KUWAITIMYCIN, A NEW ANTIBIOTIC

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A new antibiotic active against gram-positive bacteria was isolated from culture broths of *Streptomyces kuwaitinensis* nov. sp. obtained from Kuwait soil. The antimicrobial agent crystallized as dirty yellow needles that on acid hydrolysis yielded lysine, arginine, serine, glutamic acid and alanine as well as an unsaturated aliphatic acid, presumably of isohexadeca-3, 6-dienoic structure. Characteristics of the producing organism are given and consequently its systematic position among the verticillate Streptomyces species is discussed. Isolation and properties of the antibiotic, kuwaitimycin, are described.

Streptomyces AS-K-444, isolated from the soil of Kuwait, secrets in its culture broths an antibiotic that possesses substantial activity against gram-positive bacteria. The antimicrobial agent, kuwaitimycin, is freely soluble in most organic solvents but only slightly soluble in petroleum ether and water.

Characteristics of Strain AS-K-444

1. Morphological Characteristics

The morphology of the culture was microscopically observed on starch-nitrate agar and CZAPEK's agar at 30°C for 14 days. The aerial mycelium exhibits typical whorl formation (Plate 1). The electron-micrograph reveals oval hairy spores (Plate 2, a, b).

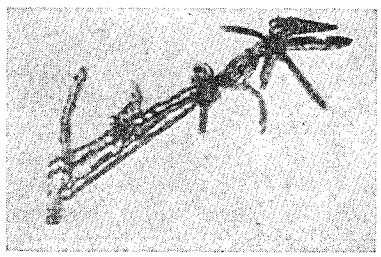
2. Cultural and Physiological Characteristics

Cultural characteristics and a summary of physiological properties of strain *S.* AS-K-444 are shown in Tables 1 and 2. The different media used in the present study were prepared according to WAKSMAN[1] and SHIRLING and GOTTLIEB[2]. Mature spores and mycelia on BENNETT's agar were used to inoculate each medium. Unless otherwise stated all cultures were incubated at 30°C for 14 days before observation.

On most media yellowish-orange vegetative growth and aerial mycelium that is white to whitish gray to pale yellow was observed. Yellowish orange to red soluble pigment was produced in some synthetic media. *Streptomyces* AS-K-444 failed to produce melanin pigment in tyrosine-agar medium.

Utilization of different carbon sources by *S.* AS-K-444 was investigated as described by PRIDHAM and GOTTLIEB[3] and the results are given in Table 3. Starch, glycerol, glucose and sucrose were readily utilized whereas fructose, maltose, lactose, rhamnose, salicin and galactose were slightly utilized. The organism failed to grow on the rest of the carbon sources tested.

Plate 1. Sporophores of *Streptomyces* AS-K-444 (×800)



From the observations aforementioned *Streptomyces* AS-K-444 may be described as follows: It forms whitish gray to pale yellow aerial hyphae with typical whorls. Orange yellow soluble pigment was occasionally produced in synthetic media. Vegetative growth on synthetic media has

Plate 2a. Spores of *Streptomyces* AS-K-444 (×10,000)

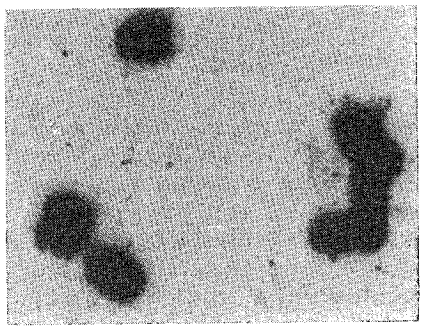


Plate 2b. Spores of *Streptomyces* AS-K-444 (×20,000)

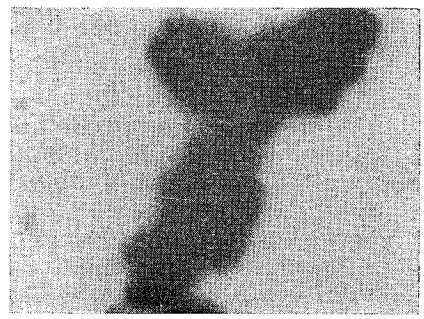


Table 1.

Media	Characters
Starch-nitrate agar	G: good; A: whitish tin powdery; Sub.: dark orange yellow; S: none
Glucose-nitrate agar	G: good; A: whitish tin powdery; Sub.: dark orange yellow S: deep orange yellow
Glucose-asparagine agar	G: weak; A: rare white; Sub.: dark red; S: dark red orange
Nutrient agar	G: Weak; A: no aerial mycelium; Sub.: creamy yellow S: none
CZAPEK's agar	G: good; A: whitish gray powdery; Sub.: orange red S: orange red.
Calcium-malate agar	G: moderate; A: white powdery; Sub.: orange S: orange
Tyrosine agar	G: weak whitish gray; A: weak whitish gray Sub: whitish S: none
Yeast malt extract agar	G: good; A: whitish gray; Sub: whitish S: none.
Glycerol-asparagin agar	G: good; A: yellowish gray powdery; Sub.: dark orange yellow; S: dark brown
BENNETT's agar	G: good; A: thin white powdery; Sub.: pale orange S: pale orange
Peptone-yeast iron-agar	G: weak; A: weak whitish; Sub.: creamy S: none
Oat meal agar	G: good; A: white greyish powdery; Sub.: pale orange S: pale orange
Milk	G: Good A: none Rapid coagulation followed by slow peptonization
Gelatin stab (15~20°C for 20 days)	G: moderate; A: none; Sub.: yellowish orange; S: none
Potato plug	G: very good, A: pallid yellow powdery, Sub.: blackish green, S: blackish green
Cellulose	No growth and no decomposition during 60 days of incubation

G: growth; A: aerial mycelium; Sub.: substrate mycelium; S: soluble pigment.

Table 2. Physiological properties of strain *S.* AS-K-444

Optimum temperature for growth	26~32°C
Optimum pH range for growth	6~8
Tyrosinase reaction	negative
Melanoid pigment	negative
Reduction of nitrate	rapid reduction
Liquefaction of gelatin	rapid
Coagulation of milk	rapid
Peptonization of milk	slow
Hydrolysis of starch	weak
Cellulose decomposition	negative
H_2S formation	negative
Antimicrobial product	kuwaitimycin

Table 3. Utilization of different carbon sources

Carbon sources	Utilization
D-Glucose	++
L-Rhamnose	+
D-Fructose	+
D-Maltose	+
Sucrose	++
L-Arabinose	− −
D-Xylose	− −
D-Sorbitol	− −
Inulin	− −
D-Lactose	+
Raffinose	− −
D-Galactose	+
Starch	+++
Glycerol	++
Trehalose	−
Salicin	+

+++=good growth; ++=moderate growth; +=feeble growth; −=no growth.

Composition of the basic medium used (g/100 ml): Carbon source, 1.0; $(NH_4)_2SO_4$, 0.264; KH_2PO_4, 0.238; K_2HPO_4, 0.565 and $MgSO_4 \cdot 7H_2O$, 0.00015.

different shades of orange. The organism is melanin negative.

3. Classification of *Streptomyces* AS-K-444

The following *Streptomyces* species of the verticillate type described by WAKSMAN[1], ISP reports by SHIRLING and GOTTLIEB[4,5,6] and other recent literature are considered related to *S.* AS-K-444: *Streptomyces cinnamomeus*[1], *S. flavopersicus*[4], *S. hachijoensis*[1,4], *S. kobenensis*[7] *S. triculaminicus*[8], *S. fervens*[1] and *S. sapporonensis*[9]. A concise comparison is given in the corresponding scheme and accordingly *S.* AS-K-444 is considered a new species belonging to the verticillate melanin-negative Cinnamomeus series[1] and the name *Streptomyces kuwaitinensis* nov. sp. is proposed.

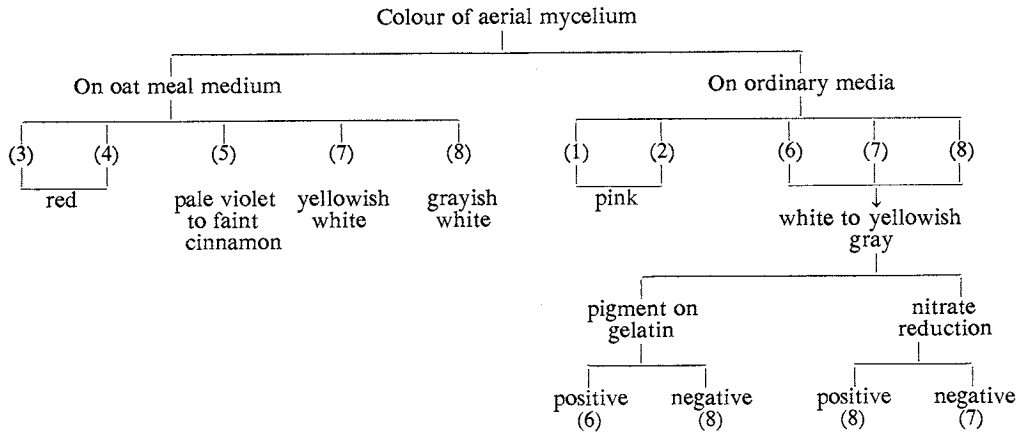
Production, Isolation and Purification of Kuwaitimycin

The production, medium contained the following ingredients (g/100 ml): glucose, 1.0; soya bean flour, 1.0; $NaNO_3$, 0.2; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.005 and KCl, 0.05. The medium was adjusted to pH 7.0 before sterilization and was then distributed in 400-ml portions among 2 liter Erlenmeyer flasks. The antibiotic titer reached a maximum of 7 mg/100 ml of broth after 6 days of incubation at 28°C on a rotary shaker at 220 r.p.m.

The broth was filtered and the filtrate extracted with ethyl acetate at pH 8.0~8.5. The extract was evaporated to dryness *in vacuo*. The brown oily residue was then dissolved in a small amount of ethyl acetate and the crude antibiotic precipitated by the addition of 10 volumes of petroleum ether (b.p. 40~60°C).

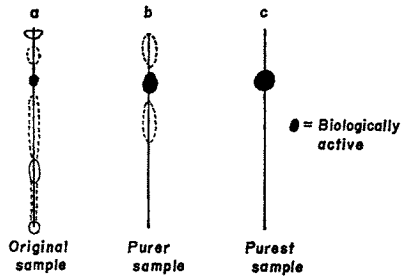
The crude antibiotic contained appreciable amounts of inactive materials as indicated by thin-layer chromatography. For purification the crude material was chromatographed on silica gel (GF 254) column. The active fraction was eluted with acetone-benzene (25:75), collected and treated with excess diethyl ether to give a yellowish brown precipitate. This precipitate was four times more active than the starting material but still contained an appreciable amount of inactive material. The antibiotic was further purified by preparative thin-layer chromatography on silica

Scheme for differentiating *Streptomyces* AS-K-444 from the related verticillate *Streptomyces* species



Case No:
(1) *S. triculaminicus*, (2) *S. fervens*, (3), *S. fivopersicus*, (4) *S. hachijoensis*, (5) *S. cinnamomeum*, (6) *S. kobenensis*, (7) *S. sapporonensis*, (8) *S.* AS-K-444.

Fig. 1. Steps of purification of kuwaitimycin on TLC with silica gel GF-254; Benzene-acetone (1:1) Detection with UV irradiation.



gel GF 254 with a solvent system composed of benzene-ethyl acetate (2:1). The active spot detected by ultraviolet irradiation was scrapped off and extracted with methanol and acetone. The extract was then concentrated *in vacuo* until near dryness when the antibiotic precipitated in a high degree of purity by slow addition of petroleum ether (b.p. 40~60°C). Samples of the antibiotic at different stages of purity were thin-layer chromatographed on silica gel GF 254 using benzene-acetone mixture (1:1) which was found suitable for comparative purposes (Fig. 1 a, b, c).

Physical and Chemical Properties of Kuwaitimycin

Kuwaitimycin is obtained as a dirty yellow crystals which melt at 158~159°C. The antibiotic is freely soluble in alcohols, ethyl and butyl acetates and acetone; to a lesser extent in benzene; and is scarcely soluble in petroleum ether, diethyl ether and water.

The infrared spectrum of kuwaitimycin in KBr (Fig. 2) showed peaks at wavenumber (cm^{-1}) 3400, 2980, 1450, 1380 and 1260. The ultraviolet spectrum (Fig. 3) in methanol showed two absorption maxima at 254 and 278 mμ ($E^{1\%}_{1cm}$ 225 and 165, respectively).

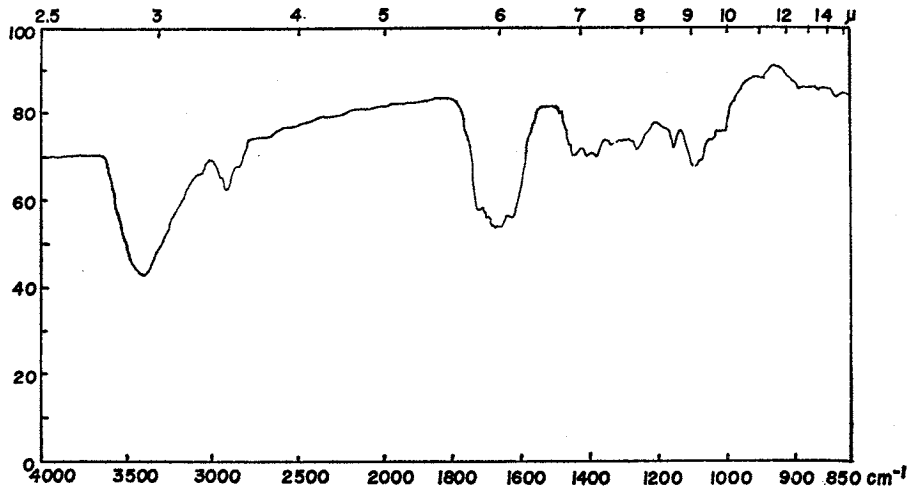
The R_f values for pure kuwaitimycin on thin-layer chromatostrips of silica gel GF 254, kieselguhr and of alumina were determined using different developing solvent systems. Location of the zones was carried out by ultraviolet light and the results are given in Table 4. A single zone possessing antimicrobial activity was always observed.

Optical rotation of kuwaitimycin was $[\alpha]_D^{23}+60°$(c *2*, ethanol).

Elemental analysis gave the following data:

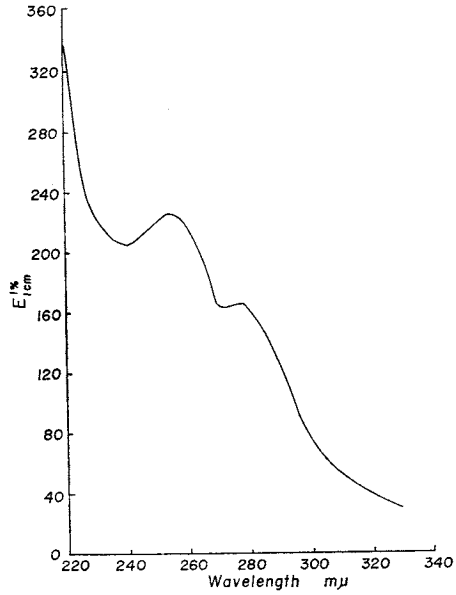
Calcd. for $(C_{13}H_{19}NO_3)_n$	C 65.82,	H 8.02,	N 5.90,	O 20.26 %
Found	C 65.90,	H 7.98,	N 5.84,	O 20.28 %

Fig. 2. Infrared spectrum of kuwaitimycin in KBr.



On hydrolysis the antibiotic with 6N HCl for 24 hours at 105°C the following amino acids could be identified by paper and thin-layer chromatography: lysine, arginine, serine, alanine and glutamic acid. Their molar proportions as determined by the amino acid analyzer are shown in Table 5. No other ninhydrin-positive products could be detected. All the amino acids were found to possess an α-configuration when the acid hydrolysate was tested as described by CRUMPLER[10].

Fig. 3. Ultraviolet spectrum of kuwaitimycin



Another portion of the antibiotic was hydrolysed at 105°C for 24 hours with 6N HCl and the hydrolysate was extracted with diethyl ether. The extract was then methylated with diazomethane in the usual way and the reaction mixture subjected to GLC (Hewlett-Backard Model 5750) on diethylene glycol succinate polymer at 160°C. A main peak at retention time 13.7 minutes was observed. Reference fatty acids were run in a similar way. The relationship between log. retention times and the carbon numbers for the individual members of each group of fatty acids (normal, unsaturated, iso and anteiso) form a straight line. For any given number of carbon atoms the unsaturated fatty acid has the highest retention time followed by the saturated number and finally by the anteiso and iso acids. The latter two acids have essentially identical retention times.

The retention times of the methyl esters of palmitic acid and the acidic moiety of kuwaitimycin were almost identical (13.5 and 13.7 minutes respectively). The latter acid strongly absorbed iodine and when hydrogenated on platinum black the retention time shifted to 11 minutes.

Table 4

Solvent systems	R_f			Remarks
	Silica gel GF 245	Alumina	Kieselguhr	
Ethanol	0.83	0.86	0.80	Single zone (active)
Ethanol-water (75:25)	0.52	0.49	0.42	〃
Acidic ethanol (pH 3.5)	0.83	0.81	0.81	〃
Alkaline ethanol (pH 8.5)	0.79	0.77	0.78	〃
Acetone	0.96	0.98	0.94	〃
Acetone-water (70:30)	0.48	0.51	0.48	〃
Acetone-benzene (1:1)	0.70	0.73	0.69	〃
Acetone-benzene (1:1) pH 3.5	0.68	0.70	0.65	〃
Acetone-benzene (1:1) pH 8.5	0.64	0.68	0.66	〃
Chloroform-petroleum ether* (1:1)	0.53	0.46	0.50	〃
Ethyl acetate-petroleum ether* (1:1)	0.60	0.52	0.57	〃

* Petroleum ether b.p. 60~80°C.

Table 5. Molar ratios of amino acids obtained after hydrolysis with 6N HCl for 24 hours at 105°C and removal of the fatty acid

	Lysine	Glutamic acid	Serine	Alanine	Arginine
A	14	1	8	2	—
B	—	1	6	—	6
C	—	2	—	2	—
D	3-4	1	3-4	1	1-2

A, B, C: moieties obtained by partial acid hydrolysis of kuwaitimycin with 1 N HCl for 3 hours at 70°C.
D: Kuwaitimycin.

Oxidation of the unidentified fatty acid by permanganate-periodate yielded malonic acid and isocapric acid ($[\alpha]_D^{21}+8°$ for the acid amide; molecular weight, found 168, calcd. 172) separable by thin-layer chromatography.

On the basis of this information the fatty acid moiety of kuwaitimycin probably has an isohexadeca-3, 6-dienoic structure.

The residual aqueous phase obtained after acid hydrolysis tested negative for carbohydrate and for amino-sugars.

Partial hydrolysis of kuwaitimycin with 1N HCl for 3 hours at 70°C gave 3 ninhydrin-positive moieties that were separated by paper chromatography using *n*-butanol-acetic acid-water (4:1:5). These moieties were separately hydrolysed with 6 N HCl for 24 hours at 105°C. Subsequently, the acid hydrolysates were extracted with diethyl ether to remove the fatty acid. The amino acid contents of the residual aqueous phases were identified by paper and thin-layer chromatography. Furthermore, quantitation of the amino acids of each fraction was carried out with Technicon TSM amino acid analyser and the results are given in Table 5.

The behaviour of the antibiotic towards different chemical tests summarized in Table 6 supports its peptidic nature.

Biological Properties

The minimum inhibitory concentrations (MIC) of kuwaitimycin for a variety of microorganisms

Table 6. Behaviour of kuwaitimycin towards different chemical tests

Chemical test	Result
Ninhydrin reagent	positive
$FeCl_3$ (neutral soln.)	no precipitate
Alkaline $KMnO_4$	reduction on cold
Acidic $KMnO_4$	reduction on cold
Biuret test	positive
MOLISCH's test	negative
Nitration	negative
Reduction to FEHLING's solution	negative
Potassium thiocyanate	negative
Potassium ferrocyanide	negative
Dilute I_2 solution in CCl_4	decolorization
Dilute Br_2 solution in CCl_4	decolorization
SAKAGUCHI's test	positive
TOLLEN's test	negative
MILLON's test	negative
ELSON MORGAN	negative

are given in Table 7. Determination of the MIC was carried out using the serial dilution method. Serial dilutions were made in 1 ml volumes of Penassay broth (Difco) for each bacterial culture tested. The tubes were inoculated with a diluted 24-hour broth culture with a resulting inoculum of approximately 10^5 bacteria per ml. The inoculated tubes were incubated at 37°C for 24 hours and then examined for visible growth.

Kuwaitimycin is primarily active against gram-positive bacteria and possesses limited activity against gram-negative ones. It possesses no significant activity against yeast and fungi.

Toxicity and Curative Tests

The LD_{50} was determined *via* the intramuscular route in the usual way using male Swiss mice (18~22 g). The median curative dose (CD_{50}) of the antibiotic for *Streptococcus pyogenes* infections in 18~22 g male mice was determined as described by PRICE *et al.*[11] In both cases the antibiotic was administered by the intramuscular route once daily for 2 days.

$$LD_{50}=34\times2 \text{ mg/kg}$$
$$CD_{50}=6.8\times2 \text{ mg/kg}$$

Discussion

Streptomyces kuwaitinensis nov. sp., isolated from the soil of Kuwait, belongs to the verticillate melanin-negative cinnamomeus series.[1] *Streptomyces kuwaitinensis* produces kuwaitimycin, a unique polypeptide antibiotic containing a novel aliphatic unsaturated iso fatty acid tentatively identified as isohexadeca-3, 6-dienoic acid. On GLC the methyl ester of this acid had almost the same retention time as that of reference palmitic acid but when reduced in presence of platinum black the product had an appreciably lower retention time. Furthermore the acid absorbs iodine. Even though the fatty acid moiety of kuwaitimycin has 16 carbon atoms while that of glumamycin[12] contains 13 carbon atoms both acids yield isocapric and malonic acids when oxidized with permanganate-periodate. This can be explained by assigning an isohexadeca-3, 6-dienoic structure to the fatty acid of kuwaitimycin that on oxidation yields two molecules of malonic acid and one molecule of

Table 7. Antimicrobial activities of kuwaitimycin

Test organisms	MIC (μg/ml)
Bacillus subtilis, D 161 streptomycin resistant	6.2
〃 〃 〃 chloramphenicol 〃	25
〃 〃 〃 cathomycin 〃	3.1
〃 〃 〃 staphylomycin 〃	6.2
〃 〃 〃 aureomycin	6.2
〃 〃 , D 166 (var. mycoides) oxytetracycline resistant	6.2
〃 〃 〃 kanamycin 〃	0.7
Bacillus cereus	3.12
〃 *anthracoid*	> 50
〃 *abortus*	6.2
〃 *anthracis*	> 50
〃 *mycoides*	25
Staphylococcus aureus, D 6 oleandomycin resistant	1.5
〃 〃 〃 streptomycin 〃	3.1
〃 〃 〃 paromomycin 〃	1.5
〃 〃 , A 55 *Micrococcus pyogenes*	0.1
〃 〃 FDA 209 P	1.6
〃 〃 (A 9606)	
〃 〃 14213	25
〃 〃 var. rose	25
Streptococcus pyogenes	0.095
Corynebacterium diphtheriae	1.56
Mycobacterium pheli	>100
Salmonella typhosa 9 D	> 50
Salmonella paratyphi A-2:a	25
〃 〃 B-4, 6:b	50
〃 〃 C-6, 7:c	25
〃 *enteritidis*	100
derby	> 50
〃 *typhimurium*	> 50
Escherichia coli: (Juhl)	100
〃 〃 A 9675	25
〃 〃 0111:B 4	> 50
〃 〃 0127	> 50
Klebsiella pneumoniae	25
〃 *pneumoniae* O-I-K 3NCTC 5056	12.5
〃 〃 NRRL B-117	50
Proteus morganii	25
Proteus mirabilis H-3	25
Proteus rettgeri	25
Haemophilus influenzae A-733	3.1
Enterobacter aerogenes 659-66-CDC	50
Enterobacter cloacae	50
Shigella equirulis H 33	50
〃 *boydii* 22854-61-CDC	50
Sarcina lutea	6.25
Saccharomyces cerevisiae NRRL Y-567	>100
Candida albicans NRRL Y-477	>100
Aspergillus niger	>100

isocapric acid. Kuwaitimycin is not acidic in nature and rather insoluble in water distinguishing it from amphomycin,[13] zaomycin,[14] crystallomycin,[15,16] aspartocin,[17,18] laspartomycin[19] and tsushimycin.[20] The fatty acid of kuwaitimycin is probably attached to the rest of the molecule through its carboxylic group *via* an amide and/or an ester linkage with the OH group of serine. The infrared spectrum of the antibiotic indicates the presence of OH, possibly NH, CH_3/CH_2, weak ester or carboxyl C=O, conjugated and/or amide C=O.

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