AN ABSTRACT OF THE DISSERTATION OF

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Title: Ascorbylation- the Biological Relevance of Covalently Bound Ascorbic Acid.

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Ascorbic acid, or vitamin C, is well known as a co-factor for proline-hydroxylase and as an anti-oxidant. However, it is also capable of forming covalent bonds, particularly in the role of a nucleophile, henceforth termed 'ascorbylation'.

The ascorbylation of electrophiles can occur under physiological conditions. Furthermore, there are a number of ascorbylated natural products found in plants and these compounds are reviewed here. Many of these natural products have biological activity. For example, the ascorbylated indole derivative ascorbigen, found in Brassica vegetables, can induce quinone reductase and thus may have a role in cancer chemoprevention.

Thus, we hypothesize that ascorbic acid has important biological relevance beyond the role of antioxidant and in the hydroxylation of proline. If ascorbic acid, which is present in cells and very high concentrations, ascorbylates electrophiles, this present a new pathway in the detoxification of toxic compounds. For example, acrolein is a toxic compound which alkylates DNA and protein, leading to cancer among other health issues. Acrolein also reacts rapidly with ascorbic acid. Once ascorbylated, acrolein is no longer electrophilic and furthermore, the ascorbyl-acrolein (AscACR) molecule is more hydrophilic and this could lead to improved elimination of the molecule. Essentially, ascorbylation could be a previously unrecognized form of phase II metabolism, analogous to glutathione conjugation. The first major goal of this research is to detect ascorbylated metabolites of electrophiles (i.e. AscACR) from biological samples.

Additionally, because ascorbylated compounds may have dramatically altered ADME characteristics compared to the non-ascorbylated compound, synthetically ascorbylated compounds may function as prodrugs. That is, a drug with poor pharmacokinetic parameters may be improved with the addition of an ascorbyl moiety, and that ascorbyl moiety may be cleaved via a retro-Michael reaction upon deliver to the site of action. The second goal of this work is to produce an ascorbylated prodrug. The candidate of choice was xanthohumol. Xanthohumol is a biologically active Michael acceptor which has very poor bioavailability due to low hydrophilicity- a characteristic which can be improved via ascorbylation.

AscACR is easily synthesized. However, it was exceedingly difficult to detect in any biological samples using LC-MS/MS. It was hypothesized originally that AscACR may be a product of oxidative stress but it could not be found in samples of human subjects exposed to oxidative stress. Samples of rats exposed to CCl_4, a well known inducer of oxidative stress, similarly contained no detectable amounts of AscACR. Even rat or cell cultures exposed to acrolein directly did not produce samples with detectable amounts of AscACR. In retrospect, this was likely due to the instability of AscACR in biological media.

However, a degradation product of AscACR was detected by LC-MS/MS in THP-1 cells exposed to acrolein diacetate (which was used to deliver acrolein intracellularly). This degradation product is produced via the hydrolysis of the lactone of AscACR, followed by decarboxylation and racemization to two stereoisomers, the mixture of which we term 5,6,7,8-tetrahydroxy-4-oxooctanal (THO). This same reaction scheme occurs with the natural product ascorbigen under certain conditions, and indeed is involved in the biosynthetic pathway of dactylose A and B.

Vitamin C adequate THP-1 cells which are exposed to acrolein (via acrolein diacetate), produce THO. Thus, biologically, ascorbylation is involved in the detoxification of electrophiles; it is a new sort of phase II metabolism (albeit without an enzyme). The ascorbylation of acrolein itself is chemical; THO is also detected in the incubation of ascorbic acid and acrolein-diacetate no cell control. However, AscACR, the precursor of THO, was only detected in the no cell

control. This is strong evidence that an enzyme, or some related biocatalyst, is involved in the conversion of AscACR to THO.

In the prodrug work, the ascorbylated form of xanthohumol (AscXN) failed to serve as a prodrug. The synthesis of AscXN was poor. AscXN itself was unstable. In a caco-2 cell study modeling intestinal absorption, AscXN failed to show any transport across the caco-2 monolayer. While AscXN failed to function as a prodrug, this may have been due to the choice in candidate, rather than failure of the hypothesis itself.

Ascorbylation- the Biological Relevance of Covalently Bound Ascorbic Acid

by
Nicholas G. Kesinger

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Chapter 1: Introduction

Ascorbylation- the Biological Relevance of Covalently Bound Ascorbic Acid

Introduction

That ascorbic acid (Vitamin C) (**Fig. 1.1**) is a unique and remarkable molecule cannot be stressed enough. Aside from the role of vitamin C as an antioxidant, its status as an essential vitamin, its place in naval history, and its controversial role in the late career of Linus Pauling in the fight against cancer and the common cold, the simple chemistry of the ascorbic acid molecule itself is unique and underappreciated.

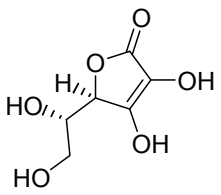


Figure 1.1: L-Ascorbic acid

The pK_a of ascorbic acid is 4.2. While many sugars and sugar derivatives (of which ascorbic acid is one) are ionized at physiological pH, the ionized functionality is invariably a carboxylate. Due to its unique electronics, in ascorbic acid, the acidic proton comes from a functional group which is essentially an enol. This means that in water, or in tissue, vitamin C exists as its conjugate base, effectively an enolate, and is capable of carrying out the nucleophilic reactions of enolates.

Consider that in organic synthesis settings, making an enolate (to carry out a Michael addition reaction, for example) typically requires -78º C, an absolutely dry aprotic polar organic solvent, a base such as lithium diisopropylamide, the conjugate acid of which has a pK_a value of approximately 36, and the enolate must be treated with an electrophile quickly and carefully because the enolate is so unstable. Ascorbate will react with Michael acceptors in water, in an open

container, on a bench at room temperature, and with no other reactant necessary.

The nucleophilic character of ascorbate has been known for decades, although this reactivity has not been widely appreciated. There have been some minor investigations of this behavior in organic synthesis laboratories (**Fig 1.2**) (Fathi, Krautheim et al., 2000; Fodor, Arnold et al., 1983; Poss & Belter, 1988). The conjugation of ascorbate with electrophiles also occurs in nature. Several secondary metabolites isolated from plants are known to contain an ascorbyl moiety, e.g. ascorbigen is an ascorbylated indole derivative found in *Brassica*.

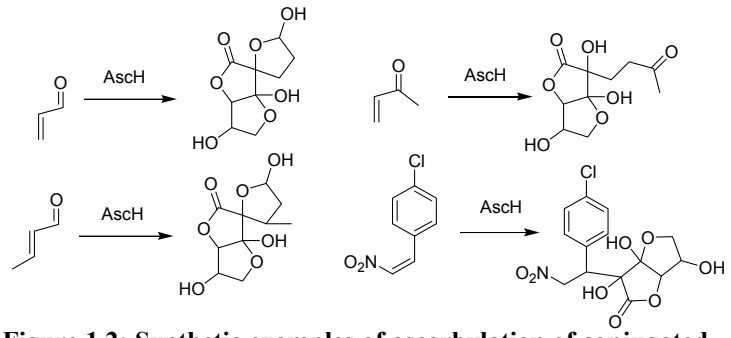


Figure 1.2: Synthetic examples of ascorbylation of conjugated alkenes. Michael additions of ascorbic acid with acrolein, crotonaldehyde, methylvinylketone, and *p*-chloro-nitrostyrene.

These natural products are the result of *in vivo* ascorbylation of electrophiles (see chapter 2). Whether the nucleophilicity of ascorbate plays any role in human biochemistry is unknown; with the exception of exploring pharmaceutical properties of ascorbigen, this question has been unexplored and ignored.

The goal of this work is to explore the question- what purpose does ascorbate, as a nucleophile, have for human biology, either naturally or by exploiting the chemistry? Does ascorbate conjugation have a role in detoxifying

electrophiles which are significant in human disease and suffering? In addition, is ascorbylation a form of secondary metabolism, catalyzed by a previously unknown enzyme, and does the bulk amount of vitamin C, quite concentrated intracellularly, detoxify electrophiles chemically?

In exploring these questions, new questions have surfaced. How are ascorbate conjugates transported and metabolized? Are they eliminated like glutathione conjugates, or absorbed like vitamin C itself? Can the transport of ascorbate conjugates be exploited, perhaps as a novel prodrug moiety?

This work examines these two main hypotheses:

1. **Ascorbate conjugation, or ascorbylation, is a pathway for the detoxification of electrophilic xenobiotics and endogenously produced compounds, analogous to Phase II metabolism.**
2. **The properties of ascorbate can be used to create novel, effective prodrugs.**

What follows is a discussion of the literature with regard to ascorbic acid, phase II metabolism, prodrug moieties, and ascorbyl moieties in natural products.

Ascorbic acid

L-Ascorbic acid (vitamin C) is a powerful anti-oxidant and essential for life in humans and many mammals, fish, and birds. The term ‘ascorbic acid’ is derived from the Greek and neo-Latin roots a- (without) and scorbutus (scurvy), for its essential role in the prevention of scurvy. Structurally, ascorbic acid is a

six-carbon γ-lactone. Due to the unusually low pK_a of one of the hydroxyl moieties of ascorbic acid, the molecule is deprotonated at neutral and biological pH, making it water soluble, and particularly hydrophilic. In most mammals, ascorbic acid is typically biosynthesized from glucose via a series of oxidative steps (Smirnoff, 2001) (**Fig 1.3**); however, humans lack functional gulonolactone oxidase activity due to mutation (Nishikimi, Fukuyama et al., 1994), and must acquire ascorbic acid from the diet. Other biosynthetic pathways have been found. In higher plants, ascorbic acid is produced via L-galactose and L-galactono-1,4-lactone, the latter being the C-3 of gluconolactone (shown in **Fig 1.3**) (Wheeler, Jones et al., 1998). Fungi do not produce ascorbic acid, but rather a five carbon homolog known as erythroascorbic acid. Erythroascorbic acid is an antioxidant and appears to serve the same function as ascorbic acid (Amako, Fujita et al., 2006; Wang, Narasaki et al., 2009).

Ascorbic acid was discovered 1927, when it was isolated by Albert Szent-Györgyi and colleagues (Szent-Györgyi won the 1937 Nobel Prize for its discovery and evidence of its anti-scorbutic properties) (Szent-Gyoergyi, 1927).

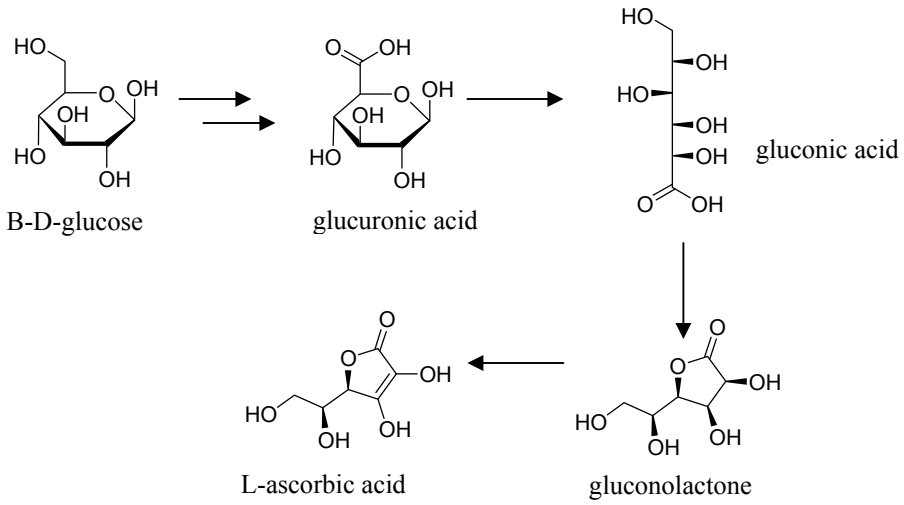


Figure 1.3: Biosynthesis of Ascorbic Acid in mammals.

The search for "anti-scorbutic" foods was a major focus of research in the prevention of scurvy, which plagued sailors in particular and malnourished people in general for all of recorded history (Hippocrates, 5th century BCE). Scurvy can cause loss of teeth, bleeding from mucous membranes, formation of sores, and ultimately death due to the inability to synthesize collagen. Ascorbic acid is essential for the hydroxylation of proline in collagen, and continued absence of ascorbic acid in the diet prevents the proper formation of collagen, leading to scurvy (Peterokofsky, 1991). It should be noted that scurvy is the disease caused as the end result of chronic ascorbic acid deficiency; it is the avitaminosis of ascorbic acid, similar to beriberi or rickets (thiamine and vitamin D, respectively). This does not mean that the inherent biological function of ascorbic acid is the

prevention of scurvy.

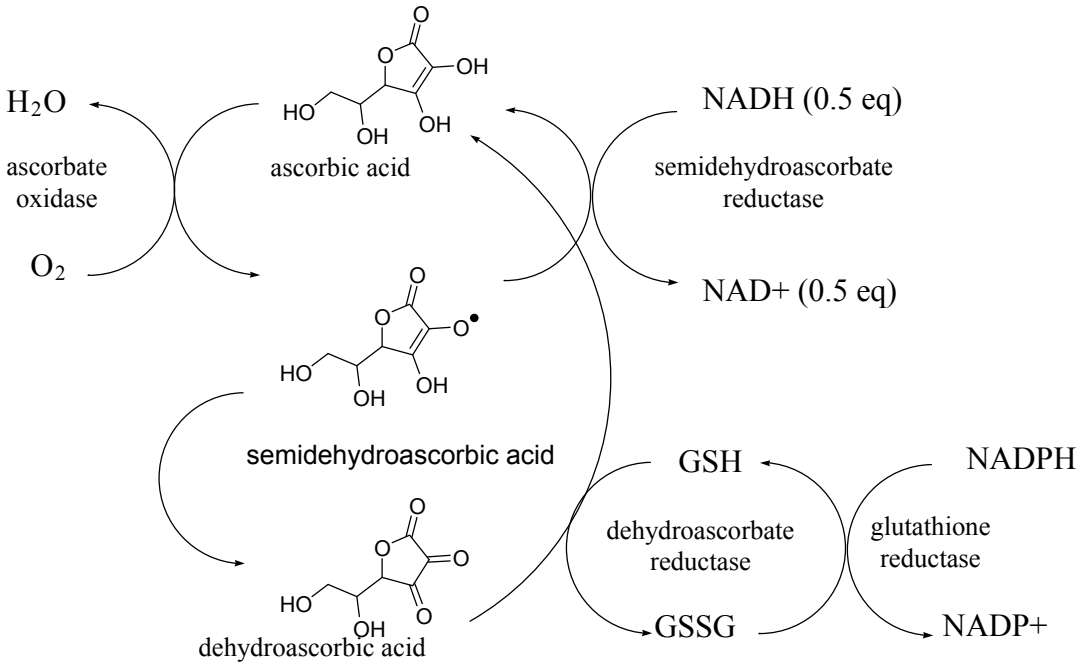


Figure 1.4: Ascorbate/Glutathione redox cycle.

Ascorbic acid also acts as an anti-oxidant by the donation of two electrons over two sequential steps. Ascorbic acid donates one electron to form semidehydroascorbic acid, a free radical. This radical is relatively stable compared to many biologically occurring radicals, such as lipid peroxidation radicals, and its stability is likely why semidehydroascorbic acid is an effective radical scavenger (Bielski, Richter et al., 1975).

Semidehydroascorbic acid can undergo a second one electron transfer to form dehydroascorbic acid, be reduced back to ascorbic acid by semidehydroascorbate reductase and NADH, or undergo disproportionation to produce ascorbic acid and dehydroascorbic acid. Semidehydroascorbic acid and

dehydroascorbic acid can be reduced back to ascorbic acid in a cycle with reduced glutathione (GSH) (**Fig 1.4**). In aqueous solutions, dehydroascorbic acid undergoes intramolecular hemiketalization with the primary alcohol to form the five membered ring, as well as hydration of the remaining ketone (Ritter, 2008). Notably, dehydroascorbic acid can also undergo hydrolysis of the lactone to 2,3-diketogulonic acid (Lewis, 1976) (**Fig 1.5**). In dehydroascorbic acid, this lactone hydrolysis leads to a series of irreversible decarboxylations (Deutsch, 2000). This loss of ascorbic acid *in vivo* can only be replaced through the diet in humans. Ascorbic acid and its oxidation products are not particularly stable in aqueous solutions and the redox cycle with glutathione, as well as other enzymatic and transport mechanisms, are essential to ensure that ascorbic acid plasma concentrations remain high, especially during instances of the absence of ascorbic acid in the diet.

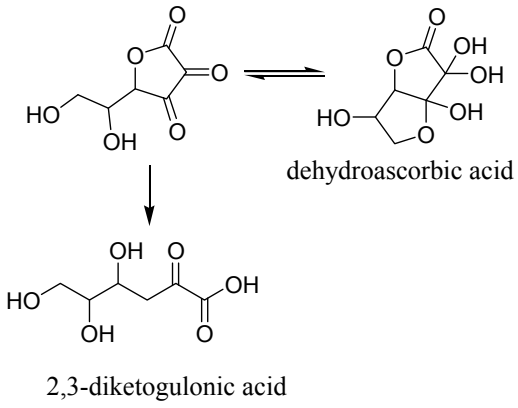


Figure 1.5: Hydration and hydrolysis of dehydroascorbic acid.

Ascorbate in proline hydroxylation

Surprisingly, while ascorbate is an essential cofactor for prolyl hydroxylase, it does not participate in the redox chemistry itself (Hoffart, Barr et al., 2006). Prolyl hydroxylase is a non-heme iron containing enzyme. Ascorbate may coordinate with the iron ion, although the catalytic cycle can proceed without it. It is α-ketoglutarate (α-KG) which is oxidatively decarboxylated to succinate, oxidizing the non-heme iron to a reactive iron-oxo species in the process. This iron-oxo species directly hydroxylates proline to 4-hydroxyproline (**Fig. 1.6**) (Pavel, Zhou et al., 1998). There is no obvious role for ascorbate. It may be

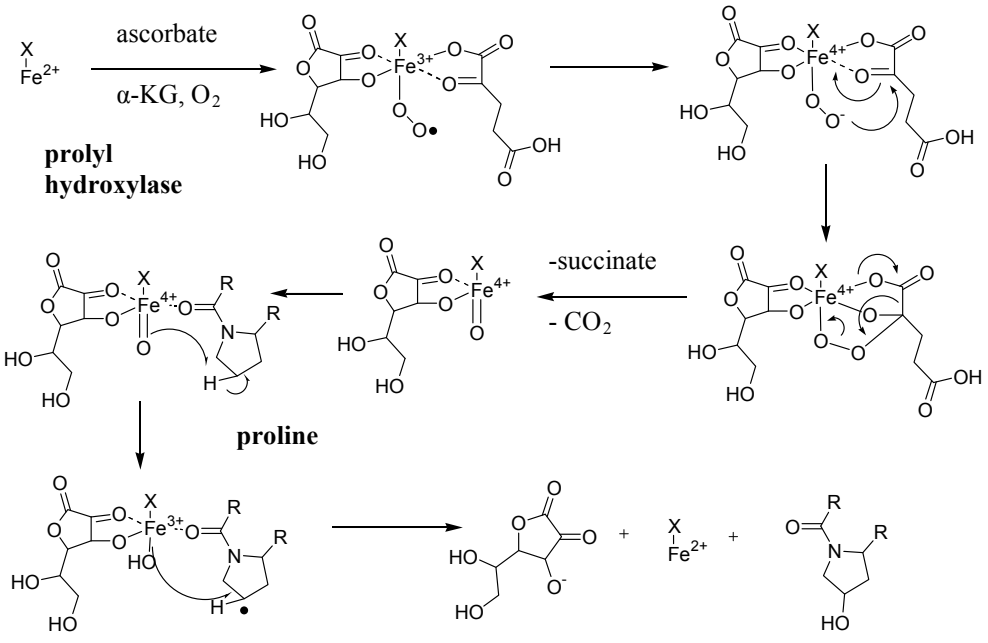


Figure 1.6: Hydroxylation of proline by prolyl hydroxylase. An activated iron-oxo species hydroxylates proline using α-ketoglutarate as a cofactor. However, ascorbate is not directly involved.

involved in the homeostasis of prolyl hydroxylase- reducing Fe(III) to Fe(II), should the heme become oxidized. However, it is not involved in the hydroxylation of proline itself.

Ascorbic acid transport

Transport of ascorbic acid across the cell membrane is accomplished by sodium-dependent vitamin C transporters (SVCT-1 and SVCT-2). The oxidized form, dehydroascorbic acid, is transported by hexose transporters GLUT1, GLUT3, and GLUT4. There is significant variation in the level at which these transporters are present in different tissues of the body. Ascorbic acid, which is present in high concentrations in foods relative to other common vitamins, is absorbed in the intestine by SVCT-1, which transports ascorbic acid across the intestinal barriers (MacDonald, Thumser et al., 2002). Bioavailability of ascorbic acid can be as high as 100%; however plasma concentration of ascorbic acid as a function of dose is sigmoidal. Oral dosage of ascorbic acid can achieve ~80 μM plasma concentration (Levine, Conry-Cantilena et al., 1996), and I.V. injections are needed to achieve higher concentration. SVCT-1 is also present in kidneys (Lee, Oh et al., 2006), where it recycles ascorbic acid back into plasma. This allows ascorbic acid plasma concentrations to remain high even when removed from the diet, and explains why development of scurvy can take months with an ascorbic acid depleted diet. While SVCT1 and SVCT2 are involved in intestinal absorption of vitamin C, they are also responsible for cellular uptake of ascorbate from plasma. Ascorbate has been found to be at millimolar concentration in

human cells (Bergsten, Amitai et al., 1990). SVCT2 is found at higher levels in certain tissues such as the central nervous system (Qiu, Li et al., 2007), adrenal glands (Patak, Willenberg et al., 2004), and the eye (Obrenovich, Fan et al., 2006). SVCT2 is present in the intestine, however its role is to transport ascorbic acid from plasma into intestinal cells, rather than involvement in intestinal absorption (Boyer, Campbell et al., 2005). Generally, it is believed that SVCT1 is responsible for homeostasis of ascorbic acid plasma concentrations, while SVCT2 is responsible for the transport of ascorbic acid to tissues prone to oxidative stress due to metabolic activity (Savini, Rossi et al., 2008).

While the facilitative hexose transporters (GLUT1, GLUT3, and GLUT4) transport dehydroascorbic acid as opposed to ascorbic acid, they are key to maintaining high intracellular concentrations of ascorbate. Dehydroascorbate is transported into the cell where it is reduced back to ascorbate by dehydroascorbate reductases (Vera, Rivas et al., 1993). Humans (and the few other mammals who cannot synthesize ascorbic acid), highly express GLUT1 in erythrocytes, which favors transport of dehydroascorbic acid over glucose. Other mammals do not express GLUT1 in erythrocytes, and it appears this expression of GLUT1 in humans co-evolved with the loss of the ability to synthesize ascorbic acid (Montel-Hagen, Kinet et al., 2008).

Ascorbate as a biological anti-oxidant

The primary function of ascorbate is as a cofactor in the hydroxylation of proline, and additionally as a cofactor in several other enzymatic reactions (Levine, Rumsey et al., 2000). However, in addition to its primary function, ascorbate is believed to be of major importance as an anti-oxidant. Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), and other free radicals contribute substantially to human disease and suffering. Lipid peroxidation products (LPO) contribute to the formation of plaques in atherosclerosis patients. Oxidation of proteins can cause a host of problems with respect to enzymatic activity, stability, and proteolysis activity. Oxidation of crystallins in the lens of the eye is known to lead to cataract formation. Radical damage of DNA can lead to mutation, and carcinogenesis. *In vitro* studies have shown that the presence of ascorbate can prevent radical oxidation of lipids, proteins, and DNA.

Ascorbate prevents the oxidation of low-density lipoproteins (LDL) and other lipids at physiological concentrations. When LDL and human plasma are exposed to a number of oxidants, such as the radical initiator 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), cigarette smoke, hydrogen peroxide, etc., ascorbate protects lipids and LDLs from oxidation. This protection is superior to other biological anti-oxidants such as α-tocopherol and ubiquinol (Frei, 1991). Studies show that treatment with ascorbate can lead to a decrease in levels of plasma F_2-isoprostanes, an important biomarker for oxidative stress (Block, Jensen et al., 2008).

Likewise, ascorbate may prevent the oxidation of proteins. Some *in vitro* studies show that ascorbate can decrease the formation of protein carbonyls, a marker of protein oxidation (Miranda, Reed et al., 2009a). However, there is really no direct epidemiological evidence for the benefit of ascorbate in the prevention of oxidation of either protein or DNA *in vivo*. This is due in part to the difficulty in measuring oxidation without the presence of ascorbate, as high concentrations are present in healthy individuals (Padayatty, Katz et al., 2003).

Vitamin C and Cancer

In the 1970s, Linus Pauling and others made the extraordinary suggestion that very large doses of vitamin C could be used to treat cancer (Cameron & Pauling, 1974). Preliminary studies involving small numbers of advanced cancer patients in a clinic trial showed promising results (Cameron & Campbell, 1974; Cameron & Pauling, 1976). However, follow-up studies conducted later showed no statistical significance between patients dosed with high concentrations of vitamin C, and the controls (Creagan, Moertel et al., 1979; Moertel, Fleming et al., 1985). Vitamin C as a treatment for cancer was largely abandoned (Levine, Espey et al., 2009). However, the problem with the attempts to reproduce the success of the early experiments was that they only provided oral doses of vitamin C to patients. The earlier experiments used oral dosage, intravenous injections, and a combination of both. As discussed earlier, plasma concentrations of vitamin C are

limited by intestinal absorption. Intravenous injection can deliver much higher concentrations of ascorbate.

Recent studies, however, have found that vitamin C at very high concentrations, higher than those delivered by oral dose, does indeed have anti-tumor activity (Verrax & Calderon, 2009). In healthy human cells, ascorbate functions as an anti-oxidant. However, in some circumstances, vitamin C can also function as a pro-oxidant (see Ch. 1 discussion section). Levine and co-workers have shown that pharmacological concentrations of ascorbate kill cancer cells, both *in vitro* and *in vivo*, by the production of hydrogen peroxide in extracellular spaces (although not in blood) and can selectively kill tumor cells (Chen, Espey et al., 2005b; Chen, Espey et al., 2008).

Phase II metabolism

The biotransformation of xenobiotics follows two main pathways- phase I metabolism and phase II metabolism. The differences between phase I and phase II metabolism correspond to the chemistry of the biotransformation. Phase I metabolism involves oxidation, reduction, and hydrolysis. Phase II metabolism involves the conjugation of xenobiotics with endogenous compounds, specifically glutathionylation, glucuronidation, acetylation, sulfation, methylation, and amino acid conjugation.

Phase I and phase II metabolism are not mutually exclusive. Phase II metabolism can follow Phase I metabolism. For example, an aliphatic double bond may undergo oxidation by the phase I enzyme cytochrome P450 to the corresponding epoxide. The epoxide, being electrophilic, may then undergo glutathione conjugation by phase II metabolism (**Fig 1.7)** (Friedman & Mottram, 2004). The opposite holds true as well, phase I enzymes can metabolize products of phase II metabolism. The anti-oxidant 2-(*t*-butyl)hydroquinone undergoes two successive rounds of both phase I and phase II metabolism (**Fig 1.8**) (Monks & Lau, 1997).

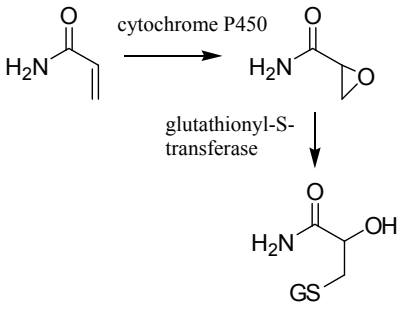


Figure 1.7: Example of sequential phase I and phase II metabolism occuring with the xenobiotic acrylamide.

Most forms of phase II metabolism (i.e. glutathionylation, glucuronidation, sulfation, and amino acid conjugation) increase the hydrophilicity of the

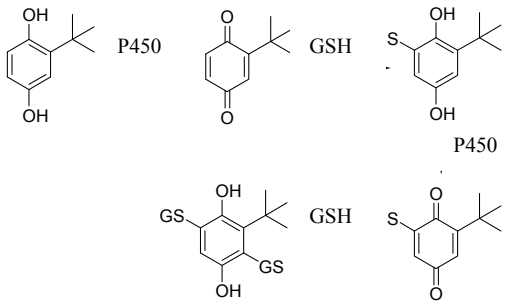


Figure 1.8: Sequential phase I metabolism and phase II metabolism of 2-(*t*-butyl)hydroquinone.

xenobiotic. This has the effect of greatly aiding the excretion of the xenobiotic in urine. With few exceptions, methylation and acetylation typically decrease the

solubility of the conjugated xenobiotic, although the conjugation can still assist in excretion of the xenobiotic in urine.

Glutathione conjugation- Many toxic xenobiotic compounds, particularly carcinogens, are electrophilic in nature. Acrolein, polycyclic aromatic hydrocarbons (PAHs), methyl iodide, polychlorinated biphenyls (PCBs), lipid peroxidation products (LPOs), and nitrosamines adduct to nucleophilic functional groups in DNA, leading to carcinogenesis. These electrophiles and others can also form adducts with proteins, impairing protein function.

Glutathione is a nucleophilic tripeptide found *in vivo* in very high intracellular concentrations, up to 10 mM (Halliwell & Gutteridge, 1999). Glutathionylation of xenobiotics is catalyzed by glutathionyl-S-transferase, although glutathione is nucleophilic enough and present at high enough levels that glutathionylation can occur without catalysis by enzymes. Glutathionylation can occur directly with the xenobiotic (e.g. the glutathionylation of acrolein), or occur after phase I metabolism. PAHs, for example, are not electrophilic but are oxidized by cytochrome P450 to an epoxide, which is electrophilic and will react with glutathione.

Most glutathionylation occurs in the liver where glutathione and glutathione-S-transferases are particularly abundant. Glutathione conjugates are further metabolized to mercapturic acids in the kidney and excreted in urine (which can be referred to as 'phase III metabolism,' the further transport and elimination of phase I and II metabolites) (Nakata, Tanaka et al., 2006).

Glutathionylation of various xenobiotics is often detected, measured, and monitored as the mercapturic acids in urine samples (Haufroid & Lison, 2005).

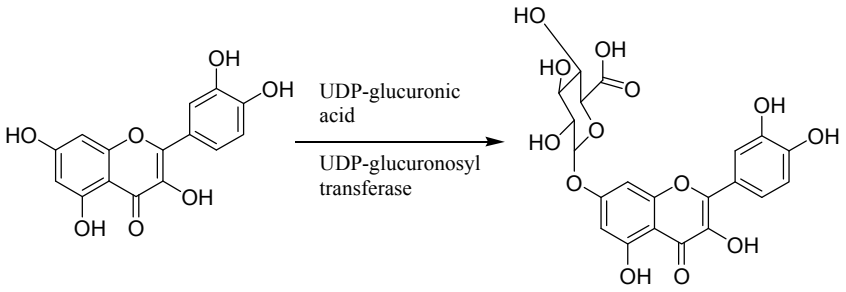


Figure 1.9: Glucuronidation of quercetin.

Glucuronidation and Sulfation- Complementary to glutathionylation is glucuronidation, which is responsible for the biotransformation of nucleophilic molecules. The glucuronide is typically conjugated with nucleophilic heteroatoms, such as the oxygen atoms in phenols (**Fig. 1.9**) (Chen, Chen et al., 2005), carboxylic acids, the nitrogen atoms in amine, amides, or pyridines, and sulfur atoms in thiols, although conjugation to carbon atoms can occur. As a sugar, the glucuronide moiety is highly polar, increasing the solubility of the conjugate and aiding excretion in the urine or bile. The pK_a of the carboxylic acid in glucuronides is around 3, ensuring ionization at physiological pH (Giroud, Carrupt et al., 1998). Glucuronides are synthesized by UDP-glucuronosyltransferases. The cofactor is UDP-glucuronic acid, which is synthesized from UDP-glucose. The concentration of free UDP-glucuronic acid is lower than glutathione, but still

fairly high at about 350 μM. The glucuronide formed is in the α- configuration, which prevents hydrolysis by β-glucuronidase activity, which is involved in the breakdown of complex carbohydrates.

Sulfation is also a phase II pathway which renders nucleophilic xenobiotics more water soluble (**Fig. 1.10**). Sulfation typically occurs with phenol and aliphatic alcohols, often conjugating with the same xenobiotic and functional group as glucuronic acid (Giroud et al., 1998). Sulfation occurs by transfer of a sulfite group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS). PAPS is synthesized from free cysteine. Due to the low concentration of free cysteine, the concentration of PAPS is lower than both glutathione and UDP-glucuronic acid. The reaction between PAPS and xenobiotics is catalyzed by sulfotranferases.

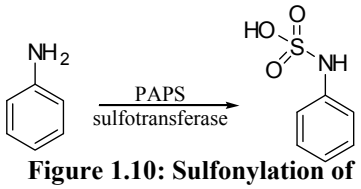


Figure 1.10: Sulfonylation of aniline to the sulfamate

There are examples of phosphorylation of xenobiotics, but these are much less common than other phase II metabolites (Testa & Kramer, 2008).

Methylation and Acetylation- As opposed to other forms of phase II metabolism, methylation and acetylation usually decrease the water solubility of xenobiotics. An exception applies to the conjugation of aromatic amines, which results in the quaternary ammonium salt (**Fig. 1.11**) (McKennis, Turnbull et al., 1963). Xenobiotics are methylated by S-adenosylmethionine (SAM), a cofactor common to many biological methylations not related to phase II metabolism. Since SAM is an electrophile, phase II methylation occurs on phenols, amines, and

the same functional groups associated with glucuronidation. Methyltransferases are responsible for these methylation reactions.

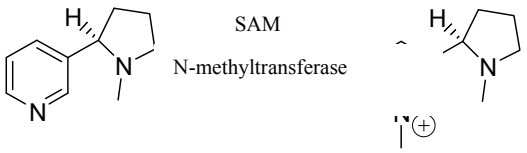


Figure 1.11: N-methylation of nicotine.

Acetylation reactions almost exclusively take place with primary aromatic amines (such as aniline), hydrazines, hydrazides, and aromatic hydroxylamines. N-acetyltransferases catalyze the acetylation reaction and use acetyl-coenzyme A (CoA) as a cofactor. Acetylation often involves the detoxification of the products of phase I metabolism of xenobiotics (Noda, Ono et al., 1995).

Prodrugs

The development and use of prodrugs is of major importance in medicine. A prodrug may be defined as a drug molecule that has been chemically modified to remove some undesired effect or to improve some transport property, and will then be transformed back to the parent molecule at the target site of action. Perhaps two iconic examples from the history of medicinal chemistry may apply- aspirin and heroin **(Fig. 1.11)**.

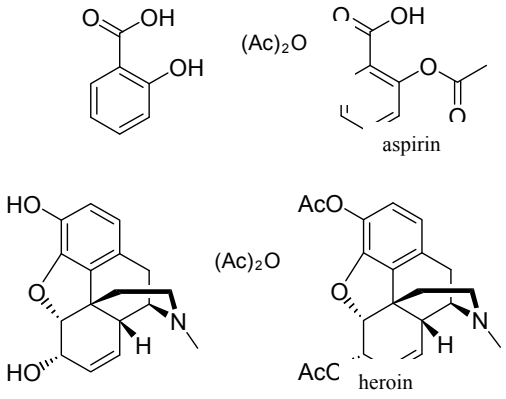


Figure 1.12: Production of aspirin and heroin, prodrugs of salicylic acid and morphine.

Salicylic acid has been used as an analgesic in traditional medicine for

thousands of years. It is a powerful anti-inflammatory. Salicylic acid inhibits cyclooxygenase (COX-2) expression by binding to C/EBPβ, a COX-2 promoter (Wu, 2003). However, its use led to undesirable side effects such as stomach bleeding and gastrointestinal ulcers. In 1897, Bayer scientists began investigating the acetate ester of salicylic acid for its less irritating side effects, and began marketing it as aspirin (Vane, 2000). Acetylsalicylic acid is hydrolyzed to salicylic acid by esterases in the plasma, after it has passed through the stomach and intestine (Gresner, Dolnik et al., 2006).

Heroin, or diacetylmorphine, is the bis acetate ester of morphine, the opiate used as a traditional analgesic and narcotic for thousands of years. Heroin was initially marketed as being less addictive than the parent drug morphine. The bioavailability is superior to morphine (Halbsquth, Rentsch et al., 2008; Perger, Rentech et al., 2008). When ingested, first pass metabolism removes both acetate groups delivering morphine to the blood stream.

However, the definition of "prodrug" may be further narrowed. Acetylsalicylic acid, non-hydrolyzed, has pharmacological properties similar to salicylic acid, although through a different mechanism- by directly acetylating COX-1 and COX-2. When given by intravenous injection, heroin does not undergo first pass metabolism, and quickly crosses the blood-brain barrier (the ability of heroin to cross the blood brain barrier is superior to morphine, and part of the reason for the illicit recreation problem of heroin abuse) where it interacts with opiate receptors. A better definition of prodrugs is given by the International

Union of Pure and Applied Chemistry: "*Prodrugs can… be viewed as containing specialized non-toxic protective groups used… to alter or eliminate undesirable properties…that undergo biotransformation before exhibiting pharmacological effects* (IUPAC, 1998)." The alteration or elimination of undesirable properties may include the increase of desired properties, such as bioavailability. In addition, while biotransformations are required in prodrugs, they do not necessarily include protective groups in the organic synthesis sense of the term.

Prodrugs are utilized for a variety of reasons. Prodrugs may be an improvement over parent drugs in terms of transport. The prodrug moiety can increase or decrease lipophilicity, improving intestinal transport, increasing permeability in the cell membrane, or improving transport across the blood-brain barrier. Prodrugs may increase patient compliance. Some prodrugs can increase the palatability of foul tasting drugs. Administration of other prodrugs may be less painful than the parent drug, allowing oral or IV dosage as opposed to intramuscular injections. Prodrugs may be directed to site-specific targets, cleaving the prodrug moiety only upon entering the target sites. Prodrugs may effectively increase the stability of parent molecules, essentially acting as protecting groups to prevent metabolism. This could involve the esterification of hydroxyl groups to prevent oxidation by phase I metabolism, or an addition to an electrophilic site to prevent glutathionylation in phase II metabolism.

There is a large number of prodrug moieties used to produce these effects, some of them uniquely tailored for specific drugs. Hexamine, for example, with

its adamantane-like structure (**Fig. 1.13)** is designed to deliver anti-microbial formaldehyde upon decomposition in the urinary tract for the treatment of urinary tract infections (Paternotte, Fan et al., 2001). However, many prodrugs use prodrug moieties that follow generalized chemical functionality, if not identical prodrug moieties. For example, many formations of prodrugs involve esterification, phosphorylation, or amidation.

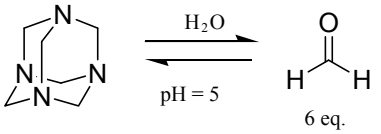


Figure 1.13: Hexamine, a unique prodrug for formaldehyde.

Esters- Perhaps the most common prodrug moieties are esters. These esters can radically alter the hydrophilicity of molecules, and are often used to alter PK/PD properties of parent molecules. For example, ampicillin, an important antibiotic, has poor intestinal absorption. A number of esterified ampicillin prodrugs have been made which have nearly 100% bioavailability while increasing the lipophilicity over the parent drug (Paternotte et al., 2001). Alternatively, increasing the hydrophilicity of a parent molecule by esterification may also improve bioavailability. There are a number of amino acid based prodrug esters, which are ionized and water soluble at physiological pH. Other uses for esterification can include protection of hydroxyl groups in the parent molecule. Propranolol is a drug commonly used to treat hypertension. However, its bioavailability is limited due to glucuronidation during first-pass metabolism. The succinate ester of propranolol (**Fig. 1.14**) both increases the

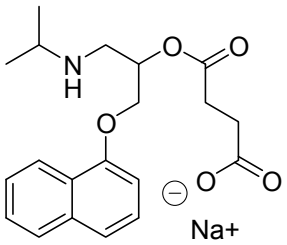


Figure 1.14: Propranolol hemisuccinate

hydrophilicity of the drug, and blocks glucuronidation, resulting in significantly higher plasma levels (Chandrasetchar, Giridhar et al., 2000). The ethyl succinate ester of erythromycin was developed for treatment of children suffering from bacterial infections. The antibiotic is notorious for its foul taste and the esterification of one of the glycone moieties of erythromycin renders the drug taste-free (Bhadra, Morris et al., 2005).

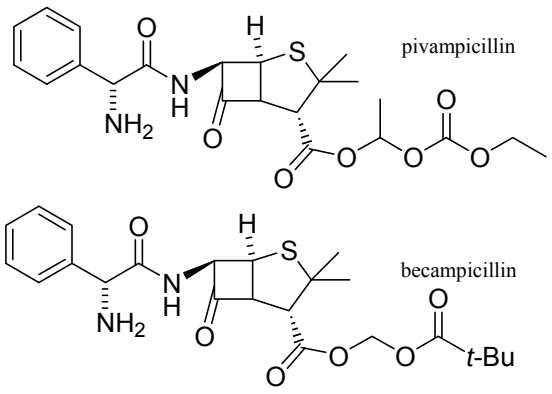


Figure 1.15: Ester prodrugs of Ampicillin

An advantage to ester based prodrugs is that many are easily hydrolyzed to the parent drugs by any number of esterases. Careful selection of which ester is employed can exploit properties of esterases. For example, the ampicillin prodrugs such as pivampicillin are designed to decrease steric hindrance in the binding site of the activating enzyme (Paternotte et al., 2001) (**Fig. 1.15**). The rate of hydrolysis of esters of propranolol depends on which ester is used as the propranolol moiety. For example, hydrolysis of propranolol acetate is faster than the propranolol pivalate (Paternotte et al., 2001).

Phosphates- The use of phosphates as pro-drug moieties is also a common approach in prodrug development, primarily by the phosphorylation of alcohols in

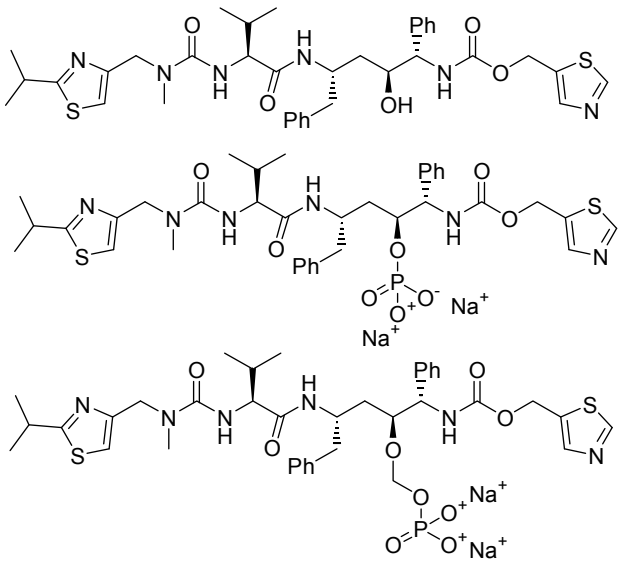


Figure 1.16: Ritonavir and phosphate prodrugs. The phosphate failed to hydrolyze *in vivo.* The -OCH_2- homolog does hydrolyze.

the parent drug. Generally, the purpose of phosphates is to improve the water solubility of the parent drug, in addition to being polar, the phosphate moiety can easily form sodium, potassium, or calcium salts. A phosphate prodrug of cyclosporin A is water-soluble and formulated for administration to the eye to treat infections (Lallemand, Varesio et al., 2007). The antiepileptic phenytoin, which is formulated in propylene glycol is notorious for causing painful injections. The phosphate ester, fosphenytoin, is water soluble and can be injected intramuscularly or intravenously, with drastically less painful (Ramsay & DeToledo, 1996).

The phosphate moiety is usually cleaved by phosphatases to provide the parent drug. If phosphatases fail to hydrolyze the phosphate group, there are strategies to homologate the phosphate ester for improved enzymatic hydrolysis.

For example, an attempt to form a phosphate-based prodrug of HIV protease inhibitors lopinavir and ritonavir resulted in compounds that were not hydrolyzed by phosphatases (DeGoey, Grampovnik et al., 2009). However, oxymethyl- and oxyethyl- phosphate homologs proved to be more water soluble than the parent molecule, were readily enzymatically cleaved, and provided the parent compound in plasma in high amounts relative to the dose of the prodrug (**Fig. 1.16**).

Lipids- A number of drugs have been coupled to lipids for use as prodrugs. These lipids could be free fatty acids, diglycerides, or phospholipids. While many strategies in prodrug development attempt to increase the hydrophilicity of drugs, the coupling of drugs to lipids holds two main advantages. Firstly, a prodrug coupled to a lipid can take advantage of absorption, distribution, and metabolic pathways of lipids. Secondly, the lipid moieties can greatly aid the molecule's passage through lipophilic barriers, such as the blood-brain barrier, or cell membranes.

While the previously discussed acetylsalicylic acid causes less gastric irritation than the parent drug, salicylic acid, there are still some issues with irritation. There have been a number of prodrugs of aspirin itself developed, including aspirin covalently linked to a diglyceride (**Fig. 1.17**). This prodrug causes less gastric irritation, and there is little hydrolysis of the lipids until reaching the small intestine (Kumar &

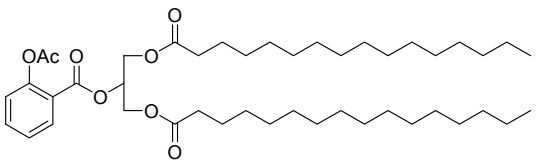


Figure 1.17: 1,3-dipalmitoyl-2-acetylsalicylateglyceride, a prodrug of aspirin

Billimora, 1978).

Carbohydrates- Sugar chemistry is sometimes used in prodrug development. Like many other prodrug moieties, sugars are very polar, can be cleaved with a number of enzymes, and are a benign leaving group. In creating a prodrug for the muscle relaxant and sedative mephenesin (**Fig. 1.18**), both sugar and lipid chemistry was used to create an amphipathic prodrug. The nature of amphipathic prodrugs, like phospholipid prodrugs, greatly enhances the ability of the prodrug to permeate the cell membrane (Martins, Mamizuka et al., 1997).

Strangely, glucuronides have been used as prodrug moieties. Glucuronidation is typically thought of as a phase II mechanism, i.e. conjugation with hydrophobic nucleophilic xenobiotics to reduce toxicity and aid in excretion. Thus, it would seem unlikely that glucuronide moieties would be useful prodrug moieties. However, they are of some considerable interest when it comes to prodrug formation of cancer chemotherapeutics. The reduced toxicity of the glucuronidated drugs lowers cytotoxicity in healthy cells, and the glucuronic acid is released in the targeted cancer cell, where β-D-glucuronidase activity can be increased. The iconic anticancer drug paclitaxel, for example, has been glucuronidated as a prodrug (**Fig. 1.19**) (Alaoui, Saha et al., 2006).

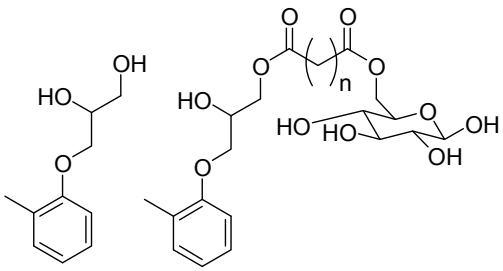


Figure 1.18: Mephenesin and glucose based amphipathic prodrug

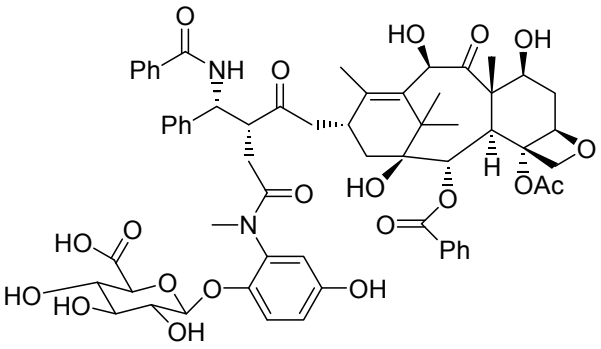


Figure 1.19: Glucuronide prodrug of paclitaxel

Discussion

In 2001, Blair and co-workers discovered that ascorbate could function as a pro-oxidant *in vitro* and induce the formation of lipid peroxidation products (LPOs) such as 4-hydroxynonenal (HNE) from the lipid hydroperoxide 13 (S)-

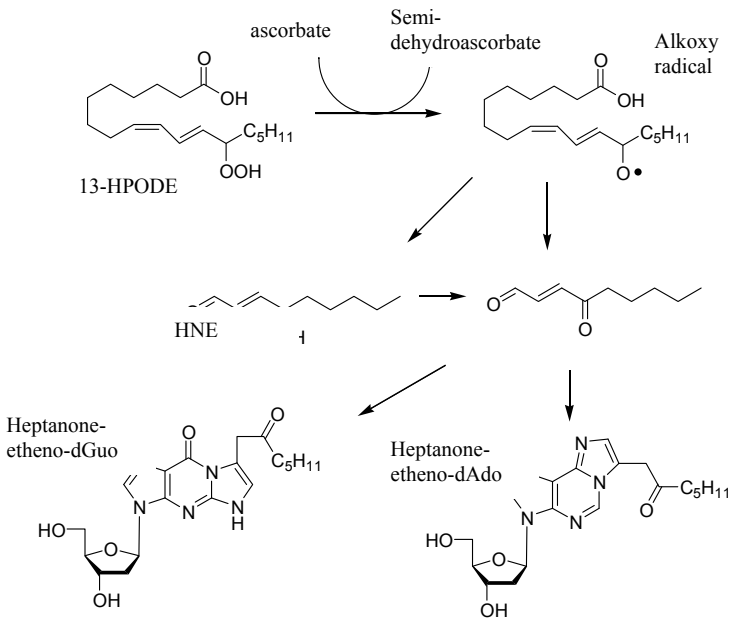


Figure 1.20: Vitamin C induced formation of HNE from HPODE, and adduction of HNE to DNA

hydroperoxy-(*Z,E)*-9,11-octadienoic acid, or (13-HPODE), a product of polyunsaturated fatty acid metabolism (Lee, Oe et al., 2001). HNE, an α,β-unsaturated aldehyde, is involved in the formation of plaques in atherosclerosis, and as Blair also discovered, can conjugate with DNA (Blair, 2008), potentially leading to cancer (**Fig. 1.20)**. This work and that of others (Halliwell, 1996; ID Podmore, 1998) indicating a pro-oxidant effect of ascorbate created interest not just in the scientific community but in the media and public (Anonymous, 2001). The latter were confused over these claims that vitamin C, which was long recognized to fight the common cold and possibly even cancer, was now carcinogenic.

Efforts in this laboratory to reproduce this result were successful; ascorbate does indeed induce the formation of HNE from HPODE *in vitro* (ascorbate does not appear to induce the formation of lipid peroxidation products in plasma) (Suh, Zhu et al., 2003). However, an unexpected product was discovered: the Michael addition product of ascorbate and HNE (**Fig. 1.21**). This conjugate is no longer electrophilic and presumably would be non-toxic, analogous to the glutathione conjugate of HNE. This means that while ascorbate, as a pro-oxidant, could cause the formation of toxic LPOs, as a nucleophile ascorbate could also be responsible for detoxification of LPOs and other electrophiles. Thus, efforts were made to ascertain the role of vitamin C in detoxification *in vivo*.

A review of the literature reveals that this is not the first time that it has been suggested that ascorbate could offer protective effects against electrophiles.

In 1973 it was reported that the simultaneous oral administration of vitamin C and nitrosamines (well established carcinogens, common in cigarette smoke) could offset the mutagenicity of the nitrosamine (Kamm, Dashman et al., 1973). The authors suggested, without much specificity, that the detoxification was due to the anti-oxidant, radical trap properties of ascorbate, perhaps not realizing the nucleophilicity of ascorbate. Indeed, even today any detoxification of compounds by ascorbate is simply assumed to involve free radical chemistry. However, in 1974 a letter appeared in Nature suggesting that the detoxification of nitrosamine with ascorbate was due to nucleophilic addition or substitution of ascorbate to the nitrosamine, and recommended further study (Edgar, 1974). To our knowledge, this suggestion went unheeded and ignored.

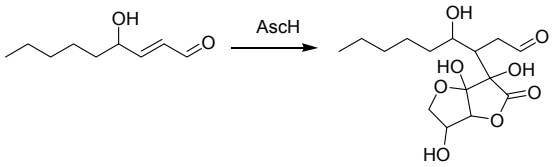


Figure 1.21: Ascorbylation of HNE

We propose that ascorbate does indeed play a role in the detoxification of some electrophiles *in vivo*. Like the phase II cofactor glutathione, ascorbate is a nucleophile. Like many phase II cofactors, ascorbate is present at remarkably high concentrations inside the cell: greater in concentration than UDP-glucuronic acid and only slightly less than glutathione. Like most phase II metabolic pathways, ascorbylation renders the reactant more polar. Ascorbyl HNE (AscHNE) is far more water soluble than HNE. While phase II metabolism is often enzymatic, catalyzed by a large variety of transferases; there is no evidence for the existence of an "ascorbyl-transferase." Glutathionylation of xenobiotics still occurs non-

enzymatically. Even if ascorbylation of xenobiotics is trivial in comparison to glutathionylation and other Phase II pathways, it seems that given the high *in vivo* concentration of ascorbate and its high *in vitro* reactivity with known, common toxins, that ascorbylation would occur *in vivo*. The first goal of this work has been to detect and measure ascorbylated compounds in biological samples.

In the course of our studies, we discovered some interesting transport properties of ascorbylated compounds (see Chapter 6). Other experiments revealed that ascorbylation, like all Michael additions, is reversible and the retro-Michael reaction can occur at physiological conditions. These and other details led us to form the hypothesis that ascorbate could function as a new prodrug moiety for drug development. Although prodrug moieties are much more numerous and varied than Phase II adducts, there are many common features of prodrug moieties that ascorbate also shares. As stated above, ascorbylation provides enhanced aqueous solubility, a trait shared by many prodrug moieties. Like all prodrug moieties, the leaving group of an ascorbylated prodrug would be pharmacologically benign. Like the lipid-based prodrugs previously discussed, an ascorbylated prodrug might utilize pathways of ascorbate transport, possibly the SVCT enzymes used to transport free ascorbic acid. While most prodrug moieties are cleaved by hydrolases, and again there is no evidence of ascorbyl-hydrolase, there are prodrugs such as hexamine which chemically deliver the active pharmaceutical parent drug. There are even examples, with the glucuronides, where the moiety is involved in both Phase II metabolism and as a prodrug moiety.

The second goal of this work has been to produce and test an ascorbylated pharmaceutically relevant compound as a prodrug.

Chapter 2: Covalent interaction of ascorbic acid with natural products

Nicholas G. Kesinger and Jan F. Stevens

Phytochemistry, in press

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.

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, Smith 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, Han et al., 1993; Packer & 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 completely incompatible with H_2O, 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 xanthydrol (Schwall, Regitz et al., 1970) (**Fig. 2.1**).

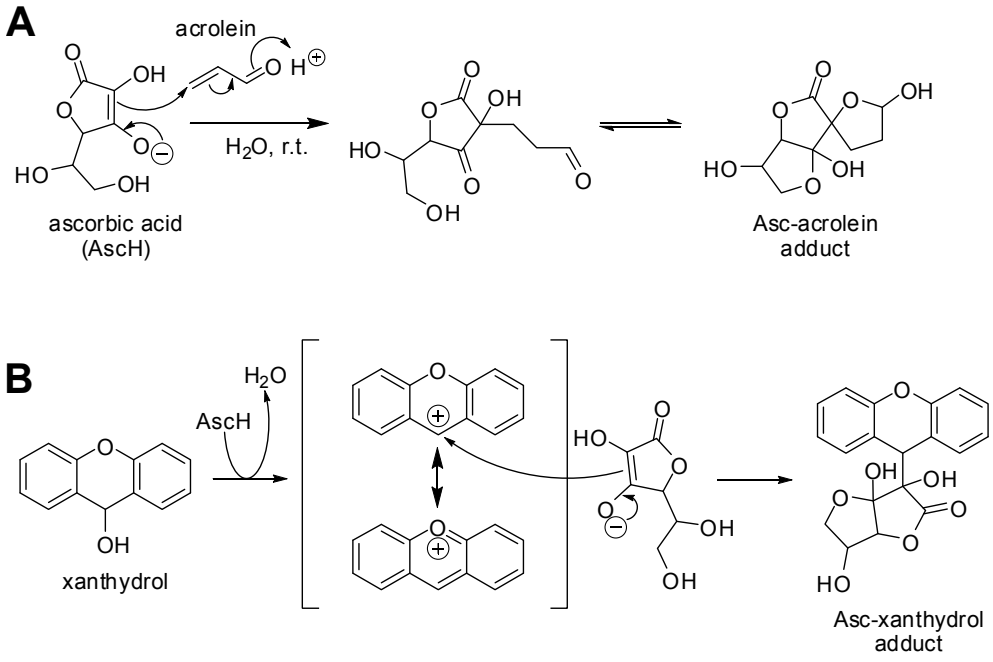
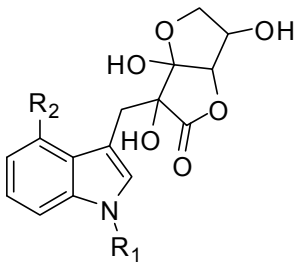


Fig.ure 2.1: Synthetic examples of nucleophilic ascorbylation via (A) Michael addition and (B) Nucleophilic substitution. The ascorbyl moiety typically forms the hemiketal upon conjugation.

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, Hansen et al., 2000) (**Fig. 2.2**).



R_1	R_2	
H	H	ascorbigen
H	OH	4-hydroxyascorbigen
H	OCH_3	4-methoxyascorbigen
OCH_3	H	neoascorbigen

Figure 2.2: Ascorbigens isolated from *Brassica* spp.

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. 2.3**). ABG concentrations can range from 5.3 mg/kg to 16 mg/kg depending on the variety (Hrncirik, Valusek et al., 2001), while total content of glucobrassicin

and its analogs can range from 100 to 1500 mg/kg (Fenwick, Haeney et al., 1989).

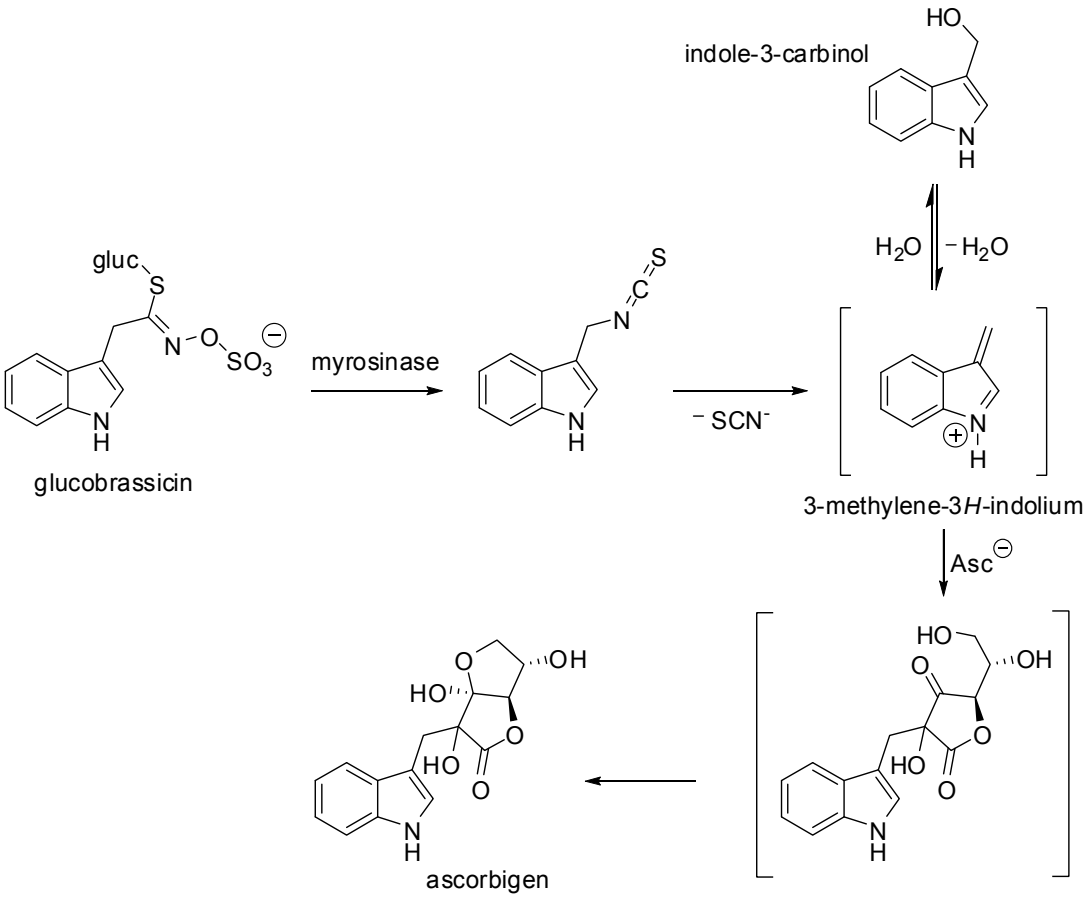


Figure 2.3. Formation of ascorbigen from glucobrassicin via indole-3-carbinol.

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, Lazhko et al., 1996) (**Fig. 2.4**). The pentose-indoles were the major metabolites found in the

blood

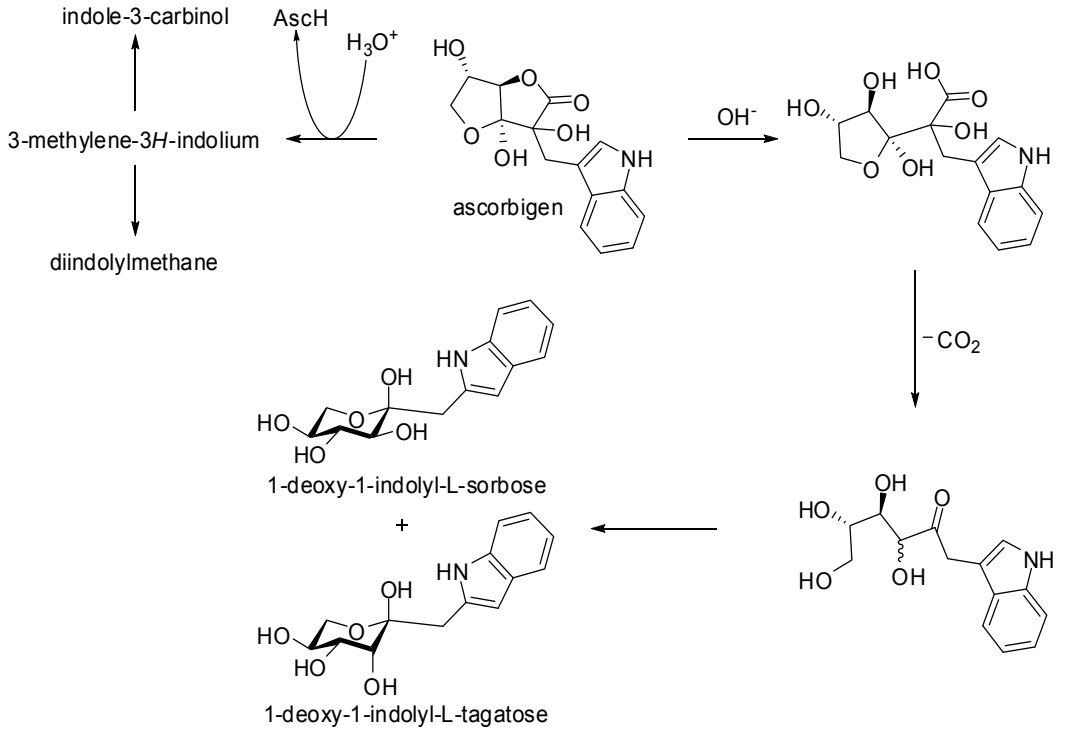


Figure 2.4: Hydrolysis and decarboxylation of ABG to deoxysorbose and deoxytagatose indoles.

serum and liver of mice after oral administration of ABG (Reznikova, Korolev et al., 2000b). During cooking, the levels of ABG can decrease. When boiled for 10 min, the amount of ABG in cabbage is reduced by 10% (Ciska, Verkerk 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, Penas et al., 2009).

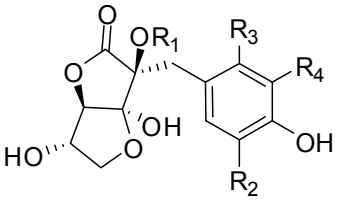
Epidemiological evidence suggests that consumption of cruciferous vegetables can lower the risk of formation of colorectal cancer (Verhoeven, Verhagen et al., 1998), but the contribution of ascorbigen to a cancer chemopreventive effect of cruciferous vegetables is unclear. Consistent with a cancer chemopreventive effect, ABG induces the carcinogen-detoxifying enzyme, NADP(H) quinone oxidoreductase 1 (NQO1) in rats (Wagner, Hug et al., 2008). On the other hand, ABG dose-dependently induced the pro-carcinogen activating enzyme, CYP1A1, in murine hepatoma 1c1c7 cells (Stephenson, Bonnesen 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 & Rimbach, 2009c). ABG also has a cytoprotective effect against glucose-induced endothelial toxicity (Joshi, Bauer 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 active Nrf2 or induce phase II metabolism (Wagner, Ernst et

al., 2009a).

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, Huebbe et al., 2008). 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 delesserine from *Delesseria sanguinea* (Yvin, Chevolot-Magueur et al., 1982), and rhodomelol and methylrhodomelol from *Polysiphonia lanosa* (**Fig. 2.5**) (Glombitza, Skupp et al., 1985).



R_1	R_2	R_3	R_4	
CH_3	H	H	H	delesserine
H	OH	Br	Br	rhodomelol
CH_3	OH	Br	Br	methylrhodomelol
H	H	H	H	4-hydroxybenzyl ascorbigen

Figure 2.5: Cresol-based ascorbyl conjugates.

Biomimetic syntheses of delesserine, 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 et al., 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 *Delesseria sanguinea* have long been known for strong anti-coagulant activity (Elsner, Liedmann et al., 1938), although this may be due to the presence of sulfated polysaccharides (Gruenewald, Groth et al., 2009) rather than delesserine. Delesserine, rhodomelol and methylrhodomelol have not been tested individually for biological activity.

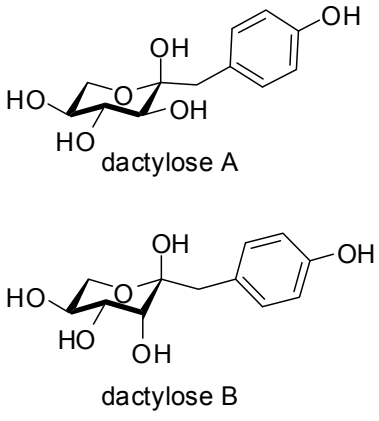


Figure 2.6: Dactylose A and B.

In addition to the indole derivatives, 4-hydroxybenzylascorbigen can also be found in

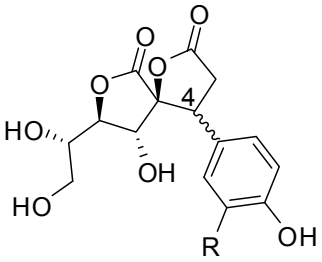
Brassica (Buskov, Hasselstrom et al., 2000). This is likely produced from the 4-hydroxybenzyl glucosinolate, glucosinalbin. Dactylose A and B (**Fig. 2.6**) have been found in *Dactylorhiza hatagirea*, a Nepalese orchid of which the bulbous roots are used traditionally as a tonic. While lacking an ascorbyl moiety, this compound derives from the hydrolysis and decarboxylation of 4-hydroxybenzylascorbigen, similar to the decomposition of ascorbigen itself. These compounds have been produced biosynthetically (Preobrazhenskaya, Rozhkov et al., 1997).

2.2. Michael additions

2.2.1. Ascorbylated coumaric acid derivatives

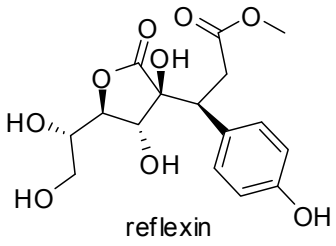
A number of ascorbylated compounds appear to be biosynthesized by Michael addition of ascorbic acid to coumaric acid (**Fig. 7**). Leucodrin was first isolated from the leaves *Leucodendron concinnum* (Proteaceae) in 1886 in a search for a quinine substitute (Rapson, 1938). It can also be found in *L. adscendens, L. stokoei, Leucospermum conocarpodendrum,* and *Leucospermum reflexum*. The structure was not confirmed until 1966 (Perold & 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, Hodgkinson et al., 1972).

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

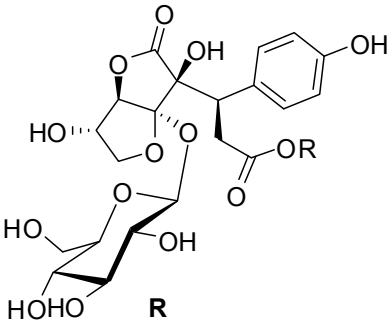


R

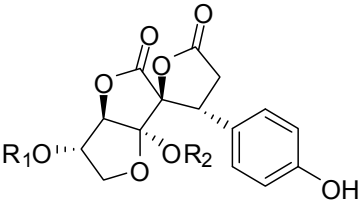
H	leucodrin (4*R*)
H	conocarpin (4*S*)
OH	leudrin (4*R)*



reflexin



R	
H	viburnolide B
Me	viburnolide C



R_1	**R_2**	
H	β-D-glucose	viburnolide A
β-D-glucose	H	dilaspirolactone
H	4,6-diacetyl-β-D-glucose	4',6'-diacetylviburnolide A
acetyl	2,4,6-triacetyl-β-D-glucose	2',4',6',12-tetraacetylviburnolide A
coumaroyl	4,6-diacetyl-β-D-glucose	4',6'-diacetyl-12-coumaroylviburnolide A
H	4-acetyl-β-D-fucose	dichotomain A
H	β-D-fucose	dichotomain B

Figure 2.7: Coumaric acid-based ascorbyl derivatives.

viburnolides A, B and C from the leaves of *V. wrightii* (Machida & 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-cumaroyl-viburnolide A (Franke, Porzel 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, Cheng et al., 2006).

Leucodrin, leudrin, reflexin, and the aglycones of dilaspirolactone, viburnolides, and dichotomains have been synthesized by Poss and co-workers (Poss et al., 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 & Stevens, 1966) (**Fig. 2.8**). The aglycone of piptoside, piptosidin, is the

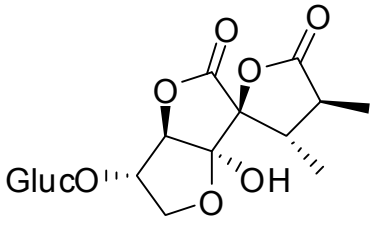


Figure 2.8. Piptoside.

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 & 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, Nonaka et al., 1986), excoecarinin B from *Excoecaria kawakamii* (Lin, Tanaka et al., 1990), mallojaponin and mallonin from *Mallotus japonicus* (Saijo, Nonaka et al., 1989), and helioscopins A and B from *Euphorbia helioscopia* (Lee, T Tanaka et al., 1990) (**Fig. 2.9**).

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, elaeocarpuscin can be synthesized by simple incubation of ascorbic acid with geraniin, a tannin distributed in a number of plant families, including Geraniaceae, Euphorbiaceae, Aceraceae, Cercidiphylaceae and Simaroubaceae (Okuda, Yoshida 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 elaeocarupsin is an intermediate in the biosynthesis of geraniin (Tanaka et al., 1986) (**Fig. 2.10**).

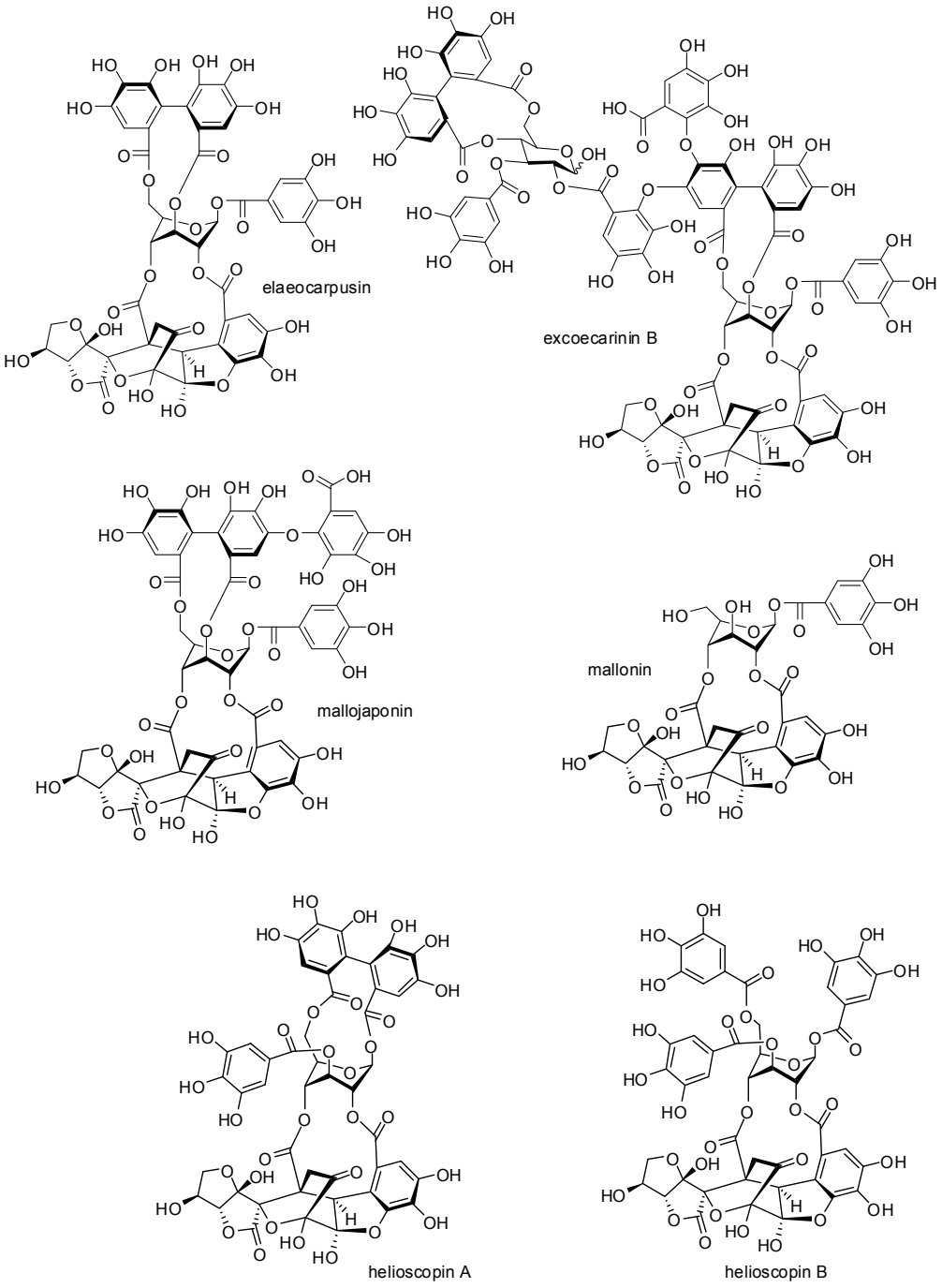


Figure 2.9: Ascorbylated hydrolyzable tannins

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). Elaeocarupsin 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, Jun et al., 2007).

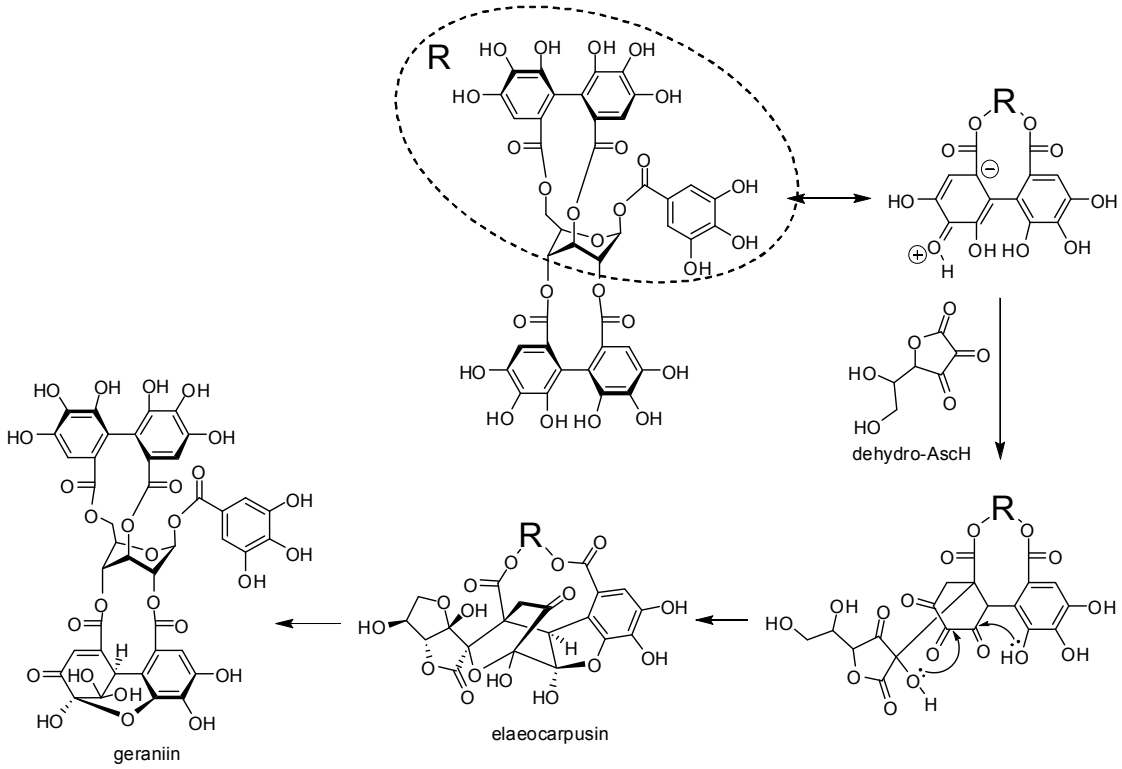


Figure 2.10: Proposed formation of geraniin from an ellagitannin via elaeocarpusin.

3.2. Ascorbylated phloroglucinol

A single ascorbylated phloroglucinol, phloroscorbinol, has been isolated as its hexaacetate from the brown alga *Sargassum spinuligerum* (Keusgen, Falk 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. 2.11**). This compound was not assayed for biological activity.

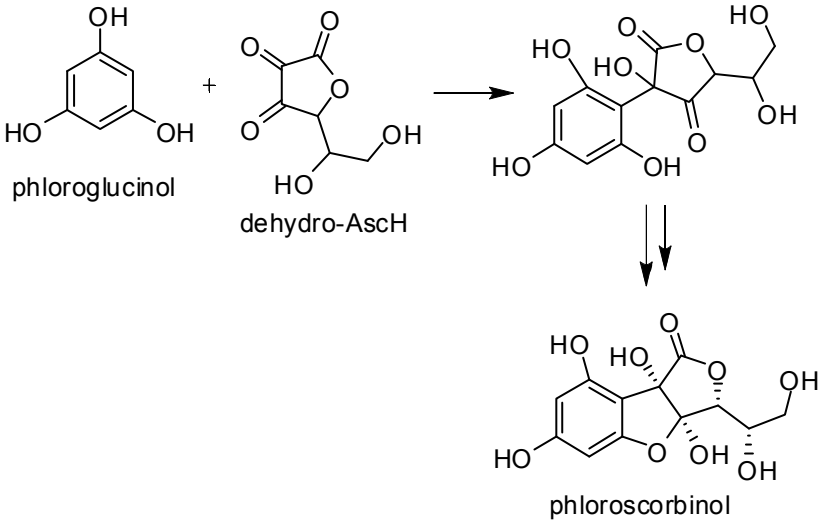


Fig 2.11: Hypothetical formation of phloroglucinol derivative found in *S. spinuligerum.*

3.3. Ascorbylated EGCG

The Nishioka group has also isolated 8-*C*-ascorbyl (-)-epigallocatechin 3-*O*-gallate (ascorbyl EGCG, **Fig. 2.12**.) from oolong tea, *Camellia sinensis* (Hashimoto, Nonaka 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.

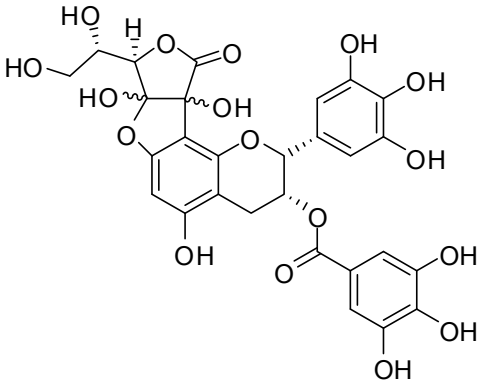


Figure 2.12: 8-*C*-ascorbyl (-)-epigallocatechin 3-*O*-gallate.

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, Matsukawa 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 IC_{50} of 36 nM for ascorbyl-EGCG against recombinant human MT1-MMP, and IC_{50}s of 25 and 0.46 μM against rhMMP-2 and rhMMP-7. For comparison, non-ascorbylated EGCG showed IC_{50} 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, Tanaka et al., 2003) and from the stem of *Dipterocarpus grandiflorus* (Ito, Tanaka 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, Kouzi et al., 2000), photochemically (He, Wu et al., 2008), and by single electron oxidation with metal reagents (Sako, Hosokawa et al., 2004). The latter work provides a mechanism for ascorbylation in shorealactone: if the dimeric resvertrol 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. 2.13**).

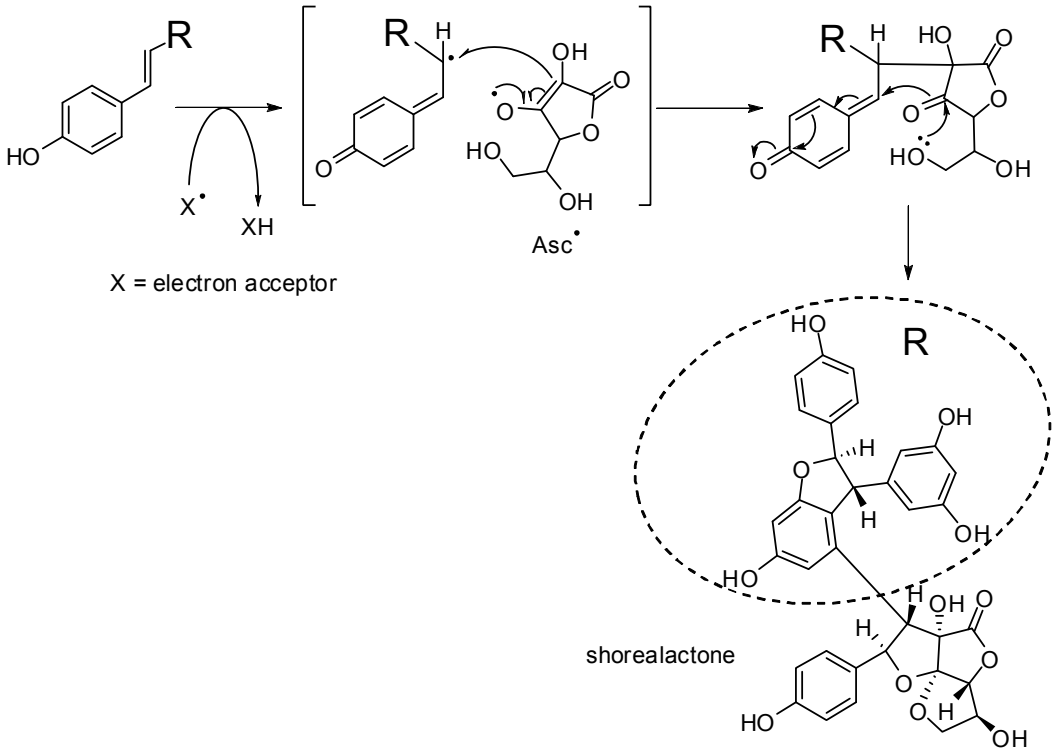


Figure 2.13: Proposed mechanism for the formation of shorealactone.

Shorealactone has been tested against type II DNA topoisomerase, which catalyzes topological changes in supercoiled or circular DNA. Shorealactone showed an IC_{50} 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, Hayashi et al., 2006).

4.2. Jolkinin

The hydrolyzable tannin jolkinin was isolated from *Euphorbia jolkinii* (Lee, Tanaka 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. 2.14**), making jolkinin unique among ascorbyl conjugates.

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

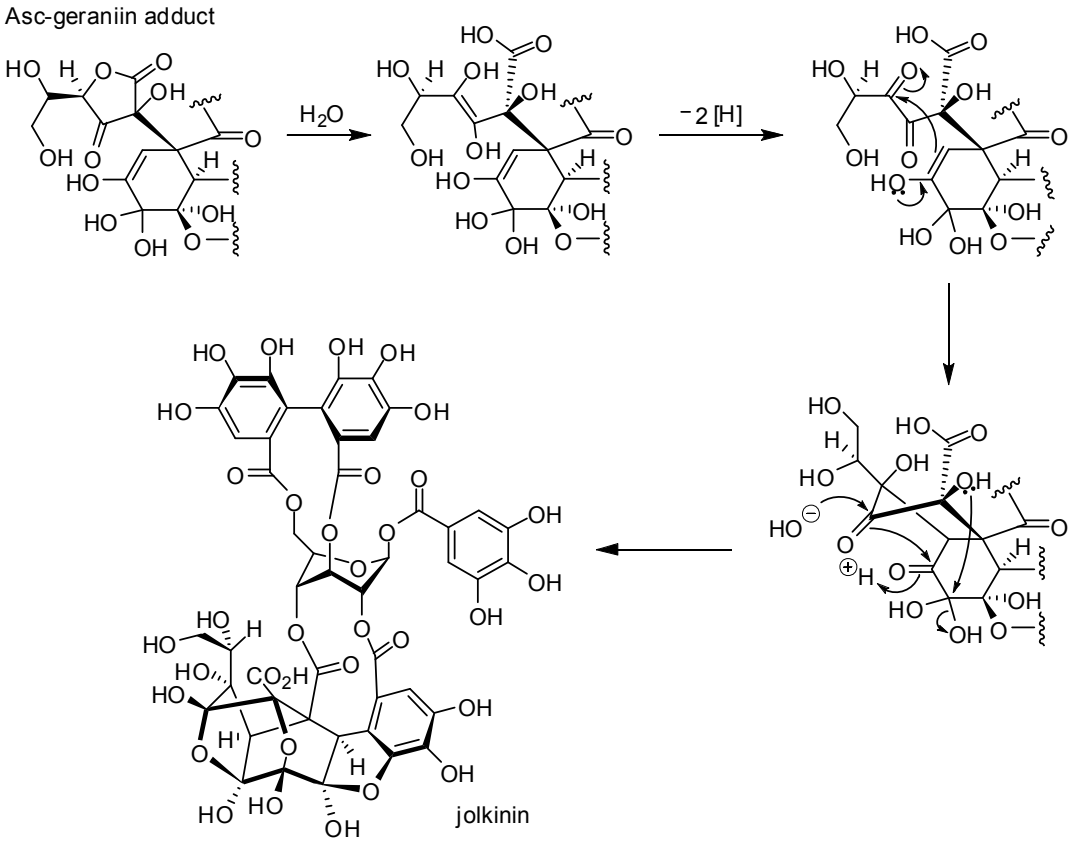


Figure 2.14: Biosynthesis of jolkinin from geraniin and ascorbic acid.

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.

Chapter 3: Ascorbyl-acrolein and Method Development

Acrolein

Acrolein, 2-propenal in IUPAC nomenclature, is a small molecule with profound toxicity. It is highly electrophilic, and readily alkylates DNA and proteins. While not currently listed as a carcinogen by the Environmental Protection Agency or the International Agency for Cancer Research, it is believed by some to have considerable carcinogenicity, and may be significant epidemiologically. A recent report implicates acrolein as the major carcinogenic contributor to cigarette smoke, over the polycyclic aromatic hydrocarbons (PAHs) and nitrosamines commonly attributed to cigarette smoke (Carmella, Chen et al., 2007). It is also the product of diesel exhaust and cooking oil fumes, and other environmental sources (Esterbauer, Schaur et al., 1991). In addition, it is produced biologically from a number of sources, although which route is the predominant *in vivo* source is subject to debate.

Acrolein can be produced from lipid peroxidation, although there is little or no evidence that this pathway is a major source of acrolein (Stevens & Maier, 2008). Acrolein may also be produced from glucose or glycosides (Yaylayan, Harty-Majors et al., 1999; Yaylayan & Keyhani, 2000b). Acrolein may also be derived from polyamines such as spermine or spermidine (**Fig. 3.1**), themselves degradation products of arginine and S-adenosylmethionine. Polyamines are particularly

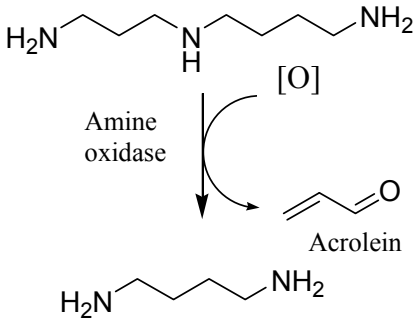


Figure 3.1: Acrolein from polyamines.

abundant in brain tissue (Shaw & Pateman, 1973) and this may be a major source of acrolein (Shaw et al., 1973). Acrolein is a known metabolite of threonine (Anderson, Hazen et al., 1997).

It is known that the major metabolic pathway of acrolein is conjugation with glutathione, oxidation or reduction to the corresponding carboxylic acid or primary alcohol, and conversion to the mercapturic acids in the kidneys (Kaye, 1973).

Ascorbyl-acrolein

In order to determine the role ascorbylation may play in the detoxification of electrophilic compounds, if any, acrolein was chosen as the candidate electrophile.

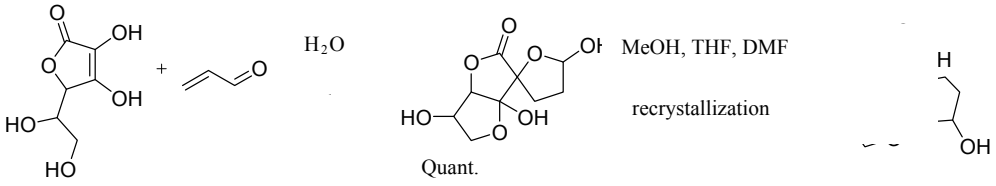


Figure 3.2: Formation of AscACR via Michael addition of ascorbic acid to acrolein.

AscACR had been synthesized previously in other laboratories (Fodor et al., 1983), although it was the subject of synthetic rather than biological research. The synthesis of AscACR is remarkably efficient (**Fig 3.2**); AscACR precipitates from the reaction mixture in quantitative yield. In contrast, AscHNE, the ascorbyl-conjugate of the lipid peroxidation product first found in our laboratory *in vitro*, has a poor yield, ~15%.

The structure of AscACR was first determined by Fodor and coworkers. However, the regiochemistry of the hemiacetal is dependent on the solvent in which the solid crystal is grown. The 6-membered fused hemiacetal ring determined by Fodor (Fodor et al., 1983), was found to be a five-membered spiro hemiacetal by x-ray crystallography (**Fig. 3.3**) when crystallized in water . The structure in solution is indeterminate (see below).

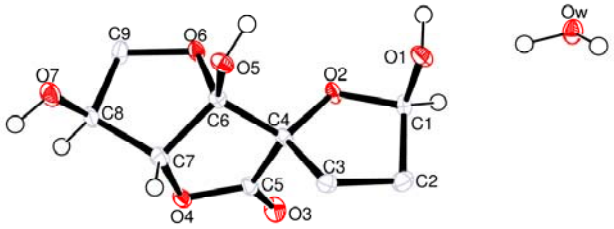


Figure 3.3: X-ray crystal structure of AscACR crystallized from water.

Due to the presence of *in vivo* acrolein, the high *in vivo* concentration of ascorbic acid, and the efficiency of this Michael addition, it was expected that AscACR would be a relevant biological product. Fodor himself conjectured that AscACR may be a metabolite of cyclophosphamide, an anti-cancer drug known to metabolize to acrolein, and that co-administration of the drug with ascorbate may have a protective effect. It has been shown that ascorbate has a protective effect in acrolein exposure (Arai, Uchida et al., 2005a; Logan, Parker et al., 2005; Nardini, Finkelstein et al., 2002), yet the possibility that ascorbylation has a protective role cancer drug administration has been ignored (see Chapter 4).

GSH/AscH Exchange of ACR

It is reasonable to question the proportion of acrolein that is ascorbylated relative to the amount of acrolein glutathionylated, given that glutathionylation of

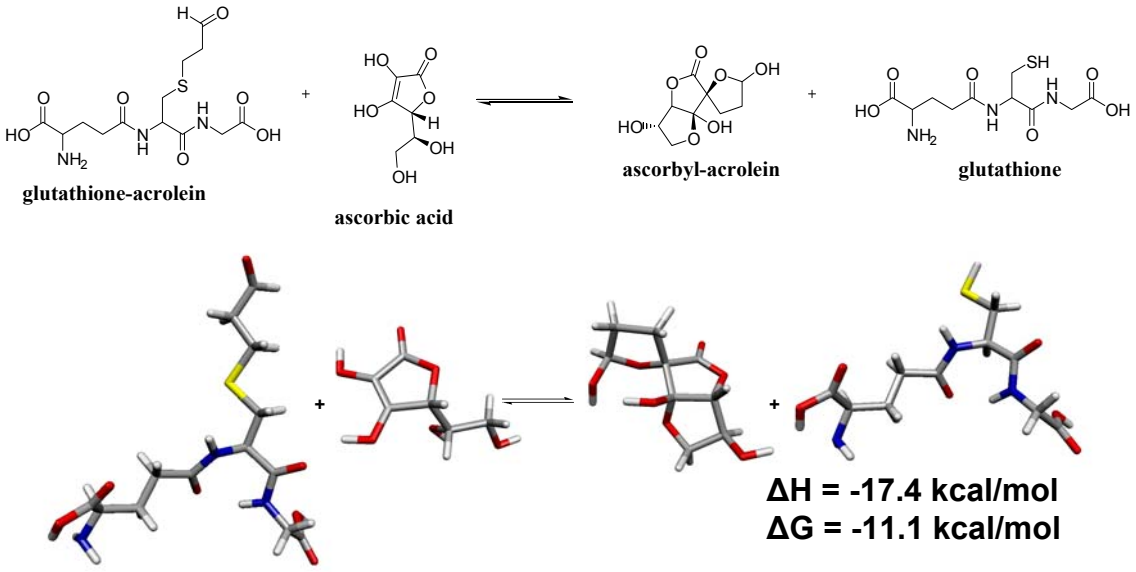


Figure 3.4: Computational studies on AscACR, thermodynamically favored over GSACR

acrolein is a well known metabolic pathway, and that glutathione is both more nucleophilic than ascorbate and present in greater concentrations *in vivo*.

Preliminary computational studies calculate that the AscACR has both lower enthalpy and Gibbs free energy, ΔH and ΔG of -17.4 and -11.1 kcal/mol respectively, than the glutathione counterpart (GSACR) (**Fig 3.4**). This should be expected, as the C-C bond formed during ascorbylation should be stronger than the C-S bond during glutathionylation. Since AscACR is thermodynamically favored over GSACR, *in vitro* exchange experiments were devised with the hypothesis that AscACR would form from GSACR in the presence of excess AscH.

Exchange Experiment Discussion

When a dilute solution of GSACR was incubated with an approximately physiological concentration of AscH, AscACR was observed to form over a time course by LC/MS (**Fig 3.5**). After roughly 24h, an equilibrium was reached where

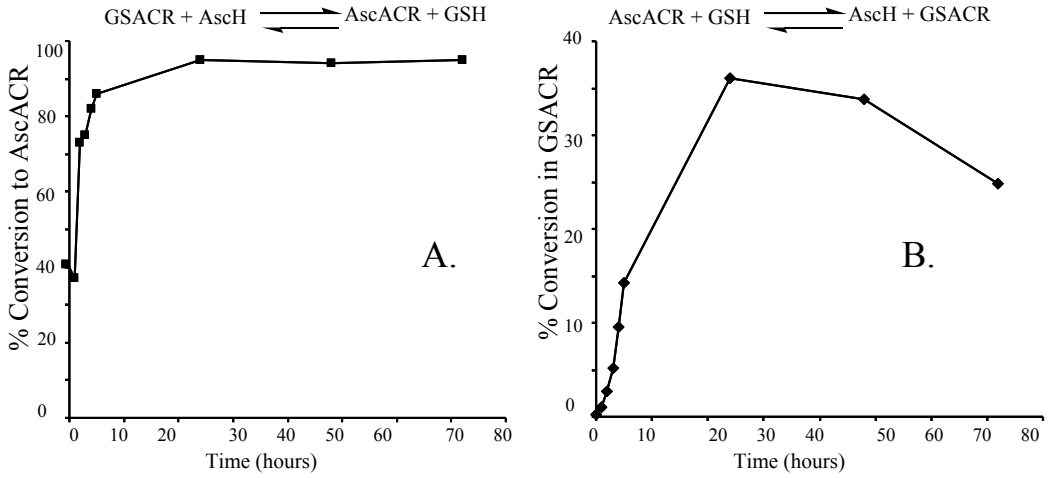


Figure 3.5: Exchange experiments, (A.) GSACR in 10 mM AscH, (B.) AscACR in 1mM GSH.

approximately 90% of the ACR was bound to ascorbate rather than glutathione. The inverse holds true for the incubation of AscACR in a high concentration of GSH, an equilibrium is again reached after 24h. It was observed that after 24h the percentage of AscACR to GSACR declined, perhaps due to degradation of AscH over a long time course. GSH and AscH were kept at a large molar excess relative to the respective conjugate. The level of AscH is originally at 10 mM to allow for some decomposition of AscH which inevitably occurs in solution. The mechanism of exchange is not determined; however, presumably it is by retro-conjugate addition and free acrolein as an intermediate.

Issues with LC method

While the synthesis of AscACR was trivial, the development of an adequate LC-MS method was not. The MS/MS spectrum and an SRM method for AscACR was acquired but due to a remarkably high polarity, no retention was observed on a conventional C-18 column running in reversed phase. Some retention is necessary for proper method development, detection and quantitation in biological samples, due to ion suppression by salts and other related chemical interferences.

Initial attempts to use a semi-preparative silica column in normal phase mode (isopropanol/hexane as a mobile phase) with Atmospheric Pressure Chemical Ionization (APCI) met with some success in providing retention. Indeed, *in vivo* AscACR was even observed in one urine sample during method development. However, this method was abandoned for reverse phase methods using conventional HPLC solvents (MeCN/H_2O) and electrospray ionization (ESI) tested with a variety of novel, commercial HPLC columns promising retention for highly polar molecules.

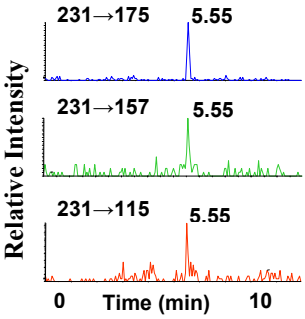


Figure 3.6: SRM traces of AscACR on a diol column.

Phenomenex Hydro-RP and Max-RP columns failed to give adequate retention of AscACR. A Thermo cyano-based column also gave no retention. A Phenomenex LUNA hilic column did provide adequate retention of AscACR; however the column was not durable and was not suitable for the routine use of

numerous samples. There was tellingly no elution of AscACR using a Phenomenex amino-based column. Unfortunately, this anomalous result was ignored when good retention was observed using a Thermo diol column (**Fig 3.6**).

The Thermo Betasil diol column gave good retention and reproducibility. This column was thereafter used as a standard for all underivatized AscACR chromatography.

Hydrolysis of AscACR

With chromatography issues solved, analysis of AscACR at low concentrations could begin. However, during the construction of calibration curves, it was noticed that AscACR disappeared below the detection limit after 24h of storage. Not suspecting that the compound would be unstable in water, a full MS scan was run on a sample, and a new peak of *m/z* 249 was detected. This new peak corresponds to the mass of AscACR + H_2O, and it was suspected that it could be the hydrate of the aldehyde moiety. An $^{16}O/^{18}O$ exchange experiment was devised where it was expected that ^{18}O should exchange with the two exchangeable oxygen atoms- the aldehyde oxygen and the ketone oxygen (hemiacetal and hemiketal in the crystal structure, respectively). Upon dissolving AscACR in $H_2{}^{18}O$, it was noticed that 3 ^{18}O atoms exchanged in AscACR, and there were a total of 4 ^{18}O in the AscACR + H_2O, indicating that the lactone is undergoing hydrolysis (**Fig. 3.7)**. The peak representing exchange of the fourth ^{18}O atom is relatively small, likely due to the rate of all four exchanges occurring in the time frame. ^{13}C labeling studies confirmed the hydrolysis of the lactone:

AscACR with the C-1 position labeled undergoes a loss of 45 Da ($^{13}CO_2$) in an MS/MS spectrum while the unlabeled AscACR shows a loss of 44 Da ($^{12}CO_2$) (See Chapter 4). This hydrolysis was unexpected. It had not been observed in the other ascorbyl-conjugates (e.g. AscHNE) in our laboratory, although this hydrolysis was not investigated with other compounds.

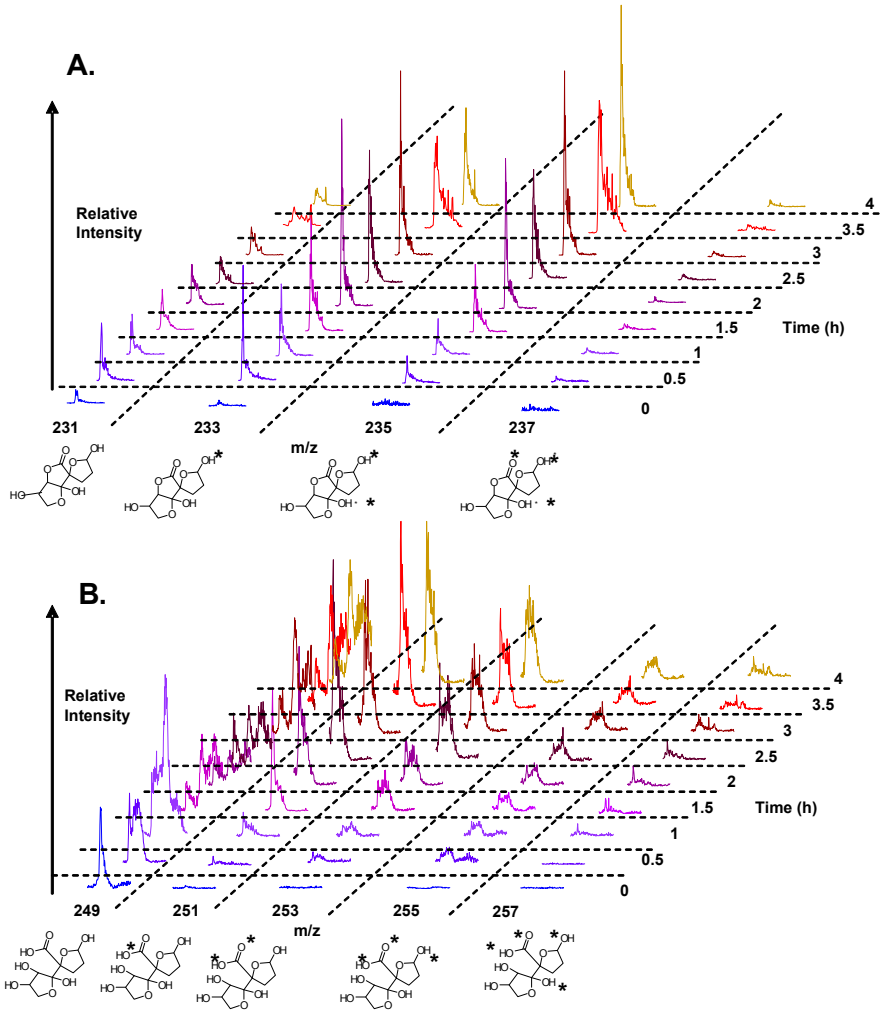


Figure 3.7: (A.) Extracted ion chromatograms of AscACR dissolved in $H_2{}^{18}O$ over a time course, representative labeled positions shown, negative ion mode. (B.) Extracted ion chromatograms of AscACR-H_2O dissolved in $H_2{}^{18}O$ over a time course, representative labeled positions shown, negative ion mode.

Derivatization of AscACR with Pentafluorobenzylhydroxylamine

While retention of AscACR had been accomplished with a diol HPLC column, peak shape was still poor. Furthermore, a difficulty found in the course of investigate AscHNE was that ascorbyl-conjugates can be difficult to distinguish from glucuronide conjugates, even with LC-SRM. It was decided that derivatizing AscACR with pentafluorobenzylhydroxylamine HCl (**Fig. 3.8**) could solve numerous experimental problems.

Firstly, the derivative shows improved chromatography. While the diol column provided some retention in normal phase for AscACR, peak shape was poor, with peak width of nearly one minute. With the large number of compounds in urine capable of giving any SRM transitions, poorly defined retention times are a disadvantage. The AscACR pentafluorobenzyl (PFB) oxime gave excellent peak shape, with a much longer retention time in reverse phase chromatography.

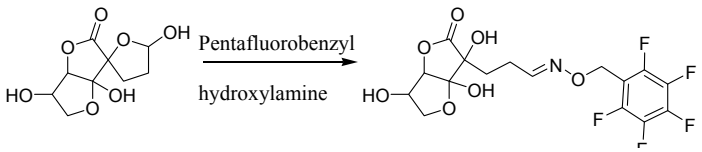


Figure 3.8: Derivatization of AscACR to give a PFB-oxime

Secondly, the AscACR PFB-oxime is far less polar than AscACR. This not only accounts for the improved chromatography, but also allows for separation from urine by liquid-liquid extraction (see below).

Thirdly, in some cases PFB-oximes can give remarkable sensitivity in LC-

MS using Atmospheric Pressure Chemical Ionization (APCI). There are reports of PFB-oximes undergoing a specific radical cleavage of the pentafluorobenzyl moiety in APCI, resulting in attomolar sensitivity (Lee, Blair, 2007). The AscACR PFB-oxime was tested with APCI and saw no such cleavage or improvement in sensitivity over ESI. This cleavage requires the formation of an $M^{\cdot -}$ ion rather than the M^- formed in the AscACR PFB-oxime. However, sensitivity was still quite good, with a detection limit in the low nanomolar range.

Fourth, pentafluorobenzyl hydroxylamine reacts selectively with carbonyls, and aldehydes in particular. Glucuronides, which can lead to false positives as they produce a fragment ion of *m/z* 175, were tested and do not react with pentafluorobenzyl hydroxylamine. Thus, the derivatization serves as method validation, both by demonstrating the functionality of the analyte, and providing an entirely new LC-SRM method.

MS methods were developed for AscACR PFB-oxime in both positive and negative ion mode, however the negative ion mode was more sensitive. Also present in the positive ion spectrum is a significant amount of the sodium adduct, which does not undergo useful fragmentation. In negative ion mode, MS/MS experiments led to fragmentation analogous to the negative ion fragmentation pattern of underivatized AscACR. The molecular ion peak at *m/z* 426 undergoes a neutral loss of 60 Da to *m/z* 366, representing the loss of the C-5 and C-6 diol from the ascorbate moiety. Also present are the fragments of *m/z* 157 and *m/z* 115, representing the loss of water and the loss of the diol moiety from the ascorbate

moiety after retro-Michael cleavage. These latter two fragments are also found in the MS/MS spectrum of the underivatizaed AscACR.

The analysis of AscACR as a standard protocol for measurement in biological samples was thus abandoned in favor of analysis for the AscACR PFB-oxime.

Elemental composition analysis of AscACR PFB-Oxime by MS

The ability to extract the AscACR PFB-oxime provided the opportunity to acquire a high-resolution accurate mass analysis of the compound. A large volume of freshly collected urine was derivatized with PFB-hydroxylamine and extracted with EtOAc. The organic layer was concentrated and the sample provided enough of the oxime to give provide an elemental composition. The analyte had a measured mass of 426.0623, 2.5 ppm error from the calculated mass of 426.0612. The empirical formula of the *in vivo* AscACR PFB-oxime, $C_{16}H_{13}F_5NO_7$ was in agreement with the correct structure, although no LC comparison could be made to a standard as the mass spectrometer was not equipped with an LC system.

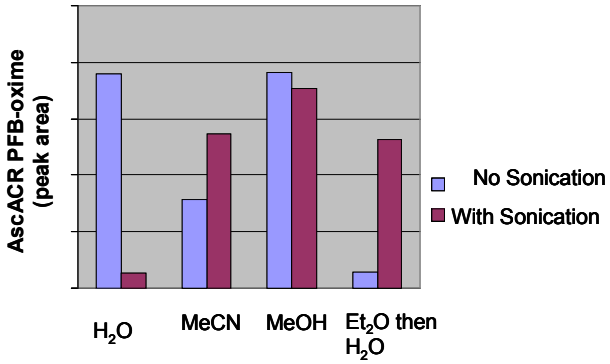


Figure 3.9: Reconstitution of AscACRPFB-oxime after liquid-liquid extraction.

Reconstitution of AscACR PFB-oxime

While PFB derivatization allowed AscACR to be detected in urine *in situ*, it was found that better signal-to-noise ratio was obtained by liquid-liquid extraction, evaporation of the organic layer, and reconstitution of the analyte in an HPLC compatible solvent such as H_2O or MeCN. An error in the experimental methods was detected, however, during attempts to create standard addition curves of derivatized AscACR in urine. There was poor reproducibility: samples spiked with derivatized $^{13}C_6$-AscACR, often showed the correct concentration of derivatized $^{13}C_6$-AscACR or no detectable amount at all.

Through a process of elimination (checking extracting solvent, volume of extracting solvent, duration of vortexing, etc.), it was determined that the solvent used for the reconstitution of the analyte after the evaporation of the organic layer was the source of the experimental error. Four sets of identical urine samples, in duplicate, were spiked with derivatized $^{13}C_6$-AscACR, extracted with EtOAc, and the organic layer evaporated (**Fig 3.9**). The samples were then reconstituted in H_2O, MeCN, MeOH, and a small volume of Et_2O, then H_2O. Water, which had previously been the solvent of choice for reconstitution, showed a large degree of variability after reconstitution, which explained the problem of reproducibility. Acetonitrile showed poor ability to reconstitute the analyte. MeOH was able to fully reconstitute the analyte and with good reproducibility and became the

reconstituting solvent for the standard method. The Et_2O followed by H_2O also had a high degree of irreproducibility. Samples were tested by sonication, but the differences appear trivial in comparison to choice of solvent.

Materials and Methods

AscACR synthesis and labeling experiments- See chapter 4.

AscACR vs. GSACR calculations- Structures were optimized in the gas phase at 25 ºC. Enthalpy and free energy values are in kcal/mol. All of the structures were computed using density functional theory (B3LYP) with the 6-31G(d) basis sets as implemented in Gaussian 03.

AscACR vs. GSACR exchange experiments- 1 μM GSACR was incubated with 10 mM AscH at 37° C under Ar in NH_4HCO_3 (pH 7.4, 25 mM). Aliquots were taken at 0, 1, 2, 3, 4, 5, 24, 48, and 72h and analyzed using an Applied Biosystems MDS Sciex hybrid triple quadrupole/ linear ion trap mass spectrometer (4000 QTrap) equipped with a TurboV electrospray source operating in negative ion mode. The opposite experiment, using 1 μM AscACR in 1 mM GSH, was otherwise identical. More AscH was utilized in the previous experiment due to the tendency for AscH to degrade in solution, even under Ar atmosphere. In both cases, a massive excess of the nucleophile was present relative to the conjugate. Neither sample was monitored for the hydrolysis/decarboxylation product discussed in Chapter 4.

LC method development- Columns tested included a Phenomenex 4μ

HydroRP column (80Å, 250 x 1.00 mm), a Thermo 5µ Betasil Diol column (100Å 150 x 2.1 mm), a Phenomenex Synergi 4µ Max RP column (80Å, 250 x 1 mm), a Phenomenex LUNA hilic 3µ column (150 x 2 mm), a Thermo HyPURITY cyano 5µ column (150 x 1 mm), and a Phenomenex LUNA NH_2 3µ column (150 x 1 mm). Columns were tested with MeCN/H_2O as a mobile phase at a wide variety of concentrations and gradients. The operating LC method for the diol column was a linear gradient of 100% B (95% MeCN/ 5% MeOH) to 95% H_2O (0.1% HCOOH) over ten min, 0.2 mL/min. The standard method for use of the LUNA hilic column was a linear gradient of 95% MeCN (solvent A- H_2O, 0.1% HCOOH) to 5% MeCN over 8 min.

Half-life determination of AscACR in urine- 10 mL of freshly collected urine was spiked with AscACR from a stock solution to a final concentration of 100 µM. 100 µL aliquots were removed and added to 100 µL solutions of pentafluorobenzylhydroxylamine HCl (200 mM) in NaOAc buffer (pH 5.5, 1M). Samples were taken over a time course, every hour for 5 hours, including time zero. Samples were made in duplicate. Analytical HPLC retention was achieved on a 2 µm Phenomenex Hydro-RP column (250 x 1 mm, Alltech) using an isocratic mobile phase of 95% H_2O (0.1% HCOOH) for one min, followed by a linear solvent gradient from 5% to 95 MeCN in H_2O (0.1% HCOOH) over 9 min, followed by isocratic 95% MeCN for 10 min at 0.1 mL/min. MS analysis was performed using SRM with an Applied Biosystems MDS Sciex hybrid triple quadrupole/ linear ion trap mass spectrometer (4000 QTrap) equipped with a

TurboV electrospray source operating in negative ion mode. Transitions measured were *m/z* 426-366, 426-157, and 426-115 *m/z* with a collision energy of -15 eV.

Elemental composition analysis of AscACR PFB-oxime- 22 mg pentafluorobenzylhydroxylamine hydrochloride (TCI America) was added to 130 mL urine. The reaction was stirred for 15 min. then extracted three times with EtOAc (150 mL). The combined organic layers were dried ($MgSO_4$), the solvent removed by rotary evaporation under reduced pressure, and redissolved in 1 mL MeOH. The sample was centrifuged for 10 minutes and the supernatant was removed for MS analysis. High resolution accurate mass data was acquired with a JEOL MS Magnetic Sector Instrument, EI, in negative ion mode.

Reconstitution experiment- A fresh urine sample was divided into eight 500 µL aliquots in 2 mL Eppendorf tubes. Each sample was spiked with $^{13}C_6$-AscACR in H_2O to a final concentration of 1 µM.

Each sample was pre-extracted 3x with EtOAc (750 µL), the organic layers were discarded. Each sample was then derivatized with 500 µL, 10 mM pentafluorobenzylhydroxylamine HCl (TCI America) in NaOAc buffer (1M, pH = 5.5). The samples were vortexed for 0.5 min and allowed to react for 1h. Each sample was extracted with EtOAc (3x 0.750 mL) and the combined organic layer evaporated in a test tube using a Zymark TurboVac evaporator under N_2 at 40° C. Two of each sample were reconstituted in 0.5 mL H_2O, 0.5 mL MeCN, 0.5 mL MeOH, and 0.1 mL Et_2O (vortexed for 1 min) then 0.4 mL H_2O. All samples were vortexed for 0.5 min. One sample of each solvent tested was additionally

sonicated for 10 min. The two samples containing ether were gently heated to remove the ether layer. Samples were analyzed using an Applied Biosystems MDS Sciex hybrid triple quadrupole/ linear ion trap mass spectrometer (3200 QTrap) equipped with a TurboV electrospray source operating in negative ion mode and positive ion mode.

Final standard procedure for analysis of AscACR in biological samples- 500 µL of sample (all volumes scaled down proportionally if less material available) spiked with ~100 nM $^{13}C_6$-labeled AscACR (see Chapter 4) as internal standard. Sample pre-extracted three times with EtOAc (750 µL). The sample is treated with 500 µL, 10 mM pentafluorobenzylhydroxylamine HCl (TCI America) in NaOAc buffer (1M, pH = 5.5). The sample is agitated and allowed to react for 1 h, then extracted three times EtOAc (750 µL). The organic layers are combined and solvent removed by evaporation. The sample is redissolved in 500 µL MeOH, vortexed, and pipeted to an auto-sampler vial for LC-MS analysis. Analytical retention was achieved using a Phenomenex Synergi Hydro-RP column (4µ, 80Å, 250 x 1 mm) in reverse phase employing an isocratic solvent mixture of 5% MeCN (0.1% HCOOH) in H_2O (0.1%) for 1 min., followed by a linear solvent gradient of 5% to 95% MeCN (0.1% HCOOH) in H_2O (0.1% HCOOH) over 9 min, followed by isocratic solvent mixture of 95% MeCN (0.1% HCOOH) in H_2O (0.1% HCOOH) for 5 min. Samples were analyzed using an Applied BiosystemsMDSSciex hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTrap) equipped with a TurboV electrospray source in negative ion mode.

MS experimental details are provided in chapter 4.

Conclusion

At the beginning of this project, the search for AscACR in biological samples was expected to be simple and fast. The project turned out to be plagued with problems, took several years, and at one point was nearly abandoned. After the lengthy development of rigorous methods for the detection of AscACR at low concentrations, despite some circumstantial evidence (e.g. HRMS of AscACR in urine, irreproducible discovery of AscACR in very fresh urine samples) AscACR could not convincingly be found in biological samples. The samples tested included the urine of smokers vs. non-smokers (AscACR as a marker of oxidative stress via lipid peroxidation), the plasma of dogs which had completed a famous endurance race known as the Iditarod (also as a model of oxidative stress), cerebrospinal fluid from Alzheimer's' patients (increased polyamine metabolism and acrolein production), the urine of rats that had been exposed to CCl_4 (an inducer of oxidative stress) or acrolein itself, and various cell studies including cells exposed to acrolein or its precursor the dimethyl acetal. AscACR could not be reproducibly detected in any of these samples.

While conducting a literature review for this dissertation, it was observed in the natural product ascorbigen (see chapter 2) that the ascorbyl-moiety can undergo hydrolysis in alkaline conditions and in serum. This was the first

precedent outside of our own observations with AscACR for such a hydrolysis. In this report, ascorbigen continues to react, undergoing decarboxylation and racemization to the indolyl-deoxyketoses. Realizing that this was a potential route for the degradation of AscACR at physiological pH, the investigation for observing the ascorbylation of acrolein in biological samples was reopened. The results of these new investigations are reported in the following chapter.

Chapter 4: Formation of a Vitamin C Conjugate of Acrolein and its Conversion into 5,6,7,8-tetrahydroxy-4-oxooctanal in THP-1 Cells

Nicholas G. Kesinger, Brandi L. Langsdorf, Cristobal L. Miranda, Jan F. Stevens

Abstract

Vitamin C (ascorbic acid) plays important roles as a biological antioxidant and as a cofactor for a number of enzymes, e.g., proline hydroxylase. Ascorbic acid has also been reported to participate in Michael addition reactions in vitro to form vitamin C conjugates with α,β-unsaturated aldehydes, such as acrolein. This study shows evidence for the formation of the vitamin C conjugate of acrolein (AscACR) in cultured human monocytic THP-1 cells exposed to acrolein diacetate and subsequent metabolic transformation of AscACR. By using ^{18}O and ^{13}C labeling in combination with liquid chromatography–tandem mass spectrometry, AscACR was shown to undergo hydrolytic conversion of the ascorbyl lactone into

an intermediate carboxylic acid. Subsequent decarboxylation of the carboxylic acid yielded 5,6,7,8-tetrahydroxy-4-oxooctanal (THO). When THP-1 cells were pretreated with ascorbic acid (1 mM, 18 hours) and then exposed to acrolein diacetate, THO was detected as its pentafluorobenzyl oxime derivative in the cells and in the medium surrounding the cells. The relative levels of THO were greater in cells than in the medium at all time points measured (3, 6, 12 and 24 hours), suggesting the involvement of enzymes catalyzing the intracellular release of acrolein and/or facilitating the hydrolytic cleavage of the ascorbyl lactone moiety. Treatment of THP-1 cells with both ascorbic acid and acrolein diacetate was required for THO formation.

Introduction

Vitamin C (ascorbic acid, AscH) acts as a cofactor for a number of 2-ketoglutarate dependent dioxygenases, including proline hydroxylase, and as a biological antioxidant (Buettner & Schafer, 2000; Gropper et al., 2009; Higdon & Frei, 2002) and prooxidant (Chen, Espey et al., 2005a; Frei & Lawson, 2008). Less known is the ability of AscH to participate in nucleophilic substitution and in Michael addition reactions. In aqueous solutions at neutral pH, AscH (pK_a = 4.2) is essentially an enolate and capable of forming C–C bonds with electrophiles, for which we here use the term ‘ascorbylation’. More than 30 ascorbylated compounds of natural (Wagner et al., 2009c) and synthetic origin (Fathi et al.,

2000) have been reported. One of these compounds is ascorbylated acrolein (AscACR), the Michael addition product of AscH and acrolein (ACR) (**Fig. 4.1**). This compound was first synthesized by Fodor and co-workers in 1983 (Fodor et al., 1983). The aldehyde group of AscACR may form a hemiacetal with either of the two oxygen atoms at positions 2 or 3, depending on the solvent used. For instance, a 5,5,5-tricyclic spiro compound is formed when AscACR is crystallized from water (Eger, Schmidt et al., 1992; Eger & Schmidt, 1989) (**Fig. 4.1**).

Humans are primarily exposed to ACR through cigarette smoking and cooking with vegetable oils in poorly ventilated kitchens (Shields, Xu et al., 1995). ACR is also produced *in vivo* as a byproduct of protein, polyamine, and glucose metabolism, and lipid peroxidation (Stevens et al., 2008). ACR itself is highly

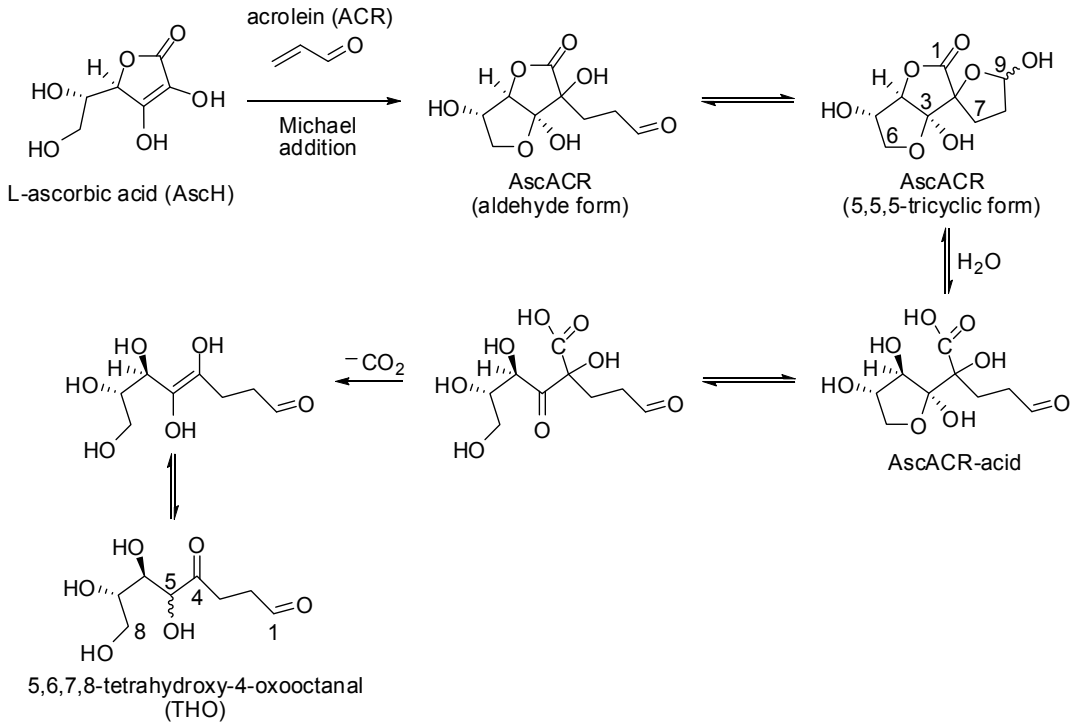


Figure 4.1: Formation of THO from AscH and ACR via AscACR and AscACR-acid. The configuration of carbon atoms 6 and 7 of THO is determined by the configuration of the corresponding carbon atoms in L-AscH. THO is likely to exist as a mixture of 5- and 6-membered cyclic hemiketals and hemiacetals in solution, but not shown here for simplicity.

electrophilic and known to adduct to proteins (Lambert, Li et al., 2007) and DNA (Chung, Young et al., 1984). Exposure to ACR has been associated with the development of lung cancer (Feng, Hu et al., 2006; Shields et al., 1995). Several research groups have reported protective effects of AscH against ACR-induced toxicity. For instance, supplementation of cultured human bronchial epithelial cells with AscH strongly inhibited ACR-induced apoptosis, which the authors attributed to a general antioxidant effect of AscH and to a 'more direct and specific effect' of AscH (Nardini et al., 2002). Arai and co-workers (Arai, Uchida et al., 2005b) demonstrated that AscH suppresses ACR modification of apolipoprotein E

in human very low density lipoprotein (VLDL) *in vitro*. The protective effect of AscH against ACR-induced neuronal damage in spinal cord white matter isolated from guinea pigs was also attributed to the antioxidant effects of AscH (Logan et al., 2005). In these studies, the reaction of AscH with ACR was ignored as a possible detoxification mechanism.

It is well documented that ACR is metabolized by (enzyme-mediated) conjugation with glutathione (GSH) (Josch, Klotz et al., 2003; Lieberman, Barrios et al., 1995a). Major metabolites of ACR found in human urine are hydroxypropyl mercapturic acid and carboxyethyl mercapturic acid (Carmella et al., 2007; Kaye, 1973). However, given the high intracellular concentrations of AscH in humans (≤ 6 mM) (Bergsten et al., 1990) and the ubiquitous presence of ACR *in vivo* (Stevens et al., 2008), we hypothesized that AscACR formation may be biologically significant. Therefore, we investigated the formation of AscACR and its fate in cell culture.

EXPERIMENTAL PROCEDURES

Chemicals

Ascorbic acid (AscH), reduced L-glutathione (GSH), and acrolein (ACR) were purchased from Sigma-Aldrich (St. Louis, MO). Pentafluorobenzyl hydroxylamine hydrochloride and acrylic acid were purchased from TCI America (Portland, OR). L-[1-^{13}C]-AscH and L-[$^{13}C_6$]-AscH were purchased from

Omicron Biochemicals (South Bend, IN). $H_2{}^{18}O$ was obtained from Cambridge Isotope Laboratories (Andover, MA). HPLC-grade MeCN and water were purchased from Honeywell, Burdick and Jackson (Muskegon, MI). Formic acid was from Fluka (Buchs, Switzerland) and K_2CO_3 was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Acrolein diacetate (allylidene diacetate) was purchased from Pfaltz & Bauer (Waterbury, CT).

Liquid Chromatography-tandem mass spectrometry

The HPLC system consisted of two Shimadzu Prominence LC-20AD pumps, a DQU-20A_5 degasser, and a Shimadzu SIL-HTc autosampler equipped with two switching valves (Shimadzu, Kyoto, Japan). Three chromatographic systems were employed. System 1 used a Thermo Betasil diol column (150 × 2.1 mm i.d.; particle size, 5 μm; pore size, 100Å; Thermo Fisher Scientific, Waltham, MA) and a linear solvent gradient from 100% solvent B (MeCN containing 0.1% HCOOH) to 5% B in solvent A (0.1% aqueous HCOOH) over 10 min at 0.2 ml/min. The first 2 min of each LC run was diverted to waste. In system 2, the HPLC column was a Synergi HydroRP C18 column (250 mm × 1 mm i.d.; particle size, 4 μm; pore size, 80Å; Phenomenex, Torrance, CA). The HPLC solvents were the same as in System 1. The column was eluted with 5% solvent B in A during the first minute, followed by a linear solvent gradient from 5% B to 95% B over 9 min and then with 95% B for 5 min. After returning to 5% B in 1 min, the column was equilibrated for 10 min before the next injection. The flow rate was 0.1 ml/min.

The column effluent was directed to the mass spectrometer between 5 and 20 min of the LC run and to a waste container during the remainder of the LC run. In system 3, chromatographic separations were achieved on a Synergi MaxRP C12 column (250 mm × 2 mm i.d.; particle size, 4 μm) (Phenomenex) at a flow rate of 0.2 ml/min. The HPLC solvents were the same as in HPLC system 1. A linear solvent gradient was used, running from 10% B to 40% B in 10 min, 40 to 90 % B over the next 2 min, held constant at 90% B for 7 min, returned to 10 % B over 1 min, and equilibrated at 10% B for 5 min before the next injection.

The LC-MS/MS instrument consisted of a hybrid triple quadrupole/linear ion trap (4000 QTrap) mass spectrometer equipped with a pneumatically assisted electrospray (Turbo V) source operated at 450 °C (Applied biosystems /MDS Sciex, Concord, Ontario, Canada). Liquid nitrogen was used as the source of heating/nebulizing, curtain, and collision gas. The spray needle was kept at -4.5 kV in the negative ion mode. Q1 mass spectra were recorded by scanning in the range *m/z* 100-300 at a cycle time of 1 s with a step size of 0.2 u. MS/MS experiments (product ion scan and selective reaction monitoring, SRM) were conducted at unit resolution for both Q1 and Q3 with collision gas set at "medium" and a collision energy of 17 eV. Peak areas were measured using Analyst 1.4.2 software (Applied Biosystems). The following LC-MS/MS characteristics were used for analysis of cell media and lysates (the first SRM transition was used for quantitative purposes, and subsequent SRMs were used for additional identity confirmation): THO-PFB oxime, t_R 10.4 min (system 2), *m/z* 400→310, *m/z*

400→167, *m/z* 400→112; $^{13}C_5$-labeled THO-PFB oxime, t_R 10.4 min (system 2), *m/z* 405→312, *m/z* 405→167, *m/z* 405→114; AscACR-PFB oxime, t_R 11.8 min (system 2), *m/z* 426→115, *m/z* 426→366, *m/z* 426→157); GSH-ACR, t_R 5.0 min (system 3), *m/z* 362→143, *m/z* 362→306, *m/z* 400→272, *m/z* 362→128, *m/z* 362→179, *m/z* 400→254; GSH-HP (system 3), t_R 5.0 min, *m/z* 364→143, *m/z* 364→128, *m/z* 364→143; GSH-AA, t_R 5.1 min (system 3), *m/z* 378→306, *m/z* 378→143, *m/z* 378→128.

Preparation of AscACR and THO

AscACR— AscACR was synthesized following the method published by Fodor (Fodor et al., 1983). ACR (1 mmol) was added to a solution of AscH (1 mmol) in water (1 ml). After 2 h stirring at room temperature, the solution was placed at 4 °C for 16 h. The resulting precipitate was filtered, washed with cold water, and dried. NMR spectra of the precipitate were in agreement with published data for AscACR (Fodor et al., 1983): ^{1}H NMR (400 MHz, CD_3OD) δ_H 5.6 (m, 1H, H-9), 4.45 and 4.44 (s, 1H, H-4), 4.29-4.25 (m, 1H, H-5), 4.22-4.14 (m, 1H, H-6), 3.88-3.83 (m, 1H, H-6), 2.3-1.8 (m, 4H, H-7 and H-8); ^{13}C NMR (100 MHz, CD_3OD) δ_C 175.6 and 175.5 (C-1), 107.3 and 106.6 (C-3), 100.5 and 100.1 (C-9), 88.1 and 87.8 (C-2), 85.76 and 85.66 (C-4), 75.3 and 75.2 (C-5), 74.2 and 74.1 (C-6), 32.7 and 32.5 (C-8), 30.4 and 29.8 (C-7). Doubling of signals is due to the presence of two anomers (Fig. 1). [$^{13}C_6$]- and [1-^{13}C]-AscACR were

produced by treating 1 mg [$^{13}C_6$]-AscH and [1-^{13}C]-AscH, respectively, with an equimolar amount of ACR in aqueous solution.

Structure Determination of AscACR by X-ray Diffraction Analysis— ACR (5 mmol) was slowly added to a stirred solution of AscH (5 mmol) in water (5 ml). The reaction mixture was stirred for 2 h at room temperature and then placed at 4 °C for 5 days. After this period, a crystalline precipitate was collected and recrystallized from water. The structure of the material was determined to be the 5,5,5-tricyclic form of AscACR (Fig. 1) by X-ray diffraction analysis (see Supplemental Data), in agreement with the structure of AscACR published by Eger and colleagues (Eger, Folkers et al., 1987).

Hydrolysis of AscACR— An aliquot of an aqueous solution of AscACR (10 µl, 100 µM) was added to 200 µl $H_2{}^{18}O$. The solution was immediately analyzed by LC-MS using HPLC system 1. The sample was analyzed at 30 minute intervals over 4 h using Q1 scanning from *m/z* 100 to 300.

Decarboxylation of AscACR Acid— AscACR (0.11 mg) was dissolved in 1 ml of an aqueous solution of K_2CO_3 (0.18 M). After 2 h of incubation at room temperature, the sample containing THO was analyzed by LC-MS using HPLC system 1.

Derivatization of THO and AscACR— A 10 µl aliquot of the above solution containing THO was treated with 1 ml of a 500 mM solution of pentafluorobenzyl hydroxylamine hydrochloride (PFBHA HCl) in NaOAc buffer (pH 5.5, 1 M) for 1 h at room temperature. Similarly, [$^{13}C_5$]-labeled THO was prepared by treating 10

µl of [$^{13}C_6$]-AscACR with aqueous K_2CO_3 (0.18 M; 200 µl) for 2 h. The reaction mixture containing [$^{13}C_5$]-labeled THO was subsequently treated with 1 ml of a 500 mM solution of PFBHA HCl in NaOAc buffer (pH 5.5, 1 M) for 1 h at room temperature. Treatment of sample solutions containing AscACR with PFBHA resulted in the conversion of AscACR into its PFB oxime. PFB oxime derivatives were analyzed by LC-MS using HPLC system 2.

Preparation of GSH Adducts

GSH adducts of ACR (GSH-ACR) and acrylic acid (GSH-AA) were prepared and characterized by LC-MS/MS following the method of Miranda et al. (Miranda, Reed et al., 2009b). Briefly, a solution of GSH (10 mM) was prepared in 0.1 M phosphate buffer (pH 8). To 100 µl aliquots were added 400 µl of the same phosphate buffer and 400 µl of water. These solutions (900 µl) were mixed with 100 µl of a 1 mM solution of ACR or acrylic acid in EtOH, and the reaction mixtures stirred for 2 h at 37 °C and then acidified to pH 3 with 1 N HCl. Work-up of the reaction mixtures utilized Strata-X solid-phase extraction (SPE) columns (60 mg; Phenomenex, Torrance, CA) that were preconditioned with 1.2 ml of MeCN containing 0.1% HCOOH and equilibrated with 1.2 mL of 0.1% aqueous HCOOH. After sample loading and washing with 0.1% aqueous HCOOH (1.2 ml), the SPE column was eluted with MeCN-0.1% aqueous HCOOH (1:1, v/v) to obtain the GSH adducts. Hydroxypropyl-*S*-GSH (GSH-HP) was prepared by

reduction of GSH-ACR adduct with 10 µl of a 5 M sodium borohydride solution in 1 N NaOH. The reaction mixture was stirred for 30 min at room temperature and then acidified to pH 3 with 1 N HCl. The reduction products were isolated by SPE as described above.

Cell culture

Metabolic Transformation of ACR(Ac)$_2$ by THP-1 Cells— THP-1 cells, obtained from the American Type Culture Collection (Manassas, VA), were grown as suspension cultures in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 95% air and 5% CO_2. The cells were pretreated with 1 mM AscH (dissolved in PBS and neutralized with sodium hydroxide) for 18 h in phenol red-free RPMI medium with supplements, and then co-treated with freshly prepared 1 mM AscH and 0.1 mM ACR(Ac)$_2$, dissolved in ethanol. Control cells were incubated with PBS and ethanol (0.1%) in phenol red-free RPMI medium with supplements. No-cell controls consisted of complete RPMI medium containing 1 mM AscH and 0.1 mM ACR(Ac)$_2$. After 3, 6, 12, and 24 h of incubation, the cells were harvested by centrifugation at 500 × *g* for 5 min and the media were collected. No-cell controls were terminated by placing

the treated media on ice and immediately frozen at -80 °C prior to analysis of ACR metabolites. The cell pellet was washed by resuspension in PBS and re-centrifugation at 500 × *g* for 5 min. The pellet was then resuspended in PBS and lysed by sonication. The experiments were conducted in replicates of five.

Derivatization of THP-1 Samples— An aliquot (200 µl) of each cell medium sample was transferred to an HPLC autosampler vial containing 50 µl PFBHA HCl (500 mM) in NaOAc buffer (pH 5.5, 1 M). Cell lysate samples were vortexed and centrifuged. An aliquot of the supernatant (150 µl) was transferred to an HPLC autosampler vial with a glass insert containing 50 µl of a 500 mM solution of PBFHA HCl in NaOAc buffer (pH 5.5, 1 M). After 1 h of incubation at room temperature, the samples were analyzed by LC-MS/MS using HPLC system 2.

LC-MS/MS Analysis of GSH Adducts in Cell Lysates and Media— Cell lysate (50 µl) and medium (400 µl) were mixed with a 2-fold volume of MeCN containing 0.1% HCOOH and centrifuged. The supernatant was analyzed by LC-MS/MS using HPLC system 3.

Calculation of Intracellular Metabolite Levels- Intracellular levels of metabolites were presented as peak areas and calculated using a THP-1 cell volume of 473 μm^3 or 4.73 $\times 10^{-7}$ µl per cell (Loke, Proudfoot et al., 2006). Cells were counted using a hemacytometer.

RESULTS

Hydrolysis of AscACR— When dissolved in water at neutral pH, AscACR produced an LC-MS signal with *m/z* 231($[M-H]^-$) that decreased over a period of hours. A simultaneous increase of a new signal with *m/z* 249 was observed that corresponds to the addition of a water molecule to AscACR (**Fig. 4.2A**). The addition of a water molecule reached equilibrium after approximately 24 h. We hypothesized that the addition of a water molecule was due to hydrolysis of the lactone moiety and not due to hydration of the aldehyde group. To test this hypothesis, AscACR was dissolved in $H_2{}^{18}O$ and the incorporation of ^{18}O was monitored. The aldehyde group of AscACR is in equilibrium with a spiro-hemiacetal moiety (**Fig. 4.1**) and is expected to incorporate one ^{18}O atom

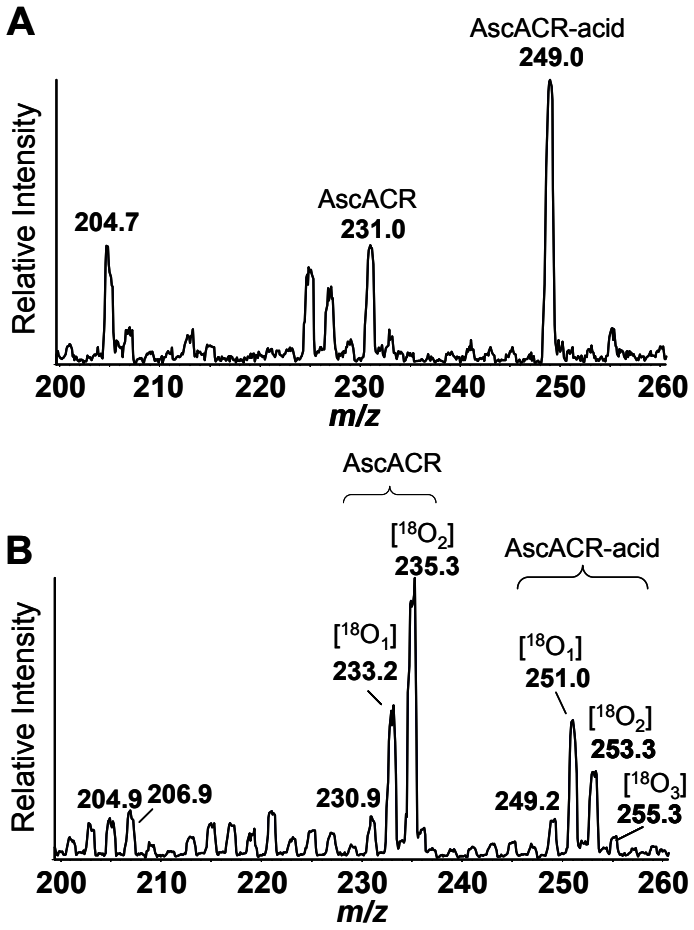


Figure 4.2: Hydrolytic conversion of AscACR into AscACR-acid monitored by LC-MS using HPLC system 1. (A) Q1 Mass spectrum of a co-eluting mixture of AscACR and AscACR-acid obtained by LC-MS of a freshly prepared solution of AscACR in water. (B) Q1 Mass spectrum of a co-eluting mixture of AscACR and AscACR-acid obtained by LC-MS analysis of a 3 h incubation of AscACR in $H_2{}^{18}O$. The ions with *m/z* 205 in panel A and with *m/z* 205 and *m/z* 207 in panel B are due to in-source fragmentation of AscACR-acid.

following $^{16}O/^{18}O$ exchange. Likewise, the oxygen atom at carbon-3 of the hemiketal group is exchangeable. After 3 h of incubation with $H_2{}^{18}O$, AscACR indeed showed incorporation of one or two ^{18}O atoms, giving rise to isotopomers with *m/z* 233 and *m/z* 235 (**Fig. 4.2B**). The molecular species with *m/z* 249 produced three ^{18}O isotopomers upon $^{16}O/^{18}O$ exchange, consistent with hydrolysis

of the lactone moiety in addition to ^{18}O incorporation at the hemiacetal and hemiketal sites.

When the H_2O addition product of AscACR with *m/z* 249 was subjected to collision-induced dissociation (CID), the MS/MS spectrum showed a fragment ion with *m/z* 205 (=249-44), consistent with loss of CO_2. The product's $^{18}O_3$-isotopomer with *m/z* 255 produced a fragment ion with *m/z* 209 (=255-46), consistent with loss of $^{18}OC^{16}O$. To identify the origin of the carbon atom that is lost as carbon dioxide, the exchange experiments were repeated with [1-^{13}C]-AscACR in unlabeled H_2O. Upon CID, the H_2O addition product of [1-^{13}C]-AscACR with *m/z* 250 produced a fragment ion with *m/z* 205 (=250-45), consistent with loss of $^{13}CO_2$ (**Fig. 4.3**).

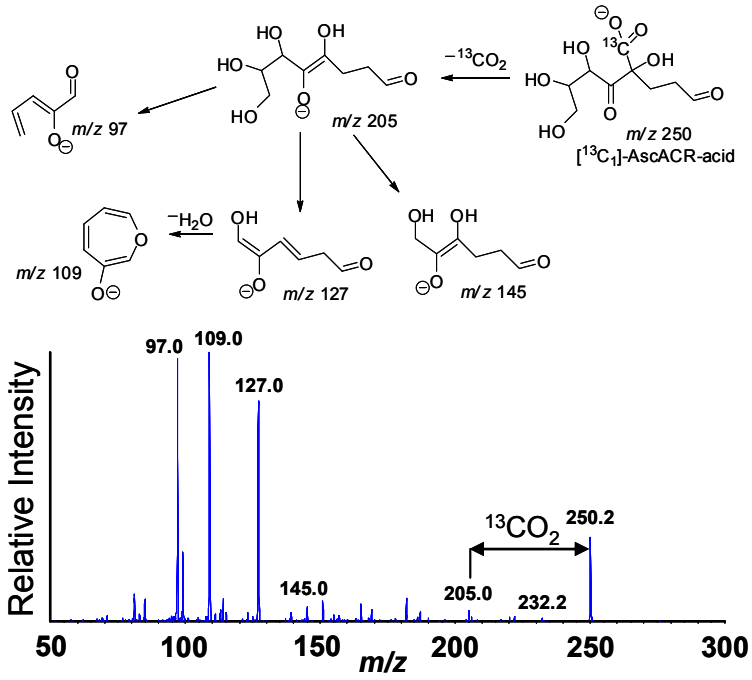


Figure 4.3: Product ion mass spectrum of the *m/z* 250 [M-H]$^-$ ion of [$^{13}C_1$]-AscACR-acid obtained by LC-MS/MS analysis of a solution of [$^{13}C_1$]-AscACR in H_2O.

Formation of AscACR-acid (**Fig. 4.1**) is consistent with the incorporation of three ^{18}O atoms in the $^{16}O/^{18}O$ exchange experiment. Hydration of the aldehyde moiety is not in agreement with the loss of $^{18}OC^{16}O$ from the [$^{18}O_3$]-isotopomer of the H_2O addition product of AscACR, because the hemiacetal and hemiketal oxygen atoms of AscACR were both exchangeable and would also be exchangeable in the H_2O addition product of AscACR, leaving the newly formed COOH group as the only possible third site of ^{18}O incorporation. Taken together, these findings indicate that AscACR undergoes hydrolysis of the lactone to form AscACR-acid.

Decarboxylation of AscACR— When AscACR was dissolved in a solution of K_2CO_3 (0.18 M), the LC-MS signal with *m/z* 231 $[M-H]^-$ rapidly declined and a new chromatographic peak with *m/z* 205 $[M-H]^-$ appeared. The newly formed species with *m/z* 205 (= 231+H_2O-CO_2) did not arise from in-source fragmentation of AscACR-acid because its retention time was different from that of AscACR-acid under the conditions of HPLC system 1. When the incubation experiment was repeated with [$^{13}C_6$]-AscACR, the corresponding new product appeared with a peak at *m/z* 210 (= 237+H_2O-$^{13}CO_2$) in the Q1 mass spectrum. These findings indicate that AscACR undergoes hydrolysis and decarboxylation in alkaline solution to form THO (Fig. 1) and that [$^{13}C_6$]-AscACR forms [$^{13}C_5$]-THO due to hydrolysis and loss of $^{13}CO_2$. The MS/MS spectrum of THO (*m/z* 205 $[M-H]^-$) shows a series of fragment ions that can be rationalized by multiple loss of H_2O neutrals and α-cleavage of enolate products (**Fig. 4.4**).

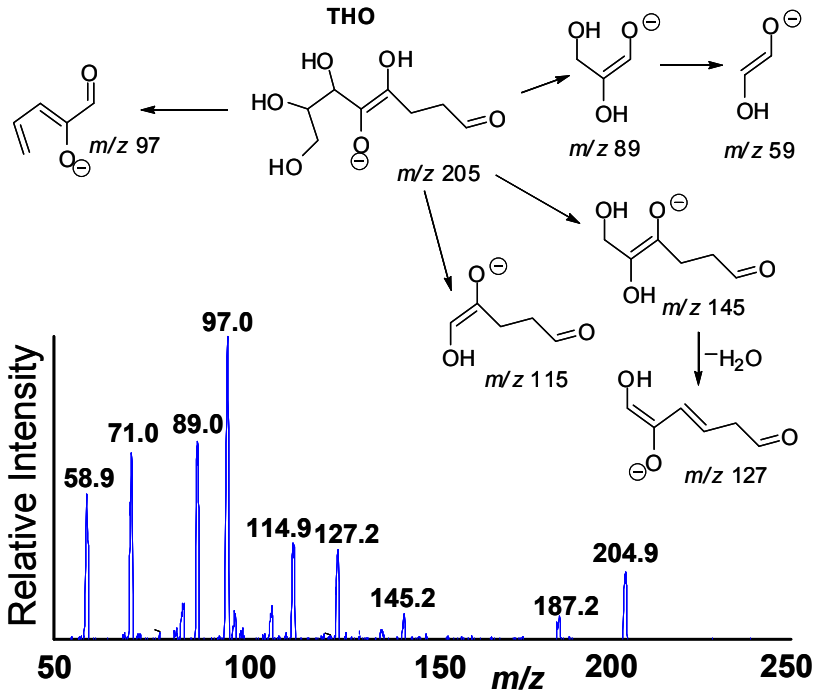


Figure 4.4: Product ion mass spectrum of the *m/z* 205 $[M-H]^-$ ion of THO obtained by LC-MS/MS analysis of a 2 h incubation of AscACR in an aqueous solution of K_2CO_3 (0.18 M).

In order to be able to analyze the hydrophilic THO by LC-MS using reversed-phase LC columns, samples containing THO were treated with pentafluorobenzyl hydroxyl amine (PFBHA) to convert THO into its PFB oxime. THO-PFB oxime was retained on a Synergy HydroRP C18 column (HPLC system 2) and showed a molecular ion with *m/z* 400 in its Q1 mass spectrum. Mass fragmentation of THO-PFB oxime gave rise to fragment ions resulting from cleavage of the oxime to form nitrile species and α-cleavage of enolate products. The odd-mass fragment with *m/z* 167 is readily identified as the pentafluorobenzyl anion (**Fig. 4.5**). In subsequent LC-MS/MS experiments, the prominent fragment ions with *m/z* 310,

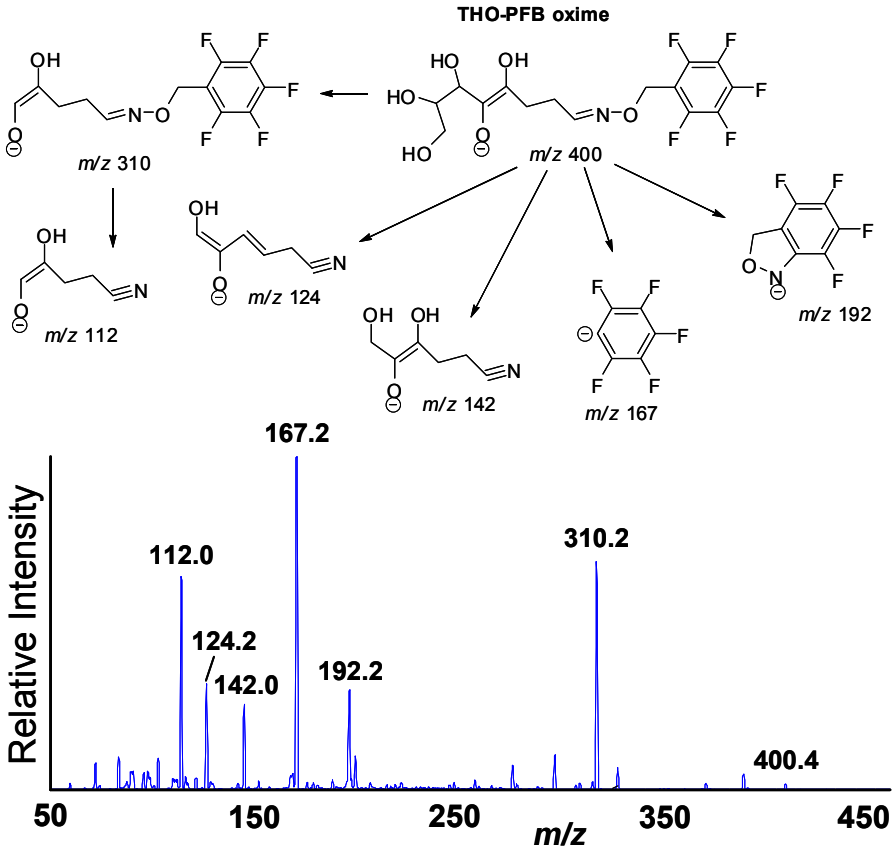


Figure 4.5: Product ion mass spectrum of the *m/z* 400 $[M-H]^-$ ion of the pentafluorobenzyl (PFB) oxime derivative of THO obtained by LC-MS/MS analysis (HPLC system 2) of an aqueous solution containing THO and treated with PFB hydroxylamine for 2 h at room temperature.

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and *m/z* 112 were chosen for selected reaction monitoring (SRM) to detect and semi-quantify THO-PFB oxime in biological samples.

Transformation of ACR(Ac)$_2$ into THO— Human monocytic THP-1 cells were pretreated with 1 mM AscH in the culture medium for 18 h and subsequently exposed to 100 μM ACR(Ac)$_2$ and freshly added AscH (1 mM). At various times following ACR(Ac)$_2$ exposure, culture medium and cell lysates were analyzed by LC-MS/MS using SRM. In the experiment of **Figure 4.6**, the samples were also

spiked with [$^{13}C_5$]-THO, prepared by alkali treatment of [$^{13}C_6$]-AscACR, and then

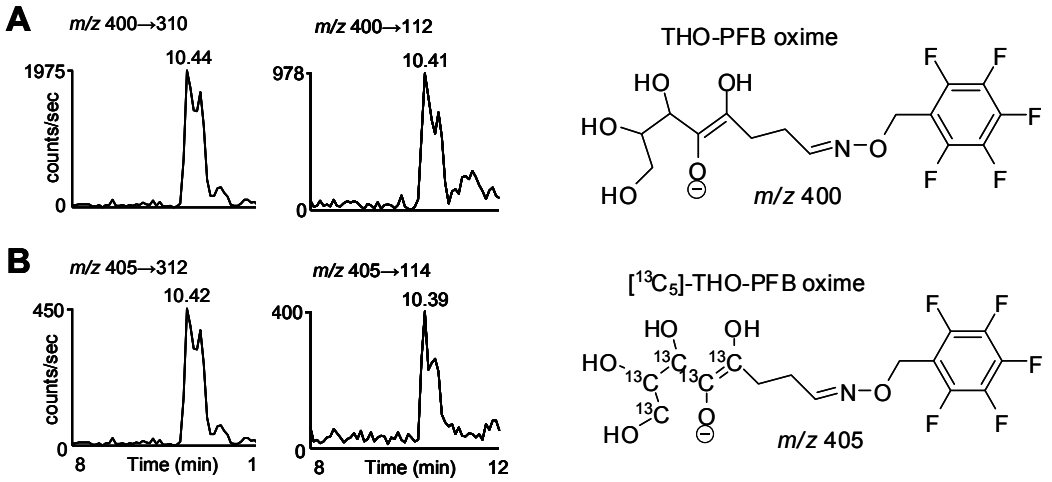

Figure 4.6: LC-MS/MS analysis of culture medium obtained from AscH-adequate THP-1 cells exposed to ACR(Ac)$_2$ for 3 h, spiked with [$^{13}C_5$]-THO, and treated with PFB hydroxylamine. (A) Detection of THO-PFB oxime by selected reaction monitoring (SRM). (B) Simultaneous detection of spiked [$^{13}C_5$]-THO-PFB oxime by using SRM. THO in the medium formed from AscH and ACR is chromatographically indistinguishable from spiked [$^{13}C_5$]-THO.

immediately treated with PBFHA. **Figure 4.6** shows the SRM ion currents for three of the most prominent ion transitions of THO-PFB oxime (**panel A**) and the corresponding SRM ion currents of [$^{13}C_5$]-THO-PFB oxime (**panel B**) in a medium sample. Panels A and B show the same chromatographic peak with a retention time of 10.4 min. We attribute the observed peak splitting to the presence of diastereoisomers (**Fig. 4.1**). The SRM ion currents shown in panel A were not observed for a solution of [$^{13}C_5$]-THO-PFB oxime alone, and neither for culture medium or cell lysate samples prepared from cells that were not pretreated

with AscH or not exposed to $ACR(Ac)_2$. These findings demonstrate that THO is formed from AscH and $ACR(Ac)_2$.

The relative levels of THO were measured by LC-SRM in culture medium and cell lysate samples prepared from AscH-pretreated THP-1 cells following $ACR(Ac)_2$ exposure. In addition, THO formation was measured in FBS-containing and FBS-lacking culture media that were co-incubated with AscH and $ACR(Ac)_2$ in the absence of cells. **Figure 4.7A** shows that the relative concentrations of THO were highest in the cells. In the medium incubations without cells, THO concentrations were higher in the presence of FBS than in the absence of FBS. The THO levels were lowest in the media surrounding the THP-1 cells.

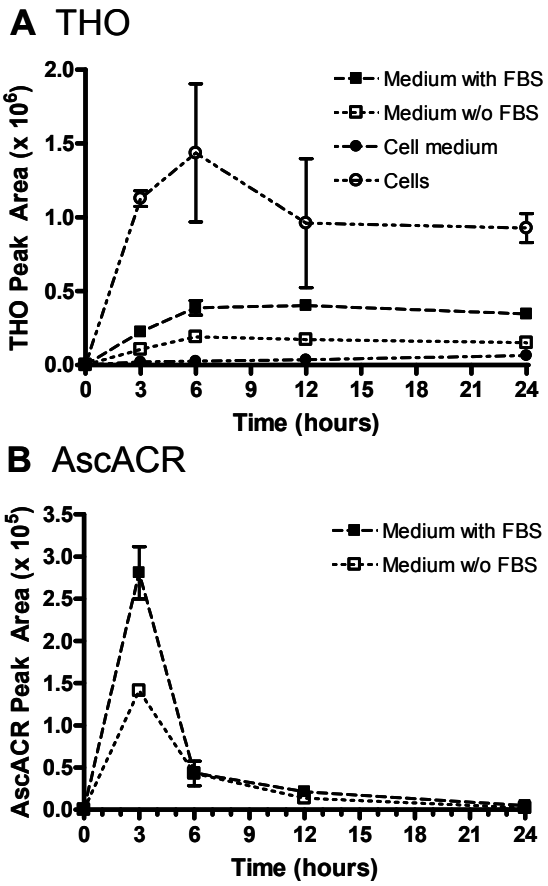

Figure 4.7: Relative concentrations of THO and AscACR following exposure to AscH and $ACR(Ac)_2$. (A) AscH-adequate THP-1 cells and the surrounding media were analyzed at various time points for AscACR and THO by LC-MS/MS using SRM. AscACR was not detected in the presence of THP-1 cells. In the absence of cells, THO (panel A) and AscACR (panel B) were both detected in FBS-containing and FBS-lacking media that were co-incubated with AscH and $ACR(Ac)_2$. Symbols represent means ± SEM of five replicates (n = 5).

Our LC-SRM method allows detection of AscACR as its PFB oxime, but it was not detected in culture medium and cell lysate samples prepared from AscH-adequate THP-1 cells exposed to $ACR(Ac)_2$. We did detect AscACR in FBS-containing and FBS-lacking culture media that were co-incubated with AscH and

$ACR(Ac)_2$ in the absence of cells. The AscACR concentration was maximal at 3 h and significantly higher in the presence of FBS (**Fig. 4.7B**).

GSH Conjugates of ACR in THP-1 Cell Lysates and Media— GSH conjugates were measured in culture medium and cell lysate samples prepared from THP-1 cells exposed to $ACR(Ac)_2$, either with or without AscH pre-incubation. GSH-ACR, GS-AA, and GSH-HP were all found to be produced following exposure of cells to $ACR(Ac)_2$. Intracellular levels of these GSH metabolites were maximal at 3 h and subsequently declined over a period 24 h. GSH-HP (**Fig. 4.8A**) was found to be the major metabolite in both the cell lysates (**Fig. 4.8B**) and the media (Fig. 8C), with a much higher concentration found in the cells relative to the media. GSH conjugates were not detected in culture medium exposed to $ACR(Ac)_2$ in the absence of cells.

DISCUSSION

With the aim to demonstrate ascorbylation of ACR in a biological environment, THP-1 cells were selected for the incubation experiments because THP-1 cells accumulate AscH at concentrations of up to 9 mM and are capable of forming GSH conjugates of 2-alkenals and their metabolites (Miranda, Reed et al., 2009c) via enzyme-mediated pathways that will compete with AscACR formation. We used $ACR(Ac)_2$ as a 'pro-drug' form of ACR to minimize adduct formation of ACR with components of the cell culture medium and to maximize intracellular

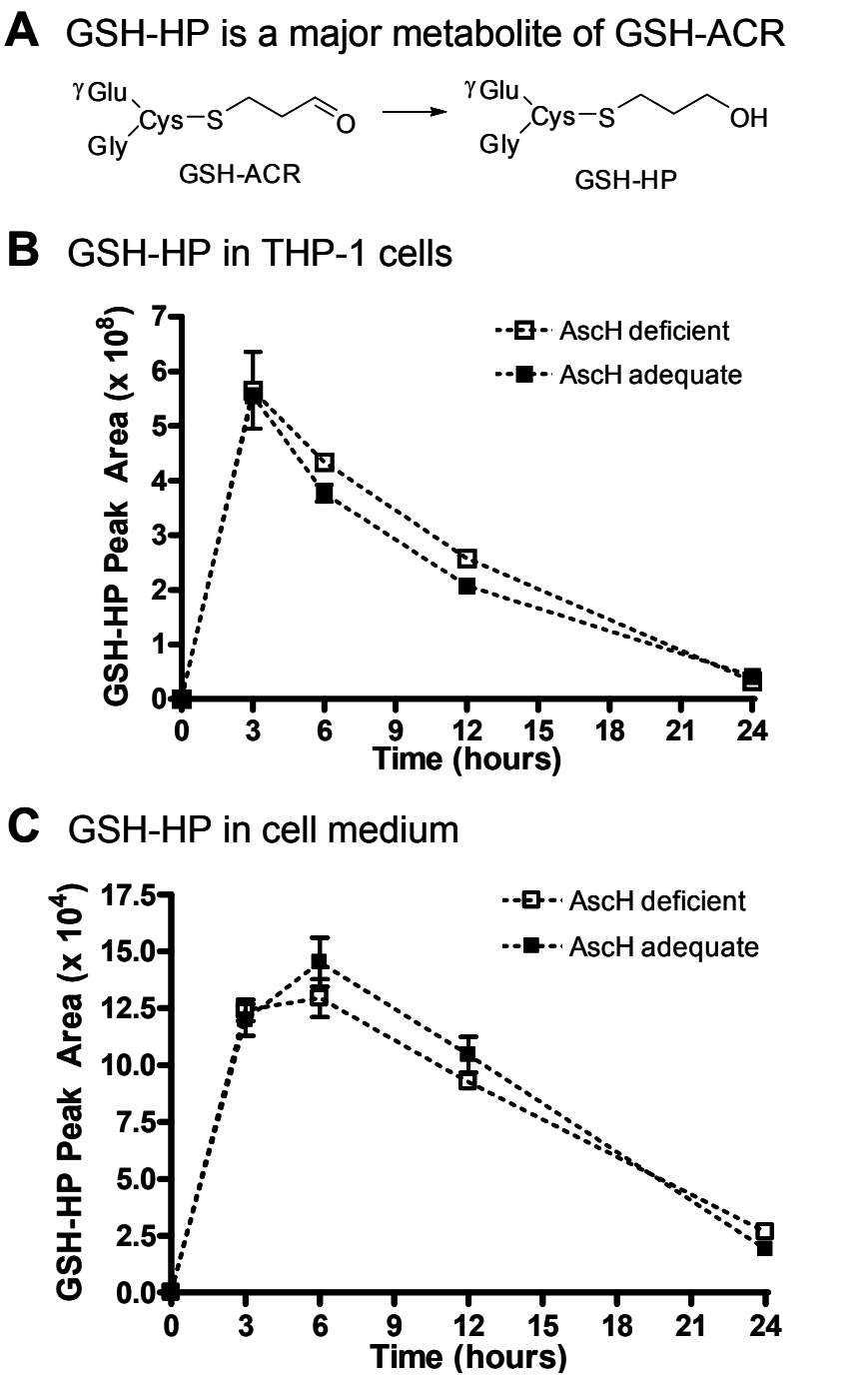

Figure 4.8: Relative concentrations of GSH-HP in THP-1 cells (panel B) and surrounding media (panel C) following exposure to $ACR(Ac)_2$, in the presence and absence of AscH. GSH-HP was measured by LC-MS/MS using SRM and HPLC system 3. Symbols represent means ± SEM of five replicates ($n = 5$).

concentrations of ACR by enzyme-mediated release of ACR from $ACR(Ac)_2$. Exposure of AscH-adequate THP-1 cells to $ACR(Ac)_2$ did not yield detectable concentrations of AscACR in the medium or cell lysates (**Fig. 4.7**). These findings suggested that 1) AscACR is not formed due to competing pathways such as GST-mediated GSH conjugation and subsequent metabolism of the GSH conjugates, or 2) AscACR is formed but not detectable due to degradation or metabolism. The second hypothesis was tested by investigating the fate of AscACR in neutral and in alkaline solution. By using ^{18}O- and ^{13}C-labeling of AscACR, we found that AscACR undergoes hydrolysis of the lactone moiety at neutral pH to form AscACR-acid (**Fig. 4.2**). The carboxyl group of AscACR-acid spontaneously dissociates from the ascorbyl moiety in the collision cell of the mass spectrometer (**Fig. 4.3**) and in alkaline solution to form THO (**Fig. 4.4**). THO was detected in the medium and lysate prepared from AscH-adequate THP-1 cells exposed to $ACR(Ac)_2$ (**Fig. 4.6**).

The most well-known and studied ascorbylated product is ascorbigen. Ascorbigen is formed during the degradation of the indole glucosinolate, glucobrassicin, in the presence of AscH and is found in many species of Brassicaceae (Kutacek, Prochazka et al., 1960; Wagner & Rimbach, 2009b). Studies by the group of Preobrazhenskaya (Preobrazhenskaya et al., 1996) have demonstrated that ascorbigen undergoes hydrolysis of the lactone moiety and subsequent decarboxylation to form a mixture of 1-indolyl-1-deoxytagatose and 1-indolyl-1-deoxysorbose. Both 1-indolyl-1-deoxyketohexoses were found in

bovine serum exposed *ex vivo* to ascorbigen and in urine of mice after i.p. administration of ascorbigen (Reznikova, Korolev et al., 2000a). The chemical transformation reported for ascorbigen in bovine serum is essentially identical to the transformation of AscACR into THO in cell culture medium (**Fig. 4.7**). The observed splitting of the chromatographic peak that represents THO (**Fig. 4.6**) may be due to the formation of cyclic hemiketal isomers, analogous to the degradation of ascorbigen in bovine serum (Reznikova et al., 2000a).

AscACR was detected in culture medium that was co-incubated with AscH and $ACR(Ac)_2$ (**Fig. 4.7B**), but not in culture medium when THP-1 cells were present. Furthermore, the maximum concentration of AscACR was higher when the culture medium contained FBS. These findings indicate that $ACR(Ac)_2$ hydrolyzes spontaneously in culture medium and that the hydrolysis is facilitated by components of FBS, presumably by enzymes with esterase activity. In the presence of cells, levels of THO in the media surrounding the cells were much lower than the intracellular concentrations of THO (**Fig. 4.7**). This suggests that AscACR is either formed in the medium but taken up by cells and subsequently metabolized intracellularly or that AscACR is formed intracellularly and metabolized intracellularly. The relatively high intracellular levels of THO and the cell-dependent disappearance of AscACR suggests that THP-1 cells produces a biocatalyst capable of converting AscACR into THO via AscACR-acid.

The detection of the major GSH metabolite, GSH-HP, and the minor metabolites, GSH-ACR and GSH-AA, in culture media and cell lysates (**Fig. 4.8**)

shows that THP-1 cells are capable of GSH conjugation of ACR and phase 1 metabolism of GSH-ACR. The intracellular concentration of GSH-HP was highest at 3 h post exposure to $ACR(Ac)_2$ and then gradually decreased over time, most likely due to excretion of GSH-HP into the medium. Cellular excretion is reflected by a delay in reaching maximum medium levels of GSH-HP at 6 h. The progressive decrease of GSH-HP in the medium is best explained by extracellular conversion of GSH-HP into Gly-Cys-HP by γ-glutamyltransferase, a membrane protein with its catalytic site faced extracellularly (Lieberman, Barrios et al., 1995b). At 6 and 12 h post exposure to $ACR(Ac)_2$, the intracellular levels of GSH-HP were slightly but significantly lower in the AscH-adequate cells compared to the Asc-deficient cells (p = 0.013 and 0.0042, resp.). This observation can be explained by the effect of AscH on preserving the export activity of the ATP-dependent multi-drug resistance-associated protein (MRP) and on maintaining ATP levels following electrophile stress in THP-1 cells (Miranda et al., 2009c). The alternative explanation for the lower cellular levels of GSH-HP in AscH-adequate cells is that AscACR formation competes with GSH conjugation of ACR. Although we are unable to distinguish between the relative contributions of the two pathways to the fate of ACR without absolute quantification of all possible metabolites of ACR, our results demonstrate that the two pathways occur concurrently.

In conclusion, we have shown that ascorbylation of ACR and subsequent transformation of AscACR into THO is a pathway for elimination of ACR that co-exists with GSH conjugation of ACR in THP-1 cells. Although AscACR formation may proceed readily without enzymes, our findings suggest that the conversion of AscACR into THO via AscACR-acid is mediated by a biocatalyst present in THP-1 cells. Ascorbylation of 2-alkenals and other electrophiles could provide a supporting detoxification pathway, especially in situations where GSH synthesis or GST activity are compromised.

Chapter 5: Linoleic Acid versus Glucose in the Formation of Acrolein

Introduction

In 2004, Medina-Navarro and co-workers reported on the *in vitro* formation of acrolein from linoleic acid and arachidonic acid (Medina-Navarro, Duran-Reyes et al., 2004). While lipids are a known source of acrolein, the glycerol moiety of triglycerides is also a logical source of acrolein (Redtenbacher, 1843; Tsukuda, Sato et al., 2007) (**Fig. 5.1**). When rape seed oil and methyl esters derived from rape seed oil are pyrolyzed, far more acrolein is produced when triglycerides are present in the rape seed oil (Pedersen, Ingemarsson et al., 1999). The formation of acrolein from free polyunsaturated fatty acids (PUFAs) requires a more complicated (Esterbauer et al., 1991) and contentious mechanism (Spiteller, Kern et al., 2001). The Medina-Navarro et al. work not only measured the formation of acrolein by GC-MS, but noticed increased formation of acrolein corresponding to the concentration of glucose, suggesting a pro-oxidant effect of glucose.

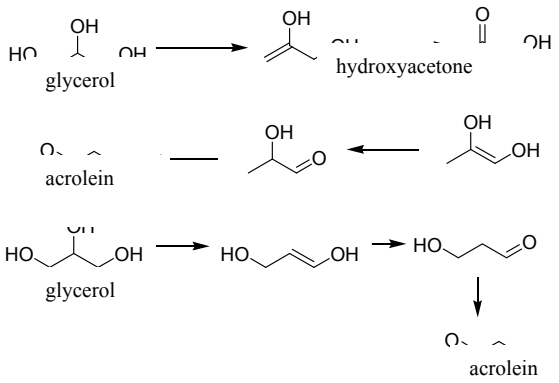

Figure 5.1: Probable mechanisms for formation of acrolein from glycerol, including hydroxyacetone as intermediate.

Medina-Navarro *et al.* neglected to consider glucose as a source of acrolein. There are several proposed mechanisms for the formation of acrolein from glucose (Yaylayan & Keyhani, 2000a) via hydroxyacetone (**Fig 5.2**), and acrolein has been detected during the pyrolysis of glucose. If the acrolein in the *in vitro* experiment is

derived from glucose, and the increased acrolein is due to increased glucose concentration rather than a pro-oxidant effect, then a simple isotopic-labeling experiment would distinguish between lipid-derived acrolein and glucose-derived

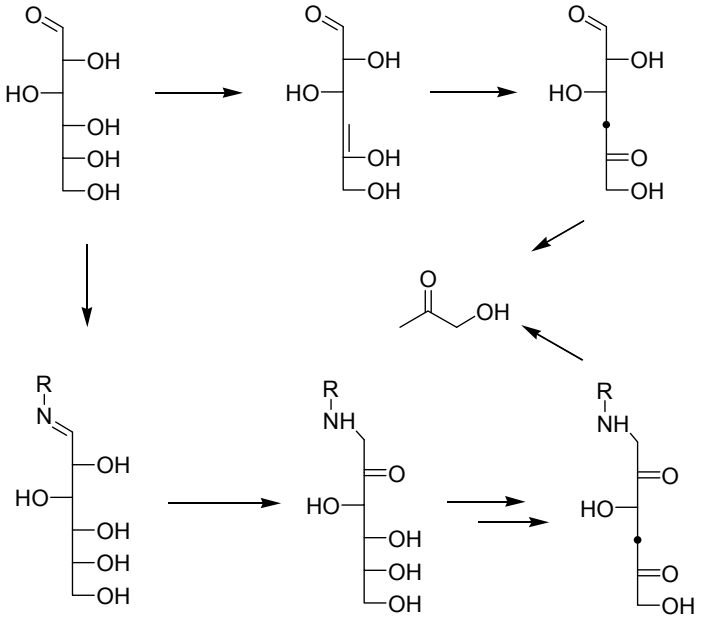

Figure 5.2: Formation of hydroxyacetone from glucose, directly or via Schiff base formation and Amadori product.

acrolein.

Methods were developed to analyze the methyl oxime derivative of labeled and unlabeled acrolein (ACR-Me oxime and $^{13}C_3$-ACR-Me oxime) by LC-MS. Also developed were methods for the detection of AscACR, 4-hydroxy-2-nonenal (HNE) and its ascorbyl derivative (AscHNE). A series of incubation experiments were designed involving the incubation of linoleic acid with universally labeled glucose. If labeled acrolein (as the $^{13}C_3$-ACR-Me oxime) could be detected, this would mean

glucose is a source of acrolein. HNE, being a lipid peroxidation product, was also measured. In a separate experiment, the incubations were conducted in the presence of AscH and samples were monitored for the formation of AscACR and AscHNE (as the methyl oximes) to determine the effect of AscH in the formation of acrolein from either linoleic acid or glucose.

Experimental Procedures

Chemicals

Ascorbic acid (AscH) and acrolein (ACR) were purchased from Sigma-Aldrich (St. Louis, MO). *O*-Methyl hydroxylamine hydrochloride, linoleic acid, and acrolein were purchased from TCI America (Portland, OR). $^{13}C_6$-glucose was purchased from Omicron Biochemicals (South Bend, IN). HPLC-grade MeCN and water were purchased from Honeywell Burdick and Jackson (Muskegon, MI). Formic acid was from Fluka (Buchs, Switzerland).

Liquid Chromatography-tandem mass spectrometry

The HPLC system consisted of two Shimadzu Prominence LC-20AD pumps, a DQU-20A_5 degasser, and a Shimadzu SIL-HTc autosampler equipped with two switching valves (Shimadzu, Kyoto, Japan). The HPLC system consisted of an HPLC column was a Synergi HydroRP C18 column (250 mm × 1 mm i.d.; particle size, 4 μm; pore size, 80Å; Phenomenex, Torrance, CA), and a linear solvent gradient from 100% solvent B (MeCN) to 5% B in solvent A (0.1% aqueous HCOOH) over 10 min at 0.2 ml/min. The column was eluted with 5% solvent B in A during the first minute, followed by a linear solvent gradient from 5% B to 95% B over 9 min and

then with 95% B for 5 min. After returning to 5% B in 1 min, the column was equilibrated for 10 min before the next injection. The flow rate was 0.1 ml/min. The column effluent was directed to the mass spectrometer between 5 and 20 min of the LC run and to a waste container during the remainder of the LC run.

The LC-MS/MS instrument consisted of a hybrid triple quadrupole/linear ion trap (3200 QTrap) mass spectrometer equipped with a pneumatically assisted electrospray (Turbo V) source operated at 450 °C (Applied biosystems /MDS Sciex, Concord, Ontario, Canada). Liquid nitrogen was used as the source of heating/nebulizing, curtain, and collision gas. The spray needle was kept at -4.5 kV in the negative ion mode. MS/MS experiments (product ion scan and selective reaction monitoring, SRM) were conducted at unit resolution for both Q1 and Q3 with collision gas set at "medium" and a collision energy of 25 eV. Peak areas were measured using Analyst 1.4.2 software (Applied Biosystems). The following LC-MS/MS characteristics were used for analysis of cell media and lysates (the first SRM transition was used for quantitative purposes, and subsequent SRMs were used for additional identity confirmation): ACR-Me oxime, t_R 10.9 min, *m/z* 86→55, *m/z* 86→43; $^{13}C_3$-labeled ACR-Me oxime, t_R 10.9 min, *m/z* 89→58, *m/z* 89→43; AscACR-Me oxime, t_R 9.4 min, *m/z* 262→230, *m/z* 262→215, *m/z* 262→150); AscHNE-Me oxime, t_R 11.7 min, *m/z* 362→177, *m/z* 362→117; HNE-Me oxime, t_R 10.8, 11.3 min, *m/z* 188→170, *m/z* 188-157.

Preparation of Methyl Oxime Derivatives

Standards were prepared by the separate addition of acrolein, AscACR (see chapter 4), HNE, and AscHNE (Miranda et al., 2009b) to 1 ml of a 200 mM solution of $MeONH_3Cl$ in NaOAc buffer (pH 5.5, 500 mM) for 16 h at room temperature (final concentration of each aldehyde being 100 μM). A standard calibration curve (in triplicate) of ACR-Me oxime was made at 10 nM, 100 nM, 1 μM, and 10 μM via serial dilution.

Linoleic acid and $^{13}C_6$-Glucose Incubation Experiments

Acrolein formation from linoleic acid with AAPH in the absence of glucose- Linoleic acid was added to 0.3 mL 3:1 phosphate buffer: MeCN in a series of seven 2 mL Eppendorf tubes. AAPH in 0.1 mL in phosphate buffer and additional phosphate buffer was added to bring the solution to a final volume of 0.5 mL, 10 mM AAPH, and 2.5 mM linoleic acid. Each tube was vortexed for 1 minute and heated in a water bath to 40° C.

Each tube was derivatized over a time course, staring at the initial time, and at every hour for six hours. The derivatization effectively halts the formation of subsequent acrolein, either by cooling the reaction mixture below 40° C or derivatizing reaction intermediates to stable species. The derivatization was performed by the addition of 400 mM *O*-methyl hydroxylamine in phosphate buffer (0.5 mL). The reaction mixture was pipeted to an injection vial for later LC-MS analysis.

Acrolein formation from linoleic acid with AAPH in the presence of glucose- Linoleic acid was added to 0.3 mL 3:1 phosphate buffer:MeCN in a series of seven 2

mL Eppendorf tubes. AAPH in 0.1 mL H_2O and $^{13}C_6$-labeled glucose (Sigma-Aldrich) in phosphate buffer was added to bring the solution to a final volume of 0.5 mL, 10 mM AAPH, 20 mM labeled glucose, and 2.5 mM linoleic acid. Each tube was vortexed for 1 minute and heated in a water bath to 40° C.

Each tube was derivatized over a time course, staring at the initial time, and at every hour for six hours. The derivatization was performed by the addition of 400 mM *O*-methyl hydroxylamine in phosphate buffer (0.5 mL). The reaction mixture was pipeted to an injection vial for later LC-MS analysis.

Acrolein formation from linoleic acid with ascorbic acid in the presence of glucose- Linoleic acid was added to 0.3 mL 3:1 phosphate buffer:MeCN in a series of seven 2 mL Eppendorf tubes. AscH (Sigma-Aldrich) in 0.1 mL phosphate buffer and $^{13}C_6$-labeled glucose (Sigma-Aldrich) in phosphate buffer was added to bring the solution to a final volume of 0.5 mL, 100 mM AscH, 20 mM labeled glucose, and 2.5 mM linoleic acid. Each tube was vortexed for 1 minute and heated in a water bath to 40° C. The reaction mixture was transferred via Pasteur pipette to injection sample vials.

Acrolein formation from linoleic acid in the presence of glucose- Linoleic acid was added to 0.3 mL 3:1 phosphate buffer:MeCN in a series of seven 2 mL Eppendorf tubes. $^{13}C_6$-labeled glucose (Sigma-Aldrich) in phosphate buffer was added to bring the solution to a final volume of 0.5 mL, 10 mM AAPH, 20 mM labeled glucose, and 2.5 mM linoleic acid. Each tube was vortexed for 1 minute and heated in a water bath to 40° C.

Each tube was derivatized over a time course, staring at the initial time, and at every hour for six hours. The derivatization was performed by the addition of 400 mM *O*-methyl hydroxylamine in phosphate buffer (0.5 mL). The reaction mixture was pipeted to an injection vial for later LC-MS analysis.

Formation of $^{13}C_3$-acrolein from $^{13}C_6$-glucose- A 500 mM solution of $^{13}C_6$-glucose in H_2O (1 mL) was placed in a round bottom flask equipped with a short path distillation head and receiving flask containing 1 mL of 400 mM *O*-methyl hydroxylamine in phosphate buffer. The distillation flask was heated by Bunsen burner under argon until all H_2O was evaporated. The flask continued to be heated until the remaining residue turned brown, then black. The flask was allowed to cool and an additional 1 mL of the derivatizing reagent was added to the flask. This was combined with the solution from the receiving flask and transferred to an autosampler vial for LC-MS analysis.

Results

Initial conditions for the reaction based on those given by Medina-Navarro gave no detectable acrolein, labeled or unlabeled, at any time point. Essentially, we were unable to reproduce the formation of acrolein from linoleic acid, based on those conditions. To force the reaction, 2,2-azobis(2-amidopropane) (AAPH), a water soluble radical initiator, was added to the reaction mixture. AAPH requires gentle heating (~40° C) to initiate radical formation. Under these conditions, formation of acrolein was observed.

All acrolein formed under these reaction conditions was entirely unlabeled. The acrolein formed only via lipid peroxidation, disproving our hypothesis that the observations made by Medina-Navarro et al. were due to acrolein formation from

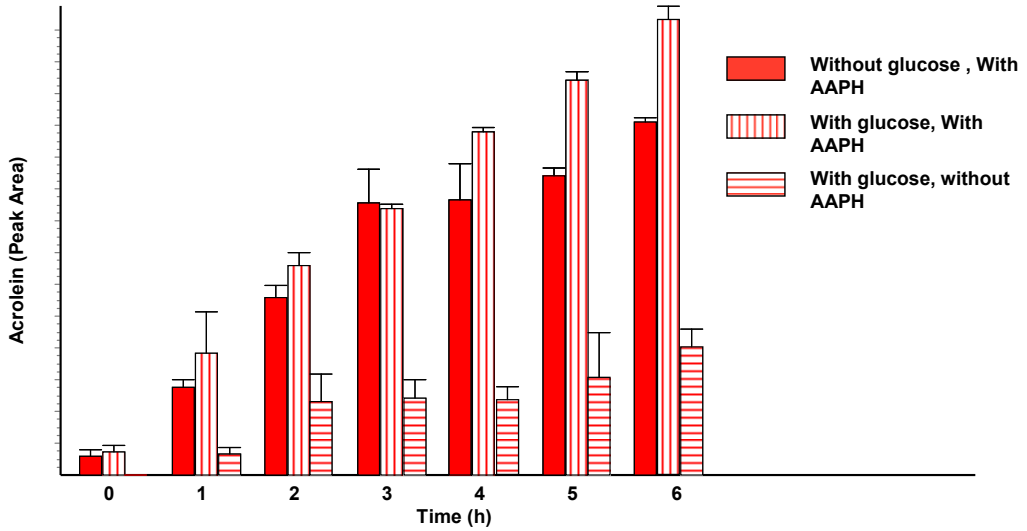

Figure 5.3: Formation of unlabeled acrolein from linoleic acid, n = 3.

glucose. However, the presence of glucose did increase the formation of acrolein from linoleic acid (**Fig 5.3**). Assuming zero-order kinetics in the formation of acrolein, linear regression of the data sets representing acrolein formation with and without glucose and with AAPH shows a significant difference in slope ($F = 8.76429$, $P = 0.0068$, $DFn = 1$, $DFd = 24$). Glucose and linoleic acid in the absence of AAPH but heated under the same conditions also produced acrolein. .

To validate the LC-SRM method for the measurement of labeled glucose, the formation of labeled acrolein was forced. Heating of an aqueous labeled glucose solution at 115º C in a sealed tube produced some decomposition of glucose, based on apparent caramelization of the reaction mixture, however no labeled acrolein was

detected after derivatization and LC-SRM analysis. However, burning of the labeled glucose was performed and after immediate derivitization, a trace amount of labeled acrolein was detected by LC-SRM with the correct transitions. Addition of roughly equimolar unlabeled acrolein standard to the labeled acrolein sample, gave peaks for both analytes with identical chromatographic characteristics.

Discussion

A new method has been developed to measure acrolein by LC-MS. The results of the Medina-Navarro experiments have been repeated using this method. The presence of glucose does increase the formation of acrolein via the lipid peroxidation of linoleic acid. Under these conditions, glucose contributes nothing to the formation of acrolein via direct degradation of glucose itself.

Chapter 6: Ascorbyl-Xanthohumol- Ascorbate as a Prodrug Moiety

Introduction

Xanthohumol is a prenylated chalcone found exclusively in hops, *Humulus lupulus,* the bittering agent used in beer production (**Fig. 6.1**). Xanthohumol is the principal prenylated flavonoid found in hops, although a number of derivatives of xanthohumol, such as isoxanthohumol and the naringenins are present in a significantly smaller amount. The principal source of human exposure to xanthohumol is from beer consumption, although it has been sold and marketed as a nutraceutical (Stevens & Page, 2004).

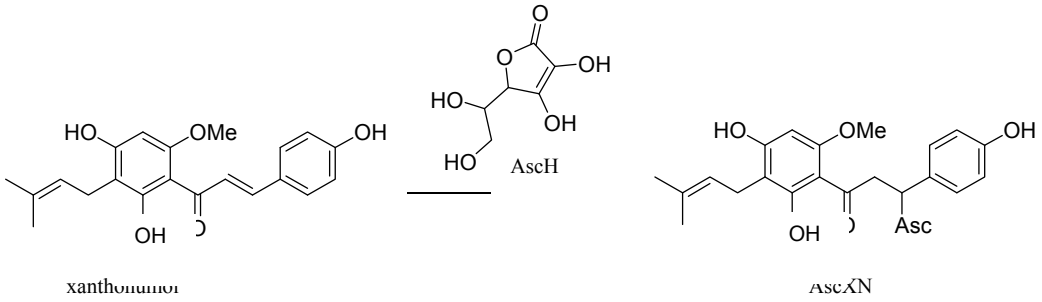

Figure 6.1: Xanthohumol and the synthesis of ascorbyl-xanthohumol.

The bioactivity of xanthohumol has received considerable interest, particularly as a cancer chemopreventative. Xanthohumol inhibits activation of the procarcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline, probably by inhibition of cytochrome P450s (Miranda, Yang et al., 2000). Xanthohumol is known to be a strong inhibitor of Cyp1A1, Cyp1A2, and Cyp1B1 (Henderson, Miranda et al., 2000), and can also inhibit NFκB activation in prostate cells (Colgate, Miranda et al., 2007). Simultaneously, xanthohumol can induce the phase II enzyme NADPH-quinone reductase and, furthermore, xanthohumol has antiproliferative and cytotoxic effects

among a number of cancer cell lines (Miranda, Stevens et al., 1999). Thus, xanthohumol can inhibit cancer-inducing phase I enzymes, induce carcinogen-metabolizing phase II enzymes, and inhibit tumor growth. In addition to these cancer chemopreventative effects, xanthohumol also has anti-inflammatory activity by inhibition of COX enzymes (Gerhauser, Alt et al., 2002) and exhibits strong anti-oxidant effects (Gerhauser et al., 2002; Miranda, Stevens et al., 2000).

Unfortunately, xanthohumol has very poor bioavailability. Only a fractional percentage of xanthohumol and its urinary metabolites were recovered from the urine of rats during a feeding study (Yilmazer, 2001). Unpublished results from our own laboratory have measured only 0.9% transport of xanthohumol across a Caco-2 cell monolayer, a common assay of intestinal absorption. This poor bioavailability of xanthohumol is due to its low solubility in aqueous solutions. It is also possible that xanthohumol can adduct to nucleophilic residues of membrane proteins, preventing transport.

Given the interesting pharmacological properties of xanthohumol and the poor bioavailability we have attempted to make prodrugs of xanthohumol. This led us to the hypothesis that an ascorbyl group might be useful as a prodrug moiety (**Fig. 6.1**). Ascorbylation drastically increases aqueous solubility of target molecules, it removes electrophilicity of the conjugated carbonyl, and in some cases it can be cleaved via retro-Michael (see ascorbigen in Chapter 2, or AscH/GSH exchange of acrolein in Chapter 3).

Experimental Procedures

Ascorbyl-xanthohumol

11 mg xanthohumol (0.032 mmol) was dissolved in 2 mL MeOH with 100 mg ascorbic acid (0.568 mmol) in a sealed tube. The tube was carefully flushed and sparged with Ar, then heated to 80° C for 16h while stirring. The reaction mixture was cooled, and AscXN purified from the reaction mixture by semi-preparatory HPLC. Separation was achieved using a Waters HPLC equipped with a Waters 996 photodiode array detector. Semi-preparative HPLC retention was achieved on a Phenomenex Ultremex 5 C_{18} column (250 x 10 mm) using a linear solvent gradient from 0% to9 5% MeCN (0.1% HCOOH) in H_2O (0.1% HCOOH) over 10 min at 0.6 mL/min. UV (MeOH) λ_{max} (log ε) 216 (2.54), 290 (1.60) nm. Fractions were combined and lyophilized to produce a white crystalline substance, 1.9 mg (10% yield).

Xanthohumol-succinate

40 mg NaH (1 mmol, 60% in mineral oil) was stirred in dry 5 mL THF for 5 min at 0° C. 30 mg xanthohumol (0.084 mmol) was added and stirred for 10 min., followed by the addition of succinic anhydride (29 mg, 3.5 eq.). The reaction was allowed to warm to r.t. and reaction appeared complete by TLC after 6h. The reaction was quenched with the addition 10 mL NH_4Cl sat. aq. The reaction was extracted with EtOAc (3x 50 mL), dried, and solvent removed by rotary evaporation. The material was redissolved in MeCN and the product purified by semi-preparative HPLC. Separation was achieved using a Waters HPLC equipped with a Waters 996

photodiode array detector. Semi-preparatory HPLC retention was achieved on a Phenomenex Ultremex 5 C_{18} column (250 x 10 mm) using a linear solvent gradient from 0% to9 5% MeCN (0.1% HCOOH) in H_2O (0.1% HCOOH) over 10 min at 0.6 mL/min.

8-(3"-methyl-3"-hydroxybutyl)-4',6-dihydroxy-5'-methoxy-flavanone **and** ***2",2"-dimethyl-3",4"-dihydro-(2H)-pyrano[2",3":3',4']2',4-dihydroxy-6'-methoxychalcone*** (**Figure 6.4 C** and **A**, respectively)-

To a solution of 12 mg xanthohumol (0.034 mmol) stirring in THF (2 mL), 1.5 eq. *p*-toluenesulfonic acid (Sigma-Aldrich) was added (0.050 mmol). The reaction mixture quickly turned a bright red color. After 4h, the reaction was quenched by the addition of 10 mL $NaHCO_3$ (sat. aq.). The reaction mixture was extracted with EtOAc (3x 20 mL), dried ($MgSO_4$), and solvent removed by rotary evaporation. The crude mixture was purified by flash chromatography (100% hexane to 80% EtOAc/hexane) to yield **Figure 4.6A** as a red crystalline solid, 6 mg, 50% yield. ^{1}H NMR, 400 MHz, δ 7.86 1H d (15.5 Hz), 7.74 1H d (15.5), 7.54 2H d (8.6), 6.86 2H d (8.6), 5.97 1H s, 3.92 3H s, 2.61 t (6.7), 1.83 t (6.7), 1.36 6H s ppm, and **Figure 4.6C** as a pale orange solid, 3 mg, 25% yield. ^{1}H NMR, 400 MHz, δ 7.36 2H d (8.3 Hz), 6.85 2H (8.3), 6.07 2H s, 5.39 1H dd (10,2.8), 3.07 dd (13, 3.0), 2.73 dd (13, 3.0), 2.59 2H m, 1.82 2H m, 1.36 6H s ppm.

Cell culture

Caco-2 cells, obtained from American Type Culture Collection (Rockville, MD, USA) were grown in Dulbecco's modified Eagle medium, 10% FBS, 0.1 mM

non-essential amino acids, and 0.01% penicillin/streptomycin at 37 ºC in air with 5% CO_2 and 95% relative humidity. The cells were seeded onto Transwell cell culture inserts and maintained until use on days 21–25 (passage 25). The integrity of the monolayers was assessed by measuring transepithelial electrical resistance (TEER) using a World Precision Instrument, EVOM (Sarasota, FL, USA).

Transport studies

Transport of xanthohumol (XN) and ascorbyl xanthohumol (AscXN) was studied as described previously (Taur & Rodriguez-Proteau, 2008) using a transport buffer consisting of Hanks' buffered salt solution (HBSS) with 10 mM HEPES and 25 mM D-glucose at a pH of 6.8 for the apical (AP) compartment and 7.4 for the basolateral (BL) compartment. The Transwell inserts were washed three times with transport buffer and then the apical (AP) and basolateral (BL) compartments were filled with 1.5 and 2.5 ml of transport buffer, respectively. After equilibration for 30 min 37 ºC, the test compounds were added either in the AP or BL compartment. XN and AscXN, at concentrations of 30 and 60 µM, respectively, were prepared in transport buffer at the appropriate pH (pH 6.8 for the AP compartment and 7.4 for the BL compartment). Aliquots (200 µl) were taken from the donor and receiver chambers initially and from the receiver chamber at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h. After 3 h, 200-µl aliquots were also taken from the donor chamber. An equal volume of fresh transport buffer (200 µl) was added to the respective chamber at each sampling time point. The experiments using XN and AscXN were performed in triplicate either in AP→BL and BL→AP direction. The collected transport buffers were analyzed

immediately for XN and metabolites.

Results

It was originally thought that the synthesis of the Michael-addition product ascorbyl-xanthohumol (AscXN) should proceed smoothly. Interestingly, the biosynthesis of xanthohumol begins with *p*-coumaroyl CoA, which becomes the enone and B-ring of xanthohumol (Stevens et al., 2004). A series of ascorbylated natural products may begin with the same precursor (see Chapter 2). However, the synthesis turned out to be anything but trivial.

One factor causing difficulty in the synthesis of AscXN is the poor hydrophilicity of the Michael acceptor and the considerable hydrophilicity of the Michael donor. Incubation of xanthohumol and AscH in H_2O produces a small amount of AscXN measurable by LC-MS, but in less than 1% yield. The screening of the reaction with a variety of organic solvents and solvent mixtures showed no improvement in product yield.

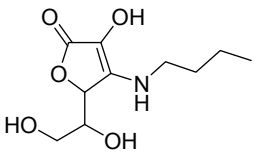

Figure 6.2: Ascorbic acid enamine derivative

Attempts to improve solubility of AscH by forming the 5,6-acetonide (Micheel & Hasse, 1936), attempts to improve the nucleophilicity of AscH by forming the enamine for a Stork-type conjugate addition (Pischetsrieder, Larisch et al., 1995) (**Fig. 6.2**), or improving the electrophilicity of xanthohumol via Knoevenagel-type addition (**Fig. 6.3**) all failed to produce any product.

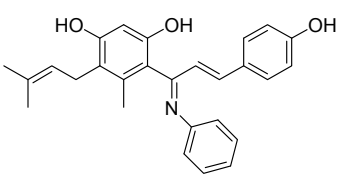

Figure 6.3: Hypothetical xanthohumol-aniline

No improvement was found by the addition of

any base to the reaction mixture. Attempts to utilize acid-catalyzed Michael additions, using either Lewis or Brønsted-Lowry acids, either failed to produce any product, or efficiently furnished the intramolecular alkene addition product between the A-ring and the isoprenyl group or a hydration of the isoprenyl double bond of isoxanthohumol (**Fig. 6.4**). NMR data of these side products match known xanthohumol metabolites measured in rat feces (Nookandeh, Frank et al., 2004).

Heating tended to produce a number of side products as well, particularly isoxanthohumol and products of Cope-rearrangement of the A-ring and isoprenyl group. However, heating of XN in MeOH with a large excess of AscH provided the best yield, approximately 10%.

AscXN was isolated as a single peak by preparative HPLC. Lyophilization produced a white crystalline mass. Unfortunately, the product is unstable and degrades to a brown oil after several hours at room temperature, and no NMR spectra were obtained. Enough material was recovered by HPLC to perform cell studies, but no further characterization. This instability may be due to the retro-Michael reaction being favored by restoration of resonance to the entire chalcone system. Model studies using chalcone showed similar difficulties in reactivity.

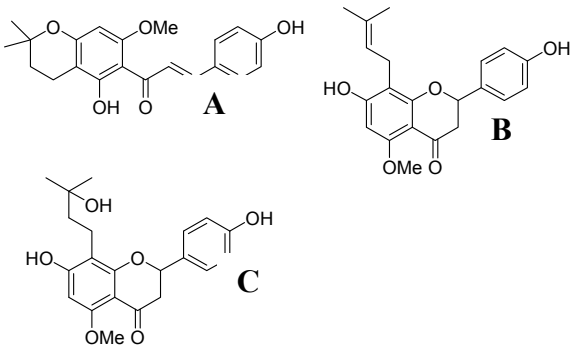

Figure 6.4: Side products formed during ascorbylation of xanthohumol, (A.) intramolecular addition of A-ring and isoprenyl, (B.) isoxanthohumol, and (C.) hydration product of isoxanthohumol.

While the product was isolated as a single peak by preparative HPLC, analytical HPLC was able to resolve two separate peaks (Peaks A & B). Without NMR, total structural identification is impossible, however based on MS/MS fragmentation (**Fig 6.5**) HPLC peak A may be the ascorbyl-conjugate of the cyclized xanthohumol produced using acidic reagents.

Transport studies of xanthohumol and ascorbyl-xanthohumol were conducted using Caco-2 cells. Caco-2 cells are an immortalized cell line which form monolayers capable of assaying gastro-intestinal absorption. Candidate drugs are applied to the apical side of the monolayer, and samples are measured on both the apical and baso-lateral side to detect transport.

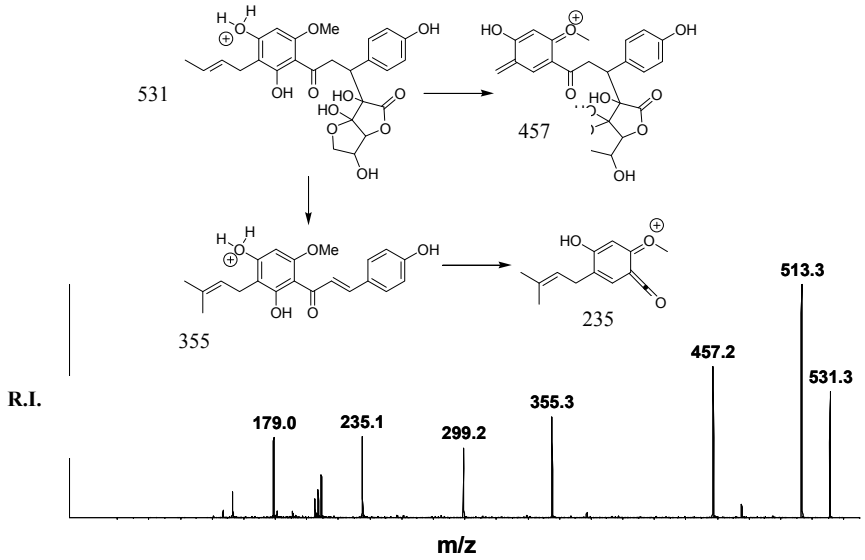

Figure 6.5: MS/MS spectrum and fragmentation of AscXN. HPLC peak A does not produce fragment peak at *m/z* 457 suggesting cyclization of isoprenyl group.

Xanthohumol shows no transport from the apical to the baso-lateral side (**Fig. 6.6**). The xanthohumol concentration decreases dramatically over a three hour time

course. This can be explained by the semi-quantitative evolution of xanthohumol-glucuronide on both sides of the monolayer, as well as possible conjugation of xanthohumol to the proteins in the cells themselves. AscXN Peak A, the suspected ascorbylated cyclization product, showed no transport from the apical side to baso-lateral side of the mono-layer. There was no loss of AscXN from the apical side, indicating that it is not being metabolized. Like xanthohumol, the AscXN peak B disappeared completely upon exposure to buffer, further demonstrating the instability of AscXN. The fate of HPLC peak B remains unknown. There was no formation of XN detected, indicating AscXN did not undergo a retro-Michael reaction.

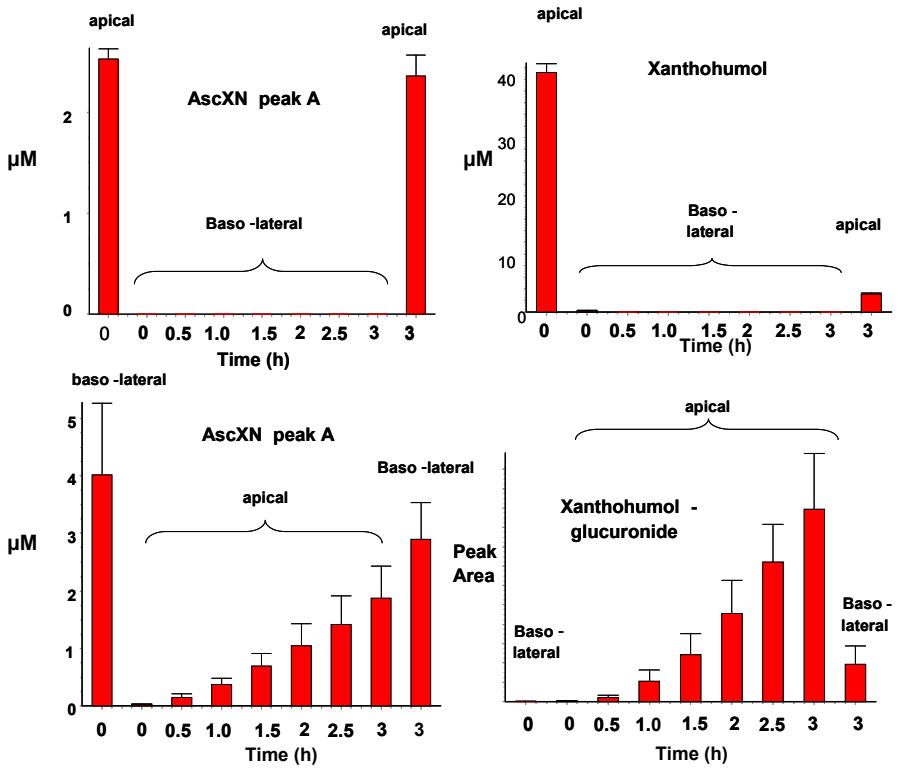

Figure 6.6: Transport studies of AscXN and XN in Caco-2 cells, n = 3.

With failure of AscXN to improve transport properties of xanthohumol, attempts were made to synthesis the succinate derivative, xanthohumol-succinate (XNSucc). Efforts to synthesize the compound were also difficult and after many attempts only a 30% reaction yield was obtained. The product was characterized by LC-MS and NMR, however it proved to be unstable, even in a -20° C freezer. Thus, no attempts were made to measure cellular transport.

Discussion

Ascorbylation of xanthohumol is not an acceptable route for creation of a xanthohumol prodrug. The synthesis is poor, the products are multiple, unstable, and of indeterminate structure, and there is no effect on transport despite the increased hydrophilicity. In retrospect it is possible that the loss of "peak A" is due to hydrolysis and decarboxylation pathway analogous to that of AscACR in cell media (see chapter 4); "peak B" appears to be relatively stable. The discovery of that pathway post-dates our work with AscXN. Based on clean syntheses of other AscACR, and cleavage of the ascorbate moiety in numerous natural products (see Chapter 2), we remain hopeful that ascorbate may have potential as a pro-drug moiety with other candidates.

Chapter 7: Conclusion

Vitamin C is indeed more than just an antioxidant and cofactor for prolyl hydroxylase. We have reviewed known natural products containing the ascorbyl moiety. These secondary metabolites can range from sources such as red algae to tea trees, and with structural complexity ranging from brominated phenols to complex hydrolyzable tannins. Bioactivities of these natural products range from low nanomolar inhibition of enzymes involved in tumor angiogenesis to induction of quinone reductase.

As for a role of ascorbate in the detoxification of electrophiles, we have shown that ascorbate is involved in the detoxification of acrolein by nucleophilic addition in THP-1 cells. However, ascorbylation represents a detoxification pathway that is previously unknown and could be of even greater importance for other electrophiles. Furthermore, AscACR itself was detected in easily measurable amounts in the no cell control but undetectable in the presence of cells, indicating there could be biocatalysts involved in the conversion of AscACR to THO. For example, lipid peroxidation produces such as 4-hydroxy-2-nonenal may undergo similar ascorbylation and conversion to the decarboxylated product. Conceivably, any electrophile may undergo this transformation; this may appear to be a new form of phase II metabolism, albeit without a “transferase” enzyme being involved in the ascorbylation.

Finally, the use of ascorbate as a prodrug moiety for xanthohumol has been explored. While this project failed to produce a useful prodrug, the hypothesis that ascorbyl moieties may be useful for such still stands. It will be essential to determine if the decarboxylation pathway is relevant for any prodrug made, as the

decarboxylation renders the retro-Michael reaction impossible. If the decarboxylation and isomerization to the corresponding deoxytagatose or deoxysorbose does not occur, then the solubility, transport properties, and ability to undergo retro-Michael cleavage should be useful characteristics of such an ascorbyl moiety.

Structure determination of AscACR by single crystal X-ray diffractometry

Determination of the crystallographic parameters, data collection and structure solution and refinement was performed as described elsewhere (1), with the following details. A well shaped crystal of dimensions 0.40x 0.30 x 0.30 mm^3 was selected and mounted on the tip of a thin glass fiber using a dab of Paratone. Crystal quality evaluation and preliminary indexing were performed from four images of 10 degrees rotation about omega, each separated from the others by 50 degrees. Proving to be a satisfactory crystal, a full set of 160 frames of 5 degrees rotation about omega was collected. The frames were integrated using the determined unit cell, using the program TwinSolve as included in Rigaku/MSC's software package CrystalClear to yield a massively redundant data set of 10934 reflections. Correction for the effects of absorption anisotropy was carried out by means of multiscans (2) as programmed in TwinSolve. Finally, a data set consisting of 1795 unique reflections in the range (-7 – 8,-13 – 13,-16 – 15) was generated with an R(merge) of 0.035. The reported unit cell was refined using all 8155 reflections with intensities greater than 10 times their e.s.d.s in the range $3.20° < \theta < 69.37°$.

The structure was solved using direct methods as programmed in SHELXS-90 (3) and the solution was refined using the program SHELXL-97 (4), followed by Fourier synthesis, which revealed the positions of the remaining atoms. All hydrogen atoms were clearly identified from the Fourier map. In order to preserve a favorable data-to-parameter ratio, the hydrogen atoms were placed in geometrically idealized positions, with the C-H distance allowed to refine. With the exception of the water of

hydration's hydrogens, all hydrogen atoms were given a displacement parameter equal to 1.5 times (methyl group) or 1.2 times (non-methyl hydrogens) the equivalent isotropic displacement parameter of the atom it is attached to. For those hydrogens belonging to the water of hydration, the hydrogen atoms were placed in the positions found from the difference Fourier map as isotropic atoms, and were allowed to fully refine. During the final cycle of least squares refinement, all non-hydrogen atoms were refined with anisotropic displacement parameters. The refined value of the absolute structure parameter (Flack parameter) (5) of 0.10(18) indicates that the model obtained accurately depicts the absolute structure of the molecule. An ORTEP (6) of the final model is given in **Figure 3.3**, with displacement ellipsoids drawn at the 50% probability level.

Supplementary Table 1. Crystal data and structure refinement for AscACR.

Identification code	FS032004	
Empirical formula	$C_9H_{14}O_8$	
Formula weight	250.20	
Temperature	100(2) K	
Wavelength	1.54180 Å	
Crystal system	Orthorhombic	
Space group	$P2_12_12_1$	
Unit cell dimensions	a = 6.9227(3) Å	α= 90°.
	b = 10.8337(5) Å	β= 90°.
	c = 13.6807(7) Å	γ = 90°.
Volume	1026.03(8) $Å^3$	
Z	4	
Density (calculated)	1.620 Mg/m^3	
Absorption coefficient	1.267 mm^{-1}	
F(000)	528	
Crystal size	0.40 x 0.30 x 0.30 mm^3	
Theta range for data collection	3.23 to 69.93°.	
Index ranges	-7<=h<=8, -13<=k<=13, -16<=l<=15	
Reflections collected	10934	
Independent reflections	1795 [R(int) = 0.035]	
Completeness to theta = 41.21°	93.0 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	1.0000 and 0.9160	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	1795 / 0 / 166	
Goodness-of-fit on F^2	1.072	
Final R indices [I>2sigma(I)]	R1 = 0.0366, wR2 = 0.0979	
R indices (all data)	R1 = 0.0367, wR2 = 0.0980	
Absolute structure parameter	0.10(18)	
Largest diff. peak and hole	0.331 and -0.202 $e.Å^{-3}$	

Supplementary Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3)
for FS032004. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
C(1)	3862(2)	7653(2)	6718(1)	14(1)
C(2)	4344(3)	8596(2)	7492(1)	17(1)
C(3)	3517(2)	9769(2)	7035(1)	14(1)
C(4)	1676(2)	9282(2)	6545(1)	11(1)
C(5)	-55(2)	9469(2)	7228(1)	13(1)
C(6)	1011(2)	10017(2)	5653(1)	11(1)
C(7)	-120(3)	11083(2)	6108(1)	13(1)
C(8)	-1718(2)	11358(2)	5376(1)	14(1)
C(9)	-2015(2)	10115(2)	4860(1)	16(1)
O(1)	5226(2)	7748(1)	5963(1)	17(1)
O(2)	1985(2)	8002(1)	6357(1)	13(1)
O(3)	-574(2)	8831(1)	7889(1)	20(1)
O(4)	-979(2)	10530(1)	6981(1)	14(1)
O(5)	2419(2)	10411(1)	5006(1)	14(1)
O(6)	-457(2)	9307(1)	5176(1)	13(1)
O(7)	-1096(2)	12252(1)	4692(1)	18(1)
OW	8624(2)	6429(1)	6175(1)	16(1)

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Supplementary Table 3. Bond lengths [Å] and angles [°] for AscACR.

C(1)-O(1)	1.403(2)	O(3)-C(5)-C(4)	128.31(16)
C(1)-O(2)	1.441(2)	O(4)-C(5)-C(4)	109.18(14)
C(1)-C(2)	1.509(2)	O(5)-C(6)-O(6)	111.97(13)
C(2)-C(3)	1.527(2)	O(5)-C(6)-C(4)	117.35(13)
C(3)-C(4)	1.534(2)	O(6)-C(6)-C(4)	107.33(13)
C(4)-O(2)	1.4265(18)	O(5)-C(6)-C(7)	112.82(13)
C(4)-C(6)	1.528(2)	O(6)-C(6)-C(7)	103.22(13)
C(4)-C(5)	1.533(2)	C(4)-C(6)-C(7)	102.86(13)
C(5)-O(3)	1.193(2)	O(4)-C(7)-C(8)	108.79(13)
C(5)-O(4)	1.358(2)	O(4)-C(7)-C(6)	103.38(12)
C(6)-O(5)	1.3846(19)	C(8)-C(7)-C(6)	104.58(14)
C(6)-O(6)	1.4317(19)	O(7)-C(8)-C(7)	110.38(14)
C(6)-C(7)	1.528(2)	O(7)-C(8)-C(9)	109.73(15)
C(7)-O(4)	1.462(2)	C(7)-C(8)-C(9)	103.20(13)
C(7)-C(8)	1.523(2)	O(6)-C(9)-C(8)	107.01(13)
C(8)-O(7)	1.413(2)	C(4)-O(2)-C(1)	109.20(13)
C(8)-C(9)	1.534(2)	C(5)-O(4)-C(7)	111.01(13)
C(9)-O(6)	1.455(2)	C(6)-O(6)-C(9)	109.76(11)

O(1)-C(1)-O(2)	109.62(14)
O(1)-C(1)-C(2)	108.53(14)
O(2)-C(1)-C(2)	105.18(14)
C(1)-C(2)-C(3)	101.18(14)
C(2)-C(3)-C(4)	101.76(13)
O(2)-C(4)-C(6)	114.06(14)
O(2)-C(4)-C(5)	110.86(13)
C(6)-C(4)-C(5)	100.51(13)
O(2)-C(4)-C(3)	106.78(13)
C(6)-C(4)-C(3)	114.84(13)
C(5)-C(4)-C(3)	109.71(13)
O(3)-C(5)-O(4)	122.51(16)

Table 4. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for AscACR.

The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2 a^{*2} U^{11} + ... + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
C(1)	11(1)	13(1)	18(1)	2(1)	-1(1)	2(1)
C(2)	16(1)	22(1)	14(1)	0(1)	0(1)	0(1)
C(3)	11(1)	16(1)	15(1)	-3(1)	0(1)	-2(1)
C(4)	10(1)	9(1)	15(1)	-1(1)	1(1)	-1(1)
C(5)	10(1)	13(1)	17(1)	-3(1)	-1(1)	1(1)
C(6)	10(1)	10(1)	14(1)	-1(1)	0(1)	-1(1)
C(7)	13(1)	13(1)	14(1)	1(1)	3(1)	1(1)
C(8)	13(1)	12(1)	17(1)	1(1)	1(1)	4(1)
C(9)	14(1)	12(1)	22(1)	2(1)	-6(1)	0(1)
O(1)	12(1)	17(1)	21(1)	-7(1)	1(1)	2(1)
O(2)	11(1)	8(1)	21(1)	-2(1)	-1(1)	2(1)
O(3)	19(1)	21(1)	20(1)	7(1)	6(1)	2(1)
O(4)	16(1)	13(1)	13(1)	0(1)	3(1)	3(1)
O(5)	15(1)	12(1)	15(1)	-1(1)	5(1)	-1(1)
O(6)	12(1)	10(1)	19(1)	-3(1)	-4(1)	1(1)
O(7)	22(1)	11(1)	21(1)	2(1)	1(1)	2(1)
OW	13(1)	14(1)	21(1)	0(1)	-3(1)	-1(1)

Supplementary Table 5. Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($Å^2 \times 10^3$) for AscACR.

	x	y	z	U(eq)
H(1)	3841	6800	6998	16
H(2A)	5756	8663	7594	21
H(2B)	3708	8400	8121	21
H(3A)	4417	10128	6550	17
H(3B)	3215	10396	7539	17
H(7)	704	11818	6257	16
H(8)	-2926	11630	5714	17
H(9A)	-3282	9758	5040	19
H(9B)	-1976	10225	4142	19
H(1A)	4988	7216	5533	25
H(5)	2796	9812	4667	21
H(7A)	-1516	12949	4856	27
HOW1	9700(40)	6770(30)	6290(20)	35(7)
HOW2	7680(50)	6980(30)	6130(20)	36(7)

Supplementary Table 6. Torsion angles [°] for AscACR.

O(1)-C(1)-C(2)-C(3)	79.37(16)	C(7)-C(8)-C(9)-O(6)	-9.24(18)
O(2)-C(1)-C(2)-C(3)	-37.88(16)	C(6)-C(4)-O(2)-C(1)	129.44(15)
C(1)-C(2)-C(3)-C(4)	37.39(16)	C(5)-C(4)-O(2)-C(1)	-117.98(14)
C(2)-C(3)-C(4)-O(2)	-24.89(17)	C(3)-C(4)-O(2)-C(1)	1.48(17)
C(2)-C(3)-C(4)-C(6)	-152.39(14)	O(1)-C(1)-O(2)-C(4)	-93.33(16)
C(2)-C(3)-C(4)-C(5)	95.32(15)	C(2)-C(1)-O(2)-C(4)	23.18(17)
O(2)-C(4)-C(5)-O(3)	34.7(2)	O(3)-C(5)-O(4)-C(7)	-176.18(16)
C(6)-C(4)-C(5)-O(3)	155.67(18)	C(4)-C(5)-O(4)-C(7)	4.45(18)
C(3)-C(4)-C(5)-O(3)	-83.0(2)	C(8)-C(7)-O(4)-C(5)	129.01(15)
O(2)-C(4)-C(5)-O(4)	-145.98(14)	C(6)-C(7)-O(4)-C(5)	18.26(18)
C(6)-C(4)-C(5)-O(4)	-25.02(16)	O(5)-C(6)-O(6)-C(9)	-91.28(16)
C(3)-C(4)-C(5)-O(4)	96.33(16)	C(4)-C(6)-O(6)-C(9)	138.56(14)
O(2)-C(4)-C(6)-O(5)	-82.33(18)	C(7)-C(6)-O(6)-C(9)	30.33(16)
C(5)-C(4)-C(6)-O(5)	159.01(14)	C(8)-C(9)-O(6)-C(6)	-13.50(18)
C(3)-C(4)-C(6)-O(5)	41.4(2)		
O(2)-C(4)-C(6)-O(6)	44.72(18)		
C(5)-C(4)-C(6)-O(6)	-73.93(15)		
C(3)-C(4)-C(6)-O(6)	168.44(13)		
O(2)-C(4)-C(6)-C(7)	153.20(14)		
C(5)-C(4)-C(6)-C(7)	34.55(15)		
C(3)-C(4)-C(6)-C(7)	-83.09(16)		
O(5)-C(6)-C(7)-O(4)	-160.50(13)		
O(6)-C(6)-C(7)-O(4)	78.46(15)		
C(4)-C(6)-C(7)-O(4)	-33.10(16)		
O(5)-C(6)-C(7)-C(8)	85.68(16)		
O(6)-C(6)-C(7)-C(8)	-35.36(15)		
C(4)-C(6)-C(7)-C(8)	-146.92(13)		
O(4)-C(7)-C(8)-O(7)	159.79(13)		
C(6)-C(7)-C(8)-O(7)	-90.27(16)		
O(4)-C(7)-C(8)-C(9)	-83.02(15)		
C(6)-C(7)-C(8)-C(9)	26.92(16)		
O(7)-C(8)-C(9)-O(6)	108.42(16)		

Supplementary Table 7. Hydrogen bonds forAscACR [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
O(1)-H(1A)...O(6)#1	0.84	1.94	2.7581(17)	164.9
O(5)-H(5)...OW#2	0.84	1.86	2.6985(17)	175.2
O(7)-H(7A)...O(5)#3	0.84	1.93	2.7636(16)	170.0
OW-HOW1...O(2)#4	0.84(3)	2.07(3)	2.8941(18)	164(3)
OW-HOW2...O(1)	0.89(3)	1.90(3)	2.7677(18)	164(3)

Symmetry transformations used to generate equivalent atoms:

#1 x+1/2,-y+3/2,-z+1 #2 x-1/2,-y+3/2,-z+1 #3 x-1/2,-y+5/2,-z+1 #4 x+1,y,z

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