UDC 547.855 : 591.2-006

2. Kuniyoshi Tanaka, Einosuke Omura, Minoru Kawashima, Junmei Watanabe, Hajime Yokotani, Homu Ito, Yukio Sugino, Motoi Ishidate, Toyoshige Araki, Yoshitomo Aramaki, and Kyo Kajiwara: Studies on Nucleic Acid Antagonists. II.* Structure-Activity Relationship in 5-Phenylazopyrimidines.

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In a previous work,* the syntheses of 31 kinds of 5-phenylazopyrimidine derivative were carried out, seeking variety in the kind and position of substituents not only in the pyrimidine ring but also in the benzene ring with consideration for their biological activity, especially their anti-tumor activity. In the present paper, relationship between the structure and activity of these compounds is discussed.

Various 5-substituted pyrimidines were first synthesized in expectation of their thymine-antagonistic activity such as seen in 5-bromo- and 5-nitrouracil, and preliminary screenings of those compounds were conducted as follows: Measurement was made of their minimum inhibitory concentrations against the growth of *Lactobacillus casei* (IFO 3069) on the PT and PFA media described by Hitchings,[1] of *Tetrahymena geleii* on a modified one of the Kidder's synthetic medium,[2] and of chick embryo fibroblast in tissue culture. As a result, it was found that 2,4,6-triamino-5-nitrosopyrimidine (Py-24) showed a remarkable growth-inhibitory effect on *L. casei*, but had no such effect on *T. geleii*. The nitroso radical of Py-24 was therefore replaced by phenylazo group and the resulting 2,4,6-triamino-5-phenylazopyrimidine (Py-41) was found to inhibit the growth of *L. casei* as well as of *T. geleii*. From these results, synthetic and biological studies of 5-phenylazopyrimidines were pushed on. First of all, in order to examine the influence of the kind of substituents in the pyrimidine ring on inhibitory activity against these microörganisms, 2-amino-4,6-dimethyl-5-phenylazopyrimidine (Py-26), 2,4-diamino-6-hydroxy-5-phenylazopyrimidine (Py-40), and 2,4-diamino-6-methyl-5-phenylazopyrimidine (Py-77) were synthesized and their activities were compared. As shown in Table I, it was found that 2,4,6-triamino compounds were the most active but the activity gradually weakened as the amino groups were substituted with hydroxyl or methyl group.

The influence of substituents in the benzene ring upon inhibitory activity against these microörganisms was then examined. When chlorine was introduced into the *para*-position of Py-41, the resulting 2,4,6-triamino-5-(4-chlorophenylazo) pyrimidine (Py-61) was as active as the original compound against both *L. casei* and *T. geleii*, while the activity, especially against *L. casei*, was much weakened in 2,4,6-triamino-5-(2-chlorophenylazo)pyrimidine (Py-62) and 2,4,6-triamino-5-(2,4-dichlorophenylazo)pyrimidine (Py-69) (Table II). As for the fluoro radical, *para*-substituted 2,4,6-triamino-5-(4-fluorophenylazo)pyrimidine (Py-90) was less active than the *meta*-substituted homolog (Py-91). A similar relationship applies also to the introduction of a nitro group, the inhibitory activity of 2,4,6-triamino-5-(3-nitrophenylazo)pyrimidine (Py-63) being equal to that of Py-41, but the *para*-substituted nitro derivative (Py-84) being almost inactive. Moreover, introduction of a substituent such as sulfonic acid, sulfonamide, carboxylic acid, phosphonic acid, or arsonic acid lowered the activity. For example, (2,4,6-triamino-5-pyrimidinylazo)benzene-4-sulfonic acid (Py-64), -2-sulfonic acid (Py-82), -4-sulfonamide (Py-72), -4-carboxylic acid (Py-74), -4-phosphonic acid

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1) G. H. Hitchings, G. H. Elion, E. A. Falco, P. B. Russel, M. B. Sherwood, H. Vanderwerff: J. Biol. Chem., **183**, 1 (1950).

2) G. W. Kidder, V. C. Dewey: *Ibid.*, **178**, 383(1949).

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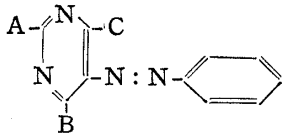
(Py-80), -3-phosphonic acid (Py-83), and -4-arsonic acid (Py-81) were all less active than their parent compound, Py-41.

TABLE I. Structure-Activity Relationship in 5-Phenylazopyrimidines (1)



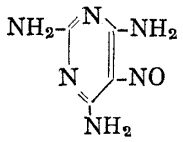
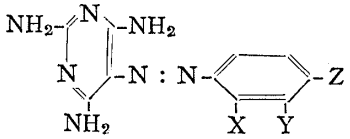
Compd. No.	Substituents A	B	C	Minimum inhibitory concn. (γ/cc.) *L. casei* in PT	*L. casei* in PFA	*T. geleii*	Chick embryo fibroblast
Py-41	NH_2	NH_2	NH_2	20	20	4	20
40	NH_2	NH_2	OH	20	20	100	200
77	NH_2	NH_2	CH_3	100	100	500	200
26	NH_2	CH_3	CH_3	500	>500	>500	200
Py-24				0.8	4	500	>200

TABLE II. Structure-Activity Relationship in 5-Phenylazopyrimidines (2)



Compd. No.	Substituents X	Y	Z	Minimum inhibitory concn. (γ/cc.) *L. casei* in PT	*L. casei* in PFA	*T. geleii*	Chick embryo fibroblast
Py-41	H	H	H	20	20	4	20
61	H	H	Cl	20	20	20	2
62	Cl	H	H	100	100	20	20
69	Cl	H	Cl	100	100	20	20
90	H	H	F	500	500		20
91	H	F	H	100	100		20
63	H	NO_2	H	20	4	20	20
84	H	H	NO_2	500	500		200
74	H	H	COOH	500	>500	>500	200
64	H	H	SO_3H	500	500	>100	>200
82	SO_3H	H	H	500	500		200
72	H	H	SO_2NH_2	100	500	500	>200
80	H	H	PO_3H_2	500	500		200
83	H	PO_3H_2	H	500	500		200
81	H	H	AsO_3H_2	500	500		>200

Inhibitory activities of 5-phenylazopyrimidines upon the growth of *L. casei* in the seven kinds of test media described by Hitchings[1] (O, OT, OFA, FA+, PO, PT, and PFA) were compared and it was found that the growth inhibitory activity shown by these compounds in the basal medium (O) diminished in the media containing thymine or folic acid, as can be seen in Table III.

TABLE III. Inhibitory Activity of 5-Phenylazopyrimidines on the Growth of *L. casei* in Seven Kinds of Hitchings' Media

i) 2,4,6-Triamino-5-phenylazopyrimidine (Py-41)

Concn. of Py-41 (γ/cc.) \ Medium	O	OT	OFA	FA+	PO	PT	PFA
100	100	100	100	100	100	87	100
20	100	76	86	16	90	0	50
4	50	20	36	17	42	0	25
0.8	12	10	0	0	0	0	13
0.16	0	5	0	0	0	0	0

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ii) 2,4,6-Triamino-5-(2-chlorophenylazo)pyrimidine (Py-62)

Concn. of Py-62 (γ/cc.) \ Medium	O	OT	OFA	FA+	PO	PT	PFA
100	100	100	100	73	88	93	96
20	80	63	63	27	78	31	42
4	80	63	45	20	44	0	21
0.8	0	0	0	0	0	0	8
0.16	0	0	0	0	0	0	0

iii) 4-(2,4,6-Triamino-5-pyrimidinylazo)benzenesulfonic Acid (Py-64)

Concn. of Py-64 (γ/cc.) \ Medium	O	OT	OFA	FA+	PO	PT	PFA
100	100	75	33	7	85	17	17
20	25	25	25	0	43	0	11
4	10	0	8	0	0	0	0
0.8	10	0	0	0	0	0	0
0.16	0	0	0	0	0	0	0

Figures indicate percentage inhibition, taking complete inhibition of growth as 100.

From this fact, the activity of these derivatives was assumed to be due to their disturbance of nucleic acid metabolism. Accordingly, one of the authors (Kawashima[3]) conducted several kinds of inhibition analyses to verify this assumption and clarified the pattern of metabolic antagonism by 5-phenylazopyrimidine derivatives. As a result, it was demonstrated that these compounds may be divided into two groups, and that the kind of substituents in the benzene ring of the 5-phenylazo radical is the determining factor for the classification, while the kind of substituent at 2-, 4-, or 6-position in the pyrimidine moiety and the position of the substituents in the benzene ring are without effect.

The inhibitory activity of these compounds on the outgrowth of chick embryo fibroblast was parallel to those on *L. casei* and *T. geleii* with some exceptions (Tables I and II).

The effect of these compounds upon the Yoshida ascites sarcoma and Ehrlich ascites carcinoma was examined by the criteria of cytological observations as well as by the life-span checking of test animals (Table IV). Compounds active on *L. casei* and *T. geleii*, such as Py-40, -41, -61, -62, -63, -69, and -70, showed no influence upon the tumor systems, while weakly active ones, such as Py-64, -72, -74, and -80, exhibited marked inhibitory effect upon them.

Further observations on the structure-activity relationship were made with several derivatives (Table V). Among the derivatives which are different from Py-64, -72, or -80 in the kind of substituents present in the pyrimidine ring, 4-(2-amino-4,6-dimethyl-5-pyrimidinylazo)benzenesulfonic acid (Py-68) showed only a slight cytological effect on Yoshida sarcoma, and the others, 4-(2,4-diamino-6-hydroxy-5-pyrimidinylazo)benzenesulfonic acid (Py-71), -sulfonamide (Py-73), 4-(2,4-diamino-6-methyl-5-pyrimidinylazo)benzenesulfonic acid (Py-78), and 4-(2,4-diamino-6-hydroxy-5-pyrimidinylazo)benzenephosphonic acid (Py-79), showed no activity. Furthermore, the compounds which, differing from Py-64 and -80, have a sulfonic acid or phosphonic acid radical at the *ortho* or *meta* position of the benzene ring, such as 2-(2,4,6-triamino-5-pyrimidinylazo)benzenesulfonic acid (Py-82) and 3-(2,4,6-triamino-5-pyrimidinylazo)benzenephosphonic acid (Py-83), did not exhibit any antitumor activity. Judging from these results, the structural requirements necessary for the compound to be effective on Yoshida sarcoma were found to be more limited than those necessary to be effective on microörganisms. It seems that to meet the former requirements, all of the 2-, 4-, and 6-positions of the pyrimidine moiety should be substituted by

3) M. Kawashima: This Bulletin, **7**, 13, 17(1959).

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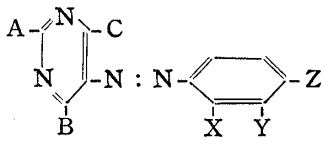
TABLE IV. Anti-tumor Effect of 5-Phenylazopyrimidines

Compd. of Py group	Toxicity LD (Mouse i.p.) (mg./kg.)	Effect on Yoshida sarcoma Cytol.	Yoshida sarcoma Life-prolong.	Ehrlich carcinoma Cytol.	Ehrlich carcinoma Life-prolong.
Py-26	>1000	±	−	±	−
37	>1000	−	−	−	−
40	>1000	−	−	±	−
41	100	−	−	−	+
58	1000	−	−	−	±
61	500	−	−	±	±
62	500~1000	−	−	−	−
63	100~500	±	−	−	−
64	>1000	+	+	±	−
65	>1000	−	−	+	±
66	>1000	±	−	−	±
68	500	−	−	−	−
69	>1000	−	−	−	−
70	>1000	−	−	−	−
71	<1000	−	−	−	−
72	>1000	+	±	±	−
73	>1000	−	−	−	−
74	1000	+	±	±	−
75	1000	−	−	−	−
77	>1000	−	−	±	−
78	1000	−	−	±	−
79	500	−	−	−	−
80	<100	+	+	−	−
81	<100	−	−	−	−
82	>1000	−	−		
83	100	−	−		
84	1000	−	−	−	−
85		−	−	−	−
86		−	−	−	−
90	<100	−	−	−	−
91	<100	−	−	−	−

amino radical and at the same time the *para*-position of the benzene ring of the 5-phenylazo radical by $-SO_3H$, $-SO_2NH_2$, -COOH, or $-PO_3H_2$. It is interesting to note that these four active compounds (Py-64, -72, -74, and -80) all belong to the second one of the two groups mentioned earlier.

TABLE V. Relationship between Structure and Anti-tumor Effect of 5-Phenylazopyrimidines



Compd. No.	A	B	C	X	Y	Z	Yoshida sarcoma Cytol.	Yoshida sarcoma Life-span
Py-64	NH_2	NH_2	NH_2	H	H	SO_3H	+	+
71	NH_2	NH_2	OH	H	H	SO_3H	−	−
78	NH_2	NH_2	CH_3	H	H	SO_3H	−	−
68	NH_2	CH_3	CH_3	H	H	SO_3H	±	−
82	NH_2	NH_2	NH_2	SO_3H	H	H	−	−
72	NH_2	NH_2	NH_2	H	H	SO_2NH_2	+	±
73	NH_2	NH_2	OH	H	H	SO_2NH_2	−	−
80	NH_2	NH_2	NH_2	H	H	PO_3H_2	+	+
79	NH_2	NH_2	OH	H	H	PO_3H_2	−	−
83	NH_2	NH_2	NH_2	H	PO_3H_2	H	−	−

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The tumor-inhibitory effect of these four compounds was further investigated,[4] and Py-64 and -80 were effective on the solid form of Ehrlich carcinoma and of sarcoma 180, respectively, in reduction of the diameter or weight of tumor, and Py-64 was also effective against mouse lymphatic leukemia SN36 and the Nitromin-resistant Yoshida sarcoma of rats in life-prolongation. The microbiological data suggesting the mechanism of inhibition of nucleic acid metabolism led to the combined administration of the active 5-phenylazopyrimidines with other antimetabolites which have so far been assumed to block other pathways of nucleic acid biosynthesis. As expected, concurrent administration of Py-64 or -80 with 6-mercaptopurine showed a marked effect upon sarcoma 180.

The preliminary report of this work was already presented[5] and pathological and pharmacological details of this study will be published later.[4,6]

Recently, Modest[7] and Timmis[8] reported that 5-phenylazopyrimidines inhibited the growth of *St. faecalis* and *Leuc. citrovorum*, acting as a folic acid antagonist. This result agrees with the present one in that 2,4,6-triaminopyrimidine derivatives were the most active, but they did not test them on transplantable tumors nor referred to the compounds belonging to the above-mentioned second group, such as Py-64 and -80.

The authors express their gratitude to Prof. T. Yoshida and Prof. M. Ishidate of the University of Tokyo for their kind instruction. They are also grateful to Dr. S. Kuwada, Director of the Laboratories, and Dr. S. Tatsuoka, head of the department, for their continued guidance and encouragement.

Experimental

Inhibitory Experiments on the Growth of *Lactobacillus casei*—The microörganism employed in this experiment was *L. casei* (IFO 3069). The composition of the seven media (O, OT, OFA, FA+, PO, PT, PFA) was described by Hitchings.[1] 5-Phenylazopyrimidine derivatives were added in 500 γ/5 cc. concentration to each medium in a tube. The mixture was diluted five-fold consecutively in the usual manner, autoclaved at 15 lb. for 5 mins., inoculated with *L. casei*, and incubated for 72 hrs. at 37°. The extent of growth of the test organism was measured by the turbidimetric procedure using the Coleman Junior spectrophotometer (650 mμ).

In Tables I and II, the growth inhibitory activities were expressed in terms of the minimal effective concentration necessary for complete inhibition. In Table III, the percentage inhibition was calculated by taking the optical density of the completely inhibited culture as 100, and that of the culture of maximal growth as 0.

Inhibitory Experiments on the Growth of *Tetrahymena geleii*—In this experiment, *T. geleii* in the modified Kidder's medium was employed as the screening system. The composition of the medium is given in Table VI. The procedure was almost the same as in the case of *L. casei*, except that the incubation was carried out at 25° for 96 hrs.

TABLE VI. Basal Medium for *Tetrahymena geleii*

Casein hydrolyzate	0.5 g.	Guanylic acid	6.0 mg.
Glucose	0.5 〃	Adenine sulfate	4.0 〃
Sodium acetate	0.2 〃	Uracil	1.0 〃
Tryptophan	20 mg.	Lipoic acid (5 : 8-)	10 γ
Cysteine	20 〃	$MgSO_4 \cdot 7H_2O$	20 mg.
Methionine	20 〃	K_2HPO_4	20 〃
Ca-pantothenate	20 γ	$CaCl_2 \cdot 2H_2O$	10 〃
Nicotinamide	20 〃	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	5 〃
Thiamine-HCl	200 〃	$FeCl_3 \cdot 6H_2O$	250 γ
Riboflavin	20 〃	$CuCl_2 \cdot 2H_2O$	1 mg.

4) K. Kajiwara, *et al.*: Gann (in press).
5) K. Tanaka, K. Kaziwara, *et al.*: Paper presented at the 15th General Meeting of the Japanese Cancer Association, Sapporo, 1956 [Proceedings; Gann, **47**, 401 (1956)].
6) Y. Aramaki, *et al.*: Ann. Repts. Takeda Research Lab., **17**, 71 (1958).
7) E. J. Modest, H. N. Schlein, G. E. Foley: Proc. Am. Assoc. Cancer Res., **2**, 134 (1956); J. Pharm. Pharmacol., **9**, 68 (1957).
8) G. M. Timmis, D. G. I. Felton, H. O. J. Collier, P. L. Huskinson: *Ibid.*, **9**, 46 (1957).

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Folic acid	2 γ	$MnCl_2 \cdot 4H_2O$	10 γ
Pyridoxine-HCl	20 〃	$ZnCl_2$	10 〃
Pyridoxal-HCl	2 〃	Tween-85	140 mg.
Pyridoxamine-HCl	2 〃	Sufficient water to make 100 cc.	
Biotin	0.1 〃		
Choline chloride	200 〃	(pH 6.8, double strength)	

Inhibitory Experiments on the Cell Multiplication of Chick Embryo Fibroblast—Minced skin-muscle tissues from 11- to 12-day-old chick embryos were fixed on the wall of 18×180 mm. test tubes by embedding in plasma clot and overlayed with 2 cc. of fluid medium, consisting of Hanks' balanced salt solution with 3% horse serum, with or without the test sample. After two days' incubation at 36°, inhibitory effect on the outgrowth of fibroblasts from the tissue fragments was examined microscopically under a low-power magnification (×80).

Inhibitory Experiments on the Growth of Yoshida Sarcoma Cells—The tumor ascites of Yoshida sarcoma cells was inoculated intraperitoneally to the "Donryu/H" rats which are known to be highly susceptible to this tumor, and bred uniformally. On the 4th day, when the tumor cells grew to a pure culture, each compound, dissolved or suspended in saline or adequate medium, was injected into the peritoneal cavity of the animal. The doses administered were the approximate LD (mouse, i.p.), its $^1/_2$, and 1/4 dose of each compound. The tumor ascites was checked at 6, 24, and 48 hrs. and on subsequent days after the injection on the Giemsa-stained specimen. Morphological changes such as karyorrhexis, vacuolisation of nucleus, denucleation, hypostainability, mitosis abnormality, as well as the infiltration of leucocyte were observed.

The life span of tumor animals was also checked together with gross pathological observations and compared with the average survival of the control tumor animal (7.53±0.167 S.E. days).

Inhibitory Experiments on the Growth of Ehrlich Ascites Carcinoma—The procedure was almost the same as in the case of Yoshida sarcoma, except that the 7 successive administrations of the drug started from the 24th hr. after transplantation, and cytological checking was made on the 8th day. Average life span of the control "ddH" mice was 14.96±0.25 days.

Summary

Among the 5-phenylazopyrimidine derivatives reported in the preceding paper,* some members showed a remarkable inhibitory effect upon the growth of *Lactobacillus casei*, *Tetrahymena geleii*, as well as chick embyro fibroblast. Others which were only slightly effective, had anti-tumor activity against Yoshida sarcoma and Ehrlich carcinoma. As a result of study on the structure-activity relationships in these compounds, it was found that 2,4,6-triamino-5-phenylazopyrimidines, in which the *para*-position of the benzene ring of the 5-phenylazo group was substituted with sulfonic acid, sulfonamide, carboxylic acid, or phosphonic acid group, were effective upon several transplantable tumors in experimental animals and their anti-tumor activity increased by a combined administration with other nucleic acid antagonists such as 6-mercaptopurine.

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