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Structure–activity studies on the side chain of a simplified analog of aplysiatoxin (aplog-1) with anti-proliferative activity

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ABSTRACT

We have recently developed a simplified analog of aplysiatoxin (aplog-1) as an activator of protein kinase C (PKC) with anti-proliferative activity like bryostain 1. To identify sites in aplog-1 that could be readily modified to optimize therapeutic performance and to develop a molecular probe for examining the analog's mode of action, substituent effects on the phenol ring were systematically examined. Whereas hydrophilic acetamido derivatives were less active than aplog-1 in inhibiting cancer cell growth and binding to PKCδ, introduction of hydrophobic bromine and iodine atoms enhanced both biological activities. The anti-proliferative activity was found to correlate closely with molecular hydrophobicity, and maximal activity was observed at a log*P* value of 4.0–4.5. On the other hand, an induction test with Epstein–Barr virus early antigen demonstrated that these derivatives have less tumor-promoting activity in vitro than aplog-1 regardless of the hydrophobicity of their substituents. These results would facilitate rapid preparation of molecular probes to examine the mechanism of the unique biological activities of aplog-1.

1. Introduction

Protein kinase C (PKC), a family of serine/threonine kinases, are a component of intracellular signaling pathways that cause differentiation, proliferation, and apoptosis.[1,2] Regulation of PKC activity has thus been recognized as an important therapeutic strategy for various cancers.[3,4] Several PKC inhibitors against the ATP-binding domain were shown to be anti-proliferative for some cancer cells.[5,6] In contrast, there exist some PKC activators that show anti-proliferative activity, representative of which is bryostatin 1 (bryo-1), a marine natural product isolated from the bryozoan *Bugula neritina*.[7,8] Although most exogenous PKC activators such as phorbol esters, teleocidins, and aplysiatoxins are strong tumor promoters,[9] bryo-1 possesses little tumor-promoting activity.[10] PKCδ, one of the PKC isozymes, plays a tumor suppressor role and is involved in apoptosis.[11,12] The unique biological activities of bryo-1 could be ascribable in part to the ability to bind to and activate PKCδ.[13] bryo-1 is under clinical trials for the treatment of several cancers.[3,8] Although some of these trials have been disappointing, bryo-1 is also expected to be promising as a therapeutic agent for Alzheimer's disease[14] and AIDS.[15] A drawback of bryo-1 is a difficulty in supply both by isolation from natural sources (only 1 g from 10 tons of *B. neritina*)[16] and organic synthesis,[17–19] though more efficient methods of producing bryo-1-related compounds have recently been reported.[20–23] Simplification of bryo-1 is a promising approach to solving this problem.[24,25]

Our approach to this issue is to focus on a skeleton other than bryo-1. As described in previous papers,[26–28] we identified a simplified analog of the tumor-promoting aplysiatoxin (ATX)[29] that behaved like bryo-1. This analog, named aplog-1 (Fig. 1), was anti-proliferative for many cancer cell lines but exhibited little tumor-promoting activity. The next step is to optimize the anti-proliferative activity of aplog-1. Introduction of a methyl group on the spiroketal moiety at position 10 drastically increased the anti-proliferative activity and the ability to bind to the C1B domain of PKCδ, whereas this derivative (10-methyl-aplog-1) showed little tumor-promoting activity both in vitro and in vivo.[30]

Another method to increase the anti-proliferative activity of aplog-1 is to modify the phenolic side chain. Previous computer-aided analyses indicated that the phenolic side chain of ATX is involved not in the specific interaction with PKC but in non-specific hydrophobic interaction with membranous lipids.[31,32] Precise structure–activity studies on the side chain would afford the optimal hydrophobicity for anti-proliferative activity and be connected to the development of molecular probes for the identification of

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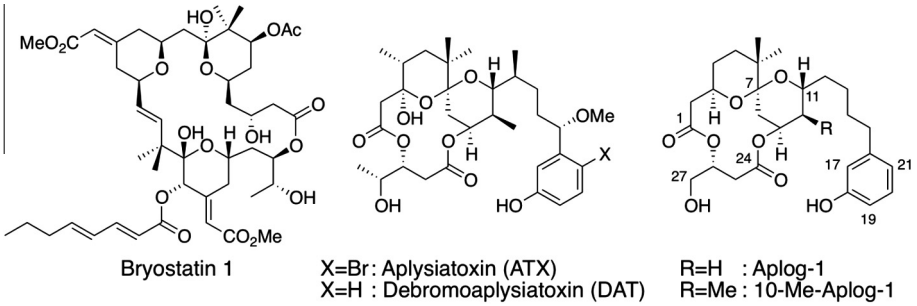


Figure 1. Structure of bryostatin 1 (bryo-1), aplysiatoxin (ATX), debromoaplysiatoxin (DAT), aplog-1, and 10-Me-aplog-1.

receptors other than PKC isozymes, since the unique biological activities of aplog-1 could not be explained only by PKC.[26–28] In fact, a molecular target of bryo-1 other than PKC has recently been reported.[33]

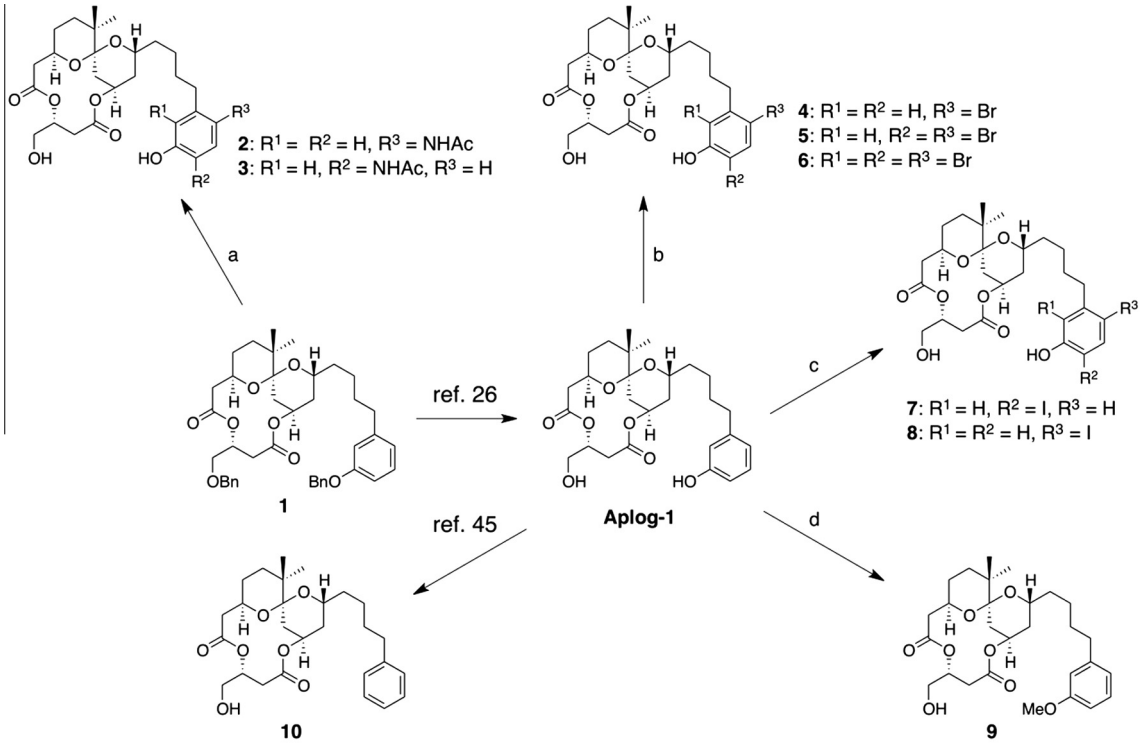
This paper deals with a comprehensive structure–activity study on the side chain of aplog-1 in binding to PKCδ C1 domains, anti-proliferative activity against 39 cancer cell lines, and tumor-promoting activity. The data clearly showed that optimal hydrophobicity exists, and that the steric requirements at this moiety are low.

2. Results and discussion

2.1. Synthesis of aplog-1 derivatives at the phenol side chain

A series of side chain derivatives of aplog-1 was synthesized from dibenzyl-aplog-1 (**1**, Bn_2-aplog-1) or aplog-1[26] (Scheme 1). These derivatives were selected based on a lowering or raising of hydrophobicity, an increase in steric hindrance around the aromatic ring, and the role of the phenolic hydroxyl group. We attempted at first the amination of aplog-1 to lower the hydrophobicity but failed because of catalytic poisoning at the debenzylation step. Subsequent acetylation followed by debenzylation gave acetamido derivatives. In brief, nitration of **1** with tetrabutylammonium nitrate and trifluoroacetic anhydride provided dibenzyl-21-NO_2-aplog-1 and dibenzyl-19-NO_2-aplog-1. 18-Crown-6 was added to increase the selectivity for the *para* position of a phenoxy ether.[34] Each nitro compound was selectively reduced to the corresponding aniline derivative, followed by acetylation. Finally, two benzyl groups in each derivative were deprotected to give acetamido-aplog-1 (**2**, **3**).

Bromination of the phenol moiety of aplog-1 was performed with the solid tribromide reagent.[35] Mono-, di-, and tri-substitutions were controlled by the amount of the tribromide reagent



(a) (i) TFAA, 18-Crown-6, tetrabutylammonium nitrate, CH_2Cl_2; (ii) $NiCl_2$-$6H_2O$, $NaBH_4$, MeOH, THF; (iii) AcCl, NEt_3, CH_2Cl_2; (iv) Pd/C, H_2, EtOH; 17% for **2**, 6% for **3** in four steps. (b) benzyltrimethylammonium tribromide, $CaCO_3$, CH_2Cl_2, MeOH, 68% for **4**, 58% for **5**, 92% for **6**. (c) benzyltrimethylammonium dichloroiodate, $CaCO_3$, CH_2Cl_2, MeOH, 29% for **7**, 21% for **8**. (d) TMS-diazomethane, MeOH, $CHCl_3$, 64%.

Scheme 1. Synthesis of **2**–**10**.

Table 1
log*P* values for aplog-1, 10-Me-aplog-1, **2**–**10**, DAT, and ATX determined by the HPLC method[38,39]

Compounds	log*P*
aplog-1	3.3
10-Me-aplog-1[a]	3.6
2	2.3
3	2.7
4	4.0
5	5.0
6	5.5
7	4.3
8	4.2
9	4.4
10	4.8
DAT	4.4
ATX	5.4

[a] Cited from Ref. 30.

added. Iodination of aplog-1 gave **7** and **8** in one step. Selective methylation of the phenolic hydroxyl group was achieved by less-explosive TMS-diazomethane[36] to give 18-OMe-aplog-1 (**9**).

2.2. Measurement of the partition coefficient (log*P*) of the aplog-1 derivatives

As expressed in 'Lipinski's rule of five',[37] the partition coefficient (log*P*) between 1-octanol and water is an important parameter for drug design. The hydrophobic side chain of tumor promoters plays a critical role in tumor promotion.[9] To analyze the relationship between biological activity and molecular hydrophobicity, a log*P* value for each derivative was estimated by the HPLC method recommended by OECD.[38,39] The retention time of each derivative on a reverse-phase column could be correlated to log*P* using appropriate reference compounds with known log*P* values.

Table 1 summarizes the log*P* values along with those of debromoaplysiatoxin (DAT) and ATX as references. Regardless of the absence of a bromine atom, DAT as well as ATX behaved as a tumor promoter in vivo.[30,40] Although all compounds contained many oxygen atoms, they showed comparatively high values, over 3.0, with the exception of **2** and **3**. This might be ascribable to the macrolactone structure where many oxygen atoms are oriented inside the ring, avoiding interaction with the solvent. Compounds **5**, **6**, **9**, and **10** showed a hydrophobicity equal to or greater than that of the tumor promoter DAT.[9] In contrast, the acetamido derivatives **2** and **3** were more hydrophilic than aplog-1, especially the p-substituted derivative **2**, which was one order of magnitude more hydrophilic than aplog-1. The hydrophobicity of aplog-1 (3.3) was almost equal to that of bryo-1 (2.9) reported by Bignami et al.[41]

2.3. Ability of the aplog-1 derivatives to bind to the C1 domains of PKCδ

As mentioned above, translocation and activation of PKCδ is considered critical to the unique biological activities of bryo-1.[13] Our group reported that aplog-1 behaved like bryo-1 in the translocation of PKCδ in CHO-K1 cells.[26] Thus, the aplog-1 derivatives synthesized in this study were subjected to evaluations of their affinity for synthetic C1 domains of PKCδ[42] by inhibition of the specific binding of [^{3}H]phorbol 12,13-dibutylate (PDBu) in the presence of phosphatidylserine as reported previously.[43,44] A binding assay using whole PKCδ reflects binding to both the C1A and C1B domains, and its instability diminishes accuracy and reproducibility. This is why we use synthetic C1A and C1B peptides (δ-C1A and δ-C1B) that are stable and inexpensive. As reported previously, binding constants of some PKC ligands in the inhibition of specific [^{3}H]PDBu-binding were almost equal to those of whole PKCδ.[30]

Table 2 summarizes K_i values of all aplog-1 derivatives. All derivatives except for **2** and **3** with log*P* values of less than 3.0 bound strongly to δ-C1B. Although these derivatives bound to δ-C1B more strongly than to δ-C1A, they showed substantial binding to δ-C1A with K_i values of about 100 nM. Hydrophobic halogen derivatives (**4**–**8**) exhibited slightly higher binding than aplog-1. Lack of the phenolic hydroxyl group hardly changed the affinity for the C1 peptides, indicating the hydroxyl group not to be critical to the binding.[45] It is remarkable that the highly bulky and hydrophobic tribromide **6** did not lose binding ability, suggesting that the aromatic side chain of aplog-1 is not close to the binding pocked of the C1 domain. This speculation is supported by computer-aided molecular modeling by several groups.[31,32]

2.4. Anti-proliferative activity of the aplog-1 derivatives

The anti-proliferative activity of the aplog-1 derivatives was evaluated by growth inhibition against a panel of 39 human cancer cell lines established by Yamori et al. as described previously.[46] The concentration required to inhibit cell growth by 50% compared to an untreated control was expressed as the GI_{50} (M). Representative cancer cell lines whose log GI_{50} was less than MG-MID are shown in Table 3. MG-MID is defined as the average of the log GI_{50} values of all 39 human cancer cell lines. For calculation of the MG-MID of **2**, the log GI_{50} was taken as −4.00 in the cell lines that showed a value of more than −4.00 (Supplementary data). The profile of anti-proliferative activities against 39 cancer cell lines of aplogs synthesized in this study was similar to that of the parent compound, DAT and 10-methyl-aplog1 (Table 3).[30]

Hydrophilic acetamido substitution (**2**, **3**) weakened the anti-proliferative activity as observed in the PKCδ binding assay; the activity of the most hydrophilic acetamide (**2**) decreased markedly. In contrast, mono-substitution with a hydrophobic halogen atom enhanced the activity of aplog-1. The bromide (**4**) was found to be slightly superior to the iodides (**7**, **8**). However, introduction of additional bromine atoms (**5**, **6**) attenuated the activity of **4**. Lack of the phenolic hydroxyl group little influenced the activity as observed in **9** and **10**. The results for the anti-proliferative activity correlated well with those for the binding affinity for the PKCδ C1 peptides.

Table 2
K_i values for inhibition of specific binding of [^{3}H]PDBu by aplog-1, 10-Me-aplog-1, DAT, and **2**–**10**

PKCδ C1 peptide	K_i (nM)												K_d (nM)
	aplog-1[a]	10-Me-aplog-1	DAT	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**[c]	PDBu[d]
δ-C1A	140	22	9.7	8700	290	66	93	79	82	110	330	130	52
δ-C1B	7.4	0.46[b]	0.20[b]	690	36	3.5	4.1	6.9	1.3	4.6	9.7	9.8	0.53

[a] Cited from Ref. 26.
[b] Cited from Ref. 30.
[c] Cited from Ref. 45.
[d] Cited from Ref. 44.

Table 3
log GI_{50} values for aplog-1, 10-Me-aplog-1, DAT, and **2**–**10** against 39 human cancer cell lines

Cancer type	Cell line	Log GI_{50} (M)											
		aplog-1[a]	10-Me-aplog-1[b]	DAT[b]	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**[c]
Breast	HBC-4	−6.33	−7.48	−6.47	>−4.00	−5.61	−7.01	−6.65	−6.06	−6.53	−6.80	−5.77	−6.28
Breast	MDA-MB-231	−5.61	−6.90	−6.03	>−4.00	−4.78	−6.33	−5.53	−4.92	−5.80	−5.81	−5.18	−5.67
CNS	SF-295	−5.06	−4.98	−5.53	−4.22	−4.88	−5.26	−5.18	−4.88	−5.39	−5.20	−4.99	−5.14
Colon	HCC2998	−5.43	−6.47	−6.09	−4.14	−4.86	−6.10	−6.05	−5.12	−5.79	−6.05	−5.10	−5.53
Lung	NCI-H460	−5.60	−7.07	−6.46	>−4.00	−4.69	−5.78	−5.91	−5.49	−5.22	−4.98	−5.59	−5.83
Lung	A549	−5.32	−6.01	−5.94	>−4.00	−4.82	−5.30	−5.31	−4.90	−5.48	−5.39	−5.31	−5.49
Melanoma	LOX-IMVI	−5.74	−6.21	−5.69	−4.13	−4.89	−6.10	−5.55	−4.93	−5.54	−5.70	−4.96	−5.17
Stomach	St-4	−5.55	−6.24	−6.44	>−4.00	−5.67	−6.02	−5.89	−5.51	−6.03	−6.37	−5.60	−6.05
Stomach	MKN45	−5.33	−4.97	−4.98	>−4.00	−4.54	−5.74	−5.43	−4.94	−4.93	−4.92	−5.02	−6.09
Prostate	PC-3	−4.96	−4.94	−4.98	>−4.00	−4.68	−5.53	−5.41	−4.88	−5.51	−5.33	−4.92	−5.26
MG-MID[d]		−4.98	−5.24	−5.22	−4.04	−4.68	−5.20	−5.17	−4.83	−5.18	−5.19	−4.95	−5.09

[a] Cited from Ref. 26.
[b] Cited from Ref. 30.
[c] Cited from Ref. 45.
[d] Average of 39 cancer cell lines. The list is shown in the Supplementary data.

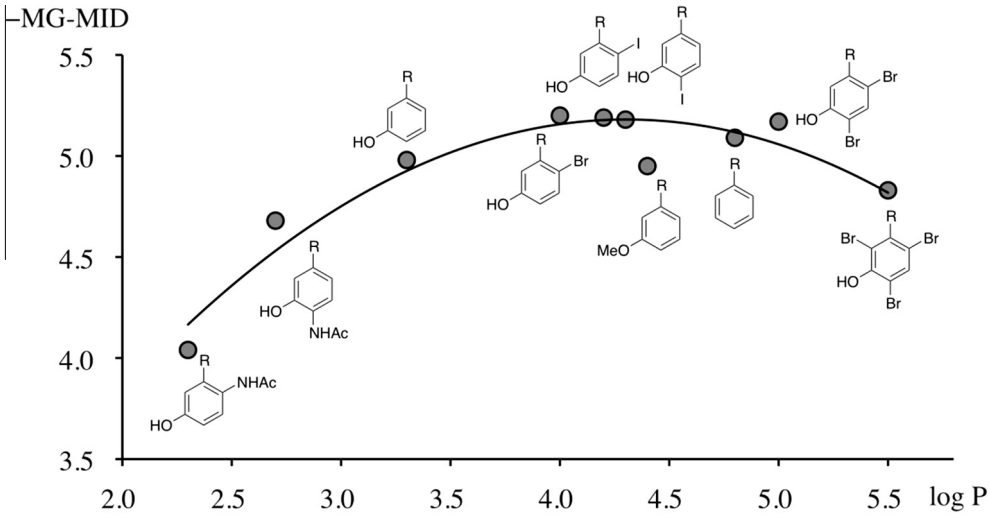


Figure 2. Correlation between anti-proliferative activity and log*P*. 'R' means the macrolide skeleton and alkyl side chain of aplog-1.

2.5. Relationship between the anti-proliferative activity and log*P* of the aplog-1 derivatives

Figure 2 shows a plot of –MG-MID against the log*P* of each aplog-1 derivative. The plot could be approximated to a quadratic curve relevant to log*P*. The least squares regression equation is shown below:

$$-\text{MG-MID} = -0.25\ (\pm 0.10)\ (\log P)^2 + 2.2\ (\pm 0.8)\ (\log P) + 0.50\ (\pm 1.5) \qquad (n = 10, s = 0.10, r = 0.95)$$

where n is the number of compounds, s is the standard deviation, and r is the correlation coefficient. Figures in parentheses are 95% confidence limits. These results indicate that the aplog-1 skeleton shows maximum anti-proliferative activity at a log*P* value of 4.0–4.5. A log*P* value greater than 4.5 decreased the activity possibly because of trapping by the cellular membrane. A log*P* value less than 3.0 also decreased the activity as observed in **2** and **3**, suggesting that these ligands could not sufficiently penetrate the cellular membrane. These results clearly indicate that the hydrophobicity of **4**, **7** and **8** is optimal, and that modification of the side chain did not drastically enhance the anti-proliferative activity of aplog-1. Recently, we have identified 10-methyl-aplog-1 with more potent anti-proliferative activity than aplog-1, where a methyl group was introduced into the spiroketal moiety at position 10.[30] Since the log*P* of 10-methyl-aplog-1 is 3.6,[30] introduction of a bromine atom into the side chain would slightly enhance the anti-proliferative activity. 10-Methyl-aplog-1 could not be incorporated in the regression analysis since the effect of modification by substituents on the phenyl ring is not similar to that of modification by the methyl group at position 10. The methyl group increased more potently the ability to bind PKCδ C1 domains and anti-proliferative activities against several cancer cell lines compared with the bromine and iodine atoms at the phenyl ring as shown in Table 3.

2.6. Tumor-promoting activity of the aplog-1 derivatives

The most critical point in developing derivatives of aplog-1 is to confirm that the structural modifications do not increase tumor-promoting activity in vivo. Since tumor promoters such as ATX and DAT[9] have higher hydrophobicity (log*P* >4.4), hydrophobic halogen substitutions at the side chain of aplog-1 might get back tumor-promoting activity. Induction of the Epstein–Barr virus early antigen (EBV-EA) is one of the most reliable assays for the evaluation of tumor-promoting activity in vitro.[47,48] EBV is activated by tumor promoters to produce early antigen (EA), that is detected by an indirect immunofluorescence technique.[49]

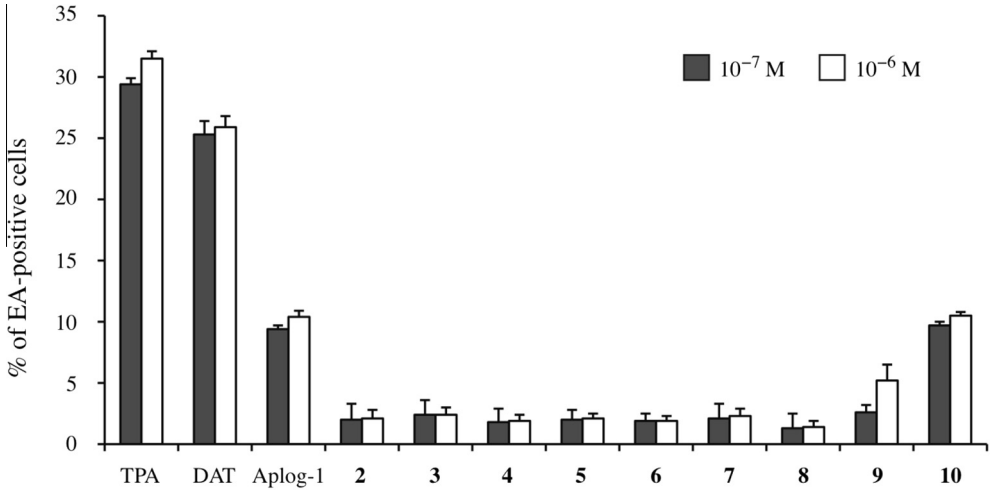


Figure 3. EBV-EA-inducing ability of TPA, DAT,[30] aplog-1,[45] **2**–**9**, and **10**.[45] Percentages of EA-positive cells are shown. Sodium *n*-butyrate (4 mM) was added to all samples to enhance the sensitivity of the Raji cells. Only 0.1% of the cells were positive for EA at 4 mM sodium *n*-butyrate. Cell viability exceeded 50% in most cases as shown in the Supplementary data. Error bars represent standard errors of the mean ($n = 3$).

As shown in Figure 3, a strong tumor promoter, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), produced 29.4% EA-positive cells at 10^{-7} M. Tumor promoters ATX and DAT also generated 20–25% EA-positive cells at this concentration as reported.[26,30] On the other hand, aplog-1[26] and its deoxy as well as methoxy analogs (**9**, **10**[45]) showed weaker EA induction even at 10^{-6} M. The hydrophilic acetamido derivatives (**2**, **3**) were almost inactive as expected. However, hydrophobic halogen substitution caused little EA induction either. Moreover, **5**–**10** with log*P* values similar to or higher than DAT hardly exhibited any EA induction even at 10^{-6} M (Table 1 and Fig. 3). This is an unexpected result, indicating the EBV-EA induction by tumor promoters ATX and DAT to be mainly caused by their macrolactone structure rather than their molecular hydrophobicity.

We have recently reported that aplog-1 and its derivatives (12,12-dimethyl-aplog-1 and 10-methyl-aplog-1) did not show any tumor-promoting activity in a two-stage carcinogenesis experiment using ICR mice even when applied at a fivefold excess compared with DAT and TPA.[28,30,50] Therefore, we examined the tumor-promoting activity of **4** with more anti-proliferative activity and less ability to induce EBV-EA using 10 male ICR mice. From a week after initiation by a single application of 390 nmol of 7,12-dimethylbenz[*a*]anthracene (DMBA), 8.5 nmol of **4** was applied twice a week from week 1 to 20. The control group was treated with DMBA and 1.7 nmol TPA twice a week. TPA-treated mice began to develop tumors in week 6 and all had tumors at week 11. On the other hand, mice given **4** did not develop tumors even at week 20. This result is consistent with that of the EBV-EA induction test, and supports the development of anti-proliferative compounds using the skeleton of aplog-1.

3. Conclusions

Our group developed aplog-1 as a simplified analog of ATX that showed anti-proliferative activity without tumor-promoting activity like bryo-1.[26] To optimize its anti-proliferative activity, the phenolic side chain was modified systematically. As shown in Figure 2, the change in anti-proliferative activity against 39 cancer cell lines was dominated by the change in the log*P* values of the aplog-1 derivatives. Although the optimal log*P* proved to be 4.0–4.5, pertinent modification at the macrolactone ring was found to be indispensable to the development of highly potent analogs of aplog-1. These attempts continue in our laboratory as exemplified by 10-methyl-aplog-1.[30]

The present results also indicated that the skeleton of aplog-1 is qualitatively different from that of DAT and ATX with tumor-promoting activity. The aplog-1 derivatives with a log*P* greater than that of DAT (4.4) did not show any tumor-promoting activity. These results support our recent conclusion that the hemiacetal hydroxyl group at position 3 and/or the methoxy group at the benzyl position of the phenolic side chain play an important role in tumor promotion.[30] DAT and ATX behave as a master key with both tumor-promoting and anti-proliferative activities, whereas aplog-1 behaves as a special key with anti-proliferative activity. The next step is to identify molecular targets of aplog-1 other than PKCδ using molecular probes derived from aplog-1 based on the present structure–activity relationship. The involvement of PKCδ in the anti-proliferative activity of the cell lines sensitive to aplog-1 (Table 3) should also be confirmed using siRNA.

4. Experimental

4.1. General remarks

The following spectroscopic and analytical instruments were used: Digital Polarimeter, DIP-1000 and P-2200 (Jasco, Tokyo, Japan); ^{1}H, ^{13}C NMR, AVANCE 400 and AVANCE III 500 (Bruker, Germany, TMS as an internal reference); HPLC, model 600E with a model 2487 UV Detector (Waters, Tokyo, Japan); HR-FAB-MS, JMS-600H and JMS-700 (JEOL, Tokyo, Japan). Reactions were monitored by TLC (TLC Silica gel 60 F_{254}, Merck). HPLC was carried out on YMC packed ODS-A AA12S05-1520WT (SH-342-5), AM-323, AQ-311, and A-023 (Yamamura Chemical Laboratory, Kyoto, Japan). Wakogel™ C-200 (silica gel, Wako Pure Chemical Industries, Osaka, Japan) was used for column chromatography. [^{3}H]PDBu (19.6 Ci/mmol) was purchased from Perkin–Elmer Life Sciences Research Products (Boston, MA, USA). The PKCδ C1 peptides were synthesized as reported previously.[42] Bn_2-aplog-1 (**1**) and aplog-1 were also synthesized as reported.[26] All other chemicals and reagents were purchased commercially and used without further purification.

4.2. Synthesis of the aplog-1 derivatives

4.2.1. Synthesis of 21-NHAc-aplog-1 (2) and 19-NHAc-aplog-1 (3)

To a solution of Bn_2-aplog-1 (**1**) (11.6 mg, 17.3 μmol) in CH_2Cl_2 (1.2 mL) and trifluoroacetic anhydride (350 μL) were added

18-Crown-6 (48.0 mg, 181 μmol, 10 equiv) and tetrabutylammonium nitrate (8.2 mg, 27.0 mmol, 1.6 equiv). After 1 h of stirring at rt, the reaction was quenched with saturated aq $NaHCO_3$ (2 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 mL × 2). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by HPLC (column: YMC-Pack ODS-A, solvent: 90% MeOH/H_2O; flow rate 8.0 mL/min; pressure: 1600 psi; UV detector: 254 nm) to afford 21-NO_2-Bn_2-aplog-1 (5.8 mg, 8.1 μmol, 47%, retention time: 22 min) and 19-NO_2-Bn_2-aplog-1 (1.5 mg, 2.1 mmol, 12%, retention time: 26 min) as clear oils.

To a solution of 21-NO_2-Bn_2-aplog-1 (19 mg, 26.6 μmol) and $NiCl_2$–$6H_2O$ (13.9 mg, 58.5 μmol, 2.2 equiv) in MeOH (4 mL) and THF (2 mL) at 4 °C was added $NaBH_4$ (5.6 mg, 148 μmol, 5.6 equiv). After 10 min of stirring at the same temperature, the solvent was removed in vacuo, and $CHCl_3$ (10 mL) and H_2O (2.5 mL) were added. The mixture was filtered through Celite, and the Celite pad was washed with $CHCl_3$ (100 mL). The combined organic layers were concentrated in vacuo to approximately 10 mL, and the organic layer was washed with saturated aq $NaHCO_3$ (10 mL). The organic layer was separated, and the aqueous layer was extracted with $CHCl_3$ (10 mL × 2). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, 10–50% EtOAc in hexane) to afford 21-NH_2-Bn_2-aplog-1 (15.0 mg, 21.9 μmol, 82%). To a solution of 21-NH_2-Bn_2-aplog-1 (15.0 mg, 21.9 μmol) and NEt_3 (6.1 μL, 43.8 μmol, 2.0 equiv) in CH_2Cl_2 (400 μL) was added acetyl chloride (6.1 μL, 88.7 μmol, 4.1 equiv) at rt. After 4 h of stirring at the same temperature, the reaction was quenched with saturated aq $NaHCO_3$ (1 mL). The resulting mixture was poured into $CHCl_3$ and water. After the organic layer was separated, the aqueous layer was extracted with $CHCl_3$ (5 mL × 2). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, 30–50% EtOAc in hexane) to afford 21-NHAc-Bn_2-aplog-1 (8.0 mg, 11.0 μmol, 50%) as clear oil. To a solution of 21-NHAc-Bn_2-aplog-1 (8.0 mg, 11.0 μmol) in EtOH (200 μL) was added a suspension of Pd/C (4.2 mg) in EtOH (300 μL) at rt. The mixture was stirred at rt under H_2 atmosphere for 4 h, and then filtered. The filtrate was concentrated in vacuo, and purified by HPLC (column: SH-342-5; solvent: 65% MeOH/H_2O; flow rate 8.0 mL/min; pressure: 2100 psi; UV detector: 254 nm; retention time: 13.9 min) to afford 21-NHAc-aplog-1 (**2**) (5.4 mg, 9.9 μmol, 90%) as a clear oil and a 7:3 mixture of conformational isomers. $[\alpha]_D$ +53.4° (*c* 0.270, EtOH, 23.5 °C). ^{1}H NMR (400 MHz, 297 K, $CDCl_3$, 0.018 M) major conformer: δ 0.85 (3H, s), 0.86 (3H, s), 1.31–1.69 (13H, m), 2.32–2.45 (3H, m), 2.49–2.63 (2H, m), 2.71–2.89 (2H, m), 3.76 (2H, m), 3.89 (1H, m), 4.26 (1H, m), 5.19 (2H, m), 6.64 (1H, dd, *J* = 8.6, 2.8 Hz), 6.76 (1H, br s, Ph-OH), 6.79 (1H, d, *J* = 2.8 Hz), 7.24 (1H, d, *J* = 8.6 Hz), 7.30 (1H, br s, NH) ppm; minor conformer: δ 0.86 (3H, s), 0.93 (3H, s), 1.31–1.69 (13H, m), 1.82 (3H, s), 2.19 (3H, s), 2.32–2.45 (3H, m), 2.49–2.63 (2H, m), 2.71–2.89 (2H, m), 3.66 (1H, dd, *J* = 11.6, 5.5 Hz), 3.76 (1H, m), 3.89 (1H, m), 4.26 (1H, m), 5.19 (1H, m), 5.27 (1H, m), 6.68 (1H, dd, *J* = 8.5, 2.8 Hz), 6.76 (1H, br s, Ph-OH), 6.86 (1H, d, *J* = 2.8 Hz), 6.99 (1H, d, *J* = 8.8 Hz), 7.54 (1H, br s, NH) ppm. ^{13}C NMR (100 MHz, 298 K, $CDCl_3$, 0.018 M) major conformer: δ 21.07, 23.71, 24.11, 25.20, 25.86, 27.19, 30.82, 33.83, 34.38, 34.46, 35.02, 36.90, 37.00, 42.91, 62.22, 63.89, 68.85, 70.61, 72.30, 100.39, 113.37, 116.02, 127.17, 127.60, 138.68, 154.90, 169.47, 169.60, 172.98 ppm; minor conformer: δ 21.10, 23.71, 24.36, 25.24, 25.86, 27.19, 30.96, 33.83, 34.38, 34.52, 35.20, 36.90, 37.00, 42.91, 61.74, 63.89, 68.75, 70.61, 72.30, 100.39, 113.67, 116.32, 128.16, 130.03, 138.68, 156.10, 169.47, 172.70, 172.98 ppm. HR-FAB-MS *m*/*z*: 570.2690 ([M+Na]$^+$, calcd for $C_{29}H_{41}NO_9Na$, 570.2679).19-NO_2-Bn_2-aplog-1 (9.0 mg, 12.6 μmol) was treated in a manner similar to that described for the synthesis of **2** to give 19-NHAc-Bn_2-aplog-1 (6.0 mg, 7.6 μmol, 60% in three steps) as a clear oil. To a solution of 19-NHAc-Bn_2-aplog-1 (6.0 mg, 7.6 μmol) in EtOH (200 μL) was added a suspension of Pd/C (4.0 mg) in EtOH (300 μL) at rt. The mixture was stirred at rt under a H_2 atmosphere for 4 h, and then filtered. The filtrate was concentrated in vacuo, and purified by HPLC (column: SH-342-5; solvent: 70% MeOH/H_2O; flow rate 8.0 mL/min; pressure: 2000 psi; UV detector: 254 nm; retention time: 21.8 min) to afford 19-NHAc-aplog-1 (**3**) (3.3 mg, 6.0 μmol, 78%) as a clear oil. $[\alpha]_D$ +42.6° (*c* 0.165, EtOH, 25.3 °C). ^{1}H NMR (400 MHz, 297 K, $CDCl_3$, 0.012 M): δ 0.87 (3H, s), 0.97 (3H, s), 1.36–1.67 (13H, m), 2.23 (3H, s), 2.42 (1H, dd, *J* = 13.0, 10.8 Hz), 2.47–2.57 (5H, m), 2.77 (2H, m), 3.75 (2H, m), 3.89 (1H, m), 4.18 (1H, m), 5.20 (2H, m), 6.69 (1H, dd, *J* = 8.1, 1.8 Hz), 6.88 (1H, d, *J* = 1.7 Hz), 7.16 (1H, d, *J* = 8.1 Hz), 7.57 (1H, br s, Ph-OH), 8.60 (1H, br s, NH) ppm. ^{13}C NMR (100 MHz, 298 K, $CDCl_3$, 0.012 M): δ 21.17, 23.93, 24.33, 25.14, 25.92, 27.23, 30.19, 34.51, 34.78, 34.81, 34.90, 36.89, 37.04, 42.75, 63.26, 64.30, 68.82, 70.59, 72.19, 100.34, 118.38, 120.64, 121.48, 123.35, 141.70, 147.83, 169.54, 169.77, 172.23 ppm. HR-FAB-MS *m*/*z*: 548.2878 (MH$^+$, calcd for $C_{29}H_{42}NO_9$, 548.2860).

4.2.2. Synthesis of 21-Br-aplog-1 (4)

To a solution of aplog-1 (3.8 mg, 7.8 μmol) in MeOH (600 μL) was added a solution of benzyltrimethylammonium tribromide (3.0 mg, 7.8 μmol, 1.0 equiv) and $CaCO_3$ (3.8 mg, 38 μmol, 4.9 equiv) in CH_2Cl_2 (1.3 mL). The mixture was stirred at rt for 3 h, and then filtered. The filtrate was concentrated in vacuo. The residue was purified by HPLC (column: SH-342-5; solvent: 80% MeOH/H_2O; flow rate 8.0 mL/min; pressure: 1700 psi; UV detector: 254 nm; retention time: 24 min) to afford 21-Br-aplog-1 (**4**) (3.0 mg, 5.3 μmol, 68%) as a clear oil. $[\alpha]_D$ +38.4° (*c* 0.114, EtOH, 15.1 °C). ^{1}H NMR (400 MHz, 298 K, $CDCl_3$, 0.011 M): δ 0.88 (3H, s), 0.96 (3H, s), 1.34–1.79 (13H, m), 2.25 (1H, br s, OH), 2.41 (1H, dd, *J* = 13.3, 10.8 Hz), 2.51 (1H, d, *J* = 15.7 Hz), 2.62 (3H, m), 2.80 (2H, m), 3.79 (2H, m), 3.94 (1H, tt, *J* = 10.7, 3.1 Hz), 4.30 (1H, m), 5.17–5.23 (2H, m), 6.60 (1H, dd, *J* = 8.6, 3.0 Hz), 6.94 (1H, d, *J* = 3.0 Hz), 6.98 (1H, br s, Ph-OH), 7.35 (1H, d, *J* = 8.6 Hz) ppm. ^{13}C NMR (100 MHz, 298 K, $CDCl_3$, 0.011 M): δ 21.18, 23.85, 25.20, 25.91, 27.18, 27.23, 33.97, 34.47, 34.92, 35.00, 36.96, 36.98, 42.89, 62.31, 64.02, 68.88, 70.68, 72.74, 100.50, 114.34, 114.61, 116.52, 133.20, 142.87, 155.87, 169.37, 173.53 ppm. HR-FAB-MS *m*/*z*: 569.1766 (MH$^+$, calcd for $C_{27}H_{38}O_8Br$, 569.1750).

4.2.3. Synthesis of 19,21-dibromo-aplog-1 (5)

To a solution of aplog-1 (6.0 mg, 12.2 μmol) and $CaCO_3$ (1.1 mg, 11.0 μmol, 0.9 equiv) in MeOH (60 μL) and CH_2Cl_2 (140 μL) was added benzyltrimethylammonium tribromide (9.5 mg, 24.4 μmol, 2.0 equiv) at rt. After 1.5 h of stirring at the same temperature, saturated aq $Na_2S_2O_3$ (330 μL) and H_2O (660 μL) were added to the mixture. The resulting mixture was extracted with EtOAc (2 mL × 4). The combined organic layers were dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, 30 to 35% EtOAc in hexane) to afford the mixture of Br_2-aplog-1 (**5**) and Br_3-aplog-1 (**6**). The mixture was purified by HPLC (column: AM-323; solvent: 85% MeOH/H_2O; flow rate 3.0 mL/min; pressure: 1800 psi; UV detector: 254 nm; retention time: 24 min) to afford 19,21-dibromo-aplog-1 (**5**) (4.6 mg, 7.1 μmol, 58%) as a clear oil. $[\alpha]_D$ +41.0° (*c* 0.807, $CHCl_3$, 15.5 °C). ^{1}H NMR (500 MHz, 295 K, $CDCl_3$, 0.014 M): δ 0.87 (3H, s), 0.95 (3H, s), 1.34–1.76 (13H, m), 2.24 (1H, t, *J* = 5.6 Hz, OH), 2.41 (1H, dd, *J* = 13.2, 10.9 Hz), 2.50 (1H, d, *J* = 15.6 Hz), 2.56–2.66 (3H, m), 2.80 (2H, m), 3.78 (2H, m), 3.92 (1H, m), 4.25 (1H, m), 5.20 (2H, m), 7.02 (1H, s), 7.03 (1H, br s, Ph-OH), 7.61

(1H, s) ppm. ^{13}C NMR (125 MHz, 298 K, $CDCl_3$, 0.014 M): δ 21.26, 24.12, 25.23, 25.91, 27.23, 27.93, 34.47, 34.53, 35.02, 36.96, 37.01, 42.86, 62.73, 64.11, 68.83, 70.69, 72.39, 77.60, 100.45, 107.29, 114.60, 117.04, 135.04, 142.89, 152.28, 169.43, 172.95 ppm. HR-FAB-MS *m*/*z*: 647.0849 (MH^+, calcd for $C_{27}H_{37}O_8Br_2$, 647.0855).

4.2.4. Synthesis of 17,19,21-tribromo-aplog-1 (6)

To a solution of aplog-1 (4.3 mg, 8.76 μmol) and $CaCO_3$ (1.3 mg, 13.1 μmol, 1.5 equiv) in MeOH (44 μL) and CH_2Cl_2 (100 μL) was added benzyltrimethylammonium tribromide (10.9 mg, 28.0 μmol, 3.2 equiv) at rt. After 1.5 h of stirring at the same temperature, saturated aq $Na_2S_2O_3$ was added to the mixture. The resulting mixture was extracted with EtOAc (2 mL × 4). The combined organic layers were dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, 35% EtOAc in hexane) to afford 17,19,21-tribromo-aplog-1 (**6**) (5.9 mg, 8.1 μmol, 92%) as a clear oil. $[\alpha]_D$ +25.7° (*c* 0.569, $CHCl_3$, 17.5 °C). ^{1}H NMR (500 MHz, 295 K, $CDCl_3$, 0.015 M): δ 0.87 (3H, s), 0.99 (3H, s), 1.34–1.67 (13H, m), 2.41 (1H, dd, *J* = 12.9, 10.9 Hz), 2.55 (2H, m), 2.71 (1H, dd, *J* = 16.6, 3.3 Hz), 2.80 (1H, dd, *J* = 16.6, 11.3 Hz), 2.92 (2H, t, *J* = 7.5 Hz), 3.71 (1H, dd, *J* = 11.9, 5.3 Hz), 3.78 (1H, dd, *J* = 11.9, 3.9 Hz), 3.89 (1H, m), 4.17 (1H, m), 5.19–5.23 (2H, m), 7.67 (1H, s) ppm. ^{13}C NMR (125 MHz, 298 K, $CDCl_3$, 0.015 M): δ 21.23, 25.20, 25.95, 27.30, 27.88, 34.60, 34.84, 35.36, 36.92, 37.02, 37.36, 42.70, 63.74, 64.47, 68.76, 70.61, 71.79, 77.60, 100.26, 107.01, 113.00, 114.79, 134.55, 141.85, 149.09, 169.58, 171.48 ppm. HR-FAB-MS *m*/*z*: 724.9947 (MH^+, calcd for $C_{27}H_{36}O_8Br_3$, 724.9960).

4.2.5. Synthesis of 19-I-aplog-1 (7) and 21-I-aplog-1 (8)

To a solution of aplog-1 (17.9 mg, 36.5 μmol) in MeOH (350 μL) and CH_2Cl_2 (600 μL) was added benzyltrimethylammonium dichloroiodate (15.9 mg, 45.7 μmol, 1.3 equiv) and $CaCO_3$ (11.3 mg, 113 μmol, 3.1 equiv). The mixture was stirred at rt for 4 h, and then filtered. The filtrate was concentrated in vacuo. The residue was purified by HPLC (column: SH-342-5; solvent: 75% MeOH/H_2O; flow rate 8.0 mL/min; pressure: 1800 psi; UV detector: 254 nm; retention time: 36 min) to afford the mixture of 19-I-aplog-1 (**7**) and 21-I-aplog-1 (**8**). The mixture was purified by HPLC (column: A-023; solvent: *i*PrOH:$CHCl_3$:hexane = 5:15:80; flow rate 3.0 mL/min; pressure: 600 psi; UV detector: 254 nm) to afford **7** (6.6 mg, 10.7 μmol, 29%; retention time: 13 min) and **8** (4.7 mg, 7.6 μmol, 21%; retention time: 17 min) as clear oils. 21-I-aplog-1 (**7**): $[\alpha]_D$ +37.7° (*c* 0.333, EtOH, 26.5 °C). ^{1}H NMR (400 MHz, 297 K, $CDCl_3$, 0.019 M): δ 0.86 (3H, s), 0.95 (3H, s), 1.34–1.69 (13H, m), 2.34 (1H, t, *J* = 5.8 Hz, OH), 2.42 (1H, dd, *J* = 13.1, 10.8 Hz), 2.47–2.59 (4H, m), 2.79 (2H, m), 3.78 (2H, m), 3.91 (1H, tt, *J* = 3.0, 10.8 Hz), 4.21 (1H, m), 5.16–5.22 (2H, m), 6.51 (1H, dd, *J* = 8.0, 2.0 Hz), 6.71 (1H, br s, Ph-OH), 6.92 (1H, d, *J* = 1.9 Hz), 7.54 (1H, d, *J* = 8.0 Hz) ppm. ^{13}C NMR (100 MHz, 298 K, $CDCl_3$, 0.019 M): δ 21.14, 24.00, 25.16, 25.91, 27.21, 29.57, 34.50, 34.50, 34.75, 34.89, 36.91, 37.02, 42.79, 62.85, 64.12, 68.78, 70.64, 72.27, 80.77, 100.35, 115.04, 122.80, 138.13, 145.44, 155.15, 169.52, 172.67 ppm. HR-FAB-MS *m*/*z*: 617.1611 (MH^+, calcd for $C_{27}H_{38}O_8I$, 617.1611). 21-I-aplog-1 (**8**): $[\alpha]_D$ +25.9° (*c* 0.235, EtOH, 25.8 °C). ^{1}H NMR (400 MHz, 297 K, $CDCl_3$, 0.015 M): δ 0.88 (3H, s), 0.96 (3H, s), 1.34–1.76 (13H, m), 2.31 (1H, t, *J* = 5.9 Hz, OH), 2.41 (1H, dd, *J* = 13.3, 10.8 Hz), 2.51 (1H, d, *J* = 15.6 Hz), 2.55–2.64 (3H, m), 2.81 (2H, m), 3.79 (2H, m), 3.94 (1H, m), 4.31 (1H, m), 5.17–5.23 (2H, m), 6.48 (1H, dd, *J* = 8.5, 3.0 Hz), 6.94 (1H, d, *J* = 2.9 Hz), 7.11 (1H, s, Ph-OH), 7.62 (1H, d, *J* = 8.5 Hz) ppm. ^{13}C NMR δ (100 MHz, 298 K, $CDCl_3$, 0.015 M): d 21.17, 23.79, 25.20, 25.91, 27.18, 27.64, 33.98, 34.47, 34.99, 36.96, 36.96, 39.84, 42.88, 62.29, 63.97, 68.87, 70.69, 72.68, 88.68, 100.50, 115.26, 116.14, 139.75, 146.16, 156.95, 169.40, 173.50 ppm. HR-FAB-MS *m*/*z*: 617.1611 (MH^+, calcd for $C_{27}H_{38}O_8I$, 617.1611).

4.2.6. Synthesis of 18-OMe-aplog-1 (9)

To a solution of aplog-1 (1.5 mg, 3.1 μmol) in MeOH (50 μL) and $CHCl_3$ (250 μL) was added 2 M TMS-diazomethane in hexane (20 μL, 40 μmol, 13 equiv) at rt. After 35 h of stirring at the same temperature, the reaction was quenched with acetic acid (20 μL). The mixture was concentrated in vacuo, and purified by HPLC (column: A-023; solvent: *i*PrOH:$CHCl_3$:hexane = 5:15:80; flow rate 3.0 mL/min; pressure: 600 psi; UV detector: 254 nm; retention time: 14.5 min) to afford 18-OMe-aplog-1 (**9**) (1.0 mg, 2.0 μmol, 64%) as a clear oil. $[\alpha]_D$ +55.2° (*c* 0.519, EtOH, 24.9 °C). ^{1}H NMR (500 MHz, 295 K, $CDCl_3$, 0.014 M): δ 0.86 (3H, s), 0.98 (3H, s), 1.34–1.68 (13H, m), 2.25 (1H, t, *J* = 5.7 Hz, OH), 2.40 (1H, dd, *J* = 12.9, 10.9 Hz), 2.49 (1H, m), 2.53 (1H, dd, *J* = 12.9, 2.8 Hz), 2.59 (2H, t, *J* = 7.8 Hz), 2.71 (1H, dd, *J* = 16.6, 3.3 Hz), 2.80 (1H, dd, *J* = 16.6, 11.3 Hz), 3.71 (1H, m), 3.77 (1H, m), 3.80 (3H, s), 3.88 (1H, m), 4.17 (1H, m), 5.18–5.23 (2H, m), 6.72 (1H, dd, *J* = 8.2, 2.5 Hz), 6.76 (1H, d, *J* = 2.3 Hz), 6.80 (1H, d, *J* = 7.6 Hz), 7.19 (1H, t, *J* = 7.9 Hz) ppm. ^{13}C NMR (125 MHz, 296 K, $CDCl_3$, 0.014 M): δ 21.20, 24.81, 25.19, 25.93, 27.29, 31.13, 34.59, 34.77, 35.51, 36.02, 36.89, 37.02, 42.01, 55.15, 63.72, 64.44, 68.79, 70.58, 71.72, 100.24, 110.87, 114.27, 120.69, 129.12, 144.61, 159.61, 169.61, 171.43 ppm. HR-FAB-MS *m*/*z*: 505.2813 (MH^+, calcd for $C_{28}H_{41}O_8$, 505.2801).

4.3. Inhibition of specific binding of [^{3}H]PDBu to PKCδ C1 peptides

The binding of [^{3}H]PDBu to the PKCδ C1 peptides was evaluated by the procedure of Sharkey and Blumberg[43] with modifications as reported previously[44] with 50 mM Tris–maleate buffer (pH 7.4 at 4 °C), 14 or 40 nM PKCδ C1 peptide, 20 nM [^{3}H]PDBu (19.6 Ci/mmol), 50 μg/mL 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (Sigma), 3 mg/mL bovine γ-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 Microsoft Excel. The inhibition constant, K_i, was calculated by the method of Sharkey and Blumberg.[43] Although we used each PKC C1 peptide at 14 or 40 nM, the concentration of the properly folded peptide was estimated to be about 3–4 nM on the basis of B_{max} values of Scatchard analyses reported previously.[44] Therefore, the concentration of free PDBu would not have drastically varied over the dose–response curve.

4.4. Anti-proliferative activity against a panel of 39 human cancer cell lines

A panel of 39 human cancer cell lines established by Yamori et al.[46] according to the NCI method with modifications was employed, and cell growth inhibitory activity was measured as reported previously.[51] 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.[52] 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). Cell growth inhibition (% growth) by each concentration of compound (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) was calculated as $100[(T - T_0)/(C - T_0)]$ using the average of duplicate points. By processing these values, each GI_{50} value, defined as $100[(T - T_0)/(C - T_0)] = 50$, was determined.

4.5. Determination of log*P* of the aplog-1 derivatives using HPLC

The HPLC method used to determine log*P* values of the aplog-1 derivatives was from the 'OECD GUIDELINE FOR TESTING OF CHEMICALS'.[39] In brief, 35% or 75% MeCN/H_2O was used for the mobile phase on a reverse-phase column AQ-311 (100 × 6.0 mm). For preparing a calibration curve, two sets of six compounds, 2-butanone (log*P*: 0.3), 4-acetylpyridine (0.5), acetanilide (1.0), phenol (1.5), acetophenone (1.7) and anisole (2.1), or phenol (1.5), anisole (2.1), 1-naphthol (2.7), cumene (3.7), *n*-butylbenzene (4.6) and triphenylamine (5.7), were used as reference compounds. Dead time was measured using unretained formamide. To confirm the accuracy of this measurement, log*P* values of **2** and **3** were also measured by the conventional 1-octanol/water shake-flask method (OECD Test Guideline 107). The results gave quite similar log*P* values.

4.6. EBV-EA induction test

Human B-lymphoblastoid Raji cells (5×10^5/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 4 mM sodium *n*-butyrate (a synergist) and 10^{-7} or 10^{-6} M of each test compound.[47,48] Each test compound was added as 2 μL of a DMSO solution along with 2 μL of DMSO; the final DMSO concentration was 0.4%. After 48 h of incubation at 37 °C, smears were made from the cell suspension, and the EBV-EA-expressing cells were stained by a conventional indirect immunofluorescence technique with an NPC patient's serum (a gift from Kobe University) and FITC-labeled anti-human IgG (DAKO, Glostrup, Denmark) as reported previously. In each assay, at least 500 cells were counted and the proportion of the EA-positive cells was recorded. Cell viability exceeded 50% in most cases as shown in Supplementary data.

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Supplementary data

Supplementary data (these data include a full version of the results of the growth inhibition assay against 39 human cancer cell lines and the EBV-EA induction test) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.03.013.

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