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Supporting Information



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# Bisaryl Hydrazones as Exchangeable Biocompatible Linkers**

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**INITIAL EXPERIMENTS – Scrambling of bisarylhydrazones in the presence of aniline and in the absence of excess monomers**

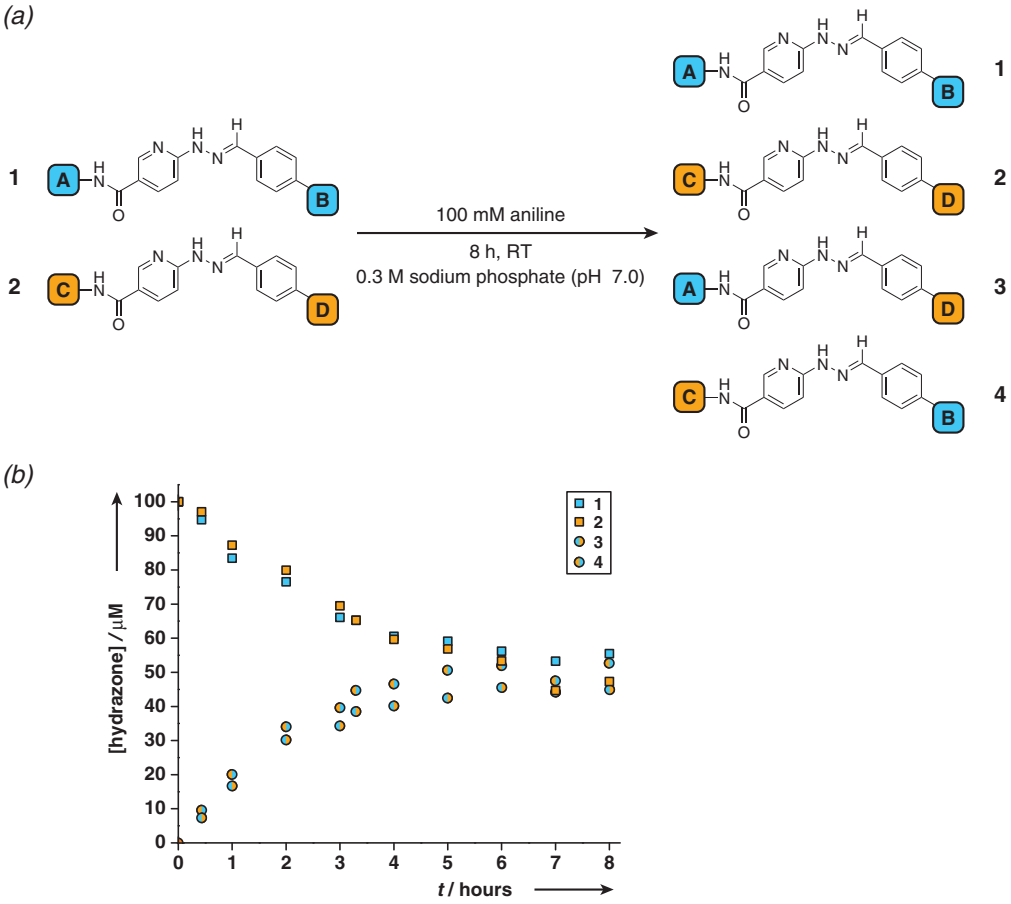



Figure 1. *(a)* Reequilibration of hydrazones **1** (**A** = FLAG, **B** = H) and **2** (**C** = biotinylated FLAG, **D** = F) through transimination in the presence of the nucleophilic catalyst aniline to a mixture of hydrazones **1**, **2**, **3**, and **4**. *(b)* Reequilibration of a mixture of **1** and **2** (100 µM each) over time in the presence of 100 mM aniline at room temperature at pH 7.0. The reaction was followed by HPLC and the ligation products were quantitated by integration (354 nm). No excess of monomers present.

**Preparation of hydrazone 1.** 100 µL of a 2 mM solution of 6-hydrazinopyridyl-FLAG and 100 µL of a 2 mM solution of benzaldehyde in 0.3 M sodium phosphate (pH 7.0) were reacted at room temperature. The reaction went to completion within 1 hour as confirmed by HPLC (220 nm and 354 nm) (gradient: 5 - 25% 9:1 *v/v* MeCN/$H_2O$ in $H_2O$, 0.1 *v*-% TFA in 15 min; flow: 3 mL/min). ESI-MS calcd. for $C_{68}H_{93}N_{20}O_{25}$ ($[M+H]^+$): 1591.6, found 1591 ± 0.7.

**Preparation of hydrazone 2.** 100 µL of a 2 mM solution of biotinylated 6-hydrazinopyridyl-FLAG and 100 µL of a 2 mM solution of *p*-fluorobenzaldehyde in 0.3 M sodium phosphate (pH 7.0) were reacted at room temperature. The reaction went to completion within 1 hour as confirmed by HPLC (220 nm and 354 nm) (gradient: 5 - 25% 9:1 *v/v* MeCN/$H_2O$ in $H_2O$, 0.1 *v*-% TFA in 15 min; flow: 3 mL/min). ESI-MS calcd. for $C_{78}H_{106}FN_{22}O_{27}S$ ($[M+H]^+$): 1835.9, found 1835.0 ± 0.7.

The reaction mixtures of hydrazone **1** (1 mM) and hydrazone **2** (1 mM) were used as such, without any further purification.

*Note:* see p7 and p8 for the synthesis and chemical structures of 6-hydrazinopyridyl-FLAG and biotinylated 6-hydrazinopyridyl-FLAG.

**Stability of a mixture of hydrazones 1 and 2 in the absence of aniline.** 50 µL of each hydrazone mixture was added to 400 µL 0.3 M sodium phosphate (pH 7.0) in an eppendorf tube to give a solution containing 100 µM of each hydrazone. A sample was taken for HPLC analysis. The solution was purged with $N_2$ and protected from

light. Samples were taken in time and analyzed by HPLC (220 nm and 354 nm) (gradient: 5 - 25% 9:1 *v/v* MeCN/$H_2O$ in $H_2O$, 0.1 *v*-% TFA in 15 min; flow: 3 mL/min) and the hydrazones were quantitated by integration at 354 nm. The hydrazone mixture was purged with $N_2$ after taking each sample. Under these conditions the hydrazones are stable for up to 22 hours and no scrambling is observed. After 47 hours ~10-15% of the hydrazones were decomposed. See previous publication for a note on the stability and storage of 6-hydrazinopyridine and its hydrazone with benzaldehyde.[1]

**Scrambling of hydrazones 1 and 2 in the presence of 100 mM aniline (Fig 1).** 250 µL of a 200 mM solution of aniline in 0.3 M sodium phosphate (pH 7.0) and 50 µL of each hydrazone mixture were subsequently added to 150 µL 0.3 M sodium phosphate (pH 7.0) to give a solution containing 100 µM of each hydrazone and 100 mM aniline. A sample was taken for HPLC analysis. The solution was purged with $N_2$ and protected from light. Samples were taken in time and analyzed by HPLC (220 nm and 354 nm) (gradient: 5 - 25% 9:1 *v/v* MeCN/$H_2O$ in $H_2O$, 0.1 *v*-% TFA in 15 min; flow: 3 mL/min) and the hydrazones were quantitated by integration at 354 nm. Transimination occurs and after 8 hours a new equilibrium is reached consisting of hydrazones **1**, **2**, **3**, and **4** at approximately 50 µM each (Fig 1). ESI-MS calcd. for hydrazone **3**: $C_{68}H_{92}FN_{20}O_{25}S$ ($[M+H]^+$): 1609.6, found 1610 ± 0.0. ESI-MS calcd. for hydrazone **4**: $C_{78}H_{107}N_{22}O_{27}S$ ($[M+H]^+$): 1817.9, found 1817.0 ± 0.7.

The almost equimolar product distribution shows that the hydrazones are of similar thermodynamic stability. The rate constant of reequilibration of $(3.5 \pm 0.2) \times 10^{-5}$ $s^{-1}$, corresponds to the rate constant of hydrolysis ($k_{-1}$) of this hydrazone in the presence

of 100 mM aniline.[1] This suggests that scrambling occurs through the direct reaction of trace free hydrazine (**A**, **C**) and free aldehyde (**B**, **D**) present in solution at equilibrium. With an $K_{eq}$ of $2.3 \times 10^6$ $M^{-1}$,[1] and a starting total concentration of 200 μM hydrazone, free monomers (**A**, **B**, **C**, and **D**) should be present at equilibrium at 4.5 μM each.

## Materials and Methods

**Solvents and starting materials.** Unless stated otherwise, all reagents and solvents were purchased from commercial sources and used without purification. ChromaLink™ Biotin[2] (CLB354) was purchased from Solulink (San Diego, CA, USA) (Fig 2). Proteins were obtained from the following sources: Human Serum Albumin (HSA, essentially fatty acid free, ~99% (agarose gel electrophoresis) or 97-99% (agarose gel electrophoresis), lyophilized powder, Sigma), aprotinin (bovine lung, crystalline, Calbiochem), cytochrome C (from bovine heart, ≥ 95% based on Mol. Wt. 12,327 basis, C2037, Sigma), myoglobin (from equine skeletal muscle 95-100%, essentially salt-free, lyophilized powder, Sigma), aldolase (lyophilized powder, Pharmacia), human activated protein C (APC, Enzyme Research Laboratories, Ltd.), human protein S (stock solution of 1 mg/mL in 20 mM Tris-HCl, 0.1 M NaCl, 1 mM benzamidine, pH 7.4, Enzyme Research Laboratories, Ltd.), human fVa (Haematologic Technologies Inc.). Avidin beads were obtained from Pierce (Pierce Avidin Agarose, support: crosslinked 6% beaded agarose, capacity: ≥ 20 µg biotin/mL of gel).

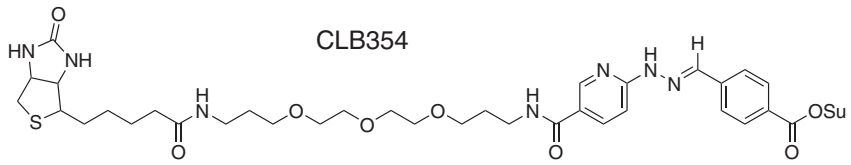



Figure 2. Structure of ChromaLink™ Biotin[2] (CLB354).

**Instrumentation.** Reversed phase high pressure liquid chromatography (HPLC) was performed on a HP1050 HPLC System (analytical HPLC) and on a Waters Delta Prep 4000 preparative chromatography system (preparative HPLC), using a Phenomenex Prodigy 5µ ODS(3) 100Å (50 × 4.60 mm) and a Phenomenex Jupiter 10µ Proteo 90Å

(250 × 21.2 mm), respectively, for separation. Electrospray ionization mass spectrometry (ESI-MS) was performed on a SCIEX API-I single quadruple mass spectrometer. UV-Vis was measured on a Genesys™ 6 spectrophotometer (Thermo Electron Corporation), except for the spectra in Fig 6 and Fig 10, which were recorded on a Cary 1-Bio dual beam UV-Vis spectrophotometer (Varian, Walnut Creek, CA). Fluorescence imaging of SDS-PAGE gels was performed on a Typhoon™ Trio variable mode imager (GE Healthcare).

**Peptide synthesis.** The peptides were obtained *via* manual solid phase peptide synthesis using an *in situ* neutralization/1*H*-benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate-(1-),3-oxide (HCTU) activation procedure for *t*Boc chemistry on a *p*-methylbenzhydrylamine (MBHA) resin.[3] 6-Hydrazinopyridyl-FLAG (Fig 3a) and aminooxyacetyl-FLAG (Fig 3c) were synthesized as previously described.[1]

**Synthesis of biotinylated 6-hydrazinopyridyl-FLAG (0.05 mmol scale) (Fig 3b).** The N-terminal *t*Boc group of *t*BocD(OcHxl)YK(2ClZ)D(OcHxl)D(OcHxl)D(OcHxl)D(OcHxl)K(2ClZ)GGGGK(Fmoc) was removed with trifluoroacetic acid (TFA) (2 × 1 min) followed by a DMF flow wash. N-terminal biotinylation was achieved by adding 0.25 mmol (61 mg) biotin in 454 μL of 0.5 M HCTU in DMF, 114 μL diisopropylethylamine (DIEA), and 454 μL DMF to the resin (30 min). After reaction the resin was washed with DMF. Then the Fmoc side chain protecting group of the C-terminal lysine residue was removed by treatment with 20 *v*-% piperidine in DMF (4 × 3 min) followed by a DMF flow wash. 0.1 mmol (25 mg) 6-*t*Boc-hydrazinonicotinic acid (6-BOC-HNA, Solulink, San Diego, CA, USA) was added to a solution of 0.1

mmol (52 mg) benzotriazole-1-yl-oxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate (PyBOP®) and 0.12 mmol ($d$ = 0.742 kgL$^{-1}$, 21 µL) DIEA in 0.5 mL of DMF and the mixture was added to the resin (45 min). The resin was washed with DMF and the *t*Boc group was removed with TFA (2 × 1 min). The resin was washed with DMF, DCM, and 1:1 *v/v* DCM/MeOH and dried under vacuum. The peptide was cleaved from the resin by HF using 4 *v*-% of anisole as a scavenger. After lyophilization, biotinylated 6-hydrazinopyridyl-FLAG was purified by RP HPLC (gradient: 5 - 30% 9:1 *v/v* MeCN/$H_2O$ in $H_2O$, 0.1 *v*-% TFA in 80 min; flow: 20 mL/min). ESI-MS calcd. for $C_{71}H_{103}N_{22}O_{27}S$ ($[M+H]^+$): 1729.8, found 1730.2.

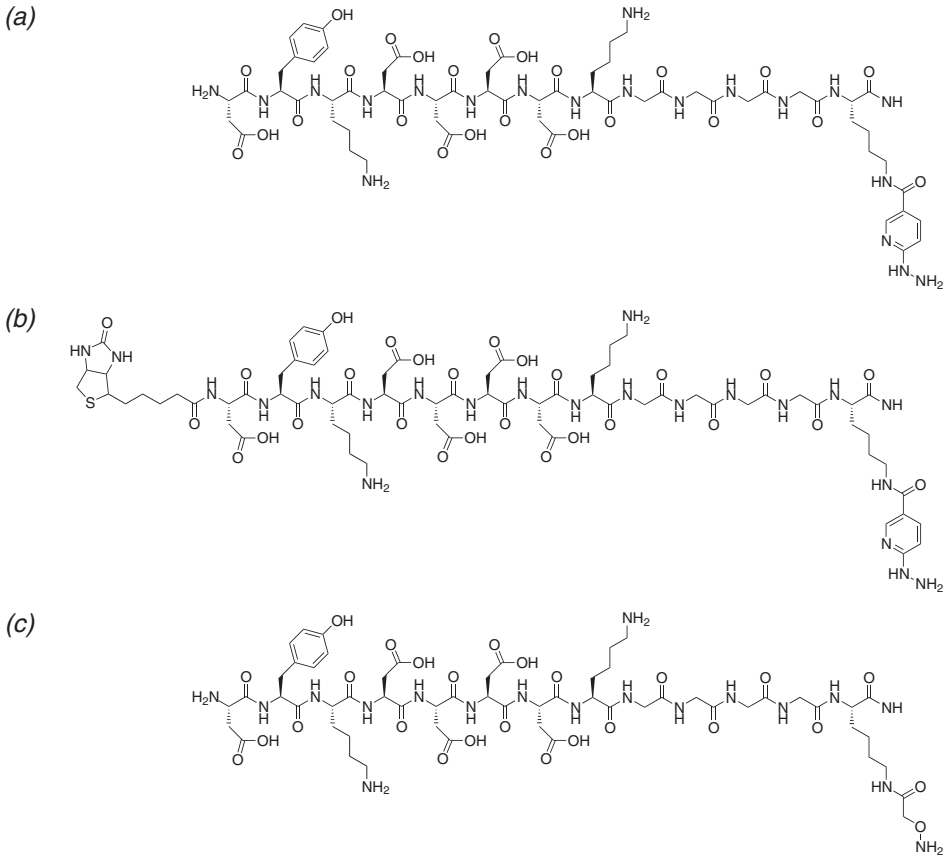



Figure 3. Chemical structures of *(a)* 6-hydrazinopyridyl-FLAG, *(b)* biotinylated 6-hydrazinopyridyl-FLAG, and *(c)* aminooxyacetyl-FLAG.

**In solution cleavage of the bisarylhydrazone of CLB354 (pH 6.0).**

The following stock solutions were prepared: **A** 1 mM CLB354 (MW 810.92, 0.81 mg/mL) in DMF; **B** 1 mM CLB354 in 1:4 *v/v* DMF/0.3 M sodium phosphate (pH 8.0) (by diluting a 5 mM solution of CLB354 (4.05 mg/mL) five times with the phosphate buffer (this stock solution was used after 2 hours); **C** 200 mM $NH_2OH$.HCl (MW 69.49, 13.9 mg/mL) in 0.1 M sodium phosphate (pH 6.0) (pH adjusted to pH 6.0 with 5 M NaOH (aq)); **D** 20 mM $NH_2OH$.HCl in 0.1 M sodium phosphate (pH 6.0) (10× dilution of **C**); **E** 200 mM aniline in 0.1 M sodium phosphate (pH 6.0).

The following reactions were performed. The final concentration of hydrazone in the reaction mixtures is 5 µM. Buffer is 0.1 M sodium phosphate (pH 6.0).

**1.** no aniline / no $NH_2OH$: 2 mL buffer + 10 µL **A** (*blank:* 10 µL DMF + 2 mL buffer)

**2.** no aniline / 10 mM $NH_2OH$: 1 mL buffer + 10 µL **A** + 1 mL **D** (*blank:* 10 µL DMF + 1 mL buffer + 1 mL **D**)

**3.** 100 mM aniline / 10 mM $NH_2OH$: 1 mL **D** + 10 µL **A** + 1 mL **E** (*blank:* 10 µL DMF + 1 mL **D** + 1 mL **E**)

**4.** no aniline / 100 mM $NH_2OH$: 1 mL buffer + 10 µL **A** + 1 mL **C** (*blank:* 10 µL DMF + 1 mL 0.1 M buffer + 1 mL **C**)

**5.** 100 mM aniline / 100 mM $NH_2OH$: 1 mL **C** + 1 mL **E** + 10 µL **A** (*blank:* 10 µL DMF + 1 mL **C** + 1 mL **E**)

**6.** 100 mM aniline / no $NH_2OH$: 1 mL buffer + 10 µL **B** + 1 mL **E** (*blank:* 10 µL 1:4 *v/v* DMF/0.3 M Na phosphate (pH 8.0) + 1 mL buffer + 1 mL **E**)

The stock solutions were combined in a quartz cuvette in the specific order stated. The reactions were followed for 2 hours by UV (354 nm, measured every minute) (Fig 4). The reaction was timed as soon as the last component was added. The reaction mixture was thoroughly mixed using a pipette and the first time point was recorded after 1 minute. Cleavage of the hydrazone with 100 mM $NH_2OH$, 100 mM aniline was also followed for 8 hours by UV (354 nm, measured every 5 minutes) to demonstrate complete cleavage of the hydrazone under these conditions (Fig 5). The concentration of hydrazone plotted in the graph is derived from the known concentration of CLB354 in the stock solution and the UV absorption measured at 354 nm at $t = 0$ minutes.

*Note:* The succinimidyl ester of CLB354 reacts instantaneously with $NH_2OH$ and more slowly with aniline at pH 6.0. This induces a small increase in the absorption at 354 nm. To analyze the reequilibration of the hydrazone of CLB354 in the absence of $NH_2OH$, but in the presence of aniline, the succinimidyl ester of CLB354 was first fully hydrolyzed at pH 8.0. Stock solution **B** was used after 2 hours. The hydrazone is stable to these conditions during this time.

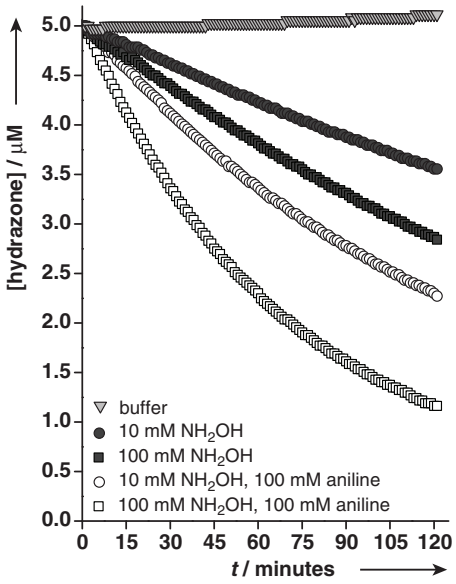



Figure 4. Cleavage (first 2 hours) of the bisarylhydrazone group of CLB354 with 10 mM or 100 mM $NH_2OH$, in the absence and presence of 100 mM aniline at pH 6.0. CLB354 in buffer (pH 6.0) alone shows the stability of the hydrazone in the absence of amines.

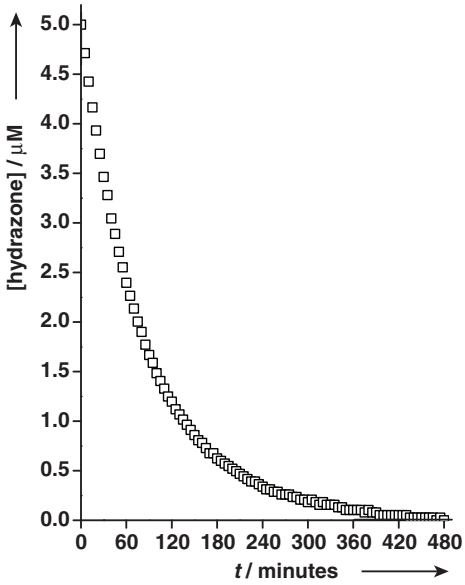



Figure 5. Cleavage of the bisarylhydrazone group of CLB354 with 100 mM $NH_2OH$ and 100 mM aniline in 0.1 M sodium phosphate (pH 6.0).

In these experiments there is no significant contribution of the aniline Schiff base of the benzaldehyde species to the absorption at 354 nm. To demonstrate this, a UV spectrum of PEGylated benzaldehyde (5 μM) was recorded in 0.1 M sodium phosphate (pH 6.0) in the presence of 100 mM aniline (Fig 6).

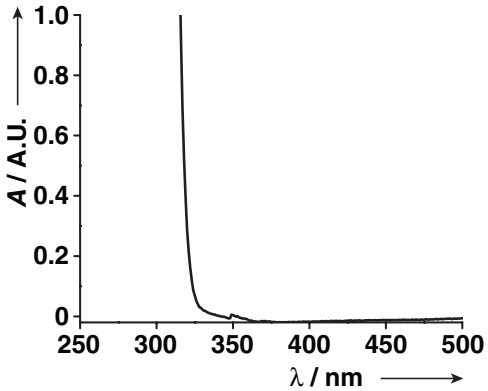



Figure 6. UV-spectrum of PEGylated benzaldehyde (4FB/PEG12-OMe (Solulink, San Diego, CA, USA), 5 μM) in 0.1 M sodium phosphate (pH 6.0) in the presence of 100 mM aniline.

CLB354 (succinimidyl ester) has an absorption maximum at 360 nm. The absorption maximum shifts to 354 nm when reacted with a primary aliphatic amine. UV spectra of CLB354 were recorded at different concentrations (1 μM, 2.5 μM, 5 μM, 10 μM, and 25 μM) in 0.1 M sodium phosphate (pH 6.0). The absorption increases linearly according to the Lambert-Beer law and there is no shift in the absorption maximum in this concentration range (Fig 7). This indicates that the hydrazone of CLB354 does not aggregate at low μM concentration.

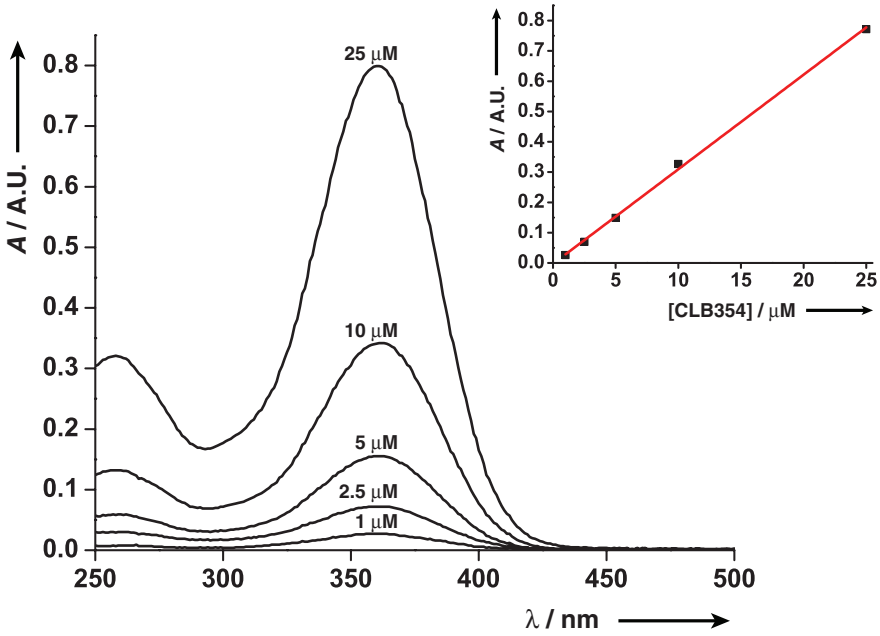



Figure 7. UV spectra of a dilution series of CLB354 in 0.1 M sodium phosphate (pH 6.0). Inset: linear fit of the absorption at 360 nm plotted against [CLB354] ($R^2$ = 0.999).

**In solution cleavage of the bisarylhydrazone of CLB354 (pH 4.6).**

Formation and hydrolysis of the bisarylhydrazone are enhanced by 1 order of magnitude at slightly acidic pH.[1] To demonstrate rapid cleavage at pH 4.6, 10 µL of a 1 mM stock solution of CLB354 in DMF was added to 2 mL of 100 mM $NH_2OH.HCl$ in 0.1 M anilinium acetate (pH 4.6) in a quartz cuvette (*blank:* 2 mL of 100 mM $NH_2OH.HCl$ in 0.1 M anilinium acetate (pH 4.6) + 10 µL DMF).

The reaction was followed for 90 minutes by UV (354 nm, measured every minute) (Fig 8). The reaction was timed as soon as CLB354 was added to the cleavage buffer. The reaction mixture was thoroughly mixed using a pipette and the first time point was taken after 1 minute. Cleavage was achieved within 1 hour.

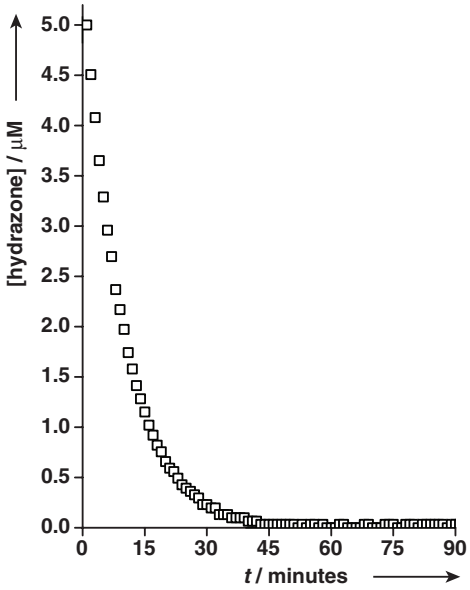



Figure 8. Cleavage of the bisarylhydrazone group of CLB354 with 100 mM $NH_2OH$ in 0.1 M anilinium acetate (pH 4.6).

As most proteins will precipitate in aqueous buffer at slightly acidic pH (pH 4-5), we chose to use 0.1 M sodium phosphate (pH 6.0) as a buffer in the pull-down assays.

## *Model studies with biotinylated HSA*

**1. Typical procedure for the synthesis of biotinylated Human Serum Albumin (HSA-CLB354$_x$; in this example x = 1.3).** 0.61 mg ($7.5 \times 10^{-4}$ mmol) of CLB354 in 50 µL of DMF was added to a solution of 5 mg ($7.7 \times 10^{-5}$ mmol) of HSA in 1 mL of 50 mM sodium phosphate/150 mM NaCl (pH 7.4). The reaction was continued for 2 hours at room temperature, protected from light. HSA-CLB354$_x$ was purified by size-exclusion chromatography over a G-25 column (length: 30 cm, width: 1 cm) using 50 mM sodium phosphate/150 mM NaCl (pH 7.4). The hydrazone of CLB354 has a distinct absorption at 354 nm ($\varepsilon$ = 29,000 $M^{-1}cm^{-1}$) when conjugated to a primary aliphatic amine.[2] Fractions containing HSA-CLB354$_x$ (determined by UV (280 and 354 nm)) were combined. A biotin content of 10.6 µM was calculated from the absorption at 354 nm. The concentration of HSA was determined with a BCA assay and found to be 7.9 µM. HSA was biotinylated on average 1.3 times with CLB354. As the labeling reaction gives a statistical distribution, a fraction of HSA remains unlabeled and is unable to bind to avidin beads.

*Note:* the degree of biotinylation varies per experiment and should always be verified. As an alternative for the BCA assay, the absorption at 280 nm can be used to determine the protein concentration, but needs to be corrected for the absorption of the hydrazone at this wavelength: $A_{280,corr} = A_{280} - (A_{354} \times 0.28)$.[4]

**2. Preparation of the protein mixture for the model study.** 1.57 mL of the HSA-CLB354$_{1.3}$ solution obtained after size exclusion chromatography was combined with 100 µL of a protein solution in 50 mM sodium phosphate/150 mM NaCl (pH 7.4)

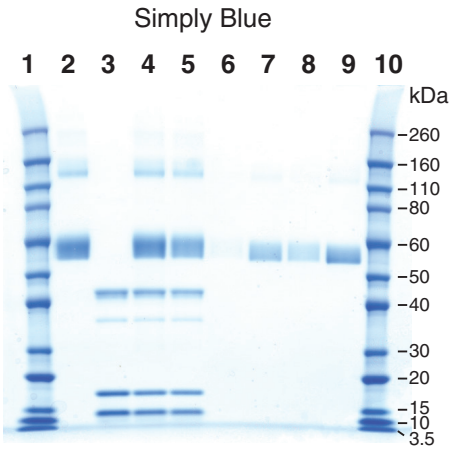
containing 1 mg/mL aprotinin, 2 mg/mL cytochrome C, 2.9 mg/mL myoglobin, 26 mg/mL aldolase. *Final concentrations:* 8 µM HSA-CLB354$_{1.3}$, 10 µM aprotinin, 10 µM cytochrome C, 10 µM myoglobin, 10 µM aldolase.

**3. Extraction of HSA-CLB354$_x$ (x = 1.3) from the protein mixture using avidin beads.** The protein solution contains 17 nmoles of CLB354 in the form of HSA-CLB354$_{1.3}$. The avidin beads have a capacity of ≥ 20 µg biotin/mL beads, which corresponds to ≥ 82 nmole/mL of beads. In order to immobilize all HSA-CLB354$_{1.3}$, 448 µL of the avidin bead slurry (capacity to bind ≥ 18 nmoles of biotin) was added to the protein mixture after washing 3 times with 50 mM Hepes, 150 mM NaCl (pH 7.4). After 30 minutes of incubation at room temperature, protected from light, while gently rotated, the beads were centrifuged, the supernatant was removed and the beads were washed with 50 mM sodium phosphate/150 mM NaCl (pH 7.4) (2 times) and 0.1 M sodium phosphate (pH 6.0) (2 times).

**4. Cleavage of the hydrazone – elution of HSA from the avidin beads.**

**4A. Dependence of the cleavage efficiency on the concentration of hydroxylamine ($NH_2OH$) and aniline at pH 6.0 (*here,* HSA-CLB354$_x$ with x = 1.3 was used) (Fig 9).** For each condition, 30 µL beads were incubated for 2 hours with 50 µL of cleavage buffer while mildly shaken. The following cleavage buffers were used: *(1)* 10 mM $NH_2OH$.HCl, *(2)* 100 mM $NH_2OH$.HCl, *(3)* 10 mM $NH_2OH$.HCl, 100 mM aniline, *(4)* 100 mM $NH_2OH$.HCl, 100 mM aniline, all in 0.1 M sodium phosphate (pH 6.0). When needed the pH of the cleavage buffer was readjusted to pH 6.0 with 5 M NaOH (aq). The beads were centrifuged and the supernatant was analyzed by SDS-PAGE. For this, 15 µL of supernatant was mixed with 1 µL of a 40 mg/mL stock

solution of iodoacetamide in water and 5 µL of SDS sample buffer. The mixture was heated for 8 minutes in boiling water and loaded onto a 4-12% NuPAGE® Novex® Bis-Tris gel (Invitrogen™). The protein bands were visualized by staining with Simply Blue™ (Invitrogen™) (Fig 9). HSA-CLB354$_{1.3}$, the protein mixture lacking HSA-CLB354$_{1.3}$, the protein mixture including HSA-CLB354$_{1.3}$, and the supernatant after immobilization on the avidin beads are references. The remaining HSA in the supernatant after immobilization is unlabeled and unable to bind to the beads.




**1:** molecular weight marker

**2:** HSA-CLB354$_{1.3}$

**3:** protein mixture without HSA-CLB354$_{1.3}$

**4:** protein mixture with HSA-CLB354$_{1.3}$ before immobilization

**5:** protein mixture with HSA-CLB354$_{1.3}$ after immobilization (supernatant)

**6:** proteins eluted with 10 mM $NH_2OH$

**7:** proteins eluted with 10 mM $NH_2OH$ and 100 mM aniline

**8:** proteins eluted with 100 mM $NH_2OH$

**9:** proteins eluted with 100 mM $NH_2OH$ and 100 mM aniline

**10:** molecular weight marker

0.1 M sodium phosphate (pH 6.0), RT, 2h
NuPAGE 4-12% Bis-Tris

Figure 9. SDS-PAGE analysis (Simply Blue™ stain) of the cleavage of immobilized HSA-CLB354$_{1.3}$ with 10 or 100 mM $NH_2OH$, in the absence or presence of 100 mM aniline, in 0.1 M sodium phosphate (pH 6.0). HSA (67 kDa), aprotinin (6.5 kDa), cytochrome C (12 kDa), myoglobin (17 kDa), aldolase (158 kDa, 4 subunits of ~40 kDa each). Novex® prestained protein standard was used as the molecular weight marker and indicates the apparent molecular weight in kDa.

**Evidence for efficient removal of the biotin tag during cleavage (Fig 10).** The biotin tag is efficiently removed from the protein after cleavage with 100 mM $NH_2OH$, 100 mM aniline in 0.1 M sodium phosphate (pH 6.0) overnight at r.t., protected from light. To demonstrate this, UV spectra of a solution of HSA-CLB354$_{1.5}$ in 50 mM Na phosphate, 150 mM NaCl (pH 7.4), of the supernatant after immobilization of HSA-CLB354$_{1.5}$ on avidin beads (Fig 10, *left*), and of the $NH_2OH$ oxime of HSA-benzaldehyde$_{1.5}$ eluted from the beads and purified by size exclusion (spin filter, Millipore, MWCO 3,000) (Fig 10, *right*) were recorded. The spectra show efficient immobilization of HSA-CLB354$_{1.5}$ on avidin beads and elution of $NH_2OH$ oxime of HSA-benzaldehyde$_{1.5}$ with > 95% removal of the biotin tag. The presence of $NH_2OH$ oxime of HSA-benzaldehyde$_{1.5}$ was confirmed by SDS-PAGE.

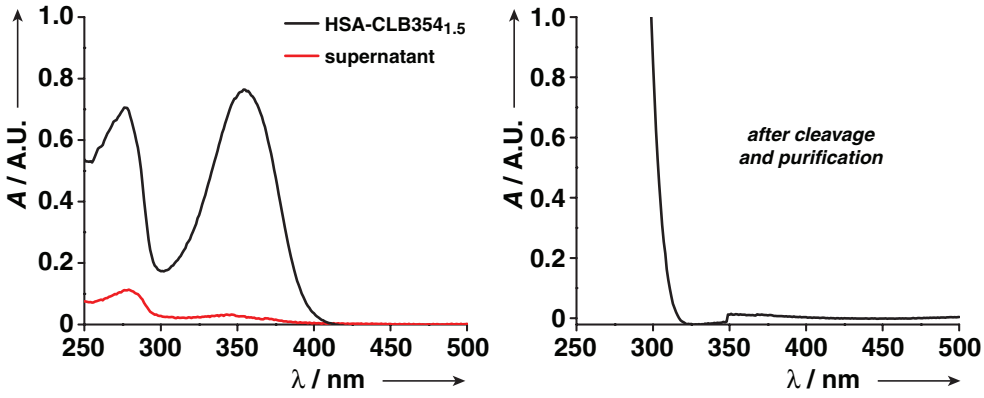
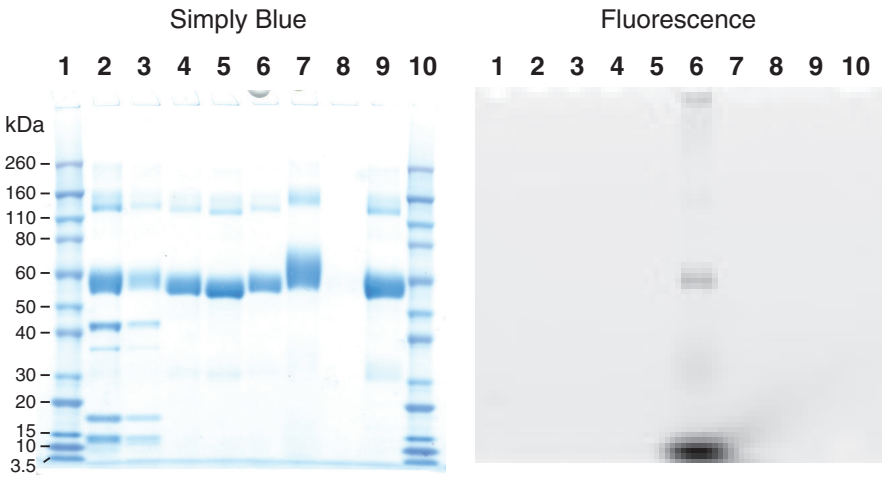



Figure 10. UV spectra of HSA-CLB354$_{1.5}$ in 50 mM Na phosphate, 150 mM NaCl (pH 7.4) ([HSA] = 18 µM, [CLB354] = 26 µM) and of the supernatant obtained after immobilization of HSA-CLB354$_{1.5}$ on avidin beads *(left)*. Residual absorption at 280 nm after immobilization can be assigned to unmodified HSA. The UV spectrum of HSA eluted from the avidin beads (the $NH_2OH$ oxime of HSA-benzaldehyde$_{1.5}$) shows no significant absorption at 354 nm *(right)*. The biotin tag is efficiently (> 95%) removed during cleavage.

**4B. Label exchange reaction (*here,* HSA-CLB354$_x$ with x = 1.3 was used) (Fig 11).** The same procedure for the loading of the avidin beads with protein mixture was performed for the label exchange. After washing, 30 µL of the beads were taken for each condition. The beads were incubated overnight at room temperature in the dark while mildly shaken with 50 µL of the following cleavage buffers: *(1)* 100 mM $NH_2OH.HCl$, *(2)* 10 mM of the fluorescent dye, Alexa Fluor® 488 $C_5$-aminooxyacetamide, bis(triethylammonium) salt (Invitrogen), or *(3)* 10 mM of the affinity tag, aminooxyacetyl-FLAG (Fig 3c), all in the presence of 100 mM aniline in 0.1 M sodium phosphate (pH 6.0). When needed the pH of the cleavage buffer was readjusted to pH 6.0 with 5 M NaOH (aq). Two control reactions were performed: incubation with buffer alone (0.1 M sodium phosphate (pH 6.0)), and an SDS boil to disrupt the biotin-avidin interaction by denaturation. The beads were centrifuged and the supernatant was analyzed by gel chromatography. For this, 15 µL of supernatant was mixed with 1 µL of a 40 mg/mL stock solution of iodoacetamide in water and 5 µL of SDS sample buffer. The mixture was heated for 8 minutes in boiling water and loaded onto a 4-12% NuPAGE® Novex® Bis-Tris gel (Invitrogen™). The protein bands were visualized by staining with Simply Blue™ (Invitrogen™) (Fig 11). The label exchange reaction with Alexa Fluor® 488 was confirmed by fluorescence imaging. The fluorescence at the bottom of the gel is excess unreacted Alexa Fluor® 488 $C_5$-aminooxyacetamide (Fig 11). The protein mixture (3× and 10× diluted), HSA-CLB354$_{1.3}$-loaded avidin beads incubated with just 0.1 M sodium phosphate (pH 6.0), and an SDS boil of HSA-CLB354$_{1.3}$-loaded avidin beads are references. The amount of labeled HSA that is retrieved from the beads was calculated relative to the amount cleaved by the SDS boil using densitometry (NIH J Image).




**1:** molecular weight marker

**2:** protein mixture (3x dilution)

**3:** protein mixture (10x dilution)

**4:** proteins eluted with 100 mM $NH_2OH$

**5:** proteins eluted with 100 mM $NH_2OH$ and 100 mM aniline

**6:** proteins eluted with 10 mM aminooxyacetyl-Alexa Fluor 488 and 100 mM aniline

**7:** proteins eluted with 10 mM aminooxyacetyl-FLAG and 100 mM aniline

**8:** buffer

**9:** proteins eluted by SDS boil

**10:** molecular weight marker

0.1 M sodium phosphate (pH 6.0), RT, overnight
NuPAGE 4-12% Bis-Tris

Figure 11. SDS-PAGE analysis (Simply Blue™ stain *(left)*, fluorescence imaging *(right)*) of the label exchange reactions on immobilized HSA-CLB354$_{1.3}$. HSA (67 kDa), aprotinin (6.5 kDa), Cytochrome C (12 kDa), myoglobin (17 kDa), aldolase (158 kDa, 4 subunits of ~40 kDa each). Novex® prestained protein standard was used as the molecular weight marker and indicates the apparent molecular weight in kDa.

**4C. Cleavage with MeO-PEG12-BA (*here,* HSA-CLB354$_x$ with x = 1.5 was used).** Avidin beads were loaded with protein mixture as described before. After washing, 50 µL of beads were taken for each condition. The beads were incubated overnight at room temperature in the dark while gently shaken with 60 µL of 10 mM MeO-PEG12-BA (4FB/PEG12-OMe, Solulink, San Diego, CA, USA) in the presence or absence of 100 mM aniline. The beads were centrifuged and the supernatant was analyzed by SDS-PAGE. 15 µL of supernatant was mixed with 1 µL of a 40 mg/mL stock solution of iodoacetamide in water and 5 µL of SDS sample buffer. The mixtures were heated for 8 minutes in boiling water and loaded onto a 4-12% NuPAGE® Novex® Bis-Tris gel (Invitrogen™). An SDS boil of the remaining beads was performed as a reference to determine the cleavage efficiency. The gel was analyzed by staining with Simply Blue™ (Invitrogen™). The amount of HSA-benzaldehyde$_{1.5}$ that was retrieved from the beads was calculated using densitometry (NIH J Image).

## *Pull-down assay from human serum with biotinylated human protein S*

**Introduction.** Recent studies have suggested that protein S might have several other functions besides its well-known activated protein C (APC) cofactor activity.[5-7] Protein S circulates in plasma in both a free form and bound to the β-chain of complement factor protein C4bP (Fig 12a).[8,9] Protein S-CLB354$_{3.4}$ was synthesized and used in a pull-down assay to retrieve its binding partners from human plasma. Protein S-CLB354$_{3.4}$ and its binding partners were pulled down with avidin beads. As anticipated, C4bP was enriched and a potential new binding partner of protein S was discovered (Fig 12b). The details of the pull-down assay are described below.

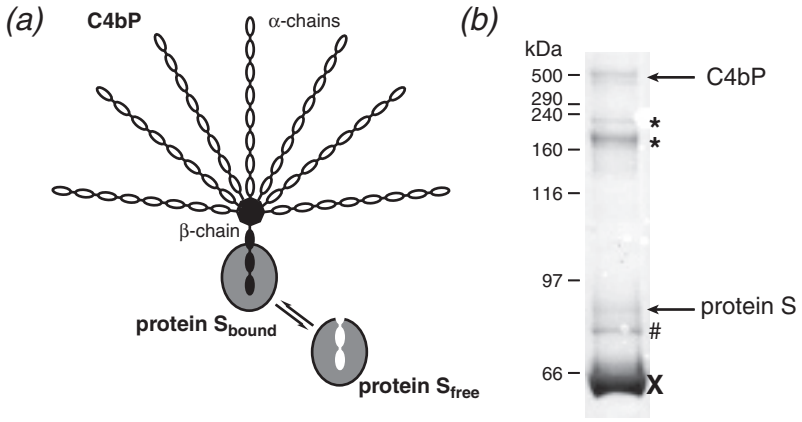



Figure 12. Pull-down assay with biotinylated Protein S to retrieve its binding partners from human plasma. *(a)* Reversible binding of protein S to the β-chain of C4bP.[8,9] *(b)* SDS-PAGE analysis (7% Tris-acetate gel, Simply Blue$^{TM}$ stain) of the proteins retrieved from human plasma with protein S-CLB354$_{3.4}$, including C4bP (~570 kDa, consisting of 7 α-chains (70 kDa each) and 1 β-chain (45 kDa)) and protein S (75 kDa). Unidentified proteins are marked (*). **#** retention correlates to C4bP α-chain (70 kDa), consistent with Western blotting; **X** retention correlates to HSA (67 kDa).

**Synthesis of biotinylated human protein S (protein S-CLB354$_x$; *here*: x = 3.4).** A stock solution of 0.5 mg ($6.17 \times 10^{-4}$ mmol) CLB354 in 50 µL DMF (final conc: 12.3 mM) was prepared. 1.1 µL of the stock solution of CLB354 (2 equivalents) were added to 500 µL of the human protein S stock solution (1 mg/mL in 20 mM Tris-HCl, 0.1 M NaCl, 1 mM benzamidine, pH 7.4, Enzyme Research Laboratories, Ltd.). The labeling reaction was continued for 2 hours at room temperature, while protected from light. Human protein S-CLB354$_x$ was purified by dialysis (MWCO 12,000-14,000) overnight at +4 ºC. The concentration of protein S and the number biotin units attached were calculated from the UV absorptions at 280 nm and 354 nm. The $\varepsilon_{0.1\%}$ = 0.95 for protein S[10] and the absorption at 280 nm was corrected for the absorption of the hydrazone: $A_{280,corr} = A_{280} - (A_{354} \times 0.28)$.[4] The concentration protein S was found to be 16.3 µM. The concentration of CLB354 was calculated from the absorption at 354 nm ($\varepsilon$ = 29,000 $M^{-1}cm^{-1}$) and found to be 55.1 µM. There are on average 3.4 biotins per protein S. *Note:* the high substitution ratio obtained suggests a lower stock concentration of proteins S than reported by the supplier.

**Incubation of human plasma with protein S-CLB354$_{3.4}$.** 270 µL of the protein S-CLB354$_{3.4}$ solution were added to 2 mL of human plasma, giving a final concentration of 1.9 µM protein S-CLB354$_{3.4}$ to compete with the 350 nM protein S present in plasma. Protein S is known to bind the β-chain of complement factor protein C4bP[8,9] in a $Ca^{2+}$ dependent manner.[9b] In particular, the off rate of this protein complex is increased by depleting the plasma from $Ca^{2+}$.[9b] To accelerate the exchange of protein S with protein S-CLB354$_{3.4}$, 5 mM of EDTA was added to scavenge $Ca^{2+}$. The plasma containing protein S-CLB354$_{3.4}$ was reequilibrated for 24

hours while mildly shaken at room temperature, protected from light. After that, 7 mM $CaCl_2$ was added to the plasma to slow down the reequilibration between protein S and C4bP.

**Recovery of protein S-CLB354$_{3.4}$ containing protein complexes from human plasma.** The plasma contains 15 nmoles of CLB354 in the form of protein S-CLB354$_{3.4}$. The avidin beads have a capacity of ≥ 20 µg biotin/mL beads, which corresponds to ≥ 82 nmole/mL beads (≥ 41 nmole/mL slurry). In order to immobilize all protein S-CLB354$_{3.4}$, 560 µL of the avidin bead slurry (capacity to bind 23 nmoles of biotin) were washed 3 times with 50 mM sodium phosphate / 150 mM NaCl (pH 7.4) and added to the human plasma. The plasma was incubated for 2 hours with the avidin beads while mildly shaken at room temperature, protected from light. The avidin beads were centrifuged, and the human plasma was removed. The beads were washed 3 times with 50 mM sodium phosphate / 500 mM NaCl (pH 7.4) (a sample of the first wash with this high salt solution was saved to use as a reference in the SDS-PAGE and Western blotting analysis later on) and subsequently 3 times with 0.1 M sodium phosphate (pH 6.0).

**Elution of the protein S-CLB354$_{3.4}$-protein complexes.** For elution 60 µL cleavage buffer containing 100 mM $NH_2OH$ and 100 mM aniline in 0.1 M sodium phosphate (pH 6.0) were added to the avidin beads. The beads were incubated overnight, while mildly shaken at room temperature, protected from light.

**Analysis of the proteins eluted from the avidin beads (Figs 13 and 14).** The avidin beads were centrifuged and the supernatant was analyzed by gel chromatography.

Three SDS-PAGE gels were run in parallel for analysis. One gel was visualized by Simply Blue™ (Invitrogen™), whereas the other two gels were analyzed for the presence of protein S and C4bP using Western blotting. For each gel, 15 µL of supernatant was mixed with 1 µL of a 40 mg/mL stock solution of iodoacetamide in water and 5 µL of SDS sample buffer. The avidin beads were washed 3 times with 0.1 M sodium phosphate (pH 6.0). An SDS boil of the remaining beads was performed as a reference. Other references are HSA, which is highly abundant in human plasma, protein S-CLB354$_{3.4}$, and activated protein C (APC, 56 kDa), which is also known to bind to protein S[11]. The mixtures were heated for 8 minutes in boiling water and loaded onto a 7% NuPAGE® Novex® Tris-acetate gel (Invitrogen™). The proteins of one gel were visualized by staining with Simply Blue™ (Invitrogen™) (Fig 13). The amount of proteins retrieved by transimination was determined by densitometry (NIH J Image), comparing the density of the protein bands eluted with 100 mM $NH_2OH$, 100 mM aniline in 0.1 M sodium phosphate (pH 6.0) (Fig 13, lane 11) with the density of the bands of the proteins that remained on the beads after the $NH_2OH$/aniline procedure, but were eluted by an SDS boil (Fig 13, lane 9).

The other two gels were transferred to a PVDF membrane and analyzed by Western blotting according to standard procedures.[12] One of the membranes was stained by using polyclonal rabbit anti-human protein S (Dako USA) as primary antibody and biotinylated donkey anti-rabbit IgG as secondary antibody to identify protein S (Fig 14, *right*), whereas the other membrane was stained by using monoclonal anti-human C4bP[13] as primary antibody and biotin-rat anti-mouse IgG as secondary antibody to identify the α-chain of C4bP (Fig 14, *left*). Subsequently, the membrane was treated with streptavidin-coupled alkaline phosphatase (SAAP, Pierce)

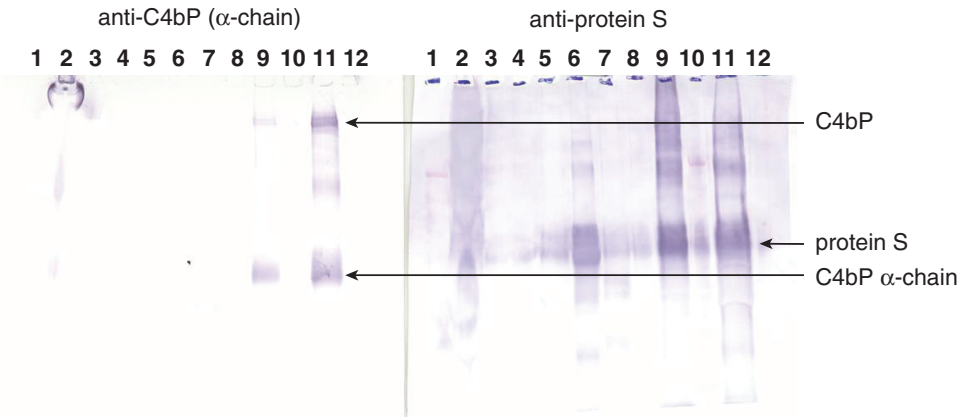
and bands were visualized by treatment with 1-Step™ BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate *p*-Toluidine) and NBT (Nitro-Blue Tetrazolium Chloride) substrate.


**1:** molecular weight marker
**2:** human plasma (first high salt wash with 50 mM Na phosphate, 500 mM NaCl (pH 7.4))
**3:** empty
**4:** molecular weight marker
**5:** HSA
**6:** protein S-CLB354$_{3.4}$
**7:** activated protein C (APC)
**8:** empty
**9:** proteins eluted by SDS boil after $NH_2OH$/aniline treatment of the beads
**10:** molecular weight marker
**11:** proteins eluted by treatment of the beads with 100 mM $NH_2OH$ and 100 mM aniline in 0.1 M sodium phosphate (pH 6.0)
**12:** empty

Figure 13. SDS-PAGE analysis (Simply Blue™ stain) of the proteins retrieved from human plasma with protein S-CLB354$_{3.4}$, including C4bP (~570 kDa, consisting of 7 α-chains (70 kDa) and 1 β-chain (45 kDa)) and protein S (75 kDa). Unidentified proteins are marked (*). # retention correlates to C4bP α-chain (70 kDa), consistent with Western blotting (see Fig 14). **X** retention correlates to HSA (67 kDa). HiMark™ stained HMW protein standard was used as the molecular weight marker and indicates the apparent molecular weight in kDa.

*Note:* in a control experiment lacking the bait protein (biotinylated protein S), avidin beads were incubated with human plasma. Human plasma was treated according to

the procedure followed for the experiment including biotinylated protein S. In this control experiment the protein bands in Fig 13 marked ***** and **X** were recovered and may be attributed to nonspecific binding of abundant plasma proteins (among them most likely HSA and IgG) to the avidin beads, but not **#**. It should be noted that we used an excess of the avidin beads to ensure full immobilization of biotinylated protein S and its binding partners. This may contribute to a relatively high background of non-specifically bound proteins. It is a challenge to remove highly abundant proteins, such as HSA and IgG, from plasma. Methods to achieve this are in development.[14] Identification of all protein bands is currently in progress and the binding of protein S to these proteins will be investigated.




**1:** molecular weight marker
**2:** human plasma (first high salt wash with 50 mM Na phosphate, 500 mM NaCl (pH 7.4))
**3:** empty
**4:** molecular weight marker
**5:** HSA
**6:** protein S-CLB354$_{3.4}$
**7:** activated protein C (APC)
**8:** empty
**9:** proteins eluted by SDS boil after $NH_2OH$/aniline treatment of the beads
**10:** molecular weight marker
**11:** proteins eluted by treatment of the beads with 100 mM $NH_2OH$ and 100 mM aniline in 0.1 M sodium phosphate (pH 6.0)
**12:** empty

Figure 14. Western blot analysis of the pull-down assay with human protein S-CLB354$_{3.4}$ with anti-C4bP (α-chain) *(left)* and anti-protein S *(right)*.

**Protein S cofactor activity assay (Fig 15).**

Activated protein C (APC) proteolytically cleaves and inactivates factor Va (fVa), a cofactor protein that dramatically enhances thrombin (fIIa) generation by the prothrombin (fII)-ase complex. Protein S is the non-enzymatic cofactor of APC in this proteolytic reaction and accelerates the inactivation of fVa by APC. To assess the cofactor activity of protein S, fVa inactivation by APC is compared in the presence and absence of protein S. To test the bio-compatibility of the cleavage buffer, protein S-CLB354 was incubated with 100 mM $NH_2OH$ and 100 mM aniline in 0.1 M sodium phosphate (pH 6.0) at room temperature overnight. The $NH_2OH$, aniline, and cleaved biotin were removed by dialysis (2 × 1000 mL) against 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$ buffer. Untreated, unlabeled protein S was used as control for these assays.

A three-step reaction was used to compare the cofactor activities of $NH_2OH$/aniline-treated protein S-CLB354 with that of untreated protein S in an APC-mediated inactivation of FVa. The first step involved the inactivation of fVa by APC/protein S in the presence of phospholipids (PL) vesicles. The second step involved the generation of thrombin (fIIa) by the prothrombinase (fXa•fVa•fII•PL) complex. The final step involved the quantification of fIIa generated by the prothrombin (FII)-ase complex with the aid of a chromogenic substrate. This APC/protein S mediated inactivation of fVa assay has been widely accepted in the coagulation field a valid assay for assessing protein S cofactor activity.[15-18]

For the first step, fVa (0.55 nM) was incubated at room temperature with PC/PS (4:1 molar ratio, ~28 μM) in 40 μl of 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$,

0.1 mM $MnCl_2$, 0.5% BSA (fII-ase buffer). At time $t$ = 0 minutes, two aliquots (1.8 µL each) of this mixture were withdrawn and tested in fII-ase assay. Controls lacking APC were performed. At $t$ = 0 minutes, human APC (0.5 nM) and protein S (80 nM) were added to the reaction mixture and subsequently 2 µL of reaction mixture was withdrawn at $t$ = 1, 2, 3, 4, 6, 8, 12, 18, 24, and 30 minutes and added to fII-ase mixture of 38 µL of fXa/PCPS (1.25 nM fXa/31 µM PC/PS) and 10 µL of fII (1.5 µM). Finally, at 2.67 minutes fIIa production by the fII-ase mixture was stopped by the addition of 15 µL of this reaction mixture to 55 µL of 50 mM Tris (pH 7.4), 100 mM NaCl, 10 mM EDTA, 0.5% BSA, and 0.02% $NaN_3$ buffer. fIIa produced was quantified by the addition of 35 µL of Pefa-Th (0.6 mM) to the mixture and color generation was monitored at 405 nm on an OPTImax tunable plate reader (Molecular Devices, Sunnyvale, CA, USA). Inactivation curves were fit according to equations described earlier (Fig 15).[17,18] Proteins S-CLB354 was found to maintain full APC cofactor activity after cleavage by overnight incubation with 100 mM $NH_2OH$, 100 mM aniline in 0.1 M sodium phosphate (pH 6.0).

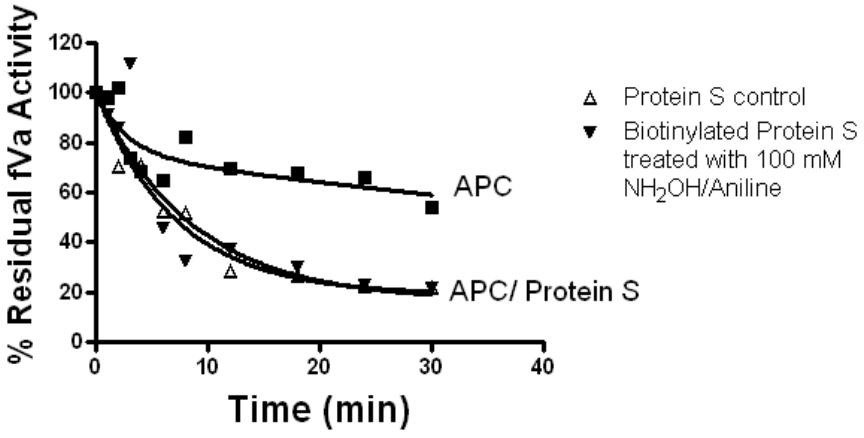



Figure 15. APC/protein S -mediated inactivation of fVa in time using protein S-CLB354 after overnight incubation with 100 mM $NH_2OH$, 100 mM aniline in 0.1 M sodium phosphate (pH 6.0). APC/protein S-mediated and APC-mediated inactivation of fVa were performed as references.

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*Note:* $\varepsilon_{0.1\%}$ is the 0.1% solution extinction coefficient and provides the absorption at 280 nm of a 1 mg/mL solution of protein measured in a 1 cm cuvette. The relationship between molar extinction coefficient ($\varepsilon_{molar}/M^{-1}cm^{-1}$) and 0.1% solution extinction coefficient ($\varepsilon_{0.1\%}$) is:

$\varepsilon_{molar} = \varepsilon_{0.1\%} \times$ molecular weight of the protein

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