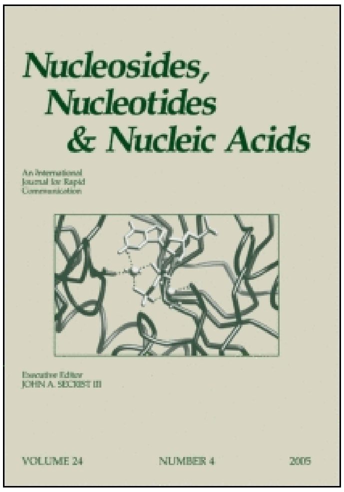

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Synthesis of 5'-Substituted Derivatives of the Pyrrolo[2,3-d]-Pyrimidine Nucleoside Sangivamycin and Their Effect on Protein Kinase A and C Activity

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SYNTHESIS OF 5'-SUBSTITUTED DERIVATIVES OF THE PYRROLO[2,3-d]-PYRIMIDINE NUCLEOSIDE SANGIVAMYCIN AND THEIR EFFECT ON PROTEIN KINASE A AND C ACTIVITY.

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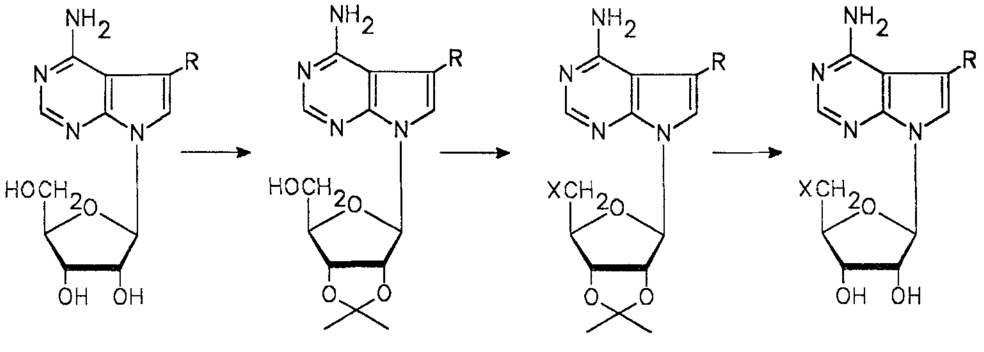
Abstract:

Under cell-free conditions, where the antibiotic sangivamycin is not phosphorylated, it is an effective inhibitor of PKC and to a lesser extent of PKA activity. In intact cells, the antibiotic is phosphorylated, thereby, extending its range of activity to other targets including DNA and RNA.To preserve selective inhibitory activity for the protein kinases, analogs potentially resistant to phosphorylation were prepared by replacing the 5'-hydroxy group with O-nitro, O-sulfamoyl, O-methanesulfonyl or azido groups. These compounds were more potent inhibitors of PKA and PKC activity than was the parent nucleoside.

Introduction

The protein kinases A (PKA) and C (PKC) participate in the transduction of various extracellular signals involved in cell proliferation and differentiation[1,2]. Because of this function, the enzymes are potential targets for inhibition of tumor growth[2,3]. Under cell-free conditions, where its phosphorylation is prevented, the nucleoside antibiotic sangivamycin was found to be an effective inhibitor of PKC activity[4]. However, in intact cells, the antibiotic is phosphorylated, and its inhibitory effects then extend to other sites as well, including RNA and DNA[5,6]. Thus, non-phosphorylatable analogs of sangivamycin have the potential for protein kinase-specific activity. Sangivamycin was found to bind to the catalytic site of PKC in place of ATP[4]. Although this binding does not depend on the presence of a phosphate group at the 5'-position, we considered it possible that the replacement of the 5'-OH group of sangivamycin with functions that can mimic the phosphate group[7] may provide



1, 5 2, 6 3a-d, 7a 4a-d, 8a

R = $CONH_2$	1	2	3a-d	4a-d
R = CN	5	6	7a	8a

a) X = ONO_2
b) X = OSO_2NH_2
c) X = OSO_2CH_3
d) X = N_3

SCHEME

derivatives that are not only refractive to phosphorylation but may bind more tightly to PKC. In this paper, we report the synthesis of a series of pyrrolopyrimidine nucleosides substituted at the 5'-positions with nitro, sulfamoyl, methanesulfonyl or azido groups, and describe the inhibitory effects these agents exert on PKA and PKC activity.

Chemistry:

Sangivamycin (**1**) was prepared from toyocamycin (**5**) as described previously[8]. The 2',3'-O-isopropylidene derivative of sangivamycin (**2**) was obtained in 95% yield by treatment of **1** with acetone/2,2-dimethoxypropane and perchloric acid, a method previously reported for preparation of **6**, the isopropylidene derivative of toyocamycin[8]. When this method was repeated for the synthesis of **6**, a less polar by-product was also obtained which could be separated by chromatography. The IR spectrum of the by-product did not show the presence of a nitrile group, but treatment with hydrogen peroxide in concentrated ammonium hydroxide solution[8] converted it to the 2',3'-O-isopropylidene derivative of sangivamycin (**2**). Use of p-toluenesulfonic acid as a catalyst in place of perchloric acid, however, gave **6** as the only product in 90% yield.

The 5'-nitro ester of sangivamycin **4a** was prepared from **2** by treatment with cold nitric acid/acetic anhydride in ethyl acetate[9], followed by removal of the isopropylidene group with concentrated formic acid. Similarly, nitration of **6** followed by acid hydrolysis of the isopropylidene group gave the 5'-nitro ester of toyocamycin (**8a**). The IR spectra of the nitro esters **4 a** and **8a** showed the characteristic absorption bands at 1290 and 1275 cm^{-1}, respectively.

Treatment of the sodium salt of **2** with sulfamoyl chloride[10] gave the 5'-sulfamoyl derivative **3b**. The isopropylidene group was removed by treatment with formic acid to yield 5'-O-sulfamoylsangivamycin (**4b**). Treatment of the sodium salt of **2** with methanesulfonyl chloride gave 2',3'-O-isopropylidene-5'-O-mesylsangivamycin (**3c**) which was converted to 5'-azido-5'-deoxy-2',3'-O-isopropylidene sangivamycin (**3d**) by displacement of the 5'-mesyl group with lithium azide in dimethylformamide. Hydrolysis with formic acid provided 5'-azido-5'-deoxysangivamycin (**4d**).

Biological Activity

The newly synthesized compounds were examined for their effects on PKA and PKC activity and on the growth of ML-1 human myeloblastic leukemia cells. As shown in the Table, sangivamycin was a more potent inhibitor of PKC than of PKA. In contrast, its derivatives were more effective inhibitors of PKA than of PKC. Furthermore, the O-nitro, O-sulfamoyl and azido derivatives of sangivamycin inhibited both PKA and PKC more potently than did sangivamycin itself. However, sangivamycin was a more potent inhibitor of ML-1 cell growth than were its derivatives. Inhibition of cell growth occurred at a much lower concentration of sangivamycin than was required for inhibition of the protein kinases, indicating that the more effective inhibition of cell growth may be related to the ability of sangivamycin to be phosphorylated, with a consequent change in its target specificity to include RNA and DNA.

Unlike sangivamycin, the pyrrolo[2,3-d]pyrimidine nucleoside toyocamycin (Table) and its 5'-O-nitro derivative were more potent inhibitors of PKA than of PKC. Nevertheless, toyocamycin was as potent an inhibitor of ML-1 cell growth as was sangivamycin, indicating that its growth inhibitory action may equally relate to its demonstrated ability to undergo phosphorylation, with a consequent extension of its target range to include RNA and DNA.

These results show that appropriate substituents at the 5'-positions of sangivamycin and toyocamycin can alter target specificity as well as extent of biological activity. These findings serve as a basis for the design and synthesis of additional, potentially more selective analogs.

Table

Effect of Pyrrolo[2,3-d]pyrimidine Nucleoside Analogs on PKC and PKA activity and on the Growth of ML-1 Cells in vitro.

			Concentration (μM) for 50 % Inhibition of		
Formula No.	R	X	PKC	PKA	ML-1
1	$CONH_2$	OH	12	70	0.03
4a	$CONH_2$	$O\text{-}NO_2$	2.7	0.13	0.4
4b	$CONH_2$	$O\text{-}SO_2NH_2$	1.4	0.01	5
4c	$CONH_2$	$O\text{-}SO_2CH_3$	38	1.6	40
4d	$CONH_2$	N_3	0.5	0.02	2
5	CN	OH	90	20	0.03
8a	CN	$O\text{-}NO_2$	12	0.7	3

Experimental Section

General:

Thin-layer chromatography was performed on EM Science silica gel glass-backed plates. Column chromatography was carried out on silica gel (230-400 mesh) from E. Merck Industries Co. All melting points were taken on a Mel-Temp capillary point block and are not corrected. ^1H NMR spectra were recorded on a Varian 390 spectrometer (90 MHz), and chemical shifts are given in ppm using tetramethylsilane as internal standard. IR spectra were recorded on Perkin-Elmer Models 457 and 710B spectrometers. Evaporations were carried out under diminished pressure at bath temperatures below 35° C. PKA from bovine heart and PKC from rat brain were purchased from Sigma and Calbiochem, respectively.

Chemistry:

4-Amino-5-carboxamido-7-(2,3-O-isopropylidene-ß-D-ribofuranosyl)pyrrolo-[2,3-d]pyrimidine (2). Perchloric acid (70%, 0.5 mL) was added to a stirred suspension of sangivamycin (**1**) (2.5 g, 8.1 mmol) in anhydrous acetone (200 mL) containing 1.5 mL of 2,2-dimethoxypropane. The mixture, which became homogeneous in 5-10 min, was stirred at room temperature for approximately 4 hr . Powdered anhydrous potassium carbonate (1.5 g) was added when TLC showed the absence of **1**, and the mixture was filtered and the solids washed with acetone (50 mL). The combined solution was evaporated to a foamy residue which was taken up in acetone (20 mL) and applied to a short column of silica gel. The column was eluted rapidly with acetone and the eluent containing the product was evaporated to give **2** (2.7 g, 95%) as a white foam. Rf= 0.5 (acetone); IR (KBr) ν_{max} 2900-3200 (OH, NH), 1600, 1050 cm^{-1}; ^{1}H NMR (methanol-d$_4$) δ 1.25 and 1.55 (6 H, 2s, isopropylidene), 3.7 (2 H, d, J= 3.5 Hz, H-5'), 4.25 (1 H, d, J= 3.2 Hz, H-4'), 5.02 (2 H, m, H-2', H-3'), 6.10 (1 H, d, J= 4.5 Hz, H-1'), 7.95 (1 H, s, H-2), 8.10 (1 H, s, H-6). Anal. Calcd. for $C_{15}H_{19}N_5O_5.H_2O$: C, 49.04; H, 5.76; N, 19.06. Found: C, 48.98; H, 5.47; N, 18.90.

4-Amino-5-carboxamido-7-(2,3-O-isopropylidene-5-O-nitro-ß-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (3a). A cold (-30°C) mixture of fuming (90 %) nitric acid (1.4 mL, 19.0 mmol) and acetic anhydride (1.4 mL, 14.0 mmol) was added dropwise to a stirred solution of **2** (660 mg, 18 mmol) in anhydrous ethyl acetate (25 mL) at -30° C. The reaction mixture was kept at -30° C for 70 min, neutralized with sodium bicarbonate, and stirred at room temperature for 20 min. The mixture was filtered and the solids washed with ethyl acetate. The combined ethyl acetate solution was washed with water, dried ($MgSO_4$), and evaporated to a solid. The residue was taken up in acetone/ethyl ether (1/1) and poured onto a silica gel column. Elution of the column with chloroform, to remove yellow-colored impurities, and with acetone/ethyl ether (1/1) followed by removal of the solvent gave product **3** as a white foamy residue (445 mg, 62 %). Rf= 0.6 (acetone/ethyl ether 1/1); IR (KBr) ν_{max} 3200 (NH), 1600 (CO amide), 1230 (ONO_2), 1060 cm^{-1}. Anal. Calcd. for $C_{15}H_{18}N_6O_7$: C, 45.68; H, 4.57; N, 21.32. Found: C, 45.44; H, 4.62; N, 21.61

4-Amino-5-carboxamido-7-(5-O-nitro-ß-D-ribofuranosyl)pyrrolo[2,3-d]-pyrimidine (4a). Compound **3a** (400 mg, 1.02 mmol) was dissolved in formic acid (88%, 10 mL) and the solution stirred at room temperature overnight. Formic acid was removed by evaporation and the residue co-evaporated with anhydrous ethanol (4 x 25 mL) to give a solid (310 mg, 82%) which was crystallized from methanol/ethyl ether. Mp 245-7 °C; Rf= 0.8 (methanol/ethyl acetate 1/4); IR (KBr) ν_{max} 3100 - 3500 (OH, NH), 1610 (CO, amide), 1290 (O-NO2) cm-1. Anal. Calcd. for $C_{12}H_{14}N_6O_7$: C, 40.67; H, 3.95; N, 23.72. Found: C, 40.59; H, 3.74; N, 23.63.

4-Amino-5-carboxamido-7-(2,3-O-isopropylidene-5-O-sulfamoyl-ß-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (3b). Sodium hydride (80% oil suspension, 120 mg, 4 mmol) was washed with hexane and added in portions to a cold (0° - 5° C) stirring solution of **2** (350 mg, 0.9 mmol) in anhydrous tetrahydrofuran (15 mL). The mixture was stirred at 0-5 °C for 15 min and was then treated with a solution of sulfamoyl chloride (500 mg, 4.3 mmol) in anhydrous tetrahydrofuran (5 mL), followed by stirring at room temperature for 4 hr. The mixture was cooled to 0° C and anhydrous ethanol (1 mL) was added followed by an aqueous solution (5 mL) of ammonium chloride (500 mg). The colorless solution was evaporated to a solid residue and, after addition of acetone (40 mL), the mixture was filtered. The residue was washed with acetone and the combined filtrate, concentrated to a small volume (5 mL), was applied to a silica gel column. The column was eluted with acetone to give a white hygroscopic solid (255 mg, 61 %). Rf = 0.65 (acetone); IR (KBr) ν_{max} 3000-3200 (NH), 1620 (CO, amide, sulfonamide), 1350 (SO_2).

4-Amino-5-carboxamido-7-(5-O-sulfamoyl-ß-D-ribofuranosyl)pyrrolo[2,3-d]-pyrimidine (4b). A solution of **3b** in formic acid (88%, 5 mL) was stirred at room temperature for 20 hr. Work-up of the mixture as described for **4a** gave **4b** as a white solid (150 mg, 77%), which was crystallized from methanol/ethanol. Rf = 0.15 (methanol/chloroform, 1/4); mp 264 - 5° C (dec); IR (KBr) ν_{max} 3200-3500 (OH, NH), 1615 (CO, amide), 1185 and 1370 (SO_2) cm^{-1}. Anal. Calcd. for $C_{12}H_{16}N_6O_7S$: C, 37.11; H, 4.12; N, 21.64. Found: C, 36.96; H, 4.40; N, 21.80.

4-Amino-5-carboxamido-7-(5-O-methanesulfonyl-2,3-O-isopropylidene-ß-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (3c). Sodium hydride (80%, 1 g, 33.0 mmol) was washed with hexane and added in portions to a stirred solution of **2** (1,4 g, 4 mmol) in anhydrous tetrahydrofuran (40 mL) at 0 - 5° C. The mixture was stirred for 15 min, and a solution of methanesulfonyl chloride (5 g, 43.6 mmol) in tetrahydrofuran (10 mL) was added dropwise. The reaction mixture was stirred at room temperature for 4 hr, cooled to 0° C, and anhydrous ethanol (5 mL) was added with vigorous stirring followed by an aqueous solution (10 mL) of ammonium chloride (5 g). The mixture was evaporated and the residue dried under reduced pressure. The light-colored residue was washed by triturating with anhydrous ethyl ether and taken up in methanol (4 mL). The solution was applied to a short column of silica gel and the column eluted with anhydrous ether to remove colored impurities, and with acetone to furnish 4-amino-5-carboxamido-7-(2,3-O-isopropylidene-5-O-mesyl-ß-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (**3c**) as a white solid (1.4 g, 82 %) which was crystallized from methanol/ether. Mp 236-8° C (dec); Rf = 0.7 (acetone); IR (KBr) ν_{max} 3000 - 3400 (NH), 1630 (CO, amide), 1345 and 1145 (SO_2); ^1H NMR (methanol-d_4) δ 1.35 and 1.65 (6 H, 2s, isopropylidene), 3.15 (3 H, s, OMs), 6.35 (1 H, d, J= 3.5 Hz, H-1'), 8.35 (2 H, br. s, H-2 and H-6). Anal. Calcd. for $C_{16}H_{21}N_5O_7S$: C, 44.97; H, 4.95; N, 16.39. Found: C, 45.15; H, 5.05; N, 16.12.

4-Amino-5-carboxamido-7-(5-azido-5-deoxy-2,3-O-isopropylidene-ß-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (3d). A stirred solution of the 5'-O-mesyl derivative **3c** (800 mg, 1.9 mmol) in anhydrous N,N-dimethylformamide (15 mL) was heated with lithium azide (1 g, 20.5 mmol) at 80 - 90° C for 1 hr, the solvent removed by evaporation, and the residue triturated with acetone (50 mL). The mixture was filtered and the solution concentrated (~25 mL) and poured onto a short column of silica gel, which was eluted with acetone to give a semisolid residue. Trituration with ethyl ether/petroleum ether gave (480 mg, 62 %) of **3d**. Rf = 0.8 (acetone); IR (KBr) ν_{max} 3200 - 3500 (NH), 2160 (N_3), 1640 (CO, amide) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.2 and 1.45 (6 H, s, isopropylidene), 3.45 (2H, d J=4 Hz, H-5'), 4.15, 4.85, and 5.20 (3 H, m, H-3', H-2', H-4'), 6.1 (1 H, br. s, H-1'), 6.65 (2 H, br. s, NH_2), 7.4 (2 H, br. s, NH_2 - carboxamide), 7.65 (1 H, s, H-6), 8.10 (1 H, s, H-2).

4-Amino-5-carboxamido-7-(5-O-methanesulfonyl--ß-D-ribofuranosyl)pyrrolo-[2,3-d]pyrimidine (4c). A solution of **3c** (500 mg, 1.2 mmol) in formic acid (60 % 20 mL) was stirred at room temperature for 18 hr. The solution was evaporated to a solid residue which was co-evaporated repeatedly with ethanol and crystallized from hot ethanol to give 340 mg (75%) of 4c. Mp 236-8 °C; R_F = 0.4 (MeOH/$CHCl_3$, 1/4); IR (KBr) 3000-3500 (OH, NH), 1680, 1350 and 1180 cm-1 (SO_2). Anal. Calcd. for $C_{13}H_{17}N_5O_7S.H_2O$: C,38.52; H, 4.69; N, 17.28. Found: C,38.38; H, 4.72; N, 17.01.

4-Amino-5-carboxamido-7-(5-azido-5-deoxy-ß-D-ribofuranosyl)pyrrolo[2,3-d]-pyrimidine (4d). Compound **3d** (400 mg, 1.06 mmol) was treated with formic acid as described for **3a** to give **4d** as an amorphous solid (210 mg, 55%). Mp 200° C; Rf = 0.55 (methanol/ethyl acetate, 1/4); IR (KBr) ν_{max} 3100 - 3400 (NH, OH), 2095 (N_3), 1610 (CO, amide). Anal. Calcd. for $C_{12}H_{14}N_8O_4$: C, 43.11; H, 4.19; N, 33.52. Found: C,43.28; H, 4.38; N, 33.87.

4-Amino-5-cyano-7-(2,3-O-isopropylidene-ß-D-ribofuranosyl)pyrrolo[2,3-d]-pyrimidine (6). p-Toluenesulfonic acid monohydrate (1.0 g, 5.5 mmol) was added to a stirred suspension of toyocamycin (**5**)(1.0 g, 3.6 mmol) in anhydrous acetone (90 mL) and 2,2-dimethoxypropane (0.7 mL). The mixture was stirred at room temperature, progression of the reaction being monitored by TLC. After 3 hr, the solution was concentrated to approximately 10 mL, diluted with chloroform (10 mL) and poured onto a short column of silica gel. Elution of the column with acetone/chloroform (1/1) and evaporation of the solvent gave 1.0 g (90%) of **6** as a white foam. Rf = 0.55 (acetone/chloroform, 1/1); IR (KBr) ν_{max} 3500-3000 (NH, OH), 2195 (CN), 1600 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.30, 1.60 (6 H, 2 s, isopropylidene), 3.85 (2H, m, H-5'), 4.45 (1H, br s, H-4'), 5.15 (2H, m, H-2' and H-3'), 5.85 (1H, d, J=3.85 Hz, H-1'), 6.25 (2H, br s, NH_2), 7.65 (1H, s, H-6), 8.20 (1H, s, H-2).

4-Amino-5-cyano-7-(2,3-O-isopropylidene-5-O-nitro-ß-D-ribofuranosyl)-pyrrolo[2,3-d]pyrimidine (7a). A cold (-38° C) mixture of fuming (90%) nitric acid (0.5 mL, 3.6 mmol) and acetic anhydride (0.5 mL, 0.5 mmol) was added dropwise to a stirred solution of **6** (250 mg, 0.75 mmol) in anhydrous ethyl acetate (10 mL) at -30° C. The reaction mixture was stirred at -30° C for 70 min, neutralized with solid sodium hydrogen carbonate and stirred at room temperature for an additional 20 min before dilution with ethyl acetate and filtration. The filtrate was washed with water, dried ($MgSO_4$) and evaporated to give a gummy residue which was taken up in chloroform and poured onto a column of silica gel. The column was eluted with chloroform to remove some colored impurities and with acetone/petroleum ether (1/1) to elute product **7a**, obtained after evaporation of the solvent as a white foamy solid (210 mg, 74%). Rf = 0.75 (acetone/petroleum ether, 1/1); IR (KBr) ν_{max} 3200 (NH_2), 2200 (CN), 1600, 1275 (NO_2); ^1H NMR ($CDCl_3$) d 1.30, 1.55 (6H, 2s, isopropylidene), 4.40 (1H, m, H-4'), 4.55 (2H, m, H-5'), 5.10, 5.25 (2H, m, H-2' and H-3'), 6.00 (1H, d, J=3.50 Hz, H-1'), 6.30 (2H, br s, NH_2), 7.55 (1H, s, H-6), 8.25 (1H, s, H-2).

4-Amino-5-cyano-7-(5-O-nitro-ß-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (8a) A solution of **7a** (150 mg, 0.41 mmol) in formic acid (60%, 4 mL) was stirred at room temperature for 18 hr. The solution was evaporated to a syrupy residue which was co-evaporated with anhydrous ethanol (2x 50 mL) to give a white solid (120 mg, 87 %). Rf = 0.68 (methanol/chloroform, 1/4). The solid was crystallized from ethanol/ether; mp 267-69° C; IR (KBr) ν_{max} 3500-2900 (NH, OH), 2195 (CN), 1600, 1270 (O-NO_2). Anal. Calcd. for $C_{12}H_{12}N_6O_6$: C, 42.85; H, 3.57; N, 25.00. Found: C, 43.01; H, 3.37; N, 25.14.

Biology:

The inhibitory effects of the compounds on ML-1 cell growth were determined by adding graded concentrations of the agents to 3 x 10^5 cells/mL of RPMI 1640 medium containing 3% FBS. Incubation was carried out at 37° C for 3 days in a 5% CO_2 atmosphere. Cell numbers were counted by hemocytometer.

Enzyme Assays. PKC was assayed by measuring the incorporation of ^{32}P from [γ - ^{32}P] ATP into histone III-S, using a previously published method[11] with minor modifications. The reaction mixture contained 20 mM Tris/HCl, pH 7.5, 0.5 mM calcium chloride, 10 mM magnesium acetate, 10 μM [γ - ^{32}P] ATP (0.5 μCi/nmole), 12 μg/mL phosphatidylserine, 12 μg/mL 1,2-dioleoylglycerol and 0.01 unit of PKC in the total volume 250 μL. The reaction, initiated by the addition of enzyme, proceeded at 30° C for 3 min and was stopped with 3 mL of ice-cold 25% TCA. The reaction mixture was applied to a Whatman GF/C filter and washed with 20 mL ice-cold 10% TCA. Filters were placed into 10 mL of CytoScint (Biomedicals) and radioactivity was measured using a Beckman liquid scintillation spectrometer, Model 1800. Protein kinase A activity was similarly assayed, except that cyclic AMP was added at a final concentration of 1 μM in place of lipids and calcium chloride.

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