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Coupling a Natural Receptor Protein with an Artificial Receptor to Afford a Semisynthetic Fluorescent Biosensor

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Abstract: An artificial receptor and a signal transducer have been engineered on a lectin (saccharide-binding protein) surface by a post-photoaffinity labeling modification method. Saccharide binding can be directly and selectively read out by the fluorescence changes of the fluorophore via photoinduced electron transfer (PET) mode. Fluorescence titration with various saccharides reveals that molecular recognition by the artificial receptor is successfully coupled to the native binding site of the lectin, producing a novel fluorescent saccharide biosensor showing modulated specificity and enhanced affinity. Designed cooperativity between artificial and native molecular recognition modules was quantitatively demonstrated by the comparison of the binding affinities, and it represents a new strategy in molecular recognition. By using appropriate artificial receptors and various native lectins, this approach may provide many new semisynthetic biosensors for saccharide derivatives such as glycolipids and glycopeptides/proteins. An extended library of lectin-based biosensors is envisioned to be useful for glycome research, a newly emerging field of the post-genomic era.

Introduction

The monitoring of chemical substances of biological importance is required in many fields of biological science and in medical diagnosis.[1−5] In glycome research, which seeks to elucidate the complicated biological functions of saccharides in the post-genomic era, it is essential to sense many saccharides in a high-throughput manner.[6−8] Although native lectins, a family of saccharide-binding proteins, have been used to qualitatively analyze carbohydrates, their low selectivity prevents the accurate high-throughput quantitation and assay of saccharides. Synthetic chemosensors may overcome these limitations, but they have not yet been successfully applied to the detection of complicated oligosaccharides. Advanced mass spectroscopic methods may be useful for the structural analysis of complex oligosaccharides,[9] but these are not so suitable to rapid and convenient sensing of saccharides in a high-throughput manner. We propose that the rational hybridization of naturally occurring receptor proteins with artificial receptor molecules represents a practical, new approach for the construction of sophisticated biosensors. In this report, we show that the introduction of an artificial sugar-binding site into a native lectin greatly enhances the binding affinity of the lectin toward specific saccharides having interaction moieties to both the lectin and the artificial receptor. As a result, one can engineer a fluorescent semisynthetic lectin with modulated saccharide selectivity.

Results and Discussion

Molecular Design of Phenylboronic Acid-Appended ConA.[10] We recently developed a new method (post-photoaffinity labeling modification: P-PALM) to incorporate a unique benzylthiol group proximal to the sugar-binding pocket of a natural lectin[11] (Concanavalin A (ConA), an α-mannoside or α-glucoside binding protein, was used in this study[12]). The benzylthiol was utilized as a reactive tag for fluorescent probes (Scheme 1). Using environmentally sensitive fluorophores (such as the Dansyl derivative, IAEDANS) as a signal transducer, we could sense α-mannoside or α-glucoside derivatives by a

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(11) (a) Hamachi, I.; Nagase, T.; Shinkai, S. *J. Am. Chem. Soc.* **2000**, *122*, 12065. (b) Nagase, T.; Shinkai, S.; Hamachi, I. *Chem. Commun.* **2001**, 229. (c) The conventional peptide digestion, HPLC separation, and tandem mass spectroscopy indicate that the photolabeling site can be assigned to Y100, a proximal amino acid in the saccharide-binding pocket. It is clear that the photoaffinity labeling process of the present P-PALM method is remarkably site-selective on a protein surface. Nagase, T.; Nakata, E.; Shinkai, S.; Hamachi, I. *Chem.−Eur. J.* **2003**, *9*, 3660.
(12) The crystal data of ConA complexed with Man-tri showed the extended interaction between Man-tri and the ConA surface using all the OH groups of Man-tri and several amino acid residues of the ConA surface. The lessened flexibility of the branched Man units of Man-tri due to such an extended interaction may be a factor for no sensing to Man-tri. Lis, H.; Sharon, N. *Chem. Rev.* **1998**, *98*, 637.

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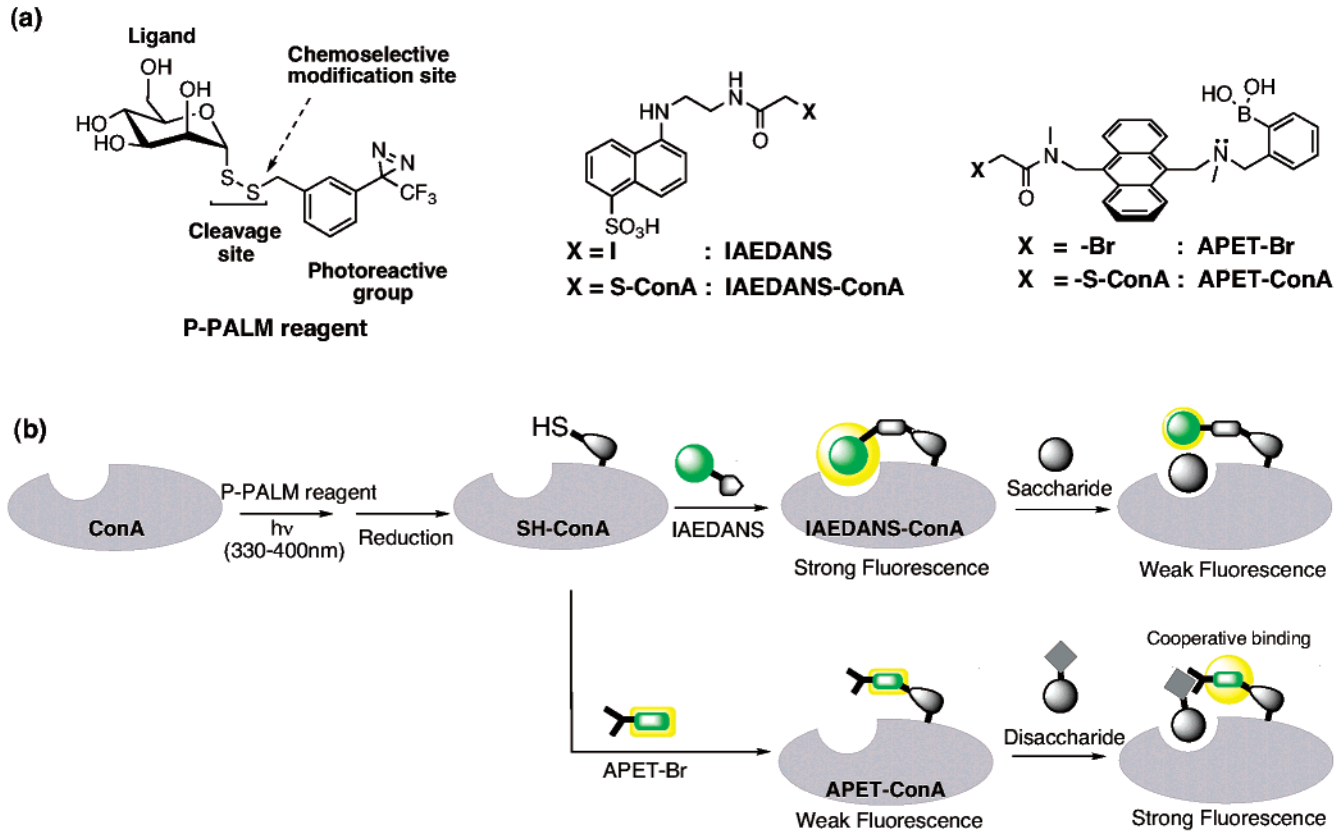
Scheme 1. Molecular Structure of a P-PALM Reagent, IAEDANS, and APET (a) and P-PALM Scheme for Semisynthesis of the Fluorescent Biosensor Discussed in This Study (b)



decrease in fluorescence intensity. This was ascribed to the DANS moiety being ejected upon saccharide binding, causing the fluorophore to move from the hydrophobic binding pocket to the bulk water. The slight red-shift of the fluorescence and decrease in the fluorescence anisotropy upon saccharide complexation reasonably supported such a mechanism. Importantly, the sugar selectivity and binding affinity of the IAEDANS-ConA evaluated by the fluorescence titration are almost identical to those of native ConA determined by isothermal calorimetry.[13]

In addition to the simple fluorescent probe, an artificial receptor can be newly introduced to the proximity of the sugar-binding pocket using the benzylthiol tag attached by P-PALM. Phenylboronic acid (PBA) is capable of binding to 1,2- or 1,3-diol derivatives. According to previous reports by us and others,[14] we equipped PBA with a fluorescent transducer motif, in which a photoinduced electron transfer (PET)-type of readout mode can operate upon complexation with a corresponding diol (Figure 1a). As shown in Scheme 2, bromoacetyl group was introduced to the receptor molecule (APET-Br) as a reactive site to ConA. 9,10-Bis-*N*-methyaminomethyl-anthracene was monoalkylated with *o*-bromomethyl-PBA, and the remaining methylamino site was acylated with bromoacetyl bromide to yield APET-Br. The resultant APET-Br was incubated with SH-ConA to afford APET-ConA via a nucleophilic substitution reaction on the ConA surface (Scheme 1b). APET-ConA was purified through gel chromatography (TOYO PEARL), and the purity was confirmed by analytical reversed-phase HPLC, in which a single ConA band having both UV absorbance and fluorescent emission was detected. In UV−visible spectroscopy of the purified APET-ConA, a strong absorbance at 257 nm and three peaks at 357, 375, and 397 nm are characteristic of the anthracene moiety (Figure 1b). The emission at 407, 432, and 454 nm and excitation spectra at 357, 375, and 397 nm of APET-ConA indicate a clear mirror image (Figure 1c), and the excitation spectrum agrees well with the absorption spectrum. These results reveal the successful attachment of the APET moiety to ConA.

Fluorescence Sensing and Saccharide Selectivity of APET-ConA. Prior to saccharide sensing, we conducted the fluorescence pH titration of APET-ConA with and without a saccharide (Figure 2a). The emission due to the anthracene moiety lessened in the basic pH region. This is ascribed to the emission quenching by the deprotonated tertiary amine group of APET. It is clear that addition of saccharide induces a basic pK_a shift of the tertiary amine, which results in retaining the fluorescence intensity at neutral pH. Consequently, the fluorescence of the anthracene moiety in APET-ConA increases following saccharide addition (Figure 2b). This is in sharp contrast to the decreased fluorescence of IAEDANS-ConA induced by saccharide binding (Figure 2c). Fluorescence intensification by sugars is direct evidence that the PBA site binds to a diol unit of an appropriate sugar and that the PET mechanism works well on a ConA surface. Table 1 summarizes the association constants of engineered ConA for various saccharides compared to native ConA. Figure 3 shows among monosaccharides, it was

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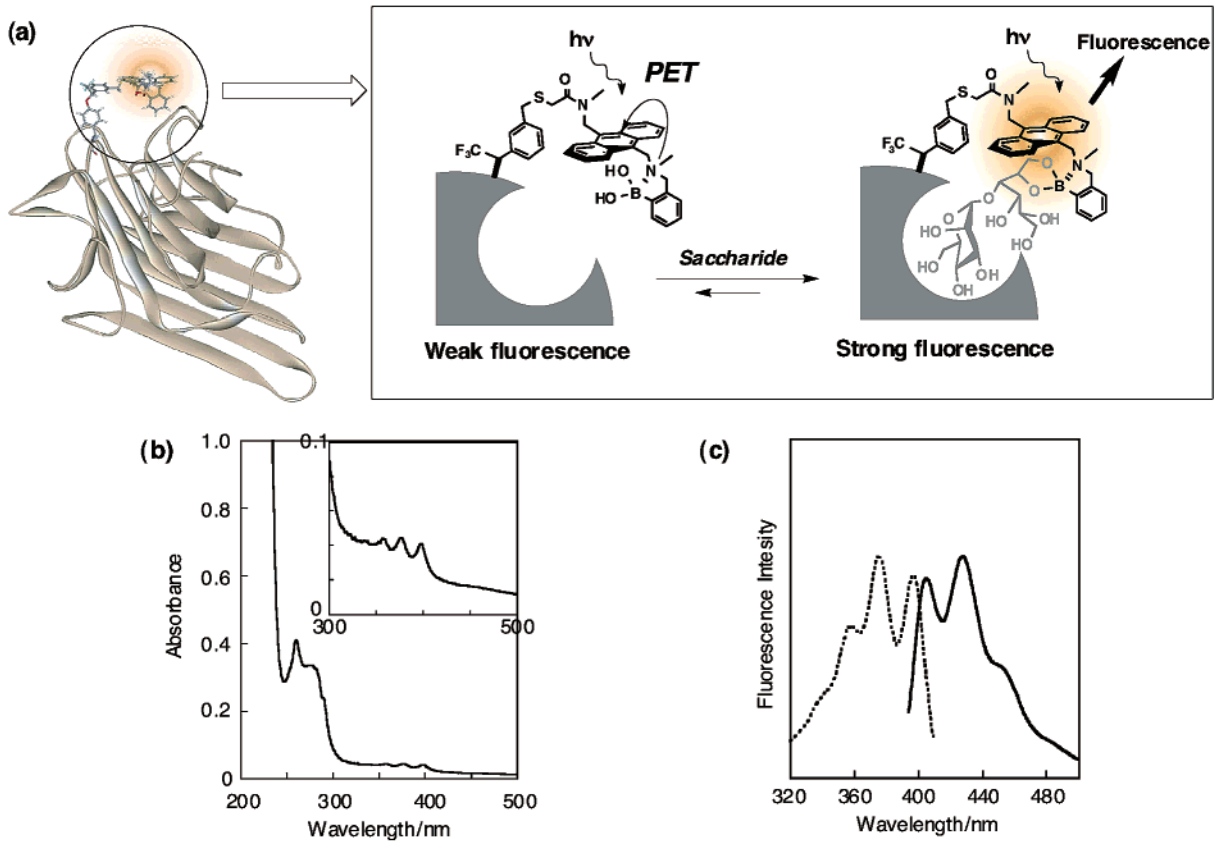
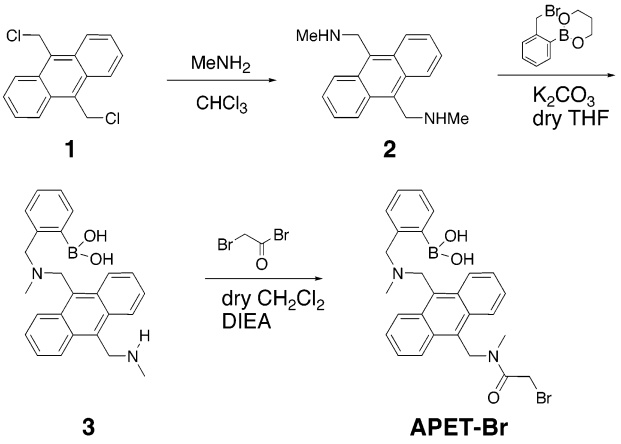


Figure 1. (a) A calculated (Insight II discover) 3D structure of APET-ConA and illustration of PET mechanism of saccharide sensing by APET-ConA. (b) UV−visible spectroscopy of the APET-ConA. (c) Excitation and emission spectra of APET-ConA. [APET-ConA] = 0.7 μM, 50 mM HEPES buffer (pH 7.5), 5 mM $CaCl_2$, 0.1 M NaCl, $T = 15 \pm 1$ °C, $\lambda_{ex} = 375$ nm, $\lambda_{em} = 425$ nm.

Scheme 2. Synthetic Route of APET-Br



found that D-fructose (Fru) and D-glucitol (Glucitol) are moderately bound to APET-ConA, showing good agreement with the binding affinity of PBA. Me-α-Man and Me-α-Glc, good ligands to native ConA, cannot be sensed by APET-ConA because of their poor affinity to the PBA site, while they can be most efficiently sensed by IAEDANS-ConA. These data indicate that any fluorescence signal from APET-ConA must be produced by a saccharide-binding event at the PBA site, not at the native ConA pocket, because the signal transducer is directly coupled to PBA via the PET mechanism (Figure 1a). Figure 3 shows the saccharide structures used in this study.

Most significantly, APET-ConA shows higher affinity and selectivity for specific oligosaccharides over monosaccharides.

Table 1. Comparison of the Association Constants of Semisynthetic ConA with Those of Native ConA and PBA

	log K^d			
saccharide	APET-ConA log $K^{APET-ConA}$	PBA log K^{PBA}	IAEDANS-ConA[e] log $K^{IAEDANS-ConA}$	native ConA[c]
Me-α-Man	*a*	*a*	3.89	4.04
Me-α-Glc	*a*	*a*	3.20	3.48
Fru	2.85	2.28	*a*	*a*
Glucitol	3.52	3.68	*a*	*b*
α-1,3-Man-bi	*a*	*a*	3.76	4.48
Isomal	4.08	*a*	3.11	3.23
Mal	*a*	*a*	2.76	3.20
Cel	*a*	*a*	*a*	*a*
Pal	5.18	2.76	3.20	*b*
Maltitol	6.84	4.51	3.00	*b*
Man-tri	*a*	*a*	5.40	5.40
Maltotriitol	5.26	4.56	3.00	*b*
Man-tetra	5.81	*a*	5.45	5.30

[a] Precise values cannot be determined because of low affinity (log $K <$ 2). [b] Not reported previously. [c] Reported values determined by isothermal titration calorimetry.[13] [d] The averaged value of at least three independent titration experiments. [e] Our previous work.[11c]

Maltitol, a disaccharide consisting of D-Glc and D-glucitol connected by an α-1,4-linkage, is most sensitively detected in the sub-micromolar range by APET-ConA. Fluorescence titration determined an association constant (log $K = 6.84$) (Figure 2c), which is approximately 3 orders of magnitude tighter binding than that by native ConA. Similarly, palatinose, a disaccharide (Pal: D-Glc-α-1,5-D-Fru), which is a poor ligand for native ConA, is effectively sensed (at the micromolar level) by APET-ConA. We found that the association constants for both disaccharides are in good agreement with the value of the

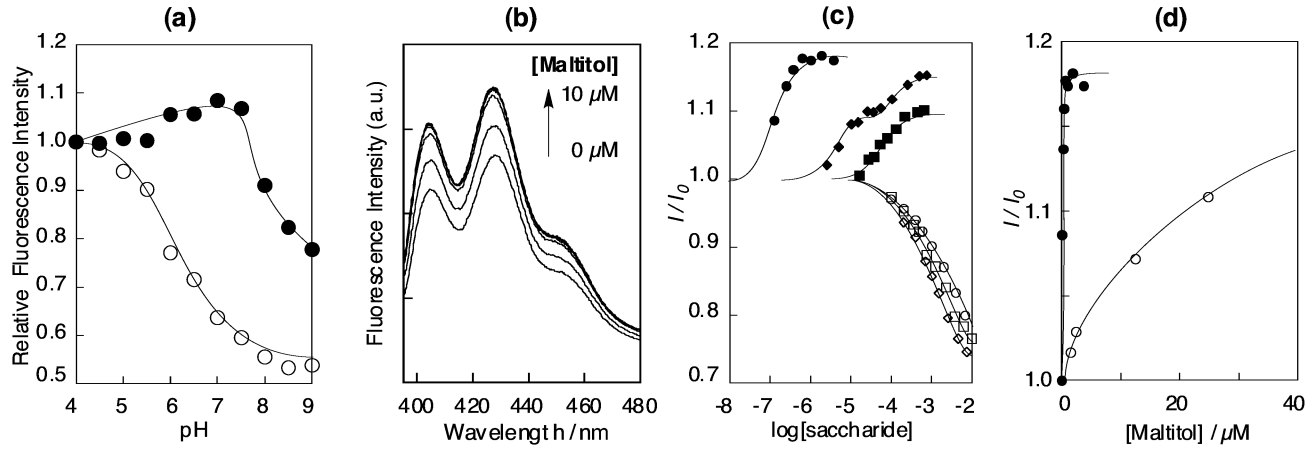


Figure 2. (a) Fluorescence pH titration profiles of APET-ConA in the absence (○) and presence (●) of 2 μM Maltitol. [APET-ConA] = 0.5 μM, 5 mM $CaCl_2$, 0.1 M NaCl, $T = 15 \pm 1$ °C, $\lambda_{ex} = 375$ nm, $\lambda_{em} = 425$ nm. (b) Fluorescence spectral change of APET-ConA upon addition of Maltitol (0−10 μM). [APET-ConA] = 0.7 μM, 50 mM HEPES buffer (pH 7.5), 5 mM $CaCl_2$, 0.1 M NaCl, $T = 15 \pm 1$ °C, $\lambda_{ex} = 375$ nm, $\lambda_{em} = 425$ nm. (c) Fluorescence titration plots of the relative intensity (I/I_0) versus saccharide concentration (log[saccharide]) for APET-ConA and IAEDANS-ConA: APET-ConA (Maltitol (●), Pal (◆), Isomal (■)) and IAEDANS-ConA (Maltitol (○), Pal (◇), Isomal (□)). (d) Fluorescence titration of APET-ConA by addition of Maltitol in the absence (●) and presence (○) of 10 mM of Me-α-Man. [APET-ConA] = 0.7 μM, 50 mM HEPES buffer (pH 7.5), 5 mM $CaCl_2$, 0.1 M NaCl, $T = 15 \pm 1$ °C, $\lambda_{ex} = 375$ nm, $\lambda_{em} = 425$ nm.

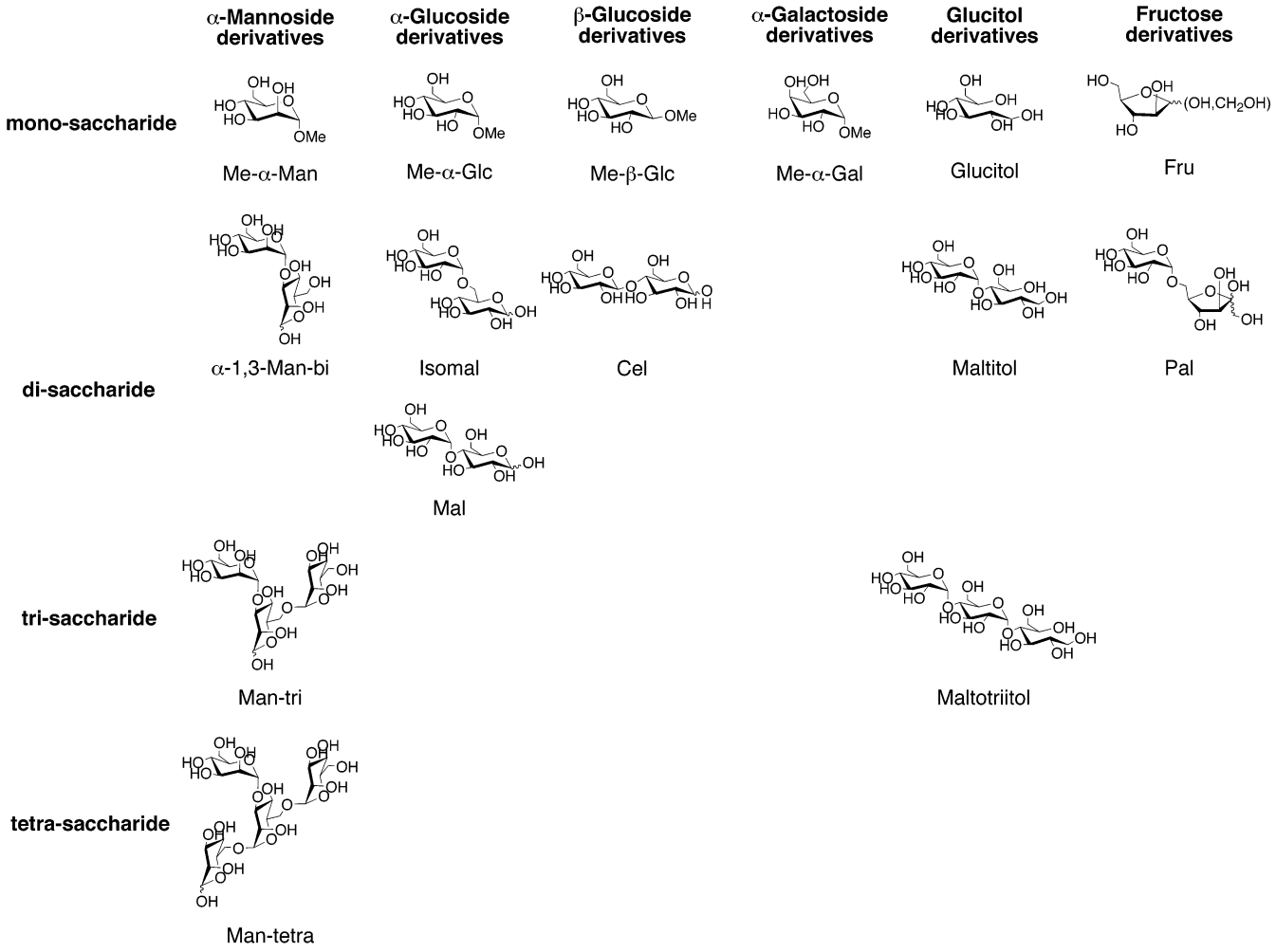


Figure 3. Saccharide structures used in this study. The abbreviation is depicted below each structure.

sum for each monosaccharide fragment, that is, log $K^{\text{APET-ConA}}$ (Maltitol) = log $K^{\text{IAEDANS-ConA}}$ (Me-α-Glc) + log K^{PBA} (Glucitol) and log $K^{\text{APET-ConA}}$ (Pal) = log $K^{\text{IAEDANS-ConA}}$ (Me-α-Glc) + log K^{PBA} (D-Fru) (see Table 1). This implies that two distinct binding sites composed of an artificial framework and a native protein scaffold successfully cooperate in APET-ConA, so as to enhance the affinity for the specific disaccharide by 10^2-10^4-fold relative to each monosaccharide fragment. The cooperative effect of Maltitol binding was confirmed by a competition assay (Figure 2d). In the presence of excess Me-

α-Man (10 mM), the apparent titration curve flattened (an apparent association constant (log $K = 4.76$)), whereas Me-β-Glc did not show such an inhibition effect. The lessened affinity can be reasonably attributed to the masking of the natural binding pocket of ConA by Me-α-Man and not by Me-β-Glc. It is clear that the hybridization of an artificial receptor to ConA successfully allows us to modulate the saccharide selectivity of a natural lectin by enhancing the affinity of the semisynthetic receptor toward the specific saccharides.

On the other hand, cellobiose (Cel: D-Glc-β-1,4-D-Glc), a β-linked disaccharide, is not fluorescently sensed, probably because of the preference of the α-configuration over β found in the native ConA pocket. In addition, regioisomers among the disaccharides composed of two D-Glc units (α-configuration) can be discriminated by APET-ConA, that is, isomaltose (Isomal) with a 1,6-connection is selectively detected but not maltose (Mal) with a 1,4-connection. In Isomal bearing a 1,6-linkage, the second glucose unit can isomerize to a furanose ring, 1,2-diol or 1,3-diol of which is favorable to binding by PBA,[14d,e] whereas such a pyranose/furanose ring isomerization cannot occur in Mal bearing 1,4-linkage. Additionally, it seems that the more flexible motion of the second glucose unit through the 1,6-connection is readily bound to the PBA site of APET-ConA. Among the more complicated oligosaccharides, 1,3- and 1,6-Mannotriose (Man-tri), which is the strongest ligand for native ConA and thus is detected by IAEDANS-ConA, is not sensed by APET-ConA. This is presumably due to all the OH groups of Man-tri interacting with the ConA surface as well as the ConA pocket[12] and being unavailable to interact with the PBA unit. Consistent with this explanation, we found that Man-tetra, which bears an additional mannose unit to Man-tri, can be sensed by APET-ConA. Although PBA itself shows the poor affinity toward Isomal and Man-tetra (log K is less than 2), APET-ConA gains a moderate affinity to these saccharides. This may be explained by the proximity effect of PBA to the native binding pocket of ConA, that is, the PBA site close to the ConA pocket effectively acts as a subsite for saccharide binding, so as to cause the PET fluorescence sensing, even though the net binding affinity is not remarkably enhanced.

Scope and Limitation. In conclusion, the rational coupling of an artificial receptor with a native receptor protein could evolve toward a novel semisynthetic receptor and sensor. As the seminal work by Norrild previously pointed out, PBA preferentially forms a tight complex with *cis*-1,2-furanose type of diol involving the C1 hydroxyl of sugars,[14d] and the hybridization of PBA to lectin has limited potential in the practical application. However, it is important that the present proof-of-principle experiment clearly demonstrates the cooperative action between an artificial and a native receptor. By using alternative approaches, host−guest chemistry has produced a number of artificial receptors such as crown ether, cyclodextrin, cyclophane, and others based on hydrogen-bonding force, etc.,[15,16] and biochemical studies have already unveiled various natural receptor proteins for the last several decades.[17] Therefore, it is envisioned that the designed hybrid of these two families will facilitate the generation of semisynthetic receptors with enhanced affinity and selectivity, not only toward a variety of simple carbohydrates, but also for other biological substances such as glycolipids, glycopeptides/proteins, and bioactive hormones, etc.[6−8]

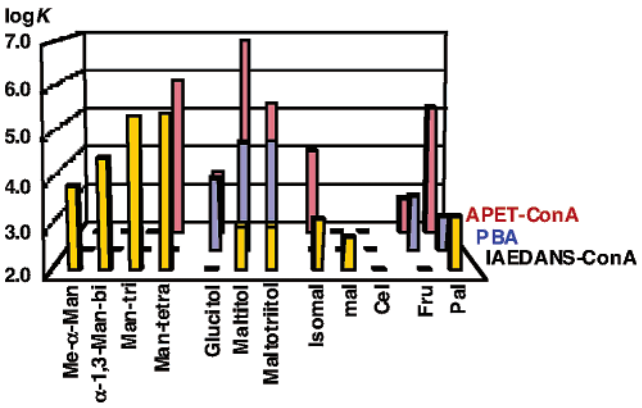


Figure 4. Summary of the binding constants evaluated by fluorescence change of various bio- or chemosensors in this study.

In cases where targets are structurally complicated and diverse, such as saccharide derivatives, it is generally difficult to determine the target structure by a typical one-to-one recognition. Instead, the pattern recognition is advantageous to such a case as Anslyn pointed out.[1] However, to carry out the pattern recognition, a set of receptors bearing various selectivity is required. In the present case, for example, IAEDANS-ConA cannot distinguish Man-tri from Man-tetra and APET-ConA can scarcely distinguish Man-tetra from Maltitol. However, comparing the sensing pattern of IAEDANS-ConA and APET-ConA enables one to distinguish the three saccharides (see the sensing pattern shown in Figure 4). Although this is still primitive, we can expect a more precise pattern recognition when a rich variety of fluorescent biosensors will be prepared according to our approach. We are currently investigating along this line.

Experimental Section

General Methods. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was recorded on PE Voyager DE-RP, where sinapinic acid (SA) was used as a matrix. UV−visible spectra were obtained on a Hitachi U-3000 spectrometer. Fluorescence spectra were recorded on a Hitachi F-4500 spectrometer. ConA was purchased from Funakoshi and used without further purification. IAEDANS was purchased from Molecular Probes. The other chemical reagents were purchased from Aldrich or Tokyo Chemical Industry.

Photoaffinity Label of ConA with P-PALM Reagent. This process was conducted according to our previous method.[11c] The second fraction was collected and mainly used in the following study.[18]

Preparation of APET-ConA. To a solution of the labeled ConA (a heterodimer consisted of the labeled monomoer and the unlabeled (native) monomer, that is, the second fraction in the affinity column) (20 μM, 10 mL) in 50 mM phosphate buffer (pH 7.5) was added Tris-carboxyethylphosphine (TCEP) (a final concentration of TCEP is 100

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(18) Since the fluorescence signal from APET-ConA is produced by a saccharide-binding event at the PBA site, not at the native ConA pocket (Figure 1a), the binding constants are evaluated on the basis of the saccharide binding at the PBA-modified ConA site. Although the other unmodified binding site (i.e., without PBA) may bind saccharides with a weak affinity, it does not affect the determination of binding constants under conditions of the excess amount of saccharide (for Benesi−Hildebrandt analysis). In case of the strong binding affinity observed at the almost stoichiometric amount of the saccharide, the affinity may be slightly underestimated because the native binding pocket binds the corresponding saccharide without fluorescence sensing so as to decrease the free concentration of the saccharide.

μM: 10 equiv for labeled ConA), and the solution was incubated for 5 h at 4 °C under nitrogen atmosphere. The reaction solution was purified by gel filtration chromatography (Bio-Gel P-30, 2 cm × 20 cm, eluent: 50 mM phosphate buffer (pH 7.5)) to give SH-ConA (12 μM, 16 mL) in quantitative yield. The thiol content in SH-ConA was determined by the Ellman method, showing that one ConA dimer has one SH group. MALDI-TOF MS (SA) demonstrated the cleavage of the thiomannose unit: *m*/*z*, calcd 25 787, obsd 25 796. The SH-ConA was quickly subjected to the next modification. To a solution of SH-ConA (20 μM, 5 mL) in 50 mM phosphate buffer (pH 7.5), 100 mM D-Glc, APET-Br (20 equiv to one SH group) in dimethyl formamide (DMF) (500 μL) was added slowly at 0 °C and incubated for 12 h at r.t. in the dark. After incubation, the solution was loaded on TOYO PEARL (1 cm × 15 cm), eluted with the buffer (C) (50 mM HEPES buffer (pH 7.5), 5 mM $CaCl_2$, 0.1 M NaCl). The protein fraction was collected, concentrated by ultrafiltration, and dialyzed against the buffer (C) to afford APET-ConA (12 μM, 8 mL) in quantitative yield. APET-ConA displays the absorbance at 280 nm due to the aromatic ring of ConA and 375 nm due to the anthracene unit. The emission maximum of APET-ConA at 407, 432, and 454 nm and the excitation maximum at 357, 375 and 397 nm are both characteristic of the anthracene chromophore. The labeling efficiency was estimated from the ratio of the corresponding absorbance to be 0.75−0.90.

Fluorescence Titration. Saccharide solution was added dropwise to a 0.7 μM APET-ConA solution [50 mM HEPES buffer (pH 7.5) containing 5 mM $CaCl_2$ and 0.1 M NaCl at 15 ± 1 °C] or to a 0.2 μM IAEDANS-ConA solution [50 mM HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.1 M NaCl at 15 ± 1 °C], and fluorescence spectra were measured. The slit widths for the excitation and emission were set to 10 nm and 10 nm, respectively. The excitation and emission wavelengths used were $\lambda_{ex} = 340$ nm and $\lambda_{em} = 484$ nm (IAEDANS-ConA) or $\lambda_{ex} = 375$ nm and $\lambda_{em} = 425$ nm (APET-ConA), respectively. Fluorescence titration curves were analyzed with the nonlinear least-squares curve-fitting method or the Benesi−Hildebrandt plot to give the association constants (log *K*) for various saccharides.[18]

pH Titration Experiments in the Absence and Presence of Saccharides. The fluorescence of APET-ConA (0.5 μM) dissolved in aqueous solution with various pH was measured. We adjusted the pH by addition of aqueous HCl or aqueous NaOH to the unbuffered APET-ConA solution. The optimal pH for the saccharide titration was determined at pH 7.5.

Synthesis of APET-Br. The following solvents were distilled under nitrogen atmosphere prior to usage: MeOH was dried over Mg and I_2. Unless otherwise stated, all of the air- or moisture-sensitive reactions were performed under nitrogen atmosphere.

PBA and 9,10-bis[(methylamino)methyl]anthracene were prepared according to literature procedure.[14a]

9-((*N*-Methyl-*N*-(*o*-boronobenzyl)amino)methyl)-10-((methylamino)methyl)anthracene (2). 2,2-Dimethylpropane-1,3-diyl(*o*-(bromomethyl)phenyl)boronate (760 mg, 3.0 mmol), 9,10-bis-((methylamino)-methyl)anthracene (880 mg, 3.33 mmol, 1.1 equiv), and potassium carbonate (1.24 g, 3.0 mmol) in anhydrous THF (40 mL) were refluxed for 3 h. The mixture was filtered, the solvent was removed in vacuo, and the residue was subjected to column chromatography ($CHCl_3$/MeOH (1% TEA): a linear gradient from 50/1 to 6/1 (v/v)) to give compound **2** as a yellow powder in 18% yield (210 mg). ^{1}H NMR ($CDCl_3$,TMS,250 MHz): δ/ppm; 8.34 (d, $J = 8.5$ Hz, 2H, Ar-*H*), 8.13 (d, $J = 8.5$ Hz, 2H, Ar-*H*), 8.11 (d, $J = 8.5$ Hz, 1H, Ar-*H*), 7.6−7.4 (m, 7H, Ar-*H*), 4.68 (s, 2H, $-CH_2-$), 4.50 (s, 2H, $-CH_2-$), 3.92 (s, 2H, $-CH_2-$), 2.65 (s, 3H, $-CH_3$), 2.21 (s, 3H, $-CH_3$). ESI-TOF MS: calcd for $C_{25}H_{28}BN_2O_2^+$ ([M + H]$^+$), 399.22; found, 399.09.

9-((*N*-Methyl-*N*-(*o*-boronobenzyl)amino)methyl)-10-((*N*-methyl-*N*-(bromoacetyl) amino) methyl)anthracene (APET-Br). The compound **2** (30 mg, 75.3 mmol), DIEA (97 mg, 753 mmol), and bromoacetylbromide (30 mg, 150 mmol, 2 equiv) in anhydrous dichloromethane (10 mL) were stirred at 0 °C for 30 min. The solvent was removed in vacuo, and the residue was subjected to column chromatography ($CHCl_3$/MeOH: a linear gradient from 20/1 to 10/1 (v/v)) to give APET-Br as a yellow powder in 82% yield (32 mg). ^{1}H NMR ($CDCl_3$, TMS, 250 MHz): δ/ppm; 8.29 (d, $J = 8.5$ Hz, 2H, Ar-*H*), 8.16 (d, $J = 8.5$ Hz, 2H, Ar-*H*), 8.05 (d, 1H, Ar−H), 7.60−7.35 (m, 7H, Ar−H), 5.68 (s, 2H, $-CH_2-$), 4.53 (s, 2H, $-CH_2-$), 4.53 (s, 2H, $-CH_2-$), 4.06 (s, 2H, Br$-CH_2-$), 3.94 (s, 2H, $-CH_2-$), 2.52 (s, 3H, N$-CH_3$), 2.24 (s, 3H, N$-CH_3$). SIMS-MS (matrix: NBA and glycerol): calcd for $C_{30}H_{32}BBrN_2O_4^+$ ([M + glycerol − $2H_2O$]$^+$), 574.576; obsd, 574.576.

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