

Ability Of Therapeutic Ozonated Water To Oxidatively Consume Orally-Active Biomolecules

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Introduction

Currently, root caries represents a challenging problem to the dental profession in view of a substantial increase in the population of elderly patients during the late 20th century. This condition is primarily ascribable to tooth demineralization processes induced by organic acids (e.g., lactic and pyruvic acids) generated by bacteria, predominantly *Streptococcus mutans* (Beighton et al. 1993, Silwood et al 2002, Lynch 2003), and recent investigations conducted by Baysan et al. (2000, 2001, 2004) have revealed that ozone (O_3) exerts a powerful bactericidal action towards this and other pathogens, together with further micro-organisms associated with primary root carious lesions. Indeed, the application of ozone in dental practices may serve as a viable, cost-effective and convenient means of treating dental caries, and it is conceivable that it could eventually replace conventional 'drilling-and-filling' procedures currently employed by dental surgeons (Holmes 2003).

In view of its powerful oxidizing actions, O_3 has a very rich chemistry and the oxidation of critical biomolecules undoubtedly accounts for its broad-spectrum biocidal properties. Indeed, ozone can attack a very wide variety of biomolecules, for example, free or protein-incorporated amino acids such as cysteine, methionine, histidine and tyrosine, carbohydrates, phenolic adducts and, of course, its well-characterized ozonylation of carbon-carbon double bonds, e.g., those of polyunsaturated fatty acids (PUFAs). Oxidation of PUFAs by ozone gives rise to the production of conjugated hydroperoxydiene and subsequently aldehydic species, the latter apparently serving as biomarkers of ozonylation. Interestingly, reaction of water-soluble, single electron-donors with ozone primarily generates the ozone radical anion, a transient adduct which, on protonation, decomposes to hydroxyl radical and dioxygen. Hence, some of the reaction products which putatively arise from the interactions of ozone with molecules present in tissues and biofluids are, at least in principle, identical to those produced from the attack of hydroxyl radical on such ROS scavengers.

In view of the clear indications for the therapeutic application of ozone in the treatment of dental diseases, a series of clinical trials involving this agent have recently been completed. Therefore, in this investigation we have employed high resolution proton (1H) nuclear magnetic resonance (NMR) spectroscopy to achieve a multicomponent evaluation of the oxidizing actions of a product containing ozonated water towards biomolecules present in intact human saliva. The therapeutic, aesthetic and biochemical significance of the results acquired are discussed in detail.

Objectives

In this study we have employed high resolution proton (1H) nuclear magnetic resonance (NMR) spectroscopy to determine the nature and extent of the oxidation of biomolecules known to be present in carious dentine, plaque and saliva before and after treatment by O_3 present in a novel ozonated water formulation (TherOzone, CA, USA).

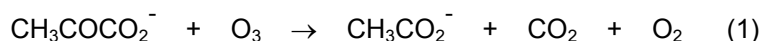
Materials and Methods

5.00 ml aliquots of aqueous solutions containing sodium pyruvate, alpha-D-glucose, L-cysteine and L-methionine (5.00 mM) and urate (0.50 mM) were prepared in 40.0 mM phosphate buffer (pH 7.00) which was rigorously deoxygenated with O_2 -free N_2 gas prior to use. 5.00 ml aliquots of these solutions were treated with 20.0 ml volumes ozonated water [TherOzone unit, CA, USA] for a period of 5 min. These experiments were conducted in triplicate. Matching de-oxygenated solutions of these biomolecules untreated with ozonated water served as controls. One-dimensional (1-D) 600 MHz proton NMR spectra of the simple chemical model systems described above were acquired on a Bruker AMX-600 spectrometer. Typical pulsing conditions were

64 FIDs using 32,768 data points, 720 pulses and a 3 s pulse repetition rate to allow full spin-lattice relaxation of the hydrogen nuclei in the samples investigated. Exponential line-broadening functions of 0.30 Hz were routinely employed for purposes of processing.

Results

5.00 mm aqueous solutions of pyruvate were treated with O₃ as described in the Materials and Methods section in order to investigate the reaction occurring between these redox-active species, and proton (1H) NMR analysis of these solutions demonstrated a marked level of oxidative decarboxylation of this alpha-keto acid anion to acetate and carbon dioxide, an observation consistent with the reaction depicted in equation 1. Indeed, these results are not unexpected, although its effective concentration in the system employed here is limited by its solubility in water, together with its rate and level of consumption by the scavenger employed, and its catalytically-promoted dissociation to dioxygen during the 5 minute treatment period. Consistent with this observation, a singlet resonance at 1.50 ppm ascribable to pyruvate hydrate (the enol form of this alpha-keto acid anion), of much lower intensity than that of the keto form at 2.388 ppm, was also removed from spectra after ozone treatment.



As expected, treatment of a 0.50 mm solution of urate with ozonated water generated allantoin as a major oxidation product (Figure 1). As expected, treatment of aqueous solutions of L-methionine with ozone confirmed oxidation to its corresponding sulphoxide under our experimental conditions (i.e., the generation of a singlet resonance at 2.725 ppm attributable to the side-chain terminal methyl groups of the sulphoxide, accompanied by marked decreases in the methionine thiomethyl group signal at 2.13 ppm) (Figure 3).

L-cysteine was also chosen for these chemical model system experiments since, (1) like methionine, it serves as a precursor to malodorous VSCs and (2) our inability to detect this thiol in human saliva by NMR analysis in view of its low concentration in the 'free' (non-protein-incorporated) state and also its complex ABX coupling pattern (i.e., no clearly-visible sharp resonances of low multiplicity). Proton NMR analysis demonstrated that exposure of aqueous solutions of L-cysteine to ozone (section 2) generated its corresponding disulphide, cystine, as a major product (data not shown). Indeed, a reference spectrum acquired on an authentic sample of L-cystine confirmed its identity [clear multiplets located at 3.20 and 3.41 ppm (AB protons) and 4.14 ppm (X proton)] (Figures 2 and 3).

Proton NMR analysis also showed that treatment of phosphate-buffered aqueous solutions of D-glucose with ozonated water in the manner described in section 2 generated formate as a major reaction product (singlet resonance at 8.46 ppm), i.e., a concentration of 1.21 mM was produced from the 4.00 mM glucose substrate, an observation consistent with previous studies conducted on the interactions of ROS (e.g., radiolytically-generated OH radical) with carbohydrates in general.

Discussion

High-resolution, high-field 1H NMR spectroscopy is a technique of much value concerning multicomponent assessments of the interactions of O₃ with human salivary biomolecules, and the oxidative decarboxylation of salivary pyruvate by O₃ serves as an important example of this which may be of some relevance to its cariostatic properties (Silwood et. al., 1999, 2002). Indeed, pyruvic acid is a very powerful proton donor (K_a = 3.20 mM) being much stronger in this capacity than lactic acid (K_a = 0.14 mM), and hence may play an important role in promoting tooth demineralization processes. Therefore, the removal of salivary pyruvate by O₃ may help suppress the development and progression of primary root carious lesions since organic acids detectable in human saliva readily diffuse into caries (in their unionized form). In view of these considerations, ozonated water may offer caries-preventative actions and experiments to investigate this further are currently in progress.

Consumption of salivary methionine and cysteine by O₃ is of great importance to oral hygiene, halitosis and periodontology since both methyl mercaptan and hydrogen sulphide are generated from these amino acids via

metabolic pathways operational in gram-negative micro-organisms. Hence, our data indicate that ozonated water has the capacity to clinically alleviate oral malodor via the direct oxidative inactivation of VSCs and their amino acid precursors.

Conclusion

In conclusion, high resolution ^1H NMR spectroscopy provides much valuable molecular information with regard to determinations of the nature and extent of salivary biomolecule consumption by ozonated water. Such information is clearly of much relevance the potential therapeutic, aesthetic and (indirectly) microbicidal actions of this product in the oral environment.

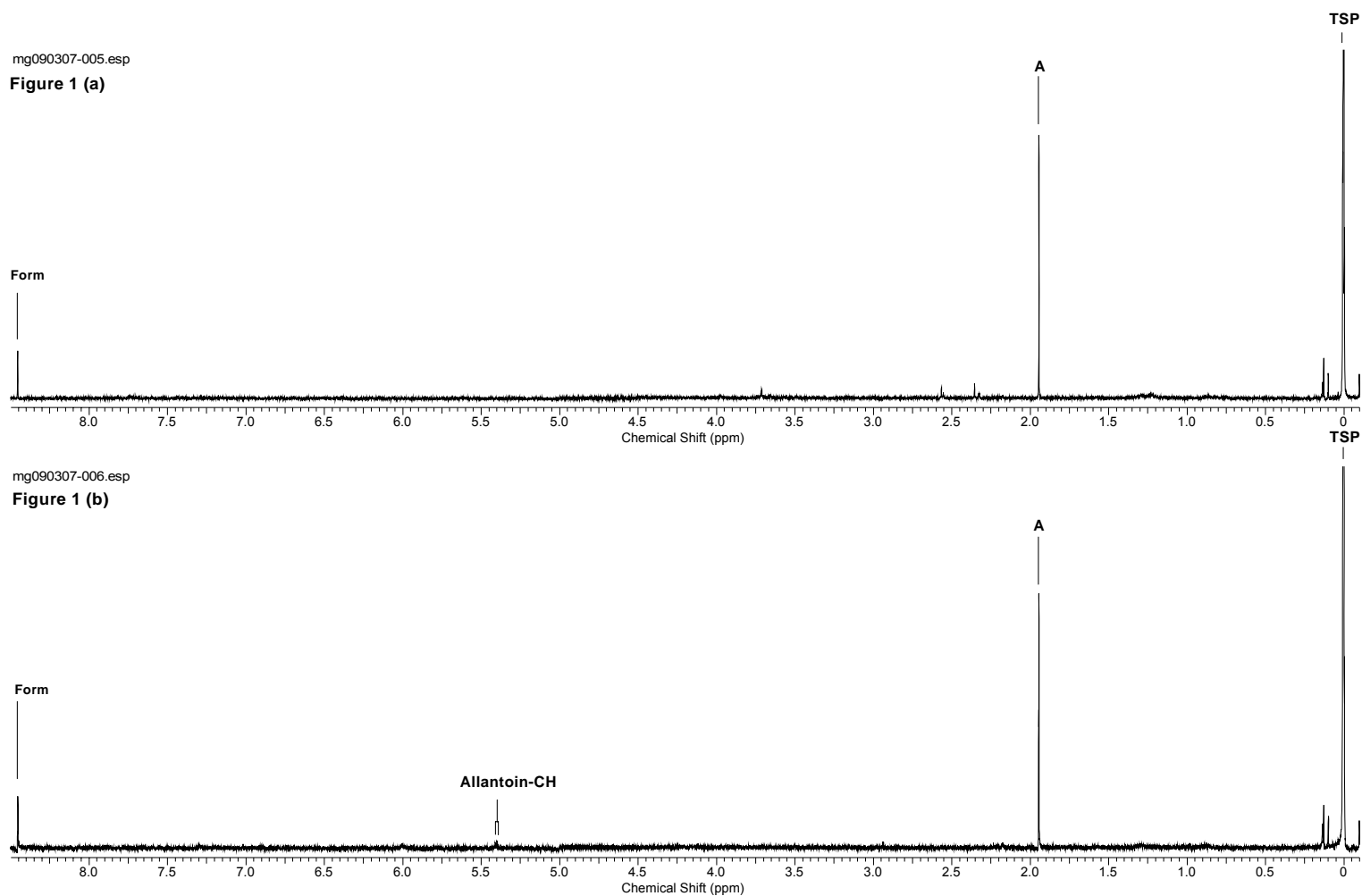


Figure 1. (a) Expanded -0.10 to 8.50 ppm region of the ^1H NMR spectrum of a 0.50 mM solution of uric acid. (b) Expanded region of the same sample treated with O_3 . Abbreviations: A. Acetate $-\text{CH}_3$; Form, formate-H; Allantoin CH. Typical spectra are shown.

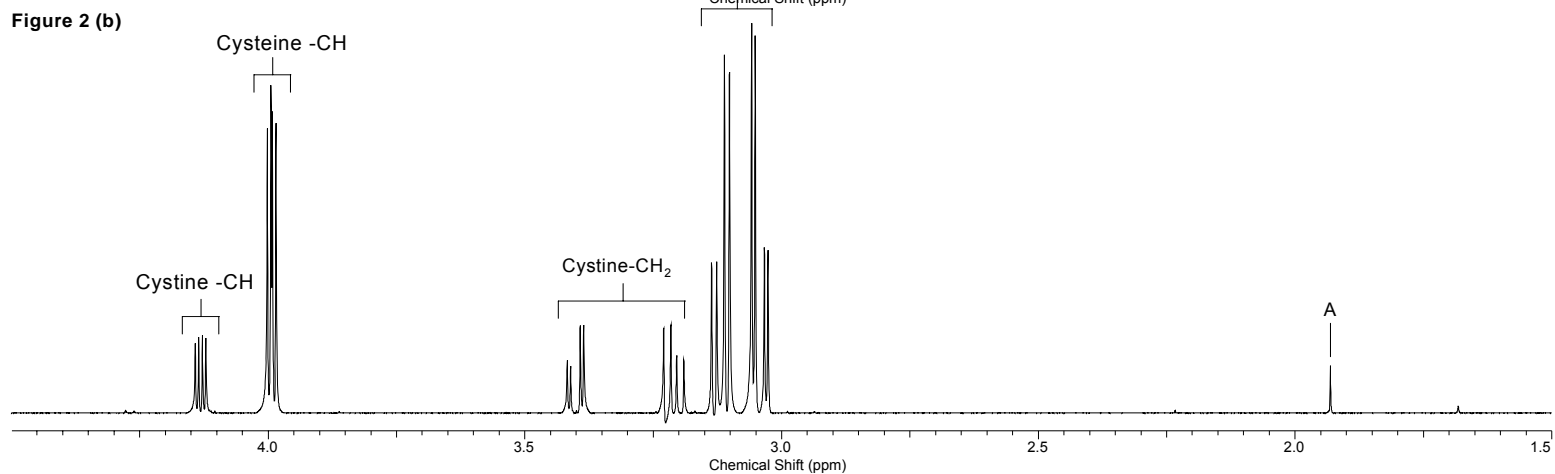
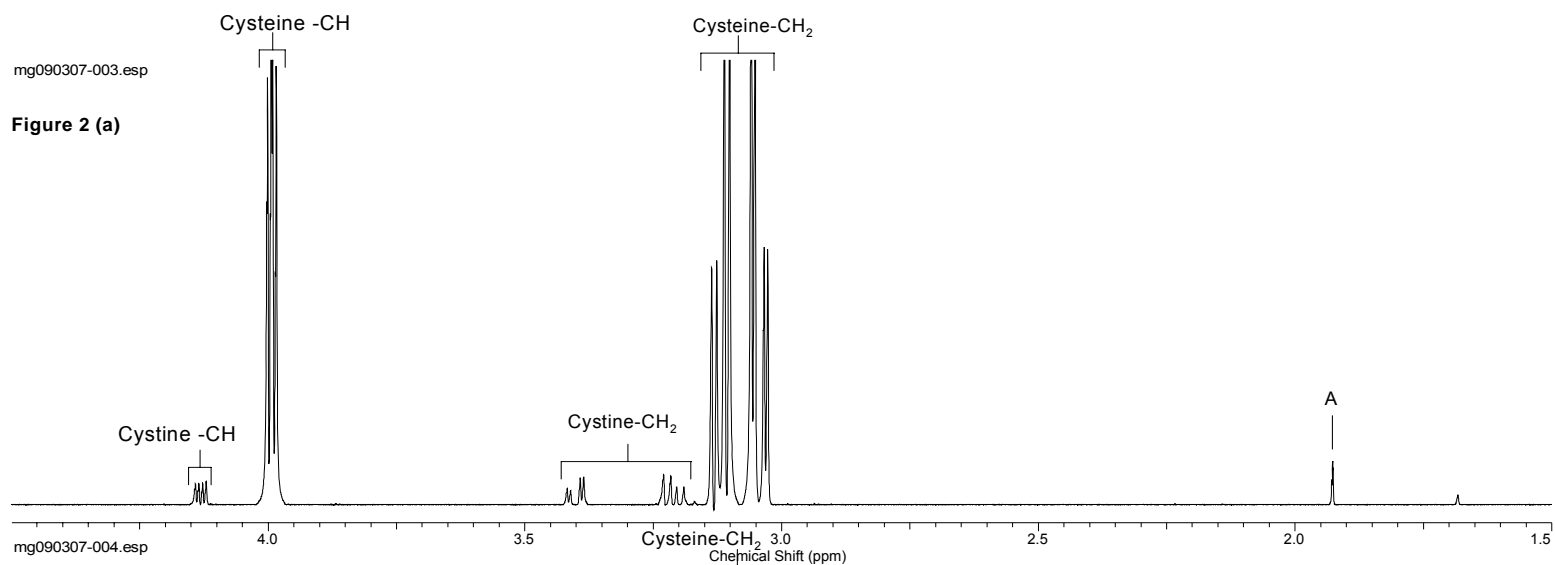


Figure 2. (a) Expanded 1.50 to 4.50 ppm region of the ^1H NMR spectrum of a 5.0 mm solution of cysteine. (b) Expanded region of the same sample treated with O_3 . The presence of minor cystine- CH_2 resonances in the control spectrum indicates a limited level of O_2 -mediated oxidation of cysteine- CH_2 occurring during the preparation of test solutions. Abbreviations: A. Acetate $-\text{CH}_3$; Form, formate-H. Typical spectra shown.

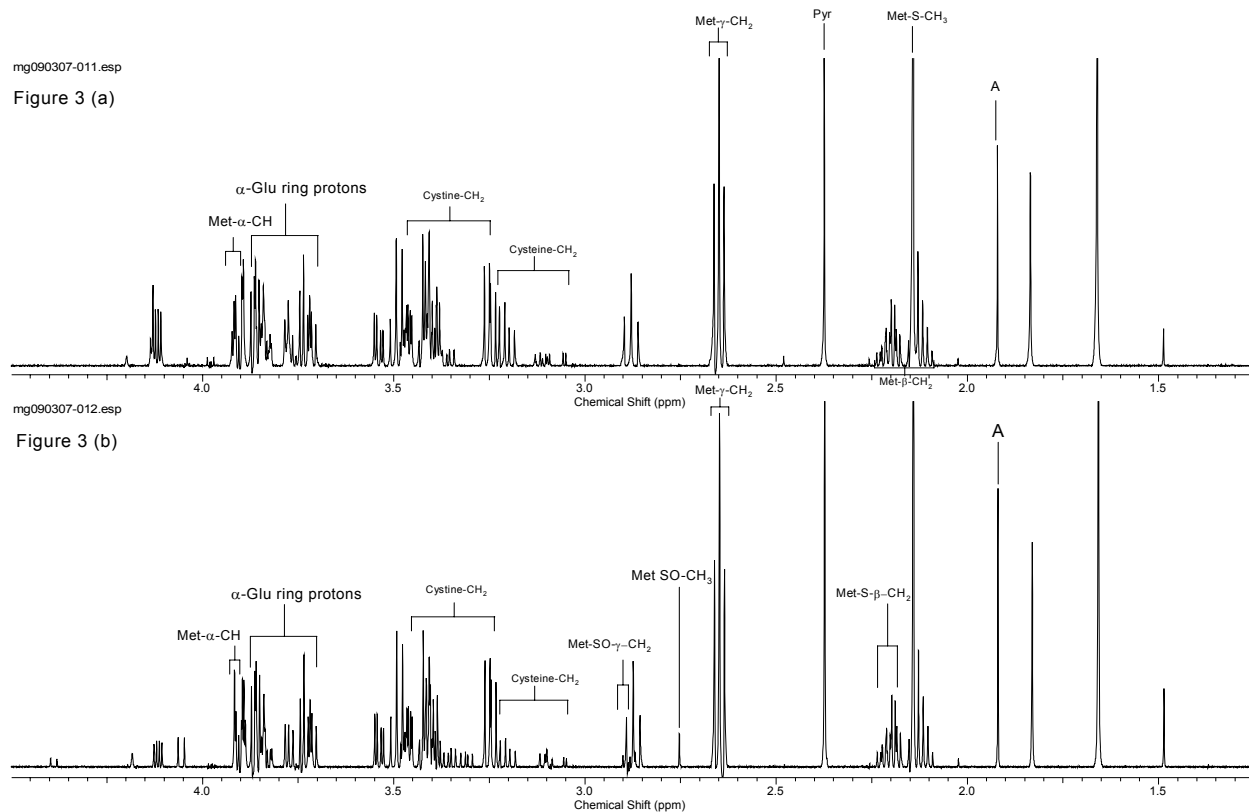


Figure 3. (a) Expanded 1.00 to 4.50 ppm region of the ^1H NMR spectrum of a phosphate-buffered aqueous solution containing 5.0 mM cysteine, methionine, glucose and pyruvate. The presence of minor cystine- CH_2 resonances in the control spectrum indicates a limited level of O_2 -mediated oxidation of cysteine- CH_2 occurring during the preparation of test solutions. A typical spectrum is shown. (b) Expanded region of the same sample treated with O_3 . Abbreviations: A. Acetate - CH_3 ; Form, formate-H; α -Glu ring protons, α -glucose ring protons; Met-S- CH_3 , - α -CH, α - CH_2 and α - CH_2 , methionine-S- CH_3 , - α -CH, α - CH_2 and α - CH_2 group protons resonances respectively; Met-SO- CH_3 , methionine sulphoxide-SO- CH_3 ; Met-SO- α - CH_2 , methionine sulphoxide- α - CH_2 group proton resonance. Typical spectra are shown.

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