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Two Antibiotics Identified as γ-Rubromycin and Crotonic Acid Produced by *Streptomyces collinus* NRC-11 Isolated from Egyptian Soils

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ABSTRACT. *Streptomyces* spp. NRC-11 was isolated from Egyptian soils. The organism was identified and shown to be identical to *Streptomyces collinus*. It produced two antibiotics in the fermentation broth. The two antibiotics were separated, purified and subjected to identification. The physico-chemical and biological properties of antibiotics are reported. Antibiotics A-1 and A-3 from filtrate and mycelium respectively, were identical to γ-rubromycin; antibiotic A-2 was identified as crotonic acid.

Rubromycins, first isolated from *Streptomyces* species by Lindenbein (1952) and Brockmann and Renneberg (1953), show strong antimicrobial activity against Gram-positive and Gram-negative bacteria. They were classified into α, β and γ-rubromycins and identified as naphthoquinone antibiotics (Brockmann *et al.* 1966, 1969, Abou-Zeid and El-Diwany 1977).

Material and Methods

Isolation of Streptomyces Species from Egyptian soils

The strain of *Streptomyces collinus* was isolated from Egyptian soils following the technique of Osman and Abou-Zeid (1968). An active strain of *Streptomyces* spp. NRC-11 was selected.

Taxonomic Studies

Taxonomic studies were performed principally by using the methods recommended by Pridham and Tressner (1974), Waksman (1957) and Arai *et al.* (1977). The studies were based on microscopic observations following 1, 2 and 3 weeks of

incubation. Also, the colour of sporulated aerial mycelia and substrate mycelia was designated mainly on the basis of seven colour series of the colour wheel made by Tressner and Backus (1963). The utilization of carbon sources were examined according to the method of Pridham and Gottlieb (1948). Hydrolysis of casein was carried out according to the method of Hastings (1903), while Conn's method (1951) was used for testing reduction of nitrate to nitrite. Decomposition of tyrosine was conducted according to the technique of Gordon and Smith (1955).

Formation of Active Antibiotics

The formation of active antibiotics was carried out by cultivation of the active strain of *Streptomyces* spp. NRC-11 in a nutrient medium containing the following ingredients (g/*l*): sucrose 20.0, sodium nitrate 2.0, disodium hydrogen phosphate 1.0, magnesium sulphate 0.5, potassium chloride 0.5, ferrous sulphate 0.01, in 1000 ml distilled water. The initial pH of the medium was adjusted to 7.0. The medium was portioned into Erlenmeyer flasks (250 ml capacity), each containing 50 ml of the medium. The flasks were plugged and sterilized at 121°C for 15 min. When the flasks attained ambient temperature (ca 25°C) they were inoculated with the active strain of *Streptomyces* spp. NRC-11 under aseptic conditions. The inoculated flasks were incubated at 30°C on a gyrorotatory shaker rotating at 200 rpm for 240 hr. When the fermentation process was completed, the fermented medium was harvested to carry out the desired analyses.

Extraction and Purification of the Active Antibiotics

The extraction and purification of active principles present in the fermentation broth and mycelia were carried out using different organic solvents (ethyl acetate, chloroform, methyl isobutyl ketone, petroleum ether and *n*-hexane) adjusted to different pH values. It was found that the mycelial extract of *Streptomyces* spp. NRC-11 inhibited the growth of *Bacillus subtilis* NRRL B-543. The mycelia of *Streptomyces* spp. NRC-11 were collected and washed several times with distilled water. The washed mycelia were dried at 20°C and ground thoroughly in a mortar. The ground dried mycelia were then extracted with acetone at pH 2.0-3.0 using an electric blender. The mixture was centrifuged at 4000 rpm and the supernatant was evaporated to dryness under vacuum to obtain the crude antibiotic material. The crude antibiotic was redissolved in ethyl acetate and filtered. The filtrate was evaporated to dryness under vacuum and the residue was dissolved in chloroform and concentrated under vacuum. *n*-Hexane was added till the solution was turbid. The turbid solution was centrifuged in glass cups and the supernatant (inactive) was discarded. The red antibiotic substance was redissolved in chloroform and petroleum ether (b.p. 40-60°C) was used for precipitation. This step was repeated several times until most purification was obtained for the antibiotic substance designated as A-3.

The extracted antibiotic substances from the fermentation broth were dissolved in ethyl acetate and filtered. The filtrate was evaporated to dryness under vacuum at 30°C and the residue was dissolved in petroleum ether (b.p. 40-60°C), filtered and concentrated to dryness under vacuum. The residue was again redissolved in petroleum ether (b.p. 40-60°C), filtered and concentrated to dryness under vacuum. The last step was repeated several times until a highly purified substance was obtained. At the final step, the solution was concentrated and crystallized at 25°C. The crystallized substance was dried under vacuum at 25°C and designated as antibiotic A-1. The clear chloroform solution was concentrated under vacuum to give a colourless substance designated antibiotic A-2. The extraction and purification procedures are shown in Fig. 1 and 2.

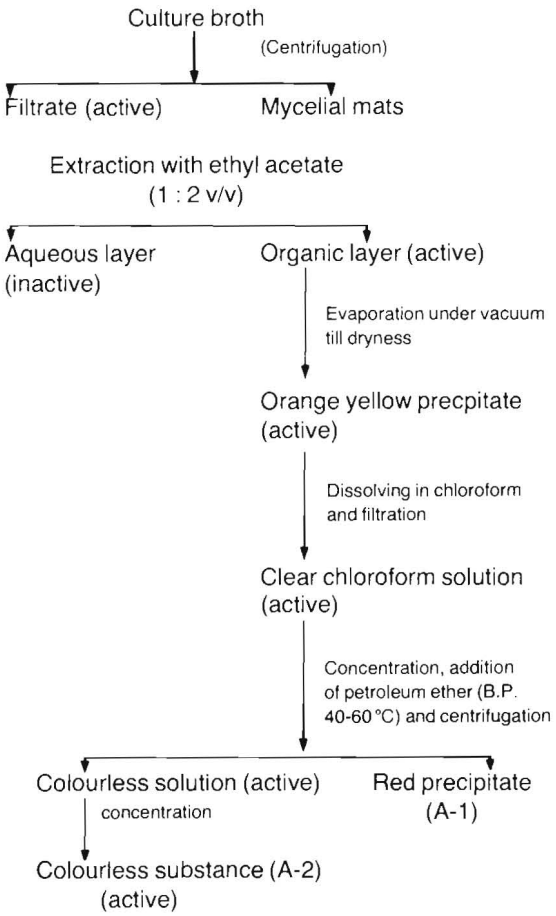

Fig. 1. Flow diagram for the isolation and purification of the active metabolites and its biological activity (against *Bacillus subtilus* NRRL B-543).

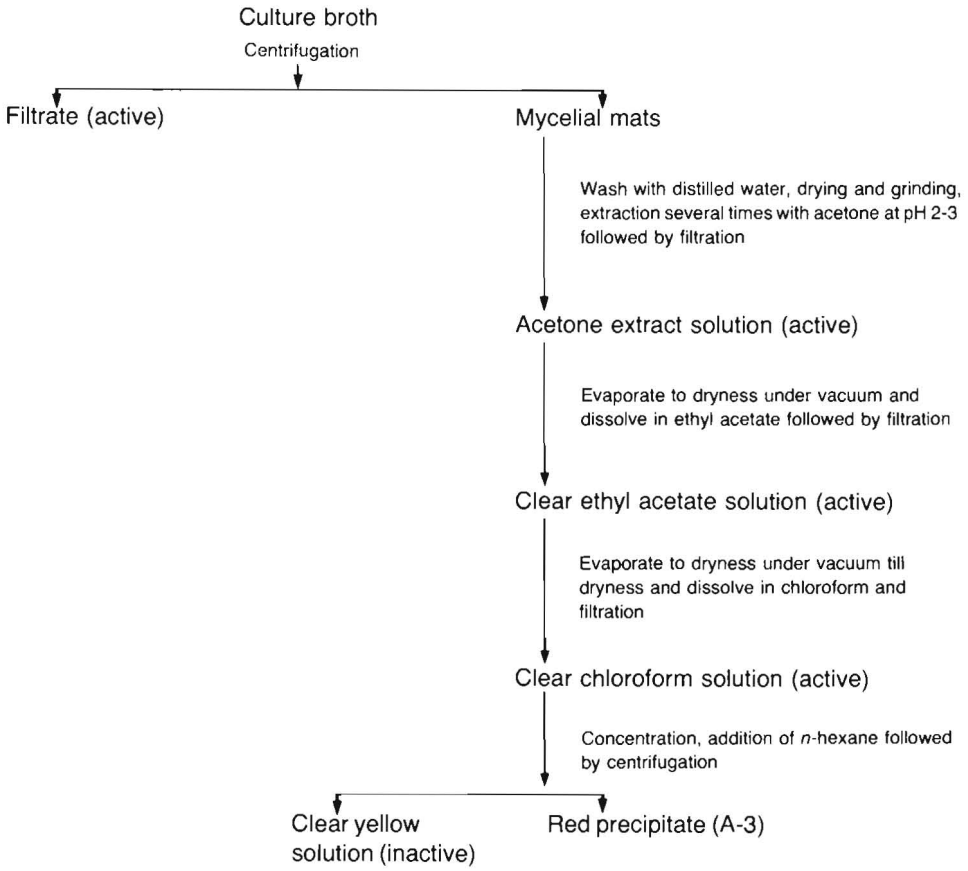

Fig. 2. Flow diagram for the isolation and purification of the active antibiotics, from the mycelial mats of *Streptomyces* spp. NRC-11 and its biological activity against *Bacillus subtilis* NRRL B-543.

Identification of the Active Antibiotics

Waksman's Key, Berdy's system and Arai's system were utilized for the identification of the active antibiotics. Chromatographic classification and identification of the active principles were carried out using the methods described by Betina (1964), Betina and Nemec (1960) and Uri (1959).

Results and Discussion

Identification of Streptomyces spp. Strain NRC-11

The results of microscopic examinations (Fig. 3 and 4), cultural characteristics, and biochemical properties of *Streptomyces* spp. NRC-11 (Table 1) show that it

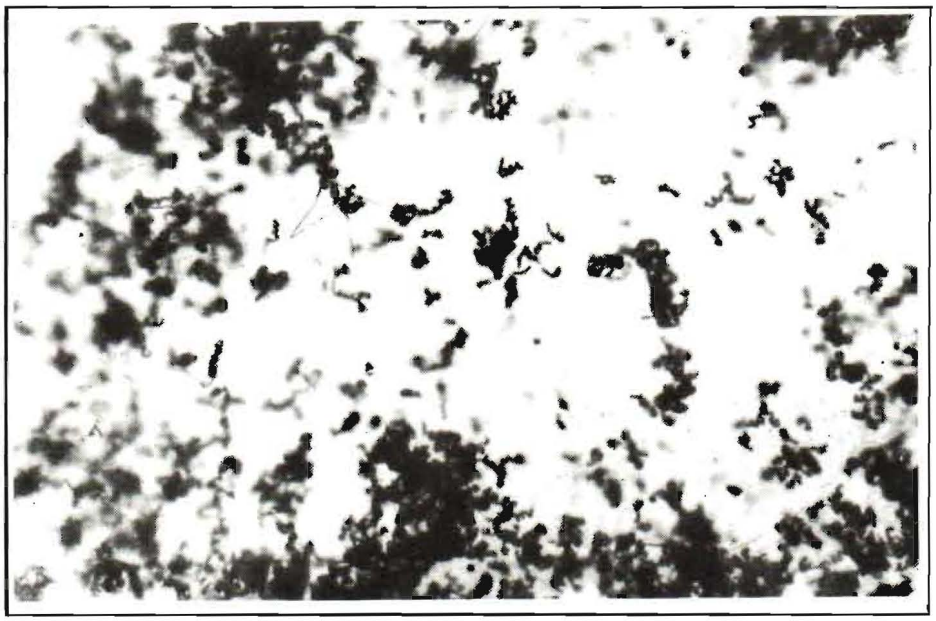

Fig. 3. Morphology of spore-bearing hyphae of *Streptomyces* sp. NRC-11.

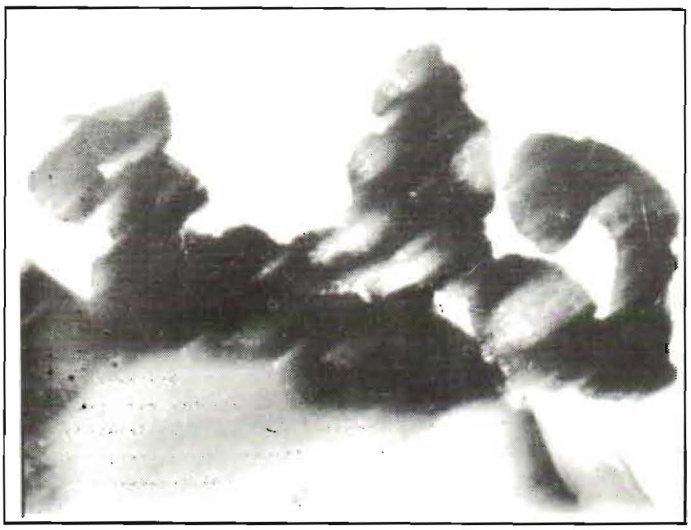

Fig. 4. Yeast malt agar: X 12000.

belongs to a group of the genus *Streptomyces* (*S*) possessing the following properties: colour of mature sporulated aerial mycelium is in the gray colour series, spore chain is in the section spiral, and spore surface is smooth.

Table 1. Cultural characteristics of isolated *Streptomyces* spp, strain NRC-11.

Medium	Criteria	7 days	14 days	21 days
1. Bennett's agar	G:	Good	Good	Good
	Am:	Yellow	Gray	Gray
	Sm:	Yellow	Yellow	Gray
	Sp:	None	None	None
2. Glucose-asparagine agar	G:	Good	Good	Good
	Am:	White	Gray	Gray
	Sm:	Red	Red	Red
	Sp:	None	None	None
3. Sucrose nitrate agar	G:	Good	Good	Good
	Am:	Gray	Gray	Gray
	Sm:	White	White	Yellow
	Sp:	None	None	None
4. Starch agar	G:	Good	Good	Good
	Am:	Gray	Gray	Dark gray
	Sm:	White	Yellow	Yellow
	Sp:	None	None	None
5. Nutrient agar	G:	Poor	Poor	Poor
	Am:	None	None	None
	Sm:	Yellow	Yellow	Yellow
	Sp:	None	None	None
6. Egg albumin	G:	Poor	Poor	Poor
	Am:	None	None	None
	Sm:	Colourless	Colourless	Colourless
	Sp:	None	None	None
7. Potato glucose agar	G:	Good	Good	Good
	Am:	White	Gray	Gray
	Sm:	Yellow	Brown	Brown
	Sp:	None	None	None
8. Glucose-yeast extract-beef extract-peptone agar	G:	Good	Good	Good
	Am;	None	None	None
	Sm:	Yellow	Yellow	Brown
	Sp:	None	None	None

Where: G: Growth
Am: Aerial mycelium
Sm: Substrate mycelium
Sp: Soluble pigments

The test microorganism utilized all carbon sources except sorbitol and cellulose (Table 2). It hydrolysed casein, coagulated milk, reduced nitrate to nitrite, and exhibited good liquefaction of gelatin. It also produced melanoid pigment when propagated on tyrosine and tryptone yeast agar (Table 3). Consequently, *S.* spp. NRC-11 was compared to *Streptomyces collinus*, *S. olivochromogenes*, *S. resistomycificus*, and *S. violaceochromogenes*. The results recorded showed that *S.* spp. NRC-11 closely resembled strain of *S. collinus*.

Table 2. Utilization of carbon sources by *Streptomyces* spp. strain NRC-11.

Carbon sources	D.u.	Carbon source	D.u.
Rhamnose	+	Arabinose	(+)
Raffinose	(+)	Salicin	+
Xylose	+	Dulcitol	+
Mannitol	+	Inulin	+
Lactose	+	Cellulose	−
Inositol	+	Phenol	(+)
Sorbitol	−	*m*-Cresol	(+)
Glucose	+	Sodium tartrate	(+)
Galactose	+	Sodium acetate	+
Starch	+	Sodium citrate	+
Sucrose	+	Sodium succinate	+
Fructose	+	Sodium formate	(+)

D.u. = Degree of utilization
(+) = Weak growth
\+ = Good growth
− = No growth

Table 3. Physiological characteristics of *Streptomyces* spp. strain NRC-11.

Physiological properties	*Streptomyces* spp. NRC-11
Liquefaction of gelatin	Good liquefaction
Hydrolysis of casein	Casein hydrolysis
Coagulation of milk	Positive
Formation of melanoid pigment:	
a) on tryptone-yeast-extract agar	Brown pigment formation
b) on tyrosine agar	Formation of brown pigment
Reduction of nitrate	Red colour

Formation of the Active Antibiotics

Optimal fermentation time period for maximal antibiotic production by *S.* spp. NRC-11 was 240 hr (Table 4).

Extraction of the Active Antibiotic Metabolites

At optimum pH, suitable solvents for extraction of the active antibiotic principles present in the fermentation broth of the strain NRC-11 (Table 5) are those giving maximal inhibition zones (I.Z.) on cultures of *Bacillus subtilis* NRRL B-543. Extraction of active antibiotic metabolites was obtained at acidic pH ranges (pH

Table 4. Effect of incubation time on the formation of antibiotic by *Streptomyces* spp. strain NRC-11.

Incubation periods (days)	Final pH value	Mycelial dry weight (mg/100 ml)	Incubation zone (mm)
2	7.6	24	13.0
3	7.8	34	15.0
4	7.8	56	16.0
5	7.5	112	17.0
6	7.7	224	18.0
7	7.7	280	20.0
8	7.7	336	22.0
9	7.6	336	23.0
10	7.7	340	24.0
11	7.7	340	24.0
12	7.7	361	24.0

Table 5. Suitable solvents and optimal pH value for extraction of active antibiotic principles from the fermentation broth of *Streptomyces* spp. strain NRC-11.

Organic solvent	Fermentation broth pH							
	2	3	4	5	6	7	8	10
	Inhibition zone (mm)							
Ethyl acetate	27.0	27.0	26.0	27.0	27.0	27.0	27.0	27.0
Chloroform	13.0	13.0	13.0	12.0	12.0	11.0	10.0	7.0
Methyl isobutyl ketone (MIBK)	0.0	0.0	11.0	10.0	10.0	10.0	0.0	0.0
Petroleum ether (40-60 °C)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
n-hexane	60.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

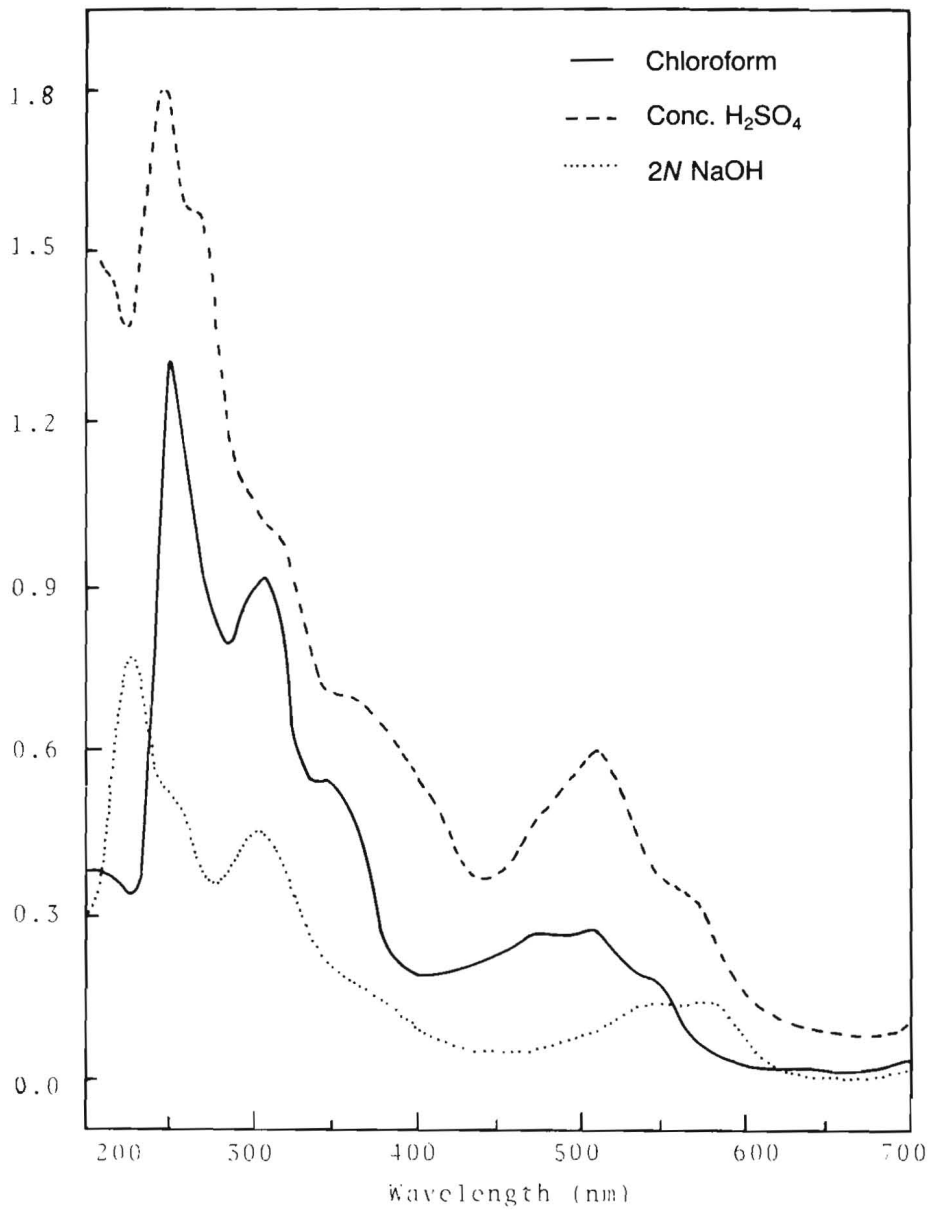

Fig. 5. Ultraviolet and visible absorption spectra of antibiotic (A-1 and A-3) in chloroform, conc. sulphuric acid and 2 *N* sodium hydroxide.

2 to 5). Ethyl acetate and chloroform were the best organic solvents for the extraction of active antibiotic metabolites. An active metabolite was also extracted from the mycelia of *S.* spp. NRC-11 with acetone at pH 2.0.

Physicochemical Properties of the Antibiotic from A-1 and A-3

The two antibiotic preparations were dark red powders, soluble in ethyl acetate, chloroform, methanol, benzene, toluene, conc. sulphuric acid and 2*N* NaOH. Their melting point ranged from 233-235°C. The elemental analysis gave C (59.18%), H (3.60%) and O (37.22%). The latter was obtained by difference. The antibiotic gave negative ninhydrin, Sakaguchi, indole and related Erlich reactions, Molisch and Fehling's reactions. However, the substance was attacked rapidly by 0.1 *N* potassium permanganate solution with formation of brown manganese dioxide. The antibiotic gave a green colour with ferric chloride solution, violet with 2 *N* NaOH solution, and a red colour with concentrated H_2SO_4. Spectroscopic properties of the two preparations were identical. The ultraviolet and visible absorption was carried out in chloroform, conc. H_2SO_4 and 2 *N* NaOH (Fig. 5). In chloroform, the maxima were observed at 250, 310, 350, 480, 510 and 550 nm.

The infrared spectra of the antibiotics (Fig. 6) show the presence of a phenolic group at 3400 cm^{-1}. The methylene (CH_2) was found at 2920 cm^{-1}, while the characteristic bands of E=O and of ester and α-pyrone appeared at 1726 and 1687 cm^{-1}. Bands also at 1605 and 1446 cm^{-1} were characteristic for chelated quinone and phenyl nucleus. The band at 1232 cm^{-1} was characteristic for (C=O) ether and aromatic methoxy group. From the data obtained, it became apparent that the substance could be related to the naphthoquinone derivative group of antibiotics.

The physicochemical and biological properties of the purified antibiotics A-1 and A-3 were identical to γ-rubromycin reported in Waksman's key, Berdy's sys-

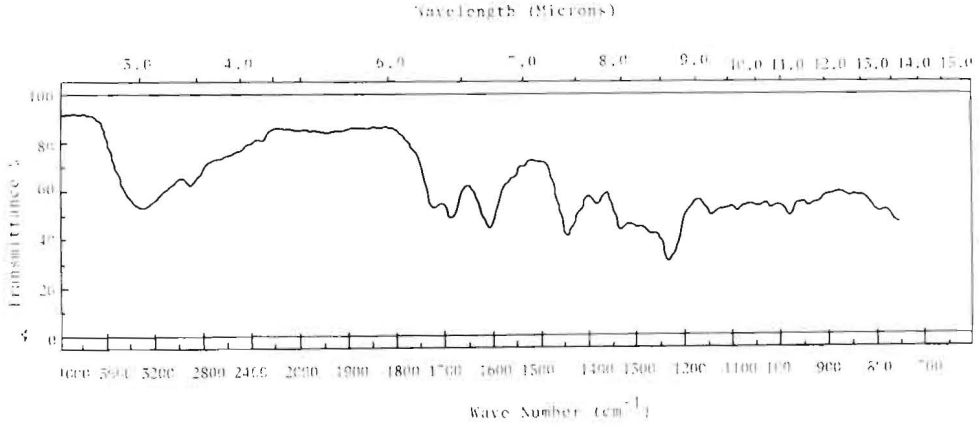

Fig. 6. Infrared spectrum of the antibiotic (A-1 & A-3) in potassium bromide

tem and Arai's system for classification and identification of antibiotics. γ-Rubromycin belongs to the group of antibiotics named rubromycins (Lindenbein 1952, Brockmann and Renneberg 1953, Brockmann *et al.* 1966 and 1969, Abou-Zeid and El-Diwany 1977).

Table 6. The R_f values of the antibiotics (A-1 and A-3) on different solvent systems.

Solvent	R_f value of active metabolite
1. Butanol saturated with water	0.37
2. Methanol-water (40:60 v/v)	0.88
3. *n*-Propanol-water (40:60 v/v)	0.66
4. Methanol- 3% ammonium chloride in water (70:30 v/v)	0.13
5. Methyl ethyl ketone-*n*-butanol-water (30:5:65 v/v/v)	0.00
6. 3% Ammonium chloride in water	0.00
7. Isoamylacetate-methanol -99% formic acid-water (65:20:5:10 v/v/v/v)	0.89
8. Isoamyl acetate-methanol -77% formic acid-water (40:20:10:30 v/v/v/v)	0.88
9. *n*-Butanol-methanol-water (40:10:50 v/v/v)	0.66
10. Methanol-*n*-hexane (60:40 v/v)	0.13
11. Benzene-Cyclohexanone- 0.15 *M* phosphate buffer, pH 7.4 (5:35:60 v/v/v)	0.65

Table 7. Antimicrobial spectrum of the active metabolites (A-1 and A-3).

Test organisms	MIC (μg/ml)
1. *Bacillus subtilis* NRRL B-543	0.002
2. *Bacillus cereus* NRRL B-469	0.002
3. *Escherichia coli* NRRL B-210	0.1
4. *Salmonella typhosae* NRRL B-573	0.1
5. *Klebsiella penumoniae* NRRL B-117	0.002
6. *Micrococcus luteus* NRRL 1018	0.01
7. *Proteus mirabilis* A-20046	0.1
8. *Candida lipolytica* NRRL E-1095	0.05
9. *Saccharomyces cerevisiae* NRRL Y-567	0.002
10. *Aspergillus niger* NRRL 599	–
11. *Candida statti*	–
12. *Bacillus megatherium* NRRL -3712	–

MIC: Minimum inhibitory concentration.

Physicochemical Properties of the Active Antibiotic Metabolite A-2

Metabolite was colourless powder, acidic, soluble in water, carbon tetrachloride, chloroform, ethyl acetate, partially soluble in methanol, and its melting point ranged 70-71°C. The elemental analysis gave C (51.89), H (7.1) and O (41.0%). The latter was obtained by difference. Sakaguchi, ferric chloride, Molisch, indole and related Erlich reactions were negative. The antibiotic substance was attacked rapidly by 1 *N* cold permanganate solution with production of brown manganese dioxide. The ultraviolet absorption spectrum shows a strong absorption maximum at 210 nm in water and in 0.02 *M* sulphuric acid.

The infrared spectrum of A-2 is diagnostic for an α-β-unsaturated carboxylic acid, and the NMR and spectrometric analysis fully confirm the identification of antibiotic A-2 as crotonic acid, *trans*-but-2-enoic acid (MacRae *et al.* 1963, Schlegel *et al.* 1961).

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التعرف على مضادين حيويين جاما ربروميسين وحمض الكروتونيك تم إنتاجهما بسلالة ستربتوميسس نوع - ١١ معزولة من التربة المصرية

أبوزيد على أبوزيد و محمد عبد الفتاح فريد

معامل المنتجات الطبيعية - المركز القومى للبحوث - الدقى - جيزة مصر

تم عزل سلالة ستربتوميسس نوع - ١١ نشطة من التربة المصرية، وبالتعرف عليها وجد أنها تشبه ستربتوميسس كولينس *(Streptomyces collinus)* وقد كونت هذه السلالة مضادين حيويين بوسط التخمر وبالتعرف عليهما بالطرق البيولوجية والخواص الطبيعية والكيميائية تبين أنهما جاما ربروميسين وحمض الكروتونيك.