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N-Acetylserotonin and 6-Hydroxymelatonin against Oxidative Stress: Implications for the Overall Protection Exerted by Melatonin

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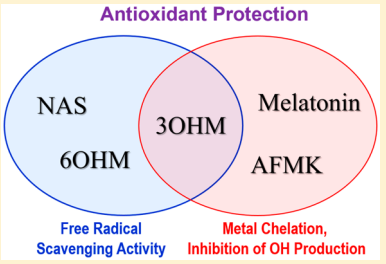
Supporting Information

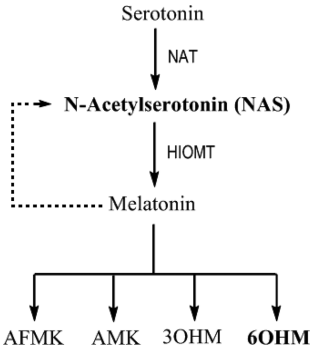
ABSTRACT: The protection exerted by *N*-acetylserotonin (NAS) and 6-hydroxymelatonin (6OHM) against oxidative stress was investigated using the density functional theory. It was found that these compounds are better peroxyl radical scavengers than melatonin itself, Trolox, caffeine, or genistein both in lipid and aqueous solutions. The related kinetic data is provided for the first time. The solvent polarity influences not only the absolute reactivity of NAS and 6OHM toward peroxyl radicals, but also their relative scavenging activity. In addition, they both fully inhibit the oxidative effects of copper-ascorbate mixtures, and $^{\bullet}$OH production via the Haber−Weiss reaction, albeit the effects on the later are only partial. On the basis of comparisons with other melatonin-related compounds, it is proposed that the role of NAS and 6OHM on the overall protection exerted by melatonin against oxidative stress is mainly related to their free radical scavenging activities. Moreover, they increase such protection. The role of the phenol moiety on such activity is demonstrated.



INTRODUCTION

N-Acetylserotonin (NAS), also known as normelatonin, is the immediate precursor of melatonin in the tryptophan metabolic pathway in mammals. Within this pathway, serotonin is converted to NAS by *N*-acetylatransferase (NAT), which is further converted to melatonin by hydroxyindole *O*-methyltransferase (HIOMT) (Scheme 1). In turn, melatonin yields several metabolites including N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), N^1-acetyl-5-methoxykynuramine (AMK), cyclic 3-hydroxymelatonin (3OHM), and 6-hydroxymelatonin (6OHM). In addition, melatonin can also be metabolized back into NAS.[1]

Scheme 1. Fragment of the Metabolic Route of Melatonin



Each of these melatonin-related compounds may play important roles in human health. In the particular case of NAS, its beneficial effects have been widely documented. It was found to offer neuroprotection by inhibiting mitochondrial death pathways and autophagy activation,[2] and to protect from oxidative stress injuries caused by H_2O_2.[3] NAS has been found to exhibit antioxidant and antiaging activities,[4] as well as to improve cognition and protect against β-amyloid-induced neurotoxicity.[5] It has also been suggested that NAS plays a protective role in preserving optimal fluidity of the biological membranes, which is attributed to its ability to reduce lipid peroxidation.[6] There is evidence supporting the protective effects of NAS against DNA damage caused by exposure to Cr^{3+} and H_2O_2,[7] and that it inhibits low-density lipoprotein oxidation induced by copper.[8,9] In addition, the potential role of NAS, and melatonin, in the treatment of multiple sclerosis has been recently highlighted.[10]

Albeit NAS is both a precursor and a metabolite of melatonin, its protective effects against oxidative damages seem to be independent from those of melatonin.[11,12]In fact, it was hypothesized that since about 15% of melatonin is demethylated into NAS *in vivo*, some of the beneficial effects of melatonin may be mediated by NAS. Moreover, it has been proposed that the antioxidant properties of NAS against *tert*-butylated hydroperoxide- and diamide-induced reactive oxygen species is higher than that of melatonin, and that 10 μM NAS is more effective than Trolox (at the same concentration) for scavenging peroxyl radicals.[13] It was also reported that NAS is more efficient than melatonin for protecting against iron

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induced lipid peroxidation,[14,15] and copper-mediated oxidation of low density lipoproteins.[8]

6OHM is the major hepatic metabolite and photodegradation product of melatonin. It is described as being efficient for protecting against oxidative damage induced by UV irradiation.[16] It significantly reduces KCN-induced superoxide anion generation, and the associated lipid peroxidation, which led to the proposal that 6OHM is a protector against KCN-induced neurotoxicity.[17] It has been reported that this melatonin metabolite scavenges singlet oxygen and the superoxide anion, thus reducing the quinolinic-acid induced oxidative neurotoxicity.[18] It has also been shown that 6OHM decreases Fe^{2+}-induced neurotoxicity,[19] and iron-induced lipid peroxidation.[20] In addition, lipo-peroxidation induced by thiobarbituric acid, which is attributed to the production of reactive oxygen species (ROS), is inhibited by 6OHM.[21] Moreover, it may be more efficient than melatonin in this capacity.

In summary, there is abundant evidence supporting the protective effects of both NAS and 6OHM against free radical and metal-induced molecular damage. However, there is still lack of information on the mechanisms that contribute to this protection, and on their site reactivity. The latter may be relevant to justify whether the presence of a phenol moiety in these compounds can be held responsible of the findings suggesting that both NAS and 6OHM are more efficient than melatonin itself as antioxidants. There is no information on the kinetics associated with their free radical scavenging activity. Additionally, while other metabolites such as AMK and AFMK have been identify to be involved in the overall protection (or scavenging cascade) exerted by melatonin,[22−24] the role of NAS and 6OHM in this context is less clear. Moreover, most of the melatonin-related compounds including melatonin itself, AMK and AFMK, are rather ineffective for scavenging peroxyl radicals ($ROO^{\bullet}$),[25,26] while they are highly biologically relevant in scavenging and reducing oxidative stress.[27] Thus, is important to establish if NAS and 6OHM are capable of scavenging these same free radicals, thereby contributing to the overall protection exerted by melatonin. Accordingly, it is the main goal of the present work to provide information on these aspects, which may widen the physico-chemical knowledge on the role of NAS and 6OHM as protectors against oxidative stress.

COMPUTATIONAL DETAILS

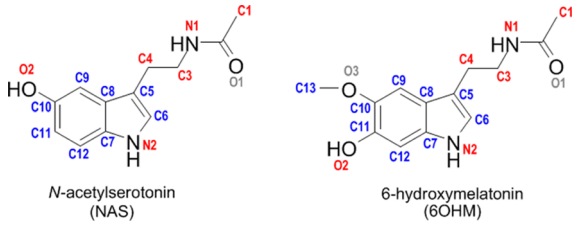
All the electronic calculations were performed with Gaussian 09 package of programs.[28] The M05-2X and M05 functionals[29] were used for geometry optimizations and frequency calculations for the systems without and with Cu, respectively. All the calculations were carried out with the 6-31+G(d) basis set and the continuum solvation model based on density (SMD).[30] The electronic energies were further improved by single point calculations with the 6-311++G(d,p) basis set. Pentyl ethanoate and water were used as solvents to mimic lipid and aqueous environments, respectively, for the free radical scavenging activity. On the other hand, the metal chelation was modeled only in aqueous solution, because this is the relevant phase for ionic species. The M05-2X functional has been chosen because it is recommended for kinetic calculations by its developers,[29] and its reliability has been independently confirmed by other authors.[31−41] It is among the best performing functionals for kinetic calculations in solution,[42] and for modeling reaction energies involving free radicals.[43] The M05 functional was chosen for the Cu involving systems because it was parametrized including both metals and nonmetals, while M05-2X has double the amount of nonlocal exchange (2×) and was parametrized mainly for nonmetals. M05 has been recommended for studies involving both metallic and nonmetallic elements, and has been reported to perform well not only for main-group thermochemistry but also for interactions with transition-metals.[29] SMD was chosen for mimicking the solvent effects because it can be consistently used for any charged or uncharged solute in any solvent or liquid medium.[30]

Local minima and transition states were identified by the number of imaginary frequencies (0 or 1, respectively). In the case of the transition states, intrinsic coordinate calculations (IRC) were performed to verify that the imaginary frequency corresponds to the proper motion along the reaction coordinate. Unrestricted calculations were used for open shell systems. Thermodynamic corrections at 298.15 K were included in the calculation of relative energies. In addition, the solvent cage effects have been considered based on the corrections proposed by Okuno,[44] and taking into account the free volume theory.[45] The rate constants (k) were calculated using the conventional transition state theory (TST)[46−48] and 1 M standard state, including zero curvature tunneling corrections (ZCT).[49] These computational details are in line with the quantum mechanics based test for overall free radical scavenging activity (QM-ORSA),[50] which has been validated by comparison with experimental results; its uncertainties have been proven to be no larger than those arising from experiments.[50]

RESULTS AND DISCUSION

Free radical scavenging activity. The structures of NAS and 6OHM, as well as their site numbering are shown in Scheme 2. Their free radical scavenging activity have been investigated using the hydroperoxyl radical ($HOO^{\bullet}$) as a model of $ROO^{\bullet}$. It is not only the simplest member of the $ROO^{\bullet}$ family but also has been suggested as crucial to the toxic side effects of aerobic respiration.[51]

Scheme 2. Structures and Site Numbering of NAS and 6OHM[a]



[a]Blue and red labels represent RAF and formal HAT reaction sites, respectively.

The free radical scavenging activity of chemical compounds may include different reaction mechanisms.[52−58] Those considered in this study are single electron transfer (SET), radical adduct formation (RAF), and formal hydrogen atom transfer (HAT). The Gibbs free energy of reaction (ΔG) for each reaction channel are reported in Table 1. It was found that the SET reactions, for both NAS and 6OHM, are largely endergonic in lipid media, since this environment does not provide the necessary solvation for the ionic species yielded by

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Table 1. Gibbs Free Energies of Reaction (ΔG, kcal/mol) at 298.15 K

	6OHM		NAS	
	PE[a]	W[a]	PE	W
SET	56.95	14.89	62.66	21.37
RAF				
C5	10.99	9.23	11.23	8.87
C6	1.11	−1.54	1.95	1.33
C9	11.28	7.52	7.90	6.72
C10	12.91	9.98	12.55	11.74
C11	8.68	5.09	13.34	10.77
C12	9.86	8.56	10.46	9.10
HAT				
C1	13.09	11.03	12.22	10.77
C3	10.40	10.57	10.36	8.78
C4	1.11	−1.35	1.34	0.42
C13	12.29	10.89		
O2	−4.20	−9.17	−3.03	−5.52
N1	21.56	22.64	22.11	27.43
N2	3.80	0.56	5.76	2.51

[a]W = aqueous solution, PE = pentyl ethanoate (lipid) solutions.

this process. In aqueous solution the endergonicity of the SET processes decreases, but still remains significant.

Most of the RAF pathways are also endergonic, with the exception of that corresponding to site C6 in 6OHM, when the reaction takes place in aqueous solution. In addition, while the adducts at sites C7 and C8 were considered at first, any attempt to locate them invariably led to structures that correspond to weak-bonded complexes rather than to the proper radical adducts (Figure 1S, Supporting Information). Therefore, they were ruled out as viable products of the reaction. Regarding the formal HAT mechanism, the reactions involving the phenolic OH are systematically predicted as exergonic. In fact, for each pair antioxidant/solvent this is the most thermochemically favored reaction pathway. In the particular case of 6OHM, formal HAT from site C4 is also exergonic when the reaction takes place in aqueous solution.

The thermochemical data was used to assess the potential viability of the different reaction pathways. Since those with $\Delta G > 0$ are reversible, the corresponding products are not expected to be observed, even if they take place at a significant rate. Therefore, they have been ruled out as viable and, consequently, are not included in the kinetic study.

The fully optimized geometries of the transition states (TS) are shown in Figures 1 and 2 for NAS and 6OHM, respectively. For both compounds the TS corresponding to the formal HAT

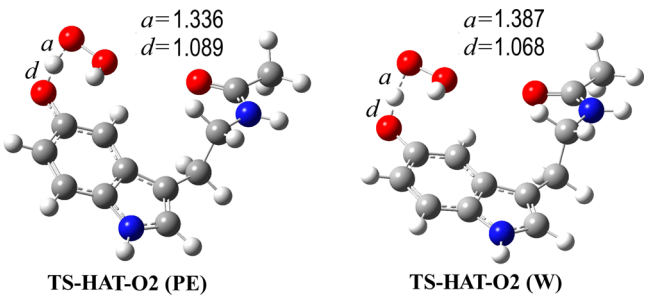

Figure 1. Optimized geometries of the transition states corresponding to NAS. Distances are reported in Å. W = aqueous solution; PE = pentyl ethanoate (lipid) solutions.

from the phenolic site is earlier in aqueous solution than in lipid, as indicated by the distances involved in the TS structures. This suggests that the polarity of the environment may influence their reactivity toward peroxyl radicals. In addition, the TS of the only viable RAF channel (site C6 in 6OHM) presents a H bond like interaction between the H in the HOO$^{\bullet}$ and the N in the pyrrole ring. For the other TSs, this interaction was not found. The imaginary frequencies for each of the located TSs are provided in Table 1S (Supporting Information).

The rate constants of each individual reaction channel, as well as the total rate coefficients for each of the studied compounds in both aqueous and lipid solution are reported in Table 2. The values of the energy barriers and the tunneling corrections used to obtain this data is provided as Supporting Information (Tables 2S and 3S). It was found that the values of the total rate coefficients increase with the polarity of the solvent, in line with the geometrical features of the TS, described above. The overall reactions of NAS and 6OHM are predicted to be about 17 and 600 times faster in aqueous than in lipid solution, respectively. In the latter NAS reacts with HOO$^{\bullet}$ about 11 times faster than 6OHM, while in water 6OHM reacts about 3 times faster than NAS. Therefore, the polarity of the media not only influences the absolute reactivity of the studied compounds toward peroxyl radicals but also their relative scavenging activity.

The total rate coefficients calculated for the reactions of NAS and 6OHM are larger than that associated with the HOO$^{\bullet}$ damage to polyunsaturated fatty acids ($1.18-3.05 \times 10^3$ M^{-1} s^{-1}).[51] This is particularly significant in aqueous solution, where NAS and 6OHM react about 3 orders of magnitude faster. It seems important to note that the reactivity of other biological targets, such as DNA and proteins, is lower than that of bis-allylic hydrogens in polyunsaturated acids, i.e., their rate coefficients are lower than the above-mentioned threshold.[59] Thus, NAS and 6OHM would also react faster than them with peroxyl radicals. Accordingly, it can be stated that these two compounds efficiently scavenge HOO$^{\bullet}$, thereby contributing to increase the overall protection exerted by melatonin against oxidative stress.

Regarding the site reactivity and the relative importance of the different reaction mechanisms, the only significant reaction is the formal HAT from the phenolic OH for NAS in both solvents, and for 6OHM in lipid solution. The only case in which there is more than one competing reaction pathway is that of 6OHM in an aqueous solution. Thus, in this case the contributions of the different mechanisms and reaction pathways to the overall HOO$^{\bullet}$ scavenging activity were further investigated by estimating the corresponding branching ratios (Γ). They were calculated as

$$\Gamma_i^{6OHM(W)} = \frac{k_i}{k_{total}} \times 100 \quad (1)$$

where i represents each individual reaction pathway. On the basis of these calculations, formal HAT from the phenolic OH is the most important pathway, with a contribution of 89.9% to the total activity. RAF at site C6 has a lower, but significant contribution (10.0%), while the relative importance of formal HAT from site C4 is negligible (0.1%). Thus, the presence of a phenol group in NAS and 6OHM, which is lacking in melatonin, AMK, and AFMK is proposed to be responsible for the increased reactivity of NAS and 6OHM toward peroxyl

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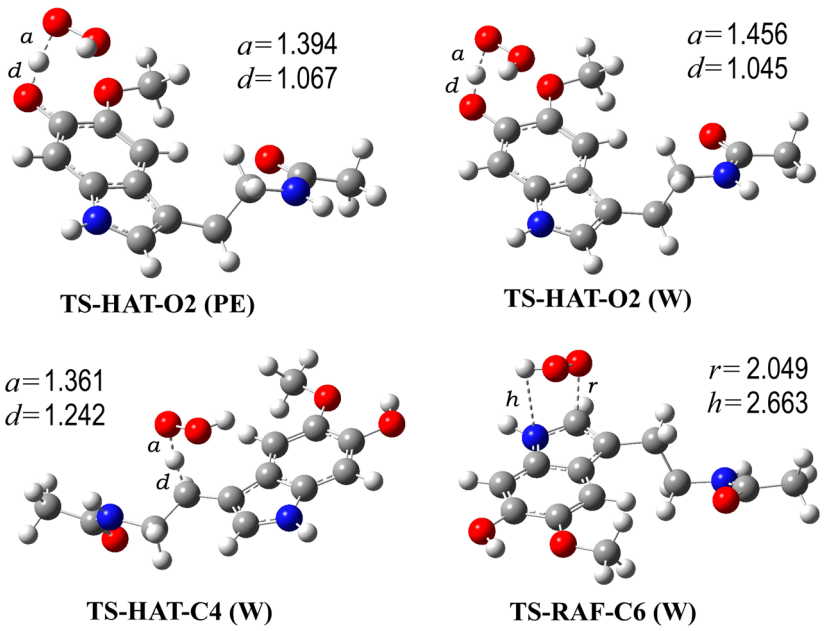

Figure 2. Optimized geometries of the transition states corresponding to 6OHM. Distances are reported in Å. W = aqueous solution; PE = pentyl ethanoate (lipid) solutions.

Table 2. Rate Constants of Each Individual Reaction Channel and total Rate Coefficients (M^{-1} s^{-1}), at 298.15 K[a]

	6OHM		NAS	
	PE[b]	W	PE	W
RAF-C6		3.62×10^{05}		
HAT-C4		5.02×10^{03}		
HAT-O2	5.81×10^{03}	3.25×10^{06}	6.70×10^{04}	1.17×10^{06}
total	5.81×10^{03}	3.62×10^{06}	6.70×10^{04}	1.17×10^{06}

[a]Details on the kinetics calculations are provided as Supporting Information. [b]W = aqueous solution; PE = pentyl ethanoate (lipid) solutions.

radicals. The only other melatonin-related compound that has been identified so far as able of scavenging peroxyl radicals is 3OHM.[60]

Metal Chelation and Inhibition of •OH Production. In addition to the free radical scavenging activity, there are other ways in which chemical compounds can protect against oxidative stress.[61] One of them involves metal chelation, which may reduce the production of •OH, which is one of the most reactive and harmful free radicals. In fact, its reactivity is so high that it would react with almost any molecule in the vicinity of its formation site. Therefore, the best way to prevent •OH from damaging biological targets is to inhibit its production. Within biological systems this radical can be formed by the Fenton reaction or by the Haber–Weiss reaction. However, the latter is so slow that it would be of no physiological importance, unless it is catalyzed by metal ions:[62]

$$M^{+q} + O_2^{\bullet -} \rightarrow M^{+(q-1)} + O_2$$

$$M^{+(q-1)} + H_2O_2 \rightarrow M^{+q} + OH^- + {}^{\bullet}OH$$

Here M can be for example iron or copper, and the second step corresponds to the Fenton reaction. Since M acts as a catalyst, thorough the overall Haber–Weiss process only $O_2^{\bullet -}$ and H_2O_2 are actually consumed, while the M^{+q} is completely regenerated. Thus, a large number of •OH can be produced with only very small amounts of M^{+q}. In addition, since Fe^{3+} and Cu^{2+} are more abundant than their reduced forms, it is likely that the first step of the Haber–Weiss process is the key reaction for •OH production, rather than a direct Fenton reaction. Thus, inhibiting this step may prevent •OH formation and the consequent oxidative damage. One possible way to achieve that is by chelating the M^{+q} ions, provided that the feasibility of the M^{+q} reduction in the chelates decreases compared to the same process for free M ions.

In the present study, we have chosen copper as the catalytic metal involved in the •OH production for several reasons. There is evidence that copper may be involved in the pathogenesis of several neurodegenerative disorders,[63] which relates to its involvement in the formation of oxidative species,[64] in particular of •OH.[63] The oxidative-induced toxicity of Cu^{2+} has been found to be greater than that of Fe^{3+} under identical experimental conditions.[65,66] There are previous studies on the chelating ability of melatonin-related compounds using copper. Thus, it would allow straightforward comparisons.

The first aspect to explore in the analyses of the potential role of NAS and 6OHM as inhibitors of the •OH production, via metal chelation, is whether the formation of the corresponding chelates is thermochemically viable. To that purpose "free" copper was modeled in its hydrated form, i.e., coordinated to water molecules, because it is more likely than the naked ion under physiological conditions. All the calculations in this section have been performed only for an aqueous solution because it is the relevant one for ionic species. Four water molecules were chosen since it was previously reported that the most likely configuration of Cu^{2+} water complexes, in the aqueous phase, corresponds to a nearly square-planar four-coordinate geometry.[67,68] The Cu^{1+} ions were modeled with the same amount of water molecules for consistency purposes, albeit in this case a two-coordinated configuration is preferred, which means that two of the water molecules are just solvating the system. This two-coordinated,

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linear, Cu^{1+} structure is consistent with previous experimental evidence.[69−71]

All the possible chelation sites in NAS and 6OHM have been explored, i.e., those involving N and O atoms (Scheme 1). In addition, the possibility of these compounds acting as both monodentate and bidentate ligands has been taken into account. Two different chelation routes have been studied, i.e., the direct chelation (DCM) and the coupled-deprotonation-chelation (CDCM) mechanisms. Since the later simultaneously involves Cu^{2+} chelation and deprotonation of the reactive site in the ligand, its equilibrium constant and Gibbs energy explicitly depend on the *p*H. More details on the CDCM processes can be found elsewhere.[61,72] In this work the reported data correspond to *p*H = 7.4.

The Gibbs energies of all the modeled chelation reactions are provided in Table 3. They were found to be >0 for all the N chelation sites, in both NAS and 6OHM, and for both chelation routes (DCM and CDCM). Therefore, these complexes are not expected to be formed to a significant extent. Conversely, some of the chelation reactions involving O sites are exergonic. They are those involving O1 via DCM, which is the only possible chelation route for this site since it has no acid protons; and the phenolic OH via CDCM. Their structures are shown in Figures 3 and 4, while those corresponding to the rest of the complexes, i.e., those yielded by endergonic reactions, are provided as Supporting Information (Figures 2S and 3S). In addition, the largest exergonicity among all the studied chelation pathways systematically involve the phenolic oxygen (site O2). Consequently they are proposed as the most likely chelation sites for the reactions of NAS and 6OHM with Cu^{2+}.

Table 3. Gibbs Free Energies (Δ*G*, kcal/mol) for the Chelation Reaction, in Aqueous Solution at *p*H = 7.4, at 298.15 K

	6OHM		NAS	
	DCM[a]	CDCM[b]	DCM	CDCM
N1	11.31	8.82	11.57	10.27
N2	8.32	3.87	9.57	3.65
N1, O1	16.26	3.52	17.01	1.06
O1	−3.19		−0.96	
O2	3.79	−4.79	5.19	−3.03
O3	4.03			
O2, O3	1.53	−8.41		

[a]DCM = formed via direct chelation mechanism. [b]CDCM = formed via coupled−deprotonation−chelation mechanism.

After identifying which chelates are viable, their potential ability to inhibit the Cu^{2+} reduction was analyzed. To that purpose, the thermochemical feasibility of the first step of the Haber−Weiss reaction was investigated. In addition, there are other chemical agents that can reduce Cu^{2+}, producing Cu^{1+}, and facilitating $^{\bullet}OH$ production via the Fenton reaction. For example, the oxidative injuries induced by the Cu-ascorbate mixture can be attributed to such a process. Accordingly the corresponding reaction was also investigated.

Considering the results reported in Table 4, the reduction of Cu^{2+} by $O_2^{\bullet-}$ can be downgraded, but not fully prevented by NAS and 6OHM. On the other hand, it was found that the Cu^{2+} reduction by ascorbate is completely turned off by all the chelates yielded via CDCM, with the phenolic O as the binding site. These are the same chelates that reduce to a larger extent the first step of the catalyzed Haber−Weiss reaction. Consequently it is proposed that both NAS and 6OHM can prevent the oxidative stress induced by Cu^{2+}−ascorbate mixtures and lower the $^{\bullet}OH$ production by sequestering Cu^{2+} ions. In addition, the phenolic moiety seems to be important for this protection, as it also is for the peroxyl radical scavenging activity.

Relative Activity. According to Halliwell and co-workers,[73,74] an antioxidant is defined as "any substance that when present at low concentrations compared to that of an oxidizable substrate would significantly delay or prevent oxidation of that substrate". Since to succeed in such a task, the antioxidant should react faster than the species to be protected, the rate constants (k) can be used as a criterion of this activity. For the reactions with $HOO^{\bullet}$, in nonpolar media NAS and 6OHM react about 215 and 19 times faster than melatonin, respectively, while in aqueous solution they react 5 and 4 orders of magnitude faster (Table 5). Therefore, both NAS and 6OHM are predicted to be better peroxyl radical scavengers than melatonin itself, regardless of the polarity of the environment.

Including other melatonin-related compounds (3OHM, AMK, and AFMK) for comparison, for which there is kinetic data available corresponding to the reactions with $HOO^{\bullet}$, it is predicted that NAS is the best peroxyl radical scavenger in the group followed by 6OHM, in lipid solution (Table 5). The order of reactivity in such an environment is predicted to be NAS > 6OHM > melatonin >3OHM > AMK > AFMK. In aqueous solution, on the other hand, the trend in peroxyl scavenging activity changes to 6OHM > 3OHM > NAS > AMK > melatonin > AFMK, with 6OHM as the best scavenger. These results support the hypothesis that, in aqueous solution, the presence of a phenol moiety in the melatonin-related compounds is the crucial structural factor in their peroxyl radical scavenging activity.

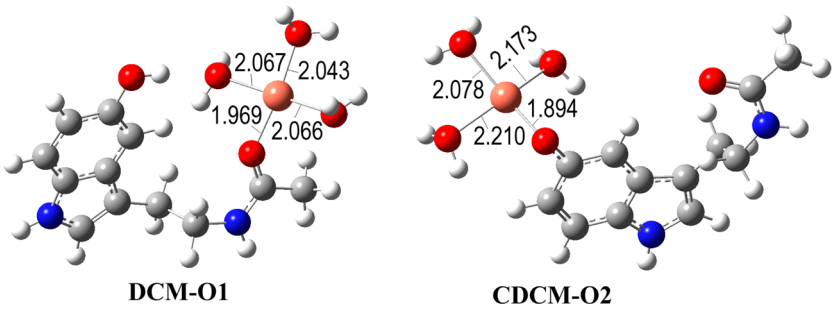

Figure 3. Optimized geometries of the Cu^{2+} complexes with NAS yielded by exergonic reactions. Distances are reported in Å.

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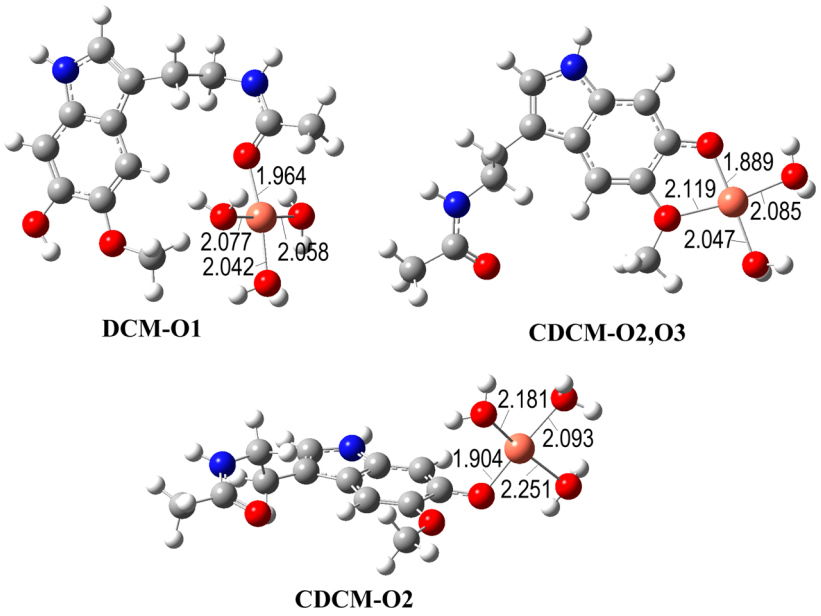

Figure 4. Optimized geometries of the stable Cu^{2+} complexes with 6OHM yielded by exergonic reactions. Distances are reported in Å.

Table 4. Gibbs Free Energies of Reaction (kcal/mol, at 298.15K) for the Reduction of Cu(II) Complexes, by $O_2^{\bullet-}$ and Ascorbate (Asc^-)

	asc^-	$O_2^{\bullet-}$
Cu^{2+}	−7.41	−32.13
NAS		
DCM-O1	−6.39	−31.11
CDCM-O2	3.57	−21.15
6OHM		
DCM-O1	−5.68	−30.40
CDCM-O2	3.88	−20.84
CDCM-O2,O3	7.00	−17.71

Table 5. Rate Constants (M^{-1} s^{-1}) for the Reactions of Other Antioxidants with $HOO^{\bullet}$, at 298.15 K, in Non-Polar (Lipid) and Aqueous Solution

	non-polar	ref	aqueous	ref
		Melatonin-Related		
melatonin	3.11×10^{02}	25	1.99×10^{01}	25
3-OHM	3.16×10^{01}	60	2.84×10^{06}	60
AMK	1.07×10^{01}	26	1.35×10^{02}	26
AFMK	4.57×10^{00}	26	4.32×10^{00}	26
		Other Antioxidants		
Trolox	3.40×10^{03}	75	8.96×10^{04}	75
ascorbic acid	5.91×10^{03}	50	9.97×10^{07}	50
resveratrol	1.31×10^{04}	58	5.62×10^{07}	58
gallic acid	5.04×10^{05}	76	8.71×10^{05}	76
caffeine	3.19×10^{01}	77	3.29×10^{-01}	77
genistein	1.21×10^{01}	78	3.33×10^{05}	78

Compared to Trolox, which is frequently used as a reference antioxidant, both NAS and 6OHM are predicted to be better peroxyl radical scavengers. They were found to react about 19 and 2 times faster than Trolox in lipid solution, and 40 and 13 times in aqueous solution, respectively (Table 5). With respect to other antioxidants, the peroxyl radical scavenging activity of NAS in lipid solution is predicted to be lower than that of gallic acid, and higher than those of resveratrol, ascorbic acid, caffeine, and genistein. In the same media the activity of 6OHM is higher than those of caffeine and genistein, and lower than the others. In aqueous solution the efficiency of the compared compounds as peroxyl radical scavengers becomes ascorbic acid > resveratrol >6OHM > NAS > gallic acid > genistein > caffeine.

Regarding the efficiency of NAS and 6OHM as inhibitors of the $^{\bullet}OH$ production, via Cu^{2+} chelation, the criteria to establish a trend are the ability of turning off the reduction of Cu^{2+} by the ascorbate ion or by $O_2^{\bullet-}$. Using any of them, NAS and 6OHM are less efficient for that purpose than 3OHM, melatonin, and AMK (in that order) which turn both reduction reactions into endergonic processes, i.e. thermochemical nonviable.[61] Their activity, on the other hand, is similar to that of AFMK. They fully inhibit the oxidative effects of the Cu−ascorbate mixture, while partially deactivate the first step of the Haber−Weiss reaction.

Role in the Antioxidant Cascade of Melatonin. The antioxidant cascade of melatonin is a very complex process that involves not only this molecule but other related compounds, such as 3OHM, AKM, AFMK, NAS, and 6OHM. On the basis of the results provided in the present study, the role of NAS and 6OHM on the overall protection exerted by melatonin against oxidative stress relates mainly to their free radical scavenging activity. On the other hand, 3OHM, melatonin itself, and AMK are more important for sequestering Cu^{2+} ions, thus inhibiting the $^{\bullet}OH$ production.

It is of interest to note that there is, consequently, a combination of protective effects provided by the melatonin family of compounds. When analyzed collectively, they are potent protectors against oxidative stress with a variety of action mechanisms. Moreover, as melatonin is metabolized the protection is renewed, increased, and diversified. Regarding their chemical antioxidant activity, they exhibit a "task-division" behavior with some of the metabolites being particularly efficient for scavenging free radicals, while others are mainly effective for metal chelation.

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CONCLUSIONS

The protection exerted by NAS and 6OHM against oxidative stress was investigated in terms of their free radical scavenging activity and their Cu^{2+} sequestering ability. It was found that both compounds efficiently scavenge $HOO^{\bullet}$, being more efficient for that purpose than melatonin itself, AMK, AFMK, Trolox, caffeine, and genistein. Therefore, it is proposed that NAS and 6OHM increase the overall protection exerted by melatonin through their higher free radical scavenging activity.

It was found that the activity of studied compounds is significantly greater in aqueous solution than in lipid media. In addition, NAS is more efficient as a peroxyl scavenger than 6OHM in lipid solution, while in aqueous solution they show the opposite trend. Therefore, the solvent polarity influences not only the absolute reactivity of NAS and 6OHM toward peroxyl radicals, but also their relative scavenging activity.

Through their metal chelating ability, they both fully inhibit the oxidative effects of the Cu-ascorbate mixture, and $^{\bullet}OH$ production via Haber−Weiss reaction, albeit the effects on the later are only partial. To that purpose NAS and 6OHM are less efficient than 3OHM, melatonin itself, and AMK.

On the basis of comparisons with other melatonin related compounds, it is thus proposed that the role of NAS and 6OHM on the overall protection exerted by melatonin against oxidative stress is mainly related to their free radical scavenging activities.

ASSOCIATED CONTENT

Supporting Information

Imaginary frequencies of the TS, Gibbs energies of activation and tunneling corrections, and detailed formulas used in the kinetic calculations, optimized geometries of the Cu^{2+} complexes yielded by endergonic reactions, and Cartesian coordinates of the TSs and Cu complexes. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.5b04920.

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Notes

The authors declare no competing financial interest.

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