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A fluorogenic and chromogenic probe that detects the esterase activity of trace copper(II)

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Abstract—Bis(picolinoyl)fluorescein, a new fluorogenic and chromogenic probe for monitoring the esterase activity of metal ions, has been synthesized. The probe is highly selective for Cu^{2+} and is applicable both to quantification of the free ion and detection of esterolytic activity of Cu bound to organic ligands or biomolecules, with a detection limit of 100 nM by fluorimetry.

Copper is an important trace metal ion in biological and environmental systems. A number of fluorescent molecular sensors for Cu^{2+} ions have been described, in which a chelating group is linked to a fluorophore; binding of paramagnetic Cu ion is signaled by fluorescence quenching.[1–3] These sensors respond to free Cu^{2+}, but might also extract the metal ion from complexes with organic ligands or biomolecules, depending on the affinity of the chelating unit. Fluorogenic probes for Cu detection are rare. An example is a rhodamine-based 'chemodosimeter' that becomes fluorescent upon redox-triggered cleavage of a hydrazide group by Cu in a nonfluorescent precursor.[4]

In a recent series of papers,[5–7] we have described the application of metal-DNA conjugates to nucleic acid sequence determination with catalytic signal amplification; the assay relies on the esterase activity of a DNA-linked Cu complex. For optimization of the system and exploration of structure–activity relationships, a sensitive probe would be useful, which allows straightforward detection of esterase activity of ligated Cu^{2+} in low concentration.

In this paper, we describe a new fluorogenic probe which does not only detect the concentration of free Cu^{2+} ions but also the *reactivity* of trace amounts of Cu^{2+} bound to organic ligands or biomolecules. Biological activity and toxicity of Cu^{2+} are functions of the tendency of complexed Cu^{2+} to react. Often, a high reactivity in a biological environment is related to the availability of free coordination sites at the metal ion, which allow interaction with biomolecular substrates, while coordinatively saturated metals tend to be less active.

The probe is a fluorescein diester which is neither colored nor fluorescent, but develops a strong absorbance at 489 nm and fluorescence emission at 513 nm when the ester groups are hydrolyzed by Cu^{2+}. Affinity of the probe to Cu^{2+} is so low that it does not compete for complexation, but, indeed, signals the reactivity of ligand-bound Cu.

While simple esters, such as diacetyl fluorescein, are frequently used in the fluorimetric detection of esterase and peptidase activities,[8] these compounds do not allow determination of hydrolase activity of metal ions since the affinity of an ester group to a metal is very low. Efficient ester hydrolysis by metal ions requires introduction of an 'anchoring' group, such as pyridyl, which brings the ester in close proximity to the metal site.[9]

The bis(picolinate) ester of fluorescein was prepared by reaction of the dye with 2 equiv of picolinoyl chloride in DMF/pyridine (Scheme 1).[23–25]

Recrystallization from acetone yields white crystals of **1**. In buffered (pH 7) solution, the compound has no electronic absorbance in the visible range and is nonfluorescent. A very slow spontaneous hydrolysis is followed by an increase of optical absorbance at 489 nm and of fluorescence emission at 513 nm.

Keywords: Cu(II) esterase activity; Detection; Fluorescence.
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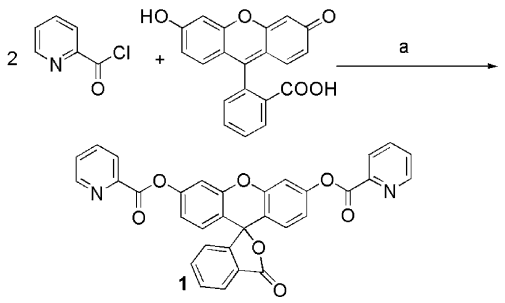


Scheme 1. Reagents and conditions: (a) anhydrous pyridine, anhydrous DMF, rt, 6 h.

Both ester bonds of **1** must be cleaved before the intense absorbance and fluorescence of fluorescein can be recorded. In enzymatic assays, the influence of the monosubstituted intermediate on kinetics is often not considered. A detailed study[10] of the two-step hydrolysis of diacetylfluorescein has revealed that significant concentrations of the monoacetylated form built up during enzymatic hydrolysis. The fluorescence emission of monoacetylfluorescein is weak, about 30 times smaller than that of fluorescein at 513 nm emission. Also, the absorbance of the monoacylated compound is significantly weaker than that of fluorescein at 490 nm. On the other hand, cleavage of the monoacetyl intermediate is faster than expected due to its accumulation at the surface of the enzyme. Since the models for accurate description of the two-step process might become rather complicated, we have not included such considerations in our kinetic analysis. We are, however, aware of the fact that we might observe the formation of both monoacylated product and of fluorescein. This is supported by the observation of a slightly increasing slope in the dA/dt or dF/dt traces (A, absorbance; F, fluorescence), compare Figure 2.

In the presence of Cu^{2+} ions, ester hydrolysis rate of **1** is strongly enhanced. Dependence of initial hydrolysis rate, determined by photometry, on Cu^{2+} concentration, is shown in Figure 1.

In the absence of copper, hydrolysis rate is 4×10^{-7} dA489/dt [s^{-1}].

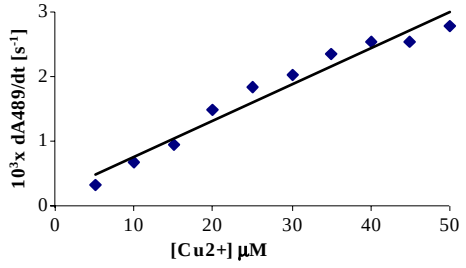


Figure 1. Hydrolysis of **1** (10 μM in H_2O), initial increase of 489 nm absorbance with time depending on $CuSO_4$ concentration. $T = 25$ °C, pH 7.0 (buffer: 10 mM morpholino N-propanesulfonic acid). Average values of two independent measurements, reproducibility ±10%.

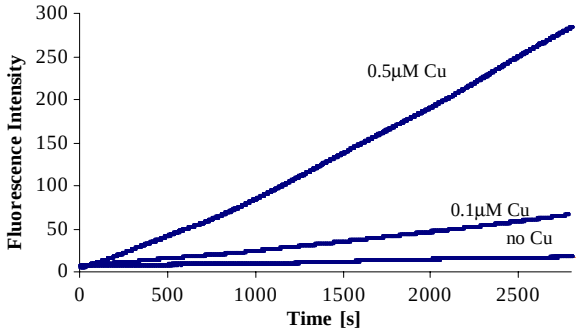


Figure 2. Hydrolysis of **1** (1 μM). Increase of fluorescence intensity with time in the absence and presence of $CuSO_4$. Excitation at 489 nm, emission at 513 nm. $T = 25$ °C, pH 7.0 (buffer: 1 mM morpholino N-propanesulfonic acid).

At higher temperatures, both hydrolysis and background rates are increased (by a factor of 2 for every 10 °C). At Cu^{2+} concentrations >50 μM, the rate becomes too fast to be determined accurately by standard photometric methods. The linear increase of rate with Cu^{2+} concentration indicates that binding sites of **1** are not saturated and interaction with the metal ion is rather weak. The formation constant of the 1:1 complex (**1**)Cu is expected to lie in the range $\log K = 2$–3. (Formation constant of the 1:1 Cu^{2+} complex of 2-Acetylpyridine: $\log K = 2.9$,[11] of 2-Pyridinecarboxamide: $\log K = 2.9$;[12] of 2-Pyridinecarboxaldehyde: $\log K = 2.7$.[13])

At Cu^{2+} concentrations <1 μM, metal ion promoted hydrolysis becomes very slow and difficult to detect by photometry. Lower Cu^{2+} concentrations down to about 0.1 μM are detectable by fluorimetry. Figure 2 compares the increase of fluorescence with time of a solution containing **1** (1 μM) in the absence and in the presence of 0.1 and 0.5 μM Cu^{2+}.

On prolonged reaction times, the reaction rate is no longer constant but decreases since the hydrolysis product picolinic acid, present in its anionic form at pH 7, is a good Cu^{2+} chelator and a strong competitive inhibitor of substrate binding.

We then investigated the esterase activity of Cu^{2+} complexes including bidentate 2,2'-bipyridine (bipy) and picolinic acid (pic), tridentate 1,4,7-triazacyclononane (tacn) and tetradentate 1,4,8,11-tetraazacyclotetradecane (cyclam), and tris(2-aminoethyl)amine (tren).

The typical coordination behavior of Cu^{2+} in such complexes is the formation of four short bonds in a tetragonal coordination plane. Additionally, one or two weak apical interactions can complete the coordination to square pyramidal or distorted octahedral.

It is evident from the crystal structures of 1:1 Cu^{2+} complexes of bipy, pic, and tacn that two of the in-plane sites are blocked by the ligand but the remaining two cis-oriented sites are available for binding of coligands.[14] The 1:1 complexes of these ligands display a significant esterase activity, although somewhat lower than free Cu^{2+}

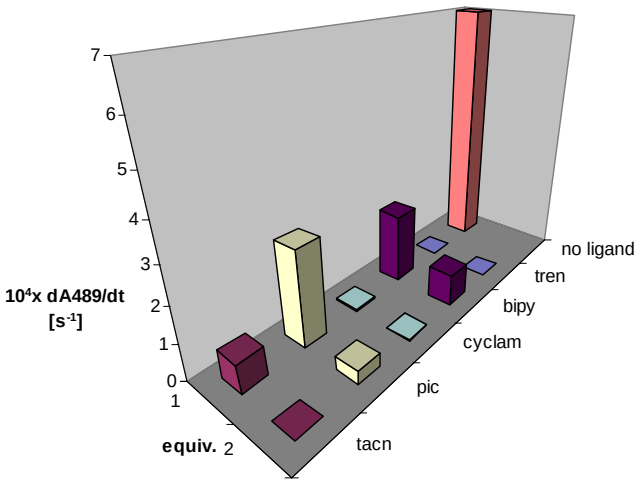
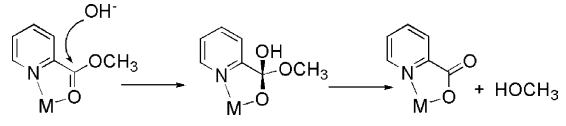


Figure 3. Cleavage of 1, comparison of the esterase activity (dA/dt) of free Cu^{2+} ions and various Cu^{2+} complexes at 1:1 and 1:2 metal-to-ligand ratio. [1] = 10 μM, [Cu^{2+}] = 10 μM, T = 25 °C, pH 7.0 (buffer: 10 mM morpholino N-propanesulfonic acid).

ions (Fig. 3). Formation constants of the 1:1 complexes are $\log K > 8$ at pH 7 in all cases, suggesting quantitative complex formation at 10 μM concentration.

When the ligand is added in excess, 2:1 complexes are formed and all four in-plane sites are blocked, resulting in loss of esterase activity. In the case of tacn, the association constant of the 2:1 complex is three orders of magnitude lower than for the 1:1 complex, but still high enough for quantitative formation of [$(tacn)_2Cu^{2+}$][15]. In the case of bipy and pic, formation of the 2:1 complexes is incomplete at 10 μM concentration due to the lower association constant $\log K = 5.3$ (for bipyCu + bipy)[16] and $\log K = 6.9$ (for picCu + pic).[17] When the concentration of these ligands is increased further equilibrium is shifted further toward the inactive 2:1 complexes and the esterase activity approaches zero.

In the case of highly efficient chelators cyclam and tren, the 1:1 complexes are inactive. The tetradentate macrocycle cyclam blocks all the four in-plane sites at Cu. Cu^{2+} complexes of tetradentate ligand tren display a trigonal bipyramidal coordination with one free apical site left for coligand coordination.[18] Since (tren)Cu is inactive, interaction of **1** with a single free site obviously does not promote hydrolysis. This is interpreted in view of the proposed mechanism[19] of picolinic ester hydrolysis by metal ions, which involves anchoring of the substrate by pyridine coordination and Lewis acid activation of the ester carbonyl by metal coordination, with the necessicity of two cis-oriented free coordination sites at the metal center (Scheme 2).



Scheme 2. Proposed mechanism of picolinate ester hydrolysis promoted by divalent transition metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}).

Before applying the probe to Cu^{2+} ions bound to a biomolecule, we have explored its cross-sensitivity to other metal ions, particularly those that are relevant biological systems. Response of **1** is highly selective to Cu^{2+}, with a discrimination factor of 200 over Ni^{2+} and Zn^{2+}, and more than 500 over Co^{2+}. Ca^{2+} and Mg^{2+} do not significantly affect the hydrolysis rate, even in 1 mM concentration. We attribute the high selectivity of **1** for Cu^{2+} to a 'double discrimination'[20] both by more effective binding and hydrolysis of this metal ion, which is the strongest Lewis acid in the series of metal ions under investigation (Fig. 4).

In the context of our studies on Cu^{2+} complexes of chemically modified oligonucleotides,[5–7,21] we have expanded the investigation to the Cu^{2+} complex of a single-stranded DNA oligonucleotide (9-mer). Association constants between Cu^{2+} ions and ss-DNA in the range $\log K = 4$–5 have been determined by spectrophotometric methods.[22] Both the phosphate backbone (by electrostatic interaction) and the nucleobases are potential Cu^{2+} binding sites.

In the preliminary experiment shown in Figure 5 we have kept the Cu^{2+} concentration constant at 10 μM and varied the DNA concentration. Ester hydrolysis was followed by photometry. Complexation by DNA strongly reduces the esterolytic activity of Cu^{2+} ions.

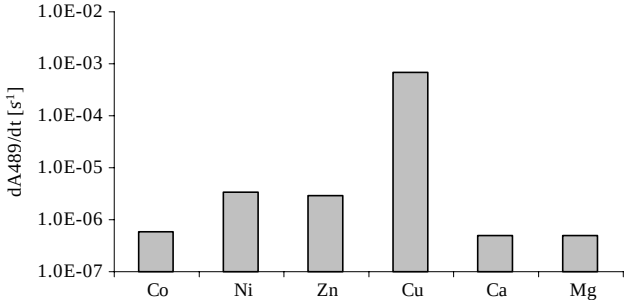


Figure 4. Cleavage of 1 (determined by photometry, dA/dt) by various divalent metal ions. [1] = 10 μM, [M^{2+}] = 10 μM, pH 7.0 (buffer: 10 mM morpholino N-propanesulfonic acid), T = 25 °C.

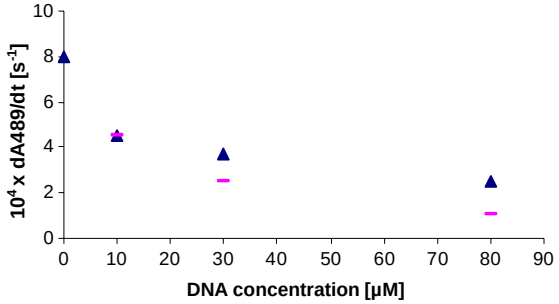


Figure 5. Triangles: hydrolysis of **1** by Cu^{2+} [10 μM] (determined by photometry, dA/dt) in the presence of a 9-mer DNA oligonucleotide (sequence: TCACAACTA) at varying concentrations. pH 7.0 (buffer: 10 mM morpholino N-propanesulfonic acid), T = 25 °C. Strokes: calculated values based on the formation of an inactive 1:1 (DNA)Cu complex with association constant $\log K = 4.9$.

With the assumption of formation of an inactive 1:1 (DNA)Cu complex, an association constant $\log K = 4.9$ is derived using kinetic data at 10 μM DNA and 10 μM Cu^{2+}. At higher DNA concentration, theoretical rates (▬, strokes) calculated on the basis of this association constant poorly fit the measured rates (▲, triangles). A model, which includes the esterase activity of (DNA)Cu (about 15% of free Cu^{2+} ions activity), is in better agreement with the experiment. This observation suggests that free in-plane coordination sites are available at DNA-bound Cu. Net reactivity of (DNA)Cu might be an average of more or less reactive Cu located at different binding sites of the oligonucleotide.

In conclusion, **1** is a fluorogenic and chromogenic probe for detection of the esterase activity of metal ions, with high selectivity for Cu^{2+}. **1** is also a substrate for complexed Cu^{2+} ions if two free in-plane coordination sites in the cis position are available at the metal. Both optical and fluorescence signals produced by Cu promoted hydrolysis are strong, the lower detection limit for Cu^{2+} ions by standard fluorescence measurement being 0.1 μM. Therefore, **1** is an ideal probe to determine the reactivity of Cu^{2+} ions bound to biomolecules, which are often available in small quantities only. We are currently preparing amino acid ester analogs of **1** in which the picolinoyl moiety is replaced by amino acyl one, to explore how biomolecules (DNA, proteins) modulate the substrate selectivity and stereoselectivity of metal ions.

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23. Synthetic procedure for **1**: fluorescein (1 g, 3 mMol) was dissolved in anhydrous pyridine. Picolinoyl chloride (4.245 g, 30 mMol) was dissolved in anhydrous DMF. Solutions were mixed and stirred for 6 h at room temperature. Solvents were removed in vacuo, residue was taken up with DMF and purified by column chromatography (8:1 EtOAc/*n*-hexane). Product was a brown powder which yielded after recrystallization 300 mg of white crystals (18%).
24. Synthesis of Picolinoyl chloride was described by Christensen in: *Molecules*, **2001**, *6*, 47–51.
25. Analytical data for **1**; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.98 (s, 1H), 7.02 (d, 1H, $J = 2.3$ Hz), 7.12 (d, 1H, $J = 2.3$ Hz), 7.17 (d, 1H, $J = 2.3$ Hz), 7.37 (d, 1H, $J = 7.5$ Hz), 7.46 (d, 2H, $J = 2.2$ Hz), 7.8 (m, 4H), 8.09 (d, 1H, $J = 1.9$ Hz), 8.13 (d, 1H, $J = 1.7$ Hz), 8.16 (d, 1H, $J = 1.6$ Hz), 8.37 (d, 2H, $J = 7.9$ Hz), 8.8 (d, 2H, $J = 4.6$ Hz). Anal. calcd for [**1**+H_2O] $C_{32}H_{20}N_2O_8$: C, 68.57; H, 3.6; N, 5.0. Found: C, 68.79; H, 3.56; N, 5.12. HRMS (ESI) calcd $C_{32}H_{18}N_2O_7+H^+$: 543.1192. Found: 543.1167.