Synthesis and application of an *N*-acylated L-homoserine lactone derivatized affinity matrix for the isolation of quorum sensing signal receptors

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Supplementary data.

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General experimental information.

All reagents were purchased from commercial sources (Aldrich, Acros, and NovaBiochem) and used without further purification. Solvents were purchased from commercial sources (Aldrich and J.T. Baker) and used as is, with the exception of dichloromethane (CH_2Cl_2), which was distilled over calcium hydride prior to use. Water was purified using a Millipore Analyzer Feed System. Affigel-10 resin and protein standards (Precision Plus, unstained) were purchased from Bio-Rad.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-300 spectrometer in deuterated solvents at 300 MHz and 75 MHz, respectively. Chemical shifts are reported in parts per million (ppm, δ) using tetramethyl silane (TMS) as a reference (0.0 ppm). Couplings are reported in hertz. Attenuated total reflectance (ATR)-IR spectra were recorded with a Bruker Tensor 27 spectrometer, outfitted with a single reflection MIRacle Horizontal ATR by Pike Technologies using a Ge crystal. Electrospray ionization (ESI) MS data for compound **6** was obtained using a Waters (Micromass) LCT™ system equipped with a time-of-flight analyzer. Samples were dissolved in methanol and sprayed with a sample cone voltage of 20.

Preparative HPLC analysis was performed using a Shimadzu system equipped with an SCL-10Avp controller, LC-10AT pump, FCV-10ALvp solvent mixer, and SPD-10MAvp UV/vis diode array detector. An Agilent C18-silica reverse-phase preparative column (7 μm, 25 mm x 250 mm) was used in the final purification of compound **6** (conditions: flow rate = 9.0 mL/min; mobile phase A = 0.1% TFA in water; mobile phase B = 0.1% TFA in acetonitrile).

Liquid chromatography-mass spectrometry (LC-MS) data were obtained using a Shimadzu LCMS-2010 system equipped with two pumps (LC-10ADvp), controller (SCL-10Avp), autoinjector (SIL-10ADvp), UV diode array detector (SPD-M10Avp), and single quadrupole analyzer (by electrospray ionization, ESI). The LC-MS was interfaced with a PC running the Shimadzu LCSolutions software package (Version 2.04 Su2-H2). A Supelco C18-silica wide-pore reverse phase column (5 μm, 15 cm x 2.1 mm) was used (conditions: flow rate = 200 μL/min; mobile phase A = 0.1% formic acid in water; mobile phase B = 0.1% formic acid in acetonitrile.)

All protein purification work was performed using a HisTrap FF 5 mL column (GE Lifesciences) and either an AKTAexplorer or an AKTAbasic chromatography system.

μLC-MS/MS analysis of sample peptides was carried out on a Waters UPLC system connected to a LTQ Velos dual pressure linear ion trap analyzer (ThermoFisher). Peptide samples were loaded onto a frit-less 100 x 165 μm fused silica capillary microcolumn packed with 10 cm of C18-silica beads (5 mm diameter, Western Analytical Products). The capillary column was connected to the UPLC system through a PEEK microcross with a platinum wire inserted into the flow-through to supply a spray voltage of 1.8 kV. The ion trap mass spectrometer was set to run with a full-mass scan between 400 and 2000 m/z, followed by an MS/MS scan of each of the highest-intensity parent ions.

Synthesis of lactone 6.

The synthesis of lactone **6** was adapted from the study reported by Amara *et al*.[1]

*Synthesis of 10-(t-butoxycarbonylamino)decanoic acid (**2**)*. In a 250-mL round-bottom flask equipped with a magnetic stirbar, 10-bromodecanoic acid (2.51 g, 10.0 mmol) was dissolved in 80 mL of aqueous ammonium hydroxide (25% NH_3). The solution was stirred for 24 h at rt. Thereafter, the mixture was concentrated *in vacuo* to yield a white solid that was used without further purification.

The crude product from the previous step was placed in a 100-mL round-bottom flask with a magnetic stirbar, and NaOH (505 mg, 12.6 mmol) and Boc anhydride (2.31 g, 10.6 mmol) were added. Water (11 mL) and *t*-butanol (5 mL) were added to the flask to dissolve the solids, and the mixture was stirred at rt for 24 h. The crude product was diluted with water (20 mL) and 1 M HCl (20 mL). The resulting mixture was extracted with ethyl acetate (30 mL, 2x) and brine (30 mL). The organic layer was isolated, dried over $MgSO_4$, and concentrated *in vacuo* to yield 2.42 g of **2** as a white solid (84% isolated yield). The ^{1}H NMR and ^{13}C NMR spectra for acid **2** were identical to those that were previously reported.[1]

*Synthesis of N-(12-(t-butoxycarbonylamino)-3-oxo-dodecanoyl)-L-homoserine lactone (**5**)*. Compound **2** (146.6 mg, 0.51 mmol), Meldrum's acid (**3**, 110.6 mg, 0.77 mmol), *N,N*'-dicyclohexylcarbodiimide (DCC, 159.7 mg, 0.77 mmol), and 4-(dimethylamino)pyridine (DMAP, 92.6 mg, 0.76 mmol) were added to a 100-mL round-bottom flask equipped with a magnetic stirbar. CH_2Cl_2 (5 mL) was added to dissolve the reagents. The flask was flushed with N_2, and the mixture was allowed to stir for 18 h at rt. Thereafter, the mixture was washed with 1 M HCl (30 mL, 2x) and brine (30 mL). The organic layer was isolated, dried over $MgSO_4$, and concentrated *in vacuo* to yield the triketone intermediate. A 25-mL aliquot of freshly distilled acetonitrile was added to resuspend the solid. Homoserine lactone hydrobromide (**4**, 109.3 mg, 0.60 mmol) and triethylamine (83.8 μL, 0.60 mmol) were added, and the resulting mixture was refluxed for 7 h. The mixture was then concentrated *in vacuo*, resuspended in CH_2Cl_2 (50 mL), and washed with 1 M HCl (30 mL, 2x) and brine (30 mL). The organic layer was isolated, dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by flash silica gel column chromatography (eluent: 60–75% ethyl acetate in hexanes) to give 164.4 mg of lactone **5** as a white solid (78% isolated yield). ^{1}H NMR: ($CDCl_3$) δ 7.75 (d, J = 6.1 Hz, 1H), 4.61 (m, 2H), 4.47 (td, J = 1.7, 9.1 Hz, 1H), 4.29 (ddd, J = 6.1, 9.3, 11.4 Hz, 1H), 3.47 (s, 2H), 3.09 (q, J = 6.8 Hz, 2H), 2.72 (dddd, J = 1.5, 6.0, 7.5, 13.5 Hz, 1H), 2.54 (t, J = 7.5 Hz, 2H), 2.27 (qd, J = 9.0, 11.8 Hz, 1H), 1.57 (m, 2H), 1.44 (bs, 11H), 1.27 (bs, 10H); ^{13}C NMR: ($CDCl_3$) δ 206.2, 177.2, 175.3, 166.9, 157.4, 66.0, 49.0, 48.9, 43.6, 33.7, 30.0, 29.3, 29.3, 29.3, 29.2, 28.9, 28.5, 26.8, 25.0, 23.3.

*Synthesis of (S)-10,12-dioxo-12-(2-oxotetrahydrofuran-3-ylamino)dodecan-1-ammonium trifluoroacetate (lactone **6**)*. Compound **5** (164.4 mg, 0.40 mmol) was placed in a 100-mL round-bottom flask equipped with a magnetic stirbar, and dissolved in CH_2Cl_2 (3 mL). Trifluoroacetic acid (TFA, 3 mL) was added, and the solution was stirred for 5 h at rt. Thereafter, the solution was concentrated *in vacuo* and methanol (1.5 mL) was added. The crude product was purified by preparative HPLC to yield 94.7 mg of lactone **6** as tan liquid (56% isolated yield). ^{1}H NMR:

(CD_3OD) δ 4.60 (dd, J = 9.3 Hz, 1H), 4.45 (td, J = 2.1, 8.7 Hz, 1H), 4.30 (ddd, J = 6.3, 8.7, 10.5 Hz, 1H), 2.91 (t, J = 7.5 Hz, 2H), 2.58 (m, 3H), 2.31 (ddd, J = 10.5, 11.1, 12.6 Hz, 1H), 1.60 (m, 4H), 1.34 (m, 10H); ^{13}C NMR: (CD_3OD) δ 206.8, 177.4, 169.7, 67.5, 50.4, 43.9, 41.0, 30.5, 30.4, 30.3, 30.2, 29.7, 28.7, 27.6, 24.6; IR (ATR): 3263, 3073, 2930, 2858, 1772, 1674, 1548, 1201, 1135, 1023, 837, 801, 722 cm^{-1}; ESI-MS m/z expected: 313.41; observed: 313.0 [$M+H^+$] (free amine).

Preparation and quantification of lactone-derivatized Affigel resin (7).

Modified resin preparation. A 1-mL slurry (50% *i*-PrOH, 50% bead; 7.5 μmol) of *N*-OSuc Affigel-10 resin was transferred to a 2-mL plastic column with 20 μ PE frit (Pierce® Centrifuge Columns/ Thermo Scientific). The solvent was drained away from the resin, and the resin was washed with DMSO (1 mL, 3x) and drained. A 10 mM solution of lactone **6** was prepared in DMSO, and a 375-μL aliquot of this solution (3.75 μmol) plus 125 μL DMSO and 50 μL DIEA were added to a 2-mL Eppendorf tube. The mixture was vortexed for 10 s, and 20 μL of this solution was collected as the "before reaction" sample of **6** for later LC-MS quantification. The solution of **6** was added to the resin in the plastic column, and the resulting slurry was mixed by repetitive pipetting. The slurry was returned to the Eppendorf tube, and the mixture was allowed to shake at rt on a LabQuake® orbital shaker for 2 h. The tube was then centrifuged, and the supernatant was collected as the "after reaction" sample of **6** for LC-MS quantification.

The slurry was transferred to a plastic column, the remaining liquid was drained out, and the resin was washed with DMSO (1 mL, 3x) and drained. To "cap" the unreacted *N*-OSuc groups on the resin, a 50 mM solution of ethanolamine was prepared in DMSO, and a 1-mL aliquot of this solution and 15 μL of DIEA were added to the resin. This slurry was poured into an Eppendorf tube, and the mixture was shaken for 2 h at rt on a LabQuake®. Thereafter, the liquid was drained, and the resin was washed with DMSO (1 mL, 2x), water (1 mL, 2x) and 0.2% NaN_3 (1 mL, 2x). The lactone-derivatized resin (**7**) was stored as a suspension in 0.2% NaN_3 at 4 °C until used.

The ethanolamine "capped" resin control was prepared in analogous fashion to resin **7**, expect it was not reacted with lactone **6**.

LC-MS coupling quantification. The "before" and "after" reaction samples of lactone **6** were analyzed LC-MS, and the peak corresponding to **6** was monitored using integration (Figure S-1). This analysis revealed that the derivatization procedure outlined above permitted quantitative coupling of lactone **6** onto Affigel-10 resin.

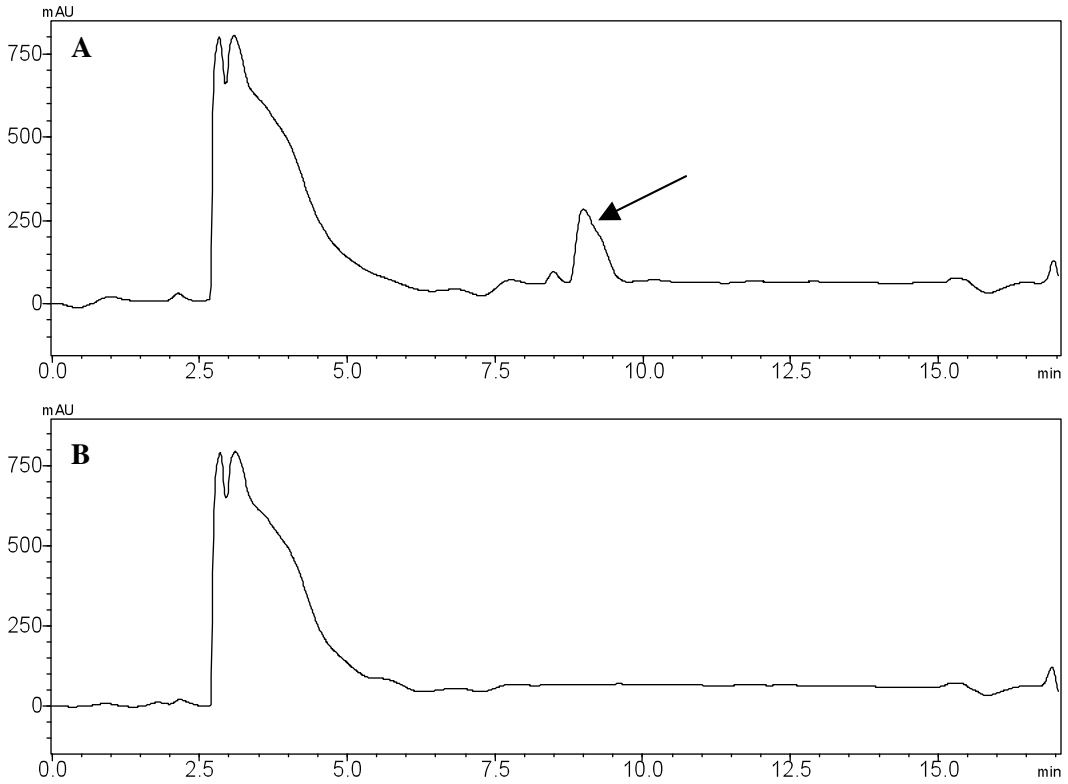

Figure S-1. LC-MS coupling quantification; (A) "Before reaction" sample of **6**; (B) "After reaction" sample of **6**. The peak indicated by the arrow corresponds to lactone **6** ([M+1] = 313.0).

QscR production and purification.

Strain information. *P. aeruginosa* PAO-T7 ($pJLQ_{his}$)[2] was grown in Luria–Bertani (LB) medium (pH 7.0) with 200 μg/mL carbenicillin at 37 °C with vigorous shaking. PAO-T7 has a chromosomal insertion containing a T7 *pol* gene, transcribed from P_{lacUV5}, and the *lacI* gene encoding Lac repressor for regulated expression of T7 *pol*. The plasmid $pJLQ_{his}$ is a T7 promoter-based expression vector that over expresses His-tagged QscR and confers resistance to carbenicillin. Growth was monitored as optical density at 600 nm (OD_{600}).

Expression and purification of QscR. *P. aeruginosa* PAO-T7($pJLQ_{his}$) was grown until OD_{600} = 0.5. The temperature was lowered to 25 °C, and shaking continued until OD_{600} = 0.6. IPTG was added so that the final concentration was 500 μM, and shaking continued for 18 h at 25 °C. Cells were harvested by centrifugation at 5,000 rpm for 15 min at 4 °C. The pellet was frozen in liquid N_2 for 10 min, and stored at -80 °C.

Harvested cells were resuspended in buffer A (50 mM K_2HPO_4, 500 mM KCl, 20 mM imidazole, 5% glycerol, pH 7.9) and allowed to thaw on ice. Cells were lysed using either a French press or Fisher Sonic Dismembrator, and the resulting lysate was clarified by centrifugation. The clarified lysate was injected onto a HisTrap FF 5 mL column that had been equilibrated with buffer A.

The column was washed with buffer A to remove any unbound sample and then the bound protein was eluted off using a linear gradient from 20 mM to 500 mM imidazole. Fractions containing QscR (as determined by SDS-PAGE; see below) were pooled, and glycerol was added such that the final concentration of glycerol was 15-20%. Samples were frozen in liquid N_2 and stored at -80 °C. Prior to incubation with resin, the sample was thawed on ice and the amount of QscR was quantified by UV-Vis using a Bradford assay (BioRad) according to the manufacturer's protocol.

For the experiments with QscR cell lysate and resin **7**, QscR was expressed as described above and harvested PAO-T7 ($pJLQ_{his}$) cells were lysed in buffer A that did not contain imidazole (50 mM K_2HPO_4, 500 mM KCl, 5% glycerol, pH 7.9). The resulting lysate was clarified by centrifugation as described above and incubated with resin **7**.

Resin 7/QscR incubation protocol.

A 1-mL slurry of resin **7** was drained of liquid in a plastic column. The resin was washed 3x with buffer (50 mM K_2HPO_4, 500 mM KCl, 5% glycerol, pH 7.9), and 1.0 mL of protein sample (purified QscR or PAO-T7 ($pJLQ_{his}$) lysate) was added. The resin/protein sample was transferred to a microcentrifuge tube and shaken on a LabQuake® at 4 °C for 2–24 h.[4] After incubation, the resin/protein sample was transferred to a new plastic column, and the supernatant was collected for subsequent analysis by SDS-PAGE. The resin was washed 4x with buffer to remove any residual protein, and each washing was collected for analysis. The resin was then resuspended in 0.5 mL of buffer, transferred to a new microcentrifuge tube and prepared for analysis by SDS-PAGE as described below. All isolated protein samples/washings were stored on ice after collection.

SDS-PAGE experimental protocol.

Gel cassette preparation. Discontinuous 12% SDS-PAGE gel cassettes were cast using a Mini-PROTEAN 3 gel casting stand (BioRad). The resolving layer consisted of 12% acrylamide, 375 mM Tris (pH 8.8), 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.1% (w/v) ammonium persulfate (APS), and 5.33 mM *N,N,N',N'*-tetramethylethylenediamine (TEMED). The resolving layer was covered with deionized H_2O and allowed to polymerize for 30 min. The water was then poured out and the stacking layer was added (5% acrylamide, 125 mM Tris (pH 6.8), 0.1% SDS, 0.1% APS, 6.66 mM TEMED). Combs were added to the cassettes, and the stacking layer was allowed to polymerize for 30 min before storage in running buffer.

Sample preparation. Protein samples (controls, supernatants, and washings) were diluted as necessary so that the total protein concentration was 1.25–2.50 mg/mL. Aliquots (20 μL per sample) were mixed with 5 μL of 4X Laemmli gel loading buffer (125 mM Tris, 8% (w/v) SDS, 40% glycerol, 2.85 M β-mercaptoethanol, 0.2% (w/v) bromophenol blue), and heat-denatured at 95 °C on a heating block for 5 min before being loaded onto a gel.

Resin samples (after washing) were resuspended in 500 μL of buffer A and 125 μL of 4X Laemmli gel loading buffer was added. The resin samples were then heated for 5 min at 95 °C, centrifuged for 3 min at 1,000 rpm, and 15 μL of the resulting supernatant were loaded onto a gel.

Gel analysis. Aliquots (15 μL) of prepared protein and resin samples were loaded into individual wells of a gel, and electrophoresis was performed at 200 V constant using a BioRad Mini-PROTEAN 3 electrophoresis module and Power-Pac power supply. The running buffer contained 25 mM Tris base, 192 mM glycine, and 0.1% SDS. After the dye front reached the bottom centimeter of the gel, the cassette was taken apart, and the gel was stained in 100 mL coomassie stain (0.1% coomassie blue, 40% ethanol, 10% acetic acid) for 1 h on a rotating shaker at rt. The coomassie stain was decanted, and the gel rinsed with deionized H_2O and destained using 100 mL of destain solution (7.5% acetic acid, 10% ethanol) with shaking at rt for 1 h or overnight.

Gels were imaged by sandwiching the gel between two sheets of transparency film and scanning in 600 dpi color on a CanoScan LiDE 30/N124OU flatbed scanner. The scanned image was cropped and desaturated using the GNU Image Manipulation Protocol (the GIMP, http://www.gimp.org).

Protein digestion protocol for mass analyses.

The procedure for in-gel protein digestion was adapted from Saveliev *et al*.[3]

Gel fragment preparation. Protein bands were excised into 1-mm pieces each and placed into microcentrifuge tubes (LoBind, Eppendorf). The gel pieces were washed with >10 volumes of Millipore water (~200 μL) for 30 s to eliminate acetic acid residue.

Destaining. Gel pieces were destained with 200 μL of 100 mM $(NH_4)HCO_3$ in 50% aq. methanol (10 min, 2x), and the supernatants were discarded. The gel pieces were dehydrated with 200 μL of 25 mM $(NH_4)HCO_3$ in 50% aq. acetonitrile for 5 min, and then once more in 100% acetonitrile for 30 s. The solutions were removed, and the gel pieces were dried for 2 min in a vacuum centrifuge at rt.

Reduction and Alkylation. Gel pieces were placed in 50 μL of freshly prepared 25 mM dithiothreitol (DTT) in 25 mM $(NH_4)HCO_3$ and the proteins were reduced for 30 min at rt. Any residual liquid was pipetted off, and 50 μL of freshly prepared 55 mM iodoacetamide (IAA) was added (450 μL of 25 mM $(NH_4)HCO_3$ in 550 mM IAA stock solution). Protein alkylation was allowed to proceed for 20 min at rt in the dark.

The liquid was pipetted off, and gel pieces were washed with >20 volumes of Millipore water (~400 μL) for 30 s to remove any residual IAA. The gel pieces were then dehydrated for 5 min with 200 μL of 25 mM $(NH_4)HCO_3$ in 50% aq. acetonitrile, and once more for 30 s in 100%

acetonitrile. The solutions were removed, and the gel pieces were dried for 2 min in a vacuum centrifuge at rt.

Trypsin Digestion. Gel pieces were rehydrated for 5 min at rt in 30 μL of the digestion solution (4 μg/mL trypsin and 0.01% ProteaseMax (Promega) in 25 mM $(NH_4)HCO_3$). The rehydrated gel pieces were overlaid with a minimal amount of 25 mM $(NH_4)HCO_3$ to keep them immersed throughout the digestion. Gel pieces were incubated at 37 °C for 2 h.

Peptide Recovery. Microcentrifuge tubes containing gel pieces were flash-spun, and the supernatant solutions were transferred into new microcentrifuge tubes. The trypsin was inactivated by adding 3 μL of 2.5% TFA. The peptide samples were purified with a C18 ZipTip prior to mass analysis.

Mass Analyses of Peptides Samples. μLC-MS/MS analysis of sample peptides following in-gel trypsin digest was carried out on a UPLC system. The MS and MS/MS data were searched against *P. aeruginosa* databases with TurboSequest (ThermoFisher) (see Table S-1 below). The analysis confirmed that the protein band observed in samples containing resin **7** contained QscR as noted in the first entry of Table S-1. A coverage of 52.74 indicates that of the 57 peptide fragments detected, 52.74% of the sequence of QscR was identified. A few nonspecific proteins were also identified, and while this analysis is not quantitative, the low coverages and low number of peptide fragments found for these additional proteins indicates that they were minor contributors to the observed protein band.

Table S-1. MS analysis of gel band extracted from experiment using PAO-T7(pJLQ$_{his}$) lysate. The first entry in the table confirms that QscR (quorum-sensing control repressor) is present. AA = amino acid.

Accession #	Coverage	# Peptides	#AAs	MW [Da]	Score	Description
15597095	52.74	57	237	27237	268.25	quorum-sensing control repressor [Pseudomonas_aeruginosa_PAO1] - [ref|NP_250589.1|]
15599469	14.72	7	231	24219	28.64	50S ribosomal protein L1 [Pseudomonas_aeruginosa_PAO1] - [ref|NP_252963.1|]
15596786	10.85	3	295	30247	18.82	succinyl-CoA synthetase alpha chain [Pseudomonas_aeruginosa_PAO1] - [ref|NP_250280.1|]
15598148	12.85	3	249	26360	13.75	electron transfer flavoprotein beta-subunit [Pseudomonas_aeruginosa_PAO1] - [ref|NP_251642.1|]
15597091	11.74	2	230	26163	3.76	hypothetical protein [Pseudomonas_aeruginosa_PAO1] - [ref|NP_250585.1|]

References and notes.

1 N. Amara, R. Mashiach, D. Amar, P. Krief, S. A. H. Spieser, M. J. Bottomley, A. Aharoni and M. M. Meijler, *J. Am. Chem. Soc.*, 2009, **131**, 10610-10619.

2 J. H. Lee, Y. Lequette and E. P. Greenberg, *Mol. Microbiol.*, 2006, **59**, 602-609.

3 S. Saveliev, D. Simpson, W. Daily, C. Woodroofe, D. Klaubert, G. Sabat, R. Bulliet and K. V. Wood, *Promega Notes*, 2008, **99**, 3-7.

4 Later experiments revealed that 2 h incubations were sufficient to give maximal protein binding.