Articles

(*S*)-13-[(Dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione (LY333531) and Related Analogues: Isozyme Selective Inhibitors of Protein Kinase C*β*

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Protein kinase C (PKC) is a family of closely related serine and threonine kinases. Overactivation of some PKC isozymes has been postulated to occur in several disease states, including diabetic complications. Selective inhibition of overactivated PKC isozymes may offer a unique therapeutic approach to disease states such as diabetic retinopathy. A novel series of 14-membered macrocycles containing a N−N′-bridged bisindolylmaleimide moiety is described. A panel of eight cloned human PKC isozymes (α, *β*I, *β*II, *γ*, *δ*, *ϵ*, *ζ*, *η*) was used to identify the series and optimize the structure and associated activity relationship. The dimethylamine analogue LY333531 (**1**), (*S*)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione, inhibits the PKC*β*I (IC_{50} = 4.7 nM) and PKC*β*II (IC_{50} = 5.9 nM) isozymes and was 76- and 61-fold selective for inhibition of PKC*β*I and PKC*β*II in comparison to PKCα, respectively. The additional analogues described in the series are also selective inhibitors of PKC*β*. LY333531 (**1**) exhibits ATP dependent competitive inhibition of PKC*β*I and is selective for PKC in comparison to other ATP dependent kinases (protein kinase A, calcium calmodulin, caesin kinase, src tyrosine kinase). The cellular activity of the series was assessed using bovine retinal capillary endothelial cells. Retinal endothelial cell dysfunction has been implicated in the development of diabetic retinopathy. Plasminogen activator activity stimulated by a phorbol ester (4*β*-phorbol 12,13-dibutyrate) in endothelial cells was inhibited by the compounds in the series with ED_{50} values ranging from 7.5 to 0.21 *μ*M. A comparison of the PKC isozyme and related ATP dependent kinase inhibition profiles is provided for the series and compared to the profile for staurosporine, a nonselective PKC inhibitor. The cellular activity of the series is compared with that of the kinase inhibitor staurosporine.

Introduction

The association between the development of diabetic complications and the level of glycemic control in diabetic patients has been demonstrated in a number of studies, including the diabetes control and complications trial (DCCT).[1] Hyperglycemia causes an elevation of diacylglycerol (DAG), an endogenous second messenger that activates protein kinase C (PKC).[2] Tissues subject to developing diabetic complications contain elevated levels of DAG.[2,3] The elevation in tissue levels of DAG takes several days to manifest itself and remains elevated several days after the tissue has been removed from a hyperglycemic environment. The hypothesis that PKC is overactivated in a diabetic state and contributes significantly to the development of diabetic complications encouraged us to initiate a program to identify a PKC selective antagonist.[4,5] Hyperactivation of PKC*β* relative to other isozymes (PKCα, *γ*, *δ*, *ϵ*, *ζ*, *η*) in tissue from diabetic animal models, such as retina and kidney glomeruli, in addition to platelets from human focused our effort on developing an isozyme selective antagonist.[6] Therapeutically, a PKC*β* selective antagonist could offer a new approach for intervention in the progression or development of diabetic retinopathy.

PKC consists of a family of closely related enzymes that function as serine and threonine kinases.[7] These enzymes were originally identified as a histone protein kinase from rat brain and activated by limited proteolysis or by calcium and lipids. Further work revealed PKC to be the molecular target for a class of tumor promoters, the phorbol esters.[8] Subsequent to the initial observations that rat brain PKC was composed of at least four isozymes (α, *β*I, *β*II, *γ*), further screening of cDNA libraries has identified an additional eight related proteins to bring the membership of the family to 12 isozymes or subtypes.[9]

A panel of eight cloned human PKC isozymes (α, *β*I,

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βII, γ, δ, ϵ, ζ, η) was used to identify and optimize a series of PKCβ selective antagonists. The panel is representative of the calcium dependent PKC isoforms (α, β, γ), the calcium independent isoforms (δ, ϵ, η), and the atypical isoforms (ζ). Until recently the isozyme selectivity of the related acyclic bisindolylmaleimide PKC inhibitors had not been realized.[10] Subsequently, some of the acyclic bisindolylmaleimides have been reported to preferentially inhibit PKCα.[11] Using assay conditions that simulate the endogenous activation of PKC with DAG and phosphatidylserine, the macrocyclic bisindolylmaleimides reported here are PKCβ selective in comparsion to the other isozymes in the panel.[12] The acyclic bisindolylmaleimides have also been reported to be competitive inhibitors of ATP binding presumably by interacting at the ATP binding site.[13] An additional criterion for selectivity is preferential inhibition of the PKCβ isozymes versus other ATP dependent kinases. An ATP dependent kinase panel consisting of four kinases (PKA, calcium calmodulin, casein kinase, src tyrosine kinase) was used in tandem with the PKC isozyme panel to demonstrate the kinase selectivity of the macrocyclic bisindolylmaleimides.

The cellular activity of the series was assessed in an endothelial cell culture assay measuring the inhibition of plasminogen activator (PA) activity.[14] Endothelial cell dysfunction is a key consideration in the development of diabetic complications and the progression of diabetic retinopathy.[15] Retinal capillary endothelial cells were treated with 4β-phorbol 12,13-dibutyrate (4β-PDBu) that stimulated PA activity. Plasminogen activator activity was determined in the cell lysate with the synthetic substrate H-D-Val-L-Leu-L-Lys-*p*-nitroaniline dihydrochloride and plasminogen. In the presence of the PKC inactive diastereomer 4α-phorbol 12,13-dibutyrate (4α-PDBu), no PA activity above the basal level was detected. Cellular toxicity of the series was determined in a parallel series of cultures using a neutral red viability assay to monitor lysosomal integrity.[16]

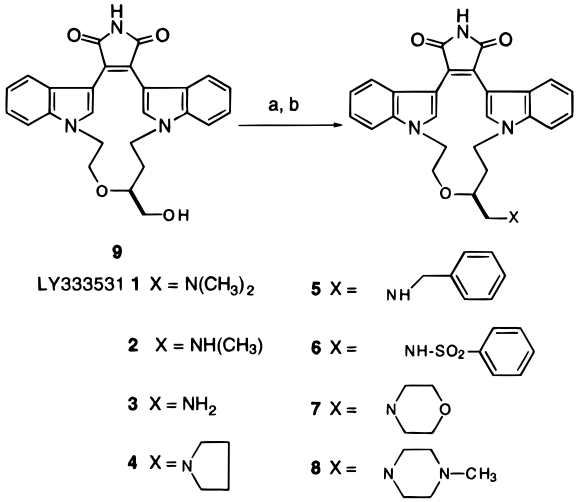
Chemistry

We recently reported the synthesis of a new structural class of protein kinase C inhibitors that are macrocyclic bisindolylmaleimides.[17] The series represented by compounds **1**−**9** are members of this structural class and possess an activity profile that could be of utility in several disease states. LY333531 (**1**), the primary amine **3**, pyrrolidine **4**, benzylamine **5**, sulfonamide **6**, morpholine **7**, and piperazine **8** were prepared from alcohol **9** (Scheme 1). Alcohol **9** was reacted with methanesulfonic anhydride to produce a mesylate that underwent displacement with dimethylamine, ammonia, pyrrolidine, the sodium salt of benzenesulfonamide, morpholine, or piperazine to yield the desired compounds **1** and **3**−**8**, respectively. The secondary amine **2** was prepared from alcohol **9** by displacement of the corresponding triflate with methylamine as formation of the *N*-methylmaleimide of **2** was observed when the corresponding mesylate of **9** was reacted with methylamine.

Biological Activity

PKC Isozyme Selectivity. LY333531 (**1**), and the additional analogues **2**−**9**, produced selective inhibition of PKCβ when assayed in the isozyme panel (Table 1).

Scheme 1[a]



[a] (a) Methanesulfonic anhydride, pyridine, CH_2Cl_2, 52%; (b) THF, amine for **1** and **3**−**8**, for **2** THF, trifluoromethanesulfonic anhydride, −78 °C, 33% aqueous methylamine in ethanol, −40 °C, 38%.

The selective low nanomolar inhibition of PKCβI and PKCβII with **1** versus other calcium dependent isozymes (PKCα and PKCγ) is remarkable given the high degree of shared homology in the ATP binding region.[18] The IC_{50} values for inhibition of the calcium independent isozymes (PKCδ and PKCϵ) and calcium dependent isozymes (PKCα and PKCγ) by **1** are comparable. The selectivity ratio (PKCx/PKCβII IC_{50} value) of **1** for the PKCβ isozymes versus PKCα, PKCγ, PKCδ, and PKCϵ is also comparable. PKCζ, an example of an atypical isoform, was least sensitive to inhibition by either the compounds in this series or staurosporine. The activity profile of methylamine **2** was similar to that of dimethylamine **1** in both activity (IC_{50} = 5 nM) and selectivity (94-fold versus PKCα) for PKCβ. Primary amine **3**, pyrrolidine **4**, morpholine **7**, and piperazine **8** were weaker inhibitors of PKCβ than either **1** or **2**. The benzylamine analogue **5** was one of the least active compounds and exhibited lower selectivity for PKCβ in comparison to the other isozymes in the panel. When the basic amine site is altered such as in sulfonamide **6**, the selectivity ratio of PKCβII versus PKCα is increased (>114-fold selectivity) relative to benzylamine **5** (20-fold selectivity). Alcohol **9** exhibited moderate isozyme selectivity. Staurosporine, an indolocarbazole, did not exhibit significant selectivity toward any of the calcium dependent isozymes (PKCα, βI, βII, γ).[19] Staurosporine also had minimal selectivity for the calcium dependent isozymes as a class versus the calcium independent (PKCδ, ϵ, η) isozymes.[20]

A number of compounds containing a bisindolylmaleimide structural component have been implicated as competitive inhibitors of ATP binding.[10,11] LY333531 (**1**) displays kinetics consistent with direct competition for ATP binding (Figure 1) to PKCβI. The ATP concentration was varied in a series of inhibition experiments of PKCβI activity with **1**. A double-reciprocal dose−response plot (Figure 1A) of data from the inhibition experiments was consistent with **1** acting as a direct competitive inhibitor with ATP for binding to PKCβI. The apparent k_i (2 nM) of **1** obtained from a Dixon plot (Figure 1B) is consistent with the observed IC_{50} value for inhibition of PKCβI (4.7 nM) and PKCβII (5.9 nM).

Table 1. PKC Isozyme IC_{50} Values[a]

compd	PKC isozyme IC_{50} (μM)							
	α	βI	βII	γ	δ	ϵ	ζ	η
1	0.36	0.0047	0.0059	0.30	0.25	0.60	>100	0.052
2	0.24	0.005	0.005	0.27	0.17	1.1	22	0.031
3	1.1	0.048	0.033	2.1	2.6	>5.0	44	0.24
4	3.5	0.12	0.044	2.2	3.3	3.8	55	0.35
5	6.3	0.33	0.31	6.5	7.8	57	54	2.5
6	>5.0	0.17	0.044	>5.0	0.88	>5.0	>5.0	1.9
7	3.5	0.049	0.038	2.5	2.0	>5.0	>5.0	1.7
8	1.8	0.030	0.024	3.2	2.8	>5.0	>5.0	2.00
9	0.72	0.073	0.028	2.0	0.69	79	8.7	0.30
staurosporine	0.045	0.023	0.019	0.11	0.028	0.018	>1.5	0.005

[a] Measurements are the average of at least three independent determinations from eight-point titration curves. The typical standard deviation was 30% of the IC_{50} value. Cloned human isozymes were used in the assay that were activated by DAG using phosphatidylserine vesicles incubated with [^{32}P]ATP and histone or myelin basic protein as a substrate.

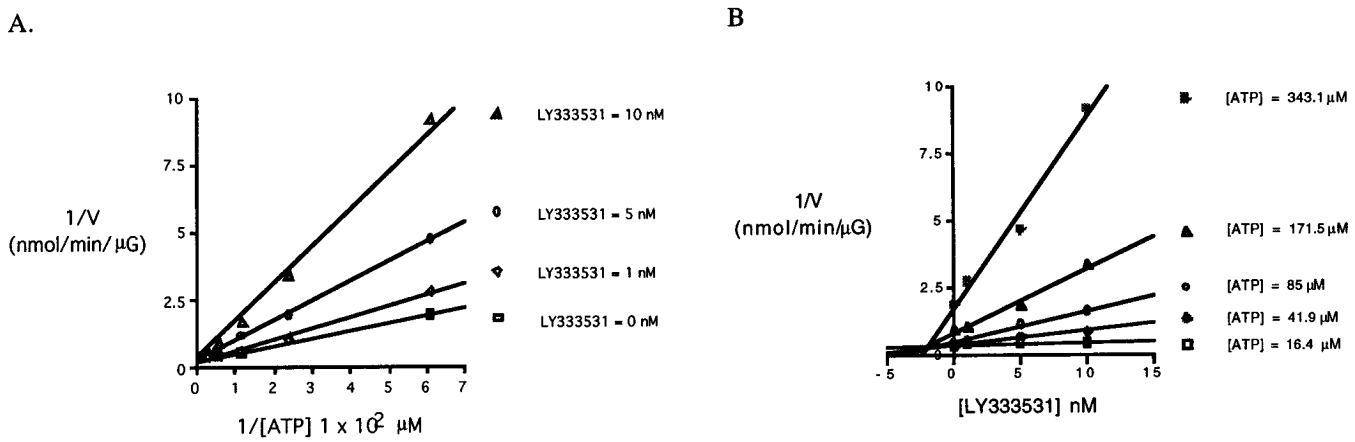


Figure 1. Lineweaver−Burk (A) and Dixon (B) plots for inhibition of PKCβI isozyme with LY333531 (**1**) (average of two independent determinations).

Table 2. ATP Dependent Kinase IC_{50} Values and IC_{50} Values for Inhibition of Rat Brain PKC[a]

compd	kinase IC_{50} (μM)				
	PKA[b]	Ca calmodulin[c]	casein K[d]	src-TK[e]	RB-PKC[f]
1	>100	6.2	>100	>100	0.32
2	49	2.2	>100	NT	0.16
3	83	2.7	>100	89	0.79
4	>100	5.3	>100	11	1.9
5	>100	4.4	>100	>100	4.2
6	>100	56	>100	>100	10
9	>100	6.0	>100	>100	0.63
staurosporine	0.10	0.004	14	0.001	0.19

[a] The values are the average of at least three independent determinations from eight-point titration curves. [b] Bovine heart cAMP dependent protein kinase catalytic subunit kinase assay. [c] Purified mammalian brain calcium calmodulin dependent protein kinase assay. [d] Purified rat brain casein protein kinase II assay. [e] src protein tyrosine kinase assay. [f] Purified rat brain protein kinase C assay.

Kinase Selectivity. The compounds **1**−**9** in the series are also PKC selective relative to other ATP dependent kinases. Comparison of IC_{50} values for inhibition of the catalytic subunit of PKA, calcium calmodulin kinase, casein type II kinase, or src tyrosine kinase indicates **1** has 3 orders of magnitude selectivity for PKCβII versus calcium calmodulin (Table 2) and was inactive against the other ATP dependent kinases tested. Secondary amine **2**, primary amine **3**, pyrrolidine **4**, benzylamine **5**, and alcohol **9** also have a similar selectivity pattern for PKC versus these kinases. The sulfonamide **6** was 1 order of magnitude less active in inhibition of calcium calmodulin kinase than the other compounds in the series. Pyrrolidine **4** was the least selective inhibitor against src tyrosine kinase. A comparison of the data from Tables 1 and 2 indicates that staurosporine is a more potent inhibitor of both calcium calmodulin kinase and src tyrosine kinase than either PKCβII or most of the other PKC isozymes tested. A high degree of species homology exists between the PKC isozymes.[21] The activity profile of the LY333531 series was optimized against the individual human PKC isozymes; however, **1**−**3** and **9** are also nanomolar potent inhibitors of rat brain PKC (RB-PKC) which consists of a mixture of isozymes from this species.

Cellular Activity. An endothelial cell-based plasminogen activator assay, coupled with a neutral red viability assay, was used to determine the cellular activity of the series. The activity of the series was measured as inhibition of the induced elevated plasminogen activator activity stimulated by β-PDBu. The dose−response curve (Figure 2) obtained for **1** indicates that these compounds effectively block PKC-mediated events such as the release of plasminogen activator with minimal cellular toxicity (left axis *verses* right axis). Cellular activity is maintained through the series with **1** having an EC_{50} in the 200 nM range (Table 3). Secondary amine **2**, pyrrolidine **4**, and piperazine **8** also have submicromolar EC_{50} values in this assay. Primary amine **3**, benzylamine **5**, sulfonamide **6**, morpholine **7**, and alcohol **9** are active in the micromolar range. None of the compounds were found to be toxic in the neutral red viability assay. Comparatively, staurosporine was toxic in the endothelial cell culture assay at the ED_{50} dose.

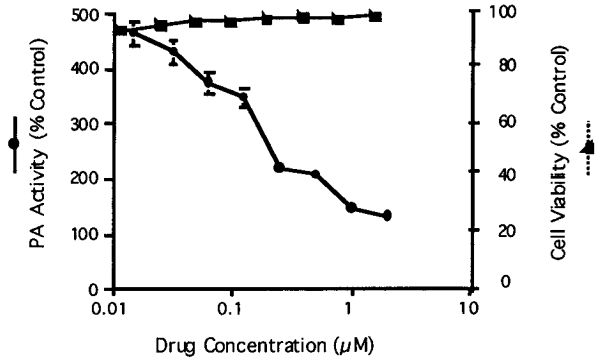


Figure 2. Dose−response of LY333531 (**1**) inhibition of β-PDBu-stimulated plasminogen activator activity (left axis). The compound blocks PA activity with minimal cellular toxicity (right axis) (average values of two determinations, see Table 3).

Table 3. Endothelial Cell-Based Plasminogen Activator Assay Activity

compd	EC_{50} (μM)[a]	$T_{ox_{50}}$ (μM)[b]	n[c]
1	0.21 (±0.08)[d]	>20	5
2	0.24 (±0.07)[d]	>20	7
3	7.5 (±1.5)[e]	>20	2
4	0.55 (±0.15)[e]	>10	2
5	4.3 (±0.81)[e]	>20	2
6	>2	>2	2
7	3.5 (+0.52)[e]	>20	2
8	0.55 (±0.23)[e]	>20	2
9	3.0 (±0.53)[e]	>20	2
staurosporine	0.02	<0.02	1

[a] EC_{50} is the effective concentration for 50% inhibition of PA activity relative to control by β-PDBu stimulation. [b] $T_{ox_{50}}$ is the concentration for 50% cell death determined by lysosomal staining in the neutral red viability assay. [c] n is the number of independent determinations. [d] σ −1 standard deviation. [e] Average value of two independent determinations assayed in duplicate.

Conclusions

In summary a novel class of macrocyclic bisindolylmaleimides has been synthesized. LY333531 and related compounds exhibit nanomolar inhibition of PKCβ and at least 60-fold selectivity for PKCβ versus PKCα. Kinase selectivity is also maintained in the series; LY333531 is several orders of magnitude more selective for inhibition of PKCβ in comparsion to other ATP dependent kinases such as calcium calmodulin (1060-fold selective) or src tyrosine kinase (2200-fold selective). In comparison, staurosporine is a more potent inhibitor of both calcium calmodulin and src tyrosine kinase than PKC.

The retinal capillary endothelial cell activity is encouraging in light of the importance of endothelial cell dysfunction in the development of diabetic retinopathy. The importance of PKC and the implications of specific isozymes in the development of diabetic complications and other disease states suggest these agents may offer a novel therapeutic approach.

Experimental Section

Melting points were determined on a MEL-TEMP II apparatus and are uncorrected. NMR spectra were recorded on a GE QE-300 spectrometer. All chemical shifts are reported in parts per million (δ) relative to tetramethylsilane. The following abbreviations are used to denote signal patterns: s = singlet, d = doublet, t = triplet, b = broad, m = multiplet. The mass spectral data were determined on VG ZAB-2SE (fast atom bombardment conditions) and VG ZAB-3F (field desorption conditions) spectrometers. Infrared spectral data were determined using a Nicolet DX10 FT-IR spectrometer. Silica gel flash chromatography was performed using Mallinckrodt SilicAR 230−400 mesh. Reverse phase chromatography was performed on a Waters HPLC system and a LC-908-G30 recycling preparative HPLC from Japan Analytical Instrument. In general, commercially available dry solvents were used. Acetone was distilled from anhydrous calcium sulfate prior to use.

(*S*)-10,11,14,15-Tetrahydro-13-(hydroxymethyl)-4,9:16,-21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]-oxadiazacyclohexadecene-1,3(2*H*)-dione (9). To a stirred solution of (*S*)-methyl 4-[(*tert*-butyldiphenylsilyl)oxy]-3-hydroxybutyrate (21.5 g, 57.7 mmol) in cyclohexane (400 mL) at ambient temperature under a nitrogen atmosphere was added allyl trichloroacetamidate (23.4 g, 115 mmol) followed by trifluoromethanesulfonic acid (1 mL, 50 μL/g alcohol) in five portions over 30 min. After stirring for 70 h, the reaction mixture was filtered to remove the precipitate followed by concentration of the filtrate *in vacuo*. The concentrate was eluted through a pad of silica gel using an ethyl acetate in hexane gradient (0−10%). The eluant was concentrated to give a mixture containing (*S*)-methyl 4-[(*tert*-butyldiphenylsilyl)oxy]-3-(allyloxy)butyrate (27 g, expected mass 24 g). NMR analysis of this mixture indicated the presence of residual imidate (ca. 10%). This mixture was used in the next reaction without further purification.

To a stirred solution of the above ester (23.8 g, 57 mmol) in anhydrous THF (1.0 L) at −75 °C under a nitrogen atmosphere was added a 1 M solution of DIBAL-H in toluene (231 mL) dropwise over 40 min. After 1.5 h, the mixture was warmed up to −10 °C and the reaction quenched with 5% water in MeOH and Celite. The suspended solids were filtered and rinsed with THF. The filtrate was concentrated and partitioned between ether and 20% citric acid. The ether layer was separated, dried, filtered, and concentrated *in vacuo* to afford the crude alcohol. The crude alcohol was eluted through a pad of silica gel with chloroform to obtain the purified alcohol (20.6 g, 93%).

The alcohol (20.6 g, 53.6 mmol) was dissolved in MeOH (500 mL) under a nitrogen atmosphere at −78 °C. Ozone was bubbled through the solution for 12 min, and excess ozone was apparent from the blue color of the solution. Nitrogen was passed through the reaction mixture displacing the excess ozone, and $NaBH_4$ (12.2 g, 321 mmol) was added to the reaction mixture. The reaction mixture was warmed to ambient temperature followed by concentration *in vacuo* to give a residue that was eluted through a pad of silica gel with ethyl acetate. Concentration of the eluant gave the diol (16.4 g, 79%) as a colorless oil.

The diol (15.7 g, 40.4 mmol) was dissolved in ether (600 mL) containing triethylamine (16.8 mL, 121 mmol) at 0 °C under a nitrogen atmosphere. To this mixture was added methanesulfonyl chloride (9.38 mL, 121 mmol). After stirring for 3 h, the reaction mixture was filtered; the filtrate was washed with water and brine, dried over $MgSO_4$, and concentrated to give the bismesylate (21.9 g, 99% yield).

To a stirred solution of the bismesylate (21.9 g) in freshly distilled dry acetone (1.4 L) were added NaI (90.4 g, 603 mmol) and $NaHCO_3$ (170 mg, 2 mmol). After 24 h at 56 °C the reaction mixture was filtered, concentrated, and partitioned between ether and 10% Na_2SO_3. The ether layer was separated, washed with brine, dried over $MgSO_4$, and concentrated *in vacuo* to give the bisiodide (*S*)-1-[(*tert*-butyldiphenylsilyl)-oxy]-2-[(2-iodoethyl)oxy]-4-iodobutane (17.9 g, 73.2%).

The above bisiodide (17.9 g, 29.4 mmol) and *N*-methylbisindolylmaleimide (10.0 g, 29.4 mmol) were dissolved in anhydrous DMF (80 mL). This solution was added over 72 h to a stirred suspension of cesium carbonate (38.3 g, 118 mmol) in anhydrous DMF (1.7 L) at 50 °C under a nitrogen atmosphere. The reaction mixture was concentrated and partitioned between $CHCl_3$ and 1 N HCl, and the aqueous layer was extracted with chloroform and ethyl acetate. The combined organic layers were washed with 1 N HCl, water, and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was used without further purification.

The above product was suspended in ethanol (700 mL) containing 5 N KOH (800 mL). The stirred suspension was heated (80 °C), and after 72 h the reaction mixture was concentrated, cooled to 0 °C, and acidified with 5 N HCl. A violet precipitate formed that was collected. The precipitate was eluted through a pad of silica gel with ethyl acetate to obtain the cyclized product (8.7 g).

The precipitate (8.7 g, 19.7 mmol) was dissolved in anhydrous DMF (1 L) followed by addition of a mixture of 1,1,1,3,3,3-hexamethyldisilazane (41.6 mL, 197 mmol) and methanol (4 mL, 98.5 mmol) under a nitrogen atmosphere. After stirring for 40 h, the DMF was removed *in vacuo*, and MeCN/1 N HCl (2:1, v/v) was added to the residue. After stirring for 1 h, the reaction mixture was concentrated and extracted with ethyl acetate, dried over $MgSO_4$, filtered, and concentrated to give a solid that was eluted through a pad of silica gel with CH_2Cl_2/MeCN (9:1) to obtaine pure (*S*)-10,11,-14,15-tetrahydro-13-(hydroxymethyl)-4,9:16,21-dimetheno-1*H*,-13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione, **9** (6.5 g, 75%), as a magenta solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.92−2.14 (m, 2H), 3.64−3.30 (m, 4H), 3.91 (m, 1H), 4.16−4.37 (m, 4H), 7.12 (t, J = 7.17 Hz, 2H), 7.18 (t, J = 8.12 Hz, 2H), 7.49 (dd, J = 19.12, 19.85 Hz, 4H), 7.80 (d, J = 8.09 Hz, 1H), 7.84 (d, J = 8.09 Hz, 1H), 10.96 (bs, 1H); IR (KBr) cm^{-1} 3446, 2931, 1703, 1470, 1288, 743; HRMS (FAB) calcd for $C_{26}H_{23}N_3O_4$ 441.1767, found 442.1771 (M^+ + 1); OR $[\alpha]_D$ −11.26° (*c* 1.0, MeOH) at 25 °C. Anal. $C_{26}H_{23}N_3O_4$-$(H_2O)_{1.5}$ C, H, N.

(*S*)-13-[(Dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*]-[1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione Monohydrochloride (1). The title compound was prepared by the following sequence of reactions. To a stirred suspension of **9**, (*S*)-10,11,14,15-tetrahydro-13-(hydroxymethyl)-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3-(2*H*)-dione (8.9 g, 20 mmol), in anhydrous CH_2Cl_2 (800 mL) under nitrogen at ambient temperature was added pyridine (4.85 mL, 60 mmol) followed by methanesulfonic anhydride (4.21 g, 24 mmol). After 16 h, the reaction mixture was washed with 0.1 N HCl and brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to afford a magenta solid that was purified by gradient elution through a column of silica gel (0−10% MeCN in CH_2Cl_2) to obtain (*S*)-10,11,14,-15-tetrahydro-13-[[(methylsulfonyl)oxy]methyl]-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione (9.4 g, 90%).

To a stirred solution of (*S*)-10,11,14,15-tetrahydro-13-[[(methylsulfonyl)oxy]methyl]-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione (2.8 g, 5.39 mmol) in THF (300 mL) was added dimethylamine (100 mL, 40% in water). The reaction vessel was sealed and heated to 50 °C. After 24 h the reaction vessel was cooled to 0 °C and the mixture concentrated *in vacuo*. The residue was eluted through a column of silica gel with a gradient of ethyl acetate to 10% triethylamine in ethyl acetate to give the free base as a violet solid (1.7 g, 67%).

The free base (1.7 g, 3.6 mmol) was suspended in methanol (300 mL) and treated with 1.0 N anhydrous HCl in ether (10 mL, 10 mmol). After stirring for 30 min, a bright orange precipitate formed and was collected. The orange cake was washed with ether and dried under vacuum to give the hydrochloride salt of (*S*)-13-[(dimethylamino)methyl]-10,11,-14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione monohydrochloride, **1** (1.6 g, 88% yield): ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.01−2.16 (m, 1H), 2.26−2.40 (m, 1H), 2.68 (s, 6H), 3.10−3.24 (m, 1H), 3.24−3.34 (m, 1H), 3.60−3.74 (m, 1H), 3.74−3.92 (m, 2H), 4.08−4.22 (m, 1H), 4.22−4.44 (m, 3H), 7.04−7.26 (m, 4H), 7.40−7.60 (m, 4H), 7.76−7.86 (m, 2H), 10.59 (bs, 1H), 10.96 (s, 1H); IR (KBr) cm^{-1} 3411.55, 3163.66, 1691.79, 1623.32, 1512.38, 1474.77, 1314.88, 1206.63; MS (FD) calcd for $C_{28}H_{29}N_4O_3Cl$ 504.5, found 468 (M^+ − HCl); OR $[\alpha]_D$ −28.7° (*c* 1.0, EtOH) at 25 °C. Anal. ($C_{28}H_{29}N_4O_3Cl$) C, H, N.

(*S*)-13-[(Monomethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione Monohydrochloride (2). To a stirred solution of (*S*)-10,11,14,15-tetrahydro-13-(hydroxymethyl)-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione (1.32 g, 3.0 mmol) in anhydrous tetrahydrofuran (120 mL) under nitrogen was added 2,4,6-collidine (1.19 mL, 9 mmol) followed by trifluoromethanesulfonic anhydride (1.52 mL, 9 mmol) at −78 °C. The reaction mixture was stirred for 40 min, at which time a solution of 33% methylamine in ethanol (19 mL, 150 mmol) was added. The reaction mixture was stirred at −78 °C for 10 min, at which time the dry ice/acetone bath was replaced with a dry ice/acetonitrile bath and the reaction mixture allowed to warm to room temperature over 18 h. The reaction mixture was concentrated *in vacuo* to yield a precipitate that was collected on a filter plate washing with ethyl acetate. The filtrate was washed with water, and the aqueous layer was back-extracted with ethyl acetate. The combined organic phases were dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give a residue. The residue and the precipitate were combined and eluted through a silica gel column with a 50% ethyl acetate/hexane to ethyl acetate and then 2% isopropylamine/ethyl acetate gradient. Removal of the eluting solvent and conversion of the free base to the hydrochloride salt was the same as described previously to give (*S*)-13-[(monomethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione monohydrochloride, **2** (520 mg, 38%), as a red powder: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.19−2.04 (m, H), 2.14−2.22 (m, 1H), 2.45−2.55 (m, 3H), 2.91−2.97 (m, 1H), 3.17−3.26 (m, 1H), 3.53−3.61 (m, 1H), 3.63−3.78 (m, 1H), 3.78−3.82 (m, 1H), 4.05−4.43 (m, 4H), 7.08 (t, J = 7.51 Hz, 2H), 7.17 (t, J = 7.48 Hz, 2H), 7.41−7.50 (m, 3H), 7.53 (d, J = 8.00 Hz, 1H), 7.77 (t, J = 7.26 Hz, 2H), 8.61 (bs, 2H), 10.91 (s, 1H); IR (KBr) cm^{-1} 2995, 2997, 2712, 1699, 1524, 1507, 1395, 1355, 748, 741; HRMS (FAB) calcd for $C_{27}H_{27}N_4O_3$ 455.2083, found 455.2086; OR $[\alpha]_D$ 80.8° (*c* 0.5, MeOH) at 25 °C. Anal. ($C_{27}H_{26}N_4O_3$·HCl) C, H, N.

(*S*)-13-(Aminomethyl)-10,11,14,15-tetrahydro4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]-oxadiazacyclohexadecene-1,3(2*H*)-dione Monohydrochloride (3). The mesylate (*S*)-10,11,14,15-tetrahydro-13-[[(methylsulfonyl)oxy]methyl]-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione (85 mg, 0.164 mmol) was dissolved in dioxane (17 mL) containing ammonium hydroxide (6 mL, concentrated) in a sealed tube. After 24 h at 60 °C, the reaction mixture was cooled to 0 °C and concentrated producing a red residue that was purified using reverse phase gel filtration HPLC (85% MeCN/H_2O, 0.01% TFA). Evaporation of the eluting solvent produced the TFA salt of (*S*)-13-(aminomethyl)-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo-[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione. The TFA salt was partitioned between ethyl acetate and 0.1 N NaOH, the ethyl acetate layer was concentrated, and the residue was dissolved in MeOH (2 mL). To this solution was added 4 N HCl/dioxane (1 mL, 1:1 by volume), and the reaction mixture was stirred for 1 h, at which time the reaction mixture was concentrated to produce (*S*)-13-(aminomethyl)-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo-[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione monohydrochloride, **3** (12 mg, 15%), as a violet solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.90−2.06 (m, 1H), 2.06−2.15 (m, 1H), 2.70−2.86 (m, 1H), 2.96−3.06 (m, 1H), 3.55−3.66 (m, 1H), 3.73−3.80 (m, 1H), 4.00−4.42 (m, 5H), 7.06 (t, J = 7.23 Hz, 2H), 7.15 (t, J = 7.32 Hz, 2H), 7.72 (d, J = 6.03 Hz, 1H), 7.76 (d, J = 6.34 Hz, 1H), 8.08 (bs, 3H), 10.91 (s, H); HRMS (FAB) calcd for $C_{26}H_{25}N_4O_3$ 441.1927, found 441.1918; OR $[\alpha]_D$ 65.5° (*c* 0.5, MeOH) at 25 °C.

(*S*)-13-(Pyrrolidinomethyl)-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,-13]oxadiazacyclohexadecene-1,3(2*H*)-dione Monohydrochloride (4). Compound **4** was prepared as described previously for compound **3** using pyrrolidine in THF (28.8 mg, 43%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.72−2.10 (m, 5H), 2.20−2.34 (m, 1H), 2.86−3.02 (m, 2H), 3.04−3.18 (m, 4H), 3.52−3.64 (m, 1H), 3.66−3.74 (m, 1H), 3.75−3.82 (m, 1H), 4.04−4.48 (m, 4H), 7.10 (t, J = 7.50 Hz, 2H), 7.18 (t, J = 7.50

Hz, 2H), 7.44 −7.53 (m, 4H), 7.81 (t, J = 7.50 Hz, 2H), 9.77 (bs, 1H), 10.96 (s, 1H); IR (KBr) cm^{-1} 3399.01, 1710.11, 1621.38, 1469.94, 1335.88, 1196.98; HRMS (FAB) calcd for $C_{30}H_{31}N_4O_3$ 495.2396, found 495.2404; OR $[\alpha]_D$ 20.3° (*c* 1.0, MeOH) at 25 °C.

(*S*)-13-[(Benzylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*]-[1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione Monohydrochloride (5). Compound **5** was prepared as described previously for compound **3** reacting the mesylate with benzylamine (45-fold excess) to yield **5** (10.2 mg, 9.3%) as a dark violet solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.00−2.07 (m, 1H), 2.19−2.22 (m, 1H), 2.80−2.91 (m, 1H), 3.11−3.15 (m, 1H), 3.50−3.64 (m, 1H), 3.70−3.77 (m, 1H), 3.81−3.88 (m, 1H), 3.99−4.42 (m, 6H), 7.11 (t, J = 9 Hz, 1H), 7.19 (t, J = 9 Hz, 1H), 7.38−7.46 (m, 6H), 7.53−7.61 (m, 5H), 7.80 (d, J = 9 Hz, 2H), 9.05 (bs, 1H), 10.96 (s, 1H); IR (KBr) cm $^{-1}$ 3020, 1711, 1468, 1193; MS (FD) calcd 567.11, found 531 (M^+ − HCl). Anal. ($C_{33}H_{31}N_4O_3\cdot H_2O$) C, H; N: calcd, 9.58; found, 9.13.

(*S*)-13-(Benzenesulfonamidomethyl)-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo-[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione Monohydrochloride (6). To a suspension of sodium hydride (264 mg, 33 mmol) in anhydrous dimethylformamide (15 mL) under nitrogen at 5 °C was added benzenesulfonamide (786 mg, 5 mmol) dissolved in anhydrous dimethylformamide (10 mL). The reaction mixture was stirred at room temperature for 2 h. The resulting sodium salt was cooled to 5 °C followed by slow addition of the mesylate (*S*)-10,11,14,15-tetrahydro-13-[[(methylsulfonyl)oxy]methyl]-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3-(2*H*)-dione (520 mg, 1 mmol) dissolved in anhydrous dimethylformamide (10 mL). The reaction mixture was heated (50 °C) for 20 h and then partitioned between 1 N HCl (200 mL) and dichloromethane (400 mL). The organic layer was separated, and the aqueous layer was further extracted with dichloromethane (2 × 100 mL). The combined organic phases were washed with brine, dried over $MgSO_4$, filtered, and evaporated *in vacuo* to give a solid that was purified by silica gel chromatography eluting the column sequentially with dichloromethane, 5% acetonitrile in dichloromethane, and finally 10% acetonitrile in dichloromethane to provide (*S*)-13-(benzenesulfonamidomethyl)-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione monohydrochloride, **6** (86 mg, 15%), as a purple solid: ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.90−1.95 (m, 1H), 2.00−2.07 (m, 1H), 2.76−2.82 (m, 1H), 2.90−2.96 (m, 1H), 3.25−3.45 (m, 2H), 3.66−3.70 (m, 1H), 3.98−4.09 (m, 2H), 4.15−4.21 (m, 1H), 4.26−4.32 (m, 1H), 7.04−7.18 (4H, m), 7.36−7.39 (m, 3H), 7.47−7.59 (m, 4H), 7.66 (t, J = 6.00 Hz, 1H), 7.71−7.78 (m, 4H), 10.88 (s, 1H); IR (KBr) cm^{-1} 1697, 3420; MS (FD) calcd for $C_{32}H_{28}N_4O_5S$ 580.67, found 580. Anal. ($C_{32}H_{28}N_4O_5S$) C, H, N.

(*S*)-13-(Morpholinomethyl)-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,-13]oxadiazacyclohexadecene-1,3(2*H*)-dione Monohydrochloride (7). As described for compound **3**, the mesylate (*S*)-10,11,14,15-tetrahydro-13-[[(methylsulfonyl)oxy]methyl]-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]-oxadiazacyclohexadecene-1,3(2*H*)-dione (150 mg, 0.29 mmol) was reacted with morpholine (0.50 mL, 20 equiv) to give a dark red solid that was converted to the its hydrochloride salt using 1 N HCl in ether to provide (*S*)-13-(morpholinomethyl)-10,-11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]-pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione monohydrochloride, **7** (109 mg, 69%), as a purple solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.99−2.04 (m, 1H), 2..23−2.30 (m, 1H), 2.95−3.03 (m, 1H), 3.18−3.37 (m, 5H), 3.51−3.88 (m, 7H), 4.07−4.39 (m, 4H), 7.06−7.11 (m, 2H), 7.17−7.18 (m, 2H), 7.39 (s, 1H), 7.42 (s, 1H), 7.47 (d, J = 8.00 Hz, 1H), 7.51 (d, J = 8.00 Hz, 1H), 7.73−7.79 (m, 2H), 10.73 (bs, 1H), 10.92 (s, 1H); IR (KBr) 1704, 2926, 3393, 3596; Anal. ($C_{30}H_{31}ClN_4O\cdot 0.5CH_3OH$) C, N; H: calcd, 5.91; found, 4.65. MS (FD) calcd for $C_{30}H_{31}ClN_4O$ 510.60 (free base), found 510.

(*S*)-13-[(*N*-Methylpiperazino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo-[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione Monohydrochloride (8). As described for compound **3**, the mesylate (*S*)-10,11,14,15-tetrahydro-13-[[(methylsulfonyl)oxy]-methyl]-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione (150 mg, 0.29 mmol) was reacted with *N*-methylpiperazine (0.64 mL, 20 equiv) to give a dark red solid that was converted to its hydrochloride salt using 1 N HCl in ether to provide (*S*)-13-[(*N*-methylpiperazino)methyl]-10,11,14,15-tetrahydro-4,9:16,-21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]-oxadiazacyclohexadecene-1,3(2*H*)-dione monohydrochloride, **8** (96 mg, 59%), as a purple solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.01−2.03 (m, 1H), 2.22−2.25 (m, 1H), 2.73 (s, 3H), 3.32−3.62 (m, 12H), 3.81−3.89 (m, 1H), 4.08−4.35 (m, 4H), 7.05−7.10 (m, 2H), 7.14−7.19 (m, 2H), 7.43−7.52 (m, 4H), 7.73−7.77 (m, 2H), 10.92 (s, 1H), 11.7 (bs, 1H); IR (KBr) cm^{-1} 1707, 2666, 3046, 3408; Anal. ($C_{31}H_{33}ClN_5O_3\cdot HCl\cdot 2H_2O$) C, H, N. MS (FD) calcd for $C_{31}H_{33}ClN_5O_3$ 523.64 (free base), found 523.

Biological Methods. Cell Culture. Retinal capillary endothelial cell cultures were initiated from bovine eyes using a modification of the procedure of Buzney.[14] Bovine eyes were transported on ice from a local abattoir. Extraocular muscle was trimmed from the eye, and the eye was bisected posterior to the ora serrata. The vitreous and anterior portion of the eye were discarded, and the neuroretina was gently dissected from the posterior eyecup. The retinas from 20 cattle were pooled and homogenized with 5 strokes of a Teflon/glass homogenizer in Hank's saline. The homogenate was passed through a 350 μm filter to remove large debris and a 210 μm filter to remove large vessels, and the microvessels were trapped on a 85 μm filter. The microvessels were resuspended in Hank's saline and digested with 7.5 mg/mL bacterial collagenase type D (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37 °C. The cells were pelleted by centrifugation (100*g*, 10 min), resuspended in 5 mL of endothelial growth media (EGM; Clonetics, San Diego, CA), and seeded in a gelatin-coated T25 flask. After 24 h the cells were trypsinized and replated in a gelatin-coated T225 flask. At 7 days and again at 14 days, the cultures were labeled with Dil-AcLDL (Stoughton, MA) and endothelial cells were separated from contaminating cell types using a fluorescent cell sorter. Cells were used between the 3rd and 10th passages. As a comparison, human dermal fibroblasts (Clonetics), between the 5th and 10th passages, also were studied.

To study PA activity retinal capillary endothelial cells were seeded into 96-well plates and grown to confluence (10^5 cells/well) in EGM containing 10% fetal bovine serum. Fibroblasts were maintained in Dulbecco's modified Eagles media (DME) supplemented with 10% fetal bovine serum. In both cell types the media were changed to DME with 10% fetal bovine serum 24 h prior to the assay. Cultures were treated with the phorbol esters 4β-phorbol 12,13-dibutyrate (4β-PDBu), 4α-phorbol 12,-13-dibutyrate (4α-PDBu), and 4β-phorbol 12-myristate 13-acetate (PMA), or the compound was dissolved in DMSO and incubated at 37 °C for 48 h. Cell toxicity was determined in a parallel series of cultures using a neutral red assay to monitor lysosomal integrity.

Plasminogen Activator Assay. Following treatment, the cells were lysed with 25 mM NH_4OH in 0.5% Triton X-100. Plasminogen activator activity was determined in the cell lysate using the synthetic substrate H-D-valyl-L-leucyl-lysine-*p*-nitroaniline dihydrochloride (Kabi, Mölndal, Sweden) using a modification of the procedure of Verheijen et al.[16] Briefly, a plasminogen−substrate solution was prepared which contained 1 mM synthetic substrate and 0.35 unit of plasminogen (Kabi)/1 mL of buffer (50 mM Tris HCl, pH 8.0, with 0.005% Tween 80). To a 50 μL aliquot of the cell lysate was added 200 μL of the plasminogen−substrate solution, the mixture was incubated at 25 °C for 45 min (endothelial cells) or 2 h (fibroblasts), and the plates were read at 410 nm. A standard curve was constructed using purified human urokinase or tPA (American Diagnostic, Greenwich, CT).

Calcium Calmodulin Dependent Protein Kinase Assay (CaM). The calcium calmodulin dependent protein kinase assay is described in *J. Neurosci.* **1983**, *3*, 818−831. The assay components were in a total volume of 250 μL: 55 mM HEPES

(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 2.75 mM dithiothreitol, 2.2 mM EGTA (ethylenebis(oxyethylenenitrilo)tetraacetic acid; used in the blank buffer), 1.1 mM calcium chloride (Sigma, St. Louis, MO; used in the control buffer), 10 mM magnesium chloride (Sigma, St. Louis, MO), 200 μg/mL histone type HL (Worthington), 10 μL of DMSO or DMSO/inhibitor, and 30 μM [γ-^{32}P]ATP (DuPont). The reaction was initiated by the addition of calcium calmodulin dependent protein kinase (isolated from rat brain homogenate), the mixture was incubated at room temperature for 10 min, and the reaction was stopped by adding 0.5 mL of ice cold trichloroacetic acid (Amresco) followed by 100 μL of 1 mg/mL bovine serum albumin (Sigma, St. Louis, MO). The precipitate was collected by vacuum filtration on glass fiber filters and quantified by counting in a beta scintillation counter.

Casein Protein Kinase II Assay (CK-II). The casein protein kinase II assay is described in *Neurochem. Res.* **1988**, *13*, 829−836. The assay components were in a total volume of 250 μL: 20 mM Tris HCl, pH 7.5, 5 mM sodium fluoride, 50 mg/mL casein (Sigma, St. Louis, MO), 10 mM magnesium chloride (Sigma, St. Louis, MO), 10 μL of DMSO or DMSO/inhibitor, and 30 μm [γ-^{32}P]ATP (DuPont). Initiation of the reaction was performed by addition of casein protein kinase II (isolated from rat brain homogenate), the mixture was incubated at room temperature for 10 min, and the reaction was stopped by the addition of 0.5 mL of ice cold trichloroacetic acid (Amresco) followed by 100 μL of 1 mg/mL bovine serum albumin (Sigma, St. Louis, MO). The precipitate was collected by vacuum filtration on glass fiber filters and quantified by counting in a beta scintillation counter.

cAMP Dependent Protein Kinase Catalytic Subunit Assay (PKA). The assay components were in a total volume of 250 μL: 20 mM HEPES (Sigma, St. Louis, MO) buffer, pH 7.5, 200 μg/mL histone type HL (Worthington), 10 mM magnesium chloride (Sigma, St. Louis, MO), 10 μL of DMSO or DMSO/inhibitor, and 30 μM [γ-^{32}P]ATP (DuPont). The reaction was initiated by addition of bovine heart cAMP dependent kinase catalytic subunit (Sigma, St. Louis, MO), the mixture was incubated at 30 °C for 10 min, and the reaction was stopped by adding 0.5 mL of ice cold trichloroacetic acid (Amresco) followed by 100 μL of 1 mg/mL bovine serum albumin (Sigma). The precipitate was collected by vacuum filtration on glass fiber filters employing a TOMTEC system and quantified by counting in a beta scintillation counter. This assay was done identically to the PKC enzyme assay except that no phospholipids or diacylglycerol was employed in the assay and the histone substrate was specific for the cAMP dependent catalytic subunit enzyme.

PKC Enzyme Assay (α, βI, βII, γ, δ, ϵ, η, ζ). Assay components in a total volume of 250 μL were as follows: vesicles consisting of 120 μg/mL phosphatidylserine (Avanti Polar Lipids) and sufficient diacylglycerol (Avanti Polar Lipids) to activate the enzyme to maximum activity in 20 mM HEPES buffer (Sigma, St. Louis, MO), pH 7.5, 940 μM calcium chloride (Sigma, St. Louis, MO) for assaying the α, βI, βII, and γ enzyme only, 1 mM EGTA for all the enzymes, 10 mM magnesium chloride (Sigma, St. Louis, MO), and 30 μM [γ-^{32}P]-ATP (DuPont). For all the enzymes either histone type HL (Worthington) or myelin basic protein was used as substrate. The assay was started by addition of protein kinase C enzyme incubated at 30 °C for 10 min and stopped by adding 0.5 mL of cold trichloroacetic acid (Amresco) followed by 100 μL of 1 mg/mL bovine serum albumin (Sigma, St. Louis, MO). The precipitate was collected by vacuum filtration on glass fiber filters employing a TOMTEC filtration system and quantified by counting in a beta scintillation counter.

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