DISCOVERY OF INHIBITORY ACTIVITY OF TENUAZONIC ACID FOR GROWTH OF HUMAN ADENOCARCINOMA-1.*

by E. A. Kaczka, C. O. Gitterman, E. L. Dulaney, M. C. Smith, D. Hendlin, H. B. Woodruff and K. Folkers

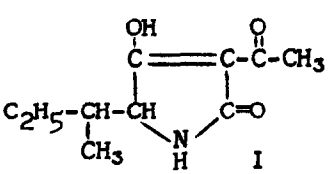
Merck Sharp & Dohme Research Laboratories
Rahway, New Jersey

Received September 16, 1963

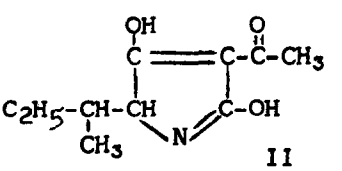
The initial search for new growth inhibitors of experimental human tumors revealed hadacidin (Kaczka et al., 1962). The continued search led to the detection of another broth, from an Alternaria isolate, which also inhibited the growth of human adenocarcinoma-1 in the embryonated egg.

Isolation of the active substance from this broth was undertaken with the biological guidance of the egg technique, and the active substance was obtained in a pure state as a viscous oil having the composition $C_{10}H_{15}NO_3$. This acidic compound and N,N'-dibenzylethylenediamine gave a water-insoluble crystalline salt $(C_{10}H_{15}NO_3)_2 \cdot C_{16}H_{20}N_2$. Titration of the acid with aqueous sodium bicarbonate and lyophilization of the aqueous solution gave the monosodium salt as a water-soluble white powder

Microanalytical data, ultraviolet, and infrared spectra for the acid were in agreement with structure I which was proposed for tenuaonic acid (Stickings, C. E., 1959), a metabolite of Alternaria tenuis.



I



II

A sample of the copper salt of tenuazonic acid was kindly furnished by Dr. C. E. Stickings of the University of London, who also informed us that the published infrared absorption bands at 1735, 1705

* Partially supported by CCNSC Contract No. SA-43-ph-3057

(shoulder), 1674 and 1630 cm^{-1} should be corrected to 1715, 1687 (shoulder) 1653 and 1619 cm^{-1}. These corrected wave lengths are in agreement within experimental deviations with those found for the acid from Alternaria. Dr. Stickings' copper salt was converted to the acid which was compared with the compound from Alternaria. Identity was demonstrated by their infrared and NMR spectra (Table I).

The interpretation of the NMR data, kindly provided by Dr. Nelson R. Trenner and Mr. Byron Arison, indicates an equilibrium mixture for the acid in chloroform. Possibly the resonance hybrids also have a hydrogen bond between the hydrogen atom and the carbonyl in the 3- and 4-substituents.

TABLE I. NMR DATA

(d = doublet, t = triplet, m = multiplet for tenuazonic acid in $CDCl_3$ solution at 60 mc.)

Proton	τ Value	Relative Area
Acidic H	1.69	
Active H	4.46	
=C-CH-N (O)	6.0 (d) 6.13 (d)	0.25-0.75 (Equilibrium or 0.20-0.80 Properties)
$-COCH_3$	7.39 7.44	
CH_2-C	8.0 (m) 8.6 (m)	
$CHCH_3$	8.83 (d) } overlaid	
CH_2CH_3	8.94 (t) }	

We also found that tenuazonic acid was a metabolite of two other widely differing genera of fungi, viz., Aspergillus and a member of the Sphaeropsidales. The pycnidial forming culture and the Aspergillus culture have been encountered only once, but numerous isolates of Alternaria were found to produce this metabolite.

The antitumor activity of tenuazonic acid in the embryonated egg against HAd-1 with comparative data of control compounds are given in Table II. The procedure for the assay has been described in literature on hadacidin (Kaczka et al., 1962; Gitterman et al., 1962). It is evident that tenuazonic acid is significantly active in this test when compared with typical known antitumor agents.

TABLE II. GROWTH RETARDATION OF HAd-1 BY TENUAZONIC ACID AND CONTROL COMPOUNDS

Compound	Dose (mg./egg)	Embryo Mortality	Growth Retardation (% Embryo	Tumor
Tenuazonic acid	0.44	5/12	1	88
	0.22	1/12	0	69
Hadacidin monosodium salt	6	3/24	3.5	61
Azaserine	1	2/9	20	62
6-Mercaptopurine	8.0	3/9	6	63

Shigeura et al. (1963) have studied the mode of inhibitory action of tenuazonic acid and have reported that it inhibits the incorporation of amino acids into microsomal protein in cell-free extracts of Ehrlich ascites and rat liver cells.

Isolation of Tenuazonic Acid.- The filtered fermentation broth is acidified to pH 1-2 and extracted with ethyl acetate. The ethyl acetate solution is extracted with 5% sodium bicarbonate; the latter solution is then acidified to pH 2 with conc. hydrochloric acid and extracted with petroleum ether (30-60°). The solvent residue is dissolved in Skellysolve B, and N,N'-dibenzylethylenediamine (DBED) is added until there is no further precipitation. The oily precipitate is dissolved in acetone and benzene is added for crystallization. Various broths contained 0.25-0.5 mg./ml. of tenuazonic acid according to approximation by ultraviolet absorption. The yields of the DBED salt

ranged about 25% of the estimated acid in the broth, and without development.

The rotation of the DBED salt in methanol is $[\alpha]^{24}_{546}$ -114° (C=0.8). The ultraviolet absorption spectrum of a methanol solution of the salt showed maxima at 240 mμ $E^{1\%}_{1\ cm}$ 360 and at 279 mμ $E^{1\%}_{1\ cm}$ 500.

The infrared spectrum of a CCl_4 solution of the acid, recorded on a Baird Infrared Spectrophotometer, showed the following characteristic bands: 3174, 3049, 1721, 1692 (Shoulder), 1663, 1630, 1458, 1383, 1330, 1294, 1232, 1110, 1098, 1034, 1018, 974, 958, 921, and 905 cm^{-1}.

The ultraviolet absorption spectrum of a ca. pH 13 solution showed maxima at 240 mμ $E^{1\%}_{1\ cm}$ 623 and 279 mμ $E^{1\%}_{1\ cm}$ 765; the spectrum of a ca. pH 2 solution showed a maximum at 277 mμ $E^{1\%}_{1\ cm}$ 640.

The DBED salt is converted to the acid by dissolving the salt in methanol and acidifying with hydrochloric acid. The solution is filtered and then evaporated to dryness *in vacuo*. The residue is extracted with petroleum ether (30-60°) and the extract evaporated *in vacuo* at ca. 30° leaving tenuazonic acid as a viscous oil.

Anal. Calcd. for $C_{10}H_{15}NO_3$ (197.228): C, 60.9; H, 7.7; N, 7.1. Found: C, 60.8; H, 7.5; N, 7.2.

Potentiometric titration of the acid with 0.1 N lithium hydroxide gave an equivalent weight of 198, $pH_{1/2}$ 3.7.

REFERENCES

Gitterman, C. O., Dulaney, E. L., Kaczka, E. A., Hendlin, D., and Woodruff, H. B., Proc. Soc. Expti. Biol. and Med., 109, 852, (1962).

Harris, S. A., Fisher, L., and Folkers, K., (1963) in press

Kaczka, E. A., Gitterman, C. O., Dulaney, E. L., and Folkers, K., Biochemistry, 1, 340 (1962).

Shigeura, H. T., and Gordon, C. N., (1963) in press.

Stickings, C. E., Biochem. J., 72, 332 (1959).