Research article

Reactions of the melatonin metabolite N^1-acetyl-5-methoxykynuramine (AMK) with the ABTS cation radical: identification of new oxidation products

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The melatonin metabolite N^1-acetyl-5-methoxykynuramine (AMK; **1**), which was previously shown to be a potent radical scavenger, was oxidized using the ABTS cation radical [ABTS = 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid)]. Several new oxidation products were obtained, which were separated by repeated chromatography and characterized by spectroscopic methods such as mass spectrometry (ESI-MS and ESI-HRMS), ^{1}H-NMR and ^{13}C-NMR, HMBC, HSQC, H,H COSY correlations and IR spectroscopy. The main products were oligomers of **1** (3 dimers, 1 trimer and 2 tetramers). In all cases, the amino group N^2 was involved in the reactions. Two of the dimers turned out to be *cis* (**2a**) and *trans* (**2b**) isomers containing an azo bond. In the other dimer (**3a**), the nitrogen atom N^2 was attached to atom C5 of the second aromatic amine, with loss of the methoxy group. In the trimer (**5**), one N^2 formed a bridge to C5 of unit B, as in the respective dimer, while this one of C had bridged to C6 of B. One of the tetramers (**6**) was composed of a trimer **5** attached to N^2 of a fourth **1** molecule via an azo bond as in **2a/b**. In the other tetramer (**7**), an additional C–C bond between rings B and C in **6** is assumed. Although oligomers of AMK may only attain low *in vivo* concentrations, the types of reactions observed shed light on the physiologically possible metabolism of AMK once reacted with a free radical. The displacement of a methoxy group, rarely seen in the oxidation of methoxylated biomolecules, underlines the reactivity of AMK (**1**). Preliminary data show that, in the presence of ABTS cation radicals, AMK (**1**) can interact with side chains of aromatic amino acids, a finding which may be crucial for understanding to date unidentified protein modification by a melatonin metabolite detected earlier in experiments with radioactively labeled melatonin.

Keywords: ABTS cation radical, AMK, kynuramines, melatonin

INTRODUCTION

The indoleamine melatonin, which is not only the principal hormone of the pineal gland, but also produced by various extrapineal sites, taken up from food, and undergoing enterohepatic cycling,[1–5] has been shown to act as a potent antioxidant.[1,5–10] These properties are based on highly complex mechanisms, which exceed direct radical scavenging and include up- and down-regulations of antioxidant and pro-oxidant enzymes, respectively, as well as mitochondrial effects. Moreover, some oxidative metabolites of melatonin seem to contribute to protection by melatonin, in particular, the 5-methoxylated kynuramines AFMK (N^1-acetyl-N^2-formyl-5-methoxykynuramine) and, even more, AMK (N^1-acetyl-5-methoxykynuramine, **1**; Fig. 1). Kynuramines represent an own class of biogenic amines and exhibit various

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biological and pharmacological effects; usually, they are formed by pyrrole ring cleavage of indoleamines. Accordingly, AFMK derives from melatonin by several enzymatic, pseudoenzymatic and radical-mediated reactions and is enzymatically deformylated to AMK.[5,6] AMK was shown to be a much more potent radical scavenger than its precursor AFMK.[11–13] Our prediction that AMK (**1**) should also possess protective properties at the mitochondrial level,[14] was confirmed by another laboratory.[15] In this context, redox reactions between AMK (**1**) and components of the electron transport chain seem to be crucial; in particular, electron donation to cytochrome *c* by AMK and electron uptake from iron-sulfur cluster *N*[2] of complex I by the deriving AMK cation radical have been discussed, a cycle assumed to diminish electron leakage.[5,14,16]

Contrary to AFMK, which preferentially undergoes two-electron transfer reactions, as shown by cyclic voltammetry,[17] AMK (**1**) easily donates single electrons.[11,12] This also becomes evident by its interaction with the ABTS cation radical [ABTS = 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid)], which can be used as a convenient test system for radical scavenging.[18] The ABTS cation radical seems also to be suitable for studies on oxidation products,[19] since this extremely long-lived radical does not readily destroy molecules by multiple interactions, and particularly not the aromatic moiety, as a highly reactive radical such as the hydroxyl radical would frequently do. Such destructions of AMK (**1**) were, in fact, observed in hydroxyl radical generating systems, as indicated by chemiluminescence from dioxetane intermediates,[11] and also directly by disappearance of any aromatic absorbance at short wavelengths (S.I. Schmidt and R. Hardeland, unpublished data). For this reason, it seemed worthwhile to investigate products from AMK (**1**) oxidation by using the less aggressive ABTS cation radical. In this study, we have identified several previously unknown compounds formed in such a reaction system.

Materials and Methods

Chemicals

AMK (**1**) was prepared from its precursor AFMK according to Kennaway *et al.*,[20] 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.*,[18] and adjusted after photometric determination, to a final concentration of 4.2 mM. Other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals used were of highest grade available. R_f values were measured on

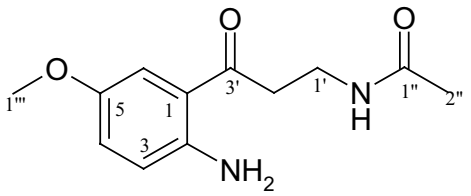

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Fig. 1. Structure of the melatonin metabolite AMK (**1**).

Polygram SIL G/UV254 (Macherey & Nagel & Co., Düren, Germany) with dichloromethane/10% methanol. Silica gel for PTLC: PF254 (Macherey & Nagel). Preparative plates were prepared with 660 g silica gel PF254 in 1.2 l of water and 60 ml of this slurry per 20 x 20 cm plate.

Equipment

^1H- 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 Inc., Palo Alto, CA, USA. Electron impact mass spectra (EI MS): Finnigan MAT95 (70 eV), Thermo Electron Corp., Bremen, Germany. Electron spray ionization mass spectrometry (ESI MS): Finnigan LCQ ion trap mass spectrometer coupled with a Flux Instruments (Basel, Switzerland) 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). Infrared spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer as KBr pellets. 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, UK) or a Perkin-Elmer Lambda 15 UV/VIS spectrometer.

Reaction, extraction and purification of products

For preparative purposes, 84.6 ml of 10 mM AMK (**1**) in water were mixed with 84.6 ml of 4.2 mM $ABTS^{\bullet+}$ and incubated for 2 min at 30°C in a water bath. After incubation, $ABTS^{\bullet+}$ was decolorized, and the solution showed a yellow color resulting from unreacted AMK (**1**) and its products. The mixture was extracted 5 times with 300 ml of ethyl acetate each time. The combined organic phases were vacuum concentrated and chro-

Table 1. Comparison of spectral data (^{1}H- and ^{13}C-NMR), HMBC and H,H COSY correlations of three dimers formed from AMK

	cis dimer 1 ([2a])					*trans* dimer 1 (**2b**)					dimer 2 (**3a**)				
No.	δ_C	δ_H	mult., J [Hz]	COSY	HMBC	δ_C	δ_H	mult., J [Hz]	COSY	HMBC	δ_C	δ_H	mult., J [Hz]	COSY	HMBC
	A					A					A				
1	134.2					142.0					119.1				
2	148.2					145.0					146.0				
3	118.8	6.47	d, 8.8	4	1, 5	121.9	7.76	d, 8.9	4	1, 2, 5	116.5	6.86	d, 8.8	4	1, 4, 5
4	118.6	6.85	dd, 2.6, 8.8	6	2	118.2	7.16	dd, 2.8, 8.9	6	2, 5, 6	124.6	6.99	dd, 2.9, 9.2	6	2, 3
5	160.1					163.6					151.6				
6	114.6	7.41	d, 2.6		2, 4, 3′	113.3	7.06	d, 2.8		2, 4, 5, 3′	115.2	7.35	d, 2.9		2, 4, 5, 3′
1′	36.1	3.58	t, 6.5		2″, 2′, 3′	35.9	3.51	t, 6.5		2″, 2′, 3′	36.4	3.24	t, 6.5		2″, 2′, 3′
2′	41.5	3.30	t, 6.5	1′	1′, 3′	45.3	3.10	t, 6.5	1′	1′, 3′	39.7	3.56	t, 6.5	1′	1′, 3′
3′	201.2			4		205.5					202.0				
1″	173.5					173.3					173.5				
2″	22.6	1.94	s	6	2″	22.5	1.87	s		2″	22.5	1.92	s		2″
1‴	56.4	3.79	s		5	56.5	3.91	s		5	56.4	3.78	s		5
	B					B					B				
1	134.2					142.0					118.6				
2	148.2					145.0					129.7				
3	118.8	6.47	d, 8.8	4	1, 5	121.9	7.76	d, 8.9	4	1, 2, 5	119.5	6.79	d, 8.8	4	1, 2
4	118.6	6.85	dd, 2.6, 8.8	6	2	118.2	7.16	dd, 2.8, 8.9	6	2, 5, 6	133.8	7.14	dd, 2.4, 8.8	6	5, 6
5	160.1					163.6					150.3				
6	114.6	7.41	d, 2.6		2, 4, 3′	113.3	7.06	d, 2.8		2, 4, 5, 3′	128.1	7.56	d, 2.4		2, 4, 5, 3′
1′	36.1	3.58	t, 6.5		2″, 2′, 3′	35.9	3.51	t, 6.5		2″, 2′, 3′	36.3	3.12	t, 6.5		2″, 3′
2′	41.5	3.30	t, 6.5	1′	1′, 3′	45.3	3.10	t, 6.5	1′	1′, 3′	39.3	3.51	t, 6.5	1′	1′, 3′
3′	201.2			4		205.5					201.4				
1″	173.5					173.3					173.4				
2″	22.6	1.94	s	6	2″	22.5	1.87	s		2″	22.5	1.89	s		2″
1‴	56.4	3.79	s		5	56.5	3.91	s		5					

For *cis* and *trans* dimer 1 (**2a/b**) both A and B have the same chemical shifts.
For dimer 2 (**3a**), A and B have the same numbering.

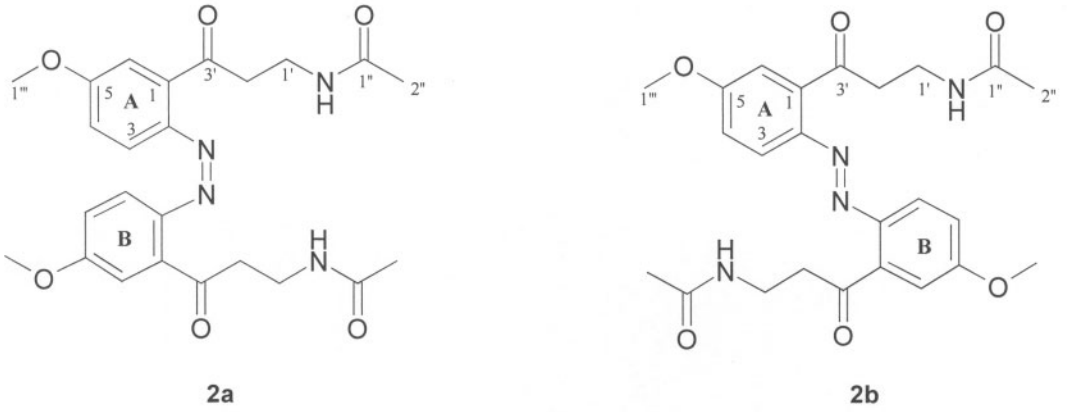

Fig. 2. Structures and atom numbering of *cis* (**2a**) and *trans* (**2b**) azo products.

matographed 3 times on preparative silica gel plates using dichloromethane/methanol (97:3) as a mobile phase. Fractions obtained from the last chromatography were further analyzed and, if necessary, purified by repeated chromatography: One fraction with R_f = 0.34–0.46 (dichloromethane:methanol, 97:3) contained a mixture of interconverting *cis* (**2a**) and *trans* (**2b**) isomers, but was otherwise pure. *Cis–trans* isomerisation at room temperature became obvious by 2-dimensional thin-layer chromatography in the same solvent. The main product (**3a**) of the following fractions was isolated by either six chromatographic steps using dichloromethane/methanol (93:7), or by chromatography on Sephadex LH-20 (44 x 1.5 cm; eluent, methanol). The last fraction contained three products, one of which (**5**) was separated by column chromatography (step 1: 60 x 2.5 cm Sephadex LH-20; eluent, methanol; step 2: 27.5 x 1 cm silica gel; eluent, ethyl acetate/methanol, 9:1), and further purified on Sephadex LH-20 (64 x 2.7 cm Sephadex LH-20; eluent, dichloromethane/methanol, 1:1). The other two compounds (**6**,**7**) were separated and purified by preparative thin-layer chromatography (PTLC) using dichloromethane/methanol (9:1), and the plate was developed 7 times.

RESULTS

All oxidation products obtained from the interaction of AMK (**1**) with $ABTS^{\bullet+}$ were identified as dimers and higher oligomers. The first type of dimers appeared as a pair of interconvertable *cis* (**2a**) and *trans* (**2b**) isomers (Fig. 2), confirmed by the presence of two separate peaks at R_t 11.41 and 13.66 min with the same mass on HPLC/MS and the slow interconversion on TLC. The ^{1}H-NMR spectrum of the mixture exhibited two sets of signals for very similar compounds in the ratio 1:5, which showed the same pattern of an 1,2,4-trisubstituted benzene-like AMK (**1**). After purification, the two isomers accounted for about 10% of the products. The ratio of *trans* and *cis* isomers found in the isolated mixture does not reflect the respective rates of formation, but rather results from the higher thermodynamical stability of the *trans* form. In both isomers, the methoxy groups and the acetylated side chains were unchanged. The ^{1}H-NMR spectrum (Table 1), the mass and the molecular formula $C_{24}H_{28}N_4O_6$ (by ESI HRMS; see Appendix) indicated them to be symmetrical dimers of **1**, which have, however, an additional double bond equivalent.

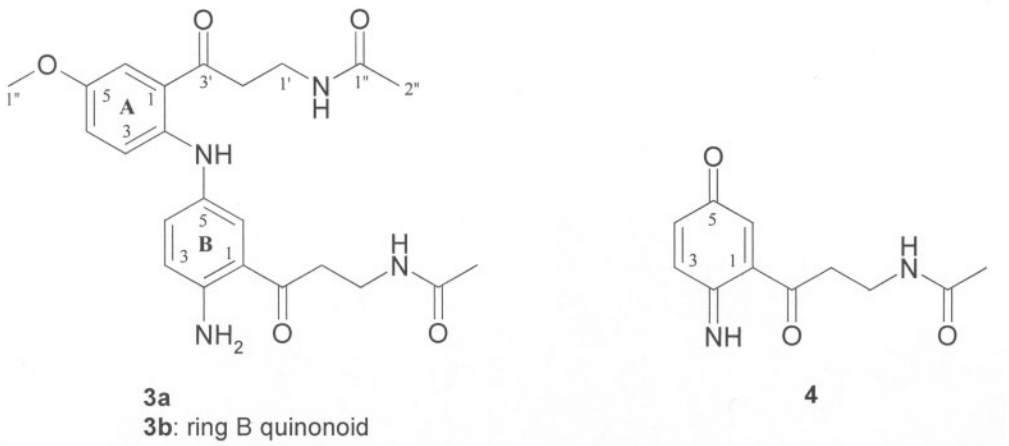

Fig. 3. Structure and atom numbering of dimer **3a** and its potential precursors **3b** (quinone-diimine in ring B instead of 1,4-diamine) and **4**.

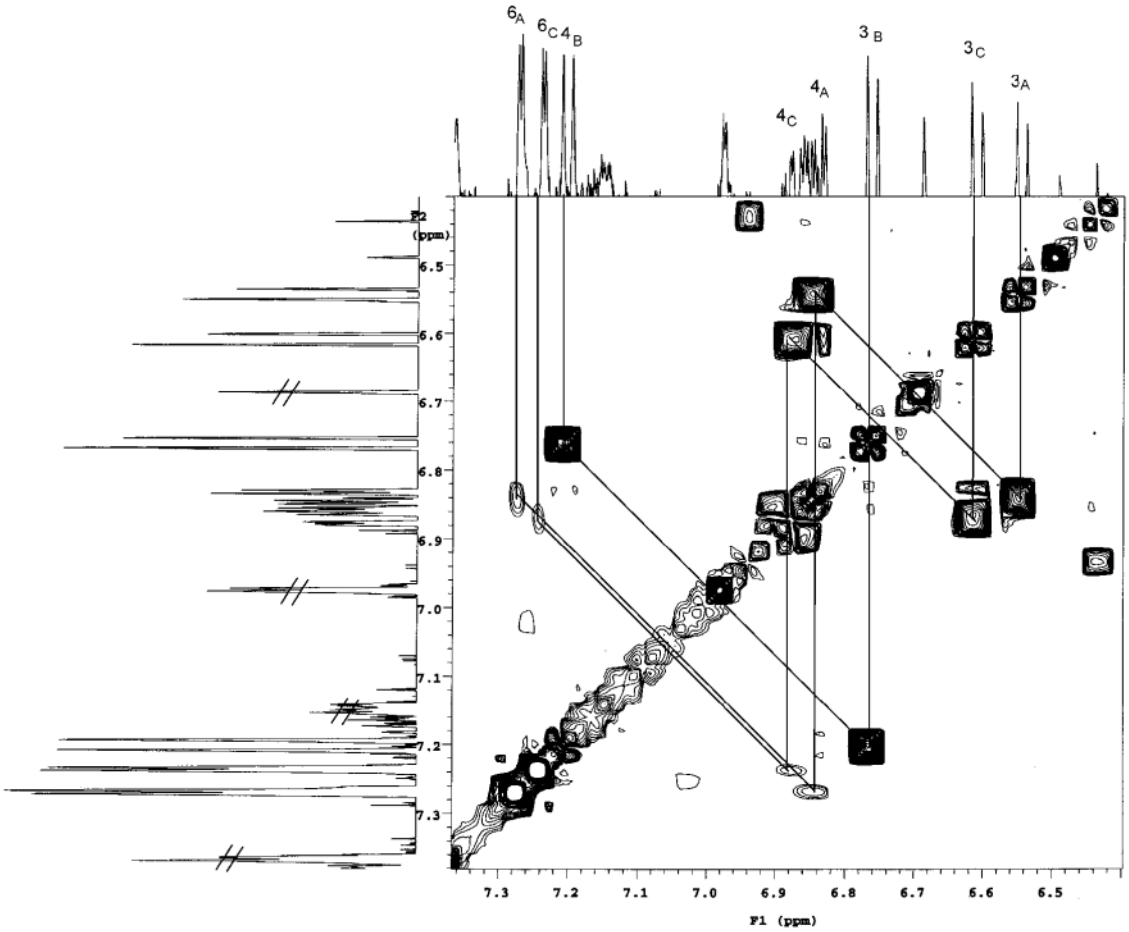

Fig. 4. H,H COSY spectrum of the trimer **5** (aromatic region). Connected lines indicate spin systems, ring signals were tentatively assigned; some impurities gave oversized signals due to the high magnification factor.

Based on these results, two molecules of **1** must be connected by an azo bond forming a highly symmetrical azo benzene with *cis* (minor isomer) and *trans* configuration, an assumption, which was further confirmed by detailed interpretation of 2-D NMR measurements. Spectral data (^{1}H- and ^{13}C-NMR) of **2a** and **2b** as well as HMBC and H,H COSY correlations are listed in Table 1. Azo compounds are usually formed via the reaction of diazonium salts with electron-rich aromates; however, the formation by oxidative dimerization of an aniline precursor is also known.[21,22]

The molecular formula of the third compound **3a** (Fig. 3), which also made up about 10% of the products, was confirmed as $C_{23}H_{28}N_4O_5$ by ESI HRMS of the pseudomolecular $[M+H]^+$ ion (Appendix), which corresponds to a dimerization of **1** under loss of methanol. This was confirmed by the ^{1}H-NMR spectrum, which indicated signals corresponding to two AMK (**1**) moieties, however, with only one methoxy group instead of two as in **2a** and **2b**. As extensive 2-D NMR measurements confirmed unchanged **1** skeletons also in this dimer (Table 1), NH_2 of **1** must have attacked C5 of a second **1** molecule under a formal displacement of the methoxy group. It can be assumed that, first, an oxidative demethylation had taken place and that the intermediate quinone-imine 4 (Fig. 3) reacted with NH_2 of the second AMK (**1**) molecule under elimination of water. A reduction of the intermediate quinone-diimine **3b**, *e.g.* by **1** or the solvent, would finally deliver **3a**.

For a further light yellow minor product, the formula $C_{35}H_{42}N_6O_8$ was derived by ESI HRMS (Appendix), which points to a trimerisation of **1**, again under loss of methanol. The purified compound accounted for about 2% of the products. The ^{1}H-NMR spectrum shows indeed only two methoxy groups, however, three ethanediyl fragments. The quartet splitting of three CH_2 signals and three acetyl singlets indicate that the acetamide ($NH–COCH_3$) fragments of three educt molecules **1** were maintained: Trimerisation must, therefore, have taken place across the aromatic rings.

The available amount was too low for ^{13}C and HMBC measurements. The ^{1}H and COSY spectra confirmed, however, two 1,2,4-trisubstituted benzene rings and two *ortho*-coupled protons. It is obvious, therefore, that the trimer contains the structural element of the dimer **3a**,

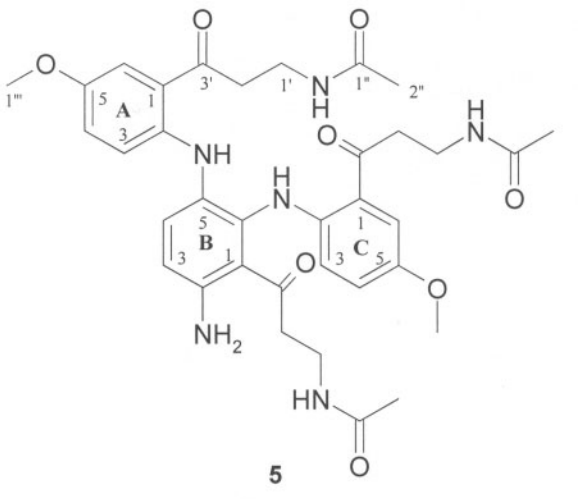

Fig. 5. Structure and atom numbering of the trimer **5**.

which is connected with a third AMK (**1**) molecule at C6 of one of the three benzene rings via a free NH_2 group. Only one of the three expectable isomers is plausible for mechanistic reasons: All intermediates in the formation of **3a** are Michael acceptors with the highest reactivity at C_B6 (*e.g.* **3b**), which would, therefore, favor the formation of **5**. Both alternative isomers would require a radical attack of NH_2 in **1** on C_A6 in **3a**, or of NH_2 in **3a** on C6 in **1**, which is less favorable. Structure **5** (Fig. 4) is, therefore, the most plausible alternative for this trimer; this agrees with the fact that the well-separated deep-field signal of C_B6 in **3a** is absent in **5**.

The ^{1}H-NMR spectrum of **6** (Fig. 5), accounting for about 2% of products, clearly revealed three methoxy and four *N*-acetyl groups, which were further confirmed by a successive loss of four acetamide fragments on ESI MS/MS. ESI HRMS of compound **6** (Appendix) indicated a molecular formula of $C_{47}H_{54}N_8O_{11}$ with 25 double bond equivalents (DBE). As each **1** molecule contributes 6 DBE, a further ring or double bond as in **2a/b** must exist.

The proton spectrum was surprisingly well separated (Fig. 6); a detailed analysis delivered three 1,2,4-trisubstituted rings and two 1H singlets of *para*-positioned protons. On the basis of the dimers (**2a/b**, **3a**) and our mechanistic considerations, a **1** molecule could attack the intermediate **4** also at position C_B4 instead of C_B6 as

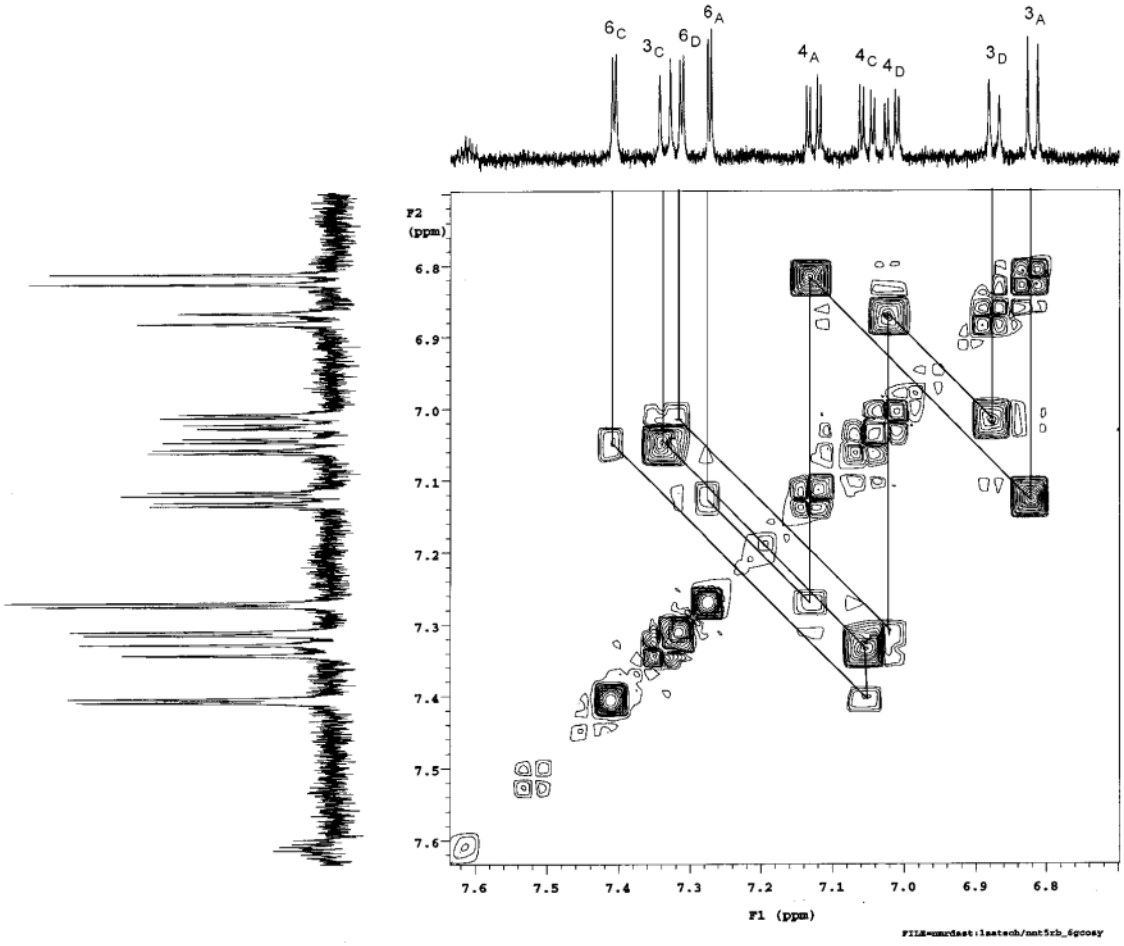

Fig. 6. H,H COSY spectrum of tetramer 1 (**6**). Connected lines indicate spin systems, ring signals were tentatively assigned.

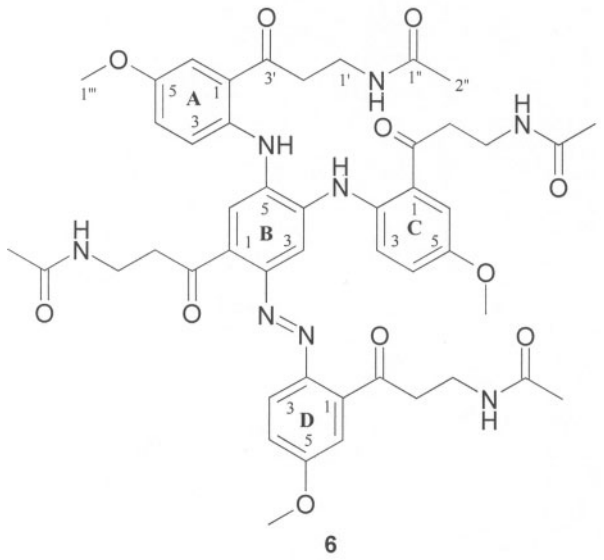

Fig. 7. Structure and atom numbering of tetramer 1 (**6**).

in **5**, and oxidative N=N bond formation with a second **1** molecule would deliver **6** (Fig. 7). All other tetrameric structures require a C–N bond formation by a less favorable attack of an unreactive amine radical on a benzene ring (*e.g.* the reaction of NH_2 in **3a** on C_A4 of **2a**). As no HMBC spectra were available, these suggested structures are, however, speculative.

A second tetramer (about 2% of products) had the formula $C_{47}H_{52}N_8O_{11}$ (from ESI HRMS) and, therefore, 1 DBE more than **6**. The high-field singlet of 3_B-H and a further *ortho*-coupled doublet were missing, and instead three (in **6**), now four *meta*-coupled doublets were visible. This is a clear indication that a ring closure in **6** had occurred between C_B3/C_C3 with formation of a carbazole, or between C_B3/C_D3 with formation of a benzo[c]cinnoline; both reactions are known from the literature. While the cyclisation of azobenzenes is, however, a typical photochemical reaction,[23,24] the carbazole ring closure occurs easily under oxidative conditions[25] and can hence be expected also with ABTS cation radicals: the most plausible structure is, therefore, **7** (Fig. 8). Only stronger oxidants should be able to form a benzo[c]cinnoline: anhydrous $FeCl_3$ is able to dimerize (*e.g.* azulene-1-azo compounds) via C-3,3′,[26] and a related reaction may occur with **6** under suitable conditions.

Discussion

Single-electron transfer reactions with phenols are responsible for the so-called phenol oxidation: a primarily formed phenoxyl radical attacks another phenol moiety or radical with formation of a peroxide or an ether bond. As these products are unstable, the dominating reaction is the dimerisation of the mesomeric oxaryl radical in *o/p*-position to dimers and higher oligomers up to highly polymer black eumelanins. In a similar way, aromatic amines are oxidized, yielding compounds very much in parallel to the phenols. It is, therefore, not surprising that the oxidation of AMK (**1**) resulted in oligomers with C–C, C–N, and N=N bonds. Our data not only confirm scavenging of ABTS cation radicals by AMK,[11] but also allow conclusions on the most reactive substituent of this kynuramine, namely, the N^2-amino residue, which participated in all di- or oligomerizations observed. Moreover, as the interaction with $ABTS^{\bullet+}$ represents a single-electron exchange reaction, formation of azo dimers implies two electron donations per AMK molecule. These findings readily explain why AMK proved to be a much more efficient reductant of $ABTS^{\bullet+}$ than its precursor AFMK.[11,12] First, the formyl substituent of AFMK largely prevents this type of reaction. In fact, comparative studies on AFMK oxidation by $ABTS^{\bullet+}$ have shown that the main product is a monomer oxidized in the aliphatic side chain (J. Rosen *et al.*, unpublished data). Second, oxidation of the aromatic amino group corresponds to the reduction of two $ABTS^{\bullet+}$ molecules.

It is remarkable that one of the dimers formed from AMK has lost one methoxy group during interaction with the nitrogen substituent in position 2. This has not been observed before in any other redox reaction of methoxylated melatonin metabolites. Regardless of the precise mechanism, this mode of adduct formation supports the previous conclusion on a reactivity higher than that of AFMK.

Without any doubt, $ABTS^{\bullet+}$ reduction represents an artificial situation. Nevertheless, its use has been successfully

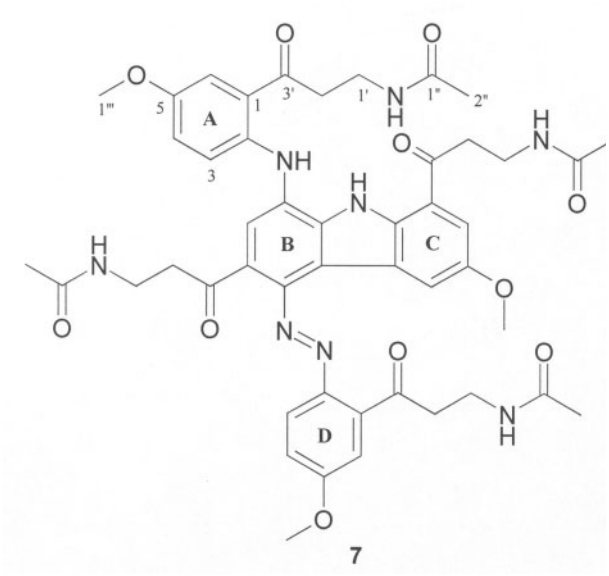

Fig. 8. Structure and atom numbering of tetramer 2 (**7**).

applied for following the conversion of melatonin and its metabolite, cyclic 3-hydroxymelatonin, to AFMK as they also occur in biological material by interactions with reactive oxygen species.[19] Moreover, $ABTS^{\bullet+}$ may serve as a model for other biologically existing aryl radicals. We believe that corresponding reactions should take place with other electron-/hydrogen-abstracting free radicals, such as hydroxyl and carbonate radicals. For experimental purposes, however, the application of these radicals is less recommendable because additional reactions take place, destroy intermediates and can even lead to cleavage of the aromatic ring (see Introduction). In fact, AMK was shown to scavenge the biologically relevant hydroxyl and carbonate radicals,[11,12] but a higher number of products and destruction of the aromatic moiety under *in vitro* conditions did not allow product analyses. Therefore, we selected the $ABTS^{\bullet+}$ as a radical not forming adducts, but reflecting the electron-abstracting reaction type known from hydroxyl and carbonate radicals.

Formation of dimers and oligomers from AMK should also be regarded as a mostly artificial process. Since melatonin concentrations in body fluids and tissues are usually in the nanomolar range, except melatonin-synthesizing or -accumulating cells,[27] bile fluid[2] and some high-melatonin organisms,[28,29] AMK should also not exceed such levels. Due to the lack of specific, highly sensitive detection procedures, physiological AMK concentrations are unknown to date, but AMK is also of particular interest in pharmacological terms, as a potent cyclooxygenase inhibitor,[30] specific down-regulator of cyclooxygenase 2 gene expression,[31] and inhibitor of neuronal NO synthase.[32] Up to now, AMK has been quantified only after administration of melatonin. When labeled melatonin was injected into the cisterna magna of rats, 35% of the radioactivity could be recovered as AMK, whereas the principal hepatic melatonin metabolite, 6-hydroxymelatonin, was not detectable.[33] Therefore, AMK appears to be a major brain metabolite of melatonin, a finding which has gained new actuality with regard to the observation that the hormone is released, via the pineal recess, to the cerebrospinal fluid of the third ventricle in concentrations about 30 times higher than those secreted into the circulation.[34] Nevertheless, AMK dimerization should be a rare reaction in biological material. However, this should by no means exclude a biological role of the reaction type studied here in an artificial *in vitro* system. It rather indicates that AMK, following interaction with an oxidant, can form adducts with other molecules carrying suitable substituents. The possibility of adduct formation from a melatonin metabolite in the course of its oxidation by free radicals has already been addressed earlier and was based on experimental data of protein modification occurring in melatonin infusion experiments.[35–37] Such reactions were, however, not detected when melatonin itself was incubated with putative interaction partners in radical-generating systems (R. Hardeland *et al*., unpublished data). Most recent experiments of this type using AMK instead demonstrated interactions with amino acid analogs carrying the relevant side chains and the formation of new compounds not deriving from any of the educts alone (C. Heer and R. Hardeland, unpublished data); these substances presumably represent adducts, but remain to be chemically characterized. This difference between melatonin and AMK now becomes plausible since, in the melatonin-oxidizing systems, the final product was mostly AFMK, which was not converted *in vitro* to AMK at substantial rates. With regard to the amazingly broad spectrum of effects exerted by melatonin, oxidative adduct formation and protein modification by AMK *in vivo* may become a relevant topic in the future.

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References

1. Hardeland R. Melatonin: multiple functions in signaling and protection. In: Altmeyer P, Hoffmann K, Stücker M. (eds) *Skin Cancer and UV Radiation*. Berlin: Springer, 1997; 186–198.
2. Tan D-X, Manchester LC, Reiter RJ *et al*. High physiological levels of melatonin in the bile of mammals. *Life Sci* 1999; **65**: 2523–2529.
3. Tan D-X, Manchester LC, Hardeland R *et al*. Melatonin – a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. *J Pineal Res* 2003; **34**: 75–78.
4. Hardeland R, Poeggeler B. Non-vertebrate melatonin. *J Pineal Res* 2003; **34**: 233–241.
5. Hardeland R. Antioxidative protection by melatonin – Multiplicity of mechanisms from radical detoxification to radical avoidance. *Endocrine* 2005; **27**: 119–130.
6. 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.
7. Tan D-X, Chen L-D, Poeggeler B *et al*. Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocr J* 1993; **1**: 57–60.
8. Reiter RJ. Oxidative damage in the central nervous system: Protection by melatonin. *Prog Neurobiol* 1998; **56**: 359–384.
9. Reiter RJ, Tan D-X, Burkhardt S. Reactive oxygen and nitrogen species and cellular and organismal decline: amelioration with melatonin. *Mech Ageing Dev* 2002; **123**: 1007–1019.
10. Reiter RJ, Tan D-X, Mayo JC *et al*. Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. *Acta Biochim Pol* 2003; **50**: 1129–1146.
11. 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 Report* 2003; **8**: 205–213.
12. Hardeland R, Ressmeyer A-R, Zelosko V *et al*. Metabolites of melatonin: formation and properties of the methoxylated kynuramines AFMK and AMK. In: Haldar C, Singh SS. (eds) *Recent Advances in Endocrinology and Reproduction: Evolutionary, Biotechnological and Clinical Applications.* Varanasi: Banaras Hindu University, 2004; 21–38.
13. Silva SO, Rodrigues MR, Carvalho SRQ *et al*. Oxidation of melatonin and its catabolites, N^1-acetyl-N^2-formyl-5-methoxykynuramine and N^1-acetyl-5-methoxykynuramine, by activated leukocytes. *J Pineal Res* 2004; **37**: 171–175.
14. Hardeland R, Coto-Montes A, Poeggeler B. Circadian rhythms, oxidative stress and antioxidative defense mechanisms. *Chronobiol Int* 2003; **20**: 921–962.
15. Acuna-Castroviejo D, Escames G, Leon J *et al*. Mitochondrial regulation by melatonin and its metabolites. *Adv Exp Med Biol* 2003; **527**: 549–557.
16. Srinivasan V, Pandi-Perumal SR, Maestroni GJM *et al*. Role of melatonin in neurodegenerative diseases. *Neurotox Res* 2005; **7**: 293–318.
17. 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.
18. 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.
19. Tan D-X, Hardeland R, Manchester LC *et al*. Mechanistic and comparative studies of melatonin and classic antioxidants in terms of their interactions with the ABTS cation radical. *J Pineal Res* 2003; **34**: 249–259.
20. 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.
21. Bamberger E, Lagutt J. Ueber das Verhalten des Phenylhydroxylamins gegen alkoholische Schwefelsäure und gegen Anilin. *Chem Ber* 1898; **31**: 1500–1508.
22. Shaabani A, Lee DG. Solvent free permanganate oxidations. *Tetrahedron Lett* 2001; **34**: 5833–5836.
23. Corma A, Garcia H., Iborra S *et al*. Cooperative effect of acid sites in the photocyclization of azobenzene within the zeolite microenvironment. *J Am Chem Soc* 1993; **115**: 2177–2180.
24. Tung C-H, Guan J-Q. Modification of photochemical reactivity by Nafion, photocyclization and photochemical *cis–trans* isomerization of azobenzene. *J Org Chem* 1996; **61**: 9417–9421.
25. Aekermark B, Oslob JD, Heuschert U. Catalytic oxidative aromatic cyclizations with palladium. *Tetrahedron Lett* 1995; **36**: 1325–1326.
26. Razus AC, Birzan L, Nae S *et al*. 1,1′-Biazulene derivatives. Synthesis and reactions. *ARKIVOC* 2002; 142-153. [Chem. Abstr. 138:303954.]
27. Tan D-X, Manchester LC, Reiter RJ *et al*. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. *Biochim Biophys Acta* 1999; **1472**: 206–214.
28. Hardeland R, Fuhrberg B. Ubiquitous melatonin – Presence and effects in unicells, plants and animals. *Trends Comp Biochem Physiol* 1996; **2**: 25–45.
29. Hardeland R. Melatonin and 5-methoxytryptamine in non-metazoans. *Reprod Nutr Dev* 1999; **39**: 399–408.
30. Kelly RW, Amato F, Seamark RF. *N*-Acetyl-5-methoxy kynurenamine, a brain metabolite of melatonin, is a potent inhibitor of prostaglandin biosynthesis. *Biochem Biophys Res Commun* 1984; **121**: 372–379.
31. Mayo JC, Sainz RM, Tan D-X *et al*. Anti-inflammatory actions of melatonin and its metabolites, N^1-acetyl-N^2-formyl-5-methoxy-kynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK), in macrophages. *J Neuroimmunol* 2005; **165**: 139–149.
32. Entrena A, Camacho ME, Carrion MD *et al*. Kynurenamines as neural nitric oxide synthase inhibitors. *J Med Chem* 2005; **48**: 8174–8181.
33. 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.
34. Tricoire H, Locatelli A, Chemineau P *et al*. Melatonin enters the cerebrospinal fluid through the pineal recess. *Endocrinology* 2002; **143**: 84–90.
35. Messner M, Hardeland R, Rodenbeck A, Huether G. Tissue retention and subcellular distribution of continuously infused melatonin in rats under near physiological conditions. *J Pineal Res* 1998; **25**: 251–259.
36. Messner M, Hardeland R, Rodenbeck A, Huether G. Effects of continuous melatonin infusions on steady-state plasma melatonin levels, metabolic fate and tissue retention in rats under near physiological conditions. *Adv Exp Med Biol* 1999; **467**: 303–313.
37. Hardeland R, Poeggeler B. Chemistry and biology of melatonin oxidation. In: Haldar C, Singaravel M, Maitra SK. (eds) *Treatise on Pineal Gland and Melatonin.* Enfield: Science Publishers (USA), 2002; 407–422.

APPENDIX

*Characteristics of the compounds **2a**, **2b**, **3a**, **5**, **6**, and **7***

cis-Dimer 1 (**2a**)

Yellow solid with R_f = 0.36 (CH_2Cl_2:CH_3OH, 9:1), violet with Ehrlich's reagent, dark red with anisaldehyde/sulfuric acid. IR (KBr, **2a/2b** mixture): ν_{max} (cm^{-1}) = 3444, 2932, 1638, 1596,1464, 1385, 1338, 1239, 1167, 1179. ^{1}H- and ^{13}C-NMR (see Table 1). (+)-ESI MS: *m/z* (%) = 491 ($[M+Na]^+$, 100), 958 ($[2M+Na]^+$, 14). (+)-ESI HRMS: *m/z* = 491.19011 $[M+Na]^+$, (calculated 491.19011 for $C_{24}H_{28}N_4O_6Na$), 469.20816 $[M+H]^+$, (calculated 469.20817 for $C_{24}H_{29}N_4O_6$).

trans-Dimer 1 (**2b**)

Yellow solid, R_f = 0.49 (CH_2Cl_2:CH_3OH, 9:1), violet with Ehrlich's reagent, dark red with anisaldehyde/sulfuric acid, rapid discoloration with Zn/HAc in MeOH. UV/Vis (MeOH, **2a/2b** mixture): λ_{max} (log ε) = 256, 369 nm. IR (KBr,

2a/2b mixture): ν_{max} (cm^{-1}) = 3444, 2932, 1638, 1596, 1464, 1385, 1338, 1239, 1167, 1179. ^{1}H- and ^{13}C-NMR (see Table 1). (+)ESI MS: *m/z* (%) = 491 ([M+Na]$^+$, 100), 958 ([2M+Na]$^+$, 78). (+)-ESI HRMS: *m/z* (%) = 491.19011 [M+Na]$^+$ (calculated 491.19011 for $C_{24}H_{28}N_4O_6Na$), 469.20816 [M+H]$^+$ (calculated 469.20817 for $C_{24}H_{29}N_4O_6$).

Dimer 2 (**3a**)

Dark yellow solid, R_f = 0.37 (CH_2Cl_2:CH_3OH, 9:1), violet with Ehrlich's reagent, brown-red with anisaldehyde/sulfuric acid, not discolored with Zn/HAc in MeOH. UV/Vis (MeOH): λ_{max} (log ε) = 232 (4.44), 295 (3.85), 411 nm (3.86). IR (KBr): ν_{max} (cm^{-1}) = 3467, 3347, 2926, 2342, 1648, 1594, 1558, 1522, 1453, 1428, 1385, 1369, 1177, 1103, 1036, 872, 839. ^{1}H- and ^{13}C-NMR (see Table 1). (+)-ESI MS: *m/z* (%) = 463 ([M+Na]$^+$, 100), 902 ([2M+Na]$^+$, 80). (+)-ESI HRMS: *m/z* = 441.21324 [M+H]+ (calculated 441.21326 for $C_{23}H_{29}N_4O_5$).

Trimer (**5**)

Light yellow solid, R_f = 0.17 (CH_2Cl_2:CH_3OH, 9:1), bright red with anisaldehyde/sulfuric acid. ^{1}H-NMR (acetone-d_6, 600 MHz): δ = 9.67, 9.53 (2 s, each 1H, 2NH), 7.27 (d, *J* = 2.9 Hz, 1H, 6-H_A), 7.24 (d, *J* = 2.9 Hz, 1H, 6-H_C), 7.20 (d, *J* = 8.6 Hz, 1H, 4-H_B), 6.87 (dd, *J* = 2.9 Hz, 9.2 Hz, 1H, 4-H_C), 6.84 (dd, *J* = 2.9 Hz, 9.1 Hz, 1H, 4-H_A), 6.76 (d, *J* = 8.6 Hz, 1H, 3H_C), 6.61 (d, *J* = 9.2 Hz, 1H, 3-H_B), 6.54 (d, *J* = 9.1 Hz, 1H, 3-H_A), 5.55 (s, 1H, NH), 3.75, 3.74 (2 s, each 3H, 2 OMe), 3.44 (q, *J* = 6.4 Hz, 2H, NH–CH_2), 3.40 (q, *J* = 6.7 Hz, 2H, NHCH_2), 3.34 (q, *J* = 7.0 Hz, 2H, NH–CH_2), 3.06 (t, *J* = 6.2 Hz, 2H, O=C–CH_2), 2.95 (t, *J* = 6.6 Hz, 2H, O=C–CH_2), 2.89 (t, *J* = 6.7 Hz, 2H, O=C–CH_2), 1.86, 1.85, 1.70 (3 s, each 3H, 3 O=C–CH_3); ring signals temptatively assigned. (+)-ESI MS: *m/z* (%) = 697 ([M+Na]$^+$, 100), 1370 [2M+Na]$^+$, 10). (+)-ESI HRMS: *m/z* = 697.29562 [M+Na]$^+$ (calculated 697.29564 for $C_{35}H_{42}N_6O_8Na$), 675.31366 [M+H]$^+$ (calculated 675.31369 for $C_{35}H_{43}N_6O_8$).

Tetramer 1 (**6**)

Yellowish brown solid, R_f = 0.15 (CH_2Cl_2:CH_3OH, 9:1), violet with anisaldehyde/sulfuric acid. ^{1}H-NMR (CD_3OD, 600 MHz): δ = 8.53 (s, 1H, 6_B-H), 7.41 (d, *J* = 3.0 Hz, 1H, 6_C-H), 7.33 (d, *J* = 9.0 Hz, 1H, 3_C-H), 7.31 (d, *J* = 2.9 Hz, 1H, 6_D-H), 7.27 (d, *J* = 2.9 Hz, 1H, 6_A-H), 7.13 (dd, *J* = 2.9 Hz, 8.7 Hz, 1H, 4_A-H), 7.05 (dd, *J* = 3.0 Hz, 9.0 Hz, 1H, 4_C-H), 7.02 (dd, *J* = 2.9 Hz, 8.8 Hz, 1H, 4_D-H), 6.87 (d, *J* = 8.8 Hz, 1H, 3_D-H), 6.82 (d, *J* = 8.7 Hz, 1H, 3_A-H), 6.10 (s, 1H, 3_B-H), 3.85, 3.83, 3.82 (3 s, each 3H, 3 OMe), 3.64, 3.55, 3.48, 3.41, 3.30, 3.26, 3.21, 3.05 (8 m, each 2H, 8 CH_2), 1.85, 1.84, 1.83, 1.74 (4 s, each 3H, 4 O=C–CH_3); ring signals temptatively assigned. ^{13}C-NMR (CD_3OD, 600 MHz): δ = 122.9 (C_C-3), 122.4 (C_A-3), 121.6 (C_D-3), 119.2 (C_C-4), 118.6 (C_A-4), 118.0 (C_D-4), 115.0 (C_C-6), 113.2 (C_D-6), 112.8 (C_A-6), 92.3 (C_B-3), 54.8, 54.8, 54.8 (3 OMe), 20.1, 20.1, 20.1, 20.1 (4 O=C–CH_3); data extracted from HMBC spectrum, C_qs not visible, CH_2 not listed. (–)-ESI MS: *m/z* (%) = 905.5 ([M-H]$^-$, 100). (+)-ESI MS: *m/z* (%) = 929 ([M+Na]$^+$, 100). (+)-ESI HRMS: *m/z* (%) = 907.39856 [M+H]$^+$ (calculated 907.39848 for $C_{47}H_{55}N_8O_{11}$).

*Tetramer 2 (**7**)*

Magenta-colored solid, R_f = 0.10 (CH_2Cl_2:CH_3OH, 9:1), pink with anisaldehyde/sulfuric acid. ^{1}H-NMR (CD_3OD, 600 MHz): δ = 8.54 (s, 1H, 6_B-H), 7.52 (d, *J* = 2.9 Hz, 1H, 6_A-H), 7.30 (dd, *J* = 2.9 Hz, 8.5 Hz, 1H, 4_D-H), 7.17 (d, *J* = 8.9 Hz, 1H, 3_D-H), 7.10 (d, *J* = 2.9 Hz, 1H, 6_D-H), 6.93 (dd, *J* = 3.1 Hz, 8.7 Hz, 1H, 4_A-H), 6.90 (d, *J* = 2.6 Hz, 1H, 6_C-H), 6.57 (d, *J* = 8.6 Hz, 1H, 3_A-H), 6.04 (d, *J* = 2.7 Hz, 1H, 4_C-H), 3.93 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.67 (s, 3H, OMe), 3.63, 3.56, 3.50, 3.45, 3.41, 3.31, 3.25, 3.19 (8 m, each 2H, 8 CH_2), 1.97, 1.93, 1.89, 1.81 (4 s, each 3H, 4 O=C–CH_3). (+)-ESI MS: *m/z* (%) = 927 ([M+Na]$^+$, 100), 905 ([M+H]$^+$, 26). (–)-ESI MS: *m/z* (%) = 903 ([M-H]$^-$, 40). (+)-ESI HRMS: *m/z* = 905.38282 [M+H]$^+$ (calculated 905.38283 for $C_{47}H_{53}N_8O_{11}$).