PROPIOXATINS A AND B, NEW ENKEPHALINASE B INHIBITORS

II. STRUCTURAL ELUCIDATION

YOSHINORI INAOKA, SHUJI TAKAHASHI† and TAKESHI KINOSHITA††

Bio-Science Research Laboratories, †Fermentation Research Laboratories and
††Analytical and Metabolic Research Laboratories,
Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

(Received for publication May 1, 1986)

The structures of propioxatins A ($C_{17}H_{29}N_3O_6$) and B ($C_{18}H_{31}N_3O_6$), new enkephalinase B inhibitors produced by *Kitasatosporia setae* SANK 60684, were determined. Both propioxatins consist of *N*-acyl-L-prolyl-L-valine. *N*-Acyl moieties of propioxatins A and B were α-propyl and α-isobutyl succinic acid β-hydroxamic acid, respectively.

In a preceding paper[1], we reported on the taxonomy of the producing organism, isolation, characterization and biochemical properties of propioxatins A and B. The producing organism was identified as *Kitasatosporia setae* SANK 60684 and the inhibitors were characterized as *N*-acyl dipeptides containing a hydroxamic acid moiety. In the present paper, the structural determination of propioxatins A and B is described.

Propioxatins A (**1**), mp 106~110°C, and B (**2**), mp 84~90°C, are water soluble substances which have only end absorption in the UV region. The molecular weights of **1** and **2** were determined by FAB-MS as 372 $(M+H)^+$ and 386 $(M+H)^+$, respectively, and the molecular formula of the trimethyl derivatives (**3**) from **1** and (**4**) from **2** obtained by treatment with diazomethane were assigned by high resolution mass spectrometry at *m/z* 413.2530 (calcd for $C_{20}H_{35}N_3O_6$: 413.2525) and *m/z* 427.2684 (calcd for $C_{21}H_{37}N_3O_6$: 427.2682), respectively. The molecular formulas of **1** and **2** were established as $C_{17}H_{29}N_3O_6$ and $C_{18}H_{31}N_3O_6$ from elementary analysis, mass and ^{13}C NMR spectral data.

Hydrolysis of **1** and **2** with 12 N HCl - CH_3COOH (1 : 1) at 105°C for 18 hours gave 1 mol each of proline and valine. No *N*-terminal amino acids were detected by Edman degradation. The high resolution mass spectra of **3** and **4** contained the same peak at *m/z* 227.1453 originating from $C_{11}H_{19}N_2O_3$. This fragment ion supported the presence of a dipeptide of prolyl-valine methyl ester or valyl-proline methyl ester, respectively. However, valine was detected as a *C*-terminal amino acid by hydrolysis with carboxypeptidase Y of **1** and **2**. In addition, a dipeptide (**5**) identical with prolyl-valine, was obtained by mild hydrolysis with water at 60°C for 5 hours. These results suggested that the dipeptide, prolyl-valine, was present in both compounds and only the *N*-acyl groups were different in **1** and **2**. Hydrolysis of **1** with 12 N HCl - CH_3COOH (1 : 1) at 105°C for 24 hours followed by treatment with diazomethane afforded a dimethyl ester (**6**), $C_9H_{16}O_4$, *m/z* 189 $(M+H)^+$. This compound was identical with α-propylsuccinic acid dimethyl ester which was prepared by reaction of α-bromo-*n*-valeric acid dimethyl ester with malonic acid diethyl ester, then decarboxylation by reflux with HCl for 3 hours and esterification with diazomethane. Mild hydrolysis of **1** as above and neutralization with NaOH gave the sodium salt (**7**) $C_7H_{10}O_3NNa_2$, FAB-MS: $QM^+=202$, together with the dipeptide (**5**). The ^1H NMR and FAB-MS spectra of **7** were very similar to *N*-hydroxy-α-propylsuccinimide. From the above results, the structure of *N*-acyl of **1** was deduced to be α-propylsuccinic

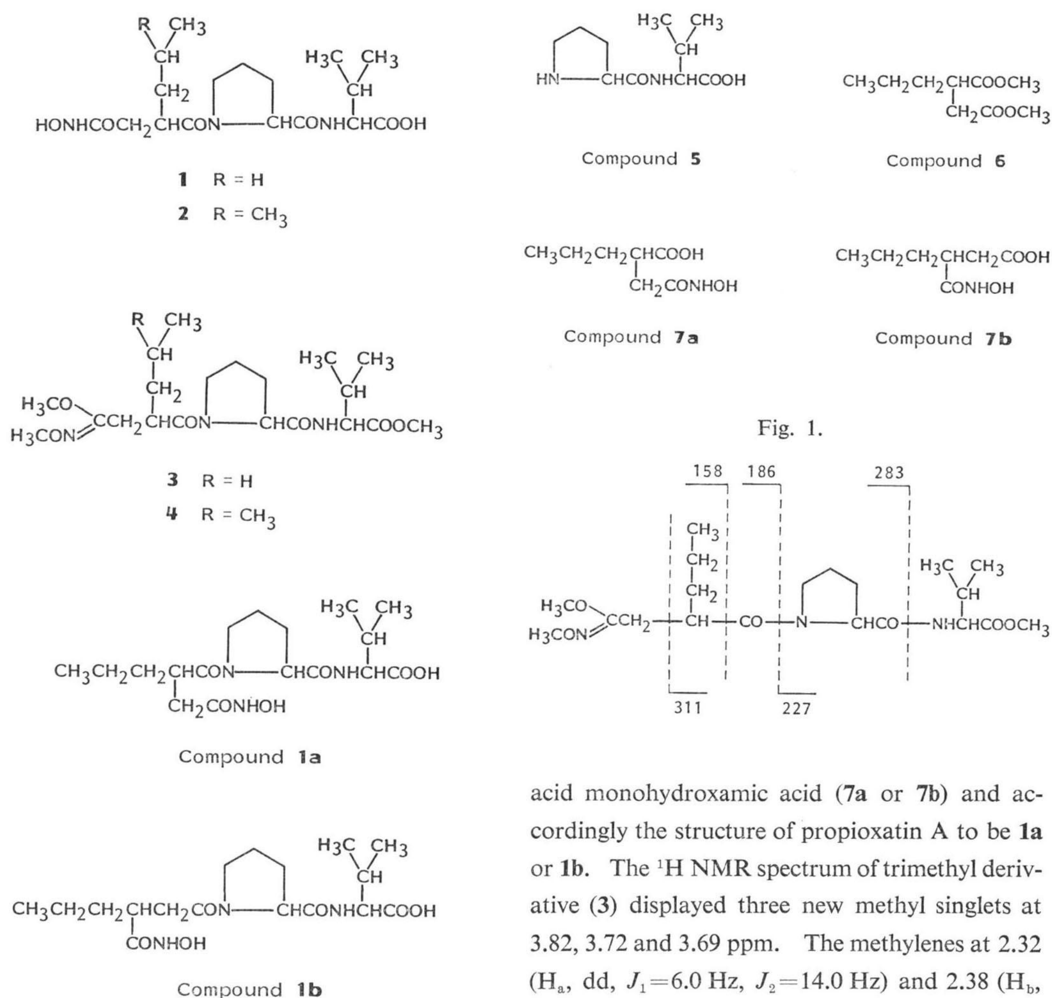

Fig. 1.

acid monohydroxamic acid (**7a** or **7b**) and accordingly the structure of propioxatin A to be **1a** or **1b**. The ^{1}H NMR spectrum of trimethyl derivative (**3**) displayed three new methyl singlets at 3.82, 3.72 and 3.69 ppm. The methylenes at 2.32 (H_a, dd, J_1=6.0 Hz, J_2=14.0 Hz) and 2.38 (H_b, dd, J_1=9.0 Hz, J_2=15.0 Hz) of **1** shifted to 2.30 (H_a, dd, J_1=16.0 Hz, J_2=3.2 Hz) and 2.88 (H_b, dd, J_1=16.0 Hz, J_2=10.0 Hz). These changes were attributed to the methylation of the hydroxamic acid. The mass spectrum of **3** showed the molecular ion at *m/z* 413 and fragment ions at *m/z* 367 (M−46, $-NHOCH_3$), 311 (M$-C_4H_8NO_2$), 283 ($C_{14}H_{23}N_2O_4$), 227 (as mentioned above), 186 (base peak, M−227) and 158 (base peak, M−227−CO). These data supported the sequence (**7a**)-proline-valine. The structure of propioxatin A was thus deduced as **1a**.

N-Acyl moiety (**8**) of propioxatin B was also obtained by mild hydrolysis similarly as in the case of **1**. The ^{1}N HMR spectrum of **8** was similar to that of **7** except for the presence of an isobutyl group instead of the propyl group. Comparing the molecular formula, ^{1}H NMR and ^{13}C NMR spectra of **2** with that of **1**, the structure of propioxatin B was determined as structure **2**.

The configurations of proline and valine in **1** and **2** were assigned on the basis of enzyme reactions and HPLC of *N*-carboxy-L-leucyl derivatives[2]. In the reactions of proline and valine with L- and D-amino acid oxidase, proline and valine were found to disappear only from the former reaction mixture and not from the latter when examined by an amino acid analyzer. Also, in a comparison of the retention times of authentic *N*-carboxy-L-leucyl derivatives of L- and D-proline and valine, the L forms

were faster than the D forms and the retention times of *N*-carboxy-L-leucyl derivatives of proline and valine in **1** were identical with that of the L-amino acids. The absolute configuration of *N*-acyl moiety, α-propylsuccinic acid monohydroxamic acid (**7**) was established to be *R* from X-ray analysis of synthetic propioxatin A di-*O*-benzyl derivative. Furthermore, the structure and stereochemistry of **1** were confirmed by its stereo-specific synthesis discussed in a subsequent paper.

Experimental

The amino acid analysis was carried out with a Hitachi 835 amino acid analyzer. UV spectra were measured on a Hitachi 320 spectrophotometer. NMR spectra were recorded on a Jeol GX-400 spectrometer using TMS as an internal standard. All mass spectral data were determined on a Jeol JMS-HX 100 spectrometer. Melting points are uncorrected. YMC pack was obtained from Yamamura Chemical Laboratories Co., Ltd. (Kyoto, Japan).

Treatment of Propioxatin A (1) with Diazomethane

A stirred solution of 100 mg of propioxatin A (**1**) in 10 ml of MeOH was treated in an ice-bath with excess diazomethane in ether overnight. After evaporation of the solvent, the residue was chromatographed on Sephadex LH-20 (200 ml) developed with $CHCl_3$ - EtOAc (1 : 1). The trimethyl ester (**3**) was obtained as an oily substance.

Hydrolysis of Propioxatins A (1) and B

A solution of 1 mg of propioxatin A (**1**) in 0.5 ml of 12 N HCl - CH_3COOH (1 : 1) was heated at 105°C for 18 hours in a sealed tube. The solvent was removed under reduced pressure. The amino acid analysis of the hydrolysate gave 1 mol each of proline and valine.

Hydrolysis of 1 with Carboxypeptidase Y

A solution of 1 mg of **1** in 1 ml of 50 mM sodium phosphate buffer, pH 6.5, was treated with carboxypeptidase Y at 37°C for 4 hours. Only valine was detected by amino acid analysis.

Mild Hydrolysis of 1 with H_2O

A solution of 5.8 mg of **1** in H_2O (1 ml) was heated at 60°C for 5 hours. After lyophilization, the crude powder was chromatographed on Sephadex G-10 (100 ml) and developed with the upper layer of BuOH - CH_3COOH - H_2O (4 : 1 : 5); 2 mg of the dipeptide (**5**) was secured from the ninhydrin positive fractions.

Isolation of α-Propylsuccinic Acid Dimethyl Ester (6)

Hydrolysis of 10 mg of **1** with 12 N HCl - CH_3COOH (1 : 1) was carried out by heating at 105°C for 24 hours. The hydrolysate was dissolved in 1 ml of MeOH and treated with diazomethane in ether. After evaporation of the solvent, the residue was determined by GC-MS as a propylsuccinic acid dimethyl ester.

Synthesis of α-Propylsuccinic Acid

To a stirred solution of 140 ml of EtOH containing 1.7 g of metal sodium, a solution of 11 g of malonic acid diethyl ester in 10 ml of EtOH was added dropwise at room temp. After stirring for 30 minutes, a solution of 12.1 g of α-bromo-*n*-valeric acid methyl ester in 10 ml of EtOH was added. The reaction mixture was stirred at room temp for 1 hour and then refluxed for 1.5 hours. After addition of H_2O and neutralization with HCl, the solution was concd to a small volume. The residue was acidified with HCl and extracted with EtOAc. The solvent layer was dried with Na_2SO_4, and evaporated to dryness, yielding 19 g of tricarboxylic acid ester. A solution of 19 g of the tri-ester in 200 ml of 10% NaOH of EtOH - H_2O (1 : 1) was refluxed for 2 hours. After removal of EtOH under reduced pressure, the residue was acidified with HCl and extracted with EtOAc. The solvent layer was treated in the usual way and evaporated to dryness. The residue (10 g) was dissolved in 350 ml of 5% HCl solution and refluxed for 6 hours, when NaCl was added and the mixture extracted with EtOAc to yield α-propylsuccinic acid (5.5 g), mp 92~93°C.

Isolation of α-Propylsuccinic Acid Monohydroxamic Acid (7)

Mild hydrolysis of 10 mg of **1** with H_2O was carried out as described above. From the hydrolysate 3 mg of the title compound (7) was obtained by HPLC (YMC pack, ODS, 15% CH_3CN - 0.2% TFA).

Oxidation of Proline and Valine in **1** and **2** with D- and L-Amino Acid Oxidase

Treatment of **1** or **2** (1.4 mg in 1 ml of 50 mM sodium phosphate buffer, pH 6.5) with carboxypeptidase Y followed by mild hydrolysis at 80°C for 4 hours released proline and valine. A 50 μl of this mixture was added into 450 μl of 0.1 M sodium phosphate buffer, pH 8.0, containing 0.1 unit of L- or D-amino acid oxidase and stirred at 37°C for 24 hours. Both proline and valine disappeared only when L-amino acid oxidase was used.

References

1) INAOKA, Y.; H. TAMAOKI, S. TAKAHASHI, R. ENOKITA & T. OKAZAKI: Propioxatins A and B, new enkephalinase B inhibitors. I. Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 39: 1368~1377, 1986

2) TAKAYA, T.; Y. KISHIDA & S. SAKAKIBARA: Determination of the optical purity of amino acids by high-performance liquid chromatography. J. Chromatogr. 215: 279~287, 1981