

## OPTIMISATION OF THE LIPASE-CATALYSED PREPARATION OF A NUCLEOSIDE PRODRUG MODEL USING AN EXPERIMENTAL DESIGN METHODOLOGY

María A. Zinni, Ernesto M. Aljinovic, Luis E. Iglesias\* and Adolfo M. Iribarren

Centro de Estudios e Investigaciones, Universidad Nacional de Quilmes, Roque Sáenz Peña 180 - (1876) Bernal, Provincia de Buenos Aires - Argentina

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OPTIMISATION OF THE LIPASE-CATALYSED PREPARATION OF A NUCLEOSIDE PRODRUG MODEL USING AN EXPERIMENTAL DESIGN METHODOLOGY. The preparation of 2', 3'-di-*O*-hexanoyluridine (**2**) by a *Candida antarctica* B lipase-catalysed alcoholysis of 2', 3', 5'-tri-*O*-hexanoyluridine (**1**) was optimised using an experimental design. At 25 °C better experimental conditions allowed an increase in the yield of **2** from 80% to 96%. In addition to the yield improvement, the volume reaction could be diminished in a factor of 5 and the reaction time significantly shortened.

Keywords: enzymatic alcoholysis; experimental design; lipases; nucleosides.

## INTRODUCTION

Nucleoside analogues are nowadays active drugs employed in the treatment of many antiviral infections and occupy an appreciable place in the chemotherapy of different diseases<sup>1-3</sup>. The regioselective acylation of pharmacological active nucleosides afford more lipophilic prodrugs of the parent compounds with improved bioavailability<sup>4-6</sup>. However, as nucleosides bear several hydroxyls groups possessing similar reactivity, regioselective transformations of these compounds by conventional chemical procedures are usually unsatisfactory. Mixtures of regioisomers are obtained, rendering poor yields of the desired product and difficulting its isolation; these facts are particularly inconvenient for a preparative purpose.

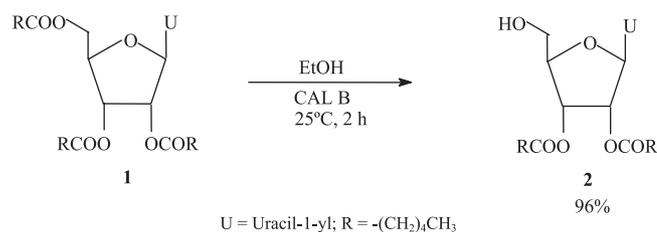
Based on the outstanding regio- and stereoselectivity of the enzymes, biocatalysis provides nowadays a convenient methodology to achieve selective chemical transformations of a wide range of substrates having very different chemical structures, through enzyme-catalysed reactions<sup>7,8</sup>. Moreover, as enzymes are environmental compatible catalysts that display their activity under mild reaction conditions and can be reused many times without a significant loss of activity, biotransformations are very promising procedures to obtain pharmaceutical compounds in a preparative scale.

In the field of nucleosides, the power of biocatalysis is well documented<sup>9-10</sup>. Biotransformations catalysed by hydrolytic enzymes provide convenient methods to achieve regioselective reactions in the sugar moiety of nucleosides. In addition to the usefulness of the described and reviewed enzymatic regioselective acylation and alkoxyacylation of nucleosides<sup>9-10</sup>, the hydrolase-catalysed deacylation of nucleosides has also been studied, mainly through enzymatic hydrolysis of peracylated deoxynucleosides<sup>11-15</sup> and ribonucleosides<sup>14,16,17</sup>.

Taking into account the excellent performance of *Candida antarctica* B lipase in organic syntheses<sup>18</sup>, and the regioselectivity displayed by this biocatalysts towards the 5' hydroxyl of nucleosides in reactions of acylation and alkoxyacylation<sup>7-10,14-16</sup>, over the last years we have been studying the *Candida antarctica* B lipase-

catalysed alcoholysis of peracylated ribonucleosides. This biotransformation has proved to be a simple and convenient access to 2', 3'-di-*O*-acylribonucleosides<sup>19-21</sup>, which can be considered as prodrug models of nucleosides. Thus, the enzymatic alcoholysis provides an improved procedure to prepare compounds with potential medicinal properties.

However, in view to obtain a scaleable process, optimisation of the experimental parameters involved in the biotransformation must be considered. With this aim in mind, we attempted to improve the previously reported<sup>21</sup> preparation of 2', 3'-di-*O*-hexanoyluridine (**2**, Scheme 1), which can be regarded as a nucleoside prodrug model due to its amphiphilic nature. Therefore, the target of the work herein reported was to optimise the enzymatic preparation of this prodrug model through an experimental design based on a response surface methodology<sup>22</sup>.



Scheme 1.

## EXPERIMENTAL PART

## General

Lipase B from *Candida antarctica* (CAL B, Novozyme<sup>®</sup> 435, 9500 PLU mg<sup>-1</sup> solid; PLU: Propyl Laurate Units) was a generous gift from Novozymes. The enzyme was used straight from the bottle.

2', 3', 5'-tri-*O*-hexanoyluridine (**1**) was obtained by treatment of uridine with hexanoic anhydride according to a previously reported protocol<sup>21</sup>.

All employed reagents and solvents were of analytical grade and obtained from commercial sources.

\*e-mail: leiglesias@unq.edu.ar

## Analytical methods

The HPLC system utilised to check the enzymatic alcoholyses consisted of a C-18 column with detection at 254 nm. A 5 min elution gradient of acetonitrile/water from 60:40 (v/v) (10 min) to 80:20 (v/v) (10 min) was utilised with a flow rate of 0.9 ml min<sup>-1</sup>. Retention times (min): **1**, 20.8 ; **2**, 7.5.

## Enzymatic reactions

All the enzymatic reactions were carried out in a temperature-controlled incubator shaker (Sontec OS 11, Argentina) at 200 rpm and at the temperatures indicated in the text.

## Enzymatic alcoholysis of **1**

According to a methodology previously reported by us<sup>21</sup>, experiments of biocatalysed alcoholyses of **1** were performed by adding CAL B (300 mg mmol<sup>-1</sup> substrate) to a suspension of the nucleoside (0.1 mmol) in absolute ethanol at the ethanol/nucleoside (E/N) mmolar ratios pointed out in the text (E/N = 260, 1.52 ml of ethanol) and shaking the resulting mixtures at 200 rpm at the indicated temperatures. At various times, samples of the biotransformations were withdrawn and after removal of the biocatalyst, acetonitrile was added to 100 µL of the supernatant up to a final volume of 1.5 mL. 20 µL of the resulting solution was analysed by HPLC.

## Experimental design

The experimental design was based on a response surface methodology, which consists of a set of mathematical and statistical methods to find combinations of experimental conditions conducting to optimum responses<sup>22</sup>. In our approach, the response was modelled by a first order polynomial obtained through a maximum slope design, on the base of regression analysis of the data. In order to ensure statistical validity, each experiment was carried out by duplicate.

## RESULTS AND DISCUSSION

As previously observed by us<sup>19-21</sup>, CAL B lipase-catalysed alcoholysis of peracylated ribonucleosides showed a high dependence of the regioselectivity on the ethanol/nucleoside (E/N) ratio. Since the reaction required very high E/N ratios and very long reaction times, for an optimised preparation of **2** the effect of three factors was taken into account: E/N ratio ( $x_1$ ), reaction time ( $x_2$ ) and temperature ( $x_3$ ). Therefore, an experimental design was followed as a function of these three variables.

A first set of experiments was conducted at two levels of the E/N ratio, two levels of temperature, including the central point of both intervals, and three reaction time levels (Table 1, Experiments 1-15). A linear regression analysis was carried out to fit a first order polynomial equation to the experimental data points, from which a second set of experiments was then performed (Table 1, Experiments 16-23). A new linear regression analysis provided the equations and statistical parameters detailed in Table 2. These equations suggested to carry out new experiments in the interval of E/N = 260-265 and in the interval of temperatures 18-27 °C, but showed no significant dependence of the product yield on the reaction time in the explored interval. Thus, we decided to perform experiments at E/N = 260 at two temperatures: 20 and 25 °C, at short reaction times. The obtained results (Table 1, Experiments 24-27) show a significant improvement of yield: 96.2% in 2 hours at 25 °C and 99.2% in 30 minutes at 20 °C, revealing that the experimental design here provided an useful tool

**Table 1.** Experimental design<sup>a</sup>

Experiment	$x_1$ <sup>b</sup>	$x_2$ (°C) <sup>c</sup>	$x_3$ (h) <sup>d</sup>	<b>2</b> (%) <sup>e</sup>	Standard deviation
1	300	30	3	82.3	0.9
2	300	50	3	84.3	4.5
3	300	30	4	88.3	1.1
4	300	50	4	87.5	0.4
5	300	30	5	89.7	0.1
6	300	50	5	89.0	0.04
7	500	40	3	72.7	3.1
8	500	40	4	78.0	9.6
9	500	40	5	83.0	0.6
10	700	30	3	51.1	3.0
11	700	50	3	57.6	14.0
12	700	30	4	62.6	2.6
13	700	50	4	67.8	15.9
14	700	30	5	65.8	6.0
15	700	50	5	72.9	15.2
16	300	30	5	90.0	0.4
17	300	40	5	85.0	1.6
18	200	30	5	88.7	0.5
19	200	40	5	78.0	0.7
20	300	30	6	89.7	0.3
21	300	40	6	87.1	0.4
22	200	30	6	89.3	0.6
23	200	40	6	76.9	0.4
24	260	20	0.5	99.2	0.9
25	260	20	2	97.8	0.4
26	260	25	2	96.2	0.3
27	260	25	5	95.8	0.7

<sup>a</sup> Typical procedure: see Experimental; <sup>b</sup>  $x_1$ : Ethanol/nucleoside (E/N) ratio; <sup>c</sup>  $x_2$ : Temperature; <sup>d</sup>  $x_3$ : Reaction time; <sup>e</sup> Determined by HPLC; in each entry, the reported value corresponds to the average yield of the experiments. In all cases, reaction mixtures consisted only of **2** and unreacted substrates.

**Table 2.** Equations fitted to the experimental data points and statistical parameters<sup>a</sup>

Factor	Equation	Typical error	P-value
$x_1$ <sup>b</sup>	$x_1 = 255.56 + 4.48 h$	1.10	0.0011
$x_2$ <sup>c</sup>	$x_2 = 5.44 + 0.62 h$	1.10	0.58
$x_3$ <sup>d</sup>	$x_3 = 34.45 - 7.40 h$	1.10	9.6 E-06

<sup>a</sup> General type of the equations:  $x_i = x_{oi} + k_i h$ ;  $k_i$ : maximum slope value;  $h$ : increment. <sup>b</sup>  $x_1$ : Ethanol/nucleoside (E/N) ratio. <sup>c</sup>  $x_2$ : Reaction time expressed in hours. <sup>d</sup>  $x_3$ : Temperature expressed in °C.

for the optimised preparation of **2**. As far as we know, this is the first report of the application of this methodology to biotransformations of nucleosides.

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