A Pradimicin-Based Staining Dye for Glycoprotein Detection

Yu Nakagawa,*[,†,‡] Shintaro Kakihara,[§] Kazue Tsuzuki,[§] Makoto Ojika,[§] Yasuhiro Igarashi,[‖] Yukishige Ito[‡,⊥]

[†]Institute for Glyco-core Research (iGCORE), Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

[‡]RIKEN Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

[§]Department of Applied Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

[‖]Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

[⊥]Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka 560-0043, Japan

Pradimicin A (PRM-A) and related compounds comprise an exceptional family of natural pigments that show Ca^{2+}-dependent recognition of D-mannose (Man). Although these compounds hold great promise as research tools in glycobiology, their practical application has been severely limited by their inherent tendency to form water-insoluble aggregates. Here, we demonstrate that the 2-hydroxyethylamide derivative (PRM-EA) of PRM-A hardly shows any aggregation in neutral aqueous media and retains binding specificity for Man. We also show that PRM-EA stains glycoproteins in dot blot assays, whereas PRM-A fails to do so, owing to severe aggregation. Significantly, PRM-EA is sensitive to glycoproteins carrying high mannose-type and hybrid-type N-linked glycans, but not to those carrying complex-type N-linked glycans. Such staining selectivity has never been observed in conventional dyes, suggesting that PRM-EA could serve as a unique staining agent for the selective detection of glycoproteins with terminal Man residues.

Pradimicins (PRMs, Figure 1) are a family of natural pigments with a common benzo[a]naphthacenequinone skeleton.[1–3] Although PRMs were originally isolated as antifungal compounds, their scientific significance has greatly increased since the discovery of their lectin-like sugar-binding property. PRMs bind D-mannose (Man) in the presence of Ca^{2+}, and completely discriminate Man from its enantiomer (L-mannose), epimers (D-glucose, D-altrose, D-talose), amino derivatives (D-mannosamine, *N*-acetyl-D-mannosamine), and other common monosaccharides (D-galactose, *N*-acetyl-D-glucosamine, L-fucose, *N*-acetylneuraminic acid).[4] In addition, and quite significantly, PRMs have the capacity to bind biologically and pathologically important glycans that contain Man residues, such as *N*-linked glycans and fungal mannans.[5–7] While significant advances have been made in the design of sugar-binding small molecules,[8–11] such specific recognition of Man residues is yet to be achieved by the existing artificial molecules, thereby highlighting the great potential of PRMs for use as unique mimics of lectins.[12–15]

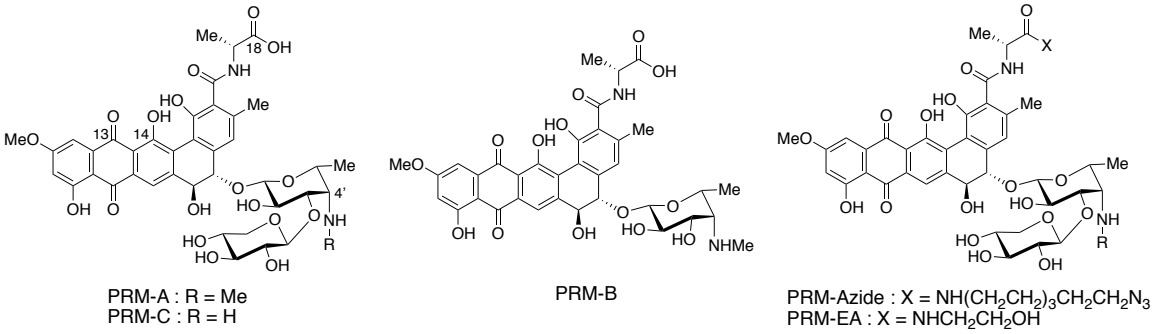
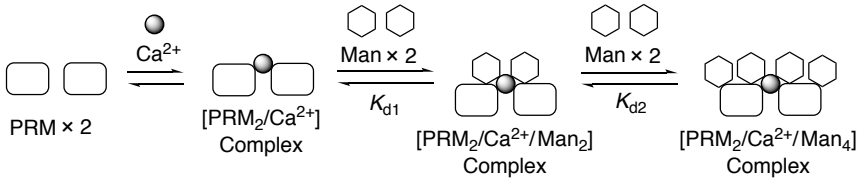


Figure 1. Structures of pradimicins (PRMs).

Despite the exceptional sugar-binding property and the commercial availability of PRMs, two fundamental problems have limited their glycobiological application. One is the lack of effective strategies for modifying PRMs to develop them as research tools. Although early reports

suggested that the C4′ amino group could be modified without abolishing Man binding activity,[16–18] the reactivity of the amino group is low, and effective derivatization at this position for glycobiological purposes is still to be developed. A recent example is the work of Enomoto *et al.*,[19] in which substitution reaction at the C4′ amino group of PRM-A with a 2-bromoacetamide derivative gave the *N*-alkylated product only in 9% yield. Another possible site of facile modification is the C18 carboxyl group, but attempts to alter this group were previously abandoned on the assumption that it may coordinate with Ca^{2+} to bind Man (Scheme 1).[3,20,21] Thus, there has been a lack of sites within the PRM molecule that could be readily and effectively modified. A more serious problem is their tendency to form aggregates. Although PRMs themselves are not cohesive, their Ca^{2+}-containing complexes (the [PRM_2/Ca^{2+}], [$PRM_2/Ca^{2+}/Man_2$], and [$PRM_2/Ca^{2+}/Man_4$] complexes in Scheme 1) readily aggregate to form water-insoluble precipitates.[4,20,22] This unfavorable propensity has been a major obstacle to their practical use in glycobiological studies.



Scheme 1. Complex formation of PRMs with Ca^{2+} and Man. Two PRM molecules are initially bridged by Ca^{2+} to form the [PRM_2/Ca^{2+}] complex, which binds two molecules of Man. The resulting [$PRM_2/Ca^{2+}/Man_2$] complex accommodates another two molecules of Man to afford the [$PRM_2/Ca^{2+}/Man_4$] complex. These three complexes have a high aggregation propensity and assemble to give water-insoluble aggregates. K_{d1} and K_{d2} represent the dissociation constants for primary and secondary Man binding, respectively.

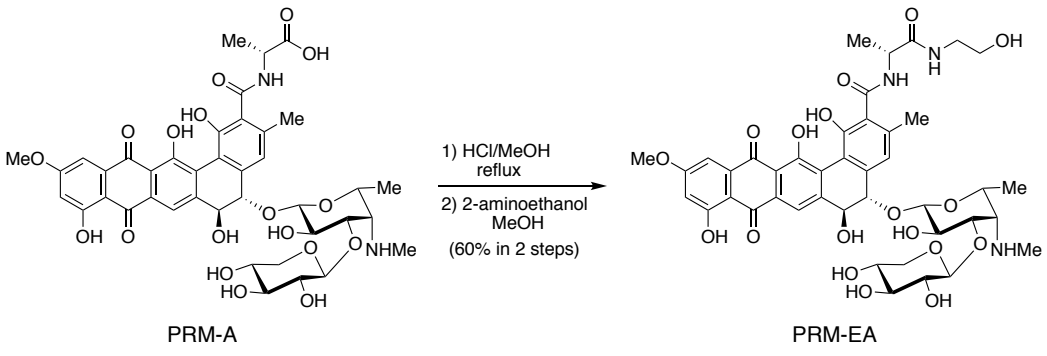
Recently, our structural studies of the [PRM_2/Ca^{2+}/Man_2] complex provided a clue as to a modifiable site within the PRM molecule.[23–26] Contrary to the previous assumption, our crystallographic analysis showed that Ca^{2+} is coordinated with the C13 carbonyl and C14 hydroxy groups of PRMs rather than with the C18 carboxyl group. Subsequent molecular modeling suggested that amide formation at the C18 carboxyl group of PRMs would not disturb their complex formation with Ca^{2+} and Man. This modification strategy was validated by the demonstration that PRM-Azide (Figure 1), an amide derivative of PRM-A,[1] retained Man binding ability.[27] This result represents a new direction in the design of PRM derivatives for glycobiological applications.

Here, we report that amide formation at the C18 carboxyl group of PRMs can also address the issue related to aggregation. Attachment of 2-aminoethanol to the carboxyl group of PRM-A via the amide linkage significantly suppresses its aggregative propensity in Ca^{2+}-containing aqueous solutions, and the resulting PRM-EA (Figure 1) retains the binding selectivity for Man over other monosaccharides observed in N-linked glycans. We also show that PRM-EA stains glycoproteins carrying high mannose-type and hybrid-type N-linked glycans in the dot blot experiments, but not those carrying complex-type N-linked glycans. This is the first demonstration that a readily prepared derivative of PRMs could serve as a unique dye for the on-membrane detection of glycoproteins with Man residues at the non-reducing ends of glycans.

RESULTS AND DISCUSSION

In our previous study, we performed fluorescent staining of fungal cells by using PRM-Azide (Figure 1).[26] During the staining experiments, we found that PRM-Azide formed fewer aggregates

in Ca^{2+}-containing aqueous media than PRM-A, raising the possibility that attachment of hydrophilic moieties to the C18 carboxyl group though the amide linkage could suppress the aggregation of PRMs. To confirm this possibility, we designed a simpler amide derivative, PRM-EA (Figure 1), in which 2-aminoethanol is connected to the C18 carboxyl group of PRM-A. PRM-EA was readily synthesized from PRM-A in two steps, consisting of methyl esterification and ester/amide exchange reaction (Scheme 2). Following its preparation, we evaluated its aggregation property using a sedimentation assay. After incubation of 0.5 or 1.0 mM PRMs in 50 mM 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (pH 7.0) containing 10 mM $CaCl_2$ (pH 7.0) at 37 °C for 15 min followed by centrifugation, the residual amounts of PRMs in the supernatant were measured by HPLC analysis (Figure 2). Under these conditions, PRM-A was rapidly precipitated and less than 5% of PRM-A was detected in the supernatant even at 0.5 mM. In sharp contrast, more than 90% of PRM-EA remained in the supernatant, and scarcely any precipitation was observed at either concentration.



Scheme 2. Synthesis of PRM-EA.

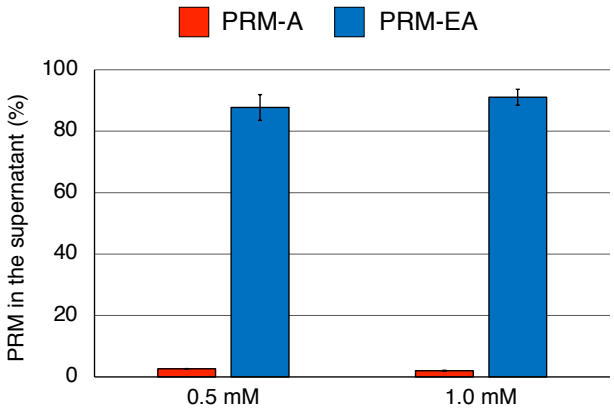


Figure 2. Aggregation of PRM-A and PRM-EA in Ca^{2+}-containing neutral aqueous media. The sedimentation assay was performed using 0.5 or 1.0 mM PRMs in 50 mM MOPS buffer (pH 7.0) containing 10 mM $CaCl_2$. The amounts of PRMs in the supernatant were estimated from HPLC peak areas and expressed as a percentage of the control.

Following the confirmation that PRM-EA is markedly less aggregative than PRM-A, we subsequently evaluated its Man binding ability by isothermal titration calorimetry (ITC). In the presence of $CaCl_2$, the titration of PRM-EA with methyl α-D-mannopyranoside (Man-OMe) provided an isotherm curve characteristic of the two-step Man binding of PRMs (Figure 3; A).[24] A control experiment in the absence of $CaCl_2$ detected almost no heat effect (Figure 3; B), supporting that PRM-EA requires the formation of the Ca^{2+} complex for Man binding. A least-squares fit of the ITC data to a two-site binding model gave the dissociation constants for primary (K_{d1} = 650 ± 60 μM) and secondary (K_{d2} = 6,300 ± 330 μM) binding (Scheme 1). These values are similar to those of PRM-Azide (K_{d1} = 510 μM, K_{d2} = 8,100 μM),[26] but higher than those of PRM-A (K_{d1} = 96 μM, K_{d2} = 3,800 μM),[24] suggesting that the amide formation of PRM-A slightly decreases binding affinity for Man. However, considering the previous demonstration that PRM-Azide binds to fungal mannans,[27] the binding affinity of PRM-EA for Man is sufficient for use in

glycobiological research. Moreover, additional ITC experiments showed that PRM-EA was responsive to Man, but not to other monosaccharides (D-glucose, D-galactose, *N*-acetyl-D-glucosamine, L-fucose, and *N*-acetylneuraminic acid methyl ester) found in biologically important glycans (Table 1). These combined results indicate that PRM-EA can selectively bind Man without significant aggregation.

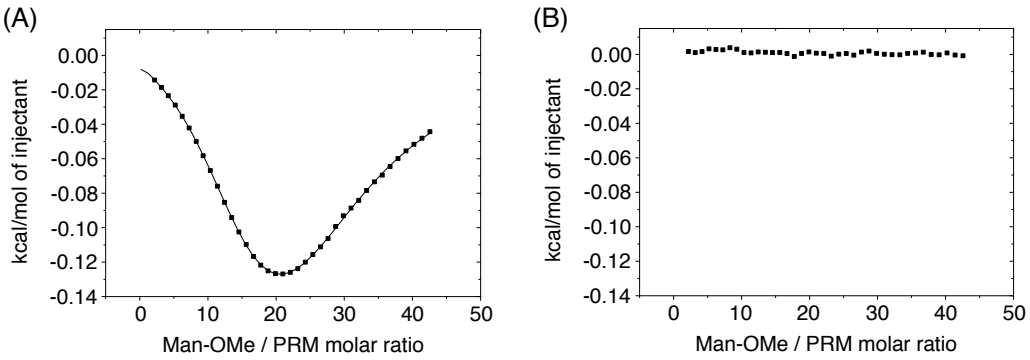


Figure 3. ITC profile for PRM-EA titrated with Man-OMe in the presence (A) and absence (B) of $CaCl_2$. The ITC experiments were performed using 1 mM PRM-EA and 200 mM Man-OMe in 50 mM MOPS buffer (pH 7.0) with or without 10 mM $CaCl_2$ at 30 °C. The solid line in (A) represents the least-squares fit of the data to a two-site binding model.

Table 1. K_d values for monosaccharides binding of PRM-EA.

Monosaccharide	K_{d1} (μM) ± Error of Fit[a]	K_{d2} (μM) ± Error of Fit[a]
Man-OMe	650 ± 60	6,300 ± 330
D-Mannose	1,600 ± 270	14,000 ± 1,100
D-Glucose	–[b]	–[b]
D-Galactose	–[b]	–[b]
N-Acetyl-D-glucosamine	–[b]	–[b]
L-Fucose	–[b]	–[b]
N-Acetylneuraminic acid[c]	–[b]	–[b]

[a]Determined by ITC experiments (see Supporting Information for the raw ITC data).

[b]Too large to be determined.

[c]*N*-Acetylneuraminic acid methyl ester was used to avoid severe pH changes during titration.

Given the encouraging Man binding ability and lower aggregation propensity of PRM-EA, we then explored its application as a staining dye for glycoproteins in a dot blot assay. Our previous studies showed that PRMs bind to Man residues at the non-reducing ends of glycans.[25,27] Thus, we initially examined whether PRM-EA could stain ovalbumin (OVA) carrying high mannose-type and hybrid-type N-linked glycans,[28] both of which possess Man residues at their non-reducing ends (Figure 4). Among the various conditions tested, clear staining of OVA was observed on polyvinylidene difluoride (PVDF) membranes when fish gelatin and 100 μM PRM-EA in 50 mM MOPS buffer (pH 7.0) containing 250 μM $CaCl_2$ were used as blocking and staining agents, respectively (Figure 5; A). Bovine serum albumin (BSA) without any glycans was not sensitive to this agent (Figure 5; B), and the addition of fungal mannan-derived polymannoses to the PRM-EA solution completely inhibited the staining of OVA (Figure 5; C). These observations collectively suggest that glycan binding of PRM-EA is responsible for this staining. In contrast, PRM-A severely aggregated in the same buffer, and failed to stain OVA despite its higher affinity for Man relative to PRM-EA (Figure 5; D), demonstrating that the lower aggregation propensity of PRM-EA is the crucial factor for staining OVA.

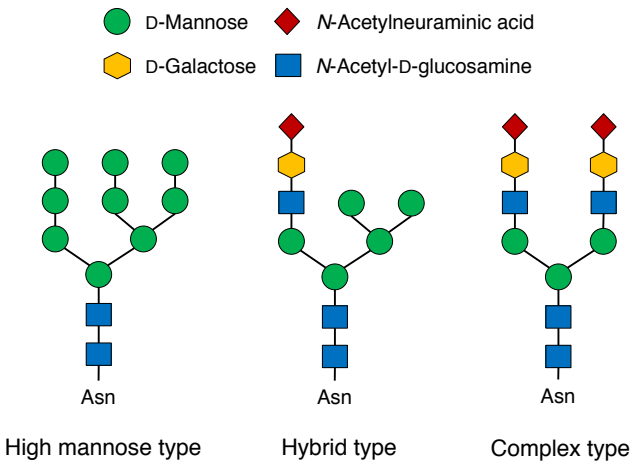


Figure 4. Representative structures of high mannose-, hybrid-, and complex-type N-linked glycans.

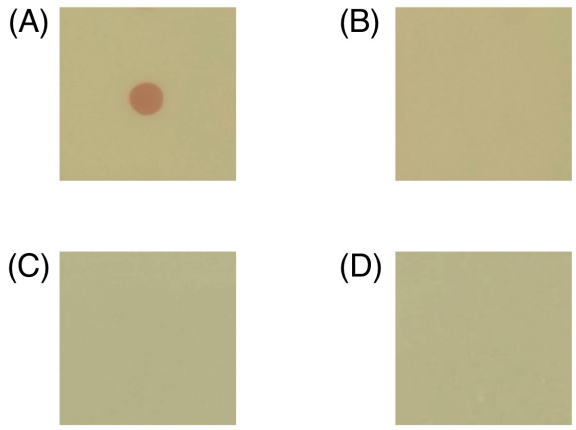


Figure 5. Dot blot assay for OVA and BSA using PRMs as staining agents. (A) 2.5 μg/spot of OVA on a PVDF membrane treated with 100 μM PRM-EA in 50 mM MOPS buffer (pH 7.0) containing 250 μM $CaCl_2$. (B) 2.5 μg/spot of BSA treated with the PRM-EA solution. (C) 2.5 μg/spot of OVA treated with the PRM-EA solution containing 1 mg/mL of cell wall mannan from *Saccharomyces cerevisiae*. (D) 2.5 μg/spot of OVA treated with 100 μM PRM-A in 50 mM MOPS buffer (pH 7.0) containing 250 μM $CaCl_2$.

The clear staining of OVA by PRM-EA further led us to perform an additional dot blot assay using well-known glycoproteins carrying different types of N-linked glycans. As shown in Figure 6, PRM-EA deeply stained bovine thyroglobulin (Tg)[29] carrying high mannose-type and complex-type N-linked glycans as well as OVA. These glycoproteins were visually detected up to 1 μg/spot on the membrane. In contrast, no staining was observed even for 5 μg/spot of human transferrin (TRF)[30] and human immunoglobulin G (IgG),[31] both of which possess only complex-type N-linked glycans with no Man residues at their non-reducing ends. These results are in good agreement with the previous finding that PRMs bind to terminal Man residues of glycans[25,27] and, more importantly, suggest that PRM-EA could be used as a staining dye for selective detection of glycoproteins with terminal Man residues.

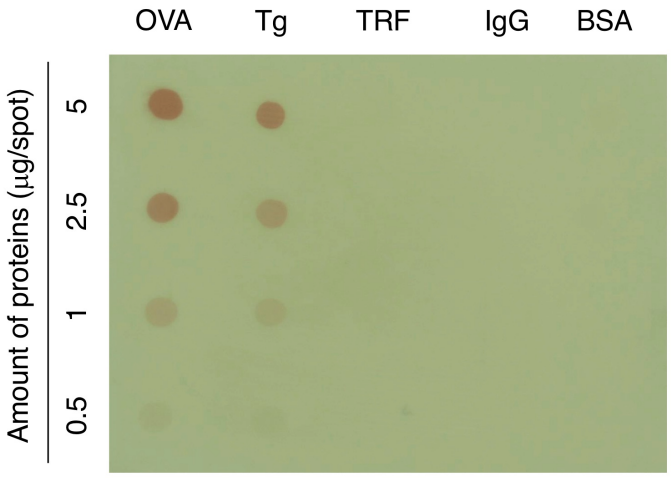


Figure 6. Dot blot assay for glycoproteins using PRM-EA as a staining agent. BSA without glycans was also spotted as a negative control (right end lane).

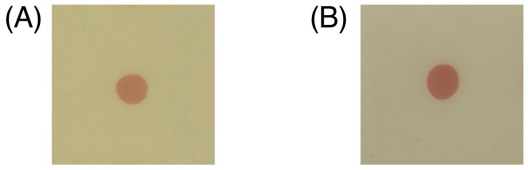


Figure 7. OVA (2.5 μg/spot) staining by the freshly prepared (A) and two-week-stored (B) solutions of 100 μM PRM-EA in 50 mM MOPS buffer (pH 7.0) containing 250 μM $CaCl_2$.

Given the possible application of PRM-EA for on-membrane detection of glycoproteins with terminal Man residues, we next examined the preservability of the staining solution, 100 μM PRM-EA in 50 mM MOPS buffer (pH 7.0) containing 250 μM $CaCl_2$. Dot blot assays demonstrated that the glycoprotein-staining ability of the solution was maintained even after storage at room temperature for two weeks (Figure 7). In accordance with this observation, HPLC analysis

confirmed that 92% of PRM-EA remained undegraded in the solution after two weeks of storage. These results indicate that the staining solution remains effective for at least two weeks at room temperature, and thus could be easy to use for glycoprotein staining experiments.

In conclusion, the present study demonstrates that PRM-EA specifically binds Man without severe aggregation and selectively stains glycoproteins having Man residues at the non-reducing ends of glycans. Such staining selectivity is not observed in the pre-existing dyes and the widely used PAS (periodic acid-Schiff) method[32] for on-membrane detection of glycoproteins, highlighting the potential of PRM-EA as an alternative to functionalized lectins,[33] such as biotinylated concanavalin A (ConA) capable of selectively staining glycoproteins with terminal Man residues. An additional advantage of using PRM-EA for glycoprotein detection is that there is no need to run chemical or enzymatic reactions. The PAS method involves glycan oxidation and subsequent Schiff base formation. Functionalized lectin-based methods also usually require chemical and/or enzymatic reactions, making the entire process time- and material-consuming. Considering these advantages over conventional staining agents along with ease of availability and long-term preservability, PRM-EA could serve as a unique dye for simple, rapid, and selective visualization of glycoproteins with terminal Man residues. One issue to be addressed is the detection limit of glycoproteins. In our dot blot assay, 1 μg/spot of Tg and OVA was slightly stained, but 0.5 μg/spot was hardly detected. This sensitivity is one or two orders of magnitude lower than those of the PAS and lectin-based methods. For example, PAS-silver staining and biotinylated ConA-based methods were reported to detect less than 0.02 μg/spot of OVA.[34,35] To improve the detection limit of PRM-EA, the development of fluorescence-labeled derivatives is currently underway and will be reported in due course.

EXPERIMENTAL SECTION

General Experimental Procedures. PRM-A was isolated from the fermentation broth of *Actinomadura* sp. TP-A0019 as reported previously[23]. HPLC was carried out using an L-2130 HPLC system (Hitachi). NMR spectra were measured on a JEOL ECX 400 (^{1}H at 400 MHz, ^{13}C at 100 MHz), a Bruker AVANCE 400 (^{1}H at 400 MHz, ^{13}C at 100 MHz) magnetic resonance spectrometer. ^{1}H chemical shifts are reported relative to the residual solvent peak (DMSO = 2.50 ppm). ^{13}C chemical shifts are reported relative to the residual deuterated solvent ^{13}C signals (DMSO = 39.52 ppm). Signal assignments were made *via* 2D spectroscopy (COSY, HSQC, and HMBC). Optical rotation data were obtained using a JASCO DIP-370 digital polarimeter. UV spectrum was measured on a JASCO V-530 spectrometer. IR data were obtained using an JASCO FT/IR-4100 spectrometer. High-resolution mass spectrum was obtained using an Applied Biosystems Mariner ESI-TOF spectrometer. Chemical reagents, proteins, and mannan from *S. cerevisiae* were purchased from commercial suppliers (Kanto Chemical, Sigma-Aldrich, TCI Chemicals, Wako Pure Chemical Industries) and were used as received without additional purification.

Synthesis and Characterization of PRM-EA. To a solution of PRM-A (TFA salt, 31.2 mg, 32.7 μmol) in MeOH (5.6 mL) was added 7% HCl/methanol (56 μL). The reaction mixture was heated at reflux for 2 hr, and then directly loaded onto a Diaion HP-20 column. After washing with water, the resin was eluted with 80% acetone/water containing 0.1% TFA and the eluate was concentrated *in vacuo* to afford PRM-A methyl ester as a TFA salt (30.4 mg, 31.4 μmol, 96%). To a solution of PRM-A methyl ester (11.5 mg, 11.9 μmol) in MeOH (0.3 mL) was added 2-aminoethanol (2.7 mL, 45.2 mmol). The reaction mixture was stirred at room temperature for 18 hr, and then directly

loaded onto a Diaion HP-20 column. After washing with water, the resin was eluted with 80% acetone/water containing 0.1% TFA and the eluate was concentrated *in vacuo*. The residue was purified by reverse-phase HPLC (column: YMC-Pack ODS-A, 5 μm, 20.0 mm ID × 250 mm; solvent: 30 min linear gradient 25–42% CH_3CN/water containing 0.1% TFA; flow rate: 8.0 mL/min; UV: 254 nm; Rt: 16.0 min) to afford PRM-EA (8.7 mg, 7.54 μmol, 63%) as a TFA salt.

Characterization data for PRM-EA. $[\alpha]^{30}_{D}$ = –744.0 (*c* 0.19, in MeOH); UV (MeOH) λ_{max} (logε): 495 nm (4.04), 322 nm (3.95), 276 nm (4.31); IR (KBr) ν_{max} 3381, 1678, 1627, 1446, 1430, 1389, 1336, 1297, 1258, 1204, 1158, 1133, 1057 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6, the numbering of PRM-EA and the NMR charts are shown in Supporting Information) δ 1.30 (3H, d, *J* = 6.7 Hz, H_3-6'), 1.33 (3H, d, *J* = 7.2 Hz, 17-Me), 2.30 (3H, s, 3-Me), 2.70 (3H, s, 4'-NMe), 3.14 (1H, m, H-5"a), 3.17 (1H, m, H-2"), 3.19 (1H, m, H-3"), 3.20 (2H, m, H_2-20), 3.34 (1H, m, H-4"), 3.47 (1H, m, H-4'), 3.48 (2H, t, *J* = 6.0 Hz, H_2-21), 3.55 (1H, br.m, H-2'), 3.78 (1H, dd, *J* = 11.3, 5.2 Hz, H-5"b), 3.93 (1H, br.q, *J* = 6.8 Hz, H-5'), 3.97 (3H, s, 11-OMe), 3.98 (1H, m, H-3'), 4.49 (1H, m, H-17), 4.49 (1H, d, *J* = 6.7 Hz, H-1"), 4.63 (1H, d, *J* = 8.7 Hz, H-5), 4.70 (1H, d, *J* = 8.7 Hz, H-6), 4.79 (1H, d, *J* = 7.7 Hz, H-1'), 6.93 (1H, d, *J* = 1.8 Hz, H-10), 7.15 (1H, s, H-4), 7.33 (1H, d, *J* = 1.8 Hz, H-12), 7.77 (1H, br.t, *J* = 5.1 Hz, 19-NH), 8.05 (1H, s, H-7), 8.27 (1H, d, *J* = 7.4 Hz, 16-NH); ^{13}C-NMR (400 MHz, DMSO-d_6) δ 15.8 (C-6'), 17.5 (17-Me), 18.9 (3-Me), 36.0 (4'-NMe), 41.5 (C-20), 48.4 (C-17), 56.2 (11-OMe), 59.6 (C-21), 63.0 (C-4'), 65.7 (C-5"), 67.2 (C-5'), 69.2 (C-4"), 69.6 (C-2'), 71.1 (C-6), 73.4 (C-2"), 75.8 (C-3"), 79.4 (C-3'), 80.5 (C-5), 103.7 (C-1'), 104.9 (C-1"), 106.7 (C-10), 107.4 (C-12), 110.0 (C-8a), 114.3 (C-14b), 115.7 (C-13a), 118.5 (C-7), 121.6 (C-4), 126.1 (C-7a), 127.7 (C-3), 131.3 (C-14a), 134.4 (C-12a), 136.7 (C-2), 137.4 (C-4a), 147.4 (C-6a), 151.2 (C-1), 157.1 (C-14), 164.6 (C-9), 165.9 (C-11), 166.7 (C-15),

172.0 (C-18), 184.9 (C-8), 187.1 (C-13); HR-ESI-MS *m/z* 884.3084 [M+H$^+$] (calcd. for $C_{42}H_{50}N_3O_{18}$, 884.3068).

Sedimentation assay. To a solution of 2.5 or 5 mM PRMs in distilled water (40 μL) in a 1.5 mL-Eppendorf tube was added 50 mM MOPS buffer (pH 7.0) containing 10 mM $CaCl_2$ (160 μL) at room temperature. The resulting mixture was incubated at 37 °C for 15 min and then at room temperature for 30 min. After centrifugation at 10,000 *g* for 5 min at 24 °C, the supernatant (30 μL) was poured into 50% CH_3CN aqueous solution containing 0.1% TFA (30 μL). The resulting solution was analyzed by reverse-phase HPLC (column: CAPCELL PAK C_{18} ACR, 5 μm, 4.6 mm ID × 250 mm; solvent: 25%–81% CH_3CN-water containing 0.1% TFA, 15-min linear gradient; flow rate: 1 mL/min; UV detection: 465 nm). The amount of non-precipitated PRM-A or PRM-EA was estimated from HPLC peak area and expressed as a percentage of the control.

ITC experiment. ITC measurements were carried out in 50 mM MOPS buffer (pH 7.0) containing 10 mM $CaCl_2$ at 30 °C using a Microcal iTC_{200} microcalorimeter (Microcal Inc., Northampton, MA). A typical titration consisted of injecting 1.0 μL of 200 mM (for Man-OMe) or 400 mM (for Man and monosaccharides used in Table 1) sugar solution (total 40 injections) into 1 mM PRM-EA with an interval of 2 min between injections at the stirrer speed of 1,000 rpm. The heat of dilution was determined under identical conditions by injecting the sugar solution into the ITC cell containing only the buffer. For every experiment, the heat of dilution was subtracted from the sample titration data before processing. The titration data were analyzed using the software provided by the manufacturer (Origin for ITC). The binding isotherm was fitted using a two-site binding model to calculate the dissociation constants (K_{d1} and K_{d2}). Raw ITC data and thermodynamic parameters are provided in Supporting Information.

Dot blot assay. Proteins were spotted on a PVDF membrane (Merk Millipore). After drying, the membrane was blocked with Fish Gelatin (TAKARA BIO Inc.) at room temperature for 10 min twice, and then washed with distilled water. The blocked membrane was incubated with 100 μM PRM-EA in 50 mM MOPS buffer (pH 7.0) containing 250 μM $CaCl_2$ at room temperature for 15 min, and then washed with the same buffer for a few minutes. The images were recorded by the digital camera (Canon IXY 630; Canon Inc.).

HPLC analysis of staining solutions. 50 μL of the freshly prepared or two-week-stored staining solution (100 μM PRM-EA in 50 mM MOPS buffer containing 250 μM $CaCl_2$) was poured into 50% CH_3CN aqueous solution containing 0.1% TFA (50 μL). The resulting solution was analyzed by reverse-phase HPLC (column: CAPCELL PAK C_{18} ACR, 5 μm, 4.6 mm ID × 250 mm; solvent: 30%–50% CH_3CN-water containing 0.1% TFA, 15-min linear gradient; flow rate: 1 mL/min; UV detection: 465 nm). The amounts of PRM-EA in the solutions were estimated from HPLC peak area.

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available.

^1H, ^{13}C, and 2D NMR spectra of PRM-EA, Raw ITC data and thermodynamic parameters for binding of PRM-EA with sugars (PDF)

AUTHOR INFORMATION

Corresponding Author

Yu Nakagawa – Institure for Glyco-core Research (iGCORE), Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan; RIKEN Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Email: yu@agr.nagoya-u.ac.jp

Authors

Shintaro Kakihara – Department of Applied Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

Kazue Tsuzuki – Department of Applied Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

Makoto Ojika – Department of Applied Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

Yasuhiro Igarashi – Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

Yukishige Ito – Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka 560-0043, Japan; RIKEN Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Notes

The authors declare no competing financial interests.

ACNOWLEDGMENTS

This work was partly supported by JSPS KAKENHI Grant (Number 19K05712 to Y.N.). We thank Prof. Takahiro Shibata and Mr. Kazushi Koga of Nagoya University for their assistance in dot blot assays and NMR measurements.

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