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Synthesis and study on hydrolytic properties of fluorescein esters

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Abstract

A series of fluorescein esters of straight chain fatty acid were synthesized and applied to the fluorometric assay of lipase. The rate of change in the fluorescence of the solution, due to the production of fluorescein, was measured and correlated with enzyme activity. The hydrolytic properties of fluorescein esters were investigated. In addition, the relationship between their hydrolytic properties and the chain length of fluorescein esters was discussed in this paper. In contrast to fluorescein dibutyrate, fluorescein dilaurate was found to be a better substrate for the assay of lipase with the higher rate of hydrolysis and better K_m value. As little as 0.0001 mg/ml of lipase can be detected with a relative accuracy of about 1.5%.

Keywords: Fluorescein esters; Fluorescein dilaurate; Spontaneous hydrolysis; Enzymatic hydrolysis

1. Introduction

Fluorescein is the most important xanthene dye widely used for labeling and sensing biomolecules [1–6]. It has a high extinction coefficient and fluorescence quantum yield in aqueous solution, and its excitation wavelength is in the visible range. However, fluorescein also has some obvious deficiencies. Particularly its hydrophilic nature limits its application to the detection of the enzyme [7–12]. Thus, some lipophilic fluorescein derivatives have been reported for this purpose.

Fluorescein esters are nonfluorescent [13], but they could be cleaved by intracellular enzymes to yield fluorescent products. The rate of change in the fluorescence of the solution, due to the production of fluorescein, is measured and correlated with enzyme activity. Therefore, fluorescein esters could be used as fluorometric determination probes to lipase [14]. In 1963, Guilbault successfully established the fluorometric method for the estimation and detection of lipase using fluorescein dibutyrate. Then, a series of fluorescein esters with 2–6 carbon chain were synthesized and examined [15–17].

On the above research basis, a series of fluorescein esters with 2–18 carbon chain were prepared and employed to evaluate the activity of the enzyme in this paper. The structures are shown in Fig. 1. The relationship between the hydrolytic properties and the chain length of fluorescein esters has been studied. The obtained results are summarized and reported here.

2. Experimental

2.1. Materials

Lipase: Sigma Company, activity 2 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.

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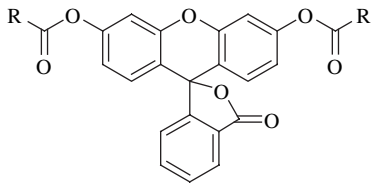


1 R= CH_3
2 R= CH_2CH_3
3 R= $(CH_2)_2CH_3$
4 R= $(CH_2)_4CH_3$
5 R= $(CH_2)_6CH_3$
6 R= $(CH_2)_8CH_3$
7 R= $(CH_2)_{10}CH_3$
8 R= $(CH_2)_{12}CH_3$
9 R= $(CH_2)_{14}CH_3$
10 R= $(CH_2)_{16}CH_3$

Fig. 1. Structural formula of fluorescein esters.

2.2. Synthesis

2.2.1. Fluorescein esters with 2–6 carbon chain (*1, 2, 3, 4*)

A suspension of fluorescein in anhydride was refluxed till the colour of the solution disappeared. The reaction mixture was cooled to 70–80 °C, and poured into 10 volumes of ice water with stirring. The precipitate was collected, washed with water and dried. The crude product was recrystallized twice from ethyl alcohol, dried to give colourless solid.

Fluorescein diacetate **1**: yield 86.1%; m.p. 202 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 2.31(s, 6H, CH_3), 6.82(dd, $J = 8.4$, 1.5 Hz, 4H), 7.09(s, 2H), 7.18–7.27(m, 1H), 7.61–7.72(m, 2H), 8.02(dd, $J = 6.6$, 1.5 Hz, 1H).
Fluorescein dipropionate **2**: yield 78.2%; m.p. 161 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 1.26(t, $J = 7.5$ Hz, 6H, CH_3), 2.61(dd, $J = 7.8$, 1.5 Hz, 4H, CH_2), 6.81(dd, $J = 8.7$, 1.8 Hz, 4H), 7.18(s, 2H), 7.17–7.26(m, 1H), 7.61–7.72(m, 2H), 8.04(dd, $J = 6.6$, 1.2 Hz, 1H).
Fluorescein dibutyrate **3**: yield 40.5%; m.p. 123 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 1.040(t, $J = 7.5$ Hz, 6H, CH_3), 1.72–1.85(m, 4H, CH_2), 2.55(t, $J = 7.2$ Hz, 4H, CH_2), 6.81(dd, $J = 8.4$, 1.8 Hz, 4H), 7.21(s, 2H), 7.16–7.19 (m, 1H), 7.60–7.71(m, 2H), 8.03(dd, $J = 6.4$, 1.5 Hz, 1H).
Fluorescein dihexanoate **4**: yield 38.4%; m.p. 100 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 0.90–0.97(m, 6H, CH_3), 1.39–1.44(m, 8H, CH_2), 1.70–1.81(m, 4H, CH_2), 2.56(t, $J = 7.5$ Hz, 4H, CH_2), 6.80(dd, $J = 8.7$, 1.5 Hz, 4H), 7.08(s, 2H), 7.17–7.21(m, 1H), 7.60–7.71(m, 2H), 8.03(dd, $J = 6.6$, 1.2 Hz, 1H).

2.2.2. Fluorescein esters with 8–18 carbon chain (*5, 6, 7, 8, 9, 10*)

A mixture of fatty acid and thionyl chloride was heated with stirring at 50 °C, kept at this temperature for 4 h and the excess thionyl chloride was removed under reduced pressure. Then the obtained acyl chloride was slowly added to fluorescein and pyridine in chloroform. The solution was stirred at 70 °C till the solution had no bright fluorescence and then concentrated. The title compounds were isolated as colourless powder by column chromatography on silica gel (ethyl acetate:petroleum ether = 10:1, $R_f = 0.8$).

Fluorescein dioctanoate **5**: yield 60.1%; m.p. 49 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 0.87–0.92(m, 6H, CH_3), 1.38–1.43(m, 16H, CH_2), 1.70(dd, $J = 7.2$, 1.2 Hz, 4H, CH_2), 2.56(t, $J = 7.5$ Hz, 4H, CH_2), 6.80(dd, $J = 8.4$, 1.5 Hz, 4H), 7.08(s, 2H), 7.17–7.19(m, 1H), 7.60–7.71(m, 2H), 8.03(dd, $J = 6.6$, 1.2 Hz, 1H).
Fluorescein didecanoate **6**: yield 53.7%; m.p. 54 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 0.90(t, $J = 6.6$ Hz, 6H, CH_3), 1.27–1.47(m, 24H, CH_2), 1.72(dd, $J = 7.2$, 1.2 Hz, 4H, CH_2), 2.56(t, $J = 7.5$ Hz, 4H, CH_2), 6.81(dd, $J = 8.7$, 1.8 Hz, 4H), 7.08(s, 2H), 7.17–7.26(m, 1H), 7.63–7.69 (m, 2H), 8.03(dd, $J = 6.6$, 1.2 Hz, 1H).
Fluorescein dilaurate **7**: yield 81.0%; m.p. 83 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 0.87(t, $J = 6.6$ Hz, 6H, CH_3), 1.26–1.43(m, 32H, CH_2), 1.73 (dd, $J = 7.5$, 1.2 Hz, 4H, CH_2), 2.56(t, $J = 7.5$ Hz, 4H, CH_2), 6.81(dd, $J = 8.7$, 1.5 Hz, 4H), 7.19(s, 2H), 7.17–7.26(m, 1H), 7.63–7.69(m, 2H), 8.03(dd, $J = 6.6$, 1.5 Hz, 1H).
Fluorescein dimyristate **8**: yield 84.8%; m.p. 73 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 0.87(t, $J = 6.6$ Hz, 6H, CH_3), 1.26–1.39(m, 40H, CH_2), 1.74(dd, $J = 7.2$, 1.2 Hz, 4H, CH_2), 2.56(t, $J = 7.5$ Hz, 4H, CH_2), 6.80(dd, $J = 8.7$, 1.5 Hz, 4H), 7.09(s, 2H), 7.19(d, $J = 6.9$ Hz, 1H), 7.63–7.69(m, 2H), 8.04(dd, $J = 6.6$, 1.2 Hz, 1H).
Fluorescein dipalmitate **9**: yield 78.6%; m.p. 69 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 0.87(t, $J = 6.6$ Hz, 6H, CH_3), 1.29–1.39(m, 48H, CH_2), 1.74(dd, $J = 7.5$, 1.5 Hz, 4H, CH_2), 2.56(t, $J = 7.5$ Hz, 4H, CH_2), 6.80(dd, $J = 8.7$, 1.5 Hz, 4H), 7.08(s, 2H), 7.19(d, $J = 7.2$ Hz, 1H), 7.61–7.69(m, 2H), 8.02(dd, $J = 6.6$, 1.2 Hz, 1H).
Fluorescein dioctadecanoate **10**: yield 90.4%; m.p. 72 °C; ^{1}H NMR(300 MHz, $CDCl_3$) δ: 0.88(t, $J = 6.6$ Hz, 6H, CH_3), 1.25–1.39(m, 56H, CH_2), 1.73(dd, $J = 7.2$, 1.5 Hz, 4H, CH_2), 2.56(t, $J = 7.5$ Hz, 4H, CH_2), 6.81(dd, $J = 8.7$, 1.5 Hz, 4H), 7.07(s, 2H), 7.18(d, $J = 6.9$ Hz, 1H), 7.63–7.69 (m, 2H), 8.03(dd, $J = 6.6$, 1.2 Hz, 1H). IR (KBr) 2919, 2850, 1768, 1614, 1497, 1243, 1221, 1136, 1105, 1084, 907, 766 cm^{-1}. MS: $m/z = 866.5(M + H)^+$.

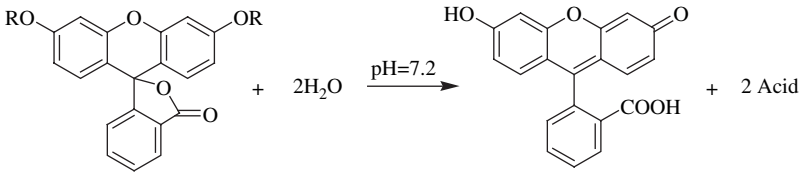
2.3. Methods

2.3.1. The spontaneous hydrolysis of fluorescein 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 492 nm. The rate of spontaneous hydrolysis could be obtained. The process of spontaneous hydrolysis of fluorescein esters is shown in Scheme 1.

2.3.2. The hydrolysis of fluorescein esters in the presence of enzyme

Tris buffer (9.8 ml) of pH 7.2 and 0.1 ml of 10^{-3} M solution of fluorescein esters were placed 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



Fluorescein ester (Nonfluorescent) Fluorescein (Fluorescent)

Scheme 1. The process of spontaneous hydrolysis of fluorescein esters.

was added, and the change in the fluorescence at λ_{ex} of 492 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 process of hydrolysis of fluorescein esters in the presence of lipase is shown in Scheme 2.

3. Results and discussion

Guibault's work has already shown that fluorescein dibutyrate appeared to be more lipophilic than fluorescein. Fluorescein dibutyrate could be used as a fluorometric substrate for the assay of enzyme. However, there is no report about fluorescein esters of long chain to this day. Actually, the longer chain fluorescein esters have the stronger lipophilicity. In order to search for a better fluorogenic substrate for lipase, we synthesized a series of fluorescein esters and evaluated them as fluorogenic substrates to the assay of lipase.

3.1. The synthesis of fluorescein esters

Fluorescein esters with short chain (including **1**, **2**, **3**, **4**) were obtained according to the reported procedure [18]. However, synthesis of fluorescein ester with long chain was not successful according to the above method. Therefore, we tried our best to prepare these compounds following method as in Scheme 3. The acyl chloride was prepared efficiently by treatment of fatty acids with thionyl chloride. The esterisation of fluorescein with the obtained acyl chloride in chloroform yielded the crude products **5**–**10** in good yield. Finally the pure products were obtained by column chromatography. The general method of synthesis of fluorescein esters with long chain has been established.

3.2. The investigation of hydrolytic properties of fluorescein esters

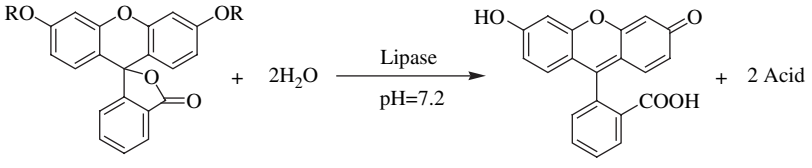
3.2.1. The rate of spontaneous hydrolysis

The spontaneous hydrolysis rate of fluorescein esters in pH = 7.2 Tris buffer was found to be in the following order: fluorescein diacetate **1** > fluorescein dipropionate **2** ≫ fluorescein dibutyrate **3** > fluorescein dihexanoate **4** ≫ fluorescein dioctanoate **5** > fluorescein didecanoate **6** ≫ fluorescein dilaurate **7** > fluorescein dimyristate **8** > fluorescein dipalmitate **9** > fluorescein dioctadecanoate **10**. The spontaneous hydrolysis of fluorescein diacetate and fluorescein dipropionate was quite faster than that of fluorescein dibutyrate. Furthermore, fluorescein esters with longer chain than butyl proceeded more slowly than fluorescein dibutyrate. As can be seen, no hydrolysis was tested in the solution of fluorescein didecanoate in four days. Fluorescein dilaurate may be stored as stock solution for one week with no apparent hydrolysis, while fluorescein dioctadecanoate may be kept for more than 10 days. In a comparison of compounds **1**–**10**, it can be seen that fluorescein esters with the longer chain which were hydrolyzed with more difficultly, were more stable.

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

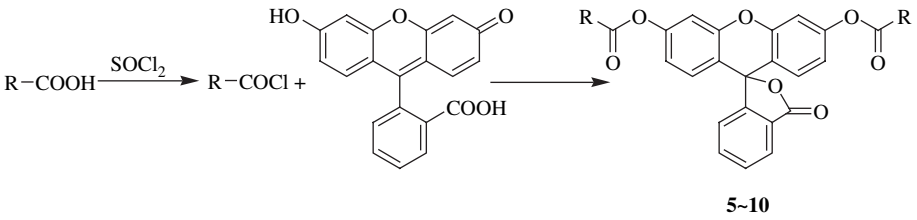
The important application of fluorescein esters is to be used as fluorogenic marker of enzyme. As we all know, fluorescein dibutyrate has been successfully applied to test the activity of lipase. However, fluorescein esters with longer chain have not been reported and not applied to the assay of lipase. Thus we studied the hydrolytic properties of a series of fluorescein esters including longer chain in the presence of lipase.

From Fig. 2, we can see that the order of the rate of enzymatic hydrolysis is not similar to that of the spontaneous hydrolysis. The rate of the enzymatic hydrolysis increases with the chain length of fluorescein esters. It is reasoned probably



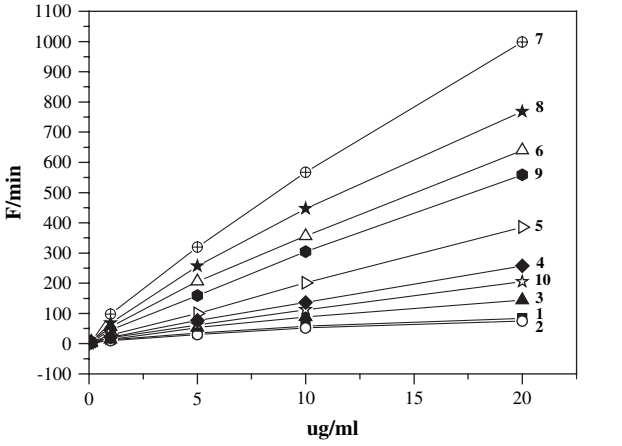
Fluorescein ester (Nonfluorescent) Fluorescein (Fluorescent)

Scheme 2. The process of hydrolysis of fluorescein esters in the presence of lipase.



Scheme 3. Synthesis of fluorescein esters with long chain of acids.

that fluorescein esters with long chain have the stronger lipophilic ability. Because of the lipophilic interaction between long chain ester and the lipophilic region of the lipase, these esters could easily and tightly bind to the enzyme while esters with short chain have less chance to approach the enzyme. Thus the order of the rate is shown as fluorescein dibutyrate **3** < fluorescein dihexanoate **4** < fluorescein dioctanoate **5** < fluorescein didecanoate **6** < fluorescein dilaurate **7**. When the chain length is larger than dodecyl, the enzymatic hydrolysis rate will decrease with increase in chain length of ester. The order of decreasing rate is that fluorescein dilaurate **7** > fluorescein dimyristate **8** > fluorescein dipalmitate **9** > fluorescein dioctadecanoate **10**. It may be explained that the longer chain 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 long chain may have effect on the elasticity of fluorescein ester and the twist of the structure which might perturb the approach of the substrate to the active site in the enzyme. It is surprising that the rate of fluorescein diacetate **1** is higher than that of fluorescein dipropionate **2**. The reason may be that **1** and **2** have so weak lipophilic ability and the rate of enzymatic hydrolysis is negligible compared to the rate of spontaneous hydrolysis. So the order of the rate is similar to that of spontaneous hydrolysis. On the contrary, if the chain of esters is longer than propyl, the enzymatic hydrolysis will play a main role, the rate of spontaneous hydrolysis is very slow enough to negligible.



1 fluorescein diacetate
2 fluorescein dipropionate
3 fluorescein dibutyrate
4 fluorescein dihexanoate
5 fluorescein dioctanoate
6 fluorescein didecanoate
7 fluorescein dilaurate
8 fluorescein dimyristate
9 fluorescein dipalmitate
10 fluorescein dioctadecanoate

Fig. 2. Plot of rate of lipase catalyzed hydrolysis of fluorescein, ΔF/min, vs enzyme concentration in μg/ml.

As shown in Fig. 2, fluorescein dilaurate was the best substrate in this series of fluorescein esters with the highest lipase hydrolysis efficiency, thus we decided to study it in detail in comparison with fluorescein dibutyrate which had been successfully applied to test the activity of lipase. The comparison between fluorescein dibutyrate and fluorescein dilaurate as substrates for lipase is shown in Table 1. From aspects of stability, fluorescein dilaurate was extremely stable in solution which had a negligible rate of spontaneous hydrolysis. This substrate may be stored as stock solution for seven days with no apparent hydrolysis. Thus the blank rate of fluorescein dilaurate was believed to be zero. However, fluorescein dibutyrate hydrolyzed in solution at a fair rate. Of the two substrates tested, fluorescein dilaurate had the better K_m value, and it showed the stronger appetency which came into being between the long chain ester and lipase. In addition, fluorescein dilaurate was more sensitive in the determination of lipase. As little as 0.1 μg/ml of lipase can be detected using this ester, compared to 2.1 μg/ml with fluorescein dibutyrate. Therefore, fluorescein dilaurate is believed as an ideal high affinity substrate for the assay of lipase by fluorescence.

4. Conclusions

A series of fluorescein esters were synthesized, especially long chain fluorescein esters were yielded by the esterisation of fluorescein with the obtained acyl chloride in chloroform in good yield. In addition, the hydrolytic properties of

Table 1
Comparison of various substrates for lipase

Substrate	Fluorescence wavelengths (nm)	Blank[a]	Lowest detectable[b] concentration (μg/ml)	K_m (mM)[c]
Fluorescein dibutyrate	$\lambda_{ex} = 492$; $\lambda_{em} = 515$	0.3	2.1	3.5×10^{-5}
Fluorescein dilaurate	Same	0.0	0.1	5×10^{-6}

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

fluorescein esters were investigated. It was seen that the length of carbon chain has an important effect on the spontaneous and the enzymatic hydrolyses. By comparison, fluorescein dilaurate 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 0.0001 mg/ml of lipase may be determined with a relative accuracy of about 1.5%. These results show that the fluorescein dilaurate may be a very sensitive fluorogenic marker of enzyme and have latent application in future.

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