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Characterisation of the non-oxidative degradation pathway of dehydroascorbic acid in slightly acidic aqueous solution

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ABSTRACT

Although L-ascorbate (vitamin C) is an important biological antioxidant, its degradation pathways in vivo remain incompletely characterised. Ascorbate is oxidised to dehydroascorbic acid, which can be either hydrolysed to diketogulonate (DKG) or further oxidised. DKG can be further degraded, oxidatively or non-oxidatively. Here we characterise DKG products formed non-enzymically and non-oxidatively at 20°C and at a slightly acidic pH typical of the plant apoplast.

High-voltage electrophoresis revealed at least five products, including two novel CPLs (epimers of 2-carboxy-L-threo-pentonolactone), which slowly interconverted with CPA (2-carboxy-L-threo-pentonate). One of the two CPLs has an exceptionally low $pK_a$. The CPL structures were supported by MS $[\text{C}_6\text{H}_7\text{O}_7]^{-}$ and by $^1\text{H}$ and $^{13}\text{C}$ NMR spectroscopy. Xylonate and its lactone also appeared.

Experiments with $[1^{-14}\text{C}]$DKG showed that all five products (including the 5-carbon xylonate and its lactone) retained DKG’s carbon-1; therefore, most xylonate arose by decarboxylation
of CPLs or CPA, one of whose –COOH groups originates from C-2 or C-3 of DKG after a ‘benzilic acid rearrangement’. Since CPLs appeared before CPA, a DKG lactone is probably the main species undergoing this rearrangement.

CPA and CPL also form non-enzymically in vivo, where they may be useful to researchers as ‘fingerprints’, or to organisms as ‘signals’, indicating a non-oxidative, slightly acidic biological compartment.

Key words
benzilic acid rearrangement
carboxypentonolactones
dehydroascorbic acid
diketogulonate
trihydroxypropyltartronic acid
vitamin C degradation

Abbreviations used: DHA, dehydroascorbic acid; DKG, 2,3-diketogulonate; CPA, 2-carboxy-L-threo-pentonate; CPL, 2-carboxy-L-threo-pentonolactone (either of the two epimers).

1. Introduction

L-Ascorbate is the major low-molecular-weight antioxidant in plant cells and an important antioxidant in animal cells (Smirnoff, 2018). Under aerobic conditions, ascorbate readily forms dehydroascorbic acid (DHA) in solution. This reaction is catalysed by the plant cell-wall enzyme, ascorbate oxidase (with water as by-product; Lin & Varner, 1991), and in the presence of minute traces of transition metal ions it also occurs non-enzymically (with H₂O₂ as by-product; Khan & Martell, 1967). In vivo, DHA can be enzymically reduced back to ascorbate by DHA reductase, with glutathione as reductant (Noctor & Foyer, 1998). Both ascorbate and DHA serve as vitamin C, important in human nutrition. Interestingly, oxidation of ascorbate to DHA is reported to enhance plant cell growth (Li et al., 2017).

If DHA is not reduced back to ascorbate, then it is chemically and/or enzymically converted to products from which (it is generally accepted) ascorbate cannot be regenerated. There are two alternative routes of such irreversible vitamin C loss: under oxidising conditions, DHA is oxidised to oxalate plus threonate (and oxalyl-threonate esters), whereas if oxidation is limited, then DHA mainly undergoes hydrolysis to form 2,3-diketo-L-gulonate (DKG). DKG exhibits the interesting ability to inhibit amyloid protein defibrillation, suggesting that ‘loss’
of ascorbate to DKG may serve beneficial roles (Yang & Zeng, 2018). DKG formation is thought to be irreversible in mammalian cells (Nishikawa et al., 2001), as DKG has been shown to have no antiscorbutic properties (Penney & Zilva, 1945). However, there is some evidence that DHA can be re-formed from DKG in vitro using non-biological agents such as aqueous hydrogen iodide (Penney & Zilva, 1945) or mercaptoethanol (Deutsch & Santhosh-Kumar, 1996).

DKG itself is an unstable compound, and has been reported to form a range of other products, including xylosone (Kang et al., 1982; Shin & Feather, 1990), xylonate, threonate and oxalate (Kagawa & Takiguchi, 1962), as well as incompletely characterised compounds. Under ‘physiological’ conditions, DKG appears to be susceptible to oxidative, reductive, and non-redox reactions:

**Oxidative:** Oxidation of DKG leads to the formation of threonate. This can occur via two sequential oxidative decarboxylation reactions, with 2-oxo-L-threo-pentonate (OTP; ‘2-keto-L-xylonate’) as intermediate (Parsons and Fry, 2012; Dewhirst and Fry, 2018), or it can occur via oxalyl-threonate esters (Dewhirst and Fry, 2018).

**Reductive:** Curiously, DKG (C₆H₈O₇ in the un-ionised form) also seems to be susceptible to reduction, forming an ascorbate-like product (‘compound 1’ of Kärkönen et al., 2017), that can generate H₂O₂ in *Picea abies* (Norway spruce) cell-suspension culture and in fresh culture medium (Kärkönen & Fry, 2006) and can also inhibit peroxidase activity (Kärkönen et al., 2017). This as-yet incompletely defined compound, with the molecular formula C₆H₆O₅, was demonstrated not to be compounds ‘C’ or ‘E’ as defined in the present manuscript (Kärkönen et al., 2017).

**Non-redox:** Non-redox products are the focus of this paper. One example is erythrulose (a non-oxidative product if the other product is oxalate), a highly reactive ketose that has been suggested to play a role in cataract formation in the human lens due to its ability to crosslink proteins (Simpson & Ortwerth, 2000). In animal cells DKG has been shown to undergo non-oxidative decarboxylation to form xylonate and lyxonate (Kanfer et al., 1960; Kagawa & Takiguchi, 1962). These compounds can then enter the pentose phosphate pathway, ultimately generating glucose 6-phosphate and lactate (Bánhegyi et al., 1997). The production of xylonate and lyxonate from DKG has not been documented in plants. Other non-redox products of DKG include its lactones (3,4-diketogulonolactone and 2,3-diketogulonolactone), which are ene-diols that may block the oxidation of yolk lipoproteins.
and so are potentially important for nutrition and health (Retsky et al., 1993; Li et al., 2001). Other C₅ catabolism products with the same redox status as DKG found in animals include l-xylusone (Shin & Feather, 1990). A further reported non-oxidative product of DKG is the C₆ branched-chain compound, CPA (2-carboxy-l-threo-pentonate) (Löwendahl & Petersson, 1976; Niemelä, 1987; Norman & Rose, 1987), assumed to be formed by a ‘benzilic acid rearrangement’ of DKG in which C-2 becomes a –COOH group attached to the former C-3, or C-3 becomes a –COOH group attached to the former C-2. CPA is the probable identity of ‘compound E’, reported to be formed from ascorbate in the apoplast of cultured Rosa cells (Green & Fry, 2005; Parsons et al., 2011).

Here we have characterised five non-redox products of DKG catabolism formed at room temperature in aqueous, slightly sub-pH7 solutions mimicking the plant apoplast (Green & Fry, 2005) and certain animal fluids such as urine, in which DKG is found (Monier & Weiss, 1953; Koshiishi et al., 1998). Two of these products (two epimers of 2-carboxy-l-threo-pentonolactone; CPLs) appear to be new compounds, whose production in vivo may offer analytically informative clues as to the redox state of the cellular compartments in which they occur as well as ‘informing’ the organism of non-oxidative, non-acidic conditions.

### 2. Materials and methods

#### 2.1. DKG preparation

Diketo-l-gulonic acid (DKG) was prepared from commercial DHA by alkaline hydrolysis in a solution containing 50 mM DHA and 60 mM NaOH. The hydrolysis was stopped after 6 minutes with a slight excess of acetic acid. The resulting solution of DKG was stored at –80°C.

Such preparations of DKG contained several other compounds [including the ‘C’ and ‘E’ previously reported by Green & Fry (2005)]. These compounds were isolated from a preparation of DKG by preparative high-voltage paper electrophoresis at pH 2.0, and subsequent elution from the paper.

#### 2.2. Anion-exchange column chromatography of DKG degradation products
A solution of freshly prepared DKG (0.5 ml, 50 mM) was passed through a 2-ml Dowex 1 anion-exchange column [formate form; pre-washed with 4 ml each of 0.5 M NaOH, 0.5 M formic acid, 2 M sodium formate, 10 mM formate (pyridinium) buffer (pH 5), then 8 ml H₂O]. Compounds were eluted with 10 × 0.5 ml H₂O, 3 × 1 ml 100 mM formic acid, 3 × 1 ml 150 mM formic acid, 3 × 1 ml 200 mM formic acid, 3 × 1 ml 250 mM formic acid, 3 × 1 ml 300 mM formic acid, 3 × 1 ml 4 M formic acid, and 3 × 1 ml 4 M trifluoroacetic acid. All fractions were dried in a SpeedVac and stored at –80°C.

2.3 High-voltage paper electrophoresis

Electrophoresis was performed on Whatman No. 3 paper at 2.5–3.5 kV for 30–70 minutes in a buffer of pH 2.0 (formic acid/acetic acid/water, 1:4:45, v/v/v) or pH 6.5 (pyridine/acetic acid/water, 33:1:300 v/v/v) (Fry, 2011). Orange G (2 µl, 10 mM) was added to all samples as an internal marker. Neutral compounds move a small distance away from the origin owing to electro-endosmosis. DKG and related compounds were stained with AgNO₃ (Fry, 2000). Electrophoretograms containing ¹⁴C-labelled compounds autoradiographed on film (Kodak BioMax MR-1 film) for 7 days.

For 2-dimensional paper electrophoresis, a single sample, along with external markers, was loaded onto Whatman No. 3 paper and subjected to electrophoresis as described above. The paper was then dried before the lane of interest was cut out. This lane was then sewn onto the origin of a new sheet of Whatman No. 3 paper, so that the compounds in the sample lane were lined up along the new origin. Further markers (internal marker Orange G, and external marker DKG products) were added. This new paper was then subjected to electrophoresis at either pH 2.0 or pH 6.5 as described previously.

2.4. Mass spectrometry

Samples were purified for mass spectrometry by preparative high-voltage paper electrophoresis followed by elution from the paper or by anion-exchange column chromatography. Samples were prepared at 2 µM concentration in 50:50 acetonitrile:water. Negative-ion electrospray ionisation mass spectrometry was carried out on a 12T SolariX Fourier transform ion cyclotron mass spectrometer (Bruker Daltonics).
2.5. Nuclear magnetic resonance spectroscopy

After purification by preparative high-voltage paper electrophoresis followed by elution from the paper, samples were dissolved in D$_2$O and Proton NMR spectra (500 MHz) and $^{13}$C NMR spectra (125 MHz) were obtained at 25°C, by use of a Bruker Avance 500 NMR spectrometer with a DCH cryoprobe optimised for $^{13}$C/$^1$H observation.

3. Results

3.1. CPLs are lactones of CPA

The incompletely characterised DKG metabolites ‘C’ and ‘E’ (Green & Fry, 2005; Parsons et al., 2011) will be shown in the present paper to be CPLs (a mixture of two epimers) and CPA respectively, and for consistency these names are used throughout. CPLs and CPA are formed non-enzymically in aqueous solution at at 20°C in slightly acidic media. The CPL spot was confirmed to be a lactonised form of CPA, as shown by experiments (Fig. S1) in which these compounds were incubated in either acid (promoting the formation of lactones) or alkali (promoting the hydrolysis of lactone rings) and then analysed by high-voltage paper electrophoresis at pH 6.5. CPL was the predominant spot during acidic incubation; alkali favoured CPA formation.

3.2. CPL comprises two isomers of \((C_6H_7O_7)^-\)

Putative CPL, obtained by preparative electrophoresis at pH 6.5, was further purified by anion-exchange chromatography and checked by paper electrophoresis at pH 2.0 (Fig. S2). Fraction 30 was highly enriched in two compounds, proposed to be epimers of CPL (Fig. S3a). Mass spectrometry of fraction 30 revealed a major peak at m/z 191.0186 (Fig. S3) agreeing with the simulated peak (m/z 191.0197; error 5.7 ppm) for \(C_6H_7O_7^-\), compatible with the CPLs being 2-carboxy-L-pentonolactones.

MS analysis of the compounds after further purification by electrophoresis at pH 2.0 (Fig. 1) supported the presence of two epimers of CPL \([(C_6H_7O_7)^-; \text{Fig. 1b,c}] and also supported CPA as \((C_6H_9O_8)^-\) (Fig. 1d). Furthermore, Cmpd 2 gave a peak corresponding to the simulated peak for xylonate and/or lyxonate, \((C_3H_5O_6)^1-\), discussed in more detail below.
3.3. NMR spectroscopy

CPLs have six carbons, all of which can be seen on the $^{13}$C-NMR spectrum (Fig. 2a). C-1 and C-2 are in the region (172–178 ppm) corresponding to carboxy groups. This is in agreement with the structure in Fig. 2a, which arbitrarily shows ‘C-1’ as the unsubstituted (ionisable) carboxy group and ‘C-2’ as the lactonised one. Epimers of CPL would have these two groups reversed. Since CPLs are formed non-enzymically, it is likely that the preparation is a mixture of the two epimers, both being lactones of CPA.

$^1$H-NMR spectroscopy (Fig. 2b) revealed four C–H bonds in the CPL preparation, corresponding to the predicted structure.

3.4. Multiple CPA-related compounds

Electrophoresis at pH 2.0 revealed that CPA and CPL samples (isolated by preparative electrophoresis at pH 6.5) contained at least five compounds, labelled CPA, CPL-a, CPL-b, Cmpd 1 and Cmpd 2 (Fig. 3a). These were individually eluted from a pH 2.0 preparative electrophoretogram and re-run analytically at pH 2.0 (Fig. 3b). Each compound proved acceptably stable during elution and re-running, migrating in the expected position (each accompanied by a trace of neutral material, probably including traces of soluble carbohydrates from the Whatman paper). Cmpd 1 was neutral, migrating to the same position as glucose and DHA. Cmpd 2 was slightly anionic at pH 2, co-migrating with threonate (and xylonate; Fig. 1a); and CPA and the CPLs were strongly anionic at pH 2 (migrating faster than the DKG marker). Cmpd 1 and the CPLs appeared to be lactones as they were lost on alkaline hydrolysis, leaving only CPA and Cmpd 2 (Fig. 3a).

3.5. CPL-a, CPL-b and Cmpd 2 each have a single ionisable group with low, moderate and high $pK_a$ respectively

Analysis by two-dimensional electrophoresis revealed some relationships between the compounds (Fig. 4). All the compounds were acceptably stable during electrophoresis and drying, as 2-D electrophoresis at pH 2.0 followed by pH 2.0 resulted in the compounds forming a single diagonal line (Fig. 4a). If interconversion between compounds had occurred
during the electrophoresis or drying then we would have seen a grid-like arrangement of silver-stained spots. Electrophoresis at pH 6.5 followed by pH 2.0 (Fig. 4b) and the converse (Fig. 4c) revealed that the spot of CPLs (‘Cmpd C’ of Green & Fry, 2005) in fact comprised CPL-a, CLP-b and Cmpd 2, all of which had co-migrated at pH 6.5 but were resolvable at pH 2.0. Since carboxylic acids are almost fully ionised at pH 6.5, these three compounds are concluded to possess the same constitution (~5–6 C atoms and one negative group, i.e. ‘C₅ 1–’ or ‘C₆ 1–’ compounds). The proposed structure of CPLs as defined in Fig. 2 has two possible epimers (2-carboxy-L-xylonolactone and 2-carboxy-L-lyxonolactone), and it is likely that the spots labelled CPL-a and CPL-b are these two epimers. At pH 6.5, CPA migrates faster than the CPLs (Green & Fry, 2005), as expected since CPA is a C₆ 2– compound whereas the CPLs are C₅ 1– compounds, which led to the conclusion that CPL is a lactonised form of CPA (Green & Fry, 2005). It is interesting that at pH 2.0, the lactone CPL-a migrates faster than the non-lactone CPA (e.g. Fig. 3), even though they possess 1 and 2 potentially ionisable groups respectively. Therefore, at pH 2.0, CPL-a carries a larger total negative charge than CPA, indicating that the single ionisable –COOH group of CPL-a has an exceptionally low pKa. The third C₅-6 1– product, Cmpd 2, despite approximately co-migrating with the CPLs at pH 6.5, had a much lower mobility at pH 2.0, indicating that it is a weak acid (high pKa) such as xylonate and/or lyxonate.

3.6. CPL-a and CPL-b are lactones of CPA, and Cmpd 1 is a lactone of Cmpd 2

All five compounds were purified by preparative electrophoresis, and each was treated with NaOH or HOAc (Fig. S4). As previously observed, CPL-a forms CPA on alkali treatment. CPL-b underwent similar changes. Furthermore, CPA formed faint spots of CPL-a and CPL-b in response to acidic treatment. The results confirmed that the CPLs and CPA are interconvertible: an acidic medium promoted the formation of the CPLs, whereas alkali (which hydrolyses lactones) promoted CPA formation. Similarly, alkali appeared to convert the non-ionic Cmpd 1 to the weakly acidic Cmpd 2, whereas acid converted Cmpd 2 to Cmpd 1, indicating that Cmpd 1 is a lactone of Cmpd 2.

3.7. All five CPA-related products retain carbon-1 of the precursor DKG
Radiolabelled CPA (prepared from $[1^{-14}C]$ascorbate via $[1^{-14}C]$DKG) provided more insight into the non-oxidative degradation pathway of DKG (Fig. 5). Treatment of $[^{14}C]$CPA with acid yielded all five expected radiolabelled compounds. These corresponded to the five AgNO$_3$-stained compounds seen on previous electrophoretograms, demonstrating that each compound retained the $^{14}C$ atom from C-1 of the original ascorbate molecule. This is as expected for CPA and the CPLs since they still contain all six carbons originating from the $[^{14}C]$ascorbate.

Cmpd 2 had a migration pattern similar to that of threonate (e.g. Fig. 3b). We hypothesised that Cmpd 2 would have a similar constitution — for example xylonate and its epimer lyxonate are reported degradation products of ascorbate (presumably formed via DKG) (Kanfer et al., 1960). As small aldonic acids with one negative charge, xylonate and lyxonate would be expected to have an electrophoretic mobility similar to that of threonate. When Cmpd 2 was electrophoresed with internal or external marker xylonate (Fig. S5), they co-migrated, supporting the identification of Cmpd 2 as xylonate and/or lyxonate.

Cmpd 2 (although presumed to be a C$_5$ compound, having lost one carbon of the CPLs or CPA, probably as CO$_2$) was also visible among the radiolabelled compounds (Fig. 5); thus at least some of the Cmpd 2 molecules also retained the $^{14}C$ atom that originated from C-1 of the precursor ascorbate molecule. The same applied to neutral Cmpd 1, which is an important observation as a radiolabelled spot cannot be a contaminating carbohydrate eluted from the Whatman paper. One possible identity for this neutral compound could be a di-lactone of CPA; however, Cmpd 1 did not yield any detectable CPA after alkaline hydrolysis (Fig. S4), so this identity is unlikely. More probably, Cmpd 1 would be one or both of the pentonolactones, xylonolactone and lyxonolactone, formed by decarboxylation of CPLs. This is supported by the observation that alkaline hydrolysis of Cmpd 1 yielded Cmpd 2 (Fig. S4).

4. Discussion

4.1. Occurrence and structures of DKG catabolites

We have further characterised the non-oxidative, non-enzymic degradation pathway of DHA, via DKG, as it occurs under slightly acidic conditions mimicking the plant apoplast (Fig. 6). This includes identification of CPA and CPLs, previously termed compounds ‘E’ and ‘C’ (Green & Fry, 2005; Parsons et al., 2011).
CPA [described as ‘2-(threo-1,2,3-trihydroxypropyl)tartronic acid’, and presumed to be formed via DKG] had previously been identified as a degradation product of DHA in near-neutral (pH 7.4) and alkaline aqueous solutions (Löwendahl & Petersson, 1976); and its production in alkali was confirmed (Niemelä, 1987). In the presence of Cu2+, DKG was also rearranged to CPA at pH 1–14, and at pH 1–7 the CPA became sequestered as an insoluble crystalline complex with Cu2+ (Norman & Rose, 1987). The possible role of traces of apoplastic Cu2+ in generating CPA/CPL in vivo would be of interest in future studies.

The formation of the lactones characterised by us as CPLs has apparently not been previously reported. In prior studies of DKG degradation in alkaline environments, any lactones would have been quickly hydrolysed. Indeed, Parsons et al. (2011) reported that, under the slightly acid conditions typical of the plant apoplast (and human urine), CPLs are formed first; CPA may have been generated mainly by subsequent hydrolysis of the lactones. CPL and CPA are interconvertible as shown in Fig. 6.

The formation of CPLs is proposed to occur via a ‘benzilic acid rearrangement’ of the vicinal diketone group of DKG. This type of rearrangement is named after the reaction by which benzil [(PhCO)2] is converted to benzilic acid (2,2-diphenyl-2-hydroxyacetic acid) (Fig. 6, inset); it also occurs during saccharinic acid formation when a reducing sugar is treated with alkali (Collins & Ferrier, 1995). If, in vivo, the CPLs are formed first (Parsons et al., 2011), it seems likely that the δ-lactone of DKG is the main species that undergoes the benzilic acid rearrangement under our slightly acidic conditions, removing one carbon from the 6-membered ring to leave a 5-membered ring comprising a mixture of the two epimeric γ-lactones (Fig. 6).

The two epimers of CPL were well resolved by electrophoresis at pH 2.0 (Figs. 1, 3, 4, 5 and S4); however, it is not clear which epimer would have the lower pKa.

CPA and/or the CPLs are also subject to decarboxylation, forming simple pentonates and their lactones. Decarboxylation of one of the two CPL epimers would yield xylonate, whereas the other would give lyxonate. Xylonate and lyxonate have previously been identified in animals as ascorbate catabolites (Kanfer et al., 1960; Kagawa & Takiguchi, 1962; Shin & Feather, 1990). Xylonolactone is a precursor of xylonate in bacteria (Buchert & Viikari, 1988). We detected xylonate and/or lyxonate by silver staining; they also retained at least some of the radioactivity originating from [1-14C]DKG. A CPL molecule, formed directly from [1-14C]DKG δ-lactone, would retain all the 14C within the ring, with the former C-2 or
C-3 becoming the new free –COOH group. If a [14C]CPL molecule formed in this way underwent decarboxylation by losing its free –COOH group, then the xylonolactone or lyxonolactone formed would retain all the 14C. On the other hand, if [14C]CPL delactonises to [14C]CPA, and then undergoes decarboxylation by losing one of its two free –COOH groups, then the xylonate or lyxonate formed will retain only about half the original radioactivity since one of the two –COOH groups is now radioactive. Either way, radioactivity of the [14C]DKG would be retained in the simple pentonic acids and their lactones though possibly with a lower specific radioactivity (Bq μmol⁻¹).

Any xylonate/lyxonate formed by direct decarboxylation of [1-14C]DKG would be completely non-radioactive, since all the 14C would then have been in the single carboxy group, lost upon decarboxylation. We therefore conclude that the simple 14C-pentonic acids detected arose via intermediary CPA and/or the CPLs and not directly from DKG.

In vivo, with [14C]DHA as substrate, [14C]CPL was found to be produced before [14C]CPA, with the yield of both being maximal under non-oxidising conditions which favour the production of [14C]DKG rather than [14C]oxalyl threonates (Parsons et al., 2011). CPL is a stable end-point of DHA catabolism in vivo (Parsons et al., 2011). The equilibrium in plant cell culture media favours the production of CPL over CPA as it is a slightly acidic environment. However, DKG is present in blood (Koshiishi et al., 1998), whose pH is typically 7.4 and so the equilibrium in this system may promote the formation of CPA.

The current study was conducted in vitro, primarily in a buffer (acetate, pH 4.5) mimicking the plant apoplast, so it is likely that the reported compounds are formed in vivo; indeed CPL and CPA have been detected in the apoplast of cultured Rosa cells (Cmpds C and E of Green & Fry, 2005). However, more work will be required to definitively establish the presence of these degradation products in intact plants and animals.

4.2. Biological relevance

The present work shows that CPA and CPL can be generated from DKG non-enzymically under slightly sub-pH7 conditions in vitro. They have been also shown to form non-enzymically in vivo in both animals (CPA; Löwendahl and Petersson, 1976) and plants (CPA and CPL; Green & Fry, 2005). They may therefore be useful to researchers as ‘fingerprints’,
and equally to the organisms in which they occur as ‘signals’, indicating a non-oxidative, non-acidic biological milieu.

It has previously been shown in living Rosa cell-suspension cultures at pH 6, that 360 μM apoplastic ascorbate is 90–95% degraded within 9 h, extracellularly, generating ~30–40 μM CPL + CPA plus other by-products (Fig. S1 of Parsons et al., 2011). The rates of formation of CPL + CPA were similar in (i) freshly prepared sterile culture medium, (ii) whole Rosa cultures, (iii) Rosa culture filtrate and (iv) heat-denatured Rosa culture filtrate — indicating that the reaction is largely non-enzymic. This appreciable yield of CPL + CPA observed in Rosa cultures indicates the biological prevalence of these compounds.

As fingerprints, it is relevant that CPA and CPL are relatively stable in vivo, for example persisting for >8 hours in Rosa cell cultures (Parsons et al., 2011), which suggests that their presence could be a lasting indicator of prior non-oxidising and sub-pH7 conditions within the organism. Likewise, Löwendahl and Petersson (1976) suggest that as CPA is formed in blood from DHA there could be a medical significance.

Biologically, CPA and CPL could potentially signal to the cell that it is not under oxidising stress and so can downregulate ascorbate production. Under oxidising conditions, DHA is principally oxidised to oxalyl threonates; only when oxidation is not prevalent does DHA persist long enough (in slightly sub-pH7 solutions) for hydrolysis to predominate and for the DKG thus formed to be further metabolised via the non-oxidative reactions reported in the present manuscript.

In more acidic solutions, the formation of CPA and CPL is not favoured. Therefore, a further possibility is that CPL and CPA formation signals that the biological compartment is less acidic than normal. Relative alkalinisation of the apoplast (favouring CPL and CPA production) is a signal of drought stress (Harting et al., 1988; Pignocchi & Foyer, 2003), to which it is valuable for the organism to be attuned.

5. Conclusion

It is well known that ascorbate can be degraded, via DKG, to DKG. However, the downstream fate of DKG remains incompletely characterised. In this work, we have characterised five DKG products, formed non-oxidatively and non-enzymically in media at slightly acidic pH. Novel products in this category include two epimers of 2-carboxy-L-threo-
pentonolactone (CPL); their free acid (CPA) was also detected. CPLs and CPA can be further decarboxylated to xylonate and/or lyxonate. Although in the present work these reactions were monitored in vitro, in the absence of enzymes, it appears very likely that the products are also formed in vivo, especially in biological compartments of a slightly acidic pH and where ROS are not so active that DKG is quickly oxidised by in competing pathways. We propose that CPLs and CPA can serve as ‘fingerprints’ (valuable analytically) and signals (valuable to the organism), reporting a cell’s redox and pH status.

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**Fig. Legends**

**Figure 1:** Mass spectrometry of DKG degradation products purified by anion-exchange column chromatography.

CPL and CPA-related compounds were separated by anion-exchange column chromatography with elution in increasing concentrations of formic acid (Fig. S2), and portions of the fractions containing the putative xylonic acid and CPL (fractions 27 and 30 respectively) were checked qualitatively by electrophoresis at pH 2.0 (a).

CPL-a, CPL-b, CPA and Cmpd 2, further purified by preparative electrophoresis at pH 2.0 were analysed using mass spectrometry. The measured spectra are shown above the simulated spectra for a compound with the formula $\left( \text{C}_{6}\text{H}_{7}\text{O} \right)^{-}$ for CPLs (b and c), $\left( \text{C}_{6}\text{H}_{8}\text{O} \right)^{-}$ for CPA (d) and $\left( \text{C}_{5}\text{H}_{6}\text{O} \right)^{-}$ for Cmpd 2 (e).

**Abbreviations:** CPA, 2-carboxy-L-threo-pentonate; CPL, 2-carboxy-L-threo-pentonolactone; DHA, dehydroascorbic acid; DKG, diketogulonate; EryR, erythrarate (meso-tartrate); ThrR, threarate (L-tartrate); XylO, xylonate.

**Figure 2:** $^{13}$C and $^1$H NMR spectra of CPL.

A purified sample of CPL (fraction 30 of Fig. S2) was analysed by $^{13}$C NMR spectroscopy (a) and by $^1$H NMR spectroscopy (b; expanded carbohydrate region). Inset: proposed structure.

**Figure 3:** At least three additional compounds accompany CPA and CPL-a.

(a) A preparation of CPA was incubated with 0.1 M NaOH or 0.2 M acetic acid (HOAc) for 16 h before being neutralised and run by electrophoresis at pH 2.0.

(b) The five compounds present in the CPA sample were separated by preparative electrophoresis, eluted from the paper in water, and re-run by analytical electrophoresis at pH 2.0.

**Abbreviations:** as in Fig. 1 plus AA, ascorbate; Glc, glucose; OG, Orange G; OxT, oxalyl threonate.

**Figure 4:** Two-Dimensional electrophoresis of CPA-related compounds.

Three identical samples containing a mixture of the five CPA-related compounds were run by electrophoresis (left to right, as depicted) at pH 2.0 or 6.5. The marker strips from this first-dimension run were cut off for staining (horizontal rectangles), then the strips containing the now separated (but unstained) analytes were cut out and sewn onto the origin of a new electrophoretogram. The new papers (with a new set of markers; vertical rectangles) were run at either the same or the other pH
such that the analytes now moved at 90° to first direction. After this second-dimension run, the whole papers were stained. Orange G was present as a marker in both dimensional runs (replenished at the new origin for the second dimension). The permutations of 2-dimensional runs were (a) pH 2.0 then pH 2.0; (b) pH 6.5 then pH 2.0; and (c) pH 2.0 then pH 6.5. In (a), all stable compounds should appear along the diagonal (dashed white line). In (b) and (c), we have traced the path of compounds in the two dimensions (dashed lines).

**Figure 5: All five CPL-related compounds retain C-1 of DKG.**

\[^{14}\text{C}]\text{CPA} \text{ (derived from } [1-{^{14}\text{C}}]\text{DKG}) \text{ was incubated in } 0.1 \text{ M NaOH or } 0.2 \text{ M acetic acid (HOAc) for 16 h. The samples were neutralised and re-run by electrophoresis at pH 2.0. The marker mixture contains a range of oxidation and hydrolysis products from } [^{14}\text{C}]\text{ascorbate (Green & Fry, 2005). An autoradiogram is shown.}\n
**Abbreviations:** as in Figs. 1 and 3 plus: cOxT, cyclic oxalyl threonate; OTP, 2-oxo-L-threo-pentonate ('2-keto-L-xylonate').

**Figure 6: Non-oxidative degradation of DHA via DKG.**

All reactions shown are non-enzymic. DKG is produced by hydrolysis of DHA and is proposed to undergo a reaction ('benzilic acid rearrangement' of the 1,2-diketone group) to form CPA and two epimers of CPL. Under slightly acidic conditions physiologically mimicking the plant apoplast or mammalian urine, the CPLs are formed before CPA (Parsons et al., 2011), suggesting that DKG \(\delta\)-lactone is the main species which undergoes the benzilic acid rearrangement, removing one carbon from the 6-membered ring to leave a mixture of the two epimeric \(\gamma\)-lactones. CPA and the CPLs are interconvertible; they are also subject to decarboxylation, forming xylonate, lyxonate and their lactones. The black asterisk (*) indicates the C atom which in some experiments was radioactive; grey asterisks imply a lower expected specific radioactivity. The classic benzilic acid rearrangement is shown in the lower left-hand box.
**Titles of Supplementary Figures**

Figure S1: CPLs and CPA can interconvert.

Figure S2: Anion-exchange purification of compound 2 and the CPLs.

Figure S3. Mass spectrometry of CPLs partially purified by anion-exchange column chromatography.

Figure S4: Alkali converts CPLs to CPA and converts Cmpd 1 to Cmpd 2.

Figure S5: Cmpd 2 co-migrates with xylonic acid on electrophoresis at pH 6.5.
Fig. 1

**a**

Orange G

CPL-a

CPL-a

CPA

CPL-b

DKG

CPL-b

CPL-a

DKG

Markers 27 30

Markers

**b**

CPL-a

Simulated (C_7H_7O_7)^{-}

m/z = 191.019726

1 - carboxy-pentonolactone epimer 1

**c**

CPL-b

Simulated (C_7H_7O_7)^{-}

m/z = 191.019726

2 - carboxy-pentonolactone epimer 2

**d**

CPA

Simulated (C_5H_9O_6)^{-}

m/z = 165.040462

Xylonic acid / Lyxonic acid

**e**

Cmpd 2

Simulated (C_5H_9O_6)^{-}

m/z = 165.040462

Cmpd 2

Neutral (DHA)

EryR

ThrR

ThrO

XylO

Neutral (DHA)
Fig. 2

(a) 

(b)
Fig. 3

(a) Electrophoresis pH 2

(b) Electrophoresis pH 2

Markers: OTA, CPL-a, CPL-b, CPA, OX1, Glc, AA, DHA, EryR, ThrO, Compound 1, Compound 2

Pre-purified by preparative electrophoresis
Fig. 4

a 2-D electrophoresis pH 2.0 / pH 2.0

b 2-D electrophoresis pH 6.5 / pH 2.0

c 2-D electrophoresis pH 2.0 / pH 6.5
Fig. 5

electrophoresis pH 2.0

Orange G

CPL-a

CPA

CPL-b

DDK

OTP

AA + DHA

Marker mixture

[14C]CPA + NaOH

[14C]CPA + HOAc

Cmpd 1

Cmpd 2
**Figure S1: CPLs and CPA can interconvert.** Partially purified aqueous solutions of CPLs and CPA were incubated for 16 h in the presence of either 0.1 M NaOH or 0.2 M acetic acid. After incubation, the samples were neutralised with either acetic acid or NaOH. Products were resolved by electrophoresis at pH 6.5 and 2.5 kV for 30 minutes, and then stained with silver nitrate. All samples contained a trace of the marker, Orange G, which was circled in pencil before silver-staining.

**Abbreviations:**
- CPA, 2-carboxy-L-threo-pentonate
- CPL, 2-carboxy-L-threo-pentonolactone
- DHA, dehydroascorbic acid
- DKG, diketogulonate
- EryR, erythrarate (*meso*-tartrate)
- Glc, glucose
- HOAc acetic acid
- OG, Orange G
- OxT, oxalyl threonate
- ThrO threonate
- ThrR, threarate (L-tartrate)
**Figure S2: Anion-exchange purification of compound 2 and the CPLs.** A solution of DKG (50 mM, containing additional degradation products) was passed through a Dowex 1 anion-exchange column (formate form). Compounds were eluted with H$_2$O, increasing concentrations of formic acid (0.1–4.0 M) and 4 M TFA. After elution and drying, the 32 collected fractions were redissolved in H$_2$O, and a small portion of each was analysed by electrophoresis at pH 2.0 (at 2.5 kV) and stained in silver nitrate. Fractions 27 and 30, enriched in compounds 2 and the CPLs respectively, were selected for further analysis.

**Abbreviations:**
- CPA, 2-carboxy-\(\text{L-threo}\)-pentonate
- CPL, 2-carboxy-\(\text{L-threo}\)-pentonolactone
- DKG, diketogulonate
- OG, Orange G
- ThrO threonate
Figure S3. Mass spectrometry of CPLs partially purified by anion-exchange column chromatography.
CPLs were partially purified as in Fig. S2, and a sample of fraction 30 was analysed by mass spectrometry.
(a, c) Measured spectra, broad and narrow range; (b, d) simulated spectra for (C₆H₇O₇)⁻.
Figure S4: Alkali converts CPLs to CPA and converts Cmpd 1 to Cmpd 2.
CPA-related compounds were eluted from a preparative electrophoretogram and then incubated in 0.1 M NaOH or 0.2 M acetic acid (HOAc), or with neither, for 4 h. After 4 h, the samples were neutralised with acetic acid or NaOH, spiked with orange G (internal marker), and then examined by analytical electrophoresis at pH 2.0 and silver-stained.

Abbreviations:
AA, ascorbate
CPA, 2-carboxy-L-threo-pentonate
CPL, 2-carboxy-L-threo-pentonolactone
DHA, dehydroascorbic acid
DKG, diketogulonate
EryR, erythrarate (meso-tartrate)
Glc, glucose
HOAc acetic acid
OG, Orange G
ThrO, threonate
ThrR, threarate (L-tartrate)
Figure S5: Cmpd 2 co-migrates with xylonic acid on electrophoresis at pH 6.5.
A sample of Cmpd 2 (eluted from a preparative electrophoretogram) was run alongside a dilution series of commercial xylonic acid (XylO). A mixture of Cmpd 2 plus XylO was also run in one lane. The paper was subjected to electrophoresis at pH 6.5 and then stained with silver nitrate.

Abbreviations:
CPA, 2-carboxy-L-threo-pentonate
CPL, 2-carboxy-L-threo-pentonolactone
OG, Orange G
XylO, xylonate
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