CL307-24, A NEW ANTIBIOTIC COMPLEX FROM *Saccharopolyspora aurantiaca* sp. nov.

II. PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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CL307-24I, the main component of the CL307-24 complex produced by *Saccharopolyspora aurantiaca* sp. nov., was found to be a potent inhibitor of yeast mitochondrial ATPase. CL307-24I displayed a high degree of activity towards some coryneform bacteria and also has been shown to possess an insecticidal activity. Its biological and physico-chemical properties clearly distinguish it from previously known ATPase inhibitors.

In the course of a screening program for insecticides from actinomycetes[1], a complex of new compounds was isolated from the fermentation broth of strain CL307-24. The taxonomy of the strain and the purification of the different components of the complex (CL307-24I, II, III, IV) have been described[2]. This paper reports the biological and physico-chemical properties of these compounds.

Materials and Methods

Antimicrobial Activities

The minimal inhibitory concentrations (MICs) of the CL307-24 compounds against some selected strains of bacteria, yeasts and filamentous fungi were determined by the conventional two-fold agar-dilution method. Bacteria were grown on Mueller-Hinton agar (Institut Pasteur Production) or on Nutrient agar (Difco) and yeasts and filamentous fungi on YP medium which consisted of yeast extract (Difco) 1%, Neopeptone (Difco) 1%, agar 1.5% and glycerol 2% or glucose 2% (YP glycerol, YP glucose), respectively. MICs were recorded after incubation of the bacteria for 1 to 3 days at 37°C, and yeasts and filamentous fungi for 2 to 6 days at 27°C.

Yeast Susceptibility Disc Assay Method

Yeast strains D273-10B/A1, D273-10B/A16 (*oli*-4), D273-10B/A21 (*oli*-1) and D273-10B/A48 (*oli*-2)[3] were kindly supplied by Dr. ANDRÉ GOFFEAU (Université Catholique de Louvain, Belgium).

YP glycerol agar medium was inoculated with a freshly prepared suspension of yeast cells. Fifty μl of a 1 mg/ml solution of oligomycin (Sigma) or CL307-24 were absorbed onto 9 mm paper discs (Schleicher and Schüll, Germany) and both types of discs were placed in duplicate on the surface of the yeast-seeded agar dishes. Incubation was carried out for 20 hours at 27°C.

Measurement of Respiration Rate and ATPase Activity of Yeast Mitochondria

Mitochondria were isolated from yeast protoplasts according to the method of GUERIN *et al*[4]. The

wild type strain of *Saccharomyces cerevisiae* (Mat alpha, *rho*+, met, his3, ura3) and the mutant strain NNY-2 (Mat alpha, met, his3, ura3, atp7::URA3) have been previously described[5].

Oxygen consumption was measured with a Clark oxygen electrode (Gilson) at 27°C in 1.5 ml of the following medium: 0.65 M mannitol, 10 mM tris-maleate pH 6.7 prepared in deionized water.

The ATPase activity of thawed yeast mitochondria was measured at basic pH (8.4) at 30°C according to the procedure of SOMLO and KRUPA[6].

Protein concentrations were determined by the method of LOWRY *et al.*[7] in the presence of SDS. Bovine serum albumin was the standard protein.

Insecticidal Activities

Musca domestica

Injection, topical application or ingestion tests on the larvae and adults have been described in a previous paper[1].

Tenebrio molitor larvae

The CL307-24 complex was dissolved in DMSO. Five μl of the solution was injected under the larval cuticle with a microsyringe; mortality was recorded after 72 hours.

Artemia salina

Artemia cysts were used in bioassay experiments. Cysts hatched 24 hours after placement into a salt solution (Artemia salts, Hobby, France) contained in a Petri dish. After 72 hours mobile nauplii were transferred into a well (Nunclon Deltabox, Nunc, Denmark) containing the CL307-24 complex dissolved in the salt solution containing DMSO at 1%. The mortality count was recorded 24 and 48 hours after transfer of the *A. salina* nauplii.

Apparatus

Infrared and ultraviolet spectra and specific rotation were determined on Perkin Elmer spectrophotometers (Perkin Elmer S.A., Bois d'Arcy, France), 683 IR, Lambda 5UV and 241 polarimeter, respectively. ^{13}C and ^{1}H NMR spectra were recorded on a Brucker WM250 (NMR Bruker, Wisembourg, France). Mass spectra by EI ionization were obtained using a Varian Matt 311a mass spectrometer.

Results

Physico-chemical Properties

The physico-chemical properties of CL307-24I are summarized in Table 1. CL307-24I was found to be a basic compound slightly soluble in highly polar or apolar solvents but freely soluble in chlorinated solvents. Electron-impact (EI) spectra revealed a highest mass ion, *m/z* 698.4339, corresponding to $C_{40}H_{62}N_2O_6S$ which is in agreement with elemental analysis (Table 1) and ^{13}C NMR spectrum (Fig. 1). The lack of characteristic absorption in the UV spectrum demonstrated the absence of conjugated double bonds or other UV absorbing chromophores. The presence of hydroxyl and carboxyl groups is suggested by the IR spectrum (Fig. 2). The ^{1}H NMR spectrum of CL307-24I is shown Fig. 3.

Table 1. Physico-chemical properties of CL307-24I.

Appearance	Amorphous white powder				
Nature	Basic				
Solubility					
Soluble in:	$CHCl_3$, dichloromethane				
Insoluble in:	Hexane, acetone, methanol, H_2O				
HREI-MS (*m/z*)					
Found:	698.4339				
Calcd:	698.4328				
Elemental analysis (%):	C	H	N	O	S
Found:	68.33	8.91	3.90	13.56	4.33
Calcd:	68.76	8.88	4.01	13.75	4.58
Molecular formula:	$C_{40}H_{62}N_2O_6S$				
$[\alpha]_D^{20}$	$-0.51°$ ($CHCl_3$)				
MP	275°C				
IR (KBr) max (cm^{-1})	3520, 3340, 2920, 1700, 1610, 1090, 1030, 750				
UV max	End absorption				

Fig. 1. ^{13}C NMR spectrum of CL307-24I in $CDCl_3$.

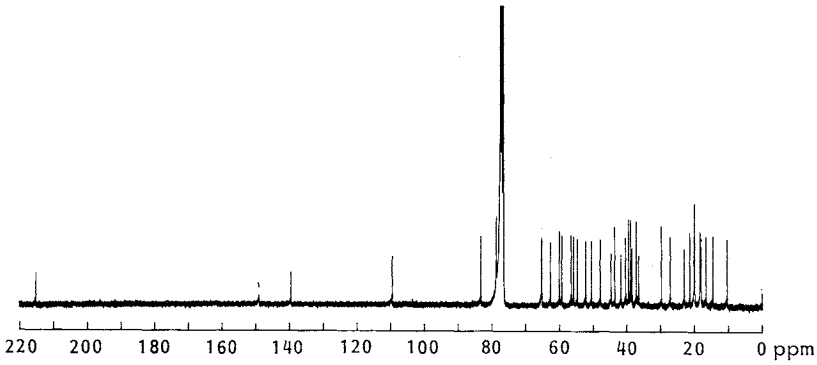


Fig. 2. IR spectrum of CL307-24I in KBr.

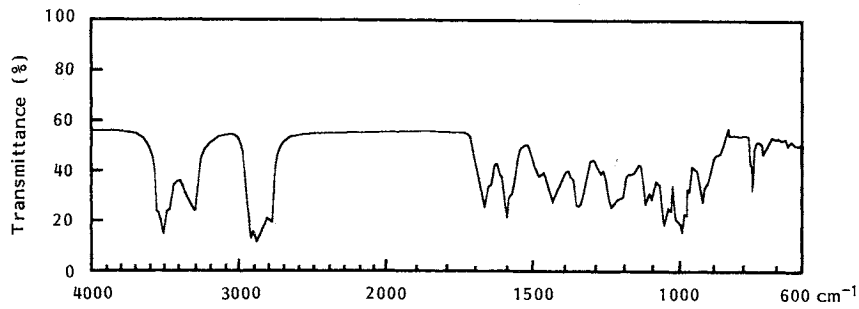
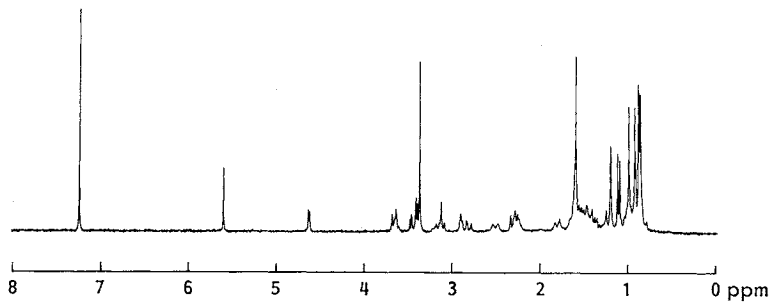


Fig. 3. ^{1}H NMR spectrum of CL307-24I in $CDCl_3$.



The physico-chemical properties of the three other minor components of the CL307-24 complex are very closely related to CL307-24I; they share the same characteristics of basicity and solubility. CL307-24II and IV have a molecular weight of 682 and CL307-24III a molecular weight of 668. UV spectra of the four components of the CL307-24 complex were identical and their IR spectra were closely related.

Biological Activities

Insecticidal Activity

The insecticidal activities of the four constituents of the CL307-24 complex are given in Table 2. CL307-24I was not active by topical application to larvae or adults, however, it was active by ingestion. Interestingly, most insecticides are active by contact[1]. The CL307-24 components also were active *vs* the

Table 2. Insecticidal activity of the four components of the CL307-24 complex.

	Musca domestica larvae		*Musca domestica* adults		*Tenebrio molitor*	*Artemia salina*[c]
	Injection[a]	Topical[a] application	Ingestion[b]	Topical[a] application	Injection[a]	
CL307-24I	0.5	>100	0.5	>100	2.5	1
CL307-24II	5	>100	10	>100	50	5
CL307-24III	0.5	>100	1	>100	5	1
CL307-24IV	5	>100	5	>100	10	5

[a] μg per insect required to kill 100% of the larvae or 100% of the adults by 72 hours.
[b] Concentration in mg/ml of suspension necessary to kill 100% of the adults by 72 hours.
[c] Concentration in μg/ml of solution necessary to kill 100% of the *A. salina* nauplii by 48 hours.

Table 3. Antimicrobial activity of CL307-24I.

Microorganisms	MIC (μg/ml)
Mueller-Hinton agar	
Escherichia coli HB101	>100
Serratia marcescens[a]	>100
Klebsiella pneumoniae[a]	>100
Proteus vulgaris[a]	>100
Citrobacter freundii[a]	>100
Comamonas terrigena ATCC 8461	>100
Bacillus subtilis ATCC 6633	>100
Streptococcus faecium D60	>100
S. faecalis D40	>100
Staphylococcus aureus RN450	>100
Nutrient agar	
Micrococcus luteus ATCC 4698	0.02
Mycobacterium smegmatis ATCC 607	50
Corynebacterium glutamicum ATCC 13287	5
Brevibacterium flavum ATCC 14067	0.05
Rhodococcus sp. ATCC 21337	0.05
Arthrobacter sp. ATCC 21858	0.05
YP glucose agar	
Aspergillus awamori NRRL363	>100
Penicillium chrysogenum[b]	>100
Sporotrichum pulverulentum F1	>100
Verticillium sp.[b]	>100
Charolopsis sp.[b]	>100
Helminthosporium sp.[b]	>100
Trichoderma reesei QM9414	50
Botrytis cinerea[b]	0.2
Alternaria alternata[b]	5
Saccharomyces cerevisiae FL200	>100
YG glycerol agar	
S. cerevisiae FL200	0.01

[a] Hospital isolates.
[b] Laboratory collection.

Fig. 4. Effect of CL307-24I on O_2 consumption by *S. cerevisiae*.

When indicated, 0.1 μM (a) or 10 μM (b) CL307-24I were added to 1.5 ml buffer.

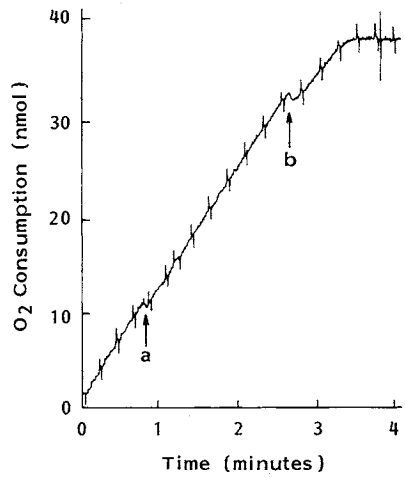


Table 4. Effect of CL307-24I on respiratory specific activities of isolated yeast mitochondria (D273-10B/A/H/U).

	nmol O_2/min/mg protein		
	−ADP	+ADP	+ADP+CCCP
None	410	890	1,300
+CL307-24I	405	414	1,150

Final concentrations of assay components were: mitochondrial protein; 0.3 mg, phosphate; 5 mM, NADH; 8 mM, ADP; 0.67 mM, CCCP; 2.6 μM, CL307-24I; 0.15 μM.

larvae of *Tenebrio molitor* and against *Artemia salina*.

The three other components of the complex displayed a 2- to 20-fold lower activity than CL307-24I towards the arthropods tested.

Antimicrobial Activities

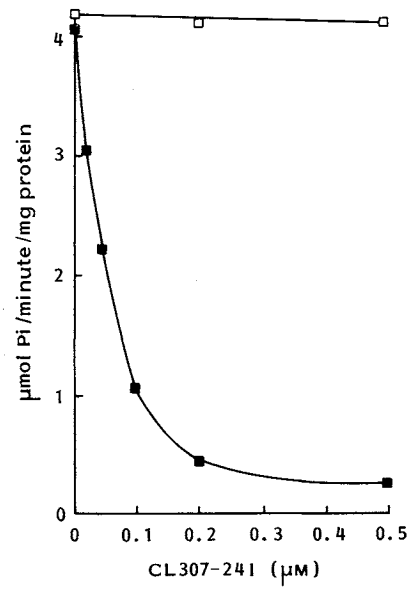
MICs of CL307-24I determined by the agar dilution method are given in Table 3. This antibiotic showed activity against fungi such as *Alternaria alternata* (5 μg/ml) or *Botrytis cinerea* (0.2 μg/ml). Significant antibacterial activities were seen against *Micrococcus luteus* ATCC4698 (0.02 μg/ml), *Brevibacterium flavum* ATCC14067, *Rhodococcus* sp. ATCC21337 and *Arthrobacter* sp. ATCC21858 (0.05 μg/ml).

Mode of Action of CL307-24I

When *Saccharomyces cerevisiae* was grown on glycerol as a sole carbon source, the MIC of CL307-24I was 0.01 μg/ml; in contrast, with glucose as a sole carbon source, the value increased to 100 μg/ml (Table 3) indicating a possible effect upon the respiratory chain. Studies of O_2 consumption by cells of *S. cerevisiae* growing in YP glycerol broth have indicated that CL307-24I had no effect upon respiration (Fig. 4). However, experiments on isolated yeast mitochondria have shown that the stimulation of respiration due to ADP addition was inhibited in the presence of CL307-24I (Table 4). The addition of the uncoupler CCCP (carboxyl cyanide *m*-chlorophenylhydrazone) maximally stimulated the respiration rate showing that the addition of the antibiotic had no effect on the respiratory chain but rather on oxidative phosphorylation in a way similar to the oligomycin effect. The ATPase activity of yeast mitochondria was measured in the presence of increasing amounts of CL307-24I (Fig. 5). The wild type mitochondria were inhibited maximally at 0.5 μM of antibiotic. Using our experimental conditions, a MIC for CL307-24I

Fig. 5. ATPase activity versus CL307-24I concentration.

■ D273-10/1/A/H/U (wild type mitochondria), □ NNY-2 (ATP 7 mutant mitochondria).



The ATPase catalyzed reaction was performed for 30 seconds with 5 mM ATP in the presence of increasing amounts of CL307-24I: the specific activity expressed as production μmol inorganic phosphate/mg of protein was measured colorimetrically.

Table 5. Activity of the CL307-24 components upon yeast strains susceptible or resistant to oligomycin.

Antibiotic	D273-10B/A1	Inhibition zone diameter (mm)		
		D273-10B/A21 (*oli*-1)	D273-10B/A16 (*oli*-4)	D273-10B/A48 (*oli*-2)
Oligomycin	24	—	—	—
CL307-24I	25	29.5	25	29
CL307-24II	19	23.5	23	23.5
CL307-24III	21	25	25	25
CL307-24IV	30	36	36	36

Each antibiotic was tested in duplicate on yeast strains using 9 mm paper disks with 50 μg antibiotic per disk.

—: No inhibition zone around the disk.

of 35 ng/ml was determined. No effect was observed when using yeast mitochondria NNY2 which were devoided of the linkage between the F1 and Fo sectors of the ATP synthase [EC 3.6.1.34][5]. This experiment showed that the hydrolysis of ATP by the catalytic sector F1 is not dependent on proton conduction through the membranous sector Fo of mutant mitochondria. As a consequence, like oligomycin, the target of CL307-24I is the Fo sector of the mitochondrial ATP synthase.

Activities of the CL307-24 components have been tested using an oligomycin-susceptible yeast strain, D273-10B/A1 and three oligomycin-resistant strains: D273-10B/A21 (*oli*-1) and D273-10B/A16 (*oli*-4) and A48 (*oli*-2)[3]. The four components of the CL307-24 complex displayed no cross-resistance with oligomycin (Table 5) indicating that the primary target of these compounds is not subunit 9 of mitochondrial ATPase (*oli*-1 locus) or subunit 6 (*oli*-2 and *oli*-4 loci).

Toxicity

Acute toxicity studies with the CL307-24 compounds were conducted in groups of five female Swiss mice (4 weeks old) treated with four graded doses of the different components of the complex. The LD50 values given intraperitoneally were: CL307-24I: 0.25 mg/kg; CL307-24II: 2.5 mg/kg; CL307-24III: 0.5 mg/kg; CL307-24IV: 0.25 mg/kg. By oral administration the acute toxicity for CL307-24I was 2.5 mg/kg.

Discussion

CL307-24 represents a complex of antibiotics highly active on houseflies, some species of coryneform bacteria and yeast growing under aerobic conditions. It also displays a relatively high acute toxicity in mice which will prevent its possible agricultural or therapeutic applications.

On the other hand, CL307-24 complex is an extremely potent inhibitor of mitochondrial ATPases, whose properties (*i.e.* basic compounds, no UV adsorption) clearly distinguish it from known ATPase inhibitors: oligomycins and venturicidins[10] as well as the newly described bafilomycins[11] and copiamycin[12] which are neutral macrolide antibiotics with typical UV spectra. In addition, CL307-24 displayed no cross-resistance with oligomycin. Other ATPase inhibitors of microbial origin which also display typical UV spectra are polyunsaturated substances (*e.g.* aurovertins and quercetin[10]) or polypeptides (*e.g.* tentoxin[10] and efrapeptins[13]). All of these latter inhibitors are fungal metabolites. Finally, consultation of the Chemical Abstracts databank has confirmed the originality of the CL307-24I molecular formula ($C_{40}H_{62}N_2O_6S$). Structural determination of CL307-24I, the major component of the complex, is in progress.

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