Amino Acid Assay

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A Spiropyran-Based Ensemble for Visual Recognition and Quantification of Cysteine and Homocysteine at Physiological Levels**

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Spiropyrans and spirooxazines belong to one of the most fascinating families of photochromic compounds; they undergo reversible structural isomerization between a colorless spiro form and a colored merocyanine upon either light, heat, or chemical stimulus.[1] In organic solvents the conversion of spiropyran into merocyanine can be induced by complexation of a metal ion with cooperative ligation of another chelating functionality attached at the 8′-position.[2] Up to now, a number of such spiropyran receptors have been designed and widely applied for the optical detection of transition-metal and alkali-metal ions.[3] It should be pointed out, however, that despite the large number of reports, spiropyran structures have rarely been used to recognize anions or small organic molecules such as biologically important amino acids. Sunamoto et al. demonstrated early on that the zwitterionic feature of the opened merocyanine should enable it to bind with a polar amino acid molecule through electrostatic interactions; this approach was utilized for the photocontrolled transfer of amino acids across bilayers and membranes.[4] Later, Inouye et al. designed a spiropyridopyran capable of binding guanosine derivatives in nonhydroxylic organic solvents by forming triple hydrogen bonds.[5] And yet, development of new spiropyrans and spirooxazines probes capable of efficient recognition and quantification of amino acids in aqueous solution remains a challenge.[1b]

Of the twenty amino acids used as building blocks for proteins, the thiol-containing amino acids play crucial roles in biological systems. For example, cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) have been proven to be linked to various human diseases.[6] At present, a wide variety of colorimetric and fluorescent probes for thiol-containing amino acids and peptides are valuable. The majority of the reported methods are based on redox chemistry or labeling with chromophores or fluorophores and a combination of separation techniques.[7] Recently, Strongin et al. as well as several others have made pioneering advances in developing highly selective probes for Cys and Hcy, as well as a thiol-quantification enzyme assay based on the covalent interaction between the probe molecule and the analyte,[8,9] which could be used for the direct assay of the amino acid content and the enzyme activity in body fluids. In addition, a sensing ensemble for Cys was also recently developed by Kim et al. based on the analyte competing for a metal receptor with a chromogenic indicator.[10] The approach showed excellent selectivity for Cys over other amino acids including Hcy. Although many reactions and various techniques have been developed for assaying Cys and Hcy,[7–10] there is still plenty of room for improvement in term of selectivity, sensitivity, and performance with a new interaction mechanism.

Here we present a new strategy for the efficient recognition and determination of Cys and Hcy in neutral aqueous solution using a spiropyran. In our proposed approach the interaction of the free spiropyran with an amino acid is comparatively weak, but the spiropyran molecule can bind a metal ion, which can interact with an amino acid ligand. The metal ion is thus expected to bind with both the spiropyran and the amino acid molecule through cooperative metal–ligand interactions. If the three components exist together, the selectivity of the approach is essentially determined by the binding affinity between the metal center and the amino acid, as well as the relative size of the analyte. In this context, we have shown that in the presence of Cu^{2+} or Hg^{2+} ions the interaction of the spiropyran with Cys or Hcy is remarkably

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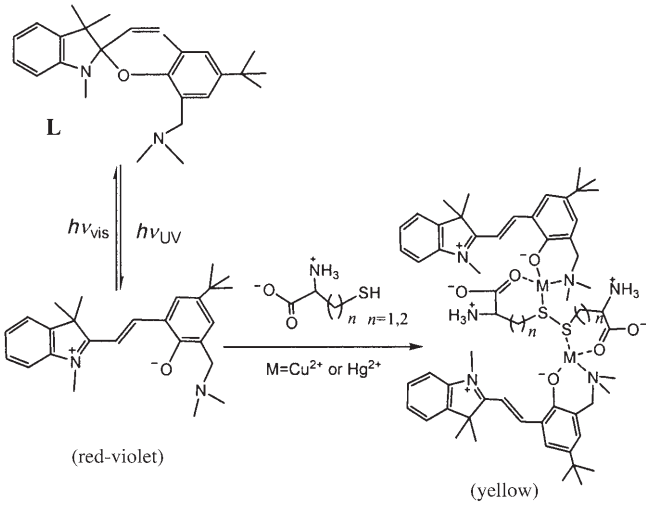
Experimental procedures, titration curves for the amino acids and anions, pH effects, fitting curves, and association constants, and mass, ^{1}H NMR, and IR spectra are available in the supporting information for this article on the WWW under http://www.angewandte.org or from the author.

selective and sensitive, resulting in appreciable changes in color and absorption properties.

We designed the free ligand (**L**) based on a 1,3′,3′-trimethylindolino-6′-*tert*-butylbenzopyran derivative with a *N*,*N*-dimethylaminomethyl moiety in the 8′-position (Scheme 1), which was employed previously for optical



Scheme 1. The free spiropyran ligand **L**, its isomerization on irradiation, and the proposed structure of the ternary complex containing **L**, Cys or Hcy, and metal ions.

recognition of Cu^{2+} ions in ethanol.[11] Figure 1 shows the changes in the absorption spectrum of **L** (1.0×10^{-4} M) in 10% aqueous ethanol at pH 7.0 in the presence of metal ions and amino acids. The absorption spectrum of the free **L** in ethanol/water is characterized by an intense absorption band centered at 532 nm ($\lg\varepsilon = 4.06\,M^{-1}cm^{-1}$) and a second band at approximately 378 nm. The UV/Vis spectrum of **L** hardly changes upon addition of 10 equiv of Cys (Figure 1, curves a and b), indicating there is little interaction between **L** and the amino acid. In contrast, when the same amount of Cys was added to the ethanol/water solution containing **L** and Cu^{2+}, or **L** and Hg^{2+}, the absorbance at 532 nm greatly decreased, while the

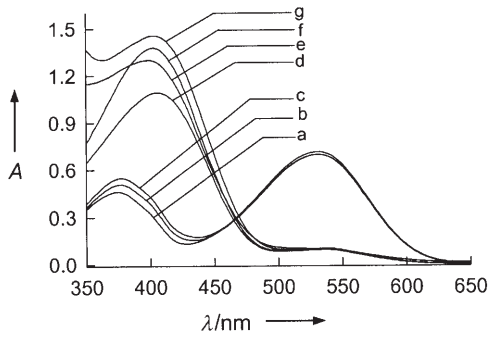


Figure 1. Effects of metal ions and amino acids on the absorption spectra of **L** in 10% aqueous ethanol solution at pH 7.0. The spectra correspond to: a) **L**, b) **L** + Cys, c) **L** + Zn^{2+} + Cys, d) **L** + Hg^{2+} + Hcy, e) **L** + Cu^{2+} + Cys, f) **L** + Hg^{2+} + Cys, and g) **L** + Cu^{2+} + Hcy. [**L**] = 1.0×10^{-4} M, [Cys] = [Hcy] = 1.0×10^{-3} M, [metal ion] = 2.5×10^{-4} M.

378-nm absorption band shifted to 405 nm concomitant with an increase in intensity (Figure 1, curves e and f). In contrast, in the presence of Zn^{2+} ions and Cys, the absorption spectrum of **L** changes only slightly (Figure 1, curve c). For Hcy, similar spectroscopic behavior was observed with both Hg^{2+} and Cu^{2+} ions (Figure 1, curves d and g), whereas the response sensitivity of **L** in the presence of Cu^{2+} ions is higher than that in the presence of Hg^{2+} ions. These results demonstrate that the interaction of **L** with Cys and Hcy is highly metal-ion-dependent. Cu^{2+} and Hg^{2+} ions can promote the binding of **L** to two different amino acids, which constitutes the basis for effective recognition of the amino acids with the proposed approach.

The selectivity of **L** in binding amino acids was studied by examining the amino acid induced changes in the color and UV/Vis spectra of ethanol/water solutions of **L** in the presence of Hg^{2+} or Cu^{2+} ions. The two metal ions show quite different effects on the color and absorption spectra of **L** in the presence of an amino acid; the most selective and distinctive binding was observed with Hg^{2+} ions. Addition of each amino acid, except glycine, to the ethanol/water solution of **L** and Cu^{2+} resulted in the shift of the absorption band of **L** and thus the color change.[12] Figure 2 shows a photograph of a

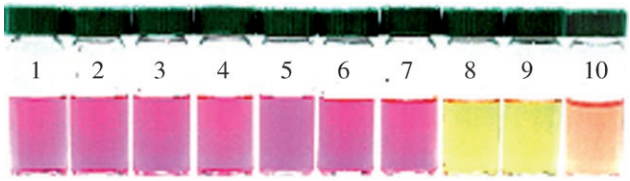


Figure 2. Color changes of the ethanol/water solution of **L**-Hg^{2+} in the presence of different amino acids. Labeled vials: 1) no amino acid, 2) Gly, 3) Leu, 4) His, 5) Glu, 6) Asp, 7) Met, 8) Cys, 9) Hcy, and 10) GSH. [**L**] = 1.0×10^{-4} M, [Hg^{2+}] = 2.5×10^{-4} M, [amino acid] = 1.0×10^{-3} M.

series of solutions of amino acids in buffered ethanol/water solution containing 1.0×10^{-4} M **L** and 2.5×10^{-4} M Hg^{2+}. Only Cys and Hcy induce a distinct color change from red-violet to yellow, which corresponds to the shift of the maximum absorption band from 532 nm to 405 nm. To quantify the spectral changes of **L** at 405 nm and 532 nm, the absorbance ratio of **L** at the two wavelengths ($R = A_{405}/A_{532}$) in the presence of 1.0×10^{-3} M of the amino acids and anions was determined.[12] Here R_0 is the value of R without the analyte. In the absence of metal ions, the values of $(R-R_0)$ are negligible except for solutions with Glu and GSH; in the presence of Cu^{2+} ions, obvious enhancements of the $(R-R_0)$ value are observed for each amino acid. The only selective enhancement of the $(R-R_0)$ value for Cys or Hcy was realized with Hg^{2+} ions. The results indicate that the **L**-Hg^{2+} system is appropriate for selective recognition of Cys and Hcy.

The dynamic response range of **L** to Cys and Hcy strongly depends on the amount of the metal ion present in the solution. For the best response to the amino acids, we optimized the metal ion concentration by holding the concentration of **L** constant (1.0×10^{-4} M) and varying the amount of Hg^{2+}. At a molar ratio of metal ions to **L** under 3.0,

both the sensitivity (response slope) and response range increase with increasing concentration of Hg^{2+}. At higher Hg^{2+} concentrations, the best response shifts from the lower concentration range to higher concentration range. Figure 3

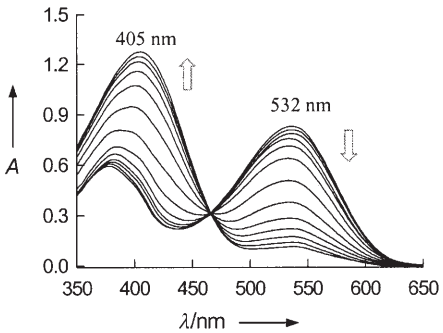


Figure 3. UV/Vis absorption spectra of **L**-Hg^{2+} in ethanol/water solution at pH 7.0 upon addition of Cys at varying concentrations. The arrows indicate the signal changes with increasing Cys concentration (0, 2.5×10^{-8}, 2.5×10^{-7}, 1.0×10^{-6}, 2.5×10^{-6}, 5.0×10^{-6}, 1.0×10^{-5}, 2.5×10^{-5}, 5.0×10^{-5}, 1.0×10^{-4}, 2.5×10^{-4}, 5.0×10^{-4}, and 1.0×10^{-3} M). [**L**] $=1.0\times10^{-4}$ M, [Hg^{2+}] $=2.5\times10^{-4}$ M.

shows the typical UV/Vis spectroscopic response of a buffered ethanol/water solution containing 1.0×10^{-4} M **L** and 2.5×10^{-4} M Hg^{2+} to increasing concentrations of Cys. At this metal ion concentration, a dramatic increase of the absorbance at 405 nm and a decrease of the absorbance at 532 nm are observed for the Cys concentration range of 5.0×10^{-6} M to 2.5×10^{-4} M. The detection limit, defined as 3σ in blank solution, is 4.0×10^{-8} M. The results suggest that our approach may be appropriate for quantification of Cys in physiological settings.[7a,8c] A similar response curve was obtained for Hcy.[12] By following the absorption change at 405 nm and a curve-fitting analysis, we estimate the stoichiometry of **L** with Cys or Hcy to be 2:2; the corresponding association constants in ethanol/water at pH 7.0 are $2.04\times10^{4}\,M^{-2}$ and $8.37\times10^{3}\,M^{-2}$,[12] respectively.

An important prerequisite for an amino acid probe is its ability to recognize a specific amino acid molecule in the presence of other biologically relevant substrates under physiological conditions. To further test the amino acid specificity of our system, complexation experiments with a range of other amino acids and anions were performed with **L**-Hg^{2+} ([**L**] $=1.0\times10^{-4}$ M, [Hg^{2+}] $=2.5\times10^{-4}$ M). The change of the absorbance ratio ($R-R_0$) was plotted against the concentration of different amino acids and anions (Figure 4). The ($R-R_0$) values increase linearly with the concentration of Hcy ($(0.1–1.0)\times10^{-3}$ M, $R^2=0.9903$) and the concentration of Cys ($(0.025–1.0)\times10^{-3}$ M, $R^2=0.9881$), which covers the upper limit of Cys concentration in normal organisms.[13] Although the system also responds to Glu as well at a concentration of 5.0×10^{-5} M, the value of ($R-R_0$) is substantially smaller than that caused by Cys or Hcy under the same conditions. The association constant of **L** with Glu in the presence of Hg^{2+} ions was $6.81\times10^{2}\,M^{-1}$. The amino acid selectivity factor for binding Cys over Glu, which was evaluated by comparing the association constants and the

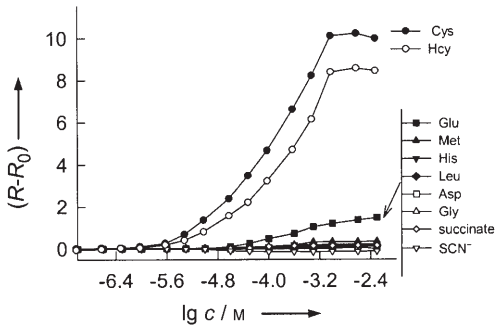


Figure 4. The dependence of the absorbance ratio of **L**-Hg^{2+} at 405 nm and 532 nm ($R=A_{405}/A_{532}$) on the increasing concentration of different amino acids and anions. R_0 is the value of R in the absence of amino acids or anions.

absorption signal changes at 405 nm with the relation $[K\Delta\varepsilon]$,[14] is approximately 206. Here K is the association constant and ε is the extinction coefficient of the amino acid complex at 405 nm. The selectivity factors of **L**-Hg^{2+} for the amino acids and anions studied here are in the order Cys > Hcy > GSH ⩾ Glu > Asp ≈ His ≈ Met > Leu ≈ Gly > succinate ≈ SCN^-. Additionally, titrating Cys in the presence of the potential biologically interfering ions Li^+, K^+, Ca^{2+}, Zn^{2+}, and SCN^- and amino acids such as Met and His^- gave a curve almost superimposable with that obtained in the presence of Cys alone (Figure 5). These results clearly indicate that our

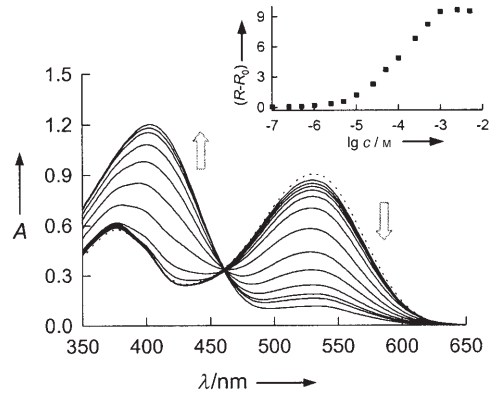


Figure 5. UV/Vis spectra of **L**-Hg^{2+} in ethanol/water solution in the presence of 1.0×10^{-3} M Li^+, Na^+, K^+, and Ca^{2+}, 1.0×10^{-4} M Zn^{2+}, and 1.0×10^{-5} M Met, His, and SCN^- with increasing amounts of Cys (1.0×10^{-7} M to 1.0×10^{-3} M). (The dashed line corresponds to the absorption spectrum of **L**-Hg^{2+} with only Cys). Inset: Absorbance ratio plotted against the logarithm of Cys concentration.

approach is not only insensitive to other cations, amino acids, and anions but also selective towards Cys and Hcy when they are present. Furthermore, it is possible to assay for Cys alone without interference from Hcy under physiological conditions. Although the response to HCy is linear in the concentration range of 1.0×10^{-4} M to 1.0×10^{-3} M, the total concentration of Hcy in human blood plasma is much lower than that of Cys.[13] In reality, Hcy would not significantly interfere with the measurement of Cys, which is important

and helpful in validation of the method to meet the selectivity requirements of a Cys assay.

The interactions of **L**, metal ion, and amino acid are pH dependent. To observe the effect of pH on the amino acid complexation, we conducted a more detailed study using an acidic solution (0.001 M HNO_3, $I = 0.1$) containing 1.0×10^{-4} M **L**, 2.5×10^{-4} M Hg^{2+}, and 1.0×10^{-4} M Cys in 10% aqueous ethanol solution, and subsequently the pH was adjusted by addition of 0.001 M NaOH. The pH values at which the complex formation and color change take place are in the range of 4.5–8.5 and thus a function of the pH of the medium. The A_{405}/A_{532} value reaches a maximum and remains constant between pH 4.5 and 8.5.[12] To further determine the complex equilibrium constant of **L** and Cys at various pH values, we performed potentiometric pH titrations of **L** with Cys in the absence and presence of Hg^{2+} ions using commercially available glass electrodes.[12] The titration data were analyzed with the "BEST" program,[15] and the resulting complex formation constants are $2.03 \times 10^2\,M^{-2}$ (without Hg^{2+}) and $1.52 \times 10^4\,M^{-2}$ (2.5×10^{-4} M Hg^{2+}), respectively. The value in the presence of Hg^{2+} ions shows little deviation from that obtained by spectrophotometric titration at pH 7.0. In the present work, we deemed pH 7.0 to be ideal.

The exact stoichiometry and mechanism of the interaction between the metal ion, the ligand **L**, and the amino acid were key aspects that were not clearly understood. We thus first studied the effect of varying the concentration of the metal ion on the absorption spectra of **L** in the ethanol/water solution with and without Cys. In the absence of Cys, the absorption spectrum of **L** in 10% aqueous ethanol solution is hardly affected upon addition of Hg^{2+} or Cu^{2+} ions. In the presence of Cys and with increasing metal ion concentration, the absorbance of **L** at 532 nm decreases significantly and that at 405 nm increases. In contrast, the effect of Zn^{2+} or Cd^{2+} ions on the absorbance of **L** at either 532 or 405 nm is negligible even in the presence of excess of Cys or Hcy. By following a curve-fitting analysis, we estimate the stoichiometry of the complex of **L** with either Cu^{2+} or Hg^{2+} ions to be 2:2. In addition, in the ESI mass spectrum of an ethanol solution containing 1.0×10^{-5} M **L**, 2.5×10^{-5} M $Cu(NO_3)_2$, and 1.0×10^{-5} M Cys, a peak at m/z 1269.4, which is assigned to $[2\mathbf{L} + 2Cu + 2Cys + 2NO_3 - 2H]^+$, is clearly observed,[12] indicating that in aqueous ethanol solution, the sulfhydryl group of Cys may be deprotonated to form a S–S-linked dimer (cystine) through a two-electron oxidation process.[16,17] To clarify whether this oxidation process is the result of dioxygen or Cu^{2+} (or Hg^{2+}) interactions, we measured the optical response of **L** to Cys under both aerobic and anaerobic conditions with and without the metal ion.[12] In the absence of Cu^{2+} or Hg^{2+} ions **L** exhibits minimal spectral changes at increasing Cys concentrations even though the analyte solution was saturated with dioxygen. On the other hand, the response curves of **L**-Cu^{2+} or **L**-Hg^{2+} to Cys under anaerobic conditions were almost the same as those obtained under aerobic conditions.[12] This results suggest that the oxidation of Cys in the present system is indeed promoted by the redox-active Cu^{2+} and Hg^{2+} ions.[16,17] Additional evidence for the formation of the S–S bridge (cystine) in the complexation process can be obtained from the spectroscopic responses of **L**-Cu^{2+} to cystine and Met (it could not form the S–S linked dimer). The response of **L**-Cu^{2+} with cystine is similar to that with Cys, whereas that with Met is considerable weaker under analogous conditions. Based on these results, we thus could presume that in the present system Cys is deprotonated at the sulfhydryl group to form a bridged dimer (cystine) through the redox-active Cu^{2+} or Hg^{2+} ions, then the metal center complexes with one cystine molecule and two **L** molecules to form a 2:1:2 (Cu^{2+}/cystine/**L**) ternary assembly. Disappearance of the carboxyl and sulfhydryl absorptions of Cys in the IR spectra, and the pronounced downfield shifts of the methylene protons as well as the upfield shifts of the *N*,*N*-dimethylamino protons of **L** in the ^1H NMR spectra indicate that the binding interactions among the metal center, **L**, and cystine occur through the phenolate hydroxy group and the dimethylamino group of **L** and the sulfhydryl groups of the amino acid.

To further elucidate the roles of the binding sites of **L** in the complex formation, a reference compound, 2-dimethylaminomethylphenol (DAMP) was examined. Under comparable conditions, DAMP exhibits a absorption maximum at 274 nm, the intensity of which is enhanced ($\approx 12\%$) upon addition of 0.5–10 equiv of Cys.[12] However, in the presence of Cu^{2+}, a great enhancement of the DAMP absorption at 274 nm was observed upon addition of 0.5–10 equiv of Cys (3.29 times the initial value). The results clearly indicate that involvement of the phenolate hydroxy group and the dimethylamino group of **L** is reasonable in complex formation. A proposed structure of the assembled complex of **L**, metal ion, and Cys is shown in Scheme 1. The formation of the copper complex would lead to the disappearance of the **L** charge-transfer band and the appearance of blue-shifted band that probably arises from a new charge-transfer involving the orbitals of the metal center, which causes the color change of **L** from red-violet to yellow ($\lg \varepsilon^{405\,nm} = 3.712\,M^{-1}\,cm^{-1}$).

In conclusion, we have proposed a new method that allows simple and efficient recognition and quantification of an amino acid in aqueous solution by a spiropyran derivative. The molecular recognition process has been proven to be sensitive to the presence of Cys and Hcy. To the best of our knowledge, this is the first spiropyran–metal receptor for amino acids, which might open up new opportunities for the application of spiropyrans and spirooxazines. Current studies in our laboratory along these lines include developing more sophisticated systems based on the same mechanism for recognition of amino acids or anions of various types and complexities.

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[1] a) R. Guglielmetti in *Photochromism*: *Molecules and Systems*, *Studies in Organic Chemistry* (Eds.: H. Dürr, H. Bouas-Laurent), Elsevier, Amsterdam, **1990**, chap. 8 and 23, and references

therein; b) G. Berkovic, V. Krongauz, V. Weiss, *Chem. Rev.* **2000**, *100*, 1741 – 1753.
[2] a) J. Phillips, A. Mueller, F. Przystal, *J. Am. Chem. Soc.* **1965**, *87*, 4020; b) L. D. Taylor, J. Nicholson, R. B. Davis, *Tetrahedron Lett.* **1967**, *8*, 1585 – 1588.
[3] Selected examples: a) M. Inouye, Y. Noguchi, K. Isagawa, *Angew. Chem.* **1994**, *106*, 1226 – 1228; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1163 – 1166; b) J. D. Winkler, C. M. Bowen, V. Michelet, *J. Am. Chem. Soc.* **1998**, *120*, 3237 – 3242; c) A. M. A. Salhin, M. Tanaka, K. Kamada, H. Ando, T. Ikeda, Y. Shibutani, S. Yajima, M. Nakamura, K. Kimura, *Eur. J. Org. Chem.* **2002**, 655 – 662; d) R. A. Kopelman, S. M. Snyder, N. L. Frank, *J. Am. Chem. Soc.* **2003**, *125*, 13684 – 13685; e) H. Sakamoto, H. Takagaki, M. Nakamura, K. Kimura, *Anal. Chem.* **2005**, *77*, 1999 – 2006.
[4] a) J. Sunamoto, K. Iwamoto, Y. Mohri, T. Kominato, *J. Am. Chem. Soc.* **1982**, *104*, 5502 – 5504; b) S. Marx-Tibbon, I. Willner, *J. Chem. Soc. Chem. Commun.* **1994**, 1261 – 1262.
[5] M. Inouye, K. Kim, T. Kitao, *J. Am. Chem. Soc.* **1992**, *114*, 778 – 780.
[6] a) F. J. T. Staal, S. W. Ela, M. Roederer, M. T. Ansderson, L. A. Herzenberg, *Lancet* **1992**, *339*, 909 – 913; b) K. S. McCully, *Nat. Med.* **1996**, *2*, 386 – 389.
[7] For recent reviews, see: a) O. Nekrrassova, N. S. Lawrence, R. G. Compton, *Talanta* **2003**, *60*, 1085 – 1095; b) R. P. Haugland, *Handbook of Fluorescent Probes and Research Products*, 9th ed., Molecular Probes, Eugene, OR, **2002**, pp. 79 – 98.
[8] a) O. Rusin, N. N. St. Luce, R. A. Agbaria, J. O. Escobedo, S. Jiang, I. M. Warner, F. B. Dawan, K. Lian, R. M. Strongin, *J. Am. Chem. Soc.* **2004**, *126*, 438 – 439; b) W. H. Wang, J. O. Escobedo, C. M. Lawrence, R. M. Strongin, *J. Am. Chem. Soc.* **2004**, *126*, 3400 – 3401; c) W. Wang, O. Rusin, X. Xu, K. K. Kim, J. O. Escobedo, S. O. Fakayode, K. A. Fletcher, M. Lowry, C. M. Schowalter, C. M. Lawrence, F. R. Fronczek, I. M. Warner, R. M. Strongin, *J. Am. Chem. Soc.* **2005**, *127*, 15949 – 15958, and references therein.
[9] H. Maeda, H. Matsuno, M. Ushida, K. Katayama, K. Saeki, N. Itoh, *Angew. Chem.* **2005**, *117*, 2982 – 2985; *Angew. Chem. Int. Ed.* **2005**, *44*, 2922 – 2925.
[10] M. S. Han, D. H. Kim, *Tetrahedron* **2004**, *60*, 11251 – 11257.
[11] N. Shao, Y. Zhang, S. M. Cheung, R. H. Yang, W. H. Chan, T. Mo, K. A. Li, F. Liu, *Anal. Chem.* **2005**, *77*, 7294 – 7303.
[12] See the Supporting Information.
[13] The total concentration of homocysteines in healthy plasma is 5–15 μM. Cysteine concentrations are typically 20–30 times that of homocysteine (see Refs. [7a], [8c]).
[14] J. Z. Zhao, T. M. Fyles, T. D. James, *Angew. Chem.* **2004**, *116*, 3543 – 3546; *Angew. Chem. Int. Ed.* **2004**, *43*, 3461 – 3464.
[15] A. E. Martell, R. J. Motekaitis, *Determination and Use of Stability Constants*, 2nd ed, VCH, New York, **1992**.
[16] M. Belcastro, T. Marino, N. Russo, M. Toscano, *J. Mass Spectrom.* **2005**, *40*, 300 – 306.
[17] F. Jalilehavand, B. O. Leung, M. Izadifard, E. Damian, *Inorg. Chem.* **2006**, *45*, 66 – 73.

