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Utilizing Reversible Copper(II) Peptide Coordination in a Sequence-Selective Luminescent Receptor

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Abstract: Although vast information about the coordination ability of amino acids and peptides to metal ions is available, little use of this has been made in the rational design of selective peptide receptors. We have combined a copper(II) nitrilotriacetato (NTA) complex with an ammonium-ion-sensitive and luminescent benzocrown ether. This compound revealed micromolar affinities and selectivities for glycine- and histidine-containing sequences, which closely resembles those of copper(II) ion peptide binding: the two free coordination sites of the copper(II) NTA complex bind to imidazole and amido nitrogen atoms, replicating the initial coordination steps of non-complexed copper(II) ions. The benzocrown ether recognizes the N-terminal amino moiety intramolecularly, and the significantly increased emission intensity signals the binding event, because only if prior coordination of the peptide has taken place is the intramolecular ammonium ion–benzocrown ether interaction of sufficient strength in water to trigger an emission signal. Intermolecular ammonium ion–benzocrown ether binding is not observed. Isothermal titration calorimetry confirmed the binding constants derived from emission titrations. Thus, as deduced from peptide coordination studies, the combination of a truncated copper(II) coordination sphere and a luminescent benzocrown ether allows for the more rational design of sequence-selective peptide receptors.

Keywords: chelates · crown compounds · fluorescence spectroscopy · molecular recognition · peptides

Introduction

The development of peptide chemosensors and the sequence-specific recognition of peptides by synthetic receptors under physiological conditions still remains a challenging task. Recently reported approaches have used porphyrins,[1] helix mimics,[2] crown ethers[3] or short peptides with guanidiniocarbonyl pyrroles[4] as binding sites for specific recognition of peptidic structures. Although peptide to metal ion coordination has been studied in detail from physicochemical and inorganic perspectives,[5] applications of reversible coordination in peptide recognition are rare.[6] Recent examples include Anslyn's[7] use of cooperative metal coordination and ion-pairing for tripeptide recognition and the application of zinc(II) dipicolylamine-stilbazoles in the specific detection of phosphorylated peptides.[8]

Systematic studies on peptide metal complexes date back to the 1950s, and potentiometric, structural[9] and spectroscopic[10] investigations have been reported in detail.[11] Copper(II), nickel(II) and zinc(II) are by far the most studied widely metal ions in peptide complexes. The ability of a peptide to act as a ligand strongly depends on its amino acid sequence, with imidazole, carboxylate, deprotonated amide and amino groups as typical donor sites. Binding specificities to metal ions have been used in the development of ion-selective electrodes[12] and fluorescent metal ion chemosensors for zinc[13] or copper[14] ions. Peptide metal complexes themselves have been utilized in DNA and RNA recognition[15] and cleavage.[16]

We have now set ourselves the goal of using the well studied metal ion complexation by peptides for the rational design of receptors specific to peptide sequences. To this end the metal ion is replaced by a stable metal complex with some labile coordination sites available for peptide binding. We reason that such metal complexes should selectively bind truncated sequences of peptides that are known to form stable complexes with the metal ion itself. To illustrate the feasibility of this approach, we have used one of the most intensely studied peptide metal complexes, consist-

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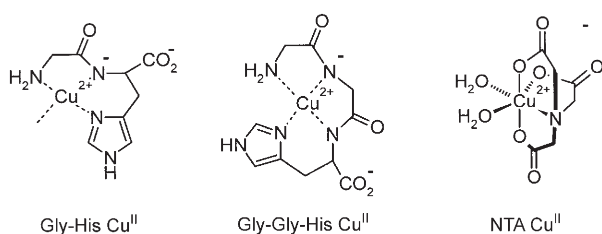
ing of histidine- and glycine-containing peptide ligands and copper(II) as the metal ion.

Results and Discussion

Receptor design: Many histidine-containing peptides form stable complexes with copper(II) ions, but the coordinating properties of the histidine residue depend significantly on the position of the residue in the peptide chain. The presence of histidine in position two in a peptide allows for the participation of the N-terminal amine, the N-1 imidazole and the amide nitrogen atoms between them in copper(II) ion coordination (Scheme 1).[5a] The presence of histidine in position three in a peptide chain leads to the cooperative formation of three fused chelate rings with copper(II) ions.[5a] In simple peptides with N-terminal histidine, the chelate of the N-terminal amino group and the imidazole nitrogen atom can be very effective, but complexes of higher ligand stoichiometry have also been discussed.[5a,17]



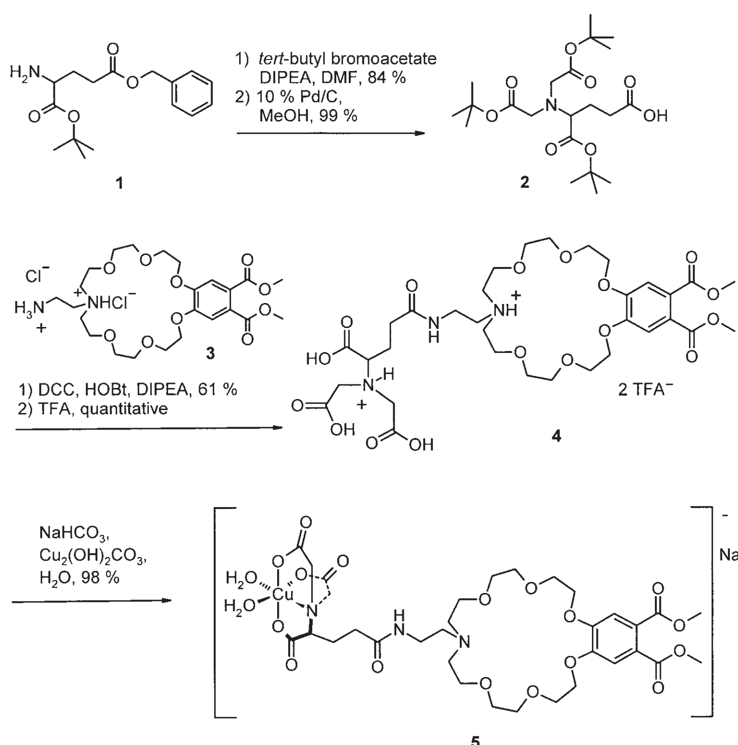
Scheme 1. Glycine-histidine copper(II) and nitrilotriacetato (NTA) copper(II) metal complexes.

Nitrilotriacetato (NTA) copper(II) complexes are stable and widely used for protein purification, thanks to their affinities towards imidazole side chains. The NTA ligand in such a complex leaves two coordination sites available for the reversible binding of donor groups.[18] The binding of a glycine-histidine peptide sequence such as GH or GGH to an NTA-Cu^{II} complex through N-imidazole and N-amide coordination, leaving the N-terminal amino group available, will therefore only proceed if a consecutive binding starting with imidazole as the primary anchoring group is assumed.

We have recently introduced luminescent benzocrown ether amino acids, which are useful molecular indicators for ammonium ions,[19] and demonstrated their ability to monitor Lys side chains in coordinated peptides.[20] A combination of such luminophores with an NTA-Cu^{II} complex should lead to a peptide-sequence-selective receptor with predictable selectivity.

Synthesis: The synthesis started from protected L-glutamic acid **1** (Scheme 2), which was converted into the NTA precursor **2**.[21] Peptide coupling under typical conditions with amino ester **3**[19] and *tert*-butyl ester cleavage gave compound **4**, which was converted into the NTA-Cu complex **5** (Cu-NTA CE) by treatment with copper carbonate. Peptides for the determination of binding selectivity were prepared in solution and by standard solid-phase methods.

Determination of peptide binding: The luminescence of the benzocrown ether moiety is strongly quenched upon coordination of the NTA ligand by copper(II),[22] but is partially restored if an ammonium ion binds to the crown ether. However, crown ether binding affinities for ammonium ions in aqueous solution at physiological pH are rather weak,[23] and interaction is therefore only observed if other parts of the peptide coordinate to the NTA-Cu^{II} complex, making the process of binding the ammonium ion intramolecular. Compound **5** will signal affinity to peptides, that coordinate to the two accessible NTA-Cu^{II} binding sites, but leaving an ammonium group available for crown ether binding.



Scheme 2. Synthesis of Cu-NTA benzocrown ether **5** (Cu-NTA CE).

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The peptide-binding selectivity of **5** was evaluated with a small rational library of di-, tri- and tetrapeptides, with some amino acid sequences resembling typical peptides for copper(II) complex formation. To select the peptide sequences with highest affinity quickly, a screening was performed in aqueous buffered solution (HEPES, 50 mM, pH 7.5) by use of a microtitre array. Five equivalents of each peptide were added to a solution of compound **5** (3.75×10^{-5} M), and the fluorescence emission intensity at 397 nm (excitation at 305 nm) was recorded. If the ratio of the observed emission intensity after and before peptide addition exceeds two, a significant interaction is expected.[24] Table 1 summarizes the results. As expected, several peptides containing His (H) and Gly (G) induced significant emission increases.[25] However, if the distance between the imidazole moiety and the N terminus becomes larger, the affinity drops. Of the peptides containing a Lys (K) residue in addition to His, only a di- and a tripeptide gave weak responses. Tetrapeptides containing a His, two Gly and variously Ala (A), Leu (L) or Gln (Q) as the fourth residue—the common sequences XaaHGG−NH_2 (Xaa = A, L, Q)—showed strong emission increases. Replacement of His by Ser (S), Glu (E) or Gln (Q) in tetrapeptides with three Gly residues led to complete loss of affinity for **5**. Cys-containing tetrapeptides with three Gly units showed weak emission responses independently of the Cys position, except when it was at the N terminus.

Table 1. Screening of the fluorescence response of **5** to small peptides.

Peptide sequence	F/F_0 > 2[a]	Peptide sequence	F/F_0 > 2[a]	Peptide sequence	F/F_0 > 2[a]
H[b]	+	**C**GGG[d]	–	**Q**GGG[d]	–
HGG[c]	+	G**C**GG[d]	+	G**Q**GG[d]	–
HGGG[d]	–	GG**C**G[d]	+	GG**Q**G[d]	–
G**H**G[d]	++[e]	GGG**C**[d]	+	GGG**Q**[d]	–
G**H**GG[d]	++	**S**GGG[d]	–	A**H**GG[d]	++
GG**H**G[d]	–	G**S**GG[d]	–	AG**H**G[d]	–
GGG**H**[d]	–	GG**S**G[d]	–	AGG**H**[d]	–
GG**H**[c]	++	GGG**S**[d]	–	L**H**GG[d]	++
G**H**[c]	++	**E**GGG[d]	–	LG**H**G[d]	–
HK[b]	+	G**E**GG[d]	–	LGG**H**[d]	–
HKGG[d]	–	GG**E**G[d]	–	Q**H**GG[d]	++
HGK[b]	+	GGG**E**[d]	–	QG**H**G[d]	–
HGKG[d]	–			QGG**H**[d]	–
HGGK[d]	–				

[a] F/F_0 is the increase in observed emission intensity after addition of 5 equiv of the peptide to compound **5**. [b] H-Xaa-OMe. [c] H-Xaa-OH. [d] H-Xaa-Xaa-Xaa-Xaa-NH_2. [e] Peptide sequences marked with ++ show a particularly strong emission increase.

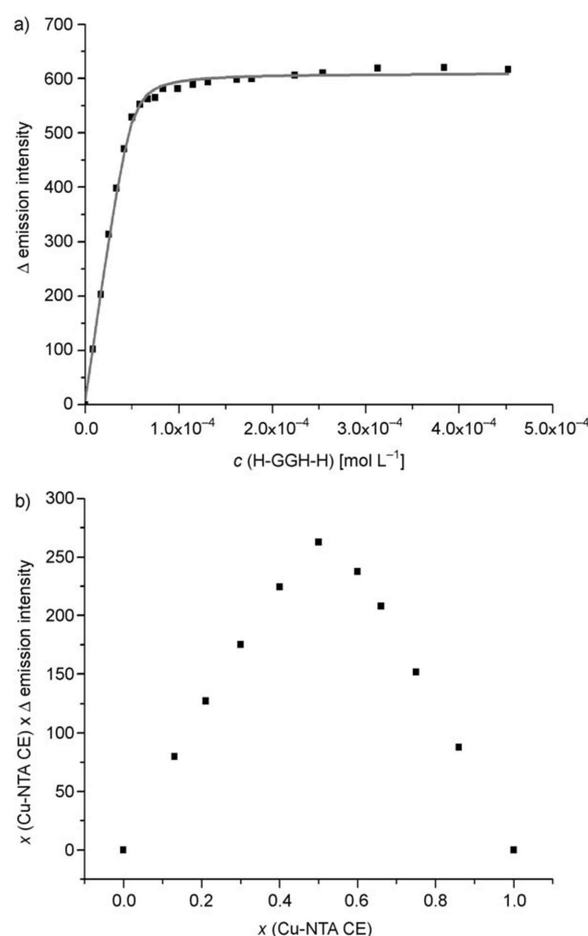

Figure 1. Emission titration (top) and Job's plot analysis (bottom) of H-Gly-Gly-His-OH and **5** in aqueous buffer.

The peptide sequences that showed significant responses in the screening assay were investigated by emission titrations in HEPES-buffered (50 mM, pH 7.5) aqueous solution. Their binding constants—log K and $K_{0.5}$ values—were derived from the titration data by nonlinear fitting methods; the stoichiometries of the binding events were determined by Job's plot analyses (see Supporting Information for data). Figure 1 shows a representative example in the form of the emission titration curve for H-GGH-OH and **5**. The peptides GGH and GHG bind to **5** with micromolar affinities, each with 1:1 stoichiometry. The binding data were confirmed by independent isothermal titration calorimetry (ITC) under the same conditions (see Supporting Information). The dipeptide GH and the tetrapeptides XaaHGG-NH_2 (Xaa = A, L, Q) show lower binding affinities, but still in the order of $\log K = 4$. Job's plot analyses of the binding of the cysteine-containing peptides indicated 2:1 stoichiometries, although the fit of the titration data to mathematical binding models was not conclusive. The remaining histidine-containing peptide sequences bind weakly to compound **5** with stoichiometries deviating from 1:1 and are therefore judged to be nonspecific binders. The data relating to the peptide sequences that bind to **5** in stoichiometric fashion and with high affinity are summarized in Table 2.

The binding data support the design concept of the receptor: only peptide sequences that form stable copper(II) complexes, and therefore bind with part of their donor sites to the Cu-NTA complex of **5**, while at the same time having an N-terminal ammonium ion available show strong and specific responses. GGH and GHG (Table 2, entries 1 and 2) show the highest affinities, as they provide optimal geometry to form six-membered chelates with the copper ion in analo-

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Table 2. Binding data for peptide sequences to **5** in aqueous buffer from emission titrations.

Entry	Peptide sequence	$K_{0.5}$[a] [μmol]	log$K$[b]
1	GG**H**[c]	24	5.8
2	G**H**G[c]	25	5.7
3	G**H**G-OMe	33	5.3
4	G**H**[c]	36	4.7
5	G**H**GG[d]	60	4.1
6	A**H**GG[d]	70	4.2
7	L**H**GG[d]	60	4.2
8	Q**H**GG[d]	187	3.7
9	Ac-G**H**G[c]	–	–

[a] Concentration of peptide needed to induce 50% of the maximal emission increase. [b] All stoichiometries determined by Job's plot analyses as 1:1, with the exception of QHGG, which shows 2:1. [c] H-(Xaa)-OH. [d] H-(Xaa)-NH_2.

gy to the initial coordination in pure copper(II) peptide complexes. The high stability of complex **5** with GHG allows its detection from solution by electrospray mass spectroscopy. On the basis of the structures of peptide copper(II) complexes, we propose the arrangement shown in Figure 2 for the association of **5** with XaaHGG peptides. The steric bulk of residues R and R′ in the tetrapeptides (entries 5–8) may account for their somewhat reduced affinities in relation to the GHG or GGH sequences. The side chains of the N-terminal amino acid in these peptides do not influence the binding affinities significantly,[26] which is in accordance with the reported tolerance of amino acids other than Gly in copper(II) complexes with XaaHG peptides.[27] The C termini of the peptides do not participate directly in the binding process, as changes from carboxylate anion to a methyl ester or glycylamide are possible. However, larger groups lead to decreases in affinity, probably due to increased steric hindrance in the coordination aggregates. The N-terminal ammonium ion is essential to trigger an emission change in **5**; N-terminal acylated peptides such as Ac-GHG (entry 9) give no emission response if added to **5**. ITC measurements revealed that Ac-GHG still shows some affinity to **5**, but that this is significantly reduced in relation to that of the non-acylated peptide (GHG: log$K=5.1$; Ac-GHG: log$K=4.0$; see Supporting Information).[28] Interestingly, it is not only the distances between the ammonium

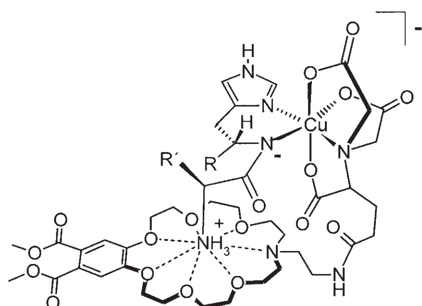

Figure 2. Proposed structures of stable peptide aggregates with compound **5** [R = CO_2^-, $CONHCH_2CO_2^-$, $CONHCH_2CO_2CH_3$, $CONHCH_2CONHCH_2CONH_2$; R′ = H, CH_3, CH_2-$CH(CH_3)_2$, $CH_2CH_2CONH_2$].

ion and the copper coordination sites within the peptide that are of importance to trigger emission output signals, but also the intervening structures. In a series of N-terminally acylated tetrapeptides the N-terminal amino acid was Lys, with an ammonium ion in its side chain (Ac-KHGG-NH_2, Ac-KGHG-NH_2, Ac-KGGH-NH_2). Although each peptide has a copper ion coordination site and an ammonium ion at a suitable distance, the more flexible connection of both functionalities does not lead to a stable aggregate with **5** with increased emission.

While the binding of histidine-containing peptides can be interpreted on the basis of known copper(II) peptide interactions, the sequence-independent effect of the cysteine-containing tetrapeptides on **5** was surprising. The interaction of cysteine with copper(II) ions has been investigated, but structural information on the interaction is very limited.[29] The cysteine thiol group strongly binds to copper(II) ions, forming polymeric species with bridging thiolate sulfur. This coordination process is accompanied by reduction of cupric ions to Cu^I ions, which in aqueous systems undergo dismutation, regenerating Cu^{II}. Analogously, the interaction of compound **5** with cysteine starts with the coordination of two thiol groups to the vacant coordination sites,[30] which explains the observed 2:1 stoichiometries of aggregates. The thiol ligands may influence the oxidation state of copper in the NTA complex,[31] but surely alter the electronic properties of the complex significantly. The UV absorption of compound **5** shows a 10 nm bathochromic shift upon addition of 5 equiv of mercaptoethanol in aqueous buffer. More significantly, in the emission spectrum a threefold increase in the emission intensity is observed (see Supporting Information). This indicates that the mechanism of emission increase in **5** produced by cysteine is different from that produced by the histidine peptides. While in histidine peptides the intramolecular ammonium ion binding to the luminescent benzocrown ether moiety partially restores the NTA-copper complex quenched emission of the fluorophore, cysteine thiol groups alter the NTA-copper complex itself and reduce its ability to quench the benzocrown ether emission. This explains the emission increase, which is independent of the position of the cysteine residue in the peptide sequence. Only peptides with cysteine as the N-terminal amino acid do not show any emission increase. Here, cysteine may form an S,N-chelate[32] with the NTA-copper complex, which still quenches the emission of the benzocrown ether fluorophore.

Conclusion

The combination of a copper(II)-NTA complex with an ammonium-ion-sensitive and luminescent benzocrown ether yields a molecular receptor that preferably binds to specific histidine-glycine peptide sequences under physiological conditions. Nearly micromolar affinities are observed for GGH and GHG; in tetrapeptides the recognition motif XaaHGG was identified, in which the N-terminal amino acid residue may vary. Only the N-terminal amino group triggers an

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emission signal; the ammonium moiety of a lysine side chain does not. Cysteine-containing peptides also trigger emission signals of **5**, but these are instead due to altered electronic properties of the copper ion cancelling its quenching of the benzocrown emission.

The selectivity and affinity of **5** in its current state may not be sufficient for direct practical use in peptide sensing, but these investigations have clearly shown that the interaction of a hybrid compound such as **5** with peptides can be explained on the basis of established coordination motifs of copper(II) ions to peptides. This offers the potential for a more rational design of sequence-selective peptide chemosensors tapping the extensively available knowledge of peptide to metal ion coordination reported over the last 50 years.

Experimental Section

General: Details of the preparation of model peptides and the spectroscopic determination of binding constants are given in the Supporting Information.

2-(Bis-*tert*-butoxycarbonylmethylamino)-pentanedioic acid 1-*tert*-butyl ester (2):[21] H-L-Glu(OBzl)-O*t*Bu·HCl (312 mg, 0.95 mmol) was dissolved under nitrogen in DMF (10 mL). DIPEA (0.8 mL, 4.73 mmol) and *tert*-butyl bromoacetate (0.6 mL, 3.78 mmol) were added to the stirred solution. The reaction mixture was heated at 80 °C overnight with continuous stirring and monitored by TLC (petrol ether/ethyl acetate 3:1). The solution was concentrated under reduced pressure, and the residue was taken up in a small amount of ethyl acetate, filtered and washed several times with petrol ether/ethyl acetate 3:1 and ethyl acetate until the filter residue was colourless. The filtrate was evaporated, and the crude product was purified by column chromatography on silica gel (petrol ether/ethyl acetate 3:1, $R_f=0.60$), yielding the title compound (418 mg, 84 %) as a light yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.42$ (s, 18 H; N-CH_2COO*t*Bu), 1.45 (s, 9 H; C^αHCOO*t*Bu), 1.82–2.07 (m, 2 H; Glu-CH_2), 2.50–2.77 (m, 2 H; Glu-CH_2), 3.38 (dd, $^3J=5.5$ Hz, 9.8 Hz, 1 H; Glu-C^αH), 3.43 (s, 4 H; N-CH_2), 5.08–5.12 (s, 2 H; Ph-CH_2), 7.26–7.38 ppm (m, 5 H; Ar-H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=25.4$ (–, 1 C; CH_2), 28.1 (+, 9 C; CH_3), 30.5 (–, 1 C; CH_2), 53.8 (–, 2 C; N-CH_2), 64.3 (+, 1 C; CH), 66.1 (–, 1 C; Ph-CH_2), 80.7 (C_{quat}, 2 C; COO*tBu*), 81.3 (C_{quat}, 1 C; COO*tBu*), 128.1 (+, 1 C; Ar-CH), 128.2 (+, 2 C; Ar-CH), 128.5 (+, 2 C; Ar-CH), 136.2 (C_{quat}, 1 C; Ar-C), 170.5 (C_{quat}, 2 C; NCH_2COO*t*Bu), 171.8 (C_{quat}, 1 C; COO*t*Bu), 173.5 ppm (C_{quat}, 1 C; COOBn); MS (ESI(+), CH_2Cl_2/MeOH+10 mmol NH_4Ac): *m*/*z* (%): 522.4 (100) $[M+H]^+$, 466.3 (3) $[M+H-C_4H_6]^+$.

The NTA bis-ester (624 mg, 1.2 mmol) was dissolved in EtOH (20 mL), to which a spatula tip of Pd/C (10 %) was added. The reaction mixture was stirred in an autoclave under 20 bar H_2 pressure for 18 h. The suspension was filtered twice and the filtrate was concentrated under reduced pressure, yielding compound **2** (513 mg, 99 %) as a colourless oil. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.43$ (s, 18 H; N-CH_2COO*t*Bu), 1.45 (s, 9 H; C^αHCOO*t*Bu), 1.78–2.07 (m, 2 H; Glu-CH_2), 2.47–2.78 (m, 2 H; Glu-CH_2), 3.36 (dd, $^3J=5.5$ Hz, 10.1 Hz, 1 H; Glu-C^αH), 3.44 ppm (s, 4 H; N-CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=25.4$ (–, 1 C; CH_2), 28.1 (+, 9 C; CH_3), 31.1 (–, 1 C; CH_2), 54.0 (–, 2 C; N-CH_2), 64.7 (+, 1 C; α-CH), 81.3 (C_{quat}, 2 C; COO*tBu*), 81.7 (C_{quat}, 1 C; COO*tBu*), 170.6 (C_{quat}, 2 C; NCH_2COO*tBu*), 171.3 (C_{quat}, 1 C; COO*tBu*), 176.2 (C_{quat}, 1 C; COOH); MS (ESI(+), CH_2Cl_2/MeOH+10 mmol NH_4Ac): *m*/*z* (%): 432.3 (100) $[M+H]^+$, 454.3 (3) $[M+Na]^+$.

Compound 4: Compound **2** (150 mg, 0.35 mmol) was dissolved under N_2 in a small amount of $CHCl_3$ and mixed with ice cooling with DIPEA (0.3 mL, 1.74 mmol), DCC (86 mg, 0.42 mmol) and HOBt (56 mg, 0.42 mmol). Then, crown ether dihydrochloride **3** (245 mg, 0.42 mmol), dissolved in a small amount of $CHCl_3$, was slowly added. The reaction mixture was allowed to warm to RT and was stirred overnight (20 h) at 40 °C. The reaction progress was monitored by TLC (ethyl acetate). After completion, the solution was diluted with ethyl acetate and the precipitated dicyclohexyl urea was filtered off over celite. The solvent was removed in vacuo, and the crude product was purified by column chromatography on silica gel ($CHCl_3$/MeOH 4:1, $R_f=0.75$, ethyl acetate), yielding the tris *tert*-butyl ester of **4** (198 mg, 60 %) as a light yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$): $\delta=1.40$ (s, 18 H; HSQC: C^1H_3), 1.41 (s, 9 H; HSQC: $C^{25}H_3$), 1.79–1.88 (m, 1 H; COSY: C^6H_2), 1.98–2.07 (m, 1 H; COSY: C^6H_2), 2.33–2.45 (m, 2 H; COSY: C^7H_2), 2.63 (br s, 2 H; COSY: $C^{11}H_2$), 2.77 (br s, 4 H; $C^{12}H_2$), 3.22–3.32 (m, 3 H; COSY: C^5H, $C^{10}H_2$), 3.36 (d, $^2J=17.2$ Hz, 1 H; C^4H_2), 3.44 (d, $^2J=17.2$ Hz, 1 H; C^4H_2), 3.57 (br s, 4 H; HMBC: $C^{13}H_2$), 3.61–3.65 (m, 4 H; HMBC: $C^{14}H_2$), 3.72–3.76 (m, 4 H; HMBC: $C^{15}H_2$), 3.85 (s, 6 H; COSY: $C^{22}H_3$), 3.88–3.91 (m, 4 H; HMBC: $C^{16}H_2$), 4.15–4.23 (m, 4 H; HMBC: $C^{17}H_2$), 6.88 (br s, 1 H; HSQC: N^9H), 7.18 ppm (s, 2 H; HSQC: C^{19}H); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta=28.1$ (+, 6 C; HSQC: C^1), 28.2 (+, 3 C; HSQC: C^{25}), 26.3 (–, 1 C; HSQC: C^6), 32.5 (–, 1 C; HSQC: C^7), 37.4 (–, 1 C; HSQC: C^{10}), 52.5 (+, 2 C; HSQC: C^{22}), 54.1 (–, 2 C; HMBC: C^4), 54.2 (–, 3 C; COSY: C^{11}, HSQC: C^{12}), 64.8 (+, 1 C; HSQC: C^5), 68.8 (–, 2 C; COSY: C^{13}), 69.5 (–, 2 C; HMBC: C^{16}), 69.7 (–, 2 C; HMBC: C^{17}), 70.6 (–, 2 C; HMBC: C^{14}), 71.1 (–, 2 C; HMBC: C^{15}), 80.7 (C_{quat}, 2 C; HMBC: C^2), 81.2 (C_{quat}, 1 C; HMBC: C^{24}), 113.7 (+, 2 C; HSQC: C^{19}), 125.4 (C_{quat}, 2 C; HMBC: C^{20}), 150.5 (C_{quat}, 2 C; HMBC: C^{18}), 167.7 (C_{quat}, 2 C; HMBC: C^{21}), 170.7 (C_{quat}, 2 C; HMBC: C^3), 171.8 (C_{quat}, 1 C; HMBC: C^8), 172.9 ppm (C_{quat}, 1 C; HMBC: C^{23}); see Supporting Information for compound numbering; IR (KBr): $\tilde{\nu}=3056$, 2976, 2931, 2823, 1724, 1658, 1600, 1520, 1363, 1138, 736 cm^{-1}; UV (MeOH): λ_{max} (log ε) = 202 (3381), 224 (3933), 267 nm (3423 $mol^{-1} dm^3 cm^{-1}$); MS (ESI(+), CH_2Cl_2/MeOH+10 mmol NH_4Ac): *m*/*z* (%): 928.5 (100) $[M+H]^+$; HRMS: *m*/*z*: calcd for $C_{45}H_{74}N_3O_{17}$: 928.5018; found: 928.5034 ± 0.0012; elemental analysis calcd (%) for $C_{45}H_{73}N_3O_{17}$: C 58.24, H 7.93, N 4.53; found C 58.42, H 8.16, N 4.97.

The tris *tert*-butyl ester of **4** (108 mg, 0.12 mmol) was suspended in trifluoroacetic acid (TFA, 4 mL). The reaction mixture was stirred at RT for 20 h and the reaction progress was monitored by TLC (ethyl acetate). TFA was evaporated, and the obtained triflate salt was redissolved in water and lyophilized, yielding **4** (118 mg, 0.12 mmol, 100 %) as a white hygroscopic solid. M.p. 60–62 °C; ^{1}H NMR (300 MHz, D_2O): $\delta=1.66$–1.84 (m, 2 H; Glu-CH_2), 1.98–2.20 (m, 2 H; Glu-CH_2), 3.13–3.25 (m, 2 H; CH_2), 3.27–3.45 (m, 6 H; CH_2), 3.51–3.94 (m, 28 H; CH_2, OMe, N-CH_2, α-CH, NH), 3.98–4.08 (m, 2 H; CH_2), 4.0–4.25 (m, 2 H; CH_2), 7.05–7.12 ppm (s, 2 H; CH); ^{13}C NMR (75 MHz, D_2O): $\delta=22.5$ (–, 1 C; CH_2), 31.6 (–, 1 C; CH_2), 33.9 (–, 1 C; CH_2), 52.2 (–, 1 C; CH_2), 53.3 (+, 2 C; CH_3), 53.6 (–, 2 C; CH_2), 54.1 (–, 2 C; N-CH_2), 63.6 (–, 2 C; CH_2), 65.7 (+, 1 C; CH), 68.1 (–, 2 C; CH_2), 69.1 (–, 2 C; CH_2), 69.8 (–, 2 C; CH_2), 69.9 (–, 2 C; CH_2), 112.6 (+, 2 C; CH), 116.3 (C_{quat}, q, $^1J_{C,F}=292.1$ Hz; CF_3COO^-), 124.5 (C_{quat}, 1 C; Ar-C), 124.7 (C_{quat}, 1 C; Ar-C), 149.58 (C_{quat}, 1 C; Ar-C), 149.62 (C_{quat}, 1 C; Ar-C), 162.7 (C_{quat}, q, $^2J_{C,F}=35.5$ Hz, CF_3COO^-), 169.2 (C_{quat}, 1 C; COOMe), 169.3 (C_{quat}, 1 C; COOMe), 170.7 (C_{quat}, 2 C; NCH_2*COOH*), 171.2 (C_{quat}, 1 C; CONH), 174.9 ppm (C_{quat}, 1 C; CH*COOH*); IR (KBr): $\tilde{\nu}=2957$, 2914, 2360, 2341, 1732, 1642, 1528, 1428, 1351, 1200, 814, 719 cm^{-1}; UV (MeOH): λ_{max} (log ε) = 202 (3860), 224 (4070), 267 nm (3587 $mol^{-1} dm^3 cm^{-1}$); MS (ESI(+), CH_2Cl_2/MeOH+10 mmol NH_4Ac): *m*/*z* (%): 796.4 (100) $[M-2H^++K^+]^-$, 780.5 (10) $[M-2H^++Na^+]^-$, 758.5 (3) $[M-H^+]$.

Glutamic acid copper NTA crown ether (Cu-NTA CE, 5): Compound **4** (164 mg, 0.17 mmol), $NaHCO_3$ (28 mg, 0.3 mmol) and $Cu_2(OH)_2CO_3$ (18.4 mg, 0.08 mmol) were dissolved in water (5 mL). The mixture was stirred at room temperature overnight, and was subsequently heated at reflux for 3 h and filtered immediately. The resulting greenish-blue solution was concentrated under reduced pressure and lyophilized, yielding **5** (185 mg, 98 %) as a turquoise solid. M.p > 175 °C (decomp); IR (KBr): $\tilde{\nu}=2929$, 2357, 2341, 1682, 1629, 1523, 1437, 1351, 1295, 1204, 1130, 801, 721 cm^{-1}; MS (ESI(+), H_2O/MeCN): *m*/*z* (%): 819.4 (100) $[M]^-$, 731.4 $[M-2CO_2]^-$, 775.3 $[M-CO_2]^-$, 901.4 $[M+2MeCN]^-$. The purity of the compound was determined by HPLC, diode array detection at 226 nm, and found to be > 97 %.

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