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The coordinating properties of *d*-biotin

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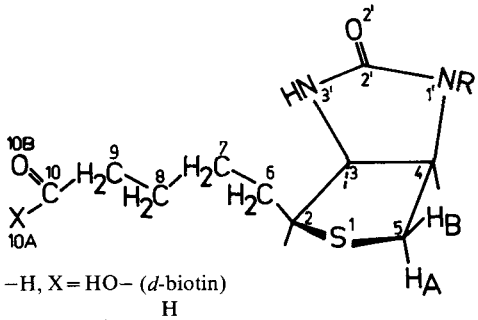
Summary. The coenzyme *d*-biotin offers in its anionic form to metal ions 3 possible binding sites: the carboxylate group of the valerate side chain, the ureido residue of the 2-imidazolidone ring, and the thioether sulfur of the tetrahydrothiophene ring; the coordinating properties of these groups are summarized and compared. Hydrogen bond formation of the ureido group has also been observed, and hydrogen bonding may possibly be important in biotin-bicarbonate recognition. The aliphatic part of the valeric acid side chain can undergo hydrophobic interactions. Such interactions and/or the stereoselective sulfur-metal ion coordination could be the means for a correct 'fixation' of the biotinyl moiety at the surface of a protein, thus creating the active enzyme-substrate complex.

Vitamin H or *d*-biotin is widely distributed in plant and animal tissues where it functions as cofactor in a variety of enzymic carbon dioxide fixation reactions[1-3]. This coenzyme is involved in vital processes which include fatty acid synthesis, glyconeogenesis, and amino acid metabolism. Its structure (**1**) has been determined by X-ray diffraction[4]: the tetrahydrothiophene ring is envelope-shaped with the sulfur atom 0.87 Å out of the plane given by the positions of the 4 carbon atoms[4], i.e. this ring puckers with the sulfur endo to the ureido ring (rather than exo)[5].



1 R = –H, X = HO– (*d*-biotin)

2 R = –H, X = $^{-}$OOC–C(H)($^{+}$NH$_3$)–(CH$_2$)$_4$–NH– (*d*-biocytin)

3 R = –COO^{-}, X = protein (CO_2 intermediate)

The carboxyl group of the valeric acid side chain of the coenzyme is covalently attached to the apoenzyme via an amide linkage with the N^{ε}-lysyl residue as in biocytin (**2**)[6]. The coenzyme functions in carboxylation, decarboxylation and transcarboxylation reactions[1-3]. The following 2-step reaction pathway is now generally accepted for carboxylases[1,7-9]:

$$\text{enzyme-biotin} + HCO_3^- + ATP \rightleftharpoons \text{enzyme-biotin-}CO_2 + ADP + \text{phosphate} \quad (1)$$

$$\text{enzyme-biotin-}CO_2 + \text{acceptor} \rightleftharpoons \text{acceptor-}CO_2 + \text{enzyme-biotin} \quad (2)$$

Strong evidence has been provided[7,10] that the enzyme-biotin-CO_2 intermediate is a species of the general structure **3**. There are indications[7] that reactions 1 and 2 occur at distinct sites on different subunits of the enzyme, and an analogous mechanism has also been proposed for biotin-dependent transcarboxylases[11]. In fact, it now seems to be generally accepted that all biotin-enzyme reactions involve 2 such basic steps during which a carboxybiotinyl intermediate forms at one site and translocates to a 2nd (distinct) site for CO_2 transfer[3].

The reactivity of such systems is very sensitive to structural alterations at the biotinyl moiety. An increase or decrease of the length of the side chain by 1 carbon, as in homobiotin or norbiotin, respectively, results with the free biotin derivatives in a 90% decrease in the carboxylation rate and in a 50% reduction in the phosphoryl transfer rate[7]. With *dl*-O-heterobiotin (oxybiotin) or *dl*-dethiobiotin (S substituted by 2 H) the carboxylation rate decreases by about 80 and 90%, respectively, while the phosphoryl transfer reaction is reduced by about 60%[7]. That both the thioether and the ureido groups are of importance during the enzymic reaction has also been shown by determining the carboxylation rates of free biotin as compared to the free biotin derivatives[12]: it turned out that dethiobiotin and a biotin without the carbonyl group in the ureido ring are inactive.

The sulfur atom is also essential for complete metabolism of this vitamin[13], however, dethiobiotin can be converted into biotin in *Aspergillus niger*[14], as well as in other fungi and bacteria. During this process, only the abstraction of some hydrogen occurs, so that the nearly intact dethiobiotin molecule is converted into biotin[14,15]. Hence, dethiobiotin can be a precursor for biotin, at least in these microorganisms, but it cannot

take over the co-enzymic role. All this suggests that the sulfur plays an important role[5] in the enzymic process.

So far, it is known that all biotin enzymes need divalent metal ions for activity. Especially reaction 1 of the carboxylases is clearly metal ion dependent[2,7]. This is not surprising as ATP, which is also involved, has a significant affinity towards metal ions[16], and possibly an O-phosphobiotin species is an enzymic intermediate[17]. Mn^{2+} is required in a pyruvate carboxylase[2] while a transcarboxylase contains tightly bound Zn^{2+} and Co^{2+}[18]; in fact, this enzyme also contains tightly bound Cu^{2+} and hence, a total of 12 metal ions per mole with a metal ion/biotin ratio of 2 (Fung et al.[19]). Metal ions, especially zinc and iron, are also essential for the biosynthesis of biotin[20].

The basic coordinating properties of *d*-biotin appear, therefore, to be of general interest[6]. From structure **1** it is evident that this molecule has 3 possible binding sites for metal ions: the carboxylate group, the ureido residue and the thioether sulfur. The coordination tendencies of these 3 groups of biotin will be summarized here and discussed in relation to the points outlined in the preceding introductory paragraphs. Appropriate hydrogen-bond formation and especially hydrophobic interactions will also be considered.

1. Carboxylate group: Basicity and formation of metal ion complexes

The acidity constants at I = 0.1 ($NaClO_4$) and 25 °C of the carboxylic acid group[21] (eq. 3)

$$HL \rightleftharpoons H^+ + L^- \quad K^H_{HL} = [HL]/([H][L]) \qquad (3)$$

in water containing 50% dioxane (v/v) show for *d*-biotin $pK^H_{HL} = 6.22$), its *d*- and *l*-sulfoxide (6.05) and sulfone (6.10) derivatives, and for *dl*-dethiobiotin (6.35) that the removal of the sulfur atom results in a slight increase in the basicity of the carboxylate group, while the oxidation of sulfur leads to a slight decrease. The results for the side-chain-modified derivatives of *d*-biotin and *dl*-dethiobiotin lead to 2 series with decreasing carboxylate basicity: *d*-homobiotin ($pK^H_{HL} = 6.29$) > *d*-biotin (6.22) > *d*-bisnorbiotin (5.86) ≫ *d*-tetranorbiotin (4.85), and *dl*-dethiobiotin (6.35) > *dl*-bisnordethiobiotin (6.11) ≫ *dl*-(and *d*-) tetranordethiobiotin (5.48). Obviously, the longer the side chain, the more basic the carboxylate group becomes. This agrees with the simple carboxylates, where the basicity decreases in the series: valerate ($pK^H_{HL} = 6.44$)[21] > propionate (6.29)[22] > acetate (6.01)[22] ≫ formate (4.75)[22]. A comparison of the acidity constants of *d*-tetranorbiotin (4.85),[21] *d*-tetranordethiobiotin (5.47),[21] and tetrahydrothiophene-2-carboxylic acid (5.58)[22b] shows that both groups, the ureido part and the thioether sulfur, decrease the basicity of the carboxylate group.

The stability of the binary 1:1 complexes (eq. 4)

$$M^{2+} + L^- \rightleftharpoons ML^+ \quad K^M_{ML} = [ML]/([M][L]) \qquad (4)$$

of Mn^{2+}, Cu^{2+}, Zn^{2+} (Sigel et al.[21]), Cd^{2+} or Pb^{2+} (Sigel et al.[23]) with *d*-biotin and its mentioned derivatives is solely governed by the basicity of the side-chain-carboxylate group. An example of the plots on which this conclusion is based[24] is given in the upper part of figure 1. For example, all values of the Cu^{2+} complexes (fig. 1, upper part) are within the accuracy of measurements on the straight

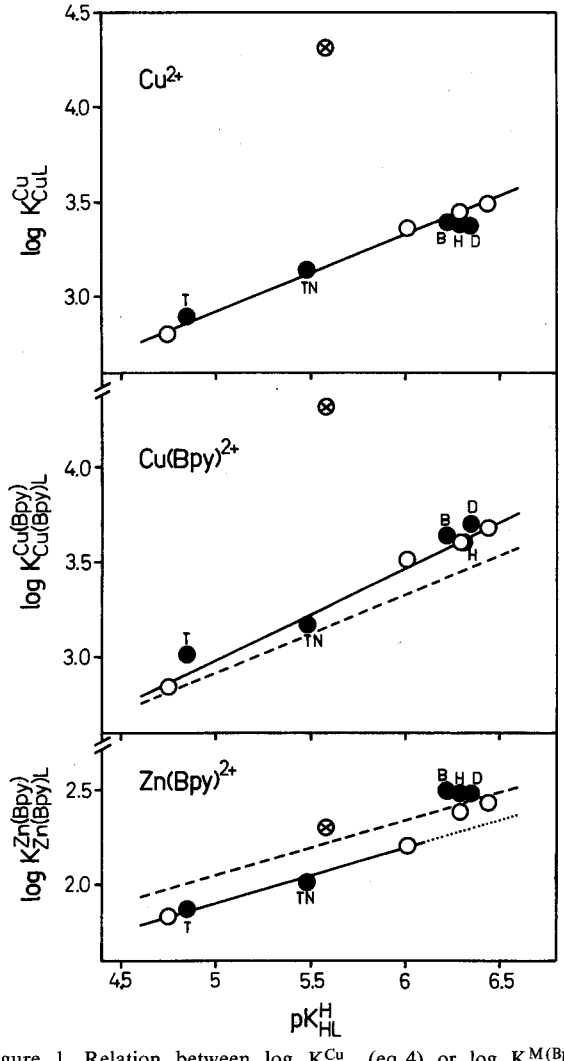


Figure 1. Relation between log K^{Cu}_{CuL} (eq. 4) or log $K^{M(Bpy)}_{M(Bpy)L}$ (eq. 5) and pK^H_{HL} (eq. 3) for the binary CuL^+ or the ternary $Cu(Bpy)L^+$ and $Zn(Bpy)L^+$ complexes of *d*-biotin (B), *dl*-dethiobiotin (D), *d*-homobiotin (H), *d*-tetranorbiotin (T) and *dl*-tetranordethiobiotin (TN) (●) or tetrahydrothiophene-2-carboxylate (⊗). The solidly drawn reference lines are due to the corresponding binary and ternary complexes formed with the monodentate carboxylates (○) (from left to right): formate, acetate, propionate, and valerate; for $Zn(Bpy)L^+$ the reference line is only drawn through the data of formate and acetate (solid part) and then tentatively extended (dotted part; cf. section 4). The broken lines refer to the binary 1:1 complexes and are given for comparison with the corresponding ternary $M(Bpy)L^+$ systems. All the plotted data (I = 0.1, $NaClO_4$; 25 °C; 50% aqueous dioxane) are taken from tables 1 and 2 of ref. 21.

'reference line' given by the simple Cu(carboxylate)$^+$ complexes. Only the Cu^{2+} complex of tetrahydrothiophene-2-carboxylate ($Thtc^-$) is about 1.16 log units more stable than expected; this shows that a chelate is formed which involves the carboxylate group and the thioether sulfur (see sections 3 and 5). Surprisingly, no increase in stability is found with *d*-tetranorbiotin, since this ligand seems, at first sight, to be similar to $Thtc^-$, differing only in the presence of the ureido bridge (see section 3.1).

For the ternary complexes formed by Cu^{2+} or Zn^{2+} and 2,2′-bipyridyl and *d*-biotinate or one of its derivatives, one obtains, as a first approximation, the same picture as that described for the binary systems, if reaction 5 is considered.

$$M(Bpy)^{2+} + L^- \rightleftharpoons M(Bpy)L^+$$

$$K^{M(Bpy)}_{M(Bpy)L} = [M(Bpy)L]/([M(Bpy)][L]) \qquad (5)$$

The data of the mixed ligand complexes with the simple carboxylates furnish the 'reference line' on which the values of most of the other systems do approximately fit, indicating again that the stability of these complexes is largely determined by the basicity of the carboxylate groups (fig. 1, middle and lower parts). There is the indication of a slight stability increase, especially for some Zn^{2+} complexes, which will be discussed in section 4, but only Cu(Bpy)$(Thtc)^+$ and Zn(Bpy)$(Thtc)^+$ are much more stable than expected on the carboxylate-basicity of $Thtc^-$; this, again, is due to the participation of the thioether group in complex formation (see sections 3 and 5).

The fact that the 'reference line' of the ternary Cu^{2+} complexes is somewhat above the line of the binary complexes, while in case of the Zn^{2+} systems it is somewhat below, is in accordance with the general experience[25-27]. In the absence of steric restrictions one obtains for the combination of a heteroaromatic N base and an O donor ligand with Cu^{2+} a *positive* value for $\Delta\log K_{Cu}$ (eq. 6), i.e. the mixed ligand complex is somewhat more stable than the binary one:

$$\Delta\log K_M = \log K^{M(Bpy)}_{M(Bpy)L} - \log K^M_{ML} = \log K^{ML}_{ML(Bpy)} - \log K^M_{M(Bpy)} \qquad (6)$$

The same ligand combination leads with Zn^{2+} and other metal ions of the second half of the 3rd series also to an enhanced, but usually less pronounced, stability[25,27], compared with the statistical expectation[28].

The acidity constant of *d*-biotin (B) and the stability constants of the corresponding binary complexes with Cu^{2+} and Zn^{2+} are smaller in water than in 50% aqueous dioxane (i.e. 0.174 mole fractions of dioxane) ($I = 0.1$; 25 °C) by about 1.7 log units. However, the constants of the simple carboxylic acids, like valeric acid and acetic acid (HAc), are influenced correspondingly:

$$pK^H_{H(B)} = 4.51 \pm 0.02;\ \log K^{Cu}_{Cu(B)} = 1.63 \pm 0.05;\ \log K^{Zn}_{Zn(B)} = 0.82 \pm 0.10 \text{ (Sigel et al.}^{21}\text{)}$$

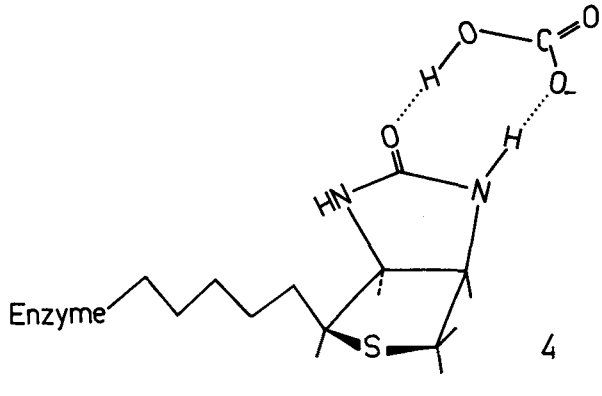
$$pK^H_{H(Ac)} = 4.54 \pm 0.01;\ \log K^{Cu}_{Cu(Ac)} = 1.81 \pm 0.02;\ \log K^{Zn}_{Zn(Ac)} = 0.86 \pm 0.06 \text{ (Banerjea et al.}^{25d}\text{)}$$

The stability of the biotin complexes in water is, again, determined by the carboxylate basicity. The same is true for *d*-biocytin (structure **2**) where complex stability is governed by the coordinating properties of the terminal amino acid residue[29].

To conclude, the dominance of the metal ion binding properties of the biotin carboxylate group (or the glycinate-like biocytin amino acid residue) does not mean that the ureido group and the thioether sulfur have no coordinating capacity at all, it only means that the nature of this capacity is not apparent under the described conditions. Moreover, it must be noted that a metal ion-carboxylate coordination could play a role only during the biosynthesis and metabolism of biotin, but not during its enzymic action because then the biotinyl moiety is amide linked to the enzyme and therefore no longer available for a metal ion coordination.

2. Ureido moiety: Hydrogen bond formation and metal ion interaction

For the formation (eq. 2) of the enzyme-biotin-CO_2 intermediate (structure **3**) some activation seems to be necessary to enhance the nucleophilicity of the involved nitrogen (N-1′)[1,8,30]: for example, through polarization of the carbonyl double bond of the urea part by the formation of a hydrogen bond or, at the extreme, protonation of the oxygen (O-2′). Indeed, for the biotin-model ethylene urea (2-imidazolidone) and phenol it has been shown by IR spectral measurements that in chloroform an intermolecular hydrogen bond is formed[31]. From the analysis of the hydrogen bonding observed in the crystal structure determinations of biotin[4] and its derivatives[3,5], a biotin-bicarbonate recognition via hydrogen bonding, as indicated in structure **4**, has been proposed[32]. This hydrogen



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bonded complex is probably relatively stable and the approach of ATP is suggested[32] to trigger the carboxylation at N-1′.

It has also been suggested[33] that *d*-biotin may be able to form an intramolecular hydrogen bond in solution between HO-10A and O-2′. This should result in a lower acidity of *d*-biotin compared, e.g., with valeric acid, but the opposite is observed: valeric acid is by a factor of about 0.5 less acidic than *d*-biotin, both in water and in 50% aqueous dioxane[21]. Thus it is unlikely that a hydrogen bond between HO-10A and O-2′ is formed; based on an NMR study the same conclusion was reached with dimethyl sulfoxide as a solvent[34].

This result, together with the observation[21] that the amide nitrogens of the ureido ring are not deprotonated in the physiological pH range, is meaningful, because it is improbable that a metal ion is much more effective than a proton in forming a macrochelate between the carbonyl (O-2′) and the carboxylate group[35]. This assumption is supported by the fact that the stabilities of the *d*-biotin and *dl*-dethiobiotin complexes are determined by the basicity of the carboxylate groups of the ligands (fig. 1, upper part). This is even true for the Mn^{2+}, Cu^{2+} and Zn^{2+} complexes of *d*-tetranorbiotin and *dl*-tetranordethiobiotin, which would be most favored for such an interaction because here 6-membered chelates could be formed. In conclusion, from the stability data[21] in 50% aqueous dioxane (or water) no indication for a metal ion/ureido interaction can be obtained.

However, NMR-line-broadening studies with Mn^{2+} or Cu^{2+} in dimethyl sulfoxide as a solvent indicate a metal ion/ureido group interaction[31]. Whether coordination to the ureido group occurs through the carbonyl oxygen or one of the nitrogens is not clear; but, since metal ions coordinate to the amide group, $-CONH-$, quite generally through the oxygen atom[35,36], it seems reasonable to assume that O coordination is also preferred in this case. In solid complexes of ethyleneurea and other urea derivatives metal ions also coordinate preferentially via the oxygen[37].

An indirect metal ion/urea group interaction has been suggested via the carbon dioxide residue of the enzyme-biotin-CO_2 intermediate (structure **3**) for metallobiotin enzymes[2,30].

3. *Thioether-sulfur coordination*

3.1. The metal ion interaction is stereoselective. The first indication for a biotin-sulfur/metal ion interaction has been obtained[21] from ^{1}H-NMR line-broadening experiments with paramagnetic metal ions[38]: the spectra of *d*-biotin in D_2O with increasing amounts of Mn^{2+} are reproduced in figure 2. As expected from section 1, the signals due to the protons of the methy-

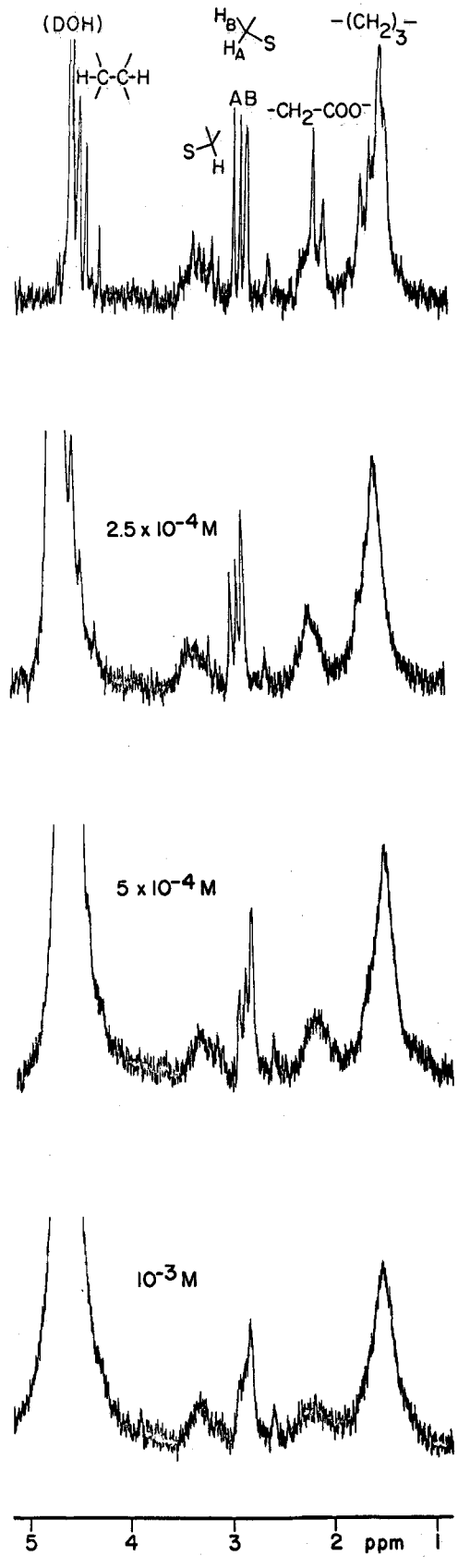
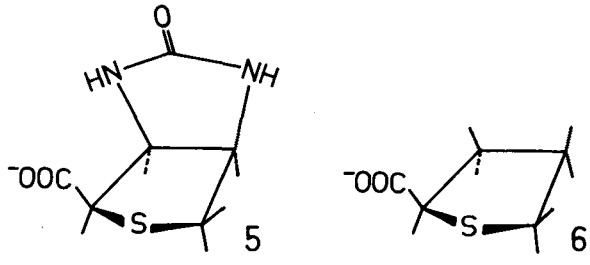


Figure 2. ^{1}H-NMR spectra of 0.12 M *d*-biotinate alone (top) and with increasing concentrations of $Mn(ClO_4)_2$ in D_2O at 37 °C and pD ~ 7.8 (reproduced by permission of the American Chemical Society, from ref. 21).

lene group neighboring the carboxylate group (structure **1**) are broadened very strongly. However, the hydrogens on C-5′ next to the sulfur are influenced too, but in an asymmetric way: the signals of H_A are more strongly broadened than of H_B. The same was observed with *d*-biotin/Cu^{2+} (Sigel et al.[21]) and *d*-biocytin/Mn^{2+} or Cu^{2+} (Griesser et al.[29]). Since the line width is dependent upon the distance of the metal ion this means that the metal ion is closer to H_A than to H_B; hence, Mn^{2+} and Cu^{2+} coordinate to the sulfur mainly from below the tetrahydrothiophene ring, i.e. *'trans'* to the ureido moiety.

This stereoselective sulfur coordination allows us now to understand the differing coordinating properties of *d*-tetranorbiotinate (structure **5**) and tetrahydrothiophene-2-carboxylate (**6**), which also further support



the described stereoselectivity. Tetrahydrothiophene-2-carboxylate forms, e.g., with Cu^{2+} or Zn^{2+} (fig. 1; sections 1 and 5) 5-membered chelates by coordinating via the carboxylate group and the thioether-sulfur. At first one would expect the same for tetranorbiotinate, but the stability of its complexes gives no indication for a chelate formation (fig. 1). This is understandable since in *d*-tetranorbiotin the carboxylate side chain points away from the side which is open for a sulfur interaction. Removal of the ureido bridge from tetranorbiotinate (**5**) results in tetrahydrothiophene-2-carboxylate (**6**) in which no steric restriction exists and therefore chelate formation occurs.

Considering the structure of *d*-biotin (**1**) the source of the stereoselective properties becomes apparent. The (C-2)-S-(C-5) angle is 89.4° (De Titta et al.[4]) and electron density maps[39] of sulfides show the electron cloud to be normal to the thioether plane[5]; this together with the distances between (C-6) ... (N-3′) and S ... (C-2′) of only 2.86 and 3.68 Å, respectively, show that the space between the ureido and the tetrahydrothiophene ring is very 'crowded'. It would be difficult to fit a partly hydrated metal ion 'between' the two 5-membered rings. Hence, it is understandable that the metal ion/sulfur coordination occurs *'trans'* to the ureido moiety; a conclusion which is further confirmed by the results given in section 3.2.

That the side below the tetrahydrothiophene ring is more open may also be seen from the kinetic preference[40] for the formation of the *d*-sulfoxide of *d*-biotin[40-42]. However, protonation of the biotin-sulfur at −60 °C in 'magic acid' (fluorosulfonic acid/antimony pentafluoride/sulfur dioxide)[43] occurs *'cis'* to the valeric acid side chain and the urea ring[44,45]; hence, the orientation of the proton differs from that of metal ions which coordinate in a *'trans'* fashion.

3.2. Stability of metal ion thioether complexes with biologically important metal ions is expected to be small. The determination of the stability constants of such weak complexes recently became possible by employing with diamagnetic metal ions ^{1}H-NMR shift measurements[47], while spectrophotometric studies[48] allow the determination of the constants for metal ion-thioether complexes in general[49].

The stability constants given in table 1 were determined[50] under conditions where the carboxylic acid group of *d*-biotin remained protonated (see section 1)[45] to prevent a carboxylate-metal ion interaction. As the neutral biotin molecule is rather insoluble in water, for the spectrophotometric experiments organic solvents had to be used. ^{1}H-NMR shift measurements could also be done in D_2O using Fourier transformation[50]. From the constants (eq. 7/table 1)

$$M^{n+} + L \rightleftharpoons ML^{n+} \qquad K^M_{ML} = [ML]/([M][L]) \qquad (7)$$

it is evident that the *d*-biotin and tetrahydrothiophene complexes are of similar, though not identical, stability (vide infra). The stabilities with the biologically meaningful metal ions Mn^{2+} and Zn^{2+} are low, but complexes are still formed. The thioether com-

Table 1. Thioether-metal ion complexes: logarithms of the stability constants of M^{n+} 1:1 complexes (eq. 7) with *d*-biotin (B) or tetrahydrothiophene (Tht) in several solvents[50]

M^{n+}	log $K^M_{M(B)}$	log $K^M_{M(Tht)}$	Solvent
Mn^{2+}	[a]	0.0 ±0.2	96% DMF (0.85)[b]; [$HClO_4$] = 1 mM; I = 1.0, $NaClO_4$; 25 °C[c]
Cu^{2+}	0.03 ± 0.11	0.16 ± 0.07	96% DMF (0.85)[b]; [$HClO_4$] = 1 mM; I = 1.0, $NaClO_4$; 25 °C[d]
Zn^{2+}	[a]	0.2 ±0.15	96% DMF (0.85)[b]; [$HClO_4$] = 1 mM; I = 1.0, $NaClO_4$; 25 °C[c]
Ag^+	1.64 ± 0.13	2.09 ± 0.05	96% DMF (0.85)[b]; [HNO_3] = 0.01 M; I = 1.0, $NaNO_3$; 25 °C[c]
Ag^+	1.45 ± 0.07	1.63 ± 0.03	~99% d_6-DMSO (0.94)[b]; [DNO_3] = 0.01 M; I = 0.5, $NaNO_3$; 34 °C[e]
Ag^+	4.20 ± 0.08	4.74 ± 0.14	D_2O; pD ~2; I = 0.5, $NaNO_3$; 27 °C[e]

[a] There is clear evidence that complex formation occurs but the errors were too large to calculate a reliable constant. [b] Mole fraction of the organic part of the (aqueous) solvent mixture: DMF, dimethylformamide; DMSO, dimethylsulfoxide. [c] Determined by spectrophotometric 'competition' experiments. [d] From direct spectrophotometric measurements. [e] From ^{1}H-NMR shift measurements.

plexes with the 'soft' Ag^+ are much more stable; a result which is expected[47,48], and in accord with the observation that Pd^{2+} and Pt^{2+} coordinate in the solid state and in solution exclusively through the sulfur to *d*-biotin[51].

From section 3.1 follows that the thioether coordination of *d*-biotin is stereoselective and that the sulfur is accessible for metal ions only from the side *'trans'* to the ureido group and the valeric acid side chain, while the sulfur of tetrahydrothiophene (Tht) is accessible from *two* sides; hence, there is a statistical factor of 2 involved. As, aside from the steric restrictions, the neighborhood of the S atom in the 2 ligands is practically the same, the $M(biotin)^{n+}$ complexes should be less stable by 0.3 log units than the $M(Tht)^{n+}$ complexes. Indeed, calculation of the differences in stability between comparable $M(biotin)^{n+}$ and $M(Tht)^{n+}$ complexes, i.e. of log $K^M_{M(Tht)}$ – log $K^M_{M(B)}$, give in average 0.33±0.10 log units from the data of table 1. Hence, this result[50] confirms the stereoselective M^{n+}/sulfur interaction in biotin as outlined in section 3.1.

The stability constants for the thioether coordination in several $M(biotin)^{2+}$ complexes in D_2O are given in table 2. From the data for $M(dimethylsulfide)^{2+}$ in H_2O it is evident that they are of comparable stability. Of further interest are the results listed for $M(Tht)^{2+}$ in 50% aqueous ethanol. Despite the fact that they are favored by a factor of 2, compared to $M(biotin)^{2+}$ complexes, it is evident from the listed stability constants that the relatively slight decrease in the activity of water by going from water as solvent to 50% aqueous ethanol (i.e. 0.24 mole fractions of ethanol) promotes the M^{2+}/thioether interaction with 'hard' metal ions very clearly. This is of interest for biological systems, because one may expect that at the surface of a protein the activity of water is reduced, and hence a metal ion/thioether interaction should be favored.

4. Hydrophobic interactions in mixed ligand complexes

It is now well established that hydrophobic interactions between aromatic and aliphatic groups[52] may be promoted by the formation of a metal ion bridge between these groups, provided that suitable metal binding sites are also available[53–55]. For the present it should be pointed out that a hydrophobic interaction has been shown to occur by ^{1}H-NMR shift measurements[54] in the ternary complexes formed between $Zn(Bpy)^{2+}$ or $Zn(Phen)^{2+}$ and propionate, butyrate or valerate; this intramolecular ligand-ligand interaction was also confirmed by the stability data obtained for the complexes of $Zn(Phen)^{2+}$ or $Cu(Phen)^{2+}$ and valerate[56].

From the lower part of figure 1 it is evident that the ternary $Zn(Bpy)^{2+}$ complexes with propionate and valerate deviate clearly due to an enhanced stability from the 'reference line'. The same is observed for the ternary complexes with *d*-biotinate and several of its derivatives. In the light of the previous results[53–56] this is clear evidence that the enhanced stability of these ternary complexes results from an intramolecular hydrophobic ligand-ligand interaction as is indicated in figure 3.

For the corresponding ternary Cu^{2+} complexes (fig. 1, middle part) a stability enhancement is less clear from the plotted data, but that the 'reference line' of the ternary complexes has an increased slope is quite evident. That the ternary Cu^{2+} and Zn^{2+} complexes have somewhat different properties is to be expected because Cu^{2+} has a square-planar (or strongly distorted octahedral), coordination sphere, while that of

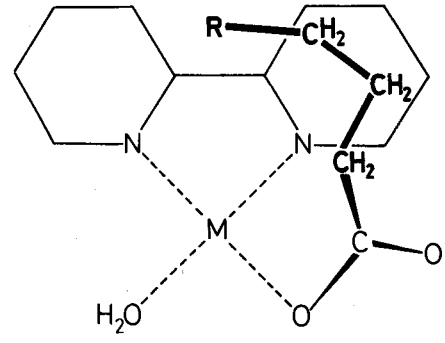


Figure 3. Tentative and simplified structure of the 'closed' isomer with the hydrophobic ligand-ligand interaction for the ternary complex of $M(Bpy)^{2+}$ and an aliphatic carboxylate ligand.

Table 2. Thioether-metal ion complexes: logarithms of the stability constants of M^{2+} 1:1 complexes (eq. 7) with *d*-biotin (B) in D_2O[a], dimethylsulfide (Dms) in H_2O[b], or tetrahydrothiophene (Tht) in 50% aqueous ethanol[c]

M^{2+}	log $K^M_{M(Dms)}$[b]	log $K^M_{M(B)}$[a]	log $K^M_{M(Tht)}$[c]
Mg^{2+}		−1.0 (+0.3/−0.6)[d]	−0.27±0.10 (cf. ref. 50)
Ca^{2+}	−1.6 (+0.2/−0.5)[d]	−1.4 (+0.3/−0.6)[d]	−0.30±0.13 (cf. ref. 48)
Cu^{2+}			0.02±0.04 (cf. ref. 48)
Zn^{2+}	−1.4 (+0.2/−0.5)[d]	−1.2 (+0.3/−0.6)[d]	−0.21±0.09 (cf. ref. 48)
Cd^{2+}	−0.3 ±0.2	−0.9 (+0.3/−0.6)[d]	−0.26±0.06 (cf. ref. 47)
Pb^{2+}	−1.05 ±0.2		0.08±0.05 (cf. ref. 47)

[a] From ref. 50: pD ~2; I=2–5, $NaClO_4$; 27 °C. [b] From ref. 47: pH ~2; I=2–4.5, $NaClO_4$ (with Pb^{2+} $NaNO_3$ was used); 34 °C. [c] From ref. 47, 48 and 50: 50% ethanol correspond to 0.24 mole fraction of ethanol; pH ~3, I=1.0, $NaClO_4$ (with Cd^{2+} and Pb^{2+} $NaNO_3$ was used); 25 °C. [d] Estimated values; the error range is given in parenthesis[47,50].

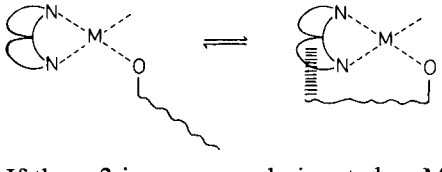
Table 3. Evidence for intramolecular hydrophobic ligand-ligand interactions in ternary M(A)(B) complexes containing a heteroaromatic N ligand (A) and a second ligand (B) with an aliphatic moiety. Estimations of the intramolecular dimensionless equilibrium constant K_I of the ternary complexes and of the percentage of the 'closed' isomer for the same systems (I = 0.1; 25 °C)[a]

No.[a]	M(A)(B)[a]	$\Delta \log K_{M/exp}$	$\Delta \log K_{M/op}$	$\Delta\Delta \log K_M$[b]	K_I (eq. 9,10)	% M(A)(B)$_{cl}$ (eq. 8)
1	Zn(Bpy)(HCOO)$^+$	−0.14	−0.12			
2	Zn(Bpy)(Ac)$^+$	−0.11				
3	Zn(Bpy)(Val)$^+$	−0.04	−0.12	0.08	0.20	17[c]
4	Zn(Bpy)(*d*-B)$^+$	0.04	−0.12	0.16	0.45	31
5	Zn(Bpy)(*d*-H)$^+$	0.05	−0.12	0.17	0.48	32
6	Zn(Bpy)(*dl*-D)$^+$	0.03	−0.12	0.15	0.41	29
7	Cu(Bpy)(HCOO)$^+$	0.04	0.10			
8	Cu(Bpy)(Ac)$^+$	0.15				
9	Cu(Bpy)(Val)$^+$	0.19	0.10	0.09	0.23	19
10	Cu(Bpy)(*d*-B)$^+$	0.25	0.10	0.15	0.41	29
11	Cu(Bpy)(*d*-H)$^+$	0.22	0.10	0.12	0.32	24
12	Cu(Bpy)(*dl*-D)$^+$	0.33	0.10	0.23	0.70	41
13	Zn(Phen)(HCOO)$^+$	−0.14	−0.15			
14	Zn(Phen)(Ac)$^+$	−0.16				
15	Zn(Phen)(Val)$^+$	−0.02	−0.15	0.13	0.35	26[c]
16	Zn(Phen)(Chac)$^+$	0.06	−0.15	0.21	0.62	38
17	Cu(Phen)(HCOO)$^+$	0.06	0.05			
18	Cu(Phen)(Ac)$^+$	0.04				
19	Cu(Phen)(Val)$^+$	0.17	0.05	0.12	0.32	24
20	Cu(Phen)(Chac)$^+$	0.26	0.05	0.21	0.62	38
21	Cu(Bpy)(Gly)$^+$	−0.35	−0.30			
22	Cu(Bpy)(Ala)$^+$	−0.26				
23	Cu(Bpy)(N$^\varepsilon$-Al)$^+$	−0.20	−0.30	0.10	0.26	21
24	Cu(Bpy)(*d*-Bct)$^+$	−0.22	−0.30	0.08	0.20	17
25	Cu(Bpy)(Leu)$^+$				~0	≤11[d]
26	Cu(Phen)(Leu)$^+$				0.15	13
27	Zn(Bpy)(Leu)$^+$				0.12	11 (~35)[e]
28	Zn(Phen)(Leu)$^+$				0.35	26 (~35)[e]

[a] The constants for $\Delta \log K_{M/exp}$ are for entries Nos 1–12 and 13–20 (50% aqueous dioxane) from ref. 21 and 56, respectively, and for Nos 21–24 (water) from ref. 29; the results of Nos 25–28 (water) are from ref. 55. Abbreviations (see also ref. 9): Ac, acetate; Ala, *L*-alaninate; B, biotin; Bct, biocytin (2); Chac, 2-cyclohexylacetate; D, dethiobiotin (=S in biotin replaced by 2 H atoms); Gly, glycinate; H, homobiotin; Leu, *L*-leucinate; N$^\varepsilon$-Al, N$^\varepsilon$-acetyl-*L*-lysinate; Val, valerate. [b] $\Delta\Delta \log K_M = \Delta \log K_{M/exp} - \Delta \log K_{M/op}$; i.e., this is the decisive parameter of equation 10. [c] The intramolecular ligand-ligand interaction, in water as solvent, has also been proven by ^{1}H-NMR (upfield shifts are observed)[54]. [d] Assuming a difference of 0.05 log units between the stability constants would have been recognized, this value is the upper limit. [e] These values were estimated from ^{1}H-NMR upfield shifts[55].

Zn^{2+} is octahedral (or tetrahedral), and this may lead to a somewhat different extent of the intramolecular ligand-ligand interaction[55].

The occurrence of a complex species with a structure similar to the one shown in figure 3, i.e., the structure which is responsible for the slight increase in stability (fig. 1, middle and lower parts) and the observed upfield shifts in the ^{1}H-NMR spectra (for propionate, butyrate and valerate see Mitchell 54), does not mean that all of the ternary M(Bpy)(L)$^+$ species exist in this folded form. In solution there is certainly an intramolecular, and therefore concentration independent, equilibrium between an 'open' and a 'closed' form (eq. 8):



(8)

If these 2 isomers are designated as M(Bpy)(L)$^+_{op}$ and M(Bpy)(L)$^+_{cl}$, the dimension-less constant of this equilibrium is defined by equation 9.

$$K_I = [M(Bpy)(L)_{cl}]/[M(Bpy)(L)_{op}] \quad (9)$$

Values of K_I may be calculated with equation 10 (for details see Fischer and Sigel[55]).

$$K_I = \frac{10^{\Delta \log K_{M/exp}}}{10^{\Delta \log K_{M/op}}} - 1 \quad (10)$$

provided that the values of $\Delta \log K_{M/exp}$ and $\Delta \log K_{M/op}$ (see eq. 6) are known.

For the present cases, the values of $\Delta \log K_{M/exp}$ for the ternary complexes of M^{2+}, Bpy, and L^- are known[21,29]. As in the M(Bpy)(HCOO)$^+$ and M(Bpy)(Ac)$^+$ complexes an intramolecular interaction either does not occur or is insignificant[54,56]. We may use the corresponding (and also experimentally determined)[21,29] values of $\Delta \log K_M$ as $\Delta \log K_{M/op}$ and hence calculate K_I for the M(Bpy)(L)$^+$ complexes. The K_I-values in turn allow the calculation of the percentage of the 'closed' isomer with the intramolecular hydrophobic interaction. The values of K_I and the resulting percentages given in table 3 can only be considered as estimates because they are derived from differences between stability constants which are connected with a certain experimental error. On the other hand, it should be pointed out that by using the

difference $\Delta\Delta$ log K_M (see footnote b in table 3) in these calculations systematic errors are, in large part, cancelled[55]. Indeed, reasonable trends are indicated; e.g. the percentage of the 'closed' isomer increases with the increasing size of the aliphatic carboxylate: valerate (No. 3, 9) < *d*-biotinate (No. 4, 10). Similarly, the hydrophobic interaction of $M(Phen)^{2+}$ with 2-cyclohexylacetate (No. 16, 20) is more pronounced than with valerate (No. 15, 19).

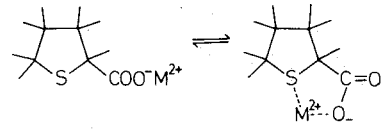
The aliphatic ligand parts of the amino acid derivatives N^{ε}-acetyl-*L*-lysinate and *d*-biocytinate (structure **2**) also lead to an intramolecular hydrophobic interaction with $M(Bpy)^{2+}$ (No. 23, 24), which is in its extent comparable to the interaction observed[55] with *L*-leucinate in corresponding ternary complexes (No. 25–28).

It is also instructive to examine the results listed in table 3 from an energetic point of view: $\Delta G° = -1.7$ kJ/mole (25 °C) was derived from theoretical calculations[52] for the hydrophobic interaction between the phenyl residue of phenylalanine and the isopropyl moiety of leucine in aqueous solution. Considering the whole range of the $\Delta\Delta$ log K_M-values (0.08–0.23 log units) listed in table 3, one calculates $\Delta G° = -0.46$ to -1.3 kJ/mole. The agreement between the theoretical and experimental data is reasonable[55], especially if one considers that the theoretical calculation[52] is based on a maximal interaction which will often not be achieved in the ternary complexes owing to the restricted mobility of the ligands; in fact, the expected gradual tailing off of the interaction toward zero is nicely seen.

5. *General conclusions*

From the observations summarized in section 2 it seems feasible that an activation of N-1′ for the formation of the enzyme-biotin-CO_2 intermediate may be facilitated by a metal ion coordination or hydrogen bond formation to O-2′, and there appears to be some evidence for this[3,30,32]. It has also been suggested that the sulfur atom is involved in hydrogen bonding[34], but there is no evidence for this[5]. Indeed, it has been observed that oxybiotin is a poor substrate for the biotin carboxylase of *Escherichia coli* while selenobiotin is a very good one[41]; if hydrogen bonding to S-1 were of importance these substrates would be expected to show the opposite properties.

It is evident from section 3.2 that the stability of the complexes formed by the biotin-sulfur and those 'hard' or 'borderline' metal ions which are important in biological systems, like Mg^{2+}, Ca^{2+}, Mn^{2+} or Zn^{2+}, is not very pronounced. However, one should not conclude that these interactions are not important: (a) we have already seen that under conditions with a reduced water activity, thioether complex stability increases for these metal ions; (b) more important, a weak interaction is enough to create specific structures. For example, this low coordination tendency allows tetrahydrothiophene-2-carboxylate in the intramolecular equilibrium 11



(11)

the formation of significant amounts of the chelated complex $M(Thtc)^{+}_{ch}$. From table 4 it is evident that the percentage of the chelated isomer depends on the metal ion involved: the whole range from traces to amounts close to 100% is covered.

It should be emphasized that the position of the intramolecular equilibrium 11 (and eq. 8 as well) is independent of the concentration and therefore exists, to a certain extent, under any condition, although a change in the water activity will alter it. This example demonstrates that thioether-sulfur may easily participate in complex formation and determine a structural arrangement if the sulfur atom is in a sterically favorable position.

So far it is not known whether or not the function of the biotinyl sulfur is to bind to a metal ion during one of the biochemical reactions, but the results summarized here show that this possibility should be considered. It is expected that different metal ions have different roles in the enzymic process and, for example, a transcarboxylase contains indeed about 12 metal ions per mole[19]. The following postulate[57] is interesting here: 'The chain linkage between the ring structure of biotin can reach a distance of 14 Å when fully extended, and therefore a structural basis is provided for the possibility of migration between

Table 4. Extent of chelate formation in binary and ternary metal ion complexes with tetrahydrothiophene-2-carboxylate (Thtc) in 50% aqueous dioxane (I = 0.1; 25 °C)[a]

M^{2+}	log $K^{M}_{M(Thtc)}$	log Δ	K_{ch}	% $M(Thtc)^{+}_{ch}$[b] (eq. 11)
Mn^{2+}	1.80 ± 0.05	~0	~0	≤20
Cu^{2+}	4.31 ± 0.03	1.16	13.5	93 ± 1
Zn^{2+}	2.35 ± 0.03	0.15	0.4	29 ± 8
Cd^{2+}	2.68 ± 0.02	0.34	1.2	54 ± 4
Pb^{2+}	3.32 ± 0.03	0.40	1.5	60 ± 5
$Cu(Bpy)^{2+}$	4.32 ± 0.03[c]	1.06	10.5	91 ± 1[d]
$Zn(Bpy)^{2+}$	2.30 ± 0.03[c]	0.23	0.7	41 ± 7[d,e]

[a] The constant for equilibrium 11 is defined by $K_{ch} = [M(Thtc)_{ch}]/[M(Thtc)_{op}] = (K^{M}_{M(Thtc)}/K^{M}_{M(Thtc)_{op}}) - 1$; for details see ref. 47. The crucial factor, $\log\Delta = \log K^{M}_{M(Thtc)} - \log K^{M}_{M(Thtc)_{op}}$, corresponds to the difference between $\log K^{M}_{M(Thtc)}$ and the reference line (see fig. 1). The last 2 entries in the table were calculated with the results of ref. 21, the others were taken from ref. 47. [b] The error limits were calculated by adding 0.02 log units to the given errors of $\log K^{M}_{M(Thtc)}$. [c,d] Value for $\log K^{M(Bpy)}_{M(Bpy)(Thtc)}$ and % $M(Bpy)(Thtc)^{+}_{ch}$, respectively. [e] From a comparison with the binary system, it appears as possible that in this ternary isomer besides the thioether also a hydrophobic interaction occurs.

physically distinct portions of the enzyme protein'. In fact, there is evidence[11] that a 'biotinyl carboxyl carrier protein acts as a shuttle' between 2 enzyme subunits. Another possible explanation[30] for 'the major role of the 14-Å arm is ... to permit carboxybiotin to traverse the gap which occurs at the interface of 3 subunits'. Hence, a weak but stereospecific biotin-sulfur/metal ion interaction could be the proper means to create the active enzyme-substrate complex. This stereospecificity would largely be lost with oxybiotin but retained with selenobiotin, because for the (C-2)-(O-1)-(C-5) angle a value of about 104° (as determined for tetrahydrofurane)[58] is expected, while for (C-2)-(Se-1)-(C-5) the angle should be close to 90° as has been determined for that of (C-2)-(S-1)-(C-5) (89.4°)[4]. The coordinating properties of a selenium atom in the given environments are also expected to be similar to those of sulfur[59].

The stereoselective correct 'fixation' of the biotinyl moiety at the surface of the protein might be further promoted, or even fully achieved, by hydrophobic interactions. From section 4 it is evident that an interaction of this type occurs in mixed ligand metal ion complexes which contain an aromatic moiety and the biotinyl group. Weak biotin-tryptophan interactions have already been suggested[42].

It appears that the dynamic features which are involved in the biotinyl-enzyme process require distinct structural conditions. But it also seems clear that these structural conditions could easily be brought about to allow a 'cyclic' process. The described sulfur-metal ion as well as the hydrophobic interactions appear to meet this condition from an energetic point of view. The whole range of the log Δ values of table 4 ($\sim$0-1.16 log units) for the metal ion-thioether interaction corresponds to $\Delta G^\circ = \sim 0$ to -6.6 kJ/mole, while for the hydrophobic interaction the following holds: $\Delta G^\circ = -0.46$ to -1.3 kJ/mole (section 4). It is therefore clear that the coordinating properties of the biotinyl moiety are very versatile indeed.

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A new solid-phase synthesis of Thymopoietin II by a mild procedure

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Summary. The solid-phase synthesis of a nonatetracontapeptide corresponding to the entire amino acid sequence of Thymopoietin II is described. Use of the recently developed, base-labile, fluorenylmethyloxycarbonyl-amino acids in combination with *tert*-butyl based side chain protecting groups and p-alkoxybenzyl ester peptide to resin linkage enabled the synthesis to be carried out under much milder reaction conditions than previously.

It is now evident that various factors are involved in the many activities of the thymus and of its cells[1]. Among these factors, Thymopoietin I and II, 2 closely related polypeptide hormones (formerly known as 'Thymin I and II') isolated from bovine thymus in 1974 by Goldstein[2], induce the selective differentiation of thymocytes from precursor