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# Trends in the search for bioactive microbial metabolites

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## SUMMARY

Bioactive microbial metabolites are attracting increasing attention as useful agents for medicine, veterinary medicine, agriculture, and as unique biochemical tools. A review of the current trends in the discovery of new metabolites shows that the number of active compounds with non-antibiotic type of activity has increased, resulting in an expansion of the variety of bioactivity of microbial metabolites. Factors that contribute to the increased rate of discovery include: development of new methods for activity measurement, exploitation of novel groups of microorganisms as sources of active compounds, new directions for chemical modification, and incorporation of newer knowledge of biotechnology into screening systems. To exemplify this, typical screening methods, and chemical and biological properties of several bioactive compounds obtained by these methods are discussed.

## INTRODUCTION

During the past 19 years my group at the Kitasato Institute has enjoyed the fortune of finding more than 100 new bioactive microbial secondary metabolites. Some of them have found practical usefulness as clinical or veterinary medicines (avermectin, nanaomycin, rokitamycin, tilmicosin, etc.) or as reagents for biochemical research (staurosporine, triacsin, etc.).

I started my antibiotic research in 1965. It was after the 'golden age' had passed, and many people working on antibiotics began to think that any further discovery of new antibiotics would be impossible. Actually, however, the number of newly discovered antibiotics and other bioactive compounds has continued to increase until today. These include thienamycin, monobactam, avermectin, FK-506, compactin, bialaphos, tunicamycin, and staurosporine, to cite only a few among numerous others. The interest and efforts directed toward the finding of these compounds have evolved new trends in this field.

Presented here is an overview of the current trends in studies on bioactive microbial metabolites and a discussion of the modern methods, as well as other factors, leading to these trends. This consideration will be useful for future success in searches for bioactive compounds. The active compounds discovered in our laboratory at earlier times and the philosophy of their discovery have been described [76]. The present article deals with the methods of discovery and the biology and chemistry of the bioactive compounds discovered more recently. Table 1 lists these compounds. Characteristic properties of some of these compounds have been reviewed [32,44,76–78,120,122].

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## CURRENT TRENDS IN STUDIES ON BIOACTIVE MICROBIAL SECONDARY METABOLITES

### *General trends*

Based on the requirements for long-lasting safety and excellent chemotherapeutic activity, the search for bioactive metabolites has been continued for the past half century. In spite of the ever-increasing difficulty faced in screening research, a steady increase has continued to date in the number of newly discovered bioactive compounds of microbial origin. On surveying the bioactive compounds reported in these decades, it is revealed that the activity profiles of bioactive compounds have changed greatly and, therefore, the methods for finding them have also changed, as characterized below:

TABLE 1

Screening methods and the new compounds found by Ōmura's group at Kitasato (1986–1991)

| Screening system | Compounds (Ref.) |
|---|---|
| Bacterial cell wall synthesis inhibitor | izupeptin [65] |
| Antifolate | 7-hydro-8-methylpteroryl-glutamylglutamie acid [64] |
| Antianaerobic | lustromycin [123], cervinomycin [66], clostomicin [84] |
| Antifungal | atpenin [55], globopeptin [121] |
| Anticancer | trienomycin [107], phenazinomycin [89], glucopiericidinol [19], furaquinocin [51b], 13-hydroxyglucopiericidin [62], okicenone [20], kazusamycin [132] |
| Cellulose synthesis inhibitor (herbicidal) | phthoramycin [88], phthoxazolin [90] |
| Insecticidal | jietacin [35], avermectin derivatives |
| Anticoccidial | diolmycin, xanthoquinodin |
| Inhibitor of | |
| Acyl-CoA synthetase | triacsin [86] |
| HMG-CoA synthase | 1233A [126] |
| Acid protease | ahpatinin [83] |
| Adenosine deaminase | adecypenol [120] |
| ACAT | purpactin [128], pyripyropene |
| Calpain | KP-1241 |
| Farnesyltransferase | OH-4652 |
| Neuritogenesis inducer | lactacystin [91] |
| Anti-platelet | aggreceride [85], aggreticin [87] |
| Antioxidant | isoflavone [18] |
| Gene technology product | mederrhodin [82], tetrahydrokalafungin [44] |
| Semisynthetic derivatives | EM-523 [101], tilmicosin [49] |

(i) The number of non-antibiotic type of active compounds has increased.
(ii) The target organisms for antimicrobial agents shifted from bacteria to other microbes.
(iii) The variety of microbial sources employed for the production of active compounds has expanded.
(iv) The application of recombinant DNA technology has increased in construction of screening systems and in breeding producing microorganisms.
(v) New biological activities have been discovered in old antibiotics or in their synthetic derivatives.

*Expansion of biological activity*

Of the above five characteristics, the increase of non-antibiotics (item i) is most significant. Fig. 1 represents a chronology taken from the Kitasato Microbial Chemistry (KMC) database on bioactive microbial secondary metabolites. Up until 1990, a total of 9046 active compounds were reported (more precisely, entered into the KMC database), of which about 67% were actinomycete products. Fig. 1 shows the numbers of antibiotic and non-antibiotic types of bioactive compounds.

It is obvious that prior to 1965 the major interest was directed toward antibiotics. Around 1965, the search for

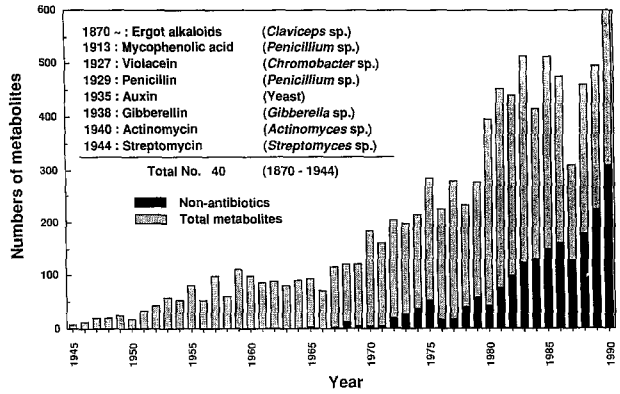



Fig. 1. Chronological list of discovery of antibiotics and other bioactive secondary metabolites.

active compounds other than antibiotics began, and was pursued continuously until 1990, when the percentage of non-antibiotics reached 53% (305/599). The increase is most marked in the most recent 5 years. Compounds in this category include inhibitors of enzymes related to disease development, immunomodulators, effectors of neurotransmission, receptor-binding agonists or antagonists, etc. It is interesting that the first microbial metabolites put

into clinical medicine were ergot alkaloids. Gibberellin is the second oldest microbial metabolite of the non-antibiotic type.

The increase in non-antibiotics has been associated with the cessation of the increase in the typical antibiotics. Simultaneously, the activity spectra of antibiotics changed (item ii). Namely, the target organisms for antibiotic activity shifted from bacteria to other microbes such as fungi, viruses, and protozoa, and other organisms such as nematodes, insects, mites, and weeds.

The continual discovery of bioactive compounds with new types of activity resulted in an expansion of horizons, and created new horizons in the studies on bioactive microbial metabolites. This is illustrated in Table 2.

This table clearly shows that microbial metabolites exhibit versatile biological activities, much more than had been expected many years ago, and, more importantly, there remain many open areas which await the discovery of new compounds. One way for further discovery may be to re-evaluate the latent activity of known compounds, as is the case with cyclosporin A. This compound was originally reported as an unattractive antifungal antibiotic, but was revived as a potent immunosuppressant more than 10 years later. Today it is one of the important adjuncts for transplantation. The latent activity of an antibiotic, once revealed, can be potentiated by chemical modification, as will be described later.

*Innovations in methodology*

The above progress was obviously brought about as a consequence of continual efforts toward methodological innovations. These include the improvement of screening systems and microbial sources, e.g., application of recombinant DNA technology, and conceptual breakthroughs in chemical modification of already existing antibiotics, as are summarized in the above items iii–v.

In order to illustrate and exemplify the above characteristics, several bioactive compounds are described below, taken from those discovered in our laboratory (Table 1).

## NEW COMPOUNDS WITH NEW TYPES OF ACTIVITY

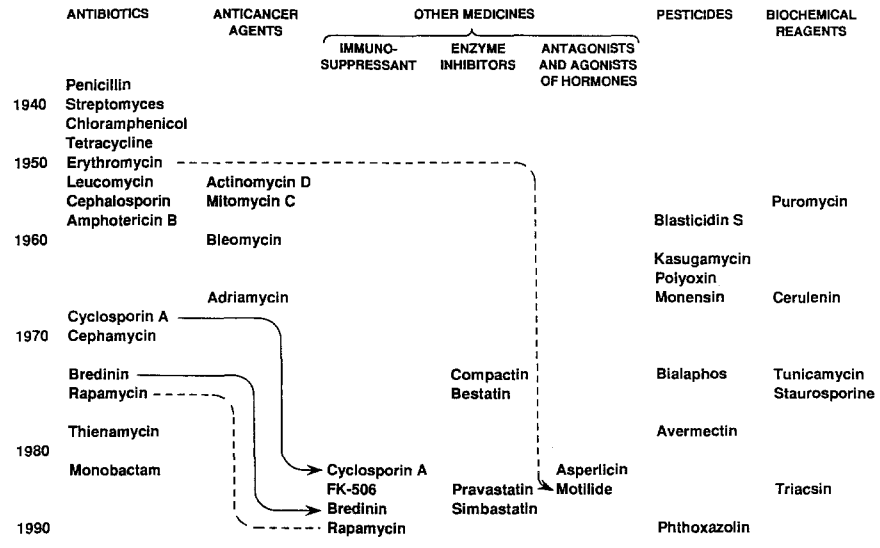
*Effectors of internal regulatory factors*

*Motilide: an agonist of the peptide hormone motilin.* Erythromycin was found as a product of *Saccharopolyspora erythraea* in 1952 [60]. This antibiotic has been widely used as a chemotherapeutic agent effective against infections by Gram-positive bacteria, especially β-lactamase-producing bacteria, and of mycoplasma. Although erythromycin shows no serious side effects, it sometimes causes gastrointestinal disorders such as nausea, vomiting and diarrhea [54]. An interesting pharmacological finding made in 1984 is that erythromycin induces gastrointestinal contraction [42].

The gastrointestinal contraction in dogs was determined by chronically implanted force transducers to which polygraphs were connected [41]. The contraction which appeared in dogs in the fasting state, occurs about every 100 min, and continues for about 25 min. It starts in the gastric body first, then moves to the gastric antrum,

TABLE 2

Expanded horizon of microbial secondary metabolites



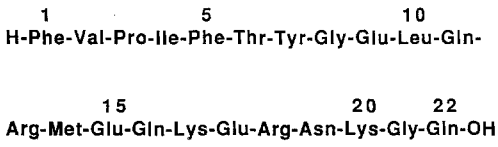



Fig. 2. Structure of motilin.

duodenum, jejunum, middle intestine and ileum. Therefore, it is called interdigestive migrating contraction.

The interdigestive migrating contractions are controlled by the gastrointestinal peptide hormone motilin which consists of 22 amino acids [6] (Fig. 2). Intravenous administration of 0.1 mg of erythromycin/kg, which is one-fiftieth of the normal erythromycin dose level when used as an antimicrobial agent, induces gastrointestinal contraction. This contraction is quite similar to natural contraction [41].

We assumed that by carrying out chemical modifications of erythromycin, it would be possible to obtain derivatives with potentiated contraction-inducing activity but with no or reduced antibacterial activity. This is a reverse approach in that an interesting but rather subsidiary activity of an antibiotic is enhanced, but its growth inhibitory activity, which has long been relied upon, is diminished. An attempt like this probably had not been made before with such an established antibiotic as erythromycin A.

Among about 250 chemically synthesized derivatives, four compounds, EM-201, and its derivatives EM-523, EM-536 and EM-574 were studied in detail. The most potent compound, EM-536, has a quarternary amine with two methyl groups and one propargyl group on the nitrogen atom. The contraction-inducing activity of EM-536 is 2890-times as great as that of erythromycin A, and it exhibits no antibacterial activity (Table 3).

Fig. 3 shows the effects of EM-523 and motilin on gastrointestinal contractile activity in fasting, conscious dogs. The contractions induced by EM-523 are quite similar to those induced by motilin [37]. EM-523 was demonstrated to act as an agonist of motilin by a receptor-binding experiment using rabbit duodenal muscle and human and rabbit antral smooth muscle membrane [14,52].

Recent studies on motilin [17,47] show that the five N-terminal amino acids are indispensable for hormone activity; these amino acids are involved in the hydrophilic region of the eight $\beta$-sheet-forming amino acids at the N-terminus of the motilin molecule. A stereomodel of EM-523, as elucidated by X-ray analysis, nicely overlaps this $\beta$-sheet. The generic name 'motilide' was proposed for a series of macrolides with motilin-agonistic activity [93]. Motilides may be useful to modulate the contractile activity, and will offer a wide variety of therapeutic approaches to the treatment of gastrointestinal disorders.

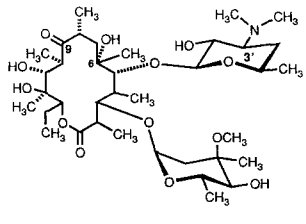
Some other antibiotics also cause 'side effects', which are most probably related to pharmacological activities of the antibiotics. The immunosuppressive effect of cyclosporin A and several anticancer antibiotics are well doc-

TABLE 3

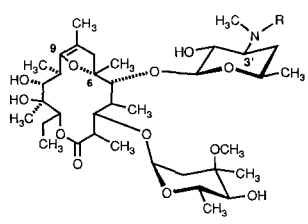
Antibacterial activity and gastrointestinal motor-stimulating (GMS) activity of motilides

| Compound | Antibacterial activity (MIC, μg/ml) [1] | GMS activity [2] |
|---|---|---|
| EM A | 0.2 | 1 |
| EM 201 | 50 | 10 |
| EM 523 | >100 | 18 |
| EM 574 | >100 | 248 |
| EM 536 | >100 | 2890 |

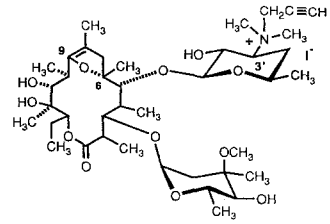
1) *Staphylococcus aureus* ATCC 6538P, agar dilution method.
2) GMS activity was estimated by 2 x 2 points pallel line assay.



Erythromycin A (EM A)



EM 201 R=$CH_3$
EM 523 R=$CH_2CH_3$
EM 574 R=$CH(CH_3)_2$



EM 536

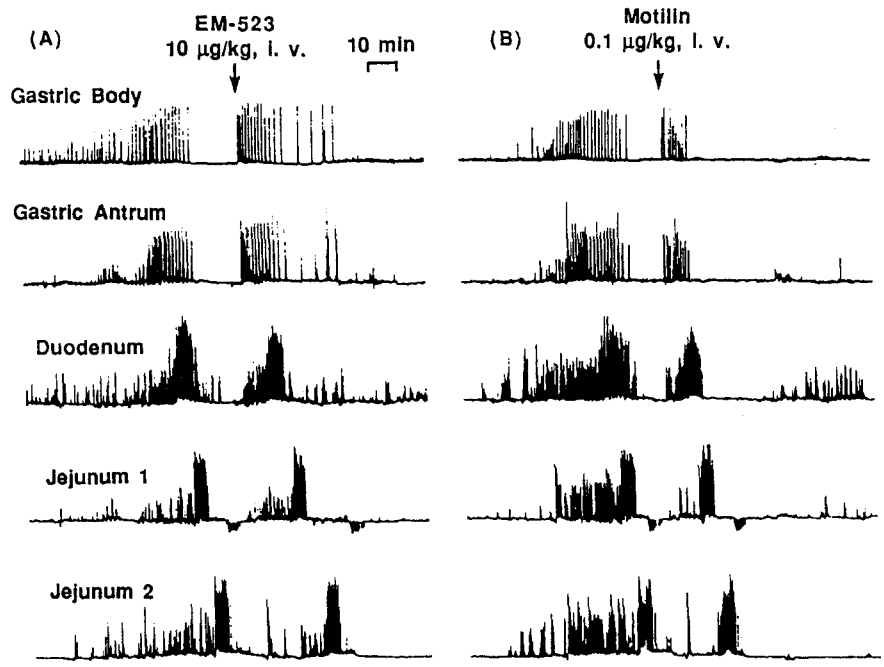



Fig. 3. Effect of EM-523 (A), and motilin (B) on gastrointestinal contractile activity during the fasting state in the conscious dog. EM-523 at 10 μg/kg and motilin at 0.1 μg/kg were used.

umented. Other examples reported recently include the inhibition of angiogenesis by herbimycin A [74] and fumagillin [38], anti-platelet activity of nanaomycin derivatives [67], and hypolipidemic activity of rifamycin derivatives [129] (see Table 4). It would be intriguing to obtain semi-synthetic derivatives of antibiotics in which the second pharmacological activity has become the major one.

*Lactacystin: a low molecular weight metabolite with neurotrophic factor-like activity.* Neurotrophic factors (NTFs) are essential for the survival and functional maintenance of nerve cells [3]. NTFs protect nerve cells from anoxia, maintain neuronal functions, control axon elongation and neuronal development, and so on. A decrease in availability of NTF causes dysfunction of the nervous system,

TABLE 4

Pharmacological activity of antibiotics

| Effect | Antibiotics |
|---|---|
| Hypotensive | erythromycin, oleandomycin, spiramycin, leucomycin, lasalocid |
| Antiplatelet | nanaomycin [67], rifampin [129] |
| Anti-coagulant | actinomycin D, cycloheximide, filipin, puromycin |
| Receptor binding antagonism | actinomycin D, (neurokinin-2 receptor binding antagonist) [10] |
| Anti-inflammatory | valinomycin, gramicidin A |
| Hypocholesterolemic | *N*-methylated neomycin, candicidin, tristreptomycylidene-diaminoguanidin 10·HCl |
| Hypolipidemic | ascofuranone |
| Relaxation of smooth mascle | dicloxacillin, aminobenzylpenicillin, chloramphenicol, spiramycin |
| Anti-osteoporosis | dimethylaminotetracycline [46] |
| Gastric contraction stimulating | erythromycin [42] |
| Angiogenesis inhibitory | fumagillin [38], herbimycin [74] |
| Cardiotonic | adriamycin, daunomycin, lasalocid |
| Blockade of neuromascle | neomycin, streptomycin, kanamycin, gentamicin, polymyxin, colistin, viomycin, bacitracin |
| Diabetogenic | streptozotocin |

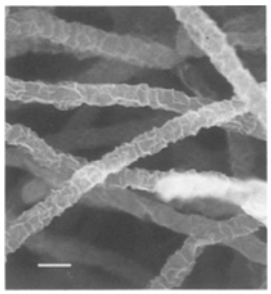
Cited from Perlman and Peruzzotti [98] and Matthews and Wade [59], and updated.

resulting in various neurological diseases including senile dementia such as Alzheimer's disease [27]. This consideration led us to speculate that substances with NTF-like activity would be useful to treat patients suffering from neurological disease.

We screened for low molecular weight compounds which induce the differentiation of Neuro 2A cells, a mouse neuroblastoma cell line. *Streptomyces* sp. OM-6519 was found to produce an active metabolite designated lactacystin (Fig. 4) [91].

Transmission electron micrography demonstrated neurite and growth cone structures of Neuro 2A cells cultured in the presence of 1.4 $\mu$M lactacystin for 4 days, suggesting that the neurite extension contains parallel arrays of microtubules and intermediate filaments. Some extensions contain mitochondria and membrane structures. The growth cone is enriched in large-cored vesicles with a diameter of 80–120 nm and contains straight microtubules and, in some cases, microtubule loops. These results demonstrate that lactacystin induces neurite formation in Neuro 2A cells.

We examined the effect of lactacystin on the intracellular cAMP levels in Neuro 2A cells, since a similar morphological change was also observed with prostaglandin $E_1$ and adenosine-isobutylmethylxanthine combination, which is known to elevate the cAMP level through a reaction of the receptor-adenylate cyclase coupling system [104]. On addition of 1.3 $\mu$M lactacystin to the medium, the growth rate of Neuro 2A cells decreased, whereas the intracellular cAMP level of the cells increased and reached a maximum level of 95–105 fmol/$10^4$ cells at 24 h after treatment, and then decreased steeply to the basal level. The transient increase of cAMP levels was dose-dependent; 2.6 and 5.3 $\mu$M lactacystin increased the cAMP level from 29.8 to 120 and 230 fmol/$10^4$ cells, respectively, after 24 h of treatment [94]. Adenylate cyclase modulators such as prostaglandin $E_1$ and a mixture of adenosine-isobutylmethylxanthine caused transient accumulation of intracellular cAMP in Neuro 2A cells within 15–30 min [104]. Thus, the slow response of Neuro 2A cells to lactacystin would not be due to direct activation of adenylate cyclase.



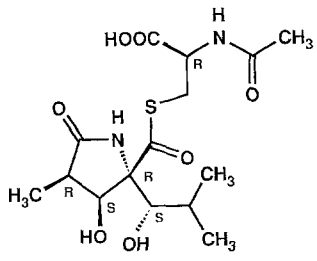



Fig. 4. Structure of lactacystin and a scanning electron micrograph of the lactacystin-producer *Streptomyces* sp. OM-6519.

In an in vitro system, lactacystin (1–100 $\mu$M) did not affect the protein kinase C activity in a rat brain preparation. Lactacystin inhibited neither thrombin nor plasminogen activator activities. Thus, lactacystin was proven to be a new type of NTF with low molecular weight, isolated from *Streptomyces* sp. This is the first microbial metabolite exhibiting neurotrophic activity, and may become a useful tool for investigating the differentiation of the nerve cells and for curing the nerve diseases associated with dysfunction of the nervous system due to a decrease in NTF.

Similar approaches are possible to obtain low molecular weight microbial metabolites exhibiting similar activities for other internal regulatory protein factors such as insulin.

*Modulators of lipid metabolism*

Lipid metabolism normally keeps a delicate balance between synthesis and degradation. When the balance is upset, hyperlipidemia such as hypercholesterolemia may occur, which, in turn, can cause atherosclerosis, hypertension, diabetes, etc. These disorders are encountered more often in industrialized societies and in association with aging. Modulators of lipid metabolism are expected to be useful in controlling these disorders.

Our screening programs for lipid metabolism modulators have discovered several inhibitors of fatty acid synthesis, such as cerulenin, and thiotetromycin [76,122]. Additional inhibitors that followed include inhibitors of acyl-CoA synthetase (triacsin), cholesterol synthesis (1233A), and of cholesterol ester formation (purpactin) [122].

*Triacsin, an inhibitor of acyl-CoA synthetase.* Long-chain fatty acid-CoA, an activated form of fatty acid, is an important intermediate in lipid biosynthesis, $\beta$-oxidation and in acyl transfer reactions. The conversion of a free long-chain fatty acid to the corresponding acyl-CoA is catalyzed by acyl-CoA synthetase. In animal tissues and cells, this is the main route supplying acyl-CoA, thus providing an important target site for drugs to control fatty acid metabolism.

In *Candida lipolytica* two distinct acyl-CoA synthetases exist, which are different from each other in their physiological role and localization. Acyl-CoA synthetase I, present in microsomes and mitochondria, is solely responsible for cellular lipid biosynthesis, whereas acyl-CoA synthetase II, localized in peroxisomes, provides an acyl-CoA that is degraded exclusively via $\beta$-oxidation, yielding acetyl-CoA (Fig. 5) [45]. Unlike in animal cells, acyl-CoA is also provided via fatty acid synthase in *C. lipolytica*.

In our screening programs two mutants of *C. lipolytica*,

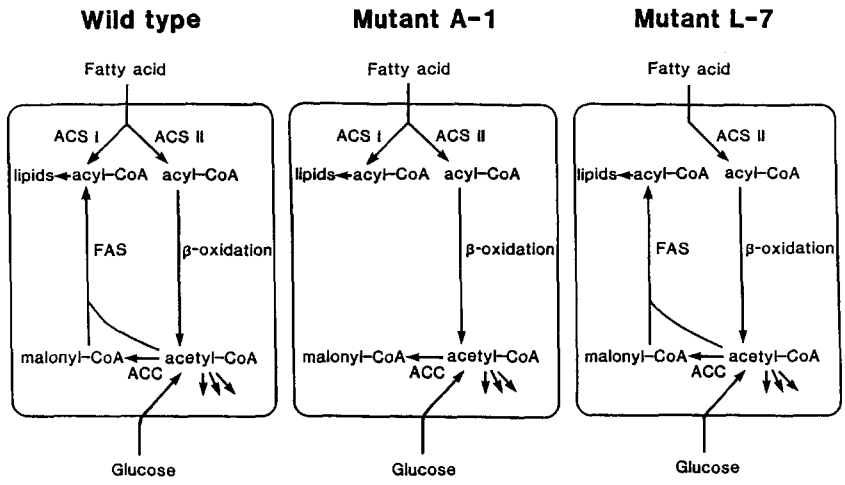



Fig. 5. Fatty acid metabolism in *Candida lipolytica* and its mutants A-1 and L-7.

strains L-7 (defective in acyl-CoA synthetase I) and strain A-1 (lacking fatty acid synthase activity) were used as test organisms. These mutant strains were grown on two media, containing either fatty acid as the sole carbon source or glucose plus a small amount of fatty acid (0.01% w/v). By examining the inhibition profiles against the two mutant strains on the two media, the sites of inhibition of unknown candidates can be assessed.

A culture of *Streptomyces* sp. SK-1894 showed inhibitory activity against strain A-1 grown in the two media but no effect on strain L-7 in either medium, indicating that the cultured broth contained acyl-CoA synthetase I inhibitors. Four active principles named triacsins A, B, C and D (Fig. 6) were isolated [86]. Among the four components, triacsin C (identical with WS-1228A) exhibited the most potent inhibition, followed by triacsin A, against bacterial and animal cell-derived acyl-CoA synthetases (Table 5)

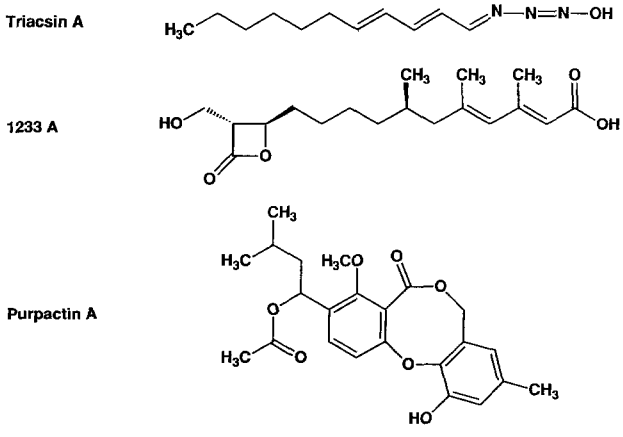



Fig. 6. Lipid metabolism modulators, triacsin A, 1233A, and purpactin A.

[124]. The inhibition appeared to be specific to long-chain fatty acids. Arachidonoyl-CoA formation from mouse fibrosarcoma cells is also sensitive to triacsin action [25]. Triacsin is the first inhibitor of acyl-CoA synthetase. It was successfully employed in demonstrating that acyl-CoA synthetase was essential in animal cells for supplying acyl-CoA, but that an additional pathway exists in bacteria, yeast and filamentous fungi [127]. Triacsin will be applied to lipid research as a unique biochemical reagent.

*1233A, an inhibitor of HMG-CoA synthase.* Mevalonate is a key intermediate in cholesterol biosynthesis. Inhibitors of this pathway would be promising as hypocholesterolemic agents. Our screening program utilized Vero cells as the test system. Cultures of soil isolates showing growth inhibition against Vero cells and reversion of the inhibition by the addition of mevalonate were selected and the active compounds characterized. *Scopulariopsis* sp. F-244 was found to produce a β-lactone, 1233A (identical with F-244 [126], and L-659, 699, Fig. 6), which was originally reported as an antifungal antibiotic produced by a strain of *Cephalosporium* sp. [1].

1233A was demonstrated to be a specific inhibitor of HMG-CoA synthase ($IC_{50}$ of 0.2 μM) [125]. Inhibitors of HMG-CoA reductase, compactin, mevinolin and their semi-synthetic derivatives [105], have been developed and commercialized as hypocholesterolemic agents.

*Purpactin, an ACAT inhibitor.* Acyl-CoA:cholesterol acyltransferase (ACAT) is concerned with cholesterol ester formation, its absorption and its accumulation, which are all accomplished in atherogenesis. Inhibitors of ACAT are expected to be effective for treatment and prevention of atherogenesis and hypercholesterolemia, although ACAT inhibitors of natural origin have not been described.

An enzyme system with rat liver microsomes was used

TABLE 5

Effect of triacsin on acyl-CoA synthetases and acetyl-CoA synthetase

| Enzyme | $IC_{50}$ ($\mu$M) | | | | |
|---|---|---|---|---|---|
| | triacsin A | triacsin B | triacsin C | triacsin D | *E,E,E*-2,4,7-undecatrienal |
| Acyl-CoA synthetase | | | | | |
| *Pseudomonas* sp. | 17 | >200 | 3.6 | >200 | – |
| Rat liver | 18 | >200 | 8.7 | >200 | – |
| Raji cells | 12 | >200 | 6.3 | >200 | n.d. |
| Acetyl-CoA synthetase | | | | | |
| *Saccharomyces cerevisiae* | – | n.d. | – | n.d. | n.d. |

>200, 40–50% inhibition at 200 $\mu$M.
–, no inhibition at 200 $\mu$M.
n.d., not determined.

in our screening program for ACAT inhibitors. A culture of *Penicillium* sp. FO-608 was found to produce an active compound. Three components were isolated and characterized, and named purpactin [128] (Fig. 6). Purpactin inhibited ACAT from rat liver microsomes and cholesterol ester formation in J774 macrophages [70].

*Inhibitors of protein phosphorylation*

Protein phosphorylation mediated by protein kinase and protein phosphatase affects biological functions of various organs or cells in the body [118]. These include neuronal function, cell growth and differentiation, cell motility, secretion and cellular metabolism, and smooth muscle contraction. Protein kinase C is the receptor protein for phorbol esters, i.e., oncogenesis promoters. Therefore, inhibitors of protein kinases with enzyme- or organ-specific activity would be of interest as medicines such as antitumor, anti-asthma, anticoagulants, anti-hypertension agents, etc.

*Staurosporine: inhibitor of protein kinases.* Staurosporine (Fig. 7) was found as a metabolite of *Saccharothrix* sp. by a chemical screening for microbial alkaloids [79]. Staurosporine was later found to be a potent inhibitor of protein kinase C. Its inhibition of protein kinase C is competitive with regard to ATP [119]. Recently, it has become recognized that staurosporine exhibits rather non-specific inhibition toward protein kinases A and B, although the inhibition against C kinase is most potent. Analogs of staurosporine, K-252a, RK-286D, RK-1409 were reported by other research groups [95,118,119].

Staurosporine attracts much attention as a unique biochemical reagent in studies of various cellular functions, and as a compound showing various pharmacological activities.

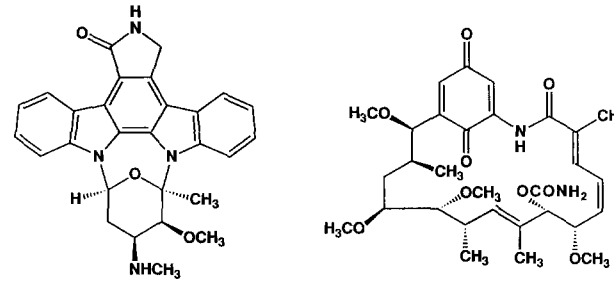



Fig. 7. Inhibitors of protein phosphorylation, staurosporine and herbimycin A.

We found that staurosporine is a potent relaxant of rabbit aortic strips contracted by various agonists [100]. This relaxing effect was slow, long lasting and non-competitive against various agonists.

Nifedipine (10 nM–10 $\mu$M) antagonized the contractile responses to prostaglandin F2$\alpha$ in normal $Ca^{2+}$ solution. On the other hand, even in $Ca^{2+}$-free solution, staurosporine (10–100 nM) potently antagonized the contractile responses. Thus, the inhibitory action of staurosporine appears to be potent and unique for intracellular calcium-dependent contractions. In i.v. administration of staurosporine to dogs, the blood flow, estimated by an electromagnetic flowmeter attached to an artery, was increased for longer than 30 min. Although staurosporine increased the blood flow, it did not affect the pulse or the contraction of cardiac muscle. The increase in blood flow caused by staurosporine may be due to a decrease in the marginal resistance [7].

Also of interest is that the alteration in platelet shape

induced by phorbol myristate acetate (PMA) was alleviated by pretreatment with staurosporine [40].

*Herbimycin A: inhibitor of tyrosine kinases.* Herbimycin A (Fig. 7) was initially found by our group as a herbicidal compound produced by a *Streptomyces* sp. [80]. Herbimycins B and C followed [43,106]. Later, it was found by Uehara et al. [130] that herbimycin A converted a transformed cell morphology to a normal cell morphology. These authors observed this using RSV (Rous sarcoma virus)-transformed rat NRK cells with a temperature-sensitive phenotype. Further studies of the reversing effect of herbimycin A indicated that herbimycin A was effective in the reversion of chicken or mammalian cells previously transformed by tyrosine kinase oncogenes such as *src*, *yes*, *fps*, *ros*, *abl*, *erb*B, but not *raf*, *ras*, and *myc* [131]. Herbimycin A reduced the total phosphotyrosine level, the phosphorylation of p36 protein, and the corresponding $p60^{src}$ kinase activity in RSV-transformed NRK cells. It was thus concluded that the RSV transformation resulted in a transformed cell morphology because of elevated phosphotyrosine levels, and that the increased *src* kinase activity was inhibited by herbimycin A so as to reverse the cell to normal morphology [131].

Erbstatin is another example of a microbial metabolite inhibiting tyrosine kinase [133].

*Other inhibitors.* Protein phosphorylation is also regulated by protein phosphatases. Okadaic acid [24] and tautomycin [57a] were shown to be effective inhibitors of protein phosphatase. These inhibitors will be useful in studying the mechanism and role of protein phosphorylation in signal transduction, carcinogenesis, and drug development.

*Agrochemicals*

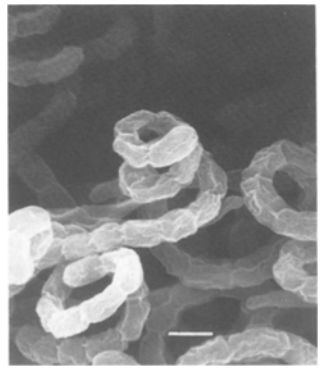
Pesticides include insecticides, herbicides, fungicides, and nematicides. They have been of great help for crop protection. Antibacterial and antiviral agents, if available, are of course also needed in agriculture. The development of improved pesticides is required for an increased production of foods. In addition to potent activity and safety, pesticides today should be biodegradable, thus providing less stress to the environment. Microbial metabolites are attracting attention as agrochemicals because of their diverse bioactivity and biodegradability [78].

*Phthoxazolin and phthoramycin: inhibitors of plant cell wall synthesis.* Cellulose exists in all plant cell walls and in the cell envelopes of some bacterial and fungal genera. Cellulose synthesis inhibitors have attracted little attention as herbicides, although coumarin, 2,6-dichloroenzonitrile (dichlobenil) [13] and isoxaben [28] were reported to have such activity.

A screening program was established for inhibitors of cellulose biosynthesis of microbial origin, based on selective growth inhibition of *Phytophthora parasitica*. This fungus is known to contain cellulose as one of the cell wall polysaccharides [4]. Microbial cultures were screened for their growth-inhibitory activity against *P. parasitica*, but with no activity against *Candida albicans*, which contains no cellulose in the cells. Phthoxazolin was discovered by this screening method as a metabolite of *Streptomyces* sp. OM-5714 [90]. The name 'phthoxazolin' was given due to its oxazole moiety and the selective activity against *Phytophthora* sp. (Fig. 8).

Phthoxazolin was selectively antimicrobial against cellulose-containing *Phytophthora parasitica* and *P. capsici*, although only moderately. No growth inhibition was observed with 200 μg phthoxazolin/ml against cellulose non-containing Gram-positive and Gram-negative bacteria such as *Staphylococcus*, *Bacillus*, *Escherichia*, *Pseudomonas*, *Acetobacter* sp., or yeasts and filamentous fungi including *Piricularia*, *Botrytis*, *Microsporum* and *Trichophyton* species.

Studies on the mode of action of phthoxazolin were carried out using *Acetobacter xylinum*, an acetic acid bacterium known to produce extracellular cellulose [23]. Phthoxazolin (10–100 μg/ml) inhibited the incorporation of



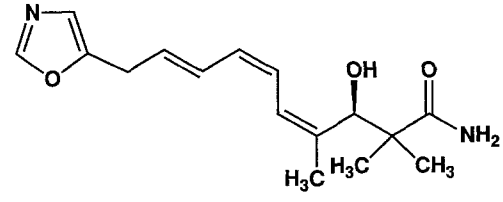



Fig. 8. Phthoxazolin, and an electron micrograph of the phthoxazolin-producing culture *Streptomyces* sp. OM-5714.

$^{14}$C-labeled glucose into the cellulose fraction of resting cells of *A. xylinum* (30–42%). It also inhibited GTP- and poly(ethylene glycol)-stimulated synthesis of cellulose by a cell-free extract of *A. xylinum* (27–69%). It has been suggested that phthoxazolin is a specific inhibitor of cellulose biosynthesis in bacterial, fungal, algal and plant systems.

Using the same screening program, phthoramycin was isolated as a metabolite of *Streptomyces* sp. WK-1875 [88]. Phthoramycin was potently herbicidal against radish seedlings. It also inhibited the growth of cellulose-containing *Phytophthora* sp. most potently, and that of a limited number of genera of cellulose non-containing fungi such as *Pyricularia* and *Mucor*, to a lesser extent. Because this compound inhibited the incorporation of $^{14}$C-labeled glucose into an alkali-insoluble glucan fraction of *A. xylinum*, it is suggested that phthoramycin is an inhibitor of cellulose biosynthesis, but the action is not specific to cellulose synthesis.

*Other enzyme inhibitors with agro-activity.* Insecticides and fungicides with interesting enzyme inhibitory activities have been reported. Allosamidin is a chitobiose analog produced by *Streptomyces* sp. with insecticidal activity [99].

A potent trehalase inhibitor, trehalostatin, was discovered by Murao et al. [63]. These compounds are expected to be useful, not as the traditional insecticides, but as a new type of insect growth regulator.

*Anticancer compounds*

Safe and excellent chemotherapeutics are still required for the control of infections by viruses, fungi and parasites. Cancer also remains largely outside of our control. In parallel with the search for new types of active metabolites described above, efforts toward finding antimicrobial chemotherapeutics and anticancer compounds are actively being continued. Pradimicins [75] and benanomicins [116] are a family of new antifungal compounds inhibiting yeast mannan biosynthesis. Calcheamicins and dinemicins are a family of extremely potent anticancer compounds produced by actinomycete strains [53,69]. They possess a diyne moiety in the molecule and resemble the neocartinostatin nucleus. Spergalin is interesting because of its antitumor activity with immuno-modulating activity [71]. We found eight new anticancer compounds in the past 5 years, three of which are described below.

*Kazusamycin.* Kazusamycins A and B (Fig. 9) were isolated from a fermentation broth of *Streptomyces* sp. 81–484 [132]. Their structures are characteristic of an unsaturated branched-chain fatty acid and a terminal $\gamma$-lactone ring. Kazusamycins showed potent cytocidal activities against L1210 and P388 leukemia cells in vitro and a broad antitumor spectrum in vivo [51]. Intraperitoneal (i.p.) injection of the antibiotic inhibited the growth of murine tumors such as sarcoma 180 (S180), P388 leukemia, and B16 melanoma. It was also active against doxorubicin (adriamycin)-resistant P388 (P388/ADM) and pulmonary metastases of Lewis lung carcinoma.

*Phenazinomycin.* Phenazinomycin (Fig. 9) was discovered in the mycelial extract of *Streptomyces* sp. WK-2057 as a dark blue powder [89]. This is the first phenazinone alkaloid possessing a sesquiterpene moiety in its structure.

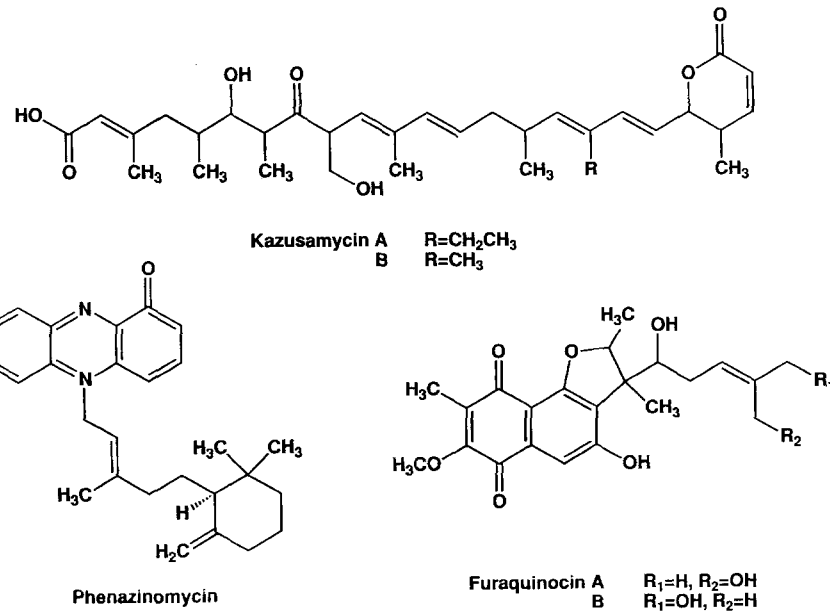



Fig. 9. Anticancer compounds, kazusamycin, phenazinomycin, and furaquinocin.

This compound showed cytocidal activity against HeLa $S_3$ cells in vitro (MIC 0.8 μg/ml) and antitumor activity against S180 tumor in vivo (ILS 130%, 22.2 mg/kg/day × 9, i.p.).

*Furaquinocins.* Two new anticancer compounds, furaquinocins A and B (Fig. 9) were isolated in a culture broth of *Streptomyces* sp. KO-3988. Furaquinocins A and B are structural isomers with the same molecular formulae. They are active in vitro against HeLa $S_3$ cells with $LC_{50}$ values of 3.1 and 1.6 μg/ml for A and B, respectively [51b], but are inactive against 16 kinds of bacteria, yeast and filamentous fungi when tested at 1 mg/ml. Biosynthetic studies showed that the carbon skeleton of furaquinocin is derived from five acetate units, two mevalonate units, and two $C_1$ units from methionine [21].

## NEW MICROBIAL SOURCES FOR PRODUCTION OF ACTIVE METABOLITES

Strains of *Streptomyces* and other genera of the *Actinomycetales* have long been the major and most fruitful microbial sources for bioactive metabolites. As the incidence of re-discovery of *Streptomyces*-derived compounds already described by other groups became more frequent, microbes other than *Streptomyces* began to be used. This approach started in the late 1960s, when gentamicin, which is a clinically useful aminoglycoside antibiotic, was discovered as a metabolite of *Micromonospora*. This is still a subject of interest today. The isolation of rare actinos, or non-*Streptomyces* actinomycetes, was greatly helped by the selective isolation techniques developed later [26,134]. These actinomycetes soon proved to be as rich a group of producers of active compounds as was *Streptomyces*, but many turned out to produce active substances which typical *Streptomyces* strains also produced. Therefore, the re-discoveries did not decrease significantly, and the use of microbes other than actinomycetes increased. Fungi and bacteria are other large groups of microbial sources. The discovery of monobactams, namely, a series of single β-lactams produced by eubacterial strains [96], promoted the search among bacteria.

Current efforts toward the isolation of different types of microorganisms include the isolation of morphologically and physiologically unusual cultures, whether *Streptomyces* or non-*Streptomyces*, by varying the conditions of culture isolation. Also challenging is the use of marine samples from which actinomycetes, bacteria or fungi are isolated, and the use of myxobacteria [56] and basidiomycetes [15]. The latter approach is increasing and has been successful in finding new compounds with novel structures. The shift of interest from *Streptomyces* to non-*Streptomyces* cultures does not necessarily mean that the *Streptomyces* genus is no longer important. In fact, new compounds are still being reported as metabolites of *Streptomyces*. Our efforts along this line led to the discovery of the genus *Kitasatosporia* [110,114].

*Kitasatosporia: discovery and characteristics*

Lechevalier and Lechevalier [57b] classified actinomycetes into nine groups according to the amino acid components of their cell walls in combination with the whole cell sugar patterns. In this chemotaxonomic classification, the stereochemistry of isomers of diaminopimelic acid (DAP), either the LL-isomer or the *meso*-isomer, is important as a criterion. Our actinomycete cultures were examined by this method for their taxonomic positions, and were found to involve 19 new species of *Streptomyces* and other genera.

One culture, KM-6054, the producer of a new macrolide antibiotic, setamycin [81b], was isolated from a soil sample taken from my garden located in Seta, Setagaya-ku, Tokyo. When examined chemotaxonomically, KM-6054 was quite unusual in that it contained nearly equal amounts of both *meso*- and LL-DAP, in spite of its abundant sporulation and microscopical resemblance to *Streptomyces* [81a]. This strain did not fall into any of the Lechevaliers' nine groups. After taxonomic studies of this culture, a new genus *Kitasatosporia* was proposed [109,110]. The major characteristics of the genus *Kitasatosporia* are described in Table 6.

The analysis of the DAP isomers of cell wall preparations has been carried out by paper chromatography. Now, a newly established HPLC method is available and is applicable to the DAP analysis of whole cell hydrolysates. In this method, DAP is converted to its glucose-isothiocyanate derivative and is detected by ultraviolet at 250 nm [112].

TABLE 6

Taxonomic properties of the genus *Kitasatosporia*

| | | |
|---|---|---|
| Morphology | | |
| AM: long spore chain | | |
| Fragmentation of VM: none | | |
| Zoospore and sporangium: none | | |
| Chemical analysis | | |
| Cell wall | | |
| amino acid: | LL-DAP (in AS and SS) | |
| | *meso*-DAP (in VM and FM) | |
| | glycine | |
| sugar: | galactose | |
| acyl type: | acetyl | |
| Whole cell | | |
| sugar: | galactose | |
| phospholipid: | PII type | |

Since our first report of *Kitasatosporia* in 1982, 14 species of *Kitasatosporia* have been reported by us and other groups, most of which produced a variety of bioactive metabolites (Table 7). Thus, this is a new and rich source for novel compounds. *Kitasatosporia* strains can be isolated effectively from soils by novobiocin resistance, abundant aerial mycelium formation on agar media, and submerged spore formation in liquid cultures [114].

*Mode of submerged spore formation in K. setae*

*K. setae* KM-6054 produces aerial mycelium and substrate mycelium on agar media. When grown in liquid media, it produces submerged spores and filamentous mycelium. In both solid and liquid cultures, LL- and *meso*-DAP are observed largely at a ratio of 1:1. A time-course study on the LL- and *meso*-DAP contents demonstrated that LL-DAP existed in aerial mycelium and submerged spores, whereas *meso*-DAP was contained in substrate mycelium (solid culture) and in filamentous mycelium (liquid culture) [81a,111]. Microautoradiographic observations (Fig. 10) suggested that, in liquid culture, small hyphal branches emerge on an elongated filamentous mycelium, which are committed to grow and release LL-DAP-containing submerged spores [113]. This mode of submerged spore formation is different from that in *Streptomyces griseus*, in which a random fragmentation of a filamentous mycelium produces submerged spores containing *meso*-DAP.

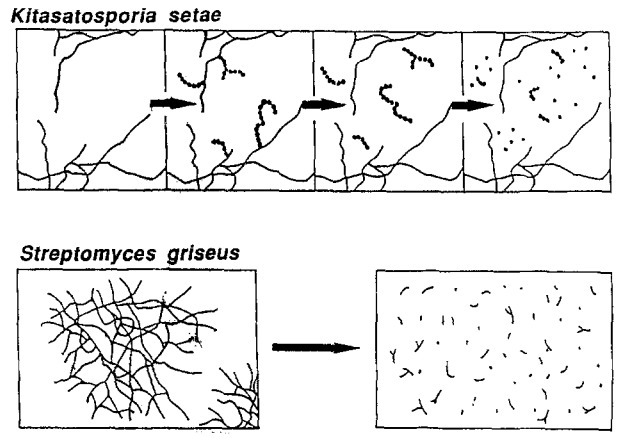



Fig. 10. Submerged spore formation in *Kitasatosporia setae* and *Streptomyces griseus*.

Table 8 is a grouping of actinomycetes according to DAP isomer type and aerial mycelium formation. There exist many open areas which await the discovery of new genera [77,109]. Classification methods for actinomycetes by 5S or 16S ribosomal RNA and ribosomal proteins have been proposed [72]. These techniques enable rapid iden-

TABLE 7

*Kitasatosporia* strains and their new metabolites

| Species | Metabolites | Activities | Ref. |
|---|---|---|---|
| *K. setae* KM-6054 | setamycin | antitrichomonal | |
| *K. griseola* AM-9660 | setamycin | antitrichomonal | |
| *K. phosalacinea* KA-338 | phosalacine | herbicidal | |
| *K. melanogena* K55-G-32 | | | |
| *K. cystarginea* RK-419 | cystargin | antifungal | |
| *K. setae* SANK60684 | propioxatin | enkephalinase B inhibitor | Inaoka et al. [36] |
| *K. griseola* MF730-N6 | terpentecin | antitumor | Tamamura et al. [117] |
| *K. clausa* 33-35-1 | | | |
| *K. kifunense* No. 9482 | kifunensine | immunomodulator | |
| *K. setae* S-9 | microbial transformation of azacarbazole | | Peczynska-Czoch et al. [97] |
| *K. brunnea* 1719-SV3 | | | |
| *K. papulosa* AB-II0 | AB-110D | antibacterial | |
| *K. grisea* AA-107 | | | |
| *K. mediocidica* NRRL B-16109 | | | |
| *Kitasatosporia* sp. No. 55 | tyrostatin | proteinase inhibitor | Oda et al. [73] |
| *K. cochleata* M-5 | | | |
| *K. kyotoensis* SAM 0170 | SUAM-2007–SUAM-20012 | proteinase inhibitor | Maeda et al. [58] |
| *Kitasatosporia* sp. SK-60 | phospholipase D | | Sawada et al. [102] |
| *Kitasatosporia* sp. F-0368 | F-0368 | agrochemical | Taguchi et al. [108] |

Cited from Takahashi [109], and updated.

TABLE 8

Grouping of genera of *Actinomycetales* by morphological and chemotaxonomical criteria

| Sporangium | Aerial mycelium | Cell wall type[a] | | | | |
|---|---|---|---|---|---|---|
| | | I | II | III | IV | Others |
| + | + | | | *Planobispora*<br>*Planomonospora*<br>*Streptoalloteichus*<br>*Streptosporangium*<br>*Spirillospora* | *Kibdelosporangium*[b] | |
| | − | | *Actoinoplanes*<br>*Ampullariella*<br>*Dactylosporangium*<br>*Pilimelia* | *Frankia* | | *Kineosporia* |
| − | + | *Intrasporangium*<br>*Nocardioides*<br>*Sporichthya*<br>*Streptomyces*<br>*Streptoverticillium* | *Glycomyces* | *Actinomadura*<br>*Actinosynnema*<br>*Microbispora*<br>*Microtetraspora*<br>*Nocardiopsis*<br>*Saccharothrix*<br>*Thermoactinomyces*<br>*Thermomonospora* | *Actinokineospora*<br>*Actinopolyspora*<br>*Pseudoamycolata*<br>*Pseudonocardia*<br>*Saccharomonospora*<br>*Saccharopolyspora* | *Kitasatosporia* |
| | − | *Terrabacter* | *Catellatospora*<br>*Micromonospora* | *Dermatophilus*<br>*Geodermatophilus* | *Amycolata*<br>*Amycolatopsis*<br>*Mycobacterium*<br>*Nocardia*<br>*Rhodococcus*<br>*Tsukamurella* | |

[a] Classification by DAP-isomers and sugars according to Lechevalier (1970).
[b] The members of this genus contain sporangium-like bodies.

tification and classification of actinomycete cultures. Taking advantages of these new techniques and selective isolation methods for specific genera [26,134], isolation of new types of actinomycetes will be realized.

## APPLICATION OF RECOMBINANT DNA TECHNOLOGY

Several applications of recombinant DNA technology to studies of bioactive microbial metabolites can be divided into two categories. In one category, an active compound-producing microorganism is genetically engineered for (i) production of new derivatives or hybrid molecules [2,30,135], (ii) an increase in titers by gene dosage effect, (iii) selective production of a useful component by activation or inactivation of specific genes [92], and (iv) studies on biosynthesis and regulatory mechanisms [29,31a,31b]. In the other category, microbes (other than those producing microorganisms), plant and animal cells are genemanipulated with or without using bioactive compounds in order to (i) produce disease-related enzymes or receptor proteins which are used in the screening of bioactive compounds, and (ii) studies on the mode of action of, or resistance to, an active compound.

More advanced forms of application included in the second category are breeding of a herbicide (or insecticide)-tolerant plant [50] and engineering of proteins or cell surface glycoconjugates. Other products of this technology, such as antisense RNA fragments, anti-viral chemotherapeutics and multivalent recombinant vaccines [68], will certainly affect studies of bioactive microbial metabolites.

We succeeded in the production of new hybrid antibiotics, mederrhodins A and B, in collaboration with Dr.

D.A. Hopwood, John Innes Institute, UK, by introducing the gene for the hydroxylation step of actinorhodin biosynthesis from *S. coelicolor* A3(2) into the medermycin-producing *Streptomyces* sp. AM-7161 [30,82]. Hydroxygranaticin and norerythromycin are also hybrid antibiotics produced by genetic recombinations [135]. Other examples of gene manipulation of active compound-producing microorganisms are described below.

*Cloning and expression of aculeacin A acylase gene from* Actinoplanes utahensis

Aculeacin A is an antifungal antibiotic of the echinocandin group [61], consisting of a cyclohexapeptide nucleus and a long fatty acid side chain (Fig. 11). It exhibits high antiyeast and antifungal activity, but loses the activity upon deacylation. An enzyme, termed aculeacin A acylase, able to hydrolyze aculeacin A between the peptide nucleus and acyl moiety, was found in the culture filtrate of *Actinoplanes utahensis* NRRL12052 [5]. The deacylated peptide (peptide nucleus) was used for creating a semisynthetic echinocandin derivative, cilofungin [9]. In order to characterize the hydrolyzing enzyme, and to use the enzyme activity as a selection marker for gene manipulation in yeast cells, we cloned the gene for aculeacin A acylase (*aac*) and examined its expression in *Streptomyces lividans*.

*Purification and characterization of aculeacin A acylase.* Purification of the enzyme from the culture filtrate of *A. utahensis* NRRL12052 was carried out by ultrafiltration and column chromatography on DEAE-cellulose, hydroxyapatite, and butyl-toyopearl 650M [115]. The final enzyme preparation gave two bands (subunits A and B) with estimated molecular weights of 55000 and 19000, respectively. The two peptides were separable by HPLC in the presence of 6 M guanidine hydrochloride, but the association of both peptides at the molecular ratio of 1:1 was required for activity.

*Cloning and analysis of aculeacin A acylase gene.* Using mixed oligodeoxyribonucleotide probes based on the amino terminal amino acid sequences of the two subunits of the acylase, overlapping clones were identified in a cosmid library of *A. utahensis* DNA [39]. Out of 32000 colonies screened, 16 showed a positive response against both probes. The restriction cleavage map of one of these clones, designated pKAA1, was elucidated and the 7.5-kb *Bgl*II-fragment of this clone was further analyzed by Southern hybridization. Then, the 7.5-kb fragment was introduced into *Streptomyces lividans* JT46 using pKU109, a pIJ101 derivative, as a vector. The resulting transformant, designated PKAA103, was confirmed to produce the same acylase protein as that of *A. utahensis* as judged by the reversed-phase HPLC profiles of the reaction product. The *aac* gene was subcloned, and the smallest fragment of 3.0 kb necessary for activity, the *Cla*I-*Pst*I fragment, was examined for its nucleotide sequence. The sequence contains one ORF of 2358 nucleotides corresponding to 786 amino acid residues, and a molecular weight of 84067. The identified ORF supports the existence of a precursor protein which would be processed to the two subunits: the B subunit from the aminoterminus and the A subunit from its carboxyterminus. Analysis with the aid of carboxypeptidase Y demonstrated that the calculated molecular weights of A and B subunits were 60300 and 19100, respectively.

*Characteristics of aculeacin A acylase produced by S. lividans.* The production of the acylase in *S. lividans* carrying pKAA103 was at a similar level as in *A. utahensis*. The purification of the enzyme was performed by the same procedure as that for the original producer, *A. utahensis*. Because most of the acylase from *A. utahensis* was bound to pigments in the culture, while *S. lividans* JT46 did not produce such pigments, the enzyme was obtained from the latter in high yield. The purified aculeacin A acylase from *S. lividans* gave five bands (molecular weights of 55000, 23000, 21000, 20500 and 19500) on SDS-PAGE (Fig. 12A). The largest peptide, with a molecular weight of 55000 was confirmed to correspond to the A subunit from *A. utahensis* by Western blot analysis using an antiserum

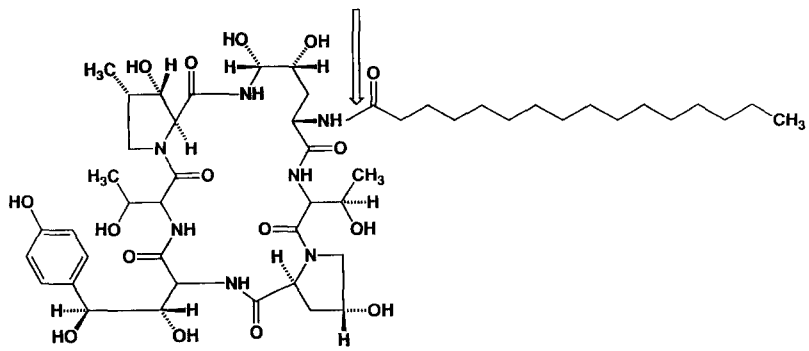


Fig. 11. Structure of aculeacin A.

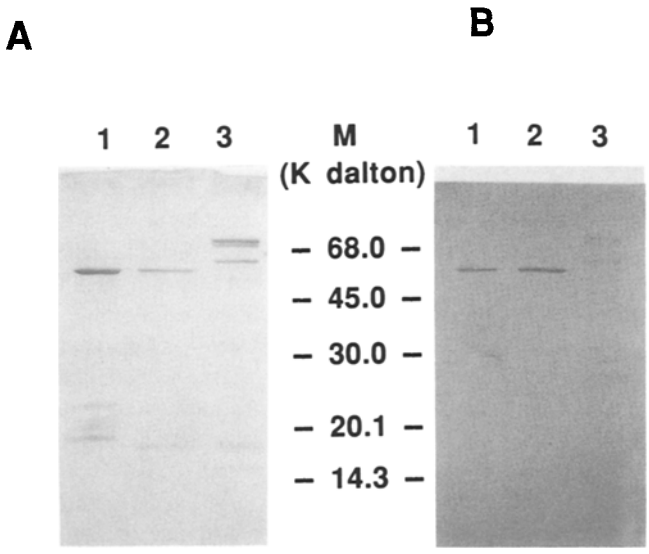
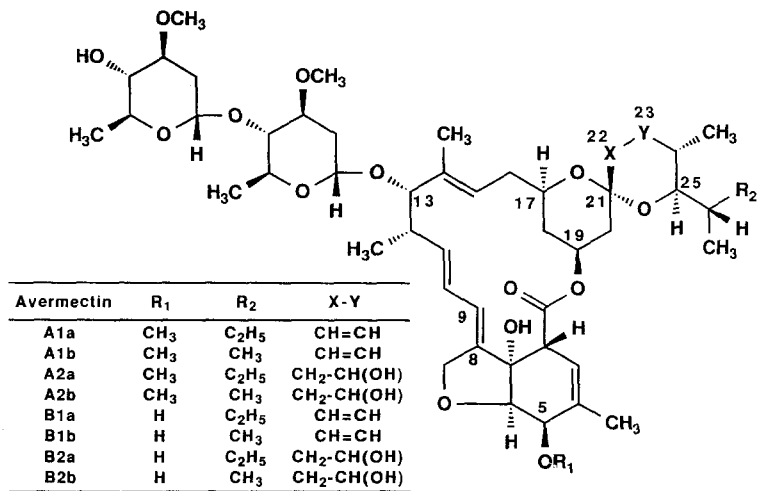



Fig. 12. Electrophoretic analysis of purified aculeacin A acylase from *Actinoplanes utahensis* expressed in *Streptomyces lividans*. A: Gel electrophoresis, B: Western blot analysis.

directed against the native acylase from *A. utahensis* (Fig. 12B). The four smaller bands did not respond to the antiserum, but were presumed to be subunits of aculeacin A acylase from *S. lividans* because the ratio of the total amount of these four peptides and the 55000 polypeptide was about 1:1. Two additional polypeptides were observed in the *A. utahensis* culture filtrate which responded to the antiserum as shown in lane 3 in Fig. 12B. Their apparent molecular weights were 87000 and 60000 on SDS-PAGE. The larger polypeptide corresponds in size to the sum of the two subunits of the acylase and is probably the precursor peptide of the two subunits.

*Increase in kalafungin production and discovery of a new kalafungin component by a gene dosage effect*

The benzoisochromanequinone antibiotics, including kalafungin, actinorhodin, and nanaomycin, show antibacterial, antifungal and antimycoplasma activities. These antibiotics are synthesized from eight acetate units via a hypothetical intermediate 'polyketide'. Kalafungin is an intermediate of actinorhodin biosynthesis in *S. coelicolor* A3(2) [12]. We examined the biosynthetic and genetic similarity with respect to kalafungin biosynthesis between actinorhodin-producing *S. coelicolor* A3(2) and kalafungin-producing *Streptomyces tanashiensis* Kala. The analysis of co-synthetic pairs of blocked mutants and the cloning of kalafungin-biosynthetic genes led to an unexpected finding that the genes involved in kalafungin biosynthesis are similar, but are not identical between the two cultures [44]. When recombinant plasmid, pKU523, which contains a 28 kb insert (the gene cluster for kalafungin biosynthesis is contained in this insert), was introduced into *kal* mutants or the wild-type strain, the production of kalafungin and dihydrokalafungin by transformants was at least twice that of wild-type strain. No increase was observed in transformants carrying a part of genes for kalafungin biosynthesis. Moreover, the transformants produced, in addition to kalafungin and dihydrokalafungin, a new compound designated tetrahydrokalafungin (Table 9). We assume that the production of the new antibiotic was caused by the 28 kb DNA fragment introduced. Tetrahydrokalafungin could be an intermediate, a shunt product, or a final product in the kalafungin biosynthetic pathway.

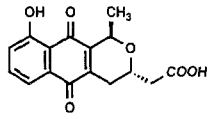
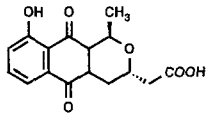



| Avermectin | $R_1$ | $R_2$ | X-Y |
|---|---|---|---|
| A1a | $CH_3$ | $C_2H_5$ | CH=CH |
| A1b | $CH_3$ | $CH_3$ | CH=CH |
| A2a | $CH_3$ | $C_2H_5$ | $CH_2$-CH(OH) |
| A2b | $CH_3$ | $CH_3$ | $CH_2$-CH(OH) |
| B1a | H | $C_2H_5$ | CH=CH |
| B1b | H | $CH_3$ | CH=CH |
| B2a | H | $C_2H_5$ | $CH_2$-CH(OH) |
| B2b | H | $CH_3$ | $CH_2$-CH(OH) |

Fig. 13. Avermectins produced by *Streptomyces avermitilis*.

TABLE 9

Physico-chemical properties of dihydrokalafungin and tetrahydrokalafungin

| | Dihydrokalafungin | Tetrahydrokalafungin |
|---|---|---|
| Appearance | yellow | pale yellow |
| SIMS m/z | 303 $(M+1)^+$ | 305 $(M+1)^+$ |
| Formula | $C_{16}H_{14}O_6$ | $C_{16}H_{16}O_6$ |
| λ max(nm) | 250, 274, 423 in $CH_3OH$ | 241, 256sh, 348 in $CH_3OH$ |
| | 280, 520 in 0.1*N* NaOH-$CH_3OH$ | 280, 520 in 0.1*N* NaOH-$CH_3OH$ |
| Structure |  |  |

*Breeding of S. avermitilis for selective production of useful avermectin components*

The anthelmintic avermectin complex produced by *S. avermitilis* consists of a family of four closely related major components, A1a, A2a, B1a, and B2a, and four structurally related minor components, A1b, A2b, B1b, and B2b (Fig. 13) [8]. A hydroxyl group at the C-5 position and the disaccharide moiety are essential for good activity. The hydrogenated product of B1 component, 22,23-dihydroavermectin B1 (ivermectin) is used as an important anthelmintic in veterinary fields and for the control of onchocerciasis in humans [22].

Studies on the biosynthesis of avermectin, independently performed by us [34] and the Merck group [11], demonstrated (i) 'A' components are derived from 'B' components through the methylation at C-5; (ii) the group '1' compounds are derived from the precursor group '2' compounds through dehydration at C-22 and C-23; (iii) the aglycone moiety is synthesized via a hypothetical intermediate 'polyketide' derived from seven acetate units, five propionate units and a branched-chain fatty acid unit [16]. The C-25 atom and its *sec*-butyl substituent of 'a' components is derived from L-isoleucine, while the 25-isopropyl substituent of 'b' components is from L-valine.

The separation of each component can be achieved by conventional chromatographic techniques. However, the industrial separation of 'a' and 'b' components is quite difficult, and commercial products of avermectin contain both 'a' and 'b' components (B1a and B1b). If the producer had the ability to accumulate 'a' components only, the industrial separation would be simpler, and each component could be made more easily.

In one approach to this goal, we attempted breeding producers that selectively produce useful components of the avermectins. By mutagenesis of the parent strain, mutant strains K2021 and K2034 were obtained (Fig. 14B and C). The genotype of K2034 was defined as *aveD*. Schulman et al. [103] has already reported this type of mutant that lacks avermectin B2 5-*O*-methyltransferase activity. The other mutant K2021 produced four 'a' components, A1a, A2a, B1a and B2a (Fig. 14C), but did not produce 'b' components. Because the biochemical and genetic characteristics of the mutation were unclear, the genotype was designated as X. The incorporation of L-valine or its keto acid counterpart into the avermectin skeleton was markedly suppressed in this mutant; therefore, the mutant accumulated 'a' components alone.

If a strain is obtained which (i) lacks the enzyme activity of avermectin 5-*O*-methyltransferase (accumulating 'B' components) and (ii) incorporates L-isoleucine or 3-methyl-2-oxovalerate into the avermectin skeleton but not L-valine or 2-oxoisovalerate (accumulating 'a' components), it would produce components 'B' and 'a', namely, avermectins B1a and B2a. Therefore, the recombination of K2034 and K2021 was carried out by protoplast fusion in the presence of poly(ethylene glycol). As expected, one of the fusants, K2038, produced components B1a and B2a only (Fig. 14D) [92]. Interestingly, genetic mapping showed that the locus of the mutation affecting the selectivity of the incorporation of branched-chain keto acids into the avermectin skeleton is significantly distant from the gene cluster for avermectin biosynthesis [32,33].

## PERSPECTIVES

Described above are the structural and biological diversities of microbial metabolites which have been exploited recently. This concept is not a new one, but has been recognized repeatedly, and is widely accepted today. In addition, we are probably dealing with less than 10% of the entire microbial population of the ecosystems. These facts suggest strongly that it is fully possible for us to find newer bioactive metabolites from microorganisms.

The discovery of new metabolites is achieved by means of effective and rational screening systems. One requirement is that the screening systems are constructed based on and integrating newer knowledge of pharmacology, basic medicine and related sciences, such as receptor biochemistry. The application of biotechnology is also important. The screening system thus set up will be mechanism based. Compound-based assay methods, gene induction assays [48], and robotic systems may be useful and will allow rapid assays.

Finally, I would like to add the importance of enthusiasm in screening research. I have experienced many times that our screening resulted in unexpected, but fortunate, findings. For example, in a screen for antimycoplasma compounds, nanaomycin was discovered, which later found usefulness as an antifungal agent. Our studies on the chemistry and biochemistry of new macrolide anti-

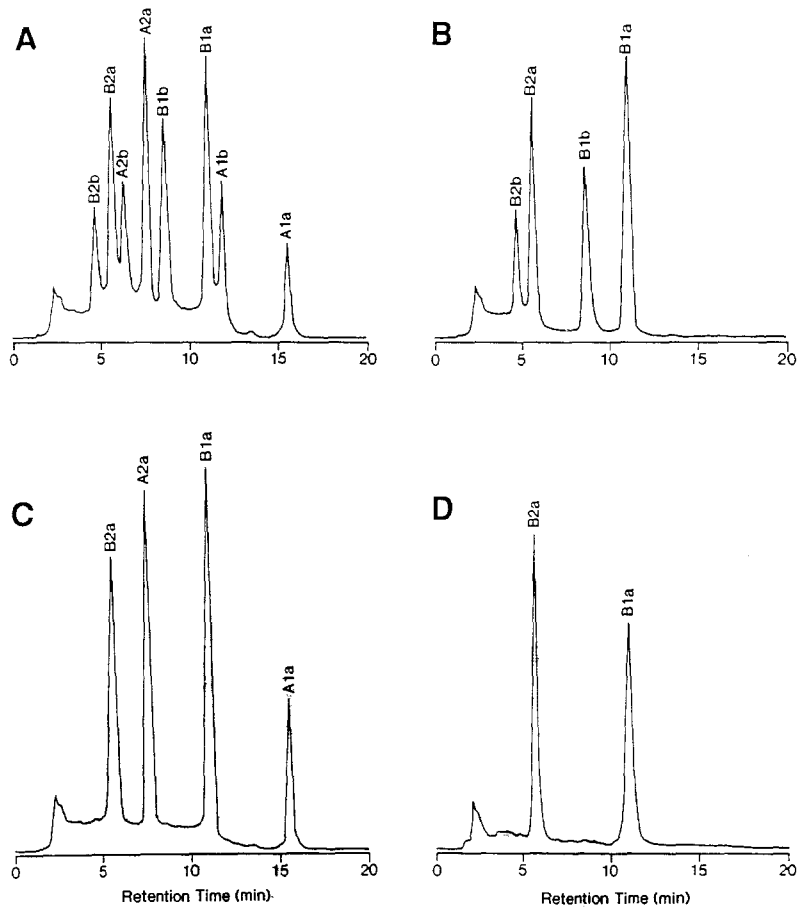



Fig. 14. Chromatograms of analytical HPLC of the mycelial extracts from (A) parent strain K139, (B) mutant strain K2034 (*ave* D), (C) mutant strain K2021 (X), (D) recombinant strain K2038 (*ave* D, X).

biotics eventually led to the discovery of motilides. Staurosporine, obtained by alkaloid screening, was found later to be a potent protein kinase inhibitor. Supporting this view, similar cases may be cited. The undesirable immunosuppression caused by the antibiotic cyclosporin A when tested as an antifungal agent led the researchers at Sandoz to develop it as an immunosuppressive agent. Biolaphos was successfully developed as a herbicide by Meiji Seika Co., after a disappointing observation of a potent plant-injuring effect when evaluating it as an antifungal antibiotic.

I believe the later discoveries were brought to us because of our enthusiasm for finding new metabolites, new activity, and new uses.

'Chance favors the prepared mind' – Louis Pasteur.

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