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Novel Coumarin-Based Fluorescent Melatonin Ligands. Design, Synthesis and Pharmacological Characterization

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Abstract: The development of a series of new fluorescent coumarin-containing melatonin analogues is presented. The combination of high-binding affinities for human melatonergic receptors (h-MT_1R and h-MT_2R) and fluorescent properties, derived from the inclusion of melatonin pharmacophoric motifs in the coumarin scaffold, yielded suitable candidates for the development of MT_1R and MT_2R fluorescent probes for imaging in biological media.

Keywords: Melatonin; GPCRs fluorescent probes; coumarins; bioisosterism.

Abbreviations

Boc, *tert*-butyloxycarbonyl protecting group; CDI, 1,1'-carbonyldiimidazole; GPCRs, G-protein-coupled receptors; HPLC-MS, liquid chromatography–mass spectrometry; MSA, methanesulphonic acid; h-MTRs, human melatonin receptors; h-MT_1R: human melatonin receptor subtype-1; h-MT_2R: human melatonin receptor subtype-2; TEA, triethylamine; TFA, trifluoroacetic acid.

Fluorescence-based techniques for studying pharmacological and biochemical processes have undergone a huge development in the last decade. Given the demanding technical set-up, hazards and expense of using radioligands for these sort of studies, fluorescent-based methodologies appear more desirable and easier to implement [1].

Melatonin is produced in the pineal gland during the dark-phase of the day-night cycle. Most of its functions are mediated by two G protein–coupled receptors (GPCRs), named MT_1 and MT_2. Beyond the regulation of the circadian rhythm, melatonergic receptors (MTRs) are involved in numerous physiological and therapeutic processes whose underlying molecular basis has not been fully elucidated [2]. Several fluorescence-spectroscopy techniques have been developed for studying different aspects of the pharmacology of MTRs [3]. Melatonin itself bears an indole moiety that confers a certain intrinsic fluorescence to the molecule, although the photochemical properties of this heterocycle are not appropriate for the development of fluorescent-based methodologies. Different melatonin ligands bearing fluorescent cores are reported in the literature, out of which only 7-azamelatonin and boron-dipyrromethene derivatives were reportedly designed and developed specifically for receptor labelling purposes [4, 5].

Coumarins are a class of fluorophores from the benzopyrone family. As a representative example, umbelliferone has an absorption wavelength above 300 nm that would prevent interference with the naturally-present tryptophan or other indole-containing biomolecules. Moreover, their generous Stokes shift and high quantum yield make coumarins suitable structures for the development of fluorescence-based imaging techniques for the visualization of metabolic processes and interactions with receptors [6, 7]. To continue the development of novel fluorescent probes capable of identifying the generally low-expressed melatonin receptors, together with our interest in the development of melatonin-based potential drugs [8, 9], we wished to integrate a suitable

fluorophore within the structure of melatonin. Herein, we report the design, synthesis, and characterization of a series of novel fluorescent-melatonin analogues in which the indole nucleus of melatonin has been bioisosterically replaced by a fluorescent 2*H*-chromen-2-one ring, with varying anchorage points of the substituents attached to it (Figure 1).

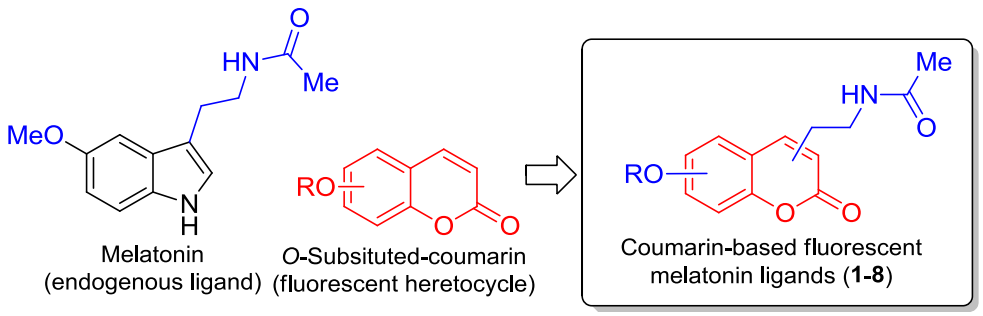
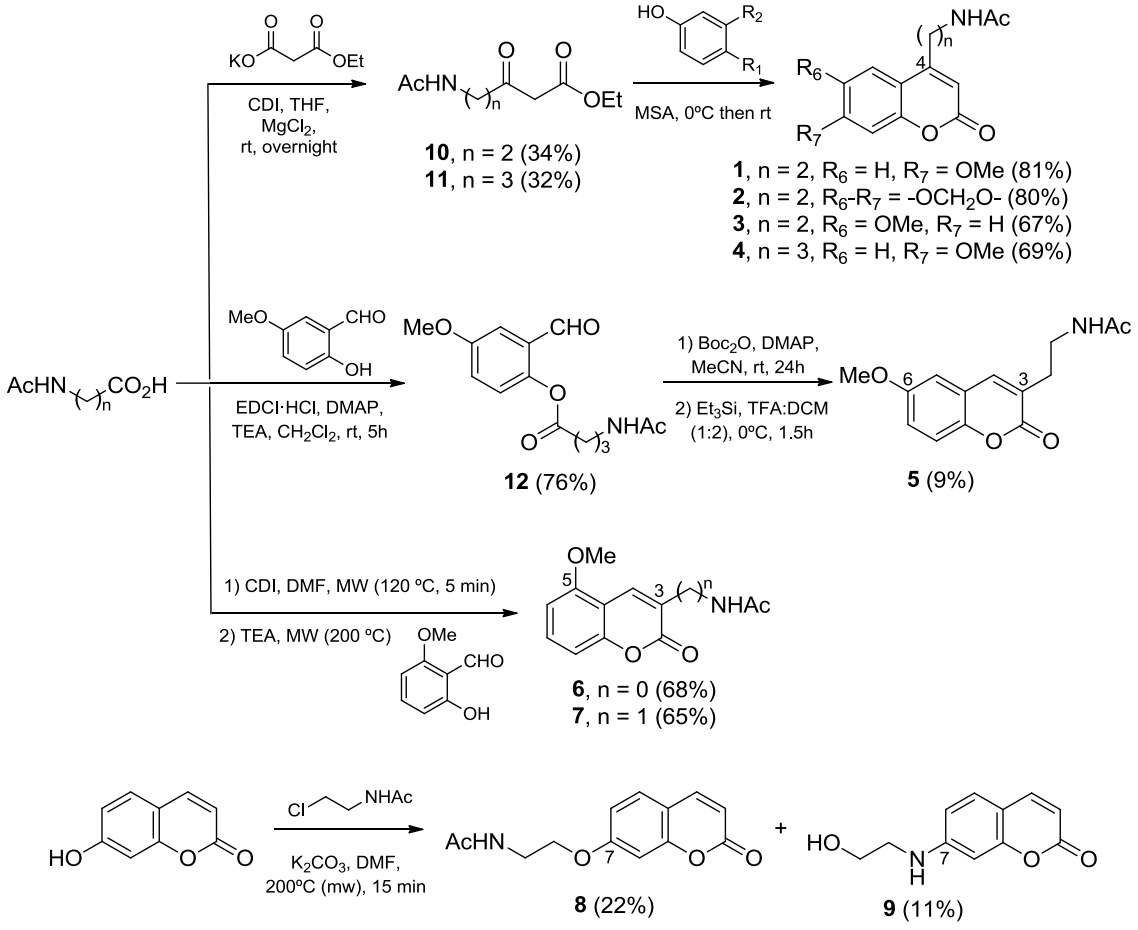


Figure 1. Structure of coumarin-based fluorescent melatonin ligands (**1**-**8**) by adding pharmacophoric groups of melatonin to the coumarin core

Coumarins bearing the alkyl acetamido chain linked to position 4 (**1**-**4**) were obtained in moderate to good yields by a Pechmann condensation between a β-ketoester (**10** or **11**) and the corresponding phenol in methanesulphonic acid (MSA) at room temperature (Scheme 1). The relative position of the alkyloxy group in the phenol greatly determined the course of the reaction; reactivity was clearly favored in *m*-alkyloxy substituted phenols, whereas *p*-alkyloxy substitution greatly retarded it. In fact, the reaction that afforded compound **3** required up to 30 days for achieving completion (see Supplementary Material for further details).

For obtaining coumarin **5**, bearing the alkyl acetamido chain in position 3 and the methoxy group in 6, an adapted Perkin condensation was employed. Preliminary attempts to promote base-catalysed intramolecular condensation of intermediate ester **12** did not led to the formation of the wanted coumarin. Conversely, 1-acetylpyrrolidin-2-one and 2-hydroxy-5-methoxybenzaldehyde were identified by HPLC-MS, suggesting that the ester group underwent a nucleophilic attack from the amidic nitrogen. In order to avoid such cyclization, **12** was protected with a *tert*-butyloxycarbonyl group (Boc) prior to its treatment with TFA. Nevertheless, in the reaction mixture a significant amount of 1-acetylpyrrolidin-2-one was also identified, revealing that undesired cyclization was only partially prevented, isolating coumarin **5** in very low yield (9%).



Scheme 1. Synthesis of new coumarin-based melatonin ligands **1**-**8**

Coumarins **6** and **7**, bearing the alkyl acetamido chain in position 3 and the methoxy group in 5, were obtained in moderate yield (65-68%) from 2-hydroxy-6-methoxybenzaldehyde and *N*-acetylglycine or *N*-acetyl-β-alanine, using 1,1'-carbonyldiimidazole (CDI) and triethylamine (TEA) in a microwave reactor at 200 ºC.

Finally, to link the acetamide chain to position 7, 7-hydroxy-2*H*-chromen-2-one (umbelliferone) was reacted with 2-chloroethylacetamide in basic medium, using a microwave reactor at 200 °C for 5 h. In addition to the expected product (**8**, 22% yield), 7-((2-hydroxyethyl)amino)-2*H*-chromen-2-one (**9**, 11% yield) was also isolated, as the result of a Smiles rearrangement, favored by the harsh conditions of basicity, pressure, and temperature that were needed to promote the primary attack of the phenolate over the chlorinated carbon.

The fluorescent properties of the coumarin-bearing compounds **1**-**8** are summarized in Table 1. Compounds **1**, **2**, **4**, and **8** showed fluorescence intensities in the same wavelength and concentration range as umbelliferone. Derivatives **3**, **6**, and **7** required higher concentration for measurable fluorescence detection; conversely, their Stokes shifts were greater than the rest of the series of compounds that in general, showed much narrower shifts than umbelliferone. The major difference between the excitation and emission wavelengths was found in coumarin **7**, better than umbelliferone itself. In the case of derivative **5**, no fluorescent emission could be recorded in the fluorimeter after excitation at its maximum UV band (368 nm).

Table 1. Photophysical properties of compounds at concentration 0.1 μM in phosphate buffer 0.1 M pH 7.4

Compd.	λ_{exc} (nm)	λ_{em} (nm)	Stokes shift (nm)	Fluorescence Intensity (A.U.)[a]
Umbelliferone	328	453	125	367
1	324	389	65	336
2	346	424	78	325
3[b]	340	435	95	320
4	322	388	66	323
6[b]	323	463	140	845
7[b]	305	467	162	724
8	324	391	67	187

[a] Fluorescence intensity expressed in arbitrary units. [b]Measured at 5 μM.

Compounds' affinity for human MT_1R and MT_2R was screened at 100 nM concentration (Table 2) [10]. Coumarin **3** showed the greatest degree of specific displacement of radioligand at both receptors (69% at h-MT_1 and 83% at h-MT_2). Its affinity constants were also determined, giving K_is in the nanomolar range: h-MT_1 (K_i, 13 ± 0.5 nM) and h-MT_2 (K_i, 3.4 ± 0.1 nM). These results confirmed that coumarin **3** strongly binds to both receptors, although with minor potency than melatonin does: h-MT_1 (K_i, 0.091 nM) and h-MT_2 (K_i, 0.15 nM) [10].

Table 2. Percentage of radioligand 2-[^{125}I]iodomelatonin displacement (%) from recombinant human MTRs, elicited by compounds at 100 nM.[a,b]

Compd.	h-MT_1	h-MT_2
1	31.8 ± 4.1	4.5
2	10.0 ± 1.4	2.3
3	69.0 ± 0.2	83.3 ± 1.2
4	43.0 ± 0.8	3.0
5	3.4	1.0
6	18.8 ± 1.8	0
7	25.1 ± 5.2	0.2
8	3.2	0.4

[a]Results are expressed as percentage inhibition ± SEM (n=3) of specific melatonin binding (for values below 10%, SEM are not given). [b]Melatonin at 100 nM gives 100 % of radioligand displacement at both MTRs.

The relative positions of both acetamido chain and alkyloxy group in the coumarin core were determinant for receptor recognition, as it could be explained by superposition studies using the bioactive conformation of melatonin. The most potent melatonergic ligand of the series **3** is able to properly reproduce the putative active conformation of natural ligand (Figure 2, left), defined by pharmacophore analysis and molecular superposition with conformationally-constrained compounds [11]. The lower binding affinity of coumarin **3** compared to melatonin is likely due to the presence of the carbonyl group in position 2, as it is known that both a 6,6-bicyclic nucleus [12] and a benzofuran oxygen [13] are well tolerated at melatonin receptors.

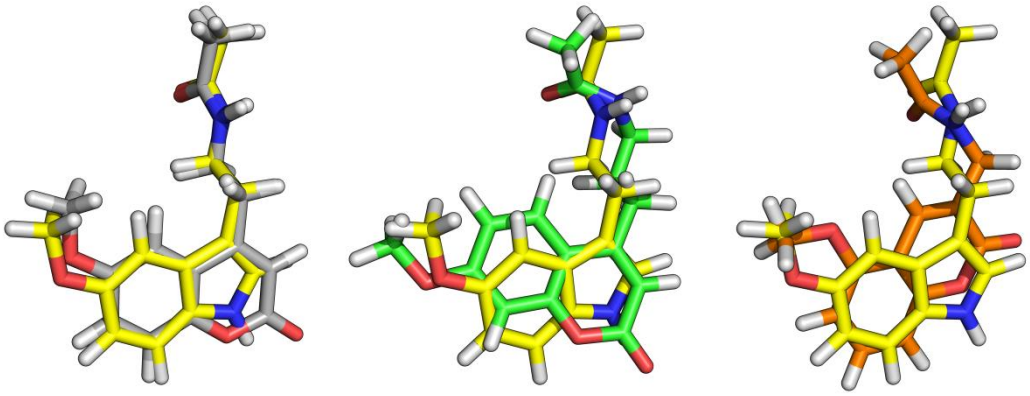


Figure 2. Melatonin (yellow carbons) in its putative active conformation, superposed to compound **3** (left, gray carbons), compound **4** (middle, green carbons) and compound **7** (right, orange carbons).

The binding drop of 7-methoxycoumarin **1** highlights the strong dependence of MTRs affinity from the presence and position of the methoxy group, in the same way that moving the methoxy group of melatonin in position 6 instead of 5 had brought more than 1.000-fold reduction in binding affinity for MTRs [14]. The compensation offered by chain elongation in compound **4** can be explained by the possibility to get a good superposition of its pharmacophoric elements on those of melatonin, with a partially folded conformation of the side chain (Figure 2, middle).

Compounds **6** and **7,** having shorter amide chains in position 3 and a methoxy group in position 5, showed very limited MT_1 binding affinity, likely due to the poor fitting to the melatonergic pharmacophore (Figure 2, right).

Compound **2** with a methylene-dioxy bridge connecting positions 6 and 7 has very low potency, likely due to an unsuitable orientation of the oxygen lone pairs, which probably hampers a proper interaction with the receptor.

Additionally the most active compounds **3** and **4** were functionally characterized in a well-established cellular model of melatonin action, cultured *Xenopus laevis* melanophores as previously described (Figure 3) [15]. Compound **4** lacked any significant effect on pigment aggregation so it had no agonist action (up to 10^{-5} M). However, **3** was a potent agonist showing close to a full agonist response (E_{max} = 89 ± 2 % compared to melatonin) with an EC_{50} = 117.8 ± 20.6 nM. At a higher dose (10^{-4} M) derivative **3** acted as a melatonin receptor antagonist as did **4** (data not shown). The drop in potency of **3** compared to melatonin (EC_{50} = 0.074 ± 0.006 nM; E_{max} = 100%) is more marked in this activity model, than in affinity in radioligand binding experiments on recombinant human melatonin receptors. Taking into account that *Xenopus laevis* melanophores express native frog melatonin receptors which are likely to be a different subtype (Mel1c), receptor-subtype and interspecies-differences could be responsible for the observed differences in potency.

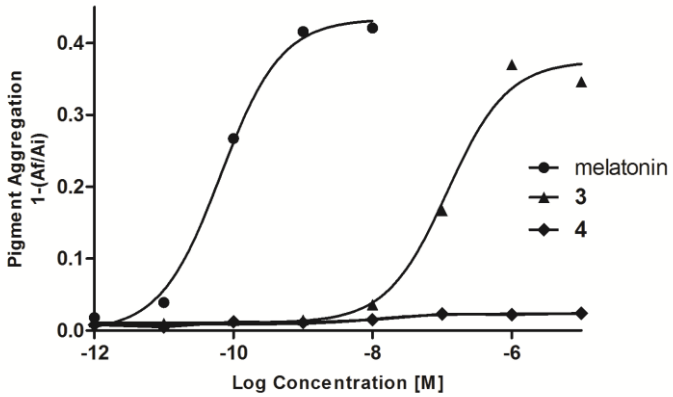


Figure 3. Concentration-response curves for melatonin, **3**, and **4** on *Xenopus leavis* melanophores. Each point on the Y axis is the mean pigment aggregation response (relative change in absorbance before [Ai] and 60 min. after [Af] drug treatment). SEM values are omitted as they were all less than the area covered by the symbol.

In summary, we have developed a series of fluorescent melatonin analogues in which the indole nucleus of melatonin has been replaced with *O*-substituted coumarin scaffolds. The relative position of the pharmacophoric substituents on the coumarin ring greatly determined the affinity for h-MT_1 and h-MT_2 and the fluorescence properties. Best binding results were obtained when the acetamido chain was attached to the coumarin position 4. Among compounds **1**-**4**, the methoxy substituent, which varying between the position 6 and 7 of the ring, affected in an inverse manner either property. *N*-(2-(6-Methoxy-2-oxo-2*H*-chromen-4-yl)ethyl)acetamide (**3**) showed the best human MT_1R / MT_2R binding, in the nanomolar range, and demonstrated its agonistic properties in *Xenopus laevis* melanophores. Thus, coumarin derivative **3** behaved as a non-selective nanomolar melatonergic fluorescent probe that could be used in cells and tissues expressing melatonin receptors.

Acknowledgements

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Supplementary data

Experimental details for the synthesis of coumarin-based compounds and intermediates. Determination of spectroscopic and biological properties.

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