ORGANIC LETTERS

2003
Vol. 5, No. 15
2659−2662

Synthesis of a Novel Radical Trapping and Carbonyl Group Trapping Anti-AGE Agent: A Pyridoxamine Analogue for Inhibiting Advanced Glycation (AGE) and Lipoxidation (ALE) End Products

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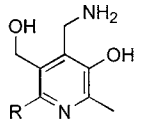
Received May 12, 2003

ABSTRACT



Pyridoxamine, PM: R = H
6-Dimethylaminopyridoxamine, dmaPM: R = $N(CH_3)_2$

Pyridoxamine is known to be an effective inhibitor of both advanced glycation (AGE) and advanced lipoxidation (ALE) end products. The synthesis of a novel multifunctional AGE and ALE inhibitor, 6-dimethylaminopyridoxamine (dmaPM, 11) is described. The 6-dimethylamino substituent increases the radical trapping ability of pyridoxamine's phenolic group. Results obtained during ribose glycations show that both the new dmaPM and a known strong radical trapping agent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), prevent intermolecular protein cross-linking more effectively than pyridoxamine (PM).

Nonenzymatic protein glycation by reducing sugars, such as glucose or ribose, (the Maillard reaction) is a complicated cascade of condensations, rearrangements, fragmentations, and oxidative modifications that lead to products collectively called advanced glycation end products (AGE).[1,2] During lipid peroxidation reactions, a number of reactive carbonyl compounds are formed by the decomposition of lipid hydroperoxides, including malondialdehyde (MDA) and hydroxynonenal (HNE). Nucleophilic proteins such as lysine can trap such lipid peroxidation products to form adducts (e.g., MDA-Lys, HNE-Lys) collectively called advanced lipoxidation end products (ALE).[2,3] Once formed, both AGE and ALE products may prevent normal protein function or recognition or stimulate potentially detrimental interactions in signaling pathways.[4] The formation of AGE and ALE has been increasingly implicated in the pathogenesis of diabetic complications, Alzheimer's disease, and atherosclerosis.[2,5] Compounds that reduce AGE or ALE formation may prove useful for limiting nonenzymatic protein modification associated with the pathology of these and other chronic age-related diseases.

(1) Abbreviations: ABAP (or AAPH), 2, 2′-azobis(2-amidinopropane) dihydrochloride; AGE, advanced glycation end products; ALE, advanced lipoxidation end products; APC, allophycocyanin; BDE, bond dissociation enthalpy; BSA, bovine serum albumin; CML, N^{ϵ}-carboxymethyllysine; DTPA, diethylenetriaminepentaacetic acid; HNE, hydroxynonenal; IP, ionization potential; MDA, malondialdehyde; PM, pyridoxamine; PN, pyridoxine; SEC, size-exclusion chromatography; Tx, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

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10.1021/ol0348147 CCC: $25.00
Published on Web 07/02/2003

Specific mechanisms for the late Maillard reaction conversion of the initial Amadori adducts to the irreversible formation of AGE involve complex sequential and parallel reactions that are believed to produce a number of reactive carbonyl compounds, especially α-dicarbonyls.[5c] AGE formation in vitro is typically accelerated by oxidative conditions (glycoxidation) catalyzed by metal ions such as Fe^{3+} or Cu^{2+}, and it is generally accepted that metals may play a role during AGE formation in vivo.[6]

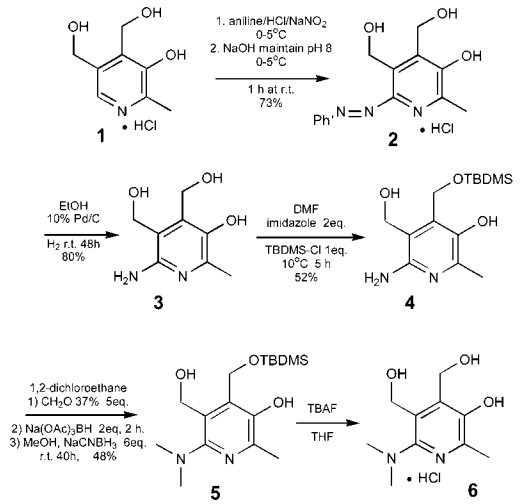
Although a variety of AGE inhibitors have been identified, their mechanisms of action are not well understood.[7] The most common feature among the AGE inhibitors known to date is the presence of a nucleophilic functionality, such as an amine or hydrazone, which can intercept reactive carbonyl compounds and prevent progression to AGE or ALE products. Other classes of inhibitors, such as metal ion chelators (diethylenetriaminepentaacetic acid (DTPA)) and radical trapping antioxidants (α-tocopherol and ascorbate) exhibit their activity by limiting oxidative acceleration of glycation.[7a] Typically, AGE inhibitors have primarily been identified using glycation models in the presence of transition metal ions. Thus, it is very difficult to discern in the literature whether some of the most promising AGE inhibitors are providing protection as a result of their carbonyl trapping or metal ion chelating activities.[8] Surprisingly, little work has been done on the effects of radical trapping antioxidants on AGE chemistry.

Pyridoxamine (PM) has been identified as an AGE inhibitor for late stage (post-Amadori) glycation and as an inhibitor of ALE.[9] For example, PM has been reported to decrease the formation of fluorescent AGE products significantly and, specifically, to decrease the yield of N^{ϵ}-carboxymethyllysine (CML).[7a,10] PM is a less active metal ion chelator than many other known AGE inhibitors, but there is evidence showing that it can trap the dicarbonyls glyoxal and methyl glyoxal.[11] The mechanism of ALE inhibition by PM has been proposed to be by the formation of PM adducts with 9- and 13-oxo-decadienoic acid intermediates formed during the peroxidation of linoleic acid, which leads to the formation of hexanoic acid amide and nonanedioic acid monoamide derivatives of PM, respectively, with substantially reduced levels of MDA-Lys and HNE-Lys.[9b]

The present work describes the synthesis of a novel multifunctional AGE and ALE inhibitor designed to address the involvement of both reactive carbonyls and radical intermediates in the complex pathways of the Maillard reaction and lipid peroxidation. Considering that the known carbonyl trapping AGE and ALE inhibitor, PM, may be found bound to important glycation or lipoxidation intermediates, we suspected that the phenol functionality might serve as a local protective agent against radical AGE and ALE intermediates. However, PM has been reported to have only a weak hydrogen atom donating (radical trapping) ability, making it only a marginally effective antioxidant.[9b,12] Electron-donating para substituents lower phenolic O−H bond dissociation enthalpies (BDE), which enhances radical trapping rates. Unfortunately, such substituents also lower the ionization potential (IP) of the phenol and can do so to such an extent that, for example, 4-dimethylaminophenol (which should be an extremely good radical trap) is of no practical value as an antioxidant because it reacts directly with dioxygen. Pratt et al.[13] have shown that strong electron-donating groups (such as dimethylamino) para to the phenolic hydroxyl group of 3-pyridinols and 5-pyrimidinols decrease the O−H BDE and greatly increase radical trapping rates but do not lower the IP to the point that there is a direct reaction with oxygen. Therefore, PM was modified by the addition of a 6-dimethylamino substituent (**11**). A pyridoxine (PN) derivative containing a 6-dimethylamino substituent (**6**) was also prepared to study the importance of the nucleophilic amine group during glycation reactions.

The synthesis of 6-dimethylaminopyridoxine hydrochloride (**6**) is given in Scheme 1.[14] The 6-aminopyridoxine hydrochloride (**3**) was synthesized by the methods of Korytnyk et al.[15] Pyridoxine hydrochloride (**1**) was reacted with diazotized

Scheme 1



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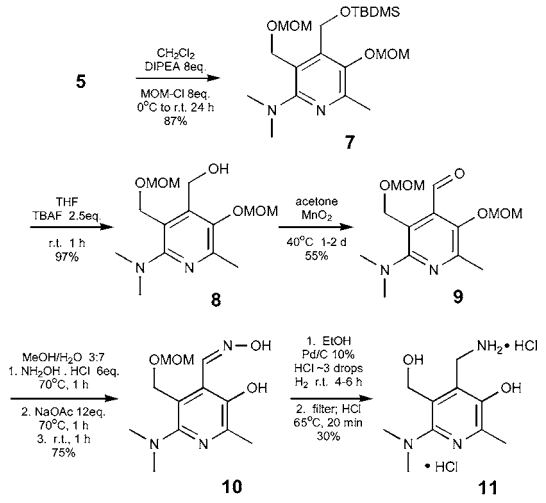
(14) See Supporting Information for details.

aniline to produce 6-phenylazopyridoxine hydrochloride (**2**), which was then hydrogenated to yield **3**. Treatement of **3** with imidazole (2 equiv) and *tert*-butyldimethylsilyl chloride (1 equiv) provided the TBDMS ether at the unexpected α^4 position, **4** in a 52% yield. The amine **4** was then alkylated by reductive amination with formaldehyde, sodium triacetoxyborohydride, and sodium cyanoborohydride. After extraction the 6-dimethylamino derivative **5** was purified by flash chromatography (**5** can also be crystallized from ethyl acetate). The position of the TBDMS ether at the α^4 position was confirmed by the X-ray crystal structure of **5**.[16] The purified compound **5** was carried forward to complete the synthesis of 6-dimethylaminopyridoxamine (**11**, Scheme 2).

Scheme 2



Compound **5** was deprotected with tetrabutylammonium fluoride (TBAF) in THF to yield **6** as a recrystallized product. The X-ray crystal structure of 6-dimethylaminopyridoxine hydrochloride **6** was also obtained.[16]

Scheme 2 shows the synthetic route to **11** from the TBDMS ether **5**. Methoxymethyl ether protecting groups were introduced at the 3-phenol and the α^5-hydroxyl groups to give **7**. The TBDMS ether was then selectively deprotected with TBAF in THF solution, and the resulting α^4-hydroxyl derivative **8** was oxidized by manganese(IV) oxide in acetone. This standard procedure for oxidation of pyridoxine to pyridoxal[15,17] was unusually slow for the conversion of **8** to the pyridoxal derivative **9**. However, these oxidation conditions generated the product cleanly and starting material was easily recovered and resubjected to the same reaction conditions. The aldehyde **9** was then converted to the oxime **10**. There was some loss of the methoxymethyl ether protecting groups during the formation of the oxime, but removal of the remaining MOM protecting groups at this stage was avoided because of the undesirable hydrolysis of the oxime under acidic conditions. Instead, the oxime **10** and other protected versions were hydrogenated to the amine in the presence of a small amount of hydrochloric acid, which catalyzed the hydrogenation, and then the methoxymethyl ether protecting groups were removed. The 6-dimethylaminopyridoxamine (**11**, or dmaPM) was purified to yield an off-white solid as a dihydrochloride salt.

The radical trapping activities of the dimethylamino derivatives dmaPN (**6**) and dmaPM (**11**) were evaluated by following the peroxyl radical induced quenching of allophycocyanin (APC) fluorescence. APC is a naturally fluorescent protein that rapidly loses its fluorescence when exposed to a source of free radicals.[18] Thermal decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) in aerated aqueous systems generates positively charged peroxyl radicals at known and reproducible rates. Radical trapping antioxidants competitively trap ABAP-derived peroxyl radicals before their reaction with the protein. Results in Figure 1 demonstrate that the novel dimethylamino derivatives

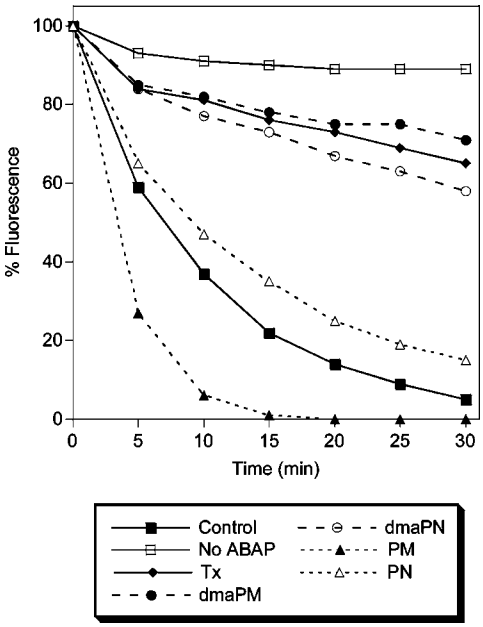


Figure 1. Radical trapping antioxidants (Tx, dmaPM, and dmaPN) retard peroxyl radical induced quenching of allophycocyanin fluorescence. Conditions were 64.4 nM APC, ABAP 12 mM, and inhibitor 50 μM, as indicated, in 50 mM phosphate buffer pH 7.4 at 37 °C under air (n = 2 with high reproducibility ± 1−2%).[22]

dmaPM (**11**) and dmaPN (**6**) are comparable in their ability to retard APC fluorescence quenching to the well-known water-soluble α-tocopherol analogue 6-hydroxy-2,5,7,8-

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(16) **Crystal Data.** The X-ray diffraction data were collected on a Bruker SMART CCD diffractometer using graphite-monochromated Mo Kα radiation at 172 K. Compound **5**: $C_{16}H_{30}N_2O_3Si$, monoclinic, space group $P2_1/c$, a = 15.6916(12), b = 8.0862(6), c = 16.9360(18) Å, β = 118.437(10)°, D_c = 1.148 g cm^{-3}, μ = 0.137 mm^{-1}, Z = 4, final residual R1(R_w) = 0.0430 (0.1149) for 3755 reflections with $I > 2\sigma(I)$, and 319 parameters. Compound **6**: $C_{10}H_{17}N_2O_3Cl$, monoclinic, space group $P2_1/c$, a = 10.4039(10), b = 12.9429(8), c = 8.9220(6) Å, β = 91.346(10)°, D_c = 1.375 g cm^{-3}, μ = 0.313 mm^{-1}, Z = 4, final residual R1(R_w) = 0.0411 (0.1069) for 2429 reflections with $I > 2\sigma(I)$, and 213 parameters. See also Supporting Information.

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tetramethylchroman-2-carboxylic acid (Trolox, or Tx). PN provided only a slight prevention of fluorescence loss, while PM was found to accelerate the loss of APC fluorescence.[19] These radical trapping comparisons were made in buffer at pH 7.4 where the dimethylamino substituent or the pyridine (for PN and PM) may be protonated. Such protonation would induce a strong electron-withdrawing effect and decrease the hydrogen atom donating ability of the phenol. Protonation of the aliphatic amine of dmaPM may also have some influence on the hydrogen atom donating ability of the phenol.[20] In working with dmaPN (**6**) and dmaPM (**11**) as hydrochloride salts, no significant decomposition was observed until the pH was adjusted with buffer. However, after a 7-day incubation at 37 °C under air in pH 7.4 phosphate buffer, ~60% dmaPM (**11**) and ~20% dmaPN (**6**) had decomposed.[21]

The novel carbonyl group trapping and radical trapping AGE inhibitor dmaPM (**11**) was also tested for its ability to act as a radical trapping antioxidant in a model glycation system. Intermolecular protein cross-links formed during the glycation of bovine serum albumin (BSA) with ribose in a metal ion free buffer[23] were determined by size-exclusion chromatography (SEC) with refractive index detection.[14] The radical trapping antioxidants dmaPM (**11**) and Tx provided the greatest inhibition of intermolecular protein cross-linking (Table 1). The carbonyl trapping inhibitor PM was less effective at preventing cross-links and PN was a poor cross-link inhibitor. The new AGE and ALE inhibitor dmaPM (**11**) was nearly as effective at preventing protein cross-linking as Tx. The radical trapping dmaPN derivative (**6**) showed less inhibition of cross-links than the carbonyl and radical group trapping dmaPM. The results from Tx experiments show that radical trapping provides better cross-link prevention than carbonyl group trapping (PM). We suggest that dmaPM (**11**) is comparable to Tx in cross-link prevention largely because of its radical trapping ability.[24] However, its carbonyl group trapping ability may also play some role in cross-link prevention.

Table 1. Radical Trapping Antioxidants Prevent Intermolecular Protein Cross-Links during Ribose Glycations[14,23]

experiment (at 7 days)	[inhibitor] (mM)	% protein cross-linked
control (0 days)	0	13.7 ± 0.5
control	0	40.3 ± 3.7
Tx	5.0	14.4 ± 1.4
Tx	1.0	15.9 ± 1.7
Tx	0.2	27.0 ± 0.1
dmaPM, **11**	5.0	14.8 ± 0.4
dmaPM, **11**	1.0	19.1 ± 1.2
dmaPM, **11**	0.2	30.5 ± 0.1
dmaPN, **6**	5.0	25.8 ± 0.6
PM	5.0	18.4 ± 0.7
PM	1.0	27.2 ± 0.1
PN	5.0	32.8 ± 0.1

Supporting Information Available: Experimental procedures for the synthesis and characterization of the compounds in Schemes 1 and 2, including data in CIF format . This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) PM incubated with APC and the absence of ABAP showed no quenching of APC fluorescence (i.e., result identical to No ABAP).

(20) We speculate that protonation of the α^4-amino group of dmaPM, **11**, may free the phenol from an intramolecular hydrogen bond and result in improved radical trapping when compared to the hydrogen bond accepting α^4-hydroxyl group of dmaPN, **6**.

(21) HPLC conditions: Discovery C18 column, solvents A (water) and B (acetonitrile) both containing 0.1% heptafluorobutyric acid delivered at 1 mL/min as gradient; time (%A) 0 min (100); 30 (40). Quantification of inhibitors was at 293 nm UV against a calibration curve.

(22) Fluorescence of allophycocyanin (E_x = 633 nm, E_m = 660 nm) at 37 °C was measured in a 1 cm cell just before addition of ABAP, and at 5 min intervals, on a Photon Technology International C-700 fluorescence lifetime system.

(23) Conditions were 10 mg/mL BSA and 50 mM ribose in 0.2 M phosphate buffer pH 7.4 containing 1 mM DTPA and 1 mM phytic acid (chelators added after buffer was stored over Chelex-100 resin for 24 h).

(24) (a) For more complete examination of dmaPM (**11**) and Tx in glycation models, see: Culbertson, S. M.; Vassilenko, E.; Morrison, L.; Ingold, K. U. manuscript submission to J. Biol. Chem. (b) For example, results from ribose derived pentosidine yields also support radical trapping activity for dmaPM, **11**, and dmaPN, **6**.