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Enantioselective Bioreduction of Prochiral Pyrimidine Base Derivatives by Boni Protect Fungicide Containing Live Cells of *Aureobasidium pullulans*

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Abstract: The enzymatic enantioselective bioreduction of prochiral 1-substituted-5-methyl-3-(2-oxo-2-phenylethyl)pyrimidine-2,4(1*H*,3*H*)-diones to corresponding chiral alcohols by Boni Protect fungicide containing live cells of *Aureobasidium pullulans* was studied. The microbe-catalyzed reduction of bulky-bulky ketones provides enantiomerically pure products (96–99% ee). In the presence of *A. pullulans* (*Aureobasidium pullulans*), one of the enantiotopic hydrides of the dihydropyridine ring coenzyme is selectively transferred to the *si* sides of the prochiral carbonyl group to give secondary alcohols with *R* configuration. The reactions were performed under various conditions in order to optimize the procedure with respect to time, solvent, and temperature. The present methodology demonstrates an alternative green way for the synthesis of chiral alcohols in a simple, economical, and eco-friendly biotransformation.

Keywords: biotransformation microbe-catalyzed reduction; prochiral ketones; stereochemistry; antifungal agent; *Aureobasidium pullulans*

1. Introduction

Biotransformation, i.e., enzyme-catalyzed reactions, have been used over millions of years in nature to carry out reactions that are complicated from a chemical point of view. Nowadays, biocatalysis has become an important method for the production of organic compounds. Biotransformations catalyzed by isolated enzymes and by whole cells of microorganisms, in terms of microorganisms growing or resting cells, are employed for the synthesis of chemicals such as pharmaceuticals, agrochemicals, and natural products [1–3].

Bioreductions of prochiral carbonyl compounds are mainly carried out using oxidoreductases, the most frequently used of which is the catalytic potential of NADH (Nicotinamide adenine dinucleotide)/NADPH (Nicotinamide adenine dinucleotide phosphate)-dependent dehydrogenases. Dehydrogenases in living organisms catalyze the reaction of the oxidation of alcohols to carbonyl compounds by mediating the transfer of a hydride ion from the cofactor on the substrate. They also have the ability to catalyze a reverse reaction in a reduction reaction. In the reduction reaction, dehydrogenases transfer the hydride ion (pro-*S* or pro-*R*) from the cofactor to one of the prochiral

sides of the carbonyl group (face *re* or *si*) to give pure enantiomeric or diastereomeric products [1,2]. The main limitation to the use of dehydrogenases is the necessity of employing expensive cofactors. In contrast, biocatalysis in the presence of living organisms in the form of tissue cultures or whole microbial cells is a very promising and effective method because the dehydrogenase, cofactor, and its regenerating system are all located within the cell [1,4–6].

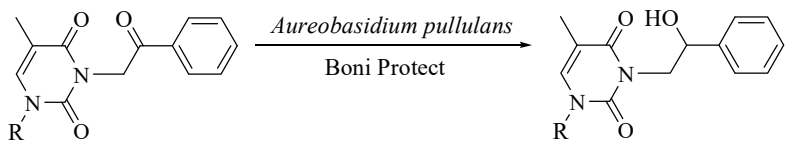
Baker's yeast is the most popular whole-cell biocatalyst for the asymmetric reduction of prochiral ketones due to its unlimited availability, ease of growing, and low cost [7]. For asymmetric bioreduction of ketones, in addition to the popular Baker's yeast, most often the cells of bacterial [8], fungal [9–13], and plant tissue [14–17] are used. The application of bioreagents in enantioselective enzymatic desymmetrization of prochiral ketones leads to a broad spectrum of chiral alcohols used as intermediates in the syntheses of many pharmaceuticals and compounds presenting biological activity [6]. Not without significance is the fact that biocatalytic reactions are conducted under moderate conditions in aqueous solutions without the use of expensive and often toxic reagents. For this reason, they are environmentally friendly, which increases the scope of their applications [1,2].

In this work, we present the microbial biotransformation of the 1-substituted-5-methyl-3-(2-oxo-2-phenylethyl)pyrimidine-2,4(1*H*,3*H*)-dione in a reduction catalyzed by Boni Protect fungicide containing live cells of *Aureobasidium pullulans*. Boni Protect is used in orchards because the microorganism contained in this antifungal preparation has an antagonistic activity against a number of phytopathogenic fungi (*Botrytis cinerea*, *Penicillium expansum*, *Monilinia laxa*, *Pezicula malicorticis*). It is used most often to combat white mold. The 3-substituted pyrimidine base derivatives exhibit biological activity, for e.g., *N*-3-substituted arabinofuranosyluracils and 4-thio analogues of *N*-3-substituted uridines possess hypnotic activity [18,19]. For this reason, the modification of these compounds in order to obtain enantiomerically pure derivatives is important from the point of view of using them as drugs.

2. Results and Discussion

Enantioselectivity and the efficiency of the microbial catalysis are mainly determined by the steric requirements of the substrate. For instance, Baker's yeast does not tolerate long-chain dialkyl ketones; however, one long alkyl chain is accepted if the methyl group has the other moiety. Highly stereoselective catalysis is achieved for the substrate with substituents of significantly different sizes [20].

The aim of the study was to use *A. pullulans* (*Aureobasidium pullulans*) to reduce the phenacyl of pyrimidine base derivatives with a prochiral carbon atom where there are two bulky substituents in the vicinity (Scheme 1). For this reason, they belong to the group of carbonyl compounds that are difficult to reduce by microbiological methods.



1 R = $CH_2CH{=}CH_2$ 1a R = $CH_2CH{=}CH_2$
2 R = CH_2CH_3 2a R = CH_2CH_3
3 R = CH_3 3a R = CH_3

Scheme 1. Bioreduction of the phenacyl of pyrimidine base derivatives (**1**–**3**) by *Aureobasidium pullulans*.

A. pullulans was earlier successfully employed as a catalyst in the bioreduction of ethyl 2-oxo-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6′-naphthalenyl)acetate and its amide [21], ethyl 4-chloro-3-oxobutanoate [22,23] and ethyl 2-methyl-3-oxobutanoate [24]. In our previous work, we also presented the selective biotransformation of unsymmetrical ketones and α,β-ketoesters in the

A. pullulans catalyzed reduction. The use of Boni Protect containing *A. pullulans* live cells resulted in alcohols and hydroxyesters with high stereoselectivity [25,26].

Compounds **1**–**3** have previously been reduced by the chiral reagents commonly used in asymmetric organic synthesis. In the presence of borane/oxazaborolidine, which was generated *in situ* from methoxyborane and (1*S*, 3*S*, 4*R*, 6*R*)-4-amino-3,7,7-trimethylbicyclo[4.1.0]heptan-3-ol, as a catalyst, the corresponding chiral alcohols (**1**a–**3**a) were obtained with a low enantiomeric excess (4–21% ee). Whereas the reduction of **1** and **3** with (*S*)-2-methyl-CBS-oxazaborolidine (CBS = Corey, Bakshi, and Shibata) gives **1**a and **3**a with 100% yield and higher enantiomeric excess (73–85% ee) [27].

We wanted to find out if the use of *A. pullulans* will enable the reduction of **1**–**3** and whether selectivity of bioreduction can be improved as a result.

The biotransformation a reaction catalyzed by *A. pullulans* was carried out in aqueous solution (phosphate buffer pH = 7.0) at 30 °C, in the presence of glucose as the energy source and with the addition of ethanol. Under these conditions, regeneration of the cofactor takes place *in situ*. First, compound 1 as a model substrate was subjected to bioreduction and the reaction was completed after 3, 5, and 6 days (Table 1, entry 1–3). We found that the process of biotransformation of the compound is very slow and in order to achieve an effective performance of greater than 50%, the reaction should be carried out for at least 144 h. The enantiomerically pure product of *R* configuration ((*R*)-1′) was obtained, regardless of at which stage the reaction was finished.

Table 1. Reduction of **1** by *Aureobasidium pullulans*.

System: Phosphate Buffer	T [h]	1 [%] [a]	1a [%] [a]	ee [%] [a]
pH = 7.0, glucose	72	64.6	35.4	99
pH = 7.0, glucose	120	51.8	48.2	99
pH = 7.0, glucose	144	39.6	60.4	99
pH = 7.0:hexane 1:1 (*v*/*v*), glucose	144	100	-	-
pH = 7.0:hexane 4:1 (*v*/*v*), glucose	144	95.9	4.1	94
pH = 7.0:hexane 88:2 (*v*/*v*), glucose	144	90.0	10.0	99
pH = 7.0:TBME 88:2 (*v*/*v*), glucose	144	96.9	3.1	99
pH = 7.0:acetonitrile 88:2 (*v*/*v*), glucose	144	98.4	1.6	99
pH = 7.0:THF 88:2 (*v*/*v*), glucose	144	98.8	1.2	99
pH = 7.0:propan-2-ol 88:2 (*v*/*v*), glucose	144	93.3	6.7	99
pH = 7.0:[BMIM][PF_6] 88:2 (*v*/*v*), glucose	144	90.3	9.7	99
pH = 7.0:[BMIM][BF_4] 88:2 (*v*/*v*), glucose	144	88.4	11.6	99
pH = 6.5, glucose	144	33.3	66.7	99
pH = 7.0, sucrose	144	24.4	75.6	99
pH = 6.5, sucrose	144	57.3	42.7	99

[a] The ee (*R*[%] − *S*[%]/*R*[%] + *S*[%]) and yield were determined by HPLC. TBME = *tert*-butyl methyl ether; THF = tetrahydrofuran; [BMIM] = butyl-3-methylimidazolium [PF_6] = hexafluorophosphate; [BF_4] = tetrafluoroborate.

In order to improve the reaction yield, it was decided to reduce 1 with the addition of organic solvents: In a two-phase system phosphate buffer (pH = 7.0): Hexane (1:1 *v*/*v*), phosphate buffer (pH = 7.0):Hexane (4:1 *v*/*v*), and with organic solvents as cosolvents (Table 1, entry 4–12). Surprisingly, regardless of the polarity of organic solvents, the performance of reduction decreased. The highest yield (11.6%) was obtained in a solution of phosphate buffer (pH = 7.0) with the addition of 2% ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]).

Further optimization of the reaction conditions concerned the change in pH of phosphate buffer solution and the introduction of sucrose as a carbon source in the pre-incubation stage. According to the literature, the optimal pH for the microorganism *A. pullulans* is slightly acidic, therefore, the reduction of **1** in an environment of pH = 6.5 was carried out [20]. The obtained results are given in Table 1 (entry 13,15).

The highest yield was obtained in a solution with pH = 7.0 with sucrose (75.6%; see HPLC analysis of reduction of **1** in this condition—Figure 1).

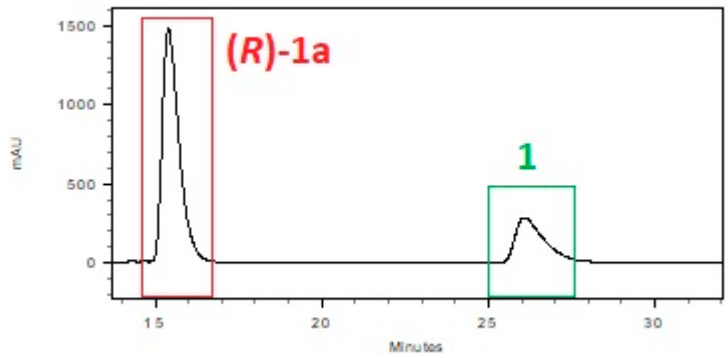


Figure 1. Reduction of **1** in phosphate buffer solution (pH = 7.0) with sucrose.

Bioreduction in aqueous solution with *A. pullulans* as a catalyst was also carried out for **2** and **3**. For compound **2**, the highest efficiency and high enantioselectivity (99% ee) were obtained in a phosphate buffer solution with pH = 7.0 in the presence of glucose, which was higher than the efficiency in the solution with pH = 6.5. However, the addition of sucrose to the reaction mixture, independent of the pH of the solution, resulted in a reduction in the reaction yield (Table 2). As in the case of **1**, the microorganism *A. pullulans* ensures excellent selectivity of biotransformation giving chiral *R*-alcohol with 96–99% ee.

Table 2. Reduction of **2** and **3** by *Aureobasidium pullulans* in different conditions.

System: Phosphate Buffer	2 [%] [a,b]	2a [%] [a,b]	ee [%] [a]	3 [%] [a,b]	3a [%] [a,b]	ee [%] [a]
pH = 7.0, glucose[c]	41.3	58.7	99	73.5	26.5	96
pH = 6.5, glucose[c]	60.4	39.6	99	91.6	8.4	99
pH = 7.0, sucrose[c]	76.8	23.2	99	86.6	13.4	99
pH = 6.5, sucrose[c]	85.3	14.7	96	94.1	5.9	96

[a] The ee and yield were determined by HPLC; [b] 144 h; [c] 30 °C.

The most difficult object to reduce was compound **3**. For this compound, as for 2, the conditions such as phosphate buffer, pH = 7.0, and glucose are the most optimal. After 144 h, the best performance was obtained, which, however, did not exceed 30%. Interestingly, lowering pH to 6.5 resulted in a decrease in performance. Under these conditions, **3**a accounted for only 10% of the reaction mixture. As a consequence, (*R*)-**3**a was obtained with low yield and high enantiomeric excess (Table 2).

Compounds **1**–**3** are structural analogues, differing in substituents in the *N*-1 position. Despite the similarity in the chemical structure, they are reduced in the same conditions at different rates. Therefore, for each of the prochiral reagents, the conditions of biocatalysis should be individually optimized. Based on the results obtained, the rate of the reaction catalyzed by *A. pullulans* and thus the efficiency is influenced by the size and chemical nature of the substituent on the first nitrogen in the pyrimidine ring. The presence of the allyl substituent increases the reaction rate.

In the next stage, biotransformation was performed at two additional temperatures to improve the efficiency of the reaction: 33 °C and 36 °C. The reaction rate increases with the increasing temperature, the enzymatic catalysis is also regulated by this dependence, but only within a certain temperature range. The optimal temperature that is characteristic of the enzyme ensures the highest activity of the biocatalyst, which results in the maximum degree of conversion. The inverse relationship applies to the selectivity of the process, in most cases the optical purity of a product decreases along with an increase in temperature [28]. We expected that as the temperature increased, the yield of chiral alcohols would increase without adversely affecting the enantioselectivity of the catalyzed reaction.

For each reagent, the two optimal conditions in which the reductions were performed at elevated temperatures were selected (Table 3). The best results were obtained for **3** at 33 °C. In a phosphate

buffer solution with pH = 7.0 with glucose (Figure 2), the yield of the reaction increased 2-fold and even 3-fold under the same conditions with sucrose added as a carbon source.

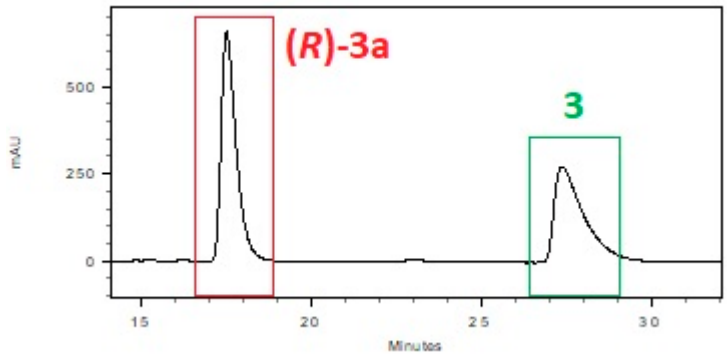


Figure 2. Reduction of **3** in phosphate buffer solution (pH = 7.0) with glucose.

Table 3. Reduction of **1**–**3** by *Aureobasidium pullulans* at 33 °C and 36 °C.

System	T [°C]	1a [%] [a]	ee [%] [a]	2a [%] [a]	ee [%] [a]	3a [%] [a]	ee [%] [a]
Phosphate buffer pH = 7.0, glucose	33 °C	Nd [b]	Nd [b]	64.1	98	55.7	99
	36 °C	Nd [b]	Nd [b]	4.4	99	3.7	99
Phosphate buffer pH = 6.5, glucose	33 °C	50.0	99	48.9	99	Nd [b]	Nd [b]
	36 °C	7.0	96	3.6	99	Nd [b]	Nd [b]
Phosphate buffer pH = 7.0, sucrose	33 °C	37.5	99	Nd [b]	Nd [b]	45.2	99
	36 °C	5.9	98	Nd [b]	Nd [b]	11.1	99

[a] The ee and yield were determined by HPLC; Nd [b]—not determined

A slight increase in yield was observed for **2** at 33 °C, and over 60% yield was obtained in the buffer solution with pH = 7.0 with glucose (Figure 3).

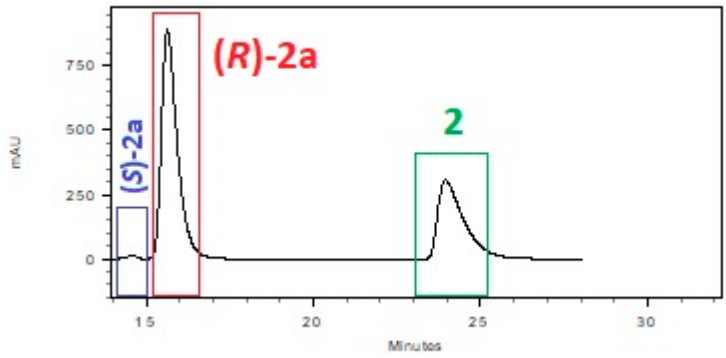


Figure 3. Reduction of **2** in phosphate buffer solution (pH = 7.0) with glucose.

In the case of **1**, a 3 °C increase in temperature resulted in a decrease in performance. A further increase in temperature to 36 °C adversely affected the conversion rate of each reagent, but did not have any practical effect on the selectivity of the process. Figure 4 shows the comparison of the yield of **1**a–**3**a alcohols at different temperatures.

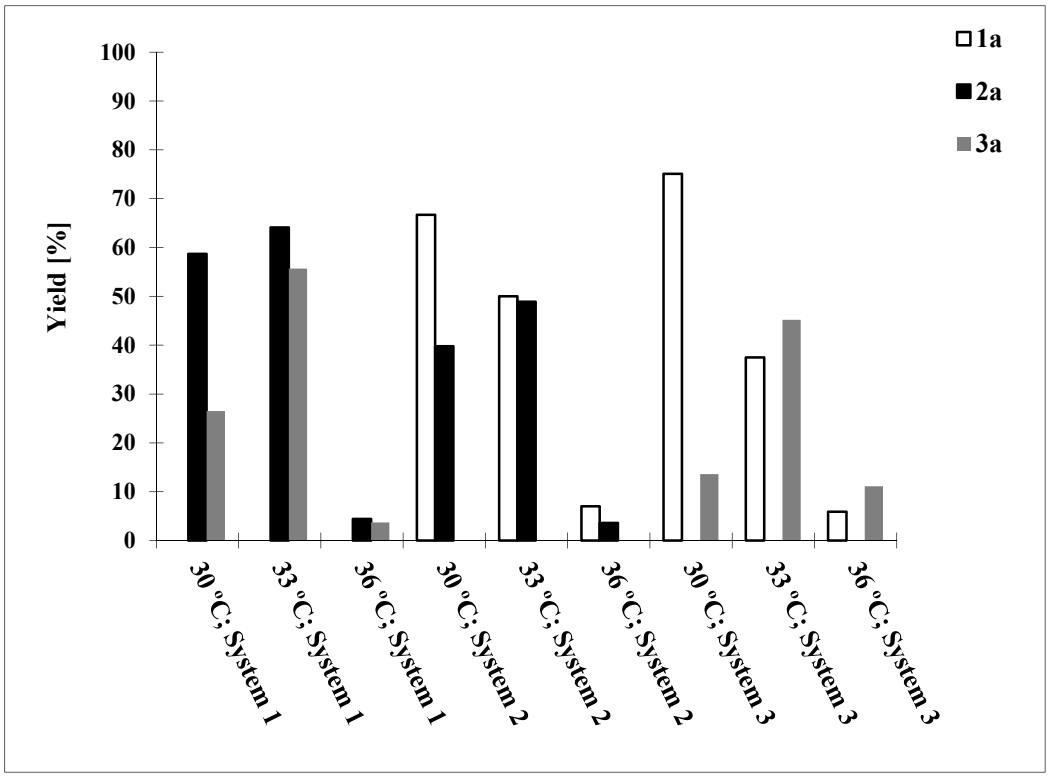


Figure 4. Comparison of **1**a–**3**a performance at 30 °C, 33 °C, and 36 °C in different conditions. System 1: Phosphate buffer pH = 7.0, glucose, System 2: Phosphate buffer pH = 6.5, glucose, System 3: Phosphate buffer pH = 7.0, sucrose. Yields were determined by HPLC.

The advantage of the microbiological method we use is a unique enantioselectivity, which combined with the ecological and economic aspect can be an interesting alternative to organocatalysis. Dehydrogenases contained in the microorganism *A. pullulans* selectively transfer one of the enantiotopic hydrogen ions of the dihydropyridine ring coenzyme to the *si* sides of the prochiral carbonyl group and provide secondary alcohols with the *R* configuration. Therefore, the Boni Protect could be considered as an efficient bioreagent for preparation of optically pure alcohols of derivatives of *N*-1 substituted thymine.

In the case of previously reduced unsymmetrical ketones (methyl ketones or bromomethyl ketones), in order to improve enantioselectivity, it was necessary to add appropriate inhibitors, i.e., additives which inhibit oxidoreductases with a specific stereopreference. Without the additives, reduction with *A. pullulans* proceeded with an enantiomeric excess ranging from 0% to 65% ee [26].

3. Materials and Methods

3.1. Analytical Methods

Nuclear magnetic resonance (NMR) spectra were performed with Bruker spectrometers (Billerica, MA, USA, 400/700 MHz). Chemical shifts are reported in δ ppm from tetramethylsilane (TMS) as an internal standard.

The enantiomeric excess of the chiral products (**1**a–**3**a) was determined by chiral stationary phase high-performance liquid chromatography (HPLC). HPLC analyses were performed on a Shimadzu SCL-10A VP, column Lux® 5µ Cellulose-3, LC Column 250 × 4.6 mm, Phenomenex (Warszawa, Poland). The mobile phase was n-hexane and propan-2-ol (60:40 *v*/*v*) at the flow rate of 0.5 mL per min. and monitored at 266 nm wavelength.

The samples were incubated in an orbital shaker (VORTEMP 1550 S2050; Equimed, Cracov, Poland).

Ketones 1–3 were obtained in our earlier work [27]. The retention times of 1–3 were 26.1 min, 24.1 min, and 27.4 min, respectively.

The structure of **1**a–**3**a was verified by ^{1}H NMR and spectra date was compared with the literature [27].

The absolute configurations of the chiral molecules were determined by various chiroptical methods (electronic circular dichroism (ECD) and vibrational circular dichroism (VCD)) [27].

3.2. Reagents and Solvents

The chemical substances of analytical grade were commercially available sucrose, glucose, ethyl acetate, ethanol, acetonitrile, *tert*-butyl methyl ether (TBME), tetrahydrofuran (THF), NaCl, $MgSO_4$, *n*-hexane for HPLC, propan-2-ol for HPLC from POCH (Polish Chemical Reagents, Gliwice, Poland), butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6], Fluka, Buchs, Switzerland), 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4], Merck, Darmstadt, Germany), and Boni Protect (Koppert Biological Systems, Wien, Austria).

3.3. General Procedure of Asymmetric Reduction by Aureobasidium pullulans

For a typical experiment, to a suspension of Boni Protect (0.5 g) in 7.5 mL of potassium phosphate buffer (pH 7.0) was added 2×10^{-4} mol glucose/sucrose, and the resulting suspension was incubated in an orbital shaker (350 rpm) at specific temperature (30 °C, 33 °C, 36 °C) for 30 min. After pre-incubation, the appropriate ketone (2.5×10^{-5} mol in 0.5 mL EtOH) was added, and stirring was continued at the same temperature. The reaction progress was monitored by Thin Layer Chromatography-TLC (the solvent system used was n-hexane:ethyl acetate 1:3 v/v). After the reaction, hyflo-super cel and ethyl acetate were added and the mixture was filtered. The celit was washed with ethyl acetate, and combined filtrates were extracted with ethyl acetate (3×20 mL). The organic phase was dried over anhydrous $MgSO_4$, and the solvent was evaporated under vacuum. After that, each reaction mixture was purified by column chromatography using n-hexane:ethyl acetate 1:3 v/v to afford the product. The enantiomeric ratios were determined on the HPLC system using a chiral column.

NMR and HPLC spectra of **1**–**3** and **1**a–**3**a were attached in the Supplementary Materials.

4. Conclusions

In summary, we have described an eco-friendly and environmentally benign asymmetric reduction system employing easily available Boni Protect fungicide as a biocatalyst. This reduction method is simple, economical (the need of costly cofactor is eliminated), and does not require the cultivation of the bioreagent. The bioreduction of different phenacyls of pyrimidine base derivatives to corresponding optically chiral alcohols has shown an exclusively (*R*) configuration. Thus, this study demonstrates an inexpensive approach in the synthesis of optically pure (*R*)-heterocyclic compounds of biological importance.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/8/7/290/s1. Figure S1: ^{1}H NMR spectra of **1**; Figure S2: ^{1}H NMR spectra of **2**; Figure S3: ^{1}H NMR spectra of **3**; Figure S4: ^{1}H NMR spectra of **1**a; Figure S5: ^{1}H NMR spectra of **2**a; Figure S6: ^{1}H NMR spectra of **3**a; Figure S7: ^{13}C NMR spectra of **1**; Figure S8: ^{13}C NMR spectra of **2**; Figure S9: ^{13}C NMR spectra of **3**; Figure S10: ^{13}C NMR spectra of **1**a; Figure S11: ^{13}C NMR spectra of **2**a; Figure S12: ^{13}C NMR spectra of **3**a; Figure S13: (*S*)-**1**a (99% ee); Figure S14: (*R*)-**1**a (96% ee); Figure S15: Reduction of **1** in phosphate buffer solution (pH = 6.5) with glucose at 30 °C; Figure S16: Reduction of **1** in phosphate buffer solution (pH = 6.5) with glucose at 33 °C; Figure S17: Reduction of **1** in phosphate buffer solution (pH = 7.0) with sucrose at 30 °C; Figure S18: Reduction of **1** in phosphate buffer solution (pH = 7.0) with sucrose at 33 °C; Figure S19: (*S*)-**2**a (99% ee); Figure S20: (*R*)-**2**a (97% ee); Figure S21: Reduction of **2** in phosphate buffer solution (pH = 7.0) with glucose at 30 °C; Figure S22: Reduction of **2** in phosphate buffer solution (pH = 7.0) with glucose at 33 °C; Figure S23: Reduction of **2** in phosphate buffer solution (pH = 6.5) with glucose at 30 °C; Figure S24: Reduction of **2** in phosphate buffer solution (pH = 6.5) with glucose at 33 °C; Figure S25: (*S*)-**3**a (99% ee); Figure S26: (*R*)-**3**a (62% ee); Figure S27: Reduction of **3** in phosphate

buffer solution (pH = 7.0) with glucose at 30 °C; Figure S28: Reduction of **3** in phosphate buffer solution (pH = 7.0) with glucose at 33 °C; Figure S29: Reduction of **3** in phosphate buffer solution (pH = 7.0) with sucrose at 30 °C; Figure S30: Reduction of **3** in phosphate buffer solution (pH = 7.0) with sucrose at 33 °C.

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Conflicts of Interest: The authors declare no conflict of interest.

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