Antitumor, Cytotoxic, and Antibacterial Activities of Tenuazonic Acid and Congeneric Tetramic Acids[1]

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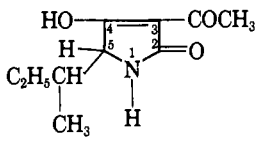
Received November 23, 1964

Sodium L-tenuazonate and several congeneric tetramates were tested for antitumor activity against the human adenocarcinoma (HAd1) growing in the embryonated egg. The D-*allo* isomer of potassium tenuazonate was no more than one-fourth as active as sodium L-tenuazonate, and because the optical purity of the D-*allo* isomer was unknown its relative activity could not be further defined. The D-isomer was inactive. Congeners substituted at the C-5 position had less than 5% of the antitumor activity of sodium L-tenuazonate. The N-methyl substituted 3-acetyl-5-*sec*-butyltetramate was 15% as active as sodium L-tenuazonate. Other substitutions at N-1 and substitutions at C-3 resulted in tetramates which were not active against HAd1. Cytotoxic activities of the C-5 substituted tetramates against KB cells correlated with antitumor activity in the egg. Substitution at the N-1 position in some instances resulted in enhanced cytotoxic activity. Antibacterial activity against *Bacillus megatarium* did not correlate with antitumor activity. The antibacterial activity of tenuazonate salts was not stereospecific. The substitution of a benzyl group for the N-hydrogen greatly enhanced antibacterial activity.

Utilization of the human tumor–egg host system for testing potential antitumor agents led to the discovery of the antitumor properties of tenuazonic acid (I).[2] Compounds structurally related to tenuazonic acid were synthesized in the Merck Sharp & Dohme Research Laboratories[3] and were tested for antitumor activity in the human tumor–egg host system. The compounds were tested also for cytotoxic activity against KB cells, and for activity against *Bacillus megaterium*. The results of studies comparing the activities of tenuazonic acid and the related compounds in the three different test systems are reported here.



I

Materials and Methods

Human Tumor–Egg Host System.—The human adenocarcinoma (HAd1)[4] was used in these studies. The procedures for testing compounds in the human tumor–egg host system have been described previously.[5]

Cell Culture System.–Cell cytotoxicity measurements were made as previously described[2] using Eagle's KB cell carcinoma.[6]

Cytotoxic activities were expressed by numbers ranging from 4 (complete cell destruction) to 0 (no cell damage). Cytotoxic readings *vs.* concentration were plotted on semilogarithmic paper for estimation of the cytotoxic end point (CE) (corresponding to a cytotoxicity reading of 2). The relative cytotoxic activity of each compound was based on a comparison of its CE value with that of sodium L-tenuazonate determined on the same day.

Microbial System.—Compounds were tested against *B. megaterium* by the agar plate diffusion method. Test compounds were dissolved in 0.05 *M* phosphate buffer at pH 6.0 and when necessary, the solutions were adjusted to pH 6.0 by the addition of 0.1 *N* HCl or 0.1 *N* NaOH. Each compound was tested at several levels. A volume of 0.06 ml. of test solution was placed on a filter paper disk (9.5 mm.) which was applied to the surface of a synthetic, glucose–citrate–salts–agar medium (part A, K_2HPO_4 7.0 g., KH_2PO_4 3.0 g., sodium citrate 0.5 g., $MgSO_4 \cdot 7H_2O$ 0.1 g., $(NH_4)_2SO_4$ 1.0 g., Bacto agar (Difco Laboratories) 20.0 g., deionized water 800 ml., adjust to pH 7.0; part B, glucose 2.0 g., deionized water 200 ml. Parts A and B were autoclaved separately at 120° for 15 min. and combined prior to use.) The synthetic glucose–citrate–salts–agar medium (10 ml.) was seeded with about 3×10^6 washed spores of *B. megaterium* and poured into 100-mm. assay plates. Plates were incubated for 24 hr. at 37° and then zones of inhibition were measured.

The level of each compound producing a zone of given diameter was compared with the level of sodium L-tenuazonate found on the same test day to produce the same size zone.

Results

Comparison of the L-, D-, and D-*allo* Isomers of Tenuazonate Salts.—Table I compares the activities of salts of the L-, D-, and D-*allo* (isotenuazonic) isomers of

TABLE I

ACTIVITIES OF SALTS OF THE L-, D-, AND D-*allo* ISOMERS OF TENUAZONIC ACID AGAINST HAd1 IN THE EMBRYONATED EGG

Isomer	Dose, mg./egg	Deaths	% growth inhibition— Embryo	Tumor
L (Na)	0.8	9/12	31	98
(Test 1)	0.48	7/12	13	97
	0.29	1/12	12	79
	0.17	1/12	3	62
	0.10	2/12	−11	72
	0.06	0/12	−1	22
	0	4/20	...	...
L (Na)	0.7	8/12	27	98
(Test 2)	0.42	3/12	9	86
	0.25	2/12	1	81
	0.15	3/12	1	62
	0.09	0/12	2	47
	0	4/20	...	...
D (K)	4.8	1/6	7	9
	1.2	0/6	−18	21
	0.3	2/6	−1	−29
	0	3/20	...	...
D-*allo* (K)	2.5	7/8	[34][a]	[80][a]
(Test 1)	0.6	1/8	7	68
	0	2/20	...	...
D-*allo* (K)	2.0	2/8	25	93
(Test 2)	1.0	0/8	31	83
	0.5	1/8	−4	61
	0	1/20	...	...

[a] Inhibitory value derived from only one survivor.

(1) This investigation was supported, in part, under National Institutes of Health Contract No. SA-43-ph-3057, by the Cancer Chemotherapy National Service Center, National Cancer Institute, Department of Health, Education and Welfare, U. S. Public Health Service, Bethesda, Md.

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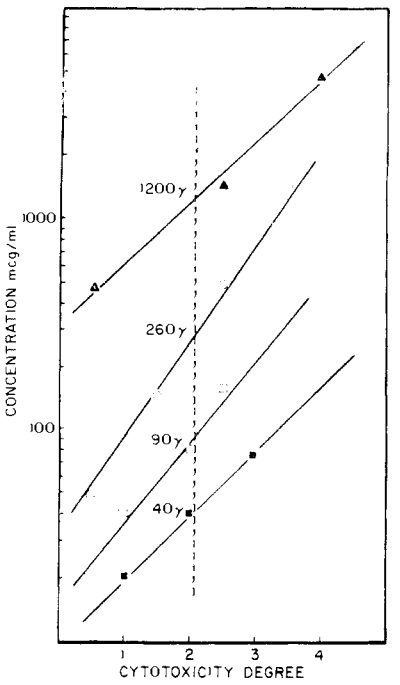

Figure 1.—Cytotoxic activities of L-, D-, and D-*allo*-tenuazonic acid salts against KB cells: expt. 1, ▲, D-tenuazonic acid (K); ■, L-tenuazonic acid (Na); expt. 2, O, D-*allo*-tenuazonic acid (K); □, L-tenuazonic acid (Na).

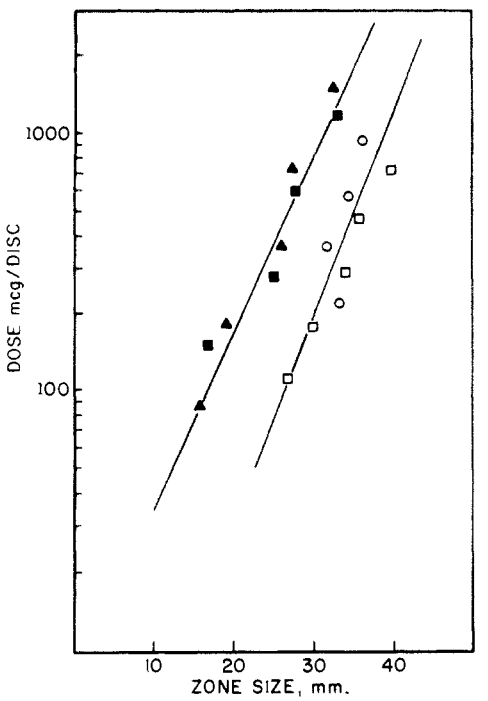

Figure 2.—Antibiotic activities of L-, D-, and D-*allo*-tenuazonic acid salts against *B. megaterium:* expt. 1, ▲, D-tenuazonic acid (K); ■, L-tenuazonic acid (Na); expt. 2, O, D-*allo*-tenuazonic acid (K); □, L-tenuazonic acid (Na).

tenuazonic acid. The L-isomer was highly inhibitory to the tumor at a dose of 0.1 mg./egg and was toxic to the embryo at 0.48 mg./egg (7/12 deaths). The D-isomer did not inhibit tumor growth nor was it toxic to the embryo at the highest level tested, 4.8 mg./egg. The D-*allo* isomer at 2.5 mg./egg was toxic (7/8 deaths) and at 0.6 mg./egg inhibited tumor growth 68%. In a second test, the D-*allo* isomer at 0.5 mg./egg inhibited tumor growth 60%. It was toxic at 1.0 mg./egg, a level which inhibited embryo growth 31%, although no deaths occurred.

From the data in Table I the ED_{60} value for sodium L-tenuazonate was estimated to be 126 γ/egg. Based on the approximate dose which inhibited HAd1 tumor growth 60%, the D-*allo* isomer was 25% as active as sodium L-tenuazonate and the D-isomer was less than 3% as active.

During the synthesis of tenuazonic acid racemization can occur with the formation of the corresponding diastereoisomer. A comparison of different samples of tenuazonates showed that high antitumor activity was associated with high negative rotation (Table II).

TABLE II

ACTIVITIES AGAINST HAd1 OF SAMPLES OF TENUAZONIC ACID SALTS WITH DIFFERENT OPTICAL ROTATIONS

Isomer	Sample	$[\alpha]_{436}^{25}$,[a] deg.	ED_{60}, γ/egg
L (Na)	9-9[b]	−165	126 (104–153)[c]
L (Na)	9-8[b]	−110	215 (186–249)[c]
L (Na)	9-6	−95	225 (176–289)[c]
D-*allo* (K)		+48	500

[a] *c* 2.0, methanol. [b] Samples 9-8 and 9-9 were kindly supplied by G. A. Stein, R. F. Czaja, and E. M. Chamberlin of the Cancer Preparations Laboratory of Merck Sharp & Dohme Research Laboratories under Contract No. ph-43-62-479 with the Cancer Chemotherapy National Service Center of the National Institutes of Health. [c] Numbers in parenthesis refer to the 95% confidence limits.

Samples 9-6 and 9-8, which were less active than 9-9, contained more of the D-*allo* isomer than did 9-9 as evidenced by optical rotation values. The isomer prepared from D-*allo*-isoleucine was of unknown optical purity.[3] Hence, part or all of its activity might be due to the presence of potassium L-tenuazonate.

The cytotoxic activities of the isomers against KB cells are shown in Figure 1. Approximate CE values for the L-, D-, and D-*allo* isomers were 40–90, 1300, and 300 γ/ml., respectively. The D-isomer was only 3% and the D-*allo* isomer was about 30% as cytotoxic as sodium L-tenuazonate.

Figure 2 shows that salts of the L-, D-, and D-*allo* isomers of tenuazonic acid were equivalent in activity against *B. megaterium*.

Comparison of the C-5 Substituted 3-Acetyltetramates.—None of the C-5 substituted 3-acetyltetramates was as active as sodium L-tenuazonate against HAd1 in the egg nor against KB cells (Table III). The 5-isopropyl-, 5-isobutyl-, and 5-benzyl-3-acetyltetramates inhibited growth of the HAd1 tumor 60%, but only at doses of 10 mg./egg or more. They were about 2% as active as sodium L-tenuazonate. Low antitumor activity was observed with the 5,5-spirotetramethylene, 5-phenyl, 5-*n*-butyl and 5-(3-amyl) congeners. No antitumor activity was observed with the remaining C-5 substituted 3-acetyltetramates. The C-5 substituted compounds also were less than 5% as cytotoxic as sodium L-tenuazonate.

The inhibitory effects of the C-5 substituted 3-acetyltetramates against *B. megaterium* did not correlate with cytotoxic and antitumor activities (Table III). The isobutyl, *n*-butyl, and *t*-butyl 5-substituted compounds were as active, and the 5-*n*-amyl compound was approximately twice as active as sodium L-tenuazonate. As already noted, salts of the L-, D-, and D-*allo* isomers of tenuazonic acid exhibited the same order of anti-

TABLE III

ACTIVITIES OF TENUAZONIC ACID CONGENERS SUBSTITUTED AT THE C-5 POSITION

Congener[a] C-5 substituent	Activity: HAd1 ED50, mg./egg	HAd1 RA,[b] %	KB CE,[c] γ/ml.	KB RA,[b] %	*B. megaterium* RA,[b] %
sec-Butyl (L)	0.125	100	40–80	100	100
sec-Butyl (D)	>4.8	<3	1300	5	100
sec-Butyl (D-*allo*)	0.5	25	270	30	90
H	>10	<2	>1500	<3	10
Methyl	>10	<2	>5000	<1	20
Dimethyl	>10	<2	>5000	<1	20
Ethyl	>10	<2	>5000	<1	20
Isopropyl	10–20	2	5000	1	50
Ethylidine	>10	<2	...	...	40
Spirotetramethylene	>10[d]	<2	1500	3	50
Phenyl	>10[d]	<2	1000	5	40
Benzyl	10	2	1500	3	50
Isobutyl	10	2	1500	3	100
n-Butyl	>10[d]	<2	1500	3	80
n-Amyl	>10	<2	1500	3	200
3′-Amyl	>8[d]	<3	1500	3	25
t-Butyl	>8	<2	1000	5	100
Trimethylene (1,5)	>10	<2	5000	1	20

[a] Compounds were tested as the water-soluble sodium or potassium salts. [b] Activity relative to that of sodium L-tenuazonate. [c] Cytotoxic end point. [d] Antitumor activity was observed only at the highest level tested; drug did not inhibit tumor growth 60%.

nate to KB cells and was 30 times as active against *B. megaterium*.

Potassium 5-(3-amyl)-N-benzyl-3-acetyltetramate was not active against HAd1 at 2.5 mg./egg but was more cytotoxic than sodium L-tenuazonate to KB cells. It was about 40 times as effective as sodium L-tenuazonate against *B. megaterium*. Potassium 5-isopropyl-N-benzyl-3-acetyltetramate was not active at 2.5 mg./egg against HAd1. Compared to sodium L-tenuazonate it was about one-half as cytotoxic to KB cells and 7 times as active against *B. megaterium*. Both the 5-(3-amyl)- and the 5-isopropyl-N-benzyltetramates were toxic to the chick embryo at 5 mg./egg. Sodium 5-phenyl-N-benzyl-3-acetyltetramate was not active against HAd1 at 4 mg./egg and was toxic to the embryo at 8 mg./egg. Its cytotoxicity to KB cells was not determined.

Sodium 5-(3,3,3-trifluoropropyl)-N-benzyl-3-acetyltetramate was about 3.5 times as cytotoxic as sodium L-tenuazonate to KB cells and 13 times as effective against *B. megaterium*. The corresponding tetramate substituted at position N-1 with hydrogen was less than 5% as cytotoxic as sodium L-tenuazonate to KB cells. It was only 1% as active as the N-benzyl compound against *B. megaterium*. Neither compound was tested against HAd1.

The enhancement of antimicrobial activity resulting from substitution at the N-1 position was observed in liquid medium studies as well as in agar plate studies.

TABLE IV

ACTIVITIES OF 3-ACETYLTETRAMATES SUBSTITUTED AT THE N-1 AND C-5 POSITIONS

Congener[a] Substituents C-5	Substituents N-1	Activity: HAd1 ED50, mg./egg	HAd1 RA,[b] %	KB CE,[c] γ/ml.	KB RA,[b] %	*B. megaterium* RA,[b] %
sec-Butyl	Methyl	1	15	500	10	500
sec-Butyl	Benzyl	>3	<10	50	100	3000
3′-Amyl	Benzyl	>2.5	<10	30	150	4000
n-Amyl	Benzyl	...	...	135	40	3000
Isopropyl	Benzyl	>2.5	<10	90	60	700
Phenyl	Benzyl	>4	<5	...	...	...
3′,3′,3′-Trifluoropropyl	Benzyl	...	...	20	350	1300
3′,3′,3′-Trifluoropropyl	H	...	...	>1000	<5	13

[a] Compounds were tested as the water-soluble sodium or potassium salts. [b] Activity relative to that of sodium L-tenuazonate. [c] Cytotoxic end point.

microbial activity. Inhibitory activities between 25 and 50% of those of sodium L-tenuazonate against *B. megaterium* were observed with isopropyl, ethylidene, spirotetramethylene, phenyl, benzyl, and 3′-amyl 5-substituted tetramates. Substitution at C-5 with hydrogen, methyl, dimethyl, ethyl, and trimethylene resulted in the greatest loss of antimicrobial activity.

Effects of N-1 Substitution on Activities of 3-Acetyltetramates.—Table IV shows the results of testing 3-acetyltetramates substituted at positions N-1 and C-5. Potassium 5-*sec*-butyl-N-methyl-3-acetyltetramate compared to sodium L-tenuazonate was about 15% as active against HAd1 and 10% as cytotoxic to KB cells. However, it was 5 times as active as sodium L-tenuazonate against *B. megaterium*. Substitution at position N-1 with benzyl resulted in a compound (sodium 5-*sec*-butyl-N-benzyl-3-acetyltetramate) which did not inhibit HAd1 tumor growth at a dose of 3 mg./egg although it was as cytotoxic as sodium L-tenuazonate. Therefore, the increase in activity observed in plates did not result merely from an increase in the rate of diffusion through the agar medium.

Table V shows the effects of substitution at the N-1, C-3, and C-5 positions. None of the four compounds at the highest levels tested was active against HAd1.

TABLE V

ACTIVITIES OF TETRAMATES SUBSTITUTED AT N-1, C-3, AND C-5 POSITIONS

Congener[a] Substituents C-5	N-1	C-3	HAd1 ED50, mg./egg	HAd1 RA,[b] %
sec-Butyl	H	H	>10	<2
sec-Butyl	H	Carboxamido	>10	<2
sec-Butyl	Benzyl	Carbethoxy	>5	<5
sec-Butyl	Benzyl	Carboxamido	>5	<5

[a] Compounds were tested as the water-soluble sodium or potassium salts. [b] Activity relative to that of sodium L-tenuazonate.

Discussion

Sodium L-tenuazonate was found to have a high degree of structural specificity against HAd1 in the egg. All changes in structure led to a marked reduction in activity.

Cytotoxic activities of the C-5 substituted congeners and of the N-methyl derivative of tenuazonic acid against KB cells correlated with antitumor activities in the egg. However, the degree of structural specificity in KB cells was not as great as in the egg tumor system, since N-benzyl compounds were as cytotoxic as L-tenuazonic acid itself.

The least structural specificity was shown in *B. megaterium*. The L-, D-, and D-*allo* isomers of tenuazonic acid had equivalent antimicrobial activities. Miller, *et al.*,[7] found a similar absence of stereospecificity in antiviral studies on sodium tenuazonate and sodium isotenuazonate. Both inhibited Echo-9, *para*influenza, vaccinia, herpes simplex, and measles viruses. However, sodium tenuazonate inhibited polio virus, but sodium isotenuazonate did not.

Some of the C-5 substituted congeners had antimicrobial activities equal to that of sodium L-tenuazonate. Substitution of a benzyl group for the N-hydrogen resulted in a striking enhancement of antimicrobial activity. This might have resulted from an increase in lipid solubility or even from different modes of action for the compounds.

Shigeura and Gordon[8] found that sodium L-tenuazonate inhibits protein synthesis in Ehrlich ascites cells and rat liver. In Ehrlich ascites cells the mode of action was shown to be an inhibition of release of protein from the ribosome. It is not known that this is the mode of action in microorganisms, nor even in other animal cells. Further, since the egg-tumor and *B. megaterium* systems studied here are of such diverse nature, a correlation of activities in the two systems would not necessarily be expected.

Acknowledgment.—The author gratefully acknowledges the technical assistance of Mrs. Barbara L. Bagdi, Mr. George W. Campbell, Mrs. Noreen A. Haag, Miss Joan E. Hitt, and Miss Dolores J. Meltz.

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Polynucleotides. III.[1] Synthesis of (3′→5′)-Linked Diribonucleoside Phosphates Containing 3- and 5-Methyluracil

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Received January 25, 1965

The occurrence of 5- and 3-methyluridine in ribonucleic acid has led us to prepare the following dibribonucleoside phosphates in order that their properties may be studied: uridylyl-(3′→5′)-3-methyluridine, 3-methyluridylyl-(3′→5′)-uridine, 3-methyluridylyl-(3′→5′)-3-methyluridine, 5-methyluridylyl-(3′→5′)-5-methyluridine, and 5-methyluridylyl-(5′→5′)-5-methyluridine. These compounds were synthesized by condensing a 3′-nucleotide blocked at the 2′- and 5′-positions by trityl groups with the 2′,3′-isopropylidene derivative of the second nucleoside. The key intermediates, 2′,5′-*O*-ditrityInucleosides were prepared by direct tritylation of the corresponding nucleosides. The location of the trityl groups in the case of 5-methyluridine was demonstrated by conversion of the blocked nucleoside to the xyloside as well as by a methylation procedure which gave 3-*O*-methylribose. The hyperchromic effect of these compounds was compared with that of uridylyl-(3′→5′)-uridine. In comparison with this compound the values for diribonucleoside phosphate methylated in the 3-position are approximately one-third higher than that for the nonmethylated derivative. The introduction of a methyl group at the 5-position causes a more than twofold increase in the hyperchromic effect. The diribonucleoside phosphates containing a 3-methyluridylyl-3′ residue are resistant to the action of pancreatic ribonuclease in contrast to the corresponding 5-methyl derivatives which are substrates for this enzyme.

The four major ribonucleosides comprise about 95–99% of the nucleosides of ribonucleic acid (RNA). The remaining percentage, particularly in the case of soluble RNA, is made up of over 20 minor nucleoside constituents[2] which appear to be distributed throughout the RNA molecule in a specific pattern.[3] The subtleties in physical and biological properties which these minor nucleosides confer upon RNA molecules are not well understood. In order to learn more about the effects of these minor constituents on RNA it is our plan to study model polyribonucleotides containing minor nucleosides. As a step toward preparation of such polynucleotides, diribonucleoside phosphates were synthesized starting from 3- and 5-methyluridine. Soluble RNA contains 0.1–0.5% of 5-methyluridine[4] and about 0.005% of 3-methyluridine.[5]

Preparation of the key intermediates, illustrated by compounds Ia and Ib, was based on an earlier approach to the synthesis of uridylyl-(3′→5′)-uridine[6] which made use of the easily prepared 2′,5′-di-*O*-trityluridine.[7] As in the case of uridine, direct tritylation of 3-methyluridine with an excess of trityl chloride afforded 2′,5′-di-*O*-trityl-3-methyluridine. Proof that the second trityl group is attached at the 2′-position is provided by the observation that methylation of the known 2′,5′-di-*O*-trityluridine affords the same

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