UDC 547.855 : 576.851.21.095.18

3. **Minoru Kawashima**: Studies on Nucleic Acid Antagonists. III.[1] Growth Inhibitory Effect of 5-Phenylazopyrimidine Derivatives on *Streptococcus faecalis*.*

(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.***)

The preceding paper of this series described the inhibitory activity of 5-phenylazopyrimidine derivatives upon the growth of *Lactobacillus casei* (IFO 3069) and *Tetrahymena geleii*.[1] It was also shown that the inhibitory activities of the pyrimidine derivatives examined were not completely parallel in these two microbiological systems. For example, 2,4,6-triamino-5-nitroso-pyrimidine (Py-24) markedly inhibited the growth of *L. casei* in Hitching's PT and PFA media but did not show any effect on the growth of *T. geleii*, while 2,4,6-triamino-5-phenyl-azopyrimidine (Py-41) inhibited the growth of these two test microörganisms. It was also demonstrated that the growth inhibition caused by these compounds was reversed on addition of thymine or folic acid on the analyzing system of Hitchings, which consisted of seven kinds of media.[1]

The present paper deals with the relationship between chemical structure of these compounds and their antagonistic properties, and with the experimental results which suggest the mechanism of their inhibitory action on the biosynthesis of nucleic acids.

Materials and Methods

The microörganism employed in this experiment was *Streptococcus faecalis* (ATCC 8043, IFO 3181) in Luckey's medium[2] for folic acid assay and the medium was supplemented with 50 mγ/100 cc. of folic acid. The composition of the basal medium is indicated in Table I.

TABLE I. Basal Medium for *Streptococcus faecalis*

Casein hydrolyzate	1.0 g.
Glucose	2.0 g.
Sodium acetate	0.4 g.
L-Cystine	20 mg.
L-Tryptophan	60 mg.
Adenine, guanine, uracil, xanthine	2.0 mg. each
Thiamine hydrochloride	40 γ
Riboflavin	40 γ
Pyridoxine hydrochloride	240 γ
Nicotinic acid	120 γ
Ca pantothenate	80 γ
Biotin	0.08 γ
Folic acid	0.05 γ
Salt solution A* and B**	1.0 cc. each
Sufficient water to make	100 cc. (pH 6.8)

* 25 g. each of KH_2PO_4 and K_2HPO_4 dissolved in sufficient water to make 250 cc. of solution.

** 10 g. $MgSO_4$, 0.5 g. NaCl, 0.5 g. $FeSO_4$, and 0.5 g. $MnSO_4$ dissolved in sufficient water to make 250 cc. of solution.

Test for Growth Inhibitory Activity—Pyrimidine derivatives were added in 500 γ/5 cc. concentration to each of the medium described above and the mixture was diluted five-fold consecutively in the usual manner.

* This paper constitutes a part of a series entitled "Studies of Nucleic Acid Antagonists" by K. Tanaka and E. Ohmura. This was read at the Kinki Local Annual Meeting of the Pharmaceutical Society of Japan, October 19, 1957.

** Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (川島 實).

1) Part. II: K. Tanaka, *et al.*: This Bulletin, **7**, 7 (1959).

2) T. D. Luckey: J. Biol. Chem., **152**, 157 (1944).

The growth inhibitory action of these compounds was expressed by the concentration necessary for complete inhibition.

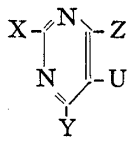
Reversal Experiments—The minimal inhibition dose of the compounds was added to 2.5-cc. aliquot of double-strength medium, a number of known metabolites was added to make the final concentrations of 10^2, 10, 1, 10^{-1}, and 10^{-2} γ/5 cc., and the total volume was made 5 cc. with distilled water.

In all instances, the mixture was autoclaved at 15 lbs. for 5 mins., inoculated, and incubated for 24 hrs. at 30°. *Str. faecalis* for the inoculum was incubated in the basal medium for 24 hrs. at 30°, and an aliquot of the culture was centrifuged, the sedimented cells were washed with saline, the washed cells were suspended in sterilized sailne, and diluted appropriately. The growth of the test organism was measured by turbidimetric procedure using the Coleman Junior spectrophotometer (650 mμ).

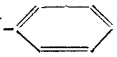
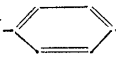
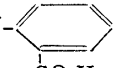
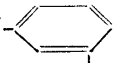
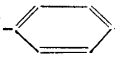
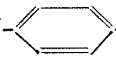
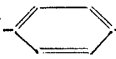
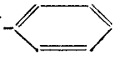
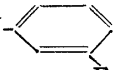
The metabolites employed in these reversal experiments were adenine, guanine, uracil, xanthine, thymine, folic acid, leucovorin, *p*-aminobenzoic acid, adenylic acid, guanylic acid, uridylic acid, cytidylic acid, uridine, cytosine, thymidine, orotic acid, and an amino acid mixture (Casamino Acid, Difco Co.).

The chemical structure of the 5-phenylazopyrimidine derivatives tested is shown in Table II.

TABLE II. 5-Phenylazopyrimidine Derivatives and their Antagonistic Properties to Thymine, Folic Acid, or Leucovorin



Compd. No.	Chemical structure				Concn. to cause complete inhibition γ/cc.	Inhibition reversed by		
	X	Y	Z	U		thymine	folic acid	leucovorin
Py-23	NH_2	NH_2	NH_2	—	100	+	+	+
Py-24	NH_2	NH_2	NH	NO	0.8	+	+	+
Py-25	NH_2	NH_2	NH_2	NH_2	20	+	+	+
Py-26	NH_2	CH_3	CH_3	-N:N-C$_6$H$_5$	500			
Py-37	OH	OH	CH_3	-N:N-C$_6$H$_5$	100			
Py-40	NH_2	NH_2	OH	-N:N-C$_6$H$_5$	20	+	−	+
Py-41	NH_2	NH_2	NH_2	-N:N-C$_6$H$_5$	20	+	−	+
Py-58	OH	CH_3	CH_3	-N:N-C$_6$H$_5$	500			
Py-61	NH_2	NH_2	NH_2	-N:N-C$_6$H$_4$-Cl	4	+	−	+
Py-62	NH_2	NH_2	NH_2	-N:N-C$_6$H$_3$(Cl)$_2$	4	+	−	+
Py-63	NH_2	NH_2	NH_2	-N:N-C$_6$H$_4$-NO_2	20	+	−	+
Py-64	NH_2	NH_2	NH_2	-N:N-C$_6$H$_4$-SO_3H	500	+	+	+
Py-68	NH_2	CH_3	CH_3	-N:N-C$_6$H$_4$-SO_3H	500			
Py-70	NH_2	CH_3	CH_3	-N:N-C$_6$H$_4$(Cl)-Cl	500			
Py-71	NH_2	NH_2	OH	-N:N-C$_6$H$_4$-SO_3H	20	+	+	+
Py-72	NH_2	NH_2	NH_2	-N:N-C$_6$H$_4$-SO_2NH_2	20	+	+	+
Py-73	NH_2	NH_2	OH	-N:N-C$_6$H$_4$-SO_2NH_2	20	+	+	+
Py-74	NH_2	NH_2	NH_2	-N:N-C$_6$H$_4$-COOH	20	+	+	+

Py-76	NH_2	NH_2	CH_3	—	20	+	+	+
Py-77	NH_2	NH_2	CH_3	-N : N- 	20	+	−	+
Py-79	NH_2	NH_2	OH	-N : N-  -PO_3H_2	500			
Py-80	NH_2	NH_2	NH_2	-N : N-  -PO_3H_2	100	+	+	+
Py-81	NH_2	NH_2	NH_2	-N : N-  -AsO_3H_2	100	+	+	+
Py-82	NH_2	NH_2	NH_2	-N : N-  SO_3H	500			
Py-83	NH_2	NH_2	NH_2	-N : N-  PO_3H_2	100	+	+	+
Py-84	NH_2	NH_2	NH_2	-N : N-  -NO_2	500			
Py-85	NH_2	NH_2	NH_2	-N : N-  -$(CH_2)_3$-COOH	500			
Py-86	NH_2	NH_2	NH_2	-N : N-  -CO-NH-COOH CH_2-CH_2-COOH	500			
Py-90	NH_2	NH_2	NH_2	-N : N-  -F	100			
Py-91	NH_2	NH_2	NH_2	-N : N-  F	20	+	−	+

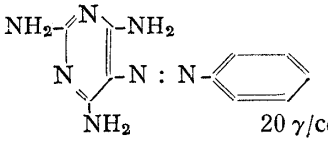
+ The growth inhibition, caused by addition of minimal effective concenration of a compound, was reversed by 1 γ/5 cc. of leucovorin or folic acid, or 100 γ/5 cc. of thymine.
− The growth inhibition failed to be reversed by 10 γ/5 cc. of folic acid.

Results

The results of the estimation of growth inhibitory activtites are listed in Table II. 2,4,6-Triamino-5-nitrosopyrimidine (Py-24) was the most effective inhibitor on the growth of *Str. faecalis*, being effective in a concentration of 0.8 γ/cc. 2,4,6-Triamino-5-(4-chlorophenylazo)pyrimidine (Py-61) and its 2-chlorophenylazo analog (Py-62) were effective for inhibition in a concentration of 4.0 γ/cc., while 2,4,5,6-tetraaminopyrimidine (Py-25), 2,4-diamino-6-hydroxy-5-phenylazopyrimidine (Py-40), and 2,4,6-triamino-5-phenylazopyrimidine (Py-41) were effective in a concentration of 20 γ/cc.

In the reversal experiments, 2,4,6-triamino-5-phenylazopyrimidine (Py-41), which is one of the typical phenylazo compound, was investigated. The growth of the test organism which was inhibited by Py-41 in 20 γ/cc. concentration was reversed completely by the addition of thymine, thymidine, or leucovorin as shown in Fig. 1, and the inhibition indices for these three metabolites were 1:0.76, 1:1.26, and 1:0.005,

Py-41



20 γ/cc.

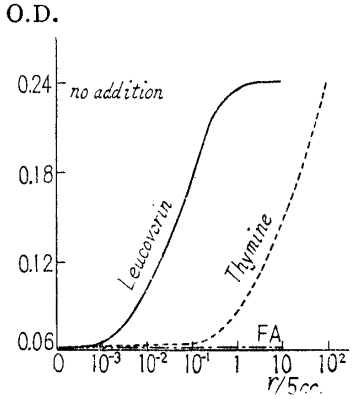
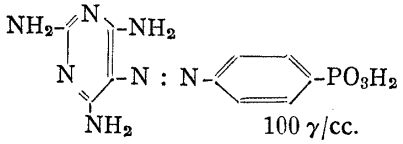


Fig. 1. Reversal Pattern of Py-41

Py-80



100 γ/cc.

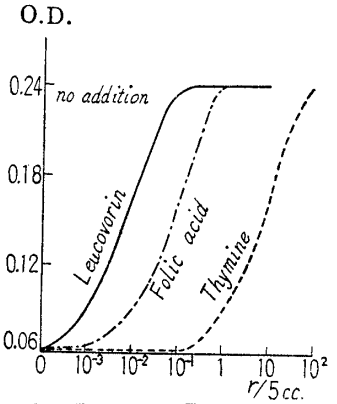


Fig. 2. Reversal Pattern of Py-80

respectively. Folic acid, PABA, amino acids, and other metabolites failed to reverse the growth inhibition.

Contrary to Py-41, the growth inhibitory activity of 4-(2,4,6-triamino-5-pyrimidylazo)benzenephosphonic acid (Py-80), which has phosphonic acid radical in the phenylazo moiety of the 5-position of pyrimidine ring, was reversed not only by thymine and leucovorin but also by folic acid (Fig. 2).

The results of the inhibition analyses of 19 kinds of 5-phenylazopyrimidine derivatives for folic acid, thymine, and leucovorin are summarized in Table III. At the same time it was noted that none of the metabolites such as PABA, orotic acid, or amino acid mixture could reverse the inhibitory activity of these compounds.

It is also noted that the 5-phenylazopyrimidine derivatives studied in the present experiments might be classified into the following two groups from the reversal patterns by thymine or leucovorin and folic acid. The first group consists of those compounds whose growth inhibitory activity is reversed by thymine or leucovorin but not by folic acid, and the second group are those compounds whose inhibitory activity is reversed by thymine or leucovorin as well as by folic acid.

The relationship between chemical structure and reversal patterns is shown as follows: The compounds which have no substituent (Py-40, 41, and 77) or those which possess Cl or NO_2 (Py-61, 62, 63, and 70) in the benzene ring of the phenylazo radical in 5-position of the pyrimidine moiety, belong to the first group, and those which have $-SO_3H$, $-SO_2NH_2$, $-COOH$, $-PO_3H_2$ in the benzene ring (Py-64, 72, 74, 80, and 81) belong to the second group. 2,4,6-Triaminopyrimidine (Py-23) and its 5-nitroso (Py-24) and 5-amino derivatives (Py-25) also belong to the second group.

It is very interesting to note that the factor determining antagonistic properties to folic acid is the kind of substituent present in the 5-position of pyrimidine ring.

Discussion

It was shown that 18 kinds of 5-phenylazopyrimidine derivatives can be divided into two groups according to their antagonistic patterns on *Str. faecalis* in inhibition analyses.

The relationship between chemical structure and these antagonistic patterns has been described. The fact that 2,4.6-triaminopyrimidine (Py-23) and its 5-nitroso derivative (Py-24), and 2,4,5,6-tetraaminopyrimidine (Py-25) belong to the second group, led to the following assumption.

These differences in antagonistic properties of 5-phenylazopyrimidine derivatives might be related to the discrepancy in their physicochemical stability.[3] It seemed that the compounds which have a substituent such as $-COOH$, $-SO_3H$, $-SO_2NH_2$, $-PO_3H_2$, or $-AsO_3H_2$ in the benzene ring of the phenylazo radical in 5-position are more unstable than the compounds which have no substituent or possess Cl or NO_2 in the phenylazo radical, and the former might cleave easily than the latter, thus showing an antagonistic pattern similar to that of compounds such as Py-23 or -24. This assumption, however, must be confirmed by further experiments.

These facts suggest that the compounds which belong to the first group specifically inhibit the conversion of folic acid to citrovorum factor (CF) in the metabolic pathway of *Str. faecalis* cell.

Hitchings[4] reported that 2,4-diamino-5-*p*-chlorophenoxy-6-ethylpyrimidine inhibited the growth of *Str. faecalis*, and the growth inhibition was reversed by leucovorin but not by folic acid.

The author is deeply indebted to Dr. S. Kuwada, Director of the Laboratories, for his permission for this investigation and its publication. The author also expresses his grateful thanks to Dr. A. Watanabe for his encouragement throughout this work.

Summary

Results of growth inhibition tests and of the reversal experiments on the growth of *Str. faecalis* by 5-phenylazopyrimidine derivatives were described.

3) H. H. Jaffi: Chem. Revs., **53**, 191(1953).
4) G. H. Hitchings: J. Biol. Chem., **199**, 43(1952).

The 5-phenylazopyrimidine derivatives were classified into following two groups on the basis of their antagonistic pattern. The first group consists of those compounds whose growth inhibitory activity is reversed by thymine or leucovorin but not by folic acid, and the second group, those compounds whose inhibitory activity is reversed by thymine or leucovorin, as well as by folic acid.

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4. **Minoru Kawashima**: Studies on Nucleic Acid Antagonists. IV.[1] Inhibitory Effect of 5-Phenylazopyrimidines on the Enzymatic Conversion of Folic Acid to Citrovorum Factor *in vitro*.*

(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.***)

In the preceding report[1] the author described the inhibitory activity of 5-phenylazopyrimidines upon the growth of *Streptococcus faecalis* and the reversal pattern by a series of metabolites. It was shown that these 5-phenylazopyrimidines are classified into two groups according to their reversal pattern by thymine, leucovorin, or folic acid.

The relatonship between their antagonistic pattern and chemical structure was also demonstrated. The possibility that the inhibitory action of the first group is due to the inhibition of the enzymatic conversion of folic acid to citrovorum factor (CF) was discussed on the basis of results reported by Hitchings.[2]

The present report deals with the inhibitory effect of 5-phenylazopyrimidines on the conversion of folic acid to CF by the chick-liver supernatant solution prepared according to Doctor's method.[3]

Material and Methods

The chemical structure and antagonistic pattern upon the growth of *Str. faecalis* of the 12 kinds of 5-phenylazopyrimidines employed in this experiment are summarized in Table I.

The liver of white Leghorn female chicks of 10 to 12 weeks old were used as the source of enzyme throughout this experiment. The chick-liver supernatant solutions were prepared in a following manner: Preparation of 20% homogenate of chick liver in 0.08*M* sodium potassium phosphate buffer (pH 6.7) was made in a Waring blendor. An aliquot of the homogenate was centrifuged for 30 mins. at 13,000*g*, the clear portion of the supernatant was decanted, and made to the volume of the starting homogenate with the same buffer.

The mixture to be incubated contained 5 cc. of the supernatant solution, 10 mg. of DL-serine, 5 mg. of DL-homocysteine, and 10 mg. of $MgCl_2$ in a total volume of 10 cc. The mixture was incubated with shaking in N_2 atmosphere for 3 hrs. at 37° and autoclaved for 30 mins. at 121°. After cool, the mixture was homogenized in a Waring blendor, made up to an appropriate volume, filtered, and the filtrate was assayed. The CF content of the incubated mixture described above was determined by using CF-assay medium, the composition of which is shown in Table II. *Leuconostoc citrovorum* (ATCC 8081) was used as the test organism and Ca-leucovorin was employed as the standard. The culture was incubated for 24 hrs. at 37° and the growth was measured by using the Coleman Junior spectrophotometer (650 mμ).

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* This paper constitutes a part of a series entitled "Studies on Nucleic Acid Antagonists" by K. Tanaka and E. Ohmura. This was read at the Kinki Local Meeting of the Pharmaceutical Society of Japan, October 19, 1957.

** Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (川島 實).

1) Part III. M. Kawashima: This Bulletin, **7**, 13(1959).
2) G. H. Hitchings: J. Biol. Chem., **183**, 1(1950).
3) V. M. Doctor: *Ibid.*, **222**, 959(1956).