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Substantially enhancing enzymatic regioselective acylation of 1-β-D-arabinofuranosylcytosine with vinyl caprylate by using a co-solvent mixture of hexane and pyridine

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ABSTRACT

Novozym 435-catalyzed regioselective acylation of 1-β-D-arabinofuranosylcytosine (ara-C) with vinyl caprylate for the preparation of its 5′-*O*-acyl derivative has been performed in six co-solvent mixtures and three pure polar solvents for the first time. Novozym 435 displayed low activity towards 1-β-D-arabinofuranosylcytosine in pure polar solvents, although those solvents can dissolve the nucleosides well. When a hexane–pyridine co-solvent system was adopted, both the initial rate and the substrate conversion were enhanced markedly. The most suitable co-solvent, initial water activity, reaction temperature and the molar ratio of vinyl caprylate to ara-C were hexane/pyridine (28/72, v/v), 0.03, 40 °C and 15, respectively. Under these conditions, the initial rate, the substrate conversion and the regioselectivity were as high as 99.0 mmol h^{-1}, 98% and >99%, respectively. The product of the Novozym 435-catalyzed reaction was characterized by ^{13}C NMR and confirmed to be 5′-*O*-octanoyl 1-β-D-arabinofuranosylcytosine.

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1. Introduction

Regioselective acylation of polyhydroxy compounds is a fundamental challenge to organic chemists. It will not only lead to a new method for introducing new protecting groups [1] but also put forward a way of obtaining nucleoside derivatives of significance in treatment of viral infections and tumors. Among these nucleoside derivatives, 5′-*O*-acyl-1-β-D-arabinofuranosylcytosine may play an important role in the therapy of acute leukemia due to its higher antitumor potency than ara-C [2,3].

Several strategies for regioselective acylation of ara-C have been reported employing conventional chemical methods, but their application is somewhat hampered by the relatively low regioselectivity, the lack of easy access to some of the key intermediates, and the environmental concerns of the process [1,3–5]. For example, the direct acylation of ara-C without prior protection catalyzed by alkaline catalyst gave preferentially 5′-*O*-acyl-1-β-D-arabinofuranosylcytosine, but the yield was only 35–70%, depending on the acyl donors used and tedious separation was required due to the low regioselectivity [6]. On the contrary, enzymatic processes are characterized by the high efficiency, mild reaction conditions, high selectivity and being environmentally friendly.

Recently, enzymes, such as lipases have proved to be effective catalysts for highly regioselective acylation of nucleosides [1]. In our laboratory, Li et al. have, for the first time, successfully performed the enzymatic acylation of ara-C with several kinds of fatty acid vinyl esters, such as vinyl acetate and vinyl propionate [7,8]. However, there has been no report on the enzymatic acylation of ara-C with vinyl caprylate (VC) so far.

In our ongoing research related to the synthesis of lipophilic derivatives of ara-C, Novozym 435 (an immobilized lipase from *Candida antarctica*, type B) has been found to be highly regioselective towards the 5′-hydroxyl group of ara-C. Here we describe the enzymatic acylation of ara-C with vinyl caprylate in a co-solvent mixture for preparation of 5′-*O*-octanoyl-ara-C, which might have a unique bioactivity (Scheme 1).

2. Materials and methods

2.1. Enzymes and chemicals

C. antarctica lipase B (CAL-B) immobilized on a macroporous acrylic support (Novozym 435, 10,000 U g^{-1}) was kindly donated by Novozymes (Denmark). 1-β-D-Arabinofuranosylcytosine (ara-C) was purchased from Aldrich (USA). All other chemicals

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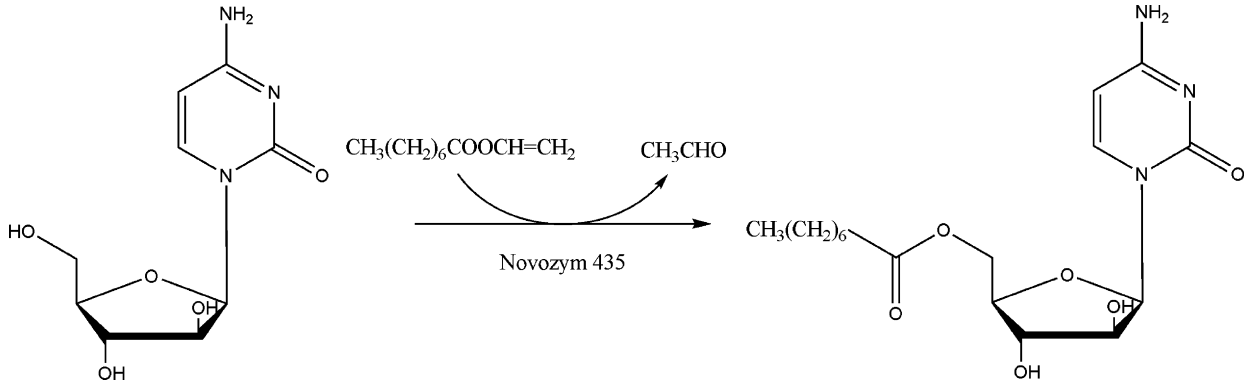

Scheme 1. Lipase-catalyzed regioselective acylation of 1-β-D-arabinofuranosylcytosine with vinyl caprylate.

were obtained from commercial sources and were of analytical grade.

2.2. General procedure for enzymatic acylation of ara-C with VC

In a typical experiment, 1 mL reaction media containing 0.02 mmol ara-C, 0.2 mmol VC and 500 U Novozym 435 were incubated with shaking at a fixed temperature stated for each experiment. Aliquots were withdrawn at specified time intervals from the reaction mixture, and then diluted 100 times with a water–methanol mixture prior to HPLC analysis. To obtain larger amounts of product for its structural characterization, the synthesis was scaled up (~0.2 mmol ara-C and 3 mmol VC). Upon completion of the reaction, the reaction mixture was filtered to remove the immobilized enzyme and evaporated under vacuum. The residue was extracted three times with ethanol and the collected solution was further evaporated to give the crude product. Separate experiments were also conducted in the various media tested with no enzyme added and the results showed that no chemical acylation reaction took place in the absence of the enzyme under the reaction conditions described above.

2.3. Control of initial water activity

The initial water activity of the reaction media, the substrates and the enzyme were controlled by gaseous equilibrium with different saturated solutions in separate closed containers at 25 °C. The following compounds were used: NaOH (a_w = 0.03), LiCl (a_w = 0.11), $MgCl_2$ (a_w = 0.33), and $Mg(NO_3)_2$ (a_w = 0.53). A molecular sieve was used to generate a nearly anhydrous reaction medium ($a_w \sim 0$).

2.4. Analytical methods

The reaction mixture was analyzed by RP-HPLC on a 4.6 mm × 250 mm (5 μm) Zorbax SB-C18 column (Agilent Technologies Co., Ltd., USA) using an Agilent G1311A pump and a UV detector at 276 nm. The mobile phase was a mixture of ammonium acetate buffer (0.01 mol L^{-1}, pH 4.27) and methanol (35/65, v/v) at a flow rate of 0.9 mL min^{-1}. The retention times for 1-β-D-arabinofuranosylcytosine and 5′-*O*-octanoyl-1-β-D-arabinofuranosylcytosine were 2.56 and 9.71 min, respectively. Regioselectivity was defined as the ratio of the HPLC peak area corresponding to the indicated product to that of all the products formed [9]. The initial rate (V_0) and the substrate conversion (C) were calculated from the HPLC data according to the following equations:

$$V_0 = \frac{C_0 - C_t}{t}$$

$$C = \frac{C_0 - C_t}{C_0}$$

where C_0 and C_t 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 assay is less than 0.5%. All reported data are averages of experiments performed at least in duplicate.

2.5. Structure determination

The position of acylation in enzymatically prepared ester was determined by ^{13}C NMR (Bruker AVANCE Digital 400 MHz Nuclear Magnetic Resonance Spectrometer, Bruker Co., Germany) at 100 MHz. DMSO-d_6 was used as a solvent and TMS was used as an internal reference. Chemical shifts were expressed in ppm shift.

3. Results and discussion

3.1. Regioselectivity of the reaction

In our detailed examination on its catalysis of ara-C acylation with vinyl caprylate in a co-solvent mixture of hexane and pyridine, Novozym 435 exhibits startling regioselectivity up to 99% towards the 5′-hydroxyl group of ara-C (Table 1).

Compared with ara-C, ^{13}C-NMR spectra of its acylated derivative exhibited additional carbon signals at δ 174.54, 23.83 and 25.89, 26.35, 30.22, 32.96, 34.91, 35.53, characteristic of octanoyl group. Moreover, C-5′ (δ 63.03) of the sugar moiety shifted downfield by 2.5 ppm and the data of its neighboring carbon atom C-4′ (δ 86.86) also showed an upfield shift of 2.84 ppm, suggesting that the acyl group was attached to the –OH at C-5′ [10]. Thus the product was identified as 5′-*O*-octanoyl-ara-C.

3.2. Effect of reaction medium

It is well known that the properties of solvents have significant effects on the catalytic behavior of enzymes. Due to the poor solubility of the hydrophilic nucleosides in most hydrophobic organic solvents [1,5,7], only polar organic solvents like pyridine and DMF are mainly used in their enzymatic acylation [11,12]. So we performed the acylation of ara-C in three commonly used polar organic solvents firstly. Although Novozym 435 displayed a higher activity

Table 1
^{13}C NMR spectral data for ara-C and the product of its acylation (δ (ppm))[a]

Carbon numbers	Ara-C	5′-*O*-Octanoyl-ara-C
2	155.91	155.30
4	166.58	166.20
5	94.24	94.44
6	145.22	145.33
Sugar moiety		
1′	87.82	88.28
2′	76.76	76.30
3′	78.21	78.63
4′	86.86	84.02
5′	63.03	65.53
Acyl moiety		
C=O		174.54
$-CH_3$		23.83
$-CH_2-$		25.89, 26.35, 30.22, 32.96, 34.91, 35.53

[a] All samples were measured in DMSO-d_6.

Table 2
Effect of organic solvents on the enzymatic acylation of ara-C with VC[a]

Solvent (v/v)	V_0 (mmol L^{-1} h^{-1})	C[b] (%)	Regioselectivity (%)
DMSO	13.1	16.0	>99
DMF	12.1	10.1	>99
Pyridine	19.1	17.6	>99
Acetonitrile–pyridine (25:75)	29.5	40.3	>99
t-Butanol–pyridine (25:75)	32.4	41.8	>99
t-Pentyl alcohol–pyridine (25:75)	36.0	59.2	>99
Tetrahydrofuran–pyridine (25:75)	46.9	63.6	>99
Isopropyl ether–pyridine (25:75)	57.4	69.3	>99
Hexane–pyridine (25:75)	65.4	73.2	>99

[a] The reaction conditions: 0.02 mmol ara-C; 0.2 mmol VC; 500 U Novozym 435; 30 °C; 200 rpm; 1 mL of the reaction medium; a_w = ~0.
[b] Maximum substrate conversion.

in pyridine than in DMSO or DMF, the initial rate and substrate conversion were obviously low (Table 2, entries 1–3). A possible reason may be that pyridine, DMSO and DMF are polar organic solvents that usually strip essential water from the enzyme molecules and thus partly inactivate the biocatalyst. In order to change the polarity of reaction media, co-solvent mixtures of organic solvents with different polarities were tested as the reaction media (Table 2, entries 4–9). Among the organic co-solvents tested, hexane–pyridine co-solvent gave the highest initial rate (65.4 mmol h^{-1}) and substrate conversion (73.2%).

3.3. Effect of hydrophobic solvent content

As can be seen in Table 3, when the volumetric concentration of hexane was below 28% in the co-solvent mixture, the higher the hexane content, the higher the initial rate and substrate conversion. Further increasing hexane content beyond 28% led to a drop in the initial rate. The less inactivation effect of a hydrophobic solvent on

Table 3
Effect of organic solvent content on the enzymatic acylation of ara-C with VC[a]

Solvent (v/v)	V_0 (mmol L^{-1} h^{-1})	C[b] (%)	Regioselectivity (%)
Hexane–pyridine (15:85)	51.7	56.7	>99
Hexane–pyridine (20:80)	60.1	71.2	>99
Hexane–pyridine (25:75)	65.4	73.2	>99
Hexane–pyridine (28:72)	70.6	76.1	>99
Hexane–pyridine (30:70)	67.3	74.4	>99

[a] The reaction conditions: 0.02 mmol ara-C; 0.2 mmol VC; 500 U Novozym 435; 30 °C; 200 rpm; 1 mL of the reaction medium; a_w = ~0.
[b] Maximum substrate conversion.

Table 4
Effect of initial water activity on Novozym 435-mediated acylation of ara-C with VC in hexane–pyridine co-solvent[a]

a_w	V_0 (mmol L^{-1} h^{-1})	C[b] (%)	Regioselectivity (%)
~0	70.6	76.1	>99
0.03	80.9	78.7	>99
0.11	60.7	53.2	>99
0.33	41.4	32.8	>99
0.53	28.1	22.7	>99

[a] The reaction conditions: 0.02 mmol ara-C; 0.2 mmol VC; 500 U Novozym 435; 30 °C; 200 rpm; 1 mL hexane–pyridine (28:72, v/v).
[b] Maximum substrate conversion.

the enzyme and the lower solubility of the substrate in it are both contributive to this. Little influence of hydrophobic solvent content on the regioselectivity was observed.

3.4. Effect of initial water activity

Generally speaking, water activity (a_w) plays a crucial role in enzymatic reactions in organic solvents [13,14]. In our case, it is of great significance to pay attention to water control since the presence of water may also promote the competitive hydrolysis of both 5′-*O*-octanoyl-ara-C and vinyl caprylate. Novozym 435-catalyzed acylation of ara-C with vinyl caprylate showed a clear a_w dependence in 28% (v/v) hexane–pyridine co-solvent system (Table 4). Both the initial rate and the substrate conversion increased with increasing a_w and reached maximum at a_w = 0.03, while rather low reaction rate and substrate conversion were observed when the reaction was carried out in medium with lower or especially higher a_w values. This is because the presence of water in the reaction medium is essential for the enzyme to keep its catalytic conformation. On the other hand, water promotes the hydrolysis reactions of both the product and the vinyl caprylate.

3.5. Effect of reaction temperature

Temperature has a great effect on the activity, selectivity and stability of a biocatalyst. As shown in Fig. 1, within the range from 20 to 40 °C, higher temperature resulted in both the higher initial reaction rate and the substrate conversion in 28% (v/v) hexane–pyridine. Further rise in temperature beyond 40 °C can pull down the reaction in above-mentioned co-solvent mixtures. The regioselectivity

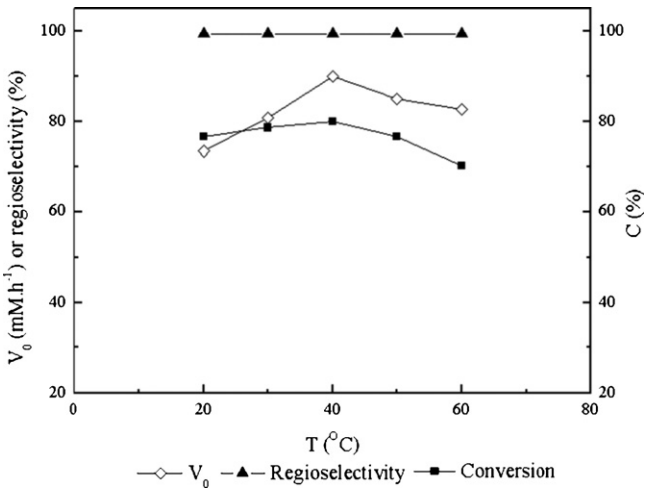

Fig. 1. Effect of reaction temperature on enzymatic acylation of ara-C with VC. The reaction conditions: 0.02 mmol ara-C; 0.2 mmol VC; 500 U Novozym 435; 200 rpm; a_w = 0.03; 1 mL hexane–pyridine (28:72, v/v).

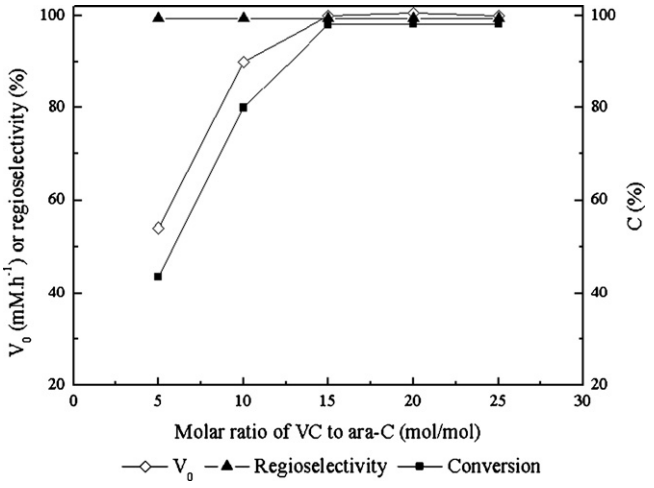

Fig. 2. Effect of the molar ratio of VC to ara-C on enzymatic acylation of ara-C with VC. The reaction conditions: 0.02 mmol ara-C; 500 U Novozym 435; 40 °C; 200 rpm; a_w = 0.03; 1 mL hexane–pyridine (28:72, v/v).

of the reaction remained above 99% at temperatures ranging from 20 to 60 °C.

3.6. Effect of molar ratio of VC to ara-C

When ara-C concentration was fixed at 20 mmol L^{-1}, the enzymatic acylation of ara-C was greatly affected by the ratio of vinyl caprylate to ara-C. As shown in Fig. 2, remarkable enhancement in both the initial rate and the substrate conversion was observed with the increasing ratio up to 15, at which the initial rate and the substrate conversion were as high as 99.0 mmol L^{-1} h^{-1} and 98%. Excessive amount of vinyl caprylate was necessary for lipase-catalyzed acylation. The reason for this is that the presence of excessive amount of vinyl caprylate inhibits the hydrolysis of the product (5′-*O*-octanoyl-ara-C). Additionally, the hydrolysis of vinyl caprylate might consume considerable amount of vinyl caprylate and lower the acylation rate and substrate conversion. The molar ratio of vinyl caprylate to ara-C had little effect on the regioselectivity of the reaction.

4. Conclusions

The results presented in this paper clearly demonstrate that co-solvent systems can boost markedly the activity of Novozym 435 in acylation of ara-C with vinyl caprylate. Using this strategy, efficiently enzymatic acylation of ara-C without prior modification was achieved, giving regioselectivity and substrate conversion exceeding 99% and 98%, respectively, which are much higher than the corresponding values of chemical processes. This finding further highlights the versatility of lipases and the potential of "solvent engineering" for modulating enzymatic reactions. Detailed investigations, however, will be necessary to gain sufficient knowledge about the impact of co-solvent mixtures on enzymes in general, and especially the interactions of various types of co-solvent mixtures with a wide variety of enzymes.

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