Isolation and Structure Determination of Novel Phosphatidylinositol Kinase Inhibitors, Echiguanines A and B, from Streptomyces sp.

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ISOLATION AND STRUCTURE DETERMINATION OF NOVEL PHOSPHATIDYLINOSITOL KINASE INHIBITORS, ECHIGUANINES A AND B, FROM *STREPTOMYCES* SP.

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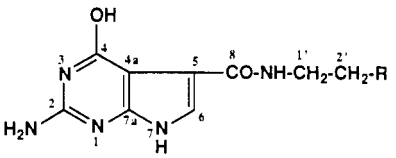
ABSTRACT.—In the course of a screening program for phosphatidylinositol kinase inhibitors, we discovered novel inhibitors, echiguanines A and B, from the culture broth of a *Streptomyces* strain MI698-50F1 (FERMP-11563). The structures of echiguanines A and B were revealed to be *N*-(2-amidinoethyl)-2-amino-4-hydroxy-7*H*-pyrrolo[2,3-*d*]pyrimidine-5-carboxamide [**1**] and *N*-(3-aminopropyl)-2-amino-4-hydroxy-7*H*-pyrrolo[2,3-*d*]pyrimidine-5-carboxamide [**2**], respectively, by nmr spectroscopy and X-ray crystallography. Echiguanines A and B inhibited phosphatidylinositol kinase of the A431 cell membrane with IC_{50}'s of 0.04 μg/ml and 0.11 μg/ml, respectively. Echiguanine A [**1**] is the most potent inhibitor of phosphatidylinositol kinase so far discovered.

A variety of mitogens (1) and oncogenes (2,3) are known to activate intracellular phosphatidylinositol turnover. For studying which phosphatidylinositol turnover is functionally significant with respect to signal transduction, specific inhibitors of the enzymes involved in the turnover should be useful. Phosphatidylinositol kinase is involved in the phosphatidylinositol turnover pathway and may be important for the regulation of phosphatidylinositol 4,5-bisphosphate levels. Therefore, we have screened inhibitors of phosphatidylinositol kinase from microbial secondary metabolites and previously isolated 2,3-dihydroxybenzoic acid (4) and toyocamycin (5).

We have isolated novel and potent inhibitors of phosphatidylinositol kinase from a *Streptomyces* strain and named them echiguanines A and B. In this paper, we describe the isolation, characterization and structure determination of echiguanines A and B.

RESULTS AND DISCUSSION

The high resolution fabms and ^{1}H- and ^{13}C-nmr data suggested that the molecular



1 $R = \overset{5'}{C}(=NH)-NH_2$

2 $R = \overset{5'}{C}H_2-NH_2$

formulas of echiguanines A and B were $C_{10}H_{13}N_7O_2$ (*m/z* 264.1227 $[M+H]^+$) and $C_{10}H_{14}N_6O_2$ (*m/z* 251.1271 $[M+H]^+$), respectively. The uv spectra of echiguanines A and B resembled each other in acidic, neutral, and alkaline solutions, indicating that they have the same chromophore. The chromophore was presumed to be 7-deazaguanine (2-amino-4-hydroxypyrrolo[2,3-*d*]pyrimidine) or 7-deazaisoguanine (2-hydroxy-4-aminopyrrolo[2,3-*d*]pyrimidine) by comparing the ^{13}C-nmr data of echiguanine A or B with those of tubercidin (6), cadeguomycin (7) and kanagawamicin (8). The results of ^{1}H and ^{13}C nmr of echiguanines A and B are compiled in Table 1.

TABLE 1. ^{1}H- and ^{13}C-nmr Data of Echiguanines A [**1**] and B [**2**] in DMSO-d_6.[a]

Position	A		B	
	δ_H	δ_C	δ_H	δ_C
2-NH_2	6.62 s	153.0 s	6.39 s	152.8 s
4-OH	9.12 s br	160.4 s	8.46 s br	160.4 s
5	—	114.1 s	—	114.3 s
6	7.24 s	122.9 d	7.23 s	122.7 d
7	9.12 s br	—	8.46 s br	—
8-NH	10.41 t ($J_{8NH,1'}$ = 12.0 Hz)	163.0 s	10.22 t ($J_{8NH,1'}$ = 11.6 Hz)	163.0 s
4a	—	95.8 s	—	95.8 s
7a	—	152.6 s	—	152.6 s
1′	3.58 dd ($J_{1',2'}$ = 13.6 Hz)	35.6 t	3.33 dd ($J_{1',2'}$ = 12.8 Hz)	35.4 t
2′	2.62 t	32.9 t	1.77 m ($J_{2',3'}$ = 15.2 Hz)	28.4 t
3′	—	168.9 s	2.82 t	37.1 t
3′-NH	11.51 s br	—	—	—
3′-NH_2	9.12 s br	—	8.46 s br	—

[a] ^{1}H chemical shifts (ppm), signal multiplicities, and coupling constants (*J* in Hz) in parentheses at 400 MHz; ^{13}C chemical shifts (ppm) and signal multipliticites at 100 MHz.

In detail, the ^{13}C-nmr signals at δ 153.0 (s) and δ 160.4 (s) in the spectrum of echiguanine A [at δ 152.8 (s) and δ 160.4 (s) of echaguanine B] revealed that -NH_2 and -OH groups link to C-2 and C-4, respectively or reversibly, of the pyrrolo[2,3-*d*]pyrimidine ring. The side chain structures of echiguanines A and B were deduced by heteronuclear multiple bond correlation (HMBC) via long-range coupling spectra. The side chain of each may be attached to the C-5 or C-6 position of the base moiety. The ^{13}C-nmr signals of C-5 and C-6 were observed at δ 114.1 and δ 122.9 of echiguanine A (at δ 114.3 and δ 122.7 of echiguanine B). Comparing these with those of sangivamycin (6), which has a -$CONH_2$ group at the C-5 position and gives signals at δ 111.3 (C-5) and δ 126.1 (C-6), suggested that the side chain of echiguanine is attached to the C-5 position. Thus, we have postulated their structures **1** and **2** as shown, and they were confirmed by X-ray crystallographic analysis.

The PLUTO (9) drawing of the molecule is shown in Figure 1. The bond lengths and the valency angles were compared with the mean values reported for seven 9-substituted guanine derivatives (10) (data not shown). A lengthening of the bonds for C-2–N-3, C-7a–C-4a, C-4–O-4, C-4a–C-5, and C-5–C-6 and shortening for N-3–C-4 were clear. These may be the consequence of the replacement of O-4 and N-5 of guanine by 4-OH and C-5 with an aliphatic structure in the echiguanine molecule.

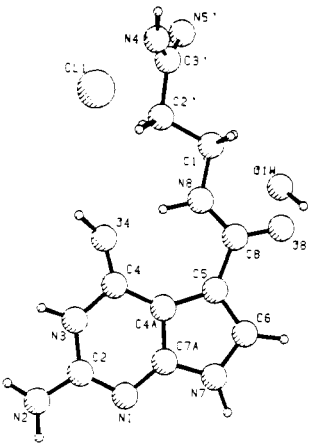


FIGURE 1. Molecular structure of echiguanine A [**1**] drawn by PLUTO.

Echiguanines A [**1**] and B [**2**] inhibited phosphatidylinositol kinase of the A431 cell membrane with IC_{50}'s of 0.04 μg/ml and 0.11 μg/ml, respectively. Structurally related compounds such as 7-deazaguanine showed only weak activity (IC_{50} 35 μg/ml), and adenine, hypoxanthine, guanine, and isoguanine did not inhibit the enzyme (IC_{50} > 100 μg/ml). Echiguanines A and B showed no antimicrobial activity. The LD_{50}'s of echiguanines A and B, when administered intravenously to mice, were >100 mg/kg.

Echiguanine A [**1**] is the most potent phosphatidylinositol kinase inhibitor ever found. It is stronger than toyocamycin (IC_{50} 3.3 μg/ml) (5), 2,3-dihydroxybenzaldehyde (IC_{50} 0.45 μg/ml) (4), quercetin (IC_{50} 1.8 μg/ml) (4), or orobol (IC_{50} 0.25 μg/ml) (4). Phosphatidylinositol kinases are classified in two types: they are phosphatidylinositol-4-kinase involved in phosphatidylinositol turnover and phosphatidylinositol-3-kinase, which is often associated with PDGF receptor (11), EGF receptor (12), and $p60^{src}$ (13). The A431 cell membrane that we used as the enzyme source has phosphatidylinositol-4-kinase (14); therefore, echiguanines are phosphatidylinositol-4-kinase inhibitors. Inhibition of phosphatidylinositol-3-kinase by echiguanines remains to be studied.

EXPERIMENTAL

MATERIALS.—A431 cells were kindly supplied by the late Dr. S. Kawai, Institute of Medical Science, University of Tokyo. [γ-^{32}P]ATP was purchased from DuPont-New England Nuclear. Si gel columns for the phosphatidylinositol kinase assay were from Analytichem International. Adenine, hypoxanthine, guanine, isoguanine, and 7-deazaguanine were obtained from Sigma Chemical Company.

PHOSPHATIDYLINOSITOL KINASE ASSAY.—Phosphatidylinositol kinase activity was measured by the method described previously (4). In brief, phosphatidylinositol, A431 cell membrane, and [γ-^{32}P]ATP with or without inhibitor were mixed in 20 mM 4-2-(hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2). The reaction mixture was incubated for 20 min at 20° and stopped by the addition of $CHCl_3$, MeOH, and 1 N HCl (4:1:2). After vigorous vortexing, the lower phase was applied to a Si gel column (1 ml) for the separation of phospholipid and unreacted [γ-^{32}P]ATP. The phosphorylated lipid then obtained was eluted with $CHCl_3$, MeOH, and 4 N NH_4OH (9:7:2), and the radioactivity was quantitated by a liquid scintillation counter.

FERMENTATION.—The *Streptomyces* strain MI698-50F1 was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of the seed medium consisting of glycerol 2.0%, dextrin 2.0%, soy peptone 1.0%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, and $CaCO_3$ 0.2%. The seed culture was incubated for 3 days at 28° on a rotary shaker (180 rpm). Part of the culture (2 ml) was transferred to a 500-ml Erlenmeyer flask that contained 110 ml of fermentation medium consisting of glycerol 2.0%, soybean meal 1.5%, K_2HPO_4 0.1%, and $CoCl_2 \cdot 6H_2O$ 0.0005%. The medium was adjusted to pH 6.2 with 1 M KH_2PO_4 prior to the addition of silicon oil as antifoam agent, and the fermentation was carried out for 8 days at 28°

on a rotary shaker (180 rpm). The echiguanine-producing strain resembled *Streptomyces toyocaensis*, *Streptomyces fasiculatus*, and/or *Streptomyces natalensis* from morphological and physiological studies. It has been deposited in the Fermentation Research Institute of Agency of Industrial Science and Technology, Tsukuba, Japan. The strain also produced toyocamycin.

ISOLATION.—The broth filtrate (27 liters) was adsorbed on Diaion HP-20 resin (3 liters), the resin was washed with distilled H_2O (9 liters), and the active components were eluted with 50% aqueous MeOH (9 liters). The eluate was concentrated in vacuo, and the concentrate was passed through a 300 ml column of reversed phase Si gel. The column was successively washed with distilled H_2O (1 liters) and 10% aqueous MeOH (1 liter) and then eluted with 30% aqueous MeOH (1 liter). After evaporation to dryness under reduced pressure, the residue was dissolved in distilled H_2O and applied to a CM-Sephadex column (Na^+, 200 ml), eluting with a gradient of 0.4 M and 1.0 M NaCl (500 ml each). The combined eluates were subjected to a column of Diaion CHP-20 (40 ml) and eluted with 0.005 N HCl/50% aqueous MeOH (100 ml). After neutralization of the eluate by Amberlite IR-45 (OH^-) anion exchange resin, the active fraction was concentrated in vacuo to give a mixture of echiguanines A and B (73 mg). The mixture was further partitioned in the solvent system EtOAc–*n*-BuOH–H_2O (4:7:10), using centrifugal partition chromatography in which the lower portion was stationary. Thus, purified echiguanines A and B were obtained at 28.6 mg and 6.7 mg, respectively.

PHYSICO-CHEMICAL PROPERTIES.—Echiguanines A and B are basic white substances. The former's color reaction was negative to ninhydrin, while the latter's was positive. Both showed positive reaction to the Rydon-Smith reagent. They were soluble in H_2O, MeOH, and DMSO, but hardly soluble in common organic solvents such as Me_2CO, EtOAc, and $CHCl_3$.

Echiguanine A melted at 215–220°, and the uv spectra showed maxima at 220 nm (ε 14740), 227 nm (ε 14740), 252 sh nm (ε 9300), and 296 nm (ε 8800) in H_2O, at 217 nm (ε 15210), 250 sh nm (ε 10420), and 281 nm (ε 7160) on 0.1 N HCl, and at 256 nm (ε 9630) and 292 nm (ε 7160) in 0.1 N NaOH. Echiguanine B decomposed at 270–280°, and its uv spectrum was approximately the same as that of echiguanine A.

The ir spectra of echiguanines A and B were as follows: echiguanine A [**1**] gave peaks at 3430–3130 br, 2350, 1700 sh, 1640, 1600, 1510, 1420, 1380, 1360, 1340, 1220, 1160, 1080, 840, 740, and 680 cm^{-1} and echiguanine B [**2**] at 3375, 3100, 2320, 1680, 1640, 1580, 1500, 1400, 1350, 1140, 820, 740, and 680 cm^{-1}.

X-RAY CRYSTALLOGRAPHIC ANALYSIS[1].—The crystals of echiguanine A were grown in an aqueous solution as colorless needles. A crystal with approximate dimensions $0.10 \times 0.08 \times 0.12$ mm was mounted on a goniometer head for the diffraction study. Unit cell and intensity data were obtained on a Philips PW1100 diffractometer using graphite monochromated CuKα radiation. The crystal of echiguanine A hydrochloride monohydrate ($C_{10}H_{13}N_7O_2 \cdot HCl \cdot H_2O$) was monoclinic and belonged to the space group *C2/c*. Its lattice constants were as follows: $a = 22.098$ (13) Å, $b = 9.929$ (6) Å, $c = 13.323$ (6) Å, $\beta = 111.10$ (5) Å, U = 2727 Å^3. The Z value, Dx and μ for CuKα were 8, 1.548 g·cm^{-3}, and 27.3 cm^{-1}, respectively. Intensities of 1326 reflections out of 3024 possible ones in the 2θ range of 6° through 156° were measured by a θ–2θ scan method. Of these, 67 were symmetry equivalent reflections. The discrepancy factor for these 67 reflections ($\Sigma||Fo|-|Fo'||/\Sigma|Fo|$, where Fo and Fo′ are for equivalent reflections) was 0.046. A total of 1259 independent reflections were used for the subsequent structure determination. The final R value was 0.08. The crystal structure was solved by the direct method and refined by the method of block-diagonal least-squares. Out of 16 hydrogen atoms, 13 were located on the difference electron-density map and included in the refinement with isotropic temperature factors. Of the remaining three hydrogen atoms, two are those of the amidino group and one belongs to the H_2O molecule. Locations of hydrogen atoms indicate that the base has a hydroxyl group at C-4 instead of the carbonyl group in guanine and protonated N-3 in this hydrogen chloride salt.

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