Natural Products

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Formation of a Dinuclear Copper(I) Complex from the *Clostridium*-Derived Antibiotic Closthioamide**

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Strictly anaerobic microorganisms, which represent the oldest living beings on earth, are ubiquitous in the environment and in the intestines of higher organisms. Despite their huge impact on human health, ecology, and industrial biotechnology, surprisingly little is known about the natural product chemistry of the oxygen-free world.[1] Until recently, no antibiotic or any other secondary metabolite was known from anaerobes. Bioinformatics analyses of genome sequences of anaerobes, however, have shown a broad biosynthetic potential, in particular for polyketides and nonribosomal peptides.[2–4] A plausible explanation for the meager metabolic profiles is that the limited energy supply under strictly anaerobic conditions causes down-regulation or even silencing of the respective genes in the absence of a particular trigger.[5] For the soil-derived bacterium *Clostridium cellulolyticum*, the cues were contained in an aqueous soil extract. Only in the presence of this complex additive, *C. cellulolyticum* cultures produced a highly unusual metabolite, named closthioamide (CTA, **1**; Figure 1).[6] CTA has a unique symmetrical structure featuring six thioamide moieties. Apart from its highly unusual structural characteristics, CTA exerts a remarkable antimicrobial potential, in particular against problematic nosocomial pathogens (including MRSA and VRE) at nanomolar concentrations.[6,7] Genetic manipulation of the producing strain allowed the isolation and characterization of seven additional thioamide congeners, which appeared to be shunt metabolites and degradation products of CTA.[8] However, the chemical function of the thioamide-rich molecule has remained a mystery. Here we report that CTA acts as a selective metal chelator and disclose the unparalleled architecture of the complex.

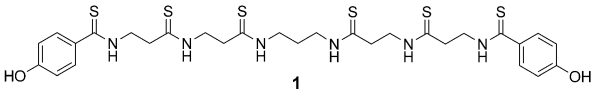

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Figure 1. Structure of closthioamide (CTA).

During our efforts to optimize CTA production rates (typically less than 0.2 mg L^{-1}), we obtained increased yields of isolated CTA when adding potassium cyanide to the cultures prior to organic workup. Thus we suspected that a portion of CTA is bound in metal complexes and that the ligand is displaceable by cyanide. Yet, a metal complex of CTA could not be detected in the broth, likely because of the low production rates, poor chromatographic performance, and strong background signals.

The structure of the symmetric polythioamide **1** strongly suggests that this compound plays a role as a metal ion ligand, in particular because the overall architecture is reminiscent of biogenetic chelators such as the siderophore desferrioxamine B.[9] In stark contrast to siderophores that bind hard Lewis acids (Fe^{3+}), the occurrence of the high number of sulfur atoms in CTA indicates a preference for soft metal ions. To identify potential metal ions that could bind to CTA, we screened mixtures of CTA and metal salts in methanol by reverse-phase thin-layer chromatography (RP-TLC), taking advantage of the strong UV absorbance of thioamides near 270 nm. By means of this assay we could narrow down the number of potential candidates, with Cu^{+}/Cu^{2+} being the most promising (Figure 2 A, series a). Only with Cu^{+}/Cu^{2+} was a quantitative consumption of CTA observed and recovery of the ligand upon cyanide addition could only be observed for Cu^{2+} (partial) and Cu^{+} (complete). In contrast, the bands for Fe^{2+}/Fe^{3+} remained unchanged, thus indicating that the thioamide has degraded (Figure 2 A, series b).

This pre-selection was confirmed by electrospray ionization (ESI-MS) and high-resolution (HR) mass spectrometry (Orbitrap) measurements. Ions of CTA–metal complexes could only be detected for the copper-containing samples. Interestingly, for both Cu^{+} and Cu^{2+} we observed two dominant peaks, $m/z = 410$ $[CTA + 2\,Cu]^{2+}$ and $m/z = 757$ $[CTA + Cu]^{+}$, each showing the characteristic isotope pattern

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Supporting information (including the Experimental Section) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201304714.

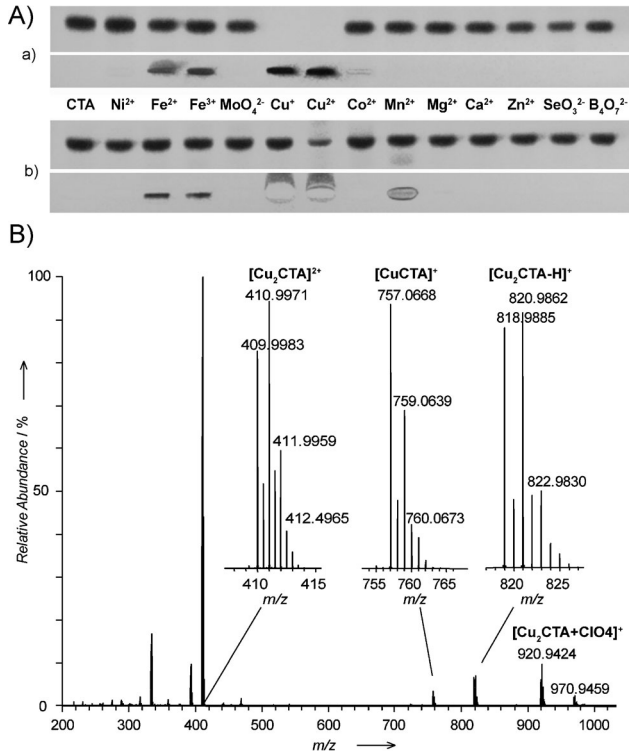


Figure 2. A) RP-TLC screening of CTA versus selected metal salts prior (a) and subsequent (b) to potassium cyanide treatment. B) HR-ESI-MS spectrum of CTA and $[Cu(CH_3CN)_4]ClO_4$ in methanol.

for one and two copper nuclei, respectively (see Figure 2B and the Supporting Information). Finally, the HRMS-derived elemental composition (Orbitrap) strongly suggests that the present complex is a copper(I) species in both samples.

Copper(II) reduction by thioamides has been reported in the context of copper-catalyzed oxidations.[10] Likewise, CTA has the ability to reduce copper(II), which rationalizes the MS data and the partial recovery of the ligand in the TLC assay. In contrast to the low stability of CTA in the presence of copper(II), we found that the copper(I) complex is stable in aqueous mixtures. Even after several days of incubation, the diagnostic peaks for the copper complex were found in the mass spectrum. The following structural studies, however, were performed in non-aqueous solvents because of the very poor solubility of CTA ($\leq 2\,\mu M$) and the corresponding complexes.

As a diamagnetic ion, copper(I) allowed NMR analyses that provided first insights into the structural properties of the complex. In the ^1H NMR spectrum, a significant downfield shift of the NH/OH protons of CTA was observed after addition of copper(I) iodide (in $[D_6]$DMSO, DMSO = dimethyl sulfoxide; see the Supporting Information). In light of the poor UV/Vis spectral differences between CTA and the respective copper(I) complex, we decided to perform a ^1H NMR titration experiment to elucidate the preferred complex stoichiometry. To avoid partial degradation of CTA, which takes place even under an inert atmosphere in the presence of trace amounts of copper(II), we added sulfur dioxide to the freshly distilled NMR solvent ($[D_6]$DMSO).

The method of continuous variation according to Job was used to get the CTA/Cu ratio in the preferred complex.[11] The curve maximum was found at a mole fraction of 0.66, thus indicating that under these conditions, two copper nuclei are coordinated by one CTA ligand (Figure 3).

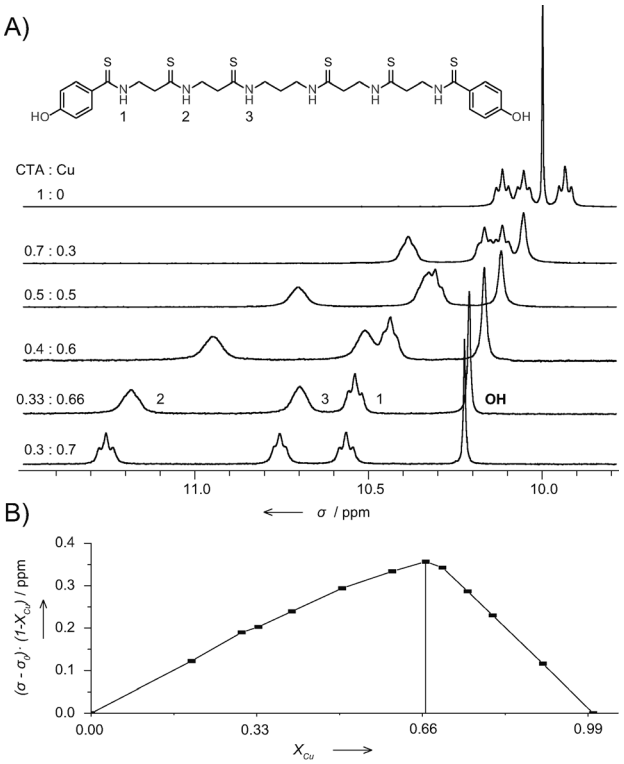
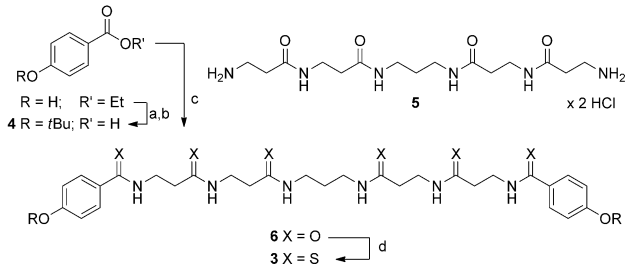


Figure 3. A) ^1H NMR titration experiment of CTA against $[Cu(CH_3CN)_4]ClO_4$; B) Job plot (NH (2) protons).

While these data suggested a monomolecular structure for the CTA–Cu complex in favor of supramolecular assemblies, the spatial structure remained a riddle. CTA provides only six thioamide donor moieties, and copper(I) usually prefers a tetrahedral coordination sphere, which is not compatible with the determined ratio. As NOESY/ROESY experiments of the complex could not provide information on the overall structure we envisaged an X-ray analysis.

Single crystals of the copper complex were obtained with O-TBDMS-substituted closthioamide (**2**), but with unsatisfying morphology. We reasoned that the *tert*-butyl substituent could be favorable because of slightly reduced steric effects and synthesized analogue **3** (Scheme 1).

Out of a total number of more than 600 different inert crystallization trials for the copper(I) complex, we finally obtained yellow hygroscopic prisms. X-ray data analysis showed an unexpected three-dimensional architecture of the complex (Figure 4). In accordance with the results obtained by the Job plot, two copper centers are involved in the coordination ($d_{Cu-Cu} = 4.1$ Å). Notably, both copper nuclei are located in the trigonal-planar environment of the thioamide sulfur atoms (Figure 4).



Scheme 1. Synthesis of $(t\mathrm{Bu})_2$CTA. a) Isobutylene, H_2SO_4, CH_2Cl_2, 0 °C, 20 h; b) KOH, H_2O, MeOH, reflux, 1.5 h, 53 % over two steps; c) EDC, HOBt, Hünig's base, DMF, RT, 15 h, 89 %; d) Lawesson's reagent, pyridine, 105 °C, 4 h, 71 %. For details, see the Supporting Information. EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride, DMF = *N*,*N*-dimethylformamide, HOBt = 1-hydroxybenzotriazole.

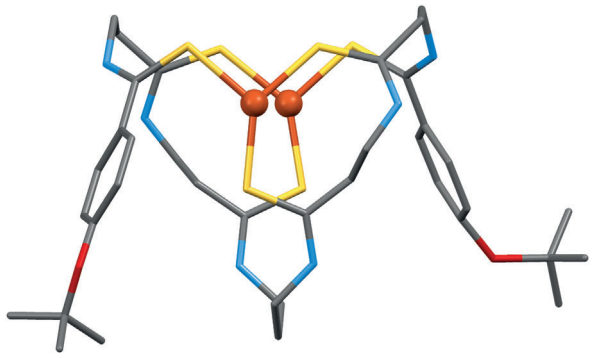


Figure 4. X-ray structure of $Cu_2(t\mathrm{Bu})_2$CTA (one dicationic species is shown; other views in the Supporting Information).

Trigonal planar copper(I) complexes have been found in a series of copper-dependent metalloproteins,[12] such as the reduced form of a Cu–Zn superoxide dismutase,[13] the binding sites of the CsoR family,[14] metallothioneins,[15] or laccases.[16,17] In contrast, trigonal planar copper(I) centers in secondary metabolite complexes are unprecedented. Another unique structural characteristic of the CTA–Cu complex is the mode of chelation. A single binding pocket for one copper ion is provided by two thioamide moieties of two adjacent β-thioalanine residues. The third donor atom is supplied by the thiobenzamide moiety, however, on the other half of the molecule. For the second copper ion, the situation is identical. In consequence, disregarding packing effects in the crystal, the overall structure has C_2 symmetry. Interestingly, the arrangement of the CTA ligand around the two copper centers is helix-like, and both copper ions stabilize the pitch. Notably, in the crystal both enantiomers are found. Helical structures of synthetic oligo β-thiopeptides in solution have been described, albeit without any chelated metal ions.[18,19]

In line with the results from physicochemical analyses, the observed trigonal planar architecture is most compatible with a copper(I) oxidation state.[20] We further substantiated this finding by EPR measurements of solutions of native and O-substituted closthioamides and $[Cu(CH_3CN)_4]ClO_4$. In all cases, the complexes were nearly EPR silent, which is in full accord with a proposed diamagnetic copper(I) complex (see the Supporting Information).

Copper(I) complexes have been described for only a few natural products, such as bleomycin,[21] penicillamine, glutathione,[22] and halichonadin.[23] Furthermore, the peptide metabolite methanobactin has been shown to be involved in copper acquisition of methane-oxidizing bacteria, which employ a copper-dependent methane monooxygenase (pMMO).[24,25] Despite their large molecular size and structural complexity, methanobactins capture only a single copper nucleus in a tetrahedral manner.[26,27] In contrast, the small backbone of CTA has the ability to accommodate two copper nuclei. Apparently, this structurally simple molecule bears only the most essential moieties for carrying two copper centers.

In contrast to methanotrophs, bacteria of the genus *Clostridium* are not known for an excessive copper metabolism and were considered as copper non-users.[28,29] To evaluate the effect of CTA on copper homeostasis, we challenged *C. cellulolyticum* with various copper levels. Yet, no increased production of CTA under copper-limiting conditions could be observed. However, a positive copper–chromazurol S (Cu–CAS) agar-plate assay[30] suggested the potency of CTA to displace CAS from its copper complex in aqueous media (see the Supporting Information). The high copper affinity of CTA was further corroborated in titration experiments under copper-limiting conditions versus the high copper(I) affinity ligand bathocuproine disulfonate (BCS; Figure 5 A). In light of the high stability constant of $[Cu(BCS)]^{3-}$,[31] the displacement of the ligand by CTA in aqueous solution further supports the role of CTA in copper complexation under physiological conditions

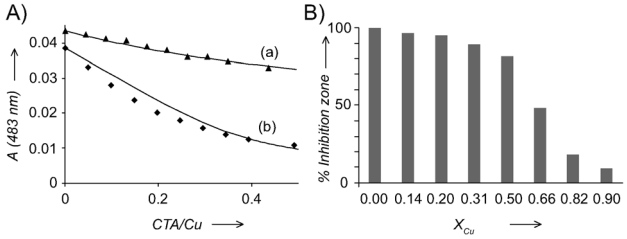


Figure 5. A) Titration experiment under copper-limiting conditions with $[Cu(BCS)_2]^{3-}$ as a competitive probe. Conditions: pH 7.0, Hepes buffer (50 mM), a) $[Cu^I]_{total} = 3.5$ μM, $[CTA]_{total} = 0$-1.5 μM, $[BCS]_{total} = 70.1$ μM; b) $[Cu^I]_{total} = 3.1$ μM, $[CTA]_{total} = 0$-1.5 μM, $[BCS]_{total} = 14.4$ μM. Traces shown were the best fits to equations 1 (a) and 2 (b; see the Supporting Information). B) Antimicrobial activity of different CTA–Cu ratios against methicillin-resistant *Staphylococcus aureus* strains (MRSA; Supporting Information).

($K_D < 10^{-15}$ M). This fact is also supported by a decreasing antimicrobial activity of CTA in the presence of copper (Figure 5 B). Overall, these findings indicate that CTA acts as a strong copper(I) chelator. A plausible scenario is the involvement of CTA in copper detoxification or selective copper reduction in the anaerobe.

In summary, we have illustrated the potential of the *Clostridium*-derived natural product closthioamide to act as a selective copper chelator using RP-TLC and HRMS analyses. NMR and X-ray structural studies have unveiled

a highly compact symmetrical arrangement of a dinuclear complex. Both copper ions are located in an unusual trigonal-planar environment of the thioamide sulfur atoms, which is unparalleled for natural products. Multiple lines of experimental evidence, a ligand recovery assay, HRMS data of the complex, NMR Job plot, the X-ray structure, and EPR measurements showed the presence copper(I) nuclei in the complex. Beyond the structural novelties, this is the first described metal complex of a secondary metabolite from the microbial anaerobic world. Competitive assays unequivocally demonstrated the formation of a CTA complex under physiological conditions and corroborated the role of CTA as a strong copper(I) chelator. The discovery of a selective copper(I) ligand may further serve as a lead for bio-inspired copper-selective chelators with therapeutic potential, since copper mis-trafficking has been implicated in various diseases, such as Menkes and Wilson's disease.[32]

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[1] S. Behnken, C. Hertweck, *Appl. Microbiol. Biotechnol.* **2012**, *96*, 61 – 67.
[2] A.-C. Letzel, S. J. Pidot, C. Hertweck, *Nat. Prod. Rep.* **2013**, *30*, 392 – 428.
[3] J. M. Winter, S. Behnken, C. Hertweck, *Curr. Opin. Chem. Biol.* **2011**, *15*, 22 – 31.
[4] S. Behnken, C. Hertweck, *PLoS ONE* **2012**, *7*, e29609.
[5] K. Scherlach, C. Hertweck, *Org. Biomol. Chem.* **2009**, *7*, 1753 – 1760.
[6] T. Lincke, S. Behnken, K. Ishida, M. Roth, C. Hertweck, *Angew. Chem.* **2010**, *122*, 2055 – 2057; *Angew. Chem. Int. Ed.* **2010**, *49*, 2011 – 2013.
[7] F. Kloss, T. Lincke, C. Hertweck, *Eur. J. Org. Chem.* **2011**, 1429 – 1431.
[8] S. Behnken, T. Lincke, F. Kloss, K. Ishida, C. Hertweck, *Angew. Chem.* **2012**, *124*, 2475 – 2478; *Angew. Chem. Int. Ed.* **2012**, *51*, 2425 – 2428.
[9] R. C. Hider, X. Kong, *Nat. Prod. Rep.* **2010**, *27*, 637 – 657.
[10] F. Shibahara, A. Suenami, A. Yoshida, T. Murai, *Chem. Commun.* **2007**, 2354 – 2356.
[11] K. Hirose in *Analytical Methods in Supramolecular Chemistry*, Wiley-VCH, Weinheim, **2007**, pp. 17 – 54.
[12] A. V. Davis, T. V. O'Halloran, *Nat. Chem. Biol.* **2008**, *4*, 148 – 151.
[13] N. L. Ogihara, H. E. Parge, P. J. Hart, M. S. Weiss, J. J. Goto, B. R. Crane, J. Tsang, K. Slater, J. A. Roe, J. S. Valentine, D. Eisenberg, J. A. Tainer, *Biochemistry* **1996**, *35*, 2316 – 2321.
[14] T. Liu, A. Ramesh, Z. Ma, S. K. Ward, L. Zhang, G. N. George, A. M. Talaat, J. C. Sacchettini, D. P. Giedroc, *Nat. Chem. Biol.* **2007**, *3*, 60 – 68.
[15] V. Calderone, B. Dolderer, H.-J. Hartmann, H. Echner, C. Luchinat, C. Del Bianco, S. Mangani, U. Weser, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 51 – 56.
[16] K. Piontek, M. Antorini, T. Choinowski, *J. Biol. Chem.* **2002**, *277*, 37663 – 37669.
[17] S. Garavaglia, M. T. Cambria, M. Miglio, S. Ragusa, V. Iacobazzi, F. Palmieri, C. D'Ambrosio, A. Scaloni, M. Rizzi, *J. Mol. Biol.* **2004**, *342*, 1519 – 1531.
[18] T. Sifferlen, M. Rueping, K. Gademann, B. Jaun, D. Seebach, *Helv. Chim. Acta* **1999**, *82*, 2067 – 2093.
[19] T. T. Tran, J. Zeng, H. Treutlein, A. W. Burgess, *J. Am. Chem. Soc.* **2002**, *124*, 5222 – 5230.
[20] R. Balamurugan, M. Palaniandavar, R. S. Gopalan, *Inorg. Chem.* **2001**, *40*, 2246 – 2255.
[21] G. M. Ehrenfeld, L. O. Rodriguez, S. M. Hecht, C. Chang, V. J. Basus, N. J. Oppenheimer, *Biochemistry* **1985**, *24*, 81 – 92.
[22] R. Österberg, R. Ligaarden, D. Persson, *J. Inorg. Biochem.* **1979**, *10*, 341 – 355.
[23] H. Ishiyama, S. Kozawa, K. Aoyama, Y. Mikami, J. Fromont, J. i. Kobayashi, *J. Nat. Prod.* **2008**, *71*, 1301 – 1303.
[24] H. J. Kim, D. W. Graham, A. A. DiSpirito, M. A. Alterman, N. Galeva, C. K. Larive, D. Asunskis, P. M. A. Sherwood, *Science* **2004**, *305*, 1612 – 1615.
[25] L. A. Behling, S. C. Hartsel, D. E. Lewis, A. A. DiSpirito, D. W. Choi, L. R. Masterson, G. Veglia, W. H. Gallagher, *J. Am. Chem. Soc.* **2008**, *130*, 12604 – 12605.
[26] N. Bandow, V. S. Gilles, B. Freesmeier, J. D. Semrau, B. Krentz, W. Gallagher, M. T. McEllistrem, S. C. Hartsel, D. W. Choi, M. S. Hargrove, T. M. Heard, L. N. Chesner, K. M. Braunreiter, B. V. Cao, M. M. Gavitt, J. Z. Hoopes, J. M. Johnson, E. M. Polster, B. D. Schoenick, A. M. Umlauf, A. A. DiSpirito, *J. Inorg. Biochem.* **2012**, *110*, 72 – 82.
[27] A. El Ghazouani, A. Baslé, J. Gray, D. W. Graham, S. J. Firbank, C. Dennison, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 8400 – 8404.
[28] M. Solioz, H. K. Abicht, M. Mermod, S. Mancini, *J. Biol. Inorg. Chem.* **2010**, *15*, 3 – 14.
[29] P. G. Ridge, Y. Zhang, V. N. Gladyshev, *PLoS ONE* **2008**, *3*, e1378.
[30] S. Yoon, A. A. Dispirito, S. M. Kraemer, J. D. Semrau, *Methods Enzymol.* **2011**, *495*, 247 – 258.
[31] Z. Xiao, J. Brose, S. Schimo, S. M. Ackland, S. La Fontaine, A. G. Wedd, *J. Biol. Chem.* **2011**, *286*, 11047 – 11055.
[32] H. Kodama, C. Fujisawa, *Metallomics* **2009**, *1*, 42 – 52.

