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Original article

Inhibition of neutrophil O_2^- production by unsymmetrical methylene derivatives of benzopyrans: their use as potential antiinflammatory agents

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Abstract – Some unsymmetrical derivatives of benzopyrans **9** were synthesized and tested to verify their PKC inhibitory activity. For this purpose, the Mannich bases of 7-hydroxycoumarins **6** were treated with 2-(dialkylamino)benzopyran-4-ones or 3-(dialkylamino)naphtho[2,1-*b*]pyran-1-ones **8** in the presence of acetic or propionic anhydride, yielding compounds **9**. Human neutrophils stimulated with either PMA and f-MLF were used as the cellular model. The efficiency of the compounds **9** was established on their capacity to reduce the O_2^- production by activated human neutrophils. Compounds **9d** and **9f**, bearing an acetoxy group in position 7 of the chromone moiety, seem to counteract the neutrophil activation efficiently. © 2001 Éditions scientifiques et médicales Elsevier SAS

Mannich bases / superoxide anion / neutrophils / 1-benzopyran-4-ones / antiinflammatory agents

1. Introduction

In previous works we showed that benzopyran-4-ones (or chromones) and naphthopyran-1-ones bearing a dialkylamine in the position adjacent to the heteroatom are potentially useful as antiinflammatory agents because their ability to inhibit the protein kinase C (PKC) dependent signal transduction pathway. In particular, these studies showed that the cited activity was prominent when the dialkylamino substituent was piperidine, diethylamine or bis(2-methoxyethyl)amine [1, 2].

Following our interest in synthetic substances that could be useful as antiinflammatory agents, we have now analyzed the PKC inhibitory activity of some of those active compounds when they are linked by means of a methylene bridge to 7-hydroxycoumarins. This type of synthesis is an application of the use of Mannich bases in acetic or propionic anhydride to form unsymmetrical methylene derivatives. In this context, the recovery of diacetyloxy derivatives of 7-hydroxycoumarins, under certain experimental conditions, may improve our understanding of the reaction mechanism.

2. Chemistry

It is known that the treatment of Mannich base derivatives with acetic anhydride usually leads to acetoxy derivatives [3]. On the contrary, we did not find any acetoxy derivative when Mannich bases of benzopyran-4-ones or naphthopyran-1-ones were treated with acetic anhydride but we did find methylene-bis-derivatives or unsymmetrical methylene derivatives such as the example shown in *figure 1*. The formation of such derivatives was determined by a balance between deamination and deaminomethylation of the Mannich bases [4]. Briefly, the Mannich base **1**, in the presence of a substrate different from that coming from its deaminomethylation but structurally similar, as the case of naphtho[2,1-*b*]pyran-1-one **2**, yields, in acetic anhydride, the unsymmetrical methylene derivative **3**. On the contrary, in the absence of a

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substrate or when the substrate was the starting compound of the Mannich base **1**, such as the chromone **4**, the symmetrical methylene derivative **5** was obtained. Further details of these reactions are given in Section 4.

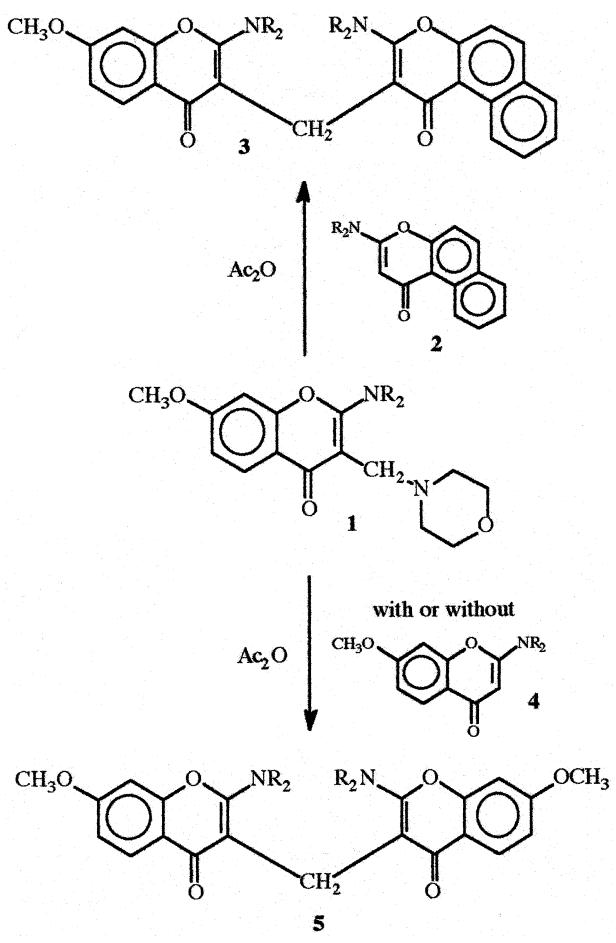


Figure 1. Methylene-bis derivatives.

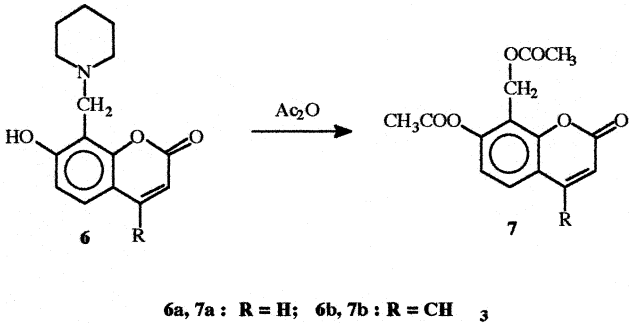


Figure 2. Diacetyl derivative.

Following our studies on unsymmetrical methylene derivatives of biologically active compounds in the benzopyran series, our interest was focused to the Mannich bases of 7-hydroxycoumarins **6**. Interestingly, the treatment of such bases **6** with acetic anhydride follows the typical route and produces the diacetyl derivatives **7** (see *figure 2*).

This preliminary observation permitted us to assume that the Mannich bases **6** are less disposed to deaminomethylation than the previously investigated Mannich bases on benzopyran-4-ones and naphtho[2,1-*b*]pyran-1-ones. Therefore, the compounds **6** seemed very interesting for the formation and the reaction study of unsymmetrical methylene derivatives.

When compounds **6** were treated with benzopyrans or naphthopyrans **8** in acetic anhydride it was possible to obtain the unsymmetrical methylene derivatives **9** in good yield (see *figure 3*). The use of propionic anhydride gave similar results to acetic anhydride. In order to counteract the formation of diacetyl derivatives **7** it is preferable to combine the anhydride and the chromonic compounds **8** first, wait for it to dissolve and than add the Mannich bases **6**.

All compounds described herein are white crystals and their structures are in agreement with elemental analyses and spectral data (see Section 5). In particular, the ^{1}H-NMR spectra of unsymmetrical derivatives in which the naphtho[2,1-*b*]pyran-1-one moiety is present retain the characteristic deshielding signal ($\delta = 10.05$) of such angular structures [5].

3. Biology

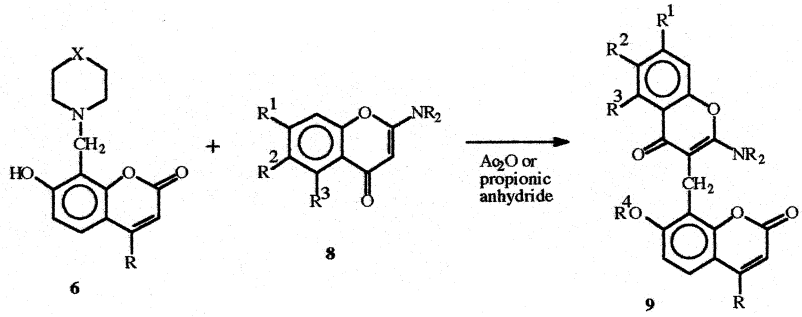
3.1. Isolation of neutrophils

Freshly collected heparinized human blood (100 mL) from healthy donors was treated with 1.6% dextran (final concentration) and left at 25–28 °C to sedimentate for 1 h. The supernatants (40 mL) were collected and layered onto 10 mL of 6% Ficoll 400 solution containing 0.17% (v/v) Urovison and centrifuged at $800 \times g$ for 20 min. The pellets containing mostly neutrophils and contaminating red cells were resuspended in 10 mL of hypotonic 0.2% NaCl. After 30 s, 10 mL of hypertonic 1.6% NaCl were added to normalize the osmotic pressure. This treatment lyses all contaminating red cells. The white cells were recovered and washed three times with 0.01 M sodium phosphate (pH 7.4), 5

mM KCl, 0.12 M NaCl, 24 mM $NaHCO_3$, 5 mM glucose. Prior to use, the cells were maintained in an ice bath in the same medium at a concentration of 15–20× 10^6 cells per mL. The cell population obtained consisted of more than 96% neutrophils, as evaluated by microscopic examination. The few remaining cells were eosinophils and monocytes.

3.2. Activation of neutrophils and assay of superoxide anion (O_2^-)

About 10^6 cells were diluted in 1 mL of 10 mM HEPES, pH 7.4, containing 0.15 M NaCl, 5.0 mM glucose, 1.0 mM Ca^{2+} and 0.625 mg mL^{-1} of cytochrome *C* (Fe^{3+}) and incubated at 37 °C for 2 min.



6 8 9

Comp.	NR_2	R	R^1	R^2	R^3	R^4
6a	--	H	--	--	--	--
6b	--	CH_3	--	--	--	--
8a	NMe_2	--	OCH_3	H	H	--
8b	NEt_2	--	OCH_3	H	H	--
8c	NEt_2	--	OH	H	H	--
8d	1-Pyrrolidinyl	--	OCH_3	H	H	--
8e	1-Piperidinyl	--	OCH_3	H	H	--
8f	$N(CH_2CH_2OCH_3)_2$	--	OCH_3	H	H	--
8g	NMe_2	--	H	benzo		--
8h	NEt_2	--	H	benzo		--
8i	1-Pyrrolidinyl	--	H	benzo		--
8j	1-Piperidinyl	--	H	benzo		--
8k	$N(CH_2CH_2OCH_3)_2$	--	H	benzo		--
9a	NMe_2	H	OCH_3	H	H	$COCH_3$
9b	NMe_2	CH_3	OCH_3	H	H	$COCH_3$
9c	NEt_2	H	OCH_3	H	H	$COCH_3$
9d	NEt_2	H	$OCOCH_3$	H	H	$COCH_3$
9e	NEt_2	CH_3	OCH_3	H	H	H
9f	NEt_2	CH_3	$OCOCH_3$	H	H	$COCH_3$
9g	NEt_2	CH_3	OCH_3	H	H	$COCH_2CH_3$

Figure 3. Unsymmetrical methylene derivatives.

9h	1-Pyrrolidinyl	H	OCH_3	H	H	H
9i	1-Pyrrolidinyl	CH_3	OCH_3	H	H	H
9j	1-Pyrrolidinyl	CH_3	OCH_3	H	H	$COCH_3$
9k	1-Piperidinyl	H	OCH_3	H	H	$COCH_3$
9l	1-Piperidinyl	CH_3	OCH_3	H	H	H
9m	1-Piperidinyl	CH_3	OCH_3	H	H	$COCH_3$
9n	$N(CH_2CH_2OCH_3)_2$	H	OCH_3	H	H	$COCH_3$
9o	$N(CH_2CH_2OCH_3)_2$	CH_3	OCH_3	H	H	$COCH_3$
9p	NMe_2	CH_3	H	b e n z o		H
9q	NMe_2	H	H	b e n z o		$COCH_3$
9r	NEt_2	H	H	b e n z o		$COCH_2CH_3$
9s	NEt_2	CH_3	H	b e n z o		$COCH_3$
9t	NEt_2	CH_3	H	b e n z o		$COCH_2CH_3$
9u	1-Pyrrolidinyl	H	H	b e n z o		H
9v	1-Pyrrolidinyl	H	H	b e n z o		$COCH_3$
9w	1-Pyrrolidinyl	CH_3	H	b e n z o		H
9x	1-Piperidinyl	H	H	b e n z o		$COCH_3$
9y	1-Piperidinyl	CH_3	H	b e n z o		$COCH_3$
9z	$N(CH_2CH_2OCH_3)_2$	CH_3	H	b e n z o		$COCH_3$

Figure 3. (*Continued*)

Phorbol myristate acetate (PMA, 100 mg) or formylmethionine–leucine–phenylalanine (f-MLF, 0.1 μM final concentration) were then added. The absorbance at 550 nm was continuously monitored for 10 min. The amount of O_2^- produced was calculated by the difference in absorbance at zero time and at the end of the reaction.

3.3. Samples

The compounds were diluted in dimethyl sulfooxide (DMSO) at standard concentration of 20 mM. When tested on neutrophils, 1 μL of standard solution was added to 1 mL of cell suspension. As a control, 1 μL of DMSO was added to the blank sample.

3.4. PKC purification

Ca^{2+} and phospholipid dependent protein kinase C (PKC) were purified from human neutrophils as described by Pontremoli et al. [6]. PKC activity was assayed using Istone type IIIS, as substrate, Ca^{2+}, phosphatidylserine, diacylglycerol and ^{32}P-ATP, as described previously [6].

4. Results and discussion

In the previous work regarding the synthesis of unsymmetrical methylene derivatives from benzopyran-4-ones it was shown that, apart from the presence of the unsymmetrical derivatives, there were small amounts of methylene-bis-derivatives of the compounds bearing the Mannich base as well as the compounds without the Mannich base. This fact was easily explained on the basis of the reversibility of the Mannich reaction [3]. On the contrary, in the present work we did not find any methylene-bis-derivatives and this absence provides further support for the scarcity of the deaminomethylation process when the Mannich bases **6** are in the presence of acetic or propionic anhydride.

Moreover, it is possible to indicate that the recovery of the diacetyl derivatives **7** or the unsymmetrical derivatives **9** is dependent on the presence of a nucleophylic reagent. This evidence permits us to give deeper insight into the reaction mechanism (see *figure 4*). The first attack of the anhydride presumably leads to the intermediate **A** which may evolve in two ways. The first one occurs in the absence of a nucleophylic

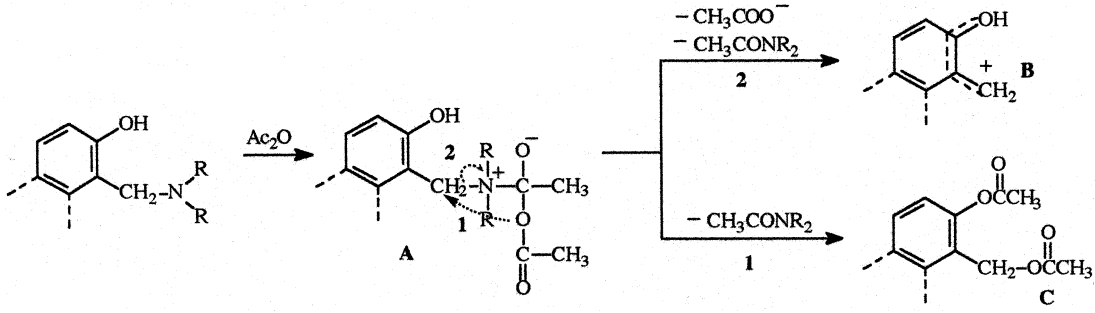


Figure 4. Proposed mechanism for the formation of acetyloxy derivative C (arrow 1) or carbocation B (arrow 2) by treatment of Mannich bases with acetic anhydride. The carbocation B may evolve in the symmetrical or unsymmetrical derivatives, according to the reaction conditions.

reagent or if the nucleophylic reagent is weak and leads to the acetoxy derivative **C** (see *figure 4*, arrow 1). The second one occurs when the nucleophylic attack is strong enough to lead to the removal of dialkylamine from the Mannich base with the formation of a stabilized carbocation **B** (see *figure 4*, arrow 2). The latter may react with position 3 of benzopyran-4-ones (or position 2 of naphthopyran-1-ones) thus forming the unsymmetrical derivatives **9**.

Interestingly, a non acylated hydroxy group is present in the formula **A**. In fact, the recovery of some compounds **9** having the R^4 substituent acylated or not, indicates that the above reaction runs quickly and its rate, in the case of a hydroxy group *ortho* to a dialkylaminomethyl group, is faster than the acylation reaction.

In fact, when the reaction leads to a derivative **9** in which the presence of the hydroxy group of the coumarin coupled with a particular dialkylamine (especially pyrrolidine and piperidine) on the benzopyran-4-one confers to the unsymmetrical dimer a high melting point, a precipitate separates out from the reaction medium in a few minutes before acylation can occur. It is sometimes possible to recover acylated and non acylated derivatives in the same reaction. On the contrary, if the products coming from the primary attack are soluble in the anhydride, the final unsymmetrical derivatives are acylated.

A further piece of evidence for the proposed reaction mechanism was the extraction, after the recovery of the unsymmetrical derivative **9**, of *N*-acetylpiperidine. A solution containing this compound was examined by GLC–MS, the main chromatographic peak presented a molecular peak at 127 and the fragmentation pattern was consistent with that of *N*-acetylpiperidine.

Another interesting observation comes from the behavior of the 7-hydroxychromone **8c** in the reaction with the Mannich bases **6**. It is known that when 7-hydroxychromones are subjected to Mannich reaction the aminomethylation occurs in position 8 (as already seen for 7-hydroxycoumarin) [7]. But in our case, the attack of the coumarin intermediate occurs in position 3 of the chromone ring, yielding **9d** and **9f**, as the acylation on the 7-hydroxy group causes the deactivation of position 8 of the chromone.

This fact means that changing the insertion of the aminomethyl group from the 7-hydroxycoumarin to the chromone is not allowed because the 7-acetyloxycoumarin cannot receive the nucleophilic attack. On the contrary, in the case of the previously studied unsymmetrical derivative **3** in *figure 1*, the Mannich base can indifferently be on the bicyclic- or tricyclic compound [4]; in fact the benzopyran-4-ones and naphtho[2,1-*b*]pyran-1-ones, lacking hydroxy groups, are inert to treatment with acetic anhydride and inactivations due to acetylation are not possible.

Another interesting consequence of the reaction between the 7-hydroxychromone **8c** and the Mannich bases **6** is the formation of 7-acetyloxy derivatives. In fact, as far as we can see, **9d** and **9f** are the first examples of derivatives of 2-(diethylamino)-7-hydroxychromone which are acylated at the 7-hydroxy group. This observation will be a source of further investigations as the 2-(diethylamino)-7-hydroxychromone, a substance endowed with notable and diverse pharmacological properties, is very resistant to the acylation [8]. On the other hand, the acylation of 7-hydroxicoumarin is very easy.

All the compounds listed in *table I* were tested for their ability to affect the production of O_2^- in human

neutrophils stimulated by chemotactic peptide f-MLF or by PMA. Some of these compounds were effective in inhibiting O_2^- production by stimulated neutrophils.

Regarding the action against the f-MLF stimulus, the tested compounds may be divided into three categories. The first one is composed of the two compounds endowed with the best activity (**9d** and **9f**); the second one comprises the 7-methoxychromone derivative **9g** and the naphthopyran derivative **9r**; the remaining part of the tested compounds comprises the third category. It is important to stress that the two best compounds (**9d** and **9f**) are the unique compounds with the 7-acetoxy group as substituent in the chromone ring.

The two compounds which are in the intermediate zone of action (**9g**, **9r**) show some mutual structural motifs, such as the coumarin moiety without the methyl group in position 4, the propionyl group as the acylating group of the 7-hydroxycoumarin, and the diethylamine in the chromone moiety.

Table I. Effects of benzopyran derivatives **9** on O_2^- production by human neutrophils stimulated with f-MLF and PMA.

Compound	Inhibition (%)	
	f-MLF	PMA
9a	0	0
9b	0	77
9c	0	12
9d	100	63
9e	0	74
9f	100	56
9g	89	92
9h	0	0
9i	0	0
9j	0	0
9k	0	0
9l	0	0
9m	0	50
9n	0	66
9o	0	30
9p	0	0
9q	0	0
9r	58	94
9s	0	46
9t	0	8
9u	0	0
9v	0	44
9w	0	0
9x	0	16
9y	0	0
9z	0	45

Neutrophil stimulation was carried out as described in Section 3. The concentration of each compound was 20 μM. The values refer to the percentage of the observed inhibition.

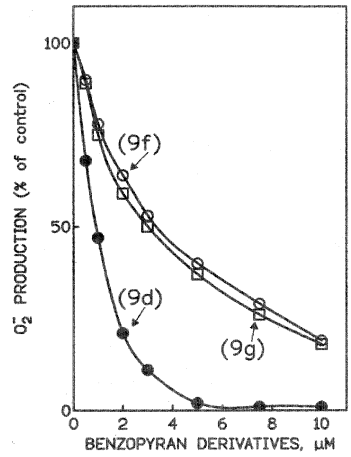


Figure 5. Effect of compounds **9d**, **9f** and **9g** on O_2^- production by human neutrophils stimulated with f-MLF. Neutrophils were stimulated with 0.1 μM as described in Section 3, in the presence of the indicated amounts of benzopyran derivatives, dissolved in 1 mL of DMSO. O_2^- production by stimulated human neutrophils in the presence of 1 mL of DMSO was used as a control and taken to be 100%.

In conclusion, from a structure–activity analysis of the two above-mentioned categories, we can conclude that in order to express biological action against the f-MLF stimulus, it is essential that the dialkylamino group on the γ-pyrone moiety is diethylamine, the γ-pyrone participates in a bicyclic ring instead of a tricyclic ring, and the α-pyrone is preferentially without the methyl group in position 4.

We cannot draw conclusions about the acyl group as we do not have data to evaluate the efficiency of the propionyl group in position 7 of the chromone.

Regarding the other stimulus (PMA), the results are not easy to interpret as there are not sharp clusters of activity, it is interesting to observe that in this case the more active compounds are **9g** and **9r**, the intermediate group with respect to f-MLF. Instead, the intermediate group with respect to PMA is comprised of compounds **9d** and **9f**, along with several others derivatives.

As the compounds **9d**, **9f** and **9g** are on the whole the more effective ones, their biological action has been analyzed in more detail (**9r** was left out due to its poor solubility).

As shown in *figure 5*, the 7-acetoxychromone derivative **9d** almost completely inhibits O_2^- production at concentration below 5 μM, with a K_{05} of ca.

1.0 μM. The 7-methoxychromone derivative **9g** and the 7-acetoxychromone derivative **9f** are two times less effective, with K_{05} values around 3.0 and 6.0 μM, respectively.

None of these compounds showing the highest inhibition of O_2^- production was effective on purified PKC activity (data not shown).

Taken together these results suggest a putative site of action for these compounds. As shown in *figure 6*, inhibition of O_2^- production by human neutrophils could be preferentially obtained by interfering at three different levels with (1) a component of the NADPH oxidase complex [9, 10]; (2) the f-MLF receptor [11, 12]; and (3) membrane associated PKC isozyme(s) [13, 14].

The last two possibilities (2 and 3) can be excluded because the neutrophil responses elicited by both f-MLF and PMA require an active membrane associated PKC [13, 14], and compounds **9d**, **9f** and **9g** do not show PKC inhibiting activity. Activation of NADPH oxidase in stimulated neutrophils require the aggregation of different protein components through a number of events, including phosphorylation and translocation from cytosol to plasma membrane [9, 10]. It seems likely that the biological activity such benzopyran derivatives can interfere with one of the processes undergoing active NADPH oxidase complex formation, on the outer surface plasma membrane [15].

The relevance of the biological effects shown by these active derivatives is due to the fact that activated neutrophils are involved, at least in part, in tissue damage characteristic of a number of human pathologies, including inflammation, ischemia and reperfusion. Further analyses are in progress to collect more data on chromone derivatives bearing different acyl substitutions on the hydroxy group in position 7.

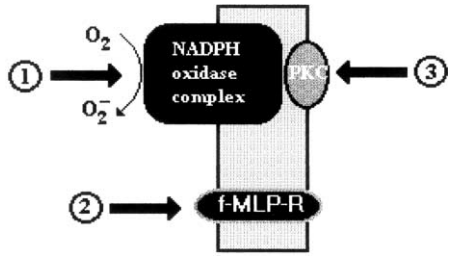


Fig. 6. Possible site of action of benzopyran derivatives on stimulated human neutrophils. f-MLF-R refers to the receptor for the chemotactic peptide f-MLF, PKC refers to Ca^{2+} lipid dependent protein kinase C. Further details are given in the text.

5. Experimental protocols

Melting points (m.p.) were determined using a Electrothermal apparatus and are uncorrected. Microanalyses were carried out on a Carlo Erba 1106 elemental analyzer. The results of elemental analysis were within ±0.3% for C and ±0.1% for H and N of the theoretical value. ^{1}H-NMR spectra were performed on a Hitachi Perkin–Elmer R 600 (60 MHz) spectrometer using TMS as internal standard ($\delta = 0$). IR spectra were recorded on a Perkin–Elmer 398 spectrophotometer.

5.1. Mannich bases ***6***

5.1.1. General method

To 20.0 mmol of 7-hydroxycoumarin (or 4-methyl-7-hydroxycoumarin) dissolved in 50 mL of ethanol, 20.0 mmol of piperidine and 2.0 mL of 40% formaldehyde were added. The resulting mixture was refluxed for 6 h. After cooling, the solvent was evaporated under reduced pressure. The pale yellow oil obtained was treated with cool acetone, leaving a white solid which was crystallized from acetone obtaining, 8-(piperidinomethyl)-7-hydroxycoumarin (**6a**), m.p. 130–131 °C, 56.4% yield; 8-(piperidinomethyl)-7-hydroxy-4-methylcoumarin (**6b**), m.p. 169–70 °C, 62.0% yield.

6a: IR (KBr) ν (cm^{-1}): 2600 (broad), 1720, 1595, 1580. ^{1}H-NMR (δ, $CDCl_3$): 1.86–1.31 (m, 6H, β+γ-piperidine CH_2), 2.87–2.29 (m, 4H, α-piperidine CH_2), 4.02 (s, 2H, CH_2 bridge), 6.19 (d, 1H, H-3), 6.75 (d, 1H, H-6), 7.30 (d, 1H, H-5), 7.64 (d, 1H, H-4), 12.09 (s, 1H, OH).

6b: IR (KBr) ν (cm^{-1}): 2600 (broad), 1722, 1600, 1580. ^{1}H-NMR (δ, $CDCl_3$): 1.96–1.37 (m, 6H, β+γ-piperidine CH_2), 2.85–2.27 (m, 7H, α-piperidine CH_2+4-CH_3), 4.05 (s, 2H, CH_2 bridge), 6.09 (s, 1H, H-3), 6.77 (d, 1H, H-6), 7.46 (d, 1H, H-5), 12.38 (s, 1H, OH).

5.2. Diacetoxy derivatives ***7***

5.2.1. General method

In a 100 mL flask, protected from moisture with a calcium chloride tube, 1.0 mmol of 8-(piperidinomethyl)-7-hydroxycoumarin (**6a**) or 8-(piperidinomethyl)-7-hydroxy-4-methylcoumarin (**6b**) was dissolved in 5 mL of freshly distilled acetic anhydride and heated at 95 °C for 1.5 h, with stirring. At the end, the cooled solution was poured onto crushed ice and water, and stirred for 1 h obtaining a white solid, which was filtered off and crystallized from ethyl acetate.

The following compounds were so obtained: 8-acetyloxymethyl-7-acetoxycoumarin (**7a**), m.p. 121–122 °C (81.3% yield) and 8-acetyloxymethyl-7-acetoxy-4-methylcoumarin (**7b**), m.p. 138–139 °C (86.2% yield).

7a: IR (KBr) ν (cm^{-1}): 1770, 1730, 1610.

^{1}H-NMR (δ, $CDCl_3$): 2.05 (s, 3H, CH_3COOCH_2), 2.38 (s, 3H, CH_3CO), 5.38 (s, 2H, CH_2 bridge), 6.43 (d, 1H, H-3), 7.11 (d, 1H, H-6), 7.41–7.90 (m, 2H, H-4, 5).

7b: IR (KBr) ν (cm^{-1}): 1760, 1730, 1605.

^{1}H-NMR (δ, $CDCl_3$): 2.06 (s, 3H, CH_3COOCH_2), 2.37 (s, 3H, CH_3CO), 2.46 (s, 3H, 4-CH_3), 5.39 (s, 2H, CH_2 bridge), 6.34 (s, 1H, H-3), 7.14 (d, 1H, H-6), 7.71 (d, 1H, H-5).

5.3. Unsymmetrical methylene derivatives ***9***

5.3.1. General method

In a 100 mL flask, protected from moisture with a calcium chloride tube, 1.0 mmol of 2-(dialkylamino)-benzopyran-1-one (**8a**–**f**) or 3-(dialkylamino)-1H-naphto[2,1-*b*]pyran-1-one (**8g**–**k**) was dissolved in 5 mL of freshly distilled acetic or propionic anhydride. Then 1.0 mmol of Mannich base (**6a** or **6b**) was added, and the mixture was heated at 95 °C for 1.5 h, with stirring. At the end, the cooled solution was poured onto crushed ice and water, and stirred for 2 h obtaining solid (case A) or oily (case B) residues. Sometimes, the solid was already obtained during the heating in the anhydride (case C) or two products, with different solubility, were isolated (case D).

In case A, the solid was collected by filtration, washed with water and crystallized from suitable solvent.

In case B, the acidic solution was alkalinized with sodium hydroxide pellets and the resulting solution was extracted three times with chloroform. The pooled organic extracts were washed with water, dried over anhydrous sodium sulfate and evaporated under reduced pressure. The oily residue obtained was treated with a small amount of ethyl acetate and the crystals which separated out were filtered and washed with cold solvent.

In case C, the final mixture was cooled and the solid obtained was filtered off.

In case D, the final mixture was cooled and a first solid was filtered off. Then the filtered solution was poured onto crushed ice and water, and stirred for 2 h obtaining a second solid.

The starting products, the anhydride used (1 for acetic anhydride, and 2 for propionic anhydride), the case (A, B, C or D) followed for each synthesised compound and the crystallization solvent are reported in brackets.

*5.3.1.1. 8-[2′-(Dimethylamino)-7′-methoxychromon-3′-yl]methyl-7-acetoxycoumarin (**9a**)*

[**6a**, **8a**; 1; C; chloroform–ethanol 1:1], m.p. 216–217 °C, 68.0% yield.

IR (KBr) ν (cm^{-1}): 1760, 1740, 1605, 1505.

^{1}H-NMR (δ, CF_3OOD): 2.60 (s, 3H, CH_3CO), 3.75 (s, 6H, NCH_3), 4.03 (s, 3H, OCH_3), 4.48 (s, 2H, CH_2 bridge), 6.64 (d, 1H, H-3), 7.85–7.10 (m, 5H, H arom.), 8.07 (d, 1H, H-5′).

*5.3.1.2. 8-[2′-(Dimethylamino)-7′-methoxychromon-3′-yl]methyl-7-acetoxy-4-methylcoumarin (**9b**)*

[**6b**, **8a**; 1; A; ethyl acetate], m.p. 178–179 °C, 67.2% yield.

IR (KBr) ν (cm^{-1}): 1760, 1735, 1605, 1550.

^{1}H-NMR (δ, $CDCl_3$): 2.15 (s, 3H, 4-CH_3), 2.42 (s, 3H, CH_3CO), 3.01 (s, 6H, NCH_3), 3.89 (s, 3H, OCH_3), 4.16 (s, 2H, CH_2 bridge), 6.28 (s, 1H, H-3), 7.61–6.68 (m, 4H, H arom.), 8.06 (d, 1H, H-5′).

*5.3.1.3. 8-[2′-(Diethylamino)-7′-methoxychromon-3′-yl]methyl-7-acetoxycoumarin (**9c**)*

[**6a**, **8b**; 1; A; ethyl acetate], m.p. 159–160 °C, 54.5% yield.

IR (KBr) ν (cm^{-1}): 1770, 1730, 1605, 1560.

^{1}H-NMR (δ, $CDCl_3$): 0.88 (t, 6H, CH_2CH_3), 2.08 (s, 3H, CH_3CO), 3.35 (q, 4H, CH_2CH_3), 3.90 (s, 3H, OCH_3), 4.20 (s, 2H, CH_2 bridge), 6.40 (d, 1H, H-3), 7.96–6.66 (m, 5H, H-6, 8, 4′, 5′, 6′), 8.16 (d, 1H, H-5′).

*5.3.1.4. 8-[2′-(Diethylamino)-7′-acetoxychromon-3’-yl]methyl-7-acetoxycoumarin (**9d**)*

[**6a**, **8c**; 1; A; ethyl acetate], m.p. 177–178 °C, 56.3% yield.

IR (KBr) ν (cm^{-1}): 1760, 1730, 1620, 1570.

^{1}H-NMR (δ, $CDCl_3$): 0.92 (t, 6H, CH_3CH_2N), 2.12 (s, 3H, 7-CH_3CO), 2.35 (s, 3H, 7′-CH_3CO), 3.31 (q, 4H, CH_3CH_2N), 4.18 (s, 2H, CH_2 bridge), 6.42 (d, 1H, H-3), 7.51–6.82 (m, 4H, H-5, 6, 6′, 8′), 7.74 (d, 1H, H-4), 8.25 (d, 1H, H-5′).

*5.3.1.5. 8-[2′-(Diethylamino)-7′-methoxychromon-3′-yl]methyl-7-hydroxy-4-methylcoumarin (**9e**)*

[**6b**, **8b**; 1; A; ethyl acetate], m.p. 179–180 °C, 58.0% yield.

IR (KBr) ν (cm^{-1}): 1765, 1730, 1610, 1590, 1560.

^{1}H-NMR (δ, $CDCl_3$): 1.34 (t, 6H, CH_2CH_3), 2.33 (s,

3H, 4-CH_3), 3.96–3.53 (m, 7H, OCH_3+CH_2CH_3), 4.03 (s, 2H, CH_2 bridge), 6.03 (s, 1H, H-3), 7.05–6.64 (m, 3H, H-6, 8, 6′), 7.36 (d, 1H, H-5′), 8.06 (d, 1H, H-5′), 11.94 (s, 1H, OH).

*5.3.1.6. 8-[2′-(Diethylamino)-7′-acetoxychromon-3′-yl]methyl-7-acetoxy-4-methylcoumarin (**9f**)*

[**6b**, **8c**; 1; A; ethyl acetate], m.p. 209–210 °C, 73.8% yield.

IR (KBr) ν (cm^{-1}): 1760, 1730, 1610, 1570.

^{1}H-NMR (δ, $CDCl_3$): 0.93 (t, 6H, CH_2CH_3), 2.10 (s, 3H, 4-CH_3), 2.54–2.25 (m, 6H, CH_3CO), 3.30 (q, 4H, CH_2CH_3), 4.14 (s, 2H, CH_2 bridge), 6.25 (s, 1H, H-3), 7.63–6.76 (m, 4H, H-5, 6, 6′, 8′), 8.22 (m, 1H, H-5′).

*5.3.1.7. 8-[2′-(Diethylamino)-7′-methoxychromon-3′-yl]methyl-7-propionyloxy-4-methylcoumarin (**9g**)*

[**6b**, **8b**; 2; B; ethyl acetate], m.p. 159–160 °C, 54.7% yield.

IR (KBr) ν (cm^{-1}): 1763, 1722, 1610, 1560.

^{1}H-NMR (δ, $CDCl_3$): 1.09–0.75 (m, 9H, NCH_2CH_3+CH_3CH_2CO), 2.57–2.15 (m, 5H, 4-CH_3+CH_3CH_2CO), 3.24 (q, 4H, NCH_2CH_3), 3.88 (s, 3H, OCH_3), 4.17 (s, 2H, CH_2 bridge), 6.27 (s, 1H, H-3), 7.63–6.69 (m, 4H, H-5, 6, 6′, 8′), 8.13 (d, 1H, H-5′).

*5.3.1.8. 8-[2′-(1-Pyrrolidinyl)-7′-methoxychromon-3′-yl]methyl-7-hydroxycoumarin (**9h**)*

[**6a**, **8d**; 1; C; chloroform–ethanol 1:1], m.p. 253–254 °C, 64.5% yield.

IR (KBr) ν (cm^{-1}): 3100 (broad), 1729, 1630, 1605, 1580, 1505.

^{1}H-NMR (δ, CF_3OOD): 2.65–2.21 (m, 4H, β-pyrrolidine CH_2), 4.03 (s, 3H, OCH_3), 4.71–4.22 (m, 6H, CH_2 bridge+α-pyrrolidine CH_2), 6.55 (d, 1H, H-3), 7.76–7.01 (m, 5H, H-4, 5, 6, 6′, 8′), 8.06 (d, 1H, H-5′).

*5.3.1.9. 8-[2′-(1-Pyrrolidinyl)-7′-methoxychromon-3′-yl]methyl-7-hydroxy-4-methylcoumarin (**9i**) and 8-[2′-(1-pyrrolidinyl)-7′-methoxychromon-3′-yl]methyl-7-acetoxy-4-methylcoumarin (**9j**)*

9i: [**6b**, **8d**; 1; D; chloroform–ethanol 1:1], m. p. 261–262 °C, 38.5% yield.

IR (KBr) ν (cm^{-1}): 2910 (broad),1726, 1624, 1605, 1566.

^{1}H-NMR (δ, CF_3COOD): 2.67–2.17 (m, 7H, 4-CH_3+β-pyrrolidine CH_2), 4.01 (s, 3H, CH_3O), 4.69–4.22 (m, 6H, CH_2 bridge+α-pyrrolidine CH_2), 6.45 (s, 1H, H-3), 8.25–7.02 (m, 5H, H arom.).

9j: [**6b**, **8d**; 1; D; ethyl acetate], m.p. 208–209 °C, 45.3% yield.

IR (KBr) ν (cm^{-1}): 1770, 1735, 1630, 1610, 1590.

^{1}H-NMR (δ, $CDCl_3$): 2.18–1.77 (m, 4H, β-pyrrolidine CH_2), 2.28 (s, 3H, CH_3CO), 2.37 (s, 3H, 4-CH_3), 4.02–3.63 (m, 7H, CH_3O+α-pyrrolidine CH_2), 4.19 (s, 2H, CH_2 bridge), 6.19 (s, 1H, H-3), 7.57–6.60 (m, 4H, H-5, 6, 6′, 8′), 8.02 (d, 1H, H-5′).

*5.3.1.10. 8-[2′-(1-Piperidinyl)-7′-methoxychromon-3′-yl]methyl-7-acetoxycoumarin (**9k**)*

[**6a**, **8e**; 1; A; ethyl acetate], m.p. 223–224 °C, 61.0% yield.

IR (KBr) ν (cm^{-1}): 1760, 1735, 1625, 1605, 1568.

^{1}H-NMR (δ, CF_3COOD): 2.39–1.78 (m, 6H, β+α-piperidine CH_2), 2.56 (s, 3H, CH_3CO), 4.53–3.62 (m, 9H, OCH_3+α-pyrrolidine CH_2+CH_2 bridge), 6.62 (d, 1H, H-3), 8.19–7.04 (m, 6H, H arom.).

*5.3.1.11. 8-[2′-(1-Piperidinyl)-7′-methoxychromon-3′-yl]methyl-7-hydroxy-4-methylcoumarin (**9l**) and 8-[2′-(1-piperidinyl)-7′-methoxychromon-3′-yl]methyl-7-acetoxy-4-methylcoumarin (**9m**)*

9l: [**6b**, **8e**; 1; D; chloroform–ethanol 1:1], m.p. 238–239 °C, 49.6% yield.

IR (KBr) ν (cm^{-1}): 2910 (broad), 1720, 1630, 1600, 1580.

^{1}H-NMR (δ, CF_3COOD): 2.28–1.78 (m, 6H, β+γ-piperidine CH_2), 2.54 (s, 3H, 4-CH_3), 4.50–3.65 (m, 9H, OCH_3+α-piperidine CH_2+CH_2 bridge), 6.45 (s, 1H, H-3), 8.18–6.88 (m, 5H, H arom.).

9m:[**6b**, **8e**; 1; D; ethyl acetate], m.p. 215–216 °C, 38.1% yield.

^{1}H-NMR (δ, $CDCl_3$): 1.70–1.33 (m, 6H, β+γ-piperidine CH_2), 2.07 (s, 3H, CH_3CO), 2.40 (s, 3H, 4-CH_3), 3.38–3.02 (m, 4H, α-piperidine CH_2), 3.88 (s, 3H, OCH_3), 4.09 (s, 2H, CH_2 bridge), 6.25 (s, 1H, H-3), 7.60–6.67 (m, 4H, H-5, 6, 6′, 8′), 8.03 (d, 1H, H-5′).

*5.3.1.12. 8-[2′-bis(2-Methoxyethylamino)-7′-methoxycrhomon-3′-yl]methyl-7-acetoxycoumarin (**9n**)*

[**6a**, **8f**; 1; A; ethyl acetate], m.p. 104–105 °C, 54.5% yield.

IR (KBr) ν (cm^{-1}): 1755, 1740, 1610, 1601, 1565.

^{1}H-NMR (δ, $CDCl_3$): 2.05 (s, 3H, CH_3CO), 3.70–2.97 (m, 14H, $NCH_2CH_2OCH_3$), 3.90 (s, 3H, OCH_3), 4.21 (s, 2H, CH_2 bridge), 6.41 (d, 1H, H-3), 7.94–6.83 (m, 5H, H-4, 5, 6, 6′, 8′), 8.12 (d, 1H, H-5′).

*5.3.1.13. 8-[2′-bis(2-Methoxyethylamino)-7′-methoxychromon-3′-yl]methyl-7-acetoxy-4-methylcoumarin (**9o**)*

[**6b**, **8f**; 1; A; ethyl acetate], m.p. 140–141 °C, 61.0% yield.

IR (KBr) ν (cm^{-1}): 1760, 1740, 1630, 1605, 1580.

^{1}H-NMR (δ, $CDCl_3$): 2.07 (s, 3H, 4-CH_3), 2.43 (s, 3H, CH_3CO), 3.70–3.07 (m, 14H, $NCH_2CH_2OCH_3$), 3.90 (s, 3H, 7-OCH_3), 4.21 (s, 2H, CH_2 bridge), 6.31 (s, 1H, H-3), 7.73–6.67 (m, 4H, H-5, 6, 6′, 8′), 8.13 (d, 1H, H-5′).

*5.3.1.14. 8-[3′-(Dimethylamino)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-hydroxy-4-methylcoumarin (**9p**)*

[**6b**, **8g**; 2; C; chloroform–ethanol 1:1], m.p. 256–257 °C, 56.8% yield.

IR (KBr) ν (cm^{-1}): 3100 (broad), 1730, 1630, 1605, 1580, 1530.

^{1}H-NMR (δ, CF_3OOD): 2.53 (s, 3H, 4-CH_3), 3.87 (s, 6H, CH_3N), 4.68 (s, 2H, CH_2 bridge), 6.49 (s, 1H, H-3), 8.05–7.20 (m, 6H, H arom.), 8.34 (d, 1H, H-5′), 9.31 (d, 1H, H-10′).

*5.3.1.15. 8-[3′-(Dimethylamino)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-acetoxycoumarin (**9q**)*

[**6a**, **8g**; 1; A; ethyl acetate], m.p. 189–190 °C, 79.5% yield.

IR (KBr) ν (cm^{-1}): 1755, 1705, 1630, 1595, 1506.

^{1}H-NMR (δ, $CDCl_3$): 2.08 (s, 3H, CH_3CO), 3.00 (s, 6H, NCH_3), 4.28 (s, 2H, CH_2 bridge), 6.36 (d, 1H, H-3), 8.20–6.83 (m, 8H, H-4+H arom.), 10.24 (d, 1H, H-10′).

*5.3.1.16. 8-[3′-(Diethylamino)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-propionyloxycoumarin (**9r**)*

[**6a**, **8h**; 2; A; ethyl acetate], m.p. 172–173 °C, 89.1% yield.

IR (KBr) ν (cm^{-1}): 1760, 1730, 1630, 1610, 1570.

^{1}H-NMR (δ, $CDCl_3$): 1.10–0.51 (m, 9H, NCH_2CH_3+CH_3CH_2CO), 2.29 (q, 2H, CH_3CH_2CO), 3.25 (q, 4H, NCH_2CH_3), 4.32 (s, 2H, CH_2 bridge), 6.40 (d, 1H, H-3), 8.31-6.74 (m, 8H, H-4+H arom.), 10.16 (d, 1H, H-10′).

*5.3.1.17. 8-[3′-(Diethylamino)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-acetoxy-4-methylcoumarin (**9s**)*

[**6b**, **8h**; 1; C; ethyl acetate], m.p. 211–212 °C, 72.4% yield.

IR (KBr) ν (cm^{-1}): 1760, 1730, 1620, 1600, 1590.

^{1}H-NMR (δ, CDCl3): 1.08–0.72 (t, 6H, NCH_2CH_3), 1.98 (s, 3H, 4-CH_3), 2.42 (s, 3H, CH_3CO), 3.27 (q, 4H, NCH_2CH_3), 4.31 (s, 2H, CH_2 bridge), 6.31 (s, 1H, H-3), 8.20–6.85 (m, 7H, H arom), 10.22 (d, 1H, H-10′).

*5.3.1.18. 8-[3′-(Diethylamino)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-propionyloxy-4-methylcoumarin (**9t**)*

[**6b**, **8h**; 2; A; ethyl acetate], m.p. 175–176 °C, 90.4% yield.

IR (KBr) ν (cm^{-1}): 1770, 1730, 1630, 1603, 1570.

^{1}H-NMR (δ, $CDCl_3$): 1.07–0.58 (m, 9H, NCH_2CH_3+$COCH_2CH_3$), 2.54–2.08 (m, 5H, 4-CH_3+CH_2CO), 3.26 (q, 4H, NCH_2CH_3), 4.32 (s, 2H, CH_2 bridge), 6.29 (s, 1H, H-3), 8.21–6.70 (m, 7H, H arom.), 10.27 (d, 1H, H-10′).

*5.3.1.19. 8-[3′-(1-Pyrrolidinyl)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-hydroxycoumarin (**9u**) and 8-[3′-(1-pyrrolidinyl)-1′H-naphtho[2′,1′-b]pyran-1′-one-2′-yl]methyl-7-acetoxycoumarin (**9v**)*

9u:[**6a**, **8i**; 1; D; chloroform–ethanol 1:1], m.p. 278–280 °C, 42.5% yield.

IR (KBr) ν (cm^{-1}): 3100 (broad), 1730, 1630, 1605, 1507.

^{1}H-NMR (δ, CF_3COOD): 2.70–2.35 (m, 4H, β-pyrrolidine CH_2), 4.82–4.19 (m, 6H, CH_2 bridge+α-pyrrolidine CH_2), 6.58 (d, 1H, H-3), 8.48–7.15 (m, 8H, H-4+H arom.), 9.40 (d, 1H, H-10′).

9v:[**6a**, **8i**; 1; D; ethyl acetate], m.p. 128–129 °C, 43.2% yield.

IR (KBr) ν (cm^{-1}): 1763, 1729, 1629, 1605, 1590.

^{1}H-NMR (δ, $CDCl_3$): 2.15–1.81 (m, 4H, β-pyrrolidine CH_2), 2.20 (s, 3H, CH_3CO), 4.01–3.62 (m, 4H, α-pyrrolidine CH_2), 4.28 (s, 2H, CH_2 bridge), 6.30 (d, 1H, H-3), 8.11–6.84 (m, 8H, H arom.), 10.15 (d, 1H, H-10′).

*5.3.1.20. 8-[3′-(1-Pyrrolidinyl)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-hydroxy-4-methylcoumarin (**9w**)*

[**6b**, **8i**; 1; C; chloroform–ethanol 1:1], m.p. 273–274 °C, 74.1% yield.

IR (KBr) ν (cm^{-1}): 3100 (broad), 1730, 1630, 1605, 1570, 1520.

^{1}H-NMR (δ, CF_3OOD): 2.70–2.26 (m, 7H, 4-CH_3+β-pyrrolidine CH_2), 4.86–4.23 (m, 6H, α-pyrrolidine CH_2+CH_2 bridge), 6.47 (s, 1H, H-3), 8.49–7.18 (m, 7H, H arom.), 9.35 (d, 1H, H-10′).

*5.3.1.21. 8-[3′-(1-Piperidinyl)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-acetoxycoumarin (**9x**)*

[**6a**, **8j**; 1; C; chloroform–ethanol 1:1], m.p. 199–200 °C, 70.1% yield.

IR (KBr) ν (cm^{-1}): 1760, 1729, 1644, 1602.

^{1}H-NMR (δ, $CDCl_3$): 1.68–1.31 (m, 6H, β+γ-piperidine CH_2), 2.00 (s, 3H, CH_3CO), 3.38–3.06 (m, 4H, α-piperidine CH_2), 4.25 (s, 2H, CH_2 bridge), 6.40 (d, 1H, H-3), 8.20–6.85 (m, 8H, H arom), 10.23 (d, 1H, H-10′).

*5.3.1.22. 8-[3′-(1-Piperidinyl)-1′H-naphtho[2′,1′-b]-pyran-1′-one-2′-yl]methyl-7-acetoxy-4-methylcoumarin (**9y**)*

[**6b**, **8j**; 1; C; chloroform–ethanol 1:1], m.p. 236–237 °C, 76.4% yield.

IR (KBr) ν (cm^{-1}): 1758, 1729, 1632, 1607.

^{1}H-NMR (δ, $CDCl_3$): 1.66–1.30 (m, 6H, β+γ-piperidine CH_2), 1.99 (s, 3H, CH_3CO), 2.40 (s, 3H, 4-CH_3), 3.39–3..02 (m, 4H, α-piperidine CH_2), 4.23 (s, 2H, CH_2 bridge), 6.25 (s, 1H, H-3), 8.12–6.89 (m, 7H, H arom.), 10.15 (d, 1H, H-10′).

*5.3.1.23. 8-[3′-Bis(2-methoxyethylamino)-1′H-naphtho[2′,1′-b]pyran-1′-one-2′-yl]methyl-7-acetoxy-4-methylcoumarin (**9z**)*

[**6b**, **8k**; 1; A; ethyl acetate], m.p. 166–167 °C, 91.1% yield.

IR (KBr) ν (cm^{-1}): 1760, 1720, 1630, 1600,1505.

^{1}H-NMR (δ, $CDCl_3$): 1.94 (s, 3H, 4-CH_3), 2.43 (s, 3H, CH_3CO), 3.80–2.87 (m, 14H, $NCH_2CH_2OCH_3$), 4.33 (s, 2H, CH_2 bridge), 6.30 (s, 1H, H-3), 8.23–6.79 (m, 7H, H arom.), 10.20 (d, 1H, H-10′).

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