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Two-step sequential synthesis of pyrimidine derivatives containing a sugar branch via combining of enzymatic Michael addition/acylation

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Abstract

A new strategy for the enzymatic synthesis of pyrimidine derivatives containing a sugar branch was developed via combining of Michael addition and acylation. The first-step reaction of pyrimidines and vinyl 3-propionyloxy propionate was catalyzed by Amano lipase M from *Mucor javanicus* in DMSO. The initial reaction rates of different pyrimidines decreased in the order of fluorouracil, uracil, thymine, in agreement with their nucleophilicity. The succeeding regioselective acylation of D-glucose and D-mannose with the Michael adducts was catalyzed by alkaline protease from *Bacillus subtilis* in pyridine. The D-glucose and D-mannose were all acylated at C-6 position. Moderate yield was obtained for each step.

Keywords: Enzymatic synthesis; Combined catalysis; Pyrimidine derivatives; Michael addition; Acylation

1. Introduction

The enzymatic synthesis has become an interesting area for organic and bioorganic chemists due to the high catalytic activity and high selectivity of enzymes under mild reaction conditions [1,2]. However, some complicated processes cannot be accomplished by single enzymatic pathway. Consequently, combination of these enzymatic pathways has attracted much attention in organic synthesis for its applications in synthesis of bioactive compounds or mimicking some consecutive biotransformation in vivo [3,4].

Recently, more and more enzymes were found to possess catalytic promiscuity, which indicates they are capable of catalyzing alternative reactions distinct from their normal biological reactions [5,6]. The study of the promiscuity of hydrolases, which are among the most used enzymes and physiologically catalyze the hydrolytic transformations involving ester and amide bonds, has become an interesting area. For instance, some lipases are able to catalyze aminolysis and ammonolysis reactions [7]. Recently, an engineered mutant of CAL B was developed to catalyze carbon–carbon bond forming reactions [8]. Our group reported alkaline protease from *Bacillus subtilis* showed a remarkable activity to catalyze Michael addition of *N*-nucleophiles to acrylates [9]. The catalytic promiscuity has expanded the applications of hydrolases in combined enzymatic reactions and a few elegant work concerned have been achieved successfully. Klaas et al. reported a combined multi-step process of deprotection, acetylation and epoxidation catalyzed by Novozym 435 [10]. A recent paper of our group reported the two-step enzymatic synthesis of imidazole derivatives containing glucose mediated by protease [11].

In the present work, we discovered that Amano lipase M from *Mucor javanicus* also has catalytic activity of Michael addition and it was employed as the first-step catalyst in the reaction of pyrimidines and vinyl 3-propionyloxy propionate. The products obtained were then used as acyl donors in the succeeding acylation of D-glucose and D-mannose under the catalysis of alkaline protease from *B. subtilis*. The finally obtained pyrimidine derivatives containing a sugar branch possess potential antitumor and antivirus activities [12,13].

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2. Experimental

2.1. Materials

Lipase from *M. javanicus* (9.9 U/mg, 1 U corresponds to the amount of enzyme which liberates 1 μmol oleinic acid from trioleoyl glycerol per minute at pH 8.0 and 37 °C), lipase form *Candida cylindracea* (1.6 U/mg, 1 U corresponds to the amount of enzyme which liberates 1 μmol oleic acid per minute at pH 8.0 and 40 °C), lipase from hog pancreas (2.4 U/mg, 1 U is the amount of immobilized enzyme which forms 1% octyl laurate from 0.5 mmol lauric acid and 1.0 mmol 1-octanol in 10 ml water-saturated isooctane in 1 h at 20 °C) and proteinase from *Aspergillus oryzae* (1.7 U/mg, one unit is the amount of enzyme which hydrolyzes 1 μmol of L-ieucine-*p*-nitroanilide per minute) were purchased from Fluka (Switzerland). Amano lipase M from *M. javanicus* (≥ 10,000 U/g enzyme activity, pH 7.0, 40 °C), lipase type VII from *Candida rugosa* (706 U/mg, one unit will hydrolyze 1.0 μequiv. of olive oil from a triglyceride in 1 h at pH 7.7 at 37 °C) and lipase from porcine pancreas (30–90 U/mg protein, one unit will hydrolyze 1.0 μequiv. of triacetin in 1 h at pH 7.7 at 37 °C) were purchased from Sigma (Steinheim, Germany). Lipase AY 30 (700–1500 U/mg solid, one unit will hydrolyze 1.0 μequiv. of olive oil from a triglyceride in 1 h at pH 7.7 at 37 °C) was purchased from Acros (New Jersey, USA). Alkaline protease from *B. subtilis* (10 U/mg, 1 U corresponds to the amount of enzyme which liberates 1 μmol folin-positive amino acids and peptides per minute at pH 7.5 and 37 °C) was obtained from Wuxi Enzyme Co. Ltd. (Wuxi, PR China). All solvents were analytical grade and were dried by storing over activated 3 Å molecular sieves before use. All other reagents were used as received.

2.2. Analytical methods

The process of reactions was monitored by TLC on silica with Petroleum ether/EtOAc (1/2 v/v) as solvent, for the first-step compounds (**3a**–**c**) and EtOAc/methanol/water (17/4/1 v/v) for the second-step compounds (**5a**–**f**). The ^{1}H and ^{13}C NMR spectra were recorded with TMS as internal standard using a Bruker AMX-500 MHz spectrometer. Chemical shifts were expressed in ppm and coupling constants (*J*) in Hz. Analytical HPLC was performed using a Agilent 1100 series with a reversed-phase Shim-Pack VP-ODS column (150 mm × 4.6 mm) and a UV detector (270 nm). IR spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer.

2.3. Synthesis of 3-(1′-uracil)-propionic acid vinyl ester (*3a*)

The reaction was initiated by adding 15 mg/ml Amano lipase M from *M. javanicus* to 20 ml DMSO containing uracil (1.5 mmol), vinyl 3-propionyloxy propionate 1 (3 mmol). The mixture was shaken at 200 rpm at 50 °C for 24 h. The reaction was terminated by filtering of the enzyme, and DMSO was evaporated in reduced pressure. The product was purified by silica gel column chromatography with an eluant consisting of petroleum ether/ethyl acetate (1/1 v/v). The yield was 54.5%. ^{1}H NMR ($CDCl_3$, δ, ppm): 9.17 (s, 1H, N(3)-H, uracil), 7.37 (d, 1H, J = 7.90, C(6)-H, uracil), 7.24 (m, 1H, O–CH=C), 5.68 (d, 1H, J = 7.80, C(5)-H, uracil), 4.93, 4.64 (m, 2H, O–C=CH_2), 4.02 (t, 2H, J = 5.85, –CH_2–N), 2.89 (t, 2H, J = 5.90, –CH_2–C=O). IR (cm^{-1}): 1732 (O–C=O) and 1642 (C=C).

2.4. Synthesis of 3-(1′-thymine)-propionic acid vinyl ester (*3b*)

3-(1′-Thymine)-propionic acid vinyl ester was synthesized by the same synthesis method as for **3a**. The yield of product was 53.5%. ^{1}H NMR ($CDCl_3$, δ, ppm): 8.9 (m, 1H, N(3)-H, thymine), 7.24 (m, 1H, O–CH=C), 7.19 (s, 1H, C(6)-H, thymine), 4.93, 4.64 (m, 2H, O–C=CH_2), 3.99 (t, 2H, J = 5.96, –CH_2–N), 2.89 (t, 2H, J = 5.97, –CH_2–C=O). IR (cm^{-1}): 1733 (O–C=O) and 1641 (C=C).

2.5. Synthesis of 3-(1′-fluorouracil)-propionic acid vinyl ester (*3c*)

3-(1′-Fluorouracil)-propionic acid vinyl ester was synthesized by the same synthesis method as for **3a**. The yield of product was 64.6%. ^{1}H NMR (DMSO-d_6, δ, ppm): 11.7 (s, 1H, N(3)-H, fluorouracil), 8.05 (d, 1H, J = 6.86, C(6)-H, fluorouracil), 7.20 (m, 1H, O–CH=C), 4.91, 4.68 (m, 2H, O–C=CH_2), 3.89 (t, 2H, J = 6.75, –CH_2–N), 2.84 (t, 2H, J = 6.74, –CH_2–C=O). ^{13}C NMR (DMSO-d_6, δ, ppm): 168.80 (C=O), 158.03, 157.82 (C-4, fluorouracil), 149.97 (C-2, fluorouracil), 141.62 (–O–CH=), 140.69, 138.88 (C-5, fluorouracil), 131.11, 130.84 (C-6, fluorouracil), 98.98 (=CH_2), 44.21 (N–CH_2–), 32.58 (–CH_2–C=O). IR (cm^{-1}): 1728 (O–C=O) and 1640 (C=C).

2.6. 6-O-[3′-(1″-Uracil)-propionyl]-D-glucose (*5a*)

The reaction was initiated by adding 15 mg/ml alkaline protease from *B. subtilis* to 20 ml Pyridine containing Michael adducts (1 mmol), sugar (0.5 mmol). The mixture was shaken at 200 rpm at 50 °C for 48 h. The reaction was terminated by filtering of the enzyme and pyridine was evaporated in reduced pressure. The product was purified by silica gel column chromatography with an eluant consisting of petroleum ethyl acetate/methanol/water (17/4/1 v/v). The yield of product was 65.7%. ^{1}H NMR (D_2O, δ, ppm): 7.59 (t, 1H, C(6)-H, uracil), 5.69 (d, 1H, C(5)-H, uracil), 5.09 (d, 0.4H, J = 3.65, H-1 of α-D-glucose), 4.54 (d, 0.6H, J = 7.96, H-1 of β-D-glucose), 4.34–4.16 (m, 2H, H-6,6′ of β-D-glucose, H-6,6′ of α-D-glucose), 3.97 (t, 2H, J = 5.81,

–CH_2–N), 3.90–3.14 (m, 4H, αH or βH of D-glucose), 2.77 (t, 2H, J = 5.97, –CH_2–C=O). ^{13}C NMR (D_2O, δ, ppm): 174.19, 174.13 (C=O), 168.64 (C-4, uracil), 153.24 (C-2, uracil), 148.93, 148.84 (C-6, uracil), 102.40 (C-5, uracil), 97.16 (C1 of β-D-glucose), 93.29 (C1 of α-D-glucose), 76.65 (C3 of β-D-glucose), 75.16 (C2 of β-D-glucose), 74.44 (C5 of β-D-glucose), 73.71 (C3 of α-D-glucose), 72.56 (C2 of α-D-glucose), 70.72 (C4 of α-D-glucose), 70.67 (C4 of β-D-glucose), 70.19 (C5 of α-D-glucose), 64.93 (C6 α,β of D-glucose), 46.19 (–CH_2–N), 33.72, 33.74 (–CH_2–C=O). IR (cm^{-1}): 3326 (O–H), 1728 and 1682 (C=O).

*2.7. 6-O-[3′-(1″-Thymine)-propionyl]-D-glucose (**5b**)*

6-*O*-[3′-(1″-Thymine)-propionyl]-D-glucose was synthesized by the same synthesis method as for **5a**. The yield of product was 75.7%. ^{1}H NMR (D_2O, δ, ppm): 7.47 (d, 1H, J = 6.41, C(6)-H, thymine), 5.09 (d, 0.4H, J = 3.67, H-1 of α-D-glucose), 4.58 (d, 0.6H, J = 7.95, H-1 of β-D-glucose), 4.39–4.18 (m, 2H, H-6,6′ of β-D-glucose, H-6,6′ of α-D-glucose), 3.99 (t, 2H, J = 6.47, –CH_2–N), 3.95–3.18 (m, 4H, αH or βH of D-glucose), 2.81 (t, 2H, J = 6.60, –CH_2–C=O), 1.82 (s, 3H, –CH_3). ^{13}C NMR (D_2O, δ, ppm): 173.04, 172.98 (C=O), 168.64 (C-4, thymine), 152.10 (C-2, thymine), 143.40, 143.35 (C-6, thymine), 110.48 (C-5, thymine), 96.00 (C1 of β-D-glucose), 92.12 (C1 of α-D-glucose), 75.47 (C3 of β-D-glucose), 73.98 (C2 of β-D-glucose), 73.28 (C5 of β-D-glucose), 72.53 (C3 of α-D-glucose), 71.39 (C2 of α-D-glucose), 69.53 (C4 of α-D-glucose), 69.48 (C4 of β-D-glucose), 69.00 (C5 of α-D-glucose), 63.72 (C6 α,β of D-glucose), 46.19 (–CH_2–N), 33.72, 33.74 (–CH_2–C=O), 11.26 (–CH_3). IR (cm^{-1}): 3338 (O–H), 1724, and 1676 (C=O).

*2.8. 6-O-[3′-(1″-Fluorouracil)-propionyl]-D-glucose (**5c**)*

6-*O*-[3′-(1″-Fluorouracil)-propionyl]-D-glucose was synthesized as the same method as for **5a**. The yield of product was 57.1%. ^{1}H NMR (D_2O, δ, ppm): 7.80 (m, 1H, C(6)-H, fluorouracil), 5.08 (d, 0.40H, J = 3.60, H-1 of α-D-glucose), 4.53 (d, 0.60H, J = 7.91, H-1 of β-D-glucose), 4.34–4.14 (m, 2H, H-6,6′ of β-D-glucose, H-6,6′ of α-D-glucose), 3.93 (t, 2H, J = 6.15, N–CH_2–), 3.90–3.13 (m, 4H, αH or βH of D-glucose), 2.75 (t, 2H, J = 5.98, –CH_2–C=O). ^{13}C NMR (D_2O, δ, ppm): 173.12, 173.07 (C=O), 160.16, 159.94 (C-4, fluorouracil), 150.84 (C-2, fluorouracil), 141.27, 139.42 (C-6, fluorouracil), 132.09–131.66 (C-5, fluorouracil), 96.21 (C1 of β-D-glucose), 92.35 (C1 of α-D-glucose), 75.69 (C3 of β-D-glucose), 74.20 (C2 of β-D-glucose), 73.48 (C5 of β-D-glucose), 72.76 (C3 of α-D-glucose), 71.61 (C2 of α-D-glucose), 69.75 (C4 of α-D-glucose), 69.71 (C4 of β-D-glucose), 69.21 (C5 of α-D-glucose), 63.99 (C6 α,β of D-glucose), 45.42 (–CH_2–N), 32.76–32.63 (–CH_2–C=O). IR (cm^{-1}): 3364 (O–H), 1714, and 1694 (C=O).

*2.9. 6-O-[3′-(1″-Uracil)-propionyl]-D-mannose (**5d**)*

6-*O*-[3′-(1″-Uracil)-propionyl]-D-mannose was synthesized as the same method as for **5a**. The yield of product was 53.1%. ^{1}H NMR (D_2O, δ, ppm): 7.60 (d, 1H, J = 7.79, C(6)-H, uracil), 5.70 (d, 1H, J = 7.74, C(5)-H, uracil), 5.05 (s, 0.6H, H-1 of α-D-mannose), 4.80 (s, 0.4H, H-1 of β-D-mannose), 4.33–4.21 (m, 2H, H-6,6′ of β-D-mannose, H-6,6′ of α-D-mannose), 3.99 (t, 2H, J = 6.06, –N–CH_2), 3.88–3.61 (m, 4H, αH or βH of D-mannose), 2.80 (t, 2H, J = 6.10, –CH_2–C=O). ^{13}C NMR (D_2O, δ, ppm): 173.18 (C=O), 166.90 (C-4, uracil), 152.19 (C-2, uracil), 147.95, 147.88 (C-6, uracil), 101.30 (C-5, uracil), 94.25 (C1 of α-D-mannose), 93.87 (C1 of β-D-mannose), 73.58 (C5 of β-D-mannose), 72.89 (C3 of β-D-mannose), 71.14 (C2 of β-D-mannose), 70.62 (C2 of α-D-mannose), 70.12 (C3 of α-D-mannose), 70.07 (C5 of α-D-mannose), 66.77 (C4 of α-D-mannose), 66.59 (C4 of β-D-mannose), 64.11 (C6 α,β of D-mannose), 45.15 (–CH_2–N), 33.72, 32.67 (–CH_2–C=O). IR (cm^{-1}): 3346 (O–H), 1722, and 1681 (C=O).

*2.10. 6-O-[3′-(1″-Thymine)-propionyl]-D-mannose (**5e**)*

6-*O*-[3′-(1″-Thymine)-propionyl]-D-mannose was synthesized as the same method as for **5a**. The yield of product was 62.8%. ^{1}H NMR (D_2O, δ, ppm): 7.40 (d, 1H, J = 3.61, C(6)-H, thymine), 5.01 (s, 0.6H, H-1 of α-D-mannose), 4.80 (s, 0.4H, H-1 of β-D-mannose), 4.28–4.16 (m, 2H, H-6,6′ of β-D-mannose, H-6,6′ of α-D-mannose), 3.91 (t, 2H, J = 6.29, –N–CH_2), 3.83–3.57 (m, 4H, αH or βH of D-mannose), 2.74 (t, 2H, J = 6.20, –CH_2–C=O). ^{13}C NMR (D_2O, δ, ppm): 173.3 (C=O), 167.17 (C-4, thymine), 152.19 (C-2, thymine), 147.95, 147.88 (C-6, thymine), 101.30 (C-5, thymine), 94.35 (C1 of α-D-mannose), 93.99 (C1 of β-D-mannose), 73.71 (C5 of β-D-mannose), 73.00 (C3 of β-D-mannose), 71.24 (C2 of β-D-mannose), 70.71 (C2 of α-D-mannose), 70.23 (C3 of α-D-mannose), 70.18 (C5 of α-D-mannose), 66.87 (C4 of α-D-mannose), 66.66 (C4 of β-D-mannose), 64.11 (C6 α,β of D-mannose), 45.15 (–CH_2–N), 33.72, 32.67 (–CH_2–C=O). IR (cm^{-1}): 3361 (O–H), 1693, and 1681 (C=O).

*2.11. 6-O-[3′-(1″-Fluorouracil)-propionyl]-D-mannose (**5f**)*

6-*O*-[3′-(1″-Fluorouracil)-propionyl]-D-mannose was synthesized as the same method as for **5a**. The yield of product was 62.9%. ^{1}H NMR (D_2O, δ, ppm): 7.82 (d, 1H, J = 5.82, C(6)-H, fluorouracil), 5.05 (s, 0.6H, H-1 of α-D-mannose), 4.80 (s, 0.4H, H-1 of β-D-mannose), 4.32–4.19 (m, 2H, H-6,6′ of β-D-mannose, H-6,6′ of α-D-mannose), 3.96 (d, 2H, –N–CH_2), 3.90–3.55 (m, 4H, αH or βH of D-mannose), 2.80 (t, 2H, J = 5.70, –CH_2–C=O). ^{13}C NMR (D_2O, δ, ppm): 173.21 (C=O), 160.53, 160.33 (C-4, fluorouracil), 151.17 (C-2, fluorouracil), 141.35, 139.51 (C-5, fluorouracil), 132.00, 131.73, 131.61 (C-6, fluorouracil),

94.39 (C1 of α-D-mannose), 94.01 (C1 of β-D-mannose), 73.71 (C5 of β-D-mannose), 73.02 (C3 of β-D-mannose), 71.27 (C2 of β-D-mannose), 70.75 (C2 of α-D-mannose), 70.24 (C3 of α-D-mannose), 70.20 (C5 of α-D-mannose), 66.89 (C4 of α-D-mannose), 66.71 (C4 of β-D-mannose), 64.26 (C6 α,β of D-mannose), 45.52, 45.46 (–CH_2–N), 33.89, 32.76, 32.70 (–CH_2–C=O). IR (cm^{-1}): 3372 (O–H), 1713, and 1694 (C=O).

3. Results and discussion

3.1. Enzymatic synthesis of pyrimidine derivatives containing a sugar branch

A new strategy for the synthesis of pyrimidine derivatives containing a sugar branch was developed via combining of Michael addition and acylation. The reaction process was shown in Fig. 1. Six pyrimidine derivatives containing a sugar branch (**5a**–**5f**) were synthesized. All compounds **3a**–**c**, **5a**–**f** 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. [14], acylation of a hydroxyl group will lead the O-acylated carbon ($^*CH_2OCOR$) downfield, while the adjacent carbon ($^*CH_2OCOR$) upfield in ^{13}C NMR. Analysis ^{13}C NMR spectra of all the products (**5a**–**5f**) revealed that esterification of D-glucose and D-mannose occurred in the primary alcohol position.

3.2. Forming mechanism of the special structure obtained in the first step

The structure of Michael adducts, in which a propenoic acid was eliminated, was not the same as expected. The interesting structure obtained in this study should be attributed to the solvent employed. In order to understand the forming mechanism of the special products, a blank experiment was performed. Shaking vinyl 3-propionyloxy propionate in DMSO alone at 50 °C for 12 h, 42.6% of it was decomposed to propenoic acid and vinyl acrylate accordingly. As the result of the chemical decomposition of vinyl 3-propionyloxy propionate, the adduct of uracil were **3a**.

When the same blank experiment was carried out in pyridine, no decomposition of vinyl 3-propionyloxy propionate was detected. As a result, the product obtained in the former work [11] was 3-((1′-imidazole)-propionyloxy)-propionic acid vinyl ester. If pyridine solvent was replaced by DMSO, vinyl 3-propionyloxy propionate was also found decomposed and the structure obtained was 3-(1′-imidazole)-propionic acid vinyl ester accordingly. All the results indicated that it was DMSO that had caused the different product between the two similar reactions.

Consequently, the ultimate product obtained in the present work was 3-(1′-uracil)-propionic acid vinyl ester but not 3-((1′-uracil)-propionyloxy)-propionic acid vinyl ester when uracil was used as nucleophile and DMSO as solvent.

3.3. Influence of enzyme on first-step reaction

The enzyme source is one of the main factors influencing enzymatic Michael addition. Eleven kinds of commercially available enzyme were screened to catalyze the reaction of uracil and vinyl 3-propionyloxy propionate (**1**). The results were compared and presented in Table 1. Results indicated that lipase from the strain of *M. javanicus* can catalyze the reaction of pyrimidines and vinyl 3-propionyloxy propionate efficiently. Alkaline protease from *B. subtilis*, which was employed as catalyst in our former work [9,11] also have strong catalytic activity for the reaction. However, some

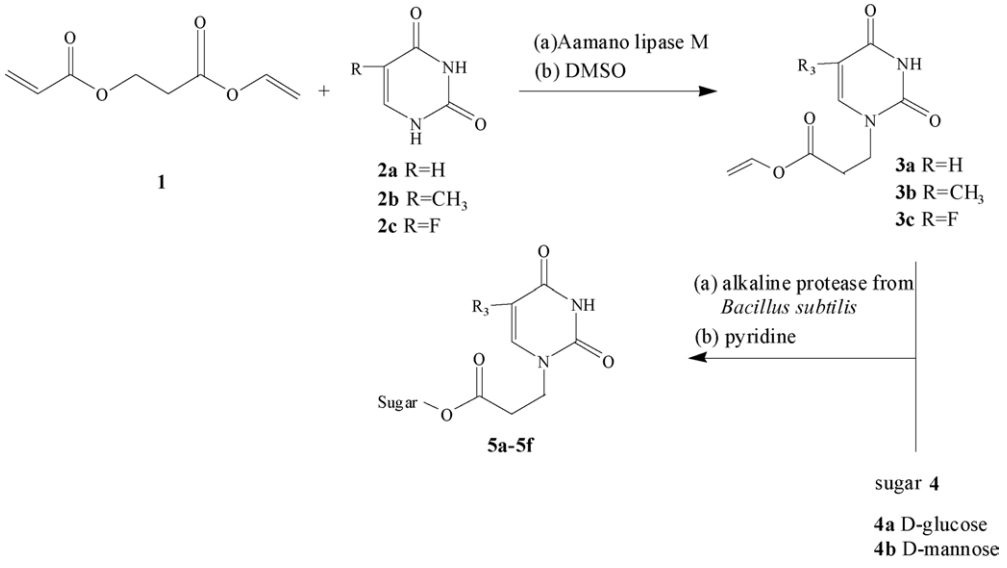

Fig. 1. Two-step enzymatic synthesis of pyrimidine derivatives containing a sugar branch.

Table 1
The effect of enzyme source to Michael addition

Entry	Enzyme source	Yield (%)[a]
1	Alkaline protease from *Bacillus subtilis*	45.5
2	Proteinase from *Aspergillus oryzae*	38.2
3	Lipase AY 30	19.1
4	Lipase from hog pancreas	39.0
5	Amano lipase M from *Mucor javanicus*	55.2
6	Lipase type VII from *Candida rugosa*	2.1
7	Lipase from *Candida cylindracea*	3.8
8	Lipase from porcine pancreas	18.9
9	Lipase from *M. javanicus*	50.8
10	–[b]	0.64

Conditions: uracil (0.075 mol), 3-propionyloxy propionate 1 (0.15 mol); 15 mg enzyme, DMSO 1 ml, 50 °C, 1 day.
[a] Determined by HPLC.
[b] Without enzyme.

lipases such as lipase AY 30, lipase from *Candida rugosa* and lipase from *Candida cylindracea* showed low reaction activity.

The reaction of vinyl 3-propionyloxy propionate with different pyrimidines in the presence of Amano lipase M from *M. javanicus* led to the corresponding Michael adduct faster than in the absence of the enzyme. We investigated the initial reaction rates in the catalysis of Amano lipase M from *M. javanicus* and blank with uracil as model substrate. The initial rate was up to 420-fold faster than the process in absence of enzyme.

3.4. Influence of solvent on first-step reaction

We also investigated the effect of organic media on enzymatic Michael addition. Some conventional solvents such as DMSO, DMF, dioxane, acetone, THF, pyridine, chloroform and *n*-hexane were screened. The yield of **3a** was about 56% after 24 h in DMSO and 43% in DMF, but no product was detected in other solvents. We further compared the different effect of DMSO and DMF in details. When fluorouracil was employed to react with **1**, the yield in DMSO was almost same as that in DMF, but to thymine, the yield in DMSO and DMF was 54 and 30%, respectively. The first-step enzymatic reaction in DMSO is faster than that in DMF.

3.5. Influence of substrate structure on first-step reaction

The structure of the substrate affected the results of the enzymatic Michael reaction. We selected uracil, thymine and fluorouracil to investigate the influence of nucleophilicity on Michael addition. The time courses of substrates **2a**–**c** were examined and the results were shown in Fig. 2. The yield in 24 h was about 65, 55 and 54%, respectively for substrates **2a**–**c**. The results were similar with that described in our former work [15].

We further calculated the initial reaction rates of different substrates. Results were shown in Table 2. The reaction rate of fluorouracil was fastest and the initial rate was up to

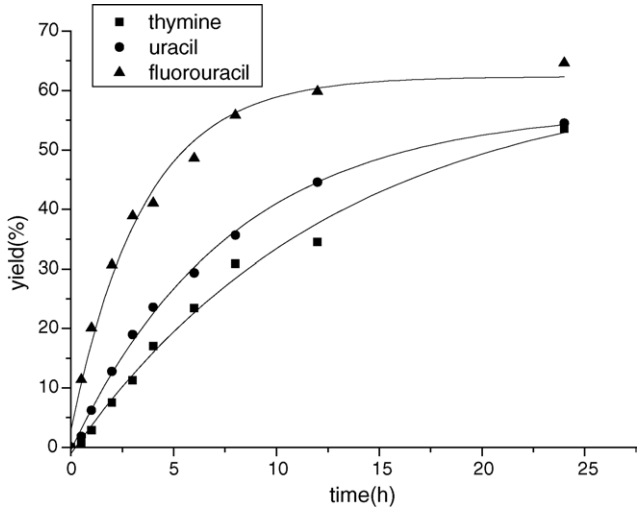

Fig. 2. Effect of substrate structure on time course of the enzymatic Michael addition of pyrimidines (75 mM) to vinyl ester **1** (150 mM) catalyzed by Amano lipase M (15 mg/ml) at 50 °C in DMSO.

12.68 mM h^{-1}. The reactivity order decreased by the following order **2c**, **2a**, **2b**, in agreement with their nucleophilicity. Accordingly, the electron withdrawing effect of nucleophile was one of the main factors affecting the Michael addition.

3.6. Influence of enzyme concentration on first-step reaction

The enzyme concentration also affected the results of the enzymatic Michael addition. Uracil was selected as model reaction. The yield under different enzyme concentration was shown in Fig. 3. From the diagram, the yield of **5a** considerably increased from 43 to 56% when the enzyme concentration was varied from 5 to 50 mg/ml and the plateau was reached at a concentration of 15 mg/ml. It was obvious that the suitable enzyme concentration would benefit the Michael addition.

3.7. The acylation of first-step products with sugars

The succeeding acylation of first-step products (**3a**–**c**) with D-glucose and D-mannose was achieved by alkaline protease from *B. subtilis* in pyridine. Anomeric mixtures were obtained for D-glucose (α:β = 40:60) and D-mannose (α:β = 60:40) in moderate yield from 57.1 to 75.7%.

The influence of acyl donors (**3a**–**c**) was not obvious. In the case of D-glucose, the yield of different acyl donors **3a**–**c** was

Table 2
The effect of substrate structure to Michael addition

Substrate	Yield (%)[a]	Initial rate (mM h^{-1})
2a	54.5	5.51
2b	53.5	3.33
2c	64.6	12.68

[a] Determined by HPLC.

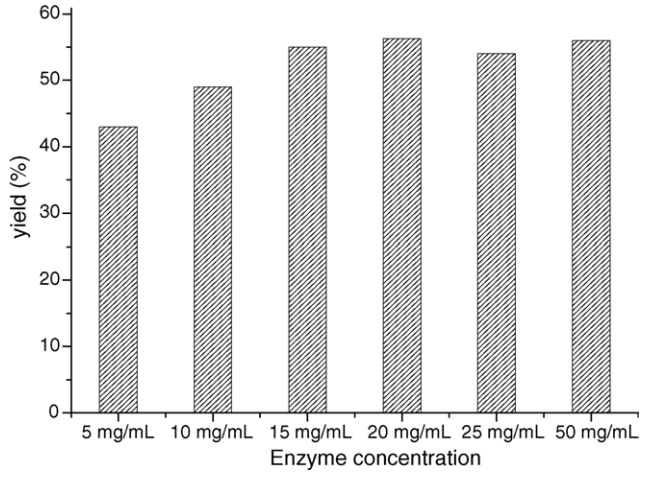

Fig. 3. Influence of enzyme concentration on enzymatic Michael addition. Conditions: pyrimidines (75 mM), vinyl ester **1** (150 mM), DMSO 1 ml, 50 °C, 24 h.

65.7, 75.7 and 57.1%, respectively. We monitored the formation of products by HPLC. Results indicated that D-glucose reacted faster than D-mannose. No product was detected in the absence of enzyme.

4. Conclusion

A new strategy for the synthesis of pyrimidine derivatives containing a sugar branch was developed via combining of Michael addition and acylation. The first-step reaction was carried out in DMSO at 50 °C with the Amano lipase M. Control experiments indicated that enzyme accelerated the reaction of uracil up to 420-fold. The influence of enzyme source, solvent, substrate structure and enzyme concentration on enzymatic Michael addition was systematically investigated. The succeeding acylation was carried out in pyridine with alkaline protease from *B. subtilis*. More applications of the combination of Michael addition/acylation are in progress.

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