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181. Akira Miyake: δ-Hydroxy-γ-oxo-L-norvaline, a New Antitubercular Antibiotic. (3). Structural Analogs; their Structure and Antitubercular Actitvity.*2

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As shown in the preceding paper,*2 HON is a relatively low-molecular amino acid having a carbonyl structure. HON exhibits activity only against pathogenic tubercle bacilli and shows no activity against pathogenic bacteria, fungi, or yeast.[1] It exhibits growth inhibiting activity against human-type tubercle bacilli only at a higher concentration than other antitubercular agents and it is equally active against the strains resistant to other common antitubercular agents as well as against those sensitive to them. The fact that these strains show no cross resistance between HON and other antitubercular agents shows that the mode of action of HON is quite different from that of other antitubercular agents.

Studies were made on the action of such anti-tumor amino acids as azaserine,[2,3] 5-oxo-6-diazo-L-norleucine,[4,5] and alazopeptin[6] on the metabolism of tumor cells and many interesting findings were obtained. Many amino acid homologs were synthesized as antimetabolites common to pathogenic bacteria and tested. As HON is active only against pathogenic tubercle bacilli, elucidation of the mode of action of this compound would make it possible to clear up the unique metabolic process of pathogenic tubercle bacilli. For this purpose, biochemical observation on the metabolic processes of microbes in detail and interpretation of the result obtained by combination doses with other antitubercular agents are thought to be useful.

Attempt was made to find the mode of action of HON by examining the change of its action against microbes by a change in its chemical structure. The points in question are 1) the optical configuration of the carbon at α-position, 2) the carbonyl at γ-position, 3) the hydroxyl group at δ-position, 4) simultaneous exchange of the carbonyl at γ-position and hydroxyl at δ-position, and 5) exchange of carboxyl and amino groups. As samples, 16 homologs were synthesized and tested. The results are shown in Table I.

1) Stereochemical Configuration of the Carbon at α-Position

Human-type *Mycobacterium tuberculosis* H 37 Rv was used as the test organism. The *in vitro* activity was tested in the Kirchner medium. DL-HON was half as active as L-HON. This showed that stereochemical configuration was essential to the antituberculosis activity of HON. The fact that L-HON and naturally occurring L-aspartic acid have the same optical configuration in the carbon atom at α-position and that the activity of L-HON was antagonized by a large amount of L-aspartic acid[1] showed that L-HON gave some effect on the test organism in the course of its utilization of L-aspartic acid. The correlation between the optical configuration and antibiotic activity was seen in the

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*2 This constitutes Part XXX of a series entitled "Studies on Antibiotics" by S. Tatsuoka. Part XXIX: This Bulletin, 8, 1074(1960).

1) K. Kanazawa, *et al.*: Am. Rev. Respiratory Diseases, **81**, 924(1960).
2) M. Potter, *et al.*: Proc. Am. Assoc. Cancer Research, **2**, 140(1956).
3) L. L. Bennett, *et al.*: Arch. Biochem. Biophys., **64**, 423(1956).
4) G. S. Tarnonski, *et al.*: Cancer Research, **17**, 1033(1957).
5) J. A. Jacquez, *et al.*: Proc. Soc. Exptl. Biol. Med., **99**, 611(1958).
6) S. E. DeVoe, *et al.*: Antibiotics Annual, 1951~1957, 730.

TABLE I.

No.	Sample	Min. growth-inhibitory concn. (γ/cc.) against *M. Tuberculosis* H 37 Rv (Kirchner medium)* at 14 days
1	L-HON	20
1	DL-HON	50
2	INAH-L-HON condensate	0.25 50 (INAH-resistant)
2	CH_2-CH-CH_2-CH-COOH OH OH NH_2 (L)	200
3	CH_2-CO-CH_2-CH-COOH SC_2H_5 NH_2 (DL)	200
3	CH_2-CO-CH_2-CH-COOH Cl NH_2 (DL)	200
3	CH_3-CO-CH_2-CH-COOH NH_2 (DL)	200
3	CH_2-CO-CH_2-CH-COOH NH_2-HCl NH_2 (DL)	200
3	 NH_2 (DL)	200
4	H_5C_2S-CH_2-C-CH_2-CH-COOH H_5C_2S SC_2H_5 NH_2 (DL)	200
4	CH=C-CH_2-CH-COOH S N NH_2 C NH_2 (DL)	200
4	CH=C-CH_2-CH-COOH NH N NH_2 C NH_2 (DL)	200
4	CH=C-CH_2-CH-COOH N NH NH_2 C SH (DL)	200
4	CH_2=CH-CH_2-CH-COOH NH_2 (DL)	200
4	CH_2=CH-CH_2-CH-COOH $NHCOC_6H_5$ (DL)	200
5	CH_2-CO-CH_2-CH-$COOCH_3$ OH NH_2 (L)	200
5	CH_2-CO-CH_2-CH-COOH OH $NHCOC_6H_5$ (L)	200

* Concentration of the H 37 Rv strain in this medium was 0.1 mg./5 cc. and the minimal inhibitory concentration of INAH and streptomycin was estimated as 0.1 γ/cc. and 5 γ/cc., respectively.

case of chloramphenicol[7] and some kinds of antibiotics of peptide type.[8]

2) Carbonyl Group at γ-Position

Condensation compound of INAH and L-HON, and γ,δ-dihydroxy-L-norvaline were tested *in vitro*. INAH-L-HON exhibited strong activity against INAH-sensitive *M. tuberculosis* H 37 Rv, but was half as active as L-HON against the INAH-resistant H 37 Rv strain. This fact, considered with the ratio of molecular weights of INAH and L-HON

7) J. Controulis, *et al.*: J. Am. Chem. Soc., **71**, 2463(1949).
8) B. F. Erlanger, *et al.*: Nature, **174**. 840(1954).

(137 : 147), showed that INAH-L-HON had not lost the activity of the original compounds and it separated into the original compounds[9,10] *in vitro* like the Schiff bases of INAH with acetone, glucose, or fructose. On the other hand, γ,δ-dihydroxy-L-norvaline showed no activity. The activity of the two compounds tested revealed that the γ-carbonyl group in L-HON was essential to its antituberculosis activity.

3) Hydroxyl Group at δ-Position

The hydroxyl group was exchanged with thiol, chlorine, hydrogen, primary amine, or diazo group and their *in vitro* activity was tested. All of these compounds showed no activity. δ-Ethylthio-γ-oxo-DL-norvaline was synthesized as a homolog of S-ethylcysteine which was twice as active as pyrazinamide against *M. tuberculosis* H 37 Rv in animal test, several times stronger than PAS as well as INAH.[11] δ-Diazo-γ-oxo-DL-norvaline was synthesized as an analog of azaserine and 6-diazo-5-oxo-L-norleucine. From the *in vitro* activity, it is concluded that the hydroxymethyl group at δ-position is essential to the activity.

4) Simultaneous Exchange of Carbonyl Group at γ-Position and Hydroxyl Group at δ-Position with Other Groups

Six compounds were tested *in vitro*, but none of them showed any activity. The result was in accord with those described in (2) and (3). β-(2-Amino-4-thiazolyl)alanine was synthesized as a homolog of 4-thiazolylalanine which was active against *Escherichia coli*.[12]

5) Change of Carbonyl and Amino Groups

The methyl ester of L-HON and N-benzoyl-L-HON were tested *in vitro* and they showed no activity. The free carbonyl and amino group were essential to the activity. Esters of penicillin were reported to be active in the animal body where the esters were hydrolyzed to free penicillin by induced esterase and they remained inactive in the animal body where no esterase was present.[13~15]

The combinations of L-HON with streptomycin and dihydrostreptomycin were tested *in vitro*, but none of them showed synergistic activity. When L-HON was administered to mice and guinea pigs which were infected with human tubercle bacilli, it showed a curative effect.[1]

Experimental*3

Condensation Compound of L-HON with INAH—To a solution of 650 mg. of INAH dissolved in 5 cc. of H_2O with stirring, 800 mg. of L-HON was added and the resultant clear solution was kept in an ice-box overnight, whereupon colorless crystals separated out. Recrystallization from hot H_2O gave colorless prisms., m.p. 190° (decomp.), insoluble in organic solvents and soluble in hot H_2O. *Anal.* Calcd. for $C_{11}H_{14}O_4N$: C, 49.62; H, 5.30; N, 21.04. Found : C, 49.22; H, 5.28; N, 20.87.

γ,δ-Dihydroxy-L-norvaline—Prepared as reported in the preceding paper[16] as (II).

δ-Ethylthio-γ-oxo-DL-norvaline—To a solution of 0.3 g. of metallic Na dissolved in 30 cc. of EtOH, 1 g. of EtSH was added under cooling and the solution was evaporated *in vacuo* to dryness. The residue was again dissolved in 10 cc. of EtOH and added to 3.5 g. of ethyl ester of N-acetyl-α-ethoxycarbonyl-δ-bromo-γ-oxo-DL-norvaline suspended in 7 cc. of EtOH under ice-cooling. The reaction proceeded exothermally and NaBr separated out. After standing overnight, the reaction mixture was heated for 0.5 hr. After cooling the mixture, H_2O was added to dissolve the precipitated NaBr and EtOH was evaporated. The resultant aqueous solution was extracted with AcOEt, the AcOEt

*3 All m.p.s are not corrected.

9) H. H. Fox, *et al.* : Science, **116**, 129(1952).
10) *Idem* : Am. Rev. Tuberc., **65**, 649(1952).
11) H. O. Brown, *et al.* : J. Am. Chem. Soc., **76**, 3860(1954).
12) E. M. Lansford, Jr., *et al.* : Arch. Biochem. Biophys., **38**, 34(1952).
13) K. Meyer, *et al.* : Science, **97**, 205(1943).
14) *Idem* : Proc. Soc. Exptl. Biol. Med., **53**, 100(1943).
15) F. H. Carpenter, *et al.* : J. Am. Chem. Soc., **70**, 2964(1948).
16) Part (1). A. Miyake : This Bulletin, 8, 1071(1960).

solution was washed with H_2O, and dehydrated over Na_2SO_4. Removal of AcOEt gave a light orange oil. Addition of Et_2O and keeping in a cold chamber gave crystals of the ethyl ester of N-acetyl-α-ethoxycabonyl-δ-ethylthio-γ-oxo-DL-norvaline. Recrystallization from EtOH gave pure plates, m.p. 84~85°. Yield, 2.5 g. (76%). *Anal.* Calcd. for $C_{14}H_{23}O_6NS$: C, 50.43; H, 6.96; N, 4.20. Found : C, 50.33; H, 6.94; N, 4.11.

A solution of 1.7 g. of this compound dissolved in 29 cc. of 10% HCl was heated for 2 hr., whereby evolution of CO_2 and dissolution of the oily starting material gradually occurred and the solution became light yellow. Evaporation of HCl solution gave an oil, which was dissolved in 5 cc. of water, decolorized with activated charcoal, and the pH was adjusted to 3.0 with 10% NH_4OH, when a colorless precipitate separated out. The precipitate was collected and washed with H_2O. Recrystallization from hot H_2O gave 800 mg. of crystals, m.p. 156° (decomp.), soluble in hot H_2O, dil. acid, and dil. alkali, and sparingly soluble in cold H_2O and alcohols. Yield, 84%. *Anal.* Calcd. for $C_7H_{13}O_3NS$: C, 43.96; H, 6.85; N, 7.32. Found : C, 44.02; H, 6.92; N, 7.12.

δ-Chloro-γ-oxo-DL-norvaline—A solution of 1 g. of the ethyl ester of N-acetyl-δ-chloro-γ-oxo-DL-norvaline dissolved in 10 cc. of 10% HCl was heated on a boiling water bath for 2 hr. After cool, the solution was evaporated to dryness. The residue was dissolved in 2 cc. of H_2O, decolorized with activated charcoal, and neutralized with pyridine, when crystals separated out. Recrystallization from hot H_2O gave 300 mg. of colorless needles, which showed no definite m.p., soluble in hot and cold H_2O up to 10 mg./cc. and insoluble in organic sclvents. Yield, 43%. *Anal.* Calcd. for $C_5H_8O_3NCl$: C, 36.27; H, 4.87; N, 8.46; Cl, 21.41. Found : C, 36.39; H, 4 83; N, 8.32; Cl, 20.87.

γ-Oxo-DL-norvaline—Prepared as reported in the preceding paper[17] as (Ⅷ). Easily soluble in water and difficultly soluble in Me_2CO.

δ-Amino-γ-oxo-DL-norvaline Monohydrochloride—Synthesized according to the method of Harrington, *et al.*[18] It darkens at 180° but shows no definite m.p. Easily soluble in H_2O. *Anal.* Calcd. for $C_5H_{11}O_3N_2Cl$: C, 32.88; H, 6.07; N, 15.34. Found : C, 32.74; H, 6.32; N, 14.43.

δ-Diazo-γ-oxo-DL-norvaline—This compound was synthesized by the Linschitz procedure,[19] and the impurities in the reaction mixture were removed by passing through a column of activated charcoal. The Ninhydrin-positive fractions of the eluates were collected. Drying under freezing conditions gave a light yellow hygroscopic powder, which was used as a sample for microbiological assay.

δ,γ,γ-Tris(ethylthio)-DL-norvaline—A solution of 400 mg. of DL-HON in 1 cc. of conc. HCl and 6 cc. of EtSH was stirred for 2 days at room temperature and the solution was evaporated to dryness. The residue was dissolved in 5 cc. of H_2O and the solution was neutralized with pyridine. Recrystallization of the colorless precipitate from hot H_2O gave colorless needles, m.p. 201° (decomp.), sparingly soluble in cold H_2O and organic solvents, and soluble in hot H_2O. *Anal.* Calcd. for $C_{11}H_{23}O_2NS_3$: C, 44.41; H, 7.79; N, 4.71. Found : C, 44.63; H, 8.12; N, 4.38.

β-(2-Amino-5-thiazolyl)alanine Monohydrochloride—A solution of 7 g. of the ethyl ester of N-acetyl-α-ethoxycarbonyl-δ-bromo-γ-oxo-DL-norvaline in 21 cc. of EtOH and 1.6 g. of thiourea was heated for 3 hr., and EtOH was distilled off to leave a yellow oil. Addition of Et_2O gave crystals, which were dissolved in H_2O and decolorized with activated charcoal. Adjustment of the pH to 7.0 with dil. NH_4OH gave 5 g. of light yellow prisms, m.p. 150°. Yield, 76%. *Anal.* Calcd. for $C_{13}H_{19}O_5N_3S$ (Ethyl ester of N-acetyl-α-(ethoxycarbonyl)-β-(2-amino-4-thiazolyl)alanine) : C, 47.40; H, 5.82; N, 12.76. Found : C, 47.04; H, 5.70; N, 12.98.

A solution of 3.5 g. of this product dissolved in 70 cc. of 10% HCl was heated for 2 hr. Evaporation of HCl gave colorless crystals, which were dissolved in a small amount of H_2O and neutralized with pyridine. Addition of EtOH gave a colorless precipitate which was recrystallized from hydr. EtOH to colorless needles, m.p. 213° (decomp.), soluble in cold water, easily soluble in hot H_2O, and insoluble in EtOH, *Anal.* Calcd. for $C_6H_{10}O_2SCl\cdot H_2O$: C, 29.81; H, 5.00; N, 17.39. Found : C, 29.94; H, 4 97; N, 16.77.

β-(2-Amino-5-imidazolyl)alanine—Synthesized according to the method of Diemair, *et al.*[20]

2-Mercaptohistidine—Synthesized according to the method of Hegedüs.[21] Did not melt below 300°. Insoluble in organic solvents and soluble in H_2O at a pH below 4.0 or above 7.0.

2-Amino-4-pentenoic Acid—In 150 cc. of EtOH, 6.9 g. of metallic Na was dissolved and 51 g. of ethyl acetamidocyanoacetate was added with stirring at 60°. To the solution, 41.8 g. of allyl bromide was added dropwise at 30~40° and kept standing for 40 hr. at room temperature. To complete the reaction the mixture was heated at 50° for 1 hr. The precipitated NaBr was dissolved by addition

17) Part (2). A. Miyake : *Ibid.*, **8**, 1074(1960).
18) C. R. Harrington, *et al.* : Biochem. J., **27**, 338(1933).
19) Y. Linschitz, *et al.* : J. Chem. Soc., **1959**, 1308.
20) W. Diemair, *et al.* : Chem. Ber., **71**, 2492(1938).
21) B. Hegedüs : Helv. Chim. Acta, **38**, 22(1955).

of H_2O, EtOH was removed by evaporation, and the resultant aqueous solution was extracted with AcOEt. The AcOEt solution was washed with H_2O, dried over Na_2SO_4, and evaporated *in vacuo* to crystals of ethyl 2-acetamido-2-cyanopentanoate, which were washed with Et_2O, m.p. 77~78°. Yield, 49 g. (78%).

A solution of 17.5 g. of this product dissolved in 18 cc. of conc. H_2SO_4 and 248 cc. of H_2O was boiled for 4 hr., cooled, a solution of 101 g. of $Ba(OH)_2$ dissolved in 250 cc. of H_2O was added, and heated again in a boiling water bath for 2 hr. The resultant $BaSO_4$ was filtered off and the filtrate was evaporated *in vacuo*. Recrystallization of residual crystals from EtOH gave 6.1 g. of needles, m.p. 246° (decomp.), easily soluble in H_2O and sparingly soluble in organic solvents. Yield, 63.5%. *Anal.* Calcd. for $C_5H_9O_2N$; C, 52.16; H, 7.88; N, 12.17. Found : C, 52.31; H, 7.72; N, 12 10.

2-Benzamido-2-pentenoic Acid—Benzoylation of 2-amino-4-pentenoic acid was carried out with BzCl in the presence of alkali, m.p. 109~110°. *Anal.* Calcd. for $C_{12}H_{13}O_3N$: C, 65.74; H, 5.98; N, 6.39. Found : C, 65.76; H, 5.96; N, 6.44.

Methyl Ester Hydrochloride of L-HON—Esterification was carried out with MeOH-HCl. After evaporation of MeOH-HCl, the reaction product was dried over NaOH and its microbiological activity was tested. Easily soluble in H_2O.

N-Benzoyl-L-HON—Prepared as reported in the preceding paper.[16]

Discussion

The mode of action of HON against pathogenic tubercle bacilli was investigated by testing the *in vitro* antituberculosis activity of 16 of its homologs. These 16 compounds were somewhat different from L-HON in the functional groups and had no *in vitro* activity. The fact suggests that every part of the constituents of L-HON is essential to its antituberculosis activity. A group of compounds which have a relatively small molecule and microbiologically active only against tubercle bacilli and inactive against common bacteria, fungi, and yeast are known which include cycloserine,[22] actithiazic acid,[23] tryptophan,[24] INAH,[25] pirazinamide,[26] and S-ethylcysteine.[11] They all lose their antituberculosis activity by a slight change in their chemical structure. L-HON is considered to be a member of this group. To elucidate the activity of these compounds, it is better to investigate the metabolic process of tubercle bacilli and to find some relationship between the metabolic products and chemicals. This method was already employed on some antibiotics.[27~29]

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Summary

Sixteen homologs of L-HON were synthesized and their *in vitro* activity against *M. tuberculosis* was tested. From such results, L-HON was classified as a member of the group which is relatively low in molecular weight and active only against *M. tuberculosis*.

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22) F. A. Kuehl, *et al.* : J. Am. Chem. Soc., **77**, 2344(1955).
23) I. A. Solomons, *et al.* : *Ibid.*, **74**, 2046(1952).
24) M. M. A. Kader, *et al.* : Experientia, **14**, 455(1958).
25) H. H. Fox, *et al.* : J. Org. Chem., **17**, 542(1952).
26) *Idem.* : J. Am. Med. Assoc., **148**, 1034(1952).
27) M. G. Harrington : J. Gen. Microbiol., **18**, 767(1958).
28) S. H. G. Allen, *et al.* : Seience, **127**, 1341(1958).
29) I. Watanabe, *et al.* : Nature, **181**, 1127(1958).