NEW PRODUCTS RELATED TO KINAMYCIN FROM
STREPTOMYCES MURAYAMAENSIS

I. TAXONOMY, PRODUCTION, ISOLATION
AND BIOLOGICAL PROPERTIES

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Six new products of *Streptomyces murayamaensis* sp. nov. Hata et Ohtani, the producer of the kinamycins, were isolated by silica gel column chromatography. The antibacterial activities of the new products, as well as that of dehydrorabelomycin and murayaquinone, previously isolated products of the same organism, were compared to the kinamycins. Three of the products had antibacterial activities similar to the kinamycins, while two others had activity only against Gram-positive bacteria. Dehydrorabelomycin and one other metabolite had no detectable antibacterial activity. The organism was found to be capable of aerial mycelium formation, with sporophores branched at regular intervals bearing square-ended spores with smooth surfaces. The culture contains L,L-diaminopimelic acid in the cell wall (Type I), is highly resistant to lysozyme, and lecithinase- and melanin-positive, suggesting a relationship with the genus *Streptoverticillium* and the *lavendulae* group of the genus *Streptomyces*.

Streptomyces murayamaensis sp. nov. Hata et Ohtani produces the kinamycins A through D (Fig. 1, 1~4), a group of antibiotics having a novel benz[*b*]tetrahydrocarbazole skeleton and *N*-cyano moiety, which were characterized by Ōmura *et al.*[1~3]. The producing culture was assigned to the genus *Streptomyces*; however, it seemed to resemble *Nocardia* because of fragmentation of the mycelium[1].

In a previous report[4] we described the isolation of dehydrorabelomycin (Fig. 1, 5) from *S. murayamaensis* and its involvement in kinamycin biosynthesis. We report here the isolation and

Fig. 1. Structure of the kinamycins A~D (1~4) and dehydrorabelomycin (5), metabolites of *Streptomyces murayamaensis*.
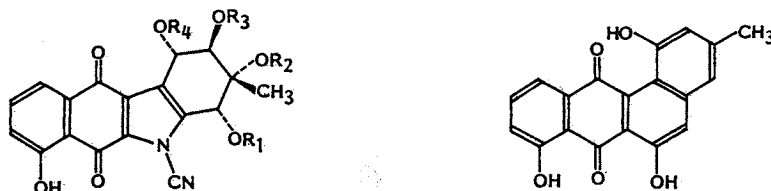
1	$R_1=R_2=R_3=Ac$	$R_4=H$
2	$R_2=Ac$	$R_1=R_3=R_4=H$
3	$R_1=R_3=R_4=Ac$	$R_2=H$
4	$R_1=R_3=Ac$	$R_2=R_4=H$
8	$R_3=Ac$	$R_1=R_2=R_4=H$
9	$R_1=Ac$	$R_2=R_3=R_4=H$
10	$R_1=R_2=R_3=R_4=H$	

bioactivity of several new products related to the kinamycins which we have obtained through manipulation of the culture conditions of *S. murayamaensis*. We have also undertaken a study of the taxonomy of the producing organism, since it does not seem to be a typical streptomycete, and much has been learned about actinomycete taxonomy since the culture was first isolated.

Materials and Methods

Microbial Cultures

The producing culture, *S. murayamaensis*, was kindly provided by Professor ŌMURA. The patent strain, *S. murayamaensis* ATCC 21414, and bacterial strains used for determination of the antibacterial spectrum of activity of the metabolites were from the American Type Culture Collection (ATCC). Other cultures used for comparison in cell wall analysis were kindly provided by A. DIETZ (Upjohn Co.) and H. PRAUSER (Jena, DDR).

Culture Maintenance

S. murayamaensis was maintained by transfer of vegetative mycelium on slants of KRAINSKY's agar (glucose 1.0%, L-asparagine 0.05%, K_2HPO_4 0.05%, agar 1.0%, pH not adjusted). Slants were incubated at $26 \sim 27°C$ for 7 days and then stored at $4°C$.

Taxonomic Studies

Growth characteristics including colonial morphology and carbohydrate utilization were determined by the methods of the International Streptomyces Project (ISP)[5]. Lysozyme resistance of *S. murayamaensis* and *Streptomyces lusitanus* was tested in YME broth (ISP-2 without agar). Egg yolk reaction (lecithinase) was tested according to NITSCH and KUTZNER[6]. Other physiological tests were done according to WILLIAMS *et al.*[7].

Cultures for study of micromorphology were grown on ISP-3, ISP-4, KRAINSKY's agar, or modified BENNETT's agar (Difco potato dextrin 1.0%, NZ-Amine Type A 0.2%, BBL beef extract 0.08%, Difco yeast extract 0.1%, KCl 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, $FeSO_4 \cdot 7H_2O$ 4 mg/liter, pH 7.4). Samples of aerial mycelium were observed by scanning electron microscopy (SEM) either on intact agar plugs or on coverslips coated with a liquid adhesive and touched to the surface of the growth. Agar plugs were critical point dried, whereas the coverslips were air dried directly from a 95% ethanol rinse. The samples were coated with gold - palladium alloy for viewing by SEM.

Analysis of whole-cell sugars and diaminopimelic acid (DAP) isomer in the cell wall was carried out according to STANECK and ROBERTS[8], using as controls *Nocardia argentinensis* ATCC 31306, *Nocardioides albus* IMET 7807, *Kibdelosporangium aridum* ATCC 39323 and *S. lusitanus* NRRL 8034.

Shake Flask Fermentations

Seed medium (glucose 2.0%, soybean meal 2.0%, NaCl 0.3% in distilled water; pH 8.0; 100 ml in a 500-ml Erlenmeyer flask) was inoculated with a loopful of vegetative mycelium from a KRAINSKY's agar slant. After incubation for 48 hours at $26 \sim 27°C$, the seed culture was used to inoculate any of the following production media (200 ml in a 1-liter Erlenmeyer flask; 5% inoculum).

Glycerol - asparagine medium consisted of glycerol 3%, L-asparagine 0.1%, $K_2HPO_4 \cdot 3H_2O$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.04% and $FeSO_4 \cdot 7H_2O$ 0.01%, in distilled water (pH not adjusted). Glycerol - ammonium sulfate medium contained glycerol 3%, $(NH_4)_2SO_4$ 0.1%, $K_2HPO_4 \cdot 3H_2O$ 0.1%, $CaCO_3$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.04% and $FeSO_4 \cdot 7H_2O$ 0.01%, in distilled water (pH not adjusted). Soybean - glycerol medium was identical to seed medium, except for the substitution of glycerol for glucose. Oatmeal - trace metals medium contained rolled oats (20 g, cooked in 500 ml of distilled water, then added to 500 ml of cold distilled water containing 2 ml trace metal salts and adjusted to pH 7.2 with NaOH). Trace metal salts solution contained, per liter of distilled water: $ZnCl_2$ 40 mg, $FeSO_4 \cdot 7H_2O$ 200 mg, $CuCl_2 \cdot 7H_2O$ 10 mg, $MnCl_2 \cdot 4H_2O$ 10 mg, H_3BO_3 5 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 10 mg and 2 ml of concentrated HCl.

All shake flask cultures were incubated at $26 \sim 27°C$ in a LabLine Rotary Incubator-Shaker at $280 \sim 300$ rpm.

Larger Scale Fermentations

Seven liters of glycerol - ammonium sulfate medium in the 10-liter vessel of a New Brunswick MicroFerm fermentor were inoculated with 200 ml of seed culture and incubated for 26 hours at 27°C at an impeller speed of 300 rpm and aeration rate of 10 liters/minute. Foaming was controlled with Antifoam A. Broth was harvested either by centrifugation with a Sharples Model T-1 continuous flow centrifuge or by acidification with 6 N HCl to pH 3 followed by filtration.

Isolation of Metabolites

Broth from a 1-liter fermentation of *S. murayamaensis* in the oatmeal - trace elements medium (5 × 200 ml in 1-liter Erlenmeyer flasks) was acidified with 6 N HCl to pH 3.0. The mycelia were removed by centrifugation (10,000 rpm, 10 minutes, IEC Model B-20A Centrifuge), resuspended in 50 ml distilled H_2O, sonicated (4°C, 5 minutes, maximum power) and recombined with the broth. The entire volume was stirred with toluene (400 ml) for 1 hour and the resulting emulsion was filtered through a Celite pad, which was then rinsed with acetone (100 ml). After further extraction of the aqueous layer with EtOAc (2 × 200 ml) the combined organics were dried (anhydrous Na_2SO_4) and concentrated *in vacuo* to give a brown gum (0.94 g).

The residue was dissolved in $CHCl_3$ (2 ml) and applied to a Silicar CC-4 column (Mallinkrodt 7086, 3 × 20 cm) packed in $CHCl_3$. Elution with $CHCl_3$ first gave a dark green (G) fraction containing 5 plus nonpolar oils (0.425 g after concentration *in vacuo*). Trituration of the oily residue with hexane gave 5 as dark green needles (5.5 mg). Continued elution with 1 % EtOAc in $CHCl_3$ gave a fraction (0.242 g) containing the two purple components (P1 and P2) and the nonpolar yellow component (Y1), along with contaminating oils. Further elution of the column with 10 % EtOAc in $CHCl_3$ yielded a mixture (0.118 g) of kinamycins A and B (1 and 2) and an unknown colorless metabolite (UK). Kinamycin D was eluted with 20 % EtOAc in $CHCl_3$, and was directly crystallized from EtOAc - hexane as an orange powder (55 mg). Finally, washing the column with EtOAc gave a mixture of the polar metabolites, PY1, PY2 and PY3 (0.123 g).

The nonpolar metabolites (P1, P2 and Y1) were further purified by column chromatography with flash grade SiO_2 (2 × 10 cm, 0.5 % Et_2O in CH_2Cl_2). The two purple components were cleanly separated and each was directly crystallized from CH_2Cl_2 - hexane. P1 (6) crystallized as dark purple needles (15 mg) and P2 crystallized as dark purple brown needles (6 mg). Y1 (7) was contaminated with oils and required further chromatographic purification through a flash SiO_2 column (2 × 10 cm, 10 % EtOAc - hexane). Fractions containing pure Y1 were combined, concentrated (12 mg) and 7 was crystallized from $CHCl_3$ - hexane.

The polar components (PY1, PY2 and PY3) were separated by column chromatography through flash grade SiO_2 (2 × 10 cm, 5 % MeOH in CH_2Cl_2). Traces of PY1 (8) eluted first, followed by PY3 (9) and finally PY2. 9 crystallized from EtOAc - hexane as an orange powder (14.5 mg) and PY2 crystallized from $CHCl_3$ - hexane as yellow orange crystals (9.4 mg).

Antibacterial Activity

The antibacterial activity spectra of the purified metabolites were determined by the agar dilution method[9] with Difco Mueller-Hinton agar. The metabolites were initially dissolved in dimethylformamide.

Results and Discussion

Taxonomy of *Streptomyces murayamaensis*

Morphology

The vegetative mycelium of *S. murayamaensis* grows abundantly on many complex and synthetic media (Table 1). The culture is strongly melanin-positive and also produces soluble pigments in many media.

Both the culture from the Kitasato University and the ATCC strain at first did not produce aerial

Table 1. Cultural characteristics of *Streptomyces murayamaensis*.

Medium	Growth: Amount, color, aerial mycelium (a.m.)	Soluble pigment
ISP-2[a]	Abundant; very dark brown; wrinkled, raised	Orange-brown
ISP-3	Moderate; dark chocolate brown; flat to slightly raised; dense, chalky, whitish a.m.	Bright yellow-orange then light brown
ISP-4	Abundant; chocolate-brown; flat; off white a.m. abundantly formed	Yellow, then light brown
CZAPEK - sucrose[b]	Very scant, colorless	None
BENNETT's (mod.)	Abundant; dark reddish to chocolate brown; wrinkled, raised	Brownish-black, then dark orange-brown
R2T (10% sucrose)[17]	Very dark brown; raised, very wrinkled; easily scraped off agar	Brownish-black
KRAINSKY (glucose - asparagine)	Moderate; very dark brown; less wrinkled; white a.m. after 17~20 days	Light brown
KRAINSKY +Mg, Fe	Same as above, except no a.m. after 20 days	Light brown

[a] Medium recommended by ISP.
[b] Medium recommended by S. A. WAKSMAN.

mycelium. However, after 3 weeks incubation of either strain on oatmeal agar, tiny isolated tufts of white aerial mycelium were observed. Subculture of the material from the Kitasato strain on oatmeal agar yielded a strain which formed the aerial mycelium more reproducibly. Formation of the aerial mycelium was facilitated by streaking the culture in parallel lines, using about a third of the area of the agar. When the entire plate was covered with growth, the aerial mycelium was again produced in isolated tiny tufts. The aerial mycelium was rather thin and chalky and tended to stick together when scraped off, so that the spore clumps were filtered out when spore suspensions were filtered through nonabsorbent cotton by the usual method[10]. The spore material in distilled water was dispersed very nicely after a few seconds sonication in a water bath sonicator (Bransonic 220). Dilution plating of the resulting suspension on KRAINSKY's, modified BENNETT's and ISP-2 agars yielded the typical wrinkled, dark brown colonies of *S. murayamaensis* at reasonably high titer (5×10^7 cfu per cm^2 oatmeal agar). Several colonies formed by the spores on KRAINSKY's agar were inoculated to the usual seed and production media, and the kinamycins were formed in high titer.

Subsequent to our initial determination of aerial mycelium formation, we have found that the subcultured strain forms aerial mycelium much more abundantly on ISP-4 (inorganic salts - starch) agar and ISP-9 agar with starch or dextrin.

On ISP-4 agar many of the sporophores consist of a long hypha which bears clusters of two to four shorter straight branches at regular intervals ("whorl" morphology; resembling the "mono-verticillate" type[5]) (Figs. 2 and 3). These sporophores resemble those of the genus *Streptoverticillium*. The spores have smooth surfaces and tend to be square-ended, except for the apical spore (Figs. 4 and 5).

S. murayamaensis resembles the nocardioform actinomycetes in its tendency to "fragment" and be lifted off the agar surface of certain media[1]. On media such as the basal medium of WILLIAMS *et al.*[7], or ISP-9 with glucose or glycerol as *C*-sources the growth was raised, wrinkled and somewhat glistening and could be lifted intact off the agar without too much difficulty. These colonies do not appear fragmented when examined *in situ* and when first placed on a microscope slide under a cover-slip; moreover, the surface is not pasty as with *Nocardioides*. "Fragmentation" can be induced with gradual pressure on the coverslip, *e.g.*, with a wooden applicator stick. The colony gradually falls

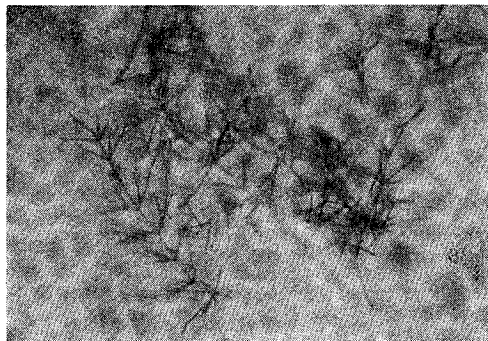Fig. 2. Photomicrograph of *Streptomyces murayamaensis* on ISP-4 agar (11 days, 26°C), ×100.

Fig. 3. Photomicrograph of *Streptomyces murayamaensis* on ISP-4 agar (11 days, 26°C), ×200.

Fig. 4. Scanning electron micrograph of *Streptomyces murayamaensis* aerial mycelium tuft on oatmeal agar (24 days, 26°C).
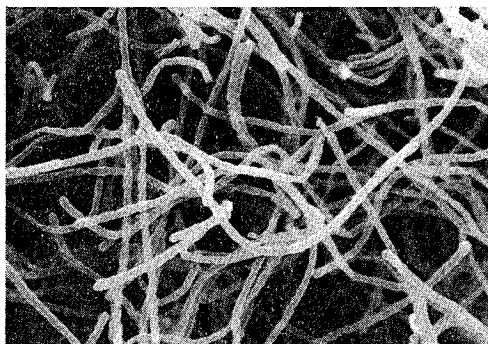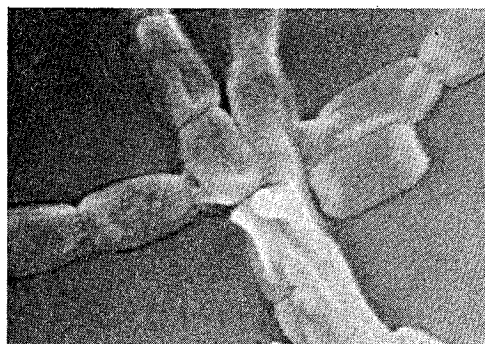
    Picture width: 30 μm.

Fig. 5. Scanning electron micrograph of *Streptomyces murayamaensis* sporophore at branch point (ISP-4 agar, 14 days, 26°C).

    Picture width: 5.25 μm.

completely apart; the mycelia break with a "popping" motion first into large fragments and eventually into smaller fragments with release of cytoplasmic contents. Eventually, the mycelium completely disintegrates. This process is more akin to autolysis than to fragmentation. On other media, *e.g.* oatmeal agar, ISP-4, or ISP-9 with no carbon source, the substrate mycelium is firmly imbedded in the agar.

    Other morphological features of the substrate mycelium were observed by phase contrast microscopy: Formation of aerial mycelium ropes (ISP-9 with glycerol, glucose, xylose); substrate mycelia with knobs (ISP-9 with xylose); swollen substrate hyphae surrounded by amorphous material (ISP-9 with glucose). Knobs and swollen hyphae, as well as autolysis, have been observed in streptomycetes during active biosynthesis of antibiotics[11].

Chemical Composition

    The analysis of whole-cell diamino acids and sugars showed *S. murayamaensis* to contain L,L-DAP and only a trace of ribose (no characteristic sugar pattern) (Type I wall).

Physiological Properties

    The ability of *S. murayamaensis* to use a number of sole carbon and nitrogen sources was tested (Tables 2 and 3). Other physiological properties of the organism are listed in Table 4.

    Two notable physiological properties which suggest a relationship of *S. murayamaensis* to the

genus *Streptoverticillium* are its lecithinase activity and high level of lysozyme resistance. All strepto-
verticillia as well as representative streptomycetes of the *lavendulae* (F-61) and *rimosus* (B-42) clusters[7]
are highly resistant to lysozyme[12,13]. Members of these groups are also highly likely to have lecithin-
ase activity[7,12]. Production of melanin pigment, for which *S. murayamaensis* is highly positive, is
not likely to be found in the *rimosus* group[7]. The "whorl" morphology of the sporophores of *S.
murayamaensis*, taken together with these physiological properties, suggest that the organism could be

Table 2. Carbon source utilization by *Streptomyces murayamaensis*.

Carbon source[a]	Utilization
D-Glucose	+
D-Fructose	+/−
L-Rhamnose	−
D-Mannitol	−
D-Mannose	−/+
i-Inositol	+
L-Arabinose	+
D-Xylose	+
Adonitol	−
Glycerol	+
Cellobiose	+
Sucrose	−
Lactose	+
Raffinose	+
Dextrin	+
Starch	+
Cellulose	−
Inulin	−
Sodium succinate (0.1%)	+
Sodium acetate (0.1%)	+

[a] Carbon sources at 1% in ISP-9 agar, unless otherwise noted.

Table 3. Nitrogen source utilization by *Streptomyces murayamaensis*.

Nitrogen source	Utilization
L-Asparagine	+
NH_4^+	+
NO_3^-	+
α-Aminobutyrate	−
L-Histidine	+
L-Hydroxyproline	+

Table 4. Other physiological characteristics of *Streptomyces murayamaensis*.

Melanin formation	+
Lecithinase (egg yolk reaction)	+
Lysozyme resistance (100 µg, 412 u/ml)	+
NaCl (7%) resistance	+
Azide (0.01%) resistance	−
Phenol (0.1%) resistance	−
Hydrolysis of xanthine	+/−
Hydrolysis of pectin	−
Neomycin (50 µg/ml) resistance	−

Fig. 6.   Purification of minor metabolites of *Streptomyces murayamaensis* by SiO_2 flash chromatography.
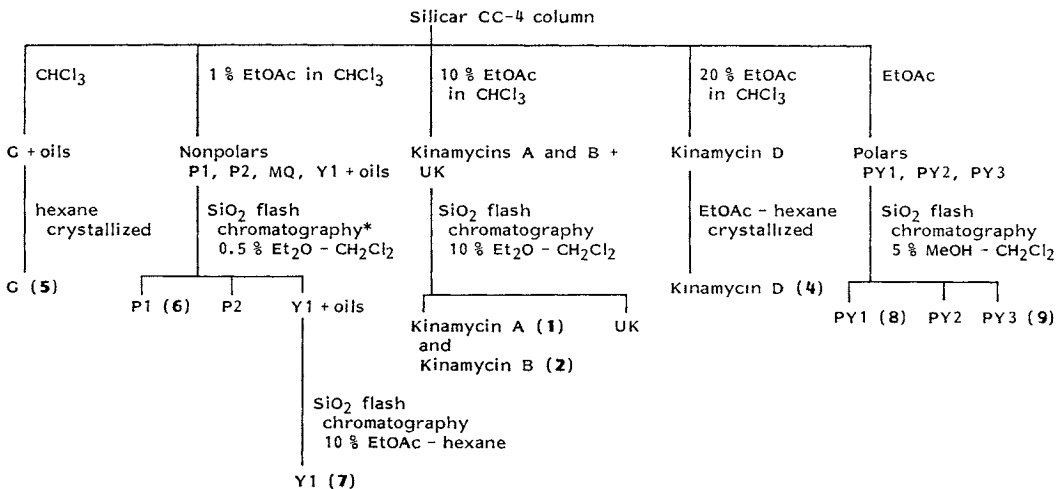
*   MQ is bound tightly, so does not interfere with isolation of P1 and P2.

reclassified as a *Streptoverticillium*[†]. Further study of the sporophores by SEM is necessary in order to distinguish whether it is the spore chains, or short spore-chain bearing hyphae, which branch off the main axis. This difference distinguishes streptoverticillia from "pseudoverticillate" streptomycetes.

Production and Isolation of Metabolites

Manipulation of the fermentation medium for *S. murayamaensis* results in the accumulation of a variety of kinamycin-related metabolites. The various metabolites were separated by chromatography on Silicar CC-4 and on flash grade silica gel, as illustrated in Fig. 6.

Of the four media, the oatmeal broth yielded the greatest amounts of the desired minor metabolites (Table 5). Murayaquinone (MQ)[14] is produced in media 1 ~ 3, while dehydrorabelomycin (G; 5)[4] is produced in all four media. The previously unknown metabolites, nonpolar (P1, P2, Y1) as well as polar (PY1, PY2, PY3), were first detected and isolated from fermentations in medium 2. It was later found that medium 4 allowed greater production of all the minor metabolites except PY1, which is most likely a degradation product of kinamycin D[15]. Proof of structures (Figs. 1 and 7) for P1 (6), P2, Y1 (7), PY1 (8), PY2 and PY3 (9) will be reported elsewhere[15]. Medium 1 was first used for early biosynthetic studies[16]. Although production of kinamycins is far greater in this medium than in any

Fig. 7. Structures of P1 (6) and Y1 (7), minor metabolites of *Streptomyces murayamaensis*.
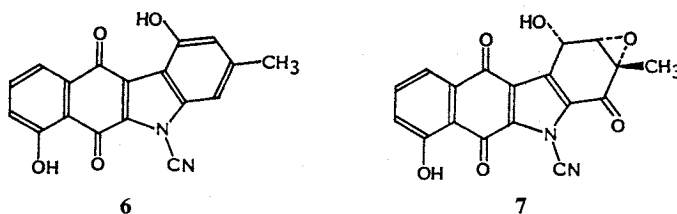

Table 5. Yields of metabolites isolated from four media.

Metabolite	Yield (mg/liter)			
	Medium 1 (Glycerol 2%, soybean meal 2%, 48 hours)	Medium 2 (Glycerol 3%, asparagine 0.1%, 48 hours)	Medium 3 (Glycerol 3%, $(NH_4)_2SO_4$ 10 mm, 26 hours)[a]	Medium 4 (Oatmeal, trace metals, 60 hours)
G (5)	5.0	6.9	1.8	5.5
P1 (6)	Trace[b]	2.8	1.1	15
MQ	5.0	2.8	2.9	Trace
P2	Trace	Trace	0.4	6.0
Y1 (7)	Trace	Trace	0.1	12
Kinamycins A (1) and B (2)+UK	63	41	41	30
Kinamycin D (4)	206 (kinamycins D and C mix)	83[c]	83[c]	99
PY1 (8)	Trace	Trace	0.1	Trace
PY2	3.0	Trace	9.9	9.4
PY3 (9)	Trace	Trace	0.6	14.5
Deacetylkinamycin (10)	—	—	Trace	—

  [a] At 35 hours TLC analysis indicates P1, P2 and Y1 have disappeared, while G and MQ have increased.
  [b] Trace: <0.05 mg/liter.
  [c] Pure kinamycin D.

  [†] One reviewer considers the evidence to be sufficient for reclassification.

Table 6. Antibacterial activities of *Streptomyces murayamaensis* metabolites.

Test organism	MIC (μg/ml)											
	KA[a]	KB[a]	KD[a]	PY1	PY3	DAK[a]	Y1	P1	G	MQ	PY2	P2
Micrococcus luteus ATCC 9341	1.0	0.2	5.0	1.0	1.0	1.0	1.0	5.0	>100	0.2	>50	12.5
Staphylococcus aureus ATCC 25923	1.0	0.2	1.0	0.2	0.2	0.2	1.0	5.0	>100	>100	>50	>25
Bacillus circulans OSU2	0.2	5.0	0.05	0.05	0.02	0.05	5.0	5.0	>100	100	>50	12.5
Streptococcus faecalis ATCC 29212	1.0	0.2	0.2	0.2	0.2	0.2	1.0	10	>100	>100	>50	>25
Bacillus subtilis ATCC 6633	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.05	5.0	>100	25	>50	1.0
Pseudomonas aeruginosa ATCC 25619	>100	>100	25	25	25	25	25	>25	>100	>100	>50	>25
Proteus vulgaris OSU1	>100	5.0	25	25	25	5.0	5.0	>25	>100	>100	>50	>25
Klebsiella pneumoniae "A"AD	>100	5.0	25	5.0	5.0	5.0	5.0	>25	>100	>100	>50	>25
Serratia marcescens ATCC 13880	>100	>100	5.0	25	100	25	25	>25	>100	>100	>50	>25
Escherichia coli ATCC 10536	>100	5.0	25	5.0	25	25	5.0	>25	>100	>100	>50	>25

Bacterial strains were grown on Difco Mueller-Hinton agar.

[a] Activities of KA, KB, KD (kinamycins A, B and D) and DAK (deacetylkinamycin) were previously determined by ŌMURA *et al.*[3].

other, the product is a mixture of kinamycins C and D which can be separated by preparative HPLC[16]. Kinamycin C is not produced in medium 2 or 3. Deacetylkinamycin (DAK; 10) had been obtained by hydrolysis of kinamycins A~D[3]; it accumulated sufficiently in medium 3 for clear identification and can be considered a natural product.

Antibacterial Activity

The activities of kinamycins A~D and deacetylkinamycin have been determined previously by ŌMURA et al.[3]. The activities of these products were determined along with those of the new metabolites for comparison (Table 6). Some of the minor metabolites had no detectable antibacterial activity (G, PY2). Murayaquinone had no activity against Gram-negative bacteria, very weak activity against Gram-positive bacteria (except for *Micrococcus luteus*). P1 and P2 were inactive against Gram-negative bacteria but showed some activity toward Gram-positive bacteria. The activity spectra of PY1, PY3 and Y1 were similar to those of the kinamycins.

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