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Direct Observation of Binding between Biotinylated Okadaic Acids and Protein Phosphatase 2A Monitored by Surface Plasmon Resonance

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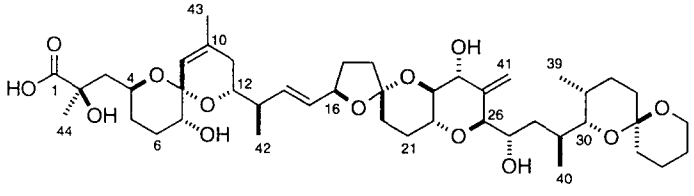
Abstract : Two biotin conjugates of okadaic acid were synthesized for evaluating their interactions with protein phosphatase 2A by surface plasmon resonance (SPR). C7-biotinylated okadaic acid revealed strong binding affinity to the enzyme, while C1-biotinylated derivative being devoid of affinity, implying that the C7-biotin conjugate is a useful tool for biochemical studies of protein phosphatase 2A.

Keywords: Okadaic acid; Surface plasmon resonance; Protein phosphatases

Reversible phosphorylation of proteins is catalyzed by both protein kinase and protein phosphatase, through which eukaryotic cells translate extracellular signals to intracellular events. Several natural products such as okadaic acid (**1**),[1] microcystin, tautomycin and calyculin A are known to bind and inhibit protein phosphatases 1 and 2A (PP2A), resulting in accumulation of phosphorylated proteins in cells of various organs.[2] The binding of these inhibitors to protein phosphatases has been mainly investigated by replacement experiments using a radioligand such as [27-^{3}H]okadaic acid[3] or by monitoring the enzymatic activity.[4] Among them, okadaic acid (**1**) is the most potent PP2A inhibitor, and now regarded as a standard reagent for biological studies of protein phosphatases.[2]



Okadaic acid (**1**)

Recent progress in optical-biosensing technologies has made it possible to investigate the kinetics of diverse interactions between biomolecules such as protein-protein, protein-peptide, and DNA-protein. Surface plasmon resonance (SPR) is one of these apparatus, by which receptor-ligand interactions are observed in real time as changes of local refractive index.[5] Once a ligand is biotinylated without loss of its binding affinity, the interaction with its receptor can be monitored using the immobilized ligand on a sensor chip via avidin-biotin binding.[6] Besides this, the biotinylation of a ligand enables us to adopt methodologies widely used in

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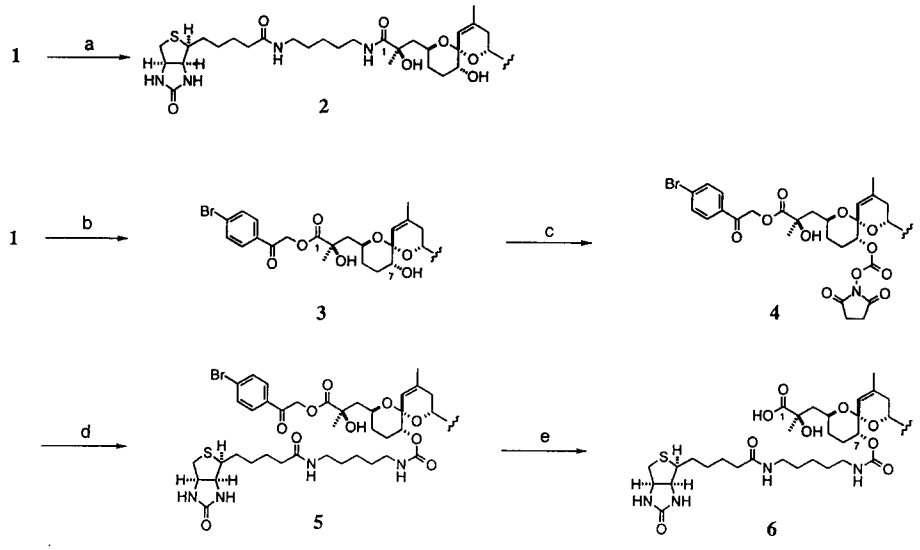
biochemistry and molecular biology including sensitive protein detection and protein capture applications.[7] Therefore, biotinylated okadaic acid would be a versatile probe not only for the study of protein phosphatases but also for specific detection and purification of okadaic acid-binding proteins other than protein phosphatases. In this study, we synthesized two biotin conjugates of okadaic acid (**Scheme**), and elucidated their binding affinity to PP2A using SPR.



Scheme. (a) 5-(biotinamido)pentylamine, HOBt, EDC•HCl, Et_3N, DMF, rt, 4h, 40%; (b) *p*-bromophenacyl bromide, Et_3N, acetone, 40℃, 3h, 93%; (c) *N,N'*-disuccinimidyl carbonate, Et_3N, CH_3CN, rt, 5h; (d) 5-(biotinamido)pentylamine, Et_3N, DMF, rt, 12h; (e) Zn, AcOH, rt, 5h, 54% in 3 steps.

Okadaic acid (**1**), isolated from a black sponge *Halichondria okadai* collected at the seashore in Kanagawa Prefecture,[1] was treated with 5-(biotinamido)pentylamine[8] in the presence of 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC•HCl) and 1-hydroxybenzotriazole (HOBt). The reaction mixture was separated by HPLC (YMC-Pack ODS-AM, AM-323, ϕ10 x 250 mm, 60% CH_3CN, 1.5 mL/min, T_r = 21.0 min), to furnish 200 μg of the C1-biotinylated okadaic acid **2** in 40% yield (FABMS, 1137.7 $[M+Na]^+$).[9]

Another conjugate was prepared by introducing a biotin moiety at one of hydroxyl groups. **1** was protected as a *p*-bromophenacyl ester **3** by treatment with *p*-bromophenacyl bromide in the presence of Et_3N (93% yield), and **3** was subjected to esterification with 5-(biotinamido)pentanoic acid at C7-OH, which is known to be the most susceptible to acylation among four hydroxyl groups in C1 esters of **1**.[10] However, Yamaguchi's method[11] or DCC-DMAP acylation[12] failed to esterify the C7-OH. Next, introduction of the biotin unit was attempted using a carbamate linkage to the C7-OH. The ester **3** was treated with *N,N'*-disuccinimidyl carbonate in the presence of Et_3N to provide activated carbonate **4**,[13] which was then coupled with 5-(biotinamido)pentylamine to provide **5** in a regioselective manner. Removal of the *p*-bromophenacyl group by Zn/AcOH, followed by HPLC purification (YMC-Pack ODS-AM, AM-323, ϕ10 x 250 mm, 70% CH_3CN, 1.5 mL/min, T_r = 37.7 min), furnished 600 μg of the C7-biotinylated okadaic acid **6** in 54% yield in three steps (FABMS, 1379.7 $[M+Na]^+$).[14]

After the biotin conjugates **2** and **6** were captured on a streptavidin-coated sensor chip (SA) of a SPR instrument (BIACORE™ X, Biacore AB) through binding between biotin and streptavidin (not shown), PP2A was introduced to a flow cell from the time zero, and then the interaction of **2** and **6** with PP2A were monitored as shown in **Fig. 1**A.[15]

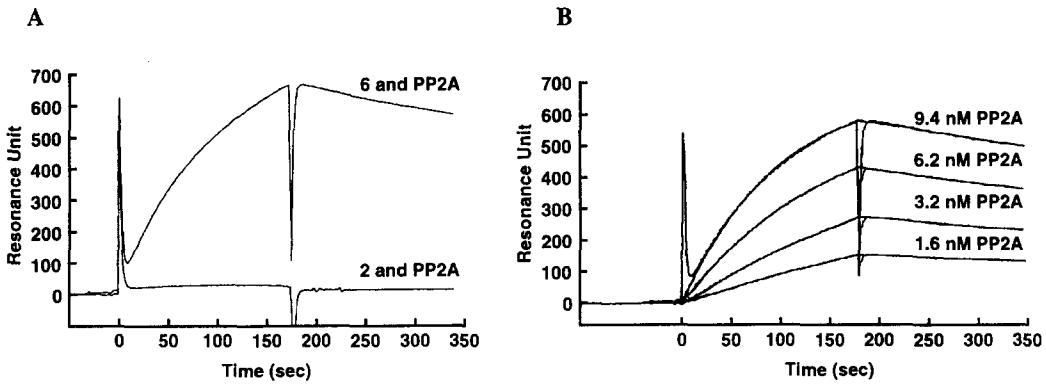


Fig. 1. Sensorgrams of the interactions between the biotin-conjugates of okadaic acid and PP2A. (A) Interactions of **2** and **6** with 9.4 nM PP2A. (B) Dependence of the binding of **6** on the concentration of PP2A.

In accordance with marked reduction of affinity reported on C1 esters of **1** to PP2A,[16] no significant change in sensorgram was observed for the chip bearing the C1-biotin conjugate **2**. On the other hand, when the C7-biotin conjugate **6** was loaded on the sensor chip, the resonance (Y-axis) kept increasing until the PP2A solution was replaced with a running buffer at 180 sec. As shown in **Fig. 1**B, this complex formation was dependent on the concentration of PP2A. Kinetic analysis of the sensorgrams revealed the association rate constant (k_a) between PP2A and the immobilized **6** to be 9.3 x 10^5 $M^{-1}s^{-1}$ and their dissociation rate constants (k_d) to be 9.6 x 10^{-4} s^{-1}. Based on the concentration of the enzyme, which was estimated from its activity on hydrolysis of *p*-nitrophenylphosphate as a substrate, the dissociation constant K_d was calculated to be 1.0 nM. These results indicate that okadaic acid was biotinylated without binding affinity to PP2A,[17] and the interaction beteen them was successfully evaluated by SPR. Thus this assay system would be applicable to the binding studies of okadaic acid derivatives to protein phosphatases in conjunction with their inhibitory action and also the search for other binding proteins of okadaic acid.

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6. Commercially available PP2A was immobilized by an amine coupling method[5a] to the dextran surface of a sensor chip (CM5), which possesses carboxylic acid groups. However, interactions of these immobilized enzymes with neither **1** nor the biotin conjugates of **1** was detected, probably because of the insignificant changes in SPR due to the much smaller molecular weight of the analyte, compared with the enzymes.
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8. 5-(Biotinamido)pentylamine was prepared by reacting biotin with *N*-(5-aminoamyl)carbamic acid *tert*-butyl ester in the presence of EDC•HCl, HOBt, and Et_3N, and removing the *tert*-butyloxy carbonyl group with trifluoroacetic acid (52% yield in 2 steps).
9. The structural resemblance between **2** and methyl okadaate[1] was evident in the ^{1}H-NMR spectra, and additional signals observed in the spectrum of **2** were attributed to a biotin moiety. Selected ^{1}H NMR ($CDCl_3$, 500 MHz) of **2**: δ 4.51 (dd, J = 7.5, 5.0 Hz, -S-CH_2-C<u>H</u>-), 4.31 (dd, J = 7.5, 4.5 Hz, -S-CH-C<u>H</u>-), 3.15 (dt, J = 4.5, 8.0 Hz, -S-C<u>H</u>-), 2.91 (d, J = 5.0, 12.5 Hz, -S-C<u>H_2</u>-), and 2.71 (d, J = 12.5 Hz, -S-C<u>H_2</u>-).
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14. Introduction of the biotin unit at C7-OH in **6** was confirmed using its precursor **5**, since the signals derived from H_2O and polar solvents such as CD_3OD, DMSO-d_6 and DMF-d_7, in which **6** was soluble, often hampered clear observation of proton signals of interest, H-7, H-24, and H-27. Selected ^{1}H NMR data ($CDCl_3$, 500 MHz) of **5**: δ 4.60 (dd, J = 11.8, 4.7 Hz, H-7), 4.24 (d, J = 10.3 Hz, H-24), and 4.13 (t, J = 10.0 Hz, H-27). The distinction in the ^{1}H NMR spectra between **1** and **5** was that one oxymethine proton of **1** (δ 3.35)[1] was deshielded by 1.25 ppm (δ 4.60) in **5**. This oxymethine proton is easily assignable to H-7 by its distinct coupling pattern, which coincided with the reported values for H-7 (11.8 and 4.2 Hz).[18] From the close spectral resemblance between **1** and **5**, the signals at δ 4.24 and δ 4.13 were assignable to H-24 and H-27, respectively, and these virtually unchanged chemical shifts between **1** and **5** ruled out their acyl substitutions.
15. A typical experimental procedure is as follows. The streptavidin immobilized sensor chip was loaded with 74 nM of **6** at a flow rate of 5 μL/min for 180 sec, which resulted in increasing resonance by 230 resonance unit (RU). After 9.4 nM PP2A (Wako Pure Chemicals) was injected at 10 μL/min for 180 sec, dissociation of the bound PP2A was monitored for 180 sec by washing the sensor chip with the running buffer. The sensor chip was regenerated by passing 100 mM Na_2CO_3 at a flow rate of 5 μL/min for 180 sec. The running buffer consisted of 150 mM NaCl, 3.4 mM EDTA, 0.01% Tween® 20, 0.02% (w/v) BSA and 10 mM Hepes/NaOH (pH 7.4). All experiments were carried out at 25 ℃.
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17. Biological acitivities of C7-OH derivatives from **1** have been previously reported.[10,16,18] Fatty acid esters at C7-OH showed very weak binding to PP2A. Compared with other derivatives such as methyl ester of **1** and decarboxylated **1**, however, these esters possess somewhat greater affinity to PP2A.[16]
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