Reactivity of Peroxynitrite with Melatonin as a Function of pH and CO_2 Content

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Keywords: Indole / Nitration / Nitrosation / Oxidation / Oxidoperoxidonitrate(1−)

Peroxynitrite is known to be a strong oxidant and a nitrating agent of aromatic phenolic or heterocyclic rings, depending on its form in aqueous solutions: the anion $ONOO^-$, its conjugate acid ONOOH, or its CO_2 adduct $ONOOCO_2^-$. Various reactions have been observed with melatonin (**1**), a tryptophan derivative, in phosphate-buffered solutions. Melatonin (**1**) is recognized as a scavenger of several strong oxidants ($HO^{\cdot}$, H_2O_2, ...) accounting for its biological and pharmacological effects. Here we describe two oxidation routes that give rise to indol-2-ones **2** (probably via a 2,3-epoxyindole) and kynuramines **6** (by cleavage of the pyrrole ring), attributable to reactions of ONOOH and $ONOO^-$, respectively, according to the effects of pH and CO_2 content. At pH = 7.6 and in the presence of CO_2, an important conversion is the cyclization of the lateral amide function, giving 3-substituted pyrroloindoles **4**. At neutral pH, therefore, all routes coexist, with a balance between indol-2-ones **2** and pyrroloindoles **4** on the one side and kynuramines **6** on the other, depending on the CO_2 content. Furthermore, under specific conditions substitutions of the hydrogen atom on the pyrrole nitrogen atom, affording the 1-nitro- (**5**) and the unstable 1-nitrosomelatonin (**7**), are among the major transformations: formation of the nitrosation product, together with that of kynuramines **6**, rises sharply when the pH of the medium increases, confirming the implication of $ONOO^-$ in their synthesis; conversely, both reaction yields decrease with increasing CO_2 content, to favor 1-nitromelatonin (**5**). Finally, nitration by aromatic substitution occurring essentially on C-4 becomes important at acidic pH, and also at pH = 7.6 over a narrow range of CO_2 concentrations. Most of the reactions are typical of the indole moiety, suggesting that melatonin (**1**) is a model of potential use for investigation of tryptophan chemistry with peroxynitrite.

Introduction

Oxidoperoxidonitrate(1−) ($ONOO^-$) is formed in vivo and in vitro from the diffusion-controlled reaction (k = 4−19 × 10^9 $M^{-1}s^{-1}$) of $^{\cdot}NO$ and $O_2^{\cdot-}$ radicals, both produced by enzymatic reactions.[1] In acid solution, isomerization affords NO_3^-, via the conjugate acid ONOOH, with a rate constant of 1 s^{-1}. The $ONOO^-$ anion is much more stable, but reacts with CO_2 (k = 3 × 10^4 $M^{-1}s^{-1}$) to produce $ONOOCO_2^-$.[2] The name "peroxynitrite" is used for the mixture of ONOOH, $ONOO^-$, and $ONOOCO_2^-$, the proportions of which depend on the pH value (pK_a = 6.8) and the presence of CO_2. It has been proposed that peroxynitrite chemistry is governed by radicals generated from ONOOH and $ONOOCO_2^-$ homolysis and their products.[3,4]

Peroxynitrite has been shown in vitro to be an oxidant of lipids, proteins, and nucleic acids, and also a nitrating agent of phenolic and heterocyclic compounds.[5,6] Under physiological conditions, it reacts with reduced transition metal containing compounds, thiols, and other natural scavengers such as urate and vitamins C and E. In specific cellular compartments, it is believed to intervene as a biological mediator during differentiation and development, or in response to stress. However, it also appears to act as a toxic agent in numerous diseases in which synthesis of both $^{\cdot}NO$ and $O_2^{\cdot-}$ is considerably increased. This occurs in infection, inflammation, and chronic degenerative processes, with markers being detectable in tissues, such as 3-nitrotyrosine residues in proteins, 8-nitroguanine in DNA, and thiyl radicals and disulfide bridges and/or linkages between peptides or proteins. These effects are related to the peroxynitrite formation and can be counteracted by administration of oxidant scavengers and/or inhibitors of NO-synthases. However, the reactivity of peroxynitrite is far from well understood. It was therefore of interest to study the reactions of peroxynitrite with a natural indole compound known to decrease the effects of oxidative stress.[7]

We investigated the interaction of peroxynitrite with the tryptophan derivative melatonin {*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]acetamide, **1**}. Melatonin is secreted in mammals by the pineal gland, following a circadian cycle, and by some other tissues such as the retina and the gastrointestinal tract.[7] This lipophilic hormone spreads throughout the body, whereas peroxynitrite is a locally formed effector. The scavenging properties of melatonin with $^{\cdot}OH$ radicals

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have been demonstrated,[8] but only a few reactions with peroxynitrite have been described. The first, well-documented, step in the reaction between melatonin and peroxynitrite is the formation of the melatoninyl radical cation through one-electron transfer to peroxynitrite.[9−11] Only a few stable products were identified by GC-MS and ^{1}H NMR.[12] In our laboratory, we have previously demonstrated the formation of unstable derivatives such as 1-hydroxy- and 1-nitrosomelatonin, with yields dependent on physicochemical factors.[13] Here we have completed the identification of a series of products and have measured their respective yields under various sets of physicochemical conditions. Taking into account the complexity and the duration of the chemical transformations of melatonin by peroxynitrite in aqueous solutions, we have distinguished different types of reactions and assigned the relevant reactive form of peroxynitrite for each of them. The results described and the proposed mechanisms afford new insights into reactions between peroxynitrite and the indole moiety.

Results and Discussion

Results

When synthetic peroxynitrite was injected into a phosphate-buffered solution of melatonin at pH = 7.6 and 25 °C, more than 13 products were detected and resolved by RP-HPLC after decay of the peroxynitrite (half-life of 1 s) (Figure 1, part A).

A tenfold excess of peroxynitrite was necessary to obtain 80% melatonin transformation at pH = 7.6. This peroxynitrite/melatonin ratio gave only 50% melatonin conversion at pH = 6.5, but complete conversion either at pH = 8−9 (even without addition of $NaHCO_3$) or at neutral pH in the presence of HCO_3^-. It is noteworthy that the melatonin was not modified by similar amounts of H_2O_2, NO_2^- or end-products of spontaneous peroxynitrite decomposition at pH = 7.6.

At pH = 7.6, the transformation products of melatonin were distributed among several fractions resolved by RP-HPLC. Analysis of the reaction mixture indicated that some peaks appeared in the following minutes and decomposed afterwards. For instance, the peak with t_R = 32 min appeared in the first few minutes and then decreased within 20 min, while the peak with t_R = 20 min appeared (see **B** in Figure 1). The latter reached its maximum after 1 h and then decayed over the following days. To preserve most of the products formed, neutral conditions were used in preparative HPLC and each collected fraction was immediately analyzed by spectrophotometry, analytical HPLC, and mass spectrometry. Analysis classified the products into five typical groups: three oxidized derivatives − pyrroloindoles, kynuramines, and indol-2-ones − and *C*-nitration and *N*-substitution melatonin derivatives. Their yields, determined by the net weights obtained at pH = 7.6 with addition of $NaHCO_3$ (100 mM), were 20, 7, 10, 5, and 16%, respectively.

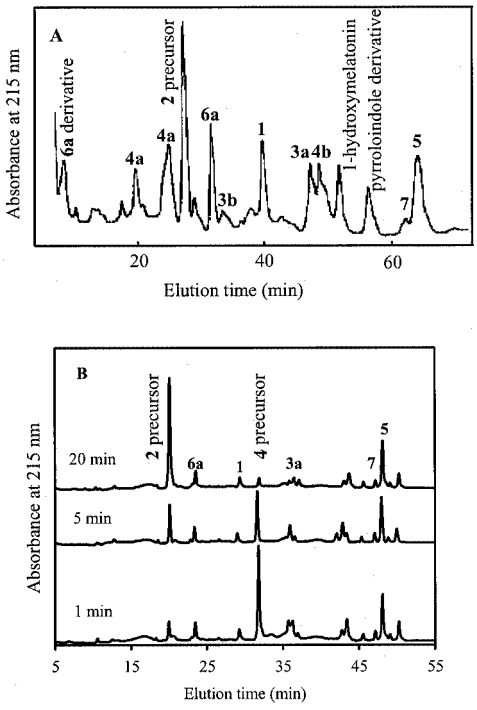


Figure 1. **A:** Typical preparative RP-HPLC profile of products of the reaction between peroxynitrite and melatonin at pH = 7.6; the reaction was carried out in a final volume of 100 mL of phosphate (0.4 M)/$NaHCO_3$ (0.1 M) buffered solution containing melatonin (2 mM) and initiated by addition of peroxynitrite (2 mmol) while stirring; the mixture was chromatographed 30 min later; **B:** the time course of the RP-HPLC analysis of the reaction mixture at pH = 7.6; peroxynitrite (10 mM) was allowed to react with melatonin (1 mM) in 1 mL of phosphate buffer and $NaHCO_3$ (60 mM); 25-μL aliquots were injected 1, 5, and 20 min afterwards; RP-HPLC methods are described in the Exp. Sect.

C-Nitromelatonin

Two stable nitration products **3a** and **3b** were identified by their characteristic absorption bands at 380 and 370 nm, by mass spectrometry, and by ^{1}H NMR. The major product, 4-nitromelatonin (**3a**), was more concentrated than **3b**, which had a low yield (below 1%) throughout the pH scale (see **A** in Figure 2). Reduction of the pH from 7.6 to 6 produced a sixfold increase in the yield of **3a**, from 1 to 6%. At pH = 7.6, nitration was favored between 10 and 20 mM $NaHCO_3$ but dramatically decreased above 40 mM (see **B** in Figure 2).

Kynuramines

Of the oxidized derivatives of melatonin, the most stable was formylkynuramine (**6a**), with close to 7% yield under preparative conditions, together with a minor deformylated product **6b**. The UV absorption spectrum shows characteristic bands at 234, 270, and 340 nm (see **A** in Figure 5). The yields of these kynuramines increased with rising pH and decreased upon addition of $NaHCO_3$ (Figure 3).

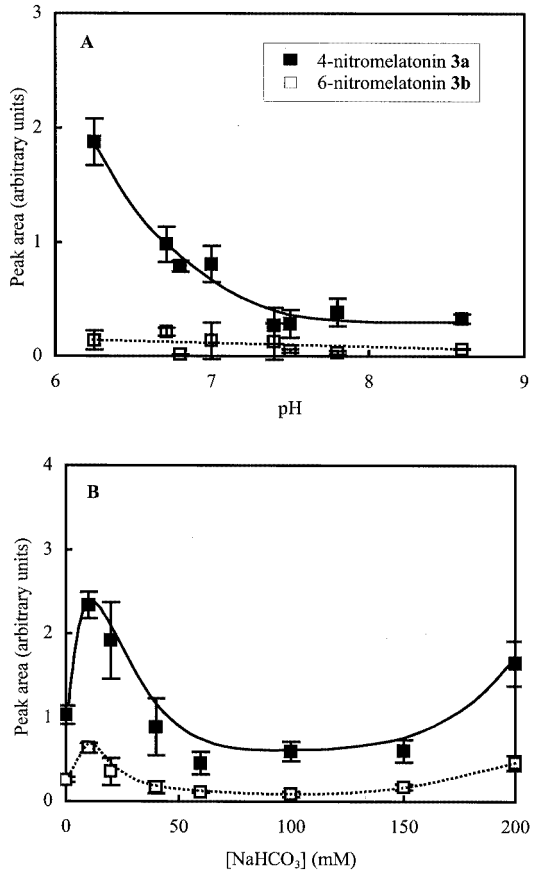


Figure 2. Effects of pH and of addition of $NaHCO_3$ on the RP-HPLC peak area of 4-nitromelatonin (**3a**, closed squares) and 6-nitromelatonin (**3b**, open squares) obtained from the reaction between peroxynitrite and melatonin; the reaction was initiated in all cases by addition of peroxynitrite; the RP-HPLC analysis conditions were as described in the Exp. Sect.; effective yields of 5 and 1% were obtained under preparative scale conditions [$ONOO^-$/melatonin (10:1), pH = 7.6, 100 mM $NaHCO_3$]; **A:** the reaction was carried out at pH = 6−9 in a final volume of 1 mL of phosphate (0.4 M) buffer that also contained $NaHCO_3$ (60 mM), melatonin (1 mM), and peroxynitrite (10 mM); **B:** the reaction was carried out at pH = 7.6 in a final volume of 1 mL of phosphate buffer that also contained $NaHCO_3$ (0−200 mM), melatonin (1 mM), and peroxynitrite (10 mM)

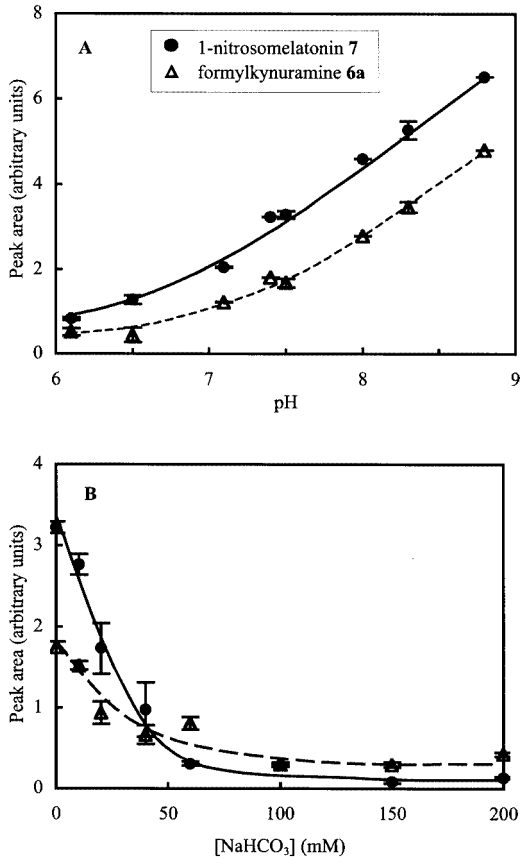


Figure 3. Effects of pH and addition of $NaHCO_3$ on the RP-HPLC peak areas of 1-nitrosomelatonin (**7**, closed circles) and formylkynuramine (**6a**, open triangles) obtained from the reaction between peroxynitrite and melatonin; effective yields of 3% for **7** and 7% for **6a** were obtained under preparative scale conditions [$ONOO^-$/melatonin (10:1), pH = 7.6, 100 mM $NaHCO_3$]; **A:** the reaction was carried out at pH = 6−9 in a final volume of 1 mL of phosphate (0.4 M) buffer, melatonin (1 mM), and peroxynitrite (10 mM) without added $NaHCO_3$; **B:** the reaction was carried out at pH = 7.6 in a final volume of 1 mL of phosphate buffer that also contained $NaHCO_3$ (0−200 mM), melatonin (1 mM), and peroxynitrite (10 mM) (see Exp. Sect. for details)

Indol-2-ones

Two other types of oxidation products were characterized: indol-2-ones **2** and pyrroloindoles **4**. In the case of indol-2-ones, a total 10% yield from melatonin was recorded. Stable 3-hydroxyindol-2-one **2a**, and two classes of corresponding dimers, a C-3−O−C-3 ether-bridged dimer **2b** and two C-3−C-3 dimers **2c**, were identified by MS, NMR, and their characteristic absorption bands were at 265 and 300 nm (see **A** in Figure 5). They derived from a labile common precursor observed as a peak with t_R = 27 and 20 min in preparative and analytical HPLC, respectively, which showed a similar absorption spectrum. MS analyses indicated addition of one oxygen atom to melatonin. According to HPLC analyses of the reaction mixture (see **B** in Figure 1), this precursor increased quickly after a few minutes of reaction, reaching a maximum within 1 h and decreasing much more slowly afterwards at pH = 7.6 ($t_{1/2}$ = 40 h) to yield the final stable products **2a**, **2b**, and **2c**.

Pyrroloindoles

Several peaks representing roughly 20% of transformation under preparative conditions (pH = 7.6, 100 mM $NaHCO_3$) contained mixtures of 3a-hydroxypyrroloindole isomers **4a** and of 3a-nitro- or more probably 3a-nitritopyrroloindole **4b** (nitrito: −O−N=O), beside others partially identified by NMR. All showed characteristic absorption bands at 235 and 300 nm (see **A** in Figure 5). Structural identification of **4a** and **4b** was based on MS and NMR results, especially on the characteristic δ value for the proton on C-8a (δ = 5−6 ppm) and on the shift in the δ value for C-3a. The compounds **4a** and **4b** are certainly formed as their respective pairs of enantiomers, but the enantiomeric ratios could not be established under our conditions.

Pyrroloindoles could not be identified in HPLC analysis of the reaction mixture. However, a transient peak (t_R = 32 min) with a similar absorption spectrum (A315/A234 = 0.21), but with an additional band at 285 nm (A285/A234 = 0.28), reached a maximum within 5 min (see **B** in Figure 1). LC-ESI-MS showed the acetonitrile adduct m/z = 341 ([M + CH_3CN + Na]$^+$) of a nitro or a nitrito derivative of melatonin and a signal m/z = 294 ([M − HNO_2 + CH_3CN + Na]$^+$) showing that NO_2 is a leaving group. Once isolated from the reaction mixture, it yielded the well-characterized pyrroloindole **4b**.

N-Substitution Products

Of the three detected *N*-substitution products, 1-hydroxymelatonin, which was described previously,[13] was obtained in very low yield (< 3%) whatever the conditions. While we can consider 1-hydroxylation almost negligible, nitration and nitrosation on the same position were highly dependent upon experimental conditions and became major transformations of melatonin under specific conditions described below.

1-Nitrosomelatonin (**7**) was described previously.[13,14] Its structure was confirmed by comparison with authentic 1-nitrosomelatonin.[13] Its absorption spectrum (see **B** in Figure 5) and its HPLC behavior were very similar to those of 1-nitromelatonin (**5**). ^{1}H NMR spectroscopy, however, showed two conformers in solution. Additional support for the structural assignments was obtained from the FT-IR spectrum, which exhibited an absorption band at 1435 cm^{-1} [ν(N−NO)], characteristic of a compound bearing a nitroso group on the nitrogen atom of an indole nucleus, in agreement with the literature.[15] The 3% yield of **7** obtained when the reaction between melatonin and peroxynitrite was carried out at pH = 7.6 in the presence of $NaHCO_3$ (100 mM) increased in the absence of additional CO_2 and with increasing pH (Figure 3). Independently of $NaHCO_3$ addition, yields reached a significant 45% at pH = 9. In the reaction mixture, **7** decomposes with a half-life of 2 h but is stable as a solid.[16]

1-Nitromelatonin (**5**) was eluted just after **7** in preparative and analytical HPLC (t_R = 68 and 48 min, respectively). MS indicated that **5** was a nitro derivative of melatonin. The ^{1}H NMR spectrum showed the same δ values as **7** but only one conformer. The IR spectrum confirmed the position of the nitro group on the pyrrole nitrogen atom by the characteristic absorption band at 1287 cm^{-1} [ν(N−NO_2)] as reported in the literature.[15] The absorption spectrum of **7** (230, 275, and 340 nm) was almost identical to that of **5** (see **B** in Figure 5). In our previous paper[13] there was confusion between these two derivatives, which have almost identical HPLC behavior and absorption spectra. Both decomposed in the reaction medium, albeit at different rates ($t_{1/2}$ = 7 d for **5**, compared to 2 h for **7**). The 10% yield obtained under preparative conditions was optimum, as addition of $NaHCO_3$ at pH = 7.6 promoted the nitration and inhibited nitrosation on the indole nitrogen atom (Figure 4). With a sufficient amount of CO_2, **5** was formed in place of **7**.

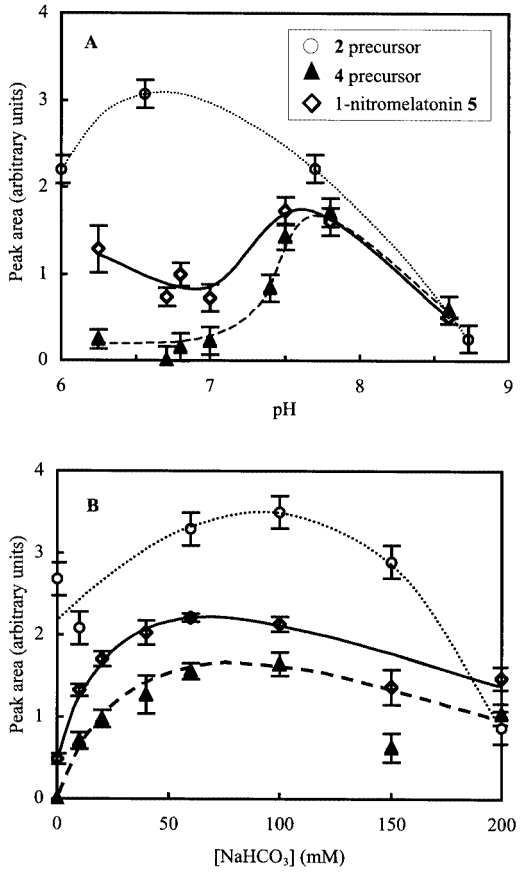
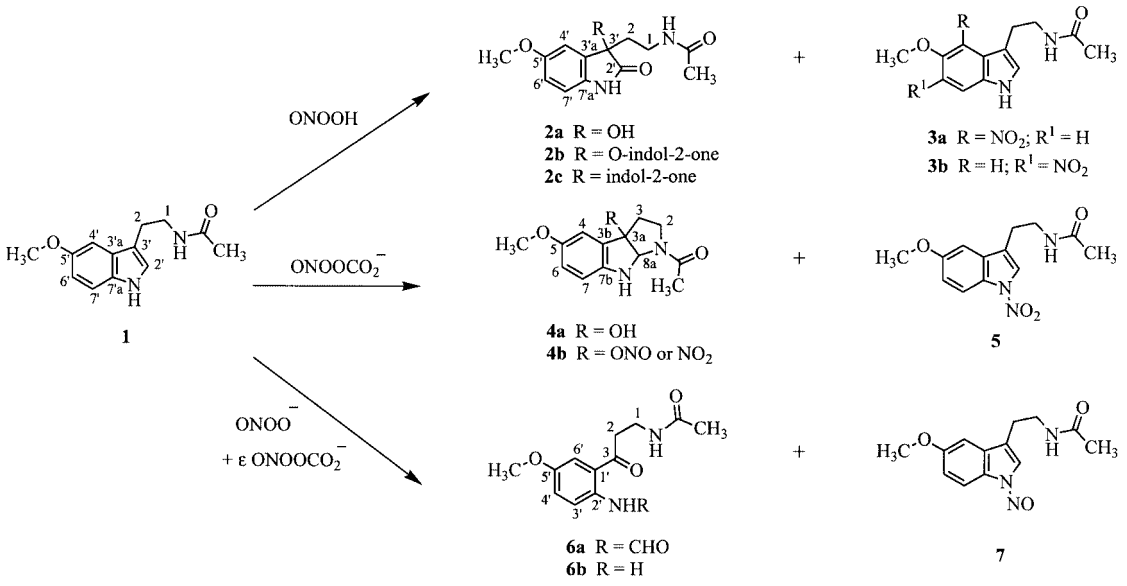


Figure 4. Effects of pH and addition of $NaHCO_3$ on the RP-HPLC peak areas of indol-2-one precursor **2** (open circles), pyrroloindole precursor **4** (closed triangles), and 1-nitromelatonin (**5**, open diamonds) obtained from the reaction between peroxynitrite and melatonin (peak areas for **2** and **4** recorded when the peaks reached their maxima); effective yields of 10% for **2**, 20% for **4**, and 13% for **5** were obtained under preparative scale conditions [$ONOO^-$/melatonin (10:1), pH = 7.6, 100 mM $NaHCO_3$]; **A**: the reaction was carried out at pH = 6−9 in a final volume of 1 mL of phosphate (0.4 M) buffer that also contained $NaHCO_3$ (60 mM), melatonin (1 mM), and peroxynitrite (10 mM); **B**: the reaction was carried out at pH = 7.6 in a final volume of 1 mL of phosphate buffer that also contained $NaHCO_3$ (0−200 mM), melatonin (1 mM), and peroxynitrite (10 mM) (see Exp. Sect. for details)

Discussion

Our results show that peroxynitrite acts upon the indole moiety simultaneously as an oxidizing, a nitrating, and a nitrosating agent. The melatonin lateral chain is only implicated in the cyclization into pyrroloindoles **4**. The different types of reactions varied with respect to the pH and CO_2 content of the medium. Three typical oxidation pathways of indole compounds (pyrroloindoles **4**, indol-2-ones **2**, and kynuramines **6**) appeared to be preponderant within the pH range of 7.5−8 in the presence of CO_2. Substitutions by nitro or nitroso groups are also important modifications; both types of nitration either on the benzene carbon atoms or on the pyrrole nitrogen atom seem to compete with the *N*-nitrosation reaction. The latter occurs at neutral pH in the absence of added bicarbonate, or at alkaline pH independently of the CO_2 content. 1-Nitrosomelatonin (**7**),



Scheme 1. Products obtained from the reaction between peroxynitrite and melatonin in phosphate (0.4 M) buffered solutions; structural assignments are presented in the Exp. Sect.

obtained by use of nitrous acid or ·NO in the presence of oxygen at pH = 7.4,[13,14] was also described by us from the reaction with peroxynitrite.[13] In contrast, the 1-nitro derivative **5** has never been reported.

The effects of pH and CO_2 allow the reactions to be divided into three classes according to the $ONOO^-$-derived reagent (ONOOH, $ONOO^-$, $ONOOCO_2^-$; Scheme 1). As $ONOOCO_2^-$ decay generates $^{\cdot}NO_2$ and $CO_3^{\cdot-}$ radicals, it is likely to induce oxidation in indol-2-ones **2** and pyrroloindoles **4** as well as nitration in various nitromelatonins.

Effect of CO_2

Oxidations to indol-2-ones **2** and pyrroloindoles **4** and *C*- and *N*-nitrations are both favored by the presence of CO_2. With regard to nitration, an equimolar amount of $NaHCO_3$ with respect to initial peroxynitrite is optimal for aromatic substitutions. Since 25 mM $NaHCO_3$ releases around 1 mM CO_2 at pH = 7.4, addition of 10 mM $NaHCO_3$ at pH = 7.6 does not allow total conversion into $ONOOCO_2^-$ from 10 mM peroxynitrite. A small amount is sufficient to induce the nitroaromatic substitutions. On the other hand, an excess of $NaHCO_3$ is necessary to obtain the maximum degree of conversion into 1-nitromelatonin (**5**), suggesting that $ONOOCO_2^-$ is the reagent of *N*-nitration.

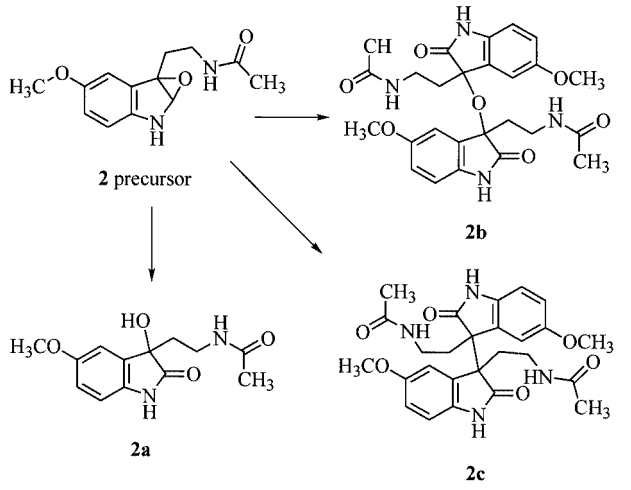
With regard to the numerous oxidation reactions achieved by peroxynitrite, CO_2 has been reported to accelerate some[2,17−19] and to inhibit others.[12,20] CO_2 concentration is probably determinant. In the case of melatonin, CO_2 promotes its transformation and, more specifically, the oxidation routes towards pyrroloindoles and indol-2-ones as well as nitrations.

Indol-2-ones and Pyrroloindoles

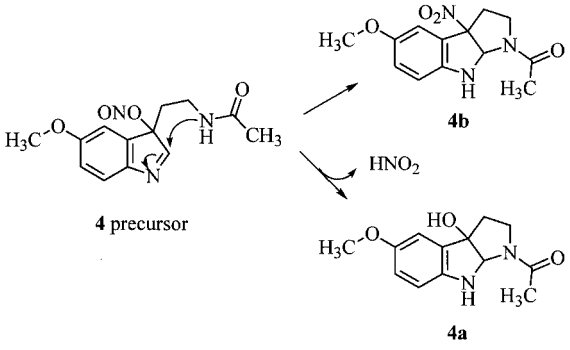
HPLC analyses of the reaction mixture over prolonged periods (minutes or days) showed that some pyrroloindoles **4** and indol-2-ones **2** are derived from corresponding precursors that are the main products observed at pH = 7.6 in the presence of CO_2 (see **B** in Figure 1). Both give substituted derivatives on C-3 of the indole ring, indicating that their precursors should have reactive functions on C-2 and/or on C-3. Hydroperoxide (−OOH), epoxide, organic nitrite (−O−N=O), or nitrosoperoxide (−OO−N=O) could be postulated. However, the formation of hydroperoxide should depend on the oxygen reaction with radicals. Such formation of hydroperoxide appears unlikely, since analysis of the whole reaction mixture performed either under air or under argon revealed identical amounts of detected products (results not shown).

The precursor of **2** corresponds, according to ESI and APCI mass spectrometry, to the addition of an oxygen atom to melatonin. Its decomposition is accelerated in the presence of ferric chloride, giving rise to the three indol-2-one derivatives **2a**, **2b**, and **2c**, identified by LC-APCI-MS. Their absorption spectra (260 and 300 nm) are similar to one another and to their precursor. We postulate that the precursor of the indol-2-ones **2** is melatonin 2,3-epoxide.

Epoxides have been proposed as short-lived transients in chemical and biological oxidations of indoles, and it has been demonstrated that they rearranged quantitatively to indol-2-ones above 0 °C.[21,22] Furthermore, oxidation of tryptophan[23] and of other molecules by peroxynitrite has been reported, with increasing yields upon CO_2 addition.[24]



The pyrroloindole precursor is more labile than the precursor of **2**. It decomposes in solution within a few minutes. Identification of the precursor of pyrroloindoles thus remains difficult, and its structure can only be investigated by MS and diode array detection coupled to HPLC. Isolated from the reaction mixture, it gives the 3-nitro- or 3-nitrito-pyrroloindole **4b**. The precursor absorption spectrum shows a 285-nm band in addition to the 235- and 295-nm bands characteristic of pyrroloindoles **4**, which are lost during the conversion, suggesting that cyclization occurs in this step. Thus, **4b** should derive from 3-nitro- or, more probably, 3-nitrito-3*H*-indole, which is a tautomer:



In the same manner, 3-(hydroperoxy)indole derived from tryptophan decays to 3-(hydroperoxy)pyrroloindole.[25]

Finally, this accounts for the fact that this NO_2 addition on C-3 occurred under the same conditions as the formation of 1-nitromelatonin (**5**) (see below).

Nitromelatonins

Nitration of aromatic rings by peroxynitrite is well documented for phenolic compounds and tyrosine residues, in which hydroxyl is oxidized in a first step, and the radical is coupled with $^{\cdot}NO_2$.[2−5] A similar mechanism could be proposed in the case of the indole ring, giving a melatoninyl radical cation delocalized on C-3 and on the pyrrole nitrogen atom [Equation (3)]. Such a mechanism would be in agreement with the formation of 1-nitromelatonin (**5**) and 3-nitro- or 3-nitrito-3*H*-indole, the postulated unstable precursor **4**, derived from melatonin. The formation of $ONOOCO_2^-$ is optimum at pH ≈ 7.5 in the presence of $NaHCO_3$ (60 mM) [Equation (1)].

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \quad (1)$$

$$ONOOCO_2^- \rightarrow {}^{\cdot}NO_2 + CO_3^{\cdot -} \quad (2)$$

$$Mel + CO_3^{\cdot -} \rightarrow Mel^{\cdot +} + CO_3^{2-} \quad (3)$$

$$Mel^{\cdot +} + {}^{\cdot}NO_2 \rightarrow NO_2Mel + H^+ \quad (4)$$

1-Nitromelatonin (**5**) is fairly stable in solution, but its slow decomposition quantitatively releases nitrite anions (results obtained by Griess test). However, the stable 4-nitro- (**3a**) and 6-nitromelatonin (**3b**) are formed mostly at acidic pH (Figure 2, part **A**) and are favored by a small amount of CO_2 (10 mM) at neutral pH (Figure 2, part **B**). A similar peroxynitrous acid dependent reaction has been described for tryptophan,[23] with a 12% yield at pH = 5.

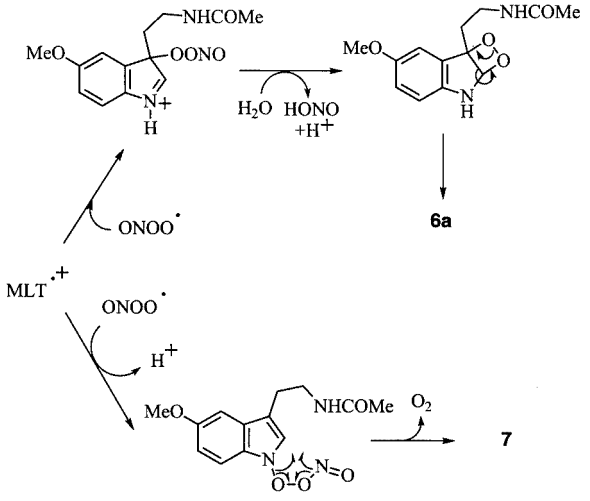
Kynuramines and 1-Nitrosomelatonin

The oxidation routes towards indol-2-ones **2** and pyrroloindoles **4** decrease in favor of kynuramines **6** with decreasing CO_2 content. In a parallel manner, the formation of 1-nitromelatonin (**5**) switches to that of 1-nitrosomelatonin (**7**). The yields of kynuramines **6** and 1-nitrosomelatonin (**7**) are 10 and 20%, respectively, at pH = 7.6. Both increase strongly with increasing pH [1-nitrosomelatonin (**7**) reaches 45% at pH = 9]. In the literature, formation of radical intermediates has been demonstrated in the course of the reaction of the indole moiety with peroxynitrite, as well as in peroxynitrite decay itself.[4,11−13] The reaction of radicals with $ONOO^-$ should be considered.[26] As the natural amount of CO_2 provided by air is sufficient to promote nitrosation and cleavage to kynuramines by $ONOO^-$, initial oxidation of $ONOO^-$ to the nitrosating radical $ONOO^{\cdot}$ has been postulated [Equation (5)]. An excess of CO_2 converts all $ONOO^-$ into $ONOOCO_2^-$ [Equation (1)], giving rise to the peroxynitrite nitration and oxidation.

$$ONOO^- + CO_3^{\cdot -} \rightarrow ONOO^{\cdot} + CO_3^{2-} \quad (5)$$

Since $ONOO^{\cdot}$ (A = 410−420 nm) and melatoninyl radicals (A = 335 and 515 nm) are transiently formed in the course of the first steps of the reactions between $CO_3^{\cdot -}$, $ONOO^-$, and melatonin,[13] the most likely common pathway is a radical mechanism[27−29] affording **6** and 1-nitrosomelatonin (**7**, Scheme 2). Addition of $ONOO^{\cdot}$ to the melatoninyl radical in the same manner as in the formation of 1- and 3-nitro derivatives is proposed, resulting either in nitrosomelatonin or in kynuramines. Likewise, kynurenines are formed by the reaction of tryptophan with superoxide,[30] which is as mild an oxidant as $ONOO^{\cdot}$. It is note-

worthy that these two melatonin derivatives have been shown to act as oxidant scavengers.[18,31,32]



Scheme 2. Proposed mechanism for the formation of formylkynuramine (**6a**) and 1-nitrosomelatonin (**7**)

The oxidized derivatives of melatonin have been already described as catabolites, or markers of oxidative stress in vivo. For instance, pyrroloindole **4a** has been detected in the urine of rats and humans and at increased levels after subjection to ionizing radiation. It has been proposed as a biomarker of oxidative stress in various diseases. Formylkynuramine **6a** has been described as being formed by dioxygenase in the brain[33] or by reaction with superoxide anion. This catabolism is recognized, together with the production of 6-sulfatomelatonin, as a part of melatonin elimination.

Transformations of melatonin are typical of indoles and could help understanding of what occurs with free tryptophan or tryptophan residues when exposed to peroxynitrite. Kynurenines and indol-2-ones (indol-2-one, 5-hydroxyindol-2-one, indole-2,3-dione) are metabolites of indole found in mammalian body fluids and tissues.[34] Some of them could be produced by peroxynitrite reactions in addition to 6-nitrotryptophan.[25,35] Treatment of proteins with peroxynitrite resulted in an extinction of the fluorescence, indicating that tryptophan residues are affected. Nitration and/or oxidation of tryptophan residues by peroxynitrite have been reported for albumin,[36] collagen,[37] and human plasma proteins. Recently, a Cu/Zn superoxide dismutase was shown to be inactivated by peroxynitrite, which modifies a specific tryptophan residue.[38] Analysis of the various reactions of peroxynitrite with 3-substituted indole might help to provide insight into peroxynitrite-dependent reactions with tryptophan residues in proteins.

Experimental Section

Chemicals and Reagents: Melatonin and isoamyl nitrite were from Sigma and Acros Organics, respectively. Melatonin was first dissolved either in DMSO or in methanol (100 mM) before dilution in a phosphate-buffered (0.4 M) solution. When melatonin was first dissolved in 5 M HCl solution and diluted in the buffer solution at pH = 7.4 according to the method of Zhang et al.,[12] the transformations of melatonin after peroxynitrite addition were similar, showing that interactions of peroxynitrite with the solvent are unlikely.

Peroxynitrite Synthesis: Peroxynitrite synthesis was performed in a two-phase system with isoamyl nitrite and hydrogen peroxide by the method of Uppu and Pryor.[39] Peroxynitrite was stored at −20 °C and its concentration was determined by measurement of the absorbance at 302 nm (ε = 1670 $M^{-1}\cdot cm^{-1}$) of samples diluted in NaOH (0.01 N). The contamination of the crude oil containing peroxynitrite (2.3 M) by H_2O_2 was estimated as 55 mM. Further treatment with MnO_2 was carried out to eliminate residual H_2O_2, but no difference in the HPLC profiles of melatonin transformations was noted for use of untreated or MnO_2-treated peroxynitrite (results not shown).

Reaction between Peroxynitrite and Melatonin: Peroxynitrite solution in NaOH (0.01 N) was added to buffered solutions of melatonin as a bolus, while stirring at room temperature. A constant 0.2 unit pH rise was recorded. Henceforth only final pH values are given. For preparative purposes, peroxynitrite (20 mM) was added to a melatonin (2 mM, 46.4 mg) solution in phosphate-buffered solutions (0.4 M, 100 mL) in the presence of $NaHCO_3$ (100 mM). The final pH was 7.6. After 30 min, the reaction products were separated by HPLC. Analytical experiments were performed with melatonin (1 mM) in phosphate-buffered solution treated with peroxynitrite at an $ONOO^-$/melatonin ratio of 10:1 in 1-mL open tubes. In a first set of experiments, the final pH was varied between 6 and 9, with or without addition of $NaHCO_3$ (60 mM). In a second series with the pH set at 7.6, $NaHCO_3$ was added up to 200 mM. When $NaHCO_3$ was used, the equilibrium between HCO_3^- and CO_2 was allowed to establish itself for 5 min before peroxynitrite addition. HPLC analyses were performed just after peroxynitrite had reacted and repeated after a few hours in order to evaluate the products' stabilities at room temperature. Each experiment was repeated at least three times independently.

UV/Vis Absorption Spectrophotometry: All absorption data were recorded with a double-beam device (Uvikon 942, Kontron Instruments) in 1-cm quartz cells.

Reversed-Phase HPLC (RP-HPLC) Devices: The preparative chromatography system consisted of a Waters Delta Prep 300 injector and a gradient pump (Waters 600E System controller) provided with a reversed-phase C18 Si, 10 μm, Waters 1000 Prep PAK column and a Waters Lambda Max Model 480 LC spectrophotometer. Fractions were collected with an ISCO-FOXY fraction separating system. Elution was carried out with a 10−30% gradient of acetonitrile for 30 min, followed by a 30−37% gradient for 15 min and a 37% isocratic mode for 25 min with a flow rate of 50 mL/min. The mixture was filtered through a 0.2 μm Acrodisc filter and injected on the column. Each fraction was analyzed (analytical RP-HPLC, MS, chemical tests, ...) and concentrated under reduced pressure and lyophilized (−50 °C, < 0.1 mbar). Analytical HPLC was used to identify products by matching their retention times with those of authentic compounds when available, with a Waters 717plus auto sampler fitted with a Waters 600E Multisolvent gradient pump, and linked to a Hypersil reversed-phase column (C18 Si, 5 μm, 250 × 4.6 mm) and a Waters 2487 Dual λ absorbance detector. The column was eluted with a 10−50% gradient of acetonitrile for 60 min at a flow rate of 1 mL/min. Waters Millenium$^{32®}$ chromatography manager software was used for data treatment. 25-μL samples of the reaction mixtures from melatonin

(1 mM) solutions were injected. Other analytical systems coupled the chromatography system described above either with a Waters 996 photodiode array detector or with a mass spectrometer.

Mass Spectrometry: All ESI-MS, LC-ESI-MS, and LC-APCI-MS (Atmospheric Pressure Chemical Ionization) measurements were performed with a quadrupole mass spectrometer (Navigator Thermofinnigan) operating in positive ionization mode. The analysis conditions for ESI-MS were: infusion flow (10 μL/min); capillary and cone voltages (3.5 kV and 20 V respectively); probe *T* 150 °C; nebulizing gas nitrogen. LC separations were performed by use of a P1000XR pump equipped with a UV 2000 absorbance detector and an AS100XR autosampler (all devices were from Thermo Separation Products). 100 μL was injected on a Hypersil reversed-phase column (C18 Si, 5 μm, 250 × 4.6 mm) with absorbance detection at 215 nm; the flow rate (1 mL/min) was split prior to the mass spectrometer so as to obtain a flow rate of 300 μL/min in the ion source. The eluents and gradient sequence are described in the HPLC section. Capillary and cone voltages were set to 3.5 kV and 20 V, respectively. The probe *T* was 180 °C for ESI and was increased to 300 °C for APCI. Nitrogen was used as nebulizing gas in both experiments. IC-MS measurements were performed at the Ecole Normale Supérieure, Paris. The instrument used was a magnetic mass spectrometer (JEOL MS 700) operating in positive chemical ionization mode with methane as ionizing gas. HR-MALDI-TOF-MS (High Resolution − Matrix Assisted Laser Desorption Ionization − Time Of Flight − Mass Spectrometry) was used in positive ionization mode with α-cyano-4-hydroxycinnamic acid (α-CHCA) as matrix and internal reference. The spectrometer was a Voyager-DE STR device from PerSeptive Biosystems.

NMR Measurements: All experiments (^{1}H, ^{13}C, H-H COSY, HMQC, HMBC, NOESY) were carried out at 300 K with a 400 MHz AMX Bruker spectrometer. Compounds were dissolved in CD_3OD. The ^{1}H and ^{13}C chemical shifts are expressed in ppm relative to $SiMe_3$. Compounds have been numbered as indicated in Scheme 1.

Nitrite Detection by Griess Reaction: In a microplate well, a fraction (50 μL) was mixed with sulfanilamide (5% in 20% HCl solution, 25 μL) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.5% in 20% HCl solution, 25 μL). Absorption at 540 nm was monitored after 20 min incubation. A linear calibration curve was obtained from $NaNO_2$ solutions (1−100 μM).

Indol-2-ones 2: The fraction (t_R = 27 min) obtained by preparative HPLC (5 mg, 10% yield) was characterized by a peak eluted at 20 min in analytical HPLC, by *m*/*z* = 271 ([M + Na]$^+$) in ESI-MS, and by an absorption spectrum exhibiting 260- and 300-nm bands with a ratio of absorbance A300/A260 = 0.17 (Figure 5, part **A**). HR-MALDI-TOF-MS: calcd. for $C_{13}H_{17}N_2O_3$ 249.1239, found 249.1242 [M + H]$^+$. Analysis of the reaction mixture by HPLC showed that this product is unstable over a period of hours. When purified or isolated in aqueous solution and incubated in the presence of ferric chloride, its decomposition gives a mixture of four stable indol-2-one derivatives, all presenting the same absorptions at 265 and 315 nm (A315/A265 = 0.2). Monomer *N*-[2-(3-hydroxy-5-methoxy-2-oxo-2,3-dihydro-1*H*-indol-3-yl)ethyl]acetamide (**2a**): t_R = 11 min in analytical HPLC; *m*/*z* = 265 ([M + H]$^+$) and 287 ([M + Na]$^+$) by LC-APCI-MS. ^{1}H NMR (CD_3OD, 27 °C): δ = 1.81 [s, 3 H, NHC(O)*Me*], 2.08 (m, 2 H, 2-H), 3.12 (m, 2 H, 1-H), 3.79 (s, 3 H, ArO*Me*), 6.79 (d, $^3J_{H,H}$ = 8.4 Hz, 1 H, 7′-H), 6.85 (dd, $J_{H,H}$ = 2.5, 8.4 Hz, 1 H, 6′-H), 7.00 (d, $^4J_{H,H}$ = 2.5 Hz, 1 H, 4′-H); δ values obtained by HMBC correlations for C-2′ and C-3′ are 182.5 and 77.5 ppm, respectively. Ether-bridged dimer **2b**: t_R = 19 min in analytical HPLC; *m*/*z* = 533 ([M + Na]$^+$) by APCI-MS. ^{1}H NMR (CD_3OD, 27 °C): δ = 1.81 [s, 3 H, NHC(O)*Me*], 2.08 (m, 2 H, 2-H), 3.12 (m, 2 H, 1-H), 3.79 (s, 3 H, ArO*Me*), 6.79 (m, 1 H, 7′-H), 6.81 (m, 1 H, 6′-H), 7.00 (m, 1 H, 4′-H) ppm; HMBC correlations give δ values for C-2′ and C-3′ 182.5 and 83.3 ppm, respectively. Two isomer dimers **2c**: t_R = 23 and 24 min in analytical HPLC; *m*/*z* = 495 ([M + H]$^+$) and 517 ([M + Na]$^+$) by LC-APCI-MS. ^{1}H NMR (CD_3OD, 27 °C): δ = 1.88 [s, 3 H, NHC(O)*Me*], 2.05−2.12 (m, 2 H, 2-H), 3.22−3.34 (m, 2 H, 1-H), 3.76 (s, 3 H, ArO*Me*), 6.78 (m, 1 H, 7′-H), 6.81 (m, 1 H, 6′-H), 6.98 (m, 1 H, 4′-H) ppm. HMBC correlations reveal ^{13}C signals at δ = 22.7 [NHC(O)*Me*], 31.1 (C-2), 37.6 (C-1), 45.7 (C-3′), 56.4 (ArO*Me*), 111.4 (C-7′), 112.5 (C-4′), 114.1 (C-6′), 132.7 (C-3′a), 137.0 (C-7′a), 157.9 (C-5′), 173.8 [NH*C*(O)Me], 182.1 (C-2′) ppm.

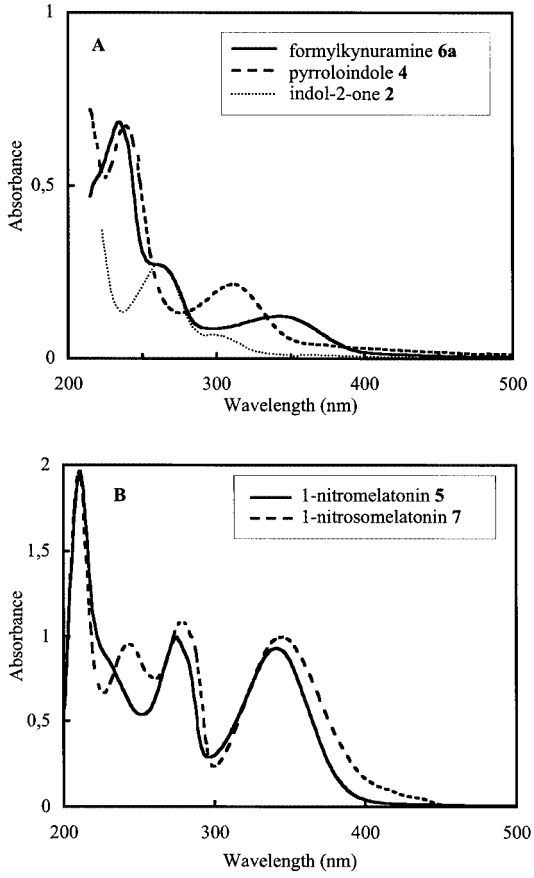


Figure 5. **A:** Absorption spectra of formylkynuramine **6a** (solid line), pyrroloindole mixture **4** (dashed line), and indol-2-one precursor **2a** (dotted line); **B:** absorption spectra of 1-nitromelatonin (**5**, solid line) and 1-nitrosomelatonin (**7**, dashed line); all spectra were recorded in methanol

***C*-Nitromelatonins 3:** 4-Nitromelatonin {*N*-[2-(5-methoxy-4-nitro-1*H*-indol-3-yl)ethyl]acetamide (**3a**), $C_{13}H_{15}N_3O_4$, MW = 277} and 6-nitromelatonin {*N*-[2-(5-methoxy-6-nitro-1*H*-indol-3-yl)ethyl]-acetamide (**3b**)} eluted with t_R = 47 min and 43 min in preparative and 37 min and 32.5 min in analytic HPLC, respectively. Yields obtained by preparative HPLC were 4.5% (2.5 mg) and 0.5% (0.3 mg), respectively. ESI-MS: *m*/*z* = 278 [M + H]$^+$, 300 [M + Na]$^+$, 316 [M + K]$^+$ and 577 [2M + Na]$^+$. HR-MALDI-TOF-MS: **3a**: calcd.

for $C_{13}H_{16}N_3O_4$ 278.1141, found 278.1148 $[M + H]^+$; **3b**: calcd. for $C_{13}H_{16}N_3O_4$ 278.1141, found 278.1148 $[M + H]^+$. Characteristic absorption bands were at 280 and 380 nm (A380/A280 = 0.4) for **3a** and at 270 and 370 nm (A370/A270 = 0.12) for **3b**. **3a**: ^{1}H NMR (CD_3OD, 27 °C): δ = 1.91 [s, 3 H, NHC(O)*Me*], 2.72 (t, $^3J_{H,H}$ = 7.1 Hz, 2 H, 2-H), 3.32 (t, $^3J_{H,H}$ = 7.1 Hz, 2 H, 1-H), 3.90 (s, 3 H, ArO*Me*), 7.04 (d, $^3J_{H,H}$ = 8.9 Hz, 1 H, 5′-H), 7.24 (s, 1 H, 2′-H), 7.49 (d, $^3J_{H,H}$ = 8.9 Hz, 1 H, 6′-H). **3b**: ^{1}H NMR (CD_3OD, 27 °C): δ = 1.90 (s, 3 H, NHC(O)*Me*), 2.93 (t, $^3J_{H,H}$ = 7.3 Hz, 2 H, 2-H), 3.45 (t, $^3J_{H,H}$ = 7.3 Hz, 2 H, 1-H), 3.96 (s, 3 H, ArO*Me*), 7.30 (s, 1 H, 4′-H), 7.36 (s, 1 H, 2′-H), 7.93 (s, 1 H, 7′-H) ppm. HMBC correlations reveal ^{13}C signals at δ = 26.1 (C-2), 41.7 (C-1), 110.7 (C-7′), 114.4 (C-3′), 130.2 (C-2′), 131.1 (C-7′a), 133.2 (C-3′a), 137.7 (C-6′), 148.8 (C-5′), 173.7 [NH*C*(O)Me] ppm.

Pyrroloindoles 4: Monomeric isomers of 1-acetyl-5-methoxy-2,3,8,8a-tetrahydropyrrolo[2,3-*b*]indol-3a(1*H*)-ol (**4a**, $C_{13}H_{16}N_2O_3$, MW = 248) were collected by preparative HPLC at t_R = 20, 25 min and 3a-nitro- or 3a-nitritopyrroloindole **4b** ($C_{13}H_{15}N_3O_4$, MW = 277) at 48 min. A total yield of 20% (10 mg) was obtained under preparative conditions. They each have two characteristic absorption bands: at 236 and 306 nm (A306/A236 = 0.33) for **4a** and at 235 and 315 nm (A315/A235 = 0.3) for **4b** (Figure 1). ESI-MS: *m*/*z* = 249 $[M + H]^+$, 271 $[M + Na]^+$, 519 $[2 M + Na]^+$ corresponding to **4a**. HR-MALDI-TOF-MS: calcd. for $C_{13}H_{16}N_2O_3$ 248.1165, found 248.1164 $[M]^+$. ^{1}H NMR of **4a** (CD_3OD, 27 °C): δ = 2.06/2.18 [s, 3 H, NHC(O)*Me*], 2.38/2.30 (m, 2 H, 3-H), 3.37−3.81/3.11−3.91 (m, 2 H, 2-H), 3.75/3.74 (s, 3 H, ArO*Me*), 5.29/5.31 (s, 1 H, 8a-H), 6.60/6.64 (d, $^3J_{H,H}$ = 8.5 Hz, 1 H, 7-H), 6.75/6.77 (m, 1 H, 6-H), 6.91/6.90 (d, $^4J_{H,H}$ = 2.5 Hz, 1 H, 4-H) ppm. HMBC correlations reveal ^{13}C signals at δ = 22.4 [NHC(O)*Me*], 38.8/38.1 (C-3), 48.5/46.5 (C-2), 56.6 (ArO*Me*), 84.4 (C-8a), 88.7/90.9 (C-3a), 110.6 (C-4), 113.1 (C-7), 117.5 (C-6), 133.3 (C-3b), 144.7 (C-7b), 156.1 (C-5), 173.3 [NH*C*(O)Me] ppm. Conformational equilibrium NOESY spots for **4a** show 2 conformers due to rotation of the acetyl group on N-1. **4b**: Eluted in analytical HPLC (t_R = 39 min), it gives a signal *m*/*z* = 300 $[M + Na]^+$ and a fragment *m*/*z* = 253 $[M - HNO_2 + Na]^+$ by ESI-MS. HR-MALDI-TOF-MS: calcd. for $C_{13}H_{16}N_3O_4$ 278.1141, found 278.1136 $[M + H]^+$. ^{1}H NMR (CD_3OD, 27 °C): δ = 2.07/2.21 [s, 3 H, NHC(O)*Me*], 2.82−3.02/2.78−2.89 (m, 2 H, 3-H), 3.35−3.96/3.00−4.12 (m, 2 H, 2-H), 3.75/3.76 (s, 3 H, ArO*Me*), 6.17/6.22 (s, 1 H, 8a-H), 6.68/6.75 (d, $^3J_{H,H}$ = 8.7 Hz, 1 H, 7-H), 6.89/6.93 (m, 1 H, 6-H), 7.04/7.03 (d, $^4J_{H,H}$ = 2.7 Hz, 1 H, 4-H) ppm. HMBC correlations reveal ^{13}C signals at δ = 23.0 [NHC(O)*Me*], 38.0/36.6 (C-3), 49.0/46.5 (C-2), 57.4 (ArO*Me*), 81.2/82.2 (C-8a), 102.9/104.7 (C-3a), 111.8/111.0 (C-4), 114.0/115.2 (C-7), 120.9 (C-6), 126.4/128.1 (C-3b), 147.7 (C-7b), 156.6/157.3 (C-5), 173.7 [NH*C*(O)Me] ppm. Conformational equilibrium NOESY spots show two conformers due to rotation of the acetyl group on N-1.

1-Nitromelatonin (5): *N*-[2-(5-Methoxy-1-nitro-1*H*-indol-3-yl)ethyl]-acetamide (**5**, $C_{13}H_{15}N_3O_4$, MW = 277) is eluted in preparative and analytical HPLC (t_R = 64 and 47 min, respectively). IC-MS: *m*/*z* = 278 $[M + H]^+$, ESI-MS confirms the molecular mass and shows the radicals *m*/*z* = 232 $[M - NO_2 + H]^+$ and 254 $[M - NO_2 + Na]^+$ due to homolytic rupture of the N−NO_2 bond. HR-MALDI-TOF-MS: calcd. for $C_{13}H_{16}N_3O_4$ 278.1141, found 278.1138 $[M + H]^+$. ^{1}H NMR shows the same δ values as **7**: ^{1}H NMR (CD_3OD, 27 °C): δ = 1.92 [s, 3 H, NHC(O)*Me*], 2.89 (t, $^3J_{H,H}$ = 7.0 Hz, 2 H, 2-H), 3.50 (t, $^3J_{H,H}$ = 7.0 Hz, 2 H, 1-H), 3.91 (s, 3 H, ArO*Me*), 7.03 (dd, $J_{H,H}$ = 2.4, 9.0 Hz, 1 H, 6′-H), 7.18 (d, $^4J_{H,H}$ = 2.4 Hz, 1 H, 4′-H), 7.85 (s, 1 H, 2′-H), 8.05 (d, $^3J_{H,H}$ = 9.0 Hz, 1 H, 7-H) ppm. HMBC correlations reveal ^{13}C signals at δ = 23.2 [NHC(O)*Me*], 26.5 (C-2), 40.5 (C-1), 57.0 (ArO*Me*), 104.8 (C-4′), 115.9 (C-6′), 117.3 (C-7′), 121.7 (C-3′), 122.6 (C-2′), 128.8 (C-7′a), 131.9 (C-3′a), 160.1 (C-5′), 174.4 [NH*C*(O)Me] ppm. IR absorption at 1287 cm^{-1} agrees with ν(N−NO_2). The absorption bands are at 230, 275 and 340 nm. Obtained with 10% yield (6.6 mg) under the preparative conditions from melatonin (2 mM), peroxynitrite (20 mM), $NaHCO_3$ (100 mM) at pH = 7.6, it decomposes in the reaction mixture very slowly ($t_{1/2}$ = 7 d) to give melatonin.

Kynuramines 6: *N*-{3-[2-(Formylamino)-5-methoxyphenyl]-3-oxopropyl}acetamide (**6a**, $C_{13}H_{16}N_2O_4$, MW = 264) is eluted with t_R = 32 and 23 min in preparative and analytic HPLC, respectively. The yield obtained by preparative HPLC was 7% (3.7 mg). It shows absorption bands at 234, 270 (A270/A234 = 0.4) and 338 nm (A338/A234 = 0.16) (Figure 5, part **A**). ESI-MS: *m*/*z* = 265 $[M + H]^+$, 287 $[M + Na]^+$, 551 $[2M + Na]^+$. HR-MALDI-TOF-MS: calcd. for $C_{13}H_{17}N_2O_4$ 265.1188, found 265.1181 $[M + H]^+$. ^{1}H NMR (CD_3OD, 27 °C): δ = 1.92 [s, 3 H, NHC(O)*Me*], 3.32 (t, $^3J_{H,H}$ = 6.3 Hz, 2 H, 2-H), 3.55 (t, $^3J_{H,H}$ = 6.3 Hz, 2 H, 1-H), 3.86 (s, 3 H, ArO*Me*), 7.19 (dd, $J_{H,H}$ = 3.0, 9.1 Hz, 1 H, 4′-H), 7.49 (d, $^4J_{H,H}$ = 3.0 Hz, 1 H, 6′-H), 8.35 [s, 1 H, ArNHC(O)*H*], 8.41 (d, $^3J_{H,H}$ = 9.1 Hz, 1 H, 3′-H) ppm. HMBC correlations reveal ^{13}C signals at δ = 22.6 [NHC(O)*Me*], 35.6 (C-1), 39.6 (C-2), 56.2 (ArO*Me*), 116.4 (C-6′), 120.6 (C-4′), 124.3 (C-3′), 125.8 (C-1′), 132.9 (C-2′), 156.9 (C-5′), 162.5 (ArNH*C*HO), 173.8 [NH*C*(O)Me], 204.0 (C-3) ppm. *N*-[4-(2-Amino-5-methoxyphenyl)-3-oxopropyl]acetamide (**6b**): **6b** is a minor product coeluting with its derivative **6a**. ESI-MS: *m*/*z* = 237 $[M + H]^+$. ^{1}H NMR (CD_3OD, 27 °C): δ = 1.92 (s, 3 H, [NHC(O)*Me*], 3.22 (t, $^3J_{H,H}$ = 6.5 Hz, 2 H, 2-H), 3.54 (t, $^3J_{H,H}$ = 6.5 Hz, 2 H, 1-H), 3.83 (s, 3 H, ArO*Me*), 7.04 (d, $^3J_{H,H}$ = 7.0 Hz, 1 H, 3′-H), 7.15 (dd, $J_{H,H}$ = 2.7, 7.0 Hz, 1 H, 4′-H), 7.42 (d, $^3J_{H,H}$ = 2.7 Hz, 1 H, 6′-H) ppm. HMBC correlations reveal ^{13}C signals at δ = 22.8 [NHC(O)*Me*], 36.7 (C-1), 40.3 (C-2), 56.8 (ArO*Me*), 116.4 (C-6′), 123.3 (C-4′), 123.8 (C-3′), 124.2 (C-1′), 138.2 (C-2′), 156.1 (C-5′), 173.5 [NH*C*(O)Me], 202.3 (C-3) ppm.

1-Nitrosomelatonin (7): Under our conditions, *N*-[2-(5-methoxy-1-nitroso-1*H*-indol-3-yl)ethyl]acetamide (**7**, $C_{13}H_{15}N_3O_3$, MW = 261) eluted just before 1-nitromelatonin (**5**), with t_R = 62 min and 46 min in preparative and analytic HPLC, respectively. The yield obtained by preparative HPLC was 2.5% (1.3 mg). Structural assignments are by comparison with the product synthesized by the Bravo method.[40] LC-ESI-MS gives the molecular mass *m*/*z* = 284 $[M + Na]^+$ and shows the radical *m*/*z* = 254 $[M - NO + Na]^+$ due to homolytic rupture of the N−NO bond. An equilibrium between two conformers due to rotation of the nitroso group is evidenced by conformational equilibrium NOESY spots. HMBC correlations reveal ^{13}C signals at δ = 23.2 [NHC(O)*Me*], 26.2 (C-2), 40.0/40.2 (C-1), 56.6 (ArO*Me*), 105.1/104.4 (C-4′), 115.9/115.1 (C-6′), 113.4/118.3 (C-7′), 126.5/124.7 (C-3′), 114.2/127.1 (C-2′), 131.8/125.5 (C-7′a), 132.9/133.2 (C-3′a), 160.5/160.9 (C-5′), 174.3 [NH*C*(O)Me] ppm. The UV absorption spectrum of **7** is very similar to that of **5**, showing a characteristic band at 243 nm besides 279 and 343 nm (Figure 5, part **B**). IR absorption at 1435 cm^{-1} corresponds to ν(N−NO).

1-Hydroxymelatonin (8): This is a minor product, described previously.[13] Eluted with t_R = 52 and 43 min in preparative and analytical HPLC, respectively, it has characteristic absorption bands at 230, 280 and 310 nm. As its formation diminishes with decreasing CO_2 content and does not vary with pH, the yield (< 3%) remains very low whatever the conditions used.

Acknowledgments

We thank Dr. Yann Henry and Dr. Olivier Laprévote for their valuable advice. Financial support was from the Centre National de la Recherche Scientifique and the Ministère de la Recherche et de la Technologie (France).

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Received June 11, 2002
[O02321]