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31. Tadashi Okabayashi, Hideo Kanô, and Yasuo Makisumi : Action of Substituted Azaindolizines on Microörganisms. I. Action on Lactic Acid Bacteria.

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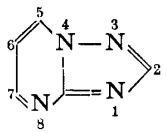
Microbiological systems are now known to offer useful tools for investigation on carcinostatic agents. Among studies on these systems, efforts have been made on the study of purine and pyrimidine analogs.[1~3] Results obtained with a vast number of compounds of this series on several microbiological systems have demonstrated that these systems give useful information on the mode of action of these analogs.

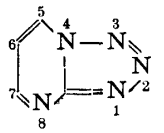
The synthesis of azaindolizine derivatives with various substituent groups has been reported by Kanô, *et al.*,[4,5] and some compounds were also found to cause a considerable damage to several experimental animal tumors.[6] Since the synthesized compound are regarded as those of condensed pyrimidine systems, and include some of isomeric compounds of purines or their analogs, there seemed to be some possibility that investigation with microörganisms generally used in the study of purine and pyrimidine antagonists might offer some clues for elucidation of their mode of action. In the present work, actions of substituted azaindolizines on *Lactobacillus casei* and *Streptococcus faecalis* were studied.

Experimental Methods

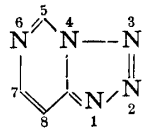
At the time of investigation, all 68 derivatives of substituted azaindolizines available were tested. The formulae of typical ring systems are shown in Chart 1.



1,3,8-triazaindolizine



1,2,3,8-tetraazaindolizine



1,2,3,6-tetraazaindolizine

Chart 1.

Compounds to be tested were dissolved in distilled water and calibrated amounts were added to media before sterilization. In cases where compounds were unstable and destroyed by heating, aqueous solution of compound was sterilized by filtration and added aseptically to the sterilized media.

The test organisms used were *Lactobacillus casei* and *Streptococcus faecalis R.* Experimental procedure for both microörganisms were the same as that described by Hitchings, *et al.*[7] except that the growth of the test organisms was measured as optical density.

Results and Discussion

Growth inhibition of lactic acid bacteria by substituted azaindolizines was determined on OFA medium for *L. casei* and on the medium described by Luckey, *et al.*[8]

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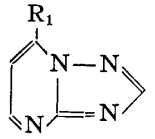
8) T.D. Luckey, *et al.* : *Ibid.*, **152**, 157(1944).

(supplemented with 1 mγ/cc. of folic acid) for *St. faecalis*. The degree of inhibition was expressed as percentage change in turbidity. When any compound exhibited complete growth inhibition the effect of addition of folic acid (F), leucovorin (L), thymine (T), and purines was also examined to observe their reversible effect. However, since the addition of purines did not show any appreciable effect on any of the agents, the results obtained with purines are not listed in the Table.

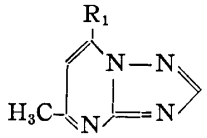
Table I. Inhibition of *L. casei* and *St. faecalis* by 5-Substituted 1,3,8-Triazaindolizines



R_1	No.	*L. casei* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	4 γ/cc.	0.8 γ/cc.	*L. casei* Reversal by folic acid	*St. faecalis* Change in turbidity[a] (%) 100 γ/cc.	*St. faecalis* Reversal by F, L, T[b]
H	03131	0				~	0	~
OH	03117	0				~	0	~
Cl	03118	−84	−50	−20	0	−	0	~
SH	03132	−98	−86	−40	0	−	0	~
NH_2	03133	0				~	0	~
NH-CH_2-furyl	03134	0				~	0	~

a) Change in turbidity compared with that of control run.
b) F, folic acid; L, leucovorin; T, thymine.
\+ Inhibition caused by the compound was reversed by F, L, or T.
− Not reversed. ~ Not tested.

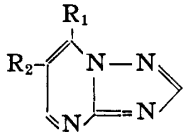
Table II. Inhibition of *L. casei* and *St. faecalis* by 5,7-Disubstituted 1,3,8-Triazaindolizines



R_1	No.	*L. casei* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	4 γ/cc.	*L. casei* Reversal by folic acid	*St. faecalis* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	*St. faecalis* Reversal by F, L, T[b]
H	0332	0			~	0		~
OH	0303	0			~	0		~
Cl	0325	50			~	0		~
NH_2	0333	0			~	0		~
$N(C_2H_5)_2$	0336	0			~	0		~
$NHNH_2$	0329	0			~	0		~
NHN=C-furyl	0335	0			~	0		~
NHN=CH-phenyl	0334	0			~	0		~
OC_2H_5	0339	0			~	0		~
SH	0326	−98	−78	−20	−	−98	−10	−
SCH_2COOH	0361	0			~	0		~
N	0337	0			~	0		~
NH-CH_2-furyl	0340	0			~	0		~
SCN	0389	−98	−90	−33	−	−25	0	~
CH_3	0314	0			~	0		~

a), *b*) Same as in Table I.

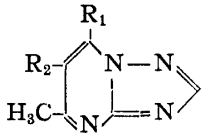
Table III. Inhibition of *L. casei* and *St. faecalis* by 5,6-Disubstituted 1,3,8-Triazaindolizines



R_1	R_2	No.	*L. casei* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	4 γ/cc.	*L. casei* Reversal by folic acid	*St. faecalis* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	*St. faecalis* Reversal by F,L,T[b]
OH	Cl	03137	0			~	0		~
〃	Br	03119	0			~	0		~
NH_2	Cl	03140	0			~	0		~
〃	Br	03136	0			~	0		~
Cl	Cl	03138	0			~	0		~
〃	Br	03120	0			~	0		~
SH	Cl	03139	−90			−	−78	0	~
〃	Br	03121	−98	−93	−73	−	−97	0	−
OH	COOH	03108	0			~	0		~
NH_2	$COOC_2H_5$	03104	0			~	−75	0	~
〃	COONa	03105	0			~	0		~
CH_3	$COOC_2H_5$	03106	0			~	−60		~
〃	COOH	03107	0			~	0		~

a), *b*) Same as in Table I.

Table IV. Inhibition of *L. casei* and *St. faecalis* by 5,6,7-Trisubstituted 1,3,8-Triazaindolizines



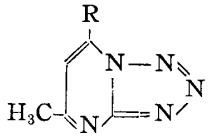
R_1	R_2	No.	*L. casei* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	4 γ/cc.	0.8 γ/cc.	*L. casei* Reversal by folic acid	*St. faecalis* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	4 γ/cc.	*St. faecalis* Reversal by F,L,T[b]
OH	Cl	0356	0				~	0			~
〃	Br	03109	0				~	0			−
NH_2	Cl	03114	−68	0			+	−69	0		+
〃	Br	03110	−70	0			+	−97	−97	−40	+
Cl	Cl	0307	0				~	0			−
〃	Br	03115	0				~	0			−
SH	Cl	0308	−95	−94	−75	−50	−	−98	−20	0	~
〃	Br	03116	−98	−93	−97	0	−	−99	0		~
SCH_3	Cl	0390	−95	−45	0		−	−30			−
H	〃	03111	0				~	0			−
Cl	C_2H_5	0391	−98	−98	−98		−	−95	−95	0	−

a), *b*) Same as in Table I.

The activities of 45 derivatives of 1,3,8-triazaindolizine are listed in Tables I to IV. Importance of the substituent and its position on the 1,3,8-triazaindolizine molecule is clearly evident from the data presented in these Tables. Compounds that have an SH, SCH_3, or SCN group in 5-position (03132, 0326, 0389, 03139, 03121, 0308, 03116, 0390) exerted some inhibition on both microörganisms. Some compounds that have a halogen substituent in 5-position (03118, 0325, 0391) also exhibited inhibition. Introduction of an amino group into 5-position also seemed to be useful (03114, 03110, 03104).

All 13 derivatives of 1,2,3,8-tetraazaindolizines, which are listed in Table V, did not show any inhibition on both lactic acid bacteria. Table VI illustrates the activity of 1,2,3,6-

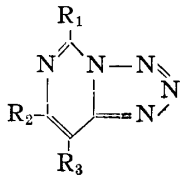
TABLE V. Activity of 5,7-Disubstituted 1,2,3,8-Tetraazaindolizines on Lactic Acid Bacteria



R	No.	*L. casei* Change in turbidity[a] (%) 100 γ/cc.	*St. faecalis* Change in turbidity[a] (%) 100 γ/cc.
OH	0316	0	0
Cl	0323	0	0
NH_2	0350	0	0
$N(C_2H_5)_2$	0342	0	0
$NHNH_2$	0338	0	0
NHN=CH-(furyl)	0345	0	0
NHN=CH-ph	0344	0	0
OC_2H_5	0351	0	0
SCH_3	0346	0	0
SCH_2COOH	0362	0	0
N(pyridyl)	0343	0	0
$NHCH_2$-(furyl)	0341	0	0
CH_3	0306	0	0

a) Same as in Table I.

TABLE VI. Inhibition of *L. casei* and *St. faecalis* by 5,7,8-Trisubstituted 1,2,3,6-Tetraazaindolizines



R_1	R_2	R_3	No.	*L. casei* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	4 γ/cc.	Reversal by folic acid	*St. faecalis* Change in turbidity[a] (%) 100 γ/cc.	20 γ/cc.	4 γ/cc.	0.8 γ/cc.	Reversal by F,L,T[b]
CH_3	OH	H	0305	0			~	0				~
〃	CH_3	〃	0315	0			~	0				~
NH_2	〃	〃	0331	0			~	−35				~
CH_3	N_3	〃	0311	0			~	0				~
〃	NH_2	CH_3	0304	0			~	0				~
〃	Cl	H	0320	0			~	0				~
H	〈_〉		0318	0			~	0				~
CH_3	〃		0302	0			~	0				~
NH_2	〃		0312	−99	−97	0	+	−98	−44			+
$NHCOCH_3$	〃		0313	−97	−97	−72	+	−93	−98	−98	−14	+

a), *b*) Same as in Table I.

tetraazaindolizines. Among this series of compounds, only amino compounds (0331, 0312, 0313) were found to have inhibitory activity.

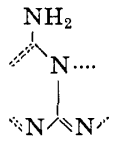
Through inspection of the data presented in Tables I to VI, the tested azaindolizines may be tentatively divided into the following three groups according to their structure and pattern of growth inhibition.

Group 1. Compounds with sulfur-containing substituents: This group involves compounds having SH, SCN, or SCH_3 group in 5-position of azaindolizine (03121, 03139, 0308,

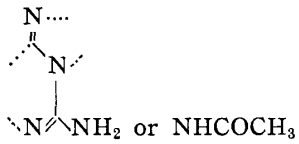
03116, 0390, 0326, 0389, 03132). Growth inhibitory activity of Group 1 compounds is in general more significant on *L. casei* than on *St. faecalis*. In the latter organism some agents did not show any growth inhibition at the highest concentration tested, while they inhibited the growth of *L. casei* at least to some extent.*[2]

It seems pertinent to exclude the possibility that Group 1 compounds act as an antimetabolite of folic acid, leucovorin, purines, and pyrimidines, since all the data presented in this investigation show that their activity is not appreciably relieved by these metabolites.

Group 2. This group involves compounds which have amino or closely related substituent in 5-position (0312, 0313, 03104, 03114, 03110, 0331). In general, compounds which belong to this group exhibited a more pronounced effect on *St. faecalis* than on *L. casei* and their activities were reduced by folic acid, leucovorin, and thymine, but not by purines. These results suggest that the structures like (a) and/or (b) would be necessary to exhibit growth inhibition and the action is antagonized by metabolites like folic acid, leucovorin, and thymine.



(a)



(b)

Group 3. Compounds with halogen in their molecule. Compounds tested in this experiment include many halogenated azaindolizines. Although some of these azaindolizines exhibited growth inhibition (0391, 0325, 03118), their effect on lactic acid bacteria was not significant. 5,6-Dichloro-7-methyl-1,3,8-triazaindolizine, which was reported by Mineshita, *et al.*[6] to have considerable antineoplastic activity on several experimental animal tumors, did not show any appreciable inhibition on lactic acid bacteria in the experimental condition employed.

The results thus obtained in this investigation indicate that there is some discrepancy between growth inhibition on lactic acid bacteria and that on animal tumors. A possible explanation for this discrepancy is that the high degree of structural change between azaindolizines and purines, pyrimidines, or their analogs is accountable for this phenomenon. The most striking difference in the structure between these azaindolizines and natural purines or their analogs is that the former belongs to the condensed [*a*]pyrimidine system and the latter to the condensed [*d*]pyrimidine system. Azaindolizines, which have no secondary amino nitrogen, lack the ability to combine with sugar or sugar phosphate to form nucleoside or nucleotide linkage, while all of naturally occurring purines, pyrimidines, and many of their antagonists are known to form nucleoside or nucleotides when they are administered to a living matter.

It is worthwhile to note that the discrepancy between carcinolytic activity and inhibitory action on the lactic acid bacteria was the most significant in Group 3 compounds, and that there seems to be mechanism other than the purine and pyrimidine inhibition. Further study on the action of this group of compounds with another microbiological system will be published in the following paper.

The authors wish to express their gratitude to Dr. Ken'ichi Takeda, Director of this Laboratory, and to Mr. Eitaro Masuo of this Laboratory, for their helpful advice and encouragement throughout

*[2] It is interesting to note that inhibitory activity of some of Group 1 compounds was antagonized by SH compounds. These results are not given in the Table and it is obscure whether or not this phenomenon gives any clue for elucidation of the mode of action of these agents.

this work. They also wish to thank Dr. Kazuo Iwai, Assistant Porfessor of Research Institute for Food Science, University of Kyoto, for the gift of leucovorin.

Summary

Sixty-eight substituted azaindolizines were tested for their activity on *Lactobacillus casei* and *Streptococcus faecalis*. These azaindolizines could be divided into three classes according to the pattern of growth inhibition, substituent present, and the position of the substituent in azaindolizine molecule. Compounds in Group 1 and 2 which have sulfur-containing substituents and halogen groups, respectively, were not affected appreciably by folic acid, leucovorin, thymine, or purines, while Group 2 compounds which have amino or closely related groups were antagonized by folic acid, leucovorin, and thymine but not by purines.

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32. Tadashi Okabayashi : Action of Substituted Azaindolizines on Microörganisms. II.[1] Action of Halogenated Azaindolizines on *Escherichia coli*.

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It has already been shown[1] that substituted azaindolizines may be divided into three groups according to their structure and their action on lactic acid bacteria. It was also demonstrated that there is a considerable discrepancy between their inhibitory action on lactic acid bacteria and their antineoplastic activities. The discrepancy between these two activities was the most prominent in Group 3 compounds which have halogens substituted in the azaindolizine ring. Since some halogenated azaindolizines were reported to cause a considerable damage to the growth of experimental animal tumors,[2] although they exhibited only a little growth inhibition of lactic acid bacteria, it seemed desirable to study their mode of action using some other appropriate microbial system. The present paper deals with the fact that halogenated azaindolizines inhibit the growth of *Escherichia coli* and that the inhibition is reversed by tyrosine and other amino acids.

Table I. Growth Inhibition of *E. coli* Strains by 7-Methyl-5,6-dichloro-1,3,8-triazaindolizine (0307)

0307(γ/cc.)	100	20	4	0.8	0.16	0
ATCC 9637	−	−	−	+	+	+
1011	−	−	−	+	+	+
O-20	−	−	−	+	+	+
287	−	−	−	+	+	+
Comm. W	−	−	−	+	+	+
Comm. MT	−	−	−	+	+	+
K-12	−	−	−	+	+	+

\+ Full growth of test organism observed. − No growth observed.

* 192 Imafuku, Amagasaki, Hyōgo-ken (岡林 直).

1) Part I. T. Okabayashi, H. Kanô, Y. Makisumi : This Bulletin, 8, 157(1960).

2) S. Mineshita, *et al.* : Ann. Repts. Shionogi Research Lab., **9**, 1(1959).