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Synthesis of monosaccharide derivatives and polymeric prodrugs of 5-fluorouridine *via* two-step enzymatic or chemo-enzymatic highly regioselective strategy

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Abstract

Efficient protocols for the selective synthesis of monosaccharide derivatives and polymeric prodrugs of 5-fluorouridine (5-FUR) have been developed. Firstly, transesterification of 5-FUR and divinyl dicarboxylates ranging from 4 to 10 carbon atoms were performed under the catalysis of *Candida antarctica* lipase acrylic resin in anhydrous THF at 50 °C, respectively. A series of vinyl 5-FUR esters were prepared, with high acylation regioselectivity at 5′-OH. The influences of reaction parameters including enzyme, solvents, molar ratio of substrates, reaction time, the carbon length of acyl donor and reaction temperature were investigated in details. And then, protease-catalyzed highly regioselective acylation of D-glucose, D-mannose and D-galactose with vinyl esters of 5-FUR gave 5-FUR-saccharide derivatives successfully. Moreover, a series of polymeric prodrugs of 5-FUR with the different linker lengths were prepared by the chemo-polymerization of vinyl 5-FUR esters in DMF initiated by azobisisobutyronitrile (AIBN).

Keywords: Enzymatic synthesis; 5-Fluorouridine; Monosaccharide; Polymerization; Prodrugs; Transesterification

1. Introduction

Nucleoside glycoconjugates and macromolecular nucleoside anticancer drugs are two attractive nucleoside derivatives with desirable properties, such as higher bioavailability and antiviral activity, potential targeting function, controlled drug release and reduced adverse effects [1–3]. For example, Hatanaka et al. [3,4] reported that polystyrene containing uridine as a nucleoside showed high inhibition against sugar transferase compared with a monomer. Saccharides play a central part in biological recognition processes as biological recognition signals and functional biomolecules. A significant number of drugs in use today rely on carbohydrates for part of their therapeutic action. It is also particularly effective for the improvement of water-solubility and dissolution behavior of parental drugs. Thus, the exploration of new approaches for the synthesis of nucleoside glycoconjugates and macromolecular nucleoside prodrugs is an interesting subject of study and will result in the discovery of a number of derivatives with potent antitumor and antiviral activities.

5-Fluorouridine (5-FUR), a cytotoxic antitumor agent, is one of the derivatives of 5-fluorouracil (5-FU), which shows higher antitumor activity than 5-FU. But its systemic toxicity has greatly restricted the clinical treatment of cancer [5]. In order to avoid the drawbacks shown by 5-FUR, various modifications have been made [5–9]. For example, the synthesis and antitumor activity of 5′-*O*-acyl-5-fluorouridine and 5′-*O*-unsaturated acyl-5-fluorouridines were investigated [6,7]. Additionally, immunoconjugates were prepared by linking 5-FUR to antiadenocarcinoma monoclonal antibody [5]. And the multiwarhead siderophore-5-fuorouridine conjugates were also reported [8].

Most of the conventional chemical modifications of nucleosides containing multiple hydroxyl groups need process of protection/deprotection and rigorous reaction conditions [10]. At these respects, enzymes as catalysts are advantageous to perform selective modifications of nucleosides with high efficiency and high selectivity. Recently, several reviews about the utility of biocatalyst for the synthesis of bioactive nucleosides or

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fine chemicals have been published [11–13]. In the past few years, several reports about the enzyme catalyzed synthesis of 5-FUR fatty acid ester have been published. For instance, Ozaki et al. prepared 5′-*O*-capryl-5-FUR using *n*-octanoic anhydride and lipase SP 435 in THF with a good yield and regioselectivity (90%), while the acylation of 5-FUR with other acyl donors yielded low regioselectivity and efficiency [14]. Zong and co-workers prepared lipophilic 5-FUR esters from acetate to stearate catalyzed by lipozyme TL IM [15]. However, all these reported 5-FUR fatty acid ester are lipophilic prodrugs with small molecular weight. To the best of our knowledge, there are no reports about the synthesis of the functional polymeric prodrug of 5-FUR and 5-FUR-saccharide conjugates *via* the two-step enzymatic or chemo-enzymatic highly regioselective synthesis strategy.

In this paper, based on our continuous interest in the enzymatic selective synthesis of nucleosides derivatives [16–19], four polymerizable vinyl esters of 5-FUR were prepared by the lipase-catalyzed regioselective acylation of 5-FUR with divinyl dicarboxylates. And the reaction conditions including enzyme sources, solvents, reaction temperature, water content, reaction time and carbon chain length of acylating agent for synthesis of 5-FUR esters were examined in details. Subsequently, the prepared 5-FUR vinyl derivatives were subjected to the second-step enzymatic acylation with monosaccharides or chemo-polymerization with AIBN. Three 5-FUR-saccharide conjugates starting from D-glucose, D-mannose and D-galactose, and four polymeric prodrugs of 5-FUR were successfully prepared.

2. Experimental

2.1. Materials

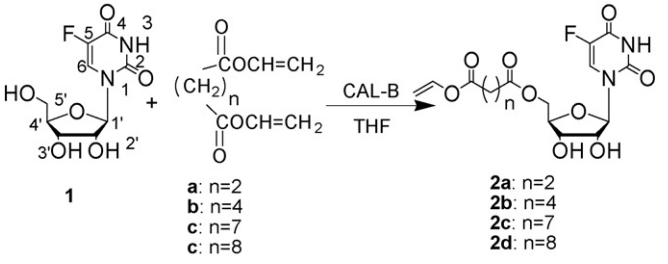
Candida antarctica lipase acrylic resin (E.C. 3.1.1.3, an immobilised preparation of lipase from *Candida antarctica* on macroporous acrylic resin, 10,000 μ/g) and Lipase Type VII from *Candida rugosa* (E.C. 3.1.1.3, powder, 706 units/mg) were purchased from Sigma. Lipozyme® (E.C. 3.1.1.1, an immobilised preparation of lipase from *Mucor miehei*, 42 μ/g), lipase from porcine pancreas (E.C. 3.1.1.3, Type II, powder, 30–90 μ/mg), lipase from *Candida cylindracea* (E.C. 3.1.1.3, powder, 2.8 μ/mg) were purchased from Fluka. Amano Lipase M, from *Mucor javanicus* (E.C. 3.1.1.3, powder, 10 μ/mg) was purchased from Aldrich. Lipase AY30 (E.C. 3.1.1.3, powder) was purchased from Acrõs. Alkaline protease from *Bacillus subtilis* (E.C. 3.4.21.14, a crude preparation of the alkaline serine protease, 100 μ/mg) was purchased from Wuxi Enzyme Co. Ltd. (Wuxi, PR China). Lipase PS "Amano" (E.C. 3.1.1.3, lyophilized powder), D-aminoacylase from *Escherichia coli* (EC 3.5.1.81, lyophilized powder) and acylase "Amano" from Aspergillus oryzae (EC 3.5.1.14, lyophilized powder) were purchased from Amano Enzyme Inc. (Japan). 5-Fluorouridine was purchased from Hefei Lifang Fine Chemicals Co. Ltd. (Hefei, PR China). Solvents were dried over 3 Å molecular sieves for 24 h prior to use. Analytical grade 5-FUR, D-glucose, D-mannose, D-galactose, azobisisobutyronitrile (AIBN) and all other chemicals were used without further purification. Divinyl succinate, divinyl adipate, divinyl azelate and divinyl sebacate were produced and purified as described in the paper [17].

2.2. Analytical methods

Reactions for the synthesis of 5-FUR esters were monitored by TLC with the developing agent consisting of petroleum ether/ethyl acetate (1/5, v/v), while 5-FUR-saccharide derivatives with ethyl acetate/methanol/water (17/8/1, v/v). ^{1}H NMR spectra were obtained on a Bruker DMX-500 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively. Spectra were run in D_2O or DMSO-d_6 with TMS as an internal standard. Infrared spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer. Analytical HPLC was performed using Shimadzu SPD-10Avp with a UV–vis detector at 260 nm and a reversed-phase column Hypersil ODS2 100 Å (5 μ, 250 mm × 4.6 mm). Elution was performed with a mixture of methanol/water at a flow rate of 1 ml/min. GPC was performed with a system equipped with refractive-index detector (Agilent 1100) and Cat. 50058 LiChrogel PS 4000 (10 μm) GPC column. The GPC columns were standardized with narrow dispersity polystyrene in molecular weights ranging from 2000 to 700,000. The mobile phase was DMF at a flow rate of 1.0 ml/min.

2.3. Synthesis of 5′-*O*-vinylsuccinyl-5-FUR (**2a**)

Scheme 1 presented the synthetic route of **2a**: the reaction was initiated by adding 50 mg CAL-B to 25 ml THF containing 1 mmol 5-FUR (**1**) and 4 mmol divinylsuccinate. The suspension was shaken at 200 rpm for 48 h at 50 °C. And the reaction was terminated by filtering off the enzyme. The filtrate was concentrated under reduced pressure. **2a** was purified from the reaction mixture by silica gel column chromatography with an eluant consisting of ethyl acetate/petroleum ether (3:1, v/v). Finally, 5′-*O*-vinylsuccinyl-5-FUR was obtained as a white powder. The yield was 68%. ^{1}H NMR (DMSO-d_6) δ = 11.90 (s, 1H, NH), 7.94 (d, 1H, J = 7.0 Hz, 6-H), 7.20 (dd, 1H, J = 6.3 Hz, J = 13.9 Hz, –O–CH=), 5.72 (t, 1H, 1′-H), 5.20–5.80 (brs, 2H, 2′, 3′-OH), 4.88 (dd, 1H, J = 1.4 Hz, J = 13.9 Hz, =CH_2), 4.65 (dd, 1H, J = 1.4 Hz, J = 6.3 Hz, =CH_2), 4.27 (m, 2H, 5′-H), 4.08 (m, 1H, 4′-H), 3.99–3.93 (m, 2H, 2′, 3′-H), 2.71 (t, 2H, O=C–CH_2), 2.65 (t, 2H, O=C–CH_2). IR (cm^{-1}): 3419 (OH), 3189 (NH), 1746,



Scheme 1. Regioselective enzymatic synthesis of vinyl 5-FUR esters.

1732, 1697, 1681 (C=O), 1664, 1645 (C=C). ^{13}C NMR data was shown in Table 1. ESI-MS (*m/z*): 411 ($M + Na^+$).

2.4. Synthesis of 5′-O-vinyladipoyl-5-FUR (**2b**)

2b was synthesized by the same method as for **2a**. The yield of product was 64%. ^{1}H NMR (DMSO-d$_6$) δ = 11.90 (brs, 1H, NH), 7.94 (d, 1H, J = 7.0 Hz, 6-H), 7.21 (dd, 1H, J = 6.3 Hz, J = 14.0 Hz, –O–CH=), 5.71 (t, 1H, 1′-H), 5.50 (brs, 1H, 2′-OH), 5.29 (brs, 1H, 3′-OH), 4.87 (dd, 1H, J = 1.4 Hz, J = 13.9 Hz, =CH$_2$), 4.64 (dd, 1H, J = 1.4 Hz, J = 13.9 Hz, =CH$_2$), 4.25 (m, 2H, 5′-H), 4.08 (m, 1H, 4′-H), 3.99 (m, 1H, 2′-H), 3.94 (m, 1H, 3′-H), 2.44 (t, 2H, O=C–CH$_2$), 2.42 (t, 2H, O=C–CH$_2$), 1.56 (m, 4H, –CH$_2$–). IR (cm^{-1}): 3435 (OH), 3145 (NH), 1758, 1707 (C=O), 1665, 1647 (C=C). ^{13}C NMR data was listed in Table 1. ESI-MS (*m/z*): 439 ($M + Na^+$).

2.5. Synthesis of 5′-O-vinylnonanedioyl-5-FUR (**2c**)

2c was synthesized by the same method as for **2a**. The yield of product was 43%. ^{1}H NMR (DMSO-d$_6$) δ = 11.90 (brs, 1H, NH), 7.94 (d, 1H, J = 7.0 Hz, 6-H), 7.22 (dd, 1H, J = 6.3 Hz, J = 14.0 Hz, –O–CH=), 5.71 (t, 1H, 1′-H), 5.51 (d, 1H, 2′-OH), 5.29 (d, 1H, 3′-OH), 4.87 (dd, 1H, J = 1.4 Hz, J = 13.9 Hz, =CH$_2$), 4.64 (dd, 1H, J = 1.4 Hz, J = 13.9 Hz, =CH$_2$), 4.24 (m, 2H, 5′-H), 4.08 (m, 1H, 4′-H), 3.98 (m, 1H, 2′-H), 3.93 (m, 1H, 3′-H), 2.41 (t, 2H, O=C–CH$_2$), 2.34 (t, 2H, O=C–CH$_2$), 1.52 (m, 4H, –CH$_2$–), 1.25 (m, 6H, –CH$_2$–); IR (cm^{-1}): 3400 (OH), 3250 (NH), 1749, 1736, 1709, 1686 (C=O), 1659, 1646 (C=C). ^{13}C NMR data was listed in Table 1. ESI-MS (*m/z*): 481 ($M + Na^+$).

2.6. Synthesis of 5′-O-vinylsebacoyl-5-FUR (**2d**)

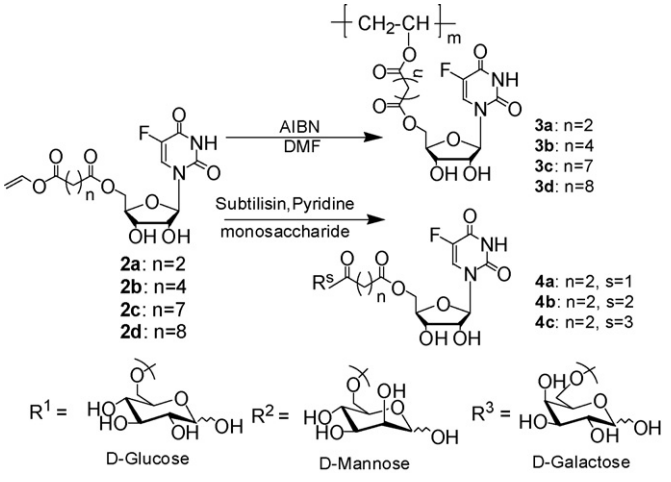
2d was synthesized by the same method as for **2a**. The yield of product was 45%. ^{1}H NMR (DMSO-d$_6$) δ = 11.90 (brs, 1H, NH), 7.93 (d, 1H, J = 7.0 Hz, 6-H), 7.21 (dd, 1H, J = 6.3 Hz, J = 14.0 Hz, –O–CH=), 5.71 (t, 1H, 1′-H), 5.20–5.80 (brs, 2H, 2′, 3′-OH), 4.88 (dd, 1H, J = 1.4 Hz, J = 13.9 Hz, =CH$_2$), 4.63 (dd, 1H, J = 1.4 Hz, J = 13.9 Hz, =CH$_2$), 4.24 (m, 2H, 5′-H), 4.07 (m, 1H, 4′-H), 3.98 (m, 1H, 2′-H), 3.93 (m, 1H, 3′-H), 2.39 (t, 2H, O=C–CH$_2$), 2.32 (t, 2H, O=C–CH$_2$), 1.52 (m, 4H, –CH$_2$–), 1.24 (m, 8H, –CH$_2$–); IR (cm^{-1}): 3441, 3410 (OH), 3189 (NH), 1752, 1735, 1697, 1679 (C=O), 1666, 1648 (C=C). ^{13}C NMR data was listed in Table 1. ESI-MS (*m/z*): 495 ($M + Na^+$).

2.7. Synthesis of poly (5′-O-vinylsuccinyl-5-FUR) (**3a**)

Synthetic route of **3a** was shown in Scheme 2: **2a** (300 mg) was dissolved in DMF (0.3 ml) and AIBN (6 mg) was added. The solution was degassed with nitrogen gas. The polymerization was continued for 4.5 h at 70 °C. The resulting product was precipitated in methanol. The precipitated material was dried under reduced pressure to give 172 mg powder. ^{1}H NMR (DMSO-d$_6$) δ = 11.85 (s, 1H, NH), 7.92 (s, 1H, 6-H), 5.72 (s, 1H, 1′-H), 5.59 (s, 1H, 2′-OH), 5.24 (s, 1H, 3′-OH), 3.36–4.14 (m, 5H, 2′, 3′, 4′, 5′-H), 2.50 (m, 5H, O=C–CH$_2$, backbone of polymer), 1.70 (m, 2H, CH$_2$ of backbone of polymer); IR (cm^{-1}): 3422 (OH), 3180 (NH), 1735, 1690 (C=O); ^{13}C NMR (DMSO-d$_6$) δ = 172.31 (C=O), 157.69, 157.48 (C-4), 149.76 (C-2), 141.56, 139.71 (C-5), 125.36 (C-6), 89.50 (C-1′), 81.60

Table 1
^{13}C NMR spectra data of polymerizable 5-FUR derivatives (**2a**, **2b**, **2c**, **2d**)

Carbon number	1	2a	2b	2c	2d
Base moiety					
2	149.80	149.79	149.74	149.69	149.75
4	157.68	157.68	157.64	157.58	157.66
	157.48	157.47	157.42	157.38	157.50
5	141.38	141.55	141.53	141.48	141.53
	139.56	139.71	139.70	139.63	139.70
6	125.53	125.63	125.53	125.45	125.64
	125.25	125.36	125.26	125.17	125.19
Sugar moiety					
1′	88.77	89.50	89.52	89.45	89.50
2′	69.94	69.92	69.93	69.89	69.93
3′	74.31	73.11	73.21	73.18	73.28
4′	85.22	81.56	81.59	81.55	81.60
5′	60.89	64.38	63.95	63.81	63.83
Acid moiety					
–CH$_2$		28.85	33.47	33.80	33.88
		28.72	33.15	33.46	33.55
			24.20	28.73	29.02
			23.95	28.71	29.00
				28.64	28.88
				24.80	28.81
				24.46	24.90
					24.55
C=O		172.62	173.04	173.20	173.26
		170.10	170.77	170.91	170.97
Vinyl group					
–O–CH=CH$_2$		141.68	141.75	141.73	141.77
		98.80	98.57	98.48	98.51



Scheme 2. Synthesis of polymeric prodrugs of 5-FUR and 5-FUR-saccharide conjugates.

(C-4′), 73.19 (C-3′), 69.98 (C-2′), 64.36 (C-5′), 44.80 (C of backbone of polymer), 28.87 (–CH_2–, and C of backbone of polymer). $M_n = 4.74 \times 10^4$, $M_w/M_n = 1.6$.

2.8. Synthesis of poly (5′-O-vinyladipoyl-5-FUR) (3b)

3b was prepared using the same methodology as for the synthesis of **3a**, and 202 mg **3b** was obtained. ^{1}H NMR (DMSO-d_6) $\delta = 11.88$ (s, 1H, NH), 7.93 (s, 1H, 6-H), 5.72 (s, 1H, 1′-H), 5.49 (s, 1H, 2′-OH), 5.28 (s, 1H, 3′-OH), 3.82–4.25 (m, 5H, 2′, 3′, 4′, 5′-H), 2.73–2.21 (m, 5H, O=C–CH_2, backbone of polymer), 1.56 (m, 6H, –CH_2–, CH_2 of backbone of polymer); IR (cm^{-1}): 3430 (OH), 3175 (NH), 1735, 1705 (C=O); ^{13}C NMR (DMSO-d_6) $\delta = 173.05$ (C=O), 157.63, 157.43 (C-4), 149.73 (C-2), 141.53, 139.70 (C-5), 125.26 (C-6), 89.55 (C-1′), 81.62 (C-4′), 73.21 (C-3′), 69.95 (C-2′), 63.95 (C-5′), 44.79 (C of backbone of polymer), 33.48 (–CH_2–, and C of backbone of polymer), 24.21 (–CH_2–); $M_n = 5.12 \times 10^4$, $M_w/M_n = 1.5$.

2.9. Synthesis of poly (5′-O-vinylnonanedioyl-5-FUR) (3c)

3c was prepared using the same methodology as for the synthesis of **3a**, and 265 mg **3c** was obtained. ^{1}H NMR (DMSO-d_6) $\delta = 11.88$ (s, 1H, NH), 7.91 (s, 1H, 6-H), 5.26–5.79 (m, 3H, 1′-H, 3′-OH, 3′-OH), 3.79–4.23 (m, 5H, 2′, 3′, 4′, 5′-H), 2.73 (m, 1H, CH_2 of backbone of polymer), 1.98–2.41 (m, 4H, O=C–CH_2–), 1.56 (m, 8H, –CH_2–, CH_2 of backbone of polymer); IR (cm^{-1}): 3445 (OH), 3180 (NH), 1730, 1702 (C=O); $M_n = 5.45 \times 10^4$, $M_w/M_n = 1.6$.

2.10. Synthesis of poly (5′-O-vinylsebacoyl-5-FUR) (3d)

3d was prepared using the same methodology as for the synthesis of **3a**, and 270 mg **3d** was obtained. ^{1}H NMR (DMSO-d_6) $\delta = 11.86$ (s, 1H, NH), 7.90 (s, 1H, 6-H), 5.27–5.71 (m, 3H, 1′-H, 3′-OH, 3′-OH), 3.83–4.30 (m, 5H, 2′, 3′, 4′, 5′-H), 2.80 (m, 1H, CH_2 of backbone of polymer), 2.17–2.31 (m, 4H, O=C–CH_2–), 1.23–1.50 (m, 10H, –CH_2–, CH_2 of backbone of polymer); IR (cm^{-1}): 3440 (OH), 3180 (NH), 1742, 1698 (C=O); $M_n = 5.51 \times 10^4$, $M_w/M_n = 1.7$.

2.11. Synthesis of 6-O-[4-(5′-O-5-FUR)-succinyl]-D-glucose (4a)

Scheme 2 presented the synthetic route of **4a**: a mixture of **2a** (200 mg, 0.5 mmol), D-glucose (180 mg, 1 mmol), alkaline protease from B. *subtilis* (100 mg), 3 ml pyridine was shaken at 200 rpm for 5 days at 50 °C. The reactions were terminated by filtering off the enzyme. Formation of the 5-FUR-D-glucose conjugate was confirmed by TLC. The product was isolated by silica gel chromatography with an eluent consisting of ethyl acetate/methanol/water (17:8:1, v/v) to give products (**4a**). The yield was 36%. ^{1}H NMR (D_2O) $\delta = 7.85$ (d, 1H, 6-H), 5.82 (t, 1H, 1′-H), 5.16 (d, 0.4H, $J = 3.7$ Hz, 1α-H), 4.61 (d, 0.6H, $J = 7.9$ Hz, 1β-H of D-glucose), 4.20–4.41 (m, 7H, 2′, 3′, 4′, 5′, 6α-H, 6β-H of D-glucose), 3.90 (m, 0.4H, 5α-H of D-glucose), 3.67 (t, 0.6H, 3α-H of D-glucose), 3.62 (m, 0.6H, 5β-H of D-glucose), 3.38–3.45 (m, 2.1H, 2α, 3β, 4α, 4β-H of D-glucose), 3.21 (t, 0.5H, 2β-H of D-glucose), 2.76 (m, 4H, O=C–CH_2–); ^{13}C NMR (D_2O) $\delta = 174.81$, 174.72 (C=O), 161.48, 161.30 (C-4), 151.82 (C-2), 142.31, 140.44 (C-5), 125.57, 125.29 (C-6), 96.42 (C-1β of D-glucose), 92.55 (C-1α of D-glucose), 90.32 (C-1′), 81.56 (C-4′), 75.99 (C-3β of D-glucose), 74.46 (C-2β of D-glucose), 74.08 (C-3′), 73.78 (C-5β of D-glucose), 73.04 (C-3α of D-glucose), 71.80 (C-2α of D-glucose), 70.06 (C-4α of D-glucose), 70.00 (C-4β of D-glucose), 69.54 (C-5α of D-glucose), 69.20 (C-2′), 64.20 (C-6α, 6β of D-glucose), 63.95 (C-5′), 29.26, 29.20 (–CH_2–); IR (KBr, cm^{-1}): 3429 (OH), 1718 (C=O); ESI-MS (*m/z*): 547 $[M + Na]^+$.

2.12. Synthesis of 6-O-[4-(5′-O-5-FUR)-succinyl]-D-mannose (4b)

4b was synthesized by the same methodology used for **4a**. The yield of product was 48%. ^{1}H NMR (D_2O) $\delta = 7.81$ (d, 1H, 6-H), 5.80 (t, 1H, 1′-H), 5.10 (d, 0.4H, $J = 3.7$, 1α-H), 4.38 (d, 0.5H, 1β-H), 4.16–4.30 (m, 7H, 2′, 3′, 4′, 5′, 6α-H, 6β-H of D-mannose), 3.57–3.87 (m, 4H, other α, β-H of D-mannose). 2.74 (m, 4H, O=C–CH_2–); ^{13}C NMR (D_2O) δ=174.88, 174.72 (C=O), 160.93, 160.74 (C-4), 151.30 (C-2), 142.12, 140.26 (C-5), 125.45, 125.34 (C-6), 94.08 (C-1β of D-mannose), 94.45 (C-1α of D-mannose), 90.38 (C-1′), 81.48 (C-4′), 73.16 (C-3β of D-mannose), 71.40 (C-2β of D-mannose), 73.15 (C-3′), 73.71 (C-5β of D-mannose), 70.37 (C-3α of D-mannose), 70.42 (C-2α of D-mannose), 67.07 (C-4α of D-mannose), 66.86 (C-4β of D-mannose), 70.88 (C-5α of D-mannose), 70.41 (C-2′), 64.32 (C-6α, 6β of D-mannose), 62.86 (C-5′), 29.09, 29.05 (–CH_2–); IR (KBr, cm^{-1}): 3408 (OH), 1717 (C=O); ESI-MS (*m/z*): 547 $[M + Na]^+$.

2.13. Synthesis of 6-O-[4-(5′-O-5-FUR)-succinyl]-D-galactose (4c)

4c was synthesized by the same methodology used for **4a**. The yield of product was 21%. ^{1}H NMR (D_2O) $\delta = 7.66$ (d, 1H, 6-H), 5.81 (m, 1H, 1′-H), 5.20 (d, 0.4H, 1α-H of D-galactose), 4.47 (d, 0.5H, 1β-H), 4.36 (m, 2H, 5′-H), 4.31–4.22 (m, 5H, 2′, 3′, 4′-H, 6-H of D-galactose), 3.49 (t, 0.5H, 2β-H of D-galactose), 3.50–4.00 (m, 3.5H, other α, β-H of D-galactose), 2.69 (m, 4H, O=C–CH_2–); ^{13}C NMR (D_2O) $\delta = 174.79$, 174.72 (C=O), 167.77, 167.59 (C-4), 157.18 (C-2), 143.30, 141.40 (C-5), 123.98, 123.88 (C-6), 96.76 (C-1β of D-galactose), 92.67 (C-1α of D-galactose), 90.18 (C-1′), 81.21 (C-4′), 72.87 (C-3β of D-galactose), 72.05 (C-2β of D-galactose), 74.09 (C-3′), 72.75 (C-5β of D-galactose), 69.24 (C-3α of D-galactose), 68.32 (C-2α of D-galactose), 69.34 (C-4α of D-galactose), 68.51 (C-4β of D-galactose), 69.08 (C-5α of D-galactose), 69.35 (C-2′), 64.48 (C-6α, 6β of D-galactose), 63.97 (C-5′), 29.29, 29.21 (–CH_2–); IR (KBr, cm^{-1}): 3427 (OH), 1716 (C=O); ESI-MS (*m/z*): 547 $[M + Na]^+$.

Table 2
Enzyme screening for synthesis of 5-FUR esters

Entry	Enzyme	Yield[a,b] (%)	5′-Selectivity[b] (%)
1	Control, no enzyme	0	–
2	Alkaline protease from *Bacillus subtilis*	27	99
3	lipase PS "Amano"	18	48
4	Lipase immobilized on acrylic resin from *Candida Antarctica* (CAL-B)	70	99
5	Lipase from *pig pancreas*	7	66
6	Lipase AY 30	17	99
7	Lipase Type VII from *Candida rugosa*	15	98
8	Lipozyme® Immobilized from *Mucor miehei,*	31	96
9	Amano lipase M from *Mucor javanicus*	14	97
10	Lipase from *Candida cylindracea*	18	99
11	PSL-C	13	15
12	Acylase Amano	15	96.1
13	D-Amino acylase	24	84.3

[a] Reaction conditions: 5-FUR (15 mg, 0.057 mmol), divinyladipate (45 mg, 0.228 mmol), 1 ml Acetone, CAL-B and PSL-C 5 mg/ml, other enzyme 10 mg/ml, 50 °C, 200 rpm, 72 h.
[b] Determined by HPLC.

3. Results and discussion

3.1. Enzymatic synthesis of polymerizable vinyl 5-FUR esters

By choosing appropriate enzymes and organic solvents, regioselective acylation at the primary hydroxyl of 5-FUR was realized and four polymerizable 5-FUR prodrugs were synthesized by transesterification of 5-FUR and divinyl dicarboxylates. The reaction route is shown in Scheme 1. The acylation position of 5-FUR was determined by ^{13}C NMR, according to the general strategy described by Yoshimoto et al. [20]: acylation of a hydroxyl group of substrate results in a downfield shift of the peak corresponding to the *O*-acylated carbon and an upfield shift of the peak corresponding to the neighboring carbon. The ^{13}C NMR data of the products **2a**–**2d** was listed in Table 1. For example, the chemical shift of the peak corresponding to C-5′ of product **2a** was downfield from 60.89 to 64.38, and that for C-4′, neighboring carbon, was upfield from 85.22 to 81.56. Therefore, from the analysis above, we can conclude that **2a** is the 5′-OH acylated product. The acylated position of other products **2b**, **2c**, **2d** can be confirmed in the similar way.

3.2. Enzyme screening for synthesis of vinyl 5-FUR esters

Enzymes derived from various sources such as bacteria, yeast and molds show different properties, including stability in organic solvent, activity and specificity. To identify the enzymes with high transesterification activity and regioselectivity for the acylation of 5-FUR in anhydrous organic solvent, thirteen commercial industrial grade lipases and proteases were screened for synthesis of 5-FUR esters. The results are presented and compared in Table 2. The corresponding control reaction in the absence of enzyme did not result in ester formation, proving that the esterification was catalyzed enzymatically. The percent conversion of the substrate catalyzed by the thirteen enzymes ranged in 7–70%. It was found that the reaction catalyzed by CAL-B had the highest yield and regioselectivity.

Many studies reporting enzymatic transesterification have been efficiently performed using immobilized lipase [21–22]. Akoh and Mutua reported that immobilization onto macroscopic surface apparently helps spread the enzyme over a larger surface area so that the enzyme is exposed to a more homogeneous substrate concentration compared the nonimmobilized enzyme [21]. As we can see from Table 2, *Candida antarctica* lipase acrylic resin, an immobilised preparation on macroporous acrylic resin, gave higher conversion than the other nonimmobilized lipase. The moderate activity of immobilized Lipozyme® in the present study might be probably ascribed to the different sources.

3.3. Influence of organic solvents on synthesis of vinyl 5-FUR esters

The performance of biocatalyst is known to be highly sensitive to the solvent. In enzymatic reactions, choosing appropriate reaction medium is of great importance. To optimize reaction conditions for enzymatic transesterification between 5-FUR and divinyl esters, eleven organic solvents were screened. As indicated in Table 3, yields varied with solvents. In the solvent with the log *P* from −0.5 to 0.46, CAL-B showed good activity. However, strong hydrophilic solvents such as DMF and DMSO can strip essential water from protein [23] after the long reaction time, therefore enzyme activities were low in these solvents. Low yields were observed in hydrophobic organic solvents such as isopropyl ether, hexane and toluene because of low solubility of 5-FUR.

3.4. Effect of reaction time and carbon chain length of acylating agent on synthesis of vinyl 5-FUR esters

It is known that in lipase-catalyzed transesterification, reactions take place *via* the formation of an acyl-enzyme intermediate [24]. As a consequence, the nature of the acyl donor has a notable effect on reactivity. As shown in Fig. 1, the reaction had the highest reaction rate when the acyl donor was divinylsuc-

Table 3
Effect of organic solvent on the synthesis of 5-FUR esters

Entry	Solvent	Log *P*	Yield[a,b] (%)	5′-Selectivity[b] (%)
1	DMSO	−1.3	N.D.	–
2	DMF	−1.0	N.D.	–
3	Dioxane	−0.5	56	95
4	Acetone	−0.23	70	99
5[c]	THF	0.46	69	99
6	Pyridine	0.65	1	16
7	*t*-Butanol	0.79	3	98
8	*t*-Amyl alcohol	1.05	N.D.	–
9	Isopropyl ether	1.9	3	97
10	Toluene	2.6	3	98
11	*n*-Hexane	3.9	N.D.	–

N.D.: not detected.
[a] Reaction conditions: 5-fluorouridine (15 mg, 0.057 mmol), divinyladipate (45 mg, 0.228 mmol), CAL-B (5 mg/ml), 1 ml solvent, 50 °C, 72 h, 200 rpm.
[b] Determined by HPLC.
[c] Reaction time: 24 h.

cinyl (C4) with the shortest carbon chain. And the reactions had higher reaction rate when acyl donor was divinyladipate (C6) than that of acyl donor with longer carbon chain (C9 or C10). In addition, after 24 h the yields of 5′-*O*-vinylsuccinyl-5-FUR and 5′-*O*-vinyladipoyl-5-FUR ester reached to the maximum, and had no obvious change when the reaction time was further prolonged.

3.5. Effect of reaction temperature on synthesis of vinyl 5-FUR esters

Temperature has a great effect on the activity, selectivity and stability of a biocatalyst and the thermodynamic equilibrium of a reaction as well. As shown in Fig. 2, within the range from 20 to 50 °C, higher temperature resulted in higher reaction rate. Further rise in temperature beyond 50 °C led to the low reaction rate. This is due to the possible inactivation of the lipase in organic solvents at high temperature. The optimal temperature was 50 °C.

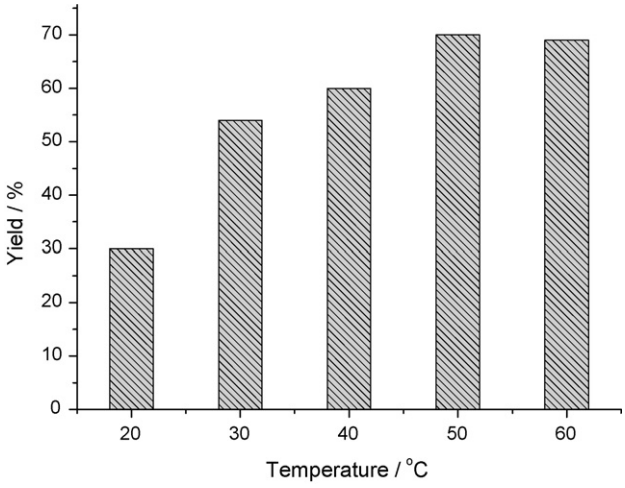


Fig. 2. The effect of temperature on the reaction. Reaction conditions: 5-FUR (15 mg, 0.057 mmol), divinyladipate (0.228 mmol), CAL-B (5 mg/ml), THF (1 ml), 200 rpm, 24 h.

3.6. Effect of water content on synthesis of vinyl 5-FUR esters

All enzymes need essentially bound water, and enzymatic activity in organic solvent depends on water content [25]. Optimal water content was required to reach the maximal activity in hydrophobic solvents. Fig. 3 showed the effect of water content in THF on transesterification of 5-FUR and divinyladipate catalyzed by CAL-B. The reaction gives high yield and regioselectivity when the water content of solvent is in the range of 0–0.04%. The optimum water content is about 0.02%. When water content increases, the hydrolysis reaction will occur because water is a competitive reactant acting against the transesterification reaction.

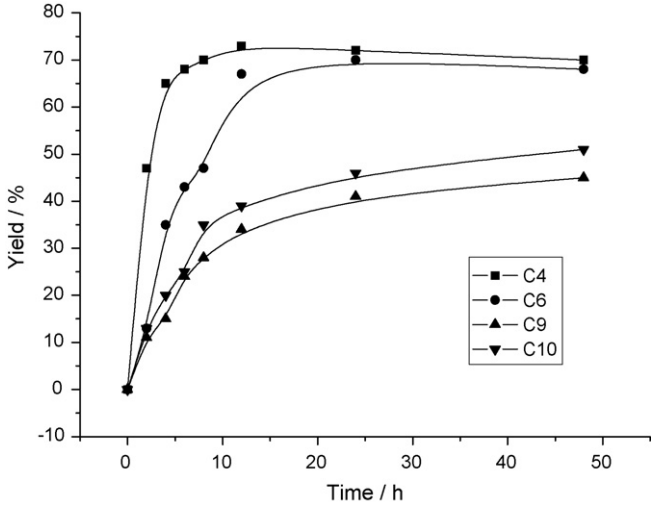


Fig. 1. The effect of reaction time and carbon chain length of acylating agents on the yield of reaction. Reaction conditions: 5-FUR (15 mg, 0.057 mmol), Divinyl dicarboxylate (0.228 mmol), CAL-B 5 mg/ml, THF (1 ml), 50 °C, 200 rpm.

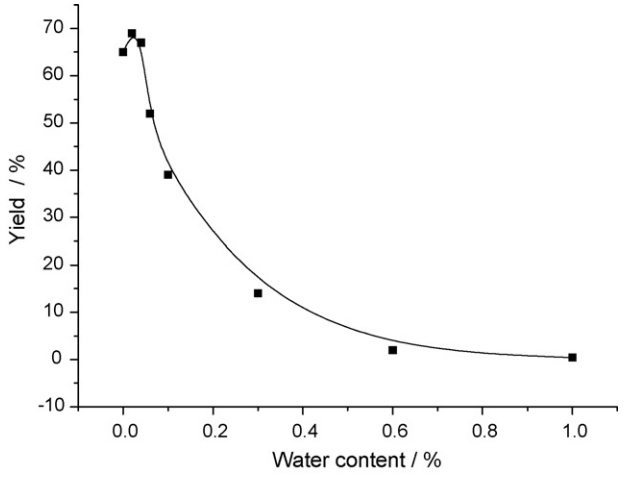


Fig. 3. Effect of water content on synthesis of 5-FUR esters. Reaction conditions: 5-FUR (15 mg, 0.057 mmol), divinyladipate (0.228 mmol), CAL-B (5 mg/ml), THF (1 ml, containing water), 200 rpm, 50 °C, 24 h. The water contents were determined by Karl Fischer method.

3.7. Synthesis of homopolymers of vinyl 5-FUR esters

The vinyl 5-FUR esters (**2a**–**2d**) were polymerized with AIBN in DMF to give polymers having molecular weight of M_n from 4.74×10^4 to 5.51×10^4 and narrow polydispersity with M_w/M_n from1.5 to 1.7. ^{13}C NMR data revealed that the peaks of vinyl group in the monomers were replaced with two peaks at 28–44 ppm which were assigned to the poly (vinyl alcohol) main chain carbons. The backbone of these polymers is poly (vinyl alcohol) which has higher hydrophilicity than polystyrene containing uridine branches reported by Hatanaka et al. [3]. Hence these polymers would be expected to have more effective application in pharmaceutical field.

3.8. Enzymatic regioselective synthesis of 5-FUR-saccharide conjugates

Synthesis of sugar esters in organic solvents is difficult due to the low solubility of sugars. Only a few solvents (e.g., pyridine or DMF) are able to dissolve both highly polar saccharide and the nonpolar fatty acid. According to our previous researches about regioselective acylation of sugars, we selected subtilisin in pyridine as the catalyst for synthesis of 5-FUR-saccharide conjugates, which had previously shown to be an efficient catalyst in the acylation of sugars [18]. The reaction route is shown in Scheme 2. Three 5-FUR derivatives containing a sugar branch (**4a**–**4c**) were synthesized. All compounds were purified by silica gel column chromatography and characterized by IR, ^{1}H NMR and ^{13}C NMR. According to the general strategy described by Yashimoto et al. [20], we concluded that acylation of D-glucose, D-mannose and D-galactose occurred in the primary alcohol position.

4. Conclusion

In the present study, we have described the selective enzymatic synthesis of four polymerizable 5-FUR derivatives with different carbon chain length. CAL-B in THF demonstrated high selectivity towards the primary hydroxyl group of 5-FUR. The influence of enzyme source, organic solvent, reaction time and chain length of acylating agent was systematically investigated. Then, 5-FUR-saccharide conjugates were synthesized from 5-FUR ester and monosaccharide following an enzymatic strategy. Furthermore, the 5-FUR esters were polymerized using AIBN as initiator. The obtained polymeric prodrugs were characterized with IR, NMR and GPC. Finally, the investigation of drug activity of 5-FUR-saccharide and controlled release of polymeric drugs are in progress.

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