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Exploring Post-translational Arginine Modification Using Chemically Synthesized Methylglyoxal Hydroimidazolones

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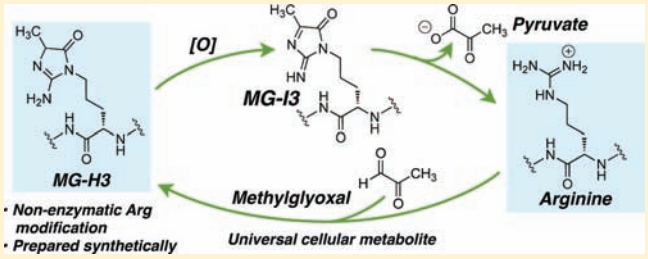
Supporting Information

ABSTRACT: The methylglyoxal-derived hydroimidazolones (MG-Hs) comprise the most prevalent class of non-enzymatic, post-translational modifications of protein arginine residues found in nature. These adducts form spontaneously in the human body, and are also present at high levels in the human diet. Despite numerous lines of evidence suggesting that MG-H−arginine adducts play critical roles in both healthy and disease physiology in humans, detailed studies of these molecules have been hindered by a lack of general synthetic strategies for their preparation in chemically homogeneous form, and on scales sufficient to enable detailed biochemical and cellular investigations. To address this limitation, we have developed efficient, multigram-scale syntheses of all MG-H−amino acid building blocks, suitably protected for solid-phase peptide synthesis, in 2−3 steps starting from inexpensive, readily available starting materials. Thus, MG-H derivatives were readily incorporated into oligopeptides site-specifically using standard solid-phase peptide synthesis. Access to synthetic MG-H-peptide adducts has enabled detailed investigations, which have revealed a series of novel and unexpected findings. First, one of the three MG-H isomers, MG-H3, was found to possess potent, pH-dependent antioxidant properties in biochemical and cellular assays intended to replicate redox processes that occur *in vivo*. Computational and mechanistic studies suggest that MG-H3-containing constructs are capable of participating in mechanistically distinct H-atom-transfer and single-electron-transfer oxidation processes. Notably, the product of MG-H3 oxidation was unexpectedly observed to disassemble into the fully unmodified arginine residue and pyruvate in aqueous solution. We believe these observations provide insight into the role(s) of MG-H−protein adducts in human physiology, and expect the synthetic reagents reported herein to enable investigations into non-enzymatic protein regulation at an unprecedented level of detail.



INTRODUCTION

Advanced glycation end-products (AGEs) comprise a structurally diverse class of post-translational protein modifications that form through non-enzymatic chemical processes in all living organisms, primarily on lysine and arginine side chains.[1,2] Out of more than eight arginine-derived AGEs reported to date, the methylglyoxal-derived hydroimidazolones (MG-Hs, Figure 1A) are believed to be the most prevalent in humans.[3] Estimates suggest that at least one MG-H adduct is present on 3−13% of the proteins found in the human body,[4,5] and that MG-H-modified arginine comprises 1.3% by weight of total enzymatic protein hydrosylate derived from alkali-treated baked goods such as pretzels.[6]

MG-H-protein adducts form both intra- and extracellularly as a mixture of three regioisomers (Figure 1A), via rapid reactions between arginine and methylglyoxal (MGO), a dicarbonyl metabolite produced by all living cells.[7] Notably, elevated levels of MGO and MG-H adducts have been shown to induce a range of cellular and subcellular effects including perturbations in cell signaling, inhibition of protein synthesis and cell growth,[8] induction of oxidative stress and pro-inflammatory cytokine release,[9−14] decreases in the adhesive properties of vascular basement membrane,[15] alterations in chaperone function,[16] and numerous other processes.[17,18] MGO concentrations have also been observed to increase in various disease states, including diabetes,[19−22] cancer,[23] cardiovascular disease,[24] and renal failure.[25] Taken together, these observations suggest that MG-H modifications may play a role in the pathophysiology of these and perhaps other illnesses.

Despite their putative biological properties and their ubiquity in humans, MG-Hs, and AGEs in general, have only been the subject of a limited number of synthetic studies. Available synthetic procedures for MG-H adducts are limited to providing products on analytical scales,[6] in very low purities,[26] or as a subset of the natural isomers.[27] The vast majority of published data regarding the biological functions of MG-Hs have been obtained using highly heterogeneous mixtures prepared via prolonged incubation of model proteins (such as albumins) with MGO.[28] Although these mixtures have

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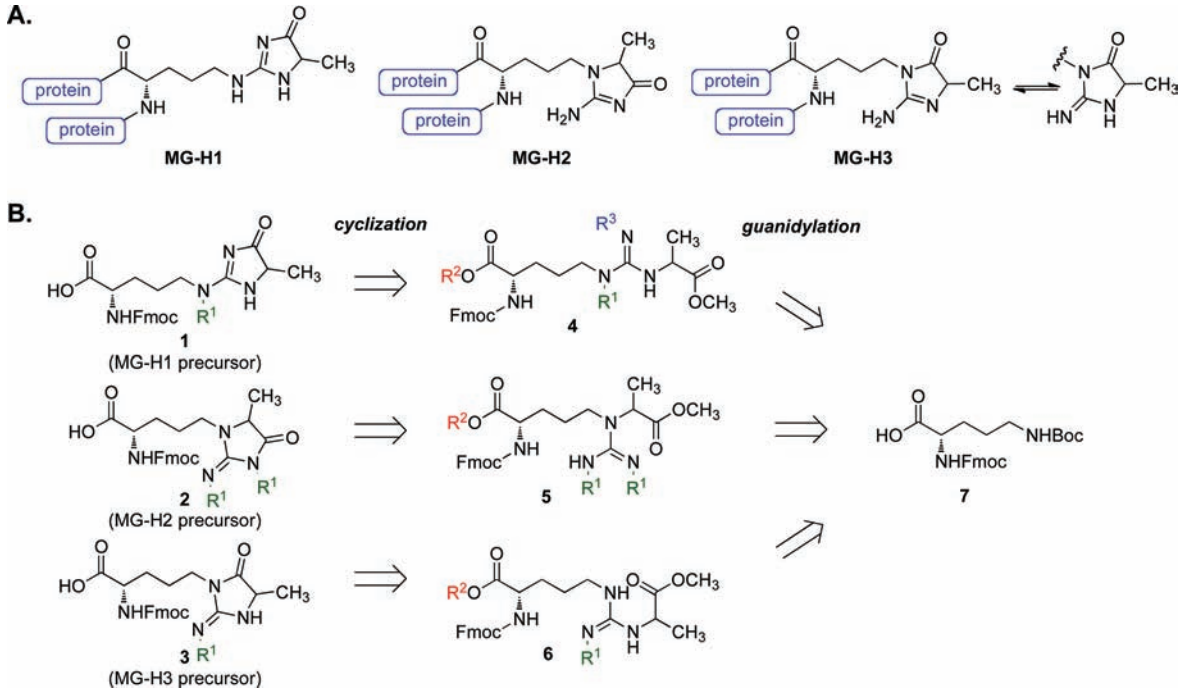
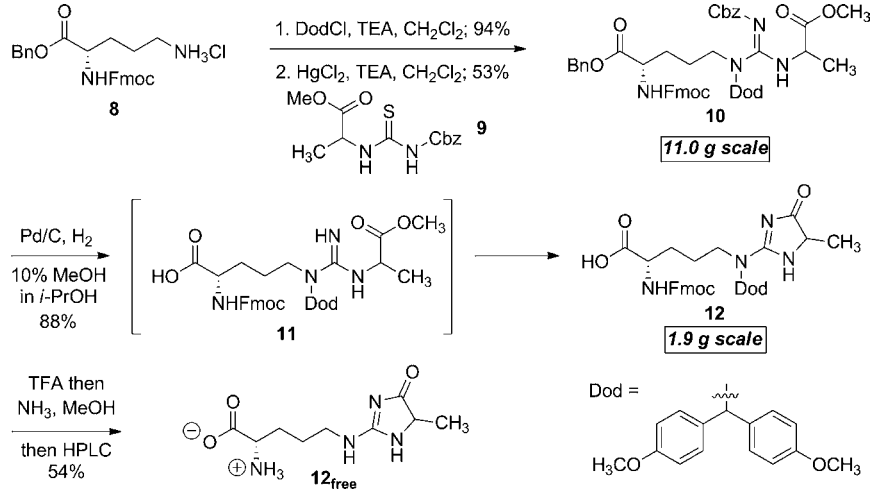


Figure 1. Stuctures and retrosynthesis of the methylglyoxal-derived hydroimidazolone (MG-H) class of AGEs. (A) The MG-Hs consist of three isomeric structures as shown, each formed through condensation of methylglyoxal with arginine. Note that MG-H3 is believed to exist as two low-energy tautomers, as shown. For simplicity, only the tautomer drawn on the left will be depicted throughout the remainder of the document. (B) Proposed "divergent" retrosynthesis of all three isomers derived from a single, readily available starting material.

Scheme 1



provided useful information regarding certain properties and functions of MGO−protein adducts, their heterogeneous nature complicates efforts to identify and characterize the specific structure(s) responsible for bioactivity.[29] Moreover, these samples are prone to contamination with varying levels of bioactive impurities (e.g. bacterial lipopolysaccharide).[30]

Here we report preparative-scale synthetic routes that for the first time afford access to the entire class of methylglyoxal-derived hydroimidazolones, as both amino acids and peptide conjugates. These routes to protected MG-H building blocks are concise (2−3 steps starting from readily accessible materials) and high yielding (44−52% overall yield), and MG-H monomers can readily be incorporated site-specifically into synthetic oligopeptides using automated Fmoc solid-phase peptide synthesis (SPPS). Critically, access to chemically characterized MG-H-containing peptide constructs has enabled us to perform biochemical and cellular evaluations, which have revealed a series of notable findings. First, we have discovered that MG-H3-containing oligopeptides are redox-active, both *in vitro* and in a cellular system. Notably, this redox activity is pH-dependent and has a molar potency comparable to that of ascorbic acid. Computational and mechanistic studies suggest that the MG-H3 core heterocycle possesses a versatile reactivity profile and is capable of participating in both one- and two-electron oxidation processes. Also, we unexpectedly found that the product of MG-H3 oxidation disassembles spontaneously in aqueous solution to provide the fully unmodified arginine side chain and pyruvate. Taken together, these data suggest that MG-H3 adducts may serve as pH-sensitive reducing agents under physiological conditions, leading to spontaneous

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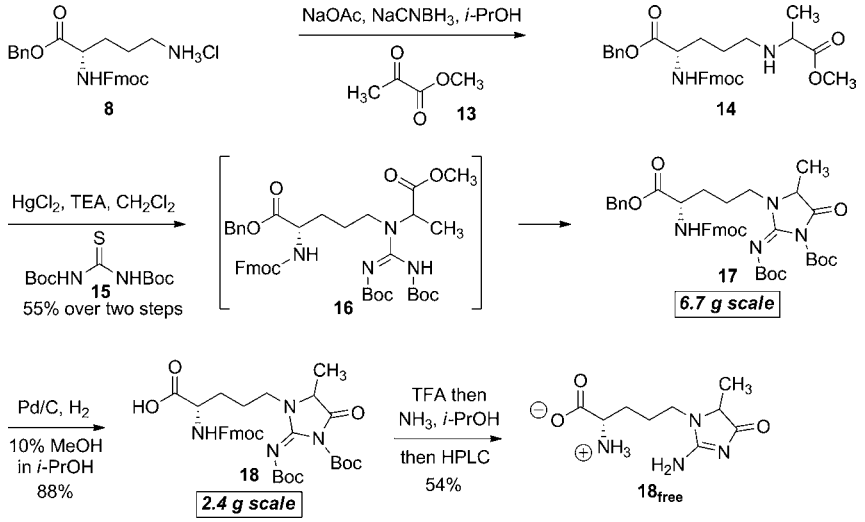
Scheme 2



deglycation of the hydroimidazolone core to regenerate the native arginine side chain. These results underscore the significant potential of chemically homogeneous MG-H adducts to facilitate chemical and biological investigations into these poorly understood species at an unprecedented level of detail.

RESULTS

Synthetic Studies. The arginine-derived hydroimidazoles MG-H1, MG-H2, and MG-H3 (Figure 1A) present substantial synthetic challenges. These compounds have been reported to undergo decomposition, spontaneous ring-opening, H/D exchange, isomerization, and rearrangement reactions.[31] Also, because MG-Hs likely exert their biological actions as peptide or protein conjugates *in vivo*, we required a synthesis that provided AGE-amino acid monomers suitably protected for Fmoc SPPS (Scheme 1). We therefore designed the retrosynthetic analysis shown in Figure 1B. We envisioned accessing protected isomeric MG-H monomers **1**–**3** via the regioselective cyclization of intermediates **4**–**6**. Regiocontrol in these ring-forming processes was anticipated to derive from the strategic placement of functional groups R^1, R^2, and R^3. We also expected that the appropriate protecting groups could be used to stabilize the acid- and base-labile hydroimidazolone ring during SPPS. Compounds **4**–**6** could then be derived from the commercial reagent N^{α}-Fmoc-N^{δ}-Boc-L-ornithine (**7**) via simple alkylation and/or guanidylation processes.[32] Due to published evidence indicating that the ring methine proton in all three MG-H isomers rapidly undergoes epimerization or exchange in water,[6,33] we decided to target these compounds as diastereomeric mixtures.

Initial synthetic efforts focused on accessing a suitably protected MG-H1–arginine adduct. Based on our preliminary studies, and prior observations suggesting that the MG-H1 isomer is kinetically disfavored,[34] we hypothesized that a blocking group on the ornithine side chain (e.g., R^1 in **4**) would be necessary to direct cyclization through the desired pathway. Furthermore, we discovered that guanidylation required the side-chain *N*-atom to be electron rich,[35] thus ruling out the majority of common *N*-protecting groups. After initial experiments, which revealed that sterically unhindered substituents on the ornithine side-chain amine (e.g., trimethoxybenzyl) led to spontaneous internal removal of the Fmoc group, we settled on the bis(4-methoxyphenyl)methyl (Dod) group (Scheme 1).[36,37] Application of this derivative to Fmoc-ornithine-OBn·HCl (**8**), followed by $HgCl_2$-promoted coupling with Cbz-thiourea (**9**), smoothly provided the corresponding guanidine (**10**) in good yield (50% over two steps).[35] Notably, this two-step sequence could be executed on preparative scales (11.0 g) without difficulty. Subjection of **10** to catalytic hydrogenolysis cleaved the Cbz and benzyl ester groups in a single operation, and the resulting intermediate (**11**) underwent spontaneous intramolecular cyclization to provide the MG-H1 building block (**12**) as the exclusive product in 88% yield. Overall, this sequence provides **12** in 43% overall yield in only three synthetic steps, starting with >12 g of ornithine derivative **8**. Removal of the remaining protecting groups in compound **12** afforded the free MG-H1–arginine adduct as a TFA salt ($\mathbf{12}_{free}$**·TFA**), whose spectral properties (^{1}H NMR, ^{13}C NMR, and HRMS) agreed well with literature values reported for a sample isolated from bakery products.[6] Assignment of the hydroimidazolone ring in **12** and $\mathbf{12}_{free}$ as the indicated tautomer was based on comparisons with previously reported data in homologous systems and detailed computational studies (Table S2).[38,39]

Having identified a successful route to the MG-H1 monomer (**12**), we next turned our attention to MG-H2 (**18**, Scheme 2). Toward this end, we developed a simple sequence consisting of reductive amination (**8** → **14**), guanidylation (**14** → **16**), and cyclization (**16** → **17**), which afforded iminohydantoin **17** in 55% overall yield. Chemoselective cleavage of the benzyl ester in **17** provided side-chain- and backbone-protected MG-H2 monomer **18** in only three synthetic steps starting from **8** and in 48% overall yield. Global protecting group removal proceeded smoothly to yield MG-H2-arginine adduct $\mathbf{18}_{free}$**·TFA**, which matched previously reported spectroscopic data.[33,40] Connectivity of $\mathbf{18}_{free}$ was also independently confirmed through HMBC experiments, which indicated strong couplings between the ring methine proton and the adjacent side-chain C-atom (Figure S5). Tautomeric forms of **18** and $\mathbf{18}_{free}$**·TFA** were assigned based on comparison to previously reported model systems,[38,39] and results of DFT calculations (Table S3).

Finally, MG-H3 precursor **22** was obtained simply by rearranging chemical transformations that had proved success-

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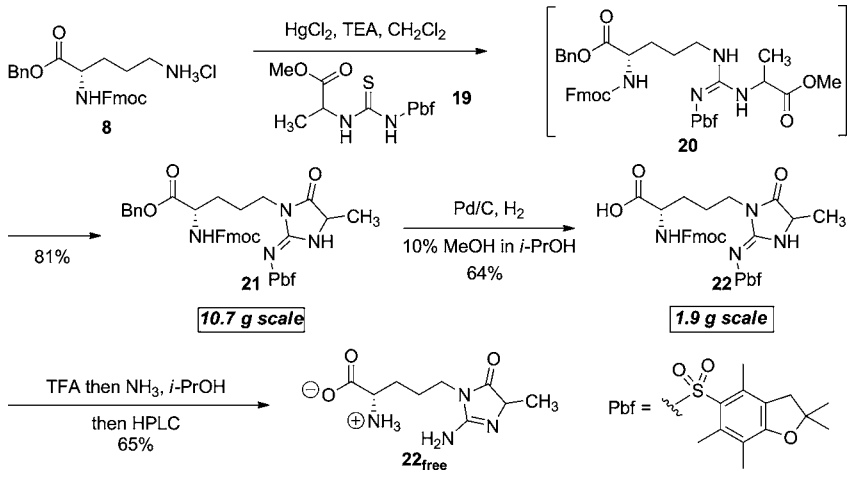
Scheme 3



ful in the routes above. This sequence (Scheme 3) began with mercury-mediated guanidylation of **8** with 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf)-derived thiourea **19**,[46] which afforded an intermediate *N,N′*-bis-alkyl-guanidine (**20**). Although, in theory, pathways for forming either MG-H1 or MG-H3 ring systems were both accessible via **20**, *in situ* cyclization provided MG-H3 precursor **21** as a single regioisomer, likely due to the steric influence of the bulky Pbf group. With **21** in hand, benzyl ester removal provided compound **22** in 64% yield. Overall, this sequence allowed us to access monomer **22** in only two steps and 52% overall yield starting from **8**. Protecting group removal provided $\mathbf{22}_{free}$**·TFA**, whose spectral properties matched previously reported values.[33,40] Notably, as for the MG-H1 series (Scheme 1), synthetic sequences for accessing both MG-H2 and MG-H3 precursors could be carried out effectively on large scales (6.7–10.7 g). Connectivity assignments in $\mathbf{22}_{free}$**·TFA** were inferred from HMBC couplings between the side-chain protons and the ring carbonyl carbon (Figure S6). However, the tautomeric preference of $\mathbf{22}_{free}$ could not be unequivocally established (see Supporting Information).[38]

With protected monomeric hydroimidazolone derivatives **12**, **18**, and **22** in hand, we set out to construct several AGE-containing oligopeptide sequences. Toward this end, we synthesized various oligopeptides, site-specifically substituting the arginine residues with the corresponding hydroimidazolones (Table 1). HSA fragments (residues 111–120 and 407–415) were chosen because of their established propensities toward glycation *in vitro*,[41,42] and were prepared in good to excellent yields, with purities universally greater than 95%.[38] Spectroscopic data for peptides **23–25** were found to match with data previously reported for these compounds.[33,40] Overall, this strategy represents the first general route for site-specifically incorporating MG-H residues into oligopeptides, is effective on large scales, and also enables straightforward access to multiply AGE-derivatized constructs (Table 1, entries 8–11).[33,40]

Although the ring stereocenters in MG-H1 and MG-H3 have been reported previously to undergo epimerization,[6] the rate of epimerization has not yet been quantified. Thus, we investigated rates of H/D exchange using constructs **23–25**. Dissolution of these peptides as the corresponding formate salts in neat D_2O revealed a gradual disappearance of the hydroimidazolone ring α-proton for all three MG-H isomers

Table 1. Synthesis of Hydroimidazolone-Containing Peptides

entry	compd	HSA fragments	peptide sequences	isolated yield (%)[a]
1	**23**	n/a	LG-(**MG-H1**)-AG	32
2	**24**	n/a	LG-(**MG-H2**)-AG	33
3	**25**	n/a	LG-(**MG-H3**)-AG	47
4	**26**	407–415	LLV-**R**-YTKKV	46
5	**27**	407–415	LLV-(**MG-H1**)-YTKKV	59
6	**28**	407–415	LLV-(**MG-H2**)-YTKKV	37
7	**29**	407–415	LLV-(**MG-H3**)-YTKKV	55
8	**30**	111–120	NLP-**R**-LV-**R**-PEV	33
9	**31**	111–120	NLP-(**MG-H1**)-LV-(**MG-H1**)-PEV	39
10	**32**	111–120	NLP-(**MG-H2**)-LV-(**MG-H2**)-PEV	36
11	**33**	111–120	NLP-(**MG-H3**)-LV-(**MG-H3**)-PEV	49

[a]Peptides were isolated in >95% purity following HPLC purification.

as monitored by ^{1}H NMR spectroscopy. The half-lives of H/D exchange in these peptides were calculated to equal 40.5, 28.5, and 15.4 h (Figures S1–S3), respectively.[38] Other workers have observed an increase in the rate of epimerization at pH 5.0 versus 7.4, suggesting that this process may occur rapidly under physiological conditions.[34] Taken together, these data suggest that MG-Hs likely exist as thermodynamic mixtures of diastereomers in the body.

Biochemical Investigations. Strong correlations between oxidative stress and AGE formation have been observed both *in vitro* and *in vivo*.[43–47] In particular, MGO–protein adducts have been suggested to induce the formation of reactive oxygen species (ROS) in tissue culture,[9–14] and treatment of bovine serum albumin (BSA) with MGO has been shown to cause free radical generation by EPR spectroscopy.[48] Interestingly, glycated proteins believed to contain MG-H adducts have also been shown to possess antioxidant properties *in vitro*.[49,50] Taken together, these observations led us to hypothesize that hydroimidazolone adducts might be capable of participating in redox processes.

To test this hypothesis, we first evaluated the redox activity of MG-H–peptide conjugates **26–29** *in vitro*, in the MTT and DPPH assay systems (Figure 2). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt

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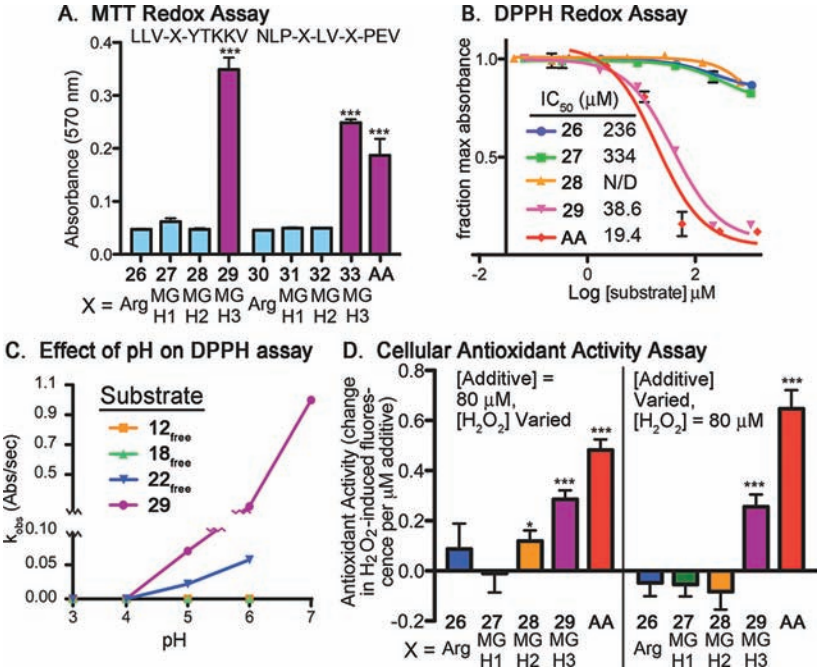

Figure 2. MG-H3-containing peptides possess antioxidant activity comparable with that of ascorbic acid (AA). (A) Peptides **26**–**29**, **30**–**33**, and AA were evaluated for their ability to reduce yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan at room temperature. Data represent the mean of triplicate experiments ± standard deviation. Data in the indicated columns (***, $P < 0.0001$) were found to differ from all other data sets in the figure using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison posthoc test. (B) Radical quenching activity of peptides **26**–**29** was determined by treatment with the stable radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH). Values represent the mean of triplicate experiments ± standard deviation. (C) Effect of pH on the observed rate of DPPH quenching by both MG-H3–peptide and MG-H3–amino acid conjugates. Note that determination of DPPH quenching rate of free MG-Hs at pH 7 was impeded by high assay background. Values represent the mean of triplicate experiments ± standard deviation. (D) Antioxidant activity of MG-H–peptide conjugates in cellular assays. RAW 264.7 macrophages were pre-incubated with dihydrorhodamine 123, a cell-permeable fluorogenic probe converted to a highly fluorescent product on exposure to various cellular oxidants, then treated with oxidant (H_2O_2) and additive (peptide conjugates or AA). Two treatment regimes were explored; in the first, oxidant concentration was varied at a fixed level of additive (left-hand panel), while in the second, additive was varied at a fixed amount of oxidant (right-hand panel). Reported "Antioxidant Activity" values reflect the extent to which each additive is capable of suppressing H_2O_2-induced fluorescence. These values (±standard error) were calculated from linear least-squares fits of fluorescence intensity data plotted versus additive concentration as detailed in the Supporting Information. *P*-values (two-tail) represent the probabilities that pair-wise differences in slope (plus versus minus additive) could have arisen from randomly chosen data points, and are reported as follows: ***, $P < 0.0001$; *, $P < 0.05$. All reported trends were observed on at least two separate occasions.

that can be reduced to a purple formazan by mitochondrial enzymes,[51] superoxide,[52] or direct electron transfer mechanisms.[53] This indicator has been used to measure cell viability,[54] superoxide production,[52] and the antioxidant activity of natural products.[55] DPPH (1,1-diphenyl-2-picryl-hydrazyl), on the other hand, is a stable radical that can be quenched by either hydrogen atom abstraction or proton-coupled electron transfer, and is commonly used to estimate the ability of substrates to serve as H-atom donors in aqueous media.[56] Peptide conjugates containing the MG-H3 modification (**29** and **33**) exhibited antioxidant activities in both MTT (Figure 2A) and DPPH (Figure 2B) assays at comparable molar potencies to ascorbic acid (vitamin C), an essential nutrient that serves as both an antioxidant[57] and enzyme cofactor.[58] Surprisingly, despite their high structural similarities to MG-H3, neither MG-H1 nor MG-H2 exhibited any redox activities under these conditions.

We also evaluated the effect of pH on the rate of DPPH quenching in the presence of various MG-H constructs (Figure 2C). Once again, MG-H3 adducts demonstrated significant activity in this assay, both as peptide conjugate **29** and as the free amino acid monomer ($\mathbf{22}_{free}$), while MG-H1 and MG-H2 constructs were inactive. Furthermore, the activity of MG-H3 conjugates was observed to increase at pH values approaching 7, which is consistent with the results of pK_a titrations and computational studies (see Table 2, below). Interestingly, pH changes were found to exert a far greater effect on DPPH reduction rate for MG-H3–peptide conjugate **29** than the corresponding free amino acid ($\mathbf{22}_{free}$). Taken together with observations in MTT assays (Figure 2A), this context-dependent pH sensitivity suggests that the chemical properties of MG-Hs may be influenced by neighboring amino acid residues, and that such effects may be important determinants of AGE effects *in vivo*.

To determine the cellular relevance of this redox behavior, we explored the effects of MG-H–peptide conjugates using a well-established cellular assay protocol.[59] Thus, RAW 264.7 macrophages were treated with dihydrorhodamine 123, a cell-permeable fluorogenic probe converted to a highly fluorescent product on exposure to cellular oxidants, in the presence of varying concentrations of oxidant (H_2O_2) and MG-H–peptide conjugates.[60] As shown in Figure 2D, MG-H3 conjugate **29** was found to possess significant cellular antioxidant activity, which was both concentration-dependent (right panel) and robust over a range of physiologically relevant H_2O_2 concentrations (left panel, see Supporting Information for more detail). Interestingly, these effects were slightly smaller in magnitude than those of ascorbic acid, perhaps due to the relatively poor membrane permeability of this specific peptide sequence

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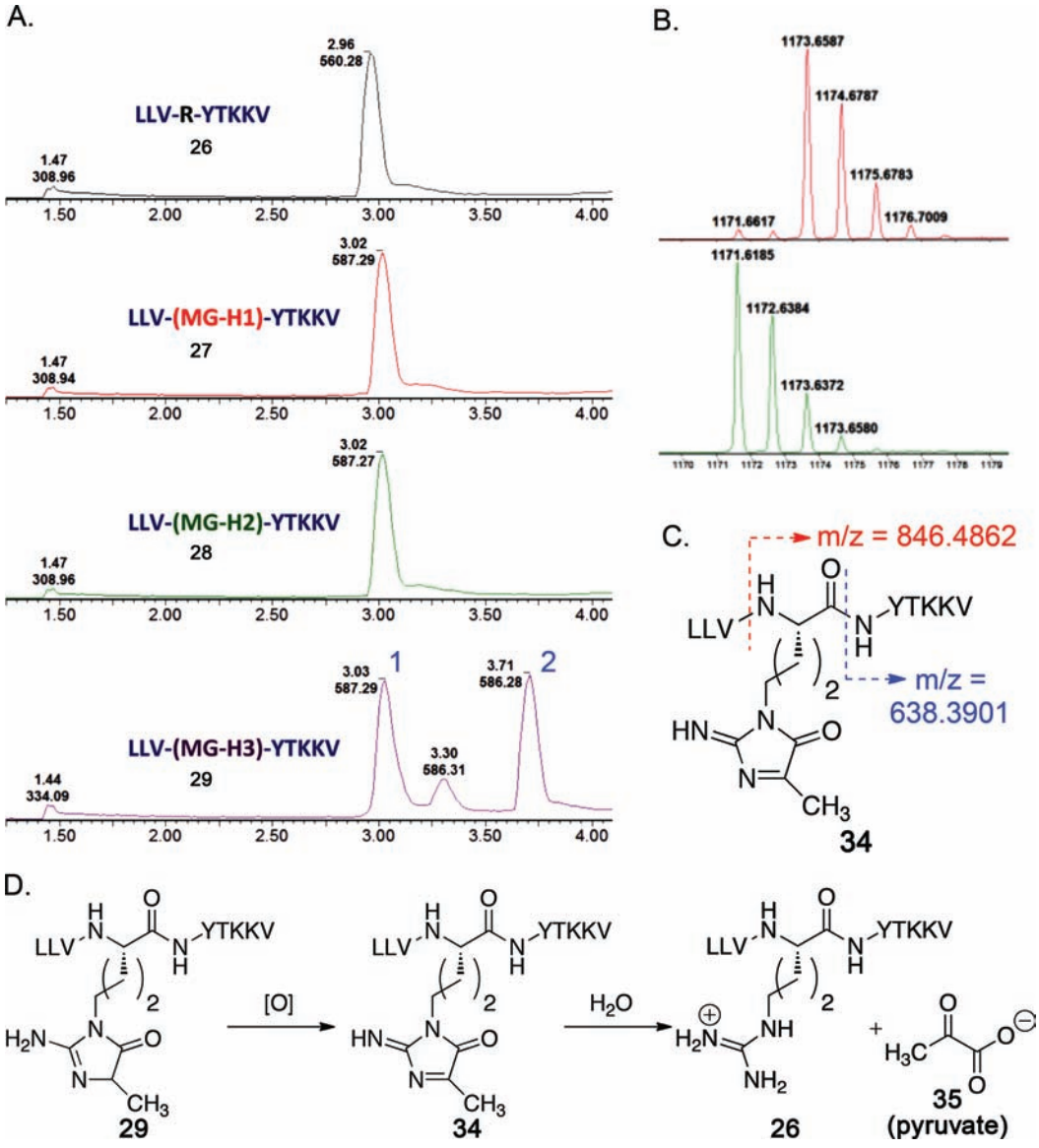

Figure 3. LC/MS and LC/MS-MS analyses of hydroimidazolone–peptide conjugates from MTT antioxidant assays. (A) LC traces of MTT assay reaction mixtures. Two new peaks are observed in assays involving peptide **29**, while experiments employing peptides **26**–**28** indicate only starting materials. (B) Mass spectra corresponding to material contained in the LC peaks labeled 1 and 2 in panel A. These experiments indicate a loss of two mass units in newly formed material versus the parent peptide **29**. (C) LC/MS-MS fragmentation analysis of material in peak 2. These data suggest that the decrease in reaction product mass originates at the MG-H3-modified residue. (D) Schematic reaction mechanism illustrating the overall transformation from MG-H3-modified peptide **29** to the corresponding Arg-modified sequence (**26**) and pyruvate (**35**) by way of imidazolone **34**.

(Figure S17). Because MGO and corresponding MG-H adducts are present physiologically both inside and outside of the cytosol, these data may underestimate the effects of MG-H3 adducts *in vivo*. Interestingly, MG-H2 conjugate **28** was found to possess detectable antioxidant effects, which were only revealed under conditions of variable H_2O_2 concentration. This observation may suggest that MG-H2 possesses cellular redox activity that emerges under conditions of high oxidative stress. As before, MG-H1 conjugate **27** was found to be inactive in this assay. Taken together, these studies demonstrate the capacity of MG-H3–peptide conjugates to serve as antioxidants in a cellular milieu in the context of physiologically relevant reactive oxygen species.

To characterize the products of redox reactions involving MG-H species, we next performed LC/MS analyses of MTT assay mixtures using peptides **26**–**29**. These experiments indicated the formation of a new species with a mass 2 units less than that of parent peptide **29** (Figure 3A,B), suggesting oxidation to the corresponding imidazolone (MG-I3, **34**).[61] Tandem mass spectrometry (MS/MS) fragmentation analysis (Figure 3C) further confirmed this decrease in mass to originate at the MG-H3 residue. Detailed NMR experiments, performed on the oxidized intermediate **34**, further support our proposed structural assignment (Figure S8). Interestingly, an analogous MG-H1-derived imidazolone was postulated in prior work to form under aerobic conditions in model reactions between MGO and BSA or model peptides;[61,62] however, subsequent studies revealed this structural assignment to be incorrect, and the adduct initially believed to be the imidazolone was actually the argpyrimidine modification.[62]

Based on the electron-deficient nature of the imidazolone oxidation product, we speculated that perhaps this material

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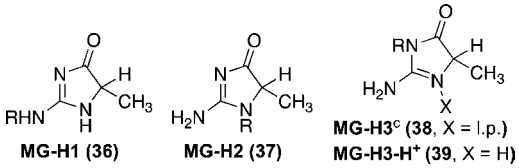
could react with arginine and/or lysine residues to form inter- or intramolecular cross-links. Formation of such cross-links is common in protein glycation processes, and has been cited as a critical contributing factor in the impact of AGE formation on protein structure and function.[63] Unexpectedly, dissolution of oxidized peptide **34** in physiological buffer led to spontaneous hydrolysis to provide the parent arginine adduct **26** and pyruvate (**35**, Figure 3D, Figures S9–S11). This hydrolysis process was found to proceed at pH 7.4 with first-order kinetics (k_{obs} = 1.84 × 10^{-5} s^{-1}; $t_{1/2}$ = 10.5 h) at a rate compatible with the lifetime of many intracellular proteins (Figure S11). Hydrolysis of imidazolone adducts to provide unmodified arginine residues is therefore kinetically competent to occur under physiological conditions.[64] Notably, these results provide a novel mechanistic framework through which MG-H3-modified proteins can directly participate in cellular redox processes and recycle back to unmodified species. Furthermore, the observation of the pyruvate byproduct in these studies supports the intermediacy of MG-I3 (**34**), and also provides evidence that MG-H adducts can non-enzymatically cleave to give rise to a metabolically useful intermediate.

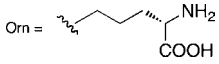
We next conducted mechanistic studies to gain insight into the origin of MG-H3's antioxidant activity. We first prepared derivatives of **29** in which the methine C–H bond in the MG-H3 core heterocycle was replaced with deuterium. Indeed, the effect of isotopic substitution on reduction rate differed substantially between the MTT and DPPH assays; a kinetic isotope effect (KIE) of 1.38 was observed using the former assay system, and 2.27 using the latter (Figures S15 and S16). The relatively large KIE observed in DPPH assays supports a mechanism involving C–H/D bond homolysis as the rate-limiting step,[65] while the relatively small KIE for the MTT assay is consistent with speculations that electron-transfer pathways are rate-limiting under these conditions.[53] Taken together, these data suggest that MG-H3 is capable of functioning as an antioxidant by way of two distinct mechanistic pathways.

To shed light on the observed reactivity differences between MG-H isomers, we performed a series of pK_a titrations and Density Functional Theory (DFT) calculations (B3LYP/6-311++G(2df, 2p), Table 2). These experiments revealed a number of intriguing features, which may provide explanations for many of MG-H3's unique properties. For example, indications that the MG-H3 core heterocycle (**38**) possesses C–H bond-dissociation energy (BDE) and ionization energy (IE) values that are *both* significantly lower than those of isomeric compounds **36** and **37** may explain the multimodal activity of MG-H3 derivatives in both MTT and DPPH assays. Furthermore, the observation that **38** possesses a pK_a value near neutrality, taken together with calculations suggesting that protonation of the core heterocycle (**39**) increases both BDE and IE, may explain the deleterious effect of decreasing pH on antioxidant activity (Figure 2C). Interestingly, the observed pK_a trends for various MG-H isomers are in agreement with experimental observations of structurally homologous isomers of creatinine.[39,66] Because the observed reactivity differences between MG-H isomers do not correlate with differences between free energy of oxidation (ΔG_{ox}) values (Table 2), we speculate that they reflect kinetic rather than thermodynamic factors.

Table 2. pK_a Values and Calculated Energies for MG-H Derivatives[a,b]



	pK_a				
compd	exptl[d]	calcd[e]	BDE[f] (kcal/mol)	IE[g] (kcal/mol)	ΔG_{ox}[h] (kcal/mol)
36	4.58	3.58	76.2	153.2	26.0
37	4.68	3.81	76.5	150.6	22.3
38	7.70	7.44	68.3	141.0	23.8
39	n/r	n/r	76.4	186.9	n/r

Orn = 

[a]R = Orn for compounds employed in pK_a measurements, and R = Me for structures used in calculations. Details of energetic calculations for relevant tautomeric and protonated forms can be found in the Supporting Information (SI). [b]All calculations were performed utilizing the Gaussian 09 program suite. Geometry optimizations were performed using the 6-31+G(d) basis set, and single-point energies determined at the 6-311++G(2df,2p) level. All calculations implemented a continuum model to account for the effects of water solvent. l.p. = lone pair. Additional details regarding pK_a titrations and calculations can be found in the SI. [c]Calculations pertaining to other MG-H3 tautomers can be found in the SI. [d]pK_a values were obtained experimentally via NMR titration. [e]pK_a values were calculated as detailed in the SI. [f]Bond dissociation energy. [g]Ionization energy. [h]Data correspond to the free energy difference between the indicated MG-H and the corresponding imidazolone oxidation product, as described in the SI.

DISCUSSION

Here we disclose robust synthetic routes that enable straightforward incorporation of all known variations of hydroimidazolone-modified arginine residues into peptides via automated Fmoc SPPS. These constructs have allowed us to undertake amongst the first detailed biochemical investigations into chemically homogeneous MG-Hs. Previously, such studies have been hampered due to limited availability of preparative-scale synthetic strategies for peptide-MG-H constructs. Our investigations have revealed a series of unexpected findings. First, using well-characterized assays to measure redox behavior, we have determined that adducts containing MG-H3 can serve as potent antioxidants *in vitro* and in cellular systems, while isomeric MG-H1 and MG-H2 conjugates are significantly less active under identical conditions. Based on kinetic isotope effects and computational results, we believe that MG-H3 conjugates can participate in redox reactions through two distinct mechanisms. In DPPH assays, we postulate this mechanism to involve rate-limiting H-atom transfer,[65] while we speculate the MTT reduction to proceed by way of rate-limiting single-electron transfer, as previously described.[53] Furthermore, results from both experimental and computational studies support that protonation of MG-H3's core heterocycle takes place within the physiological range and diminishes its reactivity, suggesting a possible role for pH fluctuations in regulating MG-H oxidation *in vivo*. Finally, we have observed that MG-H3 oxidation leads to spontaneous decomposition to pyruvate and fully unmodified arginine in aqueous solution. This result provides strong support for a previously unreported "deglycation" mechanism for MG-H-modified arginine residues, which may be relevant *in vivo*.

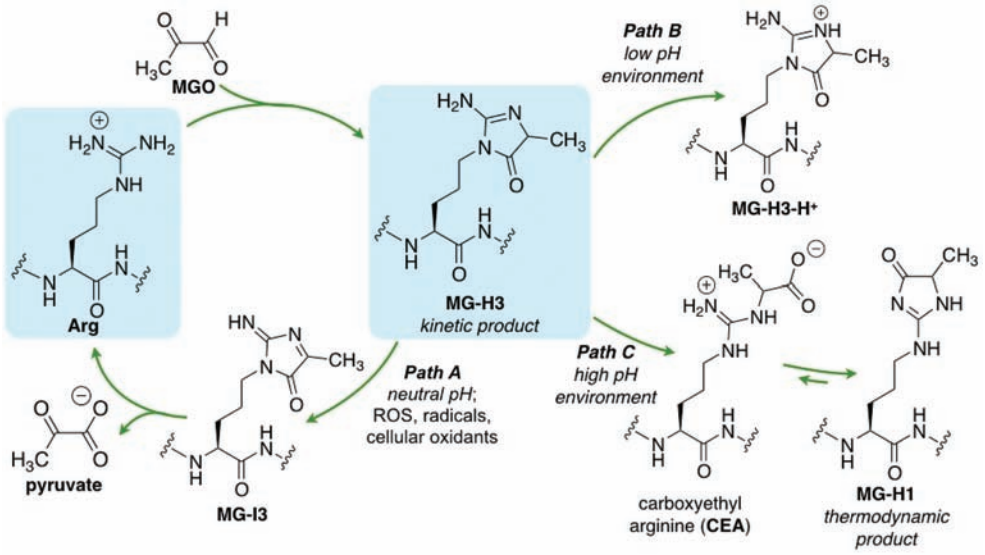


Figure 4. Proposed model for the regulation of arginine glycation. Accessible Arg side chains react with MGO to form a mixture of isomeric MG-H adducts, of which MG-H3 is believed to be the kinetic product. MG-H3 is sensitive to local perturbations in pH or redox balance. Path A: At neutral pH, MG-H3 undergoes rapid oxidative conversion to MG-I3, which then spontaneously hydrolyzes to regenerate Arg and pyruvate. Path B: At low local pH, or in proximity to acidic residues, MG-H3-H^+ is formed, and this species is expected to be relatively insensitive to changes in redox balance. Path C: At high pH or in proximity to basic residues, on the other hand, ring-opening of MG-H3 is expected to afford carboxyethylarginine (CEA) and MG-H1, both of which are predicted to be relatively insensitive to redox changes.

In light of our observations, as well as previous reports, we hypothesize that non-enzymatic post-translational modification of protein arginine residues may occur *in vivo* through the pathway shown in Figure 4. First, based on previous studies, unmodified arginine residues are hypothesized to form MG-H isomers at a rate related to MGO concentration, pH, and other protein- and context-specific variables (e.g., subcellular localization, sequence-dependent rate accelerations, etc.). Our data suggest that, once formed, MG-H3 adducts are greatly influenced by their local environment. At physiological pH, MG-H3 adducts are expected to exist as a nearly equimolar mixture of unprotonated and protonated (MG-H3-H^+) forms on the basis of pK_a determinations (Table 2). Thus, upon encountering appropriate oxidizing agents (e.g., reactive oxygen species or free radicals), unprotonated MG-H3 can undergo a net two-electron oxidation to yield MG-I3 (Figure 4, path A), which spontaneously hydrolyzes to afford arginine and pyruvate. Because oxidation is most likely a faster process than hydrolysis at neutral pH—for example, DPPH scavenging by peptide **29** has an observed half-life of approximately 6 min (Figure S11), while hydrolysis has a half-life of 10.5 h (Figures S5–S7)—the possibility exists that significant concentrations of MG-I3 can accumulate physiologically, perhaps leading to protein–protein cross-linking or oxidative stress.[67]

In situations where protonation is favored (Figure 4, path B), MG-H3 adducts are protected from oxidation (Figure 2C, Table 2) and ring-opening.[31] They are therefore believed to be quite stable—for example, the half-life of $\mathbf{22}_{free}$ is approximately 24 days in DPPH assays run at pH 4.0 (Figure 2C) and 14.8 days in hydrolysis assays conducted at pH 5.4 and 37 °C.[31] One might also speculate that the presence of a positive charge in MG-H3-H^+ serves to mitigate destabilizing effects of side-chain modification on protein structure,[68] as opposed to other MG-H modifications, which would be uncharged at neutral pH. Further studies will be necessary to investigate these possibilities in detail. Finally, in more basic environments (Figure 4, path C), MG-H3 has a half-life of approximately 20 min in pH 9.4 buffer at 37 °C,[31] and the primary products of hydrolysis are carboxyethylarginine (CEA) and MG-H1.[34] MG-H1 is also expected to be the most thermodynamically stable MG-H isomer; our calculations suggest it to be approximately 2.39 kcal/mol lower in energy than MG-H3 (Supporting Information, Table S5), a trend which is supported by previous experimental studies.[34] MG-H1 is therefore hypothesized to represent a "long-lived" modification that is susceptible neither to hydrolytic nor oxidative removal. Indeed, MG-H1 is often identified as the major MG-H formed *in vivo*,[33] perhaps because of its stability relative to MG-H3, and the scarcity of MG-H2.[33]

Also worth noting are the apparent functional similarities between MG-H3 and ascorbic acid in redox assays (Figure 2). Intriguingly, like MG-H3, ascorbic acid has been documented to serve as a reducing agent through both single-electron- and H-atom-transfer mechanisms, leaving open the possibility that these species perform similar functions *in vivo*.[69] Similarly, ascorbate's paradoxical oxidative activity, observed *in vitro* in the presence of transition metals,[70] may be mechanistically linked to pro-oxidant effects reported for MGO-modified proteins.[71] Based on reported literature values,[17,72] we estimate that concentrations of MG-H–arginine adducts can range from 100 nM to 3 μM in plasma or cytosol (higher levels are observed among diabetic patients), while ascorbate is found in the plasma at approximately 6–60 μM and in cytosol at approximately 35–500 μM.[70,73] These rough estimates suggest that MG-H3 may function as a bulk antioxidant physiologically. Also, the high cellular permeability of MGO suggests the existence of MG-H–protein adducts both intra- and extracellularly, although these modified proteins themselves may be impermeable.

In conclusion, we hypothesize that both formation and removal of MG-H adducts occur in a pH- and/or redox-regulated manner, and that these processes could be involved in normal physiology and perturbed in various disease processes.

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Validation of this theory will require follow-up studies of significant scope, which will be enabled by chemically homogeneous AGE derivatives, and have the potential to implicate MG-Hs and other AGE family members in a much broader range of life processes than previously suspected.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and compound characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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