Biotechnol Lett
DOI 10.1007/s10529-006-9053-3

ORIGINAL PAPER

# Microbial hydrolysis of acetylated nucleosides

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Received: 22 December 2005 / Accepted: 22 March 2006


**Abstract** Enzymatic hydrolysis of acetylated nucleosides using microbial whole cells has been carried out for the first time. Unlike *Candida antarctica* B lipase-catalysed alcoholysis, none of the tested microorganisms displayed a common deacetylation profile. Depending on the substrate and the biocatalyst used, 5′-selective deprotection or mixtures of mono *O*-acetylated products were obtained.

**Keywords** Deacylation · Hydrolases · Nucleosides · Whole cells

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## Introduction

Nucleoside analogues are potent pharmacological compounds displaying antiviral and antitumoral activities (De Clerq 2005). Due to their complex chemical structure, it is desirable to count on stereo- and regioselective reactions in order to avoid both by-products formation and the use of protecting groups. It is well recognized nowadays that biotransformations provide efficient procedures in organic synthesis owing to the high selectivity of enzymes; particularly, in the field of nucleosides the potential of biocatalysts has been reviewed (Utagawa 1999; Ferrero and Gotor 2000).

Over the past few years, our research group has been involved in the use of biotransformations in nucleoside chemistry. Applying biocatalytic procedures, we have synthesized purine nucleosides through biocatalysed transglycosylation employing microbial whole cells (Rogert et al. 2002; Trelles et al. 2005; Bentancor et al. 2004) and studied the enzymatic deacylation of nucleosides using isolated hydrolases. Regarding to the latter biotransformation, we found that *Candida antarctica* B lipase (CAL B) catalysed alcoholysis of acylated ribo- and deoxyribonucleosides afforded regioselectively 2′,3′-di-*O*-acyl-ribonucleosides (Iglesias et al. 2000; Zinni et al. 2002) and 3′-*O*-acetyl-2′-deoxynucleosides (Zinni et al. 2004), respectively. The interest in

regioselectively acylated nucleosides relies on their potential as prodrugs of biological active compounds due to improved bioavailability (Testa and Mayer 2003).

On the other hand, the enzymatic hydrolysis provided a mild and quantitative procedure for the full deacetylation of labile substrates (Roncaglia et al. 2001).

In order to explore new sources of hydrolases displaying wider activities than those exhibited by commercially available enzymes, we thought of interest to screen microbial whole cells for hydrolytic activity. Some recent papers deal with ester microbial hydrolysis (Kristová et al. 2005; Romano et al. 2005) but to the best of our knowledge, such a reaction has not been yet applied to acylated nucleosides. In this work, we report the results obtained in a primary screening for the microbial hydrolysis of a set of acetylated nucleosides.

## Material and methods

### Chemicals and microorganisms

Acetonitrile was dried by heating, under reflux with calcium hydride and distilled at atmospheric pressure. HPLC-grade acetonitrile was from Riedel-de Haën. Uridine, adenosine, guanosine, cytidine and inosine were of the best analytical grade (Pharma Waldhold, Sigma, Aldrich Chemicals Co. or Fluka AG). Lipase B from *Candia antarctica* (CAL B, Novozymes 435, 10,000 PLU/mg solid; PLU: propyl laurate units) was a generous gift from Novozymes (Brazil).

TLC was performed on Silicagel 60 $F_{254}$ plates (Merck) and column chromatography was carried out using silicagel Merck 60.

NMR spectra were recorded on a Bruker AC-500 spectrometer in $CDCl_3$, at 500 MHz for $^{1}H$ and 125 MHz for $^{13}C$ using TMS and $CDCl_3$ as internal standards, respectively.

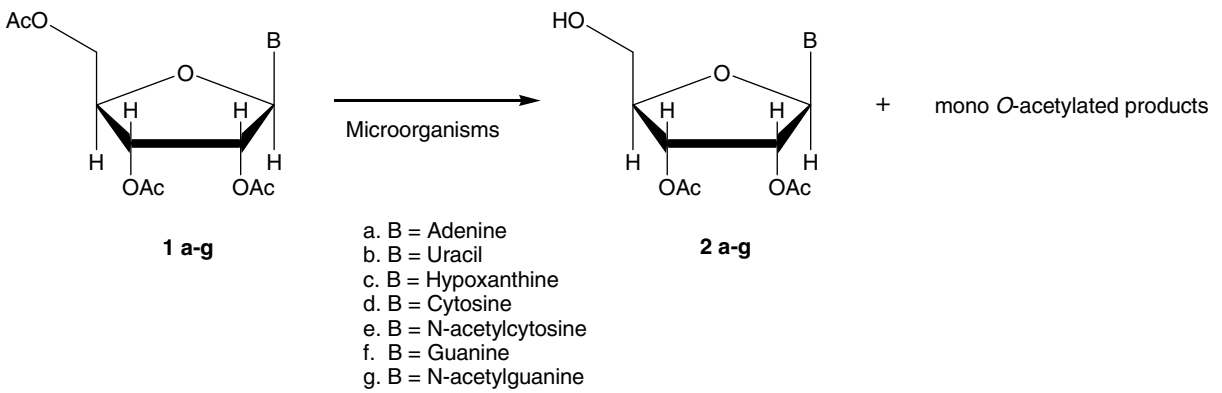
The culture media components were obtained from Merck and Difco. Most of the microorganisms were a kind gift from the Colección Española de Cultivos Tipo (CECT), Universidad de Valencia (Spain).

### Preparation of substrates

2′,3′,5′-Tri-*O*-acetylribonucleosides (**1a–d,f**) were prepared through reaction of the corresponding nucleosides with an excess of acetic anhydride and triethylamine in acetonitrile at room temperature according to previously reported protocols (Iglesias et al. 2000; Zinni et al. 2004; Matzuda et al. 1986), while the tetraacetylated compounds **1e** and **1g** were obtained by heating at 50°C (Zinni et al. 2004). (Scheme 1)

### Preparation of reference samples **2a–g**

According to protocols previously described by us (Iglesias et al. 2000; Zinni et al. 2002, 2004; Roncaglia et al. 2001), 100 mg acetylated nucleoside were dissolved in ethanol (24 ml) and CAL B (120 mg) was added. The reaction mixture was shaken at 200 rpm at 37°C; samples were taken at different times and monitored by TLC. When maximum conversion was observed,




**Scheme 1**

the reaction was stopped by filtration and ethanol evaporated. The crude mixture was chromatographically purified by silica gel column using mixtures of dichloromethane/methanol as eluting solvents. The structures of the resulting purified products were elucidated by $^{1}$H- and $^{13}$C-NMR.

### Microbial growth conditions

Microorganisms were cultured in liquid media at the below detailed optimal temperature ($T$) and time ($t$) for each genera, according to the American Type Culture Collection (ATCC): *Aeromonas* ($T$: 30°C, $t$: 1 day), *Pseudomonas* ($T$: 26°C, $t$: 1 day), *Bacillus* ($T$: 30°C, $t$: 1 day), *Achromobacter* ($T$: 30°C, $t$: 2 days), *Citrobacter* ($T$: 37°C, $t$: 1 day), *Enterobacter* ($T$: 37°C, $t$: 1 day), *Klebsiella* ($T$: 37°C, $t$: 2 days), *Escherichia* ($T$: 37°C, $t$: 1 day), *Proteus* ($T$: 37°C, $t$: 1 day), *Xanthomonas* ($T$: 26°C, $t$: 1 day), *Cellulomona* ($T$: 30°C, $t$: 1 day), *Staphylococcus* ($T$: 37°C, $t$: 1 day), *Micrococcus* ($T$: 30°C, $t$: 1 day), *Agrobacterium* ($T$: 26°C, $t$: 2 days) and *Serratia* ($T$: 26°C, $t$: 5 days) were grown in Luria Broth medium; *Erwinia* ($T$: 30°C, $t$: 1 day) and *Arthrobacter* ($T$: 26°C, $t$: 2 days) in Agar II; *Corynebacterium* ($T$: 30°C, $t$: 2 days) and *Brevibacterium* ($T$: 30°C, $t$: 2 days) in *Corynebacterium* medium, *Lactobacillus* ($T$: 37°C, $t$: 1 day) in MRS broth (oxoid CM359); *Streptomyces* ($T$: 28°C, $t$: 5 days) in *Streptomyces* medium, while *Nocardia* ($T$: 30°C, $t$: 1 day) in YEME (Bennett's agar) medium.

The saturated cultures broths were centrifuged at 12,000 *g* for 10 min and the pellets used as the biocatalysts.

### Typical procedure for whole cell-catalysed biotransformations

The biotransformations were carried out by adding a 1 mM solution of the substrate in potassium phosphate buffer (pH 7, 30 mM) to the pellets containing $1.5 \times 10^{10}$ cells, to complete a reaction volume of 3 ml. The resulting mixtures were kept at 30°C and 200 rpm in an orbital shaker. Samples (50 µl) were taken at different times, centrifuged at 12,000 *g* and the supernatants analysed by both TLC and HPLC.

### Analytical methods

TLC was performed on silica plates, using mixtures of dichloromethane/methanol as the mobile phase. HPLC was carried out employing a C-18 column with detection at 254 nm. For the analysis of the samples from the microbial hydrolysis of each substrate, the following operating conditions were used:

*2′,3′,5′-Tri-O-acetyladenosine (**1a**)* and *2′,3′,5′-tri-O-acetyluridine (**1b**)*: (1) 2 min water/acetonitrile (95:5, v/v), (2) 2 min gradient to water/acetonitrile (60:40, v/v), (3) 3 min water/acetonitrile (60:40, v/v); flow rate 1 ml min$^{-1}$.

*2′,3′,5′-Tri-O-acetylinosine (**1c**)*: (1) 3 min water/acetonitrile (95:5, v/v), (2) 2 min gradient to water/acetonitrile (60:40, v/v), (3) 1 min water/acetonitrile (60:40, v/v); flow rate 1 ml min$^{-1}$.

*2′,3′,5′-Tri-O-acetylcytidine (**1d**)* and *4-N-acetyl-2′,3′,5′-tri-O-acetylcytidine (**1e**)*: (1) 2 min water/acetonitrile (95:5, v/v), (2) 2 min gradient to water/acetonitrile (60:40, v/v), (3) 1 min water/acetonitrile (60:40, v/v); flow rate 1 ml min$^{-1}$.

*2′,3′,5′-Tri-O-acetylguanosine (**1f**)* and *2-N-acetyl-2′,3′,5′-tri-O-acetylguanosine (**1g**)*: (1) 3 min water/acetonitrile (95:5, v/v), (2) 2 min gradient to water/acetonitrile (60:40, v/v), (3) 2 min water/acetonitrile (60:40, v/v); flow rate 1 ml min$^{-1}$.

## Results and discussion

To study the deacetylation of nucleosides using bacterial whole cells, 2′,3′,5′-tri-*O*-acetyladenosine (**1a**), 2′,3′,5′-tri-*O*-acetyluridine (**1b**), 2′,3′,5′-tri-*O*-acetylinosine, (**1c**), 2′,3′,5′-tri-*O*-acetylcytidine (**1d**), 4-*N*-acetyl-2′,3′,5′-tri-*O*-acetylcytidine (**1e**), 2′,3′,5-tri-*O*-acetylguanosine (**1f**) and 2-*N*-acetyl-2′,3′,5-tri-*O*-acetylguanosine (**1g**) were used as substrates (Scheme 1).

Since bacterial whole cells had not been previously used for acylated nucleoside hydrolysis, different types of biocatalysts (wet paste or lyophilized cells) and reaction media (water or phosphate buffer) were first assayed. Since intracellular hydrolytic activity of *Bacillus stearothermophilus* has been previously reported (Molinari et al. 1999), this microorganism was tested as the biocatalyst for the deacetylation of **1b**. When

lyophilized bacteria were used, the enzymatic activity was smaller than the obtained with bacterial wet paste. Regarding to the biotransformation media, faster hydrolysis rate was reached when buffer phosphate was used instead of water. Thus, further studies were carried out using wet cell paste in phosphate buffer. Different genera and species of microorganisms from our cell collection were tested and a primary screening was carried out with all substrates in order to select the most suitable biocatalyst for each nucleoside.

The regioselective preparation of 2′,3′-di-*O*-acetylnucleosides (**2**) could only be achieved when **1a** and **1b** were used as the substrates and yields over 70% were obtained with *Cellulomona celulans* and *Klebsiella* sp. as biocatalysts, respectively (Entries 1, 2; Table 1). The choice of the microorganism had a dramatic effect on the products; to provide some examples, when the hydrolysis of **1a** was conducted using *Proteus rettgeri, Aeromonas salmonicida* and *Enterobacter cloacae* mixtures of different diacetylated compounds were formed unselectively (data not shown), while *Aeromonas salmonicida* catalysed hydrolysis of **1b** afforded a mixture of mono *O*-acetylated products in 80% yield (Entry 3).

Starting from **1c**, **1d** or **1f**, mixtures of mono-acetylated compounds (Entries 4, 5, 7) were obtained in yields higher than 50%. For these cases, *Enterobacter cloacae, Achromobacter cyicloclastes* and *Sthaphylococcus capitis* were the microorganisms, respectively selected.

Tetraacetylated compounds **1e** and **1g** were also employed as substrates, but none of the microorganisms hydrolysed the amide group. Mixtures of *N*-acetyl mono-*O*-acetylated products (Entries 6, 8) were obtained following the same reaction profile as for the biotransformations of **1d** and **1f.**

It is worthwhile mentioning that in all cases, the mono-*O*-acetylated products reported were obtained as mixtures of isomers. Since no selectivity was observed towards the formation of one isomer, probably also due to acetyl migration processes, the reported mixtures were not further characterized.

**In summary**, in this work we report for the first time the enzymatic deacetylation of nucleosides using microbial whole cells. Testing hydrolytic activities without isolating the involved enzymes may help to widen the already available enzymatic diversity. The results herein presented show that, unlike CAL B-catalysed alcoholysis, in which a systematic behaviour towards the removal of the 5′-acyl group is observed (Iglesias et al. 2000; Zinni et al. 2002, 2004; Roncaglia et al. 2001), none of the tested microorganisms displayed a common reaction profile for the deacetylation of all the assayed substrates. Moreover, it is not possible to correlate the

**Table 1** Microbial hydrolysis of acetylated nucleosides[a]

| Entry | Microorganism[b] | Substrate | Activity (μmol[c] $h^{-1}$ $1.5 \times 10^{10}$ $cells^{-1}$) | Yield[d] (% product) | Time (h) |
|---|---|---|---|---|---|
| 1 | *Celullomona celulans* | **1a** | $1.8 \times 10^{-5}$ | 79 (**2a**[e]) | 96 |
| 2 | *Klebsiella* sp. | **1b** | $1.25 \times 10^{-5}$ | 71 (**2b**[e]) | 48 |
| 3 | *Aeromonas salmonicida* | **1b** | $8.22 \times 10^{-6}$ | 80 (mixture[f]) | 144 |
| 4 | *Enterobacter cloacae* | **1c** | $5.62 \times 10^{-6}$ | 54 (mixture[f]) | 96 |
| 5 | *Achromobacter cycloclastes* | **1d** | $1.5 \times 10^{-5}$ | 62 (mixture[f]) | 48 |
| 6 | *Achromobacter cycloclastes* | **1e** | $6.25 \times 10^{-6}$ | 60 (mixture[f]) | 96 |
| 7 | *Staphylococcus capitis* | **1f** | $1.43 \times 10^{-5}$ | 60 (mixture[f]) | 144 |
| 8 | *Staphylococcus capitis* | **1g** | $3.68 \times 10^{-6}$ | 53 (mixture[f]) | 144 |

[a]Reaction mixture comprising 1 mM starting nucleoside, 30 mM potassium phosphate buffer (pH 7) and microbial cells in a total volume of 3 ml was incubated at 30°C and shaken at 200 rpm

[b]The reported microorganism corresponds to the biocatalyst affording the best yield

[c]Defined as μmoles of hydrolysed acetates

[d]Determined by HPLC

[e]Identified by comparison with reference samples

[f]A mixture of mono *O*-acetylated products was obtained

hydrolytic activity (Table 1) with regioselectivity and reaction times.

Besides, since **1g** had not been assayed in our previous studies on enzymatic alcoholysis of nucleosides, we also tested its CAL B-catalysed ethanolysis, isolating *N*-acetyl-2′,3′-di-*O*-acetylguanosine in 40% yield after 72 h at 30°C and confirming the same regioselectivity.

**Acknowledgements** This work was supported by Universidad Nacional de Quilmes and Secretaría de Ciencia y Técnica de la Nación, Argentina. ESL, LEI, JMM and AMI are research members of CONICET, Argentina.

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