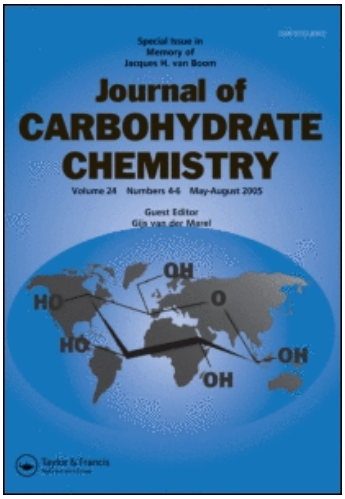

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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Hauh-Jyun[a]; Candy Chen[a]; Anthony Cerami[a]
[a] Laboratory of Medical Biochemistry, The Rockefeller University, New York

To cite this Article Hauh-Jyun, Chen, Candy and Cerami, Anthony(1993) 'Mechanism of Inhibition of Advanced Glycosylation by Aminoguanidine in Vitro', Journal of Carbohydrate Chemistry, 12: 6, 731 — 742
To link to this Article: DOI: 10.1080/07328309308019003
URL: http://dx.doi.org/10.1080/07328309308019003

J. CARBOHYDRATE CHEMISTRY, 12(6), 731-742 (1993)

MECHANISM OF INHIBITION OF ADVANCED GLYCOSYLATION BY AMINOGUANIDINE IN VITRO

Hauh-Jyun Candy Chen*[1] and Anthony Cerami[2]

Laboratory of Medical Biochemistry
The Rockefeller University
1230 York Avenue
New York, New York 10021

Received December 30, 1991 - Final Form February 17, 1993

ABSTRACT

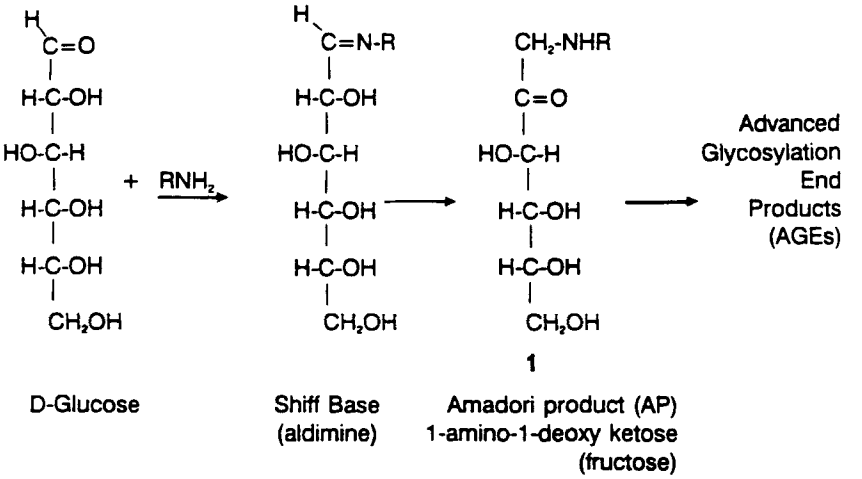
A triazine and a bishydrazone were isolated from a solution of aminoguanidine incubated with an Amadori product (1-deoxy-1-propylamino-D-fructose) under physiological conditions. The new products were characterized by NMR and mass spectrometric analysis. The mechanism of the inhibition was proposed to involve the reactive intermediate 1,4-dideoxy-1-(propylamino)-D-*glycero*-2,3-hexodiulose (i.e., 1-propylamino-1,4-dideoxyosone). The existence of this intermediate was supported by acetylation and characterization of the product. Formation of a proposed triazine and bis(amidinohydrazone) after the incubation of 1-propylamino-1,4-dideoxyosone with aminoguanidine also supported the proposed mechanism.

INTRODUCTION

About eighty years ago, Louis Maillard[3] first investigated the reaction of reducing sugars with the free amino groups of amino acids and proteins. This complicated reaction, termed the Maillard reaction, or non-enzymatic browning, is responsible for the aroma and taste in cooked or preserved foods.

The Maillard reaction is initiated by the reaction of primary amines (from amino acids, proteins and nucleic acids) with sugars to form imines (Schiff bases) which undergo further rearrangement to Amadori products[4] (AP) (Scheme 1). Further rearrangement of the Amadori product is responsible for the browning and fluorescent process which leads to the formation of advanced glycosylation end products (AGEs).

SCHEME 1

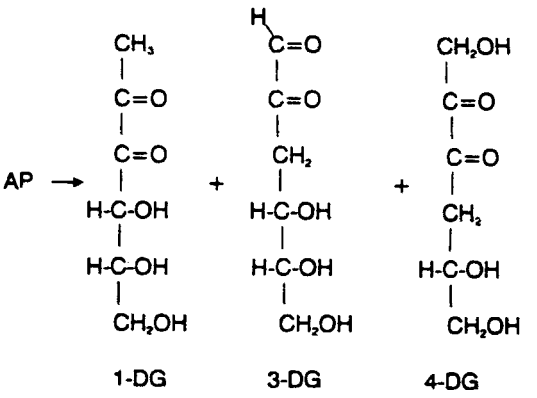


Over the last fifteen years, it has been demonstrated that the Maillard reaction also occurs *in vivo*.[5] A variety of proteins, such as hemoglobin,[6] lens crystallins, connective tissue collagen,[7] glomerular basement membrane,[8] and nerve tissue proteins[9] are modified by glucose in normal individuals. In diabetic patients, the level of Amadori

product is generally 2-3 fold higher than in non-diabetics.[10] Thus, the advanced glycosylation end products of Amadori compounds play an important role in the pathogenesis of complications in diabetes and aging.[11]

The chemistry of AGE formation is not yet well understood because of its extreme complexity. A variety of Maillard compounds have been identified from incubations prepared at high temperature or under acidic conditions.[12] There is little structural information about Maillard compounds which form under physiological conditions. In 1986 Brownlee *et al.* found aminoguanidine to be an effective inhibitor of advanced glycosylation.[13] Later, it was shown that aminoguanidine-treated diabetic animals had significantly less collagen-linked fluorescence and crosslinking in the vascular wall than the untreated ones.[14] Presently, aminoguanidine has displayed excellent efficacy in treating a variety of diabetic complications with little toxicity and is undergoing clinical efficacy trials.

SCHEME 2



The precise mechanism by which aminoguanidine prevents advanced glycosylation is unknown. Several deoxydiketoses are detected as the degradation products of the Amadori products at various pH levels and at elevated temperatures, for example, 1-deoxyglucosone[15] (**1-DG**), 3-deoxyglucosone[5,15,16] (**3-**

DG), and 4-deoxyglucosone[17] (4-DG) (Scheme 2). In most studies to date, these compounds have been obtained under non-physiological conditions (high concentrations, low pH and high temperature). For example, 3-deoxyglucosone was formed under acidic conditions.[15] Furthermore, these compounds are very unstable and proof of their existence is often obtained via chemical derivatization.

In order to minimize the side-products which are produced during the formation of the Amadori product from high concentrations of sugar, we have incubated a chemically synthesized Amadori product with aminoguanidine at physiological concentration, pH, and temperature.

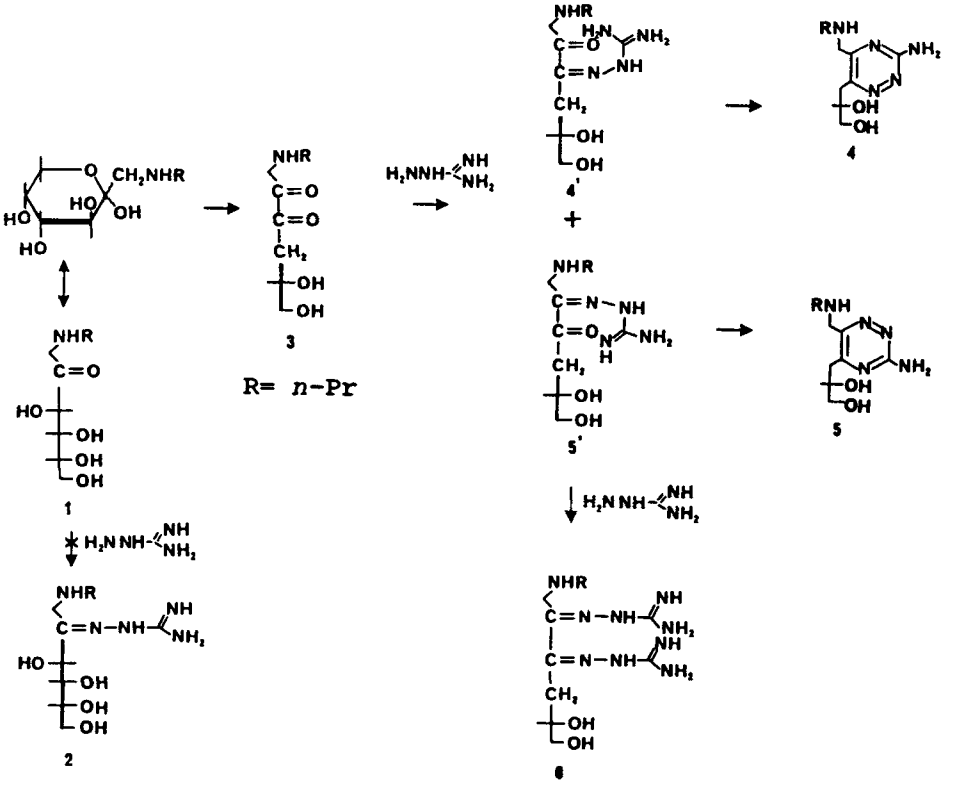
RESULTS AND DISCUSSION

The Amadori product 1-deoxy-1-propylamino-D-fructose (**1**, R= *n*-Pr) was synthesized via the substitution of the C1 hydroxyl group of D-glucose by propylamine, followed by rearrangement catalyzed by oxalic acid.[18] A white powder was obtained as an oxalate after recrystallization (dioxane/methanol).

A solution of 1-deoxy-1-propylamino-D-fructose **1** (100 mM) and aminoguanidine (100 mM) in sodium phosphate buffer (200 mM, pH 7.4) incubated in the dark at 37 °C for 34 days had an absorption maximum at 315 nm. In the absence of aminoguanidine, the incubated Amadori product showed an absorption maximum at 290 nm with a shoulder at 340 nm. Each reaction mixture was subjected to cation exchange chromatography and eluted with a salt gradient (0 to 1M NH_4OAc). Fractions were collected according to the profile recorded by UV absorption at 280 nm. Three major product peaks, P_1, P_2, and P_3, were observed in the chromatogram of the reaction mixture of the Amadori product **1** and aminoguanidine which were absent from the reaction mixture of **1** alone. The isolation of these products was difficult due to their low yields because they

were transient reactive intermediates. Attempts to isolate the intermediates by silica gel chromatography failed due to their decomposition. The starting Amadori product was the major component in the incubated solution and was separated from the products by cation exchange chromatography. The products were further purified by reverse phase preparative TLC.

SCHEME 3



It was thought that aminoguanidine might have reacted with Amadori product **1** to form an amidinohydrazone **2** which underwent further rearrangement to form the products (Scheme 3). Accordingly, the hydrazone was synthesized under mild conditions: Amadori product **1** and one equivalent of aminoguanidine hydrochloride were dissolved in 90% formic acid with stirring

at room temperature overnight. In the fast protein liquid chromatography (FPLC) system described above, this hydrazone **2** eluted similarly to P_2, but P_2 and **2** had distinct spectral properties (UV-visible and NMR) and molecular weights (determined by FAB-MS). Also, prolonged incubation of this hydrazone **2** did not generate products which coeluted with P_1 and P_3. It thus appeared that aminoguanidine reacted with a previously unanticipated rearrangement product, characterized as 1-propylamino-1,4-dideoxyosone **3** as described in the later part of this paper. Aminoguani-dine contains one amidino group and one hydrazino group. Both groups are reactive toward carbonyl functions, the latter being more reactive than the former.

The absence of peaks in the aromatic region of the ^1H NMR spectrum of P_1 ruled out the possibility of pyrrole or furan formation. The mechanism we propose is that the reactive intermediate, 1-propylamino-1,4-dideoxyosone **3**, reacts with the hydrazine moiety of one molecule of aminoguanidine to form the monohydrazone which undergoes dehydration/ring-closure to form a stable 6-membered aromatic compound P_1, a 1,2,4-triazine. The ring closure reaction must be a rapid process, since detection of this monohydrazone was not observed.

There are two possible structures of the proposed 1,2,4-triazine, resulting from the different reactivities of the two carbonyl groups in **3**. If the C-2 monohydrazone **5′** formed followed by dehydration at C-3, 3-[3-amino-6-(propylamino)-1,2,4-triazin-5-yl]-1,2-propanediol **5** would be the product. On the other hand, if the C-3 monohydrazone **4′** formed with subsequent ring closure at C-2, 3-[3-amino-5-(propylamino)-1,2,4-triazin-6-yl]-1,2-propanediol **4** would be the product. Therefore, it was the reactivity of the C-2 versus the C-3 carbonyl group in **3** which determined the structure of the product P_1. When Hirsh *et al.*[19] reacted 3-deoxyglucosone with aminoguanidine, a mixture of triazine isomers[20] in a ratio of 2:1 was observed. Thus, the C-3 carbonyl is about twice as reactive as the C-2 carbonyl, in the case of 3-deoxyglucosone.

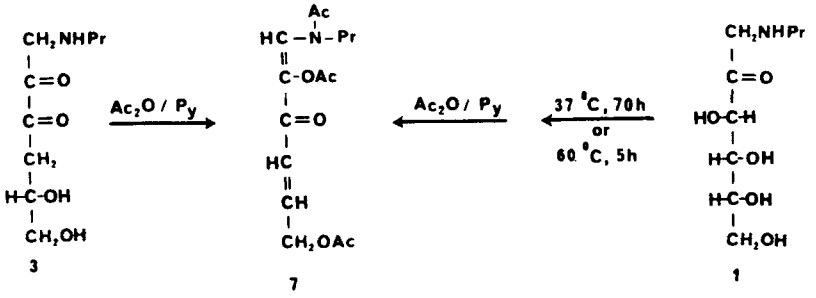
Based on the spectroscopic data (^{1}H NMR) and the chromatographic result, we were not able to distinguish the two isomers, if in fact both exist. Unfortunately, we did not obtain enough product for ^{13}C NMR and other spectroscopic analysis because the reaction was carried out at low concentration. Therefore, based upon the possibility that the reactivity of the C-3 carbonyl group of **3** is greater than that of the C-2 carbonyl group, the 3-[3-amino-5-(propylamino)-1,2,4-triazin-6-yl]-1,2-propanediol **4** is tentatively assigned to be the structure of P_1.

From NMR (^{1}H) and mass (FAB) spectral data, P_3 is proposed to be the bishydrazone **6** from the 1-propylamino-1,4-dideoxyosone (**3**) with two molecules of aminoguanidine. This is also consistent with the fact that P_3 was eluted after P_1 from the cation exchange column. Bishydrazones of aminoguanidine with dicarbonyl sugars have been reported previously.[21]

The reactive intermediate responsible for the formation of the proposed triazine and dihydrazone is proposed to be 1-propylamino-1,4-dideoxyosone **3**. It was detected by mass (FAB) spectroscopy (MW= 203) in the early fractions of the cation exchange chromatography from solutions of the Amadori product **1** incubated both with and without aminoguanidine. This reactive species decomposed after isolation on a silica gel column. Acetylation of the fractions containing **3** followed by silica gel chromatography provided the acetylated and dehydrated derivative **7**. Recently, **7** was isolated and characterized from the acetylated reaction mixture of heated Amadori product by Estendorfer *et al.*[22] (Scheme 4). All the spectroscopic data [^{1}H and ^{13}C NMR, MS (FAB, CI, EI), IR, and UV] we obtained strongly supported the structure of **7**. The product of trapping **3** with *o*-phenylenediamine was also reported.[16]

Incubation of the early fractions after FPLC with aminoguanidine for a short period of time (7 days) showed the production of P_1, P_2, and P_3 on the FPLC system described above.

SCHEME 4



From the above evidence, it is apparent that the Amadori product **1** rearranges to 1,4-dideoxyosone **3** upon incubation under physiological pH, concentration, and temperature. One or two molecules of aminoguanidine react with this intermediate to form triazine or dihydrazone products, respectively, thus preventing further advanced glycosylation.

If antibodies generated against analogues of the triazine and dihydrazone with a suitable linker (e.g., 6-aminohexanoic acid) can detect these two compounds in aminoguanidine-treated animals, it would constitute further evidence of the existence of the triazine and dihydrazone *in vivo*. Also, a quantitative test which could reveal cumulative aminoguanidine exposure would be very useful in the clinical evaluation of this potential therapeutic agent. We will try to achieve these goals in our future studies.

EXPERIMENTAL

General Procedures. ^{1}H and ^{13}C NMR spectra (chemical shifts in parts per million from the internal tetramethylsilane) were recorded either on a General Electric QE-300 or a Brucker AM 360 WB spectrometer. UV-visible spectra were measured on either a Hewlett-Packard 8450A or 8452A diode array spectrophotometer. Fast atom bombardment (FAB) mass

spectra were obtained using a VG 707E double focusing mass spectrometer and using a glycerol/0.1% acetic acid matrix. Chemical ionization (CI) mass spectra were obtained on a Hewlett-Packard model 5988A spectrometer. High resolution electron impact (EI) mass spectra were obtained on a VG-II-250 spectrometer. FT-IR spectra were performed on a Mattson model Polaris instrument.

Silica Gel 60 F_{254} (Analtech 59527) and reverse phase C_{18} (Analtech 52011 & 63527) were used for TLC and Silica Gel 60 (Fluka 60738) for column chromatography. All chemicals were reagent grade or better. Fast protein liquid chromatography (FPLC) was performed using a Pharmacia Mono S HR 16/10 column.

1-Deoxy-1-propylamino-D-fructose Oxalate (1). The synthesis was performed essentially according to the method described by F. Micheel and G. Hagemann.[18] White crystals of **1** were obtained in 65% total yield from α-D-glucose. ^{1}H NMR (D_2O) δ 0.91 (t, J=7.5 Hz, 3H), 1.66 (sext., J=7.5 Hz, 2H), 3.01 (t, J=7.5 Hz, 2H), 3.24 (s, 1H), 3.71 (m, 2H), 3.83 (dd, J=9.6, 3.6 Hz, 1H), 3.97 (m, 2H); Mass spectrum (Xe(+)-FAB) 222.

Isolation of 3-[3-Amino-5-(propylamino)-1,2,4-triazin-6-yl]-1,2-propanediol (4) (tentative structure assignment) and 1,4-Dideoxy-1-(propylamino)-D-*glycero*-2,3-hexodiulose bis (amidinohydrazone) (6). A solution containing **1** (100 mM), aminoguanidine (100 mM) and sodium phosphate (200 mM, pH 7.4) was incubated at 37 °C for 34 days. The solution was then chromatographed on a Pharmacia FPLC (Mono S, HR 16/10) with a flow rate of 3 mL/min and eluted 10 min with H_2O, 40 min with a gradient from 0 to 1M NH_4OAc, and 15 min with 1M NH_4OAc. Each fraction was collected for one minute. Fractions from 52-60 min were combined and lyophilized. The mixture was separated by preparative reverse phase TLC with MeOH/ 1M NH_4OAc (4/1). **4**: ^{1}H NMR (D_2O) δ 0.93 (t, J=7.5 Hz, 3H), 1.67 (sext., J=7.5 Hz, 2H), 3.02 (t, J=7.5 Hz, 2H), 3.18 (s, 1H), 3.5-4.3 (m, 5H), 8.50 (s, 1H); Mass spectrum (Xe(+)-FAB) 242;

$UV(H_2O)_{max}$ 315 nm. **6**: ^1H NMR (D_2O) δ 0.95 (t, J=7.5 Hz, 3H), 1.69 (sext, J=7.5 Hz, 2H), 3.04 (m, 4H), 3.36 (dd, J=12.9, 2.7 z, 1H), 3.85 (m, 2H), 7.74 (s, 1H); Mass spectrum (Xe(+)-FAB) 316; $UV(H_2O)_{max}$ 287 nm.

Synthesis of 1-Deoxy-1-(propylamino)-D-fructose Amidinohydrazone (2). To a solution of **1** (96 mg, 0.31 mmol) in 5 mL of 90% formic acid was added aminoguanidine hydrochloride (32 mg, 0.29 mmol) and the mixture was stirred at room temperature for 16 hours. The reaction mixture was concentrated and dried under reduced pressure. The monohydrazone was obtained as a brownish solid in quantitative yield. ^1H NMR (D_2O) δ 1.00 (t, J=7.5 Hz, 3H), 1.75 (sext, J=7.5 Hz, 2H), 3.10 (t, J=7.5 Hz, 2H), 3.32 (s, 1H), 3.77 (m, 2H), 3.92 (m, 1H), 4.03 (m, 2H), 8.26 (s, 1H); Mass spectrum (Xe(+)-FAB) 278; $UV(H_2O)_{max}$ 285 nm.

Detection of 1,4-Dideoxy-1-(propylamino)-D-*glycero*-2,3-hexodiulose (3). Fractions collected from 6-9 min from the Mono S chromatography of the incubation mixture of **1** and aminoguanidine hydrochloride described before were lyophilized and submitted for mass spectroscopic analysis. Mass spectrum (Xe(+)-FAB) 204. Fractions collected from the incubated mixture of **1** (sodium phosphate, pH 7.4) alone also resulted in a value of 204 by Mass (Xe(+)-FAB) spectroscopy.

Isolation of Acetylated Aminoreductone (7). To the dried fractions #6-9 (1.1 g including sodium phosphate) from the incubated mixture of **1** and AG·HCl were added acetic anhydride (11.7 mL) and pyridine (10.0 mL). The reaction was stirred at room temperature for 17 hours. The reaction mixture was then poured into ice and extracted with ethyl acetate (200 ml). The concentrated residue was purified by preparative silica gel TLC with Hexane/EtOAc/Et_3N (50/50/1) and extracted with MeOH. ^1H NMR ($CDCl_3$) δ 0.93 (t, J=7.5 Hz, 3H), 1.62 (sext, J=7.5 Hz, 2H), 2.13 (s, 3H), 2.30 (s, 3H), 2.34 (s, 3H), 3.69 (t, J=7.5 Hz, 2H), 4.77 (dd, J=4.5, 1.5 Hz, 1H), 6.67 (d, J=15.3 Hz, 1H), 6.91 (dt, J=15.3, 4.5 Hz, 1H), 7.83 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 11.10, 20.48, 20.72, 22.03, 22.38,

47.43, 63.02, 124.12, 127.75, 130.87, 140.31, 168.43, 170.48, 183.25; Mass spectrum (Xe(+)-FAB) 312; Mass spectrum (EI) 311, 269, 227, 196, 167, 154, 138, 100,70, 43; Mass spectrum (CI) 312, 298, 270, 252, 228, 210, 186, 168, 142, 102, 85; UV $(EtOH)_{max}$ 300 nm; FT-IR (KBr) 2955, 2927, 2856, 1762, 1754, 1698, 1636, 1624, 1374, 1261, 1229, 1193, 1146, 1083, 1045, 806 cm^{-1}.

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2. New address: The Picower Institute for Medical Research, 350 Community Drive, Manhasset, New York 11030.

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