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A simple procedure for the synthesis of potential 6-azauridine prodrugs by *Thermomyces lanuginosus* lipase

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ABSTRACT

Highly regioselective acylation of 6-azauridine with fatty acid vinyl esters catalyzed by the lipase from *Thermomyces lanuginosa* for the preparation of its 5′-*O*-acyl derivatives has been successfully performed for the first time. The effects of some crucial factors on the enzymatic palmitoylation of 6-azauridine were further examined. The optimum reaction medium, molar ratio of 6-azauridine to vinyl palmitate, reaction temperature and enzyme dosage were found to be anhydrous acetone, 1:7, 40 °C and 450 U/ml, under which the reaction rate, the substrate conversion and the regioselectivity were 13.3 mM/h, 98.4% and 99.0%, respectively. Moreover, the acyl recognition of the enzyme in the regioselective acylation of 6-azauridine was investigated. The results showed that although 5′-*O*-acyl derivatives of 6-azauridine were exclusively obtained with all the tested acyl donors, the enzymatic activity varied widely with different acyl donors owing to the specific structures of the lipase's active site and the acyl donors.

1. Introduction

Nucleoside chemistry represents an important research area for drug discovery. About 40 nucleoside analogs have been officially approved for clinical use as the current antiviral or antitumour agents over the last four decades [1–3]. 6-Azauridine (AzUrd), as a kind of pharmacological active analog of uridine, has been extensively applied in the treatment of psoriasis, dengue, flaviviruses and herpes viral infections [4,5]. The mechanisms of its actions have been well understood. It interferes with the RNA biosynthesis via orotidylic acid of pyrimidine precursors of nucleic acids after transformation to AzUrd 5′-phosphate in the body [6,7]. However, like other pharmacological nucleoside drugs, AzUrd suffers from low oral bioavailability because of its poor cell membrane penetrability. Moreover, AzUrd exhibits various side effects due to the break of the glycosidic bond by intestinal microorganisms, forming a less active and much more toxic metabolite, 6-azauricil [8,9]. In order to reduce the toxicity of the nucleoside drugs and retain their high bioactivity, various derivatives of AzUrd have been synthesized. It has been reported that its fatty acid ester derivatives act as the potential prodrugs with improved pharmacokinetic profile and therapeutic efficacy than AzUrd itself [8–10]. On the other hand, regioselective acylation of nucleosides with various acyl donors represents an important method of introducing protecting groups for the synthesis of a wide variety of biologically active oligonucleotides [11–13].

Nevertheless, regioselective acylation of nucleosides possessing several hydroxyl groups of similar chemical reactivity, such as AzUrd, is a fundamental challenge to organic chemists. It is usually extremely difficult to selectively acylate the desired hydroxyl of unprotected AzUrd directly via conventional chemical approaches [14,15]. Fortunately, enzymatic acylation of nucleosides is a practicable option for this purpose owing to the high selectivity, simplicity and environmental friendliness [16,17]. For examples, Gotor and co-workers [18,19] reported the enzymatic synthesis of the 3′-, 5′- and 3′, 5′-ester derivatives of some natural nucleosides. Recently, enzymatic regioselective approaches for the acylation of nucleoside analogs such as 1-β-D-arabinofuranosylcytosine [20], 5-fluorouridine [21], FUdR and its analogs [22,23], have been developed by our group. However, only one attempt has been made to prepare the ester derivatives of AzUrd enzymatically, where Zinni et al. obtained its 2′, 3′-di-*O*-acylated derivatives with good yields (80.0–99.0%) through enzymatic regioselective deacylation of the 2′,3′,5′-tri-*O*-acylated derivatives of AzUrd [10].

In addition, 5′-*O*-palmitoyl nucleosides showed improved pharmacological properties compared with the parent drugs [14,24]. Encouraged by this, we selected the palmitoylation of AzUrd as a model reaction to elucidate the characteristics of enzymatic acylation of AzUrd catalyzed by lipozyme TL IM, an inexpensive immobilized lipase from *Thermomyces lanuginose* with extensive industrial application potential in the preparation of functional lipid, biodiesel, detergent and resolution of chiral alcohols [25–27]. Furthermore, the effect of acyl donors on the reaction has been examined (Scheme 1).

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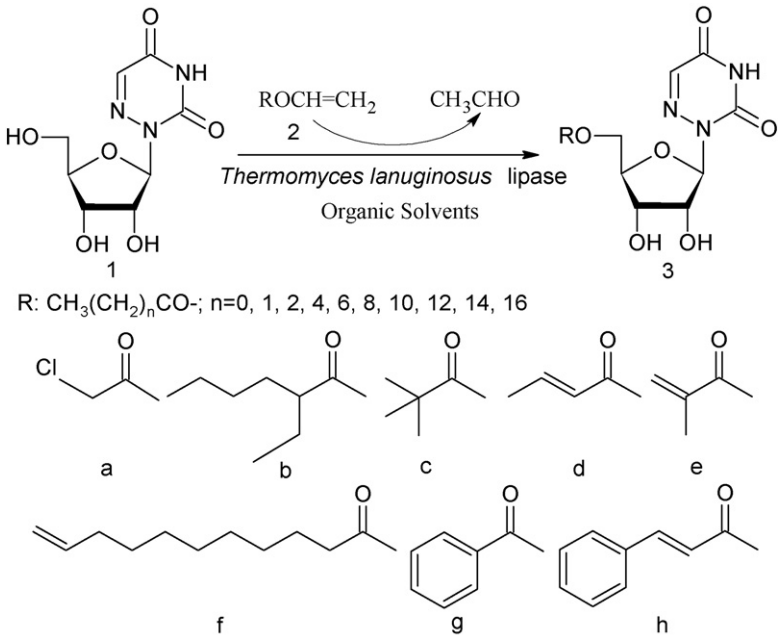

Scheme 1. Lipase-catalyzed regioselective acylation of AzUrd with various acyl donors.

2. Materials and methods

2.1. Biological and chemical materials

Lipozyme TL IM (lipase from *T. lanuginosus*, immobilized on granulated silica; 50,000 U/g) was purchased from Novozymes, Denmark. AzUrd, vinyl propionate, vinyl laurate and vinyl stearate were obtained from Aldrich. Vinyl butyrate, vinyl hexanoate, vinyl octanoate, vinyl decanoate, vinyl myristate, vinyl palmitate, vinyl pivalate, vinyl 2-ethylhexanoate, and vinyl undecenoate were from TCI. Vinyl acetate, vinyl chloroacetate, vinyl methacrylate, vinyl cinnamate, vinyl benzoate, and vinyl crotonate were purchased from Alfa Aesar. All other chemicals were also from commercial sources and of the highest purity available.

2.2. General procedure for enzymatic acylation of AzUrd

In a typical experiment, 2 ml of organic solvent containing AzUrd, a certain amount of lipozyme TL IM and a selected fatty acid vinyl ester was incubated in a 10 ml Erlenmeyer shaking flask capped with a septum in an air-bath shaker with orbital stirring (200 rpm) under predetermined reaction conditions. Aliquots were withdrawn at specified time intervals from the reaction mixture, and then diluted by 50 times with corresponding mobile phase prior to HPLC analysis. Regioselectivity was defined as the ratio of the concentration of the indicated product to that of all the products formed. The initial rate (V_0) and the maximum substrate conversion were calculated from the HPLC data according to the following equations:

$$V_0 = \frac{A_0 - A}{t}, \quad C\ (\%) = \frac{A_0 - A}{A_0} \times 100\%,$$

where A_0 and A stand for the initial substrate concentration and the substrate concentration after reaction for a certain time (t), respectively, and t represents the reaction time within which the substrate concentration decreases linearly with increasing reaction time. The average error for this determination is less than 0.5%. All reported data are averages of experiments performed at least in duplicate. Control reactions were performed by following the above procedure except that no enzyme was added, and no chemical acylation of AzUrd was detectable.

2.3. Control of the initial water activity

The organic solvents were dried with activated 4 Å molecular sieves. The enzyme, the substrates and the organic solvents were put in separate closed containers with different saturated salt solutions for 72 h at 25 °C to fix the initial water activity (a_w). The following salts were used: LiBr ($a_w = 0.07$), LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.53$), NaCl ($a_w = 0.75$), and KCl ($a_w = 0.85$) [28,29].

2.4. HPLC analysis

The reaction mixture was analyzed by RP-HPLC on a 4.6 mm × 250 mm (5 μm) Zorbax SB-C18 column (Agilent Technologies Industries Co., Ltd., USA) using an Agilent G1311A pump and a UV detector at 263 nm.

The mobile phase is a mixture of water and methanol at a flow rate of 1.0 ml/min. The volumetric ratio of water to methanol and the retention time for AzUrd and its 5′-*O*-monoester were 90/10, 2.410 and 10.032 min (acetylation), 80/20, 2.218 and 15.442 min (propionylation), 77/23, 2.363 and 12.160 min (butyrylation), 30/70, 2.277 and 11.910 min (decanoylation), 25/75, 2.183 and 13.287 min (lauroylation), 20/80, 2.229 and 16.169 min (myristoylation), 15/85, 2.208 and 15.979 min (palmitoylation), 12/88, 2.500 and 20.258 min (stearoylation), 85/15, 2.093 and 8.765 min (chloroacetylation), 60/40, 2.361 and 8.624 min (pivaloylation), 35/65, 2.376 and 7.047 min (2-ethylhexanoylation), 30/70, 2.161 and 9.900 min (undecenoylation), 68/32, 2.398 and 21.451 min (benzoylation), 60/40, 2.356 and 22.819 min (cinnamoylation), 70/30, 2.483 and 8.261 min (crotonylation), 70/30, 2.483 and 9.647 min (methacryloylation), respectively. For hexanoylation and octanoylation, the mobile phase was a mixture of water and methanol at a flow rate of 0.9 ml/min, and the volumetric ratio (water/methanol) and retention time for AzUrd and its 5′-*O*-monoester were 60/40, 2.112 and 12.078 min (hexanoylation), 38/62, 2.413 and 8.843 min (octanoylation), respectively.

2.5. Preparative-scale synthesis and structure determination of ester derivatives of AzUrd

In order to structurally characterize the products, the reactions were scaled up and the structure of the ester derivatives was determined by ^{13}C NMR and ^{1}H NMR (Bruker DRX-400 NMR Spectrometer, Bruker Co., Germany) at 100 and 400 MHz, respectively. DMSO-d_6 was used as the solvent.

2.5.1. AzUrd acetate

AzUrd (98 mg, 0.4 mmol) and vinyl acetate (241 mg, 2.8 mmol, 7 equiv.) were dissolved in anhydrous acetone (20 ml) in a 50 ml Erlenmeyer shaking flask, and lipozyme TL IM (300 mg) was added. The vessel was capped with a septum, and then was shaken on the orbital shaker (200 rpm) at 40 °C for 16 h (81% conversion by HPLC). The immobilized enzyme was filtered off after reaching the maximal conversion of 81% and the filtrate was concentrated under vacuum. The residue was purified through flash column chromatography (silica gel, 15 g). Elution with ethyl acetate/petroleum ether (16/5) afforded AzUrd acetate (88 mg, 77%) with 99.0% regioselectivity as shown by the spectral data. ^{1}H NMR: δ 12.22 (1H, br s, H_3), 7.56 (1H, s, H_5), 5.91 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.37 (1H, br s, $OH_{2'}$), 5.19 (1H, br s, $OH_{3'}$), 4.27–4.25 (1H, m, $H_{5'-1}$), 4.19 (1H, m, $H_{2'}$), 4.05–4.04 (1H, m, $H_{3'}$), 3.98–3.90 (2H, m, $H_{4'}$ + $H_{5'-2}$), 1.99 (3H, s, $H_{2''}$). ^{13}C NMR: δ 170.626 ($C_{1''}$), 156.889 (C_4), 148.619 (C_2), 136.898 (C_5), 90.185 ($C_{1'}$), 81.359 ($C_{4'}$), 72.954 ($C_{2'}$), 71.083 ($C_{3'}$), 64.643 ($C_{5'}$), 21.029 ($C_{2''}$).

2.5.2. AzUrd propionate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was vinyl propionate (280 mg, 2.8 mmol, 7 equiv.) instead of vinyl acetate and the maximum conversion of 85% was achieved in 15 h. After separation and purification (16/5, ethyl acetate/petroleum ether), AzUrd propionate (96 mg, 80%) was obtained with 99.0% regioselectivity. ^{1}H NMR: δ 12.22 (1H, br s, H_3), 7.54 (1H, s, H_5), 5.91 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.36 (1H,br s, $OH_{2'}$), 5.19 (1H, d, br s, $OH_{3'}$), 4.28–4.26 (1H, m, $H_{5'-1}$), 4.20 (1H, m, $H_{2'}$), 4.06–4.00 (1H, m, $H_{3'}$), 3.95 (2H, m, $H_{4'}$ + $H_{5'-2}$), 2.29 (2H, q, J = 7.6 Hz, $H_{2''}$), 1.01 (3H, t, J = 7.6 Hz, $H_{3''}$). ^{13}C NMR: δ 173.916 ($C_{1''}$), 156.880 (C_4), 148.598 (C_2), 136.840 (C_5), 90.216 ($C_{1'}$), 81.343 ($C_{4'}$), 72.944 ($C_{2'}$), 70.998 ($C_{3'}$), 64.463 ($C_{5'}$), 27.132 ($C_{2''}$), 9.324 ($C_{3''}$).

2.5.3. AzUrd butyrate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was vinyl butyrate (320 mg, 2.8 mmol, 7 equiv.) instead of vinyl acetate and the maximum conversion of 92% was achieved in 15 h. Purification (5/2, ethyl acetate/petroleum ether) gave AzUrd butyrate (105 mg, 83%) with 99.0% regioselectivity. ^{1}H NMR: δ 12.21 (1H, br s, H_3), 7.51 (1H, s, H_5), 5.90 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.35 (1H, br s, $OH_{2'}$), 5.17 (1H, br s, $OH_{3'}$), 4.28–4.24 (1H, m, $H_{5'-1}$), 4.21–4.20 (1H, m, $H_{2'}$), 4.05–4.04 (1H, m, $H_{3'}$), 3.97–3.92 (2H, m, $H_{4'}$ + $H_{5'-2}$), 2.23 (2H, t, J = 8.0 Hz, $H_{2''}$), 1.52–1.47 (2H, m, $H_{3''}$), 0.83 (3H, t, J = 8.0 Hz, $H_{4''}$). ^{13}C NMR: δ 173.079 ($C_{1''}$), 156.874 (C_4), 148.578 (C_2), 136.782 (C_5), 90.201 ($C_{1'}$), 81.331 ($C_{4'}$), 72.927 ($C_{2'}$), 70.970 ($C_{3'}$), 64.303 ($C_{5'}$), 35.651 ($C_{2''}$), 18.279 ($C_{3''}$) 13.773 ($C_{4''}$).

2.5.4. AzUrd hexanoate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was used as the vinyl hexanoate (398 mg, 2.8 mmol, 7 equiv.) instead of vinyl acetate and the maximum conversion of 90% was achieved in 9 h. After purification (14/5, ethyl acetate/petroleum ether), AzUrd hexanoate (119 mg, 87%) was obtained with 99.0% regioselectivity. ^{1}H NMR: δ 12.22 (1H, br s, H_3), 7.50 (1H, s, H_5), 5.88 (1H, m, $H_{1'}$), 5.36 (1H, br s, $OH_{2'}$), 5.17 (1H, br s, $OH_{3'}$), 4.26–4.23 (1H, m, $H_{5'-1}$), 4.18 (1H, m, $H_{2'}$), 4.03 (1H, m, $H_{3'}$), 3.94–3.91 (2H, m, $H_{4'}$ + $H_{5'-2}$), 2.23 (2H, t, J = 7.2 Hz, $H_{2''}$), 1.46 (2H, m, $H_{3''}$), 1.23–1.17 (4H, m, $H_{4''}$ + $H_{5''}$), 0.80 (3H, t, J = 6.8 Hz, $H_{6''}$). ^{13}C NMR: δ 173.223 ($C_{1''}$), 156.875 (C_4), 148.582 (C_2), 136.794 (C_5), 90.217 ($C_{1'}$), 81.314 ($C_{4'}$), 72.946 ($C_{2'}$), 70.969 ($C_{3'}$), 64.351 ($C_{5'}$), 33.758 ($C_{2''}$), 31.045 ($C_{4''}$), 24.487 ($C_{3''}$), 22.187 ($C_{5''}$), 14.175 ($C_{6''}$).

2.5.5. AzUrd octanoate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was vinyl octanoate (477 mg, 2.8 mmol, 7 equiv.) instead of vinyl acetate and the maximum conversion of 94% was achieved in 5 h. After purification (14/5, ethyl acetate/petroleum ether), AzUrd octanoate (134 mg, 90%) was obtained with 99.0% regioselectivity. ^{1}H NMR: δ 12.22 (1H, br s, H_3), 7.50 (1H, s, H_5), 5.87 (1H, m, $H_{1'}$), 5.35 (1H, br s, $OH_{2'}$), 5.17 (1H, br s, $OH_{3'}$), 4.24–4.22 (1H, m, $H_{5'-1}$), 4.16 (1H, m, $H_{2'}$), 4.01 (1H, m, $H_{3'}$), 3.92–3.90 (2H, m, $H_{4'}$ + $H_{5'-2}$), 2.22 (2H, t, J = 7.0 Hz, $H_{2''}$), 1.45 (2H, m, $H_{3''}$), 1.18 (8H, m, $H_{4''}$ + $H_{5''}$ + $H_{6''}$ + $H_{7''}$), 0.78 (3H, t, J = 8.0 Hz, $H_{8''}$). ^{13}C NMR: δ 173.192 ($C_{1''}$), 156.875 (C_4), 148.576 (C_2), 136.810 (C_5), 90.194 ($C_{1'}$), 81.277 ($C_{4'}$), 72.983 ($C_{2'}$), 70.994 ($C_{3'}$), 64.450 ($C_{5'}$), 33.797 ($C_{2''}$), 31.560 ($C_{6''}$), 27.055 ($C_{5''}$), 24.828 ($C_{4''}$), 22.552 ($C_{3''}$), 22.475 ($C_{7''}$), 14.333 ($C_{8''}$).

2.5.6. AzUrd decanoate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was vinyl decanoate (555 mg, 2.8 mmol, 7 equiv.) rather than vinyl acetate and the maximum conversion of 95% was achieved in 7 h. Purification (3/1, ethyl acetate/petroleum ether) afforded AzUrd decanoate (142 mg, 89%) with 99.0% regioselectivity. ^{1}H NMR: δ 12.25 (1H, br s, H_3), 7.51 (1H, s, H_5), 5.90 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.39 (1H, br s, $OH_{2'}$), 5.21 (1H, br s, $OH_{3'}$), 4.27–4.24 (1H, m, $H_{5'-1}$), 4.19 (1H, m, $H_{2'}$), 4.04 (1H, m, $H_{3'}$), 3.95–3.92 (2H, m, $H_{4'}$ + $H_{5'-2}$), 2.24 (2H, t, J = 8.0 Hz, $H_{2''}$), 1.49–1.45 (2H, m, $H_{3''}$), 1.20 (12H, m, $H_{4''}$ + $H_{5''}$ + $H_{6''}$ + $H_{7''}$ + $H_{8''}$ + $H_{9''}$), 0.82 (3H, t, J = 6.8 Hz, $H_{10''}$). ^{13}C NMR: δ 173.192 ($C_{1''}$), 156.858 (C_4), 148.561 (C_2), 136.764 (C_5), 90.229 ($C_{1'}$), 81.317 ($C_{4'}$), 72.955 ($C_{2'}$), 70.983 ($C_{3'}$), 64.426 ($C_{5'}$), 33.797 ($C_{2''}$), 31.704 ($C_{8''}$), 29.442–28.878 ($C_{4''}$ + $C_{5''}$ + $C_{6''}$ + $C_{7''}$), 24.809 ($C_{3''}$), 22.522 ($C_{9''}$), 14.327 ($C_{10''}$).

2.5.7. AzUrd laurate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that vinyl laurate (634 mg, 2.8 mmol, 7 equiv.) was used as the acyl donor instead of vinyl acetate and the maximum conversion of 95% was achieved in 8 h. After purification (3/1, ethyl acetate/petroleum ether), AzUrd laurate (150 mg, 88%) was obtained with 99.0% regioselectivity. ^{1}H NMR: δ 12.25 (1H, br s, H_3), 7.52 (1H, s, H_5), 5.90 (1H, m, $H_{1'}$), 5.38 (1H, br s, $OH_{2'}$), 5.20 (1H, br s, $OH_{3'}$), 4.27–4.25 (1H, m, $H_{5'-1}$), 4.19 (1H, m, $H_{2'}$), 4.04 (1H, m, $H_{3'}$), 3.97–3.91 (2H, $H_{4'}$ + $H_{5'-2}$), 2.25 (2H, t, J = 8.0 Hz, $H_{2''}$), 1.47 (2H, m, $H_{3''}$), 1.21 (16H, m, $H_{4''}$ + $H_{5''}$ + $H_{6''}$ + $H_{7''}$ + $H_{8''}$ + $H_{9''}$ + $H_{10''}$ + $H_{11''}$), 0.83 (3H, t, J = 6.4 Hz, $H_{12''}$). ^{13}C NMR: δ 173.174 ($C_{1''}$), 156.861 (C_4), 148.566 (C_2), 136.775 (C_5), 90.220 ($C_{1'}$), 81.314 ($C_{4'}$), 72.964 ($C_{2'}$), 70.996 ($C_{3'}$), 64.447 ($C_{5'}$), 33.798 ($C_{2''}$), 31.746 ($C_{10''}$), 29.437–28.881 ($C_{4''}$ + $C_{5''}$ + $C_{6''}$ + $C_{7''}$ + $C_{8''}$ + $C_{9''}$), 24.8142 ($C_{3''}$), 22.540 ($C_{11''}$), 14.340 ($C_{12''}$).

2.5.8. AzUrd myristate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was vinyl myristate (712 mg, 2.8 mmol, 7 equiv.) rather than vinyl acetate and the maximum conversion of 92% was achieved in 9 h. After purification (2/1, ethyl acetate/petroleum ether), AzUrd myristate (159 mg, 87%) was obtained with 99.0% regioselectivity. ^{1}H NMR: δ 12.18 (1H, br s, H_3), 7.52 (1H, s, H_5), 5.91 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.31–5.29 (2H, m, $OH_{2'}$ and $OH_{3'}$), 4.27–4.25 (1H, m, $H_{5'-1}$), 4.21–4.19 (1H, m, $H_{2'}$), 4.05–4.03 (1H, m, $H_{3'}$), 3.95–3.93 (2H, m, $H_{4'}$ + $H_{5'-2}$),

2.26 (2H, t, J = 7.4 Hz, $H_{2''}$), 1.48–1.46 (2H, m, $H_{3''}$), 1.22 (20H, m, $H_{4''} + H_{5''} + H_{6''} + H_{7''} + H_{8''} + H_{9''} + H_{10''} + H_{11''} + H_{12''} + H_{13''}$), 0.84 (3H, t, J = 6.6 Hz, $H_{14''}$). ^{13}C NMR: δ 173.139 ($C_{1''}$), 156.929 (C_4), 148.632 (C_2), 136.794 (C_5), 90.261 ($C_{1'}$), 81.316 ($C_{4'}$), 72.981 ($C_{2'}$), 71.029 ($C_{3'}$), 64.483 ($C_{5'}$), 33.806 ($C_{2''}$), 31.759 ($C_{12''}$), 29.480–28.901 ($C_{4''} + C_{5''} + C_{6''} + C_{7''} + C_{8''} + C_{9''} + C_{10''} + C_{11''}$), 24.827 ($C_{3''}$), 22.546 ($C_{13''}$), 14.350 ($C_{14''}$).

2.5.9. AzUrd palmitate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was vinyl palmitate (791 mg, 2.8 mmol, 7 equiv.) instead of vinyl acetate and the maximum conversion of 92% was achieved in 11 h. After purification (2/1, ethyl acetate/petroleum ether), AzUrd palmitate (159 mg, 82%) was obtained with 99.0% regioselectivity. ^{1}H NMR: δ 12.25 (1H, br s, H_3), 7.55 (1H, s, H_5), 5.90 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.39 (1H, br s, $OH_{2'}$), 5.21 (1H, br s, $OH_{3'}$), 4.28–4.26 (1H, m, $H_{5'-1}$), 4.19 (1H, m, $H_{2'}$), 4.05 (1H, m, $H_{3'}$), 3.98–3.92 (2H, m, $H_{4'} + H_{5'-2}$), 2.27 (2H, t, J = 8.0 Hz, $H_{2''}$), 1.50–1.47 (2H, m, $H_{3''}$), 1.23 (24H, m, $H_{4''} + H_{5''} + H_{6''} + H_{7''} + H_{8''} + H_{9''} + H_{10''} + H_{11''} + H_{12''} + H_{13''} + H_{14''} + H_{15''}$), 0.85 (3H, t, J = 8.0 Hz, $H_{16''}$). ^{13}C NMR: δ 173.137 ($C_{1''}$), 156.871 (C_4), 148.579 (C_2), 136.826 (C_5), 90.202 ($C_{1'}$), 81.282 ($C_{4'}$), 72.988 ($C_{2'}$), 71.020 ($C_{3'}$), 64.505 ($C_{5'}$), 31.778 ($C_{2''}$), 29.534–28.921 ($C_{4''} + C_{5''} + C_{6''} + C_{7''} + C_{8''} + C_{9''} + C_{10''} + C_{11''} + C_{12''} + C_{13''} + C_{14''}$), 24.838 ($C_{3''}$), 22.569 ($C_{15''}$), 14.376 ($C_{16''}$).

2.5.10. AzUrd stearate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that vinyl stearate (869 mg, 2.8 mmol, 7 equiv.) was employed as the acyl donor instead of vinyl acetate and the maximum conversion of 91% was achieved in 13 h. After purification (7/5, ethyl acetate/petroleum ether), AzUrd stearate (172 mg, 84%) was obtained with 99.0% regioselectivity.^{1}H NMR: δ 12.24 (1H, br s, H_3), 7.51 (1H, s, H_5), 5.91 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.37 (1H, br s, $OH_{2'}$), 5.18 (1H, br s, $OH_{3'}$), 4.27–4.24 (1H, m, $H_{5'-1}$), 4.20–4.19 (1H, m, $H_{2'}$), 4.05–4.03 (1H, m, $H_{3'}$), 3.95–3.92 (2H, m, $H_{4'} + H_{5'-2}$), 2.24 (2H, t, J = 8.0 Hz, $H_{2''}$), 1.47–1.46 (2H, m, $H_{3''}$), 1.23 (28H, m, $H_{4''} + H_{5''} + H_{6''} + H_{7''} + H_{8''} + H_{9''} + H_{10''} + H_{11''} + H_{12''} + H_{13''} + H_{14''} + H_{15''} + H_{16''} + H_{17''}$), 0.83 (3H, t, J = 6.6 Hz, $H_{18''}$). ^{13}C NMR: δ 173.077 ($C_{1''}$), 156.843 (C_4), 148.558 (C_2), 136.769 (C_5), 90.262 ($C_{1'}$), 81.325 ($C_{4'}$), 72.981 ($C_{2'}$), 71.049 ($C_{3'}$), 64.531 ($C_{5'}$), 33.796 ($C_{2''}$), 31.784 ($C_{16''}$), 29.540–28.947 ($C_{4''} + C_{5''} + C_{6''} + C_{7''} + C_{8''} + C_{9''} + C_{10''} + C_{11''} + C_{12''} + C_{13''} + C_{14''} + C_{15''}$), 24.830 ($C_{3''}$), 22.560 ($C_{17''}$), 14.303 ($C_{18''}$).

2.5.11. AzUrd chloroacetate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was vinyl chloroacetate (338 mg, 2.8 mmol, 7 equiv.) rather than vinyl acetate and the maximum conversion of 96% was achieved in 7 h. After purification (2/1, ethyl acetate/petroleum ether), AzUrd chloroacetate (117 mg, 91%) was obtained with 99.0% regioselectivity. ^{1}H NMR: δ 12.19 (1H, br s, H_3), 7.52 (1H, s, H_5), 5.89 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.36 (1H, br s, $OH_{2'}$), 5.20 (1H, br s, $OH_{3'}$), 4.39–4.36 (3H, m, $H_{5'-1}$), 4.33 (1H, s, $H_{2''}$), 4.17 (1H, m, $H_{2'}$), 4.08–4.01 (1H, m, $H_{3'}$), 3.98–3.96 (2H, m, $H_{4'} + H_{5'-2}$). ^{13}C NMR: δ 167.640 ($C_{1''}$), 156.895 (C_4), 148.591 (C_2), 136.955 (C_5), 90.311 ($C_{1'}$), 81.123 ($C_{4'}$), 73.028 ($C_{2'}$), 70.883 ($C_{3'}$), 65.956 ($C_{5'}$), 21.029 ($C_{2''}$).

2.5.12. AzUrd pivalate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor vinyl acetate was replaced by vinyl pivalate (513 mg, 4.0 mmol, 10 equiv.) and the maximum conversion of 18% was achieved in 45 h. Purification (2/1, ethyl acetate/petroleum ether) gave AzUrd pivalate (21 mg, 16%) with 99.0% regioselectivity. ^{1}H NMR: δ 12.24 (1H, br s, H_3), 7.51 (1H, s, H_5), 5.90 (1H, m, $H_{1'}$), 5.37 (1H, br s, $OH_{2'}$), 5.19 (1H, br s, $OH_{3'}$), 4.22–4.20 (1H, m, $H_{5'-1}$), 4.20–4.18 (1H, m, $H_{2'}$), 4.06–4.05 (1H, m, $H_{3'}$), 3.97–3.95 (2H, m, $H_{4'} + H_{5'-2}$), 1.11 (9H, s, $H_{3''}$). ^{13}C NMR: δ 177.926 ($C_{1''}$), 156.852 (C_4), 148.605 (C_2), 136.800 (C_5), 90.063 ($C_{1'}$), 81.283 ($C_{4'}$), 72.856 ($C_{2'}$), 70.764 ($C_{3'}$), 64.395 ($C_{5'}$), 38.687 ($C_{2''}$), 27.388 ($C_{3''}$), 27.358 ($C_{3''}$), 27.267 ($C_{3''}$).

2.5.13. AzUrd 2-ethylhexanoate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that vinyl 2-ethylhexanoate (681 mg, 4.0 mmol, 10 equiv.) was used as the acyl donor and the maximum conversion of 11% was obtained in 45 h. Purification (7/5, ethyl acetate/petroleum ether) afforded AzUrd 2-ethylhexanoate (15 mg, 10%) with 99.0% regioselectivity. ^{1}H NMR: δ 12.25 (1H, br s, H_3), 7.53 (1H, s, H_5), 5.90 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.39 (1H, br s, $OH_{2'}$), 5.21 (1H, br s, $OH_{3'}$), 4.27–4.25 (1H, m, $H_{5'-1}$), 4.22 (1H, m, $H_{2'}$), 4.05 (1H, m, $H_{3'}$), 3.98–3.95 (2H, m, $H_{4'} + H_{5'-2}$), 2.24–2.17 (1H, m, $H_{2''}$), 1.47–1.45 (4H, m, $H_{3''} + H_{7''}$), 1.22–1.14 (4H, m, $H_{4''} + H_{5''}$), 0.83–0.77 (6H, m, $H_{6''} + H_{8''}$). ^{13}C NMR: δ 175.089 ($C_{1''}$), 156.363 (C_4), 148.069 (C_2), 136.241 (C_5), 89.728 ($C_{1'}$), 80.800 ($C_{4'}$), 72.419 ($C_{2'}$), 70.414 ($C_{3'}$), 63.807 ($C_{5'}$), 46.330 ($C_{2''}$), 30.948 ($C_{3''}$), 28.859 ($C_{4''}$), 24.749 ($C_{7''}$), 21.936 ($C_{5''}$), 13.662 ($C_{6''}$), 11.455 ($C_{8''}$).

2.5.14. AzUrd crotonate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that vinyl crotonate (449 mg, 4.0 mmol, 10 equiv.) was used as the acyl donor and the maximum conversion of 46% was obtained in 60 h. Purification (2/1, ethyl acetate/petroleum ether) gave AzUrd crotonate (54 mg, 43%) with 99.0% regioselectivity. ^{1}H NMR: δ 12.22 (1H, br s, H_3), 7.53 (1H, s, H_5), 6.91–6.84 (1H, m, $H_{3''}$), 5.90–5.83 (2H, m, $H_{1''} + H_{2''}$), 5.37 (1H, br s, $OH_{2'}$), 5.20 (1H, br s, $OH_{3'}$), 4.33–4.30 (1H, m, $H_{5'-1}$), 4.20 (1H, m, $H_{2'}$), 4.08–4.02 (1H, m, $H_{3'}$), 4.01–3.96 (2H, m, $H_{4'} + H_{5'-2}$), 1.84 (3H, d, J = 6.8 Hz, $H_{4''}$). ^{13}C NMR: δ 165.253 ($C_{1''}$), 156.369 (C_4), 148.083 (C_2), 145.545 ($C_{3''}$), 136.322 (C_5), 121.912 ($C_{2''}$), 89.734 ($C_{1'}$), 80.881 ($C_{4'}$), 72.474 ($C_{2'}$), 70.478 ($C_{3'}$), 63.734 ($C_{5'}$), 17.601 ($C_{4''}$).

2.5.15. AzUrd methacrylate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor was vinyl methacrylate (449 mg, 4.0 mmol, 10 equiv.) rather than vinyl acetate and the maximum conversion of 67% was achieved in 50 h. After purification (2/1, ethyl acetate/petroleum ether), AzUrd methacrylate (75 mg, 60%) was obtained with 99.0% regioselectivity. ^{1}H NMR: δ 12.20 (1H, br s, H_3), 7.50 (1H, s, H_5), 6.02 (1H, m, $H_{3''-1}$), 5.90 (1H, m, $H_{1'}$), 5.67 (1H, m, $H_{3''-2}$), 5.39 (1H, br s, $OH_{2'}$), 5.22 (1H, br s, $OH_{3'}$), 4.33–4.30 (1H, m, $H_{5'-1}$), 4.21 (1H, m, $H_{2'}$), 4.09 (1H, m, $H_{3'}$), 4.06–4.01 (2H, m, $H_{4'} + H_{5'-2}$), 1.85 (3H, s, $H_{4''}$). ^{13}C NMR: δ 166.305 ($C_{1''}$), 156.359 (C_4), 148.087 (C_2), 136.327 (C_5), 135.582 ($C_{2''}$), 125.999 ($C_{3''}$), 89.656 ($C_{1'}$), 80.746 ($C_{4'}$), 72.452 ($C_{2'}$), 70.309 ($C_{3'}$), 64.137 ($C_{5'}$), 17.856 ($C_{4''}$).

2.5.16. AzUrd undecenoate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that the acyl donor vinyl acetate was replaced with vinyl undecenoate (589 mg, 2.8 mmol, 7 equiv.). The maximum conversion of 95% was achieved in 7 h and the following separation and purification (9/5, ethyl acetate/petroleum ether) gave AzUrd undecenoate (148 mg, 90%) with 99.0% regioselectivity. ^{1}H NMR: δ 12.21 (1H, br s, H_3), 7.50 (1H, s, H_5), 5.88 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.79–5.69 (1H, m, $H_{10''}$), 5.34 (1H, br s, $OH_{2'}$), 5.16 (1H, br s, $OH_{3'}$), 4.96–4.87 (1H, m, $H_{11''}$), 4.25–4.23 (1H, m, $H_{5'-1}$), 4.17–4.16 (1H, m, $H_{2'}$), 4.03–4.01 (1H, m, $H_{3'}$), 3.93–3.90 (2H, m, $H_{4'} + H_{5'-2}$), 2.23 (2H, t, J = 8.0 Hz, $H_{2''}$), 1.96–1.95 (2H, m, $H_{9''}$), 1.47–1.44 (2H, m, $H_{3''}$), 1.30–1.19 (16H, m, $H_{4''} + H_{5''} + H_{6''} + H_{7''} + H_{8''}$). ^{13}C NMR: δ 173.155 ($C_{1''}$), 156.861 (C_4), 148.578 (C_2), 139.257 ($C_{10''}$), 136.807 (C_5), 115.010 ($C_{11''}$), 90.252 ($C_{1'}$), 81.334 ($C_{4'}$), 72.90 ($C_{2'}$),

71.029 ($C_{3'}$), 64.469 ($C_{5'}$), 33.814 ($C_{9''}$), 33.619, ($C_{2''}$), 29.095–28.705 ($C_{4''} + C_{5''} + C_{6''} + C_{7''} + C_{8''}$), 24.823 ($C_{3''}$).

2.5.17. AzUrd benzoate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except the vinyl benzoate (593 mg, 4.0 mmol, 10 equiv.) was used as the acyl donor instead of vinyl acetate. The maximum conversion of 35% was obtained in 72 h and the following separation and purification (9/5, ethyl acetate/petroleum ether) gave AzUrd benzoate (45 mg, 32%) with 99.0% regioselectivity. ^{1}H NMR: δ 12.22 (1H, br s, H_3), 7.95 (2H, d, J = 8.0 Hz, $H_{3''} + H_{7''}$), 7.67–7.63 (1H, m, $H_{5''}$), 7.54–7.50 (2H, m, $H_{4''} + H_{6''}$), 7.38 (1H, s, H_5), 5.95 (1H, d, J = 4.0 Hz, $H_{1'}$), 5.42 (1H, br s, $OH_{2'}$), 5.28 (1H, br s, $OH_{3'}$), 4.54–4.50 (1H, m, $H_{5'-1}$), 4.30–4.29 (1H, m, $H_{2'}$), 4.27–4.26 (1H, m, $H_{3'}$), 4.14–4.12 (2H, m, $H_{4'} + H_{5'-2}$). ^{13}C NMR: δ 165.488 ($C_{1''}$), 156.292 (C_4), 148.082 (C_2), 136.264 (C_5), 133.349 ($C_{5''}$), 129.460 ($C_{3''} + C_{7''}$), 129.139 ($C_{2'''}$), 128.684 ($C_{4''} + C_{6''}$), 89.700 ($C_{1'}$), 80.846 ($C_{4'}$), 72.555 ($C_{2'}$), 70.350 ($C_{3'}$), 64.207 ($C_{5'}$).

2.5.18. AzUrd cinnamate

The scale-up synthesis procedure was the same as described in Section 2.5.1 except that vinyl cinnamate (697 mg, 4.0 mmol, 10 equiv.) was employed as the acyl donor instead of vinyl acetate. The maximum conversion of 81% was obtained in 72 h and the following separation and purification (2/1, ethyl acetate/petroleum ether) gave *AzUrd* cinnamate (111 mg, 74%) with 99.0% regioselectivity. ^{1}H NMR: δ 12.24 (1H, br s, H_3), 7.72–7.66 (2H, m, $H_{3''} + H_{5''} + H_{9''}$), 7.61 (1H, m $H_{7''}$), 7.50 (1H, s, H_5), 7.41–7.39 (2H, m, $H_{6''} + H_{8''}$), 6.58 (1H, d, J = 16 Hz, $H_{2''}$), 5.99–5.95 (1H, m, $H_{1'}$), 5.44 (1H, br s, $OH_{2'}$), 5.27 (1H, br s, $OH_{3'}$), 4.44–4.41 (1H, m, $H_{5'-1}$), 4.26 (1H, m, $H_{2'}$), 4.14–4.08 (3H, m, $H_{3'} + H_{4'} + H_{5'-2}$). ^{13}C NMR: δ 165.932 ($C_{1''}$), 156.344 (C_4), 148.107 (C_2), 144.764 ($C_{3''}$), 136.313 (C_5), 133.846 ($C_{7''}$), 130.493 ($C_{6''} + C_{8''}$), 128.893 ($C_{5''} + C_{9''}$), 128.283 ($C_{4''}$) 117.649 ($C_{2''}$), 89.823 ($C_{1'}$), 80.976 ($C_{4'}$), 72.513 ($C_{2'}$), 70.629 ($C_{3'}$), 64.337 ($C_{5'}$).

3. Results and discussion

3.1. Effect of reaction medium

It is well known that through solvent engineering, one can modulate enzyme activity, tailor enzyme selectivity, and alter enzyme stability. Although hydrophobic solvents are usually superior to hydrophilic ones as enzymatic reaction media [30,31], the poor solubility of AzUrd in hydrophobic solvents limits their application in enzymatic acylation of AzUrd [20,32].

As shown in Table 1, no reaction occurred in pyridine, DMF and DMSO due to the inactivation of the enzyme [33,34], although the solubility of AzUrd was high in these solvents. This is in agreement with previous reports [20,21,23]. Moderate or good conversion was observed with enzymatic acylation of AzUrd in dioxane (53.7%) or THF (77.5%). The best result was achieved with acetone being the reaction medium, affording a conversion of 88.2% and a regioselectivity of 99.0%. Obviously, there was no correlation between the log P of the solvents and the catalytic activity of the lipase in the solvents. Nevertheless, the substrate solubility seems to exert a negative influence on the enzyme activity in other solvents tested, which could be ascribed to the lower ground state energy of AzUrd in its better solvents. However, low reaction rate and conversion were observed when *tert*-butanol was used as the solvent due to the severe mass transfer limitation caused by the high viscosity of *tert*-butanol [35]. It was also worth-noting that the reaction medium showed little effect on the regioselectivity of the reaction, with 5′-*O*-palmitoyl AzUrd being the only product.

Table 2
Effect of water activity on lipozyme TL IM-catalyzed acylation of AzUrd with vinyl palmitate.

a_w	V_0 (mM/h)	Time (h)[b]	C (%)[c]	Regioselectivity
~0[a]	9.2	9.0	88.2	99.0
0.07	0.9	5.0	20.9	99.0
0.11	0.5	4.0	12.5	99.0
0.23	0.2	3.0	5.3	99.0
0.33	0.1	3.0	3.9	99.0
0.55	<0.1	2.0	2.0	99.0
0.75	<0.1	2.0	1.4	99.0
0.85	<0.1	2.0	1.1	99.0

The reactions were carried out at 35 °C, 200 rpm and various a_w values by adding 10 mM AzUrd, 50 mM vinyl palmitate, 750 U lipozyme TL IM into 2 ml acetone.
[a] Enzyme used as received, anhydrous acetone.
[b] Reaction time when the maximum conversion was achieved.
[c] Maximum substrate conversion.

3.2. Effect of water activity (a_w)

The thermodynamic water activity (a_w) is one of the crucial parameters affecting nonaqueous enzymatic processes [31,36,37]. Understandably, water plays an important role in maintaining the catalytic activity of an enzyme. On the other hand, with the increase in water activity of the reaction mixture, an enzyme will become more liable to thermal unfolding. In addition, the hydrolysis of the acylated products and the acyl donors could be enhanced by water. As a result, there exists an optimal water activity (a_w) for the enzyme to give the best catalytic performance. As can be seen in Table 2, the highest conversion of AzUrd was achieved in anhydrous acetone, suggesting that the water in the commercial enzyme preparation was enough to retain a high enzymatic activity. A higher a_w value led to a drastic decrease in the initial reaction rate and AzUrd conversion. The acceleration of the competitive hydrolysis reactions mentioned above contributed to this. Also, the enzyme displayed an absolute regioselectivity within the scope examined.

Table 1
Effect of organic solvents on lipozyme TL IM-catalyzed regioselective acylation of AzUrd with vinyl palmitate[a].

Solvent	log P	V_0 (mM/h)	Time (h)[b]	C (%)[c]	Regioselectivity (%)	Solubility of AzUrd (mM)[d]
Acetone	−0.23	9.2	9.0	88.2	99.0	17.4
THF	0.49	4.7	13.5	77.5	99.0	39.2
Dioxane	−1.10	2.8	14.0	53.7	99.0	70.8
tert-Butanol	0.60	1.3	15.0	35.5	99.0	15.3
Pyridine	0.71	n.d.	14.0	n.d.	n.d.	>1205.8
DMF	−1.00	n.d.	14.0	n.d.	n.d.	>1529.5
DMSO	−1.30	n.d.	14.0	n.d.	n.d.	>1713.2

[a] The reactions were carried out at 35 °C, 200 rpm by adding 10 mM AzUrd, 50 mM vinyl palmitate, 750 U lipozyme TL IM into 2 ml anhydrous organic solvents.
[b] Reaction time when the maximum conversion was achieved.
[c] Maximum substrate conversion.
[d] The solubility of AzUrd was determined by HPLC analyses of the saturated solutions at 30 °C.

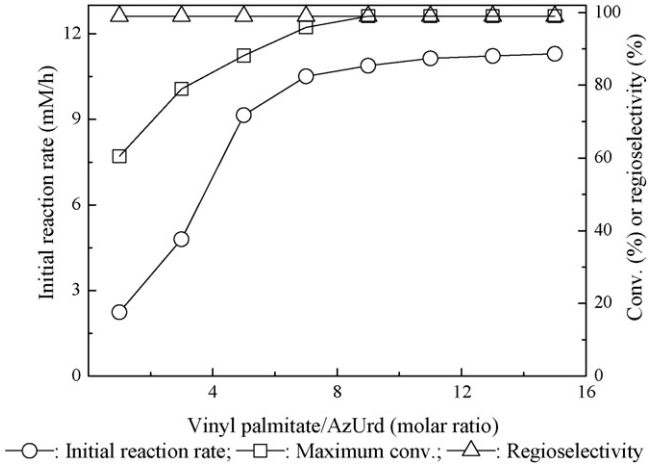

Fig. 1. Effect of the molar ratio of vinyl palmitate to AzUrd on lipozyme TL IM-catalyzed acylation of AzUrd. The reactions were carried out at 35 °C, 200 rpm by adding 10 mM AzUrd, 750 U lipozyme TL IM, various amount of vinyl palmitate into 2 ml anhydrous acetone. Reaction time (the molar ratio of vinyl palmitate to AzUrd): 6.5 h (1:1), 8.0 h (3:1), 9.0 h (5:1), 9.5 h (7:1), 9.5 h (9:1), 9.0 h (11:1), 8.5 h (13:1), 8.5 h (15:1).

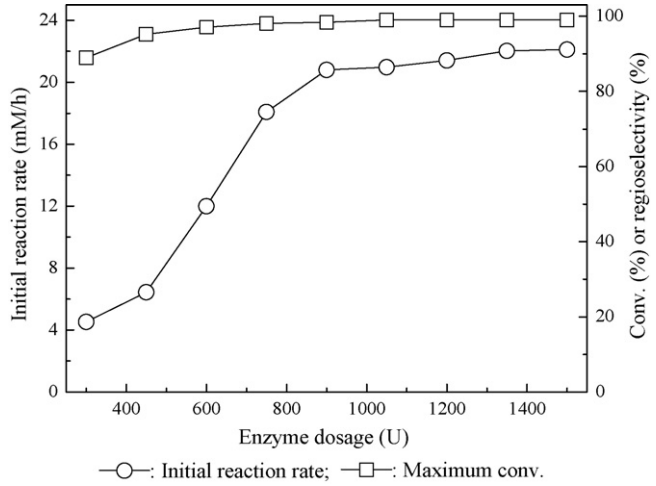

Fig. 3. Effect of enzyme dosage on lipozyme TL IM-catalyzed acylation of AzUrd with vinyl palmitate. The reactions were carried out at 40 °C, 200 rpm by adding various dosages of lipozyme TL IM, 10 mM AzUrd, 70 mM vinyl palmitate into 2 ml of anhydrous acetone. Reaction time: 15.0 h (300 U), 12.0 h (450 U), 9.0 h (600 U), 7.5 h (750 U), 6.5 h (900 U), 6.0 h (1050 U), 5.5 h (1200 U), 5.5 h (1350 U), 5.5 h (1500 U).

3.3. Effect of the molar ratio of vinyl palmitate to AzUrd

Parallel to enzymatic acylation of nucleosides with vinyl esters, the acyl donors is enzymatically hydrolyzed [38]. Accordingly, an excessive amount of acyl donors is normally required [20,21]. As depicted in Fig. 1, a striking enhancement in both the initial rate and the maximum substrate conversion was observed with increasing molar ratio of vinyl palmitate to AzUrd up to 7, the optimal ratio of vinyl palmitate to AzUrd. Additionally, change in the molar ratio of vinyl palmitate to AzUrd resulted in a small change in regioselectivity.

3.4. Effect of reaction temperature

It is well known that the higher the reaction temperature is, the more active the substrate molecules become. However, a high temperature may induce a significant conformational unfolding of the enzyme, resulting in a low initial rate. Hence, the effect of temperature on the reaction was examined. As shown in Fig. 2, the initial rate soared rapidly with the rise in the reaction temperature up to 40 °C and further ascent in temperature beyond 40 °C resulted in a drop in the initial reaction rate, suggesting the partial inactivation of the lipase in organic solvents at high temperatures. 40 °C was regarded as the optimum temperature for this reaction. Within the temperature range explored, the regioselectivity of the reaction kept 99.0% (Fig. 2).

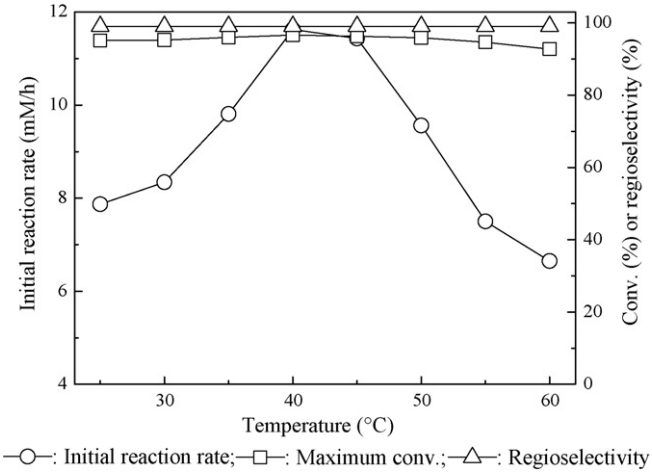

Fig. 2. Effect of the temperature on lipozyme TL IM-catalyzed acylation of AzUrd with vinyl palmitate. The reactions were carried out at 200 rpm, various temperatures from 25 to 60 °C by adding 750 U lipozyme TL IM, 10 mM AzUrd, 70 mM vinyl palmitate into 2 ml anhydrous acetone. Reaction time: 12.0 h (25 °C), 11.0 h (30 °C), 9.5 h (35 °C), 7.5 h (40 °C), 7.5 h (45 °C), 9.5 h (50 °C), 12.5 h (55 °C), 14.5 h (60 °C).

3.5. Effect of enzyme dosage and time course of reaction

As shown in Fig. 3, the reaction accelerated clearly with the increment in enzyme dosage from 500 to 900 U and then no substantial variation in initial rate occurred with further increasing enzyme dosage up to 1500 U. To gain a deeper insight into the enzymatic process, the time course of lipozyme TL IM-mediated acylation of AzUrd with vinyl palmitate was followed (Fig. 4) under the optimum conditions. As can be seen in Fig. 4, the substrate conversion underwent a steep increment in about 60 min, and then a smooth rise, possibly due to the lower concentration of the acyl donor and AzUrd. Additionally, the partial inactivation of the

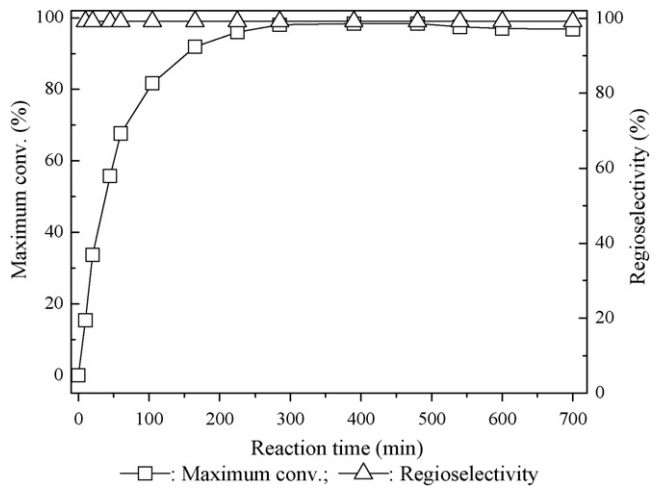

Fig. 4. Progress curve of lipozyme TL IM-catalyzed acylation of AzUrd. The reactions were carried out at 40 °C, 200 rpm by adding 900 U lipozyme TL IM, 10 mM AzUrd, and 70 mM vinyl palmitate into 2 ml of anhydrous acetone.

Table 3
Effect of various acyl donors on lipozyme TL IM-catalyzed regioselective acylation of AzUrd[a].

Entry	Acyl donor	V_0 (mM/h)	Time (h)[b]	C (%)[c]	Regioselectivity (%)
1	Vinyl acetate (C2)	3.2	15.0	87.5	99.0
2	Vinyl propionate (C3)	3.8	15.0	90.2	99.0
3	Vinyl butyrate (C4)	5.3	12.5	92.3	99.0
4	Vinyl hexanoate (C6)	12.6	7.0	96.8	99.0
5	Vinyl octanoate (C8)	22.9	4.5	>99.0	99.0
6	Vinyl decanoate (C10)	18.8	5.0	>99.0	99.0
7	Vinyl laurate (C12)	17.7	5.5	98.7	99.0
8	Vinyl myristate (C14)	15.6	6.0	98.5	99.0
9	Vinyl palmitate (C16)	13.3	6.5	98.4	99.0
10	Vinyl stearate (C18)	10.6	8.0	96.6	99.0
11	Vinyl chloroacetate	16.2	5.5	>99.0	99.0
12	Vinyl pivalate	0.1	35.0	9.4	99.0
13	Vinyl 2-ethylhexanoate	0.1	39.0	5.5	99.0
14	Vinyl crotonate	0.2	48.0	27.6	99.0
15	Vinyl methacrylate	0.4	42.0	40.1	99.0
16	Vinyl undecenoate	17.0	5.5	>99.0	99.0
17	Vinyl benzoate	0.1	72.0	23.7	99.0
18	Vinyl cinnamate	0.3	72.0	62.9	99.0

[a] The reactions were carried out at 40 °C and 200 rpm by adding 10 mM AzUrd, 70 mM acyl donor, 900 U lipozyme TL IM into 2 ml anhydrous acetone.
[b] Reaction time when the maximum conversion was achieved.
[c] Maximum substrate conversion.

enzyme by acetaldehyde from vinyl alcohol and palmitic acid from the hydrolysis of vinyl palmitate/the formed ester, was also responsible for the deceleration of the reaction [39]. After a reaction time of 6.5 h, the maximal substrate conversion was achieved, and the hydrolysis of the 5′-*O*-palmitoyl AzUrd caused a slight decline in substrate conversion thereafter.

3.6. Lipozyme TL IM-catalyzed regioselective acylation of AzUrd with various acyl donors

Lipozyme TL IM-catalyzed acylation of AzUrd with various fatty acid vinyl esters were conducted with high regioselectivity in anhydrous acetone. As identified by ^{1}H NMR and ^{13}C NMR analysis, 5′-*O*-monoesters of AzUrd were achieved exclusively from the enzymatic reaction. The reason for this is that *T. lanuginosus* lipase has a hydrophobic, crevice-like binding site with one hydrophobic lid covering it [40,41]. Owing to the enzyme's special active site, the less-hindered 5′-OH of the sugar moiety of AzUrd may enter into the active site more easily to attack the acyl-enzyme intermediate than the more-hindered 3′-OH or 2′-OH, thus resulting in the preferential formation of 5′-*O*-monoesters of AzUrd.

As shown in Table 3, Entries 1–10, the initial reaction rate and the maximum substrate conversion ascended with the elongation of chain length of the vinyl esters from C2 to C8, and then dropped (from C8 to C18), with vinyl octanoate being the most favorable acyl donor. Interestingly, the regioselectivity maintained 99.0% with all the used acyl donors. It has been reported that the favorable interactions between the hydrophobic acyl binding site of the enzyme and the medium-chain acyl group are stronger than those with a short chain [26,42]. A longer acyl donor, such as vinyl myristate (Entry 8), vinyl palmitate (Entry 9) or vinyl stearate (Entry 10), is more difficult to enter into the active site to form the first tetrahedral intermediate (generally considered as the rate-limiting step), due to the steric hindrance.

As predicted, the enzyme exhibited a poor catalytic performance in the acylation of AzUrd with branched-chain acyl donors (Entries 12 and 13). For instance, an initial rate of 0.1 mM/h and a conversion of 9.4% were obtained for the pivaloylation (Entry 12). In addition, the substrate conversion is lower with a larger α-substituent present in the acyl donor (Entry 13). The less accessibility of the enzyme's binding site for the branched-acyl donors (**2b** and **2c)** from their steric hindrance could account for this [43]. Interestingly, the enzymatic acylation rate was enhanced drastically with vinyl chloroacetate (**2a**) as the acyl donor as compared to vinyl acetate (Entry 11). The reason might be that the electron-withdrawing substituent (Cl atom) effectively lowers the charge density of the carbonyl carbon atom, facilitating the formation of the acyl-enzyme intermediate [43,44].

As can be seen in Table 3, with unsaturated acyl donors, only low initial rate and low maximum conversion could be achieved except for **2f,** while the presence of unsaturated bond in the acyl donors exerted little influence on the regioselectivity of the reaction (Entries 14–18). The low reaction rate might be attributed to the resonance effect of the conjugated unsaturated bond [45]. When the C–C double bond in the acyl moiety is far away from the carbonyl group, its effect on the enzymatic reaction is marginal (Entry 16). Surprisingly, although vinyl crotonate **2d** might be less hindered than vinyl methacrylate **2e** owing to the presence of α-methyl group in the latter, the reaction rate and the maximum conversion with the latter as the acyl donor were higher than the corresponding value with the former (Entries 14 and 15). Additionally, lipozyme TL IM-catalyzed benzoylation of AzUrd was more difficult to proceed (Entry 17), owing to the steric and the resonance effects of the rigid phenyl group. However, with the prolongation of the arm between phenyl and carbonyl, the steric strain of the rigid phenyl could be reduced, thus resulting in a high reaction rate and maximum conversion in the cinnamoylation (Entry 18) as compared to the benzoylation.

4. Conclusions

In conclusion, we have successfully synthesized various 5′-ester derivatives of AzUrd via an enzymatic acylation route. The structure of the acyl donors brings a significant impact on the catalytic performance of lipozyme TL IM. These findings will undoubtedly enrich the fundamentals of enzymology. Furthermore, the enzymatic process is highly regioselective, simple, environmentally friendly and mild as compared with the traditional chemical procedures.

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