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Novel L-amino acid ester prodrugs of azacitidine: Design, enzymatic synthesis and the investigation of release behavior

Fan Xu, Ming Zhang, Qi Wu*, Xianfu Lin*

Department of Chemistry, Zhejiang University, Hangzhou, Zhejiang, 310027 PR China

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ABSTRACT

A facile, enzymatic synthesis protocol of L-amino acid ester prodrugs of azacitidine was developed. Firstly, transesterification of azacitidine with Boc protected vinyl aminocarboxylates was performed under the catalysis of subtilisin in anhydrous pyridine at 50 °C. A series of Boc-protected amino acid-azacitidine conjugates were prepared in good regioselectivity and yield. Various factors which influence the catalytic efficiency were systematically investigated. And then, the obtained azacitidine derivatives were subjected to a deprotection process to give L-amino acid ester prodrugs of azacitidine. *In vitro* hydrolysis of prodrugs showed that the amino acid ester prodrugs had obvious sustained release characteristic. These characteristics will have potential value for clinic application.

1. Introduction

Nucleosides are fundamental building blocks for biological systems, with a wide range of biological activity [1,2]. For several decades, numerous nucleoside derivatives have been synthesized as potential anticancer or antiviral medicines [3–7]. Conjugates of nucleoside drugs with amino acids can provide higher bioavailability, antiviral activity and targeting function, lower adverse effects than their parent nucleosides [8–13]. Some examples show their promising application in the designing of prodrugs. Valine esters of ganciclovir (valganciclovir) [12] and acyclovir (valacyclovir) [13] have shown an improvement in absorption, which is attributed to the uptake via intestinal oligopeptide transporters. Fu et al. [9] described the synthesis of bis(L-amino acid) ester prodrugs of 9-[2(phosphonomethoxy)ethyl] adenine, which enhanced the anti-HBV activity of parent drug. Amidon and co-worker reported that amino acid ester prodrugs of gemcitabine [10] and floxuridine [11] could increase the oral absorption and metabolic stability remarkably compared with parent drug.

Azacitidine (5AC; 5-azacytidine; 4-amino-1-β-D-ribofuranosyl-s-triazin-2(1*H*)-one) is a nucleoside analogue of cytidine which is considered as a potential inhibitor of nucleic acid biosynthesis. This investigational antineoplastic agent can be incorporated into the RNA or DNA during transcription or replication [14–17]. It is also the first DNA hypomethylating agent approved by FDA for the treatment of myelodysplastic syndromes with the trade name Vidaza [18,19]. However, azacitidine also presents several significant drawbacks with respect to oral administration, such as sub-optimal physiochemical characteristics, hydrolytic instability, and active enzymatic degradation-all non-conducive to high passive intestinal tract absorption [20–22]. Therefore, strategies which can overcome these drawbacks would be of great benefit. In this regard, amino acid ester prodrug strategies offer maximum flexibility.

For the regioselective modification of polyfunctional compounds such as nucleoside, much attention has been focused on enzymatic reactions in organic media owing to their noticeable advantages over the chemical method. Enzymatic coupling usually can be performed under mild conditions, displays high regio- and stereoselectivity, requires minimal protection and is generally racemization free [23–33]. Moris and Gotor [33] reported an enzymatic regioselective aminoacylation of 2′-deoxynucleosides using oxime aminoacyl esters as acyl donors. Hanson et al. [32] reported on a regioselective aminoacylation of lobucavir by enzymatic transesterification using immobilized lipase from *Pseudomonas cepacia*. Tamarez and co-workers [31] developed an efficient synthetic method for aminoacyl prodrug of ribavirin via *Candida antarctica* lipase catalyzed acylation of ribavirin with the oxime ester of L-Cbz-Ala in anhydrous THF.

In this paper, we developed a facile synthesis of amino acid-azacitidine prodrugs by a regioselective enzymatic strategy combined with a deprotection process (Scheme 1). Various

* Corresponding authors. Tel.: +86 571 87951588; fax: +86 571 87952618.
E-mail addresses: llc123@zju.edu.cn (Q. Wu), xflin@zju.edu.cn (X. Lin).

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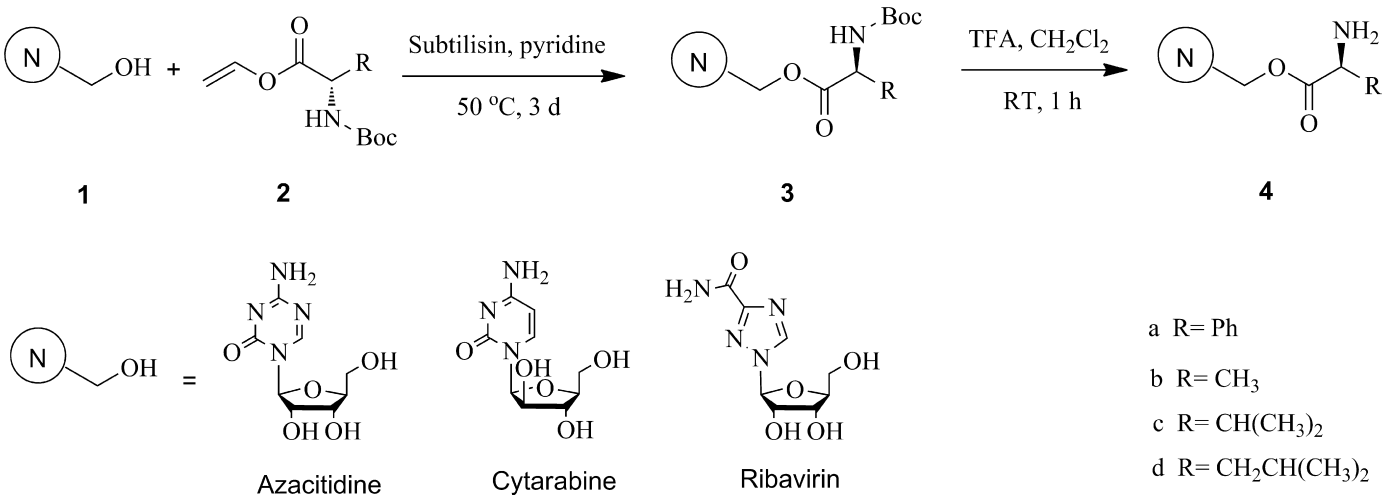
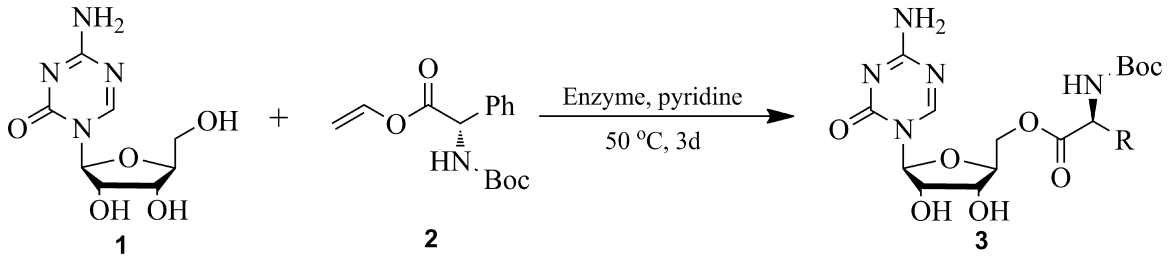


Scheme 1. Synthesis of nucleosides aminoacyl derivatives (**3**) and its amino acid ester prodrugs (**4**).



Scheme 2. Enzymatic synthesis of Boc-protected amino acid-azacitidine conjugates.

factors that influence the catalytic efficiency were evaluated systematically. A series of prodrugs of azacitidine were synthesized with good yields and then subjected to an *in vitro* hydrolysis investigation, which suggested a sustained release behavior with pseudo-first-order kinetics. The comparison of the prodrug to unmodified azacitidine in a pH 7.4 environment showed that the prodrugs could maintain a higher and more stable azacitidine concentration, which was beneficial for clinical application.

2. Results and discussion

2.1. Influence of enzyme source on the preparation of Boc-protected amino acid-azacitidine conjugates

The investigation of synthesis of L-amino acid ester prodrugs was started by the test of enzyme source for catalyzing the transesterification of azacitidine with vinyl *N*-Boc-L-phenylalanyl ester (Scheme 2). As enzymes derived from various sources such as bacteria, yeast, and molds show different properties, including stability in organic solvent, activity, and specificity, 10 commercially available enzymes were screened with pyridine as the reaction media under 50 °C. The results are presented and compared in Table 1. The position of esterification was determined by ^{1}H NMR and ^{13}C NMR spectra of the products. According to the general strategy described by Yoshimoto et al. [34], acylation of a hydroxyl group of substrate results in a downfield shift of the peak corresponding to the *O*-acylated carbon and an upfield shift of the peak corresponding to the neighboring carbon. When subtilisin (alkaline protease from *Bacillus subtilis*) was applied as the catalyst, the product was obtained with the highest yield (82%, entry 10, Table 1). According to the ^{13}C NMR spectrum data of the products, the peak of C-5′ was shifted from 60.5 to 64.3, while the peak of C-4′ was shifted from 84.8 to 80.9, indicating that the acylation occurred at the C-5′ position. The analysis of ^{1}H NMR spectra also confirmed that acylation occurred at the primary hydroxyl group of azacitidine. Thus the major product was the mono-substituted 5′-*O*-aminoacyl-azacitidine with regioselectivity up to 96.5%. The corresponding control experiment in the absence of enzyme barely gave any product with only 4% yield within 72 h. Other enzymes also failed to give any dramatic improvement over this reaction under the giving condition. Immobilized CAL-B (*C. antarctica* lipase B) usually shows great performance in transesterification reactions. Wu and his coworkers also reported a CAL-B catalyzed acylation of 5-azacytidine with fatty acid vinyl esters as the acyl reagent in a pyridine containing co-solvent system with excellent yield and regio-selectivity [27]. However, during this research, CAL-B was also failed to catalyze this reaction successfully with only a yield comparable to the control experiment(4%, entry 3, Table 1), which might be caused

Table 1
Effect of enzyme source on synthesis of azacitidine aminoacyl ester[a].

Entry	Enzyme source	Conv.[b] (%)	Yield[b] (%)
1	Control, no enzyme	4	3
2	Lipozyme® Immobilized from *Mucor miehei*	11	8
3	*Candida antarctica* lipase acrylic resin	4	4
4	Amano lipase M from *Mucor javanicus*	11	10
5	Lipase PS-C Amano I immobilized on ceramic	9	8
6	Lipase from *Candida cylindracea*	6	5
7	Lipase Type VII from *Candida rugosa*	6	5
8	Lipase from porcine pancreas	10	4
9	Lipase from hog pancreas	7	4
10	Alkaline protease from *Bacillus subtilis*	85	82
11	Protease from *Aspergillus melleus*	10	9

[a] Conditions: enzyme (15 mg), azacitidine (0.08 mmol), vinyl *N*-Boc-L-Phe (0.32 mmol), pyridine (1 mL), 50 °C, 72 h.
[b] Determined by HPLC.