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Review: Microbial synthesis of agrochemical metabolites

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The synthesis of agrochemical metabolite reference standards by microbial cultures can serve as a useful alternative to conventional chemical synthesis, particularly when the chemical synthesis is difficult. Microbially generated metabolites of agrochemicals can also be useful for *predicting* degradative pathways in animals, plants and soils *prior to* conducting animal, plant and soil metabolism studies which are required by regulatory agencies to support agrochemical registrations. Examples from the literature are used to illustrate the utility of synthesizing metabolites of agrochemicals by common microbes.

Keywords: microbial synthesis; microbial transformations; metabolite reference standards; agrochemicals

Introduction

Acceptance of agrochemical metabolism and environmental fate studies by regulatory agencies such as the United States Environmental Protection Agency [28–30] nearly always depends on the availability of analytical reference standards for identifying metabolites. Difficulty is introduced when important, albeit trace, quantities of metabolites are detected in studies which lack these standards. In those instances, possible structures of an unknown metabolite are proposed by a metabolism chemist on the basis of physicochemical and chromatographic properties, likely sites of enzymatic attack and known metabolism of similar chemicals. Based on these considerations, the putative metabolite is often provided by conventional chemical synthesis. Sometimes the synthesis may be simple, requiring a minimal number of steps; other times, it may be more complex, multi-step and difficult if not impossible to accomplish. Furthermore, this task is generally performed by synthetic chemists who are often primarily responsible for discovering new agrochemicals rather than taking time to synthesize a speculative possibility for an unknown metabolite. In the end, the aim of this often time-consuming procedure is not always met since the chemically synthesized metabolite may or may not correspond to the unknown metabolite.

The use of common microbes for the synthesis of potential metabolites of agrochemicals represents an important alternative to conventional chemical synthesis. Although well known in pharmaceutical and natural products applications [9,18,23,24], the microbial approach to synthesizing metabolites of agrochemicals has been reported less frequently. Xenobiotic metabolism by selected microbes is often remarkably similar to that in mammals, birds, fish, soil and, to some extent, plants. This similarity in metabolite profile is largely explained by the presence of the enzyme cytochrome P-450 monooxygenase in these lower organisms. This enzyme is capable of catalyzing aliphatic and aromatic hydroxylations as well as *N*-, *S*- and *O*-dealkylations on a wide range of xenobiotic substrates. Because of metabolite profiles similar to those of biological systems required for testing by the agrochemical industry (eg, in rats, goats, poultry, fish, plants and soils [28–30]), microbial cultures can be used to synthesize quantities of metabolite (ie, milligrams) sufficient to obtain spectra for identification purposes. Subsequently, these metabolites can be generated in larger quantities (ie, multimilligrams or grams) through larger-scale fermentations to serve as reference standards in support of agrochemical metabolism and environmental fate studies.

In addition to providing an alternative to chemical synthesis, the microbial synthesis approach can be used to *predict* metabolites in soil, animals and plants *prior to* conducting complicated and expensive metabolism and environmental fate studies. Thus, agrochemical metabolites identified from a small-scale microbial screen can provide a synthetic chemist with information pertinent to the nature of metabolites which would be anticipated in metabolism and environmental fate studies. The decision to prepare sufficient quantities (typically grams) of relevant metabolite reference standards by microbial or chemical syntheses can then be determined based on feasibility, time and cost considerations.

This review will refer to published papers which specifically describe the use of microbial transformations to produce metabolites of agrochemicals to either serve as analytical reference standards or to predict degradative pathways in various biological matrices.

Clomazone

Clomazone (2-[2-chlorobenzyl]-4,4-dimethyl-1,2-oxazolidin-3-one) is a herbicide developed by the FMC Corporation for use against many species of annual broad-leafed and grassy weeds in the cultivation of soybeans, peas, corn, oilseed rape, sugar cane, cassava, pumpkins and tobacco [27]. Clomazone selectively blocks both diterpene and triterpene synthesis in weeds [6]. The metabolism of cloma-

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zone has been reported in rats [31], soybeans [7] and in soil [8,14].

Liu *et al* [13] studied the microbial metabolism of clomazone to identify metabolites of the herbicide as well as to predict relevant degradative pathways in the environment. In an initial screen, 41 fungal and bacterial cultures were tested to assess their ability to generate clomazone metabolites. These cultures represented common soil organisms and included species of *Absidia, Aspergillus, Bacillus, Candida, Corynebacterium, Cunninghamella, Curvularia, Cylindrocarpon, Helicostylum, Mucor, Mycobacterium, Nocardia, Pseudomonas, Rhizopus, Rhodococcus, Sepedonium, Streptomyces* and *Syncephalastrum*. Microbial transformations were performed in liquid shake culture by use of a two-stage incubation procedure intended to obtain sufficient biomass for optimizing metabolite yields [3]. Transformations were carried out in 25 ml of soybean meal-glucose medium contained in 125-ml DeLong culture flasks containing 10 mg of clomazone added in 0.1 ml of dimethylformamide. The liquid cultures were shaken at 250 rpm at 28°C for 144 h.

The microbial screen yielded 17 cultures capable of generating clomazone metabolites. Metabolites were initially identified by high-performance liquid chromatography (HPLC), facilitated by retention time comparisons with available reference standards. A summary of metabolites produced by these cultures is presented in Figure 1. Preparative scale incubations were conducted with *Aspergillus niger* and *Cunninghamella echinulata* to generate metabolites in sufficient quantities for spectral characterization (electron impact mass spectrometry [EI/MS] and proton nuclear magnetic resonance spectroscopy [^{1}H-NMR]). Major microbial transformations included hydroxylation at the 5-methylene carbon of the isoxazolidinone ring (**2**), hydroxylation of a methyl group on the isoxazolidinone ring (**4**) and aromatic hydroxylation at position 3′ (**10**). Minor reactions included dihydroxylation of clomazone (**8**, **11**), cleavage of the isoxazolidinone N-O bond (**7**) and complete removal of the isoxazolidinone ring to form chlorobenzyl alcohol (**6**).

The metabolism of ^{14}C-[methylene]clomazone in soybean plants grown in the greenhouse at 1.1 and 2.2 kg of active ingredient per hectare was reported [7]. Identification of metabolites at 30 and 60 days post-treatment indicated that major processes included cleavage between the isoxazolidinone and aromatic rings and conjugation of the resulting chlorobenzyl alcohol moiety (**22**) to form its corresponding glycoside. Other minor metabolic routes involved monohydroxylation of clomazone on either the aromatic (**18**) or isoxazolidinone moieties (**14**, **17**) with subsequent formation of their corresponding glycosides. Identification of isolated metabolites was accomplished by interpretation of mass and ^{1}H-NMR spectra as well as by chromatographic and mass spectral comparisons to chemically synthesized reference standards, if available. A summary of clomazone metabolism in soybeans is provided in Figure 2.

The metabolism of ^{14}C-[U-phenyl]clomazone in soil at an application rate of 1.12 kg per hectare suggested a relatively slow rate of degradation (59% of the applied radioactivity remaining after 84 days corresponded to parent compound), the presence of soil-bound residues (12% of applied radioactivity) and a slow turnover to carbon dioxide (as much as 15% of the applied radioactivity at an incubation temperature of 35°C). One metabolite was detected in soil extracts but did not accumulate to any more than 5% of the applied radioactivity and was not identified [14]. In comparison, Liu *et al* [13] reported preliminary results indicating that when culture medium was inoculated with soil, ~1% of clomazone was converted to traces of **3** and **7**.

Thus, metabolites generated from microbial transformations of clomazone matched most of the metabolites (aglycones) formed in soybean plants (**2**, **3**, **4**, **6**) and to some extent, in soil (**3**, **7**). Microbial transformations of clomazone therefore potentially can be used in large-scale incubations to provide sufficient quantities of metabolites, particularly for a difficult-to-synthesize metabolite such as **4**, which was also a metabolite formed in soybeans (in its glycoside form).

Sulfonylureas

Scientists at EI du Pont de Nemours and Company have recently published several studies illustrating the microbial transformation technique for generating metabolites of sulfonylurea herbicides. Triflusulfuron methyl (methyl-2-[4-dimethylamino-6(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-lycarbamoylsulfamoyl]-*m*-toluate) is the active ingredient in a new low-use-rate sulfonylurea herbicide for postemergence weed control in sugar beets [15]. In an effort to generate preliminary information pertinent to the metabolism of this herbicide, a microbial transformation study was performed with *Streptomyces griseolus* ATCC 11796 [5]. Microbial metabolites of triflusulfuron methyl (identified by liquid chromatography/fast atom bombardment [LC/FAB] mass spectrometry) were subsequently compared with those isolated and identified from rat urine and feces.

Triflusulfuron methyl was rapidly metabolized by cytochrome P-450 monooxygenase-induced cells of *S. griseolus* in both nutrient-rich and minimal media. Nine metabolites of triflusulfuron methyl were identified in ethyl acetate extracts of culture broths. Metabolism involved ester hydrolysis, hydroxylation and dealkylation. A summary of metabolite structures and a postulated metabolic pathway in this microbe is provided in Figure 3. When compared to metabolites found in urine and/or feces of triflusulfuron methyl-treated rats, common metabolites included **23**, **24**, **25**, **26** and **29**. The authors further noted that since only major metabolites extracted from urine and feces were analyzed by LC-FAB mass spectrometry, additional minor metabolites produced by *S. griseolus* might also have been present in rat excreta. Based on the identification of triflusulfuron methyl metabolites from both *S. griseolus* and from rats, reference standards were subsequently prepared by both chemical syntheses and preparative microbial transformations for use in metabolism and residue studies required by regulatory agencies.

Microbial transformations by *S. griseolus* were reported for two other sulfonylureas, designated B8346 and T8047 [26]. The objective of this study was to microbially generate metabolites that represented regiospecific *O*-dealkylations (Figure 4). The desired compounds (**32** and **33**) were

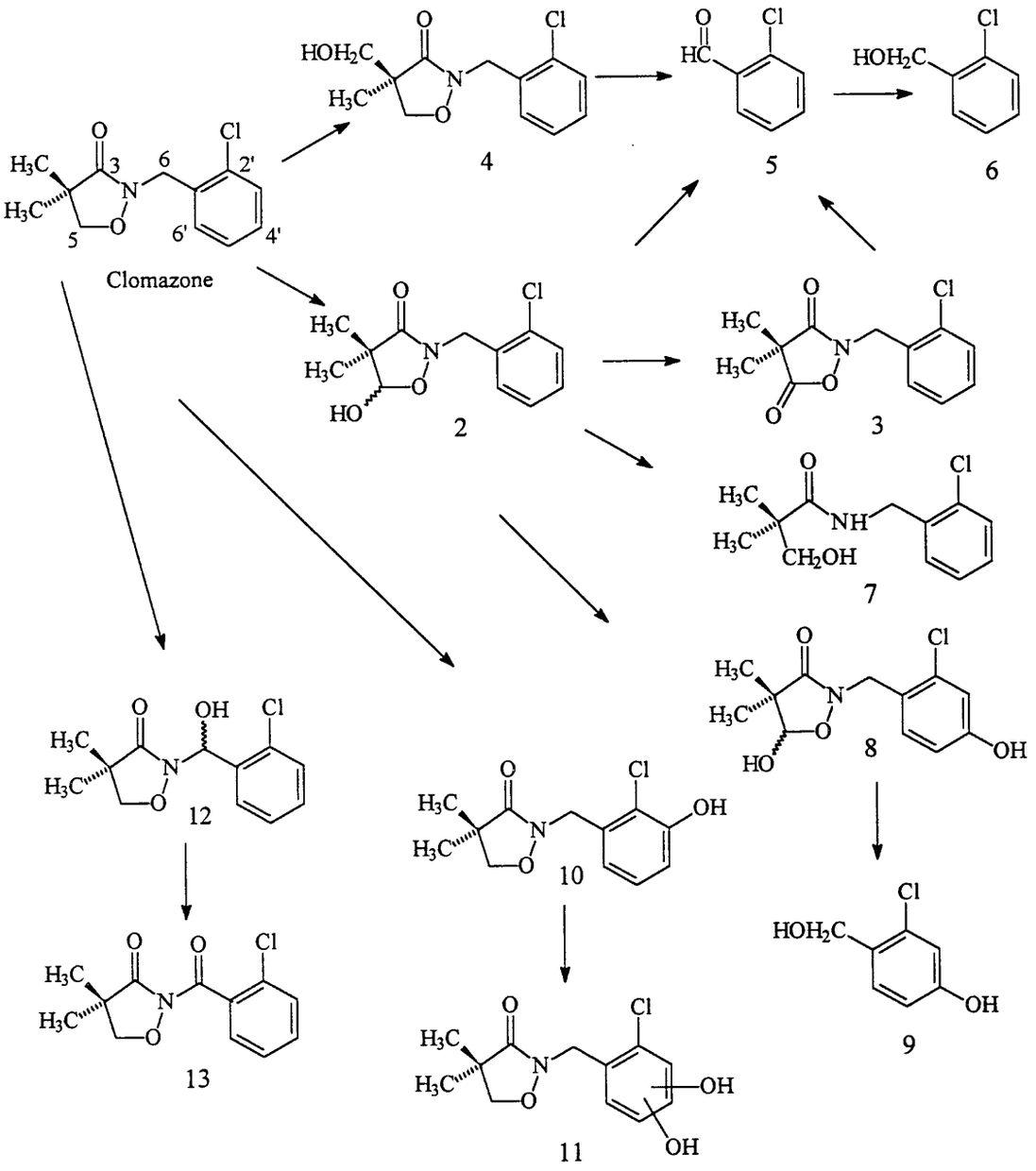

Figure 1 Postulated pathways of microbial metabolism of the herbicide clomazone based on the identification of metabolites. Metabolites, **2, 3, 4,** and **6** were also observed as aglycones in the metabolism of clomazone by soybeans (see Figure 2). Reprinted with permission from [13]. Copyright [1996] American Chemical Society.

plant metabolites of metsulfuron methyl (methyl-2-(4-methoxy-6-methyl-1,3,5-triazin-2-ylcarbamoylsulfamoyl) benzoate) and DPX-A7881, the active ingredients of Ally® and Muster® (methyl-2-[(4-ethoxy-6-methylamino-1,3,5-triazin-2-yl)carbamoylsulfamoyl]benzoate) herbicides, respectively.

The major metabolites formed in the small-scale (shake flask; 0.12 mg ml^{-1}; 25 ml medium per 125-ml Erlenmeyer flask) microbial transformations of compounds B8346 and T8047 corresponded to metabolites **32** and **33**, respectively, in yields estimated to be 80% after 24-h incubations. Metabolite identification was achieved with positive ion thermospray HPLC/mass spectrometry after prior HPLC purification of the major metabolites. The metabolites were subsequently generated in larger quantities from 2-L fermentor bioconversions at 300 and 350 mg L^{-1} initial concentrations (for compounds B8346 and T8047, respectively) with yields of 52 and 31%, respectively, after 68 h. Metabolite identities were confirmed by ^{1}H-NMR. After preparative HPLC, chemical purity was estimated to be 95% for both metabolites, based on percent of total HPLC area. Thus, *S. griseolus* was indeed capable of regiospecifically *O*-dealkylating two sulfonylureas and thereby providing sufficient quantities of metabolites difficult to synthesize chemically. Subsequently, these microbially-generated metabolites could serve as reference

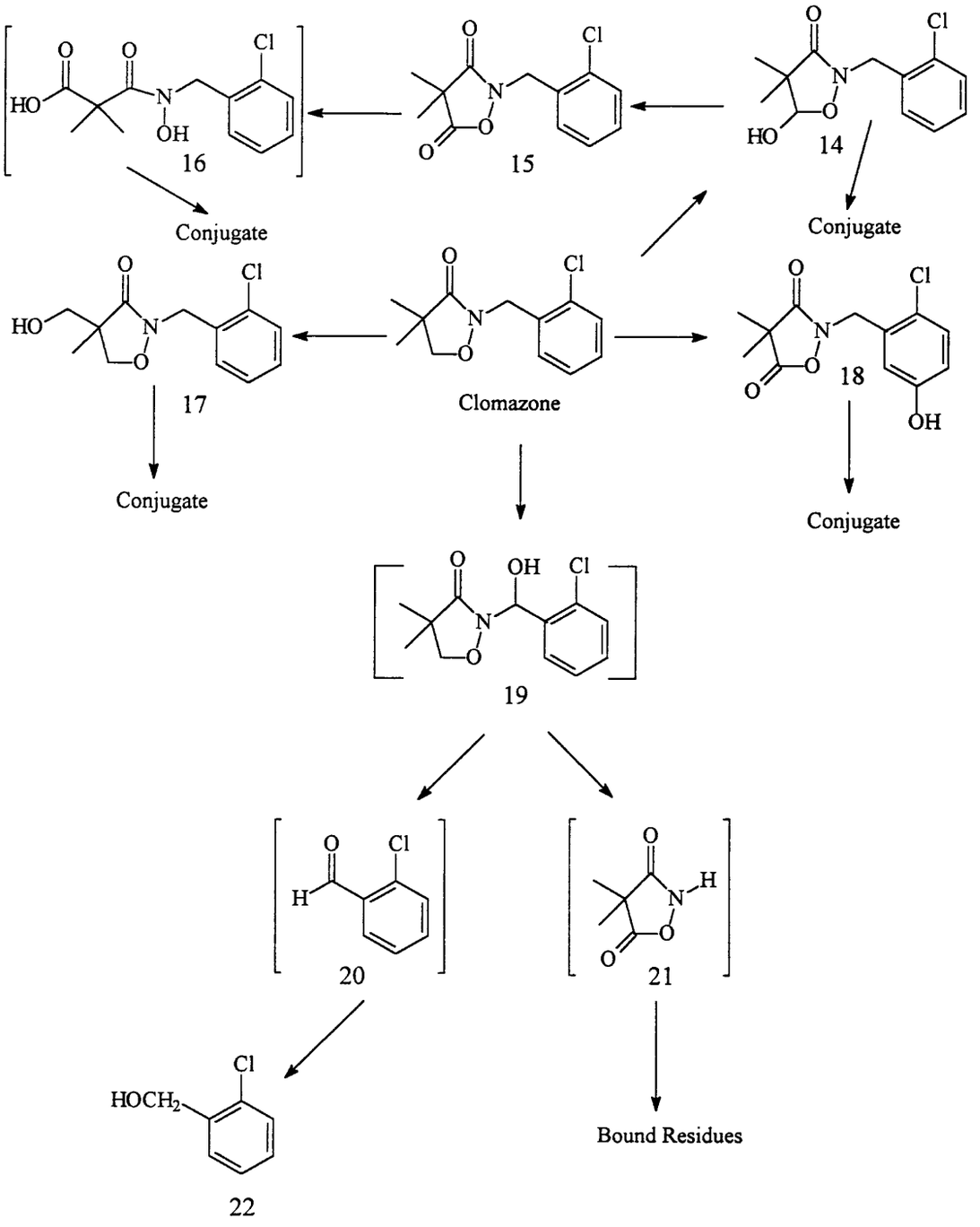

Figure 2 Postulated pathways of clomazone metabolism in soybeans based on the identification of metabolites. Reprinted with permission from [7]. Copyright [1992] American Chemical Society.

standards to aid in identifying metabolites formed in animal, plant and soil metabolism studies required by regulatory agencies.

S. griseolus again proved its usefulness in generating metabolites of chlorsulfuron (1-[2-chlorophenylsulfonyl]-3-[4-methoxy-6-methyl-1,3,5-triazin-2-yl]urea), the active ingredient in Glean® wheat herbicide, and chlorimuron ethyl (ethyl-[2-(4-chloro-6-methoxypyrimidin-2-ylcarbamoylsulfamoyl]benzoate), the active ingredient in Classic® soybean herbicide [16]. Metabolites were generated during a 4-to-48-h incubation using sulfonylurea-induced cells at substrate concentrations ranging from 100 to 160 mg L^{-1}. Metabolite identity was determined by LC/MS with continuous-flow FAB based on protonated molecular and fragment ions, which were useful for structure elucidation. (Since sulfonylurea herbicides and their metabolites are very thermolabile, they cannot be analyzed by gas chromatography (GC)/MS, and often do not give molecular ions

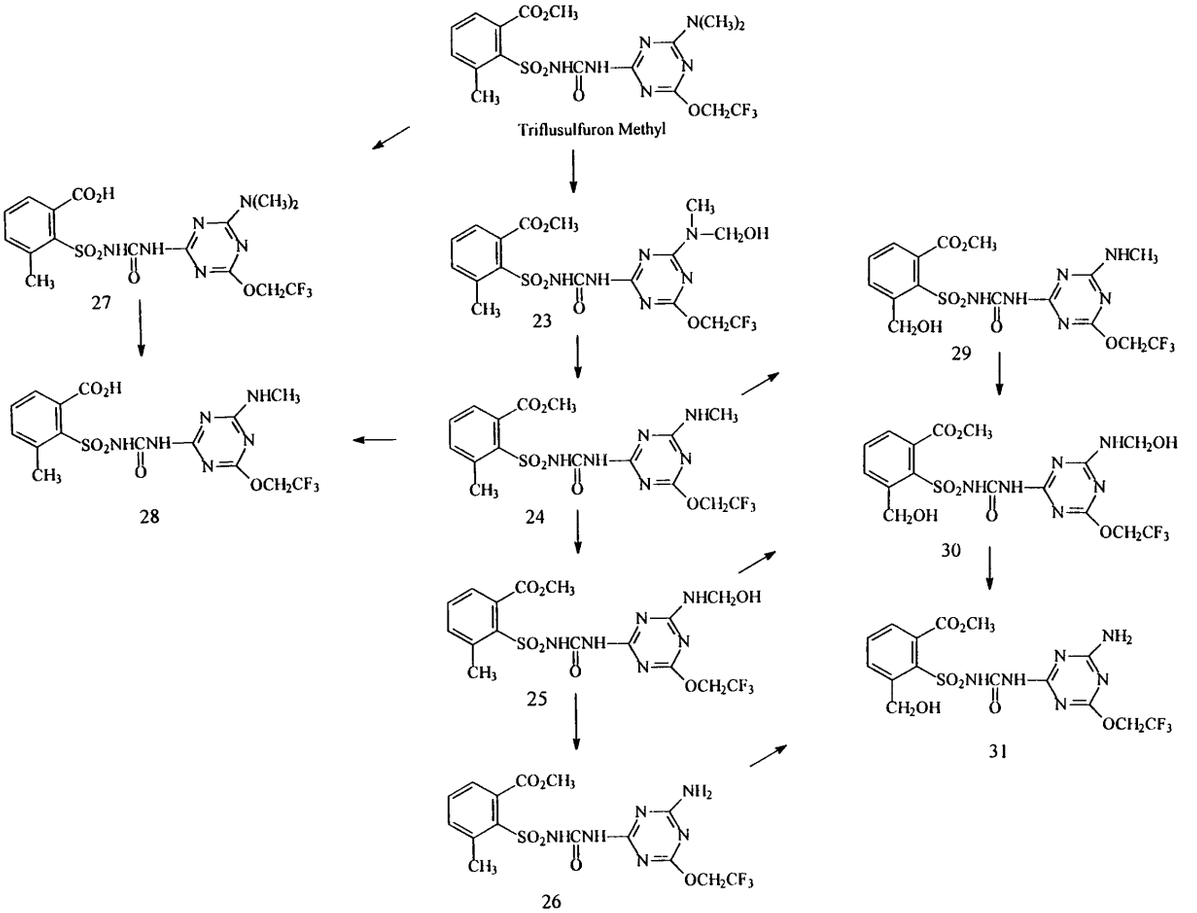

Figure 3 Postulated pathways of triflusulfuron methyl metabolism by *S. griseolus* ATCC 11796 based on the identification of metabolites. Metabolites **23, 24, 25, 26** and **29** were also observed in triflusulfuron methyl-treated rats. Reprinted with permission from [5]. Copyright [1995] American Chemical Society.

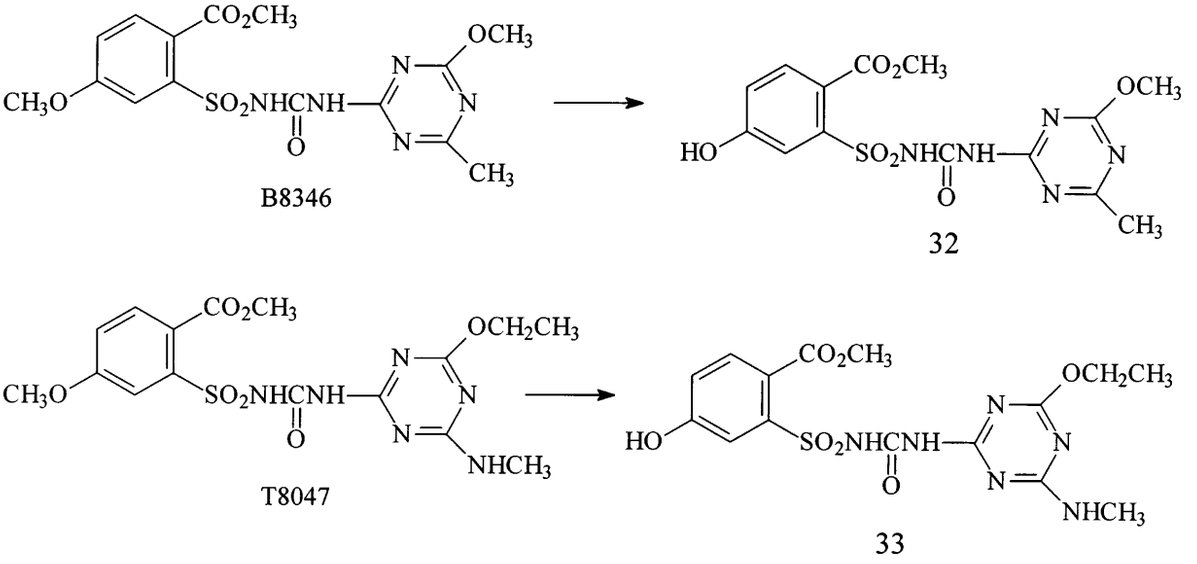

Figure 4 Desired products of microbial transformations of compounds B8346 and T8047 by *S. griseolus* ATCC 11796. Reprinted with permission from [26].

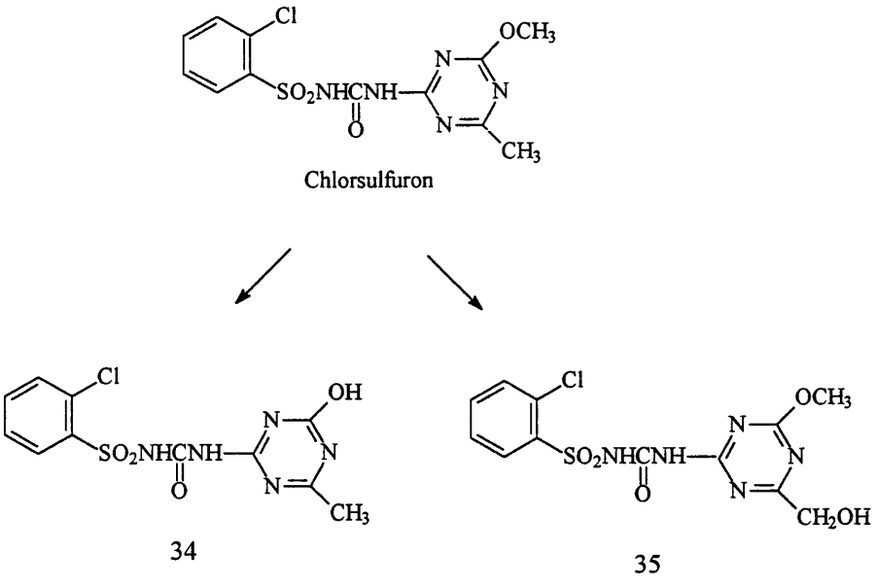

Figure 5 Metabolites formed from the biotransformation of the herbicide chlorsulfuron by *S. griseolus* ATCC 11796 [16].

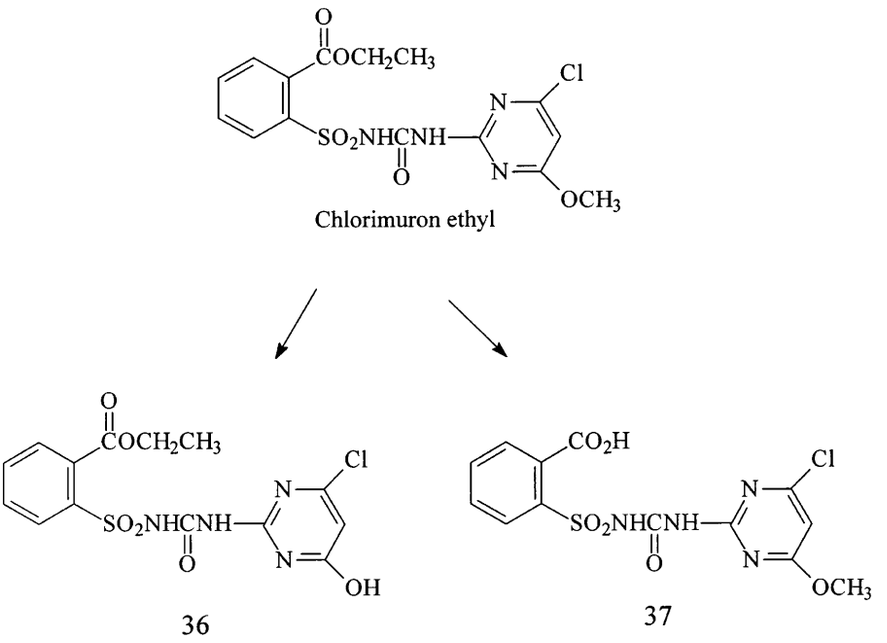

Figure 6 Metabolites formed from the biotransformation of the herbicide chlorimuron ethyl by *S. griseolus* ATCC 11796 [16].

in LC/MS with other ionization techniques such as electron impact, chemical ionization, or thermospray.) Metabolite structures and microbial pathways are presented in Figures 5 and 6 for chlorsulfuron and chlorimuron ethyl, respectively. Chlorsulfuron underwent *O*-dealkylation and methyl group hydroxylation whereas *O*-dealkylation and ester hydrolysis occurred during the chlorimuron ethyl transformation. Note that mass spectra were obtained directly from methylene chloride extracts of both culture broths without clean-up, a step(s) typically necessary in soil, animal and plant metabolism studies. Thus, the microbial approach to generating metabolites in support of metabolism and environmental fate studies is not only useful in generating significant amounts of metabolites but extensive clean-up is not often necessary.

Herbicide F5231

Schocken *et al* [20] used the microbial transformation approach to identify metabolites of a herbicide designated F5231 (1-[4-chloro-2-fluoro-5-(ethylsulfonylamino)-phenyl]-1,4-dihydro-4-(3-fluoropropyl)-5H-tetrazol-5-one), which was being considered for development by the Agricultural Chemicals Group of FMC Corporation in the late 1980s. The intention was that the microbially-generated

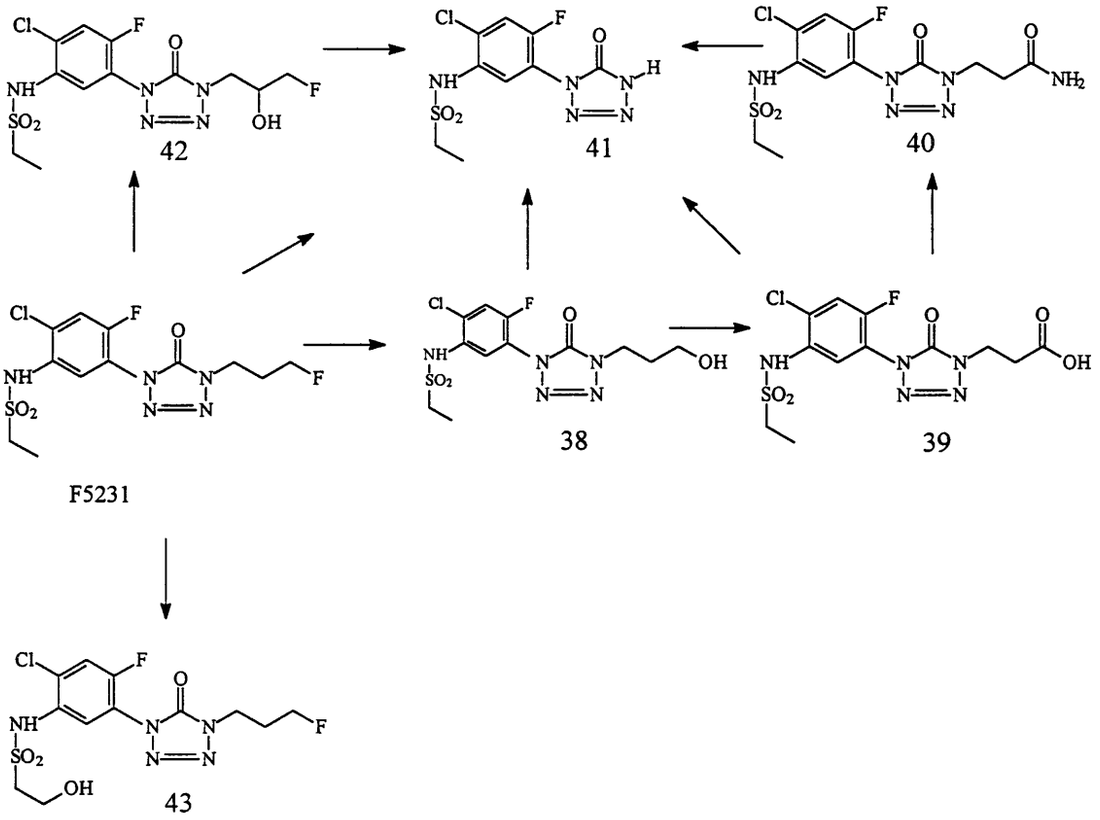

Figure 7 Postulated pathway for the metabolism of the herbicide F5231 by *Absidia pseudocylindraspora* ATCC 24169 based on the identification of metabolites. Reprinted with permission from [20]. Copyright [1989] American Society for Microbiology.

metabolites serve as reference standards for soil, plant and animal metabolism studies, which would be required for registration of the agrochemical.

The microbial transformations were carried out by the filamentous fungus *Absidia pseudocylindraspora* ATCC 24169 in a two-stage fermentation procedure [3]. Structures of the metabolites as well as a proposed bioconversion pathway are provided in Figure 7. Metabolites, generated in quantities ranging from 1 to 7 mg, were identified by mass, infrared, and nuclear magnetic resonance spectroscopies. The metabolite profile indicated that neither the aromatic nor tetrazolinone rings were modified by fungal enzymes. Instead, only sidechains (ethylsulfonylamino and fluoropropyl) of the molecule were attacked. Reactions included various aliphatic hydroxylations (**38**, **42**, **43**); an *N*-dealkylation (**41**); oxidation of a primary alcohol to a carboxylic acid (**39**); and conversion of a carboxylic acid to a carboxamide (**40**). In addition one of the metabolites identified (**38**) represented cleavage of a carbon-fluorine bond, somewhat unexpected given the relative strength of that type of bond. Confirmation of the identity of the defluorinated metabolite was accomplished with ^{19}F-NMR. Formation of the carboxamide (**40**) was also somewhat uncommon, although a similar reaction had been previously reported from the microbial transformation of the anti-inflammatory drug fenclozic acid [10]. In that study, the acetic acid side chain (analogous to the propionic acid moiety of **39**) served as the preferred site of microbial activity, and was converted to a carboxamide by eight different microbial species. The bioconversions of the vitamins biotin and desthiobiotin by the yeast *Rhodotorula flava* illustrate a similar biotransformation [21,22]. Based on the identification of metabolites produced in relatively small quantities, the authors indicated that more substantial quantities of metabolites could be produced and used as reference standards. This approach for generating metabolites of a new agrochemical can serve to *predict* metabolism and environmental fate in such important matrices as soil, plants and animals. Thus, large-scale synthesis of likely metabolite reference standards can subsequently be accomplished through either conventional chemical synthesis or through microbial synthesis.

Precocene II

Sariaslani *et al* [19] reported on microbial transformations of precocene II (6,7-dimethoxy-2,2′-dimethyl-2H-benzo[*b*]pyran), a naturally-occurring chromene known to have insecticidal properties as an anti-juvenile hormone [4]. The objective of this study was to characterize the microbial metabolism of precocene II and to compare it with insect and rat metabolism, which had been previously reported [2,11,25]. In all, a total of 52 microorganisms were screened for the ability to produce metabolites of precocene II. The microbial cultures included a variety of soil fungi (members of the genera *Aspergillus, Rhodotorula, Stysanus, Syncephalastrum, Helicostylum* and *Brevilegnia*) as well as 17 species of *Streptomyces*. The cultures were

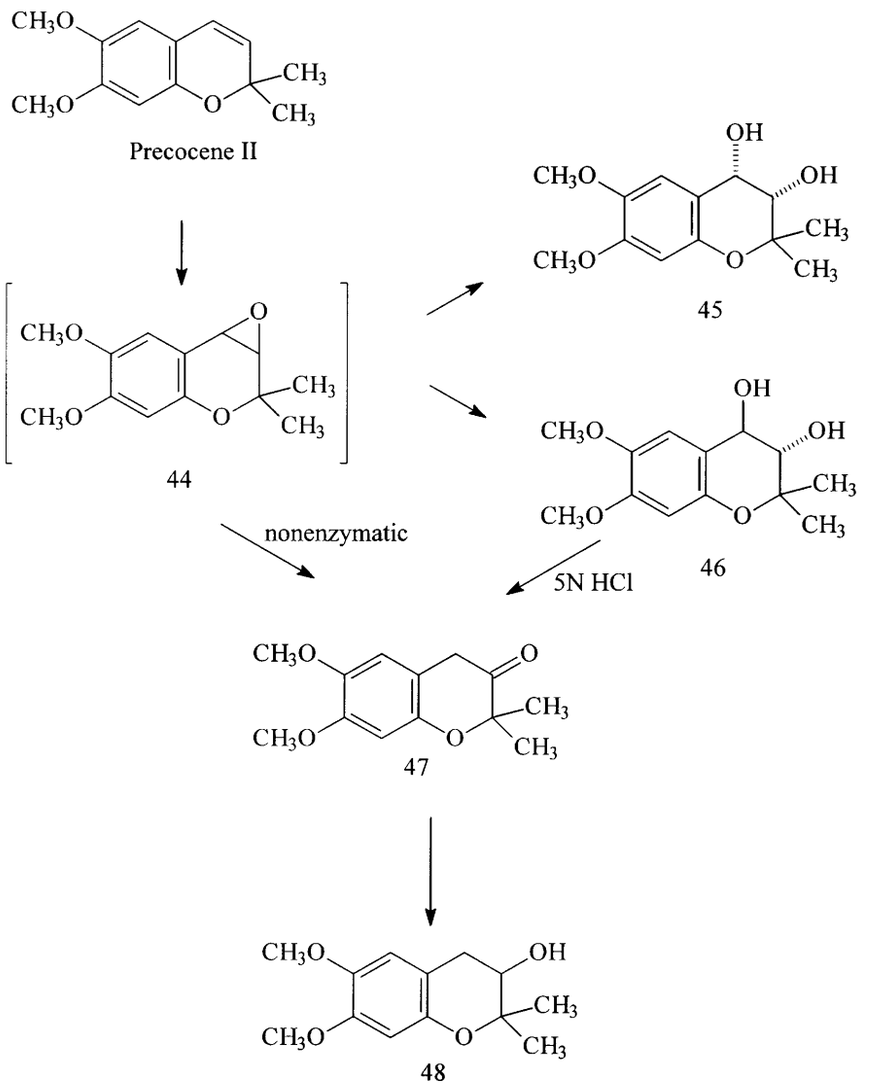

Figure 8 Postulated pathway for the bioconversion of precocene II by *S. griseus* ATCC 13273. Metabolites **45, 46** and **48** were also observed in insects [25]; **45** and **46** were observed in rats [11]. Reprinted with permission from [19]. Copyright [1987] American Society for Microbiology.

chosen for the screen based on their known abilities to transform a wide variety of xenobiotics. Twenty-three cultures produced metabolites of precocene II. The streptomycetes were found to be particularly capable of transforming precocene II. *S. griseus* ATCC 13273 was especially active, and produced the highest yields of metabolites. As a result, it was chosen for further study.

A proposed route for the bioconversion of precocene II by *S. griseus* is provided in Figure 8. The major metabolites were identified as **45**, **46** and **48**, based on ultraviolet spectroscopy, high resolution mass spectrometry (HRMS), and ^{1}H-NMR spectroscopy. Optical rotations were also recorded. The *cis*- and *trans*-precocene II-3,4-dihydrodiols (**45**, **46**) were identical (including optical rotations) to those produced by insects and rats. Use of $^{18}O_2$ and GC-HRMS indicated that only one of the atoms of molecular oxygen had been incorporated into either **45** or **46**, which suggested the presence of a monooxygenase enzyme system in *S. griseus*. The authors noted that monooxygenation of precocene II would generate a highly reactive precocene-3,4-epoxide (**44**), which upon nonenzymatic hydrolysis would account for both the *cis*- and *trans*-3,4-dihydrodiols. Precocene II-3,4-epoxide has also been reported as an initial intermediate in insects and in rat liver microsomes [1,2,11,25]. Sariaslani *et al* also noted that the reactive epoxide could rearrange non-enzymatically to form the ketone (**47**). When a separate experiment was performed in which **47** was used as a substrate for a *S. griseus* transformation, the optically active 3-chromenol (**48**) was produced. Thus, precocene II-3-ketone (**47**) was shown to be the immediate precursor of **48**, and represents a ketone reduction reaction.

Conclusions

Even though there is a relative paucity of published reports for agrochemicals, the microbial transformation approach for generating metabolite reference standards for agrochem-

ical metabolism and environmental fate studies has great utility. Not only can it serve as an alternative for conventional chemical synthesis but it can provide a synthetic chemist with information for preparing metabolite standards which are likely to be detected in metabolism and environmental fate studies.

Although not the specific focus of this review, the microbial transformation approach can also be useful for generating and subsequently evaluating the biological activities of agrochemical metabolites. An example of this application was reported by Kole *et al* [12] with the herbicide pendimethalin. Additionally, microbial transformations can potentially be used as an alternative to chemical synthesis for the preparation of a key chemical intermediate in the synthesis of an agrochemical. An example of this application was reported by Rosazza *et al* [17] with the herbicide cinmethylin.

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