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Theoretical Study of the Reaction of Vitamin B_6 with $^1O_2$

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Abstract: Singlet oxygen is known to cause oxidative stress in cells, leading to severe damage (e.g., lipid peroxidation, membrane degradation, mutagenic alterations to DNA, protein misfunctionality). Recently, pyridoxine has been discovered to be capable of quenching singlet oxygen, however, the mechanism of this reaction remains essentially unknown. In this work, we have investigated four sets of reactions: 1) 1,3-addition to a double bond connected to a hydrogen-carrying group, resulting in the formation of allylic hydroperoxides; 2) [π2+π2] 1,2-cycloaddition to an isolated double bond, resulting in the formation of 1,2-peroxides; 3) 1,4-cycloaddition to a system containing at least two conjugated double bonds, resulting in the formation of the so-called 1,4-peroxides; 4) 1,4-addition to phenols and naphthols with the formation of hydroperoxide ketones. Thermodynamically, reaction 4 and the 6(9), 3(8), and 5(8) cases of reaction 1 are the most exergonic ones, with energies ranging from -16 to -18 kcal mol^{-1}. Furthermore, reaction 4 shows the lowest barrier through the reaction path, and is predicted to be the preferred mechanism for the pyridoxine + singlet-oxygen reaction, which is in agreement with previous experimental results.

Keywords: density functional calculations · energy barriers · pyridoxine · singlet oxygen · vitamin B_6

Introduction

Pyridoxine, vitamin B_6, is one of eight water-soluble B vitamins. It is the precursor of the biologically active derivatives pyridoxal-5′-phosphate and pyridoxamine-5′-phosphate, with functional roles in a number of enzymes,[1] and plays a vital role in biological processes in many different parts of the human body (see references [2,3] and references therein).

Pyridoxine, although not classified as an antioxidant compound, has recently been shown to have highly effective antioxidant properties, although the exact mechanisms have not been clearly resolved.[4] It was proposed that this effect could be related to possible coenzymatic activities that, however, could not be observed in chemical tests. Reactive oxygen species are constantly formed in the human body and must be removed by antioxidants. A lack of balance in the oxidant–antioxidant activity is involved in many free-radical-mediated pathologies. Lately, pyridoxine-biosynthesis-deficient mutants of fungi and yeast have been shown to be sensitive to reactive oxygen species, such as singlet oxygen[5,6] and hydrogen peroxide.[7] This suggests that vitamin B_6 and its derivatives are also involved in stress tolerance in living organisms, especially in alleviating oxidative stress. In eukaryotes, it has been implied that stress resistance involves pyridoxine-dependent singlet-oxygen quenching,[8] whereby the pyridoxine itself would act as an antioxidant.[6,8] Pyridoxine was found to be the most effective of the vitamin B_6 species (pyridoxamine and their phosphate derivatives), twice as effective as pyridoxal-5-phosphate, and as effective as vitamin E.[9]

Singlet oxygen is a reactive form of dioxygen that can cause damage to lipids, amino acids, DNA, and other biologically important molecules.[10–12] Although the reaction of singlet oxygen with other vitamins has been extensively investigated,[13] the reaction of pyridoxine with singlet oxygen has been scarcely studied.[14] As mentioned above, the ability of pyridoxine to quench singlet oxygen has been demonstrated experimentally.[8] Theoretical studies of the antioxidant properties of pyridoxine have hitherto focused on other reactive oxygen species (ROS), such as $^{\bullet}OH$, $^{\bullet}OOH$ and $^{\bullet}O_2^-$ radicals.[15]

The aim of this work was to study the reaction between pyridoxine and singlet oxygen. Pyridoxine has an aromatic six-membered ring, and as described by Bobrowski et al.,[16] there are four principal types of oxygen-addition reactions to aromatic and unsaturated compounds: 1) 1,3-addition to a double bond connected to a hydrogen-carrying group, resulting in the formation of allylic hydroperoxides;

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2) [π2+π2] 1,2-cycloaddition to an isolated double bond, resulting in the formation of 1,2-peroxides; 3) 1,4-cycloaddition to a system containing at least two conjugated double bonds, resulting in the formation of the so-called 1,4-peroxides; 4) 1,4-addition to phenols and naphthols with the formation of hydroperoxide ketones. For pyridoxine and singlet oxygen, these four reactions would, in principle, occur as shown in Scheme 1. Experimental evidence has shown the product of reaction 4 to be an intermediate for the reaction of pyridoxine with singlet oxygen in methanol, in which the final product was found to be a methoxylated species.[14] However, this does not exclude the other three types of reaction of oxygen with pyridoxine.

In this work, we have studied all four reaction types by using computational quantum-chemistry techniques and by analyzing the thermodynamics and energy barriers, with the aim of giving some insight into the mechanism of the reaction of pyridoxine with singlet oxygen.

Methods

We used the hybrid B3LYP[17–19] gradient-corrected functional within density functional theory.[20,21] Structure optimizations were carried out in the gas phase by using the 6-31+G(d,p) basis set. Harmonic vibrational frequencies were determined by the analytical differentiation of gradients, in order to determine whether the structures found were minima or transition states and to extract zero-point energies and Gibbs free-energy contributions. Intrinsic reaction coordinate (IRC) calculations[22,23] were performed to assess whether the calculated transition states connect the appropriate reactants and products. Single-point calculations using the 6-311+G(2df,p) basis set and the integral equation formalism of the polarized continuum model (IEFPCM) of Tomasi and co-workers[24,25] were performed on the optimized structures to estimate the effects of bulk solvation. To take into account the influence of enthalpy and entropy, the Gibbs free-energy contributions from the gas phase were added to give the final free energies, ΔG_{aq}^{298}. It has been demonstrated elsewhere that this methodology is appropriate to study these types of reactions.[11,15]

The study of open-shell singlet systems is less trivial by using single reference determinant techniques (such as DFT). The presence of open- (diradical) as well as closed-shell (zwitterionic-like) solutions for singlet-oxygen species have been discussed in great detail elsewhere.[26,27] In the current work, only closed-shell solutions were found, despite numerous attempts to also locate open-shell species.

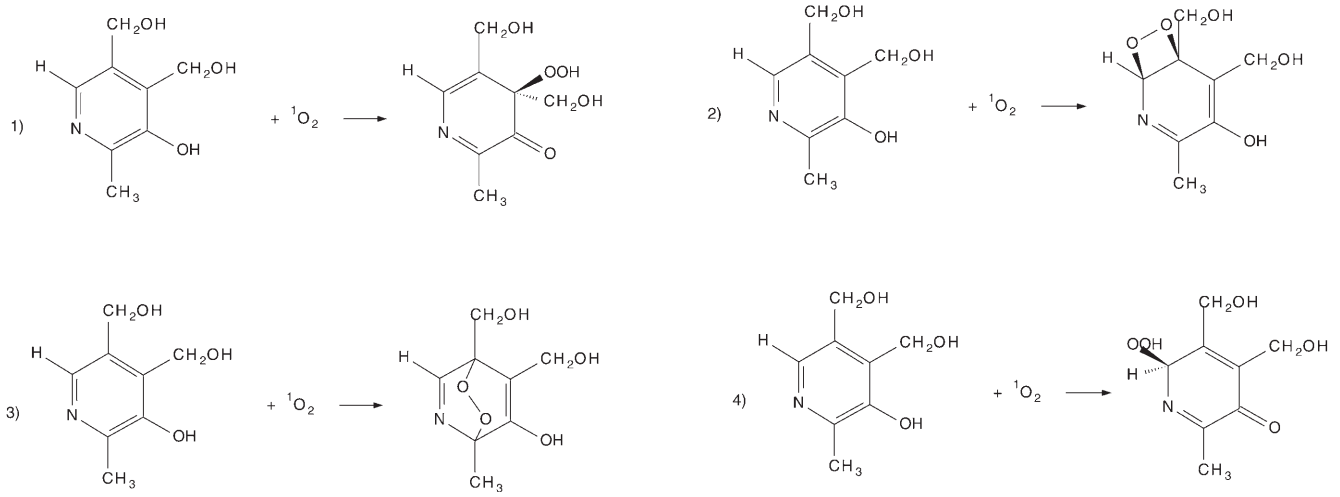
The natural bond orbital (NBO) analysis[28] was performed on all structures to determine the natural charges and diradical character of the reactant complexes, first transition states, and intermediates studied in this work.

The Gaussian 03[29] package was used throughout the study.

Results

Four reaction types, as described in the Introduction, were studied. All possible products for these reactions are discussed below, and the full reaction paths are outlined for the reactions with negative ΔG_{aq}^{298}. All energies are given relative to the energy of the optimized B_6 + the energy of the excited $O_2(^1\Delta_g)$, corrected for the free energy and including the contribution from the polarized continuum. The starting point prior to excitation, that is, B_6 + ground state $O_2(^3\Sigma_g)$, hence lies at the relative energy $-22.7\,kcal\,mol^{-1}$.[30] Throughout this study, the atoms in the pyridoxine aromatic ring are labeled as shown in Scheme 2.

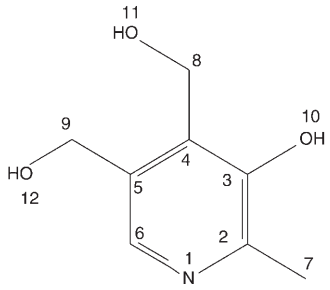
Thermodynamics of the reactions: In this section, the possible products resulting from $^1O_2$ addition to pyridoxine, as outlined in Scheme 1, are discussed. In Table 1, the ΔG_{aq}^{298} of all studied products are given. Only some of the products of reactions 1 and 4 are thermodynamically permitted. From the set of reactions labeled as 1, the most exergonic ones



Scheme 1. Possible reaction products for the pyridoxine and singlet-oxygen reactions studied.

Table 1. Gibbs free energies, ΔG^{298}_{aq}, of all possible products outlined in Scheme 1. For reaction 1, the first number is the atom to which an oxygen is bonded, and the number in parentheses indicates the atom from which the hydrogen atom is abstracted. For reactions 2 and 3, the two numbers are the atoms of the aromatic ring to which the oxygen atoms are bonded. For reaction 4, the same labeling as for reaction 1 is used. Distances between the oxygen atom of singlet oxygen and the pyridoxine atoms (R) are given in Å.

Reaction 1				Reaction 2				Reaction 3				Reaction 4		
	ΔG^{298}_{aq}	$R_{C(N)-O}$	R_{O-H}		ΔG^{298}_{aq}	$R_{C(N)-O1}$	R_{C-O2}		ΔG^{298}_{aq}	R_{C-O1}	R_{C-O2}		ΔG^{298}_{aq}	R_{C6-O1}
1(6)	26.82	1.45	1.03	6–5	0.53	1.47	1.45	6–3	−0.96	1.49	1.46	6(10)	−17.95	1.43
1(7)	11.90	1.33	0.97	5–4	12.35	1.48	1.46	5–2	1.51	1.48	1.47			
6(9)	−16.86	1.46	0.97	4–3	3.46	1.48	1.45							
5(8)	−14.19	1.47	0.97	3–2	1.14	1.47	1.46							
5(6)	42.03	1.45	0.98	1–6	43.38	1.47	1.46							
4(10)	−7.97	1.43	0.98	1–2	40.00	1.53	1.45							
4(9)	−9.41	1.47	0.98											
3(7)	−5.45	1.47	0.97											
3(8)	−18.25	1.46	0.97											
2(10)	−14.21	1.44	0.98											



Scheme 2. Pyridoxine (vitamin B_6), including atomic numbering.

are those in which the hydrogen atom is taken from either atom 8 or atom 9. This is consistent with a previous study of the attack of oxygen radicals on pyridoxine, in which it was observed that these hydrogen atoms could be abstracted without any barrier.[15] The distances between singlet-oxygen atoms and pyridoxine atoms are also given in Table 1. It may be observed that all structures show C–O bond lengths within a range of 1.43–1.48 Å and O–H bond lengths of 0.97–0.98 Å, indicative of a single bond between those atoms. These distances are discussed in more detail below.

The fact that the product from reaction 4 is one of the most exergonic is in agreement with the experimental results obtained in a previous study,[14] in which the authors investigated the reaction of pyridoxine with singlet oxygen in methanol. They found a methanol adduct of substituted 2,5-pyridinedione as product. The product of reaction 4 was found to be an intermediate, in equilibrium with another structure. However, no intermediates like the product of reaction 1 were observed experimentally, although we calculate similar ΔG^{298}_{aq} for the products. This implies that the reason for the occurrence of reaction 4-type products only could be related to a difference in kinetics between the two reaction types.

To investigate this, the full reaction path for reaction 4, and the cases 3(8), 6(9), and 5(8) of reaction 1 were studied, and the results obtained are described below.

Full reaction paths

Reaction 1: In Tables 2 and 3, the distances between the attacking species and the ring atom under attack are given along the reaction path, together with the free energies in water, ΔG^{298}_{aq}, for the 3(8), 5(8), and 6(9) reactions. For the transition state (TS), we report the corresponding imaginary frequencies. Two reaction mechanisms could be distinguish-

Table 2. Data obtained for the stepwise 6(9) case of reaction 1. Distances between the oxygen atom of singlet oxygen and the pyridoxine ring atom (R) are in Å, ΔG^{298}_{aq} values are in kcal mol^{-1}, the natural charge (q) of the oxygen molecule is in arbitrary units. For the TSs, the imaginary frequencies are in cm^{-1}. RC: reactant complex.

RC			TS1				I			
$\Delta\Delta G^{298}_{aq}$	R_{C-O}	q_{O_2}	ΔG^{298}_{aq}	freq.	R_{C-O}	q_{O_2}	ΔG^{298}_{aq}	R_{C-O}	R_{O-H}	q_{O_2}
12.68	2.78	−0.10	22.34	243.2i	1.64	−0.57	21.51	1.54	1.86	−0.61
TS2						Product				
ΔG^{298}_{aq}	freq.	R_{C-O}	R_{O-H}	R_{C-H}	q_{O_2}	ΔG^{298}_{aq}	R_{C-O}	R_{O-H}	q_{O_2}	
21.69	242.4i	1.53	1.62	1.16	−0.61	−16.86	1.46	0.97	−0.80	

Table 3. Data obtained for the concerted 5(8) and 3(8) cases of reaction 1. Distances between the oxygen atom of singlet oxygen and the pyridoxine atoms (R) are in Å, ΔG^{298}_{aq} values are in kcal mol^{-1}, the natural charge (q) of the oxygen molecule is in arbitrary units. For the TSs, the imaginary frequencies are in cm^{-1}. RC: reactant complex.

	RC			TS1					Product			
	ΔG^{298}_{aq}	R_{C-O}	q_{O_2}	ΔG^{298}_{aq}	freq.	R_{C-O}	R_{O-H}	R_{C-H}	q_{O_2}	ΔG^{298}_{aq}	R_{C-O}	R_{O-H}	q_{O_2}
5(8)	12.68	2.77	−0.11	16.37	891.4i	2.47	1.15	1.42	−0.56	−14.19	1.47	0.97	−0.80
3(8)	10.58	2.60	−0.16	16.92	1047.4i	2.27	1.20	1.36	−0.55	−18.25	1.46	0.97	−0.78

ed from the calculations; a stepwise mechanism in which O_2 first adds, followed by hydrogen abstraction by the terminal peroxyl oxygen, as in the 6(9) case, and a concerted mechanism involving simultaneous addition and hydrogen abstraction, as for 5(8) and 3(8). The structures for the stationary points along the 6(9) and 5(8) reaction pathways are given in Figures 1 and 2, respectively. The structures of 3(8) are not given because they are very similar to those of 5(8).

The stepwise reaction proceeds as follows. Firstly, the singlet oxygen approaches atom 6 in the aromatic ring, forming the reactant complex with a C–O distance of 2.78 Å. In the TS, the C–O distance is reduced to 1.64 Å, indicating a very late transition state. For the intermediate, the C–O distance is reduced to 1.54 Å, and the other oxygen atom is located 1.86 Å from the hydrogen attached to atom 9. In the second step, the hydrogen is abstracted from atom 9 by the terminal oxygen from atom 9. In the TS, the C–O distance remains constant, 1.53 Å, whereas the O–H distance is shortened to 1.62 Å. The C–H distance is elongated from 0.97 to 1.16 Å, indicative of an early TS for this step. Finally, in the product, the C–O distance is 1.46 Å, and the hydrogen is bonded to the oxygen at a distance of 0.97 Å. The ΔG^{298}_{aq} of this reaction reveals a barrier of 22.34 kcal mol^{-1} for the first step, whereas almost no barrier is observed for the second step; that is, once the peroxyl intermediate is formed, the hydrogen is very easily abstracted. The same observation was made in a previous investigation of singlet-oxygen-initiated lipid peroxidation.[11]

The concerted mechanism occurs in a very different way; in these cases, the hydrogen is first abstracted. In the reactant complexes, the C–O distances are very similar to the previous case (2.6–2.8 Å), whereas the TSs are very late, with O–H distances reduced to 1.15 and 1.20 Å, and the C–H bonds elongated to 1.42 and 1.36 Å, for the 5(8) and 3(8) cases, respectively. In both cases, the TS for hydrogen abstraction connects directly to the product. Both reactions have an energy barrier of approximately 17 kcal mol^{-1}; 5 kcal mol^{-1} lower than the concerted reaction. From a kinetic point of view, both 5(8) and 3(8) reactions are more favorable than 6(9). Upon comparison, the 3(8) product is 4 kcal mol^{-1} more stable than the 5(8) one, and we therefore predict it to be the most likely product of the two.

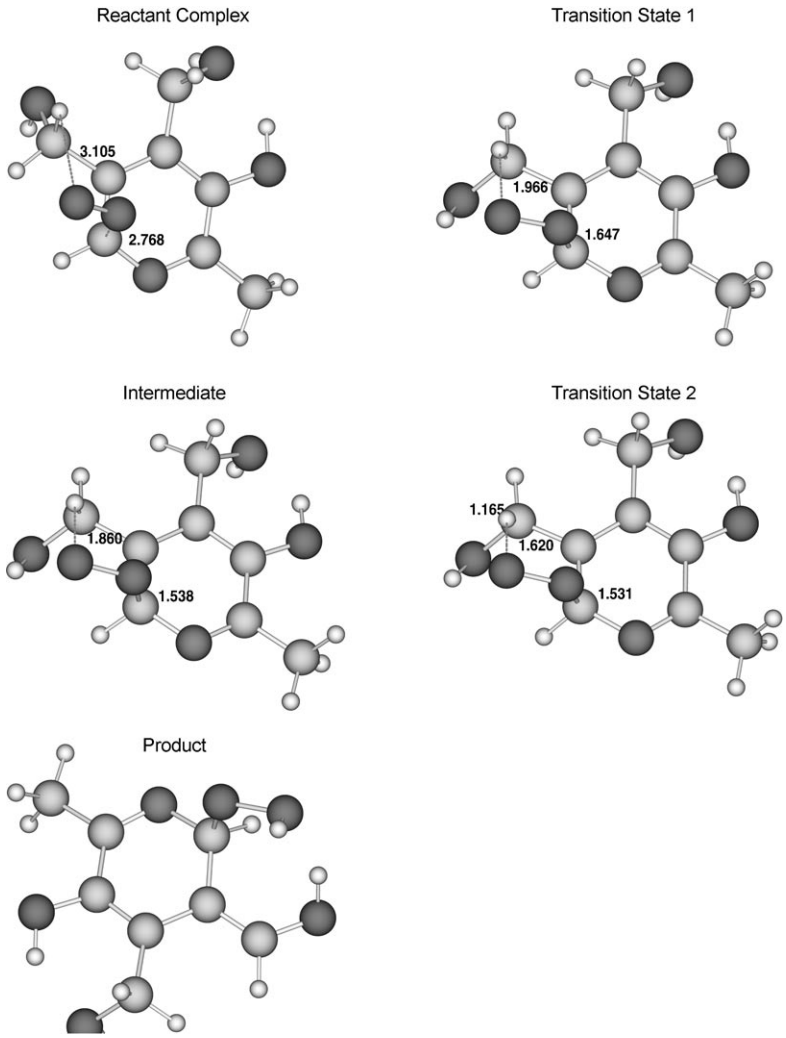


Figure 1. Structures of the stepwise 6(9) reaction from reaction 1. Light-colored atoms are those of hydrogen (small) and carbon (large). Darker atoms are those of nitrogen (the one in the ring) and oxygen. Plotted bond lengths are in Å.

The natural charges of singlet oxygen were calculated by using the NBO methodology. Both concerted and stepwise mechanisms show a large charge transfer from pyridoxine to singlet oxygen, in agreement with the zwitterionic nature of the closed-shell structures.

Reaction 4: In Table 4, the distance between the attacking oxygen and the ring atom under attack is given along the reaction path, together with the free energies in water, ΔG^{298}_{aq}. For the TS, we report the corresponding imaginary frequencies. The structures are given in Figure 3. This reaction is a two-step reaction, in which the first step involves a 1,4-cycloaddition reaction to atoms 3 and 6 of pyridoxine. In the initial reactant complex, one of the oxygen atoms, labeled as O1, interacts with atom 6 at a distance of 2.49 Å, and the other, labeled O2, is found at a distance of 2.54 Å from atom 3. In the TS, these distances are

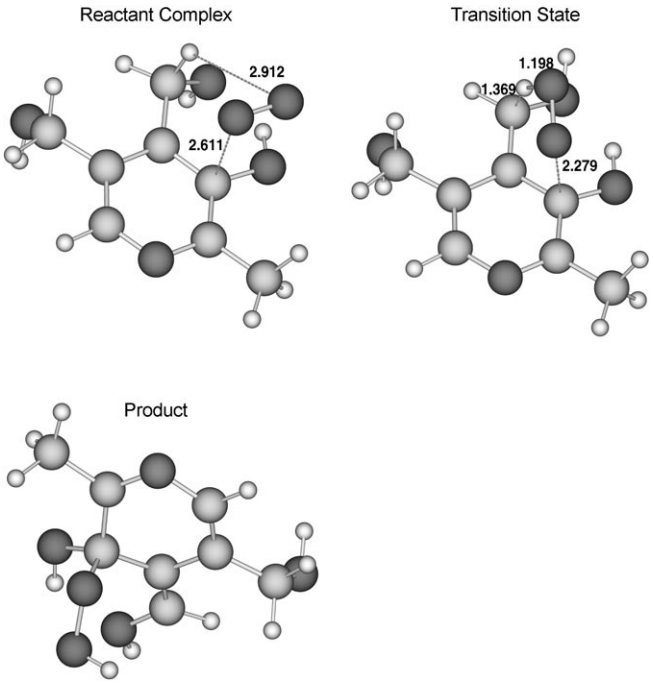


Figure 2. Structures of the concerted 5(8) reaction from reaction 1. Light-colored atoms are those of hydrogen (small) and carbon (large). Darker atoms are those of nitrogen (the one in the ring) and oxygen. Plotted bond lengths are in Å.

shortened to 1.83 and 2.08 Å, respectively. This TS connects to the 3,6-cycloadduct, in which the C–O bond lengths are further reduced to 1.48 and 1.54 Å, respectively. In the second step, the hydrogen abstraction by O2 from O10 occurs, breaking the C3–O2 bond. In the intermediate, however, the H–O2 distance (2.94 Å) is too large for hydrogen abstraction. For this reaction to occur, the assistance of an additional water molecule is needed. In the I–w (intermediate complex with a water molecule), the O2 is located 2.11 Å from the water hydrogen, H_w, and the abstracted hydrogen is 1.87 Å from the water oxygen, O_w. The C3–O2 and C6–O1 bond lengths are similar to those in the "water-free" intermediate (I) structure. In TS2, the C3–O2 bond is elongated to 2.09 Å, whereas the O2–H_w and H–O_w distances are reduced to 1.34 and 1.20 Å, respectively. The C6–O2 distance remains constant. This TS connects the intermediate with the product, P1. Because this reaction involves the exchange of hydrogen atoms between the pyridoxine moiety and water, a possible approach to verify the postulated mechanism experimentally could be by means of deuterated samples. The natural charges are large, as was observed for cases of reaction type 1, again in agreement with the zwitterionic nature of the closed-shell structures.

In reference [14], this product was found to be an intermediate in equilibrium with another structure, labeled P2 in this work. In this structure, the abstracted hydrogen is further transferred to the ring nitrogen, and O2 is bonded to C2. All attempts to locate a TS for this reaction, with and without the inclusion of additional water molecules, failed. In reference [14], the reaction was performed in methanol, which could help in the equilibrium reaction. It is not known if this equilibrium also occurs in water, but if it does, it will be a water-assisted reaction. An alternative possibility exists if pyridoxine is in its zwitterionic form (with N1 protonated and O10 deprotonated),[31] in which case the second hydrogen-transfer reaction has already occurred, and the second step involves the change from the 2,5- to the 2,6-bridging molecule.

The ΔG^{298}_{aq} of this reaction shows that the first step has a barrier of 11.51 kcal mol^{-1}, and the second step, relative to the I–w intermediate, has a barrier of 14.06 kcal mol^{-1}. These barriers are smaller than those of reaction 1 cases, as can be seen in Figure 4, and indicates that this will be the preferred reaction path for the pyridoxine reaction with singlet oxygen. This is consistent with the experimental data available.[14]

Conclusions

Four sets of reactions between pyridoxine and singlet oxygen were explored in this work: 1) 1,3-addition to a double bond connected to a hydrogen-carrying group, resulting in the formation of allylic hydroperoxides; 2) [π2+π2] 1,2-cycloaddition to an isolated double bond, resulting in the formation of 1,2-peroxides; 3) 1,4-cycloaddition to a system containing at least two conjugated double bonds, resulting in the formation of the so-called 1,4-perox-

Table 4. Data obtained for reaction 4. Distances between the two oxygen atoms of singlet oxygen (labeled O1 and O2) and the pyridoxine ring atom (R), or water-molecule atoms (labeled O_w and H_w) are in Å. Gibbs free energies in solution, ΔG^{298}_{aq} are in kcal mol^{-1}. The natural charge (q) of the oxygen molecule is in arbitrary units. For the TSs, the imaginary frequencies are in cm^{-1}. RC: reactant complex.

RC				TS1					I			
ΔG^{298}_{aq}	R_{C3-O}	R_{C6-O}	q_{O_2}	ΔG^{298}_{aq}	freq.	R_{C3-O}	R_{C6-O}	q_{O_2}	ΔG^{298}_{aq}	R_{C3-O}	R_{C6-O}	q_{O_2}
7.51	2.54	2.49	−0.26	11.51	304.3i	2.08	1.83	−0.49	−0.96	1.54	1.48	−0.61

I–w						TS2						
ΔG^{298}_{aq}	R_{C3-O2}	R_{C6-O1}	R_{O2-H_w}	R_{H-O_w}	q_{O_2}	ΔG^{298}_{aq}	freq.	R_{C3-O2}	R_{C6-O1}	R_{O2-H_w}	R_{H-O_w}	q_{O_2}
10.74	1.52	1.46	2.11	1.87	−0.66	24.80	1248.8i	2.09	1.48	1.34	1.20	−0.75

P1			P2		
ΔG^{298}_{aq}	R_{C6-O1}	q_{O_2}	ΔG^{298}_{aq}	R_{C6-O1}	R_{C2-O2}	R_{N-H}
−17.95	1.43	−0.78	−18.89	1.46	1.49	1.02

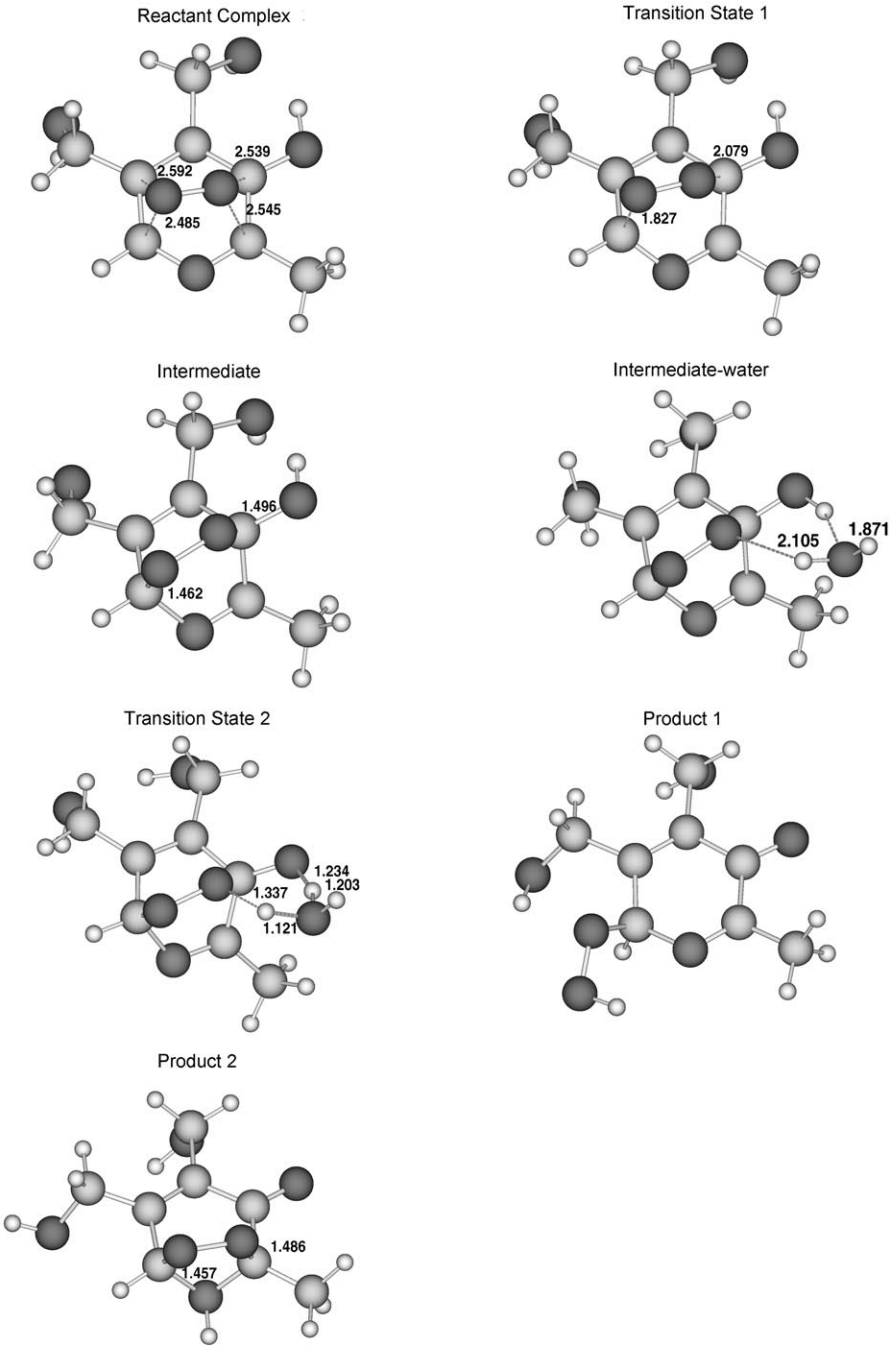


Figure 3. Structures of the reactant complex, intermediates, transition states, and products of reaction 4. Light-colored atoms are those of hydrogen (small) and carbon (large). Darker atoms are those of nitrogen (the one in the ring) and oxygen. Plotted bond lengths are in Å.

ides; 4) 1,4-addition to phenols and naphthols with the formation of hydroperoxide ketones. It was observed that, thermodynamically, only some cases of reaction type 1, namely addition to atoms 5 and 3 and abstraction of hydrogen from atoms 8, 5(8), and 3(8), and addition to atom 6 and hydrogen abstraction from 9, 6(9), and reaction 4 may occur. The high reactivity of hydrogen atoms bonded to methylene groups has been noted in previous work, involving the attack of ROS radical species to pyridoxine.[15]

For the reaction 1 cases, the 6(9) reaction occurs stepwise. Firstly, the oxygen molecule is added to a carbon atom, followed by hydrogen abstraction by the resulting peroxyl. For the other two cases, 5(8) and 3(8), the reaction occurs concertedly, with simultaneous hydrogen abstraction and addition to the carbon. These transition states occur very late, in that the hydrogen atom is almost entirely transferred while already in the TS. In the case of reaction 4, the reaction is a two-step process that requires the assistance of a water mol-

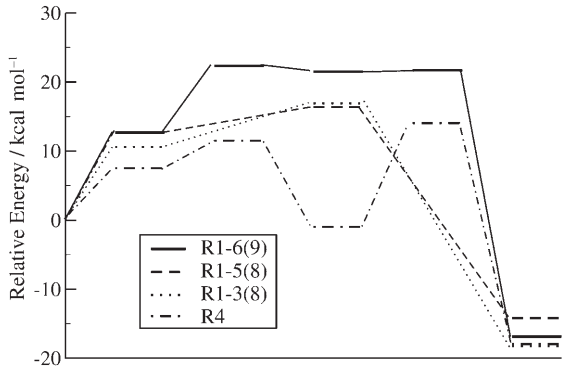


Figure 4. Reaction paths (ΔG^{298}_{aq}) for the cases of reactions 1 and 4 studied.

ecule for the second step. It was observed that the barriers of reaction 4 are smaller than those of reaction 1, suggesting that the former reaction is the most probable one. This is in agreement with experimental data.

Acknowledgements

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