Synthesis and Characterization of a Dehydrogenation Product Arising from the Oxidation of Aminoethylcysteine Ketimine Decarboxylated Dimer

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While investigating the antioxidant properties of aminoethylcysteine ketimine decarboxylated dimer (**1**) (a natural substance occurring in biological fluids such as human urine and plasma and in bovine cerebellum), a previously unreported oxidation product was obtained. This compound was identified and characterized through comparison with an authentic sample prepared via Pd-catalyzed dehydrogenation of **1**. This molecule is an example of an alternative oxidation pathway involving **1**.

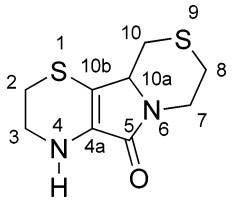
Aminoethylcysteine ketimine decarboxylated dimer (AECK-DD, Figure 1) is a natural sulfur-containing compound present in human plasma and urine[1,2] and detected in mammalian cerebellum.[3]

Recently the presence of AECK-DD in some edible vegetables has been detected.[4] As of present, no biosynthetic pathway for the formation of AECK-DD has been established, which suggests a possible external intake route via the dietary supply for the presence of this molecule in mammals. Concerning its biological role, AECK-DD has strong antioxidant activity and has been reported to interact *in vitro* with both reactive oxygen and nitrogen species (e.g., hydrogen peroxide, superoxide anion, hydroxyl radical, peroxynitrite, and its derivatives).[5,6] In addition, this molecule has been shown to protect human low-density lipoprotein and a human monocytic cell line against the oxidative stress induced by $CuCl_2$ and *tert*-butyl hydroperoxide.[7,8] Currently, the only products arising from the oxidation mixtures of AECK-DD that have been unambiguously characterized are a sulfoxide species[9] and its dimeric species.[10]

To further characterize the antioxidant properties of AECK-DD, a set of experiments were carried out with AECK-DD in the presence of $CuCl_2$/*tert*-butyl hydroperoxide and 2,2′-azo-bis-2-amidinopropane hydrochloride. The GC/MS analysis of the corresponding reaction mixtures showed the presence of a new species, **2a**, which had a mass of 226.

The decrease by two units with respect to the mass of the starting material (mass 228) suggested that the new species formed as a consequence of oxidative dehydrogenation. Indeed, the high-resolution mass spectra performed on this sample showed a fine mass value of 226.023788, which indicated a rough formula ($C_9H_{10}S_2N_2O$) compatible with the formation of a new double bond in the tricyclic structure. To confirm this hypothesis, AECK-DD was submitted to metal-mediated dehydrogenation in the presence of palladium on activated carbon (10% loading), which is a well-known method for the introduction of double bonds in aromatic and heterocyclic systems (Scheme 1).[11−14]

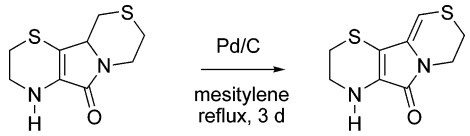
The reaction was carried out in refluxing mesitylene for 3 days, leading to the appearance of a new product, **2b**, whose mass was



1

Figure 1.

Scheme 1



1 **2b**

226 (GC/MS analysis). This new species was isolated from the reaction mixture by column chromatography (SiO_2, hexane/ethyl acetate = 7:3 v/v), and it was obtained as a bright yellow solid (40% isolated yield). The retention time of **2b** on GC/MS was identical to that of **2a** observed in the oxidation of **1**. This observation was confirmed by analyzing by GC/MS an oxidation mixture spiked with **2b**, which resulted in the appearance of just one peak with the corresponding retention time of **2a**. In addition, the GC/MS and the HRMS analyses of two distinct samples of **2a** and **2b** revealed an identical fragmentation pattern. On the basis of these results, we concluded that **2a** and **2b** were the same species.

In order to elucidate the structure of this new compound and to determine the position of the newly formed double bond in the molecule, a series of spectroscopic analyses were carried out. The UV analysis of **2b** dissolved in acetone and diluted with water (water/acetone, 280:1 v/v, 44 μM final concentration) showed a maximum absorbance at 338 nm, which corresponded to a shift of 30 nm with respect to that observed for **1** (308 nm). This difference correlates well with the Woodward−Fieser rule for the expected spectral shift observed following an extension of the conjugation in a UV-active molecule.[15,16] The ^{1}H NMR analysis of **2b** carried out in $CDCl_3$ showed an evident simplification of the spectra with respect to that reported for **1**.[10] The signal at 3.97 ppm caused by the tertiary hydrogen on carbon 10a in **1** disappeared, while a new signal at 5.58 ppm arose, suggesting the presence of an olefinic proton. The spectrum also showed the presence of four structured

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signals in the alkyl region, each of them accounting for two protons. This indicates that there are four methylene groups in the molecule, one less than in **1**. Out of all the possible isomers resulting from the introduction of a double bond into **1**, only the one reported in Scheme 1 is compatible with this spectroscopic data.

This conclusion was supported by more detailed NMR analyses. The ^{13}C NMR spectrum of **2b** ($CDCl_3$) showed the disappearance of the peak at 59.8 ppm (due to a tertiary carbon in α-position to the tertiary nitrogen in **1** and of one of the alkyl carbons in the 20−45 ppm region). On the other hand, two new carbon signals appear, one at 98.2 ppm and another in the 130s ppm region, highly indicative of the presence of two new olefinic carbons. In the heteronuclear multiple-quantum coherence spectrum (HMQC), each of the four carbons in the alkyl region (20−40 ppm) is correlated to one of the four triplets in the alkyl region of the proton spectrum (showing their secondary structure), while the peak at 98.2 ppm is correlated to the olefinic proton at 5.58. The remaining carbons (>100 ppm) are not detected, accounting for their quaternary structure. These findings are confirmed by the attached proton test spectrum (APT); whereas the quaternary carbons at >100 ppm and the secondary carbons in the alkyl region are positive, the signal at 98.2 ppm is the only negative one, making it a primary carbon. The two-dimensional $^1H-^1H$ COSY spectrum of **2b** shows two main spin systems; the signal at 3.91 ppm is correlated to the one at 3.04 ppm, while the signal at 3.64 ppm is correlated to the one at 2.99 ppm. This accounts for the presence of two couples of CH_2-CH_2 systems, which is compatible with the structure of **2b** reported in Scheme 1.

These findings also provide an explanation for why the ^1H NMR spectrum of **2b** is much simpler than that reported for **1**.[10] Indeed, the introduction of a new double bond between carbons 10 and 10a destroys the chiral center on position 10a, lowers the number of hydrogens in the molecule, and makes the two six-membered rings more similar in terms of structure.

The palladium-catalyzed dehydrogenation reaction on **1** occurs selectively on the bond between carbons 10 and 10a. From what is known about the mechanism of dehydrogenation involving cyclohexane,[17,18] it is possible that the process is triggered by an interaction between the hydrogen atoms involved and the Pd center, similar to a hydrogen bond interaction. The hydrogen atom on carbon 10a is the only tertiary one in the molecule, and the corresponding C−H bond is likely more susceptible to undergo a metal-mediated cleavage. These findings suggest that the oxidative dehydrogenation of AECK-DD (**1**) in the presence of peroxides might follow a similar path and that the reactivity of the molecular region involved in the metal-catalyzed dehydrogenation process could be very important in understanding its well-known antioxidant properties.

In conclusion, a new product following the oxidation of aminoethylcysteine ketimine decarboxylated dimer (**1**) has been observed, synthesized, and characterized through HRMS, UV, and NMR techniques. This product, which shows an additional unsaturation with respect to **1**, may arise from an oxidative dehydrogenation path that involves the carbons 10 and 10a of the tricyclic structure. Further studies are under investigation to clarify the mechanism of formation of **2b** under oxidative conditions also *in vivo*.

Experimental Section

General Experimental Procedures. Aminoethylcysteine ketimine decarboxylated dimer (**1**) was synthesized as previously reported.[19] *tert*-Butyl hydroperoxide was purchased from Sigma Chemicals Co., $CuCl_2$ was purchased from Fluka (Buchs, Switzerland), and 2−2′-azo-bis(2-amidinopropane hydrochloride) (ABAP) was purchased from Polyscience (Warrington, PA). Palladium on activated carbon (10% loading), diethylenetriaminepentaacetic acid (DTPA) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were purchased from Aldrich. Mesitylene was distilled over sodium and stored over 4 Å molecular sieves under an argon atmosphere. Purifications via column chromatography were performed on silica gel (Biosolve, 60 Å, 0.063−0.200 mm).

Instruments. GC/MS analyses were performed on an Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA). Gas chromatographic separations were carried out on an Agilent HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). Injection mode: splitless at a temperature of 260 °C. Column temperature program: 70 °C (1 min) then to 280 °C at a rate of 10 °C/min and held for 15 min. The carrier gas was helium at a constant flow of 1.0 mL/min. The spectra were obtained in electron impact mode at 70 eV ionization energy and a mass range scan from *m*/*z* 30 to 500; ion source temperature 280 °C, ion source vacuum 10^{-5} Torr.

The HMRS analysis was performed on a VG AutoSpec spectrometer. The sample (10 ng/μL in methanol) was analyzed through direct injection via a septum set at 260 °C. The spectra were obtained in electron impact mode at 70 eV ionization energy and a mass range scan from *m*/*z* 80 to 335, 0.3 s + 0.3 s delay time, centroid at 5000 RP; gas reference PFK; ion source at 250 °C, 500 μA.

NMR spectroscopy was performed using a Varian Unity Inova spectrometer, operating at 500 MHz for ^1H. All experiments were carried out at 296 K in $CDCl_3$. ^1H and ^{13}C shifts were referenced to internal $CDCl_3$ (7.26 and 77.25 ppm, respectively).

UV−vis spectra were performed on a Varian Cary 50 spectrophotometer.

Melting points were determined using a Buchi B545 melting point apparatus and they should be considered uncorrected.

Oxidation Experiments. To a solution 200 μM AECK-DD (**1**) in phosphate buffer saline (PBS) at pH 7.2 were added $CuCl_2$ (1 mM final) and *t*-BuOOH (1 mM final) or ABAP (1 mM final). The mixtures were kept at 37 °C for 60 min. Aliquots of 0.5 mL volume were taken after 5, 15, 30, and 60 min of incubation time; each of them was added with 10 μL of 10 μM DTPA and 20 μL of 2% BHT. The aliquots were extracted with chloroform (3 × 2 mL), and the organic fractions were combined and concentrated under reduced pressure. The residues were redissolved in a mixture of acetone−hexane (1:4 v/v) and the resulting solutions analyzed directly by GC/MS.

Palladium-Catalyzed Dehydrogenation of Aminoethylcysteine Ketimine Decarboxylated Dimer (1). A 100 mg (0.438 mmol) sample of aminoethylcysteine ketimine decarboxylated dimer (**1**) and 100 mg of 10% palladium on activated carbon were inserted in a Schlenk flask under argon atmosphere. The system was then briefly evacuated and backfilled with argon (3 cycles). Mesitylene (3 mL) was added, and the resulting mixture was stirred at the solvent reflux temperature for 3 days. The mixture was cooled down and filtered through a pad of Celite. The filtrate was concentrated *in vacuo* and the oily residue purified on silica gel (hexane/ethyl acetate = 7:3 v/v) to provide 39.5 mg of **2b** as a bright yellow solid (40% isolated yield). According to IUPAC nomenclature, the name of **2b** is 3,4,7,8-tetrahydro-2*H*,5*H*-pyrrolo[2,1-*c*:3,4-*b*′]bis[1,4]thiazin-5-one. The numbering scheme is reported in Figure 1, following the guidelines of the Ring Systems Handbook, Chemical Abstract Service (1993), RF 29858.

^1H NMR (500 MHz, $CDCl_3$): δ 5.58 (H_{10}, s, 1H), 3.91 (H_7, m, 2H), 3.70 (NH, bs, 1H), 3.64 (H_3, m, 2H), 3.04 (H_8, m, 2H), 2.99 (H_2, m, 2H). ^{13}C NMR (125.7 MHz, $CDCl_3$): δ 162.8 (C_5), 132.1, 130.2, 103.1 (C_{10b}), 98.2 (C_{10}), 42.3 (C_3), 39.2 (C_7), 26.4 (C_8), 25.5 (C_2). GC/MS (EI, *m*/*z*): 226 (100%), 211 (28%), 198 (11%), 180 (18%). HRMS: calcd for $C_9H_{10}N_2OS_2$ 226.023457; found 226.023788 (−1.5 ppm). Mp: 146 °C.

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Supporting Information Available: GC/MS analysis of **1** and **2a**; NMR, MS, and UV spectra of **2b**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Matarese, R. M.; Macone, A.; Antonini, R.; Maggio, A.; Antonucci, A. *J. Chromatogr. B: Biomed. Sci. Appl.* **1999**, *732*, 137−144.
(2) Matarese, R. M.; Macone, A.; Maggio, A.; Cavallini, D. *J. Chromatogr. B: Biomed. Sci. Appl.* **1996**, *683*, 269−272.
(3) Matarese, R. M.; Macone, A.; Crescentini, G.; Duprè, S.; Cavallini, D. *Neurochem. Int.* **1998**, *32*, 365−368.

(4) Macone, A.; Nardini, M.; Antonucci, A.; Maggio, A.; Matarese, R. M. *J. Agric. Food Chem.* **2002**, *50*, 2169−2172.
(5) Pecci, L.; Montefoschi, G.; Antonucci, A.; Cavallini, D. *Physiol. Chem. Phys. Med. NMR* **1995**, *27*, 223−229.
(6) Fontana, M.; Pecci, L.; Macone, A.; Cavallini, D. *Free Radical Res.* **1998**, *29*, 435−440.
(7) Matarese, R. M.; Macone, A.; Fontana, M.; Duprè, S.; Cavallini, D. *Biochem. Mol. Biol. Int.* **1998**, *46*, 829−837.
(8) Macone, A.; Matarese, R. M.; Gentili, V.; Antonucci, A.; Duprè, S.; Nardini, M. *Free Radical Res.* **2004**, *38*, 705−714.
(9) Pecci, L.; Antonucci, A.; Pinnen, F.; Cavallini, D. *Amino Acids* **2000**, *18*, 61−67.
(10) Mannina, L.; Viel, S.; Duprè, S.; Pecci, L.; Fontana, M.; Pinnen, F.; Antonucci, A.; Segre, A. L. *Tetrahedron* **2004**, *60*, 4151−4157.
(11) Fu, P. P.; Harvey, R. G. *Chem. Rev.* **1978**, *78*, 317−361.
(12) Cava, M. P.; Edie, D. L.; Saá, J. M. *J. Org. Chem.* **1975**, *40*, 3601−3602.
(13) Snyder, S. A.; Vosburg, D. A.; Jarvis, M. G.; Markgraf, J. H. *Tetrahedron* **2000**, *56*, 5329−5335.
(14) Kim, Y. H.; Choi, J. Y. *Tetrahedron Lett.* **1996**, *37*, 8771−8774.
(15) Woodward, R. B. *J. Am. Chem. Soc.* **1941**, *63*, 1123−1126.
(16) Woodward, R. B. *J. Am. Chem. Soc.* **1942**, *64*, 72−75.
(17) Raval, R.; Pemble, M. E.; Chesters, M. A. *Surf. Sci.* **1989**, *210*, 187−200.
(18) Fu, P. P.; Harvey, R. G. *Tetrahedron Lett.* **1977**, *18*, 415−418.
(19) Antonucci, A.; Pecci, L.; Coccia, R.; Fontana, M.; Cavallini, D. *Amino Acids* **1994**, *7*, 83−88.

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