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Exploring Post-Translational Arginine Modification Using Chemically Synthesized Methylglyoxal Hydroimidazolones (MG-Hs)

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Abstract

The methylglyoxal-derived hydroimidazolones (MG-Hs, Figure 1A) 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, multi-gram-scale syntheses of all MG-H–amino acid monomers 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 (SPPS).

Access to synthetic MG-H-peptide adducts has enabled detailed biochemical 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 to reflect meaningfully on 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

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Supporting Information Available: Detailed experimental procedures and compound characterizations are provided.

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 modifications comprise 1.3% by weight of total protein in heat-treated foods (e.g., baked goods, cereals, and dairy products).[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 illnesses.

Despite their putative biological properties and their ubiquity in humans, synthetic studies of the MG-Hs, and AGEs in general, are in their infancy. 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 has been obtained using highly heterogeneous mixtures prepared via prolonged incubation of model proteins (such as albumins) with MGO.[28] Although these mixtures have 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 (MG-Hs), both as amino acids and peptide conjugates. These routes 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 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 distinct 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 suggests that MG-H3 adducts may serve as pH-sensitive reducing agents under physiological conditions, leading to spontaneous de-glycation 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 through MG-H3 (Figure 1A) present substantial synthetic challenges. These compounds have been reported to decompose rapidly, and undergo 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 through R^3. We also expected that the appropriate protecting groups could be used to stabilize the acid-and base-labile hydroimidazolone ring during SPPS. Due to published evidence indicating that the ring methine proton in all three MG-H isomers rapidly undergoes epimerization or exchange in water,[6,32] we decided to target these compounds first as diastereomeric mixtures. Ultimately, **4**–**6** could be derived from the commercial reagent N^{α}-Fmoc-N^{δ}-Boc-L-ornithine (**7**) via simple alkylation and/or guanidylation processes.[33]

Initial efforts focused on a suitably protected MG-H1-arginine adduct. Based on our preliminary studies, and prior observations suggesting that the MG-H1 isomer is kinetically disfavored,[42] 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,[34] thus ruling out the majority of common *N*-protecting groups. After initial experiments, which revealed that sterically unhindered substituents (e.g., trimethoxybenzyl) led to spontaneous internal removal of the Fmoc group, we settled on the bis-(4-methoxyphenyl)-methyl (Dod) group to block the ornithine side-chain (Scheme 1).[35,36] 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).[34] 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 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, and can be conducted starting with >12 g of ornithine-derivative **8**. Two-step removal of the remaining protecting groups afforded the free MG-H1–arginine adduct as a TFA salt ($\mathbf{12_{free \cdot TFA}}$), whose spectral properties (1H-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 calculations (Table S2) and comparisons with previously reported data in homologous systems.[37,38]

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 an intermediate 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 and in 48% overall yield. Global protecting group removal proceeded smoothly to yield MG-H2-arginine adduct $\mathbf{18_{free \cdot TFA}}$, which matched previously reported spectroscopic data.[32,39] Connectivity of $\mathbf{18_{free \cdot TFA}}$ was also independently confirmed by heteronuclear HMBC coupling between the ring methine

proton and the side-chain carbon (Figure S5). Tautomeric forms of **18** and $\mathbf{18_{free}}$ were assigned based upon calculations (Table S3) and comparison to previously reported simplified systems.[37,38]

Finally, MG-H3 precursor **22** was obtained simply by rearranging chemical transformations that had proved successful 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. 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 $\mathbf{22_{free \cdot TFA}}$ whose spectral properties matched previously reported values.[32,39] 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 $\mathbf{22_{free \cdot TFA}}$ were inferred from heteronuclear 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).[37]

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 oligopeptides derived from human serum albumin (HSA), site-specifically substituting the arginine residues with the corresponding hydroimidazolone derivatives (Table 1). These included HSA fragments (residues 111–120 and 407–415), chosen because of their established propensities toward glycation *in vitro*,[40,41] and were prepared in good to excellent yields, with purities universally greater than 95%.[37] Spectroscopic data for peptides **23**–**25** was found to match perfectly with data previously reported for these compounds.[32,39] Overall, this strategy represents the first feasible route for making AGE-peptide conjugates on large scale, and also enables straightforward access to multiply AGE-derivatized constructs (Table 1, entries 8–11).[32,39]

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 a formate salt in neat D_2O revealed a gradual disappearance of the hydroimidazolone ring α-proton for all three MG-H isomers as monitored by ^1H NMR spectroscopy. The half-lives of H/D exchange in these peptides were calculated to equal 40.5, 28.5, and 15.4 hours (Figures S1–3), respectively.[37] 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.[42] Taken together with the mechanism for formation of MG-Hs from an achiral molecule (MGO), this data suggests that MG-Hs likely exist as thermodynamic mixtures of diastereomers in the body.

Biochemical Investigations

With various MG-H–peptide constructs in hand, we were intrigued by strong positive correlations between oxidative stress and AGEs formation, reported in both *in vitro* and *in vivo* assays.[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 anti-oxidant 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 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 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 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 anti-oxidant 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 (**22**$_{\mathbf{free}}$), 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 (**22**$_{\mathbf{free}}$). Taken together with observations in MTT assays (Figure 2A), this context-dependent pH sensitivity indicates the potential for neighboring amino acid residues to influence the chemical properties of AGEs, and may be an important factor in dictating the processes in which theses AGEs participate *in vivo*.

To determine the cellular relevance of this redox behavior, we explored the effects of MG-H–peptide conjugates using a well-established 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 a significant level of 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 (Supplemental 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 suggests that MG-H2 adducts may also possess cellular redox activities that emerge 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 (Figure 3A). Mass spectrometric analyses revealed this newly formed material to be two mass units less than parent peptide **29** (Figure 3B), 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 (Supplemental Figure S8). Interestingly, the 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 could react with arginine and/or lysine residues to form inter- or intramolecular crosslinks. Formation of such crosslinks 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, Supplemental Figures S9-S11). This hydrolysis process was found to proceed with first-order kinetics ($k_{obs} = 1.84 \times 10^{-5}$ s^{-1}; $t_{1/2} = 10.5$ h) at a rate compatible with the lifetime of many intracellular proteins (Supplemental 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 aimed at obtaining 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 (Supplemental Figures S15–16). 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 isomeric compounds **36** and **37** may explain the multi-modal 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.[38,66] 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.

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 among 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 completely inactive under identical conditions. Based on kinetic isotope effect data and computational results, we believe that MG-H3 conjugates 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 local pH contexts 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 novel "deglycation" mechanism for MG-H-modified arginine residues, which may also be relevant *in vivo*.

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, unmodified arginine residues react 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.), based on previous studies. Our data suggests 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), un-protonated 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 minutes (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 crosslinking or increased 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 – the $t_{1/2}$ of $\mathbf{22_{free}}$ at pH 5.4 and 37 °C is approximately 14.8 days.[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), we anticipate that MG-H3 will rapidly convert to carboxyethyl arginine (CEA) and MG-H1; previous studies have shown these two species to be the predominant products of MG-H3 hydrolytic cleavage,[42] and at pH 9.4, MG-H3 decomposes with a half-life of approximately 20 minutes.[31] 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.[42] MG-H1 is therefore expected to represent a "long-lived" modification of the arginine side-chain that is susceptible neither to hydrolytic nor oxidative removal. Indeed, MG-H1 is often identified as the major MG-H formed *in vivo*,[32] perhaps because of its stability relative to MG-H3, and the scarcity of MG-H2.

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 inside and outside of cells, although these modified proteins themselves may be impermeable.

Taken together, we hypothesize that both formation and removal of MG-H adducts can 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. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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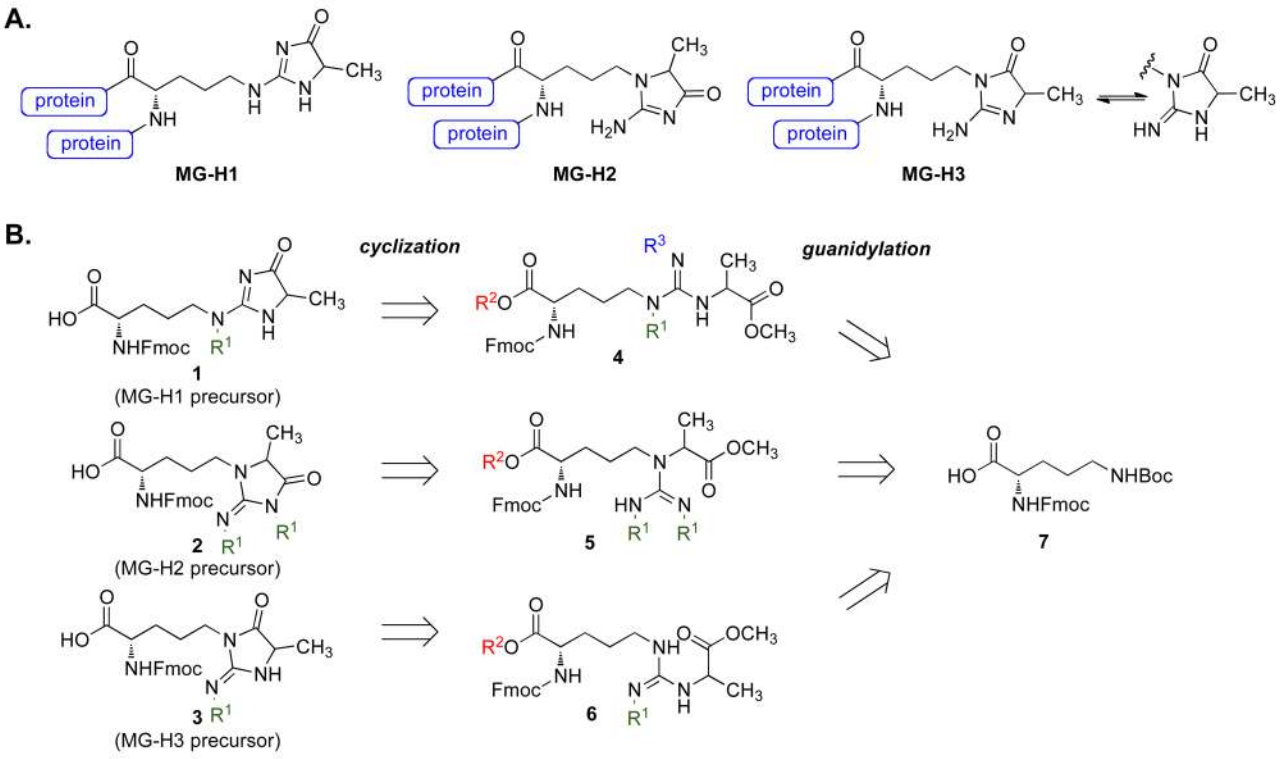


Figure 1.

Stuctures and retrosynthesis of the methylglyoxal-derived hydorimidazolone (MG-H) class of AGEs. (A) The MG-Hs consist of three isomeric structures as shown, each formed through condensation of methylglyoxal with arginine. (B) Proposed "divergent" retrosynthesis of all three isomers derived from a single, readily-available starting material.

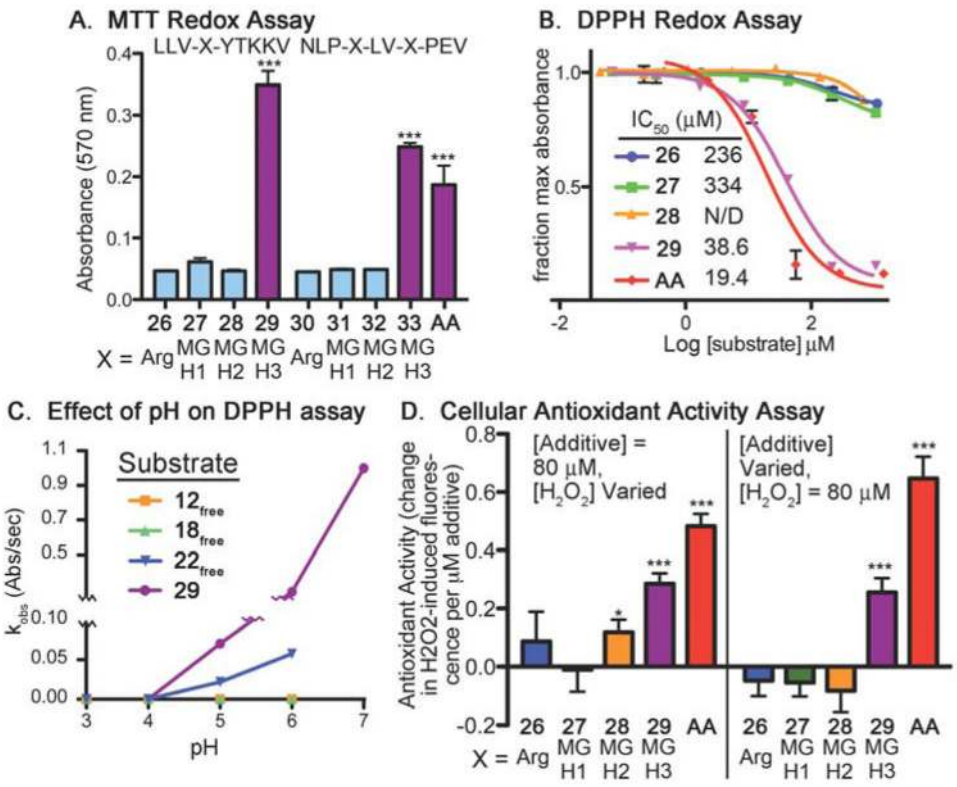


Figure 2.

MG-H3-containing peptides possess antioxidant activity comparable with ascorbic acid. (A) Peptides **26–29**, **30–33**, and ascorbic acid (abbreviated "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 represents 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.

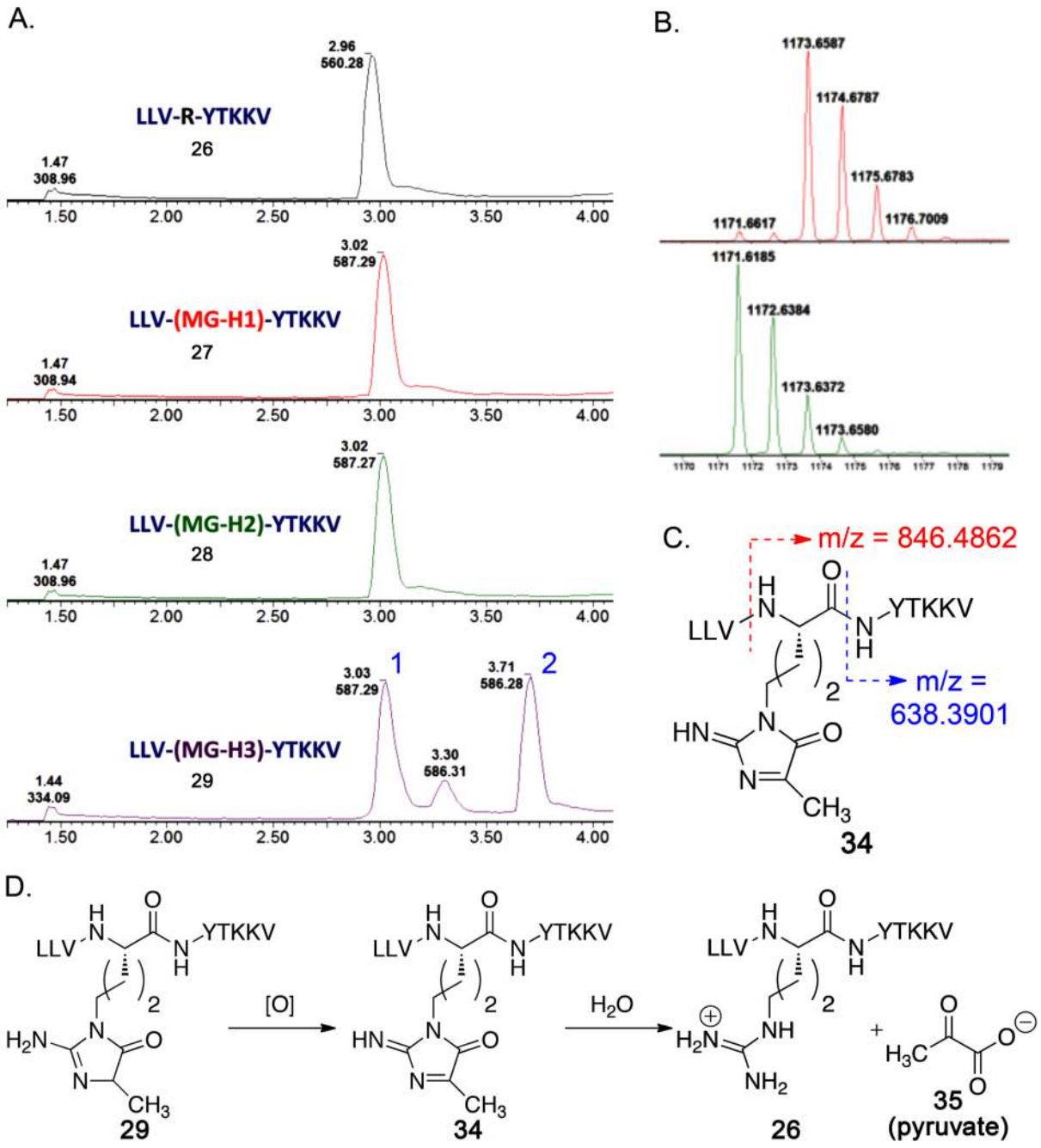


Figure 3.

LC/MS and LC/MS-MS analyses of hydroimidazolone-peptide conjugates in 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**, **27**, and **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. This data suggests 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**. For simplicity, one representitve MG-H3 tautomer is shown; the rest are provided in Scheme S4.

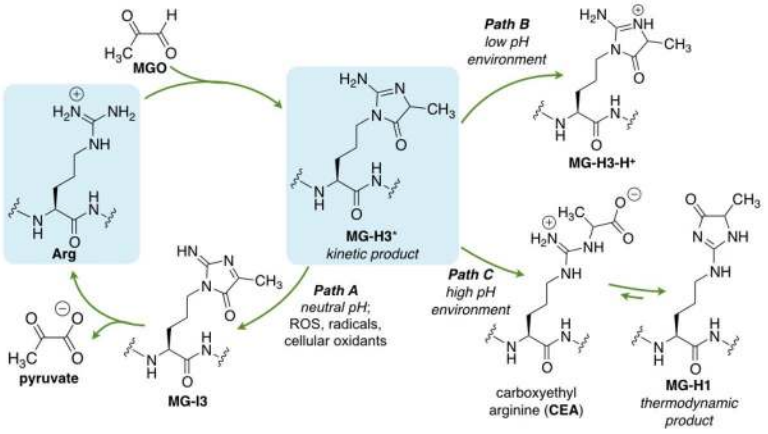
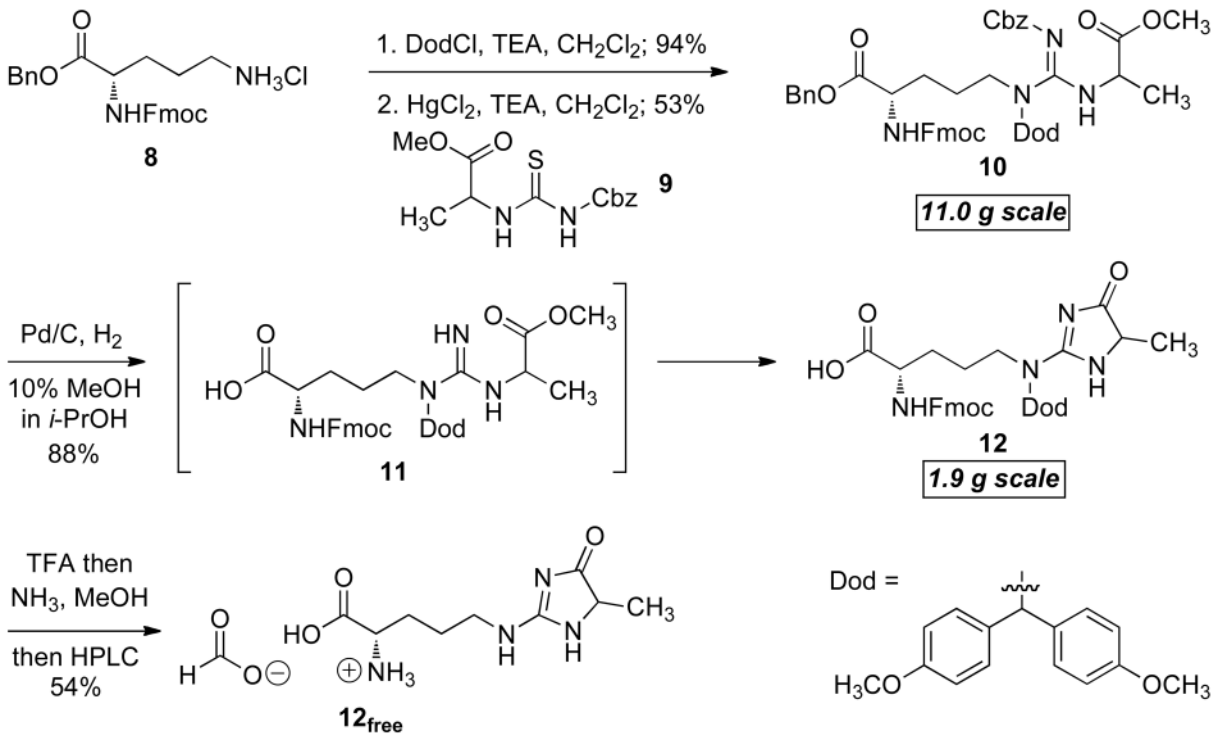
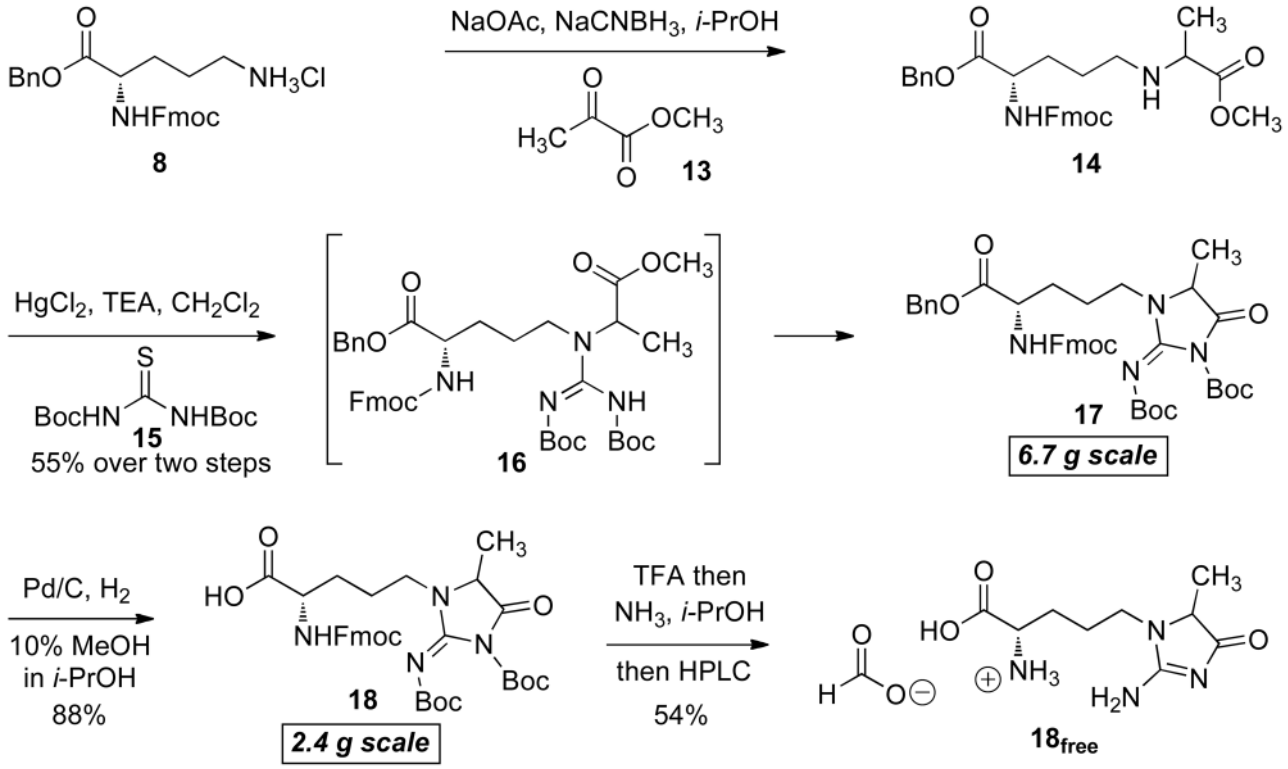


Figure 4.

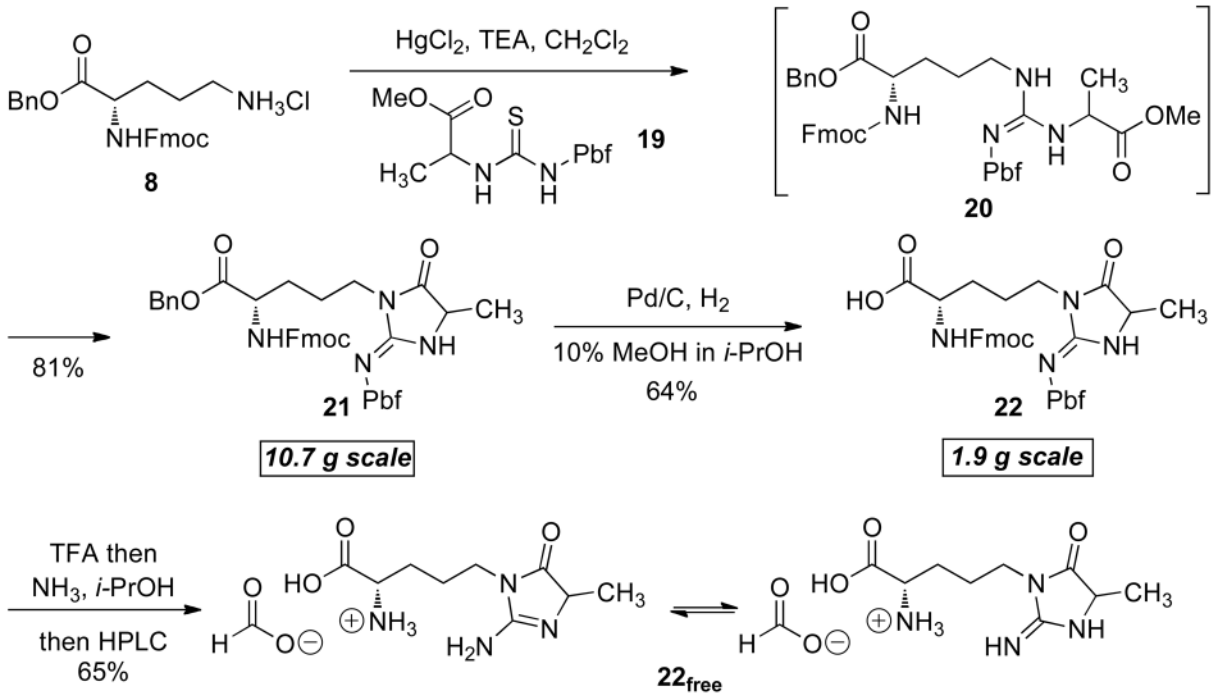
Proposed model for the regulation of arginine glycation. Accessible Arg sidechains react with MGO to form a mixture of isomeric MG-H adducts, of which MG-H3 is believed to be the kinetic product. MG-H3 is sensitive toward local perturbations in pH or redox balance. ***Path A***. At neutral pH, MG-H3 undergoes rapid oxidative conversion to MG-I3, which then spontaneously hydrolyzes to regenerate Arg plus one equivalent of pyruvate. ***Path B***. In the presence of low local pH or nearby protic residues, MG-H3-H^+ is formed, and this species is likely stable and relatively insensitive to changes in redox balance. ***Path C***. At higher local pH or in the presence of basic residues, however, ring-opening of MG-H3 affords carboxyethylarginine (CEA) and MG-H1, which both appear to be relatively environmentally insensitive. *For simplicity, only one of two likely MG-H3 tautomers is shown. Please see Supporting Information (Scheme S4) for more detail.



Scheme 1.



Scheme 2.



Scheme 3.

Table 1

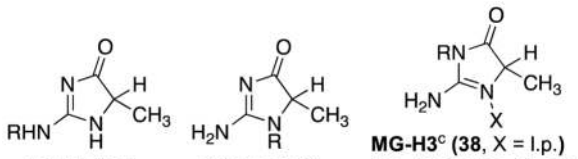
Synthesis of Hydroimidazolone-containing peptides

Entry	compound	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%

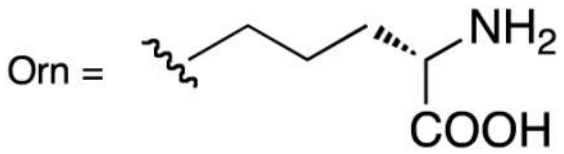
[a]Peptides were isolated in >95% purity

Table 2

pKa Values and Calculated Energies for MG-H Deriva-tives. [a,b]



Cmpd	pK_a[d]	calc'd pK_a[e]	BDE (kcal/mol)[f]	IE (kcal/mol)[g]	ΔG_{ox}(kcal/mol)[h]
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



[a] R = Orn for compounds employed in pKa measurements, and R = Me for structures used in calculations. Calculations involving the energies of relevant tautomeric and protonated forms can be found in the online supporting information.

[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 supporting information.

[c] Calculations pretaining to the other MG-H3 tautomer can be found in the supporting information.

[d] pK_a values were obtained experimentally via NMR titration.

[e] Calculated pKa, determined as detailed in the supporting information.

[f] BDE = bond dissociation energy.

[g] IE = ionization energy.

[h] Data corresponds to the free energy difference between the indicated MG-H and the corresponding imidazolone oxidation product, as described in the supporting information.