Appl Biochem Biotechnol (2008) 151:21–28
DOI 10.1007/s12010-008-8152-0

Highly Efficient Regioselective Synthesis of 5′-*O*-lauroyl-5-azacytidine Catalyzed by *Candida antarctica* Lipase B

Xi-Yu Chen · Min-Hua Zong · Wen-Yong Lou · Hong Wu

Received: 7 November 2007 / Accepted: 10 January 2008 /
Published online: 16 February 2008

Abstract Enzymatic regioselective acylation of 5-azacytidine with vinyl laurate was successfully conducted with an immobilized lipase from *Candida antarctica* type B (i.e., Novozym 435) for the first time. The acylation of 5-azacytidine took place at its primary hydroxyl group and the desired product 5′-*O*-lauroyl-5-azacytidine could be prepared with high reaction rate, high conversion, and excellent regioselectivity. The influences of several key variables on the enzymatic acylation were also systematically examined. Pyridine was found to be the best reaction medium. The optimum initial water activity, the molar ratio of vinyl laurate to 5-azacytidine and reaction temperature were 0.07, 30:1, and 50 °C, respectively. Under the optimized conditions described above, the initial reaction rate, the substrate conversion, and the regioselectivity were as high as 0.58 mM/min, 95.5%, and >99%, respectively, after a reaction time of around 5 h.

Keywords 5-Azacytidine · Novozym 435 · Organic solvent · Regioselective acylation · Vinyl laurate

Introduction

5-Azacytidine, an analogue of the natural pyrimidine nucleoside cytidine, is employed for the treatment of myelodysplastic syndrome (MDS) [1]. However, it has some disadvantages in clinical application, such as difficulty to traverse biological membranes and skin layers due to poor lipophilicity [2], spontaneous hydrolysis in aqueous solutions and rapid deamination by cytidine deaminase, etc. [1, 3–6]. In order to overcome these problems, 5′-monoester of 5-azacytidine could be used, which is primarily based on the fact that the molecules containing hydroxyl or carboxyl groups can be converted into the corresponding esters with the desired lipophilicity by the selection of an appropriate ester side chain [7].

X.-Y. Chen · M.-H. Zong (✉) · W.-Y. Lou · H. Wu
Lab of Applied Biocatalysis, South China University of Technology, Guangzhou 510640, China
e-mail: btmhzong@scut.edu.cn

5′-Monoester of 5-azacytidine could be synthesized through regioselective acylation of 5-azacytidine. On the other hand, the regioselective acylation of nucleoside is one of the important ways of introducing protecting groups as well as obtaining valuable nucleoside derivatives, and therefore will find wide applications in nucleoside chemistry.

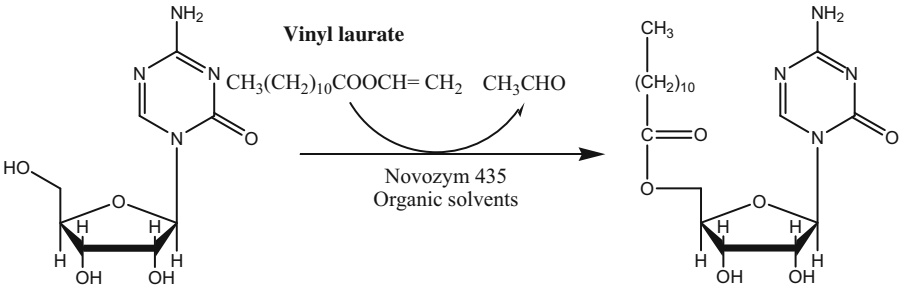
Several strategies for regioselective acylation of nucleosides have been reported using conventional chemical methods [8], but their applications are somewhat hampered due to the relatively low regioselectivity, the lack of easy access to some important intermediates, the tedious product isolation, and the environmental concerns of the process.

To date, enzymatic acylation of nucleosides in organic media has emerged as a promising procedure, due to its advantageous properties including high regioselectivity, mild reaction conditions, and environmental benign [9]. Besides, the use of organic solvents is especially advantageous when substrates or products are unstable in water. Furthermore, at a low water activity, many other water-dependent side-reactions can be prevented [10]. Ferrero and Gotor [11] have reviewed the utility of biocatalysts for the modification of nucleosides.

Various kinds of enzymes have been proven to be capable of catalyzing the acylation of nucleosides with desirable regioselectivity. Among them, Novozym 435, a commercially available lipase from *Candida antarctica* type B (CAL-B) immobilized on a macroporous resin of poly-(methyl methacrylate) (Lewatit VP OC 1600), is well recognized for its extraordinary ability to catalyze the esterification of nucleosides with substantially high regioselectivity [12, 13]. For example, Novozym 435 has been shown in our previous work to be highly active and regioselective for the enzymatic acylation of 1-β-D-arabinofuranosylcytosine [14].

Generally, fatty acid vinyl esters are preferable acyl donors in acyl transfer reactions [15]. In the course of our ongoing investigation, it was found that a great amount of undesired by-products were produced when short-chain fatty acid vinyl esters were used as acyl donors for the acylation of 5-azacytidine, while the use of long-chain fatty acid vinyl esters such as vinyl laurate yielded little by-products. Therefore, vinyl laurate, a typical long-chain fatty acid vinyl ester, is here adopted as an acyl donor for the enzymatic acylation of 5-azacytidine.

As an extension of our ongoing research program on efficient synthesis of various valuable nucleoside derivatives via enzymatic acylation, we herein for the first time report the successful regioselective acylation of 5-azacytidine with vinyl laurate catalyzed by Novozym 435 (Scheme 1) in organic solvents. The enzymatic acylation process might become a new route to the preparation of 5′-*O*-lauroyl-5-azacytidine, which is more lipophilic and might be more bio-available than 5-azacytidine. Also, the effects of several crucial factors on the enzymatic acylation are described in this paper.



Scheme 1 Novozym 435-catalyzed regioselective acylation of 5-azacytidine with vinyl laurate in organic solvents

Materials and Methods

Biological and Chemical Materials

Novozym 435 (an immobilized lipase from *Candida antarctica*, type B, 10,000 U g^{-1}) was kindly donated by Novozymes (Denmark). 5-Azacytidine and vinyl laurate were purchased from Fluka (Germany). All other chemicals were from commercial sources and were of the highest purity available.

Control of the Initial Water Activity

The reaction media, the substrate, and the enzyme were equilibrated to fixed initial water activities (α_w) over saturated salt solutions in closed containers at 25 °C separately [16–20]. The following salts were used: LiBr (α_w=0.07), LiCl (α_w=0.11), CH_3COOK (α_w=0.23), $MgCl_2$ (α_w=0.33). Molecular sieve was used to generate the nearly anhydrous reaction medium ($\alpha_w \sim 0$).

General Procedure for Enzymatic Reaction

In a typical experiment, 2 ml of pyridine containing 0.02 mmol 5-azacytidine, 0.6 mmol vinyl laurate, and 1,000 U Novozym 435 was incubated in a 10 ml Erlenmeyer shaking-flask capped with a septum at 200 rpm and 40 °C. Aliquots (20 μl) were withdrawn at specified time intervals from the reaction mixture, and then diluted by 50 times with a co-solvent mixture of water and methanol prior to HPLC analysis. To obtain larger amounts of product for its structural characterization, the synthesis was scaled up (~25 mg 5-azacytidine and 520 μl vinyl laurate). Upon the completion of the reaction, the reaction mixture was filtered to remove the immobilized enzyme and was evaporated under vacuum. The crude product was then purified by silica gel chromatography with the mixture of methanol and chloroform (25/75, *v*/*v*) as an eluant. After crystallization from ethanol, the product was obtained as a white powder (yield >90%).

HPLC Analysis

The reaction mixture was analyzed by RP-HPLC on a 4.6×250 mm (5 μm) Zorbax SB-C18 column (Agilent Technologies Industries Co., USA) using an Agilent G1311 A pump and a UV detector at 241 nm. The mobile phase was a mixture of ammonium acetate buffer (0.01 M, pH 4.27) and methanol (22/78, *v*/*v*) at a flow rate of 0.9 ml min^{-1}. The retention times for 5-azacytidine and 5′-*O*-lauroyl-5-azacytidine were 2.6 and 11.7 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 upon a certain reaction time according to the literature [21]. The initial rate (V_0) and the substrate conversion (c) were calculated from the HPLC date. The average error for this assay is less than 0.7%. All reported data are averages of experiments performed at least in duplicate.

Structure Determination

Mass spectrometric analysis in the negative ion mode was performed on an ion trap analyzer (Bruker HCTplus, Bruker Co., Germany). The capillary voltage was set at −113.5 V. ESI temperature and ion trap analyzer voltage were 300 °C and −40.0 V, respectively. The product

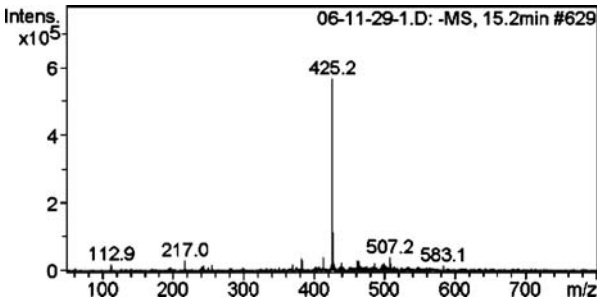


Fig. 1 Representative LC-MS/MS spectra of the main product with negative-ion mode

structure was determined by ^{13}C NMR (Bruker DRX-400 NMR Spectrometer, Bruker Co.) at 100 MHz. DMSO-d6 was used as a solvent and chemical shifts were expressed in ppm shift.

Results and Discussion

Product Characterization

As can be seen in Fig. 1, the molecular weight detected is around 425.2, which indicates that the product obtained is identical with mono lauroyl ester of 5-azacytidine (MW 426).

The ability of Novozym 435 to catalyze regioselective transformation has been exploited in the modification of polyhydroxy compounds [21, 22]. According to the published literature by Yoshimoto et al. [23], the acylation of a hydroxyl group of sugar results in a downfield shift of the peak corresponding to the *O*-acylated carbon atom and an upfield shift of the peak corresponding to the neighboring carbon atom. As evident from the data listed in Table 1, the ^{13}C NMR spectrum of the product shows a shift of 3.01 ppm on C5′ towards the lower fields as compared to the same carbon atom in the unmodified 5-azacytidine. Also, the directly neighboring carbon atom (C4′) gave a shift of about 3.27 ppm towards the higher fields due to the acylation of the hydroxyl group of C5′. In addition, 12 sharp peaks of $-CH_3$, $-CH_2$ and C=O appeared with the determinate chemical

Table 1 ^{13}C NMR spectral data for 5-azacytidine and its acylated derivative (δ, ppm)[a].

Carbon numbers	5-Azacytidine	5′-*O*-Lauroyl-5-azacytidine
Base moiety		
2	153.67	153.29
4	166.18	166.10
6	156.69	156.44
Sugar moiety		
1′	89.64	90.39
2′	74.26	73.79
3′	69.30	69.76
4′	84.65	81.38
5′	60.47	63.48
Acyl moiety		
C=O		169.54
$-CH_3$		13.97
$-CH_2$		22.54~33.91

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

Table 2 Novozym 435-catalyzed regioselective acylation of 5-azacytidine with vinyl laurate in different organic solvents[a].

Media	Solubility of 5-azacytidine (mM)[b]	lg*P*	V_0 (mM min^{-1})	C^c (%)	Regioselectivity (%)
DMSO	254.0	−1.35	0	0	0
DMF	112.9	−1.01	0	0	0
Pyridine	21.8	0.71	0.30	67.0	>99

[a] The reactions were carried out in 2 ml of different organic solvents (α_w=0.11) containing 0.02 mmol 5-azacytidine, 0.4 mmol vinyl laurate and 1000 U Novozym 435 at 200 rpm and 40 °C.

[b] The solubility of 5-azacytidine in each reaction medium was determined by HPLC analysis of the saturated solution at 30 °C.

[c] Maximum substrate conversion

shifts. So the product was proved to be 5′-*O*-lauroyl-5-azacytidine. And Novozym 435 was proved to display a startling regioselectivity up to 99% towards the 5′-hydroxyl group of 5-azacytidine.

It has been reported that *Candida antarctica* lipase B has a rather narrow and deep channel leading to an open active site [24]. The 5′-OH of the sugar moiety of 5-azacytidine may have an easier access to the active site of CAL-B to attack the acyl-enzyme intermediate than other −OH groups at C-3′ and C-2′ due to less steric hindrance, thus resulting in preferential acylation of the 5′-OH of 5-azacytidine.

Effect of Reaction Medium

One of the most troublesome limitations in the acylation of hydrophilic nucleosides is their poor solubility in most organic solvents. In fact, only polar organic solvents, such as pyridine and DMF, have been commonly used to solve the problem [25]. However, polar organic solvents usually strip the essential water off the enzyme molecules and then inactivate the biocatalyst, which greatly limits the application of enzymatic procedures in this area [10]. A less polar solvent does not inactivate the enzyme as much as a more polar one. As shown in Table 2, no reaction occurred in DMSO and DMF, although 5-azacytidine showed high solubility in these solvents. Only in pyridine could the lipase-catalyzed acylation of 5-azacytidine be efficiently carried out. Thus, pyridine was selected as the most suitable solvent for the reaction.

Table 3 Effect of initial water activity on Novozym 435-catalyzed regioselective acylation of 5-azacytidine in pyridine[a].

Initial water activity (α_w)	V_0 (mM min^{-1})	C^b (%)	Regioselectivity (%)
≈0	0.34	80.7	>99
0.07	0.39	84.4	>99
0.11	0.30	67.0	>99
0.23	0.14	14.2	>99
0.33	0.09	9.3	>99

[a] The reactions were performed in 2 ml of pyridine with different initial water activity containing 0.02 mmol 5-azacytidine, 0.4 mmol vinyl laurate and 1000 U Novozym 435 at 200 rpm and 40 °C.

[b] Maximum substrate conversion

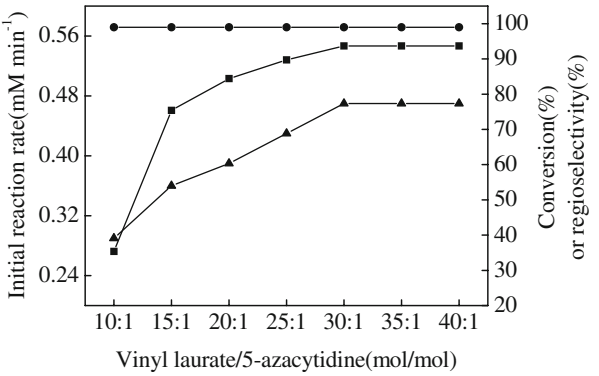


Fig. 2 Effect of the molar ratio of vinyl laurate to 5-azacytidine on the enzymatic regioselective acylation of 5-azacytidine. The reactions were performed in 2 ml of pyridine (α_w=0.07) containing 0.02 mmol 5-azacytidine, 1,000 U Novozym 435 and different amounts of vinyl laurate at 200 rpm and 40 °C. *Filled triangle*, initial reaction rate; *filled square*, conversion; *filled circle*, regioselectivity

Effect of Initial Water Activity

Generally speaking, water activity (α_w) plays a crucial role in enzymatic reactions in non-aqueous media [26–30]. In the case of the enzymatic acylation of 5-azacytidine, the presence of water may foster the competitive hydrolysis of both the desired product 5′-*O*-lauroyl-5-azacytidine and the acyl donor vinyl laurate. Therefore, it is of great importance to investigate the effect of initial water activity on the enzymatic acylation.

As shown in Table 3, Novozym 435-mediated acylation of 5-azacytidine with vinyl laurate shows a clear dependence on the α_w of the reaction system. Both the initial reaction rate and the substrate conversion increased rapidly with increasing α_w value up to 0.07, beyond which further rise in α_w value gave rise to a sharp drop in the initial reaction rate and the substrate conversion. 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 can promote the hydrolysis reactions of both the product and the acyl donor. Therefore, there exists an optimal water activity for the enzymatic acylation. The lower water activity does not provide sufficient water for the buildup of the essential water shell for the enzyme, and the higher water activity implies excessive water and thereby the lower product yield and more inactivation of the enzyme caused by the acid from the competitive hydrolysis of vinyl laurate [31, 32]. Additionally, α_w showed no significant effect on the regioselectivity, which kept above 99% within the range examined. Obviously, the optimum initial water activity for the reaction was 0.07.

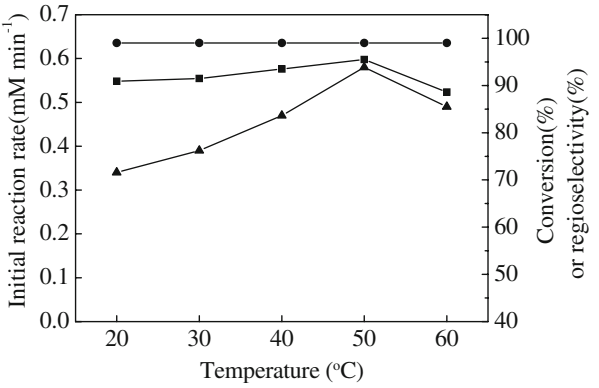


Fig. 3 Effect of reaction temperature on the enzymatic regioselective acylation of 5-azacytidine. The reactions were conducted in 2 ml of pyridine (α_w=0.07) containing 0.02 mmol 5-azacytidine, 0.6 mmol vinyl laurate and 1,000 U Novozym 435 at 200 rpm and various temperatures. *Filled triangle*, initial reaction rate; *filled square*, conversion; *filled circle*, regioselectivity

Effect of the Molar Ratio of Vinyl Laurate to 5-azacytidine

Thermodynamically, high molar ratio of vinyl laurate to 5-azacytidine may push the reaction towards the acylation of 5-azacytidine and speed up the reaction. As depicted in Fig. 2, the enzymatic acylation of 5-azacytidine was greatly affected by the molar ratio of vinyl laurate to 5-azacytidine. Remarkable enhancement in both the initial rate and the substrate conversion was observed with the increase of the ratio up to 30:1, beyond which both the initial rate and the substrate conversion showed no appreciable improvement with further increase in the molar ratio. It was also worth noting that throughout the range of molar ratio of vinyl laurate to 5-azacytidine tested, the regioselectivity manifested no variation and kept above 99%. Therefore, 30:1 was selected as the favorable molar ratio of vinyl laurate to 5-azacytidine for the enzymatic acylation. It is obvious that the excessive amount of vinyl laurate was necessary for the lipase-catalyzed acylation, which was in good accordance with our previous report [30]. Also, it has been proved experimentally that the presence of excessive amount of vinyl laurate inhibits the hydrolysis of the desired product (5′-*O*-lauroyl-5-azacytidine). In addition, the hydrolysis of vinyl laurate might consume considerable amount of vinyl laurate and lower the acylation rate and substrate conversion [32, 33].

Effect of Reaction Temperature

Temperature has great effect on the activity, selectivity and stability of a biocatalyst and the thermodynamic equilibrium of a reaction as well [34]. As show in Fig. 3, within the range from 20 to 50 °C, higher temperature resulted in both higher initial rate and higher substrate conversion. Further rise in temperature, however, led to a drastic drop in both the initial rate and substrate conversion. The regioselectivity of the reaction constantly maintained above 99% at temperatures ranging from 20 to 60 °C. The partial inactivation of the lipase in pyridine at a higher temperature (above 50 °C) may partly account for the drop in both the initial rate and the substrate conversion, which was further supported by assaying the residual activity of the enzyme after being incubated at temperatures higher than 50 °C. Thus, the optimum reaction temperature was shown to be 50 °C.

Conclusions

In summary, the regioselective acylation of 5-azacytidine with vinyl laurate could be successfully performed. Under the optimized conditions, the initial rate, the substrate conversion and the regioselectivity were as high as 0.58 mM/min, 95.5%, and >99%, respectively, after a reaction time of around 5 h. The results described here further highlights the versatility of lipases and show that the enzymatic acylation of nucleosides is a promising area.

Acknowledgement We acknowledge the National Natural Science Foundation of China (Grant No. 20676043), Science and Technology Project of Guangdong Province (Grant No. 2006A10602003; 2007B011000005), Science and Technology Project of Guangzhou (Grant No. 2007Z3-E4101), the Natural Science Foundation of Guangdong Province (Grant No. 05006571), the Doctoral Program of Higher Education (Grant No. 20070561080) and the Open Project Program of the State Key Laboratory of Catalysis, Dalian Institute of Chemical Physics, Chinese Academy of Sciences (Grant No. N-06-06) for financial support.

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