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Synthesis and Characterization of Schiff Base Chelates of Tetramic Acids as NMR Contrast Agents for Micro Imaging

O. Tietze, G. Reck, B. Schulz, and A. Zschunke

Berlin, Institut für Angewandte Analytik und Umweltchemie der Humboldt-Universität und Bundesanstalt für Materialforschung und -prüfung

K. Keller

Berlin, Institut für Pharmakologie der Freien Universität

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Abstract. Novel Cu(II) complexes derived from *N,N'*-ethylene-bis-(1',5',5'-trimethyltetramic acid-3'-acetiminato)copper (II) **aCu** [1] were evaluated as contrast agents for NMR microscopy and therefore tested for penetration into living cells (*Xenopus laevis* oocytes). By *in vitro* ^{1}H NMR relaxation (T_1) and atomic absorption spectroscopy measurements (AAS) we confirmed, that independent of the complex isomers (*Z/Z, Z/E, E/E, E/Z*) compounds with alkyl substituents in $R^1 - R^5$ exclusively were able to penetrate the plasma membranes. It is noteworthy that compounds containing the tetramic acid moieties from both a penetrating and a non-penetrating complex most likely were accumulated in the plasma membrane and/or plasma membrane-associated vesicles, which was concluded from *in vitro* NMR measurements combined with AAS trace analyses. In this context the crystal and molecular structure of the 'chimerical' $C_{17}H_{22}N_4O_4Cu \cdot 3H_2O$ **kCu** $\cdot 3H_2O$ were determined by x-ray analysis. The x-ray structural parameters are discussed in detail.

Intracellular acting contrast agents should be important tools for *in vitro* NMR microscopy. Therefore, those paramagnetics are of general interest that cross biological membranes without remarkable lesions of the cells and enhance the contrast in microscopy images by decreased T_1 times of intracellular water protons. As starting point of our investigation we favoured chelating compounds derived from tetramic acids, occuring as subunits in various natural antibiotics (e.g. ikarugamycin [2], streptolydigin [3], tenuazonic acid [4] and tirandamycin [5]). Using the membrane crossing complex *N,N'*-ethylene-bis-(1',5',5'-trimethyl-tetramic acid-3'-acetiminato)-copper(II) **aCu** [1] as lead structure, we synthesized Cu(II) complexes which differed in substitution pattern and complex isomerism (Table 1). All above mentioned complexes were soluble both in water and in organic solvents. We recently reported the structural characterization of the complexes **aCu**, **bCu**, **dCu** $\cdot H_2O$ and **eCu** [1, 6, 7]. Their corresponding ligands were investigated by NMR spectroscopy [8, 9]. The isomeric state of the complexes was proved by crystal structure analyses and was found to be without influence with regard to the penetration into living cells (e.g. *Xenopus* oocytes).

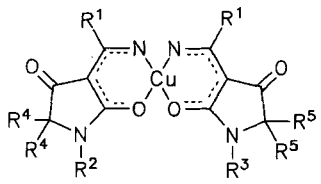
Whether the corresponding compounds penetrated, could be determined by applying the *in vitro* ^{1}H NMR spectroscopy (T_1 measurements by Inversion Recovery sequence) and atomic absorption spectroscopy (AAS), respectively. Additionally, the combination of methods allowed to differentiate between the entrance behaviour of substances within the group of non-penetrating complexes.
By *in vitro* ^{1}H NMR microscopy the results of Inversion Recovery measurements regarding the entrance of the complexes **aCu**, **bCu** and **cCu** into the oocytes could be confirmed [9-11]. The microscopy measurements have shown that the membranes of the nuclei are likewise penetrated.

Experimental

Preparation of Xenopus oocytes

Xenopus laevis oocytes were removed surgically as sections of ovarian lobes from adult female frogs under benzocaine anaesthesia [12]. After 7–8 h incubation in sterile Barth's saline at 18 °C oocytes of stage V–VI [13] were selected under a dissecting microscope according to their healthy appearance. The oocytes were defolliculated with 0.2% colla-

Table 1 List of the investigated compounds.
– m = mixture of isomers n.d. = not determined



	R^1	R^2	R^3	R^4	R^5	Isomer	Permeation	c (mmol) in Barth's medium
aCu	CH_3	CH_3	CH_3	CH_3	CH_3	*Z/Z*	Yes	10.0
bCu	CH_3	CH_2CH_3	CH_2CH_3	CH_3	CH_3	*E/E*	Yes	0.38
cCu	CH_3	CH_3	CH_2CH_3	CH_3	CH_3	n.d.	Yes	3.11
dCu	H	CH_3	CH_3	CH_3	CH_3	*E/E*	No	1.0
eCu	CH_2CH_3	CH_3	CH_3	CH_3	CH_3	*Z/E*	Yes	0.32
fCu	CH_3	H	H	CH_3	CH_3	n.d.	No	0.5
hCu	CH_3	H	CH_3	CH_3	CH_3	m	No	10.0
kCu	CH_3	H	CH_3	H	CH_3	*E/E*	No	0.51

genase (Type II, Sigma, St. Louis, MO USA) treatment for 40–50 min. Following several washing steps with ice-cold Barth's solution, the *X.* oocytes were kept in the complex solutions for defined times at 18 °C and 4 °C, respectively, and were then released from the complex by several washings.

Barth's Saline

88 mM NaCl; 1 mM KCl; 2.4 mM; 15 mM Hepes-NaOH; 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$; 0.82 mM $MgSO_4$; 10 µg/ml Benzylpenicillin (sodium salt) and 10 µg/ml Streptomycin (Sigma, St. Louis, MO, USA)

^{1}H NMR measurements

Single cells were placed in 5 mm NMR tubes, and measurements were performed at 300 MHz on a BRUKER AM 300 spectrometer using 'Inversion Recovery' as described previously [10].

Atomic absorption spectroscopy

The complex-treated *X.* oocytes (almost 50 oocytes were pooled for each sample) were breaked up (1.5 ml HNO_3, subboiling quality, 7 hours at 170 °C). Then AAS measurements were performed on a Perkin Elmer 5000 with a graphite pipe oven HGA-500 and auto sampler AS-40, or on a Perkin Elmer Zeeman 5100 PC with graphite pipe oven HGA-600 and auto sampler AS-60.

To determine the copper content the platform technique (pyrolytic coated glass–carbon platform) and the palladium/magnesium nitrate modifier have been used. The blank value of copper was less than 0.1 ng [14].

Synthesis of the Cu(II) complexes

Acyltetramic acids [15, 16] react with ethylenediamine smoothly to double Schiff bases. We obtained the corresponding Cu(II) complexes (Table 1, 2) after reaction of the Schiff bases with Cu(II) acetate hydrate in water/ethanol solution. The complexes were purified via silicagel column chromatography ($CHCl_3$:MeOH 9:1) followed by several recrystallization steps.

The reaction of two diversely substituted tetramic acids with ethylenediamine led to products containing three main components as found by thin layer chromatography.

Table 2 Analytical data of the investigated compounds

Complex	Formula	calcd. % found % C	H	N
bCu	$C_{22}H_{32}N_4O_4Cu$	55.04	6.72	11.67
	(480.1) MS 479 (M^+ ^{63}Cu)	54.94	6.55	11.67
cCu	$C_{21}H_{30}N_4O_4Cu$	54.12	6.49	12.02
	(466.0) MS 465 (M^+ ^{63}Cu)	54.25	6.73	11.39
dCu·H_2O	$C_{18}H_{24}N_4O_4Cu{\cdot}H_2O$	48.87	5.88	12.67
	(441.9) MS 423 (M^+ ^{63}Cu)	48.78	5.71	12.69
eCu	$C_{22}H_{32}N_4O_4Cu$	55.04	6.72	11.67
	(480.1) MS 479 (M^+ ^{63}Cu)	54.82	6.89	11.47
fCu·$4H_2O$	$C_{18}H_{24}N_4O_4Cu{\cdot}4H_2O$	43.58	6.50	11.29
	(496.0) MS 423 (M^+ ^{63}Cu)	44.08	6.35	11.16
hCu·H_2O	$C_{19}H_{26}N_4O_4Cu{\cdot}H_2O$	50.04	6.18	12.28
	(456.0) MS 437 (M^+ ^{63}Cu)	50.37	5.90	12.16
kCu·$3H_2O$	$C_{17}H_{22}N_4O_4Cu{\cdot}3H_2O$	43.96	6.03	12.06
	(463.9) MS 409 (M^+ ^{63}Cu)	43.75	6.20	11.88

These components were identified as two symmetrical and one asymmetrical substituted double Schiff bases. The asymmetrical ligands **c**,**h** and **k** were isolated chromatographically from the corresponding mixtures. For the compounds **h** and **k** $CHCl_3$:MeOH 9:1 could be applied. The isolation of compound **c** was rather difficult, because of the presence of direct homologues in the mixture. In this case $CHCl_3$: acetone:ethylacetate:EtOH 3:3:2:was found to be the eluent of choice. The synthetic route described above offers the possibility to create synthesis libraries.

Crystallographic Measurements and Structure Determination for the complex kCu

A dark violet crystal of **kCu**·$3H_2O$ with dimensions 0.64×0.50×0.14 mm was measured on an Enraf-Nonius CAD-4 single crystal computer-controlled diffractometer. The crystal was found to be monoclinic by automatic peak search, centre and index procedures and by cell reduction calculations. Examinations of the systematic extinctions identified the space group as $P2_1/n$.

Crystal data: $C_{17}H_{22}N_4O_4Cu \cdot 3H_2O$, $M = 463.98$ g mol^{-1}, $a = 11.161(5)$ Å, $b = 11.94(1)$ Å, $c = 15.513(8)$ Å, $\beta = 97.07(4)°$, $V = 2051.6$ Å^3, $Z = 4$, $D_c = 1.50$ g cm^{-3}, MoK_α radiation ($\lambda = 0.71073$ Å), $F(000) = 972$, $\mu(MoK_\alpha) = 11.1$ cm^{-1}.

In the range $3.0° \leq 2 \leq 50°$ 3614 reflections were measured of which 3364 with I(hkl) were considered as observed and used for structure determination.

The structure was solved by direct methods and refined by full matrix least square's procedures to minimise the quantity $\Sigma w(|F_o|-|F_c|)^2$. The weighting scheme $w=1/[(I)^2+(0.05\underline{F}^2)^2]$ was employed. Hydrogen atoms were located by difference Fourier syntheses and refined isotropically. The final residual index R was 0.037 ($R_w = 0.038$). The final difference map showed no peaks of chemical significance ($\Delta\varphi_{max} = 0.51$e Å^{-3}). The calculations were carried out with ENRAF-NONIUS-MOLEN program system [17].

Fig. 1 shows the molecular structure of the compound involving labeling of non-hydrogen atoms. Interatomic distances and bond angles [1]) indicate a wide delocalisation of the π-electrons in both moieties of the ligand molecule up to the atoms N1 and N1', respectively. However, N1-C5 and N1'-C5' distances of 1.291 Å and 1.299 Å indicate double bonds at these positions. The central Cu atom is fivefold coordinated by the atoms O1, O1', N1, N1' of the same ligand and $O2^i$ of a neighbouring molecule related by a twofold screw axis of the space group. $O2^i$ occupies at a distance of 2.591 Å the top of a nearly regular tetragonal pyramid (Fig. 2).

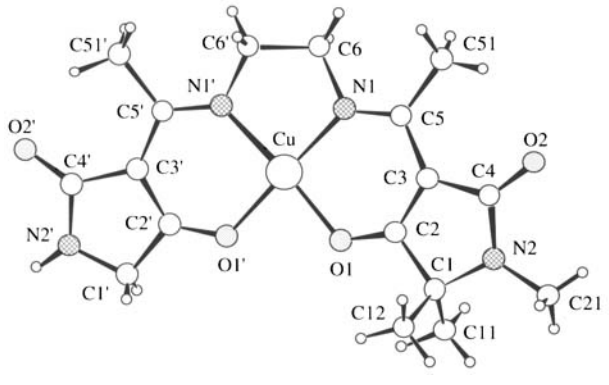


Fig. 1 *N,N'*-ethylene-(tetramic acid-1',5',5'-trimethyl tetramic acid-3,3'-acetiminato)-copper(II) **kCu** (labeling of non-hydrogen atoms)

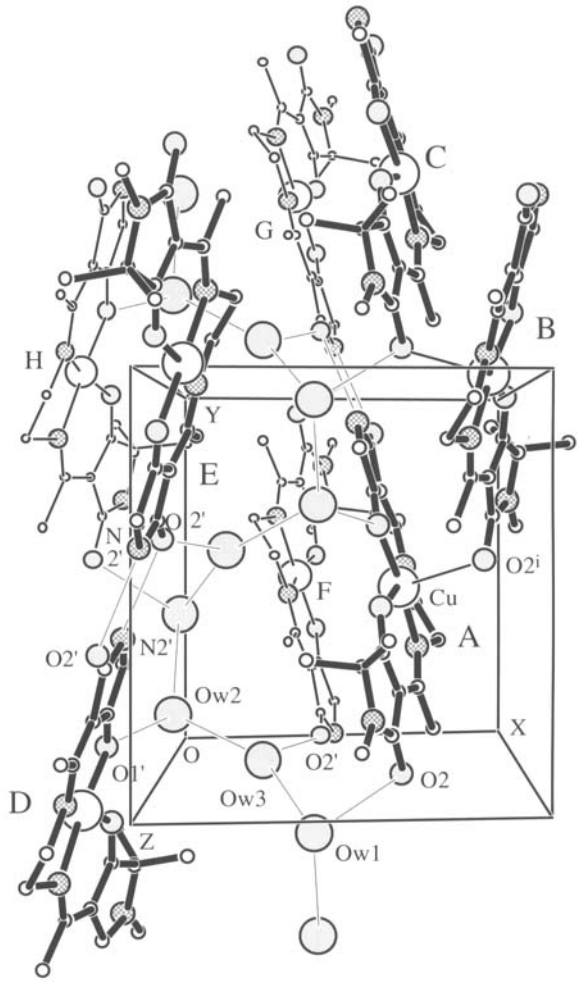


Fig. 2 Molecular packing in *N,N'*-ethylene-(tetramic acid-1',5',5'-trimethyltetramic acid-3,3'-acetiminato)-copper(II) Trihydrate **kCu**·$3H_2O$

Fig. 2 illustrates the molecular packing of **kCu**·$3H_2O$. The above mentioned participation of the O2 carbonyls of 2_1 related molecules to the Cu coordination gives rise to the formation of infinite chains of complexes in x-direction (see molecules A, B, and C in Fig. 2). Two centres of symmetry of related intermolecular hydrogen bonds N2'H–O2 (N–O = 2.886 (4) Å) form pairs (molecules D and E in Fig. 2). They are similar to base pairing in DNA and RNA, respectively.

In contrast to the recently investigated water containing Schiff base complexes K1, K2 and K3 [6] in **kCu**·$3H_2O$ none of the water molecules is involved in complex formation. The three water molecules Ow1, Ow2, and Ow3 (Fig. 2) and their 2_1 related molecules linked by hydrogen bonds form a helix in x-direction (O–O distances: 2.862(4) Å, 2.865(5) Å, and 2.813(5) Å). Surrounded complex molecules are linked to

[1]) Further details of the crystal investigation are available from the authors and the Fachinformationszentrum Karlsruhe, Gesellschaft für wissenschaftlich-technische Information mbH, D-76344 Eggenstein-Leopoldshafen, on quoting the depository number CSD...., the names of the authors and the journal citation.

the helix via hydrogen bonds Ow1-O2 (molecule A) = 2.937(4) Å, Ow2–O1' (D) = 2.964(4) Å, and Ow3–O2' (F) = 2.863 (4) Å. All these intermolecular bonds led to a very stable arrangement of water and complex molecules in the crystals of **kCu**·$3H_2O$.

Results and Discussion

The measurement of the T_1-values of the water protons in natural cells by the Inversion Recovery technique allows a qualitative detection of paramagnetic complexes dissolved in water inside the cells.

Only the complexes **aCu, bCu, cCu** (Fig. 3) and **eCu** were able to pass the cell-membranes of *X.* oocytes and could be solved in the intracellular water.

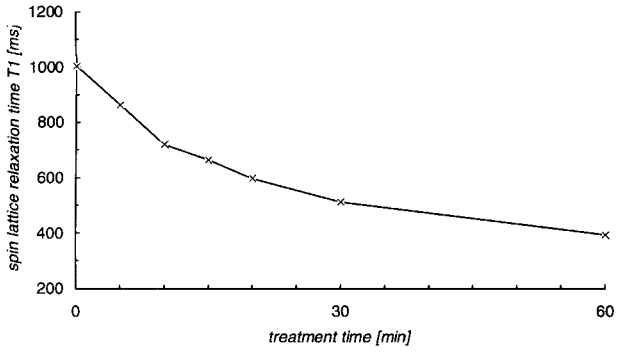


Fig. 3 Spin-lattice relaxation times (T_1) of intracellular water protons in *X.* oocytes at different times of treatment with *N,N'*-ethylene-(1-ethyl-5,5'-dimethyltetramic acid-1',5',5'-trimethyltetramic acid-3,3'-acetiminato)-copper(II) **cCu** at 18 °C ($3.11 \cdot 10^{-3}$ M). The T_1 times were detected by ^{1}H NMR Inversion Recovery (s. Experimental).

After the same procedure the complexes **dCu, fCu, hCu** and **kCu** were not detectable inside the oocytes by T_1-measurements. The AAS-measurements however registrated the Cu-contents of the whole *X.* oocyte, independent whether the complex was inside the cell or stored in the membrane-bilayer.

The non-penetrating complexes **dCu** and **fCu** were nevertheless accumulated in or at the *X.* oocytes. AAS analysis resulted in a 2.6 fold (resp. 2.7 fold) higher copper content after two hour treatment with **dCu** (resp. **fCu**) at 18 °C. At 4 °C the copper contents increased in both cases by only ~ 20%. It is unlikely that the complexes **hCu** and **kCu** were much more accumulated than **dCu** and **fCu** (see figure 4 a, b, c). Apparently the complexes **hCu** and **kCu** were trapped in the plasma membrane and/or membrane-associated vesicles and their further way into the cytoplasm might be blocked by interaction of the impermeable half of the molecule with the plasma membrane.

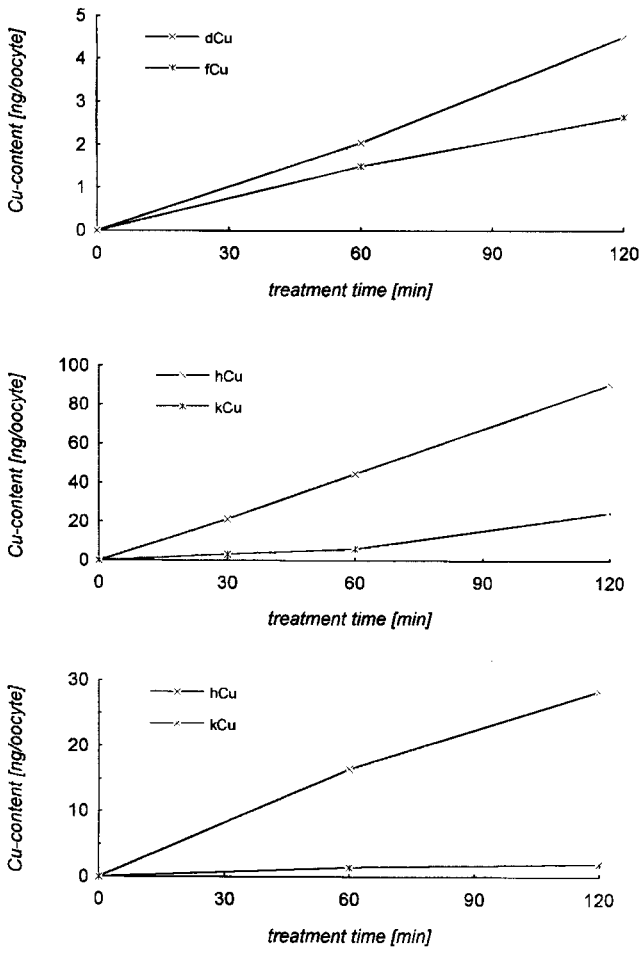


Fig. 4 AAS-measured Cu-contents after treatment of *X.* oocytes with
a) *N,N'*-ethylene-bis-(1,5',5'-trimethyltetramic acid-3'-forminato)-copper(II) **dCu** and *N,N'*-ethylene-bis-(5',5'-dimethyltetramic acid-3'-acetiminato)-copper(II) **fCu** at 18 °C
b) *N,N'*-ethylene-(5,5-dimethyltetramic acid-1',5',5'-trimethyltetramic acid-3,3'-acetiminato)-copper(II) **hCu** and *N,N'*-ethylene-(tetramic acid-1',5',5'-trimethyl tetramic acid-3,3'-acetiminato)-copper(II) **kCu** at 18 °C
c) *N,N'*-ethylene-(5,5-dimethyltetramic acid-1',5',5'-trimethyltetramic acid-3,3'-acetiminato)-copper(II) **hCu** and *N,N'*-ethylene-(tetramic acid-1',5',5'-trimethyl tetramic acid-3,3'-acetiminato)-copper(II) **kCu** at 4 °C

All membrane penetrating complexes are substituted with R^1–R^5 methyl and/or ethyl groups. The ability of penetration is independent of the location of the alkyl groups and independent of the Z/E-isomerism. Noteworthy to mention, that **aCu, bCu, cCu** and **eCu** crystallize solvent free, the non-penetrating complexes form crystal hydrates **dCu**·H_2O, **fCu**·$4H_2O$, **hCu**·H_2O, **kCu**·$3H_2O$. They contain specific hydrophilic areas which could be restrictive for membrane penetration; they were characterized by thermoanalytical estimation of dehy-

dration enthalpies [18]. From additional applications of the novel compounds on other cell systems (e.g. erythrocytes, erythrocyte ghosts), vesicular lipid membranes or synthetic membranes we expect interesting additional results.

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References

[1] A. Zschunke, U. Böttger, R. Donau, H. Bögge, A. Müller, A. Hauser, R. Lück, Z. anorg. allg. Chem. **614** (1992) 87
[2] R. C. F. Jones, R. F. Jones, Tetrahedron. Lett. **31** (1990) 3363
[3] V. J. Lee, A.R. Branfman, T. R. Herrin, K. L. Rinehart, J. Am. Chem. Soc. **100** (1978) 422
[4] M. Nolte, P. Steyn, P. Wessels, J. Chem. Soc., Perkin Trans.I **5** (1980) 1057
[5] R. K. Boeckmann, J. E. Starrett, D. G. Nickell, P. E. Sum, J. Am. Chem. Soc. **108** (1986) 5549
[6] G. Reck, B. Schulz, A. Zschunke, O.Tietze, J. Haferkorn, Z. Krist. **209** (1994) 969
[7] O. Tietze, G. Reck, B. Schulz, A. Zschunke, Z. Krist. **210** (1995)137
[8] A. Zschunke, R. Donau, U. Böttger, J. Prakt. Chem. **332** (1990) 319
[9] O. Tietze, PhD dissertation, Humboldt Universität zu Berlin (1995)
[10] S. Päuser, K. Keller, A. Zschunke, C. Mügge, Magn. Reson. Imaging **11** (1993) 419
[11] S. Päuser, PhD dissertation, Humboldt Universität zu Berlin (1993)
[12] K. Keller, M. Strube, M. Mueckler, J. Biol. Chem. **264** (1989) 18884
[13] J. N. Dumont, J. Morphol. **136** (1972) 153
[14] H. Scharf, H. J. Heinrich, Laboratory information (1994)
[15] R. N. Lacey, J. Chem. Soc. **1954**, 850
[16] A. Koppe, PhD dissertation, Humboldt Universität zu Berlin (1987)
[17] Fair C. K.: MOLEN. An Interactive Intelligent System for Crystal Structure Analysis, User Manual, Enraf-Nonius, Delft, The Netherlands (1990)
[18] O. Tietze, M. Feist, A. Zschunke, Fresenius J. Anal. Chem. **352** (1995) 532

Address for correspondence:
Prof. Dr. A. Zschunke
Institut für Angewandte Analytik und Umweltchemie
Humboldt-Universität zu Berlin
Hessische Str.1/2
D-10115 Berlin, Germany