Supplementary Material for Organic & Biomolecular Chemistry

Divalent ligand for intramolecular complex formation to streptavidin

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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-d6 (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-d6 (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_2Cl_2. The combined CH_2Cl_2 extracts were washed with water, dried over anhydrous

Na_2SO_4 and the solvent was evaporated. Distillation of the residue under reduced pressure (150°C at 0.2 torr) afforded the desired product (17.23 g, 90%). IR (film): ν_{max}/cm^{-1} 2954, 2874, 2721, 1737 and 1703. ^1H NMR (300 MHz, $CDCl_3$): δ 1.33 (s, 6H), 1.5-1.6 (m, 4H), 2.33 (m, 2H), 2.49 (m, 2H), 3.67 (s, 3H), 9.61 (s, 1H). MS (EI): m/z (rel. intensity) 214 (12, $M^{+\cdot}$), 196 (46), 186 (26), 183 (36), 143 (100), 115 (46).

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 azeotropic removal of water. After cooling, the suspension was filtered and the precipitate was washed with toluene and ethanol. Recrystallized from boiling water afforded the desired product (2.56 g, 86%) as a white solid. mp > 300°C. IR (KBr): ν_{max}/cm^{-1} 3248, 3082, 1737 and 1692. ^1H NMR (300 MHz, DMSO-d_6): 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), 6.52 (s, 2H, N*H*) and 6.99 (broad d, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 173.4, 154.5, 69.6, 65.7, 51.2, 33.6, 33.3, 33.1, 25.1, 21.3 and 21.1. MS (EI): m/z (rel. intensity) 298 (10, $M^{+\cdot}$), 267 (3), 238 (15) and 223 (100). 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 recrystallized from boiling water, affording 610 mg (2.13 mmol, 91%) of the desired compound. mp = 310°C. IR (KBr): ν_{max}/cm^{-1} 3530-2800, 1715, 1696 and 1646. ^1H NMR (300 MHz, DMSO-d_6): 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, N*H*) and 7.00 (broad d, 2H, N*H*). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.5, 154.5, 69.6, 65.8, 33.8, 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). Elemental analysis: Calcd. for $C_{12}H_{20}N_4O_4 \cdot H_2O$: C 47.67, H 7.33, N 18.53; Found: C 47.69, H 7.27, N 18.48.

***N*',*N*''-bis(6-(*tert*-butoxycarbonyl)aminohexyl)-N^{α}-benzyloxycarbonyl-L-glutamide (6)**
A solution of the bis(4-nitrophenyl)ester of *N*-benzyloxycarbonyl-L-glutamic acid (**5**) (2.314g, 4.42 mmol) in 50 mL of AcOEt was slowly added over a stirred solution of *N*-*tert*-butoxycarbonyl-1,6-hexanediamine (2.366 g, 9.36 mmol) and Et_3N (2 ml) in 50 mL of AcOEt. The mixture was allowed to react at room temperature for 48h, and then it was extracted with water (5x200 mL) and with 0.1 M HCl (2x200 mL). Subsequently the organic phase was concentrated to one fourth of the initial volume and left at 4ºC overnight. Filtration of the precipitate and washing with cold AcOEt afforded 1.648g (2.48 mmol, 56%) of the desired compound. mp = 114-115°C. R_f = 0.52 ($CHCl_3$/MeOH, 9/1). IR (KBr): ν_{max}/cm^{-1} 3307, 1689, 1649 and 1535. ^1H NMR (300 MHz., $CDCl_3$): δ 7.34 (5H, Ar-*H*), 6.88 (broad, 1H, N*H*), 6.30 (broad, 1H, N*H*), 6.23 (broad, 1H, N*H*), 5.10 (s, 2H, Ph-CH_2), 4.62 (broad, 2H, N*H*), 4.19 (m, 1H, NH-C$_\alpha$*H*-CO), 3.22 (m, 4H, CO-NH-CH_2), 3.09 (m, 4H, CH_2-NH-CO), 2.4-1.9 (m, 4H, -C$_\alpha$H-CH_2-CH_2-CO-), 1.5-1.4 (m, 26H, NH-CH$_2$-CH_2 and C(CH_3)$_3$), 1.31(m, 8H, NH-CH$_2$-CH$_2$-CH_2-). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 172.8, 171.2, 156.4, 156.1, 156.0, 136.3, 128.5, 128.1, 128.0, 78.9, 66.8, 54.2, 40.2, 40.1, 39.3, 39.2, 32.6, 29.9, 29.8, 29.4, 29.2, 28.4, 26.2, 26.1 and 26.0. FAB-MS (3-nitrobenzyl alcohol as matrix) m/z 678 $[M+H]^+$. Elemental analysis: Calcd. C 62.64, H 8.81, N 10.32; Found: C 62.51, H 8.77, N 10.33.

Compound 7
0.587g of compound **6** (0.868 mmol) was allowed to react with 20 mL of TFA at room temperature for 1 h, and then it was evaporated to dryness. The resulting product was dried under vacuum and then it was dissolved in 50 mL of anhydrous DMSO and added to a stirred solution of compound **4** (0.536g, 1.887 mmol), diphenylphosphoryl azide (0.8 ml, 3.70 mmol) and Et_3N (0.8 mL) in 50 mL of anhydrous DMSO. The mixture was allowed to react for 24 h at room temperature, and then 50 mL of water were added and the mixture was evaporated to dryness. The solid obtained was dissolved in 50 mL of MeOH and was precipitated with 300 mL of Et_2O affording a pale yellow precipitate which was subsequently purified by counter current extraction using BuOH/ AcOH/ H_2O (4/1/1) yielding 387 mg (0.383 mmol, 44%) of the desired product as a white solid. mp= 195-196 ºC. R_f = 0.25 (BuOH/ AcOH/ H_2O, 4/1/1). IR (KBr): ν_{max}/cm^{-1} 3530, 3262, 3088, 2935, 1703, 1656 and 1531. ^1H NMR (400 MHz., DMSO-d_6): δ 7.8-7.7 (4H, N*H*-CH$_2$-), 7.40-7.25 (6H, Ar-*H* + N*H*-CO-O), 7.04 (4H, NH-CO-N*H*-CH-), 6.57 (s, 4H, N*H*-CO-NH-CH-), 5.01 (s, 2H, Ph-CH_2), 3.90 (m, 1H, -NH-C$_\alpha$*H*-CO), 3.79 (t, 2H, J= 4.4 Hz, NH-C*H*-NH), 3.00 (m, 8H, CH_2-NH), 2.1-2.0 (m, 6H, CH_2-CO), 1.83 (m, 1H, NH-C$_\alpha$H-C$_\beta$*H*H-CH$_2$), 1.70 (m 1H, NH-C$_\alpha$H-C$_\beta$H*H*-CH$_2$), 1.55-1.15 (m,

28H, -C*H*$_2$-), 1.05 (s, 12H, C*H*$_3$-). ^{13}C NMR (75.4 MHz, DMSO-d$_6$): δ 171.9, 171.3, 155.8, 154.6, 137.0, 128.3, 127.8, 127.7, 69.6, 65.6, 65.4, 54.5, 38.4, 38.3, 35.2, 33.8, 33.3, 31.9, 29.2, 29.1, 29.0, 28.1, 26.1, 26.0, 21.6, 21.2.

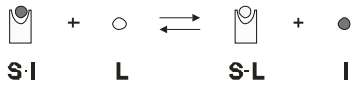
Compound 8

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 H_2 atmosphere at room temperature for 11 h. Filtration of the catalyst over Celite and evaporation of the solvent afforded 145mg (0.166 mmol, 98%) of the desired product as a withe solid. mp= 215- 216 ºC; $[\alpha]^{22}_D$ +7.2 (c 0.16, MeOH); R_f = 0.05 (BuOH/ AcOH/ H_2O, 4/1/1). IR (KBr): ν_{max}/cm^{-1} 3275, 2933, 1653 and 1522. ^{1}H NMR (500 MHz, H_2O/D_2O, 9/1, PBS pH = 6.0): δ 8.1-7.9 (4H N*H*-CH$_2$), 7.31 (s, 4H, NH-CO-N*H*-CH), 6.83 (s, 4H, N*H*-CO-NH-CH-), 4.26 (2H, NH-C*H*-NH), 3.96 (m, 1H, CO-C$_\alpha$*H*), 3.19-3.16 (m. 8H, C*H*$_2$-NH), 2.35 and 2.12 (m, 4H, CO-C$_\alpha$H-C*H*$_2$-C*H*$_2$-CO), 2.29 (t, 4H, C*H*$_2$-CO), 1.79 (m, 4H, C*H*$_2$), 1.64 (m, 4H, C*H*$_2$), 1.55-1.45 (m, 12H, C*H*$_2$-CH$_2$-NH and C*H*$_2$), 1.32 (m, 8H, C*H*$_2$-CH$_2$-CH$_2$-NH), 1.19 (s, 12H, C*H*$_3$-). ^{13}C NMR (75.4 MHz, DMSO-d$_6$): δ 171.87, 171.85, 171.83, 154.5, 69.6, 66.3, 65.7, 38.4-38.3, 35.2, 33.8, 33.3, 29.1-29.0, 26.1-25.9, 21.6, 21.1. HRMS calcd for $C_{41}H_{74}N_{13}O_8$ (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.



S: Protein subunit
I: Indicator (HABA)
L: Monovalent ligand (**4**)

Scheme 1

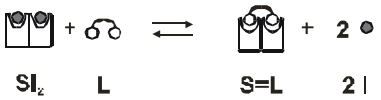
Expressions for [S], [I], [L], [SI] and [SL], derived from the 1:1 binding model[3], were used in the least-squares fitting of the calculated absorbance (A_{calc}) (eq. 1) to the experimental absorbance (A_{exp}).

$$A_{calc} = \varepsilon_{I(500)}[I] + \varepsilon_{SI(500)}[SI] \quad (1)$$

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.

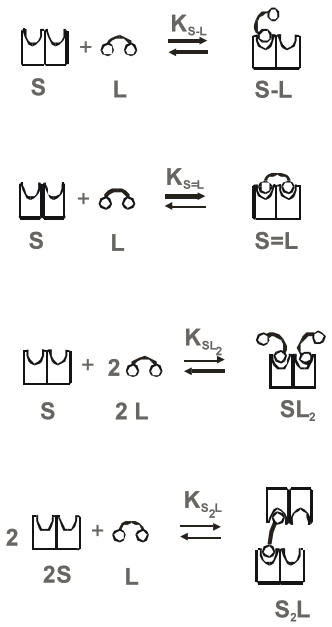
Assuming that half the protein (two proximal binding sites) behaves independently of the other half, the competitive titration could be represented as shown in scheme 2.



S: Protein bis-subunit
I: Indicator (HABA)
L: Divalent ligand (**8**)

Scheme 2

Apart from S=L, the complexes S-L, SL_2 and S_2L (scheme 3) were also considered in the fitting procedure.



Scheme 3

Consideration of these equilibria led to the use of expressions (2)-(6) in the least-squares fitting of the calculated absorbance (A_{calc}) (eq. 7) to the experimental absorbance (A_{exp}).

$$[S] = \frac{S_t}{1 + K_{SI}[I] + K_{S=L}[L] + K_{SI}K_{SI_2}[I]^2 + K_{S-L}[L]\left(1 + K_{L_2S}[L] + 2K_{S_2L}[S]\right)} \tag{2}$$

$$[I] = \frac{I_t}{1 + K_{SI}[S]\left(1 + 2K_{SI_2}[I]\right)} \tag{3}$$

$$[L] = \frac{L_t}{1 + K_{S=L}[S] + K_{S-L}[S]\left(1 + 2K_{SL_2}[L] + K_{S_2L}[S]\right)} \tag{4}$$

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

$$[SI_2] = K_{SI_2}[I][SI] \tag{6}$$

$$A_{calc} = \varepsilon_{I(500)}[I] + \varepsilon_{SI(500)}\left([SI] + 2[SI_2]\right) \tag{7}$$

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.

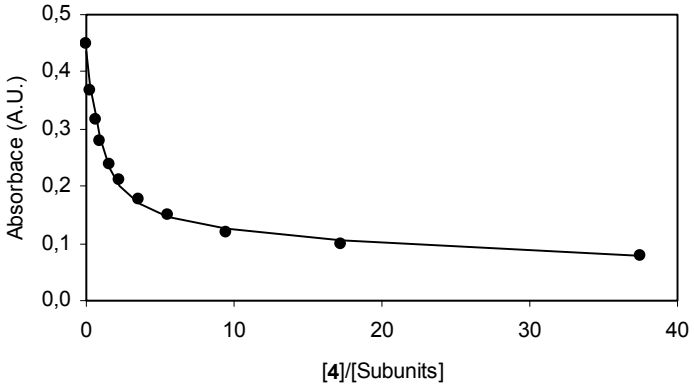


Figure 1. Absorption change at 500 nm in the titration of Av 3.0 μM (tetramer) and HABA 81 μM with ligand **4**. Phosphate buffer 0.1 M, pH = 7.3. The solid line represents the fit of the data to the (1:1) binding model.

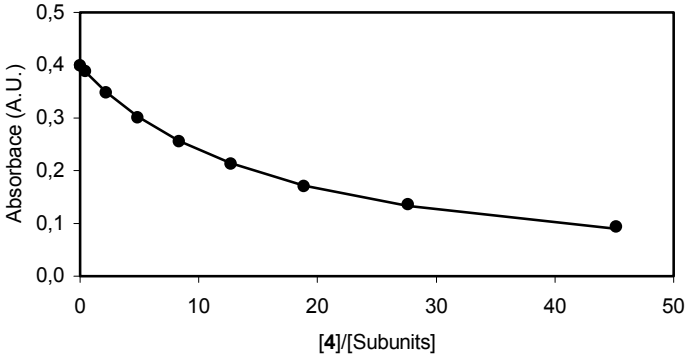


Figure 2. Absorption change at 500 nm in the titration of SAv 6.5 μM (tetramer) and HABA 76 μ M with ligand **4**. Phosphate buffer 0.1 M, pH = 7.3.

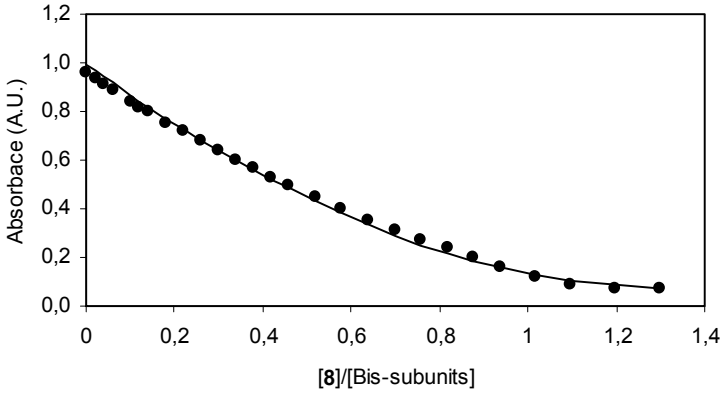


Figure 3. Absorption change at 500 nm in the titration of SAv 26 μM (tetramer) and HABA 76 μ M with ligand **8**. Phosphate buffer 0.1 M, pH = 7.3.

Calorimetric titrations

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[4]. The pH of the solutions was adjusted adding small amounts of 0.1 M KOH and 0.1 M HCl.

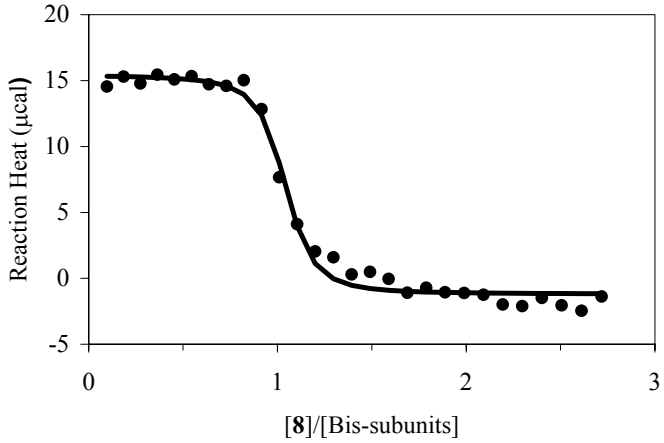


Figure 4. Isothermal titration calorimetry of a solution of Av 9.73 μM (tetramer) in 0.10 M KCl (adjusted to pH 7.3) with a solution 0.24 mM of **8** in 0.10 M KCl (adjusted to pH 7.3). The solid line represents the fit of the data to the (1:1) binding model.

References

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