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Cyclic 3-hydroxymelatonin, a key metabolite enhancing the peroxyl radical scavenging activity of melatonin

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The reactions of cyclic 3-hydroxymelatonin (3-OHM) with hydroxyl (˙OH) and hydroperoxyl (˙OOH) radicals were studied using Density Functional Theory. Two environments, mimicking lipid and aqueous solutions, have been modelled. Three mechanisms of reaction were considered: radical adduct formation (RAF), hydrogen transfer (HT), and single electron transfer (SET). Their relative importance for the free radical scavenging activity of 3-OHM was assessed. It was found that 3-OHM reacts with ˙OH at diffusion-limited rates, regardless of the polarity of the environment, which supports its excellent ˙OH radical scavenging activity. The overall reactivity of 3-OHM towards this radical was found to be similar, but slightly higher than those of melatonin and two other metabolites (N^1-acetyl-5-methoxykynuramine, AMK; and N^1-acetyl-N^2-formyl-5-methoxykynuramine, AFMK). For the reaction with ˙OOH, 3-OHM was found to react several orders of magnitude faster, in aqueous solution, than melatonin, AMK and AFMK. Furthermore, under these conditions 3-OHM was found to react with ˙OOH about 98.4 times faster than Trolox. This seems to be a very important finding since it has been proposed that melatonin, AMK and AFMK are rather ineffective as peroxyl radical scavengers, while 3-OHM is predicted to be very efficient. Therefore, it is proposed that the protective effects of melatonin against peroxyl radicals become important after being metabolized into 3-OHM. Accordingly, the results presented in this work not only support the continuous protection against oxidative stress exerted by melatonin, through its free radical scavenging cascade, but also the important role of 3-OHM on the peroxyl radical scavenging activity of melatonin.

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Introduction

Melatonin is a versatile and ubiquitous molecule with a large, and well documented, variety of beneficial effects. One of them is its protective action against oxidative stress (OS) and its related deleterious consequences.[1–9] In addition, the ability of melatonin to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) is shared by its metabolites. This a very appealing, and rare, characteristic that warranties a continuous action in living organisms. Such an "endless" protection exerted by melatonin has been referred to as the free radical scavenging cascade,[10–14] and makes this compound particularly effective, even at low concentrations, in protecting organisms from OS.[12]

There are numerous reports supporting the antioxidant activity of melatonin metabolites N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK),[15–21] and N^1-acetyl-5-methoxykynuramine (AMK).[21–31] On the other hand, studies on the free radical scavenging activity of cyclic 3-hydroxymelatonin (3-OHM) are still rather scarce. This is probably because its identification and characterization is rather recent.[32] 3-OHM is formed when melatonin reacts with the hydroxyl radical (˙OH), and has been proposed as the foot-print product of melatonin interaction with two hydroxyl radicals (˙OH).[33] It has also been found after the reactions of melatonin with other oxidants.[14,34–36] 3-OHM reportedly efficiently scavenges ˙OH[13] and $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)).[14] To our best knowledge, these are the only two previous reports on the free radical scavenging activity of 3-OHM.

There are no kinetic data reported so far for the reactions of 3-OHM with free radicals, or on the relative importance of different reaction mechanisms and reaction sites. There is no information either on the influence of the environments' polarity on its reactivity towards free radicals. Likewise, there are no data on the peroxyl radical scavenging activity of 3-OHM, which may be particularly important since melatonin, AMK, and AFMK have been proposed to be rather ineffective for scavenging hydroperoxyl radicals (˙OOH).[37,21] Consequently, it is the main goal of this work to provide new information on these aspects.

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To that purpose, we have investigated the reactions of 3-OHM with the $^{\bullet}$OH and $^{\bullet}$OOH radicals through different reaction mechanisms, mimicking lipid and aqueous media. Two different radicals have been chosen because it was previously demonstrated that the relative importance of different reaction mechanisms depends not only on the scavenger but also on the chemical nature of the radical they are reacting with.[37–39] Thermodynamic and kinetic data is provided, as well as a quantitative assessment of the contributions of the different mechanisms, and channels of reaction, to the overall reactivity of 3-OHM towards the above mentioned radicals. Comparisons with melatonin, AMK and AFMK are also provided.

Computational details

All the electronic calculations were performed with the Gaussian 09 package of programs.[40] Geometry optimizations and frequency calculations were carried out using the M05-2X functional[41] and the 6-31+G(d,p) basis set, in conjunction with the Solvent Model Density (SMD) continuum model[42] using benzene and water as solvents to mimic lipid and aqueous environments, respectively. The M05-2X functional is recommended for kinetic calculations,[41] and it has been also successfully used for that purpose.[43–46] It is also among the best performing functionals for calculating reaction energies involving free radicals.[47] SMD is considered a universal solvation model, due to its applicability to any charged or uncharged solute in any solvent or liquid medium for which a few key descriptors are known.[42] Unrestricted calculations were used for open shell systems and local minima and transition states were identified by the number of imaginary frequencies (0 or 1, respectively). In the case of the transition states, it was verified that the imaginary frequency corresponds to the expected motion along the reaction coordinate, by Intrinsic Coordinate calculations (IRC). Thermodynamic corrections at 298.15 K were included in the calculation of relative energies, which correspond to 1 M standard state. In addition, the solvent cage effects have been included according to the corrections proposed by Okuno,[48] taking into account the free volume theory.[49]

The rate constants (k) were calculated using the Conventional Transition State Theory (TST)[50–52] and 1 M standard state as:

$$k = \sigma\kappa\frac{k_B T}{h}e^{-(\Delta G^{\neq})/RT} \quad (1)$$

where k_B and h are the Boltzmann and Planck constants, $\Delta G^{\neq}$ is the Gibbs free energy of activation (calculated as the difference in Gibbs free energy between the transition state and the isolated reactants), σ represents the reaction path degeneracy accounting for the number of equivalent reaction paths, and κ accounts for tunneling corrections.

The tunneling corrections, defined as the Boltzmann average of the ratio of the quantum and the classical probabilities, were calculated using the Zero Curvature Tunneling corrections (ZCT).[53] For the electron transfer (ET) reactions, the barriers were estimated using the Marcus theory.[54,55] It relies on the transition state formalism and defines the SET activation barrier ($\Delta G_{ET}^{\neq}$) as:

$$\Delta G_{ET}^{\neq} = \frac{\lambda}{4}\left(1 + \frac{\Delta G_{ET}^{0}}{\lambda}\right)^2 \quad (2)$$

where ΔG_{ET}^{0} is the free energy of reaction and λ is a reorganization term. In this work a very simple approximation has been made in order to calculate λ:

$$\lambda \approx \Delta E_{ET} - \Delta G_{ET}^{0} \quad (3)$$

where ΔE_{ET} has been calculated as the non-adiabatic energy difference between reactants and vertical products. This approach is similar to that previously used by Nelsen and coworkers[56,57] for a large set of self-exchange reactions.

In addition, some of the calculated rate constants (k) are close to the diffusion-limit; thus, the apparent rate constant (k_{app}) cannot be directly obtained from TST calculations. In the present work the Collins-Kimball theory[58] is used to that purpose:

$$k_{app} = \frac{k_D k_{act}}{k_D + k_{act}} \quad (4)$$

where k_{act} is the thermal rate constant, obtained from TST calculations, and k_D is the steady-state Smoluchowski[59] rate constant for an irreversible bimolecular diffusion-controlled reaction:

$$k_D = 4\pi R D_{AB} N_A \quad (5)$$

where R denotes the reaction distance, N_A is the Avogadro number, and D_{AB} is the mutual diffusion coefficient of the reactants A (free radical) and B (scavenger). D_{AB} has been calculated from D_A and D_B according to ref. 60, D_A and D_B have been estimated from the Stokes–Einstein approach:[61,62]

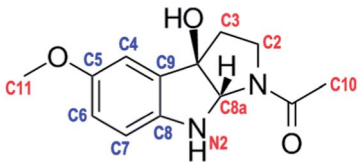
$$D = \frac{k_B T}{6\pi\eta a} \quad (6)$$

where k_B is the Boltzmann constant, T is the temperature, η denotes the viscosity of the solvent, in our case water ($\eta = 8.91 \times 10^{-4}$ Pa s) and benzene ($\eta = 6.04 \times 10^{-4}$ Pa s); and a is the radius of the solute.

The used methodology is referred to as Quantum Mechanics-based Test for Overall Free Radical Scavenging Activity (QM-ORSA), and is described in more details elsewhere,[63] where its reliability and accuracy has been demonstrated. The uncertainties arising from using this methodology have been proven to be no larger than those arising from experiments by direct comparisons with experimental values, for reactions involving diverse radicals and molecules and taking place in environments of different polarity and pH.[63]

Results and discussion

The structure and site numbering of 3-OHM are shown in Scheme 1. As it is the case for many other antioxidants,[64–70]



Scheme 1 Structure and site numbering of 3-OHM. Blue and red labels represent RAF and HT reaction sites, respectively.

different reaction mechanisms should be taken into account to assess the overall free radical scavenging activity of 3-OHM. Those considered in this work are:

-radical adduct formation (RAF):

$$\text{3-OHM} + {}^{\bullet}\text{R} \rightarrow [\text{3-OHM–R}]^{\bullet}$$

-hydrogen transfer (HT):

$$\text{3-OHM} + {}^{\bullet}\text{R} \rightarrow \text{3-OHM}_{(-\text{H})}{}^{\bullet} + \text{HR}$$

-single electron transfer (SET):

$$\text{3-OHM} + {}^{\bullet}\text{R} \rightarrow \text{3-OHM}^{+\bullet} + \text{R}^{-}$$

where $^{\bullet}$R represents the reacting radical. It should be noticed that in a previous work a hypothetical reaction pathway was proposed in which a mole of 3-OHM scavenges 2 mol of $^{\bullet}$OH, yielding AFMK as a final product.[13] Even though such mechanistic proposal escapes the purposes of the present work, it would certainly deserve further considerations.

The free radicals used to model the scavenging activity of 3-OHM are $^{\bullet}$OH and $^{\bullet}$OOH. The first one has been chosen for being the most electrophilic,[71] and reactive, of the oxygen-centered radicals with a half-life of $\sim 10^{-9}$ s,[72] and also because there is experimental evidence on the $^{\bullet}$OH scavenging activity of 3-OHM. Compared to $^{\bullet}$OH, peroxyl radicals ($^{\bullet}$OOR) are much less reactive species with half-lives in the order of seconds.[73] We have chosen $^{\bullet}$OOH because it is the simplest of the $^{\bullet}$OOR, and a good model for them when R = non-halogenated alkyl of alkenyl groups. This is because despite of the chemical differences between H and these R groups, their rate constants when reacting with organic molecules only differ moderately. For example the experimental rate constants for the reactions of linoleic acid with $^{\bullet}$OOH and $(CH_3)C(OH)OO^{\bullet}$ are $\sim 1.5 \times 10^3$ and 6×10^3 M^{-1} s^{-1}.[74,75] respectively. In the case of ascorbic acid the rate constants for its reactions with $^{\bullet}$OOH, and $CH_3OO^{\bullet}$, are 1.6×10^4, and 3×10^5 M^{-1} s^{-1},[76,77] respectively. Therefore the reactivity of these different peroxyl radicals toward organic molecules differs only to a moderate extent, in terms of kinetics. Moreover, in general the $^{\bullet}$OOH reactions are slower than those corresponding to other $^{\bullet}$OOR radicals. This means that if a particular molecule is able of efficiently scavenging $^{\bullet}$OOH, it would probably be, at least, equally efficient for scavenging other $^{\bullet}$OOR.

These radicals have been proposed to be involved in the oxidation of lipoproteins and biological membranes and are likely responsible for microvascular damage.[78] They are formed within living organisms, where they are involved in deoxyribonucleic acid (DNA) cleavage and protein backbone modification.[79] In the particular case of the $^{\bullet}$OOR studied in this work ($^{\bullet}$OOH), it was suggested to be central to the toxic side effects of aerobic respiration, and it has been pointed out that more information on the reactivity of this species is needed.[80] Since melatonin, AMK, and AFMK apparently are not efficient for scavenging this kind of free radicals, it seems important to investigate if other related molecule would efficiently scavenge $^{\bullet}$OOR.

For the reactions with $^{\bullet}$OH, all the reaction paths were found to be exergonic (Table 1), regardless of the polarity of the environment, with the exception of the SET reaction, in benzene solution. This is a logical finding because non-polar environments do not promote the necessary solvation of the intermediate ionic species yielded by this mechanism. For the $^{\bullet}$OOH reactions, on the other hand, the only thermochemically viable channel is that corresponding to HT from site N2 in aqueous solution. This is in line with the significant lower reactivity of $^{\bullet}$OOH, compared to $^{\bullet}$OH, as discussed above.

For the kinetic study we have not included the endergonic reaction paths because, even if they take place at a significant rate, they would be reversible and, therefore, the formed products would not be observed. However, they might still be significant if their products rapidly react further. This would be particularly important if the later stages are sufficiently exergonic to provide a driving force, and if their barriers of reactions are low. Due to the complexity of the biological systems, in which there are a wide variety of chemicals present, it is likely that it could be the case under such conditions. This would certainly be valid for the SET reactions in aqueous solution since they yield radical cations, which are prompt to easily, and rapidly, undergo deprotonation. In addition, slightly endergonic processes can be important when there are no exergonic

Table 1 Gibbs free energies of reaction (ΔG, kcal mol^{-1}), at 298.15 K, for the different mechanisms and reaction paths[a]

	Benzene		Water	
	$^{\bullet}$OH	$^{\bullet}$OOH	$^{\bullet}$OH	$^{\bullet}$OOH
SET	59.29	78.93	−10.26	13.24
HT				
Path 2	−25.94	6.94	−27.06	5.85
Path 3	−19.13	13.75	−21.01	11.91
Path 8a	−22.20	10.68	−22.33	10.59
Path N2	−29.06	3.82	−35.76	−2.84
Path 10	−20.00	12.88	−22.53	10.39
Path 11	−20.12	12.76	−22.00	10.92
RAF				
Path 4	−15.60	11.43	−13.83	13.68
Path 5	−18.92	9.15	−19.11	8.07
Path 6	−14.20	13.66	−15.86	10.64
Path 7	−17.50	8.78	−14.79	12.03
Path 8	−11.54	13.68	−12.46	12.98
Path 9	−21.57	9.04	−16.52	10.76

[a] The paths have been labelled according to the reaction sites presented in Scheme 1.

competing paths. This is the case of path HT-N2 for the reaction with ˙OOH in non-polar environment. Therefore, such a process has also been included in the kinetic calculations.

The optimized geometries of the transition states (TS) are shown in Fig. 1 and 2, which correspond to HT and RAF reaction paths, respectively. It was not possible to locate the TSs corresponding to the N2 path for the reaction with ˙OH in aqueous solution using full optimizations, even though it was located and characterized in a benzene solution (Fig. 1, TSN2). Using partial optimizations with frozen N–H and H–OH bond distances, we obtain a structure that presents a single imaginary frequency corresponding to the desired transition vector. Unfreezing these two distances, during a saddle point optimization, invariably led to an increase of the H–OH distance, and the corresponding reduction of the imaginary frequency and gradient, yielding the separated reactants. A relaxed scan, obtained by decreasing the H–OH distance, produces a similar result, *i.e.*, the energy drops until the H atom is completely transferred. This means that the reaction is barrier-less and strictly diffusion-controlled. In other words, every encounter is effective in producing the conversion of reactants into products.

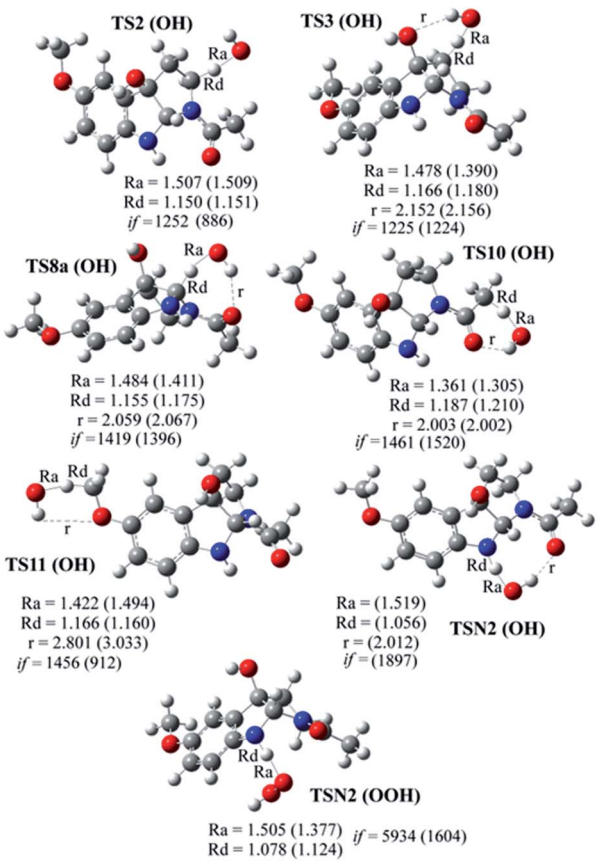


Fig. 1 Optimized geometries of the transition states corresponding to HT mechanism, in water (benzene) solution and their imaginary frequencies (*if*, cm^{-1}). R_a = distance between the transferring H and the acceptor atom, R_d = distance between the transferring H and the donor atom, r = interaction distance. The transition states have been labelled according to the reaction sites presented in Scheme 1, and the reacting radical is specified in parenthesis.

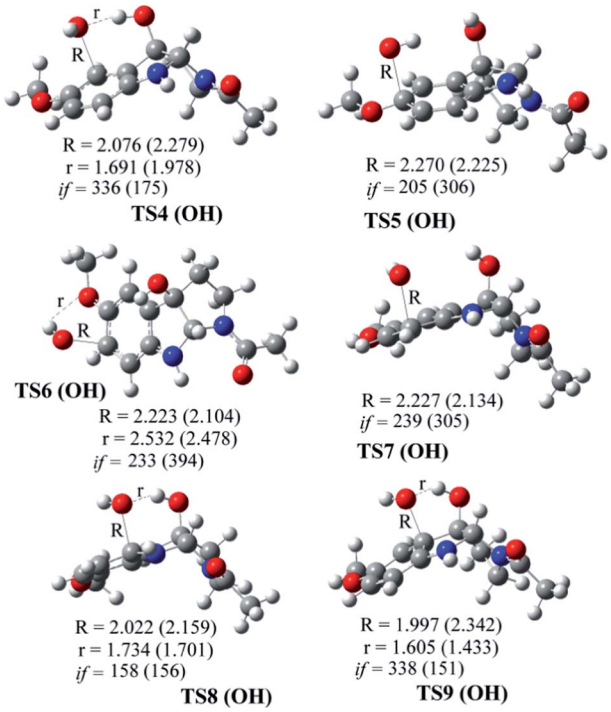


Fig. 2 Optimized geometries of the transition states corresponding to the RAF mechanism, in water (benzene) solution and their imaginary frequencies (*if*, cm^{-1}). R = distance of the forming bond, r = interaction distance. The transition states have been labelled according to the reaction sites presented in Scheme 1, and the reacting radical is specified in parenthesis.

Several of the located transition states present hydrogen bonding (HB) interactions between the reacting fragments, *i.e.*, between the radical and the 3-OHM molecule. For the HT paths they are TS3, TS8a, TS10, and TSN2, which present interaction distances (r) ranging from 2.00 Å to 2.15 Å. The r values were found to be very similar for each TS, regardless of the polarity of the environment. TS11, on the other hand, presents a much weaker interaction in aqueous solution with $r = 2.80$ Å; while in benzene solution the r value is too large for a proper HB. The earliest TS was found to be that corresponding to HT from the N2 site. In fact, it corresponds to a very early TS, which suggests that this site should be particularly reactive. In addition, the TSs in aqueous solution were found to be earlier than the corresponding ones in non-polar media, for most reaction paths. This suggests that the reactivity of 3-OHM towards oxygenated free radicals, *via* HT, increases with the polarity of the environment.

Regarding the RAF paths, transition states TS4, TS6, TS8, and TS9 also present HB interactions. In this case the r values range from 1.61 Å to 2.53 Å in aqueous solution and from 1.43 Å to 2.48 Å in benzene solution. In general the interaction distances were found to be shorter in the non-polar media, with the exception of TS4. In addition, some of the HB distances were found to be rather short, indicating strong interactions. The earliest transition states were found to be TS9 and TS6, in aqueous and benzene solutions, respectively. This indicates that the relative site reactivity, *via* RAF, might be influenced by the environment.

Table 2 Gibbs energies of activation ($\Delta G^{\neq}$, kcal mol^{-1}), at 298.15 K, for the different mechanisms and reaction paths[a]

	Benzene		Water	
	˙OH	˙OOH	˙OH	˙OOH
SET			0.02	14.12
HT				
Path 2	5.15		5.47	
Path 3	4.17		6.20	
Path 8a	2.92		3.22	
Path N2	1.29	16.85	0.00	13.54
Path 10	6.24		8.07	
Path 11	4.79		6.04	
RAF				
Path 4	~0.00		~0.00	
Path 5	1.75		~0.00	
Path 6	5.07		0.93	
Path 7	3.52		~0.00	
Path 8	~0.00		~0.00	
Path 9	~0.00		~0.00	

[a] The paths have been labelled according to the reaction sites presented in Scheme 1.

Table 3 Rate constants of the different channels of reaction, and overall rate coefficient (M^{-1} s^{-1}), at 298.15 K, for the different mechanisms and reaction paths[a]

	Benzene		Water	
	˙OH	˙OOH	˙OH	˙OOH
SET			8.17×10^9	2.77×10^2
HT				
Path 2	1.55×10^9		1.09×10^9	
Path 3	2.73×10^9		3.40×10^8	
Path 8a	3.38×10^9		2.27×10^9	
Path N2	3.64×10^9	3.16×10^1	2.48×10^9	2.84×10^6
Path 10	7.33×10^8		9.79×10^7	
Path 11	2.23×10^9		1.45×10^9	
RAF				
Path 4	2.81×10^9		1.91×10^9	
Path 5	2.80×10^9		1.91×10^9	
Path 6	1.29×10^9		1.91×10^9	
Path 7	2.59×10^9		1.91×10^9	
Path 8	2.81×10^9		1.91×10^9	
Path 9	2.81×10^9		1.91×10^9	
Overall	2.94×10^{10}	3.16×10^1	2.73×10^{10}	2.84×10^6

[a] The paths have been labelled according to the reaction sites presented in Scheme 1.

The Gibbs free energies of activation ($\Delta G^{\neq}$) for the different mechanisms and channels of reaction are reported in Table 2. For the reactions with ˙OH, several reaction paths were found to be almost barrierless and, in general, all the $\Delta G^{\neq}$ values for the reactions with this radical are low. For the paths involved in the reactions of ˙OOH, the $\Delta G^{\neq}$ values are significantly higher, which is in line with the relative low reactivity of this radical, compared to ˙OH.

The k_D values, estimated using eqn (5), for the 3-OHM + ˙OH reactions are 8.2×10^9 and 1.2×10^{10} in water and benzene, respectively. The calculated k_{act} values for the different reaction paths range from 3.9×10^8 to 2.6×10^{15} in aqueous solution, and from 9.2×10^8 to 2.8×10^{15} in benzene solution. In both cases, at least for 7 reaction paths, it was found that $k_{act} > k_D$. Therefore, it can be stated that the overall reaction of ˙OH with 3-OHM corresponds to the diffusion limit regime, regardless of the polarity of the environment (Table 3). This indicates that 3-OHM is an excellent ˙OH free radical scavenger, as is the case of melatonin, AMK, and AFM. Therefore, the protection against this radical is expected to be preserved after melatonin is metabolized. In aqueous solution, 3-OHM was found to react 1.06, 1.82, and 1.48 times faster than AMK,[21] AFMK[21] and melatonin,[37] respectively, while in benzene solution these ratios are 1.11, 2.13, and 1.32. This means that the reactivity of the four compounds towards ˙OH is similar, regardless of the environment's polarity.

Regarding the ˙OOH scavenging activity of 3-OHM, we have compared its efficiency with those of AMK, AFMK, melatonin and Trolox using previously reported data (calculated with a methodology similar to that used in this work). Based on such comparisons it is predicted that in non-polar media, 3-OHM is 2.96 and 6.90 more efficient than AMK and AFMK,[21] respectively. In contrast, it reacts about 9.85 times slower than its precursor melatonin.[37] Additionally, in such a medium, 3-OHM was found to be significantly less reactive towards ˙OOH (about 100 times) than Trolox,[81] which is frequently used as an antioxidant reference. This suggests that in non-polar media 3-OHM is rather ineffective for scavenging ˙OOH, as is also the case for melatonin, AMK and AFMK. On the contrary, in aqueous solution 3-OHM was found to react with ˙OOH about 98.4 times faster than Trolox, with an overall rate constant in the order of 10^6 M^{-1} s^{-1}. According to this value, and taking into account that the rate constants corresponding to the ˙OOH damage to polyunsaturated fatty acids are in the range 1.18–3.05×10^3 M^{-1} s^{-1},[82] it can be stated that 3-OHM is a very good ˙OOH scavenger. Considering that, usually, the rate constants of peroxyl radicals (˙OOR), with R = non-halogenated alkyl or alkenyl groups, differ only moderately it can be hypothesized that perhaps 3-OHM could also be a good scavenger for these radical species. However this point remains to be demonstrated.

This may be a very important finding since in aqueous solution melatonin, AMK and AFMK remain rather ineffective as peroxyl radical scavengers, while 3-OHM is predicted to be very efficient. In fact, the overall rate coefficient for the reaction of ˙OOH with 3-OHM was found to be orders of magnitude higher, under such conditions, than those of melatonin, AMK and AFMK. Therefore, it is proposed that in aqueous phase the protective effects of melatonin against peroxyl radical become important after it is metabolized to 3-OHM, *i.e.*, the formation of this compound seems to be crucial for the peroxyl radical scavenging activity of melatonin.

Such an increased reactivity seems to arise from the HT involving site N2. This particular reaction path was found to be responsible for more than 99% of the overall reactivity of 3-OHM towards ˙OOH in aqueous solution (Table 4). Under

Table 4 Branching ratios (Γ) of the different channels of reaction, at 298.15 K, for the different mechanisms and reaction paths[a]

	Benzene		Water	
	$^{\bullet}$OH	$^{\bullet}$OOH	$^{\bullet}$OH	$^{\bullet}$OOH
SET			29.88	0.01
HT				
Path 2	5.29		4.00	
Path 3	9.30		1.24	
Path 8a	11.51		8.31	
Path N2	12.38	100.00	9.07	99.99
Path 10	2.49		0.36	
Path 11	7.60		5.29	
RAF				
Path 4	9.57		6.98	
Path 5	9.53		6.98	
Path 6	4.39		6.97	
Path 7	8.81		6.98	
Path 8	9.57		6.98	
Path 9	9.57		6.98	
Total SET	0.00		29.88	0.01
Total HT	48.56	100.00	28.26	99.99
Total RAF	51.44	0.00	41.86	0.00

[a] The paths have been labelled according to the reaction sites presented in Scheme 1.

such conditions, the reaction barrier is about 3.3 kcal mol^{-1} lower than the equivalent one in non-polar solution. This difference alone accounts for more than two orders of magnitude in k. In addition the reaction barrier in this case was found to be very thin, the imaginary frequency of the transition state is 5933.6 cm^{-1}. Thus the tunneling correction is rather huge (κ = 3873.3). This peculiarity seems to be responsible for the rest of the increase in the rate constant. Further experiments on isotopic effects would be helpful for supporting, or refuting, this hypothesis.

In order to confirm these results, for this particular case we have also tested the possible influence of water molecules on the HT reaction from site N2. To that purpose two explicit water molecules were included, one in the vicinity of each reactant. The energy barrier calculated this way was found to be slightly higher (14.48 kcal mol^{-1} instead of 13.54 kcal mol^{-1}) than the one obtained using only SMD. This means that the difference is below the quantum chemical accuracy (1 kcal mol^{-1}). The rate constant arising from the calculations with the explicit water molecules was found to be 6.53 $\times$ 10^5 M^{-1} s^{-1}, *i.e.* 4.3 times lower than the one obtained using only the continuum model alone. This value remains significantly higher than the threshold value (1.18–3.05 $\times$ 10^3 M^{-1} s^{-1}), and supports the key role of 3-OHM on the peroxyl radical scavenging activity of melatonin. The transition state located for this reaction is shown in Fig. 3. For the reactions with $^{\bullet}$OH we have not tried the hybrid explicit/implicit solvation model since it has been previously demonstrated that for this particular radical the kinetic data does not significantly change with respect to that obtained when the pure continuum model is used.[82]

In addition, to further verify the rate constant for the HT reaction from site N2, it was also calculated using other

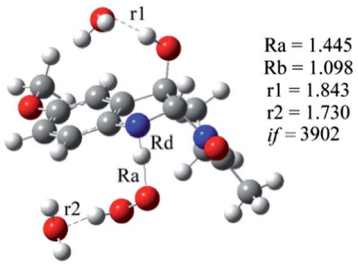


Fig. 3 Optimized geometry of the transition state corresponding to HT from site N2, including two explicit water molecules, and its imaginary frequency (*if*, cm^{-1}). R_a = distance between the transferring H and the acceptor atom, R_d = distance between the transferring H and the donor atom, r_1 and r_2 = interaction distances.

methods, known for their reliability for kinetic calculations (M06-2X and MN12SX). The values obtained with them are 6.12 $\times$ 10^6 M^{-1} s^{-1} and 1.66 $\times$ 10^7 M^{-1} s^{-1}, *i.e.* 2.2 and 14.4 times higher than the value obtained with the M05-2X functional. This means that all the methodologies used in this work support the important role of 3-OHM on the peroxyl radical scavenging activity of melatonin. Considering the values of the rate constant for this channel of reaction altogether we have estimated an average value of 1.3 $\times$ 10^7 M^{-1} s^{-1}, which indicates a fast reaction between 3-OHM and peroxyl radicals.

To investigate in detail the relative importance of the different mechanisms and paths of reaction to the overall reactivity of 3-OHM towards $^{\bullet}$OH and $^{\bullet}$OOH, the branching ratios have been estimated. They represent the percent contribution of the different paths to the overall reaction, and have been calculated as:

$$\Gamma_i = \frac{k_i}{k_{\text{overall}}} \times 100 \quad (7)$$

where k_i represents each reaction path.

We predict a wide product distribution for the reaction with $^{\bullet}$OH, regardless of the polarity of the environment (Table 4), which is in line with the high reactivity, *i.e.*, low selectivity of this radical. Regarding the relative importance of the different reaction mechanisms, in non-polar solutions it is predicted that HT and RAF are similarly important, with contributions to the whole reactivity of 3-OHM towards $^{\bullet}$OH close to 50%.

In aqueous solution the percent contributions of the different reaction mechanisms studied in this work were found to be about 40%, 30%, and 30% for RAF, SET, and HT, respectively. For the reaction with $^{\bullet}$OOH, on the other hand, HT was found to be the only relevant mechanism, regardless of the solvent polarity. Moreover, path N2 seems to be responsible for almost the entire $^{\bullet}$OOH scavenging activity of 3-OHM. This means that the presence of this site is a key structural feature on the protection of 3-OHM against the damage caused by peroxyl radicals.

Conclusions

The free radical scavenging activity of the melatonin metabolite, 3-OHM, has been investigated considering three mechanisms of reaction: radical adduct formation (RAF), hydrogen transfer

(HT), single electron transfer (SET); in environments of different polarity. For that purpose, two different free radicals were used (˙OH and ˙OOH).

It was found that 3-OHM reacts with ˙OH at diffusion-limited rates, regardless of the polarity of the environment, which supports its excellent ˙OH radical scavenging activity. The overall reactivity of 3-OHM towards this radical was found to be similar, but slightly higher than those of melatonin, AMK and AFMK. Regarding the relative importance of the different mechanisms of reaction, a wide product distribution is expected for the reaction of 3-OHM with ˙OH, regardless of the conditions under which the reactions take place. For this particular free radical, all the studied reaction mechanisms significantly contribute to the overall scavenging activity of 3-OHM.

For the reaction with ˙OOH, 3-OHM was found to react at orders of magnitude faster, in aqueous solution, than melatonin, AMK, and AFMK. Furthermore, under such conditions, 3-OHM was found to react with ˙OOH about 98.4 times faster than Trolox. This seems to be a very important finding since melatonin, AMK, and AFMK have been proposed to be rather ineffective as peroxyl radical scavengers, while 3-OHM is predicted to be very efficient. Consequently, it is proposed that the protective effects of melatonin against peroxyl radicals become important after it is metabolized to 3-OHM.

Accordingly, the results presented in this work not only support the continuous protection against OS exerted by melatonin, through the free radical scavenging cascade, but also the important role of cyclic 3-hydroxymelatonin, regarding the peroxyl radical scavenging activity of melatonin.

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