Antimicrobial Activities of Indolocarbazole and Bis-indole Protein Kinase C Inhibitors

II. Substitution on Maleimide Nitrogen with Functional Groups Bearing a Labile Hydrogen

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New compounds, structurally related to the potent protein kinase C inhibitor staurosporine, and substituted on the imide nitrogen with a functional group bearing a labile hydrogen (hydroxymethyl, amino, hydroxy), were synthesized. Their in vitro inhibitory potencies towards protein kinase C and protein kinase A showed that TV-hydroxymethyl and N-hydroxy substitution, unlike alkyl substitution, can provide efficient protein kinase C inhibitors. The antimicrobial activities of these new compounds against Streptomyces chartreusis and Streptomyces griseus, Bacillus cereus, Escherichia coli, Candida albicans and Botrytis cinerea were examined. They proved to be inactive against E. coli and two fungi. The results suggest that there is no link between in vitro inhibition of protein kinase C and inhibition of growth and sporulation of the two Streptomyces tested. Unlike indolocarbazole maleimides, bis-indole maleimides are active against the two Streptomyces species.

In a previous paper1), we reported the antimicrobial activities against Streptomyces chartreusis and Streptomyces griseus, Bacillus cereus, Escherichia coli, Candida albicans and Botrytis cinerea of compounds structurally related to the protein kinase C (PKC) inhibitors staurosporine2,3) and K-252a4) (Fig. 1). As these two potent PKC inhibitors prevent the sporulation of different Streptomyces species5), this phenomenon was also examined on structurally related analogues. No link could be found between the growth and sporulation of the two Streptomyces tested and the inhibitory potencies against the enzyme. No evident structure-activity relationships could be demonstrated for the antimicrobial activities.

Among the compounds exhibiting an antibacterial activity against the strains of Streptomyces tested, 1 (Fig. 2) substituted on the imide nitrogen with a hydroxymethyl group was found to inhibit PKC in spite of the flexibility of its framework leading usually to inactive compounds1,6). Moreover, while substitution on the imide nitrogen with a methyl or a benzyl group had previously been found to induce inactivity against PKC7,8), substitution with a hydroxymethyl group led to an inhibitory effect against PKC. AKINAGA et al.9) prepared K-252a derivatives. Of these, KT6124 (Fig. 1) substituted with an amino group on the imide nitrogen exhibits PKC inhibitory effect in vitro.

Accordingly, we investigated the syntheses, inhibitory potencies against PKC and PKA, and antimicrobial activities against the same microorganisms, of new compounds in these series, 2~7, substituted on the imide nitrogen with functional groups bearing a labile hydrogen (Fig. 2). Their frameworks are stiffer than that of 1 and were thus expected to provide a stronger PKC inhibitory effect.

Results and Discussion

The starting material for the synthesis of compounds 1~3, N-substituted with a hydroxymethyl group, was bis-indolyl-N-benzyloxymethylmaleimide 8 (Fig. 3) prepared from N-benzyloxymethyl dibromomaleimide and indolyl-MgBr.5,10) Hydrogenolysis of 8 using palladium hydroxide in ethyl acetate gave 2 in 50% yield. Photooxidation of 8 in acetonitrile with a medium pressure mercury lamp (400 W) afforded indolocarbazole 9 in 58% yield. Hydrogenolysis of 9 on Pd/C in ethanol yielded 3 (48% yield).
Fig. 1. Microbial PKC inhibitors.

Staurosporine

K-252a: $R = H; X = Y = H; Z = COOCH_3$
KT6124: $R = NH_2; X, Y = O; Z = CH_3OH$

Fig. 2. Synthetic analogs of K-252c substituted on the imide nitrogen with functional groups bearing a labile hydrogen.

1 $R = CH_2OH$
2 $R = NH_3$
3 $R = CH_2OH$
4 $R = NH_2$
6 $R = OH$

Fig. 3. Synthetic scheme for compounds 2 and 3.

Fig. 4. Synthetic scheme for compounds 4~7.
N-amino and N-hydroxy derivatives 4~7 were prepared from anhydrides 10 and 11 (Fig. 4), using hydrazine hydrate in refluxing tetrahydrofuran for the N-amino substituted compounds 4 and 5, hydroxylamine hydrochloride and N,N-diisopropylethylamine in refluxing dimethylformamide for the N-hydroxy substituted compounds 6 and 7. Bis-indoles 4 and 6 were obtained in quantitative yields, indolocarbazoles 5 and 7 in 60% yield.

Compounds 4 and 5 were found to be very unstable in the presence of acetone. They were readily converted to the corresponding imines (see experimental section: IR, H and C NMR data for imine 12 obtained from 4). This reaction unequivocally confirms the structures of 4 and 5 as N-amino compounds and not cyclic 6-membered ring hydrazides sometimes claimed to be the normal products of hydrazinolysis of cyclic imides such as phthalimide

The inhibitory potencies of 1~7 towards PKC and PKA were determined using histones Ills and Ha respectively as substrates. Isoquinoline sulfonamide inhibitor H-7 was used as reference. IC50 values are reported in Table 1. For the biological tests, 1~7 were dissolved in dimethylsulfoxide. All compounds proved to be stable in this solvent.

Of the N-hydroxy and N-amino maleimides 5~7, only N-hydroxy bis-indole 6 was an efficient PKC inhibitor.

As expected from the preliminary results obtained for 1, the more rigid maleimide 2 was much more active. Compound 3, bearing a maleimide heterocycle and an indolocarbazole framework, was inactive towards PKC. This agrees with the weaker inhibitory properties of maleimides versus maleamides in the indolocarbazole series observed previously.

Our N-substituted compounds, except 1, have lower or similar PKC inhibitory potencies compared with the parent N-unsubstituted compound. A substitution with an alkyl group on the imide nitrogen leads to inactivity, substitution with a hydroxymethyl, a hydroxy or an amino group can afford PKC inhibitors.

The antimicrobial activities of 1~7 against the microorganisms previously cited were tested. The inhibitions of growth and aerial mycelium formation were examined on the two Streptomyces species (Table 1) and compared to the inhibitions induced by staurosporine.

Compounds 1~7 had no growth inhibitory effect against two fungi (B. cinerea and C. albicans) and against E. coli, 2, 6 and 1, the strongest PKC inhibitors were active against the three Gram-positive bacteria tested. 4 (a very weak PKC inhibitor) was also active but 7, exhibiting a stronger inhibitory effect on PKC than 4, had no antibacterial activity.

<table>
<thead>
<tr>
<th>Products</th>
<th>IC50 PKC (μM)</th>
<th>IC50 PKA (μM)</th>
<th>S. chartreusis NRRL 1407</th>
<th>E. griseus ATCC 23345</th>
<th>E. coli ATCC 11303</th>
<th>B. cereus ATCC 14879</th>
<th>C. albicans IP 444</th>
<th>B. cinerea DSM 877</th>
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<tr>
<td>1</td>
<td>16</td>
<td>&gt;100</td>
<td>++</td>
<td>++ + + +</td>
<td>++</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>0.5</td>
<td>15.5</td>
<td>++ + + + + +</td>
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<td>++ + + +</td>
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<td>+ +</td>
</tr>
<tr>
<td>3</td>
<td>&gt;150</td>
<td>15.0</td>
<td>++ + +</td>
<td>++ + + + + + +</td>
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<td>++ + + + + +</td>
<td>++ + + + + + +</td>
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<td>4</td>
<td>108</td>
<td>117</td>
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<td>++ + + + +</td>
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<td>+ + + + + + + +</td>
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<tr>
<td>5</td>
<td>43.5</td>
<td>44.6</td>
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<td>++ + + + + + +</td>
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<td>++ + + + + + + +</td>
<td>++ + + + + + +</td>
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</table>

In vitro growth inhibitory effect against different microorganisms. For the two Streptomyces species: g. means inhibition of growth, sp. means inhibition of sporulation. (+ + + +), (+ + +), (+ +), (+), (±) and (−) mean very strong, strong, medium, weak, very weak and no activity. The size of zones of growth inhibition was >15 mm (+ + + +), 12~15 mm (+ + +), 10~12 mm (+ +), 7~9 mm (+), 6~7 mm (±). Data for staurosporine: S. chartreusis g. +, sp. ++, E. griseus g. ±, sp. ++.

![Fig. 5. Structures of compounds A, B, C and D.](image-url)
As previously reported, no evident correlation could be observed between the inhibitory potency towards PKC and the antimicrobial activity against the Gram-positive bacteria tested. Since staurosporine and K-252a inhibit the phosphorylation of several cell proteins in S. griseus, it seemed reasonable to correlate the inhibition of protein phosphorylation to the inhibition of sporulation. Our results suggest that the inhibition of sporulation is not necessarily linked to the inhibition of Streptomyces protein phosphorylation by PKC. It may be induced via other pathways. Ochi et al. reported a possible role of protein phosphorylation by PKC. It may be induced via other pathways.

Some substances possessing PKC inhibitory potencies in vitro are inactive against the enzyme in vivo. This is so for KT 6124, a K-252a derivative substituted on the duocarmycins to their antimicrobial activity, probably due to their ability to inhibit enzymes that damage DNA.

ICHIMURA et al. correlated the antitumor activity of ADP-ribosyltransferase in the sporulation of S. griseus. Our results suggest that the inhibition of sporulation is not necessarily due to their PKC inhibitory effect. Even if they prove to be active against the enzyme in vivo, their antibacterial activity is not necessarily due to their PKC inhibitory effect.

In conclusion, these results extend our knowledge of the structure-activity relationships in the PKC field. The bis-indoles 1, 2, 4 and 6, are active against the three Gram-positive bacteria tested, while the indolocarbazoles 3, 5 and 7 are not. This agrees with our previous findings for maleimide structures (Fig. 5). A and B have no antibacterial effects while C and D are very efficient both on growth and sporulation of the Streptomyces species tested.

### Experimental

Chromatographic purifications were performed with flash Geduran SI 60 (Merck) 0.040 - 0.063 mm.

Histones IIIa and IIa, phosphatidylserine and diacylglycerol were purchased from Sigma, [32P]ATP was from Amersham. Protein kinase A was purchased from Sigma and protein kinase C from Calbiochem.

**3,4-Bis(indol-3-yl)-1-hydroxymethyl-3-pyrrolin-2,5-dione**

3,4-Benzoxymethyl-5,7-dioxo-(5H)-6,7,13-tetrahydro-indolo[2,3-a]pyrrolo[3,4-c]carbazole 9

**6-Benzoxymethyl-5,7-dioxo-(5H)-6,7,12,13-tetrahydro-indolo[2,3-a]pyrrolo[3,4-c]carbazole 9**

**6-Hydroxymethyl-5,7-dioxo-(5H)-6,7,12,13-tetrahydro-indolo[2,3-a]pyrrolo[3,4-c]carbazole 3**

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was added H₂N–NH₂, H₂O (76 mg; 1.52 mmol; 0.074 ml). The mixture was warmed to 40~50°C for 15 minutes. The solvent was removed and the residue poured into water and extracted with ether. The organic phase was washed with brine, and dried over MgSO₄. After removal of the solvent, 4 was obtained as a red powder (104 mg; 0.30 mmol; 100% yield). HRMS (EI) calculated for C₂₀H₁₄N₄O₂ 342.1116, found 342.1117.

IR (KBr): vNH and νNH₂ 3400 cm⁻¹; vc=O 1710 cm⁻¹; mp >310°C.

¹H NMR (400 MHz, DMSO-d₆): 4.9 (2H, s, N–NH₂), 6.68 (2H, t, J = 7.7 Hz), 6.84 (2H, d, J = 7.7 Hz), 7.03 (2H, t, J = 7.7 Hz), 7.42 (2H, d, J = 7.7 Hz), 7.81 (2H, d, J = 2.3 Hz), 10.95 (2H, s, Nindole-H).

¹³C NMR (100 MHz, DMSO-d₆): 105.7, 125.3 (2C), 135.9 (Cquat.), 111.7, 119.3, 120.8, 121.6, 129.1 (Ctert.), 170.8 (C=O).

1-Hydroxy-2,3-bis(3-indolyl)-3-pyrrolin-2,5-dione 6

Anhydride 10 (300 mg; 0.91 mmol) was dissolved in DMF (20 ml). NH₂OH, HCl (317 mg; 4.57 mmol) and 7N,7N-diisopropylethylamine (590 mg; 4.57 mmol; 0.8 ml) were added. The mixture was warmed at 90~100°C for 30 minutes. After identical work-up as for 4, purification by flash chromatography (eluent - AcOEt) yielded 6 as a dark red powder (312 mg; 0.91 mmol; 99% yield).

IR (KBr): vNH and vOH 3390 cm⁻¹; vc=O 1710 cm⁻¹; mp 160°C. HRMS (EI) calcd for C₂₀H₁₃N₃O₃ 343.0957, found 343.0956.

¹H NMR (400 MHz, acetone-d₆): 6.65 (2H, t, J = 7.7 Hz), 6.94 (2H, d, J = 7.7 Hz), 6.99 (2H, t, J = 7.7 Hz), 7.42 (2H, d, J = 7.7 Hz), 7.81 (2H, d, J = 7.7 Hz), 9.55 (1H, s, N-OH), 10.85 (2H, s, Nindole-H).

¹³C NMR (100 MHz, acetone-d₆): 106.4, 125.1, 125.8, 136.3 (Cquat.), 111.6, 119.6, 121.4, 121.9, 129.2 (C tert.), 168.5 (C=O).

1-Amino-6,7,12,13-tetrahydro-5,7-dioxo-indolo[2,3-a]-pyrrolo[3,4-c]-carbazole 5

To anhydride 11 (100 mg; 0.30 mmol) in THF (20 ml) was added hydrazine monohydrate (0.076 g; 1.53 mmol; 0.075 ml). The mixture was refluxed for 4 hours. The solvent was removed and the residue washed with water. After filtration, the solid was washed with petroleum ether; 5 was obtained as a yellow powder (0.055 g; 0.16 mmol; 53% yield).

IR (KBr): vNH and vOH 3330 cm⁻¹; vc=O 1700 cm⁻¹; mp >305°C. Mass (EI) M⁺ 340 (50%).

¹H NMR (400 MHz, acetone-d₆): 7.41 (2H, t, J = 7.9 Hz), 7.60 (2H, d, J = 7.9 Hz), 7.86 (2H, d, J = 7.9 Hz), 9.06 (2H, d, J = 7.9 Hz), 11.8 (2H, s, Nindole-H).

¹³C NMR (100 MHz, acetone-d₆): 114.9, 116.0, 122.0, 128.7, 140.4 (Cquat.), 112.1, 120.3, 124.2, 126.9 (C tert.), 168.8 (C=O).

6-Hydroxy-6,7,12,13-tetrahydro-5,7-dioxo-indolo[2,3-a]-pyrrolo[3,4-c]-carbazole 7

To anhydride 11 (100 mg; 0.30 mmol) in DMF (20 ml) were added NH₂OH·HCl (106 mg; 1.53 mmol) and N,N-diisopropylethylamine (198 mg; 1.53 mmol; 0.26 ml). The mixture was warmed at 85°C for 1 hour then poured into water and extracted with AcOEt. After removal of the solvent and purification by flash chromatography (eluent - AcOEt) compound 7 was isolated as an orange solid (66 mg; 0.19 mmol; 63% yield).

IR (KBr): vNH and vOH 3400 cm⁻¹; vc=O 1695 cm⁻¹; mp >315°C. HRMS (EI) calcd for C₂₀H₁₁N₃O₃ 341.0800, found 341.0844.

¹H NMR (400 MHz, DMSO-d₆): 7.39 (2H, t, J = 7 Hz), 7.59 (2H, t, J = 7 Hz), 7.85 (2H, d, J = 7 Hz), 8.99 (2H, d, J = 7 Hz), 10.6 (1H, s, N-OH), 11.85 (2H, s, Nindole-H).

¹³C NMR (100 MHz, DMSO-d₆): 115.3, 115.7, 121.3, 129.1, 140.4 (Cquat.), 112.1, 120.3, 124.1, 127.0 (C tert.), 166.6 (C=O).

1-(N-isopropylidene-amino)-3,4-bis(3-indolyl)-3-pyrrolin-2,5-dione 12

IR (KBr): vNH 3400 cm⁻¹; vc=O and vc=N 1690 and 1700 cm⁻¹; mp >300°C.

¹H NMR (400 MHz, DMSO-d₆): 2.05 (3H, s, CH₃), 2.40 (3H, s, CH₃), 6.73 (2H, t, J = 7.5 Hz), 6.92 (2H, d, J = 8 Hz), 7.06 (2H, t, J = 7.5 Hz), 7.46 (2H, d, J = 8 Hz), 7.88 (2H, d, J = 1.4 Hz), 11.85 (2H, s, Nindole-H).

¹³C NMR (100 MHz, DMSO-d₆): 20.6 and 24.8 (CH₃), 111.9, 119.5, 121.1, 128.9 (C tert.), 165.0 (C=O).

Measurements of PKC and PKA Inhibition

Protein kinase C phosphorylation assays were performed in a reaction (80 μl) mixture containing histone IIIa (2.4 mg/ml), MgCl₂ (10 mm), CaCl₂ (0.1 mm), phosphatidylserine (10 mg/ml), diacylglycerol (10 mg/ml), ATP (10 μM), [γ³²P]ATP (10³ cpm/80 μl), Tris buffer (50 mm, pH 7.5), protein kinase C (0.5 μg/ml) and inhibitors at different concentrations.

Protein kinase A phosphorylation assays were performed in a reaction (80 μl) mixture containing histone IIa (1 mg/ml), MgCl₂ (5 mm), ATP (10 μM), [γ³²P]ATP (10⁶ cpm/80 μl), Tris buffer (50 mm, pH 7.0), protein kinase A (1 μg/ml) and inhibitors at different concentrations.

For each kinase, reactions were run at 30°C for 12 minutes and stopped with trichloroacetic acid (12% w/v) in the presence of bovine serum albumin (0.9 mg) as a carrier protein. After centrifugation (10 minutes at 3000 rpm), the pellet was dissolved in 1 ml NaOH and precipitated a second time with trichloroacetic acid. Radioactivity incorporated into histones was counted by scintillation spectrometry (Tri-Carb 4530, Packard). All experiments were carried out in triplicate.
Antimicrobial Tests

Six strains were tested: 3 Gram-positive bacteria (B. cereus ATCC 14879, S. chartreusis NRRL 11407, and S. griseus ATCC 23345), a Gram-negative bacterium (E. coli ATCC 11303), a yeast (C. albicans 444 from Pasteur Institute) and a filamentous fungus (B. cinerea DSM 877). Antibacterial activity was determined by the conventional paper disk (Durieux, N° 268, 6 mm in diameter) diffusion method using the following nutrient media: Mueller Hinton for B. cereus and E. coli, Sabouraud agar (Difco) for C. albicans and B. cinerea and Emerson agar (0.4% beef extract, 0.1% yeast extract, 0.4% peptone, 1% dextrose, 0.25% NaCl, 2% agar, pH 7.0 (Difco)) for the Streptomyces species. Growth inhibition was examined after incubation for 24 hours at 27°C (37°C for E. coli). Inhibition of sporulation was examined 3 to 5 days later. Products 1 ~ 7 were dissolved in DMSO and a paper disk containing each of the products (300 μg) was placed on agar plates.

References

11) See Beilstein, 24, 371