HETEROCYCLES, Vol. 76, No. 2, 2008, pp. 1033 - 1042. © The Japan Institute of Heterocyclic Chemistry
Received, 1st April, 2008, Accepted, 14th July, 2008, Published online, 17th July, 2008. COM-08-S(N)88

STRUCTURE-ACTIVITY RELATIONSHIP OF OKADAIC ACID, A POTENT PROTEIN PHOSPHATASES PP1 AND PP2A INHIBITOR: 24-*epi*-OKADAIC ACID AND A 18-MEMBERED LACTONE ANALOG

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Abstract - X-Ray crystallographic analysis and structure-activity relationship of okadaic acid revealed that the flexible cavity structure stabilized by intramolecular hydrogen bonds is important for its potent cytotoxicity.

Okadaic acid (OA, **1**) is a potent protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A) inhibitor [1] isolated from a black sponge *Halichondria okadai* (Figure 1).[2] OA is also well-known as a principal toxin of diarrhetic shellfish poisoning as with dinophysistoxins,[3] and a potent non-12-*O*-tertadecanoylphobol-13-acetate (non-TPA) type tumor promoter.[4] Recently, crystal structures of the OA bound to the catalytic subunit of PP1 (OA·PP1) [5] and PP2A (OA·PP2A) [6] complexes have been elucidated, in which OA has a cyclic structure stabilized by an intramolecular hydrogen bond between C-1 carbonyl group and C-24 hydroxy group. Meanwhile, glycookadaic acid (GOA, **2**) isolated from *H. okadai* has also been shown to possess a cyclic structure, which has been referred to as a "flexible cavity" model.[7] Such hydrogen bond interactions are commonly observed in the crystal structures of *o*-bromophenacyl okadaate [2] and acanthifolicin, a 9,10-episulfide OA analog.[8] To establish the importance of such cyclic conformations for OA-type compounds, we newly designed two OA analogs, 24-*epi*-OA **3** and a 18-membered lactone **4** based on flexible cavity hypothesis. Here we describe the X-ray crystallographic analysis of OA **1** itself and the structure-activity relationships of its synthetic analogs.

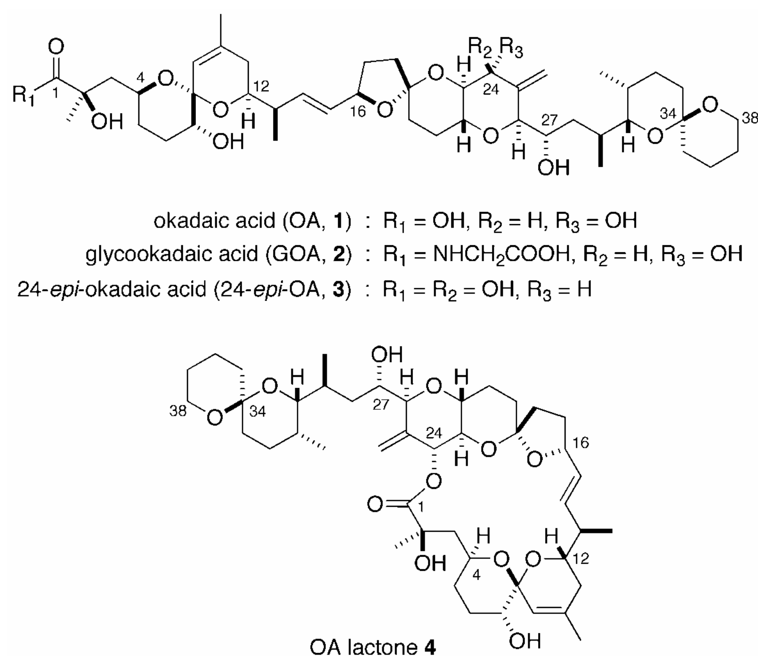


Figure 1. Structures of OA **1** and its derivatives.

First, X-ray analysis of **1** was carried out on crystals of its free acid obtained from MeOH. It formed a

18-membered pseudo-ring conformation stabilized by intramolecular hydrogen bonds between C-1 carboxyl group and C-24 hydroxy group, and also C-2 hydroxyl group and C-4 ethereal oxygen atom. Comparison of the crystal structures between free **1** and the OA bound to PP1 revealed that they have a very closed conformations (0.613 Å r.m.s.d. for overall 57 atoms) (Figure 2). The distances of two oxygen atoms on C-1 and C-24 of OA **1** free form and of the OA·PP1 complex were 2.968 and 2.847 Å, respectively. Therefore, it has been confirmed that OA **1** can bind to such enzymes with slightly little conformational changes, as suggested by the previous conformational studies.[2,7-9]

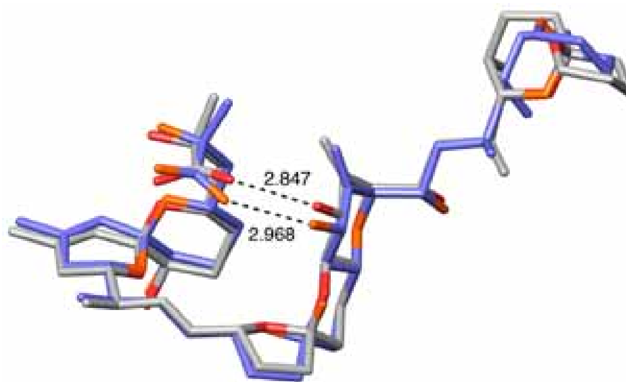
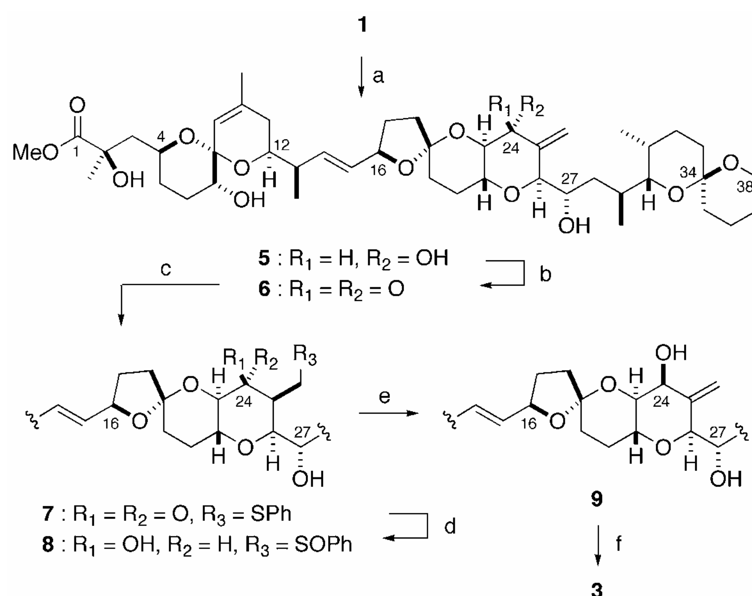


Figure 2. Superimposed crystal structures of OA **1** free acid (blue, this work) and the OA bound to PP1 (gray, PDB: 1jk7). Oxygen atoms are shown in orange and red colors. The distances of two oxygen atoms on the C-1 carboxyl group and the C-24 hydroxy group are labeled in Å.

To obtain more information on the effects of intramolecular hydrogen bonding in **1**, we began the preparation of 24-*epi*-OA **3** (Scheme 1). Since the C-24 hydroxy group in **3** is oriented in axial configuration in the fixed *trans*-fused ether rings with chair conformations, **3** may lack the C-1 to C-24 intramolecular hydrogen bonding. First, OA **1** was treated with diazometane to give methyl ester **5**, followed by MnO_2 oxidation of the allyl alcohol moiety to provide 24-keto methyl okadaate **6**. All attempts of the direct reduction of **6** were failed, but only C-24α-alcohol **5** was recovered. Meanwhile, Michael reaction of **6** with PhSNa [10] provided **7**, which was reduced by $NaBH_4$ to afford the desired C-24β-alcohol **8**.[11, 12] After $NaIO_4$ oxidation, the phenyl sulfoxy group in **8** was removed by refluxing in toluene to give **9**. Finally, methyl ester **9** was hydrolyzed to provide **3**.[13]



Scheme 1. Preparation of 24-*epi*-OA **3**. *Reagents and conditions*: (a) CH_2N_2, MeOH, rt, 98%; (b) MnO_2, CH_2Cl_2, rt, 27%; (c) PhSNa, MeOH, 0°C, 12%; (d) $NaBH_4$, MeOH, –78°C; $NaIO_4$, MeOH, 0°C; (e) toluene, reflux, 14% from **7**; (f) NaOH, H_2O–dioxane, rt, quant.

Notably, cytotoxicity of 24-*epi*-OA **3** was more than 50 times weaker than that of OA **1** against L1210 leukemia cells (Table 1). It has been shown that the inhibition of specific [^{3}H]OA binding activity of the OA glycol derivative,[14] whose C-14 olefin is dihydroxylated, is 400 times weaker than that of **1**. Thus, 24-*epi* OA **3** may also become more flexible due to the loss of intramolecular hydrogen bonding, which was considered to reduce its binding affinity with protein phosphatases as well as cytotoxicity.

Table 1. Cytotoxicity of OA **1** and its derivatives

Samples	IC$_{50}$ (μg/mL)			
	L1210 leukemia	P388 leukemia	B16 melanoma [b]	L929 fibroblast [b]
OA **1**	0.05	0.0035	0.018	0.084
GOA **2**	– [a]	–	19	28
24-*epi*-OA **3**	3.3	–	–	–
OA lactone **4**	–	> 10	–	–

[a] Test was not carried out. [b] See ref. 6.

Then we designed OA lactone **4**, possessing a fixed 18-membered macrocyclic core. A molecular modeling study using a Merck molecular force field (MMFF) for **4** indicated that the cavity structure C-1 to C-26 was mostly overlapped among the nine lowest energy conformers between 15 kJ/mol (Figure 3). In contrast, the lipophilic C-27 to C-38 part was rather flexible, due to the free rotation of sp^3-sp^3 bonds. Thus, such a rigid conformation in **4** was expected to enhance its cytotoxicity as in the case of several cyclic peptides, myclocystins [15] and nodularins.[16]

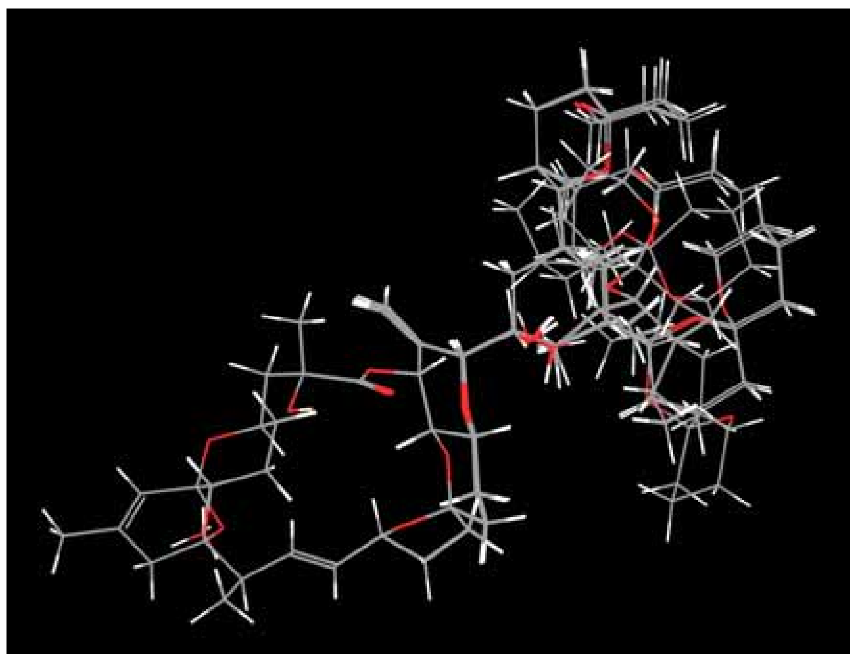
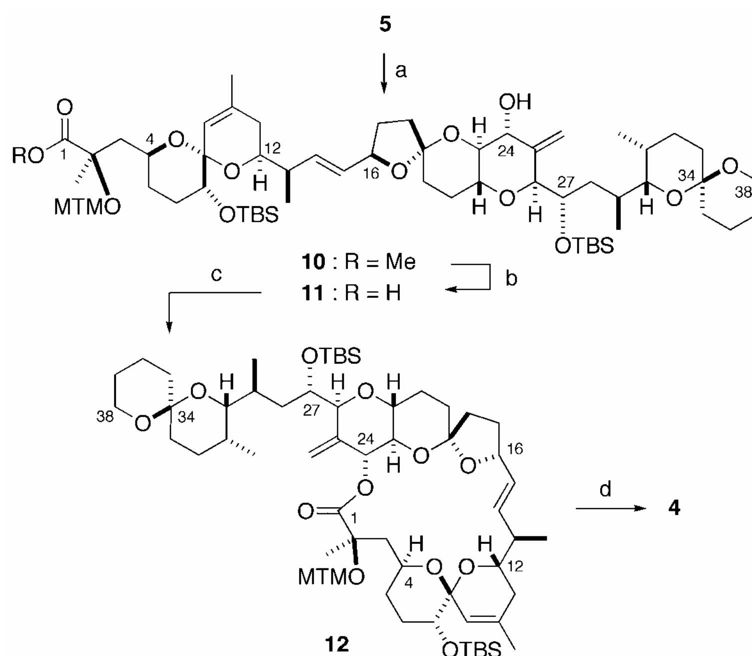


Figure 3. Superimposed structures of the nine lowest energy conformers for **4**. Models were energetically minimized via iterative application of the MMFFs (MacroModel 9.0).

Based on this assumption, lactone **4** was synthesized (Scheme 2).[17] Treatment of OA methyl ester **5** with excess amount of TBSCl afforded trisilyl ether, followed by protection of the residual tertiary alcohol with methylthiomethyl (MTM) group and selective deprotection of the C-24 TBS group using TBAF at low temperature gave **10**. Allyl alcohol **10** was hydrolyzed in basic conditions to give seco acid **11**. Yamaguchi macrolactonization of **11** using 2,4,6-trichlorobenzoyl chloride afforded **12** in 38% yield. Low-field shift of H-24 (δ 3.96 for **11** and 5.27 for **12**) in NMR spectra established the macrocyclic structure of **12**. Finally, continual deprotections of the TBS and MTM [18] groups afforded **4**.[19, 20]



Scheme 2. Preparation of OA lactone **4**. *Reagents and conditions*: (a) TBSCl, DMAP, DMF, rt; DMSO, Ac_2O, rt; TBAF, THF–DMF, –60°C to –5°C, 56%; (b) LiOH, H_2O, dioxane, rt, 90%; (c) 2,4,6-trichlorobenzoyl chloride, Et_3N, rt, then DMAP, toluene, reflux, 38%; (d) aq. HF, MeCN, rt; $Ph_3C^+BF_4^-$, CH_2Cl_2, rt, 27%.

Unlike our expectations, however, OA lactone **4** had no cytotoxicity against P388 murine leukemia cells even at 10 μg/mL. Molecular modeling studies of several PP1 inhibitors [21] and X-ray crystallographic analysis of microcystin-LR·PP1 complex [22] have suggested that a conserved acidic motif is important for PP1 inhibitors, i.e. the carboxyl group in OA, the phosphate group in calyculin A,[23] and the Glu moiety in microcystin-LR.[15] Indeed, multiple hydrogen bonds have been observed in the OA·PP1 complex between the C-1 carboxyl group and Tyr-272, and between the C-2 and C-24 hydroxy groups and two Arg residues.[5] Importance of such interactions has also been exemplified by the significant increase in K_i values by the mutation of Y272F in the catalytic subunit of PP1.[24] Thus, OA lactone **4** may lose its binding activity to PP1 as well as cytotoxicity due to the loss of its acidic nature, as with GOA **2** [6] or OA methyl ester **5**.[14]

Further biological studies on OA analogs are still an important issue, but our preliminary study revalidates that both of the terminal acidic moiety and the “flexible cavity” structure stabilized by intramolecular hydrogen bonds are essential for the OA class of compounds to be incorporated in the catalytic pocket and

to enhance their binding affinity with the enzymes. These findings should contribute further rational design of more specific protein phosphatase inhibitors.

ACKNOWLEDGEMENTS

We are grateful to Prof. Hirokazu Kawagishi (Shizuoka University) for the mass spectra analysis and to Dr. Atsushi Wakamiya (Nagoya University) for the X-ray analysis. This work was supported by the Grants-in-Aid for Creative Scientific Research (16GS0206) from JSPS.

REFERENCES AND NOTES

‡ Crystal data for **1**: $C_{44}H_{68}O_{13}$, M = 804.98, crystal dimensions 0.20 x 0.10 x 0.05 mm, orthorhombic, space group $P2_12 2_1$, a = 33.975(10) Å, b = 10.583(3) Å, c = 14.104(4) Å, V = 5071(2) Å^3, Z = 4, D_{calcd} = 1.054 g cm^{-3}, T = 100(2) K, R_{int} = 0.0592 based on 8938 reflections {$I > 2s(I)$} and 524 parameters, GOF = 1.087, R_1 = 0.0791, wR_2 = 0.2066. CCDC 691258 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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11. Phenyl sulphide **7** was obtained as a C-25β single stereoisomer. The C-25 methylthiophenyl group in **7** is oriented in an equatorial configuration in the tetrahydropyran ring with chair conformation, suggesting that thermodynamically favorable stereoisomer was formed during conjugate addition.

12. Both the MnO_2 oxidation and Michael addition reactions were slow, and considerable amounts of the starting materials were recovered.

13. Spectroscopic data for **3**: ^1H NMR ($CDCl_3/CD_3OD$ = 2/1, 400 MHz) δ 0.93 (d, J = 7.0 Hz, 3H, C29-CH_3), 1.05 (d, J = 6.2 Hz, 3H, C31-CH_3), 1.10 (d, J = 7.2 Hz, 3H, C13-CH_3), 1.28-2.04 (m, 27H), 1.30 (s, 3H, C2-CH_3), 1.72 (s, 3H, C10-CH_3), 2.20 (m, 1H, H11b), 2.33 (m, 1H, H13), 3.23 (dd, J = 2.2, 10.3 Hz, 1H, H30), 3.23 (dd, J = 3.2, 10.8 Hz, 1H, H23), 3.35 (dd, J = 5.0, 11.5 Hz, 1H, H7), 3.52 (m, 1H, H38a), 3.63 (m, 1H, H22), 3.70 (m, 1H, H38b), 3.85 (ddd, J = 3.7, 7.3, 11.0 Hz, 1H, H12), 3.94 (d, 1H, J = 9.2 Hz, 1H, H26), 4.04 (d, J = 3.2 Hz, 1H, H24), 4.07 (m, 1H, H4), 4.10 (m, 1H, H27), 4.66 (m, 1H, H16), 4.98 (br s, 1H, C25=CH_2), 5.06 (br s, 1H, C25=CH_2), 5.26 (br s, 1H, H9), 5.49 (dd, J = 7.9, 15.2 Hz, 1H, H15), 5.95 (dd, J = 8.6, 15.2 Hz, 1H, H14).

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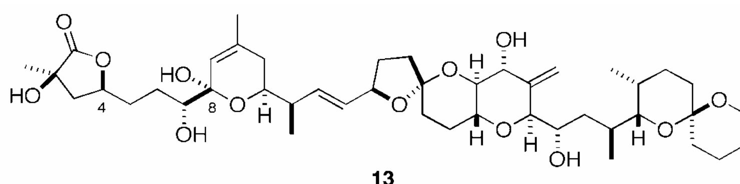
17. A direct lactonization of **1** using DCC and pyridine was attempted, but only afforded γ-lactone **13**.



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19. Spectroscopic data for **4**: NMR ($CDCl_3$, 400 MHz) δ 0.93 (d, J = 7.0 Hz, 3H, C29-CH_3), 1.05 (d, J = 6.2 Hz, 3H, C31-CH_3), 1.10 (d, J = 7.2 Hz, 3H, C13-CH_3), 1.28-2.04 (m, 27H), 1.53 (s, 3H, C2-CH_3), 1.72 (s, 3H, C10-CH_3), 2.20 (m, 2H, H11b), 2.33 (m, 1H, H13), 3.23 (dd, J = 2.2, 10.3 Hz, 1H, H30), 3.35 (dd, J = 5.0, 11.5 Hz, 1H,H7), 3.39 (dd, J = 4.7, 10.5 Hz, 1H, H-7), 3.41 (dd, J = 9.7, 9.7 Hz, 1H, H23), 3.52 (m, 1H, H38a), 3.63 (m, 1H, H22), 3.70 (m, 1H, H38b), 3.85 (ddd, J = 3.7, 7.3, 11.0 Hz, 1H, H12), 3.96 (d, J = 9.8 Hz, 1H, H-26), 4.07 (m, 1H, H4), 4.11 (br t, J = 9.9 Hz, 1H, H-27), 4.66 (m, 1H, H16), 5.02 (br s, 1H, C25=CH_2), 5.26 (br s, 1H, H9), 5.33 (br s, 1H, C25=CH_2), 5.40 (d, 1H, J = 9.9 Hz, H-24), 5.49 (dd, 1H, J = 8.1, 15.4 Hz, H-15), 5.69 (dd, J = 7.7, 15.4 Hz, 1H, H14); MS (FAB) *m/z* 809 $(M+Na)^+$.

20. During the removal of MTM group, partial degradation of the acid-labile spirocyclic moieties was observed.

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