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PA-147 (3-Carboxy-2,4-pentadienal Lactol)—a New Antibiotic[1]

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PA-147 (I) was isolated from the fermentation liquors of an unidentified *Streptomyces* strain. The antibiotic has the empirical formula $C_6H_6O_3$ and was characterized as its barium salt $(C_6H_5O_3)_2Ba\cdot H_2O$. The structure of the antibiotic was established as 3-carboxy-2,4-pentadienal lactol (Ib) and was proved by synthesis of tetrahydro-PA-147-dicarboxylic acid (IV) which is identical with ethylsuccinic acid.

During the course of our antibiotic screening program, PA-147 (I), a compound with relatively low antibacterial activity, was isolated from the fermentation filtrates of an unidentified *Streptomyces* strain.

Preliminary investigations of fermentation beers showed that the antibiotic is readily extracted from aqueous solutions at *p*H 2 to 3 by such water-immiscible solvents as methyl isobutyl ketone and ethyl acetate. PA-147 was quantitatively extracted from such solvents with a *p*H 6.8 phosphate–acetic acid buffer. Consequently this system was used as a preliminary purification step. The crude antibiotic was obtained as a viscous oil from the solvent extracts by evaporation of the solvent. The material obtained in this way still contained a considerable amount of pigment. It was soon apparent, too, that relatively rapid polymerization took place when the oil was allowed to stand at room temperature. Polymerization was very rapid at 60–80°. To free the isolate from residual pigment and other impurities, the oil finally was chromatographed on neutral aluminum oxide using ethyl acetate as the eluting solvent. The activity in the various fractions was followed by papergram analysis and bioplate assay. The chromatographic fractions so obtained contained material of 90–95% purity, determined by bioplate assay.

The infrared spectrum of this isolate indicated the presence of a five-membered ring carbonyl (5.68 μ), a hydroxyl function (2.95–3.1 μ), and a carbon-to-carbon double bond (6.25 μ). The ultraviolet spectrum had an absorption peak at 245 mμ (ϵ 7800) suggesting a substituted α,β-unsaturated carbonyl group. Titrations of PA-147 in 50% ethanol revealed a readily titratable carboxyl function. The ultraviolet spectrum of PA-147 sodium salt, taken directly in the titration solution, showed a definite shift to longer wave lengths now at 272 mμ (ϵ 20500) suggesting the formation of an $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl group (Ia).

PA-147 reacted rapidly with benzylamine to give a purple-red solid which polymerized rapidly on attempted crystallization. Attempts to form sodium, magnesium, calcium or copper salts as well as several amine salts were unsuccessful and yielded only biologically inactive solids. PA-147 was finally isolated in pure form as the crystalline barium salt, which was obtained by titration of an ethanolic solution of PA-147 with barium hydroxide and subsequent distillation of the ethanol from amyl acetate at low temperatures. The barium salt was obtained as a faintly yellow, crystalline powder, the analysis of which calculated for the empirical formula $(C_6H_5O_3)_2Ba\cdot H_2O$. This barium salt showed the characteristic infrared absorption of a carboxylate ion at 6.35 μ, a carbonyl band at 6.05 μ ($\alpha,\beta,\gamma,\delta$-unsaturated carbonyl) and a broad band at 2.9–3.2 μ attributed to –OH absorption. The doubly unsaturated carbonyl was further evident from the ultraviolet absorption peak at 272 mμ (ϵ 22000).

On hydrogenation, PA-147 readily absorbs two equivalents of hydrogen to yield tetrahydro-PA-147 (IIIb), $C_6H_{10}O_3$, as a fluid, colorless, high-boiling oil. The infrared spectrum of this compound shows absorption at 5.65 and 2.9–3.2 μ which suggests a five-membered ring lactone and a hydroxyl function. The compound readily formed a thiosemicarbazone, a semicarbazone and a 2,4-dinitrophenylhydrazone. These three derivatives still had a titratable carboxylic acid function also demonstrated by absorption peaks at 5.85 μ and 3.05 μ in the infrared spectrum. This acid function, therefore, must have been present in the lactol form (IIIb). Oxidation of tetrahydro-PA-147 (III) with nitric acid, silver oxide or hydrogen peroxide afforded a dicarboxylic acid IV with the empirical formula $C_6H_{10}O_4$, established by analysis and titration.

From the above data it was now clear that the non-carboxylic carbonyl was an $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde, giving the molecule the basic structure of a 2,4-pentadienal (Ia). Since the compound forms a five-membered lactol ring in neutral or slightly acidic solutions, the carboxyl group must, therefore, be attached at C3. It follows that PA-147 is 3-carboxy-2,4-pentadienal (Ia) existing primarily in the tautomeric lactol form Ib.

The alternate possible structure for PA-147, 3-aldehydo-2,4-pentadienoic acid (IIa), would also form a five-membered lactol ring (IIb). However, the observed ultraviolet absorption of the lactol form (245 mμ, ϵ 7800) is inconsistent with structure IIb. A structure of this type, by analogy to patulin[2] (VII), would be expected to show absorption at 270–280 mμ, ϵ 14,000 to 20,000.

The structure of tetrahydro-PA-147 dicarboxylic acid (IV), which is identical with ethylsuccinic acid, finally was verified by synthesis.[3]

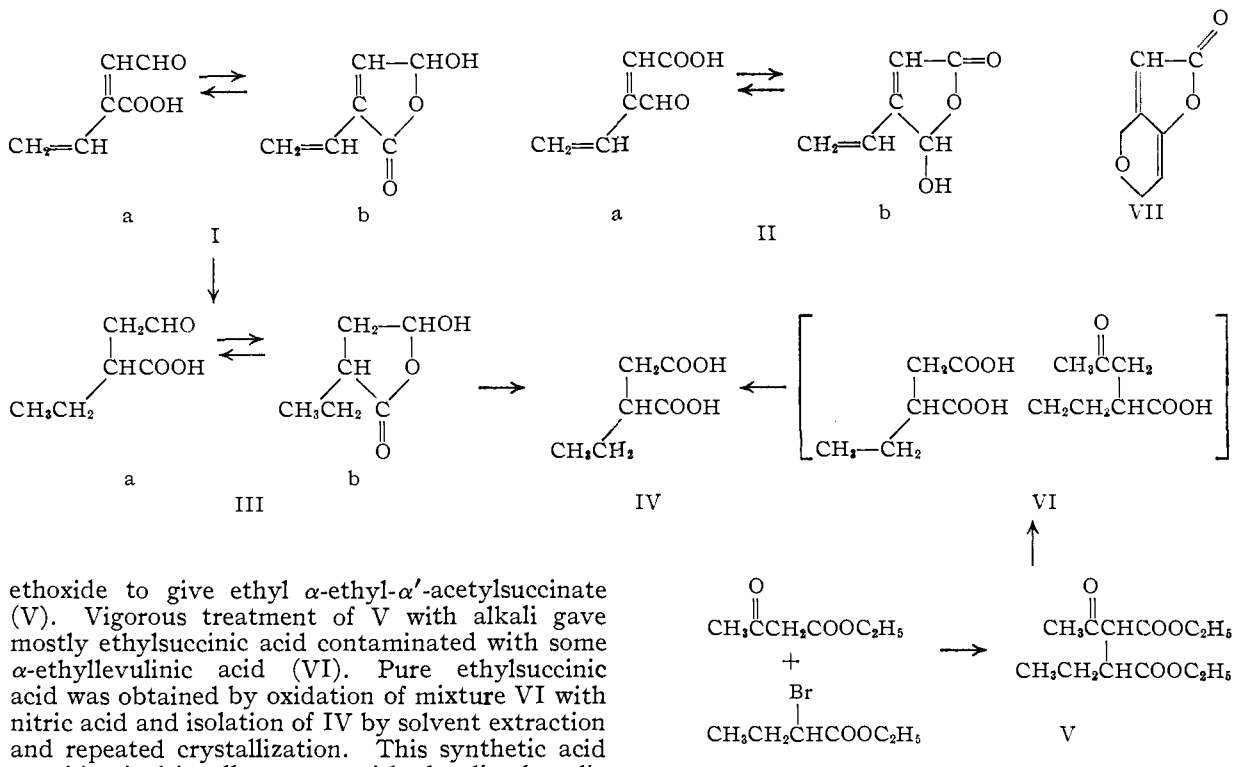
Ethyl acetoacetate and ethyl α-bromobutyrate were allowed to react in the presence of sodium

(1) Presented before the Division of Medicinal Chemistry at the 132nd Meeting of the American Chemical Society in New York, N. Y., September 8–13, 1957.

(2) R. B. Woodward and G. Singh, *Experientia*, **6**, 238 (1950); THIS JOURNAL, **72**, 1428, 5351 (1950).

(3) L. T. Thorne, *J. Chem. Soc.*, **39**, 336 (1881).

TABLE I



ethoxide to give ethyl α-ethyl-α'-acetylsuccinate (V). Vigorous treatment of V with alkali gave mostly ethylsuccinic acid contaminated with some α-ethyllevulinic acid (VI). Pure ethylsuccinic acid was obtained by oxidation of mixture VI with nitric acid and isolation of IV by solvent extraction and repeated crystallization. This synthetic acid was identical in all respects with the dicarboxylic acid IV obtained from tetrahydro-PA-147.

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Experimental

I. Isolation of PA-147. (a) **PA-147 Methyl Isobutyl Ketone Concentrate.**—Twenty gallons of broth were filtered through Supercel by suction. The filtrate was adjusted to *p*H 2 with 50% sulfuric acid. The antibiotic was extracted with two 5-gallon portions of methyl isobutyl ketone by stirring the two phase system each time for 30 minutes. The combined methyl isobutyl ketone extracts were now concentrated *in vacuo* to 1.5 liters.

Fifty milliliters of such a concentrate was taken to dryness *in vacuo* (15 mm.) at 80°. The residue was dried for 40 minutes at this temperature, giving 128 mg. of a light brown, glassy material. The residue dissolved easily in methanol or ethanol, but on repeated concentration became more and more insoluble, possibly due to polymerization. The gummy material was insoluble in acetone, benzene, Skellysolve C, chloroform and methylene chloride. After evaporation with above solvents, the residue was also completely insoluble in ethanol.

(b) **Distribution of PA-147.**—Fifty milliliters of methyl isobutyl ketone concentrate was extracted with two 30-ml. portions of *p*H 6.8 acetate buffer. The methyl isobutyl ketone layer was washed with 30 ml. of water, dried over sodium sulfate and the solvent was evaporated. A small amount of tarry material was obtained which weighed 13 mg. after drying.

The aqueous phase was adjusted to *p*H 2, and the water was extracted with two 200-ml. portions of ethyl acetate. The solvent was washed with 50 ml. of water and dried over sodium sulfate. After evaporation, 130 mg. of a glassy yellow material was obtained. Paper chromatographic analysis showed that the material consisted chiefly of PA-147 with small amounts of impurities present, detected by potassium permanganate spray.

(c) **Purification of PA-147 on Activated Aluminum Oxide.**—Ethyl acetate concentrate (100 ml.) containing approximately 3.5 g. of crude PA-147 was slurried with 2 g. of Darco G-60 for 10 minutes, and the carbon was filtered by suction through Supercel. The solution was concentrated to 15 ml. and chromatographed on 30 g. of acid-washed aluminum oxide; 50-ml. fractions were collected. The activity was followed by ultraviolet absorption. Two drops of each fraction were spotted on Whatman filter paper and viewed on the scanner screen. The intensity of the absorption was compared with the absorption of spots produced by standard solutions. Fraction 5 to 11 represented the heart cuts, containing approximately 85% of the total material. These fractions were combined and concentrated *in vacuo* to 50 ml.; 5 ml. of this concentrate gave on evaporatiou 291 mg. of a viscous oil; ultraviolet absorption λ_{max} 245 mμ, ϵ 7800; infrared absorption: 5.68 μ (5-ring carbonyl) 2.95–3.1 μ (-OH) and 6.25 μ (C=C double bond); bio-activity: 95 u./mg. ± 10 units (standard set arbitrarily at 100 u./mg. of antibiotic); $[\alpha]D$ 0 ± 2° (*c* 2 in $CHCl_3$).

(d) **Titration of PA-147 with Sodium Hydroxide.**—PA-147 (100 mg.) was dissolved in 100 ml. of 50% ethanol. Standard 0.1 *N* sodium hydroxide solution was added until the *p*H reached 7.5. The solution was then diluted to exactly 500 ml.; 1 ml. of this solution diluted tenfold (2 mg./100 ml.) showed absorption in the ultraviolet region at 272 mμ, ϵ 20500.

(e) **Benzylamine Salt of PA-147.**—Ten milliliters of ethyl acetate concentrate was taken to dryness and the oily residue was twice evaporated with 30 ml. of ether. An ethereal solution of benzylamine was added dropwise until no further precipitation took place (2.5 equivalents). In the course of a few minutes, the original colorless precipitate had turned to a deep purple-red, due to condensation of benzylamine with PA 147 (Schiff base). Although the purple material could be isolated, it resisted attempts to obtain it in crystalline form.

(f) **Barium Salt of PA-147.**—Forty milliliters of concentrate was evaporated *in vacuo* to a sirupy consistency. The residue was immediately dissolved in 50% ethanol–water and titrated to *p*H 8 with saturated barium hydroxide solution. The slightly turbid solution was filtered through Celite and carbon and to the clear filtrate was added 50 ml. of ethanol 3A and 50 ml. of amyl acetate. The faintly yellow solution was concentrated at 50° *in vacuo*. As the concentrate reached approximately 30 ml., an almost colorless crystalline

material separated, which was filtered and washed with ethyl acetate. The compound (0.87 g.) was dried for 3 hours *in vacuo* (80°, 0.05 mm.). The salt analyzed for the empirical formula $C_{12}H_{10}O_6 \cdot Ba \cdot H_2O$: Calcd. C, 35.53; H, 2.98; Ba, 33.87; H_2O, 4.4; CH_3–C, 11; CH_3CO–, 34.15. Found: C, 35.83; H, 3.12; Ba, 34.03, 33.54; H_2O (Karl Fischer), 3.10; CH_3–C, 2.24; CH_3CO–, 3.08, $[\alpha]D$ 0 ± 2° (*c* 2 in H_2O); ultraviolet absorption: λ_{max} 272 mμ, ε 2200; infrared absorption: typical absorption pattern for carboxylate ion and unsaturated carbonyl. The barium salt of PA-147 retained complete antimicrobial activity.

The barium salt was converted to the free acid by addition of one equivalent of sulfuric acid. The precipitated barium sulfate was removed by centrifuging the precipitate and decantation of the clear supernatant; ultraviolet absorption: λ_{max} 272 mμ, ε 17200.

(g) **Sodium Salt of PA-147.**—Two hundred milligrams of PA-147 Ba-salt was dissolved in 10 ml. of water. Seventy milligrams of sodium sulfate (1 equivalent) in 3 ml. of water was added and the precipitated barium sulfate was filtered by suction. Forty milliliters of ethanol 3A and 50 ml. of amyl acetate were added and the clear solution slowly was concentrated *in vacuo* at 50° (30 mm.). Some of the material separated as an oil and was re-dissolved by the addition of 10 ml. of ethanol. The solution was concentrated further to 25 ml. and ether was added slowly precipitating the semi-crystalline sodium salt of PA-147. A total yield of 135 mg. was obtained. The material had only 50% of the bioactivity of PA-147 compared on an equivalent basis.

All attempts to obtain the sodium salt by a different route were of no avail. In each case, the material obtained had no bioactivity.

II. Structure Determination of PA-147. (a) **Hydrogenation of PA-147.**—A solution of 760 mg. (6.1 mmoles) of PA-147 in 50 ml. of ethyl acetate was hydrogenated with 0.5 g. of pre-hydrogenated palladium (5%) on carbon catalyst. The compound absorbed 258.5 ml. of hydrogen (cor. 0°, 760 mm.), equivalent to 11.53 millimoles of hydrogen, calculating for 1.88 moles of H_2 per mole of compound. From the infrared spectra it could be concluded that two double bonds had been hydrogenated (no change in C=O region).

The catalyst was filtered by suction and the solvent was evaporated, giving 770 mg. of a colorless oil. The oil was distilled from a Hickman column, b.p. 120–125° (0.02 mm.). *Anal.* Calcd. for $C_6H_{10}O_3$: C, 55.37; H, 7.74; C–CH_3, 11.54. Found: C, 55.72; H, 7.72; C–CH_3, 12.21; $[\alpha]D$ 0 ± 2° (*c* 3, MeOH).

Tetrahydro-PA-147 thiosemicarbazone was prepared in the usual manner from tetrahydro-PA-147 and thiosemicarbazide and crystallized from ethanol–water, m.p. 196–198°, infrared absorption 5.88 μ (C=O) and 2.95 μ, 3.8–3.9 μ (–OH) typical of carboxylic acid.

Tetrahydro-PA-147-semicarbazone was prepared in the standard manner from tetrahydro-PA-147 and semicarbazide acetate and crystallized from ethanol–water, m.p. 158–159°. *Anal.* Calcd. for $C_7H_{13}N_3O_3$: C, 44.91; H, 7.0; N, 22.45; mol. wt., 198.19. Found: C, 44.38; H, 6.92; N, 22.52; neut. equiv., 182 (50% ethanol–water).

Tetrahydro-PA-147 2,4-dinitrophenylhydrazone was prepared in the usual manner from tetrahydro-PA-147 and 2,4-dinitrophenylhydrazine reagent solution. The derivative crystallized from ethanol–water, m.p. 157–158°; *Anal.* Calcd. for $C_{12}H_{14}N_4O_6$: C, 46.45; H, 4.54; N, 18.06; mol. wt., 310.3. Found: C, 46.41; H, 4.54; N, 18.38; neut. equiv., 324 (50% ethanol–water).

(b) **Oxidation of Tetrahydro PA-147.** (1) **With 30% Nitric Acid.**—Tetrahydro-PA-147 (800 mg.) was refluxed for 90 minutes with 50 ml. of 30% nitric acid. Strong nitrous oxide evolution indicated rapid oxidation. After 90 minutes, the solution became a clear yellow and was then concentrated *in vacuo* on the steam-bath. The residue was evaporated four times with 50 ml. of distilled water, and then distilled twice with 100 ml. of benzene–ethanol (1:1). The yellow residue (470 mg.) was crystallized from methyl isobutyl ketone, giving 70 mg. of colorless prisms, m.p. 75–78°. After one more recrystallization from ether–hexane, the compound melted at 94–95°, $[\alpha]D$ 0 ± 2° (*c* 2 in MeOH).

(2) **With Silver Oxide.**—To a slurry of silver oxide (prepared from 15 g. of silver nitrate) was added 1.0 g. of tetrahydro-PA-147 in 40 ml. of water (*p*H 8.5). The slurry was stirred for 3 days at room temperature. The silver oxide was then filtered by suction and the *p*H of the filtrate was adjusted to 1 with concd. hydrochloric acid. The aqueous phase was extracted with a total of one liter of ether. The ethereal solution was dried over sodium sulfate and the ether was evaporated. A light yellow oily residue was obtained (900 mg.) which crystallized from methyl isobutyl ketone on standing at room temperature for several days, m.p. 95–97°, mixed melting point with above acid 95–97°. The infrared spectrum clearly indicates a dicarboxylic acid, absorption at 3.35 (broad), 3.45, 3.85, 5.86 (shoulder) 5.95, 6.8, 7.05, 7.56, 7.92, 8.25, 8.43 and 8.8 μ.

(3) **Air Oxidation.**—Tetrahydro-PA-147 (500 mg.) was stirred for two weeks in 20 ml. of ethanol. After this time, the ethanol was evaporated, giving a colorless oil (500 mg.) which crystallized on standing, m.p. 95–97°, mixed melting point with above acids 95–97°. Above acids were identical in melting point, mixed melting point, infrared spectrum and paper chromatographic behavior with ethylsuccinic acid (see below).

(4) **Oxidation with 1% Hydrogen Peroxide in Ethanol.**—Tetrahydro-PA-147 (500 mg.) was dissolved in 100 ml. of 1% ethanolic hydrogen peroxide. The solution was stirred for two days at room temperature and then concentrated on the water-bath to 20 ml. Fifty milliliters of water was added; the *p*H was adjusted to 2 with 2 *N* sulfuric acid and the solution extracted with a total of 500 ml. of ethyl acetate. The ethyl acetate was dried over Na_2SO_4 and evaporated *in vacuo* leaving 250 mg. of a light yellow oil. The oil crystallized on standing in two milliliters of methyl isobutyl ketone to give 50 mg. of colorless prisms, m.p. 95–97°, identical in melting point, mixed melting point and paper chromatographic behavior with ethylsuccinic acid.

III. Synthesis of Ethylsuccinic Acid. (a) **Ethyl α-Ethyl-α-acetyl-succinate.**—Sodium (6 g.) was dissolved in 90 ml. of freshly distilled ethanol. Ethyl acetoacetate (34 g.) was added to the cooled solution, followed by 51 g. of ethyl α-bromobutyrate. The reaction mixture was kept at a gentle reflux until the reaction toward a moist litmus paper became neutral (approximately 4 hours). The ethanol was then evaporated *in vacuo* and the residue was taken up in 200 ml. of methylene chloride, washed with two 100-ml. portions of water, dried over sodium sulfate and the solvent was evaporated. A light brown, free flowing liquid was obtained (48 g.) which was distilled from a Vigreux column. Fraction 2, which has the correct boiling point, was used for the next steps without further purification.

	Temperature, °C.				
Fract.	Oil bath	Jacket	Dist.	Mm.	Wt., g.
1	130–150	65–80	70–110	28	19.0
2	210	142	152–156	25	20.7

Residue: dark brown tar 9 g.

(b) **Ethylsuccinic Acid.**—Ethyl α-ethyl-α′-acetyl-succinate (20.7 g., fract. 2) was added dropwise with stirring to a solution of 75 g. of potassium hydroxide in 35 ml. of water at 100°. After completed addition of the compound, stirring was continued for one hour. The brown solution was cooled in an ice-bath and then diluted with two volumes of water. The *p*H was adjusted to 8 with concd. hydrochloric acid and extracted with two 250-ml. portions of ether. The solvent layer was dried over sodium sulfate and evaporated to dryness. The residue (60 mg.) consisted of a yellow oil.

The aqueous layer was adjusted to *p*H 1.5 with concd. hydrochloric acid and extracted with two 350-ml. portions of methylene chloride. The solvent layer was dried over sodium sulfate and evaporated, giving 3.9 g. of an oily material, which crystallized on standing at room temperature. All attempts to crystallize ethylsuccinic acid from this residue met with failure.

Above residue was dissolved in 200 ml. of concd. nitric acid and refluxed for six hours. The acid was removed by distillation *in vacuo*, and evaporation with two 100-ml. portions of water and two 100-ml. portions of benzene–ethanol (1:1). The residue (1.5 g.) was crystallized from methyl isobutyl ketone, giving 560 mg. of colorless prisms, m.p. 95–97°.

This acid was identical with the dicarboxylic acid obtained from tetrahydro-PA-147 in melting point (95–97°), mixed melting point (95–97°), infrared spectrum and paper chromatographic migration. *Anal.* Calcd. for $C_6H_{10}O_4$: C, 49.30; H, 6.89. Found: C, 49.15; H, 6.82; $[\alpha]D$ 0 ± 2° (*c* 2 in MeOH).

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