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Reassessing the melatonin pharmacophore—Enantiomeric resolution, pharmacological activity, structure analysis, and molecular modeling of a constrained chiral melatonin analogue

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Abstract—3-(Acetylaminomethyl)-2-(ethoxycarbonyl)-6-methoxy-1,3,4,5-tetrahydrobenzo[*cd*]indole (**2**) is a rigid melatonin analogue that as a racemate displays about the same affinity and intrinsic activity of melatonin (**1**) in in vitro experiments. We report here the resolution of the racemate by preparative medium pressure liquid chromatography (MPLC) and the X-ray determination of the *R* absolute configuration of the (−)-enantiomer. The two enantiomers were separately tested as MT_1 and MT_2 ligands, and the (+)-(*S*)-**2** showed a potency comparable to that of melatonin and about three orders of magnitude greater than that of its enantiomer. The information obtained by crystallographic analysis and NMR studies about the conformational preference for **2** and by the pharmacological characterization of (*R*)-**2** and (*S*)-**2** was employed in a molecular modeling study, aimed at reassessing the melatonin receptor pharmacophore model for agonist compounds. Chiral enantioselective agonists reported in the literature were also included in the study.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT, **1**) is a tryptophan-derived hormone mainly synthesized and secreted by the pineal gland, following a circadian rhythm. It has been characterized, about 50 years ago, after its isolation from bovine pineal tissue by Lerner et al.[1,2] Extensive studies have demonstrated the role of MLT in many physiological processes, such as the regulation of circadian[3] and seasonal rhythms,[4] immune functions,[5] and retinal physiology.[6] Different potential therapeutic applications have been proposed for MLT and its derivatives.[7] They comprise the regulation of disrupted circadian rhythms and of sleep disorders,[8] the treatment of headache disorders[9] and neurodegenerative pathologies, such as Alzheimer's disease,[10] as well as the application as anticancer agents.[11]

Besides the free radical scavenging and antioxidant properties[12,13] recognized for this endogenous hormone, MLT exerts many of its effects activating high-affinity G-protein-coupled receptors, localized in the central nervous system and in peripheral tissues. The recent cloning of several G protein-coupled melatonin receptor genes has revealed at least three MLT receptor subtypes, two of which (MT_1 and MT_2) are found in mammals.[14–16] Another MLT binding site, MT_3, has recently been characterized as the hamster homologue of the human enzyme quinone reductase 2.[17] The important physiological implications and the therapeutic potential of melatonin and its derivatives have led medicinal chemists to look for the pharmacophoric requirements for MLT receptor binding and activation, in order to develop selective MT_1 and MT_2 agonists and antagonists of clinical utility. Indeed, Ramelteon (TAK-375)[18] has

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Keywords: Melatonin; Conformational analysis; Molecular modeling; Enantiomeric resolution; X-ray crystal structure; Pharmacophore model.

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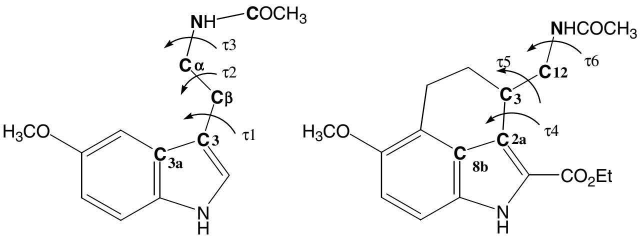
been recently approved for the treatment of insomnia, while other MLT receptor agonists are currently under clinical evaluation. Agomelatine (S-20098),[19] characterized as a melatonin-agonist and selective serotonin antagonist (MASSA), is being evaluated as an antidepressant drug[20,21] and β-methyl-6-chloromelatonin (LY-156735)[22] is under clinical investigation for the treatment of sleep disorders.[21,23]

Early structure–activity relationship (SAR) studies evidenced that both the 5-methoxy group and the C3 acetylaminoethyl side chain are important for MLT high binding affinity and that the relative spatial distance between these groups is also an important factor.[24] However, the MLT C3 side chain has a high degree of flexibility,[25] leading to uncertainty about the mode of interaction with the receptor. Considering that the synthesis of conformationally constrained analogues of natural ligands is the method of choice to investigate their bioactive conformations, we decided to apply this approach to determine how the acetylaminoethyl chain is arranged inside the binding pocket. In particular, in a previous work,[26] we synthesized and tested for their biological activity a set of MLT indole analogues in which the conformation of the C3 side chain was restricted by incorporating it into a different sized ring. Compound **2** (Fig. 1) became the most active among the series, showing, as a racemate, about the same affinity and intrinsic activity as MLT in in vitro experiments (Table 1). A pharmacophore analysis, performed on a set of conformationally constrained MLT receptor agonists including compound **2**, led us to propose a bioactive conformation of MLT in which the C3 acetylaminoethyl side chain is oriented perpendicularly to the indole ring, with a τ1*gauche*/τ2*anti* conformation (Fig. 1). However, due to the absence in our set of an enantiomerically pure, rigid ligand, the pharmacophore chirality of the models remained undetermined.

In a recent NMR conformational study, we proved the highly rigid nature of the tetrahydrobenzo[*cd*]indole scaffold of compound **2** (Fig. 1).[27] This study clearly evidenced that only one of the two possible families of conformers of **2** is present in solution, with the substituent at C3 in axial orientation. In fact, in compound **2** the crucial τ4 dihedral angle (defined by C8b–C2a–C3–C12, as indicated in Fig. 1) is restrained by the steric hindrance of the ethoxycarbonyl substituent at C2. Considering that the racemic mixture of compound **2** has proved to be equiactive to MLT,[26] we thought that new binding experiments on MLT receptors with individual enantiomers could result in a high enantioselectivity.



1 2

Figure 1. Melatonin (**1**) and its rigid analogue **2**. Torsion angles: τ1, C3a–C3–Cβ–Cα; τ2, C3–Cβ–Cα–N; τ3, Cβ–Cα–N–CO; τ4, C8b–C2a–C3–C12; τ5, C2a–C3–C12–N; and τ6, C3–C12–N–C.

Table 1. Binding affinity[a] and intrinsic activity[b] of MLT, (−)-(*R*)-**2**, (+)-(*S*)-**2**, and their racemic mixture

Compound	h-MT$_1$		h-MT$_2$	
	$K_i \pm$ SEM (nM)	IAr	$K_i \pm$ SEM (nM)	IAr
MLT	0.1396 ± 0.058	1	0.2226 ± 0.076	1
(±)-**2**	0.3275 ± 0.085	1.087	0.4976 ± 0.041	1.117
(−)-(*R*)-**2**	105.6 ± 4.2	0.396	126 ± 6.1	0.283
(+)-(*S*)-**2**	0.1816 ± 0.044	1.046	0.2876 ± 0.033	1.113

[a] K_i values were calculated from IC$_{50}$ values, obtained from competition curves by the method of Cheng and Prusoff,[51] and are means of at least three independent determinations performed in duplicate.

[b] The relative intrinsic activity values were obtained by dividing the maximal analogue-induced G-protein activation by that of MLT.

This would make the active enantiomer of **2** a suitable template for defining the topography of the MLT binding pocket. In this paper, we report the enantiomeric resolution of **2**, the pharmacological profile of the single enantiomers, and the characterization of their absolute configuration by X-ray diffractometry. Moreover, we present a molecular modeling study aimed at reassessing the pharmacophore topography for MLT receptor agonists, based on the information provided by the active enantiomer of compound **2**, and discussing the most interesting chiral enantioselective agonists reported in the literature.

2. Results and discussion

2.1. Enantiomeric resolution

Compound **2** was synthesized as already reported,[26] and its racemic mixture was resolved by medium pressure chromatography through tri-acetylcellulose (TAC) as chiral stationary phase. This chiral phase has been previously employed for the resolution of various racemates, especially aromatic pharmaceuticals,[28] and it has been successfully used for the separation of enantiomeric mixtures of MLT analogues, whose structures are closely related to that of compound **2**.[29] Moreover, the high loading capacity of TAC makes it one of the most usable chiral stationary phases for a large-scale preparation.[30] Following sample loading, the eluted fractions were initially collected on the basis of visual inspection of the chromatograms and the enantiomerically enriched batches were recycled through the same column until a complete enantiomeric separation was reached. Fractions at the beginning and at the end of the chromatogram were considered the most enantiomerically pure and their optical rotation became −54° and +54°, respectively. These optical rotation values remained constant after a second run of the same fractions through the column; thus, we kept these values as reference to evaluate the enantiomeric purity of the fractions obtained after recycling the rest of the sample. Finally, the enantiomeric purity was checked by injecting an analytical amount of sample into a TAC column of the same size and under the same conditions as used for the preparative separation. Single peak chromatograms were obtained for each enantiomer. Further details are reported in the experimental section.

2.2. Pharmacology

The racemic mixture and the isolated enantiomers of compound **2** were tested by measuring their binding affinity for human MT_1 and MT_2 receptors and their in vitro functional activity. Binding affinity was evaluated in competition experiments, using 2-[^{125}I]iodomelatonin as the labeled ligand, on cloned human MT_1 and MT_2 receptors expressed in NIH3T3 rat fibroblast cells. The relative intrinsic activity (IAr) was measured by the GTPγS test, by measuring the direct activation of the G-protein after binding of the tested compound to the cloned human MLT receptors.

The summary of in vitro pharmacological assays performed on MLT and our derivatives is reported in Table 1. Compound (+)-**2** displayed almost the same affinity and intrinsic activity as MLT and, compared to its optical isomer, has about 600 and 450 times higher affinities for MT_1 and MT_2 receptor subtypes, respectively. The eudismic ratios of these two enantiomers are among the highest reported until now for MLT receptor agonists. This indicates a highly specific interaction of the eutomer with the binding pocket, making it particularly suitable as a template for pharmacophore studies. Compound (−)-**2** was characterized by reduced potency and it behaved as a weak partial agonist at both receptor subtypes.

2.3. Crystallographic analysis

The crystal structure of compound (−)-**2** has been determined by X-ray diffraction; crystals were prepared by recrystallization from a dichloromethane–cyclohexane solution. Since the three independent molecules present in the crystallographic asymmetric unit exhibit an almost identical overall conformation, we report and discuss hereafter the molecular geometry features of just one of them.

The X-ray crystallographic analysis revealed that the chirality of the asymmetric C3 center is *R* (Fig. 2). The absolute configuration was determined by refinement of the Flack[31] parameter, based on two sets of Friedel pairs. The reported configuration yielded $x = 0.0(6)$.

A perspective view of compound *R*-(−)-**2**, showing the atom numbering scheme, is reported in Figure 2. Bond lengths and valence angles conform to those found for structurally related MLT analogues.[32] The core of the molecule is virtually planar. The nonaromatic nucleus shows an envelope conformation. The methylene atom C4 is located at −0.66(3) Å from the mean molecular plane defined by the indole ring (N1, C2, C2a, C5a, C6, C7, C8, C8a, and C8b). The methoxy group is close to the plane of the aromatic ring and the conformation about the C6–O4 bond is staggered ((−) *syn-periplanar*) with the C5a–C6–O4–C15 torsion angle of −30.2(8)°. The ethyl ester (at C2) and the acetylaminomethyl (at C3) side chains adopt fully extended conformations. The substituent at C2 lies in the same plane as the indole ring, whereas the side chain at C3 is axially oriented, with the torsion angle C8b–C2a–C3–C12 (τ4) of 97.6(4)°. Concerning the conformation of the acetylaminomethyl side chain, the torsion angles C2a–C3–C12–N2 (τ5) and C3–C12–N2–C13 (τ6) are 66.9(4)°, and −174.3(3)°, respectively. The dihedral angle between the mean planes defined by the indole ring (see above) and by the nonH atoms (C12, N2, C13, O3, and C14) of the acetylaminomethyl side chain is 111.1°. This orientation is stabilized by the *intra*-molecular hydrogen bond observed between the amide NH (N2) of the C3 side chain, acting as donor, and the carbonyl oxygen (O1) of the ethyl ester side chain, acting as acceptor (distances N2–H2: 0.84 Å; N2···O1: 2.94 Å; H2···O1: 1.86 Å; angle N2–H2···O1: 165.0°).

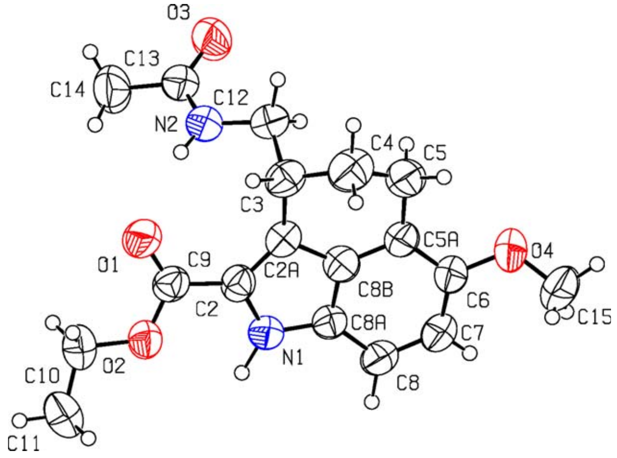


Figure 2. A perspective view of the X-ray crystal structure of compound *R*-(−)-**2**. Thermal ellipsoids are at the 50% probability level.

The molecules are arranged in the crystal as dimers. There are three strong *inter*-molecular hydrogen bonds, connecting the carbonyl O atoms (O3) of the acetylaminomethyl side chain, acting as acceptors, and the NH atoms (N1) of the indole ring, acting as donors.

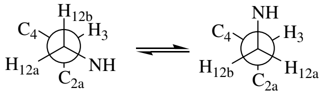
2.4. NMR analysis

The ^{1}H NMR spectrum of the carbocyclic ring and of the C-3 side chain of compound **2** has been analyzed by computer simulation and by fitting between calculated and observed frequencies in a previous paper.[27] The obtained values of the vicinal coupling constants relevant to conformational analysis in solution are given in Table 2 together with the unpublished data for the acetylaminomethyl side chain. First, the parameters of the carbocyclic fragment corroborate the axial orientation of C3 in solution, as found in the crystal structure. Second, the two vicinal couplings of H3 with the two diastereotopic hydrogens H_{12a} and H_{12b} can be interpreted by assuming an equilibrium between the two rotational isomers **I** and **II** around the C3–C12 bond and are expressed as usual by the molar averages of the parameters in the individual rotamers

Table 2. Interproton vicinal coupling constants in the carbocyclic ring and in the C3 side chain of **2**

Carbocycle		Side-chain	
H no.	J[a] (Hz)	H no.	J (Hz)
3-4α	4.93	3-12a	7.39
3-4β	2.00	12a-NH	4.71
4α-5α	4.87	3-12b	8.29
4α-5β	13.41	12b-NH	4.40
4β-5α	2.33		
4β-5β	4.65		

[a] Ref. 27.



I **II**

$$J_{\mathrm{H3\text{–}Ha}} = xJ_{\mathrm{a}} + (1-x)J_{\mathrm{g}} = 8.3,$$

$$J_{\mathrm{H3\text{–}Hb}} = xJ_{\mathrm{g}} + (1-x)J_{\mathrm{a}} = 7.4,$$

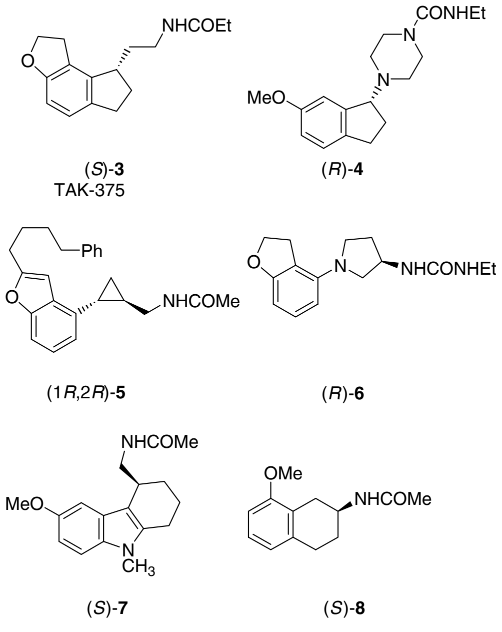
where x is the molar fraction of form **I**. By assuming torsion angles H–H of 60° and 180° for the *gauche* and *anti* arrangements and by using the Karplus–Altona[33] relationship one can estimate $J_a = 12$ Hz and $J_g = 3.6$ Hz, and consequently one obtains $x = 0.55$. This result shows that form **I** observed in the crystal state is present in solution as the major rotamer, together with a significant amount of form **II**.

Finally, the vicinal coupling constants of NH with the two diastereotopic hydrogens H_{12a} and H_{12b} are 4.7 and 4.4 Hz, respectively. In agreement with the relation of Wüthrich et al.,[34] these couplings fit well to torsional angles around ±120°, comparable to what was found in the crystal structure (−115° and 125°). However, we assume that they can be more reasonably interpreted as average values due to the presence of more conformations in solution.

2.5. Molecular modeling

MLT is characterized by a flexible acetylaminoethyl side chain,[25] that can assume different energetically accessible conformations defined by $\tau 1$ (C3a–C3–Cβ–Cα), $\tau 2$ (C3–Cβ–Cα–N), and $\tau 3$ (Cβ–Cα–N–CO) torsion angles (Fig. 1, **1**). This allows different spatial dispositions of the amide function with respect to the aromatic indole scaffold and the methoxy group. For this reason, several attempts have been made to infer the putative bioactive conformation of MLT by means of pharmacophore analysis based on conformationally constrained compounds.[26,35–39] While the proposed models agreed that MLT side chain is oriented perpendicularly to the indole ring, adopting a $\tau 1$ *gauche* disposition, two values have been proposed for $\tau 2$, corresponding to an *anti* (*a*) or a *gauche* (*g*) arrangement, leading to an extended or a folded conformation of MLT, respectively. The $\tau 2$ *a* conformation has been later supported by the synthesis of melatoninergic compounds having the ethyl side chain constrained in a 1,2-*trans*-substituted cyclopropyl ring.[40] However, no information could be obtained about $\tau 3$, due to free rotation of the amide function in all compounds examined for pharmacophore analysis. Therefore, three different values were proposed for $\tau 3$ (∼±90° and ∼180°), corresponding to molecular mechanics energy minima.[36,26] Besides the uncertainty on $\tau 3$, another still unsettled question was that of pharmacophore chirality. In fact, the spatial dispositions of the pharmacophore elements can be organized into pairs of mirror models, obtained either from specular conformations of flexible compounds or from enantiomers of chiral compounds. Each MLT conformation in a pair of specular pharmacophore models can be characterized by the value of its $\tau 1$ torsion angle, which can adopt a g^+ or a g^- arrangement, respectively. Looking at the indole ring with its benzene moiety on the left, the side chain of MLT is oriented over the plane of the indole in the g^+ models and under the plane in the g^- ones.

To solve this ambiguity, conformationally constrained chiral ligands, endowed with appropriate enantioselectivity, are needed. Some chiral MLT receptor agonists, with known absolute stereochemistry, have been reported in the literature; they comprise indane derivatives[18,41] such as Ramelteon (TAK-375, **3** in Fig. 3 and Table 3), indanyl piperazines (**4**),[42] benzoxazole and benzofuran derivatives (**5**),[43] aminopyrrolidines (**6**),[44] tetrahydrocarbazoles (**7**),[45,32] and tetralins (**8**).[36] The chiral centres in these compounds correspond to different atoms of MLT: C3 for compounds **3**, **4**, and **5**, Cα for compounds **6** and **8**, and Cβ for compounds **5** and **7**. Superimposing their 3D models in different conformations, fulfilling the requirements of previously defined pharmacophore models,[26,36] we found that none of them could clearly discriminate between the two specular models. In spite of the high potency and stereoselectivity of **3**, in this compound as **4** and **7**, the amide side chain is attached



(*S*)-**3**
TAK-375

(*R*)-**4**

(1*R*,2*R*)-**5**

(*R*)-**6**

(*S*)-**7**

(*S*)-**8**

Figure 3. Chiral melatonin receptor agonists.

Table 3. Binding affinity (nM) of chiral melatonin receptor agonists

Compound	Stereochemistry	K_i MT$_1$[a]	K_i MT$_2$[a]	K_i[b]
3	*S*	0.014	0.112	
	R	7.31		
4	*R*	200[c]	1.7[c]	
	S	>1000[c]	>1000[c]	
5	1*R*,2*R*	2.6	10	
6	*R*	0.65	0.42	
	S	7.0	2.1	
7	*S*			0.372[d]
	R			48.4[d]
8	*S*			17.3[e]
	R			3881[e]

[a] Measured on receptors expressed in recombinant cells.

[b] Measured on receptors expressed in tissues.

[c] IC_{50} value.

[d] Chicken brain membranes.

[e] Chicken retinal membranes.

to a tetrahedral atom bound to a planar aromatic carbon and it can adopt pseudo-equatorial or pseudo-axial conformations. The pseudo-equatorial ones allowed good superpositions of both the enantiomers of **3**, **4**, and **7** to both the specular conformations of MLT, although slightly better fittings to the τ1 g^+ or g^- arrangement could be observed, also depending on the value selected for τ3. As regards **5** and **6**, none of them could provide a clear discrimination between g^+ or g^- models. In fact, the stereoselectivity of compound **5** has not been clearly stated, even if the (1*R*,2*R*) isomer has been reported as the eutomer.[43b] The stereoselectivity of compound **6**, on the other hand, is limited and dependent on chemical modulation, as its amide derivatives showed a reverse stereoselectivity.[44] Moreover, these compounds suffer the same uncertainty as those previously cited, having the first rotatable bond of the side chain on the same plane as the aromatic ring. Compound **8**, with the amide function in equatorial conformation, is a nearly planar molecule too, giving a good fit to MLT in both the g^+ and g^- conformations.

In this context, the biological and structural information reported for compound **2** provides important new insights into the problem of pharmacophore chirality. Binding studies revealed the (*S*)-enantiomer as the more potent one, with greater stereoselectivity over the (*R*)-isomer. It was observed that in the crystal state the C3 side chain is in axial conformation and NMR studies showed that this conformation is exclusively present also in solution, the equatorial arrangement being hampered by the steric hindrance of the substituent at the C2 indole atom.[27] Thus, given the axial disposition of the acetylaminomethyl side chain, compound **2** allows to discriminate between specular pharmacophore models, reproducing two opposite out-of-plane conformations of τ1 by its two enantiomers. The highest affinity of (*S*)-**2** strongly supports the g^- conformation for τ1 of MLT as the active one.

In Figure 4, (*S*)-**2** is superimposed to MLT in its $\tau 1g^-$/$\tau 2a$ conformation, with τ3 fixed at 180°. The conformation selected for (*S*)-**2** is different from that found in the crystal, where the hydrogen bond between the amide NH and the ethoxycarbonyl group at C2 induces a g^- conformation for τ5. Actually, in $CDCl_3$ solution, NMR detects approximately equal amounts of both *g* and *a* conformations for τ5. Considering the high melatoninergic binding affinity of the constrained *trans*-cyclopropyl derivatives,[40] we set the torsion angle τ5, corresponding to τ2 for MLT, in its *anti* arrangement.

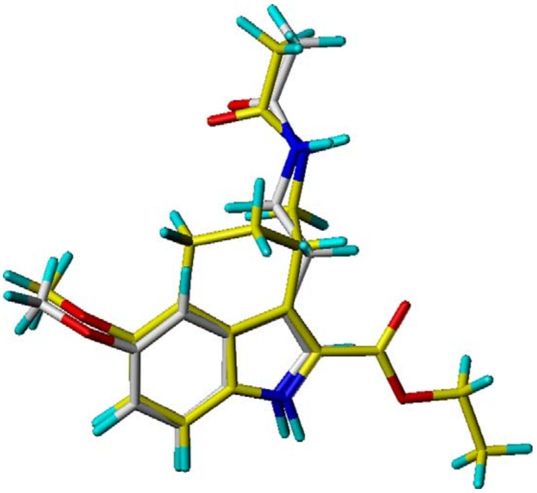


Figure 4. Superposition of MLT (white carbons) and (*S*)-**2** (yellow carbons). MLT: $\tau1 = 288°$, $\tau2 = 180°$ and $\tau3 = 180°$.

Additional comments on the comparison between our findings and previously reported data are needed. The (*S*)-enantiomer of the tetrahydrocarbazole agonist **7** has been indicated as the eutomer, based on the binding to chicken brain membrane receptors; a crystal structure has also been determined for a derivative of this compound, showing a pseudo-axial disposition of the acetylaminomethyl chain (Cambridge Structural Database[46] reference code: SEGVIV).[45,32] This finding is in contrast with our data, as it indicates the τ1 g^+ conformation of MLT as the active one. However, it should be considered that the enantioselectivity for this compound should be re-evaluated on cloned receptor subtypes. Moreover, while our hypothesis is based on experimental evidences of the axial conformation of **2** both in solution and solid state, the arrangement of the side chain of **7** has not been determined in solution, where a pseudo-equatorial orientation is most probable since this compound does not have the side chains interaction present in compound **2**: in this conformation, both enantiomers of **7** could be superimposed with a good fitting to the same conformation of MLT, leading to the above-mentioned uncertainty.

A crystal structure has also been determined for another unconstrained MLT receptor ligand (*N*-[2-(7-fluoro,1,2,3,4-tetrahydronaphthalen-1-yl)ethyl]acetamide), whose intrinsic activity, however, has not been reported. Its side chain adopts a pseudo-axial disposition (CSD reference code: PUXQOO)[47] and the more potent (*S*)-enantiomer can be easily superposed on the τ1 g^- conformation of MLT. This spatial arrangement of the τ1 g^- conformation of MLT is also consistent with the

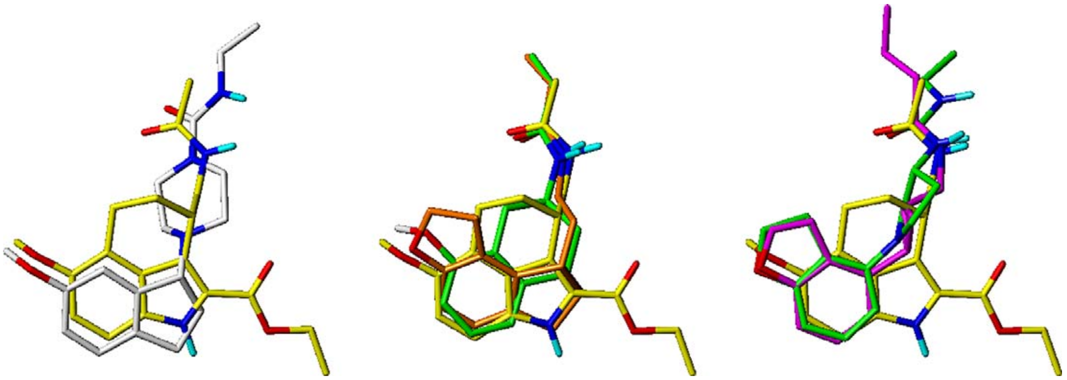


Figure 5. Left: Superposition of (*S*)-**2** (yellow carbons) and (*R*)-**4** (white carbons); middle: superposition of (*S*)-**2** (yellow carbons), (*S*)-**3** (orange carbons), and (*S*)-**8** (green carbons); right: superposition of (*S*)-**2** (yellow carbons), (1*R*,2*R*)-**5** (violet carbons), and (*R*)-**6** (green carbons). The phenylbutyl side chain of compound **5** is not shown. Only polar hydrogens are depicted.

higher affinity observed for the (*S*)-enantiomer of compounds **3** and **8**, and for the (*R*)-enantiomer of compound **4** (Fig. 5). The proposed conformation of (*S*)-**2** can also be easily superposed to the agonists (1*R*,2*R*)-**5** and (*R*)-**6** (Fig. 5), thus supporting the hypothesis that the $\tau1$ g^- is the active conformation of MLT at its membrane receptors.

3. Conclusions

The results obtained in the present paper can be summarized as follows.

The racemic mixture of **2** was resolved by MPLC on triacethylcellulose chiral stationary phase and the absolute configuration of the (−)-**2** enantiomer was determined as *R* by X-ray diffraction.

The pharmacological assays, performed on cells expressing cloned human MT_1 and MT_2 receptors, revealed the (+)-(*S*) enantiomer of **2** as the eutomer, with about 500 times greater affinity for both MLT receptors relative to its isomer. Moreover, compound (+)-**2** resulted a full agonist, while the values of relative intrinsic activity for (−)-**2** (0.396 and 0.283 on MT_1 and MT_2, respectively) indicated a reduced ability to activate MLT receptors.

The diffractometry and NMR data showed that the side chain at C3 is axially oriented, both in the crystal state and in solution. Moreover, concerning the conformation around $\tau5$, it resulted that the *gauche* form observed in the crystal state is present in solution as the major rotamer, together with a significant amount of the *anti* form.

On the basis of the experimental data obtained from **2**, molecular modeling studies allowed to refine the MLT receptor pharmacophore model for agonist compounds, solving the ambiguity of pharmacophore chirality and proposing the $\tau1$ g^- model as the active one. This model provides good superpositions for chiral enantioselective agonists reported in the literature.

4. Experimental

4.1. General methods

The melatonin constrained analogue **2** was synthesized in racemic form as already described.[26] Tri-acetylcellulose (TAC), with a particle size of 15–25 µm, was purchased from Merk (Darmstadt, Germany). Analytical grade 96% EtOH was purchased from Carlo Erba. ^1H NMR spectrum was measured in $CDCl_3$ with a Bruker Avance 500 spectrometer. The spectrum was analyzed by using the Bruker program DAISY. The two radioligands 2-[^{125}I]iodomelatonin (specific activity, 2000 Ci/mmol) and [^{35}S]GTPγS ([^{35}S]guanosine-5′-*O*-(3-thiotriphosphate); specific activity, 1000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Italy).

4.2. Preparative enantiomeric separation

A Büchi (Switzerland) borosilicate column, 3.3, with a length of 40 cm and an internal diameter of 3.0 cm was filled with a slurry of 75 g of TAC in 96% EtOH and packed following the procedure described by Copinga et al.[29]

The TAC column was conditioned with $EtOH/H_2O$ 46:54 at a flow rate of 1.5 ml/min. 70 mg of pure racemic mixture was dissolved in 1.5 ml of EtOH and the solution was loaded onto the stationary phase with a pipette washing the wall of the column three times with 1 ml of eluent ($EtOH/H_2O$ 46:54). The mobile phase ($EtOH/H_2O$ 46:54) was then eluted at a flow rate of 1.5 ml/min, corresponding to a pressure of about 10 bar.

The first enantiomer to be eluted was the levorotatory one (about 3.5 h of retention time considering a dead time (t_0) of approximately 1.5 h). The dextrorotatory isomer reached a peak level after about 4.6 h. The solvent delivery system was a Gilson 307 pump set at a flow rate of 1.5 ml/min. Detection at 254 nm was carried out using a Waters Lamba-Max Model 480 tunable absorbance detector. The chromatograms were registered with a Linseis recorder (05.80 L) (range AUFS 2.0×20 mV). Fractions were collected using a Gilson FC 2038 fraction collector (6 min/tube draining 180).

Enantiomer enriched fractions were collected into four different samples on the base of the chromatogram and of their optical rotation value. Each sample was filtered through a Millipore filter (type HA 0.45 μm), concentrated under reduced pressure, and recycled. The two almost enantiomerically pure samples of external fractions, which yielded 12 mg of the levorotatory enantiomer and 9 mg of the dextrorotatory one, were recycled one time, while the two central samples needed to be recycled twice. Enantiomerically pure fractions were collected, filtered, concentrated under reduced pressure, and recrystallized from CH_2Cl_2/cyclohexane to be used in pharmacological assays and the other analytical procedures. A sample of the levorotatory isomer was recrystallized by slow evaporation of a CH_2Cl_2/cyclohexane solution and its crystals used for X-ray diffraction. In order to check for enantiomeric purity, analytical runs were performed under the same conditions and with the same column used for preparative separation. The chromatograms showed a single peak pattern for each enantiomer. Optical properties: (−)**2** $[\alpha]_D^{25}$ −54.0 (*c* 0.05%, EtOH), (+)**2** $[\alpha]_D^{25}$ +54.5 (*c* 0.044%, EtOH); $\varepsilon_{301} = 19662.72 \pm 519$.

4.3. Pharmacology

4.3.1. Receptor binding experiments. Binding affinities of compounds were determined using 2-[^{125}I]iodomelatonin as the labeled ligand in competition experiments on cloned human MT_1 and MT_2 receptors expressed in NIH3T3 rat fibroblast cells. The characterization of NIH3T3-MT_1 and MT_2 cells has already been described in detail.[48,49] Membranes were incubated for 90 min at 37 °C in binding buffer (Tris/HCl 50 mM, pH 7.4). The final membrane concentration was 5–10 μg of protein per tube. The membrane protein level was determined in accordance with a previously reported method.[50] 2-[^{125}I]Iodomelatonin (100 pM) and different concentrations of the new compounds were incubated with the receptor preparation for 90 min at 37 °C. Nonspecific binding was assessed with 10 μM MLT; IC_{50} values were determined by nonlinear fitting strategies with the program PRISM (GraphPad SoftWare Inc., San Diego, CA). The K_i values were calculated from the IC_{50} values in accordance with the Cheng–Prusoff equation.[51] The K_i values are means of at least three independent determinations performed in duplicate.

4.3.2. Intrinsic activity determination. The intrinsic activity of the compounds was evaluated on [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) binding in NIH3T3 cells stably transfected with human MT_1 or MT_2 receptors as previously described.[48,49,52] Nonspecific binding was defined using GTPγS (10 μM). In cell lines expressing human MT_1 or MT_2 receptors, MLT produced a concentration-dependent stimulation of basal [^{35}S]GTPγS binding. The maximal G-protein activation was measured in each experiment by using MLT (100 nM). Full agonists increased the basal [^{35}S]GTPγS binding in a concentration-dependent manner, like the natural ligand MLT, whereas partial agonists increased it to a much lesser extent than that of MLT and antagonists are without effect. Compounds were added at three different concentrations (one concentration was equivalent to 100 nM MLT, a second one 10 times smaller, and a third one 10 times larger), and the percent stimulation above basal was determined. All of the measurements were performed in triplicate. The relative intrinsic activity values (IAr) were obtained by dividing the maximum ligand-induced stimulation of [^{35}S]GTPγS binding by that of MLT, as measured in the same experiment.

4.4. X-ray analysis

The structure of compound **2** was unambiguously confirmed by X-ray crystallographic analysis using synchrotron radiation data, collected at ELETTRA (XRD-1 beam line), Trieste (Italy), $\lambda = 0.8000$ Å. A marCCD detector (marUSA Inc., USA) and focusing optics were employed for the measurements. 70 images were collected at 100 K and a 3° oscillation range was used for all images. The degree of linear polarization was assumed to be 0.95 and the mosaic spread of the crystal was estimated to be 0.61°.

Raw data were indexed, integrated, scaled, and reduced using the HKL[53] and the CCP4[54] packages. Colorless needle-like crystals (25μm × 30μm × 220 μm) belong to the orthorhombic system with unit cell: $a = 12.149$ Å, $b = 18.967$ Å, $c = 22.659$ Å, space group $P2_12_12_1$, $Z = 12$, $\mu = 0.090\ \text{mm}^{-1}$ and $Dc = 1.261\ \text{g cm}^{-3}$. The intensity data were merged to give 12,811 unique reflections, merging $R = 0.046$, of which 9464 with $I \geqslant 2.0\sigma(I)$.

The structure was solved by direct methods. All nonhydrogen atoms were refined with individual anisotropic thermal parameters. Difference Fourier syntheses, using only data with $\sin\theta/\lambda \leqslant 0.5$ Å^{-1}, showed all H-atoms in configurationally feasible positions. H atoms were refined as riding atoms with the relative isotropic parameters. The final refinement, based on F^2 (all data), gave $R_1 = 0.0985$, $wR_2 = 0.2476$, $S = 1.036$, highest peak and deepest hole 0.54 and −0.21 e Å^{-3}.

All calculations were performed with the SHELX-97 program package.[55] The illustration was made with the PLATON program.[56]

Crystallographic data (excluding structure factors) for compound **2** were deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 283848. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1 EZ, UK [Fax: +44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

4.5. Molecular modeling

Molecular modeling studies were performed with Sybyl 7.0,[57] running on a Silicon Graphics O2 workstation. Minimum energy conformations of each molecule were obtained with the Tripos force field,[58] with the Powell method[59] to an energy gradient of 0.01 kcal (mol Å)$^{-1}$, with Gasteiger–Marsili charges. The force field is able to reproduce the geometry of the crystal structure with a RMS distance of 0.34 Å calculated on heavy atoms. The acetylaminomethyl side chain of compound **2**, with $\tau 5$ in *anti* conformation, was characterized by two minimum energy values for $\tau 6$, corresponding to 72° and 257°. Agonists were aligned on MLT, taken as the reference compound in its putative bioactive conformation ($\tau 1 = 288°$, $\tau 2 = 180°$, and $\tau 3 = 180°$), corresponding to our pharmacophore model B described in ref. 26. The alignment was performed by means of a rigid fit procedure, superimposing the four atoms of the amide function (only the carbonyl group for compound **4**), the centroid of the benzene portion of the indole ring, and the oxygen atom of the alkoxy group to those of MLT.

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