ANTIBIOTIC SB22484: A NOVEL COMPLEX OF THE AURODOX GROUP

I. TAXONOMY OF THE PRODUCING ORGANISM, ISOLATION OF THE ANTIBIOTICS AND CHEMICAL AND BIOLOGICAL CHARACTERIZATION

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Antibiotic SB22484 is a novel member of the aurodox type antibiotic group produced in submerged-fermentation cultures of *Streptomyces* sp. NRRL 15496.

The antibiotic complex is composed of two pairs of isomers with MW's of 752 and 766. The individual isomers, which were separated by preparative HPLC, equilibrate to a mixture of the isomer pair when left in aqueous solution.

In vitro, SB22484 antibiotics strongly inhibited neisseriae and were also active against Streptococci, *Ureaplasma urealyticum* and *Haemophilus influenzae*.

The novel aurodox type antibiotic SB22484 was isolated in the course of a discovery program for antibiotics having activity against neisseriae. During this search we found that aurodox type antibiotics were present in broths active against *Neisseria caviae* but inactive against *Staphylococcus aureus*. In fact aurodox type antibiotics characteristically show activity against anaerobes, neisseriae and Streptococci while are lacking activity against *S. aureus*[1~4]. These antibiotics inhibit bacterial protein synthesis by binding to elongation factor Tu (EF-Tu)[5,6].

This paper deals with the taxonomy of the producing strain and the isolation and characterization of SB22484.

Materials and Methods

Morphological and Cultural Characteristics of the Strain

The producing strain was isolated from a soil sample collected in a wheat field in the Valtellina valley in Italy.

Identification keys were used according to the classification of PRIDHAM *et al.*[7] and color determination according to MAERZ and PAUL[8].

The cultural characteristics of the strain on various standard media and its ability to utilize different carbon sources were determined according to SHIRLING and GOTTLIEB[9], and WAKSMAN[10]. Whole-cell analysis was carried out according to STANECK[11]. The strain was deposited in the Northern Research Center of Agricultural Research Service with the accession No. NRRL 15496.

Fermentation

A lyophilized culture of strain NRRL 15496 was transferred onto a slant of oatmeal agar. After 10 days of incubation at 28°C, the slant was used to inoculate two baffled 2,000-ml Erlenmeyer flasks containing 500 ml of seed medium (beef extract 0.5%, peptone 0.5%, enzymatically hydrolyzed casein 0.3%, yeast extract 0.5%, NaCl 0.15%, and glucose 2% in distilled water). The flasks were incubated for

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40 hours at 28°C on a rotatory shaker at 220 rpm. The culture was then transferred to a 50-liter fermenter containing 30 liters of production medium (beef extract 0.4%, peptone 0.4%, NaCl 0.25%, yeast extract 0.1%, soybean meal 1%, glucose 2.5%, and $CaCO_3$ 0.5% in tap water). The culture was fermented for 24 hours at 28°C with 500 rpm stirring and 1 v/v/minute aeration and then transferred into a 300-liter fermenter containing 200 liters of production medium. Fermentation was carried out at 28°C with 240 rpm stirring and 1 v/v/minute aeration flow. The antibiotic level was determined by paper-disk diffusion assay with *N. caviae* ATCC 14659 in brain heart infusion agar at 37°C.

HPLC of SB22484 Complex

HPLC analysis was performed on a Bakerbond (5 μm) C8 (4.6 × 250 mm) column eluted at 1.8 ml/minute flow rate with mixtures of two mobile phases, phase A: CH_3CN-THF-40 mM $HCOONH_4$ (40:40:20), phase B: CH_3CN-THF-40 mM $HCOONH_4$ (10:10:80). These mobile phases were used for both analytical and preparative HPLC separations. For analytical purposes a 20-minute linear gradient from 1:9, phases A-B to 9:1, phases A-B was used. Sample solutions of SB22484, in CH_3CN-0.1 M Na_2CO_3 (50:50), were injected and elution was followed by UV detection at 320 nm.

Isolation of Antibiotic SB22484 and Purification of the Individual Components

The filtrate (170 liters) of the harvested broth was adjusted to pH 7.0 and extracted with ethyl acetate. The crude antibiotic (9.2 g) was precipitated from the concentrated organic phase upon addition of petroleum ether. This preparation (4.5 g) was purified on a chromatographic column containing 1.5 liters of Sephadex LH-20 equilibrated and eluted with methanol. The pooled fractions containing the antibiotic were concentrated to an oily residue which was dissolved in THF. The pure antibiotic complex (960 mg) was precipitated upon addition of ethyl ether. The four individual factors which constitute the SB22484 complex were isolated by preparative HPLC using a Shimadzu LC-8A preparative chromatograph. The complex (20 mg) was dissolved in 1 ml of phase A (see above) and 1 ml of 1% (w/v) sodium bicarbonate, and the separation was performed on a Nucleosil 5 μm C18 silica gel (20 × 250 mm) column eluted at 20 ml/minute flow rate with a 20 minute linear gradient from 1:9, phases A-B to 5:5, phases A-B with UV detection at 320 nm. Fractions from repeated chromatographic runs containing the same separated factor were pooled, the organic solvents were removed under vacuum, and the residual aqueous solutions were extracted twice with ethyl acetate. The SB22484 factors were precipitated from the concentrated organic phase upon addition of ethyl ether.

Physico-chemical Analysis

The UV spectra of pH 7.0 phosphate buffer solutions of the antibiotics were recorded with a Perkin-Elmer model 320 spectrophotometer.

Liquid chromatography (LC)-MS was performed using an HP 5985 instrument working in negative ion mode, and connected with a Brownlee Labs RP18 (10 μm) HPLC column (4.6 × 250 mm). The column was eluted at 1 ml/minute flow rate with a mixture of CH_3CN-0.1 M $HCOONH_4$ (6:4).

MIC Determination

For most strains MIC were determined by microbroth dilution methodology. MIC for *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Haemophilus influenzae* were determined by agar dilution; MIC for *Clostridium perfringens*, *Mycoplasma gallisepticum*, *Ureaplasma urealyticum*, *Mycobacterium tuberculosis*, *Trichophyton mentagrophytes*, and *Trichomonas vaginalis* by macrobroth dilution. Inocula were approximately 10^4 cfu/ml, unless otherwise indicated. *M. gallisepticum* inocula were 100-fold dilutions of 48 hours cultures; *U. urealyticum* inocula were 10^4 color changing U/ml; *M. tuberculosis* inocula were from 7-day broth cultures, *T. mentagrophytes* from 7-day slants and *T. vaginalis* from 48 hours broth cultures. Incubation times were 18~24 hours except for *N. gonorrhoeae*, *N. meningitidis*, *H. influenzae*, *T. mentagrophytes*, *M. gallisepticum*, *T. vaginalis* (48 hours) and *M. tuberculosis* (7 days). All bacteria were incubated at 37°C; *Candida albicans* and *T. mentagrophytes* at 30°C. *N. gonorrhoeae* and *N. meningitidis* were incubated in a 5% CO_2 atmosphere, *C. perfringens* in an anaerobic gas mixture. Media used were Oxoid Iso-Sensitest broth (*S. aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*); Difco Todd Hewitt broth (Streptococci); Difco GC base broth+1% BBL IsoVitaleX (*N.*

caviae); GC base agar+1% IsoVitaleX+10 mg/liter hemin (*N. meningitidis*, *N. gonorrhoeae* and *H. influenzae*); Difco AC medium (*C. perfringens*); Kirschner broth+10% horse serum (*M. tuberculosis*); Difco Sabouraud broth (*T. mentagrophytes*); Difco yeast nitrogen base broth+1% glucose+0.15% asparagine (*C. albicans*); Difco PPLO broth supplemented with 1% yeast extract, 0.001% phenol red, 0.15% glucose and 10% horse serum for *M. gallisepticum*; pH 6.5 PPLO broth supplemented with 1% yeast extract 0.002% phenol red, 10% horse serum, and 0.05% urea for *U. urealyticum*; and TYM modified basal medium (Merck)+10% horse serum for *T. vaginalis*.

Bactericidal Activity

Time kill experiments with *N. gonorrhoeae* L997 were performed in Difco GC base broth+1% IsoVitaleX using an inoculum of 10^7 cfu/ml. SB22484 (five and ten times the MIC) and spectinomycin (five times the MIC) were added to stationary or growing cells of *N. gonorrhoeae*. At intervals during incubation at 37°C in a 5% CO_2 atmosphere, triplicate samples were removed and plated on Difco GC agar base+1% IsoVitaleX+10 mg/liter hemin for viable counts. The plates were incubated for 48 hours at 37°C in a 5% CO_2 atmosphere.

Experimental Infections

Each control and treatment group contained five CD-1 mice (Charles River) weighing 18~22 g. They were infected intraperitoneally with 0.5 ml of a bacterial suspension prepared by diluting overnight cultures of *S. pyogenes* or *S. pneumoniae* in sterile peptonized saline. Inocula were adjusted so that untreated animals died of septicemia within 48 hours. Animals were treated subcutaneously once daily for 3 days starting immediately after infection (*S. pyogenes*) or twice at 0 and 6 hours after infection (*S. pneumoniae*). On the 10th day, the ED_{50} in mg/kg/treatment was calculated by the Spearman and Kaerber method of literature[12] from the percentage of surviving animals at each dose.

Serum Levels in the Mouse

For each sampling time, five male and five female mice were treated subcutaneously with 100 mg/kg of SB22484. 0.5 ml of blood/animal were collected by cardiac puncture, and the pooled blood was allowed to clot. SB22484 was assayed microbiologically on *E. coli* G1625, a hyperpermeable strain of *E. coli* K-12, which is sensitive to aurodox type antibiotics (G. CARNITI and P. B. GOLDSTEIN, unpublished results).

Results

Morphological and Cultural Characteristics of Strain NRRL 15496

The strain has the general morphological features of the genus *Streptomyces*. Its whole-cell analysis showed the presence of LL-diaminopimelic acid and of no diagnostic sugars. The colonies had a yellow to deep chrome yellow vegetative mycelium and a turquoise-green aerial mycelium with sporophores arranged in spirals, coils and hooks. The spores were cylindrical with rounded ends and dimensions of $1.2 \times 2 \sim 3\ \mu m$. Based on the form of the sporophores and on the color of the aerial mycelium, the strain was assigned to the "Group Spira-Green Section". The cultural characteristics of the strain on various standard media, its physiological characteristics and its capacity to utilize different carbon sources are reported in Tables 1, 2, and 3.

Isolation of SB22484 Antibiotics and Chemical Characterization

In submerged culture, maximum antibiotic production was obtained after 40 hours of fermentation. The SB22484 antibiotics were present in the supernatant of fermentation broths as a complex of four factors with identical UV spectra which were named 1, 2, 3, and 4 according to their elution order in the reverse phase chromatographic system (Fig. 1). LC-MS analysis showed that two pairs of isomers were present (1 and 3; 2 and 4) having MW's of 752 and 766, respectively. In the fermentation broths pairs 1

Table 1. Cultural characteristics of strain NRRL 15496.

Culture medium	Vegetative mycelium	Aerial mycelium	Soluble pigments
Oatmeal agar	Abundant growth with thick and wrinkled surface, golden yellow 9/L/6	Yellow aerial mycelium with turquoise green spores	Absent
Yeast extract-malt extract agar (ISP No. 2)	Abundant growth with wrinkled surface, deep chrome yellow 9/L/7	Yellow aerial mycelium, scarce formation of turquoise green spores	Absent
Oatmeal agar (ISP No. 3)	Abundant growth with smooth surface forsythia, yellow 9/K/6	Absent	Absent
Starch agar (ISP No. 4)	Abundant growth with smooth surface, cream	Moderate formation of turquoise green spores	Absent
Glycerol-asparagine agar (ISP No. 5)	Abundant growth with thick and smooth surface, lemon yellow 9/J/2	Absent	Absent
Peptone-yeast extract-iron agar (ISP No. 6)	Moderate growth with smooth surface, colorless	Absent	Absent
Tyrosine agar (ISP No. 7)	Abundant growth with smooth surface, light hazel brown	Absent	Absent
HICKEY and TRESNER's agar	Abundant growth with wrinkled surface, yellow with green spores 9/I/3	Moderate formation of turquoise green spores	Absent
BENNETT's agar	Moderate growth with wrinkled surface, chrome lemon 9/L/3	Absent	Absent
Nutrient agar	Moderate growth with smooth surface, light cream	Absent	Absent
Potato glucose agar	Abundant growth with wrinkled surface, deep chrome 9/L/7	Scarce formation of turquoise green spores	Absent
Peptone glucose agar	Abundant growth, slightly wrinkled, light cream	Absent	Absent
CZAPEK glucose agar	Scarce growth with wrinkled and crusty surface, yellow 9/I/5	Traces of whitish aerial mycelium	Absent
CZAPEK sucrose agar	Abundant growth with thin surface	Absent	Absent
Ca-malate agar	Moderate growth with thin surface, canary yellow 10/I/1	Absent	Absent
Skim milk agar	Abundant growth with wrinkled surface, amber	Absent	Absent
Egg albumen agar	Scarce growth, straw	Absent	Absent

and 3 accounted for more than 65% of the complex. In the course of stability studies, the two isomers of each pair were found to reach a chemical equilibrium when left in aqueous solution for more than 1-day. This is characteristic of other aurodox type antibiotics we isolated in the course of our discovery program. Two isomers of kirromycin were also detected in NMR studies[13], although no structural data were reported.

Table 2. Physiological characteristics of strain NRRL 15496.

Test	Results
Starch hydrolysis	+++
H_2S formation	+
Melanin formation	−
Tyrosine hydrolysis	+
Casein hydrolysis	+/−
Calcium malate hydrolysis	−
Nitrate reduction	+/−
Litmus milk	
Coagulation	−
Peptonization	−
Gelatin liquefaction	+++

−: Negative response, +: weak positive response, ++: positive response, +++: strong positive response.

Table 3. Carbon utilization of strain NRRL 15496.

Carbon source	Growth
Inositol	−
Fructose	+++
Rhamnose	+++
Mannitol	+++
Xylose	+++
Raffinose	−
Arabinose	++
Cellulose	−
Sucrose	−
Mannose	+++
Lactose	+++
Galactose	+++
Salicin	+
Glucose	++

−: Negative response, +: weak positive response, ++: positive response, +++: strong positive response.

Fig. 1. HPLC chromatogram of SB22484 complex.

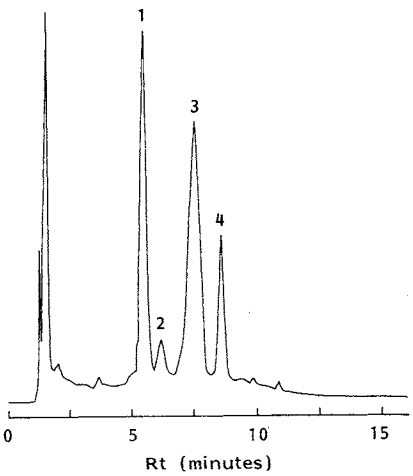
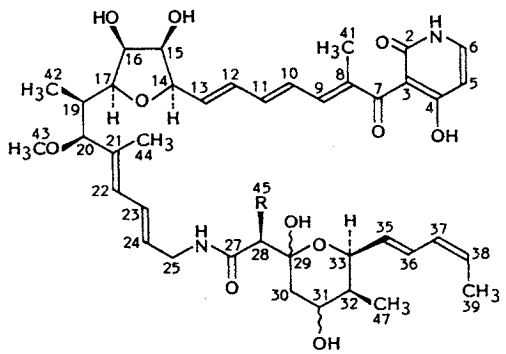


Fig. 2. Structures of SB22484 components.



Factor	Formula	MW	R	29-OH	UV λ_{max} (nm)
1	$C_{41}H_{56}O_{11}N_2$	752	CH_3	β, *R*	327, 232
2	$C_{42}H_{58}O_{11}N_2$	766	C_2H_5	β, *R*	327, 232
3	$C_{41}H_{56}O_{11}N_2$	752	CH_3	α, *S*	327, 232
4	$C_{42}H_{58}O_{11}N_2$	766	C_2H_5	α, *S*	327, 232

For SB22484, rapid workup of the eluates of the preparative HPLC separation permitted us to isolate the individual factors. The structures of the isomers, determined as described in the companion paper[14], are shown in Fig. 2.

Biological Activity

Antibacterial Activity of SB22484

As shown in Table 4, SB22484 complex is most active against *N. caviae* and *N. gonorrhoeae* (MIC 0.5 μg/ml); it is less active against Streptococci and *U. urealyticum* (MIC 4~8 μg/ml) and *H. influenzae* (MIC 32 μg/ml). MIC values are above 128 μg/ml for all the other species tested; however, in our experience, some clinical isolates of Enterococci are sensitive to aurodox type antibiotics while others, including the ATCC 7080 strain in Table 4, are resistant (F. RIPAMONTI and P. B. GOLDSTEIN, unpublished data). Against *S. pneumoniae* L44 the MIC increased from 4 μg/ml (without serum) to 16 μg/ml in presence of 50 or 70%

Table 4. Spectrum of antibacterial activity of SB22484.

Organism	MIC (μg/ml)
Staphylococcus aureus ATCC 6538	>128
Streptococcus pyogenes C 203	8
S. pneumoniae UC 41	4
S. dysgalactiae ATCC 9926	8
Enterococcus faecalis ATCC 7080	>128
Clostridium perfringens ISS 30543	>128
Escherichia coli SKF 12140	>128
Proteus vulgaris ATCC 881	>128
Pseudomonas aeruginosa ATCC 10145	>128
Haemophilus influenzae type b ATCC 9795	32
H. influenzae type d ATCC 9332	32
Neisseria caviae ATCC 14659	0.5
N. gonorrhoeae NCTC 8254	0.5
Mycobacterium tuberculosis H37Rv ATCC 9360	>128
M. gallisepticum S/6 Weybridge	>128
Ureaplasma urealyticum L1479	8
Candida albicans SKF 2270	>128
Trichophyton mentagrophytes RRL 316	>128
Trichomonas vaginalis	>128

Table 5. MIC of SB22484 and spectinomycin for 23 strains of *Neisseria gonorrhoeae*.

Antibiotic	MIC range	MIC_{50}	MIC_{90}
		μg/ml	
SB22484	0.125~8	2	8
Spectinomycin	16~>128	32	32

The isolates included penicillin-resistant strains and one spectinomycin-resistant strain.

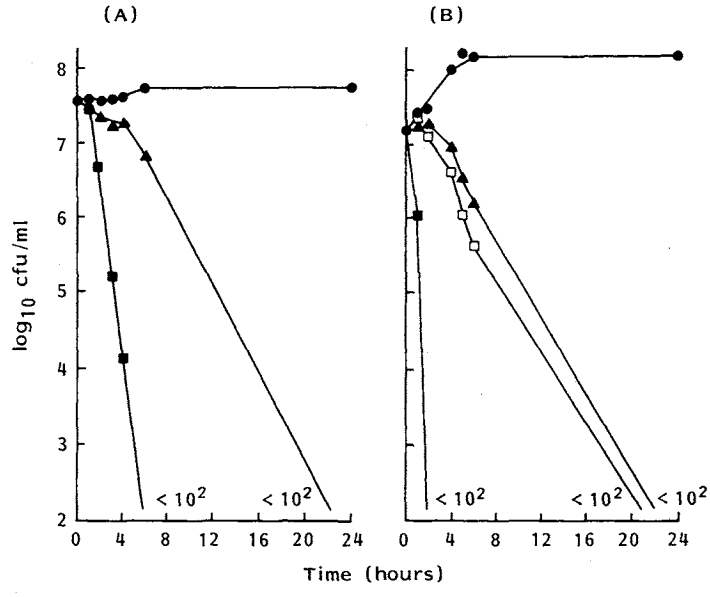
Table 6. MIC of SB22484 and spectinomycin against *Neisseria meningitidis*.

Organism	MIC (μg/ml)	
	SB22484	Spectinomycin
N. meningitidis A[a] ATCC 13077	4	32
N. meningitidis B[a] ATCC 13090	1	32
N. meningitidis C[a] ATCC 13102	2	32
N. meningitidis D[a] ATCC 13113	1	16
N. meningitidis ATCC 13804	1	8

[a] Serotype.

Fig. 3. Bactericidal activity against *Neisseria gonorrhoeae* L997.

(A) Stationary cells, (B) growing cells. ● Control, ▲ SB22484: 5 μg/ml (five times the MIC), □ SB22484: 10 μg/ml, ■ spectinomycin: 80 μg/ml (five times the MIC).
N. gonorrhoeae L997 (clinical isolate) was grown in broth +1% IsoVitaleX.



serum. There was no significant inoculum effect on the MIC for *N. gonorrhoeae* L997 determined in broth 10^4, 10^6, and 10^7 cfu/ml.

We were unable to select SB22484-resistant mutants of *N. gonorrhoeae* L997 (frequency $< 4 \times 10^{-11}$ at 10 μg/ml).

Anti-neisseria Activity of SB22484

Table 5 summarizes our MIC data for SB22484 and spectinomycin against 23 *N. gonorrhoeae* strains, 20 of which are clinical isolates, including several penicillinase producers and one spectinomycin-resistant strain. The median MIC of SB22484 was 2 μg/ml, as compared with 32 for spectinomycin. SB22484 had similar activity against *N. meningitidis*; the MIC range for 5 strains was 1~4 μg/ml, as compared with 8~32 for spectinomycin (Table 6). SB22484 was bactericidal for *N. gonorrhoeae* L997 but its action was relatively slow in comparison with spectinomycin (Fig. 3).

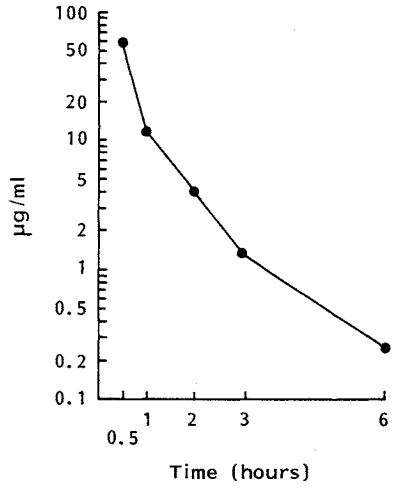
Experimental Infections

Upon subcutaneous administration, SB22484 was somewhat effective in *S. pyogenes* septicemia in the mouse (ED_{50} 174 mg/kg). It was not effective in *S. pneumoniae* septicemia at up to 300 mg/kg sc.

Serum Level in the Mouse

Serum levels in the mouse after a single subcutaneous dose of 100 mg/kg are shown in Fig. 4. The peak level in serum had occurred by the first time point (0.5 hour). Levels declined rapidly with an estimated $T_{1/2}$ of 0.77 hour. After 6 hours, the concentrations was less than 0.25 μg/ml.

Fig. 4. Serum levels in the mouse.



Discussion

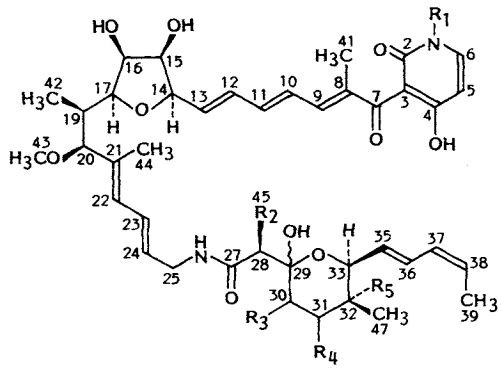
Fourteen antibiotics have been described in the literature which are related to aurodox in structure, biosynthesis and mode of action. Newly isolated antibiotics having the antimicrobial spectrum of aurodox type antibiotics were compared with reference standards of this group. Our procedure was

Table 7. Antibiotics of the aurodox group.

Group	Antibiotic	UV λ_{max} (nm)	MW
I	Kirromycin (mocimycin)[6,19]	325[a]	796
	Aurodox[4,6]	325[a]	810
	Heneicomycin[6,20]	322[c]	794
	Efrotomycin[6,21]	327[a]	1,144
	Azdimycin[22]	323[b]	861
	MSD A63A[23]	328[b]	780
II	Factumycin[24]	355[b]	778
	A73A (factumycin isomer)[25]	355[b]	778
	UK69753[26]	361[c]	1,098
III	L-681,217[27]	288[b]	659
	Phenelfamycins[28,29]	295[a]	938~1,226
	LL-E19020[30]	290[b]	1,225
IV	Dihydromocimycin[31]	233, 267, 291, 333 (br)[b]	798
	Kirrothricin[32]	231, 283, 324 (br)[b]	780

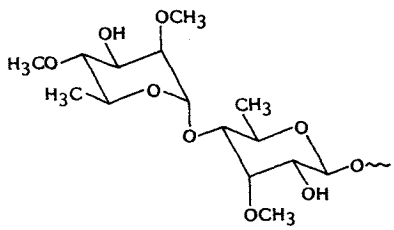
[a] Neutral pH buffers.
[b] Organic solvent.
[c] Acidic solutions.

Fig. 5. Structures of kirromycin-like antibiotics.



Antibiotic	R_1	R_2	R_3	R_4	R_5
Kirromycin	H	C_2H_5	OH	OH	CH_3
Aurodox	CH_3	C_2H_5	OH	OH	CH_3
Heneicomycin	CH_3	C_2H_5	H	OH	CH_3
Efrotomycin	CH_3	C_2H_5	OH	Sugars	CH_3
SB22484 factors 1, 3	H	CH_3	H	OH	H
SB22484 factors 2, 4	H	C_2H_5	H	OH	H

Sugars:



based on two parameters: UV absorption and MW, which usually permitted novelty assessment without the need for complete structure determination. The known aurodox type antibiotics may be divided into four groups on the basis of their UV absorption maxima (Table 7). SB22484 belongs to group I whose general structure is in Fig. 5.

The MW's of the SB22484 antibiotics, determined by LC-MS spectroscopy[15], are different from the reference standards of this group.

Comparison of the structural characteristics of the SB22484 antibiotics with those of other aurodox type compounds of group I showed that SB22484 was unique in lacking one of the two methyl groups at C-32. Moreover, factors 1 and 3 have a methyl group at C-28 instead of the ethyl group of other aurodox type antibiotics. This is noteworthy because these substituents are located in a part of the molecule known to be involved in the inhibition of protein synthesis in cell-free systems[16]. Since the antibacterial activity of SB22484 is comparable to that of other aurodox type antibiotics, these substituents are obviously not essential for inhibition of EF-Tu function.

The individual factors of the SB22484 complex have antimicrobial activity comparable to that of the complex (data not shown). SB22484 was evaluated as a potential therapeutic agent for gonococcal infections. It is active against clinical isolates of *N. gonorrhoeae* including penicillinase-producing strains and a strain resistant to spectinomycin, an antibiotic which can be used in patients allergic to penicillin and for treating penicillin-resistant gonorrhoea. SB22484 is bactericidal against *N. gonorrhoeae* although more slowly than spectinomycin. SB22484 is also active against *U. urealyticum*, a microorganism associated with nongonococcal urethritis[17], and active against *N. meningitidis*.

The low frequency of resistance to SB22484 in *N. gonorrhoeae* is probably due to the fact that *N. gonorrhoeae*, like *E. coli*, has two genes coding for the EF-Tu protein which is the target of aurodox type

antibiotics[18]. In *E. coli*, resistance to kirromycin is recessive, necessitating mutations of both the *tufA* and *tufB* genes[5].

The poor activity of SB22484 in experimental septicemias caused by sensitive microorganisms was probably due to its short $T_{1/2}$. SB22484, like other aurodox type antibiotics, showed low toxicity in the mouse ($LD_{50} > 1{,}000$ mg/kg intraperitoneally).

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