Journal of Fluorescence, Vol. 10, No. 2, 2000

Three-Dimensional Fluorescence Microscopy of Endothelial Cells Labeled with Coumarins

C. Geoffroy-Chapotot,[1] M. C. Carré,[1] F. Baros,[1] S. Muller,[2] D. Dumas,[2] J. F. Stoltz,[2] and M. L. Viriot[1]

Received October 4, 1999; accepted January 12, 2000

Fluorescent coumarins were synthesized with the objective of introducing a glucosamine part in the chemical structure, with either hydroxyl or acetyl functions. The photophysical behavior was studied in organic solvents with different polarity and viscosity. The location of the fluorescent coumarins in endothelial cells was studied using fluorescence microscopy imaging, especially with a 3-D CELLscan instrument.

KEY WORDS: Coumarin tracers; three-dimensional microscopy; endothelial cells.

INTRODUCTION

With the search for fluorescent tracers for applications in biology [1,2], particularly to develop imaging by fluorescence microscopy [3], the synthesis of coumarin derivatives (see Fig. 1) was carried out, as well as determination of the characteristics of UV–visible absorption and fluorescence emission, especially the photophysical behavior with respect to environmental modifications (polarity, viscosity, etc.). Further, fluorescent coumarins were used to label biological cells. In this report, we describe their location in endothelial cells according to their chemical structure. The specific location was determined using 3-D[3] fluorescence microscopy (CELLscan method [4]).

[1] DCPR-GRAPP, UMR 7630 CNRS-INPL, Groupe ENSIC, 1 rue Grandville, BP 451, F-54001 Nancy Cedex, France.

[2] Angiohématologie–Hémorhéologie, LEMTA, UMR 7563 CNRS-UHP-INPL, Faculté de Médecine, BP 184, F-54505 Vandœuvre-les-Nancy, France.

[3] 3-D, three-dimensional; CH_2Cl_2, methylene chloride; DCCI, 1,3-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; EtOAc, ethyl acetate; EtOH, ethanol; HOBT, 1-hydroxybenzotriazole hydrate; MeOH, methanol; MeONa, sodium methoxide.

EXPERIMENTAL

General Procedures

Chemicals and solvents were from Aldrich (Saint Quentin Fallavier, France) and SdS (Peypin, France), respectively.

Reactions were monitored by thin-layer chromatography (Kieselgel 60 F_{254}, SdS) and products were purified on silica gel column chromatography (eluents: EtOAc–CH_2Cl_2, 20/80, v/v, for **5**, R = $COCH_3$; and MeOH–CH_2Cl_2, 20/80, v/v, for **5**, R = H). The compounds were characterized by elemental and spectral analyses (IR, ^{1}H NMR).

Physicochemical Measurements

Absorption spectra were recorded with a Perkin Elmer (Lamda 2) UV–visible spectrophotometer.

Fluorescence emission spectra were obtained with a SPEX Fluorolog-2 spectrofluorometer using a 450-W xenon lamp and equipped with a thermostated cell compartment (concentration of the coumarins was in the range of $5\ 10^{-6}$ to $10^{-5}\ M$). Fluorescence quantum yields were determined using an aqueous fluorescein solution at pH 10 ($\phi_f = 0.81$ [5]) as a reference.

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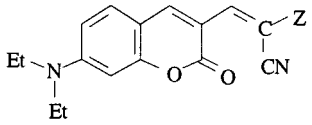

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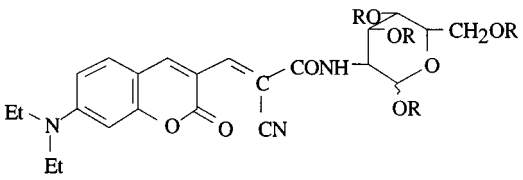
DEAC-COOEt, Z = COOEt



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DEAC-GluOAc, R = $COCH_3$

DEAC-GluOH, R = H

Fig. 1. Chemical structure of the coumarins (see Fig. 2 for the synthesis).

3-D Fluorescence Microscopy Studies

The fluorescence studies were performed with an Olympus IX-70 fluorescence inverted microscope equipped with a CELLscan optical sectionning acquisition system (Scanalytics, USA, and Bionis, France) using a ×100/0.95 NA objective (*z* scanning = 0.25 μm). This device allows 3-D image restoration and fluorescence quantification through an EPR calculation. Images were recorded with a Slow Scan Coding 12-bit CCD camera (Princeton Instruments Inc., USA). The blurring function of the optical system (PSF) was characterized by imaging a through-focus series of optical sections of a 0.17-μm-diameter fluorescent bead (Microscope Point Source Kit, Molecular Probes, USA) [6].

Endothelial Cells (ECs)

Human endothelial cell line ECV304 (ECACC No. 92091712) was used. The ECs were grown in M199 medium (GIBCO) with 10% fetal calf serum. ECs were cultured on No. 2 coverslides (70 × 44 mm) at 37°C in a humidified atmosphere (5% CO_2, 95% air).

Cell Labeling

A solution of fluorescent probes in phosphate buffer (final concentration, 5×10^{-6} *M*) was applied on the endothelial cells during 4 min. The cells were then washed twice with phosphate buffer and analyzed by microscopy.

RESULTS AND DISCUSSION

Chemical Synthesis of the Coumarins

The procedure for chemical synthesis is summarized in Fig. 2.

The addition of 4-(*N*, *N*-diethylamino)-2-hydroxy benzaldehyde, **1**, to diethyl 2-pentene dicarboxylate under Knœvenagel conditions [7] produced 7-(*N*, *N*-diethylamino)-3-(ethylacrylate)coumarin, **2**, which was further easily oxidized in 7-(*N*, *N*-diethylamino)-3-formylcoumarin, **3**.

The DEAC–COOEt tracer, **4** (*Z* = COOEt), and its acid analogoe, **4** (*Z* = COOH), were synthesized using the aldehyde **3** and 2 eq-molar of active methylene compound (ethyl cyanoacetate or cyanoacetic acid) [8].

The glucosamine derivative DEAC–GluOAc, **5** (R = $COCH_3$), was obtained by amidation of the acid **4** (*Z* = COOH) with 2-amino-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucose (whose hydroxylic functions were protected with acetyl groups to facilitate the reaction) using the coupling reagents DCCI–HOBT in DMF–CH_2Cl_2. DEAC–GluOH, **5** (R = H), with free hydroxylic functions, was obtained by deacetylation with a catalytic amount of sodium methoxide [1,2].

Fluorescence in Aprotic and Protic Organic Solvents and in Water

Absorption and fluorescence spectra of the coumarins are analogous to the spectra of other various coumarin dyes [9].

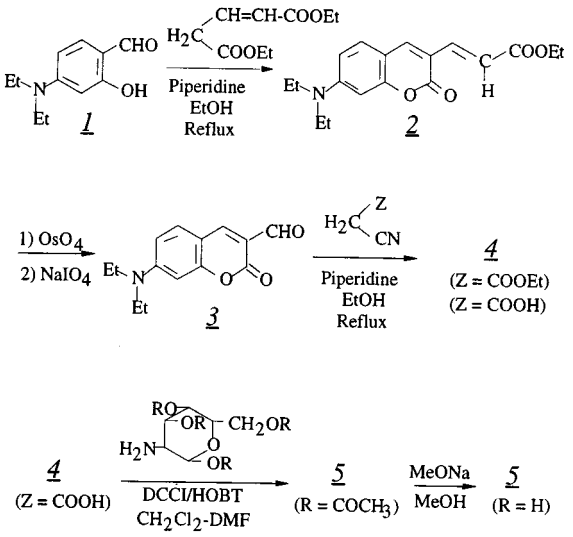


Fig. 2. Schematic steps for the synthesis of the coumarins.

Table I. Absorption and Fluorescence Emission Data of Coumarins in Solvents at 25°C[a]

	Toluene	EtOAc	EtOH	MeOH	Glycerol	Water
$E_T(30)$	33.9	38.1	51.9	55.4	57.0	63.1
η	≈0.55	0.441	≈1.1	0.547	954	0.894
			DEAC–COOEt			
$\lambda_{a\ max}$	499	498	505	503	521	510
$\lambda_{f\ max}$	531	548	562	564	574	563
Φ_f	0.03	0.075	0.17	0.19	0.85	0.007
			DEAC–GluOAc			
$\lambda_{a\ max}$	478	489	496	500	514	511
$\lambda_{f\ max}$	534	542	559	560	569	574
Φ_f	0.29	0.47	0.05	0.07	0.94	0.04
			DEAC–GluOH			
$\lambda_{a\ max}$	476	468	476	474	506	494
$\lambda_{f\ max}$	536	546	558	562	568	573
Φ_f	0.031	0.17	0.13	0.16	0.75	0.063

[a] $E_T(30)$ (kcal · mol^{-1}), polarity parameter, values defined by Reichardt [10]; η (cP), viscosity; $\lambda_{a\ max}$ (nm), absorption wavelength at its maximum; $\lambda_{f\ max}$ (nm), emission wavelength at its maximum; Φ_f, fluorescence quantum yield.

Fluorescence spectra are characteristic of donor–acceptor compounds, with only one single emission fluorescence band, which is red-shifted with increasing solvent polarity.

As an example, the increase in the wavelength of the fluorescence emission at its maximum ($\lambda_{f\ max}$) with an increase in the polarity of the solvent is reported in Fig. 3 for DEAC–GluOH (the polarity was defined by the Reichardt parameter $E_T(30)$ [10]). A classical double response was observed, according to the class of solvents, either aprotic or protic [11].

The influence of the solvent viscosity is more complex. An increase in the viscosity induces mainly an exaltation of the fluorescence efficiency. We report in Fig. 4 the variation of the fluorescence efficiency for two coumarins in glycerol when the temperature is changed, with a subsequent variation of the solvent viscosity. The representation mode is similar to that of Loutfy and Arnold one [12].

Photophysical data of coumarins in homogeneous solvents are gathered in Table I.

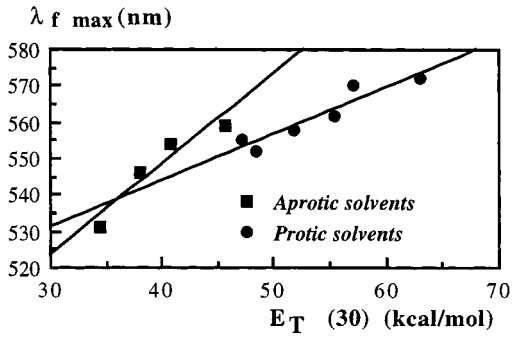


Fig. 3. Variation of $\lambda_{f\ max}$ with the solvent polarity expressed with the $E_T(30)$ parameter for DEAC–GluOH, at 25°C (λ_{ex} = 487 nm).

Fluorescence in Endothelial Cells

When applied for endothelial cell labeling, according to their structure, coumarin derivatives pre-

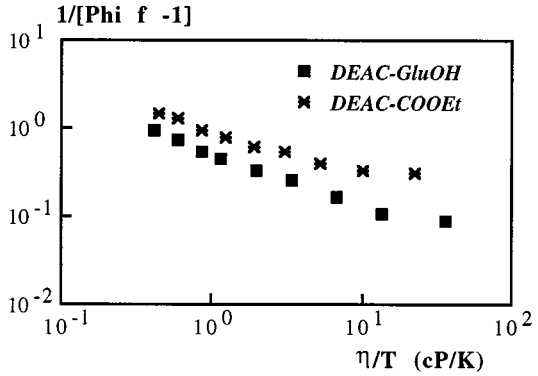


Fig. 4. Variation of $1/[\phi_f - 1]$ with the ratio η/T, for glycerol (Log scales). λ_{ex} = 487 nm for DEAC–GluOH; λ_{ex} = 506 nm for DEAC–COOEt.

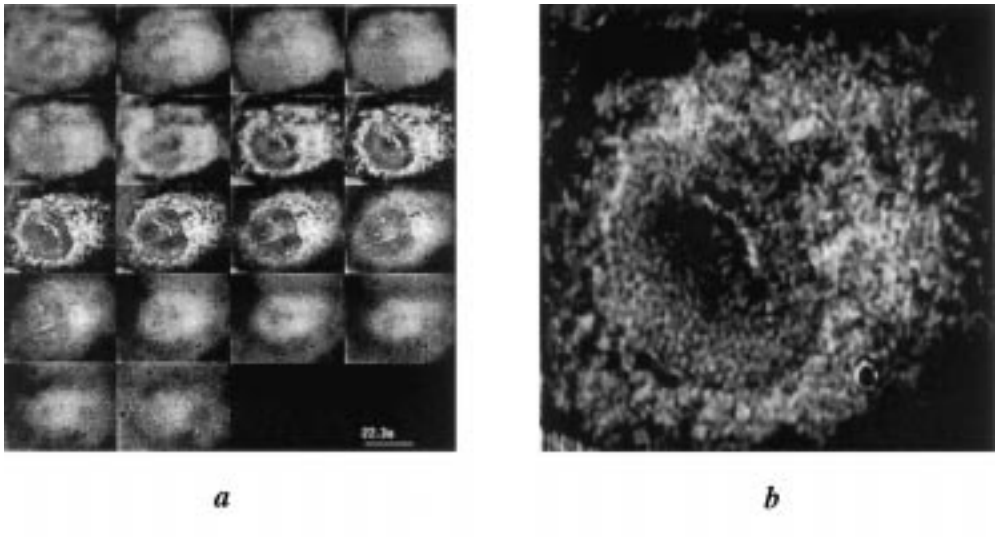


Fig. 5. Fluorescence images, CELLscan microscopy (see text).

sented some interesting location properties, which were visualized by 2-D and, especially, 3-D fluorescence microscopy imaging. In Fig. 5 we report a collection of 2-D images at sequential optical depths for an endothelial cell labeled with DEAC–GluOAc (image a), and the corresponding 3-D restoration (image b).

Using the fluorescence microscopy and double labeling, it became possible to study the location of the coumarin tracers in endothelial cells (Fig. 6).

The location of each fluorescent coumarin is clearly different and comparison with other specific fluorescent tracers seems to indicate that DEAC–COOEt is a vesicle

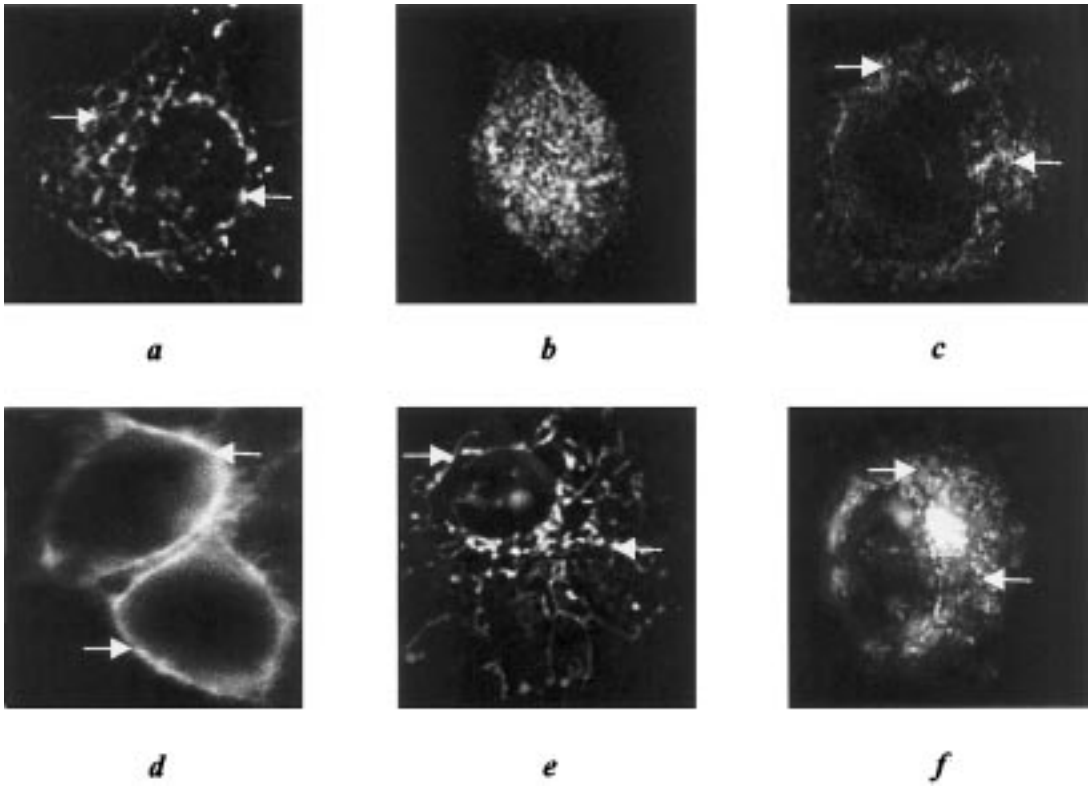
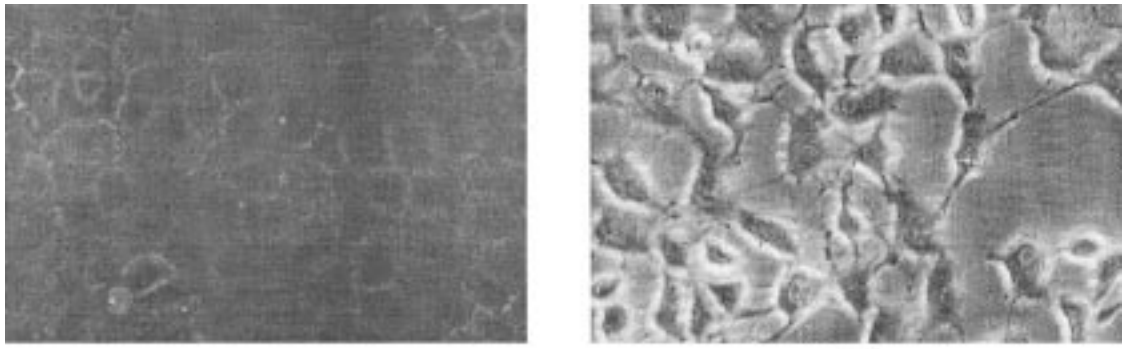


Fig. 6. Fluorescence images, CELLscan microscopy (see text).



a *b*

Fig. 7. Fluorescence images, CELLscan microscopy (see text). Monolayer of endothelial cells (a) without fluorescent tracer and (b) with a high concentration of DEAC–COOEt (10^{-5} *M*), leading to toxicity for the ECs.

membrane tracer (image a) by comparison to either TMA–DPH, which labels the whole membrane bilayer (image d), or styrylpyridinium, which labels the vesicle membrane (image e); DEAC–GluOH is a cytoplasmic tracer (image b, no specific location), and DEAC–GluOAc is a tracer of the endoplasmic reticulum (image c) by comparison to $DiOC_6$ labeling (image f).

A preliminary study was done to evaluate the toxicity of coumarins for the endothelial cell. DEAC–GluOAc does not present toxicity, on the contrary to the two other tracers, DEAC–COOEt (Fig. 7) and DEAC–GluOH, which are sligthly toxic.

CONCLUSION

Coumarin tracers are easily synthesized. They show an interesting photophysical response, i.e., a fluorescence emission wavelength in a range higher than those for intrinsic fluorescent cellular components and a fluorescence efficiency reasonably high for microscopy observations. They are also of interest for studies involving scanning near-field optical microscopy, which are under development.

ACKNOWLEDGMENTS

We thank INSERM, CNRS, MENRT, Région Lorraine, and CUGNancy for grants.

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