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Identification of an oxidation product of aminoethylcysteine ketimine dimer

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Summary. In continuation of our previous work dedicated to the detection of the oxidation products of aminoethylcysteine ketimine dimer by oxygen reactive species, we give here data for the identification of the α, β unsaturated sulfoxide as the main product of interaction of the dimer with H_2O_2. Identification has been done on the basis of mass spectrometry and NMR analyses of the product isolated by preparative chromatography.

Keywords: Amino acids – Aminoethylcysteine ketimine dimer – Hydrogen peroxide – Sulfoxide derivative – Mass spectrometry – NMR analysis

Abbreviations: HPLC: high performance liquid chromatography, TLC: thin layer chromatography, AEC: aminoethylcysteine, AECK: aminoethylcysteine ketimine, TMS: tetramethylsilane.

Introduction

Aminoethylcysteine ketimine (AECK) decarboxylated dimer (formula in Fig. 1A), hereafter simply named dimer, is the product of the spontaneous dimerization and decarboxylation of the parent ketimine (Hermann, 1961; Pecci et al., 1991). The detection of the dimer in normal human urine (Matarese et al., 1996), in bovine cerebellum (Matarese et al., 1998) and in a fraction of human plasma proteins (work in progress), could assign to this compound the role of an important biological component in the mammalian body. The biological significance of the dimer is so far supported by (i) detection of the enzymatic systems for the production of the parent aminoethylcysteine (AEC) (Costa et al., 1989); (ii) enzymatic oxidative deamination (Cavallini et al., 1982) or transamination (Costa et al., 1986; Ricci et al., 1986) of AEC yielding the ketimine form (AECK); (iii) dimerization and decarboxylation of AECK by a spontaneous process (Pecci et al., 1991). The recent finding of the occurrence of aminoethylcysteine, which represents the necessary starting product, in human urine (Yu et al., 1997) adds

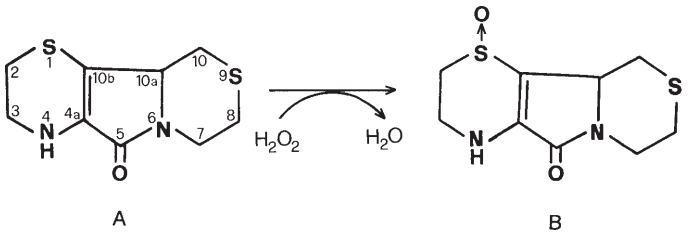

Fig. 1. Structure of the dimer (**A**) and of the dimer sulfoxide (**B**). The numbering scheme follows the recommendation of the Ring Systems Handbook, Chemical Abstract Service, (1993) RF 29858

further support to the conclusion of the dimer as an endogenous biochemical product worthy of further attention. In the search for a possible biochemical role we have found that the dimer modulates the mitochondrial activity (Pecci et al., 1994a), protects these structures from oxyradicals damage (Pecci et al., 1994b), counteracts the lipid peroxidation induced by L-dopa in the presence of iron (Pecci et al., 1995). Further, in a previous paper (Antonucci et al., 1994), we have shown that the dimer interacts with reactive oxygen species (O_2^-, $^\bullet OH$, H_2O_2) producing a number of compounds detectable by chromatographic procedures. The present paper describes the isolation and the identification of the main product of the interaction of the dimer with H_2O_2.

Materials and method

Products

The dimer was prepared as previously described (Pecci et al., 1991; Antonucci et al., 1998). Chemicals were from Fluka, from Sigma, and from Merk.

Analyses

^{1}H NMR and ^{13}C NMR were recorded on a Varian XL-300 spectrometer operating at 300 Mhz and 75.43 Mhz, respectively. Mass spectra were determined with a M-Scan's VG Autospec operating at 70 eV. High performance liquid chromatography (HPLC) analyses were carried out with a Waters chromatograph equipped with two Model 501 pumps, a Model 680 gradient controller, a U6K sample injector, a 996 Photodiode Array Detector and a Millenium chromatography manager. The column was a 4.6 × 250 mm Symmetry C18, 5 micron. The mobile phases were A: 50 mM ammonium acetate; B: Acetonitrile-water (80:20, v/v). The column was preconditioned with solvent A for 15 min before sample loading, followed by a linear gradient from A to 100% B over 30 min. Flow rate 1 ml/min at 20 °C. For preparative HPLC the column was a 7.8 × 300 mm Prep Nova-Pak HR C18, 6 micron. The mobile phases were: A, water; B, Acetonitrile-water (80:20, v/v). Linear gradient from A to 100% B over 30 min. Flow rate: 1 ml/min.

Isolation of the product of oxidation of the dimer by H_2O_2

To 22.8 mg of the dimer (0.1 mmol) dissolved in 0.5 ml of acetonitrile was added 0.4 ml of water and 0.1 ml of a 30% solution H_2O_2 (0.9 mmol). After 3 hours at room temperature the solution was injected into the HPLC preparative column. By monitoring the eluent at

308 nm, the fraction eluting between 22–24 min was collected and dried under vacuum at 40 °C. The oily residues obtained from 10 HPLC runs were pooled and further purified by preparative thin layer chromatography (TLC) on silica gel using chloroform-methanol (95:5, v/v) as eluant. The resulting solid product mp 219°–222°C on analytical TLC appears, under UV lamp, as a single spot with Rf value of 0.37.

Results

The HPLC analysis (Fig. 2) of the product of oxidation of the dimer by H_2O_2 obtained by the procedure reported in Materials and methods shows the presence of two peaks, in a ratio 4:7, eluting very close to each other with retention times (18.6 and 18.8 min) lower than that of the dimer (26 min). These two peaks correspond to those already reported (Antonucci et al., 1994) as the main products of oxidation of dimer detectable by HPLC. The UV spectra taken at the top of the two peaks appear identical with the maximum at 295 nm (Fig. 2, insert) whereas the dimer has absorption maximum at 308 nm (Hermann, 1961). Attempts to find the chromatographic conditions to separate the two peaks by preparative HPLC were unsuccessful, therefore further analyses were performed on this inseparable biased mixture. Submitted to the tests for the oxidation level of sulfur (Cavallini et al., 1959) the isolated product resulted positive to the KI + HCl test (done on filter paper), suggesting the presence of partially oxidized sulfur derivatives. These findings together with the spectroscopic data reported below are in agreement with the structure of the α, β unsaturated sulfoxide of the dimer (Fig. 1B) and

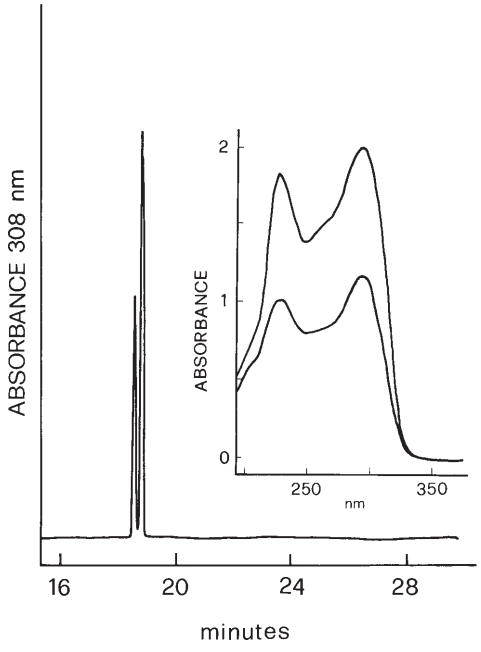

Fig. 2. HPLC elution pattern of the isolated oxidation product of the dimer. Insert: Absorbance spectra registered at the top of the two peaks

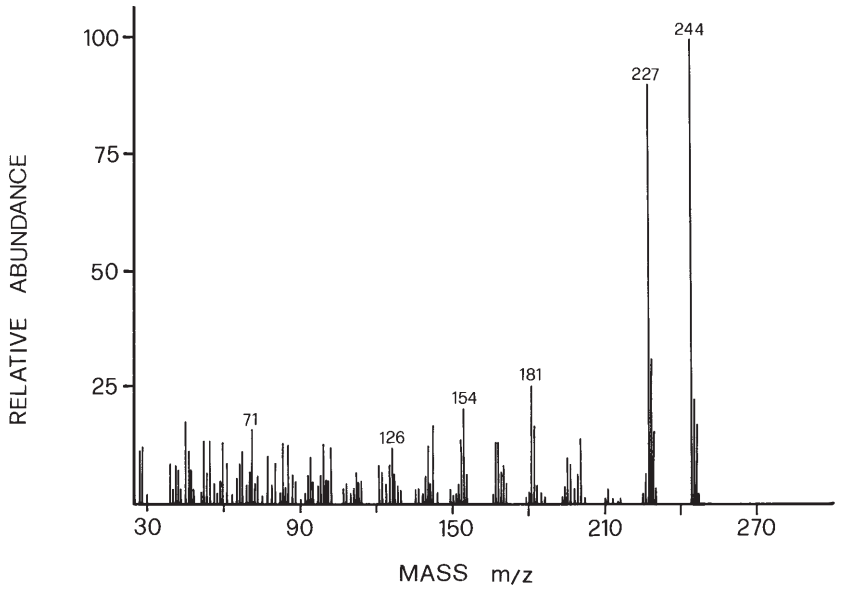

Fig. 3. Mass spectrum of the isolated oxidation product of the dimer. m/z = 244, M^+, the molecular ion; m/z = 227, [M – OH] ion

indicate that the isolated product is a mixture of diasteroisomers at the newly generated sulfur stereogenic center of the sulfoxide functionality.

In particular the mass spectrum (Fig. 3) shows the molecular ion at m/z 244 and an additional abundant peak (r. a. = 90%) corresponding to the [M − OH] ion (m/z 227). Loss of OH from the molecular ion has been reported as a characteristic fragmentation process of aliphatic and alicyclic sulfoxides (Smakman et al., 1970). IR-spectrum ($CHCl_3$) reveals an intense band at 1,040 cm^{-1} consistent with the presence of the SO group. ^{13}C-NMR spectrum in $CDCl_3$ (Fig. 4B), supported by attached proton test experiments (Patt et al., 1982), reveals the presence of two distinct sets of signals, attributable to the two diasteroisomeric forms of the sulfoxide. Another characteristic feature of the ^{13}C-NMR spectrum is represented by the chemical shifts of the six sp^3 carbon atoms: whereas four of them, corresponding to the thiazine moiety of the molecule, appear in the usual range (δ = 28.30, 33.93, 41.80, 58.70 ppm, values relative to the major diasteroisomer) as compared with the parent sulfide (δ = 28.89, 34.14, 41.67, 59.78 ppm) (Fig. 4A), the other two are shifted at lower (δ = 44.67 ppm; $\Delta\delta$ = +18.81) and higher field (δ = 31.47 ppm; $\Delta\delta$ = −9.59) than the corresponding carbon atoms in the parent sulfide. This finding is in accordance with the well known substituent-induced chemical shift effects exerted by the sulfoxide functionality (Dyer et al., 1991) and strongly suggests that S oxidation at S_1 rather than at S_9 took place during the treatment of the dimer with H_2O_2.

Discussion

The data reported above are all consistent with the sulfoxide nature of the isolated oxidation product of the dimer. The ^{13}C-NMR data indicate that S_1

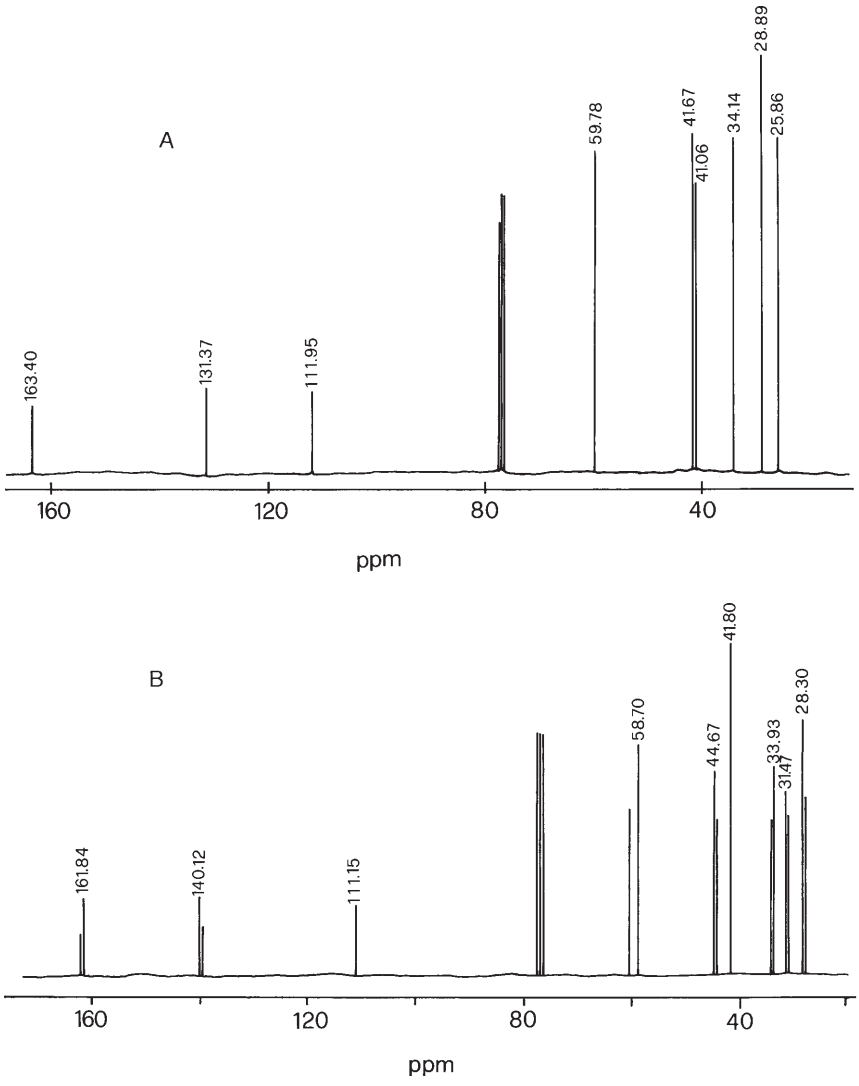

Fig. 4. ^{13}C-NMR spectra of the dimer (**A**) and of the isolated oxidation product of the dimer (**B**). Chemical shifts are given is δ ppm from TMS. In **B** δ values are relative to the major diasteroisomer

and not S_9 has to be taken as the oxidized sulfur atom. The HPLC elution pattern which appears to split in two very close narrow peaks (Fig. 2) and the ^{13}C-NMR spectrum which reveals the presence of two distinct sets of signals (Fig. 4B), indicate that the isolated product is a mixture of the two diasteroisomeric forms of the sulfoxide. These products have been already detected by HPLC in the course of oxidative reactions of the dimer where the two close peaks have been indicated OX1 and OX2 (Antonucci et al., 1994). Formation of oxidation products other than the sulfoxide has been shown by analytical procedures reported in that paper indicating that the sulfoxide is subjected to further extensive oxidation reaching the levels of cysteic acid and taurine. However, the sulfoxide appears to be the first and the longer-lived intermediate. The formation of the dimer sulfoxide as the product of oxygen

reactive species is of interest not only as a further knowledge of the chemical properties of the dimer but also because it could provide a means for investigating the involvement of the dimer in the biological defense against oxyradical damages. The dimer *itself* is excreted in human urine (Matarese et al., 1996), the eventual detection of the oxidized form in the same material and its changes in function of oxyradical exacerbations could provide an analytical support in the practical medicine.

Aknowledgements

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