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Isolation, Physico-chemical Properties and Biological Activity of Aurodox Group Antibiotics

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An isolate of *Streptomyces rochei* synonym was found to produce antibiotics with narrow antimicrobial spectrum against *Streptococcus* and *Xanthomonas* sp. Among the antibiotic complex produced by the strain, the main active compound was isolated, and its physico-chemical properties and biological activities were investigated. Molecular weight of the compound was determined to be $[M+H]^+$ 797 (FAB-MS). UV, ^1H and ^{13}C NMR, and IR spectra suggested that the compound is a kirromycin-like aurodox group antibiotic. However, the antimicrobial spectrum of the main compound was slightly different from that of kirromycin. In addition, it was newly found that kirromycin showed a selective antimicrobial activity against *Streptococcus pyogenes* and phytopathogenic *Xanthomonas* sp.

A variety of bacterial pathogens affect the respiratory tract and they are predominantly Gram-positive bacteria. *S. pyogenes* is the leading cause of streptococcal pharyngitis, so called "strep throat". *S. pyogenes* can also cause related infections of the inner ear (otitis media), the tonsils (tonsilitis), the mammary glands (mastoditis), and infections of the superficial layer of the skin (impetigo). About half of the clinical cases of severe sore throat turn out to be due to *S. pyogenes* (3). Fortunately, most respiratory bacterial pathogens thus far had responded readily to antibiotic therapy, such as cephaloridin, penicillin G and ribostamycin. However, *S. pneumonia*, *S. pyogenes* and staphylococci, organisms that cause respiratory and cutaneous infections are now resistant to virtually all of the older antibiotics (9).

Citrus canker caused by *Xanthomonas campestri* pv. *citri* is a major disease during cultivation, storage and transportation of citrus fruit. The control measures include making windbreaks of trees or netting, pruning of diseased summer and autumn shoots, and using chemical sprays. Six to seven sprays of copper are necessary to protect growth from new infection (13). Ascamycin (10), xanthostatin (4) and xanthocidin (1) were developed as *Xanthomonas*-specific antibiotics. However, the control of canker on susceptible or highly susceptible trees has not been successful to date (7). Streptomycin was employed for the control of citrus canker but it was prohibited due to its toxicity and cross-resistance problem with human pathogens.

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In the course of our screening program of antibiotics with a selective toxicity, an isolate of *Str. rochei* was found to produce antibiotics with a selective inhibitory activity against *S. pyogenes* and *Xanthomonas* sp. (6). The main active compound produced by the strain was most compatible to kirromycin (mocimycin). However, the biological activity and physico-chemical property of the compound were slightly different from those of kirromycin. In addition, it was newly found that aurodox group antibiotics showed biological activity against phytopathogenic *Xanthomonas* sp. Isolation process, physico-chemical properties and biological activity of the active compounds produced by the strain are described in this paper. The identification and fermentation of the microorganism will be mentioned elsewhere in the accompanying publication (6).

MATERIALS AND METHODS

Isolation and Purification of the Active Compounds

Culture broth (10 liters) was centrifuged to separate broth filtrate and mycelial cake (Fig. 1). The mycelial cake was extracted with 70% aqueous acetone for overnight at room temperature and concentrated under reduced pressure to eliminate acetone. The broth filtrate and the mycelial acetone extract were pooled and adsorbed to Diaion HP-10 (2.5 liters, wet volume) and the adsorbate was washed with distilled water (10 liters) and 50% aqueous MeOH (10 liters). The active compounds were subsequently eluted with 70% acetone and concentrated *in vacuo* to about 2 liters and extracted with 2 liters of EtOAc 3 times. Ethyl acetate fraction was concentrated

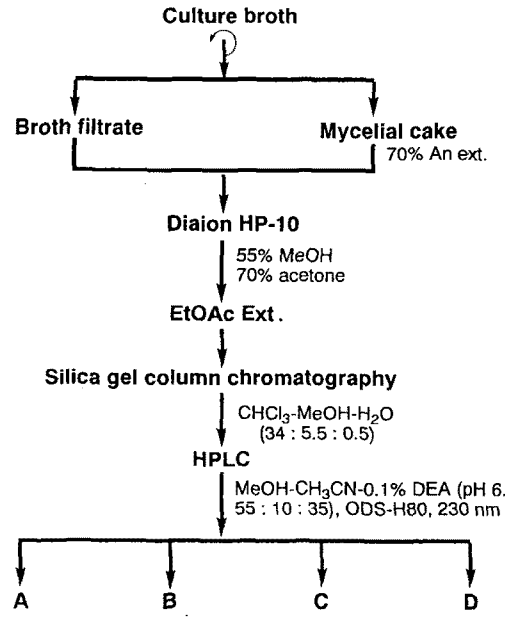

Fig. 1. Purification scheme of active compounds produced by *Streptomyces rochei* isolate.

until a oily dark brown syrup was achieved. The syrup was subjected to silica gel column chromatography with the solvent mixture of $CHCl_3$-MeOH-H_2O (34 : 5.5 : 0.5). The active fraction was further purified by HPLC with MeOH-CH_3CN-0.1% diethylamine (pH 6.7, 55 : 10 : 35, ODS-H80, YMC-pack, 30×250 mm, 220 nm) (solvent system A). pH of the diethylamine (DEA) buffer was adjusted to 6.7 with formic acid. Active fractions were concentrated *in vacuo* and subjected to MCI gel to eliminate buffer ingredients. The resin was washed with H_2O and the active compound was eluted with 70% aqueous acetone, concentrated, and lyophilized to obtain yellow amorphous powder. On the other hand, the isolation of minor active compounds by HPLC was carried out with the solvent mixture of MeOH-0.1 M phosphate buffer (pH 6.5, 65:10) (solvent system B). Paper disc agar diffusion method was employed for the tracking of active compounds.

Physico-chemical Properties

UV spectrum was recorded on Hewlett Packard spectrophotometer with the sample dissolved in MeOH. IR spectrum was measured by Jasco International infrared spectrophotometer with KBr cell. ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured by Bruker ARX-400 with the sample dissolved in CD_3OD. Tetramethylsilane was employed as the internal reference. The molecular weight was determined by FAB-MS spectrophotometer (Jeol, JMX DX-303).

Biological Activity

Minimum inhibitory concentration (MIC) was determined by the conventional agar dilution method on potato sucrose agar and Mueller Hinton agar (Difco) media for *Xanthomonas* and *Streptococcus*, respectively. *In vivo* (pot test) preventive effect of the active compound against black rot was carried out with cabbage as a host plant. Antibiotics were applied 24 h before the inoculation of pathogen and the result was calculated by the following method:

$$\text{Preventive effect (\%)} = \left(\frac{\text{No. of necrotic spots on treated plant}}{\text{No. of necrotic spots on control}}\right) \times 100$$

RESULTS AND DISCUSSION

Isolation and Purification of Active Compounds

We could identify the presence of 5 active compounds from the culture broth of *Streptomyces rochei* isolate (Fig. 2). The production yield of A was 20 mg/l culture broth. Compounds B-E were not isolated by HPLC with the solvent system A. The minor compounds were more efficiently separated by the solvent system of MeOH- 0.1 M phosphate buffer (pH 6.5).

Prolonged exposure of the active compounds to heat (80°C/30 min) in acidic or alkaline condition reduced the antimicrobial activity. However, they were heat stable at pH 7.0/80°C/30 min. Therefore, acidic or alkaline condition was excluded for the purification of the active compounds. Among the 5 active compounds (A-E), the main active compound (A) was isolated and its physico-chemical properties and biological activity were determined. Minor compounds (B-E) isolated from the HPLC solvent system B were subjected only to UV spectrum measurement due to insufficient sample size.

Physico-chemical Properties of the Active Compounds

Compound A was yellow, while compounds B-E were pale yellow amorphous powder. The active compounds were soluble in MeOH, acetone and EtOH, and insoluble in $CHCl_3$, diethylether and benzene. The main active compound was slightly soluble in H_2O. Compound A showed positive color reactions to anisaldehyde, anthrone, vanilline-sulfuric acid, but negative to Rydon-Smith, ninhydrin, Dragendorff, and 2,4-dinitrophenylhydrazin reagents. On the other hand, color reaction to bromocresol green was dubious.

UV spectrum of compound A was $\lambda_{max}^{MeOH:}$: 234 and 320 (sh) (Fig. 3). Compounds B-E shared UV absorption maxima in common with that of A; however, the shoulder at 275 nm was much lower than that observed for A. Compound B had another shoulder at 220 nm. UV absorption maxima of A was shifted to 224 and 315 (sh) nm in acidic condition and to 210, 222 (sh) and 310 (sh)

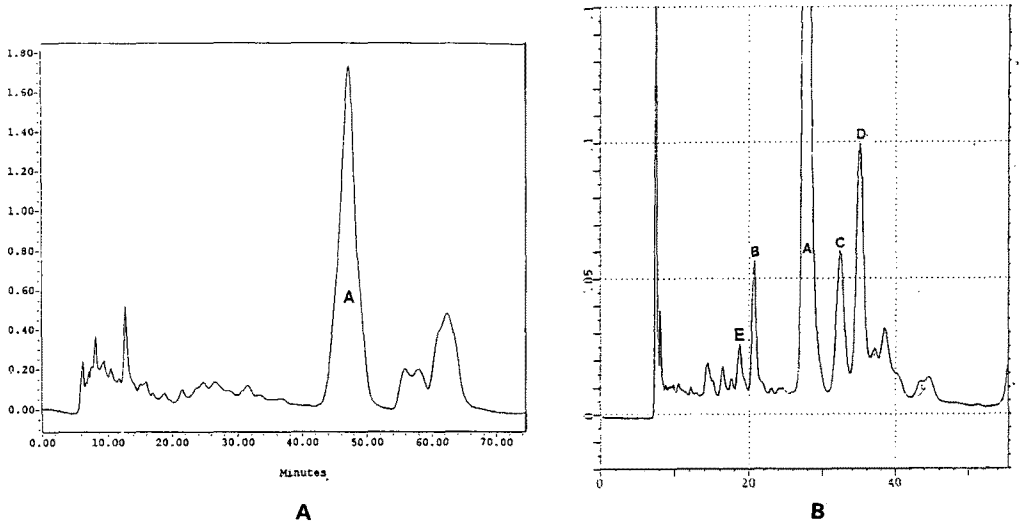

Fig. 2. HPLC profile of active compounds produced by *Strptomyces rochei* isolate.
Chromatogram of A was obtained from a solvent system of MeOH-CH_3CN-0.1% diethylamine buffer (pH 6.7, 55 : 10 : 35, ODS-H8O, YMC-pack, 231 nm) and B from MeOH-0.1 M phosphate buffer (pH 6.5, 65 : 35, ODS, Nova-pack).

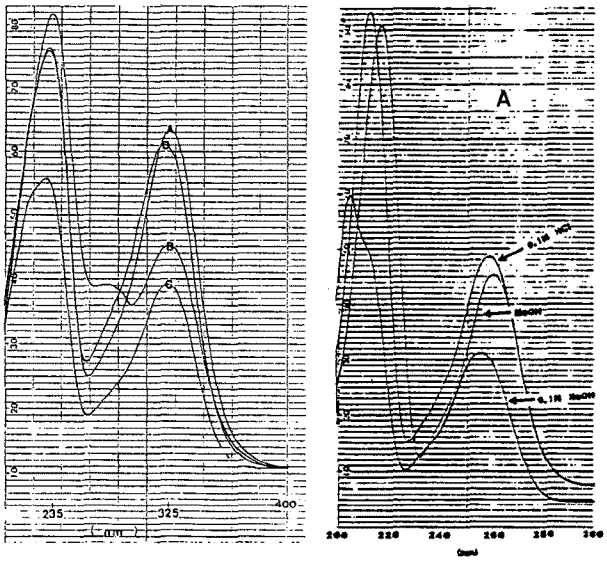

Fig. 3. UV spectra of active compounds (A-D) produced by *Streptomyces rochei* isolate.
Samples were dissolved in MeOH.

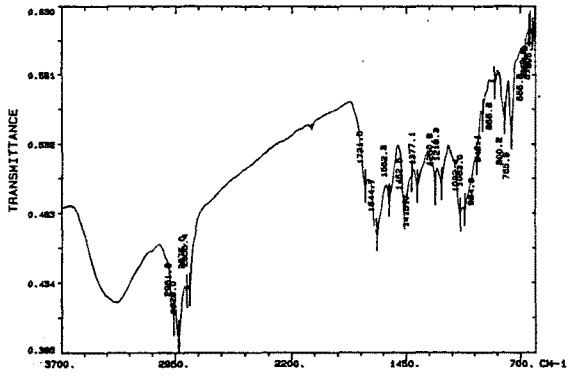

Fig. 4. Infrared spectrum of compound A.

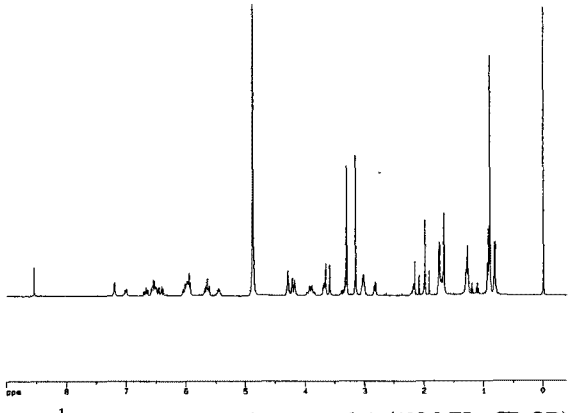

Fig. 5. ^1H NMR spectrum of compound A (400 MHz, CD_3OD).

nm in alkaline solution. IR spectrum of compound A is shown in Fig. 4. The presence of NH and OH, pyridone, amide I and sugar moieties were indicated by the bands at wavenumbers (cm^{-1}) 3650-3100, 1721, 1644, and 1092-984, respectively.

The molecular weight of compound A was determined to be $[M+H]^+$ 797 (FAB-MS). ^1H NMR spectrum of compound A is shown in Fig. 5. Signals at δ_{Hs}7.02 and 7.43 may have originated from CH in pyridone ring. Singlets at δ1.0, 3.25 and 2.0 (br.) may be assigned to CH_3 in sugar moiety, CH_3O, and olefin bound methyls, respectively. Methin signals at δ_{Hs} 5.45-6.82 indicate olefin,

Table 1. Minimum inhibitory concentration (MIC) of compound A.

Test microorganism	MIC (μg/ml)
Xanthomonas campestris pv. *citri*	4
Xanthomonas campestris pv. *oryzae*	4
Streptococcus pyogenes	0.002
Streptococcus faecium	0.002

Potato sucrose and Mueller Hinton agar media were employed for the determination of MICs of *Xanthomonas* and *Streptococcus*, respectively.

Table 2. Preventive effect of compound A against black rot.

Antibiotics	Concentration (μg/ml)		
	120	240	480
Compound A	ND*	67**	86
Streptomycin	50	74	76
Oxytetracycline	14	ND	74

*ND, Not determined. *Xanthomonas campestris* pv. *campestris* was employed for this *in vivo* (pot test) assay with cabbage as a host plant. **Preventive effect (%).

4.25-4.42 tetrahydrofuran, δ3.65-4.05 sugar moiety.

Signal at δ_C 177 and 100.4 in ^{13}C NMR spectrum (data not shown here) could be assigned to ketone and ketal, respectively. Signals at δ_{CS} 72-85 revealed the presence of tetrahydrofuran and sugar moieties. Olefinic carbons appeared at δ_{CS} 127-138.

UV spectra and other physico-chemical properties of the active compounds produced by the *Streptomyces rochei* isolate clearly place them in the aurodox group antibiotics, such as kirromycin (mocimycin) (15), aurodox (8), efrotomycin (14) and heneicomycin (5). Physico-chemical properties of compound A were compared with recently published data of aurodox group antibiotics. Compound A was most compatible with kirromycin in its molecular weight (12) and ^1H NMR assignment. However, kirromycin showed slightly different chemical shift in ^1H NMR spectrum and solubility compared to our compound A. Heneicomycin (m.w. 795), on the other hand, revealed fingerprint ^1H NMR spectrum in low magnetic field, but assignment of high magnetic field differed from that of the our compound.

Biological Activity

Compound A showed no inhibitory activity against fungi (*Rhizoctonia solani* IFO 6258, *Pyricularia oryzae* IFO 5994, *Colletotrichum lagenarium* IFO 7513, *Alternaria mali* IFO 8984, *Aspergillus niger* ATCC 9642, *Fusarium oxysporum* IFO 9761, *Glomerella cingulata* IFI 9767, *Botrytis cinerea* IFO 5365), yeast (*Candida albicans* IAM 4905, *Saccharomyces cerevisiae* IFO 1008) or algae (*Chlorella vulgaris* IFO 15941) employed in this study. It showed narrow antimicrobial spectrum against very limited genus of Gram positive and negative bacteria, such as *S. pyogenes* and *Xanthomonas* sp. at MICs of 0.002 and 4 μg/ml, respectively (Table 1). However, activity against *E. coli* AB 1157 and BE 1186, *Salmonella typhimurium* TV 119 and SL 1102, *Pseudomanas aeruginosa* IFO 13130, *Staphylococcus aureus* FDA 209P and R-209, *Mycobacterium phlei* II DIPH IFO 3158, *Bacillus subtilis* IAM 1069, *Sarcina lutea*, and *Erwinia carotovora* IFO 1008 was not observed. Antimicrobial spectrum of kirromycin was slightly different from that of compound A. Unlike the compound A, kirromycin was reported to inhibit *S. aureus*, *S. lutea*, *B. subtilis* and *E. carotovora* (15).

Preventive effect of A against the plant pathogen was not satisfactory but compatible with that of streptomycin and oxytetracycline (Table 2). However, antimicrobial activity of the compound A against *Streptococcus pyogenes* rivals that of cephaloridine (MIC, 0.001 μg/ml).

Aurodox, kirromycin and efrotomycin are intended for veterinary use, especially as growth-promoting feed additives for farm animals and as chemotherapeutic agents in the control of dysentry caused by *Treponema* infections (8).

The results of physico-chemical properties led us to conclude that the main active compound produced by *S. rochei* isolate is most probably be kirromycin. However, difference in biological activity between kirromycin and the compound A can not yet to be understood. No matter how the compound A is kirromycin, we propose that the compound A is a promissing antibiotic for the control of *S. pyogenes* and *Xanthomonas* spp. Investigation of physico-chemical properties of compounds B-E are in progress.

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