AKD-2A, B, C and D, New Antibiotics from *Streptomyces* sp. OCU-42815

Taxonomy, Fermentation, Isolation, Structure Elucidation and Biological Activity

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An antibiotic complex, AKD-2, was isolated from the mycelial cake of *Streptomyces* sp. OCU-42815. The lipophilic substances in this complex were further purified by a recycling HPLC procedure and were designated AKD-2A, C and D. AKD-2B was obtained as a mixture of AKD-$2B_1$ and AKD-$2B_2$. These substances were identified as monoglycerides having branched chain fatty acids and exhibited both antibacterial and antifungal activities.

In the course of our screening program for new antibiotics, an actinomycete strain OCU-42815 was found to produce several antibiotics. From the acetone extracts of the mycelium, four macrotetrolide antibiotics AKD-1A, B, C and D were firstly isolated and identified as nonactin, monactin, dinactin and trinactin, respectively[1~3]. Subsequently, another antibiotic complex, AKD-2, was isolated and further purified by a recycling HPLC procedure, so that monoglycerides AKD-2A, B, C and D were obtained (Fig. 1). AKD-2A had been reported as an emulsifier[4]. AKD-2B was an unknown substance consisting of AKD-$2B_1$ and AKD-$2B_2$. AKD-2C and D were also new monoglycerides.

This paper describes the taxonomy of the producing organism, fermentation, isolation, structure elucidation and antibiotic activities of these monoglycerides.

Materials and Methods

Taxonomic Studies

The organism, strain OCU-42815, was isolated from a soil sample collected at Sugimoto campus of Osaka City University.

The taxonomic studies were carried out as described by International Streptomyces Project (ISP)[5]. For the evaluation of cultural characteristics, the strain was incubated for 14~21 days at 28°C. The color recorded for mature cultures was described according to the "Color Harmony Manual"[6]. Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[7]. The type of diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al*[8].

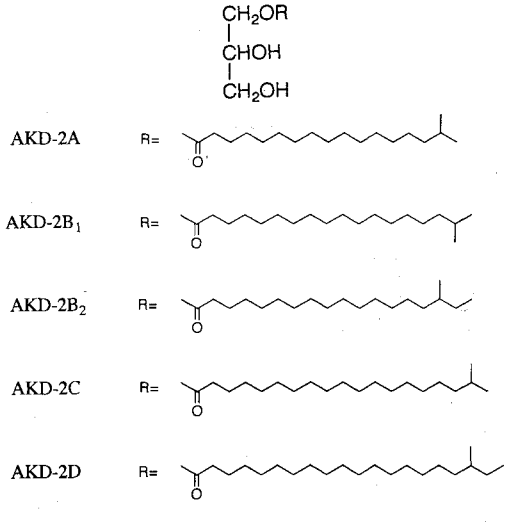
Fermentation

A loopful of the organism on mature slant culture was transferred into four 2-liter Erlenmeyer flasks each containing 500 ml of sterile seed medium. The medium contained glucose 1%, malt extract 0.5%, yeast extract 0.5% and $CaCO_3$ 0.2%. The pH was adjusted to 7.0 before sterilization. The flasks were incubated on a rotary shaker (220 rpm) for 1 day at 30°C. The resulting culture was transferred to a 100-liter sterile fermentor containing 50 liters of the seed medium. It was cultured for 1 day at 30°C with aeration at 50 liters per minute and agitation speed of 150 rpm. Fifteen liters of the seed prepared above was inoculated into a 600-liter fermentor containing 300 liters of production medium containing the same composition as the seed medium. The fermentation was carried out for 3 days at 30°C with aeration at 167 liters per minute and agitation speed of 200 rpm.

Fermentation Analysis

The growth was monitored by packed cell volume measurement. The amount of AKD-2 substances in the mycelium was quantified by HPLC using a Shimadzu LC-6A pump (column, Asahipak ODP-50; mobile phase: 88:12 acetonitrile-water; flow rate, 1.0 ml/minute; detection, UV at 220 nm). The sample for the HPLC

Fig. 1. Structures of AKD-2 substances.



assay was prepared as follows: 50 ml of the culture broth was sampled and centrifuged. The precipitated mycelium was extracted with 5 ml of acetone for 1 hour at room temperature and filtered. After removal of acetone, the residue was extracted with 1 ml of *n*-hexane. The organic layer was extracted with 1 ml of acetonitrile. After filtration, 3 μl of the filtrate of acetonitrile layer was injected into the column.

Isolation

The culture broth (300 liters) was filtered with the aid of diatomaceous earth. The mycelial cake was extracted with acetone (80 liters). After removal of acetone, the resulting aqueous solution was adjusted to pH 2 with HCl and extracted with *n*-hexane (15 liters). The organic layer was extracted with acetonitrile (15 liters). The acetonitrile extract was concentrated under reduced pressure to give 99.52 g of oily material, which was dissolved in the small amount of *n*-hexane, applied on a column of silica gel (Wakogel C-200, 5.3 liters) packed with *n*-hexane - EtOAc (7 : 3) and eluted with the same solvent (67 liters).

The first active fractions (AKD-1) were combined and concentrated under reduced pressure to afford a brownish syrup. The syrup was purified by a reversed phase HPLC using a Shimadzu LC-6AD pump (column, Capcell Pak C_{18}; 25 cm × 1.0 cm i.d.; mobile phase: 86 : 14 MeOH - water; flow rate, 3 ml/minute). This procedure yielded 0.67 g of AKD-1A (nonactin), 11.49 g of AKD-1B (monactin), 25.70 g of AKD-1C (dinactin) and 10.64 g of AKD-1D (trinactin).

Concentration of the second active fractions (AKD-2) yielded a brownish syrup. GC-Mass and NMR spectra of the crude syrup showed that AKD-2 was composed of several components of the branched fatty acid 1-monoglycerides.

Moreover, GC of more volatile AKD-2 acetates prepared by acetylation with acetic anhydride and pyridine showed it to be a mixture of more than four components, as shown in Fig. 2.

For further purification of the crude syrup of AKD-2, recycling HPLC was done on a JAI-LC-908 HPLC (Japan Analytical Industry Co., Tokyo, Japan) equipped with a JAI RI- and JAI UV-detector, operating at 220 nm. A prepacked ODS column (JAIGEL ODS-S-343, 25 cm × 2 cm i.d.) was adopted and 85% aqueous methanol was the eluting solvent at a flow rate of 3 ml per minute. This procedure yielded 153 mg of AKD-2A, 658 mg of AKD-2C and a very small amount of AKD-2D. AKD-2B was obtained as a mixture of AKD-$2B_1$ and AKD-$2B_2$ and was then characterized further by NMR and MS.

Antimicrobial Assay

The antimicrobial activity of AKD-2A, B (mixture of AKD-$2B_1$ and AKD-$2B_2$), C and D was determined by a serial broth dilution method after overnight incubation at 37°C using bouillon medium for bacteria and after 1 ~ 2 days incubation at 28°C using malt extract medium for fungi and yeasts. Test organisms used are shown in Table 6.

Results and Discussion

Taxonomy of Strain OCU-42815

The vegetative mycelium developed well without fragmentation. The aerial mycelium branched monopodially and formed spiral chains of spores with 20 to 30 spores per chain. The spores had a spiny surface and were oval in shape with size ranging from 0.8 ~ 1.0 × 0.9 ~ 1.4 μm (Fig. 3). No sclerotic granules, sporangia or zoospores were observed. The cultural characteristics observed grown on various media at 28°C for 14 ~ 21 days are shown in Table 1. The aerial mass color was in the gray color series. The color of reverse side of the colonies was colorless to dark brown.

Analysis of whole-cell hydrolysates showed the presence of LL-diaminopimelic acid. Physiological properties and utilization of carbon sources are shown in Tables 2 and 3, respectively.

Based on the taxonomic properties described above, strain OCU-42815 was considered to belong to the genus *Streptomyces*, and designated *Streptomyces* sp. OCU-42815.

Fig. 2. GC of AKD-2 acetates detected with TIM.

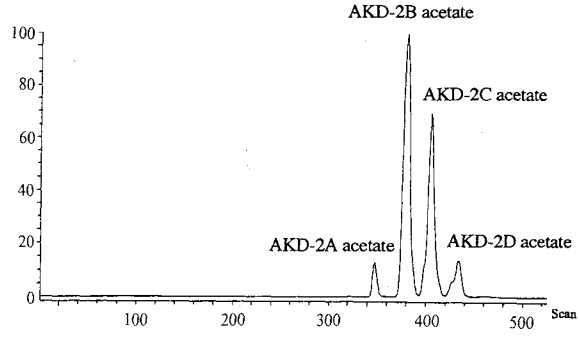


Fig. 3. Scanning electron micrograph of spore chains of strain OCU-42815.
Bar = 1.0 μm.

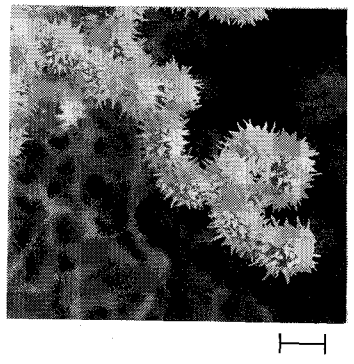


Table 1. Cultural characteristics of strain OCU-42815.

Medium	Cultural characteristics
Sucrose-nitrate agar	G: Moderate A: Moderate, gray (3fe) R: Colorless S: None
Glucose-asparagine agar	G: Moderate A: Abundant, gray (5fe) R: Colorless S: None
Glycerol-asparagine agar (ISP No. 5)	G: Good A: Abundant, gray (e) R: Colorless S: None
Inorganic salts-starch agar (ISP No. 4)	G: Good A: Abundant, gray (e-g) R: Colorless S: None
Oatmeal agar (ISP No. 3)	G: Good A: Abundant, gray (3fe-5fe) R: Colorless S: None
Yeast extract-malt extract agar (ISP No. 2)	G: Good A: Abundant, gray (5fe) R: Colorless S: None
Tyrosine agar (ISP No. 7)	G: Good A: Abundant, gray (5fe) R: Dark brown S: None
Nutrient agar	G: Moderate A: Moderate, gray (d) R: Colorless S: None
Ca-malate agar	G: Moderate A: Moderate, gray (3fe-d) R: Colorless S: None
BENNET's agar	G: Good A: Abundant, gray (5fe) R: Colorless S: None

Abbreviations: G, growth; A, aerial mycelium; R, reverse side color; S, soluble pigment.

Table 2. Physiological characteristics of strain OCU-42815.

Temperature range for growth	25~42℃
Optimum temperature for growth	37℃
Gelatin liquefaction	Negative
Milk coagulation	Negative
Milk peptonization	Positive
Starch hydrolysis	Positive
Melanoid pigment production	Negative
Nitrate reduction	Negative
NaCl tolerance	5%

Table 3. Utilization of carbon sources by strain OCU-42815.

D-Glucose	+
D-Fructose	+
D-Mannitol	+
L-Arabinose	+
myo-Inositol	+
L-Rhamnose	+
Raffinose	−
Sucrose	±

+: Utilization, −: no utilization, ±: doubtful utilization.

Fermentation and Isolation

A typical time course for the production of AKD-2A and C is shown in Fig. 4. Maximum production was observed at 3 days (AKD-2A) and 4 days (C) after cultivation.

The isolation procedure of AKD-substances is shown in Fig. 5 and in Materials and Methods section. From the mycelium of strain OCU-42815 cultured in 300 liters of the medium, 153 mg of AKD-2A, 1433 mg of AKD-2B (mixture of AKD-2B_1 and AKD-2B_2), 658 mg of AKD-2C and 5 mg of AKD-2D were obtained.

Structure Elucidation

^1H and ^{13}C NMR spectra of AKD-2C resembled those of AKD-2A that displayed signals based on 1-acylated glycerine and the branched fatty acid, as shown in Table 4. Mass spectra of AKD-2C revealed fragment ion peak $(M-(CH_2OH))^+$ at *m/e* 355 in EI-MS and at *m/z* 353 in FAB-MS but no M^+ ion characterized as 1-monoglyceride by C. B. JOHNSON and R. T. HOLMAN[9]. Moreover, in the mass spectra of the diacetate of AKD-2C, the fragment ion peak at *m/e* 354 in EI-MS and at *m/e* 355 in CI-MS were observed as heaviest ion peak together with base peak at *m/e* 159 $(\cdot CH_2\text{–}CHOAc\text{–}CH_2OAc)^+$, but M^+ ion could not be detected in both ionization mass spectra (Table 5). Similar results as in AKD-2C and AKD-2C acetate were also obtained in mass spectra of AKD-2A and AKD-2A acetate. The fragment ions at *m/e* 327 in EI-MS of AKD-2A and at *m/e* 326 in EI-MS

Fig. 4. Time course of fermentation.
□ AKD-2A, ■ AKD-2C, ● Packed cell volume, ○ pH.

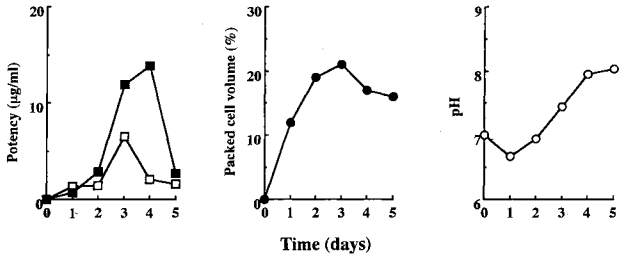


Fig. 5. Isolation procedure of AKD-substances.

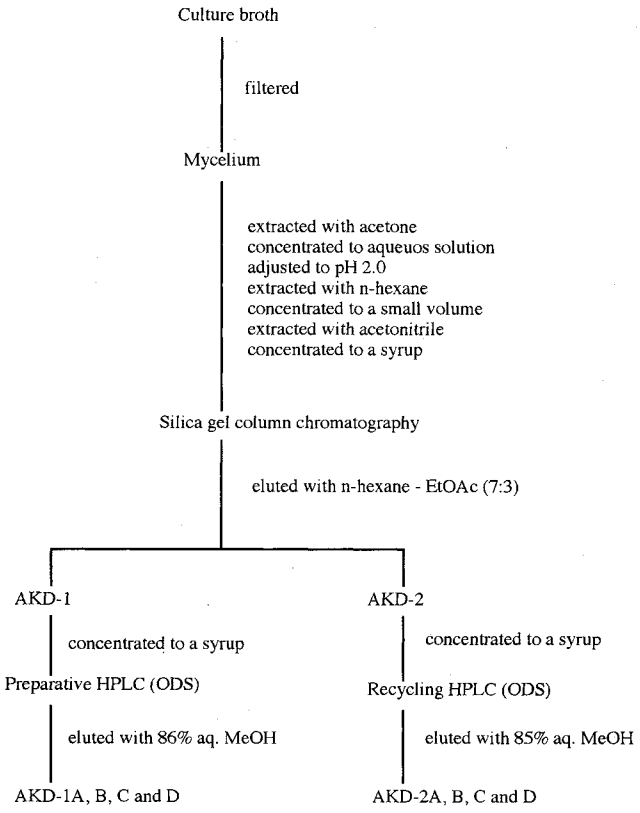


Table 4. NMR spectra of AKD-2A, B_1, B_2, C and D.

	AKD-2A		AKD-2B$_1$		AKD-2B$_2$		AKD-2C		AKD-2D	
	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
CH_3	22.66	0.85(6H,d,6.7)	22.66		11.38	0.85(3H,t,7.3)	22.66	0.85(6H,d,6.7)	11.38	0.85(3H,t,7.0)
					19.25	0.84(3H,d,6.1)			19.26	0.84(3H,d,6.1)
$\underline{CH}\text{-}CH_2$-	28.01	1.50(1H,n,6.7)	28.01	1.52(n)	34.46	1.3(overlapped)	28.01	1.50(1H,n,6.7)	34.49	
$\underline{CH}\text{-}\underline{CH_2}$-	39.11	1.13(2H,q,6.7)	39.11		36.69	1.10,1.30	39.13		36.70	
$CH_3\text{-}\underline{CH_2}$					27.13	1.3(overlapped)			27.16	
$\underline{CH_2}\text{-}CO$	34.21	2.33(2H,t,7.3)			34.21	2.34(2H,t,7.3)	34.21	2.33(2H,t,7.6)	34.22	2.34(2H,t,7.6)
$\underline{CH_2}CH_2CO$	24.97	1.62(2H,qu,7.3)			24.97	1.63(2H,qu,7.3)	24.97	1.62(2H,qu,7.6)	24.98	1.63(2H,qu,7.6)

Other common signals for all compounds.

-$(\underline{CH_2})$n	29.17-29.95	1.24-1.28
-$\underline{CH_2}$OH	63.44	3.59(1H,dd,11.3,5.8),3.67(1H,dd,11.3,4.0)
-$\underline{CH}$OH	70.39	3.91(1H,tt,5.8,4.0)
-$\underline{CH_2}$OCOR	65.27	4.14(1H,dd,11.6,5.8),4.18(1H,dd,11.6,4.6)
-$\underline{COO}$	174.28	

Table 5. Mass spectra of acetates of AKD-2A, B, C and D.

	AKD-2A acetate		AKD-2B acetate		AKD-2C acetate		AKD-2D acetate
	EI	CI	EI	CI	EI	CI	EI
m/e	326	327	340	341	354	355	368
	313	159	327	159	341	159	339
	283		311		311		325
	211		297		239		311
	159		283		159		253
			225				159
			177				
			159				

Table 6. Antimicrobial activities of AKD-2A, B, C and D.

Test organism	MIC (μg/ml)			
	AKD-2A	AKD-2B[a]	AKD-2C	AKD-2D
Escherichia coli IFO 3545	>100	>100	>100	>100
Proteus vulgaris IFO 3851	100	>100	>100	>100
Pseudomonas aeruginosa IFO 3080	100	>100	>100	>100
Serratia marcescens IFO 3046	100	>100	>100	>100
Bacillus subtilis IFO 3007	>100	>100	>100	>100
Bacillus pumilus IFO 12092	>100	>100	>100	>100
Erwinia carotovora IFO 14082	50	>100	100	>100
Micrococcus luteus IFO 3333	50	12.5	100	12.5
Staphylococcus aureus NCTC 8530	50	>100	100	>100
Staphylococcus aureus IFO 12732	>100	50	>100	>100
Streptococcus mutans IFO 13955	100	>100	50	>100
Candida albicans IFO 1061	>100	>100	100	>100
Candida rugosa IFO 1152	>100	3.13	100	100
Candida utilis OUT 6020	100	>100	100	100
Hansenula anomala IFO 0136	>100	>100	100	100
Rhodotorula rubra IFO 0001	100	>100	25	50
Saccharomyces cerevisiae IFO 0203	100	3.13	50	12.5
Saccharomyces cerevisiae IFO 0251	50	1.56	25	6.25
Schizosaccharomyces pombe IFO 0342	>100	>100	50	50
Torulaspora delbrueckii DSM 70504	100	100	12.5	100
Aspergillus niger ATCC 6275	100	50	100	100
Fusarium oxysporum IFO 7152	12.5	100	100	100
Mucor flavas IFO 9560	100	100	100	100
Mucor javanicus IFO 4569	100	100	100	100
Neurospora sitophila DSM 1130	100	100	100	100
Penicillium chrysogenum IFO 4626	50	50	50	50
Penicillium notatum Westling	50	100	100	50
Rhizopus delemar IFO 4775	25	25	50	100
Rhizopus formosaensis IFO 4732	6.25	50	50	50
Rhizopus javanicus IFO 5441	12.5	100	100	100
Rhizopus oryzae IFO 4766	50	50	100	25

[a] Mixture of AKD-2B$_1$ and AKD-2B$_2$.

and at *m/e* 327 in CI-MS of AKD-2A diacetate were observed as heaviest ion together with base peak at *m/e* 159. These spectral results were fully consistent with the proposed structures of AKD-2A (1-(16-methylheptadecanoyl)-glycerol) and AKD-2C (1-(18-methylnonadecanoyl)-glycerol) as shown in Fig. 1.

It was verified with GC-MS spectrum of AKD-2B acetate and NMR spectra of AKD-2B and its acetate that AKD-2B was the mixture of *iso*-fatty acid derivative (AKD-2B$_1$) as the minor component and *anteiso*-isomers (AKD-2B$_2$) as the main component. Specially, ^{13}C NMR spectrum of AKD-2B showed the signals at δ 11.38 (CH_3), 19.25 (CH_3), 27.13 (CH_2), 34.46 (CH) and 36.69 (CH_2) assigned to *anteiso*-isomer, and the signals at δ 22.66 ($2CH_3$), 28.01 (CH) and 39.11 (CH_2) assigned to *iso*-isomer. In GC-EI and CI-MS of AKD-2B and its acetate, two components could not be separated. However, the mass spectrum of AKD-2B acetate showed the fragment ion peak at *m/e* 311 $(340\text{-}CH_2CH_3)^+$ and 283 $(340\text{-}CH_3CH_2CHCH_3)^+$ characterized as 16-methyloctadecanoic acid, and the fragment ion peak at *m/e* 297 $(340\text{-}CH(CH_3)_2)^+$ characterized as the structure of 17-methyloctadecanoic acid. The relative ratio of *anteiso-/iso*-fatty acid in AKD-2B was 8/1 in the integration of ^1H NMR. From these spectral data, the structures of AKD-2B$_1$ (1-(17-methyloctadecanoyl)-glycerol) and AKD-2B$_2$ (1-(16-methyloctadecanoyl)-glycerol) were elucidated as formula shown in Fig. 1.

In the same manner, it was elucidated that AKD-2D was 1-(18-methyleicosanoyl)-glycerol.

Antimicrobial Activity

The antibiotic activities of AKD-2A, B (mixture of AKD-2B$_1$ and AKD-2B$_2$), C and D were determined by the serial broth dilution method. As can be seen in Table 6, AKD-2A, B, C and D showed weak activity against bacteria, yeasts and fungi. Although AKD-2A has been reported as an emulsifier, this is the first case that it acts as an antibiotic, as in the case of AKD-2B, C and D.

Monoglycerides having $C_{17}\sim C_{20}$ straight chain fatty acids did not show any antimicrobial activity at a concentration of 100 μg/ml. It has been reported that 1-monoglycerides having 12-methyltetradecanoic acid, 14-methylpentadecanoic acid and 15-methylhexadeca-

noic acid were isolated as platelet aggregation inhibitors from the culture broth of a *Streptomyces* strain and that they had no antimicrobial activity[10]. Further studies on biological properties of AKD-2 substances are now in progress.

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