Supplementary Material (ESI) for Medicinal Chemistry Communications

Electronic Supplementary Information

Interactions of natural polysulfanes with components of red blood cells

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Synthesis of compounds

Chemical reagents were purchased from Sigma-Aldrich-Fluka (Darmstadt, Germany) and used without further purification unless stated otherwise. AM, DAS, DADS and 1,9-decadiene were also purchased from Sigma Aldrich-Fluka (Darmstadt, Germany). AM and DADS were purified by distillation under reduced pressure before use. Commercial DAS and 1,9-decadiene had a purity of more than 98 % and were used without further purification. DATS, DPTS, DATTS and DPTTS were synthesized and purified according to the procedures of Derbessy *et al.* and Milligan *et al.*.[1, 2] Pure compounds were stored at -80 °C and dissolved in DMSO before use.

DAS: diallylsulfide; DADS: diallyldisulfide; DATS: diallyltrisulfide; DATTS: diallyltetrasulfide; DPTS: dipropyltrisulfide; DPTTS: dipropyltetrasulfide; AM: allylmercaptan; Dec: 1,9-decadiene.

In vitro activity assays

Buffers:

A physiological solution (buffer A) was prepared with NaCl (140 mM), KCl (7.5 mM), HEPES (10 mM) and glucose (10 mM) at pH 7.4 (pH adjusted using 0.1 M NaOH). This solution was used for the studies involving the RBCs. For the phophatidylserine (PS) exposure measurements, buffer B was prepared with NaCl (145 mM), HEPES (10 mM) and $CaCl_2$ (2.5 mM) at pH 7.4 (pH adjusted using 0.1 M NaOH).

Washing of RBCs:

The human blood samples were obtained from the Institute of Clinical Haematology and Transfusion Medicine of Saarland University Hospital. All donors had provided written informed consent under a protocol according to the regulations of the Declaration of Helsinki. Blood was centrifuged at 2,000 g for 5 min at room temperature. Plasma was removed by aspiration. The RBCs were washed 3 times in physiological solution for removing the buffy coat. Finally, the RBCs were resuspended in physiological solution and the experiments were started immediately.

Haemolysis assay:

This assay is based on the oxidation of haemoglobin and its derivatives to methaemoglobin in the presence of alkaline potassium ferricyanide (Drabkin's reagent; Merck). Methaemoglobin then reacts with potassium cyanide to form cyanomethaemoglobin. The color intensity of cyanomethaemoglobin measured at 540 nm is proportional to the total haemoglobin concentration.

DMSO was used as a solvent for preparing 1 mM stock solutions of the compounds. The experimental samples for the blood assay contained buffer A, stock solution of each compound and 2.5 % haematocrit (25 µl of the RBCs sediment after centrifugation at 12,000 g). The final concentrations of the compounds tested were as follows: 1 µM, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 250 µM and 500 µM. A negative control was used consisting of buffer A and 2.5 % haematocrit. The mixtures were vortexed well. After incubation of the RBCs with test compounds for 30 min at 37 °C in a water bath, the solutions were centrifuged at 12,000 g for 5 min. Then the supernatant was transferred into 4 ml of Drabkin's solution and incubated at room temperature for 30 min in the dark. After 30 min incubation at room temperature, the absorption at 540 nm was measured by using a UV-Vis spectrophotometer (UV mini 1240, UV-Vis Spectrophotometer, Shimadzu). At least 3 different blood samples were used for each experiment.

UV/Vis measurements

UV/Vis spectra were recorded on a CARY 50*Bio* UV-Visible spectrophotometer (Varian Inc.). A quartz cuvette was used (1 cm path length) and the spectra were recorded from 200 to 700 nm. Please note that the absorbance measured with this particular instrument is linear up to at least 4 absorbance units.

Determination of the exposure of phosphatidylserine (PS) on the outer leaflet of the RBC membrane

The presence of PS on the outer leaflet of the RBCs membrane surface is quantified based on the binding of PS with Annexin V-FITC (Invitrogen, Germany).[3-5] The compounds were prepared and incubated with the RBCs according to the procedure used in the haemolysis assay. The haematocrit value was 1.0 % instead of 2.5 %. After 30 min incubation at 37 °C in the water bath the solutions were centrifuged at 12,000 g for 5 min and then washed with buffer A. Subsequently, 400 µl of buffer B and 3 µl of Annexin V-FITC were added to each sample. The samples were mixed well and incubated at room temperature for 20 min in the dark. After incubation, the samples were put on ice and analysed with FACS (FACScalibur 4CS E4021, Becton and Dickinson and CellQuest software). The excitation and emission wavelengths of Annexin V-FITC are 488 nm and 530 nm, respectively. Annexin V-FITC is excited by an argon laser; the fluorescence is detected using a 530/30nm band pass filter. For each measurement, 30,000 RBCs were counted. At least 3 different blood samples were used for each experiment. The Annexin V positive RBCs can be calculated in percentage by comparison of positive and negative signal events with the control. Cell Quest Pro software was used for data acquisition and analysis.

Determination of Ca^{2+} uptake in RBCs

To investigate the influence of the substances on the free intracellular Ca^{2+} level in RBCs, the washed RBCs were suspended in buffer A at 1.0 % haematocrit with Fluo-4 (Molecular Probes, Germany). The cell suspension was mixed well by vortexing and incubated for 45 min at 37 °C with occasional shaking. After incubation the cells were washed 3 times with buffer A by quick centrifugation (20 s, 12,000 g) and re-suspended in buffer A. Before starting the experiment the substances and also 2 mM extracellular Ca^{2+} were added, mixed well and then the fluorescence intensity of Fluo-4 inside the RBCs was measured with FACS at room temperature. For the control, the fluorescence intensity of Fluo-4 was measured under physiological conditions in the presence of 2 mM extracellular Ca^{2+}. For each measurement, 30,000 RBCs were counted. At least 3 different blood samples were used for each experiment. The data were analysed using BD Cell Quest Pro Software.

Determination of log*P* values via HPLC-MS

The log*P* values (*P* capacity factor) of DAS, DADS, DATS, DPTS, DATTS, DPTTS, AM and Dec were determined experimentally in triplicate by RP-HPLC (ThermoScienfic Spectrasystem AS3000) on a C_{18}-boned column (Agilent Eclipse XD13-C18, pore diameter 5 μm, dimensions 4.6 X 150 mm) with a UV single wavelength detector (λ = 254 nm).[6, 7] The mobile phases were mixtures of MeOH and Millipore Q grade water. The fraction of MeOH/water was reduced gradually (10 % per run) from 100 % MeOH to 40 % MeOH. The test samples were prepared with these different percentages of MeOH/water in order to maintain the polarity of the eluent. Here, a stock solution of 10 mM of compound in HPLC-grade MeOH was prepared for each compound. This stock

solution was used to prepare 1 ml samples of compounds with a final concentration of 1 mM and different MeOH/water percentages. Before each run, the column was washed with the mobile phase for 5 min. The flow rate was 800 µl/min and the injection volume was 10 µl.

Calculated logP values:

The theoretical log*P* values of the various compounds were calculated by using an online software (ALOGPS 2.1 Program). This online software calculates the expected log*P* for a compound by nine different individual software programs and presents the average log*P* ± SD.[8, 9] In this case, the standard deviation is due to the circumstance that the different software programs result in slightly different calculated values which are subsequently averaged.

Docking studies

All ligands were constructed with SYBYL (*SYBYL 7.0* [Computer Program]. Tripos Inc., 1699 South Hanley Rd. St. Louis, MO: 2007) and subsequently energy minimized with the Tripos Force field[10] including electrostatic interactions based on calculated Gasteiger partial charges for both the ligands and the protein.[11, 12] Basis for the docking studies was the X-ray structure of the haemoglobin (from *homo sapiens*) with haem ligand in a complex with a potent anti-sickling agent, INN-298 (PDB code 3ic0, Osheiza *et al.* to be published). This structure was downloaded from the protein database (http://www.rcsb.org/pdb/home/home.do).[13] The docking studies were performed using PLANTS.[14-16] This docking program allows for some flexibility of amino acid side chains of the protein active sites during the docking procedure. In all cases, the active site was

defined by a radius of 15Å from the co-crystallized iron atom. The side chains of Leu28, His63, Val67, Phe71, Leu31, Phe42, Phe45, and Leu106 were considered to be flexible. From the resulting docking arrangements, the most favored ones were refined by energy minimization using MOE (Molecular Operating Environment, Chemical Computing Group Inc., Montreal, Canada: 2009) and subsequent prediction of the pK_i-value available in the ligX-module of MOE was carried out.

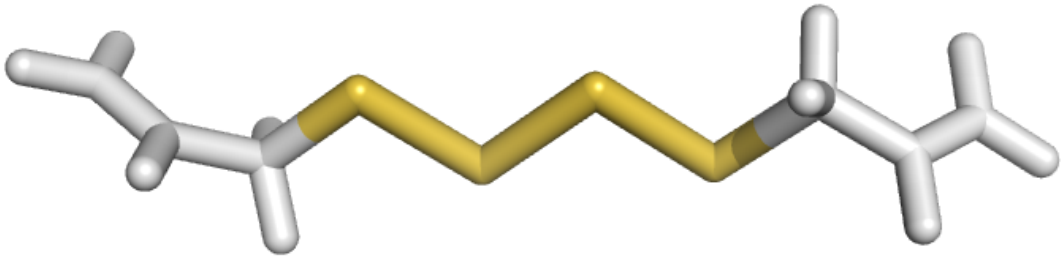
Construction of compounds

All compounds were constructed using Maestro 8.0.314, the molecular graphics surface of the Schroedinger software package (Schroedinger, LLC, 120 West 45th Street, New York, NY 10036). Subsequently, all structures were energy optimized with JAGUAR 6.0 (Jaguar 6.0. 2007. Schroedinger, LLC, 120 West 45th Street, New York, NY 10036) using DFT[17, 18] with B3LYP functionals[19-22] and HF 6-31G**+ basis sets either for closed shell systems with restricted (RODFT) or for the radicals with spin-unrestricted (UDFT) calculations allowing a maximum of 2,000 geometry optimizations until the energy change is not larger than 5.0 e^{-5} Hartree.

Figure S1: Energy minimization between the linear and the screw forms.

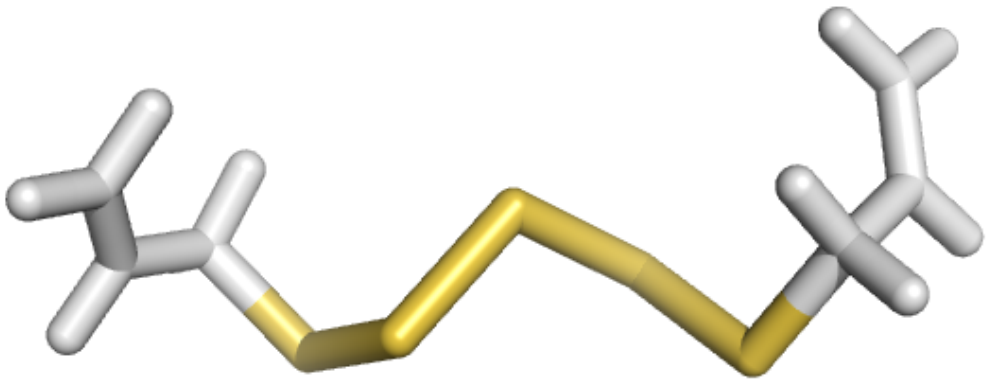
a) DATTS

For the screw form see the manuscript.

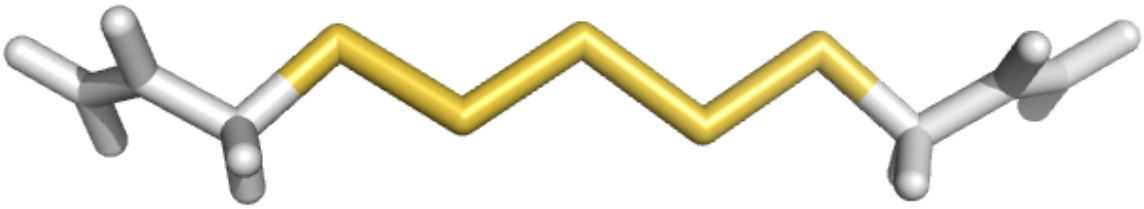


The all-*trans* conformation of DATTS which is 114 kJ/mol less stable than the most stable helical one.

b) Diallylpentasulfide

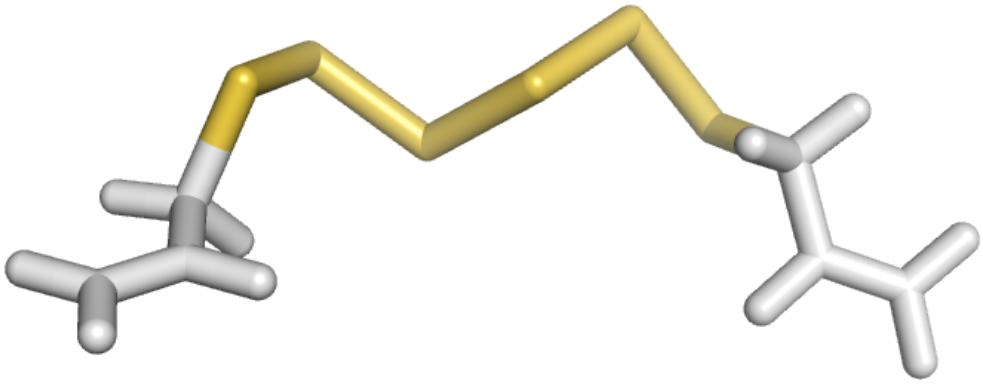


The most stable helical conformation of diallylpentasulfide. All dihedral angles have a value of -90.0°.

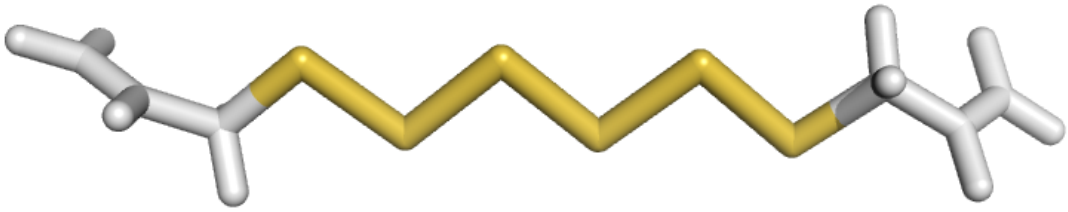


The all-*trans* conformation of diallylpentasulfide which is 157 kJ/mol less stable than the most stable helical one.

c) Diallylhexasulfide



The most stable helical conformation of diallylhexasulfide. All dihedral angles have a value of -90.0°.



The all-*trans* conformation of diallylhexasulfide which is 186 kJ/mol less stable than the most stable helical one.

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