ASPARENOMYCINS A, B AND C, NEW CARBAPENEM ANTIBIOTICS
II. ISOLATION AND CHEMICAL CHARACTERIZATION

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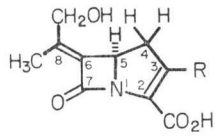
New carbapenem antibiotics named asparenomycins A, B and C were isolated from the fermentation broths of *Streptomyces tokunonensis* sp. nov. and of *Streptomyces argenteolus*. The fermentative production, isolation and physico-chemical properties of these antibiotics are described.

In recent several years, a number of β-lactam antibiotics that are characterized by having a 7-oxo-1-azabicyclo[3.2.0]hept-2-ene ring system including thienamycin[4] and its derivatives (*N*-acetylthienamycin[5], *N*-acetyldehydrothienamycin[6] and epithienamycins[7]), the olivanic acid derivatives (MM 4550[8], MC696-SY2-A[10], MM 13902[8], MM 17880[8], MM 22380[9], MM 22381[9], MM 22382[9] and MM 22383[9]), PS-5[11] and its related antibiotics (PS-6[12], PS-7[12] and PS-8[13]), and the carpetimycins A and B[14] (C-19393 S_2 and H_2[15]) have been isolated from *Streptomyces* species. This family of antibiotics has been given the name carbapenem antibiotics[15]. We also have isolated new members of this family, the asparenomycins A, B and C*, from the culture broth of *Streptomyces* strain PA-31088, identified as *Streptomyces tokunonensis* sp. nov.[2]. Asparenomycin A is the major product, and asparenomycins B and C are extremely minor products. PA-31088 also produces penicillin N (as a main product), olivanic acid derivatives and carpetimycins A and B. Asparenomycins A, B and C were isolated also from the culture broth of another strain, PA-39504, identified as *Streptomyces argenteolus*[2].

The structures of asparenomycins A, B and C are as in Fig. 1[3], the antibiotics being unique in having a substituted ethylidene side chain. In this paper, fermentative production, isolation and physico-chemical properties of these antibiotics are presented. A preliminary account of a portion of these studies was reported previously[1].

Fig. 1. Structures of asparenomycins A, B and C.



	R
Asparenomycin A	$-S(\rightarrow O)(R)-C(H)=C(H)-NHCOCH_3$
Asparenomycin B	$-S(\rightarrow O)(R)-CH_2-CH_2-NHCOCH_3$
Asparenomycin C	$-S-C(H)=C(H)-NHCOCH_3$

* Asparenomycin A was originally designated as PA-31088-IV in Japan Kokai (Patent) 55-13,628 (Aug. 10, 1980), and asparenomycin B as PA-39504-X_1 in Japan Kokai (Patent) 56-83, 489 (July 8, 1981) and asparenomycin C as PA-39504-X_3 in Japan Kokai (Patent) (in application).

Assay Procedure

Antimicrobial Assay

A mutant of *Escherichia coli* JC-2, hypersensitive to β-lactam antibiotics, was used as the test organism in a pulp disk agar diffusion method.

β-Lactamase Inhibition Assay

A modified method of the agar diffusion method devised by BROWN *et al.*[16] was used. An enzyme preparation of a penicillinase was prepared from cultured cells of *Escherichia coli* K-12, JC-411 (col El-Ap)* as the supernatant of the disrupted cells obtained by ultrasonication. An assay plate was prepared using nutrient agar containing penicillin G at a concentration of 10 μg/ml and the enzyme preparation at a concentration to just give complete hydrolysis of the substrate. The plate then was seeded with *Staphylococcus aureus* JC-1. Another enzyme preparation, a cephalosporinase, was prepared from cultured cells of *Proteus morganii*-7** and an assay plate using this enzyme preparation was prepared similarly. These assay plates must be used within 30 minutes after preparation, because of rapid hydrolysis of the substrate even at room temperature.

Pulp disks soaked with test samples were placed on the assay plate and the plate was incubated overnight. Diffusible β-lactamase inhibitors gave inhibitory zones because the presence of penicillin G protected from hydrolysis by the enzyme; the diameters of the inhibitory zones corresponded to the inhibitory activities of the test samples.

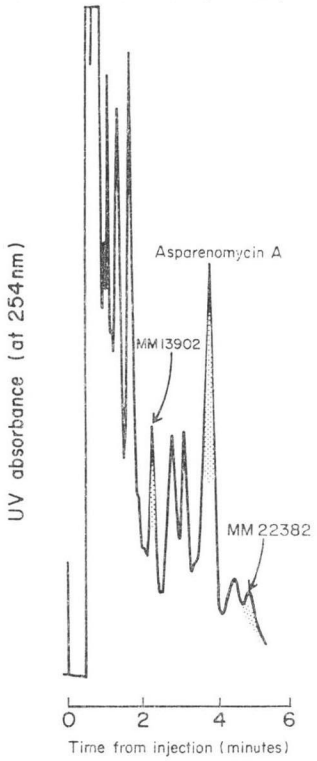
Asparenomycins exhibited relatively stronger inhibition activity to the former enzyme preparation than that to the latter enzyme preparation when compared with olivanic acid derivatives which were simultaneously produced by PA-31088. This fact was an aid in the detection of the asparenomycins during isolation process.

High Performance Liquid Chromatography (HPLC)

HPLC was carried out using a Waters Model 6000A pump with a Waters Model U6K injector. The chromatograph was monitored by a Japan Spectrooptics UVIDEC-100-II variable wave length UV spectrometer at 254 nm and 220 nm. A steel column (4×250 mm) packed with Nucleosil 10 C_{18} (Macherey-Nagel) was used at a flow rate of 2.0 ml/minute. For analysis of fermentation broths or crude samples, a gard column (Waters Co., Ltd.) packed with the same adsorbent was joined with the above column. The mobil phase used was 20 mM phosphate buffer, pH 7.0.

Samples to be analyzed were divided into two portions. One portion was chromatographed directly and the other was injected after treatment with hydroxylamine at pH 7.0 for 30 minutes at room temperature. The hydroxylamine treatment caused rapid degradation of the carbapenem antibiotics and penicillin N. By comparing the two chromatograms, hydroxylamine sensitive

Fig. 2. High performance liquid chromatography of culture filtrate from *S. tokunonensis* n. sp.
Conditions: Nucleosil 10 C_{18} column (4×250 mm). 20 mM Phosphate buffer solution, pH 7.0. Flow rate 2 ml/minute. Monitor at 254 nm.
Shadowed portions of peaks express the lost parts by hydroxylamine treatment (see text).



* The strain carrying the plasmid col El-Ap, highly resistant to ampicillin, was isolated in our laboratory.
** Clinical isolate.

peaks were easily detected and identified by comparing with those of reference samples under the same chromatographic condition. The content of asparenomycin A or other carbapenems was calculated from the peak height of standard solutions and the loss of peak height of test samples.

Since the culture broth of PA-31088 contained several carbapenem antibiotics, quantitative estimation of asparenomycin A in the broth could be attained only by this HPLC method. A typical example of the analysis of the culture broth is illustrated in Fig. 2.

Fermentation

Spores of PA-31088 were inoculated into 800 ml of a medium consisting of soluble starch 0.5%, glucose 0.5%, polypeptone 0.5%, meat extract 0.5%, yeast extract 0.25%, NaCl 0.25% (pH 7.0 before sterilization) in a 2-liter Erlenmeyer flask, and cultured at 28°C on a rotary shaker for 48 hours. The broth was then transferred into a 30-liter jar fermentor containing 20 liters of a medium consisting of tomato paste 2.4%, dextrin 2.4%, dry yeast 1.2%, $CoCl_2 \cdot 6H_2O$ 0.0006% (pH 7.0 before sterilization). Fermentation was carried out at 28°C under agitation of 300 r.p.m., aeration of 20 liters per minute and inner pressure of 0.5 kg/cm^2. Production of antibiotics was followed by the antimicrobial assay, β-lactamase inhibition assay and by the HPLC. The production of asparenomycin A reached maximum after 65 hours of fermentation. An average of several fermentation batches yielded approximately 100 μg/ml of penicillin N, 7~9 μg/ml of asparenomycin A, 3~4 μg/ml each of MM 17880 and MM 13902 and 0.5 μg/ml of MM 22382. The concentrations of asparenomycins B and C were so low, they were detectable only in partially purified preparations.

Isolation

1) Isolation of Asparenomycin A

All isolation and purification steps were carried out below 10°C, and followed by the antimicrobial and β-lactamase inhibition assays and finally confirmed by the HPLC. The harvested broth was cooled to 10°C and centrifuged by a Sharples centrifugal separator after adjusting the pH to 7.0 and the addition of sodium ethylenediaminetetraacetate (EDTA) to a concentration of 50 μg/ml. The supernatant fluid (100 liters) was passed through a column (7 liters) of an Amberlite IRA-68 (Cl^-) (Rohm and Haas Co., Ltd.) which was previously equilibrated with 10 mM phosphate buffer, pH 7.0. The major portion of the penicillin N was removed to the effluent. The column was washed with cold water and eluted with 5% NaCl in 10 mM phosphate buffer, pH 7.0, containing 10 μg/ml of EDTA. The active eluate fractions (*ca.* 12 liters) by the antimicrobial and β-lactamase inhibition assays were collected and passed through a column (7 liters) of Diaion HP-20 (Mitsubishi Kasei Kogyo Co., Ltd.). The majority of active substances were eluted with water containing 10 μg/ml of EDTA, and the active eluate (*ca.* 12 liters) was carefully adjusted to pH 5.5 with KH_2PO_4 and adsorbed on 300 g of an active carbon (Darco G-60) by batch method. The carbon cake was extracted with 60% aqueous acetone containing 10 μg/ml of EDTA under adjusting to pH 7.0 with $NaHCO_3$. Evaporation and freeze-drying gave a crude powder (20 g).

The HP-20 column was further eluted with 50% aqueous methanol containing 10 μg/ml of EDTA. Concentration and freeze-drying of the active eluate gave a crude mixture of minor products. This contained a trace amount of asparenomycin C and was used as the starting material for isolation of the antibiotic.

The crude powder was subjected to column chromatography on a Pre PAK-500/C_{18} column of a High Speed Liquid Chromatograph System 500 (Waters Co., Ltd.) with 50 mM phosphate buffer solution, pH 7.0, containing 10 μg/ml of EDTA. Approximately 4.0 g of the crude powder could be charged for

a run. The eluate fractions were monitored by the antimicrobial and β-lactamase inhibition assays. The fractions containing asparenomycin A were collected on referring to the characteristic inhibitory action of this antibiotic on the β-lactamases and the information from the HPLC analysis described above. To the collected fractions NaCl was added to 5% concentration and the solution was passed through a Diaion HP-20 column (400 ml). The column was eluted with water containing 1 μg/ml of EDTA. Concentration and freeze-drying of the active eluate gave a crude preparation of asparenomycin A (800 mg).

A trace amount of asparenomycin B was found in a crude powder obtained in a similar way from the fractions eluted just before the elution of asparenomycin A. This was used as the starting material for isolation of asparenomycin B.

The crude preparation of asparenomycin A was further chromatographed on a Sepacoal column (2.2×100 cm, Seikagaku Kogyo Co., Ltd.) packed with Diaion HP-20 AG (200~400 mesh) with 20 mM phosphate buffer solution, pH 7.0, containing 10 μg/ml of EDTA using an Altex Model 110-A pump (Mitsubishi Kasei Kogyo Co., Ltd.). Approximately 400 mg of the crude preparation could be charged for a run. The chromatogram was monitored by a Japan Spectrooptics UVILOG 5-III UV spectrometer at 254 nm. The fractions around the peak of asparenomycin A were examined for their purity by the HPLC analysis. The fractions containing exclusively asparenomycin A were collected and adsorbed on a Diaion HP-20 column (50 ml) as above. The column was eluted with distilled water, and the active eluate was freeze-dried to give a substantially pure preparation of asparenomycin A sodium salt (210 mg).

2) Isolation of Asparenomycin B

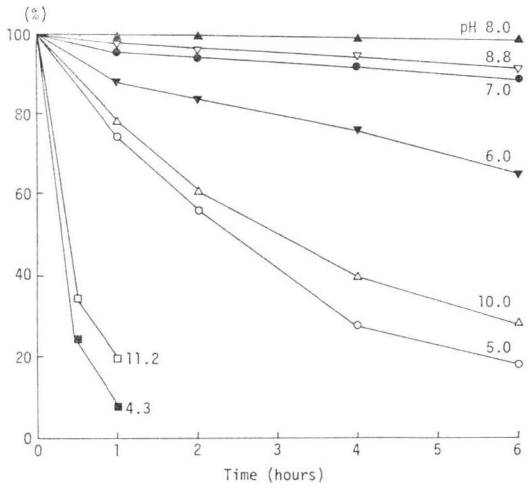
The crude powder containing asparenomycin B obtained as above were pooled from several fermentation lots. Some 2.0 g of the crude powder was chromatographed on the Sepacoal column packed with Diaion HP-20AG (200~400 mesh) in the same manner as above. The eluate fractions were monitored by the antimicrobial and β-lactamase inhibition assays. The fractions containing asparenomycin B were desalted on an HP-20 column and freeze-dried to give a crude preparation (195 mg). This preparation was further purified by chromatography on a column of QAE Sephadex A-25 (Pharmacia Fine Chemicals) with 0.1 M NaCl in 50 mM phosphate buffer, pH 7.0, containing 10 μg/ml of EDTA. The fraction of asparenomycin B was desalted on an HP-20 column and then freeze-dried to give a powder (21 mg). Final purification was achieved by preparative HPLC on a Nucleosil 10 C_{18} column (10×250 mm) with 20 mM phosphate buffer, pH 7.0, containing 10 μg/ml of EDTA. The peak fractions of this antibiotic were collected, desalted on an HP-20 column and freeze-dried to afford a substantially pure preparation of asparenomycin B sodium salt (6 mg).

3) Isolation of Asparenomycin C

Some 5.0 g of the crude mixture containing a trace amount of asparenomycin C described in the former section was fractionated by chromatography on a Pre PAK-500/C_{18} column with 10% MeOH in 50 mM phosphate buffer, pH 7.0, containing 50 μg/ml of EDTA. By pursuing the eluates by the antimicrobial and β-lactamase inhibition assays, two active fractions were found. The latter fraction was desalted on an HP-20 column and freeze-dried to give a powder (119 mg). Then the powder was chromatographed on a QAE Sephadex A-25 column with 0.2 M NaCl in 50 mM phosphate buffer, pH 7.0, containing 10 μg/ml of EDTA. By monitoring by the above assay methods, three active fractions were detected. The last fraction of the three was desalted and freeze-dried to give a crude preparation of asparenomycin C (4 mg). Preparative HPLC on a Nucleosil 10 C_{18} column (10×300 mm) with 5%

Fig. 3. Stability of asparenomycin A in aqueous solution of various pHs at 25°C.

Solutions at 100 μg/ml were used in this experiment and the remained concentrations were measured by HPLC method.



MeOH in 100 mM phosphate buffer, pH 7.0, containing 10 μg/ml of EDTA was carried out with the crude preparation, and followed desalination on an HP-20 column and freeze-drying gave a preparation of asparenomycin C sodium salt (approximately 1.0 mg), which exhibited a single peak by analysis with the HPLC method.

Table 1. Approximate Rf values of asparenomycins A, B and C in thin-layer chromatography.

Precoated cellulose plates (Eastman Chromatogram Sheet) pretreated with 10 mM phosphate buffer, pH 7.0, containing 10 μg/ml of EDTA were used.

Solvent	Rf value		
	A	B	C
80% *n*-Propanol	0.49	0.43	0.50
70% *n*-Propanol	0.70	0.65	0.70
80% Acetonitril	0.49	0.39	0.55
70% Acetonitril	0.71	0.66	0.75
n-BuOH - *i*-PrOH - H_2O (7: 7: 6)	0.67	0.61	0.67
$CHCl_3$ - EtOH - H_2O (4: 7: 2)	0.55	0.51	0.56

Table 2. High performance liquid chromatography of asparenomycins A, B and C.

Condition	Retention volume (ml)		
	A	B	C
a	14.4	11.5	60.0
b	6.0	5.2	17.8
c	39.3	27.6	n.d.*

a) Nucleosil 10 C_{18} column (4×250 mm), 20 mM phosphate buffer solution, pH 7.0, Flow rate: 2.0 ml/minute.
b) Nucleosil 10 C_{18} column (4×250 mm), 5% methanol in 20 mM phosphate buffer solution, pH 7.0, Flow rate: 2.0 ml/minute.
c) Hitachi gel #3011 column (4×500 mm), 10 mM phosphate buffer solution, pH 7.0, Flow rate: 1.0 ml/minute.
* Not determined.

Physico-chemical Properties

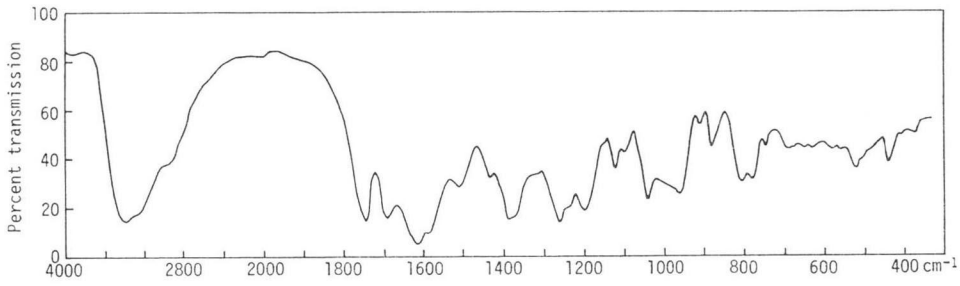
1) Asparenomycin A

Asparenomycin A sodium salt is a colorless amorphous powder and gradually decomposes over 150°C. It is soluble in water, methanol, dimethylformamide and dimethylsulfoxide, but insoluble in other common organic solvents. The antibiotic is extremely unstable in aqueous solutions of pHs below 5.0 or above 10.0. It is most stable at pH 8.0 (Fig. 3). The antibiotic is rapidly degraded by addition of hydroxylamine or cysteine in an aqueous solution of neutral pH.

Chromatographic behaviors in TLC and HPLC are presented in Tables 1 and 2. The antibiotics is visualized by spraying with EHRLICH reagent (blue), but negative to ninhydrin reaction. On paper electrophoresis with 50 mM phosphate buffer, pH 7.0, at 10 volt/cm for 3 hours, the antibiotic moved to the anode with Rm (relative mobility to penicillin N) 1.0.

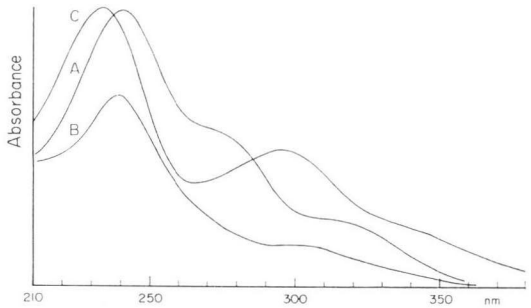
Asparenomycin A is optically active, $[\alpha]_D^{22.0}$ −210.8±5.1° (*c* 0.536, 10 mM phosphate buffer, pH 7.0); CD: $[\theta]_{390}$ 0, $[\theta]_{315}$ −22754, $[\theta]_{278}$ −102702, $[\theta]_{258.5}$ 0, $[\theta]_{243}$ +93292, $[\theta]_{190}$ +14480 (*c* 0.0458, 10 mM phosphate buffer, pH 7.0). Elemental analysis of the sodium salt: Found: C, 45.30; H, 4.82; N, 7.74; S, 7.86; Na, 5.99; Calcd. for $C_{14}H_{15}N_2O_6S \cdot Na \cdot \frac{1}{2}H_2O$: C, 45.28; H, 4.31; N, 7.55; S, 8.63; Na, 6.20. Field desorption mass spectrometry on asparenomycin A methyl ester gave a peak at *m/z* 355

Fig. 4. Infrared absorption spectrum of asparenomycin A (KBr).



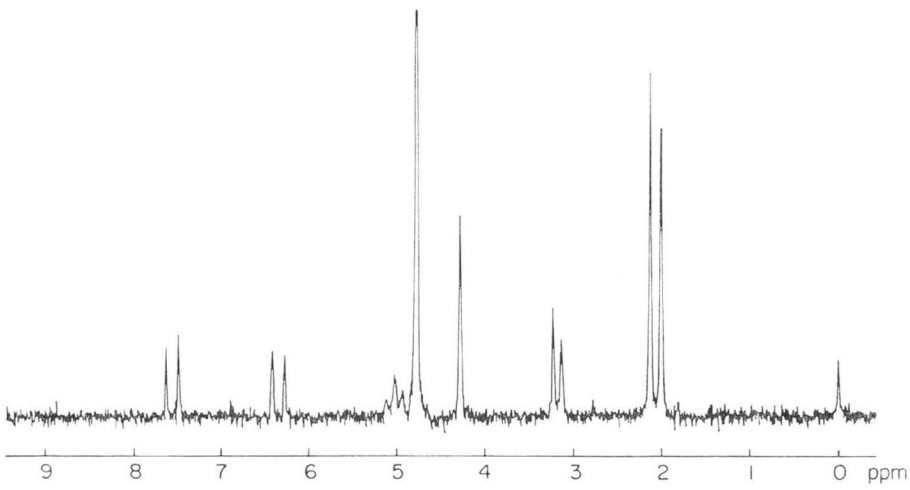
(MH$^+$). These data indicate a molecular formula $C_{14}H_{16}N_2O_6S$ for the acid form of asparenomycin A. In the IR spectrum (Fig. 4), characteristic absorptions attributable to β-lactam, amide and carboxylate were observed at 1750, 1695 and 1620 cm^{-1}

Fig. 5. Ultraviolet absorption spectra of asparenomycins A, B and C (in 10 mM phosphate buffer, pH 7.0).



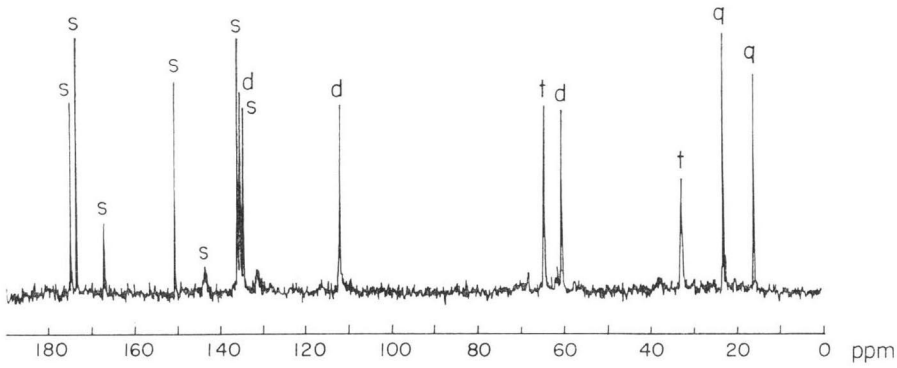
From the above properties and the biological activities (especially strong β-lactamase inhibition activities), asparenomycin A is considered to belong to the family of carbapenem antibiotics. However, in the UV spectrum (Fig. 5), characteristic absorptions, a maximum at 241 nm (ε, 21472) and two shoulders at 275 nm* and 315 nm*, which have not been reported with the hitherto known carbapenem antibiotics, are shown. The ^{1}H NMR spectrum is illustrated in Fig. 6. There are signals at δ 1.99 (3H, s, CH_3–), 2.12 (3H, s, CH_3–CO), 4.26 (2H, s, CH_2OH–), coupled signals (–CH–CH_2–) at δ 3.16 (2H, d-like) and 5.01 (1H, t-like), and signals attributable to *trans* vinyl pro-

Fig. 6. ^{1}H NMR spectrum (100 MHz) of asparenomycin A sodium salt (D_2O, internal reference DSS).



* The wave length of these shoulders previously reported[1] are corrected to this description.

Fig. 7. ^{13}C NMR spectrum (25 MHz) of asparenomycin A sodium salt (D_2O).



tons at δ 6.34 (1H, d, J=14.0) and 7.53 (1H, d, J=14.0). It is noteworthy that the spectrum lacks the signal due to 6-CH of the known carbapenem antibiotics, which usualy appears around δ 3.5~4.0. The ^{13}C NMR recorded in D_2O using CH_3CN as an internal reference by assuming δ (CH_3CN)=1.7 ppm from DSS is shown in Fig. 7. There are 14 ^{13}C signals which support the above molecular formula. It is important that there are four signals presumed to be due to $\rangle C=$. This fact is not expected from the structures of the known carbapenem antibiotics.

The above properties evidently indicated that asparenomycin A is a new member of the carbapenem antibiotics.

2) Asparenomycin B

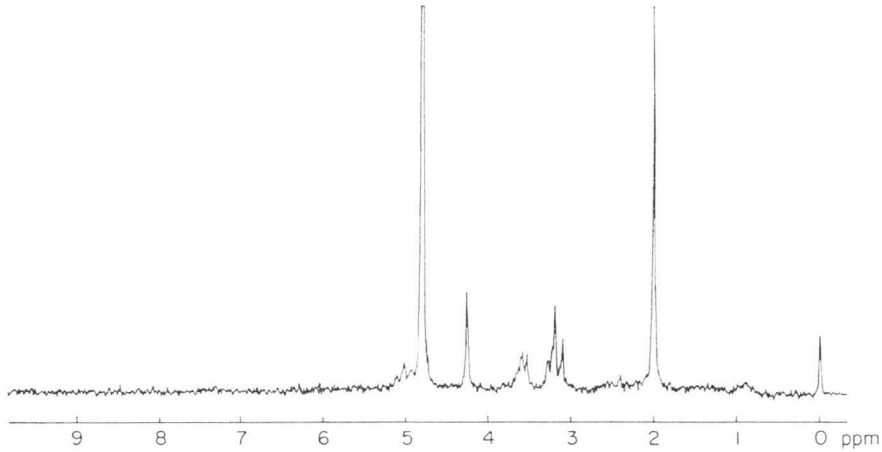
Asparenomycin B sodium salt is a colorless amorphous powder with similar solubility to that of asparenomycin A. It is also unstable in aqueous solution outside a narrow pH range. Rapid degradation by hydroxylamine or cysteine was also observed. It is similarly positive to EHRLICH reaction but negative to ninhydrin reaction and gives the same mobility as that of asparenomycin A on the paper electrophoresis. The chromatographic behaviors in TLC and HPLC are different from those of asparenomycins A and C (Tables 1 and 2).

Asparenomycin B exhibits the following CD spectrum, CD: $[\theta]_{360}$ 0, $[\theta]_{320}$ −560, $[\theta]_{267}$ −6410, $[\theta]_{245}$ 0, $[\theta]_{240}$ +1490, $[\theta]_{230}$ 0, $[\theta]_{220}$ −2300, $[\theta]_{210}$ 0 (*c* 0.0310, 10 mM phosphate buffer, pH 7.0). The UV spectrum (Fig. 5) shows a maximum at 239 nm and a shoulder at 300 nm, which also are characteristic in comparing with those of the known carbapenem antibiotics. The ^1H NMR spectrum is illustrated in Fig. 8, which is different from that of asparenomycin A essentially in the presence of signals due to four methylene protons instead of the signals of *trans* vinyl protons.

3) Asparenomycin C

Asparenomycin C is also similar to asparenomycin A in solubility, stability, color reactions and mobility on the paper electrophoresis. The chromatographic behaviors in TLC are somewhat similar to asparenomycin A, being differentiated only in solvent systems of aqueous acetonitrile (Table 1). However, the behaviors in HPLC is quite different. Since asparenomycin C is strongly retarded on the column of reversed phase under the isocratic condition for analysis of asparenomycins A and B, the mobile phase containing methanol must be used for analysis of asparenomycin C (Table 2). Two peaks at 234 nm and 296 nm are shown in the UV spectrum (Fig. 5), which is also characteristic enough to be differentiated from those of the known carbapenem antibiotics.

Fig. 8. ^1H NMR spectrum (100 MHz) of asparenomycin B sodium salt (D_2O, internal reference DSS).



The above described properties were all that were measured with the small amount of asparenomycin C available to us as natural product. However, deoxyasparenomycin A, the deoxygenated product of asparenomycin A (see Fig. 1), was identified with asparenomycin C on the basis of the fact that both specimens showed identical retention time in HPLC as described in the next publication[8]. Some additional properties measured with deoxyasparenomycin A will be presented in the succeeding paper[9].

Experimental

General Methodology

The UV absorption spectrum was measured with a Hitachi 323 spectrophotometer; IR absorption spectrum with a JASCO DS-403G spectrometer and CD spectrum with a JASCO J-40C automatic recording spectropolarimeter. FD/MS was obtained with a Hitachi RMU-8GN mass spectrometer equipped with a M-9911 field desorption ion source. ^1H NMR and ^{13}C NMR spectra were recorded with a Varian XL-100-12A spectrometer.

Asparenomycin A Methyl Ester

To a suspension of asparenomycin A sodium salt (10 mg) in dimethylformamide (1.0 ml) containing triethylamine (10 μl), methyl iodide (0.2 ml) was added. The suspension was stirred at 4°C for 16 hours. The reaction mixture was concentrated to a residue under reduced pressure, applied to precoated silica gel plates (Merck, Silica Gel GF_{254}), which were pretreated with 20 mM phosphate buffer, pH 7.0, containing 10 μg/ml of EDTA, and developed with chloroform - methanol (1: 1). A UV absorbing zone (Rf: *ca.* 0.64) was extracted with a mixture of chloroform and methanol (1: 3). Concentration to dryness under reduced pressure gave the methyl ester as a pale yellowish powder (5 mg), which gave a peak at *m/z* 355 (MH^+) in measurement of FD/MS.

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