10.1071/CH07143_ AC

Accessory Publication: Aust. J. Chem., 2007, 60(11), 829–834.

Accessory Publication

The Preparation of Solid-Supported Peptide Boronic Acids Derived from L-4-Boronophenyl Alanine and their Affinity for Alizarin

Peter J. Duggan, Daniel A. Offermann

General Synthetic Procedures:

All commercial materials were used without further purification unless otherwise stated. DMF and DMSO were stored over 4Å sieves; acetonitrile and methanol were stored over 3Å sieves; diethyl ether and THF were distilled from a sodium/benzophenone system and used immediately. ^{1}H NMR spectra were recorded on a Varian 300 MHz spectrometer and recorded in parts per million (δ) using the residual non-deuterated solvent as an internal reference. ^{13}C NMR spectra were recorded on a Varian 75 MHz spectrometer and recorded in parts per million (δ) using the residual non-deuterated solvent as an internal reference. IR spectra were recorded on a Perkin Elmer 1600 Series Fourier Transform Spectrophotometer. Liquid samples were mounted neat on NaCl plates, while solid samples were applied as a nujol (paraffin) mull. Optical rotations were determined using a Perkin Elmer 141 Polarimeter with a 1 mL cell (path length of 10 cm) at the reported temperatures and concentrations. Electrospray mass spectra were recorded on a Micromass Platform QMS Spectrometer (QMS – Quadrupole Mass Electrospray). High resolution mass spectra (HRMS) were recorded on a Bruker BioApex 47e Fourier Transform mass spectrometer. Liquid chromatography was performed using a forced flow of a mixture of solvents on silica gel 60 (0.040-0.063 mm). LC/MS analysis was performed using an Alltech Alltima C_{18} column, 150 × 4.6 mm, 5 μm particle size; Agilent 1100 series G1315A diode array detector; Waters Micromass ZMD mass spectrometer; all controlled with Micromass MassLynx software version 3.5. HPLC solvent A: 0.1 % TFA/Milli-Q water; HPLC solvent B: 0.06 % TFA/Milli-Q water. Flowrate

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was 1 mL/min with a gradient of 10-95 % (solvent B) in 15 mins. The absorbance detection range was 200-290 nm.

Solid Phase Peptide Synthesis

General

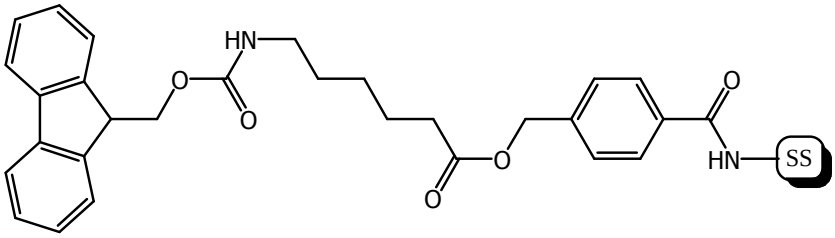
Peptides were prepared following the general procedures recommended by Mimotopes (formerly Chiron Technologies),[1] and also described by Bromfield, Cianci and Duggan[2] with the exception that the activating agent (BOP) was replaced by HBTU. All solid phase peptide synthesis was carried out using *SynPhase*™ D-series lanterns. These solid supports consisted of an acetylated methacrylic acid/dimethylacrylamide copolymer grafted to an inert polyethylene base. The solid supports were supplied without an attached linker (non-functionalised) and possessed an average loading of 8.0 μmol per unit. All amino acids used were suitably protected for standard 'Fmoc' solid phase peptide synthesis. This included Fmoc protection of the primary amine in addition to appropriate side chain protection as detailed in **Table 1**. Procedures for spacer attachment, Fmoc determination, cleavage of peptides using sonication under basic conditions and terminal *N*-capping were not reported by Bromfield[3] and are described below.

Table 1. Protected amino acids used in solid phase peptide synthesis:

Amino Acid	Protecting Group (PG)	
	α-Amine	Side Chain
Ala	Fmoc	N/A
Asp	Fmoc	*t*Bu
Arg	Fmoc	Pmc
Leu	Fmoc	N/A
BPA	Fmoc	Pin
Lys	Fmoc	BOC
Phe	Fmoc	N/A

Spacer Attachment

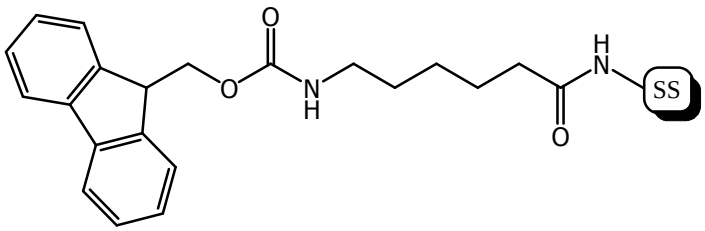
Through the HMB linker



A solution of DMAP (1.1 mg, 9 μmol) in 20 % DMF/DCM (450 μL) was prepared (designated solution A). *N*-Fmoc-6-aminohexanoic acid (31.8 mg, 90 μmol) was dissolved in 20 % DMF/DCM (450 μL), and DIC (14.1 μL, 90 μmol) was then added and the mixture allowed to stand for 2 mins (designated solution B). A lantern, bearing the HMB linker, was then immersed in solution B, then solution A was added. The combined reaction mixture (900 μL) was left to stand for 2 hrs at room temperature with occasional agitation.

The solution was then decanted and the lantern was washed with DMF (900 μL) for 5 mins, followed by DCM (900 μL) for 5 mins. The lantern, with spacer attached, was then allowed to air-dry for 30 mins at room temperature.

Directly to solid support



N-Fmoc-6-aminohexanoic acid (31.8 mg, 90 μmol) was dissolved in DMF (450 μL) (designated solution A). HOBt.H_2O (13.8 mg, 90 μmol), HBTU (34.1 mg, 90 μmol) and NMM (14.8 μL, 135 μmol) were dissolved in DMF (450 μL) (designated solution B). Solution A was then added to solution B and the combined reaction mixture (900 μL) was left to stand for 2 mins at room temperature. An Fmoc deprotected lantern was then immersed in the coupling solution and left to stand for 2 hrs at room temperature with occasional agitation. The solution was then decanted and the lantern was washed with DMF (900 μL) for 5 mins, followed by DCM (900 μL) for 5 mins. The lantern, with spacer attached, was then allowed to air-dry for 30 mins at room temperature.

Fmoc Determination

A lantern bearing an Fmoc protected peptide was immersed in 20 % piperidine/DMF (10.0 mL) cleavage solution and left to stand at room temperature for 45 mins with occasional agitation. A 1.0 mL aliquot of the reacted cleavage solution was then diluted with fresh 20 % piperidine/DMF (10 mL) and the absorbance measured (301 nm) against a blank cell also containing 20 % piperidine/DMF. The amount of Fmoc cleaved and hence the peptide loading for each solid support was calculated using **Equation 1**.

$$\mathrm{n(Fmoc)} = \left[\frac{\mathrm{A}_{301}}{\varepsilon \times l}\right] \times \mathrm{v} \times \mathrm{df}$$

Where: A_{301} = average absorbance at 301 nm; $\varepsilon = 7.8 \times 10^6$ mL.mol^{-1}.cm^{-1}; l = 1.0 cm; v = 11.0 mL; df = dilution factor = 10.

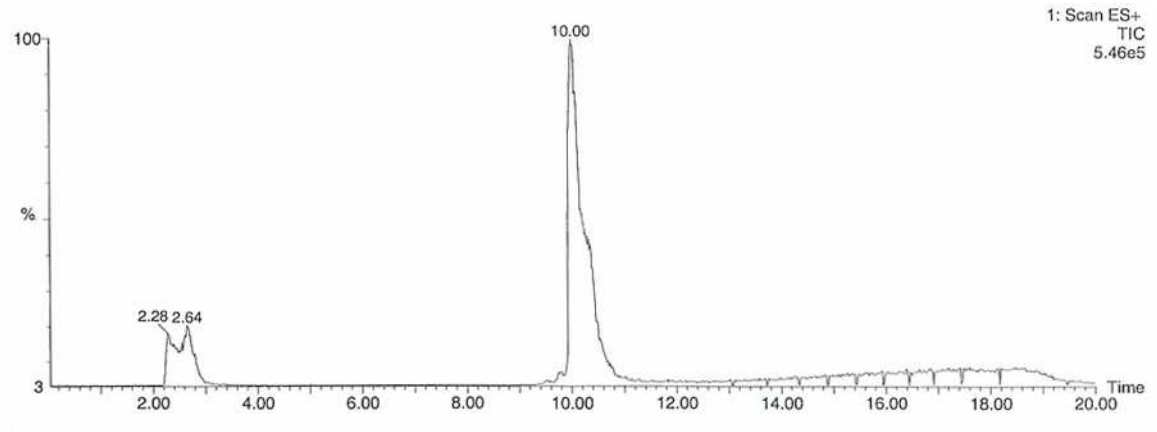
Equation 1 "Fmoc determination" calculation

Cleavage of peptides from lanterns

A lantern bearing a side chain deprotected peptide connected to the solid phase through a HMB linker was immersed in 60 % acetonitrile/0.1 M NaOH solution (900 μL) and placed in an ultrasonic bath for 30 mins at room temperature. The lantern was then removed and the cleavage solution neutralised (pH 7.0) with 2 M NaH_2PO_4. The solution was then freeze-dried to afford a mixture of the peptide and sodium phosphate salts as a white solid.

Peptide Analysis

All peptides from the initial library displayed purities in excess of 90 % *via* LC/MS. This purity refers to the organic component of the peptide cleavage mixture and does not include the accompanying inorganic salts. The total ion count (TIC) elution profiles for all peptide samples included: a minor peak (<10 %) with retention times of 2.3-2.4 mins; and a major peak (>90 %) with retention times of 8.0-10.2 mins. A typical elution profile is shown below.



*Typical LC/MS (TIC) elution profile - peptide boronic acid **4***

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Mass spectral analysis of the minor peak present in each sample failed to identify any desired peptide product, associated peptide fragments or potential deletion products, and was therefore considered to be non-peptidic in nature. Mass spectral analysis of the major peak present in each sample showed the presence of the desired peptide boronic acid. The molecular ion peaks for the free boronic acid species were rarely observed however, with the *m/z* values for a variety of corresponding boroxine species being identified. A typical mass spectral analysis together with the assignment of major *m/z* values and potential structures of the identified species is shown below.

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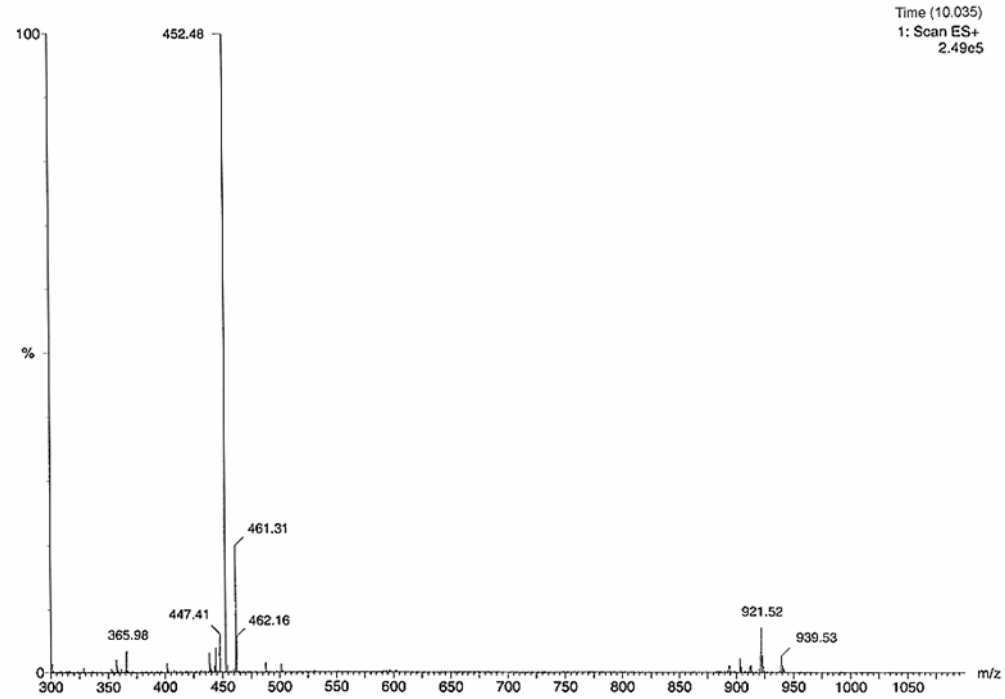
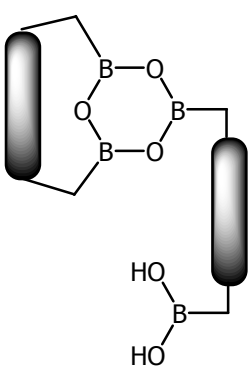
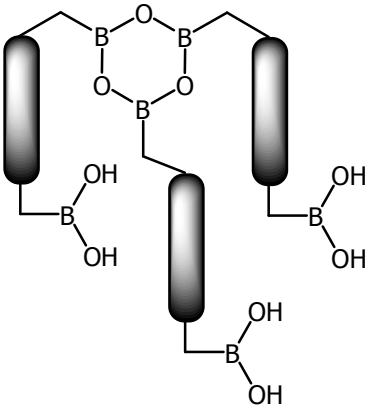


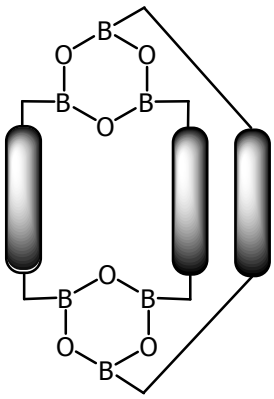
m/z	Identified Species[a]
365.98	[boroxine dimer + 5H]$^{5+}$
452.48	[macrobicyclic boroxine trimer + 6H]$^{6+}$
461.31	[boroxine trimer + 6H]$^{6+}$
921.52	[boroxine trimer + 3H]$^{3+}$
939.53	[M + H]$^{+}$



(boroxine dimer)



(boroxine trimer)



(macrobicyclic boroxine trimer)

Where:  = Peptide

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N-Terminal Capping

Acetic anhydride (25μL, 265 μmol) and DIEA (5μL, 29 μmol) were added to DMF (870 μL). A lantern bearing an Fmoc deprotected peptide, attached to the solid phase directly through the aminohexanamide spacer, was then immersed in the reaction mixture and left to stand at room temperature for 1.5 hrs with occasional agitation. The solution was then decanted and the lantern was washed with methanol (900 μL) for 2×10 mins. The lantern, with acetamide capped peptide was then allowed to air-dry for 30 mins at room temperature.

Binding Constant Determination:

General

Each peptide sequence was individually assessed for binding ability with alizarin. All binding experiments were conducted using side chain deprotected peptides bound to a lantern. 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. Blank lanterns were included in all binding experiments to assess the degree of non-specific binding to the solid support. Phenylalanine control peptides were also assessed for binding ability to determine the extent of binding due to the boronic acid moieties.

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,[3] 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,[3] and so the pH* = 10.7 for the buffer used in the binding assays.

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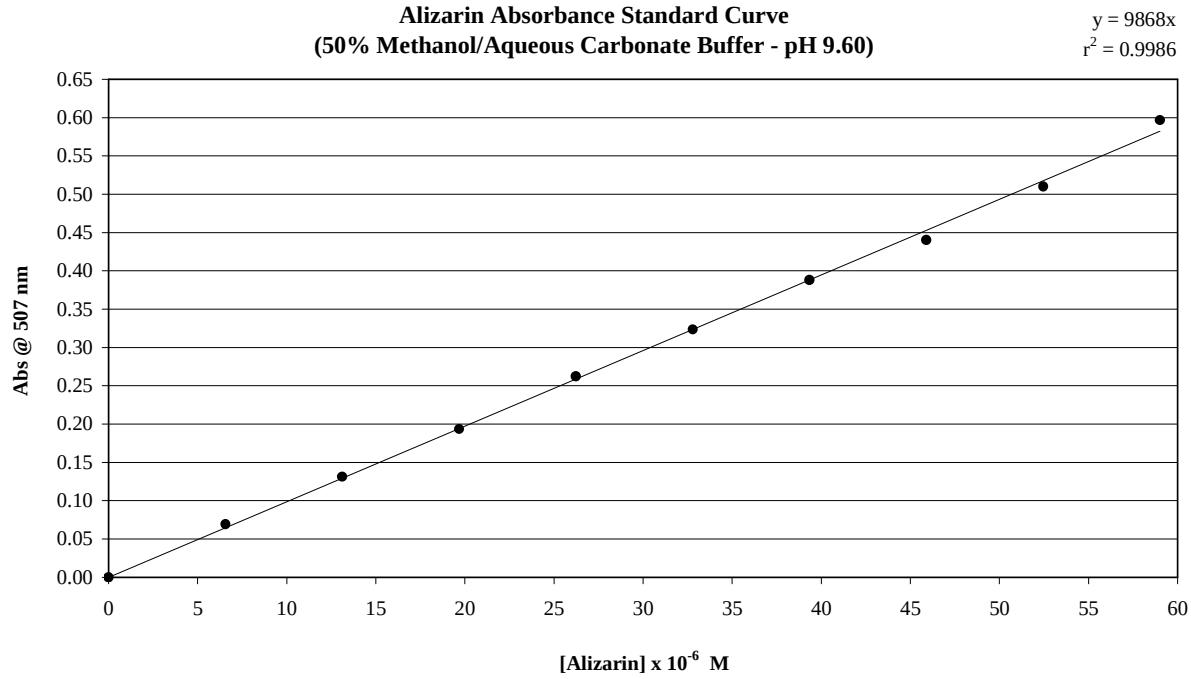
Alizarin absorbance standard curve

Standard solutions of alizarin in aqueous methanol carbonate buffer were prepared (final concentrations: 0.00, 6.56, 13.1, 19.7, 26.2, 32.8, 39.3, 45.9, 52.5 and 59.0 μM) and the absorbances measured (in triplicate) at 507 nm.



Alizarin Binding Study

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).

A lantern bearing a side chain deprotected peptide was immersed in aqueous methanol carbonate buffer (5 mL) and allowed to stand for 15 mins at room temperature. The solution was then decanted and the lantern was washed with methanol (2000 μL) for 2 × 1 min, then allowed to air-dry for 30 mins at room temperature. Commercially available alizarin (42.1

mg, 175 μmol) was dissolved in aqueous methanol carbonate buffer (2000 μL) (designated alizarin stock solution).

The aforementioned lantern 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. Alizarin 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 hrs 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. Alizarin 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 hrs at room temperature with occasional agitation. The entire process of absorbance reading, followed by addition of alizarin stock solution, and equilibration was repeated until no further alizarin binding was observed.

The concentration of bound alizarin at each point of the titration curve was then calculated by subtracting the concentration of free alizarin from the total alizarin concentration as shown in the following figure.

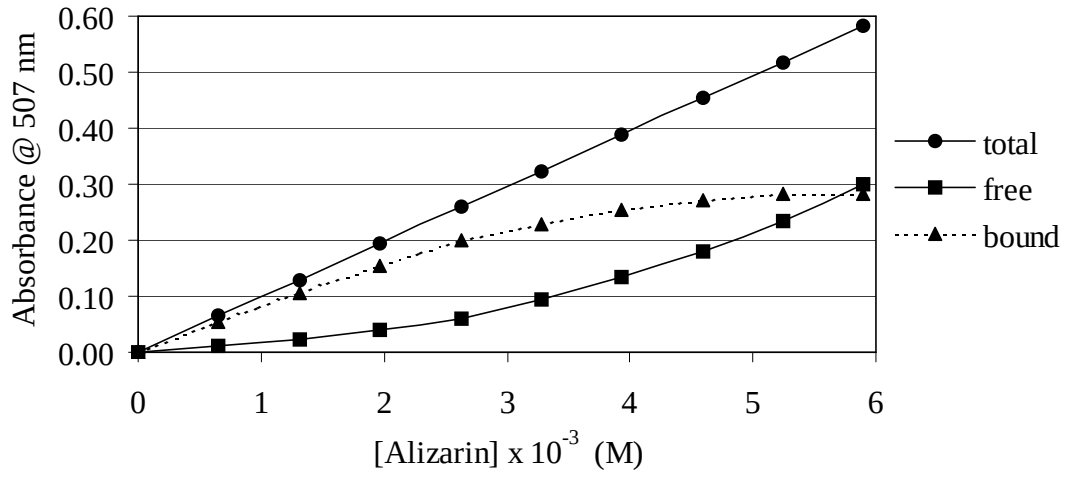


Figure showing typical determination of bound alizarin concentration – peptide boronic acid ***10***

The binding event was accompanied by an increase in colouration (dark purple) of the solid support due to the formation of the 2:1 dye/peptide diester. Alizarin addition was repeated until no further increase in binding was observed, indicated by a plateau in bound alizarin concentration.

The general equilibrium expression for alizarin binding is summarised by Equation 1.

$$H + I \leftrightarrow HI \qquad (1)$$

Where: H = free host (free peptide); I = free indicator (free alizarin); HI = bound indicator (bound alizarin).

The binding constant expression at equilibrium is shown in Equation 2.

$$K_{HI} = \frac{[HI]}{[H] \times [I]} \qquad (2)$$

Where: K_{HI} = host/indicator association constant (K_a peptide/alizarin).

The concentration of bound alizarin was then plotted against the concentration of free alizarin and a binding curve was fitted using nonlinear regression. The equation used to fit the binding isotherm is shown in Equation 3.

$$Y = \frac{B_{max} \times X}{K_d + X} \qquad (3)$$

Where: Y = bound indicator (bound alizarin) = [HI]; B_{max} = concentration of total host binding sites (asymptote); X = free indicator (free alizarin) = [I]; K_d = dissociation constant = concentration at which 50 % of binding sites are occupied.

The alizarin binding plots with nonlinear regression curves are shown below.

From the estimate of the total binding site concentration (B_{max}), it was determined that each boronic acid moiety bound a single alizarin molecule, resulting in a 2:1 alizarin/peptide binding stoichiometry. The same binding ratio was observed with all peptide boronic acids studied. Using the B_{max} estimates, the average amount of solid supported peptide was also calculated (7.2 μmol) which was slightly higher than the previous estimate obtained *via* Fmoc determination (6.5 μmol).

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Table 2: Alizarin binding constants:

Peptide	Amino Acid Sequence	K_{HI} (M^{-1})[a]
10	(Ac)BPA-BPA-Ala-Arg-Arg-Spacer-SS	1055 ± 57
11	(Ac)BPA-Arg-BPA-Ala-Arg-Spacer-SS	769 ± 36
12	(Ac)BPA-Arg-Arg-BPA-Ala-Spacer-SS	587 ± 33
13	(Ac)BPA-Arg-Ala-Arg-BPA-Spacer-SS	510 ± 33
14	(Ac)Arg-BPA-BPA-Arg-Ala-Spacer-SS	780 ± 41
15	(Ac)Arg-BPA-Arg-BPA-Ala-Spacer-SS	443 ± 26
16	(Ac)BPA-BPA-Ala-Lys-Lys-Spacer-SS	485 ± 28
17	(Ac)BPA-Lys-BPA-Ala-Lys-Spacer-SS	506 ± 31
18	(Ac)BPA-Lys-Lys-BPA-Ala-Spacer-SS	421 ± 26
19	(Ac)BPA-Lys-Ala-Lys-BPA-Spacer-SS	242 ± 24
20	(Ac)Lys-BPA-BPA-Lys-Ala-Spacer-SS	470 ± 35
21	(Ac)Lys-BPA-Lys-BPA-Ala-Spacer-SS	440 ± 23

[a] Error values were calculated from the standard error in the K_d values.

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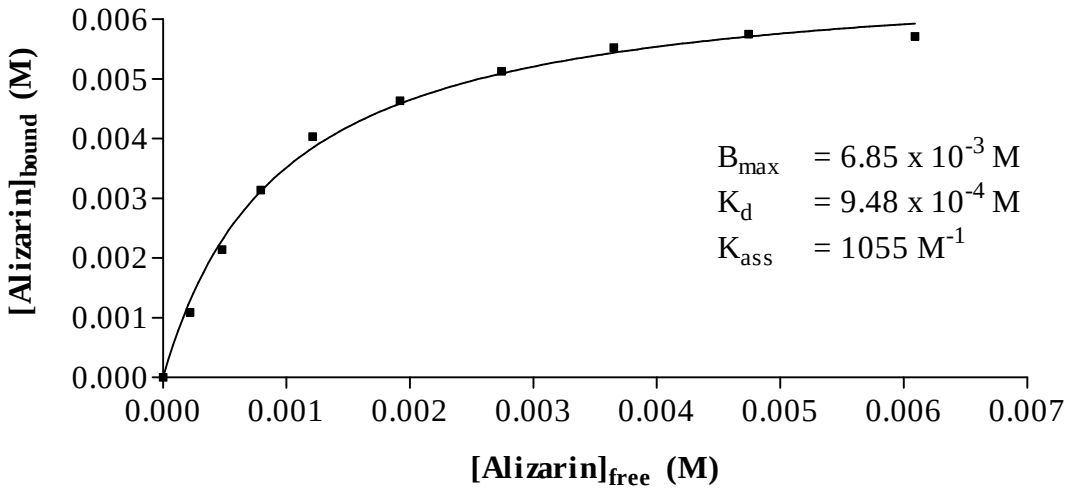
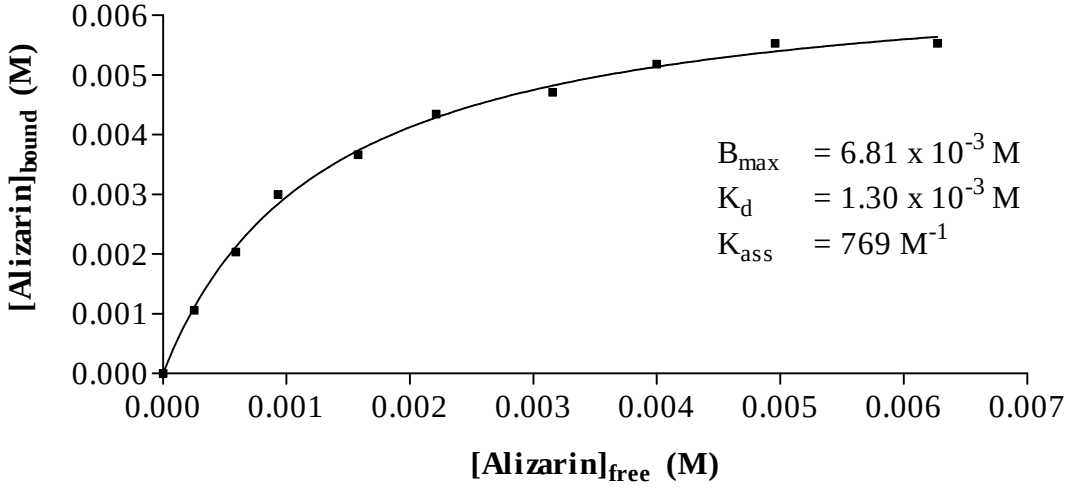
Alizarin Binding Curves

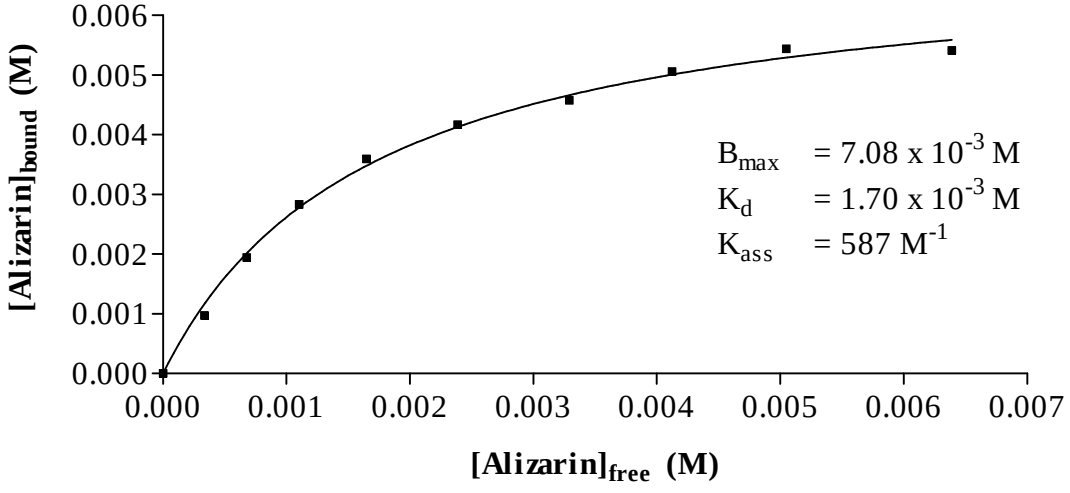
Peptide 10



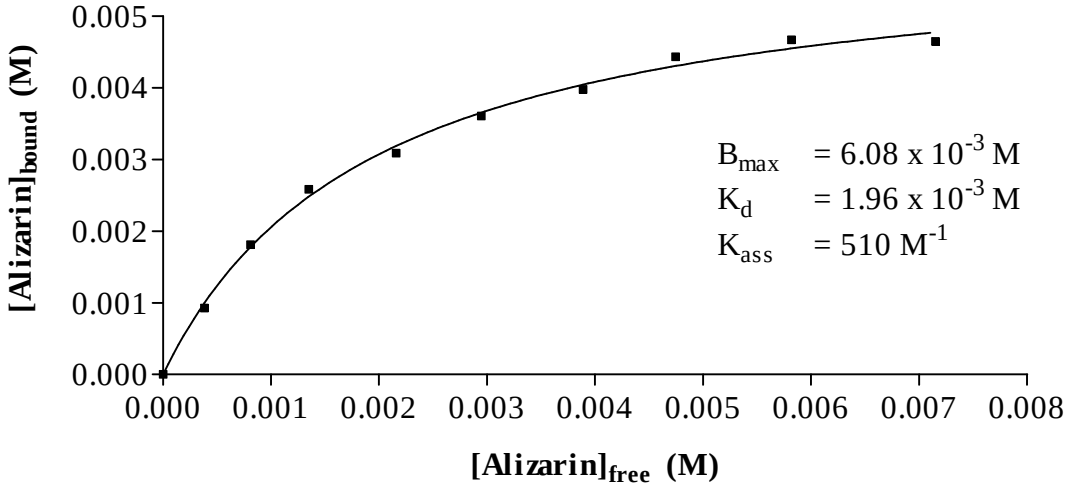
Peptide 11



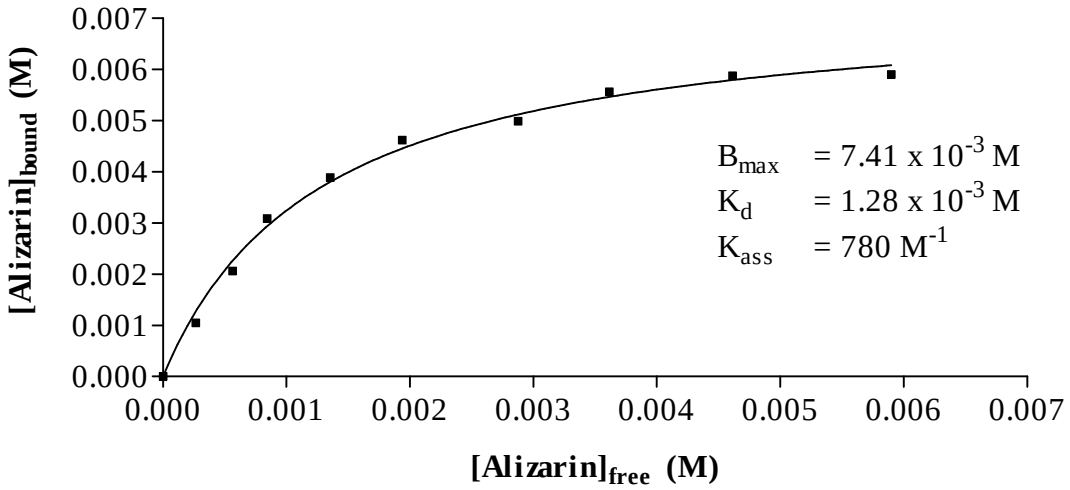
Peptide 12



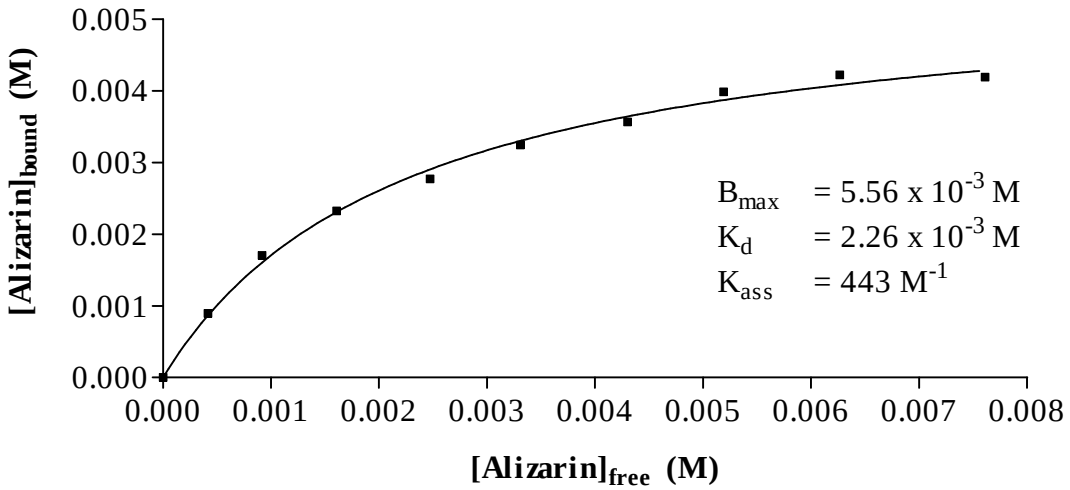
Peptide 13



Peptide 14



Peptide 15



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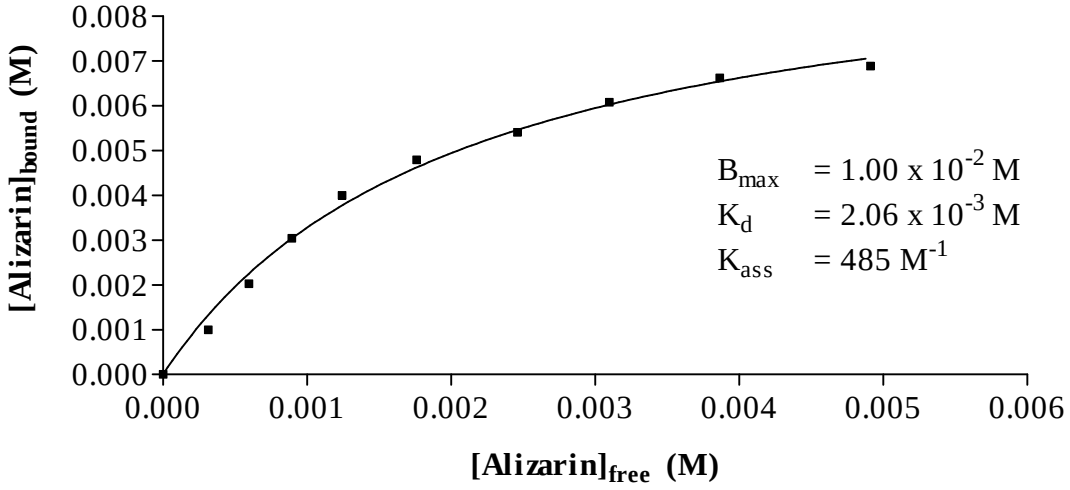
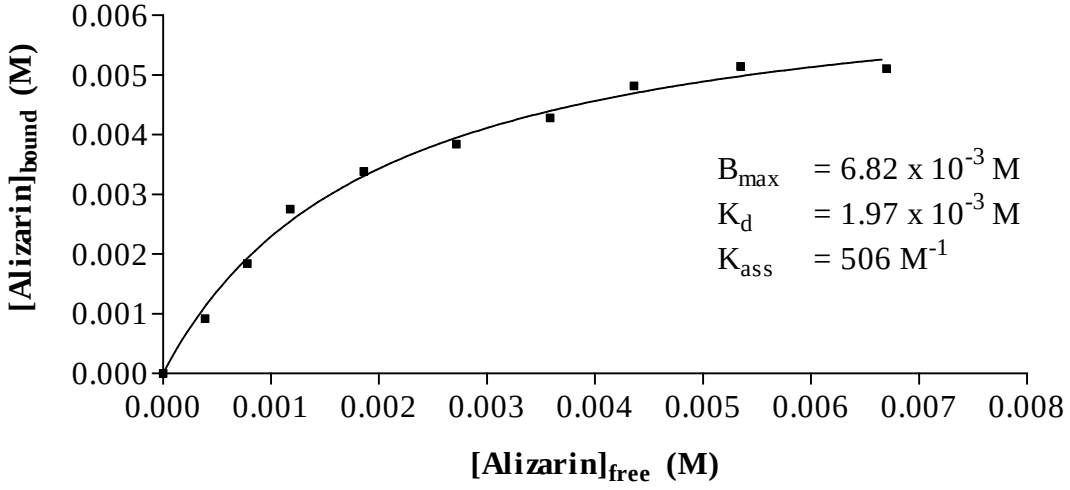
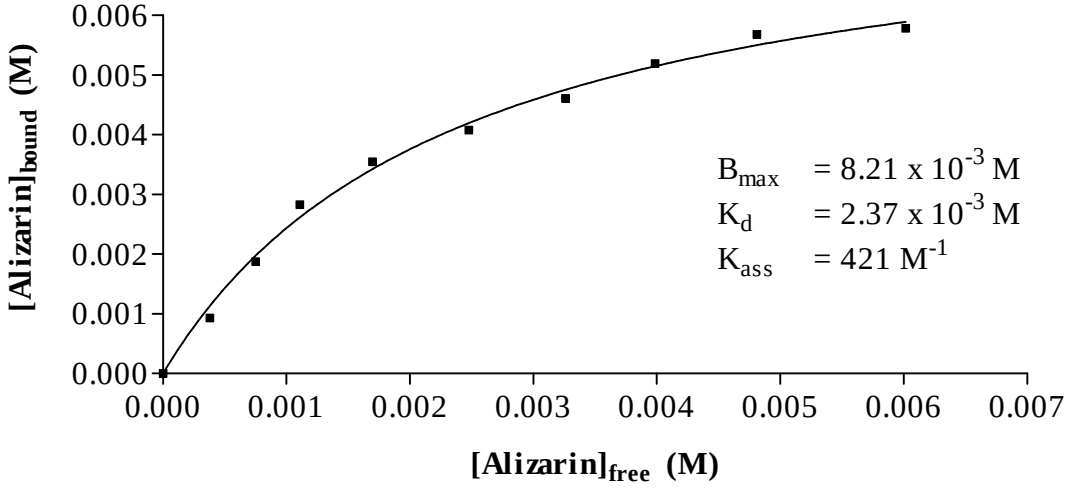
Peptide 16



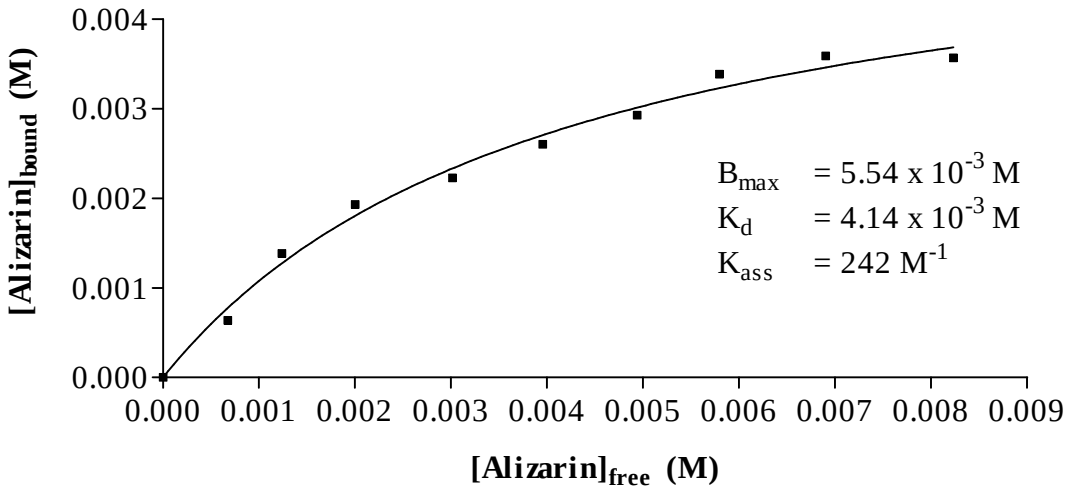
Peptide 17



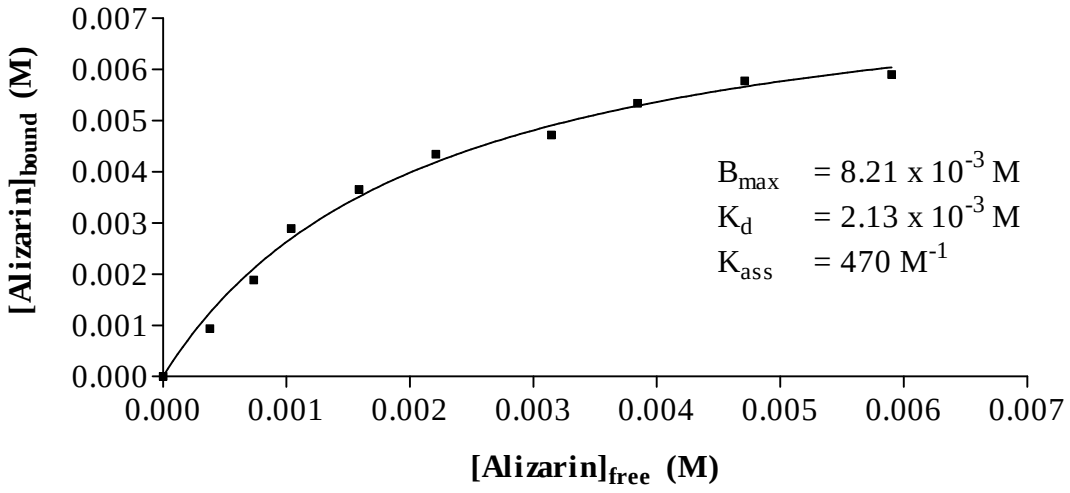
Peptide 18



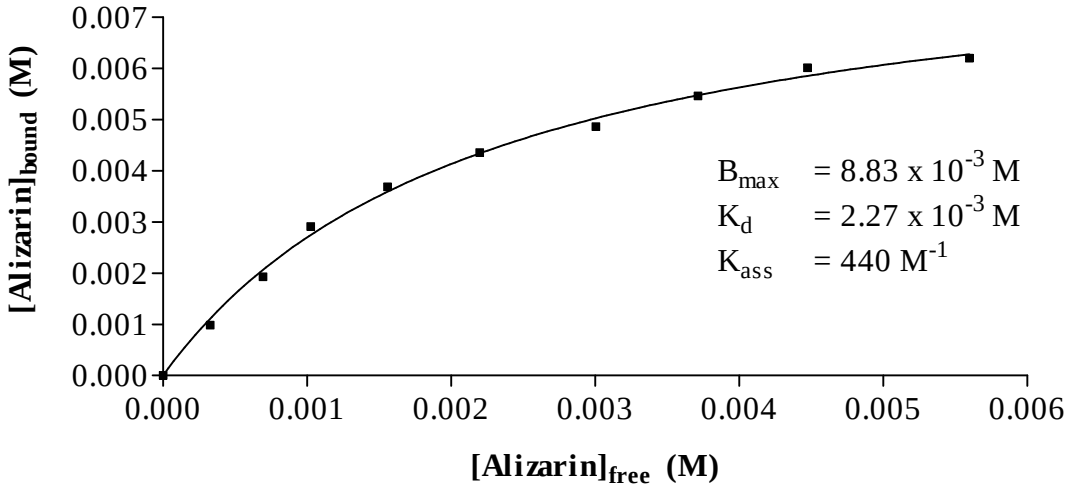
Peptide 19



Peptide 20



Peptide 21



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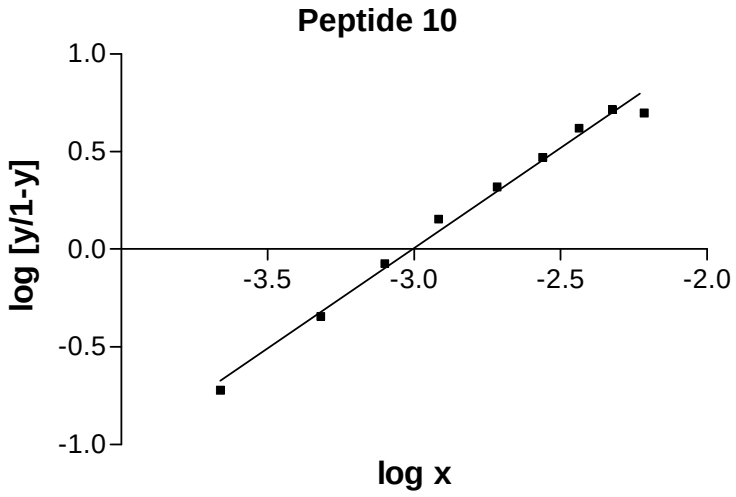
Tests for cooperativity (Hill Plots)

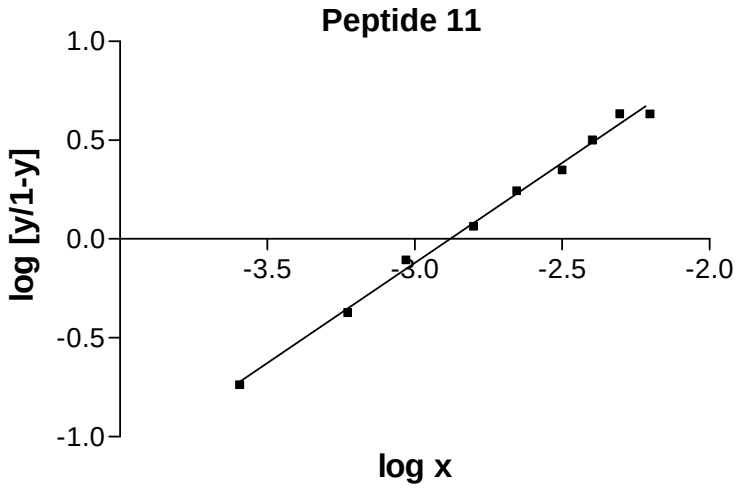
Following the method described by Connors,[4] Hill plots (logx vs log (y/1-y)) were prepared for alizarin binding to the solid supported peptide boronic acids where;

x = alizarin in solution and
y = alizarin bound to lantern, normalized using the previously calculated Bmax values.

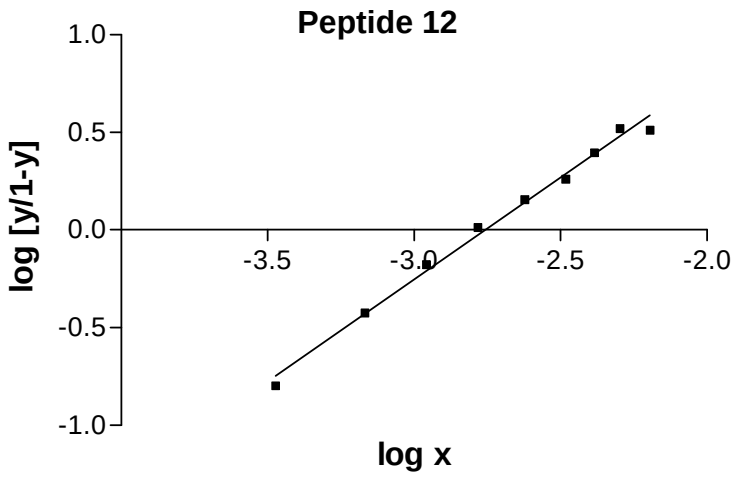
These Hill plots are shown on the following pages.



Slope	1.026 ± 0.04139	r^2	0.9887



Slope	1.013 ± 0.02892	r^2	0.9943



Slope	1.044 ± 0.03644	r^2	0.9915

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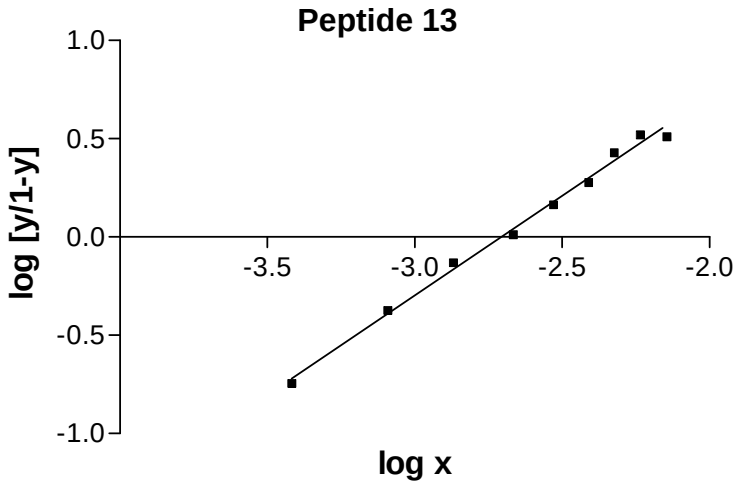
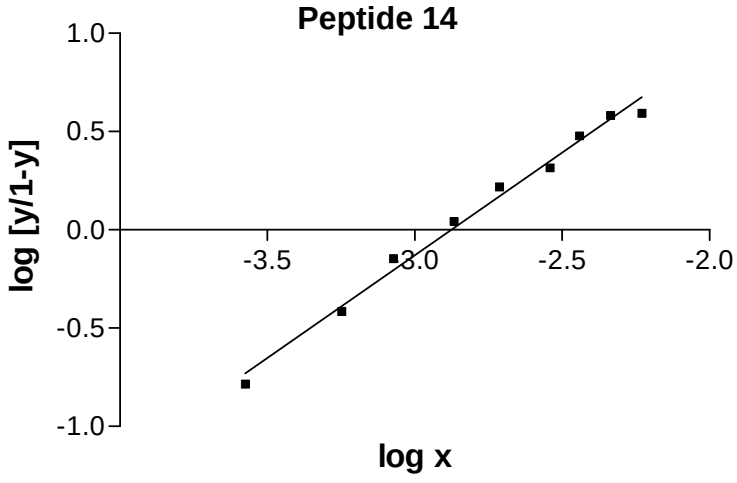
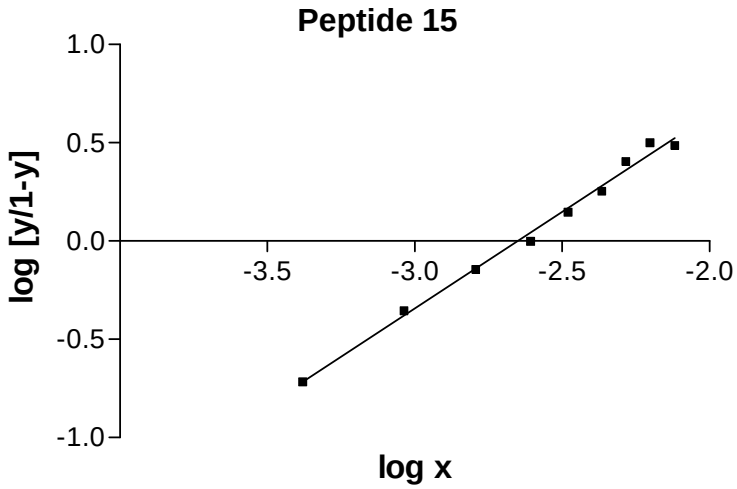


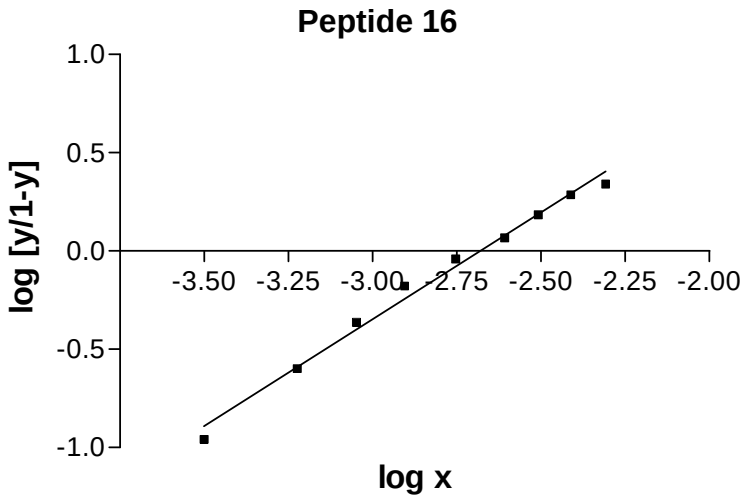
Slope	1.013 ± 0.03235	r^2	0.9929



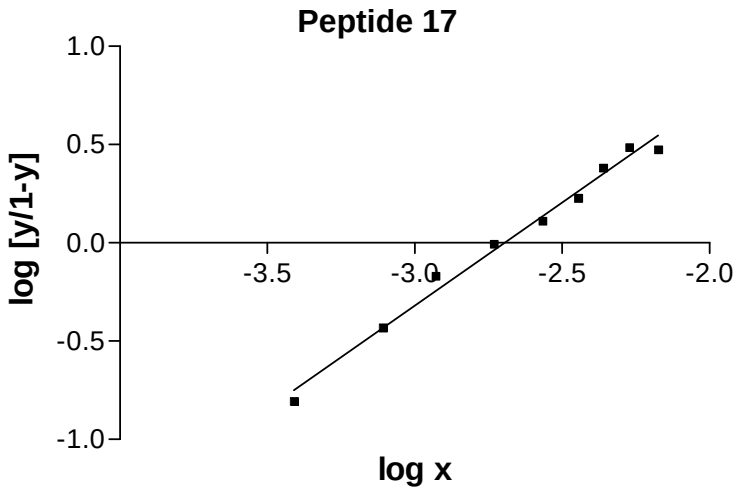
Slope	1.046 ± 0.04190	r^2	0.9889



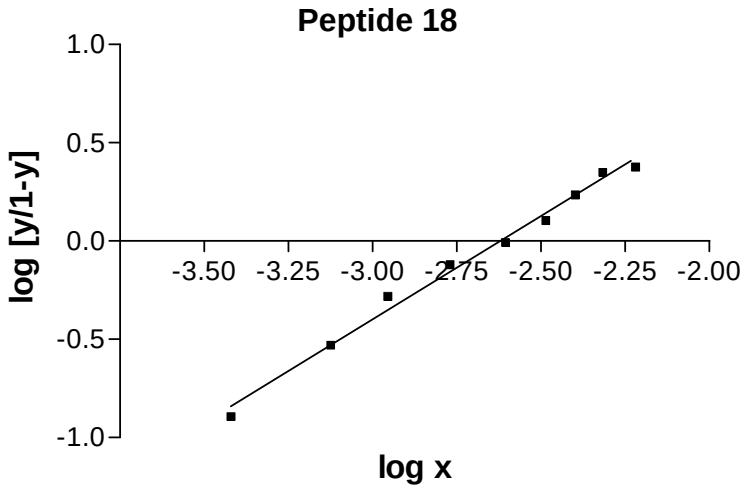
Slope	0.9844 ± 0.03326	r^2	0.9921



Slope	1.086 ± 0.04293	r^2	0.9892



Slope	1.049 ± 0.04538	r^2	0.9871



Slope	1.050 ± 0.03903	r^2	0.9904

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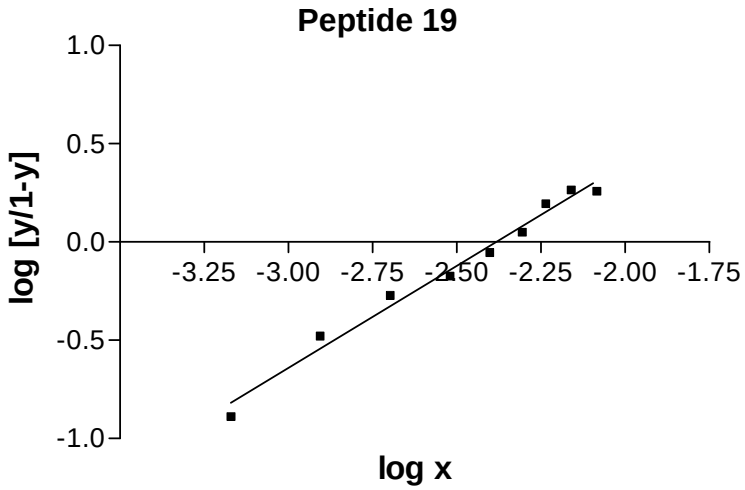
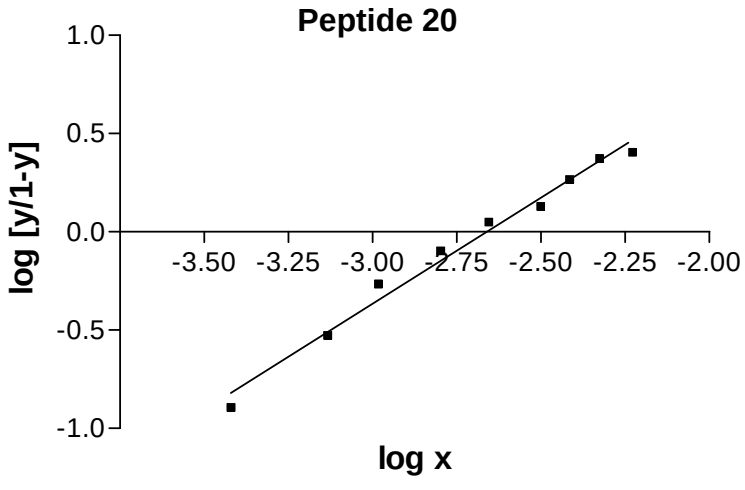
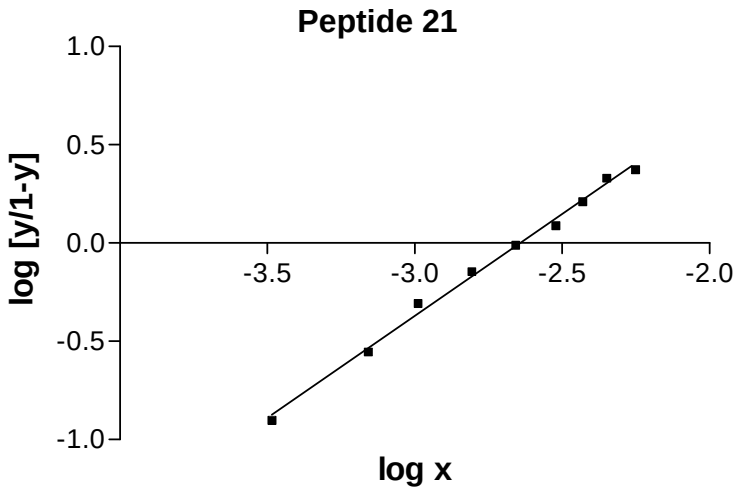


Slope	1.040 ± 0.05318	r²	0.9820



Slope	1.080 ± 0.05037	r²	0.9850



Slope	1.037 ± 0.02854	r²	0.9947

Accessory Publication

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[4] Connors, K.A., "*Binding Constants - The measurement of molecular complex stability"*, (John Wiley and sons: New York, 1987).