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Remarkably selective saccharide recognition by solid-supported peptide boronic acids

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ABSTRACT

The affinity of nine different saccharides to a library of solid-supported pentapeptide diboronic acids was measured using a competitive binding assay, which employed alizarin as the chromophoric indicator. Considerable variation in carbohydrate binding strengths was observed, with association constants in the range 60–5300 M^{-1} being recorded. Of particular note was the 7-fold preference for CMP over AMP shown by peptide **1**. Enantioselectivity was also observed, with peptide **4** showing an 8.4-fold binding preference for L-glucose over D-glucose. The remarkably selective binding characteristics of these boronic acid–peptide hybrids suggest their potential use in carbohydrate sensors and cell-specific diagnostics and therapeutics.

1. Introduction

Synthetic polyol binders capable of recognising cell surface polysaccharides have potential application in drug delivery, in diagnostics and as probes for medical imaging. Natural sugar binders, or lectins, have binding sites rich in charged and polar amino acid residues and utilise non-covalent associations such as hydrogen bonding and Van der Waal's interactions to secure their polyol substrates. A wide variety of molecular architectures have been employed in the development of synthetic polyol binders, including systems based on covalent as well as non-covalent binding. Amongst the receptors that use covalent interactions, boronic acid-containing compounds have been the most extensively explored, and have found use in artificial sugar receptors,[1] glucose sensors,[1,2] membrane transport agents,[3] and as protective agents and catalysts in synthesis.[4] Boronic acid-containing compounds designed to be small molecule mimics of lectins are now receiving heightened interest from molecular recognition researchers.[5]

The incorporation of boronic acids into peptides and polypeptides has had limited attention,[6–8] as have solid phase approaches to boronic acid receptors,[9–13] however, we recently reported a new type of hybrid peptide boronic acid sugar receptor library prepared from a protected form of 4-borono-L-phenylalanine, and determined the affinity of members of this library to the chromophoric diol, alizarin.[14,15] This hybrid system combines the covalent interactions provided by the boronic acids with the non-covalent interactions delivered by the backbone and side chains of the peptides. With the aim of developing these peptide boronic acids as selective cell surface oligosaccharide binders, we now report the results of binding studies with nine saccharide/saccharide derivatives. It has been found that our simple library of pentapeptidic diboronic acids is able to readily discriminate between the tested carbohydrates, and that variation in sequence and the nature of non-boronated peptide side chains has a marked influence on binding constants.

2. Results

2.1. Peptide boronic acid library

The solid-supported pentapeptides were prepared on *SynPhase* D-series lanterns, as previously described.[14] The principles used in the design of the peptide library have also been reported,[14] and took into account the following: (a) diboronic acids generally bind polyols more strongly and more selectively than monoboronic acids,[1,3c] whereas multiboronic acids tend to be less selective;[16,17] (b) sugar extraction and transport have been shown to be enhanced by the incorporation of cationic residues that can ion-pair with tetrahedral boronate esters;[18–20] (c) lectins use hydrogen bonding amino acid residues to secure saccharides. Thus, two boronic acids and two basic residues, lysine or arginine, were incorporated into

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Table 1
Solid-supported peptide boronic acid library[14]

	Amino acid sequence[a]
1	*N*-Ac-BPA-BPA-Ala-Arg-Arg-AHA-SS
2	*N*-Ac-BPA-Arg-BPA-Ala-Arg-AHA-SS
3	*N*-Ac-BPA-Arg-Arg-BPA-Ala-AHA-SS
4	*N*-Ac-BPA-Arg-Ala-Arg-BPA-AHA-SS
5	*N*-Ac-Arg-BPA-BPA-Arg-Ala-AHA-SS
6	*N*-Ac-Arg-BPA-Arg-BPA-Ala-AHA-SS
7	*N*-Ac-BPA-BPA-Ala-Lys-Lys-AHA-SS
8	*N*-Ac-BPA-Lys-BPA-Ala-Lys-AHA-SS
9	*N*-Ac-BPA-Lys-Lys-BPA-Ala-AHA-SS
10	*N*-Ac-BPA-Lys-Ala-Lys-BPA-AHA-SS
11	*N*-Ac-Lys-BPA-BPA-Lys-Ala-AHA-SS
12	*N*-Ac-Lys-BPA-Lys-BPA-Ala-AHA-SS

[a] BPA=4-borono-L-phenylalanine, AHA=6-aminohexanoic acid, SS=solid support.

each peptide, with the remaining position being filled with alanine. The sequences of individual library members, shown in Table 1, were carefully chosen in order to access a wide range of possible saccharide binding modes.

2.2. Binding constant determinations

The binding constants of nine saccharide/saccharide derivatives to peptides **1**–**12** were determined using a three-component competitive displacement assay in which a chromophoric indicator was first bound to the solid phase and then displaced by the substrate of interest. Saccharide binding strength was then measured by spectrophotometric quantification of the displaced indicator. Displacement methods of this general type have been known for sometime,[21] and were specifically adapted to boronic acidbased systems[22,23] and later developed into a general method for the spectrophotometric determination of solution binding constants between boronic acids and diols using ARS **13** (Fig. 1) as the indicator.[8,24] Here, this method has been further adapted to allow the determination of binding constants in a two-phase system. Alizarin **14** was used as the indicator in the current study, with absorbance being measured at the observed λ_{max} in the assay buffer, 507 nm. Binding was studied under basic conditions (50 mM sodium carbonate in 50% aqueous methanol, pH 10.7) to ensure the generation of tetrahedral boronate esters (pK_a phenyl boronic acid=8.8).[24] Under these conditions, the inherently stable tetrahedral boronates could have their stabilities further enhanced by interactions with protonated arginine side chains (pK_a side chain=12.10).[25] Scheme 1 illustrates this assay, using the displacement of alizarin **14** with CMP **16** (Fig. 2) as an example. The binding strengths of alizarin to the solid-supported peptide boronic acids **1**–**21**, needed for the calculation of polyol binding constants, have been reported[14] and are shown in Table 2.

The curves obtained from the competitive binding studies were analysed using the general methods described by Connors,[26] and further explained by Wiedenfeld,[27] and the binding constants thus obtained are shown in Table 2. The amount of alizarin displaced by the polyols (Fig. 2) in the competitive binding experiments

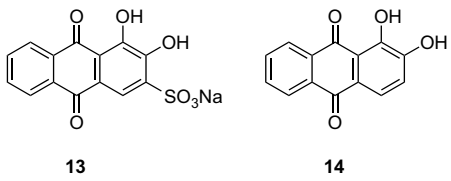


Figure 1. Structures of dyes used in competitive boronic acid binding assays; ARS **13** and alizarin **14**.

supported a 2:1 binding stoichiometry for alizarin across all peptide sequences. The same 2:1 binding stoichiometry was assumed to exist with each of the polyol substrates. The constant quantity of the solid-supported host precluded the use of the method of continuous variations (Job's method)[26] for directly determining the boronic acid–polyol stoichiometry. It is also difficult to identify cooperativity effects using this displacement assay, but previous binding studies with alizarin found no evidence of cooperativity between bound diols,[14] and this was assumed to be the case here. Thus, for a particular peptide, the two boronic acids were treated as two independent binding sites, with the measured K_a being an average of the two boronic acid association constants. The above assumptions were vindicated by the shape and excellent fit of each of the competitive binding curves.

The competitive binding assay was also performed using phenylalanine control peptides[14] in which the BPA units in the arginine series were replaced with Phe. D-Fructose was used in the displacement assay as this polyol had provided the highest average binding strength across all peptide boronic acid sequences. As expected, no significant alizarin displacement by D-fructose was observed with the phenylalanine control peptides. This indicated that essentially all polyol binding to the peptide boronic acids, at least with D-fructose and presumably with other saccharides, occurred through the boronic acid moieties.

3. Discussion

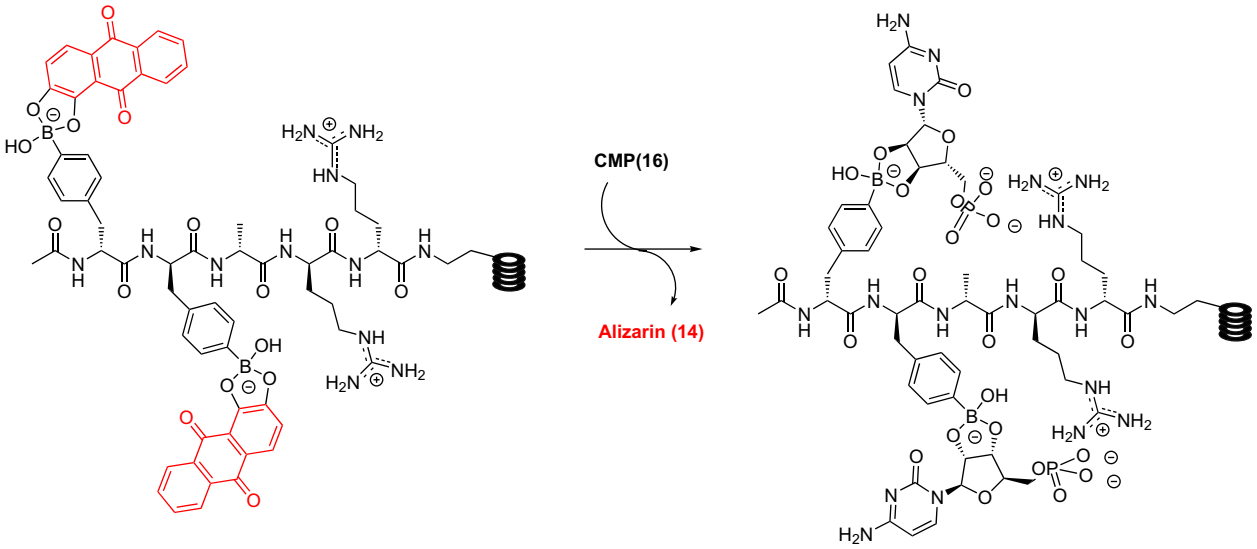
The variation in binding strength of polyols, both within and between the arginine and lysine series, and between polyols is impressive. A few selected examples are discussed here.

3.1. D-Ribose and ribonucleotides

It is instructive to compare the binding behaviour of AMP (**15**), CMP (**16**) and D-ribose (**17**), given that these compounds possess the same 2,3-diol functionality, which presumably provides the primary binding site for all three compounds to the peptide boronic acids (see Fig. 3). With the exception of peptide **6**, the least favoured substrate across all the peptide sequences was AMP, despite its potential for additional binding interactions. The increased steric bulk of this nucleotide may account for its reduced binding affinity. Four sequences **1**, **8**, **9** and **11** significantly preferred CMP, while six sequences, **3**, **4**, **5**, **7**, **10** and **12**, preferred the parent sugar, D-ribose. The preference of sequence **1** for CMP and that of **12** for D-ribose is particularly impressive, with both binding their preferred substrate approximately seven times more strongly than the next preferred ribose derivative. It is also interesting that D-ribose is preferred by so many peptides, despite the absence of a phosphate group or nucleotide base, which both offer the potential for additional binding interactions with the non-boronated side chains.

3.2. D-Fructose and D-glucose

The binding behaviour of D-fructose to the peptide boronic acid library was of particular interest given previous attempts to develop highly fructose-selective thin membranes for sugar production,[3c] and the importance of fructose interference in boronic acidbased glucose sensors.[2] As with many of the saccharides tested, the arginine series (**1**–**6**) provided stronger binding, preferring D-fructose by an average of 43% compared to the lysine series (**7**–**12**). In the arginine series, optimal substrate binding was achieved when the two BPA units were in close proximity, either adjacent or separated by one amino acid residue, with the lysine series showing a similar preference, with optimal binding occurring when the boronic acid moieties were separated by one amino acid residue (see Fig. 4).



Scheme 1. An illustration of the competitive binding assay, showing the displacement of alizarin (**14**) by CMP (**16**).

The results obtained with D-glucose are of particular importance, given the significant interest in the development of glucose sensors for the monitoring of blood sugar levels.[2] The first notable fact about the D-glucose binding data is that, contrary to that observed with other saccharides, there is a lack of a clear preference for binding to the arginine over the lysine peptides, or visa versa. For example, while binding to the arginine peptide **1** is strongly favoured over binding to its lysine analogue **7**, binding to the lysine peptide **12** is strongly favoured over binding to its arginine analogue **6**. Overall, the strongest D-glucose binding was achieved with peptide **1**, which has adjacent BPA units at positions 1 and 2, while the weakest binding was achieved by peptide **4**, which has the maximum possible spacing between the BPA units. The results of the arginine series are reminiscent of those obtained with D-fructose, in that optimal binding of D-glucose was achieved when the two BPA units were adjacent, with substrate affinity decreasing as the distance between the boronic acid moieties increased. In the lysine series, optimal D-glucose binding was obtained when the boronic acid moieties were separated by a single amino acid residue.

For the aforementioned reasons, the D-glucose–D-fructose binding selectivity shown by the peptide boronic acids was also considered a property of great importance. Of particular note are the arginine peptides **4** and **6**, which showed an 8.3 and 5.4-fold

Table 2
Binding constants (K_a, M^{-1}) of polyols (**14–23**) to solid-supported peptide boronic acids in 50% aqueous methanol, pH 10.7[a]

Peptide	14[b]	15	16	17	18	19	20	21	22	23
1	1055	747	5283	770	981	2857	3641	2518	2314	566
2	769	970	2037	1566	970	3029	2326	1954	1766	818
3	587	944	1184	3733	1522	1533	1340	2299	3826	418
4	510	721	1064	1722	1375	1997	241	2030	1474	147
5	780	465	972	1479	1132	2577	1956	2768	1746	234
6	443	1121	557	837	1027	2037	379	345	530	409
7	485	134	574	1133	487	727	1142	821	721	61
8	506	142	2542	1307	345	991	2047	1594	1126	1006
9	421	216	3104	1428	793	1449	2146	853	990	66
10	242	356	1936	3333	1357	1400	741	1170	1605	219
11	470	280	2814	1439	749	1104	1476	1219	1566	125
12	440	311	492	3669	1198	4162	2588	598	4506	140

[a] All errors were 4–10% except with peptide **10**, which had errors of 10–15%. Structures of polyols **15–23** are shown in Figure 2.
[b] From Ref. 14.

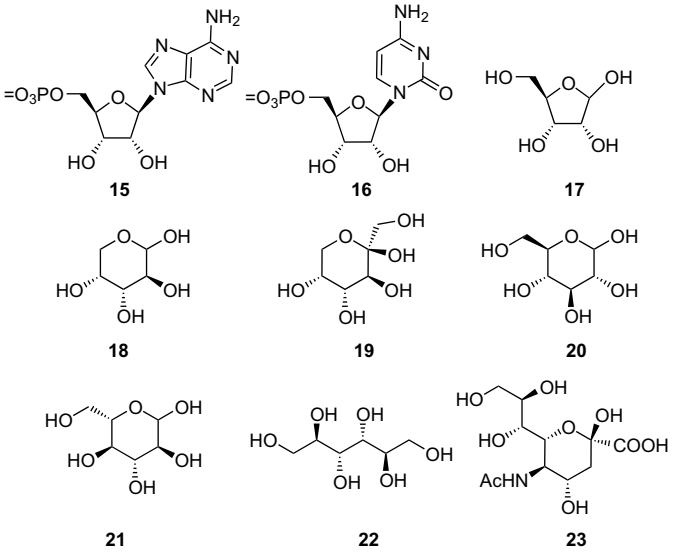


Figure 2. Structures of polyols used in binding studies; adenosine-5′-monophosphate (**15**), cytidine-5′-monophosphate (**16**), D-ribose (**17**), D-arabinose (**18**), D-fructose (**19**), D-glucose (**20**), L-glucose (**21**), D-mannitol (**22**) and sialic acid (**23**).

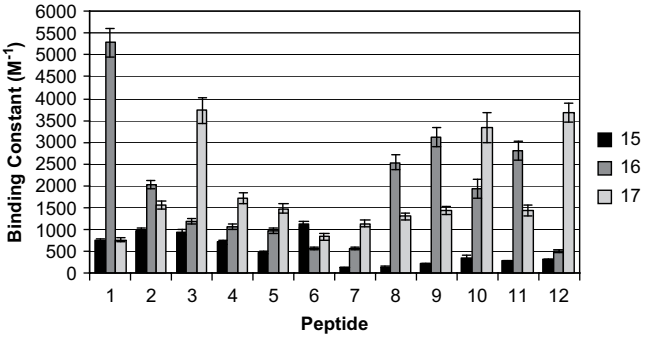


Figure 3. Comparison of the binding constants of AMP (**15**), CMP (**16**) and D-ribose (**17**) to peptide boronic acids.

preference for D-fructose, respectively, and the lysine peptide **8**, which displayed a 2.1-fold preference for D-glucose. While these observed selectivities, particularly in the latter case, are insufficient for immediate application, these results strongly suggest that with optimisation, peptide boronic acids highly selective for either D-glucose or D-fructose could be readily developed.

3.3. D-Glucose and L-glucose

The potential for these homochiral peptide boronic acids to show chiral discrimination was tested by determining their binding constants to L-glucose and comparing the results with those obtained with D-glucose (see Fig. 5). Interestingly, it was found that a number of sequences displayed significant chiral bias when binding glucose. Most notable was the arginine peptide **4**, which displayed an 8.4-fold preference for the L-enantiomer, while the lysine peptide **12** showed a 4.3-fold preference for the D-enantiomer. This is a promising result and the potential applications of such enantioselective binding are numerous.

3.4. D-Mannitol and sialic acid

Boronate esters derived from D-mannitol are amongst the most stable,[24] so it was surprising to find that only the arginine peptide **3** and the lysine peptide **12** showed particularly strong associations with this alditol. Despite possessing the same boronate-binding triol motif as D-mannitol, sialic acid shows relatively weak association with boronic acids.[24] The results obtained here with peptide boronic acids reflect this phenomenon (see Fig. 6). Four of the six peptide sequences showed stronger binding of sialic acid with arginine present rather than lysine. Once again, additional ion-pairing and hydrogen bonding interactions between the substrate and host may have contributed to this overall effect. Guanidinium ions can also strongly associate with carboxylate ions, combining an electrostatic attraction with a favourable bidentate geometry for hydrogen bonding interactions.[28] This may explain this apparent preference of the arginine series to bind sialic acid, the only saccharide tested that bears a carboxylate group. Importantly, polysialic acid is a cell surface polysaccharide involved in cancer and meningitis infections,[29] and the affinity of the arginine series for sialic acid suggests that longer peptide boronic acids bearing arginine residues may have application in the development of diagnostics and treatments for these diseases.

4. Conclusions

The primary aim of this study was to determine if changes in the sequence of peptide boronic acids would lead to variation in polyol binding strength and preference. This was observed, with significant variation in carbohydrate binding strengths being found both within and between the arginine and lysine series. Association constants fell in the range 60–5300 M^{-1}. The arginine and lysine residues were included in the peptide sequences in the expectation that their protonated forms would associate with the negatively charged boronate esters, and hydrogen bond to bound saccharides, and thus provide enhanced binding affinity and selectivity. The lysine side chains, however, are not expected to be fully protonated under the conditions of the binding assay (pK_a side chain=10.67).[25]

The arginine series generally yielded the strongest binding constants, presumably because their guanidinium side chains were fully protonated under the conditions of the assay and could additionally act as bidentate hydrogen bond donors, further stabilising boronate esters. These side chains may also be interacting with other parts of the bound sugars. Bidentate hydrogen bonding interactions between arginine side chains and diols of bound sugars are commonly observed in X-ray crystallographic structures of carbohydrate–lectin complexes.[30] There is a possibility that imine formation between the lysine side chains and the open chain forms of sugars could also have influenced binding in the lysine series, but Table 2 shows that if this did occur, it did not generally produce significant benefits to binding relative to the arginine side chain.

A comparison was made between the binding behaviour of D-ribose and its derivatives, AMP and CMP. All sequences bound AMP relatively poorly, whereas D-ribose and CMP were generally strongly associated with the peptide boronic acids. One sequence, **1**, showed a 7-fold preference for CMP over both AMP and D-ribose, and another sequence, **12**, showed a similar preference for D-ribose over CMP and AMP.

The relative binding strength of D-fructose and D-glucose to the peptide boronic acids is relevant to the development of practical applications for boronic acids, including glucose sensors and sugar permeable membranes. The 8 and 5-fold preference for D-fructose shown by the arginine peptides **4** and **6**, respectively, and the 2-fold preference for D-glucose shown by the lysine peptide **8** are of

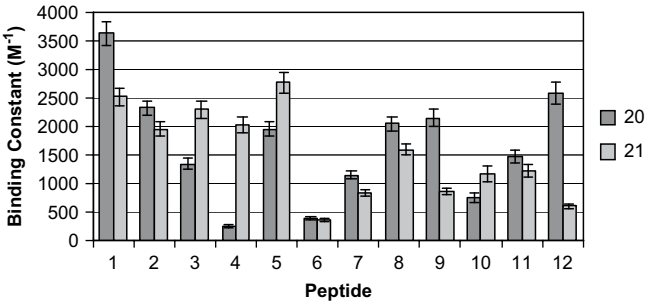


Figure 5. Comparison of the binding constants of D-glucose (**20**) and L-glucose (**21**) to peptide boronic acids.

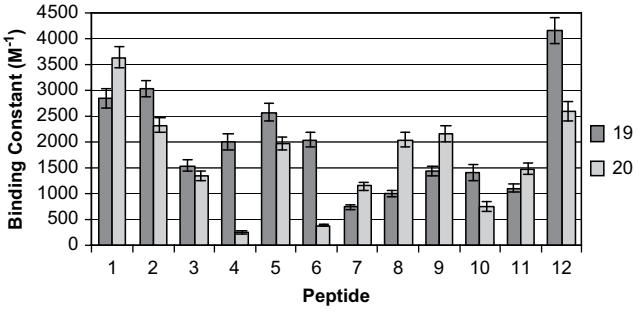


Figure 4. Comparison of the binding constants of D-fructose (**19**) and D-glucose (**20**) to peptide boronic acids.

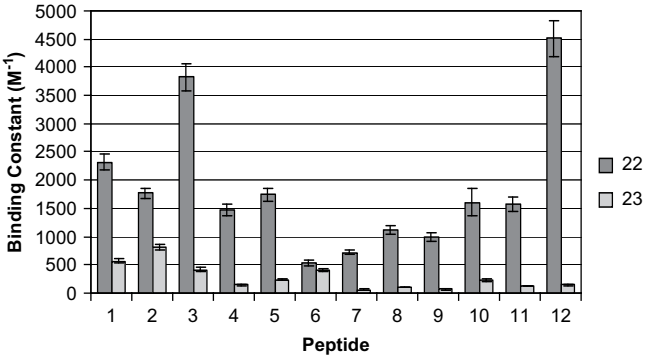


Figure 6. Comparison of the binding constants of D-mannitol (**22**) and sialic acid (**23**) to peptide boronic acids.

particular note. The amino acids used to build the peptides were all enantiomerically pure and chiral discrimination by these peptides was also observed, with a number of sequences showing a clear preference for either D-glucose or L-glucose. The arginine peptide **4** in particular had a greater than 8-fold preference for L-glucose, while the lysine peptide **12** showed a greater than 4-fold preference for D-glucose. This compares to the work of Shinkai and co-workers, who reported two chiral diboronic acid receptors based on a 1,1′-binaphthyl scaffold, with the *R* receptor showing a 1.5-fold preference for D-glucose and the *S* receptor showing a 1.3-fold preference for L-glucose.[31] More recently, Kubik and co-workers reported a diboronic acidbased on a cyclic tetrapeptide scaffold, which bound D-glucose 2.1 times as strongly as L-glucose.[32]

This study has demonstrated that boronic acid–peptide hybrids can show significant variation in carbohydrate affinity that cannot be simply attributed to the binding preference of aryl boronic acids alone. The distance between boronic acids and the nature and location of other amino acid side chains present in the peptide greatly influences saccharide affinity. There is tremendous scope for the development of highly specific carbohydrate receptors using this approach, through variation in the number and location of boronic acid side chains, the use of different boronic acid-containing amino acids and the incorporation of a wide variety of natural amino acids. Improvements to future peptide boronic acid libraries will involve the use of substituents such as electron withdrawing, aminomethyl[1,15b,33] and hydroxymethyl[34] groups so that strong affinity can be achieved at neutral pH, thus allowing physiological applications to be investigated. One of the most exciting possibilities in this area is the development of cell-specific cellular probes for diagnostic and therapeutic applications.[5]

5. Experimental

5.1. General

Each peptide sequence was individually assessed for binding ability with several polyol substrates using a competitive displacement method. For ease of comparison, all binding experiments were conducted at the same temperature (room temperature 25 °C) and pH (10.7), using comparable concentrations. Unless otherwise stated, all masses and volumes relating to binding experiments refer to a single lantern. It is important to note that all binding experiments were conducted using a two-phase system in which the peptide host and all bound species were located on the solid phase. For ease of comparison, these solid-supported entities were expressed in terms of 'concentration' by dividing the number of moles of each species by the total volume of the binding medium (2000 μL).

5.2. Aqueous methanol carbonate buffer

A 50 mM solution of $NaHCO_3$ was prepared from distilled water and the pH was adjusted to 9.60 with the addition of 1 M NaOH. The carbonate buffer was then diluted with an equal volume of methanol and thoroughly mixed to afford the 50% aqueous methanol carbonate buffer. The buffer solution was stored at 4 °C and used within 2 days.

The pH of this aqueous methanol buffer was measured to be 10.8 with a pH meter calibrated with aqueous buffers. According to Perrin and Dempsey,[35] a correction factor should be applied to obtain a pH (referred to as pH*), which reflects the actual thermodynamic equilibrium present in the mixed aqueous organic buffer. In this case the correction factor is negative 0.1,[35] and so the pH*=10.7 for the buffer used in the binding assays.

5.3. Competitive polyol binding studies

5.3.1. Alizarin saturation

A lantern bearing a side chain deprotected peptide was immersed in aqueous methanol carbonate buffer (5 mL) and allowed to stand for 15 min at room temperature before the aqueous methanol buffer was then decanted. Commercially available alizarin (9.61 mg, 40 μmol) was dissolved in aqueous methanol carbonate buffer (2000 μL). The aforementioned lantern was then immersed in the dark purple alizarin solution, sealed and left to stand for 20 h at room temperature with occasional agitation.

The alizarin solution was then decanted and the lantern was washed with methanol (2000 μL) for 2×1 min. The lantern, bearing an alizarin saturated peptide diester was then allowed to air-dry for 30 min at room temperature.

5.3.2. Polyol stock solutions

Stock solutions of each of the polyols that were to be used in the competitive binding experiments were then prepared using aqueous methanol carbonate buffer. The concentration (mM) of each polyol stock solution were **15**·H_2O: 56.16; **16**: 56.65; **17**: 56.50; **18**: 56.83; **19**: 57.75; **20**: 58.03; **21**: 57.75; **22**: 56.23; **23**: 34.65. In the case of **15**, **16** and **23**, the pH was re-adjusted to pH 10.7 with the addition of a few drops of 1.0 M NaOH.

5.3.3. Alizarin displacement experiments

A dry lantern bearing an alizarin saturated peptide diester was then placed in a 4 mL vial and fully immersed in aqueous methanol carbonate buffer (2000 μL). The first aliquots (3×10 μL) were then removed, diluted with aqueous methanol carbonate buffer (3×990 μL) and the average absorbance measured at 507 nm. Polyol stock solution (30 μL) was then added to the immersed lantern and thoroughly mixed. The vial was immediately sealed (screw-top lid) and left to equilibrate for 6 h at room temperature with occasional agitation.

The vial was then opened and the next aliquots (3×10 μL) were then removed, diluted with aqueous methanol carbonate buffer (3×990 μL) and the average absorbance measured at 507 nm. Polyol stock solution (30 μL) was again added to the immersed lantern and thoroughly mixed. The vial was immediately re-sealed and left to equilibrate for another 6 h at room temperature with occasional agitation. The entire process of absorbance reading, followed by addition of polyol stock solution, and equilibration was repeated until no further alizarin displacement was observed.

5.3.4. Expressions used for competitive binding assay[26]

The general competitive binding equilibrium is shown in Eq. 1.

$$HI + S \leftrightarrow HS + I \quad (1)$$

where S=free substrate (free polyol); HS=bound substrate (bound polyol; I=indicator (alizarin); HI=bound indicator).

The mass balance expressions for the substrate, indicator and host are shown in Eqs. 2–4, respectively.

$$S_t = [S] + [HS] \quad (2)$$

$$I_t = [I] + [HI] \quad (3)$$

$$H_t = [H] + [HS] + [HI] \quad (4)$$

where S_t=total substrate (total polyol); I_t=total indicator (total alizarin); H_t=total host (total peptide).

The indicator and substrate binding constant expressions are shown in Eqs. 5 and 6, respectively.

Table 3
Typical *Q*-ratio and *P*-value data for competitive binding of D-fructose by peptide **10**

S_t (M)	Average absorbance	[I] (M)	[HI] (M)	*Q*-ratio	*P*-value	S_t/P
8.66×10^{-4}	0.1425	2.89×10^{-3}	3.04×10^{-3}	0.95	1.89×10^{-3}	0.46
1.73×10^{-3}	0.1716	3.48×10^{-3}	2.45×10^{-3}	1.42	2.81×10^{-3}	0.62
2.60×10^{-3}	0.1906	3.86×10^{-3}	2.06×10^{-3}	1.87	3.36×10^{-3}	0.77
3.47×10^{-3}	0.2038	4.13×10^{-3}	1.79×10^{-3}	2.30	3.72×10^{-3}	0.93
4.33×10^{-3}	0.2166	4.39×10^{-3}	1.53×10^{-3}	2.86	4.06×10^{-3}	1.07
5.20×10^{-3}	0.2250	4.56×10^{-3}	1.36×10^{-3}	3.34	4.28×10^{-3}	1.22
6.93×10^{-3}	0.2359	4.78×10^{-3}	1.14×10^{-3}	4.19	4.56×10^{-3}	1.52
1.39×10^{-2}	0.2590	5.25×10^{-3}	6.76×10^{-4}	7.77	5.13×10^{-3}	2.70
2.08×10^{-2}	0.2646	5.36×10^{-3}	5.61×10^{-4}	9.55	5.26×10^{-3}	3.95
2.77×10^{-2}	0.2726	5.53×10^{-3}	3.99×10^{-4}	13.86	5.46×10^{-3}	5.08

$$K_{HI} = \frac{[HI]}{[H]\times[I]} \tag{5}$$

$$K_{HS} = \frac{[HS]}{[H]\times[S]} \tag{6}$$

where K_{HI}=host/indicator association constant (K_a peptide/alizarin); K_{HS}=host/substrate association constant (K_a peptide/polyol).

The mass balance and binding expressions were combined to afford the overall competitive binding expression shown in Eq. 7.

$$H_t = [H] + \frac{K_{HS}\times[H]\times[S_t]}{1+K_{HS}\times[H]} + \frac{K_{HI}\times[H]\times[I_t]}{1+K_{HI}\times[H]} \tag{7}$$

The *Q*-ratio was defined as the ratio of free indicator to bound indicator as shown by Eq. 8.

$$Q = \frac{[I]}{[HI]} \tag{8}$$

Substituting the *Q*-ratio into the overall competitive binding expression afforded Eq. 9.

$$H_t = \frac{1}{Q\times K_{HI}} + \frac{S_t\times K_{HS}}{Q\times K_{HI}+K_{HS}} + \frac{I_t}{Q+1} \tag{9}$$

The *P*-value was defined by Eq. 10.

$$P = \frac{S_t\times K_{HS}}{Q\times K_{HI}+K_{HS}} \tag{10}$$

The *P*-value was also defined in terms of the overall binding expression as shown in Eq. 11.

$$P = H_t - \frac{1}{Q\times K_{HI}} - \frac{I_t}{Q+1} \tag{11}$$

Rearrangement of Eq. 10 afforded the linear binding expression shown in Eq. 12.

$$\frac{S_t}{P} = \frac{K_{HI}}{K_{HS}}\times Q + 1 \tag{12}$$

The *Q*-ratios and *P*-values were calculated after each addition of polyol substrate as exemplified in Table 3, and plots of S_t/P versus Q where used to determine K_{HS}.

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Supplementary data

All binding curves and error analyses are provided. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.10.095.

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