Conformationally Restrained Melatonin Analogues: Synthesis, Binding Affinity for the Melatonin Receptor, Evaluation of the Biological Activity, and Molecular Modeling Study

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The design, synthesis, and biological profile of several indole melatonin analogues with a conformationally restricted C3 amidoethane side chain are presented. Examination of the accessible conformations of the melatonin side chain led us to explore some of its fully or partially restricted analogues, **2**−**12**, the binding affinity values of which were utilized to gain further insight on the melatonin binding site. Two pharmacophoric models have been devised for melatonin and the active compounds by conformational analysis and superimposition performed using the DISCO program. In these models, the melatonin side chain can adopt a *gauche*/*anti* conformation out of the indole plane. Another contribution of this study regards the observation of a possible binding point interaction around the C2 position of the indole, as suggested by the remarkably increased binding affinity observed in the C2-substituted analogues **6** and **9** and especially in the more rigid analogue **5**. The biological activity and the efficacy of the new compounds were tested by measuring the inhibition of the forskolin-stimulated cAMP accumulation and the GTPγS index. Both analyses demonstrated that all of the compounds were full agonists with the exception of **4** and **9**, which showed a slight reduction in efficacy and would seem to be partial agonists.

Introduction

The pineal hormone melatonin (*N*-acetyl-5-methoxytryptamine, aMT, **1**) is now recognized to be the regulator of circadian rhythmicity in humans and of seasonal breeding in different animal species.[1] Melatonin also appears to be involved in a number of physio/pathological conditions, and there is strong evidence for its use in pathologies associated with circadian rhythm disorders. The administration of aMT in humans was shown to alleviate jet lag,[2] to induce sleep,[3] and to advance the sleep rhythm of subjects with delayed sleep phase syndrome.[4] Numerous studies on elderly people and on depressed patients have also reported a decrease in overnight melatonin biosynthesis, thus suggesting a role for this hormone in the aging process[5] and in seasonal depression.[6] Recently aMT was reported to be eventually useful as an immunostimulant[7] and as coadjuvant in some antitumoral therapies.[8] Melatonin receptors are found in the CNS of mammals, and evidence for the presence of different subtypes of melatonin receptors has been reported.[9] These findings suggest that aMT should have more complex regulatory activities than those recognized to date, especially in humans, whose physiology shows few seasonal changes. Melatonin receptors have recently been cloned from *Xenopus* dermal melanophores[10] and from hamsters, sheep, and human brains.[11]

Despite major advances in our understanding of the role of aMT, the knowledge of the physiological functions of aMT receptors has been hampered by a lack of potent and selective aMT-receptor antagonists and the poor selectivity of action of the known aMT agonists.[9b]

The 3-ethylamido chain of aMT is flexible and can adopt several energetically equivalent conformations by rotation around the τ1 (C3a−C3−Cβ−Cα), τ2 (C3−Cβ−Cα−N), and τ3 (Cβ−Cα−N−CO) bonds;[12] this conformational flexibility is probably responsible for the broad spectrum of biological activities of aMT. Assuming a correlation between the lack of selectivity of aMT and the conformational freedom of the ethylamido side chain and given the fact derived in other fields that conformationally restricted compounds often lead to increased binding affinity and selectivity over receptor subtypes,[13] other authors recently reported a number of rigid aMT analogues with a conformationally restricted ethylamido side chain.[14] Structure−activity studies on aMT analogues showed that both the *N*-acyl and 5-methoxy groups are necessary for high binding affinity[15] and that the relative spatial distance between these groups is also an important factor.[14a] Thus, a knowledge of the active conformation(s) of aMT, and therefore of the spatial relationship of its presumed pharmacophoric

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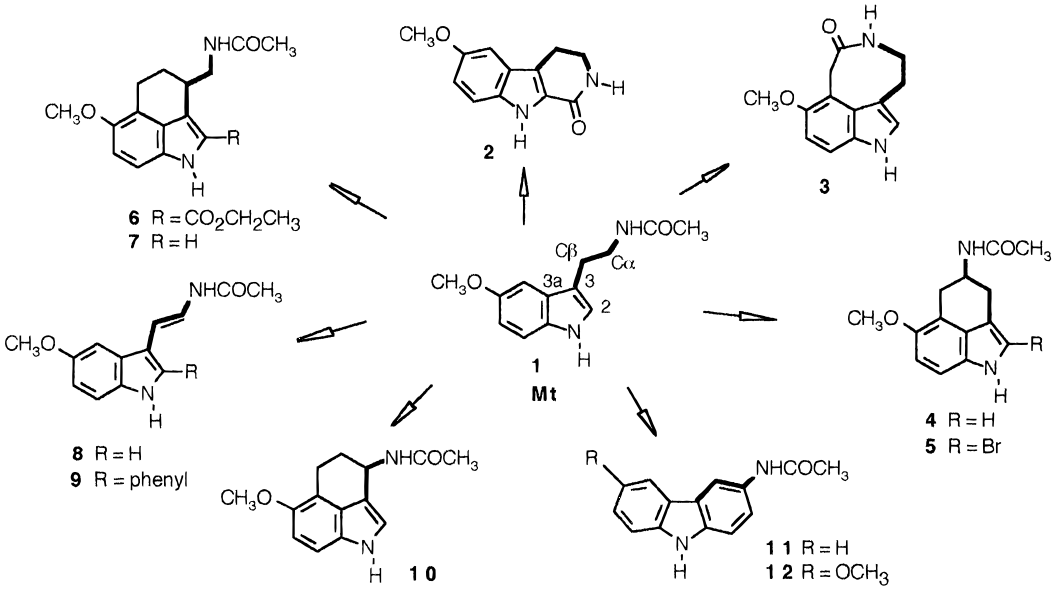


Figure 1.

Table 1. Binding Affinity[a] and Biological Activity of Compounds **2**−**16**

compd	IC_{50}	K_i	relative affinity[b]	GTPγS index[c]	cAMP index[d]	activity[e]
aMT (**1**)	2.2	0.61	1	1	1	A
2	33000 ± 3160	9100	15000	nt	nt	nt
3	4300 ± 387	1200	1950	nt	nt	nt
4	1200 ± 80	330	550	0.62	0.75	PA
5	73 ± 8	20	33	1	0.98	A
6	1.7 ± 0.2	0.48	0.77	0.85	0.9	A
7	9.9 ± 1.2	2.7	4.5	1.05	1	A
8	29 ± 2	8.0	13.2	1.25	1.18	A
9	1.4 ± 0.1	0.39	0.64	0.6	0.78	PA
10	>10000	nt	nt	nt	nt	nt
11	3000 ± 370	820	1360	0.88	0.9	A
12	230 ± 20	63	100	0.98	1.02	A
13	1300 ± 145	360	590	0.97	1	A
14	>10000	nt	nt	nt	nt	nt
15	>10000	nt	nt	nt	nt	nt
16	>10000	nt	nt	nt	nt	nt
(*S*)-29[f]			81			
30[g]			1.64			
31[h]			131			
32	7100 ± 1050	1900	3230	0.08	0	AN
2-Br-melatonin	0.13 ± 0.008	0.036	0.059	1.02	1.15	A
2-Ph-melatonin	0.062 ± 0.01	0.017	0.028	0.82	0.78	A-PA
6-Cl-melatonin	3.6 ± 0.28	0.99	1.6	0.91	1.04	A

[a] IC_{50} and K_i values are expressed in nM and are the means of 3−20 independent determinations, derived from nonlinear fitting strategies. The SEM values were below 15% of the mean. [b] Relative affinity = (IC_{50} compd/IC_{50} *aMT*) determined in parallel, in the same experiment. [c] GTPγS index = [(IC_{50} with GTPγS)/(IC_{50} without GTPγS)] compd/[(IC_{50} with GTPγS)/(IC_{50} without GTPγS)] aMT. [d] cAMP index = (percent inhibition compd)/(percent inhibition aMT) determined in parallel in the same experiment. [e] nt, not tested; A, agonist; PA, partial agonist; AN, antagonist. [f] Values from Jansen *et al.*[14e] [g] Values from Garrat *et al.*[14a] [h] Values from Gruppen *et al.*[17]

groups (*i.e.* the aromatic moiety, the ethylamido, and the methoxy substituent) is of fundamental importance both for a rational design of potent and selective new molecules and for the clarification of the topography of the aMT receptor. Therefore, along this line of thought and expanding on what had been already reported, we undertook a project aimed at exploring the possibility of limiting the conformational freedom of the 3-ethylamido side-chain in ways thus far unexplored. To this end, a variety of restricted or rigid aMT analogues with the 3-ethylamido side chain included in different structures (**2**−**12**) (Figure 1) were prepared and tested in binding assays. The biological results obtained for the most interesting compounds, *i.e.* **4**, **7**, **8**, **12** (Table 1), were submitted to a computer molecular modeling study (DISCO),[16] together with some of the previously described conformationally restricted ligands (*i.e.* (*S*)-**29**,[14e,f] **30**,[14a] **31**[17]) in order to devise possible pharmacophore models. The results of this analysis will be presented and discussed in comparison to other recently proposed models.[14e,f,15,18]

It had been reported by several groups, including ours, that appropriate substituents, either at the C2 position of the indole nucleus[15,19,20] or *ortho* to the ethylamido side chain in the naphthalenic melatonin analogues,[21] modulate the binding affinity to the melatonin receptor. We therefore decided to further investigate the consequences of suitable C2 indole substituents on the binding affinity of these new rigid analogues, in order to better understand the role of these groups in the binding to the aMT receptor. With regard to the goal of determining the bioactive conformation(s), the

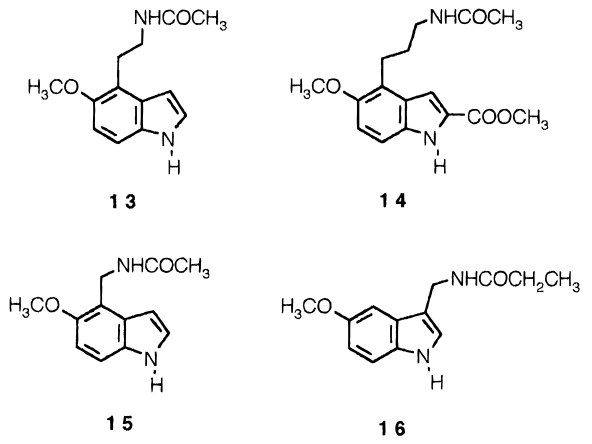


Figure 2.

accessible conformations of compounds **2**−**12** (Figure 1) were superimposed to those of aMT, looking for common relative spatial distances among the putative pharmacophoric groups.

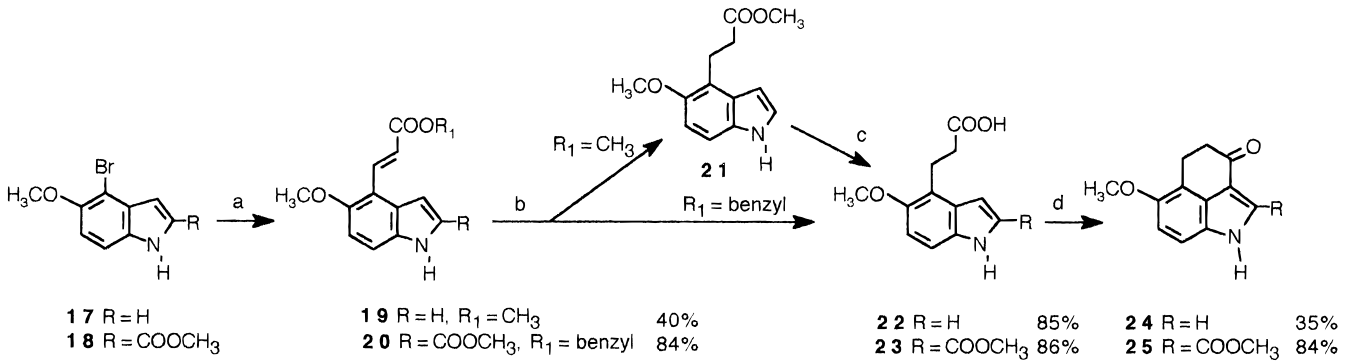
Compounds **6** and **7**, in which the C3−Cβ portion of the side chain is incorporated into a cyclic structure, and **8** and **9**, which are characterized by the presence of the Cα−Cβ double bond, represent aMT analogues with a partially constrained 3-ethylamido side chain. The carbazole derivatives **11** and **12** and compounds **4** and **5** represent two different ways in which the conformational flexibility is limited around both the τ1 and the τ2 bonds. In derivatives **4**[22]−**7**, which have a ring that bridges the α- or β-carbon of the ethylamido chain and 4-position of the indole ring, thus limiting the conformational flexibility of the side chain, the alkylamido group is conformationally free. On the contrary, in the aza tricyclic compounds **2** and **3**, formally obtained by cyclization of the acyl moiety to the 4- and 2-positions of the indole, the conformational freedom is restricted not only around the τ1 and τ2 bonds but also around the τ3 one, so that they are more rigid analogues of aMT, with the CONH group fixed in a cis conformation. Finally, we synthesized four compounds (Figure 2) with the mobile side chain attached at position 4 of the indole (**13**−**15**), or with a chain shorter than that of aMT (**16**), in order to test the hypothesis that the relative distances between the pharmacophoric points could be sufficient to determine the affinity for the aMT receptor, regardless of the attachment point of the side chain.

Although a clear stereoselectivity of the binding of conformationally restricted compounds to the melatonin receptor had recently been demonstrated,[14e,23] no attempt was made at this stage to resolve the enantiomeric mixtures. When a chiral center was present, compounds were tested as racemates.

Chemistry

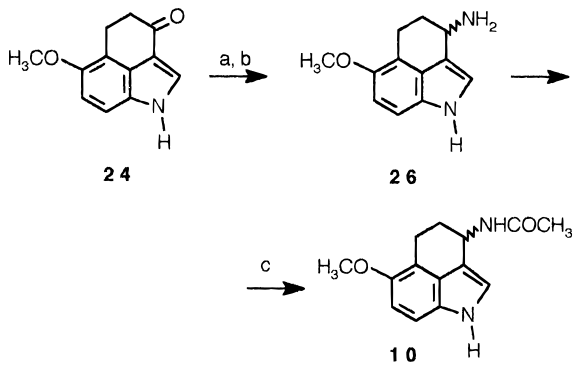
The tricyclic compounds **2**[24] and **3**[25] were synthesized according to previously reported procedures. Catalytic hydrogenation (H_2, 10% Pd−C, 50 psi) of the known 6-methoxy-4-nitro-1,3,4,5-tetrahydrobenz[*cd*]indole[26] and acetylation of the resulting crude amine provided the desired product (6-methoxy-4-acetamido-1,3,4,5-tetrahydrobenz[*cd*]indole) **4** (65% yield). Bromination of **4** with *N*-bromosuccinimide (NBS) in dioxane/AcOH (2:1) at 0 °C yielded the 2-bromo derivative **5** (52% yield). The 4-bromo-2-(carboxymethyl)-5-methoxyindole, **18** (mp 182−3 °C; 84% yield), was prepared according to a previously reported procedure for the ethyl ester derivative.[26] Key intermediates in the synthesis of new compounds **6** and **10** were the corresponding keto derivatives **24** and **25**, which were prepared as outlined in Scheme 1. Briefly, the palladium-catalyzed coupling (Heck reaction) of 4-bromo-5-methoxyindole derivatives **17**[27] and **18** with methyl or benzyl acrylate gave the derivatives **19** and **20**, to which the (*E*)-stereochemistry was assigned on the basis of the *J* value (16.5 and 16.2 Hz, respectively) reported for the ethenyl protons, in accordance with that reported for similar compounds.[28] Compounds **19** and **20** were converted to the acids **22** and **23** by catalytic hydrogenation and hydrolysis; these acids were then cyclized in polyphosphoric acid (80 °C, 80 min) to the ketones **24** and **25**. The reactions carried out on the 2-carbomethoxy derivatives **18** and **23** gave a better yield than those obtained on the unsubstituted compounds **17** and **22**. The ketone **24** was transformed in two steps (condensation with benzylamine, catalytic hydrogenation and debenzylation) to the crude amine intermediate **26**, which was acylated with acetic anhydride/TEA, obtaining the desired compound **10** in 15% overall yield (Scheme 2). As shown in Scheme 3, the ketone **25** was converted to the corresponding 2,4,6-triisopropyl hydrazone by reaction with 2,4,6-triisopropylbenzenesulfonyl hydrazide (TPSH), which was then transformed, without any previous purification, into the cyano ethyl ester derivative **27** (29% yield) by heating in ethanol with an excess of potassium cyanide using a procedure previously described for a related compound.[29] By reduction of **27** with H_2, Raney nickel in the presence of Ac_2O, we obtained the desired compound **6**. Derivative **7** was prepared by ester hydrolysis of **6** followed by decarboxylation of the corresponding acid in boiling quinoline in the presence of Cu powder. The (*E*)-3-ethenyl alkylamido derivatives **8** and **9** were synthesized in poor yields (see the Experimental Section) by reduction of the nitro group (H_2, Raney nickel, room temperature) of the corresponding (*E*)-5-methoxy-3-(2-nitroethenyl)indoles in the presence of acetic anhydride. High amounts of melatonin or 2-phenylmelatonin were isolated from this reaction. The (*E*)-stereochemistry was assigned on the basis of the coupling constant values of the ethenyl protons (J = 14.62 Hz), which were consistent with the values expected for the (*E*)-stereoisomers.[30] By catalytic hydrogenation over 10% Pd−C of 3-nitro-6-methoxy-9*H*-carbazole[31] and concomitant acylation with acetic anhydride we obtained a mixture of compounds **11** (30% yield), and **12** (30% yield) which were then separated by flash chromatography. The new compound **13** (37% yield) was prepared by reducing 4-(2-nitroethenyl)-5-methoxyindole[26] with $LiAlH_4$ and acylating the resulting crude amine with Ac_2O/TEA. The palladium-catalyzed coupling of **18** with acrylonitrile gave the derivative **28**, which was converted to the propylacetamido derivative **14** by hydrogenation over Raney nickel and N-acylation with Ac_2O (Scheme 4). By reduction of 4-cyano-5-methoxyindole[26] or 3-cyano-5-methoxyindole[32] with H_2, Raney nickel in the presence of a suitable anhydride, we obtained the corresponding alkylamidomethyl derivatives **15** and **16** (Figure 2). Compound **32**, *N*-[(2-phenyl-1*H*-indol-3-yl)ethyl]cyclobutanecarboxamide, used

Scheme 1[a]



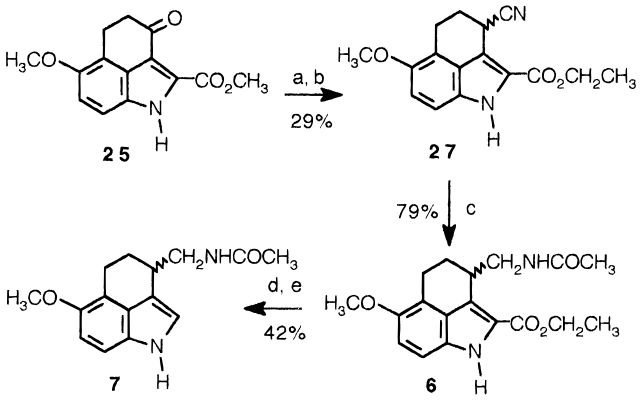
[a] Reagents: (a) $PdCl_2(PPh_3)_2$, TEA, AcOH, CH_2=$CHCOOR_1$, 120 °C, 7 h; (b) H_2, 3 atm, Pd/C (10%), THF, room temperature, 6 h; (c) 3 N KOH, MeOH, THF, room temperature, 16 h; (d) PPA, 80 °C, 80 min.

Scheme 2[a]



[a] Reagents: (a) benzylamine, *p*-TsOH·H_2O, reflux, 20 h; (b) H_2, 3 atm, Pd/C (10%), THF, 50 °C, 2 h; (c) Ac_2O, THF, TEA, room temperature (overall yield: 15%).

Scheme 3[a]



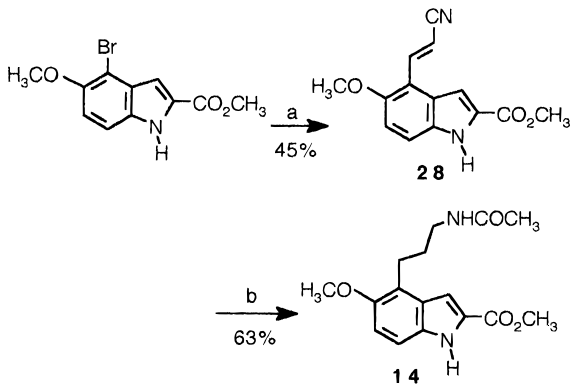
[a] Reagents: (a) TPSH, THF, room temperature, 3 h; (b) KCN, EtOH, reflux, 24 h; (c) H_2, 4 atm, Ra-Ni, THF, Ac_2O, 50 °C, 6 h; (d) 3 N KOH, MeOH, THF, room temperature, 16 h; (e) quinoline, Cu, reflux, 4 hr.

as reference antagonist, was synthesized as previously described.[33]

Pharmacology

Binding Studies and Determination of Affinity. The affinity of the analogues at the melatonin binding site in the quail optic tecta was determined in competition binding analyses using 2-[^{125}I]iodomelatonin as a labeled ligand (100 pM). There are no data in the literature indicating the type of receptor subtypes present in the quail optic tecta; however, data available for the chick[34] indicate that Mel_{1a} is expressed more abundantly than Mel_{1c}, whereas Mel_{1b} is not present.

Scheme 4[a]



[a] Reagents: (a) $PdCl_2(PPh_3)_2$, TEA, AcOH, CH_2=$CHCN$, 120 °C, 7 h; (b) H_2, 4 atm, Ra-Ni, THF, Ac_2O, 50 °C, 6 h.

Assuming a good resemblance between quail and chick brains,[35] we presume that our binding studies involved a receptor population in which the Mel_{1a} receptor subtype prevails. The IC_{50} values were determined and K_i values calculated by using nonlinear fitting strategies; compounds having low affinity ($IC_{50} > 10^{-5}$ M) were excluded from further analysis.

Biological Activity Determination. Biological activity was evaluated using two methods: (i) effects on the forskolin-stimulated cAMP accumulation in quail optic tecta explants and (ii) effects of coincubation with GTPγS (10^{-4} M) on the IC_{50} values (GTPγS index).

(i) Effects on Forskolin-Stimulated cAMP Accumulation. It is well-known that aMT inhibits forskolin-stimulated cAMP accumulation;[36] therefore, the agonist/antagonist profile of new aMT analogues can be evaluated by measuring their influence on the cAMP synthesis.

(ii) Effects of Coincubation with GTPγS on the IC_{50} Values (GTPγS Index). The choice of introducing the GTPγS index was based on the knowledge that the aMT receptor is coupled to a regulatory G-protein in its signal-transduction pathway.[35,37] It is well-known that guanine nucleotides shift a significant part of the available receptors from the state of high-affinity conformation (Rh) to a state of lower affinity conformation (Rl).[38] Agonists possess higher affinity for the Rh form, while antagonists cannot distinguish between Rh and Rl or have a higher affinity for Rl.[38,39] This is also the mechanism by which the difference between the efficacy of agonists and antagonists is explained,[40] and this method was previously used to evaluate the agonist

profile of a naphthalenic aMT ligand.[21] Assuming that under basal, unstimulated conditions there is an existing ratio of Rh/Rl < 1, the system is prevalently inactive. In that case the agonists, because of their higher affinity for the Rh, would create a shift in the equilibrium toward the Rh state, with a consequent activation of the signal-transduction pathway, while the antagonists would keep the basal conditions unaltered or at best change the ratio of Rh/Rl toward values less than basal values.[41] This type of interaction would allow prediction of the efficacy of a compound after having measured its affinity for both Rh and Rl.[42] Our prediction was that, under conditions of competition analysis (2-[^{125}I]iodomelatonin, 200 pM, see the Experimental Section), coincubation with GTPγS would result in increased IC_{50} values for an agonist (the curve would shift to the right), while the IC_{50} values for an antagonist would remain unchanged or decrease (the curve would shift to the left). The series of pilot studies using melatonin (agonist) and compound **32**[33] (antagonist) completely confirmed the prediction of this model. In the presence of GTPγS, the aMT IC_{50} values increased by 3−6 times while the IC_{50} values for compound **32** showed a 2−3 times decrease. These results completely justified the application of this new approach to assess the agonist/antagonist nature of all the compounds reported in this study.

In order to be able to compare these results with those of the cAMP analysis, a numerical index was introduced (GTPγS index) = [(IC_{50} + GTPγS)/(IC_{50} − GTPγS)]-compound/[(IC_{50} + GTPγS)/(IC_{50} − GTPγS)]aMT.

As seen in Table 1, the GTPγS index evaluation thoroughly corresponded to that obtained by using the cAMP data. The final evaluation of the compounds (agonist, partial agonist, antagonist) was made taking into consideration both indices (GTPγS index and cAMP). Compounds with indices having numerical values > 0.8 were considered full agonists; values ranging from 0.2 to 0.8 were indicative of partial agonists, and values < 0.2 were considered characteristic for the antagonists. In the few cases in which values of both indices were close to, but not within, the theoretically determined limits of the range 0.2−0.8, a double denomination was adopted, *i.e.* if a compound had a GTPγS index = 0.16 and cAMP index = 0.25, it was considered to be an antagonist−partial agonist.

Results and Discussion

The results of the biological activity determinations and binding studies are summarized in Table 1. The biological activity and the efficacy of the new compounds were tested by measuring the inhibition of the forskolin-stimulated cAMP accumulation and the GTPγS index. Both analyses demonstrated that all of the compounds were full agonists with the exception of **4** and **9** (Figures 3 and 4), which showed a slight reduction in efficacy behaving like partial agonists. The compounds synthesized exhibited a broad range of binding affinities: from as high as 0.39 nM to inactive. The affinities of compounds **6** and **7**, and **8** and **9**, with the 3-ethylamido side chain partially constrained, were the highest.

Attempts to explore full rigidity of the side chain by linking the alkylamido moiety to the 4- or 2-position of the indole ring implemented with the structures **2** or **3** led to compounds that exhibited very poor binding affinity. This could be due to the fact that the cis conformation of the amide bond is not appropriate for receptor binding, or due to an inappropriate conformation of the ethyl chain.

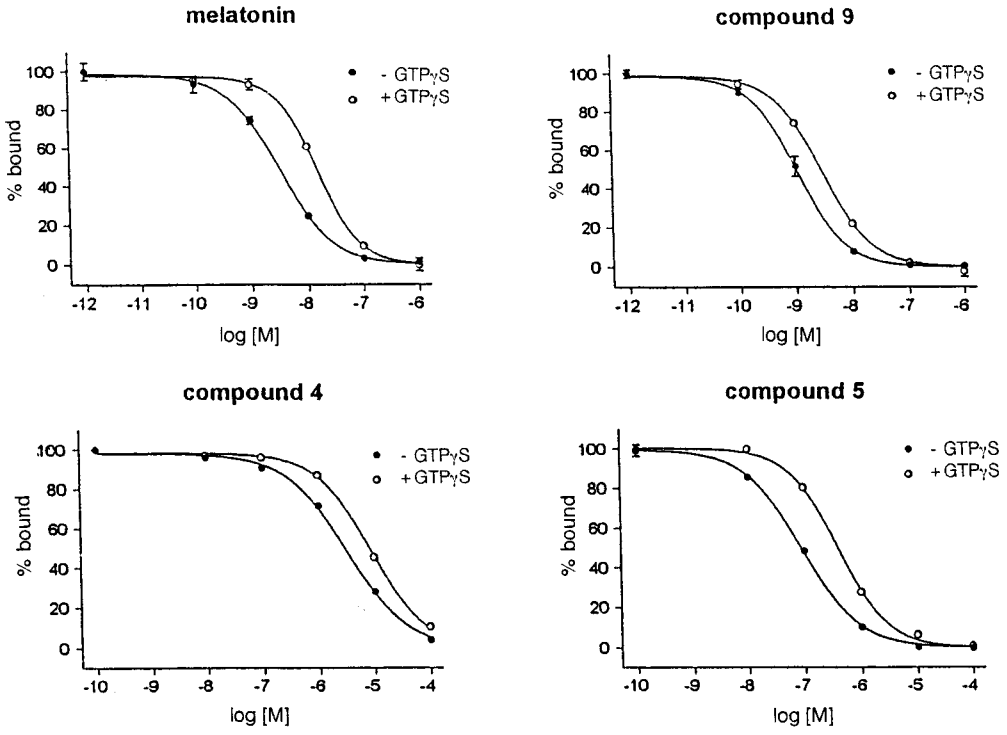
The semirigid analogues **8** and **9** provide fewer possible conformations for recognition by the receptor than aMT itself. Although in these compounds there is a rotatable bond between the indole 3-carbon and the ethenyl moiety, this chain is somewhat constrained to near-planar conformations (*s-cis*, *s-trans*) because of conjugation between the indole ring and the double bond. These compounds showed good but not optimal binding affinity. In order to determine the preferred *s-cis* or *s-trans* conformation of **8** and **9** we investigated the carbazole derivatives **11** and **12** in which the ethylamido side chain is fixed in the coplanar extended *s-cis* conformation. This transformation led to a reduction in binding affinity (*i.e.* 8 nM for **8**, compared to 63 nM for **12**). This result may indicate that the aMT receptor links preferentially to melatonin in its folded *s-trans* conformation, although it is impossible to exclude *a priori* a negative sterical interaction between the C ring of carbazole and the receptor site. Constraint of the C3−Cβ bond, which led to the derivatives **6** and **7** with the side chain partially folded toward the C4 indole position ring system, gave markedly better results than the compounds which led to the more rigid back-folded compounds **4** and **5**.

In order to build a topographical model of the binding site, the conformational space of the compounds with higher affinity was investigated, looking for a common set of distances among the hypothetical pharmacophoric points by DISCO module of SYBYL 6.3.[16] Compounds endowed with at least one hundredth of the affinity of aMT were chosen for this task, *i.e.* compounds **7**, **8**, **12**, and aMT itself. Compound **4** was also included, as its 2-Br derivative **5** is only 33 times less potent than aMT in receptor binding. Other conformationally constrained compounds with known affinity for the aMT receptor were included in the search, *i.e.* compounds (*S*)-**29**,[14e] **30**,[14a] and **31**[17] (Figure 5).

With regard to the choice of the hypothetical pharmacophoric groups, the possible anchor points were the methoxy group, the phenyl ring, and the carboxamido group. The indole NH was not considered because it is not present in compounds **29**, **30**, and **31** and in other known aMt receptor ligands, such as naphthalene derivatives.[43]

Keeping aMT as the reference compound, the search for common pharmacophoric distances was performed using DISCO on conformer databases of molecular mechanics minimized conformations. The amide and the methoxy groups were considered in fixed positions, as described in the Experimental Section. A recent paper[14e] presented a pharmacophoric model in which the methoxy groups in aMT and in **29** had opposite relative orientations. This choice was based on the observation that 6-Cl-aMT has higher receptor binding affinity than aMT itself. As we observed an opposite rank of affinity (Table 1), we decided to keep the methoxy group in the same orientation for all of the compounds.

Eight features were assigned to the pharmacophoric groups, including the aromatic centroid, the three heteroatoms, and four points in the directions of the possible hydrogen bonds. One of the two lone pair



	IC_{50}	IC_{50} + GTPγS	ratio	GTPγS index	Activity
Melatonin	3.11	15.2	4.88	1	A
9	1.07	2.99	2.79	0.57	PA
4	3110	8970	2.88	0.59	PA
5	89.5	396	4.42	0.91	A

Figure 3. Examples of the evaluation of the biological activity of the compounds by using the GTPγS index: competition experiments performed with a fixed concentration of the labelled ligand (200 pM 2-[^{125}I]iodomelatonin) and varying concentrations of the competing drug, in absence or presence of GTPγS (10^{-4} M). Ratio = (IC_{50} + GTPγS)/IC_{50}. GTPγS index = ratio compound/ratio aMT. IC_{50} values are expressed in nM. Activity: when GTPγS index < 0.2 = AN (antagonist); between 0.2 and 0.8 = PA (partial agonist); >0.8 = A (agonist).

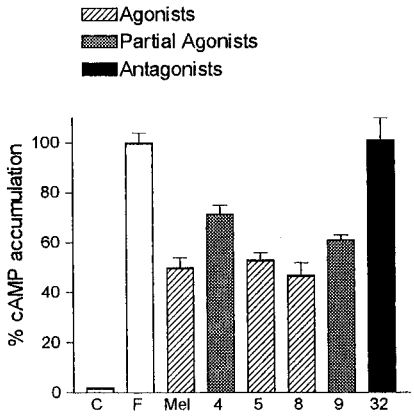


Figure 4. Examples of the evaluation of the biological activity of the compounds by measuring the effect on forskolin (10^{-5} M)-stimulated cAMP accumulation in explants from quail optic tecta. C, control; F, forskolin. Agonist: ratio of percent inhibition compound/percent inhibition aMT > 0.8. Partial agonist: ratio of percent inhibition compound/percent inhibition aMT is between 0.8 and 0.2. Antagonist: ratio of percent inhibition compound/percent inhibition aMT < 0.2.

prolongations of the carbonyl oxygen was not considered as it was found to be close to the rest of the molecule and therefore discarded as unlikely to represent a putative hydrogen bond donor site of the receptor.

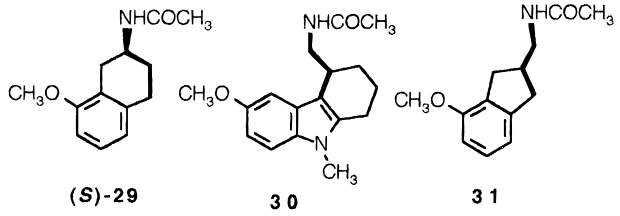


Figure 5.

Among the models belonging to the group with the lowest distance tolerance (3.0 Å), the two with the smallest union volumes were selected and named A and B. The resulting superimpositions are represented in Figure 6.

In describing the relative dispositions of the three putative pharmacophoric groups, for sake of clarity only the distances among the four points indicated in Figure 7 are reported in Table 2. In fact, these are the most informative features because the positions of amide N and O are dependent on those of their hydrogen bond direction prolongations, and the lone pair prolongations on the methoxy group are less important because its orientation is somewhat arbitrary (see the Experimental Section). The four points can be seen as the vertices of a tetrahedron, and the interpoint distances remain the same upon space reflection. Therefore, for each super-

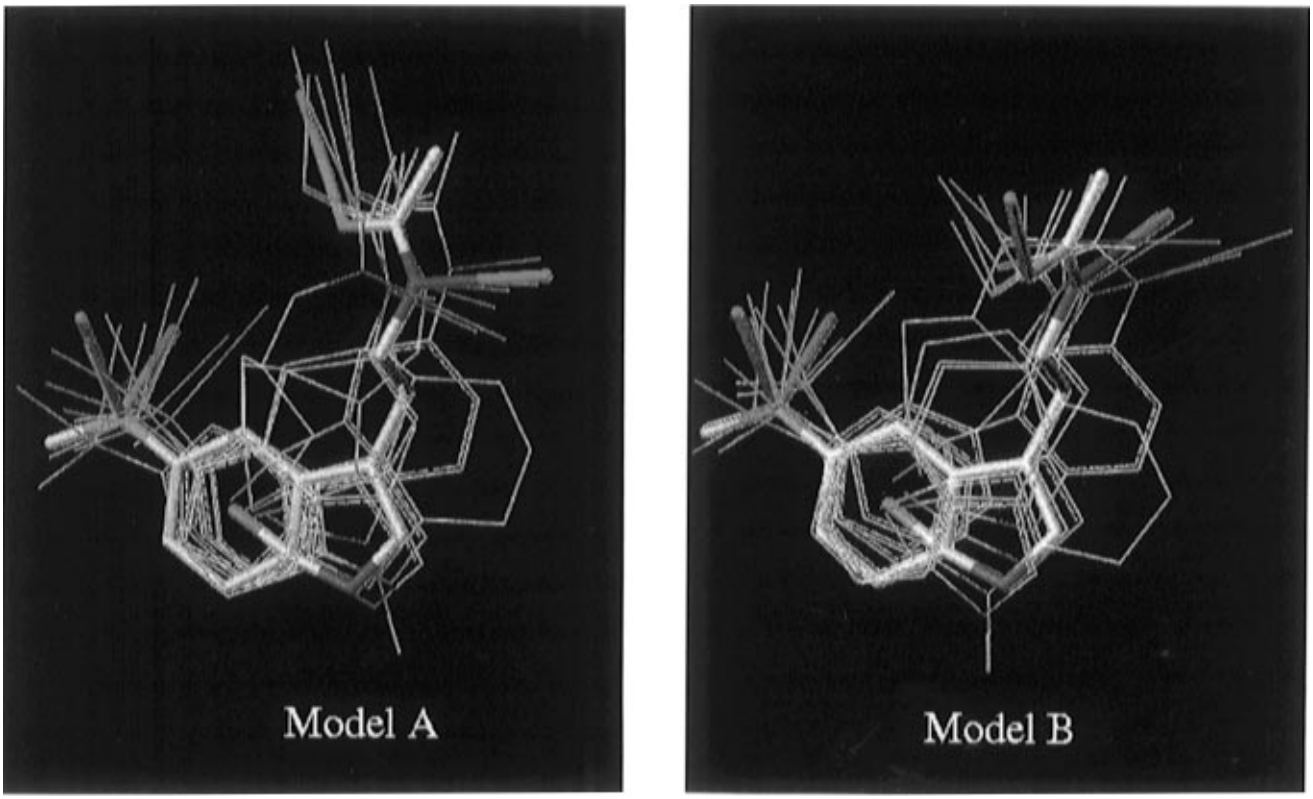


Figure 6. Superimposition of compounds with good affinity for aMT receptor, resulting from DISCO analysis, according to models A and B. Melatonin (reference compound) is highlighted in capped sticks. Lone pairs are represented in red; the prolongations of the lone pairs, the prolongations of the N−H bond, and the hydrophobic centroid are represented in purple.

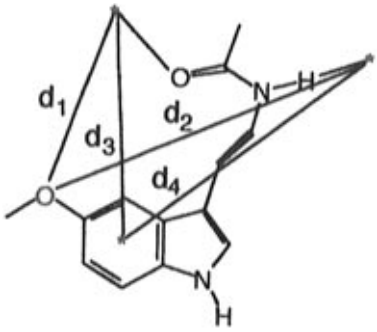


Figure 7. Pharmacophoric points employed for a synthetic description of models A and B.

Table 2. Range of Distances (Å) among the Four Pharmacophoric Points represented in Figure 7 for the Conformers Superimposed by DISCO, and Corresponding Distances for aMT (Reference Compound)

distance	melatonin[a]	all compounds	other aMt conformers
Model A			
d_1	6.70[b]	6.70−8.48	
d_2	9.71[b]	7.47−10.72	
d_3	7.64[b]	7.64− 8.67	
d_4	8.37[b]	6.34− 8.92	
Model B			
d_1	9.88[c]	7.51−10.07	9.74,[d] 9.67,[e] 7.81[f]
d_2	8.03[c]	7.08−10.72	8.24,[d] 9.21,[e] 9.21[f]
d_3	9.40[c]	7.96−9.46	8.97,[d] 8.91,[e] 8.83[f]
d_4	7.32[c]	7.01−8.91	7.72,[d] 8.16,[e] 7.81[f]

[a] Conformation used as reference. [b] $\tau1 = 79.1°$, $\tau2 = 178.6°$, $\tau3 = 78.4°$. [c] $\tau1 = 81.6°$, $\tau2 = -179.8°$, $\tau3 = -174.7°$. [d] $\tau1 = -4.2°$, $\tau2 = -175.5°$, $\tau3 = -78.4°$. [e] $\tau1 = -175.8°$, $\tau2 = 178.3°$, $\tau3 = 80.2°$. [f] $\tau1 = 0.0°$, $\tau2 = 180.0°$, $\tau3 = -179.6°$.

imposition model, a specular one exists, which for flexible compounds corresponds to specular conformations with inversion of the torsional angles, and which for rigid chiral compounds can correspond to the opposite enantiomer. For compound **29** only the (*S*)-isomer was considered, in accordance with recent findings,[14e] but it is not clearly able to discriminate between specular models as it can fit both in different conformations. In both models A and B aMT adopted a *gauche/anti* side chain conformation (A, $\tau1 = 79.1°$, $\tau2 = 178.4°$; B, $\tau1 = 81.6°$, $\tau2 = -179.8°$), whereas the $\tau3$ torsional angle was 78.3° and −174.7°, respectively (energy: B − A = 0.24 kcal/mol). Three other aMT conformers had distances d_1-d_4 within the ranges of model B: one of which ($\tau1 = -4.2°$, $\tau2 = -175.5°$; $\tau3 = -78.4°$) fit the aMT conformer of model B well, while the other two had poorer fits (see Table 2). Two pharmacophore models (afterward named G−A and G−B) were recently reported[14e,f] which, although based on a partially different set of compounds and different assumptions (methoxy group orientation, see above), are can be compared to our models. The first model, G−A (with a aMT conformation referred to as a *gauche/−gauche/−90°*), is not consistent with ours. In particular, the very active newly synthesized compound **7** gave a good fit to the aMT conformers of our models A and B (maximum interfeature distances difference of 0.90 and 0.68 Å, respectively) and a much poorer fit to the aMT conformer of G−A model (2.05 Å); moreover, the carbazole **12** did not fit the G−A model at all. The second model, G−B, of aMT conformer (*gauche/anti/−90°*) had the same chain orientation as the conformers in our models A and B, differing only in $\tau3$, which assumes the third possible minimum energy value. With respect to our superimposition protocol, the alignment of our compounds on the G−B conformer of aMT gave a union volume larger than that obtained with our models A and

B; the G−B model therefore seems less likely than A or B. Summarizing the results of the pharmacophore search, we proposed two possible pharmacophore models (A and B) on the basis of the interfeature distances and union volume of all the set compounds; the orientation of the Cα−N bond among the three alternative minimum energy values for $\tau 3$ proposed by models A, B, and G−B, remains undetermined.

The only constrained compound which did not reveal affinity for the aMT receptor, compound **10**, did not have the four pharmacophoric points within the predefined ranges. More flexible compounds, such as **13**−**16** (Figure 2), are less informative in this kind of study. Compound **13** can be easily superimposed to compounds **4** and **5**, given their topological similarity; its low affinity compared to that of **5** is probably due to the lack of a positive effect of a substituent in the 2-position of the indole. The compound with the longest chain (**14**) can accommodate its points at the distances required by models A and B, but was inactive; this seems to be due to its excessive flexibility, rather than a negative effect of the different attachment point of the chain (in C4). In fact, the other C4-substituted compound **13** retained residual affinity. The compound with the shortest chain (**15**) had no minimum-energy conformation able to fit the pharmacophore requirements and was inactive. Compound **16** is a borderline case: it can accommodate the pharmacophoric groups as required for model B, but not for model A; its low affinity does not however allow definitive statements based on this observation. In the above-mentioned compounds **2** and **3**, the amide group is located at distances from the methoxy group and from the aromatic ring which are different than those required by the two models, apart from their *cis* conformation; it is therefore impossible to assume an active *trans* conformation of aMT based only on these compounds.

We also investigated the role of appropriate C2 indole substituents in the binding affinity for the aMT receptor. In fact, we had previously reported that substitution with bromine[19] or phenyl[20] at C2 leads to a large increase in binding affinity. The favorable role of the methoxy group in the ortho position of the side chain in a naphthalenic aMT analogue had also been reported.[21] Garrat *et al.*[14a] justified the higher activity of 2-halo or 2-phenyl analogues of aMT, suggesting that the C-2 substituent plays a role in orienting the side chain toward a favorable folded conformation. In order to test this hypothesis, the molecular mechanics energy profile upon rotation of torsional angle C3a−C3−Cβ−Cα was calculated for compounds **8** and **9** by the Grid search routine of SYBYL (see the Experimental Section). The two compounds gave very similar energy *vs* torsional angle plots, with minimum energy at 180° for **8** and at 150° for **9**, and a second minimum (which fits the pharmacophoric distances) at 40° for both compounds, 1.3−1.4 kcal/mol above the global minimum. The rotational barrier was also the same in the two cases, with maximum energy around 4.5 kcal/mol above the global minimum, at 90°.

On the contrary, the remarkable increase in binding affinity observed in the C2-substituted analogues **6** and **9** and especially in the more rigid analogue **5** in comparison to the unsubstituted analogues indicate that a further binding point interaction may be the cause of the increased affinity.

The implication of the results presented in this study is that the orientation of the C3 amido side chain and the C2 indole substituent are critical determinants for receptor binding affinity. The present work extends what had been presented so far, offering additional information on the binding of melatonin for its receptor site. In particular, we demonstrate that in addition to the three pharmacophoric elements (the 5-methoxy group, 3-ethanamido side chain, and the aromatic−aromatic interaction of the indole ring), the C2 indole substituent contributes significantly to the receptor binding of this series, thus establishing a putative four-point pharmacophore. Furthermore, our results suggest that one of the preferred binding conformations of the C3-ethylamido side chain is out of the plane of the indole, *gauche*/*anti* orientated as depicted in Figure 6, and this conclusion is in agreement with recently literature data on the high-affinity compound **29**.[14a]

Experimental Section

(a) Chemical Methods. Melting points were determined on a Büchi SMP-510 capillary melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a Bruker AC 200 spectrometer; chemical shifts are reported in ppm and given in δ units. EI-MS spectra (70 eV) were taken on a Fisons Trio 1000. Only molecular ions (M^+) and base peaks are given. Infrared spectra were obtained on a Bruker FT-48 spectrometer; absorbances are reported in ν (cm^{-1}). Elemental analyses were performed on a Carlo Erba analyzer.

6-Methoxy-1-oxo-1,2,3,4-tetrahydro-β-carboline (2). Compound **2** was prepared according to the literature method.[24]

8-Methoxy-1,4,5,7-tetrahydroazocino[4,5,6-*cd*]indol-6(3*H*)-one (3). Compound **3** was prepared according to the literature method:[25] ^{1}H NMR (DMSO-d_6) δ 3.13 (t, 2H), 3.59 (q, 2H), 3.77 (s, 3H), 3.86 (s, 2H), 6.85 (d, 1H, J = 8.8 Hz), 7.13 (br s, 1H), 7.17 (d, 1H, J = 8.8 Hz), 7.35 (br t, 1H), 10.84 (br s, 1H); IR (Nujol) 3291, 3195, 1651 cm^{-1}; MS (EI) m/z 230 (M^+, 100).

(±)-4-Acetamido-6-methoxy-1,3,4,5-tetrahydrobenz[*cd*]-indole (4). A solution of 6-methoxy-4-nitro-1,3,4,5-tetrahydrobenz[*cd*]indole[26] (0.1 g, 0.43 mmol) in MeOH (6 mL) was hydrogenated over 10% Pd−C (0.03 g) at 50 psi of H_2 until hydrogen uptake ceased (∼2 h). The catalyst was filtered on Celite, and the filtrate was evaporated at reduced pressure, giving a crude residue which was dissolved in THF (4 mL) and then acylated with Ac_2O (0.05 mL, 0.68 mmol) and Et_3N (0.05 mL, 0.69 mmol) at room temperature for 3 h. The solvent was evaporated *in vacuo*, and the residue was dissolved in EtOAc, washed with a saturated $NaHCO_3$ solution and brine, dried (Na_2SO_4), and concentrated under reduced pressure. The crude product was purified by flash chromatography over silica gel using EtOAc as eluent to give **4** (0.063 g, 57%) as a white amorphous solid: ^{1}H NMR ($CDCl_3$) δ 1.94 (s, 3H), 2.85−3.10 (m, 4H), 3.86 (s, 3H), 4.76 (m, 1H), 5.52 (br d, 1H), 6.89 (d, 1H, J = 8.6 Hz), 6.93 (br s, 1H), 7.17 (d, 1H, J = 8.6 Hz), 7.79 (br s, 1H); IR (Nujol) 3380, 3280, 1639 cm^{-1}; MS (EI) m/z 244 (M^+), 185 (100). Anal. ($C_{14}H_{16}N_2O_2 \cdot {}^2/_3H_2O$) C, H, N.

(±)-4-Acetamido-2-bromo-6-methoxy-1,3,4,5-tetrahydrobenz[*cd*]indole (5). NBS (1.05 equiv) was added portion wise to an ice-cooled solution of **4** (0.24 g, 1 mmol) in AcOH (2.8 mL) and dioxane (1.4 mL). The mixture was stirred at 0 °C for 0.5 h and for 1.5 h at room temperature. The mixture was then poured onto an ice-cooled 30% NaOH solution and extracted (3×) with EtOAc, and the combined extracts were washed with brine and dried (Na_2SO_4). Evaporation of the solvent gave a crude residue which was purified by chromatography (silica gel, cyclohexane/EtOAc, 3:7, as eluent) and crystallization from EtOAc to give **5** as a beige solid (0.17 g, 53%): mp 200 °C; ^{1}H NMR ($CDCl_3$ and MeOD) δ 1.80 (s, 3H), 2.54−3.02 (m, 4H), 3.74 (s, 3H), 4.49 (m, 1H), 6.72 (d, 1H, J =

8.75 Hz), 6.98 (d, 1H, J = 8.75 Hz); IR (Nujol) 3349, 3226, 1657 cm^{-1}; MS (EI) m/z 324−322 (M^+), 263−265 (100). Anal. ($C_{14}H_{15}BrN_2O_2$) C, H, N.

Methyl (*E*)-3-(5-Methoxy-1*H*-indol-4-yl)propenoate (19). A mixture of 4-bromo-5-methoxyindole[27] (2.26 g, 10 mmol), methyl acrylate (2.58 g, 30 mmol), and bis(triphenylphosphine)palladium(II) chloride (0.7 g, 1 mmol) in AcOH (17 mL) and TEA (17 mL) was stirred for 6 h at 120 °C in a closed vessel. The mixture was cooled to room temperature and then poured onto water and CH_2Cl_2. The biphasic mixture was filtered through a pad of Celite, and the aqueous layer of the filtrate was extracted twice with CH_2Cl_2. The combined organic layers were washed with a saturated $NaHCO_3$ aqueous solution and then with brine and dried over Na_2SO_4. After removal of the solvent the desired product was chromatographed over silica gel (cyclohexane/ethyl acetate, 7:3 as eluent) to give pure **19** (0.92 g, 40%). Crystallization from CH_2Cl_2/hexane: mp 135−138 °C; ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H), 3.93 (s, 3H), 6.81 (m, 1H), 6.83 (d, 1H, J = 16.3 Hz), 6.91 (d, 1H, J = 8.9 Hz), 7.31 (t, 1H), 7.39 (dd, 1H, J = 8.91 Hz, J = 0.95 Hz), 8.32 (d, 1H J = 16.3 Hz), 8.49 (br s, 1H); IR (Nujol) 3323, 1685 cm^{-1}; MS (EI) m/z 231 (M^+, 100).

Benzyl (*E*)-3-(5-Methoxy-2-carbomethoxy-1*H*-indol-4-yl)propenoate (20). The compound **20** was prepared by the same procedure described above, using benzyl acrylate instead of methyl acrylate (yield: 3.06 g, 84%). Crystallization from MeOH: mp 181−183 °C; ^{1}H NMR ($CDCl_3$) δ 3.94 (s, 3H), 3.96 (s, 3H), 5.30 (s, 2H), 6.86 (d, 1H, J = 16.16 Hz), 7.08 (d,1H, J = 8.9 Hz), 7.40 (m, 7H), 8.31 (d, 1H, J = 16.16 Hz), 8.90 (br s, 1H); IR (Nujol) 3327, 1689 cm^{-1}; MS (EI) m/z 365 (M^+, 100%).

Methyl 3-(5-Methoxy-1*H*-indol-4-yl)propanoate (21). A solution of **19** (2.31 g, 10 mmol) in THF (150 mL) was hydrogenated over 10% Pd−C (0.23 g) at 3 atm of H_2 for 6 h at room temperature. The catalyst was filtered on Celite, and the filtrate was evaporated at reduced pressure, giving a nearly quantitative yield of the desired compound **21** as yellow solid, which was directly used without further purification: ^{1}H NMR ($CDCl_3$) δ 2.69 (t, 2H), 3.26 (t, 2H), 3.77 (s, 3H), 3.87 (s, 3H), 6.54 (m, 1H), 6.91 (d, 1H, J = 8.9 Hz), 7.19−7.25 (m, 2H), 8.17 (br s, 1H); IR (Nujol) 3320, 1700 cm^{-1}; MS (EI) m/z 233 (M^+), 160 (100).

5-Methoxy-1*H*-indole-4-propanoic Acid (22). A solution of **21** (2.33 g, 10 mmol) in THF (20 mL), MeOH (24 mL), and 3 N KOH (10 mL) was stirred at room temperature for 16 h. The solvents were removed *in vacuo*, and the residue was dissolved in water and ethyl acetate and then acidified with 6 N HCl. The layers were separated, and the organic phase was washed with brine, dried (Na_2SO_4), and evaporated *in vacuo* to obtain the desired compound **22** which was purified by crystallization from $CHCl_3$ (1.86 g, 85%): mp 152−153 °C; ^{1}H NMR ($CDCl_3$) δ 2.73 (t, 2H), 3.28 (t, 2H), 3.88 (s, 3H), 6.54 (m, 1H), 6.91 (d, 1H, J = 8.8 Hz), 7.23 (m, 2H), 8.07 (br s, 1H); IR (Nujol) 3430, 1712, 1682 cm^{-1}; MS (EI) m/z 219 (M^+), 160 (100).

5-Methoxy-2-carboxymethyl-1*H*-indole-4-propanoic Acid (23). A solution of **20** (3.65 g, 10 mmol) in THF (150 mL) was hydrogenated over 10% Pd−C (0.4 g) at 3 atm of H_2 for 16 h at room temperature. The catalyst was filtered on Celite, and the filtrate was evaporated at reduced pressure to give the title compound **23** as yellow solid, which was purified by heating in refluxing EtOAc (2.38 g, 86%): mp 209−215 °C; ^{1}H NMR ($CDCl_3$) δ 2.71 (t, 2H), 3.25 (t, 2H), 3.88 (s, 3H), 3.95 (s, 3H), 7.06 (d, 1H, J = 9.2 Hz), 7.23 (d, 1H, J = 1.59 Hz), 7.25 (d, 1H, J = 9.2 Hz), 8.88 (br s, 1H); MS (EI) m/z 277 (M^+), 186 (100); IR (Nujol) 3300, 1688, 1631 cm^{-1}.

6-Methoxy-4,5-dihydrobenz[*cd*]indol-3(1*H*)-one (24). Polyphosphoric acid (PPA) (9 g) was preheated with mechanical stirring at 80−85 °C under nitrogen atmosphere. The acid **22** (0.438 g, 2 mmol) was then added over a 5 min period, and the mixture was stirred at 80 °C for 80 min under N_2 and ice was added and left under stirring for 1 h. The crude brown solid precipitate was filtered, dissolved in hot THF (100 mL), diluted with CH_2Cl_2 (50 mL), and washed with saturated aqueous solution of $NaHCO_3$ and brine. After drying (Na_2SO_4), the solvents were evaporated under vacuum, and the crude black solid residue was purified by flash chromatography (silica gel, THF/cyclohexane, 1:1) to give **24** as a white solid (0.14 g, 35%): mp 220−230 °C dec; ^{1}H NMR (acetone-d_6) δ 2.69 (t, 2H), 3.23 (t, 2H), 3.87 (s, 3H), 6.96 (d, 1H, J = 8.8 Hz), 7.30 (d, 1H, J = 8.8 Hz), 7.77 (d, 1H, J = 1.46 Hz), 10.92 (br s, 1H); IR (Nujol) 3110, 1646, 1605 cm^{-1}; MS (EI) m/z 201 (M^+).

2-Carbomethoxy-6-methoxy-4,5-dihydrobenz[*cd*]indol-3(1*H*)-one (25). Polyphosphoric acid (PPA) (9 g) was preheated with mechanical stirring at 80−85 °C under nitrogen atmosphere. The acid **23** (0.554 g, 2 mmol) was then added over a 5 min period, the mixture was stirred at 80 °C for 60 min under N_2, and ice was added and left under stirring for 1 h.The mixture was extracted three times with $CHCl_3$, and the combined extracts were washed with 10% aqueous Na_2SO_4 solution and 5% aqueous $NaHCO_3$ solution, dried (Na_2SO_4), and concentrated under reduced pressure. The solid was triturated with EtOAc to obtain a yellow solid (0.43 g, 84%): mp 220−221 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.92 (t, 2H), 3.32 (t, 2H), 3.92 (s, 3H), 4.04 (s, 3H), 7.10 (d, 1H, J = 8.8 Hz), 7.29 (d, 1H, J = 8.8 Hz), 9.33 (br s, 1H); IR (Nujol) 3300, 1688, 1616 cm^{-1}; MS (EI) m/z 259 (M^+), 227 (100).

(±)-3-Acetamido-6-methoxy-1,3,4,5-tetrahydrobenz[*cd*]-indole (10). Under a nitrogen atmosphere a mixture of the ketone **24** (0.2 g, 1 mmol), benzylamine (0.14 g, 1.3 mmol), and *p*-toluenesulfonic acid monohydrate (0.0025 g, 0.013 mmol) in dry benzene (25 mL) was heated at reflux with a Dean−Stark water separator for 20 h. The benzene and the excess benzylamine were removed *in vacuo*, and the crude residue was dissolved in absolute EtOH (10 mL). This solution was hydrogenated at 3 atm of H_2 for 2 h at 50 °C in the presence of 10% Pd−C catalyst (0.02 g). The catalyst was filtered off, and the solvent was evaporated under reduced pressure to give the crude oily amine **26** which was then dissolved in THF (5 mL) and stirred at room temperature for 6 h with Ac_2O (0.07 mL, 0.75 mmol) in the presence of TEA (0.1 mL, 0.75 mmol). The solvent was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate, washed with $NaHCO_3$ solution followed by brine, dried (Na_2SO_4), and evaporated again. Purification by flash chromatography (silica gel; EtOAc as eluent) and crystallization from CH_2Cl_2/hexane yielded the desired product **10** as a white solid (0.037 g, 15%): mp 205−208 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.01 (s, 3H), 1.95−2.27 (m, 4H), 3.88 (s, 3H), 5.34 (m, 1H), 5.67 (br d, 1H), 6.89 (d, 1H, J = 8.9 Hz), 7.05 (d, 1H, J = 1.6 Hz), 7.15 (d, 1H, J = 8.89 Hz), 7.94 (br s, 1H); IR (Nujol) 3296, 1616 cm^{-1}; MS (EI) m/z 244 (M^+), 185 (100). Anal. ($C_{14}H_{16}N_2O_2$) C, H, N.

(±)-2-(Carboxyethyl)-3-cyano-6-methoxy-1,3,4,5-tetrahydrobenz[*cd*]indole (27). A suspension of **25** (0.26 g, 1 mmol) and TPSH (0.37 g, 1.25 mmol) in dry THF (10 mL) was stirred at room temperature under N_2 atmosphere for 3 h. The solvent was removed under vacuum, and the crude residue was dissolved in EtOH (8 mL). KCN (1.4 g; 21 mmol) was added, and the solution was heated, under reflux, for 24 h under N_2. The solvent was removed at reduced pressure, and the residue was taken up with $CHCl_3$, and H_2O. The aqueous phase was extracted three times with $CHCl_3$ and the collected organic layers were washed sequentially with a saturated solution of $NaHCO_3$, 2N HCl, H_2O, and brine, then dried (Na_2SO_4), and concentrated under vacuum. The crude product was purified by flash chromatography (silica gel, $CHCl_3$/EtOAC, 98:2) and then by crystallization from EtOAc/hexane, to obtain the desired compound **27** as a yellowish solid (0.085 g, 29%): mp 160−161 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.47 (t, 3H), 2.15 (m, 1H), 2.54 (m, 1H), 2.91 (m, 1H), 3.22 (m, 1H), 3.88 (s, 3H), 4.47 (m, 3H), 7.05 (d, 1H, J = 8.9 Hz), 7.20 (d, 1H, J = 8.9 Hz), 8.93 (br s, 1H); IR (Nujol) 3295, 2234, 1698 cm^{-1}; MS (EI) m/z 284 (M^+), 238 (100).

(±)-3-(Acetamidomethyl)-2-(carboxyethyl)-6-methoxy-1,3,4,5-tetrahydrobenz[*cd*]indole (6). A solution of **27** (0.284 g, 1 mmol) in THF (10 mL) and acetic anhydride (2 mL) was hydrogenated over Raney nickel at 4 atm of H_2 for 6 h at 50 °C. The catalyst was filtered on Celite, and the filtrate was concentrated *in vacuo* and partitioned between CH_2Cl_2 and 2 N NaOH. The organic layer was washed with brine, then dried (Na_2SO_4), and evaporated under reduced pressure to give a crude residue which was purified by flash chromatography

(silica gel; ethyl acetate as eluent) and crystallization from CH_2Cl_2/hexane to obtain **6** as a white solid (0.261 g, 79%): mp 123 °C; ^{1}H NMR ($CDCl_3$) δ 1.45 (t, 3H), 1.92 (s, 3H), 2.01 (m, 1H), 2.18 (m, 1H), 2.79 (m, 1H), 3.06 (m, 1H), 3.48 (m, 2H), 3.65 (m, 1H), 3.87 (s, 3H), 4.44 (m, 2H), 6.96 (br s, 1H), 7.05 (d, 1H, J = 8.9 Hz), 7.15 (d, 1H, J = 8.9 Hz), 8.43 (br s, 1H); IR (Nujol) 3351, 3305, 1685, 1647 cm^{-1}; MS (EI) m/z 330 (M^+), 271 (100). Anal. ($C_{18}H_{22}N_2O_4$) C, H, N.

(±)-3-(Acetamidomethyl)-6-methoxy-1,3,4,5-tetrahydrobenz[*cd*]indole (7). A solution of **6** (0.075 g, 0.23 mmol) in THF (0.5 mL), EtOH (0.5 mL), and 3 N KOH (0.2 mL) was stirrred at room temperature for 24 h. The solution was concentrated *in vacuo*, diluted with water, and acidified with 6 N HCl. The solid precipitate was filtered, washed with water, and dried at 30 °C under vacuum to obtain 0.065 g of the crude acid intermediate. This acid was dissolved in quinoline (2 mL) and heated under reflux for 4 h in the presence of 0.01 g of Cu powder in N_2 atmosphere. The mixture was acidified with 2 N HCl and extracted with CH_2Cl_2 (5 × 10 mL). The collected organic extracts were washed with 2 N HCl, $NaHCO_3$, H_2O, and brine, dried (Na_2SO_4), and evaporated *in vacuo* to obtain a crude product which was purified by flash chromatography on silica gel (ethyl acetate as eluent) to obtain the desired pure compound as white solid (0.025 g, 42%). An analytical sample was recrystallized from EtOAc/cyclohexane: mp 151−152 °C; ^{1}H NMR ($CDCl_3$) δ 1.83 (m, 1H), 1.99 (s, 3H), 2.12 (m, 1H), 3.01 (m, 2H), 3.21 (m, 1H), 3.58 (m, 2H), 3.87 (s, 3H), 5.62 (br s, 1H), 6.88 (d, 1H, J = 8.7 Hz), 6.95 (br d, 1H), 7.14 (d, 1H, J = 8.6 Hz), 7.79 (br s, 1H); IR (Nujol) 3490, 3390, 1643 cm^{-1}; MS (EI) m/z 258 (M^+), 199 (100). Anal. ($C_{15}H_{18}N_2O_2 \cdot 1.5H_2O$) C, H, N.

***(E)*-*N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethenyl]acetamide (8).** A solution of (*E*)-3-(2-nitroethenyl)-5-methoxyindole[44] (0.218 g, 1 mmol) in THF (10 mL) and acetic anhydride (2 mL) was hydrogenated over Raney nickel at 4 atm of H_2 for 10 h at room temperature. The catalyst was filtered on Celite, and the filtrate was concentrated *in vacuo* and partitioned between ethyl acetate and 2 N NaOH. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure to give a crude residue which was chromatographed (silica gel; ethyl acetate as eluent) to give 0.04 g of a mixture of the desired product **8** and its N_1-acetyl derivative (considerable amount of melatonin was also isolated). This mixture was dissolved in MeOH (8 mL) and stirred at room temperature for 4 h in the presence of 2 N NaOH (1.5 mL). The solvent was evaporated *in vacuo*, and the residue was taken up in ethyl acetate, washed with brine, and dried (Na_2SO_4). After removal of the solvent at reduced pressure, pure **8** was obtained by crystallization from EtOAc/hexane (0.023 g, 10%): mp 170−173 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.13 (s, 3H), 3.89 (s, 3H), 6.28 (d, 1H, J = 14.62 Hz), 6.87, 6.91 (dd, 1H, J = 8.58 Hz and J = 2.23 Hz), 7.20 (d, 1H, J = 2.23 Hz), 7.21 (br s, 1H), 7.27 (d, 1H J = 8.58 Hz), 7.41, 7.46 (dd, 1H J = 14.62 Hz), 8.04 (br s, 1H); IR (cm^{-1}, Nujol) 3292, 1653; MS (EI) m/z 230 (M^+, 100). Anal. ($C_{13}H_{14}N_2O_2$) C, H; N: calcd, 12.17; found, 11.67.

***(E)*-*N*-[2-(5-Methoxy-2-phenyl-1*H*-indol-3-yl)ethenyl]acetamide (9).** This compound was prepared starting from (*E*)-3-(2-nitroethenyl)-5-methoxy-2-phenylindole[20] (0.294 g, 1 mmol) according to the procedure described above (0.046 g, 15%): mp 202−204 °C; ^{1}H NMR ($CDCl_3$) δ 2.12 (s, 3H), 3.92 (s, 3H), 6.32 (d, 1H, J = 14.62 Hz), 6.89, 6.94 (dd, 1H, J = 8.58 Hz and J = 2.22 Hz), 7.19 (br s, 1H), 7.31−7.64 (m, 8H), 8.07 (br s, 1H); IR (Nujol) 3280, 1653 cm^{-1}; MS (EI) m/z 306 (M^+), 232 (100). Anal. ($C_{19}H_{18}N_2O_2$) C, H, N.

3-Acetamido-9*H*-carbazole (11) and 3-Acetamido-6-methoxy-9*H*-carbazole (12). A solution of 3-nitro-6-methoxy-9*H*-carbazole[31] (0.24 g, 1 mmol) in THF (15 mL) was hydrogenated over 10% Pd−C (0.05 g) at 4 atm of H_2 for 16 h at room temperature, then for 2 h at 50 °C. The catalyst was filtered on Celite, and the filtrate was evaporated at reduced pressure giving a crude mixture of **11** and **12**. These compounds were separated by flash chromatography (silica gel, cyclohexane/EtOAc, 3:7).

11: white solid, 0.075 g, 30%; mp 205 °C (EtOAc/hexane); ^{1}H NMR (DMSO-d_6) δ 2.06 (s, 3H), 7.11−8.02 (m, 6H), 8.33 (s, 1H), 9.91 (br s, 1H), 11.16 (br s, 1H); IR (cm^{-1}, Nujol) 3415, 3276, 1641; MS (EI) m/z 224 (M^+), 182 (100). Anal. ($C_{14}H_{12}N_2O \cdot 1.2H_2O$) C, H, N.

12: white solid, 0.081 g, 30%; mp 206 °C (EtOAc/hexane); ^{1}H NMR (DMSO-d_6) δ 2.06 (s, 3H), 3.83 (s, 3H), 6.99 (dd, 1H, J = 8.97 Hz, J = 2.56 Hz), 7.35 (m, 3H), 7.52 (d, 1H, J = 2.56 Hz), 8.35 (s, 1H), 9.88 (s, 1H), 10.94 (br s, 1H); IR (Nujol) 3378, 3182, 1668 cm^{-1}; MS (EI) m/z 254 (M^+, 100%). Anal. ($C_{15}H_{14}N_2O_2 \cdot H_2O$) C, H, N.

***N*-[2-(5-Methoxy-1*H*-indol-4-yl)ethyl]acetamide (13).** 4-(2-Nitroethenyl)-5-methoxyindole[26] (0.22 g, 1 mmol) was added portion wise to a stirred ice-cooled suspension of $LiAlH_4$ (0.23 g, 6 mmol) in dry THF (15 mL) under nitrogen and the mixture was stirred at room temperature for 5 h. After cooling at 0 °C, water was added dropwise to destroy the excess hydride, the mixture was filtered on Celite, and the filtrate was concentrated *in vacuo* and partitioned between water and ethyl acetate. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to give a crude oily amine which was then used without further purification. This crude amine was dissolved in THF (5 mL) and stirred at room temperature for 6 h with Ac_2O (0.093 mL, 1 mmol) in the presence of TEA (0.13 mL, 1 mmol). The solvent was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate, washed with $NaHCO_3$ solution followed by brine, dried (Na_2SO_4), and evaporated again. Purification by flash chromatography (silica gel; EtOAc as eluent) gave the desired product **13** (0.085 g, 37%) as yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.87 (s, 3H), 3.13 (t, 2H), 3.56 (q, 2H), 3.87 (s, 3H), 6.09 (br s, 1H), 6.47 (m, 1H), 6.90 (d, 1H J = 8.8 Hz), 7.17, 7.18 (dd, 1H, J = 3.1 Hz, J = 2.6 Hz), 7.24 (d, 1H, J = 8.8 Hz), 8.56 (br s, 1H); IR (neat) 3400, 3290, 1652 cm^{-1}; MS (EI) m/z 232 (M^+), 173 (100). Anal. ($C_{13}H_{16}N_2O_2$) C, H; N: calcd, 12.06; found, 11.61.

3-[2-(Carboxymethyl)-5-methoxy-1*H*-indol-4-yl]acrylonitrile (28). A mixture of methyl 4-bromo-5-methoxyindole-2-carboxylate (0.284 g, 1 mmol), acrylonitrile (0.16 g, 3 mmol), and bis(triphenylphosphine)palladium(II) chloride (0.07 g, 0.1 mmol) in AcOH (2 mL) and TEA (2 mL) was stirred for 6 h at 120 °C in a closed vessel. The mixture was cooled to room temperature and then poured onto water and CH_2Cl_2. The biphasic mixture was filtered through a pad of Celite and the aqueous layer of the filtrate was extracted twice with CH_2Cl_2. The combined organic layers were washed with a saturated $NaHCO_3$ aqueous solution and with brine and dried over Na_2SO_4. After removal of the solvent the desired product was chromatographed over silica gel (cyclohexane/ethyl acetate, 1:1, as eluent) to give the title compound (0.12 g, 45%) as a *cis*−*trans* mixture. It could be crystallized from MeOH: mp 225−232 °C; ^{1}H NMR (acetone-d_6) δ 3.90 (s, 3H), 3.97 (s, 3H), 6.50 (d, 1H, J = 16.8 Hz), 7.19 (d, 1H, J = 8.96 Hz), 7.44 (m, 1H), 7.62 (dd, 1H, J = 8.96 Hz, J = 0.86 Hz), 7.95 (d, 1H, J = 16.8 Hz), 11.01 (br s, 1H); IR (Nujol) 3336, 2212, 1711 cm^{-1}; MS (EI) m/z 256 (M^+, 100).

***N*-[3-(2-Carboxymethyl-5-methoxy-1*H*-indol-4-yl)propyl]acetamide (14).** A solution of **28** (0.256 g, 1 mmol) in THF (10 mL) and acetic anhydride (2 mL) was hydrogenated over Raney nickel at 4 atm of H_2 for 6 h at 50 °C. The catalyst was filtered on Celite, and the filtrate was concentrated under vacuum and partitioned between ethyl acetate and 2 N NaOH. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure to give a crude residue which was purified by flash chromatography (silica gel; ethyl acetate as eluent) and crystallization from EtOAc/hexane to obtain **14** as a white solid (0.192 g, 63%): mp 139−140 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.74 (t, 2H), 2.83 (t, 2H), 3.09 (q, 2H), 1.90 (s, 3H), 3.75 (s, 3H), 3.79 (s, 3H), 6.20 (br t, 1H), 6.92 (d, 1H, J = 8.9 Hz), 7.04 (m, 1H), 7.17 (d, 1H, J = 8.8 Hz), 9.60 (br s, 1H); IR (cm^{-1}, Nujol) 3303, 1712, 1653; MS (EI) m/z 304 (M^+), 43 (100). Anal. ($C_{16}H_{20}N_2O_4$) C, H, N.

***N*-[(5-Methoxy-1*H*-indol-4-yl)methyl]acetamide (15).** A solution of 4-cyano-5-methoxyindole[26] (0.17 g, 1 mmol) in THF (10 mL) and acetic anhydride (2 mL) was hydrogenated over Raney nickel at 4 atm of H_2 for 6 h at 50 °C. The catalyst was filtered on Celite, and the filtrate was concentrated *in vacuo* and partitioned between ethyl acetate and 2N NaOH.

The organic layer was washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure to give a crude residue which was purified by flash chromatography (silica gel; ethyl acetate as eluent) and crystallization from $CHCl_3$/hexane to obtain **15** as a white solid (0.12 g, 55%): mp 151−152 °C; ^{1}H NMR ($CDCl_3$) δ 1.96 (s, 3H), 3.89 (s, 3H), 4.75 (d, 2H), 5.90 (br s, 1H), 6.65 (m, 1H), 6.92 (d, 1H, J = 8.9 Hz), 7.25 (m, 1H), 7.32 (d, 1H, J = 8.9 Hz), 8.30 (br s, 1H); IR (Nujol) 3325, 3268, 1616 cm^{-1}; MS (EI) m/z 218 (M^+), 175 (100). Anal. ($C_{12}H_{14}N_2O_2$) C, H, N.

***N*-[(5-Methoxy-1*H*-indol-3-yl)methyl]propanamide (16).** A solution of 3-cyano-5-methoxyindole[32] (0.17 g, 1 mmol) in THF (10 mL) and acetic anhydride (2 mL) was hydrogenated over Raney nickel at 4 atm of H_2 for 6 h at 50 °C. The catalyst was filtered on Celite, and the filtrate was concentrated *in vacuo* and partitioned between ethyl acetate and 2 N NaOH. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure to give a crude residue which was purified by flash chromatography (silica gel; ethyl acetate as eluent) and crystallization from EtOAc/hexane to obtain **16** as a white solid (0.11 g, 47%): mp 157−158 °C; ^{1}H NMR ($CDCl_3$) δ 1.18 (t, 3H), 2.23 (q, 2H), 3.86 (s, 3H), 4.60 (d, 2H), 5.59 (br s, 1H), 6.87, 6.92 (dd, 1H, J = 8.8 Hz and J = 2.54 Hz), 7.08 (d, 1H, J = 2.54 Hz), 7.15 (d, 1H, J = 2.54 Hz), 7.20 (d, 1H, J = 8.8 Hz), 8.09 (br s, 1H); IR (Nujol) 3378, 3232, 1652 cm^{-1}; MS (EI) m/z 232 (M^+), 159, 160 (53.75, 53.75). Anal. ($C_{13}H_{16}N_2O_2$) C, H, N.

***N*-[(2-Phenyl-1*H*-indol-3-yl)ethyl]cyclobutanecarboxamide (32).** Compound **32**, used as reference antagonist, was synthesized as previously described.[33]

(b) Molecular Modeling. Molecular modeling was performed on a Silicon Graphics R4400 200 MHz 64 Mb RAM Indigo2 workstation, using SYBYL 6.3 software from Tripos Inc., St. Louis, MO. DISCO is a module of SYBYL.

Conformational Analyses and DISCO Model Development. Three-dimensional models of all molecules were constructed using the sketch option of the building component of Sybyl 6.3, and their geometry was subsequently optimized using the Tripos standard force field after Gasteiger−Hückel charge calculation.

Nine features were selected among those automatically assigned by DISCO in order to define the hypothetical pharmacophore; in particular, an aromatic centroid, 2 hydrogen-bond (H-B) acceptor atoms (methoxy and carbonyl oxygens), 1 H-B donor atom (amide NH), 4 H-B donor sites (dummy points in the direction of the lone pairs, 3 Å apart form the oxygen atoms), and 1 H-B acceptor site (in the direction of amide NH) were defined before subsequent calculations. Conformational databases for the compounds included in the analysis were prepared using the Multisearch option of the DISCO routine, which generates unique energy minimum conformations with different internal geometry for those atoms that determine the features. Each database was then inspected in order to verify that the conformational space was adequately represented. The carboxamido group was considered in *trans* configuration; as no constrained amide group was present in the set of selected active compounds, the *cis* configuration is also possible. Therefore, the superimposition results cannot discriminate between *cis* and *trans* configuration. The same applies for the orientation of the methoxy group which was fixed in a conformation coplanar with the indole nucleus of aMT, with the methyl group opposite the nearest bridge position. In fact, the other coplanar conformation is sterically hindered in some tricyclic compounds, as resulted from Grid searches on the O−C5 bond. This orientation is probable but not mandatory, given the unavailability of crucial information about it. A master database containing one conformer for each molecule was created and submitted to the DISCO routine; the natural agonist melatonin was selected as the reference compound. The choice of the superimposition models was based on the inclusion of at least eight features and on the lowest level of distance tolerance, in steps of 0.5 Å. Distance tolerance is the maximum allowed difference between an interfeatures distance for any conformation of each compound and the same distance for a fixed conformation of melatonin.

Grid search. The SYBYL 6.3 Grid search routine[45] was used to calculate energy profiles upon rotation of single bonds. Grid search performs rotations of the selected bond followed by energy minimization of the rest of the molecule. The standard Tripos force field with Gasteiger−Hückel charge calculation was employed, with energy minimization performed by the method of Powell and termination set at an energy gradient of 0.02 kcal/mol·Å.

(c) Melatonin Binding Assays. The source of the animals, the characterization of the melatonin receptor and the isolation of the crude membrane preparations used in the present study have been described in detail elsewhere.[35,46]

(d) Determination of the Biological Activity. Biological activity was evaluated using two methods:

(i) Effects on Forskolin-Stimulated cAMP Accumulation. The punching technique, the handling of tissue explants, and cAMP determinations have been described in detail elsewhere.[47] The concentrations of the compounds to be used were calculated on the basis of their affinity, as follows: the experimentally derived maximum effective dose of melatonin was around 10^{-7} M. Therefore, the relative affinity for each compound was determined as IC_{50} compound/IC_{50} aMT and the dose of the compound equivalent to that of melatonin (MED, melatonin equivalent dose) in terms of receptor occupancy was calculated: [MED compound = (relative affinity compound) 10^{-7}]. Afterward, two doses of each compound were assayed: one that was equal (or closest) to the calculated MED and another, greater by one order of magnitude. For example, a compound with a relative affinity of 0.025 has a MED value of 2.5×10^{-9}. Therefore the doses utilized were 10^{-9} and 10^{-8} M. This avoided the use of inadequately high or low doses in the analyses, as has been common practice to date. In all cases the two concentrations employed gave similar results, thus confirming the validity of the theoretical assumption regarding the receptor occupancy. In only a few cases, the higher dose could not be used, due to problems related to the solubility of the compounds. This was the case with compound **32**. On the basis of the data obtained, a cAMP index, assigning a rank of potency for the compounds, was calculated as follows: percent inhibition compound/percent inhibition aMT.

(ii) Effects of Coincubation with GTPγS on the IC_{50} Values (GTPγS Index). Briefly, in each experiment aMT was assayed as a reference standard, GTPγS was used in a constant concentration of 10^{-4} M and the labeled ligand was always 200 pM. The rest of the conditions of the experiment were as described elsewhere.[35,46] GTPγS index = [(IC_{50} + GTPγS)/(IC_{50} − GTPγS)]compound/[(IC_{50} + GTPγS)/(IC_{50} − GTPγS)]aMT.

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