HATOMAMICIN (YL-0358M-A), A NEW ALKALOID ANTIBIOTIC: FERMENTATION, ISOLATION, STRUCTURE, AND BIOLOGICAL PROPERTIES

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Hatomamicin, a new alkaloid antibiotic, was isolated from the culture filtrate of a strain of *Saccharopolyspora*. The antibiotic was extracted with EtOAc and purified by silica gel column chromatography. The free alkaloid was obtained as pale yellowish prisms from CH_3CN solution. It exhibits antimicrobial activity against Gram-positive organisms. The apparent molecular formula of hatomamicin was determined to be $C_{22}H_{31}NO_5$. The structure has been established by a combination of spectroscopic and X-ray crystallographic studies.

In the course of our screening for new antibiotics, a new alkaloid antibiotic hatomamicin was found. The antibiotic was produced by a strain of *Saccharopolyspora*. The strain YL-0358M was isolated from a soil sample collected at Hatoma Island, Okinawa Prefecture, Japan. The type strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, and assigned as *Saccharopolyspora* sp. YL-0358M with the accession No. FERM P-8862. Taxonomic studies of the strain are now in progress.

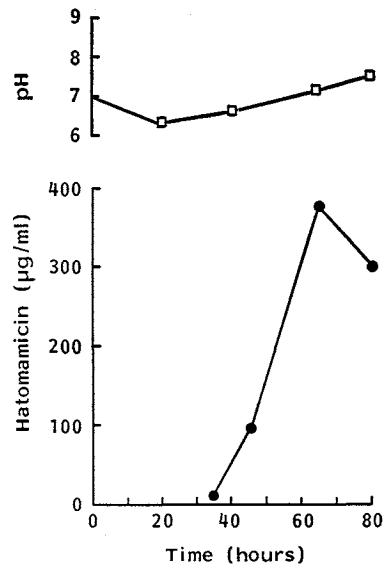
The fermentation, isolation, physico-chemical properties, structure determination and biological properties of hatomamicin are described in this paper.

Fermentation

A loopful of well-grown agar slant culture of strain YL-0358M was inoculated into a seed medium (60 ml) containing dextrin 2.0%, glucose 0.5%, Polypepton 0.5%, yeast extract 0.5%, corn steep liquor 0.5%, brain heart infusion 0.52%, and $CaCO_3$ 0.2% (pH 8.0) in a 500-ml Erlenmeyer flask, and cultured at 28°C on a rotary shaker with 7.6 cm throw at 200 rpm for 48 hours. The seed culture was then transferred at the rate of 3.0% to 80 liters of the production medium in a 100-liter jar fermenter and cultiva-

Fig. 1. Time course of the production of hatomamicin.

□ pH, ● hatomamicin.



tion was carried out for 65 hours at 28°C under aeration of 100 liters/minute, agitation of 300 rpm. The production medium contained potato starch 3.0%, wheat germ 1.0%, copra meal 1.0%, feather meal 0.2% and $CaCO_3$ 0.15% (pH 7.0). A typical time course for fermentation is shown in Fig. 1. The antibiotic production was monitored by a paper-disk assay using *Bacillus subtilis* ATCC 6633 as a test organism. Hatomamicin production started at 40 hours after inoculation, then gradually increased and reached a maximum (380 μg/ml) at 62~65 hours.

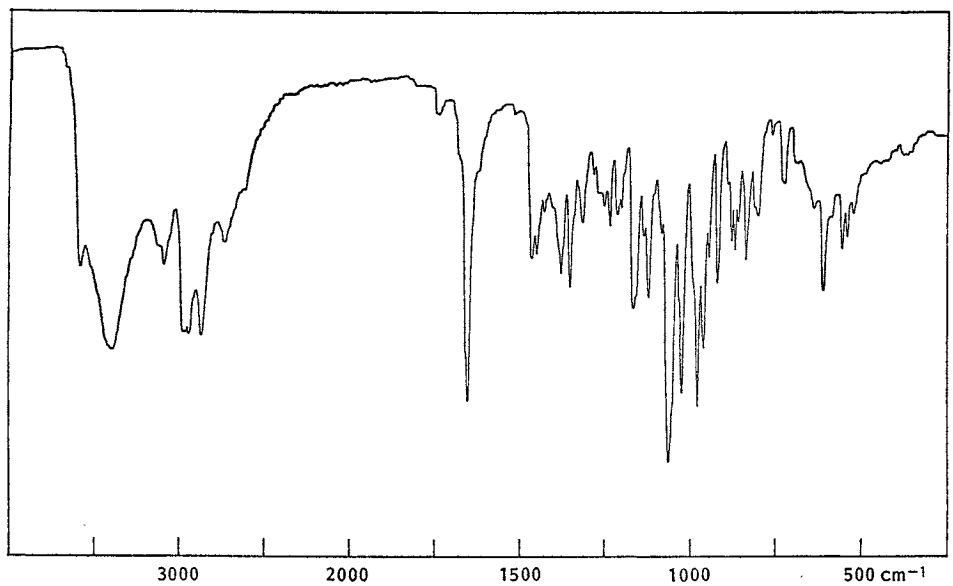
Isolation

Fermentation broth (70 liters) was filtered through a bed of filter-aid (Radiolite). The filtrate (60 liters) was adjusted to pH 8.5 with a sodium hydroxide solution and extracted with EtOAc (60 liters). The organic solute was transferred to a dilute hydrochloric acid solution (10 liters). The aqueous layer was reextracted with EtOAc at pH 8.5 and this extract was concentrated *in vacuo* to yield a brown paste (14 g). The crude material was purified by silica gel column chromatography. The column was developed with $CHCl_3$ - MeOH (50:1). Hatomamicin was monitored by TLC on Silica gel 60 F_{254} plates (Merck, Art. No. 5715) with $CHCl_3$ - MeOH (5:1). Purified active fractions were collected and concentrated *in vacuo* to give a pale yellowish powder which was crystallized from EtOAc to give hatomamicin (8 g).

Physico-chemical Properties and Structure Determination

Hatomamicin was obtained as pale yellowish prisms. It is soluble in MeOH, EtOH, CH_3CN, acetone, EtOAc, $CHCl_3$, slightly soluble in benzene and insoluble in water. The antibiotic is labile in a solution but stable in crystalline form. A solution of the compound turned gradually to reddish brown in color and finally lost biological activity. On a silica gel TLC, it gave a single spot at Rf 0.45 ($CHCl_3$ - MeOH, 9:1). The fast atom bombardment mass spectrum (FAB-MS) showed a *quasi*-molecular ion peak at m/z 390 $(M+H)^+$ in the positive ion mode. The elemental composition of this ion was indicated to be $C_{22}H_{32}NO_5$ by a high-resolution (HR)FAB-MS (Found: 390.2363, Calcd:

Fig. 2. IR spectrum of hatomamicin (KBr).



390.2446 for $C_{22}H_{32}NO_5$). The IR spectrum is shown in Fig. 2. The physico-chemical properties of hatomamicin are summarized in Table 1.

The single crystal X-ray diffraction study revealed its molecular structure. The bond lengths and angles are in good agreement with expected values. The final atomic parameters, bond distances and bond angles, and Fo-Fc table have been deposited at The Cambridge Crystallographic Data Center. The molecular structure is shown in Fig. 3.

^{13}C NMR spectrum of hatomamicin showed 22 carbon signals; 3 methyls (δ_C 10.35, 17.11 and 24.28), 5 methylenes (δ_C 21.91, 25.07, 25.42, 29.81 and 44.70), 6 methines (δ_C 59.97, 66.52, 74.18, 74.94, 75.59 and 99.13), 1 quaternary carbon atom (δ_C 55.34) and 7 olefinic carbon atoms (δ_C 118.56, 130.26, 135.08, 136.66, 137.10, 143.54 and 170.83). The ^1H NMR chemical shifts and their assignment are compatible with the molecular structure determined by the X-ray study; ^1H NMR δ 0.94 (3H, t, J= 6.8 Hz, 15-H), 1.28 (3H, d, J=6.6 Hz, 21-H), 1.30~2.40 (9H, m, 2, 14, 17, 18-H and OH), 2.15 (3H, d, J=1.2 Hz, 22-H), 2.70 (1H, br s, OH), 3.40~4.40 (6H, m, 1, 3, 13, 19, 20-H), 4.40 (1H, m, 16-H), 4.65 (1H, dd, J=9.6 and 3.1 Hz, 12-H), 5.55 (1H, dq, J=9.6 and 1.2 Hz, 11-H), 6.11 (1H, s, 9-H), 6.64 (1H, dd, J=6.1 and 1.2 Hz, 6-H), 7.63 (1H, d, J=6.1 Hz, 7-H) (proton numbering corresponds to X-ray structure numbering). The ^1H NMR spectrum is presented in Fig. 4. Thus, the molecular structure has been established as shown in Fig. 5.

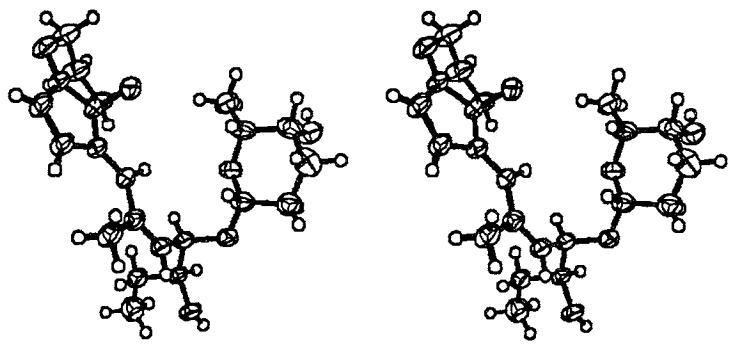
Hatomamicin consists of three parts, pyrindine ring, trideoxyhexopyranose, and 2-heptenylidene chain which connects the two rings. The 5-position of the pyrindine ring links to the 2-methyl-5-hydroxy-2-heptenylidene group. The methyl group at the 2-position of the 2-heptenylidene group is in the *trans* position relative to the six-membered ring of the pyrindine. The heptenylidene links to the 2,3,6-trideoxyhexopyranose through a glycoside bond. The trideoxyhexopyranose is identical with rhodinose.[1]

Hatomamicin is similar to the known alka-

Table 1. Physico-chemical properties of hatomamicin.

Appearance	Pale yellowish prisms
$[\alpha]_D^{25}$	+88° (c 1, MeOH)
MP	93°C
UV λ_{max}^{MeOH} nm (ε)	297 (14,200), 320 (sh, 13,600)
IR ν_{max}^{KBr} cm^{-1}	3575, 3380, 3090, 2960, 2940, 2860, 2730, 1650, 1465, 1450, 1380, 1355, 1065
FAB-MS (m/z)	390 $(M+H)^+$
Molecular formula	$C_{22}H_{31}NO_5$

Fig. 3. Stereoscopic view of hatomamicin with 50% probability ellipsoids.



The relative configuration is 3R*, 4S*, 12R*, 13S*, 16S*, 19R*, 20R*, corresponding to the crystallographic numbering scheme in Fig. 5. The absolute configuration is arbitrarily depicted to correspond to that of latumcidin.[4]

Fig. 4. ^1H NMR spectrum of hatomamicin in $CDCl_3$ at 100 MHz.

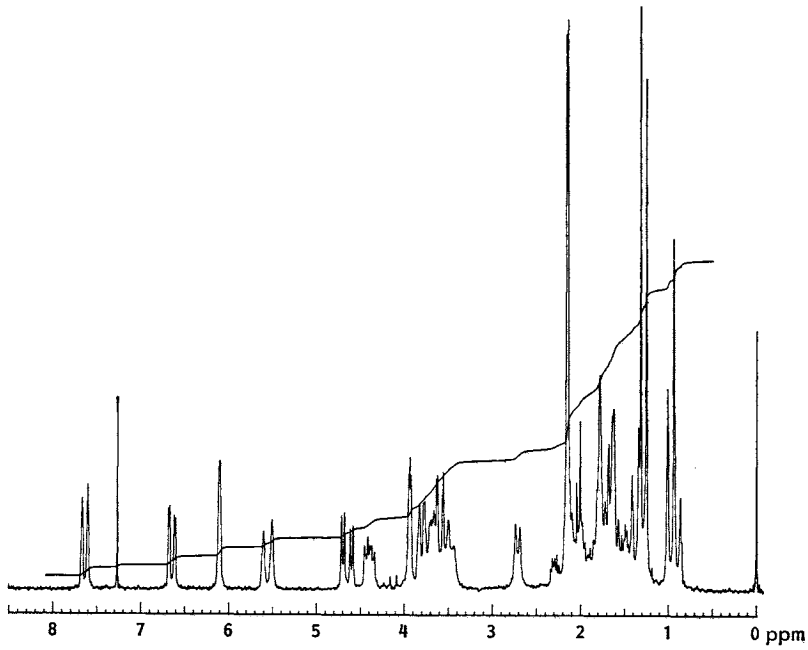
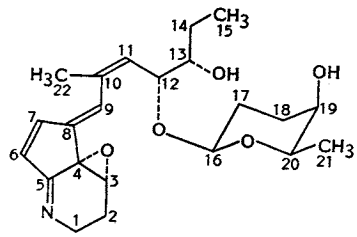


Fig. 5. Structure of hatomamicin.



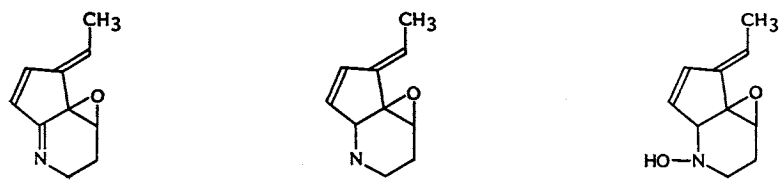
loid antibiotics such as abikoviromycin[2,3] (latumcidin[4~7]) (**1**), dihydrolatumcidin[8] (SF-973 C[9]) (**2**) and *N*-hydroxydihydroabikoviromycin[10] (**3**). Structures of these compounds are shown in Fig. 6.

Table 2. Antimicrobial spectrum of hatomamicin.

Test organisms	MIC (μg/ml)
Staphylococcus aureus FDA 209P JC-1	>100
S. epidermidis IID 866	>100
Streptococcus pyogenes Cook	25
Enterococcus faecalis IID 682	50
E. faecium CAY 09 1	100
Mycobacterium smegmatis ATCC 607	>100
Escherichia coli O-1	>100
Citrobacter freundii CAY 17 1	>100
Klebsiella pneumoniae ATCC 10031	>100
Proteus vulgalis OXK US	>100
Pseudomonas aeruginosa NCTC 10490	>100

Inoculum size: 10^6 cfu/ml, medium: Mueller-Hinton agar.

Fig. 6. Structures of abikoviromycin (**1**), dihydrolatumcidin(**2**) and *N*-hydroxydihydroabikoviromycin (**3**).



Biological Properties

The antimicrobial activity of hatomamicin was determined by a conventional agar dilution method using Mueller-Hinton medium for Gram-positive and Gram-negative organisms. MIC was expressed in terms of μg/ml after overnight incubation at 37°C. Due to poor solubility in H_2O, hatomamicin was initially dissolved in DMSO and subsequently diluted with the medium. The results are summarized in Table 2. It shows weak antimicrobial activity against Gram-positive organisms.

The cytotoxic activity of hatomamicin was determined as follows. The concentration of the antibiotic required for 50% inhibition of cell growth (IC_{50}; μg/ml) was examined by plotting the logarithm of the concentration versus the growth rate (percentage of control) of the treated cells. Hatomamicin exhibits cytotoxic activities against lymphoid leukemia L1210 and leukemia P388 with IC_{50} value of 0.26 and 0.26 μg/ml, respectively. It has been reported that the known alkaloid antibiotics possess several pharmacological activities, and it is interesting that hatomamicin is similar. Further investigation of its activities is now in progress.

Experimental

Spectroscopic Work

FAB-MS measurement was done with a Jeol JMS-DX double focusing spectrometer using glycerol matrix. IR spectra in pressed KBr disc were recorded on a Hitachi 260-50 IR spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Jeol FX-100 spectrometer with $CDCl_3$ solvent; chemical shifts are given in ppm relative to TMS (0 ppm) as an internal standard and coupling constants (J) are given in Hz.

X-Ray Crystallographic Work

Prismatic crystals were deposited from CH_3CN solution. Diffraction data were collected with graphite-monochromated CuKα radiation (λ=1.54184 Å). Accurate unit cell dimensions were derived from high-angle 2θ values. The crystal data are as follows; chemical formula $C_{22}H_{31}NO_5 \cdot 2H_2O$, monoclinic, space group C2, a=22.70(1), b=9.519(6), c=10.786(6) Å, β=90.51(5)°, Z=4, D_{calc}=1.213 gcm^{-3}.

Intensities were measured in the $\omega/2\theta$ scan mode with a scan speed of 2° (in ω) minute^{-1} and a scan width of 1.8+0.14 tan θ (in ω). Five reference reflections monitored showed no significant intensity deterioration. Of 1982 independent reflections with the range of $3° < 2\theta < 125°$, 40 weak reflections below background were considered to be zeroreflections. Corrections were made for Lorentz factor but not for absorption. The standard deviations were estimated by the equation,[11]

$$\sigma(|Fo|) = \sigma^2_p(|Fo|) + q^2Fo^2$$

where q(=0.02476) was derived from the variation of the monitored reflections and $\sigma_p(|Fo|)$ was due to counting statistics.

The structure was solved by the direct method[12] and its parameters were refined by the block-diagonal least-squares method. The quantity minimized was $\Sigma\omega(|Fo|-|Fc|)^2$ with $\omega = 1/\sigma(Fo)$. In the refinement, the zero-reflections with $|Fc| > F_{lim}$ were included by assuming $|Fo| = F_{lim}$, F_{lim} being the threshold value. A difference Fourier calculation indicated the presence of two water molecules as crystal solvent. The atomic identification was based on the thermal parameters and the geometries of bonds. Hydrogen atoms except those of water molecules were located on a difference map and were included in the refinement. The final R value was 0.041 for 1833 reflections with $|Fo| > 3\sigma(Fo)$ with the maximum shift of parameters of 0.21σ for non-hydrogen atoms. Atomic scattering factors used were taken from International Tables for X-ray Crystallography Vol. IV (Kynoch Press, Birmingham, England, 1974).

Acknowledgments

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