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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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Name

学位論文題目：
Title of Dissertation

Prediction of Binding Mode of Aplysiatoxin with Protein Kinase C and Development of a Synthetically-accessible Aplysiatoxin Analog
(アプリシアトキシンとプロテインキナーゼＣとの結合様式の予測と合成が容易なアプリシアトキシンアナログの開発)

学位論文要約：
Dissertation Summary

General introduction

Some natural products show therapeutic effect against a few intractable diseases and thus have been considered to be a promising source of therapeutic agents. However, in many cases, natural products themselves are difficult to use as therapeutic agents due to their structural complexity and multi-targeting nature. To overcome these problems, structural modification of natural products is considered to be an effective strategy.

Naturally-occurring tumor promoters, such as 12-*O*-tetradecanoylphorbol 13-acetate, teleocidin B4, and aplysiatoxin (ATX) (Figure 1), are natural products that show pleiotropic therapeutic effects against a few intractable diseases in addition to strong tumor-promoting and pro-inflammatory activities. The main cellular target of tumor promoters is protein kinase C (PKC) that plays important roles in many intracellular signal transduction involved in proliferation, differentiation, and apoptosis.[1–3] Like 1,2-diacy-*sn*-glycerol (DAG, Figure 1) that is an endogenous second messenger, tumor promoters bind to tandem C1 domains of conventional PKC (α, βI, βII, γ) and novel PKC (δ, ε, η, θ) isozymes, resulting in translocation of these isozymes to the cellular membrane fraction to lead their conformational change and activation (Figure 2).[2,3] Although some tumor promoters show cytotoxicity against several tumor cells, their therapeutic application is difficult because of their tumor-promoting and pro-inflammatory activities.

ATX and debromoaplysiatoixn (DAT, Figure 1), potent tumor promoters isolated from cyanobacteria and sea hare, are composed of a 12-membered bis-macrolactone ring and a side chain containing an aromatic ring. In terms of receptor recognition and conformational control, the structure of ATX can be divided into three regions. The recognition domain (RD) at positions 1 and 27–31 contains two ester groups and a hydroxyl group that could play some roles in the receptor recognition in a manner similar to those of DAG.[4] The conformation-controlling unit (CCU) from position 2 to 11 contains a [6,6]-spiroketal moiety and is thought to keep RD in its active conformation.[4] Therefore, ATX can be viewed as a conformation-constrained analog of flexible DAG, whereas the direction of ester linkages at position 27 of ATX and *sn*-1 position of DAG are opposite. The side chain at position 11 may play roles similar to those of hydrophobic part of naturally-occurring tumor promoters such as phorbol esters and teleocidins (Figure 1).[5]

In addition to tumor-promoting and pro-inflammatory activities, ATX and DAT also show significant anti-proliferative activity *in vitro*.[6–8] On the other hand, their simplified analogs, 10-Me-aplog-1 and 10,12-diMe-aplog-1 (Figure 1), show significant anti-proliferative activity against several human cancer cell

lines comparable to DAT, but exhibit little tumor-promoting and pro-inflammatory activities.[8,9] Thus, these analogs are promising as a potential chemotherapeutic agent. However, a more synthetically-accessible ATX analog is needed because synthesis of 10-Me-aplog-1 and 10,12-diMe-aplog-1 still requires at least 23 steps in a longest linear sequence.[9,10] Thus, the author attempts to develop a synthetically-accessible ATX analog with further simplification.

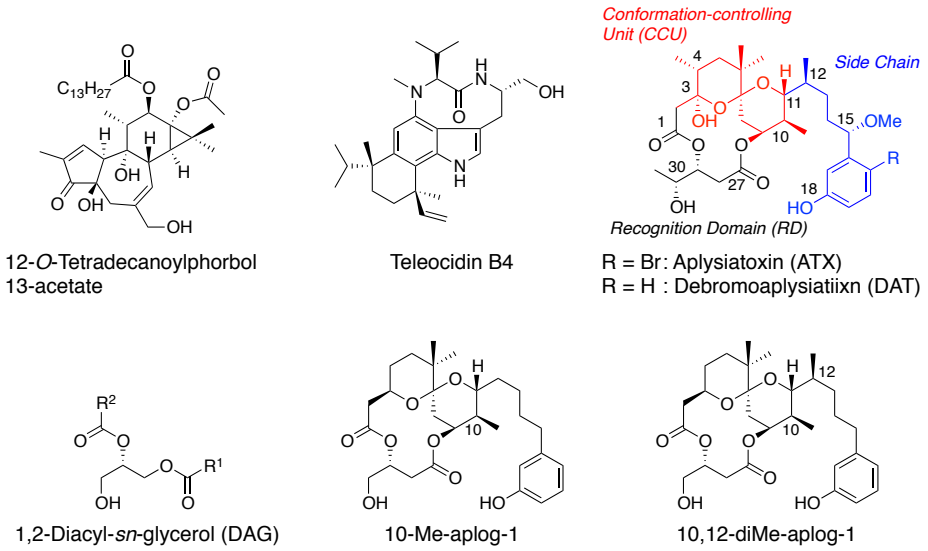

Figure 1 Structures of tumor promoters, 1,2-diacyl-*sn*-glycerol (DAG) and simplified analogs of aplysiatoxins.

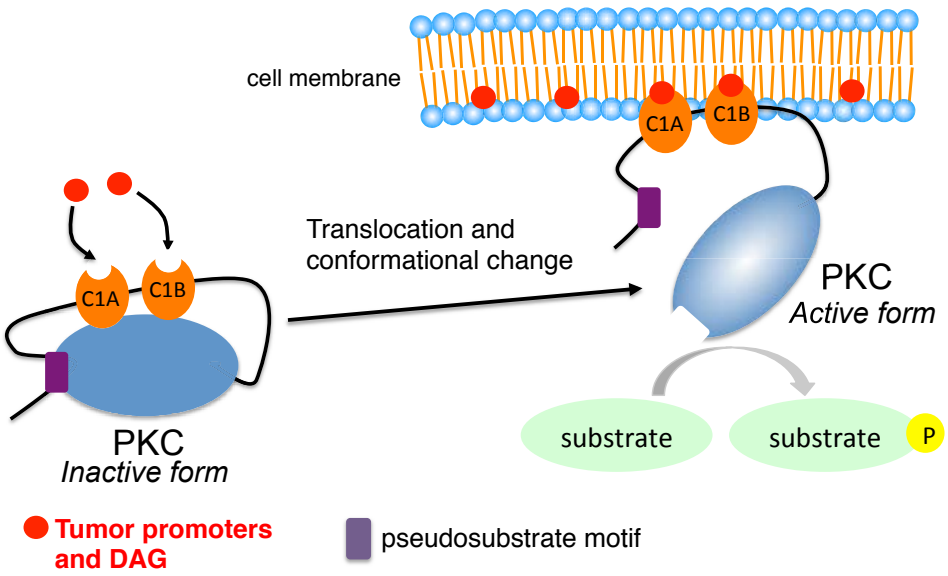

Figure 2 Activation mechanism of protein kinase C isozyme.

Prediction of binding mode of ATX with PKC through molecular simulation and structure–activity study on simplified analogs of the receptor-recognition domain

At first, the author predicted the binding mode of ATX with PKCδ C1B domain (δ-C1B). To rationally design ATX analog as anti-cancer lead compounds, knowledge about detailed interaction of ligand and target protein is needed. Kishi *et al.* reported structure–activity studies of ATX and comparisons of pharmacophoric elements between ATX and other PKC ligands.[4,5] However, a precise binding mode of ATX with the PKC C1 domains had not yet been proposed. Although X-ray crystallography and NMR-based methods are 'gold standards' for analysis of protein–ligand interaction and binding mode, it is difficult to apply these methods to the PKC ligand/C1 domain system because holo-C1 domain exists as a ternary complex of protein/ligand/phospholipid bilayer membrane. To overcome this experimental limitation, the author carried out molecular docking simulation of ATX with PKCδ C1B domain, molecular dynamics simulation (MD) of ATX/δ-C1B complex in phospholipid membrane environment, and structure–activity study using simple acyclic analogs with the receptor recognition domain of ATX.

The docking simulation provided an initial binding model where a carbonyl group at position 27, a hydroxyl group at position 30, and a phenolic hydroxyl group at position 20 of ATX were involved in intermolecular hydrogen bondings with the PKCδ C1B domain (Figure 3A). Subsequently, to refine the binding model from the docking simulation, the author carried out molecular dynamics (MD) simulation of ATX/δ-C1B complex in phospholipid membrane environment. During the 10 ns of the production run, root mean square deviation (RMSD) of backbone atoms of the protein and all atoms of the ligand from the initial structure remained at values below 2 and 1 Å, respectively, suggesting adequacy of the stability of the model.

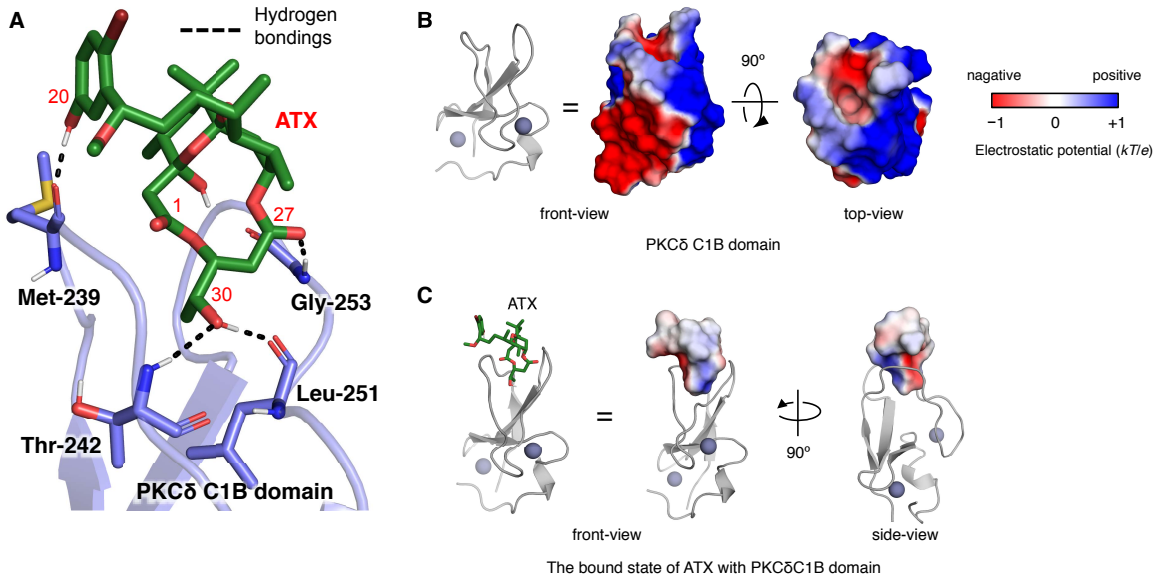

Figure 3 (A) Predicted binding model of ATX with PKCδ C1B domain through docking and MD simulations. Black dashed lines represent hydrogen bondings. Stick model colored green or purple (carbon), red (oxygen), blue (nitrogen), and yellow (sulfur). (B, C) Molecular electrostatic potential surface representation of the PKCδ C1B domain and ATX. (B) Crystal structure of the PKCδ C1B domain (PDB code: 1PTR).[11] (C) ATX in the bound state. Surfaces with negative, neutral, and positive potentials are shown in red, white, and blue, respectively.

The MD simulation provided clear insights into the role of the phenolic hydroxy group at position 20, the carbonyl group at position 27, and the hydroxy groups at position 30 of ATX in the PKC binding. However, the ester group at position 1 was not involved in any intermolecular hydrogen bond with the receptor and its role remained unclear, despite its presence in three major classes of PKC activators: aplysiatoxins, DAG, and bryostatins (Figure 4). In order to predict the role of the ester group at position 1, the author focused on molecular electrostatic potentials of ATX and the receptor. Electrostatic potential complementarity as well as shape complementarity is a key determinant of molecular recognition. A ligand binding cleft of the PKCδ C1B domain (PDB code: 1PTR)[11] was characterized by a marked positive potential of the back wall and the bottom as well as neutral to negative potential of the left frontal part (Figure 3B). As shown in Figure 3C, ATX had an electrostatic potential surface complementary to the binding cleft. The polarization of the ester group at position 1 of ATX could contribute to this complementary electrostatic potential.

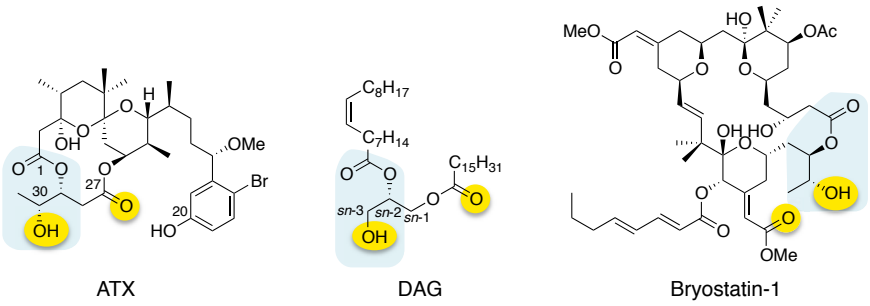

Figure 4 Structures and pharmacophoric elements of ATX, DAG, and bryostatin-1.

Although the effects of modification of the phenolic hydroxy group at position 20 and the hydroxy group at position 30 of ATX on the PKC binding have been studied previously,[4,5] the role of the two carbonyl groups in the recognition domain (RD) has not been experimentally examined. Thus, the author synthesized three acyclic analogs (**1**–**3**, Figure 5) and evaluated their ability to bind to the PKCδ C1B domain using [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) displacement assay.[12] In order to avoid possible conformational changes in the macrocyclic ring caused by these modifications, the author selected a simple acyclic analog of RD of ATX (**1**) as a reference compound because Kishi *et al.* reported that the acyclic analog of RD of ATX was capable of activating PKC.[4] The affinity of **3** lacking the carbonyl group at position 1 for δ-C1B (binding inhibition constant (K_i), 2900 nM) was approximately eight times lower than that of **1** (K_i, 370 nM). On the other hand, **2** lacking the ester group at position 27 barely bound to δ-C1B (K_i, >20,000 nM). These results suggest that the carbonyl group at position 27 was essential for receptor-recognition and the carbonyl group at position 1 also contribute to receptor recognition, which are consistent with ATX binding model from the docking simulation, the MD simulation, and the molecular electrostatic potential calculation.

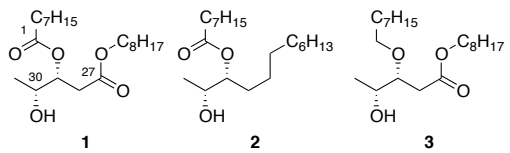

Figure 5 Structures of acyclic analogs of the recognition domain (RD) of ATX (**1**–**3**).

Synthesis, conformation, and biological activities of a des-A-ring analog of 18-deoxy-aplog-1 as a synthetically-accessible ATX analog

Subsequently, the author attempted to develop a synthetically-accessible ATX analog. The predicted binding model of ATX with PKCδ C1B domain revealed that the spiroketal moiety in CCU of ATX is not involved in hydrogen bondings with the PKCδ C1B domain. Thus, to achieve further simplification and shortening of the synthetic steps, the author focused on a spiroketal moiety in ATX. The spiroketal moiety in CCU is considered to play a pivotal role in controlling the conformation of RD and provide bulkiness to cover a binding cleft in the receptor. From the predicted binding model of ATX with the PKCδ C1B domain, the author surmised that the B-ring is more important for PKC binding than A-ring. Therefore, the author designed a des-A-ring analog (**13**, Figure 6) of 18-deoxy-aplog-1 (Figure 6)[13] as a synthetically-accessible analog.

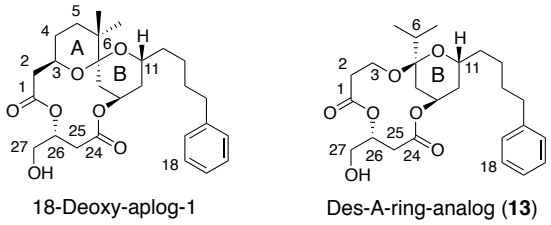

Figure 6 Structures of simplified analogs of ATX.

First, the author synthesized **13** and performed conformational analysis of **13** through NMR experiments and computational conformational search. Synthesis of **13** was achieved in a longest linear sequence of 11 steps from a commercially available starting material, which is approximately half of that of 18-deoxy-aplog-1. Conformational analysis of **13** revealed that the conformation of RD of **13** retained that of 18-deoxy-aplog-1 and that conformation of RD of aplogs was hardly affected by the removal of the A-ring. However, the conformation of CCU in **13** is slightly different from that of 18-deoxy-aplog-1 with the spiro-ring (Figure 7).

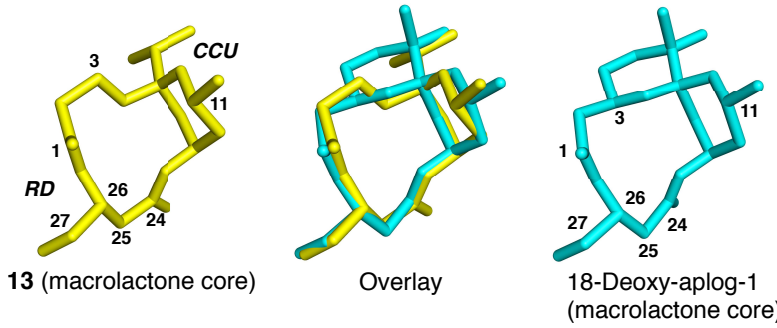

Figure 7 Three-dimensional (3D) structures of **13** and 18-deoxy-aplog-1. Left: a 3D structure of the macrolactone core in **13** (yellow). Right: a structure of the macrolactone core in 18-deoxy-aplog-1[13] (cyan) predicted from a crystal and solution structure of aplysiatoxins.[5,14] Center: overlay of both structures.

Next, the author evaluated the ability of **13** to bind to C1 domains of PKC isozymes using [^{3}H]PDBu displacement assay.[12] Table 1 lists the K_i values of **13** and 18-deoxy-aplog-1 for conventional and novel PKC isozymes. The affinity of **13** for C1B domains of novel PKCs was approximately five- to ten-times weaker than that of 18-deoxy-aplog-1, but the affinity of **13** for C1A of conventional PKC were almost equal to that of 18-deoxy-aplog-1. These results suggest that the conformation of CCU in aplogs might be important in the

recognition of C1B domains of novel PKCs. In addition, the hydrophobicity of PKC ligands is also an important factor for their binding abilities because the insertion to the phospholipid bilayer membrane is required for PKC ligands to form a stable complex with the protein. The hydrophobicity of **13** (log *P*, 4.3) is lower than that of 18-deoxy-aplog-1 (log *P*, 4.8).[15] This result implies that not only the ring conformation but also hydrophobicity at position 4 and/or 5 might be more important for the recognition of novel PKCs than that for conventional PKCs.

Table 1 Values of K_i for the inhibition of [^{3}H]PDBu binding by **13**, 18-deoxy-aplog-1

Compound	K_i (nM)						
	Conventional PKC			Novel PKC			
	α-C1A	β-C1A	γ-C1A	δ-C1B	ε-C1B	η-C1B	θ-C1B
13	100 (0)[b]	160 (10)	50 (10)	130 (20)	240 (0)	60 (0)	70 (0)
18-Deoxy-aplog-1[a]	120	140	80	9.8	37	12	8.1

[a]Cited from Ref. [13], [b]Standard deviation of at least two independent experiments.

Finally, the anti-proliferative activity of **13** against a panel of 39 human cancer cell lines (JFCR39)[16] was evaluated. Table 2 lists log GI_{50} values of **13** and 18-deoxy-aplog-1 for 11 cancer cell lines whose log GI_{50} values of 18-deoxy-aplog-1 were less than –5.00. Compound **13** did not show significant anti-proliferative activity against most of cell lines (log $GI_{50} > -5.0$), which can be attributed to the more-than-ten-fold reduction in the ability to bind to PKCδ. However, **13** exhibited significant anti-proliferative activity against NCI-H460 and MKN45 cells. In particular, the activity of **13** against NCI-H460 (log GI_{50}, −5.53) was comparable to that of 18-deoxy-aplog-1 (log GI_{50}, −5.83). This result suggests that conventional PKCs, rather than novel PKCs, were involved in the anti-proliferative activity against NCI-H460 and MKN45.

Table 2 Growth inhibitory activities of **13** and 18-deoxy-aplog-1 against several human cancer cell lines

		log GI_{50} (M)	
Cancer type	Cell line	**13**	18-Deoxy-aplog-1[a]
Breast	HBC-4	−4.98	−6.28
	MDA-MB-231	−4.90	−5.67
	BSY-1	−4.78	−5.17
CNS	SF-295	−4.94	−5.14
Colon	HCC2998	−4.86	−5.53
Lung	NCI-H460	−5.53	−5.83
	A549	−4.92	−5.49
Melanoma	LOX-IMVI	−4.99	−5.17
Stomach	St-4	−4.87	−6.05
	MKN45	−5.21	−6.09
Prostate	PC-3	−4.85	−5.26

[a]Cited from Ref. [13]

Summary and conclusion

In this study, the author attempted to develop a synthetically-accessible ATX analog with further simplification. The author first predicted binding mode of ATX with PKCδ C1B to rationally design ATX analog. The molecular docking simulation ATX with δ-C1B and molecular dynamics simulation of ATX/δ-C1B complex in phospholipid membrane environment suggest that the phenolic hydroxy group at position 20, the carbonyl group at position 27, and the hydroxy group at position 30 of ATX are involved in the intermolecular hydrogen bondings and that the ester groups at position 1 might contribute to generate molecular electrostatic potential complimentary to that of the receptor rather than hydrogen bonding. The predicted binding model of ATX with δ-C1B was consistent with structure–activity studies on aplysiatoxins reported previously and simple acyclic analogs of ATX (**1**–**3**) in this study, and provided a deeper understanding of receptor-recognition by ATX.

Based on this binding model, by simplifying the spiroketal scaffold of aplogs, the author designed the des-A-ring analog (**13**) of 18-deoxy-aplog-1 as a synthetically-accessible analog. Synthesis of **13** was achieved in a longest linear sequence of 11 steps from a commercially available starting material, which is approximately half of that of 18-deoxy-aplog-1. Conformational analysis of the des-A-ring analog **13** revealed that the conformation of RD of aplogs was hardly affected by the removal of the A-ring, but the conformation of CCU in **13** was slightly different from that of 18-deoxy-aplog-1 with the spiro-ring. This structural change selectively decreased the affinity for novel PKCs, which is somewhat surprising because nearly all structural modifications of aplogs in the previous studies increased the isozyme selectivity toward novel PKCs. Although the reason why **13** retained affinity for C1A domains of conventional PKCs is still not clear, the conformation of positions 2–7 in aplogs might be important in the recognition of C1B domains of novel PKCs. In addition, hydrophobicity at positions 4–5 might be also responsible for this phenomenon. Moreover, **13** showed selective anti-proliferative activity against NCI-H460 (lung) and MKN45 (stomach) human cancer cell lines. Because the installation of a methyl group at positions 10 and/or 12 would be promising to enhance biological activities of **13** as exemplified by 10-Me-Aplog-1 and 10,12-diMe-Aplog-1, **13** could serve as a lead compound for the development of selective chemotherapeutic agents for such types of cancer.

This study revealed that a modification of the spiroketal scaffold in the ATX analogs does not affect so much the intermolecular hydrogen bondings and that such a modification is effective not only to achieve further simplification and shortening of the synthetic steps but also to provide different selectivity among target proteins and different biological activity from the original compound. These findings also suggest that a modification of spiroketal moiety of natural products in which the spiro ring plays a role in controlling the conformation, like ATX, could be an effective strategy to achieve simplification and/or change of selectivity between multiple targets of the natural products.

References

1. Mackay, H. J.; Twelves, C. J. Targeting the protein kinase C family: are we there yet? *Nat. Rev. Cancer* **2007**, *7*, 554–562.
2. Castagna, M.; Takai, Y.; Kaibuchi, K.; Sano, K.; Kikkawa, U.; Nishizuka, Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **1982**, *257*, 784–7851.

3. Fujiki, H.; Tanaka, Y.; Miyake, R.; Kikkawa, U.; Nishizuka, Y.; Sugimura, T. Activation of calcium-activated, phospholipid-dependent protein kinase (protein kinase C) by new classes of tumor promoters: teleocidin and debromoaplysiatoxin. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 339–343.
4. Kong, F. H.; Kishi, Y.; Perez-Sala, D.; Rando, R. R. The pharmacophore of debromoaplysiatoxin responsible for protein kinase C activation. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 1973–1976.
5. Nakamura, H.; Kishi, Y.; Pajares, M. A.; Rando, R. R. Structural basis of protein kinase C activation by tumor promoters. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 9672–9676.
6. Fujiki, H.; Suganuma, M.; Nakayasu, M.; Hoshino, H.; Moore, R. E.; Sugimura, T. The third class of new tumor promoters, polyacetates (debromoaplysiatoxin and aplysiatoxin), can differentiate biological actions relevant to tumor promoters. *Gann* **1982**, *73*, 495–497.
7. Ramos, O. F.; Masucci, M. G.; Klein, E. Activation of cytotoxic activity of human blood lymphocytes by tumor-promoting compounds. *Cancer Res.* **1984**, *44*, 1857–1862.
8. Kikumori, M.; Yanagita, R. C.; Tokuda, H.; Suzuki, N.; Nagai, H.; Suenaga, K.; Irie, K. Structure–activity studies on the spiroketal moiety of a simplified analogue of debromoaplysiatoxin with antiproliferative activity. *J. Med. Chem.* **2012**, *55*, 5614–5626.
9. Kikumori, M.; Yanagita, R. C.; Tokuda, H.; Suenaga, K.; Nagai, H.; Irie, K. Structural optimization of 10-methyl-aplog-1, a simplified analog of debromoaplysiatoxin, as an anticancer lead. *Biosci. Biotechnol. Biochem.* **2016**, *80*, 221–231.
10. Kikumori, M.; Yanagita, R. C.; Irie, K. Improved and large-scale synthesis of 10-methyl-aplog-1, a potential lead for an anticancer drug. *Tetrahedron* **2014**, *70*, 9776–9782.
11. Zhang, G.; Kazanietz, M. G.; Blumberg, P. M.; Hurley, J. H. Crystal structure of the Cys2 activator-binding domain of protein kinase Cδ in complex with phorbol ester. *Cell* **1995**, *81*, 917–924.
12. Sharkey, N. A.; Blumberg, P. M. Highly lipophilic phorbol esters as inhibitors of specific [^{3}H]phorbol 12,13-dibutyrate binding. *Cancer Res.* **1985**, *45*, 19–24.
13. Yanagita, R. C.; Kamachi, H.; Tanaka, K.; Murakami, A.; Nakagawa, Y.; Tokuda, H.; Nagai, H.; Irie, K. Role of the phenolic hydroxyl group in the biological activities of simplified analogue of aplysiatoxin with antiproliferative activity. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6064–6066.
14. Moore, R. E.; Blackman, A. J.; Cheuk, C. E.; Mynderse, J. S.; Matsumoto, G. K.; Clardy, J.; Woodard, R. W.; Craig, J. C. Absolute stereochemistries of the aplysiatoxins and oscillatoxin A. *J. Org. Chem.* **1984**, *49*, 2484–2489.
15. Kamachi, H.; Tanaka, K.; Yanagita, R. C.; Murakami, A.; Murakami, K.; Tokuda, H.; Suzuki, N.; Nakagawa, Y.; Irie, K. Structure-activity studies on the side chain of a simplified analog of aplysiatoxin (aplog-1) with anti-proliferative activity. *Bioorg. Med. Chem.* **2013**, *21*, 2695–702.
16. Yamori, T.; Matsunaga, A.; Sato, S.; Yamazaki, K.; Komi, A.; Ishizu, K.; Mita, I.; Edatsugi, H.; Matsuba, Y.; Takezawa, K.; Nakanishi, O.; Kohno, H.; Nakajima, Y.; Komatsu, H.; Andoh, T.; Turuo, T. Potent antitumor activity of MS-247, a novel DNA minor groove binder, evaluated by an *in vitro* and *in vivo* human cancer cell line panel. *Cancer Res.* **1999**, *59*, 4042–4049.

List of publications

1. Ashida, Y.; Yanagita, R. C.; Takahashi, C.; Kawanami, Y.; Irie, K. Binding mode prediction of aplysiatoxin, a potent agonist of protein kinase C, through molecular simulation and structure-activity study on simplified analogs of the receptor-recognition domain. *Bioorg. Med. Chem.* **2016**, *24*, 4218–4227. DOI: 10.1016/j.bmc.2016.07.011.
2. Ashida, Y.; Yanagita, R. C.; Kawanami, Y.; Okamura, M.; Dan, S.; Irie, K. Synthesis, conformation, and biological activities of a des-A-ring analog of 18-deoxy-aplog-1, a simplified analog of debromoaplysiatoxin. *Heterocycles,* in press. DOI: 10.3987/COM-18-S(F)60.