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Formation of a dihydropyridine derivative as a potential cross-link derived from malondialdehyde in physiological systems

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Abstract Malondialdehyde is a major oxidation product of lipids which is capable of cross-linking the collagen of the cardiovascular system. Identification of cross-links usually involves degradative procedures. In this paper, we use a novel, direct, approach using nuclear magnetic resonance to identify early and labile products. Initial model studies show that malondialdehyde reacts with lysine to form a dihydropyridine derivative rather than the unstable imidopropene Schiff base previously reported. The aldehydes on the pyridine ring could react further to cross-link collagen and stiffen the aorta, thereby promoting further glycation, a process that would be accelerated in diabetes.

Key words: Glycation; Atherosclerosis; Nuclear magnetic resonance; Malondialdehyde

1. Introduction

The major oxidative breakdown product of polyunsaturated fatty acids is malondialdehyde (MDA) [1], which in turn is responsible for many of the oxidised adducts found on low density lipoprotein (LDL). Such compromised LDL is removed by macrophages which become fat-filled foam cells, ultimately causing their death. The lipid released is then deposited in the arterial intima as seen in the early stages of atheroma [2].

MDA may have additional deleterious effects on the vascular system. It has been suggested that reaction of the aldehyde groups could occur with the ε-amino groups of lysine residues in proteins [3]. Formation of such imidopropene cross-links by in vitro reaction of both aldehyde groups has been reported for polylysine and protein [4], with equivalent products observed in DNA [5]. Cross-linking of collagen, a major constituent of the vascular system with a long biological half-life, stiffens the collagen fibres and leads to reduced flexibility of the tissue. Elevated levels of MDA and glycated collagen, manifestations of long-term diabetes, have been shown in vitro to accelerate the oxidation of LDL. We have recently reported that the stiffening of the aorta, a characteristic late complication of diabetic subjects, can be correlated with the increased formation of glycation cross-links [6]. We now propose that MDA cross-linking of collagen could be partially responsible for the stiffening. However, the imidopropene cross-link is fully reversible, and may not be effective in the long term. We have therefore initiated a nuclear magnetic resonance (NMR) and electron impact mass spectroscopy (EI-MS) study to identify any potential long-term cross-link products between MDA and blocked lysine under semi-physiological conditions.

2. Materials and methods

2.1. Preparation of MDA

This was achieved using a minor modification of the protocol by Kikugawa et al. [7]. 1,1,3,3-Tetraethoxypropane (8.2 g) was stirred in 100 ml water with 40 g Dowex 50W-X8 resin for 1 h until the absorbance at 267 nm reached a maximum. After filtering and adjusting the pH to 7.0 (NaOH), the solution was rotary evaporated to approx. 3 ml, becoming a deep orange colour. Acetone was added until the water-acetone meniscus disappeared (approx. 15–20 ml), leaving pale yellow sodium MDA crystals suspended in solution. These were filtered and washed with a small amount of acetone, and then taken up in a minimum amount of water to be crystallised again to an almost white colour. The hygroscopic crystalline product was unexpectedly stable – after 1 year at −20°C the stored NaMDA was still 97% pure by NMR. All the significant impurities were identifiable MDA breakdown products [8], the main one being formic acid. The latter forms almost immediately when MDA is put into solution.

2.2. Reaction between N-carbobenzoxy-lysine (CBZ-lysine) and MDA

The reaction was undertaken with both substrates at 20 mM in 100 mM phosphate (pH 7.5, 90% H_2O, 10% D_20, 37°C) on a 500 MHz JEOL NMR instrument by taking a 32-scan spectrum at times 0, 1, 3 and 7 days. Integrals of the main products were used to find out their rates of formation, which could then be used to work out initial rate constants, as the reaction takes over a month to complete at 37°C. Previous work by HPLC separation of reaction components showed that the reaction was indifferent to 0.9% saline or a change of the buffer to 100 mM HEPES, and that CBZ-lysine was at least an order of magnitude more reactive than CBZ-arginine.

2.3. Reaction between propylamine and MDA

After checking for equivalent products with MDA at 37°C (data not shown), the NMR experiment was repeated at 60°C using propylamine instead of CBZ-lysine in order to simplify mass spectra and NMR interpretation. Spectra were taken at 0, 1, 3, 7, and 10 days. Control reactions were done with MDA alone to check its stability. Acids from auto-oxidation of MDA were detected by the change of associated nearby protons on dropping the pH to 3.75.

2.4. Mass spectroscopy of NMR mixtures

EI-MS was run on various NMR samples using an Autospec Micromass instrument. Dichloromethane was observed in most spectra as it was used to clean the needle in between runs. Most experiments were done by diluting the NMR sample in an equal volume of methanol and then running the sample. Several were done without any methanol addition.

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Abbreviations: MDA, malondialdehyde; NPMDD, *N*-propyl-4-methyl 2,6-dihydropyridine 3,5-dicarbaldehyde; β-LAA, β-lysyl aminoacrolein; CBZ-lysine, *N*-carbobenzoxy-lysine

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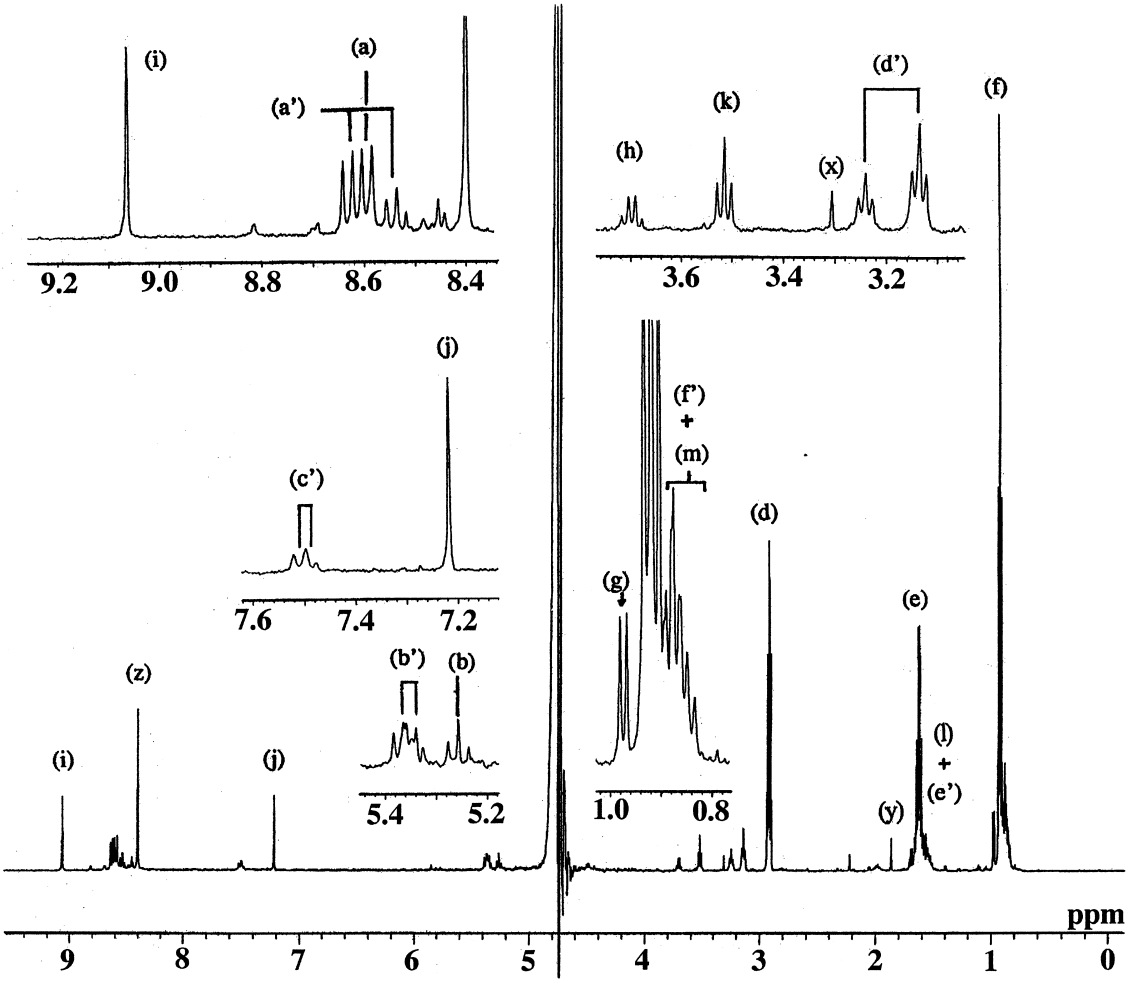


Fig. 1. 500 MHz ^{1}H NMR spectrum of propylamine (20 mM) and MDA (15 mM) left incubated at 60°C for 24 h in 100 mM phosphate buffer pH 7.5 (90% H_2O, 10% D_2O). The annotations on the diagram correspond to those used in the diagram of the proposed mechanism shown below which gives the structure of these molecules (Scheme 1).

3. Results

3.1. Identification of the products of the reaction between propylamine and MDA

Fig. 1 shows a 1-dimensional NMR spectrum taken of this reaction at 60°C, an elevated temperature to assist in determining the endpoint. Even in the strong buffer, a final pH of around 7.65 is observed at the end of the reaction. Only two major products of interest could be identified, namely β-propyl aminoacrolein (β-PAA) and *N*-propyl-4-methyl 2,6-dihydropyridine 3,5-dicarbaldehyde (NPMDD). However, substantial peaks are also seen for malonic, acetic, and formic acid. Formic acid is formed directly from the breakdown of MDA into formic acid and acetaldehyde [8], while the other acids are formed by oxidation of acetaldehyde and MDA. The inferred presence of the very reactive acetaldehyde is actually desirable as it is known to have a normal concentration of around 1 μM in blood plasma [9,10]; much higher than the 50 nM (free) and 300 nM (total) concentration estimates for MDA [11,12]. NPMDD is shown later to actually require acetaldehyde to form. These acids are seen in greater quantities when MDA is incubated alone. Thus MDA adduct formation is in direct competition with MDA breakdown as a viable fate, making it unsurprising that there is no known detoxification system for MDA.

The assignments and concentrations given in Table 1 were worked out by using integrals to associate peaks from the same molecule; referring back to the literature and matching results [13]; and by using 2D NMR to trace connectivities (data not shown). The *cis*:*trans* ratio of β-PAA is about 2:1, but the data did not show which isomer was more abundant. In particular, there was *no* evidence for the imidopropene structure mentioned earlier as proposed by several groups [3,4].

After 24 h at 60°C, most of the MDA had reacted, and the propylamine was evidently in excess. This could be expected as MDA is subject to self-scission and oxidation, and because it requires three MDA molecules and only one of propylamine to form NPMDD. From the rough concentrations given in Table 1, it is evident that β-PAA is vulnerable to oxidation on extended incubation, giving malonic acid and propylamine. β-PAA can also degrade back to its reagents or reacts with itself and acetaldehyde to eventually form more NPMDD once the concentration of MDA is low, again releasing propylamine. NPMDD is the only major long-term product, with about a 50% yield.

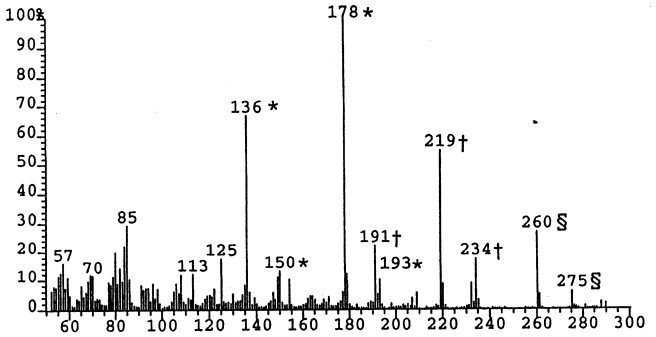


Fig. 2. Electron impact mass spectroscopy in 50% MeOH of 20 mM MDA and 20 mM propylamine incubated for 72 h at 60°C in the presence of 100 mM phosphate buffer pH 7.5. The major peaks at mass 178 and 136 were identified as $C_{10}H_{12}NO_2$ and $C_7H_6NO_2$ by measuring their mass exactly. Both of these formulae must be for molecular fragments as they cannot satisfy normal valence rules.

3.2. Evidence for further addition of propylamine to NPMDD

Fig. 2 shows EI-MS of the MDA/propylamine NMR sample after 72 h at 60°. A single mass peak is seen at 113 for β-PAA while there are small peaks at 154 and 125 that indicate trace amounts of the imidopropene cross-link. However, the much larger mass peaks at 178, 150, and 136 coincide with fragments of the mass-193 NPMDD (*); the *para*-methyl cleaved off in the former case, the propyl group cleaved off at mass 150, and both groups cleaved off to give mass 136. Two other similar series of fragment pattern are observed, one at masses 234/219/191 (†), and another at 275/260 (§). These indicate that many of the NPMDD molecules have had a second or third propylamine added on to their sparingly reactive carbaldehyde groups in the form of a Schiff base.

3.3. Reaction studies using N-acetyllysine and MDA at 37°C

To relate this work to a more physiological environment, and to obtain some idea of reaction rates, a series of NMR spectra showing the reaction between *N*-acetyllysine and MDA were run at times 0, 1, 3 and 7 days, as shown in Fig. 3.

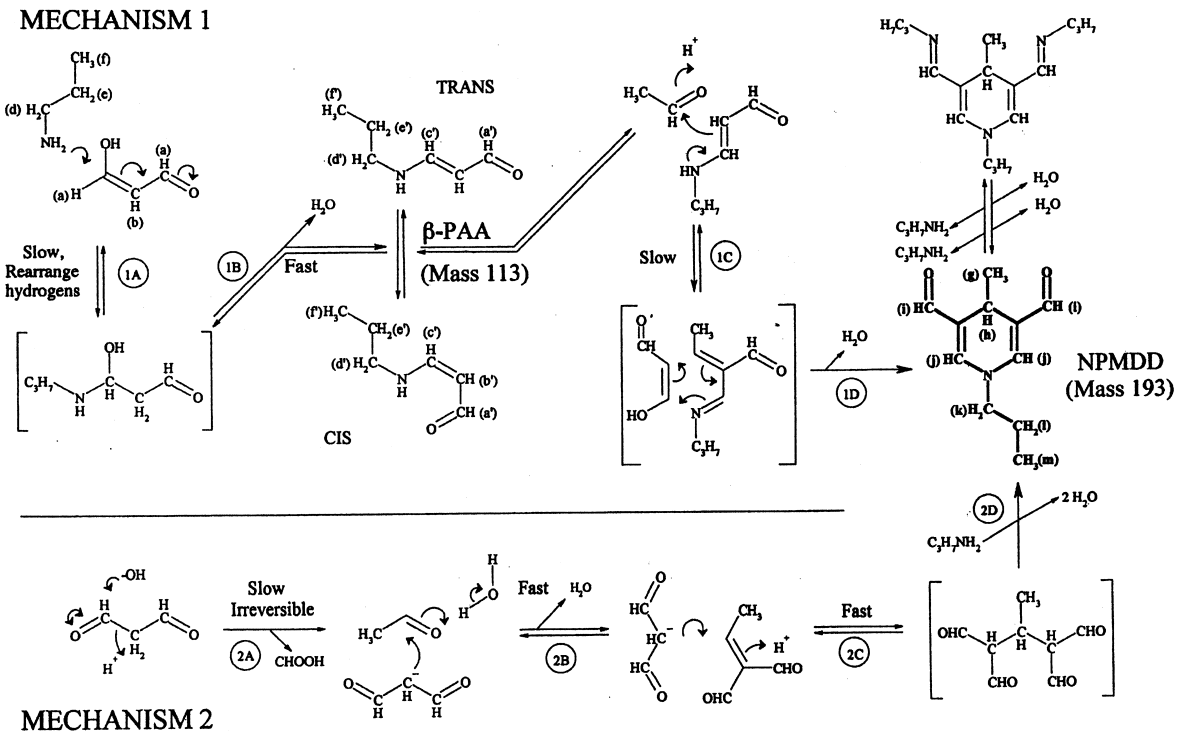
The second order rate constant, dependent on both MDA and *N*-acetyllysine for initial formation of β-lysyl-aminoacrolein (β-LAA), can be estimated at 6 mol^{-1} day^{-1}. The initial formation of *N*-lysyl-4-methyl 2,6-dihydropyridine 3,5-dicarbaldehyde (NLMDD) also fits well to a similar pseudo-second order rate of 1 mol^{-1} day^{-1}, but there is no proof of exactly what order the reaction is. If one assumes a physiological steady-state concentration of 50 nM for MDA and 10 mM for reactive lysine (an arbitrary value), these figures estimate that 25 nM β-LAA and 25 nM NLMDD form in ca. 8 and 50 days respectively. Incubation of MDA with collagen in similar conditions implies that the reaction is considerably faster in proteins as 10 mM MDA falls to trace levels (<0.5 mM) after incubation with 6 mM lysine in the form of collagen (10 mg/ml) for just 2 days at 37°C (data not shown). This could be due to a number of possible factors, such as the smaller entropy loss involved when bound lysine reacts with MDA; the catalysis of the reaction by proximal amino acids; the reaction of other collagenous components with MDA; or the local environment favouring the elimination of water that occurs on binding.

At this point, it should be noted that β-PAA has a lifetime at least three times as long in these conditions as MDA before being oxidised or reacting to give NPMDD. By inference then, most of the short-term 'MDA reserves' in physiological systems will be in the form of β-LAA, in agreement with already published results [11,12], whatever its ultimate fate may be. Most methods of measuring physiological MDA will also be measuring β-LAA.

Table 1
Temporal concentration changes of the reagents and products of a reaction between 20 mM propylamine and 15 mM MDA at 60°C in 100 mM phosphate buffer pH 7.5

Molecules observed	Concentrations at specified time			NMR chemical shift
	1 day (Fig. 1)	3 day (plots not shown)	11 day	Peak id (ppm)
MDA	1.40 mM	trace	none	a (8.59), b (5.26)
Formic acid	5.32 mM	6.05 mM	6.58 mM	z (8.41)
Malonic acid	0.22 mM	0.24 mM	0.64 mM	x (3.30)
Acetic acid	0.30 mM	0.30 mM	0.33 mM	y (1.86)
Propylamine	14.6 mM	13.7 mM	17.0 mM	d (2.92), e (1.62), f (0.92)
cis/*trans* isomers of β-PAA	2.72 mM 1.34 mM	2.06 mM 1.08 mM	0.69 mM 0.35 mM	a′ (8.63 and 8.55), b′ (7.50 and 7.49), c′ (5.37 and 5.36), d′ (3.13 and 3.24), e′ (1.57 and 1.55), f′ (0.88 and 0.87)
NPMDD	1.27 mM	2.60 mM	2.60 mM	g (0.98), h (3.70), i (9.05), j (7.22), k (3.55), l (1.69), m (0.90)

Concentrations given are approximate (±5%), having been determined from integrals of the 1D NMR spectrum. The first set of shift positions for each pair of the β-PAA assignments correspond to the higher concentration isomer. The peak annotations correspond to those used in Fig. 1 and Scheme 1.



Scheme 1. Two concurrent mechanisms for the formation of NPMDD from MDA and propylamine. The NMR assignments for protons labelled with letters are given in Figure 1.

3.4. Possible mechanisms in the MDA-primary amine reaction

Scheme 1 gives a proposed mechanism of formation of β-PAA and NPMDD at physiological pH, based on previous chemical work [13] and the current paper. Reactions 1A and 1B in Scheme 1 are favoured by the release of water and maintenance of a conjugated π system, but require an MDA molecule in its enol form as opposed to its majority (99.9%) salt form at pH 7.5 due to its acidic pK_a of 4.6. However, despite this, the formation of β-PAA is faster than that of NPMDD initially, because direct formation of NPMDD via the proposed transient intermediates (not detected in NMR spectra) requires an initial chemical cleavage of MDA into acetaldehyde and formic acid (Scheme 1, 2A). These mechanisms could be tested by changing the pH of the buffer and initial substrate concentrations. Formation of NPMDD (Scheme 1, 1D, 2D) is assumed to be irreversible as β-PAA and MDA go down to trace levels after the reaction had proceeded for 11 days at 60°C, and there is no apparent degradation of NPMDD. The exact status of its equilibrium with extra propylamines added is unknown.

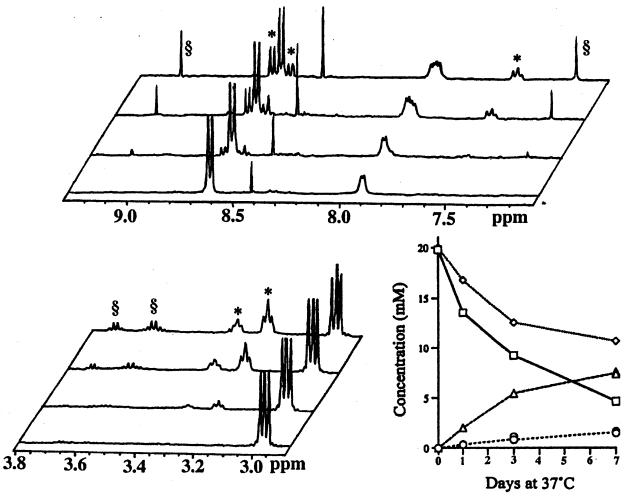


Fig. 3. Two sections of a 1D time-course NMR spectroscopic study of the reaction between *N*-acetyllysine (20 mM) and MDA (15 mM) in 100 mM phosphate buffer (pH 7.5) at 37°C (90% H_2O, 10% D_2O). *Peaks derived from β-LAA. §Peaks derived from NLMDD. The bottom right inset shows concentrations derived from integration of resolved NMR peaks from β-LAA (△, $n=2$ peaks), NLMDD (○, $n=3$ peaks), MDA (□, $n=1$ peak) and *N*-acetyllysine (◇, $n=2$ peaks). All points are plotted with no error bars shown. Annotations of peaks on the sections are as in Fig. 1.

4. Discussion

We have shown that the dihydropyridine derivative, NPMDD, is the major product of the reaction of MDA and propylamine, and that only very low levels of the imidopropene cross-link are formed by propylamine (and by inference, lysine) and MDA when they are free in solution at physiological pH, in agreement with the results of Requena et al. [4]. The native imidopropene cross-link is unlikely to be stable on a long-term basis as the formation of the link is fully reversible on addition of water, and thus will not be a major feature in cross-linking of long-lived proteins.

NPMDD (Scheme 1), or its protein equivalent, NLMDD, is both stable and has the ability to form cross-links with propylamine even when free in solution. The easy identification of NPMDD-derived cross-links between 2/3 propylamines in Fig. 2 (masses 234 and 275) implies that NPMDD forms Schiff base-derived cross-links of amine compounds that are more energetically favourable than the imidopropene deriva-

tive of β-PAA. If the same factors favouring the imidopropene cross-link in proteins also work in the favour of NLMDD, then it is reasonable to assume that any NLMDD-derived cross-link will be more stable than an imidopropene one. Even when the Schiff-base cross-link dissociates, there is no tendency for NPMDD to subsequently degrade, giving the cross-link opportunity to reform.

NLMDD will probably be present only at trace levels in vivo, because of the number of other reactive components in physiological systems that could react with β-LAA. Considering reaction 1C in Scheme 1, any aldehyde, particularly an open-chain sugar or 4-hydroxynonenal, could react to give a different substituent on the 4-position of the eventual pyridinium ring. Likewise, the Diels-Alder reaction 1D in Scheme 1 could feature any metabolite with electron-attracting groups substituted onto a double-bond instead of MDA. Maleate from the TCA cycle would be a typical example, although, again, any aldehyde in its enol form will react. Despite this variability of substituents on one side and in the para position, there will still be at least one carbaldehyde group left on the ring which could cross-link. NLMDD is unlikely to form by mechanism 2 in vivo (Scheme 1), as that requires two molecules of MDA and one of acetaldehyde to react to form the precursor, an improbable event considering their low concentrations in vivo and the number of molecules they can react with.

NLMDD would be deduced to be acid-stable from its structure, without the necessity for previous borohydride reduction. Once its separation characteristics have been determined, it should be easy to check whether it is present in acid hydrolysates, first from collagen incubated with MDA, then with collagen from diabetic mice. Subsequent work could then follow on any potential derivatives, aided by the easy fluorescent detection of the ring.

In summary, we have characterised the formation of a dihydropyridine derivative from MDA, acetaldehyde, and lysine by NMR and EI-MS in semi-physiological conditions, and have persuasive evidence that it is a potential cross-link which could contribute to the stiffening of the vascular system. A consequence of the formation of such stable cross-links would be a further reduction in turnover and increased glycation of the collagenous vascular system.

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