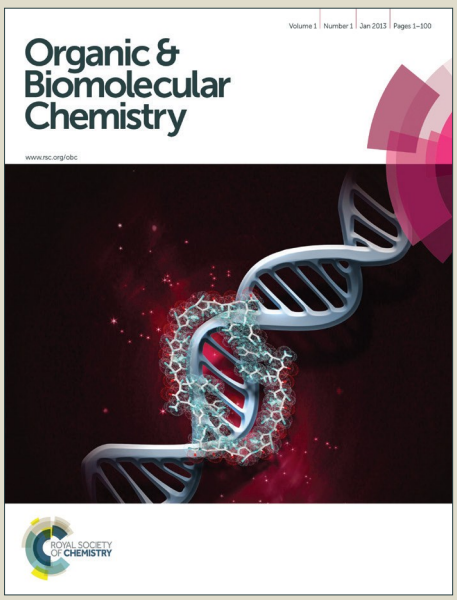
Organic & Biomolecular Chemistry

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Organic and Biomolecular Chemistry

COMMUNICATION

Arginine side-chain modification that occurs during copper-catalysed azide-alkyne click reactions resembles an advanced glycation end product

Received 29th April 2016,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Anne C. Conibear,[a] Karine Farbiarz,[a] Rupert L. Mayer,[b,c] Maria Matveenko,[a] Hanspeter Kählig[d] and Christian F. W. Becker[a]*

Dehydroascorbate is a by-product of copper-catalysed azide-alkyne click (CuAAC) reactions and also forms advanced glycation end products (AGEs) in tissues undergoing oxidative stress. Here we isolate and characterize an arginine-dehydroascorbate adduct formed during CuAAC reactions, investigate strategies for preventing its formation, and propose its biological relevance as an AGE.

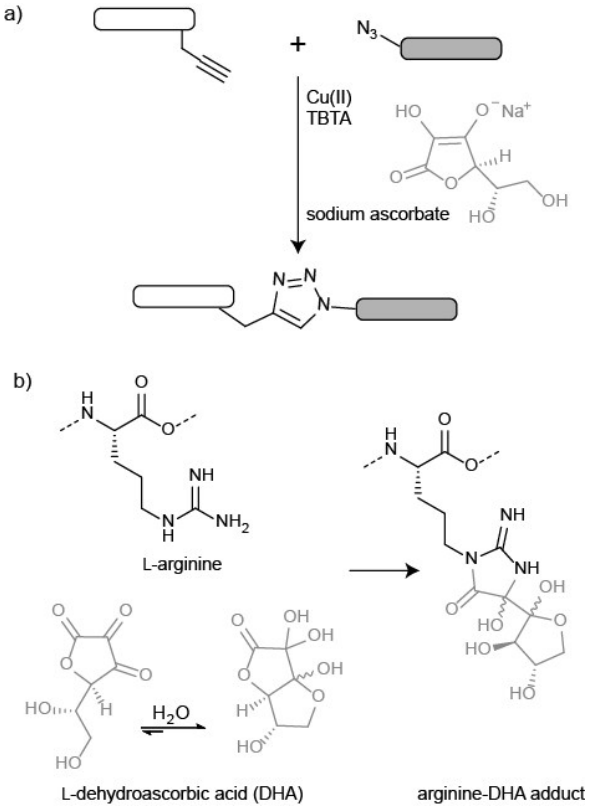
The copper-catalysed azide-alkyne click (CuAAC) ligation[1, 2] between an azide and a terminal alkyne (Scheme 1a) is one of the most widely used reactions for labeling and ligation of proteins and peptides.[3-6] Whereas the uncatalysed 1,3-dipolar cycloaddition requires heating, longer reaction times and yields a mixture of 1,4- and 1,5-disubstituted triazole regioisomers, the Cu(I)-catalysed process can be carried out at room temperature in a variety of solvents and yields only the 1,4-disubstituted 1,2,3-triazole.[1, 2] Moreover, the high selectivity of the CuAAC and the stability of the resulting 1,2,3-triazole make this reaction suitable for application in biological systems.[4]

Despite the high efficiency and selectivity of the CuAAC reaction, protein modification and degradation can occur due to the copper-mediated generation of reactive oxygen species.[7, 8] For example, oxidation of the imidazole ring of histidine side chains to 2-imidazolone was observed in the presence of Cu(II) and ascorbate, with the additional formation of a histidine-lysine crosslink if the amine moiety of a lysine side chain was nearby.[9, 10] Oxidative damage of proteins can be mitigated by a careful choice of copper-coordinating ligand;[11-13] amine-containing ligands such as *tris*[(1-benzyl-1*H*-1,2,3-triazole-4-yl)methyl]amine (TBTA),[12] or other more water-soluble derivatives,[14] are included in the reaction mixture to stabilize the Cu(I) state.

Ascorbate is widely used to reduce Cu(II) to catalytically-active Cu(I) in CuAAC reactions, generating dehydroascorbic acid (DHA) as the corresponding oxidation product. Degradation products of DHA have been reported to react with amino acid side chains.[7, 8] A five-carbon fragment of DHA was found to conjugate to the thiol group of glutathione, both isolated and in Jurkat cells,[15] and to the reduced forms of three cysteine-containing proteins, insulin B-chain, α-lactalbumin and haemoglobin.[16] DHA also fragments into the dicarbonyls glyoxal and methylglyoxal, formation of which increases in the presence of copper and which modify bovine serum albumin (BSA).[17, 18] In particular, methylglyoxal formed adducts predominantly with arginine residues on BSA and was also found to react with N^{α}-acetylarginine, -lysine and –cysteine to form fluorescent modifications.[18] In a study of lysine-arginine crosslinking by degradation products of DHA, Reihl *et al.* identified and characterised several cross-linked products of N^{α}-Boc-L-arginine and N^{α}-Boc-L-lysine and proposed pathways for their formation.[19] A conjugate of DHA and 9-ethylguanine was identified by Raza *et al.* comprising a five-membered cyclic structure that was characterised by X-ray crystallography and corresponded to a mass increase of 174 Da.[20] DHA also formed an adduct with guanosine in short DNA strands and inhibited protein expression at millimolar concentrations.[20] Whereas most studies have focused on adducts formed by DHA degradation products, this latter study suggests that DHA, and not only its degradation products, might also conjugate to guanidine and amine moieties.

[a] *University of Vienna, Faculty of Chemistry, Institute of Biological Chemistry, Währinger Straße 38, 1090 Vienna, Austria.*
[b] *University of Vienna, Faculty of Chemistry, Institute of Analytical Chemistry, Währinger Straße 38, 1090 Vienna, Austria.*
[c] *University of Vienna, Faculty of Chemistry, Mass Spectrometry Centre, Währinger Straße 38, 1090 Vienna, Austria.*
[d] *University of Vienna, Faculty of Chemistry, Institute of Organic Chemistry, Währinger Straße 38, 1090 Vienna, Austria.*
**Email: christian.becker@univie.ac.at; Phone: +43 1 4277 70510; Fax: +43 1 4277 870510.*

†Electronic Supplementary Information (ESI) available: Experimental details and additional MS, HPLC and NMR data are available in the supplementary data. See DOI: 10.1039/x0xx00000x



Scheme 1: Proposed structure of the adduct of dehydroascorbate (DHA) with arginine residues that occurs during copper-catalysed azide-alkyne click (CuAAC) reactions with ascorbate as reducing agent. a) The CuAAC reaction between a peptide or protein bearing a terminal alkyne (unshaded bar) and a peptide or protein bearing an azide (grey bar) yields a 1,4-disubstituted 1,2,3-triazole. The reaction is catalyzed by Cu(I), which is produced *in situ* by the ascorbate-mediated reduction of Cu(II) and is stabilized by polytriazole ligands such as *tris*-(benzyltriazolylmethyl)amine (TBTA). b) Proposed structure of arginine-DHA, the product of a reaction between the guanidinium group of an L-arginine side-chain with DHA (the oxidation product of L-ascorbate).

The conjugation of DHA, its degradation products and other carbohydrates to amino acid side chains is a non-enzymatic posttranslational modification (nPTM) that occurs in tissues under oxidative stress and results in compounds known as advanced glycation end products (AGEs). Accumulation of AGEs is associated with aging and several diseases and pathologies including diabetes mellitus, Alzheimer's disease, lens pigmentation and cataract formation.[21, 22] In the human lens, high levels of ascorbate led to lens browning and formation of adducts between arginine and lysine side chains in lens crystallin proteins and degradation products of DHA.[23, 24] For example, methylglyoxal was found to modify arginine and lysine residues in α-crystallin and decreased its chaperone function.[25] Analysis of some of the modifications resulting from incubation of human and calf lenses with ascorbic acid was carried out by LC-MS/MS and indicated that many of the modifications were the same as those observed in cataractous lenses.[26] In a more recent study, several lysine modification sites were identified by LC-MS and confirmed by comparison with synthetic standards containing fructoselysine.[27] The chemical structures of many AGEs and their effects on protein structure and function, however, are still unknown.

In this study, we identify a DHA modification on the side chain of arginine residues in peptides that occurs during CuAAC ligations in the presence of high concentrations of ascorbate. We propose a structure for the adduct of arginine and DHA (Scheme 1) that is consistent with the observed mass change of +174 Da and is supported by NMR characterisation of the corresponding product formed between N^{α}-Fmoc-L-arginine and DHA. Furthermore, we investigate strategies for preventing or decreasing the formation of this side-product in CuAAC reactions and discuss its possible relevance as an AGE that could form in tissues under oxidative stress.

Results and discussion

In several CuAAC reactions, which we regularly use for the chemical synthesis of biologically active proteins and peptides, we observed the formation of a side-product with a mass of 174 Da higher than the mass of the desired product. The side-product forms when one of the peptide components contains at least one arginine residue and continues to increase after the CuAAC ligation is complete, leading us to suspect a modification of the arginine side chain with DHA and to propose the structure shown in Scheme 1b.

Incubation of the arginine-containing peptide azides P1 and P2 (Figure 1a) with CuAAC reagents at the concentrations used for ligation reactions (Supplementary data) resulted in the appearance of peaks in the mass spectra corresponding to +174 Da, showing that the modification occurs in the absence of a ligation reaction. For peptide P1, which contains one arginine residue, only one adduct (P1*) was formed (Figure 1b). For peptide P2, which contains two arginine residues, two adducts (P2*) and (P2**) were formed, corresponding to the modification of one (+174 Da) or both (+ 348 Da) arginine residues respectively. Whereas the modified peptide P1* had a slightly shorter retention time in RP-HPLC than the unmodified peptide P1 and could be isolated, the modified peptides P2* and P2** co-eluted with the unmodified peptide P2 (Figure 1c). Sequencing of the modified peptide P1* by LC-MS/MS confirmed that the +174 Da modification was located on the arginine side chain. Fragmentation of the parent ion ($[M+3H]^{3+}_{calc}$ 633.97938, $[M+3H]^{3+}_{obs}$ 633.97943) gave rise to a series of b^+ and y^+ ions (Table S1) that localized the modification to the arginine-DHA fragment.

Peptide P1 incubated with sodium ascorbate alone, at the concentrations used in CuAAC reactions, was also modified (Supplementary data, Figure S1), demonstrating that copper is not required for the adduct formation. Nevertheless, the presence of the copper-TBTA complex appears to promote the adduct formation; less modified peptide P1* was formed when peptide P1 was treated with ascorbate alone than when treated with all CuAAC reagents. Direct incubation of peptide P1 with DHA did not result in modification of the peptide either in the presence or absence of the copper-TBTA complex (Figure S1). DHA is known to form several species and dimers in solution[28] and it is difficult to ascertain under CuAAC conditions whether it is ascorbate or one of the forms of DHA that initially conjugates to arginine so we refer to the modification simply as a DHA-arginine adduct.

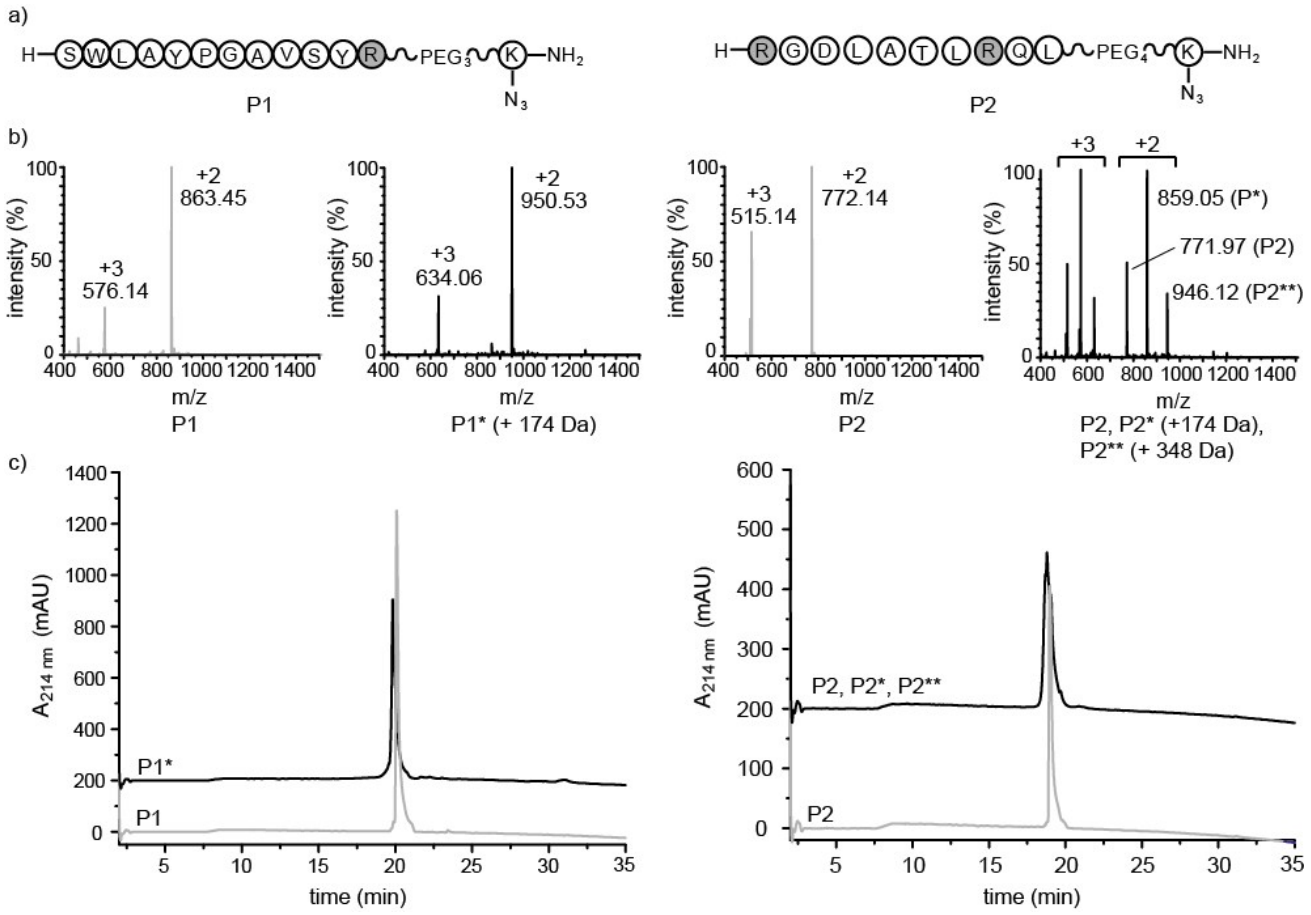


Figure 1: Formation of DHA modification on arginine-containing peptides incubated under CuAAC ligation conditions. a) Sequences of azide-functionalised peptides P1 and P2. Amino acids are represented by their one-letter codes with arginine residues shaded. The peptides contained a polyethylene glycol (PEG) linker between the peptide sequence and the azide moiety. b) Mass spectra of unmodified peptides P1 and P2 (grey spectra) and modified peptides [P1* (+ 174 Da), P2* (+ 174 Da) and P2** (+ 348 Da), black spectra]. P1 $[M+2H]^{2+}_{calc}$ 863.97, $[M+2H]^{2+}_{obs}$ 863.45, $[M+3H]^{3+}_{calc}$ 576.31, $[M+3H]^{3+}_{obs}$ 576.14; P1* $[M+2H]^{2+}_{calc}$ 950.97, $[M+2H]^{2+}_{obs}$ 950.53, $[M+3H]^{3+}_{calc}$ 634.31, $[M+3H]^{3+}_{obs}$ 634.06; P2 $[M+2H]^{2+}_{calc}$ 772.41, $[M+2H]^{2+}_{obs}$ 772.14 and 771.97, $[M+3H]^{3+}_{calc}$ 515.27, $[M+3H]^{3+}_{obs}$ 515.14; P2* $[M+2H]^{2+}_{calc}$ 859.39, $[M+2H]^{2+}_{obs}$ 859.05; P2** $[M+2H]^{2+}_{calc}$ 946.39, $[M+2H]^{2+}_{obs}$ 946.12. c) Analytical RP-HPLC traces of unmodified peptides P1 and P2 (grey traces) and modified peptides P1*, P2* and P2** (black traces, offset by 200 mAU).

Several short peptides containing arginine residues in different numbers and positions were incubated with CuAAC reagents for 60 min to determine the specificity of the DHA modification for arginine (Table 1, Figure S2). Peaks corresponding to a mass increase of 174 Da were observed for all peptides containing at least one arginine residue (P1-P5), suggesting that modification is independent of the arginine position or its flanking residues. Furthermore, peaks corresponding to a mass increase of 348 Da were observed for both peptides containing two arginine residues (P2 and P3), albeit at very low intensity. The peptide containing one arginine and one lysine residue (P5) showed evidence of only one modification and neither of the peptides without arginine (P6 and P7) were modified. Although the peptides P1-P7 were originally designed for other purposes, almost all of the naturally occurring amino acids are represented within the set. Only glutamic acid is not represented. However, the DHA modification was not observed on aspartic acid, which has a similar functional group, or on glutamine. These results demonstrate that DHA conjugates selectively to arginine under these conditions.

Comparison of the relative intensities of the $[M+2H]^{2+}$ ion peaks of the modified (P*) and unmodified (P) forms of each of the peptides suggests that the rate and/or extent of the DHA conjugation reaction varies between peptides; Whereas the modified peptide P1* peak has almost the same intensity as that of P1 after 60 min, the modified forms of P2*-P5* represent only 10-40% of the intensities of their respective unmodified peptides P2-P5 (Figure S2). This observation could be attributed to the position of the arginine residue in the peptide and further studies might reveal sequence motifs that are particularly susceptible to DHA modification.[29, 30]

Methylglyoxal also reacts specifically with arginine and this reaction has been applied for the selective modification of proteins and peptides. Whereas both lysine and cysteine are modified rapidly by methylglyoxal, the less nucleophilic arginine is modified slower but forms a more stable product, thus giving a 'thermodynamic' selectivity.[18] For example, Klok and co-workers showed that ω–methoxy poly(ethylene glycol) bearing an α-oxo-aldehyde end-group that resembles methylglyoxal selectively modified arginine residues.[31] Dawson and co-workers recently reported the synthesis of a triazolylphenylglyoxal probe that selectively reacts with arginine. They then used this reaction to attach an azide handle to proteins for

subsequent CuAAC reactions.[32] Our results suggest that DHA has a similar specificity to methylglyoxal for the modification of arginine.

Table 1: DHA adduct formation on peptides incubated with CuAAC reagents.[a]

	Sequence[b]	DHA adduct(s)	Ref.
P1	S-W-L-A-Y-P-G-A-V-S-Y-R-PEG$_3$-K(N$_3$)	yes, 1 ×	[33]
P2	R-G-D-L-A-T-L-R-Q-L-PEG$_3$-K(N$_3$)	yes, 2 ×	[34]
P3	A-L-T-R-L-R-Q-L-D-G-PEG$_3$-K(N$_3$)	yes, 2 ×	-
P4	N-C-V-I-G-Y-S-G-D-R-[SEA]	yes, 1 ×	-
P5	H-K-D-M-Q-L-G-R	yes, 1 ×	[35]
P6	Y-L-F-S-V-H-W-P-P-L-K-A	no	[36]
P7	Y-H-W-Y-G-Y-T-P-Q-N-V-I	no	[37]

[a] Peptides P1-P7 were incubated with CuAAC reagents (Supplementary data) for 60 min. Formation of a DHA adduct was determined by LC-MS analysis.

[b] Amino acids are represented by their one-letter codes with arginine residues in bold grey. P3 is a scrambled version of P2, P4 is a fragment of endothelial growth factor, K(N$_3$) represents N$^{\varepsilon}$-azidolysine, PEG represents a polyethylene glycol linker and (SEA) represents a *bis*(2-sulfanylethyl)amino linker.[38]

To confirm the structure of the DHA adduct on arginine side chains, N$^{\alpha}$-Fmoc-L-arginine was modified under standard CuAAC reaction conditions (Cu^{2+}, TBTA and ascorbate), purified, and characterized by MS, RP-HPLC and NMR. Figure 2a shows the RP-HPLC trace of N$^{\alpha}$-Fmoc-L-arginine and the reaction mixture at t = 0 and after 3 days of incubation of N$^{\alpha}$-Fmoc-L-arginine with CuAAC reagents at room temperature. A peak with retention time 21.0 min that corresponds to the mass of N$^{\alpha}$-Fmoc-L-arginine modified with DHA (N$^{\alpha}$-Fmoc-L-arginine*) appears over the 3 days but did not increase further if the reaction was left longer. Purification of N$^{\alpha}$-Fmoc-L-arginine* by RP-HPLC yielded the expected modified amino acid with MW 571.11 (Figure 2b and c). An unidentified compound having MW 441.15 that co-eluted with N$^{\alpha}$-Fmoc-L-arginine* was determined to be a contaminant in the CuAAC reagents as it was present at t = 0 and was also observed in control reactions lacking N$^{\alpha}$-Fmoc-L-arginine. As predicted, modification of N$^{\alpha}$-Fmoc-L-arginine yielded several isomers of N$^{\alpha}$-Fmoc-L-arginine* and multiple peaks with the same mass were observed in the analytical RP-HPLC chromatogram (Figure 2c). N$^{\alpha}$-Boc-L-arginine, N$^{\alpha}$-Boc-L-lysine and N$^{\alpha}$-Boc-L-glutamine were also treated with CuAAC reagents for 3 days (Figure S3). No masses corresponding to a DHA modification on either N$^{\alpha}$-Boc-L-lysine or N$^{\alpha}$-Boc-L-glutamine were observed, in agreement with the selectivity of the DHA for arginine modification. Although we observed the DHA modification on N$^{\alpha}$-Boc-L-arginine, further characterization was carried out with N$^{\alpha}$-Fmoc-L-arginine* owing to ease of purification and detection. High resolution MS (Figure S11) confirmed the predicted molecular weight ($[M+H]^+_{calc}$ 571.2035, $[M+H]^+_{obs}$ 571.2036) and elemental composition ($C_{27}H_{31}N_4O_{10}$) of the DHA-modified N$^{\alpha}$-Fmoc-L-arginine*.

High resolution NMR spectroscopy was used to further characterise N$^{\alpha}$-Fmoc-L-arginine* and verify the proposed structure of the DHA adduct. Both 1- and 2-dimensional ^{1}H, ^{13}C and ^{15}N NMR spectra were used to assign the chemical shifts of the major product (Table S2 and Figures S4-S10). Although the sample was a mixture of several compounds or isomers, all the compounds were in the same molecular mass range, as demonstrated by a diffusion weighted NMR experiment (data not shown) and the signals for the predominant N$^{\alpha}$-Fmoc-L-arginine* structure could be assigned unambiguously. A similar family of isomers was identified by Regulus *et al.* in their characterization of an adduct of a five-carbon fragment of DHA and glutathione.[15] The crystal structure of an adduct of DHA and ethyl-substituted guanine was obtained by Raza *et al.* corresponding to addition of guanine to the tricarbonyl form of DHA.[20] Although similar regio- and stereoisomers of these DHA adducts are theoretically possible, no isomers were reported in that study.

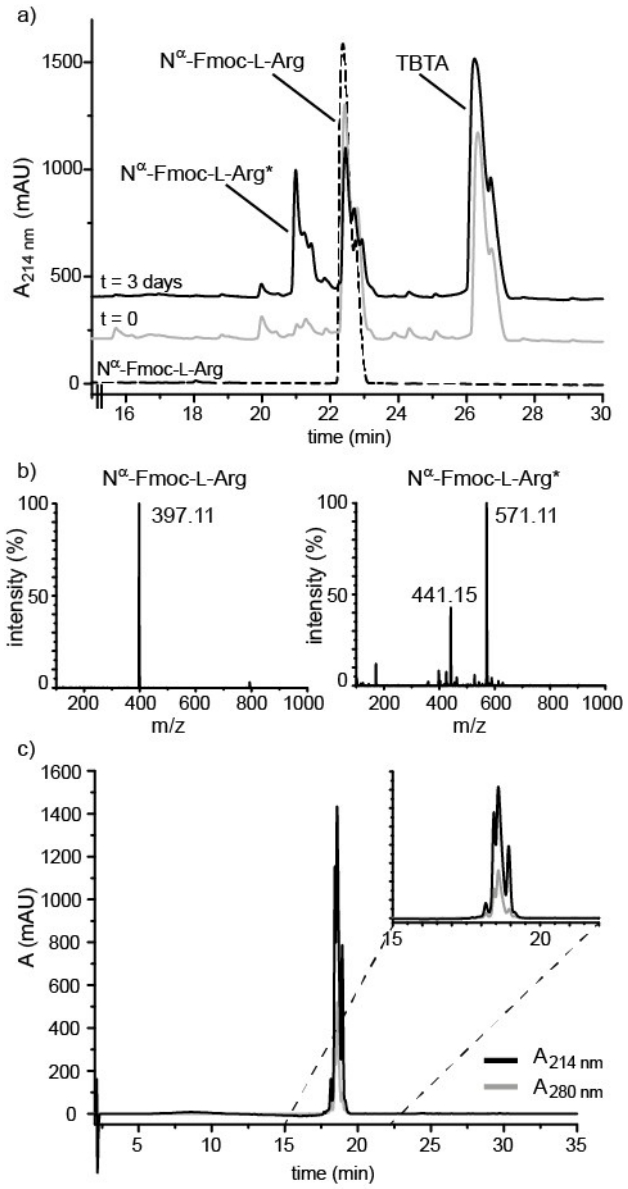


Figure 2: Modification of N$^{\alpha}$-Fmoc-L-arginine with DHA. a) RP-HPLC traces of unmodified N$^{\alpha}$-Fmoc-L-arginine (dashed line) and the CuAAC reaction mixture at t = 0 (grey line, offset by 200 mAU) and after 3 days (black line, offset by 400 mAU) showing the formation of the DHA adduct N$^{\alpha}$-Fmoc-L-arginine*. b) Mass spectra of N$^{\alpha}$-Fmoc-L-arginine, $[M+H]^+_{calc}$ 379.53, $[M+H]^+_{obs}$ 397.11 and N$^{\alpha}$-Fmoc-L-arginine*, $[M+H]^+_{calc}$ 571.64, $[M+H]^+_{obs}$ 571.11. c) Analytical RP-HPLC trace (C4 column, A_{214} trace baseline corrected) of purified N$^{\alpha}$-Fmoc-L-arginine*. Inset: multiple peaks with the same mass indicate the presence of isomers.

Based on the NMR data, we propose that the arginine-DHA structure shown in Scheme 1 is the major compound present in the NMR sample (Scheme 1, Table S2). This structure is supported by the correlations (5-C$\underline{H}_2$ to 9-C, 14-C$\underline{H}_2$ to 11-C and 8-N$\underline{H}$ to 9-C) observed in the HMBC spectra (Figure S7). Furthermore, the large chemical shift difference (0.65 ppm) of the two 14-CH_2 protons (Figure S4) suggests that these protons are part of a ring rather than a free side chain, as would be the case for guanidine attack on the tricarbonyl form of DHA. Nevertheless, nucleophilic attack of the guanidine nitrogens of arginine could also occur on the two ketones of the tricarbonyl form of DHA, or even on ascorbate, eventually giving rise to the observed structure as the thermodynamically more stable structure that prevails over time.

Although present in only small amounts, such modified arginine side-chains might influence downstream biological assays or protein properties, similarly to the well-known effects of posttranslational modifications.[39] As CuAAC reactions are often carried out on large proteins and the success of the reaction is monitored by gel electrophoresis, size-exclusion chromatography or fluorescence detection measurements, the DHA-arginine adduct formation described here might not be detected or removed because of the small difference in mass (+174 Da) between the modified and unmodified products. We therefore investigated methods of reducing or preventing the formation of the DHA-arginine adduct during CuAAC ligation reactions.

The effects of decreasing the ascorbate concentration in the CuAAC reaction mixture and of adding aminoguanidine as a scavenger were investigated in a model CuAAC ligation reaction (Figure 3a). Ligation of peptide-azide P2 and peptide-alkyne P8 under standard CuAAC reaction conditions of 40 equivalents of sodium ascorbate for 60 min yielded almost equimolar amounts of the ligation product P2-P8 and the DHA-modified ligation product P2*-P8 (Figure 3b). Decreasing the ascorbate component to 10 equivalents prevented formation of the P2*-P8 product without compromising the CuAAC ligation, demonstrating that decreasing the ascorbate concentration is an effective means of preventing DHA-arginine adduct formation. If fewer equivalents of ascorbate are used it is especially important to exclude oxygen from the reaction by degassing the solvents and flushing the reaction vessel with inert gas to prevent oxidation of Cu(I) to Cu(II). We have also observed that a large excess of ascorbate (40 or 50 equivalents) is required if the peptides contain cysteine residues, although protection of the cysteine residues might prevent their suppression of the CuAAC reaction.[7, 40] Whereas no modified product P2*-P8 was observed for the CuAAC ligation in the presence of 10 equivalents of ascorbate and 10 equivalents of aminoguanidine, some modified ligation product was observed at 40 equivalents of ascorbate even in the presence of 10 equivalents of aminoguanidine. The relative amount of modified product P2*-P8, however, was less than that formed in the reaction without aminoguanidine (Figure 3b). These results suggest that decreasing the ascorbate concentration is the most effective means of decreasing DHA-arginine adduct formation but that addition of aminoguanidine as a scavenger is also effective, especially at high ascorbate concentrations.

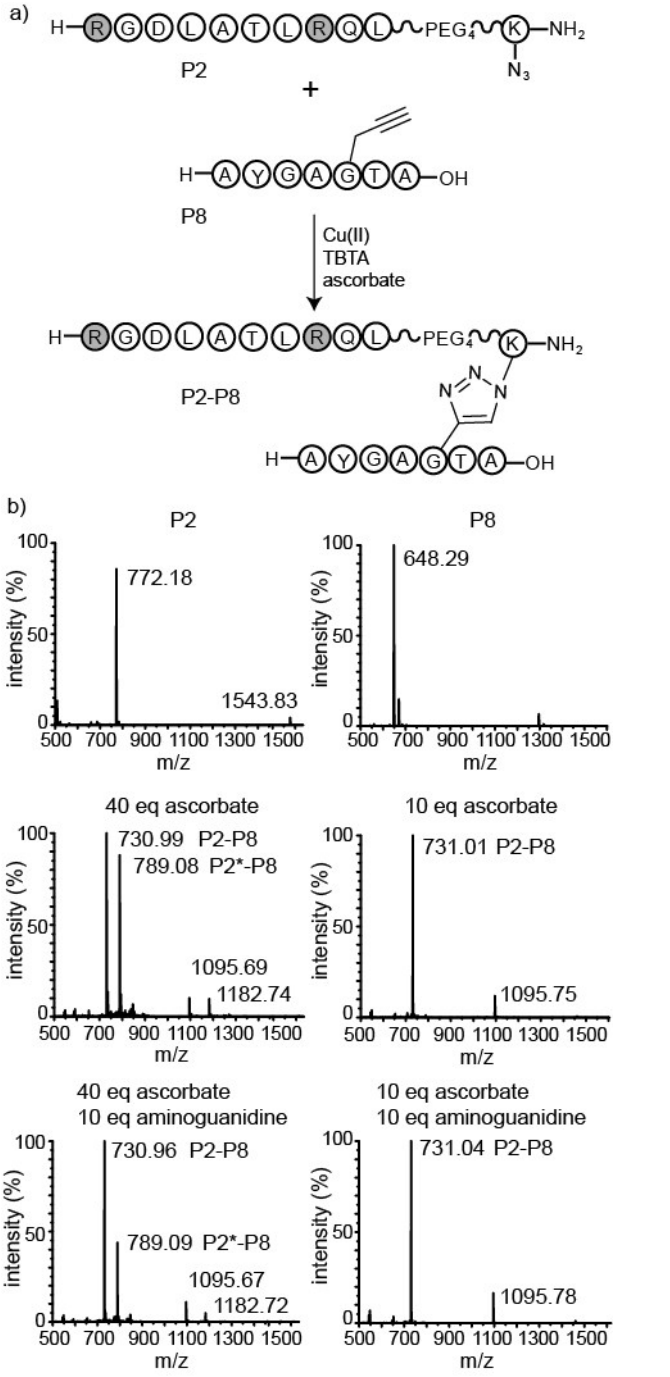


Figure 3: Model CuAAC ligations for the optimization of reaction conditions. a) CuAAC ligations of peptides P2 and P8 were carried out with either 10 or 40 equivalents of sodium ascorbate and with or without 10 equivalents of aminoguanidine. Amino acids are represented by their one-letter codes with arginine residues shaded. Peptide P2 contained a polyethylene glycol linker (PEG) between the peptide sequence and the azide moiety. b) Mass spectra of peptides P2 and P8 and the ligation products P2-P8 (unmodified) and P2*-P8 (modified). P2 $[M+H]^+_{calc}$ 1543.89, $[M+H]^+_{obs}$ 1543.83, $[M+2H]^{2+}_{calc}$ 772.41, $[M+2H]^{2+}_{obs}$ 772.18; P8 $[M+2H]^{2+}_{calc}$ 647.69, $[M+2H]^{2+}_{obs}$ 648.29; P2-P8 $[M+2H]^{2+}_{calc}$ 1096.25, $[M+2H]^{2+}_{obs}$ 1095.69, 1095.75, 1095.67 and 1095.78, $[M+3H]^{3+}_{calc}$ 731.16, $[M+3H]^{3+}_{obs}$ 730.99, 731.01, 730.96 and 731.04; P2*-P8 $[M+2H]^{2+}_{calc}$ 1183.30, $[M+2H]^{2+}_{obs}$

1182.74 and 1182.72, $[M+3H]^{3+}_{calc}$ 789.20, $[M+3H]^{3+}_{obs}$ 789.08 and 789.09.

In addition to the strategies investigated in this study, decreasing protein damage during CuAAC reactions by using alternative ligands to stabilize Cu(I) or alternative reducing agents have been proposed.[5, 8] Inclusion of excess water-soluble ligand *tris*(3-hydroxypropyl-triazolylmethyl)amine (THPTA) was found to protect *N*-benzoylhistidine from oxidative damage and, in agreement with the results presented here, aminoguanidine was found to be a modest inhibitor of ascorbate byproduct formation.[7] The recently-described *tris*(6-galactosyl-triazomethyl)amine (TGTA) ligand was found to reduce degradation of fibroblast growth factor under CuAAC conditions and the authors even suggest that pre-formation of a TGTA-Cu(I) complex might obviate the need for a Cu(II) reducing agent.[13] Hong *et al.* concluded that ascorbate is required in at least 10-fold excess to maintain the Cu(I) oxidation state and recommended that the copper-ligand complex be formed first, followed by addition of the reducing agent.[7] Alternatively, reducing agents such as *tris*(2-carboxyethyl)phosphine (TCEP) or hydrazine have been successful in some cases.[40, 41] Formation of DHA-arginine adducts can also be avoided by careful monitoring of the reaction progress so that the CuAAC reaction can be quenched as soon as it reaches completion. In the model CuAAC reactions described above, the ligations were complete within five minutes but only trace amounts of modified peptide P2*-P8 formed in this time, even in the presence of 40 equivalents of ascorbate.

The potential modification or damage of proteins during azide-alkyne click reactions can also be avoided by use of an alternative form of the reaction such as the strain-promoted azide-alkyne click ligation (SPAAC) developed by the Bertozzi group.[42] This reaction, which is driven by the ring strain of a substituted cyclooctyne, does not require a reducing agent or metal catalyst and has found wide application in protein ligation and labeling, especially in biological systems where the use of copper induces toxicity. The SPAAC, however, does have several disadvantages, including the large size, asymmetry and hydrophobicity of the alkyne moiety and its incompatibility with solid phase peptide synthesis. In contrast, the CuAAC can be used for ligation on the solid phase.[43] The two forms of the azide-alkyne click reaction, CuAAC and SPAAC, are therefore complementary and the respective advantages and disadvantages should be evaluated for each application.

Conclusions

We have shown that under CuAAC reaction conditions, DHA can form an adduct with arginine residues in proteins, giving rise to a side-product having a mass increase of 174 Da. This side-product is only detectable by strict mass spectrometric analysis and therefore might often go unnoticed during the CuAAC reaction of large biomolecules. Formation of this covalent DHA-arginine adduct, which might affect downstream biological assays and be difficult or impossible to remove, can be avoided by decreasing the concentration of ascorbate in the reaction mixture and adding aminoguanidine as a scavenger. The DHA-arginine adduct described here is of broad significance, both as a potentially notorious side-product formed in CuAAC ligations and as a possible detrimental posttranslational modification of proteins under oxidative stress.

Acknowledgements

We thank David Hoi for technical assistance. The research leading to these results has received funding from the Mahlke-Obermann Stiftung and the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 609431.

References

1. V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew Chem Int Ed Engl*, 2002, **41**, 2596-2599.
2. C. W. Tornoe, C. Christensen and M. Meldal, *J Org Chem*, 2002, **67**, 3057-3064.
3. H. C. Kolb and K. B. Sharpless, *Drug Discov Today*, 2003, **8**, 1128-1137.
4. M. Meldal and C. W. Tornoe, *Chem Rev*, 2008, **108**, 2952-3015.
5. E. Lallana, R. Riguera and E. Fernandez-Megia, *Angew Chem Int Ed Engl*, 2011, **50**, 8794-8804.
6. A. A. Ahmad Fuaad, F. Azmi, M. Skwarczynski and I. Toth, *Molecules*, 2013, **18**, 13148-13174.
7. V. Hong, S. I. Presolski, C. Ma and M. G. Finn, *Angew Chem Int Ed Engl*, 2009, **48**, 9879-9883.
8. C. S. McKay and M. G. Finn, *Chem Biol*, 2014, **21**, 1075-1101.
9. K. Uchida and S. Kawakishi, *Bioorg Chem*, 1989, **17**, 330-343.
10. Y. Liu, G. Sun, A. David and L. M. Sayre, *Chem Res Toxicol*, 2004, **17**, 110-118.
11. D. C. Kennedy, C. S. McKay, M. C. Legault, D. C. Danielson, J. A. Blake, A. F. Pegoraro, A. Stolow, Z. Mester and J. P. Pezacki, *J Am Chem Soc*, 2011, **133**, 17993-18001.
12. T. R. Chan, R. Hilgraf, K. B. Sharpless and V. V. Fokin, *Org Lett*, 2004, **6**, 2853-2855.
13. F. S. Ekholm, H. Pynnonen, A. Vilkman, J. Koponen, J. Helin and T. Satomaa, *Org Biomol Chem*, 2016, **14**, 849-852.
14. C. Besanceney-Webler, H. Jiang, T. Zheng, L. Feng, D. Soriano del Amo, W. Wang, L. M. Klivansky, F. L. Marlow, Y. Liu and P. Wu, *Angew Chem Int Ed Engl*, 2011, **50**, 8051-8056.
15. P. Regulus, J. F. Desilets, K. Klarskov and J. R. Wagner, *Free Radic Biol Med*, 2010, **49**, 984-991.
16. P. Kay, J. R. Wagner, H. Gagnon, R. Day and K. Klarskov, *Chem Res Toxicol*, 2013, **26**, 1333-1339.
17. N. Shangari, T. S. Chan, K. Chan, S. Huai Wu and P. J. O'Brien, *Mol Nutr Food Res*, 2007, **51**, 445-455.
18. T. W. Lo, M. E. Westwood, A. C. McLellan, T. Selwood and P. J. Thornalley, *J Biol Chem*, 1994, **269**, 32299-32305.
19. O. Reihl, M. O. Lederer and W. Schwack, *Carbohydr Res*, 2004, **339**, 483-491.
20. A. Raza and R. Vince, *Chembiochem*, 2011, **12**, 1015-1017.
21. P. J. Thornalley, *Chem Biol Interact*, 1998, **112**, 137-151.
22. K. Nowotny, T. Jung, A. Hohn, D. Weber and T. Grune, *Biomolecules*, 2015, **5**, 194-222.
23. R. H. Nagaraj, D. R. Sell, M. Prabhakaram, B. J. Ortwerth and V. M. Monnier, *Proc Natl Acad Sci U S A*, 1991, **88**, 10257-10261.
24. R. H. Nagaraj and V. M. Monnier, *Biochim Biophys Acta*, 1995, **15**, 75-84.
25. B. K. Derham and J. J. Harding, *Biochem J*, 2002, **364**, 711-717.
26. R. Cheng, Q. Feng and B. J. Ortwerth, *Biochim Biophys Acta*, 2006, **5**, 533-543.
27. M. Kielmas, M. Kijewska, A. Kluczyk, J. Oficjalska, B. Golebiewska, P. Stefanowicz and Z. Szewczuk, *Anal Bioanal Chem*, 2015, **407**, 2557-2567.

28. J. Hvoslef, B. Pedersen, O. Wennerström, C. R. Enzell, Å. Åkeson and G. Lundquist, *Acta Chem Scand B*, 1979, **33b**, 503-511.
29. J. Venkatraman, K. Aggarwal and P. Balaram, *Chem Biol*, 2001, **8**, 611-625.
30. M. B. Johansen, L. Kiemer and S. Brunak, *Glycobiology*, 2006, **16**, 844-853.
31. M. A. Gauthier and H. A. Klok, *Biomacromolecules*, 2011, **12**, 482-493.
32. D. A. Thompson, R. Ng and P. E. Dawson, *J Pept Sci*, 2016, **22**, 311-319.
33. M. Koolpe, M. Dail and E. B. Pasquale, *J Biol Chem*, 2002, **277**, 46974-46979.
34. T. Oyama, K. F. Sykes, K. N. Samli, J. D. Minna, S. A. Johnston and K. C. Brown, *Cancer Lett*, 2003, **202**, 219-230.
35. J. Kohl, B. Lubbers, A. Klos, W. Bautsch and M. Casaretto, *Eur J Immunol*, 1993, **23**, 646-652.
36. P. Zhao, T. Grabinski, C. Gao, R. S. Skinner, T. Giambernardi, Y. Su, E. Hudson, J. Resau, M. Gross, G. F. Vande Woude, R. Hay and B. Cao, *Clin Cancer Res*, 2007, **13**, 6049-6055.
37. Z. Li, R. Zhao, X. Wu, Y. Sun, M. Yao, J. Li, Y. Xu and J. Gu, *FASEB J*, 2005, **19**, 1978-1985.
38. N. Ollivier, J. Dheur, R. Mhidia, A. Blanpain and O. Melnyk, *Org Lett*, 2010, **12**, 5238-5241.
39. C. T. Walsh, S. Garneau-Tsodikova and G. J. Gatto, Jr., *Angew Chem Int Ed Engl*, 2005, **44**, 7342-7372.
40. M. R. Levengood, C. C. Kerwood, C. Chatterjee and W. A. van der Donk, *Chembiochem*, 2009, **10**, 911-919.
41. Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless and M. G. Finn, *J Am Chem Soc*, 2003, **125**, 3192-3193.
42. N. J. Agard, J. A. Prescher and C. R. Bertozzi, *J Am Chem Soc*, 2004, **126**, 15046-15047.
43. V. Castro, H. Rodriguez and F. Albericio, *ACS Comb Sci*, 2016, **18**, 1-14.

Table of Contents Graphic

An adduct of dehydroascorbate with arginine forms during copper-catalysed azide-alkyne click reactions and resembles an advanced glycation end product.