Synthesis and Tumor-promoting Activities of 12-Epi-phorbol-12,13-dibutyrate

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12-Epi-phorbol-12,13-dibutyrate (1), the C12-epimer of the most frequently used phorbol ester probe, phorbol-12,13-dibutyrate (PDBu), has been synthesized from phorbol in 9 steps in order to investigate the structural requirements for tumor-promoting activity. Compound 1 showed about 100-fold weaker in vitro biological activities related to in vivo tumor promotion, Epstein-Barr virus early antigen (EBV-EA)-inducing ability, superoxide (O2•−) generation-inducing ability, and binding to the protein kinase C (PKC) regulatory domain surrogate peptides. The results indicated that the β-stereochemistry at position 12 of the phorbol skeleton is important for optimal activity. Binding selectivity to each PKC C1 domain of 1 was almost equal to that of PDBu.

Key words: 12-epi-phorbol-12,13-dibutyrate; Epstein-Barr virus; phorbol ester; protein kinase C; tumor promoter

The phorbol esters are the most common potent tumor promoters found in plants.1 12-O-Tetradecanoylphorbol-13-acetate (TPA) and phorbol-12,13-dibutyrate (PDBu, Fig. 1) are typical examples and have been used extensively in the research on tumor promotion.3 Numerous phorbol ester derivatives have been synthesized during the last three decades in order to establish the structural features responsible for tumor-promoting activity.3 The studies mainly from the laboratories of Hecker, van Duuren, Blumberg, and Wender, indicated that the oxygens at positions 3/4, 9, and 20 are especially important for tumor-promoting activity. Since phorbol without the hydrophobic side chains at positions 12 and 13 is not a tumor promoter,5 these chains are also necessary for optimal activity.

The long chain ester at position 12 has been proposed to have a spatial orientation between the A and C rings3 when bound to the phorbol ester receptor, protein kinase C (PKC), the key enzyme family involved in cellular signal transduction.5 Recent investigations involving photoaffinity labeling6,7 and molecular modeling using a solution structure of the PKCα C1B domain8 have indicated that the acyl moiety at position 13 participates in the direct binding with PKC. Further interest in the role of the C-ring esters arises from the structure of membranin9 (Fig. 1), a daphnane-type compound with C12 stereochernistry opposite to that of odoracin10 and PDBu. Interestingly, membranin is a potent inducer of Epstein-Barr virus early antigen (EBV-EA) like odoracin and TPA, but it is only weakly cytotoxic against Raji cells (B lymphocytes) even at 100 μM when compared with odoracin and TPA, which are cytotoxic at less than 1 μM (Table 1).9 Moreover, the biological activities of 12-deoxy phorbol esters are noteworthy as exemplified by prostratin (Fig. 1) which is an inhibitor of the TPA-induced tumor promotion.11 Collectively, these results prompted us to prepare and examine the biological activities of 12-epi-phorbol-12,13-dibutyrate (12-epi-PDBu, 1), a potential lead for the development of isozyme selec-

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Fig. 1. Structures of Phorbol-12,13-dibutyrate (PDBu), Membranin, Prostratin, and Odoracin.

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Abbreviations: EBV-EA, Epstein-Barr virus early antigen; DMAP, 4-(dimethylamino)pyridine; PCC, pyridinium chlorochromate; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate
tive agonists of PKC. This paper describes the first synthesis and biological activities of 12-epi-PDBu (1).

**Results and Discussion**

For the synthesis of 1 (Fig. 2), the C20 hydroxyl group of phorbol was protected initially with a trityl group according to a previously reported method (63%). Phorbol-20-trityl ether (2) was then treated with butyric anhydride and triethylamine in tetrahydrofuran-dichloromethane to give 3 (78%). Pyridinium chlorochromate (PCC) oxidation of 3 gave the 12-keto compound 4 (60%). Sodium borohydride reduction of the 12-carbonyl group of 4 was attempted in the presence of cerium(III) chloride in methanol at room temperature. However, 1,4-reduction of the enone at position 3 occurred along with the reduction at position 12. To suppress the 1,4-reduction, the reduction was done at –78°C in methanol-dichloromethane to yield exclusively a 3β-hydroxyl isomer (5, 86%). The configuration at position 3 was assigned on the basis of the related work from the Wender group.19 The NOE experiments (significant NOE between C3 and C10 protons) and the analysis of the corresponding C3, C4 acetone as mentioned below. Successive sodium borohydride reduction of the 12-carbonyl group of 5 at room temperature gave only a single diastereomer (6, 49%). The hydroxyl stereochemistry at position 12 was identified as α by the NOE difference spectroscopy of 6; saturation of the C12 proton (δ 4.77, J = 7.0 Hz) in 6 caused marked increases (13% and 11%) of the C8 and C11 protons (δ 2.73 and δ 2.55), respectively. The structure of 6 was confirmed unambiguously by 13C NMR, 1H-1H COSY, NOESY, HMBC, and HMQC spectra (see the experimental section).

Treatment of 6 with butyric anhydride and 4-(dimethylamino)pyridine (DMAP) in dichloromethane gave mainly the 3- and 12-dibutanoyloxy compound, therefore the β-hydroxy groups at positions 3 and 4 were protected with acetone or phenyl boronic acid. The resultant acetone or phenyl borate was treated with butyric anhydride and DMAP in dichloromethane to give the desired 12α-esters. However, deprotection of the hydroxy groups at positions 3 and 4 using 2 M aq. HCl, p-toluenesulfonic acid, or 70% perchloric acid was unsuccessful. We focused, therefore, on the more labile boronic acid protecting group. Compound 6 was treated with 1 equiv. of boronic acid in acetonitrile. After confirming the coordination of boronic acid with 6 on silica gel TLC (i-PrOH:CHCl3:n-hexane = 4:16:80), acetonitrile was removed. The resultant residue in dichloromethane was then treated with butyric anhydride, DMAP, and triethylamine, followed by aqueous work-up to yield 7 (20%). Thirty-six percent of unreacted 6 was recovered. PCC oxidation of 7 (27%) followed by deprotection of the C20 trityl group (66%) gave 12-epi-PDBu (1). The 1H NMR spectrum of 1 in deuteriochloroform at room temperature showed broad signals, which sharpened at 50°C. Comparison of the 1H NMR spectra of 1 with that of PDBu indicated a high field shift and coupling con-

Fig. 2. Synthetic Scheme of 12-epi-PDBu (1).
12-Epi-phorbol-12,13-dibutyrate

Table 1. EBV-EA-inducing Activities of 12-Epi-PDBu (I)

<table>
<thead>
<tr>
<th>Compound</th>
<th>10^{-6} M</th>
<th>10^{-5} M</th>
<th>10^{-4} M</th>
<th>10^{-3} M</th>
<th>10^{-2} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDBu</td>
<td>4.4(0.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-Epi-PDBu (I)</td>
<td>12.7(0.6)</td>
<td>26.5(0.0)</td>
<td>26.9(1.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td></td>
<td>32.8</td>
<td>22.1(9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Odoracin</td>
<td>29.5</td>
<td>34.0</td>
<td>22.1(9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membranein</td>
<td>25.6</td>
<td>32.0</td>
<td>29.6(9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.5</td>
<td>12.4</td>
<td>5.2</td>
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<td></td>
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</table>

Table 2. Superoxide Generation-inducing Activities of 12-Epi-PDBu (I)

<table>
<thead>
<tr>
<th>Compound</th>
<th>10^{-5} M</th>
<th>10^{-4} M</th>
<th>10^{-3} M</th>
<th>10^{-2} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA (control)</td>
<td></td>
<td></td>
<td>1.67(0.01)</td>
<td></td>
</tr>
<tr>
<td>PDBu</td>
<td>0.00(0.0)</td>
<td>0.22(0.03)</td>
<td>1.62(0.03)</td>
<td>1.62(0.04)</td>
</tr>
<tr>
<td>12-Epi-PDBu (I)</td>
<td>0.03(0.01)</td>
<td>0.17(0.01)</td>
<td>1.24(0.03)</td>
<td>1.63(0.04)</td>
</tr>
</tbody>
</table>

Table 3. Kᵣ Values for Inhibition of the Specific Binding to PKC C1 Domains of [³²H]PDBu by 12-Epi-PDBu (I)

<table>
<thead>
<tr>
<th>PKC C1 Peptide</th>
<th>12-Epi-PDBu (I)</th>
<th>PDBu</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-C1A (72-mer)</td>
<td>49.8(13.1)</td>
<td>1.55(0.37)</td>
<td>32.1</td>
</tr>
<tr>
<td>β-C1B</td>
<td>1.418</td>
<td>26.8(0.71)</td>
<td>52.9</td>
</tr>
<tr>
<td>β-C1A (72-mer)</td>
<td>33.1(1.1)</td>
<td>1.80(0.06)</td>
<td>18.4</td>
</tr>
<tr>
<td>γ-C1B</td>
<td>61.9(14.9)</td>
<td>1.97(0.04)</td>
<td>31.4</td>
</tr>
<tr>
<td>δ-C1A</td>
<td>84.5(18.0)</td>
<td>2.91(0.52)</td>
<td>29.0</td>
</tr>
<tr>
<td>δ-C1B</td>
<td>65.7(7.3)</td>
<td>1.58(0.08)</td>
<td>41.5</td>
</tr>
<tr>
<td>δ-C1A</td>
<td>2,360</td>
<td>107(26.0)</td>
<td>22.1</td>
</tr>
<tr>
<td>δ-C1B</td>
<td>31.5(4.3)</td>
<td>1.03(0.06)</td>
<td>30.6</td>
</tr>
<tr>
<td>ε-C1A</td>
<td>381(16.3)</td>
<td>14.9(1.6)</td>
<td>25.6</td>
</tr>
<tr>
<td>ε-C1B</td>
<td>59.8(19.9)</td>
<td>1.35(0.11)</td>
<td>44.3</td>
</tr>
<tr>
<td>η-C1A</td>
<td>234(44.8)</td>
<td>10.2(2.1)</td>
<td>22.9</td>
</tr>
<tr>
<td>η-C1B</td>
<td>22.5(6.2)</td>
<td>0.95(0.12)</td>
<td>23.7</td>
</tr>
<tr>
<td>θ-C1A</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;200</td>
<td></td>
</tr>
<tr>
<td>θ-C1B</td>
<td>42.1(2.8)</td>
<td>1.54(0.08)</td>
<td>27.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ratio: Kᵣ (12-epi-PDBu)/Kᵣ (PDBu).
<sup>b</sup> Ten residues from both N and C-terminus of the previous α-C1A and β-C1A<sup>c</sup> were elongated since the solubility of the original 52-mer peptides was extremely low.
<sup>c</sup> Standard deviation of at least two separate experiments.
<sup>d</sup> Not tested. The Kᵣ value of [³²H]PDBu to θ-C1A could not be measured because of its very weak binding affinity.

const change for the C12 proton signal of I (δ 5.06, J = 7.1 Hz for I; δ 5.57, J = 10.3 Hz for PDBu), in accord with the inverted stereochemistry at C12.

The biological activities of I were examined by three in vitro bioassays that correlate with in vivo tumor promotion: Epstein-Barr virus early antigen (EBV-EA)-inducing ability,<sup>14,15</sup> superoxide (O₂⁻) generation-inducing ability,<sup>16,17</sup> and binding to the PKC regulatory domain surrogate peptides.<sup>18,19</sup> Tables 1, 2, and 3 summarize the results of these assays.

EBVs are under the strict control of the host human lymphoblastoid Raji cells. They are activated by tumor promoters like phorbol esters to produce the early antigen (EA). The EBV-EA-inducing activity is expressed as the percentage of EA-positive cells.<sup>14</sup> Under our experimental conditions, about 30% of maximum EA-induction was observed with typical tumor promoters.<sup>14,20</sup> Superoxide generation is triggered by the potent tumor promoter TPA in epithelial cells and leukocytes through the xanthine oxidase and NADPH oxidase systems, respective.<sup>21,22</sup> We selected the O₂⁻ generation-inducing ability as another in vitro bioassay for tumor promotion. The activity is expressed as the level of O₂⁻ production, and under our experimental conditions, TPA produced 1.67 nmol/ml-min of O₂⁻ at 10⁻⁷ M.

PDBu showed potent EA-induction at 10⁻⁷ M. 12-Epi-PDBu (I) showed maximum EA-induction at only 10⁻⁵ M. Both PDBu and 12-epi-PDBu (I) did not show cytotoxicity against Raji cells at 50 μM. A similar relationship was observed in the superoxide generation induction test. The concentration of the maximum induction of PDBu was 10⁻⁷ M, while that of I was 10⁻⁵ M. These results indicate that I might be 100-fold weaker as a tumor promoter than PDBu and that the stereochemistry at the C12 position of the phorbol esters is important for optimal tumor-promoting activities.

Finally, the binding affinity of I to the regulatory domains (C1A and C1B) of all PKC isozymes (α, β, γ, δ, ε, η, θ) was evaluated by inhibition of the specific binding of [³²H]PDBu. The phorbol esters activate PKC by binding to the cysteine-rich C1 domains designated as C1A and C1B.<sup>23,24</sup> We previ-
ously reported the solid-phase synthesis, folding, and binding properties of individual C1A and C1B domains of all PKC isoforms. All C1B peptides were folded upon zinc treatment to afford PKC C1 domain surrogates which have strong PDBu binding ability comparable to native PKC isoforms. Although the PDBu binding affinity of the C1A peptides was weak under the previous assay condition at 30°C incubation, we have recently found that some peptides (γ-C1A, ε-C1A, η-C1A, and γ-C1B) suffered from temperature-dependent inactivation. The dissociation constants (Kd) of [3H]PDBu to these peptides were measured during 4°C incubation. The C1A peptides, the PDBu binding of which could not be detected previously, are α-C1A and β-C1A. Recent studies found that the C1A and C1B domains of PKCα play equivalent roles in translocation in response to tumor promoters, suggesting that C1A of PKCα should bind strongly to PDBu. Since the solubility of 52-mer α-C1A and β-C1A peptides was extremely low compared with other C1A peptides, ten residues from both N and C-termini of α-C1A (37–88) and β-C1A (37–88) were added to achieve good solubility since the ten residues contain several acidic and basic amino acids. The 72-mer C1 peptides of mouse PKCα and β [α-C1A-long (27–98) and β-C1A-long (27–98)] were synthesized in a stepwise fashion on Fmoc-Gly-PEG-PS resin using the Fmoc chemistry as previously reported. The resultant α-C1A-long and β-C1A-long showed good solubility and bound to PDBu with high affinity, and their Kd for [3H]PDBu at 4°C were 1.1 and 1.3 nM, respectively. Overall, we measured the dissociation constants of [3H]PDBu (Kd) to all C1 peptides except for θ-C1A, enabling us to measure the binding constants (Kb) of other tumor promoters than PDBu by inhibition of specific [3H]PDBu binding.

Dose-response curves were plotted for each compound, and the concentration at which 50% of the specific [3H]PDBu binding was inhibited (IC50) was calculated. The binding K50, were calculated from the Kd of PDBu by the method of Sharkey and Blumberg. Table 3 gives the results of these analyses. PDBu had potent binding affinities to most of the PKC C1 peptides except for δ-C1A and ϑ-C1A. 12-Epi-PDBu (1) bound to these peptides with affinities 20–50 fold less than PDBu. These data correspond well with the results of the EBV-EA induction and the superoxide generation induction tests. The binding selectivity to each PKC C1 domain of 1 was almost equal to that of PDBu as exemplified by the data in Table 3, indicating that inversion of the C12 configuration of PDBu did not significantly change the PKC isozyme selectivity.

In conclusion, we have synthesized 12-epi-PDBu (1) for the first time, and examined its biological activities related to in vivo tumor promotion. The results clearly showed that the β-stereochemistry at position 12 of the phorbol skeleton is important for optimal activities.

**Experimental**

*General remarks.* The following spectroscopic and analytical instruments were used: UV, Shimadzu UV-2200A; Digital Polarimeter, Jasco DIP-1000; 1H NMR, Bruker ARX500 and AC300 (ref. TMS); HPLC, Waters Model 600E with Model 484 UV detector; FAB-MS, JEOL JMS-600H (matrix, m-nitrobenzylalcohol), HPLC was carried out on a YMC packed SH-342-5 (ODS, 20 mm i.d. × 150 mm), AM-323 (ODS, 10 mm i.d. × 250 mm), and A-023 (silica gel, 10 mm i.d. × 250 mm) column (Yamamura Chemical Laboratory). Wako C-100 and C-200 gel (silica gel, Wako Pure Chemical Industries) and YMC A60-350/250 gel (ODS, Yamamura Chemical Laboratory) were used for column chromatography. [3H]PDBu was purchased from NEN Research Products. Phorbol was isolated from croton oil by the method reported previously.

**Synthesis of [1αR-(1αc, 1ββ, 4αβ, 7αα, 7ββ, 8αα, 9αα]-1, 1α, 1β, 4α, 5α, 7α, 7β, 8, 9-decahydro-4α, 7b-dihydroxy-3-(hydroxymethyl)-1, 1, 6, 8-tetramethyl-5-oxo-9αH-cyclopropa(3, 4)benz(1, 2-e)azulene-9α-dibutanylo ester [12-Epi-PDBu (1)].**

**Synthesis of 3.** Phorbol 20-tritylether (2) was synthesized by the method reported previously in 63% yield. Compound 2 (861 mg, 1.42 mmol) dissolved in dry CH2Cl2 (20 ml) and THF (20 ml) was stirred with butyric anhydride (3.49 ml, 21.3 mmol) and triethylamine (2.96 ml, 21.3 mmol) at room temperature for 9 h. After evaporation of CH2Cl2, the reaction mixture was diluted with EtOAc (100 ml), followed by washing with 1 M aqueous HCl and adding water (100 ml). The solution was stirred for 0.5 h to decompose butyric anhydride, and extracted with EtOAc. The EtOAc layer was dried over Na2SO4 and evaporated in vacuo to dryness. The concentrate was purified by column chromatography on Wakogel C-200 using 25% EtOAc in hexane to give 3 (751 mg, 1.11 mmol) in 78% yield. Compound 3, oil, [α]D +64.3° (c = 0.325, MeOH), UV λmax (MeOH) nm (ε): 259 (3,700, sh.), 1H NMR δ (CDCl3, 0.29 nM, 27°C) ppm: 0.97 (3H, t, J = 7.4 Hz, H4-2'), 0.99 (1H, d, J = 5.4 Hz, H-14), 1.06 (3H, d, J = 6.4 Hz, H5-18), 1.22 (3H, s, H3-16 or 17), 1.26 (3H, s, H3-16 or 17), 1.67 (2H, m, H2-3'), 1.77 (3H, m, H2-19), 1.98 (dq, J = 9.7, 6.4 Hz, H-7), 2.35 (2H, t, J = 7.4 Hz, H2-2'), 2.41 (1H, d, J = 19.0 Hz, H-5a), 2.43 (1H, br.s, OH), 2.48 (1H, d, J = 19.0 Hz, H-5b), 3.07 (1H, s, H-10), 3.11 (1H, t, J = 5.3 Hz, H-8), 3.54 (1H, d, J = 13.5 Hz, H20a), 3.56 (1H, d, J = 13.5 Hz, H-20b), 3.97 (1H, dd, J = 9.7, 3.4 Hz, H-12), 5.59 (1H, d, J = 4.3 Hz, H-7), 7.22–7.43 (15H, m, trityl), 7.56 (1H, s, H-1). HR-FAB-MS m/z: 677.3481 (MH+)
calcd. for C_{24}H_{29}O_{7}, 677.3478).

Synthesis of 4. Compound 3 (751 mg, 1.11 mmol) was stirred with PCC (479 mg, 2.22 mmol) and sodium acetate (36.4 mg, 0.444 mmol) in CH_{2}Cl_{2} (38 ml) at room temperature for 3 h. After extraction with EtOAc, the EtOAc layer was dried over Na_{2}SO_{4} and evaporated in vacuo to dryness. The residue was purified by column chromatography on Wakogel C-200 using 15% EtOAc in hexane to give 4 (447 mg, 0.663 mmol) in 60% yield. Compound 4, oil, [α]_{D}^{25} + 94.2° (c = 0.320, MeOH). UV \lambda_{max} (MeOH) nm (e): 252 (4.100, sh.). \textsuperscript{1}H NMR δ (CDCl_{3}, 0.022 m, 27°C) ppm: 0.98 (3H, t, J = 7.4 Hz, H_{-4'}), 1.17 (3H, d, J = 6.8 Hz, H_{-18}), 1.22 (3H, s, H_{-12} or 17), 1.36 (3H, s, H_{-16} or 17), 1.44 (1H, d, J = 5.3 Hz, H_{-14}), 1.69 (2H, m, H_{-3'}), 1.79 (3H, dd, J = 2.8, 1.2 Hz, H_{-9'}), 2.15 (2H, s, OH), 2.24 (2H, m, H_{-2'}), 2.43 (1H, d, J = 19.1 Hz, H_{-5a}), 2.56 (1H, d, J = 19.1 Hz, H_{-5b}), 2.89 (1H, q, J = 6.7 Hz, H_{-11}), 3.25 (1H, s, H_{-10}), 3.54 (1H, t, J = 5.5 Hz, H_{-8}), 3.55 (2H, s, H_{-20}), 5.50 (1H, s, OH), 5.73 (1H, d, J = 4.2 Hz, H_{-7}), 7.21-7.44 (15H, m, trityl), 7.53 (1H, s, H-1).

HR-FAB-MS m/z: 675.3332 (MH\textsuperscript{+}, calcd. for C_{24}H_{29}O_{7}, 675.3321).

Synthesis of 5. Compound 4 (447 mg, 0.663 mmol) was dissolved in CH_{2}Cl_{2} (16 ml) and MeOH (16 ml). After addition of CeCl_{3} \cdot 7H_{2}O (495 mg, 1.33 mmol), the mixture was cooled to -78°C. NaBH_{4} (50.3 mg, 1.33 mmol) was added to the solution and stirring at -78°C for 40 min, acetone (5 ml) was added to the reaction mixture, which was extracted with CHCl_{3}. The CHCl_{3} layer was dried over Na_{2}SO_{4} and evaporated in vacuo to dryness. The concentrate was purified by column chromatography on Wakogel C-200 using 20% EtOAc in hexane, followed by HPLC on YMC-AM-323 using 65% CH_{2}CN to give 6 (62.1 mg, 0.9016 mmol) in 49% yield. Compound 6, oil, [α]_{D}^{25} + 4.95° (c = 0.372, MeOH). UV \lambda_{max} (MeOH) nm (e): 259 (700, sh.). \textsuperscript{1}H NMR δ (CDCl_{3}, 0.024 m, 27°C) ppm: 0.95 (1H, d, J = 9.0 Hz, H-14), 0.97 (3H, t, J = 7.4 Hz, H_{-4'}), 1.08 (3H, s, H_{-16} or 17), 1.12 (3H, d, J = 7.4 Hz, H_{-18}), 1.23 (3H, s, H_{-16} or 17), 1.69 (2H, m, H_{-3'}), 1.73 (3H, br.s, H_{-19}), 1.85 (1H, s, OH-9), 2.16 (1H, d, J = 7.0 Hz, OH-3), 2.17 (1H, s, OH-12), 2.37 (2H, t, J = 7.5 Hz, H_{-2'}), 2.47 (1H, d, J = 18.0 Hz, H-5a), 2.55 (1H, br.d, J = 18.0 Hz, H-5b), 2.56 (1H, m, H-11), 2.59 (1H, s, OH-4), 2.73 (2H, m, J = 8, 10), 3.52 (1H, d, J = 11.4 Hz, H-20a), 3.55 (1H, d, J = 11.4 Hz, H-20b), 4.19 (1H, d, J = 7.0 Hz, H-3), 4.77 (1H, d, J = 7.0 Hz, H-12), 5.57 (1H, d, J = 3.7 Hz, H-7), 5.60 (1H, d, J = 1.3 Hz, H-1), 7.21-7.44 (15H, m, trityl). \textsuperscript{13}C NMR δ (CDCl_{3}, 0.024 m, 27°C) ppm: 11.35 (C-18), 13.51 (C-19), 13.71 (C-1'), 15.82 (C-16 or 17), 18.48 (C-3'), 21.66 (C-16 or 17), 25.19 (C-15), 32.06 (C-14), 36.29 (C-2'), 36.93 (C-8), 37.49 (C-11), 42.63 (C-5), 59.06 (C-10), 60.97 (C-13), 69.26 (C-20), 69.73 (C-12), 79.87 (C-9), 80.03 (C-4), 86.24 (C-3'), 86.92 (trityl), 125.20 (C-1), 126.97 (trityl), 127.76 (trityl), 128.25 (C-7), 128.73 (trityl), 137.25 (C-6), 139.02 (C-2'), 144.21 (trityl), 172.55 (C-1'). HR-FAB-MS m/z: 678.3586 (M\textsuperscript{+}, calcd. for C_{45}H_{50}O_{17}, 678.3556).

Synthesis of 7. Compound 6 (38 mg, 55.9 \mu mol) dissolved in anhydrous CH_{2}CN (1.5 ml) was stirred with H_{2}BO_{3} (3.4 mg, 55 \mu mol) at room temperature for 15 min. The reaction mixture was filtered, and the filtrate was evaporated in vacuo to dryness. The residue dissolved in CH_{2}Cl_{2} (0.4 ml) was stirred with butyric anhydride (18.1 \mu l, 111 \mu mol), DMAP (13.7 mg, 111 \mu mol), and triethylamine (15.4 \mu l, 111 \mu mol) at room temperature for 2 h. CHCl_{3} (3 ml) and water (3 ml) were added to the solution, which was stirred for 20 min. The mixture was extracted with CHCl_{3}. The CHCl_{3} layer was dried over Na_{2}SO_{4} and evaporated in vacuo to dryness. The concentrate was purified by column chromatography on Wakogel C-200 using 20% EtOAc in hexane, followed by HPLC on YMC-AM-323 using 85% MeOH to give 7 (8.4 mg, 11 \mu mol) in 20% yield. Unreacted 6 (13.5 mg, 19.9 \mu mol, 36%) was recovered. Compound 7, oil, [α]_{D}^{25} - 13.6° (c = 0.550, MeOH). \textsuperscript{1}H NMR δ (CDCl_{3}, 0.013 m, 52°C) ppm: 0.95 (3H, t, J = 7.4 Hz, H_{-4'} or 4*), 0.97 (3H, t, J = 7.4 Hz, H_{-4'} or 4*), 1.06 (3H, d, J = 7.3 Hz, H_{-18}), 1.11 (3H, s, H_{-16} or 17), 1.16 (1H, br.s, H_{-14}), 1.21 (3H, s, H_{-16} or 17), 1.58-1.69 (4H, m, H_{-3'} and 3*), 1.72 (3H, dd, J = 2.7, 1.3 Hz, H_{-19}), 1.96 (1H, d, J = 7.3 Hz, OH-3), 2.25 (4H, q, J = 7.6 Hz, H_{-2'} and 2*), 2.42 (1H, m, H-11), 2.49.
(2H, br.s, H_{2-5}), 2.56 (1H, s, OH), 2.78 (2H, m, H-8 and 10), 3.53 (1H, d, J=11.4 Hz, H-20a), 3.59 (1H, d, J=11.4 Hz, H-20b), 4.19 (1H, d, J=7.3 Hz, H-3), 4.99 (1H, d, J=7.1 Hz, H-12), 5.58 (1H, d, J=5.4 Hz, H-7), 5.62 (1H, d, J=1.3 Hz, H-1), 7.21-7.45 (15H, m, trityl). HR-FAB-MS m/z: 771.3894 (MNa^+), calcd. for C_{39}H_{41}O_{8}, 771.3873.

Synthesis of 8. Compound 7 (19.9 mg, 26.3 μmol) dissolved in CHCl_{3} (1.4 ml) was stirred with PCC (6.0 mg, 28.2 μmol) and sodium acetate (1.0 mg, 12.1 μmol) at room temperature for 30 min. After extraction with CHCl_{3}, the CHCl_{3} layer was dried over Na_{2}SO_{4} and evaporated in vacuo to dryness. The residue was purified by column chromatography on Wakogel C-200 using 20% EtOAc in hexane, followed by HPLC on YMC-AM-323 using 85% MeOH and on YMC-A-023 using hexane, CHCl_{3}, and I-PrOH (80:19:25:0.75) to give 8 (5.4 mg, 7.2 μmol) in 27% yield. Compound 8, oil, [ε]_225^0=12.9° (c=0.226, MeOH). UV λ_{max} (MeOH) nm (ε): 221 (20.400), 230 (13,700). ^1H NMR δ (CDCl_{3}, 0.014 m, 52°C) ppm: 0.93-1.00 (9H, m, H-18, 4', 4''), 1.14 (3H, s, H_{16} or 17), 1.15 (4H, br.s, H_{16} or 17 and H-14), 1.58-1.71 (4H, m, H-3' and 3''), 1.78 (3H, dd, J=2.7, 1.3 Hz, H-19), 1.95 (1H, s, OH), 2.20-2.29 (4H, m, H-2' and 2''), 2.38 (2H, m, H-5a and H-11), 2.49 (1H, br.d, J=19.0 Hz, H-5b), 2.97 (1H, t, J=6.4 Hz, H-8), 3.11 (1H, br.s, H-10), 3.55 (2H, s, H-2), 5.07 (1H, d, J=7.0 Hz, H-12), 5.61 (1H, d, J=4.0 Hz, H-7), 7.18-7.43 (15H, m, trityl), 7.48 (1H, s, H-1). HR-FAB-MS m/z: 747.3931 (MH^+), calcd. for C_{39}H_{41}O_{8}, 747.3896.

Synthesis of 9. Compound 8 (5.4 mg, 7.2 μmol) was stirred with 70% HClO_{4} (3.7 μl) in MeOH (1.1 ml) at room temperature for 45 min. After addition of saturated NaHCO_{3} (1 ml), the reaction mixture was extracted with EtOAc. The EtOAc layer was dried over Na_{2}SO_{4} and evaporated in vacuo to dryness. The residue was purified by HPLC on YMC-AM-323 using 70% MeOH to give 12-epi-PDBu (1, 2.4 mg, 4.7 μmol) in 66% yield. 12-epi-PDBu (1), oil, [ε]_225^0=10.0° (c=0.076, MeOH). UV λ_{max} (MeOH) nm (ε): 228 (5,800). ^1H NMR δ (CDCl_{3}, 0.010 m, 52°C) ppm: 0.96 (3H, t, J=7.4 Hz, H_{4'-4'}), 0.97 (3H, t, J=7.4 Hz, H_{4'-4'}), 0.98 (3H, d, J=7.2 Hz, H_{18}), 1.15 (3H, s, H_{16} or 17), 1.17 (3H, s, H_{16} or 17), 1.22 (1H, d, J=7.4 Hz, H-14), 1.65 (4H, m, H-2' and 3''), 1.79 (3H, dd, J=2.8, 1.4 Hz, H-19), 2.08 (1H, s, OH), 2.23-2.30 (4H, m, H-2' and 3''), 2.40 (1H, q, J=7.2 Hz, H-11), 2.42 (1H, d, J=19.3 Hz, H-5a), 2.53 (1H, d, J=19.3 Hz, H-5b), 3.02 (1H, br.t, J=6.6 Hz, H-8), 3.12 (1H, br.s, H-10), 4.01 (2H, br.s, H-20), 5.06 (1H, d, J=7.1 Hz, H-12), 5.67 (1H, d, J=4.0 Hz, H-7), 7.49 (1H, s, H-1). HR-FAB-MS m/z: 505.2805 (MH^+), calcd. for C_{39}H_{38}O_{8}, 505.2801.

Epstein-Barr virus early antigen induction test. The EBV-EA induction test was done in the Raji cell (nonproducer) system with sodium n-butyrate (3 mM) by the method reported previously.14

Superoxide generation test. The superoxide generation test in differentiated HL-60 cells was done by the method reported previously16,17 without inhibitors.

PDBu-binding assay of the PKC surrogate peptides. The [^{3}H]PDBu binding to the PKC surrogate peptides was evaluated using the procedure of Sharkey and Blumberg19 with modifications as reported previously.18 The assay mixture (250 μl) contained 50 mm Tris-maleate buffer (pH 7.4 at 4°C), 5-20 nm PCK C1 peptide, 20-40 nm [^{3}H]PDBu (20 Ci/mmol), 50 μg / ml 1,2-di(cis-9-octadecenyl)-sn-glycero-3-phospho-l-serine, 3 mg/ml bovine γ-globulin, and various concentrations of an inhibitor. Final ethanol concentration was 2%. The samples were incubated at 4°C for 20 min instead of the 30°C of the previous method18 to avoid the decrease of the specific [^{3}H]PDBu binding of some PKC C1 peptides.25 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. The binding constant, K_{b}, was calculated by the method of Sharkey and Blumberg.19

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