NEW POLYENIC ANTIBIOTICS ACTIVE AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

VIII. CONSTRUCTION OF SYNTHETIC MEDIUM FOR PRODUCTION OF MONO-CHLORO-CONGENERS OF ENACYLOXINS

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New antibiotics enacyloxins (ENXs) are a family of non-lactonic polyene antibiotics produced by *Frateuria* sp. W-315. For the production of antibiotics, we had to employ two-step fermentations, the first is the production of spent medium of *Neurospora crassa* and the second is the production of antibiotics by *Frateuria*. To simplify the production of antibiotics, systematic analyses have been done on the spent medium, and factors which affect the production of antibiotics characterized. From the above results, we constructed a new medium for antibiotic production. Moreover, we could get a new antibiotic named enacyloxin IIIa (**1**), $C_{33}H_{48}O_{11}NCl$ (*m/z* 669). **1** was deduced to be one of the congeners of enacyloxins because it was similar to ENX IIa or ENX IVa both in biological and physico-chemical properties. Chlorine of **1** could be replaced by bromine, biosynthetically, and the resultant bromine-containing antibiotic also showed an antibacterial activity comparable to **1**.

In the production of enacyloxins[1~3] (former name; AB-315), we had to employ two-step fermentations. That is, the first step is the production of spent medium of *Neurospora crassa* and the second is production of antibiotics by *Frateuria* (formerly *Gluconobacter*) grown in the spent medium. With these two-step fermentation methods, we obtained a family of enacyloxins and have isolated at least 7 congeners of enacyloxins including enacyloxins IIa[2] and IVa[3]. Since two-step fermentations are rather hard to carry out on a large scale, we searched for a new medium to be able to carry out one step fermentations for production of the antibiotics. For this reason, we tried to find out the factors which stimulate the production of enacyloxins in the spend medium of *N. crassa*. In this paper, we described the factors which stimulate the production of the antibiotics and the properties of the new antibiotics in the newly designed synthetic medium.

Materials and Methods

Production of Spent Medium of *N. crassa*

Spent medium of *N. crassa* IFO 6068 was prepared according to our previous paper[1] using Czapek-Dox medium consisting of: Sucrose, 20 g; $NaNO_3$, 3 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g and $FeSO_4 \cdot 7H_2O$, 0.018 g in 1 liter of distilled water, pH 6.5. After *N. crassa* was grown in 1 liter of Czapek-Dox medium in 3-liter shaking flask at 30°C for 5 days on the reciprocal shaker, the broth was filtered through Toyo filter paper No. 2 (Advantec Toyo, Tokyo).

Assay of Stimulatory Effects of Factors on the Production of Antibiotics

Assay for stimulatory effects of factors on the production of antibiotics was carried out using the

spent medium of *N. crassa* or KING's A medium: Polypeptone, 20 g; glycerol, 10 g; K_2SO_4, 10 g and $MgCl_2 \cdot 6H_2O$, 1.4 g in 1 liter of distilled water, pH 7.2. After *Frateuria* sp. was grown in the spent medium or KING's A medium both with or without factors, antibiotics formed in the culture supernatant were determined by measuring absorption of the supernatant at 370 nm, because the antibiotics dissolved in water have an absorption maximum at that wavelength.

Analysis of the Spent Medium by Ion Exchange Column Chromatography

A column of Dowex 1-X2 (5 × 40 cm, diameter × height, CH_3COO^- form) was used for the analysis. Three liters of spent medium was applied to the column which was then eluted with 6 liters of 0.01 N HCl. Eluate was evaporated to dryness *in vacuo*, dissolved in 30 ml of distilled water, stored at −20°C until use and designated as "adsorbed material."

Separation of Factors in "Adsorbed Material" by Gel-filtration Using Bio-gel P-2

"Adsorbed material" was subjected to gel-filtration using Bio-gel P-2 (Bio-Rad., U.S.A.). Ten ml of "adsorbed material" was charged on the column (2 × 150 cm, diameter × height) and eluted with distilled water; flow rate, 75 ml/hour and 9.5 ml fractions were collected. Stimulation of antibiotic production by factors was examined using 50 ml of KING's A medium supplemented with 5 ml of each fraction of gel-filtrate and was measured for optical density at 370 nm. Sugars, amino or imino compounds and protein in each fraction were determined by phenol-sulfuric acid method[4]), ninhydrin reaction and optical density at 280 nm, respectively. Gel-filtration was also carried out with the spent medium of *N. crassa* in a small scale.

Identification of Sugars Derived from Gel-filtration by Paper Chromatography

Ascending paper chromatography was carried out using solvent system of *n*-BuOH - pyridine - H_2O (6 : 4 : 3), with Toyo filter paper No. 50 (40 × 40 cm). Twice developed paper was soaked in silver nitrate solution for color development.

Estimation of Glucose, Fructose and Oligosaccharide in the Spent Medium, "Adsorbed Material" and Gel-filtration Fractions

Glucose and fructose were estimated according to the modified method of Willstätter-Schudel[5]) and cysteine-carbazole method[6]), respectively. Estimation of di- and tri-saccharides was carried out by the phenol-sulfuric acid method[4]).

Analysis of the Antibiotics Formed by HPLC

Analysis of antibiotics formed by HPLC was performed using Tosoh CCPD 8000 system with ODS 120A column using a solvent system of acetonitrile - H_2O - formic acid (1,000 : 1,200 : 6.6).

Production of Antibiotics in Modified Czapek-Dox Medium

Production of antibiotics in modified Czapek-Dox medium was carried out aerobically in 100 ml of the medium in a 500-ml volume shake flask at 30°C for 48 hours. Antibiotics excreted into the culture fluid were extracted with diethyl ether under acidic condition. Other procedures for purification are the same as described in our previous paper[3]).

Others

UV and IR spectra were measured using a Hitachi 124 spectrophotometer and a Jasco IRA-1 spectrometer, respectively.

Results and Discussion

Effects of "Adsorbed Material" on Production of Antibiotics

In the previous paper[7]), it was shown that the addition of a small amount of Na-propionate to slightly modified Czapek-Dox medium stimulated production of antibiotics. In order to confirm the effect of volatile fatty acids, volatile fatty acid fraction was obtained from the spent medium by steam distillation and was

added to the new spent medium or slightly modified Czapek-Dox medium and then incubated with *Frateuria* sp. to produce antibiotics. No stimulatory effects on production of antibiotics could be detected with the distilled fraction. Therefore, we looked elsewhere to find what kinds of materials were needed for the production of antibiotics.

In the early period of this work, we assumed that the compounds which are effective for production of antibiotics may be formed by *N. crassa* in small amounts. Extraction of the spent medium of *N. crassa* with diethyl ether under acidic, neutral and alkaline conditions was carried out, and the each of ether fractions was added to the new spent medium or slightly modified Czapek-Dox medium to examine their effects on the production of antibiotics. The ether extract under acidic condition was shown to stimulate the production of antibiotics to a small extent, but the unextracted water layer still had an activity to stimulate the production of antibiotics, suggesting that this stimulating effect is, at least, attributable to the unextractable substance(s) (data not shown). Next, we treated the spent medium to adsorb on anion exchange resin of Dowex 1-X2, and "adsorbed material" was obtained by eluting the resins with 0.01 N HCl. As shown in Table 1, when "adsorbed material" was added to the spent medium or KING's A medium, production of antibiotics was obviously stimulated together with a slight increase in bacterial growth. As the antibiotics were fluorescent pigments, we used KING's A medium, which is well known as pyocyanin-producing medium, as a basal medium for production of antibiotics.

Analysis of "Adsorbed Material" by Gel-filtration

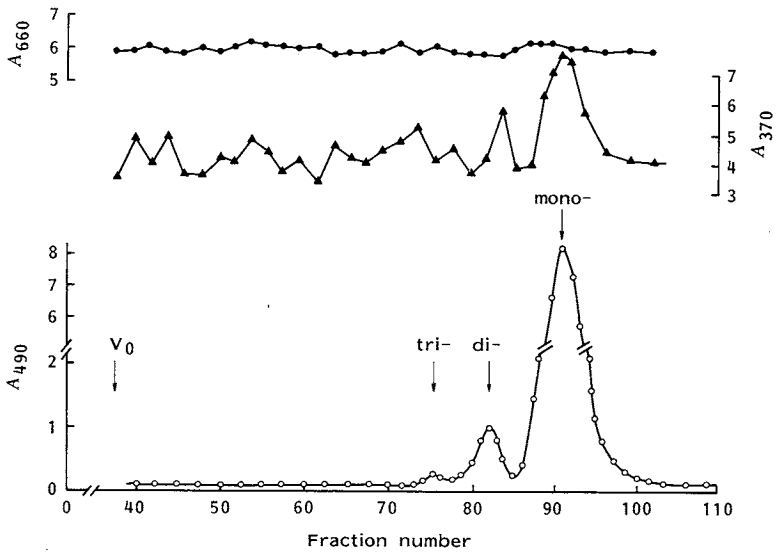
In the preliminary experiments, it was shown that "adsorbed material" contained considerable amounts of neutral sugars, so we performed gel-filtration for the fractionation of stimulatory factors. Although anion exchange resin Dowex 1 was thought not to adsorb neutral sugars, neutral sugars were eluted from the column of Dowex 1 with 0.01 N HCl after it was washed with distilled water. Probably, washing of the column with distilled water after charging of the sample was not sufficient and sugars remained on the column to be later eluted by dil HCl. The results are shown in Fig. 1. By the determination of sugars using the phenol-sulfuric acid method (shown as A_{490} in Fig. 1), a major peak and other small peaks were detected. From the retention time of standard sugars, the main peak (fraction number 86~94) was shown to correspond to mono-saccharides, and a neighboring peak (fraction number 80~84) and the smallest peak (fraction number 76), corresponded to di- and tri-saccharides, respectively. The stimulatory effects of these three fractions on production of antibiotic which is indicated as value of A_{370} was examined using KING's A medium as basal medium, and the main peak was found to be the most active. A slightly stimulative effect was observed with fraction number 82, but other fractions had no appreciable stimulating activity.

Table 1. Stimulatory effects of adsorbed material on production of antibiotics.

Basal medium	"Adsorbed materials"	Bacterial growth (A_{660})	Antibiotics produced (A_{370})	Ratio
Spent medium		6.64	14.0	1.00
Spent medium	+0.5 ml	8.60	22.6	1.61
Spent medium	+1.0 ml	8.76	24.6	1.76
KING's A medium		6.30	4.73	1.00
KING's A medium	+0.5 ml	6.70	8.08	1.71

One hundred ml of the spent medium of *Neurospora crassa* or KING's A medium with or without "adsorbed material" as indicated was incubated for 48 hours at 30°C aerobically and the production of antibiotics was examined by measuring the optical density of the culture supernatant at 370 nm.

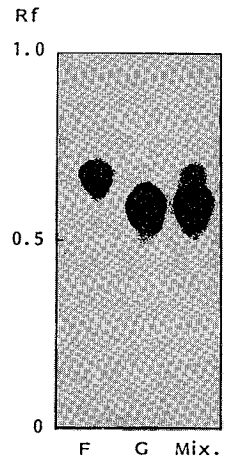
Fig. 1. Separation of "adsorbed material" by gel-filtration.



Bacterial growth (A_{660}) was not affected by the addition of these fractions. A small amount of both ninhydrin-reactive materials and materials showing an absorption at 280 nm were eluted earlier than tri-saccharide fraction (fraction number 76) (data not shown).

Fig. 2. Identification of mono-saccharides in the "adsorbed material" by paper chromatography.

F: Fructose; G: glucose; Mix: mixture of fraction numbers from 86 to 94.



Estimation of Mono-saccharide Eluted from Bio-Gel P-2

To identify mono-saccharides in the eluate of gel-filtration, a combined fraction from 86 to 94 was subjected to ascending paper chromatography. As illustrated in Fig. 2, two spots were separated and upper and lower spots were shown to correspond to fructose and glucose, respectively. Since Czapek-Dox medium employed for cultivation of *N. crassa* contains sucrose as a sole carbon source, sucrose might be split to glucose and fructose by invertase of *N. crassa* and then these mono-saccharides utilized for production of antibiotics. If the recoveries of glucose and fructose in the spent medium were the same during the adsorption to and elution from Dowex 1, fructose would be utilized preferentially, because more glucose was detected in the eluate from Dowex 1.

Effects of Sugars on Growth of *N. crassa* and Production of Antibiotics by *Frateuria* sp.

In order to examine the effects of sugars such as sucrose, fructose and glucose, *N. crassa* was cultivated in each medium containing one of the above mentioned sugars as a sole carbon source, and the resultant

Table 2. Growth of *Neurospora crassa* in the medium containing various kinds of sugars and production of antibiotics in these spent media by *Frateuria*.

Carbon source	Growth of *N. crassa* (Dry cell weight) (mg/100 ml)	Antibiotics production	
		A_{660}	A_{370}
Sucrose	28.7	2.62	2.74
Glucose	7.0	3.35	3.42
Fructose	25.2	1.00	2.00

Czapek-Dox medium was used with or without replacement of sucrose (2%) by glucose (1%) or fructose (1%). Each two flasks containing 100 ml of medium were inoculated with *N. crassa* and incubated. After 5 days of incubation, one flask was used for production of antibiotics and the other for estimation of growth. Production of antibiotics by each medium was performed for 48 hours.

Table 3. Sugar compositions in the spent medium and "adsorbed material".

Sugar	In the spent medium (%)	In "adsorbed materials" (%)
Glucose	80	84
Fructose	11	12
Sucrose	8	4
Tri-saccharide	1	—

The value shows as glucose equivalent one.

Table 4. Effects of glucose on production of antibiotics.

	A_{660}	A_{370}	Ratio (A_{370})
KING's A	6.7	9.58	1.00
KING's A + glucose 10 mg	6.7	10.86	1.13
KING's A + glucose 20 mg	6.5	15.56	1.62
KING's A + glucose 50 mg	6.7	17.47	1.82
KING's A + glucose 10 mg + fructose 10 mg	6.6	11.75	1.22

Fifty ml of KING's A medium was supplemented with glucose or fructose as indicated.

spent media were used for production of antibiotics. The results are shown in Table 2. Growth of *N. crassa* estimated by dry weight was regarded to be dependent on fructose rather than glucose and both growth and production of antibiotics by *Frateuria* sp. appeared to be much better in the presence of glucose than fructose.

Determination of Sugar Composition in the Spent Medium and "Adsorbed Material"

To confirm the effects of sugars on production of antibiotics, sugar compositions of the spent medium and "adsorbed material" were analyzed. As shown in Table 3, sugar compositions of the spent medium and "adsorbed material" were similar. Moreover, it is interesting that glucose accounted for more than 80% of total sugar in the spent medium of *N. crassa*. In conclusion, *N. crassa* utilized fructose preferentially, and the resultant spent medium which is rich in glucose was used for production of antibiotics by *Frateuria* sp.

Effects of Glucose on the Production of Antibiotics

From the results described above, glucose was considered to stimulate production of antibiotics. We examined the effects of various concentrations of glucose on production of antibiotics. As shown in Table 4, production of antibiotic was stimulated in accordance with the increase of glucose concentration, and fructose was considerably less effective than glucose.

Analysis of "Adsorbed Material"

In the preliminary experiments, we detected organic acids in addition to sugars in "adsorbed material." Then, we analyzed sugars and organic acids by phenol-sulfuric acid method and by HPLC using Shodex Ionpac C-811, respectively. A half ml of "adsorbed material" contains: Total sugar, 27.5 mg; malic acid, 5 mg; succinic acid, 2.5 mg and fumaric acid, 0.25 mg.

Reconstruction of "Adsorbed Material"

From the above results, we reconstructed "adsorbed material" with identified sugars and organic acids

and examined their effect on the production of antibiotics. As shown in Table 5, addition of glucose, fructose, DL-malic acid, succinic acid and fumaric acid with the similar amounts as in "adsorbed material" showed similar stimulative activity as "adsorbed material".

Table 5. Reconstruction of "adsorbed material" with sugars and organic acids assayed by production of antibiotics.

Medium Addition	A_{660}	A_{370}	Ratio (A_{370})
KING's A	5.8	2.79	1.00
KING's A + glucose (G)	5.8	4.22	1.51
KING's A + fructose (F)	5.9	3.46	1.24
KING's A + G + F	5.9	4.95	1.77
KING's A + organic acids + G + F	6.2	7.28	2.61
KING's A + "adsorbed materials" (0.5 ml)	6.8	5.37	1.91

Fifty ml of KING's A medium was supplemented with: Glucose, 18 mg; fructose, 8 mg and organic acids including, DL-malic acid, 5 mg; succinic acid, 2.5 mg; fumaric acid, 0.25 mg as indicated in Table 5.

Construction of Simple Synthetic Medium for Production of Antibiotics

To construct a new simple synthetic medium, we modified Czapek-Dox medium. From taxonomic studies on the bacterium[7], it was shown that sucrose and $NaNO_3$ were not utilized for *Frateuria*. The first modifications were changes of sucrose to glucose, $NaNO_3$ to $(NH_4)_2SO_4$ and addition of 0.2% of $CaCO_3$ for pH stabilization (pH 7.2). Essentially no production of antibiotics and less bacterial growth were seen when pH was lowered to under 6.8. The second modification was a change of $(NH_4)_2SO_4$ to urea and we observed an increase in antibiotic production by this change. Urea may provide a small amount of ammonium ion continuously by its degradation. We did not add organic acids because we prefer the simple medium. In conclusion, the composition of a new synthetic medium named modified Czapek-Dox medium is as follows: Glucose, 20 g; urea, 3 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.018 g and $CaCO_3$, 2 g pH 7.0 in 1 liter of distilled water.

HPLC Profiles of Antibiotics

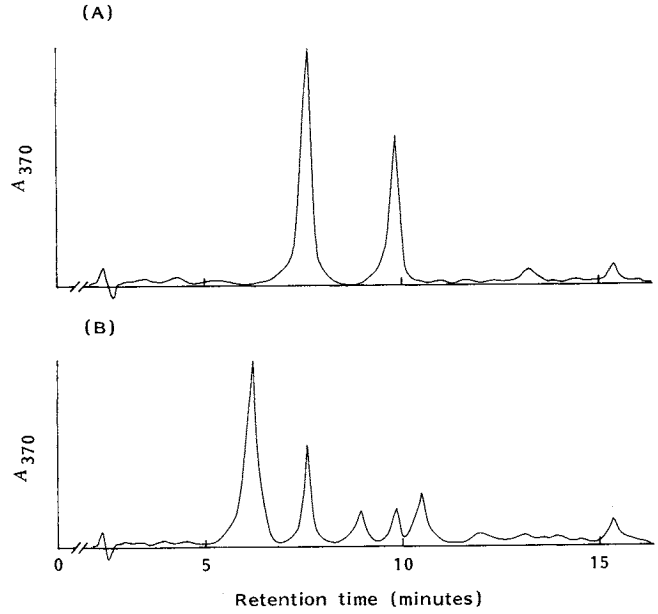
The culture supernatant of *Frateuria* sp. grown in the modified Czapek-Dox medium was analyzed using reversed phase HPLC in comparison with that grown in the spent medium of *N. crassa*. In Fig. 3(A), there are two big peaks which have been already identified as enacyloxin IVa (ENX IVa) (left in Fig. 3(A)) and enacyloxin IIa (ENX IIa) (right in Fig. 3(A)), but as shown in Fig. 3(B), several unknown peaks could be observed in addition to these peaks. To make clear whether these unknown peaks are newly produced in the modified medium or only degradative products of known ENXs, we tried to isolate these unknown peaks. Isolation and purification were carried out according to our previous papers[1,2]. We describe here the properties of the biggest peak named enacyloxin IIIa (**1**).

Biological and Physico-chemical Properties of **1**

Compound **1** was active against Gram-positive and Gram-negative bacteria, slightly active against fungi, but inactive against yeasts. MIC values against *Escherichia coli* K-12 and *Bacillus megaterium* were 5.5 μg/ml and 2.5 μg/ml, respectively. Antifungal activity towards *Pyricularia oryzae* was shown to be more than 200 μg/ml as MIC. FAB-MS spectrum of **1** as shown in Fig. 4(A) was apparently different from that of authentic ENX IIa shown in Fig. 4(B). This observation suggested that **1** might have one chlorine atom in the molecule in contrast to two-chlorine-containing ENX IIa. Mass numbers of 714 and 692 seemed to correspond to $(M-H^+ +2Na^+)$ and $(M+Na^+)$, respectively. If this is the case, the mass number of molecular **1** will be 669. For further investigation, we tried to get antibiotics produced in the modified Czapek-Dox medium in which KCl is replaced by KBr. Fermentation, isolation and purification were carried out similarly as those for **1**. FAB-MS profile of antibiotic corresponding to **1** (HPLC) showed *m/z*

Fig. 3. HPLC profiles of antibiotics.

(A) Culture supernatant from the spent medium. (B) Culture supernatant from the modified Czapek-Dox medium.



Ten μl of culture supernatant was injected. Flow rate: 1 ml/minute.

Fig. 4. MS spectra of **1** (A) and ENX IIa (B).

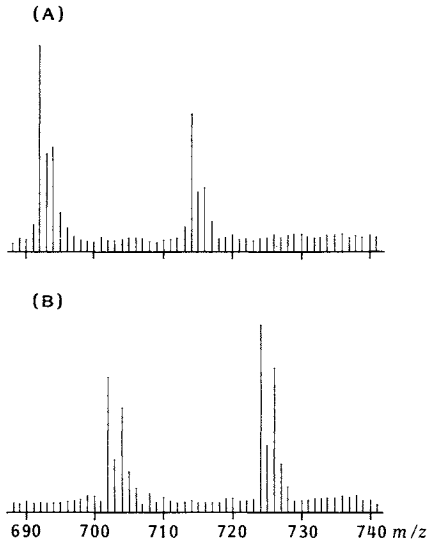


Fig. 5. MS spectrum of bromine-containing antibiotic corresponding to **1**.

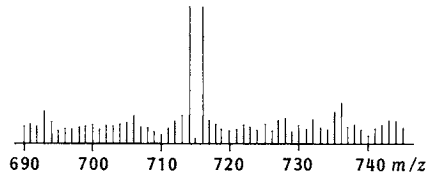
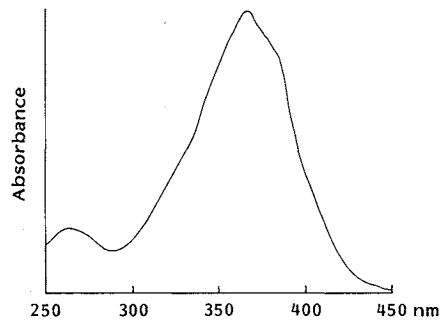
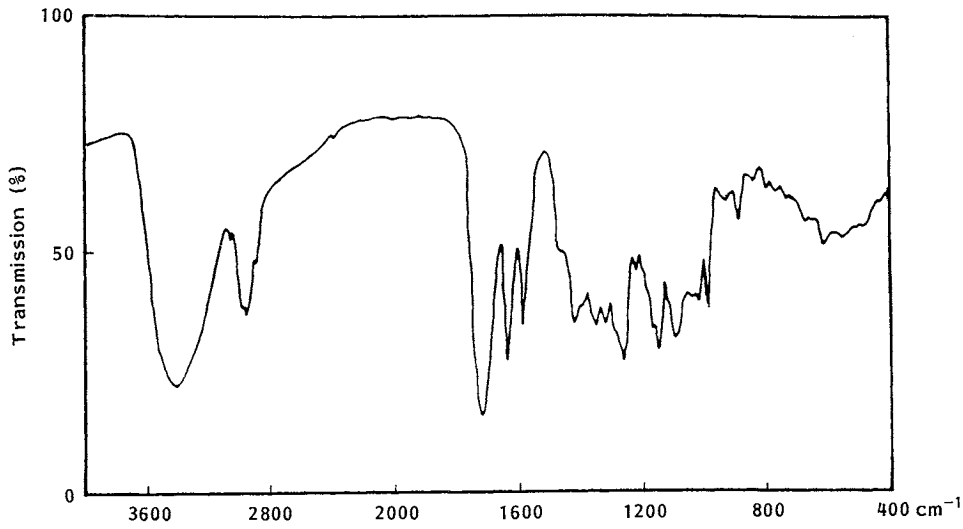


Fig. 6. UV spectrum of **1** (95% MeOH).



values of 714 ($M+H^+$) and 716, a typical profile obtained from the molecule containing one bromine atom as shown in Fig. 5. These values suggest that one atom of bromine was incorporated into **1** instead of chlorine. From these results, we can conclude that **1** should have one chlorine atom in the molecule

Fig. 7. IR spectrum of **1** (KBr).



suggesting **1** as a new antibiotic. Antibacteial activity of bromine-containing antibiotic is comparable to that of **1**.

UV and IR spectra of **1** shown in Figs. 6 and 7, respectively were quite similar to those of ENX IIa[2] and ENX IVa[3]. Summarized values were as follows: UV λ_{max}^{MeOH} nm 268, 365 and 383, IR(KBr) cm^{-1} 3400, 1710, 1620, 1575 and 975. HRFAB-MS revealed *m/z* 692.2796 $(M+Na)^+$ (calcd for $C_{33}H_{48}O_{11}NClNa$ = 692.2814 $(M+Na)^+$). In conclusion, we assigned chemical formula of **1** as $C_{33}H_{48}O_{11}NCl$.

In the early phase of fermentation in the modified Czapek-Dox medium, HPLC of the product showed substantially one peak of **1** suggesting **1** should be a real product in fermentation (data not shown). One of the unidentified peaks other than **1** was isolated and named enacyloxin Ia (ENX Ia), which also has one chlorine atom in the molecule (unpublished data). The structural elucidation of **1** and ENX Ia will be reported elsewhere.

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References

1) WATANABE, T.; K. IZAKI & H. TAKAHASHI: New polyenic antibiotics active against Gram-positive and -negative bacteria. I. Isolation and purification of antibiotics produced by *Gluconobacter* sp. W-315. J. Antibiotics 35: 1141~1147, 1982
2) WATANABE, T.; T. SUGIYAMA, M. TAKAHASHI, J. SHIMA, K. YAMASHITA, K. IZAKI, K. FURIHATA & H. SETO: The structure of enacyloxin II, a novel linear polyenic antibiotic produced by *Gluconobacter* sp. W-315. Agric. Biol. Chem. 54: 259~261, 1990
3) WATANABE, T.; J. SHIMA, K. IZAKI & T. SUGIYAMA: New polyenic antibiotics active against Gram-positive and Gram-negative bacteria. VII. Isolation and structure of enacyloxin IVa, a possible biosynthetic intermediate of enacyloxin IIa. J. Antibiotics 45: 575~576, 1992
4) DUBOIS, M.; K. A. GILLES, J. K. HAMILTON, P. A. REBERS & F. SMITH: Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350~356, 1956

5) OSODO, K.; H. KAZUMI, M. KOTAKA & H. SHITOMI: Determination of glucose in fruits by micro hypoiodite method. Nippon Nogei-Kagaku Kaishi (in Japanese) 33: 166~170, 1959
6) DISCHE, Z. & E. BORENFREUND: A new spectrophotometric method for the detection and determination of keto sugars and trioses. J. Biol. Chem. 192: 583~587, 1951
7) WATANABE, T.; K. IZAKI & H. TAKAHASHI: New polyenic antibiotics active against Gram-positive and -negative bacteria. II. Screening of antibiotic producers and taxonomical properties of *Gluconobacter* sp. W-315. J. Antibiotics 35: 1148~1155, 1982