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Mapping of the Primary Mannose Binding Site of Pradimicin A

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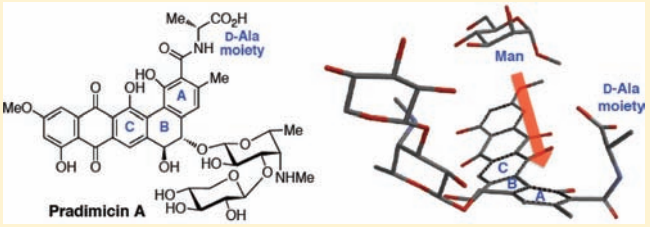
Supporting Information

ABSTRACT: Pradimicin A (PRM-A) is an actinomycete-derived antibiotic with the lectin-like property of being able to recognize D-mannopyranoside (Man) in the presence of Ca^{2+} ion. PRM-A and its derivatives have been attracting a great deal of attention as the only family of natural carbohydrate receptors with nonpeptidic skeleton and, more recently, as conceptually novel drug candidates for human immunodeficiency virus (HIV). Despite its scientific interest and potential therapeutic importance, understanding how PRM-A recognizes Man has been severely limited. Conventional interaction analysis of PRM-A with Man in solution has been frustrated by aggregation of PRM-A and the three-component equilibrium consisting of the [PRM-A_2/Ca^{2+}], [PRM-A_2/Ca^{2+}/Man_2], [PRM-A_2/Ca^{2+}/Man_4] complexes, and their mixed oligomers. In this Article, we demonstrate the interaction analysis of PRM-A with methyl α-D-mannopyranoside (Man-OMe) in the solid state, which benefits from aggregate-forming propensity of PRM-A and eliminates the problem associated with the complicated equilibrium in solution. Isothermal titration calorimetry (ITC) analysis and coprecipitation experiments revealed that the primary Man binding of PRM-A is markedly tighter than the secondary one, leading to preparation of the solid aggregate solely composed of the [PRM-A_2/Ca^{2+}/Man-OMe_2] complex. The simple 1:1 complexes of biosynthetically ^{13}C-enriched PRM-As and [$^{13}C_6$]Man-OMe facilitated the analysis of the primary Man binding of PRM-A by two-dimensional dipolar-assisted rotational resonance (2D-DARR), which clearly identified that the cavity consisted of D-alanine moiety and ABC rings of PRM-A is the Man binding site. Interestingly, the proposed Man binding site of PRM-A seems to resemble the typical architecture of artificial carbohydrate receptors.



INTRODUCTION

Pradimicins and benanomicins are a structurally and biologically unique class of antibiotics derived from actinomycetes.[1] Pradimicin A (PRM-A, Figure 1), an original member of pradimicins, was first isolated from *Actinomadura hibisca* P157-2 in 1988.[2] PRM-A exhibits potent antimicrobial activities against fungi and yeasts both in vitro and in vivo. The most important and interesting feature of PRM-A is its lectin-like ability to recognize D-mannopyranosides (Man) in the presence of Ca^{2+} ion.[1,3] PRM-A and its derivatives have been found to discriminate Man from other hexoses including D-allose, D-altrose, D-glactose, D-glucose, D-gulose, D-idose, D-talose, and even L-mannose.[4] This Man-specific recognition by nonpeptidic compounds has attracted a great deal of attention in the fields of natural product chemistry, carbohydrate chemistry, and biological chemistry. Although synthetic carbohydrate receptors have been progressively developed in the field of supramolecular chemistry[5] as exemplified by Davis's "synthetic lectins" for β-pyranosides of D-glucose and *N*-acetyl-D-glucosamine,[6] the design of receptors with preference for Man is still an open challenge. The most effective receptors reported to date are chiral diaminopyrrolic tripodal receptors developed by Roelens et al.[7] Their receptors displayed substantial selectivity for *n*-octyl mannopyranoside in acetonitrile relative to *n*-octyl glycosides of D-glucose, D-galactose, and *N*-acetyl-D-glucosamine. However, yet to date there has been no report of water-soluble Man receptors of synthetic origin, and PRM-A and its related compounds are the only family of nonpeptidic Man receptors, which are active under physiologically relevant conditions.

Coupled with its Man binding ability, PRM-A as well as several Man-specific lectins have been recognized as conceptually novel drug candidates for human immunodeficiency virus (HIV).[8] They have been found to show the dual mode of antiviral action, blockage of virus entry and triggering the action of immune system by exposing cryptic immunogenic epitopes of the virus surface. Both of these antiviral effects are ascribed to their specific binding to Man residues of glycans on the viral envelope. Because typical lectins of proteinic nature are intrinsically unfavorable as drug candidates, because of their limited supply, poor oral bioavailability, susceptibility to proteases, and potential risk of initiating unfavorable immune response,

Received: August 18, 2011
Published: September 25, 2011

nonpeptidic PRM-A is more promising as a lead for the unprecedented anti-HIV drugs.

Given its special scientific interest and potential therapeutic importance, it is highly desirable to establish the molecular basis of Man recognition by PRM-A. Earlier studies using a PRM-A derivative with improved water solubility suggested that PRM-A recognizes hydroxyl groups at positions 2, 3, 4, and 6 of Man to form the ternary PRM-A/Ca^{2+}/Man complex with a molar component ratio of 2:1:4.[4,9] Subsequent spectroscopic analysis of the complex-forming process showed that one molecule of PRM-A binds two molecules of Man in two separate steps; the binary [PRM-A_2/Ca^{2+}] complex initially binds two molecules of Man to form the ternary [PRM-A_2/Ca^{2+}/Man_2] complex, which then incorporates another two molecules of Man to form the ultimate ternary [PRM-A_2/Ca^{2+}/Man_4] complex.[10] The existence of the [PRM-A_2/Ca^{2+}] complex in aqueous solutions was confirmed by several NMR studies.[11] Structure−activity relationship (SAR) studies by antimicrobial, biochemical, and physicochemical evaluations of naturally occurring and semisynthetic derivatives of PRM-A revealed the importance of D-alanine and D-thomosamine moieties for Man recognition together with the possible involvement of the carboxyl group of PRM-A for Ca^{2+} binding.[1,9,12] However, a clue to understand how PRM-A recognizes Man has been severely limited. The essence of the problem lies in aggregation of PRM-A in the presence of Ca^{2+} ion and complicated three-component equilibrium consisting of the [PRM-A_2/Ca^{2+}], [PRM-A_2/Ca^{2+}/Man_2], and [PRM-A_2/Ca^{2+}/Man_4] complexes, and their mixed oligomers in solution (Scheme 1), which have collectively frustrated conventional X-ray crystallographic and solution NMR analyses.

This situation led us to explore a conceptually novel analytical strategy in the solid state, which benefits from aggregate-forming propensity of PRM-A and eliminates the problem associated with the complicated equilibrium in solution. We recently performed bipartite solid-state NMR experiments using the solid aggregates of PRM-A with methyl α-D-mannopyranoside (Man-OMe).[13] The results led us to propose that PRM-A binds at least one molecule of Man in a Ca^{2+}-mediated manner through its carboxylate group. In this Article, we advance the interaction analysis of PRM-A with Man-OMe in the solid state. A combination of isothermal titration calorimetry (ITC) analysis and coprecipitation experiments allowed us to confirm that the primary Man binding of PRM-A is Ca^{2+}-mediated, and that the solid aggregate solely composed of the [PRM-A_2/Ca^{2+}/Man-OMe_2] complex is successfully prepared through an extensive washing process. Simple 1:1 complexes of biosynthetically ^{13}C-enriched PRM-As with [$^{13}C_6$]Man-OMe facilitated two-dimensional dipolar-assisted rotational resonance (2D-DARR)[14] analyses, which clearly identified the primary Man binding site of PRM-A. The present results suggest the unexpected similarity between PRM-A and artificial carbohydrate receptors.

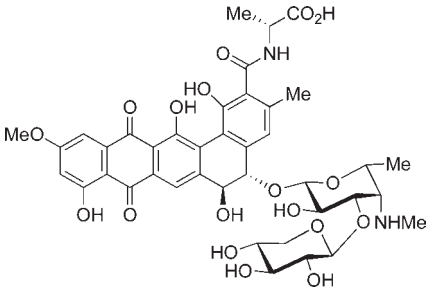


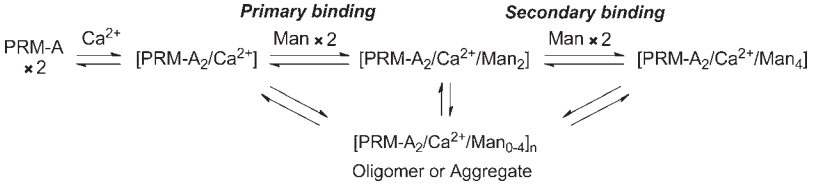
Figure 1. Structure of pradimicin A (PRM-A).

RESULTS AND DISCUSSION

Analysis of Two-Step Man Binding of PRM-A by Isothermal Titration Calorimetry (ITC) and Coprecipitation Experiments. In the previous study, we estimated the PRM-A:Man-OMe ratio in the ternary PRM-A/Ca^{2+}/Man-OMe aggregate to be 1:1 even when an excess amount of Man-OMe was used for aggregate formation.[13] The value was at variance with the previously proposed stoichiometry (1:2) of the ultimate [PRM-A_2/Ca^{2+}/Man-OMe_4] complex.[9] To consolidate these seemingly conflicting results, we assumed that PRM-A might possess two Man binding sites with different affinities and Man-OMe would have been released from the weaker binding site during the washing process of the aggregate. This hypothesis is based on the previous spectroscopic study of Fujikawa et al.,[10] in which a PRM-A derivative was shown to bind two molecules of Man-OMe in two separate steps. Although the binding constant (K_a) for the secondary Man binding was estimated to be in the range of 600−1000 M^{-1}, the K_a for the primary Man binding was not obtained due to minute absorbance change. Thus, as a prerequisite for interaction analysis in the solid state, ITC analysis of Man binding of PRM-A was performed to confirm the existence of two Man binding sites with different affinities.

ITC is a powerful technique to characterize directly the thermodynamics of molecular interactions. In an ITC experiment, binding of two molecules is determined by direct heat change with the environment. In the case of the binding analysis of PRM-A with Man-OMe, ITC experiments should be run under the conditions that can provide enough heat effects and minimize aggregation of PRM-A. Among various conditions examined, the best titration curve was obtained when 1 mM PRM-A and 100 mM Man-OMe in 50 mM 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (pH 7.0) containing 10 mM $CaCl_2$ were used as the titrate and titrant solutions, respectively. The obtained ITC profile is shown in Figure 2A. The isotherm curve was fitted well to a 1:2 binding model, supporting the existence of two Man binding sites in PRM-A. Control experiments without Ca^{2+} ion or with methyl α-D-glucopyranoside (Glc-OMe) in place of Man-OMe confirmed that the characteristic heat effects truly originated from specific complex formation of PRM-A with Man-OMe and Ca^{2+} ion (Figure 2B,C). The K_a

Scheme 1. Complex-Forming Equilibrium of PRM-A with Ca^{2+} and Man



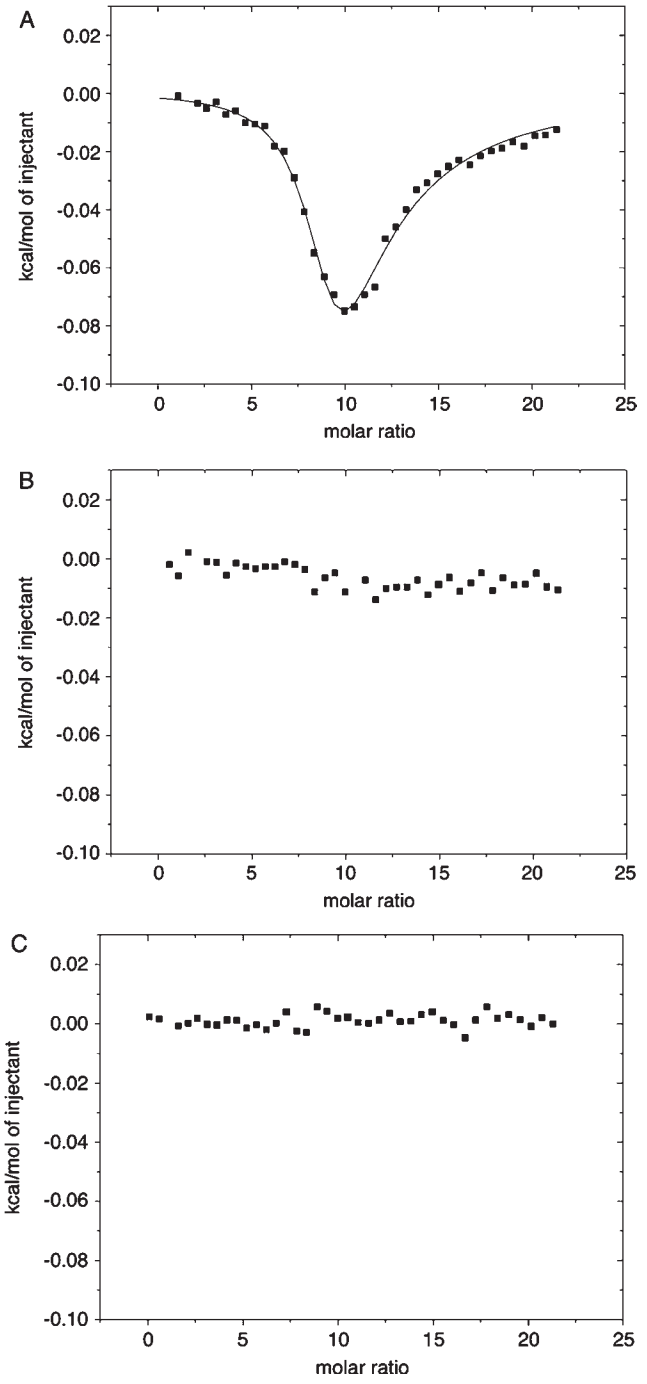


Figure 2. ITC profiles for PRM-A titrated with (A) Man-OMe in the presence of $CaCl_2$, (B) Man-OMe in the absence of $CaCl_2$, and (C) Glc-OMe in the presence of $CaCl_2$. The experiments were performed using 1 mM PRM-A and 100 mM Man-OMe or Glc-OMe in 50 mM MOPS buffer (pH 7.0) with or without 10 mM $CaCl_2$ at 30 °C. The solid line in (A) represents the least-squares fit of the data to a two sets of sites binding model.

values for the primary and secondary Man-OMe binding were provided to be 10 400 ± 1660 and 263 ± 27 M^{-1},[15] respectively, indicating that the primary Man binding is about 40 times stronger than the secondary one.

Having confirmed that PRM-A possesses two Man binding sites with significantly different affinities, we next examined the

Table 1. Quantification of Man-OMe and Glc-OMe Coprecipitated with PRM-A and Ca^{2+} Ion

	molar ratio relative to PRM-A[a]	
number of washing times	Man-OMe	Glc-OMe
0	2.35 (0.10)[b]	0.34 (0.03)
1	1.45 (0.05)	<0.1
2	1.08 (0.09)	<0.1
3	1.10 (0.04)	<0.1
4	1.07 (0.09)	<0.1

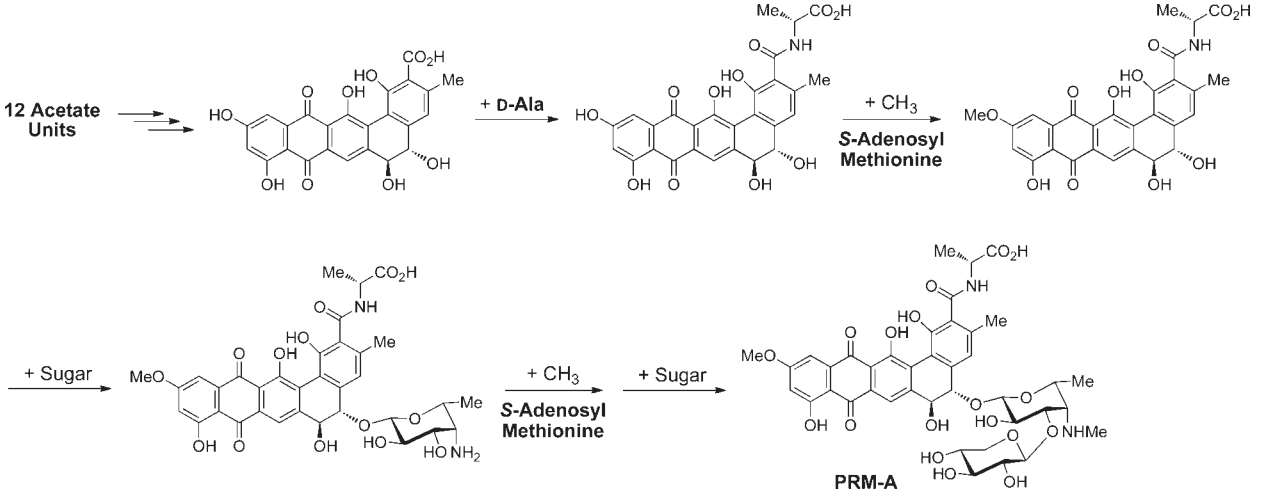
[a] The ratios were calculated by solution ^{1}H NMR on the basis of integration values of PRM-A and Man-OMe or Glc-OMe after dissociation of the aggregate by acid treatment. [b] Standard deviation of at least three separate experiments.

effect of washing on Man-OMe incorporation in the PRM-A/Ca^{2+} aggregate. Table 1 summarizes the molar ratio of Man-OMe relative to PRM-A in the aggregate after 0−4 times washing with an aqueous $CaCl_2$ solution. Experiments using Glc-OMe instead of Man-OMe were similarly conducted to evaluate nonspecific binding. Without washing, the aggregate apparently consisted of the 1:2 complex of PRM-A and Man-OMe, that is, [PRM-A_2/Ca^{2+}/Man-OMe_4], with a small amount of Man-OMe that was nonspecifically absorbed on the aggregate. Although the molar ratio of Man-OMe stepwise decreased to the equimolar amount after the first and second washings, the ratio was maintained after the third and forth washings. The realistic implication of these results is that the extensive washing of the aggregate results in a release of Man-OMe from the secondary Man binding site to produce the 1:1 complex of PRM-A and Man-OMe, that is, [PRM-A_2/Ca^{2+}/Man-OMe_2]. Taken together, the ITC and coprecipitation experiments support our hypothesis and, as a result, indicate that our proposed Ca^{2+}-mediated binding[13] is applicable to the primary Man binding of PRM-A. It is also practically important that the aggregate solely composed of the [PRM-A_2/Ca^{2+}/Man-OMe_2] complex was found to be prepared through the extensive washing process. Taking advantage of this finding, we conducted further analysis of the primary Man binding of PRM-A in the solid state.

Biosynthetic Preparation of ^{13}C-Enriched PRM-As for Solid-State NMR Experiments. Our analytical strategy in the solid state relies on two-dimensional dipolar-assisted rotational resonance (2D-DARR)[14] methodology. DARR, also known as RF assisted diffusion (RAD),[16] is a technique of solid-state NMR spectroscopy to detect weak ^{13}C−^{13}C couplings in the presence of strong couplings derived from directly bound carbons.[17] In 2D-DARR spectra, dipolar interactions between ^{13}C nuclei that are located within 6 Å can be detected as cross peaks, allowing us to evaluate intermolecular close contacts between PRM-A and Man-OMe. Therefore, selective ^{13}C-enrichment of PRM-A is necessary for enhancing the spectral sensitivity as well as facilitating data interpretation.

While Suzuki's total synthesis of PRM-A is a marvelous achievement,[18] it is unlikely to be applicable to the preparation of ^{13}C-labeled PRM-A due to a large number of synthetic steps. One possible approach is semisynthesis via detachment of the D-alanine or disaccharide moieties of PRM-A followed by introduction of ^{13}C-enriched counterparts. However, earlier studies have revealed that chemical modification of these moieties is arduous and material-consuming.[12,19] On the other hand, the biosynthetic approach would be more promising in light of its

Scheme 2. Outline of the Putative Biosynthetic Pathway of PRM-A



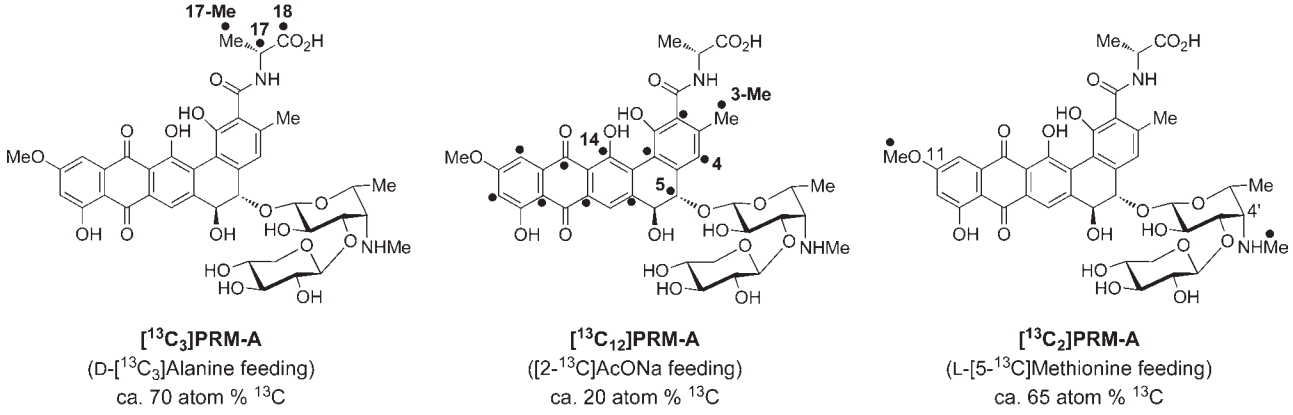


Figure 3. Biosynthetically ^{13}C-enriched PRM-As. Black circles indicate ^{13}C-enriched positions. The ^{13}C-population was calculated by solution ^{1}H NMR on the basis of integration values of proton signals split with $^{1}H-^{13}C$ couplings.

potential to facilitate the production of the ^{13}C-enriched PRM-As. In the study on the biosynthesis of PRM-A (Scheme 2), D-alanine and AcONa were shown to be incorporated into D-alanine and benzo[*a*]naphthacenequinone moieties of PRM-A, respectively.[20] Moreover, 11-*O*- and 4′-*N*-methyl groups are expected to be introduced from L-methionine via *S*-adenosyl methionine,[21] collectively indicating that the biosynthetic approach could realize ^{13}C-labeling of the whole region of PRM-A in a site-selective manner. Thus, we took advantage of the biosynthesis of PRM-A using *Actinomadura* sp. TP-A0019 for the preparation of ^{13}C-enriched PRM-As.

Effective ^{13}C-enrichment of D-alanine moiety of PRM-A (ca. 70 atom % ^{13}C, Figure 3) was achieved by feeding of exogenous D-[$^{13}C_3$]alanine with D-cycloserine, which inhibits the supply of endogenous D-alanine.[13] On the other hand, a similar strategy was not employed for feeding experiments with ^{13}C-labeled AcONa and L-methionine because disrupted supply of endogenous AcONa and L-methionine results in growth inhibition of the actinomycete. Thus, ^{13}C-labeling by feeding with excessive amounts of ^{13}C-labeled substances was performed (Supporting Information). Whereas four additions of 100 mg of [2-^{13}C]AcONa in intervals of 1 day to the culture broth (100 mL) inhibited growth of the actinomycete, those of 50 mg did not interfere with the growth and PRM-A production of *Actinomadura* sp. TP-A0019. The solution ^{13}C NMR spectrum of the obtained PRM-A showed a moderate level of ^{13}C-enrichment (ca. 20 atom % ^{13}C, Figure 3) of the benzo[*a*]naphthacenequinone moiety (Supporting Information).

In the case of feeding of L-[5-$^{13}CH_3$]methionine, reasonable ^{13}C-enrichment (ca. 45 atom % ^{13}C) of both 11-*O*- and 4′-*N*-methyl groups was observed when 100 mg of L-[5-$^{13}CH_3$]-methionine was added to the culture broth (100 mL) before incubation. This observation provided the first experimental evidence that the 4′-*N*-methyl group of PRM-A is derived from L-methionine. The ^{13}C-incorporation was found to be enhanced through additional feeding of L-[5-$^{13}CH_3$]methionine during incubation. The maximum ^{13}C-enrichment (ca. 65 atom % ^{13}C, Figure 3) was obtained when incubation started in the presence of 100 mg of L-[5-$^{13}CH_3$]methionine, and three additions of 50 mg were made in intervals of 1 day (Supporting Information).

Detection of Close Interactions of PRM-A with Man-OMe by Two-Dimensional Dipolar-Assisted Rotational Resonance (2D-DARR). With the ^{13}C-enriched PRM-As ([$^{13}C_3$]-, [$^{13}C_{12}$]-, and [$^{13}C_2$]PRM-As) in hand, we prepared the solid aggregates of their [PRM-A_2/Ca^{2+}/Man-OMe_2] complexes using [$^{13}C_6$]Man-OMe[22] according to our standardized procedure.

dx.doi.org/10.1021/ja207816h |*J. Am. Chem. Soc.* 2011, 133, 17485–17493

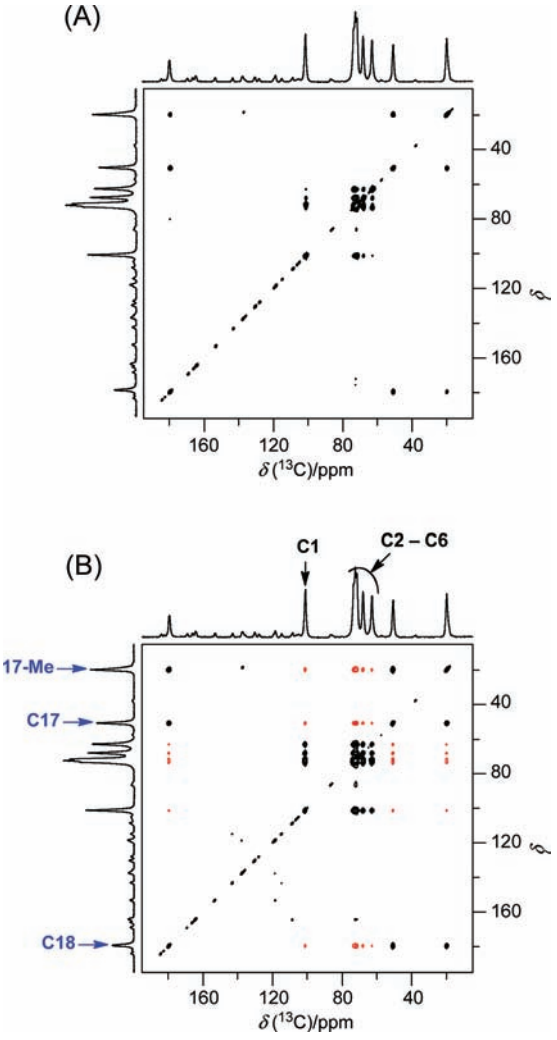


Figure 4. 2D-DARR spectra of the [$^{13}C_3$]PRM-A_2/Ca^{2+}/[$^{13}C_6$]Man-OMe_2 complex at the mixing times of (A) 20 ms and (B) 500 ms. Blue and black arrows represent ^{13}C signals for [$^{13}C_3$]PRM-A and [$^{13}C_6$]Man-OMe, respectively. Intermolecular cross peaks are shown in red.

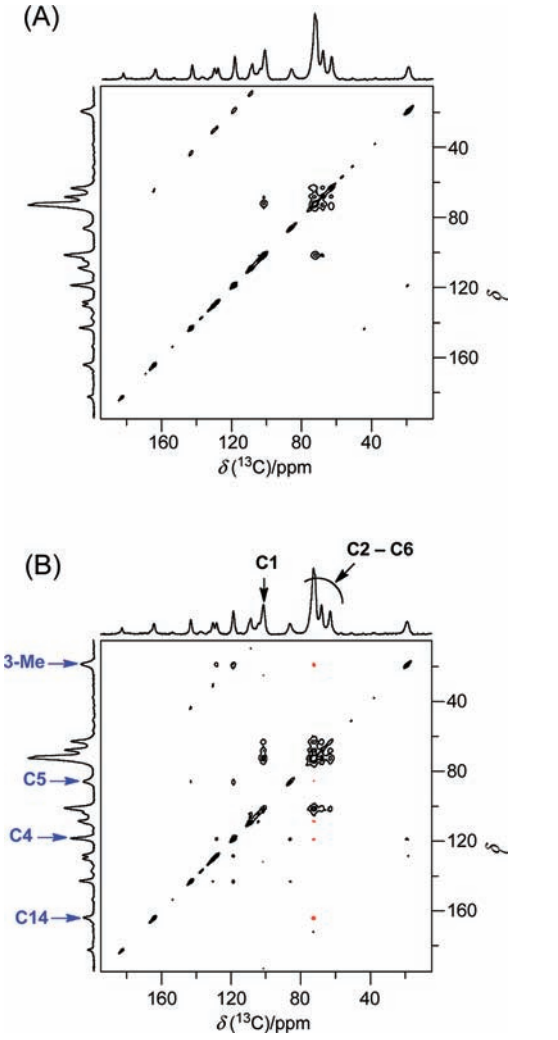


Figure 5. 2D-DARR spectra of the [$^{13}C_{12}$]PRM-A_2/Ca^{2+}/[$^{13}C_6$]Man-OMe_2 complex at the mixing times of (A) 20 ms and (B) 500 ms. Blue and black arrows represent ^{13}C signals for [$^{13}C_{12}$]PRM-A and [$^{13}C_6$]Man-OMe, respectively. Intermolecular cross peaks are shown in red. The ^{13}C signal at 108.9 ppm could not be assigned due to signal overlapping.

2D-DARR spectra of three complexes are shown in Figures 4−6. Signal assignments were based on solution ^{13}C NMR spectra of ^{13}C-enriched PRM-As and, in the case of [$^{13}C_{12}$]PRM-A, 2D-DARR spectra of its [PRM-A_2/Ca^{2+}/Man-OMe_2] complex using nonlabeled Man-OMe (Supporting Information).

While only intramolecular cross peaks were observed at the mixing time of 20 ms in all complexes (Figures 4−6A), the spectra of the complexes of [$^{13}C_3$]- and [$^{13}C_{12}$]PRM-As at the mixing time of 500 ms showed intermolecular cross peaks. Symmetric cross peaks were clearly observed between carbon signals for the D-alanine moiety of PRM-A (δ 20.0 for 17-Me, 50.8 for C17, 179.8 for C18) and those for Man-OMe (δ 63.0, 68.2, 71−76 for C2−C6, 101.4 for C1) in the complex of [$^{13}C_3$]PRM-A with [$^{13}C_6$]Man-OMe (Figure 4B).[13] On the other hand, asymmetric cross peaks were detected between four carbon signals of PRM-A (δ 18.9 for 3-Me, δ 86.1 for C5, δ 118.8 for C4, δ 164.3 for C14) and C2−C6 signals of Man-OMe in the complex of [$^{13}C_{12}$]-PRM-A with [$^{13}C_6$]Man-OMe (Figure 5B). The asymmetry of cross peaks is mainly ascribed to nonuniform signal enhancement by cross-polarization (CP) and low ^{13}C-enrichment (ca. 20 atom % ^{13}C) of [$^{13}C_{12}$]PRM-A.[23] Although intensities of these intermolecular cross peaks were weak, we were still able to confirm that they were actually "true" cross peaks by the mixing time dependency. The absence of these cross peaks at the mixing time of 20 ms (Figure 5A) clearly indicates that they arose from $^{13}C-^{13}C$ dipolar interaction reintroduced by DARR during mixing time.

In contrast to the above two complexes, the ternary complex of [$^{13}C_2$]PRM-A with [$^{13}C_6$]Man-OMe showed no intermolecular cross peak even at the mixing time of 500 ms despite the high ^{13}C-enrichment (ca. 65 atom % ^{13}C) of [$^{13}C_2$]PRM-A (Figure 6B). This observation eliminates the possibility that the intermolecular cross peaks observed in the complexes of [$^{13}C_3$]PRM-A and [$^{13}C_{12}$]PRM-A are simply derived from nonspecific binding of Man-OMe to PRM-A or accidental proximity in the solid sample. The coprecipitation experiments described above also support that nonspecific binding of Man-OMe is negligible. Consequently, it is confirmed that the intermolecular cross peaks truly arise from specific close interactions, and Man-OMe is located within 6 Å of the D-alanine moiety, 3-Me, C4, C5, and C14 of PRM-A in the [PRM-A_2/Ca^{2+}/Man-OMe_2] complex.

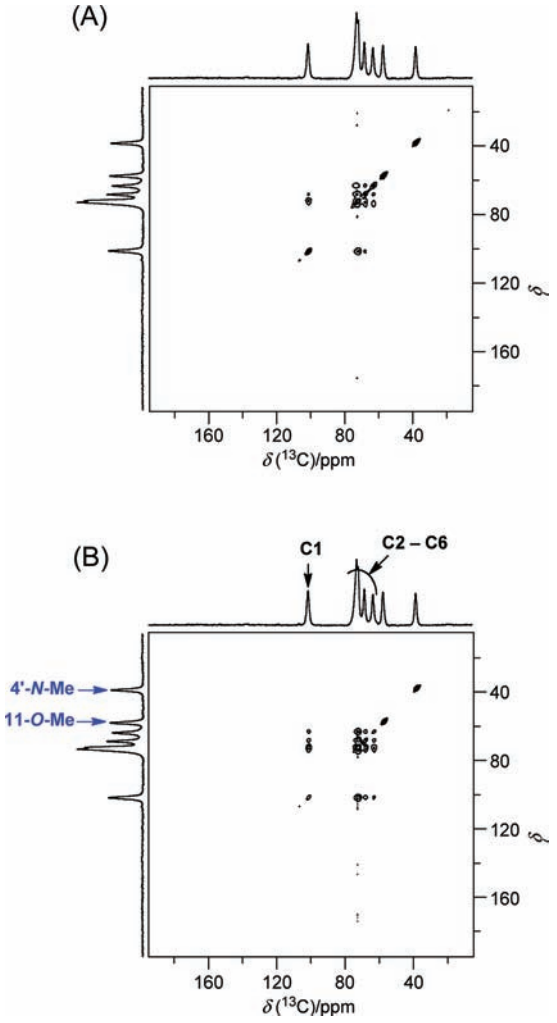


Figure 6. 2D-DARR spectra of the $[^{13}C_2]$PRM-A_2/Ca^{2+}/$[^{13}C_6]$Man-OMe_2 complex at the mixing times of (A) 20 ms and (B) 500 ms. Blue and black arrows represent ^{13}C signals for $[^{13}C_2]$PRM-A and $[^{13}C_6]$Man-OMe, respectively. No intermolecular cross peak was detected.

These DARR data are well explained when the D-alanine moiety and the C14 carbon atom are oriented on the same face of the A ring. In such a conformation as shown in Figure 7A, the close contacts of the D-alanine moiety, 3-Me, C4, C5, and C14 with Man-OMe are realized by placing Man-OMe above the A ring of PRM-A, suggesting that a cavity consisting of the D-alanine moiety and ABC rings is a possible Man binding site. This is in good agreement with the previous SAR studies, which showed that the antifungal activity of PRM-A is sensitive to chemical modification of the D-alanine moiety and A ring.[12,24] Replacement of D-alanine with L-alanine or other D-amino acids except D-serine diminished the antifungal activity. The 1-*O*-methyl derivative also showed significantly reduced activity, and the 4-Br, NO_2, and NH_2 derivatives were found to be completely inactive. Because the antifungal activity correlates well with the Man binding ability,[1,25] all of these results corroborate our conclusion that the D-alanine moiety and A ring are critical constituents of the Man binding site of PRM-A.

Regarding the disaccharide moiety of PRM-A, previous studies indicated the possibility that the 2′-OH group is more important for Man binding than the 4′-$NHCH_3$ group and the D-xylose moiety.[12,26] Our DARR experiments suggest that the 4′-*N*-methyl group is apparently distant from the Man, supporting that the 4′-$NHCH_3$ group is not involved in Man binding. These findings are also explained by positing that the dissacharide moiety is oriented on the same face as the D-alanine moiety and the C14 carbon atom (Figure 7A). While the 4′-$NHCH_3$ group and D-xylose moiety are oriented outward from the binding cavity, the 2′-OH group is located close to the A ring. Assuming that the 2′-OH group also forms a part of the binding cavity, we propose the model for the primary Man binding of PRM-A (Figure 7B, left). In addition to the Ca^{2+}-mediated interaction with Man through the carboxylate group, hydrogen bonds with Man would be formed at the 14-OH and 2′-OH groups of PRM-A. Because CH/π interaction is well appreciated to be critical as an attracting force in carbohydrate recognition,[27] it is quite likely that the close contact (<6 Å) of the A ring with Man is derived from CH/π interaction. To date, a number of artificial carbohydrate receptors have been designed using arene-based tripodal architectures (Figure 7B, right).[5,7,28] These types of receptors incorporate three hydrophilic moieties interconnected by an arene spacer, and realize three-dimensional recognition of sugars by providing both surfaces of hydrogen-bonding and CH/π interaction. It is particularly interesting that PRM-A, the naturally occurring carbohydrate receptor, seems to share a similar architecture; the A ring provides the hydrophobic core for CH/π interaction, and the D-alanine, anthraquinone, and disaccharide moieties serve as hydrogen-bonding substituents. The highly sophisticated tripodal structure of PRM-A possibly provides a unique guide to realize artificial Man recognition in water.

CONCLUSION

In the present work, we investigated the primary Man binding of PRM-A in the solid state. The key to our analytical strategy is the use of the solid aggregate solely composed of the [PRM-A_2/Ca^{2+}/Man-OMe_2] complex, which enabled us to analyze a simple 1:1 interaction of PRM-A and Man with avoidance of the problem associated with the complicated equilibrium in solution. The 2D-DARR experiments using the [PRM-A_2/Ca^{2+}/Man-OMe_2] complexes of biosynthetically ^{13}C-enriched PRM-As with $[^{13}C_6]$Man-OMe successfully detected the close interactions of the D-alanine moiety, 3-Me, C4, C5, and C14 of PRM-A with Man-OMe. These close contacts are simultaneously compatible when Man is located on the same face of the A ring as the D-alanine moiety and the C14 carbon atom. This is the first solid evidence that the cavity consisting of D-alanine moiety and ABC rings is the primary Man binding site of PRM-A. This finding, coupled with our previous result, led us to propose a model for the primary Man binding of PRM-A (Figure 7B). The Ca^{2+}-coodination, hydrogen-bonding, and CH/π interaction might be involved in the interaction of PRM-A with Man. Quite interestingly, the architecture of the primary Man binding site of PRM-A is conceptually similar to those of artificial tripodal receptors, which have been a mainstay of the molecular recognition in the supramolecular chemistry. The present study provides an important step toward the full elucidation of the molecular basis of Man recognition by PRM-A and may open the way to a new generation of artificial Man receptors.

EXPERIMENTAL SECTION

General Remarks. PRM-A was isolated from the fermentation broth of *Actinomyces* sp. TP-A0019. Enriched D-$[^{13}C_3]$alanine (>99 atom % ^{13}C),

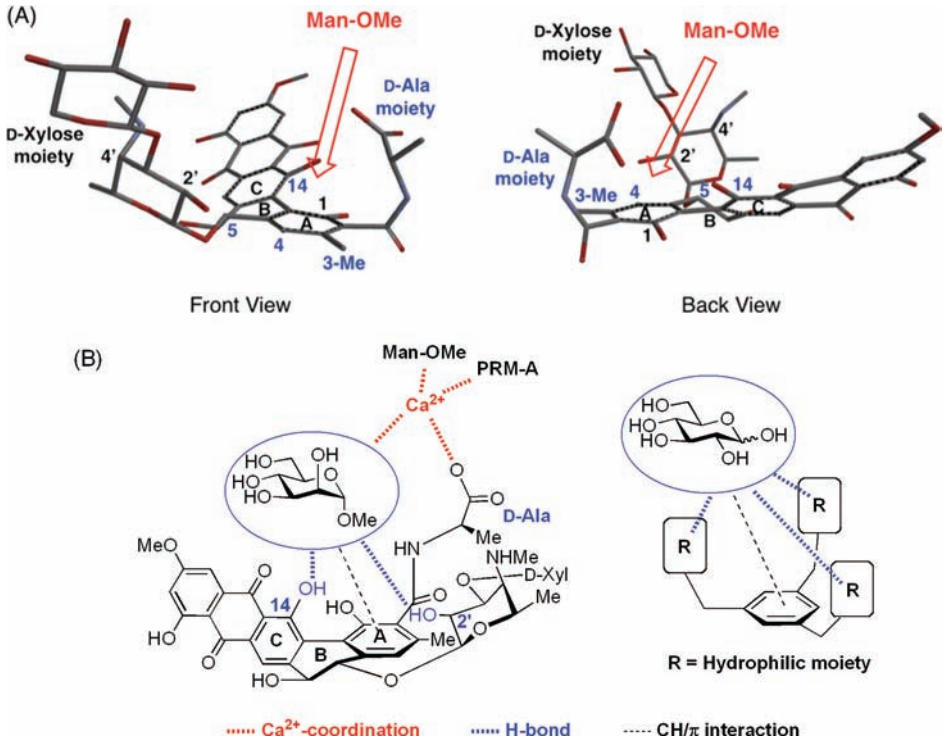


Figure 7. (A) Possible Man binding conformation of PRM-A. The energy-minimized conformation of monomeric PRM-A without Ca^{2+} ion was obtained by using Spartan'10 (Wave function, Inc., Irvine, CA). Carbon, oxygen, and nitrogen atoms are shown in gray, red, and blue, respectively. Hydrogen atoms are omitted for clarity. (B) Model for the primary Man binding of PRM-A (left) and typical architecture of tripodal carbohydrate receptors (right).

[2-^{13}C]AcONa (>99 atom % ^{13}C), L-[5-$^{13}CH_3$]methionine (>99 atom % ^{13}C), and D-[$^{13}C_6$]mannose (98 atom % ^{13}C) were purchased from Taiyo Nippon Sanso Co. (Tokyo, Japan). All other chemicals and reagents were purchased from chemical companies and used without further purification.

Isothermal Titration Calorimetry Analysis. Isothermal titration calorimetry (ITC) measurements were carried out at 30 °C using a Microcal iTC$_{200}$ microcalorimeter (Microcal Inc., Northampton, MA). The measurements were conducted in 50 mM MOPS buffer (pH 7.0) with or without 10 mM $CaCl_2$. The samples were prepared in the same buffer solution and degassed by sonication before use. A typical titration consisted of injecting 1.0 μL of 100 mM Man-OMe or Glc-OMe (total 40 injections) into 1 mM PRM-A with an interval of 5 min between injections. To achieve a homogeneous mixing and minimize the aggregation of PRM-A in the cell, the stirrer speed was kept constant at 1500 rpm. The heat of dilution was determined under identical conditions by injecting the 100 mM Man-OMe or Glc-OMe into the ITC cell containing only the buffer. For every experiment, the heat of dilution was determined and 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 two sets of sites model to calculate the binding constants. The accuracy of the calculated values was checked by three independent titrations, and uncertainties are expressed as standard deviations of the three experiments.

Coprecipitation Experiment. To a solution of PRM-A·TFA (2.9 mg, 3.0 μmol) in distilled water (300 μL) in a 1.5 mL Eppendolf tube were added 100 mM aqueous solutions of $CaCl_2$ (300 μL, 30.0 μmol, 10 equiv) and Man-OMe or Glc-OMe (750 μL, 75 μmol, 25 equiv) at room temperature. The pH of the solution was adjusted to 4.5 with 1 N NaOH, and the resulting mixture was incubated at 60 °C for 2 h and 4 °C for 1 h. After centrifugation at 10 000 rpm (9170g) for 10 min at 4 °C, the supernatant was removed by aspiration. The precipitate was washed 0−4 times with aqueous 100 mM $CaCl_2$ by repeating the following procedure: addition of aqueous $CaCl_2$ (1.0 mL), shaking with a Boltex mixer for a few seconds, centrifugation at 10 000 rpm (9170g) for 5 min at 4 °C, and aspiration of the supernatant. The tip of the tube was cut off and put in a 50 mL Falcon tube. The precipitate in the tip of the tube was dissolved in 50% CH_3CN/H_2O containing 0.1% TFA (2 mL) by sonication. The resulting solution was dried in vacuo to afford a mixture of free PRM-A and Man-OMe or Glc-OMe, the ratio of which was determined by integration values in solution ^{1}H NMR spectra (in DMSO-d_6 at 60 °C, JEOL ECX 400 spectrometer) as reported previously.[13]

Preparation of ^{13}C-Enriched PRM-As. Fermentation: *Actinomyces* sp. TP-A0019 was grown at 30 °C for 10 days on ISP-4 medium containing 0.2% yeast extract. The seed culture was incubated for 6 days at 30 °C in V22 medium (20 mL) in a 50 mL Erlenmeyer flask, and aliquot (3.0 mL) was used as inocula to start fermentation. Each feeding experiment was carried out in A3M medium (100 mL) in a 50 mL Erlenmeyer flask shaken at 180 rpm for 6 days at 30 °C. All ^{13}C-enriched compounds and D-cycloserine were dissolved in distilled water (D-[$^{13}C_3$]-alanine 100 mg/mL; D-cycloserine 1.0 mg/mL; [2-^{13}C]AcONa 100 mg/mL; L-[5-$^{13}CH_3$]methionine 50 mg/mL). Each solution was sterilized by filtration, and aliquot (2.0 mL for D-[$^{13}C_3$]alanine and L-[5-$^{13}CH_3$]-methionine, 1.0 mL for D-cycloserine, 0.5 mL for [2-^{13}C]AcONa) was added to the medium before incubation. For feeding of [2-^{13}C]AcONa and L-[5-$^{13}CH_3$]methionine, additions of aliquot (0.5 mL × 4 for [2-^{13}C]AcONa, 1.0 mL × 3 for L-[5-$^{13}CH_3$]methionine) were continued in intervals of 1 day during incubation. The compositions of the V22 and A3M media are as follows: V22 medium; soluble starch 1.0%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, triptone 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3% (pH 7.0); A3M medium; glucose 0.5%, glycerol 2.0%, soluble starch 2.0%, Pharmamedia 1.5%, yeast extract 0.3%, Diaion HP-20 1.0% (pH 7.0).

dx.doi.org/10.1021/ja207816h |*J. Am. Chem. Soc.* 2011, 133, 17485–17493

Harvesting and purification of ^{13}C-enriched PRM-As: After incubation, CH_3CN (100 mL) was added to the culture broth, and the mixture was shaken at 180 rpm for 2 h. The mycelia were separated by centrifugation at 12 000 rpm (11 040*g*) for 20 min. The supernatant was concentrated in vacuo, and the residue was adsorbed on a column of Diaion HP-20. After washing with water, the resin was eluted with 70% acetone in water containing 0.1% TFA, and the eluate was concentrated in vacuo. The residue was purified by column chromatography (gel, Cosmosil 140C_{18}-OPN; solvent, 20%→30% CH_3CN containing 0.1% TFA) to give crude ^{13}C-enriched PRM-As. Further purification was carried out by normal-phase HPLC (column, TSK gel Amide-80, 10 μm, 21.5 mm ID × 300 mm; solvent, 50 min linear gradient 95%→63.5% CH_3CN containing 0.1% TFA; flow rate, 6.0 mL/min; UV, 254 nm; retention time, 39.0 min) followed by reverse-phase HPLC (column: YMC-Pack ODS-A, 5 μm, 20.0 mm ID × 250 mm; solvent, 40 min linear gradient 25%→37% CH_3CN containing 0.1% TFA; flow rate, 8.0 mL/min; UV, 254 nm; retention time, 32.5 min) to afford pure ^{13}C-enriched PRM-As (11.2 mg for [$^{13}C_3$]PRM-A, 16.0 mg for [$^{13}C_{12}$]PRM-A, 11.7 mg for [$^{13}C_2$]PRM-A) as a TFA salt. ^{13}C chemical shifts of the ^{13}C-enriched PRM-As were identical to those of PRM-A (Supporting Information).

Preparation of the Solid Aggregate Composed of Exclusively the [PRM-A_2/Ca^{2+}/Man-OMe_2] Complex Using ^{13}C-Enriched PRM-A and [$^{13}C_6$]Man-OMe. To a 10 mM solution of ^{13}C-enriched PRM-A·TFA in distilled water were added 50 mM $CaCl_2$ (10 equiv) and 100 mM [$^{13}C_6$]Man-OMe (25 equiv) at room temperature. The pH of the solution was adjusted to 4.5 with 1 N NaOH, and the resulting mixture was incubated at 60 °C for 2 h and 4 °C for 1 h. After centrifugation at 10 000 rpm (9840*g*) for 20 min at 4 °C, the supernatant was removed by decantation. The precipitate was washed two times with 50 mM $CaCl_2$ and then dried in vacuo to afford the solid aggregate solely composed of the [PRM-A_2/Ca^{2+}/Man-OMe_2] complex as a red powder.

Two-Dimensional Dipolar-Assisted Rotational Resonance (2D-DARR) Experiment. 2D-DARR experiments were carried out at 14 T (150 MHz for ^{13}C) with a JEOL ECA600 spectrometer and a custom-fabricated probe with a Chemagnetics 3.2 mm spinning system at a MAS frequency of 15 kHz and room temperature. Variable-amplitude CP (VACP) and two-pulse phase-modulated (TPPM) decoupling were used. Pulse sequence parameters were contact time of 1 ms, proton decoupling power of 70 kHz, t_1 increments of 33 μs, number of scans of 16 per increment, t_1 points of 256, mixing time of 20 or 500 ms, and pulse delay of 3 s. Chemical shifts were calibrated in ppm relative to TMS by taking the ^{13}C chemical shift for the methine ^{13}C of solid adamantane (29.5 ppm) as an external reference standard.

■ ASSOCIATED CONTENT

Supporting Information. Full results of feeding experiments with [2-^{13}C]AcONa and L-[5-$^{13}CH_3$]methionine, solution 1D-^{13}C NMR spectra of ^{13}C-enriched PRM-As, and signal assignment of the [$^{13}C_{12}$]PRM-A_2/Ca^{2+}/[$^{13}C_6$]Man-OMe_2 complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

Y.M. is a Research Fellow of the Japan Society for the Promotion of Science. We thank Prof. Toshikazu Oki for his generous support, and Ms. Akemi Takahashi and Ms. Satoko Shirahata for technical assistance. This research was partly supported by an Incentive Research Grant from RIKEN and a MEXT Grant-in-Aid for Young Scientists (B) (22780109).

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