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Journal of Molecular Catalysis B: Enzymatic 29 (2004) 3–7

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Benzimidazole as deazapurine analogue for microbial transglycosylation

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Received 3 July 2003; received in revised form 23 September 2003; accepted 15 October 2003

Available online 12 April 2004

Abstract

Benzimidazole nucleosides are compounds with interesting pharmacological properties. Although chemical synthesis of these analogues has been carried out, only few derivatives have been obtained so far using biotransformations. We report herein the biocatalysed preparation of benzimidazole ribo- and 2′-deoxyribonucleosides by different microbial whole cells that afforded yields between 75 and 85%.
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Keywords: Benzimidazole; Whole cells; Microbial transglycosylation; Nucleoside analogues

1. Introduction

Benzimidazole derivatives have received attention in medicinal chemistry because they are involved in different biological activities such as inhibition of phosphodiesterase IV [1] and proton pumps [2] and also display antiarrythmic [3], antihelmintic [4] and antiviral [5] properties.

Benzimidazole nucleosides are particularly attractive as potential antiviral agents because of their stability toward some enzymes involved in nucleosides inactivation e.g. adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP).

Different modifications on this heterocycle have been reported [6] with the aim of decreasing the cytotoxicity and increasing bioavailability [7]. For example, polyhalogenated benzimidazole nucleosides [8] and modified benzimidazole L-nucleosides [9] have been used for treating herpes and citomegalovirus infection. Although these compounds have been synthesised by chemical methods, the yields achieved were low because many stereo- and regiocontrolled steps are involved [10–13]. Therefore, biotransformations provide an interesting alternative route. It has already been shown that benzimidazole derivatives have good affinity for PNP [14] but on the contrary, the corresponding ribosides are poor substrates for the phosphorolysis due to the higher stability of their glycosidic bond compared to those of purine nucleosides [15]. Benzimidazole ribo- and 2′-deoxyribosides have previously been synthesised in high yields using *Escherichia coli* as the biocatalyst and guanosine and 2′-deoxyguanosine as the pentafuranosyl donors, respectively [14]. Since the low solubility of these starting materials may be a disadvantage for preparative purposes, we decided to apply our screening methodology to the search of microorganisms capable of using readily available and soluble reagents such as uridine and thymidine for the synthesis of benzimidazole nucleosides (Scheme 1). We also report herein the optimisation of some experimental conditions involved in the preparation of these compounds.

2. Experimental

2.1. Chemicals and microorganisms

Nucleosides and bases were purchased from Sigma, Aldrich Chemicals Co. or Fluka AG. The culture media components were obtained from Merck and Difco. HPLC grade methanol and acetonitrile were from Riedel-de Haën. Most of the microorganisms were supplied by the Sociedad Española de Microbiología.

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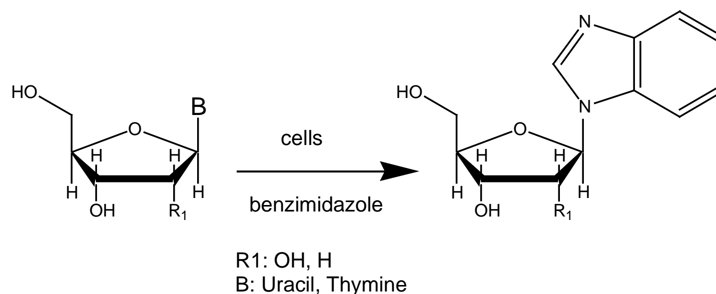
doi:10.1016/j.molcatb.2003.10.007



Scheme 1.

2.2. Growth conditions

All the microorganisms used in this study belong to our cell collection. The strains were cultured in liquid medium, at the below detailed optimal temperature (*T*) and time (*t*), until saturation conditions. The saturated culture broths were collected, centrifuged at 12 000 × *g* for 10 min and the pellets used as the biocatalyst.

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*: 30 °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), *Xanthomona* (*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) strains 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 Agar *Lactobacillus* (*T*: 37 °C, *t*: 1 day) in Mrs Broth (oxoid CM359) medium; *Thermoactinomyces* (*T*: 50 °C, *t*: 2 days) in Agar triptona soya (oxoid M131); *Streptomyces* (*T*: 28 °C, *t*: 5 days) in Streptomyces medium; *Norcardia* (*T*: 30 °C, *t*: 1 day) and *Thermonospora* (*T*: 50 °C, *t*: 2 days) in YEME (Bennett's agar) medium, while *Alicyclobacillus* (*T*: 55 °C, *t*: 3 days) in *Alicyclobacillus acidocaldorius* medium.

2.3. Biotransformation (standard conditions)

The standard reaction mixture comprising: wet cell paste containing 9×10^8 cells, 10 mM of uridine or thymidine, 10 mM of benzimidazole and 30 mM pH 7 potassium phosphate buffer (final volume 500 μl), was stirred at 200 rpm and 60 or 45 °C. Samples were centrifuged at 10 000 × *g* for 30 s and the supernatants were analysed by both TLC and HPLC. The products were further characterised by MS.

2.4. Analytical methods

In all cases, TLC was performed on silicagel plates and a mixture of chloroform:methanol (85:15, v/v) was used as the mobile phase. For semiquantitative analysis, 5 μl calibrated capillary tubes were used to apply the samples on the adsorbent and spot intensities were compared with standard concentration solutions of products.

HPLC analysis was performed with a C-18 column (250 mm × 4 mm) at a flow rate of 0.9 ml min^{-1}. The UV detector was set at 254 nm and the column was operated at room temperature. The other operating conditions were as follows: 3 min water/methanol (85:5, v/v), 7 min gradient to water/methanol (40:60, v/v), and 5 min water/methanol (40:60, v/v).

Mass spectra of isolated ribo- and 2′-deoxyribobenzimidazole nucleosides were carried out using ZAB-VSEQ hybrid mass spectrometer (Fisons, VG Analytical, Manchester, UK) with BEqQ geometry, working only with B and E sectors, at an acceleration voltage of 8 kV. FAB spectra were obtained with a Cesium ion gun, accelerated at 25 kV, affording the expected molecular mass.

3. Results and discussion

We have recently developed screening methodologies for the search of microbial whole cells capable of synthesising natural and modified purine nucleosides from available precursors [16–18]. The enzymatic synthesis involves the activity of nucleoside pyrophosphorylases that make purine–pyrimidine interchange in one or two steps, depending on the selected microorganisms.

E. coli BMT-1D/1A [14] has been previously used to produce benzimidazole riboside and 2′-deoxyriboside from guanosine and deoxyguanosine in 70 and 90% yield, respectively. In the present work, available and more soluble nucleosides, uridine and thymidine, are used as reagents.

The results of the screening for benzimidazole riboside are shown in Table 1. Although more than 100 strains were screened, only those that produced the target nucleosides are included in the table.

Aeromonas hidrophila CECT 4226 was selected as the best producer and it was further used to study the optimisation of experimental conditions.

The influence of the temperature was assessed carrying out the biotransformation at 25, 37, 45 and 60 °C. Fig. 1 shows the time course of reaction at the tested conditions. The velocity as a function of the temperature is also shown.

Table 1
Results of screenings for the preparation of benzimidazole nucleosides

Microorganisms	Benzimidazole riboside, *T*: 60 °C		Benzimidazole 2′-deoxyriboside, *T*: 45 °C	
	Yield (%)	Time (h)	Yield (%)	Time (h)
Pseudomonas putida (CECT324)	0	–	77	5
Citrobacter koseri (CECT856)	6	3	78	24
A. hidrophila (CECT4226)	70	24	60	24
Klebsiella sp. (CECT367)	31	24	63	24
Chromobacter violacium (CECT4226)	61	24	82	24
Streptomyces badius (CECT3275)	6	24	83	24
Enterobacter aerogenes (CECT684)	13	5	75	24
Proteus rettgeri (CECT171)	26	24	35	24
Aeromonas salmonicida (CECT896)	56	24	61	24
A. hidrophila (CECT839)	12	5	ND	–
Proteus vulgaris (CECT174)	15	5	82	5
Citrobacter freundii (CECT401)	66	48	65	24
Streptomyces sp. (CECT3145)	6	5	ND	–
S. rubidae (CECT868)	17	24	84	5
E. coli (CECT105)	3	3	ND	–
Erwinia amylovora (CECT222)	15	3	74	24
Bacillus cereus (CECT193)	2,7	3	ND	–
Enterobacter cloacae (CECT194)	23	2	81	24
A. hidrophila (CECT4225)	8	3	0	–
Arthrobacter oxydans (CECT387)	0	–	84	24
E. coli BL21 (ATCC 47092)	3	3	62	5
Staphylococcus capitis (CECT233)	4	3	84	24
Pseudomonas stuzeri (CECT930)	0	–	72	5
Micrococus luteus (CECT241)	6	3	0	–

The optimal temperature found was 60 °C, as for most of the purine ribosides reported in the literature [4,19].

Another studied variable was the nucleoside:base (N:B) ratio (Table 2). The results show that both yield and reaction rate do not significantly depend on the N:B proportion. Therefore, in order to avoid reagent excess, the selected condition was 1:1. This result is also an improvement respect to

Table 2
Effect of uridine:benzimidazole ratio (N:B) on the production of benzimidazole riboside

(N:B)ratio	Yield (%)	Time (h)
1:1	78	32
2:1	74	24
3:1	70	24

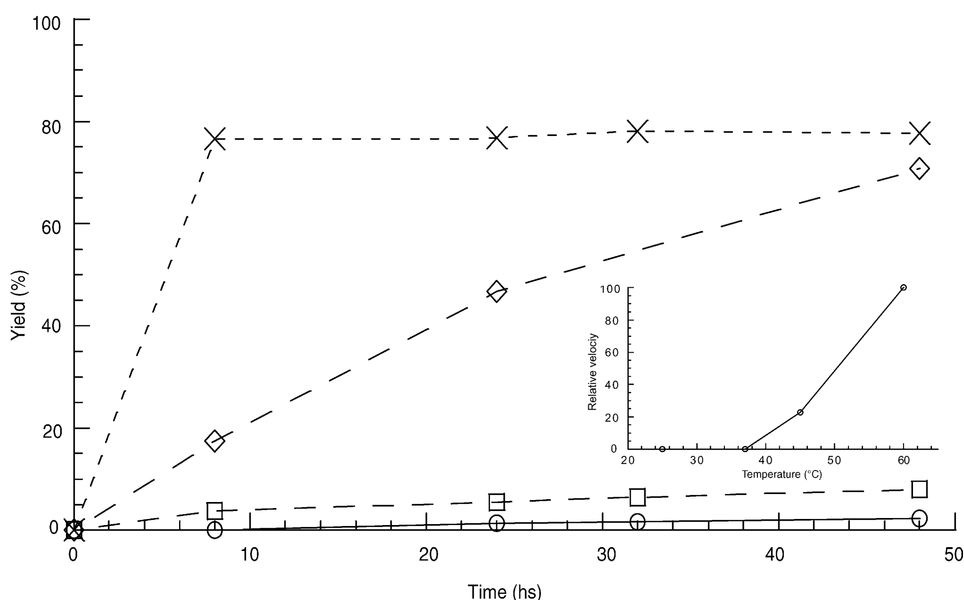


Fig. 1. Influence of temperature on benzimidazole riboside production: 25 °C (○), 37 °C (□), 45 °C (◇), 60 °C (×). Relative velocities vs. temperature are given in the insert.

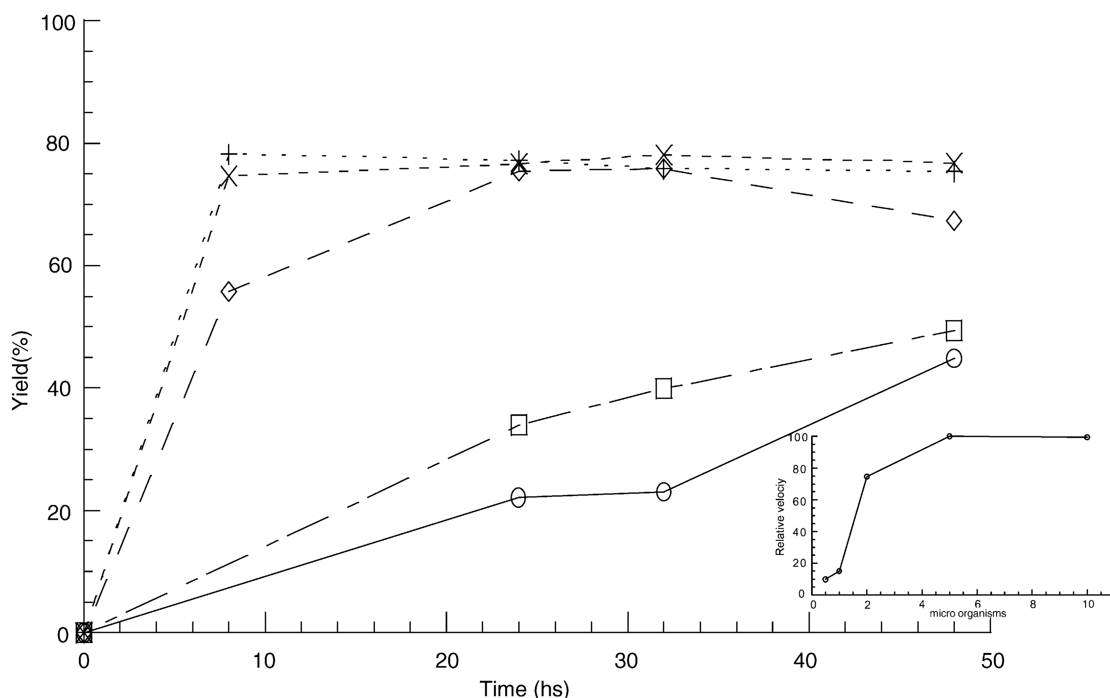


Fig. 2. Influence of amount of biocatalyst on benzimidazole riboside production: 9×10^7 cells (○), 1.8×10^8 cells (□), 3.6×10^8 cells (◇), 9×10^8 cells (×), 1.8×10^9 cells (+). Relative velocities vs. cell number are given in the insert.

the previously reported biocatalysed preparation [14] since it involved a 3:1 nucleoside:benzimidazole ratio, probably due to the fact that only PNP catalyses this reaction and this enzyme is more active for synthesis rather than phosphorolysis [20].

The influence of time and amount of biocatalyst was also evaluated (Fig. 2). Long reaction times were required in order to achieve high yields when small quantities of the biocatalyst were employed. When this number of cells was increased, the reaction took place faster reaching the velocity a plateau at 9×10^8 cells. Considering productivity concerns the selected conditions were 9×10^8 cells and 8 h reaction (75% yield).

The dependence of reagent concentration on the velocity was also assessed (Fig. 3). Increasing this parameter from 10 to 75 mM, which is the solubility limit, the time necessary

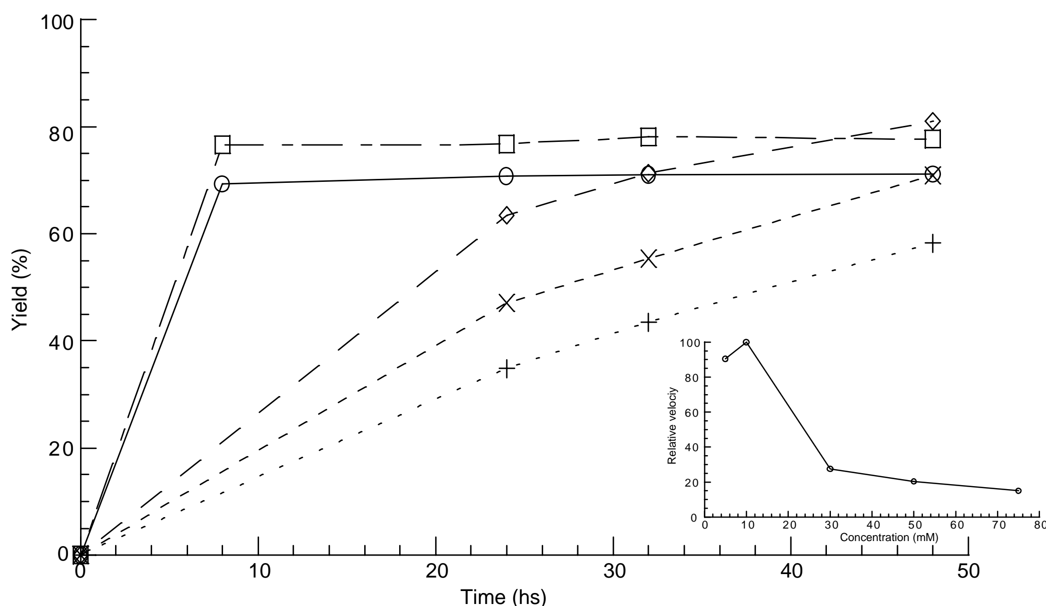


Fig. 3. Influence of reagent concentration on benzimidazole riboside production: 5 mM (○), 10 mM (□), 30 mM (◇), 50 mM (×), 75 mM (+). Relative velocities vs. reagent concentration are given in the insert.

for achieving similar maximal conversions also increased. This result is probably due to an insufficient amount of biocatalyst. However, the increase of cell number afforded aggregates that produced a decrease in the number of effective cells (data not shown).

In order to find the strain with the best transglycosylation activity for benzimidazole 2′-deoxyriboside, a screening was performed starting from thymidine (Table 1). In this case, the used temperature was 45 °C to avoid thymidine phosphorylase (TP) inhibition. The selected microorganism was *Serratia rubidae* that afforded the product in 84% yield in 5 h.

Other microorganisms containing *N-trans*-2′-deoxyribosylases that selectively accept as substrates 2′-deoxynucleosides, such as *Lactobacillus*, did not afford the expected product since benzimidazole was not an efficient base acceptor.

4. Conclusions

In this paper, the preparation of benzimidazole nucleosides using microbial transglycosylation is reported.

Although the synthesis of the ribo- and 2′-deoxyribonucleosides gave similar yields to those reported previously, in this work more soluble precursors and an equimolecular proportion of base to sugar donor were employed.

Further productivity improvements through biocatalyst immobilisation and analysis of the biological activity of these deazapurine analogues are at present being carried out.

Acknowledgements

This work was supported by Universidad Nacional de Quilmes and Secretaría de Ciencia y Técnica de la Nación, Argentina. AMI is research member of CONICET, Argentina.

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