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Synthesis and biological activities of the amide derivative of aplog-1, a simplified analog of aplysiatoxin with anti-proliferative and cytotoxic activities

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Synthesis and biological activities of the amide derivative of aplog-1, a simplified analog of aplysiatoxin with anti-proliferative and cytotoxic activities

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Aplog-1 is a simplified analog of the tumorpromoting aplysiatoxin with anti-proliferative and cytotoxic activities against several cancer cell lines. Our recent findings have suggested that protein kinase Co (PKCo) could be one of the target proteins of aplog-1. In this study, we synthesized amideaplog-1 (3), in which the C-1 ester group was replaced with an amide group, to improve chemical stability in vivo. Unfortunately, 3 exhibited seventyfold weaker binding affinity to the C1B domain of PKCo than that of aplog-1, and negligible antiproliferative and cytotoxic activities even at 10^{-4} M. A conformational analysis and density functional theory calculations indicated that the stable conformation of 3 differed from that of aplog-1. Since 27-methyl and 27-methoxy derivatives (1, 2) without the ability to bind to PKC isozymes exhibited marked anti-proliferative and cytotoxic activities at 10⁻⁴ M, 3 may be an inactive control to identify the target proteins of aplogs.

Key words: aplysiatoxin; anti-proliferative; protein kinase C; tumor promoter

Aplysiatoxin (ATX) is a potent tumor promoter that has been isolated from the digestive gland of the sea hare *Stylocheilus longicauda*.¹⁾ ATX strongly binds to and activates protein kinase C (PKC) isozymes, as well as 12-*O*-tetradecanoylpholbol 13-acetate (TPA) and teleocidin B-4.^{2,3)} Since PKC is a family of serine/threonine kinases that play pivotal roles in cellular signal transduction including proliferation, differentiation, and apoptosis,^{4–6)} tumor promoters may become therapeutic agents for intractable diseases such as cancer, Alzheimer's disease (AD), and acquired immune deficiency syndrome (AIDS). However, difficulties are associated with their application to therapeutic uses due to their potent tumor-promoting and inflammatory activities.^{7,8)}

Bryostatin-1 (bryo-1)⁹⁾ which was isolated from the marine bryozoan Bugula neritina, is a unique PKC activator that does not exhibit tumor-promoting or inflammatory activity. Bryo-1 has been reported to have significant anti-cancer and anti-proliferative activities, and these have been attributed to activation of the PKC δ isozyme,^{10–12}) which plays a tumor suppressor role and is involved in apoptosis.¹³⁻¹⁵ Bryo-1 is also expected to become a therapeutic candidate for AD^{16} and AIDS.¹⁷⁾ Despite its potential as a new medicinal lead, further studies on its mode of action and structural optimization have been hampered due to its limited availability from natural sources and synthetic complexity. A functional oriented synthesis of the simplified analogs of bryo-1 was recently conducted address these issues.^{18,19)}

As an alternative approach, we developed aplog-1, a simplified analog of ATX.²⁰⁾ Aplog-1, supplied in only 27 steps via standard reactions, was not tumor-promoting or inflammatory, but was anti-proliferative even though it has the skeleton of tumor-promoting ATX. The antiproliferative activity of aplog-1 against several cancer cell lines was previously shown to be similar to that of bryo-1.20) Furthermore, aplog-1 behaved in a similar manner to bryo-1 rather than TPA for the translocation of GFP-tagged PKC8 using CHO-K1 cells; aplog-1 as well as bryo-1 translocated GFP-tagged PKCS to the nuclear membrane and perinuclear region rather than to the plasma membrane, unlike TPA.²⁰⁾ To examine the contribution of PKCS to the anti-proliferative activity of aplog-1, we recently synthesized 27-methyl and 27methoxy derivatives (1, 2) that lacked the ability to bind to PKC δ and evaluated their anti-proliferative activities against 39 human cancer cell lines.²¹⁾ Compounds 1 and 2 only exhibited weak anti-proliferative activities against all the human cancer cell lines tested,²¹⁾ suggesting that the activation of PKCS was involved in growth inhibitory activities against several cancer cell lines that were at least sensitive to aplog-1.

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The next step is to evaluate and improve the anti-proliferative activity of aplog-1 in vivo. When a bioactive compound is applied to in vivo studies, its chemical stability is critical to the drug efficacy. Compounds with ester groups are generally susceptible to hydrolysis by esterases in vivo. For example, epothilone B²²⁾ showed cytotoxicity by inhibiting the depolymerization of microtubules,²³⁾ but its efficacy was limited in vivo due to its ester group. Ixabepilone is a derivative of epothilone B, in which the ester group is replaced with an amide group. Ixabepilone was approved as an anti-breast cancer agent because of its potent cytotoxicity in vivo.^{24,25)} Aplog-1 has two ester linkages at C-1 and C-24 in the macrolactone ring. We previously reported that benzolactams, the simplified analogs of teleocidins, bound to PKC isozymes more strongly than their lactone counterparts, benzolactones, $^{26,27)}$ which prompted us to develop a new derivative of aplog-1 with a more stable amide linkage. We herein described the synthesis of amide-aplog-1 (3), in which the ester group at C-1 of aplog-1 was replaced with an amide group, along with several of its biological activities such as PKCS binding, in vitro tumorpromoting, anti-proliferative, and cytotoxic activities. We chose to replace the C-1 ester group because this site and benzolactones (Fig. 1) share a common hydrophilic substructure, -C(=O)-O-CHR-CH₂OH, with an inversed stereochemistry, and the amide proton of 3 was expected to be at a spatial position similar to that of the hydrogen atom in the hemiacetal 3-OH group of ATX.

Results and discussion

The synthesis of **3** was accomplished in a convergent approach from a spiroketal $(6)^{20}$ and carboxylic acid (**5**), as shown in Scheme 1. Compound **4** was prepared from *Z*-D-Asp(O*t*Bu)-OH as reported previously,²⁸⁾ and the subsequent removal of the *t*-butyl ester with trifluoroacetic acid (TFA) afforded the carboxylic acid (**5**). The spiroketal **6** was synthesized from *m*-hydroxycinnamic acid in a similar manner to the synthesis of aplog-1.²⁰⁾

Yamaguchi's esterification²⁹⁾ of **6** with **5** provided **7**. Oxidative cleavage of the olefin group, followed by esterification with *N*-hydroxysuccinimide, gave an activated ester. Immediately after deprotection of the benzyl (Bn) and carbobenzyloxy (Cbz) groups by catalytic hydrogenation, lactamization occurred to give **3** in a single step. The yield (23%) in two steps) was not good because the Bn group resisted the catalytic hydrogenation.

We initially evaluated the ability of 3 to bind to PKC δ using a synthetic PKC δ -C1B peptide (δ -C1B),³⁰ which is a major binding site and plays a predominant role in the activation of PKC δ by PKC ligands such as bryo-1, ATX, and TPA. The concentration required to cause 50% inhibition (IC₅₀) of [³H]phorbol 12,13-dibutyrate (PDBu) was measured using a competitive binding assay.^{31,32)} Affinity for δ -C1B was expressed as a K_i value calculated from the IC₅₀ value of **3** and the K_d value of [³H]PDBu, as reported by Sharkey and Blumberg.³¹⁾ Table 1 lists the K_i value of **3** for δ -C1B along with those of aplog-1 and its 27-methyl and 27-methoxy derivatives (1 and 2). As previously reported,^{21,33}) the stereochemistry at position 27 was critical for PKC binding, and the free hydroxyl group at position 27 was indispensable. Although the amide analog 3 fulfilled these structural requirements at position 27 for PKCS binding, the affinity of 3 ($K_i = 520$ nM) was approximately two orders of magnitude weaker than that of aplog-1 ($K_i = 7.4$ nM).

Regarding PKC activators such as 1,2-diacyl-*sn*-glycerol (DAG) and indolactam-V (Fig. 1), the replacement of an ester group with an amide group and *vice versa* significantly affected their abilities to bind to PKC isozymes by changing their conformation. The replacement of either the *sn*-1 or *sn*-2 ester group of DAG, an endogenous second messenger, with an amide group markedly reduced its ability to activate PKC isozymes.³⁴⁾ The conformationally constrained analogs of DAG (DAG-lactones) with similar modifications developed by Marquez and colleagues also showed approximately ninety-fold to two hundred-fold lower binding affinities for PKC α than those of their ester counterparts.³⁵⁾ On the other hand, amide-to-ester



Fig. 1. Structures of bryostatin-1, aplysiatoxin, its simplified analogs (1-3), teleocidin-B4, indolactam-V, and its 8-membered analogs.



Scheme 1. (a) TFA, dichloromethane (95%); (b) 5, 2,4,6-trichlorobenzoyl chloride, Et₃ N, DMAP, toluene (80%); (c) KMnO₄, NaIO₄, *t*-BuOH, pH 7 buffer (74%); (d) *N*-Hydroxysuccinimide, DCC, MeCN; (e) 20% Pd(OH)₂-C, MeOH (23% in two steps).

Table 1. K_i values for the inhibition of [³H]PDBu-binding by aplog-1, 1, 2, and 3.

	$K_{\rm i}$ (nM)			
	Aplog-1 ^a	1 ^b	2 ^b	3
δ–C1B peptide	7.4	>2,500	>2,500	520 (19)

^aCited from Ref. 20.

^bCited from Ref. 21.

^cValues in parentheses represent the standard deviation from triplicate experiments.

modification in the indolactam-V analogs gave opposite results. A lactone analog of the nine-membered indolactam-V, the core structure of teleocidins, showed a different conformational preference and was completely inactive,³⁶⁾ while a lactone analog of the eight-membered benzolactam-V8 took a ring conformation similar to that of benzolactam-V8 with *cis*-amide, and exhibited twenty to ninety-fold lower affinity for PKC C1 domains than the corresponding benzolactam-V8 analog.²⁷⁾ Therefore, both ester and amide analogs could bind to PKC isozymes if it adopted an appropriate conformation.

The strong binding ability of ATX to PKC isozymes was attributed to the rigid conformation of the macrocyclic ring, which included a hydrophilic pharmacophore,³³⁾ and an NMR analysis of aplog-1²⁰⁾ indicated that its preferred macrocyclic ring conformation was similar to that of 3-deoxy-debromo-ATX (e.g., $J_{2,3} = 10.8$ and 2.8 Hz for aplog-1; 11 and 3 Hz for 3deoxy-debromo-ATX).³³⁾ In the case of 3, an NOE correlation between NH-26 and H-11 was observed in a 2D NOESY NMR experiment in CDCl₃ (Supplemental Fig. 2), suggesting that the conformation of 3 differed to those of ATX³³) and aplog-1, in which the distance between these atoms could be more than 4 Å. Since ATX and aplogs were involved in the hydrophobic environment when bound to PKCδ-C1B in the presence of phosphatidylserine, the conformation of 3 in CDCl₃ would reflect a conformation in a ternary complex of 3, PKCδ-C1B, and phosphatidylserine membrane. In order to clarify the effects of conformational changes in 3 on decreases in PKCS binding, we performed a conformational search followed by density functional theory (DFT) calculations to estimate the relative stabilities of possible conformers.

A set of possible conformations of the macrolactone core structure of 3 was generated by the simulated annealing method, and we chose three possible conformers: the global-minimum with a trans-amide, an ATX-like conformation with a trans-amide, and a conformation with a cis-amide because the active conformation of indolactam compounds is known to be a cis-amide form.^{37,38)} Side chains at C-11 were attached to them and dihedral angles in the side chain were manually rotated to search for an energetically stable orientation, in which the 18-OH group is involved in intramolecular hydrogen bonding as suggested by its sharp ¹H NMR signal in CDCl₃. The candidate structures were pre-optimized using the molecular mechanics method with the MMFF94s force field, and the final DFT geometry optimizations were then performed at the ω B97X-D/6–31G* level of theory.³⁹⁾ Fig. 2 shows the resulting three possible conformers of 3 (A-C) and their relative ω B97X-D/6–31G* energies.

As described above, conformers A and B had a trans-amide bond, while C had a cis one. Conformer B resembles a stable conformation of ATX.33) In conformers A and B, NH-26 and H-11 were spatially close (2.06 Å in A, 2.61 Å in B), which is consistent with the NOE correlation. Furthermore, the sharp and downfield-shifted ¹H NMR signal of NH-26 (7.45 ppm) in CDCl₃ could be explained by intramolecular hydrogen bonding between N-H and an oxygen atom in these conformers. DFT calculations showed that conformer A had the lowest energy, and differences in energies between A-B and A-C were 1.365 and 13.259 kcal mol^{-1} , respectively. These results suggested that 3 existed as conformer A in CDCl₃. Since conformer B resembled but significantly diverged from the stable conformation of ATX due to the rotation of amide plane by 45° (based on N-C-1-C-2-C-3 dihedral angle), the difference in energies between conformer A and the ATX-like active conformation would be more than 1.365 kcal mol⁻¹. Thus, a seventy-fold decrease in the binding ability of 3 from aplog-1, which is equivalent to a 2.34 kcal mol⁻¹ change in free energy, could be ascribable mainly to the change in the conformational preference.

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Fig. 2. Cross-eyed stereo views of possible conformations of 3 (A–C) and their relative energies at the ω B97X-D/6–31G* level. Note: Dashed lines represent hydrogen bonding.

We then evaluated the tumor-promoting activity of **3** *in vitro* by testing the induction of Epstein-Barr virus early antigen (EBV-EA) production.^{40,41} EBV is activated by treating cells with tumor promoters such as TPA in order to produce EA, which can be detected by an indirect immunofluorescence technique. Aplog-1 and its C-27 derivatives (**1** and **2**) induced EA production more weakly than the potent tumor promoter TPA. The ability of **3** to induce EA production was weaker than those of **1** and **2** without the ability to bind to PKC isozymes^{21,33} (Fig. 3). Although EA production is considered to be related to the activation of PKC isozymes,⁴² this result suggested that some part of the induction of EA caused by aplog-1 and its derivatives could be attributed to other mechanisms.

The anti-proliferative activity of **3** was evaluated with a panel of 39 human cancer cell lines established by Yamori and colleagues^{44,45)} The results for HBC-4 and NCI-H460 were shown in Fig. 4 as typical



Fig. 3. EBV-EA production induced by TPA, aplog-1, 1, 2, and 3. Notes: The percentages of EA-positive cells are shown. Sodium *n*-butyrate (4 mM) was added to all samples to enhance the sensitivity of Raji cells. Only 0.1% of cells were positive for EA at 4 mM sodium *n*-butyrate. The final concentration of DMSO was 0.4%. Cell viability exceeded 60%. Error bars show the standard error of the mean (n = 3). ^aCited from Ref. 43. ^bCited from Ref. 21.

examples, because aplog-1 showed stronger growth inhibitory activity against these cell lines. Similar results were observed in MDA-MB-231, SNB-78, HCC2998, A549, LOX-IMVI, and St-4 cell lines (Supplemental Table 1). Cell growth was estimated by the sulforhodamine B assay and expressed as a percentage of the control without **3**. As expected from the weak binding affinity for PKC δ , **3** hardly inhibited their growth at low concentrations (<10⁻⁵ M) as well as **1** and **2**. Furthermore, **3** exhibited weak cytotoxicity even at 10⁻⁴ M, whereas aplog-1, **1**, and **2** induced cell death regardless of their binding affinities for PKC δ at the same concentration, 10⁻⁴ M. This result suggests that other targets may be involved in the cytotoxicity of aplogs at 10⁻⁴ M.

In summary, we synthesized a new amide derivative of aplog-1 (3) in order to improve the stability of aplog-1 against esterases and pH changes in vivo. Although the very weak binding of 3 to the C1B domain of PKCS could be detected, 3 showed weak anti-proliferative activity against the 39 cancer cell lines examined, even at 10^{-4} M (Supplemental Table 1), and hardly induced the production of EBV-EA. In contrast, aplog derivatives without binding affinity to PKC δ and its isozymes $(1, 2)^{21}$ retained these activities at 10⁻⁴ M. These results suggest that some of the antiproliferative and cytotoxic activities of aplogs were attributed to unidentified targets other than PKC isozymes. A conformational analysis and DFT calculations indicated that the stable conformation of 3 differed from that of aplog-1. This conformational change could prevent 3 from binding to PKCS and other target proteins responsible for anti-proliferative activity and EBV-EA induction. Given that conformational changes in PKC ligands could affect not only biological activities, but also cellular targets, these effects must be taken into account to successfully derivatize the skeletons of aplogs in the future.

We recently developed aplog-based molecular probes and are currently attempting to identify its target proteins other than PKC isozymes. As described above,

4



Fig. 4. Effects of aplog-1, 1, 2, and 3 on the growth of human cancer cell lines: HBC-4 (breast) and NCI-H460 (non-small cell lung). Note: Cell growth was expressed as a percentage of the control (media only). The results were presented as the average of duplicate points.

27-methyl and 27-methoxy derivatives (1, 2) did not exhibit the ability to bind to PKC isozymes, but had marked anti-proliferative and cytotoxic activities at 10^{-4} M, indicating that 1 and 2 were not suitable as inactive controls for the target analysis. Since 3 exhibited weak binding affinity for PKC, but little cytotoxic activity even at 10^{-4} M, it would be suitable as an inactive control for the identification of targets other than PKC isozymes.

Experimental

The following spectroscopic and analytical instruments were used: digital polarimeter, DIP-1000 (Jasco, Tokyo, Japan); ¹H and ¹³C NMR, Avance III 400, Avance III 500, and Avance II 800 (reference TMS, Bruker, Germany); HPLC, model 600E with a model 2487 UV detector (Waters, Tokyo, Japan); and HR-FAB-MS, JMS-600H (JEOL, Tokyo, Japan), and JMS-700 (JEOL, Tokyo, Japan). HPLC was carried out on a YMC-packed ODS-A AA12S05-2510WT (Yamamura Chemical Laboratory, Kyoto, Japan). Wakogel[®] C-200 (silica gel, Wako Pure Chemical Laboratory, Osaka, Japan) was used for column chromatography. [³H] PDBu (18.7 Ci/mmol) was custom-synthesized by Perkin-Elmer Life Sciences Research Products (Boston, MA). The PKCo C1B peptide was synthesized as reported previously.³⁰⁾ All other chemicals and reagents were purchased from chemical companies and used without further purification.

Synthesis of 3. Compound 4 was prepared as reported previously.²⁸⁾ $[\alpha]_D + 13.2^\circ$ (c = 0.44, MeOH, 10.9 °C). TFA (0.9 mL) was added to a solution of 4 (35.8 mg, 89.7 µmol) in dichloromethane (0.9 mL) at 0 °C. After 4 h of stirring at room temperature, the reaction mixture was concentrated in vacuo to afford 5 (29.3 mg, 85.4 μ mol, 95%) as a clear oil. ¹H NMR (500 MHz, CDCl₃, 0.015 M) ppm: δ 2.70 (2H, d, J = 5.9 Hz), 3.55–3.59 (2H, m), 4.23 (1H, br. s), 4.50 (2H, s), 5.09 (2H, s), 5.41 (1H, br. d, J = 8.1 Hz), 7.27–7.37 (10H, m); ¹³C NMR (125 MHz, CDCl₃, 0.015 M) ppm: & 35.8, 47.8, 66.9, 70.8, 73.4, 127.7 (2C), 127.9 (2C), 128.1, 128.2, 128.5 (2C), 128.6 (2C), 136.4, 137.7, 155.9, 174.1; HR-EI-MS m/z: 343.1427 $([M]^+$ Calcd. for C₁₉H₂₁NO₅ 343.1420) $[\alpha]_D$ + 16.1° $(c = 0.15, \text{ CHCl}_3, 10.3 \text{ °C}).$

2,4,6-trichlorobenzoyl chloride (28.0 µL, 179 µmol, 1.7 equiv.) was added to a solution of 5 (51.1 mg, 149 µmol, 1.4 equiv.) and Et₃ N (24.9 µL, 179 µmol, 1.7 equiv.) in toluene (1.1 mL) at room temperature. After 3 h of stirring at room temperature, the supernatant of the suspension was added to a solution of 6^{20} (50.0 mg, 105 µmol) and DMAP (27.0 mg, 221 µmol, 2.1 equiv.) in toluene (1.1 mL) at room temperature. The mixture was stirred at 50 °C for 2 h and then poured into H_2O (5.0 mL). The mixture was extracted with EtOAc (5 mL x 3). The combined organic layers were washed with brine, dried over Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, $10\% \rightarrow 20\%$ EtOAc/hexane) to afford 7 (67.8 mg, 84.4 µmol, 80%) as a clear oil. ¹H NMR (500 MHz, CDCl₃, 0.0027 M) ppm: δ 0.86 (3H, s), 0.95 (3H, s), 1.33-1.49 (9H, m), 1.57-1.66 (4H, m), 2.19 (1H, br. d, J = 15.5 Hz), 2.26 (1H, m), 2.35 (1H, m), 2.57 (2H, t, J = 7.8 Hz), 2.61 (1H, dd, J = 15.8, 8.0 Hz), 2.69 (1H, dd, J = 5.8 Hz), 3.49-3.60 (4H, m), 4.16-4.21 (2H, m), 4.47-4.52 (2H, m), 4.95–5.02 (2H, m), 5.04 (2H, s), 5.06–5.09 (3H, m), 5.56 (1H, br. d, J = 8.8 Hz), 5.80 (1H, m), 6.78– 6.82 (3H, m), 7.18 (1H, t, J = 7.8 Hz), 7.27–7.45 (14H, m); ¹³C NMR (125 MHz, CDCl₃, 0.0027 M) ppm: δ 22.0, 24.8, 25.3, 26.1, 27.2, 31.3, 33.7, 34.5, 35.6, 36.0, 36.5, 36.8, 40.9, 48.0, 63.9, 66.7, 68.4, 70.0, 71.1, 71.7, 73.3, 100.1, 111.8, 115.2, 116.6, 121.2, 127.5 (2C), 127.6 (2C), 127.8, 127.9 (2C), 128.1, 128.1, 128.4 (2C), 128.5 (2C), 128.6 (2C), 129.2, 135.4, 136.6, 137.3, 137.9, 144.6, 155.8, 158.9, 171.3; HR-FAB-MS (matrix, *m*-nitrobenzyl alcohol) *m/z*: 826.4324 $([M + Na]^{+})$ Calcd. for C₅₀H₆₁NO₈Na 826.4295) $[\alpha]_{\rm D}$ + 19.7° (c = 0.14, CHCl₃, 28.9 °C).

KMnO₄ (13.1 mg, 83.2 µmol, 1 equiv.) was added to a suspension of NaIO₄ (143 mg, 0.666 mmol, 8 equiv.) in pH 7.2 phosphate buffer (6.9 mL) in one portion. After 5 min of stirring at room temperature under an Ar atmosphere, the mixture was added to a solution of 7 (66.8 mg, 83.2 µmol) in *t*-BuOH (6.9 mL). The reaction mixture was stirred at room temperature for 1 h, and the reaction was quenched with Na₂S₂O₃ (39.5 mg). The organic layer was separated, and the aqueous layer was extracted with EtOAc (20 mL × 3). The combined organic layer was washed with brine, dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, 20% EtOAc/hexane containing 1% AcOH) to afford a carboxylic acid (50.8 mg, 61.9 μ mol, 74%) as a clear oil.

N,N'-dicyclohexylcarbodiimide (7.3 mg, 35.4 µmol, 1.5 equiv.) in MeCN (0.20 mL) was added to a solution of the carboxylic acid (19.4 mg, 23.6 µmol) and Nhydroxysuccinimide (5.4 mg, 47.2 µmol, 2 equiv.) in MeCN (0.20 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 10 h, then concentrated in vacuo. The residue was purified by column chromatography (silica gel, $5\% \rightarrow 10\% \rightarrow 20\% \rightarrow 30\%$ EtOAc/hexane) to afford a crude activated ester (28.1 mg). A solution of the activated ester (26.7 mg) in MeOH (1.0 mL) was added to 20% Pd(OH)₂-C (wet support, Aldrich) (7.8 mg) in a flask at room temperature. The mixture was vigorously stirred under a H₂ atmosphere at room temperature for 4.5 h. The mixture was filtered, and the filtrate was concentrated in vacuo. The residue was dissolved in MeOH (0.6 mL), and again added to 20% Pd (OH)₂-C (wet support, Aldrich) (5.9 mg) in a flask at room temperature. The mixture was vigorously stirred under a H_2 atmosphere at room temperature for 2.5 h. The mixture was filtered, and the filtrate was concentrated in vacuo. This procedure was repeated two times and a total of 38.5 mg (46.9 µmol) of the carboxylic acid was reacted. These residues were purified by HPLC (column, YMC-Pack ODS-A AA12S05-2510WT; solvent MeOH/H₂O = 75:25, flow rate 3.0 mL/min; pressure, 2100 psi; UV detector 254 nm; retention time, 20.1 min) to afford **3** (5.2 mg, 10.6 μ mol, 23%) as a clear oil. ¹H NMR (400 MHz, CDCl₃, 0.0036 M) ppm: δ 0.96 (3H, s, H₃₋₂₃), 1.00 (3H, s, H₃₋₂₂), 1.38–1.59 (9H, m, H₂₋₄, H₂₋₅, H-10a, H₂₋₁₂, H₂₋₁₃), 1.64 (2H, m, H₂₋₁₄), 1.71 (1H, dd, J = 15.5, 3.7 Hz, H-8 α), 1.79 (1H, m, H-10b), 2.28 $(1H, dd, J = 15.1, 1.1 Hz, H-2a), 2.46 (1H, m, H-8\beta),$ 2.55-2.65 (4H, m, H-2b, H₂₋₁₅, H-25a), 2.95 (1H, dd, J = 16.5, 10.7 Hz, H-25b), 3.70–3.87 (4H, m, H-3, H-26, H_{2-27}), 4.09 (1H, m, H-11), 4.45 (1H, dd, J = 7.6, 5.0 Hz, OH), 5.21 (1H, m, H-9), 6.27 (1H, s, Ph-OH), 6.65-6.74 (3H, m, H-17, H-19, H-21), 7.13 (1H, t, J = 7.8 Hz, H-20), 7.45 (1H, br. d, J = 4.7 Hz, NH); ¹³C NMR (125 MHz, CDCl₃, 0.0029 M) ppm: δ 21.5 (C-22), 24.2 (C-13), 25.6 (C-8), 25.9 (C-23), 27.8 (C-4), 30.2 (C-14), 34.2 (C-5 or 10 or 12), 34.4 (C-5 or 10 or 12), 34.7 (C-5 or 10 or 12), 35.3 (C-15), 36.3 (C-25), 37.3 (C-6), 43.8 (C-2), 51.6 (C-26), 63.9 (C-11), 64.5 (C-27), 68.5 (C-9), 71.3 (C-3), 101.3 (C-7), 112.8 (C-19), 115.1 (C-17), 120.6 (C-21), 129.4 (C-20), 144.4 (C-16), 156.3 (C-18), 170.3 (C-24), 173.4 (C-1); HR-FAB-MS (matrix, m-nitrobenzyl alcohol) m/ z: 490.2812 ($[M + H]^+$ Calcd. for $C_{27}H_{40}NO_7$ 490.2805) $[\alpha]_{D}$ + 38.9° (*c* = 0.074, CHCl₃, 9.6 °C).

Inhibition of specific binding of $[{}^{3}H]PDBu$ to the *PKC* δ -*C1B peptide*. The binding of $[{}^{3}H]PDBu$ to the δ -C1B peptide was evaluated by the procedure of Sharkey and Blumberg³¹) with modifications as reported previously³²) using 50 mM Tris-maleate buffer (pH 7.4 at 4 °C), 13.8 nM δ -C1B peptide, 20 nM $[{}^{3}H]$ PDBu (18.7 Ci/mmol), 50 µg/mL 1,2-dioleoyl-*sn*-glyce-ro-3-phospho-L-serine sodium salt (Sigma), 3 mg/mL bovine γ -globulin, and various concentrations of an inhibitor. Binding affinity was evaluated based on the

concentration required to inhibit the specific binding of $[{}^{3}\text{H}]\text{PDBu}$ by 50%, the IC₅₀, which was calculated by a computer program with a probit procedure.⁴⁶⁾ The inhibition constant, K_{i} , was calculated by the method of Sharkey and Blumberg.³¹⁾

EBV-EA induction test. Human B-lymphoblastoid Raji cells $(5 \times 10^{5}/\text{mL})$ were incubated at 37 °C under a 5% CO₂ atmosphere in 1 mL of RPMI 1640 medium (supplemented with 10% fetal bovine serum) with 4 mM sodium *n*-butyrate (a synergist) and 10, 100, or 1000 nM of each test compound. Each test compound was added as 2 µL of a DMSO solution (5, 50, and 500 μ M stock solution) along with 2 μ L of DMSO; the final DMSO concentration was 0.4%. After 48 h of incubation, smears were made from the cell suspension, and the EBV-EA-expressing cells were stained by a conventional indirect immunofluorescence technique with the serum of an NPC patient's (a gift from Kobe University, Japan) and FITC-labeled anti-human IgG (DAKO, Glostrup, Denmark) as reported previously.⁴¹⁾ At least 500 cells were counted in each assay and the proportion of EA-positive cells was recorded. Cell viability exceeded 60% in all experiments.

Measurements of cell growth inhibition. A panel of 39 human cancer cell lines established by Yamori and colleagues⁴⁴⁾ according to the NCI method with modifications was employed, and cell growth inhibitory activity was measured as reported previously.45) In brief, cells were plated on 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. Absorbance for the control well (C) and test well (T)was measured at 525 nm along with that for the test well at time 0 (T_0). Cell growth inhibition (% growth) by each concentration of the drug $(10^{-8}, 10^{-7}, 10^{-6}, 10^{-6})$ 10^{-5} , and 10^{-4} M) was calculated as $100[(T - T_0)/$ $(C - T_0)$] using the average of duplicate points.

Conformational search of 3. The generation of a conformer library of **3** by a simulated annealing method under a vacuum was performed using the GROMACS program⁴⁷⁾ (version 4.6.5) with a general AMBER force field.⁴⁸⁾ The side chain at C11 of **3** was replaced with a methyl group. All bonds were constrained using the LINCS algorithm. The annealing temperature was initially set to 1,500 K in order to surpass the *cis-trans* isomerization barrier in this system and the temperature was kept constant for 1 ps. The temperature was linearly dropped to 100 K over 1 ps and then to 0 K over 1 ps, and kept at the same temperature for 1 ps. This 5-ps cycle was repeated 200 times to give the conformer library.

Three possible conformers were selected from this library: the global-minimum with *trans*-amide, ATX-like conformation with *trans*-amide, and a conformation with *cis*-amide. The side chains at C-11 were attached to them, and dihedral angles in the side

chain were manually rotated to search for energetically stable orientation. The candidate structures were pre-optimized using the molecular mechanics method with the MMFF94s force field as implemented in Avogadro⁴⁹⁾ (version 1.1.1) and then optimized using the DFT method at the level of ω B97X-D/6–31G*³⁹⁾ employing Gaussian 09.⁵⁰⁾ The obtained geometries were characterized as minimum structures on the basis of their harmonic vibrational frequencies and number of imaginary frequencies.

Supplemental material

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.1002452.

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