K-252b, c AND d, POTENT INHIBITORS OF PROTEIN KINASE C FROM MICROBIAL ORIGIN

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Nocardiopsis sp. K-290 was found to produce novel metabolites, designated K-252b, c and d, which were structurally related to K-252a. These compounds were isolated from the culture broth and the physico-chemical and biochemical properties were examined. The compounds strongly inhibited protein kinase C. IC_{50} values (the concentrations causing 50% inhibition) for the effects of K-252b, c and d on the rat brain enzyme were 38.3, 214, and 337 nM, respectively.

In a previous paper[1], we reported that *Nocardiopsis* sp. K-252 produced a potent and new protein kinase C inhibitor, K-252a (**1**). In the present investigation, we have isolated three compounds, designated K-252b (**2**), c (**3**) and d (**4**), which are structurally related to K-252a, from the culture broth of *Nocardiopsis* sp. K-290. Similar to K-252a, these compounds strongly inhibited protein kinase C. This paper deals with fermentation, isolation and purification, and some biochemical properties of K-252b, c and d. Structural elucidation studies will be described in a succeeding paper[2].

Materials and Methods

Microorganisms

Nocardiopsis sp. K-290, isolated from a soil of Tama-shi, Tokyo, Japan, was employed in the present studies. Taxonomy of the strain will be described in a separate paper.

Fermentation

The fermentation was initiated by transferring a loopful of the organism grown on an agar slant into large test tubes (20×200 mm), each containing 15 ml of the following seed medium; glucose 1%, soluble starch 1%, Bacto-Tryptone (Difco Laboratories) 0.5%, yeast extract (Daigo Eiyo Chemicals Ltd.) 0.5%, beef extract (Kyokuto Pharmaceutical Industry Ltd.) 0.3% and $CaCO_3$ 0.3%, pH 7.2. The test tubes were incubated on a reciprocating shaker (300 rpm) for 9 days at 28°C. A 10% transfer of this culture growth was used to inoculate 300-ml Erlenmeyer flasks, each containing 40 ml of the following fermentation medium; glucose 0.5%, soluble starch 3%, soybean meal 3%, corn steep liquor 0.5%, yeast extract 0.5% and $CaCO_3$ 0.3%, pH 7.2~7.4. The flasks were incubated on a rotary shaker (200 rpm) for 8 days at 28°C.

Enzyme Assay

Protein kinase C was prepared from rat brain according to a modification of the method of KIKKAWA *et al.*[3] as described in a previous paper[1]. Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE) and calmodulin of bovine brain were prepared according to the method of KAKIUCHI *et al.*[4] with some modifications[1]. Bovine heart calmodulin, Ca^{2+}/calmodulin-dependent PDE and Ca^{2+}/calmodulin-independent PDE were purchased from Sigma Chemical Co. Ca^{2+}/calmodulin-activated activity, basal activity (the activity in the absence of Ca^{2+} and calmodulin) and Ca^{2+}/

calmodulin-independent activity of various PDE's, and the activity of protein kinase C were measured as described previously[1].

Preparation of K-252b Sodium Salt

K-252b (1.16 g) was dissolved in 0.1 N $NaHCO_3$ solution (400 ml), adsorbed on a column of Diaion HP-20ss (46 ml), washed with H_2O (200 ml), and then eluted with 60% MeOH. The eluate was evaporated to remove MeOH and then lyophilized to yield a pale yellow powder of K-252b sodium salt.

Results

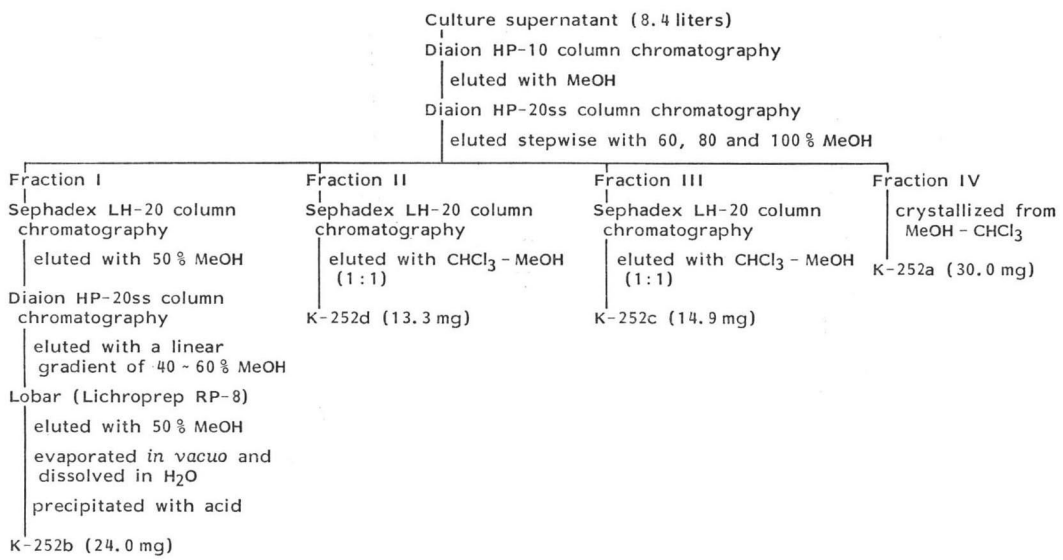
Nocardiopsis sp. K-290 was found to produce several compounds, the UV spectra of which were similar to K-252a (Fig. 2a). One of them gave the same Rf values as K-252a on TLC developed with various solvent systems[1]. The behaviors of other three compounds, designated K-252b, c and d, on TLC were different from those of K-252a. The production of these compounds (K-252 compounds) reached maximum levels in 8 days of cultivation in 300-ml Erlenmeyer flasks.

Isolation and Purification

The isolation procedure of K-252 compounds from the culture broth of *Nocardiopsis* sp. K-290 is schematically shown in Fig. 1. The culture supernatant (8.4 liters) was passed through a column of Diaion HP-10 (1 liter, Mitsubishi Chemical Industries Ltd.). The adsorbed materials were eluted with methanol. The eluate (2 liters) was concentrated *in vacuo* to remove methanol, and applied to a column of Diaion HP-20ss (85 ml, Mitsubishi Chemical Industries Ltd.). The adsorbed materials were eluted stepwise with 60, 80 and 100% methanol (250 ml each). K-252b, d, c and a were eluted at elution volumes from 0 to 250 ml (fraction I), from 432 to 532 ml (fraction II), from 550 to 577 ml (fraction III), and from 578 to 750 ml (fraction IV), respectively.

Fraction I was concentrated *in vacuo* and applied to a column of Sephadex LH-20 (55 ml, Pharmacia Fine Chemicals). Elution was performed with 50% methanol. Fractions containing K-252b

Fig. 1. Purification procedure of K-252a, b, c and d.

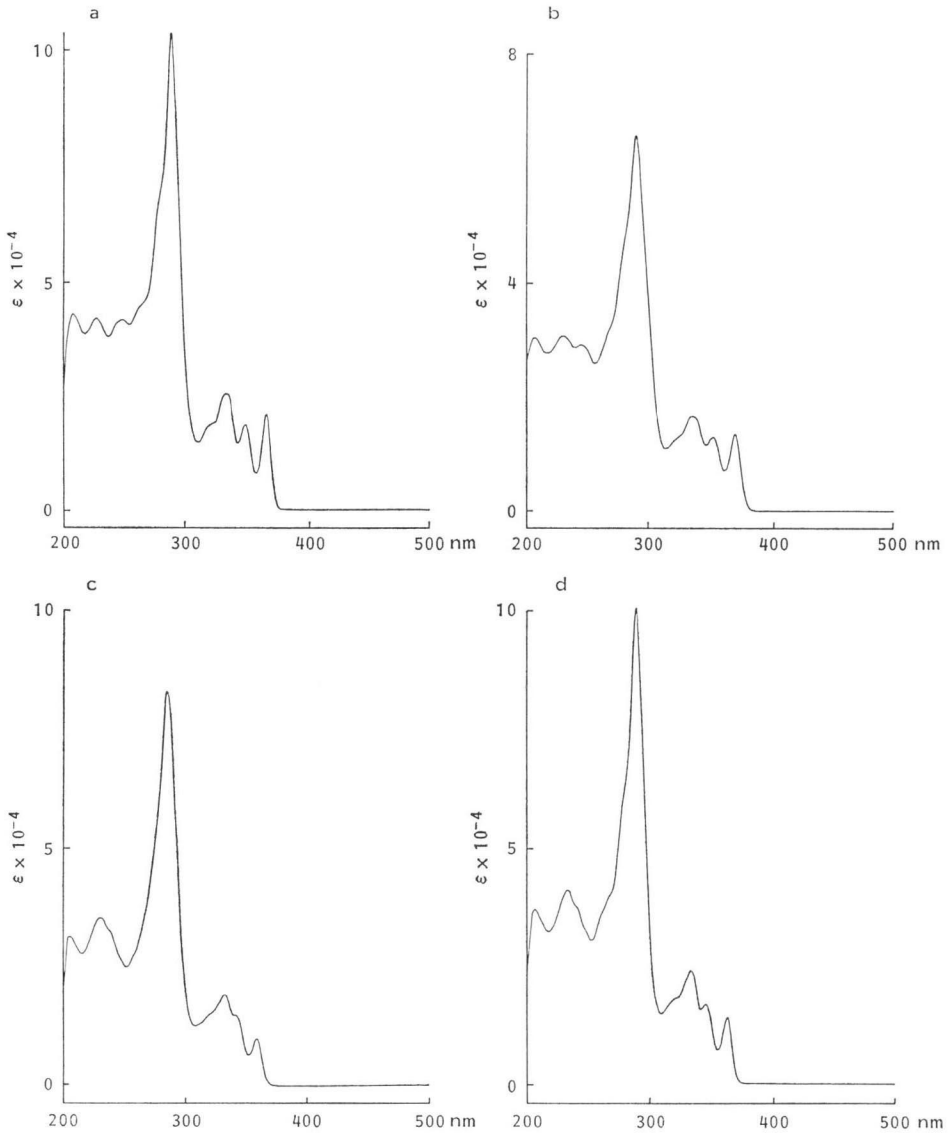


were combined and evaporated to dryness (47.4 mg, brown powder).

The powder was dissolved in 40% methanol (20 ml), adsorbed on a column of Diaion HP-20ss (33 ml), and the adsorbed components were eluted with a linear gradient between 40% methanol (150 ml) and 60% methanol (157 ml). The fractions containing K-252b were concentrated *in vacuo* and applied to a Lobar column (Lichroprep RP-8, size B, Merck). The elution was performed with 50% methanol. Fractions containing K-252b were combined and evaporated to yield a pale yellow powder. The powder was dissolved in water and then 0.1 N HCl was added to precipitate K-252b (24.0 mg).

Fraction II was concentrated *in vacuo*, applied to a column of Sephadex LH-20 (55 ml), and developed with chloroform - methanol (1 : 1). Fractions containing K-252d were combined and evapo-

Fig. 2. UV spectra of K-252a, b, c and d.
a; K-252a, b; K-252b, c; K-252c, d; K-252d.
K-252a, c and d were dissolved in MeOH and K-252b in 0.01 N Na_2CO_3.



rated to yield a white powder of K-252d (13.3 mg).

Fraction III was concentrated and chromatographed on a column of Sephadex LH-20 (55 ml) using chloroform - methanol (1 : 1) as a developing solvent. Fractions containing K-252c were combined and evaporated to yield a brown powder of K-252c (14.9 mg).

Fraction IV was concentrated *in vacuo* to dryness. The yellow powder thus obtained was crystallized from methanol - chloroform to yield pale yellow crystalline K-252a (30.0 mg).

Physico-chemical Properties

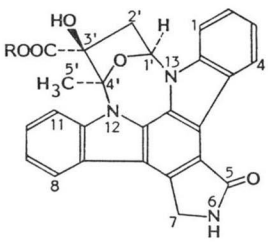
Physico-chemical properties of K-252b, c and d are summarized in Table 1. From their UV spectra (Fig. 2), these compounds were suggested to possess the same chromophore as K-252a. The structure of K-252b, c and d were determined to be **2**, **3** and **4** (Fig. 3), respectively, on the basis of various spectral data[2]. K-252b and d are new compounds, whereas K-252c is identical with the

Table 1. Physico-chemical properties of K-252b, c and d.

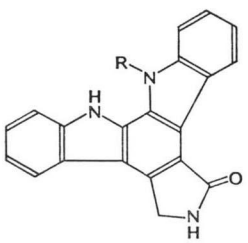
	K-252b	K-252c	K-252d
Appearance	Pale yellow powder	Pale yellow needles	Pale yellow needles
MP	262~266°C (dec)	>300°C	240~245°C (dec)
$[\alpha]_D^{20}$	+97° (*c* 0.6, DMF)		+35° (*c* 0.4, MeOH)
Color test			
Ninhydrin	Negative	Negative	Negative
$FeCl_3$	Negative	Negative	Negative
Anisaldehyde	Positive	Negative	Negative
Solubility			
H_2O	Insoluble	Insoluble	Insoluble
0.01 N HCl	Insoluble	Insoluble	Insoluble
0.01 N Na_2CO_3	Soluble	Insoluble	Insoluble
MeOH	Soluble	Slightly soluble	Soluble
$CHCl_3$	Insoluble	Slightly soluble	Soluble
DMSO	Soluble	Soluble	Soluble
Rf value			
system I[a]	0.00	0.36	0.18
system II[b]	0.59	0.29	0.38

[a] Plate: Silica gel 60 (Merck, 5631), solvent; $CHCl_3$ - MeOH (9 : 1).
[b] Plate: RP-18 (Merck, 13724), solvent; 80% MeOH.

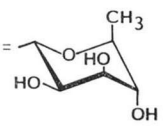
Fig. 3. Structures of K-252a (**1**), b (**2**), c (**3**) and d (**4**).



K-252a (**1**) R = CH_3
K-252b (**2**) R = H



K-252c (**3**) R = H



K-252d (**4**) R =

staurosporine[5,6] aglycon synthesized by SARSTEDT and WINTERFELDT[7]. However, there appear to be no other reports on the isolation of K-252c from microbial culture.

Biochemical Properties

K-252b, c and d inhibited protein kinase C in a dose dependent manner (Table 2). The most potent compound was K-252b with an IC_{50} value of 38.3 nM under the condition used. The IC_{50} values of K-252c and d were 214 and 337 nM, respectively.

These compounds also inhibited Ca^{2+}/calmodulin-activated PDE. The IC_{50} values of K-252b for the brain and heart enzymes were 10.9 and 12.5 μM, respectively. In contrast with K-252a[1], K-252b inhibited the basal activity as well as Ca^{2+}/calmodulin-activated activity of PDE from bovine brain and heart. Furthermore, K-252b inhibited Ca^{2+}/calmodulin-independent PDE from bovine heart with comparable potencies to Ca^{2+}/calmodulin-dependent PDE's. These data suggested the loss of calmodulin selectivity in K-252b.

The inhibition by K-252c and d of Ca^{2+}/calmodulin-dependent PDE was weak with IC_{50}

Table 2. Inhibitory effects of K-252a, b, c and d on protein kinase C.

Protein kinase C activity was measured as described in the previous paper[1].

The reaction mixture contained, in a final volume of 0.25 ml, Tris-HCl buffer (pH 7.5) 20 mM, [γ-^{32}P]ATP 5 μM, $Mg(CH_3COO)_2$ 10 mM, histone H1 0.2 mg/ml, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid 4 mM, $CaCl_2$ 3 mM, phosphatidylserine 0.08 mg/ml, 1,3-diolein 3.2 mg/ml and enzyme 0.16 mg/ml.

Compound	IC_{50} (nM)
K-252b[a,b]	38.3
K-252c[c]	214
K-252d[d]	337
K-252a[c]	32.9

[a] K-252b sodium salt, prepared from K-252b as described in Materials and Methods.
[b] dissolved in H_2O.
[c] dissolved in DMSO.
[d] dissolved in MeOH.

Table 3. Inhibitory effects of K-252a, b, c and d on various cyclic nucleotide phosphodiesterases.

Phosphodiesterase activities were measured as described in the previous paper[1].

The reaction mixture contained, in a final volume of 0.5 ml, imidazole - HCl buffer (pH 6.9) 80 mM, $MgSO_4$ 3 mM, dithiothreitol 0.3 mM, NaCl 100 mM, cyclic AMP 1.2 mM as a substrate, the enzyme, and other additives as indicated.

Enzyme	CaM*	IC_{50} (μM)[e]			
		K-252b[f]	K-252c	K-252d	K-252a
Bovine brain CaM-PDE**	+[a]	10.9	297	46.2	2.9
	−[b]	70.9	—	>200	>200
Bovine heart CaM-PDE**	+[c]	12.5	—	—	1.3
Bovine heart CaM-independent PDE	−[d]	22.9	—	—	97.5

[a] The activity of bovine brain Ca^{2+}/calmodulin-dependent phosphodiesterase (26 mU/ml) in the presence of 50 μM $CaCl_2$ and 4 U/ml calmodulin.
[b] The activity of bovine brain Ca^{2+}/calmodulin-dependent phosphodiesterase (18.6 mU/ml) in the presence of 3 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (basal activity).
[c] The activity of bovine heart Ca^{2+}/calmodulin-dependent phosphodiesterase (40 mU/ml) in the presence of 50 μM $CaCl_2$ and 2.5 U/ml calmodulin.
[d] The activity of bovine heart Ca^{2+}/calmodulin-independent phosphodiesterase (25 mU/ml) in the presence of 3 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.
[e] These compounds were dissolved in the same solvents as in Table 2.
[f] K-252b sodium salt, prepared from K-252b as described in Materials and Methods.
—: Not determined.
* Calmodulin. ** Ca^{2+}/calmodulin-dependent phosphodiesterase.

values for the bovine brain enzyme of 297 μM for K-252c and 46.2 μM for K-252d.

These compounds at 100 μg/ml showed no inhibitory activity against *Candida albicans* KY5011, *Enterococcus faecalis* KY4280, *Pseudomonas aeruginosa* KY4276, *Staphylococcus aureus* KY4779, *Escherichia coli* KY4271, *Bacillus subtilis* KY4773, *Proteus vulgaris* KY4277, *Shigella sonnei* KY4281, *Salmonella typhosa* KY4278, and *Klebsiella pneumoniae* KY4275.

Discussion

The occurrence of the unusual indole carbazole system has been recently reported in the natural products arcyriaflavins B and C, isolated from the slime mold *Arcyria denudata*[8], the antibiotic staurosporine produced by *Streptomyces* sp.[5,6] and the antitumor antibiotic rebeccamycin isolated from *Nocardia aerocolonigenes*[9]. In a previous paper[1], we described a novel indole carbazole-containing compound K-252a (**1**) isolated from *Nocardiopsis* sp. K-252. The present studies demonstrate the isolation of new metabolites K-252b (**2**) and d (**4**) which contain the K-252a aglycon, and K-252c, the aglycon itself.

Similar to K-252a, K-252b, c and d are potent inhibitors of protein kinase C. Retention of the inhibitory activity for the enzyme in K-252c and d may indicate that the aglycon moiety of K-252 compounds is important for the inhibition of the enzyme activity. The results strongly suggest that the other indole carbazole compounds described above may also inhibit protein kinase C. Actually, TAMAOKI *et al.* in our laboratories have recently observed that staurosporine also strongly inhibits protein kinase C (T. TAMAOKI, H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA, submitted to Biochem. Biophys. Res. Commun.).

Unlike K-252a, K-252b appeared to lose the calmodulin selectivity: not only Ca^{2+}/calmodulin-dependent activity but also the basal activity of Ca^{2+}/calmodulin-dependent PDE or the activity of Ca^{2+}/calmodulin-independent PDE were inhibited by K-252b. K-252c and d inhibited Ca^{2+}/calmodulin-dependent PDE, but their effects were minimal. Thus, K-252 compounds proved to be different from each other in potency and selectivity for inhibition of calmodulin-dependent activity.

We have found that K-252b, c and d seriously affect the function of platelets, mast cells, and several other cells and tissues. These newly developed compounds should facilitate studies on the physiological role of protein kinase C and calmodulin in Ca^{2+}-messenger system.

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