Characterization of a Triazine (2-[(3-Amino-1,2,4-triazin-5-yl)methylene]-hydrazinecarboximideamide) Formed from Glucose and Aminoguanidine under Oxidative Conditions

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Summary: A novel reaction product, 2-[(3-amino-1,2,4-triazin-5-yl)methylene]hydrazinecarboximideamide, has been identified as a product of reaction between glucose and aminoguanidine under aerobic conditions.

Glycation (nonenzymatic glycosylation) of proteins is the first step in a complex series of reactions between reducing sugars and amino groups on protein, known collectively as the Maillard or browning reaction. This reaction takes place in vivo, leading ultimately to the development of brown, fluorescent, crosslinked, and insoluble tissue proteins.[1] The initial Schiff base adduct between glucose and protein rearranges to form a more stable Amadori product (AP), which can undergo a variety of reactions to yield poorly characterized advanced glycation end products, or AGEs.[2] In long-lived tissue proteins, these AGEs accumulate as a function of time, and at an increased rate in diabetes, as a result of elevated blood glucose levels.[3] Because the accumulation of AGE products is thought to contribute to the development of pathophysiologies associated with aging and diabetes,[2] there is considerable interest in the design and evaluation of pharmacological agents to inhibit AGE formation. Among these, aminoguanidine (AG) has been reported to be a highly effective inhibitor of the formation of fluorescent products and crosslinks in arterial collagen via the Maillard reaction both in vitro and in vivo.[4] AG is thought to act by trapping reactive dicarbonyl intermediates formed either by the direct oxidation of glucose or from the rearrangement of the AP. We have investigated the effects of AG on the browning and crosslinking of protein by glucose under physiological conditions (pH 7.4, 37 °C) and report here the isolation and characterization of a novel compound, 2-[(3-amino-1,2,4-triazin-5-yl)methylene]hydrazinecarboximideamide, or aminoguanidine triazine (AGT) (Figure 1), arising from the reaction of glucose with AG under oxidative conditions.

When rat tail collagen was incubated with glucose (250 mM) and AG (25 mM) in 200 mM phosphate buffer, the formation of characteristic Maillard reaction products (such as carboxymethyllysine and pentosidine) and collagen-linked fluorescence was inhibited[2] and the supernatant developed a yellow color in a time-dependent fashion. Color development was dependent on the glucose concentration, phosphate concentration (or more specifically, the trace concentrations of metal ions in the buffer), and required oxygen, but did not require the presence of protein. On the basis of these observations, a 10-L reaction was set up without protein in order to isolate a quantity of the colored product(s) sufficient for characterization. After 43 days, a 4-L aliquot of this reaction was removed. The λ_{max} of the reaction mixture was 322 nm, with an absorbance of 5.0.

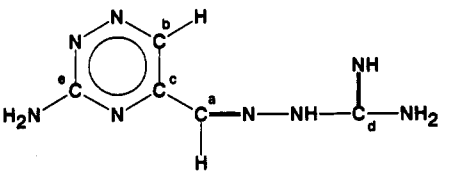

Figure 1. Structure of AGT.

AGT (60 mg yield; 0.7% based on AG) was isolated by sequential preparative reversed-phase and cation-exchange chromatography, followed by desalting on a C-18 reversed-phase column. The resulting product was characterized by ^{1}H and ^{13}C NMR, and high-resolution FAB and ionspray mass spectrometry, and found to have the structure shown in Figure 1. AGT had a UV absorbance maximum at 286 nm. ^{1}H NMR in D_2O showed only two resonances, both singlets of equal abundance, at δ = 8.0 and 9.0 ppm, corresponding to the nonexchangeable protons at C-6 of the triazine ring and the methylene carbon, respectively. The assignment at C-6 is based on comparison with the ^{1}H NMR of the model compound 3-amino-1,2,4-triazine, which gave two resonances, both doublets, at δ = 8.5 and 8.3 ppm, assigned to the protons at the 5 and 6 positions of the triazine ring, respectively. Assignment of the methylene proton in AGT at δ = 9.0 ppm agrees well with the assignment in a series of guanylhydrazines prepared by Desideri et al. (δ = 8.7 ppm).[5] Proton-decoupled ^{13}C NMR of AGT in D_2O showed five resonances of equal intensity, at δ = 136, 142, 155.8, 156, and 161.8 ppm, assigned to carbons a, b, c, d, and e, respectively, as shown in Figure 1. The protonation of the carbons was confirmed by INEPT analysis, revealing that carbons a and b were singly protonated. The ^{13}C NMR spectrum of 3-amino-1,2,4-triazine yielded three resonances, at δ = 141.0, 153.3, and 163.6 ppm, corresponding to carbons 6, 5, and 3 of the triazine ring,[6] confirming our assignments for carbons b, c, and e in AGT. ^{13}C NMR of AG showed a single resonance at 160 ppm, confirming the assignment of the guanadinyl carbon in AGT. ^{1}H-coupled ^{13}C NMR of AGT revealed J_4 coupling between the carbon at position 3 and both the proton at position 6 of the triazine ring and the methylene

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proton attached at position 5, confirming substitution at the 5 position of the triazine ring.

High-resolution FAB-MS, in the positive ion mode, gave rise to a single ion of m/z = 181.0955 amu. The predicted mass for the formula $C_5H_9N_8$, corresponding to the protonated form of AGT, is 181.0950 amu, in excellent agreement with the experimentally derived value (2.7 ppm error). Subsequent analysis by ionspray MS to obtain fragmentation data gave ions of m/z = 164 (26%, loss of amine), 137 (62%, loss of guanadinyl group), 122 (base peak, loss of urea), and 95 (24%, loss of methylenehydrazinecarboximideamide), further supporting the structure of AGT.

While we have reported the formation of triazines from dicarbonyl precursors[7] and have proposed triazine formation to be the mechanism by which AG exerts its inhibitory effect,[8] to our knowledge this is the first report of the formation of a triazine by reaction of glucose with AG. The same compound, AGT, is formed during AG inhibition of the browning of collagen by glucose. Further work is in progress to determine the actual pathway leading to formation of AGT.

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Supplementary Material Available: Experimental procedures and spectra (8 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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