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Molecules of Interest

Hydroxynitrile glucosides

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ABSTRACT

β- and γ-Hydroxynitrile glucosides are structurally related to cyanogenic glucosides (α-hydroxynitrile glucosides) but do not give rise to hydrogen cyanide release upon hydrolysis. Structural similarities and frequent co-occurrence suggest that the biosynthetic pathways for these compounds share common features. Based on available literature data we propose that oximes produced by CYP79 orthologs are common intermediates and that their conversion into β- and γ-hydroxynitrile glucosides is mediated by evolutionary diversified multifunctional orthologs to CYP71E1. We designate these as $CYP71_{\beta\gamma}$ and $CYP71_{\alpha\beta\gamma}$; in combination with the classical $CYP71_{\alpha}$ (CYP71E1 and orthologs) these are able to hydroxylate any of the carbon atoms present in the amino acid and oxime derived nitriles. Subsequent dehydration reactions and hydroxylations and a final glycosylation step afford the unsaturated β- and γ-hydroxynitrile glucosides. This scheme would explain the distribution patterns of α-, β- and γ-hydroxynitrile glucosides found in plants. The possible biological functions of these hydroxynitriles are discussed.

1. Introduction

Hydroxynitrile glucosides are a group of plant secondary metabolites. The most common members of the group are the cyanogenic glucosides, which are β-glucosides of α-hydroxynitriles. They are among the oldest and most well studied plant defense compounds, found in pteridophytes, gymnosperms and angiosperms, including many food and fodder crops (Bak et al., 2006). Upon hydrolysis of the cyanogenic glucosides, the unstable α-hydroxynitriles readily release hydrogen cyanide (HCN) (Fig. 1), which is generally toxic to respiring organisms (Teuscher and Lindequist, 1994). Among the plants harboring cyanogenic glucosides several also produce β- and γ-hydroxynitrile glucosides (Fig. 2), which do not release HCN upon hydrolysis. Little is known about how and why the plants produce β- and γ-hydroxynitrile glucosides. Because of the striking structural similarities of α-, β- and γ-hydroxynitrile glucosides and a high frequency of co-occurrence it has been proposed that the compounds are biosynthetically related (e.g. reviewed by Lechtenberg and Nahrstedt (1999)). Recent research has indeed established a biosynthetic connection between α-, β- and γ-hydroxynitrile glucosides but the details remain unclear (Bjarnholt et al., 2008; Forslund et al., 2004; Morant et al., 2007). The hydrolysis of cyanogenic glucosides is facilitated by specific β-glucosidases, which are physically separated from the substrate in intact plant cells (Gruhnert et al., 1994; Kakes, 1985; Saunders and Conn, 1978; Thayer and Conn, 1981). Upon cell disruption by e.g. herbivores, cyanogenic glucosides and β-glucosidases are intermixed resulting in cyanogenesis i.e. release of HCN. Recently, it has also been demonstrated that cyanogenic β-glucosidases can hydrolyze β- and γ-hydroxynitrile glucosides *in vivo* (Morant et al., 2008; Nielsen et al., 2006). This review compiles the current knowledge on β- and γ-hydroxynitriles biosynthesis and their possible biological significance.

At least 2650 plant species from 130 families have been reported to be cyanogenic. Around 30 naturally occurring cyanogenic glucosides have been identified (latest reviews by Lechtenberg and Nahrstedt (1999) and Moller and Seigler (1999)), and the number continues to grow with two new cyanogenic glucosides recently identified (Jaroszewski et al., 2002; Nakamura et al., 2007). In addition to the cyanogenic mono-glucosides, some plants produce di-glucosides. A large number of derivatives have also been identified but the most commonly encountered are the non-modified mono-glucosidic core structures (Lechtenberg and Nahrstedt, 1999; Moller and Seigler, 1999). In all known cases but one, the sugar moiety directly attached to the α-hydroxyl group is D-glucose. The single exception carries a D-allose moiety (Christensen and Jaroszewski, 2001). Cyanogenic glucosides are derived from the amino acids L-valine (val), L-isoleucine (ile), L-leucine (leu), L-phenylalanine (phe), L-tyrosine (tyr) or from the non-protein amino acid L-2-(2′-cyclopentenyl)glycine (cpg). One exception is apparently derived from nicotinic acid (Lechtenberg and Nahrstedt, 1999; Moller and Seigler, 1999). Each plant species is usually restricted to the

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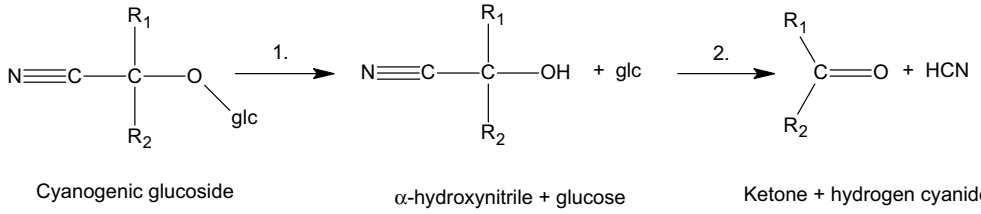


Fig. 1. Structure and degradation pathway of cyanogenic glucosides. Step 1 in the degradation pathway is catalyzed by β-glucosidases, step 2 proceeds spontaneously or catalyzed by α-hydroxynitrile lyases.

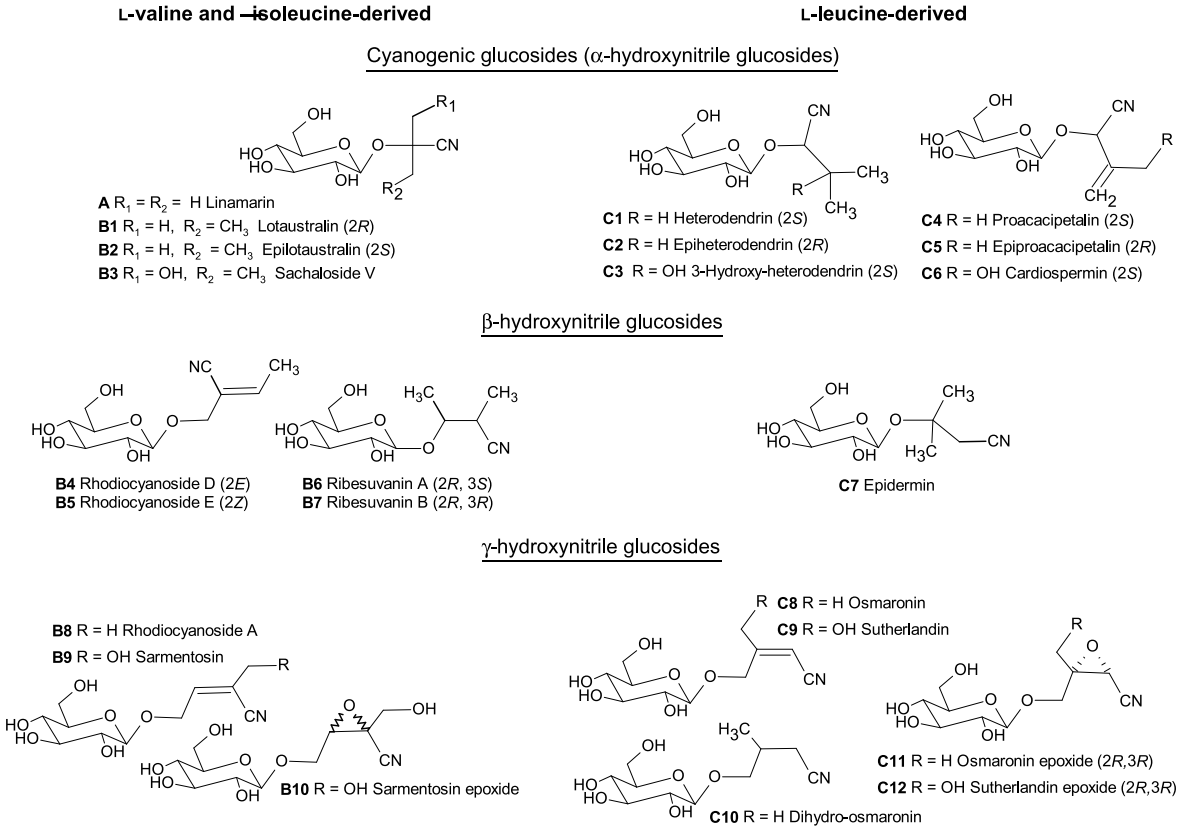


Fig. 2. Aliphatic hydroxynitrile glucosides. Aliphatic α-, β- and γ-hydroxynitrile glucosides co-occurring in *Lotus*: **A**, **B1** and **B4**–**B8** (Bjarnholt et al., 2008; Forslund et al., 2004), *Ribes*: **B1**, **B2** and **B4**–**B9** (Bjarnholt et al., 2008), *Rhodiola*: **B1**–**B9** (Bjarnholt et al., 2008; Fan et al., 2001; Nakamura et al., 2007; Yoshikawa et al., 1996, 1997; Yousef et al., 2006), *H. vulgare*: **C2** and **C7**–**C10** (Nielsen et al., 2002; Pourmohseni et al., 1993), *Rosaceae*: **C1**–**C2**, **C6** and **C7**–**C12** (Lechtenberg et al., 1994, 1996; Nahrstedt and Scheid, 1981), *Acacia*: **C4** and **C9** (Swenson et al., 1987).

use of a single amino acid precursor for biosynthesis of cyanogenic glucosides. However, valine- and isoleucine-derived cyanogenic glucosides commonly co-occur, and these in turn co-occur with those derived from cyclopentenylglycine in a few cases (Jaroszewski et al., 1988b, 2002; Olafsdottir et al., 1989).

Several plants producing either isoleucine- or leucine-derived cyanogenic glucosides contain a suite of structurally related β- and γ-hydroxynitrile glucosides (Fig. 2). In species of *Lotus* (Fabaceae), *Rhodiola* (Crassulaceae), *Ribes* (Grossulariaceae) and possibly *Jatropha* (Euphorbiaceae), the isoleucine-derived cyanogenic glucoside lotaustralin (**B1**) co-occurs with a range of β- and γ-hydroxynitrile glucosides, namely rhodiocyanides A, D and E, ribesuvanin A and B (**B4**–**B8**) and sometimes sarmentosin (**B9**) and the hydroxylated cyanogenic glucoside sachaloside V (**B3**) (Fig. 2) (Bjarnholt et al., 2008; Fan et al., 2001; Forslund et al., 2004; Nakamura et al., 2007; Yoshikawa et al., 1996, 1997; Yousef et al., 2006). A corresponding range of compounds, epidermin, osmaronin, sutherlandin and dihydroosmaronin (**C7**–**C10**), co-occur with the leucine-derived cyanogenic glucoside epi-heterodendrin (**C2**) in *Hordeum vulgare* L. (barley, Poaceae) and members of the Rosaceae. The latter also contain heterodendrin (**C1**), cardiospermin (**C6**), osmaronin epoxide (**C11**) and sutherlandin epoxide (**C12**) (Bjarnholt et al., 2008; Lechtenberg et al., 1994, 1996; Nahrstedt and Scheid, 1981; Nielsen et al., 2002; Pourmohseni et al., 1993; Swenson et al., 1987). **C9** was originally isolated along with the cyanogenic glucoside proacacipetalin (**C4**) from *Acacia sutherlandii* F. Muell. (Fabaceae) and the remaining cyanogenic glucosides have been found in various other *Acacia* species (Brimer et al., 1981; Ettlinger et al., 1977; Jaroszewski, 1986; Maslin et al., 1988; Seigler et al., 1975, 1983). Sarmentosin epoxide (**B10**) was isolated alone from *Sedum cepea* L. (Crassulaceae) (Nahrstedt et al., 1982).

2. The β- and γ-hydroxynitrile glucosides have evolved from the cyanogenic glucoside biosynthetic pathway

2.1. Cyanogenic glucoside biosynthesis

Cyanogenic glucosides isolated from the pteridophytes, gymnosperms and earliest angiosperms are aromatic whereas the later angiosperms contain both aromatic and aliphatic cyanogenic glucosides (Bak et al., 2006). Thus, most likely the biosynthetic pathway for aliphatic cyanogenic glucosides has evolved from that of the aromatic compounds. Accordingly, the characteristics of all identified cyanogenic glucoside biosynthetic enzymes and intermediates strongly suggest that the biosynthetic pathway is conserved throughout the plant kingdom. The biosynthesis is catalyzed by two multifunctional cytochromes P450 (P450) and a UDPG-glucosyl transferase (UGT) as demonstrated in detail for *Sorghum bicolor* (L.) Moench (sorghum, Poaceae), which produces the cyanogenic glucoside dhurrin (**D**) (Fig. 3). The first P450, CYP79A1, catalyzes a multi-step conversion of tyrosine into the corresponding *Z*-oxime (tyr-ox) (Koch et al., 1995; Sibbesen et al., 1994, 1995). This *Z*-oxime is further metabolized by a second multifunctional P450, CYP71E1, to form the corresponding α-hydroxynitrile (Bak et al., 1998; Kahn et al., 1997) which is finally glucosylated by UGT85B1 to form **D** (Hansen et al., 2003; Jones et al., 1999; Thorsoe et al., 2005). In *Triglochin maritima* (seaside arrowgrass, Juncaginaceae) the CYP79A1 orthologs, CYP79E1 and CYP79E2, catalyze the same conversion of tyrosine into tyr-ox in the biosynthesis of cyanogenic taxiphyllin and triglochinin (Nielsen and Møller, 1999, 2000). In the case of **A**- and **B1**-producing *Manihot esculenta* Crantz (cassava, Euphorbiaceae) it has been demonstrated that CYP79D1 and CYP79D2 convert valine and isoleucine into the corresponding oximes (val- and ile-ox) (Andersen et al., 2000). The same is the case for CYP79D3 and CYP79D4 in *L. japonicus* (Forslund et al., 2004). In *M. esculenta*, CYP71E7 has been shown to metabolize val- and ile-ox into the **A** and **B1** α-hydroxynitriles (Morant et al., submitted for publication). No UGT85B1 orthologs have been identified, cloned and expressed but soluble UDP-glucose:α-hydroxynitrile-β-D-glucosyl transferases purified from *M. esculenta*, *Linum usitatissimum* L. (flax, Linaceae) and *T. maritima* have been demonstrated to glucosylate the expected substrates (Hahlbrock and Conn, 1970; Hosel and Schiel, 1984; Mederacke et al., 1996). Several other data derived from microsomal studies or from administration of substrates to detached leaves show that cyanogenic plants metabolize amino acids, oximes or nitriles into the expected oximes, α-hydroxynitriles and/or cyanogenic glucosides, e.g. for phenylalanine (Hahlbrock et al., 1968; Tapper and Butler, 1971),

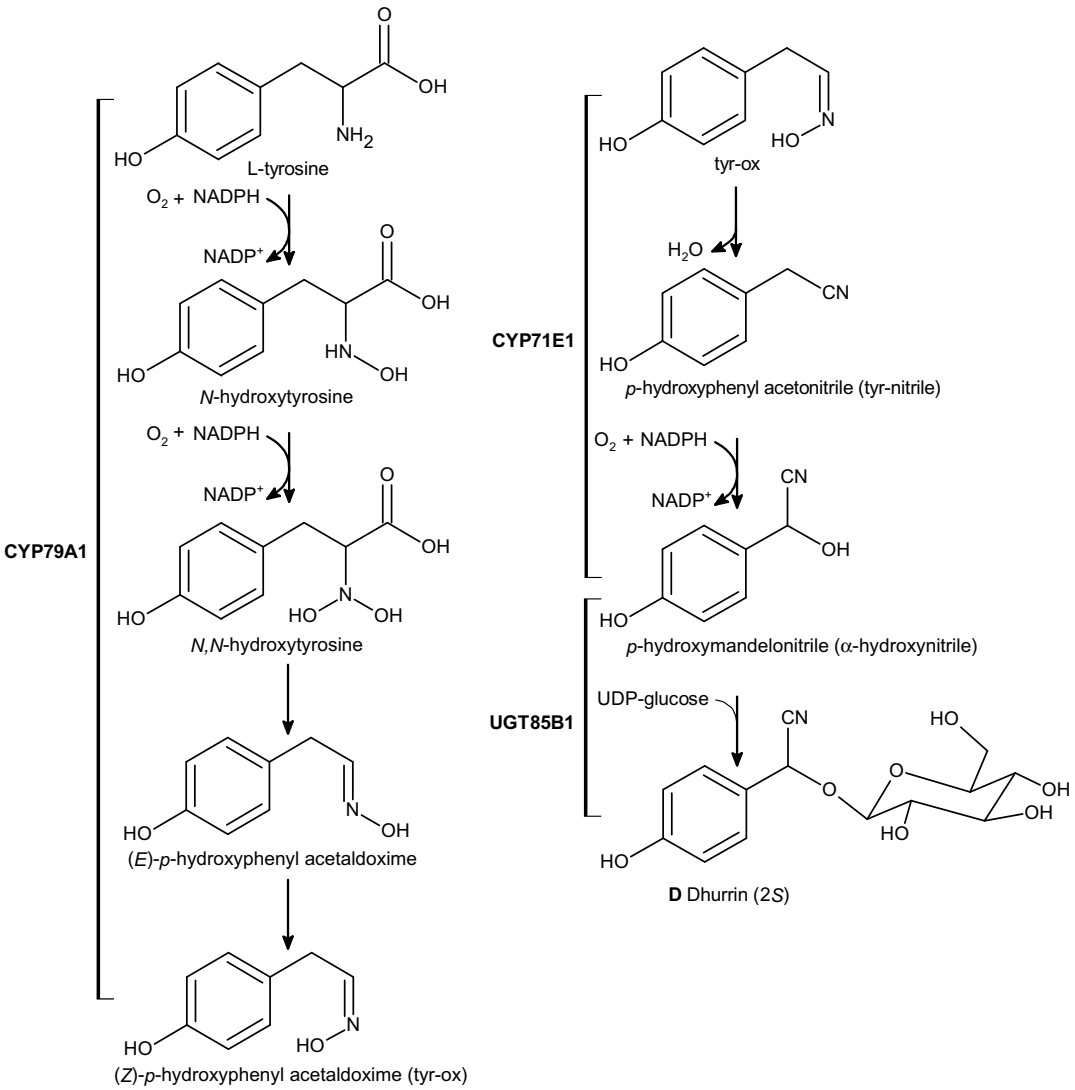


Fig. 3. The biosynthetic pathway for dhurrin production in *S. bicolor* (Hansen et al., 2003; Jones et al., 1999; Kahn et al., 1997, 1999; Sibbesen et al., 1994, 1995).

cyclopentenylglycine (Jaroszewski et al., 1996; Olafsdottir et al., 1992; Tober and Conn, 1985) and leucine (Butterfield et al., 1975; Ettlinger et al., 1977; Nielsen et al., 2002).

2.2. Evidence of close relationship between α-, β- and γ-hydroxynitrile glucoside biosynthesis

An investigation of the co-occurrence of isoleucine-derived α-, β- and γ-hydroxynitrile glucosides in *Lotus*, *Ribes* and *Rhodiola* species demonstrated that the presence of β- and γ-hydroxynitrile glucosides **B4**–**B9** was accompanied by presence of lotaustralin in all 13 species analyzed (Bjarnholt et al., 2008). A parallel result was obtained on the co-occurrence of the leucine-derived α-hydroxynitrile glucoside epi-heterodendrin (**C2**) and the β- and γ-hydroxynitrile glucosides **C7**–**C10** (Fig. 2) in all eight Rosaceae species studied by Lechtenberg et al. (1996). It was previously established that isoleucine is the common amino acid precursor in the biosynthesis of the α-hydroxynitrile glucoside **B1** and β- and γ-hydroxynitrile glucosides **B4** and **B8** in *L. japonicus* (Regel) K. Larsen (Japanese birdsfoot trefoil) (Forslund et al., 2004). In addition we have demonstrated that isoleucine is the common precursor of the entire group of **B** glucosides in *Ribes uva-crispa* L. (gooseberry) and that leucine is the common precursor of the **C** glucosides in *H. vulgare* (Bjarnholt et al., 2008) (Fig. 2). Furthermore, *L. japonicus* transformed with an RNAi construct targeted against CYP79D3 and CYP79D4 displayed a transient decrease in the level of all hydroxynitrile glucosides (Morant et al., 2007). This means that the ile-ox produced by CYP79D3 and CYP79D4 is most likely a shared intermediate in the biosynthesis of α-, β- and γ-hydroxynitrile glucosides. Further downstream the biosynthetic pathways bifurcate; in *L. japonicus* the presence or absence of all β- and γ-hydroxynitrile glucosides is determined by a single recessive genetic trait, which does not affect presence or absence of **B1** (Bjarnholt et al., 2008).

2.3. A proposed route for biosynthesis of β- and γ-hydroxynitrile glucosides

Nielsen et al. (2002) suggested that the biosynthesis of the non-cyanogenic β- and γ-hydroxynitrile glucosides reflects evolutionary diversification of the second P450 enzyme in the general cyanogenic glucosides biosynthetic pathway, such as CYP71E1 in *S. bicolor*, CYP71E7 in *M. esculenta* and their putative orthologs in other plant species, here henceforth referred to as CYP71$_\alpha$. These P450 enzymes catalyze a dehydration of the oximes to produce the corresponding nitriles and a C-hydroxylation to produce the α-hydroxynitriles (Fig. 3) (Bak et al., 1998; Halkier and Møller, 1990; Kahn et al., 1997; Shimada and Conn, 1977). The order of reactions was determined to be tyr-ox → tyr-nitrile → **D** α-hydroxynitrile in *S. bicolor*, as demonstrated by the inability of the microsomal system to metabolize or produce the alternative intermediate, tyrosine-derived α-hydroxy oxime (Shimada and Conn, 1977). This has been indirectly verified for other plant species by demonstration of metabolic conversion of oximes or nitriles into corresponding nitriles or α-hydroxynitriles and glucosides (Collinge and Hughes, 1984; Jaroszewski et al., 1996; Koch et al., 1992; Nielsen and Møller, 1999; Olafsdottir et al., 1992). In the suggested pathway for synthesis of the β- and γ-hydroxynitrile glucosides the putative CYP71$_\alpha$ paralog, a multifunctional CYP71$_{\alpha\beta\gamma}$, is thought to be able to hydroxylate any of the carbon atoms in the nitrile intermediate (Nielsen et al., 2002). The putative α-, β- and γ-hydroxynitriles formed are subsequently either glucosylated to produce the saturated hydroxynitrile glucosides or undergo a C–C dehydration reaction followed by another C-hydroxylation to afford the unsaturated β- and γ-hydroxynitriles which can then be glucosylated. Production of sarmentosin, sutherlandin and the epoxides (**B9**, **C9**, **B10**, **C11**, **C12**) (Fig. 2) requires one or two additional hydroxylation steps. This biosynthetic scheme was originally suggested for *H. vulgare* in which the occurring hydroxynitrile glucosides represent C-hydroxylation at all possible positions of the leucine-derived nitrile (Nielsen et al., 2002). Adopting this scheme, the recent discovery of ribesuvanin A and B (**B6** and **B7**) means that the hydroxynitrile glucosides found to co-exist in *Ribes*, *Rhodiola* and *Lotus* likewise represent hydroxylations of all possible carbon atoms in the isoleucine-derived nitrile as illustrated in Fig. 4 (Bjarnholt et al., 2008).

2.4. Evolution of cyanogenic glucoside biosynthetic enzymes

All known CYP79A1 orthologs have been shown to be highly substrate specific for the amino acid corresponding to the cyanogenic glucosides found in the plant in which the enzyme was identified (Andersen et al., 2000; Forslund et al., 2004; Kahn et al., 1999). On the contrary, all known and putative CYP71$_\alpha$ orthologs are more or less promiscuous enzymes able to produce α-hydroxynitriles from oximes and/or nitriles derived from several of the six known amino acid precursors and even some artificial nitriles. The *S. bicolor* CYP71E1 assayed as recombinant protein and in microsomal preparations was specific to the aromatic amino acids but conversion of phe-ox was only three times less effective than of the native **D** precursor, tyr-ox (Kahn et al., 1999). The *M. esculenta* CYP71E7 recombinant protein and microsomal preparations converted val-ox, ile-ox, tyr-ox and phe-ox into α-hydroxynitriles (Koch et al., 1992; Morant et al., submitted for publication). This supports the notion that the aliphatic cyanogenic glucosides are evolved from the aromatic pathway. Putative CYP71$_\alpha$ orthologs from plants producing aliphatic cyanogenic glucosides have also been shown to accept a range of aliphatic substrates. In *H. vulgare* microsomes, ile-ox was a competitive inhibitor of the conversion of leu-ox to the α-hydroxynitrile (Nielsen et al., 2002). *Turnera angustifolia* Mill. (Turneraceae) contains unsaturated cyanogenic glucosides that are all derived from cyclopentenylglycine. However, administration of val- and ile-nitriles and saturated cpg-nitrile to detached leaves led to production of the corresponding cyanogenic glucosides (Jaroszewski et al., 1996). Leaves of *Passiflora morifolia* Mast. (Passifloraceae) which normally contain valine- and isoleucine-derived **A**, **B1** and **B2**, produced leucine-derived **C1** and **C2** and cyclopentenylglycine-derived tetraphyllin A and deidaclin (**E1**, **E8**) (Fig. 5) upon administration of the corresponding nitriles (Jaroszewski et al., 1996).

The biosynthetic pathway for cyanogenic glucosides is highly channeled (Jørgensen et al., 2005b; Nielsen et al., 2008). This presumably explains why the CYP71$_\alpha$ orthologs have not been under selection pressure to evolve high substrate specificity. The assembly of the three enzymes and the required P450 reductase into a supramolecular protein complex (metabolon) ensure channeling of intermediates from one enzyme to the next (Jørgensen et al., 2005b; Nielsen et al., 2008). This optimizes the rate of biosynthesis, restricts access of inhibitory molecules to the active site, prevents release of reactive and toxic intermediates and provides a means to regulate metabolic cross-talk between biosynthetic pathways, which share enzymes or intermediates (Jørgensen et al., 2005b). The organization of the biosynthetic pathway in a metabolon ensures that the CYP71$_\alpha$ and UGT85B1 orthologs are not introduced to other than the intended substrates. Substrate specificity is then essentially determined at the CYP79 level. Accordingly, the specificity of UGT85B1 and putative orthologs for modifications of the substrate backbone structure is also low. The UGT85B1 willingly accepted the phenylalanine-derived α-hydroxynitrile with an efficiency of 78% of that for the native substrate, the tyrosine-derived

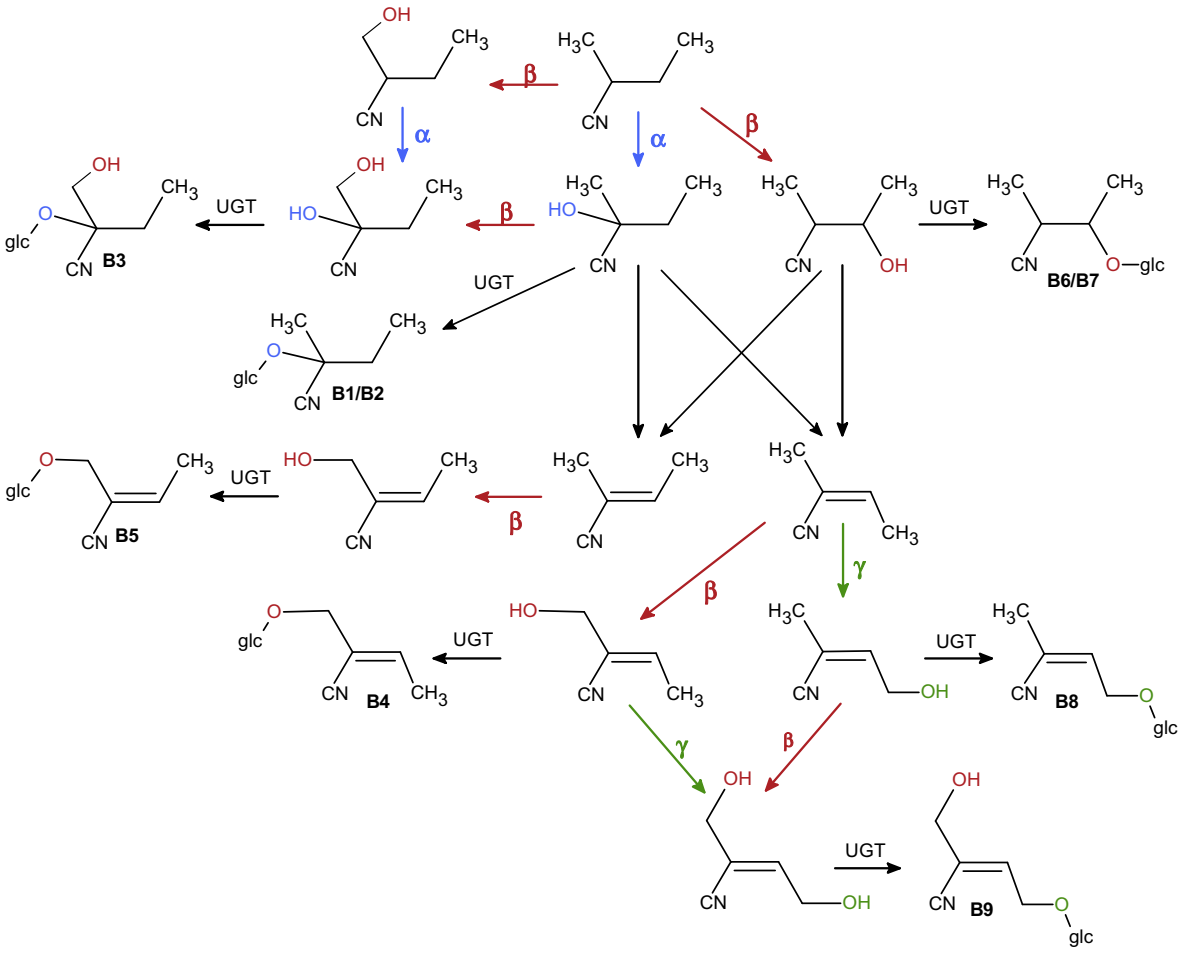
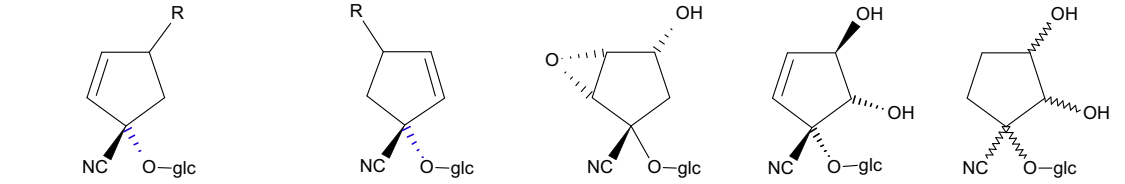


Fig. 4. Proposed reaction scheme for the putative CYP71$_{\alpha\beta\gamma}$-enzymes.



E1 R = H Tetraphyllin A (1*S*)
E2 R = OH Tetraphyllin B (1*S*,4*S*)
E3 R = OH Epivolkenin (1*S*,4*R*)
E4 R = OSO_3^- **E2** sulfate (1*S*,4*S*)
E5-7 R = variable sugar residues different from glucose: Passibiflorin, passicapsin, passitrifasciatin (1*S*,4*R*)

E8 R = H Deidaclin (1*R*)
E9 R = OH Volkenin (1*R*,4*R*)
E10 R = OH Taraktophyllin (1*R*,4*S*)

E11 Suberin A (1*R*,2*R*,3*R*,4*R*)

E12 Gynocardin (1*S*,4*S*,5*R*)

E13 Passiguatemalin

Fig. 5. Cyclopentenylglycine-derived cyanogenic glucosides from *Passiflora*. From Jaroszewski et al. (2002).

α-hydroxynitrile (Jones et al., 1999). UGT85B1 did also glucosylate aliphatic α-hydroxynitriles, albeit with low efficiency, as well as a number of aromatic and aliphatic structurally related compounds with variable efficiency (Hansen et al., 2003; Jones et al., 1999). For biosynthesis of cyanogenic glucosides from non-native nitriles, *T. angustifolia* and *P. morifolia* would obviously also have to possess enzymes that glucosylated the α-hydroxynitrile intermediates (Jaroszewski et al., 1996). The glucosyltransferase from *L. usitatissimum* converted both native and non-native aliphatic α-hydroxynitriles to cyanogenic glucosides and to a somewhat lower extent also aromatic substrates (Hahlbrock and Conn, 1970).

Evolutionary expansion of the group of CYP79A1 orthologs has continuously extended the range of amino acids serving as substrates for cyanogenic glucoside biosynthesis. However, the high substrate and product specificity of each individual CYP79A1 ortholog has been retained through this diversification. On the other hand, the CYP71$_\alpha$ orthologs have acquired broader substrate specificity through evolution. Furthermore, a functional evolution of the CYP71$_\alpha$ at the level of nitrile hydroxylations and dehydrations is indicated by multiple examples of diversification of the core cyanogenic glucoside structure, which are described in the following.

2.5. Cyclopentenylglycine-derived cyanogenic glucosides

The existence of cyanogenic glucosides derived from cyclopentenylglycine amino acids is presumably associated with occurrence of a CYP79A1 ortholog specific for these substrates. However, the large number of different cyclopentenylglycine-derived cyanogenic glucosides identified (reviewed by Lechtenberg and Nahrstedt (1999) and Jaroszewski et al. (2002)) (Fig. 5) indicates diversification and expansion of the general biosynthetic pathway, which does not appear to be associated with the putative CYP79A1 ortholog. The occurrence of cyclopentenylglycine-derived cyanogenic glucosides is restricted to plants of the families Turneraceae, Kiggelariaceae (Flacourtiaceae), Passifloraceae, Malesherbiaceae and Achariaceae. This coincides with the limited prevalence of the amino acid precursor(s) (Clausen et al., 2002; Olafsdottir et al., 1992; Wellendorph et al., 2001) as is illustrated by the fact that although *M. esculenta* microsomes can use cyclopentenylglycine as a substrate for α-hydroxynitrile formation (Koch et al., 1992), the plant does not produce the cyclopentenylglycine-derived cyanogenic glucosides *in vivo* (Lykkesfeldt and Møller, 1995). Indeed, some species of the five genera producing cyclopentenylglycine-derived cyanogenic glucosides (**E** glucosides, Fig. 5) contain **A**, **B1** and **B2**; in at least two species they co-occur (Andersen et al., 1998; Jaroszewski et al., 2002; Olafsdottir et al., 1990). This strongly suggests that the **E** glucosides are derived from the **A**/**B** biosynthetic pathway.

The large variation in cyclopentenylglycine-derived cyanogenic glucoside structures appears to be the result of diversification at several levels of the biosynthetic pathway. One type of variation is caused by secondary modifications of the core structures such as sulfurylation (**E4**) and additional glycosylations resulting in compounds with unusual sugar moieties (**E5**–**E7**) (Jaroszewski et al., 1988a, 2002). It is possible that the differences in stereochemistry among the **E** glucosides are directly linked to the stereochemistry of the parent amino acid but this issue has not been settled. In some cases, cyclopentenylglycine has been isolated as a mix of different epimers. The ratios between the epimers corresponded to the ratios between the corresponding cyanogenic glucoside epimers isolated from the very same plants (Clausen et al., 2001, 2002; Wellendorph et al., 2001). In other cases only one epimer of the amino acid has been identified in plants that harbor both epimers of the cyanogenic glucosides (Clausen et al., 2001). The biosynthetic origin of hydroxylated **E** glucosides has also not been resolved. In one study, radiolabelled cyclopentenylglycine was administered to *T. ulmifolia* which produced the non-hydroxylated **E1** and **E8** as well as the hydroxylated glucosides, tetraphyllin B (**E2**) and volkenin (**E9**) (Tober and Conn, 1985). The radiolabel was incorporated into **E1** and **E8** but not into the hydroxylated **E2** and **E9** (Tober and Conn, 1985). In a different study a hydroxylated cyclopentenylglycine, 2-(2′-hydroxy-3′-cyclopentenyl)glycine was isolated from plants producing **E** glucosides (Clausen et al., 2002). However, its structure does not correspond to the known hydroxylated cyanogenic glucosides which are all apparently derived from 2-(2′-cyclopentenyl)glycine.

In summary, it has not been demonstrated that non-hydroxylated cyclopentenylglycine is in fact the precursor of the hydroxylated **E** glucosides. On the other hand, no studies have documented the presence of hydroxylated amino acids that would serve as precursors for formation of the hydroxylated **E** glucosides without additional hydroxylations. The required additional hydroxylations may be envisioned to proceed either as secondary modifications of the cyanogenic glucosides or at the oxime intermediate level by the operation of a CYP71$_{\alpha\beta\gamma}$ enzyme. Cyclopentenylglycine-derived β- and γ-hydroxynitrile glucosides have never been identified but this may be because the search for new compounds has been based on the ability of the examined plants or plant extracts to release HCN.

2.6. Cyanolipids

Cyanolipids are a small group of natural products found in the seed oils of Sapindaceae and Hippocastanaceae (e.g. reviewed by Seigler (1991) and Mikolajczak (1977)). The cyanolipids are fatty acid esters of four different hydroxynitriles (Fig. 6). Two of these are α-hydroxynitriles corresponding to the leucine-derived glucosides proacacipetalin (**C4**) and cardiospermin (**C6**). The other two are γ-hydroxynitriles corresponding to osmaronin (**C8**) and sutherlandin (**C9**). In addition to the cyanolipids, the α- and γ-hydroxynitrile glucosides **C1**, **C3**–**C6** and **C9** (Fig. 2) have been isolated from aerial tissues of Sapindaceaeous species (Brimer et al., 1981; Ettlinger et al., 1977; Jaroszewski, 1986; Mikolajczak, 1977; Seigler et al., 1974, 1983; Swenson et al., 1987). In a few studies, it has been documented that the cyanolipids and hydroxynitrile glucosides co-exist and that their occurrence might be biosynthetically connected (Mikolajczak, 1977; Selmar et al., 1990). Leucine was demonstrated to be the most likely precursor of the γ-hydroxynitrile lipids **C15**–**C16** in *Koelreuteria paniculata* Laxm. (Sapindaceae) and of **C14** in *Cardiospermum grandiflorum* forma *hirsutum* (both Sapindaceae) (Seigler and Butterfield, 1976; Seigler and Kennard, 1977). Enzymatic breakdown of **C13**–**C14** produces the α-hydroxynitriles as evident from HCN release detected upon wounding (Selmar et al., 1990). Out of 17 investigated species, the α- and γ-hydroxynitrile lipids were only reported to co-occur in five (Mikolajczak, 1977). Nevertheless, cyanolipids most likely represent yet a different variation derived from the biosynthetic pathway of the hydroxynitrile glucosides found in the same species, with esterification to lipids possibly serving specific purposes, e.g. with respect to storage, transport or herbivore uptake.

3. To be or not to be CYP71$_{\alpha\beta\gamma}$

3.1. Evolution in the CYP71 family

All characterized members of the CYP79 family convert amino acids to the corresponding *Z*-oximes. This functionality is thus conserved across the biosynthetic pathways for cyanogenic glucosides

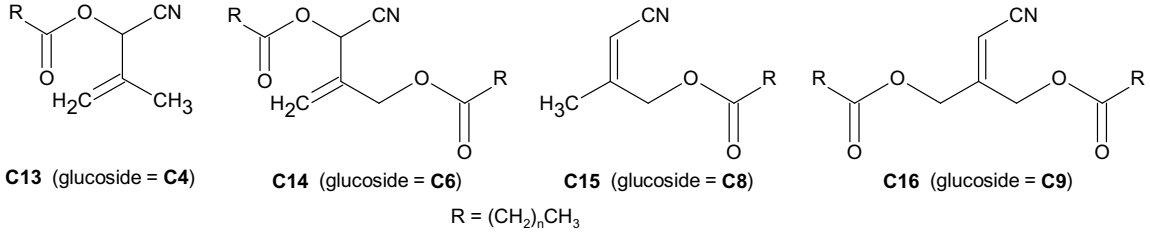


Fig. 6. Cyanolipids isolated from the Sapindaceae.

and glucosinolates (Bak et al., 2006; Nafisi et al., 2006). In contrast, the CYP71 family is functionally diverse and enzymes within this family play key roles in emergence and evolution of several different classes of natural products (e.g. reviewed by Nielsen and Møller (2005)).

In biosynthesis of the grass specific benzoxazinoid defense compounds, DIBOA/DIMBOA and their glucosides, the first four enzymes converting indole to DIBOA by four consecutive hydroxylations are all members of the CYP71C subfamily (Frey et al., 1997; Glawischnig et al., 1999). Although the individual enzymes are highly specific for each their substrate, they have evolved from gene duplications from a common precursor early in the evolution of grasses (Gierl and Frey, 2001; Glawischnig et al., 1999).

The enzymes belonging to the CYP71D subfamily hydroxylate a range of different types of substrates in the biosyntheses of structurally diverse natural products such as alkaloids, flavonoids and terpenoids (Nielsen and Møller, 2005). In *Mentha* species, CYP71D18 and CYP71D13/D15 catalyze stereo- and regio-specific hydroxylations of (−)-4*S*-limonene at, respectively, the C6- and C3-positions to form intermediates in (−)-menthol and (−)-carvone biosynthesis (Lupien et al., 1999; Schalk and Croteau, 2000). Site-directed mutagenesis of CYP71D18 showed that substitution of a single amino acid residue changed the regiospecificity of the enzyme from a limonene-C6-hydroxylase to afford a limonene-C3-hydroxylase with catalytic efficiency comparable to CYP71D13 and CYP71D15 (Schalk and Croteau, 2000). A very interesting member of the CYP71D subfamily is CYP71D20, the 5-epi-aristolochene-1,3-dihydroxylase (EA-1,3-dihydroxylase) from *Nicotiana tabacum* L. (Solanaceae). This enzyme performs two successive stereo- and regio-specific hydroxylations of its sesquiterpene substrate (EA) to produce the phytoalexin capsidiol found in several solanaceous species (Ralston et al., 2001; Takahashi et al., 2005). The enzyme will apparently di-hydroxylate other substrates with backbone structures and functional groups similar to EA (Greenhagen et al., 2003) in analogy to the properties of the CYP71$_\alpha$ enzymes. Substitution of a single amino acid residue by site-directed mutagenesis resulted in a highly promiscuous enzyme producing a mixture of stereo- and regio-unspecific 1-, 2- or 3-hydroxy-EAs and the corresponding ketones (Takahashi et al., 2005). While the capacity for performing two hydroxylations of the same substrate was lost, the overall catalytic efficiency of the mutated enzyme for conversion of EA into a total of seven different monooxygenated products (alcohols and ketones) was two-fold higher than that of the original conversion into capsidiol (Takahashi et al., 2005).

The oxime-metabolizing enzymes of the Brassicales involved in glucosinolate biosynthesis constitute another example of the diversity and plasticity of the CYP71 family (reviewed by Bak et al. (2006) and Nafisi et al. (2006)). The oximes are produced by CYP79s most likely recruited from the cyanogenic glucoside biosynthetic pathway (Bak et al., 2006; Nafisi et al., 2006). In *A. thaliana*, the aliphatic, aromatic and indole-derived oximes are metabolized by CYP83A1 and CYP83B1 (Bak et al., 2001; Bak and Feyereisen, 2001; Hansen et al., 2001; Naur et al., 2003). The oximes are N-oxidized into either aci-nitro- or nitrile oxide-intermediates to which a cysteine residue is subsequently coupled by an unidentified enzyme (Hansen et al., 2001). According to their amino acid sequence identity to CYP71E1 and the general phylogenetic clustering of CYP83s and CYP71s, the CYP83s do in fact belong to the CYP71 family but for historical reasons the CYP83 family has been retained (Bak et al., 2006). Accordingly, it is possible that the CYP83s are CYP71$_\alpha$ paralogs recruited from the cyanogenic glucosides pathway. However, the mechanistics of the CYP83A1/B1 and CYP71$_\alpha$ catalyzed reactions and any possible evolutionary connection remain to be resolved (Bak et al., 2006). The *A. thaliana* CYP71A13 of the camalexin biosynthetic pathway is a monofunctional enzyme, which catalyzes the dehydration of the tryptophan-derived oxime to form the corresponding nitrile in a reaction homologous to the first reaction of the multifunctional CYP71$_\alpha$ in the cyanogenic glucoside pathway (Nafisi et al., 2007). No evolutionary connection has been established between CYP71E1 and CYP71A13 either. However, the occurrence of the phytoalexin camalexin is restricted to one family (Brassicaceae) of the glucosinolate-producing Brassicales (Nafisi et al., 2006). Since the Brassicales order does not contain cyanogenic glucosides it seems that CYP71A13 cannot have evolved directly from CYP71E1.

3.2. Multifunctionality in plant natural products biosynthesis

Multifunctional P450s are not uncommon in the biosynthetic pathways resulting in the formation of natural products in plants. The *A. thaliana* CYP701A3 catalyzes two or three consecutive C-hydroxylations of ent-kaurene to form ent-kaurenoic acid in the biosynthesis of giberellin (Helliwell et al., 1999). CYP88A orthologs from *A. thaliana* and *H. vulgare* catalyze two consecutive C-hydroxylations, a C–O dehydration and another C-hydroxylation of ent-kaurenoic acid to form the di-acid gibberellin GA_{12} (Helliwell et al., 2001). CYP720B1 from *Pinus taeda* (Loblolly pine, Pinaceae) performs a similar reaction with various diterpenoids as substrates to produce diterpene resin acids (Ro et al., 2005). Although the enzyme was indeed found to be capable of converting terpenes to carboxylic acids, the activity was much higher when the initial substrate was a terpenol or terpenal (Ro et al., 2005). This perhaps indicates that CYP720B1 is half-way in the evolutionary process of becoming a fully multifunctional enzyme capable of introducing the whole carboxylic acid functional group into a terpene with high activity. Evolutionarily this would represent achieving stronger binding of the terpene while upholding the ability to retain the more polar mono- and di-hydroxylated products within the active site of the enzyme to facilitate the subsequent C-hydroxylations. A similar evolution has been proposed for CYP79A1 which mediates two consecutive *N*-hydroxylations of tyrosine to produce *N*-hydroxytyrosine and *N*,*N*-dihydroxytyrosine. The latter is converted into *Z*-*p*-hydroxyphenylacetaldoxime which within the same active site may be further hydroxylated to produce 1-nitro-2-(*p*-hydroxyphenyl)ethane (Halkier and Møller, 1990). Likewise, CYP71D20 which produces the di-hydroxylated capsidiol, may have evolved from a more promiscuous mono-hydroxylating enzyme as illustrated by the site-directed mutagenesis study described above. It was demonstrated that the enzyme has greater affinity for the mono-hydroxylated product than for the initial sesquiterpene substrate (Takahashi et al., 2005). Substrate docking models and point mutation studies indicated that the amino acid residue determining the stronger affinity for the second hydroxylation also played a role in facilitating the substrate regio- and stereo-specificity in the first hydroxylation (Takahashi et al., 2005). This illustrates how small changes in the amino acid sequence of an enzyme can simultaneously provide multifunctionality and enhance substrate specificity.

The flavonoid 3′,5′-hydroxylases (F3′5′Hs) are CYP75 family enzymes which hydroxylate two different C-atoms of the same substrate much like the CYP71D20 synthesis of capsidiol (Menting et al., 1994; Seitz et al., 2006). Phylogenetic analyses indicate that the F3'5'H's have evolved from the 3′-flavonoid mono-hydroxylases (F3′Hs) (Seitz et al., 2006). Interestingly, phylogenetic analyses also showed that the three identified F3′5′Hs of the Asteraceae are closer related to the known F3′Hs than they are to the F3′5′Hs found in the rest of the plant kingdom, which all fall into a different cluster together. This suggests that the F3′5′Hs have evolved at least twice from the same enzymatic precursor (Seitz et al., 2006).

3.3. Indications of $CYP_{\alpha\beta\gamma}$ activity in biosynthesis of β- and γ-hydroxynitrile glucosides

Given the history of family CYP71 evolution as outlined above, it is difficult to imagine that the pathway for synthesis of β- and γ-hydroxynitrile glucosides should not have evolved at least in part from $CYP71_{\alpha}$ paralogs. The simplest explanation for the genetic inheritance of β- and γ-hydroxynitrile glucosides formation observed in *L. japonicus* is the existence of a CYP71A13 ortholog as the single oxime-metabolizing enzyme in the pathway, providing nitrile substrates for subsequent hydroxylating and dehydrating enzyme(s). However, as demonstrated in detail for the proposed pathway of isoleucine-derived hydroxynitrile glucosides in Fig. 4 all the known leucine- and isoleucine-derived compounds can also be accounted for by the action of a single $CYP71_{\alpha\beta\gamma}$ enzyme. The hypothesized presence of two paralogs in *L. japonicus*, $CYP71_{\alpha}$ and $CYP71_{\alpha\beta\gamma}$, would also explain the demonstrated inheritance of presence or absence of the β- and γ-hydroxynitrile glucosides (Bjarnholt et al., 2008; Forslund et al., 2004; Morant et al., 2007). However, as the β-hydroxylated α-hydroxynitrile glucoside **B3** has not been identified in *L. japonicus*, the compounds known to exist in this plant (Fig. 2) can in fact be explained by the presence of two enzymes of distinct functions, $CYP71_{\alpha}$ and $CYP71_{\beta\gamma}$, acting independently (Fig. 4). This also satisfies the observed inheritance in *L. japonicus* and the variation seen in *Rhodiola kirilowii* (Regel) Maxim. where one accession lacked α-hydroxynitrile glucosides (Bjarnholt et al., 2008). The entire set of hydroxynitrile glucosides found to co-exist in *H. vulgare* can also be explained by the action of a putative $CYP71_{\beta\gamma}$ acting in parallel with a $CYP71_{\alpha}$ (not shown) (Nielsen et al., 2002). In fact, the only leucine- and isoleucine-derived hydroxynitrile glucosides described here which are not accounted for by the existence of two parallel oxime-metabolizing enzymes, are the hydroxylated and/or unsaturated α-hydroxynitrile glucosides, **B3** from *Rhodiola* (Nakamura et al., 2007) and **C3**–**C6** from *Acacia* (Brimer et al., 1981; Ettlinger et al., 1977; Seigler et al., 1974). These require α-hydroxylation as well as β- or γ-hydroxylation and/or β-γ-desaturation. Combinations of distinct $CYP71_{\alpha}$, $CYP71_{\beta}$ and $CYP71_{\gamma}$ paralogs acting in series such as documented for the CYP71Cs of the benzoxazinoid pathway would explain all the compounds including **B3** and **C3**–**C6**. A single gene regulating the expression of all these CYP71 paralogs, e.g. a transcription factor, could then account for the observed inheritance in *L. japonicus*. However, the diverse array of compounds co-occurring in different combinations would require that between one and three CYP71 paralogs operate in different orders at the same time (Figs. 2 and 4). A more evident explanation is that **B3** and **C3**–**C6** result from secondary modifications either before or after the glucosylation reaction. Such a pathway for secondary modifications operating in *Rhodiola* and *Ribes* may also account for the lack of hydroxynitrile glucosides with multiple hydroxylations (**B3** and **B9**) in *Lotus* species (Bjarnholt et al., 2008). Similar pathways could be responsible for the epoxides **B10** and **C11**–**C12** in Rosaceae and *Sedum* species (Lechtenberg et al., 1996; Nahrstedt et al., 1982).

In conclusion, the most straight forward explanation for a unifying route enabling synthesis of the entire set of hydroxynitrile glucosides and taking into account the inheritance and variation patterns remains the existence of a $CYP71_{\alpha\beta\gamma}$ or $CYP71_{\beta\gamma}$ operating in parallel with a $CYP71_{\alpha}$, in some plant species possibly accompanied by enzymes catalyzing secondary modifications. As previously mentioned, the mechanistic details of the $CYP71_{\alpha}$ reaction have not been resolved. As demonstrated for the dihydroxylating CYP71D20 there could be a tight coupling between the functions needed to position the initial substrate (oxime) for the first reaction (oxime dehydration) and retention/positioning of the intermediate (nitrile) for the second reaction (nitrile α-hydroxylation). Under assumption of such a functional linkage between the different steps, it is interesting to note the similar geometry of the proposed substrates and intermediates in the $CYP71_{\alpha}$ and the $CYP71_{\gamma}$ reactions (Fig. 7). The $CYP71_{\alpha}$ enzymes hydroxylate the C-atom which becomes allylic with respect to the newly formed triple bond (Fig. 7). As demonstrated in Fig. 7, the proposed $CYP71_{\gamma}$ likewise hydroxylates the allylic C-atom hypothetically resulting from dehydration of the α-hydroxynitrile by the same enzyme. The molecular distance between the abstracted hydroxyl group and the added one is approximately the same. If the proposed tight coupling between the two reactions exists, this would indicate that the ability to produce γ-hydroxynitriles from α-hydroxynitriles would require a relatively small change of the $CYP71_{\alpha}$ enzyme.

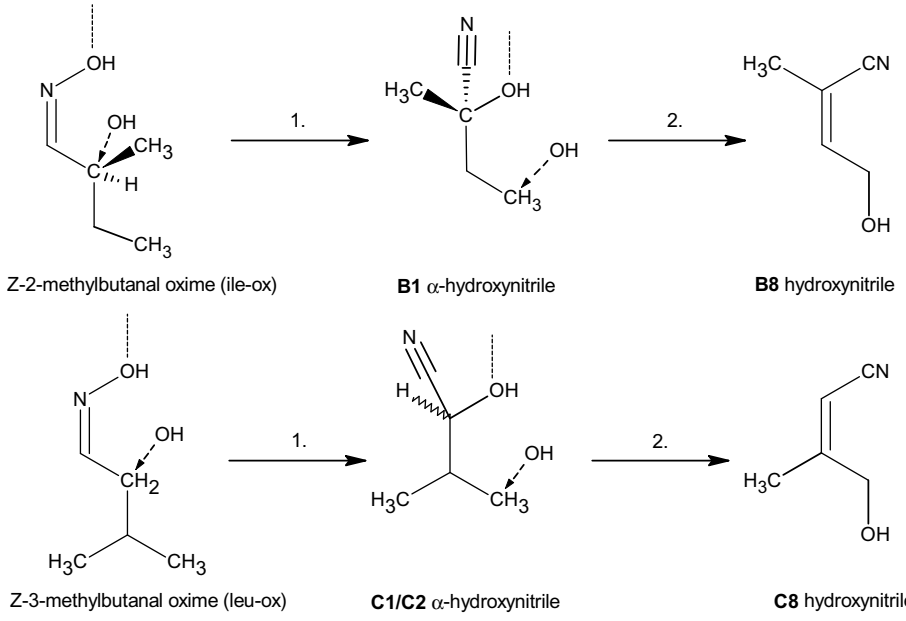


Fig. 7. Proposed $CYP71_{\alpha\beta\gamma}$ facilitated synthesis of unsaturated γ-hydroxynitriles. (1) The oxime is dehydrated and then hydroxylated to produce the α-hydroxynitrile which is released and repositioned. (2) The α-hydroxynitrile is dehydrated and then hydroxylated at the γ-position.

Thus, if the enzymes involved in β- and γ-hydroxynitriles have indeed evolved from CYP71$_{\alpha}$, it is reasonable to assume that α-β unsaturated γ-hydroxynitriles would be the most dominant product. This is consistent with the observation that these constitute 45–75% of total hydroxynitrile glucosides in the majority of *H. vulgare*, *Ribes*, *Lotus* and *Rhodiola* species and cultivars (Bjarnholt et al., 2008; Nielsen et al., 2002). In the Rosaceae, the unsaturated γ-hydroxynitriles and their epoxide derivates were likewise reported to make up 88–100% of total hydroxynitrile glucosides (Lechtenberg et al., 1996). Clearly, the saturated and/or β-hydroxylated hydroxynitriles would require further evolution, but a similar hypothetical mechanism of C–C dehydration of an initially β- or γ-hydroxylated nitrile and hydroxylation of the allylic C-atom would lead to formation of **B4**–**B5** and **C4**–**C6**. It should be mentioned that known P450 mediated oxime dehydration is thought to proceed via initial binding of the nitrogen atom to the iron atom of the heme group (Boucher et al., 1994; Konishi et al., 2006; Morant et al., submitted for publication). The mechanism for C–C dehydration proposed here does not attempt to address whether N–Fe-binding is also a prerequisite for this reaction and whether binding of the α-hydroxynitrile in the CYP71 active site could facilitate the dehydration.

3.4. Evolutionary relationship between plants harboring putative CYP71$_{(\alpha)\beta\gamma}$ enzymes

While *H. vulgare* belongs to the monocots (Poales), the remaining plants producing the β- and γ-hydroxynitrile glucosides and lipids described here are all found in the core eudicots (Soltis and Soltis, 2004; The Angiosperm Phylogeny Group, 2003). Identification of the genes responsible for β- and γ-hydroxynitrile glucosides biosynthesis will disclose whether they have a common ancestor, which was present prior to the segregation of monocots and eudicots. If so, β- and γ-hydroxynitrile glucosides would be expected to be produced in older eudicots as well. Currently, the isoleucine-derived β- and γ-hydroxynitrile glucosides have only been found to occur in the monophylic order Saxifragales (Grossulariaceae and Crassulariaceae), in Fabales (Fabaceae) and in Malphigiales (Euphorbiaceae) (both eurosids I). The latter order also harbors the cyclopentenylglycine-derived compounds, albeit in different families. The leucine-derived compounds are found in Fabales (Fabaceae), Sapindales (Sapindaceae and Hippocastanaceae) (eurosids II) and Rosales (Rosaceae) (eurosids I).

It has been argued that the Saxifragales are closer related to the rosids clade than the two are to the asterids clade (Soltis and Soltis, 2004; The Angiosperm Phylogeny Group, 2003) (Fig. 8A). The proposed tight phylogenetic relationship between Saxifragales and rosids is interesting because aliphatic cyanogenic glucosides do occur in the asterids whereas the β- and γ-hydroxynitrile glucosides have not yet been identified in this clade (Lechtenberg and Nahrstedt, 1999). If scheme A (Fig. 8) for the connection between Saxifragales and rosids is accepted, it implies that the biosynthetic pathway for production of β- and γ-hydroxynitrile glucosides have evolved twice in evolution; once in the Poales after the divergence of monocots and eudicots and once after divergence of rosids and asterids, but before divergence of Saxifragales and rosids. As previously mentioned, this is also speculated for the aliphatic cyanogenic glucosides and the glucosinolates (Bak et al., 2006) and is more or less established for the F3′5′Hs (Seitz et al., 2006). The occurrence of a series of seemingly tyrosine-derived γ-hydroxynitrile glucosides further complicates the overall picture. These compounds have been identified in families scattered all over the eudicots including older eudicots, rosids and asterids (e.g. reviewed by Lechtenberg and Nahrstedt (1999)). Although a biosynthetic relationship between these apparently tyrosine-derived γ-hydroxynitrile glucosides and the tyrosine-derived cyanogenic glucosides has been proposed, the experimental evidence for such a relationship is lacking. The biosynthetic precursors have not been identified, the compounds have only rarely been reported to co-occur with cyanogenic glucosides, and their putative biosynthesis involves multiple hydroxylations of the aromatic ring as well as subsequent methylations, reactions that would require more substantial evolution of the biosynthetic pathway (Seigler et al., 2005).

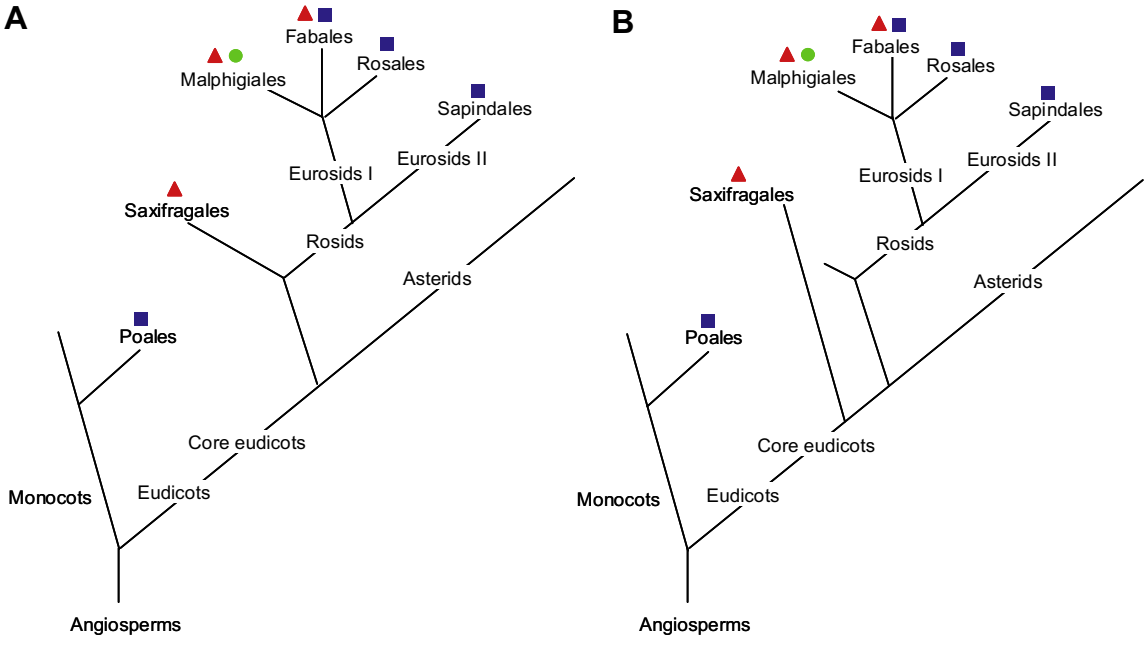


Fig. 8. Simple schematic of the evolutionary relationship between families harboring β- and γ-hydroxynitrile glucosides or lipids and cyclopentenyl cyanogenic glucosides. Only orders and families referred to in the text are shown. Evolutionary distances are not implied. Red triangles = isoleucine-derived, blue squares = leucine-derived, green circles = cyclopentenylglycine-derived. (A) The relation between Saxifragales and rosids according to The Angiosperm Phylogeny Group (2003). (B) The relation according to Soltis and Soltis (2004). (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

4. Biological function of hydroxynitrile glucosides and lipids

4.1. Cyanogenic glucosides and cyanolipids

While it is plausible that cyanogenesis has originally evolved for the purpose of plant defense, the role and functions of cyanogenic glucosides in plants today is mottled. It is clear that specialist plant pathogens and herbivores have co-evolved to circumvent cyanogenesis based defense strategies. Their countermeasures are numerous, including feeding strategies to avoid HCN release, unique HCN detoxification systems, or even sequestration enabling herbivores to utilize HCN or cyanogenic glucosides in defense, communication or primary metabolism (Engler et al., 2000; Gleadow and Woodrow, 2002; Seigler, 1991; Zagrobelny et al., 2004, 2007b). Cyanogenic glucosides may also play a role in plants as storage forms of reduced nitrogen. The biosynthesis of **D** in *S. bicolor* is transcriptionally induced by application of NO_3^- fertilizer to 5 weeks old plants (Busk and Møller, 2002). In *M. esculenta* the concentration of **A** and **B1** increases in the shoot apex upon supply of NO_3^- to cuttings from 6 weeks old plants (Jørgensen et al., 2005a). The recovery of the nitrogen stored in cyanogenic glucosides has been suggested to proceed via the initial β-glucosidase mediated hydrolysis followed by assimilation of the released HCN by the general pathway operating in plants for detoxification of HCN, e.g. produced during ethylene biosynthesis (Peiser et al., 1984). The HCN is incorporated into β-cyanoalanine by β-cyanoalanine synthase (CAS) (Floss et al., 1965; Miller and Conn, 1980; Watanabe et al., 2008). The level of CAS activity is generally higher in plants producing cyanogenic glucosides compared to non-cyanogenic plants (Miller and Conn, 1980). The β-cyanoalanine is subsequently converted to asparagine and aspartic acid + NH_3 by the combined action of nitrile hydratases/hydrolases (NIT4) (Castric et al., 1972; Piotrowski et al., 2001; Piotrowski and Volmer, 2006). Indications of turnover of cyanogenic glucoside nitrogen into primary metabolism via the HCN–CAS–NIT4 pathway have been observed in *Lotus tenuis* L. and *L. arabicus* L. (Abrol and Conn, 1966). Upon administration of L-[U-^{14}C]-valine to apical leaves, 2.7–9.7% of the radiolabel was incorporated into **A** while another 0.4–1.3% was recovered in asparagine. Most of the label was found in the amide C-atom, consistent with incorporation via the HCN–CAS–NIT4 pathway (Abrol and Conn, 1966). It was recently discovered that grasses (Poaceae) possess different NIT4 isoforms (NIT4A and NIT4B) which are individually inactive but form active heteromers. The heteromers may hydrolyze other nitriles than β-cyanoalanine to release NH_3 (Jenrich et al., 2007). For the *S. bicolor* NIT4A/NIT4B2 heteromer the best substrate found was the tyr-nitrile, which was also demonstrated to be a possible product from an alternative degradation pathway for **D**. The operation of such a catabolic pathway would enable grasses to utilize the nitrogen atom of the cyanogenic glucoside without release of toxic HCN.

Cyanogenic di-glucosides have been suggested to serve as transport/storage forms of cyanogenic glucosides and/or reduced nitrogen (Lieberei et al., 1985; Sánchez-Pérez et al., 2008; Selmar et al., 1988; Swain and Poulton, 1994). In some species which accumulate cyanogenic (di-)glucosides in seeds the glucosides have been demonstrated to be degraded during germination without release of HCN to the surroundings (Lieberei et al., 1985; Swain and Poulton, 1994). It was suggested that the cyanogenic (di-)glucosides from the seeds were transported and metabolized to supply nitrogen to the developing seedling where the highest CAS activity was located (Lieberei et al., 1985; Swain and Poulton, 1994). About 20% of the nitrogen stored in seed of *Ungnadia speciosa* Endl. (Sapindaceae) is bound in the cyanogenic cyanolipid **C13** (Selmar et al., 1990). During germination, the cyanolipid disappeared from the seed while the corresponding glucoside **C4** was formed in the seedling. However, only a quarter of the nitrogen bound in cyanolipid was recovered as proacacipetalin; the rest was assumed to be utilized for growth as no HCN was released to the surroundings (Selmar et al., 1990). When L-[U^{14}C]-leucine was administered to fruits of *K. paniculata*, accumulation of radiolabelled cyanolipid **C15**–**C16** in the seeds only occurred if fruits and seeds were immature at the time of amino acid administration. There was no significant turnover of radiolabelled cyanolipids in mature seeds (Seigler and Butterfield, 1976). It appears evident from these data that the cyanolipids are storage and/or transport forms of nitrogen and of the hydroxynitrile glucosides found in the same plants. The distinction between secondary and primary metabolism will vanish as we begin to understand the intimate and important roles of secondary metabolites in plants.

4.2. β- and γ-hydroxynitrile glucosides in H. vulgare

The hydroxynitrile glucosides of *H. vulgare* accumulate in the leaf epidermal cells. This is the site of attack, growth and reproduction of the fungus *Blumeria graminis* f. sp. hordei, the causal agent of the economically important disease powdery mildew (Nielsen et al., 2002). There appears to be no β-glucosidase activity in the *H. vulgare* leaves and it has been shown that intact **C2** stimulates germination of the *B. graminis* spores. Conversely, germination in the presence of the β- and γ-hydroxynitrile glucosides **C7**–**C10** (Fig. 2) did not differ from the control (Nielsen et al., 2006). All compounds stimulated further development of the germinated spores but the cyanogenic **C2** was the best stimulant; the poorest stimulation was seen on γ-hydroxynitrile glucoside **C10** which constitutes 35–47% of hydroxynitrile glucosides in four commercial *H. vulgare* cultivars (Nielsen et al., 2002, 2006). When the compounds were hydrolyzed before addition of spores, germination was not induced; among the germinated spores, development was stimulated by **C2** α-hydroxynitrile but not by the β- and γ-hydroxynitriles (Nielsen et al., 2006). Thus, the ability of *H. vulgare* to produce β- and γ-hydroxynitrile glucosides instead of α-hydroxynitrile glucosides may prevent attack by the fungus. However, no clear connection has been established between hydroxynitrile glucosides concentration and susceptibility to powdery mildew. Nielsen et al. (2006) did not see any correlation between concentration and susceptibility in a study of near isogenic lines where the same set of resistance related genes was expected to be present and similarly expressed.

4.3. Alternative nitrogen storage or defense compounds?

With the discovery of the grass NIT4A/NIT4B heteromer-activities it is evident that the β- and γ-hydroxynitrile glucosides may serve as alternative storage compounds for nitrogen. The expansion of the cyanogenic glucoside biosynthetic pathway to produce β- and γ-hydroxynitrile glucosides would provide the host plant with storage compounds which do not release toxic HCN during nitrogen re-assimilation. It would also reduce the nutritious value of the plants to pests that have evolved the ability to exploit HCN in their metabolism. Jenrich et al. (2007) suggested that *S. bicolor* has evolved an alternative catabolic pathway for the cyanogenic glucoside **D**, which results in formation of the nitrile serving as a substrate for the *Sb*NIT4A/NIT4B2 heteromer. This is consistent with the idea that *S. bicolor* still appears to depend on cyanogenesis for defense (Seigler, 1991). On the contrary, *Lotus* species are the preferred host plants for *Zygaena* moths and larvae, which sequester **A** and **B1** for their own defense, signaling and primary metabolism (Zagrobelny et al., 2004, 2007b). In a study of this interaction, larvae of *Z. filipendulae* were reared either on *L. corniculatus* which only contains **A** and **B1** or on *L. japonicus* harboring

α- and β-/γ-hydroxynitrile glucosides in a 60:40 ratio ((**A** + **B1**):(**B4** + **B8**) (Fig. 2)) (Zagrobelny et al., 2007a). Although the total concentrations of hydroxynitrile glucosides in the two plants were the same, the larvae developed poorly on *L. japonicus* compared to *L. corniculatus*. Newly hatched larvae did not even survive when reared on an accession of *L. corniculatus* devoid of hydroxynitrile glucosides (Zagrobelny et al., 2007a). Accordingly, *L. japonicus* has nothing to lose in the battle against this specific herbivore by abolishing the cyanogenesis based defense strategy. The same may be the case for *H. vulgare* which has apparently lost its β-glucosidases and hence capability for cyanogenesis during cultivation (Nielsen et al., 2006).

A few aliphatic γ-hydroxynitrile glucosides and related products have also been found in arthropods, adding yet another dimension to the discussion. The isoleucine-derived **B9** may turn out to be quite common in moths and butterflies with reported findings in species of *Abraxas*, in *Parnassius phoebus* and possibly a zygaenid moth (Nishida et al., 1994; Nishida and Rothschild, 1995). The magpie moth, *Abraxas grossulariata* L. has been described as "a pest where currants and gooseberries are cultivated", i.e. *Ribes* species (Nishida et al., 1994). It appears that **B9** may be *de novo* synthesized by *Abraxas* as the moths and pupae contained high amounts of the compound independent of whether they were reared on *Prunus* or *Euonymus* species (respectively, Rosaceae and Celastraceae) (Nishida et al., 1994). While *Euonymus* may contain **B9** (Nishida and Rothschild, 1995), *Prunus* has to date exclusively been reported to contain phenylalanine-derived cyanogenic glucosides and di-glucosides. The butterfly *P. phoebus* feed on *Sedum* spp. (Crassulaceae) which also contain **B9** and **B10** (Nahrstedt et al., 1982; Nishida and Rothschild, 1995). Larvae of *Z. philipendulae* sequestered β- and γ-hydroxynitrile glucosides **B4** and **B8** from *L. japonicus* along with the α-hydroxynitrile glucosides **A** and **B1**, but **B4** and **B8** were not transferred from the last instar larvae to the adult moths (Zagrobelny et al., 2007a). On the other hand, the *Abraxas* larvae and moths were stated to be strongly repellent to predators and **B9** was reported to be bitter, which could imply a defensive role (Nishida et al., 1994). Likewise, several true bugs contain the leucine-derived sutherlandin (**C9**). Larvae and adults of *Jadera* species and *Leptocoris isolata* (scentless plant bugs, Rhopalidae) feed on Sapindaceae seeds which contain the leucine-derived cyanolipids (Aldrich et al., 1990; Braekman et al., 1982). When larvae and bugs of *Jadera* were fed seeds containing the cyanogenic cyanolipid **C14** their body fluids contained the corresponding cyanogenic glucoside **C6**. When the seeds contained the γ-hydroxynitrile cyanolipids **C15**–**C16**, they contained the corresponding γ-hydroxynitrile glucosides **C8** and **C9** (Aldrich et al., 1990). "Experienced predators" would not eat the larvae while "naive predators" would eat them once, only to avoid them and the adult bugs the next time offered (Aldrich et al., 1990). The *L. isolata* larvae were collected in the field and analyzed without previous controlled feeding. Larvae and adults contained **C6** and apparently **C9** (stereochemistry was not assigned) (Braekman et al., 1982). Although **C6** was reported to be the strongest repellent to predating ants, **C9** was reported to deter ants effectively just as stated for the isoleucine-derived homolog **B9** (Braekman et al., 1982; Nishida et al., 1994).

Upon feeding on the seeds containing cyanolipids, the *Jadera* larvae and bugs also accumulated the lactone **F1** (Aldrich et al., 1990) (Fig. 9). Likewise, the *L. isolata* adults contained a lactone glucoside **F5** (Braekman et al., 1982). Fig. 9 shows how **F1** could be formed from **C8**. The suggested formation proceeds in analogy to the *Sb*NIT4A/NIT4B2 pathway via hydrolysis of the hydroxynitriles to form γ-hydroxy acids which spontaneously esterify to form the lactones (Carey, 1992). None of the known leucine-derived hydroxynitrile glucosides directly explain the lactone glucoside **F5** also found in *L. isolata* (Braekman et al., 1982). The authors could not determine the stereochemistry of the **C9** isomer isolated from *L. isolata* and it was later suggested that it was in fact the *E*-isomer (Swenson et al., 1987). This does not explain the presence of **F5**, as the hydroxynitrile of the *E*-isomer of **C9** would also lead to formation of **F2**. Conversely, the lactone glucoside **F5** can be formed upon migration of the double bond in **C6** followed by nitrile hydrolysis and lactone formation (Braekman et al., 1982) (Fig. 9). Such a double bond migration yielded the lactone **F4** when **C14**, the cyanolipid corresponding to **C6**, was hydrolyzed with HCl/MeOH (Mikolajczak, 1977). Likewise, dilute base causes double bond migration in **C4** (Ettlinger et al., 1977). The *Jadera* larvae were shown to be attracted by the **F1** lactone (Aldrich et al., 1990), indicating that the compound may also exist in its food plant, e.g. as a result of nitrogen utilization by the nitrilase pathway. Furanones (e.g. lactones) with multiple hydroxyl-, methyl- and ethyl-substituents are widely abundant in plants. They are important aroma compounds in e.g. strawberry, raspberry, pineapple and tomato, where they are mainly thought to be produced from carbohydrates (reviewed by Slaughter (1999)). Furanones from plants, algae and fungi have been reported to posses antibacterial and antifungal effects (Slaughter, 1999; Sung et al., 2007) and one furanone apparently functions as a pheromone in a cockroach (*Eurycolis florionda* (Walker)) (Slaughter, 1999). The lactones in Fig. 9 have not been described. The reported strong odor of the **F4** lactone was burnt sugar or caramel (Mikolajczak, 1977) where as the **F6** lactone has a rotting wood-/fungi-like odor (purchased from *Aldrich*).

In conclusion, the β- and γ-hydroxynitrile glucosides do appear to play a significant biological role in plants and herbivores with respect to nitrogen storage and metabolism, defense and/or signaling. In the case of cyanogenic glucosides, their deterrent effect on generalist herbivores is reflected in the utilization of these compounds by arthropods for their own defense. Conversely, the deterring effects on predators of arthropods containing γ-hydroxynitrile glucosides most likely reflect similar effects on generalist herbivores of plants containing the compounds. The presence of the lactones strongly supports that a *Sb*NIT4A/NIT4B2 analogous γ-hydroxynitrile hydrolysis occurs, in plants, arthropods or both. The existence of the lactone glucoside **F5** in the *L. isolata* adults complicates matters, as it indicates that the putative nitrilases can act on either intact glucosides or on α-hydroxynitriles. An explanation may be that the suggested double bond migration stabilizes the α-hydroxynitrile to prevent HCN release and make it available to NIT4B hydrolysis, with the glucoside being a detoxification product. Studies of lactone formation and nitrogen metabolism in plants harboring γ-hydroxynitrile glucosides and their interactions with herbivores will provide more insight into the questions raised here.

5. Biomedicinal effects of hydroxynitrile glucosides

Hydroxynitrile glucosides or plants producing them have frequently been ascribed effects in traditional herbal medicine. Herbal medicine is based on use of either crude plant extracts comprising many different constituents or plant extracts enriched in specific classes of compounds. In a few cases, α-, β- and γ-hydroxynitrile glucosides have indeed been isolated from plants and ascribed pharmacological effects based on animal *in vitro* and *in vivo* assays. *Rhodiola* species are used in traditional herbal medicine across the world (Garcia et al., 2003; Kelly, 2001; Yoshikawa et al., 1996, 1997). *Rhodiola rosea* L. is one of three herbs recognized as "adaptogens", expressing that it has a general positive "adaptive" effect on the body in case of stress (Kelly, 2001; Narimanian et al., 2005). Other specific health effects are also attributed to *R. rosea* and other *Rhodiola* species. Yoshikawa et al. (1996, 1997) isolated

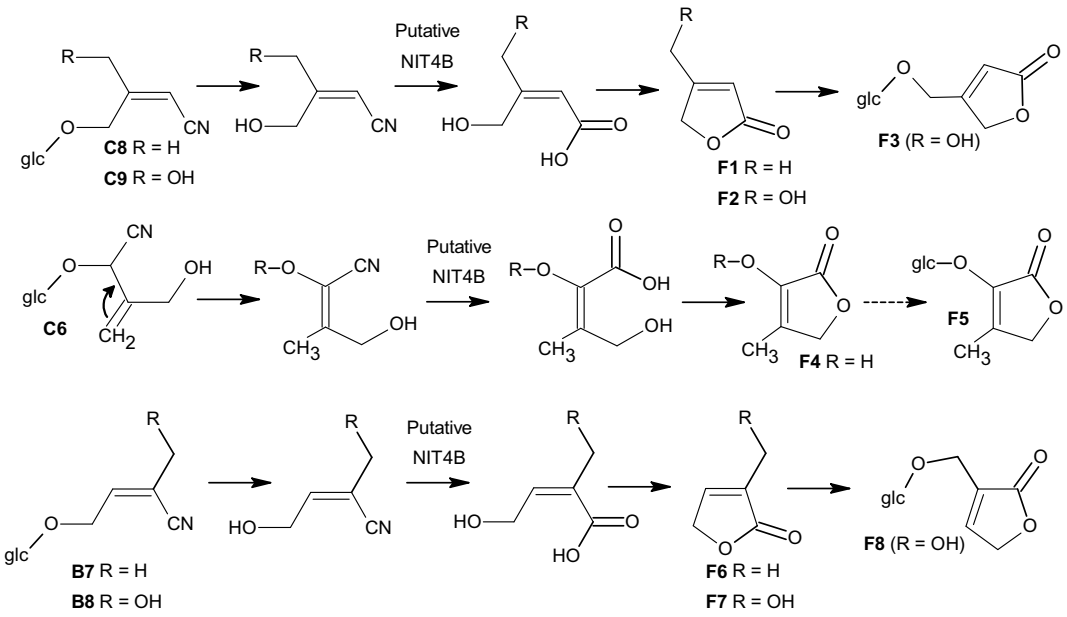


Fig. 9. Suggested formation of lactones and derivatives from γ-hydroxynitrile compounds.

and identified **B4** and **B8** from *Rhodiola* species in an attempt to identify components which could explain the anti-inflammatory, anti-cough and anti-asthma effects ascribed to these plants. They demonstrated that the plant extracts as well as all isolated hydroxynitrile glucosides (**B1**, **B4**, **B8** and **C1**) inhibited histamine release from rat peritoneal cells in allergic response. *Rhodiola* species are also ascribed effects in treatment of Alzheimers disease and as a memory and learning enhancer. However, no studies in which hydroxynitrile glucosides were isolated and tested have shown this type of effects of any of the compounds (Fan et al., 1999, 2001; Mook-Jung et al., 2002).

The γ-hydroxynitrile glucoside **B9** was first isolated from *Sedum sarmentosum* Bunge (Lechtenberg and Nahrstedt, 1999). Herbal extracts from *S. sarmentosum* are widely applied in traditional medicine as treatment against chronic viral hepatitis and **B9** was demonstrated to possess immunosuppressive effects in mice and rats (Zhu et al., 1996). Zhang et al. (2002) speculated that the effect of **B9** is based on the structure of the intact glucoside and synthesized a range of stabilized sarmentosin analogs where the glucose moiety was substituted by more stable structures. An effect of these compounds against mouse lymphocyte proliferation was recorded. The reported anti-cancer effects of *Sedum* is thought to be based on the effect against chronic viral hepatitis (Kang et al., 2000). *Rhodiola* species have also frequently been ascribed a direct or indirect effect in cancer treatment and a few successful studies involve preparations which could possibly contain the hydroxynitrile glucosides (Bogdashin et al., 1990; Majewska et al., 2006; Seo et al., 2001; Udintsev and Schakhov, 1991). The cyanogenic glucoside **A** has come the longest way as a potential anti-cancer drug using an enzyme/pro-drug activation approach (Cortes et al., 1998, 2002; Kousparou et al., 2002). The potential treatment relies on HCN release following hydrolysis of **A**. Mammalian cells do not possess the required β-glucosidase activity so this is introduced to the cancer cells either by gene therapy or antibody mediated targeting (Cortes et al., 1998, 2002; Kousparou et al., 2002). Subsequent administration of **A** results in HCN release in the tumor, whereas the healthy tissue remains unharmed by the intact **A**. The approach has been shown to be efficient on rat tumors *in vivo* (Cortes et al., 1998) and on human cancer cells *in vitro* (Cortes et al., 2002; Kousparou et al., 2002).

van den Berg et al. (1995) isolated **B8** (under the name "multifidin") from the latex of *J. multifida*. The latex is used in folk medicine for treatment of infected wounds (Kosasi et al., 1989). The active components have not been identified but Kosasi et al. (1989) found immunomodulating activity in a water soluble fraction of latex, which would contain the highly polar hydroxynitrile glucosides if present (the authors ascribed the effect to an anthocyanin). Mcgaw and Eloff (2005) and Aiyelaagbe (2001) found antimicrobial effects of leaves or root and bark extracts of *J. multifida*. Similar anti-microbial or anti-inflammatory activities have also been ascribed to extracts from species of *Rhodiola* (Abidov et al., 2004; Ming et al., 2005; Narimanian et al., 2005), *Sedum* (Garcia et al., 2003; Kim et al., 2004) and *Ribes* (Barak et al., 2002; Declume, 1989; Werlein et al., 2005) likely to contain hydroxynitrile glucosides.

6. Conclusions – are the γ-hydroxynitrile glucosides and lipids the endpoint of hydroxynitrile evolution?

The strong dominance of γ-hydroxynitrile glucosides and lipids in plants as described above and by Bjarnholt et al. (2008) implies a biological function. While the plants are clearly capable of producing β-hydroxynitriles, serving the hypothetical purpose of being non-cyanogenic feeding deterrents or nitrogen storage compounds, the majority of non-cyanogenic compounds are γ-hydroxynitrile glucosides. In the Sapindaceae γ-hydroxynitrile glucosides and lipids are apparently the only alternative to α-hydroxynitrile compounds. As hypothesized above, this may be the result of a pathway where the new function of the CYP71$_\alpha$ paralog is to metabolize the α-hydroxynitrile in a manner similar to the oxime. Identification of a beneficial biological function of the lactones will explain this preferential production of γ-hydroxynitrile glucosides and lipids. While β-hydroxyacids will lead to unstable four-membered rings (if formed at all), γ-hydroxyacids form stable five-membered rings.

Most of the plant species here described, including *Ribes* species, *H. vulgare* and the Rosaceae are completely dominated by the hydroxylated γ-hydroxynitrile glucosides (**B9**, **B10**, **C9**, **C11**, **C12**), which require one or two extra hydroxylations to be formed. These compounds are also found in moths and butterflies and

furthermore, various aryl esters of them have been identified in *Ribes*, *Rhodiola* and *Sorbaria* (Rosaceae) (Kim and Zee, 2000; Lu et al., 2002; Schwarz and Hofmann, 2007; Yoshikawa et al., 1996). As postulated for the di-glucosides, these esters may be transport forms, protected from β-glucosidase mediated degradation (Lieberei et al., 1985; Selmar et al., 1988; Swain and Poulton, 1994). In *Ribes*, the esters have been identified in seeds of *Ribes nigrum* (Lu et al., 2002) and in extracts of *R. rubrum* fruit pulp most likely containing seeds (Schwarz and Hofmann, 2007). This is consistent with storage of nitrogen or cyanogenic glucosides for the next generation as suggested for cyanolipids in Sapindaceae (Selmar et al., 1990) and the di-glucosides in *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. (Lieberei et al., 1985; Selmar et al., 1988), *Prunus serotina* (Swain and Poulton, 1994) and *Prunus dulcis* (Miller) D. A. Webb. (Sánchez-Pérez et al., 2008). Investigation of β- and γ-hydroxynitrile glucosides tissue- and developmental-wise localization, possible transport and turnover will provide more insight into the role of the compounds in nitrogen-metabolism.

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