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Pseudoprolines: Targeting a *cis* Conformation in a Mimetic of the gp120 V3 Loop of HIV-1**

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Pseudoprolines (ΨPro) have been introduced recently as synthetic proline analogues readily obtained by cyclocondensation of the amino acids cysteine, threonine, or serine with aldehydes or ketones.[1] Their application as structure-disrupting, solubilizing protecting groups in solid-phase peptide synthesis[2, 3] was followed by conformational investigations concerning the ΨPro preceding peptide bond.[4, 5] In fact, the propensity of the amino acid proline for forming a Xaa_{i-1}-Pro_i

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cis peptide bond can be strongly enhanced by the introduction of a pseudoproline residue, depending on its C(2) substituents. In particular, the 2,2-dimethylated thiazolidine derivative can induce up to 100% *cis* conformation in the preceding peptide bond in model di- and tripeptides.[4, 5] We herein report the first example of this class of pseudoprolines as mimetics of biologically relevant *cis*-prolyl conformations.

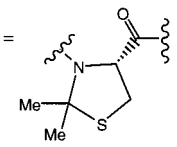
An interesting target for the introduction of a *cis*-inducing pseudoproline unit is the V3 loop of the envelope protein gp120 of immunodeficiency virus type 1 (HIV-1), which has been the subject of numerous studies since it was shown to correspond to the major HIV-1 neutralizing epitope.[6–8] Proteolytic cleavage of V3, after binding of gp120 to the cell surface receptor CD4, has been suggested as being a prerequisite for viral infection.[9, 10] A generally conserved tetrapeptide motif Gly-Pro-Gly-Arg is situated at the tip of the V3 loop, forming a type II β-turn.[11] Johnson et al. proposed that a *trans*/*cis* isomerization towards a type VI β-turn with a *cis*-proline peptide bond is a necessary conformational change for cleavage and subsequent fusion.[12] Other indications of the importance of conformational changes in gp120 preceding infection have recently appeared,[13, 14] and a type VI β-turn conformation has been observed in a peptide derived from the HIV-1_{IIIB} V3 loop when bound to an anti-gp120 antibody.[15] Here, we have targeted the proposed infection-active *cis* conformation by preparing pseudoproline-containing, *cis*-constrained V3 loop analogues as immunogens.

The eleven-residue cyclic loop structure cyclo(-Arg-His-Ile-Gly-Xaa-Gly-Arg-Ala-Phe-Cys-Tyr-) with a sequence based on the HIV-1_{MN} V3 variant and containing the tetrapeptide motif Gly-Xaa-Gly-Arg, was selected as a base for the mimetic (Figure 1). A cysteine was incorporated as a useful linker site for immunological studies. Three cyclic V3-peptides were synthesized, a constrained pseudoproline-containing mimetic (Xaa = Cys($\Psi^{Me,Me}$pro), **I**^{c}), a proline-containing loop analogue (Xaa = Pro, **II**^{c}) similar to the native structure, and a peptide containing Aib (α-aminoisobutyric acid) replacing Pro (Xaa = Aib, **III**^{c}). In addition, the corresponding linear peptides **I**^{l} and **II**^{l} (Figure 1) were prepared for determining the impact of the cyclic structure upon the immunogenic properties of the peptides. In Figure 2, the Gly-ΨPro and Gly-Pro regions of peptides **I**^{c} and **II**^{c} are represented, showing the *cis* and *trans* peptide bond.

cyclo(-Arg-His-Ile-Gly-Xaa-Gly-Arg-Ala-Phe-Cys-Tyr-) **I**^{c}, **II**^{c}, **III**^{c}

Ac-His-Ile-Gly-Xaa-Gly-Arg-Ala-Phe-Cys-NH_2 **I**^{l}, **II**^{l}

I Xaa =



II Xaa = Pro

III Xaa = Aib (α-aminoisobutyric acid)

Figure 1. Sequences of cyclic (**I**^{c}, **II**^{c}, **III**^{c}) and linear (**I**^{l}, **II**^{l}) peptides.

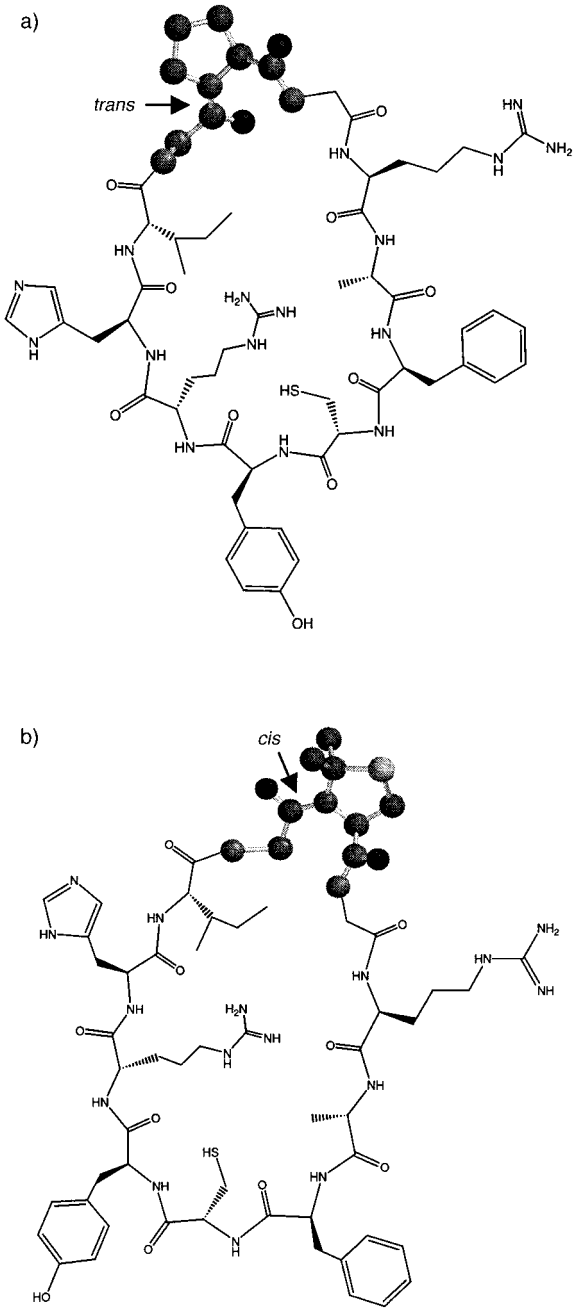


Figure 2. Model of the conformational change induced by the presence of the pseudoproline. a) Gly–Pro region of peptide **II**^{c} forming a *trans* peptide bond; b) Gly–Cys($\Psi^{Me,Me}$pro) region of peptide **I**^{c} forming a *cis* peptide bond.

Peptides **I**–**III**, obtained by solid-phase peptide synthesis,[16] were characterized by mass spectrometry and by one- and two-dimensional NMR spectroscopy. 2D ^{1}H NMR TOCSY, COSY-DQF, and ROESY data allowed complete attribution of proton chemical shifts for peptides **I** and **II**. Three conformers were observed for the cyclic ΨPro-containing peptide **I**^{c}. By integration of the β-methyl signals of Ile, their ratio was determined to be 80:10:10. In the ROESY experiment, the major conformer showed the typical pattern expected for a *cis* amide bond between Gly_{i-1} and Cys_i-($\Psi^{Me,Me}$pro), that is, $\alpha H_{i-1}-\alpha H_i$ and $\alpha H'_{i-1}-\alpha H_i$ cross peaks. The corresponding region is represented in Figure 3 a. One of

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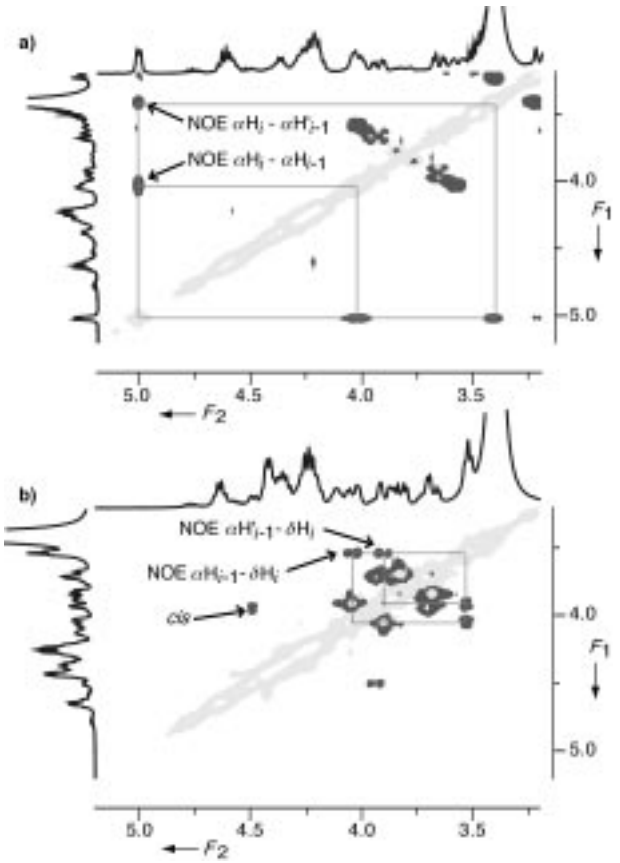

Figure 3. Expanded regions of the 2D ^{1}H-NMR ROESY spectra of the V3 loop analogues in DMSO-D_6 at 305 K (400 MHz, $\tau_m = 200$ ms). a) ΨPro-containing peptide **I**^{c} with NOE connectivities $\alpha H_i - \alpha H_{i-1}$ and $\alpha H_i - \alpha H'_{i-1}$ between Gly_{i-1} and Cys_i($\Psi^{Me,Me}$pro); b) Pro-containing peptide **II**^{c} with NOE connectivities $\alpha H_{i-1} - \delta H_i$ and $\alpha H'_{i-1} - \delta H_i$ between Gly_{i-1} and Pro_i.

the minor conformers was expected to be the corresponding *trans* form with a *trans* peptide bond between Gly_{i-1} and Cys_i-($\Psi^{Me,Me}$pro) but with characteristic NOE connectivities $\alpha H_{i-1} - \delta H_i$ and $\alpha H'_{i-1}$-δH_i could not be observed because of the dimethylation of the δ-position. Spectra of the cyclic Pro-containing peptide **II**^{c} showed three conformers present at a ratio of 80:15:5. In the major conformer, the Gly-Pro peptide bond was shown to be *trans* as proved by the characteristic NOE connectivities $\alpha H_{i-1} - \delta H_i$ and $\alpha H'_{i-1}$-δH_i between Gly_{i-1} and Pro_i (Figure 3b). A set of minor resonances could be attributed to the corresponding *cis* conformer with cross peaks $\alpha H_{i-1} - \alpha H_i$ and $\alpha H'_{i-1}$-αH_i between Gly_{i-1} and Pro_i (also shown in Figure 3b) which shows the inherent propensity of the amino acid proline for a $Xaa_{i-1} - Pro_i$ *cis* peptide bond. The high conformational flexibility of Aib-containing peptide **III**^{c} resulted in very undefined spectra, which didn't allow for complete attribution of proton chemical shifts. NMR spectra of the linear peptide **I**^{l} interestingly showed a single conformation with a *cis* bond preceding the pseudoproline residue. For the linear peptide **II**^{l}, two conformations were observed at a ratio of 30:70, the minor conformer containing a *cis* Gly–Pro bond and the major conformer having a *trans* bond preceding proline.

Antibodies were raised against peptides **I**^{c} and **II**^{c}. In order to obtain an immunogenic preparation, both loop analogues were coupled to the carrier protein BSA (bovine serum albumin) via linkage with 3-(*N*-maleimido)propionic acid hydroxysuccinimide ester. Balb/c mice were immunized with the two bioconjugates according to standard protocols.[17] After one boost, the mice were bled and the obtained antisera were tested by ELISA (enzyme-linked immunosorbent assay) using covalent linkage of the cyclic peptide antigens to the plate surface, which allows sensitive testing conditions. In Figure 4, the immunological response of the antisera towards

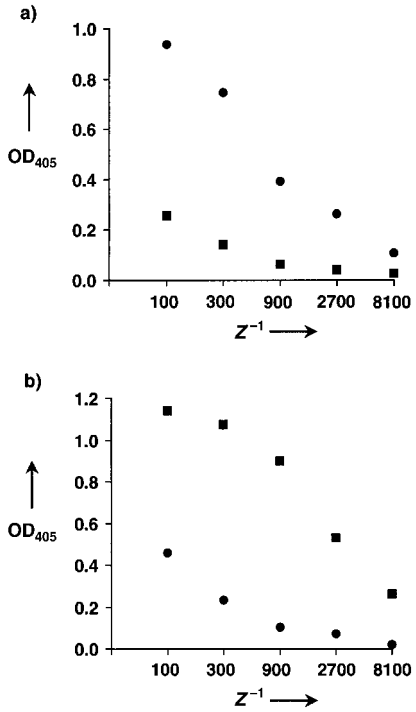

Figure 4. Specificity assays showing recognition of sera from mice immunized with ΨPro-containing peptide **I**^{c} (●) and Pro-containing peptide **II**^{c} (■). a) Coating peptide **I**^{c} ($c = 10\ \mu g\,mL^{-1}$); b) Coating peptide **II**^{c} ($c = 10\ \mu g\,mL^{-1}$). Z = serum dilution, OD_{405} = optical density at 405 nm.

the haptens is represented. In both cases, the polyclonal antibodies show a high recognition for the corresponding immobilized hapten, but not for the foreign loop-peptide. In order to confirm this result, competition assays were carried out in which the sera were allowed to preincubate with peptides **I**^{c} or **II**^{c} for 30 minutes before addition to the immobilized hapten. The results with **I**^{c} as the plate antigen are shown in Figure 5. They demonstrate that the antigenic reaction is strongly inhibited with increasing concentration of **I**^{c}, compared to the much weaker influence of **II**^{c}, confirming the high selectivity between the *cis*-constrained and the unconstrained peptides.

Monoclonal antibodies (mAbs) were raised by fusion of spleen cells from the immunized animals with myeloma cells of an Ag8 strain.[17, 18] Hybridoma cells which produced antibodies with the desired specificity were propagated and cloned, and the monoclonal antibodies purified. Monoclonal antibodies selected against **I**^{c} and **II**^{c} were tested for their recognition towards the cyclic and linear peptides. Both confirm the results obtained with polyclonal antibodies, specifically recognizing the corresponding cyclic antigen. As

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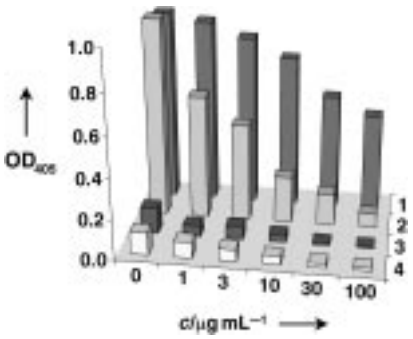

Figure 5. Competition assay showing inhibition of the antigenic reaction with increasing concentration of peptide **I**^{c}. Peptide **II**^{c} inhibits the antigen reaction considerably less. The plate antigen is **I**^{c} coated at a concentration of 10 μg mL^{-1}. 1: Serum anti-**I**^{c} + peptide **II**^{c}; 2: Serum anti-**I**^{c} + peptide **I**^{c}; 3: Serum anti-**II**^{c} + peptide **II**^{c}; 4: Serum anti **II**^{c} + peptide **I**^{c}. c = concentration of competitive peptide.

shown in Figure 6, mAbΨPro238.13 selected against the cyclic ΨPro-containing peptide **I**^{c} recognizes the Pro-containing peptide **II**^{c} to about 15%, in harmony with the degree of *cis* content in **II**^{c} determined by NMR. As a further indication of the presence of an mAb directed at *cis* amide bonds,

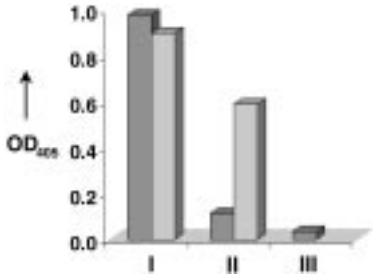

Figure 6. Recognition of monoclonal antibody mAbΨPro238.13 selected against **I**^{c} towards cyclic (c, dark bars) and linear (l, light bars) peptides. I: ΨPro-containing peptides **I**^{c} and **I**^{l}; II: Pro-containing peptides **II**^{c} and **II**^{l}; III: Aib-containing peptide **III**^{c}.

no recognition was observed towards reference peptide **III**^{c}, which contained Aib as a structural mimetic of the C(2) dimethyl substituted ΨPro residue in peptide **I** (Figure 6). Most notably, the mAb directed towards the cyclic *cis*-containing peptide **I**^{c} shows also a high recognition for the corresponding linear peptide **I**^{l}. Due to the substantial differences of the cyclic and linear peptides with respect to their conformational flexibility and overall shape, the recognition of both species by mAbΨPro238.13 points to the common *cis* amide bond as a recognition site. This observation is further validated by a partial recognition of the linear Pro-containing peptide **II**^{l}, which can be only rationalized by a *trans* to *cis* isomerization of the Gly–Pro bond of the flexible peptide **II**^{l} upon interaction with the *cis*-directed antibody. Most notably, this isomerization occurs to a much lower degree within the conformationally constrained cyclic peptide **II**^{c}, resulting in a substantially lower recognition by the *cis* bond directed antibody. This difference in recognition of monoclonal antibody anti-**I**^{c} towards the cyclic and linear peptides **II**^{c} and **II**^{l} which both contain the Gly–Pro chemical entity clearly indicates a conformational specificity.

In conclusion, a 2,2-dimethylated thiazolidine (ΨPro) derivative has been introduced into a biologically relevant cyclized peptide sequence from the V3 loop of HIV-1 which has induced a *cis* peptide bond and represents a conformation that is proposed to play a crucial role in the HIV infection process. Furthermore, immunological results demonstrate that antibodies have been raised which selectively distinguish between the *cis* and the *trans* conformation of Xaa-Pro imide bonds in linear and cyclic peptides, offering interesting perspectives for applying the Ψ-proline concept as a diagnostic tool for the detection of conformational changes during biological processes.

Experimental Section

Peptides were synthesized according to standard procedures of SPPS (solid-phase peptide synthesis)[16] by the Fmoc (9-fluorenylmethoxycarbonyl) strategy on Sasrin (super acid sensitive resin)[19] or Sieber amide resin.[20] The pseudoproline, a cysteine derived 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid (Cys($\Psi^{Me,Me}$pro)), was introduced during SPPS as a preformed dipeptide building block[3] (for experimental details, see Supporting Information). The products were characterized by ESI-MS (electrospray ionization mass spectrometry) and 1D and 2D ^{1}H NMR TOCSY, COSY-DQF and ROESY spectroscopy. Spectra were recorded at 400 MHz (τ_m = 200 ms) in DMSO-D_6 at 305 K (see Supporting Information).

The immunogenic construct was prepared by coupling the linker molecule 3-(*N*-maleimido)propionic acid hydroxysuccinimide ester to the carrier protein BSA in 0.1 M phosphate buffer (pH 7.0)/dioxane (5/1; v/v). After dialysis against 0.1 M phosphate buffer (pH 7.0) (molecular weight cut-off 2000), peptides **I**^{c} or **II**^{c} were added and left to incubate for 1 h before dialysis against water and lyophilization of the product. The number of V3-peptides coupled to each molecule of BSA was determined by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) to lie between 10 and 30.

For the elicitation of antibodies, antigen (15 μg in 25 μL of phosphate-buffered saline, PBS) was mixed with Titer Max Adjuvant (25 μL) and injected subcutaneously (base of tail) into eight week old Balb/c mice. Mice immunized with **I**^{c} were boosted after 5 weeks, and all mice were bled after 9 weeks.

ELISA-plates used for covalent linkage of thiol groups were AquaBind Combiplates from BioConcept. Coating was performed by incubating peptide solutions (10 μg mL^{-1}) in 0.2 mM carbonate buffer (pH 9.5) overnight at 4 °C. After washing the plates with PBS/0.05% Tween 20, the surface was blocked with a 5% solution of milk powder in PBS. Serum dilutions were prepared in PBS/0.05% Tween 20 and incubated for two hours at room temperature. Plates were washed with PBS/0.05% Tween 20 and incubation was effected with a conjugate of anti-mouse antibodies and alkaline phosphatase in PBS/0.05% Tween 20 (1:1000, 1.5 h). After washing, a premixed substrate solution (*p*-nitrophenyl phosphate) was added to the plates and, after 30 min, the absorbance was read at 405 nm.

Myeloma cells used for fusion were of the x63Ag8.653 cell line.[18] Fusion, propagation of hybridoma cells, cloning, and purification of the monoclonal antibodies was performed following standard protocols.[17]

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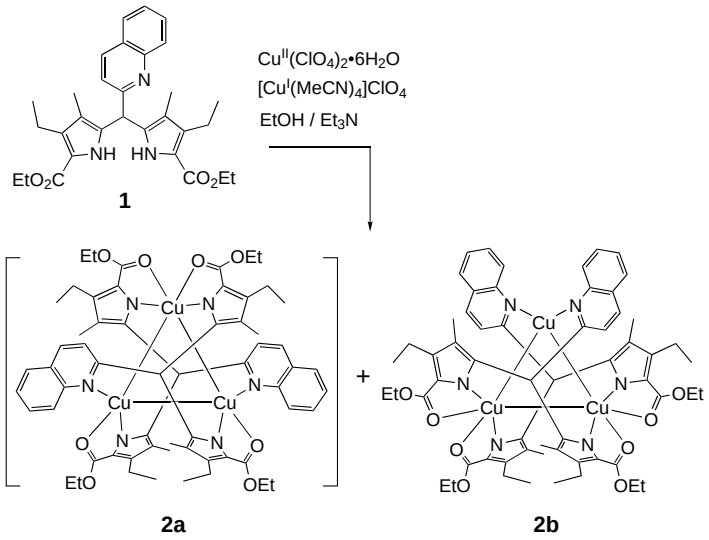
A Triangular Mixed-Valent $Cu^{II}Cu^{I}Cu^{I}$ Cluster Supported by the Tripod Ligand 2-Quinolyl-2,2′-dipyrrolylmethane**

Jun-ichiro Setsune,* Tatsuo Yokoyama, Setsuo Muraoka, Hong-wei Huang, and Takeshi Sakurai

Trinuclear copper complexes are attracting increasing attention as models for the metal sites of multicopper oxidases.[1, 2] While triangular Cu_3 complexes with C_{3v} symmetry are well known,[3] those with lower symmetry are important because the metallosites of multicopper oxidases consist of two type-3 Cu sites and one type-2 Cu site.[1] We report here that triangular Cu_3 cluster complexes with a $Cu^{II}Cu^{I}Cu^{I}$ mixed-valent state are obtained by using the mixed tripod ligand 2-quinolyl-2,2′-dipyrrolylmethane. The X-ray structure and spectroscopic properties of the Cu_3 complex indicate that two Cu ions interact strongly with one another.

We have shown that 2-pyridyl-2,2′-[3,3′-dimethyl-4,4′-diethyl-5,5′-bis(ethoxycarbonyl)]dipyrrolylmethane occupies three coordination sites of the basal plane of a square pyramidal Cu^{II} center through a pyridine N atom, a pyrrole N atom, and the conjugated carbonyl O atom.[4] This tridentate coordination mode would cause steric hindrance to the fourth coordination site of the Cu^{II} basal plane in the case of 2-quinolyl-2,2′-[3,3′-dimethyl-4,4′-diethyl-5,5′-bis(ethoxycarbonyl)]dipyrrolylmethane (**1**). Hence, the three arms of the tripod ligand **1** would not converge to a single metal center. When a mixture of the ligand **1** (0.194 mmol), $Cu(ClO_4)_2 \cdot 6H_2O$ (0.194 mmol), Et_3N (0.73 mmol), and EtOH (6 mL) was stirred at room temperature for 1 h under argon, the dark green Cu complex **2a** and the light green Cu complex **2b** were obtained in 7% and 17% yield, respectively (Scheme 1). On



Scheme 1. Formation of Cu_3 cluster complexes.

the basis of FAB-MS ($m/z = 1189$) and analytical data, **2a** and **2b** are isomeric and composed of two doubly deprotonated ligands and three Cu atoms. Therefore, the total charge of the Cu_3 cluster seems to be +4. Indeed, the reaction of **1**, $Cu^{II}(ClO_4)_2 \cdot 6H_2O$, and $[Cu^{I}(MeCN)_4]ClO_4$ in the molar ratio of 1.0/0.5/1.0 gave improved yields of **2a** and **2b**.[5]

Single-crystal X-ray analysis of **2b** showed a triangular Cu_3 cluster with Cu–Cu distances of 2.596(2), 2.597(2), and 2.574(3) Å that is sandwiched between two tripod ligands (Figure 1). The Cu(1) and Cu(2) sites are ligated by two anionic pyrrole nitrogen atoms and two weakly coordinating carbonyl oxygen atoms. The Cu(3) site is ligated only by two

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