Supplementary data

General Techniques.

$^1$H NMR spectra were recorded on a Bruker-AC300 (300 MHz) or Bruker Digital DMX-500 (500 MHz) in the indicated solvent. Chemical shifts were reported in part per million (ppm) relative to tetramethylsilane (0.0 ppm), CDCl$_3$ (7.26 ppm), DMSO-d$_6$ (2.5 ppm) or dioxane (3.76 ppm) as an internal standard. NMR multiplicities are reported using the following abbreviations: s: singlet; d: doublet, t: triplet; m: multiplet. J values are given in Hz. $^{13}$C NMR spectra were recorded on a Bruker-AC300 (75 MHz). Chemical shifts were given in part per million (ppm) relative to CDCl$_3$ (77.0 ppm) or DMSO-d$_6$ (39.5 ppm) as an internal standard.

Infrared spectra were recorded on a Perkin-Elmer 681. UV-Vis spectra were recorded on a Varian CARY 50. Mass spectra were recorded on a ThermoFinnigan MAT900S spectrometer for ESI spectra and a VG 7070 spectrometer for FAB spectra. High resolution mass spectrum (ESI-TOF) were recorded in a Bruker BIOTOF II spectrometer by the Mass Spectrometry Service of the University of Santiago de Compostela.

Analytical thin layer chromatography was performed on Merck Silica Gel 60 F254 plates and visualized with UV light and developed by exposition to Cl$_2$ (gas) previous to soaking into a solution of 4,4’-(methylenebis(N,N-dimethylaniline)).

Melting points were determined in a variable temperature optical microscope and are uncorrected.

Optical rotation was measured using a 1 mL cell with a 1 dm length on a Perkin Elmer 241 MC polarimeter

Anhydrous DMSO was obtained by stirring overnight DMSO over BaO under dry N$_2$ and distillation prior to use. All the other solvents were purified according to standard procedures described in the literature. Commercial reagents were used without further purification. N-(2-methyl-1-propenyl)morpholine (3) was prepared by condensation of morpholine and isobutyraldehyde, as described by Benzing.$^1$ Methyl 5-(chloroformyl)pentanoate was prepared as described by Morgan and Walton.$^2$

Methyl, 7,7-dimethyl-6,8-dioxooctanoate (2)

N-(2-methyl-1-propenyl)morpholine$^1$ (13.21 g, 93.5 mmol) was added dropwise to methyl 5-(chloroformyl)pentanoate (1)$^2$ (16 g, 89.6 mmol) at room temperature, under nitrogen atmosphere and with stirring. When the addition was complete, the mixture was warmed to 45°C and allowed to react at this temperature for two hours. To the resulting reaction mixture, saturated aqueous NaHCO$_3$ solution was added, and the aqueous phase was repeatedly extracted with CH$_2$Cl$_2$. The combined CH$_2$Cl$_2$ extracts were washed with water, dried over anhydrous
Methyl 5-(9,9-dimethyl-3,7-dioxo-2,4,6,8-tetraaza[3.3.1]bicyclonon-1-yl)pentanoate (3)
A mixture of methyl, 7,7-dimethyl-6,8-dioxooctanoate (2) (2.14 g, 10 mmol), urea (1.80 g, 30 mmol), toluene (40 mL) and trifluoroacetic acid (0.4 mL) was refluxed in an inert atmosphere for 10 h with azotropic removal of water. After cooling, the suspension was filtered, washed with cold water and reccrystallized from boiling water. Recrystallization of the desired product (2.56 g, 86%) as a white solid. mp > 300°C. IR (KBr): νmax/cm−1 3530-2800, 1715, 1696 and 1646. 1H NMR (300 MHz, DMSO-d6): δ 1.06 (s, 6H), 1.4-1.5 (m, 6H), 2.20 (m, 2H), 3.79 (t, 1H, J = 4.5 Hz), 6.54 (s, 2H, NH), and 7.00 (broad d, 2H, NH). 13C NMR (100 MHz, DMSO-d6): δ 174.5, 154.5, 69.6, 65.8, 33.8, 33.6, 33.2, 25.2, 21.5 and 21.2. FAB-MS (3-nitrobenzyl alcohol as matrix): m/z 307 (M+Na), 285 (M+H). Elemental analysis: Calcd.: C 52.31, H 7.46, N 18.34; Found: C 52.34, H 7.43, N 18.41.

5-(9,9-dimethyl-3,7-dioxo-2,4,6,8-tetraaza[3.3.1]bicyclonon-1-yl)pentanoic acid (4)
700 mg (2.35 mmol) of compound 3 in 50 mL of 2M NaOH were heated to reflux for 3h. After cooling and acidification to pH = 1, the white precipitate was filtered, washed with cold water and reccrystallized from boiling water, affording 610 mg (2.13 mmol, 91%) of the desired compound. mp = 310°C. IR (KBr): νmax/cm−1 3300, 2954, 2874, 2721, 1737 and 1692. 1H NMR (300 MHz, DMSO-d6): δ 1.06 (s, 6H), 1.3-1.6 (m, 6H), 2.30 (t, 2H, J = 6.8 Hz), 3.59 (s, 3H), 3.78 (t, 1H, J = 4.5 Hz), 6.54 (s, 2H, NH) and 6.99 (broad d, 2H, NH). 13C NMR (75 MHz, DMSO-d6): δ 173.4, 154.5, 69.6, 65.7, 33.6, 33.3, 33.1, 25.1, 21.3 and 21.1. MS (EI): m/z (rel. intensity) 298 (10, M+), 267 (3), 238 (15) and 223 (100). Elemental analysis: Calcd.: C 35.8, 33.6, 33.3, 25.2, 21.5 and 21.2. FAB-MS (3-nitrobenzyl alcohol as matrix): m/z 307 (M+Na), 285 (M+H).
**Compound 8**

To a solution of 175 mg (0.173 mmol) of compound 7 in 20 mL of MeOH, 10 mg of 10% Pd/C were added and the mixture was kept under H₂ atmosphere at room temperature for 11 h. Filtration of the catalyst over Celite and evaporation of the solvent afforded 145 mg (0.166 mmol, 98%) of the desired product as a white solid. mp = 215-216 ºC; [α]D +7.2 (c 0.16, MeOH); Rᶠ = 0.05 (BuOH/ AcOH/ H₂O, 4/1/1). IR (KBr): νₓ max/cm⁻¹ 3275, 2933, 1653 and 1522. ¹H NMR (500 MHz, H₂O/D₂O, 9/1, PBS pH = 6.0): δ 8.1-7.9 (4H NH-CH₂), 7.31 (s, 4H, NH-CO-NH-CH), 6.83 (s, 4H, NH-CO-NH-CH), 4.26 (2H, NH-C₆H₄), 3.19-3.16 (m, 8H, CH₃-NH), 2.35 and 2.12 (m, 4H, CO-C₆H₄-CH₂-CH₂-CO), 2.29 (t, 4H, CH₂-CO), 1.79 (m, 4H, CH₂), 1.64 (m, 4H, CH₂), 1.55-1.45 (m, 12H, CH₃-CH₂-NH and CH₂), 1.32 (m, 8H, CH₂-CH₂-NH), 1.19 (s, 12H, CH₂-). ¹³C NMR (75.4 MHz, DMSO-d₆): δ 171.87, 171.85, 171.83, 154.5, 69.6, 66.3, 65.7, 38.4-38.3, 35.2, 33.8, 29.1-29.0, 26.1-25.9, 21.6, 21.1. HRMS calcd for C₄₁H₇₄N₁₃O₈ (MH⁺) 876.5783, found 876.5780.

**Determination of the binding constant by spectrophotometric competitive titration.**

**a. Binding model for the monovalent complexes.**

Assuming that the four identical binding sites of both Av and SAv behave independently, the competitive titration could be represented as shown in scheme 1.

![Scheme 1](image1.png)

Expressions for [S], [I], [L], [SI] and [SL], derived from the 1:1 binding model¹, were used in the least-squares fitting of the calculated absorbance (Aᵦ calc) (eq. 1) to the experimental absorbance (Aᵦ exp).

\[
A_{calc} = ε_{I(500)}[I] + ε_{SI(500)}[SI]
\]

(1)

This fitting procedure led to the optimal value for the binding constant between S and L. The reasonably good fit between the experimental and calculated curves supports our assumption of independent behaviour between binding sites.

**b. Binding model for the divalent complexes.**

Assuming that half the protein (two proximal binding sites) behaves independently of the other half, the competitive titration could be represented as shown in scheme 2.

![Scheme 2](image2.png)

Apart from S=L, the complexes S-L, SL₂ and S₂L (scheme 3) were also considered in the fitting procedure.
Consideration of these equilibria led to the use of expressions (2)-(6) in the least-squares fitting of the calculated absorbance \( A_{\text{calc}} \) (eq. 7) to the experimental absorbance \( A_{\text{exp}} \).

\[
[S] = \frac{S_I}{1 + K_{\text{SL}} [I] + K_{S-L} [L] + K_{\text{SL}_2} [I]^2 + K_{S-L} [L] \left[ 1 + K_{L-S} [L] + 2K_{S,L} [S] \right]} \tag{2}
\]

\[
[I] = \frac{I_I}{1 + K_{\text{SL}} [S] \left[ 1 + 2K_{\text{SL}_2} [I] \right]} \tag{3}
\]

\[
[L] = \frac{L_I}{1 + K_{S-L} [S] + K_{S-L} [S] \left[ 1 + 2K_{\text{SL}_2} [L] + K_{S,L} [S] \right]} \tag{4}
\]

\[
[SI] = K_{\text{SL}} [S] [I] \tag{5}
\]

\[
[SI_2] = K_{\text{SL}_2} [I] [SI] \tag{6}
\]

\[
A_{\text{calc}} = \varepsilon_{[I,500]} [I] + \varepsilon_{\text{SI}[500]} ([SI] + 2[SI_2]) \tag{7}
\]
S_t, I_t and L_t are the total concentrations of S, I and L respectively. This fitting procedure led to the optimal value for the binding constant of the divalent complex (K_{S=L}). Incorporation of the complexes S-L, SL_2 and S_2L into the fitting procedure resulted to have only little influence on the value obtained for K_{S=L}.

c. General experimental procedure.
Spectrophotometric competitive titrations were performed on a 1.5mL cell of 1cm pathlength. Aliquots of a 0.1-2.0 mM solution of the ligand in phosphate buffer (pH=7.3) were added to a 3-30 μM solution of the protein and 70-100 μM of HABA in phosphate buffer (pH=7.3). UV-Vis absorption spectra were recorded five minutes after each addition from 650 to 300 nm, and the changes in absorbance at 500nm and 348nm (due to the displacement of the protein-bound dye by the ligand) were fitted to the corresponding 1:1 binding model assuming independent binding.

![Graph](image1.png)

*Figure 1. Absorption change at 500 nm in the titration of Av 3.0 μM (tetramer) and HABA 81 μM with ligand 4. Phosphate buffer 0.1 M, pH = 7.3. The solid line represents the fit of the data to the (1:1) binding model.*

![Graph](image2.png)

*Figure 2. Absorption change at 500 nm in the titration of SAv 6.5 μM (tetramer) and HABA 76 μM with ligand 4. Phosphate buffer 0.1 M, pH = 7.3.*
Calorimetric titrations were carried out using a Microcal VP-ITC instrument with a cell volume of 1.4115 mL. The titrations were performed by adding 10 µL aliquots of a 0.2-0.8 mM aqueous solution of the ligand (pH 7.3) to a 5-33 µM solution of the protein (pH 7.3) in the calorimetric cell, and monitoring the heat change after each addition. All isothermal titration calorimetry experiments were conducted in 100 mM KCl in the absence of buffer to avoid heat effects due to ionization of buffer components. The pH of the solutions was adjusted adding small amounts of 0.1 M KOH and 0.1 M HCl.

References
1 E. Benzing, Angew. Chem. 1959, 71, 521.