Synthesis and Characterization of New Photolabile Phorbol Esters for Affinity Labeling of Protein Kinase C

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Three new photolabile phorbol esters (**3**, **6**, and **9**) with a diazoacetyl group at positions 3, 12, and 13, respectively, have been synthesized from phorbol (**1a**) and found to bind with significant affinity to the peptide (peptide C) incorporating the phorbol ester-binding domain of protein kinase C. As required for its use as a protein kinase C receptor probe, the solution photochemistry of **3** led predominantly to insertion products. Tritium labeling of these probes at C-20 was accomplished by oxidation with activated manganese dioxide to the C-20 aldehyde and subsequent ^{3}H-labeled sodium borohydride reduction. Initial affinity labeling studies showed that probes **6** and **9** labeled peptide C, suggesting that the introduction of a photolabile group into position 12 or 13 of the phorbol esters could be used to identify the structural features of PKC involved in its binding to activators.

Introduction

Protein kinase C (PKC) is a phospholipid-dependent serine/threonine kinase that plays a central role in cellular signal transduction.[1] Under normal conditions, PKC is transiently activated by diacylglycerol (DAG), a second messenger released by phosphatidylinositol turnover. Several exogenous compounds, most notably the tumor-promoting phorbol esters, teleocidins, and aplysiatoxins, also bind to and activate PKC.[2] While their binding is competitive with DAG, these agents elicit biochemical and physiological responses (e.g., tumor promotion) that differ markedly from those obtained through DAG activation. In further contrast to DAG, these agents bind to PKC with higher affinities and are not rapidly metabolized, resulting in persistent, abnormal PKC activation.

While DAG and tumor promoters differ significantly in structure, their association with a common PKC receptor domain suggests that they could employ a common subset of structural features in binding to PKC.[3] Computer modeling and structure-activity studies have provided significant information on the *structural features of the activators* involved in PKC binding.[3−9] However, the *structural features of the activator binding site* on PKC itself are not known for the PKC-activator−phospholipid aggregate[10] even though this information is indispensable for understanding the activation mechanism of PKC and developing new medicinal leads based on modulation of PKC signaling.

As indicated by cloning and sequence analysis, PKC is a family of closely related isozymes. Thus far, 11 members of this family have been characterized.[11−14] The main subspecies of PKC (α, βI, βII, and γ) are single polypeptides with four conserved (C_1−C_4) and five variable (V_1−V_5) regions (Figure 1).[11] While recent studies have demonstrated that a cysteine-rich tandem repeat in the C_1 region is necessary and sufficient for phorbol ester binding,[15−18] the specific amino acids in this sequence with which phorbol esters interact have not yet been identified.

Photoaffinity labeling serves as a promising approach to the identification of the PKC subunits directly involved in DAG or phorbol ester binding. However, previous photoaffinity labeling studies[19,20] using photolabile phorbol esters and mouse brain or rat epidermal membrane

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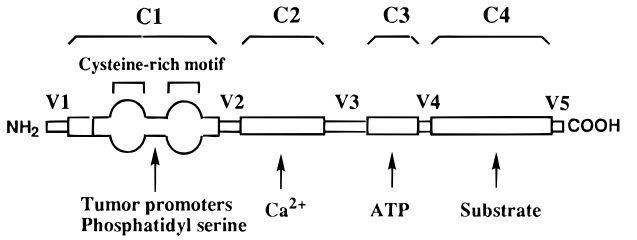
(1) Nishizuka, Y. *Nature* **1984**, *308*, 693−698.
(2) Fujiki, H.; Sugimura, T. *Adv. Cancer Res.* **1987**, *49*, 223−264.
(3) Wender, P. A.; Cribbs, C. M. Computer assisted molecular design related to the protein kinase C receptor. In *Advances in Medicinal Chemistry*; Maryanoff, C. A., Maryanoff, B. E., Eds; JAI Press, Inc.: London, 1992; Vol. 1, pp 1−53.
(4) Jeffrey, A. M.; Liskamp, R. M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 241−245.
(5) Wender, P. A.; Koehler, K. G.; Sharkey, N. A.; Dell'Aquila, M. L.; Blumberg, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4214−4218.
(6) Itai, A.; Kato, Y.; Tomioka, N.; Iitaka, Y.; Endo, Y.; Hasegawa, M.; Shudo, K.; Fujiki, H.; Sakai, S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 3688−3692.
(7) Wender, P. A.; Cribbs, C. M.; Koehler, K. F.; Sharkey, N. A.; Herald, C. L.; Kamano, Y.; Pettit, G. R.; Blumberg, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7197−7201.
(8) Thomson, C.; Wilkie, J. *Carcinogenesis* **1989**, *10*, 531−540.
(9) Nakamura, H.; Kishi, Y.; Pajares, M. A.; Rando, R. R. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9672−9676. Rando, R. R.; Kishi, Y. *Biochemistry* **1992**, *31*, 2211−2218.
(10) Since completion of this work, an important crystal structure of the Cys2 activator binding domain of PKCδ in complex with phorbol ester but without phospholipid has been reported (Zhang, G.; Kazanietz, M. G.; Blumberg, P. M.; Hurley, J. H. *Cell* **1995**, *81*, 917−924). The relevancy of this structure to the solution structure of the PKC−phorbol ester−phospholipid aggregate remains to be determined (see: *Chem. Eng. News* **1995**, 21−25).
(11) Nishizuka, Y. *Nature* **1988**, *334*, 661−665 and references cited therein.
(12) Osada, S.; Mizuno, K.; Saido, T. C.; Akita, Y.; Suzuki, K.; Kuroki, T.; Ohno, S. *J. Biol. Chem.* **1990**, *265*, 22434−22440.
(13) Bacher, N.; Zisman, Y.; Berent, E.; Livneh, E. *Mol. Cell. Biol.* **1991**, *11*, 126−133.
(14) Ono, Y.; Fujii, T.; Ogita, K.; Kikkawa, U.; Igarashi, K.; Nishizuka, Y. *J. Biol. Chem.* **1988**, *263*, 6927−6932.
(15) Ono, Y.; Fujii, T.; Igarashi, K.; Kuno, T.; Tanaka, C.; Kikkawa, U.; Nishizuka, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 4868−4871.
(16) Burns, D. J.; Bell, R. M. *J. Biol. Chem.* **1991**, *266*, 18330−18338.
(17) Quest, A. F. G.; Bardes, E. S. G.; Bell, R. M. *J. Biol. Chem.* **1994**, *269*, 2961−2970.
(18) Wender, P. A.; Irie, K.; Miller, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 239−243. Irie, K.; Koizumi, F.; Iwata, Y.; Ishii, T.; Yanai, Y.; Nakamura, Y.; Ohigashi, H.; Wender, P. A. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 453−458.
(19) Delclos, K. B.; Yeh, E.; Blumberg, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3054−3058.
(20) Schmidt, R.; Heck, K.; Sorg, B.; Hecker, E. *Cancer Lett.* **1985**, *26*, 97−111.

0022-3263/96/1961-2164$12.00/0



Peptide C

H_2N-HKFRLHSYSSPTFCDHCGSLLYGLVHQGMKCSCCEMNVHRRCVRSVPSLCG-COOH
(101 … 151)

Figure 1. Structure of rat brain PKC γ and peptide C.

fractions did not result in the specific labeling of any receptor proteins. While our recent studies[21] in this area using photolabile teleocidin derivatives and mouse epidermal particulate fraction resulted in the specific labeling of two proteins of *ca*. 30 and 50 kD, no specific labeling was detected in the 70−80 kD region corresponding to PKC. The failure of these attempts to label PKC is presumably due in part to the nonproductive positioning of the photolabile groups in the bound complex precluding their interaction with PKC, the insufficient reactivity of the labeling functionality (nitrenes derived from aryl azides), or the extremely low content of PKC in these membrane fractions. In order to address these issues, we have now synthesized new photolabeling agents, phorbol esters (**3**, **6**, and **9**), that incorporate a carbene precursor in the form of a diazoacetyl group at positions 3, 12, and 13 of the phorbol skeleton. The synthesis and photochemistry of **3** have recently been described in a preliminary publication.[22] This paper is a full report on the synthesis and binding of **3** and the related photolabile phorbol esters **6** and **9** and an initial report on their use in the photoaffinity labeling of the peptide incorporating the phorbol ester-binding domain of protein kinase C, peptide C (Figure 1), recently identified in our work.[18]

Results

Synthesis of Photolabile Phorbol Esters. There are six positions for attachment of a photolabile diazoacetyl group to phorbol: positions 3, 4, 9, 12, 13, and 20. Because the free hydroxyl groups at positions 4 and 20 are necessary for PKC binding[1,3,23] and derivatization of position 9 is sterically difficult, positions 3, 12, and 13 represent the prime sites for attachment of an affinity label.[23] Esters **3**, **6**, and **9** were, therefore, targeted as photoaffinity analogues of phorbol 12,13-dibutyrate (PDBu), a widely studied PKC binder and activator.[24]

The C3 β-hydroxy stereoisomer of **3** was selected for initial study because previous work in our laboratory indicated that a 3β-hydroxyl derivative (**11**) bound strongly to PKC while the corresponding 3α-isomer did not.[25] For the synthesis of **3**, phorbol (**1a**, caution[26]) was treated with butyric anhydride, DMAP, and triethylamine to give phorbol 12,13,20-tributyrate (**1b**, 43%).[27] Sodium borohydride reduction of **1b** in the presence of cerium(III) chloride, followed by treatment with tetra-*n*-butylammonium fluoride (TBAF), gave *exclusively one alcohol isomer* (**2a**, 78%), which was assigned the 3β-hydroxy stereochemistry on the basis of mechanistic considerations and its conversion to a C3,C4 acetonide.[25] It is noteworthy that the *complementary stereoisomer* (**2b**) was obtained by reduction of **1b** with sodium triacetoxyborohydride.[25,28] These assignments were confirmed by NOE difference spectroscopy of **2a** and **2b**. Saturation of the C3 proton (δ 4.21) in **2a** caused a marked enhancement (22.3%) of the C10 signal (δ 2.98). A significant enhancement of the C3 signal (13.6%) was also observed when the C10 proton in **2a** was saturated. No NOE enhancement was detected for these protons (C3 and C10) in **2b**.

Conversion of **2a** to the diazoacetyl derivative **3** was initially attempted by employing the method of Sen *et al.*,[29] who synthesized a diazoacetyl derivative of retinal from a glyoxylic acid (*p*-toluenesulfonyl)hydrazone.[30] However, a similar approach applied to **2a** gave none of the desired product. Because base decomposed the tosylhydrazone,[31] **2a** was treated with glyoxylic acid tosylhydrazone in the presence of only DCC (without DMAP and triethylamine), afterwhich triethylamine was added.[32] However, this variation proved to be unsuccessful.

An alternative route to the diazoacetyl derivative **3** based on modification of a glycine ester[33] was next examined. In this case, compound **2a** was condensed with Boc-glycine using DCC, DMAP, and triethylamine to give the glycinate **2c** (77%). The Boc group of **2c** was cleaved by 1 N HCl in acetic acid. Treatment of the resulting amine with sodium nitrite and sulfuric acid in water−dichloromethane provided the diazo ester **2d** (40%). Since the diazoacetyl group was acid labile, various alkaline conditions (potassium carbonate, lithium hydroxide, barium hydroxide and ammonium hydroxide in methanol) were examined in order to selectively cleave the C20 butanoyl group. This proved to be most readily achieved with barium hydroxide in methanol which gave the desired diazoacetyl derivative **3** in 54% yield.

The synthesis of **6**, incorporating a diazoacetyl group at position 12, was carried out in a fashion similar to that involved in the preparation of **3**. Phorbol (**1a**) was first selectively butanoylated at positions 13 and 20 with butyric anhydride and triethylamine to give **4** (52%).[34] Condensation of **4** with Boc-glycine gave the glycinate **5a** (79%). After removal of the Boc group of **5a**, the

(21) Irie, K.; Okuno, S.; Koizumi, F.; Koshimizu, K.; Nishino, H.; Iwashima, A. *Tetrahedron* **1993**, *49*, 10817−10830.
(22) Wender, P. A.; Irie, K.; Miller, B. L. *J. Org. Chem.* **1993**, *58*, 4179−4181.
(23) Hecker, E.; Adolf, W.; Hergenhahn, M.; Schmidt, R.; Sorg, B. Irritant Diterpene Ester Promoters of Mouse Skin: Contributions to Etiologies of Environmental Cancer and to Biochemical Mechanisms of Carcinogenesis. In *Cellular Interactions by Environmental Tumor Promoters*; Fujiki, H., Hecker, E., Moore, R. E., Sugimura, T., Weinstein, I. B., Eds.; Japan Sci. Soc., Tokyo; VNU Science: Utrecht, 1984; pp 3−36.
(24) Driedger, P. E.; Blumberg, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 567−571.
(25) Lee, H. Y. Ph.D. dissertation, Stanford University, 1988.
(26) Caution: Phorbol esters are potent tumor promoters and powerful irritants and should be handled with care.
(27) The structures of all compounds (>98% purity) were confirmed by UV, IR where relevant, ^{1}H NMR, and FAB-MS or high-resolution (HR) FAB-MS.
(28) Gribble, G. W.; Nutaitis, C. F. *Org. Prep. Proc.* **1985**, *17*, 317. Evans, D. A.; Di Mare, M. *J. Am. Chem. Soc.* **1986**, *108*, 2476−2478 and references cited therein.
(29) Sen, R.; Carriker, J. D.; Balogh-Nair, V.; Nakanishi, K. *J. Am. Chem. Soc.* **1982**, *104*, 3214−3216.
(30) Blankey, C. J.; Sauter, F. J.; House, H. O. *Org. Synth.* **1969**, *49*, 22−27.
(31) Corey, E. J.; Myers, A. G. *Tetrahedron Lett.* **1984**, *25*, 3559−3562.
(32) Ouihia, A.; Rene, L.; Badet, B. *Tetrahedron Lett.* **1992**, *33*, 5509−5510.
(33) Singh, A.; Thornton, E. R.; Westheimer, F. H. *J. Biol. Chem.* **1962**, 237, PC3006−3008.
(34) Sorg, B.; Fürstenberger, G.; Berry, D. L.; Hecker, E.; Marks, F. *J. Lipid Res.* **1982**, *23*, 443−447.

resultant amine was diazotized by the above-mentioned procedure to yield **5b** (57%), which was selectively hydrolyzed to give **6** (59%).

The synthesis of the third affinity labeling candidate (**9**), bearing a C-13 diazoacetyl group, required a slight modification of the procedure used for **6**. Phorbol-20 trityl ether[34] (**1c**) was condensed with Boc-glycine to give the C13-glycinate **7a** (33%). After cleavage of the trityl group by perchloric acid (84%), the resultant C20 alcohol (**7b**) was butanoylated to give **8a** (86%). Compound **8a** was subjected to acid treatment followed by diazotization to give **8b** (20%). The desired diazoacetyl compound **9** was obtained by the alkaline treatment of **8b** (67%).

Photolytic Fate of the Photolabile Phorbol Ester 3. As a prelude to PKC photoaffinity labeling, the photolytic fate of **3** in methanol as a model nucleophile and its ability to serve as a labeling agent were examined. At room temperature, methanol solution of **3** (1 mg/mL) under nitrogen was irradiated at 253.7 nm using a Rayonet photochemical reactor. The diazo ester was completely consumed within 5 min, giving one major product (**10**) and four minor products (**11**−**14**). The isolated yields of these compounds (**10**−**14**) were 43%, 14%, 12%, 11%, and 8%, respectively, indicating >50% solvent insertion products and <20% of compounds derived from the often favored Wolff rearrangement.[35] The major compound (**10**) proved to be the direct insertion product [^{1}H NMR ($CDCl_3$) δ 3.49 (s, 3H, OCO-$CH_2O\mathit{CH_3}$), 4.15 (s, 2H, OCO$\mathit{CH_2}$OCH$_3$), 5.59 (d, J = 1.3 Hz, 1H, H-3)]. Compound **11** was 3-deoxo-3β-hydroxyphorbol 12,13-dibutyrate,[25] which presumably results from hydrolysis of the C3 ester group during the photolysis and/or purification steps. The relatively low yield of **11** indicates that the ester group at position 3 is moderately stable to UV irradiation. Compound **12** was also a solvent-trapping product [^{1}H NMR ($CDCl_3$) δ 2.39 (t, J = 6.3 Hz, 1H, OCOCH$_2$CH$_2\mathit{OH}$), 2.70 (m, 2H, OCO$\mathit{CH_2}$CH$_2$OH), 3.95 (m, 2H, OCOCH$_2\mathit{CH_2}$OH), 5.57 (s, 1H, H-3)], arising from carbene insertion into the CH bond of methanol. Compounds **13** and **14** are derived from Wolff rearrangement and subsequent inter- and intramolecular capture of the resultant ketene. The ^{1}H NMR of **13** in deuteriochloroform exhibited a new methylene resonance [δ 4.23 (d, J = 16.7 Hz, 1H) and δ 4.41 (d, J = 16.7 Hz, 1H)], an ester methyl group [δ 3.80 (s, 3H)], and an upfield C3 hydrogen [δ 4.04 (s, 1H)] in accord with the assigned structure. The ^{1}H NMR of **14** in deuteriochloroform also showed new methylene signals [δ 4.07 (d, J = 17.6 Hz, 1H) and δ 4.25 (d, J = 17.6 Hz, 1H)] and an upfield C3 hydrogen [δ 4.45 (s, 1H)]. A signal corresponding to the hydroxyl group at position 4 was lacking.

Tritium Labeling of the Photolabile Phorbol Esters (3, 6, and 9). Because the diazo ester linkage to the phorbol ring system is relatively stable, the phorbol moiety was selected as a convenient subunit for radioisotopic labeling as required for identification and analysis of photolabeled PKC. Tritium incorporation into the probes **3**, **6**, and **9** was accomplished by oxidation with activated manganese dioxide to give the C-20 aldehydes and subsequent reduction with tritium-labeled sodium borohydride.[36] The specific radioactivity of the resultant probes was between 75 and 90 mCi/mmol when 359 mCi/mmol tritium-labeled sodium borohydride was used. The radiochemical purity of these tritiated probes determined by TLC and HPLC was found to be >98%. The yield of the reduction was between 40% and 50%.

Binding Affinity of the Photolabile Phorbol Esters (3, 6, and 9) to Peptide C. While a several micromole sample of pure PKC isozyme is necessary for the photoaffinity labeling, such amounts of pure peptide are not easily obtainable from natural sources. However, since the cysteine-rich sequence in the PKC regulatory domain is sufficient for phorbol ester binding,[15,16] we have explored the solid phase synthesis and use of truncated versions of PKC as more readily available surrogates of the phorbol ester binding (activator) domain of PKC.[18] Among the peptides synthesized, peptide C (Figure 1), incorporating amino acids 101−151 of the second cysteine-rich repeat of rat brain PKC γ, bound [^{3}H]PDBu with significant affinity (K_d = 41.4 nM) and only in the presence of phosphatidylserine as is found for PKC itself.[18] Moreover, like PKC itself, this peptide also bound 1,2-dioctanoyl-*sn*-glycerol and teleocidin B-4 and exhibited an ability to differentiate phorbol ester from its C-4 epimer (the former binds to both PKC and peptide C while the latter binds to neither). These findings indicate that this peptide can be used as a model of regulatory domain of PKC for photoaffinity labeling and other studies.

The binding affinities of probes **3**, **6**, and **9** to peptide C were measured in two ways: a rapid filtration procedure using a glass-fiber filter which had been treated with a cationic polymer, poly(ethylenimine),[37] and a poly(ethyleneglycol) precipitation assay.[38] We tested both assays using [^{3}H]PDBu and peptide C in the presence of phosphatidylserine. γ-Globulin (3 mg/mL) was added to the reaction mixture to precipitate peptide C.[38] The addition of γ-globulin did not affect significantly the [^{3}H]-PDBu-peptide C binding affinity in the filtration assay. Although the level of nonspecific binding was higher in the precipitation assay than in the filtration assay, the precipitation assay gave good reproducibility. Systematic studies on the [^{3}H]PDBu-peptide C binding using the precipitation assay were, therefore, performed in order to examine a number of binding variables such as concentration, the stoichiometry of [^{3}H]PDBu binding with peptide C, and the type of phospholipid required for optimal binding. Among various conditions examined, 20 nM [^{3}H]PDBu and 100 nM peptide C in 50 mM Tris−HCl (pH 7.4) in the presence of 50 μg/mL of 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine and 3 mg/mL γ-globulin gave the best results. Under these conditions, *ca*. 60 000 dpm of the total binding and *ca*. 6 000 dpm of the nonspecific binding was detected from 250 μL of the assay mixture.

Table 1 shows that the photoaffinity probes **3**, **6**, and **9** as well as PKC activators 1,2-dioctanoyl-*sn*-glycerol, (−)-indolactam-V, and teleocidin B-4 significantly bind to peptide C as determined by the inhibition of the specific binding of [^{3}H]PDBu to peptide C. The binding affinity was evaluated by the logarithm of the concentration required to cause 50% inhibition of the specific binding of [^{3}H]PDBu, pIC_{50}. IC_{50} values were calculated by a computer program (SAS: statistical analysis system) with a probit (probability unit) procedure.[39] 4α-Phorbol

(35) Shafer, J.; Baronowsky, P.; Laursen, R.; Finn, F.; Westheimer, F. H. *J. Biol. Chem.* **1966**, *241*, 421−427.
(36) Kreibich, G.; Hecker, E. *Z. Krebsforsch.* **1970**, *74*, 448−456.
(37) Tanaka, Y.; Miyake, R.; Kikkawa, U.; Nishizuka, Y. *J. Biochem.* **1986**, *99*, 257−261.
(38) Sharkey, N. A.; Blumberg, P. M. *Cancer Res.* **1985**, *45*, 19−24.

Table 1. Inhibition of the Specific [^{3}H]PDBu Binding to Peptide C by the Photoaffinity Probes (3, 6, and 9)[a]

compd	95% fiducial limits[b]			pIC_{50} [mouse epidermis][c] (log 1/M)
	pIC_{50} (log 1/M)	lower	upper	
3	5.23	5.11	5.36	*ca.* 5.00
6	6.24	6.12	6.36	NT[d]
9	6.43	6.31	6.56	NT
phorbol 12,13-dibutyrate	7.50	6.65	8.31	NT
1,2-dioctanoyl-*sn*-glycerol	5.35	5.24	5.46	NT
(−)-indolactam-V	5.61	5.45	5.75	5.97
teleocidin B-4	8.06	7.94	8.18	8.71
4α-phorbol 12,13-didecanoate	<4.00			NT

[a] This assay was carried out by the poly(ethyleneglycol) precipitation method.[38] The assay solution (250 μL) consisted of 50 mM Tris−HCl (pH 7.4 at 25 °C), 3 mg/mL bovine γ-globulin, 20 nM [^{3}H]PDBu (20 Ci/mmol), 50 μg/mL 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, 100 nM peptide C, and various concentrations of an inhibitor. Nonspecific binding was determined in the presence of a 500-fold excess of nonradioactive PDBu. The final dimethyl sulfoxide concentration was 2%. Each point was measured in triplicate with less than 10% variation. [b] These limits were calculated by the probit analysis.[39] [c] Data from the inhibition of the specific [^{3}H]-12-*O*-tetradecanoylphorbol 13-acetate (TPA) binding to the mouse epidermal particulate fraction.[21] [d] Not tested.

Table 2. Binding of the Tritiated Photolabile Phorbol Esters (3, 6, and 9) to Peptide C[a]

photoaffinity probe	total binding (SEM) (pmol)	nonspecific binding (SEM) (pmol)	specific binding (pmol)
3	47.3 (5.4)	14.3 (0.3)	32.9
6	36.0 (8.1)	7.8 (0.3)	28.2
9	47.6 (4.8)	7.8 (1.0)	39.8

[a] This assay was carried out by the poly(ethyleneglycol) precipitation method.[38] The assay solution (250 μL) consisted of 50 mM Tris−HCl (pH 7.4 at 25 °C), 3 mg/mL bovine γ-globulin, 100 μg/mL 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, 3 μM peptide C, and 2 μM tritiated **3**, **6**, or **9** (85.4, 79.0, and 79.3 mCi/mmol, respectively). Nonspecific binding was determined in the presence of 500-fold excess of nonradioactive PDBu.

12,13-didecanoate, a phorbol ester derivative that does not bind to PKC, did not bind to peptide C. These structure−activity relationships are thus very close to those observed for PKC itself.[1,2,23] Furthermore, a good correlation between the binding assay using peptide C and the conventional receptor assay using the mouse epidermal particulate fraction[40,41] was observed.

Photoaffinity Labeling of Peptide C by the Tritiated Photolabile Phorbol Esters (3, 6, and 9). Peptide C (3 μM) was incubated with 2 μM of a tritium-labeled photoaffinity probe (**3**, **6**, or **9**) in the presence of 100 μg/mL of 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine and 3 mg/mL of bovine γ-globulin at 30 °C for 20 min. Table 2 summarizes the total and the nonspecific binding under these conditions. The specific binding of **3**, **6**, and **9** was 32.9, 28.2, and 39.8 pmol, respectively, which corresponded to 6.6, 5.6, and 8.0% of the total photolabile probes added in the reaction mixture. Concentrations of peptide C of more than 5 μM gave poor binding, possibly because of aggregation and/or precipitation of peptide C from the reaction mixture. When the peptide C concentration was fixed at 3 μM, a 2 μM probe concentration gave the best specific to nonspecific binding ratio. The concentration of the phospholipid of 100 μg/mL gave the highest specific binding.

After a 5-min incubation at 0 °C, the reaction mixture was irradiated by a UV lamp for 10 s under argon. Irradiation for 10 s under the same conditions decomposed more than 80% of the probes, but did not significantly decrease the specific binding of [^{3}H]PDBu to peptide C (data not shown). Significant modification of peptide C was observed with the 30-s irradiation. The photolabeled peptide C was precipitated by addition of 35% poly(ethyleneglycol). After centrifugation, the precipitate was subjected to SDS gel electrophoresis, which was carried out by the method of Swank and Munkres[42] with slight modifications.

As shown in Figure 2, specific photolabeling peaks from **6** and **9** were detected in the higher molecular weight region than peptide C, suggesting that peptide C was photolabeled by these probes. Although the molecular weight of these peaks deduced from the marker proteins (8.2−10.6 kD) differs slightly from that of the phorbol−peptide C adducts (6.3 kD), the branched molecular shape of these adducts would reduce their mobility since the intrinsic charge and shape could be relatively more important in determining the mobility of small proteins in SDS gels than of large proteins. In fact, at least 20% deviation is generally observed in the SDS−PAGE with proteins smaller than 10 kD.[42] No significant peak in this region (8.2−10.6 kD) was detected in the absence of peptide C or 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine (data not shown), indicating that these specific photolabeling peaks are not ascribable to the photolabeled γ-globulin and/or unidentified impurities. The specific photolabeling yield of peptide C by **6** and **9** was determined to be 2.9% and 1.9%, respectively.

In contrast to the results with probes **6** and **9**, no significant peak was observed in the molecular weight region higher than peptide C when photolabeling was conducted with **3**. Only a peak in the molecular weight region lower than 2.5 kD was observed. A significant peak in this region (<2.5 kD) was also detected in the absence of peptide C or the phospholipid (data not shown). Moreover, similar peaks in this region (<2.5 kD) were also faintly detected when photolabeling was performed with **6** and **9** as shown in Figure 2, suggesting that the peak observed in the photoaffinity labeling with **3** is not due to the peptide C−ligand adduct, but rather to a complex with the phospholipid and/or the degradation products of **3**.

Discussion

Three new photolabile phorbol esters (**3**, **6**, and **9**) incorporating a diazoacetyl group at positions 3, 12, and 13 have been synthesized from phorbol (**1a**). The diazoacetyl group is a carbene precursor with minimum steric requirements and is photoactivatable at 254 nm. Although this wavelength is potentially damaging to proteins, carbene generation is sufficiently rapid to minimize degradation and the derived carbene is sufficiently reactive to readily enter lipophilic as well as hydrophilic regions. This character seems to be especially important for photolabeling of PKC since previous photoaffinity labeling using aryl azide derivatives of phorbol esters and

(39) James, H. G. Probit procedure. In *SAS User's Guide*; Jane, T. H., Kathryn, A. C., Eds.; SAS Institute: Cary, NC, 1979; pp 357−360.
(40) Ashendel, C. L.; Boutwell, R. K. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 543−549.
(41) Hergenhahn, M.; Hecker, E. *Carcinogenesis* **1981**, *2*, 1277−1281.
(42) Swank, R. T.; Munkres, K. D. *Anal. Biochem.* **1971**, *39*, 462−477.

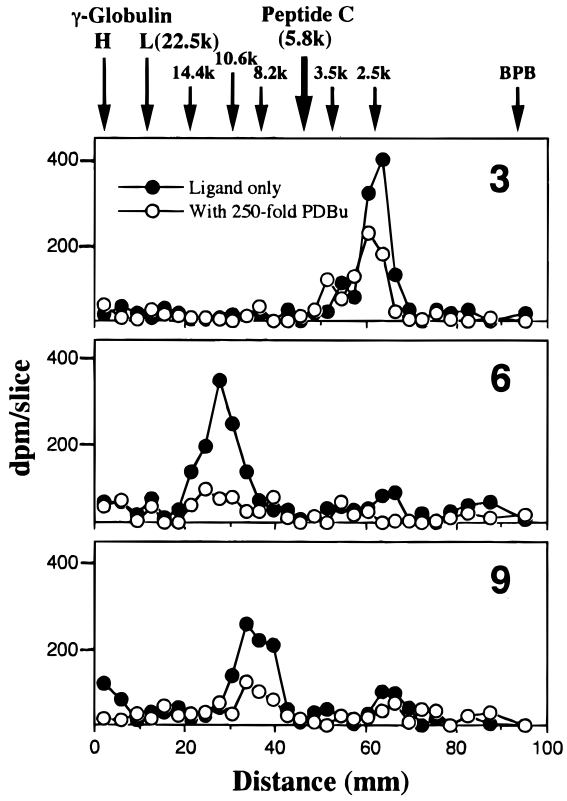


Figure 2. SDS gel electrophoresis of peptide C photolabeled with the tritiated photolabile phorbol esters (**3**, **6**, and **9**) whose specific radioactivities were 85.4, 79.0, and 79.3 mCi/mmol, respectively. This is the result of one experiment when all probes were tested simultaneously. A similar result was obtained in another experiment. Peptide C (3 μM) incubated with each tritium probe (2 μM) in the presence of 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine (100 μg/mL) and bovine γ-globulin (3 mg/mL) at 30 °C for 20 min was irradiated in the absence (●) or in the presence (○) of nonradioactive PDBu (500 μM). After precipitation with 35% poly(ethyleneglycol) followed by centrifugation, the pellet was analyzed by SDS−PAGE. The total percentage concentration of acrylamide and bisacrylamide was 13.75%. The percentage concentration of the cross linker relative to the total concentration was 9.09%. Urea (8 M) was added to the gel to achieve a superior resolution. The radioactivity in the various regions of the gel was determined by slicing the gel into 3 mm fractions. Molecular masses (in kD in the top section) of the marker proteins (myoglobin fragments), peptide C (MW 5772), and bovine γ-globulin are indicated by arrows.

teleocidins failed to photolabel PKC.[19−21] The most serious drawback of the diazoacetyl group as a photoaffinity label is its propensity for Wolff rearrangement to a ketene.[35] However, a model experiment using **3** in methanol gave only 20% Wolff rearrangement products even in a hydrophilic solvent which accelerates this rearrangement, establishing its potential as a photoaffinity label.

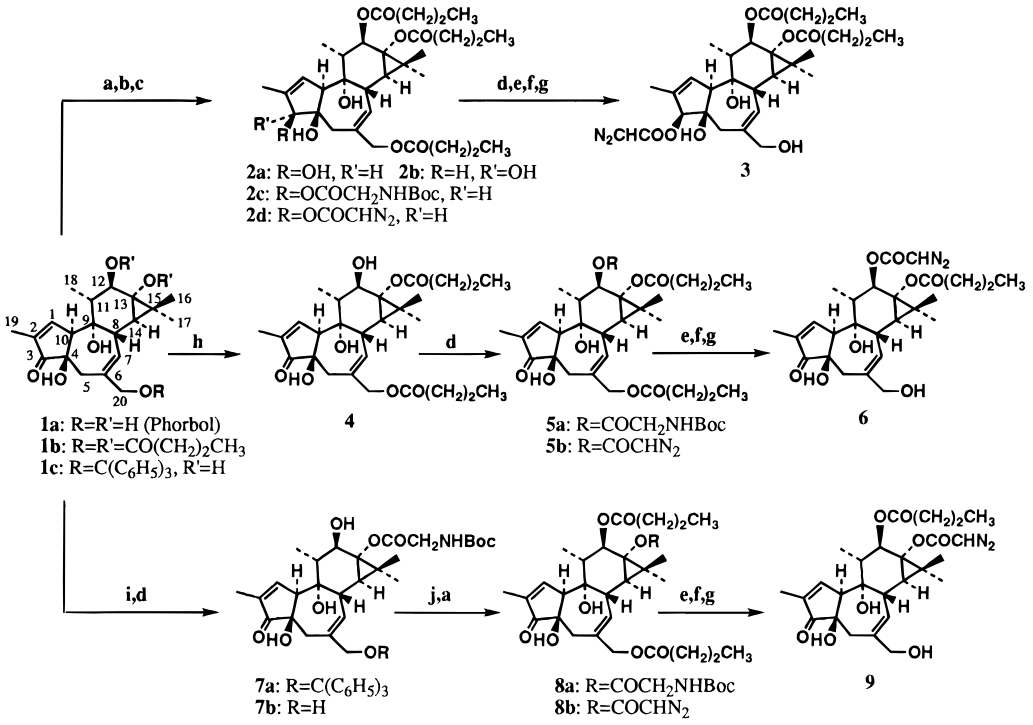
Milligram quantities of a PKC isozyme necessary for photoaffinity labeling cannot be easily obtained from natural sources. To obtain a pure and abundant sample of the PKC regulatory domain and to simplify the analysis of the photolabeled PKC, we have synthesized peptide C, a model peptide incorporating the phorbol ester binding domain of rat brain PKC γ.[18] The similar binding behavior of peptide C and native PKC with phorbol esters and the NMR behavior of peptide C indicate that peptide C emulates the structural features of the regulatory domain of PKC γ in its ability to recognize activators and could thus be used to investigate the structural requirements for PKC activation by phorbol ester-type tumor promoters.[18] Moreover, a fairly good correlation was observed between the binding assay using peptide C and the conventional receptor assay using the mouse epidermal particulate fraction (Table 1).[40,41] As such, this peptide C assay can be used for various studies in place of the PKC assay, a procedure which typically requires sacrificing a large number of mice. In addition, the particulate fraction used in the conventional receptor assay degrades on long term storage and the IC_{50} values determined by this assay can vary significantly depending on the particulate fraction prepared. Since peptide C is stable under argon at 4 °C and readily available in milligram quantities, the binding assay using peptide C is a new and convenient method to evaluate the potential tumor-promoting activity of the phorbol ester-type tumor promoters and more generally to obtain information on the structure of the PKC regulatory domain.

The three photoaffinity phorbol esters (**3**, **6**, and **9**) bind to peptide C with high affinities. Of these probes, **6** and **9** showed approximately 10-fold stronger binding affinity than **3**, suggesting that groups at position 3 affect binding, either directly or through indirect influence on the adjacent, C4 hydroxyl group. While the binding of these probes was 10−100-fold weaker than that of PDBu, it is sufficient for their use in the photoaffinity labeling. This was confirmed by using the corresponding tritium labeled probes; 2 μM tritium-labeled **3**, **6** or **9** specifically bound to 3 μM peptide C in the presence of the phospholipid (Table 2).

Photoaffinity labeling of peptide C by tritium-labeled **6** and **9** with a diazoacetyl group at positions 12 and 13, respectively, resulted in the specific photolabeling of peptide C. On the other hand, no specific photolabeling on peptide C was observed using tritium-labeled **3**. Previous structure−activity[23] and molecular modeling studies[3−9] suggested that oxygens at C-9, C-20, and C-3 or C-4 of the phorbol esters interact with PKC and that the ester groups at C-12 and C-13 interact with phosphatidylserine. On the basis of these studies, attachment of a diazoacetyl group at position 3 rather than at position 12 or 13 could allow the photoaffinity labeling of PKC. However, the present labeling results suggest that the C3 position of the phorbol esters could be positioned between PKC and the phospholipids rather than deeply embedded in either. Thus, the oxygen at C-3 could be involved in interaction with the polar serine moiety of the phosphatidylserine molecule, and the ester groups at C-12 and C-13 could interact with both the nonpolar group of the phosphatidylserine and peptide C. Alternatively, the 10-fold lower binding affinity of **3** to peptide C relative to that of **6** and **9** might be another reason for the failure of the photolabeling of peptide C by **3**.

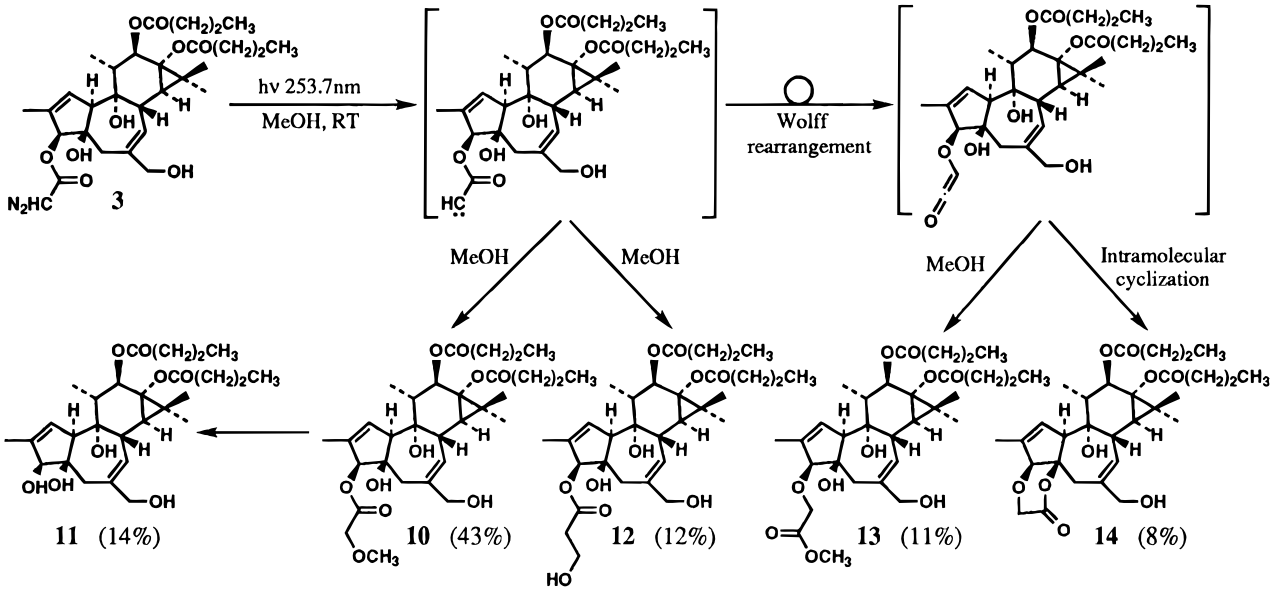
The specific labeling yield of peptide C by **6** and **9** was 2.9% and 1.9%, respectively. Although this value is not high enough at present to determine the labeled amino acid residue(s) of peptide C, as the first demonstration of the photolabeling of the PKC regulatory domain (peptide C) using photolabile phorbol esters, it suggests that this approach could lead to identification of the amino acid residues with which phorbol esters interact in the solution aggregate. Since **6**, with a diazoacetyl group at position 12, photolabels peptide C, the previous failure[19,20] to photolabel PKC in the mouse brain or rat epidermal particulate fraction using phorbol probes with an aryl azide group at position 12 might be mainly

Scheme 1. Synthesis of photolabile phorbol esters (3, 6, and 9)[a]



[a] Key: (a) $[CH_3(CH_2)_2CO]_2O$, DMAP, NEt_3, CH_2Cl_2; (b) $NaBH_4$, $CeCl_3$, MeOH; (c) TBAF, THF; (d) Boc-Gly, DMAP, NEt_3, DCC, THF; (e) 1 N HCl, AcOH; (f) $NaNO_2$, $H_2O-CH_2Cl_2$, H_2SO_4; (g) $Ba(OH)_2$, MeOH; (h) $[CH_3(CH_2)_2CO]_2O$, NEt_3, $THF-CH_2Cl_2$; (i) $(C_6H_5)_3CCl$, pyridine; (j) $HClO_4$, MeOH.

Scheme 2. Photolysis of 3 in Methanol



ascribable to the scale required for studies with native PKC, the orientation of the affinity functionality, and/or the reactivity of the nitrene.

Conclusion

In summary, we have synthesized three new photolabile phorbol esters with a diazoacetyl group at positions 3, 12, and 13 (**3**, **6**, and **9**). Esters **6** and **9** photolabeled peptide C, a newly introduced and readily available peptide corresponding to the activator domain of PKC.[18] The failure of **3** to photolabel peptide C suggests that the ketone group at C3 of the phorbol esters could be positioned near the polar domain of phosphatidylserine in the bound complex. Although the labeling yields obtained with **6** and **9** were not high enough to determine the labeled amino acid residue(s) of peptide C, the present result shows that introduction of a photolabile group into position 12 or 13 can be exploited for the photoaffinity labeling of peptide C. Further studies are directed at enhancing the labeling yield of peptide C by these newly designed photoaffinity probes. A significant drawback of the diazoacetyl group for PKC photoaffinity labeling proved to be its hydrophilic character which weakens the binding affinity of the probe to PKC. Aryldiazirines are one of the most promising candidates for new probes

because the diazirine-derived protein complex is fairly stable[43] and because the hydrophobic character of the diazirine would enhance the binding affinity of the probe to peptide C. The synthesis and photoaffinity labeling of peptide C with the new photoaffinity probes having the aryl diazirine moiety at position 12 or 13 are in progress.

Experimental Section

General Methods. HPLC was carried out on YMC packed A-023 (silica gel, 10 mm i.d. × 250 mm), AQ-323 (ODS, 10 mm i.d. × 250 mm) and A-311 (ODS, 6 mm i.d. × 100 mm) columns (Yamamura Chemical Laboratory) and a μ-Bondasphere C_{18} (19 mm i.d. × 150 mm) column (Waters Associates). Kieselgel 60 (Merck), Wakogel C-100 and C-200 (silica gel, Wako Pure Chemical Industries), and YMC gel A60-350/250 (ODS, Yamamura Chemical Laboratory) were used for column chromatography.

[^{3}H]$NaBH_4$ (359.8 mCi/mmol) and [^{3}H]PDBu (20 Ci/mmol) were purchased from NEN Research Products. Teleocidin B-4 and (−)-indolactam-V were isolated from *Streptoverticillium blastmyceticum* NA34-17 as reported previously.[44] All other chemicals and reagents were purchased from commercial sources.

Synthesis of the Photoaffinity Probe 3. Butyric anhydride (0.5 mL, 3.06 mmol) was treated with phorbol (**1a**, 200 mg, 0.549 mmol) and DMAP (234 mg, 1.92 mmol) in dry triethylamine (0.5 mL, 3.59 mmol) and CH_2Cl_2 (9 mL) at room temperature for 18 h. After being partitioned between CH_2Cl_2 and water, the CH_2Cl_2 layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness. The CH_2Cl_2 extracts were purified by flash column chromatography on Kieselgel 60 with 15% EtOAc in hexane to give **1b** (136.4 mg, 0.238 mmol) in 43% yield: IR (neat) 3405, 2966, 2878, 1734, 1716, 1177 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 0.087 M) δ 0.87−0.98 (m, 12H), 1.03 (d, J = 5.2 Hz, 1H), 1.20 (s, 3H), 1.23 (s, 3H), 1.58−1.71 (m, 6H), 1.77 (brs, 3H), 2.13 (m, 1H), 2.25−2.34 (m, 6H), 2.41 (d, J = 18.7 Hz, 1H), 2.54 (brd, J = 18.7 Hz, 1H), 3.23 (m, 2H), 4.46 (m, 2H), 5.41 (d, J = 10.4 Hz, 1H), 5.68 (brs, 1H, OH), 5.72 (brd, J = 4.1 Hz, 1H), 7.60 (m, 1H); HR-FAB-MS m/z 573.3085 ([M − H]$^-$, calcd for $C_{32}H_{45}O_9$, 573.3064).

Compound **1b** (54.0 mg, 0.094 mmol) and $CeCl_3 \cdot 7H_2O$ (70.0 mg, 0.188 mmol) were dissolved in MeOH (5 mL). To the solution was added $NaBH_4$ (7.1 mg, 0.188 mmol) portionwise. The reaction mixture was stirred at room temperature for 2 h. After being quenched with water (1 mL), the solution was concentrated *in vacuo* and extracted with CH_2Cl_2. After drying over Na_2SO_4, the CH_2Cl_2 extracts were dissolved in THF (2 mL) and 1 M TBAF in THF (0.4 mL) and stirred at room temperature for 1.5 h. After addition of 5% HCl (0.5 mL), the reaction mixture was extracted with CH_2Cl_2. The CH_2Cl_2 extracts were purified by flash column chromatography on Kieselgel 60 with 20% EtOAc in hexane to give **2a** (42.1 mg, 0.073 mmol) in 78% yield: IR (neat) 3429, 2965, 2878, 1738, 1715, 1462, 1178, 1083 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 0.10 M) δ 0.92−1.00 (m, 12H), 1.04 (d, J = 5.0 Hz, 1H), 1.20 (s, 3H), 1.27 (s, 3H), 1.61−1.72 (m, 9H), 2.13 (m, 1H), 2.28−2.35 (m, 6H), 2.52 (brd, J = 17.1 Hz, 1H), 2.60 (d, J = 17.1 Hz, 1H), 2.77 (m, 2H, H-8 and OH), 2.98 (m, 1H), 4.21 (brs, 1H), 4.51 (m, 2H), 5.42 (d, J = 10.4 Hz, 1H), 5.44 (brs, 1H, OH), 5.74 (m, 2H); HR-FAB-MS m/z 575.3250 ([M − H]$^-$, calcd for $C_{32}H_{47}O_9$, 575.3220).

Dry triethylamine (0.21 mL, 1.50 mmol), Boc-glycine (262.1 mg, 1.50 mmol), DCC (308.7 mg, 1.50 mmol), and DMAP (182.9 mg, 1.50 mmol) were added to an anhydrous THF solution (14.4 mL) of **2a** (431.2 mg, 0.75 mmol). After being stirred for 17 h, the reaction mixture was treated with water (2 mL) and extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness. The EtOAc extracts were purified by flash column chromatography on Kieselgel 60 with 16% EtOAc in hexane to give **2c** (425.0 mg, 0.580 mmol) in 77% yield; IR (neat) 3397, 2967, 2878, 1736, 1719, 1518, 1452, 1368, 1251, 1172, 980, 870 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 0.010 M) δ 0.93−0.99 (m, 12H), 1.02 (d, J = 4.9 Hz, 1H), 1.19 (s, 3H), 1.24 (s, 3H), 1.45 (s, 9H), 1.61−1.72 (m, 9H), 2.15 (m, 1H), 2.21−2.39 (m, 6H), 2.53 (brd, J = 17.6 Hz, 1H), 2.55 (brs, 1H, OH), 2.75 (d, J = 17.6 Hz, 1H), 2.78 (m, 1H), 3.06 (m, 1H), 3.98 (m, 2H), 4.49 (m, 2H), 5.07 (brm, 1H), 5.41 (d, J = 10.3 Hz, 1H), 5.43 (brs, 1H, OH), 5.54 (d, J = 1.3 Hz, 1H), 5.73 (brd, J = 6.2 Hz, 1H), 5.89 (d, J = 1.4 Hz, 1H); HR-FAB-MS m/z 732.3929 ([M − H]$^-$, calcd for $C_{39}H_{59}NO_{12}$, 732.3959).

Compound **2c** (66.0 mg, 0.090 mmol) was dissolved in 1 N HCl and AcOH (0.8 mL). After being stirred for 20 min at room temperature, the solution was neutralized with 5% $NaHCO_3$ and extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue (3-deoxo-3β-glycyloxyphorbol 12,13,20-tributyrate) was dissolved in water (0.5 mL) and CH_2Cl_2 (0.6 mL) and diazotized under nitrogen with $NaNO_2$ (10.8 mg, 0.16 mmol) and 12% (w/w) H_2SO_4 (50 μl) at −5 °C. The reaction mixture was stirred at 0 °C for 10 min. After neutralization with 5% $NaHCO_3$ in water, the mixture was extracted with CH_2Cl_2 and dried over Na_2SO_4. The CH_2Cl_2 extracts were purified by flash column chromatography on Kieselgel 60 with 20% EtOAc in hexane to give **2d** (23.1 mg, 0.036 mmol) in 40% yield: IR (neat) 3423, 2966, 2878, 2114 (CHN_2), 1732, 1699, 1459, 1382, 1250, 1180, 1086, 1070, 979, 876, 737 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 0.008 M) δ 0.93−0.99 (m, 12H), 1.03 (d, J = 4.9 Hz, 1H), 1.20 (s, 3H), 1.25 (s, 3H), 1.62−1.72 (m, 9H), 2.13 (m, 1H), 2.24 (brs, 1H, OH), 2.25−2.37 (m, 6H), 2.54 (dd, J = 17.3, 1.9 Hz, 1H), 2.71 (brt, J = 5.9 Hz, 1H), 2.78 (d, J = 17.3 Hz, 1H), 3.05 (m, 1H), 4.51 (m, 2H), 4.88 (brs, 1H, CHN_2), 5.42 (d, J = 10.3 Hz, 1H), 5.44 (brs, 1H, OH), 5.55 (d, J = 1.3 Hz, 1H), 5.73 (brd, J = 6.9 Hz, 1H), 5.88 (d, J = 1.6 Hz, 1H); HR-FAB-MS m/z 643.3197 ([M − H]$^-$, calcd for $C_{34}H_{47}N_2O_{10}$, 643.3231).

Saturated $Ba(OH)_2$ methanol solution (0.35 mL) was added to the methanol solution (5 mL) of **2d** (54.4 mg, 0.084 mmol). After being stirred for 3 h at room temperature, the mixture was partitioned between EtOAc and water. The EtOAc extracts were purified by flash column chromatography on Kieselgel 60 with 35% EtOAc in hexane to give **3** (26.0 mg, 0.045 mmol) in 54% yield: UV λ_{max} (MeOH) nm (ϵ) 248.5 (15 700); IR (neat) 3416, 2966, 2877, 2115 (CHN_2), 1718, 1459, 1382, 1251, 1181, 1069, 980, 874, 738 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, 0.019 M) δ 0.93−0.99 (m, 9H, H_3-18, H_3-butyrate), 1.04 (d, J = 4.9 Hz, 1H, H-14), 1.19 (s, 3H, H_3-16 or -17), 1.24 (s, 3H, H_3-16 or -17), 1.62 (t, J = 1.1 Hz, 3H, H_3-18), 1.68 (m, 4H, H_2-butyrate), 1.72 (m, 1H, OH-20), 2.13 (m, 1H, H-11), 2.23 (brs, 1H, OH-9), 2.30 (m, 4H, H_2-butyrate), 2.54 (brd, J = 17.4 Hz, 1H, H-5a), 2.73 (t, J = 5.8 Hz, 1H, H-8), 2.80 (d, J = 17.4 Hz, 1H, H-5b), 3.03 (d, J = 2.2 Hz, 1H, H-10), 4.07 (d, J = 5.9 Hz, 2H, H-20), 4.89 (brs, 1H, CHN_2), 5.41 (d, J = 10.3 Hz, 1H, H-12), 5.43 (brs, 1H, OH-4), 5.54 (d, J = 1.0 Hz, 1H, H-3), 5.67 (d, J = 4.6 Hz, 1H, H-7), 5.87 (d, J = 1.5 Hz, 1H, H-1), these assignments were derived from a ^{1}H−^{1}H COSY spectrum; HR-FAB-MS m/z 573.2831 ([M − H]$^-$, calcd for $C_{30}H_{41}N_2O_9$, 573.2812).

Reduction of 1b by Sodium Triacetoxyborohydride. Compound **1b** (62.6 mg, 0.11 mmol) was dissolved in dry THF (4.3 mL). To the solution was added $NaBH(OAc)_3$ (113.8 mg, 0.537 mmol). The reaction mixture was stirred at room temperature for 3 h. After addition of 5% $NaHCO_3$ solution (3 mL), the reaction mixture was extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness. The EtOAc extracts were purified by flash column chromatography on Kieselgel 60 using 30% EtOAc in hexane to give **2b** (36.5 mg, 0.063 mmol) as colorless needles in a 58% yield: IR (neat) 3428, 2965, 2877, 1735, 1720, 1459, 1377, 1264, 1182, 1087, 980, 914, 867 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 0.080 M) δ 0.93−0.99 (m, 12H), 1.06 (d, J = 4.9 Hz, 1H), 1.20 (s, 3H), 1.27 (s, 3H), 1.62−1.72 (m, 6H), 1.81 (d, J = 1.5 Hz, 3H), 2.07 (m, 2H, H-11, OH), 2.22−2.41 (m, 7H), 2.62 (brt, J = 6.0 Hz, 1H), 2.94 (d, J = 16.5 Hz, 1H), 3.13 (m, 1H),

(43) Hatanaka, Y.; Hashimoto, M.; Kurihara, H.; Nakayama, H.; Kanaoka, Y. *J. Org. Chem.* **1994**, *59*, 383−387 and references cited therein.

(44) Irie, K.; Koshimizu, K. *Mem. Coll. Agric., Kyoto Univ.* **1988**, *132*, 1−59.

3.74 (d, J = 8.3 Hz, 1H), 4.59 (m, 2H), 5.43 (d, J = 10.4 Hz, 1H), 5.49 (brs, 1H, OH), 5.69 (brd, J = 6.5 Hz, 1H), 5.91 (s, 1H). The ^{1}H NMR of **2b** was identical with that reported previously.[25]

Synthesis of the Photoaffinity Probe 6. Phorbol (**1a**, 500 mg, 1.37 mmol) was mixed with triethylamine (2.87 mL, 20.6 mmol) and butyric anhydride (3.37 mL, 20.6 mmol) in dry CH_2Cl_2 (13.6 mL) and dry THF (13.6 mL). After the reaction mixture was stirred for 19 h at room temperature, EtOAc (*ca.*100 mL) was added, and the mixture was then washed with 1 M HCl. The solution was stirred with water (60 mL) for 2 h and extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4 and concentrated to dryness *in vacuo*. The concentrates were purified by column chromatography on Wakogel C-200 using 20% EtOAc in hexane to give **4** (362.3 mg, 0.72 mmol) in 52% yield: UV λ_{max} (MeOH) nm (ϵ) 234 (5000); ^{1}H NMR δ (500 MHz, $CDCl_3$, 0.069 M) δ 0.91−1.03 (m, 10H), 1.22 (s, 3H), 1.24 (s, 3H), 1.65 (m, 4H), 1.77 (d, J = 1.7 Hz, 3H), 2.01 (m, 1H), 2.29 (t, J = 7.4 Hz, 2H), 2.34 (t, J = 7.4 Hz, 2H), 2.38 (d, J = 19.1 Hz, 1H), 2.53 (brd, J = 19.1 Hz, 1H), 3.13 (m, 2H, H-10, OH), 3.20 (brt, J = 5.3 Hz, 1H), 3.48 (s, 1H, OH), 3.96 (dd, J = 9.7, 2.9 Hz, 1H), 4.44 (d, J = 12.4 Hz, 1H), 4.49 (d, J = 12.4 Hz, 1H), 5.68 (d, J = 4.1 Hz, 1H), 7.57 (s, 1H); FAB-MS m/z 505 (MH^+).

Compound **4** (345 mg, 0.68 mmol) was condensed with Boc-glycine by a method similar to that used in the synthesis of **2c**. After 39 h of stirring, the crude product was purified by column chromatography on Wakogel C-200 using 20% EtOAc in hexane to give **5a** (358 mg, 0.54 mmol) in 79% yield: UV λ_{max} (MeOH) nm (ϵ) 232 (9100); ^{1}H NMR (500 MHz, $CDCl_3$, 0.11 M) δ 0.90 (d, J = 6.5 Hz, 3H), 0.93 (t, J = 7.4 Hz, 3H), 0.96 (t, J = 7.4 Hz, 3H), 1.06 (d, J = 5.3 Hz, 1H), 1.22 (s, 3H), 1.23 (s, 3H), 1.45 (s, 9H), 1.65 (m, 4H), 1.78 (m, 3H), 2.17 (m, 1H), 2.31 (m, 4H), 2.42 (d, J = 19.1 Hz, 1H), 2.55 (brd, J = 19.1 Hz, 1H), 2.64 (brs, 1H, OH), 3.25 (s, 2H), 3.93 (m, 2H), 4.45 (d, J = 11.3 Hz, 1H), 4.49 (d, J = 11.3 Hz, 1H), 5.05 (brs, 1H, NH), 5.44 (d, J = 10.3 Hz, 1H), 5.63 (brs, 1H, OH), 5.72 (d, J = 4.0 Hz, 1H), 7.59 (s, 1H); FAB-MS m/z 684 ($[M + Na]^+$).

Compound **5b** (43 mg, 0.075 mmol) was obtained from **5a** (86 mg, 0.13 mmol) in a manner similar to that described for **2d** in 57% yield: UV λ_{max} (MeOH) nm (ϵ) 249 (20 800); ^{1}H NMR (500 MHz, $CDCl_3$, 0.038 M) δ 0.92−0.97 (m, 9H), 1.05 (d, J = 5.2 Hz, 1H), 1.21 (s, 3H), 1.24 (s, 3H), 1.60−1.70 (m, 4H), 1.78 (m, 3H), 2.13 (m, 1H), 2.27−2.37 (m, 5H, H_2-butyrate, OH), 2.42 (d, J = 19.1 Hz, 1H), 2.53 (brd, J = 19.1 Hz, 1H), 3.23 (m, 2H), 4.45 (d, J = 12.4 Hz, 1H), 4.49 (d, J = 12.4 Hz, 1H), 4.74 (brs, 1H, CHN_2), 5.45 (d, J = 10.3 Hz, 1H), 5.66 (brs, 1H, OH), 5.72 (d, J = 4.0 Hz, 1H), 7.61 (s, 1H); FAB-MS m/z 573 (MH^+).

Deprotection of the C-20 butanoyl group of **5b** (89.2 mg, 0.156 mmol) was carried out by the method employed in the synthesis of **3**. The crude product was purified by HPLC on μ-Bondasphere using 63% MeOH followed by YMC A-023 using 5% 2-PrOH in hexane to give **6** (46.1 mg, 0.0918 mmol) in 59% yield: UV λ_{max} (MeOH) nm (ϵ) 249 (18 800); IR (neat) 3350, 2900, 2105 (CHN_2), 1695, 1375, 1190, 1000 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, 0.0016 M) δ 0.93 (d, J = 6.6 Hz, 3H, H_3-18), 0.96 (t, J = 7.4 Hz, 3H, H_3-butyrate), 1.08 (d, J = 5.2 Hz, 1H, H-14), 1.21 (s, 3H, H_3-16 or -17), 1.23 (s, 3H, H_3-16 or -17), 1.67 (m, 2H, H_2-butyrate), 1.78 (m, 3H, H_3-19), 2.14 (m, 1H, H-11), 2.19 (brs, 1H, OH-9), 2.27−2.37 (m, 2H, H_2-butyrate), 2.48 (d, J = 19.0 Hz, 1H, H-5a), 2.55 (brd, J = 19.0 Hz, 1H, H-5b), 3.24 (m, 2H, H-8, 10), 3.99 (dd, J = 13.1, 6.2 Hz, 1H, H-20a), 4.04 (dd, J = 13.1, 5.5 Hz, 1H, H-20b), 4.73 (brs, 1H, CHN_2), 5.46 (d, J = 10.2 Hz, 1H, H-12), 5.66 (brs, 1H, OH-4), 5.69 (d, J = 5.6 Hz, 1H, H-7), 7.60 (s, 1H, H-1), these assignments were derived from a ^{1}H−^{1}H COSY spectrum; HR-FAB-MS m/z 503.2361 (MH^+), calcd for $C_{26}H_{35}N_2O_8$, 503.2393.

Synthesis of the Photoaffinity Probe 9. Phorbol (**1a**, 1 g, 2.74 mmol) was reacted with trityl chloride (2.3 g, 8.25 mmol) in pyridine (22.5 mL) at room temperature for 47 h. The reaction mixture was partitioned between EtOAc and saturated NaCl solution. The EtOAc layer was washed with 1 M HCl, dried over Na_2SO_4, and evaporated to dryness *in vacuo*. The concentrates were purified by column chromatography on Wakogel C-200 with EtOAc and increasing amounts of hexane to give **1c** (1.02 g, 1.68 mmol) in 61% yield. Compound **1c** (454 mg, 0.75 mmol) was stirred with Boc-glycine (131 mg, 0.75 mmol), DCC (310 mg, 1.50 mmol), triethylamine (0.21 mL, 1.50 mmol), and DMAP (183 mg, 1.50 mmol) in dry THF (32.8 mL) at room temperature for 5 h. After addition of water (10 mL), the mixture was extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4 and evaporated *in vacuo* to dryness. The concentrates were purified by column chromatography on Wakogel C-200 using 30% EtOAc in hexane, followed by YMC gel using 70% MeOH to give **7a** (187 mg, 0.24 mmol) in 33% yield: UV λ_{max} (MeOH) nm (ϵ) 256 (2800), 207 (29 200); ^{1}H NMR (500 MHz, $CDCl_3$, 0.055 M) δ 1.03 (m, 4H), 1.22 (s, 3H), 1.24 (s, 3H), 1.45 (s, 9H), 1.76 (d, J = 1.6 Hz, 3H), 1.99 (m, 1H), 2.36 (d, J = 18.9 Hz, 1H), 2.48 (d, J = 18.9 Hz, 1H), 2.71 (brs, 1H, OH), 3.04 (brs, 1H), 3.11 (m, 2H, H-8, OH), 3.38 (brs, 1H, OH), 3.55 (m, 2H), 3.97 (m, 3H), 5.05 (brs, 1H, NH), 5.58 (d, J = 4.3 Hz, 1H), 7.21−7.43 (m, 15H), 7.54 (s, 1H); FAB-MS m/z 786 ($[M + Na]^+$).

Compound **7a** (179 mg, 0.24 mmol) was stirred with $HClO_4$ (0.06 mL) in MeOH (18 mL) at room temperature for 20 min. After addition of saturated $NaHCO_3$ (20 mL), the reaction mixture was extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness. The concentrates were purified by column chromatography on Wakogel C-100 using hexane and increasing amounts of EtOAc to give **7b** (103 mg, 0.198 mmol) in 84% yield: UV λ_{max} (MeOH) nm (ϵ) 231 (4800); ^{1}H NMR (500 MHz, $CDCl_3$, 0.027 M) δ 1.04 (d, J = 6.4 Hz, 3H), 1.08 (d, J = 5.4 Hz, 1H), 1.22 (s, 3H), 1.24 (s, 3H), 1.49 (s, 9H), 1.77 (m, 3H), 2.01 (m, 1H), 2.44 (d, J = 19.0 Hz, 1H), 2.53 (brd, J = 19.0 Hz, 1H), 3.08 (brs, 2H, OH), 3.15 (s, 1H), 3.21 (t, J = 5.2 Hz, 1H), 3.99 (m, 6H), 5.18 (brt, J = 5.5 Hz, 1H, NH), 5.63 (d, J = 4.6 Hz, 1H), 7.56 (s, 1H); FAB-MS m/z 522 (MH^+).

Compound **7b** (103 mg, 0.198 mmol) was stirred with butyric anhydride (0.175 mL, 1.07 mmol), triethylamine (0.175 mL, 1.26 mmol), and DMAP (84.9 mg, 0.69 mmol) in dry CH_2Cl_2 (3 mL) at room temperature for 2 h. The reaction mixture was partitioned between EtOAc and water. The EtOAc layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness. The concentrates were purified by column chromatography on Wakogel C-200 using 20% EtOAc in hexane to give **8a** (113 mg, 0.17 mmol) in 86% yield: UV λ_{max} (MeOH) nm (ϵ) 230 (9100); ^{1}H NMR (500 MHz, $CDCl_3$, 0.012 M) δ 0.89 (d, J = 6.4 Hz, 3H), 0.94 (d, J = 7.4 Hz, 3H), 0.96 (d, J = 7.4 Hz, 3H), 1.15 (d, J = 5.1 Hz, 1H), 1.21 (s, 3H), 1.24 (s, 3H), 1.44 (s, 9H), 1.62−1.71 (m, 4H), 1.79 (m, 3H), 2.16 (m, 1H), 2.26 (brs, 1H, OH), 2.27−2.36 (m, 4H), 2.42 (d, J = 19.0 Hz, 1H), 2.53 (brd, J = 19.0 Hz, 1H), 3.25 (m, 2H), 3.89 (dd, J = 18.3, 5.1 Hz, 1H), 3.99 (dd, J = 18.3, 6.5 Hz, 1H), 4.44 (d, J = 12.6 Hz, 1H), 4.50 (d, J = 12.6 Hz, 1H), 5.02 (m, 1H, NH), 5.29 (brs, 1H, OH), 5.36 (d, J = 10.4 Hz, 1H), 5.71 (d, J = 4.8 Hz, 1H), 7.59 (s, 1H); FAB-MS m/z 662 (MH^+).

Compound **8b** (19.0 mg, 0.033 mmol) was obtained from **8a** (109 mg, 0.16 mmol) in a manner similar to that described for **2d** in 20% yield: UV λ_{max} (MeOH) nm (ϵ) 252 (20 300); IR (neat) 3370, 2950, 2105 (CHN_2), 1730, 1700, 1670, 1455, 1370, 1342, 1255, 1180 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, 0.066 M) δ 0.91 (d, J = 6.5 Hz, 3H), 0.93 (t, J = 7.5 Hz, 3H), 0.96 (d, J = 7.5 Hz, 3H), 1.16 (d, J = 5.2 Hz, 1H), 1.20 (s, 3H), 1.24 (s, 3H), 1.60−1.71 (m, 6H), 1.78 (m, 3H), 2.14 (dq, J = 10.3, 6.5 Hz, 1H), 2.26−2.36 (m, 4H), 2.36 (brs, 1H, OH), 2.42 (d, J = 19.0 Hz, 1H), 2.54 (brd, J = 19.0 Hz, 1H), 3.24 (m, 2H), 4.45 (d, J = 12.4 Hz, 1H), 4.49 (d, J = 12.4 Hz, 1H), 4.83 (brs, 1H, CHN_2), 5.50 (d, J = 10.3 Hz, 1H), 5.66 (brs, 1H, OH), 5.73 (d, J = 4.1 Hz, 1H), 7.61 (s, 1H); FAB-MS m/z 573 (MH^+).

The C-20 butanoyl group of **8b** (13.3 mg, 0.023 mmol) was deprotected by the method employed in the synthesis of **3**. The crude product was purified by HPLC on μ-Bondasphere using 63% MeOH, followed by HPLC on YMC A-023 using 5% 2-PrOH in hexane to give **9** (7.8 mg, 0.016 mmol) in 67% yield: UV λ_{max} (MeOH) nm (ϵ) 252 (19 400); IR (neat) 3370, 2950, 2105 (CHN_2), 1730, 1700, 1670, 1475, 1255, 1190, 750 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, 0.031 M) δ 0.91 (d, J = 6.5 Hz, 3H, H_3-18), 0.96 (t, J = 7.4 Hz, 3H, H_3-butyrate), 1.17 (d, J = 5.2 Hz, 1H, H-14), 1.20 (s, 3H, H_3-16 or -17), 1.24 (s, 3H, H_3-16 or -17), 1.68 (m, 2H, H_2-butyrate), 1.77 (m, 3H, H_3-19),

1.81 (brs, 1H, OH-20), 2.15 (m, 1H, H-11), 2.31 (m, 2H, H_2-butyrate), 2.45 (brs, 1H, OH-9), 2.49 (d, J = 19.0 Hz, 1H, H-5a), 2.56 (brd, J = 19.0 Hz, 1H, H-5b), 3.25 (m, 2H, H-8, 10), 3.99 (brd, J = 12.9 Hz, 1H, H-20a), 4.04 (brd, J = 12.9 Hz, 1H, H-20b), 4.82 (brs, 1H, CHN_2), 5.50 (d, J = 10.3 Hz, 1H, H-12), 5.68 (brs, 1H, OH-4), 5.69 (d, J = 4.8 Hz, 1H, H-7), 7.59 (s, 1H, H-1), these assignments were derived from a $^1H-^1H$ COSY spectrum; HR-FAB-MS m/z 525.2180 ([M + Na],$^+$ calcd for $C_{26}H_{34}N_2O_8Na$, 525.2213).

Photolysis of the Photoaffinity Probe 3 in Methanol. Compound **3** (20.0 mg) was dissolved in 20 mL of MeOH (spectrophotometric grade) in a quartz tube. After being purged with nitrogen for 1 h, the solution was irradiated at 253.7 nm using a Rayonet photochemical reactor (cat No. RPR100) at room temperature for 5 min. After evaporation *in vacuo*, the residue was purified by flash column chromatography on Kieselgel 60 using EtOAc and hexane, followed by flash chromatography on ODS gel using methanol in water to give compounds **10**−**14**.

Compound **10** (43% yield): IR (neat) 3420, 2964, 2877, 1736, 1719, 1380, 1261, 1190, 1127, 981, 874 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 0.0067 M) δ 0.92−1.00 (m, 9H), 1.05 (d, J = 5.0 Hz, 1H), 1.19 (s, 3H), 1.24 (s, 3H), 1.62−1.72 (m, 7H), 2.12 (m, 1H), 2.26 (s, 1H, OH), 2.22−2.40 (m, 4H), 2.53 (brd, J = 17.4 Hz, 1H), 2.72 (brt, J = 5.5 Hz, 1H), 2.77 (d, J = 17.4 Hz, 1H), 3.05 (m, 1H), 3.49 (s, 3H), 4.07 (brs, 2H), 4.15 (s, 2H), 5.42 (d, J = 10.2 Hz, 1H), 5.46 (brs, 1H, OH), 5.59 (d, J = 1.3 Hz, 1H), 5.68 (brd, J = 6.3 Hz, 1H), 5.90 (d, J = 1.6 Hz, 1H); HR-FAB-MS m/z 601.3014 ([M + Na]$^+$, calcd for $C_{31}H_{46}O_{10}$-Na, 601.2989).

Compound **11** (14% yield): IR (neat) 3356, 2964, 2877, 1736, 1716, 1460, 1376, 1264, 1192, 1081, 981, 866 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 0.0042 M) δ 0.92−1.00 (m, 9H), 1.04 (d, J = 5.0 Hz, 1H), 1.20 (s, 3H), 1.27 (s, 3H), 1.61−1.72 (m, 7H), 2.13 (m, 1H), 2.24−2.40 (m, 4H), 2.52 (brd, J = 17.1 Hz, 1H), 2.67 (d, J = 17.1 Hz, 1H), 2.69 (s, 1H, OH), 2.77 (brt, J = 5.9 Hz, 1H), 2.96 (m, 1H), 4.06 (m, 2H), 4.25 (brd, J = 6.6 Hz, 1H), 5.42 (d, J = 10.4 Hz, 1H), 5.47 (brs, 1H, OH), 5.67 (brd, J = 7.0 Hz, 1H), 5.75 (d, J = 1.6 Hz, 1H). The ^1H NMR of **11** was identical with that of authentic 3-deoxo-3β-hydroxyphorbol 12,13-dibutyrate.[25]

Compound **12** (12% yield): IR (neat) 3383, 2966, 2878, 1735, 1718, 1376, 1252, 1178, 1070, 981, 870 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 0.0046 M) δ 0.92−1.00 (m, 9H), 1.04 (d, J = 5.0 Hz, 1H), 1.19 (s, 3H), 1.24 (s, 3H), 1.61−1.72 (m, 7H), 2.13 (m, 1H), 2.22−2.34 (m, 4H), 2.39 (t, J = 6.3 Hz, 1H), 2.45 (s, 1H, OH), 2.51 (dd, J = 17.4, 1.7 Hz, 1H), 2.70 (m, 2H), 2.75 (m, 1H), 2.79 (d, J = 17.4 Hz, 1H), 3.04 (m, 1H), 3.95 (m, 2H), 4.07 (m, 2H), 5.42 (d, J = 10.4 Hz, 1H), 5.46 (brs, 1H, OH), 5.57 (s, 1H), 5.67 (br.d. J = 5.5 Hz, 1H), 5.89 (d, J = 1.6 Hz, 1H); HR-FAB-MS m/z 601.3002 ([M + Na]$^+$, calcd for $C_{31}H_{46}O_{10}$-Na, 601.2989).

Compound **13** (11% yield): IR (neat) 3431, 2960, 2877, 1735, 1719, 1376, 1184, 1132, 979, 873 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 0.0035 M) δ 0.92−1.01 (m, 10H), 1.19 (s, 3H), 1.27 (s, 3H), 1.61−1.71 (m, 7H), 2.18−2.39 (m, 5H), 2.61 (d, J = 17.6 Hz, 1H), 2.69 (brd, J = 17.6 Hz, 1H), 2.98 (m, 1H), 3.03 (brt, J = 6.0 Hz, 1H), 3.80 (s, 3H), 3.83 (s, 1H, OH), 4.04 (m, 3H), 4.23 (d, J = 16.7 Hz, 1H), 4.41 (d, J = 16.7 Hz, 1H), 5.39 (d, J = 10.2 Hz, 1H), 5.40 (brs, 1H, OH), 5.65 (brd, J = 5.7 Hz, 1H), 5.76 (d, J = 1.3 Hz, 1H); HR-FAB-MS m/z 601.2975 ([M + Na]$^+$, calcd for $C_{31}H_{46}O_{10}Na$, 601.2989).

Compound **14** (8% yield): IR (neat) 3406, 2964, 2876, 1736, 1718, 1265, 1192, 985 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 0.0039 M) δ 0.88 (d, J = 6.6 Hz, 3H), 0.93−1.00 (m, 6H), 1.06 (d, J = 4.7 Hz, 1H), 1.20 (s, 3H), 1.33 (s, 3H), 1.61−1.74 (m, 7H), 2.16 (m, 1H), 2.25−2.40 (m, 4H), 2.58 (brt, J = 5.7 Hz, 1H), 2.86 (dd, J = 17.4, 1.9 Hz, 1H), 2.99 (d, J = 17.4 Hz, 1H), 3.12 (m, 1H), 4.07 (d, J = 17.6 Hz, 1H), 4.10 (m, 2H), 4.25 (d, J = 17.6 Hz, 1H), 4.45 (s, 1H), 5.43 (d, J = 10.5 Hz, 1H), 5.64 (s, 1H, OH), 5.75 (dd, J = 6.7, 1.8 Hz, 1H), 6.00 (d, J = 1.7 Hz, 1H); HR-FAB-MS m/z 547.2892 (MH$^+$, calcd for $C_{30}H_{43}O_9$, 547.2907).

Tritium Labeling of the Photoaffinity Probes 3, 6, and 9. These probes (**3**, **6**, and **9**) were tritium labeled by oxidation with activated manganese dioxide to give the C-20 aldehyde, followed by reduction with tritium-labeled sodium borohydride.

Compound **3** (5.5 mg, 0.0096 mmol) was dissolved in CH_2Cl_2 (3 mL). To the solution was added 36.5 mg (0.42 mmol) of activated MnO_2 (Aldrich). After being stirred for 30 min at room temperature, the mixture was filtered and evaporated *in vacuo* to dryness. The concentrates were purified by HPLC on YMC AQ-323 using 80% MeOH to give the C-20 aldehyde (**15**, 3.9 mg, 0.0068 mmol) in 71% yield: IR (neat) 3408, 2965, 2877, 2114 (CHN_2), 1718, 1686, 1380, 1248, 1180, 1063, 982, 883 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 0.023 M) δ 0.94−1.01 (m, 9H), 1.19 (d, J = 4.9 Hz, 1H), 1.23 (s, 3H), 1.26 (s, 3H), 1.61−1.73 (m, 7H), 2.21 (m, 1H), 2.28−2.40 (m, 4H), 2.43 (brd, J = 18.4 Hz, 1H), 2.46 (s, 1H, OH), 2.87 (m, 1H), 3.23 (brt, J = 5.8 Hz, 1H), 3.45 (d, J = 18.4 Hz, 1H), 4.90 (brs, 1H, CHN_2), 5.44 (d, J = 10.3 Hz, 1H), 5.52 (s, 1H), 5.67 (s, 1H), 5.83 (d, J = 1.7 Hz, 1H), 6.72 (dd, J = 6.7, 2.6 Hz, 1H), 9.46 (s, 1H, CHO); FAB-MS m/z 595 ([M + Na]$^+$).

Compound **15** (430 μg, 0.752 μmol) was dissolved in EtOH (100 μL). To the solution, were added 17.2 μL of H_2O and 2.84 μL (0.188 μmol) of a 0.01 N NaOH solution of [^{3}H]$NaBH_4$ (2.5 mg/mL, 359 mCi/mmol). After 1 min, the reaction mixture was concentrated to 30 μL by an argon stream for 10 min in the dark. The sample was purified by HPLC with a radio detector on YMC A-311 using 70% MeOH to give tritium-labeled **3** as a 70% MeOH solution (268.4 μM, 85.4 mCi/mmol).

Tritium labeling of **6** was carried out by a method similar to that mentioned above. The crude product obtained from the oxidation of **6** (25.8 mg, 0.0514 mmol) was purified by HPLC on YMC A-023 using 1% 2-PrOH in hexane, followed by HPLC on μ-Bondasphere C_{18} using 65% MeOH to give the C-20 aldehyde (**16**, 10.4 mg, 0.0208 mmol) in 40% yield: UV λ_{max} (MeOH) nm 327 (1000), 246 (28 300); IR (neat) 3450, 2950, 2105 (CHN_2), 1700, 1470, 1200, 770 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, 0.008 M) δ 0.94 (d, J = 6.5 Hz, 3H), 0.98 (t, J = 7.4 Hz, 3H), 1.22 (s, 3H), 1.24 (d, J = 5.2 Hz, 1H), 1.28 (s, 3H), 1.69 (m, 2H), 1.79 (m, 3H), 2.20 (m, 1H), 2.37 (m, 2H), 2.45 (brs, 1H, OH), 2.47 (brd, J = 17.3 Hz, 1H), 2.93 (d, J = 17.3 Hz, 1H), 3.06 (brs, 1H), 3.62 (t, J = 5.3 Hz, 1H), 4.75 (brs, 1H, CHN_2), 5.48 (d, J = 10.3 Hz, 1H), 5.89 (brs, 1H, OH), 6.72 (dd, J = 5.8, 2.2 Hz, 1H), 7.56 (m, 1H), 9.44 (s, 1H, CHO); FAB-MS m/z 501 (MH$^+$). Compound **16** was tritiated by a method similar to that mentioned above to give tritium-labeled **6** as a 60% MeOH solution (381.5 μM, 79.0 mCi/mmol).

Tritium labeling of **9** was carried out by a method similar to that mentioned above. The crude product obtained from the oxidation of **9** (5.9 mg, 0.012 mmol) was purified by HPLC on μ-Bondasphere C_{18} using 65% MeOH to give the C-20 aldehyde (**17**, 2.8 mg, 0.0056 mmol) in 47% yield: UV λ_{max} (MeOH) nm 335 (2400), 250 (37 000); ^1H NMR (500 MHz, $CDCl_3$, 0.008 M) δ 0.92 (d, J = 6.5 Hz, 3H), 0.96 (t, J = 7.4 Hz, 3H), 1.24 (s, 3H), 1.25 (s, 3H), 1.34 (d, J = 5.3 Hz, 1H), 1.68 (m, 2H), 1.79 (m, 3H), 2.21 (m, 1H), 2.32 (m, 2H), 2.44 (brs, 1H, OH), 2.46 (brd, J = 19.5 Hz, 1H), 2.94 (d, J = 19.5 Hz, 1H), 3.07 (brs, 1H), 3.62 (t, J = 5.3 Hz, 1H), 4.86 (brs, 1H, CHN_2), 5.53 (d, J = 10.3 Hz, 1H), 5.91 (brs, 1H, OH), 6.73 (dd, J = 5.8, 2.2 Hz, 1H), 7.56 (m, 1H), 9.44 (s, 1H, CHO); FAB-MS m/z 501 (MH$^+$). Compound **17** was tritiated by a method similar to that mentioned above to give tritium-labeled **9** as a 60% MeOH solution (352.2 μM, 79.3 mCi/mmol).

The radiochemical purity of these tritiated probes (**3**, **6**, and **9**) determined by TLC on silica gel (Empore 3M with fluorescent indicator, cat. No. 412001) using 50% EtOAc in hexane and by HPLC equipped with a radio detector on YMC A-311 using 60−70% MeOH was found to be >98%.

Synthesis of Peptide C. Peptide C was synthesized by the Protein and Nucleic Acid Facility, Stanford University, with a Milligen/Biosearch 9050 automated peptide synthesizer using the (fluorenylmethoxycarbonyl)polyamide strategy.[45] Crude material was purified by HPLC using μ-Bondasphere C_{18} with elution by a 120 min linear gradient of 10−50% CH_3CN in 0.1% trifluoroacetic acid (TFA). The peak corresponding to peptide C (t_R = 105 min) was collected, and the solution was concentrated *in vacuo* under 30 °C. Lyophilization of this sample gave peptide C as a TFA salt. The purified sample

(45) Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis: A Practical Approach*; IRL: Oxford; 1984.

exhibited satisfactory amino acid and mass spectral analyses (electrospray ionization: average mass 5772.2; calcd for $C_{244}H_{383}N_{77}O_{68}S_9$, 5771.8).

Inhibition of Specific [^{3}H]PDBu Binding to Peptide C. The standard mixture (250 μL) contained in a 1.5 mL of Eppendorf tube 50 mM Tris−HCl (pH 7.4 at 25 °C), 100 nM peptide C, 20 nM [^{3}H]PDBu (20 Ci/mmol), 50 μg/mL 1,2-di-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, 3 mg/mL bovine γ-globlin, and various concentrations of an inhibitor. Final DMSO concentration was 2%. The phosphatidylserine was suspended in 50 mM Tris−HCl (pH 7.4) by sonication (30 s) and added to the above reaction mixture. Total binding was measured in the absence of the inhibitor, and nonspecific binding was measured in the presence of 10 μM nonradioactive PDBu. Specific binding represents the difference between the total and the nonspecific binding. The samples were then incubated at 30 °C for 20 min. After the mixture was cooled at 0 °C for 5 min, 187 μL of 35% (w/w) poly(ethyleneglycol) (average molecular weight, 8000) was added to the tubes and the mixture vigorously stirred. The tubes were incubated at 0 °C for 15 min and centrifuged for 20 min at 12 000 rpm in an Eppendorf microcentrifuge at 4 °C. The supernatant of each tube was removed by aspiration, and the tube was blotted with a Kimwipe. The tip of the tube was cut off, and the radioactivity in the pellet was measured to determine the bound [^{3}H]PDBu. In each experiment, each point represents the average of at least triplicate determinations. Binding affinity was evaluated by the concentration required to cause 50% inhibition of the specific binding, IC_{50}, which was calculated by a computer program (SAS) with a probit procedure.[39]

Binding of the Tritiated Photolabile Phorbol Esters (3, 6, and 9) to Peptide C. Binding of the tritiated photolabile phorbol esters (**3**, **6**, and **9**) to peptide C was measured by a method similar to that mentioned above. The assay solution (250 μL) consisted of 50 mM Tris−HCl (pH 7.4 at 25 °C), 3 mg/mL bovine γ-globulin, 100 μg/mL 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, 3 μM peptide C, and 2 μM tritiated **3**, **6**, or **9** (85.4, 79.0, and 79.3 mCi/mmol, respectively). Nonspecific binding was determined in the presence of a 500-fold excess of nonradioactive PDBu. PDBu was added as a DMSO solution, and the final DMSO concentration was 2%.

Photoaffinity Labeling of Peptide C with the Photoaffinity Probes 3, 6, and 9. Peptide C (3 μM) was incubated in an Eppendorf tube (1.5 mL) at 30 °C for 20 min with tritiated **3**, **6**, or **9** (2 μM) in the presence or absence of a 250-fold excess (500 μM) of PDBu in 750 μL of 50 mM Tris−HCl (pH 7.4 at 25 °C) containing 100 μg/mL 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine and 3 mg/mL bovine γ-globlin. PDBu was added as a DMSO solution, and the final DMSO concentration was 2%. After an additional 5 min incubation at 0 °C, the reaction mixture was poured on a 2.3 cm diameter petri dish. The solution was stirred and irradiated by a UV lamp (8 W, 250−400 nm, PUV-1, TOPCON) for 10 s from 3 cm distance under argon. A 650 μL aliquot of the solution was then transferred to an Eppendorf tube (1.5 mL), and 486 μL of 35% (w/w) polyethyleneglycol was added. After vigorous stirring, the tube was allowed to stand at 0 °C for 15 min and then was centrifuged at 12 000 rpm for 20 min at 4 °C. The supernatant was removed by aspiration, followed by careful removal by blotting with a Kimwipe. The resultant precipitate was dissolved in a sample solution (200 μL), which consisted of 10.5 mM Tris−H_3PO_4 (pH 6.8), 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 8 M urea, and 0.1% (w/v) BPB. Twenty microliters of the sample solution was analyzed on a 2-mm thick polyacrylamide slab gel. The separation gel (T = 13.75%, C = 9.09%) consisted of 12.5% (w/v) acrylamide (monomer), 1.25% (w/v) *N*,*N*′-methylenebisacrylamide, 0.1% (w/v) SDS, 0.075% (v/v) TEMED, 0.02% (w/v) ammonium persulfate, 8 M urea, 0.165 M Tris, and 0.052 M H_3PO_4 at a final pH of 6.83 (25 °C). The stacking gel (T = 4.14%, C = 9.09%) consisted of 3.76% (w/v) acrylamide (monomer), 0.376% (w/v) *N*,*N*′-methylenebisacrylamide, 0.1% (w/v) SDS, 0.075% (v/v) TEMED, 0.02% (w/v) ammonium persulfate, 8 M urea, 0.165 M Tris, and 0.052 M H_3PO_4 at a final pH of 6.83 (25 °C). Reservoir solutions contained 0.1% SDS plus 0.1 M H_3PO_4 adjusted to pH 6.83 with Tris (25°C). Myoglobin fragments (Sigma) were used as molecular weight markers. After electrophoresis, quantitation of the radioactivity bound to peptide C was carried out by slicing the gels. The 3 mm gel slices were digested by overnight incubation in 1 mL of 15% H_2O_2 at 55−60 °C, and the radioactivities were counted in 10 mL of scintillation fluid.

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Supporting Information Available: Copies of the ^{1}H NMR spectra of **3**, **6**, and **9** (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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