Synthesis and Biological Evaluation of 12-Aminoacylphorboids[||]

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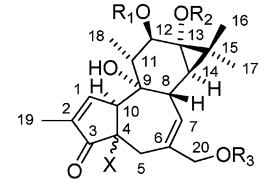
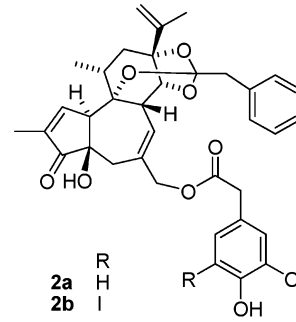
Spurred by the paradoxical anti-inflammatory activity of some aminoacylphorbol derivatives, the naturally occurring and epimeric *N,N*-dimethylvalinoyl-4α-4-deoxyphorbol derivatives **3b** and **3d** have been prepared from 4α-4-deoxyphorbol (**3e**), a byproduct of the isolation of phorbol from Croton oil and a phorboid polyol so far largely overlooked in terms of biological activity. The configuration of the side chain stereocenter was confirmed for both natural products, and to investigate the side chain structure−activity relationships within this class of compounds, their corresponding *N,N*-dimethylglycinate (**3g**) and nor (**3h**) and di-nor derivatives (**3i**, **3j**) were also prepared. By using a PKC-sensitive model of HIV-1 latency (activation of HIV- gene expression in Jurkat-LAT-GFP cells), it was found that both **3b** and **3d** can activate PKC-dependent responses, while a series of experiments with isoform-specific PKC inhibitors showed that these compounds target PKCα and -*δ*. Both *N,N*-dimethylation and the presence of side chain α-substitution were critical for activity. Selective PKC binding, rather than COX inhibition, might explain the paradoxical anti-inflammatory activity of extracts containing aminoacylphorboids in the mouse ear edema assay.

Over the past few years, there has been a growing awareness that the phorbol core is a privileged structure for bioactivity, which can be directed to different targets, or even reversed from agonist to antagonist, by modification of the acyl decoration of the basic polyol or by isomerization of the A,B-ring junction. Thus, acylation of the 20-hydroxy group with homovanillic acid redirects the activity of phorbol 12-phenylacetate-13-acetate (**1a**) from various PKC enzyme isoforms to the structurally unrelated ion channel TRPV1, generating the species-selective vanilloid ligand PPAHV (**1b**),[1] while epimerization at C-4 turns the ultrapotent PKC activator phorbol didecanoate (**1c**) into a TRPV4 ligand (4α-PDD, **1d**) devoid of PKC affinity,[2] and iodination of the side chain of resiniferatoxin (**2a**) converts this ultrapotent TRPV1 agonist into an ultrapotent TRPV1 antagonist (I-RTX, **2b**).[3]

In this context, our attention was piqued by the discovery of the potent COX-inhibiting activity of two aminoacylphorboids isolated from a medicinal Mexican *Croton* species, one of the many "sangre de drago" plants of South American ethnomedicine.[4] The discovery of these compounds came from an unexpected and somewhat paradoxical observation, namely, the inhibition of the phorbol ester-induced mouse ear edema formation by extracts from *Croton ciliatoglandulifer* Ortega (Euphorbiaceae). Surprisingly, fractionation of the active hexane extract afforded as anti-inflammatory principles a pair of 4α-phorboids bearing modified valine residues at the 12-hydroxy position (**3a** and **3b**). While the structure of **3a** is incompatible with its spectroscopic data and requires revision to its corresponding phorbol (and not 4α-phorbol) derivative (**3c**),[5] that of the *R*(D)-*N,N*-dimethylvalinoyl ester **3b** seems correct and shows several intriguing features. Thus, **3b** is the first compound from the 4α-4-deoxyphorbol series to show bioactivity. Remarkably, this activity is directed to a paradoxical end-point (COX inhibition) for phorbol esters, the archetypal obnoxious and irritant compounds,



	R_1	R_2	R_3	X
1a	PhAc	Ac	H	β-OH
1b	PhAc	Ac	HMV	β-OH
1c	Dec	Dec	H	β-OH
1d	Dec	Dec	H	α-OH



	R
2a	H
2b	I

PhAc = $C_6H_5CH_2CO$-

HMV = 3-OMe-4-OH-$C_6H_3CH_2CO$-

Dec = $nC_9H_{19}CO$-

and is critically dependent on the configuration at C-2′, with the side chain epimer from the *S*(L)-series (**3d**) being inactive. Finally, acylation by an amino acid and not by a fatty acid is also unusual, although not unprecedented, in phorboids.[6] 4α-4-Deoxyphorbol (**3e**) is available as a byproduct from the isolation of phorbol from Croton oil (*Croton tiglium* L.). Armed with a good supply of crude fractions containing a mixture of **3e** and phorbol from previous studies in this area,[7] we have embarked on a study of the biological profile of the 12-valinyl esters of 13-acetyl-4α-4-deoxyphorbol. To shed light on the structure−activity relationships and assess the biological relevance of the side chain nitrogen decoration and of the α-alkyl substitution, the synthesis of analogues characterized

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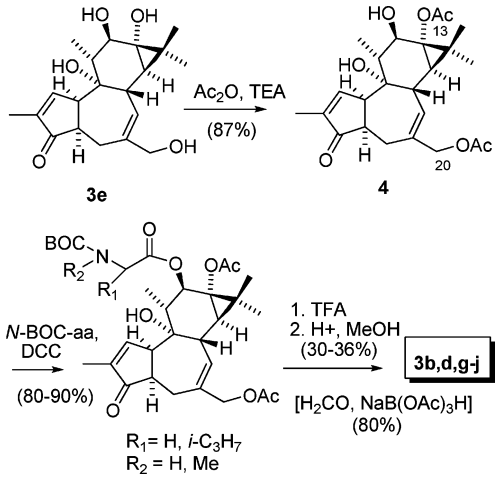
Scheme 1. Synthesis of the 12-Acetyl-13-aminoacylphorboids **3b**,**d**,**g**−**j**



by the stepwise removal of the *N*-methyls and of the α-isopropyl group was planned.

4α-4-Deoxyphorbol (**3e**) was purified from the mother liquor of phorbol after conversion to a triacetate (**3f**). While phorbol and its 4α-4-deoxy derivative are difficult to separate, their 12,13,20-triacetyl derivatives could be satisfactorily separated by gravity column chromatography on normal silica gel. After rehydrolysis, further acylative elaboration (Scheme 1) was done according to an established protocol for phorbol derivatives, namely, (a) selective acetylation of the 13- and 20-hydroxy groups, (b) esterification with a *N*-BOC-protected amino acid (BOC = *tert*-butoxycarbonyl), and (c) final stepwise deprotection of the amino group (TFA in CH_2Cl_2) and of the 20-hydroxy group ($HClO_4$ in methanol).[7] The failure to remove both acid-sensitive protecting groups (*N*-BOC and 20-acetyl) in a single step was not surprising, since different mechanisms are involved (protonolysis for the removal of the *N*-BOC-group and transesterification for the removal of the 20-acetyl). D- and L-*N*-BOC-valines are commercially available, as is the *N*-methyl derivative of L-*N*-BOC-valine, and esterification of 4α-4-deoxyphorbol-13,20-diacetate with these amino acids was uneventful. The *N,N*-dimethyl decoration had to be introduced at the stage of 12,13-phorboid diesters, because of the failure of all our attempts to prepare enantiopure *N,N*-dimethylvalines from the reaction of dimethylamine and their corresponding and commercially available enantiopure bromoisovalerates. Despite the risk of having to run a somewhat capricious reaction like the reductive *N,N*-dialkylation at the level of multifunctional compounds, we were, however, delighted to discover that the ring A enone system of the terpenoid core of valinoylphorboids was stable under the conditions of reductive dimethylation (aqueous formaldehyde and sodium triacetoxyborohydride). Overall, this semisynthetic route to aminoacylphorboids seems robust and amenable to the preparation of further analogues. Semisynthetic **3b** and **3d** had NMR spectra identical to those reported for the natural products, thus confirming their structural assignment based on molecular mechanic calculations and NMR experiments.[4]

Long-chain phorbol esters require a 4β-hydroxy group to bind PKC,[8] but, surprisingly, this hydroxy is redundant for PKC binding in short-chain phorbol esters from the 4β-deoxy series.[8] Intrigued by these observations, we investigated the activity of our 4αH-deoxyphorbol esters in a PKC-sensitive model of HIV-1 latency, namely, the activation of HIV gene expression in Jurkat-LAT-GFP cells.[9] Surprisingly, it was found that the naturally occurring *N,N*-dimethylvalinates **3b** and **3d**, but not their semisynthetic analogues **3g**−**j**, could induce HIV-1 reactivation from latency (Figure 1). Pretreatment with the inhibitors Gö6976 (classical PKCs inhibitor) and Gö6983 (pan-PKC inhibitor) strongly inhibited GFP expression induced by **3b** and **3d**, confirming the PKC dependence of this

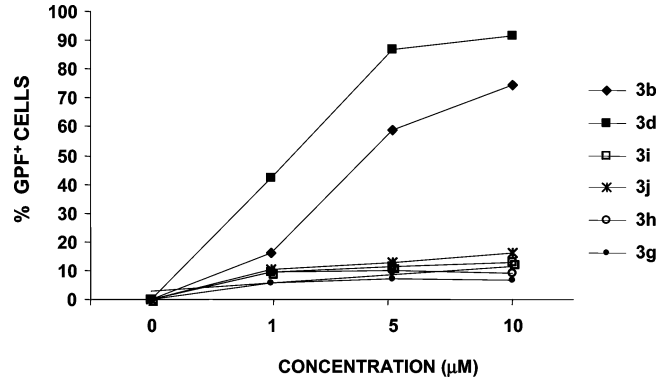
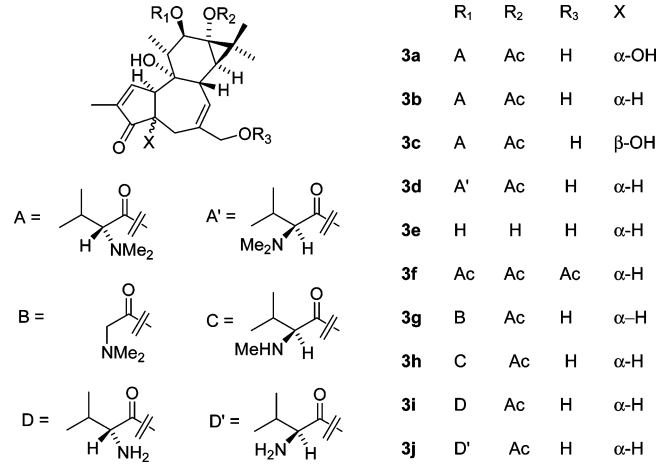


Figure 1. Effects of nitrogen-containing 4-deoxyphorbol esters in HIV-1 reactivation from latency. Jurkat LAT-GFP cells were stimulated with the compounds at the indicated concentrations for 6 h and next analyzed by flow cytometry. Results are represented as the percentage of GFP positive cells ± SD of four different experiments.



	R_1	R_2	R_3	X
3a	A	Ac	H	α-OH
3b	A	Ac	H	α-H
3c	A	Ac	H	β-OH
3d	A'	Ac	H	α-H
3e	H	H	H	α-H
3f	Ac	Ac	Ac	α-H
3g	B	Ac	H	α–H
3h	C	Ac	H	α-H
3i	D	Ac	H	α-H
3j	D'	Ac	H	α-H

response (Figure 2). Remarkably, the structure−activity relationships seem very strict, since both the *N.N*-dimethyl groups (cf. **3b**,**d** vs **3e**) and the side chain α-substitution (cf. **3b**,**d** vs **3g**) are critical for activity.

To investigate the PKC activity at a molecular level, the ability of compounds **3d** and **3g**, as representative of a positive and a negative probe, respectively, to activate PKCs (α and δ isotypes) in vitro[10] was used. A substrate peptide that can be efficiently phosphorylated by both PKCα and PKCδ was employed.[12] Only **3d** could activate both PKC isoenzymes in a concentration-dependent manner (Figure 3A). Since PKCs are also regulated by phosphorylation, and activated PKCδ exhibits a critical phorphorylation of Thr-505 in the activation loop,[10] we used a specific antibody antiphospho PKCδ (Thr505) to confirm the results of the biochemical assay. In full accordance with the previous observations, **3d**, as well as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), could activate PKCδ (Figure 3B), while PKCδ phosphorylation was undetectable with **3g**. Compound **3b** has been reported to inhibit COX-1 and COX-2 in a recombinant assay.[4] Using this approach, we indeed detected a marginal COX-2-inhibiting activity[12] in all compounds assayed. However, these compounds did not inhibit the release of PGE_2 in activated primary monocytes,[12] strongly suggesting that the activity observed in the recombinant assay is probably due to an artifact.

Taken together, our results suggest that the natural *N,N*-dimethylvalinoylphorboids **3b** and **3d** are potent and isoform-specific activators of PKC, whose binding to the C1 pocket of this enzyme could explain the paradoxical inhibitory activity of extracts containing these compounds in the TPA mouse ear edema assay.

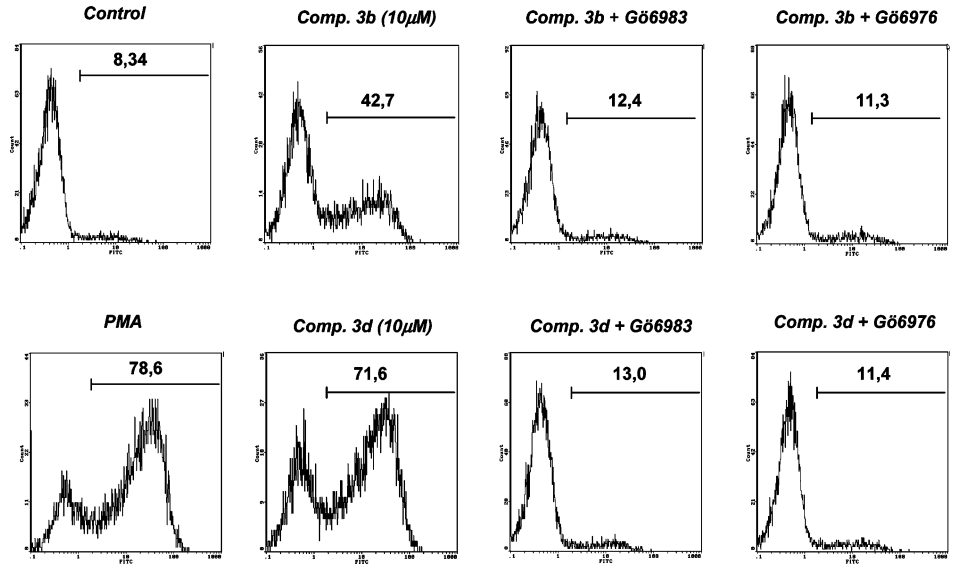


Figure 2. Compounds **3b** and **3d** antagonize HIV-1 latency through a PKC-dependent pathway. Jurkat LAT-GFP cells were pretreated with the indicated inhibitors (1 μM) for 30 min and then stimulated with **3b** and **3b** (10 μM) for 6 h. The percentage of GFP+ cells was measured by flow cytometry. Histograms represent one out three independent experiments, and the percentage of GFP positive cells is indicated.

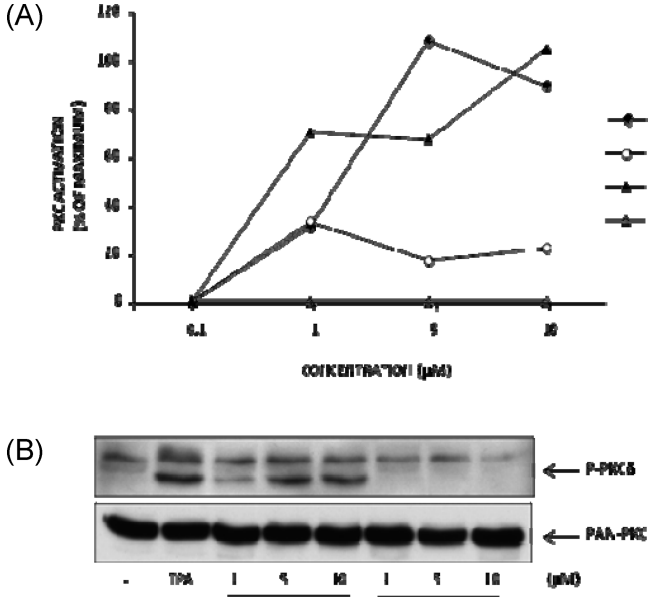


Figure 3. Activation of PKCα and PKCδ by **3d** and **3 g**. (A) PKCα and PKCδ activities were assayed in the presence of phospholipid vesicles (20% phosphatidylserine, 80% phosphatidylcholine) and increasing concentration of **3d** and **3g**. Results are expressed as percentage of the activation observed with 1 μM TPA (100%) and represent the mean ± SE of triplicate determination in a single experiment. Two additional experiments gave similar results. (B) TPA and **3d** phosphorylate PKCδ. Jurkat LAT-GFP cells were incubated with either TPA (1 μM) or increasing concentrations of **3d** and **3g** during 15 min. Total PKCs and phosphorylated PKCδ (Thr505) were analyzed using specific antibodies by western blot.

PKC functional activity was found for the first time in 4α-4-deoxyphorbol esters, qualifying these compounds as interesting and so far overlooked noninflammatory probes to study PKC functions and to design small molecules capable of modulating this enzyme in a therapeutically useful way.

Experimental Section

General Experimental Procedures. IR spectra were obtained on a Shimadzu DR 8001 spectrophotometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained at room temperature with a JEOL Eclipse spectrometer. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. CIMS were taken on a VG Prospec (FISONS) mass spectrometer. Silica gel 60 (70−230 mesh) was used for gravity column chromatography. Reactions were monitored by TLC on Merck 60 F_{254} (0.25 mm) plates and were visualized by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. Organic phases were dried with Na_2SO_4 before evaporation.

12,13,20-Triacetyl-4α-4-deoxyphorbol (3f). Phorbol was obtained from Croton oil as previously reported.[7] Crude semisolid phorbol (ca. 0.1% from the oil) obtained from the gravity column chromatography purification of the defattened oil hydrolysate was triturated with EtOAc and filtered. The filtrate (ca. 44% of the crude phorbol) was a ca. 3:1 mixture of phorbol and 4α-4-deoxyphorbol (R_f 0.17 in 9:1 EtOAc−MeOH for both compounds). To 5 g of this crude mixture were added Ac_2O (110 mL) and cat. DMAP (ca. 100 mg). After stirring at room temperature for 1 h, the reaction was worked up by the addition of MeOH (ca. 75 mL) to destroy the excess acetic anhydride and next washed with 2 N H_2SO_4 (5 × ca. 50 mL) and brine. The organic phase was dried, filtered, and evaporated to afford a semisolid residue, which was fractionated by gravity column chromatography on silica gel (ca. 35 g) to afford 12,13,20-triacetylphorbol (petroleum ether−EtOAc, 8:2, as eluant, 2 g) and 12,13,20-triacetyl-4α-4deoxyphorbol (**3f**, petroleum ether−EtOAc, 7:3, as eluant, 600 mg): white powder; IR (liquid film) ν_{max} 3416, 2950, 1738, 1432, 1375, 1320, 1022, 978 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.96 (1H, brs, H-1), 5.43 (1H, d, J = 10.7 Hz, H-12), 5.15 (1H, brs, OH-10), 5.08 (1H, brs, H-7), 4.45 (1H, brs, J = 12.6 Hz H-20a), 4.32 (1H, brd, J = 12.6 Hz, H-20b), 3.46 (1H, m, H-10), 3.44 (1H, brdd, J = 11.0 Hz, H-5a), 2,.72 (1H, m, H-4), 2.45 (1H, brdd, J = 11.0 Hz, H-5b), 2.06 (9H, s, OAc), 1.90 (1H, m, H-8), 1.75 (3H, brs, H-19), 1.70 (1H, m, H-11), 1.24 (3H, s, H-17), 1.17 (3H, d, J = 7.0 Hz, H-18), 1.07 (3H, s, H-16), 0.78 (1H, d, J = 5.2 Hz, H-14); CIMS m/z 475 $[M + 1]^+$ $[C_{26}H_{34}O_8 + H]^+$.

4α-4-Deoxyphorbol (3e). 12,13,20-Triacetyl-4α-4-deoxyphorbol (**3f**, 100 mg; 0,23 mmol) was dissolved in 0.01 N NaOMe (2.0 mL), and the solution was stirred at room temperature, maintaining the pH below 12 with the addition of further amounts of 0.01 N NaOMe. After 1 h, the reaction was worked up by neutralization with acetic acid, filtration, and evaporation. The residue was purified by gravity column chromatography on silica gel (2 g; petroleum ether−EtOAc gradient, from

7:3 to pure EtOAc) to afford 4α-4-deoxyphorbol as a white powder (80 mg): IR (liquid film) ν_{max} 3345, 2933, 1704, 1454, 1378, 1331, 1067, 1018 cm^{-1}; ^1H NMR (300 MHz, MeOH-d_4) δ 7.29 (1H, brs, H-1), 5.13 (1H, brs, H-7), 4.02 (1H, d, J = 10.0 Hz, H-12), 3.84 (2H, brs, H-20a,b), 3.49 (1H, brs, H-10), 3.18 (1H, brd, J = 15.0 Hz, H-5a), 2.69 (1H, m, H-4), 2.26 (1H, brd, J = 15.0 Hz, H-5b), 1.84 (1H, m, H-8), 1.71 (3H, brs, H-19), 1.59 (1H, m, H-11), 1.23 (3H, s, H-17), 1.21 (3H, d, J = 7.0 Hz, H-18), 1.13 (3H, s, H-16), 0.52 (1H, d, J = 5.3 Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$) δ 212.8 (s, C-3), 155.8 (d, C-1), 130.4 (s, C-2), 137.3 (s, C-6), 126.2 (d, C-7), 78.2 (s, C-9), 74.8 (d, C-12), 69.2 (t, C-20), 65.1 (s, C-13), 49.6 (d, C-4), 47.4 (d, C-10), 43.0 (d, C-11), 40.5 (d, C-8), 37.2 (d, C-14), 25.9 (t, C-5), 25.1 (s, C-15), 16.3 (t, C-16), 11.9 (t, C-18), 10.4 (t, C-19); CIMS m/z 349 $[M + 1]^+$ $[C_{20}H_{28}O_5 + H]^+$.

13,20-Diacetyl-4α-4-deoxyphorbol (4). To a solution of 100 mg of 4α-4deoxyphorbol (**3e**, 0,23 mM) in 1:1 (v/v) CH_2Cl_2−THF (5 mL) were added an excess of TEA (1.64 mL, 18 mM, 8 molar equiv) and Ac_2O (2.1 mL, 16 mM, 8 molar equiv). After stirring at room temperature for 1.5 h, the reaction was worked up by the addition of methanol (ca. 1.5 mL) to quench the excess Ac_2O and washed (×5) with 2 N H_2SO_4 and with brine. The organic layer was dried, filtered, and evaporated. The residue was purified by gravity column chromatography on silica gel (5 g; petroleum ether−EtOAc eluant, from 6:4 to 4:6) to afford 94 mg (87%) of **4** as a white solid: ^1H NMR (300 MHz, $CDCl_3$) δ 6.96 (1H, brs, H-1), 5.43 (1H, d, J = 10.7 Hz, H-12), 5.15 (1H, brs, OH-10), 5.08 (1H, brs, H-7), 4.39 (2H, brs, J = 27.2 Hz H-20a,b), 3.46 (1H, m, H-10), 3.44 (1H, brdd, J = 11.0 Hz, H-5a), 2.72 (1H, m, H-4), 2.45 (1H, brdd, J = 11.0 Hz, H-5b), 2.06 (6H, s, OAc), 1.90 (1H, m, H-8), 1.75 (3H, brs, H-19), 1.70 (1H, m, H-11), 1.24 (3H, s, H-17), 1.17 (3H, d, J = 7.0 Hz, H-18), 1.07 (3H, s, H-16), 0.78 (1H, d, J = 5.2 Hz, H-14); CIMS m/z 433 $[M + 1]^+$ $[C_{24}H_{32}O_7 + H]^+$.

General Protocol for the Esterification of 13,20-Diacetyl-4α-4-deoxyphorbol (4) with BOC-Protected Amino Acids. To a solution of 13,20-diacetyl-4α-4-deoxyphorbol (**4**, 100 mg, 0.23 mmol) in 3:1 toluene−CH_2Cl_2 (20 mL) was added a solution of an *N*-BOC-amino acid (for the synthesis of **3g** and **3j**) or an *N*-methyl-*N*-BOC-amino acid (for the synthesis of **3h**) (1.5 molar equiv) and DCC (1.5 molar equiv) in 3:1 toluene−CH_2Cl_2 (20 mL). After stirring at room temperature for 1 h, the reaction was worked up by filtration with toluene. The organic phase was sequentially washed with 2 N H_2SO_4, saturated $NaHCO_3$, and brine. After drying, the organic phase was filtered and evaporated. The residue was purified by gravity column chromatography on silica gel (10 g, petroleum ether−EtOAc, 8:2 to 7:3, as eluant) to afford 13,20-diacetyl-12-(*N*-BOC-aminoacyl)-4α-4-deoxyphorbols as white powders (yield 80−90%).

General Protocol for the *N*-Deprotection of 12-(*N*-BOC-aminoacyl)-13,20-diacetyl-4α-4-deoxyphorbols. To a solution of 100 mg of a 12-(*N*-BOC-aminoacyl)-13,20-diacetyl-4α-4-deoxyphorbol in CH_2Cl_2 (5 mL) was added trifluoroacetic acid (TFA) (0.25 mL, 5% of the substrate solution). After stirring at room temperature for 8 h, the reaction was worked up by filtration and washing with toluene. The filtrate was washed with saturated $NaHCO_3$ and next with brine. After drying, filtration, and evaporation, the residue was purified by gravity column chromatography on silica gel (10 g, petroleum ether−EtOAc gradient, from 6:4 to pure EtOAc, as eluant) to afford the corresponding 13,20-diacetyl-12-aminoacyl-4α-4-deoxyphorbol (ca. 60% yield).

General Protocol for the Chemoselective 20-Deacetylation of 12-Aminoacyl-13,20-diacetyl-4α-4-deoxyphorbols. A solution of 100 mg of a 13,20-diacetyl-12-aminoacyl-4α-4-deoxyphorbol in MeOH (ca. 5 mL) was brought to pH ca. 0.5 by the dropwise addition of $HClO_4$. After stirring at room temperature for 24 h, the reaction was worked up by neutralization with solid sodium acetate, filtration, and removal of methanol. The residue was dissolved in dichloromethane, and the organic phase was washed with 5% $NaHCO_3$ and brine. After drying and evaporation, the residue was purified by gravity column chromatography on silica gel (10 g, EtOAc−MeOH gradient, from 5:% to 2:8, as eluant) to afford the corresponding 13-acetyl-12-aminoacyl-4α-4-deoxyphorbol in 50−60% yield.

General Protocol for the *N*,*N*-Dimethylation of 12-Aminoacyl-13,20-diacetyl-4α-4-deoxyphorbols. To a solution of a 12-aminoacyl-13-acetyl-4α-4-deoxyphorbol (0−20 mM, ca. 100 mg) in acetonitrile (570 μL) was added 36.5% aqueous formaldehyde (164 μL). After stirring at room temperature for 5 min, sodium triacetoxyborohydride (161 mg, 0.76 mmol, 3.8 equiv) and glacial acetic acid (38 μL) were added, and stirring was continued for 1 h. The reaction was next worked up by dilution with saturated $NaHCO_3$, and the organic layer was washed with brine. After drying and evaporation, the residue was purified by gravity column chromatography on silica gel (10 g, EtOAc−MeOH gradient, from 6:4 to 2:8, as eluant) to afford the *N*,*N*-dimethyl derivatives **3b**, **3d**, and **3g** (ca. 80% yield).

***N*,*N*-Dimethyl-12-(*R*-valinoyl)-13-acetyl-4α-4-deoxyphorbol (3b).** ^1H NMR (300 MHz, $CDCl_3$) δ 7.06 (1H, brs, H-1), 5.50 (1H, d, J = 10.5 Hz, H-12), 5.16 (1H, brs, H-7), 5.12 (1H, s, OH-9), 4.00 (1H, brd, J = 10.8 Hz H-20a), 3.90 (1H, brd, J = 10.8 Hz, H-20b), 3.58 (1H, m, H-10), 3.44 (1H, brdd, J = 16.8 Hz, H-5a), 2.79 (1H, brs, H-4), 2.45 (1H, brdd, J = 15.6 Hz, H-5b), 2.29 (6H, s, *N*-Me_2), 2.06 (3H, s, OAc), 2.02 (1H, m, H-3′), 1.97 (1H, brs, H-8), 1.79 (3H, brs, H-19), 1.71 (1H, m, H-11), 1.21 (3H, s, H-16), 1.20 (3H, d, J = 3.9 Hz, H-18), 1.13 (3H, s, H-17), 1.04 (1H, d, J = 6.6 Hz, H-4′), 0.98 (3H, d, J = 6.6 Hz, H-5′), 0.80 (1H, d, J = 5.1 Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$) δ 212.8 (s, C-3), 155.9 (d, C-1), 173.4 (s, OAc), 172.1 (s, C-1′), 155.9 (d, C-1), 143.4 (s, C-2), 137.4 (s, C-6), 126.3 (d, C-7), 78.2 (s, C-9), 74.8 (d, C-12), 74.5 (d, C-2′), 69.3 (t, C-20), 65.3 (s, C-13), 49.6 (d, C-4), 47.4 (d, C-10), 42.6 (d, C-11), 41.4 (q, *N*-Me_2), 40.6 (d, C-8), 36.8 (d, C-14), 28.2 (d, C-3′), 25.0 (t, C-5), 25.2 (s, C-15), 24.0 (q, C-17), 21.0 (q, OAc), 19.6 (t, C-5′), 19.3 (t, C-4′), 16.3 (q, C-16), 12.0 (q, C-18), 10.4 (q, C-19); CIMS m/z 518 $[M + 1]^+$ $[C_{29}H_{43}NO_7 + H]^+$.

***N*,*N*-Dimethyl-12-(*S*-valinoyl)-13-acetyl-4α-4-deoxyphorbol (3d).** ^1H NMR (300 MHz, $CDCl_3$) δ 7.04 (1H, brs, H-1), 5.67 (1H, d, J = 10.5 Hz, H-12), 5.09 (1H, s, OH-9), 5.11 (1H, brs, H-7), 4.01 (1H, brd, J = 11.7 Hz, H-20a), 3.89 (1H, brd, J = 11.7 Hz, H-20b), 3.48 (1H, m, H-10) 3.40 (1H, brdd, J = 16.8 Hz, H-5a), 2.80 (1H, brs, H-4), 2.48 (1H, brdd, J = 20.0 Hz, H-5b), 2.35 (6H, s, *N*-Me_2), 2.10 (3H, s, OAc), 2.77 (1H, m, Hz, H-2′), 1.98 (1H, brs, H-8), 2.02 (1H, m, H-3′), 1.78 (3H, brs, H-19), 1.71 (1H, m, H-11), 1.13 (3H, s, H-17), 1.20 (3H, d, J = 13.5 Hz, H-18), 1.20 (3H, s, H-16), 1.00 (3H, d, J = 6.9 Hz, H-4′), 0.92 (3H, d, J = 6.6 Hz, H-5′), 0.82 (1H, d, J = 5.1 Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$) δ 212.8 (s, C-3), 155.7 (d, C-1), 173.4 (s, OAc), 171.3 (s, C-1′), 155.7 (d, C-1), 143.4 (s, C-2), 137.2 (s, C-6), 126.3 (d, C-7), 78.2 (s, C-9), 74.8 (d, C-12), 74.7 (d, C-2′), 69.3 (t, C-20), 65.3 (s, C-13), 49.6 (d, C-4), 47.3 (d, C-10), 42.7 (d, C-11), 41.3 (q, *N*-Me_2), 40.6 (d, C-8), 36.8 (d, C-14), 27.3 (d, C-3′), 24.8 (t, C-5), 25.2 (s, C-15), 24.0 (q, C-17), 21.1 (q, OAc), 19.7 (t, C-5′), 19.2 (t, C-4′), 16.3 (q, C-16), 11.9 (q, C-18), 10.4 (q, C-19); CIMS m/z 518 $[M + 1]^+$ $[C_{29}H_{43}NO_7 + H]^+$.

***N*,*N*-Dimethyl-12-glycinoyl-13-acetyl-4α-4-deoxyphorbol (3g).** ^1H NMR (300 MHz, $CDCl_3$) δ 7.01 (1H, brs, H-1), 5.52 (1H, d, J = 10.5 Hz, H-12), 5.09 (1H, s, OH-9), 5.11 (1H, brs, H-7), 4.00 (1H, brd, J = 12.6 Hz H-20a), 3.88 (1H, brd, J = 12.6 Hz, H-20b), 3.49 (1H, m, H-10), 3.42 (2H, brdd, J = 18.0 Hz, H-5a,b), 3.27 (1H, d, J = 16.2 Hz, H-2′a), 3.16 (1H, d, J = 15.9 Hz, H-2′b), 2.78 (1H, brs, H-4), 2.38 (6H, s, *N*-Me_2), 2.06 (3H, s, OAc), 1.96 (1H, brs, H-8), 1.77 (3H, brs, H-19), 1.71 (1H, m, H-11), 1.16 (3H, s, H-17), 1.19 (3H, s, H-16), 0.82 (1H, d, J = 5.1 Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$) δ 212.8 (s, C-3), 173.3 (s, OAc), 170.4 (s, C-1′), 155.8 (d, C-1), 143.4 (s, C-2), 137.3 (s, C-6), 126.2 (d, C-7), 78.2 (s, C-9), 74.8 (d, C-12), 69.3 (t, C-20), 65.1 (s, C-13), 60.6 (t, C-2′), 49.6 (d, C-4), 47.4 (d, C-10), 45.2 (q, *N*-Me_2), 43.0 (d, C-11), 40.6 (d, C-8), 37.1 (d, C-14), 25.9 (t, C-5), 25.1 (s, C-15), 24.1 (q, C-17), 21.0 (q, OAc), 16.3 (q, C-16), 11.9 (q, C-18), 10.4 (q, C-19); CIMS m/z 476 $[M + 1]^+$ $[C_{26}H_{37}NO_7 + H]^+$.

***N*-Methyl-12-(*S*-valinoyl)-13-acetyl-4α-4-deoxyphorbol (3h).** ^1H NMR (300 MHz, $CDCl_3$) δ 7.05 (1H, brs, H-1), 5.52 (1H, d, J = 10.2 Hz, H-12), 5.14 (1H, s, OH-9), 5.11 (1H, brs, H-7), 4.00 (1H, brd, J = 10.8 Hz, H-20a), 3.90 (1H, brd, J = 10.8 Hz, H-20b), 3.50 (1H, m, H-10), 3.45 (1H, brdd, J = 17.7 Hz, H-5a), 2.80 (1H, m, H-4), 2.48 (1H, brdd, J = 21.0 Hz, H-5b), 2.43 (3H, s, *N*-Me), 2.06 (3H, s, OAc), 2.89 (1H, d, J = 6.9 Hz, H-2′), 1.96 (1H, brs, H-8), 1.89 (1H, m, H-3′), 1.78 (3H, brs, H-19), 1.73 (1H, m, H-11), 1.14 (3H, s, H-17), 1.12 (3H, d, J = 6.0 Hz, H-18), 1.20 (3H, s, H-16), 1.00 (3H, d, J = 6.9 Hz, H-4′), 1.00 (3H, d, J = 6.6 Hz, H-5′), 0.82 (1H, d, J = 5.4 Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$) δ 212.8 (s, C-3), 174.8 (s, OAc), 173.4 (s, C-1′), 155.8 (d, C-1), 143.5 (s, C-2), 137.3 (s, C-6), 126.1 (d, C-7), 78.2 (s, C-9), 75.9 (d, C-12), 70.4 (d, C-2′), 69.2 (t, C-20), 65.3 (s, C-13), 49.6 (d, C-4), 47.3 (d, C-10), 42.8 (d, C-11), 40.6 (d, C-8), 37.1 (d, C-14), 35.0 (q, *N*-Me), 31.4 (d, C-3′), 25.0 (t, C-5), 25.2 (s, C-15), 24.0 (q, C-17), 21.0 (q, OAc), 19.3 (t, C-5′), 19.0 (t, C-4′), 16.4

(q, C-16), 12.0 (q, C-18), 10.4 (q, C-19); CIMS *m/z* 504 $[M + 1]^+$ $[C_{28}H_{41}NO_7 + H]^+$.

12-(*R*-Valinoyl)-13-acetyl-4α-4-deoxyphorbol (3i). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.03 (1H, brs, H-1), 5.67 (1H, d, $J = 10.5$ Hz, H-12), 5.11 (1H, brs, H-7), 4.01 (1H, brd, $J = 12.9$ Hz, H-20a), 3.89 (H, brd, $J = 12.3$ Hz, H-20b), 3.50 (1H, m, H-10), 3.40 (1H, brdd, $J = 20.0$ Hz, H-5a), 2.79 (1H, m, H-4), 2.49 (1H, brdd, $J = 20.4$ Hz, H-5b), 2.10 (1H, m, Hz, H-2′), 2.05 (3H, s, OAc), 1.96 (1H, brs, H-8), 1.81 (1H, brs, H-3′), 1.78 (3H, brs, H-19), 1.74 (1H, m, H-11), 1.15 (3H, s, H-17), 1.10 (3H, d, $J = 6.6$ Hz, H-18), 1.21 (3H, s, H-16), 1.05 (3H, d, $J = 6.9$ Hz, H-4′), 0.95 (3H, d, $J = 6.9$ Hz, H-5′), 0.82 (1H, d, $J = 4.8$ Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$) δ 212.8 (s, C-3), 175.6 (s, OAc), 173.4 (s, C-1′), 155.8 (d, C-1), 143.4 (s, C-2), 137.3 (s, C-6), 126.2 (d, C-7), 78.2 (s, C-9), 76.0 (d, C-12), 69.2 (t, C-20), 65.1 (s, C-13), 59.6 (d, C-2′), 49.6 (d, C-4), 47.3 (d, C-10), 42.9 (d, C-11), 40.5 (d, C-8), 39.1 (d, C-3′), 37.0 (d, C-14), 24.9 (t, C-5), 25.2 (s, C-15), 24.1 (q, C-17), 21.0 (q, OAc), 19.2 (t, C-5′), 16.6 (t, C-4′), 16.4 (q, C-16), 12.0 (q, C-18), 10.4 (q, C-19); CIMS *m/z* 490 $[M + 1]^+$ $[C_{27}H_{39}NO_7 + H]^+$.

12-(*S*-Valinoyl)-13-acetyl-4α-4-deoxyphorbol (3j). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.04 (1H, brs, H-1), 5.67 (1H, d, $J = 10.5$ Hz, H-12), 5.12 (1H, brd, $J = 5.4$ Hz H-7), 4.01 (1H, brd, $J = 12.6$ Hz, H-20a), 3.89 (1H, brd, $J = 12.6$ Hz, H-20b), 3.49 (1H, m, H-10), 3.44 (1H, brdd, $J = 16.0$ Hz, H-5a), 2.82 (1H, m, H-4), 2.47 (1H, brdd, $J = 20.7$ Hz, H-5b), 2.04 (3H, s, OAc), 2.02 (1H, m, Hz, H-2′), 1.96 (1H, m, H-8), 1.81 (1H, brs, H-3′), 1.78 (3H, brs, H-19), 1.74 (1H, m, H-11), 1.16 (3H, s, H-17), 1.19 (3H, s, H-16), 1.11 (3H, d, $J = 7.0$ Hz, H-18), 1.03 (3H, d, $J = 6.9$ Hz, H-4′), 0.94 (3H, d, $J = 6.9$ Hz, H-5′), 0.82 (1H, d, $J = 4.8$ Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$) δ 212.8 (s, C-3), 175.6 (s, OAc), 173.4 (s, C-1′), 155.6 (d, C-1), 143.4 (s, C-2), 137.6 (s, C-6), 126.2 (d, C-7), 78.2 (s, C-9), 76.0 (d, C-12), 69.2 (t, C-20), 65.1 (s, C-13), 59.6 (d, C-2′), 49.6 (d, C-4), 47.3 (d, C-10), 42.9 (d, C-11), 40.5 (d, C-8), 39.1 (d, C-3′), 37.0 (d, C-14), 24.9 (t, C-5), 25.2 (s, C-15), 24.1 (q, C-17), 21.0 (q, OAc), 19.2 (t, C-5′), 16.6 (t, C-4′), 16.4 (q, C-16), 12.0 (q, C-18), 10.4 (q, C-19); CIMS *m/z* 490 $[M + 1]^+$ $[C_{27}H_{39}NO_7 + H]^+$.

Cells and Reagents. Jurkat-LTR-GFP is a Jurkat-derived clone infected latently with a recombinant virus containing the GFP gene driven by the HIV-LTR promoter.[9] The cells were stimulated with the indicated compounds for 6 h, and the GFP expression was analyzed by flow cytometry in an EPIC XL flow cytometer (Coulter, Hialeah, FL). Ten thousand gated events were collected per sample, and the fluorescence pattern was determined. The antibodies anti-panPKCs and antiphospho-PKCδ (Thr505) were from Cell Signaling Technology (Danvers, MA). The inhibitors Go6983 and Go6976 were from Calbiochem (EMD Biosciences, Inc. Darmstadt, Germany). [γ-^{32}P]ATP (3000 Ci/mmol) was from MP Biomedicals (Irvine, CA). The inhibition of COX-2 activity and the liberation of PGE_2 from activated primary monocytes were evaluated as reported previously.[12]

Western Blots. Jurkat-LAT-GFP cells (106 cells/ml) were stimulated with the indicated compounds. Cells were then washed with PBS and proteins extracted in 50 μL of lysis buffer (20 mM HEPES pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4, 5 mM NaFl, 1 mM DTT, leupeptin 1 μg/mL, pepstatin 0.5 μg/mL, aprotinin 0.5 μg/mL, and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA), and 30 μg of proteins was boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4 °C) for 1 h. Blots were blocked in TBS solution containing 0.1% Tween 20 and 5% nonfat dry milk overnight at 4 °C, and immunodetection of specific proteins was carried out with primary antibodies using an ECL system (GE Healthcare).

PKC Kinase Assay. In vitro activation of recombinant PKC α and -δ (Invitrogen) was assayed by measuring the incorporation of P32 from ATP into the specific PKC substrate peptide [QKRPSQRSKYL]. PKC activation was measured using the PKC Assay Kit (Millipore, MA) following the instructions of the manufacturer. Compounds **3d** and **3g** were added at the indicated concentrations from the appropriate DMSO stocks; the final concentration of the diluents did not exceed 0.2%. The assay tubes were incubated for 10 min at 30 °C, and the reaction was stopped by chilling on ice. Aliquots of 25 μL were spotted onto P81 phosphocellulose papers followed by washing three times in 0.5% phosphoric acid and once with acetone. The filters were transferred to scintillation vials, and the bound radioactivity was read in a scintillation counter. In each single experiment, each ligand concentration was assayed in triplicate and a dose−response curve was plotted.

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