Pyridindolols K1 and K2, New Alkaloids from *Streptomyces* sp. K93-0711

YONG-PIL KIM, SATOSHI TAKAMATSU, MASAHIKO HAYASHI,
KANKI KOMIYAMA and SATOSHI ŌMURA*

The Kitasato Institute,
5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

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Two new alkaloids with the β-carboline skeleton, pyridindolols K1 ($C_{18}H_{18}N_2O_5$) and K2 ($C_{16}H_{16}N_2O_4$), were isolated from the culture broth of *Streptomyces* sp. K93-0711. The structure of pyridindolols were established by spectroscopic investigations and chemical transformations. Pyridindolol K2 inhibited the adhesion of HL-60 cells to LPS-activated HUVEC monolayer (IC_{50} = 75 μg/ml).

Chemical screening for secondary metabolites of microbial origin has led to the discovery of natural products with different types of structures and biological activities[1]. In the course of selective screening for amines using special staining reagents on TLC, new β-carboline derivatives, termed pyridindolols K1 (**1**) and K2 (**2**), were isolated from the culture broth of *Streptomyces* sp. K93-0711 together with a known alkaloid, pyridindolol (**3**) reported as an β-galactosidase inhibitor (Fig. 1). This paper describes the isolation, physico-chemical properties, structural determination including the absolute configuration, and biological characteristics of **1** and **2**.

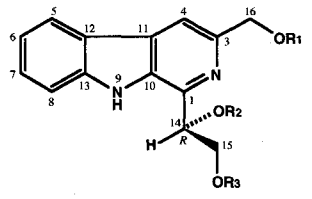
Results and Discussion

Microbial strain K93-0711 was originally obteined in a screening program aimed at the inhibitor of interleukin-6 activity, madindoline. Taxonomic studies, fermentation of the producing strain and extraction of the cultured broth were reported in a previous paper[2]. As described previously, *Streptomyces* sp. K93-0711 was cultured in a jar fermenter for 4 days. Isolation of **1**~**3** under guidance of the purple-colored reaction with Ehrlich reagent on TLC, is shown in Fig. 2. Finally, three alkaloid compounds (**1**~**3**) were isolated at yields of 45 mg, 300 mg and 32 mg, respectively. In the UV spectra (Fig. 3), three compounds showed similar spectra. The characteristic absorption maxima near 346, 290 and 238 nm suggested that these compounds might be β-carboline derivatives[3]. Among the three alkaloids, **3** was identified as pyridindolol by spectroscopic analyses[4,5]. The physico-chemical properties of **1** and **2** are summarized in Table 1.

Compound **1** was isolated as a colorless oil. The molecular formula of **1** was determined as ($C_{18}H_{18}N_2O_5$) by the HR-FAB-MS. The IR absorptions at 1740 and 1728 cm^{-1} of **1** showed the presence of ester groups. In the ^{13}C NMR spectrum of **1** (Table 2), the chemical shifts of carbon signals showed a similar pattern to those of **3**, except for two sets of acetoxy groups observed at δ 171.0 *s*, δ 21.1 *q*, δ 172.9 *s* and δ 21.1 *q*. In the ^1H NMR spectrum of **1** (Table 3), two methyl signals observed at δ 2.10 (3H, *s*) and δ 2.15 (3H, *s*) were also appeared as singlets when compared with those of **3**. From these results, compound **1** was presumed to be a diacetate of pyridindolol (**3**). In the HMBC spectrum of **1** (Fig. 4), the connections between C-16 and C-18 *via* C-17, and C-15 and C-20 *via* C-19 were clarified. Therefore, the structure of **1** was determined as 15,16-*O*-diacetylpyridindolol as shown in Fig. 1.

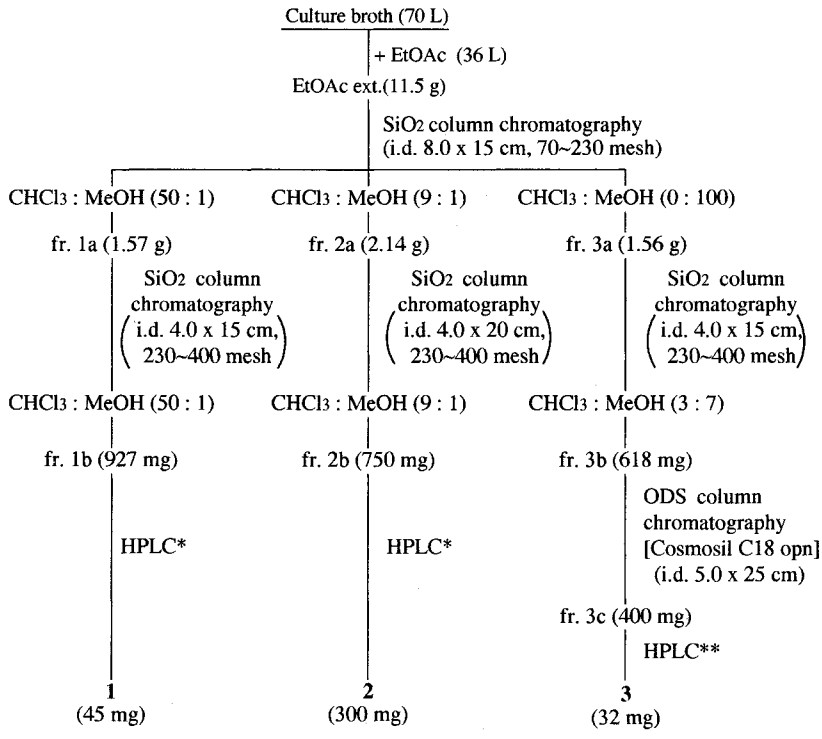
The molecular formula of **2** was assigned as ($C_{16}H_{16}N_2O_4$) based on the HR-FAB-MS. Comparison of molecular formulas of **1** and **2** indicated that **2** might be a monoacetate of pyridindolol (**3**). The IR spectrum (1741 cm^{-1}), ^{13}C NMR (δ 172.7 *s*, δ 20.9 *q*) and ^1H NMR (δ 2.25, 3H, *s*) also showed that compound **2** was

Fig. 1. Strucures of **1**~**5**.



1: R_1=-CO-CH_3, R_2=H, R_3=-CO-CH_3
2: R_1=-CO-CH_3, R_2=H, R_3=H
3: R_1=H, R_2=H, R_3=H
4: R_1=-CO-CH_3, R_2=-CO-CH_3, R_3=-CO-CH_3
5: R_1=-CO-CH_3, R_2=-Bz-dma, R_3=-Bz-dma
(Bz-dma: *p*-Dimethylaminobenzoyl)

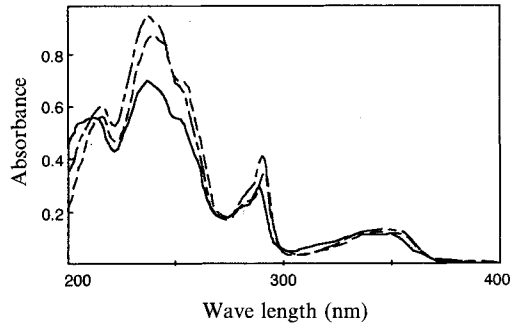
Fig. 2. Extraction and isolation of **1**, **2** and **3**.



HPLC (Senshu Pak Pegasil ODS; i.d. 2 x 25 cm; Detection, UV at 210 nm, flow rate; 7 ml/minute) were performed with CH_3CN : H_2O (4 : 6, v/v)* or MeOH : H_2O (4 : 6, v/v)**.

Fig. 3. UV spectra of **1**, **2**, and **3** (*c* 0.001%, MeOH).

1: ——, **2**: – – –, **3**: – – – –.



a monoacetate of **3** (Tables 1, 2 and 3). The carbon signal of **2** at δ 68.4 (*t*, C-16) shifted downfield in the range of 2.3 ppm compared with that of **3**. The proton signal at C-16 (δ 5.32, 2H, *br.s*) of **2** also shifted downfield (Δ 0.58 ppm). Final elucidation of the structure of **2** was performed using the HMBC experiment. The connectivity between C-4 and C-18 was clearly demonstrated through analysis of the HMBC spectrum of **2** (Fig. 4). From these results, the structure of **2** was determined as 16-*O*-acetylpyridindolol (**2**) (Fig. 1).

The absolute structures of **1** and **2** were determined by chemical transformation to the acetate and by the benzoate chirality method. Acetylation of **1**, **2** and **3** with acetic anhydride in pyridine at room temperature gave triacetate of **3**. All spectra (mass, IR and ^1H NMR) were in good agreement with those reported for pyridindolol (**3**)[4,5]. On the other hand, compound **2** was transformed to 14,15-*O*-di(*p*-dimethylaminobenzoate) (**5**)[6]. In the CD spectrum, **5** showed a negative Cotton effect at 353 nm ($\Delta\varepsilon$ −1.6) and positive Cotton effects at 303 nm ($\Delta\varepsilon$+6.3), 267 nm ($\Delta\varepsilon$+0.74) and 233 nm ($\Delta\varepsilon$+1.4). These effects showed that the stereochemistry of **2** was 14*R*. Therefore, the absolute structures of **1** and **2** were the same as that of **3**, the stereochemistry of which was clarified by X-ray crystallographic techniques using the bromine derivative[4].

As the result of screening for amines, we isolated two new alkaloids, named pyridindolol K1 (**1**) and K2 (**2**), with the known alkaloid pyridindolol (**3**). Compound **3**, a metabolite of actinomycetes, was found to be an inhibitor of bovine liver β-galactosidase in acidic conditions[7,8]. Among the three alkaloids, **2** showed

Table 1. Physico-chemical data of **1** and **2**.

	1	**2**
Appearance	Colorless oil	Colorless needles
Molecular weight	342	300
Molecular formula	$C_{18}H_{18}N_2O_5$	$C_{16}H_{16}N_2O_4$
MP	—	123 ~ 124 °C
Positive FAB-MS (*m/z*)	343 $(M+H)^+$	301 $(M+H)^+$
Positive HR-FAB-MS (*m/z*)	Obsd. 343.1215 ($C_{18}H_{19}N_2O_5$)	Obsd. 301.1206 ($C_{16}H_{17}N_2O_4$)
	Calcd. 343.1216	Calcd. 301.1188
$[\alpha]_D^{20}$ (MeOH)	− 16.0° (*c*. 0.23)	− 35.0° (*c*. 0.40)
UV λ_{max}^{MeOH} nm (log ε)	356 sh. (3.28), 346 (3.35), 290 (3.75), 282 sh. (3.62), 262 sh. (3.87), 254 sh. (4.03), 247 sh. (4.08), 238 (4.14), 215 (4.04), 205 sh. (4.02).	356 sh. (3.48), 343 (3.53), 291 (4.02), 282 sh. (3.84), 262 sh. (4.12), 254 sh. (4.32), 245 sh. (4.39), 238 (4.42), 215 (4.23).
IR ν_{max}^{KBr} cm^{-1}	3410, 2927, 1740, 1728, 1628, 1248.	3388, 3282, 1741, 1628, 1363, 1250.
Color reaction		
50% H_2SO_4 + Δ	Positive	Positive
Iodine	Positive	Positive
Dragendorff's reagent	Positive	Positive
Ehrlich's reagent + Δ	Positive	Positive
Ninhydrin reagent	Negative	Negative

Table 2. ^{13}C NMR chemical shifts of **1**, **2** and **3**.

No.	*M*	1*	2**	3**
1.	*s*	140.1	145.7	149.4
3.	*s*	143.3	144.1	142.8
4.	*d*	113.7	114.3	112.1
5.	*d*	121.8	122.5	122.4
6.	*d*	120.3	120.7	120.5
7.	*d*	128.8	129.6	129.5
8.	*d*	112.0	113.1	112.9
10.	*s*	132.4	134.7	134.3
11.	*s*	130.4	131.6	131.9
12.	*s*	121.2	112.0	122.2
13.	*s*	140.8	142.8	145.0
14.	*d*	71.6	76.1	75.9
15.	*t*	70.4	67.0	67.0
16.	*t*	67.4	68.4	66.1
17.	*s*	171.0	172.7	—
18.	*q*	21.1	20.9	—
19.	*s*	172.9	—	—
20.	*q*	21.1	—	—

M: Multiplicity, *in $CDCl_3$, **in CD_3OD.

inhibitory activity on the adhesion of HL-60 cells to LPS-activated HUVEC monolayer ($IC_{50}=75\,\mu g/ml$), but **1** and **3** did not show at the concentration of 200 μg/ml. Compounds **1** and **2** showed no antimicrobiological activities against Gram-positive and Gram-negative bacteria, fungi or yeast at a concentration of 1 mg/ml. Growth inhibitory activity of pyridindolols was examined against mammalian cells *in vitro*. Although the cells were continuously exposed to the compounds **1** and **2** for 3 days, the growth of B16 melanoma, P388 leukemia, and Shionogi carcinoma (SC-115) was not affected at a concentration of 100 μg/ml of **1** or **2** (data not shown). No acute toxicity was observed when **1** or **2** was injected intraperitoneally into BDF1 mice at 50 mg/kg for 5 days (data not shown). Therefore, it is of interest to consider the structure-activity relationships between pyridindolols and anti-adhesion activity. Although **2** showed the inhibitory activity of cell adhesion, the activity on the other adhesion molecules will be examined.

Experimental

General

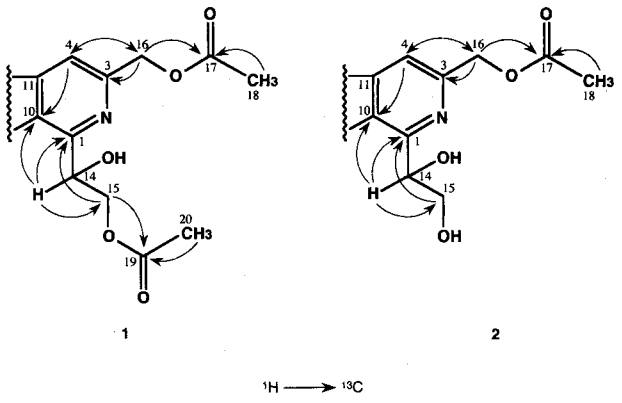
UV spectra were recorded on a Shimadzu model UV-160A spectrophotometer. IR spectra were obtained with a Horiba model Fourier transform infrared spectrophotometer FT-210. MS were obtained with a JEOL model JMS DX-300 mass spectrometer. ^1H (270 MHz) and ^{13}C (67.8 MHz) NMR spectra were recorded on a JEOL JNM-EX 270. CD spectra were

Table 3. ^{1}H NMR chemical shifts of **1**, **2** and **3**.

No.	1*	2**	3**
4.	7.93 (1H, *s*)	8.04 (1H, *s*)	7.97 (1H, *s*)
5.	8.07 (1H, *d*, *J*=7.9)	8.15 (1H, *d*, *J*=8.0)	8.05 (1H, *d*, *J*=7.9)
6.	7.23 (1H, *td*, *J*=7.9, 4.0)	7.23 (1H, *t*, *J*=7.5)	7.13 (1H, *td*, *J*=7.9, 1.3)
7.	7.51 (1H, *br. d*, *J*=3.6)	7.53 (1H, *dd*, *J*=8.0, 1.0)	7.42 (1H, *td*, *J*=7.9, 1.3)
8.	7.51 (1H, *br. d*, *J*=3.6)	7.60 (1H, *d*, *J*=8.0)	7.50 (1H, *br. d*, *J*=7.9)
9.	9.67 (1H, *br. s*)	–	–
14.	5.35 (1H, *dd*, *J*=7.6, 2.0)	5.20 (1H, *br. s*)	5.10 (1H, *dd*, *J*=6.3, 4.6)
15.	4.81 (1H, *dd*, *J*=12.0, 2.0)	3.97 (2H, *br. s*)	3.88 (1H, *dd*, *J*=11.2, 4.6)
	4.10 (1H, *dd*, *J*=7.9)		3.82 (1H, *dd*, *J*=11.2, 6.3)
16.	5.31 (2H, *s*)	5.32 (2H, *br. s*)	4.74 (2H, *s*)
18.	2.10 (3H, *s*)	2.25 (3H, *s*)	–
20.	2.15 (3H, *s*)	–	–

M: Multiplicity (*J* value in Hz), * in $CDCl_3$, ** in CD_3OD.

Fig. 4. Key ^{1}H-^{13}C long range couplings detected by HMBC experiments (J=8 Hz) of **1** ($CDCl_3$) and **2** (CD_3OD).



measured on a JASCO J-720 spectropolarimeter in MeOH. Analytical HPLC was carried out with CH_3CN-H_2O (4:6, v/v) using a Senshu Pak Pegasil ODS (5 μm, i.d. 4.6×250 mm) column employing a UV monitoring system (210 nm) at a flow rate of 0.8 ml/minute. Preparative HPLC was performed using a Senshu Pak Pegasil ODS (5 μm, i.d. 20×250 mm) column with a solvent system of CH_3CN-H_2O (4:6, v/v) or CH_3OH-H_2O (4:6, v/v) at 7 ml/minute.

Acetylation of 1~3

Each compound, **1** (5.0 mg), **2** (5.2 mg) and **3** (7.0 mg), was dissolved in 1 ml of pyridine. Acetic anhydride (2 ml) was added gradually to the solution. After stirring for 24 hours at room temperature, the products were extracted with $CHCl_3$ and purified by column chromatography on silica gel with $CHCl_3$: acetone (9:1, v/v). Triacetate of **3** (**4**) was obtained with a yield of 85% (4.8 mg), 96% (6.4 mg) or 48% (5.0 mg) from **1**, **2** and **3**.

Triacetate of pyridindolol (**4**): UV λ_{max}^{MeOH} nm (log ε): 356 (3.48 sh.), 348 (3.57), 291 (4.03), 282 (3.82 sh.), 262 (4.15 sh.), 254 (4.29 sh.), 245 (4.37 sh.), 238 (4.40), 214 (4.27); $[\alpha]_D^{27}$ −27.5° (*c* 0.125, $CHCl_3$); positive HR-FAB-MS: $C_{20}H_{21}O_6N_2$ ($[M+1]^+$, *m/z* 385.1409, calcd 385.1362); ^{1}H NMR ($CDCl_3$) δ ppm: 9.20 (1H, *dd*, *J*=15.8 and 5.3 Hz, H-13), 6.75 (1H, *dd*, *J*=15.8 and 6.3 Hz, H-7), 5.95 (1H, dd, *J*=15.8 and 1.3 Hz, H-12), 5.94 (1H, *dd*, *J*=15.8 and 1.3 Hz, H-6), 5.36 (1H, *m*, H-14), 5.32 (1H, *m*, H-3), 5.27 (1H, *m*, H-8), 5.08 (1H, *m*, H-15), 5.07 (1H, *m*, H-9), 2.60 (2H, dd, *J*=5.3 and 1.0 Hz, H-2), 2.12 (6H, *s*, acetyl methyl), 1.36 (3H, *d*, *J*=10.5 Hz, 9-Me), 1.34 (3H, *d*, *J*=10.9 Hz, 3-Me), 1.28 (3H, *d*, *J*=10.2 Hz, 15-Me).

Hydrolysis of 1 and 2

Hydrolysis of **1** (5.5 mg) and **2** (8.0 mg) in 1 M NaOMe/MeOH (1 hour at room temperature) provided pyridindolol (**3**) at a yield of 80% (3.3 mg) or 77% (5.3 mg). Synthetic **3** prepared from **2** was identified as a pyridindolol by comparison of MS, IR and ^{1}H NMR data reported previously[4,5].

Benzoylation of 2[6]

The mixture of **2** (5 mg) [CD nm ($\Delta\varepsilon$), 262 (+1.9), 241 (−1.6), 223 (+1.7)], *p*-(dimethylamino)benzoic acid (3.9 mg), and tri-*n*-butylamine (10 mg) in CH_2Cl_2 (4 ml) was refluxed with 2-chloro-1-methylpyridinium *p*-toluenesulfonate (8 mg) for 3 hours. The purification of a crude reaction product by preparative TLC with $CHCl_3$: acetone (9:1, v/v) gave **5** at a yield of 40% (4 mg).

14,15-*O*-Di(*p*-dimethylaminobenzoyl)pyridindolol (**5**): positive FAB-MS: $[M+H]^+$ 595; ^{1}H NMR ($CDCl_3$) δ ppm: 10.27 (1H, *s*, H-9), 8.14 (1H, *d*, *J*=8.1 Hz, H-5), 8.00 (1H, *s*, H-4), 7.98 (4H, *d*, benzoyl *ortho* position), 7.58 (1H, *m*, H-8), 7.31 (1H, *m*, H-7), 6.67 (4H, *d*, *J*=8.9 Hz, benzoyl *meta* position), 6.06 (1H, *td*, *J*=6.6 and 2.0 Hz, H-6), 5.53 (1H, *dd*, *J*=5.6 and 3.0 Hz, H-14), 5.40 (2H, *s*, H-16), 5.08 (1H, *dd*, *J*=12.2 and 3.0 Hz,

H-15a), 4.35 (1H, *d*, $J=12.0$ and 6.9 Hz, H-15b), 3.07 (12H, *s*, *N*-methyl), 2.18 (3H, s, H-18); CD nm ($\Delta\varepsilon$), 353 (−1.6), 303 (+6.3), 267 (+0.74), 233 (+1.4).

Cell Adhesion Assay[9]

HUVEC (human umbilical vein endothelial cells) were stimulated with 50 ng/ml of LPS for 5 hours in 96-well plates. After washing two times, test materials at various concentrations were added and incubated for 10 minutes. HL-60 cells (human leukemia) were prelabeled with CFSE (carboxyfluorescein diacetate succinimyl ester) by the method of BRONNER-FRASER[10]. Then, the CFSE-labeled HL-60 cells (1.0×10^6/0.1 ml/well) suspended in RPMI medium supplemented with 10% FCS were added and incubated for 30 minutes at 37°C. After removing the fluid and non-adherent cells by centrifugation, the number of attached cells (fluorescence intensity) to HUVEC was measured by Fluoroskan II (Dainippon Pharmaceutical Co., Ltd., Osaka).

Antimicrobial Activity Tests[11]

The antimicrobial spectra of the test materials were determined using 6 mm paper disks (ADVANTEC). Test organisms were as follows: bacteria, *Staphylococcus aureus* KB 210 (ATCC 6538p), *Micrococcus luteus* KB40 (ATCC 9341), *Bacillus subtilis* KB27 (PCI 219), *Mycobacterium smegmatis* KB 42 (ATCC 607), *Escherichia coli* KB 8 (NIHJ), *Escherichia coli* KB176 (NIHJ JC-2), *Pseudomonas aeruginosa* KB 105 (PCI 602), *Xanthomonas oryzae* KB 88, *Bacteroides fragilis* KB169 (ATCC 23745), *Acholeplasma laidlawii* PG 8; fungi, *Aspergillus niger* KF 103 (ATCC 6275), *Piricularia oryzae* KF 180, *Mucor racemosus* KF 223; yeast, *Candida albicans* KF 1 and *Saccharomyces sake* KF 26. Bacteria were grown on Mueller-Hinton agar medium (Difco) and fungi or yeast were grown on potato-broth agar medium. Antimicrobial activity was observed after 24 hours of incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Cytotoxic Activity Tests[12]

Three strains of established mammalian cells were maintained in monolayers or in suspension in EAGLE's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) or RPMI 1640 medium supplemented with 10% FCS, respectively. To determine the cytotoxicity of pyridindolols, cells suspended in 200 μl of the medium were plated in a 96-well culture plate (Corning) and incubated for 24 hours at 37°C in a 5% CO_2-95% air atmosphere. Five microliters of medium containing a different concentration of pyridindolols was added to each well. After 72 hours of incubation, the cell growth was evaluated by the method of ALLEY *et al.*[13].

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