C for 30 min, then a 0.05 M $Na_2B_4O_7 \cdot 10H_2O$–Na_2CO_3 buffer (pH 10.0, 3.5 ml) was added. The resulting mixture was centrifuged at 2000 *g* for 10 min at 20 °C. The relative fluorescence intensity (RFI) of the solution was measured, *e.g.*, at 425 nm (excitation (Ex) at 360 nm) for **3a** or 520 nm (Ex at 398 nm) for **4a**. For the blank, 0.1 M barbital buffer (pH 8.0) was added instead of the indicated volume of lipase solution.

The relation between the concentration of the phenol (*e.g.*, **2a** or **4a**) and RFI was examined according to the standard procedure except that ester and lipase were replaced by the corresponding phenol and water, respectively. Linear relationships were obtained in the range of 4.88×10^{-8}—1.56×10^{-6} M (**3a**) and 4.88×10^{-8}—3.13×10^{-6} M (**4a**).

Results and Discussion

Preparation of the Fatty Acid Esters of Phenols (1a—4a)

The phenols (**1a**—**4a**) could be converted to various aliphatic carboxylic acid esters by the acid anhydride or *N*,*N*′-carbonyldiimidazole method. The esters prepared are shown in Table I with the correpsonding phenols.

Electronic Absorption and Fluorescence Spectral Properties of the Phenols and the Esters

As shown in Table I, it has become apparent that the vinylogues (**2a**, **4a**, and their esters) show red shifts (33—39 nm) relative to the corresponding compounds in the UV spectra.

Fluorescence spectra of the phenols and the esters were measured both in EtOH and in an alkaline ethanol solution (pH 10) (Table I). Generally, the emission maxima of the phenols and the esters in the alkaline solution were at longer wavelengths than in EtOH. For the use of these esters as synthetic substrates, it is required that the emission maxima of the phenols liberated in the enzyme reaction are well separated from those of the corresponding esters. Therefore, the esters (**1**—**4**, R = acyl) might be suitable as substrates for the assay of lipase activity by fluorometry.

Evaluation of Esters as Substrates for Measurement of Enzyme Activity

The suitability of these esters for use as substrates for the fluorometric assay of lipase activity was examined. As a representative, **3e** was used to establish the evaluation method, with porcine pancreatic lipase as the enzyme. The effect of buffers [0.1 M barbital (pH 6.0—8.2) and 0.1 M phosphate (pH 7.5—9.0)] was examined and the optimal pH was found to be near 8.0 in both cases. We used 0.1 M barbital buffer (pH 8.0) which showed a larger RFI than that of 0.1 M phosphate buffer. The effect of the incubation time on RFI was examined, and a linear relation was obtained in the range of 10—60 min at 37 °C. The effect of concentration of **3e** on lipase activity was examined and a maximum RFI was obtained at 60 μM. The apparent K_m value for **3e** obtained from Lineweaver–Burk plots was 2.27×10^{-5} M and thus the substrate concentration was set at 3.00×10^{-4} M. For six kinds of ester (**3d**—**g** and **4d**, **e**), K_m

TABLE I. Spectral Data for Phenols and Their Carboxylic Acid Esters

Compound	UV (nm) EtOH		Excitation Maximum (nm)	Emission Maximum (nm)	Excitation Maximum (nm)	Emission Maximum (nm)
	λ_{max}	log ε	(Ethanol solution)		(Alkaline solution)[a]	
1a	308	4.60	310	354	326	408
1b	304	4.54	313	353	326	407
1d	304	4.28	311	352	335	407
2a	343	4.50	343	405	360	470
2d	337	4.42	343	386	288	430
3a	319	4.39	326	376	360	425
3b	300	4.03	325	362	350	425
3c	301	4.31	325	365	350	425
3d	301	4.18	320	360	315	378
3e	301	3.81	314	362	330	380
3f	300	3.92	314	360	330	380
3g	301	3.82	315	365	290	370
4a	358	4.43	310	370	398	520
4b	335	4.62	330	407	290	355
4d	336	4.52	330	405	305	422
4e	338	4.15	332	407	330	414

a) EtOH: (0.05 M $Na_2B_4O_7 \cdot 10H_2O$–Na_2CO_3 (pH 10.0)) = 10 : 90 (v/v).

TABLE II. Relative Reaction Velocity, K_m Value, and V_{max} for Substrates in the Reaction of Lipase

Substrate	Relative reaction velocity[a] (M/min)	K_m (M)	V_{max} (M/min)	V_{max}/K_m (min^{-1})
3d	4.17×10^{-9}	1.79×10^{-5}	6.17×10^{-9}	3.45×10^{-4}
3e	5.33×10^{-9}	2.27×10^{-5}	2.46×10^{-9}	1.08×10^{-4}
3f	1.83×10^{-9}	7.25×10^{-6}	8.17×10^{-10}	1.13×10^{-4}
3g	6.17×10^{-10}	2.50×10^{-6}	3.34×10^{-10}	1.34×10^{-4}
4d	1.60×10^{-8}	4.55×10^{-5}	5.67×10^{-9}	1.25×10^{-4}
4e	9.67×10^{-9}	8.33×10^{-5}	3.27×10^{-9}	3.93×10^{-5}

a) Relative reaction velocity was estimated from the amount of phenol liberated according to the procedure described in Experimental. All substrate concentrations were fixed at 2.00×10^{-4} M.

values and relative reaction velocities at 2.00×10^{-4} M are summarized together with the maximum velocities (V_{max}) in Table II. Among the substrates, **3d**, **4d**, and **4e** showed larger V_{max} values than the others. The order of hydrolysis rates was as follows: **4d** > **4e** > **3e** > **3d** > **3f** > **3g**. From the above results, **3d**, **4d**, and **4e** seem to be preferable as substrates for lipase. Though V_{max}/K_m for **4e** was the smallest owing to its large K_m value, **4e** should be useful as a substrate because of its relatively large reaction velocity. The reactivity of lipase appears to be practically independent of the carbon chain length of the esters. A linear relationship between the amount of lipase and the amount of **3a** hydrolyzed was obtained in the range of 0—75 U/tube, and an amount of lipase of 62.5 U/tube was selected for the evaluation procedure. Fluorescence stability of **3a** produced in the enzyme reaction was also checked; the RFI was constant for at least 180 min.

In conclusion, it is apparent that the fluorogenic esters listed in Table II can be used as substrates for assay of lipase activity. However, before using these esters in this way, it is necessary to examine the effects of various other enzymes on them. The application of the study to practical samples is now under study.

Acknowledgments We are grateful to Nippon Shoji Co., Ltd. for gifts of fatty acids, and to Mr. A. Shinji for technical assistance.

References and Notes

1) Part XI of "Development and Application of Organic Reagents for Analysis." For Part X, see K. Nakashima, C. Umekawa, H. Yoshida, S. Nakatsuji, and S. Akiyama, *J. Chromatogr.*, **414**, 11 (1987).
2) S. Kuroda, *Med. Technol.*, **12**, 31 (1984) and references cited therein.
3) *a*) F. J. Whitaker, *Clin. Chim. Acta*, **44**, 133 (1975); *b*) G. G. Guilbault, J. Hieserman, and M. H. Sadar, *Anal. Lett.*, **2**, 185 (1969); *c*) S. Kamachi, K. Wakabayashi, M. Yamaguchi and Y. Ohkura, *Anal. Chim. Acta*, **149**, 255 (1983).
4) All products, including the corresponding phenols and their methyl ethers, gave satisfactory spectral data (IR, ^{1}H-NMR, MS).
5) S. Akiyama, H. Akimoto, S. Nakatsuji, and K. Nakashima, *Bull. Chem. Soc. Jpn.*, **58**, 2192 (1985).
6) S. Nakano, H. Taniguchi, and K. Mikoshiba, *Yakugaku Zasshi*, **93**, 344 (1973).
7) H. Gilman, J. L. Towle, and R. K. Ingram, *J. Org. Chem.*, **21**, 595 (1956).
8) U. Marshall and M. C. Whiting, *J. Chem. Soc.*, 4082 (1956).