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The High Affinity Melatonin Binding Site Probed with Conformationally Restricted Ligands—I. Pharmacophore and Minireceptor Models

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Abstract—The affinities of enantiomers of conformationally restricted melatonin analogues for the ML-1 and ML-2 putative melatonin receptor subtypes are reported. Most ligands exhibited reversed stereoselectivity when competing with ^{125}I 2-iodomelatonin binding to chicken retinal (ML-1) and hamster brain (ML-2) membranes, further supporting the biochemical and pharmacological differences reported for these two sites. Based on the data for the ML-1 site and thorough conformational analyses of several ligands, two pharmacophore models were derived using the program APOLLO. The pharmacophoric elements included were putative receptor points from the amide NH, the amide CO, and the methoxy-O, together with the normal through the phenyl ring. The large drop in ML-1 affinity observed for 4-methoxy-2-acetamido-indan (**6a**) could not be explained from either of these models. Minireceptors were subsequently built around the two pharmacophores using Yak. Analysis of the resulting ligand–minireceptor interactions offered an explanation for the low affinity of **6a** and allowed one of the pharmacophore models to be selected for use in future drug design. Copyright © 1996 Elsevier Science Ltd

Introduction

The determination of the primary amino acid sequence of a melatonin receptor[1,2] can be considered a first step towards understanding the interaction of ligands with this binding site. However, in the absence of experimental data concerning the 3-D structure of the receptor or the actual ligand–receptor interaction, the development of a model to describe the binding mode of ligands still relies on a thorough analysis of the available ligands. With respect to the melatonin binding site, classical structure–activity relationship studies have shown the importance of the amide functionality and the methoxy moiety for affinity and agonistic activity (see Fig. 1 for the structure of melatonin).[3–8]

The availability of conformationally restricted ligands, in which the methoxy and amide moieties have a (relatively) fixed 3-D relationship, would allow probing the binding site for acceptable positions of these pharmacophoric groups. Series of such ligands have been developed, but until now only racemic mixtures were tested for affinity towards the melatonin binding site.[9,10] In this study, the binding affinity for a series of enantiomers was determined and these data, together with conformational analyses of the ligands **1**, **5**, and **6** (Fig. 1), were used to develop pharmacophore models. Since the affinities of the rigidified ligands were lower than the affinity of the natural ligand melatonin, the previously described ligands **7**[11,12] and **8**[10] (Fig. 1) were also considered; the higher affinities of these compounds coincide with a higher degree of conformational freedom. Ligand **9**[10] (Fig. 1) was included since it represents yet another restricted framework.

In order to study intermolecular interactions between these ligands and putative active site residues and to verify the pharmacophore models, minireceptors were generated in the minireceptor/pseudoreceptor modeling program Yak.[13–15] This program allows for the construction of a peptidic mini- or pseudoreceptor around any molecular ensemble of interest (like a pharmacophore). A minireceptor consists of an unconnected set of residues interacting with this ensemble; in a pseudoreceptor, a fully-linked binding pocket is created. The construction of a binding pocket in Yak relies upon the directionality of ligand–receptor interactions. This directionality is used to identify ligand functional groups relevant for receptor binding, to position and orient residues in 3-D space and to optimize the ligand–minireceptor complex.

Key words: melatonin, receptor, modeling, pharmacophore, minireceptor.

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Methods

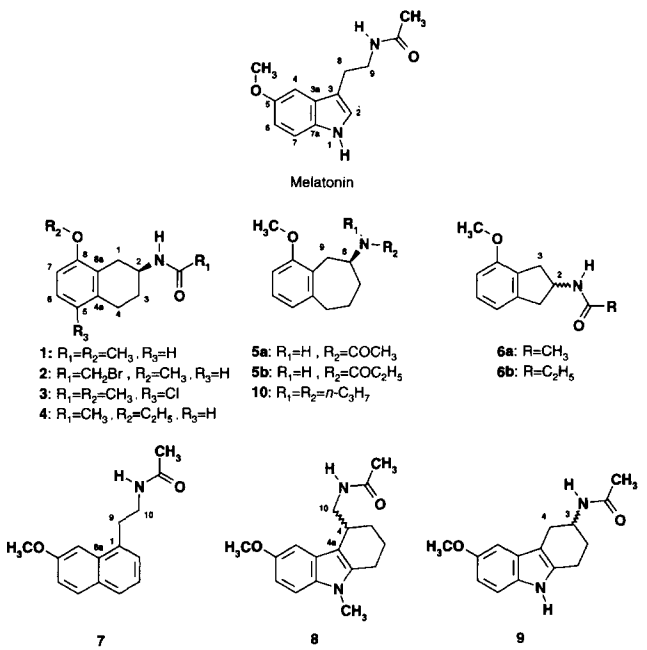
Receptor binding

The enantiomers were obtained by semipreparative enantiomeric separation on triacetylcellulose columns as described elsewhere,[16] or by synthesis from chiral precursors in the case of **1**.[17] Melatonin was shown to bind to two pharmacologically distinct receptors, the ML-1 and ML-2 subtypes.[18,19] The affinities (K_i) of the enantiomers for the ML-1 melatonin receptor were evaluated by competition with ^{125}I 2-iodomelatonin binding to chicken retinal membranes. Experiments were performed as described by Dubocovich and Takahashi,[20] with the exception that the samples were incubated at 25 °C during 1 h. The affinity of the enantiomers for the ML-2 binding site was determined by competition with ^{125}I 2-iodomelatonin binding to hamster brain membranes.[21] The K_i values were calculated by the method of Cheng and Prusoff from IC_{50} values obtained from competition curves fitted using the INPLOT program.[22]

Conformational analyses

Conformational analyses were performed within MacroModel version 4.5[23] using the MM2* force field and the Monte Carlo (MC) search protocol,[24] essentially as described previously.[25] In short, starting structures were built from standard fragments in MacroModel and minimized. To search conformational space, 1000 MC steps were taken on each input structure. Starting geometries for each step were selected with the usage-directed method and the number of dihedral angles changed in each step was randomly varied between 2 and n-1 (n = total number of variable dihedral angles) or between 1 and 3 when $n = 3$ or 4. In the analyses of **1**, **5**, **6**, **8**, and **9**, a ring-opening was defined for the non-aromatic ring to allow dihedral angles within this ring to be included as variables; ring closure distances were limited to values between 0.5 and 2.5 Å. The minimization protocol consisted of a maximum of 100 steps using the Truncated Newton Conjugate Gradient minimizer, followed by the Full Matrix Newton Raphson minimizer (until convergence: 0.002 kcal/Å mol). An energy cutoff of 12 kcal/mol was applied to the search results.

Alternative sets of systematically generated conformations were obtained using the MULTIC protocol from MacroModel with a dihedral angle resolution of 15° (and the default nonbonded distance cutoff of 1.5 Å).[26] For melatonin, rotation was performed around τ_1, τ_2, and τ_N, whereas for **1**, **5a**, **6a**, and **9**, τ_N was rotated, starting from the various ring conformations identified in the MC search. Note that in all experiments described below using MULTIC sets of conformations, **5a** was used instead of **5b**, since the latter contained an additional torsion angle. The resulting conformations were stored after calculating their energies.



Compound	τ_1	τ_2	τ_N
Melatonin	C3a-C3-C8-C9	C3-C8-C9-N	C8-C9-N-CO
1			C1-C2-N-CO
5			C9-C8-N-CO
6			C3-C2-N-CO
7	C8a-C1-C9-C10	C1-C9-C10-N	C9-C10-N-CO
8		C4a-C4-C10-N	C4-C10-N-CO
9			C4-C3-N-CO

Figure 1. Structures and conformational definitions of compounds showing the configurations as used in the pharmacophore searches (both enantiomers were included for **6**, **8**, and **9**).

Pharmacophore searches

The input for the pharmacophore searches consisted of a set of conformations for each ligand from either the MC search or the MULTIC procedure. The module VECADD from the program APOLLO[27–31] was used to add extension vectors from hydrogen bond donating or accepting groups towards putative receptor points or to define the centroid and normal through a plane for each conformation in those sets. A minimum density of vectors was specified, representing the ideal position for hydrogen bonding (alternatively, the program allows for the addition of multiple vectors as a cone emanating from the atom of interest). The module RMSFIT was then used to identify those conformations of the different ligands that exhibited the best overall least-squares fit of specified points: the extension vectors from the N—H, C=O and methoxy-O, together with the top and bottom of the normal through the phenyl ring. All points were weighted equally. The two different receptor points from the carbonyl-oxygen and the methoxy-oxygen were defined as choices: the program treats all different combinations of these points, thereby recognizing that different ligands can use different lone pairs to form a hydrogen bond with the same receptor point. When fitting the normal through the phenyl ring, two entries were included in which both the top and bottom of the normal were defined as choices. This allows the top of the normal of one ligand to match the bottom of the normal of another ligand. The energies of the conformations were used together with the root mean square

(rms) deviations to score the matches: high scores correspond to low-energy conformations in a match with low rms deviations. In cases where geometrical differences between matches were small (when using systematically generated MULTIC sets), matches were selected based on this score; specific matches were extracted using the MMDFIT module.

Minireceptor generation

Conformations identified from the pharmacophore searches in APOLLO were transferred to Yak V3.8-GL[32] after calculating atomic charges by a single-point AM1 calculation[33] (MOPAC program package version 5.0, accessed through Sybyl[34]). K_i values were used to estimate free energies of binding and the program ESOLV (supplied with Yak and based on an algorithm developed by Still et al.[35]) produced free energies of ligand solvation. These two values are used by Yak to derive mapping weights for the ligands which are used to guide the placement of the residues. In the minireceptor approach an attempt is made to generate a minireceptor in which the differences in internal binding energy of a series of ligands with the minireceptor residues, corrected for the free energy of solvation, correspond to the observed differences in free energy of binding.

Melatonin was used as a primary ligand, determining the initial placement of selected residues. Addition of a residue to the endpoint of a vector, originating from a selected anchorpoint of the primary ligand, was followed by global rotation: a rigid body rotation of the residue around the new interaction in 15° increments. The position with the lowest total energy was selected. Subsequently, side-chain relaxation was performed: a systematic search in 5° increments with respect to the side-chain torsion angles of the residue (leaving the interaction intact). The dihedral angles yielding the lowest energies were selected. This step was skipped in cases where bad steric interactions between the residue interaction point and (some of) the ligands were observed and alternatively, all rotamers available in Yak (from the Ponder/Richards side-chain rotamer library) were individually examined. A final step consisted of an energy minimization of the whole residue with respect to translational, rotational, and torsional (side-chain) degrees of freedom. When different rotamers were examined or the residue contained multiple interaction sites, the rotamer/site that yielded the strongest interactions with the ligands was selected.

Results and Discussion

Receptor binding

The results of the receptor binding experiments are shown in Table 1. Like melatonin, the conformationally restricted compounds **1–6** had a lower affinity for the ML-2 sites, as compared with the ML-1 sites. It is especially noteworthy that for all compounds except **5b** the stereoselectivity for the ML-1 and ML-2 sites was reversed. This reversed stereoselectivity further supports the contention that ^{125}I 2-iodomelatonin binds to two biochemically and pharmacologically distinct sites in the chicken retina (ML-1) and hamster brain (ML-2).[18,19] Furthermore, these data suggest that melatonin interacts with these sites in two different conformations. The fact that the reversal of stereoselectivity was not observed for **5b** could be related to the fact that of the restricted analogues examined, this compound has the highest conformational flexibility; one enantiomer could therefore mimic both bioactive melatonin conformations. In the remainder, only the interaction of ligands with the ML-1 sites will be considered since the functional role of the ML-2 sites is not yet clear.[18,19]

Absolute configuration

The absolute configuration of the enantiomers of **1** has been determined previously: (*S*)-**1** has a negative optical rotation.[17] This enantiomer also had a negative ^1L_b band in the CD spectrum and it was the first enantiomer to elute from the triacetylcellulose columns used in the semipreparative separation.[16] Likewise, the other tetralin compounds **2–4** combined a negative optical rotation with a negative ^1L_b band in the CD spectrum.[16] Together with the fact that for all four tetralins the minus enantiomer eluted first from the

Table 1. Competition of enantiomers with ^{125}I 2-iodomelatonin binding to chicken retinal (ML-1) and hamster brain (ML-2) membranes

Compound	Chicken retina K_i (nM)[a]			Hamster brain K_i (nM)[b]		
	(+)	(−)	(+)/(−)	(+)	(−)	(+)/(−)
Melatonin		0.39 ± 0.05			73 ± 7	
1	3881 ± 710	17.3 ± 1.2	224	1776 ± 161	7070 ± 559	0.3
2	143 ± 44	1.67 ± 0.50	86	1729 ± 268	9914 ± 2320	0.2
3	96.8 ± 6.6	50.4 ± 6.7	2	146 ± 22	1892 ± 180	0.1
4	367 ± 83	81.5 ± 0.3	5	1664 ± 319	15877 ± 1759	0.1
5b	180 ± 34	17.4 ± 2.9	10	21157 ± 4904	4964 ± 809	4
6b	530 ± 74	> 10000	< 0.1	7336 ± 1360	4091 ± 258	2

[a]Means ± SEM of four separate determinations performed in duplicate; melatonin and **1** were assayed 10 and three times (in duplicate), respectively.
[b]Means ± SEM of three separate determinations performed in duplicate; melatonin was assayed six times (in duplicate).

triacetylcellulose columns and exhibited highest ML-1 affinity, this strengthens the assumption that these compounds all have the *S*-stereochemistry associated with a negative optical rotation. In the following, only the *S*-enantiomer of **1** was considered.

Replacement of the acetamido side-chain in (*S*)-**1** with an *N,N*-dipropylamine did not alter the sign of the optical rotation, nor the sign of the ^1L_b band.[36] With respect to **5b**, it was observed that the analogue (*S*)-**10** had a negative optical rotation and showed a negative ^1L_b band in the CD spectrum.[37] Since (−)**5b** also showed a negative ^1L_b band in the CD spectrum,[16] this compound could be expected to have the *S*-stereochemistry. It should also be noted that (−)-**5b** eluted first from the triacetylcellulose columns and had a higher affinity for the ML-1 sites than the corresponding plus enantiomer; this makes it likely that the stereochemistry of (−)-**5b** is comparable to that of (−)-**1**. It was therefore decided to only use the *S*-enantiomers of **5a** and **5b** in the pharmacophore searches.

The stereochemistry of **6b** is less easily deduced since in this case the plus enantiomer eluted first from the triacetylcellulose columns and exhibited highest affinity for the ML-1 sites. This makes it tempting to assume that (+)-**6b** has the same sense of stereochemistry as (−)-**1** and (−)-**5b**, namely *S*. It was decided to include both enantiomers in the development of a pharmacophore.

As no information was available on the stereoselectivity of **8** and **9**, both enantiomers of these ligands were included in the analyses below.

Methoxy group orientation

In all conformational analyses, the dihedral angle defining the orientation of the aromatic methoxy group was allowed to vary. For melatonin, minimum energy conformations were identified with C4—C5—O—C at both 180 and 0°. For compounds **5** some conformations were identified in which the methoxy methyl group was oriented perpendicular to the aromatic ring plane. Also, for **1** and **5** one conformer was identified, in which the methoxy methyl group pointed towards the nonaromatic ring; this orientation was observed in several conformations identified for **6**. The conformations with the methoxy methyl group perpendicular to the aromatic ring, or pointing towards the nonaromatic ring, all had higher energies.

It was previously shown that affinity for the melatonin binding site increased with substitution on melatonin position 6 with Cl;[5,20] a similar Cl substitution on position 7 of compound **1** resulted in a fivefold decrease in affinity.[17] These observations can be explained by the effect of the Cl substitution on the orientation of the methoxy group. In order to determine the rotation barrier and the location of the minima for the dihedral angle τ_{OCH3}, $C_{aromatic}$—$C_{aromatic}$—O—C, AM1-calculations[33] defining this dihedral angle as a reaction coordinate were performed (MOPAC program package version 5.0, accessed through Sybyl[34]); model compounds without the amide side-chain were used (**11–13**).

The results in Figure 2 show that the Cl substituent at position 6 of the indole **11** forces the methoxy group into 0° or ±100° orientations. Due to the proximity of Cl, the plot of the methoxy-substituted tetralin **12** shows a maximum at the 0° orientation (5.9 kcal/mol higher in energy) and a preference for the planar 180° orientation. With an adjacent Cl substituent, the methoxy methyl group of the tetralin **13** is forced out of the plane of the aromatic ring and prefers a perpendicular orientation. Since in the other active compounds the methoxy group can easily assume planar orientations and since the binding site does seem to have room for bulky substituents next to the methoxy group, a likely explanation for the drop in affinity for 7Cl-**1** is the fact that the methoxy methyl group is forced to adopt an unfavorable orientation.

Based on these observations, the output from the conformational searches was filtered to only retain those conformations in which the methoxy group was in the plane of the phenyl ring and oriented in a manner consistent with the above findings: melatonin C4—C5—O—C at 0° and compounds **1**, **5**, and **6** with the methoxy methyl pointing away from the nonaromatic ring (as in Fig. 1). These sets of conformations were used throughout the experiments described herein. For compounds **7–9** the output was not filtered

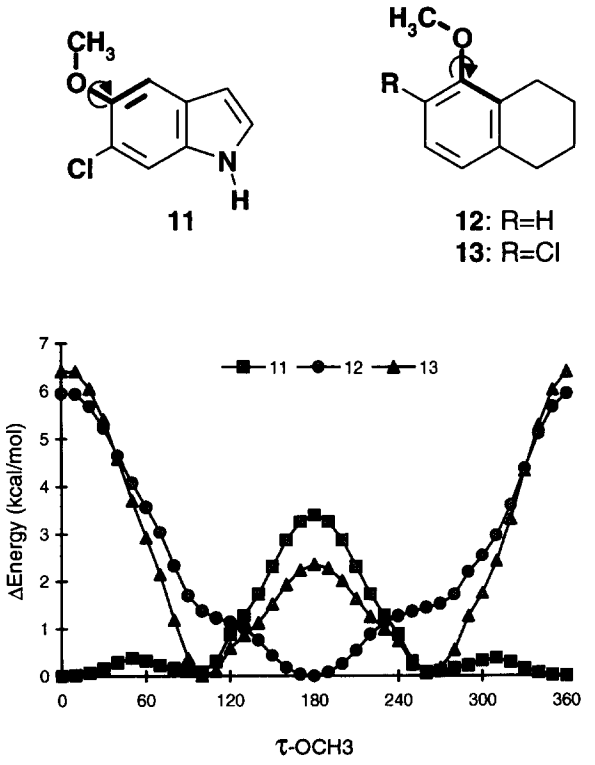


Figure 2. Barriers to rotation around the aromatic methoxy group from AM1 calculations for the disubstituted indole **11** and the tetralins **12** and **13**. The dihedral angles that were defined as reaction coordinates are represented in bold.

with respect to the methoxy group orientation, since no information is available concerning the effect of substituents next to the methoxy group. Therefore, two planar orientations of the methoxy group (τ_{OCH3} at 180 and 0°, respectively) were present for each conformation of these compounds.

Conformational analyses

The results of the MC searches with respect to the number of minimum energy conformations, their energy window and basic conformational characteristics are summarized in Tables 2(a) and 2(b). The number of systematically generated conformations obtained from the MULTIC procedure is also listed in Table 2(a). Although conformational analyses for melatonin and **1** have been described previously,[25] MC searches were repeated in the present version of MacroModel.

In the following a new code will be used to describe the conformations of **1** and **5**: the ring conformation is mentioned (chair, boat or twist), with the orientation of the amide side-chain as superscript (ax = pseudoaxial and eq = pseudoequatorial) and the sign of τ_N as subscript (negative values correspond to the hydrogen of the chiral centre eclipsing the carbonyl-C).

Vacuum versus solvent

The MacroModel package offers the possibility to mimic the presence of solvent in a continuum model

Table 2(a). Results from Monte Carlo and MULTIC searches

Compound	Monte Carlo		MULTIC	
	No. of minimum energy conformations	ΔE[a] (kcal/mol)	No. of starting conformations	No. of allowed conformations
Melatonin	18[b]	1.5	1	9667
1	9	5.6	4	87
5a[c]	11[d]	6.3	6	120
5b[c]	33[e]	7.4		
6a	7	3.4	2	48
7	36[f]	3.6		
8	26	3.6		
9	8	3.2	4[g]	88

[a]Energy window spanned by the minimum energy conformations.
[b]Additionally, a conformation with $\tau_1/\tau_2/\tau_N$ in an *anti/anti*/eclipsed orientation ($\Delta E = 3.7$ kcal/mol) was recognized as a true minimum; this structure was identified as a saddlepoint before.[25]
[c]Monte Carlo searches were performed taking 2500 steps.
[d]In addition, a conformation with a $\Delta E = 9.9$ kcal/mol was found, which has a distorted, twist-like conformation of its nonaromatic ring.
[e]The conformations identified for **5a** are now found in triplicate due to the +/− *gauche* and *anti* behavior of the propionamide side-chain torsion N—C(=O)—C—C; again (3) distorted twist-like ring structures were found, having ΔE values between 10.6 and 11.0 kcal/mol.
[f]Additionally, a conformation with $\tau_1/\tau_2/\tau_N$ in an *anti/anti*/eclipsed orientation ($\Delta E = 5.5$ kcal/mol) was identified as a true minimum.
[g]Two methoxy group orientations and two ring conformations.

Table 2(b). Conformational characteristics observed in MC sets

Compound	τ_1	τ_2	Nonaromatic ring[a]	τ_N[b]
Melatonin[c]	+/−*gauche*, *anti*	+/− *gauche*, *anti*	—	+/− 90°
1[c]	—	—	Chair, boat; ax/eq (4)	−120, 60°
5	—	—	Chair, boat, twist; ax/eq (6)[d]	−120, 60°
6	—	—	Envelope, ax/eq (2)	−120, 60°[e]
7	+/− 90°, *anti*[f]	+/− *gauche*, *anti*	—	+/− 90°
8	—	+/−*gauche*, *anti*	Chair, ax/eq (2)	+/− 90°
9	—	—	Chair, ax/eq (2)	−120, 60°

[a]Labels 'ax' and 'eq' denote pseudoaxial and pseudoequatorial orientations of the side-chains, respectively; the number of observed ring conformations is listed in parentheses.
[b]The behavior around τ_N for melatonin, **7** and **8** places the planar amide moiety perpendicular to the RCH—CH_2 chain. In the remaining compounds, the hydrogen at the chiral centre eclipses either the carbonyl-C (τ_N around −120°) or the N—H (τ_N around 60°).[25]
[c]See also ref 25.
[d]Comparable to the characteristics previously described for benzocycloheptene-containing compounds.[43]
[e]Two additional τ_N values were identified for the pseudoequatorial ring conformation, orienting the C2—H perpendicular to the NH (H—C2—N—H +/− 90°).
[f]The τ_1 behavior of **7** resembled the expected preference for perpendicular orientations better than it did in melatonin. Although also here an *anti* orientation was observed, these conformations had higher relative energies (ΔE values of 3 kcal/mol compared with 1 kcal/mol in melatonin). It was argued before that the deviation of melatonin in this respect might be due to the double-bond character of the bond between C2 and C3, which favors an eclipsed orientation of the attached ethyl moiety.[25] Noteworthy in this respect is also the X-ray conformation of **7**,[44] which indeed exhibits a perpendicular orientation around τ_1, in contrast to the *anti* orientation observed in the X-ray conformation of melatonin.[45–47]

when performing conformational searches and minimizations.[35] To be able to compare the results from calculations with NMR data, one would assume that the former should be obtained using a solvation model. An MC search for **1** was performed using both the chloroform and in vacuo models. From the resulting conformational manifolds, Boltzmann averaged proton–proton coupling constants were calculated using a modified Karplus equation;[38] these values are listed in Table 3. The NMR characteristics of **1** were reported previously and the proton–proton coupling constants from the chloroform experiment are also included in Table 3. From this table it appears that neither of the two sets of conformations gave a close correlation with the measured coupling constants. One reason for this might be that the previously described intramolecular interaction between the amide NH and the aromatic system[25] perturbs the energy calculations, also when a solvent environment is mimicked by a continuum model.

The geometries of the conformations identified in both the in vacuo and the chloroform searches were the same with the exception of the boat$^{ax}_{+}$, which was only identified in the in vacuo search. The absolute and relative energies of the conformations differed but the ordering of the first three geometries was the same. Since the chloroform solvation model did not enhance the correlation between the calculations and the NMR data and, since in the in vacuo calculations more minimum energy conformations were identified, it seemed justified to continue working with conformations minimized in vacuo.

Pharmacophore searches

Extension vectors from the N—H, C=O and methoxy-O, together with the top and bottom of the normal through the phenyl ring, were used to identify good matches for the MC sets of conformations for melatonin, **1** and **5b**. For the methoxy-O, extension vectors in the direction of the sp^3 orbitals were used; the sp^2 orbitals did not yield any good matches due to the fact that the methyl groups for melatonin and **1** and **5** were pointing in opposite directions (see discussion on methoxy group orientation above). Searches were performed by stepwise increasing the rms cutoff value and the first matches occurred at a cutoff of 0.7 Å. These used the chair$^{ax}_{-}$ conformation of **1** and the twist$^{ax}_{-}$ conformation of **5b**; only one melatonin conformation was used: $\tau_1/\tau_2/\tau_N = gauche/\text{-}gauche/-90°$, conformation *1*. When going up to an allowed cutoff of 1.0 Å, a total of two minimum energy melatonin conformations were found to match the conformationally restricted compounds **1** and **5b**. Melatonin conformation *1* matched chair$^{ax}_{-}$ and boat$^{ax}_{-}$ of **1** and twist$^{ax}_{-}$ and chair$^{ax}_{-}$ of **5b**. Melatonin conformation *2* ($\tau_1/\tau_2/\tau_N = \text{-}gauche/gauche/-90°$) matched the chair$^{ax}_{-}$ and boat$^{ax}_{-}$ conformations of **1** and the chair$^{ax}_{-}$ and twist$^{ax}_{-}$ conformations of **5b**.

Including the MC conformations of **9** in the pharmacophore search resulted in only one match being identified at a 1.0 Å rms cutoff level: model A (Fig. 3). This match used melatonin conformation *1*, chair$^{ax}_{-}$ for **1** and twist$^{ax}_{-}$ for **5b**, as occurred in the best matches without **9**. The amide moieties of **1** and **5b** occupy similar regions in space that deviate from the position occupied by melatonin (Fig. 3); the amide moiety of **9** occupies yet another unique position. The structure used for **9** in this match had the *S*-configuration, a pseudoaxial orientation of the amide side-chain and the methoxy group in the opposite direction compared with melatonin. The ΔE values and rms deviations with respect to melatonin of the conformations used in this model are listed in Table 4.

It could be argued that consideration of only minimum energy conformations does not do just to the flexibility of the ligands (melatonin: τ_1, τ_2 and τ_N; **1**, **5** and **9**: τ_N) and overemphasizes orientations generated by the force field. In order to examine whether consideration of non-minimized, systematically generated conforma-

Table 3. Experimental and calculated *J* coupling constants for compound **1**

Vicinal protons[a]	NMR[b]	MacroModel conformational analysis[c]			
		In vacuo		$CHCl_3$ model	
	J (Hz)	*J* (Hz)	ΔJ (Hz)[d]	*J* (Hz)	ΔJ (Hz)[d]
1α-2β	8.0	7.1	−0.9	7.8	−0.2
1β-2β	5.5	5.0	−0.5	4.8	−0.7
3α-2β	8.5	6.1	−2.4	5.9	−2.6
3β-2β	3.0	6.3	+3.3	6.1	+3.1
3α-4α	6.2	5.3	−0.9	4.6	−1.6
3α-4β	8.6	6.1	−2.5	6.1	−2.5
3β-4α	5.9	8.6	+2.7	6.2	+0.3
3β-4β	5.9	4.2	−1.7	7.4	+1.5

[a]Numbers refer to the atom number of the carbon atom to which the hydrogen is attached; labels α and β refer to hydrogens *cis* and *trans*, respectively, to the amido group.
[b]Experiments performed in $CDCl_3$ at 298 K.
[c]Boltzmann averaged coupling constants calculated at 298 K.
[d]*J*(calculated) − *J*(experimental).

Figure 3. Pharmacophore model A including melatonin (colored by atom-type, nitrogen: blue, oxygen: red), **1** (yellow) and *S*-**8** (magenta).

tions could improve the fit and yield again matches with pseudoequatorially oriented side-chains, conformations obtained using the MULTIC protocol were subjected to pharmacophore searches. In a first run, the same points as above were matched at a 0.5 Å rms cutoff for melatonin and **1**. Only 99 melatonin conformations matched conformations of the tetralin compound; these were subsequently used in the second run to select matches with the MULTIC conformations of both **1** and **5a**. This procedure identified 34 melatonin conformations, 17 conformations of **1** and 15 of **5a**. All four ring conformations of **1** were present in good matches, the chair^{ax} yielded the best ones; chair^{ax}, boat^{eq} and twist^{ax} occurred for **5a**, the latter being present in the best matches.

The 34 melatonin geometries could be subdivided into two families: 29 conformations matched pseudoaxially oriented geometries of **1** and **5a** (chair^{ax}/boat^{ax} and chair^{ax}/twist^{ax}, respectively). The remaining five melatonin conformations matched the restricted compounds with a pseudoequatorial substituent (chair^{eq}/boat^{eq} for **1** and boat^{eq} for **5a**). However, the latter melatonin conformations had ΔE values of at least 12.9 kcal/mol. Of the set of melatonin conformations matching pseudoaxial structures, only 11 had ΔE values smaller than 10 kcal/mol (in fact between 1.5 and 6.9 kcal/mol); these could all be described by a *gauche*/-*gauche*/-90°-like specification for $\tau_1/\tau_2/\tau_N$, as melatonin conformation *1* identified above. These conformations occurred in good matches with chair^{ax}/boat^{ax} conformations of **1** and twist^{ax} conformations of **5a**. The best matches found above using minimum energy MC conformations, therefore, correspond to the ones found using the MULTIC systematically generated series and consist of melatonin conformation *1* and pseudoaxially oriented conformations of **1** (chair) and **5** (twist).

Table 4. Specifications of conformations used in pharmacophore models A and B

Compound	Model A (axial)		Model B (equatorial)	
	ΔE^a (kcal/mol)	rms[b]	ΔE^a (kcal/mol)	rms[b]
Melatonin	0.0	—	1.1	—
S-**1**	0.2	0.73	7.6	0.98
S-**5a**			20.5	0.90
S-**5b**	6.4	0.87		
S-**9**	0.2	0.87		
R-**9**			1.1	0.51
7	0.0	0.26	1.5	0.43
S-**8**	0.2	0.20	0.4	0.19
S-**6**	0.0	0.71	6.6	0.89
R-**6**			1.1	0.93

[a]Compared with the global minimum identified in the respective MC searches.
[b]rms value determined by fitting the analogues onto melatonin, using the extension vectors from the N—H, C=O and methoxy-O, together with the top and bottom of the normal through the phenyl ring.

In order to obtain matches of pseudoequatorially oriented structures that used feasible melatonin conformations, it was decided to use systematically generated MULTIC sets of conformations for the conformationally restricted compounds and minimum energy MC conformations for melatonin. Up to an allowed rms cutoff of 0.9 Å, only matches comparable to model A were identified when considering melatonin, **1**, **5a**, and **9**. An additional melatonin conformation *3* ($\tau_1/\tau_2/\tau_N$=*gauche*/*gauche*/+90°) was used, but these matches had the methoxy moieties at different positions, using for one molecule the extension vector above the plane and for another molecule the extension vector below the plane; this also caused a marked deviation in the orientation of the phenyl ring planes. A pseudoequatorially oriented match with proper location of the methoxy groups was obtained at a 1.0 Å cutoff, using melatonin conformation *4* ($\tau_1/\tau_2/\tau_N$=*gauche*/*anti*/-90°): model B (Fig. 4, Table 4).

Boat conformations were used for **1** and **5a**, whereas for **9** the *R*-configuration was selected with a pseudoequatorial amide side-chain orientation and the methoxy group in the opposite direction compared with melatonin. The amide moieties of **1** and **5b** occupy similar regions in space that deviate from the positions occupied by both melatonin and *R*-**9**. Although the relative energies of the conformations used for **1** and **5a** in this model were high, it should be realized that nonminimized conformations were used. It will be shown below that minimization of these ligands in the presence of a minireceptor relieved much of the strain.

Pharmacophore probing

Ligands **7** and **8** have K_i values for the ML-1 site comparable to melatonin and should be able to fit well into a pharmacophore model. A first check of models A and B deduced above consisted of finding comparable matches of minimum energy MC conformations of these ligands with the now postulated bioactive melatonin conformations. Both **7** and *S*-**8** overlapped very well with melatonin in either of the two models; the amide moieties occupied the same regions in space (Figs 3 and 4; Table 4).

The K_i value of racemic **6a** for the ML-1 site was >10.000 nM;[39] the K_i values for the enantiomers of the propionamide analogue **6b** listed in Table 1 were high as well. Any pharmacophore developed to explain the affinity of melatonin and compounds **1** and **5** should consider the large drop in affinity when the nonaromatic ring is contracted. However, both models A and B could also be derived with the enantiomers of **6a** included in the pharmacophore searches. Again, for model A minimum energy MC conformations were used, whereas for model B MULTIC sets were systematically generated. Low-energy conformations of **6a** were used that also had a reasonable rms deviation with respect to melatonin when compared with the other ligands (Table 4). The enantiomer selected for **6a** in model A had the *S*-configuration; both *R*- and *S*-**6a** fitted into model B.

Minireceptor generation

Based on the APOLLO generated fits, no choice could be made between the two models and neither of them could explain the large drop in affinity observed for **6**. It was therefore decided to build a minireceptor in order to be able to investigate specific intermolecular interactions. In Table 5 the values used for the free energies of binding and solvation are listed.

A preliminary homology-based model of the melatonin receptor was built[40] based on the crystal structure of BacterioRhodopsin[41] and the primary amino acid sequence of the melatonin binding site.[1] This model indicated the presence of two serine residues in helix three and one histidine residue in helix five on positions that could interact with the NH and CO of the amide moiety and the methoxy-O of melatonin, respectively. In the mammalian sequences that were published after this work was completed,[2] these residues were conserved. The residues were positioned around both model A and model B, including melatonin, **1**, **5**, **7**, **8**, and **9**, using the approach described in Materials and Methods. It should be noted that no attempt was made to mimic a helical relationship between the two serines; the position of the main-chain atoms was simply accepted as obtained through energy minimization. The epsilon tautomer was selected for the histidine residue, since it was less hindered by the deviating methyl groups of the various ligands. The resulting minireceptors were reminimized in Yak after deletion of **1**, **5**, and **9** to obtain an orientation optimized for interaction with the high affinity ligands.

Since Yak does not allow the conformations of ligands to be optimized, each ligand was minimized separately in MacroModel in the presence of the minireceptor residues. This was especially relevant for model B,

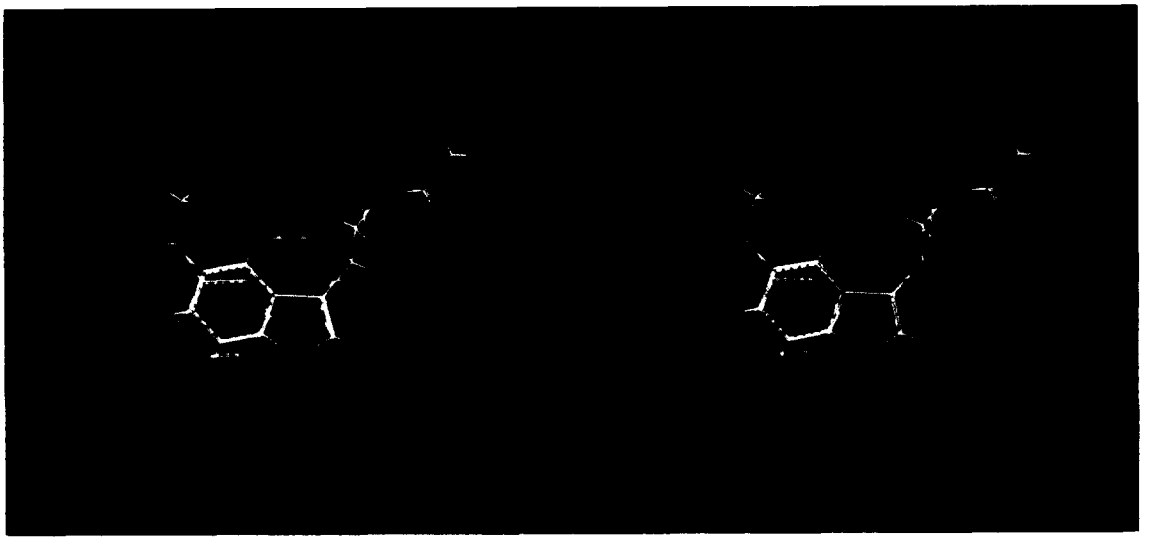


Figure 4. Pharmacophore model B including melatonin (colored by atom-type, nitrogen: blue, oxygen: red), **1** (yellow) and *S*-**8** (magenta).

where nonminimized conformations were used. The residues were constrained (force constant of 120 kcal/Å), except for the hydroxyl H and lone pairs of the two serine residues. The latter was necessary to allow ligands to interact with those residues from different positions (as was allowed in the APOLLO generated matches, see Figs 3 and 4). In these models, **6a** was minimized starting from the conformations that matched models A and B (described above). This procedure changed the orientation of the ligands and, since MacroModel is less well suited to study directionality of interactions (and Yak was specifically designed to do just that), the conformations and orientations obtained were again transferred to the minireceptor in Yak. A final minimization of the ligands in the presence of the three residues was then performed, considering translational and rotational degrees of freedom of the ligands only. In cases where a serine hydroxyl-H had rotated in MacroModel, the same rotation (dihedral C—C—O—H) was applied to the minireceptor in Yak.

Table 5. Free energies of binding and solvation

Compound	$\Delta G°$[a] (kcal/mol)	$\Delta G°$(solv.) model A[b] (kcal/mol)	$\Delta G°$(solv.) model B[b] (kcal/mol)
Melatonin	−12.8	−7.8	−7.3
7	−12.8[c]	−5.5	−5.3
S-**8**	−12.7[d]	−8.0	−7.6
S-**1**	−10.6	−4.7	−4.6
S-**5**[e]	−10.6	−4.1	−4.2
9	−9.5[d]	−7.3	−7.2

[a]$\Delta G°$ was derived from K_i values using $\Delta G° = -RT\ln K$ ($T = 298$ K).
[b]Free energies of solvation from the program ESOLV.
[c]K_i value reported[11] corrected for 4.3-fold higher K_i value of melatonin in present study.
[d]Racemic K_i values reported[10] divided by two.
[e]For both models A and B the free energy of binding was based on the K_i of *S*-**5b**.

Figure 5 shows the result for the minireceptor from model A and optimized ligands melatonin, **1** and *S*-**6a**. It is clear that **1** and *S*-**6a** overlapped perfectly and could reach the minireceptor residues equally well. The strength of the hydrogen bonds formed by *S*-**6a** was even higher than the strength of those formed by **1** (with histidine-H, serine-O and serine-H: −1.4, −3.6 and −4.4 kcal/mol versus −0.8, −2.8 and −3.7 kcal/mol, respectively).

The results for model B are visualized in Figure 6; H-bond strengths and relative energies of all optimized ligands are given in Table 6. With respect to the orientation of *R*-**6a** in this minireceptor, two possibilities were observed. During the procedure outlined above, orientation I was obtained, in which the carbonyl of *R*-**6a** interacted with the serine-H as melatonin did. The NH of *R*-**6a** in this case was located between the N—H of melatonin and the N—H of **1**; the methoxy-O was displaced, compared with both melatonin and **1** [Fig. 6(a)]. Rotation of the serine C—C—O—H interacting with the carbonyl to mimic the orientation of the minireceptor of **1** prior to the MacroModel minimization of *R*-**6a** resulted in orientation II [Fig. 6(b)]. The amide moieties of **1** and *R*-**6a** overlapped well, but again the methoxy-O was in a different location. Also, the phenyl ring of *R*-**6a** had rotated significantly away from the melatonin orientation, occupying a substantial additional volume. Including *S*-**6a** in the minireceptor resulted in an orientation of the amide between the

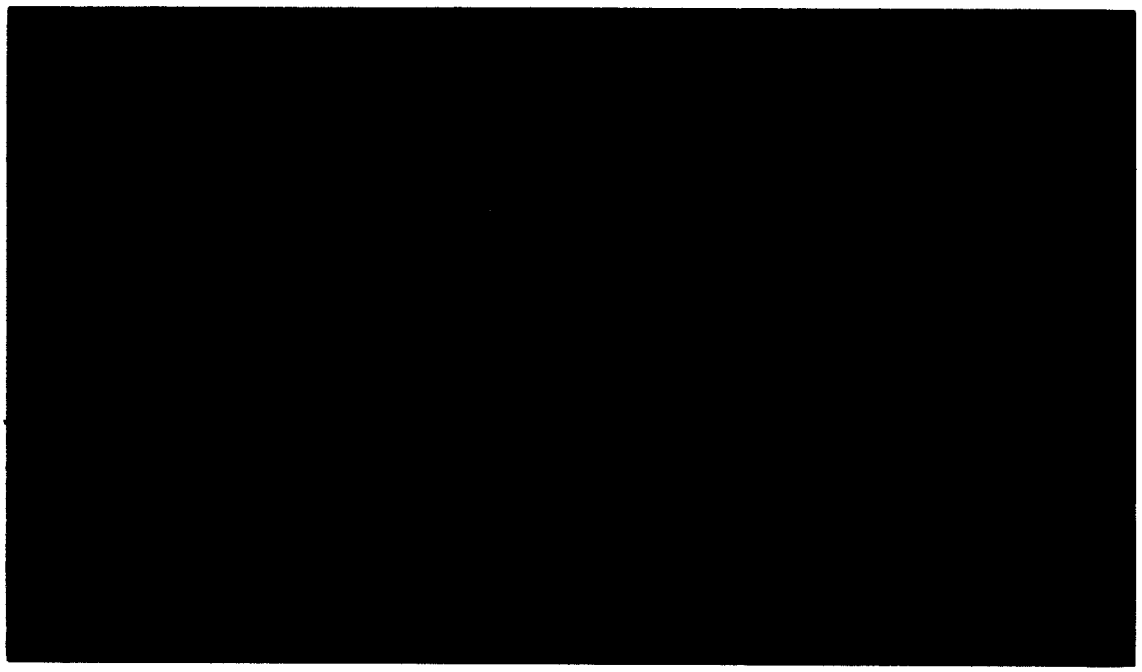


Figure 5. Minireceptor derived from pharmacophore model A including melatonin (green), **1** (yellow) and *S*-**6a** (red). The amide NH of **1** and *S*-**6a** occupies a different position compared with the amide NH of melatonin which has caused the interacting serine-OH to rotate. The residues are coloured by atom-type, nitrogen: blue, oxygen: red.

positions occupied by this moiety in melatonin and **1**; also the methoxy-O occupied a slightly different position (rotation of the serine C—C—O—H interacting with the carbonyl prior to MacroModel minimization, as described above for *R*-**6a**, did not influence this orientation).

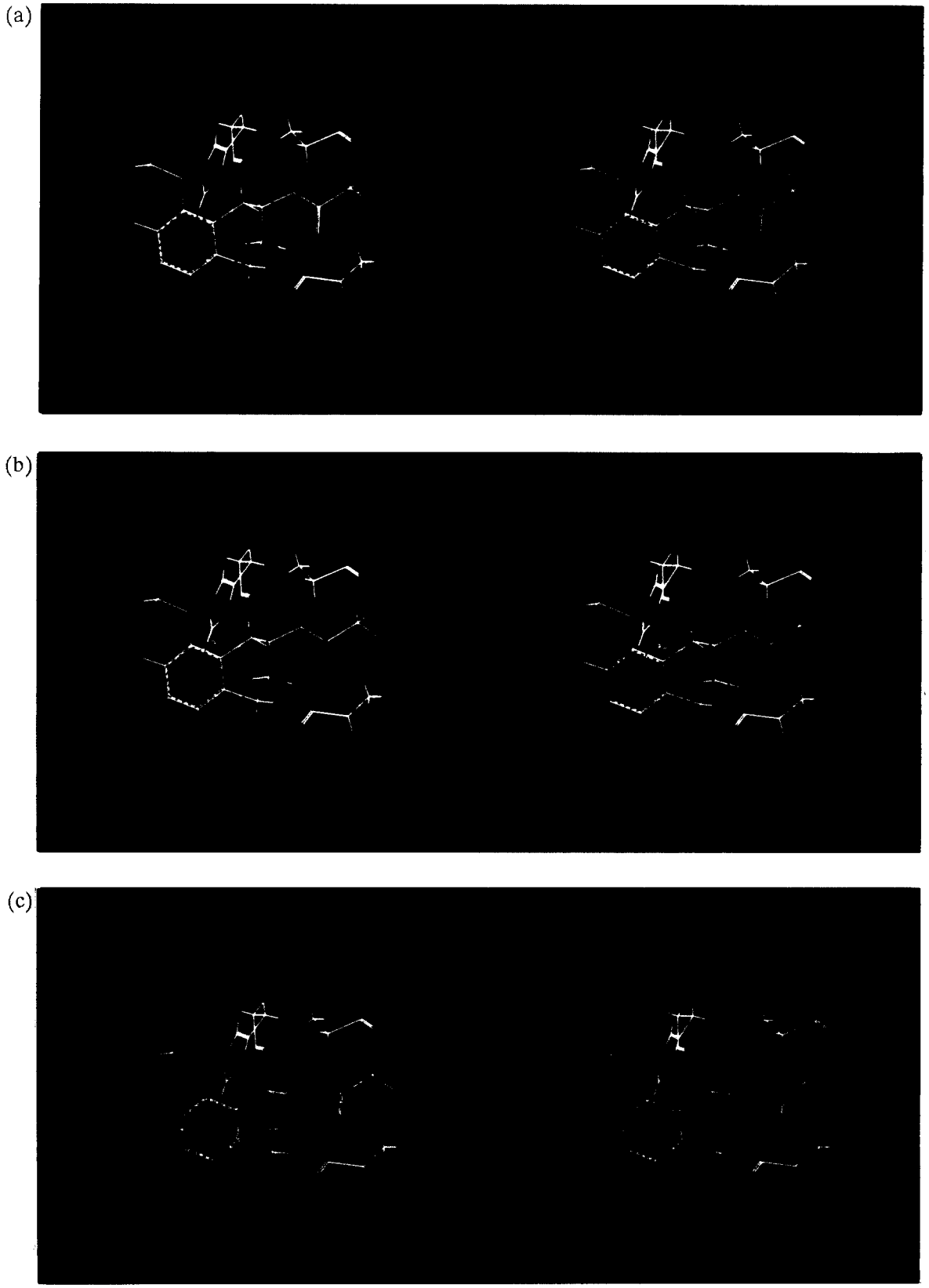


Figure 6. Minireceptor derived from pharmacophore model B including melatonin (green), **1** (yellow) and: 6a: *R*-**6a** in position I (red); 6b: *R*-**6a** in position II (red); 6c: *R*-**9** (light blue). The residues are colored by atom-type, nitrogen: blue, oxygen: red. The different positions of the amide NH and amide CO of the different ligands have caused both serine-OH moieties to rotate.

Table 6. Hydrogen bond strengths and ΔE values of ligands with respect to the Yak minireceptor derived from model B

Compound	His-H···OCH_3 (kcal/mol)	Ser-O···HN (kcal/mol)	Ser-H···O=C (kcal/mol)	ΔE[a] (kcal/mol)
Melatonin	−3.2	−2.8	−4.3	2.2
7	−3.0	−2.9	−4.5	2.0
S-**8**	−3.1	−2.7	−4.3	0.8
S-**1**	−3.1	−2.2	−4.1	5.5
S-**5a**	−2.4	−2.5	−4.4	6.5
R-**9**	−2.9	−2.4	−4.5	1.2
R-**6a** I[b]	−1.4	−3.5	−2.7	1.4
R-**6a** II[c]	−2.2	−1.9	−3.6	0.2
S-**6a**	−2.1	−3.3	−0.7	2.0

[a]Compared with the global minimum identified in the respective MC searches.
[b]Amide positioned like in melatonin (see text).
[c]Amide positioned like in **1** (see text).

As shown in Table 6, neither the *S*-enantiomer nor the *R*-enantiomer (in either of the two positions) of **6a** was able to interact with the minireceptor residues as strongly as the other ligands, providing an explanation for the drop in affinity. The fact that **1** and **5** exhibit a lower affinity, compared with melatonin, might be related to the relatively high ΔE values of the conformations involved. The ΔE value for *R*-**9** seems reasonable, but as shown in Figure 6(c), the methoxy-O is not in the same location as that of the other ligands. According to Yak, this location yielded a good hydrogen bond, but this deviation may still result in a lower affinity for **9**. With respect to the ΔE value of melatonin, it should be noted that the indole N—H was tilted out of the plane during minimization in the presence of the minireceptor, which increased the ΔE of this conformation.

The minireceptor derived in this study differs from the model recently put forward by Sugden et al.[42] in the selection of putative active site residues, but agrees with the suggestion of these authors that hydrogen bonding is likely to be involved at both the 5-methoxy and amide moieties. However, the present model also includes a hydrogen bond interaction at the amide CO function, which seems appropriate due to the fact that an amine alone is not recognized by the melatonin receptor.[3,4,7,8] Site-directed mutagenesis studies might be able to verify the involvement of the proposed residues in binding melatonin and its analogues.

Conclusion

Radioligand binding data for enantiomers indicated a difference between the previously distinguished ML-1 and ML-2 sites. For the ML-1 binding site, two possible pharmacophore models were derived from matching extension vectors from the amide N—H, the amide C=O and the methoxy-O, together with the normal through the phenyl ring, for sets of possible conformations of melatonin, **1**, **5**, **7**, **8**, and **9**. Model A used pseudoaxially oriented conformations of the rigid compounds and was obtained using minimized conformations from MC searches. Model B was found at higher rms values and consisted of pseudoequatorially oriented conformations of the rigid compounds. This model could only be derived using systematically generated conformations of **1**, **5a**, and **9**.

Minireceptors were built around both models to investigate putative ligand–residue interactions. Based on the observed interactions and considering the drop in affinity for **6**, model B is selected as a model to be used in future drug design. The minireceptor approach is highly recommended to investigate the directionality of interactions. The combination of this approach with the pharmacophore searches, using if necessary systematically generated sets of conformations, appears well suited to search for feasible interaction modes of series of ligands.

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