The reactivity of $[Fe^{II}(CN)_4(CO)_2]^{2-}$ and its use as a building block for solid-state materials are under active investigation.

Experimental Section

$Na_2(dmf)_4$-**1**: An aqueous solution (50 mL) of NaCN (0.417g, 8.51 mmol) was added dropwise to an aqueous solution (50 mL) of $FeCl_2 \cdot 4H_2O$ (0.422 g, 2.12 mmol) under a CO atmosphere. The reaction mixture was stirred under a CO atmosphere for 18 h. The water was removed under vacuum, and the residue was extracted with 30 mL of DMF. The product (0.520 g, 44% yield) was obtained by the addition of diethyl ether, filtration, and washing with cold ethanol.

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Synthesis, Biological, and Immunological Properties of Cyclic Peptides from *Plasmodium Falciparum* Merozoite Surface Protein-1**

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Malaria is the most important parasitic disease in humans, and in 1997 a malaria risk of varying degrees existed in 100 countries. In 92 of these, transmission included the malignant (*Plasmodium falciparum*) form of the disease, which causes 1.5 to 2.7 million deaths each year.

In spite of intensive efforts, not a single vaccine against a human parasitic disease is currently commercially available, and only one vaccine candidate, namely Spf66,[1] reached phase III clinical trials. Most efforts to develop synthetic peptide vaccines have focused on the use of short, linear B-cell epitopes to elicit the desired immune response. However, linear peptides are inherently flexible molecules and are proteolytically unstable in serum.[2] Conformationally restricted cyclic peptides have the potential to preserve the original secondary structure of the native proteins and in many cases this has been shown to constitute an important feature for a peptide to behave as a good immunogen.[3] Furthermore, it has long been known that the cyclization of a peptide may induce higher binding affinities and a greater degree of specificity in peptide–receptor interactions.[4]

The invasion process of malaria merozoites into human red blood cells (RBCs) is a crucial step in the life cycle of the parasite in the vertebrate host and gives rise to the blood stage part of the parasite cycle, which is responsible for the clinical manifestations of malaria.[5] The rationale of our approach was the identification of small, structurally defined peptide structures that mimic the interaction of the parasitic Merozoite Surface Protein 1 (MSP-1) from *P. falciparum* with the human RBC. Induction of antibodies to such mimics may be useful for studying and blocking these interactions.

To better characterize ligand–receptor interactions that are involved in the invasion process of RBCs, we studied the effect of the introduction of conformational constraints into a linear lead peptide. In parallel, we also studied the effect of peptide cyclization on epitope immunogenicity.

For this purpose, the central part of the 42-61 N-Terminal MSP-1 fragment (Peptide 1513: GYSLFQKEKMVL-NEGTSGTA) was cyclized by using a modified version of the backbone cyclization method.[6] The peptide 1513 plays a decisive role in the recognition of RBC receptors by malaria

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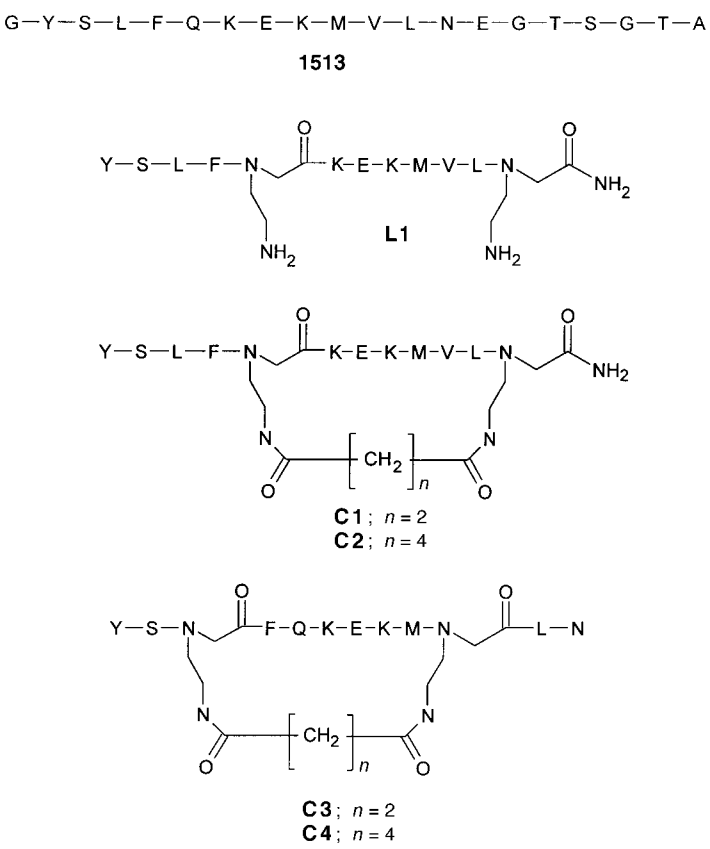
parasites, and previous studies[7] showed that this linear peptide binds to RBCs with a 200 nM affinity.

To determine which amino acid (AA) residues are essential for ligand–receptor interactions within the linear lead peptide, we substituted each AA by alanine (Alan scan) and determined the RBC binding affinity of the resulting peptides by measuring their ability to inhibit the binding of the original peptide (binding inhibition assay). The alanine analogues were synthesized by Boc-SPPS (SPPS = solid-phase peptide synthesis, Boc = *tert*-butoxycarbonyl) by using 4-methylbenzhydrylamine (4-MBHA) resin and *N*,*N*′-dicyclohexylcarbodiimide/1-hydroxy-1*H*-benzotriazole activation. Free peptides were obtained by cleavage with HF, purified by dialysis and analyzed by RP-HPLC and MALDI-TOF MS (matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry). The lead peptide 1513 was radiolabeled with $Na^{125}I$ by using the methodology of Yamamura et al.[8] Human RBCs (2×10^7) were incubated for one hour at room temperature with ^{125}I-radiolabeled peptide 1513 (10 nM) in the absence or presence of each Ala analogue (at 10, 100, and 800 nM) as described earlier.[9] Results obtained by using 100-nM unlabeled peptide 1513 are shown in Figure 1. Analogues with substitutions in the central part (…Q**K**E**KM**VL…), especially of the AAs highlighted in bold, were not able to inhibit the binding of the original peptide, demonstrating the importance of these residues for the binding process.

Based on these results, we designed a set of cyclic peptides that contain the essential AAs involved in the ligand–receptor interaction of MSP-1 with the RBC. To introduce



Scheme 1. Linear and cyclic peptide analogues of the AA 42-61 N-terminal MSP-1 fragment.

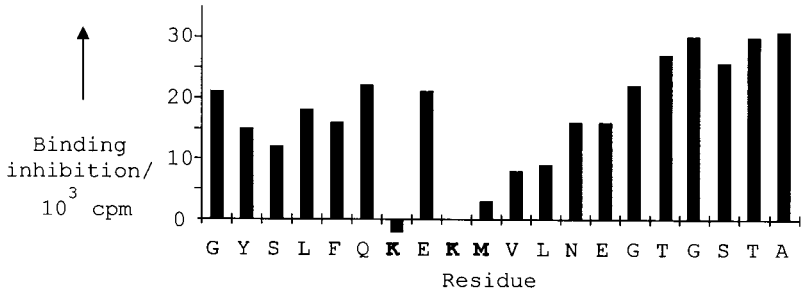


Figure 1. Inhibition of RBC binding of peptide 1513 by its Ala analogues (Ala-Scan).

conformational restraints, peptide cyclization was accomplished by the introduction of two aminoethylglycine subunits, which in all cases are separated by seven AAs. These two subunits were linked by diacid bridges of varying length, resulting in cyclic analogues **C1**–**C4** and the linear control peptide **L1** (Scheme 1).

Fmoc-*N*-(2-aminoethyl)-Boc-glycine (**1**; Fmoc = 9-fluorenylmethoxycarbonyl), which is necessary for the cyclization of resin-supported peptides, was obtained from the previously described[10] Fmoc-*N*-(2-aminoethyl)glycine-*tert*-butylester (**5**) in two steps (Scheme 2). First, the carboxy group was deprotected with 100% TFA, and then a Boc group was introduced at the secondary amine functionality by the reaction of **6** with Boc anhydride in an acetone/water mixture. The bridging monomer **2** was obtained by the reaction of succinic anhydride and 9-fluorenylmethanol (FmOH). Monoesterification of excess DCC/DMAP-activated adipic acid with 9-FmOH yielded bridging monomer **3** (Scheme 2).

All cyclic peptides were obtained by using a solid-phase variation of the backbone-to-backbone cyclization strategy developed by Bitain et al.[6] The peptides were synthesized on a 4-MBHA resin by using a combined Boc/Fmoc strategy (Scheme 3). The peptide chains were elongated by a Boc strategy with HATU activation, whereas the introduction of the bridging elements as well as peptide cyclization was performed by a Fmoc cycle and HATU activation. The peptides were cleaved from the solid support by liquid HF following a low/high protocol. Crude peptides were purified by RP-HPLC and analyzed by RP-HPLC and MALDI-TOF MS. All peptides were obtained in >80% yield and high purity (>95%).

Peptide polymers for immunization studies were obtained from monomeric peptides carrying additional N- and C-terminal CG or GC residues, respectively. The polymerization was accomplished by cysteine oxidation with oxygen at pH 7.4. Peptides for RBC binding studies were radiolabeled with $Na^{125}I$ by using the chloramine T method.[8] To determine binding dissociation constants (K_D), 2×10^7 human RBCs were incubated with increasing concentrations of radiolabeled peptide, as previously described.[9] The affinity constants (K_D) and Hill coefficients were calculated by using Scatchard

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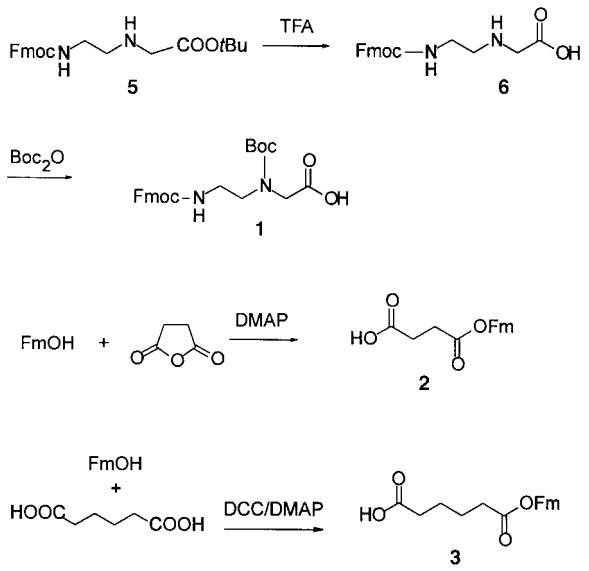
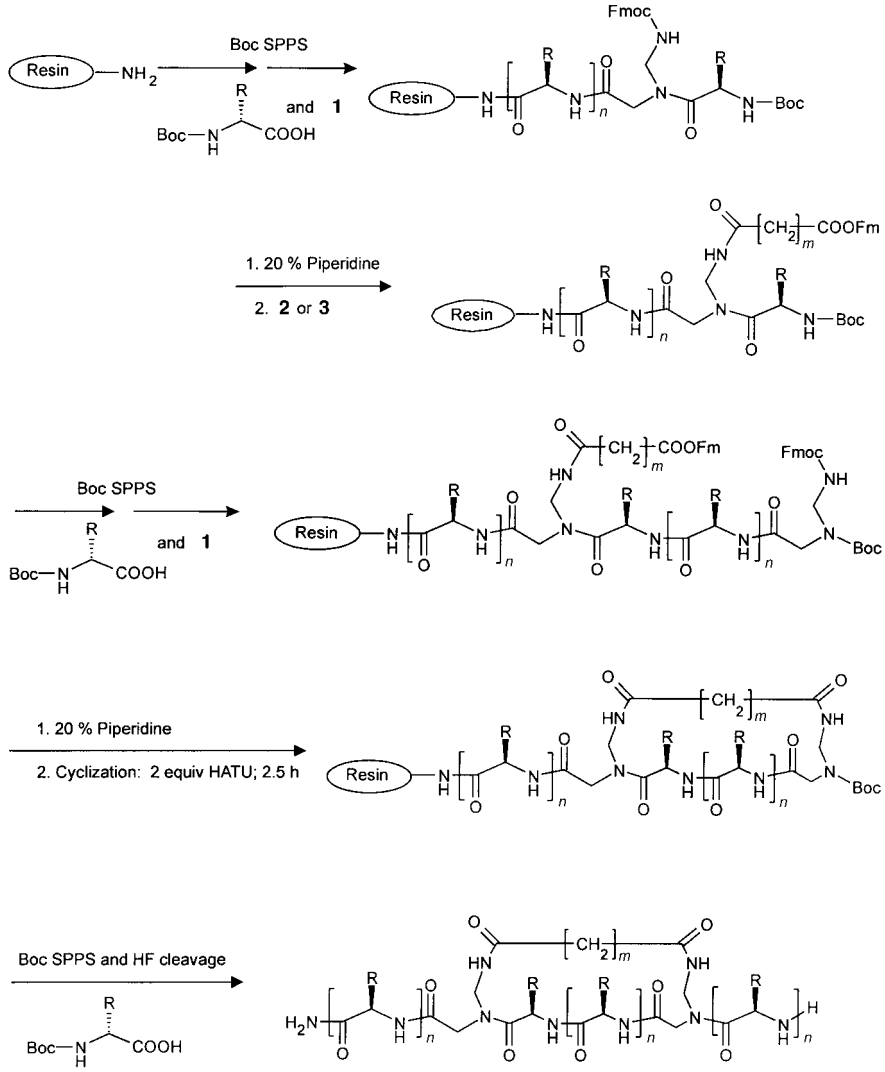


Scheme 2. Synthesis of the modified AA **1**, and of bridging alkyldiacid derivatives **2** and **3** for the SPPS of N-backbone cyclic peptides.



Scheme 3. General strategy for the synthesis of N-backbone cyclic peptide analogues of the AA 42-61 N-terminal MSP-1 fragment. HATU = *N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]-pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate.

analysis.[9] All cyclic peptides showed high binding affinities to RBCs. Cyclic peptide **C1** ($K_D = 470$ nM) had the highest affinity, and its corresponding linear control **L1** had the lowest ($K_D = 2.75$ μM) (Figure 2).

The interaction of the linear peptide 1513 and its cyclic analogues with receptors on the RBC surface was further analyzed by gel autoradiography. For this purpose, RBC membrane preparations from the binding assay were used. Radiolabeled peptides were cross-linked with receptors on the RBC by using bis(sulfosuccinimidylsuberate) (10 μM) as described earlier.[11]

Membrane proteins were separated by using SDS PAGE (SDS PAGE = sodium sulfate dodecyl polyacrylamide gel electrophoresis) and the resulting gel was used to expose a Kodak X-OMAT film, which was developed autoradiographically. Figure 3 shows that RBC membrane preparations with cyclic peptides displayed only one sharp radioactive band with an approximate molecular weight of 19 kDa. In contrast, the linear peptide 1513 preparation showed a diffuse band at the same molecular weight, indicating that cyclic peptides interact with higher specificity with the RBC membrane.

For the characterization of the immunogenicity of the cyclic peptides, *Aotus* monkeys were immunized with each of the polymeric peptide 1513 analogues as well as with the polymeric parent peptide and the linear control **L1**. Four monkeys per group were immunized by subcutaneous inoculation of three 125-μg peptide doses at 20-day intervals. Antibody titres determined in sera of the immunized monkeys by ELISA (ELISA = enzyme-linked immunosorbent assay) showed that none of the linear peptides induced a humoral immune response, whereas all cyclic analogues induced high antibody titres (ranging from 1:16 000 to 1:256 000). Immune sera from all four *Aotus* monkeys immunized with the cyclic peptide **C2** were able to recognize in Western blots with blood stage parasite lysates MSP-1 processing products of a molecular weight of 42 and 83 kDa (Figure 4), which contain the peptide 1513 sequence and are displayed on the surface of the parasites in the late schizont stage.[12]

To further substantiate that the cyclic peptide **C2** can induce parasite-binding antibodies, spleen cells of **C2**-immunized mice were used to generate B cell hybridomas by the polyethylene glycol fusion method.[13] Of the eight **C2**-specific monoclonal antibodies identified by ELISA, two (designated S1.2 and S1.7) exhibited binding to the N-terminal portion of MSP-1 (AA 34-595 from *P. falciparum* strain MAD-20 recombi-

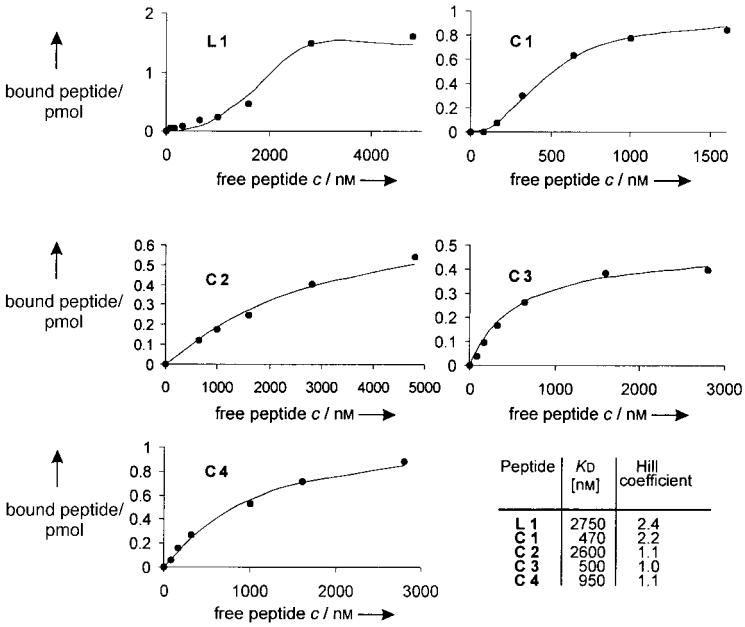


Figure 2. RBC binding of cyclic peptides C1 – C4 and of the linear peptide L1. Saturation curves, K_D values, and Hill coefficients shown in the table were calculated by using Scatchard analysis.

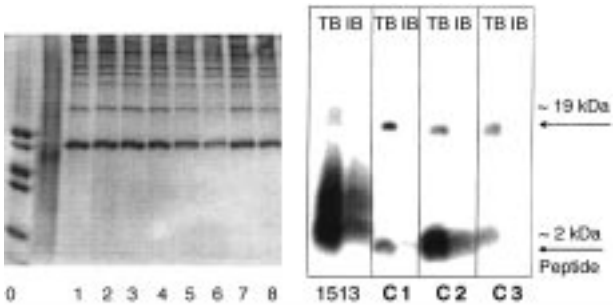


Figure 3. Analysis of peptide binding to RBC cell-surface proteins by SDS PAGE and autoradiography. RBCs were incubated with radiolabeled peptides 1513, **C1**, **C2**, and **C3**. After cross-linking with bis(sulfosuccinimidylsuberate) (10 μM), membrane proteins were separated by SDS PAGE (left side). The corresponding gel autoradiography is shown on the right side. 0 = MWM (molecular weight marker), 1 = 1513 TB (total binding), 2 = 1513 IB (inhibited binding, with 100-fold excess of unlabeled peptide), 3 = **C1** TB, 4 = **C1** IB, 4 = **C2** TB, 5 = **C2** TB, 6 = **C2** IB, 7 = **C3** TB, 8 = **C3** IB.

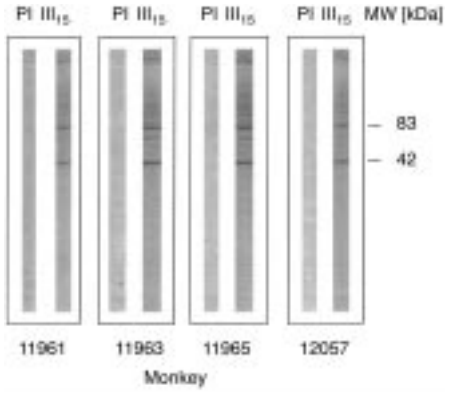


Figure 4. Western blot analysis of solubilized antigens obtained from late stage *P. falciparum* schizonts by using sera from *Aotus* monkeys immunized with cyclic peptide **C2**. PI = pre-immune sera; III_{15} = sera taken 15 days after third dose.

nantly expressed in *E. coli*) in ELISA (data not shown). The same two monoclonal antibodies also exhibited binding to malaria blood stage parasites in an indirect immunofluorescence assay (Figure 5C), whereas the other six **C2**-specific monoclonal antibodies did not bind (Figure 5A).

In summary, the small cyclic peptides presented in this work are able to effectively mimic an important structural element of MSP-1. The 12-mer cyclic peptides **C1** – **C3** showed an increased specificity in ligand – receptor interactions with a 19 kDa receptor on the RBC membrane, when compared with the larger 20-mer parent peptide. The ability of antibodies induced against cyclic peptides to recognize *P. falciparum* MSP-1 on the cell surface of blood stage parasites indicates that they are an effective structural mimic of a functionally important MSP-1 epitope. Cyclic peptides may thus be useful in the design of epitope-focussed synthetic peptide vaccines.

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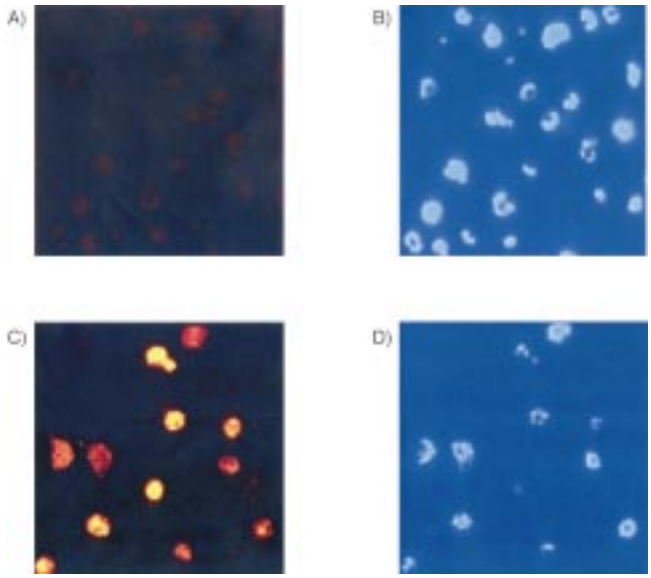

Figure 5. Indirect immunofluorescence staining of *P. falciparum* blood stage parasites by using Cy-3 conjugated goat anti-mouse IgM-specific antibodies for detection of bound antibody. As a control, parasite DNA was counter-stained with Hoechst 33285 (B and D) as described.[13] C) Shows staining of parasites by the anti-**C2** monoclonal antibody S1.2. In contrast, monoclonal antibody S1.8 did not bind to MSP-1 (A).

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