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6-Diazo-5-oxo-L-norleucine, A New Tumor-inhibitory Substance. II.[1] Isolation and Characterization[2]

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RECEIVED FEBRUARY 2, 1956

A new tumor-inhibitory antibiotic, 6-diazo-5-oxo-L-norleucine, has been isolated in crystalline form from culture broths of an unidentified *Streptomyces*. Characteristic physical properties of the compound have been determined. The structure of the antibiotic was proven by periodic acid oxidation to L-glutamic acid and Wolff rearrangement to α-aminoadipic acid.

A new tumor-inhibitory antibiotic, 6-diazo-5-oxo-L-norleucine, has been isolated from culture broths of an unidentified *Streptomyces* found in a sample of Peruvian soil.

Preliminary studies on culture filtrates showed that the compound was very sensitive to heat and *p*H. Stability studies at room temperature demonstrated the necessity of experimenting within the *p*H range of 4–8, the optimum range being 4.5–6.5. Extraction of the culture filtrates with several classes of organic solvents was unsuccessful. The Amberlites IR-120, IRC-50, IR-45 and IRA-400 either failed to adsorb or else destroyed the compound. However, the compound possessing antitumor activity was adsorbed on activated alumina from 90% aqueous ethanol solution or on activated carbon from water solution; elution was accomplished by aqueous alcohols or aqueous acetone.

The most feasible conditions for the purification of the antibiotic were: (1) adsorption from 90% aqueous ethanol onto alumina adjusted to *p*H 5.5–6.5, and elution with 25% aqueous ethanol; (2) carbon chromatography of the best fractions from the alumina on Darco G-60, using 1% aqueous acetone as both solvent and developer; (3) crystallization of the best carbon fractions from aqueous alcohol or aqueous acetone.

From aqueous methanol, 6-diazo-5-oxo-L-norleucine is obtained as fine, light yellow-green needles; the compound possesses no sharp melting point, but merely decomposes at 145–155° with gas evolution. It is very soluble in water, in aqueous solutions of methanol, ethanol and acetone, but only slightly soluble in absolute alcohols. Stability data of the crystalline antibiotic are given in Fig. 1. The specific rotation is $[\alpha]^{26}D$ +21° (*c*, 5.4% in water). In *p*H 7.0 phosphate buffer, the compound exhibits a characteristic ultraviolet absorption spectrum (Fig. 2) with maxima at 274 mμ ($E^{1\%}_{1\ cm.}$ 683) and 244 mμ ($E^{1\%}_{1\ cm.}$ 376). Alkali or acid do not effect a hypsochromic or bathochromic shift. In 0.1 *N* alkali, there is a gradual decrease of the characteristic ultraviolet absorption with a concomitant decrease in activity against *Torulopsis albida*. There is a 50% loss of ultraviolet absorption in 2 hours, and a 98.5% loss after 19 hours. In 0.1 *N* hydrochloric acid, both the ultraviolet absorption and microbiological activities are completely destroyed within one hour. The infrared absorption spectrum, as shown in Fig. 3, indicates strong absorption at 3.19, 3.32, 4.66, 6.14, 6.30, 6.59 and 7.18 μ. The antibiotic gives positive ninhydrin and Tollens tests. Gas is liberated upon treating an aqueous solution of the compound with strong acids.

Elemental analyses show that the antibiotic possesses the empirical formula $C_6H_9N_3O_3$. Molecular weight determination by titration gives a value of 171 with $pK'a$ values of 2.1 and 8.95 in water. The ultraviolet absorption spectrum is characteristic of diazoketones. The diazo grouping is confirmed by acid liberation of $^2/_3$ of the total nitrogen and also by the strong absorption band at 4.66 μ in the infrared. The presence of an amino acid is evidenced by the positive ninhydrin test, the typical $pK'a$ values, and the infrared absorption at 3.19, 3.78, 6.30 and 6.59 μ.

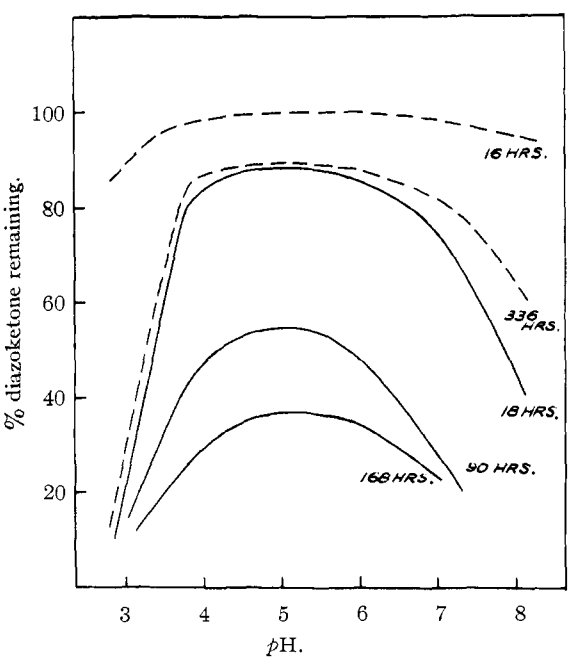

Fig. 1.—Stability of 6-diazo-5-oxo-L-norleucine in aqueous buffered solutions (concentration 32 γ/ml.): ---, 5°; —, 30°.

Degradative studies employing periodic acid oxidation gave 0.96 mole of L-glutamic acid per mole of antibiotic. This is evidence for the presence of a straight chain molecule; however, since glutamic acid contains two carboxyl groups, two diazoke-

(1) For the first communication of this series, see J. Ehrlich, G. L. Coffey, M. W. Fisher, A. B. Hillegas, D. L. Kohberger, H. E. Machamer, W. A. Rightsel and F. R. Roegner, *Antibiotics & Chemotherapy*, paper in press.

(2) Presented before the Division of Medicinal Chemistry at the 129th National Meeting of the American Chemical Society, Dallas, Texas, April 10, 1956.

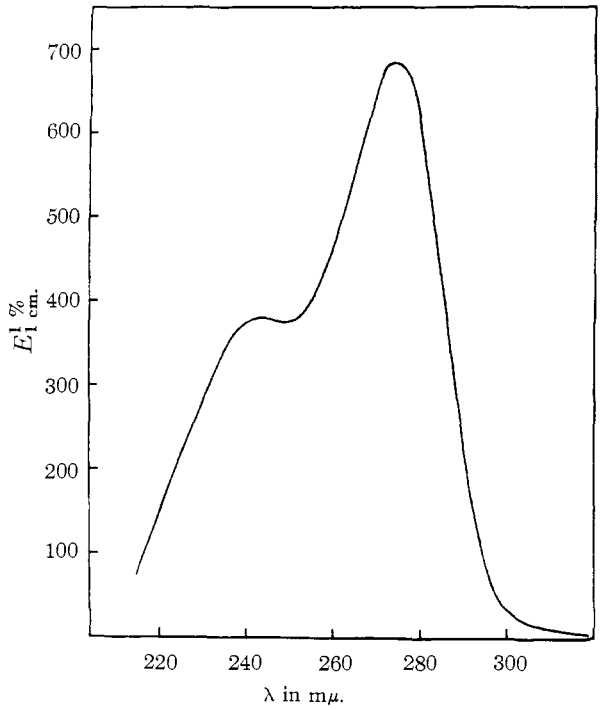

Fig. 2.—Ultraviolet absorption spectrum of 6-diazo-5-oxo-L-norleucine in *p*H 7.0 phosphate buffer.

tones are possible, namely, 6-diazo-5-oxo-L-norleucine and 6-diazo-5-oxo-4-aminohexanoic acid. The Wolff rearrangement of the antibiotic to α-aminoadipic acid showed conclusively that the structure was 6-diazo-5-oxo-L-norleucine since 6-diazo-5-oxo-4-aminohexanoic acid would rearrange to β-aminoadipic acid.

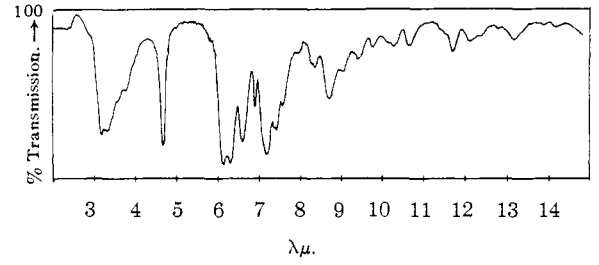

Fig. 3.—Infrared spectrum of 6-diazo-5-oxo-L-norleucine in compressed potassium bromide.

Experimental

Assay.—In the preliminary studies, 6-diazo-5-oxo-L-norleucine was assayed against Crocker mouse sarcoma 180 in mice.[3] In subsequent studies, this activity was correlated with the inhibitory action of the antibiotic against *Torulopsis albida* NRRL Y1400[1]; the microbiological activity, in turn, was correlated with the ultraviolet absorption at 274 and 244 mμ. When the crystalline antibiotic was assayed against the beer standard, it was found to possess approximately 650 *T. albida* units/mg.

Isolation of Crude 6-Diazo-5-oxo-L-norleucine.—The fermentation broth (41.5 liters), adjusted to *p*H 6.8 with sulfuric acid, was treated with 1% (w./v.) Hyflo Super-Cel, and the slurry was filtered through a pad of this filter-aid. The clear filtrate, containing 1,391,500 *T. albida* units, was concentrated in a Precision circulating still under vacuum at <35° to 1920 ml.; the concentrate was then diluted to 19.2 liters with 95% ethanol No. 3A. The precipitated solid was filtered and discarded; the filtrate, now containing 595,200 *T. albida* units, was reserved for chromatography on alumina.

Alumina (2.3 kg.) was adjusted to *p*H 6.4 with hydrochloric acid, washed with water and activated for 4 hours at 200°. The adsorbent was slurried in 90% aqueous ethanol[4] and added to a four-inch Pyrex glass column; the prepared column had a holdup volume of 2.3 liters and a gravity flow rate of 2 liters/kg. Al_2O_3 per hr. The filtrate, containing the antibiotic, was percolated through the alumina, and the column was washed with approximately 1 holdup volume of 90% aqueous ethanol and 5 holdup volumes of 75% aqueous ethanol. The antibiotic was then eluted with 7 holdup volumes of 25% aqueous ethanol and the eluate collected in 1-liter fractions. The first six fractions of eluate were concentrated severally *in vacuo*, and dried from the frozen state. Only these fractions were of sufficient purity to give the desired results in the subsequent carbon chromatography.

The recovery of *T. albida* inhibitory activity from the alumina chromatography varied from 51–87%, depending on the quality of the starting material used. The best fractions obtained by this procedure contained 35–38 *T. albida* units/mg. solid, or 5–6% 6-diazo-5-oxo-L-norleucine.

Since the antibiotic is so sensitive to *p*H, the *p*H of solutions of the compound as well as the *p*H of the alumina used for the preliminary purification are important factors in determining yields and the over-all efficiency of the process. Best results were obtained by using alumina adjusted to *p*H 5.5–6.5 and also by maintaining aqueous solutions of the antibiotic between *p*H 4.5 and 6.5.

Some of the fractions of the 25% aqueous eluate exhibited a maximum at 271–272 mμ; however, fractions from alumina columns do not always exhibit a direct correlation between *T. albida* inhibitory activity and ultraviolet absorption at 270–275 mμ. Those fractions which exhibited a maximum in this range but which possessed very little antimicrobial activity were discarded.

Carbon Column Chromatography.—A mixture of 780 g. of Darco G-60 and 780 g. of Celite 545 was slurried in 1% aqueous acetone and the slurry added to a 5-foot Pyrex glass column, having a diameter of four inches; size of packed column 4″ × 22″; holdup volume 4 liters; rate of flow 1750 ml./hr.

Ninety-five grams of crude alumina fractions, containing 2.16 g. of pure material (1,402,500 *T. albida* units), was dissolved in 858 ml. of 1% aqueous acetone; the solution, *p*H 6.0, was percolated through the packed carbon column which was then developed and eluted with 20.5 liters of 1% aqueous acetone. Fractions of 500 ml. were collected. The seventeenth to the twentieth fractions inclusive were concentrated individually *in vacuo* and each concentrate dried from the frozen state.

Since at this stage of purification there is good correlation between the ultraviolet absorption at 274 mμ and the *anti-T. albida* activity, the eluates were first analyzed during collection by determination of the ultraviolet absorption; later these analyses were confirmed by microbiological assay *vs. T. albida*. The recovery of *T. albida* inhibitory activity from the carbon chromatography varied from 55–71%, depending on the quality of the starting material used. The better fractions assayed from 52–63% pure 6-diazo-5-oxo-L-norleucine.

Crystallization of 6-Diazo-5-oxo-L-norleucine.—The dried solids, obtained from the seventeenth to the twentieth fractions from the carbon column, were crystallized severally from 95% aqueous methanol; a total yield of 232 mg. of crystalline antibiotic was obtained. This yield amounted to 10.8% of the charge on the carbon column and to 0.7% of the antibiotic present in the filtered fermentation broth. Recrystallization of 151 mg. of once-crystallized material from 95% aqueous methanol gave 34.5 mg. (23% yield) of fine, light yellow-green needles.

Anal. Calcd. for $C_6H_9N_3O_3$: C, 42.10; H, 5.30; N, 24.55; diazo N, 16.37; mol. wt., 171.16. Found: C, 42.16; H, 5.70; N, 24.07; diazo N, 16.01; mol. wt., 171 (titration).

(3) D. A. Clarke, H. C. Reilly and C. C. Stock, Abstracts of Papers 129th Meeting, American Chemical Society, Dallas, Texas, April, 1956, p. 12-N.

(4) Nine volumes of 95% ethanol No. 3A and one volume of water has been called arbitrarily 90% ethanol.

Paper Chromatography and Paper Ionophoresis.—The antibiotic gave satisfactory R_f values with Whatman No. 1 paper when developed descendingly in aqueous alcohol mixtures, for example, (1) ethanol–water (3:1), R_f 0.58; (2) isopropyl alcohol–water (7:3), R_f 0.54; (3) *t*-butyl alcohol–water (7:3), R_f 0.51; and (4) *n*-butanol–ethanol–water (1:1:1), R_f 0.52. In paper electrophoresis using the Durrum[5] type apparatus, the antibiotic showed very little movement in 0.1 *M* *p*H 4 sodium acetate, 0.05 *M* *p*H 6.8 phosphate buffer, and 0.1 *M* *p*H 8.6 veronal buffer for four hours at 460–480 volts and 2.4–2.6 milliamp.

Degradation of 6-Diazo-5-oxo-L-norleucine to L-Glutamic Acid.—The antibiotic (26 mg.) was dissolved in 10 ml. of 0.2 *M* periodic acid and the solution was left at room temperature for 16 hours. The periodate solution was diluted to 60 ml. with water and percolated through a column of 5 ml. of Dowex 50WX8, hydrogen cycle. The resin column was washed with water and the basic compounds eluted with 0.5 *M* ammonium hydroxide. The ninhydrin-positive fractions (ammonia-free) were combined and percolated through a column of 5 ml. of Amberlite IR-45 (OH^-). The resin was washed with water and the dicarboxylic amino acid eluted with 0.5 *M* hydrochloric acid. A quantitative ninhydrin determination[6] on the acid eluate gave 0.96 mole of an amino acid in terms of glutamic acid per mole of antibiotic. The acid eluate from the Amberlite IR-45 was chromatographed on Dowex 50WX8, and the free amino acid eluted with 0.5 *M* ammonium hydroxide. The ninhydrin-positive fractions (ammonia-free) were combined, evaporated *in vacuo*, and the residue crystallized twice from aqueous methanol.

Anal. Calcd. for $C_5H_9NO_4$: N, 9.52. Found: N, 9.67. pK'_a of standard glutamic acid in water: 2.2, 4.4, 9.8; mol. wt., 149. Found (unknown): 2.2, 4.35, 9.8; mol. wt., 150.

The degradation product could not be differentiated from a standard sample of glutamic acid either by means of paper chromatography in three separate systems (1) methyl ethyl ketone–propionic acid–water (75:25:30),[7] (2) *n*-butanol–methanol–water (2:2:1),[8] and (3) pyridine–acetic acid–water (50:35:15),[9] or by means of paper electrophoresis using 0.25 *N* acetic acid and 0.1 *M* *p*H 8.6 veronal buffer. The infrared curve of the degradation product was identical with a standard sample of L-glutamic acid. A microbiological assay for L-glutamic acid using the method of Hier[10] showed that the amino acid possessed the "L"-configuration.

(5) E. L. Durrum, THIS JOURNAL, **72**, 2943 (1950).

(6) E. C. Cocking and E. W. Yemm, *Biochem. J.*, **58**, XII (1954).

(7) R. A. Clayton and F. M. Strong, *Anal. Chem.*, **26**, 1362 (1954).

(8) T. L. Hardy, D. O. Holland and J. H. C. Naylor, *ibid.*, **27**, 971 (1955).

(9) P. Decker and W. Riffart, *Chem. Ztg.*, **74**, 261 (1950).

(10) S. W. Hier, C. E. Graham, R. Freides and D. Klein, *J. Biol. Chem.*, **161**, 705 (1945).

Degradation of 6-Diazo-5-oxo-L-norleucine to α-Aminoadipic Acid (Wolff Rearrangement[11]).—A slurry of 20.5 mg. of the antibiotic in 1 ml. of dioxane was added to a solution of 0.3 ml. of concentrated ammonium hydroxide and 0.1 ml. of 10% silver nitrate in a 15-ml. centrifuge tube equipped with a reflux condenser. The solution was heated for 0.5 hour on the steam-bath. At that time another 0.2 ml. of concentrated ammonium hydroxide was added to the reaction mixture and the solution heated for an additional 2.5 hours.

The reaction mixture was centrifuged and the precipitate was extracted with 2 ml. of hot water; the wash and original supernatant were combined, and evaporated to dryness *in vacuo*. The residue was dissolved in 3 ml. of 3 *M* hydrochloric acid and the solution was heated on the steam-bath for 3 hours to hydrolyze the amide of the aminoadipic acid.

The resulting light brown solution was evaporated *in vacuo* to remove the hydrochloric acid, and the residue was successively chromatographed on resin columns (5 ml. each) of Dowex 50WX8, Amberlite IR-45 and Dowex 50WX8 as in the case of the periodate oxidation product of the diazoketone. The eluate from the second Dowex 50WX8 column, containing the amino acid, was evaporated *in vacuo* and the residue (16.4 mg.; theoretical yield of α-aminoadipic acid is 19.3 mg.) crystallized from aqueous methanol to give 7.2 mg. of crystalline amino acid.

Anal. Calcd. for $C_6H_{11}NO_4$: N, 8.69. Found: N, 8.38.

The amino acid could not be differentiated from a standard sample of α-aminoadipic acid by a combination of paper chromatography and paper electrophoresis, using the same procedure as was used for the identification of glutamic acid from the periodate oxidation. The infrared spectrum was identical with a standard sample of α-aminoadipic acid.

Acknowledgments.—The authors are indebted to Dr. C. C. Stock and associates of the Sloan–Kettering Institute for Cancer Research, New York, N. Y., for the assay of various fractions against Crocker sarcoma 180 in mice. We also wish to thank Dr. John Ehrlich and associates for laboratory quantities of culture filtrates and for microbiological assays; Dr. Harry M. Crooks, Jr., for his advice and interest in the structure determination; Dr. John M. Vandenbelt and associates for ultraviolet and pK'_a determinations; Mr. R. Bruce Scott for the infrared spectra; Mr. C. E. Childs and associates for microanalyses; Dr. H. E. Machamer and associates for pilot plant quantities of culture filtrates; and Dr. O. D. Bird for the microbiological determination of L-glutamic acid.

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(11) A. Burger and S. Avakian, *J. Org. Chem.*, **5**, 606 (1940).