Pergamon

Tetrahedron 56 (2000) 9003–9014

TETRAHEDRON

Development of Biotin–Avidin Technology to Investigate Okadaic Acid-Promoted Cell Signaling Pathway

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Received 17 May 2000; accepted 24 July 2000

Abstract—Four biotin conjugates of okadaic acid were synthesized for evaluating their interactions with protein phosphatase 2A (PP2A) by surface plasmon resonance (SPR). C7-biotinylated okadaic acid exhibited the strongest binding affinity to the enzyme, while C1-biotinylated derivative was devoid of affinity. C24- or C27-biotinylated okadaic acid showed moderate affinity to the enzyme. In the wake of this finding, a biotinyl photoaffinity probe was introduced into 7-OH of okadaic acid. Photoaffinity labeling followed by SDS-PAGE analysis indicated that the okadaic acid derivative clearly labeled PP2A. Furthermore, three proteins were also labeled in crude extracts of a marine sponge *Halichondria okadai*. All these results imply that the C7-biotin conjugate is a versatile reagent for biochemical studies of okadaic acid-binding proteins including PP2A.

Introduction

Reversible phosphorylation of proteins is catalyzed by both protein kinases and protein phosphatases, through which intracellular events are regulated in eukaryotic cells according to extracellular signals and their transduction. Several natural products such as okadaic acid (Fig. 1, **1**),[1] microcystin-LR, tautomycin and calyculin A are known to bind to protein phosphatases 1 (PP1) and 2A (PP2A), and inhibit their enzymatic activity, 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 inhibitor of PP2A, and now regarded as a standard reagent for biological studies of protein phosphatases.[2]

The structure–activity relationship of okadaic acid has been investigated in detail by many groups.[5] Substitution at the C1 carboxylic acid or 24-OH greatly reduces the activity. Other structural changes such as hydrogenation at C14–C15 olefinic bond or deoxidation at C2, both of which affect the pseudocyclic conformation formed by hydrogen bonding between C1 carboxylic acid and 24-OH, also reduce the potency. While these preceding studies convince importance of the pseudocyclic structure for its recognition by PP2A, it still remains vague which side of this structure is recognized except for an observation on okadaic acid acylated at 7-OH retaining a moderate binding ability to PP2A.

Recently, optical-biosensing techniques have become potential methods to investigate kinetics of diverse interactions between two or more molecules including proteins, peptides, nucleic acids, carbohydrates, lipids, and low molecular weight molecules such as signaling substances and drug candidates.[6] Surface plasmon resonance (SPR) is one of these apparatus. Once a ligand is immobilized to a sensor plate, binding with its receptor can be monitored in real time as the local change in refractive index of a solution passing near the surface of a sensor plate, or the change in mass concentration of the surface layer. This change is reflected on resonance between the evanescent wave of the probe light, irradiated from the other side of the sensor plate at the total reflection condition, and free electron plasmon at the surface of the plate made from gold foil. In order to evaluate applicability of this method on more precise studies on the molecular mechanism of okadaic acid inhibiting action of the protein phosphatase, immobilization of okadaic acid was undertaken.

The immobilization of a ligand can be achieved by in situ formation of a covalent bond such as amide, disulfide, and

Keywords: surface plasmon resonance; photoaffinity labeling; okadaic acid; protein phosphatase.
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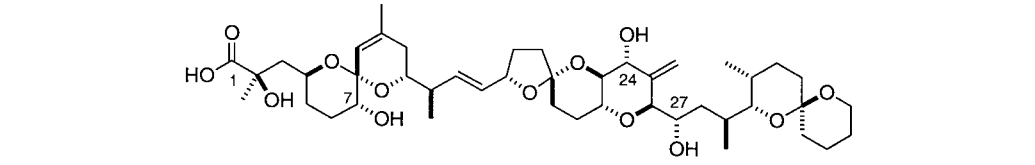
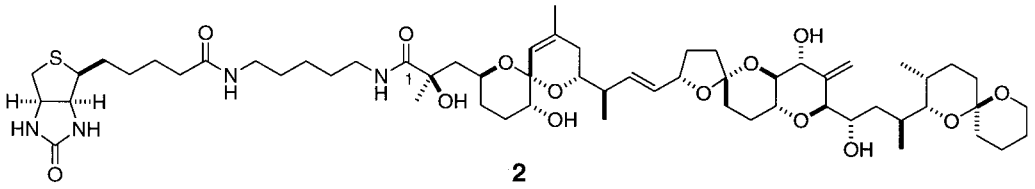
PII: S0040-4020(00)00752-3



1 (okadaic acid)



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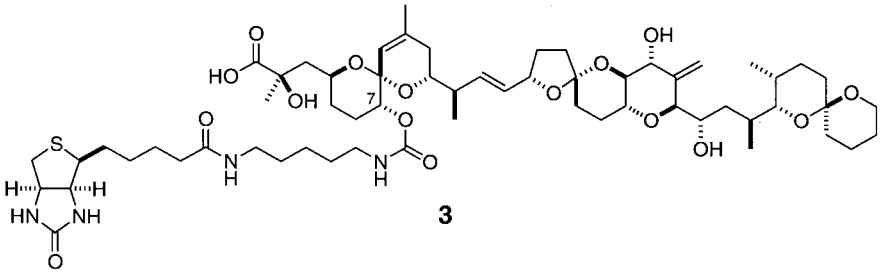

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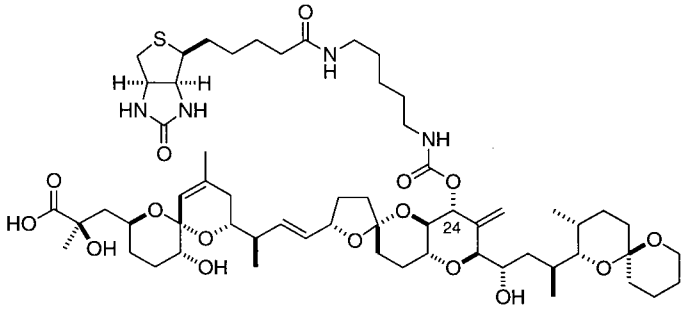

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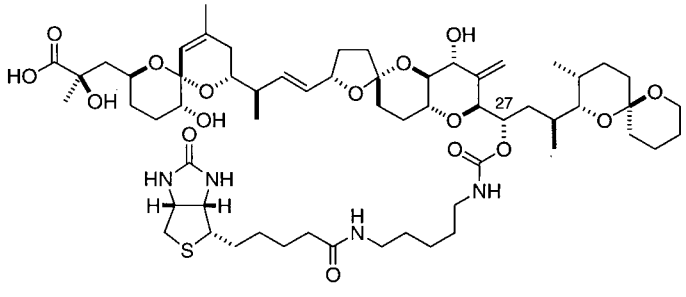

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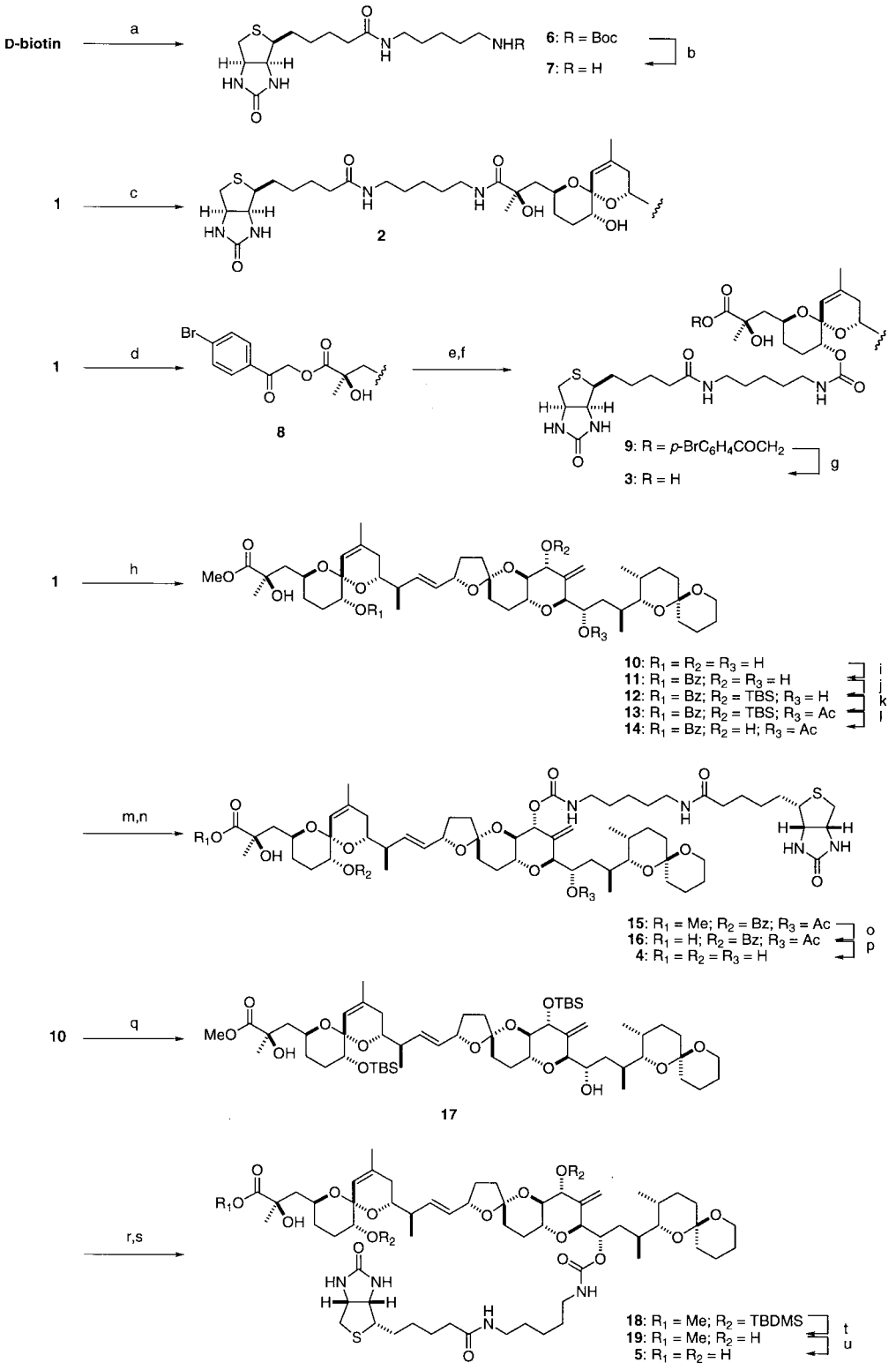
Figure 1. Okadaic acid and its biotinylated derivatives.

hydrazide, or a strong noncovalent bonding such as biotin–avidin complexation with a suitable kind and length of a spacer attached at a desired site of the ligand molecule. Since optimization of the solid-phase reaction for covalent immobilization would require a large excess of okadaic acid derivatives and possibly be costing expenses for wasted sensor plates, the non-covalent method was adopted in the present study. Namely, okadaic acid was biotinylated in liquid phase at its C1, C7, C24, or C27 positions through a linker chosen according to precedents (Fig. 1), and these derivatives were respectively immobilized to the same sensor plate where streptavidin was pre-immobilized.

Besides the utility in SPR sensing, biotinylation of a ligand enables us to adopt methodologies widely used in biochemistry and molecular biology such as sensitive detection of proteins or protein capture applications.[7] Among them, photoaffinity labeling has become a powerful tool for



Scheme 1. (a)*t*-Butyl *N*-(5-aminopentyl)carbamate, EDC·HCl, HOBt, Et_3N, DMF, rt, 52%; (b) CF_3CO_2H–CH_2Cl_2 (1:2), rt, quantitative; (c) **7**, EDC·HCl, HOBt, DMF, rt, 40%; (d) *p*-$BrC_6H_4COCH_2Br$, Et_3N, acetone, 40°C, 94%; (e) *N*,*N*′-Disuccinimidyl carbonate, Et_3N, DMF; (f) **7**, Et_3N, DMF, rt; (g) Zn/AcOH, rt, 54% in 3 steps; (h) $TMSCHN_2$, C_6H_6–MeOH (1:1), rt, quantitative; (i) BzCl, Et_3N, CH_2Cl_2, rt; (j) TBDMSCl, imidazole, DMF, rt; (k) Ac_2O, pyridine, rt, 68% in 3 steps; (l) 47% aq. HF–CH_3CN (1:4), rt, 85%; (m) *N*,*N*′-Disuccinimidyl carbonate, Et_3N, CH_3CN, rt; (n) **7**, Et_3N, DMF, rt, 85% in 2 steps; (o) 1 M aq. NaOH–THF (1:1), 40°C; (p) 5% NaOH/MeOH, 60°C; (q) TBDMSCl, imidazole, DMF, rt, 95%; (r) *N*,*N*′-Disuccinimidyl carbonate, Et_3N, CH_3CN, rt; (s) **6**, Et_3N, DMF, rt, 28% in 2 steps; (t) 47% aq. HF–CH_3CN (1:4), rt; (u) 1 M aq. NaOH–THF (1:1), 40°C, 71% in 2 steps.

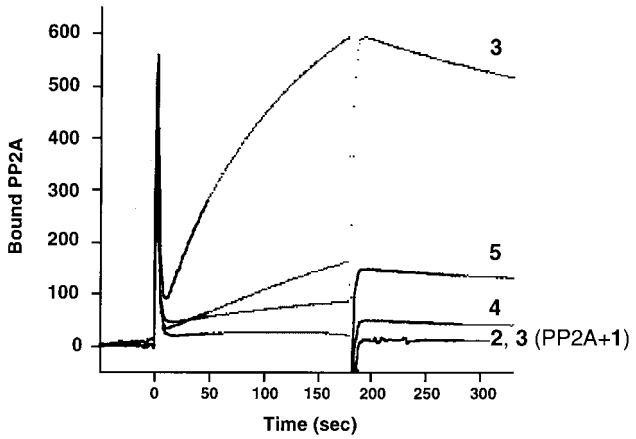


Figure 2. Interaction of biotinylated okadaic acids and protein phosphatase 2A. A streptavidin-immobilized sensor chip was loaded with the biotinylated okadaic acid **2**, **3**, **4** or **5** (100 nM) in 100 μL of a running buffer at a flow rate of 5.0 μL min^{-1} for 180 s. After a PP2A solution (12.5 nM) in the running buffer containing 0.02% w/v BSA was let flow at 10 μL min^{-1} for 180 s, dissociation of the bound protein was monitored for 180 s by washing the sensor chip with the same buffer. The sensor chip was regenerated by passing 100 mM Na_2CO_3 at a flow rate of 5.0 μL min^{-1} for 180 s. All experiments were carried out at 25°C.

investigating ligand/receptor interactions at various labels. To screen target molecules or identify binding domains, radiochemical probes bearing ^3H or ^{125}I were commonly used before.[8] Because of requirements for streamlining the tedious process, however, detection techniques with fluorescence or chemiluminescence have been developed, acquiring similar sensitivity to those for the radioligands to date. In this course, a new photoaffinity ligand **20** (Scheme 2) was devised by Hatanaka et al., and successfully used in identification of binding domain on GalT (β-1,4-galactosyltransferase).[9] This tridentate ligand bears a biotin moiety for detection, a 3-trifluoromethyl-3-diazirinyl moiety for photolabeling, and a carboxyl group for linkage to ligands. The photoreactive moiety has provided outstanding photocrosslinking results,[10] because irradiation at 360 nm would not destroy proteins, and it is also stable to mild reductive, acidic, and basic conditions. In their study, the binding proteins on an SDS-PAGE gel were captured by streptavidin conjugated to horseradish peroxidase (HRP), and thus were detected by chemiluminescence. After a proteolytic digestion, the peptidic fragments were subjected to amino terminal sequence analysis, and the labeled sites were confined within fragments of ten amino acid levels. In this article, we report our design and synthesis of biotinylated okadaic acids (Fig. 1) for SPR measurements, and then photoaffinity probe based on the result from the above. Labeling experiments using this probe successfully labeled PP2A, as well as a few proteins in crude extracts of a marine sponge *Halichondria okadai*, known to contain high content of okadaic acid. These new proteins are suspected to be involved in self-resistance mechanism of the sponge against okadaic acid.

Results and Discussion

Synthesis of biotinylated okadaic acids

To insert a spacer between biotin and okadaic acid, which is generally needed to ease the biotin for complex formation, D-biotin was treated with *t*-butyl *N*-(5-aminopentyl)carbamate in the presence of EDC·HCl and Et_3N, and deprotection in an acidic condition gave *N*-biotinyl-1,5-pentanediamine **7** (Scheme 1).

Okadaic acid **1** was reacted with freshly prepared **7** in the presence of EDC·HCl and HOBt to furnish **2** in 40% yield.[11] The structural resemblance between **2** and methyl okadaate **10** was evident in the ^1H NMR spectra, and additional signals observed in the spectrum of **2** at δ 4.51 (–S–CH_2–C*H*–), 4.31 (–S–CH–C*H*–), 3.15 (–S–C*H*–), and 2.91/2.71 (–S–CH_2–) were attributed to the biotin moiety.

Since nucleophilicity of 7-OH is known to be higher than the other secondary hydroxyl groups, selective biotinylation at this position was envisaged by controlling quantity and concentration of reagents.[12] Okadaic acid biotinylated at 7-OH (**3**) was synthesized as follows.[11] After the carboxyl group in okadaic acid was protected as the *p*-bromophenacyl ester (**8**), the ester was reacted with *N*,*N*′-disuccinimidyl carbonate in the presence of Et_3N to give an intermediate mixed carbonate.[13] Without further purification, this intermediate was treated with **7** in the presence of Et_3N, and deprotected under Zn/AcOH at 40°C to afford **3**. The reaction of **8** with N,N′-disuccinimidyl carbonate was turned out not to be so selective, and thus needs to be controlled in relatively diluted condition at this stage. Otherwise, the following reaction with **7** and deprotection of *p*-bromophenacyl substituent resulted in simultaneous production of regioisomers such as **4** or **5**, which was observed by LC/MS analysis (data not shown). Introduction of the biotin unit at 7-OH in **3** was confirmed by ^1H NMR spectroscopy, where 7-H, 24-H, and 27-H were assigned by tracing the COSY spectrum. The signal for 7-H in **3** appeared at δ 4.57, being downshifted from that in **1** at δ 3.35.[1] Since **1** and **3** exhibited the closely resembling spectra except for the above signals for 7-H, the signals at δ 4.07 and δ 4.05 assigned to 24-H and 27-H, respectively, indicated that neither of corresponding hydroxyls is acylated.

24-*O*-Biotinylated okadaic acid (**4**) was synthesized based on difference in nucleophilicity between 24-OH and 27-OH, which has been indicated in a modified preparation of 27-[^3H]okadaic acid via methyl 7-*O*,24-*O*-disilylokadaic acid, vide infra (Ojika, M. Personal communication). Namely, the 7-, 24-, and 27-hydroxyl groups in methyl okadaate (**10**) were protected as benzoate, TBDMS ether, and acetate, respectively in this order, to yield **13**. Selective deprotection at 24-OH in **13** by aq. HF–AcOH, carbonation of 24-OH in **14** with *N*,*N*′-disuccinimidyl carbonate and Et_3N, and substitution by **7** furnished **15**. Successive deprotection with aq. NaOH–THF followed by NaOH/MeOH produced **4**. Introduction of the biotin unit at 24-OH in **4** was confirmed by the ^1H NMR signal at δ 5.31 for 24-H, instead of that at δ 4.12 in **1**.[1] Virtually unchanged chemical shifts of 7-H and 27-H at δ 3.37 and δ 4.15, respectively, from **1** ruled out their acylations.

27-*O*-Biotinylated okadaic acid **5** was synthesized from methyl okadaate (**10**). After protection of the 7- and 24-hydroxyl groups as TBDMS ethers, the disilyl ether **17** was then treated with *N*,*N*′-disuccinimidyl carbonate and

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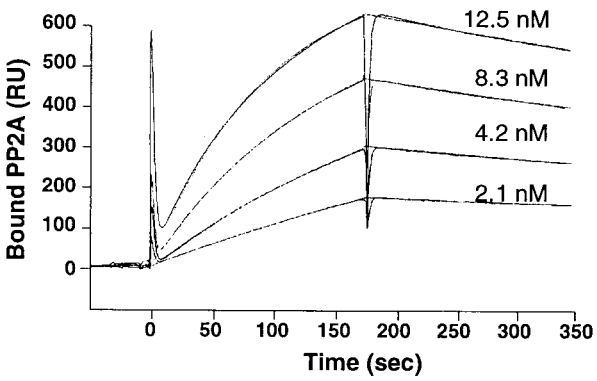

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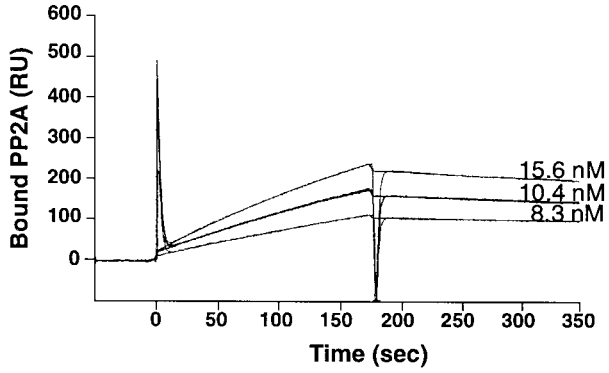


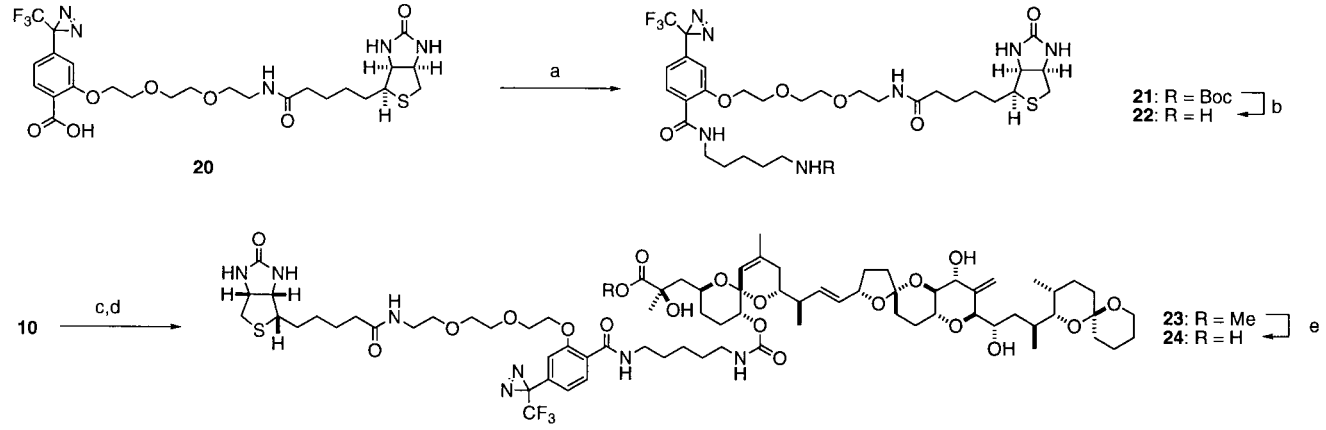
(C)

Immoblized ligand	PP2A (nM)	k_a ($M^{-1}s^{-1}$) x 10^{-5}	k_d (s^{-1}) x 10^4	K_d (nM)	R_{max} (RU)
3	2.1	9.04	8.45	0.94	759
	4.2	9.00	10.00	1.11	768
	8.3	9.33	10.60	1.14	734
	12.5	10.0	9.47	0.95	777
5	8.3	1.49	7.61	5.11	663
	10.4	1.80	9.26	5.14	719
	15.6	1.95	8.32	4.27	659

Figure 3. Dependency of the SPR sensorgram on the concentration of PP2A in the running buffer with the sensor chip with immobilized **3** (A) or **5** (B), and their derived kinetic parameters (C). Experimental conditions are the same as in Fig. 2.

Et_3N, and the mixed carbonate was reacted with **7** to give **18**. Deprotection with aq. HF–AcOH and alkaline hydrolysis gave **5**, where introduction of the biotin unit at 27-OH was confirmed by the ^1H NMR signal at δ 5.32 for 27-H, instead of that at δ 4.08 in **1**.[1] The otherwise close spectrum of **5** to **1** gave the signals at δ 3.33 and δ 4.09 for 7-H and 24-H, respectively.

Each of the four biotin conjugates prepared above (**2**–**5**) gave a single spot on silica gel thin-layer chromatogram



Scheme 2. (a) *t*-Butyl *N*-(5-aminopentyl)carbamate, EDC·HCl, HOBt, DMF, rt, 86%; (b) TFA–CH_2Cl_2 (1:1), rt, 71%; (c) *N*,*N*′-Disuccinimidyl carbonate, Et_3N, CH_3CN, rt; (d) **22**, Et_3N, DMF, rt, quantitative in 2 steps; (e) 1 M aq. NaOH–THF (1:1), 40°C, 75%.

(10% methanol/chloroform) at different R_f values. This and their ^1H NMR spectra justified purity of these samples, which were used in the following analysis.

Surface plasmon resonance analysis

Each biotin conjugate was captured on a streptavidin-coated sensor chip (SA) placed in a flow cell of an SPR instrument (BIACORE™ X, Biacore AB) through binding between biotin and streptavidin, and the unbound conjugate was washed out by the running buffer (data not shown). After an equilibrium was reached, PP2A was introduced to the flow cell from the time zero onward, and then the interaction between immobilized okadaic acid and the enzyme was monitored as the change in resonance unit (Fig. 2).[11]

In accordance with marked reduction in affinity to PP2A previously reported on C1 esters of okadaic acid (**2**), no significant change in sensorgram was observed for the chip bearing the C1-biotin conjugate. On the other hand, when the C7-biotin conjugate (**3**) was loaded on the sensor chip, the change of resonance (*Y*-axis) kept increasing until the PP2A solution was replaced with the running buffer at 180 s. The PP2A solution preincubated with **1** did not cause any change in the refractive index on the sensorgram, confirming this binding to be specific. C24-Biotin conjugate (**4**) and C27-biotin conjugate (**5**) exhibited moderate binding affinity to PP2A. The affinity of the immobilized conjugates to PP2A was in the order of **3**>**5**>**4**>**2**.

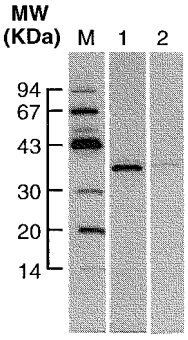


Figure 4. Electrophoregram of photolabeled PP2A by photoaffinity probe **24**. A PP2A solution (100 μL) in the absence (lane 1) or presence (lane 2) of okadaic acid **1** (12.5 μM) was incubated with **24** (330 nM) for 1 h, and irradiated at 360 nm for 1 h. After the SDS-PAGE gel was blotted to a nitrocellulose membrane, the membrane was immersed in T-PBS buffer containing HRP-conjugated streptavidin for 1 h, and chemiluminescence was exposed to an autoradiography film.

As shown in Fig. 3, 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 **3** and **5** to be 9.3×10^5 M^{-1} s^{-1} and 1.8×10^5 M^{-1} s^{-1}, and their dissociation rate constants (k_d) to be 9.6×10^{-4} s^{-1} and 8.4×10^{-4} s^{-1}, respectively. They gave rise to the calculated dissociation constants in equilibrium (K_d) of 1.0 nM for **3** and 4.8 nM for **5**. Substantial close values of calculated K_d and maximum binding (R_{max}), i.e. the amount of the immobilized ligand, regardless of varied PP2A concentrations warrant reliability of these kinetic data. These results indicate that these two biotinylated okadaic acids retained binding affinity to PP2A to different degrees,[14] and the extent of interaction was successfully evaluated by SPR, supporting the previous observation on structure–activity relationships in reference to the derivatized position. Thus this assay system would be applicable to the binding studies of the okadaic acid derivatives to other protein phosphatase preparations in conjunction with their inhibitory action and also the search for other binding proteins of okadaic acid as described below.

Photoaffinity labeling

Based on the above results, a biotinyl photoaffinity probe **20**[10a] was introduced into 7-OH of okadaic acid (Scheme 2). First to insert a spacer between okadaic acid and the probe, **20** was conjugated with *t*-butyl *N*-(5-aminopentyl)carbamate, and deprotection with trifluoroacetic acid gave **22**. Methyl okadaate (**10**) was activated with *N*,*N*′-disuccinimidyl carbonate in the presence of Et_3N, and then treated with **22**. Deprotection in aqueous basic condition and purification by flash chromatography afforded okadaic acid bearing the photoaffinity probe (**24**). The substitution at 7-OH was confirmed in the same manner as the above with observation of the 7-H NMR signal at δ 4.60.

After the compound **24** was incubated with PP2A in a buffer

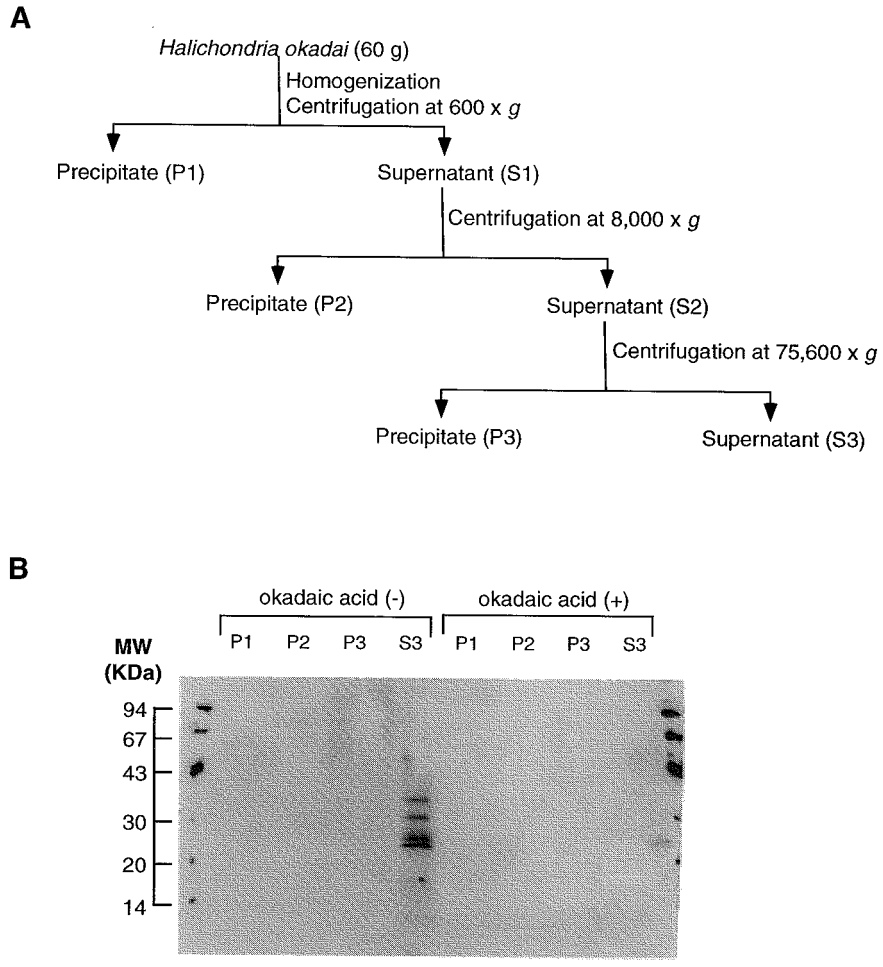


Figure 5. Electrophoregram of photolabeled proteins in *H. okadai* by photoaffinity probe **24**. Proteins from crude extracts from *H. okadai* (60 g) were separated by stepwise centrifugation to give: (A) three precipitates (P1–P3) and one supernatant (S3), (B) and protein solutions P1–P3 and S3 in the absence or presence of okadaic acid (12.5 μM) were photolabeled in the same manner as in Fig. 4.

solution at 4°C for 1 h, the reaction mixture was irradiated at 365 nm for 40 min. To identify possible labeling due to nonspecific binding, the same procedure was repeated in the presence of excess okadaic acid. Each mixture was solubilized in aqueous SDS, and subjected to SDS-PAGE. Proteins on the gel were blotted to a nitrocellulose membrane, and biotin conjugates were bound to horseradish peroxidase (HRP)-conjugated streptavidin. The membrane was then incubated with a substrate of the enzyme, and the chemiluminescence was exposed to a film. As shown in Fig. 4, the probe was found to photolabel PP2A (ca. 37 kDa for the catalytic subunit) in lane 1 but not in lane 2. The comparison between the two lanes indicated that the binding of **24** to PP2A is completely competitive. Though Nishiwaki et al. reported that methyl 7-*O*-(4-azidobenzoyl)-27-[^{3}H]okadaate labeled the catalytic subunit of PP2A, the amino acid sequence of the labeled domain has not been clarified.[3b] Since the present results were obtained by a radiochemical-free detection of labeled proteins, LC-MS/MS techniques will be applicable to precise identification of the labeled region.[9,15]

This photoaffinity labeling was next applied to possible identification of okadaic acid-binding protein(s) hitherto unknown in a sponge *Halichondria okadai*, from which okadaic acid was originally isolated.[1] As widely accepted at the present, okadaic acid must not originate from the sponge but from symbiotic or ingested microbe. Since both phosphorylation and dephosphorylation of proteins are inevitable process in cell signaling pathway, amino acid sequences for the catalytic subunit of protein phosphatases are highly conserved.[16] To consider toxicity of okadaic acid, a simple question has been raised how the sponge protects itself from such a potent phosphatase inhibitor. Putting aside the biological roles of okadaic acid in the sponge which has been obscure so far in spite of a lot of investigations,[17] we suspected the marine sponge has binding proteins to reduce free concentration of okadaic acid in their cells, and carried out photoaffinity labeling experiments by using crude extracts from the sponge.

Sixty grams of *H. okadai* was homogenized in Waring blender, and centrifuged at 600×*g* for 10 min. The obtained supernatant was successively centrifuged at 8000×*g* for 10 min and 75,600×*g* for 1 h (Fig. 5A). The fractionated precipitates (P1–P3) and one supernatant (S3) were subjected to photoaffinity labeling experiments. As shown in Fig. 5B, three proteins in S3 were specifically labeled by the ligand, while labeling was not observed in the other fractions. Ammonium sulfate precipitation, ion exchange

chromatography, hydrophobic gel chromatography, and gel filtration chromatography so far made us purify two okadaic acid binding proteins. Further characterizations of these proteins, including sequential analysis, are currently underway and will be reported elsewhere in due course.

In conclusion at the present, we believe that the okadaic acid photoaffinity probe **24**, together with 7-biotinylated okadaic acid **3**, are evidently versatile reagents to investigate okadaic acid binding proteins.

Experimental

Materials

The marine sponge *Halichondria okadai* was collected off at the coast of Aburatsubo Bay in Kanagawa Prefecture. Okadaic acid was isolated from the sponge as described in the literature.[1b] Protein phosphatase 2A was purchased from Wako Pure Chemicals. BIACORE™ X (Biacore AB, Uppsala) was used as the surface plasmon resonance apparatus. Sensor plates SA, with preimmobilized streptavidin, were purchased from Biacore AB. Stepwise syntheses of biotinylated okadaic acids were monitored by ^{1}H NMR spectroscopy (500 MHz) on Brucker DRX-500 or JEOL A-500, and FABMS on JEOL SX102L or ESIMS on Micromass Q-tof 2. Chemical shifts in the NMR data are referenced to $CHCl_3$ at δ 7.24, or CHD_2OD at δ 3.30. Photocrosslinking was performed by an XX-15L lamp (UVP, Inc.). Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F254 precoated plates (0.25 mm thickness; E. Merck). Column chromatography in the normal phase was on 230–400 mesh silica gel 60 (E. Merck), and that in the reversed-phase on YMC gel ODS-AM 120-S50 (YMC). Other reagents and solvents were used as supplied from the vendors unless otherwise noted.

Synthesis

***t*-Butyl 5-biotinamidopentylcarbamate (6).** A solution of D-biotin (150 mg, 610 μmol), *t*-butyl *N*-(5-aminopentyl)-carbamate (148 mg, 732 μmol), HOBt (100 mg, 732 μmol), and EDC·HCl (141 mg, 732 μmol) in DMF (2.5 mL) was treated with Et_3N (1 mL), and stirred at room temperature for 3 h. After the solution was concentrated under vacuum, it was diluted with EtOAc (100 mL), and washed with brine (20 mL×2). Drying over $MgSO_4$, concentration, and flash chromatography (5–10% MeOH/$CHCl_3$) gave **6** (136 mg, 52%): ^{1}H NMR ($CDCl_3$) δ 6.42 (br, 1H), 6.25 (br, 1H), 5.60 (br, 1H), 4.69 (br, 1H), 4.49 (dd, *J*=7.7, 5.0 Hz, 1H), 4.29 (dd, *J*=7.5, 4.8 Hz, 1H), 3.19 (td, *J*=6.7, 6.2 Hz, 2H), 3.12 (td, *J*=7.5, 4.6 Hz, 1H), 3.08 (brtd, *J*=6.6, 6.3 Hz, 2H), 2.88 (dd, *J*=12.8, 4.9 Hz, 1H), 2.71 (d, *J*=12.8 Hz, 1H), 2.18 (t, *J*=7.5 Hz, 2H), 1.75–1.61 (m, 6H), 1.51–1.43 (m, 6H), 1.41 (s, 9H).

5-Biotinamidopentylamine (7). To a solution of **6** (136 mg) in CH_2Cl_2 (2.0 mL), trifluoroacetic acid (1.0 mL) was added dropwise at 0°C, and the mixture was stirred at room temperature for 2 h. After the solvent was removed under vacuum, reversed-phase chromatography (0–50% aq. MeOH) followed by lyophilization gave **7** as its trifluoroacetic salt (136.8 mg, quantitative): ^{1}H NMR (CD_3OD) δ 4.45 (dd, *J*=8.0, 5.5 Hz, 1H), 4.26 (dd, *J*=7.5, 4.5 Hz, 1H), 3.18–3.12 (m, 3H), 2.90–2.86 (m, 3H), 2.66 (d, *J*=13.0 Hz, 1H), 2.16 (t, *J*=7.5 Hz, 2H), 1.71–1.48 (m, 8H), 1.42–1.33 (m, 4H).

***N*-(5-Biotinamidopentyl)okadaamide (2).** A solution of okadaic acid (**1**; 500 μg), **7** (1.0 mg), HOBt (1.0 mg), EDC·HCl (1.0 mg) in DMF (50 μL) was treated with Et_3N (50 μL), and stirred for 4 h at room temperature. After the solvent was removed under vacuum, the concentrate was dissolved in H_2O (500 μL), and extracted with EtOAc (500 μL×3). The organic layer was concentrated, and subjected to reversed-phase HPLC (YMC Pack ODS-AM 250, ϕ 10×250 mm, 60% aq. CH_3CN). The eluate at 30–33 mL were collected based on absorption at 210 nm: ^{1}H NMR ($CDCl_3$) δ 5.45 (dd, *J*=15.0, 8.0 Hz, 1H), 5.39 (s, 1H), 5.35 (dd, *J*=15.0, 9.0 Hz, 1H), 5.31 (s, 1H), 5.04 (s, 1H), 4.51 (m, 2H), 4.31 (dd, *J*=7.5, 4.0 Hz, 1H), 4.09–4.03 (m, 2H), 3.97–3.93 (m, 2H), 3.72 (m, 1H), 3.67–3.60 (m, 2H), 3.53 (m, 1H), 3.38–3.28 (m, 4H), 3.26 (dd, *J*=10.5, 2.0 Hz, 1H), 3.15 (td, *J*=8.0, 4.5 Hz, 1H), 3.08 (br, 2H), 2.91 (dd, *J*=12.5, 5.0 Hz, 1H), 2.71 (d, *J*=13.0 Hz, 1H), 2.33 (t, *J*=7.5 Hz, 2H), 2.30–1.20 (m, 49H), 1.04 (d, *J*=6.5 Hz, 3H), 0.94 (d, *J*=6.5 Hz, 3H), 0.90 (d, *J*=7.0 Hz, 3H); HRMS (FAB) calcd for $C_{59}H_{94}O_{14}SN_4Na$ ($[M+Na]^+$) 1137.6385, found 1137.6371.

***p*-Bromophenacyl okadaate (8).** A solution of **1** (3.0 mg) and bromophenacyl bromide (2.0 mg) in 4.6% Et_3N/acetone (500 μL) was stirred at 40°C for 3 h. Concentration and flash chromatography (0–5% MeOH/$CHCl_3$) gave *p*-bromophenacyl okadaate **8** (3.5 mg, 94%): ^{1}H NMR ($CDCl_3$) δ 7.76 (dt, *J*=8.5, 2.0 Hz, 2H), 7.64 (dt, *J*=8.5, 2.0 Hz, 2H), 5.56 (dd, *J*=15.0, 7.5 Hz, 1H), 5.50 (d, *J*=16.5 Hz, 1H), 5.43 (dd, *J*=15.0, 7.5 Hz, 1H), 5.34 (s, 1H), 5.32 (s, 1H), 5.23 (d, *J*=16.5 Hz, 1H), 5.03 (s, 1H), 4.41 (td, *J*=8.0, 7.0 Hz, 1H), 4.13–4.02 (m, 3H), 3.91 (d, *J*=9.5 Hz, 1H), 3.74 (m, 1H), 3.65 (td, *J*=11.0, 3.0 Hz, 1H), 3.59–3.50 (m, 2H), 3.46–3.38 (m, 2H), 3.26 (dd, *J*=10.0, 2.0 Hz, 1H), 2.30–1.23 (m, 37H), 1.03 (d, *J*=7.0 Hz, 3H), 0.99 (d, *J*=6.5 Hz, 3H), 0.90 (d, *J*=7.0 Hz, 3H); FABMS 1023.4, 1025.4 $[M+Na]^+$.

***p*-Bromophenacyl 7-*O*-(5-biotinamidopentylcarbamoyl)-okadaate (9).** A solution of **8** (1 mg) and *N*,*N*′-disuccinimidyl carbonate (2 mg) in CH_3CN (50 μL) was treated with Et_3N (5 μL), and stirred at room temperature for 5 h. After the reaction was quenched with saturated aq. $NaHCO_3$ (20 μL), the solvent was removed under stream of N_2 gas. The residue was suspended in saturated aq. $NaHCO_3$ (500 μL), and extracted with EtOAc (500 μL×3). The organic layer was washed with brine (200 μL), and the solvent was evaporated under vacuum. The mixed carbonate in DMF (50 μL) was added dropwise with 5-biotinamidoamylamine (**7**; 10 mg) in 2.8% Et_3N/DMF (150 μL), and the mixture was stirred at room temperature for 12 h. After the solvent was evaporated under vacuum, the residue suspended in saturated aq. $NaHCO_3$ (500 μL) was extracted with EtOAc (500 μL×3). Washing with brine (200 μL), concentration, and flash chromatography (0–10% MeOH/$CHCl_3$) gave **9**: ^{1}H NMR ($CDCl_3$) δ 7.75 (dt, *J*=8.5, 2.0 Hz, 2H), 7.63 (dt, *J*=8.5, 2.0 Hz, 2H), 5.62 (dd, *J*=15.0, 7.5 Hz,

1H), 5.50 (d, *J*=16.5 Hz, 1H), 5.45 (dd, *J*=15.0, 8.5 Hz, 1H), 5.34 (s, 1H), 5.32 (s, 1H), 5.23 (d, *J*=16.5 Hz, 1H), 5.03 (s, 1H), 4.60 (dd, *J*=11.8, 4.2 Hz, 1H), 4.54 (dd, *J*=8.0, 5.5 Hz, 1H), 4.34 (dd, *J*=7.5, 4.5 Hz, 1H), 4.24 (d, *J*=10.0 Hz, 1H), 4.13 (d, *J*=10.0 Hz, 1H), 4.06 (m, 1H), 3.91 (d, *J*=10.0 Hz, 1H), 3.85–3.45 (m, 4H), 3.45–3.35 (m, 3H), 3.26 (dd, *J*=10.0, 2.0 Hz, 1H), 3.20–3.12 (m, 3H), 2.91 (dd, *J*=12.5, 5.0 Hz, 1H), 2.72 (d, *J*=13.0 Hz, 1H), 2.36–1.18 (m, 51H), 1.03 (d, *J*=7.0 Hz, 3H), 0.99 (d, *J*=6.5 Hz, 3H), 0.90 (d, *J*=7.0 Hz, 3H); FABMS 1377.7, 1379.7 $[M+Na]^+$.

7-*O*-(5-Biotinamidopentylcarbamoyl)okadaic acid (3). A solution of **9** in AcOH (100 μL) was stirred over activated Zn powder (20 mg) at room temperature for 5 h. The Zn powder was filtered, and the solvent was evaporated from the filtrate. The residue suspended in H_2O (500 μL) was extracted with 1% AcOH/EtOAc (500 μL). Concentration and flash chromatography ($CHCl_3$–MeOH–AcOH, 87:10:3) gave **3** (625 μg, 54% in 3 steps): ^1H NMR ($CDCl_3$, 500 MHz) δ 5.63 (dd, *J*=15.0, 8.5 Hz, 1H), 5.52–5.46 (m, 2H), 5.36 (s, 1H), 5.23 (s, 1H), 5.03 (s, 1H), 4.57 (dd, *J*=12.0, 4.5 Hz, 1H), 4.52–4.45 (m, 2H), 4.31 (dd, *J*=7.5, 4.5 Hz, 1H), 4.12–4.02 (m, 3H), 3.91 (d, *J*=10.0 Hz, 1H), 3.75–3.45 (m, 4H), 3.42–3.32 (m, 3H), 3.27 (dd, *J*=10.0, 2.0 Hz, 1H), 3.20–3.10 (m, 3H), 2.91 (dd, *J*=12.5, 5.0 Hz, 1H), 2.72 (d, *J*=13.0 Hz, 1H), 2.36–1.18 (m, 51H), 1.03 (d, *J*=6.5 Hz, 3H), 0.99 (d, *J*=6.5 Hz, 3H), 0.90 (d, *J*=7.0 Hz, 3H); HRMS (FAB) calcd for $C_{60}H_{94}O_{16}SN_4Na$ ($[M+Na]^+$) 1181.6283, found 1181.6283.

Methyl okadaate (10). A solution of **1** (3.0 mg) in benzene–MeOH (150 μL, 1:1) was treated with 2.0 M $TMSCHN_2$ in hexane (18 μL), and stirred at room temperature for 10 min. The reaction was terminated with AcOH (3 drops). Concentration and flash chromatography (5% MeOH/$CHCl_3$) gave methyl ester **10** (3.2 mg, quantitative): ^1H NMR ($CDCl_3$) δ 5.54 (dd, *J*=15.0, 8.0 Hz, 1H), 5.48 (dd, *J*=15.0, 7.0 Hz, 1H), 5.38 (s, 1H), 5.28 (s, 1H), 5.04 (s, 1H), 4.46 (dd, *J*=8.5, 7.0 Hz, 1H), 4.11–4.01 (m, 2H), 3.95–3.88 (m, 2H), 3.79 (s, 3H), 3.67–3.51 (m, 4H), 3.42–3.35 (m, 2H), 3.27 (dd, *J*=10.0, 2.0 Hz, 1H), 2.32–1.22 (m, 37H), 1.04 (d, *J*=6.5 Hz, 3H), 1.01 (d, *J*=6.5 Hz, 3H), 0.90 (d, *J*=6.5 Hz, 3H); FABMS 841.5 $[M+Na]^+$.

Methyl 27-*O*-acetyl-7-*O*-benzoyl-24-*O*-(*t*-butyldimethylsilyl)okadaate (13). A solution of methyl ester **10** (500 μg) and Et_3N (5 μL) in CH_2Cl_2 (50 μL) was treated with benzoyl chloride (5 μL) in CH_2Cl_2 (50 μL), and stirred at room temperature for 20 h. After the solution was cooled to 0°C, MeOH (20 μL) was added to terminate the reaction. It was diluted with $CHCl_3$ (500 μL), washed with H_2O (500 μL), and the solvent was evaporated to afford **11** to be directly used in the next step. To a solution of the above concentrate (**11**) and imidazole (11.5 mg) in DMF (100 μL), *t*-butyldimethylsilyl chloride (12.6 mg) was added portionwise at 0°C, and the solution was stirred at room temperature for 1 h. The reaction was terminated with H_2O (500 μL), and the mixture was extracted with 25% EtOAc/hexane (500 μL). Evaporation of the solvent under vacuum gave **12**, which was again used in the following reaction without further purification.

A solution of **12** in pyridine (100 μL) was treated with Ac_2O (5 μL) at 0°C, and stirred at room temperature for 14 h. After the reaction was quenched with MeOH (1 μL), the solution was diluted with H_2O (500 μL), and extracted with 25% EtOAc/hexane (500 μL×3). Concentration and flash chromatography (25% EtOAc/hexane) gave **13** (470 μg, 68% in 3 steps): ^1H NMR ($CDCl_3$) δ 7.92 (d, *J*=7.0 Hz, 2H), 7.50 (t, *J*=7.5 Hz, 1H), 7.39 (t, *J*=7.5 Hz, 2H), 5.75 (dd, *J*=15.5, 7.5 Hz, 1H), 5.55 (d, *J*=16.0, 7.0 Hz, 1H), 5.52 (t, *J*=10.0 Hz, 1H), 5.36 (s, 1H), 5.21 (s, 1H), 5.02 (s, 1H), 4.92 (dd, *J*=11.5, 4.5 Hz, 1H), 4.62 (br, 1H), 4.51 (td, *J*=9.0, 7.0 Hz, 1H), 4.12–4.02 (m, 4H), 3.83 (m, 1H), 3.74 (s, 3H), 3.72–3.52 (m, 3H), 3.38 (t, *J*=10 Hz, 1H), 3.26 (dd, *J*=10.0, 2.0 Hz, 1H), 2.06 (s, 3H), 2.42–1.24 (m, 37H), 1.13 (d, *J*=7.0 Hz, 3H), 1.04 (d, *J*=6.5 Hz, 3H), 0.90 (s, 3H), 0.87 (d, *J*=6.5 Hz, 3H), 0.08 (s, 3H) 0.05 (s, 3H); FABMS *m/z* 1101.5 $[M+Na]^+$.

24-*O*-(5-Biotinamidopentylcarbamoyl)okadaic acid (4). A solution of **13** (470 μg) in 47% aq. HF–AcOH (100 μL, 1:4) was stirred at room temperature for 75 min. The reaction was quenched with saturated aq. $NaHCO_3$ (500 μL), and the product was extracted with $CHCl_3$ (500 μL×3). Flash chromatography of the concentrate (25% AcOEt/hexane) gave alcohol **14** (360 μg, 85%).

A solution of alcohol **14** (360 μg) and *N*,*N*′-disuccinimidyl carbonate (40 mg) in CH_3CN (100 μL) was treated with Et_3N (20 μL), and stirred at room temperature for 24 h. After evaporation of the solvent under stream of N_2 gas, the residue suspended in saturated aq. $NaHCO_3$ (750 μL) was extracted with EtOAc (750 μL×3). The organic layer was washed with brine (500 μL), and the solvent was evaporated under vacuum. To a solution of the concentrate in DMF (20 μL) was added a solution of amine **7** (10 mg) in 20% Et_3N/DMF (100 μL), and the mixture was stirred at room temperature for 7 h. After the solvent was removed under stream of N_2 gas, the residue was partitioned between H_2O (750 μL) and EtOAc (750 μL). Flash chromatography (5% MeOH/$CHCl_3$) of the organic concentrate afforded **15** (425 μg, 85% in 2 steps).

A solution of **15** (425 μg) in THF (50 μL) was added with 1 M aq. NaOH (50 μL) at 0°C, and stirred at 40°C for 3 h. It was cooled to 0°C, and then neutralized with saturated aq. NH_4Cl (500 μL). The solution was extracted with 1% AcOH/EtOAc (500 μL×5), and the solvent was removed to give **16**: ^1H NMR ($CDCl_3$) δ 7.91 (d, *J*=7.0 Hz, 2H), 7.51 (t, *J*=7.5 Hz, 1H), 7.38 (t, *J*=7.5 Hz, 2H), 6.38 (br, 1H), 6.32 (br, 1H), 5.97 (br, 1H), 5.67 (dd, *J*=15.0, 8.5 Hz, 1H), 5.57 (t, *J*=10.0 Hz, 1H), 5.51 (dd, *J*=15.0, 8.5 Hz, 1H), 5.28–5.20 (m, 3H), 5.18 (s, 1H), 5.06 (br, 1H), 5.01 (s, 1H), 4.90 (dd, *J*=11.5, 4.5 Hz, 1H), 4.52 (dd, *J*=7.5, 4.5 Hz, 1H), 4.42 (td, *J*=9.0, 7.0 Hz, 1H), 4.38 (d, *J*=7.5, 4.5 Hz, 1H), 4.20 (t, *J*=10.5 Hz, 1H), 4.09 (d, *J*=10 Hz, 1H), 3.82 (td, *J*=10.0, 4.5 Hz, 1H), 3.51 (m, 1H), 3.45 (m, 1H), 3.44 (t, *J*=10.0 Hz, 1H), 3.29 (dd, *J*=11.0, 2.0 Hz, 1H), 3.15 (brtd, *J*=6.5, 5.5 Hz), 3.08 (m, 1H), 2.90 (dd, *J*=13.0, 5.0 Hz, 1H), 2.69 (d, *J*=13.0 Hz, 1H), 2.32 (t, *J*=7.5 Hz, 2H), 2.07 (s, 3H), 2.25–1.15 (m, 49H), 1.06 (d, *J*=6.5 Hz, 3H), 1.02 (d, *J*=6.5 Hz, 3H), 0.86 (d, *J*=7.0 Hz, 3H).

A solution of the methyl ester **16** in 5% w/v NaOH/MeOH (100 μL) was stirred at 60°C for 3 h. The solution was neutralized with AcOH (3 drops) at 0°C, and the solvent was removed under stream of N_2. The concentrate was diluted with H_2O (500 μL), extracted with EtOAc (500 μL×5), and the solvent was evaporated under vacuum to afford **4** (260 μg, 71% in 2 steps): ^{1}H NMR ($CDCl_3$) δ 6.43 (br, 2H), 5.93 (br, 1H), 5.82 (br, 1H), d 5.65 (dd, *J*=15.0, 9.0 Hz, 1H), 5.42 (dd, *J*=15.0, 9.0 Hz, 1H), 5.33 (m, 1H), 5.31 (d, *J*=9.0 Hz)), 5.18 (s, 1H), 5.05 (s, 1H), 4.99 (s, 1H), 4.50 (dd, *J*=7.5, 4.5 Hz, 1H), 4.31 (m, 2H), 4.15 (t, *J*=9.5 Hz, 1H), 4.02 (t, *J*=11.0 Hz, 1H), 3.91 (d, *J*=10.5 Hz, 1H), 3.70–3.60 (m, 3H), 3.52–3.43 (m, 4H), 3.37 (dd, *J*=11.5, 4.5 Hz, 1H), 3.27 (dd, *J*=11.0, 2.0 Hz, 1H), 3.14 (m, 1H), 3.06 (m, 1H), 2.90 (dd, *J*=13.0, 5.0 Hz, 1H), 2.69 (d, *J*=13.0 Hz, 1H), 2.33 (t, *J*=7.5 Hz, 2H), 2.30–1.20 (m, 49H), 1.07 (d, *J*=6.5 Hz, 3H), 0.99 (d, *J*=6.5 Hz, 3H), 0.90 (d, *J*=7.0 Hz, 3H); HRMS (FAB) calcd for $C_{60}H_{94}O_{16}SN_4Na$ ($[M+Na]^+$) 1181.6283, found 1181.6293.

Methyl 7-*O*,24-*O*-bis(*t*-butyldimethylsilyl)okadaate (17). A solution of methyl okadaate (**10**; 2.0 mg) and imidazole (11.5 mg) in DMF (100 μL) was added portionwise with *t*-butyldimethylsilyl chloride (12.6 mg) at 0°C, and stirred at room temperature for 2 h. The reaction was quenched with H_2O (1.0 mL), and extracted with 25% EtOAc/hexane (700 μL×4). Concentration and flash chromatography (25% EtOAc/hexane) gave **17** (2.9 mg, 95%): ^{1}H NMR ($CDCl_3$) δ 5.70 (dd, *J*=15.5, 7.5 Hz, 1H), 5.49 (dd, *J*=15.5, 7.0 Hz, 1H), 5.36 (s, 1H), 5.12 (s, 1H), 5.00 (s, 1H), 4.89 (s, 1H), 4.47 (td, *J*=7.5, 7.0 Hz, 1H), 4.10–4.04 (m, 2H), 3.95–3.88 (m, 2H), 3.73 (m, 1H), 3.72 (s, 3H), 3.65 (td, *J*=11.5, 2.5 Hz, 1H), 3.54–3.50 (m, 2H), 3.45–3.41 (m, 2H), 3.27 (dd, *J*=10.0, 2.0 Hz, 1H), 2.35–1.23 (m, 37H), 1.07 (d, *J*=6.5 Hz, 3H), 1.04 (d, *J*=7.0 Hz, 3H), 0.92 (s, 9H), 0.90 (d, *J*=7.0 Hz, 3H), 0.77 (s, 9H), 0.08 (s, 3H), 0.05 (s, 3H), −0.04 (s, 3H), −0.08 (s, 3H); FABMS *m*/*z* 1069.5 $[M+Na]^+$.

Methyl 27-*O*-(5-biotinamidopentylcarbamoyl)-7-*O*,24-*O*-bis(*t*-butyldimethylsilyl)okadaate (18). A solution of **17** (2.9 mg) and *N*,*N*′-disuccinimidyl carbonate (20 mg) in CH_3CN (100 μL) was treated with Et_3N (20 μL), and stirred at room temperature for 18 h. After evaporation under stream of N_2, the residue was diluted with saturated aq. $NaHCO_3$, and extracted with EtOAc (1 mL×5). Washing with brine (1.0 mL) and concentration gave a mixed carbonate.

To a solution of the above concentrate in DMF (20 μL) was added 5-biotinamidopentylamine (**7**; 10 mg) in Et_3N–DMF (90 μL, 1:8). After stirred at room temperature for 43 h, the solvent was evaporated under stream of N_2 gas, and the residue suspended in H_2O (1.0 mL) was extracted with EtOAc (1.0 mL×5). Concentration and flash chromatography (5% MeOH/$CHCl_3$) gave **18** (900 μg, 28% in 2 steps): ^{1}H NMR ($CDCl_3$) δ 5.69 (dd, *J*=15.5, 7.5 Hz, 1H), 5.50 (dd, *J*=15.5, 7.5 Hz, 1H), 5.40–5.32 (m, 2H), 5.12 (s, 1H), 5.00 (s, 1H), 4.51 (dd, *J*=7.5, 5.0 Hz, 1H), 4.45 (td, *J*=15.0, 7.5 Hz, 1H), 4.33 (dd, *J*=7.5, 5.0 Hz, 1H), 4.09–4.04 (m, 2H), 3.92 (brt, *J*=11.5 Hz, 1H), 3.75 (m, 1H), 3.70 (m, 1H), 3.62 (td, *J*=11.5, 2.0 Hz, 1H), 3.53 (m, 1H), 3.42 (dd, *J*=11.0, 4.0 Hz, 1H), 3.36 (t, *J*=9.5 Hz, 1H), 3.28–3.23 (m, 3H), 3.20–3.13 (m, 3H), 2.93 (dd, *J*=13.0, 5.0 Hz, 1H), 2.72 (d, *J*=12.0 Hz, 1H), 2.39–2.30 (m, 3H), 2.25–1.20 (m, 48H), 1.07 (m, 6H), 0.91 (m, 9H), 0.86 (d, *J*=6.5 Hz, 3H), 0.77 (s, 9H), 0.08 (s, 3H), 0.05 (s, 3H), −0.05 (s, 3H), −0.09 (s, 3H); FABMS *m*/*z* 1423.9 $[M+Na]^+$.

27-*O*-(5-Biotinamidopentylcarbamoyl)okadaic acid (5). A solution of **18** in 47% aq. HF–CH_3CN (40 μL, 1:4) was stirred at room temperature for 45 min, and the solution was neutralized with saturated aq. $NaHCO_3$ (750 μL). Extraction with EtOAc (750 μL×4) and concentration gave **19**, which was used in the following reaction without further purification: ^{1}H NMR ($CDCl_3$) δ 5.56 (dd, *J*=15.0, 8.0 Hz, 1H), 5.47 (dd, *J*=15.0, 8.0 Hz, 1H), 5.38 (s, 1H), 5.34 (m, 1H), 5.30 (s, 1H), 5.04 (s, 1H), 4.51–4.47 (m, 2H), 4.32 (dd, *J*=7.5, 5.0 Hz, 1H), 4.12–4.07 (m, 2H), 3.94 (t, *J*=10.5 Hz, 1H), 3.79 (m, 1H), 3.72 (s, 3H), 3.65–3.45 (m, 3H), 3.37–3.34 (m, 2H), 3.30–3.15 (m, 5H), 3.09 (m, 1H), 2.91 (dd, *J*=13.0, 5.0 Hz, 1H), 2.71 (d, *J*=13.0 Hz, 1H), 2.33 (t, *J*=7.5 Hz, 2H), 2.30–1.20 (m, 49H), 1.06 (d, *J*=6.5 Hz, 3H), 1.01 (d, *J*=6.5 Hz, 3H), 0.86 (d, *J*=7.0, 3H); FABMS *m*/*z* 1195.6 $[M+Na]^+$.

To a solution of **19** in THF (500 μL) was added 1 M aq. NaOH (500 μL), and the mixture was stirred at 40°C for 3 h. The solution was neutralized with saturated aq. NH_4Cl (750 μL) at 0°C, and extracted with 1% AcOH/EtOAc (750 μL×4). The solvent was removed under vacuum to give **5** (480 μg, 71% in 2 steps): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.65 (dd, *J*=15.0, 8.0 Hz, 1H), 5.43 (dd, *J*=15.0, 8.0 Hz, 1H), 5.40 (s, 1H), 5.36 (m, 1H), 5.29 (s, 1H), 5.07 (s, 1H), 4.57 (td, *J*=7.5, 4.5 Hz, 1H), 4.49 (dd, *J*=7.5, 5.0 Hz, 1H), 4.37 (dd, *J*=7.5, 5.0 Hz, 1H), 4.11 (d, *J*=10.0 Hz, 1H), 4.09 (d, *J*=10.0 Hz, 1H), 3.94 (t, *J*=11.5 Hz, 2H), 3.77 (m, 1H), 3.64–3.51 (m, 3H), 3.38–3.27 (m, 5H), 3.23 (td, *J*=6.6, 6.3, 2H), 3.14 (m, 1H), 2.91 (dd, *J*=13.0, 5.0 Hz, 1H), 2.71 (d, *J*=13.0 Hz, 1H), 2.33 (t, *J*=7.5 Hz, 2H), 2.20–1.20 (m, 49H), 1.06 (d, *J*=6.5 Hz, 3H), 1.01 (d, *J*=6.5 Hz, 3H), 0.85 (d, *J*=7.0 Hz, 3H); HRMS (FAB) calcd for $C_{60}H_{94}O_{16}N_4SNa$ ($[M+Na]^+$) 1181.6283, found 1181.6268.

5-[2-(8-Biotinamido-3,6-dioxaoctyl)-4-(3-trifluoromethyl-3*H*-diazirin-3-yl)benzamido]pentylamine (22). A solution of benzoic acid **20** (5 mg, 82 μmol), *t*-butyl *N*-(5-aminopentyl)carbamate (20 mg, 99 μmol), HOBt (13 mg, 95 μmol) and EDC (30 mg, 156 μmol) in DMF (150 μL) was treated with Et_3N (20 μL, 156 μmol) at 0°C, and stirred at room temperature for 72 h. After the solvent was evaporated under vacuum, the residue dissolved in EtOAc (1.0 mL) was washed with brine (1.0 mL). Concentration and flash chromatography ($CHCl_3$–MeOH–AcOH, 80:17:3) gave **21** (5.63 mg, 86%): ^{1}H NMR ($CDCl_3$) δ 8.17 (d, *J*=8.0 Hz, 1H), 8.00 (br, 1H), 6.88 (d, *J*=8.5 Hz, 1H), 6.67 (s, 1H), 6.32 (br, 1H), 5.89 (br, 1H), 5.10 (br, 1H), 4.77 (br, 1H), 4.45 (dd, *J*=8.0, 5.0 Hz, 1H), 4.30–4.20 (m, 3H), 3.87 (t, *J*=4.5 Hz, 2H), 3.67 (br, 2H), 3.61 (br, 2H), 3.51 (t, *J*=5.0 Hz, 2H), 3.45–3.35 (m, 4H), 3.10 (br, 3H), 2.88 (dd, *J*=13.0, 5.0 Hz, 1H), 2.70 (d, *J*=12.5 Hz, 1H), 2.15 (t, *J*=7.5 Hz, 2H), 1.75–1.45 (m, 12H), 1.40 (s, 9H); FABMS *m*/*z* 810.38 $[M+Na]^+$.

A solution of amine **21** in CH_2Cl_2 (150 μL) was treated with trifluoroacetic acid (150 μL) at 0°C, and stirred at room temperature for 30 min. Concentration and flash chromatography ($CHCl_3$–MeOH–AcOH, 80:17:3) gave **22** (3.45 mg, 71%): ^{1}H NMR (CD_3OD) δ 7.96 (d, *J*=8.5 Hz, 1H), 7.03 (d, *J*=8.0 Hz, 1H), 6.86 (s, 1H), 4.46 (dd, *J*=7.5, 5.0 Hz, 1H), 4.30–4.20 (m, 3H), 3.90 (br, 2H), 3.71 (br, 2H), 3.63 (br, 2H), 3.51 (br, 2H), 3.42 (br, 2H), 3.35–3.25 (m, 2H), 3.15 (m, 1H), 2.95–2.87 (m, 3H), 2.69 (d, *J*=12.5 Hz, 1H), 2.17 (t, *J*=7.5 Hz, 2H), 1.75–1.40 (m, 12H).

Methyl 7-*O*-[5-[2-(8-biotinamido-3,6-dioxaoctyl)-4-(3-trifluoromethyl-3*H*-diazirin-3-yl)benzamido]pentylcarbamoyl]okadaate (23). A solution of methyl okadaate (**10**; 500 μg) and *N*,*N*′-disuccinimidyl carbonate (8 mg, 31 μmol) in CH_3CN (100 μL) was treated with Et_3N (20 μL, 156 μmol) at room temperature, and stirred for 3.5 h. After the reaction was quenched with saturated aq. $NaHCO_3$ (100 μL), the solution was concentrated under stream of N_2 gas, diluted again with saturated aq. $NaHCO_3$ (500 μL), and extracted with EtOAc (750 μL×5). Washing with brine (500 μL) and concentration gave a mixed carbonate.

To a solution of the mixed carbamate in DMF (50 μL) was added **22** (3.4 mg) in 10% Et_3N/DMF (110 μL), and the mixture was stirred at room temperature for 29 h. After the solvent was evaporated under vacuum, the concentrate was diluted with H_2O (500 μL), and extracted with EtOAc (700 μL×3). Concentration and flash chromatography (10% MeOH/$CHCl_3$) gave **23** (900 μg, quantitative): ^{1}H NMR ($CDCl_3$) δ 8.17 (d, *J*=8.0 Hz, 1H), 6.88 (d, *J*=8.0 Hz, 1H), 6.68 (s, 1H), 5.58 (dd, *J*=14.5, 7.5 Hz, 1H), 5.49 (dd, *J*=14.5, 7.5 Hz, 1H), 5.37 (s, 1H), 5.23 (s, 1H), 5.03 (s, 1H), 4.59 (dd, *J*=12.0, 4.5), 4.51–4.45 (m, 2H), 4.30 (br, 1H), 4.25 (t, *J*=4.5 Hz, 2H), 4.10 (d, *J*=10 Hz, 1H), 4.06 (t, 9.5 Hz, 1H), 4.00 (t, *J*=10 Hz, 1H), 3.93 (d, *J*=10.0 Hz, 1H), 3.88 (t, *J*=4.5 Hz, 2H), 3.77 (s, 3H), 3.69–3.37 (m, 15H), 3.27 (dd, *J*=10.0, 2.0 Hz, 1H), 3.15–3.02 (m, 3H), 2.90 (dd, *J*=13.0, 5.0 Hz, 1H), 2.71 (d, *J*=12.5 Hz, 1H), 2.33 (t, *J*=7.5 Hz, 2H), 2.40–1.20 (m, 40H), 1.04 (d, *J*=6.5 Hz, 3H), 1.00 (d, *J*=7.0 Hz, 3H), 0.91 (d, *J*=6.50 Hz, 3H).

7-*O*-[5-[2-(8-Biotinamido-3,6-dioxaoctyl)-4-(3-trifluoromethyl-3*H*-diazirin-3-yl)benzamido]pentylcarbamoyl]-okadaic acid (24). A solution of methyl ester **23** (950 μg) in THF (100 μL) was treated with 1 M aq. NaOH (100 μL), and stirred at 40°C for 5 h. The solution was neutralized with AcOH (5 drops) at 0°C, and the solvent was removed under stream of N_2 gas. The concentrate was diluted with H_2O (500 μL), extracted with EtOAc (750 μL×3), and then the organic layer was concentrated to give **24** (710 μg, 75%): ^{1}H NMR ($CDCl_3$) δ 8.17 (d, *J*=8.5 Hz, 1H), 6.88 (d, *J*=8.5 Hz, 1H), 6.68 (s, 1H), 5.67 (dd, *J*=15.0, 8.5 Hz, 1H), 5.48 (dd, *J*=15.0, 8.0 Hz, 1H), 5.40 (s, 1H), 5.24 (s, 1H), 5.03 (s, 1H), 4.60 (dd, *J*=12.5, 5.0 Hz, 1H), 4.55–4.48 (m, 2H), 4.31 (br, 1H), 4.25 (t, *J*=4.5 Hz, 2H), 4.10–4.04 (m, 3H), 3.92 (d, *J*=9.5 Hz, 1H), 3.88 (t, *J*=4.5 Hz, 2H), 3.70–3.30 (m, 15H), 3.27 (dd, *J*=10.0, 2.0 Hz, 1H), 3.15–3.02 (m, 3H), 2.91 (dd, *J*=13.0, 5.0 Hz, 1H), 2.71 (d, *J*=12.5 Hz, 1H), 2.33 (t, *J*=7.5 Hz, 2H), 2.30–1.20 (m, 49H), 1.03 (d, *J*=6.5 Hz, 3H), 0.98 (d, *J*=7.0 Hz, 3H), 0.90 (d, *J*=6.50 Hz, 3H); ESIMS *m*/*z* 1517.6 $[M-H]^-$.

SPR analysis on interaction of immobilized okadaic acids with protein phosphatase 2A

SPR analysis carried out on BIACORE™ X were as following. A streptavidin-immobilized sensor chip was loaded in a flow cell, and contacted with a solution of a biotinylated okadaic acid (4.3 nmol) in a running buffer (100 μL) consisting of 150 mM NaCl, 3.4 mM EDTA, 0.01% Tween® 20, and 10 mM Hepes–NaOH (pH 7.4) at a flow rate of 5.0 μL min^{-1} for 180 s. After a protein solution of a known concentration in the running buffer containing 0.02% (w/v) BSA was injected at 10 μL min^{-1} for 180 s, dissociation of the bound PP2A was monitored for 180 s by washing the sensor chip with the same buffer. The sensor chip was regenerated to be free of bound PP2A by passing 100 mM Na_2CO_3 at a flow rate of 5.0 μL min^{-1} for 180 s. All experiments were carried out at 25°C.

Data transformation and interaction analyses were performed by BIAevaluation™ software equipped to the instrument, with algorithms for numerical integration and global analysis linked to the software. With this software, nonlinear curve fittings were performed to calculate kinetic parameters of k_a and k_d.

Photoaffinity labeling experiments

A protein solution (100 μL) was incubated with **24** (330 nM) for 1 h, and irradiated at 360 nm by an XX-15L lamp (UVP, Inc.) for 1 h. After the proteins were denatured with a buffer consisting of 2.0% SDS, 10% glycerol, 5.0% w/v dithiothreitol, bromophenol blue (a tracking dye, 10 μ mL^{-1}), and 50 mM Tris–HCl (pH 6,8), they were subjected to SDS-PAGE (Ready Gel J 10–20%, BioRad), and electroblotted to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia). The membrane was blocked by 2.0% BSA in T-PBS buffer (PBS buffer containing 0.1% Tween® 20) for 1 h, and washed with the buffer (15 min×1, and 5 min×2). It was then immersed in T-PBS buffer containing horseradish peroxidase (HRP)-conjugated streptavidin (Amersham Pharmacia) for 1 h, and washed with the same buffer (15 min×1, and 5 min×2). After the membrane was reacted with a substrate for the enzyme in aq. H_2O_2 (ECL Western Blotting Reagent, Amersham Pharmacia), chemiluminescence was exposed to an autoradiography film (Hyperfilm-ECL, Amersham Pharmacia) for a desired period, and the film was developed as per manufacturer's recommendation.

Extraction of proteins from a marine sponge *Halichondria okadai*

A marine sponge *Halichondria okadai* (60 g, wet wt.), collected at the coast of Aburatsubo Bay in Kanagawa Prefecture, was washed with a buffer consisting of 540 mM NaCl, 7 mM $(NH_4)_2SO_4$, 0.2 mM Na_2CO_3, 10 mM KCl, 20 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ mL^{-1} leupeptin hemisulfate monohydrate, 1 μ mL^{-1} pepstatin A, 0.5 mM dithiothreitol, and 20 mM Tris–HCl (pH 8.2), cut into small pieces with a razor's edge,

and homogenized in a Waring blender with the same buffer (60 mL). The homogenate was filtered, and then the filtrate was centrifuged at 600×*g* for 10 min. The supernatant was successively centrifuged at 8000×*g* for 10 min, and then 75,600×*g* for 90 min.

Acknowledgements

This work is supported in part by Grant-In-Aids from the Ministry of Education, Science, Sports and Culture of Japan (EYS10780349 to K. K.; SRPA08281105 and SR12470504 to Y. H.). The authors are grateful to Dr M. Ojika of Nagoya University and Dr M. Sasaki in our laboratory of The University of Tokyo for discussion on the derivatization of okadaic acid, to Dr J. Inagawa of Biacore (K. K.) for valuable discussions and suggestions on SPR analysis, Ms M. Izumikawa in our laboratory of The University of Tokyo for mass spectrometry, and to Messrs H. Kusuoku, S. Matsuoka, H. Hori, and K. Otsuki in our laboratory of The University of Tokyo for their help in collecting *H. okadai*.

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