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Bicyclic melatonin receptor agonists containing a ring-junction nitrogen: Synthesis, biological evaluation, and molecular modeling of the putative bioactive conformation

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Abstract—Employing 1,3-dipolar cycloaddition for the synthesis of the 7a-azaindole nucleus, analogues of melatonin have been synthesized and tested against human and amphibian melatonin receptors. Introducing a phenyl substituent in position 2 of the heterocyclic moiety significantly increased binding affinity to both the MT_1 and MT_2 receptors. Shifting the methoxy group from position 5 to 2 of the 7a-azaindole ring led to a substantial reduction of MT_1 binding when MT_2 recognition was maintained. We theoretically investigated the hypothesis whether the 2-methoxy function of the azamelatonin analogue **27** is able to mimic the 5-methoxy group of the neurohormone by directing its 2-methoxy function toward the methoxy binding site. DFT calculations and experimental binding differences of analogue compounds indicate that the energy gained by forming the methoxy-specific hydrogen-bond interaction should exceed the energy required for adopting an alternative conformation.

1. Introduction

In recent years, the pineal hormone melatonin, which acts on specific G-protein coupled receptors, has become the focus of considerable interest for its implication in a wide variety of functions involving the circadian, visual, neuroendocrine, reproductive, cerebrovascular, and immune systems.[1] There is also a growing literature on the anti-oxidant actions of melatonin. The synthesis of melatonin and its secretion at night from the pineal gland are controlled by a circadian clock within the hypothalamic suprachiasmatic nuclei (SCN)[2] and are synchronized by environmental light.[3] Melatonin therefore has a chronobiotic function in that it works to alter the timing of physiological and behavioral processes through its action. Cloning studies identified three G-protein coupled receptors. Like all GPCRs, melatonin receptors have seven hydrophobic regions that are thought to form α-helical transmembrane (TM) domains connected by intra- and extracellular hydrophilic loops. The MT_1 and MT_2 melatonin receptor subtypes[4] are present in humans and other mammals, and their activation leads to the inhibition of adenylyl cyclase through activation of a G_i protein.[5,6] An additional subtype (termed Mel_{1c} receptor), not detected in mammals, has been cloned from chicken, *Xenopus*, and zebrafish.[7] In mammals, melatonin receptors are expressed in the brain, with considerable variation in location and density of expression between species, and also in some peripheral organs.[8] The MT_1 subtype is present within the pars tuberalis of the pituitary and the hypothalamic SCN, while the MT_2 subtype is mainly expressed in the retina. This diversity and the differences in tissue distribution suggest different functional physiological roles for each receptor subtype. For example, there is evidence suggesting that selective MT_2 receptor agonists should be particularly useful for the treatment of sleep and chronobiotic disorders, including jet lag and work shift syndrome.[9] Therefore, current research goals include the design of subtype-selective melatonin receptor agonists and antagonists, which recently led to a binding hypothesis for known MT_2 selective antagonists.[10]

Keywords: Melatonin receptor agonist; Bioactive conformation; Subtype selectivity; 7a-Azaindole.
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The ability of melatonin to alter circadian rhythm synchronization implies its potential use in the treatment of conditions associated with problems in this area. However, the use of melatonin as a drug is limited by its short biological half-life (15–20 min) and its poor bioavailability. In order to circumvent these problems, several potent ligands have been designed by employing bioisosteric replacement of the indole moiety by naphthalene and benzofuran analogues of types **1**[11] and **2**,[12] respectively (Chart 1). The naphthalene derivative agomelatine (**1**, R^1 = Me) is currently in phase III clinical trials as a new antidepressant agent.[13]

As previously shown for the indole nucleus, introducing a phenyl group in position 2 as well as lengthening the *N*-acyl side chain by up to two carbon atoms are both effective ways to increase the binding affinity at both subtypes.[14] Based on extended SAR data of a series of tryptamines, it has been concluded that the 5-methoxy group is not an essential requirement for biological activity; however, it clearly shows major interactions with a specific binding site in the melatonin receptor for a broad variety of ligands.[15] Moreover, it has been reported that appropriate substituents *ortho* to the ethylamido side chain in naphthalenic melatonin analogues modulate the binding affinity to the melatonin receptor.[16] Thus, we were further interested in the effects of 'switching' the methoxy group from position 5 of the pyrazolo[1,5-*a*]pyridine scaffold to position 2. Recently, we have been able to demonstrate by in vitro[17–19] and in vivo[20,21] studies in the field of dopamine receptor ligands that the pyrazolo[1,5-*a*]pyridine moiety is a valuable heterocyclic bioisostere of indoles. Therefore, we were intrigued to determine whether this finding holds true for other GPCRs like the melatonin receptors as well. A further aim of this study was to increase or modify both, the binding affinity toward the two receptor subtypes and the receptor activation. Taking advantage of the beneficial pharmacokinetics of the pyrazolo[1,5-*a*]pyridine bearing antiallergic agent ibudilast[22] and the antiplatelet agent KC-764,[23] we herein report the synthesis of azamelatonins of type **3**, their binding properties at the recombinant MT_1 and MT_2 subtypes determined in radioligand competition experiments and their intrinsic activity using the pigment aggregation assay on a clonal *Xenopus* melanophore cell line.

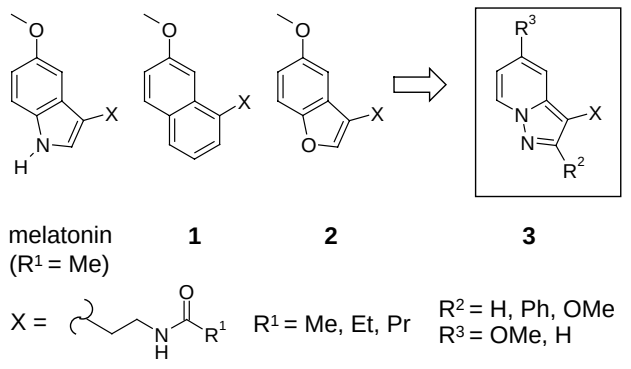
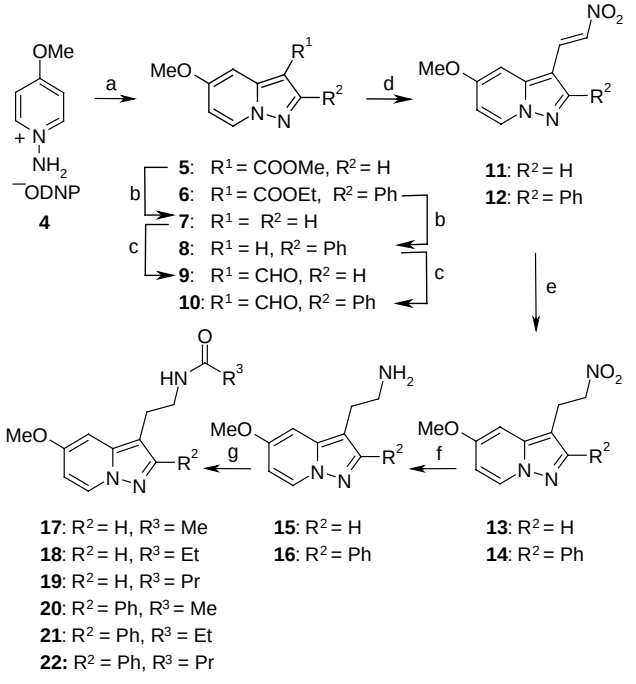


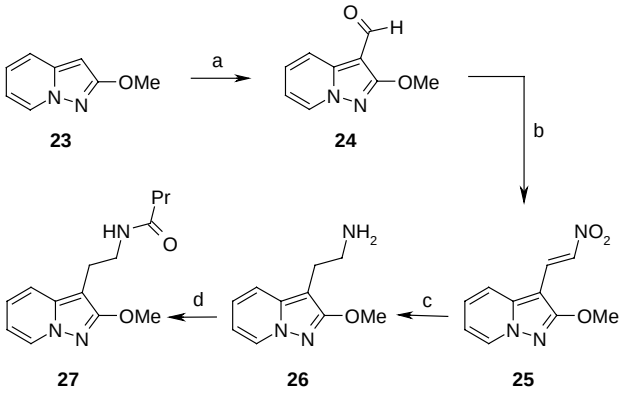
Chart 1.

2. Chemistry

As an extension of our previous efforts,[24] the 5-methoxy substituted target derivatives **17**–**22** were prepared according to the route shown in Scheme 1. Taking advantage of a recently reported highly efficient synthesis of *O*-(2,4-dinitrophenyl)hydroxylamine (NH_2-ODNP) by Legault and Charette,[25] we were able to prepare the *N*-aminopyridinium salt **4** derived from 4-methoxypyridine in 86% yield, thus, avoiding drawbacks exhibited by the widely used *N*-aminating reagents hydroxylamine-*O*-sulfonic acid[26] and *O*-mesitylenesulfonylhydroxylamine.[27] 1,3-Dipolar cycloaddition of **4** with methyl propiolate and ethyl phenylpropiolate, respectively, under oxidative conditions, followed by hydrolysis and decarboxylation of the respective ester intermediates **5** and **6**, furnished the pyrazolo[1,5-*a*]pyridine derivatives **7** and **8** in good yields. Subsequent formylation under Vilsmeier–Haack conditions gave the aldehydes **9** and **10**, respectively, followed by a mild Knoevenagel condensation with nitromethane at room temperature, which resulted in the formation of nitroalkenes **11** and **12**. After all attempts of direct reduction with $LiAlH_4$ provided only very modest yields of the product, reduction of the α,β-unsaturated nitroalkenes to the desired saturated amines **15** and **16** was performed with $NaBH_4$, followed by reduction with tin powder in HOAc. Finally, the ethylamino functions were acylated with the appropriate anhydrides to furnish the target compounds **17**–**22**.



Scheme 1. Reagents and conditions: (a) methyl propiolate or ethyl phenylpropiolate, K_2CO_3, air-O_2, DMF, rt, 16 h (43% for **5**; 35% for **6**); (b) H_2SO_4 (v/v 50%), 80 °C (3 h, 83% for **7**; 16 h, 80% for **8**); (c) $POCl_3$, DMF, rt, 1 h (91% for **9**; 94% for **10**); (d) CH_3NO_2, CH_3NH_3Cl, CH_3CO_2K, MeOH, rt (3 days, 84% for **11**; 10 days, 74% for **12**); (e) $NaBH_4$, MeOH, rt, 50 min (78% for **13**; 75% for **14**); (f) Sn, HOAc, EtOH, rt, 3 days (60% for **15**; 59% for **16**); (g) $(R^3CO)_2O$, Et_3N, THF, 0 °C to rt, 3 h (89% for **17**; 85% for **18**; 82% for **19**; 84% for **20**; 87% for **21**; 84% for **22**).



Scheme 2. Reagents and conditions: (a) $POCl_3$, DMF, rt, 1 h (98%); (b) CH_3NO_2, HOAc, NH_4OAc, ultrasound, 22 °C, 6 h (54%); (c) $LiAlH_4$, THF, 0 °C to rt, 2 h (45%); (d) butyric anhydride, Et_3N, THF, 0 °C to rt, 3 h (72%).

The 2-methoxy substituted target derivative **27** was prepared as outlined in Scheme 2, starting from 2-methoxypyrazolo[1,5-*a*]pyridine (**23**) that was prepared according to the method described by Ochi et al.[28] After formylation furnished the aldehyde **24**, application of an ultrasound promoted Knoevenagel condensation[29] with nitromethane gave the nitroalkene **25** in moderate yield. Subsequent reduction with $LiAlH_4$ and acylation with butyric anhydride led to the target molecule **27**.

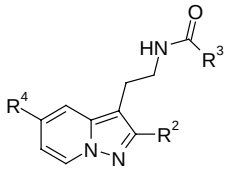
3. Results and discussion

3.1. Receptor binding and functional assay

The binding affinity of melatonin and its 7a-aza analogues (**17**–**22** and **27**) was determined in competition radioligand binding assays using 2-[^{125}I]-iodomelatonin as described previously[30] employing recombinant human MT_1 and MT_2 subtypes expressed in NIH 3T3 cells. Ligand efficacy of the test compounds was assessed in a well-established model system of melatonin action, the pigment aggregation response of *Xenopus laevis* melanophores.[31,32]

The results of both tests as well as MT_1/MT_2 selectivity ratios of the new compounds are reported in Table 1. The analysis demonstrated that all synthesized compounds were full agonists in the functional assay. Further examination of the results, especially with respect to the modifications at the C-2 and C-5 positions and in the acyl side chain, can be summarized to the following points: (1) As exemplified by the analogue **17** the pyrazolo[1,5-*a*]pyridine moiety is capable of serving as an useful bioisostere for the melatonin indole nucleus, thus, corroborating the previously reported insight that the proton-donor NH indole may not be essential for the anchoring of a ligand to the melatonin receptor.[33,34] The reduced affinity of **17** when compared to the natural neurohormone might be due to the polarization of the aromatic system that is caused by the additional nitrogen atom. (2) The positive effect of a 2-phenyl substitution already described for melatonin itself and some analogues[14] is also observed in our series and affects both MT_1 and MT_2 subtypes, leading to the agonists **20**–**22**, which were almost equipotent to melatonin in the *X. laevis* melanophore assay. This increase in potency can be ascribed to both the increased population of the active conformation for binding to the melatonin receptor and to the presence of an auxiliary binding site around the C-2 position,[35] which obviously exist in both receptor subtypes. For comparison, the affinity and efficacy of previously reported 2-phenyl melatonin analogues[14] were investigated in both hMT_1 and hMT_2 receptors as well as in the *X. laevis* melanophore aggregation model. The results obtained in both test systems for **20**–**22** are very similar to their respective melatonin-derived homologues, which leads us to the presumption that the mutual structural elements are 'privileged' for subtype-unselective MT receptor recognition and activation.

Table 1. Binding affinity of melatonin and the pyrazolo[1,5-*a*]pyridines **17**–**22** and **27** on human MT_1 and MT_2 receptors and their agonist activity in the *Xenopus laevis* melanophore assay



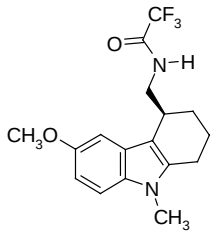
Compound	R^2	R^3	R^4	Receptor binding (K_i, nM)[a]		Selectivity (MT_1/MT_2)	*Xenopus* melanophores (EC_{50}, nM)[b]
				hMT_1	hMT_2		
Melatonin				0.45 ± 0.05	0.30 ± 0.02	1.5	0.02 ± 0.003
17	H	Me	OMe	12.3 ± 3.7	4.01 ± 0.09	3.1	10.2 ± 0.29
18	H	Et	OMe	14.5 ± 1.9	2.84 ± 0.06	5.1	9.06 ± 0.85
19	H	Pr	OMe	7.61 ± 0.45	0.52 ± 0.12	14.6	1.81 ± 0.04
20	Ph	Me	OMe	0.58 ± 0.04	0.40 ± 0.03	1.5	0.09 ± 0.006
21	Ph	Et	OMe	1.86 ± 0.09	0.38 ± 0.07	4.9	0.06 ± 0.001
22	Ph	Pr	OMe	1.37 ± 0.10	0.39 ± 0.10	3.5	0.03 ± 0.001
27	OMe	Pr	H	118 ± 8.2	1.55 ± 0.51	76	253 ± 6.9

[a] K_i values are means ± SEM of three concentration–response curves.
[b] EC50 values are means ± SEM of three concentration–response curves.

(3) At each of the cloned receptor subtypes, the effect of lengthening the *N*-acyl side chain from one carbon atom to three carbons was different. While the binding affinities at the MT_2 receptor increased on going from acetyl to butanoyl as the acylating group, no such consistent effect could be observed at the MT_1 receptor. (4) As shown by compound **27**, shifting the methoxy group from position 5 to 2 of the pyrazolo[1,5-*a*]pyridine moiety can modulate the affinity and selectivity of these ligands, resulting in a K_i value of 1.55 nM at the MT_2 receptor and a MT_1/MT_2 selectivity ratio of 76. Taking advantage of the results derived from the 5-methoxy series, we decided to prepare the butanoyl derivative **27** to gain maximum binding at the MT_2 receptor. Interestingly, the comparison of the obtained data for compounds **19** and **27** reveals that switching the 5-methoxy group to position 2 has a much greater effect on potency in the functional pigment aggregation assay in melanophores and on binding affinity to the MT_1 receptor subtype than it does on binding affinity to the MT_2 receptor subtype. One possible explanation is that both the Mel_{1c} receptor of lower vertebrates, which mediates pigment aggregation in *Xenopus* melanophores,[36] and the human MT_1 receptor subtype may be more reliant on hydrogen bonding to the 5-methoxy oxygen than the human MT_2 receptor subtype. We have made similar observations for melatonin and *N*-acetyltryptamine, where upon removal of the 5-methoxy group the affinity is clearly more adversely affected at the MT_1 (~1550-fold decrease) and the Mel_{1c} subtypes (~1380-fold decrease) than at the MT_2 subtype (~187-fold decrease).[37]

3.2. Theoretical investigations

Based on the high MT_2 affinity of **27**, we were intrigued by the question of how **27** is able to compensate the omission of the 5-methoxy group, which is usually found to have a strong adverse effect as stated before. In principle, two different explanations are conceivable: Instead of the 5-methoxy, the 2-methoxy group could bind to the specific 'methoxy binding site' (mbs) or the 2-methoxy group could bind to an 'auxiliary binding site' (abs) close to position 2, which is known to accept even phenyl moieties of ligands such as **20**–**22**. These two states resemble a discrimination of two different binding situations for a naphthyl ligand postulated by Langlois et al.[16] In order to investigate, whether **27** is able to adopt a conformation allowing it to mimic the regular interaction of the missing 5-methoxy group, we performed an extensive conformational sampling of **27** using SYBYL6.9[38] multisearch (10,000 searches yielding 203 unique conformational clusters) and the implemented TRIPOS force field. The most favorable conformer, which is able to interact with the mbs, was selected by a DISCO pharmacophore search (>1 acceptor atoms, >1 donor sites, 1 donor atom, 1 acceptor site, and 2 hydrophobic centroids), using the crystal structure (CSD-ID of the (*R*)-enantiomer: SEGVIJ) of the high affinity rigidized tricyclic ligand **28** (Chart 2) after inversion to the (*S*)-enantiomer as a structural template.[39] The inversion was performed since this configuration proved to show a higher MT receptor binding in the corresponding series. For comparison, the most reasonable



28

Chart 2.

conformer, which is expected to direct its 2-methoxy group conformations in favor of the abs toward the abs, was chosen from a similar DISCO run (1 acceptor atom, 1 donor site, 1 donor atom, 1 acceptor site, and 2 hydrophobic centroids). Subsequently, both conformers of **27** were subjected to a series of DFT calculations[40] (see Table 2) giving more reliable, relaxed structures. As we had no indications for the correct conformation of the propyl side chain from the structural template **28**, we only included a methyl side chain for both conformations in our quantum chemical calculations. The final conformers derived from these calculations were again submitted to a DISCO alignment yielding the final pharmacophoric superpositions as depicted in Figure 1. For the abs aligned conformer (Fig. 1A), a six point 'pharmacophore model' with an rmsd fit of 0.238 was retrieved, while for the mbs aligned conformer (Fig. 1B), a nine-point 'pharmacophore model' with an rmsd fit of 1.312 was obtained. Within the DFT calculations, increasing basis set levels show a narrowing gap between both aligned conformers, which directs the 2-methoxy group toward the auxiliary binding site. At the highest level of calculation an energy difference of 1.54 kcal/mol was found, which we try to evaluate subsequently considering the relative binding contributions of the auxiliary and methoxy binding site. Due to the fundamental thermodynamic relationship between the difference in the free energy of binding $\Delta\Delta G$ and the difference in the logarithmic inhibition constants ΔpK_i given by the equation

$$\Delta\Delta G = RT \times \ln 10 \times \Delta pK_i,$$

we can assess the relative binding contribution of the mbs, for instance, from the already cited example of melatonin and *N*-acetyltryptamine.[37] While the loss of the 5-methoxy group in *N*-acetyltryptamine decreases its binding affinity at the MT_1 receptor by ~1550-fold equaling a reduction of the binding energy by about 4.4 kcal/mol, the MT_2 receptor seems to be less sensitive to the absence of this interaction, showing a ~187-fold attenuated receptor binding, which equals a reduction in binding energy of about 3.1 kcal/mol (Fig. 2). On the other hand, we can estimate from the introduction of a phenyl moiety in position 2 of melatonin, leading to a ~3.5- and 3.2-fold increase of affinity at MT_1 and MT_2 receptors, respectively,[37] that additional binding interactions in the magnitude of 0.7 kcal/mol are formed between the substituent and the abs in both subtypes and that the abs tolerates large substituents up to the size of a phenyl moiety very well. Taking also a series of 2-halo analogues into consideration, we can conclude

Table 2. Quantum chemical calculations on the abs and mbs aligned conformations of **27**

Method/basis	Calcd type	*E* [hartree]		ΔE [kcal/mol] mbs − abs
		Abs aligned	Mbs aligned	
B3LYP/3-21G	OPT	−776.706687984	−776.700447881	+3.92
B3LYP/6-31G(d)	OPT	−781.005469098	−781.002460477	+1.88
B3LYP/6-311+G(d,p)	OPT	−781.222977630	−781.220374865	+1.63
B3LYP/6-311++G(2df,p)	SP	−781.265971776	−781.263512658	+1.54

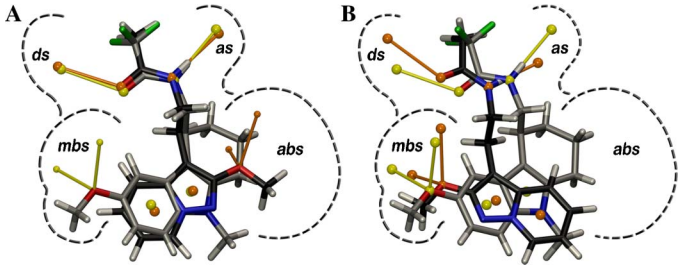


Figure 1. Comparison of the two different final DISCO pharmacophore models, involving the auxiliary binding site (abs) oriented conformation (A) and the methoxy binding site (mbs) oriented conformation (B) of **27** (carbons colored in black) fitted on the rigidized tricyclic subtype-unselective agonist **28** (carbons colored in gray). For both models, specific acceptor and donor binding sites (as and ds) adjacent to the amidoethane side chain have been included leading to 6 and 9 pharmacophoric points (with an rmsd fit of 0.238 and 1.312) satisfied in models A and B, respectively. The picture was prepared with VMD1.8.2.

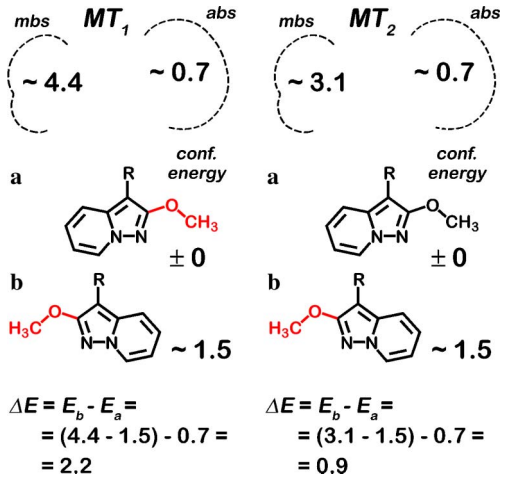


Figure 2. Estimation of the more favorable conformer of **27** based on the energy contents of its receptor interactions and conformational strain. The values shown in this figure are absolute numbers of relative interaction energies given in kilo calories per mole, which were deduced from characteristic binding differences of melatonin, *N*-acetyltryptamine, and 2-substituted melatonin derivatives[37] or obtained by quantum chemical calculations.

that the order of magnitude of the interactions with the abs is quite consistent for 2-substituents with different sizes and hydrophobicities.[37] Furthermore, none of these substituents in position 2 is capable of inducing MT_1/MT_2 selectivity. Thus, one possible explanation for the selectivity-inducing properties of the 2-methoxy substituent in **27** could be a putative subtype-specific interaction with significant energy content between the methoxy group and the MT_2-abs, which should not be present in MT_1. Except for this hypothesis involving such an MT_2 specific interaction partner in the abs, which is then likely to favor the abs aligned conformation of **27** (Fig. 1A), evaluation of the assessable relative binding contributions of both abs and mbs (Fig. 2) indicates that the mbs aligned conformer of **27** (Fig. 1B) should yield more favorable interactions. Summing up the previously estimated interaction energies, we have gathered evidence that the methoxy-mbs interaction energy exceeds the typical abs-interaction energy and the calculated energy effort for adopting the mbs-aligned conformation (Fig. 1B) by about 2.2 and 0.9 kcal/mol for MT_1 and MT_2, respectively (Fig. 2). Although such a purely ligand-based relative comparison of binding affinities can of course not be regarded as a final proof for this conclusion, it casts a new light on the relative energy difference of both conformers and provides the opportunity to rank this conformational energy versus interaction energies with the receptor. In the more probable mbs binding hypothesis, MT_2 selectivity of **27** could be based upon different capabilities of MT_1 and MT_2 to adapt either to the slight displacement of the side chain–amide interactions or to the steric requirements and altered π-interaction patterns of the heteroaromatic moiety.

4. Conclusion

In conclusion, we were able to show that the pyrazolo[1,5-*a*]pyridine moiety can serve as a useful bioisostere of indole in the search for melatonin receptor agonists. In general, most of the structure–affinity relationships previously described for indole-derived melatonin analogues[37] are found to be consistent with our series, especially the effects exhibited by lengthening of the *N*-acyl side chain and introduction of a 2-phenyl substituent. However, we found that by shifting the 5-methoxy group into position 2 of the aromatic core and, at the same time, keeping the *N*-butanoyl side chain, a MT_2 selective agonist with MT_1/MT_2 selectivity ratio of 76

and good binding affinity (1.55 nM) can be produced. This may provide a starting point for the development of additional selective agonists, which are useful tools in defining the physiological roles of the receptor subtypes. Furthermore, considering the indole ring of melatonin as the structural site of catabolic inactivation,[41] it could be assumed that the reported pyrazolo[1,5-*a*]pyridines are metabolically more stable when compared with melatonin, as indicated by the pharmacokinetics of the pyrazolo[1,5-*a*]pyridine bearing antiallergic agent ibudilast[22] and the antiplatelet agent KC-764.[23]

5. Experimental

All reactions were carried out under nitrogen atmosphere, except 1.3-dipolar cycloadditions and ester hydrolyses. Solvents were purified and dried by standard procedures. All reagents were of commercial quality and used as purchased. MS were run on a Finnegan MAT TSQ 70 spectrometer by EI (70 eV) with solid inlet. The ^{1}H NMR spectra were obtained on a Bruker AM 360 (360 MHz) spectrometer, if not otherwise stated in $CDCl_3$ relative to TMS. IR spectra were performed on a Jasco FT/IR 410 spectrometer. Purification by chromatography was performed using Silica Gel 60, TLC analyses were performed using Merck 60 F_{254} aluminum sheets and analyzed by UV light (254 nm) or in the presence of iodine. CHN elementary analyses were performed at the Department of Organic Chemistry of the Friedrich Alexander University.

5.1. *N*-Amino-(4-methoxy)pyridinium 2,4-dinitrophenolate (4)

A mixture of 4-methoxypyridine (1.5 g; 14 mmol) and *O*-(2,4-dinitrophenyl)hydroxylamine[25] (3.1 g; 15.1 mmol) in MeCN (9 ml) was stirred at 40 °C for 24 h. After the addition of Et_2O, the resulting yellow-orange solid was collected and dried yielding **4** (3.7 g; 86%). Mp 139 °C; IR 3197, 3095, 1536, 1507, 1257, 738 cm^{-1}; ^{1}H NMR $(CD_3)_2SO$ δ 4.01 (s, 3H), 6.35 (d, J = 9.8 Hz, 1H), 7.52–7.56 (m, 2H), 7.76 (br s, 2H), 7.80 (dd, J = 9.8 Hz, 3.2 Hz, 1H), 8.60 (d, J = 3.2 Hz), 8.66–8.69 (m, 2H); EIMS 184 (M^+)–$C_6H_4N_2O_5$, 124 (M^+)–$C_6H_9N_2O$.

5.2. Methyl 5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxylate (5)

Methyl propiolate (945 mg; 11.2 mmol) was added dropwise to a mixture of **4** (3.1 g; 10.2 mmol), K_2CO_3 (2 g; 14.5 mmol), and DMF (22 ml) and the reaction mixture was stirred vigorously at rt for 24 h. The suspension was filtered over Celite and the filtrate evaporated. The residue was dissolved in EtO_2, solution was washed three times with water, dried ($MgSO_4$), and evaporated. The crude product was purified by flash chromatography (hexane–EtOAc, 9:1) to afford **5** (903 mg; 43%) as a light yellow oil. IR 1699, 1536, 1278, 1051, 773 cm^{-1}; ^{1}H NMR δ 3.90 (s, 3H), 3.93 (s, 3H), 6.62 (dd, J = 7.5 Hz, 2.8 Hz, 1H), 7.42 (dd, J = 2.8 Hz, 0.7 Hz, 1H), 8.28 (s, 1H), 8.32 (dd, J = 7.5 Hz, 0.7 Hz, 1H); EIMS 206 (M^+); Anal. ($C_{10}H_{10}N_2O_3$): C, H, N.

5.3. Ethyl 5-methoxy-2-phenylpyrazolo[1,5-*a*]pyridine-3-carboxylate (6)

This compound was prepared as described for **5** using **4** (3 g; 9.7 mmol) and K_2CO_3 (1.9 g; 13.7 mmol) in DMF (21 ml) and ethyl phenylpropiolate (1.8 g; 10.3 mmol) to afford **6** (1 g, 35%) as a light yellow oil. IR 1702, 1683, 1289, 1050 cm^{-1}; ^{1}H NMR δ 1.26 (t, J = 7.1 Hz, 3H), 3.95 (s, 3H), 4.28 (q, J = 7.1 Hz, 2H), 6.63 (dd, J = 7.5 Hz, 2.8 Hz, 1H), 7.45–7.41 (m, 3H), 7.53 (br d, J = 2.8 Hz, 1H), 7.72–7.75 (m, 2H), 8.33 (br d, J = 7.5 Hz, 1H); EIMS 296 (M^+); Anal. ($C_{17}H_{16}N_2O_3$): C, H, N.

5.4. 5-Methoxypyrazolo[1,5-*a*]pyridine (7)

A suspension of **5** (5 g; 24 mmol) in 50% H_2SO_4 (40 ml) was heated at 80 °C for 3 h. After cooling to rt and then to 0 °C, the solution was neutralized with a 5 N NaOH solution, extracted with EtO_2, extract dried ($MgSO_4$), and evaporated. The residue was purified by flash chromatography (hexane–EtOAc, 8:2) to give **7** (3 g, 83%) as a colorless oil. IR 2937, 1646, 1340, 1024 cm^{-1}; ^{1}H NMR δ 3.84 (s, 3H), 6.32 (br d, J = 2.5 Hz, 1H), 6.44 (dd, J = 7.5 Hz, 2.8 Hz, 1H), 6.74 (d, J = 2.8 Hz, 1H), 7.85 (d, J = 2.5 Hz, 1H), 8.28 (br d, J = 7.5 Hz, 1H); EIMS 148 (M+); Anal. ($C_8H_8N_2O$): C, H, N.

5.5. 5-Methoxy-2-phenylpyrazolo[1,5-*a*]pyridine (8)

This compound was prepared as described for **7** but heating for 16 h using **6** (1 g; 3.4 mmol) in 50% H_2SO_4 (10 ml) to afford **8** (605 mg; 80%) as a colorless oil after purification by flash chromatography (hexane–EtOAc, 8:2). IR 2928, 1649, 1420, 1028 cm^{-1}; ^{1}H NMR δ 3.85 (s, 3H), 6.43 (dd, J = 7.5 Hz, 2.8 Hz, 1H), 6.61 (br s, 1H), 6.72 (br d, J = 2.8 Hz), 7.33–7.46 (m, 3H), 7.92–7.94 (m, 2H), 8.29 (br d, J = 7.5 Hz, 1H); EIMS 224 (M+); Anal. ($C_{14}H_{12}N_2O$): C, H, N.

5.6. 5-Methoxypyrazolo[1,5-*a*]pyridine-3-carbaldehyde (9)

To a solution of $POCl_3$ (1.6 g; 10.4 mmol) in DMF (4 ml), **7** (500 mg; 3.4 mmol) dissolved in DMF (1 ml) was slowly added. The mixture was stirred at rt for 1 h and after cooling to 0 °C it was diluted with H_2O, made alkaline with 2 N NaOH, and extracted with $CHCl_3$. After drying ($MgSO_4$), the extract was evaporated and the residue was purified by flash chromatography (hexane–EtOAc, 1:1) to afford **9** (541 mg; 91%) as a white solid. Mp 93 °C; IR 1662, 1280, 1201, 831 cm^{-1}; ^{1}H NMR δ 3.96 (s, 3H), 6.72 (dd, J = 7.5 Hz, 2.8 Hz, 1H), 7.59 (d, J = 2.8 Hz, 1H), 8.27 (s, 1H), 8.37 (d, J = 7.5 Hz, 1H), 9.96 (s, 1H); EIMS 176 (M+); Anal. ($C_9H_8N_2O_2$): C, H, N.

5.7. 5-Methoxy-2-phenylpyrazolo[1,5-*a*]pyridine-3-carbaldehyde (10)

This compound was prepared as described for **9** using **8** (440 mg; 2 mmol) and $POCl_3$ (919 mg; 6 mmol) in DMF (2.5 ml) to afford **10** (463 mg; 94%) as a white solid after purification by flash chromatography (hexane–EtOAc,

1:1). Mp 92 °C; IR 1735, 1437, 1243, 700 cm^{-1}; ^1H NMR δ 3.98 (s, 3H), 6.74 (dd, J = 7.5 Hz, 2.8 Hz, 1H), 7.50–7.56 (m, 3H), 7.74–7.77 (m, 3H), 8.39 (br d, J = 7.5 Hz, 1H), 10.04 (s, 1H); EIMS 252 (M+); Anal. ($C_{15}H_{12}N_2O_2$): C, H, N.

5.8. 5-Methoxy-3-(2-nitroethenyl)-pyrazolo[1,5-*a*]pyridine (11)

To a solution of **9** (400 mg, 2.3 mmol) in MeOH (4 ml) were added nitromethane (150 mg; 2.5 mmol), methylamine hydrochloride (57.5 mg; 0.85 mmol), and potassium acetate (57.5 mg; 0.59 mmol), and the reaction mixture was stirred at rt for 3 days. The precipitate was collected, washed with a small amount of MeOH and hexane, and dried to afford **11** (418 mg; 84%) as maroon prisms. Mp 157 °C; IR 1646, 1538, 1490, 1344, 1214, 833 cm^{-1}; ^1H NMR $(CD_3)_2SO$ δ 3.96 (s, 3H), 6.82 (dd, J = 7.5 Hz, 2.5 Hz, 1H), 7.64 (br d, J = 2.5 Hz, 1H), 8.10 (d, J = 13.1 Hz, 1H), 8.48 (d, J = 13.1 Hz, 1H), 8.60 (s, 1H), 8.71 (d, J = 7.5 Hz, 1H); EIMS 219 (M+); Anal. ($C_{10}H_9N_3O_3$): C, H, N.

5.9. 5-Methoxy-3-(2-nitroethenyl)-2-phenylpyrazolo[1,5-*a*]pyridine (12)

This compound was prepared as described for **11** using **10** (463 mg; 1.8 mmol), nitromethane (113 mg; 1.9 mmol), methylamine hydrochloride (50 mg; 0.74 mmol), and potassium acetate (50 mg; 0.51 mmol), and the reaction mixture was stirred at rt for 10 days, yielding **12** (400 mg; 74%) as orange crystals. Mp 166 °C; IR 1740, 1648, 1457, 1316, 1072, 769 cm^{-1}; ^1H NMR δ 4.01 (s, 3H), 6.73 (dd, J = 7.5 Hz, 2.6 Hz, 1H), 6.92 (d, J = 2.6 Hz, 1H), 7.50 (d, J = 13.6 Hz, 1H), 7.50–7.56 (m, 3H), 7.64–7.70 (m, 2H), 8.33 (d, J = 13.6 Hz, 1H), 8.42 (d, J = 7.5 Hz, 1H); EIMS 295 (M+); Anal. ($C_{16}H_{13}N_3O_3$): C, H, N.

5.10. 5-Methoxy-3-(2-nitroethyl)-pyrazolo[1,5-*a*]pyridine (13)

A mixture of **11** (300 mg; 1.4 mmol) and MeOH (14 ml) was stirred during the portionwise addition (20 min) of $NaBH_4$ (164.5 mg; 4.4 mmol). The solution was stirred for 30 min after the addition of $NaBH_4$ was completed, then adjusted to pH 5 with HOAc, and concentrated. The residue was diluted with H_2O and extracted three times with CH_2Cl_2. The extracts were combined, washed with H_2O, dried ($MgSO_4$), concentrated, and purified by flash chromatography (hexane–EtOAc, 3:1) to give **13** (236 mg; 78%) as light yellow crystals. Mp 140 °C; IR 1650, 1544, 1459, 1238, 1014, 829 cm^{-1}; ^1H NMR δ 3.41 (t, J = 7.1 Hz, 2H), 3.88 (s, 3H), 4.60 (t, J = 7.1 Hz, 2H), 6.46 (dd, J = 7.5 Hz, 2.5 Hz, 1H), 6.63 (d, J = 2.5 Hz, 1H), 7.74 (s, 1H), 8.24 (br d, J = 7.5 Hz, 1H); EIMS 221 (M+); Anal. ($C_{10}H_{11}N_3O_3$): C, H, N.

5.11. 5-Methoxy-3-(2-nitroethyl)-2-phenylpyrazolo[1,5-*a*]pyridine (14)

This compound was prepared as described for **13** using **12** (50 mg; 0.17 mmol) in MeOH (5 ml) and $NaBH_4$ (20 mg; 0.53 mmol), yielding **14** (38 mg; 75%) after purification by flash chromatography (hexane–EtOAc, 9:1) as light yellow crystals. Mp 152 °C; IR 1646, 1543, 1459, 1428, 1021, 700 cm^{-1}; ^1H NMR δ 3.57 (t, J = 7.5 Hz, 2H), 3.90 (s, 3H), 4.47 (t, J = 7.5 Hz, 2H), 6.48 (dd, J = 7.5 Hz, 2.5 Hz, 1H), 6.65 (d, J = 2.5 Hz, 1H), 7.40–7.51 (m, 3H), 7.64–7.67 (m, 2H), 8.26 (d, J = 7.5 Hz, 1H); EIMS 297 (M+); Anal. ($C_{16}H_{15}N_3O_3$): C, H, N.

5.12. 2-(5-Methoxypyrazolo[1,5-*a*]pyridine-3-yl)-ethylamine (15)

To a solution of **13** (250 mg; 1.1 mmol) in EtOH (12 ml), tin powder (0.3 g) and HOAc (3 ml) were added and the mixture was stirred for 3 days at rt. The solid particles were filtered off and the filtrate evaporated. The residue was diluted with H_2O, made alkaline with 2 N NaOH, and extracted five times with $CHCl_3$. The combined extracts were washed with saturated sodium chloride solution, dried ($MgSO_4$), concentrated, and purified by flash chromatography (CH_2Cl_2–MeOH, 8:2) to give **15** (130 mg; 60%) as a colorless oil. IR 2935, 1649, 1559, 1244, 1018, 813 cm^{-1}; ^1H NMR δ 2.81 (t, J = 6.7 Hz, 2H), 2.98 (t, J = 6.7 Hz, 2H), 3.85 (s, 3H), 6.42 (dd, J = 7.5 Hz, 2.5 Hz, 1H), 6.65 (d, J = 2.5 Hz, 1H), 7.74 (s, 1H), 8.23 (d, J = 7.5 Hz, 1H); EIMS 191 (M+); Anal. ($C_{10}H_{13}N_3O$): C, H, N.

5.13. 2-(5-Methoxy-2-phenylpyrazolo[1,5-*a*]pyridine-3-yl)-ethylamine (16)

This compound was prepared as described for **15** using **14** (200 mg; 0.67 mmol) in EtOH (6 ml), tin powder (150 mg), and HOAc (1.5 ml) yielding **16** (108 mg; 60%) after purification by flash chromatography (CH_2Cl_2–MeOH, 8:2) as a colorless oil. IR 2931, 1645, 1249, 1222, 1022, 700 cm^{-1}; ^1H NMR δ 2.91–3.01 (m, 4H), 3.88 (s, 3H), 6.43 (dd, J = 7.5 Hz, 2.7 Hz, 1H), 6.69 (d, J = 2.7 Hz, 1H), 7.36–7.48 (m, 3H), 7.70–7.73 (m, 2H), 8.26 (d, J = 7.5 Hz, 1H); EIMS 267 (M+); Anal. ($C_{16}H_{17}N_3O$): C, H, N.

5.14. *N*-[2-(5-Methoxypyrazolo[1,5-*a*]pyridine-3-yl)-ethyl]-acetamide (17)

To a cooled solution (0 °C) of **15** (18.4 mg, 0.1 mmol) in THF (1.5 ml) were added Et_3N (34 mg; 0.34 mmol) and acetic anhydride (33.8 mg; 0.34 mmol). The ice bath was removed and the solution was stirred for 3 h. The solvent was evaporated in vacuo, and the residue was taken up in ethyl acetate and washed with H_2O, saturated aqueous $NaHCO_3$, and brine. The organic phase was dried ($MgSO_4$), concentrated in vacuo, and purified by flash chromatography (CH_2Cl_2–MeOH, 95:5) to afford **17** (20 mg; 89%) as colorless crystals. Mp 122 °C; IR 1650, 1560, 1459, 1241, 808 cm^{-1}; ^1H NMR δ 1.94 (s, 3H), 2.89 (t, J = 6.9 Hz, 2H), 3.51 (dt, J = 6.9 Hz, 6.5 Hz, 2H), 3.86 (s, 3H), 5.56 (br s, 1H), 6.43 (dd, J = 7.5 Hz, 2.5 Hz, 1H), 6.66 (d, J = 2.5 Hz, 1H), 7.71 (s, 1H), 8.23 (br d, J = 7.5 Hz, 1H); EIMS 233 (M+); Anal. ($C_{12}H_{15}N_3O_2$): C, H, N.

5.15. *N*-[2-(5-Methoxypyrazolo[1,5-*a*]pyridine-3-yl)-ethyl]-propionamide (18)

This compound was prepared as described for **17** using **15** (26.5 mg; 0.14 mmol) in THF (1.5 ml), Et_3N (41.2 mg; 0.41 mmol), and propionic anhydride (53.4 mg; 0.41 mmol) yielding **18** (25 mg; 85%) after purification by flash chromatography (CH_2Cl_2–MeOH, 95:5) as colorless crystals. Mp 109 °C; IR 1647, 1560, 1457, 1244, 808 cm^{-1}; ^1H NMR δ 1.13 (t, J = 7.6 Hz, 3H), 2.16 (q, J = 7.6 Hz, 2H), 2.92 (t, J = 6.8 Hz, 2H), 3.55 (dt, J = 6.8 Hz, 6.2 Hz, 2H), 3.86 (s, 3H), 5.50 (br s, 1H), 6.43 (dd, J = 7.5 Hz, 2.7 Hz, 1H), 6.65 (br d, J = 2.7 Hz, 1H), 7.71 (s, 1H), 8.23 (br d, J = 7.5 Hz, 1H); EIMS 247 (M+); Anal. ($C_{13}H_{17}N_3O_2$): C, H, N.

5.16. *N*-[2-(5-Methoxypyrazolo[1,5-*a*]pyridine-3-yl)-ethyl]-butyramide (19)

This compound was prepared as described for **17** using **15** (23 mg; 0.12 mmol) in THF (1.5 ml), Et_3N (47.3 mg; 0.47 mmol), and butyric anhydride (75 mg; 0.47 mmol) yielding **19** (30 mg; 82%) after purification by flash chromatography (CH_2Cl_2–MeOH, 95:5) as colorless crystals. Mp 102 °C; IR 1649, 1537, 1456, 1246, 811 cm^{-1}; ^1H NMR δ 0.92 (t, J = 7.4 Hz, 3H), 1.58–1.68 (m, 2H), 2.10 (t, J = 7.6 Hz, 2H), 2.88 (t, J = 6.8 Hz, 2H), 3.52 (dt, J = 6.8 Hz, 6.2 Hz, 2H), 3.86 (s, 3H), 5.51 (br s, 1H), 6.44 (dd, J = 7.5 Hz, 2.7 Hz, 1H), 6.66 (br d, J = 2.7 Hz, 1H), 7.71 (s, 1H), 8.24 (br d, J = 7.5 Hz, 1H); EIMS 261 (M+); Anal. ($C_{14}H_{19}N_3O_2$): C, H, N.

5.17. *N*-[2-(5-Methoxy-2-phenylpyrazolo[1,5-*a*]pyridine-3-yl)-ethyl]-acetamide (20)

This compound was prepared as described for **17** using **16** (20 mg; 0.08 mmol) in THF (1.5 ml), Et_3N (25.6 mg; 0.26 mmol), and acetic anhydride (25.4 mg; 0.26 mmol) yielding **20** (19 mg; 84%) after purification by flash chromatography (CH_2Cl_2–MeOH, 95:5) as colorless crystals. Mp 134 °C; IR 1645, 1457, 1429, 1248, 700 cm^{-1}; ^1H NMR δ 1.80 (s, 3H), 3.06 (t, J = 6.9 Hz, 2H), 3.43 (dt, J = 6.9 Hz, 6.5 Hz, 2H), 3.89 (s, 3H), 5.43 (br s, 1H), 6.46 (dd, J = 7.5 Hz, 2.7 Hz, 1H), 6.74 (d, J = 2.7 Hz, 1H), 7.37–7.49 (m, 3H), 7.69–7.72 (m, 2H), 8.27 (br d, J = 7.5 Hz, 1H); EIMS 309 (M+); Anal. ($C_{18}H_{19}N_3O_2$): C, H, N.

5.18. *N*-[2-(5-Methoxy-2-phenylpyrazolo[1,5-*a*]pyridine-3-yl)-ethyl]-propionamide (21)

This compound was prepared as described for **17** using **16** (20 mg; 0.08 mmol) in THF (1.5 ml), Et_3N (25.6 mg; 0.26 mmol), and propionic anhydride (33.4 mg; 0.26 mmol) yielding **21** (21 mg; 87%) after purification by flash chromatography (CH_2Cl_2–MeOH, 95:5) as colorless crystals. Mp 129 °C; IR 1645, 1457, 1223, 1022, 699 cm^{-1}; ^1H NMR δ 1.04 (t, J = 7.6 Hz, 3H), 2.01 (q, J = 7.6 Hz, 2H), 3.07 (t, J = 7.0 Hz, 2H), 3.44 (dt, J = 7.0 Hz, 6.5 Hz, 2H), 3.89 (s, 3H), 5.41 (br s, 1H), 6.45 (dd, J = 7.5 Hz, 2.7 Hz, 1H), 6.73 (br d, J = 2.7 Hz, 1H), 7.37–7.49 (m, 3H), 7.69–7.73 (m, 2H), 8.26 (br d, J = 7.5 Hz, 1H); EIMS 323 (M+); Anal. ($C_{19}H_{21}N_3O_2$): C, H, N.

5.19. *N*-[2-(5-Methoxy-2-phenylpyrazolo[1,5-*a*]pyridine-3-yl)-ethyl]-butyramide (22)

This compound was prepared as described for **17** using **16** (23 mg; 0.09 mmol) in THF (1.5 ml), Et_3N (33.9 mg; 0.34 mmol), and butyric anhydride (53.8 mg; 0.34 mmol) yielding **22** (24 mg; 84%) after purification by flash chromatography (CH_2Cl_2–MeOH, 95:5) as colorless crystals. Mp 123 °C; IR 1645, 1457, 1248, 1022, 699 cm^{-1}; ^1H NMR δ 0.87 (t, J = 7.4 Hz, 3H), 1.48–1.59 (m, 2H), 1.96 (t, J = 7.6 Hz, 2H), 3.06 (t, J = 6.8 Hz, 2H), 3.44 (dt, J = 7.0 Hz, 6.2 Hz, 2H), 3.89 (s, 3H), 5.40 (br s, 1H), 6.46 (dd, J = 7.5 Hz, 2.7 Hz, 1H), 6.66 (br d, J = 2.7 Hz, 1H), 7.37–7.49 (m, 3H), 7.69–7.73 (m, 2H), 8.27 (br d, J = 7.5 Hz, 1H); EIMS 337 (M+); Anal. ($C_{20}H_{23}N_3O_2$): C, H, N.

5.20. 2-Methoxypyrazolo[1,5-*a*]pyridine-3-carbaldehyde (24)

This compound was prepared as described for **9** using **23**[28] (120.5 mg; 0.81 mmol) and $POCl_3$ (383 mg; 2.5 mmol) in DMF (1 ml) to afford **24** (140 mg; 98%) as a white solid after purification by flash chromatography (hexane–EtOAc, 1:1). Mp 85 °C; IR 1654, 1631, 1511, 1411, 1064, 759 cm^{-1}; ^1H NMR δ 4.13 (s, 3H), 6.94 (ddd, J = 7.1 Hz, 6.7 Hz, 1.4 Hz, 1H), 7.47 (ddd, J = 8.9 Hz, 7.1 Hz, 1.4 Hz, 1H), 8.12–8.15 (br d, J = 8.9 Hz, 1H), 8.31–8.34 (br d, J = 6.7 Hz, 1H), 9.92 (s, 1H); EIMS 176 (M+); Anal. ($C_9H_8N_2O_2$): C, H, N.

5.21. 2-Methoxy-3-(2-nitroethenyl)-pyrazolo[1,5-*a*]pyridine (25)

A mixture of **24** (100 mg; 0.57 mmol), nitromethane (417 mg; 6.9 mmol), HOAc (0.1 ml), and ammonium acetate (94.4 mg; 1.2 mmol) was sonicated at 22 °C for 6 h. After addition of CH_2Cl_2, the organic phase was washed with H_2O and brine, dried ($MgSO_4$), and concentrated. The residue was purified by flash chromatography (hexane–EtOAc, 8:2) to give **25** (67 mg; 54%) as a yellow solid. Mp 120 °C; IR 1639, 1608, 1542, 1461, 1207, 829 cm^{-1}; ^1H NMR $(CD_3)_2SO$ δ 4.12 (s, 3H), 7.10 (ddd, J = 7.2 Hz, 6.7 Hz, 1.2 Hz, 1H), 7.59 (ddd, J = 8.9 Hz, 7.2 Hz, 1.2 Hz, 1H), 7.75 (d, J = 13.1 Hz, 1H), 8.11 (d, J = 8.9 Hz, 1H), 8.34 (d, J = 13.1 Hz, 1H), 8.73 (d, J = 6.7 Hz, 1H); EIMS 219 (M+); Anal. ($C_{10}H_9N_3O_3$): C, H, N.

5.22. 2-(2-Methoxypyrazolo[1,5-*a*]pyridine-3-yl)-ethylamine (26)

A suspension of **25** (40 mg, 0.18 mmol) in THF (2 ml) was treated with $LiAlH_4$ (1 M in THF; 183 µl; 0.18 mmol) at 0 °C and the reaction mixture was stirred at rt for 2 h. After the addition of H_2O (0.2 ml), the suspension was filtered and washed with EtO_2. The filtrate was dried ($MgSO_4$), evaporated, and the residue was purified by flash chromatography (CH_2Cl_2–MeOH, 95:5) to give **26** (16 mg, 45%) as a colorless oil. IR

2923, 1641, 1515, 1409, 1342 cm^{-1}; ^1H NMR δ 2.73 (t, $J = 6.74$, 2H), 2.93 (t, $J = 6.74$ Hz, 2H), 4.04 (s, 1H), 6.53 (ddd, $J = 7.1$ Hz, 6.65 Hz, 1.24 Hz, 1H), 7.00 (ddd, $J = 8.96$ Hz, 6.65 Hz, 1.24 Hz, 1H), 7.23–7.24 (m, 1H), 8.18–8.20 (br d, $J = 7.1$ Hz, 1H); EIMS 191 (M+); Anal. ($C_{10}H_{13}N_3O$): C, H, N.

5.23. *N*-[2-(2-Methoxypyrazolo[1,5-*a*]pyridine-3-yl)-ethyl]-butyramide (27)

This compound was prepared as described for **17** using **26** (18 mg; 0.09 mmol) in THF (1.5 ml), Et_3N (37 mg; 0.37 mmol), and butyric anhydride (58.7 mg; 0.37 mmol) yielding **27** (26 mg; 72%) after purification by flash chromatography (CH_2Cl_2–MeOH, 95:5) as colorless crystals. Mp 115 °C; IR 1636, 1518, 1409, 1214, 744 cm^{-1}; ^1H NMR δ 0.91 (t, $J = 7.4$ Hz, 3H), 1.56–1.67 (m, 2H), 2.09 (t, $J = 7.5$ Hz, 2H), 2.80 (t, $J = 6.6$ Hz, 2H), 3.46 (dt, $J = 6.6$ Hz, 5.8 Hz, 2H), 4.05 (s, 3H), 5.70 (br s, 1H), 6.55 (ddd, $J = 6.9$ Hz, 6.8 Hz, 1.4 Hz, 1H), 7.02 (ddd, $J = 8.9$ Hz, 6.8 Hz, 1.1 Hz, 1H), 7.24 (br d, $J = 8.9$ Hz, 1H), 8.19 (br d, $J = 6.9$ Hz, 1H); EIMS 261 (M+); Anal. ($C_{14}H_{19}N_3O_2$): C, H, N.

5.24. Melatonin receptor binding studies

NIH3T3 cells expressing recombinant human MT_1 and MT_2 receptors were cultured, harvested, and lysed as described previously.[30] Membrane aliquots were stored in liquid nitrogen until used. For competition assays, 2-[^{125}I]-iodomelatonin (70 pM for hMT_1; 110 pM for hMT_2) was incubated with NIH3T3 cell membranes at room temperature (~21 °C) for 90 min in a 96-well Multiscreen plate with a PVDF membrane (Millipore, Whatman Ltd, Maidstone, Kent, UK) pre-treated with 2% v/v polyethylenimine. Binding assays were terminated by the rapid addition of 100 μl ice-cold buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4) to each well and immediate vacuum filtration. Each well was then rinsed with a further 2× 100 μl buffer. Membrane-bound 2-[^{125}I]-iodomelatonin trapped on each filter was counted (Cobra II Auto-Gamma, PACKARD). All assays were done on triplicate homogenate aliquots. Specific binding was calculated by subtracting non-specific binding (defined using 1 μM melatonin) from total binding. Preliminary studies showed that equilibrium is reached under the binding conditions used, and saturation experiments gave K_d values close to those reported previously (hMT_1, 81 pM; hMT_2, 131 pM).

5.25. *Xenopus* melanophore pigment aggregation assay

Melanophores were grown as described previously.[30] Briefly, flat-bottomed 96-well cell culture plates containing ~5×10^3 melanophores/well were plated at least 2 days prior to use in pigment aggregation experiments. One hour prior to all concentration–response experiments, growth medium (0.7× L-15 medium containing 15% heat-inactivated FCS, 100 iu/ml penicillin, and 100 μg/ml streptomycin) in each well was aspirated and replaced with 0.7× L-15 medium (containing 1 mg/ml bovine serum albumin) and plates were left in room light. In 0.7× L-15, pigment remained fully dispersed. The change in distribution of pigment within melanophores was quantitated using a Bio-Tek microtitre plate reader (model EL3115, Anachem, Luton, UK) by measuring the change in absorbance (630 nm) before and after drug treatment. The fractional change in absorbance, $1 - (A_f/A_i)$, where A_i is the initial absorbance before drug treatment and A_f is the final absorbance, was calculated to determine agonist activity. To test for antagonist activity, melatonin (1 nM) was then added and absorbance was read again after 60 min (A_a). Antagonist response was calculated as $1 - (A_a/A_i)$. All drugs were freshly prepared from 10 mM stock solutions in DMSO kept at −20 °C. The maximal concentration of solvent was 1% v/v which did not cause pigment redistribution in melanophores.

5.26. Data analysis

In receptor binding studies, the four parameter logistic equation was used for curve-fitting concentration–response data to obtain the concentration of compounds producing 50% inhibition of the specific 2-[^{125}I]-iodomelatonin binding (for determination of K_i). The concentration of agonists producing 50% of maximal pigment aggregation (EC_{50}), or antagonists inhibiting melatonin-induced aggregation by 50% (IC_{50}), was determined in the same way.

5.27. Modeling

Conformational sampling of 27 using SYBYL6.9 multisearch[38] and pharmacophore modeling using DISCO[38] was performed on a SGI Octane 2 workstation. DFT calculations were performed on an 8 Intel Xeon processor Linux cluster using Gaussian98.[40]

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