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Covalent interaction of ascorbic acid with natural products

Nicholas G. Kesinger and Jan F. Stevens*

Department of Pharmaceutical Sciences and the Linus Pauling Institute, Oregon State University, Corvallis, OR 97331

Abstract

While ascorbic acid (Vitamin C) is mostly known as a cofactor for proline hydroxylase and as a biological antioxidant, it also forms covalent bonds with natural products which we here refer to as 'ascorbylation'. A number of natural products containing an ascorbate moiety has been isolated and characterized from a variety of biological sources, ranging from marine algae to flowering plants. Most of these compounds are formed as a result of nucleophilic substitution or addition by ascorbate, e.g. the ascorbigens from *Brassica* species are ascorbylated indole derivatives. Some ascorbylated tannins appear to be formed from electrophilic addition to dehydroascorbic acid. There are also examples of annulations of ascorbate with dietary polyphenols, e.g., epigallocatechin gallate (EGCG) and resveratrol derivatives. Herein is a survey of thirty-three ascorbylated natural products and their reported biological activities.

Keywords

ascorbic acid; ascorbate; ascorbylation; ascorbigen; resveratrol; hydrolyzable tannins

1. Introduction

Ascorbic acid is well known for its ability to prevent scurvy in humans by acting as a cofactor for proline hydroxylase, an enzyme involved in collagen synthesis (Gropper et al., 2009). Ascorbic acid acts also as a biological antioxidant by donating an electron to free radical species, such as tocopherol radical, thereby interrupting the radical chain reaction in biological membranes (Constantinescu et al., 1993; Packer and Obermuller-Jevic, 2002). A less appreciated role for ascorbic acid is its capacity to form covalent bonds, in particular as a nucleophile. By electronic structure searching using SciFinder Scholar®, we conducted a survey of natural products which contain a covalently bound ascorbyl moiety. Included are the sources of these compounds, their structure, their mechanism of formation, and their reported biological activities.

2. Ascorbate as nucleophile

At neutral or physiological pH, ascorbic acid (pK_a 4.2) is essentially an enolate and capable of reacting as a nucleophile. Typical enolates are formed at low temperatures and are

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*Corresponding author. 203 Pharmacy Building, 1601 SW Jefferson, Oregon State University, Corvallis, Oregon 97331. Tel. +1-541-737-9534; Fax +1-541-737-3999; fred.stevens@oregonstate.edu.

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completely incompatible with H₂O, but ascorbate can have similar reactivity at room temperature in aqueous solutions. At room temperature, these reactions are under thermodynamic rather than kinetic control. Intermediate adducts can dissociate or form stable products by subsequent reactions, e.g., oxidation, dehydration, or hemiacetal or hemiketal formation. For instance, C-alkylated products can be formed via Michael addition, such as the reaction of ascorbate with acrolein (Fodor et al., 1983), or via S_N1 substitution provided the carbocation is sufficiently stabilized via resonance (such as in the reaction of ascorbate with xanthidrol (Schwall et al., 1970) (Fig. 1).

2.1. Substitution reactions with ascorbate

2.1.1. Ascorbigens—The most studied and recognized of the ascorbylated natural products is the glucobrassicin-derived ascorbigen (ABG), which is found in cruciferous vegetables belonging to *Brassica* (broccoli, cauliflower, cabbage, etc.). Derivatives of ABG have also been found in various *Brassica* species, including 4-hydroxyascorbigen, 4-methoxyascorbigen, and (the *N*-methoxy derivative) neoascorbigen (Buskov et al., 2000a) (Fig. 2).

The glucosinolate, glucobrassicin, is degraded by myrosinase to the corresponding isothiocyanate, which forms indole-3-carbinol by elimination of thiocyanate and water addition to the resulting 3-methylene-3*H*-indolium intermediate. ABG is produced when ascorbate reacts with the methylene-3*H*-indolium intermediate, either directly or, more likely, via indole-3-carbinol (Fig. 3). ABG concentrations can range from 5.3 mg/kg to 16 mg/kg depending on the variety (Hrncirik et al., 2001), while total content of glucobrassicin and its analogs can range from 100 to 1500 mg/kg (Fenwick et al., 1989).

ABG is a reactive compound and in acidic media ascorbic acid is cleaved to produce methylene-3*H*-indolium and ascorbate, or a series of indole dimers. In basic media, the lactone of the ascorbyl moiety is hydrolyzed and undergoes decarboxylation to produce deoxyketose-indoles (Preobrazhenskaya et al., 1996) (Fig. 4). The pentose-indoles were the major metabolites found in the blood serum and liver of mice after oral administration of ABG (Reznikova et al., 2000). During cooking of cruciferous vegetables, the levels of ABG can decrease. When boiled for 10 min, the amount of ABG in cabbage is reduced by 10% (Ciska et al., 2009). This degradation appears to be due to elimination of the ascorbyl moiety, as deduced by the simultaneous increase of ABG degradation products such as indole-3-carbinol. Conversely, when cabbage is allowed to ferment (e.g. during the production of sauerkraut or kimchi), the concentration of ABG may be 10- to 20-fold higher compared to raw cabbage. This may be due to the pH (4.2-7.0) of the material being optimum for allowing the ascorbate ion to undergo nucleophilic addition to the indolium intermediate of glucobrassicin degradation (Martinez-Villaluenga et al., 2009).

Epidemiological evidence suggests that consumption of cruciferous vegetables can lower the risk of formation of colorectal cancer (Verhoeven et al., 1998), but the contribution of ascorbigen to a cancer chemopreventive effect of cruciferous vegetables is unclear. Consistent with a cancer chemopreventive effect, ABG induces the carcinogen-detoxifying enzyme, NADP(H) quinone oxidoreductase 1 (NQO1) in rats (Wagner et al., 2008b). On the other hand, ABG dose-dependently induced the pro-carcinogen activating enzyme, CYP1A1, in murine hepatoma 1c1c7 cells (Stephenson et al., 1999). The authors attributed the induction of CYP1A1 by ABG in whole cells to the formation of the ABG degradation product, indolo[3,2-*b*]carbazole, a known potent CYP1A1 inducer. The same study showed inhibition of CYP1A1 enzyme activity by ABG in the microsomal fraction of the cells (Stephenson et al., 1999). These activities of ascorbigen and related indoles has recently been reviewed elsewhere (Wagner and Rimbach, 2009). ABG also has a cytoprotective effect against glucose-induced endothelial toxicity (Joshi et al., 2008). While consumption of cruciferous vegetables may

activate the transcription factor Nrf2 and therefore induce phase II metabolism, this activity is due to the presence of the isothiocyanate, sulforaphane. ABG or its cleavage products, indole-3-carbinol and ascorbic acid, do not activate Nrf2 or induce phase II metabolism (Wagner et al., 2009).

ABG may function as antioxidant. In a recent study using cultured human keratinocytes, Wagner and co-workers found that ABG was a potent free radical scavenger, while ascorbic acid itself (a potent antioxidant) showed no effect (Wagner et al., 2008a). However, in a no cell control, ascorbic acid was shown to effectively scavenge free radicals whereas ABG was ineffective. The mechanism by which ABG acts as an antioxidant in cells, as opposed to ascorbic acid, is not known. It is conceivable that the more lipophilic ABG functions as a “prodrug” carrier of ascorbic acid across the cell membrane and that ABG provides an intracellular source of ascorbic acid upon cleavage.

2.1.2. Ascorbylated cresols—Three natural products have been isolated from red algae containing an ascorbyl moiety and a cresol-like skeleton with varying substitution. These are delessierine from *Delessieria sanguinea* (Yvin et al., 1982), and rhodomelol and methylrhodomelol from *Polysiphonia lanosa* (Fig. 5) (Glombitza et al., 1985).

Biomimetic syntheses of delessierine, methylrhodomelol and rhodomelol have been achieved by the Poss group in simple single step incubations of ascorbic acid or 2-*O*-methylascorbic acid with the corresponding *p*-hydroxybenzyl alcohol (Poss and Belter, 1988). These reactions undergo an S_N1 mechanism, and require the *p*-hydroxy group to stabilize the carbocation intermediate. Model studies using *o*- and *m*-hydroxybenzyl alcohols gave no substitution products.

Extracts of *Delessieria sanguinea* have long been known for strong anti-coagulant activity (Elsner et al., 1938), although this may be due to the presence of sulfated polysaccharides (Gruenewald et al., 2009) rather than delessierine. Delessierine, rhodomelol and methylrhodomelol have not been tested individually for biological activity.

In addition to the indole derivatives, 4-hydroxybenzylascorbigen can also be found in *Brassica* (Buskov et al., 2000b). This is likely produced from the 4-hydroxybenzyl glucosinolate, glucosinabin. Dactylose A and B (Fig. 6) have been found in *Dactylorhiza hatagirea*, a Nepalese orchid of which the bulbous roots are used traditionally as a tonic. Although lacking an ascorbyl moiety, both compounds derive from the hydrolysis and decarboxylation of 4-hydroxybenzylascorbigen, similar to the decomposition of ascorbic acid itself. These compounds have been produced synthetically from 4-hydroxybenzyl alcohol and L-ascorbic acid (Preobrazhenskaya et al., 1997).

2.2. Michael additions

2.2.1. Ascorbylated coumaric acid derivatives—A number of ascorbylated compounds appear to be biosynthesized by Michael addition of ascorbic acid to coumaric acid (Fig. 7). Leucodrin was first isolated from the leaves *Leucodendron concinnum* (Proteaceae) in 1886 in a search for a quinine substitute (Rapson, 1938). It can also be found in *L. adscendens*, *L. stokoei*, *Leucospermum conocarpodendrum*, and *Leucospermum reflexum*. The structure was not confirmed until 1966 (Perold and Pachler, 1966). The Perold group has also isolated conocarpin (an epimer of leucodrin) from *L. conocarpodendrum*, leudrin from various *Leucodendron* species, and reflexin (not to be confused with the flavanone of the same name) from *L. reflexum*, although the latter may be an artifact of extraction (Perold et al., 1972).

Reports of ascorbylated coumaric acid glycosides include dilaspirolactone from the leaves of the shrub *Viburnum dilatatum* (Iwagawa and Hase, 1984) and viburnolides A, B and C from

the leaves of *V. wrightii* (Machida and Kikuchi, 1994). Several derivatized viburnolides were found in *Gnidia socotrana* (Thymelaeaceae) from Yemen: 4',6'-diacetyl-viburnolide A, 2',4',6',12-tetraacetyl-viburnolide A, and 4',6'-diacetyl-12-coumaroyl-viburnolide A (Franke et al., 2002).

Dichotomains A and B have recently been isolated and characterized from the fronds of the fern *Dicranopteris dichotoma*. Structurally these are fucose analogs of dilaspirolactone. Both dichotomains were tested for cytotoxicity in airway epithelial A549 cells but showed no activity. Dichotomain B showed minimal cytotoxicity against C8166 T-lymphocytes using the MTT assay and showed weak anti-HIV-1 activity (Xiao-Li et al., 2006).

Leucodrin, leudrin, reflexin, and the aglycones of dilaspirolactone, viburnolides, and dichotomains have been synthesized by Poss and co-workers (Poss and Belter, 1988). The same synthetic strategy was used for the ascorbylation of cresols, i.e., substitution with derivatized *p*-hydroxybenzyl alcohols, rather than a biomimetic approach of a Michael addition to coumaric acid derivatives.

2.2.2. Piptoside—Piptoside was first isolated in 1966 from *Piptocalyx moorei*, of Trimeniaceae (Riggs and Stevens, 1966) (Fig. 8). The aglycone of piptoside, piptosidin, is the Michael addition product of ascorbic acid with either of the *E/Z* isomers, tiglic or angelic acid. Piptosidin has been synthesized via Michael addition with ascorbic acid and tigloyl cyanide (Poss and Smyth, 1987). There are no reports of either piptoside or piptosidin having been screened for bioactivity.

3. Dehydroascorbate as Electrophile

3.1. Tannins

A series of hydrolyzable tannins incorporating an ascorbyl moiety have been isolated from a variety of sources. Much of this work has been performed by the Nishioka group. These compounds include elaeocarpusin from the evergreen tree, *Elaeocarpus sylvestris* (Tanaka et al., 1986), excoecarinin B from *Excoecaria kawakamii* (Lin et al., 1990), mallojaponin and mallonin from *Mallotus japonicus* (Saijo et al., 1989), and helioscopins A and B from *Euphorbia helioscopia* (Lee et al., 1990) (Fig. 9).

In all cases, the ascorbyl moiety is bound to a dehydrohexahydroxydiphenoyl ester functionality. On first appearance, it would seem that the ascorbyl moiety is formed from simple Michael addition of ascorbate to the corresponding cyclohexenone moiety. Indeed, elaeocarpusin can be synthesized by simple incubation of ascorbic acid with geraniin, a tannin distributed in a number of plant families, including Geraniaceae, Euphorbiaceae, Aceraceae, Cercidiphyllaceae and Simaroubaceae (Okuda et al., 1986). However, Nishioka proposes an alternative biosynthetic pathway for elaeocarpusin. The dehydroxydiphenol ester group in compounds such as geraniin is believed to be produced via enzymatic dehydrogenation of the hexahydroxydiphenoyl esters in ellagitannins (Haslam, 1982). Nishioka proposes that the hexahydroxydiphenoyl group, as a nucleophile, attacks dehydroascorbate, and that elaeocarpusin is an intermediate in the biosynthesis of geraniin (Tanaka et al., 1986) (Fig. 10).

Elaeocarpusin has been found to be cytotoxic against PRMI-7951 melanoma cells, although it showed no activity against tumor cells derived from lung carcinoma, ileocecal adenocarcinoma, epidermoid carcinoma of the nasopharynx, or medulloblastoma tissues (Kashiwada, 1992). Elaeocarpusin and helioscopin B have been found to be inhibitory against prolyl endopeptidase (PEP). PEP is important in the inactivation of proline-containing neuropeptides, and may play a role in the formation of amyloid plaques in Alzheimer's patients (Lee et al., 2007).

3.2. Ascorbylated phloroglucinol

A single ascorbylated phloroglucinol, phloroscorbinol, has been isolated as its hexaacetate from the brown alga *Sargassum spinuligerum* (Keusgen et al., 1997). Due to the chemical instability of phloroscorbinol, the extract was peracetylated prior to isolation. No mechanism for the biosynthesis of phloroscorbinol was provided. However, the simplest hypothesis may involve the aromatic electrophilic substitution of phloroglucinol to C-2 of dehydroascorbate, followed by hemiketalization (Fig. 11). This compound was not assayed for biological activity.

3.3. Ascorbylated EGCG

The Nishioka group has also isolated 8-*C*-ascorbyl (-)-epigallocatechin 3-*O*-gallate (ascorbyl EGCG, Fig. 12.) from oolong tea, *Camellia sinensis* (Hashimoto et al., 1989). Given the stereo-electronics of the EGCG moiety, we believe ascorbyl EGCG is formed from aromatic electrophilic substitution with dehydroascorbate, analogous to phloroscorbinol.

Oku and co-workers investigated 47 polyphenols, including ascorbyl EGCG and EGCG, for their potency to inhibit the metalloproteinases, MT1-MMP, MMP-2 and MMP-7 (Oku et al., 2003). These metalloproteinases play key roles in angiogenesis, tumor cell migration, and in metastasis. Membrane-type 1 matrix metalloproteinase (MT1-MMP) catalyzes the conversion of proMMP-2 into active MMP-2. Using a fluorogenic peptide cleavage assay, the authors recorded an IC₅₀ of 36 nM for ascorbyl-EGCG against recombinant human MT1-MMP, and IC₅₀s of 25 and 0.46 μM against rhMMP-2 and rhMMP-7. For comparison, non-ascorbylated EGCG showed IC₅₀ values of 19 nM, >100 μM, and >100 μM against MT1-MMP, MMP-2, and MMP-7, respectively. The authors conclude that tea polyphenols may interfere with tumor angiogenesis by inhibiting metalloproteinases (Oku et al., 2003).

4. Annulations

There are two ascorbylated natural products which defy categorization based on mechanism of formation: shorealactone and jolkinin.

4.1. Shorealactone

The ascorbyl-resveratrol derivative shorealactone has been isolated from the bark of *Shorea hemsleyana* (Ito et al., 2003) and from the stem of *Dipterocarpus grandiflorus* (Ito et al., 2004). In the latter isolation, the authors also found several resveratrol oligomers.

Shorealactone is formed from an annulation of ascorbate to the double bond of a resveratrol moiety in a resveratrol dimer, forming a tetrahydrofuran ring. Given the structural similarity of this ring compared to the tetrahydrofurans formed during the oligomerization of resveratrol, it is reasonable to assume that the ascorbylation follows a similar mechanism. The oligomerization of stilbenoids has been well studied; it can proceed enzymatically (Cichewicz et al., 2000), photochemically (He et al., 2008), and by single electron oxidation with metal reagents (Sako et al., 2004). The latter work provides a mechanism for ascorbylation in shorealactone: if the dimeric resveratrol precursor donates a single electron to dehydroascorbate or other electron acceptor, semidehydroascorbate (ascorbyl radical) can react with the resveratrol radical to form a C-C bond (Fig. 13).

Shorealactone has been tested against type II DNA topoisomerase, which catalyzes topological changes in supercoiled or circular DNA. Shorealactone showed an IC₅₀ value of 11 μM in inhibiting topoisomerase II, only slightly higher than daunorubicin (8.5 μM), a topoisomerase II inhibitor used as an anti-cancer drug (Yamada et al., 2006).

4.2. Jolkinin

The hydrolyzable tannin jolkinin was isolated from *Euphorbia jolkinii* (Lee et al., 2004). Its structure is identical to elaeocarpusin, except for the ascorbyl moiety. Nishioka and colleagues proposed a biosynthesis in which the ascorbyl moiety of an ascorbyl-geraniin adduct undergoes hydrolysis, oxidation, intramolecular aldol reaction and hemiketalization (Lee et al., 2004) (Fig. 14), making jolkinin unique among ascorbyl conjugates.

Jolkinin exhibited strong inhibitory activity against PEP (Lee et al., 2007).

5. Conclusion

We have found 33 examples of ascorbylated natural products via electronic structure searches using SciFinder Scholar® and a review of the literature. These range from the well known ascorbigen to the relatively obscure piptoside. Some of these show promising cancer-related activity (ascorbyl-EGCG, jolkinin) but many have yet to be tested. Given the high concentration of ascorbic acid in many plants (as well as other living organisms), it may be that these 33 compounds are only the tip of the iceberg of ascorbylated natural products.

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Biography

Nicholas G. Kesinger, Ph.D. Nicholas Kesinger received a B.S. in chemistry from Western Washington University in Bellingham, Washington, in 2001. He joined the Department of Pharmaceutical Sciences at Oregon State University in 2005, where he graduated with a Ph.D. in Pharmacy in 2009. His Ph.D. studies were focused on the biological significance of the covalent interaction of vitamin C with naturally occurring electrophiles.

Jan Frederik Stevens, Ph.D. Jan Frederik ('Fred') Stevens received his M.Sc. in pharmacy (1988), pharmacy license (1990), and Ph.D. in medicinal chemistry (1995) from Groningen University, The Netherlands. He received postdoctoral training at Oregon State University (1995-1999), the Free University of Amsterdam (1999-2000), and the Leibniz Institute for Plant Biochemistry, Halle/Saale, Germany (2000-2002). He joined the faculty at Oregon State University (OSU), Corvallis, in 2002. Dr. Stevens is currently an Associate Professor of Medicinal Chemistry in the Department of Pharmaceutical Sciences at OSU. Dr. Stevens is affiliated with the Linus Pauling Institute as one of 12 Principal Investigators. He has authored or co-authored 57 articles in peer-review journals, 15 of which were published in *Phytochemistry*.

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Fig. 1. Synthetic examples of nucleophilic ascorbylation via (A) Michael addition and (B) Nucleophilic substitution. The ascorbyl moiety typically forms the hemiketal upon conjugation.

Fig. 2.
Ascorbigens isolated from *Brassica* spp.

Fig. 3.
Formation of ascorbigen from glucobrassicin via indole-3-carbinol.

Fig. 4.
pH-Dependent degradation of ascorbigen.

Fig. 5.
Cresol-based ascorbyl conjugates.

Fig. 6.
Dactylose A and B.

RH leucodrin (4*R*)H conocarpin (4*S*)OH leudrin (4*R*) **R₁****R₂**

H β-D-glucose

β-D-glucose H

H 4,6-diacetyl-β-D-glucose

acetyl 2,4,6-triacetyl-β-D-glucose

coumaroyl 4,6-diacetyl-β-D-glucose

H 4-acetyl-β-D-fucose

H β-D-fucose

reflexin

H viburnolide B

Me viburnolide C

Fig. 7.

Coumaric acid-based ascorbyl derivatives.

Fig. 8.
Piptoside.

Fig. 9.
Ascorbylated hydrolyzable tannins.

Fig. 10.
Proposed formation of geraniin from an ellagitannin via elaeocarpusin.

Fig. 11.
Hypothetical formation of a phloroglucinol derivative found in *S. spinuligerum*.

Fig. 12.
8-*C*-ascorbyl (-)-epigallocatechin 3-*O*-gallate.

Fig. 13.
Proposed mechanism for the formation of shorealactone.

Fig. 14.
Biosynthesis of jolkinin from geraniin and ascorbic acid.