Research Article

An Efficient Chemoenzymatic Process for Preparation of Ribavirin

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Ribavirin is an important antiviral drug, which is used for treatment of many diseases. The pilot-scale chemoenzymatic process for synthesis of the active pharmaceutical ingredient Ribavirin was developed with 32% overall yield and more than 99.5% purity. The described method includes the chemical synthesis of 1,2,4-triazole-3-carboxamide, which is a key intermediate and enzyme-catalyzed transglycosylation reaction for preparation of the desired product. 1,2,4-Triazole-3-carboxamide was synthesized from 5-amino-1,2,4-triazole-3-carboxylic acid by classical Chipen-Grinshtein method. Isolated from *E. coli* BL21(DE3)/pERPUPHHO1 strain the purine nucleoside phosphorylase was used as a biocatalytical system. All steps of this process were optimized and scaled.

1. Introduction

Antiviral properties of 1-β-D-ribofuranosyl-1-H-1,2,4-triazole-3-carboxamide or Ribavirin were described in 1972 for the first time [1]. Since that moment it is used in the treatment of influenza, severe respiratory syncytial virus (RSV) infection, and Lassa fever virus infection [1]. In combination with interferon-α, it is a current standard for hepatitis C virus (HCV) therapy [1]. This disease was determined by World Health Organization (WHO) as one of the most important problems of health care. Chronic form of HVC is the main reason for causing liver cirrhosis and hepatocellular carcinoma [2]. There are 150 million infected people in the world and every year their number increases by about 3-4 millions [3]. Therefore, the development of the reliable and effective technology for Ribavirin production is a topical problem.

All the methods for synthesis of Ribavirin available at the moment could be divided into chemical [4–7] and microbiological [8–16] approaches. Typical procedure for chemical synthesis includes condensation of the 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose **1** with 1,2,4-triazolecarboxylic acid esters **2** followed by treatment of the resulting compound **3** with methanolic ammonia solution (Scheme 1). Starting material, 1,2,3,5-tetra-O-acetyl-β-D-furanose **1**, can be prepared under acid-catalyzed acetylation of inosine, or of the other natural occurring nucleosides. In general, chemical methods have pure yield, high consumption of starting materials, and reagents, and an amount of toxic waste must be utilized.

That is why microbiological methods are considered more efficient. Nucleoside phosphorylase (NP) plays the key role in these methods. It catalyzes transfer of sugar moiety from one heterocyclic base to another. The bacterial [9, 14] or isolated enzymes are used for nucleoside synthesis [17, 18]. The main disadvantages of this approach are low productivity and high costs of enzymes and starting materials.

Our technology of Ribavirinsynthesis combines the microbiological method for generation of the target structure with the chemical synthesis of key intermediates. This approach gives high level of reliability and efficiency while producing less toxic waste.

2. Experimental

2.1. Chemicals and Reagents. The starting reagents and solvents were obtained from Sigma-Aldrich Company and used
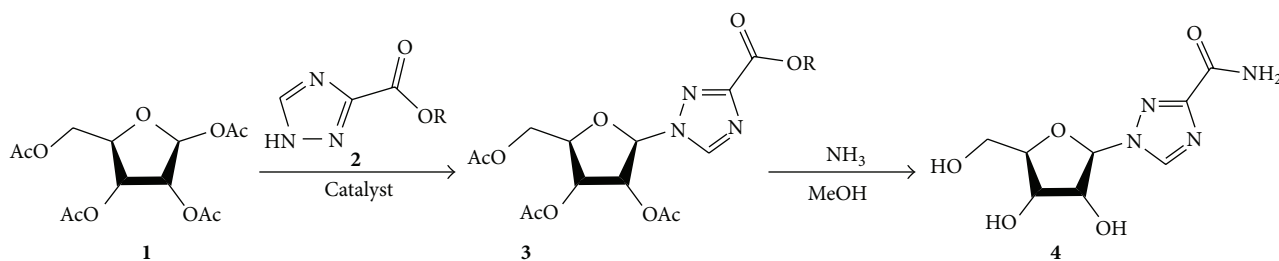
SCHEME 1

as received. Purine nucleoside phosphorylase (protein concentration determined by Bradford assay, 15 mg/mL; activity, 52 units/mg of protein) was obtained in the group of recombinant proteins (Institute of Bioorganic Chemistry, Russian Academy of Sciences, supervisor Dr. Esipov) [19].

2.2. *Methods.* The reactions were monitored by high-performance liquid chromatography (HPLC). Analysis was performed on Shimadzu LC 20 Prominence apparatus and Waters Xbridge C18, 50 mm × 4.6 mm column with water-acetonitrile eluent. The purity of compounds and the yield of the target products were also determined by HPLC. ^{1}H NMR spectra were recorded on a Bruker DRX500 (500 MHz) and were determined in DMOS-d_6. ^{13}C NMR spectra were recorded at 125 MHz. Chemical shifts are reported in parts per million relative to the peak of tetramethylsilane (TMS) (0.00 ppm). Melting points were measured on Mel-Temp 3.0.

2.3. *Preparation of 1,2,4-Triazole-3-carboxylic Acid (7).* To a suspension of 8.25 kg (64.5 mol) 5-amino-1,2,4-triazole-3-carboxylic acid **5** in 50.0 L water 1.9 L concentrated hydrochloric acid was added. The suspension was heated at 70°C to dissolve. The solution was cooled to room temperature and 30 kg of ice was added. Further ice-cold solution of 8.75 kg (126.8 mol) sodium nitrite in 19.0 L of water was added. Following the addition the reaction mixture was stirred 30 min at 0–10°C and 40 min at room temperature. The resulting suspension was filtered and washed in ice water (2 × 250 mL) to give 11.25 kg of diazonium salt **6**, which was used in the next step without further purification.

To a suspension of 3.75 kg (21.25 mol) diazonium salt **6** in 25.0 L of methanol 10 g of sodium borohydride was added at 0–4°C (exothermic). The reaction mixture was cooled to 0–4°C and procedure was repeated two times. After completion, the reaction mixture was refluxed 5 min, cooled to 0–2°C over 20–25 min, and filtered. The precipitate was washed with 6.5 L of cool methanol and 4.75 kg (65%) of 1,2,4-triazole-3-carboxylic acid **7** was obtained, as a white solid, mp 136-137°C. ^{1}H NMR (500 MHz, DMOS-d_6) δ: 8.67 (1H, s), 14.01–14.52 (1H, bs). ^{13}C NMR (125 MHz, DMOS-d_6) δ: 148.5, 151.9, 165.6. Found, %: C 31.44; H 2.79; N 36.98. Calculate, %: C 31.87; H 2.67; N 37.16.

2.4. *Preparation of 1,2,4-Triazole-3-carboxylic Acid Methyl Ester (2).* A hydrogen chloride gas was purged through the cooled to 0–2°C suspension of 4.75 kg (41.9 mol) 1,2,4-triazole-3-carboxylic acid **7** in 35.0 L of methanol over 8 h.

After completion, reaction mixture was stirred at room temperature for over 48 h. The resulting suspension was filtered and washed with 3.5 L cool methanol. Product was obtained as a white solid, yield 4.68 kg (88%), mp 185-186°C. ^{1}H NMR (500 MHz, DMOS-d_6) δ: 3.85 (3H, s), 8.73 (1H, s), 14.65 (1H, bs). ^{13}C NMR (125 MHz, DMOS-d_6) δ: 52.1, 145.2, 145.5, 160.2. Found, %: C 37.69; H 4.09; N 33.16. Calculate, %: C 37.80; H 3.97; N 33.06.

2.5. *Preparation of 1,2,4-Triazole-3-carboxamide (8).* To 25.0 L of (162.2 mol) 25% aqueous ammonia 4.68 kg (36.8 mol) 1,2,4-triazole-3-carboxylic acid methyl ester **2** was added while stirring. The reaction mixture was refluxed 30 min, cooled to 40°C, and evaporated at reduced pressure to volume of 15.0 L. The resulting suspension was cooled to 0–5°C over 2 h and filtered. After recrystallization from water, 3.51 kg (85%) of 1,2,4-triazole-3-carboxamide **8** was obtained, as a white solid, mp 224–228°C. ^{1}H NMR (500 MHz, DMOS-d_6) δ: 7.49 (1H, s), 7.73 (1H, s), 8.65 (1H, s), 14.26–14.98 (1H, bs). ^{13}C NMR (125 MHz, DMOS-d_6) δ: 144.6, 156.4, 160.1. Found, %: C 32.20; H 3.49; N 49.86. Calculate, %: C 32.15; H 3.60; N 49.98.

2.6. *Preparation of 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin, 4).* 20.4 g of potassium dihydrophosphate was dissolved in 10 L of distilled water. The pH of the solution was adjusted to 7.0 with 5 N solution of potassium hydroxide. To the buffer solution 7.8 g (0.07 mol) 1,2,4-triazole-3-carboxamide **8**, 42.5 g (0.15 mol) guanosine **9**, and 30 mL of PNP (15 mg/mL, 52 ea/mg of protein) solution were added. The reaction mixture was thermostated at 52–55°C over 7 d. The resulting solution was concentrated at reduced pressure to 1/2 of volume and cooled to 4–8°C. The precipitated guanine with guanosine traces were filtered out. The filtrate was passed through ion exchange resin (eluted with water). Solvent was evaporated at reduced pressure and 12.2 g (71%) of ribavirin was obtained with 98.6% purity. After recrystallization from ethanol, 11.1 g (65%) of ribavirin was obtained, as white solid, mp 175–178°C. Purity is 99.5% by HPLC (Figure 1 and Table 3). ^{1}H NMR (500 MHz, DMOS-d_6) δ: 3.48–3.52 (1H, m), 3.60–3.65 (1H, m), 3.93 (1H, m), 4.12 (1H, m), 4.37 (1H, m), 4.90 (1H, t, J = 5.5 Hz), 5.19 (1H, d, J = 5.6 Hz), 5.57 (1H, d, J = 5.7 Hz), 5.82 (1H, d, J = 3.8 Hz), 7.59 (1H, s), 7.80 (1H, s), 8.86 (1H, s), 14.26–14.98 (1H, bs). ^{13}C NMR (125 MHz, DMOS-d_6) δ: 61.1, 69.8, 74.5, 85.5, 92.0, 145.0, 151.4, 156.1. Found, %: C 39.41; H 4.83; N 22.99. Calculate, %: C 39.35; H 4.95; N 22.94.
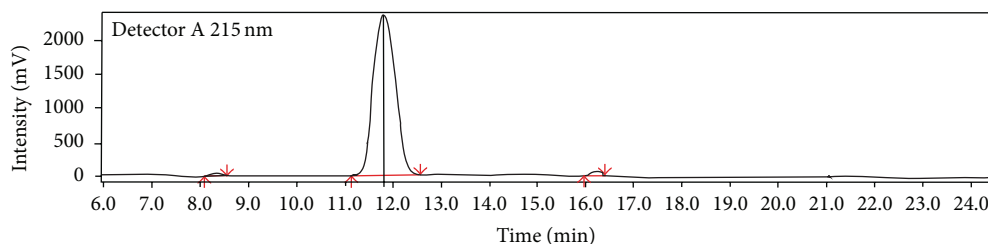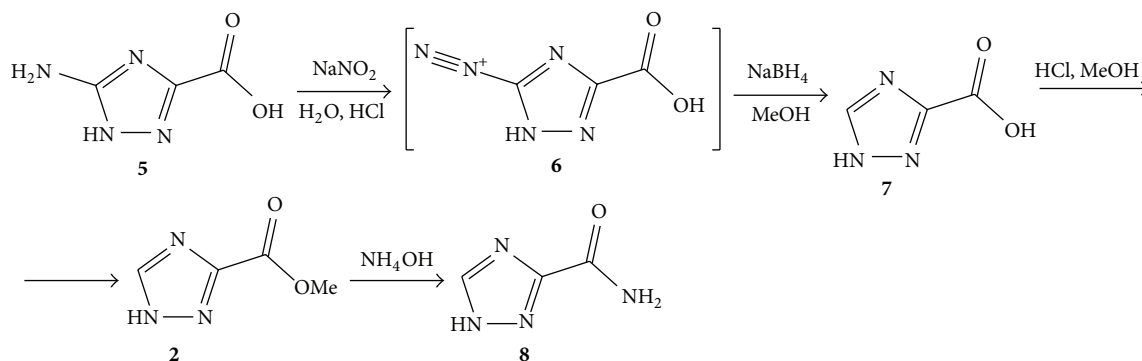
FIGURE 1: Typical chromatogram of the obtained Ribavirin.

SCHEME 2

TABLE 1: Optimization of transglycosylation condition.

No.	Nucleoside	Ratio TCA/nucleoside	Time, h	Yield of Ribavirin, %
1	Guo	1 : 1	120	50
2	Guo	1 : 1	360	84
3	Ino	1 : 1	120	45
4	Ino	1 : 1	360	60
5	Guo	1 : 2	120	97
6	Guo	1 : 3	120	97
7	Guo	1 : 5	120	98
8	Guo	1 : 15	120	99

TABLE 2: The Ribavirin synthesis with different biocatalytical systems.

Donor	Biocatalyst	Conversion, %	Reference
Uridine	*A. hydrophila* CECT4226	65	[9]
Guanosine	GA-*E. coli* BMT 4D/1A	70	[10]
Uridine	pET-30a-*udp-deoD*	84	[14]
Uridine	ATCC 12407	86	[16]
Guanosine	BL21(DE3)/pERPUPHHO1	97	This study

3. Results and Discussion

It was previously reported that 1,2,4-triazolecarboxylic acid esters are not PNP substrate [20]. Therefore, only 1,2,4-triazole-3-carboxamide **8** (TCA) could be used as a free heterocyclic base in Ribavirin synthesis. Thereby our first goal was to find out the most convenient and efficient way for chemical synthesis of TCA **8**, which could be used in industry. After analysis of the literature we have chosen the classical Chipen-Grinshtein method [21], represented in Scheme 2.

The entire process is suitable for kg-scale preparation. The yields of intermediates at each stage were not less than 65%, and the overall yield of TCA **8** was 49%.

There are two described approaches to microbiological synthesis of Ribavirin **4** from TCA: enzymatic (exogenic) [9] and fermentative (endogenic) [8]. In keys of exogenic approach all the components of the reaction systems should be added separately. Transglycosylation reaction, that is,

transfer glycoside moiety from nucleoside (donor) to free purine base (acceptor), is an example of this kind of reactions. During endogenic catalysis the substrate should be added to bacterial culture [8].

Transglycosylation reaction (Scheme 3) was chosen, because it is better studied and easy to control. Purified enzyme, the purine nucleoside phosphorylase (PNP), which was isolated from *Escherichia coli* (*E. coli*) BL21(DE3)/pER-PUPHHO1 strain, was used as a biocatalytical system.

Natural nucleoside guanosine (Guo) **9** was chosen as ribose donor, because of sedimentation of product guanine (Gua) **10** during the reaction, causing the equilibrium shift in favor of the required product. In addition, Guo **9** is commercially available, which is an important fact for the industrial application. The optimal ratio of Guo **9** to TCA **8** was also studied. The results are shown in Table 1.

In accordance with this data, the 97% conversion of TCA **8** into the Ribavirin **4** can be achieved when using 2 mol of Guo **9** per one mol of **8**. Further increase of Guo **9** excess did not improve the conversion rate. In addition, comparison with described biocatalysts is shown in Table 2.
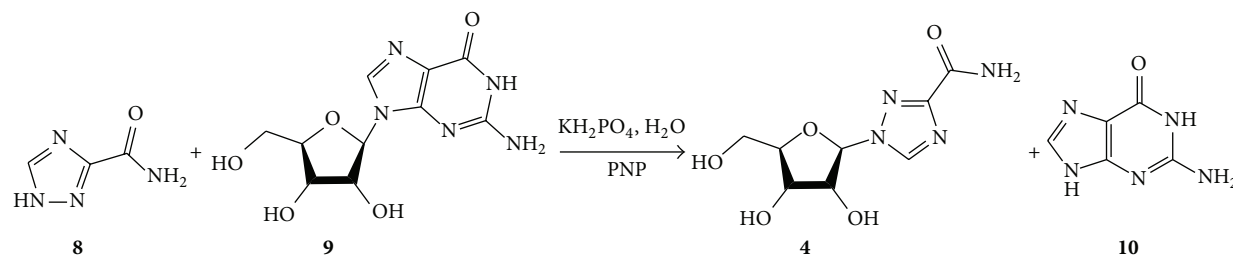
Scheme 3

Table 3

Peak#	Ret. Time	Area	Area%	Height	Mark	Conc.	Unit	ID#	Name
1	8.351	15549	0.024	946	M	0.024			
2	11.804	64132634	99.950	2160366		99.950			
3	16.180	16757	0.026	2019	M	0.026			
Total		64164940	100.00	2163331		100.00			

The process was scaled from 0.5 L volume to 20 L volume. In order to achieve 97% conversion the reaction time was extended from 120 to 168 h. Residual TCA **8** was removed during chromatographic purification and recrystallization. Typical chromatogram of the obtained Ribavirin is shown in Figure 1 and Table 3. TCA **8** (0.434%, RT 16.180 min) and Guo **9** (0.026%, RT 8.351 min) is main impurity.

The use of soluble enzyme is the main limitation for industrial application of this process. Therefore, further efforts will be focused on the development of immobilized enzyme. Recently, Rivero et al. reported successful immobilization of *E. coli ATCC 12407* in agarose matrix [16]. Moreover, immobilization on fused silica Open Tubular Capillary [22], aldehyde-agarose [23], and MagReSyn epoxide microspheres [24, 25], in calcium alginate, agar, and k-carrageenan matrix [18], was described in the literature. Thus, we believe, that the immobilisation of our enzyme is possible.

4. Conclusions

To conclude, we have found an efficient combined chemoenzymatic method of Ribavirin synthesis from commercially available materials. We have adapted previously reported laboratory protocol of 1,2,4-triazole-3-carboxamide chemical synthesis to kilograms scale. Purity of the final product is sufficient to use as an active pharmaceutical ingredient. The reported approach is superior in comparison with the earlier chemical methods and approaches. In the future, we plan to immobilize the enzyme for further improvement of the technology.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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