Enantioselective Fluorescence Quenching by a Chiral Copper(II) Complex in Ligand Exchange Equilibria

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The first case of enantioselective fluorescence quenching of dansyl(DNS)-amino acids (**2**) by a chiral complex, bis-(L-phenylalanylamidato)copper(II) (**1**) in aqueous solution is reported. The nature of the quenching, whether static or dynamic, has been investigated by time-resolved fluorescence techniques. The fluorescence lifetimes of the unquenched D- and L-DNS-phenylalanine were found to be the same as the quenched ones: 90% of the total fluorescence intensity for all samples was due to a 8.9 ns component. Thus, the decrease in the fluorescence intensities was ascribed to ground state interactions between the dansyl moiety and the copper complex (static quenching). From the Stern–Volmer plot it was possible to calculate the formation constants of the ternary diastereomeric complexes formed according to a ligand exchange reaction. The difference between the logarithms of the stability constants of these complexes accounts for the enantioselectivity observed in aqueous solutions. The present results are relevant also for the understanding of the mechanism of chiral recognition in HPLC using chiral copper(II) complexes as additives to the eluent (Chiral Ligand Exchange Chromatography), and must be taken into account for correct quantitative determination.

Chiral discrimination is currently a field of great interest both from the mechanistic[1] and the application[2] points of view. The nature and the magnitude of the binding and repulsive interactions occurring between the chiral selector and the enantiomers are studied by means of spectroscopic methods, thermodynamics of diastereomeric complex formation and chromatography.

In Chiral Ligand Exchange Chromatography (CLEC), first introduced by Davankov,[3] the separation of two optical isomers is accomplished by inducing repetitive diastereomeric interactions with a chiral metal complex bound to the column stationary phase. Enantioselectivity is essentially the result of the difference between the free energies of association of the diastereomeric ternary complexes $[-\Delta_{R,S}(\Delta G^{\circ})]$ thus formed. In a more versatile version of this method, Gil-Av and coworkers[4] proposed the utilization of reversed-phase columns and chiral metal(II) complexes added to the mobile phase. In these systems enantioselectivity appears to be a complex function of discriminating interactions occurring both in the mobile and in the stationary phase. It generally involves not only ligand-exchange equilibria, but also phase distribution of the selector–enantiomer adducts, unless the chiral selector resides entirely in one of the phases.[5]

In order to study the enantioselectivity effects, we have recently utilized chiral copper(II) complexes of L-amino acid amides, which enabled us to separate most naturally occurring D- and L-amino acids as dansyl (DNS) derivatives on commercial reversed-phase columns (RP-C_{18}).[6]

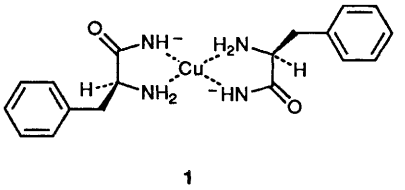
With our selectors, but also with others reported in the literature,[7] we noticed a different fluorescence response of the two dansyl-amino acid enantiomers, thus implying caution in the evaluation of enantiomeric excess by this method (chiral eluents and fluorescence detection). On the other hand enantioselectivity in fluorescence represents an intriguing photophysical phenomenon which can eventually provide information about the interactions and equilibria responsible for chiral discrimination.

Only a few examples of enantioselective photophysical behaviour due to intermolecular interactions are reported in the literature.[8] In particular, chiral discrimination in the formation of excimers was reported by Fendler[9] and enantioselective fluorescence quenching of (*R*)-1,1′-binaphthyl by chiral amines was studied by Irie.[10] More recently, Avnir and Ottolenghi[11] reported quenching of a binaphthyl-modified silica surface as a model for chiral recognition in chromatographic systems.

Copper(II) is known to induce fluorescence quenching of DNS-amino acids and dipeptides.[12] We now report the first case of enantioselective fluorescence quenching by a chiral copper(II) complex in aqueous solution. It is the purpose of the present paper to investigate the nature of the quenching, whether static or dynamic, in order to obtain information about the interactions responsible for chiral recognition. The results may be relevant for a better understanding of the mechanism of CLEC.

Results

The chiral complex bis(L-phenylalanylamidato)copper(II) [Cu-(L-PheNH)$_2$] **1** is used as additive to the aqueous mobile phase in HPLC (reversed phase) for the enantiomeric separation of DNS-amino acids **2**, as previously described.[6]



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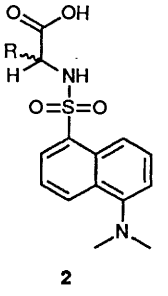

2

The observed elution order is $k'_L < k'_D$ for apolar amino acids and $k'_D < k'_L$ for polar ones. Although racemic mixtures of DNS-amino acids are injected, the peak areas of the D-enantiomers, obtained by a fluorescence detector, are always

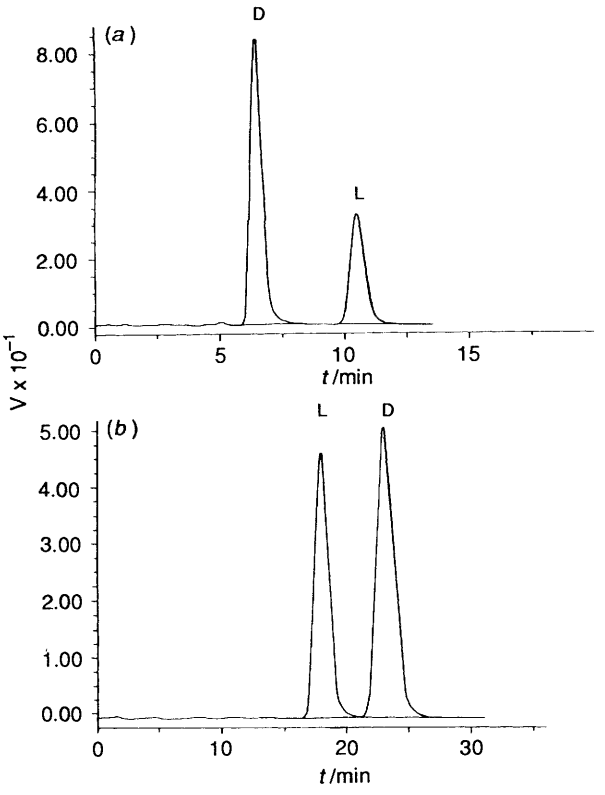

Fig. 1 Enantiomeric separation of DNS-glutamic acid (*a*) and DNS-phenylalanine (*b*) by HPLC (reversed phase) by using [Cu(L-Phe-NH)$_2$] **1** (2×10^{-3} mol dm^{-3}) as additive to the eluent (H_2O–CH_3CN, 70:30; pH = 7.5; 0.3 mol dm^{-3} CH_3COONa)

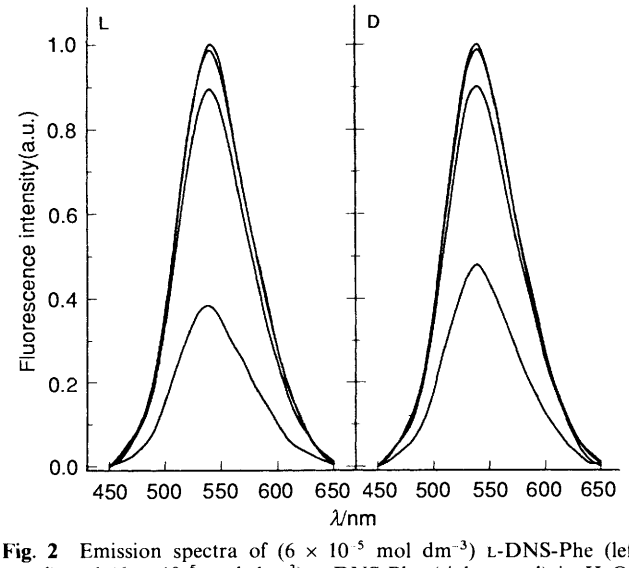

Fig. 2 Emission spectra of (6×10^{-5} mol dm^{-3}) L-DNS-Phe (left panel) and (6×10^{-5} mol dm^{-3}) D-DNS-Phe (right panel) in H_2O–CH_3CN, 80:20; from top to bottom: in the absence of quenchers; in the presence of (6×10^{-5} mol dm^{-3}) copper(II); in the presence of (6×10^{-5} mol dm^{-3}) Cu(Phe-NH)$_2$; in the presence of (36×10^{-5} mol dm^{-3}) Cu(Phe-NH)$_2$. Control spectra of D- and L-DNS-Phe were normalized to the same intensity. Excitation wavelength was 330 nm.

higher than those of the corresponding L-enantiomers, independently of the elution order. In Fig. 1 chiral separation of racemic DNS-glutamic acid (DNS-Glu) and DNS-phenylalanine (DNS-Phe) are reported.

The effect is more pronounced for polar amino acids, especially for D-DNS-aspartic acid (D-DNS-Asp) and D-DNS-Glu, which show response factors almost double those of the corresponding L-forms.

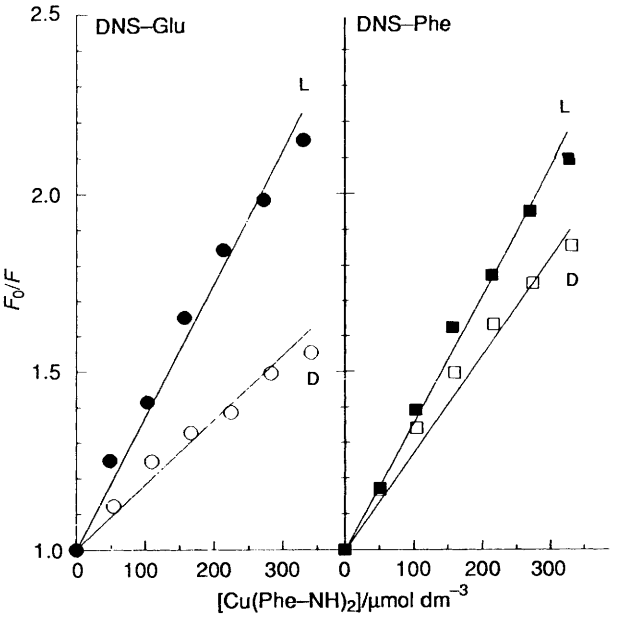

Fig. 3 Stern–Volmer plots of fluorescence quenching of D- and L-DNS-Glu (6×10^{-5} mol dm^{-3}) (left panel) and D- and L-DNS-Phe (6×10^{-5} mol dm^{-3}) (right panel) upon addition of increasing amount of complex **1** in H_2O–CH_3CN, 80:20, buffered at pH 7.5 with 0.3 mol dm^{-3} sodium acetate. D-Enantiomers are represented by open symbols, L-enantiomers by filled symbols.

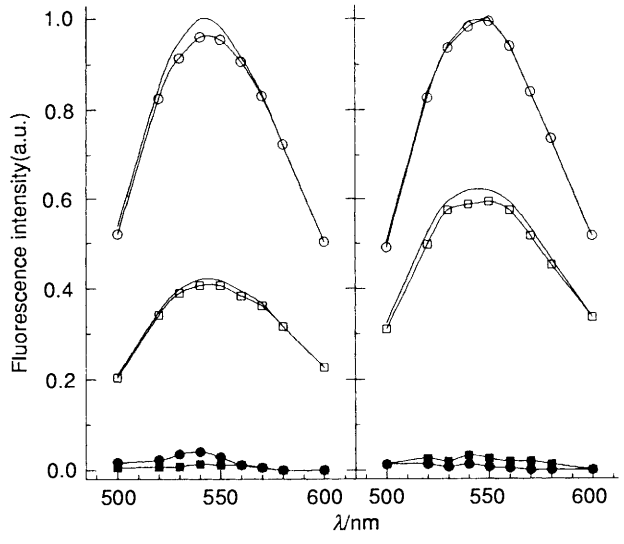

Fig. 4 Decay associated spectra of (6×10^{-5} mol dm^{-3}) L-DNS-Phe (left panel) and of (6×10^{-5} mol dm^{-3}) D-DNS-Phe (right panel) in CH_3CN–H_2O, 60:40, at pH 7.5, 0.3 mol dm^{-3} CH_3COONa, in the absence (circles) and in the presence of (18×10^{-5} mol dm^{-3}) Cu(Phe-NH)$_2$ (squares). Open symbols refer to the long lived component [8.9 (0.1) ns] and filled symbols to the short lived component [3.4 (1.0) ns]. The lines above the open symbols represent the static emission spectra of the various species. The larger standard error (in parentheses) associated with the short lived component is mainly due to the low fluorescence intensity of a small amount of the emitting species.

In order to investigate the origin of this effect in the HPLC peak response, excitation and emission fluorescence spectra of D- and L-DNS-Glu and D- and L-DNS-Phe were recorded in water–acetonitrile solutions in the absence and in the presence of copper(II) (as copper acetate) and of the chiral copper(II) complex **1**. In this medium both dansyl substrates have absorption bands near 330 nm, which give rise to fluorescence emission at 545 nm. Results for D- and L-DNS-Phe are reported in Fig. 2.

Upon addition of complex **1** (up to a six-fold excess) to the DNS-amino acid enantiomers, the following features are

Table 1 Logarithms of the formation constants (log K_D and log K_L) of the ternary mixed complexes [Cu(L-PheNH)$_2$(DNS-AAH$_{-1}$)] as calculated from fluorescence measurements at 21 °C; pH = 7.5; 0.3 mol dm^{-3} CH_3COONa

AA	Q	CH_3CN(%)	log K_D	log K_L	Δlog $K_{D,L}$
DNS-Phe	Cu^{2+}	40	3.14(2)	3.15(1)	0.01
DNS-Phe	Cu(L-Phe-NH)$_2$	40	3.59(3)	3.69(3)	0.10
DNS-Phe	Cu(L-Phe-NH)$_2$	20	3.44(3)	3.55(3)	0.11
DNS-Glu	Cu(L-Phe-NH)$_2$	20	3.24(3)	3.57(3)	0.33

observed: (i) the fluorescence intensity of both enantiomers is markedly decreased, and to a larger extent than was observed with the copper(II) ion alone; (ii) the fluorescence intensity of the D-enantiomer is always higher than that of the L-enantiomer; (iii) the shapes and maxima of the emission and excitation spectra are unchanged as compared with those obtained in the absence of complex **1**; (iv) the difference in the fluorescence intensity between D- and L-DNS-Glu is larger than that between D- and L-DNS-Phe, as is clear from the Stern–Volmer plot reported in Fig. 3.

In order to determine the type of the quenching mechanism, whether static or dynamic,[13] we measured the fluorescence lifetimes of D- and L-DNS-Phe alone and in the presence of either copper(II) ion or of the chiral complex **1**, in H_2O–CH_3CN (60:40). A biexponential function $F_t = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}$ was used for the analysis of fluorescence decay data. In all cases, more than 90% of the observed fluorescence is found to be due to a component having a 8.9 ns lifetime, which remains unchanged upon addition of either the copper(II) ion or of the [Cu(L-PheNH)$_2$] complex. As shown in Fig. 4, the fluorescence intensity decreases to a different extent for D- and L-DNS-Phe, whereas the fluorescence lifetime of the excited state is unaffected for both enantiomers, suggesting that static quenching is most likely to occur, and that the fluorescence observed is that due to the unquenched fluorophores ($\tau_0 = \tau$).

It is thus possible to achieve a quantitative measure of the affinity of complex **1** for the dansyl-amino acid by applying the Stern–Volmer equation[13] [eqn. (1)] where K is the formation

$$F_0/F = 1 + K[Q] \quad (1)$$

constant of the complex between the fluorophore and the quencher, F_0 and F are the fluorescence intensities of the fluorophore (DNS-AA) in the absence and in the presence of the quencher **1** respectively and [Q] is the concentration of the free quencher. Fig. 3 reports the Stern–Volmer plots for D- and L-DNS-Glu and D- and L-DNS-Phe under the same conditions (H_2O–CH_3CN, 80:20; pH = 7.5; 0.3 mol dm^{-3} CH_3COONa). The difference in the fluorescence of the two enantiomers increases linearly upon addition of increasing quantities of complex **1**.

Table 1 reports the logarithms of the Stern–Volmer constants for D- and L-DNS-Phe and D- and L-DNS-Glu under various conditions. As expected, the stability constants are practically identical for D- and L-DNS-Phe in the presence of the bare copper(II) ion, whereas they are significantly different in the presence of the chiral complex **1**.

Complex **1** shows a much higher enantioselectivity towards the polar DNS-Glu (Δlog $K_{D,L}$ = 0.33) than the apolar DNS-Phe (Δlog $K_{D,L}$ = 0.11), under the same conditions. This is mainly due to the less favourable association constant of D-DNS-Glu relative to that of D-DNS-Phe, whereas the association of the L-enantiomers is almost the same for both amino acids.

Upon decreasing solvent polarity (from 20% to 40% CH_3CN) the association constants of both D- and L-DNS-Phe increase, and thus the selectivity remains practically unchanged (Δlog $K_{D,L}$ = 0.10).

Discussion

Nature of the Enantioselective Effect.—The molecular mechanism of fluorescence quenching can be either dynamic or static.[13] In the former the excited state of the fluorophore undergoes radiationless decay, losing energy by collision with the quencher: as the concentration of the quencher increases, the probability of collision increases and hence both the fluorescence intensity and the excited state lifetime decrease. On the contrary, in the latter a non-fluorescent fluorophore–quencher adduct is formed in the ground state and only residual fluorescence due to the uncomplexed fluorophore is observed: since the emitting species is always the same, no change in the excited state lifetime is observed.

Time-resolved spectra of D- and L-DNS-Phe performed in the presence and in the absence of excess **1** show that the 90% of the total fluorescence intensity for all samples is due to the 8.9 ns component and that the excited state lifetime remains essentially unchanged in all cases, although the fluorescence intensity is strongly decreased. From these results it is clear that only static quenching is involved and that non-fluorescent ternary complexes are most likely to be formed. Although we cannot exclude the possibility that mixed complexes with residual fluorescence could eventually be present, nevertheless, it seems very unlikely that they would have the same spectroscopic properties as the uncomplexed species.

If only static quenching is present, then the Stern–Volmer constants correspond to the association constants between the fluorophore (DNS-AA) and the quencher **1**.

Ligand Exchange Equilibria.—We have previously reported[14] the equilibria of formation of the binary species formed by copper(II) and several L-amino acid amides in aqueous solution. The predominant species present in the pH range 7–8 were found to be [Cu(PheNH)]$^+$, [Cu(PheNH)(PheNH$_2$)]$^+$ and [Cu(PheNH)$_2$]. The last species, which is practically unique at pH > 9, has been characterized by UV–VIS and CD spectroscopy. In particular, with L-phenylalaninamide, the species [Cu(PheNH)$_2$] was isolated as crystals and its structure was determined by X-ray crystallography.[15]

However, species distribution diagrams obtained with the program SPE© indicated that, with low copper concentrations (6×10^{-5} mol dm^{-3}) and a 1:2 metal to ligand ratio, the [Cu(PheNH)]$^+$ is the major species present at pH = 7.5, owing to the dissociation equilibria of eqns. (2) and (3).

$$[Cu(PheNH)_2] + H^+ \rightleftharpoons [Cu(PheNH)]^+ + PheNH_2 \quad (2)$$

$$[Cu(PheNH_2)(PheNH)]^+ \rightleftharpoons [Cu(PheNH)]^+ + PheNH_2 \quad (3)$$

It is feasible that the species involved in the formation of diastereomeric mixed complex is mainly the 1:1 copper(II) complex of the deprotonated amide [Cu(PheNH)]$^+$.

On the other hand, DNS-glycine was shown[16] to form binary complexes with copper(II), mainly of the 1:1 type at pH = 7.5. The major species is [Cu(DNS-AAH$_{-1}$)] in which the dansyl amino acid is bound to the copper(II) ion *via* the carboxylate

and the deprotonated sulfonamide nitrogen, as observed in the solid state by X-ray crystallography.[17]

Therefore, we can assume that equilibrium (4) occurs, leading to formation of ternary complexes of the type [Cu(PheNH)-(DNS-AAH$_{-1}$)].

$$\text{DNS-AA}^- + [\text{Cu(PheNH)}]^+ \overset{K_e}{\rightleftharpoons} [\text{Cu(PheNH)(DNS-AAH}_{-1})]^- + \text{H}^+ \quad (4)$$

The Stern–Volmer constant therefore, corresponds to the conditional constant

$$K' = K_e/[\text{H}^+].$$

Thus, the observed enantioselectivity in fluorescence is accounted for by the different stability of the diastereomeric complexes formed.

On the other hand, under the conditions utilized in HPLC (higher concentration of **1**), the species [Cu(PheNH)$_2$] and [Cu(PheNH)(PheNH$_2$)]$^+$ are present in higher percentages, so that the formation of the ternary diastereomeric complexes can be actually viewed as a 'ligand exchange' reaction, which is the sum of equilibria (3) and (4) or (2) and (4). Since the only contribution which gives rise to enantioselectivity comes from equilibrium (4), the fluorescence experiments should account for the overall enantioselectivity observed both in diluted and in concentrated solution.

The association constants are almost the same for both the L-enantiomers (log K_L = 3.57 for L-DNS-Glu and log K_L = 3.55 for L-DNS-Phe in 20% H_2O–CH_3CN), whereas they are different for the D-enantiomers (log K_D = 3.24 for D-DNS-Glu and log K_D = 3.44 for D-DNS-Phe). This effect might be accounted for by a less unfavourable interaction between the D-phenylalanine aromatic side chain and the dansyl moiety, than that occurring with D-glutamic acid. In a less polar solvent (40% CH_3CN) both diastereomeric complexes are more stable, as expected, so that the enantioselectivity remains practically the same.

Chromatographic Implications.—In most cases the chromatographic separation is accounted for by a number of equilibria occurring both in the mobile and in the stationary phase, together with partition equilibria of the different species between the phases.[5] The present equilibria in aqueous solutions do not account for the overall enantioselectivity observed in the chromatographic system. In fact, if this were the case, we should observe the same elution order for polar and apolar amino acids. Instead, while the relative stabilities are consistent with the elution order for DNS-Phe ($k'_D > k'_L$), the most stable ternary complex being eluted first, the opposite occurs for DNS-Glu ($k'_D < k'_L$).

Therefore, our results indicate that the contribution to enantioselectivity in the stationary phase is in most cases the key point and must not be neglected.

In particular, for DNS-Glu, we can infer that the stability constants of the diastereomeric ternary complexes adsorbed on the stationary phase should be in the order $K_D < K_L$.

In conclusion, indeed, enantioselective fluorescence quenching may be induced by chiral copper(II) complexes. The results with complex **1** are consistent with the formation of diastereomeric ternary complexes between **1** itself and the enantiomers, allowing us to give a quantitative measurement of the enantioselectivity in aqueous solution.

Moreover, on the basis of the present results, caution must be used when performing quantitative determination of enantiomers with copper(II) complexes added to the eluent.

Experimental

Melting points are uncorrected. Routine ^{1}H and ^{13}C NMR spectra were obtained on Bruker AC 100 or CXP 200 spectrometers. $[\alpha]_D$ values were obtained on a Rudolph III polarimeter. Mass spectra were obtained by a Finnigan 1020 automated GC–MS. HPLC measurements were performed on a WATERS liquid chromatograph, equipped with an automatic sample injector WISP Waters 712 and controlled by the software MAXIMA 820©. The detector was a WATERS 470 fluorimeter with excitation wavelength at 330 nm and emission wavelength at 545 nm. The column (RP_{18} Spherisorb ODS 3 μm) was thermostatted at 21 °C with an automatic temperature controller WATERS TCM.

UV absorption spectra were recorded on a KONTRON UVIKON 860 spectrophotometer. Fluorescence spectra were recorded on a JASCO FP 770 or on a Perkin-Elmer MPF 44A instrument in a 0.2 × 1 cm quartz cell. Temperature was controlled by BRAUN THERMOMIX 1441-FRIGOMIX 1495 UNITS.

All solvents used were HPLC grade; doubly distilled water was produced by Millipore Alpha-Q equipment. Monohydrate copper(II) acetate (RPE-ACS grade) was obtained from Carlo Erba (Milan, Italy). D- and L-DNS-phenylalanine (analytical grade SIGMA) were used without further purification. D- and L-DNS-glutamic acid were synthesized according to a modified literature procedure.[18] D- or L-glutamic acid (1 g, 6.99 mmol) and Li_2CO_3 (5.31 g, 70 mmol) were dissolved in 500 cm^3 of doubly distilled water; the pH was adjusted to 9.5 with LiOH under a nitrogen flux for several minutes. To the mixture cooled to 0 °C by an ice bath, dansyl chloride (2.70 g, 10 mmol) in acetonitrile (250 cm^3) was added dropwise under magnetic stirring. After the addition, the reaction mixture was allowed to stand in the dark at room temperature for 2.5 h, and then extracted three times with diethyl ether in order to remove the unreacted dansyl chloride. The aqueous layer was adjusted to pH = 4 and repeatedly extracted with diethyl ether. The organic solvent was evaporated at room temperature, and 1.6 g of the product were recovered (60% yield). The optical purity was checked by optical rotation and by HPLC analysis: D-DNS-Glu: m.p. = 166–167 °C, $[\alpha]_D = -24.4$, $[\alpha]_{546} = -32.1$; L-DNS-Glu: m.p. = 166–167 °C, $[\alpha]_D = +25.0$, $[\alpha]_{546} = +32.6$.

Spectroscopic data (^{1}H and ^{13}C NMR, IR, UV and mass spectra) were consistent with those obtained with an authentic specimen of commercial L-DNS-Glu (Analytical grade SIGMA).

Chromatographic Experiments.—The mobile phase was prepared by dissolving L-phenylalaninamide (4×10^{-3} mol dm^{-3}) and copper acetate (2×10^{-3} mol dm^{-3}) in aqueous sodium acetate (0.3 mol dm^{-3}). Acetonitrile was added to the desired percentage; the pH was adjusted to 7.5 with KOH (1 mol dm^{-3}). The eluent was filtered and degassed under reduced pressure.

Fluorescence Measurements.—Solutions for fluorescence measurements were prepared by mixing standard solutions of weighted dansyl-amino acids and of the complex **1** with a KLOEHN syringe and diluting with the desired ionic medium to the appropriate volume. Concentration of the DNS-amino acids in the samples was 6×10^{-5} mol dm^{-3}. Absorbances were in the range 0.08–0.11 at 330 nm, and 0.04–0.06 at 545 nm. Oxygen was not removed, since the quantum yield of the dansyl moiety has been reported[19] to be unaffected by it; moreover, only relative fluorescence and lifetimes were compared.

Time-resolved Experiments.—Fluorescence decays were obtained using a single-photon-counting fluorimeter that uses a

N_2 pulsed lamp as a source (Edinburgh Instrument mod. F199, Edinburgh, GB) (modified in order to allow N_2 to flow at a rate of 1 dm^3 min^{-1}), Jasco and Farrand monochromators, Philips XP2020Q photomultiplier as a fast detector. Fast NIM electronics was from EG & G (Oak Ridge, TN, USA), Tennelec (Oak Ridge, TN, USA), and Silena (Milan, Italy). Decays were acquired in a Silena BS27N multichannel analyser (512 channels, channel width 0.16446 ns) and their acquisition was alternated with that of the instrumental decay function (lamp) in order to correct for lamp fluctuations and drift. Each sample was excited at 330 nm and emission was detected at 9 different wavelengths (500 nm, 520 nm, 530 nm, 540 nm, 550 nm, 560 nm, 570 nm, 580 nm, 600 nm). Every single experimental decay is a convolution of a multiexponential decay function with the instrument response function.

The best fitting of experimental data was obtained by using a biexponential model:

$$F_t = \alpha_1 e^{-t/\tau}1 + \alpha_2 e^{-t/\tau}2.$$

The analysis was carried out using a global approach,[20] linking together the lifetimes at the different wavelengths, and it was optimized for the wavelength dependence of the photomultiplier response.[21] The fitting was evaluated by the χ^2 value (always lower than 1.5) and by the randomness of the residuals. Decay associated spectra (DAS) were obtained from the fractional fluorescence of every single component (τ_i) as: $F_i = \alpha_i\tau_i/\Sigma_i\alpha_i\tau_i$ normalized for the static emission spectrum.

Acknowledgements

This research was supported by the Italian CNR (Rome) (*Progetto Finalizzato Chimica Fine II*) and by MURST (*Ministero dell'Università e della Ricerca Scientifica e Tecnologica*). We are particularly grateful to Professor F. Dallavalle, *Istituto di Chimica Generale ed Inorganica* of the University of Parma for useful discussions.

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Paper 2/01450E
Received 18*th March* 1992
Accepted 30*th June* 1992