Structures of Ezomycins A\textsubscript{1} and A\textsubscript{2} \textsuperscript{\dagger}

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The structures of ezomycins A\textsubscript{1} and A\textsubscript{2}, antifungal antibiotics produced by a strain of *Streptomyces*, were determined as 1 and 2, respectively, by degradative and spectrometric studies.

Ezomycin complex is an antifungal antibiotic produced by a strain of *Streptomyces* and is active against very limited species of phytopathogenic fungi such as *Sclerotinia* and *Botrytis* sp.\textsuperscript{1} We have reported preliminarily the isolation of eight components designated as ezomycins A\textsubscript{1} (1), A\textsubscript{2} (2), B\textsubscript{1}, B\textsubscript{2}, C\textsubscript{1}, C\textsubscript{2}, D\textsubscript{1}, and D\textsubscript{2} together with their structural elucidation.\textsuperscript{2-5} In this paper we wish to present the details of our experimental results leading to the structural determination of 1 and 2.

As previously reported,\textsuperscript{4} 1 C\textsubscript{38}H\textsubscript{58}N\textsubscript{8}O\textsubscript{15}S\textsubscript{5}H\textsubscript{2}O is composed of 2 C\textsubscript{19}H\textsubscript{26}N\textsubscript{6}O\textsubscript{12}H\textsubscript{2}O and L-cystathionine (3). Acid hydrolysis of 2 gives ezoaminuroic acid (4) C\textsubscript{6}H\textsubscript{11}NO\textsubscript{5} and cytosine.

Alkaline hydrolysis of 1 gave anhydrodeaminonucleoside A (5) C\textsubscript{13}H\textsubscript{14}N\textsubscript{4}O\textsubscript{8}, \(\lambda\text{max}=261\) nm, pKa' 9.9, accompanied by conversion of the cytosine moiety of 1 into uracil. The presence of ureido group in 5 was suggested by a positive p-dimethylaminobenzaldehyde test.\textsuperscript{6} All of the signals in the PMR spectrum of 5 were confirmed by spin-decoupling experiments as shown in Table I. Esterification of 5 with MeOH-HCl, followed by acetylation, yielded methyl ester-monooacetate (6) as the main product together with methyl ester-diacetate (7). Downfield shift of the signal due to H-2' in the PMR spectrum of 6 indicated the occurrence of acetylation at the C-2' hydroxyl. The location of the ureido group at C-5' was deduced from spin-decoupling experiments which revealed that the broad multiplet due to H-5' (4.7, W\textsubscript{1/2}=18) was coupled with an amide proton (6.48, d, 9.0) as well as with H-4' (4.17, dd, 11) and H-6' (6.04, d, 6.0). Six of the eight oxygen atoms in 5 were ascribed to a uracil, a ureido, a carboxyl and a hydroxyl, and the remaining two were supposed to constitute ether linkages. The chemical shift of an olefinic proton (H-6', 5.86, d, 5.9) and pKa' value (3.8) suggest the presence in 5 of an \(\alpha\)-alkoxy-\(\alpha\),\(\beta\)-unsaturated carboxylic acid moiety which must be formed by \(\beta\)-elimination of 4 during alkaline hydrolysis of 1. This was substantiated by close consistency of the UV maximum (238 nm) of 5 with that (239 nm) of methyl (methyl 4-deoxy-\(\beta\)-L-threo-hex-4-enopyranosid) uronate,\textsuperscript{7} having the same chromophoric system. This elimination seems to be similar to the alkaline hydrolysis of carbohydrates such as hyaluronic acid and chondroitin sulfates, resulting in the production of \(\alpha\),\(\beta\)-unsaturated carboxylic acids.\textsuperscript{8}

To satisfy the hydrogen deficiency in 5 the sugar part of 5 should be bicyclic. Thus, the planar structure of 5 was deduced as illustrated in Fig. 1. Diacetate 7 showed a UV maximum similar to that of 6, but the former was negative to the p-dimethylaminobenzaldehyde test. This suggests the presence of the second acetyl in 7 at the ureido group.

Mass spectra of trimethylsilyl derivatives of 5 and its methyl ester also support the above assignment. Trimethylsilylation of 5 gave a pentatrimethylsilyl derivative (M\textsuperscript{+} m/e 714); a similar treatment of the methyl ester of 5 gave a tetratrimethylsilyl derivative (M\textsuperscript{+} m/e 656).

\textsuperscript{\dagger} Studies on Ezomycins, Antifungal Antibiotics. Part VII. Preceding paper, see Reference 3).
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*Chemical shifts are expressed in δ values and coupling constants in Hz. Splitting are shown as s (singlet), d (doublet), dd (doublet of doublet), t (triplet), and m (multiplet).*
Oxidation of 1 with 2 molar equivalents of NaIO₄, followed by treatment with phenylhydrazine, yielded glyoxal bisphenyl-hydrazone, lactam-aminohemiacetal (8) and nucleoside A (9): C₁₃H₁₇N₅O₈; ƒÉ0.05NHCl max nm (ƒÃ) 212.5 (9800), 278.5 (13,000), ƒÉ0.05NNaOH max 230 (sh., 7200), 271 (8400). Compound 9 showed a positive p-dimethylaminobenzaldehyde test. Esterification of 9 with MeOH-HCl followed by acetylation gave methyl ester-tetraacetate (10): C₂₂H₂₇N₅O₁₂; ƒÉMeOn max nm (r) 212 (20,700), 249 (18,700), 297 (8400). In the PMR spectrum of 10 (Table I) signals ascribed to H-2' and H-6' were observed in a lower field by more than 1 ppm as compared with those of 9. This indicates the occurrence of acetylation at two hydroxyls at C-2' and C-6' in 9. Further, introduction of other acetyl groups to the ureido and cytosine functions was deduced from the negative coloration with the p-dimethylaminobenzaldehyde test and UV maximum (297 nm) of 10. In the PMR spectrum of 10 (Table I) signals ascribed to H-2' and H-6' were observed in a lower field by more than 1 ppm as compared with those of 9. This indicates the occurrence of acetylation at two hydroxyls at C-2' and C-6' in 9. Further, introduction of other acetyl groups to the ureido and cytosine functions was deduced from the negative coloration with the p-dimethylaminobenzaldehyde test and UV maximum (297 nm) of 10. In the spectrum in DMSO-d₆ (Table I), the doublet (4.70, 1H, 1.8) was ascribed to H-7'. The broad multiplet (4.51, 1H, W₁/₂=11) was assigned to H-5', because the signal became narrow (W₁/₂=6.5) on addition of D₂O. Accordingly, one of the lowfield two-proton signals (5.49) was ascribed to H-6' (5.65, dd, 1.8, 3.0, in CDCl₃-MeOH-d₄=3:1) and another to H-2' (5.55, d, 5.6, in CDCl₃-MeOH-d₄=3:1). Thus, structure 9 was assigned to nucleoside A apart from its stereostructure, and 2 was postulated to have a structure in which 4 is attached to C-6' of 9 through a glycosyl linkage.

In the spectra of 2, 5, 6, 7, 9, and 10, the anomic proton (H-1') was always observed as a sharp singlet. This suggests that the furanose ring is fixed in a C-2' exo- or C-3' endo-conformation, and H-1' and H-2' are oriented to each other in trans.⁹-¹¹. The large vicinal coupling constant (J₃',₄'=11) indicates a 1,2-trans-diaxial relationship between H-3' and H-4' and only a 2',3'-trans configuration satisfies these requirements. Based on the foregoing, comprehensive inspection of the PMR spectra using Dreiding model gives the two possible stereostructures 9 and 9' (Fig. 2), for nucleoside A as follows.

The bulky carboxyl group is probably oriented equatorially. If the 6-membered ring assumes a boat-conformation (structure 9'), the hydroxyl at C-6' should be oriented pseudo-axially because of the small J₆',₇'=1.8. In this case the proton at C-3' is sterically close to the C-6' hydroxyl. This relationship is expected to cause a considerable paramagnetic effect on the chemical shift of H-3'.¹² However, the characteristic signal due to H-3' always appears in a relatively higher field than other ring-protons (Table I). Thus, possible structure 9' was considered less favourable. On the other hand, if the 6-membered ring assumes a chair-conformation (structure 9), the hydroxyl at C-6' should be axial because of the small J₆',₇'=1.8. The small J₆',₅'=4.0~4.6 indicates an axial orientation of the
ureido group. These inspections lead to formulation of structure 9 for nucleoside A.

![CD Spectra of 1, 2, 9, and Cytidine in H2O.](image)

From CD spectra of 1, 2 and 9 (Fig. 3), the configuration of the 1-substituted cytosine in these compounds is determined as β.13) The structure of 9 was therefore deduced to be 1-(3′,7′-anhydro-5′-deoxy-5′-ureido-β-D-threo-β-D-allooctofuranosyluronic acid)-cytosine. In the PMR spectrum of 2, H-1′′ appeared at 4.74 as a doublet ($J_{1′′,2′′}=6.5$). This large coupling constant favoured β-glycoside linkage.14) Accordingly, the structure of ezomycin A₂ was identified as 2 (Fig. 4).

Hydrogenation of 1 over Raney nickel catalyst liberated alanine, indicating that L-cystathionine is attached to 2 in the homocystein part.

![The lactam-aminohemiacetal (8), [α]_D^27=-43.2°, obtained by the periodate oxidation of 1 described above, gave a pair of anomeric proton signals at 5.60 (0.5H, t, 6.0) and 5.80 (0.5H, d, 6.0) in its PMR spectrum. On mild alkaline hydrolysis 8 gave cystathionine sulfoxide (11) in a good yield, while 8 considerably resisted to acid hydrolysis to yield a small amount of 11. Reduction of 8 with NaBH₄ gave N-(2′,4′-dihydroxybutyryl)-L-cystathionine sulfoxide (12). In the PMR spectrum of 12 the pair of anomeric proton signals disappeared and a triplet (4.15, 2H, t, 6.5) ascribable to a hydroxymethyl group having two vicinal hydrogens was observed. Accordingly, a lactam-aminohemiacetal structure* was assigned to 8 as depicted in Fig. 5. Compound 12 was readily hydrolyzed with 0.5 N HCl at 90°C in 30 min to yield L-

![Fig. 5.](image)

* Formation of 8 on the periodate oxidation is compared with the liberation of pyrrolidine sugar on a controlled cleavage of 1-acetamido-1-deoxy-o-ribitol.13)
cystathionine sulfoxide (11) C$_7$H$_4$N$_2$O$_5$S and α-hydroxybutyrolactone (13): C$_5$H$_6$O$_3$; M$^+$ m/e 102; [6]$^2_30^1$ + 5800. The absolute configuration of 13 was determined as S by the positive Cotton effect at 222 nm. This confirmed the β-configuration of 4.$^{16}$ Thus, the structure of ezomycin A$_1$ was proposed as 1.

On the way of periodate oxidation of 1, an unexpected reaction was observed. Compound 12 was hydrolyzed very easily with 0.5 N HCl at 80°C in 20 min, whereas 8 resisted to hydrolysis under the same reaction conditions. The facile hydrolysis of the former is rationalized by the participation of C-4' hydroxyl in the hydrolysis as shown in Fig. 5. On the other hand, the resistance of 8 to this hydrolysis is ascribable to the stabilization by the formation of the aminohemiacetal structure.

As that of a natural antibiotic, structure 1 for ezomycin A$_1$ is of considerable interest. Ezomycin A$_1$ is the first naturally occurring nucleoside antibiotic which contains a novel bicyclic anhydrooctose uronic acid.*

**EXPERIMENTAL**

Melting points were determined on a hot stage and uncorrected. The UV, IR and PMR spectra were recorded on a Hitachi 124 spectrophotometer, a Perkin-Elmer 521 grating infrared spectrophotometer and a Varian HA-100D NMR spectrometer, respectively. The solvent, 2% ND$_3$ in D$_2$O, for PMR spectra determination was prepared by diluting 20% ND$_3$ in D$_2$O. Tetramethylsilane was used as an external standard. Mass spectra measurements were performed on a JEOL JMS-OISG spectrometer. The trimethylsilylation of samples was carried out as follows. Each sample (50~100 µg) was treated in a sealed capillary at 80°C for 30 min with 10~20 µl of N,O-bis-trimethylsilyl trifluoroacetamide(BSTFA)-trimethylchlorosilane(TMCS)pyridine (10:1:5) which was prepared just before use. The reaction mixture was immediately submitted to evaporation for 10 min. The resulting suspension of the Avicel in n-BuOH was added to make a suspension. The water of the mixture was removed by evaporation. The resulting suspension of the Avicel in n-BuOH was introduced onto a column (1.6 x 60 cm) of Avicel which was packed with n-BuOH and n-BuOH was added to make a suspension. The sample solution was absorbed with a small amount of Avicel and n-BuOH was added to make a suspension. The water of the mixture was removed by evaporation. The resulting suspension of the Avicel in n-BuOH was introduced onto a column (1.6 x 60 cm) of Avicel which was packed with n-BuOH and AcOH-H$_2$O (7:1:2). The column was eluted with the same solvent. The fractions showing UV absorption were combined, concentrated and kept at 5°C overnight to produce needles (30 mg) of [α]$_{D}^{25}$ +218° (c=1.2, 1 N NH$_4$OH); mp > 257°C; [6]$^2_30^1$ (257, 1000); [6]$^2_30^1$ (1000, 10,700); [6]$^2_30^1$ (10,700, 257, 257). M $^+$ for C$_{13}$H$_{16}$N$_4$O$_8$(TMS)$_5$, 641, 597, 584, 569, 551, 541, 526, 501, 472, 451, 429, 341, 298, 257, 241, 185, 169; Found: C, 43.86; H, 4.02; N, 15.61%. Calcd. for C$_{13}$H$_{16}$N$_4$O$_8$: C, 44.07; H, 3.98; N, 15.82%.

**Esterification of 5 with methanolic hydrogen chloride followed by acetylation**

A mixture of 5 (18 mg) and dry methanol (10 ml) containing 5% HCl was refluxed for 30 min and kept at r.t. overnight to yield needles (14 mg) of methyl ester of 5: [α]$_{D}^{25}$ +218° (c=1.2, 1 N NH$_4$OH); mp > 257°C (dec.); [α]$_{D}^{25}$ (257, 1000); [α]$_{D}^{25}$ (1000, 10,700); [α]$_{D}^{25}$ (10,700, 257, 257). M $^+$ for C$_{13}$H$_{16}$N$_4$O$_8$(TMS)$_5$, 641, 597, 584, 569, 551, 541, 526, 501, 472, 451, 429, 341, 298, 257, 241, 185, 169; Found: C, 43.86; H, 4.02; N, 15.61%. Calcd. for C$_{13}$H$_{16}$N$_4$O$_8$: C, 44.07; H, 3.98; N, 15.82%.

* Recently Isono et al. reported isolation and structural determination of octosyl acids A, B and C which also have bicyclic anhydrooctose uronic acids as their sugar parts.$^{17}$
A mixture of 9 (15 mg) and dry MeOH (10 ml) containing 5% HCl was heated under reflux for 1 hr, and then evaporated with occasional additions of dry methanol to remove remaining HCl to yield crude methyl ester of 9. After drying over NaOH pellets, the methanol to remove remaining HCl to yield crude and then evaporated with occasional additions of dry ethylene glycol, the reaction mixture was concentrated and applied onto a Sephadex G-15 column (2 x 90 cm), which was developed with water. The fractions showing UV absorption and positive coloration with ninhydrin were combined and reacted with 1 ml of acetic anhydride (0.5 ml) at r.t. for 48 hr. The reaction mixture was kept at 5°C for 48 hr. After treatment with 2 drops of ethylene glycol, the reaction mixture was concentrated and applied onto a Sephadex G-15 column (2 x 90 cm), which was developed with water. The fractions showing UV absorption and positive coloration with ninhydrin were combined and reacted with 1 ml of a phenylhydrazine reagent (1 g of phenylhydrazine was filled up to 10 ml with acetic acid) at pH 1 with stirring at r.t. for 4 hr. The ethyl acetate extracts of the reaction mixture gave an orange-yellow material after usual work-up. Crystallization from benzene-hexane gave orange crystals. The product was identified as glyoxal bisphenylhydrazone by comparison of its IR with that of the authentic sample.

The water layer after extraction with ethyl acetate was neutralized with 2 N NaOH, concentrated and applied onto a column (2 x 95 cm) of DEAE-Sephadex A-25 packed with 0.2 M pyridine-acetate buffer (pH 4.8). The column was developed with the same buffer (fraction size, 15 ml). Fractions (No. 51~58) positive to ninhydrin were combined, concentrated and lyophilized to yield white powder (85 mg) of 8: [a]$_D^{20}$ = -43.2° (c=0.50, H$_2$O); PMR $\delta$ (CDCl$_3$-McOH-d$_4$-3:1, 60 MHz) 2.18, 2.30, 3.91 (3H each, s), 5.6~5.8 (3H), 6.34 (1H, $s$, 6.0), 7.50 (1H, d, 7.6).

Fractions (No. 76~87) were combined and concentrated to a small volume. After addition of a small volume of MeOH the solution was kept at 5°C to give white crystals (95 mg). Recrystallization from benzene-hexane followed by acetylation to preparative TLC (silica gel GF$_{254}$, 0.5 mm thickness; CHCl$_3$-MeOH, 14: 1) to yield 13 mg of 10 as the main product. Crystallization from acetone-ethanol gave white powder of 10: mp $>280°C$ (dec.); [a]$_D^{20}$ +68.7 (c=0.15, CHCl$_3$-MeOH=1: 1); $\lambda_{MeOH}^{max}$ nm (c) 212 (20,700), 249 (18,700), 297 (8400), PMR (Table 1); Found: C, 47.04, H, 4.93; N, 35.89%. Calcd. for C$_{22}$H$_{27}$N$_6$O$_{12}$: C, 47.74; H, 4.92; N, 12.65%.

**Treatment of 1 with Raney nickel catalyst**

A water solution (1 ml) containing 3.5 mg of 1 was treated at 70°C for 1 hr with 0.5 ml of Raney nickel freshly prepared by the method of Driel et al.\textsuperscript{18} After removal of the catalyst by centrifugation, the supernatant was subjected to TLC analysis (silica gel G and cellulose layer, solvent C). Alanine was detected as the sole amino acid in the reaction mixture.

**Sodium borohydride reduction of 8 followed by acid hydrolysis**

A solution of 8 (27 mg) in 8 ml of water was treated with NaBH$_4$ (15 mg) at r.t. for 1.5 hr. After addition of 1 N acetic acid to destroy the excess NaBH$_4$, the reaction mixture was neutralized with dil. NH$_3$:OH, concentrated and applied onto a Sephadex G-10 column (1.5 x 90 cm). The column was developed with water (fraction size, 4 ml). Fractions (No. 24~26) were combined and lyophilized to yield white powder (25 mg) of 12: PMR $\delta$(D$_2$O) 2.3 (2H, $m$), 2.65 (2H, $m$), 3.45 (2H, $t$, 7.5), 3.82 (2H, $d$, 6.0), 4.15 (2H, $t$, 6.5), 4.72 (2H, $m$).

A mixture of 12 (25 mg) and 0.5 N HCl (4 ml) was heated at 90°C for 30 min. After evaporation to dryness, the residue was dissolved in water (2 ml) and extracted with ethyl acetate. The combined extracts were concentrated to a small volume, mixed with a small amount of silica gel and introduced onto a column (1.0 x 20 cm) of silica gel G packed with CHCl$_3$. The column was developed with 2% MeOH in CHCl$_3$. Fractions showing a spot positive to a KMnO$_4$ reagent on TLC were combined and evaporated to dryness to give 2 mg of 13: MS m/e (relative intensity) 104 (M$^+$, 1.5), 90 (3.7), 85 (6.3), 83 (9.3), 75 (19.6), 58 (15.9), 57 (27.8), 45 (14.1), 32 (44.4), 31 (83.3), 29 (13.0), 28 (17.8), 18 (100.0); CD (MeOH) [a]$_{252}$ +5800.

The water layer after extraction with ethyl acetate was adsorbed on Avicel (0.5 g). The powder was suspended in n-BuOH and applied onto a column (2 x 50 cm) of Avicel packed with n-BuOH-AcOH-water (4: 1: 2). The column was developed with the same solvent (fraction size, 10 ml). Fractions (No. 30~44) were combined and evaporated to dryness. The residue (8 mg) was crystallized from aqueous-ethanol to give a white powder (5 mg) of 11: mp $>210°C$ (dec.); [a]$_D^{20}$ +10.2° (c=0.52, 0.1 N NaOH); PMR $\delta$(D$_2$O) 2.42 (2H, $m$), 3.22 (2H, $m$), 3.53 (2H, $d$, 6.0), 4.62 (2H, $m$), 5.65 (0.5H, $t$, 6.0), 7.50 (0.5H, $d$, 6.0).
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6.0), 3.98 (1H, t, 6.0), 4.32 (1H, m); Found: C, 35.25; H, 5.86; N, 11.47. Caled. for C$_7$H$_{14}$N$_2$O$_5$S: C, 35.28; H, 5.92; N, 11.76%; MS m/e 526 [M$^+$ for C$_7$H$_{14}$N$_2$O$_5$S (TMS)$_4$], 511, 409, 354, 294, 232, 218, 160.

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