Structure of Enteromycin. II. Derivatives and Products in Various Reactions of Dimethoxyenteromycin*

By Kōmei MIZUNO

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Dehydroxytetrahydrodemethoxyenteromycin (XV)[1] obtained by the catalytic reduction of enteromycin (I) or demethoxyenteromycin (XII) seems to have the same skeleton as that of the latter two. And from the course of changes of I, XII appears to be in the closest relation with I. In the present work, the structure of XV was established, and the functional group of XII and its derivatives were investigated in detail.

The infrared spectrum (Fig. 1) of XV exhibited the absorptions of NH, COOH and $COO^- \cdot NH_3^+$ (2100, 1620, 1370 cm^{-1}), but its ultraviolet spectrum showed no specific absorption at the long wave region beyond λ 220 mμ. Also, an amino group was detected in this compound by azotometry. Since the compound was considered to be a saturated peptide in view of the above facts, it was hydrolyzed with hydrochloric acid and the product was subjected to paper chromatography, detecting glycine (XXIII) and β-alanine (XXV). The DNP derivatives[2] of both amino acids were also confirmed by paper chromatography. Hydrolysis of the DNP derivative of XV produced no DNP-amino acid, but since β-alanine was obtained as a free amino acid, XV was.

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1) K. Mizuno, This Bulletin, **34**, 1419 (1961).

2) W. W. Bromer, *J. Am. Chem. Soc.*, **79**, 2794 (1957).

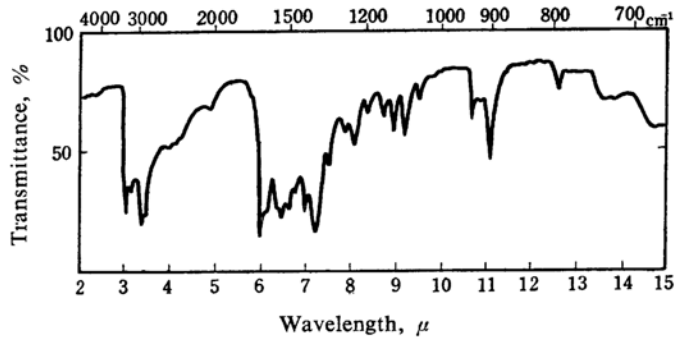
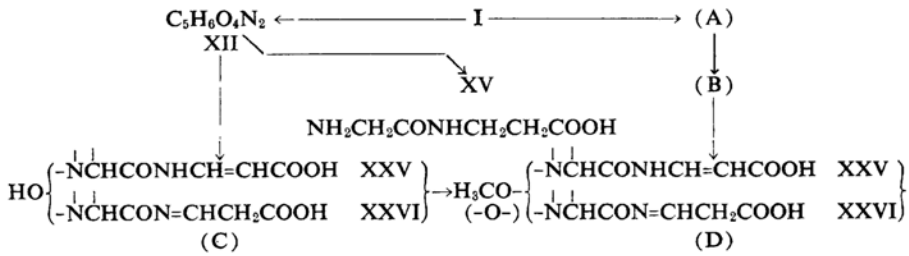


Fig. 1. Infrared absorption spectrum of dehydroxytetrahydrodemethoxyenteromycin (glycyl-β-alanine) (XV).



assumed to be glycyl-β-alanine. So *N*-formylglycine was condensed with β-alanine ethyl ester in the presence of dicyclohexylcarbodiimide and the resulting *N*-formylglycyl-β-alanine ethyl ester was hydrolyzed with sodium hydroxide to give *N*-formyl-β-alanine. The product was deformylated by the method of Miyamoto[3] et al., and the infrared spectrum, melting point (decomp.), and R_f value in paper chromatography of the resultant product were found to be in full accord with those of XV. From these results the formula C was presumed for XII and the formula D for I.

The two acidic groups[1] in XII were confirmed by acetylation and methylation of XII. The product was obtained as prisms (XVIII) [m. p. 181°C (decomp.), $C_7H_8O_5N_2$] negative to the Barton reaction, and since its infrared spectrum (Fig. 2) showed a clear absorption of >NO–COR[4] (1775 cm^{-1}), it was called *O*-acetyldemethoxyenteromycin.

Reaction of XII with diazomethane products, one of which (XIX) [m. p. 159°C, $C_7H_{10}O_4N_2$] was obtained as prisms and had a methoxy group and the other (XX) [m. p. 137°C, $C_7H_{10}O_4N_2$] was yielded as needles and had two methoxy groups. As the infrared spectra (Figs. 3 and 4) of both products showed the absorption of COOR (1710 cm^{-1}), they were called *N*-methyldemethoxyenteromycin methyl ester and *O*-methyldemethoxyenteromycin methyl ester, respectively.

From the formation of these compounds, it is obvious that XII representable by the formula C has a phenolic hydroxyl group in addition to a free carboxyl group. Decomposition of XII with hydrochloric acid produced acetaldehyde (V), carbon dioxide (VI), ammonia (VII), and glyoxylic acid (VIII), but not formaldehyde, and boiling of XII with water also afforded no formaldehyde, and therefore the structure of XII would be almost the same as that of I, except the special functional[1] group including a methoxy group. Amidation of XII gave a compound (XXI) m. p. 168°C (decomp.), $C_5H_9O_4N_3$] as needles, which positive to the ninhydrin reaction and reverted to the original compound by treatment with heat, water, or diluted acetic acid. And as the infrared spectrum (Fig. 5) of this product exhibited the absorption characteristic of amides, it was called demethoxyenteromycin amide. Also similarity of the ultraviolet spectrum of the product to that of XII suggested that it has a structure considerably different from that of entromycin amide (XIV).

Finally the course of the change of XII to XV by catalytic reduction was investigated in detail. Namely, catalytic reduction of XII on palladium-carbon first yielded prisms (XXII) [m. p. 203°C (decomp.), $C_5H_8O_3N_2$] which were sparingly soluble in cold water and gave a

3) M. Miyamoto et al., *J. Pharm. Soc. Japan* (*Yakugaku Zasshi*), in press.
4) H. Bredereck et al., *Chem. Ber.*, **89**, 1533 (1956).

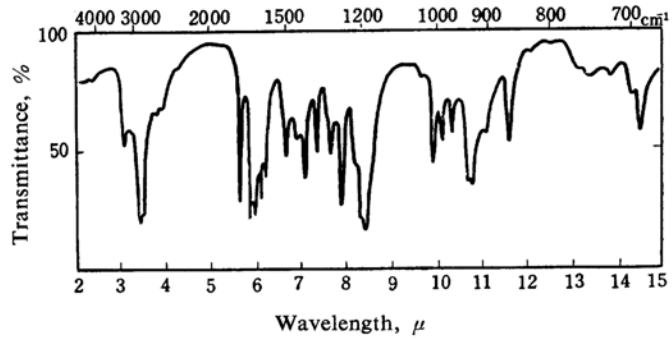


Fig. 2. Infrared absorption spectrum of *O*-acetyldemethoxyenteromycin (XVIII).

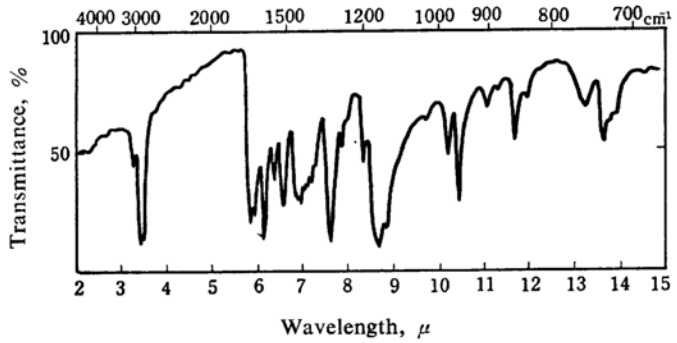


Fig. 3. Infrared absorption spectrum of *N*-methyldemethoxyenteromycin methyl ester (XIX).

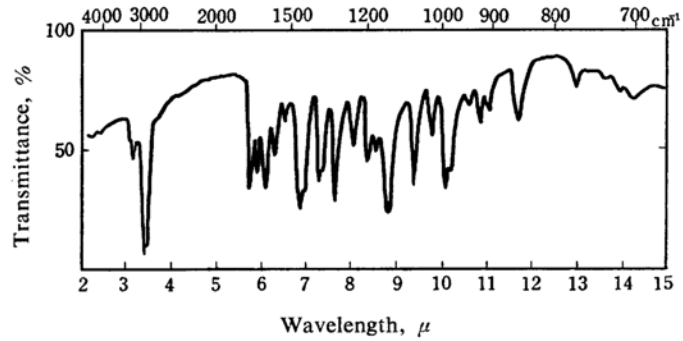


Fig. 4. Infrared absorption spectrum of *O*-methyldemethoxyenteromycin methyl ester (XX).

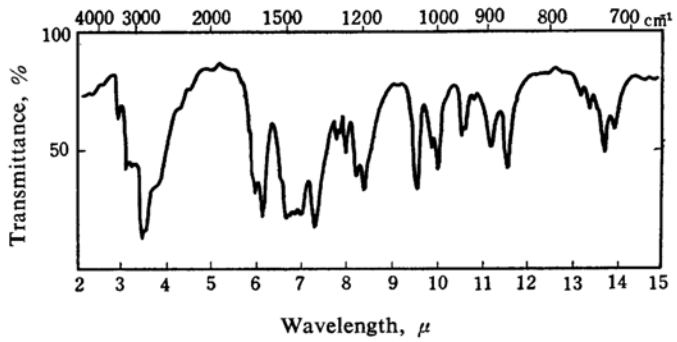


Fig. 5. Infrared absorption spectrum of demethoxyenteromycin amide (XXI).

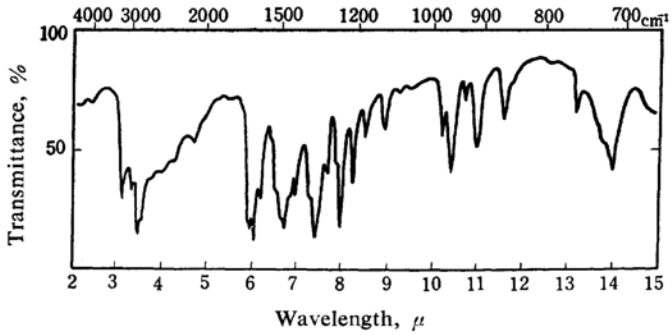


Fig. 6. Infrared absorption spectrum of dehydroxydihydrodemethoxyenteromycin (XXII).

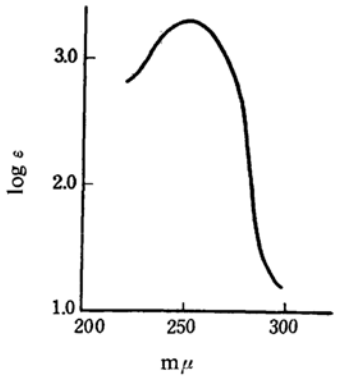
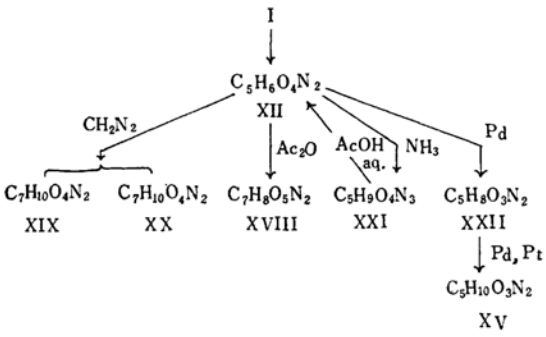


Fig. 7. Ultraviolet absorption spectrum of XXII in water.

yellow ninhydrin reaction, and further reduction resulted in the formation of glycyl-β-alanine (XV). From its molecular formula, XXII was called dehydroxydihydrodemethoxyenteromycin, and since its infrared spectrum (Fig. 6) exhibited the absorptions of NH, COOH and $COO^- \cdot NH_3^+$, it was considered to be a peptide. The ultraviolet spectrum (Fig. 7) of the compound showed a single absorption maximum (ε_{max} 2000) at λ_{max} 250 mμ.



Experimental

Hydrolysis of Dehydroxytetrahydrodemethoxyenteromycin (XV).—A solution of 10 mg. of XV in 1 cc. of 6 N hydrochloric acid was heated at 110°C for 5 hr. in a sealed tube, the air in which was replaced with nitrogen. The reaction mixture was evaporated to dryness, the residue was dissolved in 1 cc. of water, and part of the solution was subjected to paper chromatography according to the method described in Report I, giving two spots at R_f 0.42 and 0.65. The former spot colored brownish pink with ninhydrin and the latter, blue, and they were identified as glycine and β-alanine, respectively. Five tenths cc. of the above aqueous solution was adjusted to pH 8 with 30 mg. of sodium hydrogen carbonate, the mixture was allowed to react with a solution of 8 mg. of 1-fluoro-2,4-dinitrobenzene in 0.5 cc. of ethanol, and the product was extracted by the method of Bromer[2]. The product was developed in the dark on filter paper, Whatman No. 1, with the upper layer of *n*-butanol-0.1% aqueous ammonia (1 : 1), giving two yellow spots at R_f 0.28~0.33 and 0.37~0.40. They were identified as DNP-glycine and DNP-β-alanine. Each of the two spots was extracted with 4 cc. of hot water, and the absorption of the extracts was measured, finding that the two products were contained in the material in equimolecular amounts.

Hydrolysis of DNP-Dehydroxytetrahydrodemethoxyenteromycin.—To a solution of 10 mg. XV in 2 cc. of water was added a solution of 7 mg. of 1-fluoro-2,4-dinitrobenzene in 2 cc. of ethanol, followed by 20 mg. each of sodium hydrogen carbonate and sodium carbonate, and the mixture was treated as in the preceding experiment. The reaction mixture was extracted first with ether at acid pH to remove 2,4-dinitrophenol and then with isobutanol to obtain the DNP-derivative of the peptide compound. Part of the product was subjected to two dimensional paper chromatography; namely it was, developed first with the same solvent system as in the preceding experiment to detect a yellow spot at R_f 0.26, which migrated to R_f 0.26 in the second development with a solution of sodium dihydrogen phosphate (1 M) and disodium hydrogen phosphate (0.5 M) in 1 l. of water. The above DNP-derivative was hydrolyzed by heating with 0.5 cc. of 10 N hydrochloric acid at 100°C for 3 hr. and the ether soluble portion of the product was chromatographed as above, detecting the spot of 2,4-dinitrophenol at R_f 0.44~0.49 (in the first development) and 0.26 (in the second development) and the spot of 2,4-dinitroanilin at 0.85~0.95 (in the first development) and 0.00 (in the second development), but no spot of DNP-glycine was observed. The acid solution from which the ether solube portion had been separated was evaporated to dryness and the residue was chromatographed as in the preceding experiment, whereupon a spot coloring blue with ninhydrin was detected at R_f 0.65, which was identified as β-alanine.

Synthesis of Glycyl-β-alanine.—To a solution of 2.1 g. of *N*-formylglycine in 20 cc. of ethanol was added 2.4 g. of β-alanine ethyl ester, followed by

4.2 g. of dicyclohexylcarbodiimide, and the mixture was allowed to stand overnight with cooling. The separated urea derivative was filtered off, the filtrate was evaporated under reduced pressure, and the residue was dissolved in 20 cc. of ethyl acetate. The solution was filtered and the needle-like crystals, m. p. 72°C (ca. 2.5 g.) obtained from the filtrate was purified by recrystallization from ethyl acetate. The product melted at 75°C.

Found: C, 47.81; H, 7.09; N, 14.13. Calcd. for $C_8H_{14}O_4N_2$ (*N*-formylglycyl-β-alanine ethyl ester): C, 47.52; H, 6.97; N, 13.85%.

One gram of the product dissolved in 20 cc. of dioxane was hydrolyzed by standing with 6~7 cc. of 1 N sodium hydroxide at room temperature, the reaction mixture was passed through a column of Amberlite IR-120 (H-form) to remove sodium ion, and the effluent was concentrated under diminished pressure to give white crystals, which were recrystallized from ethanol in prisms, m. p. 121°C (600 mg.).

Found: C, 41.68; H, 5.81; N, 15.84. Calcd. for $C_6H_6O_4N_2$ (*N*-formylglycyl-β-alanine): C, 41.38; H, 5.79; N, 16.09%.

To a solution of 450 mg. of the product in 2 cc. of methanol was added 0.25 cc. of hydrazine hydrate (80%), and the mixture was heated for 5 hr. on the water bath. The resulting crystals were washed with methanol and recrystallized from aqueous methanol, yielding 250 mg. of prisms, m. p. 225°C (decomp.). The infrared spectrum, R_f value in paper chromatography and coloration in the ninhydrin reaction of the product were in complete agreement with those of XV described in Report I.

Found: C, 40.75; H, 7.10; N, 18.84. Calcd. $C_5H_{10}O_3N_2$ (glycyl-β-alanine): C, 41.09; H, 6.90; N, 19.17%.

***O*-Acetyldemethoxyenteromycin (XVIII).**—To a solution of 300 mg. of demethoxyenteromycin (XII) in 15 cc. of hot water was added dropwise 2 cc. of acetic anhydride at 55~60°C over a period of about 15 min. with vigorous stirring. The mixture once became clear, but white crystals m. p. 180°C (decomp.) (ca. 320 mg.), gradually separated there from, which were recrystallized from methanol in prisms, m. p. 181°C (250 mg.). The product is negative to the ferric chloride-potassium ferricyanide reaction and resists ester exchange with methanol and decomposes when dried at a temperature over 70°C in vacuo.

Found: C, 42.33; H, 4.33; N, 13.97; $-COCH_3$, 20.50. Calcd. for $C_7H_8O_5N_2$: C, 42.00; H, 4.03; N, 14.00; $-COCH_3$, 21.50%.

***N*-Methyldemethoxyenteromycin Methyl Ester (XIX).**—To a solution of 600 mg. of XII in 30 cc. of ethanol was added a solution of diazomethane (produced from 7 g. of nitrosomethylurea) in 80 cc. of ether and the mixture was left standing at room temperature for 10 hr. The solvent was distilled off and the resulting white crystals, m. p. 120°C (150 mg.) were recrystallized from 1.5 cc. of methanol in needles (XIX), m. p. 159°C (ca. 60 mg.). This product is negative to the ferric chloride-potassium ferricyanide reaction.

Found: C, 45.16; H, 5.21; N, 15.22; $O-CH_3$, 16.38. Calcd. for $C_7H_{10}O_4N_2$: C, 45.16; H, 5.41; N, 15.05; $O-CH_3$, 16.67%.

***O*-Methyldemethoxyenteromycin Methyl Ester (XX).**—The mother liquor from XIX was concentrated to 0.7 cc. and left standing, separating 70 mg. of needles, which after crystallization from methanol melted at 137°C. Like XIX, the product is negative to the phenolic OH reaction, and its molecular weight is 180±20 when measured by the Rast method, using camphor as solvent.

Found: C, 45.15; H, 5.45; N, 15.04; $O-CH_3$, 32.45. Calcd. for $C_7H_{10}O_4N_2$: C, 45.16; H, 5.41; N, 15.05; $O-CH_3$, 33.30%.

Decomposition of Demethoxyenteromycin with Hydrochloric Acid.—According to the method described in Report I, a mixture of 100 mg. of XII and 3 cc. of 1 N hydrochloric acid was heated in the atmosphere of nitrogen at 100°C for about one hour in flask A, which was connected with vessel B containing pure water and vessel C containing baryta water. The reaction mixture changed from brown to redish brown and plenty of barium carbonate deposited. After the reaction, the aldehyde in the vessel B was converted to the 2,4-DNPH derivative with a solution of 2,4-dinitrophenylhydrazine in hydrochloric acid, and the product was chromatographed as before, detecting a single spot of the 2,4-DNPH of acetaldehyde at R_f 0.38.

A solution of 2,4-dinitrophenylhydrazine in hydrochloric acid was added to one half of the contents of the flask A, the resulting 2,4-DNPH derivative was extracted with ethyl acetate, and the extract was shaken with sodium hydrogen carbonate solution. The aqueous layer was made acid and extracted again with ethyl acetate, and the extract, after washing with water and drying, was concentrated under reduced pressure to give yellow crystals, which on paper chromatography exhibited the spot of the 2,4-DNPH derivative of glyoxylic acid at R_f 0.42. The remaining half of the contents of the flask A was made alkaline and steam-distilled, and the distillate neutralized with hydrochloric acid was evaporated to dryness, affording white crystals, which, when paper chromatographed as described in Report I, gave the spot of ammonium chloride at R_f 0.14.

Demethoxyenteromycin Amide (XXI).—An amount of 500 mg. of XII was dissolved in 100 cc. of methanol saturated with ammonia and the solution, after standing overnight, was concentrated in the atmosphere of nitrogen at low temperature under reduced pressure, giving about 200 mg. of a residue which became light yellow prisms melting at 168°C (decomp.) after washing with a mixture of acetone and ethanol. The same treatment of the mother liquor yielded 250 mg. of unchanged XII. The product, m. p. 168°, colors blue with ninhydrin and is positive to the ferric chloride-potassium ferricyanide reaction. It produces XII quantitatively when treated with diluted acetic acid or hot water.

Found: C, 34.54; H, 5.49; N, 23.93. Calcd. for $C_5H_9O_4N_3$: C, 34.28; H, 5.18; N, 23.99%.

Dehydroxydihydrodemethoxyenteromycin (XXII).—A 200 mg. portion of XII dissolved in 20 cc. of pure methanol was reduced on 150 mg. of palladium

carbon (1 : 10) until about 2 mol. of hydrogen was absorbed. The catalyst was filtered and washed with hot water, the combined washing and filtrate were concentrated under reduced pressure, and methanol was added to the concentrate, giving about 150 mg. of crystals. The product was treated with a little cold water and the insoluble portion was recrystallized from water, yielding 70 mg. of prisms, m. p. 203°C (decomp.), which colored yellow with ninhydrin and gave a spot at R_f 0.63 on paper chromatography[2].

Found: C, 41.46; H, 5.79; N, 19.48; $-NH_2$, 11.06. Calcd. for $C_5H_8O_3N_2$: C, 41.66; H, 5.59; N, 19.44; $-NH_2$, 9.86%.

Catalytic reduction of a solution of 30 mg. of XXII in 10 cc. of a mixture of glacial acetic acid and methanol on about 30 mg. of Adams platinum oxide (or palladium-carbon) afforded about 20 mg. of glycyl-β-alanine.

Summary

The structure of dehydroxytetrahydrodemethoxyenteromycin was established to be glycyl-β-alanine. Demethoxyenteromycin yielded *O*-acetyldemethoxyenteromycin by acetylation, *N*-methyl- and *O*-methyldemethoxyenteromycins by methylation, and demethoxyenteromycin amide by amidation. Catalytic reduction of demethoxyenteromycin gave first dehydroxydihydrodemethoxyenteromycin and then glycyl-β-alanine. Acid decomposition products of demethoxyenteromycin were the same those of enteromycin, except methyliodide.

Research Laboratories
Takeda Chemical Ind., Ltd.
Higashiyodogawa-ku, Osaka