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Synthesis of fluorescein aromatic esters in the presence of P_2O_5/SiO_2 as catalyst and their hydrolysis studies in the presence of lipase

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ABSTRACT

A series of fluorescein aryl esters were synthesized by the esterification of fluorescein with carboxylic acids in the presence of P_2O_5/SiO_2 and their hydrolytic properties were investigated. The rate of hydrolysis in the presence or absence of lipase, due to the increase of fluorescein concentration, was measured by monitoring of fluorescence of the solution and correlated with enzyme activity. In addition, the substitute effect on the aromatic ring of fluorescein esters was studied. In contrast to fluorescein diacetate or dibutyrate, fluorescein dibenzoate and fluorescein bis(4-methylbenzoate) were found to be better substrates for the fluorometric assay of lipase with the higher rate of hydrolysis and better K_m value, respectively. As little as 9.3 ng mL^{-1} of lipase can be detected with fluorescein bis(4-methylbenzoate).

1. Introduction

Xanthene-based dyes such as rhodamines and fluorescein are widely used for labeling and sensing biomolecules [1,2]. They have high extinction coefficient and fluorescence quantum yield in aqueous solution, and their excitation and emission wavelengths are in the visible range. Brightness and biocompatibility of fluoresceins are interested to many researchers and many derivatives of fluoresceins are reported [3]. However, fluorescein also has some obvious disadvantages, particularly its hydrophilic nature limits its applications for detection of enzymes [4–6]. Fluorescein esters due to the locked spiro lactone structures are nonfluorescent [7], but they could be easily cleaved by intracellular enzymes to yield fluorescent products. Thus some lipophilic fluorescein esters have been reported for this purpose [8]. Kramer and Guilbault [9] successfully established the fluorometric method for the estimation and detection of lipase using fluorescein dibutyrate ester. A series of fluorescein esters with 2–18 carbon chains were examined [8–10]. However, the spontaneous hydrolysis rate of fluorescein esters was found to be decreased by the chain length of fluorescein esters, whereas, the rate of the enzymatic hydrolysis increased with the chain length of fluorescein esters. It is obvious that fluorescein esters with long chains have the stronger lipophilic ability. *p*-Guanidinobenzoate esters of fluorescein which were designed and synthesized as active site titrant of serine protease [11] is the only reported fluorescein aryl ester.

Fluorescein esters were prepared by either reaction of fluorescein with anhydride in the presence of a base [12,13] or with an alcohol in the presence of diethyl azodicarboxylate/triphenylphosphine or dicyclohexylcarbodiimide [14]. However, a few anhydrides are available and other method needs to activation of carboxylic acid. Considering the above reports, we wish to report the synthesis of a series of aromatic esters of fluorescein from carboxylic acids in the presence of P_2O_5/SiO_2. In order to search for a fluorogenic substrate for lipase, we evaluate the fluorogenic behavior of fluorescein aryl esters by spontaneous and enzymatic hydrolysis.

2. Experimental

2.1. General

Chemicals were either prepared in our laboratories or purchased from Merck, Fluka and Aldrich Chemical Companies. All yields refer to the isolated products. The products were characterized by comparison of their physical data with those of known samples or by their reported spectral data. Melting points were recorded on an Electro thermal type 9100 melting point apparatus.

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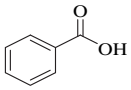
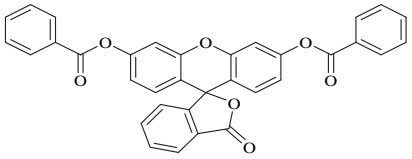
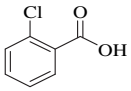
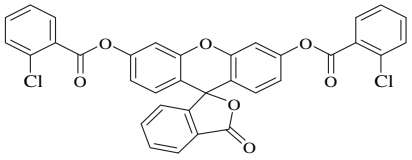
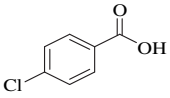
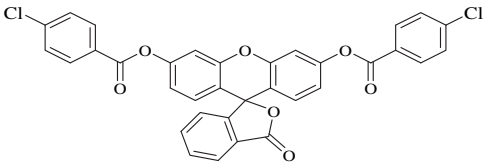
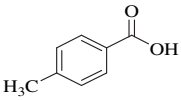
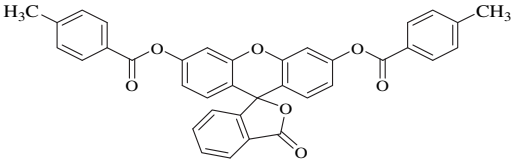
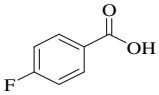
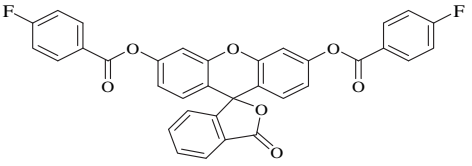
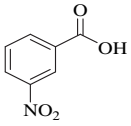
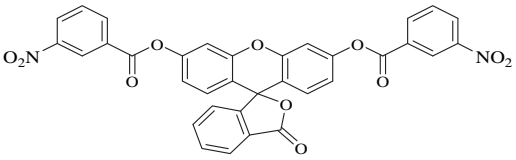
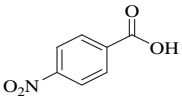
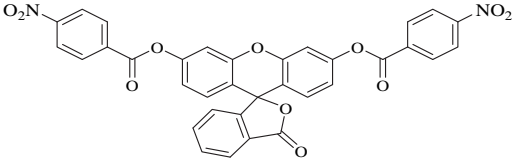
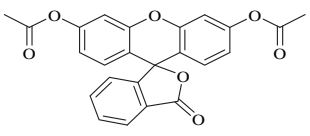
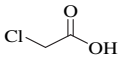
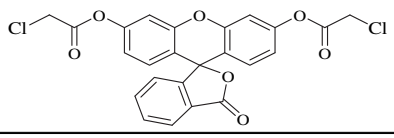
Table 1
One-pot preparation of fluorescein esters from fluorescein and carboxylic acids by means of P_2O_5/SiO_2 as catalyst[a].

Entry	Acid	Time (h)	Product	Yield (%)
1		4		90
2		4		93
3		4		87
4		4		89
5		4		93
6		4		95
7		4		90
8		2		94
9		1		92

[a] Isolated yield.

IR spectra were recorded on a Shimadzu–IR 470 spectrophotometer. The ^{1}H NMR (100 MHz) spectra were recorded on a Bruker AC 100 spectrometer. Chemical shifts are reported in ppm downfield from TMS as internal standard; coupling constance J are given in Hertz. Elemental analyses were obtained on a Thermo Finnigan Flash EA micro- analyzer. Silica gel 60 (230–400 mesh) was purchased from Merck. P_2O_5/SiO_2 was prepared according to previously reported procedure [15,16].

Lipase: Sigma Company, activity 1302 units per mg.

Buffer: Tris(hydroxymethyl)aminomethane buffer, 0.1 M, pH 7.2, was prepared by dissolving the pure compound in distilled water and adjusting the pH with 0.1 M hydrochloric acid.

Working standard of fluorescein esters: fluorescein esters were, respectively, dissolved in methylcellosolve as 10^{-3} M solution.

2.2. General procedure for the synthesis of compounds **3a**–**3i**

A mixture of fluorescein (**1**) (1.66 g, 5 mmol), carboxylic acid (**2**) (10 mmol) and P_2O_5/SiO_2 (0.02 mol) was grounded in a mortar for 10 min. and then, was heated at 60 °C for 4 h. After the completion, the reaction mixture was treated with CH_2Cl_2 (50 mL), stirred for one hour, and the catalyst filtered off. The organic layer was washed with water (25 mL), dried over Na_2SO_4 and concentrated to afford crude product. The crystalline pure product was obtained by further recrystallization in methanol (Table 1).

2.3. Spectral data of the prepared compounds

2.3.1. Fluorescein dibenzoate (**3a**)

Yield 90%; m.p. 208–210 °C; ^{1}H NMR: (CD_3COCD_3, ppm) δ: 8.2 (dd, 4H, J_1 = 7.0 Hz, J_2 = 2 Hz), 7.4–8.1 (m, 12H), 7.1 (dd, 2H, J_1 = 8 Hz, J_2 = 2 Hz), 7.0 (d, 2H, J = 8 Hz). IR (KBr disc) ν: 1770, 1750, 1610, 1450, 1425, 1255, 1240, 1155, 1110, 1055 cm^{-1}.

2.3.2. Fluorescein di(2-chlorobenzoate) (**3b**)

Yield 93%; m.p. 188–189 °C; ^{1}H NMR: ($CDCl_3$, ppm) δ: 8.0–8.2 (m, 3H), 7.7 (dt, 2H, J_1 = 7 Hz, J_2 = 1.5 Hz), 7.1–7.6 (m, 8H), 6.9 (m, 5H). IR (KBr disc) ν: 1775, 1750, 1620, 1470, 1275, 1255, 1230, 1150, 1075 cm^{-1}.

2.3.3. Fluorescein di(4-chlorobenzoate) (**3c**)

Yield 87%; m.p. 241–242 °C; ^{1}H NMR: ($CDCl_3$, ppm) δ: 8.15 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.5 Hz), 8.05 (d, 4H, J = 8 Hz), 7.55–7.8 (m, 2H), 7.5 (d, 4H, J = 8 Hz), 7.25(m, 3H), 6.9(m, 4H). IR (KBr disc) ν: 3100, 1765, 1745, 1615, 1595, 1545, 1460, 1260, 1250, 1160, 1105, 1075, 1050 cm^{-1}.

2.3.4. Fluorescein di(4-methylbenzoate) (**3d**)

Yield 189%; m.p. 89–190 °C; ^{1}H NMR: ($CDCl_3$, ppm) δ: 8.1 (d, 4H, J = 8 Hz), 8.05 (m, 1H), 7.7 (m, 2H), 7.30 (d, 4H, J = X Hz), 7.2 (m, 3H), 6.9 (m, 4H), 2.45 (s, 6H). IR (KBr disc) ν: 1765, 1745, 1610, 1585, 1500, 1470, 1260, 1245, 1155, 1110, 1055 cm^{-1}.

2.3.5. Fluorescein di(4- fluorobenzoate) (**3e**)

Yield 93%; m.p. 234–235 °C; ^{1}H NMR: ($CDCl_3$, ppm) δ: 8.0 (ABq, 8H), 7.9 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.5 Hz), 7.7 (m, 3H), 7.2–7.6 (m, 2H), 6.9 (m, 4H). IR (KBr disc) ν: 1770, 1745, 1615, 1595, 1480, 1460, 1290, 1280, 1245, 1190, 1155, 1110, 1050 cm^{-1}.

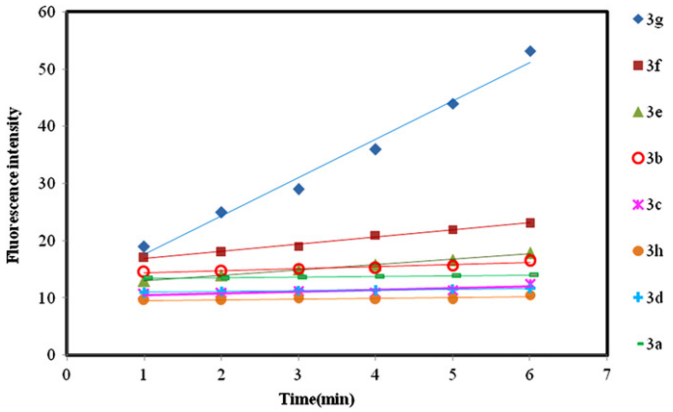


Fig. 1. The spontaneous hydrolysis of fluorescein esters in pH 7.2 Tris buffer.

2.3.6. Fluorescein di(3-nitrobenzoate) (**3f**)

Yield 95%; m.p. 168–169 °C; ^{1}H NMR: (CD_3COCD_3, ppm) δ: 8.9 (t, 2H, J = 1.5 Hz), 8.60 (m, 4H), 7.7–8.1 (m, 5H), 7.45 (d, 2H, J = 1.5 Hz), 7.4 (m, 1H), 7.2 (dd, 2H, J_1 = 8 Hz, J_2 = 1.5 Hz), 7.05 (d, 2H, J = 8 Hz). IR (KBr disc) ν: 1765, 1750, 1610, 1540, 1495, 1390, 1295, 1250, 1150, 1120 cm^{-1}.

2.3.7. Fluorescein di(4-nitrobenzoate) (**3g**)

Yield 90%; m.p. 178–180 °C; ^{1}H NMR: (CD_3COCD_3, ppm) δ: 8.45 (m, 8H), 7.95 (m, 1H), 7.8 (m, 2H), 7.5 (m, 2H), 7.2 (dd, 2H, J_1 = 8 Hz, J_2 = 2 Hz), 7.1 (d, 2H, J = 8 Hz). IR (KBr disc) ν: 1765, 1750, 1615, 1530, 1450, 1420, 1380, 1240, 1160 cm^{-1}.

2.3.8. Fluorescein diacetate (**3h**)

Yield 94%; m.p. 202–203 °C; ^{1}H NMR: ($CDCl_3$, ppm) δ: 8.00 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.5 Hz), 7.65 (m, 2H), 7.15 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.5 Hz), 7.1 (d, 2H, J = 1.5 Hz), 6.8 (m, 4H), 2.29 (s, 6H). IR (KBr disc) ν: 1770, 1750, 1615, 1495, 1460, 1285, 1245, 1210, 1155, 1110, 1090 cm^{-1}.

2.3.9. Fluorescein di(2-chloroacetate) (**3i**)

Yield 92%; m.p. 193–194 °C; ^{1}H NMR: (CD_3COCD_3, ppm) δ: 8.10 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.5 Hz), 7.8 (m, 2H), 7.40 (dd, 1H, J_1 = 7.5 Hz, J_2 = 1.5 Hz), 7.30 (d, 2H, J = 1.5 Hz), 7.0 (m, 4H), 4.6(s, 4H). IR (KBr disc) ν: 2900, 1775, 1750, 1625, 1580, 1470, 1290, 1230, 1220, 1150, 1120, 1085 cm^{-1}.

2.4. The spontaneous hydrolysis of fluorescein aryl esters

In a 10 mL volumetric flask, place accurately measured volume of various substrates (0.1 mL) and pH 7.2 Tris buffer (9.9 mL). Measure the rate of change in the fluorescence with time (ΔF/min) at λ_{ex} of 456 nm and λ_{em} of 510 nm. The rate of spontaneous hydrolysis could be obtained. The results of spontaneous hydrolysis of fluorescein esters are shown in Fig. 1 and Table 2.

Table 2
Comparison of the rates of enzymatic and spontaneous hydrolysis of fluorescein esters in pH 7.2 Tris buffer.

Compounds	3a	3b	3c	3d	3e	3f	3g	3h
R_e(min^{-1})	23.739	13.794	22.139	14.630	35.971	9.137	25.202	21.572
R_s(min^{-1})	0.112	0.368	0.298	0.13	0.945	1.236	6.764	0.146
R_e/R_s	200	35	74	112.5	40	7.5	4	150

R_e: The rate of enzymatic hydrolysis; R_s: The rates of spontaneous hydrolysis.

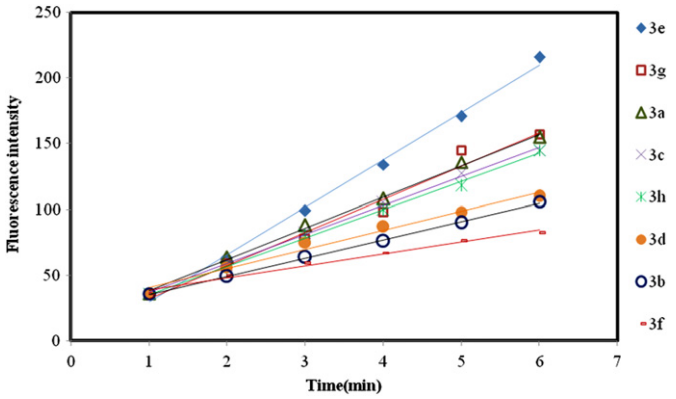


Fig. 2. The hydrolysis of fluorescein esters in the presence of lipase in pH 7.2 Tris buffer.

2.5. The hydrolysis of fluorescein aryl esters in the presence of lipase

Tris buffer (9.8 mL) of pH 7.2 and 0.1 mL of 10^{-3} M solution of fluorescein esters were put in a 10 mL volumetric flask and the instrument was adjusted to read zero. At the zero time, 0.1 mL of lipase solution with certain concentration was added, and the change in the fluorescence at λ_{ex} of 456 nm with time, ΔF/min, was recorded for over a period of 6 min. A plot of ΔF/min vs lipase concentration was made. The enzymatic hydrolysis results of fluorescein aryl esters in the presence of lipase are shown in Figs 2–8 and Tables 2 and 3.

3. Results and discussion

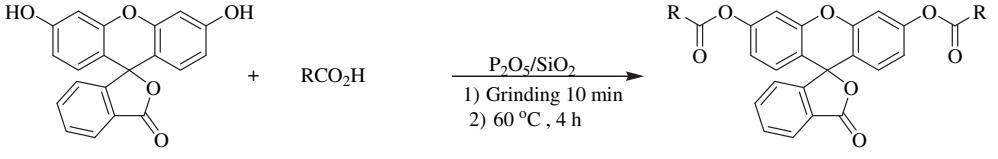
3.1. Direct synthesis of fluorescein esters

P_2O_5/SiO_2 is a relatively new reagent which introduced firstly by our research group for esterification of phenols [15] and alcohols [16]. We decided to synthesis fluorescein esters from fluorescein and carboxylic acids by means of P_2O_5/SiO_2 as catalyst. Thus, a mixture of fluorescein (**1**), carboxylic acid (**2**) and P_2O_5/SiO_2 was grounded in a mortar for 10 min. and then, was heated in 60 °C for 5 h. After a simple workup, fluorescein esters (**3a**–**h**) were obtained in 87–95% yields. Absorption of the released water by P_2O_5/SiO_2 plays a vital role in the completion of reaction (Scheme 1). In our method fluorescein esters (**3**) were washed with CH_2Cl_2 from reaction mixture and the catalyst regenerated by heating in an oven for further re-use. Aliphatic and aromatic carboxylic acids were reacted successfully in this condition and the corresponding fluorescein esters were obtained in high yields (Table 1). However, aliphatic carboxylic acids such as acetic acid and 2-chloroacetic acid were reacted with fluorescein in shorter reaction times.

The products were characterized by ^{1}H NMR, IR, and melting points and in the cases of known compounds were compared with authentic samples. The OH group's signals were omitted in ^{1}H NMR and IR spectra and strong absorption bands were appeared in IR spectra at 1765 and 1745 cm^{-1} due to ester and lactone carbonyl groups, respectively. In ^{1}H NMR spectra of aromatic esters a signal was appeared in 8–8.2 ppm corresponded to the *ortho* protons of aromatic rings of esters.

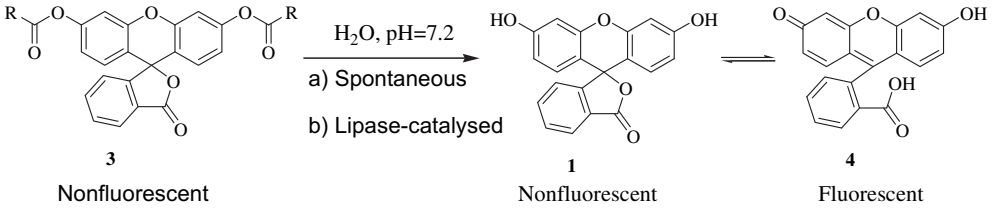
3.2. The investigation of hydrolytic properties of fluorescein esters

Kramer and Guilbault [9] successfully established the fluorometric method for the estimation and detection of lipase using fluorescein dibutyrate. Recently, Ge et al. [10] have been reported that fluorescein dilaurate is a better substrate for the lipase assay owing to its higher rate of hydrolysis and better K_m value, which was correlated with the stronger lipophilicity of its longer chain. However, there is no report about fluorescein aryl esters to this day. Actually, the longer chain fluorescein esters have the stronger lipophilicity, but the chains longer than laurate cannot approach easily with enzyme active site. We suggest that a small aryl group may be approach easily to the active site in the enzyme. In order to search for a fluorogenic substrate for lipase, we evaluated the fluorogenic behavior of fluorescein aryl esters (**3a**–**3g**) to the assay of lipase and the results were compared with fluorescein diacetate (**3h**) (Scheme 2).



a) R= C_6H_5
b) R= 2-Cl-C_6H_4
c) R= 4-Cl-C_6H_4
d) R= 4-Me-C_6H_4
e) R= 4-F-C_6H_4
f) R= 3-NO_2-C_6H_4
g) R= 4-NO_2-C_6H_4
h) R= CH_3
i) R= CH_2Cl

Scheme 1. Preparation of fluorescein aromatic esters in the presence of P_2O_5/SiO_2.



Scheme 2. Hydrolysis of fluorescein aryl esters in the presence or absence of lipase.

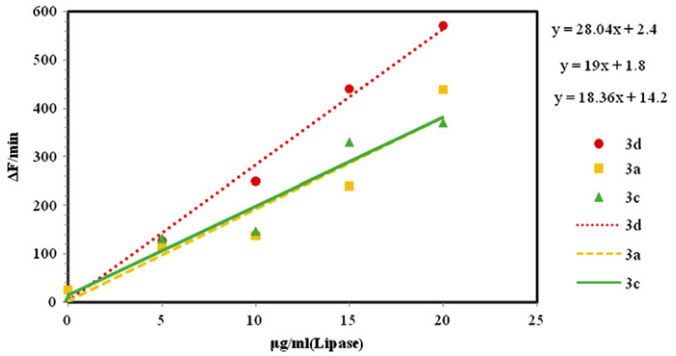


Fig. 3. The hydrolysis of fluorescein esters in the presence of different concentration of lipase in pH 7.2 Tris buffer.

3.2.1. *The rate of spontaneous hydrolysis*

The spontaneous hydrolysis rate of fluorescein esters in pH 7.2 Tris buffer was investigated by fluorometric method (Fig. 1), which found to be in the following order:

Fluorescein dibenzoate **3a** < fluorescein bis(4-methylbenzoate) **3d** < fluorescein diacetate **3h** < fluorescein bis(4-chlorobenzoate) **3c** < fluorescein bis(2-chlorobenzoate) **3b** << fluorescein bis(4-fluorobenzoate) **3e** < fluorescein bis(3-nitrobenzoate) **3f** << fluorescein bis(4-nitrobenzoate) **3g**. The rate of the spontaneous hydrolysis of fluorescein diesters increases in the presence of electron-withdrawing groups on the aryl rings. The spontaneous hydrolysis of fluorescein dibenzoate **3a** and fluorescein bis(4-methylbenzoate) **3d** were as slow as fluorescein diacetate **3h** and even slower than that of fluorescein dibutyrate [9,10]. It seems to be appropriate ester as model for the assay of enzymes. Furthermore, fluorescein esters with electron-withdrawing groups such as **3e**–**3g**, proceeded more rapidly than fluorescein dibenzoate **3a**. Fluorescein dibenzoate **3a**, fluorescein bis(4-methylbenzoate) **3d** and fluorescein bis(4-chlorobenzoate) **3c** hydrolyzed very slowly, whereas, fluorescein bis(4-nitrobenzoate) **3g** and fluorescein bis(2-chloroacetate) **3i** (not shown) hydrolyzed more rapidly. In a comparison of compounds **3a**–**3i**, it can be seen that fluorescein dibenzoate **3a**, fluorescein bis(4-methylbenzoate) **3d** and fluorescein bis(4-chlorobenzoate) **3c** which were hydrolyzed with more difficultly, were more stable in aqueous solutions.

3.2.2. *The rate of hydrolysis in the presence of the enzyme*

The importance of fluorescein esters are their applications as fluorogenic probe of enzyme. As we all know, fluorescein dibutyrate has been successfully applied to test the activity of lipase and elongation of ester chain increased this ability to the assay of lipase. However, the synthesis and the application of fluorescein esters with aryl groups are not reported. So we decided to study the

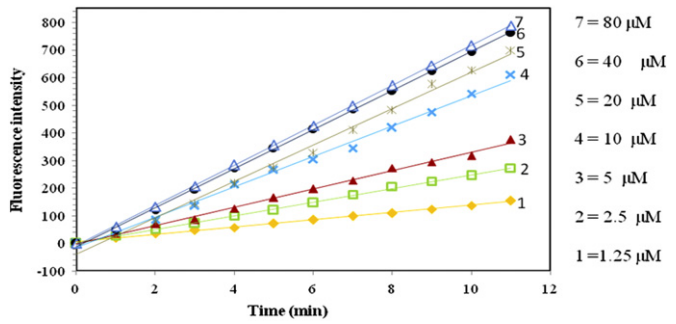


Fig. 4. The hydrolysis of different concentration of fluorescein dibenzoate **3a** in the presence of certain concentration of lipase (10 μg mL^{-1}) in pH 7.2 Tris buffer.

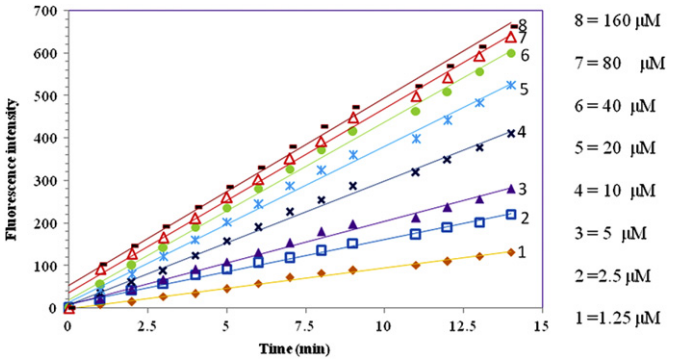


Fig. 5. The hydrolysis of different concentration of fluorescein bis(4-chlorobenzoate) **3c** in the presence of certain concentration of lipase (10 μg mL^{-1}) in pH 7.2 Tris buffer.

synthesis and the hydrolytic properties of a series of fluorescein aryl esters in the presence of lipase.

As shown in Fig. 2, the order of the rate of enzymatic hydrolysis is not similar to that of the spontaneous hydrolysis. The rate of the enzymatic hydrolysis increases for all substituents and fluorescein bis(4-fluorobenzoate) **3e** has maximum rate and fluorescein bis(3-nitrobenzoate) **3f** has minimum rate. Thus the order of the hydrolysis rate of fluorescein esters in the presence of lipase is shown as: Fluorescein bis(4-fluorobenzoate) **3e** >> fluorescein bis(4-nitrobenzoate) **3g** > fluorescein dibenzoate **3a** > fluorescein bis(4-chlorobenzoate) **3c** > fluorescein diacetate **3h** >> fluorescein

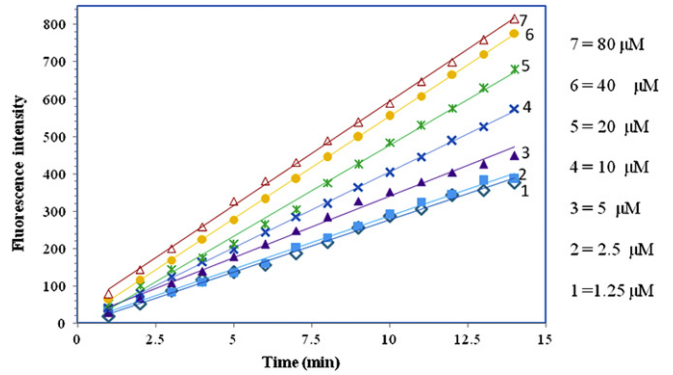


Fig. 6. The hydrolysis of different concentration of fluorescein bis(4-methylbenzoate) **3d** in the presence of certain concentration of lipase (10 μg mL^{-1}) in pH 7.2 Tris buffer.

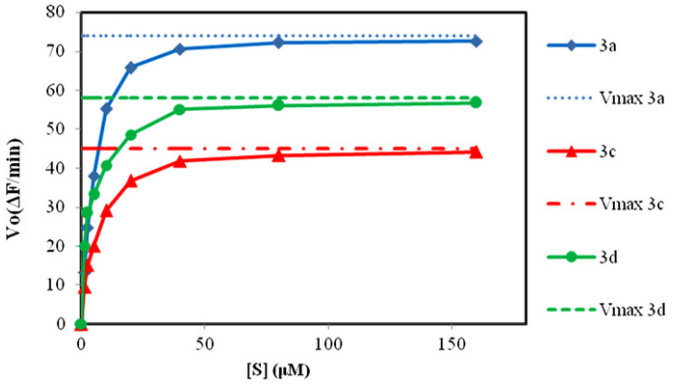


Fig. 7. Plot of rates of lipase catalyzed hydrolysis (Δ*F*/min) *vs* of fluorescein dibenzoate **3a**, fluorescein bis(4-chlorobenzoate) **3c**, and fluorescein bis(4-methylbenzoate) **3d** concentrations (μM) in the presence of certain concentration of lipase (10 μg mL^{-1}) in pH 7.2 Tris buffer.

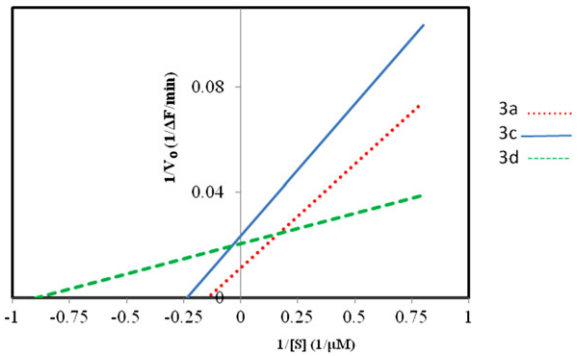
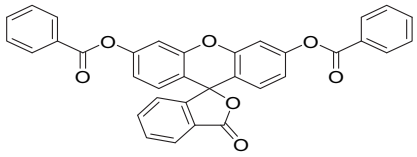
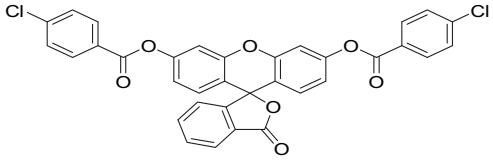
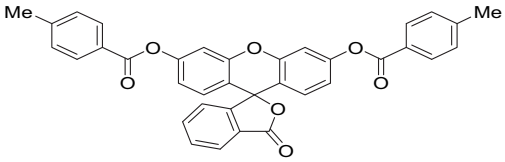
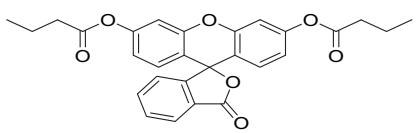


Fig. 8. Lineweaver–Burk plot of $(1/V_0)$ versus $(1/[S])$ of fluorescein dibenzoate **3a**, fluorescein bis(4-chlorobenzoate) **3c**, and fluorescein bis(4-methylbenzoate) **3d**.

bis(4-methylbenzoate) **3d** > fluorescein bis(2-chlorobenzoate) **3b** >> fluorescein bis(3-nitrobenzoate) **3f**. Accordingly, *p*-substituted phenyl ring (H, Cl, Me, F, and NO_2) have the high rates of hydrolysis. **3b** and **3f** with substitution in *o*- or *m*-positions have the lowest rates of hydrolysis. It is reasoned probably that fluorescein esters with or without *p*-substitution have the stronger lipophilic ability, these esters could easily and tightly bind to the enzyme while esters with *o*- or *m*- substitution have less chance to approach the enzyme. It may be explained that the *o*- or *m*-substitution can add to the steric hindrance and therefore is disfavorable for the interaction between esters and enzyme, resulting in the decreasing rate of enzymatic hydrolysis. Furthermore, the *p*-substitution may have effect on the elasticity of fluorescein ester which might perturb the approach of the substrate to the active site in the enzyme. It is surprising that the rate of fluorescein dibenzoate **3a** is higher than that of fluorescein diacetate **3h** and also than fluorescein bis(4-methylbenzoate) **3d**. The reason may be that the phenyl rings have so much lipophilic ability and the rate of enzymatic hydrolysis is increase seriously compared to the rate of spontaneous hydrolysis.

In another approach, comparison of the rates of enzymatic and spontaneous hydrolysis (R_e/R_s) for these esters showed that lipase hydrolysis acceleration are 150 for fluorescein diacetate **3h** and 200 for fluorescein dibenzoate **3a** (Table 2). This rate acceleration decreases to 4 for fluorescein bis(4-nitrobenzoate) **3g**. Fluorescein bis(4-fluorobenzoate) **3e** which have high rate of hydrolysis in the presence of lipase, possess only a 40 times rate acceleration in comparison to its spontaneous hydrolysis. Actually, the rate acceleration of benzoate esters with electron-withdrawing groups

Table 3
Comparison of various substrates for lipase assay.

Substrate	K_m (μM)[a]	V_{max}[b]	Lowest detectable concentration[c] ng mL^{-1}	Fluorescence wavelengths	Rate of spontaneous hydrolysis K_s (ΔF/min)[d]	Lit
 **3a**	7.04	83.3	12	$\lambda_{ex} = 456$ $\lambda_{em} = 510$	0.112	[e]
 **3c**	4.3	41.7	32	$\lambda_{ex} = 456$ $\lambda_{em} = 510$	0.298	[e]
 **3d**	1.1	50	9.3	$\lambda_{ex} = 456$ $\lambda_{em} = 510$	0.13	[e]
 (Fluorescein dibutyrate)	0.035	–	210	$\lambda_{ex} = 492$ $\lambda_{em} = 515$	0.3	9,10

[a] K_m is Michaelis constant for the enzyme–substrate reaction. K_m value was obtained by the method of Lineweaver–Burk.
[b] Maximum rate of enzymatic hydrolysis expressed in Δ fluorescence units per minute.
[c] The lowest detectable concentration of lipase reported was that concentration required to give an enzymatic rate twice that of the spontaneous rate.
[d] Rate of spontaneous hydrolysis expressed in Δ fluorescence units per minute.
[e] Present work.

in the presence of lipase is only about 4–40 times. Whereas, the rate acceleration of fluorescein bis(4-chlorobenzoate) **3c** and fluorescein bis(4-methylbenzoate) **3d** are 74 and 112 times, respectively. The rate acceleration of fluorescein bis(4-chlorobenzoate) **3c** rather than fluorescein bis(2-chlorobenzoate) **3b** is twofold which shows **3c** easily and tightly binds to the enzyme active site.

Thus, the order of the relative rate accelerations by lipase (R_e/R_s) are: Fluorescein dibenzoate **3a** > fluorescein diacetate **3h** > fluorescein bis(4-methylbenzoate) **3d** > fluorescein bis(4-chlorobenzoate) **3c** > fluorescein bis(4-fluorobenzoate) **3e** > fluorescein bis(2-chlorobenzoate) **3b** >> fluorescein bis(3-nitrobenzoate) **3f** >> fluorescein bis(4-nitrobenzoate) **3g**.

As shown in Figs 1 and 2, fluorescein dibenzoate **3a** and fluorescein bis(4-methylbenzoate) **3d** were the best substrates in this series of fluorescein esters with the highest lipase rate acceleration, thus we decided to study **3a**, **3c** and **3d** in details in comparison with fluorescein dibutyrate which had been successfully applied to test the activity of lipase [9,10].

The rate of lipase catalyzed hydrolysis of the fluorescein esters **3a**, **3c** and **3d** (ΔF/min) in different enzyme concentrations was studied (Fig. 3). Also, the rates of hydrolysis of different concentrations of the fluorescein esters in the presence of constant concentration of lipase (10 μg mL^{-1}) were shown in Figs. 4–6. As shown in Fig. 3, fluorescein bis(4-methylbenzoate) **3d** was the best substrate in these aryl ester series with the highest lipase hydrolysis efficiency. Accordingly, as little as 9.3 ng mL^{-1} of lipase can be detected using this ester, compared to 210 ng mL^{-1} with fluorescein dibutyrate [9,10]. Also, fluorescein dibenzoate **3a** and fluorescein bis(4-chlorobenzoate) **3c** can be detected 12 and 32 ng mL^{-1} of lipase, respectively. According to Figs. 4–6, we was drawn the plot of V_0 versus [S] and also, $1/V_0$ versus 1/[S] in Figs. 7 and 8. The results are shown in Table 3 and compared with a well known lipophilic substrate, fluorescein dibutyrate. Of the three substrates tested, fluorescein bis(4-methylbenzoate) **3d** had the better K_m value, and it showed the stronger appetency which came into being between the alkyl-substituted aryl ester and lipase. In addition, **3d** was more sensitive in the determination of lipase. As little as 9.3 ng mL^{-1} of lipase can be detected using this ester, compared to 210 ng mL^{-1} with fluorescein dibutyrate. Therefore, fluorescein diaryl esters are believed as an ideal high affinity substrate for the assay of lipase by fluorescence. The change in aryl rings or elongation of alkyl substitution are under investigation in our laboratories.

4. Conclusions

A series of fluorescein aryl esters were synthesized, by the esterification of fluorescein with the carboxylic acids in the presence of P_2O_5/SiO_2 under solvent-free conditions in high yields. The hydrolytic properties of fluorescein aryl esters were investigated in the presence or absence of lipase. It was seen that the presence of an electron-withdrawing group in aryl ester has an important effect on the spontaneous hydrolysis. Although, lipase accelerates all substrate hydrolysis, the rate acceleration is higher for **3a**, **3d** and **3c.** By comparison, fluorescein bis(4-methylbenzoate) **3d** was found to be the better substrate for the assay of lipase with the higher rate of hydrolysis and better K_m value. As little as 9.3 ng mL^{-1} of lipase, may be determined with this substrate. These results show that the fluorescein aryl esters may be a very sensitive fluorogenic marker of enzyme and have some latent applications in future.

Acknowledgements

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