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Grau de Química

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Synthesis and characterization of new coumarin-based caging groups.

Síntesi i caracterització de nous grups protectors basats en cumarina.

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Research is to see what everybody else has seen,
and to think what nobody else has thought.

Albert Szent-Gyorgyi

Voldria agrair al Dr. Vicente Marchán Sancho per la seva gran orientació i dedicació durant tota la recerca.

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Per acabar, un agraïment no menys sincer a la meva família i amics per tot el suport proporcionat dia a dia.

A tots, moltes gràcies.

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1. SUMMARY

Light is an ideal trigger for controlling the outcome of molecular processes with high spatiotemporal precision and without causing damage to the living system. Owing to these promising properties, the incorporation of photoremovable protecting groups (PPGs; also commonly referred as caging groups) in key positions of the molecule whose activity has to be inhibited temporarily, have found widespread attention in recent years. Among molecules with suitable characteristics to be used as PPGs, coumarin-based COUPY fluorophores are particularly appealing owing to their interesting spectroscopic and photophysical properties, such as absorption and emission in the far-red/near-infrared (NIR) region.

In this work, we have focused on the synthesis and characterization of two new COUPY-caged model compounds with the aim of exploring the development of a new family of caging groups with red-shifted absorption and emission.

Keywords: COUPY fluorophores, caged compounds, caging groups, coumarin, photoactivation.

2. RESUM

La llum és un estímul ideal per regular processos moleculars amb una elevada precisió espai-temporal i bona compatibilitat amb els sistemes biològics. Gràcies a aquestes prometedores propietats, la incorporació de grups protectors fotolàbils (PPGs) en posicions clau de molècules, l'activitat de les quals es vol inhibir temporalment, ha aconseguit una gran atenció en el últims anys. Entre els compostos amb característiques adequades per actuar com a PPGs, són particularment interessants els fluoròfors COUPY basats en cumarina, ja que presenten propietats espectroscòpiques i fotofísiques encoratjadores, com absorció i emissió a la zona entre el vermell llunyà i l'infraroig proper (NIR).

En aquest treball, ens hem centrat en la síntesi i la caracterització de dos nous models de compostos protegits amb derivats COUPY amb l'objectiu de desenvolupar una nova família de grups protectors fotolàbils que presentin l'absorció i l'emissió desplaçada cap el vermell.

Paraules clau: fluoròfors COUPY, compostos fotoactivables, grups protectors fotolàbils, cumarina, fotoactivació.

3. INTRODUCTION

3.1. PHOTOLABILE PROTECTING GROUPS AND CAGED COMPOUNDS

The use of photocleavable protecting groups (PPGs or caging groups) has received widespread attention in recent years since an exquisite control of complex biological processes can be achieved by using caged analogs of bioactive compounds, such as peptides, neurotransmitters and antibiotics,[1] which can be prepared by modifying an essential functionality with the appropriate PPG. Activation of the resulting caged compound requires irradiation with light of the suitable wavelength and intensity to trigger the unmasking of such functionality and for instance, the recovery of the biological activity of the parent compound (Figure 1). Therefore, caged compounds are inert analogues of biologically active molecules whose activity has been disabled through the attachment of a caging group via a photolabile chemical bond.[2]

Besides offering high spatiotemporal precision, light does not contaminate the living system, and its wavelength and intensity can be precisely regulated to make it fully compatible with living systems. For these reasons, PPGs have found applications in several fields, from organic synthesis to materials science and biology.[3]

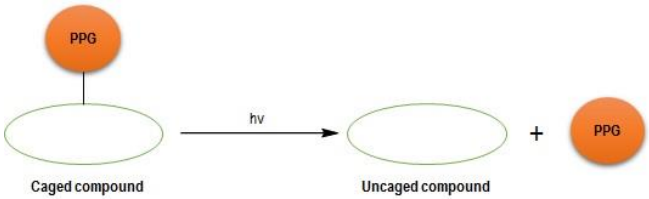


Figure 1. Schematic representation of light-triggered deprotection of a caged compound.

Depending on the final application, PPGs need to fulfill some of the following requirements:[4]

i) Uncaging should occur at wavelengths well above 365 nm to minimize absorption by endogenous molecules and to avoid damage to the biological entity.

ii) The photoreaction should be fast, occur with high release efficiency and with no side-reactions.

iii) PPGs should be soluble in water as well as the resulting caged compounds, they have to show affinity to specific target components and/or pass through barriers.

iv) The photochemical by-products must be biocompatible.

3.2. COUMARIN DERIVATIVES AS CAGING GROUPS

Numerous photocleavable protecting groups have been described to date. [4] However, most of them are photoactivated by UV light which should be avoided given its known phototoxicity and capacity to interact with endogenous biomolecules, as well as its poor capacity of penetration in biological tissues. Among the large number of PPGs, coumarin derivatives fulfill some of the criteria for an ideal caging group: they are relatively easy to synthesize from cheap available precursors and amenable to structural modifications both to facilitate the attachment of the compound to be caged through different types of bonds (for example, ester, amide, carbamate or carbonate) and to improve their photophysical and photochemical properties.

Among coumarin-based PPGs described to date, those possessing an *N,N*-dialkylamino group at position 7 have larger molar extinction coefficients at longer wavelengths than their analogs with hydroxy or methoxy substituents at that position (Figure 2 and Table 1). The higher electron-donating ability of the *N,N*-dialkylamino causes a large bathochromic shift due to a large charge-transfer in the excited state. Recently, the modification of the carbonyl group of the lactone function of the coumarin moiety through thionation has been proved to be a valuable way to red-shift the absorption and emission maximum, leading to blue absorption ($\lambda_{max} \sim 470$ nm) and green light emission ($\lambda_{em} \sim 550$ nm) in the case of coumarin NdiEt-tcBA.[5] Further red-shifted absorption is possible by introduction of electron-withdrawing groups at the 2- or 3-positions of the coumarin skeleton (Figure 2).[5] In fact, extending the conjugation of the system at position 2 has been reported to induce similar bathochromic effects in both absorption and emission in several systems.[5][6]

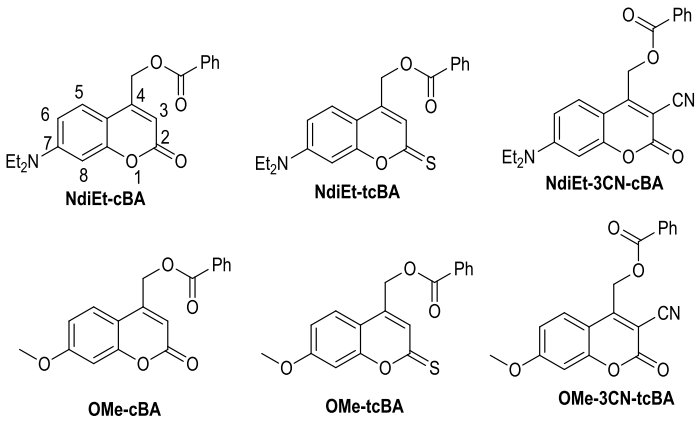
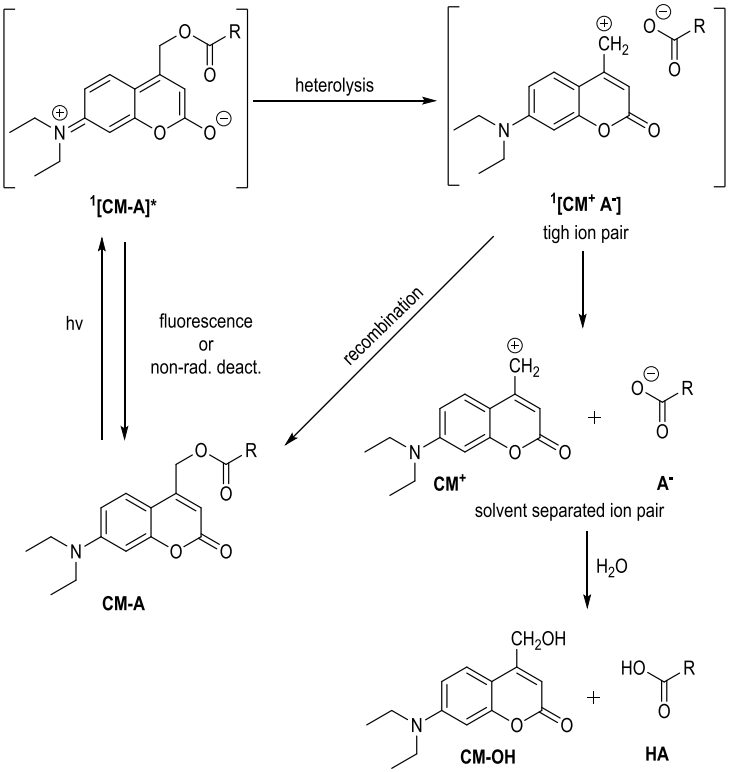


Figure 2. Structure of some coumarin derivatives.

Entry	Compound	λ_{max} [nm]	ε (λ_{max}) [mM^{-1} cm^{-1}]
1	OMe-cBA	323	13.5
2	NdiEt-cBA	385	24
3	OMe-tcBA	398	17
4	OMe-3CN-tcBA	427	18.5
5	NdiEt-3CN-cBA	443	26
6	NdiEt-tcBA	472	31

Table 1. Absorption maximum for different coumarin derivatives.[5]

(Coumarin-4-yl)methyl derivatives have been successfully applied to mask the biological activity of many functionalities via ester bond (CM-A). As shown in Scheme 1, after absorption of a photon by CM-A, relaxation to the lowest excited singlet state, ^{1}[CM-A]*, occurs. Deactivation of ^{1}[CM-A]* by means of fluorescence and non-radiative processes competes with heterolytic bond cleavage forming a tight ion pair, ^{1}[CM^+ A^-], which can either recombine to lead back to ground-state CM-A or escape from the solvent cage and react with water to give (coumarin-4-yl)methyl alcohol (CM-OH) (when using water as a solvent) and the corresponding uncaged carboxylic acid (HA).[1],[4]



Scheme 1. Mechanism for the photocleavage of (coumarin-4-yl)methyl esters.

According to this mechanism of photocleavage of coumarinylmethyl esters, stabilization of the carbocation CM^+ (by incorporation of electron-donating substituents at the coumarin skeleton) and of the released carboxylate (by decreasing its basicity) have a positive effect on the photocleavage rate of coumarinylmethyl esters.

3.3. COUMARIN-BASED FLUOROPHORES (COUPY DYES) AS CAGING GROUPS

As shown in Figure 3, our research group has recently synthesized a series of dicyanocoumarin-caged model compounds,[7] DEAdcCM (**1a**-**4d**) and DEAdcCE (**5e**-**8h**), with the aim of exploring how the uncaging process is influenced by the chemical structure of the PPG (the influence of the stability of the carbocation intermediate) and the nature of the leaving group (aliphatic or aromatic carboxylic acid or amine).

As shown in Table 2, the maximum absorption wavelength and the molar extinction coefficient of ester and carbamate derivatives are very similar. Moreover, esterification either with benzoic acid or with acetic acid caused a slight redshift (about 5-11 nm) with respect to the parent coumarin alcohol derivatives (**4d** and **8h**), and in all cases the presence of the methyl group on the coumarin skeleton caused an additional redshift compared with non-methylated analogs (compare **1a** and **5e**). A similar bathochromic effect is observed for the emission wavelength for the caged compounds compared with the corresponding coumarin alcohol derivatives.

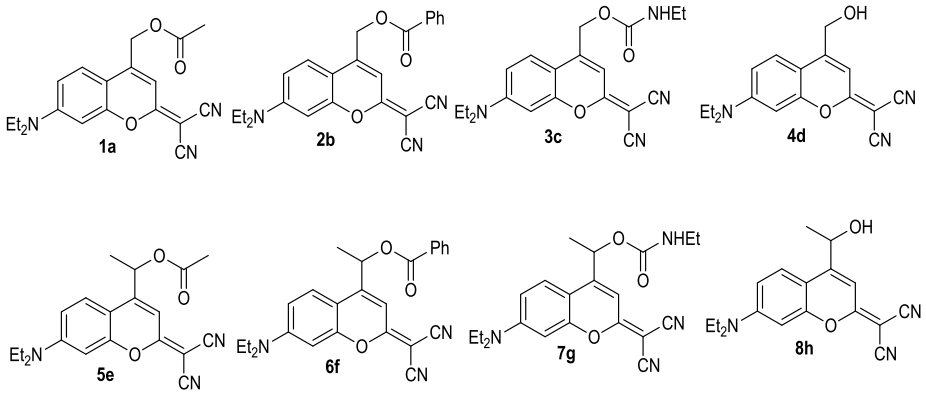


Figure 3. Dicyanocoumarin-caged compounds: DEAdcCM (**1a**-**4d**) and DEAdcCE (**5e**-**8h**).

Entry	Absorption		Emission	
	λ_{max} [nm]	ε (λ_{max}) [mM^{-1} cm^{-1}]	λ_{Em} [nm]	Stokes shift [cm^{-1}]
1a	486	30.4	556	2633
2b	487	32.5	558	2613
3c	489	31.2	549	2193
4d	489	32.0	548	2202
5e	485	32.8	555	2600
6f	488	33.0	544	2067
7g	478	32.5	545	2572
8h	482	33.0	539	2194

Table 2. Photophysical properties of coumarin.[7]

Furthermore, DEAdcCM (**1a**-**4d**) and DEAdcCE (**5e**-**8h**) compounds were studied to evaluate if green light could be used to deprotect them. As shown in Figure 4, the concentration of dicyanocoumarin-caged compounds decreased gradually with time upon irradiation with green light, being dependent on the structure of the coumarin chromophore and on the nature of the leaving group.

Entry	Leaving group
1a	AcOH
2b	PhCOOH
3c	$EtNH_2$
5e	AcOH
6f	PhCOOH
7g	$EtNH_2$

Table 3.

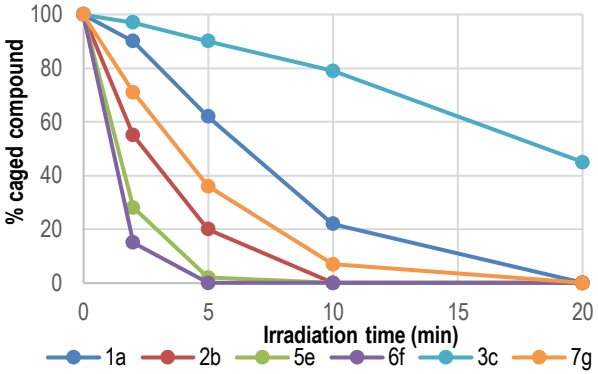


Figure 4. Plot of the temporal evolution of the amount of caged compounds after irradiation with green light. [7]

Overall, these results from photolysis experiments revealed the important influence of:

1. The structure of the dicyanocoumarin caging group.
2. The nature of the leaving group.
3. The type of bond photocleaved (ester or carbamate), on the time necessary to release the carboxylic acid or the amine from the corresponding caged compounds.

From these results, it is worth noting that photolysis of DEAdcCE-caged compounds (**5e**, **6f** and **7g)** is much faster than that of DEAdcCM (**1a**, **2b** and **3c**). This result can be attributed to the different stability of the carbocation intermediates (primary or secondary) generated during photoheterolysis (Scheme 1) of the ester or carbamate bonds. For instance, the release of acetic acid from **5e** was almost complete after 5 min of irradiation whereas it required 20 min to completely uncage compound **1a**. Similar trends are found when comparing **2b** and **6f**, and **3c** and **7g** (Figure 4). Hence, the incorporation of a methyl group in the coumarin moiety in a position adjacent to the bond to be photocleaved allows to accelerate the uncaging process due to the stabilization of the coumarinyl carbocation intermediate.

On the basis of all these precedents, in this work we have focused on the design and synthesis of new coumarin-based caging groups that could be removed by irradiation with visible light. As a starting point, we have selected a new family of coumarin-based fluorophores recently developed in our research group, nicknamed COUPY,[8] in which one cyano group in the dicyanomethylenecoumarin was replaced with a pyridine (Figure 5) in order to increase the π-conjugation and the push-pull character of the chromophore, which resulted in a large bathochromic shift in absorption and emission bands. The electron-deficient pyridine heterocycle offers the possibility of tuning its electronic properties through *N*-alkylation, which is an important advantage since the resulting positive charge on the nitrogen atom would lead to an increased electron-withdrawing effect and, consequently, to a large intramolecular charge-transfer effect along the coumarin skeleton.

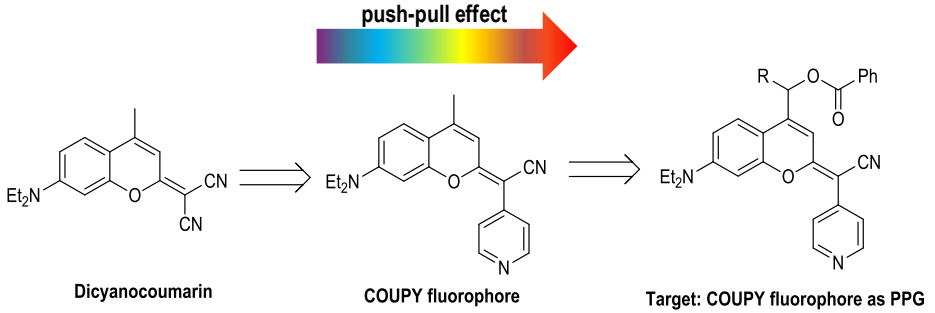


Figure 5. Rational design of COUPY-based caging.

COUPY dyes shows promising photophysical properties, including emission in the far-red/near-infrared (NIR) region, large Stokes shifts, high photostability and brightness.[8] As previously started, emission at longer wavelengths exhibits several appealing features, such as deep tissue penetration while minimizing photodamage to living cells compared to UV and blue light. In conclusion, COUPY fluorophores are very promising candidates to cage biological molecules.

Taking into account the potential applications of courmarin-based fluorophores, in this research work we have focused on transforming COUPY dyes into PPGs that could be removed with far-red/NIR light. As shown in Figure 6, a methyl group adjacent to the bond to be

photocleaved was incorporated with the aim of studying how the uncaging process is influenced by the chemical structure of the coumarin chromophore (R=H or Me) since a secondary carbocation would be generated upon photolysis instead of a primary carbocation, which would facilitate the uncaging process by increasing the stability of the intermediate CM^+ (Scheme 1). The structures of the two target COUPY caged model compounds are shown in Figure 6 and Table 4.

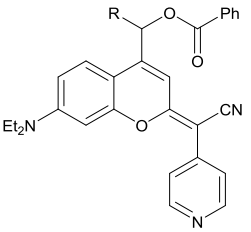


Figure 6. Structure of COUPY-caged model compounds.

Compound	R
1	H
2	CH_3

Table 4. Summary of COUPY-caged model compounds synthesized in this work.

4. OBJECTIVES

In this work we have focused on the synthesis and characterization of two new (coumarin-4-yl)methyl derivatives (**1** and **2**) to investigate the goal of transforming coumarin-based COUPY fluorophores into PPGs removable with red light.

The specific objectives of this work were:

1) Synthesis and characterization of thiocoumarin precursors **3** and **4**.
2) Synthesis and characterization of compounds **1** and **2** as models of COUPY-caged carboxylic acids.
3) Study of the rotamers of coumarins **1** and **2** by 2D NOESY NMR experiments.

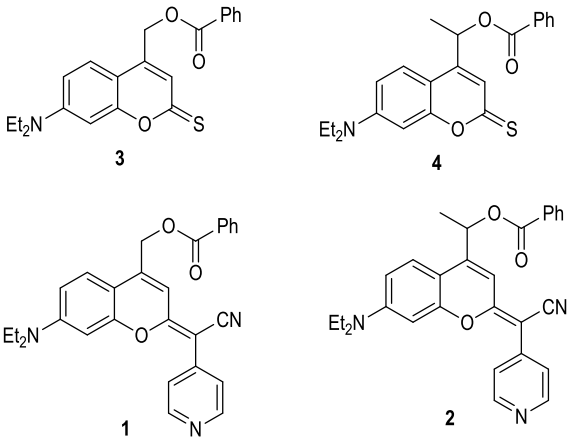


Figure 7. Structure of target COUPY-caged model compounds.

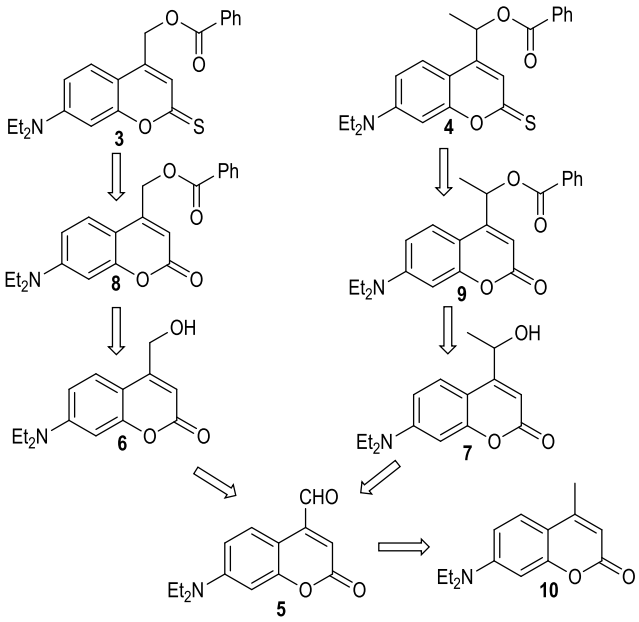
5. RESULTS AND DISCUSSION

5.1. SYNTHESIS OF 4-(BENZOYLOXYMETHYL)-7-(*N,N*-DIETHYLAMINO)THIOCOUMARIN DERIVATIVES (3 AND 4)

As shown in Scheme 2, the thiocoumarin derivatives 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)thiocoumarin (**3**) and 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-thiocoumarin (**4**) were synthesized through thionation of the carbonyl group of the lactone function of 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)coumarin (**8**) and 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)coumarin (**9**), respectively, with Lawesson's reagent.[8],[9] The benzoate derivatives (**8** and **9**) were obtained by using two different methods:

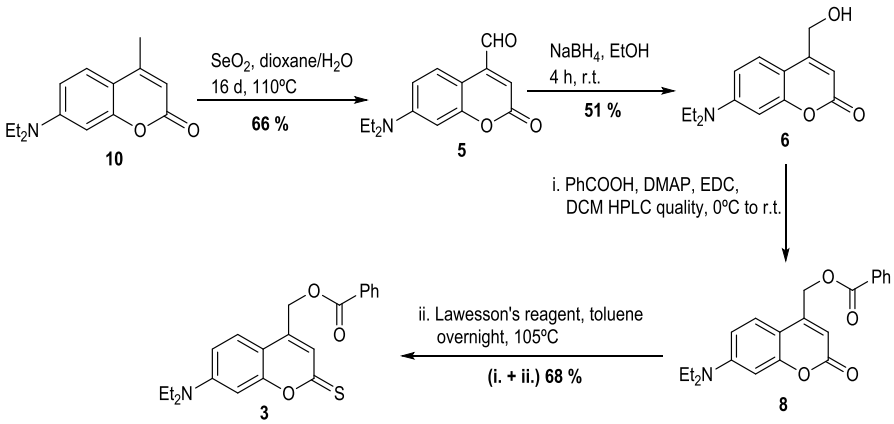
1- Compound **8** was obtained by esterification of the primary alcohol function of 7-(*N,N*-diethylamino)-4-(hydroxymethyl)coumarin (**6**), which can be prepared by reduction of the aldehyde function of compound **5** with $NaBH_4$. [7],[8]
2- Compound **9** was obtained by esterification of the secondary alcohol function of 7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)coumarin (**7**), which can be prepared by methylation of the aldehyde function of compound **5** through Grignard reaction. [7],[8]

The aldehyde derivative 4-carbaldehyde-7-(*N,N*-diethylamino)coumarin (**5**) was obtained from the commercially available 7-(*N,N*-diethylamino)-4-methylcoumarin (**10**) by oxidation of the allylic position with SeO_2.[8],[10]



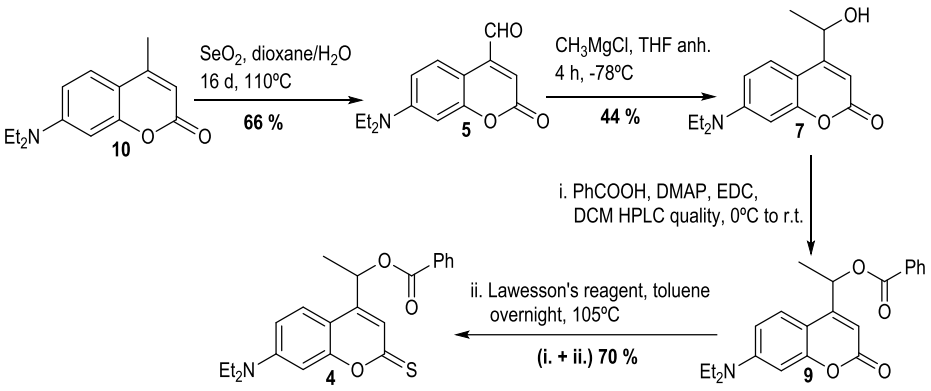
Scheme 2. Retrosynthetic analysis for the preparation of thiocoumarin **3** and **4** from **10**.

The synthesis route followed for the preparation of 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)thiocoumarin (**3**) is shown in Scheme 3.



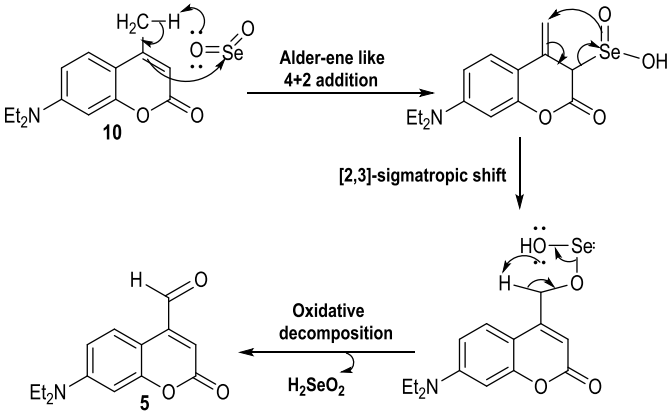
Scheme 3. Synthetic route followed for the preparation of **3**.

The synthesis route followed for the preparation of 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-thiocoumarin (**4**) is shown in Scheme 4.



Scheme 4. Synthetic route followed for the preparation of **4**.

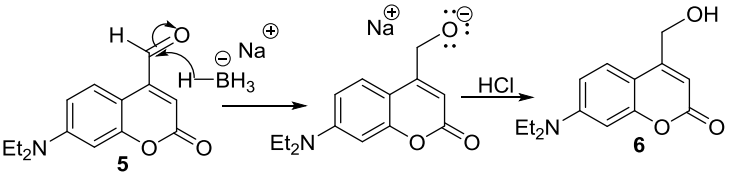
Starting with the commercially available coumarin **10**, oxidation with SeO_2 in dioxane/water mixture during 16 days at 110ºC conduced to the aldehyde **5** (as shown in Scheme 5), which was purified by silica column chromatography and characterized by ^{1}H-NMR.



Scheme 5. Mechanism for the oxidation of **10** with SeO_2.

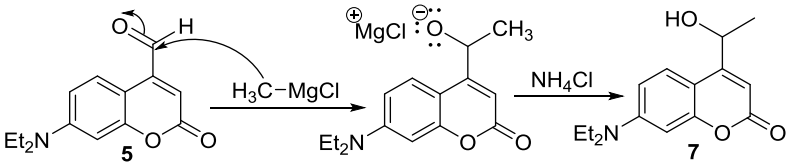
Then, the aldehyde group was reduced by using two different methods to obtain compounds **6** and **7**:

1- Compound **6**: The aldehyde group was reduced with $NaBH_4$ in EtOH for 4 h at r.t. to generate the coumarin alcohol derivative **6**, which was isolated by silica column chromatography and characterized by ^{1}H-NMR.



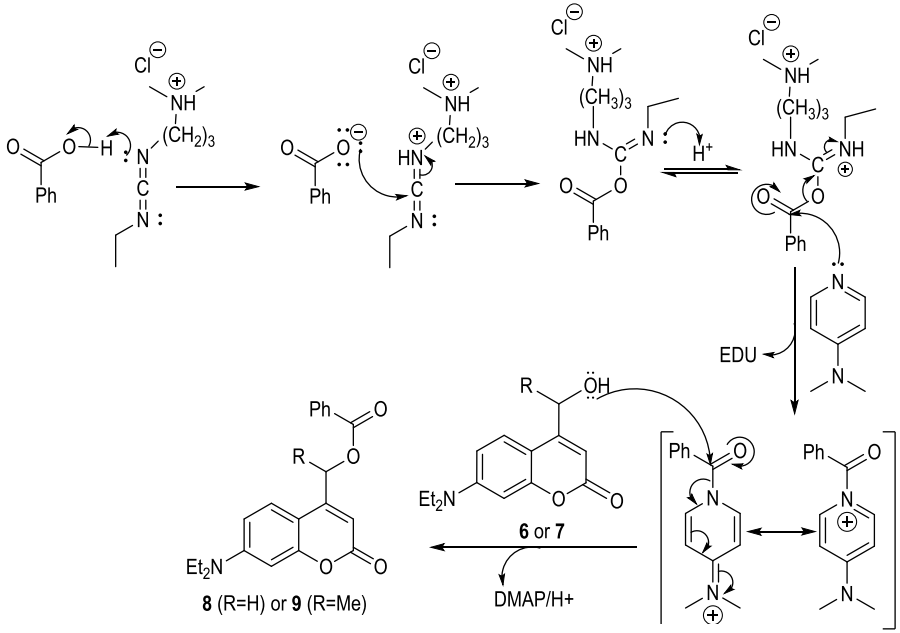
Scheme 6. Mechanism for the reduction of **5** with $NaBH_4$.

2- Compound **7**: The aldehyde group was reacted with CH_3MgCl in anhydrous THF for 4 h at -78ºC to generate the coumarin alcohol derivative **7**, which was isolated by silica column chromatography and characterized by ^{1}H-NMR.



Scheme 7. Mechanism for the Grignard reaction of **5**.

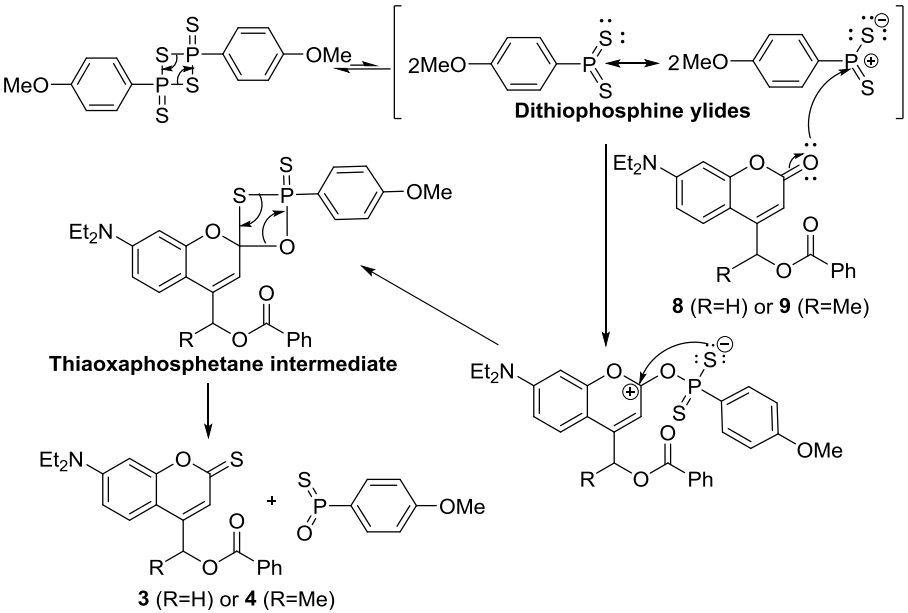
The following step involved protection of the primary and secondary alcohol function of **6** and **7**, respectively, by esterification with benzoic acid using EDC as coupling agent and DMAP as catalyst to give compounds **8** and **9** (as shown in Scheme 8), respectively, which were used without further purification in the next step.



Scheme 8. Mechanism for the esterification reaction of **6** or **7**.

Finally, both thiocoumarin derivatives, 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)thiocoumarin (**3**) and 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-thiocoumarin (**4**) were obtained by reaction of **8** and **9,** respectively, with Lawesson's reagent overnight in toluene at 105ºC. According to the known higher reactivity of Lawesson's reagent for lactones than for esters, thionation occurred in the carbonyl group of the lactone rather in the ester.[9] Thiocoumarin derivatives **3** and **4** were purified by silica column chromatography and characterized by ^{1}H-NMR.

Lawesson's reagent has a four-membered ring of alternating phosphorus and sulphur atoms. Upon heating, the central four-membered ring is opened to form two more reactive dithiophosphine ylides, which react with a carbonyl group to generate a thiaoxaphosphetane intermediate. Then, the formation of a stable P=O bond forces the cycloreversion step (Scheme 9).



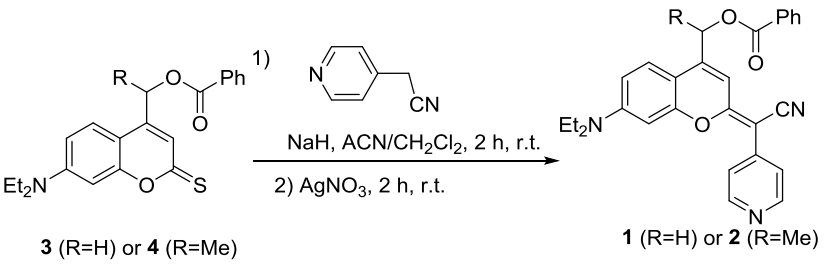
Scheme 9. Mechanism for the thionation of **8** or **9** by using Lawesson's reagent.

5.2. SYNTHESIS OF 4-(BENZOYLOXYMETHYL)-2-(CYANO(4-PYRIDINE)METHYLENE)-7-(*N,N*-DIETHYLAMINO)COUMARIN DERIVATIVES (1 AND 2)

As shown in Scheme 10, condensation of 4-pyridylacetonitrile with thiocarbonyl group of thiocoumarins **3** and **4** afforded the target compounds, 4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)coumarin (**1**) and 4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-coumarin (**2**), which were isolated by column chromatography and fully characterized by ^{1}H and ^{13}C NMR, UV-Vis and 2D NOESY NMR, and their purity was assessed by HPLC analysis.

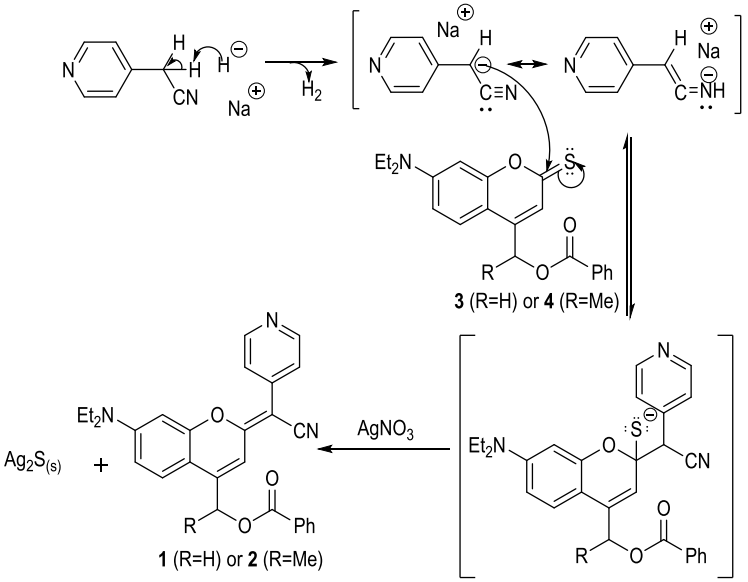
Entry	Compound	R	Yield [%]
1	4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)coumarin	H	53
2	4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-coumarin	CH_3	60

Table 5.



Scheme 10. Synthesis of COUPY caged model compounds (**1** and **2**).

First, 4-pyridylacetonitrile was deprotonated with NaH in anhydrous ACN under an argon atmosphere and protected from light for 15 min. Afterwards, 4-pyridylacetonitrile carbanion acts as a nucleophile and attacks thiocarbonyl group forming a tetrahedral intermediate. Finally, addition of $AgNO_3$ conduces to the formation of the target compounds **1** and **2** by facilitating the removal of the sulphur atom.



Scheme 11. Proposed mechanism for the condensation of 4-pyridylacetonitrile with **3** or **4**.

5.3. STUDY OF THE ROTAMERS OF TARGET COUMARINS BY 2D NOESY NMR EXPERIMENTS

Interestingly, the ^{1}H-NMR spectra (Appendix 1) of both COUPY-caged model compounds (**1** and **2**) showed the presence of two sets of proton signals in $CDCl_3$ and in DMSO-d_6 in an ~90:10 ratio and the same duplicity was found in their ^{13}C-NMR spectra. Full assignment of the ^{1}H-NMR spectra by using 2D NOESY NMR experiments confirmed the presence of two species in slow equilibrium in solution on the chemical shift time scale.

As shown in Figures 9 and 10, the presence of chemical exchange cross-peaks in the 2D NOESY spectra accounts for the existence of *E* and *Z* interconverting rotamers around the exocyclic C=C bond (Figure 8). It should be mentioned that noticeable exchange cross-peaks were observed only in DMSO-d_6 and not in $CDCl_3$.

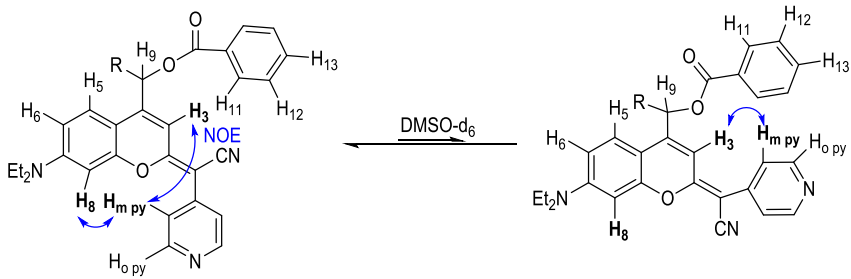


Figure 8. Structures of the *E* and *Z* rotamers of coumarin **1** (R=H_9) or **2** (R=Me, H_{10}) with some diagnostic NOE cross-peaks indicated.

The existence of rotamers instead of diastereomers can be attributed to the strong push−pull character of the compounds: the exocyclic C=C bond connecting C-2 of the coumarin moiety and C-4 of the pyridine cannot be considered a pure double bond, but a double bond with partial single bond character due to the strong electronic delocalization along the π-conjugated system, from the electron-donating NEt_2 group to the pyridine and nitrile.

The presence of diagnostic NOE cross-peaks, such as the one between the H_3 proton of the coumarin in the minor rotamer and the meta protons of the pyridine, ($H_{m\ py}$, Figures 9 and 10), enabled us to conclude that the *E* rotamer was the major one.

Having established by NMR the existence of rotamers in solution for the two coumarin scaffolds, in the following two sections the aromatic region of 2D NOESY NMR spectra of the compounds are shown together with a list of the chemical shifts of several diagnostic protons.

5.3.1. Characterization by 2D NOESY NMR of compound 2

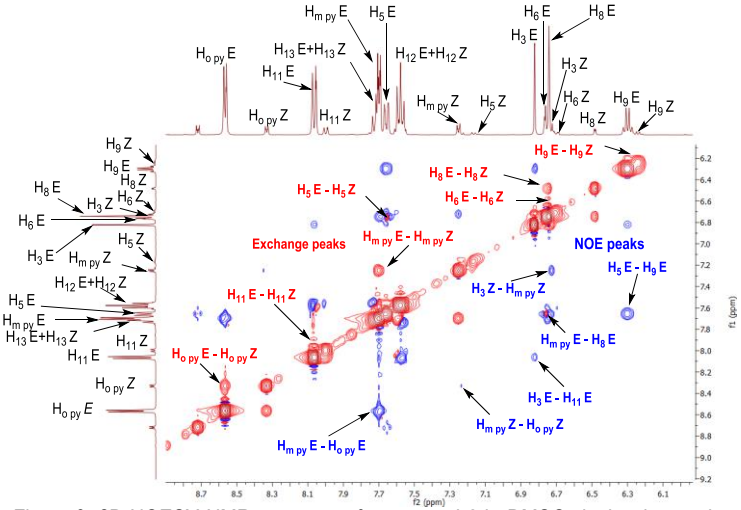


Figure 9. 2D NOESY NMR spectrum of compound **2** in DMSO-d_6 showing exchange cross-peaks (red) between rotamers resonances and NOE cross-peaks (blue).

Proton	δ (ppm)		Proton	δ (ppm)	
	E rotamer	*Z* rotamer		*E* rotamer	*Z* rotamer
$H_{o\ py}$	**8.57**	**8.33**	**H_3**	**6.82**	**6.72**
H_{11}	8.06	8.00	**H_6**	6.76	6.70
$H_{m\ py}$	**7.71**	**7.25**	**H_8**	**6.74**	**6.49**
H_{13}	7.73	7.73	**H_9**	6.30	6.24
H_5	**7.65**	**7.16**	**H_{10}**	1.69	1.62
H_{12}	7.57	7.57			

Table 6. Chemical shifts of compound **2** in DMSO-d_6.

As shown in Table 6 and Figure 9, protons $H_{o\ py}$, $H_{m\ py}$, H_5, H_3 and H_8 appear at lower chemical shifts in *Z* rotamer than in the *E* rotamer. This fact can be explained considering the three dimensional structure adopted by each rotamer (Figure 8). In *Z* rotamer, pyridine protons are

closer to the phenyl group, causing that these protons appear more shielded. Similarly, protons H_5 and H_3 appear at higher field in *Z* rotamer. Moreover, NOE cross-peak between H_8-$H_{m\ py}$ only can be seen in the *E* rotamer because in Z rotamer H_8 is far away from pyridine group.

Finally, in Figure 10 is shown the 2D NOESY NMR spectrum of compound **2** in $CDCl_3$.

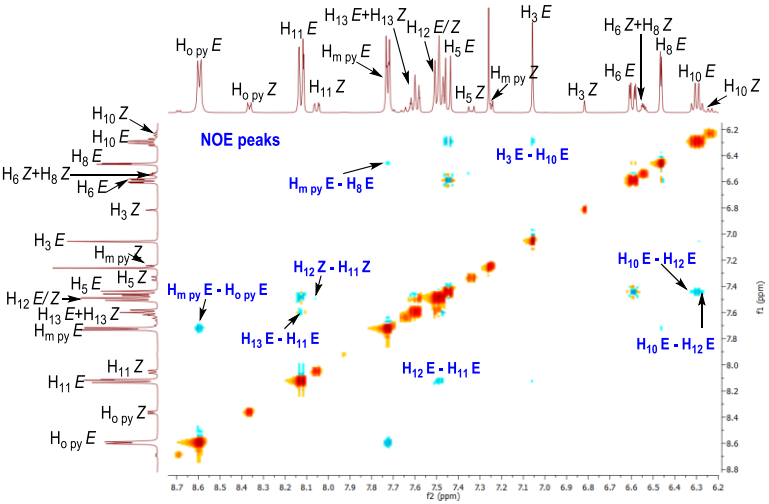


Figure 10. 2D NOESY spectrum NMR of compound **2** in $CDCl_3$ showing NOE cross-peaks (blue).

Proton	δ (ppm)		Proton	δ (ppm)	
	***E* rotamer**	***Z* rotamer**		***E* rotamer**	***Z* rotamer**
$H_{o\ py}$	**8.60**	**8.36**	**H_3**	**7.05**	**6.83**
H_{11}	8.13	8.05	**H_6**	6.59	6.55
$H_{m\ py}$	**7.73**	**7.25**	**H_8**	6.46	6.55
H_{13}	7.60	7.60	**H_9**	**6.30**	**6.24**
H_{12}	7.49	7.49	**H_{10}**	1.75	1.66
H_5	**7.45**	**7.32**			

5.3.2. Characterization by 2D NOESY NMR of compound 1

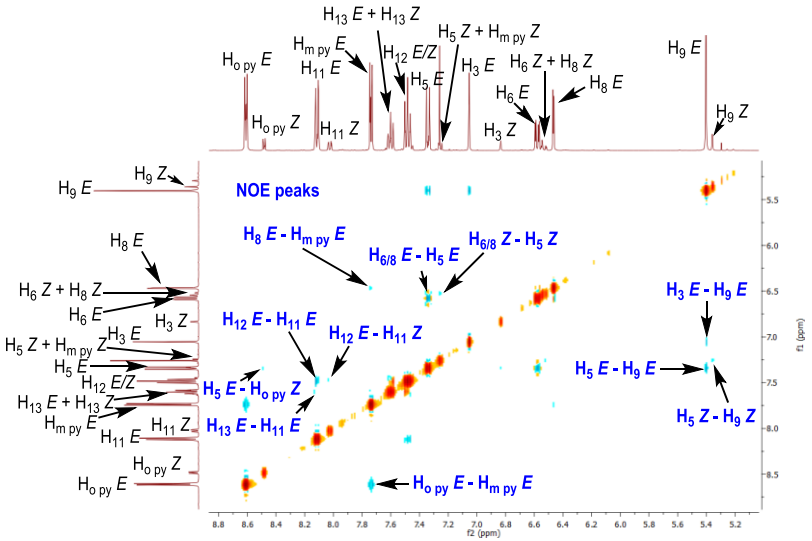


Figure 11. 2D NOESY spectrum NMR of compound **1** in $CDCl_3$ showing NOE cross-peaks (blue).

Proton	δ (ppm)		Proton	δ (ppm)	
	E rotamer	*Z* rotamer		*E* rotamer	*Z* rotamer
$H_{o\ py}$	**8.61**	**8.48**	H_5	**7.34**	**7.25**
H_{11}	8.12	8.03	H_3	**7.05**	**6.84**
$H_{m\ py}$	**7.74**	**7.25**	H_6	6.58	6.53
H_{13}	7.60	7.60	H_8	**6.47**	**6.53**
H_{12}	7.48	7.48	H_9	5.40	5.36

Table 8. Chemical shifts of compound **1** in $CDCl_3$.

Although it was not possible to record the NMR spectra of compound **1** in DMSO-d_6 due to solubility problems, 2D NOESY NMR spectra in $CDCl_3$ allowed us to study the two rotamers in solution. Despite lacking of exchanging cross-peaks, chemical shifts of *Z* rotamer could be assigned according to the results obtained for compound **2**, as well as their proton integration and NOE cross-peaks of compound **1** (Figure 11).

6. CONCLUSIONS

Two thiocoumarin derivatives, compounds **3** and **4**, have been satisfactorily synthesized in 4 steps (overall yields: 23 % and 20 %, respectively) from commercially available coumarin and characterized by spectroscopic techniques. The two target COUPY-caged benzoic acid derivatives, compounds **1** and **2**, were prepared from the corresponding thiocoumarin precursors by condensation with 4-pyridilacetonitrile, and were fully characterized by ^{1}H and ^{13}C NMR and HPLC-MS. In addition, 2D NOESY NMR experiments allowed us to demonstrate the existence of rotamers in an ~90:10 ratio (*E* rotamer and *Z* rotamer, respectively) due to rotation around the exocyclic double bond in the compounds, which cannot be considered a pure double bond due to π-delocalization.

Overall, in this research work we have developed a novel family of caging groups based on COUPY fluorophores with encouraging photophysical properties owing to their increased push-pull character, which will allow to perform uncaging with the biologically-compatible red light.

7. EXPERIMENTAL SECTION

7.1. MATERIALS AND METHODS

7.1.1. Reagents and solvents

All reagents were supplied by Sigma Aldrich, Alfa Aesar, Fluorochem or Acros. The solvents and its quality and supplier are indicated in the following table:

Entry	Solvent	Quality	Supplier
1	Acetonitrile(a)	HPLC quality	Scharlau
2	CH_2Cl_2	HPLC quality	Fisher
3	CH_2Cl_2	Synthesis quality	SDS
4	1,4-dioxane(b)	Synthesis quality	SDS
5	EtOH absolute	Synthesis quality	Panreac
6	Hexane	Synthesis quality	SDS
7	H_2O	Deionized	-
8	MeOH	Synthesis quality	Scharlau
9	Toluene	Synthesis quality	Scharlau

(a) ACN was dried by standing over 4Å molecular sieves
(b) DMF was dried by standing over 4Å molecular sieves and bubbled with nitrogen to remove volatile amines.

Table 9.

7.1.2. Nuclear magnetic resonance spectroscopy (NMR)

All NMR spectra were recorded at 25ºC on a Varian Mercury 400MHz, using $CDCl_3$ containing 0.03% (v/v) of TMS, except 2D NOESY NMR spectra which were recorded both in DMSO-d_6 and $CDCl_3$. Coupling constants (J) are given in Hz and the following abbreviations were used to indicate multiplicities: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet.

7.1.3. Chromatographic techniques

7.1.3.1. High-performance liquid chromatography (HPLC)

Reversed-phase HPLC analyses were carried out on a Waters instrument equipped with a diode array detector. The detection was carried out within the following range: 200-800 nm.

A Jupiter 4u Proteo column (Phenomenex, 250 x 4.6 mm, 4 µm, 90 Å) was used at a constant flow (1 mL/min) using the following solvents: A (Formic acid 0.1% in H_2O), B (Formic acid 0.1% in ACN).

7.1.3.2. Thin layer chromatography (TLC)

TLC analyses were performed on aluminium plates coated with 0.2 µm thick layer of silica gel (60 F, 245 nm, Merck). TLC was visualized directly under an UV lamp (245 nm and 365 nm).

7.1.3.3. Column Chromatography

Flash column chromatography was carried out with silica gel Chromatogel 60 Å (35-75 µm) from SDS.

7.1.4. Mass spectrometry

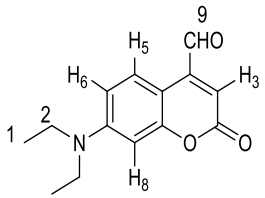
7.1.4.1. Electrospray ionization mass spectrometry (ESI-MS)

Electrospray ionization mass spectrometry analyses were carried out on a HPLC Waters 2695 equipped with a Micromass ZQ quadrupole analyzer and UV-Vis detector.

7.2. SYNTHESIS OF 4-(BENZOYLOXYMETHYL)-7-(*N,N*-DIETHYLAMINO)THIOCOUMARIN (3) AND 4-(BENZOYLOXYMETHYL)-7-(*N,N*-DIETHYLAMINO)-4-(1-HYDROXYETH-1-YL)-2-THIOCOUMARIN (4)

7.2.1. Synthesis of 4-carbaldehyde-7-(N,N-diethylamino)coumarin (5)

Selenium dioxide (22 g, 195 mmol) was added to a solution of 7-(*N,N*-diethylamino)-4-methylcoumarin (**5**) (15 g, 64.9 mmol) in a mixture of 1,4-dioxane (500 mL) and deionized H_2O (15 mL). The reaction mixture was stirred under reflux for 16 days at 110ºC. After removal of the solvent under reduced pressure, the crude residue was dissolved in methanol and filtered through Celite, which was washed with methanol. Then, the solvent was removed under reduced pressure and the product was purified by column chromatography (silica gel, 50-100% CH_2Cl_2 in hexane) to give 10.5 g (66% yield) of a red solid.

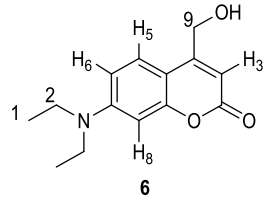


5

Red solid.
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 10.04 (s, 1H, H_9), 8.33 (d, J_{5-6} = 9.2 Hz, 1H, H_5), 6.66 (dd, J_{6-5} = 9.2 Hz, J_{6-8} = 2.5 Hz , 1H, H_6), 6.57 (d, J_{8-6}=2.5 Hz , 1H, H_8, 6.48 (s, 1H, H_3), 3.43 (q, J_{2-1} = 7.2 Hz, 4H, H_2), 1.21 (t, J_{1-2} = 7.2 Hz, 6H, H_1).
Rf (5% MeOH in CH_2Cl_2) = 0.55

7.2.2. Synthesis of 7-(*N,N*-diethylamino)-4-(hydroxymethyl)coumarin (6)

Sodium borohydride (672.9 mg, 17.79 mmol) was added to a solution of 4-carbaldehyde-7-(*N,N*-diethylamino)coumarin (**5**) (4.39 g, 17.79 mmols) in EtOH absolute (300 mL). The reaction mixture was stirred for 4 h at room temperature. After acidification of the mixture with HCl 1M (80 mL), the solution was diluted with deionized H_2O (50 mL). Then, the solution was extracted with CH_2Cl_2 (3 x 30 mL). The combined organic layers were washed with deionized water (20 mL) and dried over anhydrous $MgSO_4$ and filtered. After removal of the solvent under reduced pressure, the product was purified by column chromatography (silica gel, 50-100% CH_2Cl_2 in hexane and 0-1.5% MeOH in CH_2Cl_2) to give 2.25 g (51% yield) of a yellow solid.



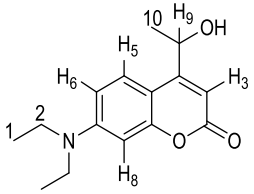
6

Yellow solid.
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.34 (d, J_{5-6}=9.1 Hz, 1H, H_5), 6.63 (dd, J_{6-5} = 9.1 Hz, J_{6-8} = 2.5 Hz, 1H, H_6), 6.56 (d, J_{8-6} = 2.5 Hz, 1H, H_8), 6.27 (t, J_{3-9}= 1.4 Hz, 1H, H_3), 4.84 (d, J_{9-3} = 1.4 Hz, 2H, H_9), 3.41 (q, J_{2-1} = 7.2 Hz, 4H, H_2), 1.21 (t, J_{1-2} = 7.2 Hz, 6H, H_1).
Rf (5% MeOH in CH_2Cl_2) = 0.32

7.2.3. Synthesis of 7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)coumarin (7)

A solution of 4-carbaldehyde-7-(*N,N*-diethylamino)coumarin (**5**) (5.78 g, 23.46 mmols) in anhydrous THF (120 mL) was cooled at -78ºC under argon atmosphere. Then, a solution of CH_3MgCl in dry THF (24 mL, 70.38 mmol) was added dropwise in the dark and the reaction mixture was stirred for 4 h. After addition of a saturated solution of NH_4Cl (200 mL), the mixture was brought to room temperature. The solution was extracted six times with ethyl acetate (150 mL first and then 80 mL). The combined organic layers were dried over $NaSO_4$ and filtered. After removal of the solvent under vacuum, the product was purified by column chromatography (silica

gel, 50-100% CH_2Cl_2 in hexane and 0-1.5% MeOH in CH_2Cl_2) to give 2.72 g (44% yield) of a yellow solid.



7

Yellow solid.
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm):
7.42 (d, $J_{5\text{-}6}$ = 9.0 Hz, 1H, H_5), 6.57 (dd, $J_{6\text{-}5}$ = 9.0 Hz, $J_{6\text{-}8}$ = 2.6 Hz, 1H, H_6), 6.52 (d, $J_{8\text{-}6}$ = 2.6 Hz, 1H, H_8), 6.27 (d, $J_{3\text{-}9}$ = 0.5 Hz, 1H, H_3), 5.13 (dd, $J_{9\text{-}10}$ = 6.9 Hz, $J_{9\text{-}3}$ = 0.5 Hz 1H, H_9), 3.41 (q, $J_{2\text{-}1}$ = 7.0 Hz, 4H, H_2), 1.57 (d, $J_{10\text{-}9}$ = 6.9 Hz, 3H,H_{10}), 1.21 (t, $J_{1\text{-}2}$ = 7.0 Hz, 6H, H_1).
Rf (5% MeOH in CH_2Cl_2) = 0,33

7.2.4. Synthesis of 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)coumarin (8) and 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)coumarin (9)

These reactions were carried out in parallel and the amount of each reagent are indicated in the following tables.

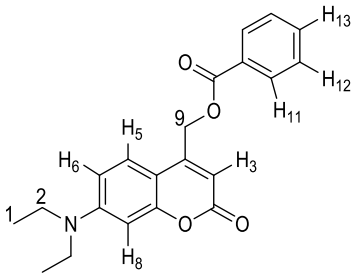
For compound 8	4-(benzoyloxymethyl)-7-(N,N-diethylamino)coumarin (6)	Benzoic acid	EDC	DMAP
Mass quantity [g**]**	2.98	1.80	2.00	1.61
Molar quantity [mmol**]**	12.00	14.42	13.20	13.20

Table 10.

For compound 9	4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)coumarin (7)	Benzoic acid	EDC	DMAP
Mass quantity [g**]**	2.72	1.60	1.80	1.40
Molar quantity [mmol**]**	10.38	12.46	11.42	11.42

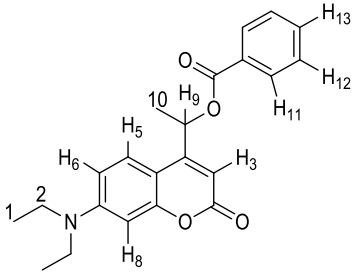
Table 11.

The corresponding coumarin derivative (**8** or **9**), benzoic acid, DMAP and EDC were cooled at 0ºC under an argon atmosphere and then dissolved in CH_2Cl_2 (100 mL, HPLC quality). The mixture was stirred at 0ºC for 15 min and 17 h at room temperature. The solution was washed with saturated NH_4Cl (100 mL), 5 % aqueous $NaHCO_3$ (1 x 100 mL, then 2 x 50 mL) and deionized water (100 mL). The organic layer was dried over anhydrous $MgSO_4$ and filtered. After removal of the solvent under reduced pressure, the corresponding product obtained was used without further purification in the next step.



8

Yellow solid.
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 8.12 (dd, J_{11-12} = 8.2 Hz, J_{11-13}= 1.1 Hz, 2H, H_{11}), 7.62 (tt, J_{13-12} = 9.1 Hz, J_{13-11} = 1.1 Hz, 1H, H_{13}), 7.49 (t, J_{12-11} = 8.2 Hz, J_{12-13} = 9.1 Hz, 2H, H_{12}), 7.37 (d, J_{5-6} = 9.0 Hz, 1H, H_5), 6.60 (dd, J_{6-5} = 9.0 Hz, J_{6-8} = 2.6 Hz, 1H, H_6), 6.54 (d, J_{8-6} = 2.6 Hz, 1H, H_8), 6.26 (t, J_{3-9} = 1.3 Hz, 1H, H_3), 5.47 (d, J_{9-3} = 1.3 Hz, 2H, H_9), 3.42 (q, J_{2-1} = 7.0 Hz, 4H, H_2), 1.22 (t, J_{1-2} = 7.0 Hz, 6H, H_1).
Rf (5% MeOH in CH_2Cl_2) = 0.64



9

Orange-brown oil.
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 8.10 (dd, J_{11-12} = 8.3, J_{11-13} = 1.1 Hz, 2H, H_{11}), 7.62 (tt, J_{13-12} = 9.1 Hz, J_{13-11} = 1.1 Hz, 1H, H_{13}), 7.49 (t, J_{12-11} = 8.3 Hz, J_{12-13} = 9.1 Hz, 2H, H_{12}), 7.37 (d, J_{5-6} = 9.0 Hz, 1H, H_5), 6.61 (dd, J_{6-5} = 9.0 Hz, J_{6-8} = 2.7 Hz, 1H, H_6), 6.53 (d, J_{8-6} = 2.7 Hz, 1H, H_8), 6.32 (q, J_{9-10} = 6.6 Hz, 1H, H_9), 6.25 (s, 1H, H_3), 3.42 (q, J_{2-1} = 7.0 Hz, 4H, H_2), 1.72 (d, J_{10-9} = 6.6 Hz, 3H, H_{10}), 1.21 (t, J_{1-2} = 7.0 Hz, 6H, H_1).
Rf (5% MeOH in CH_2Cl_2) = 0.69

7.2.5. Synthesis of 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)thiocoumarin (3) and 4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-thiocoumarin (4)

These reactions were carried out in parallel and the amount of each reagent are indicated in the following tables.

For compound 3	**4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)coumarin (8)**	**Lawesson's reagent**
Mass quantity [g]	4.22	3.88
Molar quantity [mmol]	12.00	9.60

Table 12.

For compound 4	**4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)coumarin (9)**	**Lawesson's reagent**
Mass quantity [g]	3.788	3.360
Molar quantity [mmol]	10.379	8.300

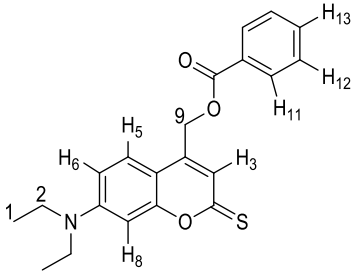
Table 13.

Lawesson's reagent was added to a solution of the corresponding coumarin derivative (**3** or **4**) crude in toluene (100 mL) under an argon atmosphere, and the mixture was stirred under reflux at 105ºC in the dark overnight. After removal of the solvent under vacuum, the product was purified by column chromatography (silica gel, 25-65% CH_2Cl_2 in hexane). In the following table are shown the results obtained of both products.

Entry	Compound	Mass synthesized [g]	Yield[(a)] [%]
3	4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)thiocoumarin	2.96	68
4	4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-thiocoumarin	2.73	70

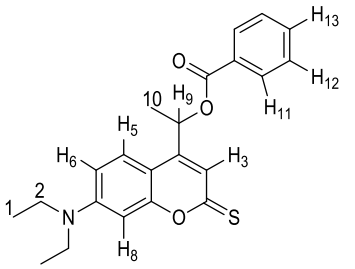
(a) Yield for the two steps

Table 14.



3

Yellow solid.
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 8.11 (dd, J_{11-12} = 8.3 Hz, J_{11-13}= 1.3 Hz, 2H, H_{11}), 7.62 (tt, J_{13-12} = 9.1 Hz, J_{13-11} = 1.3 Hz, 1H, H_{13}), 7.48 (m, 2H, H5, H_{12}), 7.17 (s, 1H, H_3), 6.80 (m, 2H, H_6, H_8), 5.44 (s, 1H, H_9), 3.42 (q, J_{2-1} = 7.1 Hz, 4H, H_2), 1.22 (t, J_{1-2} = 7.1 Hz, 6H, H_1).
Rf (CH_2Cl_2) = 0.60



4

Yellow solid.
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 8.09 (dd, J_{11-12} = 8.1, J_{11-13} = 1.1 Hz, 2H, H_{11}), 7.62 (tt, J_{13-12} = 9.1 Hz, J_{13-11} = 1.1 Hz, 1H, H_{13}), 7.55 (d, J_{5-6} = 10.0 Hz, 1H, H_5), 7.48 (t, J_{12-11} = 8.1 Hz, J_{12-13} = 9.1 Hz, 2H, H_{12}), 7.16 (s, 1H, H_3), 6.70-6.68 (m, 2H, H_6, H_8), 6.32 (q, J_{9-10} = 6.6 Hz, 1H, H_9), 3.43 (q, J_{2-1} = 7.1 Hz, 4H, H_2), 1.71 (d, J_{10-9} = 6.6 Hz, 3H, H_{10}), 1.23 (t, J_{1-2} = 7.1 Hz, 6H, H_1).
Rf (CH_2Cl_2) = 0.64

7.3. SYNTHESIS OF 4-(BENZOYLOXYMETHYL)-2-(CYANO(4-PYRIDINE)METHYLENE)-7-(*N,N*-DIETHYLAMINO)COUMARIN (1) AND 4-(BENZOYLOXYMETHYL)-2-(CYANO(4-PYRIDINE)METHYLENE)-7-(*N,N*-DIETHYLAMINO)-4-(1-HYDROXYETH-1-YL)-2-COUMARIN (2)

These reactions were carried out in parallel and the amount of each reagent are indicated in the following tables.

For compound 1	4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)thiocoumarin (3)	4-pyridylacetonitrile	NaH	$AgNO_3$
Mass quantity [g]	0.500	0.40	0.21	0.57
Molar quantity [mmol]	1.362	2.60	5.18	3.41

Table 15.

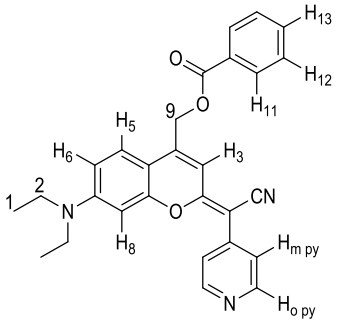
For compound 2	4-(benzoyloxymethyl)-7-(*N,N*-diethylamino)thiocoumarin (4)	4-pyridylacetonitrile	NaH	$AgNO_3$
Mass quantity [g]	0.500	0.40	0.21	0.56
Molar quantity [mmol]	1.311	2.62	5.24	3.28

Table 16.

Firstly, the reagents NaH, 4-pyridylacetonitrile and the corresponding coumarin derivative (**1** or **2**) were dried putting them at the desiccator for 2 h. Secondly, the reaction took place as described below. A solution of NaH and 4-pyridylacetonitrile in ACN (30 mL, HPLC quality) was stirred at room temperature for 15 min. Then, a solution of the corresponding coumarin derivative (**1** or **2**) in ACN/CH_2Cl_2 (1:1, 50 mL) was added dropwise under argon atmosphere to the solution prepared previously. The reaction mixture was stirred at room temperature in the dark for 2 h. Next, $AgNO_3$ was added and stirring was kept for 2 h. After removal the solvent under reduced pressure, the product was purified by column chromatography (silica gel, 50-100% CH_2Cl_2 in hexane and then, 0.1-2.5% MeOH in CH_2Cl_2). In Table 17 are shown the results obtained of both products.

Entry	Compound	Mass synthesized [mg]	Yield [%]
1	4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)coumarin	322	53
2	4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-coumarin	369	60

Table 17.



1

Yellow solid.

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm):

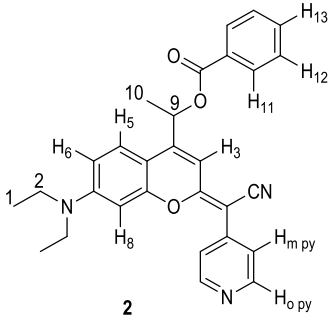
Rotamer E: 8.61 (dd, $J_{Ho\,py-\,Hm\,py}$ = 4.7 Hz, $J_{H'o\,py-\,Hm\,py}$ = 1.6 Hz, 2H, $H_{o\,py}$, $H'_{o\,py}$), 8.12 (dd, J_{11-12} = 8.4 Hz, J_{11-13}= 1.3 Hz, 2H, H_{11}), 7.74 (dd, $J_{Hm\,py-\,Ho\,py}$ = 4.7 Hz, $J_{H'mpy-\,Ho\,py}$ = 1.6 Hz, 2H, $H_{m\,py}$, $H'_{m\,py}$), 7.60 (tt, J_{13-12} = 9.1 Hz, J_{13-11} = 1.3 Hz, 1H, H_{13}), 7.48 (t, J_{12-11}= 8.4 Hz, 2H, H_{12}), 7.34 (d, J_{5-6} = 9.0 Hz, 1H, H_5), 7.05 (d, J_{3-9} = 1.0 Hz, 1H, H_3), 6.58 (dd, J_{6-5}= 9.0 Hz, J_{6-8}= 2.5 Hz, 1H, H_6), 6.47 (d, J_{8-6}= 2.5 Hz, 1H, H_8), 5.40 (d, J_{9-3} = 1.0 Hz, 2H, H_9), 3.42 (q, J_{2-1} = 7.0 Hz, 4H, H_2), 1.22 (t, J_{1-2} = 7.0 Hz, 6H, H_1).

Rotàmer Z: 8.48 (dd, $J_{Hm\,py-\,Ho\,py}$ = 4.5 Hz, $J_{H'm\,py-\,Ho\,py}$ = 1.6 Hz, 2H, $H_{m\,py}$, $H'_{m\,py}$), 8.03 (dd, J_{11-12} = 8.4 Hz, J_{11-13}= 1.3 Hz, 2H, H_{11}), 7.60 (tt, J_{13-12} = 9.1 Hz, J_{13-11} = 1.3 Hz, 1H, H_{13}), 7.48 (t, J_{12-11}= 8.4 Hz, J_{12-13}= 9.1 Hz, 2H, H_{12}), 7.25 (d, J_{5-6} = 12 Hz, 1H, H_5), 6.83 (d, J_{3-9} = 1.0 Hz 1H, H_3), 6.53 (m, 2H, H_6, H_8), 5.36 (d, J_{9-3} = 1.0 Hz, 2H, H_9), 3.42 (q, J_{2-1} = 7.0 Hz, 4H, H_2), 1.22 (t, J_{1-2} = 7.0 Hz, 6H, H_1).

^{1}H NMR ($CDCl_3$, 101 MHz) δ (ppm): 165.9, 162.6, 154.6, 150.7, 149.9, 140.5, 140.2, 133.6, 129.8, 129.3, 128.6, 124.8, 111.4, 109.3, 107.15, 97.2, 62.3, 44.7, 12.5

HPLC: Rt = 11.02 min (analytical gradient: 30-100% in 30 min; A: 0.1% formic acid in H_2O, B: 0.1% formic acid in ACN). MS (ESI): m/z calcd. for $C_{28}H_{72}N_3O_3$ $[M+H]^+$ 452.04; found 451.19

Rf (5% MeOH in CH_2Cl_2) = 0.36



2

Orange solid.

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm):

Rotamer E: 8.60 (dd, $J_{Ho\ py-\ Hm\ py}$ = 6.2 Hz, 2H, $H_{o\ py,}$), 8.13 (dd, J_{11-12} = 8.11 Hz, J_{11-13}= 1.0 Hz, 2H, H_{11}), 7.73 (dd, $J_{Hm\ py-\ Ho\ py}$ = 4.7 Hz, $J_{H'm\ py-\ Ho\ py}$ = 1.6 Hz, 2H, $H_{m\ py,}$ $H'_{m\ py}$), 7.60 (tt, J_{13-12} = 8.0 Hz, J_{13-11} = 1.0 Hz, 1H, H_{13}), 7.49 (t, J_{12-11}= 7.6 Hz, 2H, H_{12}), 7.45 (d, J_{5-6} = 9.1 Hz, 1H, H_5), 7.05 (s, 1H, H_3), 6.59 (dd, J_{6-5}= 9.1 Hz, J_{6-8}= 2.5 Hz, 1H, H_6), 6.46 (d, J_{8-6}= 2.5 Hz, 1H, H_8), 6.30 (q, J_{9-10} = 6.6 Hz, 1H, H_9), 5.40 (s, 1H, 2H, H_9), 3.42 (q, J_{2-1} = 7.0 Hz, 4H, H_2), 1.75 (d, J_{10-9} = 6.6 Hz, 3H, H_{10}), 1.24 (t, J_{1-2} = 7.0 Hz, 6H, H_1).

Rotamer Z: 8.36 (dd, $J_{Ho\ py-\ Hm\ py}$ = 6.1 Hz, 2H, $H_{o\ py,}$), 8.05 (dd, J_{11-12} = 8.15 Hz, J_{11-13}= 1.1 Hz, 2H, H_{11}), 7.60 (m, 1H, H_{13}), 7.49 (m, 2H, H_{12}), 7.32 (d, J_{5-6} = 9.6 Hz, 1H, H_5), 7.25 (m, 2H, $H_{m\ py}$), 6.83 (s, 1H, H_3), 6.55 (m, 2H, H_6, H_8), 6.24 (q, J_{9-10} = 6.5 Hz, 1H, H_9), 1.66 (d, J_{10-9} = 6.6 Hz, 3H, H_{10}).

^{1}H NMR ($CDCl_3$, 101 MHz) δ (ppm): 165.6, 163.0, 154.8, 150.6, 149.8, 146.4, 140.7, 133.4, 129.8, 129.6, 128.7, 128.6, 124.9, 120.9, 119.3, 109.3, 109.1, 106.6, 97.3, 83.6, 68.2, 44.7, 20.9, 12.5.

HPLC: Rt =11.48 min (analytical gradient: 30-100% in 30 min; A: 0.1% formic acid in H_2O, B: 0.1% formic acid in ACN). MS (ESI): m/z calcd. for $C_{29}H_{74}N_3O_3$ $[M+H]^+$ 466.01; found 465.20

Rf (5% MeOH in CH_2Cl_2) = 0.32

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9. ACRONYMS

ACN: acetonitrile

Calcd.: calculated

DCM: dichloromethane

DMAP: 4-dimethylaminopyridine

EDC: *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide

EDU: EDC hydrolyzed urea derivative

ESI-MS: electrospray ionization mass spectrometry

HPLC: high-performance liquid chromatography

NMR: nuclear magnetic resonance

PPG: photolabile protecting group

Rf: retention factor in TLC

Rt: retention time in HPLC

THF: tetrahydrofuran

TLC: thin layer chromatography

TMS: trimethylsilane

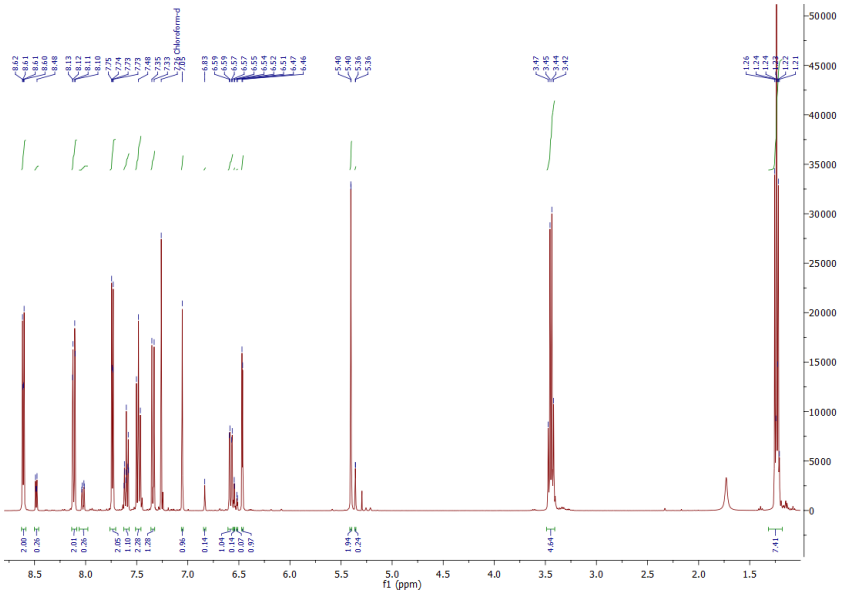
UV: ultraviolet

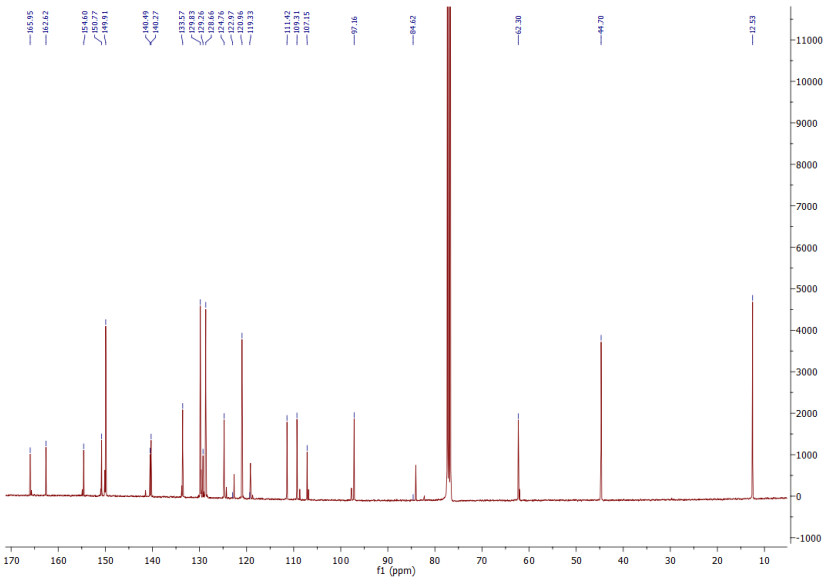
Vis: visible

APPENDICES

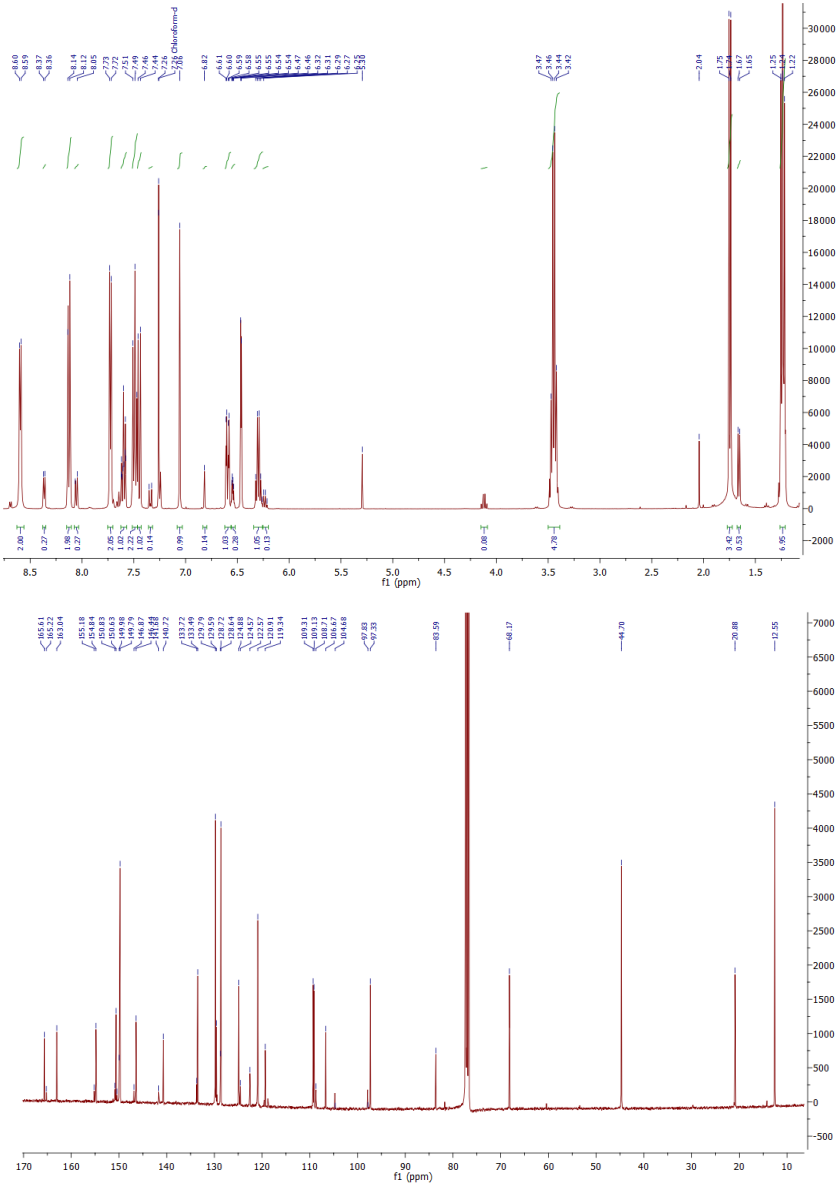
APPENDIX 1: ^{1}H AND ^{13}C NMR SPECTRA OF THE COMPOUNDS

4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N*,*N*-diethylamino)coumarin (1)





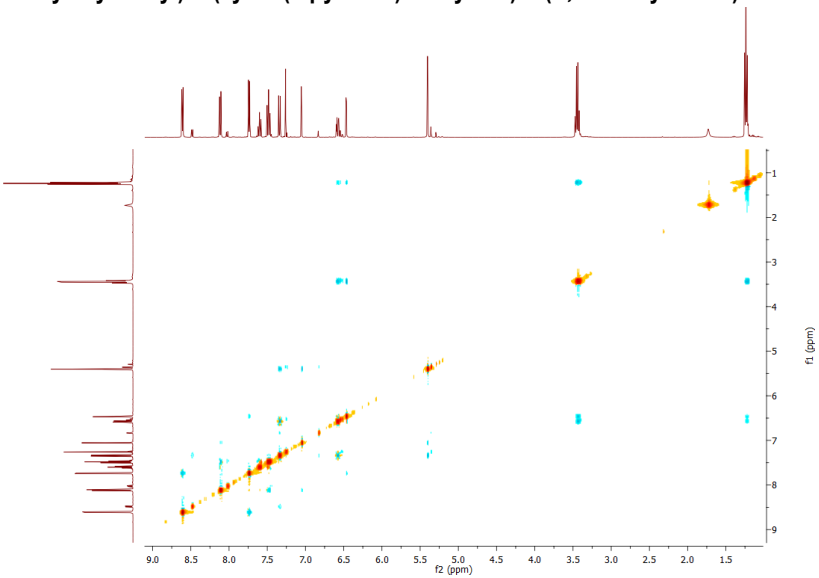
4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-coumarin (2)



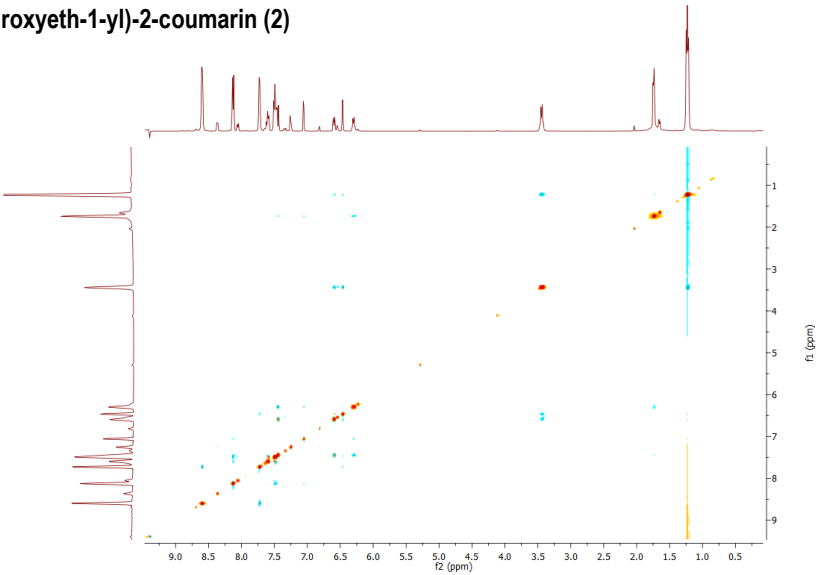
APPENDIX 2: 2D NOESY NMR IN DMSO-D_6 AND $CDCL_3$

2D NOESY NMR in $CDCl_3$

4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)coumarin (1)



4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N,N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-coumarin (2)



2D NOESY NMR in DMSO-d_6

4-(benzoyloxymethyl)-2-(cyano(4-pyridine)methylene)-7-(*N*,*N*-diethylamino)-4-(1-hydroxyeth-1-yl)-2-coumarin (2)