NOVEL ANTIBIOTICS NAPYRADIOMYCINS
PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY

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Novel antibacterial antibiotics napyradiomycins A, B1, B2, B3, C1 and C2 have been isolated from the culture broth of *Chainia rubra* MG802-AF1. In this paper, taxonomy of the producer, and production, isolation, physico-chemical properties and biological activities of napyradiomycins are reported. They contain the naphtopyran chromophore and halogens, and inhibit the growth of Gram-positive bacteria including drug-resistant strains.

In the course of screening for new antibiotics from *Actinomycetes*, we found new antibiotics, which inhibited the growth of Gram-positive bacteria and which were produced by *Chainia rubra* MG802-AF1. We named them napyradiomycins A, B1, B2, B3, C1 and C2. In this paper, the production, isolation, physico-chemical properties and biological activities of napyradiomycins are described together with the taxonomy of the napyradiomycins-producing strain.

Taxonomy of the Napyradiomycins-producing Strain

Strain MG802-AF1 was isolated from a soil sample collected in Niigata Prefecture, Japan. Morphological and physiological properties of the organism were examined according to SHIRLING and GOTTLIEB[1]; several other tests were also used.

Strain MG802-AF1 produces aerial mycelia forming hook to spiral chains of spores with more than 10 spores per chain. The spores are 0.4~0.5 by 0.6~0.7 μm in size with very short spines on surface. The sclerotia are observed abundant on calcium - malate agar and are 5~50 μm in size. Aerial mass color of the colony is white to brownish white. Growth color is pale orange to pale reddish orange on oatmeal agar and pale yellow, pale brown to grayish brown on various media. Melanoid pigments are not formed, but a faint orange or reddish brown pigment is produced in most media.

The whole-cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid. Based on its characteristics, strain MG802-AF1 is considered to belong to the genus *Chainia*. Twelve species of *Chainia* were listed in Approved Lists of Bacterial Names[2], and among them eleven species have been described in ISP reports[3] as *Streptomyces*. Among the known species of *Chainia* and *Streptomyces*, *C. rubra* is recognized to be similar to the strain MG802-AF1 except for the utilization of raffinose as shown in Table 1. This difference is not sufficient to designate the strain MG802-AF1 as a new species. Therefore, the strain is considered to be the member of *C. rubra*. Strain MG802-AF1 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession number of FERM P-8022.

Production and Isolation

The strain *C. rubra* MG802-AF1 was precultured in a 500-ml Erlenmeyer flask containing 110 ml

Table 1. Comparison of taxonomic characteristics of strain MG802-AF1 and *Chainia rubra*.

	MG802-AF1	*Chainia rubra* IMC A-012 (ATCC 177)
Spore chain morphology	Hooks - spirals	Hooks - spirals
Spore surface	Spiny with very short spines	Smooth (?) or spiny (?) with very short spines
Shape and size of spores	Spherical or subspherical $0.4\sim0.5\times0.6\sim0.7$ μm	Spherical $0.5\sim0.6\times0.6\sim0.7$ μm
Sclerotia	Abundant	Abundant
Aerial mass color	White to brownish white	White to brownish white
Color of vegetative growth	Pale orange to pale reddish orange on oatmeal agar	Pale orange to pale reddish orange on oatmeal agar
	Pale yellow, pale brown to grayish brown on various media	Pale yellowish brown, pale brown to grayish red brown on various media
Soluble pigment	Faint orange or reddish brown	Faint orange or reddish brown
Melanin formation	Negative	Negative
Liquefaction of gelatin	Positive	Positive
Hydrolysis of starch	Positive	Positive
Coagulation of skim milk	Positive	Positive
Peptonization of skim milk	Positive	Positive
Nitrate reduction	Positive	Positive
Carbon utilization		
D-Glucose	+	+
L-Arabinose	+	+
D-Xylose	+	+
D-Fructose	+	+
Sucrose	−	−
Inositol	−	−
L-Rhamnose	+	+
Raffinose	+	−
D-Mannitol	+	+

+ Utilized, − not utilized.

of medium described in the paragraph of Experimental on a rotary shaker at 27°C for 5 days. It (3 ml) was inoculated into 500-ml Erlenmeyer flask containing 110 ml of the same medium and shake-cultured at 27°C for 3 days.

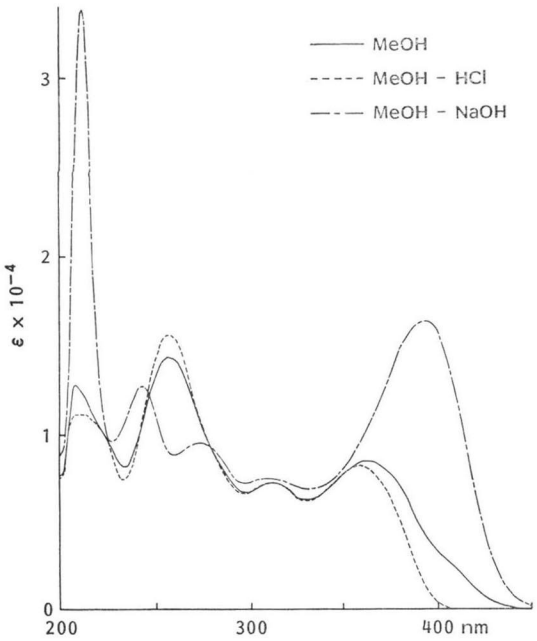
The culture filtrate was adjusted to pH 8.0 and extracted with butyl acetate. The mycelia were extracted with methanol. After concentrating the extract, the napyradiomycins were extracted with butyl acetate at pH 8.0. The extracts of the culture filtrate and the mycelia were combined and concentrated under reduced pressure to give a brownish oil. The residual oil was dissolved in toluene and charged on silica gel column. After washing the column with toluene, the napyradiomycins were eluted with toluene - ethyl acetate (50: 1). The active eluate was concentrated, dissolved in methanol and subjected to Sephadex LH-20 column chromatography using methanol. Napyradiomycin A was thus obtained.

The fractions containing A were concentrated and subjected to silica gel TLC with chloroform - methanol (40 : 1). A was detected by UV light and its band was extracted with methanol. The extract was concentrated to dryness. The oily material thus obtained contained Na^+. In order to obtain the metal free A, it was extracted with the mixture of chloroform and water at pH 3.0. The chloroform layer was concentrated and A was further purified by Sephadex LH-20 chromatography with methanol. The active eluate was concentrated to give the yellow brownish powder of pure A.

Table 2. Physico-chemical properties of napyradiomycins.

	A	B1	B2	B3	C1	C2
Appearance	Yellow brownish powder	Yellow brownish powder	Pale yellow needles	Yellow brownish powder	Yellow brownish powder	Yellow brownish powder
Anal Found	C 61.44, H 6.41, O 16.31, Cl 15.16				C 61.82, H 5.82, O 16.36	
Calcd	C 62.37, H 6.28, O 16.62, Cl 14.73				C 62.64, H 5.89, O 16.69, Cl 14.79	
FD-MS (*m/z*)	480, 482, 484	514, 516, 518, 520	478, 480, 482	558, 560, 562, 564	478, 480, 482	512, 514, 516, 518
HR-MS Found	480.1464 (M^+)	478.1318 (M^+-HCl)		522.0805 (M^+-HCl)		512.0920 (M^+)
Calcd	480.1468	478.1312		522.0807		512.0922
Molecular formula	$C_{25}H_{30}O_5Cl_2$	$C_{25}H_{29}O_5Cl_3$	$C_{25}H_{23}O_5Cl_2$	$C_{25}H_{29}O_5Cl_2Br$	$C_{25}H_{28}O_5Cl_2$	$C_{25}H_{27}O_5Cl_3$
Optical rotation	$[\alpha]_D^{27}$ +41° (*c* 0.5, EtOH)	$[\alpha]_D^{27}$ −96° (*c* 0.5, MeOH)	$[\alpha]_D^{22}$ −146° (*c* 0.5, EtOH)	$[\alpha]_D^{29}$ −85° (*c* 0.5, MeOH)	$[\alpha]_D^{27}$ +4.8° (*c* 0.5, EtOH)	$[\alpha]_D^{21}$ +72° (*c* 0.5, EtOH)
MP (°C)	33~38	115~121	208~212 (dec)	105~115	110~120	81~90
UV λ_{max}^{MeOH} nm (log ε)	205 (4.25), 251 (4.14), 270 (sh 4.04), 295 (sh 3.87), 360 (3.80), 400 (sh 3.24)	204 (4.03), 252 (4.20), 270 (sh 4.00), 300 (3.88), 360 (3.86), 400 (sh 3.29)	206 (4.11), 220 (sh 4.01), 256 (4.16), 280 (sh 3.96), 312 (3.86), 362 (3.92), 400 (sh 3.53)	204 (4.13), 251 (4.19), 270 (sh 4.02), 298 (3.97), 363 (3.91), 400 (sh 3.59)	205 (4.22), 274 (4.23), 320 (sh 3.78), 343 (3.82)	206 (4.22), 271 (4.23), 340 (3.84)
$\lambda_{max}^{MeOH-HCl}$ nm (log ε)	205 (4.23), 250 (4.18), 270 (sh 4.07), 359 (3.81)	204 (3.99), 251 (4.24), 270 (sh 4.02), 305 (3.81), 357 (3.85)	208 (4.04), 220 (sh 4.01), 256 (4.19), 311 (3.86), 357 (3.91)	205 (4.09), 252 (4.27), 273 (sh 4.02), 305 (3.85), 358 (3.88)	206 (4.20), 275 (4.25), 345 (3.84)	208 (4.22), 272 (4.25), 340 (3.85)
$\lambda_{max}^{MeOH-NaOH}$ nm (log ε)	208 (4.54), 263 (3.99), 298 (4.18), 385 (4.00)	206 (4.53), 245 (sh 3.94), 260 (4.00), 296 (4.13), 383 (4.08)	209 (4.53), 241 (4.10), 272 (3.97), 309 (3.87), 393 (4.21)	207 (4.53), 245 (sh 3.97), 261 (4.03), 296 (4.15), 383 (4.09)	208 (4.54), 311 (4.28), 397 (3.99)	209 (4.49), 259 (3.98), 311 (4.18), 404 (4.05)
IR (KBr) cm^{-1}	3400, 2920, 1700, 1640, 1610, 1380, 1250, 1070, 870, 730	3430, 2990, 1710, 1620, 1380, 1260, 1090, 1020, 880, 760	3400, 2980, 1710, 1620, 1370, 1300, 1260, 1180, 1150, 1050, 870	3400, 2990, 1700, 1620, 1370, 1260, 1090, 1020, 880, 740	3400, 2930, 1700, 1640, 1600, 1430, 1380, 1280, 1080, 990, 710	3400, 2930, 1700, 1640, 1600, 1440, 1390, 1360, 1290, 1080, 990, 800

Fig. 1. UV spectra of napyradiomycin B2.



The fractions containing napyradiomycins except A were concentrated and subjected to a chromatography on silica gel TLC developed with chloroform - methanol (40:1). The bands of napyradiomycins B and C detected by UV light were cut and eluted with methanol. Each eluate was dried and extracted with chloroform at pH 3.0. B thus obtained was further separated to B1, B2 and B3 by HPLC with methanol - water (85:15) (see Experimental). B1, B2 and B3 containing fractions were concentrated and chromatographed on Sephadex LH-20 with methanol. B1 and B3 were obtained as yellow brownish powder. B2 was obtained as pale yellow needles from methanol. C was further separated to C1 and C2 by Sephadex LH-20 chromatography with methanol. After the concentration of the eluates, pure C1 and C2 were obtained as yellow brownish powder.

Physico-chemical Properties

The napyradiomycins are soluble in methanol, ethyl acetate and chloroform but insoluble in water and hexane. They show positive color reactions with $KMnO_4$, anisaldehyde-H_2SO_4 and Gibbs reagent (A, B1, B2, B3 and C2 on TLC showed gray spots and C1 showed orange). Other physico-chemical properties are shown in Table 2. The UV and IR spectra of B2 are shown in Figs. 1 and 2. The IR

Fig. 2. IR spectrum of napyradiomycin B2 (KBr).

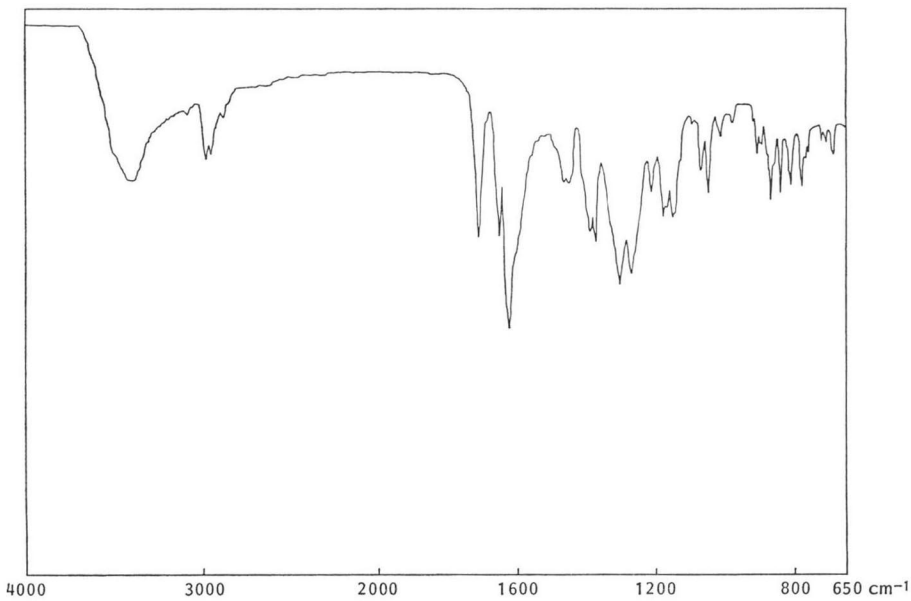


Table 3. Antimicrobial activities of napyradiomycins.

Test organisms	MIC (μg/ml)					
	A	B1	B2	B3	C1	C2
Staphylococcus aureus FDA 209P	1.56	1.56	6.25	0.78	12.5	25
S. aureus Smith	3.12	1.56	6.25	1.56	25	50
S. aureus MS8710*	3.12	1.56	6.25	0.78	12.5	50
S. aureus MS9610**	3.12	1.56	6.25	0.78	12.5	50
Micrococcus luteus FDA 16	3.12	1.56	6.25	1.56	12.5	25
M. luteus IFO 3333	1.56	1.56	3.12	1.56	12.5	50
M. luteus PC I1001	3.12	3.12	12.5	1.56	12.5	50
Bacillus anthracis	1.56	0.78	3.12	0.78	12.5	12.5
B. subtilis NRRL B-558	3.12	1.56	6.25	1.56	12.5	25
B. subtilis PCI 219	3.12	1.56	6.25	1.56	12.5	50
B. cereus ATCC 10702	3.12	1.56	6.25	1.56	12.5	25
Corynebacterium bovis 1810	1.56	1.56	6.25	1.56	12.5	12.5
Escherichia coli NIHJ	>100	>100	>50	>100	>100	>50
E. coli K-12	>100	>100	>50	>100	>100	>50
E. coli ML1629	>100	>100	>50	>100	>100	>50
Shigella dysenteriae JS11910	>100	>100	>50	>100	>100	>50
S. flexineri 4bJS11811	>100	>100	>50	>100	>100	>50
S. sonnei JS11746	>100	>100	>50	>100	>100	>50
Salmonella typhi T-63	>100	>100	>50	>100	>100	>50
S. enteritidis 1891	>100	>100	>50	>100	>100	>50
Proteus vulgaris OX19	>100	>100	>50	>100	>100	>50
P. mirabilis IFM OM-9	>100	>100	>50	>100	>100	>50
P. rettgeri GN311	>100	>100	>50	>100	>100	>50
P. rettgeri GN466	>100	>100	>50	>100	>100	>50
Serratia marcescens	>100	>100	>50	>100	>100	>50
Pseudomonas aeruginosa A3	100	>50	>25	>50	>50	>25
Klebsiella pneumoniae PCI 602	>100	>100	>50	>100	>100	>50
Mycobacterium smegmatis ATCC 607	12.5	1.56	6.25	3.12	25	50

Mueller-Hinton agar, 37°C.

* Resistant to penicillin, tetracycline, erythromycin and leucomycin.

** Resistant to penicillin, tetracycline, erythromycin, leucomycin, streptomycin, oleandomycin and josamycin.

spectra of the napyradiomycins were almost similar to one another. The molecular formulas of A, B1, B2, B3, C1 and C2 were established by field desorption mass spectrometry (FD-MS), high resolution mass spectrometry (HR-MS) and elemental analysis. The number of halogen atoms of the napyradiomycins were confirmed by the ratios of isotopic ion peaks of FD-MS and/or elemental analysis. The napyradiomycins have novel unique structures as will be reported in next paper.

Biological Activities

The antimicrobial activities of napyradiomycins A, B1, B2, B3, C1 and C2 are shown in Tables 3 and 4. They inhibit the growth of Gram-positive bacteria including multiple drug-resistant strains such as *Staphylococcus aureus* MS8710 and MS9610, but are not active against most Gram-negative bacteria and fungi.

The LD_{50} (ip) of A, B1, B2, B3, C1 and C2 in mice (their suspension in physiological saline was injected) were >250, 125~250, >100, >125, >250 and >100 mg/kg, respectively.

The IC_{50} of A, B1 and C1 in inhibiting the growth of L-1210 cells *in vitro* were 2.7, 2.2 and 9.2 μg/ml, respectively.

Table 4. Antimicrobial activities of napyradiomycins A, B1 and C1.

Test organisms	Medium	MIC (μg/ml)		
		A	B1	C1
Aeromonas punctata IAM 1646	a	>100	>100	>100
A. salmonecida ATCC 14174	a	>100	>50	>50
Aeromonas sp. (KT-444)	a	>100	>50	>100
Vibrio anguillarm NCMB6	a	>100	>50	>100
Pseudomonas fluorescens	a	>100	>50	>50
P. lachrymans	a	>100	>50	>50
Erwinia aroideae	a	>100	>100	>100
Candida tropicalis F-1	b	>100	>100	>100
C. pseudotropicalis F-2	b	>100	>100	>100
C. albicans 3147	b	>100	>100	>100
Candida Yu-1200	b	>100	>100	>100
C. krusei F-5	b	>100	>100	>100
Saccharomyces cerevisiae F-7	b	>100	>100	>100
Cryptococcus neoformans F-10	b	>100	>100	>100
Helminthosporium oryzae	b	>100	>50	>100
Pyricularia oryzae	b	100	>100	>100
Pellicularia filamentosa Sasakii	b	100	>50	>50
Xanthomonas citri	b	>100	>100	>100
X. oryzae	b	100	50	>100
Aspergillus niger F-16	b	>100	>100	>100
Trichophyton asteroides 429	b	100	>100	>100
T. mentagrophytes	b	>50	>100	>100

a; Mueller-Hinton agar, 27°C, b; nutrient agar + glucose 1%, 27°C.

Experimental

Production and Isolation of Napyradiomycins

C. rubra MG802-AF1 grown on a yeast - starch agar slant was inoculated into 500-ml Erlenmeyer flasks containing 110 ml of the medium [Bacto-Soytone (Difco) 1.0%, galactose 2.0%, corn steep liquor 0.5%, dextrin 2.0%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, silicon oil (Shin-Etsu Chemical Industry, KM-70) 0.03%, pH 7.4] and shake-cultured on a rotary shaker (180 rpm, 8 cm) at 27°C for 5 days. Each 3 ml of the cultured broth was inoculated into 500-ml Erlenmeyer flasks containing 110 ml of the same medium and cultured for 3 days similarly. Antibacterial activity was assayed by cup-assay or paper-disk method using *Micrococcus luteus* IFO 3333.

The cultured broth was filtered and the filtrate (7.1 liters, pH 7.4) adjusted to pH 8.0 with 1 N NaOH was extracted with equal volume of BuOAc. The mycelium mass was extracted with 2.0 liters of MeOH, and the extract was concentrated to dryness under reduced pressure. To this residue, 2.0 liters of BuOAc and 2.0 liters of H_2O were added. After extracting under pH 8.0 (1 N NaOH) by shaking, BuOAc extracts of the culture filtrate and mycelia were combined and concentrated under reduced pressure to give 1.5 g of brownish oil. The residual oil was dissolved in the minimal volume of toluene and poured on a column of silica gel (Merck, 7734, 65 g). The column was washed with 0.5 liter of toluene and then eluted with 1.5 liters of toluene - EtOAc (50: 1). Active fractions were combined and concentrated to dryness under reduced pressure to give 430 mg of brownish oil. The residue was dissolved in MeOH and charged on a 300-ml column of Sephadex LH-20 (Pharmacia, 2.1×87 cm). The column was developed with MeOH. Napyradiomycin A was first eluted to separate from the mixture of B and C. They were detected by UV light on silica gel TLC (Merck, 5715, developed with $CHCl_3$ - MeOH, 40 : 1). The fractions containing A (Rf 0.47) was collected and concentrated to dryness under reduced pressure to give 280 mg of brownish oil. Sixty-six mg of the mixture of B and C was obtained similarly as a brownish oil. A (55 mg) was developed on 5 plates (20×20 cm) of silica gel TLC (Merck, 5715) with $CHCl_3$ - MeOH (40 : 1), and A detected by UV light was eluted with MeOH and concentrated

to dryness under reduced pressure. To this dried material, $CHCl_3$ and H_2O were added, pH was adjusted to 3.0 and the mixture was shaken. The $CHCl_3$ layer was dehydrated with anhydrous Na_2SO_4 and concentrated under reduced pressure to give 52.1 mg of metal free A. The crude A was dissolved in MeOH and further purified by Sephadex LH-20 column (200 ml, 2.1 × 58 cm) chromatography developed with MeOH. The fractions showed the single spot on a silica gel TLC developed with $CHCl_3$ - MeOH (40:1) were collected and concentrated under reduced pressure to give 45.2 mg of yellow brownish powder of pure A.

The mixture (66.0 mg) of B and C described above was subjected to 4 plates of silica gel TLC (Merck, 5715, 20×20 cm) with $CHCl_3$ - MeOH (40:1). After B (Rf 0.40~0.44) and C (Rf 0.50~0.52) detected by UV light were eluted with MeOH, the metal free B (35.2 mg) and C (22.3 mg) were obtained by $CHCl_3$ extraction at pH 3.0 similarly as in the case of A.

B (35.2 mg) was charged on HPLC (Macherey-Nagel Co., Nucleosil $5C_{18}$, 20×300 mm) and eluted with MeOH - H_2O (85: 15) at the flow rate of 7.5 ml/minute. B1, B2 and B3 were eluted at 45, 30 and 63 minutes, respectively. Each fraction was concentrated under reduced pressure and rechromatographed using Sephadex LH-20 column (200 ml, 2.1×58 cm) to yield 6.8 mg of B1, 1.5 mg of B2 and 4.3 mg of B3.

C (22.3 mg) obtained above was dissolved in MeOH, and rechromatographed on Sephadex LH-20 column (200 ml, 2.1×58 cm). C1 and C2 were eluted successively with MeOH to yield 13.2 mg of C1 and 4.1 mg of C2.

Melting point was measured by Micro melting point apparatus MP-S3 (Yanagimoto Seisakusyo Co., Japan). The mass spectra (FD-MS and HR-MS) were determined by Hitachi M-80H spectrometer.

The UV and IR spectra were measured by Hitachi 220S and 260-10 Spectrophotometer, respectively. Optical rotations were taken by Perkin-Elmer 241 Polarimeter using micro-cell (light path 10 cm).

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