# On the Role of Structural Zinc in Bis(Cysteinyl) Protein Sequences**

**Axel Meißner, Wolfgang Haehnel, and Heinrich Vahrenkamp***

**Abstract**: Besides its functional role in many hydrolytic metalloenzymes, zinc acts as a structural component by being attached to bis(cysteinyl) protein sequences in some of the same enzymes, and in other metalloproteins and zinc fingers, and by being an essential constituent in metallothioneins. It is not always obvious whether the zinc-binding proteins are preorganized for the incorporation of the metal or whether the zinc ion provides the structurizing power and stability for the observed peptide conformations. We have addressed the coordination chemistry aspects of this question by synthesizing zinc complexes of small model peptides and by determining their structures in solution by 2D NMR spectroscopy. The peptides chosen were of the terminally protected bis(cysteinyl) type: Cys-Cys, Cys-Gly-Cys, Cys-Phe-Cys, and Cys-Gly-Ile-Cys. The zinc ions fold these peptides into structures that can be superimposed on those of the natural proteins.

**Keywords**
NMR spectroscopy · peptides · protein structures · structure elucidation · zinc

## Introduction

The best-known role of zinc in biological processes is its function in the active centers of hydrolytic enzymes.[2, 3] In recent times, however, zinc has been found to be equally important as a structural component, assisting in the folding of proteins.[4]

One of the most common protein motifs for the binding of the so-called structural zinc is the bis(cysteinyl) sequence Cys-$X_n$-Cys. It occurs in alcohol dehydrogenase,[5] in many zinc fingers,[6, 7] zinc twists, and zinc clusters,[8–10] as well as in metallothioneins.[11, 12] As a rule the two cysteine residues coordinating one zinc ion are separated by only one to three amino acid residues, and in metallothioneins they may even be immediate neighbors. Often the binding sites for structural zinc occur in $\beta$-turn or pseudo-$\beta$-turn like regions.[13]

The question of whether the folding or the immediate metal environment of these proteins are induced by the zinc ions or whether the preorganization of the proteins provides a singularly favorable environment for the structural zinc cannot always be answered. While it could be shown that zinc finger fragments or metallothioneins are converted from random coils to ordered folds upon addition of zinc, this is not obvious for fully intact transcription factors or enzymes containing structural zinc, because the structure of the metal-free protein is not known.

Model studies may help to uncover the folding and binding mechanism, as has been shown for the design of protein–zinc binding sites[14, 15] as well as for the metal ion assisted helix formation of small polypeptides.[16] We have approached this field with simple coordination compounds of zinc with aminethiols[17] and cysteine-containing peptides.[18] The aim of our studies is to find thermodynamic and geometrical factors determining the composition, structure, and immediate zinc environment of complexes of chelating N and S (e.g. peptide) ligands. In turn these factors may help in the construction of peptides that induce specific protein folds when complexed to "structural" zinc. This would allow coordination chemistry to contribute to protein design.

In addition to underlining the high affinity between zinc and thiolates and the very marked preference for $ZnN_2S_2$ coordination, our studies so far have shown that, even with highly voluminous or electronegative substiuents on the thiolates, it is nearly impossible to obtain non-oligomeric (i.e. not Zn-S-Zn bridged) complexes of alkanethiols (namely, cysteine derivatives) in the absence of nitrogen donors. When investigating the zinc coordination chemistry of bis(cysteinyl)peptides with the purpose of modeling structural zinc sites, we therefore ensured their monomeric and stable nature by incorporation of a nitrogen coligand. The four peptides used, **1**–**4**, were protected at

Bz-Cys-Cys-OEt **1** Bz-Cys-Gly-Cys-OEt **2**

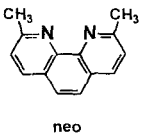
Bz-Cys-Phe-Cys-OEt **3** Bz-Cys-Gly-Ile-Cys-OEt **4**

both the N- and C-termini to prevent coordination other than by the thiolate functions. They are minimal representations of the bis(cysteinyl) protein sequences with 0, 1, and 2 spacer amino acid residues between the two cysteines. The protecting groups were N-benzoyl (Bz) for the N-terminus and ethoxy (OEt)

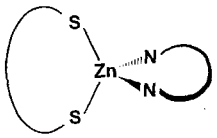
[*] Prof. Dr. H. Vahrenkamp, Dr. A. Meißner
Institut für Anorganische und Analytische Chemie der Universität Freiburg
Albertstr. 21, D-79104 Freiburg (Germany)
Fax: Int. code +(761)203-6001
Prof. Dr. W. Haehnel
Institut für Biochemie der Pflanzen der Universität Freiburg
Schänzlestr. 1, D-79104 Freiburg (Germany)
Fax: Int. code +(761)203-2601

[**] Zinc complexes of amino acids and peptides, Part 10. Part 9, ref. [1].

for the C-terminus. The *o*-phenanthroline derivative neocuproine (neo), with its two nitrogen donor funtions, was found to be an ideal coligand. With this ligand combination, principally simple mononuclear complexes of the type (pept)Zn(neo) were accessible.



neo



(pept)Zn(neo)

## Results and Discussion

The peptides **1**–**4** were obtained starting from the cystine derivative [H-Cys-OEt]$_2$·2HOTos. Stepwise coupling with the N-protected amino acids Boc-Gly-OH, Boc-Phe-OH, Boc-Ile-OH, and [Bz-Cys-OH]$_2$, respectively, was performed by using the mixed anhydride technique. The resulting bis(cystine) peptides were reduced to the bis(cysteine) peptides **1**–**4** with dithioerythritol. The products obtained were of analytical purity, and further purification by HPLC was not required. They were fully characterized (IR, NMR, MS, and elemental analyses; Experimental Procedure).

The zinc complexes of the type (pept)Zn(neo) were obtained in a two-step procedure. After deprotonation with sodium ethoxide, the peptides were treated with zinc perchlorate in ethanol to give the Zn(SR)$_2$ compounds **1a**–**4a**. Their reactions with equimolar amounts of neocuproine produced the desired complexes **1b**–**4b**.

| | |
|---|---|
| [(Bz-Cys-Cys-OEt)Zn]$_x$ **1a** | [(Bz-Cys-Cys-OEt)Zn(neo)] **1b** |
| [(Bz-Cys-Gly-Cys-OEt)Zn]$_x$ **2a** | [(Bz-Cys-Gly-Cys-OEt)Zn(neo)] **2b** |
| [(Bz-Cys-Phe-Cys-OEt)Zn]$_x$ **3a** | [(Bz-Cys-Phe-Cys-OEt)Zn(neo)] **3b** |
| [(Bz-Cys-Gly-Ile-Cys-OEt)Zn]$_x$ **4a** | [(Bz-Cys-Gly-Ile-Cys-OEt)Zn(neo)] **4b** |

Compounds **1a**–**4a** slowly precipitated from the reaction solutions. After precipitation they were practically insoluble in all solvents except strong acids, thereby indicating their possible oligomeric nature. Therefore they were isolated only for analytical purposes. For the formation of **1b**–**4b** they were treated in situ with neocuproine. Complexes **1b**–**4b** were also isolated by precipitation from ethanol. Their solubility in solvents of lower polarity (see NMR experiments) indicates, however, that they are monomeric. Their identity was verified by analytical and spectroscopic data.

Proof for the monomeric and mononuclear nature of **1b**–**4b** was obtained by ESI-MS. The mass of peptides **1**, **3**, and **4** was accurate to within $\pm 0.1$ of the calculated mass as detected by a quadrupole mass spectrometer equipped with an electrospray interface.[19] Protonation of the ligands decreased the stability of the zinc complexes at low pH and made it difficult to detect the intact complexes in the presence of acid introduced with the eluent used for chromatographic purifications. However, complexes **1b**, **3b**, and **4b** dissolved in $CHCl_3$/acetonitrile (1:1 *v*/*v*), yielded the masses 627.1, 774.3, and 797.9, respectively, for the intact protonated complexes (with $^1H$, $^{13}C$, $^{32}S$, and $^{64}Zn$) when acid-free methanol was used as eluent. This indicates a remarkable stability of these zinc complexes. In contrast, in previous studies with zinc-finger proteins[20,21] and the copper(I)-containing protein plastocyanin[22] only the masses of the metal-free apoproteins were observed. The zinc isotopes 64, 66, 67, 68, and 70 incorporated in complexes **1b**, **3b**, and **4b** were resolved as individual peaks in each of the three mass spectra showing relative intensities in good agreement with the natural abundance of the isotopes. This provides clear evidence for the monomeric character of these complexes and excludes the possibility of dimers, such as $(MH^+)_2^{2+}$. Thus the combined advantages of electrospray ionization and the presence of uncharged molecular complexes have allowed an unprecedentedly exact determination of their molecular masses.

The complexes **1b**–**4b** could not be obtained as single crystals for X-ray structure determinations. We therefore applied 2D NMR methods to determine their structures in solution. Conventional COSY and NOESY techniques were applied for data acquisition and the extraction of torsional angles.[23] The structures were solved by the procedure of dynamical simulated annealing using the program XPLOR[24] (for details, see Experimental Procedure). For reference purposes the NMR data of the peptides **1**–**4** were obtained in the same way. They showed, as expected, that the peptides have no preferred conformations, that is, they exist as random coils in solution. Typically their $J_{HN}$ coupling constants are all close to 7.0 Hz, and the NOESY spectra show for each cysteinyl function cross-signals relating the NH protons with both diastereotopic $C_\beta H_2$ protons. This indicates that the peptide backbone as well as the side chains for **1**–**4** are randomly oriented. In contrast, in complexes **1b**–**4b** the same peptides have well-defined conformations.

In the following graphical representations of the complex structures, wire models of several of the "best" (see Experimental Procedure) solutions are superimposed by using the central peptide linkage as the anchor. This shows that there is rather good definition in the conformation of the peptide backbone and a high degree of freedom for the terminal protecting groups and the peptide side chains. According to the NMR data the Zn–neocuproine unit has considerable freedom through a rocking motion with respect to the $ZnS_2$ plane. The Zn–N distances remained close to 2.00 Å and the Zn–S distances close to 2.30 Å during the refinement. With the N-Zn-N angle constrained to 80°, the S-Zn-S angles refined at $133 \pm 2°$ and the N-Zn-S angles at $108 \pm 6°$.

The complex of **1** (Figure 1) contains a nine-membered chelate ring. Its key features are the *cis* configuration of the peptide bond and the absence of stabilizing hydrogen bonds.

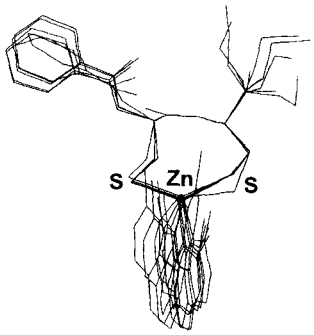


Figure 1. Wire model of complex **1b**.

The complexes of **2** (Figure 2) and **3** (Figure 3) both have twelve-membered chelate rings and *trans* configurations of all



0947-6539/97/0302-0262 $ 15.00+.25/0 Chem. Eur. J. 1997, 3, No. 2

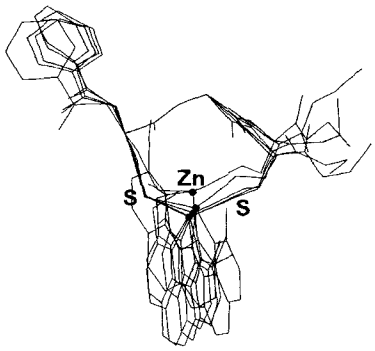



Figure 2. Wire model of complex **2b**.

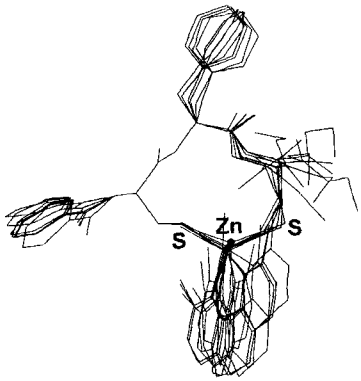



Figure 3. Wire model of complex **3b**.

the peptide bonds. NMR data and molecular geometry indicate that the NH proton of the C-terminal cysteine is involved in a hydrogen bond to one or both of the sulfur atoms.

The complex of **4** (Figure 4) contains a fifteen-membered chelate ring. All the peptide bonds are again in a *trans* configuration. The peptide forms a loop which is very similar to a type I $\beta$-turn,[13] however, without a hydrogen bond between CO of the N-terminal and NH of the C-terminal cysteines.

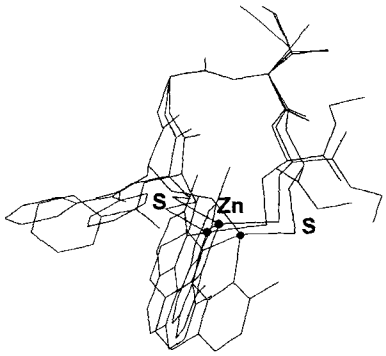



Figure 4. Wire model of complex **4b**.

Figure 5 shows the structures of all four complex molecules as ball-and-stick models in the SCHAKAL format, obtained by averaging the wire models displayed in Figures 1–4. This representation facilitates visualization, but also gives the false impression that the accuracy of the structure determinations is of X-ray quality.

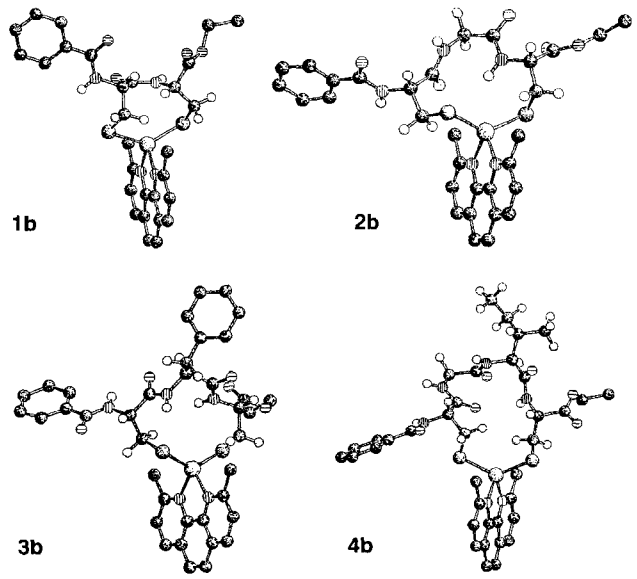



Figure 5. Ball-and-stick models of the average structures of **1b**, **2b**, **3b**, and **4b**.

The coordination environment of the zinc ions in the average structures of the four complexes is given in Table 1. For reference one zinc complex with a very simple $ZnN_2S_2$ environment (**A** = Zn(*N*-methyl imidazole)$_2$(SPh)$_2$[25]) and one neocuproine–Zn(SPh)$_2$ complex (**B** = Zn(neo)(SPh)$_2$[26]) are included. The consistency of bond lengths and angles among the four peptide complexes is noticeable. While for the bond lengths this is an expression of the imposed restaints on their variability (see Experimental Procedure), such is not the case for the bond angles, which were allowed to vary within a wide range. The distances and angles are in a reasonably good agreement with those of reference complex **B** in which the neocuproine ligand also enforces a very small N-Zn-N angle, allowing the S-Zn-S angle to become very large. Reference complex **A** adopts a more "natural" disposition of the ligands around zinc, with bond angles closer to the tetrahedral value, but bond lengths again reasonably close to those in **1b–4b**.

Table 1. Bond lengths [Å] and angles [°] at the zinc ions in complexes **1b–4b** and reference compounds.

| | **1b** | **2b** | **3b** | **4b** | **A** | **B** |
|---|---|---|---|---|---|---|
| Zn–S$^1$ | 2.29 | 2.30 | 2.30 | 2.30 | 2.30 | 2.26 |
| Zn–S$^2$ | 2.30 | 2.30 | 2.30 | 2.30 | 2.29 | 2.26 |
| Zn–N$^1$ | 2.01 | 2.01 | 2.01 | 2.00 | 2.05 | 2.09 |
| Zn–N$^2$ | 1.99 | 2.01 | 2.00 | 2.00 | 2.03 | 2.09 |
| S$^1$-Zn-S$^2$ | 134.7 | 135.6 | 131.4 | 133.4 | 109.4 | 134.5 |
| S$^1$-Zn-N$^1$ | 108.9 | 110.0 | 114.1 | 110.4 | 110.6 | 106.7 |
| S$^1$-Zn-N$^2$ | 103.1 | 101.8 | 103.2 | 102.0 | 111.1 | 107.7 |
| S$^2$-Zn-N$^1$ | 105.2 | 105.4 | 105.8 | 106.3 | 108.1 | 107.7 |
| S$^2$-Zn-N$^2$ | 111.4 | 110.1 | 110.2 | 111.9 | 115.3 | 106.7 |
| N$^1$-Zn-N$^2$ | 80.3 | 80.2 | 80.0 | 80.1 | 101.9 | 80.2 |

The structural results show that the zinc ion, assisted by the neocuproine ligand, is able to assemble the peptide ligands such that quite unusual chelate ring sizes (9, 12, 15) are formed, although this limits the conformational freedom of the peptides, which exist as random coils in the absence of zinc. The molecular dynamics calculations show that the zinc ion does not impose

conformational strain, that is, the structures are compatible with the "natural" situation of the corresponding amino acid sequences in proteins.

The degree of this compatibility can be evaluated by superimposing the zinc peptide units of the four complexes on those of their natural equivalents, as shown in Figure 6 for the Cys–Cys

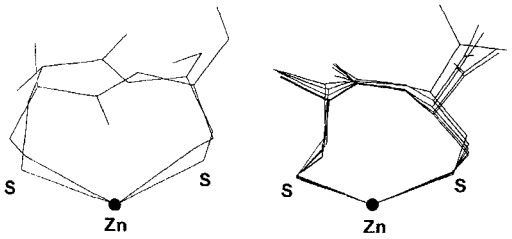



Figure 6. The Zn(CysCys) units of rat liver metallothionein-II (left) and of the five "best" structures of the Zn(CysCys) complex **1b** (right) projected onto the $ZnS_2$ plane.

complex **1b** and two Zn(CysCys) units in rat liver metallothionein-II.[12] Again, the zinc ion is used as anchor. While a general similarity is to be expected due to the relatively small chelate ring size, the differences are nevertheless obvious. They result from the fact that the peptide bond in the complex has a *cis* arrangement and that in the metallothionein a *trans* arrangement. A direct comparison is not possible because the Cys–Cys units in the metallothionein are both coordinated to one additional zinc ion.

The two Zn(Cys-X-Cys) complexes of peptides **2** and **3** find their natural equivalent in three of the seven zinc-coordinating Cys-X-Cys units of the metallothioneins, namely, the Cys-Ser-Cys units, which each bind to only one zinc ion.[12] In order to align them with the complexes of **2** and **3** a different mode of projection is employed. In Figure 7 the $Cys^1$–X amide bond is

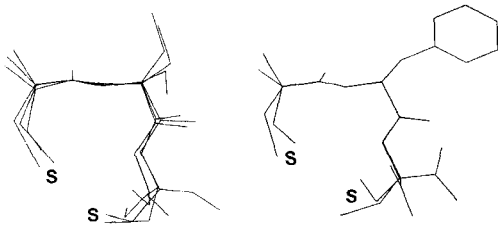



Figure 7. The Cys-Ser-Cys units of three zinc-binding sites in rat liver metallothionein-II (left) and the Cys-X-Cys units of the zinc complexes **2b** and **3b** (right) projected onto the plane of the $Cys^1$-X amide bond.

used as the anchor and as the plane of projection, and the zinc ions are omitted. The reason for this lies in the large variation in the orientations of the cysteinyl side chains (i.e., the S-$C_\beta$-$C_\alpha$-C dihedral angles) in the five species. Except for this conformational freedom found in the three natural and the two synthetic examples, the shapes of the five tripeptide "ligands" are very similar.

The zinc binding units Cys-X-X-Cys exemplified by **4b** occur in metallothioneins[11, 12] as well as in zinc fingers,[6, 27–29] and in many other "structural" sites of proteins.[2–4] Three of those that are obtainable from the protein data banks (two from the zinc finger ZIF 268[28] and one from rat liver metallothionein-II[12]) are depicted in Figure 8 for comparison with the corresponding fragment of the complex of **4b**. As for **2b** and **3b** the amide bond between $Cys^1$ and $X^2$ is used as the anchor and plane of projection, because the real protein structures and the

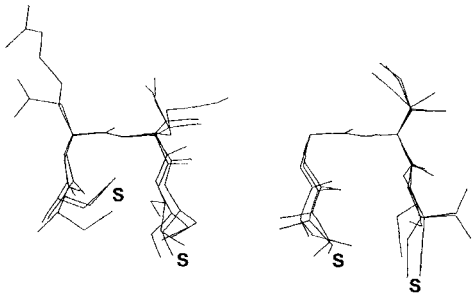



Figure 8. Superpositions of the zinc binding Cys-Arg-Ile-Cys and Cys-Asp-Ile-Cys units from ZIF 268 and the Cys-Ala-Lys-Cys unit from rat liver metallothionein-II (left) and of the three "best" structures of **4b** in its zinc complex (right) projected onto the plane of the $Cys^1$–$X^2$ amide bond.

best solutions of the model structure exert their largest conformational freedom in the cysteine side chains. On the one hand, this seems to cause a large flexibility of the zinc ion position if viewed from the anchoring point on the other side of the chelate ring; on the other, it again reveals a marked similarity between the shapes of the chelate rings formed by the natural and the synthetic Cys-X-X-Cys ligands.

The three comparisons between the natural bis(cysteinyl) zinc binding sites and their model structures presented here reveal typical differences and striking similarities. The main differences lie in the variable orientations of the cysteinyl $CH_2$–S side chains and S–Zn bonds with respect to the plane of the peptide loop. In the natural structures this reflects the conformational freedom of these units whose orientation is determined more by extraloop rather than intraloop interactions. In the different solutions obtained by the computational process for the structures of each model complex, it reflects the fact that the $C_\beta$-S-Zn-S dihedral angles are not constrained by the NMR data. Another reason for the differences between the natural and the model structures lies in the different modes of hydrogen bonding. In the model structures only intraloop hydrogen bonds are possible and become visible for **2b** and **3b** (see above). In the natural structures various extraloop hydrogen bonds exist in addition to the same intraloop interaction as in the models.[11, 12]

Taking these limitations into account, the geometrical fit between the natural examples and the model complexes is remarkably high. Figures 7 and 8 demonstrate this by using a central amide unit as the plane of projection. It thus becomes clear that the conformations of the three or four peptide constituents are highly conserved and that only the side chains (including the thiolate side chains) are free to orient themselves.

The peptide conformations found in complexes **1b**–**4b** are not the minimum-energy conformations of the free peptides. The NMR data of the free peptides **1**–**4**, in agreement with molecular mechanics calculations, indicate that they exist in



0947-6539/97/0302-0264 $ 15.00+.25/0 Chem. Eur. J. 1997, 3, No. 2

solution as random coils. Thus, the binding to the zinc ions enforces the loop structure on the peptides and is the structurizing influence generating the observed conformations. The similarity between the model complexes and their natural equivalents suggests that the same mechanism occurs in the proteins. This allows the further conclusion that "structural zinc" not only stabilizes the structure, but is also the essential component for the folding of the corresponding protein segments.

## Experimental Procedure

The general experimental techniques and spectroscopic methods were as described previously [30]. The peptide derivatives $[H\text{-}Cys\text{-}OEt]_2 \cdot 2HOTos$, $[Bz\text{-}Cys\text{-}OH]_2$, Boc-Gly-OH, Boc-Phe-OH, and Boc-Ile-OH were prepared by standard procedures from the commercially available amino acids cystine, glycine, phenylalanine, and isoleucine. All solvents were free of water and oxygen. All manipulations were carried out under an atmosphere of nitrogen (99.99%).

**Peptide syntheses**: The following standard procedures were applied:

*A (coupling)*: The cystinyl component (5.00 mmol, as its HOTos (tosylate) or HOTf (triflate) adduct) was dissolved in DMF (20 mL) and treated with *N*-methylmorpholine (NMM) (1.10 mL, 10.0 mmol). In a second flask the coupling component (Bz or Boc amino acid, 10.00 mmol) was dissolved in THF (50 mL), cooled to −15 °C, and treated with NMM (1.10 mL, 10.0 mmol). After 15 min this second solution was treated with chloroformic acid isobutyl ester (CAIBE) (1.30 mL, 10.0 mmol). After 2 min the first solution was added to the second, and the mixture stirred for 16 h. The precipitate was then removed with a G3 frit and washed with THF (20 mL). The combined filtrates were concentrated to 25 mL in vacuo and treated with water (80 mL) to precipitate the crude product. The voluminous precipitate was separated with a G3 frit and washed with water (3 × 15 mL) and 5% $NaHCO_3$ solution (3 × 15 mL). It was then washed with 15 mL portions of water until the filtrate had a neutral pH. The colorless product was dried under an oil pump vacuum.

*B (coupling)*: Like A until the precipitation of the crude product. The oily crude product was taken up into ethyl acetate (100 mL), and the aqueous phase was extracted with ethyl acetate (50 mL). The combined ethyl acetate phases were washed with water (3 × 50 mL), HCl solution (pH = 3) (3 × 50 mL), water (3 × 50 mL), 4% $NaHCO_3$ solution (3 × 50 mL), and finally 50 mL portions of water until the filtrate had a neutral pH. The ethyl acetate phase was dried over $Na_2SO_4$ and evaporated to dryness under an oil pump vacuum.

*C (removal of Boc groups)*: The Boc-protected peptide (5.00 mmol) was dissolved in water-free trifluoroacetic acid (TFA) (50 mL) whereupon a vigorous gas evolution occurred. After 1 h the TFA was removed in vacuo. The oily yellow residue was triturated with diethyl ether until it was transformed into a colorless solid. This was filtered off over a G3 frit, washed with ether, and dried in vacuo.

*D (disulfide cleavage)*: The cystine derivative (5.00 mmol) was suspended in water (100 mL) and treated with dithioerythritol (DTE) (1.31 g, 8.50 mmol). The pH was adjusted to 8.5 by addition of 0.2 M NaOH. Acetonitrile (100 mL) was then added, and the solution refluxed 6 h. After cooling to room temperature the resulting clear solution was acidified with TFA and evaporated to dryness. The remaining solid was washed with ether (30 mL) and cold water (5 mL) and then dried under an oil pump vacuum.

**1**: From $[Bz\text{-}Cys\text{-}OH]_2$ (3.36 g, 7.50 mmol) and $[H\text{-}Cys\text{-}OEt]_2 \cdot 2HOTos$ (4.81 g, 7.50 mmol), according to procedure A with NMM (2 × 1.65 mL) and CAIBE (1.95 mL), producing $[Bz\text{-}Cys\text{-}Cys\text{-}OEt]_2$ (4.51 g, 85%). Cleavage according to procedure D; $[Bz\text{-}Cys\text{-}Cys\text{-}OEt]_2$ (4.36 g, 12.30 mmol) with DTE (3.79 g, 24.6 mmol) yielded **1** (4.05 g, 92%) as a colorless powder, m.p. 198–200 °C. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 3360 (s, NH), 2546 (w, SH), 1737 (s), 1644 (s), 1525 (s, ester, amide). $^1H$ NMR ($CDCl_3$): $\delta$ = 7.84 (d, $J_{om}$ = 8.0 Hz, 2H, *o*-H Bz); 7.60–7.41 (m, 3H, *m,p*-H Bz); 7.26 (d, $J_{\alpha N}$ = 7.2 Hz, 1H, NH-$^1$Cys); 7.21 (d, $J_{\alpha N}$ = 8.4 Hz, 1H, NH-$^2$Cys); 4.95–4.80 (m, 2H, $C_\alpha$H-$^1$Cys, $C_\alpha$H-$^2$Cys); 4.27 (q, $J_{et}$ = 7.1 Hz, 2H, $CH_2$-Et); 3.22 (ddd, $J_{\alpha\beta}$ = 4.8 Hz, $J_{\beta\gamma}$ = 7.8 Hz, $^2J_{\beta\beta'}$ = 13.9 Hz, 1H, $C_\beta$H-$^1$Cys); 3.04 (dd, $J_{\alpha\beta}$ = 4.4 Hz, $J_{\beta\gamma}$ = 8.9 Hz, 2H, $C_\beta$H-$^2$Cys); 2.90 (ddd, $J_{\alpha\beta'}$ = 6.7 Hz, $J_{\beta'\gamma}$ = 10.1 Hz, $^2J_{\beta\beta'}$ = 13.9 Hz, 1H, $C_\beta$H'-$^1$Cys); 1.86 (dd, $J_{\beta\gamma}$ = 8.0 Hz, $J_{\beta'\gamma}$ = 10.1 Hz, 1H, SH-$^1$Cys); 1.45 (t, $J_{\beta\gamma}$ = 8.9 Hz, 1H, SH-$^2$Cys); 1.31 (t, $J_{et}$ = 7.1 Hz, 3H, $CH_3$-Et). $C_{15}H_{20}N_2O_4S_2$ (356.5): $MH^+$ found: 357.1 (ESI-MS).

**2**: From $[H\text{-}Cys\text{-}OEt]_2 \cdot 2HOTos$ (6.41 g, 10.00 mmol) and Boc-Gly-OH (3.82 g, 20.00 mmol), procedure B, yielding $[Boc\text{-}Gly\text{-}Cys\text{-}OEt]_2$ (5.80 g, 95%). This product (5.80 g, 9.50 mmol), treated according to procedure C, yielded $[H\text{-}Gly\text{-}Cys\text{-}OEt]_2 \cdot 2TFA$ (5.11 g, 84%). This product (4.11 g, 6.43 mmol) and $[Bz\text{-}Cys\text{-}OH]_2$ (2.87 g, 6.43 mmol), treated according to procedure A, yielded $[Bz\text{-}Cys\text{-}Gly\text{-}Cys\text{-}OEt]_2$ (5.01 g, 94%). This product (4.89 g, 11.89 mmol) and DTE (3.67 g, 23.77 mmol), according to procedure D, yielded **2** (3.54 g, 72%) as a colorless powder, m.p. 136–137 °C. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 3308 (s), 3249 (m, NH), 2548 (w, SH), 1730 (s), 1676 (s), 1632 (s), 1578 (m), 1534 (s, ester, amide). $^1H$ NMR ($CDCl_3$): $\delta$ = 7.87–7.82 (m, 2H, *o*-H Bz); 7.48 ($m_c$, 3H, *m,p*-H Bz); 7.24 (d, $J_{\alpha N}$ = 7.1 Hz, 1H, NH-$^1$Cys); 7.16 (t, $J_{\alpha N}$ = 5.4 Hz, 1H, NH-Gly); 6.96 (d, $J_{\alpha N}$ = 6.8 Hz, 1H, NH-$^3$Cys); 4.85 (ddd, $J_{\alpha N}$ = 7.1 Hz, $J_{\alpha\beta}$ = 4.4 Hz, $J_{\alpha\beta'}$ = 6.5 Hz, 1H, $C_\alpha$H-$^1$Cys); 4.83 (dd, $J_{\alpha N}$ = 6.8 Hz, $J_{\alpha\beta}$ = 4.4 Hz, 1H, $C_\alpha$H-$^3$Cys); 4.22 (q, $J_{et}$ = 7.1 Hz, 2H, $CH_2$-Et); 4.08 (dd, $J_{\alpha N}$ = 5.7 Hz, $^2J_{\alpha\alpha'}$ = 16.8 Hz, 1H, $C_\alpha$H-Gly); 4.03 (d, $J_{\alpha' N}$ = 5.7 Hz, $^2J_{\alpha\alpha'}$ = 16.8 Hz, 1H, $C_\alpha$H'-Gly); 3.25 (ddd, $J_{\alpha\beta}$ = 4.4 Hz, $^2J_{\beta\beta'}$ = 13.9 Hz, $J_{\beta\gamma}$ = 7.7 Hz, 1H, $C_\beta$H-$^1$Cys); 3.01 (dd, $J_{\alpha\beta}$ = 4.4 Hz, $J_{\beta\gamma}$ = 8.4 Hz, 2H, $C_\beta$H-$^3$Cys); 2.89 (ddd, $J_{\alpha\beta'}$ = 6.5 Hz, $J_{\beta'\gamma}$ = 10.2 Hz, $^2J_{\beta\beta'}$ = 13.9 Hz, 1H, $C_\beta$H'-$^1$Cys); 1.87 (dd, $J_{\beta\gamma}$ = 7.7 Hz, $J_{\beta'\gamma}$ = 10.2 Hz, 1H, SH-$^1$Cys); 1.42 (t, $J_{\beta\gamma}$ = 8.4 Hz, 1H, SH-$^3$Cys); 1.28 (t, $J_{et}$ = 7.1 Hz, 3H, $CH_3$-Et). $C_{17}H_{23}N_3O_5S_2$ (413.5): calcd C 48.37, H 5.60, N 10.16; found: C 48.58, H 5.24, N 9.79.

**3**: From $[H\text{-}Cys\text{-}OEt]_2 \cdot 2HOTos$ (3.20 g, 5.00 mmol) and Boc-Phe-OH (2.65 g, 10.00 mmol), procedure B, yielding $[Boc\text{-}Phe\text{-}Cys\text{-}OEt]_2$ (3.34 g, 84%). This product (3.34 g, 4.22 mmol), treated according to procedure C, yielded $[H\text{-}Phe\text{-}Cys\text{-}OEt]_2 \cdot 2TFA$ (3.36 g, 97%). This product (3.15 g, 4.17 mmol) and $[Bz\text{-}Cys\text{-}OH]_2$ (1.87 g, 4.17 mmol), treated according to procedure A, yielded $[Bz\text{-}Cys\text{-}Phe\text{-}Cys\text{-}OEt]_2$ (3.72 g, 89%). This product (3.70 g, 7.38 mmol) and DTE (2.39 g, 15.50 mmol), according to procedure D, yielded **3** (3.06 g, 82%) as a colorless powder, m.p. 186–188 °C (dec.). IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 3484 (sh), 3280 (s, NH), 2561 (w, SH), 1738 (s), 1628 (s), 1579 (m), 1529 (s, ester, amide). $^1H$ NMR ($CDCl_3$): $\delta$ = 7.77 (d, $J_{om}$ = 7.5 Hz, 2H, *o*-H Bz); 7.60–7.40 (m, 3H, *m,p*-H Bz); 7.16 ($m_c$, 6H, Aryl-H Phe, NH-$^1$Cys); 7.05 (d, $J_{\alpha N}$ = 7.8 Hz, 1H, NH-Phe); 6.99 (d, $J_{\alpha N}$ = 7.5 Hz, 1H, NH-$^3$Cys); 4.88–4.72 (m, 3H, $C_\alpha$H-$^1$Cys, $C_\alpha$H-Phe, $C_\alpha$H-$^3$Cys); 4.22 (q, $J_{et}$ = 7.0 Hz, 2H, $CH_2$-Et); 3.22–3.04 (m, 1H, $C_\beta$H-$^1$Cys); 3.13 ($m_c$, 2H, $C_\beta$H-Phe); 2.97 ($m_c$, 2H, $C_\beta$H-$^3$Cys); 2.87–2.72 (m, 1H, $C_\beta$H'-$^1$Cys); 1.68 (dd, $J_{\beta\gamma}$ = 7.7 Hz, $J_{\beta'\gamma}$ = 10.2 Hz, 1H, SH-$^1$Cys); 1.39 (t, $J_{\beta\gamma}$ = 9.0 Hz, 1H, SH-$^3$Cys); 1.29 (t, $J_{et}$ = 7.1 Hz, 3H, $CH_3$-Et). $C_{24}H_{29}N_3O_5S_2$ (503.6): calcd C 57.23, H 5.80, N 8.35; found: C 56.24, H 5.75, N 7.87; $MH^+$ found: 504.2 (ESI-MS).

**4**: From $[H\text{-}Cys\text{-}OEt]_2 \cdot 2HOTos$ (4.81 g, 7.50 mmol) and Boc-Ile-OH (3.47 g, 15.00 mmol), procedure B, yielding $[Boc\text{-}Ile\text{-}Cys\text{-}OEt]_2$ (4.69 g, 86%). This product (4.57 g, 6.33 mmol), treated according to procedure C, yielded $[H\text{-}Ile\text{-}Cys\text{-}OEt]_2 \cdot 2TFA$ (3.52 g, 74%). This product (3.52 g, 4.68 mmol), and Boc-Gly-OH (1.64 g, 9.36 mmol), treated according to procedure A, yielded $[Boc\text{-}Gly\text{-}Ile\text{-}Cys\text{-}OEt]_2$ (3.76 g, 96%). This product (3.75 g, 4.48 mmol), treated according to procedure C, yielded $[H\text{-}Gly\text{-}Ile\text{-}Cys\text{-}OEt]_2 \cdot 2TFA$ (3.85 g, 99%). This product (3.85 g, 4.45 mmol), and $[Bz\text{-}Cys\text{-}OH]_2$ (2.00 g, 4.45 mmol), treated according to procedure A, yielded $[Bz\text{-}Cys\text{-}Gly\text{-}Ile\text{-}Cys\text{-}OEt]_2$ (3.02 g, 64%). This product (2.90 g, 5.53 mmol) and DTE (3.09 g, 20.00 mmol), treated according to procedure D, yielded **4** (2.40 g, 83%) as a colorless powder, m.p. 162–164 °C (decomp.). IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 3304 (s, NH), 2565 (w, SH), 1729 (s), 1631 (s), 1578 (w), 1542 (s, ester, amide). $^1H$ NMR ([D$_6$]DMSO): $\delta$ = 8.51 (d, $J_{\alpha N}$ = 7.6 Hz, 1H, NH-$^1$Cys); 8.35 (d, $J_{\alpha N}$ = 8.5 Hz, 1H, NH-$^4$Cys); 8.29 (t, $J_{\alpha N}$ = 5.2 Hz, 1H, NH-Gly); 7.90 (d, $J_{om}$ = 8.1 Hz, 2H, *o*-H Bz); 7.72 (d, $J_{\alpha N}$ = 7.9 Hz, 1H, NH-Ile); 7.47 ($m_c$, 3H, *m,p*-H Bz); 4.53 ($m_c$, 1H, $C_\alpha$H-$^1$Cys); 4.40 ($m_c$, 1H, $C_\alpha$H-$^4$Cys); 4.25 ($m_c$, 1H, $C_\alpha$H-Ile); 4.10 (q, $J_{et}$ = 7.1 Hz, 2H, $CH_2$-Et); 3.81–3.62 (m, 2H, $C_\alpha$H-Gly); 3.28 ($m_c$, 2H, SH-Cys); 3.07–2.64 (m, 4H, $C_\beta$H-Cys); 1.72 ($m_c$, 1H, $C_\beta$H-Ile); 1.41 ($m_c$, 1H, $C_\gamma$H-Ile); 1.17 (t, $J_{et}$ = 7.1 Hz, 3H, $CH_3$-Et); 1.07 ($m_c$, 1H, $C_\gamma$H-Ile); 0.82 (d, $J_{\beta\gamma}$ = 6.7 Hz, 3H, $C_\gamma H_3$-Ile); 0.79 (t, $J_{\gamma\delta}$ = 5.7 Hz, 3H, $C_\delta$H-Ile). $C_{23}H_{34}N_4O_6S_2$ (526.7): calcd C 52.45, H 6.51, N 10.64; found: C 52.06, H 6.44, N 10.62; $MH^+$ found: 527.2 (ESI-MS).

**Compounds Zn(SR)$_2$** (general procedure): The peptide was suspended in ethanol (50 mL per mmol), and a 0.2 M solution of sodium ethoxide (2 equiv)

in ethanol was added with stirring, to produce a clear solution. $Zn(ClO_4)_2 \cdot 6H_2O$ (1 equiv) in ethanol (5 mL per mmol) was slowly added with stirring. The solution was heated to 80 °C for a few minutes and then cooled slowly to room temperature. A colorless precipitate was formed. The volume of the mixture was reduced by one half in vacuo, and then the mixture was held at −15 °C for 20 h. The colorless precipitate, consisting of compounds **1a**–**4a**, was filtered off, washed with cold ethanol and dried in vacuo.

**1a**: From **1** (1.07 g, 3.00 mmol) and $Zn(ClO_4)_2 \cdot 6H_2O$ (1.12 g, 3.00 mmol). Yield 0.65 g (52%), m.p. > 250 °C (decomp.). IR (KBr, cm$^{-1}$): $\tilde{\nu}$ = 3422 (w), 3062 (s, NH), 1735 (s), 1645 (s), 1512 (s, ester, amide). $C_{15}H_{18}N_2O_4S_2Zn$ (419.8): calcd C 42.91, H 4.32, N 6.67, Zn 15.57; found: C 41.95, H 4.12, N 6.44, Zn 14.94.

**2a**: From **2** (0.10 g, 0.24 mmol) and $Zn(ClO_4)_2 \cdot 6H_2O$ (0.090 g, 0.24 mmol). Yield 0.070 g (61%), m.p. 190–192 °C (decomp.). IR (KBr, cm$^{-1}$): $\tilde{\nu}$ = 3142 (w), 3060 (w, NH), 1733 (m), 1652 (s), 1521 (s, ester, amide). $C_{17}H_{21}N_3O_5S_2Zn$ (476.9): calcd C 42.82, H 4.44, N 8.81, Zn 13.71; found: C 42.43, H 4.16, N 8.56, Zn 13.54.

**3a**: From **3** (0.10 g, 0.20 mmol) and $Zn(ClO_4)_2 \cdot 6H_2O$ (0.074 g, 0.20 mmol). Yield 0.074 g (65%), m.p. 204–206 °C (decomp.). IR (KBr, cm$^{-1}$): $\tilde{\nu}$ = 3394 (s), 3373 (s), 3310 (s, NH), 1728 (s), 1646 (s), 1521 (s, ester, amide). $C_{24}H_{27}N_3O_5S_2Zn$ (567.0): calcd C 50.84, H 4.80, N 7.41, Zn 11.53; found: C 50.15, H 4.37, N 7.55, Zn 11.12.

**4a**: From **4** (0.53 g, 1.00 mmol) and $Zn(ClO_4)_2 \cdot 6H_2O$ (0.37 g, 1.00 mmol). Yield 0.41 g (69%), m.p. 163–165 °C (decomp.). IR (KBr, cm$^{-1}$): $\tilde{\nu}$ = 3420 (s), 3345 (m, NH), 1738 (s), 1639 (s), 1522 (s, ester, amide). $C_{23}H_{33}N_4O_6S_2Zn$ (591.0): calcd C 46.74, H 5.63, N 9.48, Zn 11.06; found: C 46.54, H 5.22, N 9.31, Zn 10.87.

**Complexes (pept)Zn(neo)** (general procedure): The peptide was suspended in ethanol (50 mL per mmol) and treated with 2 equiv of a 0.2 M solution of sodium ethoxide in ethanol. The clear solution was treated dropwise with vigorous stirring with 1 equiv of $Zn(ClO_4)_2 \cdot 6H_2O$ in ethanol (5 mL). Subsequently 1 equiv of neocuproine (neo) in ethanol (10 mL) was added. The resulting suspension was heated under reflux for 2 h and then stirred overnight at room temperature. The mixture was then reduced to one half of its volume in vacuo and kept at −15 °C for 3 d. The colorless precipitate (complexes **1b**–**4b**) was filtered off, washed with cold ethanol (5 × 10 mL) and dried in vacuo. In order to remove some remaining $[Zn(SR)_2]_x$ the residue was taken up into a minimum amount of $CHCl_3$. The mixture was then filtered through a membrane filter and concentrated to dryness again.

**1b**: From **1** (1.07 g, 3.00 mmol), $Zn(ClO_4)_2 \cdot 6H_2O$ (1.12 g, 3.00 mmol), and neo (0.68 g, 3.00 mmol). Yield 0.65 g (34%), m.p. > 250 °C (decomp.). IR (KBr, cm$^{-1}$): $\tilde{\nu}$ = 3408 (s, NH), 1731 (m), 1650 (s), 1508 (s, ester, amide). $^1$H NMR ($CDCl_3/CD_3CN$): $\delta$ = 8.78 (d, $J$ = 8.5 Hz, 2H, H4′7′-neo); 8.31 (d, $J_{\alpha N}$ = 9.0 Hz, 1H, NH-$^1$Cys); 8.29 (d, $J_{\alpha N}$ = 7.7 Hz, 1H, NH-$^2$Cys); 8.15 (s, 2H, H5′6′-neo); 7.98 (d, $J$ = 8.5 Hz, 2H, H3′8′-neo); 7.85 (m$_c$, 2H, *m*-H Bz); 7.46–7.39 (m, 3H, *o,p*-H Bz); 4.61 (ddd, $J_{\alpha N}$ = 9.0 Hz, $J_{\alpha\beta}$ = 4.0 Hz, $J_{\alpha\beta'}$ = 4.8 Hz, 1H, C$_\alpha$H-$^1$Cys); 4.39 (ddd, $J_{\alpha N}$ = 7.7 Hz, $J_{\alpha\beta}$ = 4.2 Hz, $J_{\alpha\beta'}$ = 10.9 Hz, 1H, C$_\alpha$H-$^2$Cys); 4.12 (q, $J$ = 7.1 Hz, 2H, $CH_2$-OEt); 3.25 (dd, $J_{\alpha\beta}$ = 4.0 Hz, $^2J_{\beta\beta'}$ = 14.1 Hz, 1H, C$_\beta$H-$^1$Cys); 3.08 (s, 6H, 2′9′$CH_3$-neo); 3.04 (dd, $J_{\alpha\beta}$ = 4.2 Hz, $^2J_{\beta\beta'}$ = 12.3 Hz, 1H, C$_\beta$H-$^2$Cys); 3.02 (dd, $J_{\alpha\beta'}$ = 10.9 Hz, $^2J_{\beta\beta'}$ = 12.3 Hz, 1H, C$_{\beta'}$H-$^2$Cys); 2.98 (dd, $J_{\alpha\beta'}$ = 4.8 Hz, $^2J_{\beta\beta'}$ = 14.1 Hz, 1H, C$_{\beta'}$H-$^1$Cys); 1.22 (t, $J$ = 7.1 Hz, 3H, $CH_3$-OEt). $C_{29}H_{30}N_4O_4S_2Zn$ (628.1): calcd C 55.46, H 4.81, N 8.92, Zn 10.41; found: C 54.62, H 4.54, N 8.66, Zn 10.22; MH$^+$ found: 627.1 (ESI-MS).

**2b**: From **2** (0.19 g, 0.46 mmol), $Zn(ClO_4)_2 \cdot 6H_2O$ (0.17 g, 0.46 mmol), and neo (0.10 g, 0.46 mmol). Yield 0.19 g (60%), m.p. 192–194 °C (decomp.). IR (KBr, cm$^{-1}$): $\tilde{\nu}$ = 3402 (s, NH), 1733 (s), 1652 (s), 1511 (s, ester, amide). $^1$H NMR ($CDCl_3/CD_3CN$): $\delta$ = 8.93 (d, $J_{\alpha N}$ = 5.8 Hz, 1H, NH-$^3$Cys); 8.47 (d, $J_{3'4'}$ = 8.3 Hz, 1H, H4′-neo); 8.43 (d, $J_{7'8'}$ = 8.3 Hz, 1H, H7′-neo); 7.93 (s, 2H, H5′6′-neo); 7.87 (m$_c$, 2H, *o*-H Bz); 7.83 (d, $J_{3'4'}$ = 8.3 Hz, 1H, H3′-neo); 7.75 (d, $J_{7'8'}$ = 8.3 Hz, 1H, H8′-neo); 7.55 (dd, $J_{\alpha N}$ = 7.9 Hz, $J_{\alpha N}$ = 5.4 Hz, 1H, NH-$^2$Gly); 7.53 (m$_c$, 2H, *p*-H Bz); 7.46 (m$_c$, 2H, *m*-H Bz); 7.45 (d, $J_{\alpha N}$ = 7.1 Hz, 1H, NH-$^1$Cys); 4.65 (ddd, $J_{\alpha N}$ = 7.1 Hz, $J_{\alpha\beta}$ = 2.9 Hz, $J_{\alpha\beta'}$ = 5.8 Hz, 1H, C$_\alpha$H-$^1$Cys); 4.61 (ddd, $J_{\alpha N}$ = 5.8 Hz, $J_{\alpha\beta}$ = 6.6 Hz, $J_{\alpha\beta'}$ = 4.6 Hz, 1H, C$_\alpha$H-$^3$Cys); 4.61 (dd, $J_{\alpha N}$ = 7.9 Hz, $^2J_{\alpha\alpha'}$ = 17.2 Hz, 1H, C$_\alpha$H-$^2$Gly); 4.20 (q, $J$ = 7.1 Hz, 2H, $CH_2$-OEt); 3.88 (dd, $J_{\alpha N}$ = 5.4 Hz, $^2J_{\alpha\alpha'}$ = 17.2 Hz, 1H, C$_{\alpha'}$H-$^2$Gly); 3.29 (s, 3H, 2′$CH_3$-neo); 3.17 (s, 3H, 9′$CH_3$-neo); 3.14 (dd, $J_{\alpha\beta}$ = 6.6 Hz, $^2J_{\beta\beta'}$ = 12.6 Hz, 1H, C$_\beta$H-$^3$Cys); 3.08 (dd, $J_{\alpha\beta'}$ = 4.6 Hz, $^2J_{\beta\beta'}$ = 12.6 Hz, 1H, C$_{\beta'}$H-$^3$Cys); 2.82 (dd, $J_{\alpha\beta}$ = 2.9 Hz, $^2J_{\beta\beta'}$ = 12.5 Hz, 1H, C$_\beta$H-$^1$Cys); 2.54 (dd, $J_{\alpha\beta'}$ = 5.8 Hz, $^2J_{\beta\beta'}$ = 12.5 Hz, 1H, C$_{\beta'}$H-$^1$Cys); 1.29 (t, $J$ = 7.1 Hz, 3H, $CH_3$-OEt). $C_{31}H_{33}N_5O_5S_2Zn$ (685.2): calcd C 54.34, H 4.85, N 10.22, Zn 9.54; found: C 53.86, H 4.77, N 10.34, Zn 9.36.

**3b**: From **3** (0.50 g, 1.00 mmol), $Zn(ClO_4)_2 \cdot 6H_2O$ (0.37 g, 1.00 mmol), and neo (0.23 g, 1.00 mmol). Yield 0.46 g (60%), m.p. 200–202 °C (decomp.). IR (KBr, cm$^{-1}$): $\tilde{\nu}$ = 3500 (s), 3399 (s, NH), 1733 (s), 1652 (s), 1511 (s, ester, amide). $^1$H NMR ($CDCl_3/C_3CN$): $\delta$ = 8.60 (d, $J_{\alpha N}$ = 6.6 Hz, 1H, NH-$^3$Cys); 8.52 (d, $J$ = 8.6 Hz, 2H, H4′7′-neo); 7.96 (s, 2H, H5′6′-neo); 7.79 (d, $J$ = 8.6 Hz, 2H, H3′8′-neo); 7.73 (d, $J_{\alpha N}$ = 10.1 Hz, 1H, NH-$^2$Phe); 7.64 (m$_c$, 2H, *o*-H Bz); 7.43 (m$_c$, 2H, *p*-H Bz); 7.37 (m$_c$, 2H, *m*-H Bz); 7.33 (s, 5H, Aryl-Phe); 7.15 (d, $J_{\alpha N}$ = 7.0 Hz, 1H, NH-$^1$Cys); 4.96 (ddd, $J_{\alpha N}$ = 10.1 Hz, $J_{\alpha\beta}$ = 4.3 Hz, $J_{\alpha\beta'}$ = 9.4 Hz, 1H, C$_\alpha$H-$^2$Phe); 4.55 (ddd, $J_{\alpha N}$ = 6.6 Hz, $J_{\alpha\beta}$ = 6.2 Hz, $J_{\alpha\beta'}$ = 3.1 Hz, 1H, C$_\alpha$H-$^3$Cys); 4.42 (ddd, $J_{\alpha N}$ = 7.0 Hz, $J_{\alpha\beta}$ = 3.9 Hz, $J_{\alpha\beta'}$ = 9.4 Hz, 1H, C$_\alpha$H-$^1$Cys); 4.14 (q, $J$ = 7.0 Hz, 2H, $CH_2$-OEt); 3.37 (dd, $J_{\alpha\beta}$ = 4.3 Hz, $^2J_{\beta\beta'}$ = 14.0 Hz, 1H, C$_\beta$H-$^2$Phe); 3.16 (dd, $J_{\alpha\beta}$ = 6.2 Hz, $^2J_{\beta\beta'}$ = 14.0 Hz, 1H, C$_\beta$H-$^3$Cys); 3.01 (s, 6H, 2′9′$CH_3$-neo); 2.97 (dd, $J_{\alpha\beta}$ = 9.4 Hz, $^2J_{\beta\beta'}$ = 14.0 Hz, 1H, C$_{\beta'}$H-$^2$Phe); 2.94 (dd, $J_{\alpha\beta'}$ = 3.1 Hz, $^2J_{\beta\beta'}$ = 14.0 Hz, 1H, C$_{\beta'}$H-$^3$Cys); 2.78 (dd, $J_{\alpha\beta}$ = 3.9 Hz, $^2J_{\beta\beta'}$ = 12.1 Hz, 1H, C$_\beta$H-$^1$Cys); 2.61 (dd, $J_{\alpha\beta'}$ = 9.4 Hz, $^2J_{\beta\beta'}$ = 12.1 Hz, 1H, C$_{\beta'}$H-$^1$Cys); 1.23 (t, $J$ = 7.0 Hz, 3H, $CH_3$-OEt). $C_{38}H_{39}N_5O_5S_2Zn$ (775.2): calcd C 58.87, H 5.07, N 9.04, Zn 8.43; found: C 57.16, H 5.21, N 7.97, Zn 8.59; MH$^+$ found: 774.3 (ESI-MS).

**4b**: From **4** (0.53 g, 1.00 mmol), $Zn(ClO_4)_2 \cdot 6H_2O$ (0.37 g, 1.00 mmol), and neo (0.23 g, 1.00 mmol). Yield 0.40 g (50%), m.p. > 250 °C (decomp.). IR (KBr, cm$^{-1}$): $\tilde{\nu}$ = 3397 (s, NH), 1737 (s), 1653 (s), 1509 (s, ester, amide). $^1$H NMR ($CDCl_3/CD_3CN$): $\delta$ = 8.56 (d, $J_{3'4'}$ = 8.5 Hz, 1H, H4′-neo); 8.50 (d, $J_{7'8'}$ = 8.7 Hz, 1H, H7′-neo); 7.99 (s, 2H, H5′6′-neo); 7.85 (d, $J_{\alpha N}$ = 10.0 Hz, 1H, NH-$^3$Ile); 7.80 (d, $J_{3'4'}$ = 8.5 Hz, 1H, H3′-neo); 7.74 (m$_c$, 2H, *m*-H Bz); 7.71 (d, $J_{7'8'}$ = 8.7 Hz, 1H, H8′-neo); 7.41 (dd, $J_{\alpha N}$ = 6.5 Hz, $J_{\alpha' N}$ = 6.1 Hz, 1H, NH-$^2$Gly); 7.40–7.35 (m, 3H, *o,p*-H Bz); 7.36 (d, $J_{\alpha N}$ = 6.1 Hz, 1H, NH-$^4$Cys); 7.24 (d, $J_{\alpha N}$ = 7.4 Hz, 1H, NH-$^1$Cys); 4.52 (ddd, $J_{\alpha N}$ = 7.4 Hz, $J_{\alpha\beta}$ = 4.3 Hz, $J_{\alpha\beta'}$ = 6.5 Hz, 1H, C$_\alpha$H-$^1$Cys); 4.43 (dd, $J_{\alpha N}$ = 10.0 Hz, $J_{\alpha\beta}$ = 7.8, 1H, C$_\alpha$H-Ile); 4.28 (ddd, $J_{\alpha N}$ = 6.1 Hz, $J_{\alpha\beta}$ = 3.5 Hz, $J_{\alpha\beta'}$ = 9.1 Hz, 1H, C$_\alpha$H-$^4$Cys); 4.12 (q, $J$ = 7.1 Hz, 2H, $CH_2$-OEt); 4.02 (dd, $J_{\alpha N}$ = 6.5 Hz, $^2J_{\alpha\alpha'}$ = 16.9 Hz, 1H, C$_\alpha$H-$^2$Gly); 3.80 (dd, $J_{\alpha N}$ = 6.1 Hz, $^2J_{\alpha\alpha'}$ = 16.9 Hz, 1H, C$_{\alpha'}$H-$^2$Gly); 3.11 (s, 3H, 2′$CH_3$-neo); 3.07 (s, 3H, 9′$CH_3$-neo); 3.03 (dd, $J_{\alpha\beta}$ = 3.5 Hz, $^2J_{\beta\beta'}$ = 13.3 Hz, 1H, C$_\beta$H-$^4$Cys); 2.95 (dd, $J_{\alpha\beta'}$ = 9.1 Hz, $^2J_{\beta\beta'}$ = 13.3 Hz, 1H, C$_{\beta'}$H-$^4$Cys); 2.83 (dd, $J_{\alpha\beta}$ = 4.3 Hz, $^2J_{\beta\beta'}$ = 12.6 Hz, 1H, C$_\beta$H-$^1$Cys); 2.51 (dd, $J_{\alpha\beta'}$ = 6.5 Hz, $^2J_{\beta\beta'}$ = 12.6 Hz, 1H, C$_{\beta'}$H-$^1$Cys); 2.01 (m$_c$, 1H, C$_\beta$H-$^3$Ile); 1.58 (m$_c$, 1H, C$_\gamma$H-$^3$Ile); 1.29 (m$_c$, 1H, C$_\gamma$H-$^3$Ile); 1.21 (t, $J$ = 7.1 Hz, 3H, $CH_3$-OEt); 0.98 (t, $J_{\beta\gamma}$ = 6.9 Hz, 3H, C$_\gamma$H$_3$-$^3$Ile); 0.93 (dd, $J_{\gamma\delta}$ = 7.4 Hz, $J_{\gamma'\delta}$ = 8.2 Hz, 3H, C$_\delta$H-$^3$Ile). $C_{37}H_{44}N_6O_6S_2Zn$ (798.3): calcd C 55.67, H 5.56, N 10.53, Zn 8.19; found: C 55.12, H 5.34, N 10.45, Zn 7.98; MH$^+$ found: 797.9 (ESI-MS).

**Mass spectrometry**: Mass spectra were recorded on a tandem quadrupole instrument (TSQ 700, Finnigan) equipped with an electrospray interface. The samples obtained from the chromatographic separations were freed from the eluent in vacuo and dissolved in chloroform/acetonitrile (1:1). These solutions (2–3 μL) were injected by means of a syringe pump at 5 μL min$^{-1}$. The scan range of $m/z$ was from 150 to 1000 and could be extended to 2000. The $m/z$ values given for **1**, **3**, **4**, **1b**, **3b**, and **4b** are those for the peaks resulting from the isotopes $^1$H, $^{12}$C, $^{32}$S, and $^{64}$Zn.

**Structure determinations**: The samples of complexes **1b**–**4b** were dissolved in $CDCl_3/CD_3CN$ (1:1), the solutions were degassed in vacuo and handled in sealed 5 mm test tubes. DQF-COSY spectra were recorded on a Bruker AC200, NOESY spectra on a Varian Unity 300 machine. The number of NOE values obtained was 18 for **1b**, 23 for **2b** and **3b**, and 25 for **4b**. Of these, the nontrivial ones were 7 for **1b**, 8 for **2b**, 9 for **3b**, and 11 for **4b**. The number of dihedral angles extracted from the $^3J$ values was 6 for **1b**, 8 for **2b**, 9 for **3b**, and 10 for **4b**. The distances extracted from the NOESY spectra were calibrated by using the known distances within the aromatic neocuproine ligands. The NOESY spectra were thus used to confirm the assigned peptide sequences and to distinguish between the two cysteine units. The choice between the alternative torsional angles $\phi$ and $\chi$ extracted from the DQF-COSY spectra was aided by the distance values from the NOESY spectra and by the fact that both cysteine units are coordinated to the zinc ion, thereby making the peptides torsionally constrained chelate ligands [23]. The peptide geometries thus obtained and the known geometry of the neocuproine



0947-6539/97/0302-0266 $ 15.00+.25/0 Chem. Eur. J. 1997, 3, No. 2

ligand provided the basic structures of the complexes for the subsequent molecular mechanics calculations.

The calculations were performed by applying the procedure of hybrid distance geometry dynamical simulated annealing [31]. using the program XPLOR [24]. In order to include all parts of the complexes in the computations the molecular mechanics parameters of the terminal protecting groups (benzoyl, ethoxy) and the neocuproine ligand had to be defined based on their known geometries. Furthermore, starting values for the Zn–N (2.00 Å) and Zn–S (2.30 Å) distances as well as for the angles N-Zn-N (80.0°), N-Zn-S (109.5°), and S-Zn-S (135.0°) had to be assigned based on our experience with $ZnN_2S_2$ coordinated complexes [16,17,32]. These distances were allowed to vary by $\pm 0.02$ Å and the angles by $\pm 20°$. The neocuproine ligand was constrained to planarity. Within the peptide moieties the bond lengths were allowed to differ by $\pm 0.01$ Å and the bond angles by $\pm 2°$ from their standard values.

The refinement of the structures was performed by using the simulated annealing protocol of the program XPLOR [24]. In the first cycle the peptides were disconnected from the zinc ion for 4000 steps of a 1000 K molecular dynamics calculation. In the second cycle the Zn(neocuproine) unit was attached to the peptides again, and for the subsequent 2000 steps at 1000 K the NMR-derived distances and angles were taken into consideration. In the third cycle (3000 steps) the fictitious temperature was reduced to 100 K in 50 K decrements. This three-cycle procedure was performed 20 times yielding 20 different structures.

The whole sequence was then repeated for all 20 structures, but without disconnecting the peptides and the Zn(neocuproine) units and using a higher weighting for the NMR-derived distances and angles. The structures with unrealistic deviations from the experimental distances and angles (i.e., atomic distances differing by more than $\pm 0.5$ Å from the values obtained from the NOE's and dihedral angles differing by more than $\pm 5°$ from those derived from the $^3J$ values) were eliminated, leaving 5–8 "good" structures. The wire models representing superpositions of these "good" structures are shown above as Figures 1–4.

The rigidity of the complex structures thus obtained, that is, the precision of the determination of the solution structures, is obvious from the figures. This can be assessed quantitatively from the RMSD values for all atoms, obtained from the XPLOR program by comparing the individual structures with their common average structure. In the specific cases of complexes **1b–4b**, the RMSD values for the peptides are the smallest, ranging from 0.2 to 2 Å, followed by those for the protecting groups (benzoyl, ethoxy), ranging from 0.5 to 3.3 Å. Those for the neocuproine ligand are by far the largest, ranging from 1.5 to 5.5 Å, reflecting the lack of NMR-derived constraints for a rocking motion of the neocuproine ligand with respect to the $ZnS_2$ plane. The drawings were produced by the SCHAKAL program [33], after converting the atomic coordinates of all structures from the protein data bank (.pdb) format to the SCHAKAL (.dat) format.

**Acknowledgments:** This work was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie. We thank Dr. W. Deck and Dr. V. Brecht for help with the NMR, C. Vonrhein for computing instructions, P. Wlasak for recording the mass spectra, and F. Bitgül for preparative contributions.

Received: July 19, 1996 [F416]

[1] M. Ruf, R. Burth, K. Weis, H. Vahrenkamp, *Chem. Ber.* **1996**, *129*, 1251–1257.
[2] B. L. Vallee, D. S. Auld, *Acc. Chem. Res.* **1993**, *26*, 543–551.
[3] L. Bertini, C. Luchinat, W. Maret, M. Zeppezauer, *Zinc Enzymes*, Birkhäuser, Boston, **1986**.
[4] D. W. Christianson, *Adv. Protein Chem.* **1991**, *42*, 281–355.
[5] For a recent discussion see E. Magonet, P. Hayen, D. Delforge, F. Delaire, J. Remacle, *Biochem. J.* **1992**, *287*, 361–365.
[6] A. Klug, D. Rhodes, *Trends Biochem. Sci.* **1987**, *12*, 464–469. For a tabulation see B. A. Krizek, B. T. Amann, V. J. Kilfoil, D. L. Merkle, J. M. Berg, *J. Am. Chem. Soc.* **1991**, *113*, 4518–4523.
[7] T. H. Gibson, J. P. M. Postma, R. S. Brown, P. Argos, *Protein Eng.* **1988**, *2*, 209–218.
[8] B. F. Luisi, W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, P. B. Sigler, *Nature* **1991**, *352*, 497–505.
[9] B. L. Vallee, J. E. Coleman, D. S. Auld, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 999–1003.
[10] R. Marmorstein, M. Carey, M. Ptashne, S. C. Harrison, *Nature* **1992**, *356*, 408–414.
[11] M. J. Stillman, C. F. Shaw, K. T. Suzuki, *Metallothioneins* ,VCH, Weinheim, **1992**.
[12] A. H. Robins, D. E. McRee, M. Williamson, S. A. Collett, N. H. Xuong, W. F. Forey, B. C. Wang, C. D. Stout, *J. Mol. Biol.* **1991**, *221*, 1269–1293.
[13] J. S. Richardson, *Adv. Protein Chem.* **1981**, *34*, 167–339.
[14] J. A. Ippolito, D. W. Christianson, *Biochemistry* **1994**, *33*, 15241–15249.
[15] J. M. Berg, *Acc. Chem. Res.* **1995**, *28*, 14–19.
[16] M. R. Ghadiri, C. Choi, *J. Am. Chem. Soc.* **1990**, *112*, 1630–1632.
[17] U. Brand, H. Vahrenkamp, *Inorg. Chem.* **1995**, *34*, 3285–3293. *ibid.* **1996**, *35*, 1083–1086.
[18] H. Albrich, H. Vahrenkamp, *Chem. Ber.* **1994**, *127*, 1223–1233. A Meißner, P. Gockel, H. Vahrenkamp, *ibid.* **1994**, *127*, 1235–1241.
[19] C. G. Edmonds, R. D. Smith, *Methods Enzymol.* **1990**, *193*, 412–431.
[20] C. Khoo, R. J. Cousins, *Biochem. J.* **1993**, *299*, 445–450.
[21] A. Martin-Ponthieu, D. Wouters-Tyrou, B. Pudlo, E. Buisine, P. Sautière, *Eur. J. Biochem.* **1994**, *220*, 463–468.
[22] W. Haehnel, M. Hippler, unpublished results.
[23] *NMR and Biomolecular Structure* (Ed.: I. Bertini), VCH, Weinheim, **1991**. *NMR of Macromolecules* (Ed.: G. C. K. Roberts), Oxford University Press, Oxford, **1993**. *Two-Dimensional NMR Spectroscopy: Applications for Chemists and Biochemists* (Eds.: W. R. Croasman, R. M. K. Carlson), VCH, Weinheim, 2nd ed., **1994**.
[24] A. T. Brünger, XPLOR, Version 3.1, *A System for X-Ray Crystallography and NMR*, Yale University, New Haven, **1992**.
[25] D. T. Corwin, S. A. Koch, *Inorg. Chem.* **1988**, *27*, 493–496.
[26] K. J. Jordan, W. F. Wucholtz, G. A. Crosby, *Inorg. Chem.* **1991**, *30*, 4588–4593.
[27] R. E. Klevit, J. R. Herriott, S. Harvath, *Proteins* **1990**, *7*, 215–226.
[28] N. Pavletich, C. Pabo, *Science* **1991**, *252*, 809–817.
[29] J. G. Omichinski, G. M. Clare, M. Robien, K. Sakaguchi, E. Appella, A. M. Gronenborn, *Biochemistry* **1992**, *31*, 3907–3917.
[30] M. Förster, R. Burth, A. K. Powell, T. Eiche, H. Vahrenkamp, *Chem. Ber.* **1993**, *126*, 2643–2648.
[31] P. J. Kraulis, G. M. Clore, M. Nilges, T. A. Jones, G. Pettersson, J. Knowles, A. M. Gronenborn, *Biochemistry* **1989**, *28*, 7241–7257, and references therein.
[32] M. Melnik, K. Györyova, J. Skorsepa, C. E. Holloway, *J. Coord. Chem.* **1995**, *35*, 179–279.
[33] E. Keller, SCHAKAL-93, Universität Freiburg, **1993**.