THE REVISED STRUCTURE OF BOTTROMYCIN A2

Sir:

During the studies on application of chemical ionization (CI) mass spectrometry to antibiotic research, we found that the fragmentation pattern of the spectrum of bottromycin A2 cannot be explained by its proposed structure[1] (Fig. 1). This prompted us to reinvestigate its chemical structure. In this communication, the revised structure of bottromycin A2 deduced mainly by mass spectrometric analyses, is presented.

The CI mass spectrum of bottromycin A2 together with the electron impact (EI) mass spectrum are shown in Fig. 2. There exist only five significant peaks over *m/e* 100 in the CI mass spectrum [*m/e* 823 (M + 1), 654, 476, 348 and 170]. These peaks can be assigned to the fragment ions of the proposed structure shown in Fig. 1. For a linear structure of bottromycin A2, one would anticipate additional fragment ions, due to the cleavage of other peptide bonds. Thus, the CI mass spectrum suggests that the fragment ion *m/e* 476 is cyclic. The molecular formula of bottromycin A2 can be assigned to $C_{42}H_{62}N_8O_7S$ by high resolution EI mass spectrometry, observed: *m/e* 822.4411, error −4.7 millimass units (Hitachi RMU-7M spectrometer with 002 data processing system), which is in accord with earlier report[1].

The ^{1}H-NMR spectrum of bottromycin A2 indicated that there is no olefinic proton. The resonance at δ 6.9 (doublet, J = 9 Hz) in $CDCl_3$, which was originally assigned to the olefinic proton of Δ^1-isocaproic acid moiety in the proposed structure, can be assigned to an NH proton, because it rapidly disappeared by addition of D_2O. The ^{13}C-NMR spectrum (Fig. 3) also confirmed the absence of such olefinic carbons. The ^{1}H- and ^{13}C-NMR spectra rather suggested the presence of two tertiary butyl groups. There is an 18 H-singlet peak at δ 1.00 in the ^{1}H-NMR spectrum and 2 quaternary carbon resonances at δ 33.1 and 35.6, and a very strong methyl carbon resonance at δ 27.9, of which intensity is about 6 times stronger than a nearby methyl carbon resonance, in the ^{13}C-NMR spectrum.

Fig. 1. The previous structure of bottromycin A2

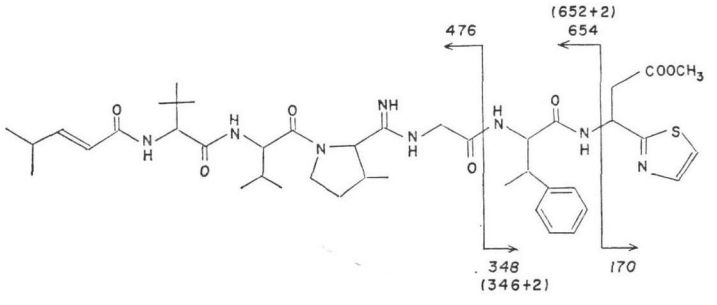


Fig. 2. The CI and EI mass spectra of bottromycin A2 (Hitachi RMU-6M spectrometer)

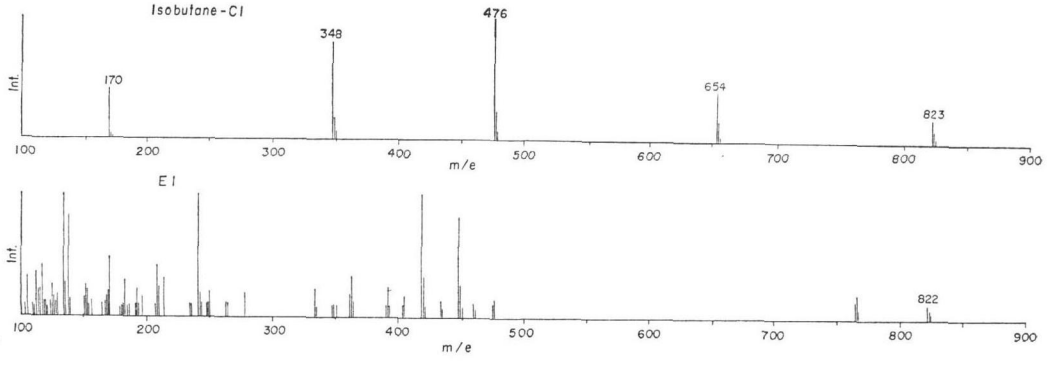
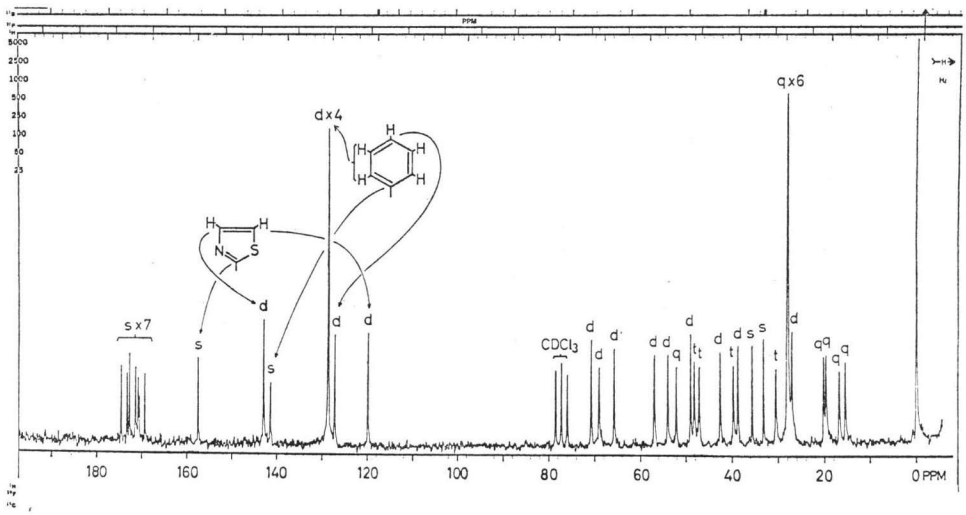
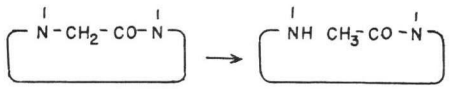


Fig. 3. The ^{13}C-NMR spectrum of bottromycin A2 at 25.2 MHz (the symbols s, d, t and q mean singlet, doublet, triplet and quartet under off-resonance decoupling)



Total acid hydrolysis of bottromycin A2 gives each one mole of 3-methyl-3-phenyl-L-alanine[2], 3,3-dimethyl-2-aminobutyric acid (DMAB)[3], L-valine[3,4], 3-(2-thiazolyl)-β-alanine[2], *cis*-3-methyl-L-proline[3] and glycine[4]. Partial acid hydrolysis (1 N HCl, 110°C, 6.5 hours) gives two peptides. One is 3-methyl-3-phenylalanyl-3-(2-thiazolyl)-β-alanine[2] and the other is a tetrapeptide composed of DMAB, valine, 3-methylproline and glycine. The molecular formula of the tetrapeptide was assigned to $C_{25}H_{41}N_5O_4$ by high resolution mass spectrometry, observed: *m/e* 475.3118, error –3.8 millimass units. The CI mass spectrum showed only the quasimolecular ion (*m/e* 476) which also suggested the cyclic structure. The tetrapeptide is a dehydration product which is deduced from its molecular formula. A free carboxyl function, which can be expected to be formed by acid hydrolysis, is not present in the tetrapeptide. Therefore, the carboxyl group seems to be concerned with the dehydration. The tetrapeptide has a weak basic group (pKa 2.75 in methanol - water, 3:2), which should be derived from the basic function of bottromycin A2 (pKa 8.1~8.3 in the same measurement condition described above). These relations can be interpreted by imidazolone formation between an amidine and the above-mentioned carboxyl functions during acid hydrolysis.

The tetrapeptide was treated with sodium metal in liquid ammonia in the presence of trace amount of methanol at —33°C for 8 minutes to intend to cleave the amino peptide bond of the 3-methylprolyl residue reductively[5]. One of the major reaction products was isolated by silica gel chromatography using $CHCl_3$ – *i*-PrOH(8:1), yield 39%. It was found to be a diastereoisomeric mixture, because it could be separated into two spots by paper electrophoresis using AcOH - HCOOH - H_2O(75: 25: 900) but they showed the same fragmentation pattern in the mass spectrometry, which showed that they are the tetrahydroderivatives of the tetrapeptide, M^+: *m/e* 479. By acid hydrolysis they gave DMAB, valine, and 3-methylproline, but not glycine. The ^{1}H-NMR spectrum suggested that an N-acetyl group, of which detailed discussion will be described later, was formed by reductive ring-opening at the glycine moiety* (See scheme below).

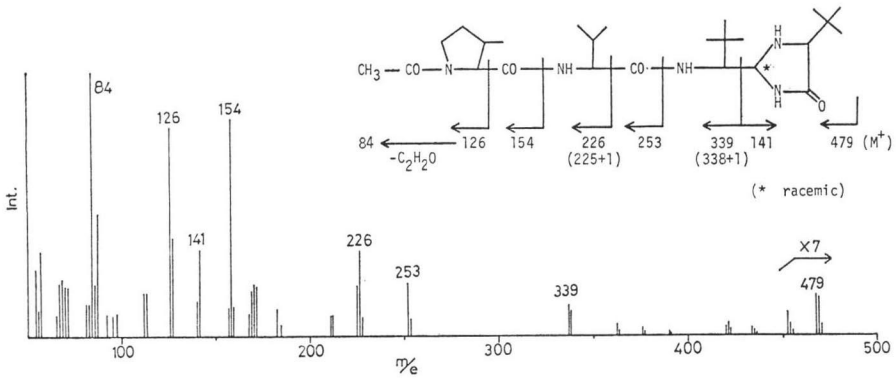


The EI mass spectrum of the tetrahydroderivative (Fig. 4) established the peptide sequence as acetyl-3-methylprolyl-valyl-NH-CH-. The assign-

t-C_4H_9

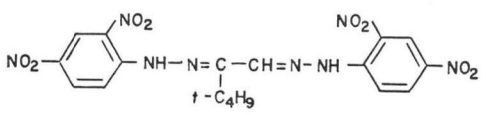
* The expected peptide, which is formed by cleavage at the amino peptide bond of the 3-methylprolyl residue, is obtained in better yield under longer reaction period.

Fig. 4. The EI mass spectrum of the tetrahydrotetrapeptide



ment of the fragment ions was verified by their elemental compositions obtained by high resolution mass spectrometry (Table 1). The rest of the molecule should be an imidazolidone containing another *t*-butyl group (*m/e* 141, see Fig. 4 and Table 1), which is derived by reduction of the imidazolone in the tetrapeptide.

The imidazolidone is a potential aldehyde. Thus, the tetrahydroderivative was hydrolyzed in 6 N HCl in the presence of 2,4-dinitrophenylhydrazine to catch the yielded aldehyde. The hydrolyzate contained an orange-colored substance together with DMAB, valine and 3-methylproline. The molecular formula, $C_{18}H_{18}N_8O_8$, of the colored substance was established by high resolution mass spectrometry, observed: *m/e* 474.1274, error + 2.8 millimass units. The ^{1}H-NMR spectrum in $CDCl_3$ showed the presence of a *t*-butyl [δ 1.38 (9H, singlet)], a methine [δ 7.94 (1H, singlet)], two 2,4-dinitrophenyl (6 protons of a pair of 1,2,4-substituted benzene at δ 8.20, 8.28, 8.38, 8.50, 9.13 and 9.15) and two hydrogen-bonded NH groups [δ 11.41 (1H, singlet), 13.31 (1H, singlet)]. Thus, the structure of the colored substance was determined to be the osazone of *t*-butylglyoxal.



If the tetrahydroderivative has a latent 2-amino-3,3-dimethylbutyraldehyde moiety, it will give 3,3-dimethyl-2-oxo-butyl alcohol by prototropy and deaminative hydration during acid hydrolysis. The osazone of *t*-butylglyoxal is derived from the latter in the presence of the phenylhydrazine. The similar reaction is reported by TATSUTA *et al*[6]., that is: 2-oxo-3-phenylpropyl alcohol is yielded from the phenylalaninal moiety of chymostatin by acid hydrolysis. Thus, the presence of the imidazolidone was confirmed by isolation of the osazone, and the structure of the tetrahydroderivative was established as shown in Fig. 4.

Table 1. The elemental compositions of the fragment ions shown in Fig. 4

m/e (observed)	Elemental composition	Error (millimass unit)
84.0809	$C_5H_{10}N_1$	−0.2
126.0901	$C_7H_{12}N_1O_1$	−1.6
141.1028	$C_7H_{13}N_2O_1$	+0.1
154.0864	$C_8H_{12}N_1O_2$	−0.2
255.1552	$C_{12}H_{21}N_2O_2$	−4.9
226.1687	$C_{12}H_{22}N_2O_2$	+0.7
253.1547	$C_{13}H_{21}N_2O_3$	−0.3
339.2474	$C_{18}H_{33}N_3O_3$	−4.5
479.3451	$C_{25}H_{45}N_5O_4$	−1.7

The ^{1}H-NMR spectrum of the diastereoisomeric mixture of the tetrahydroderivative showed that the N-acetyl group appeared separately at δ 1.83 and 1.92 with almost equal intensity, though the acetyl group is far from the racemic carbon. It suggests the presence of intramolecular hydrogen-bondings to keep these groups in proximity. Formation of the fragment ions *m/e* 226 (225+1) and 339 (338+1) also could be explained by a strong intramolecular hydrogen-bonding between the carbonyl group of 3-

methylprolyl moiety and an NH proton of the imidazolidone with ten-atoms ring.

The structures of the tetrapeptide and bottromycin A2 are presented as shown in Fig. 5*. It must be noticed that the source of DMAB derived by acid hydrolysis of the tetrapeptide is different from that of its tetrahydroderivative. The CI mass spectrum of bottromycin B2[7] gave five significant peaks at *m/e* 809 (M+1), 640, 462, 348 and 170. Therefore, the structure of B2 is assigned to be the structure shown in Fig. 5, in which the 3-methylprolyl moiety is substituted by prolyl moiety[7]. For the structures of the other components[7,8] of bottromycins, the reinvestigation should be necessary. But the samples are not available now.

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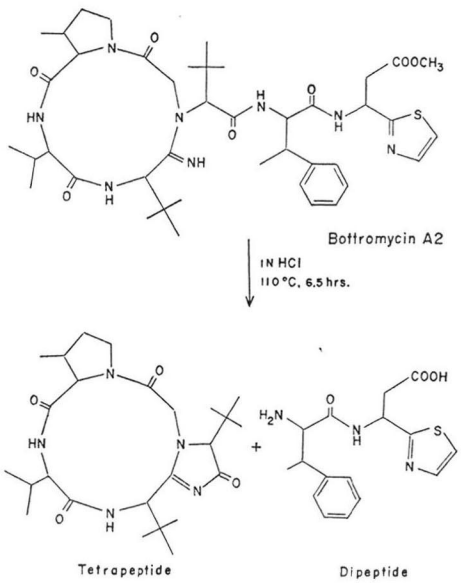
(Received July 9, 1976)

Fig. 5. The revised structure of bottromycin A2 and its mild acid hydrolysis products.



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* Δ^1-Isocaproic acid unit in bottromycin A2 and the tetrapeptide in the previous structures was deduced from the isolation of isobutylaldehyde by ozonolysis. This isobutylaldehyde might be introduced from the previously used apparatus. The reexamination did not give any volatile carbonyl compound by the ozonolysis of bottromycin A2 and the tetrapeptide.

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