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Mechanistic Advances in Plant Natural Product Enzymes

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Summary of Recent Advances

The biosynthetic pathways of plant natural products offer an abundance of knowledge to scientists in many fields. Synthetic chemists can be inspired by the synthetic strategies that nature uses to construct these compounds. Chemical and biological engineers are working to reprogram these biosynthetic pathways to more efficiently produce valuable products. Finally, biochemists and enzymologists are interested in the detailed mechanisms of the complex transformations involved in construction of these natural products. Study of biosynthetic enzymes and pathways therefore has a wide-ranging impact. In recent years, many plant biosynthetic pathways have been characterized, particularly the pathways that are responsible for alkaloid biosynthesis. Here we highlight recently studied alkaloid biosynthetic enzymes that catalyze production of numerous complex medicinal compounds, as well as the specifier proteins in glucosinosolate biosynthesis, whose structure and mechanism of action are just beginning to be unraveled.

Introduction

Many natural products derived from plants are highly successful pharmaceuticals (Figure 1).[1-4] *Taxus brevifolia* (Pacific Yew) and *Catharanthus roseus* (Madagascar periwinkle) produce the anticancer compounds taxol[5] and vincristine,[6-7] while *Artemisia annua* (sweet wormwood) yields the antimalarial artemisinin.[8] However, these products are frequently produced in low yield in the plants, and the complex structure of these compounds often makes total synthesis costly or impractical on a large scale. Many plant derived compounds are therefore extremely expensive or inaccessible. Understanding the biosynthetic pathways of plant natural product metabolism opens up new approaches for production. Organic chemists can formulate new synthetic approaches to the molecules based on nature's method of production. Moreover, biochemists can engineer biosynthetic pathways to increase production of the medicinal compounds. An understanding of the biosynthetic pathways is of obvious importance and the basis of understanding these pathways lies with the enzymes catalyzing the reactions.

As the genetic tools to identify new plant enzymes has improved, enzymology of plant biosynthesis has flourished, though there is still much to be learned. This review explores significant and recent (after 2006) mechanistic advances in enzymes involved in plant natural products. Specifically, the biosynthetic enzymes involved in the production of nitrogen containing natural products- alkaloids- have been a particularly fruitful area of study. We

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examine the alkaloid biosynthetic enzymes strictosidine synthase, norcoclaurine synthase, and berberine bridge enzyme, and specifier proteins utilized in glucosinolate biosynthesis.

Strictosidine Synthase

Strictosidine synthase,[9-11] originally detected in *Catharanthus roseus* and *Rauwolfia serpentina* 30 years ago,[12,13] is one of the most well-characterized alkaloid biosynthetic enzymes. Strictosidine synthase catalyzes the first committed step of monoterpene indole alkaloid biosynthesis by catalyzing a Pictet-Spengler condensation[14-15] between tryptamine and secologanin to yield strictosidine (Figure 2a). This central biosynthetic intermediate is converted into many alkaloids including vincristine (Figure 1). The crystal structure of strictosidine synthase was recently solved,[16-17] and this structure has paved the way for mechanistic studies, as well as protein design efforts. Structures of strictosidine synthase (*R. serpentina*) complexed with tryptamine, secologanin, and strictosidine have all been reported[16,18] and the residues of the substrate binding pockets have been clearly identified.[16,18]

Glu309 (*R. serpentina*) has been shown by both this structural analysis and by site-directed mutagenesis experiments to be the key residue required for acid- base catalysis in the Pictet-Spengler reaction.[16,19,20] Glu309 likely hydrogen-bonds to the primary amino group of tryptamine, ensuring that it is neutral and therefore nucleophilic. This primary amine attacks the aldehyde of secologanin to form an iminium ion (Figure 2a). To form the strictosidine product, the indole moiety then attacks at the iminium in an electrophilic aromatic substitution reaction creating the (*S*)-stereocenter. In one possible reaction pathway (Pathway A), the C-2 carbon of tryptamine attacks the iminium to form a six-membered ring and a tertiary carbocation. Glu309 then deprotonates, restoring aromaticity, and creating strictosidine. (Figure 2a)

Alternatively (Pathway B), the C-3 carbon attacks the iminium to form a spirocyclic intermediate, which would then rearrange to form a six-membered ring (Figure 2a). *Ab initio* calculations find that the transition state of the spirocyclic intermediate is higher in energy than the transition state for direct C-2 attack.[20] Moreover, the rearrangement of the spirocyclic intermediate to the six-membered ring was found to be energetically unfavorable.[20] Therefore, if the spirocyclic intermediate does form, it is likely a non-productive intermediate, and will simply revert back to the iminium starting material. A tryptamine substrate with a deuterium label at the C-2 position displayed a primary kinetic isotope effect, indicating that all steps prior to re-aromatization are reversible.[20]

Several substrate specificity and redesign studies for strictosidine synthase have been carried out.[3,16-23] Strictosidine synthase will turn over only a few variants of the secologanin substrate, though the enzyme accepts a number of substituted tryptamine analogs with modest steric and electronic perturbations.[19,22,23] The active site of this enzyme can be mutated to accommodate more sterically demanding substrate analogues.[19,23] These substrates, which are not turned over by the natural strictosidine synthase enzyme, are nevertheless turned over by downstream biosynthetic enzymes,[19] suggesting that strictosidine synthase is a "gate-keeper" of substrate specificity in this pathway. Engineered variants of strictosidine synthase can therefore be incorporated into the biosynthetic pathway to produce new alkaloid analogs.[24]

Norcoclaurine Synthase

Norcoclaurine synthase is utilized in the first committed step of the benzylisoquinoline biosynthetic pathway.[25-27] This enzyme, like strictosidine synthase, catalyzes a Pictet-Spengler condensation. Norcoclaurine synthase uses dopamine and 4-hydroxyphenylacetaldeyde (4-HPAA) to yield (S)-norcoclaurine, a central biosynthetic

intermediate in the benzylisoquinoline pathway (Figure 2b).[25-27] Norcoclaurine synthase has been recently cloned from both *Thalictrum flavum*[28-29] and *Coptis japonica*.[30] Notably, in *C. japonica*, two entirely different enzymes catalyzing the reaction were cloned and isolated.[30] One shows strong sequence similarity to the enzyme identified from *T. flavum*, while the other appears to be similar to a 2-oxoglutarate-dependent dioxygenases requiring ferrous ion.[30] It remains to be determined what specific *in planta* roles these two norcoclaurine enzymes have. Notably, neither of the norcoclaurine synthase sequences is at all similar to strictosidine synthase,[31] despite the fact that all of these enzymes catalyze a Pictet-Spengler reaction.

The enzyme identified from *T. flavum*, which shows homology to Bet v 1 allergen and pathogenesis-related (PR)10 proteins,[29,31-33] is the best-characterized of the norcoclaurine synthase enzymes. Norcoclaurine synthase displays sigmoidal saturation kinetics with the dopamine substrate, indicating that there is positive cooperativity in the binding of dopamine.[29] Enzymes exhibiting this type of kinetic property often have a regulatory role in metabolism, suggesting that norcoclaurine synthase may play a role in regulating the metabolic flux of the benzylisoquinoline pathway.

The crystal structure of *T. flavum* norcoclaurine synthase has recently been solved in complex with dopamine and a nonreactive aldehyde, 4-hydroxybenzylaldehyde.[31] The enzyme appears to be a homodimer, where each monomer contains an active site in which the dopamine and aldehyde substrates form a stacked configuration. Interactions of the phenolic hydroxyl groups of the substrates with tyrosine and aspartate residues appear to keep the substrates oriented.

The placement of Lys122, Tyr108, and Glu110 relative to the orientation of the substrates in the active site[31] suggests that these residues may be involved in the catalytic mechanism. Although mutations of Tyr108 and Glu110 result in some loss of enzyme catalysis, mutation of Lys122 to Ala results in a complete loss of activity,[34] suggesting that Lys122 is the key catalytic residue of the enzyme. Positively charged Lys122 is proposed to polarize the carbonyl of the aldehyde substrate, making it more susceptible to nucleophilic attack by the primary amine. Lys122 may also protonate the resulting carbinolamine, driving the loss of water to form the iminium species (Figure 2b). Following iminium ion formation, the dopamine moiety is deprotonated at the C-2 hydroxyl group, which helps drive the attack on the iminium ion to form the norcoclaurine skeleton. Formation of this phenolate ion has been proposed to be mechanistically essential since analogues lacking a C-2 hydroxyl group are not substrates.[34] However, the residues involved in C-2 hydroxyl group deprotonation, or stabilization of the resulting phenolate ion, have not been definitively identified, though Tyr108 may be involved. Glu110 or water could then deprotonate at C-5 to rearomatize the intermediate to form (S)-norcoclaurine. As with strictosidine synthase, a primary kinetic isotope effect with deuterated dopamine was also noted.[34]

Berberine Bridge Enzyme

The berberine bridge enzyme also acts in the benzylisoquinoline alkaloid pathway, and serves to channel (S)-reticuline into the berberine type alkaloids,[35] rather than the morphine type alkaloids (Figure 1). This enzyme catalyzes the regioselective formation of a carbon-carbon bond between the N-methyl group and the C2′ carbon of the phenolic ring of (S)-reticuline to create (S)-scoulerine (Figure 3).[35,36] Notably, this reaction is not readily accomplished by synthetic means. Berberine bridge enzyme (*Eschscholzia californica*) has been mechanistically studied in detail, and has been recently shown to contain covalent linkages to the FAD co-factor at residues His104 and Cys166 (Figure 3).[37,38] Although mutation of His104 to Ala or Thr failed to yield soluble enzyme for mechanistic analysis, the Cys166Ala mutant was readily isolated.[38] This Cys166A mutant has a significantly decreased oxidation potential compared

to the wild type enzyme, indicating that the bicovalent attachment tunes the redox properties of the enzyme.[38]

The X-ray structure of the berberine bridge enzyme in complex with (S)- reticuline was recently elucidated.[36] A variety of protein-substrate interactions were noted, but most importantly Glu417 appears to hydrogen bond to the C3′ OH group of (S)-reticuline.[36] Since substrates lacking the C3′-OH group are not turned over by the enzyme,[39] the C3′ hydroxyl group likely plays a critical role in the mechanism. It is proposed that Glu417 deprotonates the C3′-OH group, thereby enhancing the nucleophilicity of the C2′ carbon so that it can attack the N-methyl group and form the "berberine bridge".[36] Consistent with this hypothesis, the Glu417Gln mutant displays a 1500 fold decrease in the rate of (S)-scoulerine formation.[36] In addition to the attack of the C2′ carbon on the N-methyl group, the enzyme also catalyzes transfer of a hydride from the N-methyl to FAD, thereby reducing the flavin cofactor. The Glu417Gln mutant does not appear to catalyze flavin reduction,[36] suggesting that the attack of C2′ onto the N-methyl and the transfer of hydride to the flavin are coupled processes. Following berberine bridge formation, an unidentified base deprotonates at the C-2 position to restore aromaticity and form (S)-scoulerine (Figure 3).

Specifier Proteins

Specifier proteins are enzymes that work in concert with myrosinases to produce products derived from glucosinolate natural products.[40] Glucosinolates are deglucosylated by myrosinases to yield a reactive aglycone intermediate (Figure 4).[41] In the absence of specifier proteins, the deglycosylated glucosinolates undergo a spontaneous Lossen-type rearrangement[42] to yield isothiocyanates,[43,44] which are believed to protect plants from herbivore and pathogen attack.[43,45-48] In the presence of specifier proteins however, the glucosinolate hydrolysis intermediate is directed away from isothiocyanate formation towards the production of epithionitriles, nitriles, and thiocyantes, depending on the structure of the specific glucosinolate (Figure 4).[40,43] The biological functions of the epithionitriles, nitriles, and thiocyanates are not currently known, but are assumed to have defensive functions.[43,45,46,48] Importantly, the specifier proteins influence the enzymatic reaction by producing different products, but these specifier proteins do not display hydrolytic activity, and can only function in the presence of myrosinase.[40,43]

Rigorous identification and biochemical characterization of the specifier proteins has only recently begun. Two iron-dependent specifier proteins, epithiospecifier protein[49-52] and thiocyanate-forming protein,[53,54] which share high levels of sequence identity, have so far been identified from *Arabidopsis thaliana*,[51] *Brassica napus*,[50] *Brassica oleracea*,[52] and *Lepidium sativum*.[43,53] Each specifier protein contain Kelch motifs[52,55] that are known to play a role in protein-protein interactions.[56] It is therefore logical to speculate that the specifier proteins physically interact with the myrosinases to direct formation of alternative products from the glusosinolate aglycone. However, stable interactions among myrosinases and specifier proteins have not been detected.[48] Evaluation of how the specifier protein:myrosinase ratio affects the ratio and rate of product formation suggests that the specifier proteins may play a catalytic role, and do not simply act as cofactors for myrosinase.[55] The pronounced substrate specificities of the different specifier proteins[40] also suggest that these proteins may be catalytic enzymes. However, much work is needed to understand, on the molecular level, exactly how the specifier proteins control product formation. These specifier proteins, which both control and expand the diversity of products that result from glucosinolate hydrolysis, represent an exciting area in plant-derived natural product biosynthesis.

Conclusions

The alkaloid biosynthetic enzymes and the specifier proteins of the glucosinolates represent two distinct classes of plant proteins involved in nitrogen containing natural product biosynthesis. Strictosidine synthase, norcoclaurine synthase, and berberine bridge enzyme are each well-studied proteins with explicitly defined catalytic functions. Recent structural analyses and mechanistic studies of these three enzymes have spectacularly demonstrated how nature catalyzes complex biochemical transformations in alkaloid natural product biosynthesis. In contrast, the catalytic function of the specifier proteins remains cryptic, though these proteins are clearly required for correct product formation. Additional mechanistic studies using site-directed mutagenesis, structural analysis and isotope labeling will undoubtedly elucidate the chemical mechanisms of these intriguing enzymes. The selected examples described in this review demonstrates the impressive progress that has been made in plant biosynthetic studies in recent years, but also highlights the complexities in plant natural product biosynthesis that remain to elucidated.

Acknowledgements

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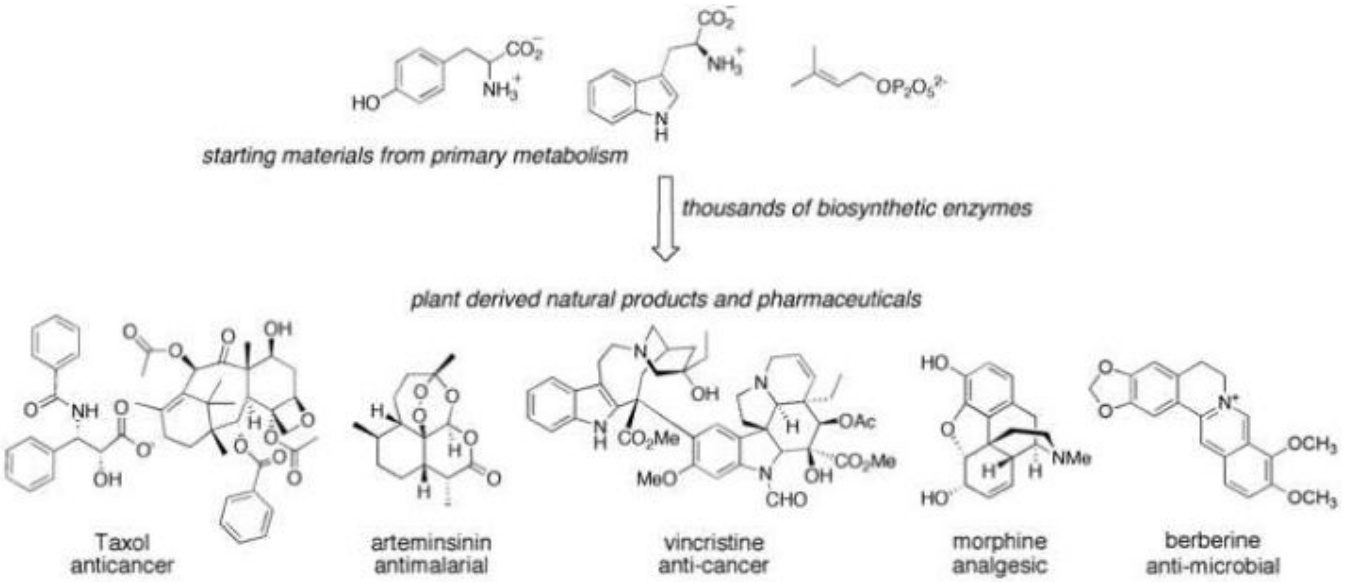

Figure 1.

Enzymes from natural product biosynthetic pathways convert simple starting materials from primary metabolism into complex natural products. Representative pharmaceutically important compounds derived from plants are shown.

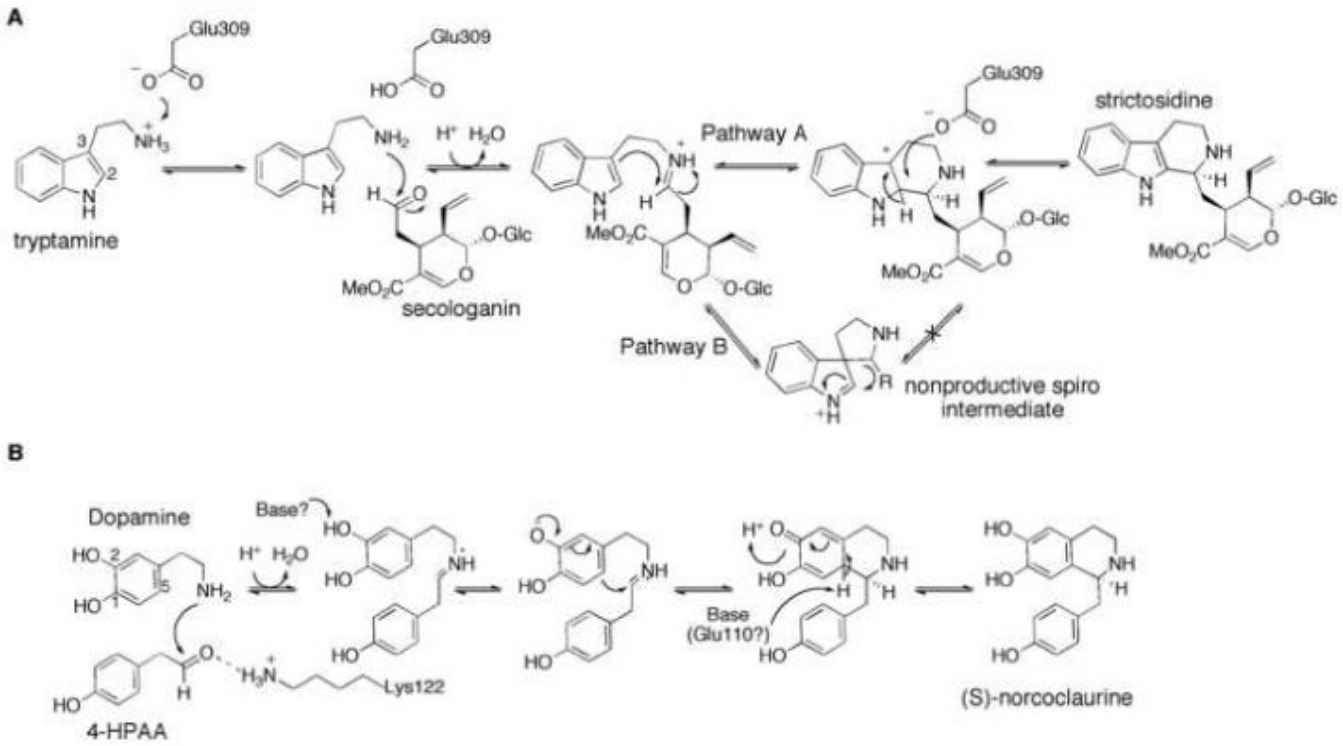

Figure 2.

Enzymes that catalyze the Pictet-Spengler reaction are shown. **A.** Strictosidine synthase catalyzes the condensation of secologanin and trypamine to form an iminium species. The nucleophilic indole then attacks the iminium, and deprotonation leads to the strictosidine product. Computational studies suggest that pathway A is favored. Glu309 is believed to be responsible for all acid-base catalysis. **B.** Norcoclaurine synthase catalyzes iminium formation between dopamine and 4-HPAA (4-hydroxyphenylacetaldehyde). Lys122 is proposed to enhance the electrophilicity of the aldehyde so that iminium formation is facilitated. The C-2 hydroxyl group may be deprotonated prior to cyclization of the aromatic group onto the iminium. However, it is not clear which protein residues assist in this step. Glu110 may be involved in deprotonation of the final reaction intermediate to lead to formation of the product, (S)-norcoclaurine.

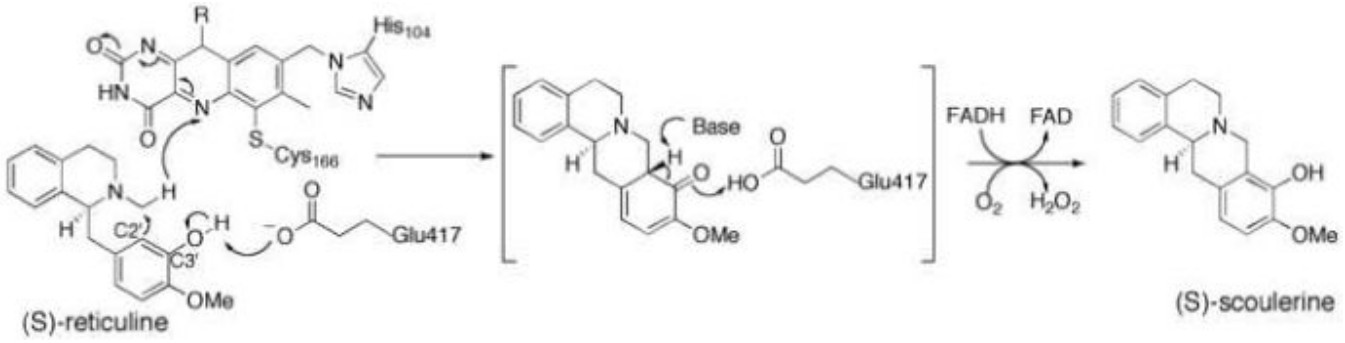

Figure 3.

The berberine bridge enzyme catalyzes carbon-carbon bond formation between the N-methyl group and the C-2′ carbon of (S)-reticuline. Deprotonation of the C-3′ hydroxyl group is critical for enhancing the nucleophilicity of the C-2′ carbon for attack on the N-methyl group. As the carbon-carbon bond is formed, a hydride from the N-methyl group is transferred to the flavin. A base, not yet definitively identified, deprotonates the reaction intermediate to yield (S)-scoulerine.

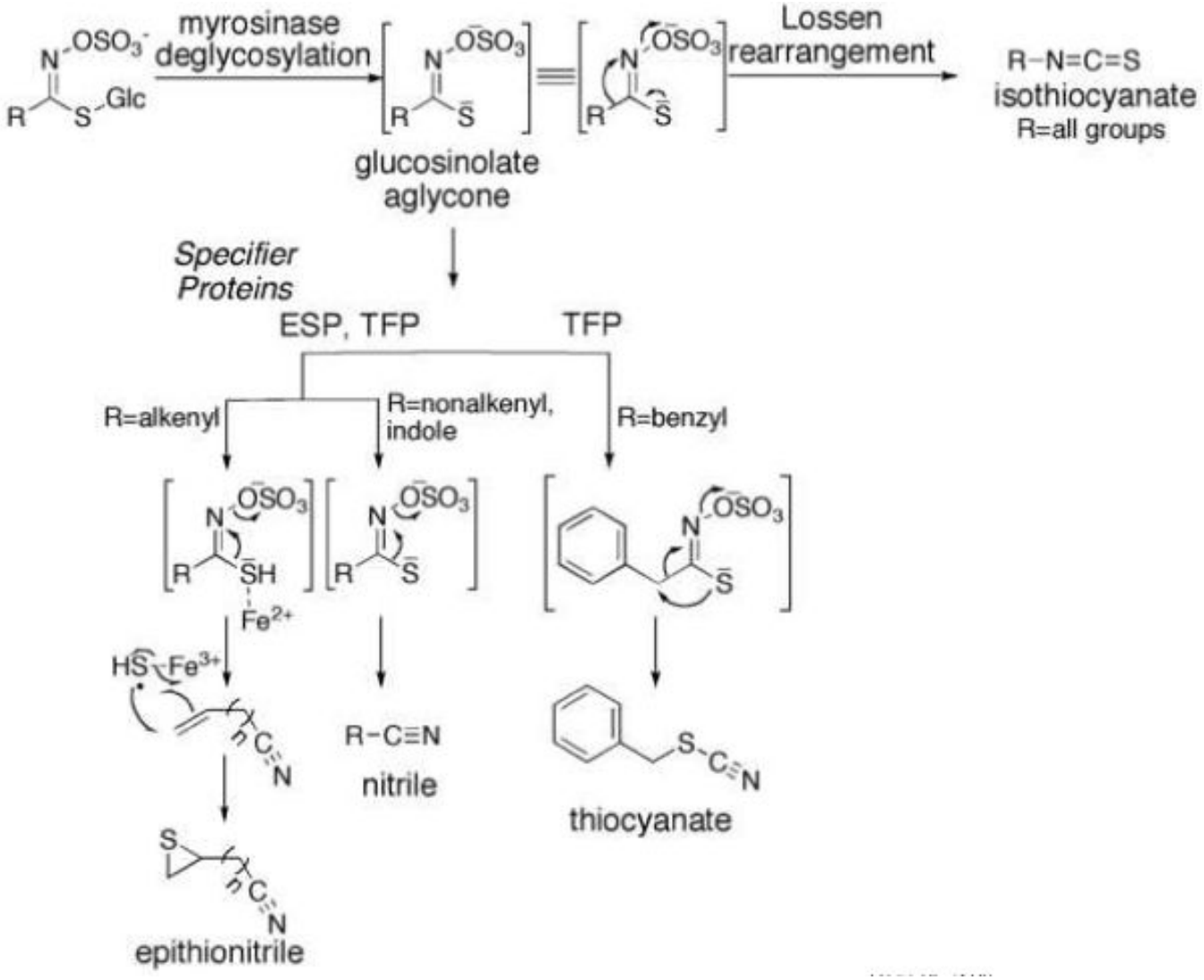

Figure 4.

Glucoinosolates are deglycosylated by myrosinase, and the resulting aglycone product rearranges to form isothiocyanates. However, when specifier proteins such as epithiospecifier protein (ESP) and thiocyanate-forming protein (TFP) the reaction pathway changes to yield epithionitriles, nitriles and thicyanates. The mechanistic details of these specifier proteins remain to be elucidated.