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Review

Enzymatic preparation of nucleoside antibiotics [1]

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Abstract

Microbial nucleoside transformation has been applied to the chemical process to produce biologically active nucleosides. Adenine arabinoside (ara-A), ribavirin, 2′-amino-2′-deoxyadenosine, 2′,3′-dideoxyinosine (ddI), and some other nucleosides with antiviral activity have been prepared through this process. *Enterobacter aerogenes*, *Brevibacterium acetylicum*, *Erwinia herbicola*, and *Escherichia coli* are selected as the best producers for their corresponding nucleosides. The transformation involves *N*-pentose transfer reaction. Inorganic phosphate was an essential co-factor to complete the reaction, and pentose 1-phosphate was isolated as an intermediate from the reaction mixture. Nucleoside phosphorylases were isolated from crude extract of the microorganisms and shown to be involved in the transformation. The transformation was catalyzed at a high temperature range of 50°C–65°C under the neutral pH range.

Keywords: Nucleoside transformation; Adenine arabinoside (ara-A); Ribavirin; Dideoxyinosine (ddI); Nucleoside phosphorylase; *N*-Pentose transfer

1. Introduction

Many nucleoside analogs have been found occurring naturally or have been synthesized chemically. Extensive attempts have been made to develop selective antiviral or antitumor agents that specifically suppress viral or tumor-cell replications without affecting normal cell metabolism.

Recently, it has been shown that some virus-infected cells produce virus-specific enzymes such as thymidine kinase [1] or DNA polymerase [2]. These virus-producing enzymes have been proven to differ from host's corresponding enzymes in terms of substrate specificities.

Thus, the compounds that are recognized by their virus-producing enzymes, but not by host indigenous enzymes, would be promising antiviral agents and become the target compounds for drug synthesis.

Among the newly developed nucleoside analogs, some compounds exhibit a distinct selective antiviral activity.

In recent years, a great deal of effort has been concentrated to the synthesis of nucleoside analogs with the aim of improving chemotherapy of various virus infected diseases. The chemical synthesis of nucleoside analogs often requires difficult and time-consuming multistep processes. The most ordinary methods are the sugar-based coupling reaction and modification

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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of naturally occurring nucleosides. However, difficulties are involved in these approaches due to the formations of undesirable anomers and some isomers. Low-yield, laborious, and time consuming processes are also too difficult in a practical application to industry.

Therefore, microbial enzymes, as a bio-catalyst in the chemical process, have been applied to the synthesis of stereo-specific nucleosides.

2. Synthesis of adenine arabinoside

Adenine arabinoside (ara-A) has been pharmaceutically used as an anti-herpes agent [3]. This compound was first synthesized by Lee et al. by chemical method [4], and it was later found in culture medium of *Streptomyces antibioticus* [5].

Microbial enzymes that catalyze *trans*-arabinosylation reaction have been looked for and applied in the chemical process to produce ara-A from uridine as shown in Fig. 1 [6,7]. Uracil arabinoside (ara-U) can be prepared from uridine through chemical reaction with ethylene carbonate followed by the hydrolysis of cyclouridine (cyclo-U) by acid [8].

2.1. Screening of adenine arabinoside producers

Microorganisms obtained from type culture collections and isolated natural samples were incubated at 30°C for 24 h in an appropriate medium (pH 7.0) containing meat extract and yeast extract. Cells were harvested by centrifugation and washed twice with 0.1 M potassium phosphate buffer (pH 7.0). The obtained cell paste was mixed with ara-U and adenine in potassium phosphate buffer and incubated at 60°C for 15 h. After the reaction, the supernatant was obtained and the reaction products were analyzed by high performance liquid chromatography (HPLC).

Table 1
Ara-A producers

Strain	Ara-A formed (mg/l)
Pseudomonas stuzeri AJ 2251	113
Flavobacterium gasogenes AJ 2425	110
F. proteus AJ 2508	104
Achromobacter lacticum AJ 2384	85
Salmonella typhimurium AJ 2635	115
Citrobacter intermedium AJ 2620	170
C. freundii AJ 2619	245
Escherichia coli AJ 2594	1850
E. freundii AJ 2605	1020
E. aurescens AJ 2610	650
Enterobacter aerogenes AJ 11125	2050
Ent. cloacae AJ 2656	1660
Ent. liquifaciens AJ 2661	350
Aeromonas hydrophila AJ 2623	845
Aer. salmonicida AJ 2926	1240
Aer. punctata AJ 2625	440
Serratia marcescens AJ 2703	860
S. rubefaciens AJ 2712	650
Erwinia carotovora AJ 2753	780
Erw. herbicola AJ 2196	1560
Proteus vulgaris AJ 2764	135
Pro. rettgeri AJ 2769	355
Pro. mirabilis AJ 2773	105
Xanthomonas campestris AJ 2797	265
Bacterium cadaveris AJ 3264	320

As shown in Table 1, many bacteria were found to produce ara-A under the reaction con-

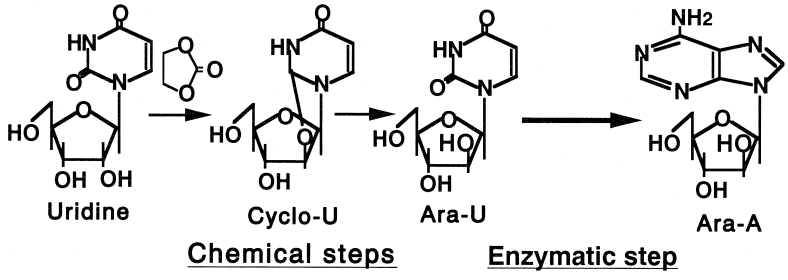


Fig. 1. Chemo-enzymatic synthesis of ara-A.

dition that temperature was at 60°C, but not at 30°C, a physiological growth temperature. *Enterobacter aerogenes* was selected as the best producer of ara-A and subjected to further study. The optimal temperature and pH for ara-A synthesis were 60°C–65°C and 7.0, respectively.

The reaction temperature is very important and a key to the synthesis of ara-A. As shown in Fig. 2, hypoxantine can be formed as by-product in a reaxtion mixture because adenine deaminase of microorganisms which is active in a temperature range of 30°C–50°C. Few ara-A could be detected under these conditions.

A high reaction temperature has additional advantages, such as preventing from contamination of other bacteria during the reaction process and increasing the substrate solubility in the reaction mixture, following by accelerating the reaction. More than 90% of adenine transformed to ara-A under the optimal reaction conditions.

Due to the poor solubility of ara-A, extremely pure crystals of ara-A precipitated in the reaction mixture, and final could be obtained only by recrystallization in water.

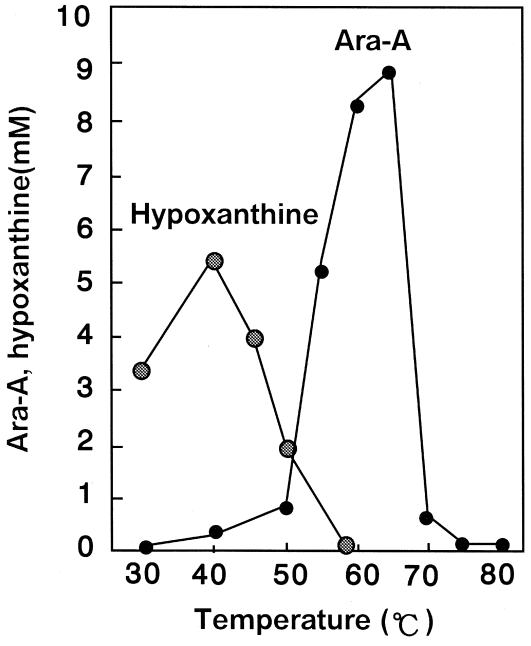


Fig. 2. Effect of temperature on ara-A production.

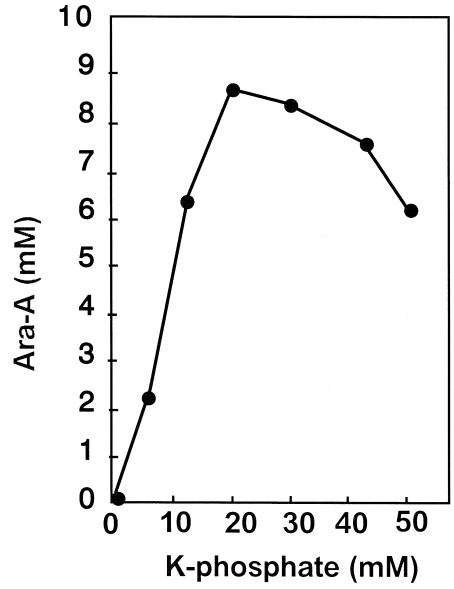


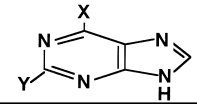
Fig. 3. Effect of K-phosphate concentrations on ara-A production.

2.2. Effect of inorganic phosphate on ara-A synthesis

As shown in Fig. 3, none of ara-A was formed without inorganic phosphate. Its optimal concentration was 20–30 mM for nucleoside phosphorylase in the *trans*-arabinosylation reaction.

For the purpose of investigating the mechanism of *trans*-arabinosylation, arabinose 1-phosphate (ara-1-P) was isolated as barium salt

Table 2
Substrate specificity



X	Y	Yield (%)
NH_2	H	92
NH_2	CH_3	72
NH_2	NH_2	83
OH	H	50
OH	NH_2	8
OH	Cl	40
OH	CH_3	55
H	NH_2	61

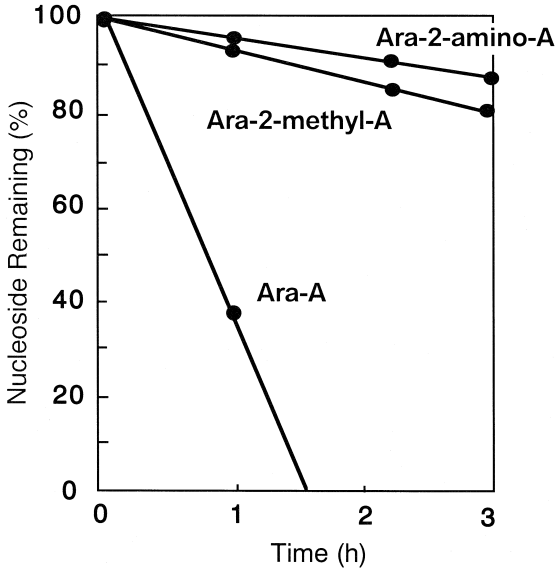


Fig. 4. Stability of ara-A analogs in mice blood. Ara-2-amino-A:2-aminoadenine arabinoside. Ara-2-methyl-A:2-methyladenine arabinoside.

from the reaction mixture. Then it has been clarified that nucleoside phosphorylases are involved in the *trans*-arabinosylation reaction, and ara-1-P is an intermediate of the reaction for ara-A formation [9].

3. Synthesis of purine arabinosides

Table 2 shows the substrate specificity of the *trans*-arabinosylation reaction by *Ent. aerogenes* [10]. As shown in Table 2, many nucleosides could be synthesized by this reaction, with good yield, except for the synthesis of guanine arabinoside (ara-G).

2-Chlorohypoxanthine arabinoside is an important compound as a useful intermediate for the preparation of ara-G analogs [11].

For the synthesis of ara-G, when guanosine was used instead of guanine as the base donor, forty five percent of guanosine was transformed to ara-G from guanosine and ara-U. The higher solubility of guanosine, compared with guanine may be the reason of this high yield.

Among the purine arabinosides, 2-substituted ara-A showed a good stability against adenosine deaminase in blood. For example, Fig. 4 shows the stability of 2-substituted ara-A in mice blood. The acute degradation of ara-A in blood to form hypoxanthine arabinoside was caused through adenosine deaminase, but 2-substituted ara-A showed excellent stability in mice blood. The weak point of ara-A as a therapeutic agent is its instability in blood, and these new compounds are expected to show promising drugs against virus-infected disease.

4. Synthesis of 2′-amino-2′-deoxynucleosides

Some 2′-amino nucleosides had been synthesized chemically [12] or found in the culture broth of microorganisms. First 2′-amino-2′-deoxyguanosine (2AG) was isolated from *Aerobacter* in 1974 [13], and then 2′-deoxy-2′-aminoadenosine (2AA) from *Actinomadura* [14] and *Actinomyces* [15] in 1979 and 1980. These nucleosides showed interesting biological activities and were seemed to be the targets for enzymatic synthesis (Fig. 5).

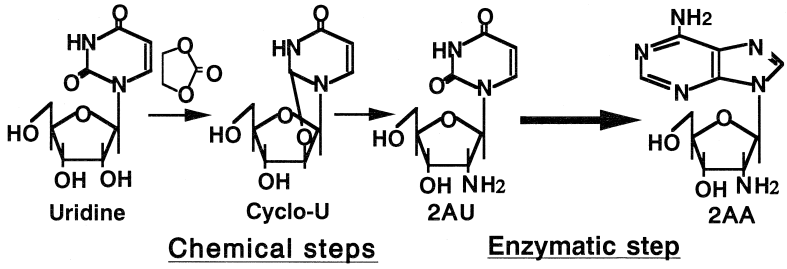


Fig. 5. Chemo-enzymatic synthesis of 2AA.

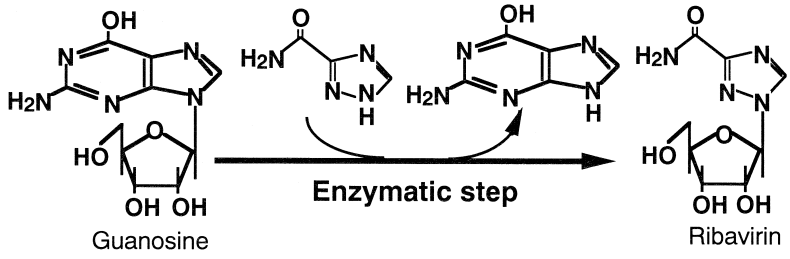


Fig. 6. Enzymatic synthesis of ribavirin.

2′-Deoxy-2′-aminouridine (2AU) was synthesized as a substrate by a method reported by Verheyden et al. [16]. Cyclouridine (cyclo-U) is a common chemical intermediate for both ara-U and 2AU synthesis.

Microorganisms were prepared by the same method as ara-A synthesis. Adenine, 2AU, and cell paste were mixed in phosphate buffer (pH 7.0), and kept at 60°C for 15 h. The 2AA formed in the reaction mixture was analyzed by HPLC.

It was found that many bacteria could catalyze transaminoribosylation reaction at 60°C [17,18]. *Erwinia herbicola* was selected as the best producer and used for further investigation. The optimal temperature and pH were virtually the same as those of transarabinosylation as described in Section 2.

2′-Amino-2′-deoxy-2-chloroinosine is a useful compound for a chemical reaction to synthesize some new 2′-amino-2′-deoxyguanosine analogs [19].

5. Synthesis of ribavirin

Ribavirin is an antiviral agent with a wide antiviral spectra not only for DNA viruses, but also for RNA viruses. This compound was first

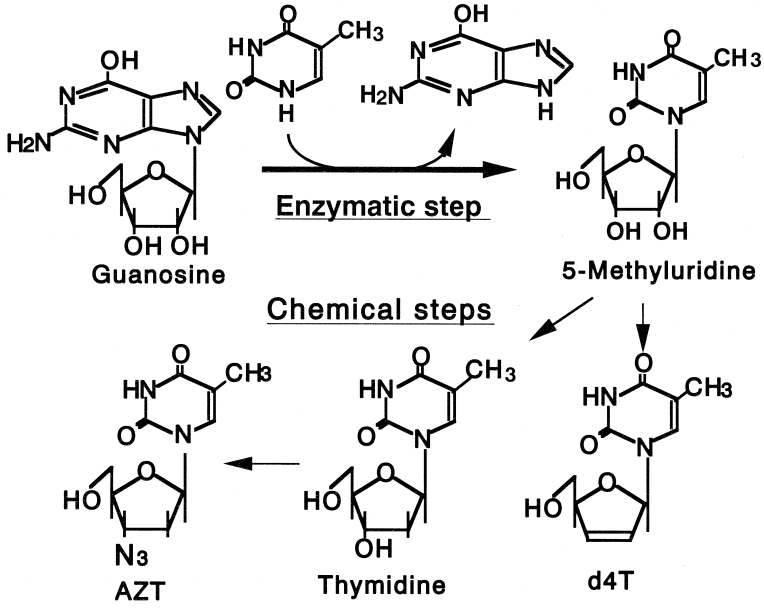


Fig. 7. Chemo-enzymatic synthesis of d4T and AZT.

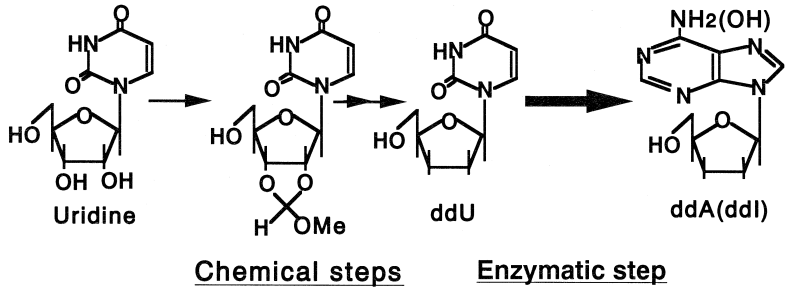


Fig. 8. Chemo-enzymatic synthesis of ddA(ddI).

synthesized by a chemical method [20], but the yield was not sufficient for a practical production. The microbial enzyme were applied to the synthesis of ribavirin [21,22]. Guanosine was selected as the best ribose donor, and triazole carboxyl amide (TCA), an artificial base, was the ribose acceptor for ribavirin synthesis (Fig. 6). After the screening of microorganisms, ribavirin production was carried out at 60°C with *Brevibacterium acetylicum* selected as the best producer.

In the course of the reaction, guanine, the base of guanosin, precipitated in the reaction mixture due to its poor solubility. Therefore, the reaction inclined towards the synthetic of ribavirin.

6. Synthesis of methyluridine

Methyluridine is an important intermediate for the chemical synthesis of d4T and AZT,

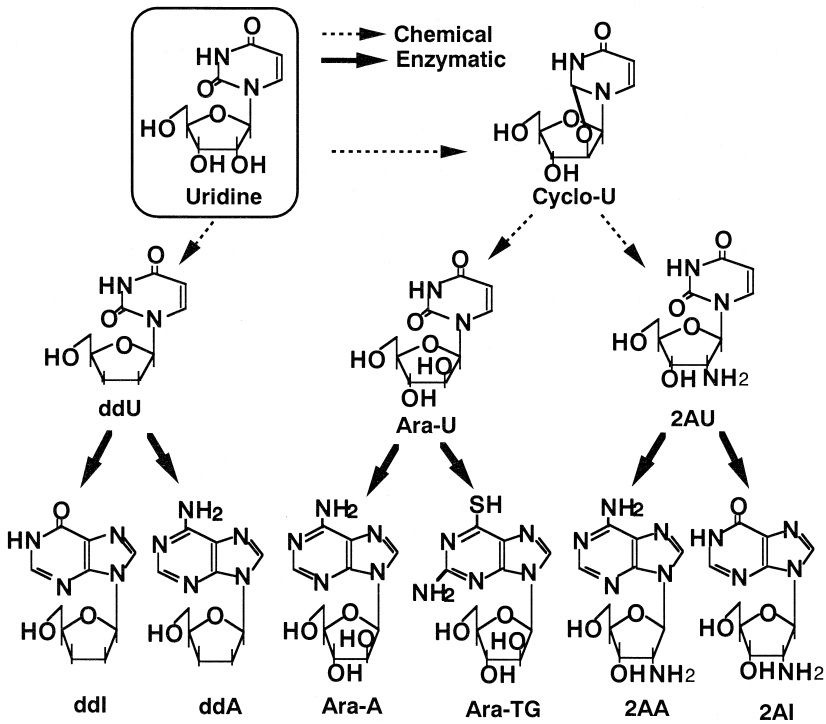


Fig. 9. Chemo-enzymatic synthesis of nucleoside antibiotics (1).

which are clinically used as anti-HIV agents [23,24].

On the preparation of methyluridine, guanosine and thymine were used as substrates for the screening as the same reason of ribavirin synthesis. *Erw. carotovora* was found to be the best producer of methyluridine [25]. Azidothymidine and d4T may be synthesized through a chemical reaction [26], as shown in Fig. 7.

7. Synthesis of ddI

2′,3′-Dideoxyinosine (ddI) and 2′,3′-dideoxyadenosine (ddA) are promising anti-HIV agents [27]. 2′,3′-dideoxyuridine (ddU) was selected as a substrate for the screening to form ddA by the *trans*-2′,3′-dideoxyribose reaction [28].

ddU can be synthesized from uridine by the chemical method [29]. Screening was carried out at 50°C. *Escherichia coli* was a best producer of ddA and ddI (Fig. 8). The optimal temperature for ddI synthesis was approximately 50°C, which is slightly lower than that of other *trans*-pentose reactions.

8. Discussion

This review describes the microbial transformation of nucleosides. This transformation has been applied to the preparation of biologically active nucleosides, which are mainly used as antiviral agents. This transformation generally involves a *N*-pentose transfer reaction. Various microorganisms can catalyze this reaction. As mentioned above, *Ent. aerogenes* is the best producer for ara-A synthesis, *Erw. herbicola* is

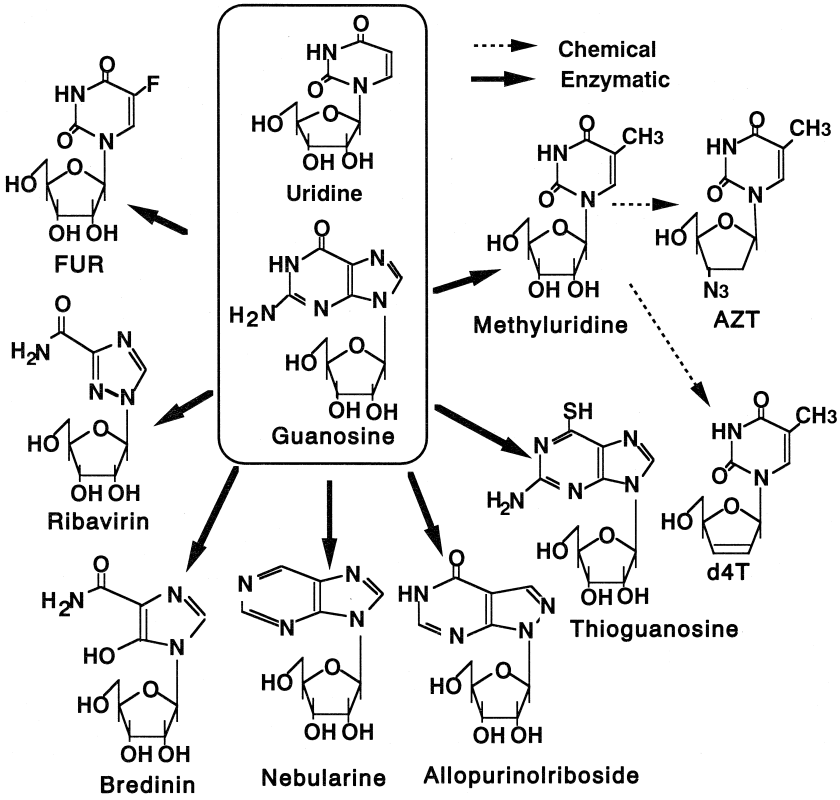


Fig. 10. Chemo-enzymatic synthesis of nucleoside antibiotics (2).

the best for 2′-amino-2′-deoxyadenosine, *B. acetylicum* is the best for ribavirin, and *E. coli* is the best for ddI. These microorganisms were selected through screenings with the reaction mixtures containing their corresponding substrates. These facts indicates there must be many types of microbial enzymes with some specificities that catalyze various types of *trans*-pentose reactions to form new types of nucleoside analogs.

Figs. 9 and 10 show summaries of nucleoside analog syntheses through the combination with the chemical process and the biological process.

Uridine can be conveniently modified in the sugar moiety by the chemical reaction to synthesize their 2′ or 3′-modified uridine analogs. Uridine is an abundant nucleoside in nature and was selected as a starting raw material for the synthesis of pentose-modified nucleosides as shown in Fig. 9.

Uridine and guanosine were selected as the ribose donors to synthesize base-modified, biologically active nucleosides or ribonucleosides intermediate for the chemical modification as shown in Fig. 10. Guanine formed from guanosine is a by-product of the reaction and precipitates in the reaction mixture due to its poor solubility. This poor solubility promote the reaction towards the synthetic direction of another nucleoside.

The application of microbial enzymes to the synthesis of biologically interesting nucleosides shows great promises on the benefits of its simplicity and high stereospecificity.

References

[1] S. Kit, Molec. Cell Biochem. 4 (1976) 161.
[2] A. Weissbach, S.C.L. Hong, J. Aucker, R. Muller, J. Biol. Chem. 248 (1973) 6270.
[3] S.S. Cohen, Medical Biology 54 (1976) 229.
[4] W.W. Lee, A. Benitez, L. Goodman, B.R. Baker, J. Am. Chem. Soc. 82 (1960) 2648.
[5] Park, Davis, Belgian Patent, 671,557 (1967).
[6] T. Utagawa, H. Morisawa, T. Miyoshi, F. Yoshinaga, A. Yamazaki, K. Mitugi, FEBS Lett. 109 (1980) 261.
[7] T. Utagawa, H. Morisawa, F. Yoshinaga, A. Yamazaki, K. Mitugi, Y. Hirose, Agric. Biol. Chem. 49 (1985) 1053.
[8] H. Komura, T. Yoshino, Y. Ishido, Bull. Chem. Soc. (Japanese) 46 (1973) 550.
[9] T. Utagawa, H. Morisawa, S. Yamanaka, A. Yamazaki, F. Yoshinaga, Y. Hirose, Agric. Biol. Chem. 49 (1985) 2425.
[10] T. Utagawa, H. Morisawa, A. Yamazaki, S. Yamanaka, F. Yoshinaga, Y. Hirose, Agric. Biol. Chem. 49 (1985) 2167.
[11] H. Morisawa, T. Utagawa, T. Miyoshi, F. Yoshinaga, A. Yamazaki, K. Mitsugi, Tetrahedron Letters 21 (1980) 479.
[12] M.L. Wolfrom, M.W. Wikley, J. Org. Chem. 32 (1967) 1823.
[13] T. Nakanishi, F. Tomita, T. Suzuki, Agric. Biol. Chem. 38 (1974) 2465.
[14] Y. Iwai, A. Nakagawa, A. Nagai, K. Matsuyama, Y. Takahasi, M. Matsushita, A. Hirano, S. Omura, J. Antibiotics 32 (1979) 1367.
[15] N. Okawa, H. Nakayama, K. Ikeda, K. Furihata, A. Shimazu, N. Otake, H. Yonehara, Agric. Biol. Chem. 44 (1980) 1671.
[16] J.P.H. Verheyden, D. Eagner, F.G. Moffat, J. Org. Chem. 36 (1971) 250.
[17] T. Utagawa, H. Morisawa, S. Yamanaka, A. Yamazaki, Y. Hirose, Agric. Biol. Chem. 49 (1985) 2711.
[18] T. Utagawa, H. Morisawa, T. Nakamatsu, A. Yamazaki, S. Yamanaka, FEBS Letters 119 (1980) 101.
[19] H. Morisawa, T. Utagawa, S. Yamanaka, A. Yamazaki, Tetrahedron Letters 21 (1980) 479.
[20] R.W. Sidwell, J.H. Huffman, G.P. Khare, J.T. Witkowski, R.K. Robins, Science 177 (1972) 705.
[21] T. Utagawa, H. Morisawa, S. Yamanaka, A. Yamazaki, Y. Hirose, Agric. Biol. Chem. 50 (1986) 121.
[22] H. Shirae, K. Yokozeki, K. Kubota, Agric. Biol. Chem. 52 (1988) 1777.
[23] H. Mitsuya, K.J. Weihold, P.A. Furman, M.H. St. Clair, S.N. Lehrman, R.C. Gallo, D. Bolognesi, D.W. Barry, S. Broder, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 7096.
[24] S. Broder, Med. Res. Rev. 10 (1990) 410.
[25] M. Ishii, H. Shirae, K. Yokozeki, Agric. Biol. Chem. 53 (1989) 3209.
[26] H. Shiragami, T. Ineyama, Y. Uchida, K. Izawa, Nucleoside and Nucleotide 15 (1996) 47.
[27] H. Mitsuya, S. Broder, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 1911.
[28] H. Shirae, K. Kobayashi, H. Shiragami, Y. Irie, N. Yasuda, K. Yokozeki, Appl. Environ. Microbiol. 55 (1989) 419.
[29] H. Shiragami, Y. Irie, H. Shirae, K. Yokozeki, N. Yasuda, J. Org. Chem. 53 (1988) 5170.