Amino Acids (2003) 25: 227–232
DOI 10.1007/s00726-003-0013-0

Amino Acids
Printed in Austria

Tyrosine oxidation products: analysis and biological relevance

Review Article

C. Giulivi[1,2], **N. J. Traaseth**[1], and **K. J. A. Davies**[3]

[1] Department of Chemistry and
[2] Department of Biochemistry and Molecular Biology, University of Minnesota, Duluth, Minnesota, U.S.A.
[3] Ethel Percy Andrus Gerontology Center, and Division of Molecular & Computational Biology,
The University of Southern California, Los Angeles, California, U.S.A.

Received February 28, 2003
Accepted May 7, 2003
Published online July 29, 2003;

Summary. Dityrosine is found in several proteins as a product of UV irradiation, γ-irradiation, aging, exposure to oxygen free radicals, nitrogen dioxide, peroxynitrite, and lipid hydroperoxides. Interest of dityrosine in proteins is based on its potential as a specific marker for oxidatively damaged proteins and their selective proteolysis, hence it could be used as a marker for oxidative stress. Dityrosine is also the product of normal post-translational processes affecting specific structural proteins. Since post-translational modification of a given amino acid in a protein is equivalent to the substitution of that residue by an analogue, it has been proposed that the covalent modification of amino acids may serve as a "marking" step for protein degradation.

Keywords: Dityrosine – Oxygen free radicals – Proteolysis – Oxidative stress – Protein damage

Introduction

Exposure of proteins to oxygen and nitrogen free radicals may alter every level of protein structure from primary to quaternary (if multimeric proteins) (Davies, 1987; Davies and Delsignore, 1987; Davies et al., 1987a, b; Giulivi and Davies, 1993, 1994a; Malencik et al., 1996). Histidine, proline, arginine, lysine, methionine, and cysteine residues are particularly sensitive to site-specific metal-catalyzed oxidation. Levine (1983) showed that some amino acid residues are converted to carbonyl derivatives, especially from proline, arginine, lysine, and histidine residues (Levine, 1983; Giulivi and Davies, 1994a). It has been reported that the formation of carbonyl compounds can be used as a measure of oxygen radical-mediated protein damage under various pathophysiological conditions (Stadtman, 1990; Giulivi and Davies, 1994a). Consequently, several sensitive methods for the determination of protein carbonyl groups have been developed (Levine, 1984; Ahn et al., 1987; Levine et al., 1990; Climent et al., 1989; Giulivi and Davies, 1994a). Although carbonyl assays can be used as a measure of protein oxidation, it must be noted that these moieties may also be introduced into proteins by mechanisms that do not involve the oxidation of amino acid residues. For example, α–β unsaturated alkenals produced during lipid peroxidation may react with sulfhydryl groups of proteins to form stable covalent thioether adducts carrying carbonyl groups (Esterbauer and Zollner, 1989; Giulivi and Davies, 1994a). Similarly, Schiff bases obtained by reaction of reducing sugars (e.g., glucose) with the ε-amino group of lysyl residues in proteins may, upon Amadori rearrangement, yield ketoamine protein conjugates (Cerami et al., 1987; Giulivi and Davies, 1994a).

It is noteworthy that aromatic amino acids in proteins are not major sites of oxidation by metal-catalyzed oxidation systems, whereas phenylalanine, tryptophan, and tyrosine are the preferred targets by radicals produced during γ-radiolysis. These differences in target specificity result from the chemical structure of some amino acids with putative binding sites for metals (e.g., ε-amino of lysine). In the presence of oxygen, considerable peptide cleavage occurs, with concomitant formation of carbonyl groups. Under hypoxic (conditions similar to those found in biological systems) or anoxic conditions, hydroxyl radical produced during radiolysis leads to extensive protein–protein crosslinkage via tyrosine–tyrosine bonding (dityrosine),

and possibly other amino acid cross-links as well (e.g., disulfide bridge).

Thus, specific post-translational modifications of amino acids in proteins is necessary to assess cellular or organismal injury under different oxidative stress conditions. This modification should exhibit the following characteristics: *i*) it should increase in proteins exposed to oxidative stress under conditions similar to those found in biological systems (10–50 μM oxygen, pH, negligible concentration of free metals, among others), *ii*) it should be a final, stable product preferably derived from aromatic residues because of their low abundance compared to the non-aromatic ones, *iii*) it should be detectable by simple, widely available lab procedures, and possibly *iv*) it should be able to serve as a specific marker for protein degradation. Since post-translational modification of a given amino acid in a protein is equivalent to the substitution of that residue by an analogue, it has been proposed that the covalent modification of amino acids may serve as a ''marking'' step for protein degradation. The only protein modification obtained under various conditions of oxidative stress and consistent with these points, was the formation of dityrosine. We, therefore, proposed an alternative method for assessing protein oxidative damage by measuring dityrosine formation and release (Giulivi and Davies, 1993).

Dityrosine as a biomarker for oxidative stress

What is dityrosine?

Dityrosine was first recognized over 40 years ago (Gross and Sizer, 1959). Dityrosine is the product of normal post-translational processes affecting specific structural proteins. It is an unusual amino acid that is distinguished by the intense 420-nm fluorescence, measurable upon excitation within either 315 nm (alkaline solutions) or 284 nm (acidic solutions) absorption bands (Giulivi and Davies, 1994a).

Renewed interest in its occurrence in proteins is based on its potential as a specific marker for oxidatively damaged proteins and their selective proteolysis (Giulivi and Davies, 1994a; Giulivi and Davies, 2001). Indeed, dityrosine can be found in several proteins as a product of UV irradiation (Lehrer and Fasman, 1967; Giulivi and Davies, 1994a), γ-irradiation (Boguta and Dancewicz, 1981; Giulivi and Davies, 1994a), aging (García-Castiñeiras et al., 1978; Giulivi and Davies, 1994a), exposure to oxygen free radicals (Davies, 1987; Tew and Ortiz de Montellano, 1988; Giulivi and Davies, 1993, 1994a, b), nitrogen dioxide, peroxynitrite, and lipid hydroperoxides (Giulivi and Davies, 1994a). Enzymatic formation of dityrosine has been found to promote protein crosslinking after incubation of proteins with peroxidases (Wagley et al., 1950; Sizer, 1953; Aeschbach et al., 1976; Foerder and Shapiro, 1977; Giulivi and Davies, 1994a).

Mechanism of dityrosine formation in oxidatively modified proteins

The mechanism of dityrosine formation begins with the generation of a tyrosyl radical, radical isomerization followed by diradical reaction, and finally enolization (Fig. 1; Giulivi and Davies, 1994a). The overall rate constant for this process was reported to be $4 \times 10^8\,M^{-1}\,s^{-1}$ (Giulivi and Davies, 1994a).

Thus, the initial step in the formation of dityrosine involves the formation of tyrosine radical. The reduction potential of the tyrosine$O^{\cdot}$/tyrosineOH couple is 0.88 V (Giulivi and Cadenas, 1998) indicating that the coupled reaction needs to be highly oxidative to be thermodynamically favorable. In this regard, hydroxyl radical, oxoferryl moiety, porphyrin π cation radical, peroxynitrite, nitrogen dioxide, and nitryl cation, are some of the species that fulfill this requirement (Davies et al., 1987; Giulivi and Davies, 1994a). Once the tyrosyl radical is formed, the production of dityrosine involves two monomeric molecules of tyrosine-containing proteins, joined by intermolecular cross-linking, thus resulting in the formation of protein dimers detectable by SDS-PAGE under reducing conditions (Giulivi and Davies, 2001). Although the mechanisms for tyrosine radical formation in myo- and hemo-globins seem to be similar on a molecular basis, dityrosine cross-linking in myoglobins only occurs in protein derived from sperm whale (Tew and Ortiz de Montellano, 1992; Giulivi and Cadenas, 1998). This myoglobin has an essential tyrosine-151 which elicits the dimerization of myoglobin (Tew and Ortiz de Montellano, 1989; Giulivi and Davies, 2001) indicating a steric or conformational requirement for its occurrence. The release of dityrosine from hemoglobins seems to be independent of the hemoprotein source (bovine, human or rabbit) probably owed to the presence of conserved tyrosines in the primary structure. Several reports have shown the formation of dityrosine in different proteins exposed to a variety of oxidants (UV light, γ-irradiation, oxygen radicals), supporting a wider use of dityrosine release as a marker for oxidative stress (Giulivi and Davies, 2001).

Dityrosine has also been proposed as a marker of organismal oxidative stress (Heinecke, 2002). In this regard, dityrosine concentrations were found 100-fold higher in

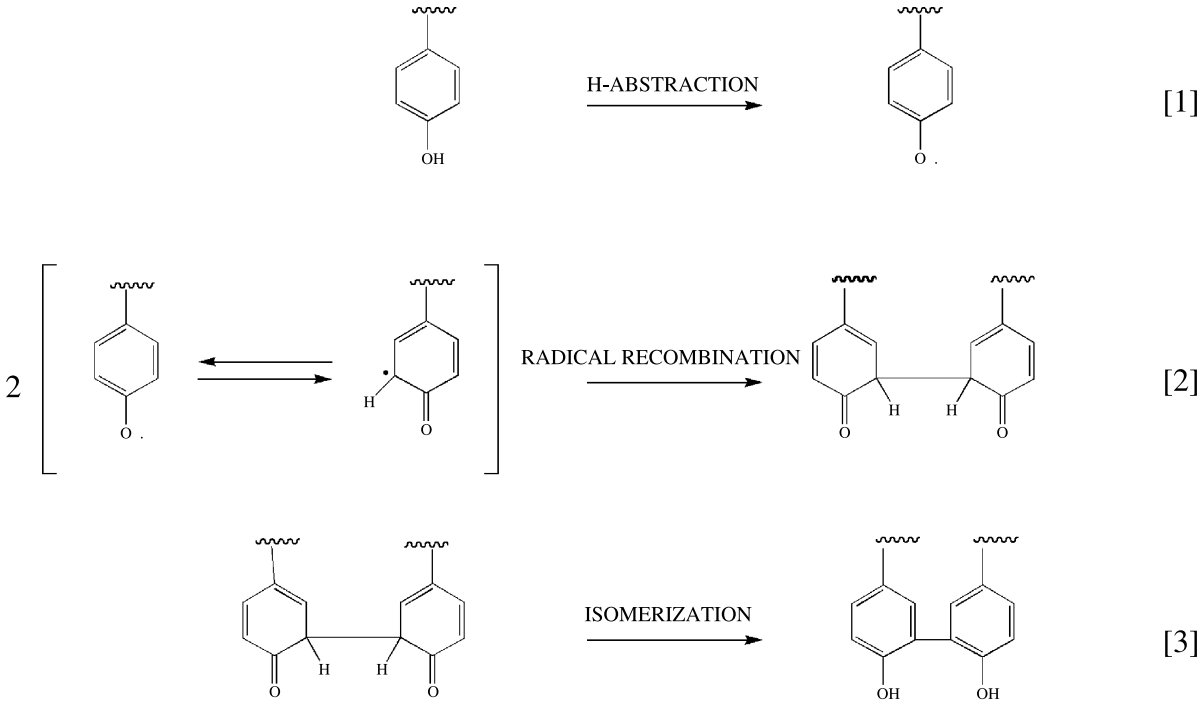

Fig. 1. Mechanism of dityrosine formation

low-density lipoproteins isolated from atherosclerotic lesions than in normal ones. Also analysis showed that humans suffering from systemic bacterial infections had twice the concentration of dityrosine in urine than those of healthy individuals (Heinecke, 2002).

Characterization of tyrosine oxidation products in oxidatively modified proteins

The tyrosyl radical may dissipate by pathways other than those involving intermolecular diradical crosslinking of tyrosine. Indeed, covalent binding of tyrosine to the heme group in myoglobin or addition of oxygen have been proposed as alternative pathways (Tew and Ortiz de Montellano, 1992).

The formation of tyrosine oxidation products (not dityrosine) might involve cyclization, decarboxylation, and further oxidation steps on either the protein or fragments released from the protein (Fig. 2; Giulivi and Davies, 2001). In our experience, high performance liquid chromatography (HPLC) analysis with fluorescent detection of both pronase-treated fragments-released from H_2O_2-exposed hemoglobin- and of pronase-treated hemoglobin-exposed to H_2O_2 and freed of fragments-pointed to the occurrence of products derived from oxidatively modified tyrosine (Giulivi and Davies, 1994a). Co-elution of the products in the pronase-treated fragments with synthetic standards, indicated that dopamine, dopamine quinone, and 5,6-dihydroxyindol were the major products of tyrosine oxidation present in this fraction, whereas DOPA, and 5,6-dihydroxy-3-oxo-indol, were minor products (Giulivi and Davies, 1993). These compounds were further characterized by using different chromatographic conditions suitable for dopachrome-related products, with electrochemical detection (Leonard et al., 1988; Segura-Aguilar and Lind, 1989; Giulivi and Davies, 2001).

Specificity of dityrosine as a product of free radical modification

Tyrosine and dityrosine can be analyzed by HPLC with diode array detection. A typical chromatogram is shown in Fig. 3. Tyrosine and dityrosine elute at 4.8 and 5.6 minutes, respectively. For measuring dityrosine release from proteins pre-exposed to oxygen radicals, a proteolytic or acid digestion of the oxidatively modified protein is followed by HPLC analysis with fluorescence or diode array detection (Giulivi and Davies, 1993). Dityrosine, along with other tyrosine oxidation products were identified by HPLC in pronase digested H_2O_2-treated oxyhemoglobin (free from fragments). Importantly, no dityrosine was detectable in pronase digests of either control hemoglobin

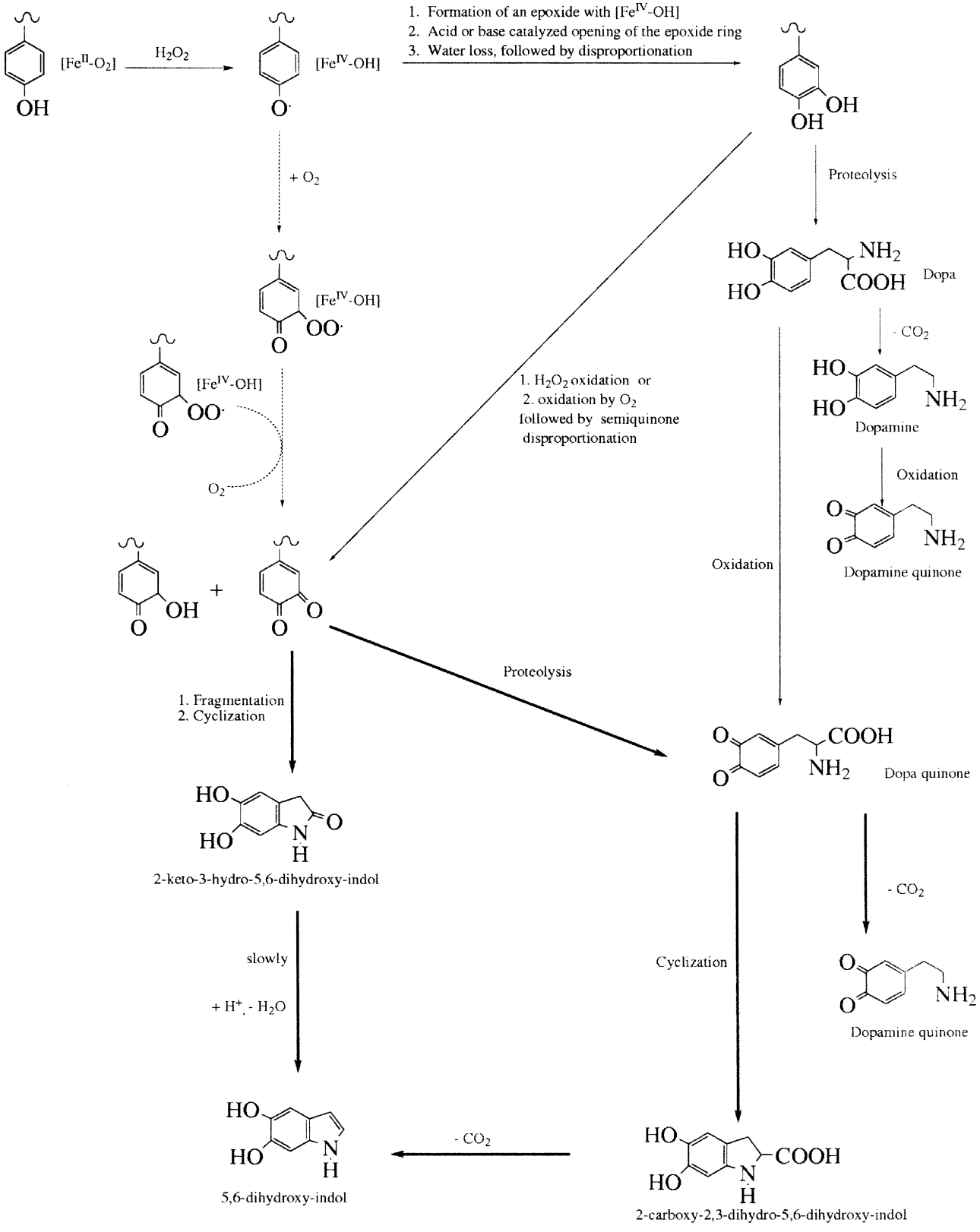

Fig. 2. Proposed pathway for oxidation products of tyrosine

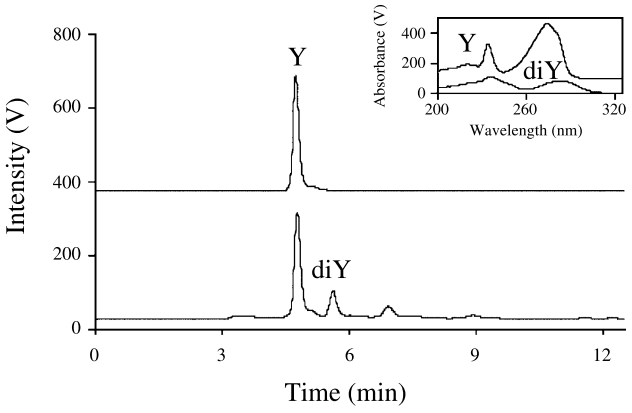

Fig. 3. Reversed-phase HPLC analysis of tyrosine and dityrosine. Dityrosine and tyrosine were analyzed by HPLC using a C18 column (4.60×250 mm). The mobile phase consisted of 4% methanol, 50 mM sodium citrate, 50 mM acetic acid (pH 3.1) set at 1 ml/min. Dityrosine was synthesized as described before (Giulivi and Davies, 1994a). Inset; Absorption spectra of tyrosine and dityrosine. Under these experimental conditions, the λ_{max} for tyrosine are 234 and 274 nm, whereas those for dityrosine are 236 and 284 nm

(not exposed to oxygen radicals) or protein fragments, whereas dityrosine was clearly present in pronase digests of H_2O_2-exposed hemoglobin, free from fragments (Giulivi and Davies, 1993). These results were then extended to other experimental conditions, thus allowing us to conclude that the formation of dityrosine in a protein exposed to oxygen free radicals can serve as a marker of oxidatively modified proteins.

Incubation of oxidatively modified proteins with proteolytic enzymes resulted in increased proteolysis in which the modified proteins underwent selective digestion (Levine, 1983; Dean and Pollak, 1985; Rivett, 1985; Stadtman and Wittenberger, 1985; Davies, 1987; Davies and Delsignore, 1987; Davies et al., 1987a, b; Taylor and Davies, 1987; Davies and Lin, 1988a, b; Marcillat et al., 1988; Giulivi and Davies, 1993, 1994a). Using the technique described before, i.e., HPLC with fluorescence or diode array detection, we measured dityrosine release, (after proteolysis) from oxyhemoglobin or red blood cells that had been exposed to H_2O_2 (Giulivi and Davies, 1993, 1994a, b). This release was detected in H_2O_2-treated oxyhemoglobin (but not in control hemoglobin), increased by proteases, and inhibited by protease inhibitors. Thus, dityrosine release can be considered not only as a marker for protein oxidative damage but also as an endogenous marker for the selective degradation of oxidatively modified proteins (Giulivi and Davies, 1994a).

Conclusions

Several oxidation products of tyrosine (obtained after exposing proteins or intact red blood cells to a flux of H_2O_2; Giulivi and Davies, 2001) are released by fragmentation or proteolysis after complicated reactions involving cyclization, H-abstraction, oxidation and interaction with proximal amino acids (Giulivi and Davies, 2001). However, dityrosine should be considered a specific marker for selective proteolysis (although a minor product in quantitative terms) because its release occurs only after an oxidative stress (exposure to H_2O_2) and subsequent proteolysis. In addition, it can be considered a marker for organisms exposed to oxidative stress, like atherosclerosis, acute inflammation, systemic bacterial infections (Heinecke, 2002), and lens cataracts (García-Castiñeiras et al., 1978).

Dityrosine release can be measured by HPLC with (Malencik and Anderson, 1987; Giulivi and Davies, 2001) or without (Malencik et al., 1996; Giulivi and Davies, 2001) derivatization, or by high efficiency thin layer chromatography, taking into account that the latter technique is at least 100-times less sensitive than the other two (Giulivi and Davies, 2001). Moreover, dityrosine has the advantage over the other oxidation products (which are sensitive to oxygen and to high pH) of being a stable compound because once the 3′–3′ carbon–carbon bond is formed it is resistant to hydrolysis by other lytic enzymes (Giulivi and Davies, 1993, 2001). Dityrosine can also be measured quantitatively and definitively by stable isotope dilution gas chromatography-mass spectrometry demonstrated by Heinecke and co-workers (Leeuwenburgh et al., 1997; Heinecke, 1999). Unfortunately, the expense and expertise required for stable isotope dilution gas chromatography-mass spectrometry analysis (Leeuwenburgh et al., 1997; Heinecke, 1999) may put this technique beyond the reach of some investigators.

Finally, identification and quantification of dityrosine is and will continue to be an invaluable tool at evaluating the role oxidative stress has on proteins, cells, and entire organisms.

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Authors' address: Dr. Cecilia Giulivi, Department of Chemistry, University of Minnesota, 10 University Dr., Duluth, MN 55812, U.S.A., Fax: 218-726-7590, E-mail: cgiulivi@d.umn.edu