Reactions of the melatonin metabolite AMK (N^1-acetyl-5-methoxykynuramine) with reactive nitrogen species: Formation of novel compounds, 3-acetamidomethyl-6-methoxycinnolinone and 3-nitro-AMK

Abstract: The melatonin metabolite AMK (N^1-acetyl-5-methoxykynuramine) was found to be unstable on air when adsorbed on a thin-layer silica gel chromatography plate, a result being in good agreement with the relatively high reactivity of this compound. Three novel main products were separated from the reaction mixture and identified by mass spectrometry and NMR data as (i) 3-acetamidomethyl-6-methoxycinnolinone (= AMMC), (ii) 3-nitro-AMK (= N^1-acetyl-5-methoxy-3-nitrokynuramine = AMNK), and (iii) *N*-[2-(6-methoxyquinazolin-4-yl)-ethyl]-acetamide (MQA). AMMC and AMNK are shown to be non-enzymatically formed also in solution, by NO in the first case, and by a mixture of peroxynitrite and hydrogen carbonate, in the second one. Three different NO donors, PAPA-NONOate, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and sodium nitroprussiate led to essentially the same results, with regard to a highly preferential formation of AMMC; AMNK was not detected in these reaction systems. Competition experiments with the NO scavenger *N*-acetylcysteine indicate a somewhat lower reactivity compared to the competitor. Peroxynitrite led to AMNK formation in the presence of physiological concentrations of hydrogen carbonate at pH 7.4, but not in its absence, indicating that nitration involves a mixture of carbonate radicals and NO_2, formed from the peroxynitrite-CO_2 adduct. No AMMC was detected after AMK exposure to peroxynitrite. Both AMNK and AMMC exhibited a much lower reactivity towards ABTS cation radicals than AMK. In a competition assay for hydroxyl radicals, AMMC showed prooxidant properties, whereas AMNK was a moderate antioxidant. AMMC and AMNK should represent relatively stable physiological products, although their rates of synthesis are still unknown and may be low. Formation of these compounds may contribute to the disappearance of AMK from tissues and body fluids.

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Introduction

To date, our knowledge on the kynuric pathway of melatonin catabolism only extends to the second metabolite following pyrrole ring cleavage, N^1-acetyl-5-methoxykynuramine (AMK, **1a**) [1-5]. In the pioneering work on its discovery, Hirata *et al*. [6] had shown that AMK (**1a**) can be detected in the urine, after both intracisternal and intravenous injection. Thereafter, the metabolism of this pathway was poorly investigated, partially because the commercially unavailable compound was not easily accessible, partially because the kynuric route was considered irrelevant compared to that of 6-hydroxylation/conjugation and, perhaps, also because its excretion may have led to the impression that the pathway would neessarily terminate with AMK (**1a**) as a urinary metabolite.

However, such a view was by no means justified since it neglected several decisive facts. The urinary appearance was observed after administration of a pharmacological dose of melatonin [6]. Therefore, physiological concentrations of melatonin would not necessarily lead to substantial urinary levels of this kynuramine, and, generally, excretion does not exclude catabolism of another fraction of the same compound. On the other hand, AMK (**1a**) had been shown to be the principal melatonin metabolite in the central nervous system: after injection of labeled melatonin into the *cisterna magna*, more than one third of the radioactivity was recovered as urinary AMK (**1a**) [6]. Under these conditions, AMK (**1a**) was the only product formed from melatonin in the brain and no 6-hydroxymelatonin was detected [6]. A tendency of judging on melatonin degradation mainly on the basis of the circulating hormone

and its hepatic metabolism may have kept researchers from thoroughly investigating the alternative kynuric pathway.

Meanwhile, our knowledge on tissue melatonin has considerably increased, although many substantial gaps remain to be filled. Release of comparably high amounts of melatonin to the central nervous system *via* the pineal recess [7,8] may attract new attention to the methoxylated kynuramines formed by brain metabolism. Moreover, the amounts of tissue melatonin exceed by far those in the pineal gland and in the circulation. For instance, the gastrointestinal tract contains several hundred times more of melatonin than the pineal [9-11]. Extrahepatic 6-hydroxylation of melatonin should be of minor importance, and that fraction of the hormone which is neither released to the circulation nor to the gastrointestinal lumen should be metabolized differently, e.g. *via* the kynuric pathway.

The precursor of AMK (**1a**), N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK, **1b**), can be formed enzymatically; however, in addition, various reactions involving free radicals, photocatalytic mechanisms or pseudoenzymatic processes have been described which also lead to this compound [2,5,12,13]. AFMK has therefore gained a new relevance as a product formed under enhanced oxidative pressure or oxidative stress. Two different enzymatic reactions are known to convert AFMK (**1b**) into AMK (**1a**), one catalyzed by an unspecific aryl formamidase [2,5] and a second one by hemoperoxidase („catalase“) [4]. Again, by the second reaction, the kynuric pathway is linked to oxidative metabolism and may be relevant in the context of antioxidative defense.

Recently, AMK (**1a**) was reported to be a potent radical scavenger, surpassing by far its precursor AFMK [14]. It had not been the primary intention of that study to focus on protection, although such effects were demonstrable *in vitro*, but the results had characterized the compound as being highly reactive compared to other metabolites, so that the possibility of non-enzymatic conversion into further compounds appeared to be realistic. In fact, we observed that AMK (**1a**) underwent reactions when exposed to the air on a large surface. In the present study, we have characterized three major products, all being biologically and chemically novel, and we can demonstrate that at least two of them are probably formed in vertebrates by interactions with NO or peroxynitrite-derived radicals, respectively.

Table 1. Chromatographic properties of AMMC (**4**), AMNK (**3**) and MQA (**2**)

	R_f values of products in thin-layer chromatography		
Fluid phase	AMMC	AMNK	MQA
Ethylacetate/methanol 9:1	0.23	0.50	n.d.
Dichloromethane/methanol 92:8	0.32	0.62	0.37
Butanol/glacial acetic acid/ water 16:1:2	0.59	n.d.	0.42

n.d.: not determined

Materials and methods

Chemicals

AMK (**1a**) was prepared from melatonin *via* AFMK (**1b**) according to Kennaway *et al*. [15]. The identity was confirmed by electrospray ionization mass spectrometry (ESI MS) and ^{1}H and ^{13}C NMR spectra. ABTS [2,2´-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid)], SNAP (*S*-nitroso-*N*-acetylpenicillamine), PAPA NONOate [3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine] and L-*N*-acetylcysteine were purchased from Sigma, Taufkirchen, Germany, peroxynitrite from Alexis Deutschland, Grünberg, Germany, sodium nitroprussiate from Riedel de Haen, Hanover, Germany. All other chemicals were from Merck, Darmstadt, Germany. All chemicals used were of highest grade available. R_f values were measured on Polygram SIL G/UV$_{254}$ (Macherey & Nagel & Co., Düren, Germany) with dichloromethane/10 % methanol when not stated otherwise. Silica gel for PTLC: PF$_{254}$ (M & N). Preparative plates were prepared with 660 g silica gel PF$_{254}$/L and 60 mL of this slurry per 20 × 20 cm plate).

NMR and mass spectrometry equipment

^{1}H and ^{13}C 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 Unity 200 (50.3 MHz for ^{13}C), Varian Inc., Palo Alto, CA, USA. Electron impact mass spectra (EI MS): Finnigan MAT95 (70 eV), Thermo Electron Corp., Bremen, Germany; perfluorokerosene was used as reference substance in HREI MS. Electron spray ionization mass spectrometry (ESI HRMS): Finnigan LCQ ion trap mass spectrometer coupled with a Flux Instruments (Basel, Swizerland) quaternary pump Rheos 4000 and a HP 1100 HPLC (nucleosil column EC 125/2, 100-5, C 18) 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).

Other analytical equipment

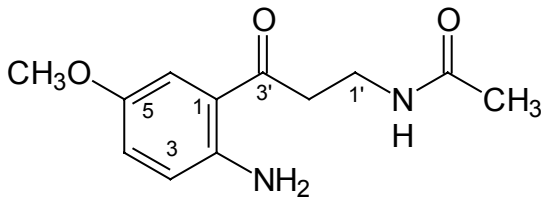
Chromatograms were analyzed at 254 and 366 nm by means of a UV lamp (DESAGA, Heidelberg, Germany). Isolated products were studied photometrically, using Ultraspec II (LKB Biochrom, Cambridge, England) or PMZ (Zeiss, Oberkochen, Germany) photometers, and/or fluorometrically by means of an Aminco-Bowman (Silver Spring, MD, USA) spectrophotofluorometer, model JA-8965-E, equipped with ellipsoidal condensing system, operated in the ratio mode. This equipment was also used for following AMMC (**4**) formation in reaction systems containing organic NO donors.

Formation of products from AMK

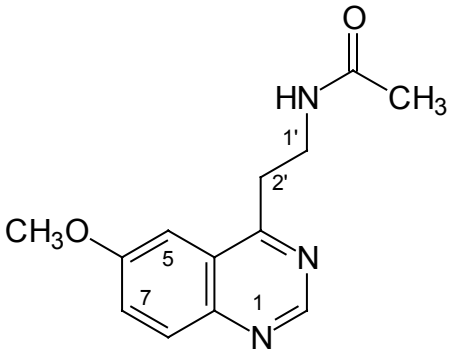
A 5 mM solution of AMK (**1a**) in ethyl acetate was dispensed uniformly on 20 × 20 cm silica gel plates (silica gel 60 F_{254}, on aluminum sheets, type 1.05554.0001, Merck, Darmstadt, Germany; about 14 – 17 mg AMK per plate) and kept in the dark at 25 °C for several days in a steady air stream (ventilator). The experiments were conducted in a room in which no chemicals were stored and which was distant from the main laboratory. The time course of reactions was studied over periods of up to 155 h.

In a typical experiment, 205 mg AMK were applied to 12 TLC plates and exposed to the air stream for about 95 h. Thereafter, the silica gel was removed from the aluminum sheets, extracted with dichloromethane/10% methanol (2 times; about 15 mL/10 mg educt each; 5 min of sonication) and filtered; the solution was brought to dryness, the residue re-dissolved in dichloromethane/10% methanol and separated on 5 preparative TLC plates (20 × 20 cm). Developing with dichloromethane/8% methanol afforded three main and three minor product zones, besides residual AMK. The main zones of AMMC (**4**, colorless with blue UV fluorescence, R_f = 0.32), MQA (**2**, colorless, blue fluorescent, R_f = 0.37), the starting material AMK (**1a**, yellow, non-fluorescent, R_f = 0.55), and AMNK (**3**, dark yellow, non-fluorescent, R_f = 0.62) were eluted with dichloromethane/10% methanol, the solutions evaporated to dryness and the residues re-chromatographed by PTLC (dichloromethane/8% methanol) and then by column chromatography on Sephadex LH-20 (methanol or dichloromethane/methanol 1:1). In the case of AMMC (**4**), impurities were removed by washing with dichloromethane. As AMMC (**4**) was only slightly soluble in methanol or dichloromethane/50% methanol, the ^{1}H NMR spectra were measured in trifluoroacetic acid. The pure compounds were re-crystallized. AMK (**1a**) was consumed to about 46%).

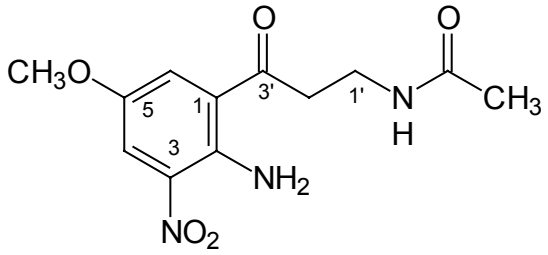
3-Acetamidomethyl-6-methoxycinnolinone (AMMC, **4**; Fig. 1*)*: 9 mg (4.3% of starting material; 9.4% of converted AMK, **1a**), from $CHCl_3$/MeOH brownish needles due to slight impurities, m.p. 233 °C; for chromatographic properties see Table 1. UV/Vis (MeOH): λ_{max} (log ε) = 239.5 (3.90), 293 (3.26), 350 (3.56), 361 nm (3.57). Fluorescence (EtOH, λ_{excit} = 366 nm): λ_{max} = 404 nm. ^{1}H NMR (CD_2Cl_2/MeOD, 300.1 MHz): δ = 7.57 (d, J = 9 Hz, 1 H, 8-H), 7.50 (d, J = 3 Hz, 1 H, 5-H), 7.38 (dd, J = 9, 3 Hz, 1 H, 7-H), 3.90 (s, 3 H, OMe), 2.00 (s, 3 H, Me); CH_2 not assigned. ^{1}H NMR (TFA, 300.1 MHz): δ = 8.01 (d, J = 9.7 Hz, 1 H, 8-H), 7.71 (d br, J = 9.4 Hz, 1 H, 7-H), 7.57 (s, 1 H, 5-H), 4.79 (s, 2 H, CH_2), 3.92 (s, 3 H, OCH_3), 2.18 (s, 3 H, CH_3). ^{13}C NMR (TFA, 75.5 MHz): δ = 180.7 (CO-4), 164.8 (CO-NH), 161.9 (C-6), 142.7 (C-8a), 141.8 (C-3), 134.9 (C-7), 125.7 (C-4a), 121.9 (C-8), 100.7 (C-5), 57.7 (OMe), 42.3 (CH_2), 22.0 (Me). (+)-ESI MS: *m/z* (%) = 270.2 ($[M + Na]^+$, 64), 517.0 ($[2 M + Na]^+$, 100); (-)-ESI MS: *m/z* = 246.2 ($[M - H]^-$); (+)-HRESI MS: 248.10293 $[M + H]^+$, (calcd. 248.1297 for $C_{12}H_{14}N_3O_3$), 270.08493 $[M + Na]^+$ (calcd. 270.08492 for $C_{12}H_{13}N_3O_3Na$).



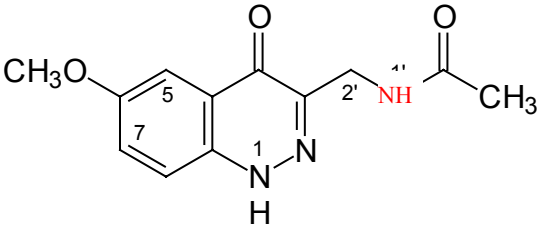
1a: AMK
1b: AFMK, NH-CHO instead of NH_2



2: MQA



3: AMNK



4: AMMC

Fig. 1. Structures of N^1-acetyl-5-methoxykynuramine (AMK, **1a**), N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK, **1b**), *N*-[2-(6-methoxyquinazolin-4-yl)-ethyl]-acetamide (MQA, **2**), N^1-acetyl-5-methoxy-3-nitrokynuramine = 3-nitro-AMK (AMNK, **3**), and 3-acetamidomethyl-6-methoxycinnolinone (=AMMC,**4**)

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N-[3-(2-Amino-5-methoxy-3-nitrophenyl)-3-oxopropyl]-acetamide (N^1-acetyl-5-methoxy--3-nitrokynuramine (3-nitro-AMK, AMNK, **3**; Fig. 1*)*: 3.4 mg (1.7 % of starting material; 3.6% of converted AMK, **1a**), orange-colored solid after evaporation, not fluorescent, m.p. 190 °C; for chromatographic properties see Table 1. UV/Vis (MeOH): λ_{max} (log ε) = 217 (4.31), 452.5 nm (3.78). ^{1}H NMR (DMSO, 599.7 MHz): δ = 8.50 (s, 2 H, NH_2), 7.93 (d, *J* = 3.1 Hz, 1 H, 6-H), 7.86 (s br, 1 H, NH), 7.84 (d, *J* = 3.0 Hz, 1 H, 4-H), 3.81 (s, 3 H, OCH_3), 3.35 (q, *J* = 6.6 Hz, 2 H, 1'-CH_2), 3.20 (t, *J* = 6.6 Hz, 2 H, 2'-CH_2), 1.77 (s, 3 H, CH_3); ^{13}C NMR (DMSO, 150.8 MHz): δ = 200.8 (CO), 169.2 ($COCH_3$), 147.0 (C-5), 141.7 (C-2), 132.0 (C-1), 128.7 (CH, C-6), 121.7 (C-3), 114.9 (CH, C-4), 56.1 (OCH_3), 39.4 (CH_2, C-2'), 34.5 (CH_2, C-1'), 22.4 (CH_3). EI MS (70 eV): *m/z* (%) = 43.0 (41), 44.0 (6), 121.1 (6), 149.1 (10), 163.1 (5), 175.1 (23), 195.1 (14), 204.1 (33), 221.1 (96), 222.1 (100), 246.2 (6), 264.2 (6), 281.2 (75); (+)-ESI MS: *m/z* (%) = 304.1 ($[M + Na]^+$, 36), 584.9 ($[2 M + Na]^+$, 100); (-)-ESI MS: *m/z* = 280.0 ($[M - H]^-$); (+)-HRESI MS: *m/z* = 282.10851 $[M + H]^+$ (calcd. 282.10845 for $C_{12}H_{16}N_3O_5$), 304.09041 $[M + Na]^+$ (calcd. 304.09040 for $C_{12}H_{15}N_3O_5Na$).

N-[2-(6-Methoxyquinazolin-4-yl)-ethyl]-acetamide (MQA, **2**; Fig. 1*)*: 55.4 mg (27% of starting material; 58.8% of converted AMK, **1a**), ochre-colored needles from CH_2Cl_2/cyclohexane, m.p. 138 °C; for chromatographic properties see Table 1. UV/Vis (MeOH): λ_{max} (log ε) = 229 (4.54), 329 nm (3.57). Fluorescence (EtOH, λ_{excit} = 333 nm): λ_{max} = 388 nm. ^{1}H NMR ($CDCl_3$, 300.1 MHz): δ = 9.06 (s, 1 H, 2-H), 7.90 (d, *J* = 9.2 Hz, 1 H, 8-H), 7.50 (dd, *J* = 9.2, 2.7 Hz, 1 H, 7-H), 7.31 (d,

J = 2.7 Hz, 1 H, 5-H), 6.53 (s br, 1 H, NH), 3.95 (s, 3 H, OCH_3), 3.84 (q, *J* = 6.1 Hz, 2 H, 1'-CH_2), 3.43 (t, *J* = 6.2 Hz, 2 H, 2'-CH_2), 1.94 (s, 3 H, CH_3); ^{13}C NMR/APT ($CDCl_3$, 50.3 MHz): δ = 170.5 (CO), 166.8 (C_q-4), 158.6 (C_q-6), 152.2 (CH-2), 145.7 (C_q-8a), 130.4 (CH-8), 126.95 (CH-7), 125.2 (C_q-4a), 101.6 (CH-5), 55.9 (OCH_3), 36.9 (CH_2-1'), 33.7 (CH_2-2'), 23.4 (CH_3). EI MS (70 eV): *m/z* (%) = 43.0 (42), 132.1 (6), 159.1 (13), 171.1 (26), 174.1(32), 186.1 (90), 202.2 (32), 230.2 (7), 245.2 ($[M]^+$, 100); (+)-ESI MS: *m/z* (%) = 268.2 $[M + Na]^+$ (15), 513.0 ($[2 M + Na]^+$, 100); (+)-HRESI MS: 246.12376 $[M + H]^+$ (calcd. 246.12371 for $C_{13}H_{16}N_3O_2$), 268.10574 $[M + Na]^+$ (calcd. 268.10565 for $C_{13}H_{15}N_3O_2Na$).

Product formation in solution

AMMC (**4**) formation under the influence of inorganically generated NO was carried out in analogy to the procedure by Connor *et al*. [16], using 22.6 mg AMK (**1a**) in 2 mL water and 5 mL 1 M HCl, to which 7.24 mg $NaNO_2$ in 2 mL water were slowly added at –5°C, followed by 30 min of stirring at room temperature. Work-up was performed as described for the preparative procedure.

AMMC (**4**) formation using nitroprussiate or organic NO donors was studied in the following systems: (i) 0.12 mL of 2 mM AMK (**1a**) in ethanol, 0.12 mL 10 mM sodium nitroprussiate in potassium phosphate buffer 50 mM, alternatively pH 7.4 or pH 6.0, and 0.96 mL of the respective phosphate buffer; NO liberation was induced by irradiation with visible light (thin-walled glass tubes; Fiolax, outer diameter 12 mm; wall 0.4-0.5 mm: Schott, Mainz, Germany), using a slide projector (FA 150 Liesegang, Düsseldorf, Germany, with projector bulb Philips type 7158), at a photon flux rate of 1400 µmol photons m^{-2} s^{-1}. Throughout incubation, mixtures were continually shaken (GFL laboratory shaker, type 3016, Burgwedel, Germany); in order to avoid heating, the equipment was kept under the steady air stream of a ventilator; the entire laboratory was kept at 25 °C. (ii) 0.1 mL of 2 mM AMK (**1a**) in ethanol, 0.1 mL SNAP, either in potassium phosphate buffer, 50 mM, pH 7.4 or in sodium acetate buffer 50 mM pH 5.0, and 0.8 mL of the respective buffer; incubation in darkness, at ca. 22 °C. (iii) 0.1 mL of 2 mM AMK (**1a**) (alternatively other concentrations, see below: Fig. 4) in ethanol, 0.2 mL of 10 mM PAPA NONOate in 0.01 M NaOH, 0.7 mL potassium phosphate buffer 0.1 M pH 7.4; incubation in darkness, at 22 °C. Competition experiments with L-*N*-acetylcysteine (NAC) were carried out in systems (ii) and (iii), at final concentrations indicated in Fig. 6 (see below); 0.1 mM buffer was replaced by the same volume of NAC in respective buffer.

AMNK (**3**) formation was studied in a reaction mixture consisting of 0.75 mL of 2 mM AMK (**1a**) in ethanol, 0.75 mL of 210 mM $NaHCO_3$ in potassium phosphate buffer 0.4 M pH 7.4, 2.25 mL potassium phosphate buffer 0.4 M pH 7.4, and 1.5 mL 20 mM peroxynitrite in 0.01 M KOH; the reaction was started by addition of peroxynitrite. In additional experiments, the concentration of peroxynitrite was varied by adding 2.8, 5.6, 12 and 13.8 mM of this compound to the system. 2.8 and 5.6 mM peroxynitrite were also used in the absence of $NaHCO_3$, by replacing this component by buffer.

Product analyses in experiments with organic NO donors or peroxynitrite were carried out by extracting reaction mixtures with 5-fold volumes of ethyl acetate, under continuous shaking for 15 min (GFL laboratory shaker, type 3016, Burgwedel, Germany), followed by evaporation to a small volume which was suitable for thin-layer chromatography (fluid phases were used according to chromatographic properties of compounds of interest; cf. Table 1).

Radical scavenging assays

Reduction of ABTS cation radicals was tested according to Re *et al*. [17]. Scavenging of hydroxyl radicals was tested in an ABTS competition assay, as previously described [14,18]. Depending on the absorption spectra of compounds tested, wavelengths of 420 nm or 734 nm were used for measuring the ABTS cation radical concentration. Measurements were mostly carried out in triplicate.

Results

When adsorbed on thin-layer chromatography plates, AMK (**1a**) turned within a few days in the dark into several products, some of which showed strong blue fluorescence or were highly colored. Three main products were formed, which were separated on silica gel and analyzed by MS and NMR techniques. All compounds had higher molecular weight than the starting material and were therefore not simple degradation products.

The compound $C_{12}H_{13}N_3O_3$ (**4**) [determined by (+)-ESI HRMS of the $[M+H]^+$ signal] with the lowest mobility on TLC was very sparingly soluble in the usual solvents. The ^{1}H NMR spectrum in a CH_2Cl_2/MeOH mixture showed clearly that the substituent pattern of the benzene ring in **1a** was unchanged; also the methoxy and the N-acetyl group were still present. In trifluoroacetic acid (TFA), the reso-

lution was lower, however, the solubility was good enough even for a ^{13}C NMR spectrum. Whereas the ethanediyl side chain of **1a** was forming two triplets in protic solvents, in the new product only a single methylene group was visible, which was responsible for a 2H singlet at 4.84. Only two structures are in accordance with these data, **4** and the isomeric seven-membered 1,4-dihydrobenzo[c][1,2]diazepin-5-one, however, due to mechanistic considerations, the latter was excluded: The formation of **4** can easily be explained by an interaction of **1** with NO *via* an intermediate nitrosoketone or the isomeric diazohydroxide, respectively, which cyclizes by release of water. Incubation of AMK (**1a**) in an NO-generating system (HNO_2 at acidic pH) [16]) delivered indeed **4**; this procedure confirmed the mechanistic considerations and the chemical identity of the product (mass spectrometry and NMR).

According to various ESI MS measurements, the second, dark yellow compound had the formula $C_{12}H_{15}N_3O_5$, which is equivalent to AMK – H + NO_2. As the side chain was unchanged due to the ^1H NMR spectrum with one 2H triplet and one 2H quartet, and as only two aromatic protons in *meta* position were visible, this compound was clearly the nitro-AMK (**3**). The ^{13}C NMR data and HMBC measurements (Fig. 2) were confirming this assignment. The identity of the product was further proven by nitration of AMK (**1a**) with HNO_3, which afforded **3** as the main product.

The ^1H NMR data of the third compound indicated that the side chain and the substituent pattern of the benzene ring were again the same as in **1a**, however, an additional CH singlet was visible at δ 9.06 and 152.2, respectively, and the keto carbonyl signal of the starting material **1a** was missing. The presence of an additional carbon atom as compared with **1a** was confirmed by the empirical formula $C_{13}H_{15}N_3O_2$ [(+)-ESI HRMS]. Due to these data and the HMBC couplings (Fig. 2), the product was identified as *N*-[2-(6-methoxyquinazolin-4-yl)-ethyl]-acetamide (abbreviated MQA, **2**; Fig. 1). Formally, it is the condensation product of AFMK (**1b**) with ammonia, however, was also formed when **1b** was definitely absent.

None of these compounds has been listed in the Chemical Abstracts. No evidence for such products was found in extensive biomedical literature searches. To the best of our knowledge, the three compounds are novel.

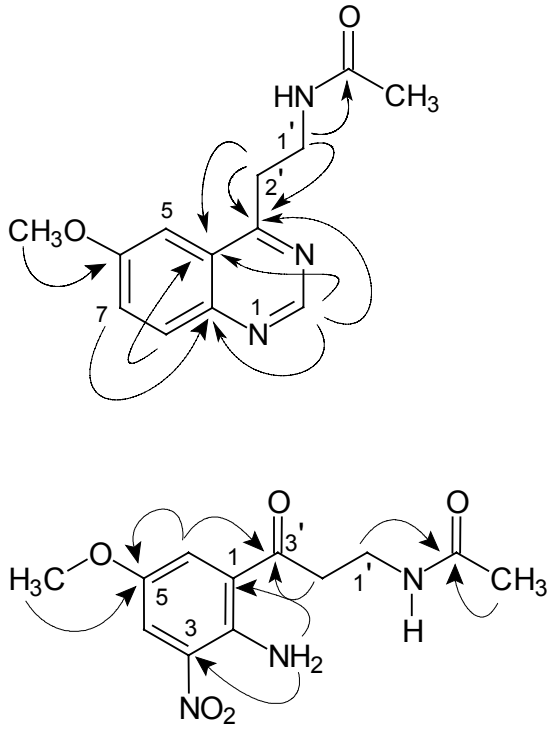

Fig. 2. Selected HMBC correlations in MQA (**2**, left formula) and AMNK (**3**, right formula).

The next step in our investigation was to identify conditions under which these products were formed from AMK (**1a**) and biologically relevant reaction partners; their formation on TLC plates will be the theme of further investigations. To date, we have not found any non-enzymatic reaction of MQA (**2**) formation from AMK (**1a**), which could have any physiological significance. The possibility that MQA (**2**) might alternatively derive from AFMK – which was absent from our AMK (**1a**) preparations – by interactions with ammonia/ammonium ions was tested too, but results were not yet sufficiently conclusive (details not shown).

However, the formation of AMMC (**4**), already shown by NO generation from nitrite, was easily demonstrable by using various NO donors frequently applied in biological experiments. Quantitative studies were conducted by following AMMC (**4**) production on the basis of its fluorescence and absorbance properties (Fig. 3). Three different NO donors of entirely different chemical nature readily caused AMMC (**4**) formation from AMK (**1a**), namely, (i) sodium nitroprussiate (SNP) liberating NO under light exposure, (ii) *S*-nitroso-*N*-acetylpenicillamine (SNAP), and (iii) 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA NONOate) (Fig. 4). With SNP as an NO source, no strong differences between pH 7.4 and 6.0 were observed,

whereas NO formation and, therefore, AMMC (**4**) production by SNAP was much more effective at acidic (5.0) than at physiological pH. Using light-exposed SNP as an NO donor, AMMC (**4**) formation was also possible in ethanolic solution, however, at lower rates (details not shown). For studies in aqueous solution at pH 7.4, PAPA NONOate turned out to be most suitable. Chromatographic product analyses revealed that AMMC (**4**) was the only substance formed in higher quantities, under any of these conditions; usually, only one additional, very faint band appeared (details not shown). AMMC (**4**) re-eluted from chromatography plates showed the spectral properties of the synthesized compound. The amount of AMMC (**4**) produced depended on the AMK (**1a**) concentration (Fig. 5).

Since AMMC (**4**) was easily formed by interaction of AMK (**1a**) and NO, we tested the relative scavenging capacity of AMK (**1a**) for NO, using *N*-acetylcysteine (NAC) as a competitor. In experiments with SNAP as an NO donor, AMK (**1a**) and NAC were approximately equally potent (Fig. 6A). Since these reactions had to be conducted at acidic pH, additional runs were carried out at pH 7.4 using PAPA NONOate. However, the combination of this NO donor and NAC led to very low levels of fluorescence, possibly caused by a quenching effect. Therefore, competition between AMK (**1a**) and NAC for NO was also tested photometrically at 400 nm (Fig. 6B), a wavelength at which AMK (**1a**) but not AMMC (**4**) absorbs (cf. Fig. 3). Although this wavelength does not coincide with the maximum of absorbance, competition experiments were possible and revealed a somewhat higher NO affinity of NAC, compared to AMK (**1a**), at physiological pH (Fig. 6B).

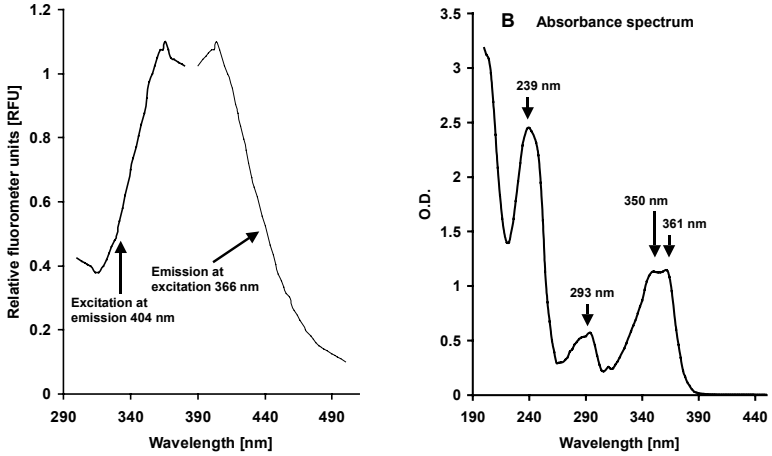

Fig. 3. Fluorescence and absorbance spectra of AMMC (**4**). A: Fluorescence in ethanol as solvent. B: Optical density (O.D.) in methanol as solvent; the spectrum is calculated for a concentration of 0.3 mM (measurements were made at concentrations sufficing for linearity according to Lambert-Beer's law).

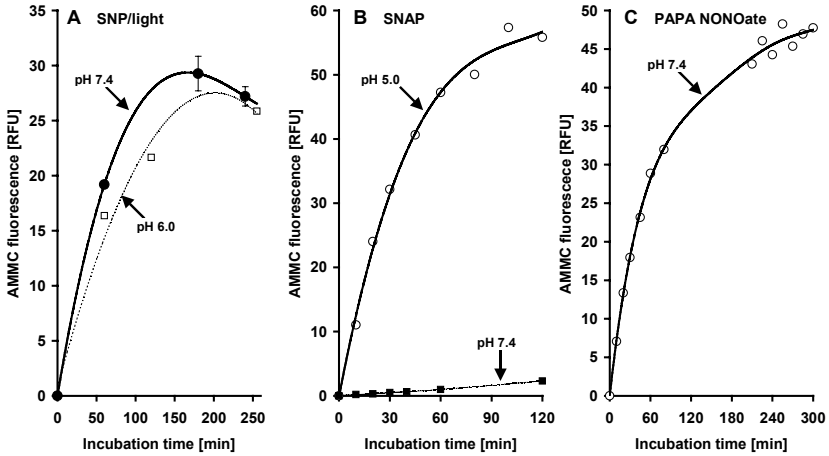

Fig. 4. AMMC (**4**) formation from AMK (**1a**) using three different NO donors. SNP = sodium nitroprussiate; SNAP = *S*-nitroso-*N*-acetylpenicillamine, PAPA NONOate = 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine. Fluorescence was measured at 366 nm excitation and 404 nm emission. Vertical lines: s.e.m. (sometimes too small for graphic representation).

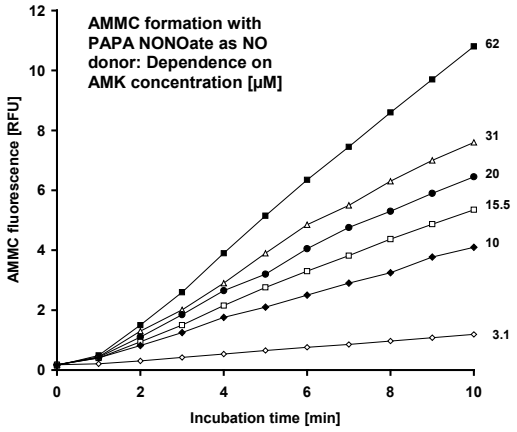

Fig. 5. Dependence of AMMC (**4**) formation on AMK concentration. Numbers on the right: µM AMK. Other details as in Fig. 4.

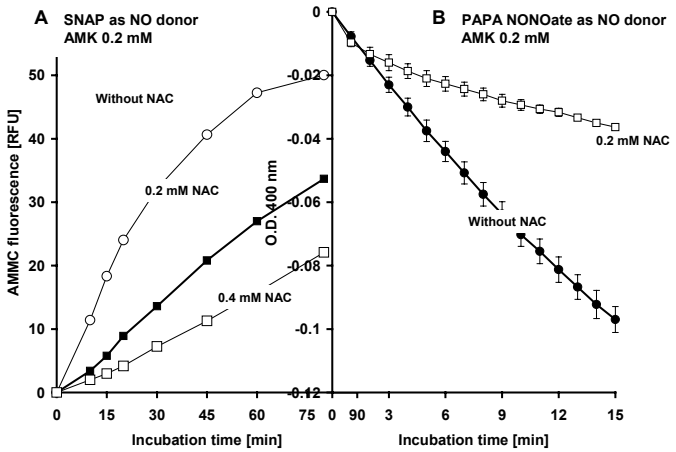

Fig. 6. Competition between AMK and *N*-acetylcysteine (NAC) for NO, using (A) SNAP at pH 5.0 or (B) PAPA NONOate at pH 7.4. Fluorescence measurements as in Figs. 3 and 4. Changes in absorbance at 400 nm reflect AMK consumption [AMMC (**4**) does not absorb at this wavelenght]. Other details as in Fig. 4.

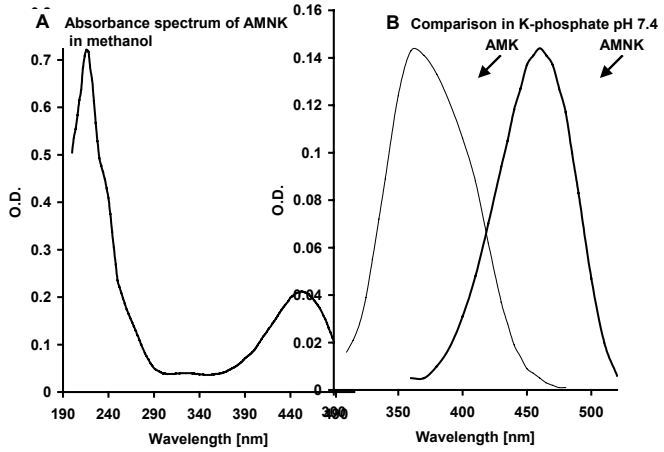

Fig. 7. Absorbance spectra of AMNK (**3**). A: Full spectrum in methanol as solvent. B: Section of spectrum which is relevant for distinction to AMK, in potassium phosphate buffer; this curve was obtained from AMNK (**3**) formed in the peroxynitrite/hydrogen carbonate system and re-eluted from a chromatogram.

Formation of AMNK (**3**) from AMK (**1a**) was possible when combining peroxynitrite with physiological concentrations of hydrogen carbonate (30 mM). Since peroxynitrite reacts practically instantaneously, no curves can be presented for the time course of AMNK (**3**) production. Directly upon addition of peroxynitrite to the reaction mixture, the characteristic orange color of AMNK (**3**) became visible. AMNK (**3**) was the only product formed in high quantities. If peroxynitrite was given in excess (12 mM or higher), AMNK (**3**) was largely destroyed. When AMNK (**3**) was separated from residual AMK (**1a**) by thin-layer chromatography (dichloromethane/methanol 92:8), the re-eluted product exhibited the spectral properties of the authentic compound (Fig. 7), being in accordance with the results of chemical nitration by HNO_3 (see above). If peroxynitrite (2.8 or 5.6 mM) was used in the absence of hydrogen carbonate, no orange color became visible and no substantial amounts of AMNK (**3**) were present in product analyses; however, AMK (**1a**) was partially destroyed (details not shown).

In order to test for the stability of AMMC (**4**) and AMNK (**3**) in the presence of free radicals, we incubated the two compounds in two reaction systems, one containing a radical of low reactivity, the ABTS cation radical, the other one generating the highly reactive hydroxyl radical *via* the Fenton reaction. The reactivity towards ABTS cation radicals was almost negligible in the case of AMMC (**4**), and comparably low in the case of AMNK (**3**), which was a much poorer reductant of the cation radicals than its precursor, AMK (**1a**, Fig. 8). In a competition assay for hydroxyl radicals, using ABTS as the competitor, AMNK (**3**) exhibited moderate reactivity, which was somewhat less than that of AMK (**1a**), but still in the same order of magnitude [cf. ref. 14] (Fig. 9). In the same system, AMMC (**4**) showed a strong prooxidant effect, to an extent, which is rarely seen in comparable experiments with other compounds (Fig. 9). The increase in optical density, as measured in the presence of AMMC (**4**), was due to a rise in the concentration of ABTS cation radicals, which were measured in this case at 734 nm, i.e. far apart from the absorbance of AMK-derived products, and which was visible by the naked eye as a higher intensity of the green colored cation radical. In these experiments, both AMMC (**4**) and AMNK (**3**) appear to be relatively stable end products in the presence of low-reactivity radicals. Not surprisingly, they are, however, attacked by the highly reactive hydroxyl radical.

Discussion

To the best of our knowledge, the three compounds formed from AMK (1a) on air-exposed TLC plates have not been described in chemical literature nor have they been found in biological material. The conditions under which they originated in the initial experiments on the plates were highly artificial, with regard to a large surface, eventual polarization on the silica, presence of traces of other compounds in the air or on the plate. Nevertheless, their formation is in good agreement with the high reactivity of AMK (**1a**), as observed in an earlier study [14]. This is the first work to identify and characterize metabolites from AMK (**1a**).

To date we have not been able to find any non-enzymatic reaction by which the quinazoline derivative could be formed physiologically. However, the two other compounds, AMMC (**4**) and AMNK (**3**), may well be produced in biological material because the reaction partners used for their generation in liquid environments, i.e. reactive nitrogen species as well as CO_2, generated from its equilibrium with hydrogen carbonate, are present in tissues and body fluids in quantities sufficient for making their physiological formation highly likely. At least, AMMC (**4**) and AMNK (**3**) must be regarded as natural compounds arising from the kynuric degradation pathway of melatonin metabolism endogenously.

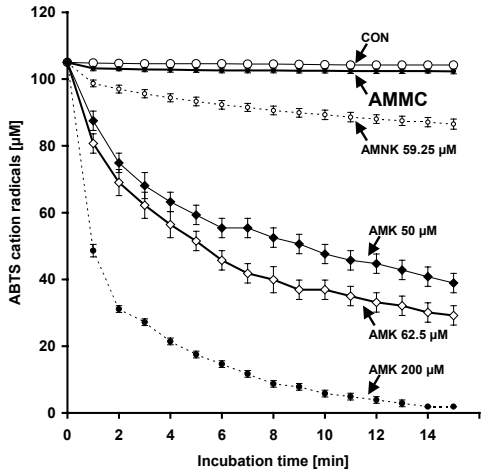

Fig. 8. Comparison of the capacities of AMK (**1a**), AMNK (**3**) and AMMC (**4**) to reduce ABTS cation radicals. CON = control, in the absence of these compounds. Vertical lines: s.e.m. (sometimes too small for graphic representation).

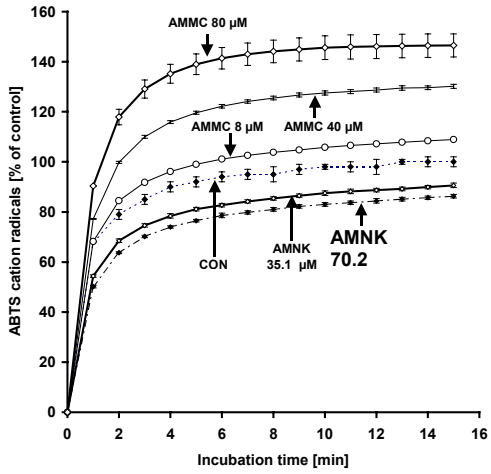

Fig. 9. Moderate antioxidant behavior of AMNK (**3**) and prooxidant effects of AMMC (**4**) in an ABTS competition assay for hydroxyl radicals. Other details as in Fig. 8.

Three chemically different NO donors, PAPA NONOate, SNAP and sodium nitroprussiate, applied under different conditions, at physiological pH 7.4, at acidic pH 5.0 or 6.0, and in ethanol, consistently led to the formation of AMMC (**4**) as the principal product; other compounds, if at all, were only present in minute quantities. The use of ethanol as a solvent for AMK (**1a**) also excluded the involvement of peroxynitrite-derived hydroxyl radicals. The nitration product, AMNK (**3**), was notably not produced under the influence of the NO donors. The application of peroxynitrite, in the presence of near-physiological concentrations of hydrogen carbonate, almost exclusively resulted in AMNK (**3**) formation. No AMMC (**4**) was found under these conditions, so that decomposition of peroxynitrite to NO should not have been of relevance. Since AMNK (**3**) was not produced by peroxynitrite in the absence of hydrogen carbonate, one can conclude that AMK (**1a**) was not primarily nitrated by the subsequent actions of hydroxyl radicals and NO_2, as formed by autolysis of ONOOH, but rather by the alternative nitration mixture, carbonate radicals ($CO_3^{\bullet -}$) and NO_2, which is generated by autolysis of the peroxynitrite-CO_2 adduct ($ONOOCO_2^-$) [19,20]. Moreover, the presence of hydrogen carbonate prevented to some extent direct actions of hydroxyl radicals on AMK (**1a**), if generated by decomposition of ONOOH, because •OH is largely scavenged by HCO_3^-, thereby forming water and $CO_3^{\bullet -}$. Apart from the particular finding of AMK (**1a**) nitration, these results shed a light on the frequently underrated relevance of HCO_3^- and CO_2 in the physiology and pathophysiology of radical reactions. Considerable rises of conversion rates were also achieved by addition of HCO_3^- to systems oxidizing melatonin [5,21-23] or caffeic acid [24]. AMK (**1a**) was found to be easily attacked by $CO_3^{\bullet -}$ [5,14] but, in the absence of NO_2, to be degraded mainly to colorless, non-fluorescent oxidation products [V Zelosko, R Hardeland, unpubl. data]. These findings demonstrate the high specificity and selectivity of the reactions.

AMMC (**4**) and AMNK (**3**), resulting from interactions with reactive nitrogen species, may be classified as melatonin metabolites produced under enhanced oxidative pressure or manifest oxidative stress. This is not only a matter of their formation from AMK (**1a**), but also relates to the implication of free radicals in the first step of the pathway, pyrrole-ring cleavage. AFMK is either formed non-enzymatically from melatonin by reactive oxygen species or enzymatically, e.g., by in-

doleamine 2,3-dioxygenase, in a reaction involving a superoxide anion as cosubstrate [1-3,5,6,12,13]. In the brain, indoleamine 2,3-dioxygenase is induced by proinflammatory cytokines [25-27] and, thereby, also related to a particular situation of enhanced oxidative metabolism. AMK (**1a**) is obviously easily formed, especially as it has been recovered in higher quantities than AFMK after injection of melatonin [6]. AMMC (**4**) and AMNK (**3**) represent products expected to be primarily formed in situations in which both reactive oxygen and nitrogen species are elevated. This aspect should be considered in the future with regard to melatonin administration in pertinent pathophysiological situations, e.g. during reperfusion when reactive oxygen species are rising, but also HCO_3^-, CO_2 and NO are still high, the latter having been released during vain attempts of improving circulation. Another case may be neuronal excitotoxicity, in which reactive oxygen species are produced as a consequence of calcium-dependent mitochondrial dysfunction in conjunction with calcium-stimulated NO synthesis.

Despite the likelihood of physiological AMMC (**4**) and AMNK (**3**) formation, the biological rates at which these processes occur are as yet unknown. Their determination is not easily possible, because physiological levels of AMK (**1a**) have never been measured, and judgments on the basis of AMK (**1a**) administration would be questionable. Starting with subnanomolar or nanomolar concentrations of melatonin in body fluids or tissues, respectively, one should not expect AMK (**1a**), AMMC (**4**) or AMNK (**3**) to substantially exceed such levels, even if the last two should turn out to be slowly metabolized or only removed with delay. Since AMK (**1a**) is also easily oxidized by other free-radical reactions [14], the two compounds may only represent a fraction of the total of AMK (**1a**) products. With regard to its presumably low concentration, AMK (**1a**) should not be primarily regarded as an effective scavenger in a defense system against reactive nitrogen species. Moreover, our competition experiments revealed that the affinity of AMK (**1a**) to NO is somewhat lower than that of *N*-acetylcysteine. However, since it is in the same order of magnitude, AMMC (**4**) formation is physiologically likely. From a general point of view, these findings do not rule out a possible biological relevance of AMMC (**4**) and/or AMNK (**3**), especially as long as their pharmacology has not been studied. This should be done particularly for AMMC (**4**). Although this family of substances, the cinnolines, was believed to be absent from biological material, several members of this class are pharmacologically active and have already been used as drugs. For instance, some of these substances exerted antiallergic, antitumor or neurotropic effects, including anxiolytic actions [28-32]. Although any judgment on eventual effects of AMMC (**4**) and AMNK (**3**) would be premature, their roles should primarily be sought in properties of putative signaling molecules or of antagonists. Whether such a signaling function might contribute to the broad spectrum of protective effects exerted by melatonin, is an open but intriguing question, which deserves further study.

This investigation is the first work to conclusively demonstrate that the kynuric pathway of melatonin catabolism extends beyond the kynuramine AMK (**1a**), yielding metabolites of potential importance. Our findings confirm that AMK is not an end product of melatonin metabolism, but continues to react with nitrogen species.

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References

1. HARDELAND R, FUHRBERG B, BEHRMANN G, BALZER I. Sleep-latency reducing pineal hormone melatonin as a scavenger of free radicals: hemin-catalysed formation of N^1-acetyl-N^2-formyl-5-methoxykynuramine. Sleep Res 1993; **22**:621.
2. HARDELAND R, REITER RJ, POEGGELER B, TAN D-X. The significance of the metabolism of the neurohormone melatonin: antioxidative protection and formation of bioactive substances. Neurosci Biobehav Rev 1993; **17**:347-357.
3. HARDELAND R, FUHRBERG B. Ubiquitous melatonin - Presence and effects in unicells, plants and animals. Trends Comp Biochem Physiol 1996; **2**:25-45.
4. TAN D-X, MANCHESTER LC, REITER RJ *et al*. Significance of melatonin in antioxidative defense system: Reactions and products. Biol Signals Recept 2000; **9**:137-159.
5. HARDELAND R, RESSMEYER A-R, ZELOSKO V *et al*. Metabolites of melatonin: Formation and properties of the methoxylated kynuramines AFMK and AMK. In: Recent Advances in Endocri-

nology and Reproduction: Evolutionary, Biotechnological and Clinical Applications. Haldar C, Singh SS. eds., Banaras Hindu University, Varanasi, 2004; pp. 21-38.

6. HIRATA F, HAYAISHI O, TOKUYAMA T, SENOH S. *In vitro* and *in vivo* formation of two new metabolites of melatonin. J Biol Chem 1974; **249**:1311-1313.
7. TRICOIRE H, LOCATELLI A, CHEMINEAU P, MALPAUX B. Melatonin enters the cerebrospinal fluid through the pineal recess. Endocrinology 2002; **143**:84-90.
8. REITER RJ, TAN D-X. Role of CSF in the transport of melatonin. J Pineal Res 2002; **33**:6.
9. HUETHER G. The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. Experientia 1993; **49**:665-670.
10. BUBENIK GA. Localization, physiological significance and possible clinical implication of gastrointestinal melatonin. Biol Signals Recept 2001; **10**:350-366.
11. BUBENIK GA. Gastrointestinal melatonin: localization, function and clinical relevance. Dig Dis Sci 2002; **47**:2336-2348.
12. TAN D-X, MANCHESTER LC, BURKHARDT S *et al*. N^1-Acetyl-N^2-formyl-5-methoxykynuramine, a biogenic amine and melatonin metabolite, functions as a potent antioxidant. FASEB J 2001; **15**:2294-2296.
13. TAN, D-X, REITER RJ, MANCHESTER LC *et al*. Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical svavenger. Curr Top Med Chem 2002; **2**:181-197.
14. RESSMEYER A-R, MAYO JC, ZELOSKO V *et al*. Antioxidant properties of the melatonin metabolite N^1-acetyl-5-methoxykynuramine (AMK): scavenging of free radicals and prevention of protein destruction. Redox Rep 2003; **8**:205-213.
15. KENNAWAY DJ, HUGEL HM, CLARKE S *et al*. Structure-activity studies of melatonin analogues in prepubertal male rats. Aust J Biol Sci 1988; **41**:393-400.
16. CONNOR DT, YOUNG PA, VON STRANDTMANN M. Heterocyclic synthesis with N-oxides. Preparation of pyridine N-oxide substituted chromones, chromanones, coumarins, quinolones, dihydroquinolones and cinnolones. J Heterocyclic Chem 1977; **14**:143-145.
17. RE R, PELLEGRINI N, PROTEGGENTE A *et al*. Antioxidant activity applying an improved ABTS cation radical decolorization assay. Free Radic Biol Med 1999; **26**:1231-1237.
18. POEGGELER B, THUERMANN S, DOSE A *et al*. Melatonin's unique radical scavenging properties – Roles of its functional substituents as revealed by a comparison with its structural analogs. J Pineal Res 2002; **33**:20-30.
19. SQUADRITO GL, PRYOR WA. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. Free Radic Biol Med 1998; **25**:392-403.
20. BLANCHARD B, POMPON D, DUCROCQ C. Nitrosation of melatonin by nitric oxide and peroxynitrite. J Pineal Res 2000; **29**:184-192.
21. HARDELAND R, NIEBERGALL R, SCHOENKE M, POEGGELER B. Carbonate radicals as initiators of melatonin oxidation: Chemiluminescence and formation of oxidation products. In: Actions and Redox Properties of Melatonin and Other Aromatic Amino Acid Metabolites. Hardeland R, ed., Cuvillier, Göttingen, 2001; pp. 49-55.
22. HARDELAND R, POEGGELER B, NIEBERGALL R, ZELOSKO V. Oxidation of melatonin by carbonate radicals and chemiluminescence emitted during pyrrole ring cleavage. J Pineal Res 2003; **34**:17-25.
23. HARDELAND R, POEGGELER B, BURKHARDT S *et al*. Oxidation chemistry of melatonin: New aspects of radical reactions. In: Neuroendocrine System and Pineal Gland with Special Reference to Lifestock. Haldar C, Singh SS, eds. Banaras Hindu University, Varanasi, 2003; pp. 27-35.
24. HARDELAND R, NIEBERGALL R. Effects of bicarbonate on the oxidation of caffeic acid: Role of carbonate radicals? In: Actions and Redox Properties of Melatonin and Other Aromatic Amino Acid Metabolites. Hardeland R, ed., Cuvillier, Göttingen, 2001; pp. 58-61.
25. ALBERATI-GIANI D, RICCIARDI-CASTAGNOLI P, KÖHLER C, CESURA AM. Regulation of the kynurenine pathway by IFN-γ in murine cloned macrophages and microglial cells. Adv Exp Med Biol 1996; **398**:171-175.
26. SAITO K, SEISHIMA M, NOMA A *et al*. Cytokine and drug modulation of kynurenine pathway metabolism by blood mononuclear cells. Adv Exp Med Biol 1996; **398**:161-165.
27. MCKENZIE CR, GONZÁLEZ RG, KNIEP E *et al*. Cytokine mediated regulation of inter-

feron-gamma-induced IDO activation. Adv Exp Med Biol 1999; **467**:533-539.

28. HOLLAND D, JONES G, MARSHALL PW, TRINGHAM GD. [i]Cinnoline-3-propionic acids,a new series of orally active antiallergic substances. J Med Chem 1976; **19**:1225-1228.
29. PATEL JB, MEINERS BA, SALAMA AI *et al*. A novel potent non-benzodiazepine anxioselective agent with reduced dependence liability: ICI 198,256. Prog Clin Biol Res 1990; **361**:483-488.
30. STANCZAK A, KWAPISZEWSKI W, SZADOWSKA A, PAKULSKA W. Synthesis and action of the central nervous system of some N2-substituted cinnoline derivatives. Pharmazie 1994; **49**:406-412.
31. STANCZAK A, PAKULSKA W, PIETRZAK B, LEWGOWD W. Comparison of pharmacophore cinnoline and quinoline systems on the basis of computer calculation and pharmacological screening of their condensed systems. Pharmazie 2001; **56**:501-505.
32. RUCHELMAN AL, SINGH SK, RAY A *et al*. 11H-Isoquino[4,3-c]cinnolin-12-ones; novel anticancer agents with potent topoisomerase I-targeting activity and cytotoxicity. Bioorg Med Chem 2004; **12**:795-806.