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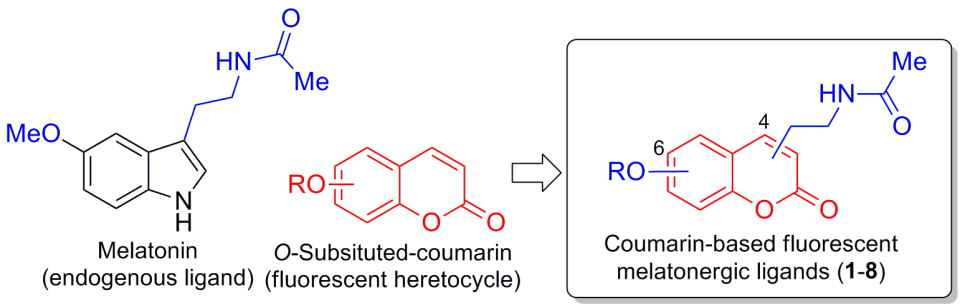

3: 6-methoxy, 4-ethylacetamide
h-MT_1, K_i = 13 ± 0.5 nM
h-MT_2, K_i = 3.4 ± 0.1 nM

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

Mario de la Fuente Revenga,[1] Clara Herrera-Arozamena,[1] Nerea Fernández-Sáez,[1] Gema Barco,[1] Itxaso García-Orue,[1] David Sugden,[2] Silvia Rivara,[3] and María Isabel Rodríguez-Franco*,[1]

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Abstract: The design and synthesis 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 elements 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; MTRs, melatonergic 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 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. Besides the regulation of circadian rhythms, melatonergic receptors (MTRs) are involved in numerous physiological 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 GPCRs [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 emission wavelength above 450 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 receptor ligands. 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).

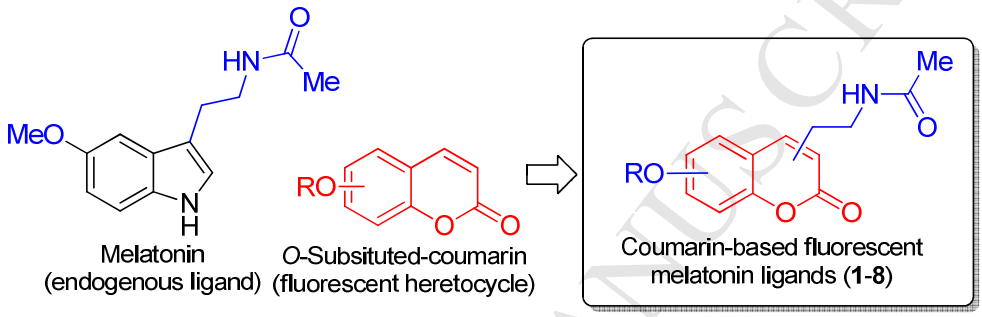
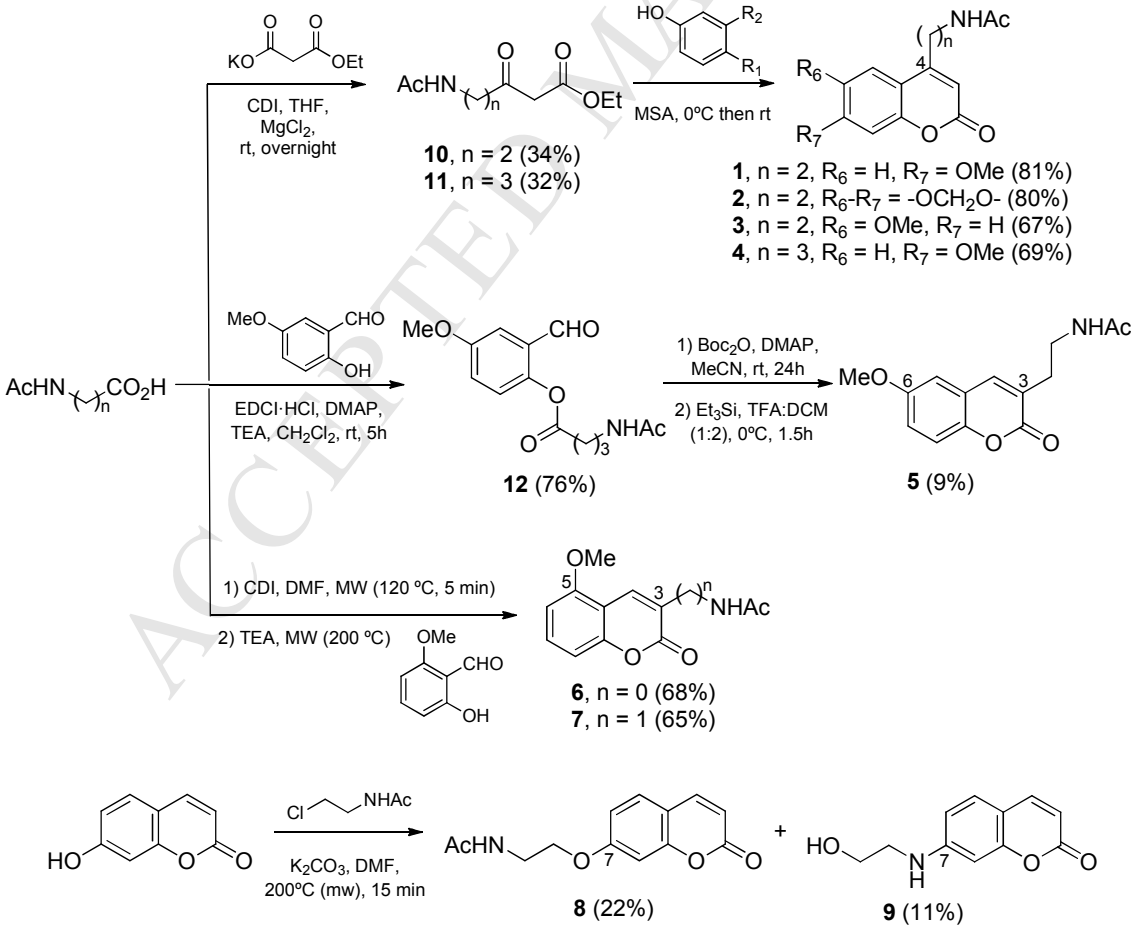


Figure 1. Structure of coumarin-based fluorescent melatonin ligands (**1**-**8**) designed by adding pharmacophoric elements of melatonin to the coumarin core.

Coumarins bearing the acetylaminoalkyl 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 desired 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 amide nitrogen. In order to avoid such cyclization, **12** was protected with a *tert*-butyloxycarbonyl group (Boc) prior to its treatment with trifluoroacetic acid (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 other compounds in the series that, in general, showed much narrower shifts than umbelliferone. The major difference between the excitation and emission wavelengths was found in coumarin **7**, greater 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 lower potency than melatonin: 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 the acetamido chain and alkyloxy group on 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 the 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.

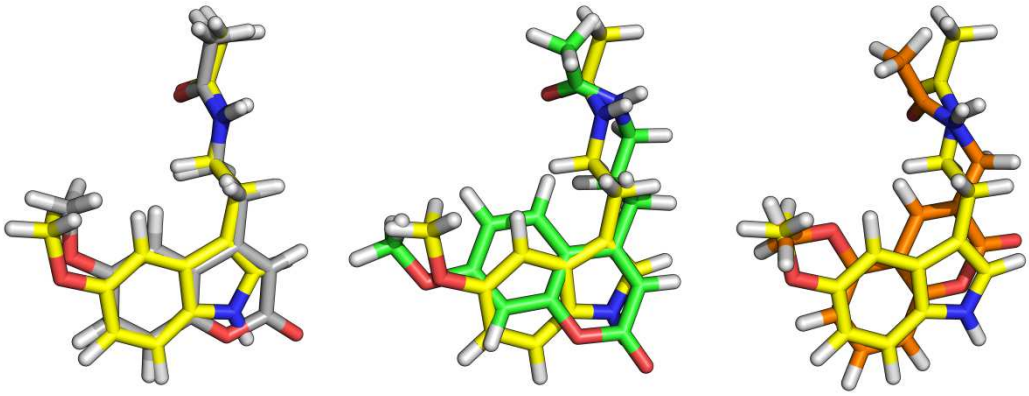


Figure 2. Melatonin (yellow carbons) in its putative active conformation, superposed to compound **3** (left, gray carbons), compound **4** (middle, green carbons) and compound **7** (right, orange carbons).

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 from position 6 to position 5 had brought more than 1.000-fold reduction in binding affinity [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, probably related 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, with no agonist action up to 10^{-5} M concentration. On the other hand, **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 radioligand binding experiments on recombinant h-MTRs. 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.

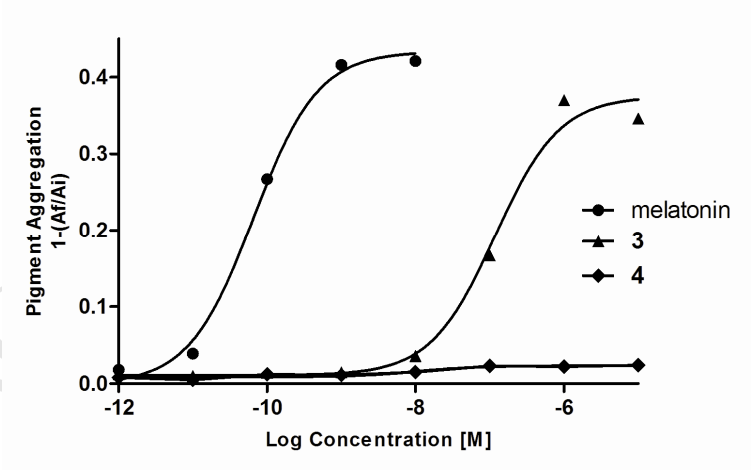


Figure 3. Concentration-response curves for melatonin, **3**, and **4** on *Xenopus laevis* melanophores. Each point on the Y axis is the mean pigment aggregation response (relative change in absorbance before [Ai] and 60 min. after [Af] drug treatment). SEM values are omitted as they were all less than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

In summary, we have designed 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 elements 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, either in position 6 or 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 nonselective nanomolar melatonergic fluorescent probe that could be used for imaging in cells and tissues expressing melatonin receptors.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO, Grant SAF2012-31035), Fundación de Investigación Médica Mutua Madrileña Automovilística (Grant AP103952012) and Consejo Superior de Investigaciones Científicas (CSIC, Grant PIE-201280E074). M.F.R. was the recipient of a JAE-Predoctoral Contract (Grant JAE-Pre-2009-106) and I.G.-O. of a fellowship for Research Starting (Grant JAE-Intro-2012-0047), both from the Program "Junta para la Ampliación de Estudios" co-financed by the CSIC and the European Social Fund.

Supplementary data

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

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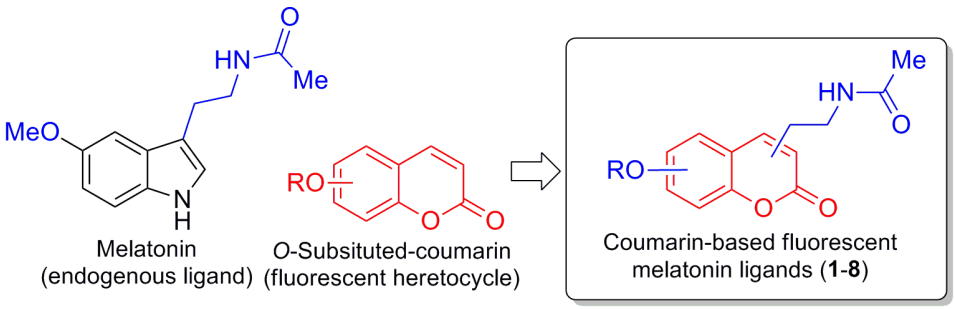
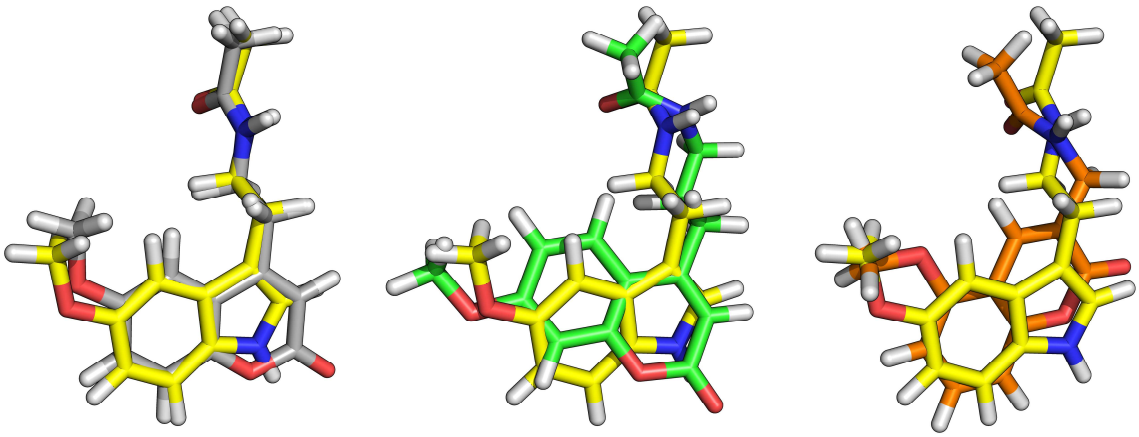
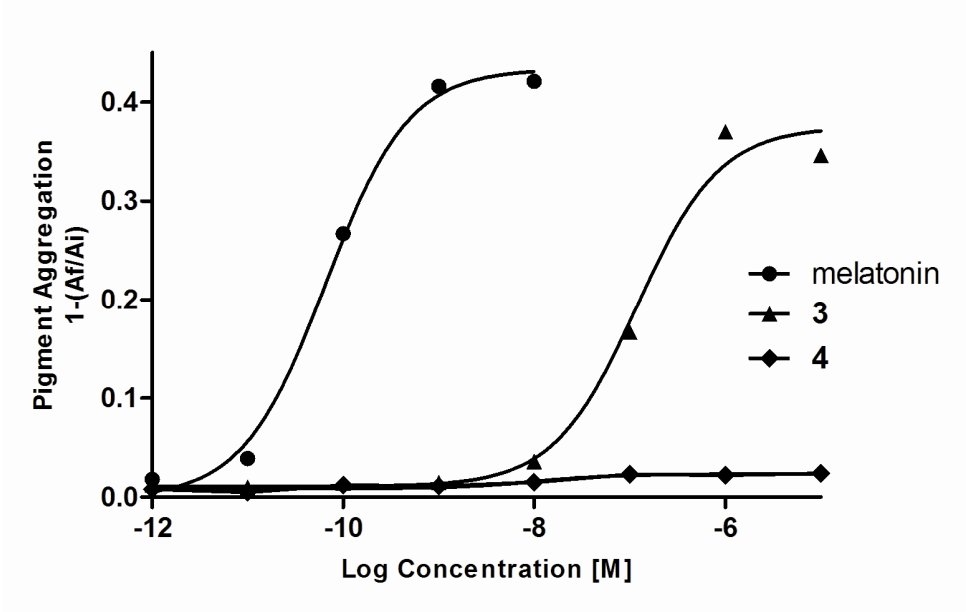


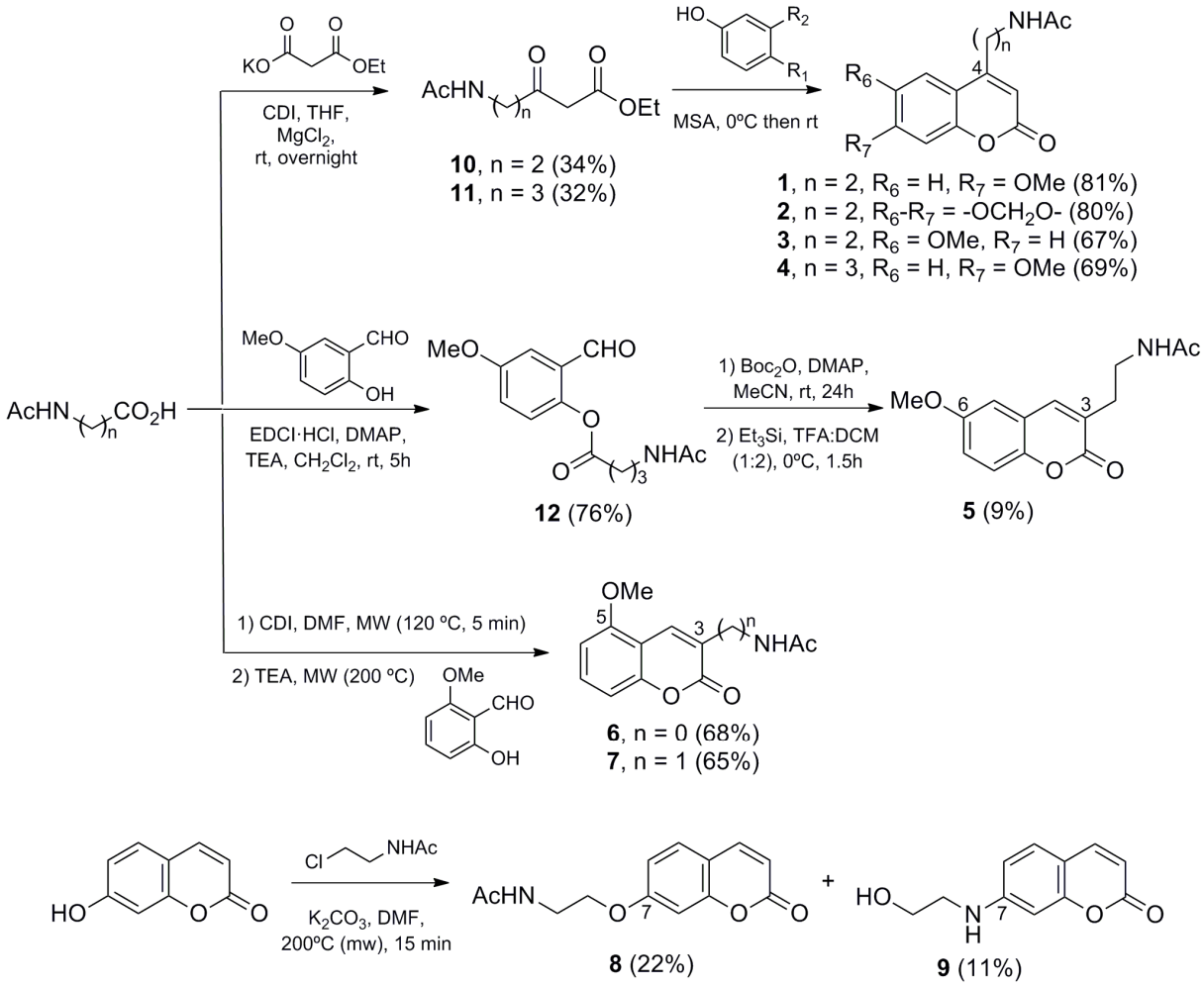
Melatonin (endogenous ligand)

O-Subsituted-coumarin (fluorescent heretocycle)

Coumarin-based fluorescent melatonin ligands (**1**-**8**)






CDI, THF, MgCl2, rt, overnight
10, n = 2 (34%)
11, n = 3 (32%)
MSA, 0ºC then rt
1, n = 2, R6 = H, R7 = OMe (81%)
2, n = 2, R6-R7 = -OCH2O- (80%)
3, n = 2, R6 = OMe, R7 = H (67%)
4, n = 3, R6 = H, R7 = OMe (69%)
EDCI·HCl, DMAP, TEA, CH2Cl2, rt, 5h
12 (76%)
1) Boc2O, DMAP, MeCN, rt, 24h
2) Et3Si, TFA:DCM (1:2), 0ºC, 1.5h
5 (9%)
1) CDI, DMF, MW (120 ºC, 5 min)
2) TEA, MW (200 ºC)
6, n = 0 (68%)
7, n = 1 (65%)
K2CO3, DMF, 200ºC (mw), 15 min
8 (22%)
9 (11%)

Highlights for

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

- New coumarin-based fluorescent ligands at human melatonergic receptors (h-MTRs)
- *N*-(2-(6-Methoxy-2-oxo-2*H*-chromen-4-yl)ethyl)acetamide: nanomolar affinity at h-MTRs
- Coumarin – melatonin superposition studies: a rationale for h-MTRs affinities

Supplementary data for

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

Mario de la Fuente Revenga,[1] Clara Herrera-Arozamena,[1] Nerea Fernández-Sáez,[1] Gema Barco,[1] Itxaso García-Orue,[1] David Sugden,[2] Silvia Rivara,[3] and María Isabel Rodríguez-Franco*,[1]

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EXPERIMENTAL SECTION

Chemistry. **General Methods**. Reagents and solvents were purchased from common commercial suppliers, mostly Sigma-Aldrich, and were used without further purification. Analytical thin-layer chromatography (TLC) was carried out using Merck silica gel 60 F254 plates, and the compounds were visualized under UV-light (λ = 254 or 365 nm) and/or stained with phosphomolybdic acid 10% wt. in ethanol. Automatized chromatographic separation was carried out in an IsoleraOne (Biotage) using different silica Si_{50} cartridges from Agilent Technologies. High-performance liquid chromatography was performed on a Waters analytical HPLC-MS (Alliance Watters 2690) equipped with a SunFire C_{18} 4.6 x 50 mm column, a UV photodiode array detector (λ = 214–274 nm) and quadrupole mass spectrometer (Micromass ZQ). HPLC analyses were used to confirm the purity of all compounds (>95%) and were performed on Waters 6000 equipment, at a flow rate of 1.0 mL/min, with a UV photodiode array detector (λ = 214–274 nm), and using a Delta Pak C_{18} 5 μm, 300 Å column. The elution was performed in a gradient mixture of MeCN/water.

Melting points (uncorrected) were determined in a MP70 apparatus (Mettler Toledo). ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 solutions using the following NMR spectrometers: Varian INOVA-300, Varian INOVA-400, Varian Mercury-400 or Varian Unity-500. Chemical shifts are reported in δ scale (ppm) relative to internal Me_4Si. *J* values are given in hertz, and spin multiplicities are expressed as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet) or m (multiplet). High Resolution Mass Spectra (HRMS) were obtained by electron spray ionization (ESI) in positive mode using a Hewlett-Packard MSD 1100 spectrometer. Reactions under microwave irradiation (MW) were performed in a Biotage Initiator 2.5 reactor.

Ethyl 5-acetamido-3-oxopentanoate (**10**). *N*-acetyl-β-alanine (1 g, 7.62 mmol) and carbonyldiimidazole (CDI, 1.5 g, 9.3 mmol) were dissolved and stirred for 1 h in tetrahydrofurane (THF, 26 mL). Potassium monoethylmalonate (2 g, 11.43 mmol) and magnesium chloride ($MgCl_2$, 0.72 g, 7.62 mmol) were then added. The reaction was stirred overnight at rt. Water was then added and the mixture extracted with EtOAc (5 x 20 mL), the organics were dried over $MgSO_4$ and solvent removed. The crude was chromatographed (DCM : MeOH gradient) to afford **10** (530 mg, 34%) as a pure oil. HPLC-MS (*m/z*) [MH^+] 202.14. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.02 (s, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.51 (d, *J* = 5.9 Hz, 2H), 3.46 (s, 2H), 2.81 (d, *J* = 5.9 Hz, 2H), 1.95 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H).

Ethyl 6-acetamido-3-oxohexanoate (**11**). Following the method described for **7**, from 4-acetamidobutanoic acid (2.5 g, 17.2 mmol), **11** (1.18 g, 32% yield) was obtained as a yellow oil. HPLC-MS (*m/z*) [MH^+] 216.26. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.88 (s, 1H), 4.19 (q, *J* = 7.1 Hz, 2H), 3.44 (s, 2H), 3.25 (q, *J* = 6.6 Hz, 3H), 2.61 (d, *J* = 6.7 Hz, 2H), 1.96 (s, 3H), 1.81 (p, *J* = 6.7 Hz, 2H), 1.27 (t, *J* = 7.1 Hz, 3H).

Pechmann condensantion. General procedure. Intermediate **10** (1 eq.) was mixed with the corresponding phenol (1.5 eq.) and methanesulfonic acid (MSA, 25 eq.) was added at 0ºC, and then stirred at rt. Reaction evolution was followed by TLC. Reaction times were highly dependent on phenol substituents. When the reaction reached completion, sat. aq. $NaHCO_3$ was added over the mixture and the aqueous phase extracted several times with EtOAc. After drying the organics over $MgSO_4$, and removal of the solvent, the resulting crude was purified to afford the corresponding 2-oxo-2*H*-chromen acetamide.

***N*-(2-(7-Methoxy-2-oxo-2*H*-chromen-4-yl)ethyl)acetamide** (**1**)**.** Obtained from **10** and resorcinol in 84% yield as a white solid of mp 164-166 ºC. Reaction time: 1h. Crude chromatographed (DCM:MeOH gradient). HPLC purity 99% (230 to 400 nm). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68 (d, *J* = 8.9 Hz, 1H), 6.88 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.79 (d, *J* = 2.5 Hz, 1H), 6.11 (s, 1H), 5.91 (s, 1H), 3.86 (s, 3H), 3.56 (d, *J* = 7.0 Hz, 2H), 2.97 (d, *J* = 7.0 Hz, 2H), 1.99 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.53, 162.80, 162.50, 161.17, 155.58, 153.56, 125.64, 112.60, 111.63, 101.07, 55.78, 38.66, 32.00, 23.25. HRMS (ESI^+): *m/z* calcd for $C_{14}H_{15}NO_4$ $(M)^+$ 261.1005, found 261.1001.

***N*-(2-(6-Oxo-6*H*-[1,3]dioxolo[4,5-*g*]chromen-8-yl)ethyl)acetamide** (**2**). Obtained from **10** and resorcinol in 80% yield as a white solid of mp 196-198 ºC. Reaction time: 30 min. Crude chromatographed in DCM:MeOH gradient. HPLC purity 100% (230 to 400 nm). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (t, *J* = 5.6 Hz, 1H), 7.42 (s, 1H), 7.09 (s, 1H), 6.17 (s, 2H), 3.31 (q, *J* = 6.8 Hz, 2H), 2.84 (t, *J* = 6.8 Hz, 2H), 1.78 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.51, 160.18, 154.26, 150.74, 150.36, 144.74, 112.70, 111.40, 102.62, 102.60, 98.10, 37.55, 31.80, 22.59. HRMS (ESI^+): *m/z* calcd for $C_{14}H_{13}NO_5$ $(M)^+$ 275.0794, found 275.0793.

***N*-(2-(6-Methoxy-2-oxo-2*H*-chromen-4-yl)ethyl)acetamide** (**3**). Obtained from **10** and 4-methoxyphenol. Reaction time: 30 days. The compound was crystallised from EtOH:Et_2O and isolated as a white powder of mp 206-207 ºC. Yield: 67%. HPLC purity 100% (230 to 400 nm). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.09 (t, *J* = 5.8 Hz, 1H), 7.42 (d, *J* = 2.9 Hz, 1H), 7.36 (d, *J* = 9.0 Hz, 1H), 7.22 (dd, *J* = 9.0, 2.9 Hz, 1H), 6.36 (s, 1H), 3.86 (s, 3H), 3.36 (q, *J* = 6.8 Hz, 2H), 2.93 (d, *J* = 6.8 Hz, 2H), 1.79 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.54, 159.88, 155.69, 153.64, 147.50, 119.39, 119.30, 117.75, 114.80, 107.74, 55.83, 37.52, 31.36, 22.54. HRMS (ESI^+): *m/z* calcd for $C_{14}H_{15}NO_4$ $(M+H)^+$ 261.1001, found 261.0999.

In an attempt to improve the synthesis of **3**, a wide variety of methodologies reported for the synthesis of coumarins via Pechmann condensation were tested, namely $TiCl_4$ [1], 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate) (Selectfluor®) [2], $BiCl_3$ [3], TsOH [4], $ZrCl_4$ [5], $Bi(NO_3)_3 \cdot H_2O$ [6], H_2SO_4 [7], and MSA [8]. Among them, only MSA catalysis was able to afford the desired compound, despite the extremely low rate of the reaction turnover.

***N*-(3-(7-Methoxy-2-oxo-2*H*-chromen-4-yl)propyl)acetamide** (**4**). Obtained from **11** and resorcinol in 69% yield as an off-white solid of mp 99.5 – 101.5 ºC. Reaction time: 30 min. Crude chromatographed (DCM : MeOH gradient). HPLC purity 100% (230 to 400 nm). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.93 (t, *J* = 5.5 Hz, 1H), 7.72 (d, *J* = 8.8 Hz, 1H), 7.00 (d, *J* = 2.6 Hz, 1H), 6.97 (dd, *J* = 8.8, 2.6 Hz, 1H), 6.20 (s, 1H), 3.86 (s, 3H), 3.12 (q, *J* = 6.7 Hz, 2H), 2.83 – 2.65 (m, 2H), 1.81 (s, 3H), 1.77 – 1.68 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.80, 162.95, 160.91, 157.12, 155.74, 126.79, 112.88, 110.96, 101.63, 56.60, 38.67, 29.11, 28.58, 23.32. HRMS (ESI^+): *m/z* calcd for $C_{15}H_{17}NO_4$ $(M+H)^+$ 275.1158, found 275.1164.

2-Formyl-4-methoxyphenyl 4-acetamidobutanoate (**12**). 1-Acetamidobutyric acid (960 mg, 6.62 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl, 1.65 g, 8.61 mmol), dimethylaminopyridine (DMAP, 162 mg, 1.32 mmol) and triethylamine (TEA, 1.48 ml, 10.60 mmol) were dissolved in dichloromethane (DCM, 60 mL) and then 2-hydroxy-5-methoxybenzaldehyde (1 g, 6.62 mmol) was added. The reaction was kept stirring at room temperature (rt) for 5h, then HCl 0.1M was added till pH stayed acidic. The aqueous phase was extracted with ethylacetate (EtOAc, 3 x 40 mL). The organics were pooled together, dried over $MgSO_4$, solvent evaporated and the resulting crude was chromatographed (DCM: MeOH) to afford intermediate **12** (1.40 g, 76%) as a white solid. HPLC-MS (*m/z*) 100% (230 to 400 nm)

[MH+] 280.15. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.03 (s, 1H), 7.34 (d, *J* = 3.1 Hz, 1H), 7.17 (dd, *J* = 8.9, 3.1 Hz, 1H), 7.11 (d, *J* = 8.9 Hz, 1H), 5.85 (s, 1H), 3.87 (s, 3H), 3.40 (q, *J* = 6.2 Hz, 2H), 2.72 (t, *J* = 7.3 Hz, 2H), 2.18 – 1.87 (m, 5H).

***N*-(2-(6-Methoxy-2-oxo-2*H*-chromen-3-yl)ethyl)acetamide** (**5**). Intermediate **12** (500 mg, 1.79 mmol), di-*tert*-butyldicarbonate (Boc_2O, 699 mg, 1.79 mmol) and DMAP (547 mg, 2.69 mmol) were dissolved in anhydrous acetonitrile (MeCN, 5.5 mL). The mixture was stirred at rt for 24h. The solvent was removed to afford a crude that was chromatographed (hexane : EtOAc) to give 230 mg of a mixture of Boc-protected-**5** (HPLC-MS (*m/z*) [MH+] 362.35, A_{max} 286 nm) and 1-acetylpyrrolidin-2-one (HPLC-MS (m/z), [MH+] 127.79, no abs. 230 to 400 nm) in a proportion of ca. 1:2 as determined by ^{1}H-NMR. This mixture was dissolved in triethylsilane (253 µL, 1.6 mmol), treated at 0 ºC with trifluoroacetic acid (TFA) and DCM (1.8 mL, 1:2) and then, allowed to reach rt. After 90 min the reaction was finished and DCM partially removed. The mixture was then added over a cooled sat. solution of ammonia in MeOH (6 mL). Solvent and excess of ammonia were removed under vacuum and the solid mixture of salts and organics chromatographed (DCM : MeOH) to afford **5** (40 mg, 9% two steps) as a yellow shiny solid of mp 205 – 208 ºC. HPLC purity 95% (230 to 400 nm). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.66 (s, 1H), 7.71 (t, *J* = 2.9 Hz, 1H), 6.95 (d, *J* = 2.4 Hz, 1H), 6.87 – 6.83 (m, 2H), 3.72 (d, *J* = 7.2 Hz, 2H), 3.70 (s, 3H), 3.02 (td, *J* = 7.2, 2.9 Hz, 2H), 2.46 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.30, 169.04, 152.32, 151.42, 130.43, 128.43, 122.33, 117.69, 116.93, 113.52, 55.81, 42.56, 25.21, 22.89. HRMS (ESI^+): *m/z* calcd for $C_{14}H_{15}NO_4$ $(M)^+$ 261.1001, found 261.0995.

General procedure for coumarins 6 and 7. Corresponding acid (2 eq) and CDI (2.1 eq) were dissolved in the minimum amount of anhydrous DMF and microwaved at 120 ºC for 5 minutes. 2-Hydroxy-6-methoxybenzaldehyde (1 eq) and TEA (1 eq) were

added and the reaction was heated at 200 ºC during the required time. The solvent was removed under vacuum, ethyl acetate was added and the mixture was washed with $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The crude was purified by chromatographed (DCM to DCM/ethyl acetate) to afford the corresponding coumarin.

***N*-(5-Methoxy-2-oxo-2*H*-chromen-3-yl)acetamide (6).** Obtained from *N*-acetylglycine in 68% yield as a pale yellow solid of mp 271-274 ºC. Overall reaction time: 12 min. HPLC purity 100% (230 to 400 nm). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.93 (s, 1H), 8.01 (s, 1H), 7.36 (t, *J* = 8.3 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 1H), 6.75 (d, *J* = 8.2 Hz, 1H), 3.94 (s, 3H), 2.23 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ = 169.13, 159.01, 156.10, 150.98, 130.17, 122.72, 119.03, 110.52, 108.81, 105.85, 56.12, 24.90. HRMS (ESI^+): *m/z* calcd for $C_{12}H_{11}NO_4$ $(M+H)^+$ 233.06881, found 233.06828.

***N*-((5-Methoxy-2-oxo-2*H*-chromen-3-yl)methyl)acetamide (7)** Obtained from *N*-acetyl-β-alanine in 65% yield as a white solid of mp 192-195 ºC. Overall reaction time: 50 min. HPLC purity 98% (230 to 400 nm). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (d, *J* = 0.8 Hz, 1H), 7.42 (t, *J* = 8.4 Hz, 1H), 6.91 (dt, *J* = 8.4, 0.7 Hz, 1H), 6.71 (dd, *J* = 8.3, 0.8 Hz, 1H), 4.32 (dd, *J* = 6.4, 0.7 Hz, 2H), 3.92 (s, 3H), 1.99 (s, 3H).^{13}C NMR (126 MHz, $CDCl_3$) δ 170.10, 162.01, 156.26, 154.42, 136.26, 132.06, 123.03, 109.77, 108.81, 105.25, 55.96, 39.90, 23.33. HRMS (ESI^+): *m/z* calcd for $C_{13}H_{13}NO_4$ $(M+H)^+$ 247.08446, found 247.08458.

Reaction of umbelliferone and 2-chloroethylacetamide. Four microwave vials were filled with umbelliferone (60 mg, 0.37 mmol), 2-chloroethylacetamide (112 µL, 1.1 mmol), potassium iodide (cat. amount), K_2CO_3 (77 mg, 0.55 mmol) and anhydrous dimethylformamide (DMF, 1 mL). The vials were microwaved at 200 ºC for 5 h. After

addition of water (1 mL) to each vial they were pooled together. The mixture was extracted with EtOAc (3 x 5 mL). The organics were dried over $MgSO_4$ and solvent removed under vacuum to afford a crude that was chromatographed twice (DCM : MeOH, EtOAc : MeOH), to afford **8** (80 mg, 22%) and **9** (33 mg, 11%).

***N*-(2-((2-Oxo-2*H*-chromen-7-yl)oxy)ethyl)acetamide** (**8**). White solid of mp 141 - 147ºC. HPLC purity 98% (230 to 400 nm) ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.13 (s, 1H), 7.98 (d, *J* = 9.4 Hz, 1H), 7.62 (d, *J* = 8.6 Hz, 1H), 6.99 (d, *J* = 2.4 Hz, 1H), 6.94 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.28 (d, *J* = 9.4 Hz, 1H), 4.07 (t, *J* = 5.6 Hz, 2H), 3.41 (q, *J* = 5.6 Hz, 2H), 1.81 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.99, 161.99, 160.74, 155.80, 144.78, 129.97, 113.14, 113.01, 112.88, 101.67, 67.56, 38.42, 22.98. HRMS (ESI$^+$): *m/z* calcd for $C_{13}H_{13}NO_4$ $(M+H)^+$ 247.0852, found 247.0845.

7-((2-Hydroxyethyl)amino)-2*H*-chromen-2-one (**9**). Yellow solid of mp 141 - 144ºC. HPLC purity 97% (230 to 400 nm). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.79 (d, *J* = 9.3 Hz, 1H), 7.33 (d, *J* = 8.6 Hz, 1H), 6.73 (t, *J* = 5.6 Hz, 1H), 6.61 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.45 (d, *J* = 2.2 Hz, 1H), 5.96 (d, *J* = 9.3 Hz, 1H), 4.78 (t, *J* = 5.4 Hz, 1H), 3.56 (q, *J* = 5.7 Hz, 2H), 3.17 (q, *J* = 5.8 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 161.03, 156.44, 152.77, 144.78, 129.07, 110.49, 108.10, 107.86, 96.17, 59.34, 45.12. HRMS (ESI$^+$): *m/z* calcd for $C_{11}H_{11}NO_3$ $(M+H)^+$ 205.0739, found 205.0747.

Spectroscopic Data. Stock solutions (10 mM) in DMSO of the compounds were serially diluted in phosphate buffer 0.1M. Absorption spectra (50 µM) were recorded on a Multiskan Spectrum (Thermo Electron Co.) spectrophotometer. Excitation and emission spectra (0.1 µM, except for **3**, 5 µM) were recorded on a Perkin-Elmer LS50B spectrofluorimeter using a Hëllma Quartz SUPRASIL (10mm) cuvette.

Radioligand displacement studies at human melatonergic receptors (hMT_1 and hMT_2). 2-[^{125}I]Iodomelatonin binding assay conditions, materials and references can be found in the online catalogue of CEREP [9, 10]. Briefly, chinese hamster ovary cells membranes expressing recombinant human MT_1 (hML_{1A}) or MT_2 (hML_{1B}) receptors were incubated in the presence or absence of the test compound (100 nM) and 2-[^{125}I]melatonin (0.01 nM for MT_1, 0.05 nM for MT_2) in a buffer containing 50 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$ for 60 min at 22 ºC or 120 min at 37 ºC respectively in duplicates. Nonspecific binding was defined by melatonin (1 μM) in both cases. Bound radioactivity was separated by vacuum filtration using 96-sample cell harvester (Unifilter, Packard) through GF/B filters (previously soaked in 0.3% polyethylenimine), which were rinsed several times with ice-cold 50 mM Tris-HCl buffer. The filters were dried, and then, counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. Filters were counted by liquid scintillation spectrometry. The results are expressed as a percent of control specific binding

For affinity constant determination the same procedure was carried out with eight test compound concentrations in duplicates (ranging from 0.1nM-3μM). The IC_{50} values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (n_H) were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting. This analysis was performed using software developed at CEREP (Hill software) and validated by comparison with data generated by the commercial software SigmaPlot® 4.0 for Windows® (© 1997 by SPSS Inc.). The inhibition constants (K_i) were calculated using the Cheng Prusoff equation: $K_i = IC_{50}/(1 + ([L]/K_D)$, where [L] is the

concentration of radioligand used in the assay and K_D, the dissociation constant of the radioligand in the assay and K_D the dissociation affinity constant for either receptor. A Scatchard plot was used to determine the K_D.

Pigment aggregation response at *Xenopus laevis* melanophores. Compounds **3** and **4** were functionally characterized in a well-established cellular model of melatonin action, cultured *Xenopus laevis* melanophores as previously described [11]. In these cells, melatonin triggers an aggregation of melanin, normally distributed throughout the cell, towards the cell centre. This response is accurately quantified in melanophores grown in 96-well plates by measuring the change in light (630 nm) absorbance of the cells as the pigment concentrates at the centre of each cell. Each well of the plate contains ~5000 cells, allowing construction of reliable concentration-response curves. Briefly, ligand effect was determined by incubation of cells with a series of 10-fold concentrations (10^{-12} to 10^{-5} M) of compound. Absorbance in each well is measured before (Ai) and 60 min after (Af) the addition of melatonergic ligand. The fractional change in absorbance in triplicate wells at each concentration is calculated (1-Af/Ai) and plotted *vs*. ligand concentration (log [product]). The half maximal effective concentration of a given compound (EC_{50}) was calculated using nonlinear curve fitting (GraphPad Prism).

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