Interaction of $V^{IV}O$, $V^{V}O_2$ and Cu^{II} with a Peptide Analogue SalGly-L-Ala

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The formation of complexes of a tripeptide analogue, salicylglycyl-L-alanine [$HOC_6H_4C(O)NHCH_2C(O)NHCH(CH_3)$-COOH, H_2SalGly-L-Ala], was studied with Cu^{II}, $V^{IV}O$ and $V^{V}O_2$ in aqueous solution using pH-potentiometric and spectroscopic (UV/Vis, CD, EPR and ^{51}V NMR) techniques. The results demonstrated the ambidentate character of the ligand. The metal ion-induced deprotonation and subsequent coordination of the two neighbouring amide groups was shown to occur in a cooperative way in the pH range 5–6. At pH ≈ 6.5 a complex with an (O^-, $2 \times CON^-$, COO^-) binding set becomes predominant for both Cu^{II} and $V^{IV}O$. The affinity of the phenolate-O^- for $V^{IV}O$ is high and the ligand is bound to this metal ion even at pH values greater than 10. Conversely, the affinity for $V^{V}O_2$ is significantly lower and no interaction between this metal oxo-ion and the ligand could be detected in the pH range 2–12. In contrast, with the dipeptide analogue H_2SalGly, chelation involving the deprotonated amide-N^- could be unambiguously detected.

Introduction

Vanadium is an important trace metal with a great variety of physiological effects, including its interference with various phosphate-metabolizing reactions.[1] This suggests interactions between vanadium ions and protein side-chain donors. Peptides are not the best molecules for modeling metal ion binding to proteins, since the specially arranged side-chain donor groups of the amino acid units, determined by the tertiary structure of the proteins, are usually the primary bindings sites of proteins, while in the oligopeptides the N- and C- terminal donors are the basic sites for metal ion coordination. However, peptides are the most closely related models for proteins, and in some cases proteins may have specific terminal metal ion bindings sites as in the case of albumin.[2] For $V^{IV}O$ one of the known biologically important interactions with peptides is that with the important cell constituent and natural tripeptide, glutathione (GSH). In fact it is believed that GSH may play a role in the redox transformations of vanadium in the cells.[3] The equilibria in the systems $V^{IV}O^{2+}$–GSH and $V^{IV}O^{2+}$–GSSG have been recently studied.[4–5]

In order that simple di- or tripeptides bind metal ions strongly enough, the amide-N^- should be involved in coordination, but in some cases suitable anchoring donors should be available to promote amide-NH deprotonation and subsequent coordination. Various metal ions were found to engage in this type of bonding, such as Pt^{II}, Pd^{II}, Cu^{II}, Ni^{II},[6] $V^{V}O_2$ [7] and $V^{IV}O$.[8] The usual anchoring donor for binding metal ions, including Cu^{II}, and inducing amide deprotonation is the terminal NH_2 group,[6] although in some cases the terminal COO^- or the side chain thiolate-S^- or imidazol-N also proved to be efficient at inducing this process.[6] For $V^{IV}O^{2+}$, the terminal NH_2 is not a suitable anchor,[9] but phenolate proved to be an efficient anchoring donor in cases such as H_2salGly (2-OH-hippuric acid) where the p*K* for the amide-NH deprotonation/coordination of 4.76 was found to be similar to values seen for $V^{IV}O$ and Cu^{II}.[8,10]

In this work the binding capability of the tripeptide analogue H_2SalGly-L-Ala (**1**) containing a phenolate anchoring donor has been studied, and the solution speciations and binding modes of $V^{IV}O$, $V^{V}O_2$ and Cu^{II} complexes of **1** are reported. In some cases complexes were obtained in the solid state and characterized.

Results and Discussion

The tripeptide analogue H_2SalGly-L-Ala (**1**) was synthesized by the procedure described in the Exp. Sect. Its for-

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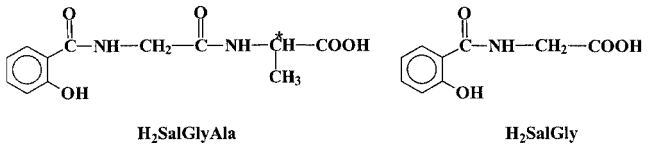
mula together with that of H_2SalGly are included in Scheme 1.



Scheme 1

Interactions of SalGly-L-Ala with Cu^{II} and $V^{IV}O$

The protonation and $V^{IV}O$ and Cu^{II} complex formation constants are in Table 1. H_2SalGly-L-Ala contains two dissociable protons: on the phenolic OH and the terminal COOH groups. The log K_{OH} value of 7.81 can be assigned to the less acidic phenolic OH and the log K value of 3.37 to the terminal COOH group. These data are similar to those of the corresponding dipeptide analogue, H_2SalGly (log K_{OH} = 8.16 and log K_{COOH} = 3.37).[8]

Table 1. Protonation (log K) and Cu^{II} and $V^{IV}O$ complex formation constants (log β with 3 S.D. values in parentheses) of the complexes of SalGly-L-Ala at T = 25 °C and I = 0.2 M (KCl)

Species	Cu^{II}	$V^{IV}O$
log K(phen-O^-)	7.81(1)	
log K(COO^-)	3.37(2)	
MLH	9.49(8)	9.98(11)
ML	4.45(5)	6.43(5)
MLH_{-1}	−0.49(6)	1.06(8)
MLH_{-2}	−6.64(5)	−4.31(6)
No. of points	297	245
Fitting[a] (mL)	0.0017	0.0032
pK(MLH)	5.03	3.55
pK(ML)	4.92	5.37
pK(MLH_{-1})	6.16	5.37

[a] Average difference between the experimental and calculated titration curves expressed in mL of the titrant.

Based on earlier speciation studies of the corresponding dipeptide H_2SalGly[8,10] and also Gly-like tripeptides,[6] the pH-metric titration curves measured for the Cu^{II}− and $V^{IV}O$−SalGly-L-Ala systems were evaluated by assuming 1:1 complex formation at the different states of protonation. Whenever 2:1 complexes were included in addition to the 1:1 complexes, they were always rejected by the PSEQUAD computer program. The best fit between the experimental and the calculated titration curves was obtained with the set of species listed in Table 1. The concentration distribution curves of the complexes formed in the Cu^{II}−SalGly-L-Ala and $V^{IV}O$−SalGly-L-Ala systems are depicted in Figures 1 and 2.

In agreement with the speciation curves the visible spectra of the metal-ligand systems (e.g. Figure 3 for the Cu^{II} system) start to deviate from that of the corresponding aqua ions $[Cu(H_2O)_6]^{2+}$ or $[VO(H_2O)_5]^{2+}$ only at pH > 3, where

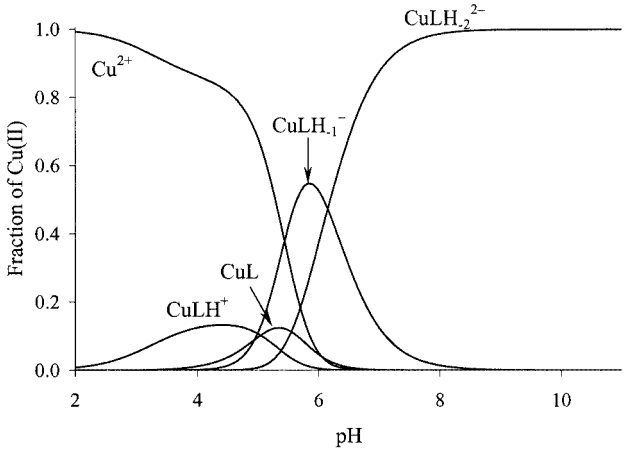


Figure 1. Species distribution curves as a function of pH for the Cu^{II}−SalGly-L-Ala system at a 1:2 ratio and c_{Cu} = 0.002 M

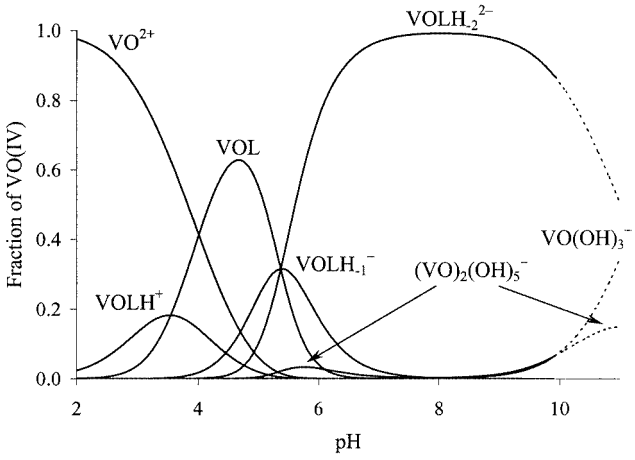


Figure 2. Species distribution curves as a function of pH for the $V^{IV}O$−SalGly-L-Ala system at a 1:2 ratio and c_{VO} = 0.002 M. Dashed lines indicate a higher uncertainty and thus only the tendency of the speciation as a result of slow pH equilibration (see Exp. Sect.)

the formation of the protonated species $[MLH]^+$ is indicated by the speciation curves. It can also be seen in Figure 3 that as complex formation proceeds, the d-d transition of the Cu^{II}-ligand system is shifted to higher energies and becomes constant at a pH of around 7.5, indicating the exclusive formation of a single species $[CuLH_{-2}]^{2-}$ (λ_{max} = 545 nm, ε = 240 $M^{-1}\cdot cm^{-1}$). At the same time in the corresponding $V^{IV}O$ system the visible spectra remain almost constant in the pH range 6.5−8.0 ($[VOLH_{-2}]^{2-}$: λ_{max} = 960 nm (ε = 120 $M^{-1}\cdot cm^{-1}$) and 690 nm (ε = 115 $M^{-1}\cdot cm^{-1}$). However, at higher pH the intensity of the absorption decreases as the ligand is displaced by OH^-. The CD spectra depicted in Figures 4 and 5 are in full agreement with these observations.

At pH < 5 the CD spectra of the $V^{IV}O$−SalGly-L-Ala system have relatively low intensities (species $[VOLH]^+$ and [VOL]). At pH > 5 their intensities increase (parallel with the increase in the visible absorption), and three peaks develop in the CD spectra at λ_{max} 760 nm (Δε < 0), 610 nm

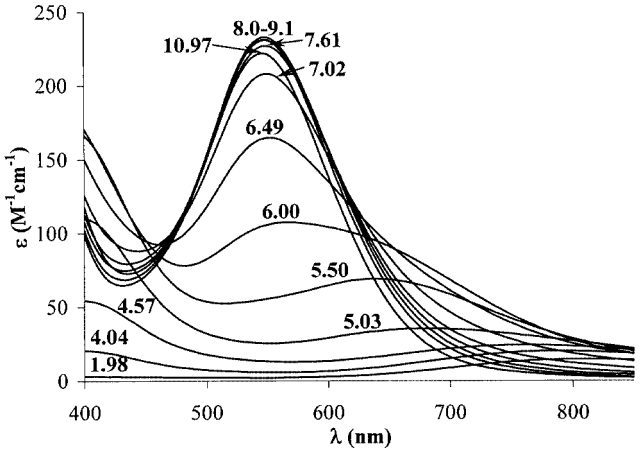


Figure 3. Visible absorption spectra of the Cu^{II}−SalGly-L-Ala system at a 1:2 metal ion to ligand ratio at several pH values and c_{Cu} = 0.006 M

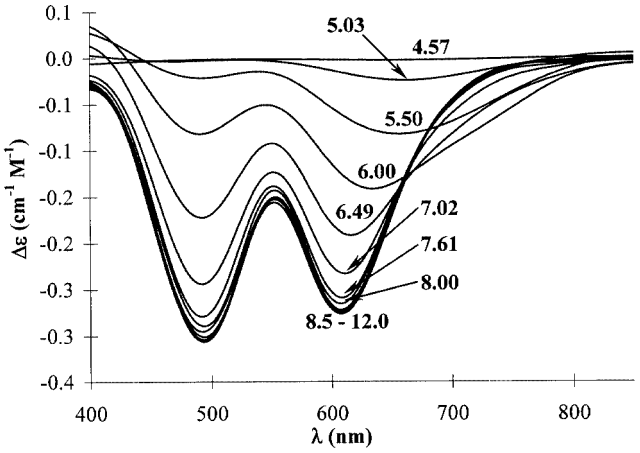


Figure 4. CD spectra of the Cu^{II}−SalGly-L-Ala system at a 1:2 metal ion to ligand ratio at several pH values and c_{Cu} = 0.006 M

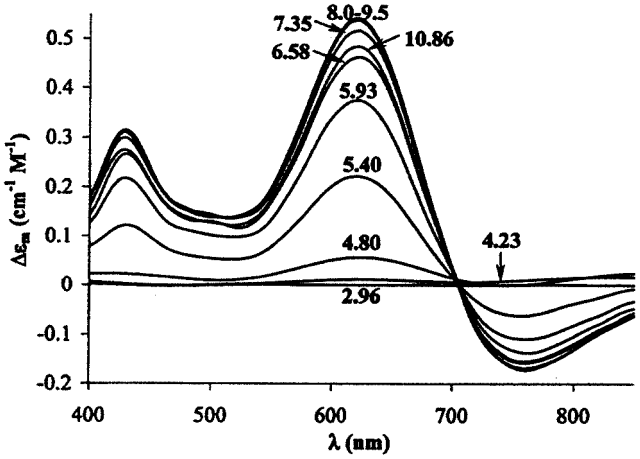


Figure 5. CD spectra of the $V^{IV}O$−SalGly-L-Ala system at a 1:2 metal ion to ligand ratio at several pH values and c_{VO} = 0.0045 M

($\Delta\varepsilon > 0$) and at 415 nm ($\Delta\varepsilon > 0$) (Figure 5), indicating coordination of the amide N^- group close to the chiral centre. Similarly, in the Cu^{II}−SalGly-L-Ala system the visible band at λ = 545 nm which is apparent by a pH of approximately 7 (see Figure 3), corresponds to two bands in the CD spectra at 490 nm ($\Delta\varepsilon = -0.3$ $M^{-1}\cdot cm^{-1}$) and at 610 nm ($\Delta\varepsilon = -0.28$ $M^{-1}\cdot cm^{-1}$). The significant blue shift in the absorption spectra and the intense CD bands indicate coordination of the amide N^- in the corresponding complex. In the CD spectrum of the GlyGly-L-Ala−Cu^{II} solutions, a band with λ_{max} at 475 nm ($\Delta\varepsilon = -0.8$ $M^{-1}\cdot cm^{-1}$) was observed.[11] The different CD spectra obtained here confirm that in the Cu^{II} complexes formed with **1**, which predominate at pH > 5, the phenolate O^- is bound to the metal ion.

The visible and CD spectra obtained at different pH values were used to calculate the spectrum of each individual species formed in the Cu^{II}− and $V^{IV}O$−SalGly-L-Ala systems, using the PSEQUAD computer program. Hydrolysis and observable increases in the absorbances of the very slightly cloudy solutions, especially in the case of the $V^{IV}O$−SalGly-L-Ala system, precluded the use of visible spectroscopic data to determine individual spectra precisely. CD data were more suitable for such a treatment. The fact that reasonable CD spectra could be simulated for each species (see Figure 6) confirms that the speciation models proposed and the stability constants calculated from the pH-metric measurements are correct.

It is worth noting here that significant changes and increases in the CD intensities can be observed only upon appearance of the $[MLH_{-2}]^{2-}$ species in both systems, and this is reflected in the calculated CD spectra of $[MLH_{-2}]^{2-}$. This indicates that coordination of the closest donor atom to the chiral centre (the first amide N^- from the C terminus) presumably occurs only in this species.

The EPR spectra help further to elucidate the binding modes of the complexes formed in the two systems. For the $V^{IV}O$ systems, Chasteen[12] introduced an additivity rule to estimate the hyperfine coupling constants $A_{\parallel}^{est}$ ($A_{\parallel}^{est} = \Sigma A_{\parallel,i}$), based on the contributions $A_{\parallel,i}$ of each of the four equatorial donor groups, its estimated uncertainty being $\pm$ 3 × 10^{-4} cm^{-1}. Most of the $A_{\parallel,i}$ parameters were presented by Chasteen[12] while the others were presented by Cornman et al. [$A_{\parallel}(N^-)$ = 34−35 × 10^{-4} cm^{-1}],[13] Tasiopoulous et al. [$A_{\parallel}(N^-)$ = 34−43 × 10^{-4} cm^{-1}],[14] Hamstra et al. [$A_{\parallel}(O_{amide})$ = 43−44 × 10^{-4} cm^{-1}],[15] and Costa Pessoa et al. [$A_{\parallel}(N^-)$ dipeptides = 34−36 × 10^{-4} cm^{-1} [16] and $A_{\parallel}(COO^-)$ = 42 × 10^{-4} cm^{-1}].[17] As can be seen, there is a rather high uncertainty in the reported $A_{\parallel}(N^-)$ values: 36±4 × 10^{-4} cm^{-1}. We chose the value 39 × 10^{-4} cm^{-1} for CON^-, which is within the uncertainty range. This was chosen without any theoretical precedent but because it gave a better agreement with the experimental coupling constants of the present system. Although a similar additivity rule has not been developed for Cu^{II} complexes, the large amount of EPR data available in the literature also allows us to make similar estimates for the coordinating donors in the corresponding Cu^{II} complexes. A rigorous comparison of the EPR spectra with the pH-metrically determined speciation curves may indicate some differences in the relative amount of some of the species at given pH values. This may be explained by the differences in the temperature, which was

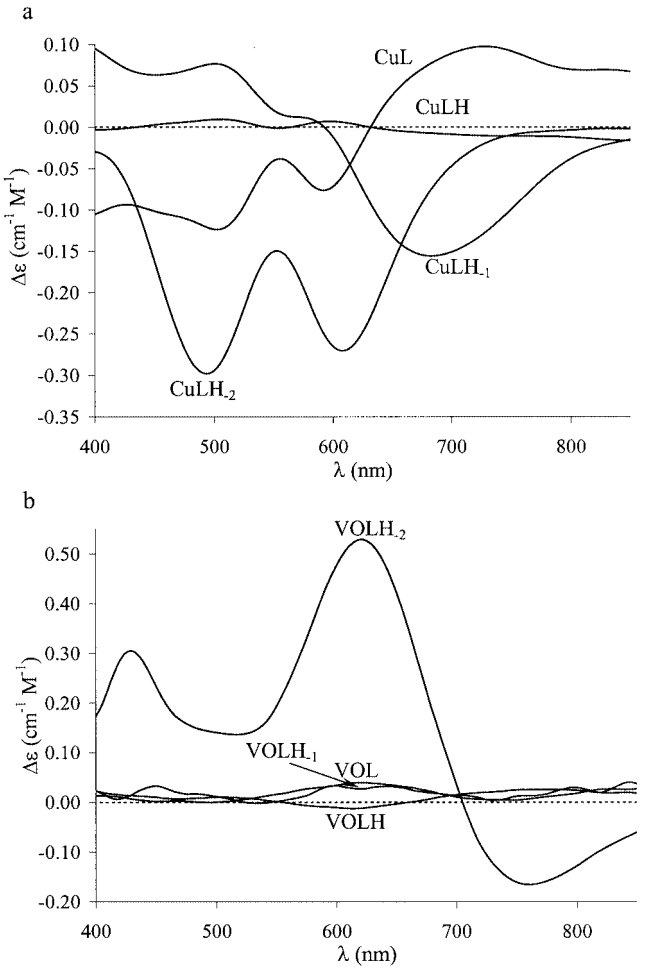


Figure 6. Simulated CD spectra for each individual species formed in (a) the Cu^{II}−SalGly-L-Ala system and (b) the $V^{IV}O$−SalGly-L-Ala system, using the program PSEQUAD

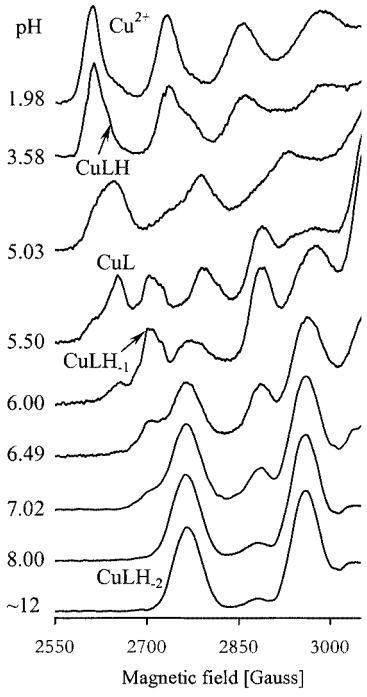


Figure 7. Low-field region of frozen EPR spectra (X-band) recorded at 77 K on the Cu^{II}−SalGly-L-Ala system at a 1:2 metal ion to ligand ratio at different pH values, c_{Cu} = 0.006 M

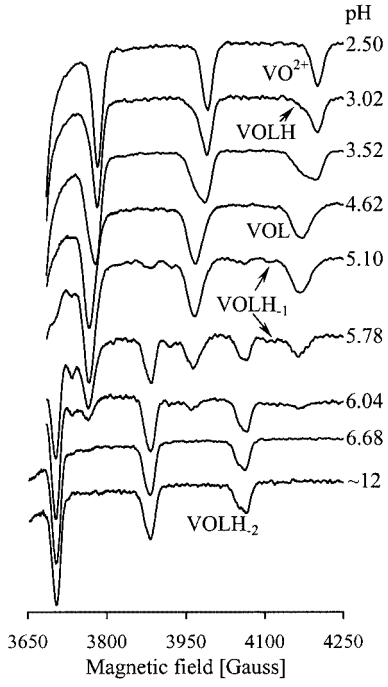


Figure 8. High-field region of frozen EPR spectra (X-band) recorded at 77 K on the $V^{IV}O$−SalGly-L-Ala system at a 1:2 metal ion to ligand ratio at different pH values, c_{VO} = 0.0045 M

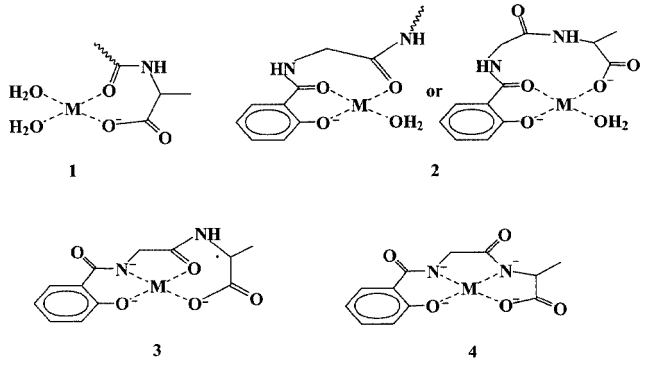
25 °C for pH-metry and ca. −3 °C for EPR (the approximate freezing point of the sample), and also in the concentration of the metal ion. For this reason EPR is used only in a quantitative way to confirm the formation of each of the species assumed from the pH-metric speciation measurements. Figures 7 and 8 depict the low-field region (high-field in the case of $V^{IV}O$) of some of the frozen solution EPR spectra of the two systems at different pH values. Table 2 summarizes the EPR parameters obtained from simulation of the spectra by using the computer program of Rockenbauer,[18] and also the most likely binding modes of the complexes. For the $V^{IV}O$ system, CD and EPR spectra were also recorded at pH = 5.5 and c_L = 10 mM, with a variable c_{VO} concentration always confirming the presence of the MLH_{-1} species (see Figure S1, Supporting Information, see also the footnote on the first page of this article).

The pH-metric speciation curves (Figures 1 and 2) indicate that complex formation starts at a pH of about 3 in both systems with a protonated species $[MLH]^+$. In this species, like the corresponding complexes of the dipeptide analogue H_2SalGly, coordination at the terminal COO^- through (COO^-, CONH) chelation is most likely (see structure 1, Scheme 2). This binding mode explains why the observed EPR and visible spectra at pH around 3 show only minor differences from those of the aqua ions. In fact no significant differences are expected in the EPR and visible

Table 2. Calculated EPR parameters ($g_{\|}$ and $A_{\|}$) for the complexes formed in the Cu^{II}– and $V^{IV}O$–SalGly-L-Ala systems

Species	Cu^{II}		$V^{IV}O$			Equatorial binding set
	$g_{\|}$	$A_{\|}$ 10^4 [cm^{-1}]	$g_{\|}$	$A_{\|}$ 10^4 [cm^{-1}]	$A_{\|}^{est}$	
MLH	2.380	147	1.934	180	178	(COO^-, CONH)
ML	2.358	148	1.937	176	171–174	(O^-, 2 × CONH) or (O^-, CONH, COO^-) or (O^-, CONH)
MLH_{-1}	2.270	178	1.945	165	164	(O^-, CON^-, CONH, COO^-)
MLH_{-2}	2.209	192	1.958	159	159	(O^-, 2 × CON^-, COO^-)

spectra for such binding modes when compared with the aqua ions.[16,19]



Scheme 2

Three protons are liberated from these complexes with stepwise p*K* values of 5.03, 4.92 and 6.16 for the Cu^{II} complexes, and 3.55, 5.37 and 5.37 for the $V^{IV}O$ complexes. The first deprotonation process, leading to the species [ML] in both systems, is attributable to the phenolic OH group of the metal-bound ligand. This indicates a rearrangement to (O^-, 2 × CONH) or (O^-, CONH, COO^-) binding modes (see structure 2, Scheme 2), as supported by the spectral changes. The EPR parameters (see Table 2) are characteristic of pure O-coordination, and agree well with those in the corresponding complex formed with H_2SalGly[8] and other aromatic hydroxycarboxylic acids.[20]

The next two p*K* values can be assigned to the deprotonation/coordination of the two amides, yielding (O^-, CON^-, CONH, COO^-) and (O^-, CON^-, CON^-, COO^-) joint chelate systems (structures 3 and 4, Scheme 2) respectively. As the simultaneous equatorial coordination of the second amide-O and the terminal COO^- may be sterically hindered, the tridentate (O^-, CON^-, COO^-) binding mode of the ligand (see Scheme 2) is also possible in species $VOLH_{-1}$. Due to the coordination of the two amide nitrogens, superhyperfine coupling (five lines with shf $A_{N\perp}$=15.5 G) can be observed in the perpendicular region of the EPR spectrum of the $CuLH_{-2}$ species. In the case of $V^{IV}O$, the EPR parameters estimated by the additivity rule are 164 ×10^{-4} cm^{-1} and 159 × 10^{-4} cm^{-1} which are in reasonable agreement with the experimental hyperfine coupling constants (Table 2). Since the species $[VOLH_{-1}]^-$, with one deprotonated amide N^-, could hardly be detected in the pH range 5–6 at a comparable metal ion to ligand ratio, EPR measurements in the wide L:M range of 20–1 were also carried out (see Exp. Sect.). Under such conditions the species was clearly detected by the appearance of a distinct signal (see Figure S1, Supporting Information). As mentioned above, the fact that significant increases in the $|\Delta\varepsilon|$ CD parameters are only observed for the fully deprotonated complex $[MLH_{-2}]^{2-}$ indicates the following sequence of amide deprotonation: first that is closer to the phenolate O^- then that is closer to the COO^-, the latter being closer to the chiral centre. Interestingly, the opposite sequence was observed for the organotin(IV)–peptide complexes,[16] which pointed to the C-terminal COO^- group being the primary anchor. With $V^{IV}O$ and Cu^{II}, the phenolate O^- seems to be the primary anchor. In the case of Gly-type tripeptides, the N-terminal site is the primary binder for Cu^{II}.[6] At pH > 10, a significant decrease in the intensities of the EPR signals was observed in the $V^{IV}O$–SalGly-L-Ala system (to compensate for this, the receiver gain and the modulation were increased) indicating the partial hydrolysis of the metal ion through formation of the EPR silent hydroxo-bridged species (represented by $[(VO)_2(OH)_5]^-$ in the speciation diagram).

Compounds in the Solid State

The tripeptide analogue H_2SalGly-L-Ala **1** was synthesized by the procedure described in the Exp. Sect. Complex **2**, formulated as VO(SalGly-L-Ala)·3.2H_2O·0.2EtOH, was prepared from solutions containing $V^{IV}O$ and H_2SalGly-L-Ala at a pH of around 5.5, i.e. where we expect the [VOL] stoichiometry to be predominant. Elemental analysis (see Exp. Sect.) indicates that the amide is not deprotonated, and the CD spectrum of **2** in a KBr disk shows a band pattern and a λ_{max} value similar to those of the VOL species shown in Figure 6b. The binding in the solid state presumably involves O^-, CONH, COO^- and H_2O, and eventually another amide O in the axial position. In frozen solution the experimental $A_{\|}$ is around 175–176 × 10^{-4} cm^{-1}, which corresponds either to (i) (O^-, CONH, COO^-, $H_2O)_{equatorial}$ coordination ($A_{\|}^{est}$ = 38.9 + 43.7 + 42.1 + 45.6 = 170 × 10^{-4} cm^{-1}) or to (O^-, CONH, 2 × $H_2O)_{equatorial}$ coordination ($A_{\|}^{est}$ = 38.9 + 43.7 + 45.6 + 45.6 = 174 × 10^{-4} cm^{-1}).

Complex **3**, formulated as K[Cu(SalGly-L-Ala)]·3.2H_2O, was also prepared from aqueous/ethanolic solutions at pH = 6. The elemental analysis agrees with the formulation of $CuLH_{-1}$ for this complex and the CD spectrum of **3** in a KBr disk shows a band pattern and a λ_{max} value close to those of the $CuLH_{-1}$ species (see Figure S2, Supporting

Table 3. IR bands [cm^{-1}] and assignments of H_2SalGly-L-Ala and its VO and Cu complexes prepared in this work (vs: very strong, s: strong, m: medium, w: weak, vw: very weak; *: bands emerging from a broad band)

	Ligand **1**	Complex **2**	Complex **3**
Hydrogen bonded O−H and N−H	Broad band (2500−3500)	Broad band (2500−3600)	Broad band (2400−3700 with a few weak peaks emerging from it
ν(N−H)	3325 vs*	3346 m*	−
Aromatic C−H strech	3000 w*	3020 w*	≈3000 vw*
Aliphatic C−H strech	2932 w*	2990 w*	≈2950 vw*
Other amide bands	3072 vw* ,[23][24] 2613 vw*[a], 2499 vw*[a], 2411 vw*[a], 2290 vw*[a]	A few very weak bands	
$\nu(C{=}O)_{COOH}$	1730 s	−	−
Amide I (C=O)	1661 s, 1632 m (?)	1638 m-s* [22,p.72]	1605 s* [22]
Amide II	1547 s	1541 s	1534 s*
C=C Ring stretch	1593 s	1593 s*	≈1600 s*
$\nu_{as}(COO^-)$		1574 s* [22,p.72−73]	1572 s*
$\nu_s(COO^-)$		1370 m-s	1400 m-s (?)
$\nu(C{-}O_{Phe})$	1344 m-s (?)	1236 m-s	1256 s
ν(V=O)	−	966s	−
C−H Out-of-plane deformation	758 m-s	758 m-s	762 s

[a] These are multiple combination bands due to symmetrical, asymmetrical and overtone N−H bands.[21−24]

Information). The elemental analysis and spectra of this Cu^{II} complex are therefore consistent with a binding of the ligand through O^-, COO^- and CON^-. No individual ν(N−H) band could be detected emerging from the broad band in the 2400−3700 cm^{-1} range.

A comparison of the FTIR spectroscopic data for compounds **1**−**3** is summarised in Table 3. The data for **2** and **3** agree with the binding modes proposed. The assignments are based on Refs. 21−25, and by comparison with some $V^{IV}O$ and Cu^{II} complexes formed with the reduced Schiff base ligands derived from the reaction of salicylaldehyde and GlyGly or GlyGlyGly.[26] All compounds present broad bands in the wavenumber range corresponding to hydrogen-bonded O−H and N−H. In the case of **1** a strong ν(N−H) band emerges from this broad band as well as several other weak or very weak bands (Table 3). This is characteristic of compounds containing secondary amide groups.[23,24] The strong $\nu(C{=}O)_{COOH}$ band at 1730 cm^{-1} in the FTIR of **1** is absent in its $V^{IV}O$ and Cu^{II} complexes. For **2**, a narrow band emerges at 3346 cm^{-1} and a second weaker one at 3454 cm^{-1}, presumably also due to ν(N−H) of the amide groups.[21,23] All our efforts to characterise other solid compounds from these metal-ligand systems, particularly those of oxovanadium with a deprotonated amide, were unsuccessful. Either no solid could be isolated or when precipitation was achieved, no reasonable formulation could be found.

Interactions of H_2SalGly and H_2SalGly-L-Ala with V^VO_2

Vanadate(V)-promoted deprotonation of the amide NH and the formation of stable complexes with dipeptides through (NH_2, CON^-, COO^-) coordination was first detected by Rehder[7] using ^{51}V NMR spectroscopy (δ = −505−510 ppm). Pettersson et al.[27,28] gave complete speciation descriptions of V^VO_2 with dipeptides such as ProAla, AlaGly and AlaHis. A single complex species of moderate stability (at approx. tenfold excess of ligand ca. 50% of the vanadate was bound to the peptide) was formed with the above binding mode in fairly slow processes (completion of complex titration took several hours). The chemical shift values were as follows: δ = −495 ppm for ProAla, δ = −512 ppm for AlaGly and δ = −518 ppm for AlaHis.[27,28]

As phenolate proved to be a more efficient anchoring donor to promote amide deprotonation with $V^{IV}O$, we were also interested in studying this interaction with vanadate(V). The ^{51}V NMR spectrum of the vanadate(V)−SalGly system (c_VV= 0.003 M) displayed an extra peak (δ = −508.5 ppm) as well as the resonances of the V1 (δ = −561.8 ppm), V2 (δ = −574.8 ppm), V4 (δ = −579.2 ppm) and V5 (δ = −593.7 ppm) oxoanions. At a pH of around 7 the maximum amount of the complex was around 54 % with a tenfold excess of ligand (see Figure 9). The spectrum was identical after 24 h. This is in agreement with the earlier literature findings (vide supra), and an (O^-, CON^-,COO^-) binding mode has been suggested for the complex detected.[7,27−29] Interestingly, no new peaks were observed in the spectrum of the vanadate(V)−SalGly-L-Ala system which also had a tenfold excess of ligand (see Figure S3, Supporting Information), whether at pH 7 or 8. In both cases the polyoxoanions predominated in the system and the spectra were practically unchanged after 24 h.

In the case of tripeptides, amide deprotonation seems to occur at higher pH values when compared with dipeptides (e.g. for the corresponding Cu^{II} systems pK(CuA) = 4.40 for Cu^{II}−SalGly and pK(CuA) = 4.92 and pK($CuAH_{-1}$) = 6.16 for SalGly-L-Ala), where the likelihood of hydrolysis of the copper(II) is higher. This sensitive equilibrium between the metal-peptide complex and the binary (hydroxo)metal complex is finely shifted to the direction of peptide coordination with dipeptides, but to the direction of

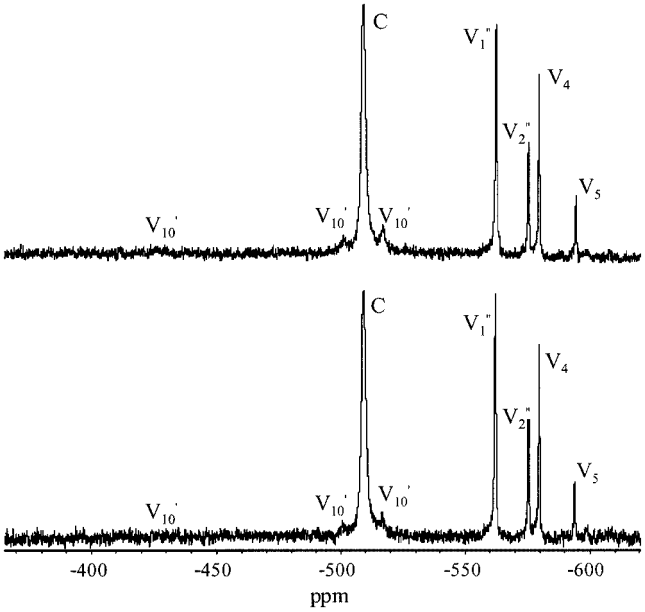


Figure 9. ^{51}V NMR spectra measured at pH ≈ 7 of a solution of the V^{V}−SalGly system at a 1:10 metal ion to ligand ratio. (a) in freshly prepared solution, (b) 24 h later, c_V = 0.003 M

oxoanion formation in the presence of tripeptides. Similar observations were made with organotin(IV)−di and tripeptide systems.[29]

Conclusions

The speciation and solution structural investigations discussed above strongly indicate that both the terminal COO^- and the phenolate O^- can behave as anchoring donors to chelate this tripeptide-analogue ligand. Coordination at a pH of around 3 starts at the terminal carboxylate through chelation with the neighbouring amide carbonyl. At a pH of approximately 4.5 the binding mode also involves the terminal phenolate and this induces deprotonation of the two amide-NH groups in a consecutive but rather cooperative way in the pH range 5−6. With the phenolate O^- anchor $V^{IV}O$ seems to be slightly more efficient in promoting deprotonation than Cu^{II} (the value of ΣpK_{amides} is 10.74 for $V^{IV}O$, and 11.08 for Cu^{II}), MLH_{-2} being the most important stoichiometry in the pH range 6−10, corresponding to an $(O^-, 2 \times CON^-, COO^-)_{equatorial}$ binding set. While Cu^{II} is able to promote amide deprotonation with normal tripeptides with about the same efficiency (ΣpK_{amides} is 11.82[8]), $V^{IV}O$ cannot induce deprotonation of the amide because of its low affinity for primary amine donors and the metal ion precipitates as $VO(OH)_2$. Interestingly, phenolate O^- did not prove to be such an efficient anchoring donor with vanadate(V) or $V^{V}O_2$ as with $V^{IV}O$. Amide deprotonation could not be detected by ^{51}V NMR spectroscopy in the case of the tripeptide analogue H_2SalGly-L-Ala. With the dipeptide analogue H_2SalGly, a species with an (O^-, N^-, COO^-) binding mode could be detected in the pH range 7−8. This is in full accordance with earlier literature reports concerning the interaction of vanadate^{V} with various dipeptides.[7,27−28] However, the moderate affinity of vanadate^{V} for amides, even with the more hard and probably more efficient phenolate O^- anchor, is reflected in the fact that even with a tenfold excess of ligand only around 50% of the metal ions are bound to SalGly, the rest being in the various oxoionic forms.

The considerably weaker ability of vanadium(V) to induce amide deprotonation, compared with vanadium(IV), can probably be explained by electrostatic ($V^{IV}O^{2+}$ vs. $V^{V}O_2^+$ towards LH_{-2}^{4-}) and steric arguments (there are 4 equatorial positions free around $V^{IV}O^{2+}$, while only 3 in the case of $V^{V}O_2^+$ because of the *cis* arrangement of the two oxo groups).

Experimental Section

Synthesis of H_2SalGly-L-Ala (1): The compound was synthesized by two different routes: (i) through the stepwise build up of the pseudopeptide from the individual components via the classical liquid phase method, (ii) through the Merrifield solid-phase approach.

Route 1

Z-GlyOH: To an ice-cold solution of glycine (7.5 g, 100 mmol) in 1 M NaOH (100 mL) was added benzyl chloroformate (16 mL, 100 mmol), dropwise with stirring. The pH of the solution was maintained at 10−10.5 by addition of 1 M NaOH. The mixture was stirred for 2 h and then extracted with diethyl ether. The aqueous layer was acidified to a pH ≈ 1 and the solid which precipitated was filtered, washed with water and dried. Yield (17.9 g, 86%), m.p. 119−120 °C (ref.[30] m.p. 120−121 °C). NMR ([D$_6$]DMSO): δ = 3.66 (d, J = 6 Hz, 2 H, CH_2Gly), 5.03 (s, 2 H, CH_2Ar), 7.34 (m, 5 H, Ar), 7.55 (t, J = 6 Hz, 1 H, NH), 12.25 (br. s, 1 H, COOH) ppm.

L-AlaOEt·HCl: Thionyl chloride (12.5 mL) was added dropwise to stirred and cooled (−10 °C) ethanol (50 mL), followed by the addition of L-Ala (4.46 g, 50 mmol). The temperature of the solution was increased and maintained at 40 °C for 4 h. The solvent was removed and crystallization from methanol/ethyl ether yielded 4.65 g (86%) of white crystals melting at 76 °C (ref.[31] m.p. 76 °C). NMR ([D$_6$]DMSO): δ = 1.22 (t, J = 7.2 Hz, 3 H, CH_2CH_3), 1.40 (d, J = 7 Hz, 3 H, CH_3 Ala), 4.00 (q, J = 7.2 Hz, 2 H, CH_2CH_3), 4.17 (apparent q, J = 7.2 Hz, 1 H, αCH Ala), 8.66 (br. s, 3 H, NH_3^+) ppm.

Z-Gly-L-AlaOEt: Z-GlyOH (4.2 g, 20 mmol) was dissolved in ethyl acetate (50 mL) and DCC (4.3 g, 21 mmol) was added after cooling to 0 °C. The mixture was stirred for 30 min whereupon L-AlaOEt·HCl (3.1 g, 20 mmol) and triethylamine (2.8 mL, 20 mmol) were added. The reaction mixture was stirred at room temperature overnight. The insoluble material was filtered off and the solution was washed successively with 1 M NaOH, 1 M HCl, and saturated NaCl. After drying with $MgSO_4$ the solvents were removed under reduced pressure. The oil obtained was crystallized from ethyl acetate/petroleum ether. The white crystals were filtered and dried (4.52 g, 73%), m.p. 60−64 °C; (ref.[31] m.p. 60−62 °C). NMR ($CDCl_3$): δ = 1.28 (t, J = 8 Hz, 3 H, CH_2CH_3), 1.40 (d, J = 7 Hz, 3 H, CH_3 Ala), 3.89−3.92 (m, 2 H, CH_2Gly), 4.19 (q, J = 7.2 Hz,

2 H, CH_2CH_3), 4.52−4.60 (m, 1 H, αCH Ala), 5.13 (s, 2 H, CH_2Ar), 5.57 (br. s, 1 H, NH), 6.71 (br. s, 1 H, NH), 7.36−7.33 (m, 5 H, Ar) ppm.

HGly-L-AlaOEt·HBr: Z-Gly-L-AlaOEt (3.08 g, 10 mmol) was treated with 40% HBr/acetic acid (15 mL) at room temperature for 1 h with occasional stirring and protection from moisture. Diethyl ether was added and after cooling for a few hours the precipitated solid was filtered, washed with diethyl ether and dried. The chromatographically pure compound was used in the next step without further purification. Yield (2.42 g, 94%). NMR ([D_6]DMSO): δ = 1.20 (t, J = 7.2 Hz, 3 H, CH_2CH_3), 1.30 (d, J = 7.2 Hz, 3 H, CH_3 Ala), 3.57 (d, J = 3.3 Hz, 2 H, CH_2 Gly), 4.10 (q, J = 7.2 Hz, 2 H, CH_2CH_3), 4.23−4.32 (m, 1 H, αCH Ala), 8.01 (br. s, 3 H, NH_3^+), 8.70 (d, J = 7.2 Hz, 1 H, NH Ala) ppm.

HSalGly-L-AlaOEt: Salicylic acid (0.69 g, 5 mmol) was dissolved in ethyl acetate (20 mL) and the solution cooled to 0 °C. DCC (1.08 g, 5.2 mmol), HGlyAla·OEt·HBr (1.28 g, 5 mmol) and triethylamine (0.9 mL, 5 mmol) were then added successively. The reaction mixture was stirred at room temperature overnight. The insoluble material was filtered off and the solvents removed under reduced pressure. The residue was dissolved in a minimum amount of acetone and stored at 0 °C for 4 h. The precipitated solid was filtered off (dicyclohexylurea) and the solution concentrated. The oil obtained was crystallized from ethyl acetate/petroleum ether. (0.808 g, first crop, 55%, m.p. 104−106 °C). NMR ([D_6]DMSO): δ = 1.28 (d, J = 7.5 Hz, 3 H, CH_3 Ala), 1.17 (t, J = 7.2 Hz, 3 H, CH_2CH_3), 4.07 (q, J = 7.2 Hz, 2 H, CH_2CH_3), 3.94 (m, 2 H, CH_2 Gly), 4.20 (m, 1 H, αCH Ala), 6.91 (tap, J = 7.8 Hz, 2 H, 3-H and 5-H), 7.40 (tap, J = 8 Hz, 1 H, 4-H) 7.86 (dd, J = 1.2 Hz and 8 Hz,1 H, 6-H), 8.46,(d, J = 8.4 Hz, 1 H, NH Ala), 9.04 (t, J = 6 Hz, 1 H, NH Gly), 12.25 (br. s, 1 H, OH) ppm. $C_{14}H_{18}N_2O_5$ (294.3): calcd. C 57.14, H 6.16, N 9.52; found C 57.6, H 6.1, N 9.7.

H_2Sal-L-GlyAla (1): HSalGly-L-AlaOEt (0.734 g, 2.5 mmol) was dissolved in methanol (5 mL) and 1 M NaOH (5.5 mL, 5.5 mmol) was added. The mixture was stirred at room temperature for 4 h and 1 M HCl (2.5 mL, 2.5 mmol) was then added. The methanol was removed under reduced pressure and the solution thus obtained cooled in an ice bath and acidified with 1 M HCl (3 mL) with vigorous stirring. The white solid precipitated was filtered off, washed with water and dried (0.581 g, 87%), m.p. 197−199 °C. NMR ([D_6]DMSO): δ = 1.28 (d, J = 7.5 Hz, 3 H, CH_3 Ala), 3.94 (m, 2 H, CH_2 Gly), 4.20 (m, 1 H, αCH Ala), 6.91 (tap, J = 7.8 Hz, 2 H, 3-H and 5-H), 7.39 (tap, J = 7.8 Hz, 1 H, 4-H) 7.86 (dd, J = 1.2 Hz and 8 Hz, 1 H, 6-H), 8.34 (d, J = 7.5 Hz, 1 H, NH Ala), 9.03 (t, J = 6 Hz, 1 H, NH Gly), 12.20(s, 1 H, OH), 12.40 (br. s, 1 H, OH) ppm. $C_{12}H_{14}N_2O_5$ (266.3): calcd. C 54.13, H 5.30, N 10.52; found C 54.17, H 5.41, N 10.42.

Route 2

The dipeptide chain was elongated on an HMP resin (1.1 mmol/g). In the first step, Fmoc-Ala−OH (5.47, 17.6 mmol) was coupled to the resin (4 g, 4.4 mmol) in the presence of HOBt (2.38 g, 17.6 mmol), DCC (3.63 g, 17.6 mmol) and dimethylaminopyridine (1.07 g, 8.8 mmol). After removing the Fmoc protecting group by treatment with piperidine, the resultant free amino function was acylated with Fmoc−Gly−OH (2.97 g, 10 mmol). After completion of the dipeptide unit, the *N*-terminal Fmoc-protecting group was removed as previously described and the resultant primary amino group was acylated with salicylic acid (2.76 g, 20 mmol). The phenolic hydroxy group of the salicylic acid residue was unprotected. All the couplings were performed with *N*,*N*′-dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole. Amino acid or salicylic acid incorporation was monitored using the ninhydrin test. The completed peptide resins were treated with TFA/dichloromethane/water (35:60:5, vol/vol), at 0 °C for 1 h. The solvents were removed and the resultant free product was dissolved in 10% aqueous acetic acid, filtered and lyophilised (798 mg). The crude peptide derivative (100 mg) was purified by reverse-phase HPLC using a Nucleosil 7C-18 column (16 × 250 mm). The solvent system used was the following: 0.1% TFA in water, 0.1% TFA, 80% acetonitrile in water, gradient: 0% → 0% B over 10 min, then 10% → 30% over 50 min, flow 3.5 mL/min, detection at 226 nm. The appropriate fractions were combined and lyophilised. Peptide purity was above 97% (HPLC) and the measured Mw (227) value was in good agreement with the calculated one. NMR (D_2O): δ = 1.38 (d, J = 7.2 Hz, 9 Hz, 3 H, CH_3 Ala), 4.11 (s, 2 H, CH_2 Gly), 4.34 (m, 1 H, αCH Ala), 6.99 (tap, J = 7.8 Hz, 2 H, 3-H and 5-H), 7.46 (tap, J = 7.8 Hz, 1 H, 4-H) 7.50 (dd, J = 1.5 Hz, 2 and 9.46 Hz, 1 H, 6-H) ppm.

Synthesis of the Complexes

VO(SalGly-L-Ala)·3.2H_2O·0.2EtOH (2): To a solution of H_2SalGly-L-Ala (1 mmol) and sodium acetate−trihydrate (2 mmol) in ethanol/water (2.5:5 mL) was slowly added a solution of $VOSO_4$·5H_2O (1 mmol) in water (1 mL). The mixture was divided in two equal parts (**A** and **B**). The pH of solution **A** was adjusted to 5.5. A blue-green solid precipitated which was collected by filtration and washed with ethanol/water (1:2), diethyl ether and dried. Yield: 40% (80 mg). $C_{12}H_{12}N_2O_6V$·3.2H_2O·0.2EtOH: calcd. C 37.42, H 4.86, N 7.04; found C 37.4, H 5.3, N 7.00.

K[Cu(SalGly-L-Ala)]·3.2H_2O (3): To a solution of H_2SalGly-L-Ala (0.5 mmol) and KOH (0.5 mmol) in water (10 mL), was slowly added $Cu(CH_3COO)_2$·H_2O (0.5 mmol) in ethanol (5 mL). The pH was adjusted to 6 with 2 M KOH and kept at ≈ 45 °C for 1 h. A green precipitate was collected by filtration, washed with ethanol/water (1:2), ethanol and diethyl ether and dried. Yield: 42% (90 mg). $C_{12}H_{11}CuKN_2O_5$·3.2H_2O: calcd. C 34.03, H 4.14, N 6.61; found C 33.7, H 3.7, N 6.2.

Physical and Spectroscopic Studies

Melting points were recorded by using a Gallenkamp melting point apparatus and are uncorrected. NMR spectroscopic data were recorded either with a Varian Unity Plus 300 Spectrometer (for the several steps of the synthesis of **1**) in the solvent indicated, or with a Varian Unity-500 Spectrometer operating at frequencies of 131.404 MHz and 499.824 MHz, respectively, using a 5-mm broad-band probe at 25.0±0.5 °C. ^{51}V NMR chemical shifts were referenced to an external $VOCl_3$ solution at δ = 0 ppm. The ^{51}V NMR acquisition parameters were as follows: 33 kHz spectral width, 30-μs pulse width, 1 s acquisition time and 10 Hz line broadening. IR spectra were recorded with a BioRad FTS-3000 MX FTIR spectrometer. CD spectra were recorded with a JASCO-720 spectropolarimeter with a red-sensitive photomultiplier (EXWL-308). Visible spectra were recorded with a Hitachi U-2000 spectrophotometer. X-band (9.44 GHz) EPR spectra were usually recorded at 77 K (on glasses made by freezing solutions in liquid nitrogen) with a Bruker ESP 300E X-band spectrometer. Mass spectra were recorded with a Finnigan TSQ-7000 tandem quadrupole mass spectrometer equipped with an electrospray ion source. The HPLC apparatus was made by Knauer (Berlin, Germany).

All samples for the pH-potentiometric and spectrophotometric measurements were prepared under inert conditions (under high purity nitrogen or argon). For potentiometric measurements the

ionic strength was adjusted to 0.2 M KCl and the temperature maintained at 25.0±0.1 °C. For the spectral measurements the temperature was maintained at 25.0±0.3 °C with circulating water.

pH Measurements for Spectroscopic Studies: For preparation of the solutions and pH calibrations a special double-walled glass vessel was used with entrances for the combined electrode (Radiometer ''Red Rod'' pHC2015−8), thermometer, nitrogen and reagents (e.g. base). A computerized system developed locally (IBM-PCXT 286 computer) was used to control the titration conditions for pH calibrations. The E.M.F measurements were made with a Denver Model 15 pH meter.

pH-Potentiometric Titrations: Stock solutions of $V^{IV}O$ and vanadate(V) for the pH-metric titrations were prepared and standardised as described earlier.[32] The purity of the ligand was checked and the exact concentration of its solutions was determined by the appropriate Gran plot.[33] The stability constants of the proton and metal complexes of the ligand were determined by pH-metric titration of 10.0 cm^3 samples. The ligand concentrations were in the range of 0.001 to 0.002 M and the metal ion to ligand molar ratio varied from 1:1 to 1:3. Titrations were performed from pH 2.0 up to pH 11 with KOH solution of known concentration (ca. 0.2 M) under purified argon unless very extensive hydrolysis or slow equilibration was observed. The reproducibility of titration points included in the evaluation was within 0.005 pH units in the whole pH range. The pH was measured with an Orion-710A precision digital pH meter equipped with an Orion Ross 8103BN type combined glass electrode, calibrated for hydrogen ion concentration as described earlier.[34] The ionic product of water, pK_w is 13.76. The concentration stability constants $\beta_{pqr} = [M_pL_qH_r] / [M]^p[L]^q[H]^r$ were calculated with the aid of the PSEQUAD computer program.[35] The formation of the hydroxo complexes of $V^{IV}O$ was taken into account. The following species were assumed: $[VO^{IV}(OH)]^+$ ($\log \beta_{10-1} = -5.94$), $[(V^{IV}O)_2(OH)_2]^{2+}$ ($\log \beta_{20-2} = -6.95$). Stability constants were calculated from the data of Henry et al.[36] and corrected for the different ionic strengths by using the Davies equation. $[(V^{IV}O)_2(OH)_5]^-$ ($\log \beta_{20-5} = -22.5$) and $[V^{IV}O(OH)_3]^-$ ($\log \beta_{10-3} = -18.0$).[37]

Spectroscopic Measurements: Unless otherwise stated, by visible (Vis) and circular dichroism (CD) spectra we mean a representation of ε_m or $\Delta\varepsilon_m$ values vs. λ [ε_m = absorption/(bc_M) and $\Delta\varepsilon_m$ = differential absorption/(bc_M) where b = optical path and c_M=total $V^{IV}O$ or Cu^{II} concentration]. The spectral range covered is normally 400−900 (Vis) and 400−1000 nm (CD). All measurements and operations of the spectropolarimeter were computer controlled. For the CD spectra a Fast Fourier-Transform noise-reduction routine (JASCO) was used without affecting peak shapes. For the $V^{IV}O$ system, the EPR, Vis and CD spectra were recorded by varying the pH with an approximately fixed total vanadium and ligand concentration, at L/M ratios of 1.76 ($c_{VO} \approx 0.003$ M) and 2.00 ($c_{VO} \approx 0.0045$ M), and at pH = 5.5 and $c_{ligand} \approx 0.010$ mM . L/M ratios were varied in the range of 20−1 by addition of $V^{IV}O$ stock solution. For the Cu^{II} system, the EPR, Vis and CD spectra were recorded varying the pH with $c_{Cu} \approx 0.006$ M and L/M = 2.

CD spectra of Solid Complexes: Samples of compounds **2** and **3** were prepared as described previously,[38] dispersed in KBr disks, and placed between two microscope slides. One or two such paired microscope slides were placed in the sample compartment. Each final spectrum is the average of 4 to 6 individual spectra, recorded as described above.

Supporting Information: Additional EPR, CD and ^{51}V NMR spectra for the $V^{IV}O$−SalGly-L-Ala and $V^{V}O_2$−SalGly-L-Ala systems are available (see also the footnote on the first page of this article).

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