15. Metal Complexes with Macrocyclic Ligands

Part XXXII[1])

Reactivity Studies of the Pendant Carboxylic Group in a Macrocyclic Cu^{2+} Complex Towards Amide Formation and Its Use as a Protein-Labelling Agent

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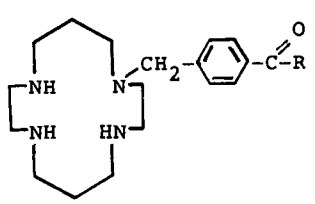
The Cu^{2+} complex of **1**, having a non-coordinating carboxylic group, can be reacted under the typical conditions of peptide formation with amines such as 2-methylpropylamine or (pyrid-2-yl)methylamine to give, after removal of Cu^{2+} with CN^-, the amides **4** and **5**. The Cu^{2+} ion is of paramount importance since it protects the four amino groups of the macrocycle so that the amide condensation can specifically be done with the exogenous amine. It is also shown that the Cu^{2+} complex of **1** can be covalently attached to bovine serum albumin (BSA), thus opening the possibility to use this compound as a labelling agent for proteins and antibodies.

Introduction. – Functionalized macrocycles are interesting in many respects [2]. On the one hand, they are ideal ligands for the coordination chemist to study changes in structure and stability when donor groups are introduced as pendant side chains [3]. On the other hand, these compounds allow to study metal-promoted reactions since the substrate, covalently attached through the side chain of the macrocycle, can be brought in close vicinity of the metal ion so that the formation of a pseudo metal-substrate complex takes place [4]. Examples of metal-induced hydrolytic cleavages of a nitrile [5] and of carboxylate and phosphonate esters [6] have been described. It has been shown that the metal ion activates the functional group through polarisation and/or orients the nucleophile and the functional group to be hydrolyzed in the transition state.

In continuation of such studies on the chemistry of functional groups on the side chain of tetraazamacrocyles, we have now investigated the reactivity of the carboxylic group in the Cu^{2+} complex of **1** in connection to its ability to form amide bonds. The



1 R = OH[a])

4 R = $(CH_3)_2CHC_2NH$

5 R = (pyrid-2-yl)CH_2NH

[a]) For the structures of complexes **2** and **3**, see systematic names in the *Experimental*.

[1]) Part XXXI, see [1].

model studies with low molecular weight amines have then been extended to reactions with bovine serum albumin (BSA), to study the possibility of using this compound as a labelling agent for proteins and antibodies in particular.

Experimental. – *General.* IR spectra (cm^{-1}): *Perkin Elmer 157G;* KBr pills. ^{1}H-NMR spectra: *Varian-EM-360* spectrometer using sodium 3-(trimethylsilyl)propanesulfonate as internal standard.

4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]benzoic Acid Tetrahydrochloride (**1**·4 HCl) was prepared as described [7].

{4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]benzoic Acid}copper(II) Dichloride (**2**). An aq. soln. of **1** (1.0 g, 2 mmol) and $CuCl_2 \cdot 2\,H_2O$ (0.34 g, 2 mmol) in H_2O, adjusted to pH 5 with 0.5M NaOH, was kept at 50° for 2 h. After the pH was set to 1 with 1M HCl, the filtered soln. was kept in a dessicator over $CaCl_2$ until crystals of the product were formed: 0.37 g (38%) of **2**. IR (KBr): 1700 (COOH). Anal. calc. for $C_{18}H_{30}Cl_2CuN_4O_2 \cdot 0.9\,H_2O$ (484.95): C 44.58, H 6.61, Cl 14.62, Cu 13.10, N 11.55, H_2O 3.30; found: C 44.55, H 6.78, Cl 14.55, Cu 13.00, N 11.54, H_2O 3.30.

{4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]benzoato}copper(II) Perchlorate (**3**). A soln. of **1** (2.0 g, 4.1 mmol) in H_2O (20 ml) was reacted for 10 min with $CuCl_2 \cdot 2\,H_2O$ (0.7 g, 4.1 mmol) and NaOH (0.5 g) at 100°. After addition of NaOH (0.5 g), so much $NaClO_4$ was given to the hot soln. until the product began to crystallize. Slow cooling to r.t. gave **3** which was recrystallized from H_2O at pH 10: 1.29 g (60%) of **3**. IR (KBr): 1590, 1550 (COO^-). Anal. calc. for $C_{18}H_{29}ClCuN_4O_6 \cdot 1.5\,H_2O$ (523.44): C 41.30, H 6.16, Cl 6.77, Cu 12.14, N 10.70; found: C 41.66, H 6.22, Cl 6.59, Cu 11.90, N 10.64.

N^1*-(2-Methylpropyl)-4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzamide Tetrahydrochloride* (**4**·4 HCl). A mixture of Et_3N (3.75 ml), **2** (1.0 g, 1.8 mmol), and chloroacetonitrile (0.81 ml, 9 mmol) was refluxed for 1 h. After addition of 2-methylpropylamine (1.5 ml, 15.6 mmol), the soln. was refluxed for an additional 2 h. The volatile components were evaporated, the residue was taken up in H_2O (20 ml) and 1M NaOH (3 ml) and extracted with CH_2Cl_2 (5 × 50 ml). To the aq. phase, EtOH (20 ml) and NaCN (0.53 g, 10.8 mmol) were added and refluxed for 0.5 h. After evaporation of EtOH, the aq. soln. was extracted with CH_2Cl_2 (5 × 50 ml). From the combined dried org. fractions, a yellowish oil was obtained after evaporation. Treatment of it with EtOH (10 ml) and conc. HCl soln. (3 ml) gave the hydrochloride which was recrystallized from H_2O/EtOH/HCl: 0.31 g (31%) of **4**·4 HCl. IR (KBr): 1630 (CONH). ^{1}H-NMR (D_2O): 0.85 (*d*, 2 CH_3); 1.4–2.5 (*m*, 2 CH_2, CH); 3.0–3.8 (*m*, 9 CH_2N); 4.45 (*s*, CH_2Ar); 7.4–7.8 (*m*, 4 arom. H). Anal. calc. for $C_{22}H_{43}Cl_4N_5O \cdot 1.15\,H_2O$ (556.16): C 47.51, H 8.20, Cl 25.50, N 12.59, H_2O 3.72; found: C 47.74, H 8.10, Cl 25.24, N 12.62, H_2O 4.0.

N^1*-[(Pyrid-2-yl)methyl]-4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]benzamide Pentahydrochloride* (**5**·5 HCl). To a refluxing soln. of **3** (2.62 g, 5 mmol) and (pyrid-2-yl)methylamine dihydrochloride (3.55 g, 19.6 mmol) in dry MeCN (150 ml), dicyclohexylcarbodiimide (DCC; 4.27 g, 20.2 mmol) in dry MeCN (80 ml) was added within 0.5 h. After refluxing for additional 2 h, the soln. was cooled to r.t. and left overnight. To destroy excess DCC, H_2O (20 ml) was added. After evaporation of MeCN, the remaining soln. was filtered. The filtrate, extracted with CH_2Cl_2 (4 × 100 ml), was made alkaline by addition of NaOH (1.67 g) and extracted again with CH_2Cl_2 (4 × 100 ml). The aq. phase mixed with EtOH (20 ml) was then treated with NaCN (1.67 g, 34 mmol) and refluxed for 0.5 h, whereby the blue color disappeared. After cooling to r.t., most of the solvent was evaporated and the remaining soln. extracted with CH_2Cl_2 (5 × 50 ml). From the org. phase, the product was obtained after evaporation, by treatment with HCl/EtOH. Recrystallization from H_2O/EtOH/HCl gave 1.46 g (46%) of **5**·5 HCl. IR (KBr): 1650 (CONH). ^{1}H-NMR (D_2O): 2.0–2.2 (*m*, 2 CH_2); 3.1–3.7 (*m*, 8 CH_2N); 4.3 (*s*, CH_2Ar); 4.7 (*s*, CH_2Ar); 7.5–8.6 (*m*, 8 arom. H). Anal. calc. for $C_{24}H_{36}N_6O \cdot 4.6\,HCl \cdot 2.5\,H_2O$ (638.40): C 45.15, H 7.17, Cl 25.71, N 13.16, H_2O 7.05; found: C 45.30, H 7.27, Cl 25.79, N 13.35, H_2O 7.28.

Bovine Serum Albumin (BSA) Modification Procedures. Method A. In a soln. of **2** (0.31 g, 0.64 mmol) in 0.1M phosphate buffer pH 6.5 (20 ml), *N*-[3-(dimethylamino)propyl]-*N'*-ethyl carbodiimide hydrochloride (1 g, 5.2 mmol) and BSA (0.22 g, $3.28 \cdot 10^{-3}$ mmol) were carefully dissolved and incubated for 1 d at r.t. The purification over a *Sephadex G25* column (1.8 cm × 18 cm) with 0.1M phosphate buffer pH 6.5 gave two pale violet fractions, the first being the modified BSA. This fraction was run over a second *Sephadex G25* column to remove the salts and was then lyophilized: pale violet protein which contained *ca.* 32 Cu-macrocyclic (Cu(mac)) units attached to it, as determined from the absorptivity at 520 nm.

Method B. A mixture of 6.6 mM $CuCl_2$ (1 ml), 6.6 mM **1** (1 ml), and 0.1M acetate buffer pH 5 (10 ml) was heated to 50° for 20 min. After addition of 0.1M HCl (10 ml), the pale violet soln. was evaporated, and to remove all traces of solvent, it was dried at 100°/11 Torr for 15 min. The solid was taken up in 13.2 mM DCC in DMF (5 ml) and the resulting soln. stirred for 15 min. Then, 6.6 mM *N*-hydroxysuccinimide in DMF (5 ml) was added and the mixture kept for 1.5 h at r.t. The DMF was then removed by distillation at 0.01 Torr. The residue (active ester) was

dissolved in 0.1M phosphate buffer pH 6.7 (10 ml), filtered, and reacted with BSA (44 mg, $6.6 \cdot 10^{-4}$ mmol), dissolved in the same buffer (10 ml) for 2 h at r.t. To purify the product, 3 ml of the mixture were run over a *Sephadex G 25* column (1.8 cm × 18 cm) using 0.1M phosphate buffer pH 6.7. Spectrophotometric detection at 280 nm indicated two fractions, the modified BSA and the excess of unreacted Cu^{2+} complex. Both fractions exhibit a VIS spectrum with λ_{max} 520 nm. From the absorptivity at 520 nm, the BSA/Cu(mac) ratio was determined to be 1:3.

Method C. A soln. of *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (36.2 mg, 0.20 mmol), **1** (18.1 mg, 0.037 mmol), and *N*-hydroxysuccinimide (8.5 mg, 0.075 mmol) in 0.1M phosphate buffer pH 6.7 (1.1 ml) was kept at r.t. for 1 h. To this, BSA (7.5 mg, $1.1 \cdot 10^{-4}$ mmol), dissolved in 0.1M phosphate buffer pH 6.7 (2 ml), was added and reacted for 1 h. The purification was achieved by running the mixture over a *Sephadex G 25* column (1.8 cm × 18 cm) with 0.1M phosphate buffer pH 6.7. To the fraction containing the protein, three portions of 0.01M $CuSO_4$ (100 µl each) were added, and the increase of the absorptivity at 520 nm was followed. From it, the BSA/Cu(mac) ratio was determined to be 1:6.

Measurements. pH Titrations were run at 25° using the titrator described previously [8]. Typical concentrations were: [**5**] = $8.2 \cdot 10^{-3}$M, [Cu^{2+}] = 0, $8.2 \cdot 10^{-3}$M, or $1.6 \cdot 10^{-2}$M, [KNO_3] = 0.5M.

The ratio BSA/Cu(mac) was determined spectrophotometrically at 520 nm in 4-cm cuvettes on a *Cary 118 C* spectrophotometer, using the molar absorptivity of the Cu^{2+} complex $\varepsilon = 100 M^{-1}cm^{-1}$.

The modified BSA was analyzed in a *SDS* polyacrylamide gradient gel (*SDS-PAGE*, 7–12%) under non-reducing conditions according to *Lämmli* [9]. The electrophoresis was performed using a *LKB 2001* electrophoresis chamber and a *Biowerk* power supply at pH 8.3 (0.375M *Tris* buffer). The protein bands were visualized using *Coomassie* brillant blue *(Merck)* staining.

Discussion. – *Syntheses.* We have previously reported that mono-*N*-functionalized polyazamacrocycles carrying a carboxylic group in their side chain can easily be prepared [7]. A structural study of the Cu^{2+} complexes of the *o*- and *p*-toluic acid derivatives has shown that the carboxylic and carboxylate group of the *o*-derivative bind axially to the Cu^{2+} ion [10], whereas in the case of the *p*-compound, no interaction between the carboxylate and the metal ion could be detected [7], as expected from the configuration.

We now took advantage of this observation and investigated the reactivity of the carboxylic group in the *p*-toluic derivative **1**. Before activating the carboxylic group in order to synthesize amides, we had to protect the amino functions of the macrocycle. For this purpose, we used Cu^{2+} which by complexation protected the four N-atoms of **1** at once. The introduction of the Cu^{2+} ion is, therefore, of paramount importance since it allows to selectively react the carboxylic group with exogenous amines and thus prevents intra- and/or intermolecular amide formation.

The model studies with 2-methylpropylamine and (pyrid-2-yl)methylamine indicate that the typical methods used in peptide chemistry [11] are also successful in our case. So, the activation of the carboxylic group through cyanomethyl ester formation in MeCN gave the expected amide **4** in 31% yield. Similarly, the use of DCC as an activating reagent and the condensation with (pyrid-2-yl)methylamine yielded the corresponding amide **5** (46%). In order to obtain the free ligand, the Cu^{2+} was taken out of the macrocycle by reaction with CN^- in both cases.

Potentiometric Studies. Beside the macrocyclic unit, compound **5** contains as an additional coordinating group the pyridine N-atom of the side chain. It was, therefore, interesting to investigate the equilibria of this ligand with an excess of Cu^{2+} to see whether it can form 2:1 species. Thus **5** · 5 HCl was titrated with NaOH, first in the absence, then in the presence of Cu^{2+}. The protonation constants, obtained by fitting the potentiometric curves with the computer program TITFIT [12], are given in the *Table*. The free ligand has, as all tetraazacyclotetradecane derivatives, two high and two low $\log K_H$ values [13]. In addition, we found a $\log K_H$ at 4.49 for the pyridinium proton.

Table. *Protonation Constants of L and* $[CuL]^{2+}$ (L = **5**). $T = 25°$, $I = 0.5$M (KNO_3).

	$\log K_{H,1}$	$\log K_{H,2}$	$\log K_{H,3}$	$\log K_{H,4}$
L	11.46(5)	9.28(7)	4.49(8)	< 2
$[CuL]^{2+}$			4.45(3)	

If 1 equiv. of Cu^{2+} was added to **5**, a relatively slow reaction took place in which Cu^{2+} was incorporated into the macrocycle to give the kinetically stable Cu^{2+} macrocyclic moiety $[CuL]^{2+}$. Thus, the stability of this species could not be determined. The titration of an acid solution of this complex with NaOH still revealed a deprotonation step with $\log K_H = 4.45$ *(Eqn. 1)*. Since this value was practically unchanged with respect to that in the free ligand, it indicated that either the charges of the two protons in the case of the free ligand or of the Cu^{2+} in the macrocycle practically do not influence the acidity of the pyridinium group or that their influence is very similar. The pH titration of $[CuLH]^{3+}$ in the presence of an additional equivalent of Cu^{2+} showed a depression of the pyridinium buffer region *(Figure)* and thus indicated that the second equivalent of Cu^{2+} interacts

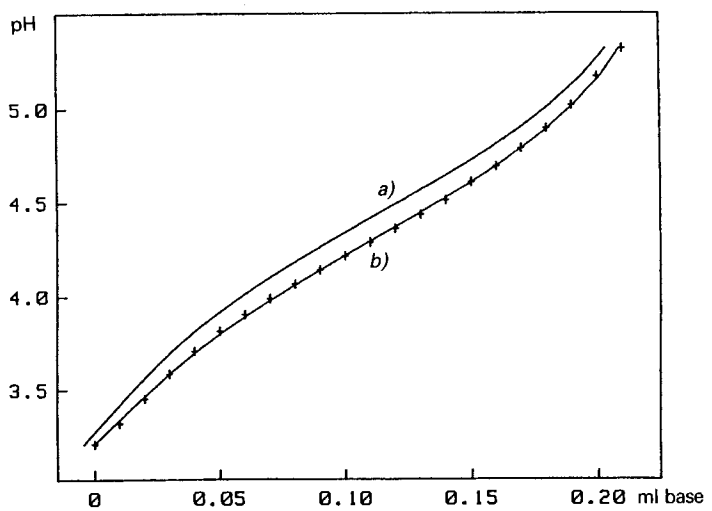


Figure. *Titration of* $8 \cdot 10^{-3}$ M $[CuLH]^{3+}$ (L = **5**) a) *in the absence and* b) *in the presence of* $8 \cdot 10^{-3}$ M Cu^{2+}. $T = 25°$, $I = 0.5$M (KNO_3).

with the pyridine group giving a weak complex $[Cu_2L]^{4+}$ *(Eqn. 2)* with $\log K = 1.67$, a value which can be compared with the stability of the Cu^{2+} complex of 2-methylpyridine ($\log K = 1.30$ [14]). Above pH 5.4, $Cu(OH)_2$ precipitated so that it was not possible to observe the amide deprotonation, in accordance with previous observations that only Cu^{2+}-amide complexes in which a five-membered chelate ring is formed can be deprotonated at the amide function [15].

$$[CuL]^{2+} + H^+ \rightleftharpoons [CuLH]^{3+},\ K_H = [[CuLH]^{3+}]/([[CuL]^{2+}] \cdot [H^+]) \quad (1)$$

$$[CuL]^{2+} + Cu^{2+} \rightleftharpoons [Cu_2L]^{4+},\ K = [[Cu_2L]^{4+}]/([[CuL]^{2+}] \cdot [Cu^{2+}]) \quad (2)$$

Bovine Serum Albumin Modifications. After the model reactions with low molecular weight amines had shown that it was possible to form amides with the side-chain carboxylic group of the Cu^{2+} complex of **1**, we investigated whether it would be possible to use this complex as a labelling agent for proteins. To do this, we tested three different methods. In a first series of experiments, we reacted BSA in phosphate buffer pH 6.5 with complex **2** using the H_2O-soluble *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide. Depending on the excess of Cu^{2+} complex and on the reaction time, up to 30 macrocyclic units could be covalently attached to BSA. However, HPLC control and *SDS-PAGE* electrophoresis showed that partial cross-linking had occurred since the carbodiimide, being in large excess, also activated the carboxylic groups of the protein, so that it reacted with itself. The use of the *N*-hydroxysuccimide ester with BSA gave better results in regard to cross-linking. In both cases, the number of Cu^{2+}-macrocyclic units attached to BSA was measured spectrophotometrically using the 520-nm band typical for the Cu^{2+}-macrocyclic chromophore.

In a last series of experiments, we studied the post-labelling technique. In this case, we reacted the *N*-hydroxysuccimide ester of **1** with the protein, purified the protein, and then added the Cu^{2+} as last reactand, whereby the typical color of the Cu^{2+} macrocyclic moiety developed. These examples clearly indicate that the Cu^{2+} complex with **1** is a useful labelling reagent. The applications of it in labelling antibodies will be published elsewhere [16].

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