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Chemical and Biological Studies on *1,2*-Dihydro-*s*-triazines. IV. Activity in Pteroylglutamic Acid-*Streptococcus faecaelis* No. 8043 Systems. (20477)

G. E. FOLEY. (Introduced by Harry Eagle)

From the Laboratories of Microbiology, The Children's Cancer Research Foundation and the Department of Pathology, Harvard Medical School, at Children's Medical Center, Boston, Mass.

Preliminary communications from these laboratories reported the synthesis(1,2) of an hitherto undescribed class of compounds, the *1,2*-dihydro-*s*-triazines which exhibit significant anti-malarial*(1-3), anti-tumor(3), and anti-vitamin(1,4) activity. Details of the synthesis, chemical characterization and identification of these compounds are recorded elsewhere(2,5). During the course of these studies, certain derivatives of this series also have been synthesized independently by others in connection with studies on the identification of the active metabolite of paludrine(6,7).

The biological activity of the symmetrical dihydrotriazines was first observed in these laboratories early in 1950 when the parent-compound of the series (D-19 • HCl) was studied in routine screening assays† for microbiological activity. Since that time, more than 250 samples of 71 derivatives of this series have been studied. The purpose of the present communication is to report the activity of these compounds in *Streptococcus faecalis* No. 8043‡-pteroylglutamic acid (PGA) assay systems.

Methods. PGA assays(8) using *Streptococcus faecalis* were done in Difco PGA Assay Medium or the casein hydrolysate medium prepared as described by Tepley and Elvehjem(9). The basal medium was diluted (usually 1:1) with glass-distilled water to yield uniformly reproducible optical densities of 4.0-6.0 x 10 from minimal inocula with the addition of PGA and no growth in the absence of PGA. Media were handled in chemically clean glassware, dispensed in 10 ml volumes in 22 x 175 mm optically standardized tubes, closed with loose fitting aluminum caps and autoclaved 5 minutes at 15 lb pressure. Crystalline PGA, other metabolites and the various dihydrotriazines were prepared in appropriate concentrations in sterile glass-distilled water and added aseptically to the cooled sterile medium to the desired final concentration. Assays were incubated at 37°C for 24 hours. Optical density was read di-

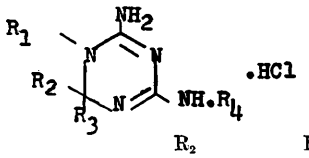
* We are indebted to J. H. Williams and his colleagues of the Lederle Laboratories, Pearl River, N. Y. for the anti-malarial assays.

† The routine screening program, which comprises several standard microbiological assay systems as well as studies with a number of miscellaneous bacteria and fungi was organized in 1947 as one part of a broad program in the chemotherapy of cancer initiated by Sidney Farber.

‡ A. T. C. C. No. 8043.

TABLE I. Inhibition of *Streptococcus faecalis* #8043-PGA Assay Systems by Ketone Derivatives of Dihydrotriazine Series.

Structure (1)



Compound	R_1	R_2	R_3	Inhibition index vs. PGA .01 γ/ml	Inhibition index vs. PGA .001 γ/ml
D-19 (2)	4'-carbethoxyphenyl	methyl	methyl	37.5	325.0
D-20 (2,3,4,5)	4'-chlorophenyl	"	"	9.5	95.0
D-22 (6)	4'-carboxyphenyl	"	"	350000	3500000
D-23 (3,5)	phenyl	"	"	10.0	110.0
D-27 (2)	4'-carboxamidophenyl	"	"	1625	6870
D-32 (1)	phenyl	"	"	33.3	114.0
D-38	2'-tolyl	"	"	4000	12000
D-40	4'-chlorophenyl	pentamethylene		16000	80000
D-43	"	methyl	ethyl	260.0	495.0
D-53	2',4'-dichlorophenyl	"	methyl	15000	34000
D-54 (3,7)	3',4'-dichlorophenyl	"	"	10.0	35.0
D-56	2',5'-dichlorophenyl	"	"	330000	2000000
D-64	4'-chlorophenyl	tetramethylene		460.0	100.0
D-65	4'-bromophenyl	methyl	methyl	12.0	40.0
D-66	phenyl	"	ethyl	240.0	640.0
D-68	2'-chlorophenyl	"	methyl	14100	72400
D-69	3'-chlorophenyl	"	"	32.5	75.0
D-72	2'-tolyl	tetramethylene		310000	800000
D-73	4'-nitrophenyl	methyl	methyl	470.0	4000
D-75	3',4'-dichlorophenyl	"	ethyl	37.5	250.0
D-81 (8)	4'-(4,6-diamino-1,2-dihydro-2,2-dimethyl-1-*s*-triazinyl)-phenyl	"	methyl	375000	2250000
D-82	2'-bromophenyl	methyl	methyl	164000	1520000
D-83	4'-tolyl	"	"	40.0	340.0
D-87	4'-chlorophenyl	"	*n*-propyl	400	1800

(1) R_4 = H except in the case of compound D-32 • HCl where $R_4 = CH_3$.
(2) Free base microbiologically active.
(3) Dihydrochloride microbiologically active.
(4) Nitrite, nitrate and bicarbonate microbiologically active.
(5) Picrate microbiologically active.
(6) Inactive un-blocked hydrochloride (see text).
(7) Sample received through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England showed similar microbiological activity.
(8) As the dihydrochloride.

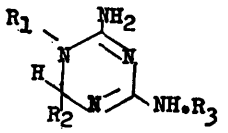
rectly in a Lumetron colorimeter adjusted so that medium blanks read zero versus the 650 mμ filter.

The test compounds were titrated from 1000 γ/ml or more to the minimal inhibiting dose in repeated serial assays in the presence of 0.01 and 0.001 γ/ml of PGA, amounts which represent excess and minimal concentrations supporting maximal growth under these experimental conditions. PGA and medium controls were included in each experiment. Assays in which low values in the PGA controls or growth in the medium controls occurred were discarded. The 50% minimal inhibiting dose§ (10) for the excess and minimal concentrations of PGA was interpolated

§ The 50% minimal inhibiting dose and inhibition index are relative values and do not necessarily bear any direct relationship to the minimal inhibiting dose required for the complete inhibition of a given inoculum in a given assay system. Although subject to somewhat less variation than "complete inhibition", variations in procedure and the basal medium are reflected in the mathematical interpolation of the 50% inhibition index, hence such values may not be exactly reproducible under other experimental conditions. However, the relative relationships among a series of inhibitors should be comparable at these 50% values irrespective of variation in assay systems.

TABLE II. Inhibition of *Streptococcus faecalis* #8043-PGA Assay Systems by Aldehyde Derivatives of Dihydrotriazine Series.

Structure (1)



•HCl

Compound	R_1	R_2	Inhibition index vs. PGA .01 γ/ml	.001 γ/ml
D-31	phenyl	*n*-propyl	1500	2500
D-34 (2)	"	phenyl	2500	6200
D-37	4'-chlorophenyl	"	380	880
D-45 (3)	"	methyl	220	750
D-47	"	2'-chlorophenyl	550	750
D-48	"	*n*-propyl	235	1750
D-49	4'-anisyl	"	480	4800
D-55	3',4'-dichlorophenyl	phenyl	62	440
D-57	2',4'-dichlorophenyl	"	13000	110000
D-58	2',5'-dichlorophenyl	"	7500	75000
D-59	4'-chlorophenyl	*i*-propyl	1050	4400
D-60 (2,3)	"	ethyl	200	500
D-62	3',4'-dichlorophenyl	*i*-propyl	365	990
D-63 (3)	"	ethyl	40	104
D-67	"	methyl	32.5	125
D-76	"	*n*-propyl	95	350
D-84	4'-chlorophenyl	*n*-hexyl	45	220
D-86 (1)	"	phenyl	4200	6200
D-88	2'-tolyl	*n*-propyl	640000	200000

(1) $R_3 = H$ except in compound D-86 • HCl where $R_3 = CH_3$.
(2) Free base also microbiologically active.
(3) Dihydrochloride also microbiologically active.

from a plot of the data obtained from such titrations.

Inhibition analyses(11) were done in systems containing 0.01 γ/ml of PGA, to which were added the minimal concentrations of the respective dihydrotriazines which completely inhibited growth as determined by previous titration in the presence of excess concentrations of PGA. Various metabolites were then titrated in these inhibited systems to determine their ability to reverse dihydrotriazine inhibition. Those derivatives characterized by a 50% inhibition index of less than 100 were studied under these experimental conditions. Similar assays inhibited with 4-aminopteroylglutamic acid (4-APGA) also were done for purposes of comparison.

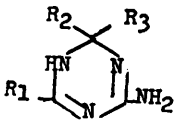
Results. The 50% inhibition indices obtained with 2,2-di-substituted and 2-monosubstituted derivatives are presented in Tables I and II. Microbiological activity varies considerably with substitution in the molecule in either series. Maximum activity is obtained with 2,2-dimethyl substitutions, *e.g.*, D-20 • HCl ($R_2 = R_3 = CH_3$) exhibits greater activity than its isomeric derivative D-60 • HCl ($R_2 = C_2H_5$) (Table II) or D-43 • HCl ($R_2 = CH_3$, $R_3 = C_2H_5$) (Table I). If the structure is otherwise unaltered microbiological activity is enhanced by *para*-or-*meta*-halogen substitution in the phenyl ring and activity is further increased by 3',4'-di-halogen-substitution, as illustrated by a comparison of the microbiological activity of compounds D-23 • HCl, D-20 • HCl, D-69 • HCl, D-65 • HCl, and D-54 • HCl‖ (Table I).

Substitution of a sufficiently large group at the *ortho*-position of the phenyl ring (*e.g.*, Cl in compound D-68 • HCl) results in reduced microbiological activity and similarly, the presence of a large blocking group at the 2-position of the triazine ring (*e.g.*, penta-

‖ Two samples of this compound were used in these studies, one synthesized in these laboratories by Dr. E. J. Modest and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

TABLE III. Relative Inactivity of Isomers of Certain Dihydrotriazines in *Streptococcus faecalis* #8043-PGA Assay Systems.

Structure (1)



Compound	R_1	R_2	R_3	Inhibition index vs. PGA .01 γ/ml	Inhibition index vs. PGA .001 γ/ml
DX-19	4'-carbethoxyanilino	methyl	methyl	140000	980000
DX-20	4'-chloroanilino	"	"	6400	52000
DX-20 • HNO_2	"	"	"	32500	325000
DX-20 • HCl	"	"	"	56000	375000
DX-20 • 2HCl	"	"	"	5800	35000
DX-22	4'-carboxyanilino	"	"	188000	1130000
DX-23	anilino	"	"	600000	6000000
DX-29 (1)	"	"	"	75000	750000
DX-30 • HCl	N-methylanilino	"	"	200000	2000000
DX-34	anilino	H	phenyl	156000	1560000
DX-35 • HCl (1)	4'-chloroanilino	methyl	methyl	20000	200000
DX-60	"	H	ethyl	49000	500000

(1) In compound DX-35, NH_2 is replaced by $NHCH_3$; in compound DX-29, NH_2 is replaced by $N(CH_3)_2$.

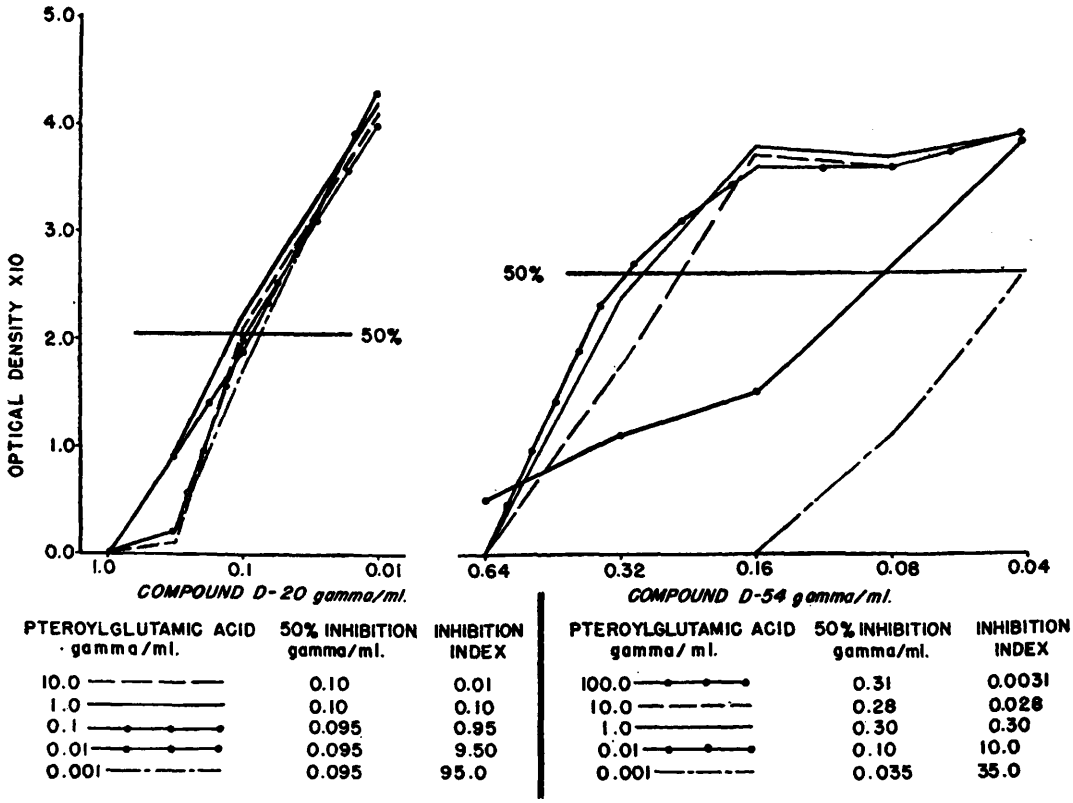
methylene in compound D-40 • HCl) also diminishes microbiological activity (Table I). All hydrochlorides studied thus far which do not possess either of these structural features have exhibited microbiological activity except the *para*-carboxy derivative D-22 • HCl (Table I).

The free bases prepared by careful neutralization of the corresponding dihydrotriazine hydrochlorides also exhibit microbiological activity (Tables I, II) while the isomeric (DX) compounds prepared by rearrangement of the corresponding (D • HCl) structures under appropriate conditions(1,2,5) are relatively inert in *Streptococcus faecalis*-PGA systems (Table III). A more detailed study of the relation of chemical structure to microbiological activity, as well as the reduced activity resulting from substitution at the *ortho*-position of the phenyl ring and the 2-position of the triazine ring will be presented in subsequent reports.

Inspection of the 50% inhibition indices (Tables I, II) indicates that these dihydrotriazines exhibit a non-competitive antagonism(12). The 50% inhibition indices computed over a wide range of concentrations of PGA (*e.g.*, Fig. 1) further illustrate the minimal effect of increasing concentrations of PGA on the 50% minimal inhibiting dose. The results of other experiments in which excesses of PGA were added to the basal medium containing 0.01 γ/ml of PGA and inhibitory concentrations of dihydrotriazine indicate that if at all, inhibition is reversible by PGA only within a narrow range of concentration of inhibitor. The magnitude of this range varies somewhat with different derivatives but in each instance concentrations of dihydrotriazine slightly in excess of the minimal inhibiting doses are uniformly irreversible by excess PGA.

The results of inhibition analyses with the 2,2-di-substituted derivatives exhibiting most activity versus PGA (D-20 • HCl, D-23 • HCl, D-54 • HCl) and a derivative of moderate activity (D-19 • HCl) together with the most active (D-63 • HCl) and least active (D-76 • HCl) 2-mono-substituted derivatives are summarized in Table IV. The inhibition induced by all derivatives was reversible by dihydropteroylglutamic acid¶ (dihydro-PGA)(13), N^{10}-formylpteroylglutamic acid¶ (N^{10}-formyl-PGA)(14), leucovorin¶(15), and citrovorum factor¶ (CF)(16) but not by PGA. The concentrations of the various metabolites required for the reversal of different derivatives varied but did not appear to be directly related to the 50% inhibition index vs PGA.

¶ We wish to express our appreciation to the Lederle Laboratories, Pearl River, N. Y., for generous samples of these compounds.



PTEROYLGLUTAMIC ACID · gamma/ml.	50% INHIBITION gamma/ml.	INHIBITION INDEX
10.0	0.10	0.01
1.0	0.10	0.10
0.1	0.095	0.95
0.01	0.095	9.50
0.001	0.095	95.0

PTEROYLGLUTAMIC ACID gamma/ml.	50% INHIBITION gamma/ml.	INHIBITION INDEX
100.0	0.31	0.0031
10.0	0.28	0.028
1.0	0.30	0.30
0.01	0.10	10.0
0.001	0.035	35.0

FIG. 1.
Effect of increasing concentrations of PGA on 50% minimal inhibiting dose of dihydrotriazine.

Similar studies with the purines, pyrimidines, nucleotides and nucleosides indicate that unlike 4-APGA(17) the dihydrotriazines are not reversed by excesses of adenine and guanine or their derivatives but are partially reversed by excess uracil or uridylic acid, desoxyribonucleic acid and more completely by thymine (Table IV).

In general, excess concentrations of other vitamins have no effect on the inhibition of *Streptococcus faecalis* by these dihydrotriazines. However, certain derivatives of this series exhibit considerable microbiological activity versus *Lactobacillus arabinosus* No. 17-5** in niacin assay systems(1). The inhibition of *Streptococcus faecalis* by such compounds is partially reversed by high concentrations of niacin; for example, D-54 • HCl in concentrations circa 1.0 γ/ml inhibits *Lactobacillus arabinosus* in niacin assay systems, while concentrations circa 100 γ/ml of niacin partially reverse D-54 • HCl inhibition in *Streptococcus faecalis*-PGA assay systems. Detailed studies of the inhibition observed in *Lactobacillus arabinosus* and other *Lactobacillus* assay systems will be reported elsewhere.

Discussion. The microbiological activity of these symmetrical dihydrotriazines in *Streptococcus faecalis* systems differs from that exhibited by structural analogs of PGA in that inhibition is essentially non-competitive over a wide range of concentrations of PGA and is irreversible by excess PGA. Subsequent to the original communication from these laboratories(1) several classes of compounds bearing a formal structural relationship to these dihydrotriazines, namely aryl-diamino-pyrimidines(18,19),-*as*-triazines(19) and -guanazoles(19) also have been reported to be non-competitive inhibitors of PGA in *Streptococcus faecalis* systems. Consideration of the biologically active configuration of the sym-

** *Lactobacillus plantarum*, A.T.C.C. No. 8014.

TABLE IV. Reversal of Dihydrotriazine Inhibition in *Streptococcus faecalis* #8043-PGA Assay Systems.

Metabolite	Concentration in γ/ml reversing inhibition of: D-19·HCl	D-20·HCl	D-23·HCl	D-54·HCl	D-63·HCl	D-76·HCl	4-APGA
	γ/ml						
	1.25	.60	.60	.3	2.0	4.0	.1
Pteroylglutamic acids							
Pteroylglutamic acid	.0	.0	.0	.0	.0	.0	*.1*
Dihydropteroylglutamic acid	.01	1.0	1.0	10.0	1.0	1.0	.1
N^{10}-formylpteroylglutamic acid	.01	1.0	20.0	20.0	1.0	1.0	.1
Leucovorin	.00001	.0001	.0001	.0001	.0001	.00001	.000005
Citrovorum factor	.0000005	.00005	.00005	.00005	.0005	.00005	.00005
Purines*							
Adenine	.0	.0	.0	.0	.0	.0	*100.0*‡
Guanine	.0	.0	.0	.0	.0	.0	*100.0*‡
Pyrimidines*							
Thymine	1.0	1.0	10.0	10.0	1.0	1.0	10.0
Uracil	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Cytosine	.0	.0	.0	.0	.0	.0	.0
Nucleotides*†							
Adenylic acid (yeast)	.0	.0	.0	.0	.0	.0	*500.0*‡
Guanylic acid	.0	.0	.0	.0	.0	.0	*500.0*‡
Cytidylic acid	.0	.0	.0	.0	.0	.0	.0
Uridylic acid‡	500.0	500.0	500.0	500.0	500.0	500.0	1000.0
Nucleosides*†							
Adenosine	.0	.0	.0	.0	.0	.0	*1000.0*‡
Guanosine	.0	.0	.0	.0	.0	.0	*1000.0*‡
Cytidine	.0	.0	.0	.0	.0	.0	.0
Uridine	.0	.0	.0	.0	.0	.0	.0
Nucleic acids*							
Ribose	.0	.0	.0	.0	.0	.0	.0
Desoxyribose‡	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* Tested in concentrations 100-.1 γ/ml.
† Thymidylic acid and thymidine not tested.
‡ Circa 50—75% reversal.
Addition of PABA to 100 γ/ml had no sparing effect in these systems.
Similar results were obtained with sample of dichloro-derivative (D-54·HCl) supplied by Imperial Chemical Industries, Ltd.

metrical dihydrotriazines in relation to these other types of compounds will be reported elsewhere.

It has become generally accepted that CF is the physiologically active form of PGA as suggested by Sauberlich(20) and that PGA is concerned with the utilization of thymine or thymidine in the synthesis of nucleic acids (21-24). CF(25,26) and thymidine(23,26) have been shown to replace PGA and to reverse inhibition produced by analogs of PGA in microbiological systems. These and other microbiological studies, toxicity studies in mice(25,26) and more recent *in vitro* observations on liver slices(27) suggest that the inhibition produced by analogs of PGA is due at least in part to interference with the conversion of PGA to CF in biological systems.

The ability of reduced PGA and formylated PGA as well as CF and thymine to reverse dihydrotriazine inhibition suggests that like 4-APGA, the dihydrotriazines may interfere with the conversion of PGA to CF. However, the dihydrotriazines differ from 4-APGA in that non-competitive inhibition by the former is not relieved by adenine or guanine and is not reversed by PGA. In this respect, the behavior of the dihydrotriazines in *Streptococcus faecalis* systems resembles that of 4-APGA and other analogs of PGA in mammals in that once inhibition (or toxicity) has occurred, it is not readily reversed by PGA

(28) but can be reversed with CF(25,26) and nucleic acids(17). Preliminary experiments indicate that the temporal sequence of exposure to inhibitor and metabolite may be of importance in the dihydrotriazine inhibition of *Streptococcus faecalis* as has been observed with 4-APGA in mammals(29).

Summary. A series of 71 *1,2*-dihydro-*s*-triazines have been studied in *Streptococcus faecalis* No. 8043-pteroylglutamic acid assay systems. The active derivatives exhibit noncompetitive inhibition over a wide range of concentrations of PGA and differ from 4-aminopteroylglutamic acid in that inhibition is not relieved by adenine or guanine and is irreversible with pteroylglutamic acid. Differences in microbiological activity could be correlated with certain variations in structure and substitution in the molecule with the series. Maximum activity is obtained with 2,2-dimethyl substitution in the triazine ring together with *para-* and *meta*-halogen substituents in the phenyl ring. The inhibitory effect of these compounds on *Streptococcus faecalis* No. 8043 is reversed by appropriate concentrations of dihydropteroylglutamic acid, N^{10}-formylpteroylglutamic acid, synthetic and natural citrovorum factor and thymine, suggesting that these compounds interfere with the conversion of pteroylglutamic acid to citrovorum factor.

The invaluable technical assistance of Brittmarie Lindsjo, Carolyn Hall, Janet Smith and Edward Botan during the course of these studies is gratefully acknowledged.

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