ABIERIXIN, A NEW POLYETHER ANTIBIOTIC

PRODUCTION, STRUCTURAL DETERMINATION AND BIOLOGICAL ACTIVITIES

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A new polyether antibiotic, abierixin, was found in the mycelium of a culture broth of nigericin-producing *Streptomyces albus* NRRL B-1865. Abierixin was extracted with organic solvents and purified by column chromatography and HPLC.

The structure of abierixin was determined by FAB/MS/MS and CI/MS/MS and ^{1}H and ^{13}C NMR spectrometries.

Abierixin exhibited weak antimicrobial and ionophorous activities, low toxicity but good anticoccidial activity.

Nigericin biosynthesis from abierixin is discussed.

The majority of polyether antibiotics are capable of complexing and transporting monovalent cations. They assume considerable commercial importance as feed additives, acting as anticoccidial agents and enhancing feed efficiency in ruminants.

Abierixin (**1**), a new polyether antibiotic, was found in the mycelium of a culture broth of nigericin-producing *Streptomyces albus* NRRL B-1865.

In the present report, the production, isolation, structural determination and biological properties of abierixin are described.

Materials and Methods

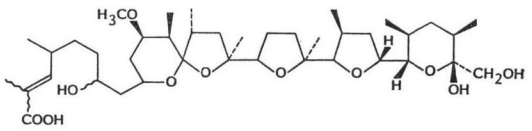
Melting-points were measured with Reichert hotplate microscope and are uncorrected.

IR spectra were recorded with a Perkin-Elmer 327 spectrometer in KBr.

UV spectra were determined with a Cary-15 spectrophotometer in EtOH.

NMR spectra were recorded on a Brucker W2 200 SY instrument at 200 MHz for the ^{1}H spectra and 50.33 MHz for the ^{13}C spectra. In all cases TMS was used as internal standard.

Column chromatography was performed on Merck Silica Gel 60 (0.063 ~ 0.200 mm). Column chromatography was also carried out using the flash-chromatography[1] with Merck Silica Gel 60 (0.025 ~ 0.040 mm). The purification of abierixin was achieved by HPLC (Waters Associated Prep LC/system 500).



Abierixin (**1**)

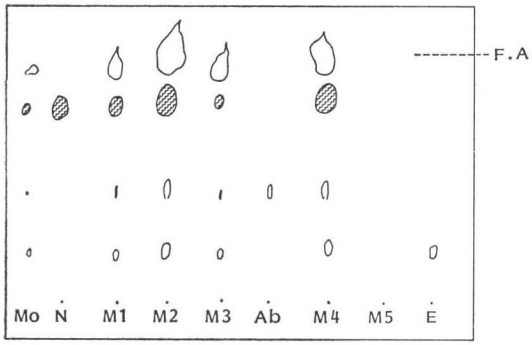
Production and Isolation

Production

The primary goal of this study was the improvement of fermentation yields of the new carboxylic

Table 1. Evaluation of several fatty acids methyl esters as supplements in basal medium 1.

Fatty acids added (10 ml/liter)	pH	Mycelium (weight g/liter)	EtOAc extract (weight mg/liter)
Mo: none	8.6	4.0	290
M1: myristate	8.1	6.3	1,300
M2: palmitate	7.9	8.5	3,800
M3: stearate	8.8	2.7	400
M4: oleate	8.3	8.0	3,000
M5: linoleate	6.6	—	—



F.A.: Fatty acids complex, N: Nigericin, Ab: Abierixin, E: Elaiophylin, TLC (Merck Co., Ltd., Silica gel 60F-204, solvent $CHCl_3$ - MeOH, 9: 1).

polyether antibiotic, abierixin. The original culture of *S. albus* NRRL B-1865 in medium 1 (glucose 1%, meat extract 0.1% and Casamino Acids (Difco) 0.4%) for 4 days, produced approximatively 100 mg/liter of antibiotics (mainly nigericin) and trace of abierixin. A variation in the levels of the constituants of the medium 1 and addition of several minerals effected only minor changes in the titer of antibiotics.

Addition of oils or fatty acids to the fermentation medium has, in the past, positively affected the production of some polyether antibiotics[2,3]. In our experiments, several fatty acids methyl esters have been tested to determine the contribution of chain length and double bond location in the production of abierixin (Table 1). The methyl esters of oleic acid and palmitic acid markedly increased the titers of nigericin and abierixin (over 700% with addition of 1% methyl oleate after 7 days).

The fermentation process for antibiotics production is as following. Seven ml of frozen mycelia were inoculated into 100 ml of the seed medium consisting of glucose 3%, corn-steep 0.5%, soybean meal 0.5% and $CaCO_3$ 0.5% in tap water adjusted to pH 7 before autoclaving. This culture was carried out at 27°C for three days on a shaker and then transferred to 1 liter of the same medium in a 2-liter fermentor. After 24 hours, this seed culture was transferred into a 20-liter fermentor containing 15 liters of the fermentation medium consisting of glucose 1%, meat extract 0.1%, yeast extract 0.1%, Casamino Acids (Difco) 0.4% and methyl oleate 1% (pH 7.2 before sterilization). The fermentation was run with aeration (0.5 liter/minute/liter of fermentation medium) and agitation (650 rpm).

Isolation

Fifteen liters of the culture broth from one 20-liter fermentor was filtered yielding 285 g of mycelial cake which was extracted by the following procedure. The mycelial cake was extracted with EtOH (300 ml/liter of fermentation broth) during 2 hours after using an Ultra-Turax homogenizer. Then, the cells were removed by filtration and the EtOH extract was concentrated under reduced pressure. The residual syrup was eluted with 0.1 N HCl and extracted twice in one-fifteenth volume of EtOAc. After drying over anhydrous sodium sulfate, the EtOAc extract was concentrated under reduced pressure to give 75 g of oily product. This material was chromatographed on a column of silica gel using cyclohexane - EtOAc with increasing amounts of EtOAc as eluting solvent. The eluate containing abierixin was concentrated and chromatographed on a flash-chromatography column using MeOH - $CHCl_3$ with increasing amounts of MeOH as eluting solvent. The separation of abierixin from the antibiotic complex was achieved by HPLC. Two grams of crude abierixin was dissolved in 2 ml of

$CHCl_3$ and injected into Prep PAK 500 column and eluted with 1 liter of $CHCl_3$ at a flow rate of 50 ml/minute, 3 liters of $CHCl_3$ - CH_3OH, 9.5: 0.5, at 100 ml/minute and 4 liters of $CHCl_3$ - CH_3OH, 9: 1, at 100 ml/minute. The eluates were monitored by a refractive index detector.

Structural Determination

Abierixin Na-salt was obtained as a colorless powder, soluble in organic solvents but not soluble in H_2O. Abierixin was isolated from the Na-salt by passage through a flash-chromatography column with the same solvents as above acidified by 5% acetic acid. The molecular formula of abierixin was estimated to be $C_{40}H_{68}O_{11}\cdot H_2O$. These results as well as other physico-chemical properties are summarized in Table 2.

The IR spectrum of abierixin (Fig. 1) was similar to the spectra of other polyether carboxylic antibiotics. It showed absorptions at 3700~3150, 1715 and 1115~1020 cm^{-1} corresponding to hydroxyl, $\alpha\beta$-unsaturated carboxylic acid and ether functions, respectively. The UV spectrum (Fig. 2) of abierixin showed an absorption peak at λ_{max}^{EtOH} 217 nm (ε 10,400), confirming the presence of an $\alpha\beta$-unsaturated carboxylic acid in this compound.

The 200 MHz ^1H NMR spectrum of abierixin (Fig. 3) suggests the presence of one methoxy group characterized by one singlet at 3.36 ppm. The unsaturated character of abierixin is further supported by the presence of one doublet at δ 6.65 ppm (1H, J=10.6 Hz) which was assigned to an ethylenic proton-C3 coupled to a proton-C4. The proton-C4 is also coupled with the 38-methyl[4] characterized by one doublet at δ 1.83 ppm (3H, J=1.45 Hz).

The techniques of FAB (fast atom bombardment)[5] mass spectrometry and MS/MS using collisionally activated dissociation (CAD)[6] were helpful in characterizing the structure of abierixin. We

Table 2. Physico-chemical properties of abierixin.

Nature	Colorless powder
MP	83~85°C (Na-salt; 120~123°C)
$[\alpha]_D^{25}$	+45° (c 0.03, CH_3OH)
Elementary analysis: Found	C 64.41, H 9.46, O 25.60
Calcd ($C_{40}H_{68}O_{11}\cdot H_2O$)	C 64.66, H 9.49, O 25.84
Solubility: Soluble in	MeOH, EtOAc, $CHCl_3$
Insoluble in	H_2O
Rf (Silica gel F 254 Merck)	0.25, $CHCl_3$ - MeOH (9: 1)
UV λ_{max}^{EtOH}	217 (ε 10,400), 275 nm (ε 3,080)
IR ν_{max}^{KBr}	3700~3150, 1715, 1115~1020 cm^{-1}

Fig. 1. IR spectrum of abierixin in KBr.

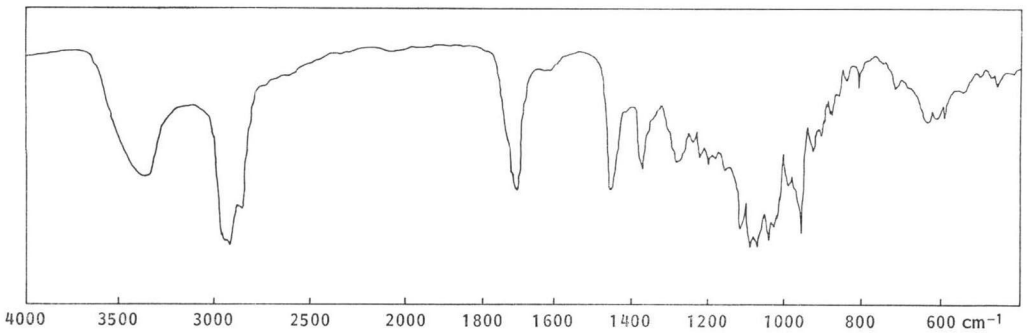
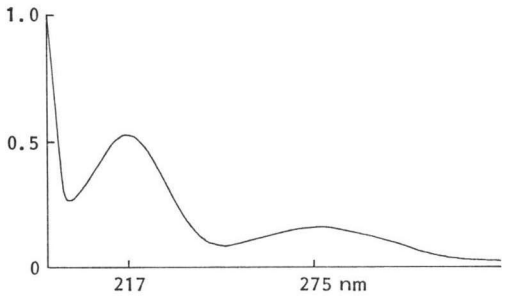


Fig. 2. UV spectrum of abierixin in ethanol.



report here success in obtaining both molecular ions and structural information using these techniques in comparison with nigericin (**2**) (Scheme 1). The molecular weight of 724 amu for abierixin and nigericin has been determined. Indeed in the positive FAB/MS, diagnostic cationized molecules m/z 747 $(M+Na)^+$ (Fig. 4) and m/z 763 $(M+K)^+$ (Fig. 5) were detected without observation of abundant fragment ions. In the negative mode (Fig. 6), only the deprotonated molecule $(M-H)^-$ at m/z 723 was observed confirming the molecular weight. Thus abierixin and nigericin are isomers.

The studies of metastable decompositions induced by collisions (Mass-analysed Ion Kinetic Energy using collisionally activated dissociation, MIKE/CAD, spectra of molecular species) gave more information about the relative structure of abierixin and nigericin. The MIKE/CAD spectra of both $(M+Na)^+$ isomeric ions present a strong difference on the small neutral loss (absence of CO_2 in the case of abierixin) while the partial "fingerprints" below m/z 650 range are identical for both ions

Fig. 3. ^1H NMR spectrum of abierixin in $CDCl_3$ (200 MHz).

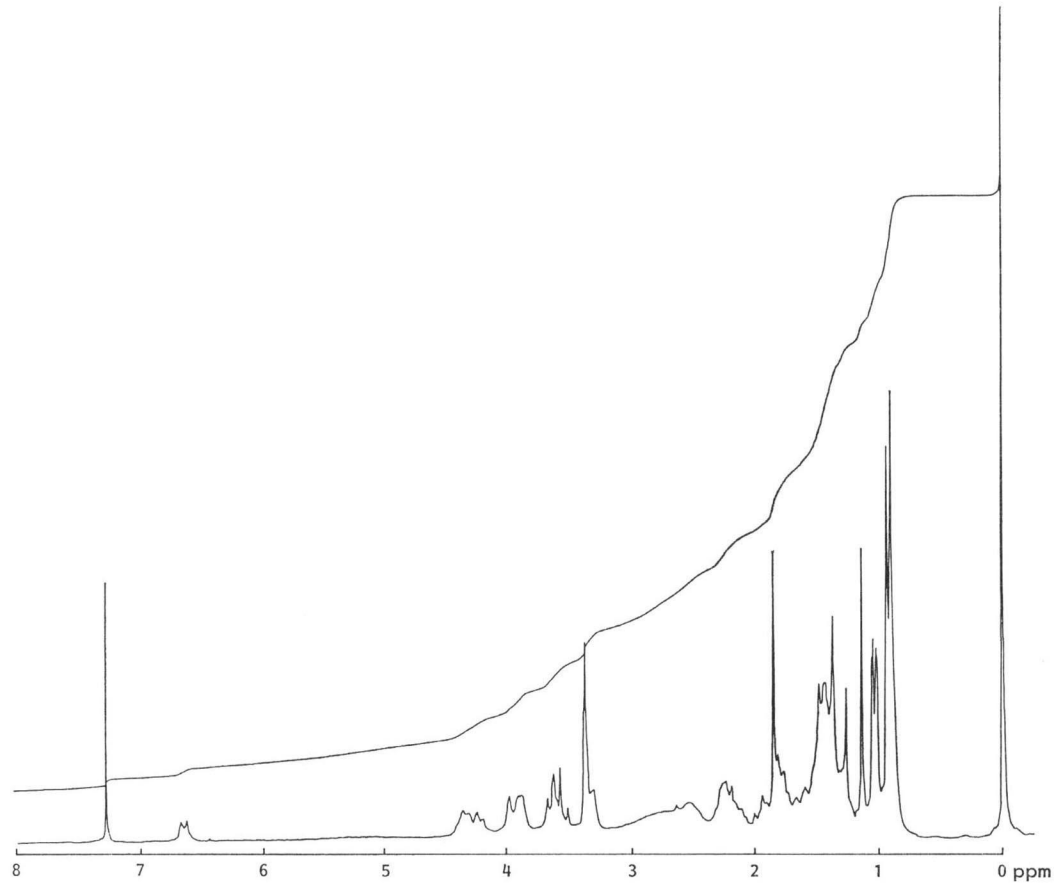


Fig. 4. Positive FAB/MS of (a) nigericin and (b) abierixin using thioglycerol+NaI as matrix.

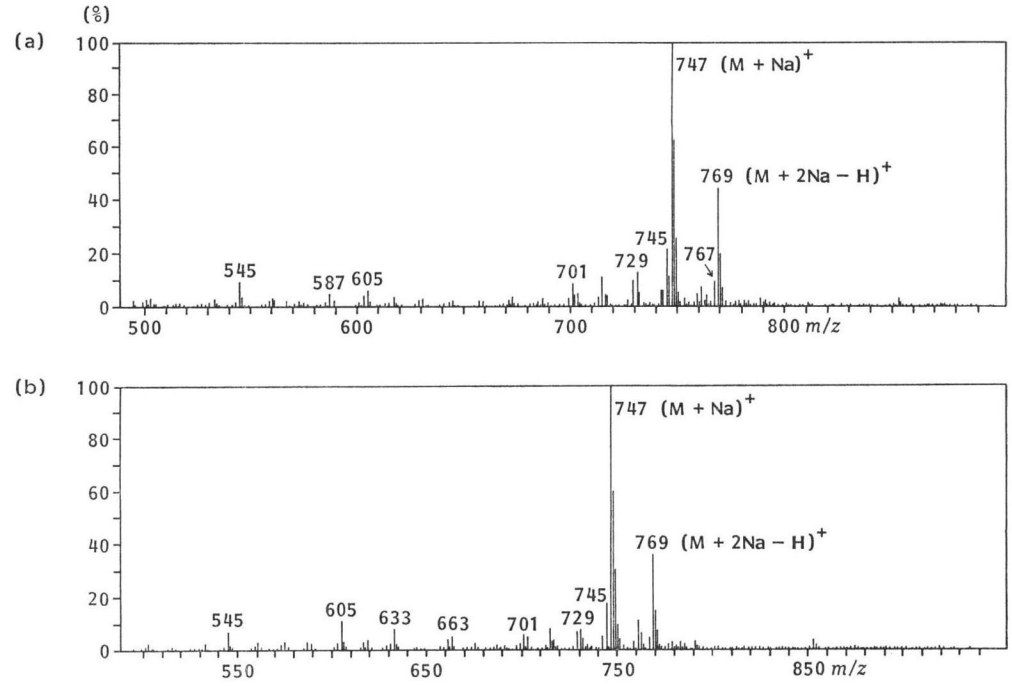
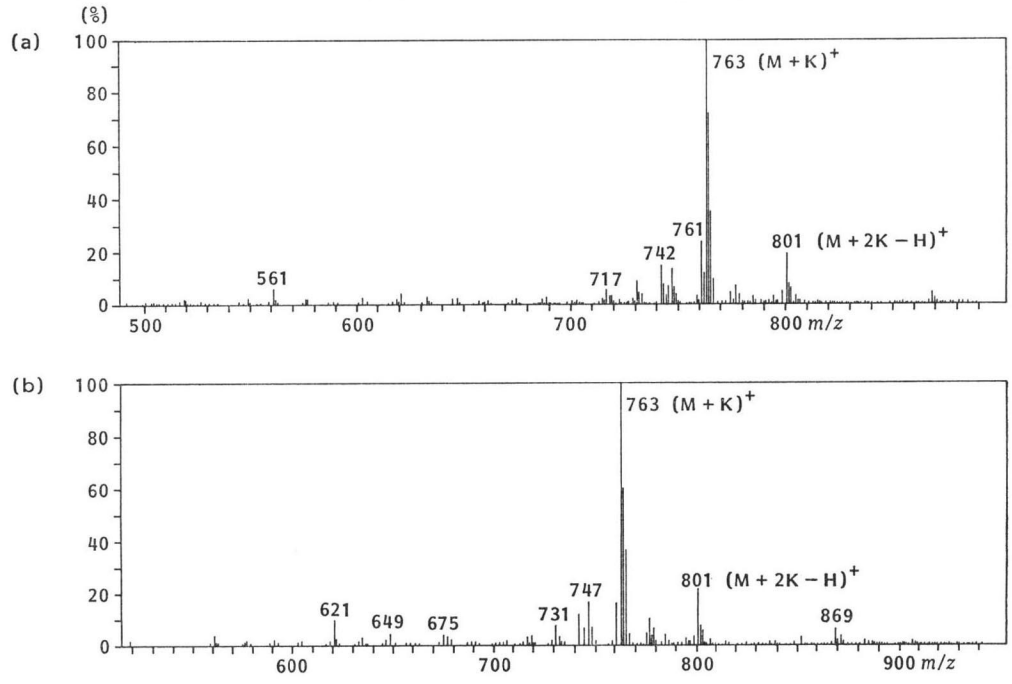


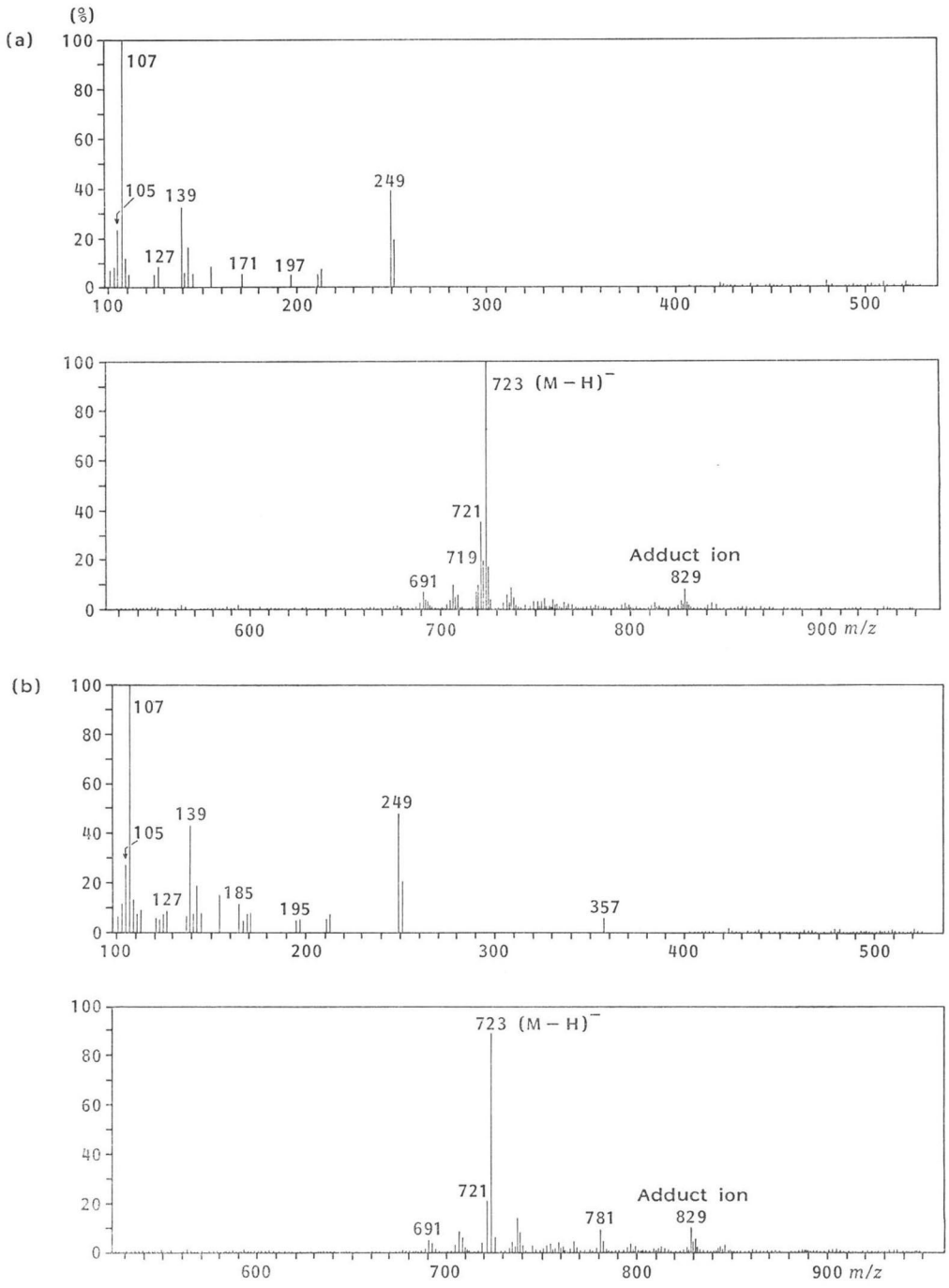
Fig. 5. Positive FAB/MS of (a) nigericin and (b) abierixin using thioglycerol+KI as matrix.



formed under FAB conditions.

Furthermore, with CI/NH_3 techniques[7], the $(M+NH_4-H_2O)^+$ species can be detected (low yield). The analysis of the respective MIKE/CAD spectra indicate the structural difference at the acidic site.

Fig. 6. Negative FAB/MS of (a) nigericin and (b) abierixin.



A large consecutive loss of (CH_3OH+2H_2O) was detected in abierixin and only elimination of (CH_3OH+H_2O) in nigericin. The large fragments involving the non-linear chain of abierixin and nigericin present identical MIKE/CAD spectra indicating that the stereochemistry of the various asymmetric centers are same (to be published).

Fig. 7. J modulated spin echo ^{13}C NMR spectrum of abierixin in $CDCl_3$ (50 MHz).

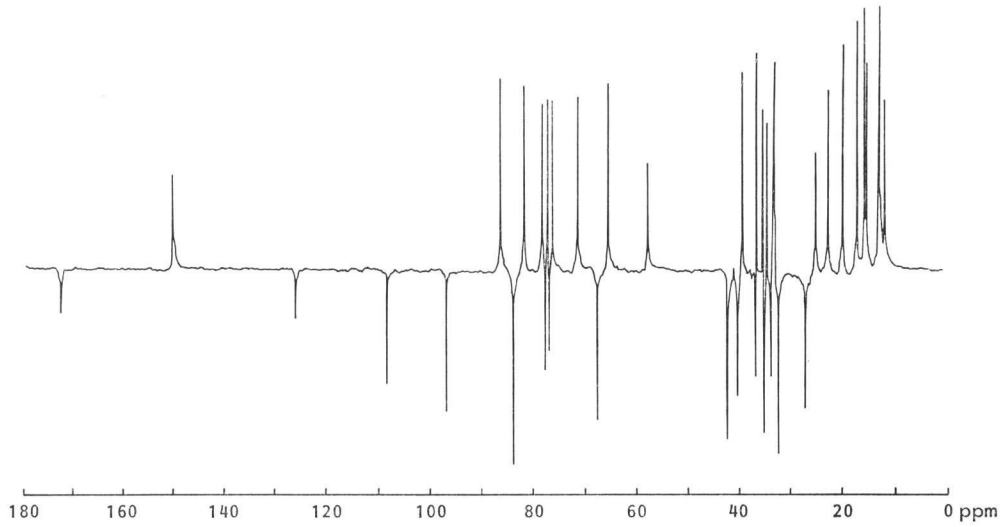


Table 3. Chemical shifts of nigericin and abierixin in $CDCl_3$.

No.	Nigericin		Abierixin		No.	Nigericin		Abierixin	
	Function-ality	δ	δ	Function-ality		Function-ality	δ	δ	Function-ality
1	C(=O)O	177.5	172.07	C(=O)O	12	CH	36.9	36.9	CH_2
					8	CH_2	35.8	36.8	CH
			149.7	$H\diagdown C=$	22	CH	35.4	35.5	CH
			125.9	=C				35.2	CH_2
								35.0	CH_2
13	C(=O)O	108.2	108.3	C(=O)O				34.8	CH
								34.4	CH_2
29	C(=O)O	97.0	97.3	C(=O)O				34.4	CH
21	CH–O	85.3	86.5	CH–O				33.3	CH
20	C–O	83.4	84.1	C–O	10	CH_2	32.6	32.9	CH_2
16	C–O	82.4	84.1	C–O	23	CH_2	32.2	32.3	CH_2
17	CH–O	81.5	81.8	CH–O	26	CH	31.8		
11	CH–O	79.3	78.3	CH–O	19	CH_2	30.7		
25	CH–O	78.0	77.3	CH–O	4	CH	37.9		
24	CH–O	74.5	76.3	CH–O	35	CH_3–CO	27.5	27.3	CH_2
3	CH–O	72.9			5	CH_2	26.1		
7	CH–O	69.0	71.4	CH–O	18	CH_2	25.7	25.2	CH_3
30	CH_2OH	68.3	67.7	CH_2OH	6	CH_2	23.4	23.0	CH_3
9	CH–O	60.4	65.5	CH–O	34	CH_3–CO	22.7	19.9	CH_3
40	O–CH_3	57.4	58.0	O–CH_3	32	CH_3	17.3	17.4	CH_3
2	CH	44.2			31	CH_3	16.4		
15	CH_2	42.5	42.3	CH_2	33	CH_3	16.3	15.9	CH_3
			40.3	CH_2	39	CH_3	15.6	15.0	CH_3
14	CH	39.0	39.6	CH	36	CH_3	13.2	13.2	CH_3
27	CH_2	37.4			37	CH_3	13.1	12.9	CH_3
28	CH	37.2			38	CH_3	10.8	12.2	CH_3

These results are coherent with the presence of an $\alpha\beta$-unsaturated carboxylic acid and an additional hydroxyl group likely at C-7.

The ^{13}C NMR spectrum of abierixin gives supplementary informations (Fig. 7). All the 40 carbons

were observed. The carbons bounded or not bounded with an oxygen in comparison with nigericin[8] are shown in Table 3. The signals at δ 72.9 and δ 44.2 ppm, which were assigned to carbons 2 and 3 respectively in nigericin, were absent for abierixin. Two new signals at δ 149.7 and 125.9 ppm appeared, indicating that the doubly bounded carbons of abierixin are 2 and 3 respectively.

These results confirm the proposed structure of abierixin (**1**). To explain the molecular formula ($C_{40}H_{70}O_{12}$) of abierixin, it must be due to the fact that this product is a monohydrate $C_{40}H_{68}O_{11}\cdot H_2O$.

Biological Properties

A first approximation of the monovalent cation transport by abierixin and nigericin, produced in the same culture broth, was made in a U-tube system[9]. The results at pH 9 are given in Table 4. The cations K^+ and Na^+ are transported across the chloroform barrier from one aqueous phase to another at a faster rate by nigericin than by abierixin.

The minimal inhibitory concentration (MIC) of abierixin and other polyether antibiotics against *Bacillus cereus* were determined by a serial two-fold dilution using Mueller-Hinton broth. As shown in Table 5, abierixin is more than two orders of magnitude less active than monensin and nigericin.

The acute toxicity of abierixin in mice was examined. The LD_{50} values of monensin and abierixin were 80~100 mg/kg and 600 mg/kg respectively, *per os*.

The anticoccidial evaluation of abierixin was carried out with 17 days-old chickens infected with *Eimeria tenella* oocysts. The test was continued for 8 days. Abierixin, at 40 ppm, was effective in reducing the mortality of chickens and increasing average body weight of treated infected chickens compared to untreated infected controls[10].

Discussion

Nigericin (**2**) and abierixin (**1**) (Scheme 1) have a very close structural correlation. Acid or base

Table 4. Ionophorous activity of abierixin and nigericin at pH 9.

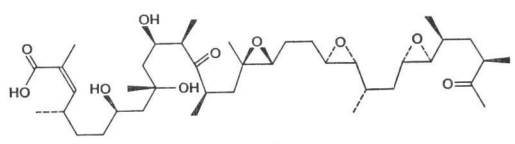
	K^+ (mol/s/cm^2)	Na^+ (mol/s/cm^2)
Nigericin	3×10^{-10}	3×10^{-10}
Abierixin	1.5×10^{-10}	0.5×10^{-10}

The U-tube system was filled with a chloroformic solution (9 ml) of antibiotic (1×10^{-4} M). Five ml of an aqueous tricine-buffer containing 10^{-2} M KCl or NaCl was placed in one arm of the U-tube and an equal volume of the same buffer-solution in the other arm. The pH of the only solution containing the cation is modified by the addition of $(CH_3)_4NOH\cdot 5H_2O$.

Table 5. Antibacterial activity related to *Bacillus cereus*.

Antibiotic	MIC (μg/ml)
Abierixin	25
Monensin	>0.05
Nigericin	0.05
Grisorixin	3.12

Scheme 1.



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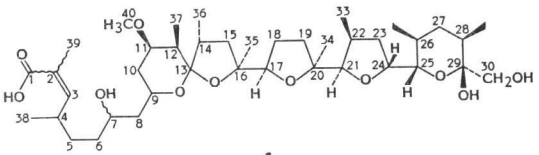

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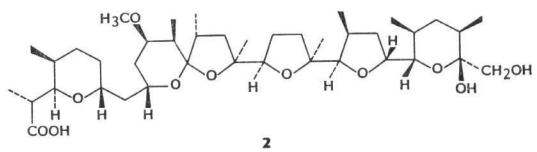

2

treatment of nigericin did not however give abierixin. Thus nigericin has not converted into abierixin on isolation.

It is quite plausible that abierixin is the ultimate metabolic precursor of nigericin. According to the proposed model for nigericin biosynthesis[11], a polyene would be first converted to a triepoxyde **3** (Scheme 1). Attack of the C-9 hydroxyl of **3** at the C-13 carbonyl carbon would initiate a cascade of ring closures to generate all five ether rings of abierixin with the observed stereochemistry. Then abierixin would give nigericin by a MICHAEL reaction. The ring closure by attack of the C-7 hydroxyl of abierixin at the C-3 double bond would be possible by the presence of the conjugated carboxylic group. Configurations of nigericin carbons 2, 3 and 7 are *R*, *S* and *R* respectively. If abierixin is the last precursor of nigericin, the stereochemistry of C-7 and double bond of abierixin must be *R* and *Z* respectively. For further confirmations, experiments are in progress and the results will be reported elsewhere.

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