Dyes and Pigments 80 (2009) 115–120

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

The synthesis, photophysical properties and energy transfer of a coumarin-based bichromophoric compound

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A R T I C L E I N F O

Article history:
Received 15 February 2008
Received in revised form 16 May 2008
Accepted 28 May 2008
Available online 7 June 2008

Keywords:
Fluorescent probes
Coumarins
Bichromophoric compound
Methyl red
Energy transfers

A B S T R A C T

The energy transfer between two coumarin compounds having donor emission/acceptor absorption spectra overlap was studied firstly in solution by applying an excess of the acceptor. The observed transfer was then confirmed in the bichromophoric compound resulting from the covalent binding of the two fluorophores via a short alkyl chain.

1. Introduction

Multiplexing of fluorescent labels is frequently required in biology and more particularly in molecular biology. Firstly applied in the Sanger sequencing, it is now well adapted for PCR technology. Among the different probes used in this technology, molecular beacons (MB) first described by Tyagi and Kramer [1] are largely developed as seen by the numerous works published during the last decade [2,3]. MB are hairpin-shaped oligonucleotides carrying at their extremity both a fluorophore and a quencher in close proximity. In this conformation, energy emitted by the excited fluorophore is absorbed by the quencher and dissipated. By interaction with its specific target oligonucleotide, the structure opens and the resulting fluorescence measured is then directly proportional to the quantity of the DNA fragment present. Many fluorescent probes and adapted quenchers are either commercially available or synthesized. Amongst them, we were interested in the wavelength-shifting chromophores described by the pioneers of this method [4]. The authors have developed this kind of compounds to access fluorophores with various colors being excited by a common monochromatic source. Our goal aims at combining two fluorophores in structures showing various fluorescence emission fields but being quenched by a common quencher (Scheme 1).

In previous work, we have shown that fluorophores belonging to molecular rotors could be applied in various fields and among them, coumarin dyes remain interesting probes [5,6]. Indeed, due to their separated photophysical fields, we are intended to combine two different coumarin compounds to evidence energy transfer. The two coumarins chosen have been already studied in bichromophores' structures [7–9], but their association is different as the way to link them. We describe herein the synthesis of a bichromophoric compound **1** (NCouOCou) trying to obtain structures that could be later applied in molecular biology. Compound **1** (NCouOCou) was synthesized from the two different coumarin compounds designed, respectively, by **2** (HOCou) and **3** (NCou) (Fig. 1).

The energy transfer was firstly performed in solution between the two free coumarins by using a method tested previously in our laboratory to demonstrate the fluorescence quenching of fluorescein by iodide anions [10]. The transfer was then confirmed in the bichromophoric compound. Moreover, methyl red (MR), a dabcyl isomer, has been successfully tested as quencher.

The interesting photophysical properties found show that this strategy could be further extended to achieve a series of compounds with different wavelengths range.

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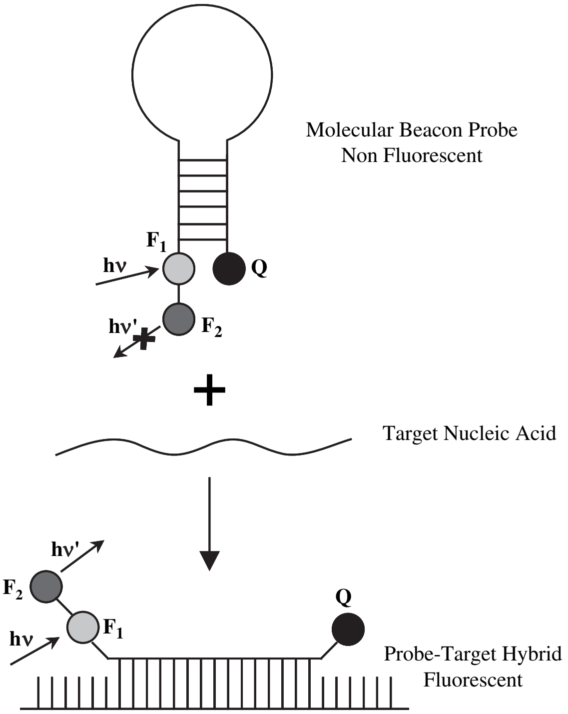
doi:10.1016/j.dyepig.2008.05.010



Scheme 1. A schematic representation of a molecular beacon with a bichromophore (donor F_1/acceptor F_2) on one end and a donor quencher on the other end.

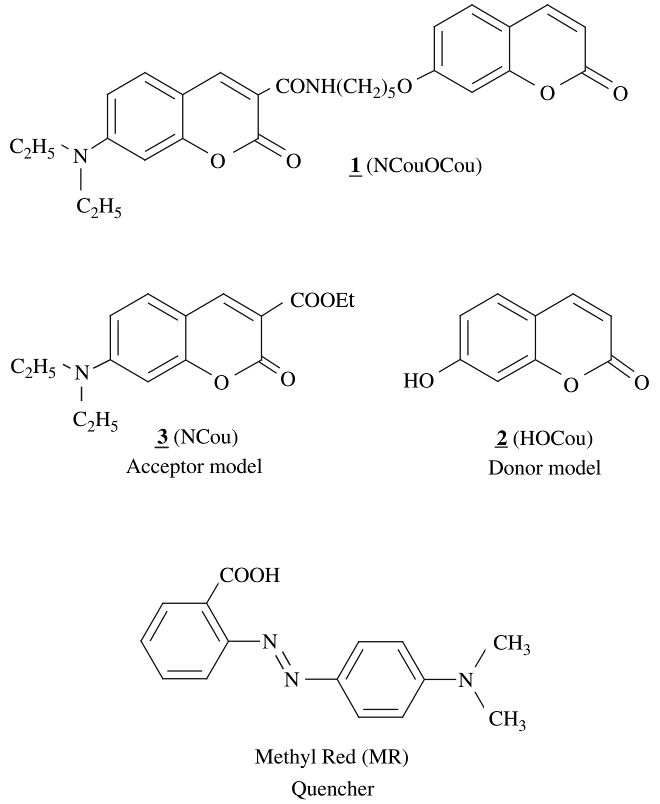


Fig. 1. Structures of the investigated compounds: three fluorescent dyes **1–3** and methyl red (MR) quencher.

2. Experimental

2.1. Materials and instrumentation

Chemicals and solvents were purchased from Aldrich and SdS. Thin layer chromatography was performed using silica gel plates (60 F254, Merck). Liquid chromatography was performed using silica gel (70–200 µm, chromagel, SdS).

^1H NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 300 spectrometer; chemical shifts (δ, ppm) were referenced internally to solvent signal. UV/vis absorption spectra were recorded on a Lambda EZ210 spectrophotometer (Perkin–Elmer). Fluorescence excitation and emission spectra were obtained with a Spex-Fluorolog-3 (HORIBA, Jobin Yvon) spectrofluorimeter. Measurement of fluorescence decays was performed by way of the single photon counting (SPC) technique [11] using a self-assembly apparatus. Excitation was provided by a pulsed lamp nF900 (Edinburgh Instruments) filled with hydrogen. The acquisition chain comprises a microchannels' plate photomultiplier R2287U (Hamamatsu) whose signal, after amplification and discrimination, is directed to a time–amplitude converter 2145/1467 (Canberra) in the same time than a synchronisation signal issued from a photomultiplier coupled by fiber optic to the lamp. Acquisition of the histogram of the fluorescence decay was then performed by a multichannels' analyser coupled to a microcomputer. Analysis of experimental decays was made with a homemade software using a deconvolution–reconvolution method with gold number dichotomy [12]. This method requires an assumption on the analytical equation governing decays (mono- or bi-exponential, or else …).

2.2. Syntheses

7-Hydroxycoumarin **2** (HOCou) was a commercial sample. Compound **4** (NCouCOOH) was synthesized by Knoevenagel condensation of *Meldrum's* acid with 4-(diethylamino)salicylaldehyde according to Ref. [13]. Brominated coumarin compound **5** was prepared following the protocols described elsewhere [5].

2.2.1. 7-[5-(7-diethylaminocoumarin-3-carboxamido) pentyloxy]coumarin *1* (NCouOCou)

Equimolar ratio (0.25 mmol) of **2** (HOCou) (40.5 mg) and brominated coumarin **5** (102.25 mg) was added to DMF (0.5 mL). K_2CO_3 (155.25 mg, 0.75 mmol, 3 equiv) was then added and the mixture was strongly stirred at room temperature for 14 h. H_2O was added to the mixture and the solution was extracted with CH_2Cl_2. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness to give crude compound. Chromatography on silica gel ($EtOH/CH_2Cl_2$ 0.5–3%, v/v) gave **1** (NCouOCou) as a yellow solid.

Yield: 102 mg, 0.208 mmol, 83%; m.p. 139 °C. ^1H NMR ($CDCl_3$) δ_H (ppm): 1.25 (6H, t, $-N(CH_2CH_3)_2$), 1.47–1.95 (6H, m, $-CONHCH_2(CH_2)_3CH_2OAr$), 3.47 (6H, m, $-N(CH_2CH_3)_2$ and $-CONHCH_2(CH_2)_3CH_2OAr$), 4.03 (2H, t, $-CONHCH_2(CH_2)_3CH_2OAr$), 6.23 (1H, d, $J = 9.6$ Hz, H_3 **2** (HOCou)), 6.53 (1H, s, H_8 **3** (NCou)), 6.75 (1H, d, $J = 8.4$ Hz, H_6 **3** (NCou)), 6.82 (2H, m, H_6, H_8 **2** (HOCou)), 7.36 (1H, d, $J = 7.2$ Hz, H_5 **2** (HOCou)), 7.43 (1H, d, $J = 8.7$ Hz, H_5 **3** (NCou)), 7.62 (1H, d, $J = 9.6$ Hz, H_4 **2** (HOCou)), 8.71 (1H, s, H_4 **3** (NCou)), 8.84 (1H, br, –CONH). FAB-HRMS: m/z calcd for $C_{28}H_{30}N_2O_6$ 513.20016 $[M + Na]^+$, found 513.2008 $[M + Na]^+$.

2.3. Photophysical study

The fluorescence quantum yields (ϕ_f) were determined in ethanol using fluorescein as reference ($\phi_f = 0.79$ at 25 °C) [14], by exciting fluorescein (R) and the sample (S) of unknown quantum yield at the same wavelength at weak absorbance (respectively, A_R and A_S). Quantum yield ϕ_f was then obtained from the rate of the surfaces of the emission spectra (S_R and S_S), according to

$$\phi_f = 0.79 \times \frac{(S_S/A_S)}{(S_R/A_R)}$$

For the energy transfer between the two coumarins, the **2** (HOCou) solution (A) in ethanol was 5×10^{-6} M. The second solution (B) was composed with the same concentration of **2** (HOCou) *i.e.* 5×10^{-6} M and a greater concentration of **3** (NCou) at 4×10^{-5} M. Nine samples were prepared by mixing volumes of solution B starting from 0.3 mL and subsequent increasing by 0.3 mL level completed at 3 mL by the solution A. By this way, concentration of **2** (HOCou) remained constant, while the concentration of **3** (NCou) varied from 4×10^{-6} M to 4×10^{-5} M.

For the quenching study with MR, the **1** (NCouOCou) solution in ethanol was 5×10^{-6} M. The second one comprised the same concentration of **1** (NCouOCou) *i.e.* 5×10^{-6} M and a greater concentration of MR at 4×10^{-5} M. By mixing the two solutions as above, nine samples were obtained with **1** (NCouOCou) concentration constant and MR concentrations ranging from 4×10^{-6} M to 4×10^{-5} M.

3. Results and discussion

3.1. Choice of the probes

After having analysed different spectra of commercially available or synthesized fluorophores, the study started with two coumarinic compounds which should give access to a bichromophore with planned properties. In Fig. 1, are shown the two base coumarinic structures on which the photophysical characterization was investigated separately. The first one acting as the donor was the commercial umbelliferone: 7-hydroxycoumarin **2** (HOCou); the second, acceptor part is an amino coumarin derivative **3** (NCou) already studied in our laboratory [5,6]. Fig. 2 illustrates respective excitation and emission spectra in ethanol of coumarins **2** (HOCou) and **3** (NCou): a good overlap of the donor emission spectrum with the acceptor absorption one is observed, which is predictable of a transfer possibility. At this stage, this fact was very promising for the target of our study, therefore the coupling synthesis between the two compounds was approached.

3.2. Synthesis of the coumarin-based bichromophore 1 (NCouOCou)

The best way to obtain the bichromophoric compound **1** (NCouOCou) is shown in Scheme 2. The commercial 7-hydroxycoumarin **2** (HOCou) acting as donor was used as such in the final coupling step. The acceptor part needed some modification steps: the base coumarin was first prepared by a protocol leading directly to the corresponding coumarin acid derivative **4** at very high yield as reported [13]. Compound **5**, alkylbrominated derivative of **3** (NCou), was synthezised in two steps as reported in Ref. [5]. In the last step, alkylation of the phenoxy moiety of the donor coumarin **2** (HOCou) with **5** was performed in the presence of K_2CO_3 in DMF, to yield 83% of the bicoumarinic compound **1** (NCouOCou).

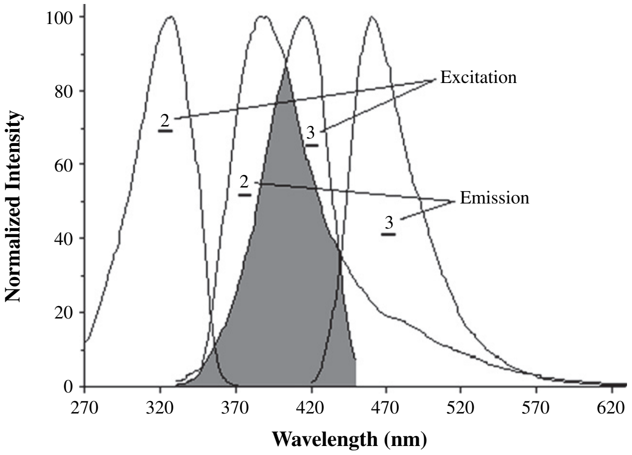


Fig. 2. Normalized fluorescence excitation and emission spectra of **2** (HOCou) and **3** (NCou) in EtOH at RT; visualisation of the overlap between donor emission and acceptor excitation.

3.3. Energy transfer studies

As reported in Refs. [15,16], the mechanism of energy transfer between a donor molecule D and an acceptor molecule A can be radiative or not.

Considering the radiative transfer where an emitted photon from D is absorbed by A, in addition to an overlap of the donor emission and acceptor absorption spectra, a large excess of A is required to quantify this effect. In this case, the shape of the donor emission spectrum is modified in the region of spectral overlap.

In the non-radiative case, the interaction can be of dipole–dipole nature operating at distances up to 80–100 Å. Here, the donor emission spectrum shape is not affected.

Measurement of fluorescence decays is a complementary way to determine if the transfer is radiative or not, and in the last case to calculate some kinetics parameters (such as the yield of transfer).

In the simple case of a donor molecule with a mono-exponential decay

$$D \exp(-t/\tau_d)$$

where t is time and τ_d the lifetime of donor molecule, two situations can occur:

- a radiative transfer, where the donor decay becomes

$$D' \exp(-t/\tau_d)$$

(with $D' < D$, but the SPC technique cannot determine the absolute values of pre-exponential factors),

- a non-radiative transfer, where the donor decay is now

$$D'' \exp\left(-t/\tau'_d\right)$$

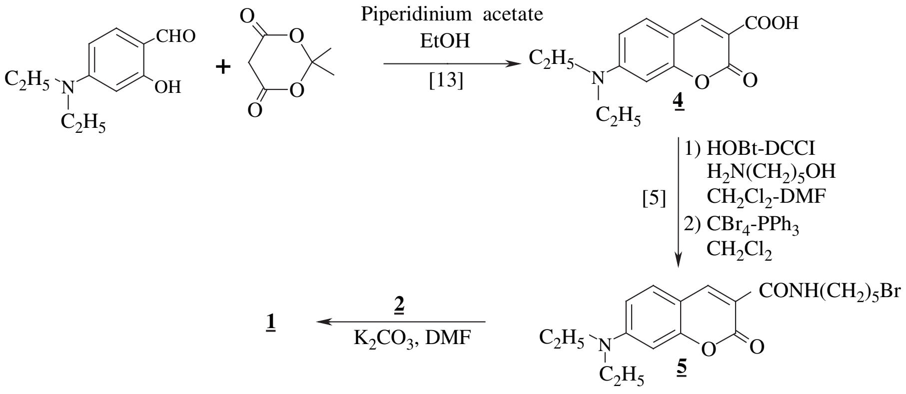
(with $\tau'_d < \tau_d$) and the acceptor one is such as $A \exp(-t/\tau_a) - A''' \exp(-t/\tau'_d)$ (with τ_a the lifetime of acceptor).

In order to discriminate the two effects, a steady-state study on the three compounds and some pulsed excitation analyses have been performed.

3.3.1. Study of the two free coumarins 2 (HOCou) and 3 (NCou)

3.3.1.1. Steady-state study. To approach the transfer in solution between the two dyes, we have followed a method already used in our laboratory [10] and largely argumented [15,16]. For this purpose, a series of nine solutions were prepared (see Section 2). Fig. 3 reports emission spectra of the two dyes after excitation at 320 nm of these obtained different solutions.

Obviously, we observe the classical features of an energy transfer, that is a large decrease of the fluorescence of the donor (localized initially at 390 nm) and a concomitant increase of the acceptor band at 456 nm. We can see a modification of the donor spectra in the overlap region blue wavelength shifted from 390 to 373 nm, occurring in the same time which is in favour of a radiative transfer, as already mentioned by Valeur [15].



Scheme 2. Synthesis of 7-[5-(7-diethylaminocoumarin-3-carboxamido)pentyloxy]coumarin **1** (NCouOCou).

The efficiency of the energy transfer Φ_T may be assessed by using the following relation [15]

$$\Phi_T = 1 - I_D/I_0$$

where I_D is the fluorescence intensity of the donor in the presence of acceptor, I_0 the fluorescence intensity of the donor without acceptor.

The values of Φ_T calculated as a function of acceptor concentration and gathered in Table 1 indicate clearly an increase of energy transfer efficiency Φ_T correlated with **3** (NCou) acceptor concentration. The fluorescence of the acceptor was evaluated by subtracting from the rough signal, the spectra of both direct excitations of the donor and acceptor.

3.3.1.2. Pulsed excitation study. In the case of transfer from **2** (HOCou) to **3** (NCou) in solution, the lifetime of **2** (HOCou) (UV excitation, emission between 380 and 420 nm, concentration 5×10^{-6} M) was measured as 0.78 ns without **3** (NCou) and as 0.79 and 0.77 ns in the presence of **3** (NCou) at concentrations of 10^{-5} M and 6×10^{-5} M, respectively. These values are similar (according to the accuracy of our apparatus) and confirm the results observed above in accordance with a radiative transfer.

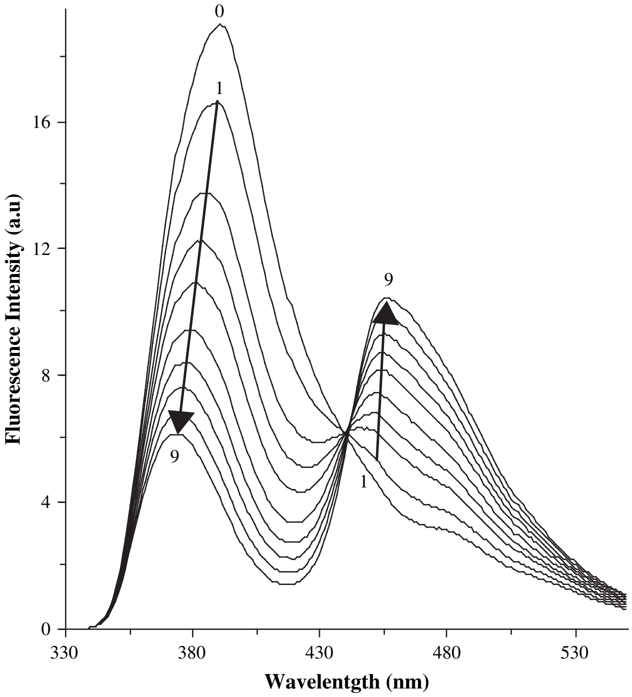


Fig. 3. Energy transfer from **2** (HOCou) to **3** (NCou) in EtOH at RT ($\lambda_{exc} = 320$ nm) by increasing acceptor **3** (NCou) concentration (0: **2** (HOCou) alone, 5×10^{-6} M; 1–9: **2** (HOCou), 5×10^{-6} M with **3** (NCou) varying from 4×10^{-6} M to 4×10^{-5} M).

3.3.2. Study of the bichromophoric compound 1 (NCouOCou)

3.3.2.1. UV/vis absorption spectra. As reported in Fig. 4, it appears that the bichromophoric compound shows the characteristics of the two initial free compounds.

Indeed, the absorption spectrum of **1** (NCouOCou) displays two maxima signals at 321 nm ($\varepsilon = 18{,}700$ L mol^{-1} cm^{-1}) and at 418 nm ($\varepsilon = 46{,}000$ L mol^{-1} cm^{-1}), respectively (Table 2), confirming the presence of two independent fluorophores, as required for an energy transfer [17,18].

3.3.2.2. Emissive properties. The excitation at 320 nm (absorption domain of **2** (HOCou)) was realized on the three compounds (Fig. 5).

For **2** (HOCou), we can observe fluorescence emission at 390 nm. For the bichromophoric compound **1** (NCouOCou), the signal from **3** (NCou) part at 460 nm is the only one visible. This is in favour that a transfer occurs from **2** (HOCou) to **3** (NCou) inside the molecule. For compound **3** (NCou), a direct irradiation at this wavelength leads to a very low signal. By applying the relation given previously, the efficiency of the transfer was calculated equal to 98%. The main photophysical data are collected in Table 2.

3.3.2.3. Fluorescence lifetimes. In this case, where the two fluorophores donor/acceptor are linked by a short alkyl chain, the donor fluorescence is very weak as shown in Fig. 5 and consequently, the sensitivity of our apparatus is not sufficient to allow acquisition of the corresponding fluorescence decay. Decay of the assumed acceptor is clearly bi-exponential ($\exp(-t/0.37) + 0.06\exp(-t/1.71)$), with t in ns, but with two positive pre-exponential factors and time constants independent of the excitation wavelength

Table 1
Efficiency of energy transfer Φ_T from **2** (HOCou) to **3** (NCou) as a function of **3** (NCou) concentration

Sr no.	Concentration of **3** (NCou) (10^{-6} M)	Φ_T
1	4.0	0.13
2	9.3	0.30
3	13.3	0.39
4	17.3	0.47
5	22.7	0.57
6	26.7	0.64
7	30.7	0.69
8	36.0	0.74
9	40.0	0.78

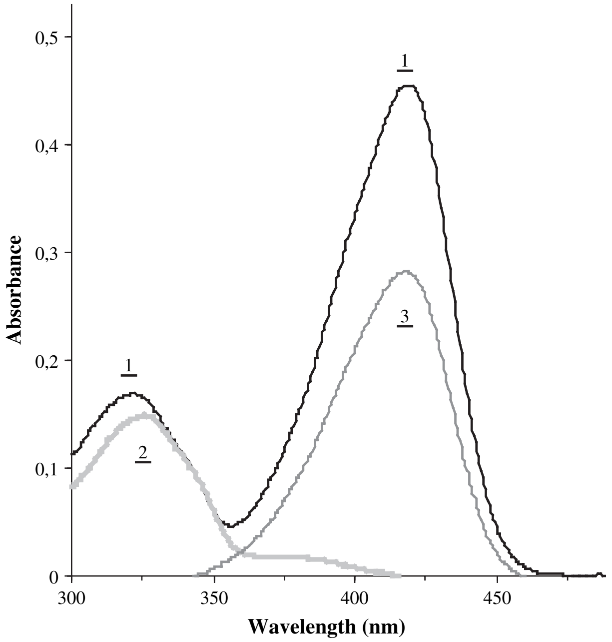


Fig. 4. UV/vis absorption spectra of **1**–**3** as equimolar solutions in EtOH (10^{-5} M) at RT.

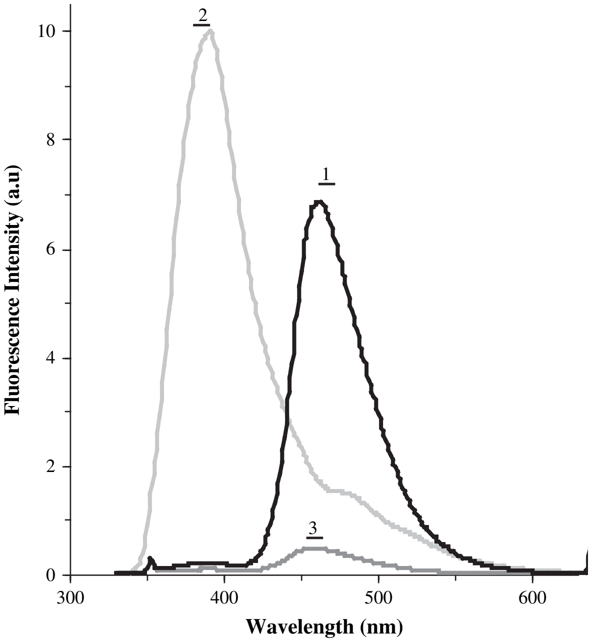


Fig. 5. Fluorescence spectra of **1**–**3** as equimolar solutions in EtOH at RT (2.5×10^{-6} M; $\lambda_{exc} = 320$ nm).

(330 nm for transfer, 380–420 nm for direct excitation). Absence of a decay term with negative factor when exciting at 330 nm is simply due to the very small non-measurable value of the donor lifetime as mentioned above. Nevertheless, these observations are in accordance with a non-radiative transfer, even if in this particular case, experiments in pulsed excitation don't give more information than the one obtained in continuous excitation.

3.3.2.4. Quenching study by methyl red. As the objective is to use such compounds in order to label molecular beacon oligonucleotides used in PCR technology, we have studied the behavior of **1** (NCouOCou) in the presence of methyl red (MR), a structural isomer of dabcyl. This quencher has already been included in molecular beacons' structure [19], we also used it, because its absorption properties seem suitable for our quenching study, it is not fluorescent and commercially available at low price.

The quenching effect was experienced by the same way as described in preceding study. Here the "mother" solutions were one with a given concentration of **1** (NCouOCou) without MR and the second one with the same concentration of **1** (NCouOCou) and a maximal given concentration of MR, in excess compared to **1** (NCouOCou).

The emission spectra of **1** (NCouOCou) in the presence of increasing quantities of MR are reported in Fig. 6. It is interesting to mention that we observe a significant diminution of fluorescence emission of the compound as a consequence of an inhibiting effect from MR, the greatest MR concentration leading to 82% of quenching.

The fluorescence spectra exhibit a weak blue shift indicating that a part of the fluorescence decrease is due to radiative transfer. But, the shape of the spectra is not deeply modified which is in favour of a quenching (non-radiative) mechanism. Slope at origin of the Stern–Volmer plot (inset of Fig. 6) gives a value of the constant nearby 5400 L mol^{-1}, indicative of a moderate efficiency of this quenching reaction conducted in solution between free compounds.

Table 2
Spectroscopic characteristics of compounds **1**–**3** in ethanol

Compound	λ_{abs} (nm)	ε (L mol^{-1} cm^{-1})	λ_{fl} (nm)	Stokes shift (nm)	Φ_{fl}[a] (%)
1 (NCouOCou)	321	18,700			
	418	46,000	461	140	0.24
2 (HOCou)	326	16,300	390	64	0.28
3 (NCou)	414	35,200	458	44	0.11

[a] Values calculated referring to fluorescein ϕ_f ($\phi_f = 0.79$ at 25 °C, in ethanol) [14].

4. Conclusion

We have shown that the covalent binding of two coumarin fluorophores with different photophysical properties and with

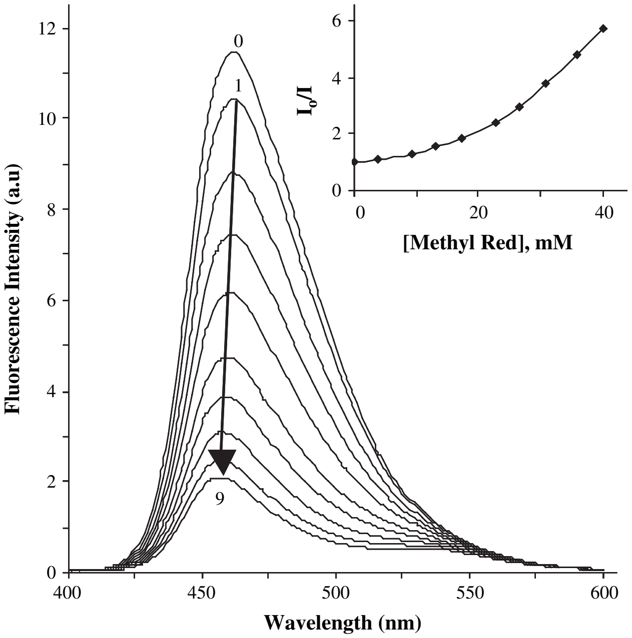


Fig. 6. Fluorescence spectra of **1** (NCouOCou) quenched by methyl red (MR) in EtOH at RT ($\lambda_{exc} = 320$ nm); (0: **1** (NCouOCou) alone, 5×10^{-6} M; 1–9: **1** (NCouOCou), 5×10^{-6} M with MR from 4×10^{-6} M to 4×10^{-5} M); inset of the figure shows the Stern–Volmer plot of the variation of fluorescence intensity versus the MR concentration.

efficient overlap of emission and absorption spectra led to an interesting compound. The length of the linker binding covalently the two molecules is appropriate for a good energy transfer between them. This effect was evidenced by the different steady-state studies conducted on the three compounds and corroborated by time-resolved experiments. Whatever the nature of the transfer is, *i.e.* radiative or not, it remains an interesting feature for our objective. Furthermore, it appears that this kind of bichromophoric compound easily synthezised could find application where great wavelength shifts are researched. Work is in progress with the synthesis of bichromophoric compounds comprising in their structure suitable functions for their grafting on oligonucleotides.

Acknowledgments

The authors acknowledge INSERM (Institut National de la Santé et de la Recherche Médicale), CNRS (Centre National de la Recherche Scientifique) and Cancéropôle du Grand-Est for financial support.

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