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AGROACTIVE COMPOUNDS OF MICROBIAL ORIGIN

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ABSTRACT

Microbial metabolites attract increasing attention as potential pesticides. They are expected to overcome the resistance and pollution that have accompanied the use of synthetic pesticides. Several microbial metabolites, such as avermectin, have proved useful as agroactive agents. In this review, we attempt to identify newer agroactive microbial metabolites with feasible activity or interesting action sites from those reported in recent years. In addition, microbial and chemical modifications of existing microbial agrochemicals are discussed to illustrate the usefulness of these technologies in potentiating agroactivity and stability. We discuss the possibility of future discovery of excellent microbial agrochemicals, and the importance of efforts to promote positive public perception and public acceptance of pesticide chemicals.

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INTRODUCTION

Pesticides have been of great help to crop production. They protect crop plants from injuries and damage caused by harmful insects and mites, infectious fungi and bacteria, and invasive weeds. Without pesticides, crop production decreases by an estimated 20–40%. This includes decreases in amounts at harvest and additional losses occurring after the harvest and during transportation. The harmful organims are almost countless: insects and mites number at least 5000 species, fungi at least 8000, bacteria at least 70, and viruses at least 500. In addition, 1800 weed species may be harmful, 200 of which account for 95% of damage (45).

The world market for pesticides had increased to $17.9 billion US in 1986, increasing further to $20.4 billion in 1988 (end-user-value basis), according to the 1990 *Wood Mackenzie Report* (77a), a well-known annual report on agroindustry and the world market for agrochemicals and veterinary products. Of global sales in 1988, herbicides comprised 43.6%, insecticides 29.7%, fungicides 20.5%, and others 6.1%.

The development of pesticides has not been without problems. One of the most serious problems arising in association with pesticide use has been the adverse effects of residual chemicals on environmental ecosystems. Another problem is the emergence of pesticide-resistant insects and fungi. In view of the very strict requirements for approval from regulatory authorities, and the efforts of industry, the former problem should soon be less serious, if appropriate ways of use are followed. However, the general public does not recognize the fact that recently approved agrochemicals are far safer than early ones (28). In addition, even with those compounds approved recently, uncontrolled (repeated and heavy) pesiticide use currently practiced in some areas, and possible misuses, will likely pollute ground water. Therefore, public concern today is still focused on safety of pesticides or alternative measures of pest control.

Here lies a dilemma. Although public concern over safety runs high, current and future problems involving many uncontrolled crop-harming insects, mites, and fungi urgently require a solution. Besides, the expected increase in global population in the coming twenty-first century requires an increase in crop production by about 30% above the present level (25, 28).

Interest in microbial metabolites as pesticides arises from attempts to solve the above dilemma. Microbial cultures are a treasure box, as is often mentioned and borne out by pharmacoactive drugs and antibiotics. Microbial metabolites are expected to conquer the resistance and pollution likely caused by synthetic pesticides. The merits of microbial metabolites as pesticides would be: (*a*) They are versatile in structure and activity. An unexpected structure with new agroactivity showing no cross-resistance is very likely to

be identified through effective screening systems, which have progressed greatly in the long history of antibiotic screening. And (*b*) they are biodegradable. They degrade usually within a month, or even a few days, when exposed to firm soils. Thus they are expected to stress and pollute ecosystems less.

Pesticides of microbial origin introduced into field applications 30 years ago include blasticidin S, polyoxin, kasugamycin, validamycin, and mildiomycin as fungicides, and tetranactin as a miticide. Recent examples include avermectin, milbemycin, and bialaphos. The excellent activity of these compounds suggests that other desirable pesticides will be discovered from microbial metabolites.

This review presents an overview of the discovery of agroactive compounds from microorganisms reported in the past 10 years, emphasizing the methods of screening and sites of action of these active compounds. We attempt to identify areas of agroactivity of new and growing interest in the hope that screening of microbial metabolites will be more successful if efforts are focused toward particular types of agroactivity in which synthetic chemicals were ineffective.

Use of antibiotics as crop protectants has been reviewed (59, 83, 85) as has the use of microbial products with herbicidal activity (4, 45). Reviews on bioactive microbial metabolites and the methods for their screening (100, 102, 103, 117) have also appeared. For progress in pesticide sciences, readers can refer to the books published on the occasion of the International Conference of Pesticide Chemistry, held every four years (see 32 for the latest issue, 1991). A book describing strategies and methods for screening of bioactive microbial metabolites appeared very recently (104). This is a good source of information, screening methods, and techniques for insecticides, herbicides, and fungicides, as well as other pharmacoactives and antibiotics.

DISCOVERY OF AGROACTIVE COMPOUNDS FROM MICROORGANISMS

Current Trends in the Search for Agroactive Microbial Metabolites

A survey of recent literature indicates a continual increase in the discovery of new agroactive compounds from microorganisms. Most of them were obtained by conventional activity-monitoring biological screening with *Streptomyces* spp. as the major microbial source. Chemical-screening techniques were adopted less frequently. Fundamentally, the increased discovery comes from an enhanced interest toward microbial metabolites as potential pesticides.

More importantly, it arises from keen needs for excellent pesticides. Technically, the increase is a consequence of the following:

1. Establishment and improvement of screening technologies.
   a. Exploitation of novel microbial groups as sources for production of active compounds. Basidiomycetes, blue-green algae, and myxobacteria were shown to be rich producers of agroactive metabolites.
   b. Development of unique fermentation techniques and their application to screening programs.
   c. Development of new methods for detection of agroactivity. Unconventional test organisms were introduced to find new compounds.
2. Application of genetic techniques for breeding mutants that produce an altered spectrum of metabolites. Microbial conversion and mutational biosynthetic techniques were applied.
3. Progress in chemistry and biochemistry of pesticide sciences and its application to the synthetic design and the construction of new screening methods.
4. Others. The chemical basis of plant-pathogen interaction was elucidated, and many compounds likely to be involved in the interaction were identified. In addition, interesting pharmacological activities were uncovered for several agroactive microbial metabolites. These findings stimulated the interest in microbial metabolites.

From the standpoint of agrochemical discovery, construction and improvement of screening methods (1a–c) are most important and are described in some detail below.

NOVEL MICROBIAL SOURCES FOR AGROCHEMICAL PRODUCTION *Streptomyces* spp. have been, and remain, the most fruitful source of microorganisms for all types of bioactive metabolites, including agroactives. In fact, about 60% of new insecticides and herbicides reported in the past five years are of *Streptomyces* origin. With *Streptomyces* spp., however, the frequency of rediscovery of known compounds has become fairly high. In an effort to reduce this replication, the use of organisms other than *Streptomyces* spp., which include rare actinomycetes, fungi, basidiomycetes, and other taxa, has increased steadily.

Rare actinomycetes We (113, 114) discovered that *Kitasatosporia,* a strain of the order *Actinomycetales* produces setamycin, an insecticidal 16-membered macrolide (123). This genus is characterized by its abundant aerial mycelia formation and by unique amino acid composition in the cell walls. The cell walls of aerial spores and submerged spores contain LL-diaminopime-

lic acid, the isomeric type of which is of taxonomic importance, whereas aerial and submerged mycelia contain the *meso*-isomer. Members of this genus produce phosalacine, an herbicidal tripeptide (108, 140); cystargin, an antifungal peptide (156); and a variety of antibiotics (103). Strains of *Kitasatosporia* and other species of rare actinomycetes and bacteria can be isolated from soil samples with selective isolation techniques (37, 40, 139).

Bacidiomycetes and filamentous fungi Anke and his group focus their attention on bacidiomycete cultures for identifying bioactive metabolites (2). Strains of basidiomycetes produce many new antifungal agents with agrochemical interest, such as oudemansin (3) produced by a strain of *Oudemansiella radicata,* aleurodiscal (77) produced by a strain of *Aleurodiscus mirabilis,* strobilurin (2) produced by a strain of *Strobilurus* sp., pilatin (43) produced by a strain of *Flagelloscypha pilatii,* and herbicidal agents such as pereniporin (68) produced by a strain of *Perenniporia madullaenpanis*. Xerulin and dihydroxerulin produced by a strain of *Xerula melanotricha* are unique in structure and activity. These compounds possess an α,β-unsaturated γ-lactone and conjugated poly-ene-yne moieties. Xerulin inhibits cholesterol biosynthesis in HeLa cells (72).

Blue-green algae, Myxobacteria, *and red algae* Blue-green algae, such as *Anabaena* spp., produce a variety of antifungal compounds (30, 86). Another blue-green alga, *Lyngbya majuscula,* produced malyngolide, a δ-lactone with a long alkyl chain (48). Myxobacterial strains were found to produce antifungal, antibacterial, and cytocidal compounds (127). Among them, phenoxan (74), ambruticin, and pyrrolnitrin exhibit agricultural antifungal activity. A methanol extract of the red alga *Laurencia nipponica* was used to isolate a family of insecticides, *Z*-leurentin, *Z*-isoleurentin, and deoxyprepaciferol (157).

The isolation, growth, and preservation of these new groups of microorganisms requires specific techniques different from those used for *Streptomyces* spp. None of their products so far identified has reached a marketable stage. Nevertheless, these results appear to warrant further screening.

UNIQUE FERMENTATION TECHNIQUES The uniqueness of the fermentation technique is another factor that often favors discovery of new compounds (144). A medium containing a high content of inorganic phosphate was employed for production of an antifungal compound, FR-900848 (159). This medium refutes a widely accepted notion that a low content of inorganic phosphate favors antibiotic production. FR-900848 is a nucleoside, and an unusual linear chain of five cyclopropane rings is attached to its sugar moiety. It showed potent in vitro activity against *Sclerotinia arachidis, Fusarium oxysporum,* and *Penicillium chrysogenum*. Our laboratory (79, 115, 116)

developed phosphate ion– and ammonium ion–depressed fermentation using trapping agents such as the minerals allophane and zeolite, respectively. This fermentation technique led to the discovery of phthoxazolin (Figure 3, below) (119), jietacin (52, 112) (Figure 2, below), globopeptin (145), and others (101).

TARGETED AGROACTIVITY AND NEW DETECTION METHODS Synthetic chemicals are in use or are under development in many areas of crop protection. Microbial metabolites as pesticides are later developments. In view of this situation, and the possible advantages of microbial metabolites over synthetic chemicals, targeted agroactivities and screening methods are of strategic importance in their screening. Two approaches were taken in the choice of target activity and therefore in constructing and improving screening methods, as illustrated in Tables 1–3.

In one approach, efforts centered on compounds of possible use in existing areas of agroactivity where synthetic chemicals are available but must be improved. The new microbial metabolites discovered were expected to serve as new leads, because of novel structure and activity with no cross-resistance and low residual secondary effects. In this approach, improved or modified screening methods are critical to obtaining better compounds. New compounds thus discovered are jietacin (112), discovered by using the pine wood nematode; altemicidin (138) and dioxapyrrolomycin (20), detected using brine shrimp; and allosamidin (130), found as an enzyme inhibitor for chitinase. This category also includes a new herbicidal inhibitor of starch synthesis, 6241-B substance (67).

In the second approach, attention was focused toward compounds with possible uses in novel agrochemical areas, namely, compounds with activity toward pests for which previous synthetic chemicals were either not effective or insufficiently effective. The resultant compounds were expected to open a new area of pesticide application, as did avermectin. Examples of these include inhibition of cellulose biosynthesis (phthoxazolin) (119) and growth inhibition of *Phytophthora* (phthoramycin) (118) and *Puccinia* (rustmicin) (96).

Microbial products already put into practice are concentrated in a few areas, namely, antifungal agents for rice-disease control and miticides. The use of microbial products in these areas is well established, while products for other areas are under development. Consideration of the pest to be targeted and the site of action of the compounds may help select the most appropriate screening method for finding novel agroactive microbial metabolites.

Described below are recently discovered microbial metabolites with feasible activity, or with interesting action sites. In order to exemplify the current trends, selected methods of screening are also discussed.

Table 1 Insecticides of chemical and microbial origins

Harmful insects	Insecticides	
	Chemical[b]	Microbial
Hemiptera		
Brown planthopper, green rice leafhopper, aphids	**Organophosphorus,** buprofezin, **carbamates,** imidacloprid, pyrethroids	
Lepidoptera		
Rice stem borer, corn borer, potato tuberworm, tobacco cutworm, cabbage armyworm, diamondback moth, apple leaf miner	**Organophosphorus, carbamates,** pyrethroids, benzoylphenylureas	(Avermectin = EMA)
Diptera		
Rice leaf miner, wheat thigh chloropid fly, seed maggot	**Organophosphorus, carbamates,** pyrethroids	
Coleoptera		
Soybean beetle, cucurbit leaf beetle, 28-spotted lady beetle	**Organophosphorus, carbamates**	
Acarina		
Kanzawa spider mite, two-spotted spider mite, citrus red mite	Benzilates, organophosphorus, pyrethroids, hexythiazox	Avermectin, milbemycin, polynactins (allosamidin)

[a] Commercialized compounds are listed. Compounds of recent discovery with noticeable activity are included in parentheses.
[b] Compounds showing decreased efficacies due to resistance are in bold.

Insecticides

AVERMECTINS AND MILBEMYCINS Avermectin (Figure 1) represents a family of fused 16-membered macrolides produced by *Streptomyces avermitilis* (11, 12, 29). The potent nematicidal, insecticidal, and acaricidal activities were discovered by a joint research effort of Merck, Sharp and Dohme Research Laboratories and the Kitasato Institute (135). Soon after the introduction of an avermectin derivative, ivermectin, to field applications as a veterinary drug, the marked antiectoparasitic effect was demonstrated in sheep, cattle, horse, pig, dog, and so on. Ivermectin is currently also used as a human drug for control of onchocerciasis, or African river blindness, one of the most serious endemics caused by a microfilaria, *Onchocerca volvulus* (12, 13).

Avermectin is a neuroactive substance with a fast-acting effect. It inhibits

Table 2 Agricultural fungicides of chemical and microbial origins

Fungal diseases	Fungicides[a]	
	Chemical[b]	Microbial
Phycomycetes		
Late blight	Fosetyl, dithiocarbamates, **acylalanines**	
Downy mildew	Fosetyl, dithiocarbamates, **acylalanines,** Bordeaux mixtures	(Phthoramycin, valclavam)
Ascomycetes		
Powdery mildew	**Triazoles, benzimidazoles**	Mildiomycin
Bitter rot	**Benzimidazoles,** dithiocarbamates	
Scab	**Benzimidazoles, triazoles,** dithiocarbamates	
Brown rot	**Benzimidazoles, dicarboximides**	
Basidiomycetes		
Rust	**Triazoles,** anilides, dithiocarbamates	(Rustmicin)
Sheath blight	Anilides	Validamycin (Dapiramicin)
Fungi imperfecti		
Blast	Organophosphorus, probenazole, tricyclazole, isoprothiolane	Blasticidin S Kasugamycin
Anthracnose	**Benzimidazoles,** dithiocarbamates	
Gray mold	**Benzimidazoles, dicarboximides**	
Leaf mold	**Benzimidazoles,** dithiocarbamates	
Leaf spot	Dithiocarbamates, dicarboximides, Bordeaux mixtures	**Polyoxins**
Wilt diseases	Benzimidazoles	

[a,b] See footnotes to Table 1.

Table 3 Herbicides and plant growth regulators (PGR) of chemical and microbial origins and their action sites.

Action site	Herbicides or PGRs[a]	
	Chemical	Microbial
Photosynthesis and respiration	Triazines, acylanilides, pyrazolate, diphenylethers, dinitrophenols	(6241-B)
Biosynthesis or function of:		
Auxin	2,4-D	
Gibberellin	Triazoles	Gibberellin
Fatty acid	Aryloxyphenoxypropionates, cyclohexanediones	
Amino acid	Sulfonylureas, Imidazolinones, glyphosate, phosphinothricin	Bialaphos (Phosalacine, vulgamycin)
Cellulose	(Isoxaben)	(Phthoxazolin)

[a] See footnote a to Table 1.

signal transmission at the GABA (γ-aminobutyric acid) receptor level, although the precise mechanism of inhibition is not yet defined. In *Ascaris* sp., avermectin functioned as a GABA receptor agonist, stimulating GABA release from presynaptic inhibitory membranes. Kass et al (62) proposed that in the presence of avermectin, chloride ion channels remain open, allowing chloride and sodium ions to flow out. The resulting ion imbalance results in the blockade of signal transmission. Recently, avermectin-binding proteins were detected. By using photoaffinity labeling techniques with a biologically active, radiolabeled azidoavermectin derivative, Rohrer et al (129a) identified three (53, 47, and 8 kDa) specific avermectin-binding polypeptides in membranes of a nematode, *Caenorhabditis elegans,* and one (~47 kDa) in an insect, *Drosophila melanogaster*. Studies on these polypeptides, including cloning, are expected to lead to a better understanding on the mode of avermectin action.

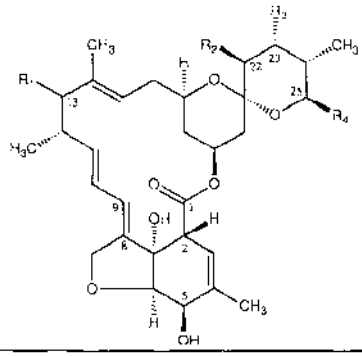
In parallel to the veterinary and medical uses of an avermectin derivative, agricultural usefulness of avermectin itself was also examined. Avermectin showed extremely high efficacies at low rates of applications. It is used as an acaricide in agriculture and horticulture and as an antiarthropod agent for both crop and noncrop applications (76). It is active against mites, leafminers, thrips, armyworms, aphids, and psyllids. Mites (Tetranychidae and Eriophyidae) were highly sensitive with IC_{90} values of 0.02–0.24 ppm, a level some orders of magnitude lower than that for existing synthetic acaricides. It also kills eggs of a tetranychid spider mite. Avermectin's effects on Lepidoptera (hornworms and armyworms) vary somewhat: an LC_{50} of 0.02 ppm for tobacco hornworm and 6 ppm for southern armyworm. Lasota & Dybas (76) suggest that avermectin use in agriculture does not adversely affect beneficial arthropods and agricultural ecosystems [see also Campbell (12), chapters 11–14]. A controversial interpretation of these effects was reported recently (136).

Milbemycins are a structurally related family of compounds produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus* (142). They are 16-membered macrolides very similar to the aglycone of the avermectins but lacking the disaccharide moiety attached to the C-13 hydroxyl of the aglycone (Figure 1). Milbemycins are available commercially as an agricultural acaricide and as a veterinary antiparasite. An oxime derivative has appeared on the market as an antiparasite for dog (151).

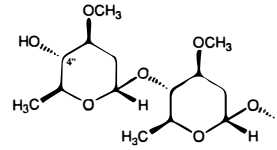
Avermectin's uses have had a positive impact on the chemistry and biochemistry of natural products, including avermectin-milbemycin itself. The compound undoubtedly encouraged those who were searching for the utility of natural products, supporting the strategy of pest control by microbial metabolites. The *Journal of Antibiotics* published four papers in 1980 on microbial metabolites with insecticidal or herbicidal activity. This number

increased to 7 in 1986, and 22 in 1992. Discoveries of antifungal compounds with agrochemical activity also increased.

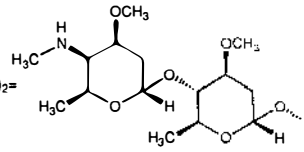
To date, attempts to isolate new avermectin analogues directly from soil microorganisms have been unsuccessful. A later section describes products of microbial and chemical modifications. On the other hand, new milbemycins, totaling 27 components, have been discovered; Figure 1 depicts some of them. The first paper on milbemycins by Takiguchi et al (142) described ten members of the α series and three members of the β series. From a mutant of the original milbemycin-producing culture, Mishima et al (84) isolated five new members of the α series, (D, F, G, J, K) and two members of the β series (E, H). Four VM compounds corresponding to the milbemycin α series were discovered from *Streptomyces* sp. E 255 (47). VM-44857 (Figure 1) showed in vivo efficacy against *Trichostrongylus* spp. at 0.25 mg/kg. Naturally acquired roundworm infections by *Haemonchus*, *Trichostrongylus*, and *Chabertia* spp. were cleared by more than 99% (with a few cases of 96% and 80%) at 0.2 mg/kg. In comparative testing, ivermectin afforded 100% clearance at the same dose level. Another four compounds, LL-F28249 substances named nemadectins, were isolated from *Streptomyces*



(Ole)$_2$=



MA(Ole)$_2$=



	R_1	R_2	R_3	R_4
Avermectin B_{1a}	$(Ole)_2$[1)]	CH=CH		$CH(CH_3)CH_2CH_3$
Ivermectin	$(Ole)_2$	H	H	$CH(CH_3)CH_2CH_3$ (80%), $CH(CH_3)_2$ (20%)
4"-Epi-methylamino-4"-deoxy-avermectin B_1 (EMA)	$MA(Ole)_2$[2)]	H	H	$CH(CH_3)CH_2CH_3$ (80%), $CH(CH_3)_2$ (20%)
Milbemycin D	H	H	H	$CH(CH_3)_2$
Nemadectin α	H	H	OH	$C(CH_3)=CHCH(CH_3)_2$
VM 44857	H	H	H	$C(CH_3)=CHCH_3$
UK 78624	$OCOCH(CH_3)_2$	OH	H	$C(CH_3)=CHCH_3$

1) $(Ole)_2$: α-L-oleandrosyl-α-L-oleandrosyloxy, 2) $MA(Ole)_2$: 4"-epi-methylamino-4-deoxy-$(Ole)_2$

Figure 1 Structures of avermectins and new milbemycin homolgues discovered recently.

cyaneogriseus (15, 150). They were compounds of the milbemycin α series. Nemadectin α at a single dose of 0.2 mg/kg [given orally (or)] was effective in vivo against *Trichostrongylus colubriformis* in gerbils.

One of the curious discoveries resulting from these screenings was the isolation C-13–substituted derivatives from natural sources, such as avermectin aglycone. Unfortunately, the above milbemycin components bore no hydroxyl at the C-13 position. Haxel et al (39) of the Pfizer group discovered a series of 12 C-13 β-acyloxylated molecules, known as UK substances, produced by *Streptomyces hygroscopicus* ATCC 53718. UK-78624 showed in vitro more than 95% killing of *C. elegans* at 10 ng/ml. Thus, a total of 40 milbemycin components were added to the arsenal of chemicals available for pest control.

The C-13 acyloxyl group of UK substances exhibited a β configuration, in contrast to the α configuration of the C-13 hydroxyl in avermectin, the oxygen of which originated biosynthetically from propionate (14). Haxel et al suggested that the C-13 oxygen of the former UK compounds was derived oxidatively from 13 unsubstituted intermediates in *S. hygroscopicus* ATCC 53718. They verified this hypothesis by the conversion of nemadectin γ, which is not a product of ATCC 53718, to 13β-isobutyloxylated nemadectin γ by *S. hygroscopicus* ATCC 53718 (39). No biosynthetic experiments with molecular oxygen were reported.

NEW INSECTICIDES DETECTED BY NEWLY CONSTRUCTED SCREENING METHODS

Otoguro et al (122, 152) devised a screening method for antinematode activity in which the pine wood nematode *Bursaphelenchus lignicolus* was used as the test organism, instead of the conventionally employed free-living nematode, *C. elegans* (128). Nematicidal activity was assayed by counting motile nematodes that could actively pass through a thin filter paper. These authors found jietacins (Figure 2) from a *Streptomyces* strain that was isolated from a soil sample collected at the Jie-tai temple in Beijing. The compound showed an in vitro activity comparable to that of avermectin. This assay system could detect avermectin B_1 (IC_{50} 0.5 μg/ml), as well as gramicidin S (0.1 μg/ml), aureothin (0.5 μg/ml), colistin (1.0 μg/ml), and staurosporine (3.5 μg/ml).

Jietacin is unique in structure in that the molecule possesses an α,β-unsaturated azoxy moiety. Synthetic derivatives of jietacin suggested that the vinyl-azoxy moiety was essential for activity, but the carbonyl moiety could be reduced to a hydroxyl, or to a methylene functionality (152).

Several insecticides were detected by using brine shrimp. Eggs of the brine shrimp *Artemia salina* are available commercially (e.g. at a pet shop). Eggs hatch out within 1–2 days in 3–5% aqueous NaCl solution, or in artificial sea water (which is better and is also commercially available), with or without forced aeration at room temperature (below 28°C). They are then ready for

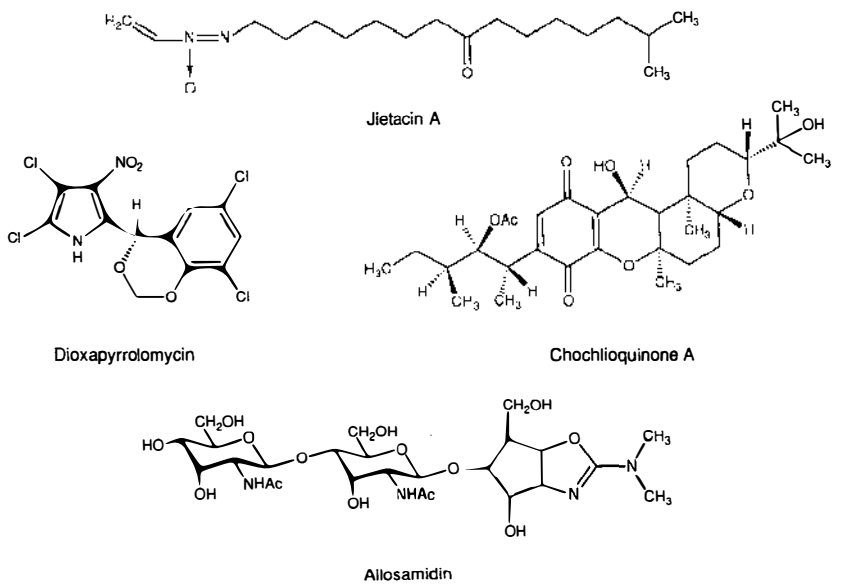


Figure 2 Insecticidal microbial metabolites discovered recently.

use in an insecticide assay. Blizzard et al (9) took advantage of this simplicity to evaluate hundreds of semisynthetic avermectins. The result was roughly parallel to that from a conventional assay method, which uses two-spotted spider mites (*Tetranychus urticae*) and kidney bean leaves and takes 1–3 days. Also using brine shrimp, Conder et al (20) identified dioxapyrrolomycin as an insecticidal compound produced by a strain of *Streptomyces* sp. (95).

ALLOSAMIDIN AND OTHER INSECTICIDES Many other insect-active compounds have been identified from microorganisms. In many cases, the screening methods were conventional or were not reported in detail. Some of them are in an early stage of evaluation. These are chochlioquinone A (131), paraherquamide and recently described related compounds (8), okaramines (41), aculeximycin (88), W-719 substance (126), allosamidins (97), all insecticides, and the miticides AB-3217-A substance (61) and bagougeramin (139).

Chochlioquinone A (Figure 2), which is produced by *Helminthosporium sativum* and was originally discovered as a metabolite of the plant pathogen *Cochliobolus miyabeanus,* is a competitive inhibitor of [^{3}H]-ivermectin binding to membranes of *C. elegans* (131).

A synthetic chemical, buprofezin, was postulated to be a chitinase inhibitor,

but allosamidin is the first such compound obtained from a microorganism. Chitin is a homopolymer of *N*-acetylglucosamine with a β-1,4 linkage. It is involved in the envelope of insects and fungi. Mammalian cells do not contain chitin; hence, chitin synthesis or degradation have attracted much attention as a promising target site for insecticidal and fungicidal agents (18, 58). Sakuda et al (97, 130) exploited allosamidins (Figure 2), which are produced by *Streptomyces* No. 1713. A chitinase preparation from the silkworm (*Bombyx mori*) was used in screening. Allosamidins are oligosaccharides composed of an *N*-acetylyglucosamine dimer (chitobiose) and a new bicyclic aminocyclitol named allosamizolin. Allosamidin and demethylallosamidin inhibited chitinases from *B. mori* equally (IC_{50} of 0.20 and 0.25 μg/ml, respectively). Allosamidin inhibited ecdysis of *B. mori* and *Leucania* sp. when injected at 2–4 μg/insect. No contact inhibition was observed. *Saccharomyces cerevisiae* cells exposed to allosamidin were heavily attached to each other.

Herbicides

Herbicides compose the largest portion (44%) of total pesticide sales. Currently applied herbicides consist mostly of synthetic chemicals. A few of them were designed with microbial and other natural products as a model. NK-049, for example, was synthesized based on the structure of anisomycin, an herbicidal metabolite from *Streptomyces* sp.

Bialaphos is the first microbial product put into practice as an herbicide. Keys to this great success were the potent and broad-spectrum activity, the high fermentation yield achieved during the development of this compound (141), and the basic studies on biochemistry and genetics of bialaphos biosynthesis and resistance (38, 73). It is an inhibitor of glutamine synthetase.

Discoveries of new herbicidal microbial metabolites increased recently, but are slower than discoveries of insecticides and fungicides (4, 45). Many phytotoxins and plant-growth regulators were identified that were produced by plant-pathogenic fungi as chemicals likely to be involved in plant-pathogen interactions. However, these toxins are not included in the above reckoning, because the relevant plants are beneficial ones like rice or ornamentals and thus the reported phytotoxins have less herbicidal value. Phytotoxins produced by weed-parasitic fungi have more potential as herbicides (146). An example is alteichin produced by *Alternaria eichorniae* (129).

NEW HERBICIDAL COMPOUNDS WITH PROMISING ACTIVITY Three new microbial metabolites showed promising activity. Hydantocidin (Figure 3) produced by *S. hygroscopicus* SANK 13584 is a unique sugar-spiro-hydantoin with potent herbicidal activity (82, 93). Chinese cabbage seeds in small test tubes were used for herbicidal assays. A spore suspension of *S. hygroscopicus* was used as inoculant for constant fermentation yields. It inhibited nonselectively

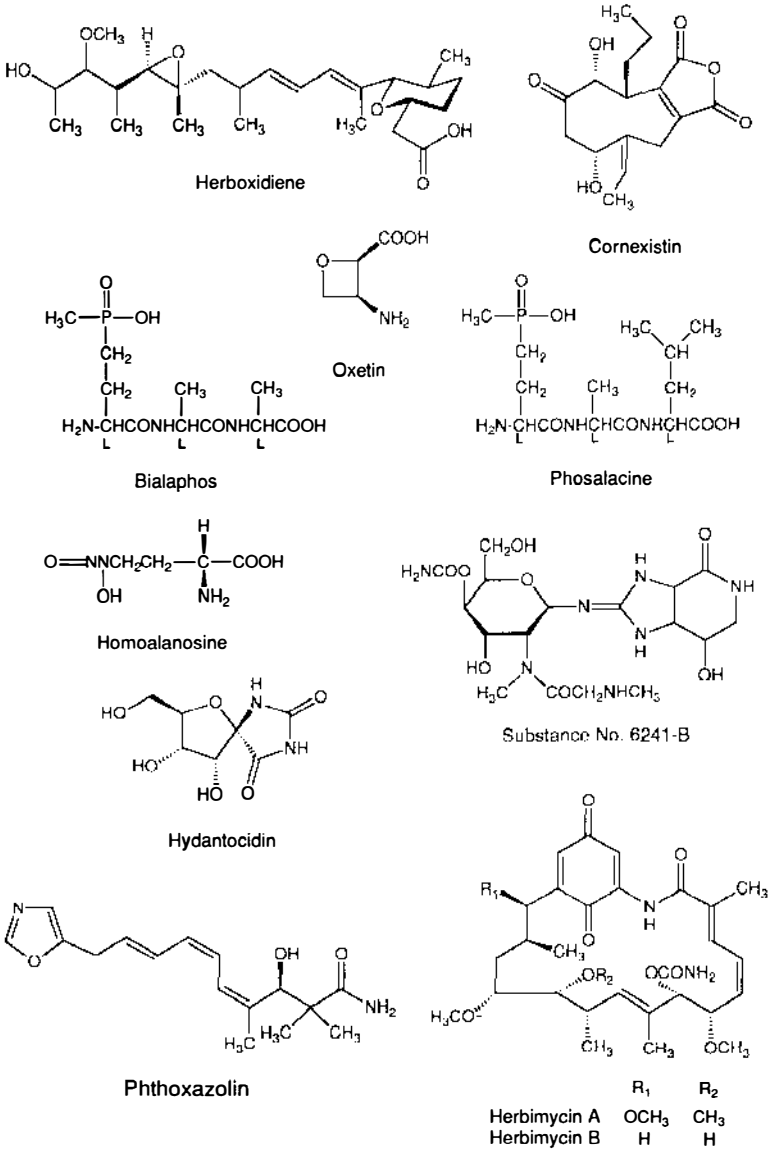


Figure 3 Recently discovered herbicidal microbial metabolites. Bialaphos is included as reference.

the growth of mono- and dicotyledonous annuals and perennials. Under pot-test conditions, the effect of hydantocidin at 500 ppm was comparable to glyphosate (at 500 ppm) and somewhat superior to bialaphos (at 500 ppm). Deoxy derivatives were synthesized but showed no activity (82).

Cornexistin is a lipophilic herbicidal metabolite with a nonadride structure (Figure 3) isolated from *Paecilomyces variotii* SANK 21086 (94). Pot tests demonstrated activity against common annual species of weeds on postemergence treatment at 100 and 500 ppm. Corn seedlings tolerated 500 ppm of cornexistin well. Thus, cornexistin is probably useful for postemergence weed control in corn fields. A weak herbicidal effect of the structurally related rubratoxin B was reported earlier (94).

Herboxidiene was discovered as an herbicidal metabolite of *Streptomyces chromofuscus* (81). Postemergence treatment demonstrated a selective effect at 69 g/ha. Many weeds (rape, wild buckwheat, morning glory) were well controlled, whereas rice, soybean, and wheat exhibited little or no sensitivity. Fermentation yields have been increased by about 20-fold, reaching 160 mg/liter. Screening method and mode of action were not reported for these compounds.

HERBICIDAL COMPOUNDS WITH INTERESTING MODES OF ACTION Herbicidal screening usually requires more effort and a longer time period for the assay than does antifungal screening. A conventional spray-assay in-pot test requires two weeks or so, which implies a smaller number of test samples per year, and perhaps less chance of discovering new compounds. These difficulties may account, at least in part, for the observed slow discovery of new herbicidal compounds from microorganisms.

A gap between in vitro effect and in vivo efficacy also hampers herbicidal screening. In addition, rediscovery confounds screening, especially when a random screening technique is employed. In fact, many known compounds have been observed more than once with herbicidal activity, such as several nucleosides including aristeromycin, coaristeromycin, 5-deoxy-toyocamycin, coformycin, Ara-A, toyocamycin, tubercidin, sangivamycin (4, 55), isoxazole-4-carboxylate, harman and norharman, arabenoic acid, α-methylene-β-alanine (56, 57, 71, 158), homoalanosine (34), and so on.

An alternative approach to finding new herbicides would be biorational mechanism–based in vitro screening, followed by in vivo pot tests, the latter of which is absolutely necessary. Microbiological assay methods or enzyme inhibition assays (149) are convenient when available. This approach enables herbicidal assays to be smaller in apparatus size and larger in number of test samples. Recent trials along this line led to the discovery of new herbicides of interesting modes of action. These are amino acid–synthesis inhibitors, cellulose-biosynthesis inhibitors, and starch-synthesis inhibitors.

Ōmura et al (64, 105, 108, 109) found the glutamine antagonists, phosalacine and oxetin, both with potent herbicidal activity (Figure 3). A bacterium, *Bacillus subtilis,* grown in a synthetic medium (Davis' medium) was employed for the initial detection of glutamine antagonism. Phosalacine is a tripeptide, phosphinothrycyl (PT)-alanylleucine, similar to bialaphos (PT-Ala-Ala). The original culture of bialaphos-producing *S. hygroscopicus* SF-1293 produced, besides bialaphos, PT-Ala, PT-Gly-Ala, and PT-Ala-Val (54). Another strain of *S. hygroscopicus* produced trialaphos (PT-Ala-Ala-Ala) (63). These compounds are probably also glutamine antagonists.

L-Homoalanosine is a synthetic analogue of L-alanosine, an ecdysis inhibitor of microbial origin (80). L-Homoalanosine was also found in a culture of a *Streptomyces* sp. and showed selective herbicidal activity at 400 g/ha. It is an aspartic acid and/or glutamic acid antagonist.

Vulgamycin, a lipophilic metabolite with no structural similarity to amino acids reported earlier, was found to act as an isoleucine antagonist. It showed selective protection of cotton, barley, and maize from weeds on postemergence treatment at 125 and 500 g/ha (6). In *E. coli,* vulgamycin inhibited acetolactate synthase (isozyme I). In a cell suspension of *Catharanthus roseus,* chlorotic symptoms caused by vulgamycin (10 μg/ml) reverted upon supplementation with isoleucine plus valine. L-Methionine-reversible chlorosis was also observed with a *Streptomyces* product, 2-amino-1-hydroxyl-cyclobutane-1-acetic acid (5). Herbicidal effects of aromatic amino acid analogues, 2,5-dihydrophenylalanine (54) and 1,2,4-triazolealanine (53) were reported.

Kishore & Shah (70) pointed out four pathways to amino acid groups as feasible target sites of herbicides: glutamate and glutamine, branched-chain amino acids, aromatic amino acids, and histidine. Apparently other pathways are also important, although the mechanism by which amino acids reverse chlorosis is not known.

A cellulose biosynthesis inhibitor, phthoxazolin (Figure 3), was discovered by our group as a potent herbicide (119). Cellulose resides in all the plant systems, but not in mammalian cells. Cellulose biosynthesis therefore is one of the promising target sites with excellent selective toxicity. Ōmura et al employed the fungus *Phytophthora parasitica* as a test organism. This fungus contains cellulose in the cell walls, but common fungi such as *Candida, Pyricularia* spp., etc, do not. The active microbial cultures showed anti-*Phytophthora* activity, but no growth inhibition against common fungi. Phthoxazolin showed potent herbicidal activity in pot tests and inhibited cell-free cellulose synthesis. This is the first microbial metabolite isolated as a cellulose-biosynthesis inhibitor. Recently, a synthetic chemical, isoxaben, was shown to have this activity in *Arabidopsis thaliana* (42).

Photolinked starch synthesis occurs only in plants and limited genera of bacteria (photosynthetic bacteria). This is another target site warranting

excellent selective toxicity for herbicides. In fact, half of synthetic herbicides currently available in the marketplace are photosynthetic electron-transfer inhibitors. A new inhibitor of starch synthesis, 6241-B substance (Figure 3), of *Streptomyces* origin was exploited as the first example of this class of microbial herbicides (67). This substance showed at 500 ppm selective herbicidal activity against barnyard millet (*Panicum crus-galli*) with no toxicity to rice. The screening was based on the initial detection of de novo starch-synthesis inhibition in leaf segments of barnyard millet, followed by determination of O_2 evolution from cells of a green alga, *Scenedesmus obliquus*.

Fungicides

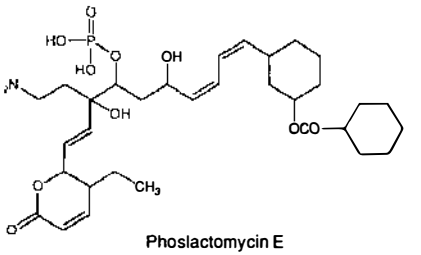
Fungal diseases of crop plants are extremely numerous. Because of narrow host-pathogen specificity, different antifungal pesticides are required for individual diseases. Hence, many kinds of fungicides are available (see Table 2). However, the effectiveness of antifungal agents changes (7) because of the emergence of resistance, and antifungal agents that control fungal diseases are still limited. A few serious fungal diseases remain uncontrolled today.

A great many antifungal metabolites have been isolated from microorganisms. Many of them were studied mainly as medicines; some were evaluated as agricultural fungicides. This section discusses those with promising crop-protection action in pot tests.

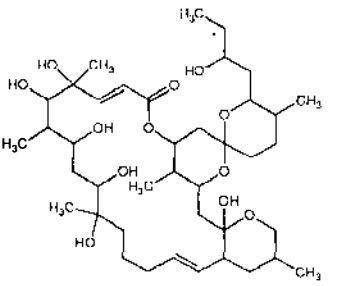
ANTIFUNGAL COMPOUNDS WITH FEASIBLE ACTIVITY A family of phosphate ester antifungal agents, phoslactomycins, was isolated from *Streptomyces nigrescens* (Figure 4) (35). Structurally related phosphazomycins (148) have different substituents on the terminal cyclohexane ring. Phoslactomycin E exhibited potent in vitro activity (MIC 0.3–3.0 μg/ml) against *Botrytis cinerea, Rhizoctonia solani*, and *Alternaria kikuchiana*. Phoslactomycin E controlled *Botrytis* infection at 10 ppm with no phytotoxicity in pot tests, whereas other derivatives were somewhat phytotoxic to wheat.

A 7-deaza purine nucleoside analogue, dapiramicin (98) (Figure 4), produced by a *Micromonospora* sp. showed weak in vitro antifungal activity against *Rhizoctonia, Pyricularia*, and *Botrytis* spp., which was monitored by observing mycelial elongation of *Rhizoctonia solani* on pieces of water agar under a microscope. However, dapiramicin potently suppressed rice-sheath blight caused by *R. solani*. The efficacy (95% protection at 50 ppm) was comparable to validamycin.

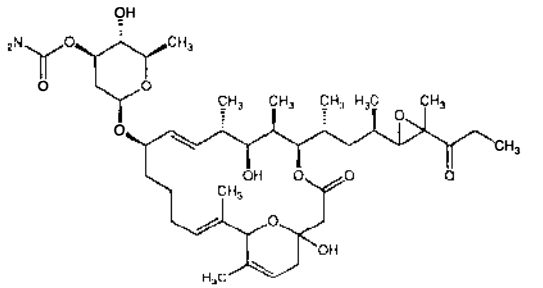
Our group (111, 120) discovered irumamycin (Figure 4), a 20-membered macrolide produced by a *Streptomyces flavus* subsp. *irumaensis*. It showed potent in vivo and in vitro activity against *Pyricularia* and *Botrytis*, and it



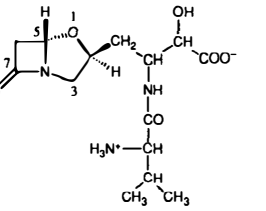
Phoslactomycin E



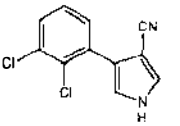
Phthoramycin



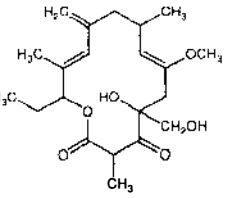
Irumamycin



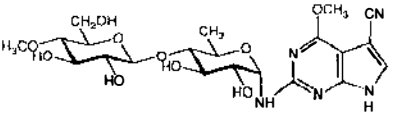
Valclavam



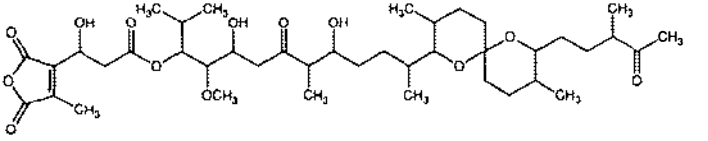
Fenpiconil



Rustmicin



Dapiramicin



Tautomycin

Figure 4 Recently discovered fungicidal microbial metabolites and their synthetic derivatives.

tolerated sunlight. The application of irumamycin to *Botrytis* control is under study.

FUNGAL CELL-WALL INHIBITORS AND OTHER ANTIFUNGAL COMPOUNDS WITH NOVEL TYPE OF AGROACTIVITY Fungal cell walls provide one of the best target sites for selective toxicity of fungicides. Polyoxins represent this class of fungicides and have been used in agricultural fields (58). The emergence of polyoxin-resistant fungal strains have restricted the effect of polyoxins.

Nikkomycins are structurally related nucleosides with antifungal, insecticidal, and miticidal activities (27). Unfortunately, the development of nikkomycins as an agricultural agent has been discontinued because of lower efficacy than expected. In a search for new nikkomycin components, mutants of *Streptomyces tendae* were found to produce new nikkomycins: K_2, K_x, O_z, O_x, ϕ_z, ϕ_J, W_z, W_x, S_z, S_x, S_{oz}, S_{ox} (10, 132).

Peptide antifungal agents such as globopeptin (145), neopeptin (153), and cystargin (156) inhibit cell-wall glycan biosynthesis and induce swollen morphology of plant-pathogenic fungal cells. Asperfuran (124) was isolated as an inhibitor of chitin synthase from *Coprinus* sp.

Widely spread fungal diseases of crop plants that have not been controlled well include, among many others, potato late blight (caused by *Phytophthora infestans*), grape downy mildew (*Plasmopara viticola*), barley stem rust (*Puccinia graminis*), and apple scab (*Venturia inaequalis*). Bacterial infection by *Erwinia* spp. has also been poorly controlled. Microbial metabolites active against these pathogens are of agricultural interest and importance (19, 133). Omura (90, 118) found a 22-membered macrolide named phthoramycin, which was active against *Phytophthora* spp. A related glycoside, cytovaricin (69), and a stereoisomer, kaimonolide (44), were also reported. The latter compound was herbicidal. Phthoramycin is now under evaluation as a pesticide.

Many antifungal β-lactams, valclavam, clavamycin, Ro 22-5417, and hydroxyethylclavam (89, 125) have been discovered. Valclavam (Figure 4) showed potent in vitro activity against an oomycete, *Pythium*, and other phytopathogenic fungi. Antibacterial activity varied. Unfortunately, an immediate application of valclavam as fungicide could not be achieved because of its instability; either of two stereoisomers can occupy the C-5 position. Valclavam exhibited 5S configuration, whereas 5R configuration was evident in clavulanic acid, other antibacterial β-lactamase inhibitors, and penicillins. Hydroxyethylclavam inhibited RNA synthesis in *S. cerevisiae*, but acted as a methionine antagonist in *E. coli* and *B. subtilis*.

New 14-membered macrolides, rustmicin and neorustmicin, were isolated from *Micromonospora chalcea* (Figure 4) (96). Galbonolides isolated independently from *Streptomyces galbus* were identical to rustmicins (26).

Rustmicin potently inhibited the proliferation of the wheat stem rust fungus *Puccinia graminis* in a greenhouse. This is the first microbial metabolite that shows anti-*Puccinia* activity in vitro and in vivo. An in vitro assay method for this obligate parasite was developed and used in the screening.

MICROBIAL AND CHEMICAL MODIFICATIONS OF COMMERCIAL MICROBIAL AGROCHEMICALS

Investigators have used chemical and microbial methods to modify agroactive microbial metabolites to obtain derivatives with potentiated activity and increased photostability. Many semisynthetic avermectin derivatives have been synthesized and evaluated (12, 21, 22, 24, 87). Among them, 4″-*epi*-methylamino-4″-deoxy avermectin B_1 (EMA) (Figure 1) exhibited a dramatically altered activity spectrum (24). EMA was one tenth as active against spider mites as avermectin B_1, but 1500 times more active against armyworms.

The biosynthetic pathway to avermectins (12, 49–51), its regulation (99), and selective production (106) have been elucidated at the biochemical and genetic levels. Studies on aglycone biosynthesis showed that the C-25 substituents originated from isobutyrate or sec-valerate provided by valine or isoleucine metabolism, respectively. A mutant, *S. avermitilis* ATCC 53568, was isolated that was defective in the ability to synthesize precursors for aglycone synthesis (23, 36). This mutant restored avermectin production when the culture medium was supplemented with the precursor fatty acids. Dutton et al (23) applied a mutational biosynthesis technique using this mutant to prepare avermectin analogues. Feeding about 800 fatty acids to cultures of ATCC 53568 yielded 17 new avermectins possessing an altered substitutent at C-25 that included 4-tetrahydropyranyl, cyclohexyl, 2(or 3)-thienyl, 1-methylbut-1-enyl. All showed 100% killing of *C. elegans* at 0.1 μg/ml (23). By similar techniques, analogues with a sec-valeryl, or a sec-hexyl substituent at C-25, were obtained (16).

Chemical modification of milbemycin D led to the discovery of 5-keto-5-oxime derivatives with increased activity against a microfilaria, *Dirofilaria immitis* in dogs (151).

Microbial conversion of milbemycins allows hydroxylation at C-13. This conversion can be accomplished using various microorganisms, including *Streptomyces violascens* ATCC 31560, *Streptomyces cavourensis* SANK 67386, *Amycolata autotrophica* ATCC 35204, *Cunninghamella echinulata* ATCC 9244, and *Syncephalastrum racemosus* SANK 62672 (91, 92, 147). Thus milbemycins A_3, A_4, D, and nemadectin α were monohydroxylated at C-13, and in some cases dihydroxylated at C-13 and C-24, 28, 29, or 30. *Amycolatopsis mediterranei* IFO 13415 hydroxylated 22,23-dihydro-avermectin B_{1a} at C-30.

The photostability of avermectins is very poor, as it is for other macrolides with a diene-lactone ring system. Avermectin loses activity within one day under sunlight. Conversion of avermectin to an 8,9-epoxide increased the stability significantly without decreasing the miticidal activity (24). The same modification of 13-deoxyaglycone, a milbemycin, resulted in a loss of activity.

Pyrrolnitrin is an antifungal metabolite from a *Pseudomonas* species. It is also photolabile. Nyfeler (28) synthesized a stable derivative, fenpiconil (Figure 4), by replacing Cl and NO_2 with CN and Cl, respectively. Fenpiconil is now under development as a seed treatment.

By feeding 5-fluorocytosine to a culture of the blasticidin S–producing *Streptomyces griseochromogenes,* Kawashima et al (65) obtained 5-fluoroblasticidin S. Antifungal activity did not change. The fluorinated product is expected to show long-lasting activity in field applications because of resistance to enzymatic 4-deamination, a mechanism of blasticidin S inactivation caused by blasticidin S–resistant bacteria (60). Fluorovulgamycins were prepared in a similar manner (66).

UNCOVERED PHARMACOLOGICAL ACTIVITY OF AGROACTIVE MICROBIAL METABOLITES

Several compounds identified as insecticides, herbicides, or fungicides were later found to exhibit entirely different, but pharmacologically interesting, activities (102).

Herbimycins, an ansamycin group of compounds produced by a *Streptomyces* species, were discovered by our group (33, 34, 110, 134) as herbicidal, antitumor, and antiviral compounds. Recently herbimycin A (Figure 3) was demonstrated to be an inhibitor of oncogene function, inhibiting protein-tyrosine kinase. Herbimycin A was effective in restoring normal cell morphology from abnormal NRK cells that were previously transformed by tyrosine kinase oncogenes such as *src, ras, myc,* etc (154). Antitumor activity of herbimycin A and its semisynthetic derivatives are under evaluation (46, 155).

Tautomycin (Figure 4) was initially reported by Isono's group as an antifungal compound of agrochemical interest (17). Later studies revealed that it induced bleb formation in K562 cells. The bleb formation resulted from inhibition of protein phosphatase by tautomycin and other phosphatase inhibitors (75).

Sangivamycin is an herbicidal nucleoside with a 7-deaza adenosine structure. Osada et al (121) showed that sangivamycin inhibited protein kinase C in K562 cells. The inhibition was less potent than that of staurosporine (107), a microbial alkaloid with insecticidal activity (122) and the most potent

inhibitor of protein kinase C (143). Tubercidin and toyocamycin are related 7-deaza adenosines, but exhibited weaker inhibition than sangivamycin.

Dioxapyrrolomycin (Figure 2) and related pyrrolomycins are substance P antagonists (78). Substance P and neurokinin A are neuropeptides of the tachykinin family. Substance P was proposed to be a neurotransmitter of pain or an internal mediator in inflamatory and immune responses.

How the above activities are related to the initially described agroactivity of the compounds is not known. Nevertheless, these examples demonstrate the diversity in activity and structure of microbial secondary metabolites, and at the same time, the importance of screening technology.

PERSPECTIVES

The avermectin story encourages optimism about further discovery of excellent pesticides from microorganisms. This review has described many microbial metabolites exhibiting a variety of activities and structures. The described compounds probably include candidates for the next success story, as supported by the considerations below.

The number of microorganisms, and the secondary metabolites they produce, is almost uncountable. About 180,000 species have been identified, but an estimated 5 to 10 times more species probably exist. Applied microbiology is currently dealing with only 10% or less of the total microbial population. To date, 16,500 individual microbial metabolites have been isolated and characterized. One percent of them, 130–150 compounds, have found practical uses. Ten or so are used in agriculture, several tens in veterinary fields, and all the rest in humans. In medicines, approximately 20% of sales come from microbial compounds, to which semisynthetic penicillins and cephalosporins contribute much. One wonders why microbial metabolites do not exceed this rate in the pesticide market. In any case, approximately one practicable pesticide is found at a rate of 10^{-2}, an efficiency two orders of magnitude higher than the corresponding rate, 10^{-4}, for synthetic agrochemicals (45). One should note that the 10 already commercialized agricultural compounds have been selected out, not from 16,500, but from a smaller number of agroactive microbial metabolites discovered in pesticidal screening. Obviously, a great many agroactive compounds produced by a huge number of microorganisms await discovery. An immediate question is how to identify them.

In the development of a biological screening program, one considers the target pest to be controlled first, then the mode of action of newly discovered compounds. Based on such considerations, combined with an estimate of need, impact, market size, etc, appropriate screens are constructed and

optimized. Tables 1–3 suggest examples of the pests to be tackled by future screening programs. In choosing the expected sites of action exhibited by the compounds discovered, selective toxicity is the major concern. In this sense, targets of action of possible new inhibitors include chitin synthesis and metabolism; cellulose biosynthesis; amino acid biosynthesis, in paticular that of essential amino acids; sterol biosynthesis and metabolism; photosynthetic reactions; and so on. These metabolic pathways represent fundamental structural and physiological differences between harmful insects, mites, fungi, weeds, and humans. The advantage of cell envelopes as targets of pesticide action is well documented (18, 58). In addition, insects and mites are not capable of de novo synthesis of cholesterol. Insects therefore require phytosterols, from which they derive ecdysteroids for growth and reproduction (137). These insect-specific steps are rational targets for selective toxicity, but no microbial agrochemicals of this class have been reported. Alternate uses of two pesticides with different modes of action is one way to reduce the emergence of resistant pests. Therefore, microbial metabolites with varying actions are necessary. Compounds with multiple action sites may be of interest because a low frequency of resistant pests should result.

Biological pest control is based on the chemicals involved in plant-pest and natural enemy–pest interactions (1, 31, 146). Despite its potentially great advantages, biochemical pest control plays only a compensatory role for chemical means because it is too strict in host or pest specificity and the efficacy is insufficient to protect crops from simultaneous attacks by multiple types of harmful pests. This technology will find more utility in future plant factories, where gene-engineered plants are grown in clean houses.

Pesticide chemistry has progressed by unexpected discovery, concentrated research, basic science, and interdisciplinary cooperation. Further progress will come from these sources. Of particular importance is the cooperation of microbiologists, plant physiologists, biochemists, entomologists, and chemists.

Finally, both industry and academia must exert more effort to promote positive public perception and public acceptance of pesticide chemicals (28). One way can be a campaign about roles of pesticides in crop production, correct uses, production process, and regulation, and about the global necessity and ecological safety of modern pesticides of both microbial and synthetic origins. These efforts should lead to an increase in the variety of chemicals available for field applications, as well as increase general interest in microbial pesticides. The shortage of research scientists and research funds in pesticide sciences would be solved in this way. In total, these efforts should accelerate progress in pest control.

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