AZ-GE-11 A NEW PEPTIDE ANTIBIOTIC ACTIVE AGAINST GRAM-POSITIVE BACTERIA

II. Fermentation, Isolation and Characterization.

B.M. Haroun*, A.M. Ragab**, El-Said A. Ghazal* and Zeinab K. Abd El Aziz**

*Botany Department, Faculty of Science, Al-Azhar University
*Botany Dept.,Faculty of Science for Girls,Al-Azhar Univ.

ABSTRACT

An active metabolite biosynthesized by an actinomycete isolate previously identified as Streptomyces AZ-GE-11(1) has been isolated from the culture beer by solvent extraction and purified by paper and column chromatography. The elemental analyses revealed that AZ-GE-11 has the molecular formula $C_{55}H_{78}O_{13}S_2$ and belongs to the peptide group of antibiotics. Maximum u.v. absorption band at 207 nm was recorded. The biological activities of the antibiotic revealed that it is active in vitro mainly against Gram-positive bacteria and slight or no activity was detected against Gram-negative ones.

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1. AZ = Al-Azhar University.
2. G = Faculty of Science for Girls.
3. E = Al-Ezaziah.

INTRODUCTION

Search for new antibiotics has been widely performed since about 40 years ago, and new are still being found. However, the possibility of discovering new antibiotics merely by random screening is reduced nowadays, and new approaches are required for finding new antibiotics efficiently.

On screening for new antibiotics, three major factors must be considered, i.e., detection methods, selection of producing microorganisms and cultivation methods.

In the course of a screening programme for new antibiotics, an actinomycete strain -11- identified as Streptomyces AZ-GE-11 attracted our attention due to its high activity on the tested Gram-positive bacteria and large productivity.

MATERIALS AND METHODS

Microorganisms:

Streptomyces AZ-GE-11 was used for antibiotic biosynthesis. On the other hand, Bacillus subtilis ATCC, 7972 and Staphylococcus aureus ATCC, 6538 P were used as test organisms for AZ-GE-11 bioassay.

Media and culture conditions:

Assay medium:

Nutrient agar medium composed of (g/1): Peptone, 5.0;

beef extract, 3.0; NaCl, 5.0, agar, 15.0 and distilled water 100 ml. The Ph was adjusted at 7.0.

Sporulation medium:

Consists of the following ingredients (g/1): Soluble starch, 10.0; $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4.YH_2$, 1.0; NaC1, 1.0 $(NH_4)_2SO_4$, 2.0; $CaCO_3$, distilled water 1000 ml. The final pH of the medium was adjusted at 7.0 before sterilization.

Seed medium:

Consts of yeast extract, 10.0 gm; dextrose, 10 gm; tap water, 1 liter and pH 7.4. The medium was sterilized, inoculated with spores of Streptomyces AZ-GE-11 and incubated for 72 hours at 30°C on a rotary shaker of 200 r.p.m. The previous medium was used to inoculate the production medium.

Production medium:

Consists of (g/1): Yeast extract, 4.0; malt extract, 10.0; glucose, 4.0; tap water, 1000.0 ml and pH 7-7.3 before sterilization. One ml of the seed medium previously described, was used for inoculating 50 ml of the fermentation medium in a 250 ml conical flask. The inoculated culture was incubated on a rotary shaker of 200 r.p.m. for 4 days at 30°C.

Bioassay of AZ-GE 11 in fermentation broth:

The antibiotic AZ-GE 11 in fermentation broth was bioassessed using the paper disc diffusion method and Bacillus subtilis ATCC 7972 and Staphylococcus aureus ATCC 6538 P as an indicator test organism.

A slant of nutrient agar was inoculated by a loopful of the test organism and incubated for 24 hours at 37°C. Five ml of sterile distilled water was added to the culture tube and mixed by vortec mixer.

Two drops were used for inoculating 100 ml semisolid sterile nutrient agar medium and poured after mixing in sterile petri dishes. After solidification, the paper disks contain the active material-adhered on the surface of seeded plates.

Determination of the minimum inhibitory concentration (MIC):

Minimum inhibitory concentration (MIC) werer determined using the agar plate dilution technique. Nutrient agar medium was used in MIC determination. Representative test organisms were used including Gram positive, Gram negative bacteria and some unicellular and filamentous fungi.

Chromatography:

Paper, thin layer and column chromatography were used for AZ-GE 11 purification.

Bioautography:

It is conducted by preparing nutrient agar medium, seeded by the test organism, flooded over the glass sheet of the tray and left to cool under aseptic conditions. The developed Whatman No. 1 chromatographic strips containing the antibiotic(s) material and flooded over the seeded agar plate, left for half an hour in a refrigerator for diffusion and then incubated at 37°C for 18 hours.

Chemical reactions:

Different chemical reactions were carried out on the antibiotic AZ-GE 11 to demonstrate the specific chemical groups of the antibiotic for helping in clarifying the chemical nature of the antibiotic.

General instrumentation:

Infrared (IR) absorption spectrum was recorded on Beckman IR-12 spectrophotometer. The ultraviolet (u.v.) spectrum was taken in methanol on a Beckman spectrophotometer and NMR spectrometer.

RESULTS

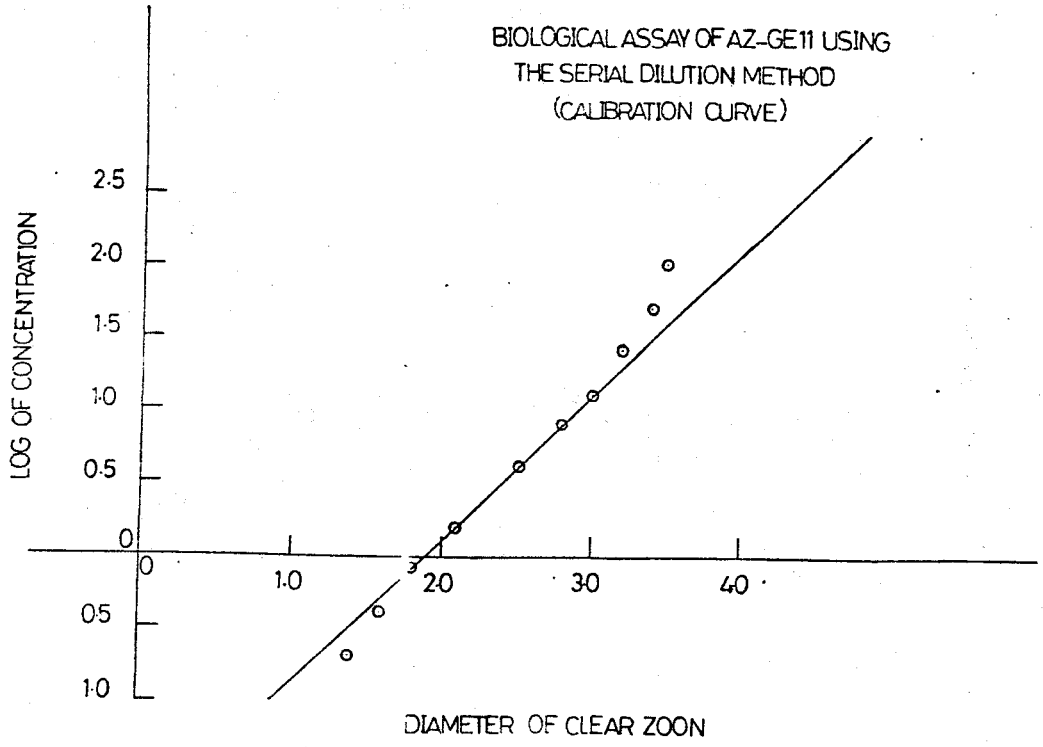
I. Fermentation, isolation and purification:

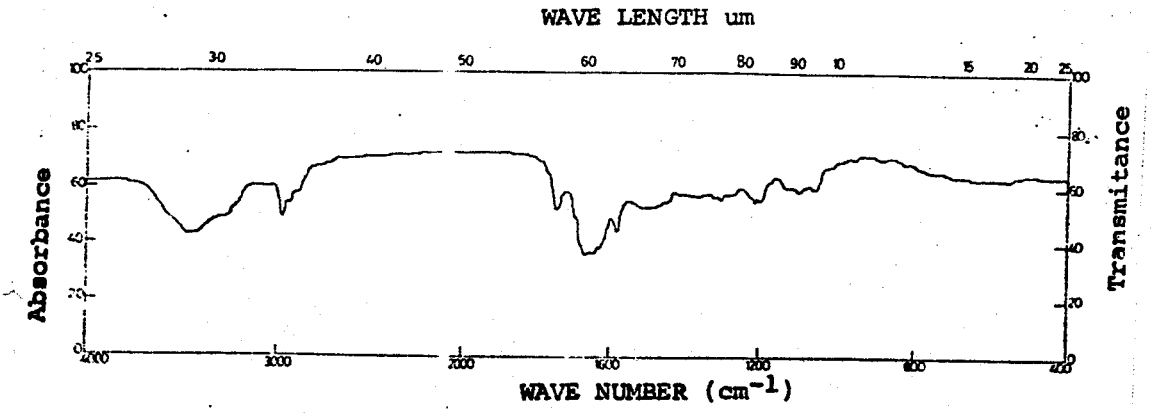
A. Production of AZ-GE 11 antibiotics:

Fifty ml of the yeast extract-dextrose (YD) liquid mediumwas inoculated by Streptomyces AZ-GE 11 in 250 ml conical flasks. The flasks werer incubated at 30°C for 3 days on a rotary shaker of 200 r.p.m. One ml of the previous culture (seed culture) was used to inoculate fifty ml (4%) of the fermentation medium as previously described. Maximum antibiotic biosyunthesis was recorded after 6 days at 30°C on rotary shaer of 200 r.p.m. The relation between antibiotic biosynthesis, mycelial dry weight and incubation time is illustrated in Fig. (1).

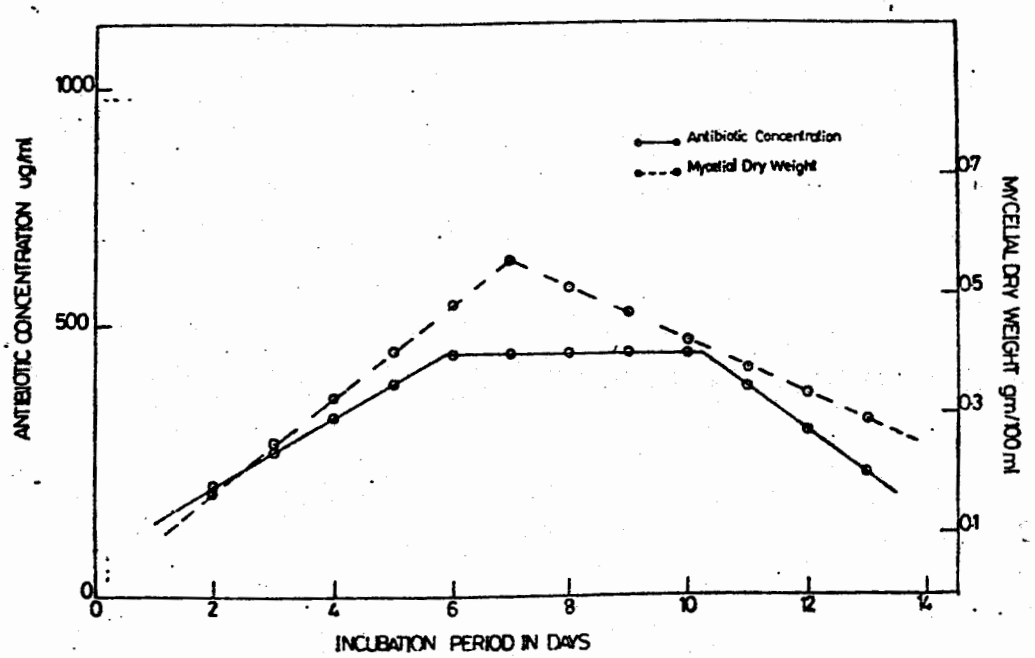
B. Isolation and purification of AZ-GE 11:

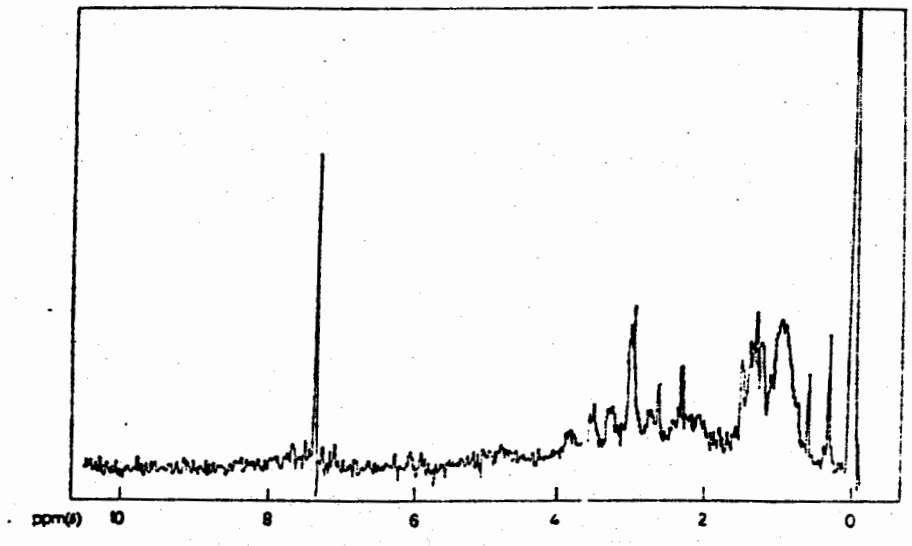
The culture filtrate was extracted by a mixture of chloroform; ethyl acetate (1:2[4] v/v) at pH 7.0. The organic layer was collected, evaporated using a rotary evaporator in presence of a water aspirotor to give a reduced pressure and to decrease the point at which evaporation was carried, so as not to exceed 40°C. The residue was collected as crude antibiotic in the form of a viscous syrup. The crude residue was purified by loading on a Whatman No. 1 paper chromatographic sheets and developed by ethyl alcohol/water. The yellow band at Rf 0.8 was collected, eluted with chloroform and concentrated. Excess petroleum ether (B.P. 40/60°C) was added to the concentrated solution where a yellow pigment was





INFRARED ABSORPTION SPECTRUM OF AZ-GE-11 ANTIBIOTIC





H NMR SPECTRUM OF ANTIBIOTIC AZ-GE 11

precipitated. The mixture was centrifuged at 3000 r.p.m. for 10 minutes. The precipitate was dissolved in the least amount of chloroform and reprecipitated by excess petroleum ether. The process of dissolving and precipitating of the antibiotic was repeated to get rid of the inactive coloured substances. The antibiotic was collected as an orange yellow powder and purified using a column of sephadex LH 20 and elluted by chloroform: methanol 9:1 (v/v). The active fructions following the void volume were collected, mixed, concentrated under reduced pressure and precipitated as previously described.

To be sure that the antibiotic is one component, Whatman No. 1 chromatographic paper strips, 8.0 mm width were loaded with the purified antibiotic and developed by various solvents. After developing, the strips were dried and bioautographed using Staphylococcus aureus ATCC 6538 P as the test organism. After 18 hours incubation at 37°C, one inhibition zone was always detected, indicating that the antibiotic is one component.

II. Physicochemical Properties of AZ-GE-11:

The active substance is obtained as a yellow amorphus powder of no characteristic odour and melts with decomposition at 180°C.

Elemental analyses found (%): C, 58.9; H, 6.86; N, 10.7; O, 17.86 and S, 5.68. From the previous data, the molecular formula was calculated and found to be $C_{55}H_{78}N_7O_{13}S_2$. The antibiotic is freely soluble in ethanol, methanol, butanol,

chloroform, ethyl acetate, butyl acetate, isopropyl alcohol, acetone; sparingly soluble in water; N. hexane and insoluble in petroleum ether and hexane. The behaviour of AZ-GE-11 towards various chemical tests is illustrated in Table 1.

Migration of the antibiotic on paper chromatograms using different solvents is illustrated in Table 2.

Spectroscopic analyses of AZ-GE 11:

The ultraviolet absorption spectrum of the antibiotic AZ-GE 11 exhibit maximum absorption band at 207 nm and shoulder at 429.4 nm. The IR spectrum of the antibiotic pelleted in KBr showed characteristic maximum bands at 1720, 1605, 1555, 1430, 1250 and 1160. Proton Nuclear Magnetic Resonance spectra H. NMR is illustrate in Figs 3, 4, and 5 respectively.

The antibiotic was hydrolyzedby 6N Hcl at 110°C in a sealed tube for 24 hours and the product was chromatographically investigated for its contents of amino acids after evaporation and desalting under vacuum and dissolving in 10% isopropyl alcohol. Seven amino acids were identified, namely: arginine, thronine, proline, methionine, phenyl alanine. Alkaline hydrolysis of the antibiotic revealed the presence of tryptophan and tyrosine.

Antimicrobial activity:

The antimicrobial activity of the antibiotic AZ-GE 11 are sshown in Table 3. This test was carried out by paper disk

Table 1: Characteristic chemical reactions of AZ-GE-11

No.	Chemical reaction	Result	Remarks
1	Biuret reaction	+ve	Proteins and their larger hydrolytic products are present
2	Xanthoprotein reaction	+ve	Aromatic compounds are present
3	Sakagu ki reaction	+ve	Arginine is present
4	Ehrlisch reaction	+ve	Indolic group is present
5	Millon's reaction	+ve	Tyrosine is present
6	Hopkins-cole reaction	+ve	Tryptophan is present
7	Iead sulphide reaction	+ve	Amino acids containing sulphur are present
8	Bendict	-ve	Absence of reducing sugars
9	Fehling	-ve	No free sugars
10	Ninhydrin	-ve	No NH_2 group free
11	Ferric chloride reaction	-ve	Diketones absent
12	Nitroprusside reaction	+ve	Sulphur is present
13	Molish test	-ve	No sugar moiety is present

Table 2: Bioautographic mobility of antibiotic AZ-GE-11 using precoated silica gel and Whatman No.1 paper strips on S. aureus.

No:	Developing solvent	R_f
1	Chloroform	1.00
2	Petrolium ether-chloroform	1.00
3	N-hexand	0.00
4	Isopropanol-water	0.90
5	Chloroform-ethyl acetate	0.80
6	Ethyl alchol	0.80
7	Isopropyl alchol	0.80
8	Ethyl alchol-water	0.80
9	Etyl acetate	0.70
10	Water	0.00
11	Petrolium ether	0.00
12	Acetone	1.00
13	Acedic chloroform	0.50
14	Alkaline chloroform	0.60
15	Butyl alchol	0.90
16	Butyl acetate	0.87
17	Methyl alchol	0.85

methods using nutrient agar medium for bacterial growth and Dox medium for fungal growth. AZ-GE 11 showed no antibacterial activity against Gram-negative bacteria and fungi, but is inhibitory to Gram-positive bacteria.

DISCUSSION

AZ-GE 11 was isolated from the culture fluid of Streptomyces AZ-GE 11 by solvent extraction. AZ-GE 11 is a neutral, yellow polypeptide antibiotic. It is different from many known macromolecular antibiotics such as plurallen[2], iyomycin complex[3] carzinocidin[4], melanomycin[5], A-280[6], peptinomycin[7] and lymphomycin[8]. AZ-GE 11 is different from the previous antibiotics in its composition, spectrum and biological activity.

AZ-GE 11 strongly inhibited the growth of Gram positive bacteria and little or no activity was recorded when Gram-negative bacteria and fungi were tested. So AZ-GE 11 is considered to be a new peptide antibiotic and was given the name AZ-GE 11.

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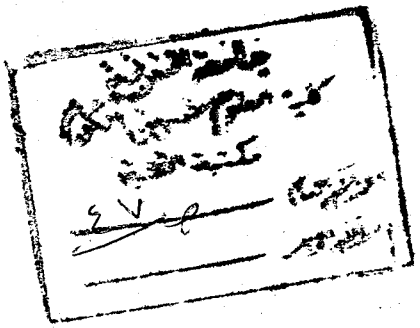

أ ذ ـ ج أ ـ ١١ مضاد حيوى جديد
ضد البكتريا الموجبة لصبغة جرام

٢) انتاج وعزل وصفات المضاد

بكرى محمد هارون * أحمد محمد رجب ، السعيد عبده محمد غزال زينب خالد عبدالعزيز *
كلية العلوم للبنات ـ كلية العلوم للبنين
جامعة الأزهر ـ مدينة نصر ـ القاهرة

لقد تم عزل مضاد حيوى ببتيدى جديد أ ذ ـ ج أ ـ ١١ من كائن ن جنس ستربتوميسيس أ ذ ـ ج أ ـ ١١ بواسطة الاستخلاص بالمذيبات العضوية تم تنقية بوسائل مختلفة مثل استخدام ورق الكروماتوجرافى وعامود الكروماتوجرافي .

وقد أمكن حساب المعادلة الجزئية له بعد أن تم تحليل دقيق للعناصر الموجودة بـــــــه فوجدت ك 55 يد78 ب 9 أ 13 كب 2 ، كما وجد أن المضاد الاحيائى يحتوى على أحمـــــاض أمينية كالتالي الارجنين والمثايونين البرولين ، الثريونين التيروزين ، الفينيل الانين ، التربتوفان وقد وجد أن للمضاد الاحيائى نشاط كبير ضد البكتريا الموجبة لصبغة جرام وخاصـــــة البكتريا العنقودية وليس له تأثيرا ملحوظا على البكتريا سالبة الجرام أو الفطريات .