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134. Shoshiro Nakamura and Hamao Umezawa : The Structure of Bottromycin A_2, a New Component of Bottromycins[1~4]

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The main active component of bottromycins has been isolated from the fermentation broth of *Streptomyces* No. 3668-L2[1] and designated as bottromycin A_2. The former bottromycin A is designated as bottromycin A_1.[2] Isolation and characterization of bottromycin A_2 will be reported in the next paper.

Bottromycin A_1 and A_2 are closely related to bottromycin, $C_{38}H_{57\sim61}O_{7\sim8}N_7S$, isolated by Waisvisz, *et al.*[5~8] in chemical and antimicrobial properties. Bottromycin A_2 has one titrable basic group (pKa 8.1~8.3 in methanol-water=3:2) and the molecular weight is 810~840 by the potentiometric titration. The antibiotic shows negative ninhydrin reaction, negative Sanger decomposition and gives one mole of nitrogen gas by Van Slyke method. Hydrochloric acid hydrolysis of bottromycin A_2 gives each one mole of 3-methyl-3-phenyl-L-alanine,[2,6~8] 3,3-dimethyl-2-aminobutyric acid[*2,2](DMAB), L-valine, 3-(2-thiazolyl)-β-alanine,[2,6~8] *cis*-3-methyl-L-proline[2] and glycine. Acetic anhydride decomposition of bottromycin A_2 yields N-acetyl-3-methyl-3-phenylalanyl-3-(2-thiazolyl)-β-alanine methyl ester.[1,6,7]

The partial structure of bottromycin A_1 was reported in the previous communication[3] as shown in the structure 1 and structural studies of bottromycin A_2 is described in detail in this paper.

Negative ninhydrin reaction and negative Sanger decomposition of bottromycin A_2 suggest that the amino group of the N-terminal amino acid must be acylated in the antibiotic as shown in bottromycin A_1. Ozonolysis of bottromycin A_2 gives isobutylaldehyde. Therefore, pivaric acid in bottromycin A_1 replaced by Δ^1-isocaproic acid in bottromycin A_2 and the difference of one carbon atom between their molecular formulae can be attributed to the difference between pivalic acid and Δ^1-isocaproic acid.

Hydrolysis of bottromycin A_2 with 2.5*N* sodium hydroxide for 16 days at room temperature yields ammonia. The neutralization of the hydrolyzate with cation exchange resin IRC-50 (H-type) and the elution from the resin with 1*N* hydrochloric acid yield two ninhydrin positive peptides. The peptides are separated on a cellulose powder column with butanol-acetic acid-water=100:12:100 (upper phase). The first eluted peptide is hydrolyzed to 3-methyl-3-phenylalanine and 3-(2-thiazolyl)-β-alanine and the structure is reasonably posturated to be 3-methyl-3-phenylalanyl-3-(2-thiazolyl)-β-alanine by considering the acetic anhydride decomposition product of bottromycin A_2. Another peptide yields DMAB, valine, 3-methylproline and glycine by hydrochloric

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*2 This amino acid was reported to have L-configulation, but now under investigation to determine L- or D-configulation.

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7) J. M. Waisvisz, M. G. van der Hoeven, B. te Nijenhuis : *Ibid.*, 79, 4524 (1957).
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Table I. Rf Values of the PTH Amino Acids

PTH-DMAB	PTH-Val	PTH-MePro	PTH-Gly
0.66[a]	0.48[a]	0.44[a]	0.11[a]
0.71[b]	0.68[b]	0.58[b]	0.35[b]

a) Toyo Roshi No. 51 filter paper (pyridine *n*-heptane=3:7)
b) Silica Gel G (AcOEt-*n*-heptane=1:1)

acid hydrolysis. The C-terminal amino acid of the peptide is shown to be glycine by the hydrazinolysis. The phenythiohydantion (PTH) amino acids obtained from the peptide show the Rf values as shown in the Table I by the paper and thin-layer chromatographies. The first PTH amino acid from the peptide by Edman degradation[9] is identified as PTH-DMAB and the second one is shown to be PTH-valine by the paper and thin-layer chromatographies. Therefore, the structure of the tetrapeptide is elucidated to be 3,3-dimethyl-2-aminobutylyl-valyl-3-methylprolyl-glycine.

Δ^1-isocaproic acid, the tetrapeptide and 3-methyl-3-phenylalanyl-3-(2-thiazolyl)-β-alanine methyl ester are linked by an amide and peptide bonds in bottromycin A_2 and the molecular formula is calculated to be $C_{42}H_{61}O_8N_7S$ for this acylpeptide methyl ester. The formula $C_{42}H_{62}O_7N_8S$ has been established for bottromycin A_2. Then, in bottromycin A_2 one of the oxygen atoms in the acylpeptide methyl ester must be replaced by an imino group to form an amidine group with an adjacent nitrogen atom or to form an imino ether group with the methoxyl group. Existance of an amidine group in bottromycin A_2 is also suggested by the infrared spectrum (1690 cm^{-1} in $CHCl_3$),[10] the pKa value 8.1~8.3 and generation of ammonia by the alkaline hydrolysis. One mole of nitrogen gas from bottromycin A_2 by Van Slyke method can be attributed to a tautomeric form of this amidine group.

The position of the amidine group is shown by the hydrolyzed product of bottromycin A_2 with 1*N* hydrochloric acid. Two peptides are isolated from the hydrolyzate by the silica gel column chromatography. The one, $C_{25}H_{43}O_5N_5$, m.p. 238~239°, is hydrolyzed with constant boiling hydrochloric acid to DMAB, valine, 3-methylproline and glycine. This peptide is considered to be Δ^1-isocaproyltetrapeptide because of negative ninhydrin reaction and isolation of isobutylaldehyde by ozonolysis. The peptide is also shown to contain the amidine group by the molecular formula. The other, $C_{16}H_{19}O_3N_3S \cdot 1/2H_2O$, m.p. 190~194°, is hydrolyzed with constant boiling hydrochloric acid to 3-methyl-3-phenylalanine and 3-(2-thiazolyl)-β-alanine.

The structure of the Δ^1-isocaproyltetrapeptide can be presumed to be one of the structures 2-I, 2-II, 2-III and 2-IV as shown in the structure 2. The author has applied the pKa rule[11] to elucidate the structures of amidinomycin,[12,13] telomycin,[14] and netropsin.[15] The structure of the Δ^1-isocaproyltetrapeptide could be discussed on the basis of the pKa rule. Potentiometric titration of the Δ^1-isocaproyltetrapeptide shows pKa value 2.75 in methanol-water=3:2. The pKa values of noformicin[16] are measured to be 9.9 and higher than 11.5 in the same solvent. The value 9.9 can be assigned to the cyclic

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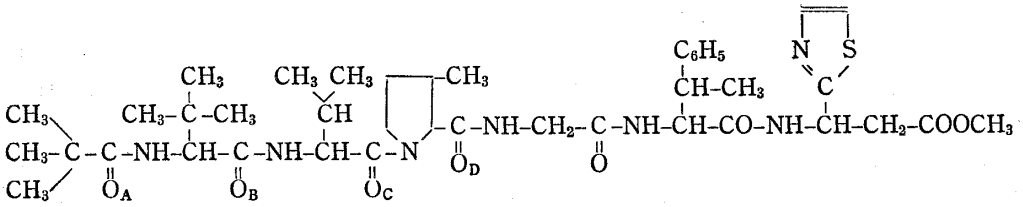
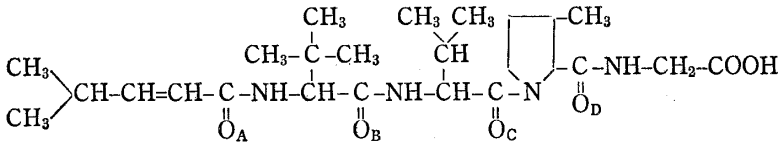
Structure 1 : The Partial Structure of Bottromycin A_1



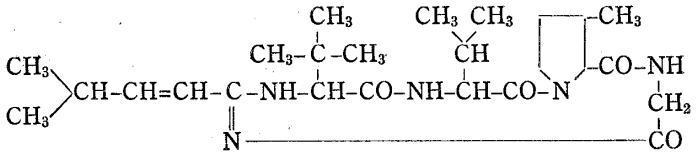
>C=O at A, B, C or D is replaced by >C=NH in bottromycin A_1.

Structure 2 :

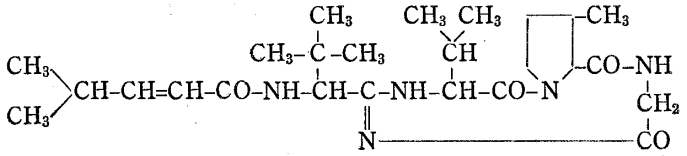


>C=O at A, B, C or D is replaced by >C=NH in structure 2-I, 2-II, 2-III or 2-IV.

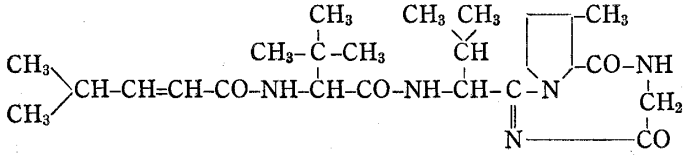
Structure 3-I :



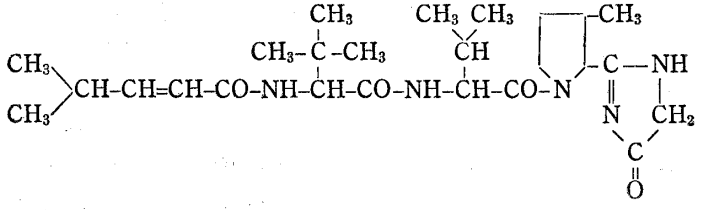
Structure 3-II :



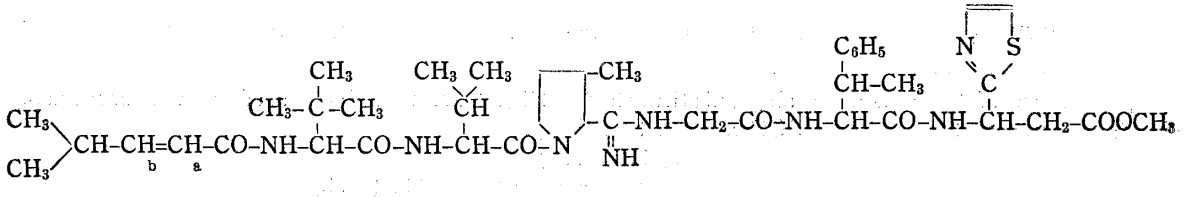
Structure 3-III :



Structure 3-IV :



Structure 4 : The Structure of Bottromycin A_2



disubstituted amidine group which has a carboxamide group on the α-carbon atom and the value higher than 11.5 to the other amidine group. The pKa value of the none-cyclic dialkylsubstituted amidine group which has a carboxamide group on the α-carbon atom could be assumed to be around 9.9~9.6 by considering the pKa-increasing effect of cyclization.

When the oxygen atom at A-position is replaced by the imino group (Structure 2-I), the value 9.9~9.6 would be decreased to around 8.9~8.6 by the pKa-decreasing effect of -CH=CH- (ΔpKa≒1)*3,17) which is one carbon atom away from the protonizing nitrogen atom. In the structures 2-II and 2-III, the value 9.9~9.6 should be decreased approximately to 8.4~8.1 by the acylated amino group*3,17) which is two carbon atoms away from the protonizing nitrogen atom. The pKa value of the none-cyclic dialkyl substituted amidine would be roughly predicted to be 12.3~12.0 and this value should be decreased approximately to 9.9~9.6 in the last case (Structure 2-IV) by the pKa-decreasing effects of the acylated amino group (ΔpKa=1.5)*3 and the C-terminal carboxyl group (ΔpKa=0.9).*3

Since the pKa value of a C-terminal carboxyl group in a tetrapeptide[11] is around 3.4 if no other pKa-decreasing substituent is located on the C-terminal amino acid moiety, the value due to the C-terminal carboxyl group can be predicted to be around 3.4 in the structures 2-I, 2-II and 2-III. The value 2.3*4 is roughly predicted for the carboxyl group in the structure 2-IV.

The value 2.75 of the Δ^1-isocaproyltetrapeptide can not be attributed to the carboxyl group of the C-terminal glycine and any of the pKa value due to the disubstituted amidine group can not be observed in the peptide. As the most reasonable explanation for the pKa studies of the Δ^1-isocaproyltetrapeptide and bottromycin A_2, the both imino and C-terminal carboxyl groups in the Δ^1-isocaproyltetrapeptide must be connected to form a ring compound during the hydrochloric acid hydrolysis. This explanation is also supported by the molecular weight determination of the Δ^1-isocaproyltetrapeptide by mass spectrometry ($C_{25}H_{41}O_4N_5$, m/e=475) and by liberation of one mole of crystallizing water from the peptide in vaccum at 150° over phosphorus pentoxide.

4-Imidazolones[18] can be synthesized from corresponding imino ether and amino acid ethyl ester *via* amidino acid, and formation of a cyclic peptide by the acid hydrolysis of bottromycin A_2 seems to be less favorable. The Δ^1-isocaproyltetrapeptide has a shoulder at 225~230 mμ (ε 5,900) in methanol. On the other hand, a maximum should be observed around 250~260 mμ due to -CH=CH-C=N-CO- for the structure 3-I. The structure 3-III is excluded by generation of one mole of Van Slyke nitrogen gas from bottromycin A_2. The infrared spectrum of the Δ^1-isocaproyltetrapeptide shows the band (1730 cm^{-1} in Nujol) which can be assigned to a carbonyl group of a smaller ring amide. The pKa value 2.75 of the Δ^1-isocaproyltetrapeptide is not contradictious for the acylated amino 4-imidazolone ring as shown in the structure 3-IV. Shimomura, *et al.* has reported pKa values of trisubstituted 5-imidazolidones as 2.7~4.8 and these values are decreased about 0.3~0.4 in cases of 5-imidazolones.[19] Thus, the structure of the Δ^1-isocaproyltetrapeptide can be drawn as shown in the structure 3-IV.

Consequently, the most reasonable structure of bottromycin A_2 is proposed as shown in the structure 4. The pKa value of the amidine group in the structure of bottromycin A_2 can be predicted to be 8.4~8.1 from the same reason for the structures

*3 pKa values of the pKa-decreasing substituents for NR_2H^+ are tentatively used for the discussion.
*4 ΔpKa value of NR_2H^+ for -COOH is used tentatively.

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2-II and 2-III of the Δ^1-isocaproyltetrapeptide and the predicted value is in good agreement with the experimental value (8.1~8.3) of bottromycin A_2.

The nuclear magnetic resonance (NMR) spectrum of bottromycin A_2 (in $CDCl_3$) shows the doublet at τ 3.1 (J=9 c.p.s.) which could be assigned to the proton on the C_a of Δ^1-isocaproic acid moiety (Structure 4). The qualtet due to the proton on the C_b would be arround at τ 2.7, though it is covered by the signals of the phenyl group and of chloroform contaminated.

Experimental

Ozonolysis of Bottromycin A_2——To a solution of bottromycin A_2 (100 mg.) in $CHCl_3$ (30 ml.) was bubbled O_3 at 0°. The reaction mixture was evaporated *in vacuo* and the residue was steam-distilled with Zn powder and AcOH. The distillate was collected in the solution of 2,4-dinitrophenylhydrazine. Thus, the reaction product was precipitated as 2,4-dinitrophenylhydrazone. Recrystallization from aqueous MeOH gave orange needles, m.p. 181~182° (12 mg.). *Anal.* Calcd. for $C_{10}H_{12}O_4N_4$: C, 47.62; H, 4.80; N, 22.22. Found : C, 47.07; H, 4.56; N, 22.20.

The orange needles were identified as 2,4-dinitrophenylhydrazone of isobutylaldehyde by admixture with the authentic specimen and by the IR spectra.

Alkaline Hydrolysis of Bottromycin A_2——Bottromycin A_2 (600 mg.) was dissolved in acetone (20 ml.), 1 *N* NaOH (10 ml.) was added and allowed to stand for 1 hr. at room temperature. Acetone was removed *in vacuo* and NaOH (1.6 g.) in H_2O (11 ml.) was added to the reaction mixture and kept to stand at room temperature for 16 days. During the hydrolysis, NH_3 was generated and identified as NH_4Cl. After the hydrolysis, the reaction mixture was passed through a column of IRC-50 (H-type). The IRC-50 column was treated with 1*N* HCl and the eluate was evaporated to dryness *in vacuo*. The residue was desalted with MeOH and dried *in vacuo*. The mixture showed two ninhydrin positive spots, Rf 0.22 and 0.53, by the paper chromatography using the upper phase of BuOH-AcOH-H_2O=100:12:100. The mixture was purified by the cellulose powder column chromatography with the same solvent system. Each fraction was applied to the paper chromatography. The fraction showing Rf 0.53 and 0.22 were respectively collected and dried *in vacuo*. The dipeptide (16 mg.) was recovered from the fractions of Rf 0.53 and the tetrapeptide (38 mg.) from the fractions of Rf 0.22.

Hydrochloric Acid Hydrolysis of the Di- and Tetrapeptide——Two mg. of the dipeptide (Rf 0.53) was hydrolyzed with constant boiling HCl (1 ml.) in a sealed tube at 120° for 17 hr. The hydrolyzate was evaporated *in vacuo* to dryness and neutralized with IR 4 B (OH-type) after dissolved in a small amount of H_2O. 3-Methyl-3-phenylalanine and 3-(2-thiazolyl)-β-alanine were shown in the hydrolyzate by the paper chromatography.

The same procedure was applied for the tetrapetide and DMAB, valine, 3-methylproline and glycine were shown in the hydrolyzate.

Hydrazinolysis of the Tetrapeptide——The tetrapeptide (10 mg.) in H_2O (5 ml.) was neutralized by passing through a column of IR 4B (OH-type) and dried *in vacuo*. The dried peptide was decomposed with 1 ml. of anhydrous hydrazine (freshly distilled over BaO) for 7 hr. at 100° in a sealed tube. Hydrazine was removed *in vacuo* over H_2SO_4 and the residue was stirred with benzaldehyde after addition of 1 ml. of H_2O for 2 hr. at room temperature. The reaction mixture was extracted with each 2 ml. of AcOEt to remove impurities and the water phase was condensed. Glycine was shown by the paper chromatography as the C-terminal amino acid of the tetrapeptide.

Edman Degradation of the Tetrapeptide——Phenylisothiocyanate (0.1 ml.) was added to a solution of the tetrapeptide (3.5 mg.) in dioxane (2 ml.) and H_2O (2 ml.). The mixture was adjusted to pH 8.7~9.0 by addition of 0.1 *N* NaOH under stirring at 40° until no more NaOH was consumed. After 2 hr., the mixture was extracted 5 times with cyclohexane and 5 times with benzene to remove excess of the reagent. The water phase was dried *in vacuo* over NaOH. The remaining PTC-peptide was stirred in 3 *N* HCl (20 ml.) for 9 hr. at 50°. The end of cyclalization was checked by the UV maxima at 240 and 270 mμ. The reaction mixture was extracted twice with ether and the ether extracts were combined and evaporated to recover the PTH-N-terminal amino acid. The azide method was used for the paper chromatography to detect the PTH-amino acid. The PTH-amino acid was detected under UV-light by the thin-layer chromatography after spraying a solution of fluorescein. The PTH-amino acid was identified as PTH-DMAB by the paper and thin-layer chromatographies. The water phase was dried over NaOH *in vacuo* after extraction with AcOEt to recover the remaining tripeptide. The residue was forewarded to second Edman degradation. The same procedure was repeated to get the PTC-tripeptide and the PTH-amino acid was recovered from the PTC-tripeptide by 3 hr. treatment at 50° with 3*N* HCl. The second PTH-amino acid was determined to be valine by the same method above mentioned.

Synthesis of PTH-DMAB and PTH-3-Methylproline——DMAB (5 mg.), Na_2CO_3 (5 mg.) and phenylisothiocyanate (0.01 ml.) were stirred in 50% aqueous dioxane (2 ml.) at 40° for 2 hr. Thenafter, the reaction

mixture was extracted with cyclohexane and benzene. The water phase was evaporated to dryness *in vacuo*. The residue was kept at 40° for 3 hr. after addition of 1 *N* HCl (2 ml.). The PHT-DMAB precipitated was washed with a small amount of H_2O and dried.

The same method as above was employed to make PTH-3-methylproline. The PTH-3-methylproline was extracted with AcOEt after treatment with 1*N* HCl and dried.

Hydrochloric Acid Hydrolysis of Bottromycin A_2——Bottromycin A_2 (1 g.) was refluxed in 1*N* HCl (30 ml.) for 7 hr. The hydrolyzate was evaporated to dryness *in vacuo*, and the residue was dissolved in aqueous MeOH and passed through a column of IR 4B (OH type) to remove HCl. The eluate was dried *in vacuo* and purified by silica gel column chromatography. The Δ^1-isocaproyltetrapeptide was recovered from MeOH-AcOEt (2:8) eluate. Recrystallized from MeOH and ether, white prisms, m.p. 237~239°. Dried at 60° *in vacuo*. *Anal.* Calcd. for $C_{25}H_{43}O_5N_5$: C, 60.82; H, 8.78; O, 16.21; N, 14.19. Found: C, 60.85; H, 9.02; O, 16.06; N, 13.74. Dried at 150° *in vacuo*. *Anal.* Calcd. for $C_{25}H_{41}O_4N_5$: C, 63.13; H, 8.69; O, 13.46; N, 14.73. Found: C, 62.79; H, 9.10; O, 13.70; N, 14.50.

The dipeptide was eluted by MeOH-AcOEt=1:1 from the silica gel column and crystallized from MeOH, m.p. 190~194°. *Anal.* Calcd. for $C_{16}H_{19}O_3N_3S \cdot 1/2H_2O$. C, 56.11; H, 5.85; N, 12.28; S, 9.38. Found: C, 56.17; H, 5.90; N, 12.58; S, 9.21.

The dipeptide was hydrolyzed to 3-methyl-3-phenylalanine and 3-(2-thiazolyl)-β-alanine with constant boiling HCl at 120° for 17 hr.

Ozonolysis of the Δ^1-Isocaproyltetrapeptide——The Δ^1-isocaproyltetrapeptide (50 mg.) in $CHCl_3$ (20 ml.) and MeOH (2 ml.) was oxidized with O_3 at 0°. The same procedure before mentioned was traced and 2,4-dinitrophenylhydrazone of isobutylaldehyde was isolated.

Summary

Bottromycin A_2 is hydrolyzed to tetrapeptide and dipeptide by alkaline hydrolysis. The structures of both peptides are determined to be 3,3-dimethyl-2-aminobutylyl-valyl-3-methylprolylglycine and 3-methyl-3-phenylalanyl-3-(2-thiazolyl)-β-alanine.

Hydrochloric acid hydrolysis of bottromycin A_2 gives Δ^1-isocaproyltetrapeptide and the structure is discussed on the bases of pKa studies. The structure of bottromycin A_2 is proposed from the results above described.

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135. Toshio Kinoshita,*1,*2 Morizo Ishidate,*3 and Zenzo Tamura*1: 3-Ketoglucuronic Acid. I. Synthesis of 3-Ketoglucuronic Acid *4

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Recently, Imai and his co-workers[1] noted in their studies on nitric acid oxidations of starch that the oxidized product of starch contains some keto-sugar moieties. By periodate oxidation and subsequent acid hydrolysis of the product they[2] obtained

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*4 Preliminary communication of this work was published: M. Ishidate, Z. Tamura, T. Kinoshita: This Bulletin, **10**, 1258 (1962); **13**, 99 (1965).
1) Y. Imai: Yakugaku Zasshi, **81**, 1109 (1961); Y. Imai, Y. Hirasaka: *Ibid.*, **81**, 1362 (1961).
2) Y. Imai; *Ibid.*, **81**, 1115 (1961).