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Carbohydrate–Lipid Interactions: Affinities of Methylmannose Polysaccharides for Lipids in Aqueous Solution

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Abstract: The interactions between 3-*O*-methyl-mannose polysaccharides (MMPs), extracted from *Mycobacterium smegmatis* (consisting of a mixture of MMP-10, -11, -12 and -13) or obtained by chemical synthesis (MMP-5_s, -8_s, -11_s and -14_s), and linear saturated and unsaturated fatty acids (FAs), and a commercial mixture of naphthenic acids (NAs) in aqueous solution at 25 °C and pH 8.5 were quantified by electrospray ionization mass spectrometry (ESI-MS). Association constants (K_a) for MMP binding to four FAs (myristic acid, palmitic acid, stearic acid and *trans*-parinaric acid) were measured by using an indirect ESI-MS assay, the "proxy protein" method. The K_a values are in the 10^4–$10^5\,M^{-1}$ range and, based on results obtained for the binding of the synthetic MMPs with palmitic acid, increase with the size of the carbohydrate. Notably, the measured affinity of the extracted MMPs for *trans*-parinaric acid is two orders of magnitude smaller than the reported value, which was determined by using a fluorescence assay. Using a newly developed competitive binding assay, referred to as the "proxy protein/proxy ligand" ESI-MS method, it was shown that MMPs bind specifically to NAs in aqueous solution, with apparent affinities of approximately $(5\times10^4)\,M^{-1}$ for the mixture of NAs tested. This represents the first demonstration that MMPs can bind to hydrophobic species more complex than those containing linear alkyl/alkenyl chains. Moreover, the approach developed here represents a novel method for probing carbohydrate–lipid interactions.

Keywords: carbohydrates · electrospray ionization mass spectrometry · lipids · naphthenic acids · polysaccharides

Introduction

Polymethylated polysaccharides (PMPs) are cytoplasmic glycans produced by mycobacteria that contain 10–20 carbohydrate residues.[1,2] These species have been postulated to play a role in regulating lipid metabolism in the organisms that produce them, through the formation of complexes with long-chain fatty acids and their activated coenzyme-A (CoA) derivatives.[3,4] For example, PMPs have been shown to tolerize mycobacteria to the high concentration of the long-chain acyl-CoA derivatives needed for the synthesis of mycolic acids, archetypal mycobacterial lipids that are essential for viability.[5] In another study, these species have been demonstrated to regulate fatty acid chain lengths of fatty acids produced by Type I fatty acid synthetase in *Mycobacterium smegmatis*.[6]

One class of PMPs are 3-*O*-methyl-mannose polysaccharides (MMPs), a family of molecules that contain 10–13 mannopyranose (Man*p*) moieties.[7] In MMPs, every Man*p* residue, except that at the non-reducing terminus, is methylated on O-3 (Figure 1). When present in an aqueous solution containing lipids, for example, long-chain linear fatty acids (FAs), MMPs have been suggested to adopt a helical conformation in which the methyl groups are on the inside of the helix, thus providing a hydrophobic channel capable of lipid binding, and a hydrophilic exterior.[8] Using fluorescence measurements, very high association constants (K_a) of $(1.0\times10^7)\,M^{-1}$ and $(2.5\times10^6)\,M^{-1}$, have been reported for MMP binding to the important biochemical intermediate palmitoyl CoA and the polyene parinaric acid, respectively.[9,10]

The remarkably high lipid affinities reported for MMPs raise the interesting possibility of using these polysaccharides as a sorbent to remove hydrophobic contaminants from aqueous solutions. In this regard, of particular current relevance are the tailings ponds accumulating as a byproduct of the bitumen extraction process in the Athabasca oil sands in Northern Alberta. Tailings pond waters (TPW) are an increasing environmental concern and the primary toxic components are believed to be naphthenic acids (NAs, Figure 1), a complex mixture of alkyl-substituted cyclic and acyclic aliphatic carboxylic acids.[11] These compounds have the general molecular formula, $C_nH_{2n+Z}O_2$, where Z is 0 or a negative even integer. There is considerable interest in remediation of TPW, and carbohydrate sorbents, such as dimethylaminoethyl-cellulose[12] and β-cyclodextrin,[13] have been reported for this purpose. However, a distinct advantage of MMPs, compared to these other carbohydrates, is their inherently high affinity for hydrophobic molecules.

We describe here the results of a quantitative study of the interactions between MMPs, either extracted from mycobac-

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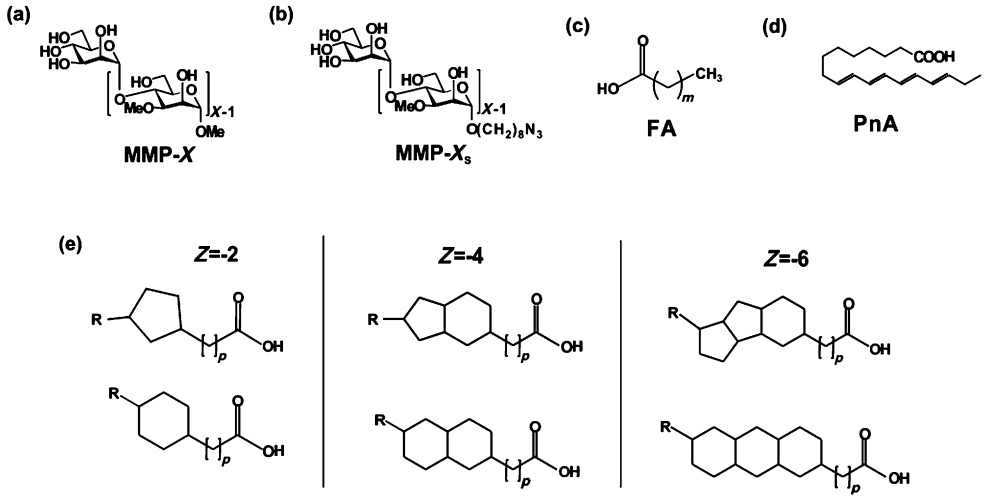


Figure 1. Structures of: a) extracted MMP-X; $X=10$, 11, 12, 13; b) synthetic MMP-X_s; $X=5$, 8, 11, 14; c) FAs, $m=12$ (MA), 14 (PA), 16 (SA); d) PnA; e) representative structures of NAs; R = alkyl group.

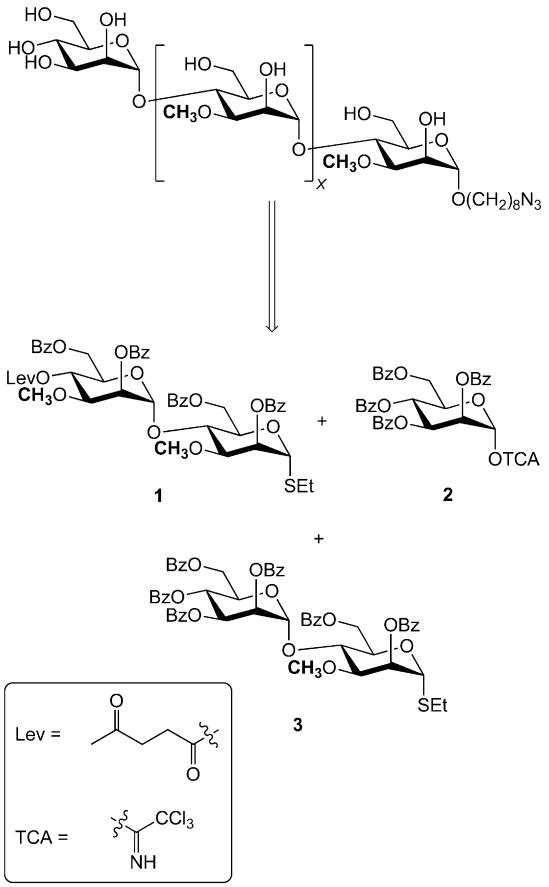
teria (a mixture of MMP-10, -11, -12 and -13) or pure species produced by chemical syntheses (MMP-5_s, -8_s, -11_s and -14_s) and linear, saturated and unsaturated FAs, and a commercial mixture of NAs in aqueous solution at 25 °C and pH 8.5. Both direct and indirect (competitive) electrospray ionization mass spectrometry (ESI-MS) assays were used to quantify the interactions between the MMPs and the FAs and NAs. However, due to the occurrence of in-source dissociation, the direct ESI-MS assay[14–17] was found to significantly underestimate the strength of the interactions. The affinities for the individual MMPs and the mixture of extracted MMPs for four FAs, myristic acid, palmitic acid, stearic acid and *trans*-parinaric acid (Figure 1), were measured by using the "proxy protein" ESI-MS method.[18] Notably, the affinity measured for *trans*-parinaric acid is two orders of magnitude smaller than the reported value, which was determined by using a fluorescence assay.[9] The interactions between the MMPs and the commercial mixture of NAs were investigated by using a newly developed competitive binding assay referred to as the "proxy protein/proxy ligand" ESI-MS method. The results of these measurements show, for the first time, that MMPs do bind specifically to NAs, a complex mixture of linear and cyclic carboxylic acids, in aqueous solution, with apparent affinities of approximately $(5\times10^4)\,\text{M}^{-1}$.

Results and Discussion

Synthesis of MMPs: Despite the intriguing biophysical properties of PMPs, the preparation of structurally defined analogues of these glycans has received little attention. Indeed, to date, only a handful of reports have addressed the chemical synthesis of PMPs or related analogues.[19–23] With regard to the MMPs, previous synthetic work has reported the preparation of analogues in which all of the mannopyranose moieties are methylated.[19] These compounds thus differ slightly from the natural glycans, in which the residue at the non-reducing terminus is unmethylated. Using an iterative approach, a homologous series of MMP derivatives containing 5–20 monosaccharide residues was prepared.[19] In the initial report on the synthesis of these compounds,[19a] the glycosyl donor contained a non-participating protecting group, which, given the required 1,2-*trans* stereochemistry in the products, necessitated careful control of reaction conditions to ensure good glycosylation stereoselectivities. Purification of the compounds proved problematic in some cases and, in a subsequent synthesis,[19b] donors employing participating acyl groups were employed thus providing significantly improved stereocontrol.

Mindful of this previous work, we endeavored to develop a synthetic approach employing donors that contain an ester moiety adjacent to the glycosylation site, and which would lead to products that could be deprotected in a single step under non-hydrogenolytic conditions. The final target compounds were synthesized as 8-azidooctyl glycosides to enable their possible conjugation to, for example, solid supports or protein carriers either through reduction to the amine and subsequent amidation, or through click chemistry. It was, therefore, envisioned that MMP-5_s, -8_s, -11_s and -14_s could be synthesized starting from three building blocks, **1**–**3** (Scheme 1). The strategy was built around species containing solely acyl-based protecting groups, and with a levulinate ester serving as the requisite temporary protecting group needed to facilitate chain extension.

Monosaccharide tricholoroacetimidate imidate **2** was prepared as previously reported[24] and disaccharide thioglycosides **1** and **3** were synthesized as described in the Supporting Information (Scheme S1 in the Supporting Information). With these precursors in hand, they were assembled into the oligosaccharide targets. The preparation of MMP-5_s and -8_s is outlined in Scheme 2. The synthesis of MMP-11_s and -14_s followed similar routes and details can be found in the Supporting Information (Schemes S2 and S3). To synthesize



Scheme 1. Retrosynthetic analysis for MMP-X_s targets (where $X=5$, 8, 11 and 14).

MMP-5_s (Scheme 2a), disaccharide thioglycoside **1** was first coupled with 8-azidooctanol by using activation with NIS and silver triflate.[25] This reaction afforded the expected disaccharide **4** in 84% yield. The levulinoyl ester was then cleaved by treatment with hydrazine acetate, giving an 86% yield of the corresponding alcohol **5**. Subsequent glycosylation of this compound, again by using thioglycoside **1** and NIS/AgOTf, afforded **6**, which was then deprotected with hydrazine acetate. The product, tetrasaccharide alcohol **7**, was obtained in 71% overall yield in two steps from **5**. The final monosaccharide residue was incorporated into **7** by using imidate **2**, under the promotion of TMSOTf. Final treatment of the pentasaccharide product **8** with sodium methoxide removed all of the benzoate esters resulting in MMP-5_s (65% yield in two steps from **5**).

The preparation of the octasaccharide target MMP-8_s (Scheme 2b) was achieved from tetrasaccharide **7**, an intermediate in the synthesis of MMP-5_s. First, the tetrasaccharide was elongated to a hexasaccharide (**9**) by glycosylation with thioglycoside **1**. Subsequent cleavage of the levulinate group (yielding **10**) was followed by glycosylation with thioglycoside **3** affording ocatasaccharide **11** in 85% yield over the three steps from **7**. Final treatment of **11** with sodium methoxide afforded MMP-8_s in 97% yield.

Binding measurements: MMPs isolated from mycobacteria are a homologous mixture of species with 10–13 Man*p* residues. Both MALDI-MS and ESI-MS were used to analyze the MMPs, which were extracted from *M. smegmatis*. Shown in Figure 2a is a representative MALDI mass spectrum ob-

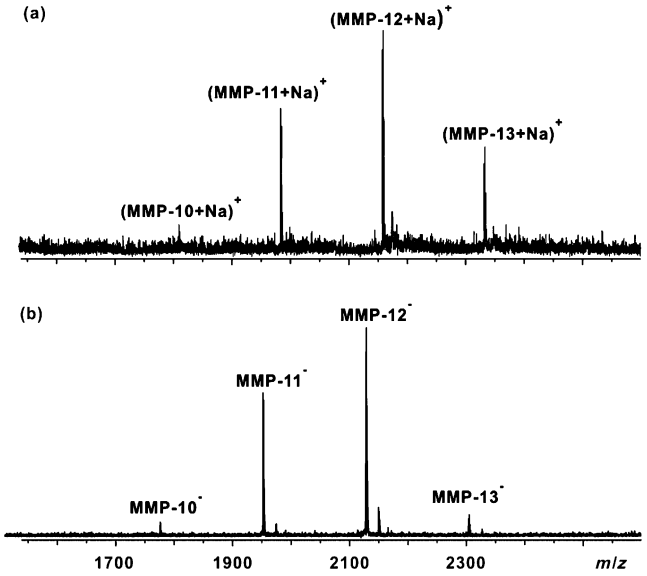


Figure 2. a) MALDI mass spectrum obtained for solution of extracted MMP mixture in positive ion mode; b) ESI mass spectrum obtained for an aqueous ammonium acetate (10 mM, pH 8.5) solution of extracted MMPs (50 μM) in negative ion mode.

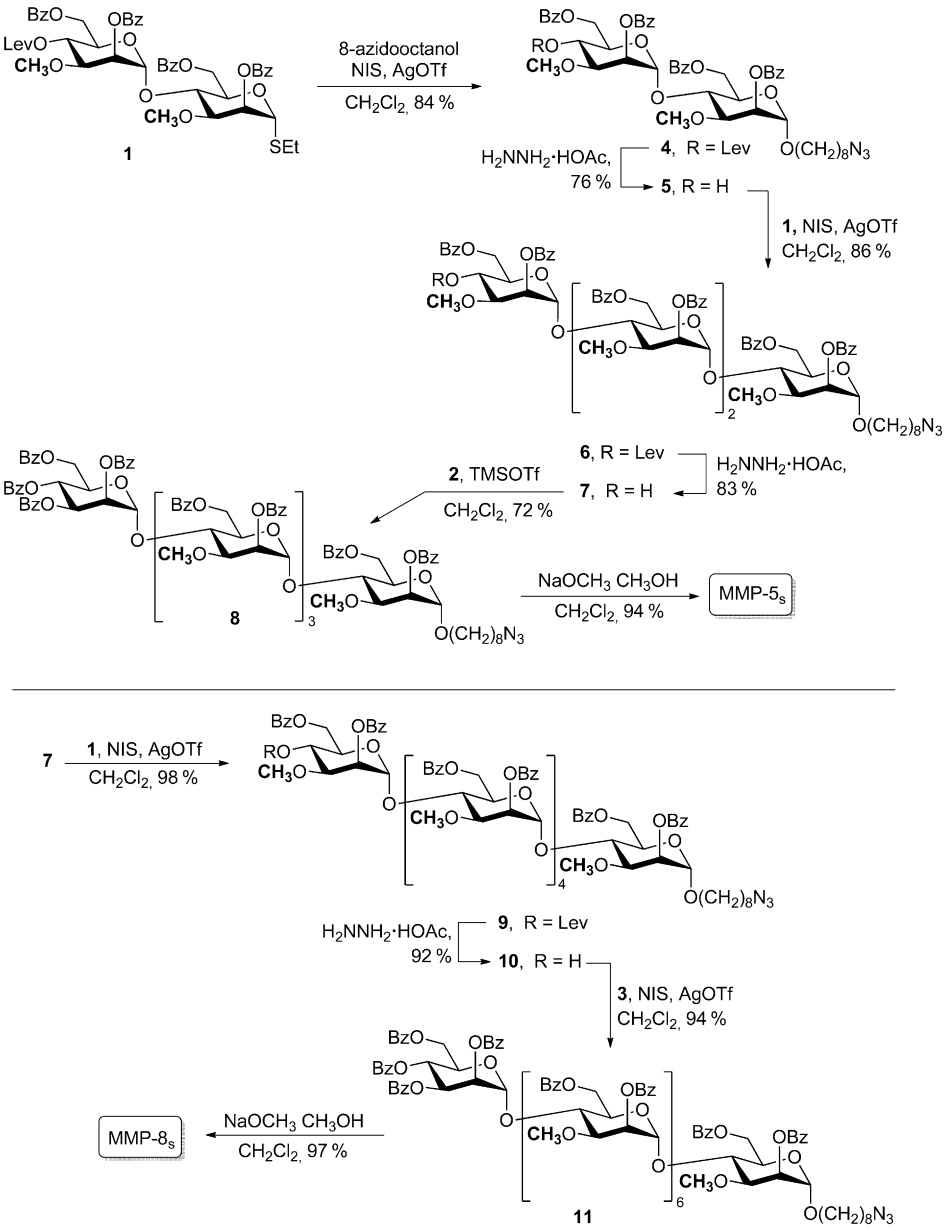
tained in positive ion mode; an ESI mass spectrum obtained in negative ion mode is shown in Figure 2b. Analysis of the mass spectra reveals that MMP-10, -11, -12 and -13 represent the major components of the MMP mixture. The distributions of MMPs in the MALDI and ESI mass spectra are reasonably similar, with MMP-12 and -11 being the two most abundant species. The fractional abundance ($f_{\text{MMP-}X}$) of each of the four MMP-X species (where $X=10$–13) was estimated from the distribution of MMPs established from replicate ESI mass spectra, Equation (1):

$$f_{\text{MMP-}X} = \text{Ab(MMP-}X)/\Sigma\text{Ab(MMP-}X) \quad (1)$$

Assuming similar ESI response factors for the four MMPs, the $f_{\text{MMP-}X}$ values are 0.04 ± 0.01 ($X=10$), 0.37 ± 0.01 (11), 0.53 ± 0.02 (12) and 0.06 ± 0.01 (13). From these values and the molecular weights of individual MMP-X species ($M_{\text{W MMP-}X}$), the weighted average M_{W} ($M_{\text{W MMP}}$) of the MMP mixture was calculated:

$$M_{\text{W MMP}} = \Sigma f_{\text{MMP-}X}(M_{\text{W MMP-}X}) \quad (2)$$

The NA sample used in this work consisted of a mixture of alkyl-substituted cyclic and acyclic aliphatic carboxylic acids. Shown in Figure 3 is an ESI mass spectrum acquired in negative ion mode for an aqueous solution of NAs at pH 8.5 and 25°C. Inspection of the mass spectrum reveals



Scheme 2. Synthesis of MMP-5_s and MMP-8_s targets.

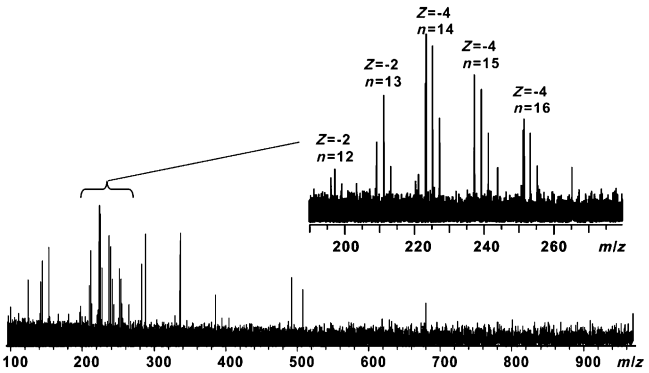


Figure 3. ESI mass spectrum obtained for aqueous ammonium acetate (10 mM, pH 8.5) solution of NAs (160 μM) in negative ion mode.

ion signals corresponding predominantly to species belonging to the $C_nH_{2n+Z}O_2$ series with $n=12$ ($Z=-2$), 13 ($Z=-2$), 14 ($Z=-4$), 15 ($Z=-4$), and 16 ($Z=-4$). The average molecular weight of the NA sample was calculated following the same procedure as used for the MMPs.

The interactions between the extracted MMPs and the components of the NA mixture were initially investigated by using the direct ESI-MS assay. Shown in Figure 4a is an illustrative ESI mass spectrum acquired in negative ion mode for an aqueous solution of MMP (100 μM), NA (100 μM) and imidazole (10 mM), which was added to reduce the extent of in-source dissociation.[14] Signals corresponding to $(MMP+NA)^-$ ions consisting of the major components of the NA mixture were detected. Curiously, the complexes were composed exclusively of MMP-11 and no complexes containing the MMP-12, most abundant MMP, were detected. This result, on its own, suggests that NA binding may be dependent on the size of the MMPs, with the smaller MMPs (i.e., MMP-10) exhibiting higher affinities. However, it is also possible that, despite the presence of the stabilizing additive imidazole in solution, the $(MMP+NA)^-$ ions composed of the longer MMPs undergo in-source dissociation, such that the ESI mass spectrum does not accurately reflect solution composition. The possibility of in-source dissociation notwithstanding, the apparent K_a (i.e., $K_{a,app}$) was determined to be $(1\times10^3)\,M^{-1}$, based on the relative abundance of all free MMP ions and the ligand bound MMP-11 ions.

With the goal of testing the reliability of the direct ESI-MS assay for quantifying the interactions between MMPs and hydrophobic ligands, binding measurements were performed on solutions containing the mixture of extracted MMPs and PnA or PA. As noted above, MMPs are reported to bind to long-chain FAs and their CoA derivatives with high affinity in aqueous solution; for example, an affinity of $(2.5\times10^6)\,M^{-1}$ has been measured for PnA by using fluores-

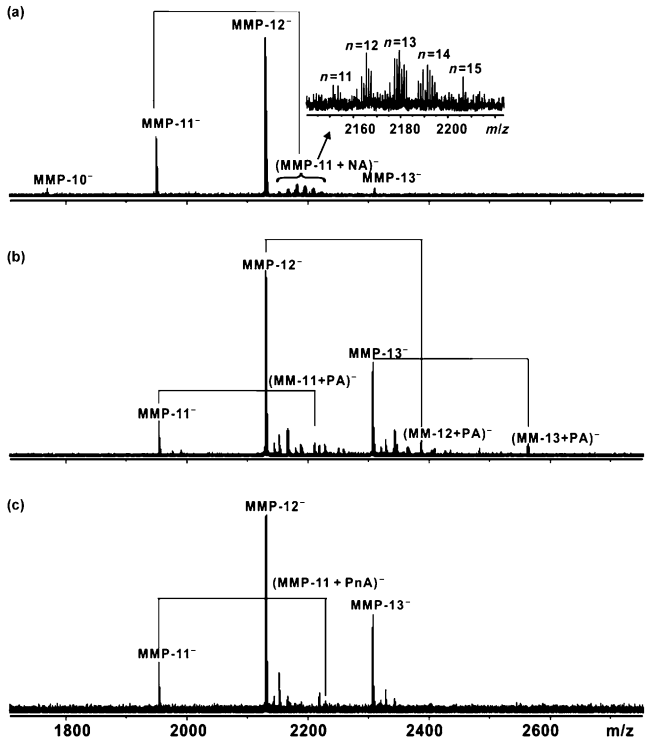


Figure 4. ESI mass spectra obtained in negative ion mode for aqueous ammonium acetate (10 mM, pH 8.5) solutions of: a) MMP (100 μM) with NA (100 μM) imidazole (10 mM); b) MMP (75 μM) with PA (106 μM), imidazole (10 mM); and c) MMP (75 μM) with PnA (96 μM), imidazole (10 mM). The solution temperature for all measurements was 25 °C.

cence spectroscopy.[9] Shown in Figure 4b and c are illustrative ESI mass spectra acquired in negative ion mode for aqueous solutions of MMP (75 μM) with PA (106 μM) and PnA (96 μM), respectively. ESI-MS analysis of the solution containing PA reveals signal corresponding to $(MMP+PA)^-$ ions consisting of each of the three major MMP species, that is, $X=11$–13 (Figure 4b). This result suggests that hydrophobic ligand binding to the MMPs is, in fact, not strongly dependent on the size of the MMP. However, the $K_{a,app}$ calculated from the mass spectral data, $(3\times10^3)\,M^{-1}$, is quite low compared to previously reported values. In contrast, ESI-MS analysis of the solution containing PnA reveals only signal corresponding to complex composed of MMP-11, similar to the results obtained for the NA sample (Figure 4c). The $K_{a,app}$ determined for the interaction with PnA, $(4\times10^2)\,M^{-1}$, is approximately four-orders of magnitude smaller than the reported value.[9] Taken together, these results strongly suggest that the gaseous deprotonated $(MMP+L)^-$ ions composed of PA, PnA or the NAs, are prone to in-source dissociation, and that the addition of imidazole is insufficient to maintain the integrity of the complexes.

To demonstrate conclusively the occurrence of in-source dissociation an indirect assay, based on the recently developed "proxy protein" ESI-MS method,[18] was employed to quantify the interactions between the MMPs and PA and PnA. As noted above, this assay involves the use of a P_{proxy}, which binds specifically to the ligand of interest with known affinity and for which the gaseous ions of the corresponding $(P_{proxy}+L)$ complex are resistant to in-source dissociation. For these measurements, the protein Lg, which possesses a large hydrophobic cavity that can accommodate a wide variety of hydrophobic ligands,[26,27] was utilized. We recently demonstrated that the interactions between Lg and long-chain FAs, such as PA, can be quantified directly by ESI-MS measurements.[14] For example, shown in Figure 5a is an ESI

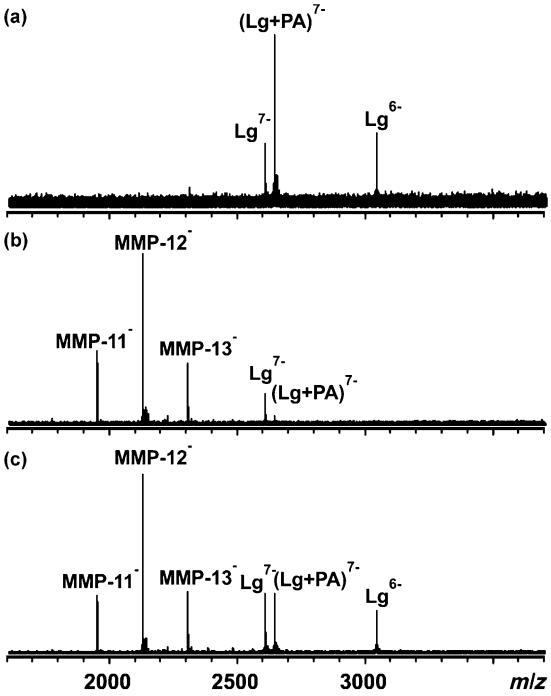


Figure 5. ESI mass spectra obtained in negative ion mode for aqueous ammonium acetate (10 mM, pH 8.5) solutions of: a) Lg (12 μM), PA (10 μM), imidazole (10 mM); b) Lg (12 μM), PA (10 μM), MMP (65 μM), imidazole (10 mM); and c) Lg (12 μM), PA (10 μM), MMP (65 μM), NA (74 μM), imidazole (10 mM). The solution temperature for all measurements was 25 °C.

mass spectrum acquired in negative ion mode for solution containing Lg (12 μM) and PA (10 μM) at pH 8.5 and 25 °C. Ions corresponding to free Lg, that is, Lg^{q-}, at $q=6$ and 7, were detected together with PA-bound Lg, $(Lg+PA)^{7-}$. From the relative abundance of ligand-bound and free Lg, a K_a of $(3.5\times10^5)\,M^{-1}$ was obtained (Table 1). This value is in good agreement with values reported previously for this interaction.[14]

Shown in Figure 5b is an ESI mass spectrum acquired in negative ion mode for a solution of Lg (12 μM), PA (10 μM) and MMP mixture (65 μM) at pH 8.5 and 25 °C. Although no $(MMP+PA)^-$ ions were detected at these concentrations, the addition of the MMPs to solution resulted in a dramatic decrease in the relative abundance of the $(Lg+PA)^{7-}$ ion. This observation confirms that the MMPs do bind PA under these conditions. Following the procedure described in the Experimental Section, a K_a value of $(1.1\times10^5)\,M^{-1}$ was established for the interaction between the MMPs and PA. This

Table 1. Apparent association constants ($K_{a,app}$ [M^{-1}]) for a mixture of MMPs[a] binding to L (L = PA, SA, MA, PnA and NA), determined by the "direct" ESI-MS assay, the "proxy protein" ESI-MS assay, and the "proxy protein/proxy ligand" ESI-MS assay, at 25 °C and pH 8.5.[b]

L	Direct ESI-MS	Proxy protein ESI-MS	Proxy protein/proxy ligand ESI-MS
MA	–	$(4.9\pm0.9)\times10^4$	–
PA	$(2.5\pm0.3)\times10^3$	$(1.1\pm0.2)\times10^5$	–
SA	–	$(2.0\pm0.4)\times10^5$	–
PnA	$(3.9\pm0.1)\times10^2$	$(4.3\pm0.6)\times10^4$	–
NA	$(1.1\pm0.3)\times10^3$	–	$(4.7\pm0.9)\times10^4$ (L_{proxy} = PA) $(5.2\pm0.7)\times10^4$ (L_{proxy} = SA) $(4.0\pm0.9)\times10^4$ (L_{proxy} = MA) $(4.5\pm0.6)\times10^4$ (L_{proxy} = PnA) $(4.6\pm0.5)\times10^4$ (average)

[a] MMPs extracted from *M. smegmatis*. [b] Errors correspond to one standard deviation.

value is approximately 100-times larger than the value established from the direct ESI-MS measurements. Using this competitive assay, MMP binding measurements were performed on three other FAs, MA, SA and PnA. The results of the binding measurements are listed in Table 1. Notably, the K_a value measured for PnA, $(4.3\times10^4)\,M^{-1}$, is also significantly larger than that obtained from direct ESI-MS measurements. Taken together, these results confirm that the deprotonated $(MMP+FA)^-$ and, presumably, $(MMP+NA)^-$ ions are prone to gas-phase dissociation during ESI-MS analysis, and that an indirect measurement of these interactions is required. It is also worth noting that the K_a value measured for PnA is significantly smaller than the reported value, which was measured by using a fluorescence assay.[9] The reason for this discrepancy is not known but may be due to an error in the concentration of the MMP mixture used in the earlier study. Specifically, an overestimation of the MMP concentration due to incomplete removal of Tween (see the Experimental Section), which was used for culturing the bacteria from which these glycans were isolated, would lead to an overestimation in the K_a value. In addition, this fluorescence assay has been noted[28] to be highly sensitive to the presence of oxygen and trace amount of impurities, which are not expected to be limitations of the ESI-MS assay we have developed.

In principle, the proxy protein ESI-MS method could also be used to quantify the interactions between the MMPs and the NAs. However, ESI-MS measurements performed on solutions of Lg and the NA mixture revealed no evidence of (Lg+NA) complexes (data not shown). Therefore, an alternative approach, the "proxy protein/proxy ligand" ESI-MS assay, was used. As noted above, this assay employs a P_{proxy} and an L_{proxy}. Both L and L_{proxy} bind competitively to the MMPs, whereas only L_{proxy} binds to P_{proxy}. Furthermore, the gaseous ions of the $(P_{proxy}+L_{proxy})$ complex are resistant to in-source dissociation. Therefore, from the change in relative abundance of $(P_{proxy}+L_{proxy})$ complex, determined from direct ESI-MS measurements, the affinity of the (MMP+L) complex can be determined.

Figure 5c shows an ESI mass spectrum acquired in negative ion mode for a solution of Lg (12 μM), PA (10 μM), MMPs (65 μM) and NAs (74 μM) at pH 8.5 and 25 °C. Although no ions corresponding to the (MMP+NA) were detected at these concentrations, the addition of the NAs to the solution resulted in a marked increase in the relative abundance of the $(Lg+PA)^{7-}$ ion, from 0.30 (Figure 5b) to 0.55 (Figure 5c). This increase is consistent with the presence of specific interactions between the MMPs and NAs. Analysis of the ESI-MS data by using the approach described in the Experimental Section yields a $K_{a,app}$ of $(4.7\pm0.9)\times10^4\,M^{-1}$ for the interactions between the MMPs and NAs. Analogous measurements were performed by using MA, SA, or PnA as L_{proxy}. In all cases, there is good agreement between the K_a values determined for the interactions between the MMPs and NAs (Table 1). Based on all four data sets, an average K_a value of $(4.6\pm0.5)\times10^4\,M^{-1}$ was established.

These studies represent the first demonstration that MMPs can bind to cyclic or branched lipids. This finding is, perhaps, not surprising given that the inner diameter of the proposed helical structure of the MMPs is comparable to the cavity present in β-cyclodextrin, which binds to an array of branched and aromatic lipophilic molecules, albeit with affinities lower than that reported here for the NA–MMP interaction.[29,30] Moreover, in contrast to the cyclodextrins, which have cavities of defined size constrained by their cyclic nature, the acyclic structure of MMPs should allow sufficient plasticity for the structure to "breathe", thus allowing binding to species other than simple fatty acids and their CoA derivatives.

The data presented above suggest that MMPs extracted from mycobacteria could, in principle, be used as a sorbent for the removal of NAs from TPW. However, the use of MMPs extracted from bacteria is impractical given the low yields in which they can be isolated. Instead, implementation of this strategy requires obtaining MMPs by other methods. One option is chemical synthesis and ideally purification columns based upon these materials would be constructed by using the shortest MMP that effectively binds to NAs. Another advantage of synthetic glycans over the natural materials, which are terminated at the reducing end with a methyl group, is that they can be prepared with a functional group (e.g., an azide or its amine counterpart) to facilitate their conjugation to a solid support. To establish the influence of size on the affinities of MMPs for NAs, the series of synthetic MMP-X_s, where X = 5, 8, 11, and 14, described above (Figure 1) were evaluated for their ability to bind to NAs. Using the proxy protein (where P_{proxy} = Lg) and proxy protein/proxy ligand ESI-MS assays (where P_{proxy} = Lg and L_{proxy} = PA) the affinities of the individual synthetic MMPs for PA and the commercial mixture of NAs, respectively, were measured at pH 8.5 and 25 °C (Table 2). It can be seen that, for both PA and the NAs, the value of K_a increases modestly with the length of MMP. The effect is more pronounced for PA binding, with the affinity increasing from $(1.8\pm0.5)\times10^4$ (MMP-5_s) to $(8.8\pm0.3)\times10^4\,M^{-1}$ (MMP-14_s);

Table 2. Association constants (K_a, M^{-1}) measured for the binding of synthetic MMP-X_s binding to PA (by using proxy protein ESI-MS method, P_{proxy} = Lg), and to NAs (by using the proxy protein/proxy ligand ESI-MS assay, P_{proxy} = Lg, L_{proxy} = PA) at 25 °C and pH 8.5.[a]

MMP-X_s	K_a [M^{-1}]	
	PA	NA
$X=5$	$(1.8\pm0.5)\times10^4$	$(3.6\pm0.5)\times10^4$
$X=8$	$(2.6\pm0.5)\times10^4$	$(3.9\pm0.6)\times10^4$
$X=11$	$(3.7\pm0.2)\times10^4$	$(4.5\pm0.5)\times10^4$
$X=14$	$(8.8\pm0.3)\times10^4$	$(7.6\pm0.7)\times10^4$

[a] Errors correspond to one standard deviation.

for the NAs, the values range from $(3.6\pm0.5)\times10^4$ to $(7.6\pm0.7)\times10^4\,M^{-1}$. Notably, the affinities measured for synthetic MMPs for the NAs are very similar to apparent value measured for the extracted MMPs. Based on these results, it is predicted that columns containing immobilized MMPs as short as five monosaccharide residues (MMP-5_s) would be nearly as effective as those containing significantly longer MMPs.

Conclusion

In summary, the interactions between MMPs, extracted from *M. smegmatis* or produced synthetically, and linear saturated and unsaturated FAs, and a commercial mixture of NAs, in aqueous solution (25 °C, pH 8.5) were quantified by ESI-MS. Association constants for the binding of the MMPs to four FAs, MA, PA, SA and PnA, were measured by using the "proxy protein" ESI-MS assay. The measured K_a values range from about 10^4 to approximately $10^5\,M^{-1}$, and increase with the size of the MMP. Notably, the apparent affinity measured for the mixture of extracted MMPs and PnA is significantly smaller (by a factor of 100) than the reported value. A newly developed competitive binding assay, referred to as the "proxy protein/proxy ligand" ESI-MS method, was used to quantify the interactions between the MMPs and the commercial NA mixture. These results demonstrate, for the first time, that MMPs bind specifically to cyclic, branched-chain lipids. Moreover, we have demonstrated that at least, some of the NAs in the mixture bind to the MMPs with apparent affinities of approximately $(5\times10^4)\,M^{-1}$, which in turns suggests that these glycans may have potential in the remediation of TPW. In preliminary investigations, we have demonstrated that the polystyrene beads functionalized with MMP-5_s do indeed bind to NAs in TPW and that the treated water has significantly reduced toxicity. Finally, the ESI-MS method developed here represents an attractive approach for probing carbohydrate–lipid interactions. In particular, it does not suffer from the aforementioned limitations of the previously developed fluorescence assay,[9,10] We also view it as superior to a more recently-reported UV-based binding assay, which requires deconvolution of complex equilibria involving lipid aggregates.[28]

Experimental Section

MMPs, proteins and assay solutions: The mixture of MMPs (consisting of species with 10–13 Man*p* residues, named MMP-10, -11, -12 and -13, respectively, Figure 1) were extracted and purified from *M. smegmatis* as described by Hindsgaul and Ballou.[31] Key steps in the purification were affinity chromatography by using a silica-based absorbent functionalized with palmitic acid followed by treatment of the eluate with decolorizing charcoal. Although earlier work[31] had reported the MMPs could be obtained pure following a single affinity chromatography-decolorization sequence, in our hands, multiple column passages were required to remove the polyoxyethylenesorbitan monooleate (Tween) that was used in the media for growing the bacteria. The presence of Tween was obvious in the mass spectra obtained while carrying out our investigations. However, given the similar chemical shifts of the hydrogen atoms in Tween and the MMPs, this impurity cannot be readily seen by ^{1}H NMR spectroscopy, which had been used by Hindsgaul and Ballou to characterize their isolated materials.[31]

The synthesis of MMP-5_s, -8_s, -11_s and -14_s was carried out as described in the Results and Discussion and in the Supporting Information. All experimental data for the synthetic intermediates and final compounds can be found in the Supporting Information.

Bovine β-lactoglobulin (Lg, monomer M_W 18281 Da), palmitic acid (PA, 256.4 Da), myristic acid (MA, 228.4 Da), and stearic acid (SA, 284.8 Da) were purchased from Sigma–Aldrich Canada (Oakville). *trans*-Parinaric acid (PnA, M_W 276.4 Da) was purchased from Cayman Chemical (Ann Arbor, MI). A sample of commercially available Merichem naphthenic acids (NA) was generously provided by Professor Greg G. Goss (Department of Biological Sciences, University of Alberta). The structures of the MMPs, FAs and NAs are shown in Figure 1.

Stock solutions of MMPs were prepared by dissolving extracted and purified MMPs into Milli-Q water to yield a final concentration of 3 mM. The stock solutions of FAs and NAs were prepared by dissolving a known mass of each compound or mixture of compounds into methanol (MeOH). Lg was dissolved and exchanged directly into Milli-Q water, by using an Amicon microconcentrator with a MW cutoff of 10 kDa. The concentration of the Lg solution was determined by lyophilizing a known volume of the filtrate and measuring the mass of the protein. The protein stock solution was stored at −20 °C. The ESI solutions were prepared from the stock solutions. For the binding measurements, imidazole (10 mM) was also added to reduce the occurrence of in-source dissociation.[14–16] Ammonium acetate buffer was added into the ESI solution to a final concentration of 10 mM. Aqueous ammonium hydroxide was added to adjust the pH of the solution to 8.5.

Mass spectrometry: Isolated MMPs were analyzed by matrix-assisted laser desorption ionization (MALDI) MS in positive ion mode by using a Voyager Elite MALDI time-of-flight (ToF) mass spectrometer (AB Sciex, Framingham, MA). A solution of 2,5-dihydroxybenzoic acid (DHB, 20 mg mL^{-1}) in 3:1 H_2O/MeOH was used as the matrix solution. An aqueous solution of MMPs was mixed with the matrix solution at a 1:1 ratio. An aliquot (0.7 μL) of the mixture was loaded onto the MALDI target plate by using a micropipette and allowed to dry. A small volume of an NaCl (1 mM) solution was added to the spots to improve ionization efficiency. Analysis of the MMPs by ESI-MS, as well as the ESI-MS binding measurements, were performed in negative ion mode by using a 9.4 Tesla Apex II Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker, Billerica, MA). Nanoflow ESI was performed by using borosilicate tubes (1.0 mm o.d., 0.68 mm i.d.), pulled to approximately 5 μm o.d. at one end by using a P-2000 micropipette puller (Sutter Instruments, Novato, CA). Details of the instrumental parameters used for the binding measurements are given elsewhere.[14–16]

ESI-MS binding measurements: Both direct and competitive ESI-MS assays were used to quantify the K_a values for the interaction between MMPs and FA and NA ligands (L), Equation (3):

$$MMP + L \rightleftharpoons (MMP + L) \quad (3)$$

A brief description of each assay is given below.

1) Direct ESI-MS assay: The direct ES-MS assay is based on the quantification of the abundance (Ab) of ligand-bound and unbound ions in the gas phase,[14,16,32–36] for example, $(MMP+L)^-$ and MMP^-, respectively. The measured abundance ratio (R) is assumed to be equivalent to the equilibrium concentration ratio of bound and free MMP ions in solution, Equation (4):

$$\frac{[MMP+L]_{eq}}{[MMP]_{eq}} = \frac{Ab((MMP+L)^-)}{Ab((MMP)^-)} = R \quad (4)$$

From the measured R value and initial concentrations of MMP and L (i.e., $[MMP]_o$ and $[L]_o$, respectively), the association constant $K_{a,MMP}$ can be calculated by using Equation (5):

$$K_{a,MMP} = \frac{R}{[L]_o - \frac{R}{1+R}[MMP]_o} \quad (5)$$

2) Proxy protein ESI-MS assay: A competitive binding assay,[18] employing a proxy protein (P_{proxy}) that binds specifically to L, Equation (6):

$$P_{proxy} + L \rightleftharpoons (P_{proxy} + L) \quad (6)$$

was also employed to quantify the interactions of FAs with the MMPs. Bovine Lg, which is known to bind reasonably strongly to long-chain FAs in basic solutions and forms kinetically-stable gaseous $(Lg+FA)^{q-}$ ions,[14] served as P_{proxy} for these measurements. The concentration of free L in solution is determined from the ratio (R_{proxy}) of ligand-bound and unbound P_{proxy} measured by ESI-MS and the known affinity of P_{proxy} for L ($K_{a,proxy}$), Equations (7) and (8):

$$\frac{[P_{proxy}+L]_{eq}}{[P_{proxy}]_{eq}} = \frac{\sum_q Ab((P_{proxy}+L)^{q-})}{\sum_q Ab((P_{proxy})^{q-})} = R_{proxy} \quad (7)$$

$$K_{a,proxy} = \frac{[P_{proxy}+L]_{eq}}{[P_{proxy}]_{eq}[L]_{eq}} = \frac{R_{proxy}}{[L]_{eq}} \quad (8)$$

and the concentration of $(P_{proxy}+L)$ can be calculated from Equation (9):

$$[P_{proxy}+L]_{eq} = [P_{proxy}]_o \frac{R_{proxy}}{1+R_{proxy}} \quad (9)$$

where $[P_{proxy}]_o$ is the initial concentration of P_{proxy}. From mass balance considerations, the concentration of $(MMP+L)$ and MMP can be calculated by using Equation (10) and (11):

$$[MMP+L]_{eq} = [L]_o - [L]_{eq} - [P_{proxy}+L]_{eq} \quad (10)$$

$$[MMP]_{eq} = [MMP]_o - [MMP+L]_{eq} \quad (11)$$

The value of $K_{a,MMP}$ can then be calculated by from Equation (12):

$$K_{a,MMP} = \frac{[MMP+L]_{eq}}{[L]_{eq}[MMP]_{eq}} \quad (12)$$

3) Proxy protein/proxy ligand ESI-MS assay: A newly developed ESI-MS method was ultimately used to determine the affinities of the MMPs for NAs. This assay incorporates both a P_{proxy} and a proxy ligand (L_{proxy}) to establish the K_a for the MMPs binding to L. Both L and L_{proxy} bind to the MMPs, with association constants $K_{a,(MMP+L)}$ and $K_{a,(MMP+Lproxy)}$; whereas only L_{proxy} binds to P_{proxy}, with the association constant $K_{a,proxy}$, Equations (3), (13) and (14):

$$P_{proxy} + L_{proxy} \rightleftharpoons (P_{proxy} + L_{proxy}) \quad (13)$$

$$MMP + L_{proxy} \rightleftharpoons (MMP + L_{proxy}) \quad (14)$$

The procedure for determining K_a for the interaction between MMP and L is similar to that described above. The concentration of free L_{proxy} can be determined from the ratio (R_{proxy}) of L_{proxy}-bound and unbound P_{proxy} measured by ESI-MS and the known affinity ($K_{a,proxy}$) of P_{proxy} for L_{proxy}, Equations (7) and (8). From the values of $[L_{proxy}]_{eq}$ and of $[P_{proxy}+L_{proxy}]_{eq}$, the concentration of MMP bound L_{proxy} can be found from the equation of mass balance, Equation (15):

$$[MMP+L_{proxy}]_{eq} = [L_{proxy}]_o - [L_{proxy}]_{eq} - [P_{proxy}+L_{proxy}]_{eq} \quad (15)$$

The concentration of free MMP in solution can be determined from Equation (16):

$$[MMP]_{eq} = \frac{[MMP+L_{proxy}]_{eq}}{K_{a,(MMP+L_{proxy})}[L_{proxy}]_{eq}} \quad (16)$$

and the concentration of MMP bound to L can be found from Equation (17):

$$[MMP+L]_{eq} = [MMP]_o - [MMP]_{eq} - [MMP+L_{proxy}]_{eq} \quad (17)$$

Finally, $K_{a,(MMP+L)}$ can be calculated by using Equation (12).

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Carbohydrates

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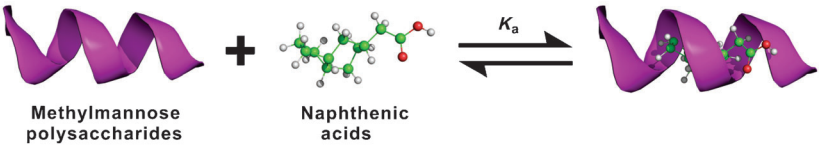
Carbohydrate–Lipid Interactions: Affinities of Methylmannose Polysaccharides for Lipids in Aqueous Solution



Mixing sugar and oil: The interactions between 3-*O*-methyl-mannose polysaccharides, extracted from *Mycobacterium smegmatis* or obtained by chemical synthesis, with fatty acids and a commercial mixture of naphthenic acids (see scheme) were quantified by electrospray ionization mass spectrometry.