



Short communication

Thermomyces lanuginosus lipase-catalyzed regioselective acylation of nucleosides: Enzyme substrate recognition

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ABSTRACT

Substrate recognition of *Thermomyces lanuginosus* lipase in the acylation of nucleosides was revealed through rational substrate engineering for the first time. *T. lanuginosus* lipase displayed higher catalytic activities and excellent 5'-regioselectivities (94–99%) in the acylation of ribonucleosides **1f–1j** as compared to those in the acylation of 2'-deoxynucleosides **1a–1e**. The higher reaction rates and excellent 5'-regioselectivities might derive from a favorable hydrogen bonding between the 2'-hydroxyl group of **1f–1j** and phenolic hydroxyl group of Tyr21 present in the hydrophilic region of the lipase.

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1. Introduction

Natural nucleosides serve as the building blocks for the biological synthesis of DNA or RNA in the cells. Their analogs such as 1-β-D-arabinofuranosylcytosine (Gunji et al., 1991) and halogenated pyrimidine nucleosides (Heidelberger and Ansfield, 1963; Prusoff, 1959) display antitumor or antiviral bioactivities. However, most of nucleoside drugs exhibited low oral bioavailability in the clinical treatment, due to the low lipid solubility and poor permeability across the cell membrane (Chabner et al., 2001). Additionally, various side effects of these drugs were associated with its clinical application (Grem, 2000). Many efforts have been made to overcome these limitations by chemists. Chemical modification of sugar moiety is one of the successful strategies (Berezovskaya and Chudinov, 2005; De Clercq and Field, 2006). It has been demonstrated that the ester derivatives displayed higher chemotherapeutic efficacy than the parent drugs (Landowski et al., 2005). Valganciclovir (De Clercq, 2004) and valaciclovir (Crooks, 1995) are two classical examples which act as the antiviral alternatives to ganciclovir and acyclovir, respectively. Nevertheless, it is difficult to selectively acylate the desired hydroxyl group of nucleosides through traditional organic synthesis methods owing to the presence of two or three hydroxyl groups with similar chemical

reactivity in the nucleosides. Enzymatic regioselective acylation of the nucleosides has received increasing attentions in synthetic chemistry, due to its simplicity, exquisite selectivity, high efficiency and being environmentally friendly (Diaz-Rodriguez et al., 2005; Liu et al., 2007).

Thermomyces lanuginosus lipase (TLL) is a glycosylated hydrolase with a molecular weight of 30 kDa and an optimum pH of 11–12 (Neves Petersen et al., 2001). The X-ray crystallographic studies have revealed that the active site of TLL comprises two subsites: (a) a hydrophobic region, into which the acyl moiety binds; (b) a hydrophilic region, into which the alcohol moiety fits (Lawson et al., 1994). Although enzymatic regioselective acylation of nucleosides has been well established (Ferrero and Gotor, 2000), there are few reports regarding TLL-catalyzed acylation of nucleosides in the literatures (Wang et al., 2007). Previously, enzymatic regioselective approaches for the acylation of nucleoside analogs such as 1-β-D-arabinofuranosylcytosine (Li et al., 2006a, 2006b, 2006c), 5-fluorouridine (Wang et al., 2007), FUDR and its analogs (Li et al., 2007, 2008a, 2008b, 2009), were developed by our group. In the present work, we continued to focus our interest on the substrate recognition of TLL in the acylation of nucleosides by means of rational substrate engineering (Fig. 1).

The solvent is one of the key factors on the reaction in nonaqueous biocatalysis. The catalytic activity and selectivity of the enzyme such as the enantioselectivity (Tawaki and Klibanov, 1992) and regioselectivity (Rubio et al., 1991) as well as the thermodynamic equilibrium of the enzymatic reaction (Arcos et al., 2001; Otero et al., 2001) could be manipulated by the reaction medium, which is

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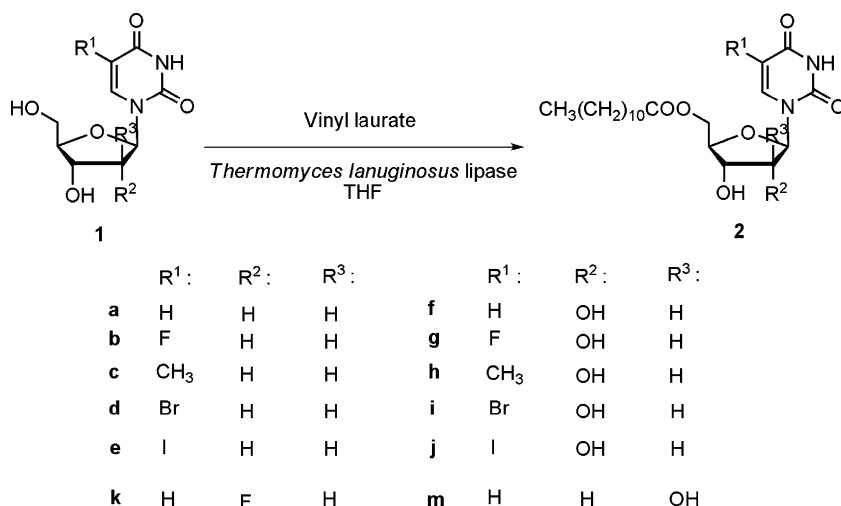


Fig. 1. TLL-catalyzed lauroylation of nucleosides.

known as 'solvent engineering'. Because of the polar nature, nucleosides are poorly soluble in hydrophobic organic solvents, a group of friendly media for the enzyme, while hydrophilic organic solvents usually inactivate the enzyme. The optimal reaction medium was thus screened with TLL-mediated lauroylation of floxuridine **1b** as a model reaction (Table 1). In a previous report, it was demonstrated that TLL displayed no catalytic activity in the acylation of 5-fluorouridine **1g** in highly polar solvents such as pyridine, DMF and DMSO (Wang et al., 2007). Therefore, four less polar solvents were examined. As shown in Table 1, high catalytic activities were observed in the tested solvents as well as good substrate solubility. Unfortunately, TLL exhibited unsatisfactory 5'-regioselectivities (51–71%) in the lauroylation of floxuridine. Among the four solvents, the best result was achieved in THF.

Next, the substrate recognition of TLL was studied with the lauroylation as a model reaction (Table 2). As shown in Table 2, TLL displayed high catalytic activities in the acylation of 2'-deoxynucleosides **1a–1e**, and high substrate conversions (>99%) were achieved within the reaction time of 1.5–3.0 h (entries 1–5). Nevertheless, the 5'-regioselectivities of the enzymatic reactions were low to moderate (49–77%). In addition, the reaction rate and the 5'-regioselectivity showed a clear dependence on the R¹ group of 2'-deoxynucleosides. Both the enzymatic reaction rate and the 5'-regioselectivity decreased with increasing bulk of R¹ group (Table 2, entries 1–5). For example, the highest selectivity was obtained for the enzymatic acylation of 2'-deoxyuridine **1a** with R¹ = H (77%, entry 1), lower for the acylation of floxuridine **1b** with R¹ = F (71%, entry 2), even lower for the acylation of **1c** with R¹ = CH₃ (50%, entry 3) and **1d** with R¹ = Br (59%, entry 4), and the lowest for the acylation of **1e** with R¹ = I (49%, entry 5). The reason might be that the increasing size of R¹ group results in unfavorable steric strain, destabilizing

the conformation of 5'-acylation transition state and increasing the activation energy of the enzymatic reaction.

Interestingly, TLL displayed higher catalytic activities and excellent 5'-regioselectivities (94–>99%) in the acylation of ribonucleosides **1f–1j** as compared to those in the acylation of 2'-deoxynucleosides **1a–1e** (Table 2, entries 1–5 and 6–10). Moreover, the effects of R¹ group on the 5'-regioselectivity in the acylation of ribonucleosides **1f–1j** closely resembled those in the acylation of 2'-deoxynucleosides **1a–1e**. For example, the enzymatic lauroylation of uridine **1f** afforded the highest 5'-regioselectivity (>99%) among **1f–1j** (corresponding to 2'-deoxyuridine **1a** with R¹ = H among **1a–1e**), while the lowest (94%) for the acylation of 5-iodouridine **1j** (corresponding to idoxuridine **1e** with R¹ = I). The unique difference of the structure between the two groups of nucleosides **1a–1e** and **1f–1j** lies in the 2'-substituent. As shown in Table 2, the 2'-hydroxyl group might be the origin of the higher reaction rates and excellent 5'-regioselectivities in TLL-mediated lauroylation of ribonucleosides **1f–1j**. The X-ray crystallographic study has revealed that TLL has a tyrosine residue Tyr21 in the hydrophilic region of the active site, into which the alcohol moiety binds, corresponding to Tyr28 in *Rhizomucor miehei* lipase (Lawson et al., 1994). In addition, the regions of the active sites and the lids are closely similar in the two homologous lipases (Brady et al., 1990; Derewenda et al., 1994). The molecular dynamic simulations of *R. miehei* lipase have revealed that the phenolic hydroxyl group of Tyr28 of the enzyme contributes to the stabilization of the transition state (Norin et al., 1994). It is well known that the hydroxyl group is a good hydrogen bond acceptor or donor. As a result, it is easy to make a hydrogen bond interaction between the 2'-hydroxyl group of ribonucleosides **1f–1j** and phenolic hydroxyl group of Tyr21 of TLL. The extra hydrogen bond might be responsible for the higher reaction rates

Table 1
Effect of organic solvents on TLL-catalyzed lauroylation of floxuridine **1b**^a.

Organic solvent	Solubility ^b (mM)	Time (h)	Conversion ^c (%)	Regioselectivity ^d (%)		
				5'-Ester	3'-Ester	3',5'-Diester
Acetonitrile	40.4	1.0	99	59	2	39
Acetone	81.9	1.5	99	53	14	33
THF	154.9	2.0	99	71	16	13
t-Butanol	31.9	2.0	96	51	11	38

^a The reaction was initiated by adding 60 U TLL (433 U/g) into anhydrous organic solvent (2 mL) containing 0.04 mmol floxuridine and 0.24 mmol vinyl laurate and then the mixture incubated at 40 °C, 250 rpm.

^b At 25 °C.

^c Determined by HPLC analysis using SB-C18 column.

^d Defined as the ratio of the concentration of the desired product to that of all the products, and determined by HPLC analysis using SB-C18 column.

Table 2
Effect of substrate structure on TLL-catalyzed lauroylation of nucleosides^a.

Entry	Nucleoside	Time (h)	Conversion ^b (%)	5'-Regioselectivity ^c (%)
1	1a	1.5	>99	77
2	1b	2.0	>99	71
3	1c	2.0	>99	50
4	1d	2.5	>99	59
5	1e	3.0	>99	49
6	1f	1.5	>99	>99
7	1g	1.5	>99	98
8	1h	1.0	>99	96
9	1i	1.0	99	97
10	1j	1.5	>99	94
11	1k	3.5	99	92
12	1m	1.5	>99	89

^a The reaction was initiated by adding 60 U TLL (433 U/g) into anhydrous THF (2 mL) containing 0.04 mmol nucleoside and 0.24 mmol vinyl laurate and then the mixture incubated at 40 °C, 250 rpm.

^b Determined by HPLC analysis using SB-C18 column.

^c Defined as the ratio of the concentration of the desired product to that of all the products, and determined by HPLC analysis using SB-C18 column.

and excellent 5'-regioselectivities in the enzymatic acylation of ribonucleosides **1f–1j**. Likewise, Gotor and co-workers have proposed that the excellent regioselectivity of *Candida antarctica* lipase B toward 5'-hydroxyl group of thymidine comes from an extra remote interaction between the base moiety of thymidine and the large hydrophobic pocket of the enzyme (Lavandera et al., 2005). Kazlauskas and co-workers attributed the high enantioselectivity of *Pseudomonas cepacia* lipase toward 2-phenoxy-1-propanol to an extra hydrogen bond between the phenoxy oxygen of the substrate to the phenolic OH of Tyr29 of the enzyme (Tuomi and Kazlauskas, 1999).

A slightly lower, yet good 5'-regioselectivity (89%) was achieved in the enzymatic acylation of 1-β-D-arabinofuranosyluracil **1m** (Table 2, entry 12) as compared to that in the acylation of uridine **1f**. The X-ray crystallographic studies have indicated that the conformation of 2'-hydroxyl group of **1m** differs from that of **1f** (Green et al., 1975; Tollin et al., 1973). The distance between the 2'-hydroxyl group of **1m** and phenolic hydroxyl group of Tyr21 of the enzyme might be beyond the range required for a hydrogen bond interaction. As a result, a weaker close interaction rather than hydrogen bond interaction occurred, thus leading to a lower regioselectivity. As can be seen in Table 2, entry 11, in spite of the absence of an extra hydrogen bond between F atom and phenolic hydroxyl group of Tyr21 of the enzyme, TLL still showed a good 5'-regioselectivity (92%) in the acylation of 2'-fluoro-2'-deoxyuridine **1k**. The unexpected fact could be attributed to the inductive effect of 2'-F atom. The F atom with strong electronegativity would withdraw the electrons of the adjacent 3'-hydroxyl group, which decreases remarkably the nucleophilicity of 3'-hydroxyl group (Smith and March, 2007). In contrast, the reactivity of 5'-hydroxyl group was affected marginally due to its position far away from the F atom.

In summary, the catalytic activities and regioselectivities of TLL showed a clear dependence on the substrate structure, especially R¹, R² and R³ group in the acylation of nucleosides. A deep insight was casted into the interactions between the enzyme and the substrates (nucleosides) in the catalytic pathway. The findings would provide a guide to control and maximize the regioselectivity of the synthetically useful enzyme via the chemical modification or protein engineering approaches.

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Appendix A. Supplementary data

HPLC analysis conditions, retention time and characterization data and NMR spectra of the compounds are available as supplementary data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2009.02.003.

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