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Development of a Functional *cis*-Prolyl Bond Biomimetic and Mechanistic Implications for Nickel Superoxide Dismutase

Daniel Tietze,[a] Marco Tischler,[a] Stephan Voigt,[a] Diana Imhof,[b] Oliver Ohlenschläger,[c] Matthias Görlach,[c] and Gerd Buntkowsky*[a]

Abstract: During recent years several peptide-based Ni superoxide dismutase (NiSOD) models have been developed. These NiSOD models show an important structural difference compared to the native NiSOD enzyme, which could cause a completely different mechanism of superoxide dismutation. In the native enzyme the peptide bond between Leu4 and Pro5 is *cis*-configured, while the NiSOD models exhibit a *trans*-configured peptide bond between these two residues. To shed light on how the configuration of this single peptide bond influences the activity of the NiSOD model peptides, a new *cis*-prolyl bond surrogate was developed. As surrogate we chose a leucine/alanine-based disubstituted 1,2,3-triazole, which was incorporated into the NiSOD model peptide replacing residues Leu4 and Pro5. The yielded 1,5-disubstituted triazole nickel peptide exhibited high SOD activity, which was approximately the same activity as its parent *trans*-configured analogue. Hence, the conformation of the prolyl peptide bond apparently has of minor importance for the catalytic activity of the metallopeptides as postulated in literature. Furthermore, it is shown that the triazole metallopeptide is forming a stable cyanide adduct as a substrate analogue model complex.

Keywords: biomimetic synthesis · enzyme catalysis · nickel · superoxide dismutase

Introduction

Superoxide anions ($O_2^{\bullet -}$) are generated as toxic by-products of the respiratory chain in aerobic organisms.[1] If not eliminated, they can cause severe metabolic malfunctions and damage biological macromolecules like DNA by peroxidation.[2] Superoxide dismutases (SOD; EC 1.15.1.1) disproportionate the superoxide anion radical by converting it into hydrogen peroxide and oxygen with a rate near the diffusion limit ($k_{cat} > 2 \times 10^9\,M^{-1}s^{-1}$).[3–5] Today four independent classes of SODs are known. They contain either a dinuclear Cu, Zn, a mononuclear Fe, Mn, or a mononuclear Ni cofactor, respectively.[2,6–8] NiSOD, the most recent member of this class of enzymes, was identified in *Streptomyces* in 1996 and later on also in several marine cyanobacteria.[6,7] Recently, an in silico analysis has been performed to search for sequences encoding NiSOD proteins. It has revealed the presence of homologous proteins in actinobacteria, proteobacteria, chlamydiae, and eukarya (green algae).[9] The NiSOD enzymes cycle by means of a ping-pong mechanism between Ni^{II} and Ni^{III} oxidation states during catalysis.[4,10,11] First investigations concerning the mode of action of different NiSOD mutants made by Maroney et al.[12] and Wuerges et al.[11] demonstrated that the axial His1 imidazole is vitally important. As soon as His1 is replaced by Ala or Asp for example, the catalytic activity of the native enzyme is completely lost.[3,11] The active site of NiSOD is conserved within the first six N-terminal residues (HCXXPC) and coordinates the nickel ion in a "hooklike" manner.[10] This structural feature made it possible to develop peptide-based model substances. Shearer et al.[13–15] and Weston et al.[16] independently took advantage of it and prepared several metallopeptide NiSOD models that were based on the first 12, 9, 7, or 6 residues from the N-terminus of the active form of *S. coelicolor* NiSOD. In contrast to all other inorganic

[a] Dr. D. Tietze, M. Tischler, S. Voigt, Prof. G. Buntkowsky
Technische Universität Darmstadt
Eduard-Zintl-Institut für Anorganische und Physikalische Chemie
Petersenstr. 22, 64287 Darmstadt (Germany)
Fax: (+49) 6151-164347
E-mail: gerd.buntkowsky@chemie.tu-darmstadt.de

[b] Dr. D. Imhof
Friedrich-Schiller-Universität Jena
Zentrum für Molekulare Biomedizin
Institut für Biochemie und Biophysik
Hans-Knöll-Str. 2, 07745 Jena (Germany)

[c] Dr. O. Ohlenschläger, Dr. M. Görlach
Leibniz-Institut für Altersforschung/Fritz-Lipmann-Institut
Beutenbergstr. 11, 07745 Jena (Germany)

model compounds, these peptide-based NiSOD model compounds exhibit high catalytic activity that is only about one order of magnitude lower as compared to the native enzyme.[14,15] These model compounds allow a systematic study of the mode of action of the enzyme. Two models were discussed in literature that differ in the binding of the substrate. Depending on whether the substrate is bound in the first coordination sphere of the nickel ion or not they are called inner-sphere or outer-sphere electron transfer (ET) mechanisms. Recently some of us have been able to synthesize and characterize a metallopeptide substrate model complex employing cyanide as a substrate analogue inhibitor that clearly supports the inner-sphere ET mechanism.[17]

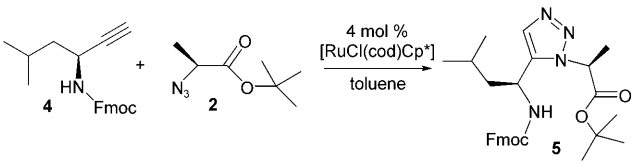
To reveal the role of the N-terminal histidine residue of the native enzyme, several mutants of the metallopeptide NiSOD models were studied.[11,13–16,18] In these mutants His1 was replaced by Ala or Asp. Two different results were reported for the replacement of His1 by Ala (H1A). In one study a reduction of the SOD activity by more than one order of magnitude[14] was observed, while another work reported only a 10% reduction of SOD activity compared to the parent metallopeptide.[16] Another important feature of the NiSOD model peptides was revealed by their liquid-state NMR structure. In contrast to the *cis*-configured Leu4–Pro5 peptide bond in the native enzyme, they carry a *trans* peptide bond in this position.[16] Moreover, density functional theory calculations indicated that the carbonyl group of the *trans*-configured Leu4–Pro5 peptide bond probably adopts the role of the fifth ligand and forces substrates to approach the nickel center from the opposite side as compared to the native enzyme.[16] As a consequence, these conclusions would reduce the comparability of the proposed catalytic mechanism of the synthetic metallopeptides with their *trans*-configurations and the native NiSOD enzyme. Thus a *cis*-configured prolyl isomer of the NiSOD model peptide is needed. Such a prolyl *cis/-trans* isomerization (CIT) is "undoubtedly one of the finest ways to tune the physical and chemical properties of biological macromolecules."[19] In biological systems CIT is mainly performed by light irradiation or peptidyl prolyl isomerases (PPIases).[20–22]

Thus the systematic synthesis of a *cis* peptide bond or of full synthetic surrogates is of general interest. For example, the active form of the conotoxin δ-EVIA from marine cone snail *Conus ermineus* shows a CIT of the peptide bond between Leu12 and Pro13 with the *cis* isomer supposed to be the bioactive form. However, during standard solid-phase peptide synthesis the *trans* conformation is strongly preferred and it is practically impossible to enforce the formation of a *cis* peptide bond. A possible solution for this particular problem is mimicking the *cis* peptide bond by a suitable surrogate. Such a strategy was indeed successfully applied for selective activity measurements of both isomers of conotoxin δ-EVIA, where a thiazolidine-based *cis*-peptide-bond surrogate was reported.[23,24] A similar approach was introduced by Tam et al.[25] Instead of Asn113–Pro114 of bovine pancreatic ribonuclease (RNase A; 124 residues), they incorporated several disubstituted 1,2,3-triazoles as fully synthetic *cis*-peptide-bond surrogates.

These results prompted us to apply the same strategy to the NiSOD and to develop a synthetic surrogate that can be employed as an experimental model for the investigation of the *cis*-configured protein. We inserted a *cis*-peptide-bond surrogate, based on Tam's strategy, into the heptamer metallopeptide carrying the first seven residues of the N-terminus of the active form of *S. coelicolor* NiSOD. The main questions to be solved were the influence of this bond on the conformation of the metallopeptide and on the catalytic mechanism and efficacy of the resulting peptide. We intended to investigate the comparability of the mechanism of $O_2^{\bullet-}$ degradation and the ability to form stable cyanide adduct as a substrate model for NiSOD.

Results and Discussion

In order to synthesize the disubstituted 1,2,3-triazole, we employed the Huisgen 1,3-dipolar cycloaddition reaction.[26] According to earlier observations that the 1,5-disubstituted triazole better matches the criteria of mimicking a *cis*-prolyl bond than the 1,4-disubstituted triazole, we decided to use a Ru^{II} catalyst, which will exclusively yield the 1,5-disubstituted triazole (Scheme 1).[27,28] Based on the amino acid sequence of the NiSOD model peptides, Leu4 and Pro5 were subjected to replacement by the 1,5-disubstituted triazole (Figure 1) based on leucine and alanine. As mentioned above, Tam's incorporated triazole components were based either on Ala/Ala or Asn/Ala.



Scheme 1. Ru^{II}-catalyzed 1,3-dipolar cycloaddition of the amino alkyne **4** and the azido acid **2** to yield the triazole component **5**.

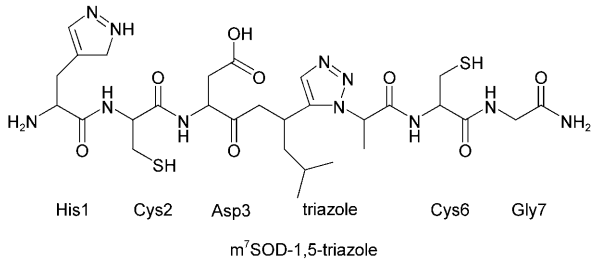


Figure 1. Scheme of the heptamer NiSOD model peptide showing the 1,5-disubstituted triazole as a Leu4-Pro5 surrogate.

To the best of our knowledge this is the first time that this Leu/Ala-based triazole group has been incorporated into a peptide. The cycloaddition was promoted by the catalyst

[RuCl(cod)Cp*] (Scheme 1; cod = cyclooctdiene, Cp* = pentamethylcyclopentadienyl). The triazole component was obtained in high yields (77%).

The model peptide (H-HCD-triazole-CG-NH_2, m^7SOD-triazole) was prepared by a standard solid-phase peptide synthesis (SPPS) protocol according to the Fmoc strategy. The HPLC chromatograms of the crude peptide showed two main components in a 58:42% ratio. Both main components were purified by semipreparative HPLC and one of the two main components was identified as the desired product (m^7SOD-1,5-triazole) by mass spectrometry and 2D liquid-state NMR experiments (ROESY, HSQC, Supporting Information).

To yield the nickel metallopeptide, an aqueous $NiCl_2$ solution was added to the triazole peptide. Before adding the $NiCl_2$ to the solution of the m^7SOD-1,5-triazole, the peptide content was determined using Ellman's reagent.[29] The formation of the metallopeptide by adding one equivalent of $NiCl_2$ to the peptide solution was pH-dependent as described earlier.[15] During titration with 0.1 M NaOH starting at a pH around 3 (pH of the lyophilized peptide) up to a pH of 7.8, the color of the solution changed from colorless to light pink. The color change occurred between pH 5.5 and 6. The triazole peptide coordinates only a single Ni^{II} ion, as determined by ESI mass spectrometry. The preparation of the cyanide adducts of the metallopeptide (Ni(m^7SOD-1,5-triazole)) was carried out as follows. Upon addition of 1.5 equivalents of KCN to the pink metallopeptide solution, that is, according to the peptide content, the color changed immediately to light yellow as we reported earlier.[17] The resulting UV/Vis spectrum (Figure 2) of Ni(m^7SOD-1,5-triazole) recorded in the region from 12500 to 50000 cm^{-1} (800–200 nm) was in good agreement with those obtained for other NiSOD metallopeptides (Ni(mSOD)).[14,15,17] The band at 22000 cm^{-1} (458 nm; ε = 413 $M^{-1}cm^{-1}$) in the electronic absorption spectra was identified by Shearer[13,30] and Fiedler[31] as a dd-Ni($3d_{xy}$)/S→Ni-($3d_{x^2-y^2}$)/S/N(σ)* transition, which is characteristic for square planar $Ni^{II}N_2S_2$ complexes. The UV region in Figure 2 shows only one broad band centered around 38167 cm^{-1} (262 nm; ε = 13528 $M^{-1}cm^{-1}$) and a shoulder at 44843 cm^{-1} (223 nm).

The formation of the cyanide adduct of the triazole metallopeptide was assessed by ESI mass spectrometry and UV/Vis spectroscopy. Upon the addition of KCN to the metallopeptide to generate the cyanide adduct, nearly all transitions were shifted into the UV region (Figure 3). In contrast to the cyanide-free metallopeptide of Ni(m^7SOD-1,5-triazole) we observed only one transition at 24096 cm^{-1} (415 nm; ε = 293 $M^{-1}cm^{-1}$) in the visible region. The most intense band was shifted from 38167 to 39682 cm^{-1} (252 nm; ε = 18178 $M^{-1}cm^{-1}$) and showed, in contrast to the broad band of the cyanide-free metallopeptide, a very distinct shoulder around 35460 cm^{-1} (282 nm). The shoulder at 44558 cm^{-1} (224 nm) was shifted to 45500 cm^{-1} (220 nm). The spectra of the triazole cyanide adduct are in good agreement with those of Ni(CN)(mSOD), which were published in our previous work.[17] Only the maxima of the typical transitions of the cyanide adducts in the visible region were slightly different (410 nm (Ni(CN)(mSOD), 415 nm (Ni(CN)(m^7SOD-1,5-triazole)).

These UV/Vis data indicate that the m^7SOD-1,5-triazole behaves like the NiSOD model peptides described earlier.[14–17] The triazole peptide coordinates one single Ni ion and perfectly matches the spectroscopic properties of the native enzyme. These properties make it suitable as a NiSOD model substance. Furthermore, Ni(m^7SOD-1,5-triazole) is able to bind a single CN ion as a substrate analogue as shown for Ni(mSOD).[17]

As mentioned above the focus of this work is to investigate in which way the activity of the new triazole NiSOD model peptide was influenced by the replacement of Leu4 and Pro5 by our *cis*-prolyl-bond surrogate. Therefore, the activity data for this new peptide were recorded by applying the McCord–Fridovich test. According to literature data some problems[15,16] were described for the well-established McCord–Fridovich test and needed to be solved initially.[32] If the activity of peptide-based NiSOD models was determined by the standard McCord–Fridovich procedure, the destruction of the model peptides was already observed after 30–90 s. This degradation was apparently determined by $O_2^{\bullet-}$ or H_2O_2.[15,16] In order to quantify the catalytic activity of our met-

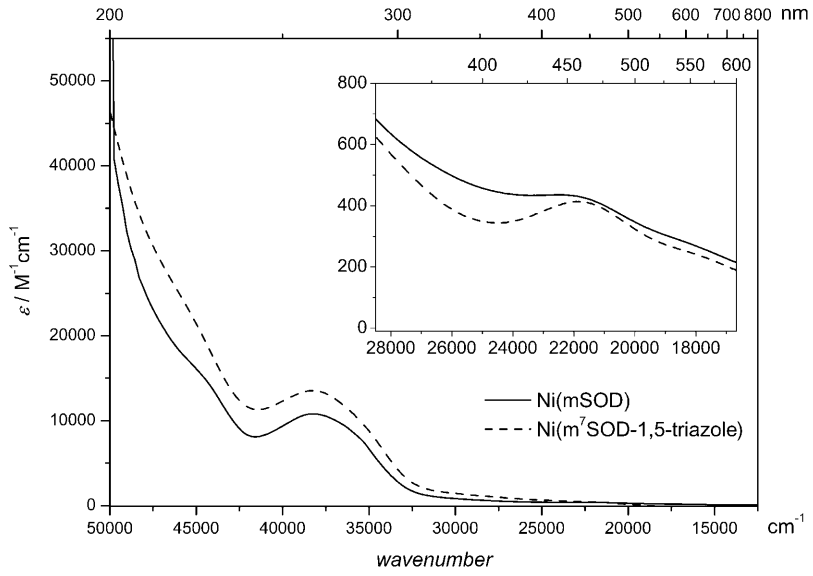


Figure 2. The UV/Vis spectra of the metallopeptides Ni(mSOD) (——) and Ni(m^7SOD-1,5-triazole) (-----) (water/NaOH, pH 7.8, 25 °C). The inset displays an enlargement of the region from 16600–28500 cm^{-1}.

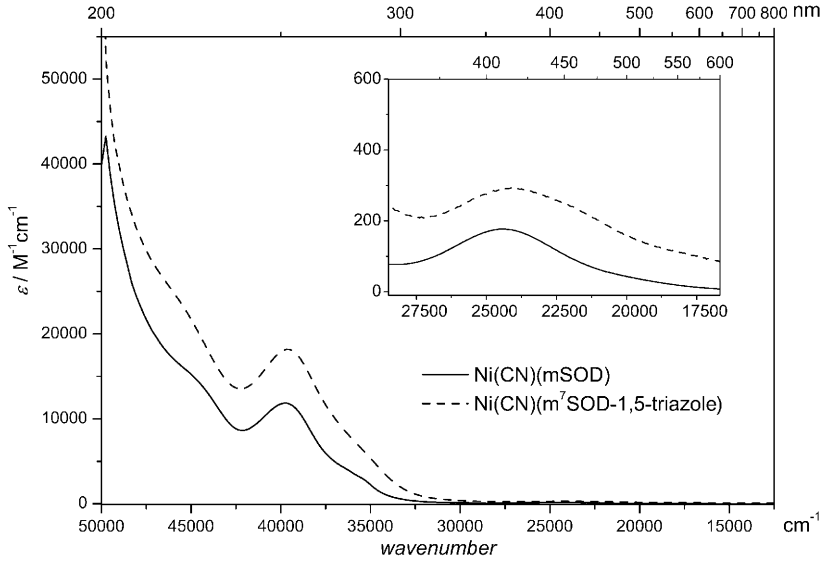


Figure 3. UV/Vis spectra of the triazole metallopeptide cyanide adduct (-----) compared to the published Ni(CN)(mSOD) (——) spectrum; (water/NaOH, pH 7.8, 25 °C). The inset displays an enlargement of the region from 16600–28500 cm^{-1}.

allopeptides (described above), we modified this assay according to a literature derived procedure, which was originally developed for the observation of direct decay kinetics of $O_2^{\bullet-}$.[33] Therefore, the addition of catalase (CAT) to the reaction mixture was used to protect the catalytically active species from being destroyed through H_2O_2. As evident from Figures 4 and 5 Ni(m^{7}SOD) (the parent heptamer metallopeptide) was protected by CAT very effectively from being destroyed (more details are presented in the Experimental Section).

In addition to the "triazole" metallopeptide, we also quantified the activity of the parent heptamer metallopeptide (Ni(m^{7}SOD); m^{7}SOD = H-HCDLPCG-NH_2). The results are presented in Table 1. An example of an activity measurement is depicted in Figure 5b. If one compares the activity of the *cis*-prolyl-bond NiSOD biomimetic (Ni(m^{7}SOD-1,5-triazole), 2090 U μmol^{-1}) with its parent metallopeptide (Ni(m^{7}SOD), 1940 U μmol^{-1}), it is evident that the activities for both metallopeptides are nearly equal. Furthermore, the literature activity value of the nonametallopeptide (Ni(mSOD), 2100 U μmol^{-1}) coincides with the triazole peptide value.

These results are a clear indication that the configuration of the peptide bond between leucine and proline has only a minor importance for the catalytic activity of the metallopeptides.

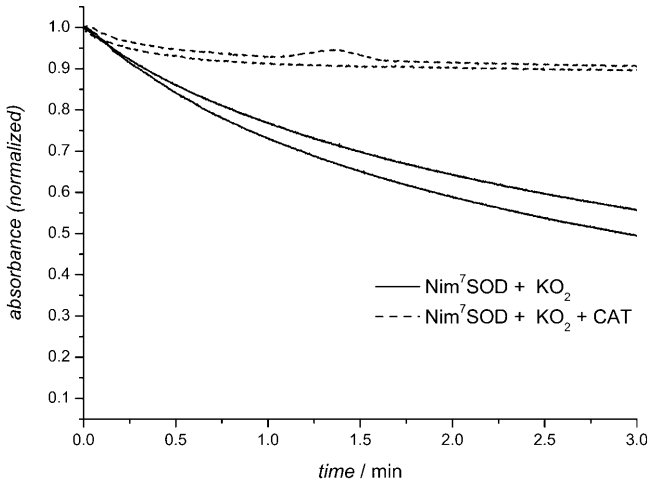


Figure 4. Degradation process of Ni(m^{7}SOD) observed at 458 nm in the presence of KO_2 (aq) (KH_2PO_4/Na_2HPO_4, 50 mM, pH 7.8, 25 °C). Every experiment was done twice to show that the amounts of KO_2 given to the reaction medium were constant. The "artifact" in the top curve was caused by an oxygen bubble in the cuvette.

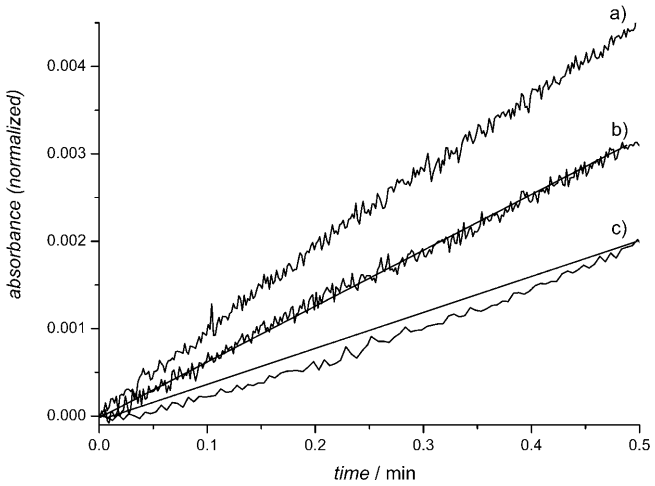


Figure 5. Quantitative activity measurements for Ni(m^{7}SOD): a) reference measurement for the formazan production without a SOD active substance for measurement b only. b) Ni(m^{7}SOD) + CAT and c) Ni(m^{7}SOD) without CAT according to the modified and the unmodified McCord activity test. The straight black line was fitted manually to illustrate the linear and nonlinear trend of the measurements. More details and the principle of the test are presented in the experimental section.

Conclusion

The synthesis of a 1,5-disubstituted 1,2,3-triazole as a *cis*-peptide-bond surrogate and its insertion into a small peptide were successfully performed. The peptide, containing the 1,5-disubstituted triazole instead of Leu4 and Pro5, coordi-

Table 1. Catalytic activity of the "triazole" metallopeptide compared to its parent metallopeptide Ni(m^{7}SOD) derived from our modified "McCord activity assay" and to published data of Ni(mSOD).[16] The activity for NiSOD *S. coelicolor* is given per subunit.

	Activity [U μmol^{-1}]
Ni(mSOD)	≈2100[16]
Ni(m^{7}SOD)-1,5-triazole	≈2090
Ni(m^{7}SOD)	≈1940
NiSOD *S. coelicolor*	≈45292[6]

nated one Ni ion. The resulting nickel-containing peptide also forms a stable substrate adduct with a single cyanide ion as a substrate analogue. The activity of the "triazole" metallopeptide was examined by a modified McCord–Fridovich test. The "triazole" metallopeptide is SOD active. Compared to the parent heptamer metallopeptide Ni(m^{7}SOD), exhibiting the *trans*-configured Leu4–Pro5 peptide bond as mentioned at the beginning, Ni(m^{7}SOD-1,5-triazole) showed nearly the same activity.

Consequences on the mode of action of NiSOD: The results of our studies indicate that a previously proposed influence[16] of the conformation of the prolyl peptide bond on the catalytic activity of the metallopeptides seems to be of minor importance. In particular the conformational change cannot account for the higher catalytic activity of the native enzyme as compared to the metallopeptides. Thus the mechanistic results for the superoxide dismutation derived from the NiSOD *trans*-metallopeptides are valid for the mechanism of the NiSOD enzyme.

Experimental Section

General: UV/Vis spectra were recorded on a Varian Cary 5000 UV-Vis-NIR spectrophotometer using quartz cuvettes with 1 cm path length. All solutions were prepared from double-distilled water at pH 7.8. Mass spectra were recorded using a SSQ 710, Finigan MAT instrument. The purity of the peptide and the labeled amino acid derivatives was assessed by analytical HPLC (high pressure liquid chromatography) by using a Shimadzu LC-10AT system equipped with a reversed phase Vydac 218TP column (25×4.6 mm, 5 μm) and gradients of 0–30% (gradient A) eluent B over 30 min, 0–60% (gradient B) eluent B over 60 min and 0–40% (gradient D) eluent B over 40 min (A: 0.1% trifluoroacetic acid (TFA) in water; B: 0.1% TFA in acetonitrile) at a flow rate of 1 mL min^{-1}. For the purification of the crude peptide a semi preparative Shimadzu LC-8 A HPLC system equipped with a Eurospher 100 C18 column (Knauer, 250×32 mm, 5 μm) was used. Elution was performed with gradients of 10–60% (gradient C) eluent B over 120 min (A: 0.1 % TFA in water; B: 0.1 % TFA in 9:1 aceonitrile/water) at a flow rate of 10 mL min^{-1}. All chromatograms were recorded at 220 nm using a Shimadzu UV/Vis detector. NMR spectra were recorded on a Bruker Avance III spectrometer with a proton frequency of 600 MHz. The samples were dissolved in 90% H_2O/10% D_2O from freeze dried peptide powder. Data were acquired and processed with Topspin (Bruker, Rheinstetten, D). The proton resonance assignment was performed by using a combination of 2D ^{1}H,^{1}H-ROESY and ^{1}H,^{13}C-HSQC experiments. ROESY experiments were acquired with 100 and 120 ms mixing time.

Activity measurements: The principle of this test is xanthine oxidase (XOD) oxidized xanthine to generate $O_2^{\bullet-}$ at a low and constant (steady state) rate. Free $O_2^{\bullet-}$ then reduces nitroblue tetrazolium chloride (NBT), which can be monitored photometrically at 560 nm. If an added substance is SOD-active, it competes for $O_2^{\bullet-}$, thus inhibiting the reduction of NBT. The measured rise (m) in the extinction is lower than the control reaction. The SOD-activity (A) is defined as the half-limited reduction of NBT and measured by a reduction in the slope (the linear part is used) and set relative to the concentration of the substance (U μmol^{-1}): $A = 2(m_{control} - m_{substance})/m_{control}$.

The test was modified according to the procedure of Bolann et al., which was developed for the study of direct decay kinetics of $O_2^{\bullet-}$.[33] Knowing that CAT is often contaminated with SOD, it has been tested by a qualitative assay[16] for SOD activity. Catalase from *Aspergillus niger* purchased from SERVA® Electrophoresis showed no SOD activity and was used for our activity assay.

General procedure: In the case of testing peptides for SOD activity, a 2 mM peptide solution (phosphate buffer, 50 mM, pH 7.8) was used for each peptide. The control experiment was carried out as follows: NBT solution (0.5 mL), CAT (SERVA® Electrophoresis) solution (100 μL) and xanthine solution (0.6 mL) were added to phosphate buffer solution (1.4 mL), see Table 2 for standard and buffer solutions. Before the measurement was started XOD (0.804 U mg^{-1}) solution (0.4 mL) was added. The absorption was measured over a period of 30 s at 560 nm (Figure 5).

The activity measurements were carried out in analogy to the control experiment, except phosphate buffer (0.1 mL) was replaced by the peptide solution (0.1 mL). Every measurement was performed two times at room temperature.

XOD inhibition control experiment was carried out as follows: CAT solution (100 μL) and xanthine solution (0.6 mL) were added to phosphate buffer solution (1.8 mL). Before the measurement was started XOD solution (0.4 mL) and peptide solution (0.1 mL) were added. The absorption was measured over 60 s at 290 nm.

Table 2. Standard and buffer solutions for activity measurements.

phosphate buffer	KH_2PO_4/Na_2HPO_4, 50 mM, pH 7.8
NBT	10 mg (1.22×10^{-5} mol) in 10 mL H_2O (dest.)
xanthine	12 mg xanthine (7.8×10^{-5} mol) in 100 mL phosphate buffer
xanthine oxidase (stock solution)	5 mg XOD (8.04 U mg^{-1}) in 5 mL phosphate buffer
catalase (*A. niger*)	2 mg CAT (2660 U mg^{-1}) in 10 mL phosphate buffer

Stability measurements: Stability measuremnts of Ni(m^{7}SOD) were carried out in phosphate buffer (KH_2PO_4/Na_2HPO_4, 50 mM) at a pH value of 7.8. Ni(m^{7}SOD) (300 μL; 0.2 mM, phosphate buffer 50 mM, pH 7.8) and catalase (300 μL; 532 U ml^{-1}, phosphate buffer 50 mM, pH 7.8) were added to phosphate buffer (2.4 mL) and mixed in a quartz cuvette (path length 1 cm). Before the measurement started an ice-cold aqueous solution of KO_2 (10 μL; 8 mg mL^{-1}, NaOH 50 mM) was added to the sample. The absorption was recorded at 458 nm over a period of 3 min (Figure 4).

Synthesis of (2*S*)-2-azido-propanoic acid (1): Sodium azide (5 g, 76.9 mmol) was dissolved in distilled H_2O (13 mL) with CH_2Cl_2 (22 mL) and cooled on an ice bath. Triflyl anhydride (2.5 mL, 14.9 mmol) was added slowly over 5 min with stirring continued for 2 h. The mixture was placed in a separating funnel and the CH_2Cl_2 phase was removed. The aqueous portion was extracted with CH_2Cl_2 (2×20 mL). The organic fractions, containing the triflyl azide, were pooled and washed once with saturated Na_2CO_3 and used without further purification. L-Alanine (716.3 mg, 8.0 mmol) was combined with K_2CO_3 (1.63 g, 11.8 mmol) and $Cu^{II}SO_4$ pentahydrate (26 mg, 0.104 mmol), distilled H_2O (25 mL) and CH_3OH (50 mL). The triflyl azide in CH_2Cl_2 was added and the mixture was stirred at ambient temperature and pressure overnight. Subsequently, the organic solvents were removed under reduced pressure and the aqueous slurry was diluted with H_2O (150 mL). This was acidified to pH 6 with conc. HCl and diluted with 0.25 M, pH 6.2 phosphate buffer (150 mL) and extracted with EtOAc (4×50 mL) to remove sulfonamide by-product. The aqueous phase was then acidified to pH 2 with conc. HCl. The product was obtained from another round of EtOAc extrac-

tions (3×50 mL). These EtOAc extracts were combined, dried over $MgSO_4$(s) and evaporated to dryness giving (2S)-2-azido-propanoic acid as a pale oil in 88% yield with no need for further purification. ^{1}H NMR (200 MHz, CD_2Cl_2): δ=10.36 (s, 1H), 4.06 (q, J=7.2 Hz, 1H), 1.52 ppm (d, J=7.2 Hz, 3H); ^{13}C NMR (50 MHz, CD_2Cl_2): δ=177.1, 57.5, 16.9 ppm.

Synthesis of *tert*-butanol-(2*S*)-(2-azido)-propanolate (2): Compound **1** (914 mg, 7.9 mmol) was dissolved in anhydrous CH_2Cl_2 (60 mL) and cooled to −78°C with a dry ice/acetone bath. Isobutylene (50 mL) was condensed with a cold finger (−78°C) in a separate flask and both substances were poured together into an autoclave. Concentrated H_2SO_4 (4 drops) was added to the resulting mixture, and the autoclave was sealed and allowed to slowly warm to room temperature. The reaction mixture was allowed to stir at room temperature for 72 h, after which the reaction was vented carefully. Saturated $NaHCO_3$ was added, and the organic layer was separated and washed with brine. The organic extracts were dried over anhydrous $MgSO_4$(s) and filtered, and the filtrate was concentrated to yield a light-yellow oil that was purified by flash chromatography (silica gel, 1:1 ethyl acetate/hexanes) to give **2** as a colorless oil in 12% yield. ^{1}H NMR (200 MHz, CD_2Cl_2): δ=3.78 (q, J=7.2 Hz, 1H), 1.48 (s, 9H), 1.40 ppm (d, J=7.2 Hz, 3H); ^{13}C NMR (50 MHz, CD_2Cl_2): δ=170.5, 82.8, 58.3, 28.1, 16.9 ppm; MS (ESI) (+ve ion): m/z (%): 194 (100) $[C_7H_{13}N_3O_2Na]^+$.

Synthesis of (3*S*)-3-(*N*-Boc)-amino-5-methyl-1-hexyne (3): (*N*-Boc)-L-leucine-*N*′-methoxy-*N*′-methylamide (2.6 g, 9.4 mmol) was dissolved in anhydrous CH_2Cl_2 (75 mL), and the resulting solution was cooled to −78°C with a dry ice/acetone bath. Diisobutylaluminum hydride (1 M in CH_2Cl_2, 12 mL, 12 mmol) was added dropwise, and the resulting mixture was stirred at −78°C for 40 min. Excess hydride was quenched by the addition of anhydrous MeOH (20 mL) and the resulting solution was warmed to 0°C with an ice/water bath. Potassium carbonate (2.8 g, 20 mmol) and the Bestmann–Ohira reagent (2.18 g, 11.3 mmol) were added to the reaction mixture. The resulting solution was stirred at room temperature for 18 h. The solvents were removed under reduced pressure, and the crude residue was dissolved in EtOAc (100 mL) and water (100 mL). The layers were partitioned, and the organic extracts were washed with brine and dried over anhydrous $MgSO_4$(s). The solvent was removed under reduced pressure, and the pale oil was purified by column chromatography (silica gel 60 0.040–0.063 mm, 230–300 mesh, eluent $CHCl_3$/CH_3OH 100:1 v/v) to give the desired alkyne in 24% yield. ^{1}H NMR (200 MHz, CD_2Cl_2): δ=2.31 (d, J=2.2 Hz, 1H), 1.78 (m, J=6.8 Hz, 1H), 1.53 (t, J=7.6 Hz, 2H), 1.44 (s, 9H), 0.95 ppm (d, J=6.6 Hz, 6H); ^{13}C NMR (50 MHz, CD_2Cl_2) δ=155.1, 84.5, 80, 70.7, 45.5, 41.5, 28.5, 25.3, 22.7, 22.2 ppm.

Synthesis of (3*S*)-3-(*N*-Fmoc)-amino-5-methyl-1-hexyne) (4): Alkyne **3** (396.5 mg, 1.45 mmol) was dissolved in 50% TFA in CH_2Cl_2 (3 mL) and stirred at room temperature for 1 h. The solvent was removed under reduced pressure to yield a pale oil. This oil was dissolved in 50% Acetone in H_2O (5 mL) and Na_2CO_3 (161 mg, 1.5 mmol) was added. Fmoc-OSu (508.7 mg, 1.5 mmol) was added stepwise, while the pH was kept at 9–10 by addition of 1 M aq Na_2CO_3. After stirring overnight, EtOAc (6 mL) was added and the mixture was acidified with 6 M HCl. The organic layer was separated, washed with H_2O (4×50 mL) and dried over $MgSO_4$(s). The organic solvents were removed under reduced pressure and the crude residue was purified by flash-chromatography (silica gel, eluent EtOAc/hexanes 1/1 v/v) to give alkyne **4** as a crude, white residue in 62% yield. ^{1}H NMR (200 MHz, CD_2Cl_2): δ=7.79 (d, J=6.8 Hz, 2H), 7.63 (d, J=7.2 Hz, 2H), 7.4 (m, 4H), 5.00 (s, 1H), 4.4 (m, 3H), 4.24 (t, J=6.6 Hz, 1H), 2.34 (d, J=2.4 Hz, 1H), 1.78 (m, 1H), 1.56 (t, J=7.4 Hz, 2H), 0.95 ppm (d, J=6.6 Hz, 6H); ^{13}C NMR (50 MHz, CD_2Cl_2) δ=155.6, 144.3, 141.7, 128.0, 127.4, 125.4, 120.3, 84, 71.2, 67.1, 47.7, 45.3, 42.1, 25.3, 22.6, 22.1 ppm; MS (ESI) (+ve ion): m/z (%): 356.2 (100) $[C_{22}H_{13}NO_2Na]^+$.

Synthesis of 1-(L-alanine-*tert*-butanoate)-5-(Fmoc-L-leucine)-1,2,3-triazole (5): Compounds **2** (49 mg, 0.29 mmol) and **4** (96.2 mg, 0.29 mmol) were dissolved in dry, degassed toluene (2 mL) under an inert atmosphere of Ar(g). Chloro-(1,5-cyclooctadiene)-(pentamethylcyclopentadienyl)ruthenium(II) (10.4 mg, 24.6 μmol) was added and the resulting mixture was stirred overnight. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (silica gel 60 0.040–0.063 mm; 230–300 mesh, eluent 2% CH_3OH in CH_2Cl_2 v/v) to give triazole **5** as an amorphous, beige residue in 77% yield. ^{1}H NMR (200 MHz, CD_2Cl_2): δ=7.57 (d, J=7.4 Hz, 2H), 7.55 (m, 3H), 7.37 (m, 4H), 4.90 (m, 2H), 4.62 (q, J=4.2 Hz, 1H), 4.19 (m, 2H), 1.86 (d, J=7.2 Hz, 3H), 1.68–1.41 (m, 4H), 1.36 (s, 9H), 0.94 ppm (d, J=5.4 Hz, 6H); ^{13}C NMR (50 MHz, CD_2Cl_2) δ=168.4, 155.9, 144.1, 141.7, 138.9, 131.6, 128.1, 127.4, 125.3, 125.2, 120.3, 83.1, 67.0, 57.1, 47.7, 44.2, 43.6, 27.9, 25.0, 22.9, 22.0, 17.7 ppm; MS (ESI) (+ve ion): m/z (%): 527.2 (100) $[C_{29}H_{36}N_4O_4Na]^+$.

Synthesis of 1-(L-alanine)-5-(Fmoc-L-leucine)-1,2,3-triazole (6): Compound **5** (52.5 mg, 0.104 mmol) was dissolved in 50% v/v TFA in CH_2Cl_2 (1 mL). The reaction mixture was stirred until all the starting material was consumed (5 h), as judged by TLC. The solvent was removed under reduced pressure and excess TFA was removed as an azeotrope with toluene to give compound **6**.

Synthesis of H-HCD-triazole-CG-NH$_2$ (m^{7}SOD-1,5-triazole) and H-HCDLPCG-NH$_2$ (m^{7}SOD): The heptapeptide was synthesized by using standard manual Fmoc SPPS on Rink amide MBHA (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-4-methylbenzhydrylamine) resin with a loading capacity of 0.7 mmol g^{-1} and with 1-hydroxy-1*H*-benzotriazole (HOBt)/*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexylfluoro-phosphate (HBTU; 4 equiv each relative to loading capacity) or diisopropylcarbodiimide (DIC; 4 equiv relative to loading capacity) coupling reagents. The base diisopropylethylamine (DIEA) was used in twofold excess compared to the amino acids and coupling reagents. Cleavage from the resin was achieved with 95% TFA, 2.5% triisopropylsilane, and 2.5% water. The peptide was precipitated in cold diethyl ether, washed several times, and freeze-dried prior to purification by semipreparative HPLC and storage at −80°C.

Data for m^{7}SOD: HPLC: t_R=24.3 min (analytical, gradient A); 42 min (preparative, gradient C); MS (ESI) (+ve ion): m/z (%): 743 (100) $[M+H]^+$, 781 (30) $[M+K]^+$; MS (ESI) (−ve ion): m/z: 741 (100) $[M-H]^-$, 780 (15) $[M+K]^+$.

Data for m^{7}SOD-1,5-triazole: HPLC: t_R=25.7 min (analytical, gradient B); 42.7 min ("1,5-triazole"), 53.2 min ("1,4-triazole") (preparative, gradient C); MS ESI (+ve ion): m/z (%): 741.2 (100) $[M]^+$.

Synthesis of Ni(m^{7}SOD-1,5-triazole) and Ni(m^{7}SOD): m^{7}SOD (9.8 mg, peptide content 75% as determined by Ellman's reagent) was dissolved in water (10 mL) and $NiCl_2$ solution (0.0185 M, 0.541 mL, 1 equiv) was added according to the peptide content calculated by the Ellman's test.[29] During the process of adjusting the pH to 7.8 using a computer controlled titrator (0.1 M NaOH; Methrom, Titrino DMS 716) a slight pink solution was formed and afterwards freeze-dried. The procedure for the "triazole" peptides was made in analogy to that described above.

Data for Ni(m^{7}SOD): MS (ESI) (−ve ion): m/z (%): 797 (100) $[M]^-$.

Data for Ni(m^{7}SOD-1,5-triazole): HPLC: t_R=23.6 min (analytical, gradient D); MS (ESI) (−ve ion): m/z (%):795 (100) $[M]^-$; UV/Vis (water/NaOH, pH 7.8): λ_{max} (ε)=458 (413), 262 (13528 L mol^{-1} cm^{-1}), 223 nm (sh).

Synthesis of Ni(^{13}CN)(m^{7}SOD-1,5-triazole): A freshly prepared K^{13}CN (0.2 M, water, 98% enriched, Chemotrade Chemiehandelsgesellschaft mbH, Leipzig) solution (24.8 μL) was added to the metallopeptide solution (3 mL, 0.83 mM). Afterwards the pH value of the metallopeptide solution was adjusted to 7.8. After addition of K^{13}CN a slight yellow solution was formed in water as well as in buffer solution. MS (ESI) (−ve ion): m/z (%):823.3 (100) $[M+H]^-$, 845.2 (80) $[M+Na]^-$; UV/Vis (water/NaOH, pH 7.8): λ_{max} (ε)=415 (293), 282 (sh), 252 (18178 L mol^{-1} cm^{-1}), 220 nm (sh).

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