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Electronic Supplementary Information

Lectin Recognition of a New SOD Mimic Bioconjugate Studied with Surface Plasmon Resonance Imaging.

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General

Reagents were obtained from commercial suppliers and used without further purification. Dithiobis(succinimidyl propionate) (DTSP) and *Ricinus communis agglutinin* (RCA) were obtained from Sigma-Aldrich. Methoxypoly(ethylene glycol) amine (mPEG-NH_2, MW=5000) was obtained from Nektar Therapeutics AL, USA. Ultra-pure water (Milli-Q Element, Millipore) was used for all the experiments. Phosphate buffered saline (PBS) solutions at pH = 7.4, (NaCl 137mM, KCl 2.7 mM, phosphate buffered 10mM, Amresco) were used in SPR imaging experiments.

TLC was carried out on silica gel plates (Merck 60-F254) or Al_2O_3 (Alugram ALOX N/UV_{254} 0.2 mm Macherey-nagel). Galactose derivatives were detected by anisaldehyde reagent and **1** by a $CuSO_4$ solution. The N,N-Bis-(p-toluensulfonyl)-3,6-diazoethane-1,8-di-p-toluensolfonate, 2-bromoethyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside, 1,4,7,10,13-Pentaazacyclo pentadecane were synthesized as reported in the literature.[1,2]

NMR experiments

^{1}H NMR spectra were recorded at 25°C with a Varian Inova 500 spectrometer at 499.883 MHz. The ^{1}H NMR spectra were measured by using standard pulse programs from the Varian library. In all cases the length of the 90° pulse was c.a. 7 μs. The 2D spectra (COSY, TOCSY, HSQC) were acquired using 1K data points, 256 increments and a relaxation delay of 1.5 s.

Synthesis of 1-(2-ethoxy-β-D-galactopyranosyl)-1,4,7,10,13-Pentaazacyclopentadecane (2). 1,4,7,10,13-pentaazacyclopentadecane (170 mg; 0.79 mmol) was added to a solution of the 2-bromoethyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (180 mg, 0.395 mmol) in anhydrous DMF (1 ml). The reaction was carried out at 60°C under stirring and under nitrogen for 20 hours. The solvent was evaporated and the acetylated **2** was isolated by chromatography using a R_P8 column (2.5x16cm) and a linear gradient of water (0.1 M CH_3COONH_4) → MeOH (total

volume 500 ml). The acetylated **2** was isolated and characterized. TLC: SiO_2 R_f = 0.11 (eluent: $PrOH/H_2O/NH_3$ 5:3:3). Yield 62%.

ESI-MS m/z = 590.3 (M+1). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 5.37 (dd; 1H; H-4); 5.13 (dd; 1H; H-3); 4.91 (m; 1H; H-2; $J_{1,2}$= 8.0 Hz; $J_{2,3}$= 18.0 Hz); 4.71 (d; 1H; H-1; $J_{1,2}$ = 8.0 Hz); 4.66-4.06 (m; 3H; H-5,H-6,H-6'); 3.96-3.93 (m; 1H; H of CH_2 in α to O); 3.70-3.69 (m; 1H; H of CH_2 in α to O); 3.25-2.20 (m; 22H; H of **1** moiety, CH_2 in β to O); 2.10-1.85 (s; 12H; CH_3).

The product **2** was obtained by hydrolysis of acetyl groups in NH_3 (10%) for 2h.

ESI-MS m/z = 422.3. Elemental Analysis calcd. for $C_{18}H_{39}O_6N_5 \cdot 2H_2O$ C 47.2, H 9.5, N 15.3; found C 46.9, H 9.3, N 15.1

^{1}H NMR (500 MHz,D_2O, pD= 5) δ (ppm): 4.31 (d; 1H; H-1; $J_{1,2}$ =7.80 Hz); 3.95 (ddd; 1H; H of CH_2 in α to O, $J_{aa'}$ = 10.78 Hz, $J_{ab'}$ = 6.89 Hz, J_{ab} = 3.90 Hz); 3,81 (d; 1H; H-4); 3.69-3.58 (m; 3H; H-5,H-6', H of CH_2 in α to O) 3.55-3.52 (m; 2H; H-3, H-6); 3.42 (dd; 1H; H-2, $J_{1,2}$ =7.80, $J_{2,3}$ = 8.80 Hz); 3.14-3.12 (m; 4H; some H of **1** moiety); 3.10-3.06 (m; 8H; CH_2 of [15]ane N_5 moiety); 3.03 (s; 4H; CH_2 of [15]ane N_5 moiety); 2.82-2.77 (m; 6H; other CH_2 of [15]ane N_5 moiety, CH_2 in β to O).

^{13}C (125 MHz, D_2O, pD= 5) δ (ppm): 103.2 (C-1), 75.4 (C-5), 73.0 (C-3), 71.0 (C-2), 68.8 (C-4), 68.0 (C of ethylenic chain in α to O), 61.3 (C-6), 51.5 (C of ethylenic chain in β to O), 53.5, 50.0, 49.0, 48.1, 45.8, 45.7, 45.4, 45.1 (C of macrocycle moiety)

Synthesis of [Mn(2)Cl_2]:

The **2** (50 mg; 0.118 mmol) was solubilized in 2 ml MeOH anhydrous and $MnCl_2$ (14.94 mg, 0.100 mmol) was added.

The reaction mixture was refluxed for 3 hours under stirring and under nitrogen. The MeOH was filtered, evaporated and acetone was added. [Mn(**2**)]Cl_2 was collected as white solid by filtration. Elemental Analysis calcd. for $C_{18}H_{39}O_6N_5MnCl_2 \cdot 2H_2O$ C 37.1, H 7.5, N 12.0; found C 36.8, H 7.4, N 11.8.

ESI-MS m/z = 511.1 $[Mn(\mathbf{2})^{35}Cl]^+$, 513.0 $[Mn(\mathbf{2})^{37}Cl]^+$.

Synthesis of 2-azidoethyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside

NaN_3 (1.43 g, 0.022 mol) was added to 2-bromoethyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (1.0 g, 2.20 mmol) in anhydrous DMF (10 ml) and the reaction was carried out at 70°C under stirring for 3 h.

The solvent was evaporated and the product was isolated by chromatography on silica gel using CH_2Cl_2/CH_3COCH_3 24:1 as eluent. TLC = 0.70 (eluent CH_2Cl_2/CH_3COCH_3 24:1).

Reaction yield 94%.

ESI-MS m/z = 418.4 (M+1). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 5.41 (dd; 1H; H-4, $J_{3,4}$ = 3.36 Hz, $J_{4,5}$ = 1.1 Hz); 5.25 (dd; 1H; H-2, $J_{1,2}$ = 8.01 Hz, $J_{2,3}$ 10.60 Hz); 5.04 (dd; 1H; H-3, $J_{2,3}$ 10.59 Hz, $J_{3,4}$ 3.36 Hz); 4.57 (d; 1H; H-1; $J_{1,2}$ = 8.01 Hz); 4.20 (dd; 1H; H-6, $J_{6,6'}$ = 11.89, $J_{6,5}$ = 6.60 Hz), 4.14 (dd, 1H, H-6', $J_{6,6'}$ = 11.89, $J_{6',5}$ = 6.72); 4.07(ddd; 1H; H of the CH_2 in β to azido group, $J_{aa'}$ = 10.60 Hz, $J_{ab'}$ = 4.65 Hz, J_{ab} = 3.36 Hz); 3.93 (td; 1H; H-5, $J_{5,6}$ 6.72 Hz, $J_{4,5}$ = 1.0 Hz); 3.70 (ddd; 1H; H of CH_2 in α to N_3, $J_{aa'}$ = 10.70 Hz, $J_{a'b}$ = 8.53 Hz, $J_{a'b'}$ = 3.36 Hz); 3.52 (ddd; 1H; H of the CH_2 in α to N_3, $J_{bb'}$ = 13.40 Hz, $J_{a'b}$ = 8.53 Hz, J_{ab} = 3.36 Hz); 3.31 (ddd; 1H; H of CH_2 in β to N_3 $J_{bb'}$ = 13.43 Hz, $J_{ab'}$ = 4.65 Hz, $J_{a'b'}$ = 3.36 Hz); 2.17(s; 3H; CH_3), 2.08 (s; 3H; CH_3), 2.06 (s; 3H; CH_3), 2.01 (s; 3H; CH_3).

Synthesis of 2-aminoethyl-β-D-galactopyranoside (3)

Triphenylposphine (3.24 g, 12.36 mmol) was added to 2-azidoethyl-2,3,4,6-tetraacetyl-β-D-galactopyranoside (0.86 g) in anhydrous DMF (8ml). The reaction was carried out at 25°C. After 3 h NH_3 (30%, 3ml) was added and the reaction was left under stirring for 12 hours.

After the solvent elimination, water was added to precipitate the $(Ph)_3P$. The mixture was filtered and the filtrate was concentrated. The final product deacethylated was isolated by a CM

Sephadex C-25 (NH_4^+ form) column, using a linear gradient H_2O → NH_4HCO_3 (0.3 M). **3** was isolated in 30% yield. Rf = 0.10 (eluent 1-PrOH/H_2O/AcOEt /NH_3 5:3:2:1).

Elemental Analysis calcd. for $C_8H_{17}O_6N \cdot 2H_2O$ C 37.1, H 8.2, N 5.4; found C 36.8, H 8.1, N 5.3

ESI-MS m/z = 224.23 (M+1).

^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.31(dd; 1H; H-1; $J_{1,2}$= 7.89 Hz); 3.88 (m; 1H; H of CH_2 in β to NH_2); 3.82 (m; 1H; H-4); 3.72-3.60 (m; 3H; H-5, H-6, H of CH_2 in β to NH_2); 3.60 (m, 1H, H-6'); 3.56 (dd, 1H, H-3, $J_{2,3}$ = 9.88 Hz, $J_{3,4}$ = 3.29 Hz); 3.44 (dd; 1H; H-2, $J_{2,3}$ = 9.88 Hz, $J_{1,2}$ = 7.89 Hz); 2.84 (s; 2H; H of CH_2 in α to NH_2).

Superoxide Dismutase Assay

SOD-like activity was determined by the indirect method of cytochrome c.[3] Solutions containing the manganese complex in phosphate buffer (1 10^{-2} M, pH 7.4) were used. Superoxide anion was enzymatically generated by the xanthine-xanthine oxidase system and spectrophotometrically detected by monitoring the formation of reduced cytochrome c at 550 nm.

The reaction mixture was composed by cytochrome c 50 μM, Xantine 50 μM, catalase 30 μg/ml in phosphate buffer 10 mM, at pH 7.4. An appropriate amount of xanthine oxidase was added to 2 mL reaction mixture to produce a ΔA_{550nm} min^{-1} of 0.024. This corresponded to a O_2^- production rate of 1.1 μM min^{-1}. The cytochrome reduction rate was measured in the presence and in the absence of the investigated complex for 600 sec. All measurements were carried out at 25±0.2 °C using 1x1 cm thermostatted cuvettes in which solutions were magnetically stirred. In separate experiments urate production by xanthine oxidase was spectrophotometrically monitored at 295 nm, ruling out any inhibition of xanthine oxidase activity.

The I_{50} (the concentration which causes the 50% inhibition of cytochrome c reduction) of the complexes at pH 7.4 was determined as reported elsewhere.[4] Three determinations were carried out. The standard deviation was about 15%.

Gold surface functionalization and patterning

Bare gold chips (Au 450Å, Cr 50Å, evaporated on SF-10 borosilicate glass. GWC Technologies, MD, USA.) were immersed in a 4 mM DTSP solution in DMSO for at least 48 hours under inert atmosphere at room temperature.[5] The modified chips were then thoroughly rinsed with DMSO, water and ethanol, respectively and then dried under a stream of nitrogen. The DTSP modified gold surface was patterned by manually spotting 1μl of 10^{-2} M phosphate buffered saline solutions (PBS, pH=7.4) of **1**, **2** and **3** by using a micro-tip equipped pipette. The anchoring of the three compounds to the surface through the amine coupling reaction was completed in 2 hour at 25° C. The spotted chip was then soaked at room temperature for 2 hours in a mPEG-NH_2 solution (4 mM in 0.1M TEA, pH=8) in order to improve the array resistance to protein nonspecific interactions. Unreacted N-succinimidyl ester moieties on the chip surface were deactivated by further soaking the chip into a 1 M ethanolamine solution at pH=8.5. Chips used to study the RCA_{120} interaction with the Mn(II) complexes were also refluxed for 3 hours under nitrogen in a $MnCl_2$ 1 mM methanol solution and subsequently rinsed with methanol, water and ethanol, respectively.

Surface plasmon resonance imaging experiments

The SPR imaging experiments were carried out by using an SPR imager apparatus (GWC Technologies, MD, USA) equipped with a white light source and an SF-10 prism.[6] A narrow band-pass filter (800 nm) was placed before the CCD camera used to obtain SPR images.

SPR imaging binding experiments were carried out by using a 60 μl flow cell (GWC Technologies, MD, USA) and a Masterflex L/S (Cole-Parmer, USA) peristaltic pump.

SPR images were collected by using the V^{++} software (version 4.0, Digital Optics Limited, Auckland, New Zealand) and further analyzed using the software package Image J 1.32j (National Institutes of Health, USA). SPR imaging provides data as pixel intensity units on a 0-255 scale. As specified by the manufacturer data are converted in percent reflectivity (%R), or Δ%R in the case of difference images, by using the formula:

%R = 100* (0.85Ip/Is)

where Ip and Is refer to the reflected light intensity detected using p- and s-polarized light, respectively. Kinetics experiments were carried out by sequentially acquiring 15 frames averaged SPR images with time intervals of 3 seconds. Kinetics data were obtained by plotting the normalized difference in percent reflectivity (Δ%R) from selected regions of interest (ROIs) of the array as a function of time. Procedures have been described in detail elsewhere.[7] ROI data were normalized to the average of the Δ %R measured for mPEG background ROIs adjacent to each array element. SPR chip images where acquires with intervals of 3 seconds.

SPR imaging kinetics experiments were carried out at 25°C by flowing (flow rate 500 μl min^{-1}) RCA_{120} solutions in PBS at different concentration (2.5 10^{-7}M, 5.0 10^{-7}M and 7.5 10^{-7} M). PBS was used as the running buffer. Solutions of **3** (25 mM) and HCl (0.1 M) were used to regenerate the surface after each interaction..

The ClampXP data analysis software[8] was used to obtain the rate constants k_a and k_d and the equilibrium constants K_A. On the basis of an hypothetical interaction model the software operates a non linear global curve fitting of the adsorption/desorption kinetics data by using the function resulting from the numerical integration of the differential rate equations for the selected model. Constants were calculated by both locally and globally fitting kinetic curves obtained at the different RCA_{120} concentrations. Global fittings that produced the reported values for the rate and equilibrium constants were obtained with a residual randomly scattered around the baseline with an amplitude lower than ± 0.1 Δ%R roughly equivalent to the typical instrumental noise of the SPRimager apparatus.

Experimental SPR data were fitted on the basis of a bivalent model.[9] In the model, the binding process is described as follows:

$$\mathbf{a} + \mathbf{b} \underset{k_{d1}}{\overset{k_{a1}}{\rightleftarrows}} \mathbf{ab} \qquad\qquad \mathbf{a} + \mathbf{bb} \underset{k_{d2}}{\overset{k_{a2}}{\rightleftarrows}} \mathbf{abb}$$

where **a** would be one RCA_{120} molecule, **b** a galactose unit onto the chip surface, **ab** the complex formed and **abb** the complex formed between two galactose unit on the chip surface and one RCA_{120} molecule. Rate equations describing the interaction model are:

$$\frac{d[b]}{dt} = -\left(k_{a1}[a][b] - k_{d1}[ab]\right) \qquad (1)$$

$$\frac{d[ab]}{dt} = k_{a1}[a][b] - k_{d1}[ab] \qquad (2)$$

$$\frac{d[bb]}{dt} = -\left(k_{a1}[a][bb] - k_{d1}[abb]\right) \qquad (3)$$

$$\frac{d[abb]}{dt} = k_{a1}[a][bb] - k_{d1}[abb] \qquad (4)$$

It is clear from the above equations that desorption of RCA_{120} from the glycoconjugate modified surface, obtained when RCA_{120} solution is replaced with the buffer ([**a**]=0), is not dependent on the initial RCA_{120} concentration. As a consequence of that similar dissociation curves are observed when the change in percent reflectivity as a function of time normalized data are

compared. Figure 1 compares the normalized Δ%R vs time data for the dissociation phase of RCA_{120} from **2**.

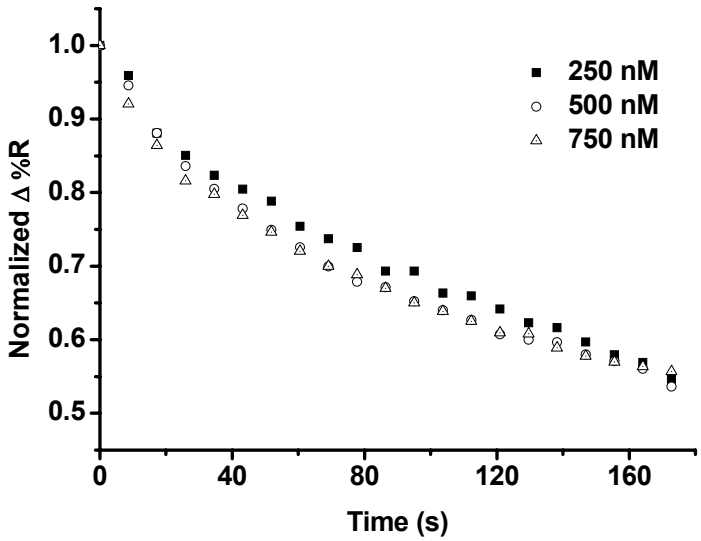

Figure 1

The ClampXP software automatically generates the differential rate equations for the described model and integrates them numerically using a semi-implicit extrapolation method. The quality of the non linear fitting of the experimental data is evaluated by calculating the residual Ssq that represents the sum of the squared difference between the measured and simulated data.

Figure 2 shows kinetics data and curve-fit for the interaction between **3** and RCA_{120}.

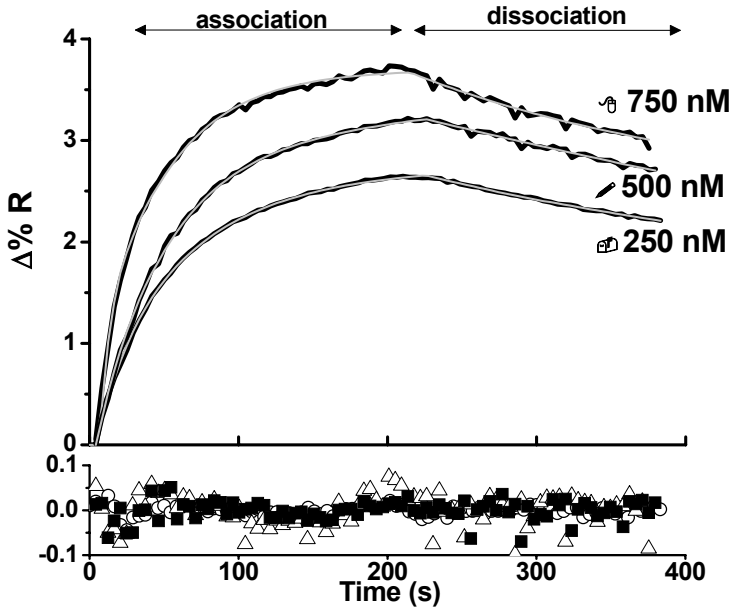

Figure 2

Equilibrium adsorption isotherms for the RCA_{120} interacting with **2** and **3** also support the deviation from a Langmuir model which assumes a 1:1 interaction between RCA_{120} and the glycoconjugate derivative. In the ideal Langmuir model all receptor sites are energetically equivalent, independent and available for binding. Equilibrium SPR imaging measurements were performed by introducing aliquots of increasing RCA_{120} concentrations over the array surface and measuring the Δ%R after 45 min, for each concentration. Signals due to adsorption generally reached a steady-state value after 15-20 min. It has been previously demonstrated that SPR imaging can be used to quantify the amount of material absorbing to a gold surface and to obtain protein surface coverage values.[10]

Figures 3 and 4 show the adsorption isotherms for the binding of RCA_{120} to **2** and **3** respectively. They have been obtained by plotting the relative lectin surface coverage at equilibrium as a function of the lectin concentration.

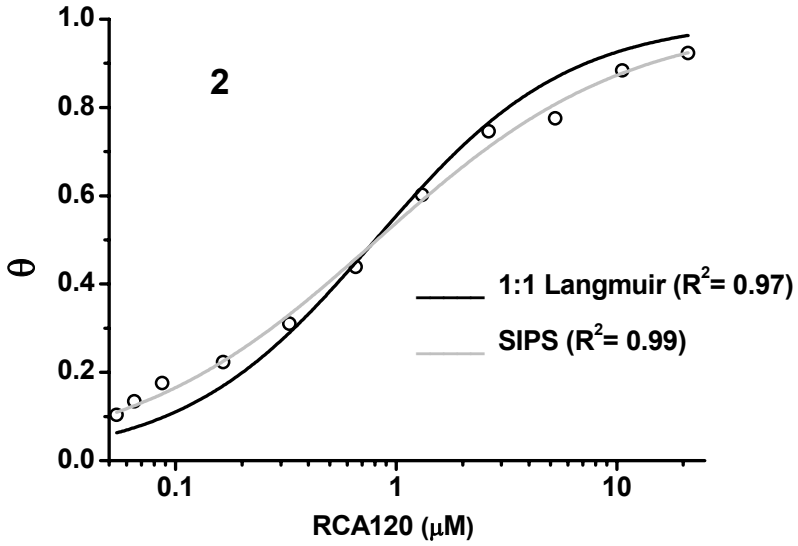

Figure 3

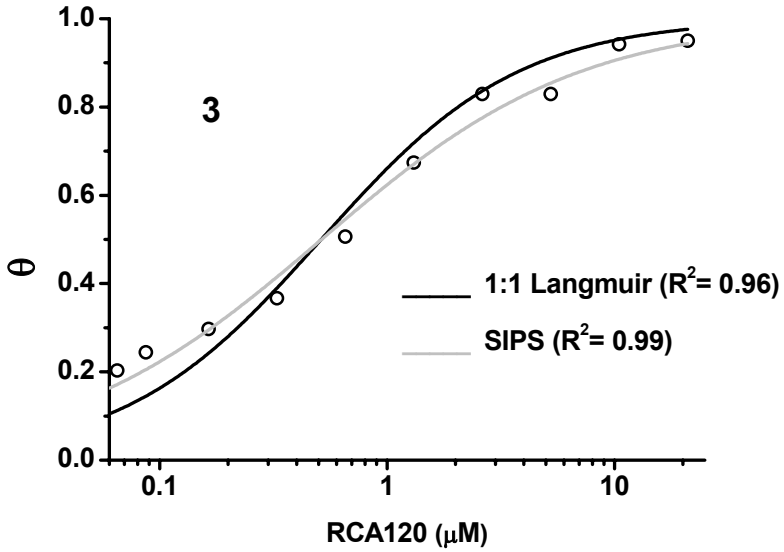

Figure 4

The solid lines in the Figures 3 and 4 were obtained by fitting the data to a Langmuir isotherm and a Sips isotherm.[11] The Sips adsorption isotherm has the form

$$\theta = \frac{(K_{Ads}\,[RCA\ I])^a}{1 + (K_{Ads}\,[RCA\ I])^a}$$

where θ is the lectin surface coverage (given by $\Delta\%R/\Delta\%R_{max}$), $[RCA_{120}]$ is the concentration of the lectin solution, K_{Ads} is the adsorption coefficient and **a** is the heterogeneity index. **a** represents a pseudo-Gaussian distribution of binding energies of set width. The Sips isotherm includes heterogeneity in the interaction model (more than one single binding energy) and reduces to the Langmuir isotherm when **a**=1 (only one single binding energy). Sips isotherm better fits adsorption data by providing K_{Ads} = 1.2 10^6 M^{-1} (**a**= 0.77) for the RCA_{120}/ **2** interaction and K_{Ads} = 1.9 10^6 M^{-1} (**a**= 0.76) for the RCA_{120}/ **3** interaction.

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