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Identification and Structural Determination of a Potent P-Selectin Inhibitor

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Abstract—Small quantities of a potent P-selectin inhibitor, 2 ($IC_{50} = 0.2\,\mu M$), were isolated and an initial structure proposed based on 1D proton NMR. A reterosynthetic analysis of the proposed structure led us to a total synthesis of **2**. NMR studies using the 2-D homo-TOCSY and NOESY and 2-D hetero-HMQC helped to confirm the structure of **2**.

Introduction

During an inflammatory response, the initial rolling of leukocytes is arbitrated by the selectins, which are a family of calcium-dependent, cell adhesion molecules. One member of the family, P-selectin is expressed on the activated endothelium at sites of inflammation. The natural ligand for P-selectin is PSGL-1, a complex homodimeric glycoprotein with a carbohydrate subunit, sialyl Lewis X (sLex), which is the minimal lactosaminoglycan carbohydrate epitope for all the selectins.[1,2] Several acute diseases, such as stroke and reperfusion injury, and chronic diseases, such as psoriasis and rheumatoid arthritis, are the result of the excess recruitment of leukocytes.[3–10] The development of a small molecule inhibitor that blocks the interaction of selectins with their natural ligands has become an attractive therapeutic approach.

As part of our ongoing effort to develop a P-selectin antagonist, we have done random screening of several compound libraries in search of a small non-oligosaccharide molecule that can inhibit the P-selectin/PSGL-1 interaction. A high-throughput ELISA-based assay was used. The ELISA evaluates the binding of a PSGL-1 fusion protein, which consists of the functional N-terminal region of PSGL-1 fused to the Fc region of IgG1, to immobilized soluble P-selectin, which is pre-incubated with test compound.

Results and Discussion

Several antagonists of P-selectin were discovered. One of the most potent inhibitors was **ZZZ21322**. The structure provided by the supplier is shown in Table 1 and will be referred to as **1**. The initial assay results showed **ZZZ21322** to have an IC_{50} of 0.4 μm. Compound **1** was synthesized using a known procedure,[11] shown in Scheme 1, and was assayed. The pure sample of **1** was found to have an IC_{50} of 5 μm. The reduced activity of **1** lead to an analysis of the sample of **ZZZ21322** that was obtained from the supplier. The sample was found to be a multicomponent mixture by HPLC and NMR. The mixture was separated on a reverse-phase C18 column (90 Å, 25×1 cm, flow rate 3 mL/min) eluting with a gradient starting from 50% of solvent A (0.1%TFA/H_2O) to 62% solvent B (0.1%TFA in 90% CH_3OH/10% H_2O v/v) in 120 min. Analysis of HPLC fractions showed that the major component of the mixture was **1**, which eluted between 51 and 55 min.

Evaluation of the HPLC fractions in our P-selectin assay showed that the most potent compound (**2**) eluted between 70 and 74 min. This compound was a minor component in the crude mixture. Compound **2** was found to be more potent than the **ZZZ21322** mixture and **1**, having an IC_{50} of 0.2 μm.

One-dimensional proton NMR spectra[12] of **1** and **2** were compared. The proton NMR spectrum of **1**, shows that the molecule is axisymmetric with a center olefinic carbon atom. The NMR spectrum appears very simple and each peak set contains double proton signals, except the

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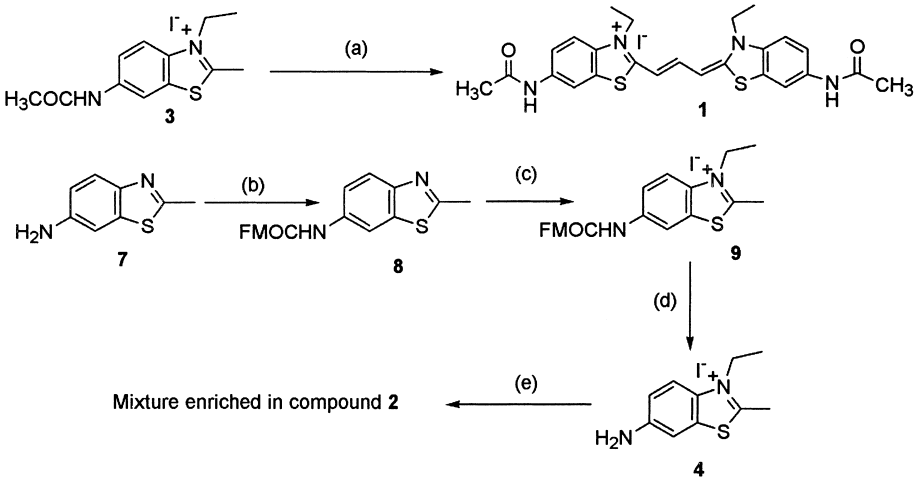
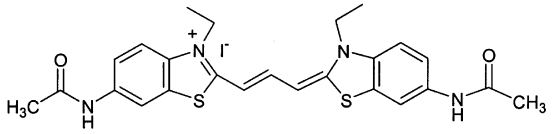


Scheme 1. Conditions: (a) $HC(OEt)_3$, pyridine, reflux; (b) FMOCCl, $NaHCO_3$, H_2O, dioxane; (c) Etl, 110 °C; (d) piperidine, DMF; (e) **3**, $HC(OEt)_3$, nitrobenzene, 130 °C.

Table 1. In vitro inhibition of P-selectin to sLex containing PSGL-1

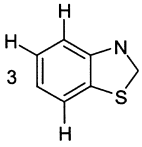


1

Sample	Assay (IC_{50}) PSGL-1(148.Fc)/Psel	Purity
ZZZ21322	0.4 μm	Impure: HPLC and NMR show several peaks
1	5 μm	Pure
2	0.2 μm	Pure
SLex	7 mM	Pure

Molecular weight: 681.2140 (HRMS, FAB)

Composition:

	C	H	N	O	S
2 CH_3CONH	4	8	2	2	0
3 CH_2CH_3	6	15	0	0	0
3 	21	9	3	0	3
3 	5	4	0	0	0
NH	0	1	1	0	0
TOTAL	36	37	6	2	3

Molecular formula: $C_{36}H_{37}N_6O_2S_3^+ / C_{36}H_{38}N_6O_2S_3^{++}$

US = (36+1) - (37-6-1)/2 = 22 (+1 charge)

or (36+1) - (38-6-1)/2 = 22 (+2 charge)

Figure 1. MS data analysis.

middle olefinic proton, which integrates to one. There are four isolated spin systems in each half of the molecule. These are one ethyl, one *N*-acetyl, the aromatic AMX and olefinic AX spin systems. The center olefinic proton (δ 7.67) is hidden in the aromatic proton region. However on expanding this region a triplet ($J = 12.8$ Hz) can be seen. The 1-D spectrum of **2** is complicated, but has peaks similar to those found in the 1-D spectrum of **1**. From the spectrum, it is clear that **2** has a non-symmetric structure. Further NMR studies at this point were impossible due to sample limitation. An MS analysis (Fig. 1) and 1-D NMR data indicated that **2** contained three benzothiazole units. This information indicated two possible structures for **2**, which are shown in Figure 2.

Based on the reported synthetic route of compound **1**, we proposed a mechanism for the formation of **2**, which is shown in Figure 2. In the proposed structure the three benzothiazole units are connected in a linear fashion. The two end benzothiazole units (A and C) come from the *N*-acetylamino-2-methylbenzothiazolium salt (**3**). They have similar chemical shifts and spin coupling pattern as compound **1**. We reasoned that the middle benzothiazole unit (B) must come from amino-2-methylbenzothiazolium salt (**4**), which could result from deacetylation of **3** during the reaction[13] or incomplete acetylation of **7** during the synthesis of **3**. The linkage between the benzothiazole units A and B is formed from the condensation of one molecule of triethylortho-formate ($HC(OEt)_3$) with benzothiazoles **3** and **4**. The free 6-amino group of intermediate **5** then reacts with another molecule of triethylorthoformate forming the intermediate **6**, which is attacked by the nucleophillic anion of benzothiazolium salt (**3**) to furnish the proposed compound. The proposed mechanism could provide a route to higher order forms of **2**. The presence or absence of these forms in the crude reaction mixture was not investigated since none of the other HPLC fractions had activity comparable to compound **2**.

Thus the aminobenzothiazolium salt (**4**) might be one of the key components in the formation of active compound

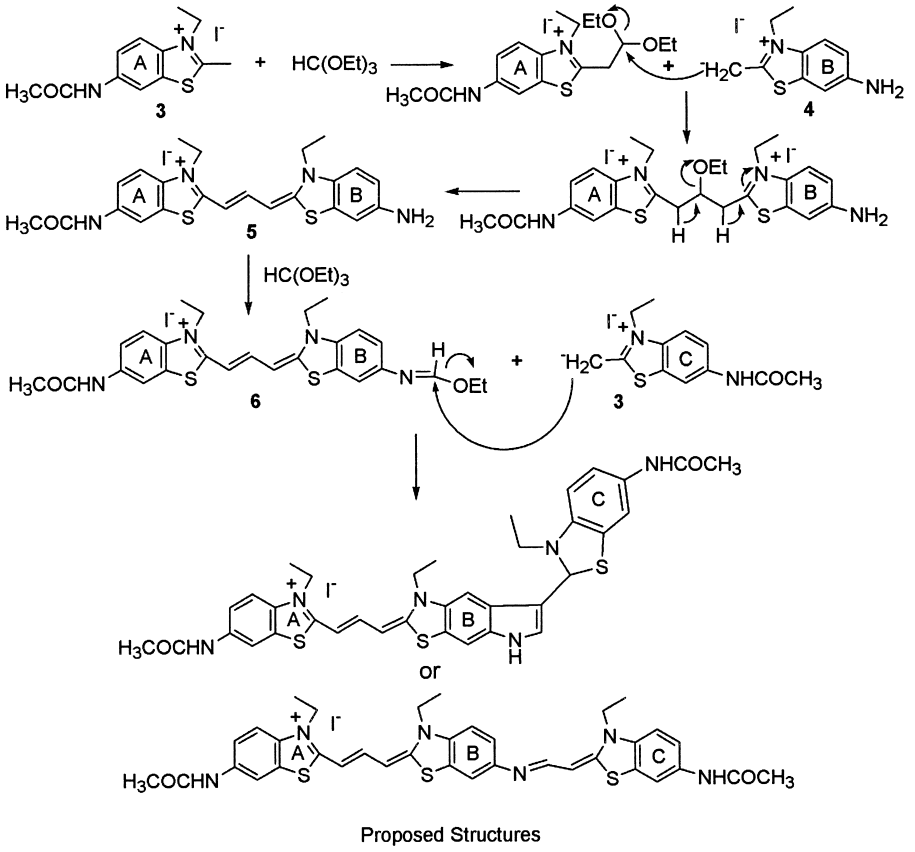


Figure 2. Proposed mechanism for formation of **2**.

2. Based on this analysis, we designed a synthesis shown in Scheme 1. The amino group in 2-methyl-benzothiazol-6-ylamine (**7**)[14] was protected with 9-fluorenylmethyl carbamate (Fmoc). The protected benzothiazole (**8**) was converted to the *N*-ethyl salt **9** and then the Fmoc protective group removed using piperidine to provide the required compound (**4**). Final reaction of acetylaminobenzothiazolium salt (**3**),[11] aminobenzothiazolium salt (**4**) and triethylortho-formate in nitrobenzene provided a mixture enriched in the potent compound **2**, which proved to be identical by HPLC and activity to the compound isolated earlier from **ZZZ21322**.

An HPLC analysis of the crude reaction mixture showed the presence of **2** along with some **1** and other impurities. Compound **2** was purified by preparative reverse phase HPLC. With more material in hand, several 2-D NMR and 1-D NMR experiments with pH titration were performed.[12] The 2-D TOCSY data was used to assign three aromatic rings (A, B, and C), three *N*-acetyl groups, three ethyl groups and two olefinic spin systems. The 2-D NOESY revealed the structural relationship between these units (Fig. 3). The one bond heteronuclear (^{13}C–^{1}H) correlation spectrum (2-D HMQC; Fig. 4) was used to assign protons which were in crowded aromatic

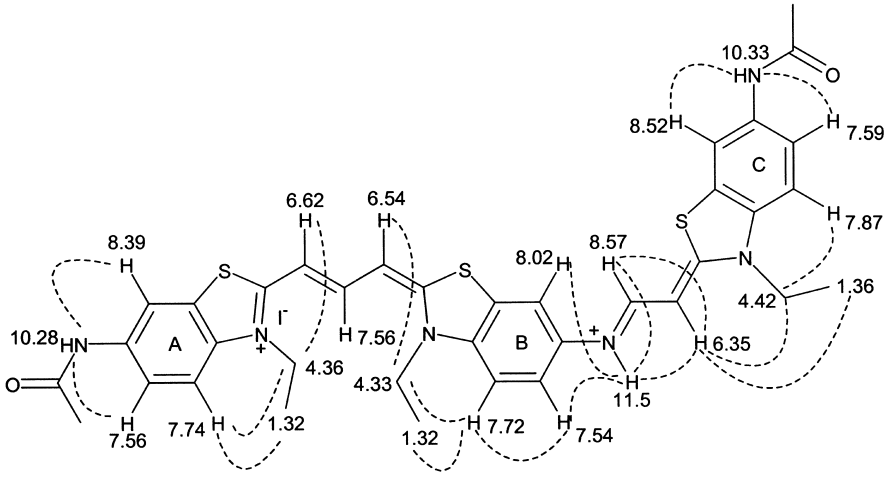


Figure 3. 2-D NOESY correlation in **2**.

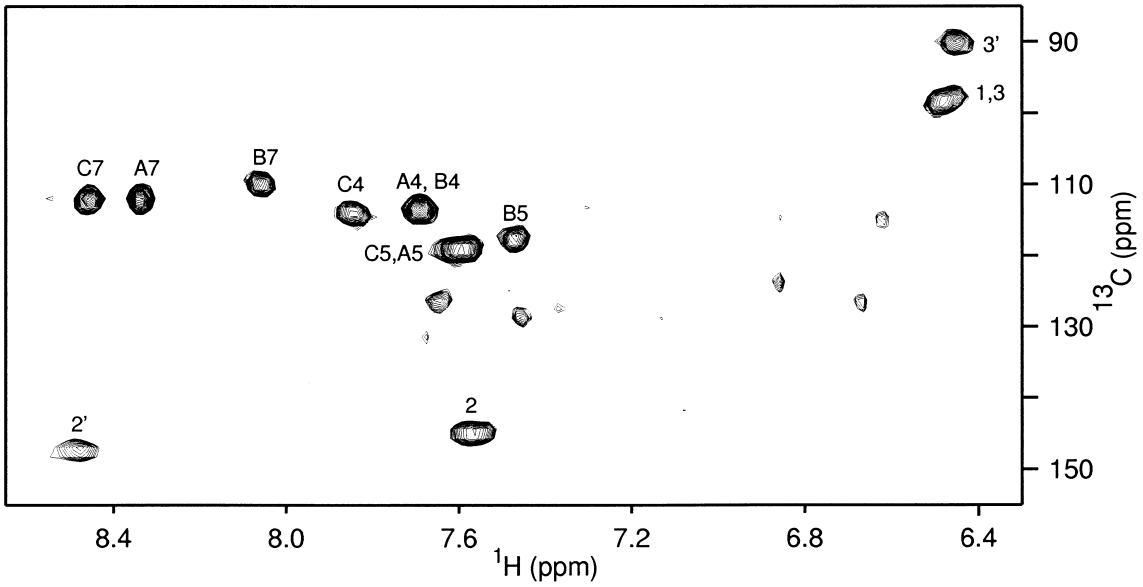


Figure 4. 2-D HMQC for compound **2** (downfield region; numbering is shown in Fig. 6).

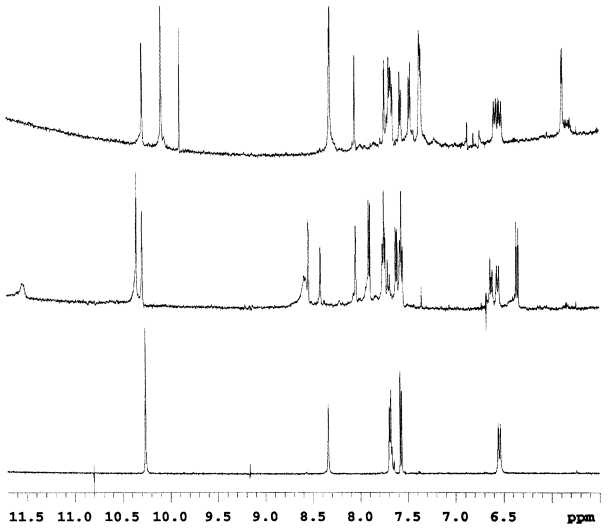


Figure 5. 1-D Proton spectra of compound **1** (bottom) and compound **2** at pH ~6.6 (middle) and pH ~8 (top).

regions and hard to decipher by TOCSY and NOESY. Long range heteronuclear (^{13}C–^{1}H) correlation spectrum (2-D HMBC) confirmed their relationship further and the final structure was established. It is interesting to note that 1-D proton spectra of **2** are concentration- and pH-dependent. The compound is a dye; it is highly colored and shares electronic charges across the whole molecule. The molecule stacks due to high conjugation, especially in more concentrated solutions (a 3 mM solution in DMSO shows very broad peaks). Compound **2** has a particularly labile NH proton when compared to **1**. The assignment of this iminium proton (δ 11.5; Fig. 5) was done by pH titration. When the pH was changed from acidic (pH 6.6) to basic (pH 8.1) the peak for this proton moved upfield and finally disappeared at pH ~10. This behavior was found to be reversible. The 2-D NOESY at low pH showed the correlation of the iminium proton with the most upfield olefinic proton (δ 8.57) and two aromatic protons (δ 8.02, 7.54; Fig. 3). Thus the imino N is protonated only at lower pH. This also explains the pH dependence of the 1-D spectrum of **2**.

Conclusion

All of the above data led to the confirmation of the proposed structure of an extremely potent P-selectin inhibitor **2** (Fig. 6). Compound **2** was found to be inactive in an E-selectin/PSGL-1 assay at concentrations of 200 μM. It was also inactive in numerous non-selectin based assays. This compound is undergoing further biological evaluation.

Experimental

***N*-(2-Methyl-benzothiazolyl-6-yl)-9-fluorenylmethyl carbamate (8).** 9-Fluorenylmethyl chloroformate (2.26 g, 8.72 mmol), 2-methyl-benzothiazol-6-ylamine (**7**, 1.3 g,

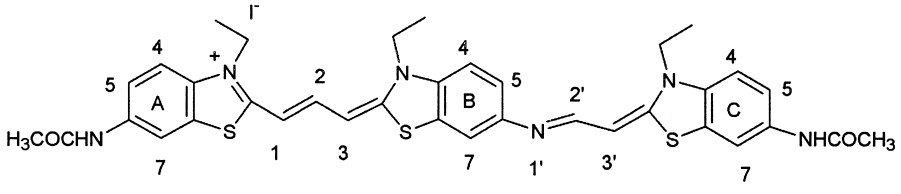


Figure 6. Final structure of compound **2**.

7.93 mmol)[14] and sodium bicarbonate (2.67 g, 31.72 mmol) were dissolved in a mixture of dioxane (8 mL) and water (6.7 mL). The reaction was stirred at rt for 15 h and then poured into water. The aqueous phase was extracted with ethyl acetate (3×20 mL). The organic extracts were dried ($MgSO_4$), filtered and concentrated in vacuo to give the crude product. Purification by flash silica gel chromatography (EtOAc/hexane, 1/3) and further crystallization from ethyl acetate/hexane furnished the carbamate **8** in 70% yield (2.14 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.81 (s, 3H, CH_3), 4.30 (t, 1H, J = 6.3 Hz, $CH(Ar)_2$), 4.60 (d, 2H, J = 6.2 Hz, OCH_2), 6.80 (br s, 1H, NH), 7.18 (br d, 1H, Ar), 7.34 (t, 2H, J = 7.1 Hz, Ar), 7.43 (t, 2H, J = 7.2 Hz, Ar), 7.64 (d, 2H, J = 7.6 Hz, Ar), 7.80 (d, 2H, J = 7.8 Hz, Ar), 7.83 (d, 1H, J = 9.2 Hz, Ar), 8.15 (br s, 1H, Ar).

6-(9-Fluorenylmethyl carbamate)-3-ethyl-2-methyl-benzothiazolium iodide (9). Ethyl iodide (8.3 mL, 104 mmol) and carbamate **8** (1 g, 2.59 mmol) were heated in a sealed tube at 110 °C overnite. The reaction was then cooled and diluted with ethyl acetate. The mixture was sonicated for 30 min and filtered. The solid obtained was the required salt **9** (1.2 g, 86%): ^{1}H NMR (300 MHz, DMSO) δ 1.43 (t, 3H, J = 6.9 Hz, CH_2CH_3), 3.14 (s, 3H, CH_3), 4.36 (t, 1H, J = 6.3 Hz, $CH(Ar)_2$), 4.60 (d, 2H, J = 6.1 Hz, OCH_2), 4.68 (q, 2H, J = 7.0 Hz, CH_2), 7.36 (t, 2H, J = 7.4 Hz, Ar), 7.44 (t, 2H, J = 7.4 Hz, Ar), 7.76 (m, 3H, Ar), 7.93 (d, 2H, J = 7.6 Hz, Ar), 8.23 (d, 1H, J = 8.9 Hz, Ar), 8.61 (br s, 1H, Ar), 10.32 (s, 1H, NH).

6-Amino-3-ethyl-2-methyl-benzothiazolium iodide (4).[15] Piperidine (0.89 mL, 9 mmol) and benzothiazolium salt (**9**, 0.96 g, 1.77 mmol)[14] were dissolved in DMF (4.4 mL). The reaction was stirred at rt for 1.5 h and then poured into a mixture of ethyl acetate (88 mL) and water (6.7 mL). The reaction was filtered and concentrated in vacuo to give the crude product. Sonicating the crude product with ethyl acetate for 30 min furnished the salt **4** in 79% yield (0.45 g): ^{1}H NMR (300 MHz, DMSO) δ 1.40 (t, 3H, J = 7.1 Hz, CH_2CH_3), 3.04 (s, 3H, CH_3), 4.60 (q, 2H, J = 7.1 Hz, CH_2), 6.06 (s, 2H, NH_2), 7.04 (dd, 1H, J = 1.8, 9.0 Hz, H-5), 7.29 (d, 2H, J = 1.8 Hz, H-7), 7.94 (d, 1H, J = 9.0 Hz, H-4).

Compound 2

N-Acetylaminobenzothiazolium salt (**3**, 0.135 g, 0.38 mmol),[11] aminobenzothiazolium salt (**4**, 0.06 g, 0.19 mmol) and triethylortho-formate (0.063 mL, 0.38 mmol) were dissolved in nitrobenzene (1 mL) and stirred at 130 °C for 1 h. The reaction was cooled and ether (15 mL) was added. The resulting suspension was stirred for 10 min and decanted. This process was repeated two more times. The residue obtained was purified by preparative reverse-phase HPLC using a C18 column (90 Å, 25 cm×2 in, flow rate 60 mL/min) eluting with a gradient starting from 50% of solvent A (0.1%TFA/H_2O) to 63% solvent B (0.1%TFA in 90% CH_3OH/10% H_2O v/v) in 88 min. The final compound obtained was a purple solid (0.051 g, 40%). ^{1}H NMR (600 MHz, DMSO) δ 1.32 (t, 6H, J = 7.4 Hz, 2×CH_2CH_3 (ring A, ring B)), 1.36 (t, 3H, J = 7.4 Hz, CH_2CH_3 (ring C)), 2.07 (s, 3H, $COCH_3$), 2.08 (s, 3H, $COCH_3$), 4.33 (q, 2H, J = 7.4 Hz, NCH_2 (ring B)), 4.36 (q, 2H, J = 7.4 Hz, NCH_2 (ring A)), 4.42 (q, 2H, J = 7.4 Hz, NCH_2, [ring C]), 6.35 (d, 1H, J = 12.5 Hz, $H_{3'}$), 6.54 (d, 1H, J = 11.8 Hz, H_3), 6.62 (d, 1H, J = 13.3 Hz, H_1), 7.54 (d, 1H, J = 8.0 Hz, H_{B5}), 7.56 (d, 1H, J = 8.0 Hz, H_{A5}), 7.56 (dd, 1H, J = 13.3, 11.8 Hz, H_2), 7.59 (d, 1H, J = 8.5 Hz, H_{C5}), 7.72 (d, 1H, J = 8.0 Hz, H_{B4}), 7.74 (d, 1H, J = 8.0 Hz, H_{A4}), 7.87 (d, 1H, J = 8.5 Hz, H_{C4}), 8.02 (s, 1H, H_{B7}), 8.39 (s, 1H, H_{A7}), 8.52 (s, 1H, H_{C7}), 8.57 (br s, 1H, $H_{2'}$), 10.28 (s, 1H, $NHCOCH_3$, [ring A]), 10.33 (s, 1H, $NHCOCH_3$, (ring C)), 11.5 (br s, 1H, $NH^+_{11'}$). ^{13}C NMR (150 MHz, DMSO) δ 12.26 (2×CH_2CH_3 (ring A, ring B)), 12.20 (CH_2CH_3 (ring C)), 23.64 ($COCH_3$), 23.69 ($COCH_3$), 41.14 NCH_2 (ring A, ring B)), 42.00 NCH_2 (ring C)), 89.94 ($C_{3'}$) 97.70 (C_1), 97.76 (C_3), 109.61 (C_{B7}), 111.92 (C_{A7}), 111.94 (C_{C7}), 113.23 (C_{A4}, C_{B4}), 114.02 (C_{C4}), 117.30 (C_{B5}), 118.39 (C_{A5}), 119.59 (C_{C5}), 144.87 (C_2), 147.10 ($C_{2'}$). HRMS (FAB) calcd for $C_{36}H_{37}N_6O_2S_3$ $(M)^+$: 681.21401, found: 681.21400.

ELISA assay

P-selectin (a soluble construct consisting of the entire extracellular domain expressed and purified from transfected CHO cells) was suspended at a concentration of 5 μg/mL in coating buffer (10 mM MOPS, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.5) and applied to a 96-well microtiter plate (50 μL/well). After overnight incubation at 4 °C, the wells were washed four times with wash buffer (coating buffer containing 0.05% Tween 20). Wells were then treated with blocking buffer (wash buffer + 0.1% gelatin) for 60 min at rt. After removal of blocking buffer, a PSGL-1/IgG construct $(148.Fc)^2$ suspended at 1 μg/mL in wash buffer was applied (100 μL/well) in the presence or absence of inhibitor and incubated for 30 min at rt. After washing of the microtiter plate wells, a complex of biotinylated goat antihuman IgG/streptavidin alkaline phosphatase (both from Caltag, each at 1/1000 dilution, precomplexed for 60 min) in wash buffer was applied and reacted for 60 min at rt. The wells were washed and reacted with PNPP (Pierce) at a concentration of 1 mg/mL in 10 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5. The absorbance at 405 nm was recorded after approximately 20 min. IC_{50} values were calculated by determining the concentration of inhibitor which decreased UV absorbance of uninhibited controls (typically OD of 2.0) by 50% after subtracting non-specific binding signals in control wells (typically less than 0.1 OD).

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12. All spectra were carried out on a Varian Unit + 600 spectrometer with triple-resonance probe with an actively shielded Z-gradient coil and pulsed field gradient accessories. All samples were dissolved in DMSO-d_6 or 10% DMSO-d_6 aqueous solution, with concentration at 0.2–0.4 mM, recorded at 25 or/and 35 °C. The chemical shifts are expressed relative to DMSO for ^{1}H NMR (at δ 2.5) and ^{13}C (at δ 39.91). NOESY, TOCSY (total correlation spectroscopy MLEV17), HMQC (heteronuclear multiple quantum coherence) and HMBC (heteronuclear multiple bond coherence) experiments were performed using standard Varian and homemade PFG pulse sequences.[16] For TOCSY experiments, 30 ms of spinlocking time and for NOESY experiments 600 ms of spin mixing time was used. All 2-D experiments were collected through States TPPI phase cycling. With the exception of 1-D proton NMR, where Varian VNMR software on the SUN computer was used, all 2-D data were processed and displayed on SGI workstation using the processing program packages NMRDraw and NMRPipe. A 60° phase-shift sine-bell weighting function and zero-filling were used in each dimension prior to Fourier transformation. All data analysis, spectra peak picking and plotting were performed with the psc and pipp programs.[17]
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