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# A Simple Analogue of Tumor-Promoting Aplysiatoxin Is an Antineoplastic Agent Rather Than a Tumor Promoter: Development of a Synthetically Accessible Protein Kinase C Activator with Bryostatin-like Activity

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***Abstract:*** Protein kinase C (PKC) is widely recognized as a therapeutic target in intractable diseases such as cancer, Alzheimer's disease (AD), and acquired immune deficiency syndrome (AIDS). While inhibition of PKC is a general therapeutic strategy for the treatment of cancer, PKC activators are potential therapeutic agents for AD and AIDS. However, concerns have been raised about their therapeutic use since PKC activators such as phorbol esters exhibit potent tumor-promoting activities. Naturally occurring bryostatin 1 (bryo-1), prostratin, and 12-deoxyphorbol 13-phenylacetate (DPP) are fascinating PKC activators without tumor-promoting activities. Bryo-1 is currently in clinical trials for the treatment of cancer and is also effective against AD. Prostratin and DPP are attractive candidates for the adjunctive treatment of human immunodeficiency virus (HIV) infection. However, their limited availability from natural sources and synthetic complexity have hampered further development as therapeutic agents. We report here easy access (22 steps) to a simple analogue (**1**) of the tumor-promoting aplysiatoxin (ATX) as a novel PKC activator with anticancer and anti-tumor-promoting activities. Anticancer activities of **1** against several human cancer cell lines were comparable to those of bryo-1. Moreover, **1** as well as bryo-1 significantly inhibited the Epstein−Barr virus early antigen (EBV-EA) induction by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA), whereas ATX strongly induced EBV-EA. This inhibitory effect is characteristic of antitumor promoters. Compound **1** as well as bryo-1 displayed significant binding and activation of PKC$\delta$ and induced its translocation to the nuclear membrane in CHO-K1 cells. This study provides a synthetically accessible PKC activator with bryo-1-like activities, which could be another therapeutic lead for cancer, AD, and AIDS.

## Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases that play pivotal roles in cellular signal transduction including differentiation, proliferation, and apoptosis.[1,2] PKC participates in carcinogenesis and malignant transformation, suggesting it to be a potential target for cancer therapy.[3,4] Inhibition of PKC activity is a general therapeutic strategy for the treatment of cancer, and PKC inhibitors, including ATP-competitive compounds such as UCN-01 and LY317615 and antisense oligonucleotides, have been identified as anticancer drugs.[5] Recent investigations have revealed that PKC activators are also potential therapeutic agents for Alzheimer's disease (AD) and acquired immune deficiency syndrome (AIDS). Naturally occurring 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and artificial PKC activators with a benzolactam skeleton reduced levels of A$\beta$, a causative protein for AD.[6,7] TPA is also reported to activate latent human immunodeficiency virus (HIV) and thus represents a possible adjuvant for antivirus therapy.[8] However, their therapeutic uses have been limited by strong tumor-promoting activities.

Bryostatins and 12-deoxyphorbol derivatives are fascinating PKC activators without tumor-promoting activities. Bryostatins are a family of structurally complex marine macrolides that exhibit unique profiles of biological effects, including anti-tumor-promoting and antineoplastic activities.[9] Bryostatin 1

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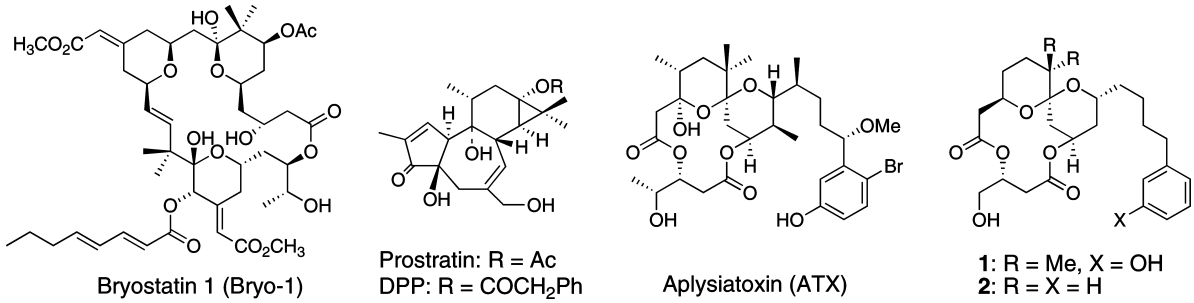


***Figure 1.*** Structures of bryostatin 1 (bryo-1), 12-deoxyphorbol esters, aplysiatoxin (ATX), and simplified analogues (**1** and **2**) of ATX.

(bryo-1),[10] the lead member of this family, is currently in clinical trials for the treatment of cancer and has been shown to be effective in reducing levels of A$\beta$ similar to TPA.[11−13] More recently, bryo-1 has been revealed to have cognitive restorative and antidepressant effects, and its therapeutic potential as a central nervous system (CNS) drug has attracted much attention.[14] Prostratin and 12-deoxyphorbol 13-phenylacetate (DPP) are non-tumor-promoting natural products with a phorbol skeleton.[15] These less hydrophobic phorbol derivatives induce HIV expression in latently infected cell lines and primary cells and downregulate the activity of HIV receptors to decrease infections of new targets.[16,17]

Despite the promising biological properties of bryo-1 and 12-deoxyphorbol derivatives, their limited availability from natural sources and synthetic complexity have hampered further studies on their mode of action and structural optimization as therapeutic agents. Recently, excellent practical methods of synthesizing bryo-16 and 12-deoxyphorbol derivatives have been reported by Trost and Dong[18] and Wender et al.,[19] respectively. Wender's group has also succeeded in developing simplified analogues of bryo-1 via function-oriented synthesis.[20,21] These simplified analogues were at least as potent as the bryostatins in both binding to PKC and inhibiting growth of several human cancer cell lines. These efforts undoubtedly provide a promising way to address issues regarding the supply and therapeutic optimization of bryo-1 and 12-deoxyphorbol derivatives.

Given the therapeutic importance of PKC activators, the identification of synthetically more accessible compounds with bryo-1- or 12-deoxyphorbol-like activities is highly desirable. In this article, we report that a simple and less lipophilic analogue (**1**) of the tumor-promoting aplysiatoxin (ATX, Figure 1)[22] is a possible candidate for another non-tumor-promoting PKC activator with anticancer activities. The analogue **1**, easily synthesized in only 22 steps via standard reactions, displayed growth inhibition of human cancer cell lines comparable to that of bryo-1 and suppressed significantly Epstein−Barr virus early antigen (EBV-EA) induction by TPA, one of the biological activities characteristic of anti-tumor promoters. Binding and translocation profiles of **1** for PKC$\delta$, a member of the PKC family associated with biological effects of bryo-1, are also described.

## Results and Discussion

**Design and Synthesis of Simple ATX Analogues.** ATX is a polyacetate-type tumor promoter isolated from the sea hare *Stylocheilus longicauda*.[22] An interesting feature of ATX is the role of the bromine atom on the phenol ring. Loss of this atom did not affect the binding to PKC but reduced the tumor-promoting activity of ATX.[23,24] Although the role of the bromine atom remains unclear, it was proposed from molecular modeling that the phenolic side chain of ATX might play a role analogous to that of the ester chain of phorbol esters.[25] Lipophilicity of the C-13 side chain is a pivotal factor in determining the biological profile of 12-deoxyphorbol esters. The less lipophilic DPP with a phenylacetyl group at position 13 is a potent anti-tumor promoter,[26,27] whereas the more lipophilic 12-deoxyphorbol 13-tetradecanoate derivative displays tumor-promoting activity.[28] Interestingly, these compounds are similar in their affinity for PKC.[28] These results suggest that the lipophilicity of the bromine atom might be responsible for the tumor-promoting activity of ATX, and that ATX could be a 12-deoxyphorol class compound rather than a typical tumor promoter whose lipophilicity is critical for binding PKC as well as the tumor-promoting activity.

This hypothesis led us to generate simple and less lipophilic analogues (**1** and **2**) of ATX as potential candidates for new synthetically accessible PKC activators with anti-tumor-promoting and anticancer activities. With the consideration that consecutive stereogenic centers of ATX were problematic in

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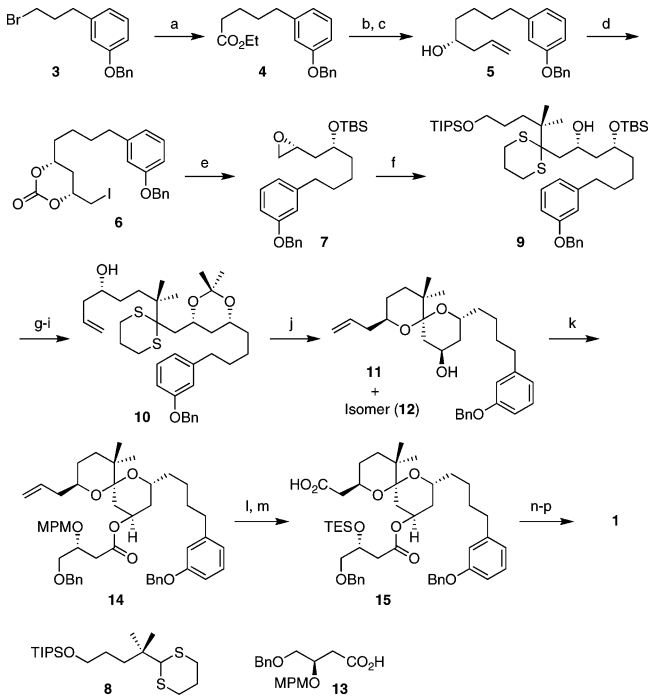
***Scheme 1.*** Synthesis of **1**[a]




[a] (a) (1) Diethyl malonate, NaH, DMF; (2) LiCl, DMSO, $H_2O$; 76% in two steps. (b) DIBAL, toluene, 77%. (c) Allyl-$SnBu_3$, Ti(O*i*-Pr)$_4$, (*S*)-BINOL, 4 Å MS, $CH_2Cl_2$, 85%. (d) (1) $(Boc)_2O$, NaHMDS, THF; (2) IBr, toluene; 51% in two steps. (e) (1) $K_2CO_3$, MeOH; (2) TBSCl, imidazole, DMAP, DMF; 74% in two steps. (f) **8**, *n*-BuLi, THF, 91%. (g) (1) TsOH·$H_2O$, $CH_3CN$, THF; (2) 2,2-dimethoxypropane, CSA, $CH_2Cl_2$; 77% in two steps. (h) $SO_3$·Pyr, $Et_3N$, DMSO, $CH_2Cl_2$, 90%. (i) Allyl-$SnBu_3$, Ti(O*i*-Pr)$_4$, (*S*)-BINOL, 4 Å MS, $CH_2Cl_2$, 71%. (j) (1) TsOH·$H_2O$, $H_2O$, $CH_3CN$, THF; (2) $Hg(ClO_4)_2$·$6H_2O$, $CH_3CN$, $CH_2Cl_2$, $H_2O$; **11**, 49%; **12**, 34%. (k) **13**, TCB-Cl, $Et_3N$, DMAP, toluene, 96%. (l) (1) DDQ, $CH_2Cl_2$, $H_2O$; (2) TESCl, imidazole, THF; 81% in two steps. (m) $KMnO_4$, $NaIO_4$, *t*-BuOH, pH 7 buffer, 63%. (n) HF·Pyr, pyridine, THF, 90%. (o) TCB-Cl, $Et_3N$, DMAP, toluene, 87%. (p) $H_2$, Pd−C, MeOH, 97%.

its total synthesis,[29−32] the chiral methyl and methoxy groups were removed not only to decrease lipophilicity but also to increase synthetic accessibility. The labile hemiacetal hydroxyl group[33] was also replaced with a hydrogen atom to increase the chemical stability of the analogues. While **1** retains the geminal dimethyl substituents at the spiroketal moiety and the phenolic hydroxyl group at the side chain, **2** lacks both. The lipophilicity of **2** estimated roughly by use of ChemBioDraw version 11.0 was similar to that of **1** and lower than that of ATX (*C* log *P* of **1**, 2.3; of **2**, 1.9; and of ATX, 4.3).

Synthesis of **1** began with substitution of the known bromide (**3**)[34] with diethyl malonate and subsequent decarbalkoxylation (Scheme 1). Reduction of the ester (**4**) followed by asymmetric Keck allylation[35] provided **5**. Stereoselective iodocarbonate cyclization was performed by the two-step sequence of Duan and Smith.[36] The resulting **6** was converted into an epoxy alcohol, whose hydroxyl group was protected as a *tert*-butyldimethylsilyl (TBS) ether. Coupling of **7** with the dithiane (**8**) was achieved by the protocol of Ide and Nakata.[37] Hydrolysis of the silyl ethers of **9**, followed by O-isopropylidenation and oxidation of the primary alcohol, provided an aldehyde, which was converted to the homoallyl alcohol (**10**). Detachment of the isopropylidene acetal and subsequent hydrolytic cleavage of the dithiane moiety rapidly generated a 1.4:1 mixture of spiroketals (**11** and **12**), whose configurations were determined by nuclear Overhauser enchancement values (NOEs) as shown in the Supporting Information. Treatment of the undesired diastereomer (**12**) with pyridinium *p*-toluenesulfonate (PPTS) provided a 1:1.8 mixture of **11** and **12**. Repeating this equilibrium reaction twice gave **11** in 72% yield. Condensation of **11** with the carboxylic acid (**13**) was achieved by the method of Yamaguchi and co-workers.[38] Oxidative cleavage of the olefin group of **14** gave a complex mixture due to cleavage of the 4-methoxybenzyl (MPM) group. Among the various protective groups examined, protection with a triethylsilyl (TES) group successfully furnished carboxylic acid (**15**) in three steps from **14**. Deprotection of the TES group, lactonization, and removal of two benzyl groups provided **1** in 22 steps with an overall yield of 1.9%.

Synthesis of **2** started with the stereoselective iodocarbonate cyclization of the known allyl alcohol (**16**)[39] to give **17**, which was converted to the epoxide (**18**) (Scheme 2). Three-component linchpin coupling of TBS-dithiane according to Smith et al.[40] with **18** and commercially available **19**, followed by hydrolysis of the silyl ethers and protection of the 1,3-diol, gave **20**. The subsequent construction of the spiroketals and the lactone ring was achieved by procedures similar to those for **1**. Compound **2** was obtained in 18 steps with an overall yield of 4.5%.

**Anticancer and Anti-Tumor-Promoting Activities of the Simple ATX Analogues.** The analogues (**1** and **2**) were initially evaluated for their anticancer activities by growth inhibition assays against human cancer cell lines in vitro. The panel of 39 human cancer cell lines established by Yamori et al.[41] according to the NCI method with modifications was employed, and cell growth inhibition was measured as reported previously.[42] To our delight, **1** displayed significant anticancer activities; log $GI_{50}$ values of **1** were comparable to those of bryo-1[43] in several cancer cell lines (Table 1). The anticancer activities against the other 30 cell lines are shown in the

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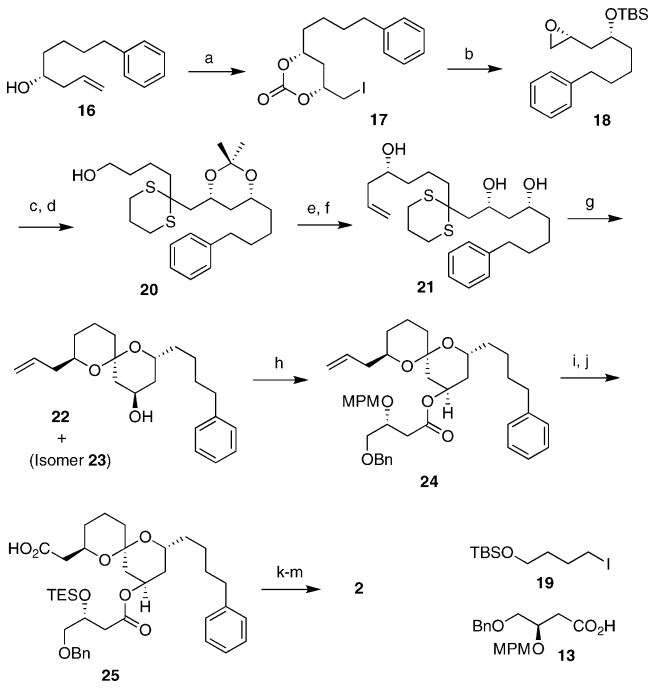
***Scheme 2.*** Synthesis of **2**[a]




[a] (a) (1) $(Boc)_2O$, NaHMDS, THF; (2) IBr, toluene; 77% in two steps. (b) (1) $K_2CO_3$, MeOH; (2) TBSCl, imidazole, DMAP, DMF; 74% in two steps. (c) (1) 2-TBS-1,3-dithiane, *t*-BuLi, $Et_2O$, HMPA, **19**; (2) TsOH·$H_2O$, $H_2O$, $CH_3CN$, THF; 63% in two steps. (d) 2,2-Dimethoxypropane, CSA, $CH_2Cl_2$, 87%. (e) $SO_3$·Pyr, $Et_3N$, DMSO, $CH_2Cl_2$, 79%. (f) (1) Allyl-$SnBu_3$, Ti(O*i*-Pr)$_4$, $TiCl_4$, $Ag_2O$, (*S*)-BINOL, $CH_2Cl_2$; (2) TsOH·$H_2O$, $H_2O$, $CH_3CN$, THF; 90% in two steps. (g) (1) $CH_3I$, $CH_3CN$, $NaHCO_3$, $H_2O$; (2) PPTS, $CH_2Cl_2$, MeOH; **22**, 34% in two steps; **23**, 64% in two steps; (h) **13**, TCB-Cl, $Et_3N$, DMAP, toluene, 96%. (i) (1) DDQ, $CH_2Cl_2$, $H_2O$; (2) TESCl, imidazole, THF; 80% in two steps. (j) $KMnO_4$, $NaIO_4$, *t*-BuOH, pH 7 buffer, 80%. (k) HF·Pyr, pyridine, THF, 99%. (l) TCB-Cl, $Et_3N$, DMAP, toluene, 80%. (m) $H_2$, Pd−C, MeOH, 97%.

***Table 1.*** Growth Inhibition of **1**, **2**, and Bryo-1 against Human Cancer Cell Lines

| | | log $GI_{50}$ (M) | | |
|---|---|---|---|---|
| cancer type | cell line | **1** | **2** | bryo-1[a] |
| breast | HBC-4 | −6.33 | −5.32 | NT[b] |
| breast | MDA-MB-231 | −5.61 | −4.55 | −5.20 |
| CNS | SF-295 | −5.06 | −4.57 | −5.20 |
| colon | HCC2998 | −5.43 | −4.57 | −5.30 |
| lung | NCI-H460 | −5.60 | −4.70 | −5.60 |
| lung | A549 | −5.32 | −4.48 | −5.20 |
| melanoma | LOX-IMVI | −5.74 | −4.66 | NT |
| ovarian | OVCAR-5 | −4.95 | −4.25 | >−5 |
| prostate | PC-3 | −4.96 | −4.50 | −5.30 |

[a] Cited from ref 43. [b] Not tested.

Supporting Information. Significant growth inhibitory activities were found in many tumor cells, especially in breast cancer cell line HBC-4 (log $GI_{50}$ −6.33) and melanoma cell line LOX-IMVI (log $GI_{50}$ −5.74). On the other hand, **2** without the geminal dimethyl group and the phenolic hydroxyl group showed inhibitory activities that were approximately 1 order of magnitude lower than those of **1**, indicating that the anticancer activities of ATX analogues do not simply depend on molecular hydrophobicity since **1** and **2** were estimated to be similar in hydrophobicity. Practical implications are that the geminal dimethyl group and/or the phenolic hydroxyl group of **1** would play specific roles in obtaining significant anticancer activities.

Encouraged by the promising results from the growth inhibition assays with cancer cell lines, we evaluated the tumor-promoting and anti-tumor-promoting activities of **1** by testing induction of EBV-EA. Epstein−Barr viruses (EBVs) are under the strict control of the host human B-lymphoblastoid Raji cells. They are activated when treated with chemicals such as tumor promoters and produce early antigen (EA),[44] which can be detected by an indirect immunofluorescence technique.[45] The EBV-EA-inducing activity is expressed as the percentage of EA-positive cells; potent tumor promoters like TPA induced maximal proportions of EA-positive cells (25∼30%) at 10∼100 nM.[46] In this assay, the potent tumor promoter ATX induced 22% EA-positive cells at 100 nM like TPA (Figure 2). On the other hand, **1** induced only weak EBV-EA induction by itself at 100 nM and markedly inhibited 100 nM TPA-induced EA in a manner similar to bryo-1.[47] Only weak EBV-EA induction (about 10% EA-positive cells) was observed even at 1 $\mu$M **1** (data not shown). These results indicate that **1**, like bryo-1, could block the tumor-promoting effect of TPA and would thus be a unique anti-tumor promoter with the skeleton of the tumor-promoting ATX.

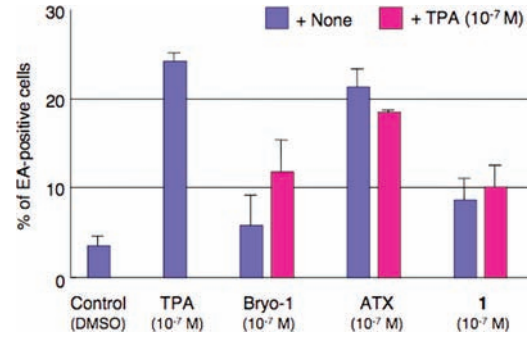



***Figure 2.*** EBV-EA-inducing ability of TPA, bryo-1, ATX, and **1**. Percentages of EA-positive cells are shown. Sodium *n*-butyrate (3 mM) was added to all samples to enhance the sensitivity of Raji cells. The final concentration of dimethyl sulfoxide (DMSO) was 0.5%. Viability of the cells exceeded 80% in all experiments. Error bars represent standard deviation values from at least two separate experiments.

**Binding and Activation Profiles of the Simple ATX Analogues for PKC$\delta$.** The activation of PKC$\delta$, one of the PKC isozymes, is likely to be responsible for the unique biological activities of bryo-1.[48] Therefore, we evaluated the binding and activation profiles of **1** along with **2** for PKC$\delta$ to explore the mechanism responsible for the anticancer activities of **1**. PKC$\delta$ has two C1 domains (C1A and C1B) that bind bryo-1, tumor promoters, and endogenous diacylglycerols. Blumberg and co-workers[48] reported that prostratin, DPP, and bryo-1 with anti-tumor-promoting and anticancer activities were dependent on both C1 domains of PKC$\delta$, whereas tumor promoters like TPA and indolactams preferred the C1B domain. We previously established a C1 peptide library of all PKC isozyme C1 domains by solid-phase synthesis followed by zinc folding.[49,50] By use of the PKC$\delta$ C1 peptides ($\delta$-C1A and $\delta$-C1B), the concentration

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***Table 2.*** $K_i$ Values for the Inhibition of [$^3$H]PDBu's Binding by **1**, **2**, Bryo-1, ATX, PDBu, and Indolactam-V

| PKCδ and its C1 peptides | $K_i$ (nM) | | | | | |
|---|---|---|---|---|---|---|
| | **1** | **2** | bryo-1[a] | ATX | PDBu[b] | indolactam-V[c] |
| δ-C1A | 140 (10)[d] | 6800 (800) | 5.3 | 12 (0.0) | 110 | 1900 |
| δ-C1B | 7.4 (1.1) | 170 (10) | 0.60 | 0.41 (0.01) | 1.0 | 11 |
| ratio[e] | 19 | 40 | 8.8 | 29 | 110 | 170 |
| whole PKCδ | 15 (1.0) | 400 (40) | 0.84[f] | 3.0 (0.1) | 0.71[g] | 12 |

[a] Cited from ref 52. [b] Cited from ref 53. [c] Cited from ref 54. [d] Standard deviation from at least three separate experiments. [e] $K_i$ for δ-C1A/$K_i$ for δ-C1B. [f] Cited from ref 55. [g] Cited from ref 56.

required to cause 50% inhibition, $IC_{50}$, of the binding to [$^3$H]phorbol 12,13-dibutyrate (PDBu) was measured. The affinity of **1** and **2** for δ-C1A and δ-C1B was expressed as $K_i$ values calculated from the $IC_{50}$ and the $K_d$ values of [$^3$H]PDBu as reported by Sharkey and Blumberg.[51]

Table 2 summarizes these results along with $K_i$ values for whole PKCδ. Compound **1** displayed significant affinity for PKCδ C1 peptides ($K_i$ for δ-C1A and δ-C1B: 140 and 7.4 nM). In contrast, the binding of **2** was 20 ∼ 50 times weaker than that of **1**, suggesting that the geminal dimethyl group and/or the phenolic hydroxyl group of **1** specifically interacts with PKCδ. Most importantly, the relative affinity of ATX and its analogues (**1** and **2**) for δ-C1B was markedly lower than that of other tumor promoters; the ratio ($K_i$[C1A]/$K_i$[C1B]) of **1** (19) was slightly higher than that of bryo-1 (8.8) but markedly lower than that of a tumor promoter like PDBu (110) or indolactam-V (170).[52−56] Considering the relatively low affinity of 12-deoxyphorbol esters for δ-C1B,[48] these results support our hypothesis that ATX could be a 12-deoxyphorbol class compound rather than a typical tumor promoter and also suggest the relatively diminished affinity for δ-C1B and less hydrophobicity of **1** to be responsible for its anti-tumor-promoting and anticancer activities.

PKC's activation is intimately coupled with its translocation from the cytosol to the membranous fraction.[57] The binding of a PKC activator like a tumor promoter to an inactive PKC in the cytoplasm increases the affinity of the kinase for membranes and induces its translocation to the plasma membrane. Association with the membrane drives the release of the autoinhibitory sequence from the kinase domain to activate the enzyme. This conjunction of translocation and activation has led to the widespread use of translocation as a surrogate measure of PKC's activation.

Blumberg and co-workers[58] reported that bryo-1 with anticancer activities induced translocation of PKCδ to the nuclear membrane rather than the plasma membrane. For the evaluation of PKCδ's translocation by the simple ATX analogues (**1** and **2**) in living CHO-K1 cells, a translocation assay using green fluorescent protein (GFP) -tagged PKCδ (PKCδ-GFP) was carried out. As shown in Figure 3, bryo-1 induced significant translocation of PKCδ-GFP not only to the plasma membrane but also to the nuclear membrane in our system (Figure 3a). Stimulation by **1** at 1 μM induced the translocation of PKCδ-GFP to the plasma membrane (Figure 3b). Interestingly, a higher concentration (10 μM) of **1** induced significant translocation of PKCδ-GFP to the nuclear membrane in addition to the plasma membrane (Figure 3c). In contrast, stimulation by **2** at 1 μM had no effect on the localization of PKCδ-GFP (Figure 3d). Although **2** at 10 μM was able to induce the translocation of PKCδ-GFP to the plasma membrane, no significant translocation to the nuclear membrane in response to **2** was observed (Figure 3e). Overall, the correlation between anticancer activity and translocation to the nuclear membrane reported by Blumberg and co-workers[58] was also observed for **1**, suggesting **1** to be a bryo-1-like compound with anticancer activities.

Finally, to confirm the ability of **1** to activate PKCδ, an in vitro kinase assay using PKCδ-GFP was carried out. As shown in Figure 4, in the presence of bryo-1 at 1 μM, the kinase activity of PKCδ-GFP increased about 120% compared with when DMSO only was in the system. Compound **1** at 1 μM had little effect on the kinase activity of PKCδ-GFP. However, at 10 and 50 μM, it enhanced the activity 30% and 60%, respectively. Compound **2** did not enhance the activity even at 50 μM (data not shown). As mentioned above, bryo-1 at 1 μM translocated PKCδ-GFP to the perinuclear region and nuclear membrane whereas **1** at 10 μM had a similar translocation profile to bryo-1, indicating that **1** is actually able to activate PKCδ. These results suggest that the anticancer activities of **1** are ascribable at least in part to the ability to bind, translocate, and activate PKCδ but cannot be explained solely by the signal transduction via PKCδ, since **1** as well as bryo-1 showed similar anticancer activities in several cancer cells (Table 1).

## Conclusion

Despite the growing therapeutic potential of PKC activators in the treatment of intractable diseases, most PKC activators are plagued by tumor-promoting activity. Non-tumor-promoting bryo-1 and 12-deoxyphorbol esters show promising biological responses, but their low natural abundance and structural complexity have limited their widespread use as therapeutic agents. In this study, we identified synthetically accessible simple ATX analogues (**1** and **2**) as new anticancer compounds. Analogue **1**, obtained in only 22 steps via standard reactions, exhibited substantial anticancer activities comparable to bryo-1 in several cancer cell lines. Moreover, the EBV-EA induction test demonstrated **1** to be an antitumor promoter like bryo-1. Binding and activating assays of **1** for PKCδ, an important enzyme responsible for the biological response of bryo-1,[48] suggest that the anticancer effect of **1** could be partly associated with the activation of PKCδ. Analogue **1**, which can be prepared in reliable quantities and tuned for function, would thus be a new therapeutic lead for cancer, Alzheimer's disease, and AIDS. An analysis of its mode of action and the identification of superior analogues are in progress.

## Experimental Section

**Synthesis of the Simple ATX Analogues (1 and 2).** Experimental procedures with detailed spectroscopic data are shown in the Supporting Information.

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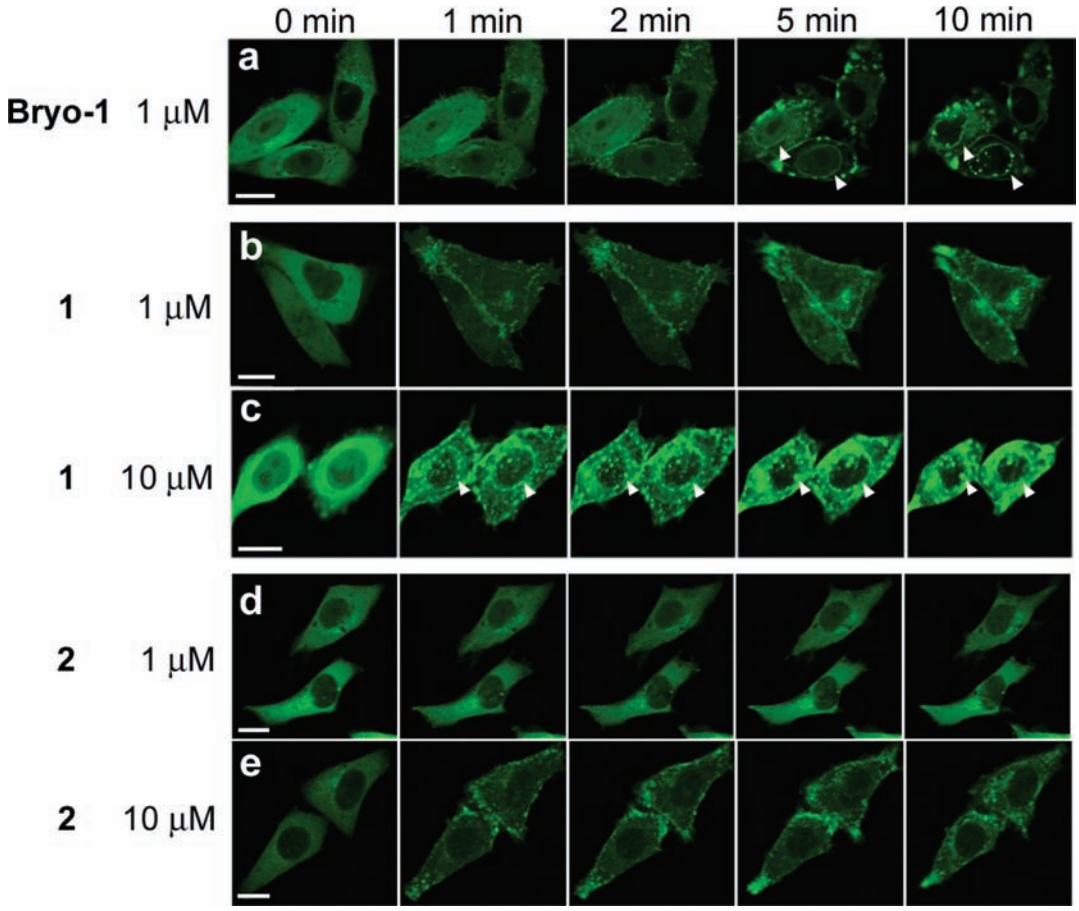


***Figure 3.*** Translocation of PKC$\delta$-GFP in CHO-K1 cells induced by **1**, **2**, and bryo-1. Fluorescent images of CHO-K1 cells expressing PKC$\delta$-GFP after 0, 1, 2, 5, and 10 min of treatment with each compound are shown. Arrowheads indicate translocation to the perinuclear and nuclear membranes. Scale bar: 10 $\mu$m. (a) Bryo-1 at 1 $\mu$M; (b) **1** at 1 $\mu$M; (c) **1** at 10 $\mu$M; (d) **2** at 1 $\mu$M; (e) **2** at 10 $\mu$M.

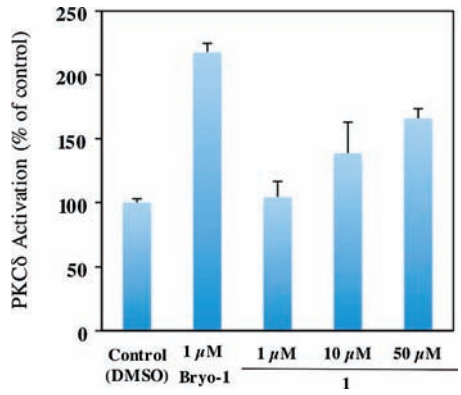


***Figure 4.*** Kinase activity of PKC$\delta$ stimulated by bryo-1 and **1** at the indicated concentrations. Immunoprecipitated PKC$\delta$-GFP was used for the assay. Activity levels were evaluated by the incorporation of $^{32}P_i$ into Ac-MBP(4−14) by use of [$\gamma$-$^{32}$P]ATP and expressed as a percentage relative to the control (DMSO). The final DMSO concentration was 10%. Error bars represent the standard deviation from triplicate experiments.

**Measurements of Cell Growth Inhibition.** The panel of 39 human cancer cell lines established by Yamori et al.[41] according to the NCI method with modifications was employed, and cell growth inhibition was measured as reported previously.[42] In brief, the cells were plated in 96-well plates in RPMI 1640 medium supplemented with 5% fetal bovine serum and allowed to attach overnight. The cells were incubated with each test compound for 48 h. Cell growth was estimated by the sulforhodamine B assay. The 50% growth inhibition ($GI_{50}$) parameter was calculated as reported previously.[59] Absorbance for the control well ($C$) and the test well ($T$) were measured at 525 nm along with that for the test well at time 0 ($T_0$). The $GI_{50}$ was calculated as $100[(T - T_0)/(C - T_0)] = 50$.

**EBV-EA Induction Test.** Human B-lymphoblastoid Raji cells ($5 \times 10^5$ cells/mL) were incubated at 37 °C under a 5% $CO_2$ atmosphere in 1 mL of RPMI 1640 medium (supplemented with 10% fetal bovine serum) with 3 mM sodium $n$-butyrate (a synergist) and 100 nM test compound in the presence or absence of 100 nM TPA. TPA was added as 5 $\mu$L of a 2% EtOH/phosphate-buffered saline (PBS) solution of the 20 $\mu$M stock solution. Since each test compound was added as 5 $\mu$L of a DMSO solution of each 20 $\mu$M stock solution, the final DMSO concentration was 0.5%. After incubation for 48 h, smears were made from the cell suspension, and the EBV-EA-expressing cells were stained by a conventional indirect immunofluorescence technique with mouse anti-EBV-EA monoclonal antibody (Chemicon International Inc., Temecula, CA) and fluorescein isothiocyanate (FITC) -labeled antimouse IgG (DAKO, Glostrup, Denmark) as reported previously.[60] In each assay, at least 500 cells were counted and the proportion of the EA-positive cells was recorded. Test compounds [**1**, bryo-1 (Sigma), and ATX[61]] did not induce any cell death during the experiment at 100 nM. Cell viability exceeded 80% in each experiment.

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**Inhibition of Specific Binding of [$^3$H]PDBu to PKC$\delta$ C1 Peptides.** The binding of [$^3$H]PDBu to the PKC$\delta$ C1 peptides was evaluated by the procedure of Sharkey and Blumberg[51] with modifications as reported previously[49,50] with 50 mM Tris-maleate buffer (pH 7.4 at 4 °C), 10 ∼ 40 nM PKC$\delta$ C1 peptides, 20 nM [$^3$H]PDBu (19.6 Ci/mmol, Perkin-Elmer Life Sciences), 50 $\mu$g/mL 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (Sigma), 3 mg/mL bovine $\gamma$-globulin (Sigma), and various concentrations of inhibitors. Binding affinity was evaluated on the basis of the concentration required to cause 50% inhibition of the specific binding of [$^3$H]PDBu, $IC_{50}$, which was calculated with PriProbit 1.63 software.[62] The inhibition constant, $K_i$, was calculated by the method of Sharkey and Blumberg.[51] Although we used each PKC C1 peptide in the range of 10 ∼ 40 nM, the concentration of the properly folded peptide was estimated to be about 3 nM on the basis of $B_{max}$ values of Scatchard analyses reported previously.[50] Therefore, the concentration of free PDBu will not markedly vary over the dose−response curve.

**Translocation of PKC$\delta$.** CHO-K1 cells transfected with GFP-tagged PKC$\delta$ were cultured for 16 ∼ 48 h for maximal fluorescence. The medium was then replaced with Ringer's solution composed of 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), and 10 mM glucose at pH 7.3. Translocation of the GFP-tagged PKC$\delta$ was triggered by addition of a test compound as Ringer's solution (final DMSO concentration 0.01 ∼ 0.1%) to obtain the appropriate final concentration. All experiments were performed at 37 °C. The fluorescence of GFP was monitored by confocal laser scanning fluorescence microscopy (Carl Zeiss, Jena, Germany) at 488 nm argon excitation with a 505 ∼ 550 nm band-pass barrier filter for GFP.

**Immunoprecipitation and Kinase Assay of GFP-Tagged PKC$\delta$.** COS-7 cells ($1.1 \times 10^6$) were seeded on 6-cm-diameter dishes and cultured for 24 h. GFP-tagged PKC$\delta$ was transiently introduced into the COS-7 cells by lipofection using FuGENE 6 transfection reagent (Roche) according to the manufacturer's directions. After cultivation for 24 h, semiconfluent cells were harvested by centrifugation at 800*g* and washed once with PBS. The cell pellets were resuspended in 200 $\mu$L of homogenate buffer, consisting of 20 mM Tris-HCl (pH 7.4), 250 mM sucrose, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM phenylmethanesulfonyl fluoride, 200 $\mu$g/mL leupeptin, and 1% Triton-X, and were sonicated twice for 20 s each at output setting 6 by use of Handy Sonic (Tomy). The lysate was centrifuged at 17400*g* for 15 min and the supernatant was used for the immunoprecipitation. Samples were incubated with rabbit serum containing anti-GFP antibodies for 1 h at 4 °C and then with protein G−Sepharose (Amersham Biosciences) for an additional 1 h. The beads were collected by centrifugation at 800*g*, washed five times in 1 mL of the homogenate buffer, and suspended in 240 $\mu$L of 20 mM Tris-HCl buffer (pH 7.4). The kinase assay of GFP-tagged PKC$\delta$ was performed as described previously.[63] The kinase activity of the immunoprecipitate (10 $\mu$L of the suspended pellet) stimulated by various PKC activators (final DMSO concentration 10%) was assayed by measuring the incorporation of $^{32}P_i$ into Ac-MBP(4−14) (Gibco BRL Life Technologies) from [$\gamma$-$^{32}$P]ATP in the presence of 8 $\mu$g/mL of 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (Sigma).

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**Supporting Information Available:** Experimental procedures with spectroscopic data for the simple ATX analogues (**1** and **2**) and their anticancer activities. This material is available free of charge via the Internet at http://pubs.acs.org.

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