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Ascorbic acid conjugates isolated from the phloem of *Cucurbitaceae*

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ABSTRACT

Analysis of phloem exudates from the fruit of *Cucurbitaceae* revealed the presence of several compounds with UV–visible absorption spectra identical to that of L-ascorbic acid. In *Cucurbita pepo* L. (zucchini), the compounds could be isolated from phloem exudates collected from aerial parts of the plant but were not detected in whole tissue homogenates. The compounds isolated from the phloem exudates of *C. pepo* fruit were eluted from strong anion exchange resin in the same fraction as L-ascorbic acid and were oxidised by ascorbate oxidase (E.C. 1.10.3.3). The major compound purified from *C. pepo* fruit exudates demonstrated similar redox properties to L-ascorbic acid and synthetic 6-*O*-glucosyl-L-ascorbic acid (6-GlcAsA) but differed from those of 2-*O*-glucosyl-L-ascorbic acid (2-GlcAsA) isolated from the fruit of *Lycium barbarum* L. Parent and fragment ion masses of the compound were consistent with hexosyl-ascorbate in which the hexose moiety was attached to C5 or C6 of AsA. Acid hydrolysis of the major *C. pepo* compound resulted in the formation of L-ascorbic acid and glucose. The purified compound yielded a proton NMR spectrum that was almost identical to that of synthetic 6-GlcAsA. A series of L-ascorbic acid conjugates have, therefore, been identified in the phloem of *Cucurbitaceae* and the most abundant conjugate has been identified as 6-GlcAsA. The potential role of such conjugates in the long-distance transport of L-ascorbic acid is discussed.

1. Introduction

Over the last decade significant advances have been made in the understanding of the physiology and metabolism of L-ascorbic acid (AsA) in plants. The *de novo* biosynthetic pathway was identified in 1998 (Wheeler et al., 1998) and all of the pathway enzymes have now been identified and the corresponding genes cloned (Østergaard et al., 1997; Keller et al., 1999; Wolucka et al., 2001; Gatzek et al., 2002; Laing et al., 2004; Dowdle et al., 2007). Advances have been made in understanding the pathways of ascorbate breakdown (Green and Fry, 2005) and the role of AsA as a precursor for organic acids (DeBolt et al., 2007). A number of advances have also been made in understanding the role of ascorbate in plant physiology aided by the availability of the AsA deficient vtc mutants of *Arabidopsis thaliana* that have revealed roles for AsA in developmental processes such as flowering, senescence and morphogenesis (Pavet et al., 2005; Attolico and De Tullio, 2006; Barth et al., 2006; Olmos et al., 2006). The vtc mutants have also aided the analysis of the role of AsA in the modulation of plant responses to both biotic (Pavet et al., 2005) and abiotic (Pignocchi and Foyer, 2003) stresses.

In many plant tissues the concentration and redox status of the AsA pool is tightly controlled. For example, with the exception of the apoplast, the aerial parts of plants have a highly reduced AsA pool with dehydroascorbic acid (DHA) representing less than 10% of the total (Noctor, 2006) while in root tissues the AsA pool is more oxidised with up to 30% DHA (Córdoba-Pedregosa et al., 2003). The mechanisms controlling AsA concentration and redox status are still largely mysterious with large areas of uncertainty concerning the genetic and biochemical controls of pathway flux (Hancock and Viola, 2005). Further uncertainty exists regarding the control of distribution of AsA at the whole plant level. Several studies have indicated that long-distance AsA transport via the phloem can occur (Franceschi and Tarlyn, 2002; Tedone et al., 2004) however, in the only systematic study undertaken to date, AsA accumulation in blackcurrant (*Ribes nigrum*) fruit was correlated with *in situ* synthesis and the contribution of AsA transport from source tissues was negligible (Hancock et al., 2007). It remains unclear to what extent AsA transport contributes to sink accumulation in other species and organs. Although the phloem is capable of synthesising AsA from sugars (Hancock et al., 2003), radiolabelling evidence also suggests active uptake of AsA in source leaf phloem (Franceschi and Tarlyn, 2002; Tedone et al., 2004) although the transporters are yet to be identified. These experiments were undertaken in apoplastic phloem loaders and currently no information is available regarding the uptake of AsA by the phloem of symplastic loaders.

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During research to gain greater insight into the roles of phloem AsA, a number of compounds with physicochemical properties similar to those of authentic AsA were isolated. In the present work, the presence of such compounds in several *Cucurbitaceae* species are documented and detailed physicochemical analysis of the most abundant AsA analogue from *Cucurbita pepo* was undertaken.

2. Results

2.1. ***Cucurbitaceae*** *phloem exudates contain multiple AsA analogues*

Analysis of deproteinated phloem exudates from several species of *Cucurbitaceae* by cation interaction HPLC, revealed the presence of several peaks with identical absorption spectra to AsA but different retention times. Retention times and relative peak areas of compounds with maximum absorbance 245 ± 3 nm from several *Cucurbitaceae* fruit are presented in Table 1. Only AsA was detected in *C. maxima* however, in all other species analysed at least one additional peak was detected and in several species a number of additional peaks were observed. For the majority of species sampled, AsA was the most abundant peak however, in *C. maxima* × *C. moschata* the peak at 9.0 min was almost four times as abundant as AsA and in *Cucumis sativus* (cucumber) the peak eluting at 11.7 min was slightly more abundant than AsA.

Due to the relatively large volumes of exudate that could be collected from *C. pepo* (zucchini) fruit, the presence of at least three of the four unidentified peaks and the ready availability of fruit from local markets throughout the entire year, further characterisation was undertaken on exudates from this species. To enrich for putative AsA analogues, crude exudates were collected into an equal volume of 10% $HClO_4$ containing 10 mM TCEP. After neutralisation with 5 M K_2CO_3, sample supernatants were applied to strong anion exchange cartridges and AsA and putative analogues were eluted with 300 mM formic acid. Following purification and concentration an additional peak was observed eluting after 13.5 min (Fig. 1).

Confirmation that the peaks eluting in the 300 mM formic acid fraction were related to AsA was achieved following incubation with ascorbate oxidase (E.C. 1.10.3.3) which resulted in the removal of all peaks (Fig. 2). Ascorbate oxidase specifically oxidises AsA, its 5-carbon analogue D-erythroascorbate (Hancock et al., 2000) and a few closely related glycosyl conjugates (Loewus, 1999).

The distribution of AsA related compounds within plant tissues was quantified in zucchini plants grown under glass. The most abundant analogue (Fig. 1, peak 2) was detected in trace amounts in flowers but could not be detected in any other whole tissue extract (Table 2). On the contrary, AsA was found in all tissues examined at concentrations similar to those reported in previous studies (Ranieri et al., 1993; Logan et al., 1998). Both AsA and its most abundant analogue were found in phloem exudates collected from fruit, leaf petioles and stems. In comparison to our previous findings in locally purchased zucchini (Hancock et al., 2003), phloem AsA concentrations were approximately 10-fold lower. This may be related to the growing conditions of the fruit which failed to exude as strongly as those obtained commercially. The position of phloem sampling had little effect on the AsA concentration determined with similar values recorded whether sampled from the stem, the leaf petiole or from fruit. In contrast, the concentration of the major analogue was dependent on the part of the plant from which phloem was sampled with low concentrations in phloem exudates from the stem, intermediate concentrations in fruit exudates and high concentrations in leaf petiole exudates.

2.2. The major zucchini analogue is a glucoside

Acid hydrolysis resulted in the progressive conversion of the primary analogue isolated from zucchini phloem to AsA as determined by cation interaction HPLC with diode array detection (Fig. 3, panel A). The other reaction product was glucose as determined by anion exchange HPLC with pulsed amperometric detection (Fig. 3, panel B). These data indicate that the major AsA analogue present in the phloem of zucchini is an L-ascorbic acid glucoside.

2.3. The most abundant zucchini conjugate has physicochemical properties similar to AsA and 6-GlcAsA but different from 2-GlcAsA

To further characterise the major AsA glucoside isolated from zucchini fruit exudate (Fig. 1, peak 2), its absorption spectrum was compared with that of AsA, synthetic 6-*O*-β-D-glucosyl AsA (6-GlcAsA) and 2-*O*-β-D-glucosyl AsA (2-GlcAsA) isolated from *Lycium barbarum* L. fruit (Toyoda-Ono et al., 2004). AsA, 6-GlcAsA and the glucoside isolated from zucchini fruit exudates all showed similar absorbance spectra at both pH 2.0 and pH 7.6 with λ_{max} close to 243 nm and 265 nm, respectively. On the contrary, 2-GlcAsA had lower absorbance maxima of 235 and 259 nm at acid and alkaline pH (Table 3).

AsA, 6-GlcAsA and the unknown zucchini glucoside demonstrated a strong capacity to reduce dichlorophenolindophenol (DCPIP). On the contrary, 2-GlcAsA demonstrated a very weak capacity to reduce DCPIP (Table 4). The mechanism of DCPIP reduction by AsA involves both the C2 and C3 hydroxyl groups (Rao et al., 1987) suggesting that these groups were unmodified in the zucchini analogue.

AsA was readily oxidised by both H_2O_2 and ascorbate oxidase while synthetic 6-GlcAsA and the zucchini analogue showed an intermediate oxidation rate approximately 40% of that of AsA. 2-GlcAsA was completely resistant to oxidation by either H_2O_2 or

Table 1
Relative concentration of compounds with similar optical properties to AsA isolated from *Cucurbitaceae* phloem

Species	Peak retention (min)				
	7.7	9.0	10.0	11.7	12.7
C. maxima × *C. moschata*	0.25 ± 0.01	3.69 ± 0.04	0.14 ± 0.02	ND	1.00 ± 0.03
Cucurbita moschata	ND	0.38 ± 0.05	ND	ND	1.00 ± 0.05
C. maxima	ND	ND	ND	ND	1.00
C. pepo (zucchini)	0.03 ± 0.01	0.50 ± 0.01	0.03 ± 0.01	ND	1.00 ± 0.01
C. pepo (squash)	ND	0.73 ± 0.01	0.01 ± 0.0	ND	1.00 ± 0.01
Cucumis sativus	ND	ND	ND	1.29 ± 0.05	1.00 ± 0.05

Phloem exudates from a number of *Cucurbitaceae* species were harvested from cut fruits and stabilised by dilution into an equal volume of 10% metaphosphoric acid containing 10 mM TCEP. Protein precipitates were removed by centrifugation and the supernatant filtered through a 0.22 μm filter and injected onto a Coregel 64H cation exchange column. UV traces were collected at 245 nm and absorbance spectra of individual peaks monitored between 200 and 600 nm using a UVD 340U Diode array detector. Under the conditions described authentic AsA eluted at 12.7 min and had a maximum absorbance at 245 nm. Compounds with similar optical properties were defined as those with a λ_{max} 245 ± 3 nm. Values are presented relative to AsA ± SE (n = 3). ND = peak not detected.

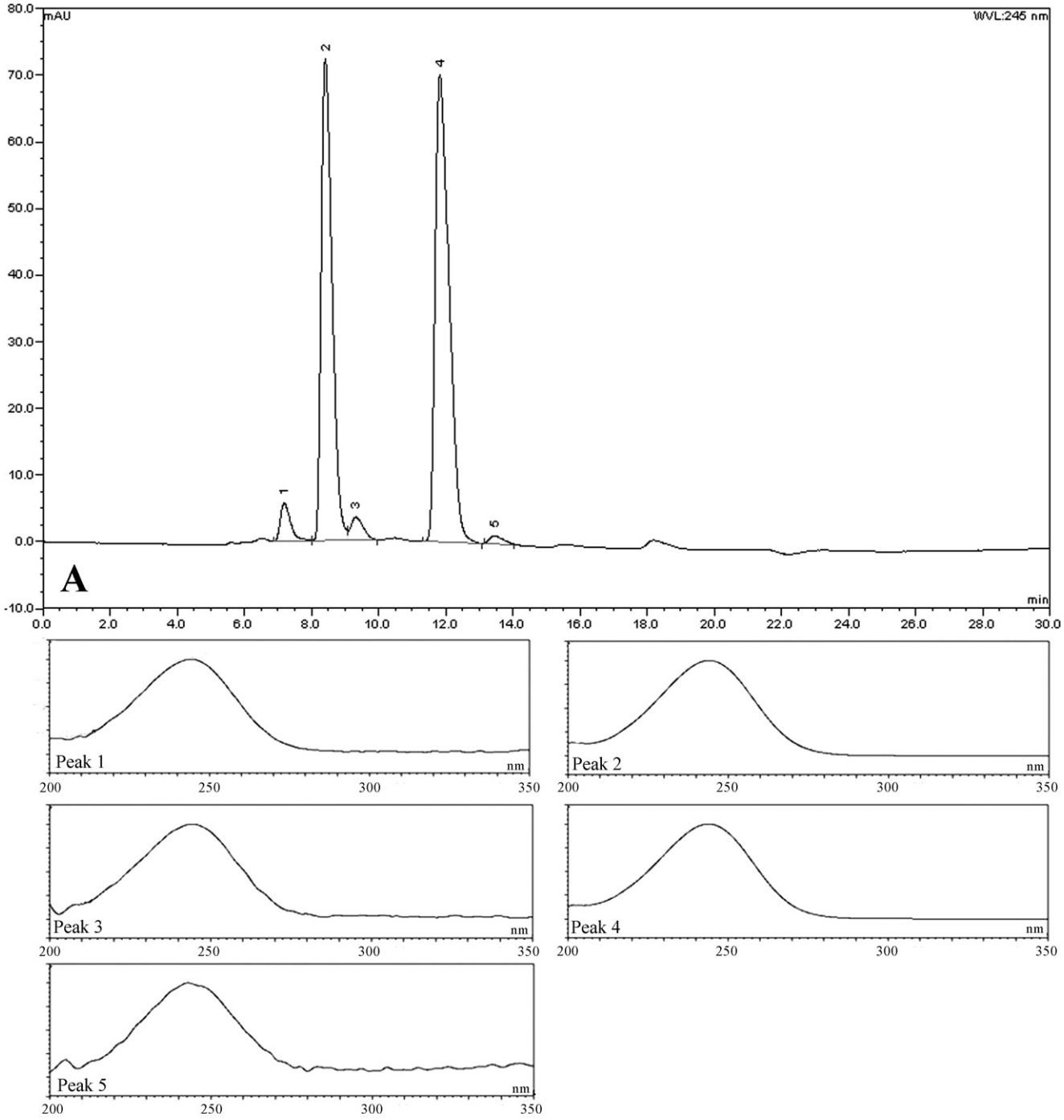

Fig. 1. HPLC traces of partially purified zucchini phloem exudates. Phloem exudates were collected from zucchini fruit into an equal volume of 10% $HClO_4$ containing 10 mM TCEP. Samples were centrifuged and the supernatant neutralised with 5 M K_2CO_3. Following centrifugation to remove insoluble $KClO_4$, sample supernatants (500 μl) were applied to SAX cartridges (100 mg resin, acetate form) and washed with distilled H_2O. Samples were eluted in 4 ml 300 mM formic acid, lyophilized, resuspended in 200 μl distilled H_2O and injected onto a Coregel 64H cation interaction column as described in the text. Panel A shows the HPLC chromatogram recorded at 245 nm. Peaks with absorbance maxima in the region 245 nm are numbered and the absorbance spectra are shown. Peak 4 co-eluted with authentic AsA.

ascorbate oxidase (Table 4). H_2O_2 oxidation of AsA proceeds through the abstraction of a proton from the hydroxyl group at C2 to form the ascorbate free radical, water and hydroxyl radical (Isbell and Frush, 1979). This suggests that the zucchini AsA glucoside had a free hydroxyl group at the C2 position. Similarly, hydrogen bonding between the hydroxyl group at position 2 of AsA and a histidine residue at the active site is required for the interaction of AsA with ascorbate oxidase (Caselllla et al., 1999). A further hydrogen bond is required between the hydroxyl group at C3 of AsA and tryptophan 362 of ascorbate oxidase, providing further support for a lack of modification at C2 or C3 of the zucchini glucoside. On the contrary, ascorbate oxidase is highly tolerant of substitutions of C6 with bromyl, deoxy and phenyl modifications well tolerated (Wimalasena and Dharmasena, 1994).

The rates of auto-oxidation of AsA and analogues were examined at pH 7.0 in the temperature range 30–70 °C. AsA, synthetic 6-GlcAsA and the zucchini glucoside all showed strong temperature dependent auto-oxidation kinetics while auto-oxidation was barely detected in 2-GlcAsA (Table 4).

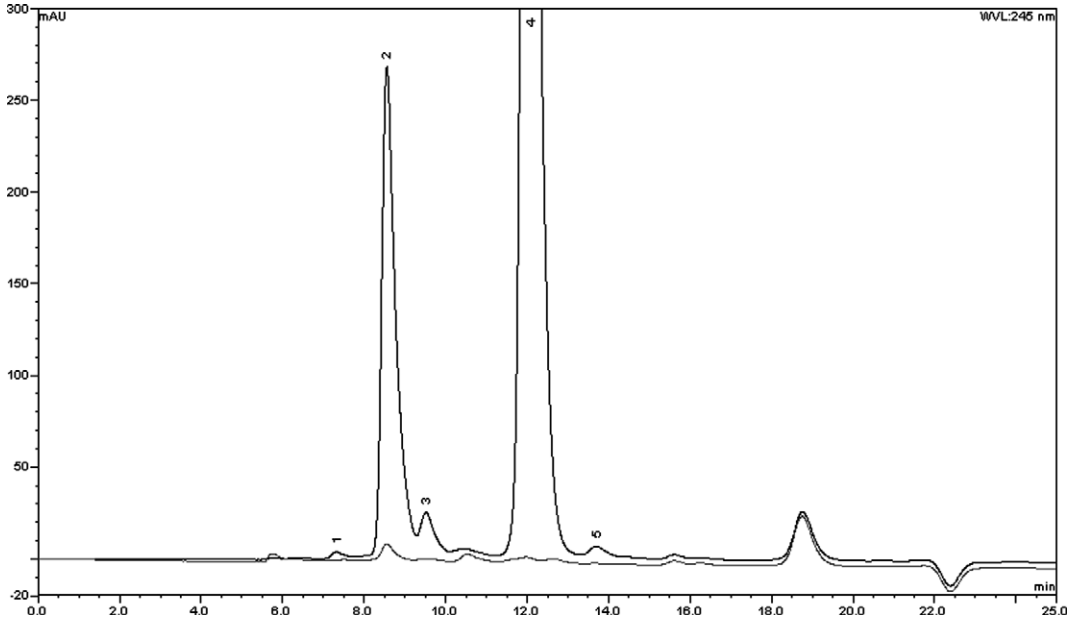

Fig. 2. Treatment of zucchini phloem exudates with ascorbate oxidase. Partially purified zucchini phloem exudates were incubated at 30 °C in either 50 mM sodium phosphate buffer pH 5.6 (black trace) or phosphate buffer + 100 U ml^{-1} ascorbate oxidase (grey trace) for 3 h. The reaction was stopped by boiling for 1 min after which samples were cooled on ice, centrifuged (16,000g, 5 min, 1 °C), filtered (0.22 μm) and injected onto a Coregel 64H cation interaction column. Traces were recorded at 245 nm and peaks with absorption maxima 245 ± 3 nm are numbered. Under the conditions described auto-oxidation of peaks was negligible.

Table 2
Distribution of major AsA analogue in tissues of zucchini

Tissue	Concentration (mg gFW^{-1})	
	AsA analogue	AsA
Root tissue	ND	0.030 ± 0.006
Stem tissue	ND	1.297 ± 0.245
Stem exudates	0.016 ± 0.005	0.043 ± 0.013
Leaf blade tissue	ND	1.458 ± 0.113
Leaf vascular tissue	ND	0.286 ± 0.051
Leaf petiole exudate	0.098 ± 0.011	0.059 ± 0.020
Flowers	0.001 ± 0.000	0.269 ± 0.055
Fruit tissue	ND	0.140 ± 0.011
Fruit exudate	0.040 ± 0.011	0.060 ± 0.010

Courgette plants were grown to maturity under glass, tissue exudates were sampled by making a transverse cut and collecting exuded material using a positive displacement pipette. Samples were immediately transferred to an approximately equal volume of ice cold 10% MPA containing 10 mM TCEP in order to precipitate proteins and reduce AsA and its analogue. Whole tissues were ground to a powder in liquid N_2 and extracted in 19 volumes 5% MPA containing 5 mM TCEP. AsA and its analogue were separated by cation interaction HPLC and quantified using a standard curve constructed against AsA. Values are given as mean ± SE (n = 3). ND = not detected.

AsA, 6-GlcAsA and the zucchini glucoside were able to rapidly quench the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation while radical quenching by 2-GlcAsA showed a slower reaction kinetic (Fig. 4). Similar results were obtained using the 1-diphenyl-picrylhydrazyl radical as a substrate (data not shown). In both reactions, AsA donates hydrogen from the hydroxyl groups at C2 and/or C3 (Takebayashi et al., 2003, 2006). The observation that the zucchini glucoside was as effective a radical scavenger as AsA and 6-GlcAsA but more effective than 2-GlcAsA further supports the hypothesis that it is unsubstituted at either C2 or C3.

2.4. Mass spectra of the primary zucchini analogue are consistent with a 5- or 6-hexosyl ascorbate

AsA, synthetic 6-GlcAsA and 2-GlcAsA purified from fruit of *L. barbarum* were subject to direct injection electrospray MS–MS. AsA (m/z 175) produced a major fragment ion with m/z 115 consistent with loss of C5 and C6 from the compound to give $[M-C_2H_5O_2]^-$ (Fig. 5). 2-GlcAsA gave a parent ion $[M-H]^-$ at m/z 337 and a fragment ion again consistent with loss of C5 and C6 from AsA at m/z 277. On the contrary, 6-GlcAsA had the same parent ion mass $[M-H]^-$ at m/z 337 but the major fragment ion had m/z 115 consistent with loss of the C5–C6 fragment of AsA carrying the glucosyl decoration $[M-C_8H_{14}O_7]^-$. The zucchini glucoside showed an identical mass spectrum to 6-GlcAsA (Fig. 5) consistent with it being a conjugated AsA carrying a hexosyl moiety at C5 or C6.

2.5. NMR analysis

The ^1H NMR spectra of the zucchini glucoside was compared with that of synthetic 6-GlcAsA. Comparison of the spectra show clear similarities (Fig. 6) with the exception that a number of additional peaks were observed in the sample prepared from zucchini exudates. It is proposed that these additional peaks represent impurities that were not removed from the more complex matrix of the exudates during purification.

3. Discussion

The data presented here provides clear evidence for a series of novel L-ascorbic acid analogues from the phloem exudates of *Cucurbitaceae*. The most abundant analogue from zucchini was selected for structural characterisation due to the year round availability of the fruit in local markets and its relative abundance in phloem exudates. The analogue was determined to be 6-GlcAsA, and represents the first time this compound has been isolated from plants. Previously, a 2-*O*-β-D-glucosyl AsA was isolated from the fruit of *Lycium barbarum* (Toyoda-Ono et al., 2004) however the structural and physicochemical differences between the two compounds suggest they fulfill different functions. The analogue isolated from zucchini phloem exudates has greater structural similarities to a number of AsA analogues isolated from fungi. Although fungi do not synthesise AsA in the absence of exogenous

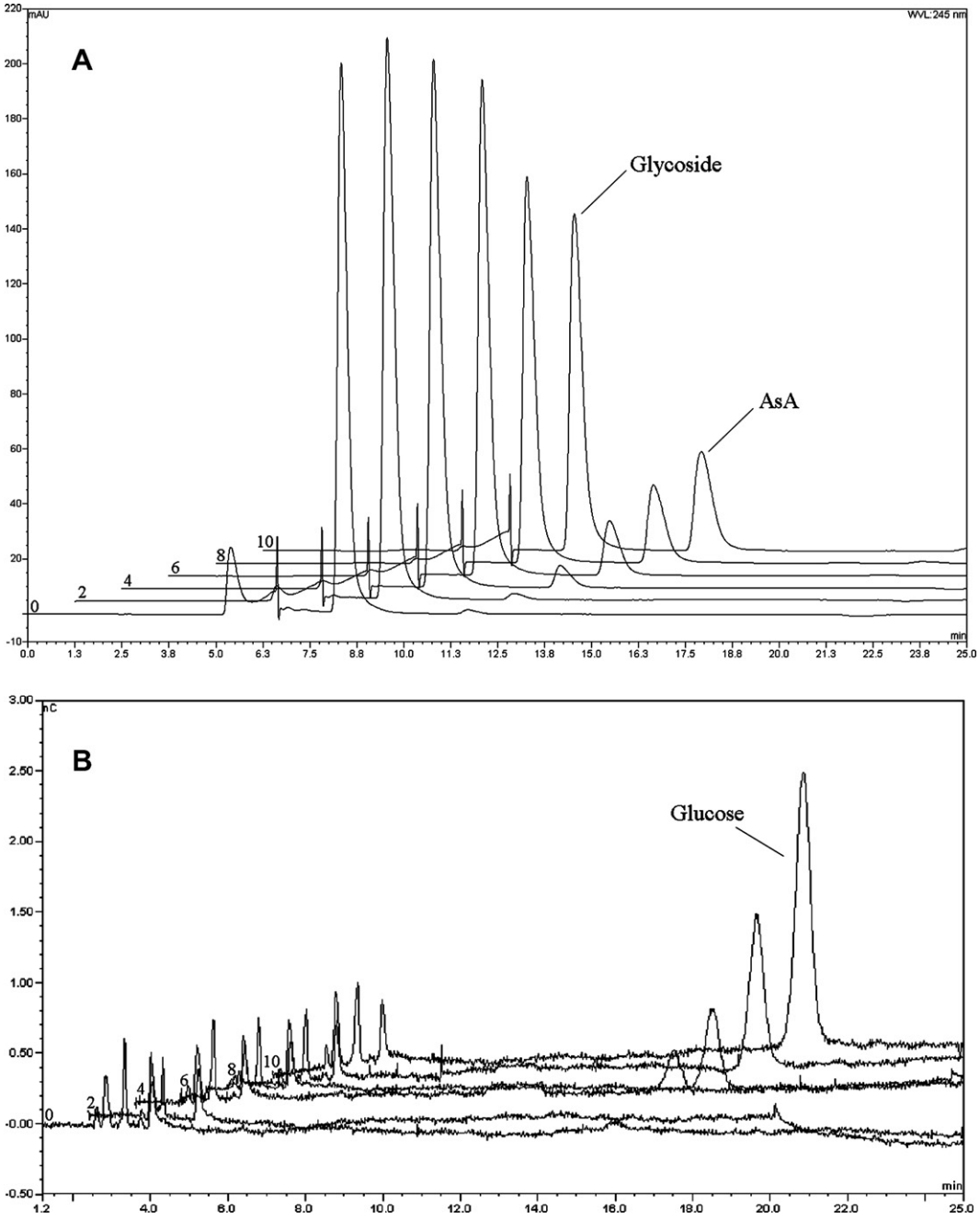

Fig. 3. Acid hydrolysis of primary AsA analogue from zucchini. Purified analogue was redissolved in 3 M HCl and hydrolysis continued at 100 °C for up to 10 min after which the reaction was stopped by plunging samples into liquid N_2. Samples were lyophilized, resuspended in distilled H_2O and injected onto either cation interaction resin for the detection of AsA and related compounds by diode array detection or anion exchange resin for the detection of sugars and related compounds by pulsed amperometry. HPLC traces are shown offset by 2% and 5% in the detector response and time axes, respectively. Numbers to left of individual traces show the length of time (min) that samples were subjected to hydrolysis. Panel A – traces obtained by UV detection at 245 nm from a cation interaction resin. Peaks corresponding to the major zucchini phloem exudate (glycoside) and AsA are shown. Panel B – traces obtained by pulsed amperometric detection from an anion exchange resin, a peak co-eluting with glucose is shown.

Table 3
UV–vis absorption characteristics of AsA and associated analogues

Compound	λ_{max} pH 2.0	λ_{max} pH 7.6	OD_{max} pH 7.6/OD_{max} pH 2.0
AsA	243 nm	265 nm	1.43
2-GlcAsA	235 nm	259 nm	1.94
6-GlcAsA	243 nm	265 nm	1.28
Zucchini analogue	242 nm	265 nm	1.50

Spectra of 28 μM solutions of AsA or analogues were recorded between 400 and 200 nm in either 8 mM H_2SO_4 (pH 2.0) or 50 mM tris HCl (pH 7.6) in quartz cuvettes using a 1 cm light path.

precursors (Hancock and Viola, 2001), they synthesise closely related compounds such as D-erythroascorbic acid (EAsA) and 6-deoxy-L-ascorbic acid (AsAH) (Loewus, 1999). These compounds are frequently conjugated to sugars and EAsA and AsAH glycosides have been found in *Sclerotinia sclerotiorum* (Keates et al., 1998), *Phycomyces blakesleeanus* (Baroja-Mazo et al., 2005) and several species of edible mushroom (Okamura, 1994). Structural analysis of such compounds has always revealed the sugar decoration to be on the C5 of EAsA or AsAH. It is currently unknown how the compounds are synthesised but the fact that structurally similar

Table 4
Redox properties of AsA and related compounds

Reaction	AsA	2-GlcAsA	6-GlcAsA	Zucchini analogue
DCPIP reduction (nmol min^{-1})	9.33 ± 0.43	0.05 ± 0.00	11.30 ± 0.62	9.77 ± 0.69
H_2O_2 oxidation (nmol min^{-1})	8.13 ± 0.59	0.00 ± 0.00	7.18 ± 0.88	3.22 ± 0.96
AO oxidation (nmol min^{-1})	4.30 ± 0.42	0.00 ± 0.00	2.30 ± 0.06	1.63 ± 0.03
Auto-oxidation (pmol min^{-1} $°C^{-1}$)	5.62 (0.99)	0.02 (0.50)	4.58 (0.97)	3.37 (0.99)

One milliliter of 28 μM solutions of AsA or related compounds were reacted with DCPIP, H_2O_2 or ascorbate oxidase (E.C. 1.10.3.3) as described in materials and methods. DCPIP reduction was monitored by recording the change in absorbance at 522 nm and oxidation of AsA and related compounds was monitored by the change in absorbance at 265 nm. Reaction rates are presented as nmol min^{-1} ±SE ($n = 3$). Auto-oxidation is presented as the change in rate (pmol min^{-1}) of auto-oxidation per 1 °C temperature increase and figures in brackets represent the R^2 value of the regression line.

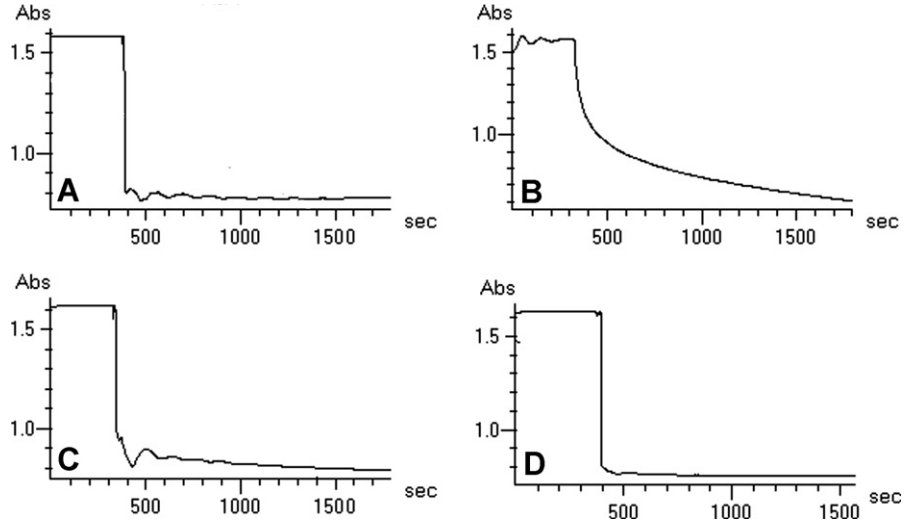

Fig. 4. Reaction kinetics of AsA and analogues with $ABTS^{\cdot+}$ radical. $ABTS^{\cdot+}$ radical was produced by reaction with H_2O_2 and horse radish peroxidase and diluted into 50 mM sodium citrate buffer pH 4.0. The radical quenching reaction was started by the addition of 28 μM AsA or analogue and the reaction monitored by following the solution absorbance at 750 nm. Panel A – AsA, B – 2-*O*-GlcAsA isolated from *L. barbarum* fruit, C – synthetic 6-*O*-GlcAsA, D – Zucchini analogue (Fig. 1, peak 2).

compounds have been isolated from specific tissues in the *Cucurbitaceae* may point to common biosynthetic mechanisms.

The *Cucurbitaceae* are symplastic phloem loaders characterised by a high number of plasmodesmatal connections between leaf mesophyll and companion cells (Gamalei, 1991), low carbohydrate to amino acid ratios in the phloem (Richardson et al., 1982) and the transport of raffinose series oligosaccharides (RFOs; Richardson et al., 1984). It is believed that the transport of RFOs is obligate in symplastic loaders where sugar accumulation in source phloem occurs by the synthesis of RFOs from sucrose and galactinol in the intermediary cells which are closely associated with the sieve elements. The synthesis of RFOs represents a mechanism for the concentration of sugars in the source phloem. It is proposed that sucrose is able to diffuse from the leaf mesophyll into the intermediary cells via plasmodesmata but that the larger RFOs synthesised in the intermediary cells are unable to diffuse back into the mesophyll (Turgeon, 1996). It is interesting to speculate that a similar mechanism may be required for the loading of AsA into source phloem and that the AsA analogues identified in the current work have a similar transport function to RFOs. If this speculation is correct, overexpression of the conjugating enzyme in companion cells could provide a mechanism for enhancing the AsA content of *Cucurbitaceae* fruits.

4. Conclusions

A series of novel AsA analogues have been identified in the phloem of *Cucurbitaceae*. Structural analysis of the most abundant analogue from zucchini revealed it to be an ascorbate glucoside. It is postulated that AsA conjugates may play a role in phloem loading of AsA in *Cucurbitaceae* and other symplastic loaders. Future work should be targeted at identification of the substrates for conjugation and enzyme(s) responsible for catalysing the reaction.

5. Experimental

5.1. Plant material

Fruits of *Cucurbita moschata*, *C. maxima*, *C. pepo*, *C. maxima* x *C. moschata* and *Cucumis sativus* were obtained from a local market. Partially dried *Lycium barbarum* fruit were obtained from a local Chinese herbalist. *C. pepo* plants (cv. Defender) were grown from seed in unheated glasshouses as previously described (Hancock et al., 2003).

5.2. Enzymatic synthesis of AsA glucosides

6-*O*-(β-D-Glucopyranosyl)AsA was synthesized from AsA and cellobiose catalysed by cellulase as described by Toyada-Ono et al. (2005). 300 mM cellobiose was reacted with 200 mM AsA in 10 mM acetate buffer pH 3.0 in a 5 ml reaction containing 1600 U cellulase from *Trichoderma viridae* (Sigma–Aldrich Co. Ltd., Dorset, UK) at 30 °C for 24 h. The reaction was terminated by boiling for 3 min and denatured protein was removed by centrifugation (10 min, 10,000*g*, 1 °C). 50 μl 4080 indicator (VWR, Leicestershire, UK) were added and sample pH was raised to 6–7 by the addition of 1 M NaOH. 2.5 ml aliquots were applied to 500 mg SAX cartridges (Alltech, Lancashire, UK) and the cartridges were washed with 10 ml

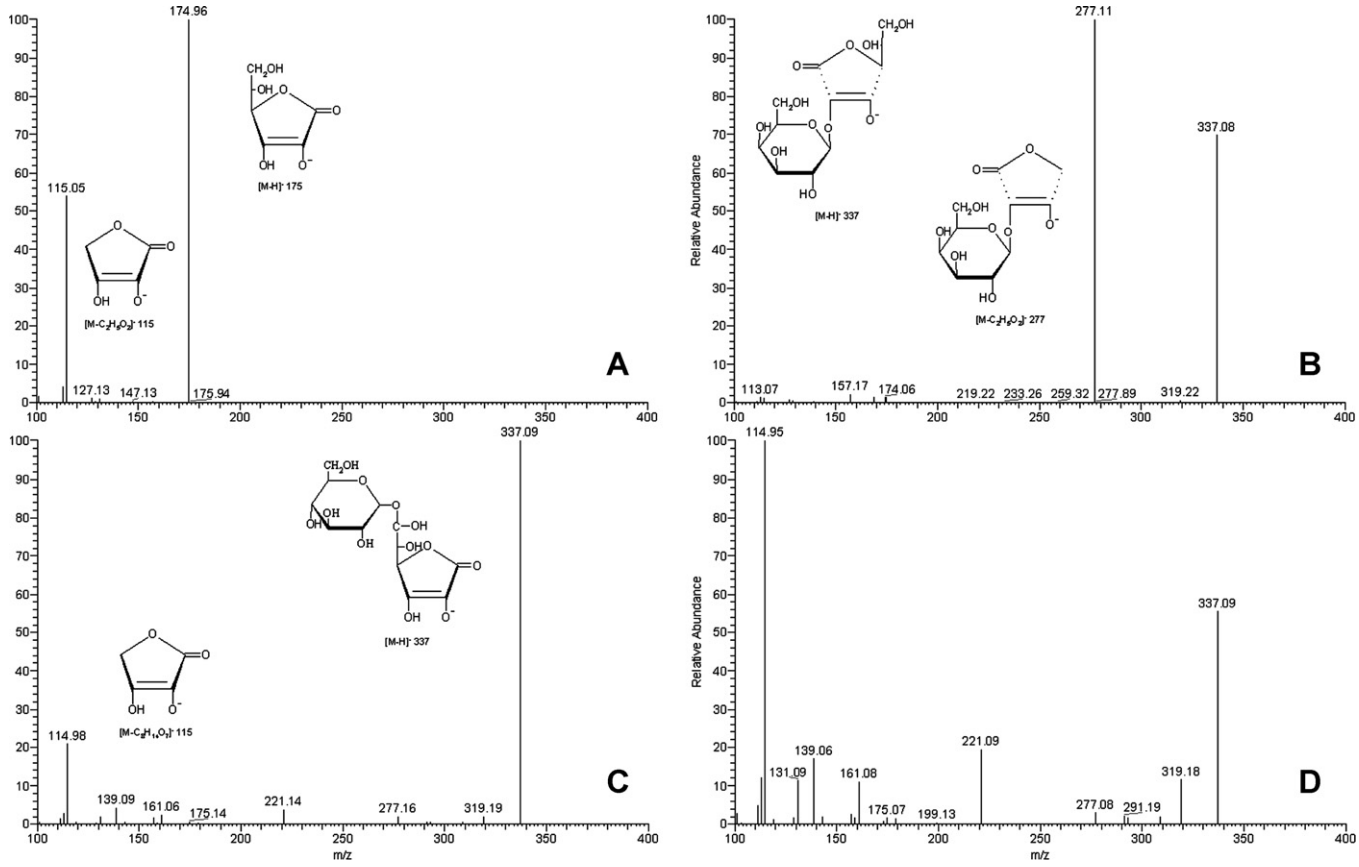

Fig. 5. MS/MS of AsA and related conjugates. AsA (panel A), 2-GlcAsA isolated from *L. barbarum* fruit (panel B), synthetic 6-GlcAsA (panel C) and the major analogue isolated from zucchini phloem (panel D) were subjected to MS/MS as described in the text. Insets represent the presumed parent and major fragment ions.

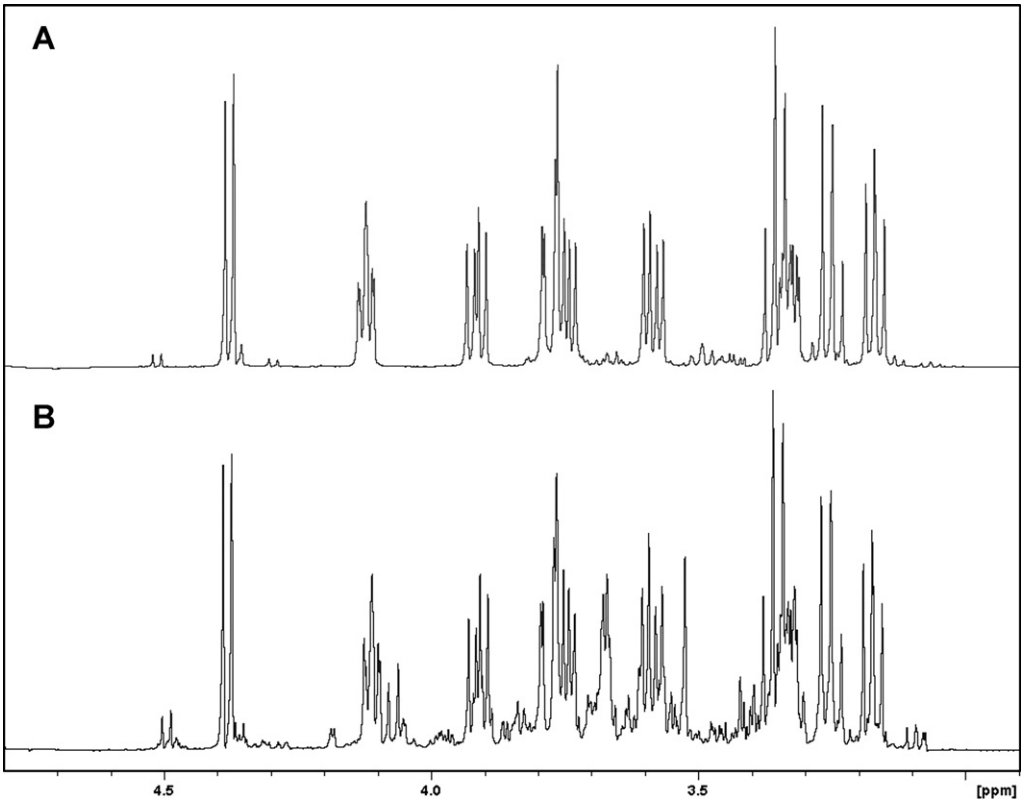

Fig. 6. ^{1}H NMR spectra of AsA conjugates. Synthetic 6-GlcAsA (panel A) and the major zucchini analogue (panel B) were prepared and purified as described in the text. Samples were resuspended in D_2O and spectra acquired using presaturation to suppress remaining water signals.

distilled H_2O. AsA glucosides were eluted with 10 ml 300 mM formic acid and lyophilized. 6-*O*-(β-D-glucopyranosyl)AsA was further purified by cation interaction HPLC as described below.

5.3. Isolation and purification of AsA analogues

For the extraction of AsA analogues from partially dried *L. barbarum* fruit, samples were ground to a powder in liquid N_2 and extracted 5:1 (v/w) in 5% perchloric acid (PCA) containing 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). AsA analogues were extracted from vascular exudates of *Cucurbitaceae* by collection from cut fruit directly into an equal volume of 10% PCA containing 10 mM TCEP. All samples were centrifuged (10,000*g*, 10 min, 1 °C) and the supernatant transferred to a fresh tube. 4080 indicator was added (VWR, Leicestershire, UK) to a concentration of 10 μl ml^{-1} and the sample brought to pH 6–7 with 5 M K_2CO_3. Samples were placed on ice for 30 min and centrifuged (10,000*g*, 10 min, 1 °C) to remove precipitated $KClO_4$. 2.5 ml aliquots of sample supernatants were applied to 500 mg strong anion exchange (SAX) cartridges (acetate form, Alltech, Lancashire, UK) and the cartridges were washed with 10 ml distilled H_2O. AsA analogues were eluted with 10 ml 300 mM formic acid and the samples lyophilised. Samples were resuspended in an appropriate quantity of distilled H_2O and injected onto a Coregel 64H 300 × 7.8 mm HPLC column (Transgenomic, CA, USA) with a 4 × 3 mm carbo H^+ guard cartridge (Phenomenex, Cheshire, UK) using a Gynkotech Gina 50 (Dionex, Surrey, UK) cooled autosampler maintained at 5 °C. The column was maintained at 50 °C in a STH585 column oven (Dionex) and the mobile phase was 8 mM formic acid with a flow rate of 0.6 ml min^{-1} maintained using a Gynkotech P580 quaternary gradient pump (Dionex). AsA analogues were detected by their absorbance at 245 nm using a UVD340U diode array detector (Dionex) and fractions containing AsA analogues were collected manually. Individual fractions were lyophilised and resuspended in an appropriate volume of distilled water. Analogue purity was monitored by re-injection onto a Coregel 64 H column as described above with the exception that the mobile phase was 8 mM H_2SO_4 and absorbance was monitored in the range 200–600 nm.

5.4. Acid hydrolysis of AsA analogues

Equal volumes of solutions of AsA analogues and 6 M HCl were combined and heated at 100 °C for 0–10 min. Reactions were stopped by transferring the reaction tubes to liquid N_2 and the samples were lyophilised and resuspended in appropriate volumes of distilled H_2O. Remaining analogue and AsA were detected by UV absorbance at 245 nm after HPLC on a Coregel 64 H column using 8 mM H_2SO_4 as the mobile phase. Sugars were separated by HPAEC on a 4 × 250 mm Carbopak PA-1 column (Dionex) with a 4 × 50 mm guard column of the same resin. The mobile phase was 16 mM NaOH pumped at a flow rate of 1 ml min^{-1} by a Dionex GP40 gradient pump and 1 M NaOH was added post-column with a post-column pneumatic controller set at 5.5 bar. The column was washed for 10 min with 200 mM NaOH and re-equilibrated in 16 mM NaOH for 10 min between samples. Sugars were detected by pulsed amperometric detection with an ED40 electrochemical detector with a gold working electrode at a potential of 0.1 V.

5.5. Spectrophotometry

All spectrophotometry was undertaken on a Hitachi U3010 spectrophotometer (Hitachi High-Technologies, London, UK) equipped with a 12 cuvette sample changer maintained at 30 °C by a Grant W14 circulating water bath (Grant Instruments Ltd., Cambridgeshire, UK). Data analysis was achieved using UV solutions version 1.2.

Absorbance spectra were recorded between 400 and 200 nm using 28 μM AsA or analogues dissolved in either 50 mM tris HCl (pH 7.6) or 8 mM H_2SO_4 (pH 2.0). Appropriate background spectra were recorded and subtracted from the sample spectra.

Reduction of dichlorophenol indophenol (DCPIP) was undertaken as described by Baroja-Mazo et al. (2005). 28 μM AsA or analogues were dissolved into 50 mM tris HCl pH 7.6 and 0.04% (w/v) DCPIP in 50% (v/v) ethanol was added to give a final concentration of 0.8 μg ml^{-1}. Reactions were undertaken at 30 °C and DCPIP reduction monitored by the change in absorbance at 522 nm. The rate of reduction was calculated based on an extinction coefficient for DCPIP of 19100 M^{-1} cm^{-1}.

Oxidation of AsA and analogues by H_2O_2 was monitored by determination of the change in absorbance at 265 nm of a 28 μM solution of the appropriate compound in 50 mM tris HCl pH 7.6 after the addition of H_2O_2 to a final concentration of 88 mM. Reactions were undertaken at 30 °C and the rate of AsA or analogue oxidation was calculated based on an extinction coefficient of 15100 M^{-1} cm^{-1}.

Oxidation of AsA and analogues by ascorbate oxidase (E.C. 1.10.3.3; Roche Applied Science, Sussex, UK) was monitored by determination of the change in absorbance at 265 nm of a 28 μM solution of the appropriate compound in 100 mM sodium phosphate buffer pH 5.6 after the addition of 0.017 U ml^{-1} of enzyme. Reactions were undertaken at 30 °C and oxidation rates calculated as for H_2O_2 oxidation.

Auto-oxidation was undertaken using 28 μM AsA or analogue in 50 mM tris HCl pH 7.0 at 30, 40, 50, 60 and 70 °C. Oxidation rates were calculated as described above.

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{\cdot+}$) was generated using horse radish peroxidase as described by Takebayashi et al. (2003) by dissolving 12 mM ABTS with 5 mM H_2O_2 and 1 U ml^{-1} horse radish peroxidase (E.C. 1.11.1.7) in 10 mM sodium citrate buffer pH 4.0 and incubating for 30 min at room temperature. Radical scavenging activity was determined by mixing 10 μl $ABTS^{\cdot+}$ solution with an appropriate volume of 50 mM sodium citrate buffer pH 4.0. The reaction was started by the addition of AsA or AsA analogue to a final concentration of 28 μM to give a total reaction volume of 1 ml. Radical scavenging was determined by measurement of the change in absorption of the reaction mixture at 750 nm.

Scavenging of 1,1-diphenyl-picrylhydrazyl (DPPH) radical was determined by measuring the change in absorbance at 524 nm of a 100 μM solution of DPPH in 60% ethanol/40% sodium citrate buffer pH 4.0 after the addition of AsA or AsA analogue to a final concentration of 28 μM (Takebayashi et al., 2006).

5.6. Mass spectrometry

Negative ion mode mass spectra were recorded following direct injection of aqueous solutions into a LTQ XL mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) fitted with an electrospray ionisation interface using a syringe pump set to deliver 10 μl min^{-1}. Nitrogen was used as sheath and auxiliary gas. Transfer capillary temperature was maintained at 275 °C and a capillary voltage of 3.5 kV was used. The mass spectrometer was tuned prior to use with 1 mg ml^{-1} AsA using TunePlus version 2.2 software. MS2 data was recorded following isolation and fragmentation of the major parent ion by collision induced dissociation and the mass spectrum was collected over 1 min.

5.7. NMR spectroscopy

^1H NMR spectra were acquired using a Bruker AVANCE II FT NMR spectrometer (Bruker U.K., Coventry) resonating at 500.13 MHz for protons. Samples were dissolved in D_2O and spec-

tra were acquired using presaturation to suppress any remaining water.

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