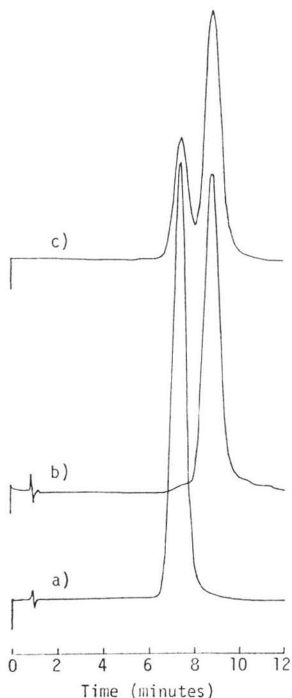
CHEMISTRY OF BLEOMYCIN. XXVII CLEOMYCIN, A NEW FAMILY OF BLEOMYCIN-PHLEOMYCIN GROUP

Sir:

In the course of screening of a highly-productive strain of bleomycin, it was found that a mutant, which was obtained by treatment of a bleomycin-producing strain by UV-irradiation, produced a new type of bleomycin together with known bleomycins. This new type of bleomycin was named cleomycin on the basis of a structural feature that it contains a cyclopropane ring. In this communication, we report isolation, properties and structure determination of cleomycin.

The mutant which produces cleomycin has the same morphological characters as the original strain. Cleomycin was isolated from the culture filtrate by the similar procedures as described previously[1)] for the isolation of bleomycin. The elution profile from the CM-Sephadex column used to separate the congeners was apparently the same as in the case of bleomycins (Fig. 1-b in reference 1). However, each peak of the chromatogram contained two components which were separated by a reverse phase high pressure liquid chromatography (HPLC). As an example, HPLC separation of the bleomycin B2 fraction of the CM-Sephadex chromatography is shown in Fig. 1. The fast eluted substance was identified to be bleomycin B2 and the later eluted one was found to be a new type of bleomycin which has same terminal amine, agmatine, as bleomycin B2. Thus, cleomycins A1, B1′, demethyl-A2, A2, B2, B4 and A5, which corresponded to bleomycins A1, B1′, demethyl-A2, A2, B2, B4 and A5[1)], respectively, were identified.

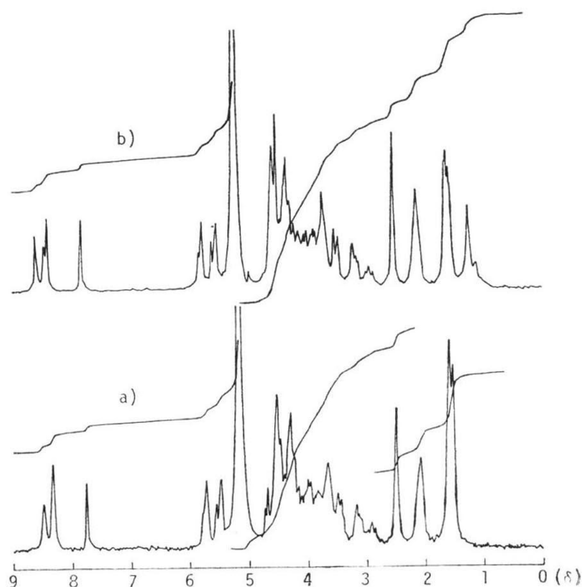
Fig. 1. Reverse phase liquid chromatography of bleomycin (BLM) B2 and cleomycin (CLM) B2.



a) BLM B2, b) CLM B2, c) mixture of BLM B2 and CLM B2. Column: Radial PAK A (8× 100 mm). Solvent: acetate buffer (4% AcOK, 2% AcOH) - MeOH (7:3), 3 ml/min. Detection UV (292 nm).

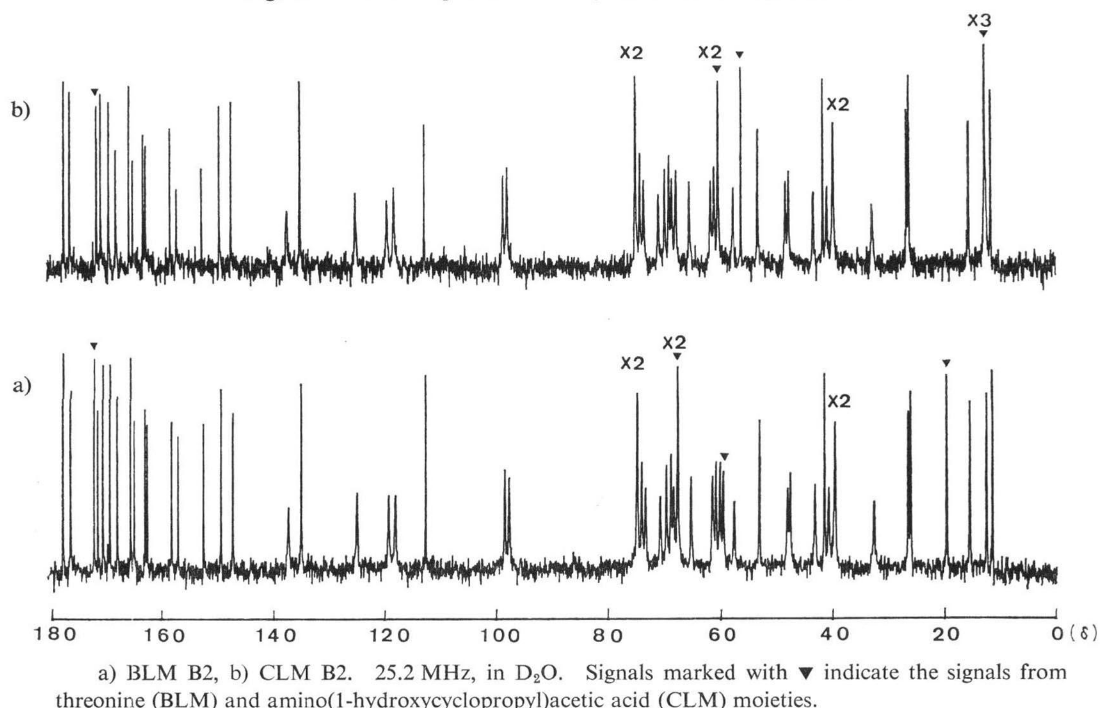
Cleomycin B2 was preparatively separated from bleomycin B2 by reverse phase column chromatography: Lobar column packed with Lichroprep RP-8 (E. Merck), eluted with 1% $AcONH_4$ - MeOH (4:1). Cleomycin B2 thus obtained showed the same UV spectrum as that of bleomycin B2. On acid hydrolysis, cleomycin

Fig. 2. ^{1}H-NMR spectra of bleomycin B2 and cleomycin B2.



a) BLM B2, b) CLM B2. 100 MHz, in D_2O, external TMS reference. Slight difference in the pDs of the solutions caused apparent difference of the spectra in the lower field (imidazole protons).

Fig. 3. ^{13}C-NMR spectra of bleomycin B2 and cleomycin B2.



a) BLM B2, b) CLM B2. 25.2 MHz, in D_2O. Signals marked with ▼ indicate the signals from threonine (BLM) and amino(1-hydroxycyclopropyl)acetic acid (CLM) moieties.

B2 gave the same ninhydrin-positive products as bleomycin B2 except for threonine. Thus, the acid hydrolysate of cleomycin B2 contained β-aminoalanine[2)], 3-amino-3-(4-amino-6-carboxy-5-methylpyrimidin-2-yl)propionic acid[3)], β-hydroxyhistidine[4)], 4-amino-3-hydroxy-2-methylpentanoic acid[5)] and 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylic acid[6)], which are present in all bleomycins, and agmatine[7)]. However, the hydrolysate neither contained threonine[2)], nor any ninhydrin-positive product replacing the threonine.

The ^{1}H-NMR spectrum of cleomycin B2 recorded at 100 MHz in D_2O solution was compared with that of bleomycin B2 (Fig. 2). There were the signals of 6 protons emanated from 2 C-methyl groups at δ *ca.* 1.6 (external TMS reference) in the spectrum of cleomycin B2, while there were 3 C-methyl signals at this region in the spectrum of bleomycin B2. The doublet signal at δ 4.70 of the α-methine of the threonine moiety of bleomycin was also missing from the spectrum of cleomycin. These results were in accord with the absence of threonine in the acid hydrolysate of cleomycin. In the spectrum of cleomycin there were signals of 4 protons centered at δ 1.2 and a singlet signal at δ 4.44, which were not present in the spectrum of bleomycin. The former signals appeared to be emanated from the ethylene group contained in a cyclopropane ring as judged from the chemical shift and coupling pattern. The rest of the spectrum of cleomycin B2 was very similar to that of bleomycin B2.

The ^{13}C-NMR spectrum of cleomycin B2 was also compared with that of bleomycin B2 (Fig. 3). All chemical shifts in the ^{13}C-NMR spectrum of bleomycin have already been assigned by us[8,9)]. Four signals at δ 172.7 (CO), 68.0 (β-CH), 59.9 (α-CH) and 19.8 (CH_3) originating from the threonine moiety of bleomycin were missing in the spectrum of cleomycin, in which, however, there were four corresponding signals at δ 171.9, 56.2, 60.4 and 12.7. This suggested the presence of an amino acid residue replacing threonine, although acid hydrolysis of cleomycin did not give a new ninhydrin-positive spot. The remaining 51 signals in the spectrum of cleomycin B2 corresponded to the rest 51 signals in the spectrum of bleomycin B2 within 0.1 ppm shift except for the carbonyl signal of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety (δ 178.3 in bleomycin, δ 178.0 in cleomycin). This carbonyl group of bleomycin is directly connected

Scheme 1.

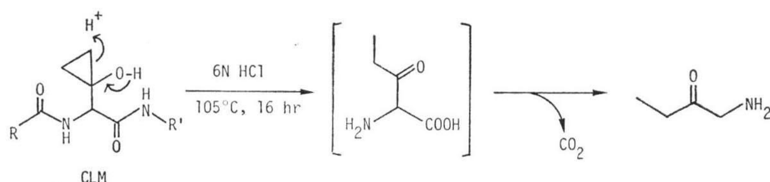
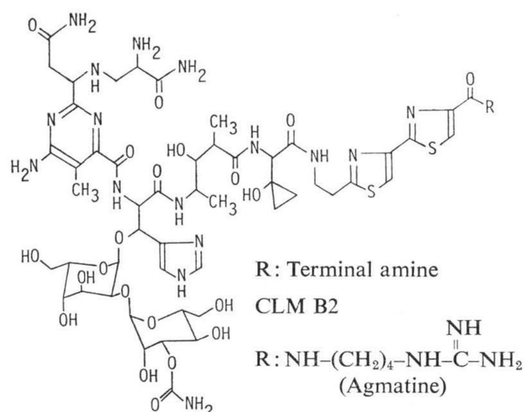


Fig. 4. Structure of cleomycin.

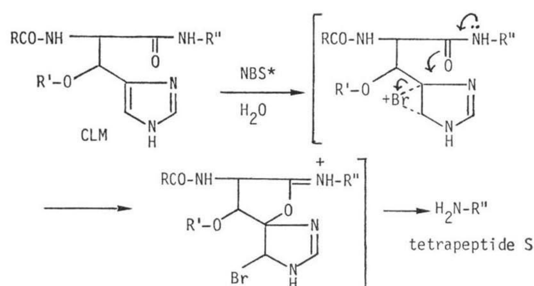


with the amino group of threonine. Therefore, the chemical shift difference of 0.3 ppm between both carbonyl signals seems to be caused by the difference of the adjoining amino acid. Thus, cleomycin B2 is different from bleomycin B2 only with respect to threonine moiety.

The four ^{13}C signals emanated from this new amino acid residue of cleomycin were analyzed by the ^{1}H off-resonance spectrum [δ 171.9, singlet (CO); 60.4, doublet (CH); 56.2, singlet (non-hydrogen-bearing sp^3-C); 12.7, triplet (CH_2)]. The signal at δ 12.7 appeared to be emanated from two equivalent methylenes of the ethylene group contained in a cyclopropane ring and the signal at δ 56.2 from the 1-carbon of a 1-hydroxycyclopropyl group. Thus, amino (1-hydroxycyclopropyl) acetic acid was deduced as the new amino acid in cleomycin.

If this amino acid is present in cleomycin, 1-amino-2-butanone will be formed by acid hydrolysis (Scheme 1). 1-Amino-2-butanone was prepared from propionic anhydride and glycine by DAKIN-WEST reaction[10]. It gave a characteristic yellow spot by ninhydrin reaction on cellulose thin-layer chromatogram (Rf 0.71, developing solvent; PrOH - pyridine - AcOH - H_2O, 15: 10: 3: 12). However, 1-amino-2-butanone was not detected on the thin-layer

Scheme 2.



*N-bromosuccinimide R, R′, R″: see Fig. 4

chromatogram of the acid hydrolysate of cleomycin. We assume that the 1-amino-2-butanone yielded by acid hydrolysis of cleomycin underwent some reaction with the concomitantly liberated sugars to give tarry substances.

After a failed attempt to get a sugarless peptide fragment containing the intact new amino acid of cleomycin by mild acid hydrolysis, the desired peptide fragment was obtained by oxidative cleavage of cleomycin B2 with N-bromosuccinimide[11] (Scheme 2). Total acid hydrolysis of the peptide (tetrapeptide S[11] of cleomycin) gave 1-amino-2-butanone together with 4-amino-3-hydroxy-2-methylpentanoic acid, 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylic acid and agmatine. The ^{1}H-NMR spectrum of the tetrapeptide showed a singlet methine signal at δ 4.41 and a 4-protons signal centered at δ 1.2, which indicated the existence of an intact amino-(1-hydroxycyclopropyl)acetic acid moiety in the tetrapeptide. The ^{13}C-NMR spectrum also indicated the existence of an intact amino(1-hydroxycyclopropyl)acetic acid moiety; δ 171.5 (CO), 60.4 (α-CH), 56.0 (1-hydroxycyclopropyl-1-C), 12.7 $\times$ 2 (ethylene contained in cyclopropane ring). The chemical shifts of the rest 20 signals from other three components of the tetrapeptide corresponded to those of the tetrapeptide S of bleomycin within 0.1 ppm of the chemical shift except for the carbonyl group of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety [δ 177.2 (tetra-

peptide S of bleomycin), 176.9 (tetrapeptide S of cleomycin)]. Therefore, the presence of amino(1-hydroxycyclopropyl)acetic acid moiety in cleomycin instead of the threonine residue of bleomycin was proved. The proposed structure of cleomycin is shown in Fig. 4.

The biological activity of cleomycin B2 is almost the same as that of bleomycin B2. It should be noted that YA-56[12], a family of bleomycin-phleomycin group, contains in the place of the L-threonine of bleomycin L-β-hydroxyvaline, which is formally a hydrogenolysis product of the amino(1-hydroxycyclopropyl)acetic acid. Therefore, the stereochemistry of the amino (1-hydroxycyclopropyl)acetic acid of cleomycin should be *S*.

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