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Interactions of melatonin and its metabolites with the ABTS cation radical: extension of the radical scavenger cascade and formation of a novel class of oxidation products, C2-substituted 3-indolinones

Abstract: Melatonin had previously been shown to reduce up to four 2,2'-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) cation radicals (ABTS•$^+$) via a scavenger cascade ending with N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK). However, when melatonin is added to the reaction system in much lower quantities than ABTS•$^+$, the number of radicals scavenged per melatonin molecule is considerably higher and can attain a value of ten. Under conditions allowing for such a stoichiometry, novel products have been detected which derive from AFMK (**1**). These were separated by repeated chromatography and the major compounds were characterized by spectroscopic methods, such as mass spectrometry (HPLC-MS, EI-MS and ESI-HRMS), ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR, heteronuclear multiple bond connectivity (HMBC) correlations. The identified substances are formed by re-cyclization and represent 3-indolinones carrying the side chain at C2; the *N*-formyl group can be maintained, but deformylated analogs seem to be also generated, according to MS. The primary product from AFMK (**1**) is *N*-(1-formyl-5-methoxy-3-oxo-2,3-dihydro-1H-indol-2-ylidenemethyl)-acetamide (**2**), which is obtained after purification as E- and Z-isomers (**2a**, **2b**); a secondary product has been identified as *N*-(1-formyl-2-hydroxy-5-methoxy-3-oxo-2,3-dihydro-1H-indol-2-ylmethyl)-acetamide (**3**). When H_2O_2 is added to the ABTS•$^+$ reaction mixture in quantities not already leading to substantial reduction of this radical, compound **3** is isolated as the major product, whereas **2a** and **2b** are virtually absent. The substances formed differ from all previously known oxidation products which derive from melatonin and are, among these, the first 3-indolinones. Moreover, the aliphatic side chain at C2 is reminiscent of other substances which have been synthesized in the search for melatonin receptor ligands.

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Introduction

Radical scavenging is a well-established property of melatonin, which has been repeatedly reviewed [1–6]. Although antioxidative protection exceeds by far the direct interactions with free radicals, the melatonin molecule is particularly effective in detoxifying reactive oxygen species (ROS) for two reasons: (i) the high affinity to and selectivity for certain radicals, in particular, the hydroxyl radical (•OH) [4–6], and (ii) the generation of a scavenger cascade, in which primary and secondary products formed by radical reactions contribute to the elimination of radicals. This has been studied in detail by using the ABTS cation radical (ABTS•$^+$), a substance which displays the exceptional property of a free radical to be relatively long-lived and stable for days [7], so that it can be applied as a useful tool for studying electron donation by radical scavengers. In contrast with •OH, this test radical has the advantage of a uniform primary reaction consisting in the abstraction of an electron and of not destroying the benzene moiety, when given in excess. In initial studies on the interactions of melatonin with ABTS•$^+$, it turned out that four radicals can be eliminated per melatonin molecule, in a reaction chain leading to the formation of cyclic 3-hydroxymelatonin (c3OHM) and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK; **1**) [8].

The substituted kynuramine AFMK (**1**) is also capable of interacting with hydroxyl radicals [9, 10]. For these reasons, the scavenger cascade should comprise this latter compound, too, and the number of radicals eliminated should be higher than four. However, AFMK (**1**) is a poorer scavenger than melatonin or its deformylated metabolite, N^1-acetyl-5-methoxykynuramine (AMK) [6, 11–13]. AMK formation from AFMK (**1**) was observed in the presence of hemoperoxidase [4, 6, 14], but is usually negligible in the majority of oxidation systems. Other oxidation products

from AFMK (**1**) have remained unknown to date. In hydroxyl radical-generating systems, AFMK (**1**) is destroyed to several products which are unstable in the presence of these radicals and can, therefore, be identified only with difficulty. For this reason, we have studied the oxidation of AFMK (**1**) by ABTS$\bullet^+$, which allowed us to characterize new compounds formed in the extended scavenger cascade. Correspondingly, we can demonstrate that the number of radicals scavenged per molecule of melatonin is considerably higher than previously known, if ABTS$\bullet^+$ is given in excess.

Materials and methods

Chemicals

AFMK (**1**) was prepared according to Kennaway et al. [15], with slight modifications. 2,2′-Azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was obtained from Fluka, Buchs, Switzerland. ABTS cation radicals (ABTS$^{\bullet+}$) were prepared according to Re et al. [7] and adjusted after photometric determination, to the desired final concentration. Melatonin was obtained from Sigma (Taufkirchen, Germany). Other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals used were of highest grade available. R_f values were measured on thin-layer chromatography (TLC) sheets, with silica gel 60 F_{254} on aluminum, no. 1.05554.0001 (Merck), using ethyl acetate/methanol 9.5:0.5. Silica gel for preparative thin-layer chromatography (PTLC): PF_{254} (Macherey & Nagel, Düren, Germany). Preparative plates were prepared with 660 g of silica gel PF_{254} in 1.2 L of water and 60 mL of this slurry per 20 × 20-cm plate.

Equipment

Electron impact mass spectra (EI-MS): Finnigan MAT95 (70 eV); Thermo Electron Corp., Bremen, Germany; high-performance liquid chromatography-mass spectrometry (HPLC-MS): electron spray ionization mass spectrometry (ESI-MS) on Finnigan LCQ ion trap mass spectrometer coupled with Flux Instruments (Basel, Switzerland) quaternary pump Rheos 4000 and a HP 1100 HPLC (Synergi 4 μm Max-RP column, 150 × 2 mm, C 12; Phenomenex, Aschaffenburg, Germany) with autosampler (Jasco 851-AS; Jasco Inc., Easton, MD, USA) and a Diode Array Detector (Finnigan Surveyor LC System); High-resolution mass spectra (HR-MS) were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra: Varian Inova 600 (599.7 MHz; 150.8 MHz for ^{13}C), Varian Mercury 300 (300.1 MHz; 75.5 MHz for ^{13}C), Varian Inc., Palo Alto, CA, USA.

Chromatograms were analyzed at 254 and 366 nm by means of a UV lamp (DESAGA, Heidelberg, Germany). Isolated products were studied photometrically, using an Ultraspec II (LKB Biochrom, Cambridge, England) or a Perkin-Elmer Lambda 15 UV/VIS spectrometer. Studies on ABTS$^{\bullet+}$ reduction were carried out in a temperature-controlled PMZ photometer (Zeiss, Oberkochen, Germany). Fluorescence spectra were measured using an Aminco-Bowman (Silver Spring, MD, USA) spectrophotofluorometer, model JA-8965-E, equipped with ellipsoidal condensing system, operated in the ratio mode.

Chemiluminescence was measured by means of a temperature-controlled scintillation spectrometer (Packard Instrument model 3330; Downers Grove, IL, USA).

Reaction systems and chromatography

The capacity of ABTS$\bullet^+$ scavenging was investigated in a variant of the procedure by Re et al. [7]. In order to perform calculations of stoichiometry, the ABTS$\bullet^+$ reagent was adjusted to a concentration of 1.6 mM, which gives a final concentration of 40 μM in the reaction system [950 μL water, 25 μL ABTS$\bullet^+$ reagent, 25 μL melatonin 0.32, 0.16, 0.08 or 0.04 mM (final concentrations 8, 4, 2 or 1 μM)].

The ABTS competition assay for determination of •OH scavenging was carried out as the pH 5.0 variant [11] of a procedure described earlier [16]. Radicals were generated by a Fenton reagent, and the reaction was followed by measuring ABTS$\bullet^+$; final concentrations used: ABTS 0.1 mM; compound **3** 0.1 or 0.25 mM, respectively. To avoid absorbance changes owing to the decrease of compound **3**, measurements were carried out at 734 nm.

Chemiluminescence emitted during oxidation of compound **3** was measured using a hemin-catalyzed H_2O_2 system [16] consisting of 0.7 mL of 0.1 M glycylglycine/NaOH buffer, pH 8.0, 0.1 mL of 4 mM compound **3**, 0.1 mL of hemin chloride 50 μM, and 0.1 mL of 10% H_2O_2. Determinations were carried out at 20 °C, operated in the noncoincidence mode at window and gain settings optimized for chemiluminescence.

For initial product analyses, 1 mL of melatonin, 3 mM in ethanol, was reacted with 7.5 mL of ABTS$\bullet^+$ 4 mM and 1.5 mL of water for 24 hr at 20 °C. Mixtures were extracted with 20 mL of ethyl acetate, concentrated by evaporation and chromatographed on TLC plates, using ethyl acetate/methanol 9.5:0.5, in the majority of runs. In corresponding experiments with AFMK (**1**) and its products, compounds **2a**/**2b** or **3**, same volumes and amounts of ABTS$\bullet^+$ and water were used, while melatonin was replaced by 3 mM of AFMK (**1**), or ca. 1.5 mM of products **2a**/**2b** (as mixture) or **3** (higher concentrations could not be used for reasons of available amounts).

Reaction systems for preparative purposes consisted of 225 mL of ABTS$\bullet^+$ 4 mM, 30 mL of melatonin or AFMK (**1**) 3 mM in ethanol and 45 mL of water. After incubation at 20 °C for 24 hr, mixtures were extracted with 600 mL of ethyl acetate, concentrated by evaporation and chromatographed on PTLC plates, using ethyl acetate/methanol 9.5:0.5 as fluid phase. Bands were re-eluted in ethyl acetate/methanol 9.5:0.5, concentrated and purified on Sephadex LH 20 (column 64.0 × 1.5 cm; packed with 49 cm Sephadex LH 20), using dichloromethane/methanol 1:1. While compound **3** eluted as a homogeneous fraction, products of another band were separated by repeated chromatography on this column into three fractions, containing compounds **2a**/**2b** (isomer mixture), **4** and **5a**/**5b** (presumably also an isomer mixture). Isomers of fraction **2a**/**2b** were separated by HPLC-MS.

In a variant of the ABTS$^{\bullet+}$ system containing H_2O_2, reaction mixtures were composed of 4.6 mL of ABTS$^{\bullet+}$ 4.2 mM, 0.15 mL of AFMK 3 mM in ethanol, 0.25 mL of H_2O_2 0.2%, or, for production compound **3**, 552 mL of ABTS$^{\bullet+}$ 4.2 mM, 18 mL of AFMK 3 mM in ethanol, 30 mL of H_2O_2 0.2%. The amount of H_2O_2 added proved to be small enough to not rapidly reduce the ABTS$^{\bullet+}$ by superoxide anions deriving from the peroxide, so that incubations of about 6 hr were possible.

Results

The incubation of melatonin in the presence of an excess of ABTS$^{\bullet+}$ (melatonin/ABTS$^{\bullet+}$ ratio 1:5, 1:10, 1:20 or 1:40) revealed that the number of radicals scavenged per molecule of melatonin increased considerably with a decreasing ratio of these reactants (Fig. 1), from about 4.7 at 1:5 to approximately 10 at 1:40. In other words, the stoichiometry of radical scavenging per melatonin molecule becomes only apparent if the concentration of ABTS$^{\bullet+}$ is not too much lowered during the incubation period.

Such a finding leads to the conclusion that AFMK (**1**), the substance previously thought to be the end product in the ABTS$^{\bullet+}$ system, is also attacked by this radical and should give rise to other compounds. In fact, preliminary product analyses by TLC showed that new substances became apparent when melatonin was incubated with an excess of ABTS$^{\bullet+}$ for a sufficient period of time (Table 1). Apart from c3OHM and AFMK, substances which were expected, two other major bands were present. The band at $R_f = 0.59$ was further separated by Sephadex chromatography, using dichloromethane/methanol 1:1, into three fractions (Table 1). On HPLC-MS, one of these turned out to be composed of two isomers (**2a**/**2b**); for another one, we assume a corresponding isomery (**5a**/**5b**; see Discussion). The other main band was found to be homogenous when re-chromatographed. AMK was notably not present in substantial amounts. The assumption that the new compounds were derived from AFMK (**1**) was confirmed by incubating this kynuramine with ABTS$^{\bullet+}$, at a molar ratio of 1:10. Again, the same major bands became apparent (Table 1).

The compounds present in these bands were isolated, purified and analyzed by MS (HPLC-MS, EI-MS and ESI-HRMS). Molecular masses were determined to be 278 for compound **3** (Fig. 2), 260 for compounds **2a**/**2b**, and 232 for **4** and **5a**/**5b**. In the course of further analyses, the material with mass 260 turned out to be a mixture of two isomers.

The substances with masses of 260 (compounds **2a** and **2b**) and 278 (compound **3**), representing the most abundant products from AFMK (**1**), were further analyzed by spectroscopic methods. The characteristics of these molecules are summarized as:

Compounds 2a/2b

Pale yellow solid, $R_f = 0.59$ (ethyl acetate/methanol 9.5:0.5). – **UV/Vis**, in HPLC eluent (gradient 10–100% MeOH/0.5% formic acid in water), λ_{max} **2a** (at retention time ca. 60% MeOH): 242, 338 nm; λ_{max} **2b** (at retention time ca. 72% MeOH): 245, 384 nm. — **(+)-ESI HR-MS:** $m/z = 261.08705$ $[M+H]^+$, (calcd. 261.08698 for $C_{13}H_{13}N_2O_4$), 283.09263 $[M+Na]^+$, (calcd. 283.06893 for $C_{13}H_{12}N_2O_4Na$). — **EI-MS**: m/z (%) = 260 (100), 232 (93), 217 (7), 191 (83), 162 (10), 149 (16), 134 (17), 122 (10), 106 (7), 95 (3), 82 (23), 59 (4), 54 (32), 43 (6). — **^{1}H-NMR** ($CDCl_3$, 600 MHz, 2nd value for *cis* isomer 2a): $\delta_H = 9.96/9.39$ (brs, 1 H, N-H), 8.50/8.63 (d, $J = 9.1$ Hz, 1 H-7), 8.38/8.38 (d, $J = 1.8$ Hz, 1 H, CHO), 7.65/7.63 (brs, =C*H*-NH), 7.37/7.34 (d, $J = 3.0$ Hz, 1 H, H-4), 7.15/7.12 (dd, $J = 3.0$ u. 9.1 Hz, 1 H, H-6), 3.83/3.83 (s, 3 H, OMe), 2.62/2.62 (s, 3 H, O=C-CH_3). — **^{13}C-NMR** ($CDCl_3$, 150 MHz; values of quaternary C signals were obtained from HMBC spectra): $\delta_c = 182.5$ (C_q, C=O, C-3), 166.3 (C_q, O=C-CH_3, C-1″), 159.2 (C_q, CHO), 154.9 (C_q, C-5), 137.5 (CH, C-1′), 131.5 (C_q, C-7a), 124.2 (C-7), 124.1 (C-3a), 119.5 (C-6), 116.0 (C-4), 55.7 (OMe), 14.5 (CH_3, C-2″); the signal of C-2 was not visible, but is expected at δ 120 ± 15.

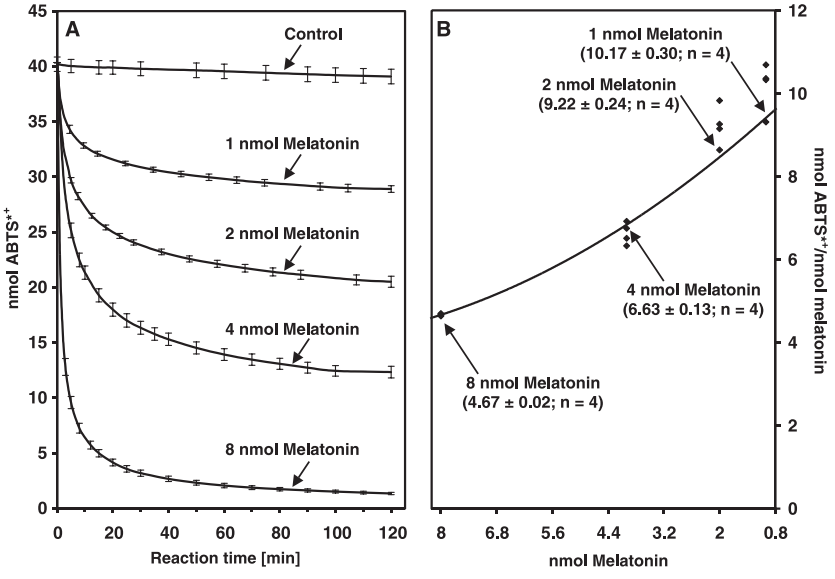


Fig. 1. Concentration dependence of ABTS$^{\bullet+}$ reduction by melatonin. (A) Time course of ABTS$^{\bullet+}$ reduction by different amounts of melatonin added at start of reaction. Vertical lines: S.E.M. (B) Stoichiometry of ABTS$^{\bullet+}$ scavenged per melatonin molecule, at different initial amounts of the indoleamine. Datapoints: values from single determinations; in parentheses: ABTS$^{\bullet+}$ scavenged per melatonin, as means ± S.E.M. for four parallels; line: second-order polynomic regression.

Table 1. Overview of major products obtained from the ABTS•$^+$ reaction system with melatonin and its different metabolites, after separation by thin-layer chromatography (R_f values for ethyl acetate/methanol 9.5:0.5)

Band	R_f	Fluorescence (excitation at 254 or 366 nm)	Educt				
			Melatonin	AFMK	AFMK, with H_2O_2	**2a**/**2b**	**3**
(Unknown)	0.91	254	–	(+)	–	(+)	(+)
(Unknown)	0.80	254	(+)	–	–	–	–
2a/**b**[a];**4**;**5a**/**b**	0.59	overlapping; **2**, **4**, and **5** separated by dichloromethane/methanol 1:1[c]					
		366	+	++	–	res.[b]	–
(Unknown)	0.49	366	(+)	–	–	–	–
Melatonin	0.45	254	res.[b]	–	–	–	–
3	0.41	366	+++	++++	+++++	++	res.[b]
AFMK (**1**)	0.24	366	+	res.[b]	res.[b]	–	–
c3OHM	0.16	254	++	–	–	–	–

[a]Separated by high-performance liquid chromatography-mass spectrometry; [b]res., residual educt; [c]separated on column by dichloromethane/methanol 1:1; **5** should exist in two isomeric forms (**5a**/**5b**), assignments and structures of **4** and **5a**/**5b** provisional. Semiquantitative evaluation of bands are indicated by plus signs.
AFMK, N^1-acetyl-N^2-formyl-5-methoxykynuramine.

Compound 3

Pale yellow solid, $R_f = 0.41$ (ethyl acetate/methanol 9.5:0.5). - **UV/Vis**, in HPLC eluent (gradient 10–100% MeOH/0.5% formic acid in water), at retention time ca. 43% MeOH: λ_{max} 248, 384 nm; in water adjusted to pH 5.0: λ_{max} 245, 381 nm. – Fluorescence (EtOH), λ_{excit} 378 nm, λ_{emiss}484 nm (Fig. 3). – **(+)-ESI HR-MS:** m/z = 279.09765 $[M+H]^+$, (calcd. 279.09755 for $C_{13}H_{15}N_2O_5$), 301.07975 $[M+Na]^+$, (calcd. 301.07949 for $C_{13}H_{14}N_2O_5Na$). **-EI-MS**: m/z (%) = 278 (5), 250 (6), 221 (2), 207 (25), 179 (100), 178 (15), 136 (5), 79 (4), 72 (14), 43 (38). – **^{1}H-NMR** (CD_3OD, 600 MHz): δ_H = 8.68 (s, 1 H, CHO), 8.26 (d, J = 8.9 Hz, 1 H, H-7), 7.35 (dd, J = 8.9 and 2.8 Hz, 1 H, H-6), 7.22 (d, J = 2.7 Hz, 1 H, H-4), 3.89, 3.64 (d_{AB}, J = 14.1 Hz, 2 H, CH_2-NH), 3.84 (s, 3 H, OMe), 1.71 (s, 3 H, O=C-CH_3). – **^{1}H-NMR** ($CDCl_3$, 600 MHz): δ_H = 8.71 (s, 1 H, CHO), 8.27 (d, J = 9.0, 1 H, H-7), 7.27 (dd, J = 9.0 and 2.9 Hz, 1 H, H-6), 7.14 (d, J = 2.7 Hz, 1 H, H-4), 6.17 (brs, 1 H, N-H), 3.81 (s, 3 H, OMe), 3.73, 3.67 (ABX, J_{AB} = 16.2, $J_{AX/BX}$ 7.0 Hz, 1 H, CH_2-NH), 2.08 (s, 3 H, O=C-CH_3). – **^{13}C-NMR** (CD_3OD, 150.8 MHz): δ_C = 198.5 (C_q, C=O, C-3), 173.7 (C_q, O=C-CH_3, C-1″), 160.9 (C_q, CHO), 158.6 (C_q, C-5), 146.4 (C_q, C-7a), 127.0 (C-6), 124.1 (C-3a), 119.5 (C-7), 106.7 (C-4), 90.1 (C-OH, C-2), 56.3 (OMe), 45.2 (CH_2-NH, C-1′), 22.1 (CH_3, C-2″). [Correction added after publication 15 September 2006: in the preceding sentence in the second-last term, (CH_2-NH, C-1) was corrected to (CH_2-NH, C-1′)]

From these data, the following molecular structures are deduced. HMBC correlations are shown for compound **2b** in Fig. 4 and for compound **3** in Fig. 5. Compounds **2a** and **2b** are Z- and E-isomers (**2a** = Z; **2b** = E) of *N*-(1-formyl-5-methoxy-3-oxo-2,3-dihydro-1H-indol-2-ylidenemethyl)-acetamide, whereas compound **3** is *N*-(1-formyl-2-hydroxy-5-methoxy-3-oxo-2,3-dihydro-1H-indol-2-ylmethyl)-acetamide. Structures and atom numbering are presented in Fig. 6.

These oxidation products are obviously formed by re-cyclization of an intermediate deriving from AFMK. The formyl group is maintained during the cyclization process (for deformylated analogs see next). For this reason, the side chain at C2 of the resulting 3-indolinone is by one C-atom shorter than in melatonin.

To further identify the sequence of formation, compounds **2a**/**2b** (not separated) and **3** were incubated with ABTS•$^+$. Thereafter, compound **3** had been formed from **2a**/**2b**, whereas **3** did not generate **2a**/**2b** (Table 1). Therefore, the C2-hydroxylated product is obviously formed from a precursor unsaturated in the aliphatic chain, **2a** or **2b** or, perhaps, either of them. When AFMK (**1**) was incubated with ABTS•$^+$ in the presence of 0.01% H_2O_2, the 2-hydroxylated compound **3** was detected as the only product present in relevant quantities (Table 1), whereas **2a**/**2b** were practically undetectable.

To test whether compound **3** further extends the radical scavenger cascade, the purified substance was tested in the ABTS•$^+$ reduction assay. Compound **3** turned out to be a very poor electron donor to ABTS•$^+$: 1 nmol of **3** scavenged much less than 1 nmol ABTS•$^+$ (details not shown). However, this does not imply a general incapability of **3** to interact with free radicals. In an ABTS competition assay for hydroxyl radicals, **3** proved to be relatively efficient. At equimolar concentrations, **3** exhibited about 20% of the scavenging capacity of high-affinity scavenger ABTS, which easily interacts with •OH; at 2.5-fold concentration, compound **3** diminished ABTS•$^+$ formation by about 49% (Fig. 7). The inhibitory action of **3** was obviously not because of iron chelation, as we did not detect any spectral changes upon addition of Fe^{2+} or Fe^{3+} to **3** (details not shown). Radical scavenging by **3** was corroborated by measurements of chemiluminescence, as emitted during oxidation of this compound in a hemin-catalyzed H_2O_2 system. However, compound **3** did obviously not form a strong emitter, so that light emission remained moderate (median of first 10-min interval: 26,000 cpm). Product analysis after 2 hr of incubation revealed the presence of a substance which may represent an oxidation product and is different from **4** and **5a**/**5b** (see next).

Further compounds were obtained from extracts of the ABTS•$^+$ reaction system, substances which were present in

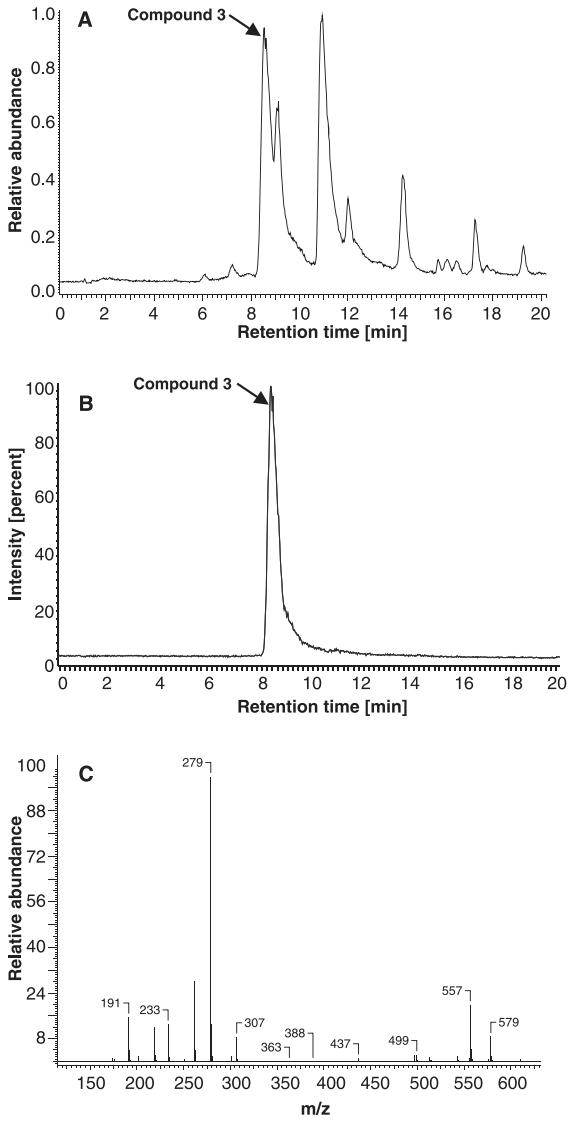


Fig. 2. High-performance liquid chromatography-mass spectrometry (HPLC-MS) characteristics of compound **3**. (A) HPLC of extract from reaction mixture. (B) HPLC for the mass $[M+H]^+ = 279$ (compound **3**). (C) Mass spectrum $[M+H]^+$, for peak of compound **3**, at retention time in (B).

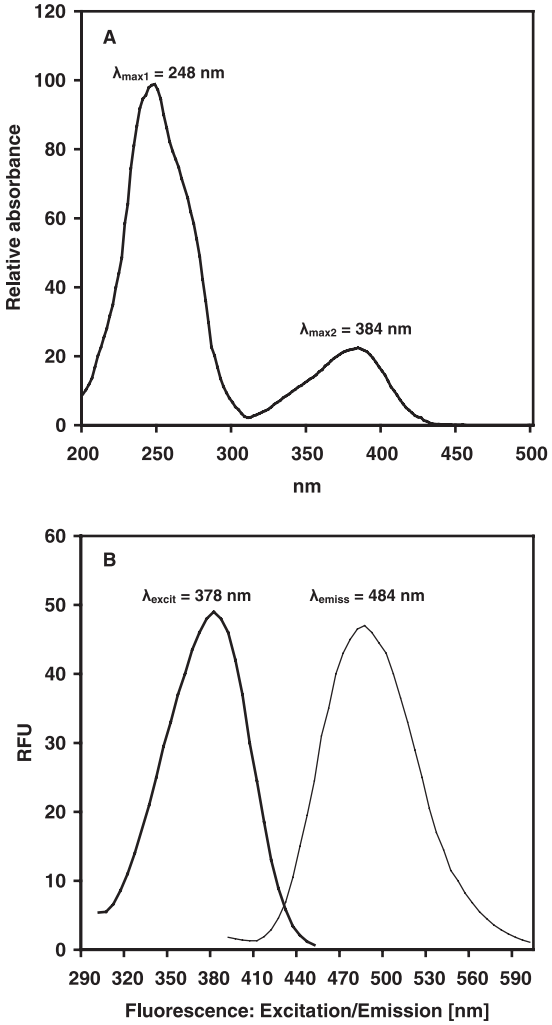


Fig. 3. Absorbance (A) and fluorescence (B) spectra of compound **3**. (A) Measurements from high-performance liquid chromatography (HPLC) run (eluent see current text). (B) Measurements in ethanol. RFU: relative fluorometer units.

the TLC band at $R_f = 0.59$. After separation by column chromatography on Sephadex, distinct fractions of yellow and red daylight colors were obtained. These substances exhibited fluorescence (in ethanol; yellow compound: excitation maximum 343 nm, emission maximum 389 nm; red compound: excitation maximum 287 nm, emission maximum 372 nm). However, the quantities were only sufficient for MS, not for NMR analyses. The masses were determined in both cases to be 232, which indicates that these substances are deformylated analogs of **2a**/**2b**.

Discussion

The efficacy of melatonin as an antioxidant agent has multiple reasons. Apart from signaling mechanisms controlling the expression of anti- and prooxidant enzymes and various effects reducing radical formation [6, 17], the scavenger cascade, which detoxifies several free radicals per melatonin molecule, can be of importance in those experimental systems in which direct scavenging is of decisive importance. Although an extension of this cascade could be predicted on the basis of results concerning interactions of AFMK (**1**) with •OH [10], the results presented here exceed that what might have been expected on the basis of previous knowledge. Although ABTS•$^+$ is nothing more but a model radical, its usefulness in identifying sequential steps of product formation has been demonstrated [8]. However, stoichiometric proportions can be only identified as long as the, compared with other free radicals, relatively inert ABTS•$^+$ is not limiting and, thus, cannot be determined under conditions leading to almost total consumption of the test radical. At a melatonin/ABTS•$^+$ ratio of 1:40, about ten radicals are reduced, a finding indicating a much higher direct antioxidant efficacy of melatonin than previously thought. Because AFMK (**1**) is a comparably poor scavenger of ABTS•$^+$, an extended

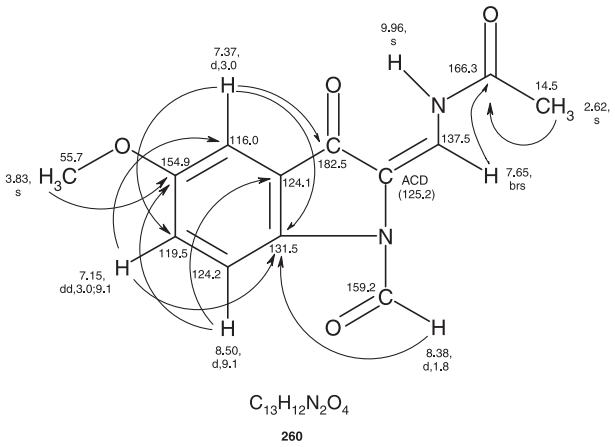


Fig. 4. HMBC correlations for indolinone **2b**, from ^{1}H-NMR in $CDCl_3$.

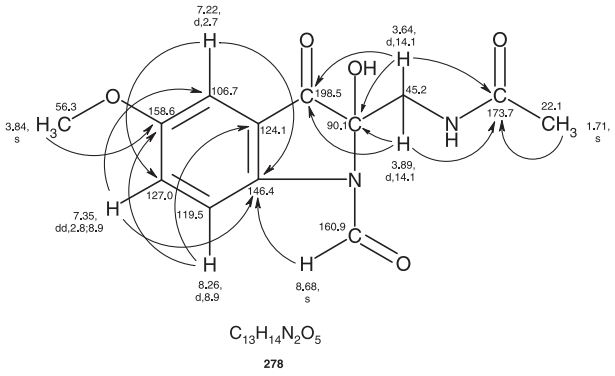


Fig. 5. HMBC correlations for indolinone **3**, from ^{1}H-NMR in CD_3OD.

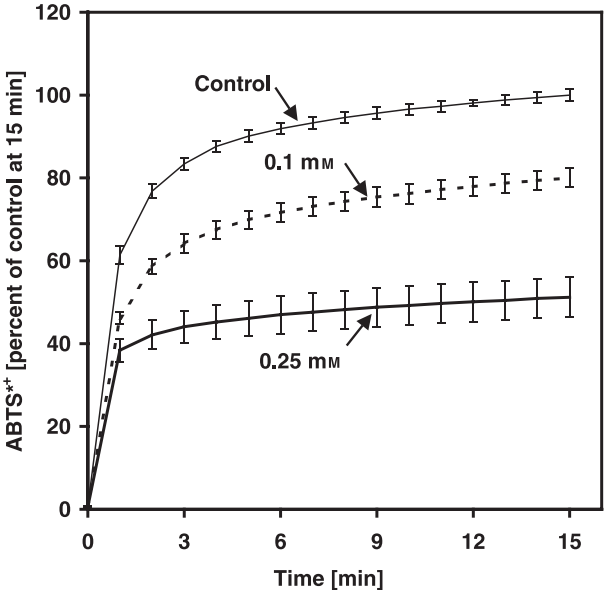
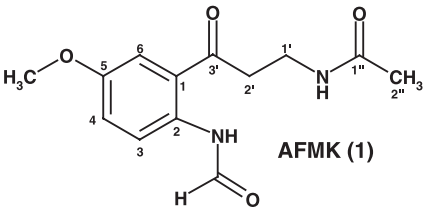
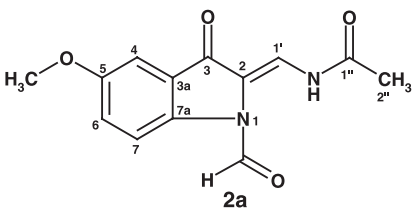
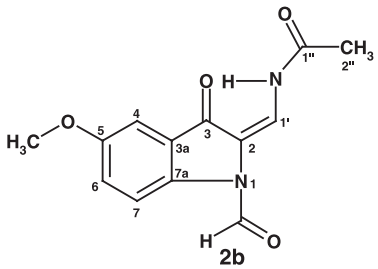


Fig. 7. Scavenging of •OH by compound **3** in the ABTS competition assay. Data are presented as means ± S.E.M.; control values for 15 min are set at 100%. ABTS concentration in the assay was 0.1 mM. Neither ABTS•$^+$ reduction nor iron chelation contributed to the lower values obtained in the presence of compound **3**.

period of incubation is required for obtaining the value of 10 reduced radicals, but about seven reduced radicals are obtained within 15 min with 1 μM of melatonin and 40 μM of ABTS•$^+$ (data of Fig. 1). This does not indicate poor relevance with regard to physiological conditions, as AFMK (**1**) easily interacts with •OH [9, 10] and, according to our observations, is readily destroyed in reaction systems generating this radical.







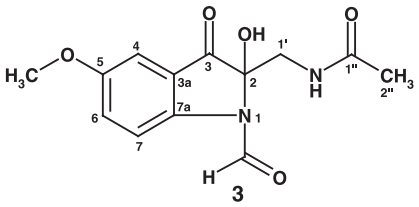


Fig. 6. Structures and atom numbering of the educt, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) (**1**), and three products, the indolinones **2a**/**2b** [Z-, E-isomers of *N*-(1-formyl-5-methoxy-3-oxo-2,3-dihydro-1H-indol-2-ylidenemethyl)-acetamide] and **3** [*N*-(1-formyl-2-hydroxy-5-methoxy-3-oxo-2,3-dihydro-1H-indol-2-ylmethyl)-acetamide].

Product analyses revealed that the major oxidation products were entirely new compounds, which had not been chemically described before. Notably, the known metabolite AMK was not present in detectable amounts. This should not be because of a more rapid conversion of AMK, which is a more efficient ABTS•$^+$ reductant than AFMK (**1**) [11, 12], as recently identified products formed from AMK by interaction with ABTS•$^+$ [18] were likewise absent. Instead, we detected products which are formed by re-cyclization of AFMK (**1**), and are also produced when this kynuramine is incubated with ABTS•$^+$. Cyclization causes formation of 3-indolinone (**2a**/**2b**), in which the oxo group otherwise characteristic for kynuramines or kynurenines is maintained, but which carries the side chain at C2. This side chain is by one C-atom shorter than in melatonin and its physiologically occurring analogs, because the *N*-formyl group of AFMK (**1**) does not take part in the re-cyclization.

The C2-substituted 3-indolinones must not be confused with C3-substituted 2-indolinones formed in other reaction systems directly from melatonin, e.g., by scavenging of 2 •OH and subsequent ketone formation from the resulting 2-hydroxymelatonin [6, 19, 20], which is one of the alternate products of melatonin hydroxylation by free radicals.

To what extent the new compounds **2a**, **2b** and **3** are formed under physiological conditions remains to be investigated. Homologous reactions of AFMK (**1**) with other electron-abstracting free radicals are likely, but reaction rates and further metabolism will have to be determined in the future. The C2-substituted 3-indolinones discovered may also be of interest in another context. Similar compounds carrying the aliphatic side chain at C2 have been synthesized in the search for melatonin-receptor ligands and for melatonin-related antioxidants [21–24]. The major differences to those chemically designed compounds concern the presence of a carbonyl group at C3 and the aliphatic side chain, which is by one C-atom shorter. Nevertheless, it will be an intriguing question as to whether the new products **2a**, **2b** and **3** deriving from AFMK (**1**) also display properties of receptor ligands.

Compounds **2a** and **2b** represent E-, Z-isomers which are apparent at the same time during isolation, **2b** being present in higher quantities than **2a**. Because such isomers should be assumed to be interconvertable, it will be difficult to judge which of them is initially formed. The E-isomer (**2b**), which should be stabilized by a hydrogen bond between the acetamido nitrogen and the oxygen at C3, represents presumably the favored structure. The sequence of formation concerning the unsaturated products **2a**/**2b** and the saturated, C2-hydroxylated compound **3** is unequivocal. In the ABTS•$^+$ reaction system, the **2a**/**2b** mixture gives **3**, but not the reverse. Moreover, attempts of removing water from **3** by concentrated H_2SO_4 did not lead to **2a**/**2b**, but rather to an entirely different substance (details not shown).

Additional compounds have been obtained from extracts of the ABTS•$^+$ reaction system, which appear to be deformylated, according to MS. Whether these colored, fluorescent products may result from deformylation of **2a**/**2b** or, in conjunction with the removal of water, from **3**, or by another mechanism is currently unknown. Likely structures (**4**, **5a**, **5b**) are depicted in Fig. 8, where **5a** and **5b** represent again E-/Z-isomers.

With regard to the scavenger cascade, the formation of 3-indolinones **2a**/**2b** by re-cyclization of an intermediate from AFMK (**1**) should already contribute considerably to the scavenging capacity. From melatonin to AFMK (**1**), four radicals can be scavenged in the ABTS•$^+$ system [8]. From AFMK (**1**) to **2a**/**2b**, four electrons are lost and, although the details of the mechanism leading to **2a**/**2b** are not fully understood, this balance may be equivalent to four additional radicals scavenged per melatonin molecule.

As scavenging of up to ten ABTS•$^+$ was demonstrated (Fig. 1), the question remains which additional steps contribute to the reduction of these radicals. The conversion of **2a**/**2b** to indolinone **3** can be understood as water uptake and may not appear in the balance. However, the presence of higher quantities of indolinone **3** and the virtual absence of **2a/2b** in reaction systems containing H_2O_2 might, with due caution, indicate an involvement of redox mechanisms, though. Compound **3** turned out to be a relatively poor scavenger of ABTS•$^+$, a finding explaining why this substance remained stable in the reaction system and was present as the major product from AFMK. Therefore, the scavenger cascade is practically terminated in the ABTS•$^+$ system with compound **3**. Up to this step, only eight radicals scavenged per melatonin molecule are explained, and, thus, the remaining two radicals must be because other reactions in the system. This problem cannot be easily solved, because compounds **2a/2b** are not very

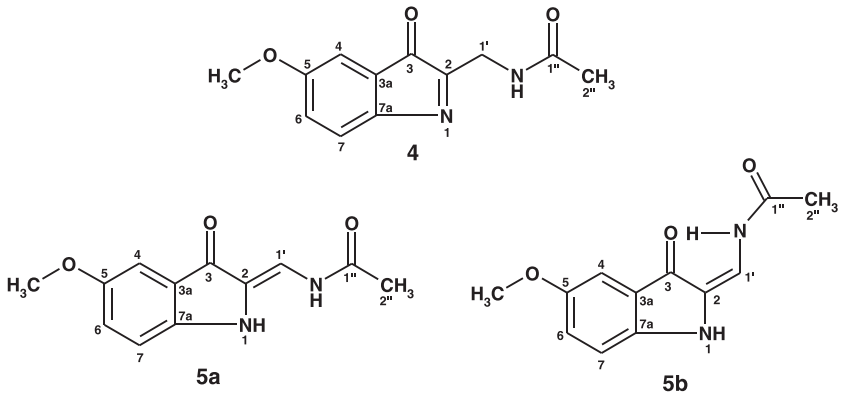


Fig. 8. Presumed structures of deformylated products **4**, **5a** and **5b**.

stable in solution. This holds even more for compounds **4**, **5a**/**5b**, which may derive from **2a**/**2b**, but can be isolated only in low amounts. Moreover, they should be assumed to be interconvertable. Electron donation by these substances to free radicals, in analogy to results obtained with methoxylated indoleamines [1–5], do not seem unlikely with regard to the presence of an NH group in the five-atom ring of **5a**/**5b**.

Termination of the scavenger cascade in the ABTS$\bullet^+$ system is, however, not equivalent to the situation in vivo or in systems generating other free radicals of higher reactivity. In fact, we show that compound **3** does interact with •OH at substantial rates in the ABTS competition assay, despite its low reactivity toward ABTS$\bullet^+$. Therefore, the scavenger cascade may be even longer in vivo and continue with oxidation of the indolinone **3**.

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