A COMPARISON OF ANTIBACTERIAL COMPOUNDS SECRETED BY A MUTANT STRAIN OF THE ENTOMOPATHOGENIC FUNGUS BEAUVERIA BASSIANA

By

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DEDICATION

This thesis is dedicated to my family who have all shown me their love and patience which helped me to endure: my wife Kristy, and my children Toria and Andrea; and my parents, Jacob and Bernice Boon, who have waited so long.

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ENTOMOPATHOGENIC FUNGUS

BEAUVERIA BASSIANA

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NOMENCLATURE

Abs	absorbance
GAC	glucose-ammonium nitrate-citrate
"M" antibiotic	migrating antibiotic
NCB	neopeptone citrate broth
"O" antibiotic	origin antibiotic
OD	optical density
P-10	Bio-Rad P-10 gel filtration
R_f	$\frac{\text{distance from base line traveled by compound}}{\text{distance from base line traveled by solvent}}$
SFB	Sabourand's fructose broth
TCA	tricarboxcylic acid
TSA	tryptic soy agar

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

According to the usually accepted definition, antibiotics are substances, produced by microorganisms, small amounts of which inhibit growth of microorganisms. Many kinds of approaches to isolate new antibiotics have been made and a large number of antibiotics have been discovered in the past four decades. However, additional antibiotics are needed for the treatment of diseases caused by drug-resistant microbes, of super infections, and of opportunistic infections. Unfortunately, finding new antibiotics has become more and more difficult.

It was once commonplace to demonstrate the existence of, isolate, purify, and determine many properties of hundreds of antimicrobial compounds a year. The slowing of the rate of discovery of new antibiotics may be because nearly all have already been found, or, more likely, that the most convenient environments containing antibiotic-producing organisms have already been thoroughly screened. Since there remains a need for new antibiotics that can perform specific functions, it is worthwhile to learn some of the properties of new antimicrobial compounds to determine if they have any potential value as therapeutic agents. It was with this goal in mind that further study of the antimicrobial factors of Champlin (1981) and Adefarati (1983) was undertaken.

These factors were the object of study in an ongoing program at Oklahoma State University on the use of the entomopathogenic fungus Beauveria bassiana as an insect control agent. The ability to control insect pests without the use of large amounts of toxic chemicals has been of concern to environmentally-oriented scientists, and others, for some time.

There are many approaches to using biological agents to control insects. The use of entomopathogenic microorganisms (fungi, bacteria, and viruses) has been shown to be effective in a significant number of cases. Beauveria bassiana is such an organism. It is a member of the group Deuteromycetes (not known to produce spores by a sexual process).

This fungus has been known to cause death of certain insects (notably the silkworm) for many years (Kobayasi, 1977). At about the same time its entomopathogenicity was noted, it was also observed that insects dying from B. bassiana infection were somehow preserved and protected from bacterial decomposition, and would remain in a "mummified" state. This suggests that the fungus produces antimicrobial compounds, which may be related or unrelated to its entomopathogenic activity. Several authors have commented on the apparent antimicrobial activity of B. bassiana (Kodaira, 1961; Kobyashi, 1977; Walstad et al., 1970).

B. bassiana has long been of interest as a potential agent of biological insect control. Grula and his students (1978) showed that the fungus could penetrate the integuments of the corn earworm (Heliothis zea) and that such infections produced a significant number of deaths in infected larvae.

As of 1981, only two compounds with antimicrobial activity had been demonstrated and partially characterized. One of these substances is beauvericin, a methanol-soluble compound found in the hyphae of B. bassiana. It has been shown to have toxic effects on mosquito larvae (Hamill et al., 1969).

It was thought that beauvericin might be the basis for the pathogenicity of B. bassiana for the corn earworm. Champlin and Grula (1979), however, found that beauvericin had no involvement in this entomopathogenicity, since no trace of the antimicrobial substance was found in the hemolymph of moribund larvae, and intrahemocaelic injections of up to 6 ug of synthetic beauvericin failed to induce any deleterious effect.

In attempts to demonstrate other virulence-related factors in culture filtrate of B. bassiana, use was made of its antibacterial activity against a Bacillus species isolated from soil. Many soil isolates of Bacillus were screened for susceptibility to B. bassiana antibacterial factors, and the most sensitive strain was chosen as an assay organism. In subsequent work with the antibacterial factors themselves, this strain was used (see the Materials and Methods section of this thesis).

Most of the references to toxic products of B. bassiana were related to its entomopathogenicity, but more recently there have been some references to an antibacterial substance isolated from B. bassiana which was a red pigmented compound found in submerged culture filtrates. It was called oosporein, and was identified as a dibenzoquinone similar to others found in many fungi (Vining et al., 1962). Certain specific growth conditions were needed to induce oosporein production. It was isolated by lowering the pH to 3.0, extracting with ethyl acetate, and crystallizing from methanol.

Beauvericin, which has been studied in our laboratory from the standpoint of insect pathogenicity, is the only other toxic substance produced by B. bassiana which has been studied extensively. It is not released extracellularly, but is retained within the fungal membranes. It is a cyclic hexadepsipeptide ionophore consisting of three residues each of D-a-hydroxy-isovaleric acid and N-methyl-L-phenylalanine in an alternating sequence. It functions as an

ionophore, transporting mono- and divalent cations (similar to the activity of neutral ionophores such as valinomycin and polymyxin B (Ouchinnikov et al., 1971), which render biological membranes permeable to cations. Beauvericin has been shown to be toxic toward the Bacillus sp. used as the assay organism in this work (Champlin and Grula, 1979).

In the initial phases of this project, mutants were obtained by the ultraviolet irradiation of a wild-type strain of B. bassiana, and selecting the survivors for high proteolytic activity. This property was thought, at one time, to be related to entomopathogenicity. One of the mutants showing unusually high proteolytic activity (as indicated by zones of clearing on a casein agar plate) was selected for further study on the mechanisms of entomopathogenicity. In another aspect of the project, Champlin (1981) began examining various mutants for the production of antibacterial substances in culture filtrates. It was quickly shown there was no red pigment, and antibacterial substances were excreted into the medium, differentiating them from beauvericin and oosporein. Champlin and Grula (1979) did not find any toxic methanol-soluble substances in the spent growth medium, or in the hemolymph of infected and moribund larvae.

In further studies on the antibacterial properties of the spent medium, Champlin (1981) found, using paper chromatography and bioautography, two substances in the spent B. bassiana medium which were toxic for the soil isolate Bacillus No. 4. Bioautography is a method of visualizing the antibiotic on paper chromatography by transferring strips of the chromatogram to pyrex plates which contain a seeded agar medium. Zones of inhibition are revealed on the agar after 24 hours and the strips are removed. One of these migrated to an R_f of 0.80 in a solvent system consisting of water, isopropanol and butanol (65:22.5:12.5), while the other remained at the origin. These were referred to

as the "migrating" (M) antibiotic and the "origin" (O) antibiotic, respectively. The problem then became one of separation and further characterization of each of these substances.

It was shown by Champlin (1981) that by adjusting the composition of the growth medium, selective production of either the origin or the migrating antibiotic, with very little of the other type was possible. By controlling the time of incubation and harvest, one or the other could be produced exclusively. A mixture of L-alanine, L-phenylalanine, and L-valine, plus basal salts and minerals, resulted in only the origin antibiotic up to 96 hr. A glucose-NH_4Cl-citrate medium resulted in a significant amount of migrating antibiotic, but very little origin antibiotic, up to 96 hr.

Even though citrate could not be utilized as a sole source of carbon and energy, its addition to all of the media studied resulted in approximately a threefold enhancement of migrating antibiotic activity. The three amino acids produced approximately a twofold increase in the amount of origin antibiotic. Pyruvate and three other TCA cycle intermediates, in combination with glucose, also stimulated the production of migrating antibiotic to about the same degree as citrate.

The separation and characterization of the two antibiotics by Champlin and Adefarati becomes important because both arrived at a migrating compound but each compound differed in its mode of action. Champlin's compound was bacteriolytic while Adefarati's was bacteriostatic. The work for this thesis was begun on the assumption that there was only one "M" antibiotic, but it became apparent after some experimentation that we were dealing with two different compounds.

As Champlin (1981) described them, the origin antibiotic is methanol-insoluble and the M antibiotic is methanol-soluble. The pH of the medium is

important in determining the extractability of the M antibiotic by polar as compared with less polar solvents. Migrating antibiotic is methanol-extractable from lyophilized spent Sabouraud's fructose broth (pH 2.5) but not from neopeptone citrate broth (NCB) (pH 6.5).

Migrating antibiotic can be partially extracted with n-butanol from H_2O used to dissolve an NCB precipitate which has lowered pH to 2.0. None can be extracted if the pH remains unchanged. Both of these observations indicate the active molecule is a weak acid, nononized at a low pH, and therefore extractable by relatively nonpolar organic solvents. Conversely, increasing the pH (to about 6.5) renders the molecule ionized, and therefore extractable by the polar solvent water.

In all extractions, and other procedures designed to purify the antibiotic, all fractions were assayed by disc agar diffusion, and by chromatography followed by bioautography. It was evident early in this work that pH of the medium in which the M antibiotic was dissolved was very important in determining its biological properties. Champlin (1981) showed that migration of the M antibiotic in the solvent system used was unchanged over a range of pH from 2.45 to 11.8; however, culture filtrate (spent medium) samples from Sabouraud's fructose broth (SFB-final pH 2.8) yielded a much larger and clearer zone of inhibition than did that from neopeptone-citrate broth (NCB-final pH 5.5). Approximately the same degree of growth in both media had been reached.

Acetone precipitates of NCB culture filtrates, when extracted with an aqueous solution, showed enhanced M antibiotic activity if treated with dilute HCl, as compared with an equal volume of water. Acid rendered the M antibiotic somewhat methanol-soluble, indicating again that a low pH repressed the ionization of an acidic group on the molecule. In addition, n-butanol can partially extract M antibiotic activity from a water extract of the NCB

acetone precipitate if the pH was lowered from the normal 6.5 to 2.0. None is extracted by n-butanol if the pH of the water extract is unchanged.

According to Champlin (1981), bioautography of the n-butanol partitioned antibiotic revealed a much larger and clearer zone of inhibition after development of the paper chromatograms in the Heathcote-Jones I solvent system than occurred after development in the antibiotic solvent system. Heathcote-Jones I contains 4% formic acid.

Additional work done by Champlin on isolation of the antibiotics from spent medium emphasized that the acetone precipitate from 96 hr NCB culture filtrates contains both M and O antibiotics, and can be washed with methanol without extraction of either factor. Evaporation of the methanol supernatant leaves a yellow-oily residue. (The latter is reminiscent of the description given by Adefarati (1984) for his factor.)

To circumvent contamination of spent medium by peptides, Champlin, in further attempts to purify the M antibiotic, grew B. bassiana in glucose-NH_4Cl-citrate (GAC) medium. He showed that a portion of the total amount of activity present could be extracted from the water extract if the pH were lowered from the initial 6.5 to 2.5. The facts that acidification made the M antibiotic extractable by a relatively nonpolar solvent (butanol) is in keeping with the hypothesis that it is a weak acid, and that only a portion of the activity was extractable, is compatible with the hypothesis that there were actually two distinct antimicrobial substances in the water extract of the acetone precipitate, only one of which had acidic properties.

These experimental procedures are being scrutinized in some detail, because another student, Adefarati (1984), continued work on the isolation and characterization of the M antibiotic. He arrived at a product which, while

relatively pure, was quite different in some of its properties from the least impure preparation of the M antibiotic that Champlin had described.

The procedure devised for extraction of the M antibiotic from spent medium filtrates (144 hr cultures) omitted the step of acetone precipitation. The crude filtrate was lyophilized and then reconstituted at a 10X concentration (pH 6.4). The pH of the 10X solution was adjusted to 2.5 with 3N HCl, and it was extracted with n-butanol. The n-butanol phase was concentrated five-fold by evaporation and it was re-extracted with water (pH 6.4). The higher pH enabled the molecule to ionize after which it entered the aqueous phase, which was then lyophilized to dryness and stored at -20 C. Some properties of this preparation are as follows: (1) no detectable ninhydrin-positive compounds, or citrate, are present; (2) it has an R_f value of about 0.8 in the antibiotic solvent system; and (3) it possesses a small amount of reducing sugar which exhibits an R_f value similar to that of the antibiotic. Champlin concluded that the M antibiotic either contained a carbohydrate moiety, or that some glucose remained as an impurity from the original medium. It became of interest to identify this carbohydrate.

Champlin (1981) tried gel filtration, using concentrated water extracts of lyophilized GAC medium culture filtrates to separate the migrating antibiotic from the origin antibiotic. Passing this through a Sephadex G-25 column produced fractions containing only M antibiotic. They were pooled and lyophilized, yielding a white granular powder. (Note: depending on molecular weight, these fractions could contain either the acid, or the lytic factor, or both.)

Champlin (1981) performed his lysis experiments on the G-25 eluate fractions that had M antibiotic activity, as determined by bioautography. Use of the GAC medium precluded the presence of the O antibiotic. Water solutions of this powder (200 mg/ml) were tested using the disc antibiotic assay against a

number of different bacterial species. It is interesting to note that, at this point, Champlin indicated that the zones of inhibition obtained against certain of the organisms revealed the possible presence of a heretofore undetected third antibacterial factor. Several of the bacteria tested showed large zones of only partial growth inhibition (a hazy zone) at 12 hr. In cases where both clear and hazy zones existed with the same organism, the margin between the two was distinct rather than gradual. Organisms showing a hazy zone include both Gram-negative and Gram-positive species (10 species altogether), while those showing a clear zone were Bacillus No. 4, Bacillus cereus, and Microaccus lysodeikticus (all Gram-positive). Only Bacillus No. 4 showed both a hazy and a clear zone.

The two distinct zones can only be interpreted as indicating two antimicrobial substances, with different diffusion coefficients. After 12 hr incubation, the hazy zone tended to disappear, indicating that it slowed but did not completely prevent growth of the susceptible species. One aspect of the hypothetical antibiotic producing the hazy zone, which indicates it is neither the acid factor of Adefarati (1984) nor the lytic factor of Champlin (1981), is the fact that addition of glucose to the medium (in the cases of S. lactis and S. faecalis) antagonizes, rather than stimulates, its antibiotic activity.

Isolation Procedure Used by Adefarati

The fractionation procedure used by Adefarati (1984) to isolate a migrating antibacterial factor from spent medium will be summarized and discussed briefly. One key point in Adefarati's procedure is that he discarded the acetone precipitate (obtained in one of the initial fractionation steps) applied to the spent medium. Champlin showed that, from NCB (neopeptone-citrate-broth, final pH 5.5) culture filtrate, acetone would quantitatively precipitate the

migrating antibiotic. In later work with GAC medium, Champlin omitted the acetone precipitation, and used an n-butanol extraction of a culture filtrate lyophilizate, adjusted to pH 2.5.

Adefarati used the GAC medium throughout. At the time of completion of growth and removal of cells, pH of the spent medium was near neutrality. He showed that by lowering the pH of the spent medium to approximately 3.0, an antibacterial factor had been produced which was extractable with organic solvents. This factor had an R_f value of about 0.80 in the antibiotic solvent system, very close to Champlin's M antibiotic. Adefarati achieved initial separation by adding five volumes of acetone to one volume of spent medium (GAC) at about pH 3.0. The precipitate was discarded since the acetone supernatant apparently contained all or most of the activity.

One type of observation mentioned in which Champlin and Adefarati give diametrically opposed results regarding the extractability of the antimicrobial factors from spent culture media. Champlin reported that from spent Sabouraud's fructose broth (SFB) cultures (pH 2.5), the migrating antibiotic was soluble in methanol but only slightly soluble in ethanol, whereas Adefarati found general movement of activity from aqueous to the organic phase at pH 3.0.

Adefarati further purified the factor in the acetone supernatant by evaporating off the acetone, lyophilizing, and redissolving in one-tenth (of the original) volume of glass-distilled water. This solution was extracted with 10X the volume of isoamyl alcohol, followed by re-extracting back into one-tenth the volume of water. Alternatively, the isoamyl alcohol could be evaporated off, the residue dissolved in 10 ml water, and lyophilized to dryness. Adefarati stated that the above procedure "yielded a yellowish, oily substance which had been earlier found to have relatively high antibiotic activity." The oily product

was water-soluble. Extraction of the aqueous solution with chloroform would remove the yellow color while full activity was retained.

Thus, the most purified form achieved by Adefarati differed significantly in melting point (an oily liquid as compared to a white powder at room temperature). Furthermore, there was a large difference in lytic activity toward Bacillus No. 4 between these two preparations. Champlin's antibiotic quickly caused a very precipitous drop of OD in exponential phase cultures of Bacillus No. 4, and complete lysis within 2.5 hr. The O antibiotic resulted in cessation of growth with only a slight lowering of the optical density.

Nongrowing cells were only slightly affected by the lytic activity. These observations, coupled with microscopic examination of the cells during lysis led Champlin to the conclusion that the M antibiotic is not acting directly on the cell wall, but that the wall is in some way being degraded via activation of the constitutive autolytic system resulting as a secondary effect of membrane disorganization. As a test of this hypothesis, Champlin compared a known "membrane antibiotic," polymyxin B, with his M antibiotic and found its action to be very similar to that of the M antibiotic.

Adefarati, on the other hand, using his preparation of M antibiotic from isoamyl alcohol extracts of GAC acetone supernatant showed, a leveling off of growth of exponential phase cells after about 2 hr of contact with the preparation by the cells. Resting cells showed a slight decrease in optical density over about 3 hr.

In both cases, it is very difficult to relate the activity (any kind of activity) to a known concentration of pure active substance; but the very large difference in lytic activity seen by the two investigators in what was presumably the same substance is not reasonably explained as simply a quantitative difference. Some discrepancy in the separation procedures must be resolved.

In addition to the large difference in lytic activity, it appears to be important to compare the products of the two earlier investigators regarding other properties of their preparations. Effects of pH extremes on antibiotic activity, antibacterial spectrum, solubilities, and effect of growth medium composition on yield of antibiotic as shown by Champlin (1981) will be noted unless otherwise stated.

Champlin observed that low pH values in culture filtrate samples affected both the degree of inhibition observed in bioautography, and solubility characteristics of molecule. The antibiotic from SFB (final pH 2.8) yielded a much larger and clearer zone of inhibition than that from NCB (final pH 5.5).

Adefarati, using a disc assay of a solution adjusted to different pH values, showed an optimum pH of 2.5 to 3.5 for eight organisms. In both cases, the active compound appears to be a weak acid, which would be expected to show maximum activity in the nonionized (protonated) form. The value of the pKa is similar in both cases.

A low pH was found to render the M antibiotic more soluble in organic solvents such as methanol and n-butanol (Champlin, 1981). His M antibiotic could be partially extracted with n-butanol from an aqueous extract of the NCB acetone precipitate _if_ the extract pH was lowered to 2.0, but was not extracted if the pH remained at 6.5.

It appears at this point that the pH of the spent medium at the time of acetone precipitation is a key for resolving the differences between the results and interpretation of Champlin and Adefarati. Champlin found that acetone completely precipitated the antibiotic from NCB broth at pH 6.5. Adefarati observed that if the pH of the spent medium was 3.0, acetone still produced a precipitate, but much more activity was found in the acetone supernatant than in the precipitate. The first step used by Adefarati in his purification process

was adjustment of the pH of the GAC spent medium to 3.0 with HCl. Adefarati used the GAC medium with aspartic acid added. He showed, using ^{14}C labeled compounds, that citrate was the compound most efficiently incorporated into the antibiotic, thereby indicating the possibility that it was a direct precursor. Some carbon from aspartate was also incorporated, but very little carbon from glucose. Champlin also observed that citrate in the growth medium enhanced production of the antibiotic.

In comparing the data of the two authors on the antimicrobial spectra of their respective preparations, many differences are apparent. Champlin (p. 88, Table XI) may have been dealing with two factors in his M antibiotic, because he describes clear and hazy zones (presumably concentric around the disc). Only one organism (Bacillus No. 4) was susceptible to both. The factor producing the hazy zone was active against three Bacillus species: S. aureus, Streptococcus lactis, Streptococcus faecalis, but not M. lysodeikticus and Erwinia carotovaro. The clear zone factor was active against M. lysodeikticus and B. cereus as well as Bacillus No. 4. Unfortunately, Champlin did not mention the pH at which he performed his tests.

Adefarati, testing at pH 2.5, found activity against a number of species of Clostridium, Bacillus No. 4, E. carotovora, E. coli, S. aureus, Pseudomonas aeruginosa, and M. Lysodeikticus.

The most reasonable conclusion at this point is that Champlin's preparations actually contained two quite different factors, one of which was an acid, probably the same as Adefarati's factor, and was soluble in organic solvents (including acetone) at a low pH (2.5-3.5). The other was the lytic factor, which was completely precipitated by acetone from spent medium at a range of pH values from 6.5 to 3.0. Therefore, Adefarati missed the lytic activity, since he discarded the acetone precipitate. If the original acetone precipitation was

done at a high pH, the lytic factor could be extracted from the precipitate with water and then partially extracted by an organic solvent if the water extract were acidified. These observations are compatible with the hypothesis that the lytic factor is a weak acid.

Adefarati's tests on his relatively pure factor, particularly using nuclear magnetic resonance, and infra-red and mass spectroscopy, indicated that he had a tricarboxylic acid, of the empirical formula $C_6H_8O_7$, and containing a methyl group. Therefore, it was not citric acid, although the molecule was similar in certain respects to citric acid, notably molecular weight.

Initially, our goal was to study further the factor isolated by Adefarati, particularly its antimicrobial spectrum and its mode of action. Not long after beginning the study, it was realized that Champlin's migrating (M) antibiotic and Adefarati's tricarboxylic acid were quite different and distinct entities, and the direction of research was changed to concentrate more on the purification, primary characterization, and mode of action of the lytic antibiotic.

CHAPTER II

MATERIALS AND METHODS

Antibiotic Production

In order to facilitate the isolation and purification of the factors, a chemically defined medium containing glucose, NH_4Cl, citrate, and three amino acids had been devised (Champlin, 1981). Later it was learned that NH_4Cl could serve as a sole nitrogen source, and the amino acids were omitted for the production of the migrating antibiotic. This medium was then primarily used by Adefarati (1984). Its composition is found in Table 1. Citrate and a high final pH in the medium (which the trisodium citrate helped to maintain) insured a good yield of the tricarboxylic acid factor.

The addition of citrate was shown by Champlin to increase the production of both antibiotics about threefold. In this medium, lacking peptones or amino acids, production of origin antibiotic was very low. Citrate was thought to have a stimulatory effect on M antibiotic apart from, and in addition to, its buffering effect. This medium is referred to throughout this thesis as GAC (glucose-ammonia-citrate) medium (see Table 1).

One hundred ml of GAC in 250 ml flasks was inoculated with two loopfulls of 15-day-old B. bassiana spores from a SDA slant. The flasks were put on a gyrotory shaker (New Brunswick Scientific Company, model V) with a rotational speed of 180 rpm in a 25 C room.

Table 1. Composition of Medium

Ingredients	g/100 ml
Glucose	1.000
Trisodium Citrate	2.580
$MgSO_4 \cdot 7H_2O$	0.003
NH_4Cl	0.100
K_2HPO_4	0.174
KH_2PO_4	0.136
20 ml trace minerals (Grula, 1960)	
47 ml glass distilled water	
pH to 6.8 with 3N HCl	

Glucose was added after the medium had been autoclaved for 15 min at 15 lb and cooled to room temperature.

Cultures were grown for 180 hr, the time at which maximal migrating antibiotic production occurred (Champlin, 1981). The fungal mass was removed by centrifugation using a Sorvall RC2-8 at 10,000 rpm with a GSA rotor. The supernatant was filtered using a 0.45 μ millipore system and divided into 50.0 ml portions. Each batch was tested for antibiotic activity with the antibiotic disc assay. The 50 ml in a vial were frozen on a slant and lyophilized for further experimentation or purification. The lyophilizates were stored at 0 C.

Test Organism

The strain of B. bassiana used for antibiotic production was selected from variety of UV-induced mutants selected on the basis of their ability to produce large amounts of extracellular proteolytic enzymes when plated on Sabouraud Dextrose agar (SDA, Difco) with casein (Grula et al., 1978). Fifteen strains isolated on the basis of high proteolytic activity were screened for their ability to produce extracellular antibiotics using Bacillus No. 4 as assay organism, and the mutant strain showing the highest activity was selected for further study. The strain was designated number 7 but was later referred to as cc-1.

All cultures of B. bassiana strains were maintained on slants of SDA supplemented with 1% yeast extract (Difco) at 25 C. Conidiospore formation was complete in 14 to 21 days and the slants were then stored at 2 to 4 C until use. These cultures were used as the inoculum for antibiotic production. Stock cultures were transferred every two weeks.

Assay Organism

The assay organism used with all antibiotic assays was a Bacillus sp., isolated from soil, which was found to be the most sensitive when cross-streaked against B. bassiana on SDA. The strain is aerobic, gram positive, and spore-

forming. Morphologically, the cells are long rods that form chains. This strain of Bacillus does not correspond with any species described in the 8th edition of Bergey's Manual of Determinitive Bacteriology (Smith and Gordon, 1957; Gibson and Gordon, 1974). A detailed description of the organism is found in Table 1 in Champlin's thesis (1981). The organism has been designated Bacillus No. 4 because it was the fourth culture to be tested for sensitivity to B. bassiana.

Bacillus No. 4 was used for all bioautography and antibiotic disc assay on SDA. It was found to be not only sensitive to the extracellular products of Beauveria bassiana but to synthetic beauvericin. It was maintained on Tryptic Soy Agar (TSA, Difco) slants and grown at 30 C. After 15 to 18 hr of growth the slants were refrigerated at 2 to 4 C. Stock cultures of the bacterium were transferred to a fresh TSA slant every two weeks.

Agar Diffusion Assay

Agar diffusion assays (disc assay) were performed on the spent medium, and at each step in the fractionation of the spent medium. Since the M antibiotic(s) from B. bassiana filtrate displayed more activity at a lower pH, SDA (pH 5.6) was used for the antibiotic disc assay. 56.3 grams (g) of SDA per liter was used instead of the usual 65 g, which would allow for increased diffusibility of the antibiotic. The medium was steamed to dissolve the agar and 12 ml portions were placed in bottles. Each bottle was autoclaved and stored at 2 to 4 C until use.

To perform the assays, the SDA was melted and cooled to 45 C in a water bath and seeded with 0.35 ml of washed cells of Bacillus No. 4. The cells were harvested at 12 to 14 hr from an SDA slant, suspended in saline (0.85% NaCl), and adjusted to an optical density (OD) of 0.500 at 540 nm using a Bausch and Lomb Spectronic 88 spectrophotometer. Each suspension was sonicated in a water bath

sonicator (Laboratory Supplies Company, Inc.; model G112SP1T) for two min before being pipetted into the melted and cooled SDA, in order to disperse the cells evenly. The seeded agar was then poured into petri plates and gently swirled to insure even dispersion of bacteria. The agar was allowed to solidify, after which a 13 mm Whatman No. 1 paper disc was impregnated with the antibiotic solution by dipping one edge of the disc into the solution until the opposite edge of the disc was moist. It was then carefully laid on the surface of the seeded agar. In the case of gel filtration fractions, 6 mm Whatman paper No. 1 discs were used to reduce the number of petri plates needed. All plates were incubated right side up at 30 C for 18 hr.

Paper Chromatography

Paper chromatography, combined with bioautography as described above, was used to monitor activity found in B. bassiana culture filtrates (spent medium) or fractions thereof obtained by various fractionating techniques. It was also used in the analysis of the purity and chemical composition of the lytic antibiotic through the use of certain sprays.

Concentrated samples of 15 to 30 µl were applied to an 8" x 8" square of Whatman No. 1 chromatography paper which was divided into seven 1-in. lanes with a half inch left on each side of the paper. After the depositions were dried with hot air from a hair drier, the square was rolled into a cylinder with depositions on the bottom and the two sides were stapled along the half-inch strips. The bottom of the cylinder was then placed in a large pickle jar with 100 ml of a solvent mixture of isopropanol, water, and n-butanol (22.5:65.0:12.5). Solvent mixture was allowed to equilibrate for 1 hr. The running time for the solvent was about 6.0 to 6.5 hr at 25 C at which time the solvent front was marked and the cylinder was removed from the solvent and allowed to dry.

The dried chromatograms were then analyzed for antibiotic activity by bioautography or were sprayed with reagents specific for the detection of certain types of compounds. For the detection of free amino acids and peptides, ninhydrin at a concentration of 0.4% in acetone was sprayed on the chromatograms, which was incubated for 3 min at 100 C. Lipid detection was achieved by spraying the chromatograms with Rhodamine B6 which will flouresce a red-violet color with lipids on a pale pink background under UV light.

Tailing probably resulted from the high polarity of the antibiotic compound and the interaction of hydrogen bonding with cellulose. The same solvent system was used with both Whatman No. 1 and Whatman GS-81 paper. Therefore, silica gel loaded absorbent paper was used to reduce tailing.

Since there was an indication from alkaline potassium permanganate sprays of paper chromatographs that the migrating antibiotic may be some type of carbohydrate, the detection reagents for sugars were employed and are presented in Table 2.

Bioautographic Assay

The bioautographic assay was coupled with paper chromatography to detect the presence or absence of antibiotic activity in all samples. Sabouraud's dextrose agar (Difco) was made up in 190 ml dilution bottles at 90 ml per bottle at a concentration of 56.3 per liter. Bottles of agar were autoclaved at 15 lb for 15 min and stored at 2 to 4 C until used. To perform an assay, the agar was melted and cooled to 45 C. A Bacillus No. 4 suspension was prepared in a manner similar to that for the disc assay (with the following modifications): an 18- to 20-hr-old culture agar slant of Bacillus No. 4 was washed with 5.0 ml of 0.85% saline; the 5 ml were sonicated to disperse cells more evenly and break any chain formation. The suspension was then diluted to an OD540 of 0.80; 3 ml of this

Table 2. Detection Reagents for Sugars

Anisidine Preparation: 1 g of p-anisidine hydrochloride in 10 ml of methanol. Dilute with n-butanol to 100 ml. Add 0.1 g of sodium dithionite and shake well.
Procedure: Spray solution and heat for 10 min at 130 C.
Results: Aldohexoses, green-brown; ketohexoses, yellow; uronic acid, red; 2-deoxyaldoses, gray-brown.

p-Anisidine-Phthalic Acid Preparation: 1.23 g p-anisidine and 1.66 g phthalic acid in 100 ml methanol.
Procedure: Spray solution and heat for 10 min at 130 C.
Results: Hexoses, green; pentoses, red-violet; methyl pentoses, yellow-green; uronic acid, brown.

Anthrone Reagent Preparation: 0.3 g anthrone in 10 ml of glacial acetic acid and add to the solution 20 ml of 96% by volume ethanol, 3.0 ml of concentrated phosphoric acid, and 1.0 ml of water.
Procedure: Spray solution and heat for 5 to 6 min at 110 C.
Results: Ketoses and oligosaccharides containing ketoses as yellow spots.

Ninhydrin Preparation: 0.1% ninhydrin in acetone.
Procedure: Dip solution and heat for 1 to 2 min at 80 C.
Results: Free amino sugars give a reddish-purple color; amino uronic acids give a light brown color at first, turning purple on standing.

Periodate-Anisidin Preparation: Solution a: 1.0 g p-anisidine in 100 ml of 70% ethanol. Solution b: 10 ml of 0.1 M sodium periodate in 100 ml of acetone.
Procedure: Spray with solution a; heat 5 to 10 min at 105 C; dip in solution b.
Results: Spots detected on a brownish background. Polyols, white; sugar acids (uronic acids, red to brown; aldonic acids, white); 2-deoxy sugars, yellow; amino sugars, brown or yellow; pentoses and hexoses, red fluorescence in UV light.

Vanillin Preparation: 1.0% solution vanillin in concentrated H2SO4.
Procedure: Spray with solution; heat 4 to 5 min at 110 C.
Results: Polyether, brown spots.

Bromcresol Green Preparation: a. 0.1% bromcresol green in 95% ethanol; add 1N NaOH until color becomes blue-green. b. Color reagent--mix solution a and acetone (1:4).
Procedure: Draw dry chromatogram through reagent. Lay paper on another sheet of paper.
Results: Acids give yellow spots on green background; basic components give blue spots.

Source: J. Sherma, Handbook of chromatography, 1972, p. 119.

suspension were added to the 90 ml of melted SDA. Two of the seeded SDA bottles were poured into a sterile 22 x 34 baking dish, which was covered with sterile aluminum foil. The agar was allowed to solidify; each strip from the chromatogram was cut to a width of 2.54 cm and carefully laid upon the agar surface. The dishes were incubated at 25 C for 20 hr.

A zone of inhibition will appear under the strip wherever an antibiotic to which Bacillus No. 4 is sensitive has migrated on the chromatograph. This will give a characteristic (R_f) for that particular antibiotic under the test conditions.

Gel Filtration

Three types of gels were used to isolate and purify the migrating lytic antibiotic from the acetone precipitate of GAC which contained high levels of the migrating antibiotic and small amounts of the origin antibiotic. Sephadex G-25 (50 to 150 µm; fractionation range 100 to 5000) was used for desalting and excluding higher and lower molecular weight compounds from the antibiotic fraction. Sephadex G-75 (40 to 120 µm), with a fractionation range of 3000 to 80,000 daltons, was useful in determining which gel to use so that the molecular weight of the migrating antibiotic will fall within the linear part of a selectivity curve. Bio-Rad Biogel P-10 (or older type P20) with an exclusion range of 1500 to 20,000 (40 to 80 µm hydrated bead diameter) was used for the purification of the migrating lytic antibiotic by fractionation of antibacterial G-25 preparations. The choice of using Bio-Rad P gels over Sephadex gels was because their polyacrylamide structure makes them more hydrophobic and essentially free of charge which would give better separation of polar compounds such as carbohydrates.

The amount of gel needed was determined by the volume of the column used and the hydrated bed volume given for each gel. Twice the amount of

water was added to a particular gel and the mixture was heated in an Arnold steamer for 1 hr for G-25 and P-10, and 3 hr for G-75 to swell the beads. The gels were then allowed to settle and cool overnight. After hydration, half of the top aqueous layer supernatant was decanted and degassed for one hour. A smooth slurry was formed by adding a small amount of degassed water to the hydrated gel and gently pouring into a funnel fixed on the top of the column. Once the gel had settled 2 to 5 cm, the column was allowed to flow 0.5 to 0.7 ml/min with a minimum hydrostatic head that would not damage the beads. As the column became more packed, the hydrostatic head was increased. After packing, degassed water was added to fill the column; a Whatman No. 1 paper disc filter was gently placed on the beds' surface to protect the gel. A reservoir of degassed water was attached to the top of the column and two bed volumes of water was passed through the column to ensure complete packing of the gel.

The void volume (Vo) of each gel and column was determined by applying a blue dextran or cytochrome C. This also gives evidence of nonuniform resistance of the gel by skewing or uneven migration of the colored zone as it traverses the bed. Blue dextran was added at 2 mg/ml concentration and cytochrome C at 6 mg/ml concentration.

The layering of colored proteins and samples on the gel's surface was done by draining the water on the top part of the column to 6.0 cm from the gel. The samples were gently applied with a 2.0 ml pipette. The concentrations of both blue dextran and cytochrome C were around 100 mg/ml. Samples contained a 3.0% solution of sucrose to make it viscous enough to layer the samples under the water. The flow rate allowed the sample to drain into the top portion of the gel bed. Additional water was added to the column from the reservoir attached to the column. Flow rates and hydrostatic heads were adjusted according to the gel and column used. Fractions were collected with an automatic fraction

collector at 3.0 ml per tube after the Vo had passed. Specifications of each gel and column are presented in Table 3.

All fractions collected were monitored for antibiotic activity with the antibiotic disc assay. All fractions with activity were pooled, frozen, and then lyophilized. These samples were stored below 5 C. Monitoring of blue dextran, colored proteins, cytochrome C, and dinitrophenylalanine (for final volume) was conducted on a Beckman model 24 spectophotometer at 280, 620, 410, and 360 nm, respectively.

Treatment of Growing Cells With Antibiotics

Champlin (1981) showed that the migrating antibiotic was highly lytic when used to treat exponentially growing cells of Bacillus No. 4. Nongrowing (stationary phase) cells also showed a decrease in optical density, but not as large as that with growing cells.

In determining the lytic activity of various fractions, I used an 18- to 20-hr culture of Bacillus No. 4 grown on a TSA slant at 30 C. It was washed with 5.0 ml of sterile saline, the bacterial suspension was subjected briefly to a waterbath sonicator to break up cell clumps and chains, and diluted to an OD540 of 0.500. One milliliter of this inoculum was transferred to 100 ml of sterilized TSB in a 250 ml Erlenmeyer side arm flask. The side arm of the flask was an 18 x 150 mm tube, which provided a means of measuring the OD without removing a sample. The flask was placed on a shaking rotational instead of reciprocal waterbath at 30 C at an rpm of 180. The OD of the culture was monitored every hour and every half hour when cells entered the exponential growth phase.

As the cells reached the exponential phase (about OD540 of 0.300 to 0.400) 4.5 ml portions were placed in sterile 18 x 150 tubes. The desired antibiotic concentration was added in 0.50 ml amounts to bring the total volume to 5.0

Table 3. Gel and Column Specifications

Parameter	Value
Sephadex G-25	
Column Length	40 cm (BioRad)
Diameter	25 mm
Packing Height	32 cm
Hydrostatic Head	30 cm
Void Volume	53 ml
Sample Conc.	2.0 ml[1]
Sephadex G-75	
Column Length	60 cm
Diameter	15 mm
Packing Height	48 cm
Flow Rate	1.0 ml/min
Void Volume	75 ml
Volume Total	225 ml
Sample Conc.	2.0 ml[2]
Bio-Gel P-10	
Column Length	100 cm
Diameter	15 mm
Packing Height	75 cm
Flow Rate	0.5 ml/min
Void Volume	70 ml
Sample Conc.	2.0 ml

[1] 100 mg/ml of GAC acetone precipitate.

[2] 100 mg/ml of GAC acetone precipitate.

[3] 50 mg/ml of G-25 lyophilized G-25 active fractions.

ml. One-half ml of sterile water was added to the control to adjust for the dilution factor. An OD540 reading of each tube was taken immediately; each tube was then replaced in the shaking waterbath. Readings were repeated every 15 min for 2 to 3 hr.

n-Butyl Alcohol and Isoamyl Alcohol Extractions

n-Butanol extraction of lyophilized GAC filtrates was a biphasic partitioning procedure designed to purify the migrating antibiotic. Five milliliters of glass-distilled water were added to rehydrate a lyophilized 50 ml sample of GAC filtrate. The rehydrated sample was placed in a 50 ml conical centrifuge tube, and the pH was adjusted to 2.5 with 3 N HCl to which 25 ml of n-butanol was added. The tube was vortexed for 1 min at room temperature, and the phases were allowed to separate. After separation the butanol phase was removed and subjected to evaporation by either flowing nitrogen gas or air with negative pressure until 5.0 ml remained. Five milliliters of glass-distilled water were added to the butanol phase; the tube was vortexed for 1 min at room temperature. The aqueous layer which contained the migrating antibiotic was removed and lyophilized.

Purification of the migrating antibiotic by Adefarati (1984) was done by acetone precipitation of the GAC filtrate and extracting the acetone supernatant with isoamyl alcohol. Adefarati's procedure was as follows.

The pH of the filtered B. bassiana growth medium was lowered to 3.0 with 6N HCl. Acetone was added at five times the volume of filtrate. The mixture was stirred for 24 hr at 4 C. After this point a precipitate was formed which was discarded. It is possible that in discarding this precipitate, Adefarati may have discarded the lytic factor described by Champlin. Champlin (1981) found that acetone would completely precipitate the lytic factor from neopeptane

citrate broth at pH 5.5. Absolute methanol would not extract the lytic factor from the acetone precipitate but water would. The acetone was removed from the supernatant by a rotary evaporator (Rotorvapor R, Brinkmann Buchi Type KRvr 65/45) on speed 2 at 50 C. The acetone was recycled and the aqueous phase was lyophilized in 50.0 ml aliquots. Five milliliters of glass-distilled water were added to reconstitute the lyophilized fractions after which 50 ml (10X) volume of isoamyl alcohol was added. The mixture was mixed on a gyrotor in a 250 ml Erlenmeyer flask for 30 min at 180 rpm and allowed to separate into the immiscible phases. The isoamyl alcohol phase, which contains a migrating antimicrobial compound, was removed and evaporated by negative pressure with a vacuum in a 250 ml Erlenmeyer flask. The residue after evaporation was redissolved in 5.0 ml of water and lyophilized to dryness, yielding a dark yellow oily substance.

Methanol Fractionation of Antibiotics From GAC Filtrate and GAC Acetone Precipitate

Since the M antibiotic is slightly soluble in methanol, methanol was used to extract the M antibiotic from the GAC spent filtrate, the GAC acetone precipitate, and the GAC spent filtrate acidified to a pH of 2.8 which was similar to the procedure used by Champlin.

A 50 ml sample of lyophilized spent GAC filtrate or a 150 mg sample of GAC acetone precipitate was brought up in 3.0 ml of absolute methanol and the pH was lowered to 2.8 with 1.0 N HCL. Additional methanol was added to bring the volume to 5.0 ml. Samples tested without pH adjustment were brought up in 5.0 ml of absolute methanol.

All samples were then agitated on a vortex mixer for one minute and the insoluble material was removed by centrifugation on a clinical centrifuge. 5.0

ml of ddH_2O were added to the supernatant and allowed to separate, at which point 5.0 ml of chloroform were added. The top aqueous layer was removed which contained the M antibiotic.

CHAPTER III

RESULTS

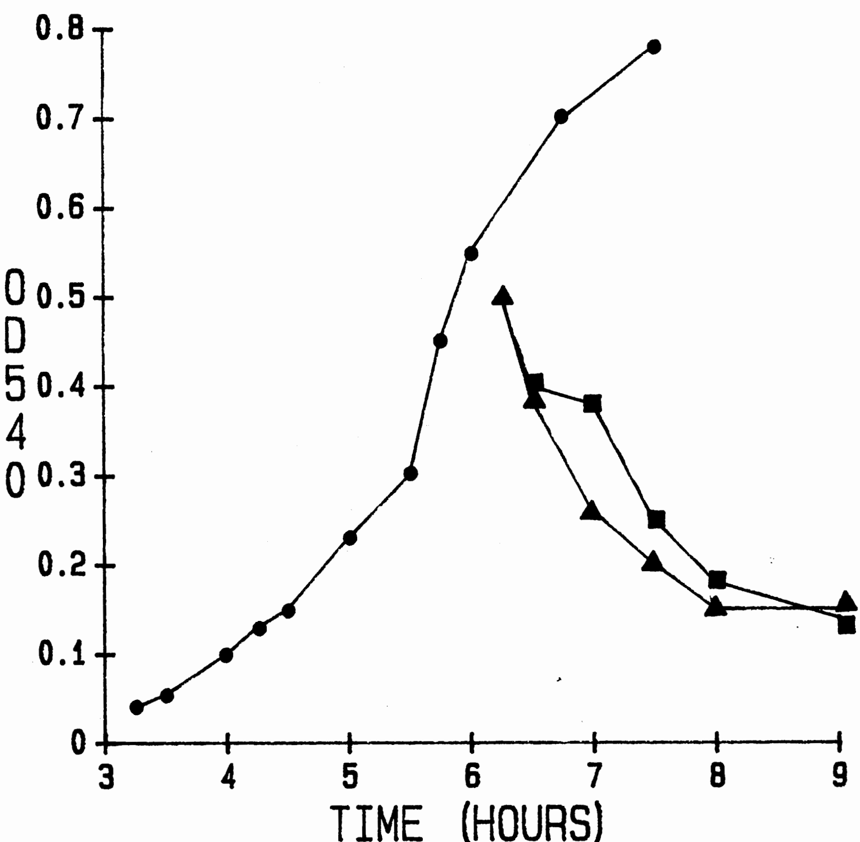
Treatments of Growing Bacillus Cells With Different Isolated Fractions of Spent GAC Filtrate

I first tested the lytic activity of the GAC filtrate against Bacillus No. 4 grown in tryptic soy broth (TSB) at 30 C. Results are given in Figure 1. At an OD of 0.500, the culture was divided into sterile 18 X 150 mm tubes, after which a final concentration of 10 mg/ml or 1.0 mg/ml of lyophilized GAC crude filtrate was added to their respective tubes. There was an immediate drop in OD which continued for two hours and then leveled at an absorbance of 0.100. The pH of the TSB at the final reading was 6.4. The results of Champlin using G-25 gel filtered M antibiotic were similar, in which Bacillus No. 4 was grown in a defined medium, including glucose and aspartate, at 25 C.

Adefarati, on the other hand, detected an M antibiotic that was isolated in the acetone supernatant of GAC, which was then lyophilized and purified further with an isoamyl alcohol extraction of the lyophilized and reconstituted supernatant. Figure 2 shows the isoamyl alcohol-extracted antibiotic to be bacteriostatic against log-phase Bacillus No. 4. The acetone precipitate of GAC, however, exhibited a dramatic lytic activity. At a final concentration of 8 mg/ml, the lytic activity is greater than the GAC culture filtrate, shown in Figure 1, which was added at a final concentration of 10 mg/ml. The final pH of

Figure 1. Treatment of growing *Bacillus sp.* cells with lyophilized filtrates of GAC extracted with water

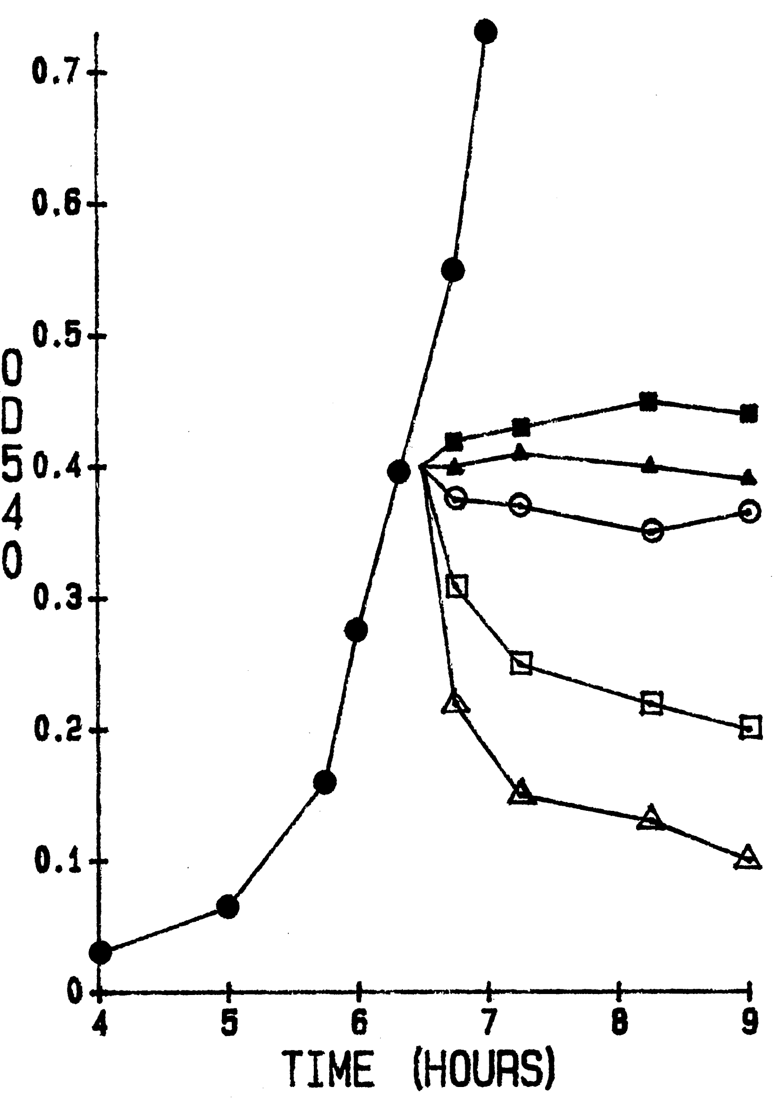
●, Control; ▲, 10 mg/ml GAC; ■, 1 mg/ml



0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD540
3
4
5
6
7
8
9
TIME (HOURS)

Figure 2. Treatment of growing Bacillus sp. with lyophilized solvent extracts. All lyophilizates were brought in water and added at a final concentration of 10 mg/ml to a growing culture of Bacillus sp. in TBS at an absorbance of 0.400.

■ isoamyl extract of acetone supernatant
▲ n-butanol, extract of GAC
○ water extract of butanol phase
□ methanol extract of acetone precipitate
△ acetone precipitate of GAC
● control


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD540
4
5
6
7
8
9
TIME (HOURS)

the Bacillus No. 4 culture, which was lysed by the acetone precipitate of the GAC culture, was 5.6 and the final absorbance reading was below 0.10. The M antibiotic extracted from GAC acetone precipitate with methanol exhibited similar lytic characteristics but with less activity, indicating either a loss in antibiotic concentration than the acetone precipitate or a loss in activity due to pH.

Other solvent extractions which contained migrating antibiotic previously done by Champlin were also observed for activity against growing cells. The butanol extract of GAC crude culture filtrate showed some bacteriostatic activity (i.e., growth inhibition without lysis).

To further illustrate the two different type of migrating antibiotic, which were both present in the GAC culture filtrate, 100 ml filtrate was concentrated fivefold by lyophilization followed by reconstitution in 20 ml water. Dialysis of this concentrate was used to separate the two factors. Since the antimicrobial factor isolated by Adefarati had been shown, using NMR and mass spectroscopy to be or to resemble an isomer of citric acid, it should have a molecular weight around 300. On the other hand, Champlin showed that his migrating factor eluted close to or with the void volume from a Sephadex G-25 gel; this indicated that the molecular weight of the substance was equal to or above 5000. Therefore, it was decided to try dialysis tubing that would retain any molecule with a molecular weight greater than 12,000. After dialysis was complete, the concentrated dialyzed sample and dialysate at 10 mg/ml were paper chromatographed to confirm the presence of a migrating antibiotic, then tested against growing Bacillus No. 4 cells for activity. Chromatography of the dialyzed sample from inside the bag showed a strong presence of M antibiotic with similar tailing seen with the acetone precipitate while the weaker zone of the dialysate GAC was more compact. This lack of activity with the dialysate may be caused by the

higher pH of 6.13. As seen in Figure 3, the graph of the dialyzed sample versus dialysate illustrates that the sample with the higher molecular weight contains the lytic activity while the dialysate shows little activity.

Chromatography of Acetone Precipitate of Spent Medium

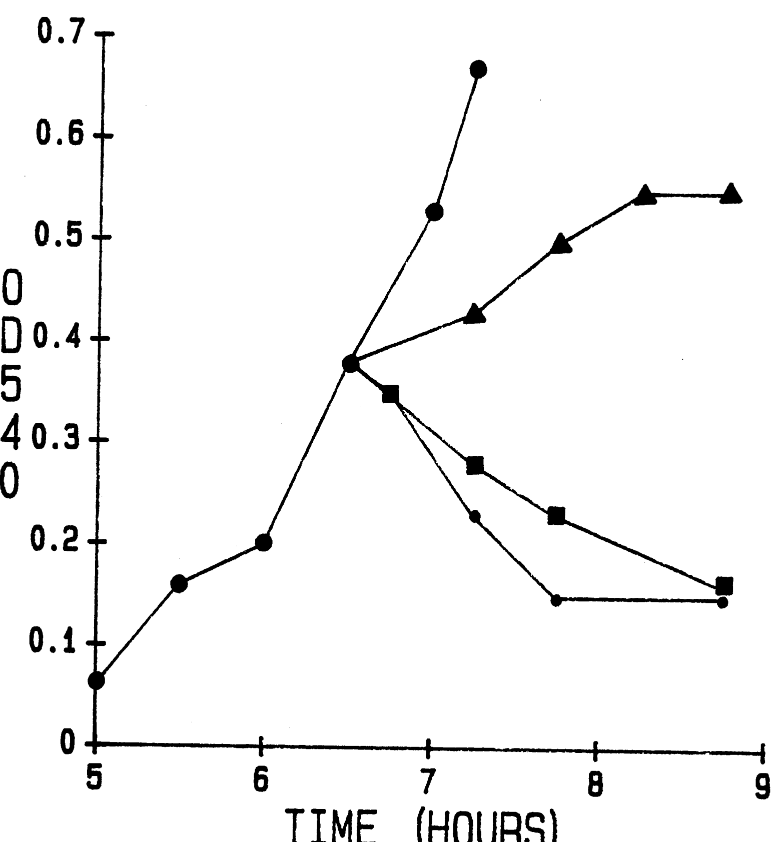
Since the evidence of lytic activity in the crude acetone precipitate of the GAC spent medium contrasted with the lack of such activity in the isoamyl alcohol extract of the acetone supernatant pointed to the existence of two extracellular antimicrobial factors produced by B. bassiana, it appeared to be useful to compare the migrations of these two putative factors in paper chromatography. It may well be that the confusion concerning the nature and properties of the factor(s) arose because they had very similar or identical R_f values in the chromatographic system used.

The following fractions were run on paper chromatograms using the solvent system: water, n-butanol 2-propanol (65:12.5: 22.5): (1) GAC spent medium; (2) acetone precipitate of spent medium, G-25 filtered; (3) isoamyl alcohol extract of acetone precipitate; (4) n-butanol extract of GAC spent medium; and (5) trisodium citrate. The latter was tested because the factor of Adefarati (nonlytic, acid-activated) resembled citric acid; mass spectrometry evidence showed it was a possible isomer of citrate.

Migration was monitored using bioautography with Bacillus No. 4. Diagrams of chromatograms are given in Figure 4 and R_f values in Table 4. It is apparent that both the "origin" and the migrating "M" factor were present in the crude spent medium and in the acetone precipitate. The reason for the heading of the origin antibiotic is unknown, but probably is because of impurities in the preparation. Champlin, in developing solvent systems for separation of the two factors obtained in SFB, found that by increasing polarity of the solvent system,

Figure 3. Treatment of Bacillus sp. with dialyzed and dialyzate lyphilized samples of GAC culture filtrate. The samples were dissolved in glass-distilled water and added to growing Bacillus sp. cells at a final concentration of 10 mg/ml.

▲, GAC dialyzate; ■, GAC dialyzed;
•, GAC acetone precipitate; ●, control



0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD540
5
6
7
8
9
TIME (HOURS)

Figure 4. Paper chromatography of various fractions obtained from B. Bassiana spent medium. Medium components were spotted with 20 µl of a 10 mg/ml solution whereas citrate (pH 3.0) was spotted with 20 µl of a 40 mg/ml solution. The solvent system used was water:n-butanol:isopropanol (65:12.5:22.5).

GAC, spent culture filtrate; APPT, GAC acetone precipitate; G-25, gel filtered acetone precipitate; I, isoamyl extract of acetone supernatant; C, trisodium citrate (sigma) pH 3.0; B, n-butanol extract of GAC.

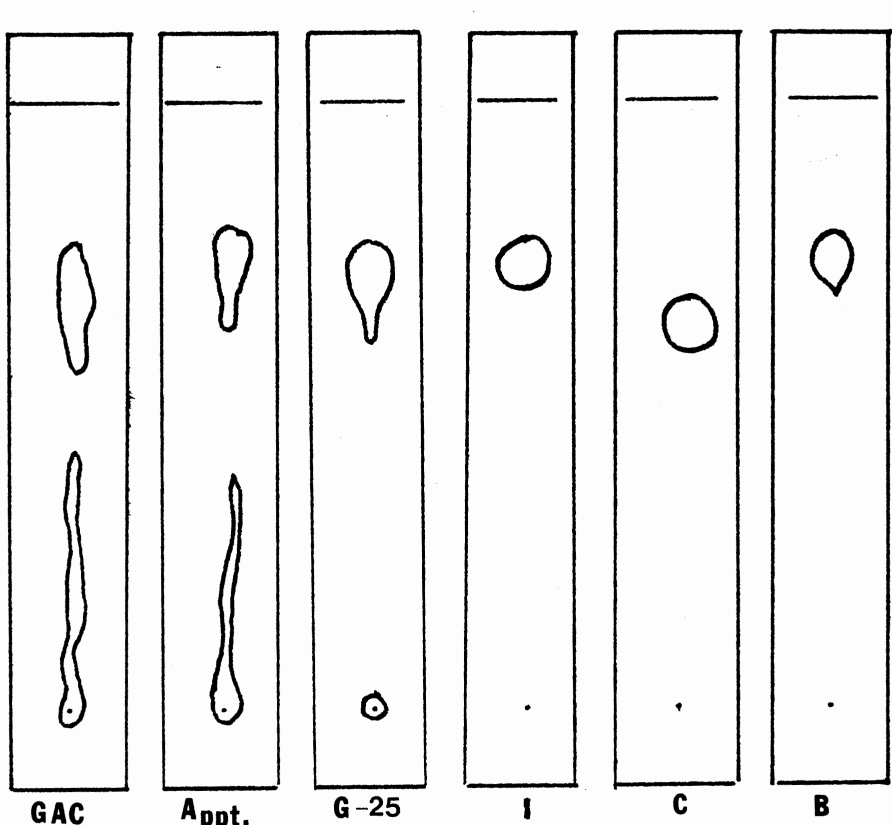

GAC
Appt.
G-25
I
C
B

Table 4. R_f values of active migrating samples on bioautography

Sample	R_f
GAC crude filtrate	0.80
Acetone precipitate[1]	0.78
Isoamyl extraction[2]	0.79
G-25[3]	0.80
Butanol Extraction[4]	0.80
Citrate	0.69

All samples were paper chromatographed at 10 mg/ml except for citrate which was at 40 mg/ml.

[1]Acetone precipitate of GAC.

[2]Isoamyl extraction of the acetone precipitate of GAC.

[3]G-25 of acetone precipitate.

[4]Butanol extraction of GAC.

he got better separation with 65:12.5:22.5 (water:n-propanol:isopropanol); separation was complete and there was no tailing or heading. The different medium may be responsible for the difference observed here (lack of clean-cut separation using the culture filtrate).

It was shown, however, that passage of the acetone precipitate through G-25 Sephadex removes whatever is causing the heading. The most interesting aspect of this chromatography is the fact that the isoamyl alcohol extract (presumably containing only the nonlytic factor) has almost exactly the same R_f values as the lytic factor present in the acetone precipitate of the spent medium.

This chromatogram is of interest for another reason: the citrate ion has a distinctly different R_f from the isoamyl alcohol extracted material. This substance, partially purified, was acidic in nature, and was shown to have a low molecular weight. It had been speculated in the past that the substance was actually citric acid, but the degree of separation from citric acid achieved on this chromatogram should rule that possibility out.

Although the isoamyl alcohol-extracted substance and the G-25 filtrate have essentially the same R_f, the shape and appearance of the two spots including the slight tacking on G-25 are different. This is compatible with the idea that they are different substances. Agar diffusion (disc) assays were performed with citrate, and with the isoamyl alcohol extract at pH 3.0 and at pH 5.0. Results are given in Table 5.

Data given in Table 5 provided strong evidence that the acid-activated antimicrobial substance of Adefarati is not citric acid. It could be an isomer of or related to citric acid. Chemically, the molecule is an acid, and its specific activity against Bacillus No. 4 is considerably higher than that of citrate.

Table 5. Effect of pH of preparations on activity shown in antibiotic disc diffusion assay

Substance	Concentration (mg/ml)	Sample pH	Zone Diameter (mm)
GAC spent culture medium	10	6.8	3
GAC acetone PPT	10	5.6	3
Isoamyl extract of acetone supernatant	10	3.0	23
Isoamyl extract of acetone supernatant	10	5.0	5
Citrate	40	3.0	20
Citrate	40	5.0	0
G-25 gel filtered acetone PPT	10	6.4	4
n-butanol extract of acetone PPT	10	5.0	10
P-10 gel filtered[1]	10	6.0	6

[1]P-10 gel filtered sample was derived from G-25 gel filtered sample.

The zones produced by the lytic antibiotic were very small and greatly undulated at the periphery (zones produced by the acid factor were nearly circular, with entire edges). No hazy secondary zones as described by Champlin (1981) were seen. The small irregular zones produced by the G-25 AP fraction could indicate that the active compound is a rather large molecule which has difficulty diffusing through the agar. One other conclusion is evident from the data in Table 5: the lytic factor produces a much smaller zone in the disc assay than does the acid factor. At a pH of 5.0, the activity of the isoamyl extract produces a zone of only 5 mm in diameter, which, if one extrapolates to higher pH values, would be very small (less than 3 min) at pH 6.8.

To my knowledge, there has been no test of lytic activity of the acetone precipitate component of the M antibiotic, as opposed to zone diameter on a disc agar diffusion assay, as a function of pH. Therefore, the lytic activity of the GAC spent medium acetone precipitate, redissolved in water, was tested for pH dependency of activity by adjusting it to at pH values of 2.8, 7.0, and 9.0. Results are shown in Figure 5.

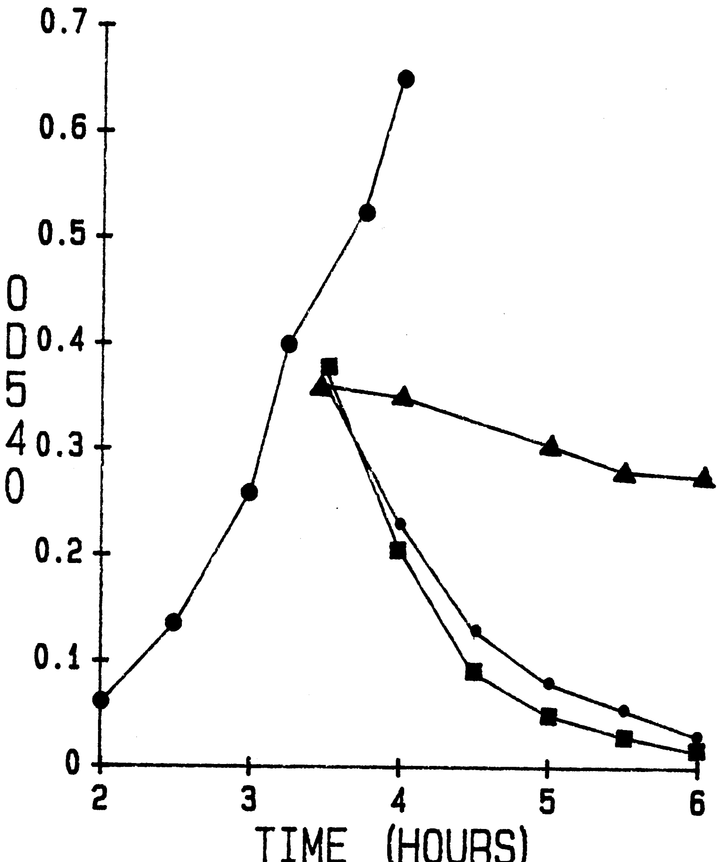
In contrast to the factor in isoamyl alcohol extract, lowering the pH of the medium to 2.8 to 3.0 markedly reduces the lytic activity of the substance. A high pH (9.0) does not affect activity. After lysis had taken place at pH 7.0 and 9.0, the final pH values had decreased to 7.35 and 6.74, respectively.

Purification and Further Characterization of the Lytic Antibiotic

Since the acid component of Champlin's M antibiotic has already been fairly well characterized (Adefarati, 1984), whereas the lytic component had never previously been recognized as a separate entity, we believed that further study and characterization of this substance should be done.

Figure 5. Effect of pH on the lytic activity of the GAC acetone precipitate on growing *Bacillus sp.* in TBS at 37 C.

▲, pH 3; ●, pH 7; ■, pH 9


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD540
2
3
4
5
6
TIME (HOURS)

Since the molecule passes through a G-25 Sephadex column within the void volume, its molecular weight should be fairly high--5000 or above. Sephadex G-25 is a gel used mostly for desalting of crude preparations, since its fractionation range is given as 100 to 5000. In order to further isolate the lytic factor, a longer column, prepared with G-75 was used. The fractionation range of molecular weights is given as 3000 to 50,000.

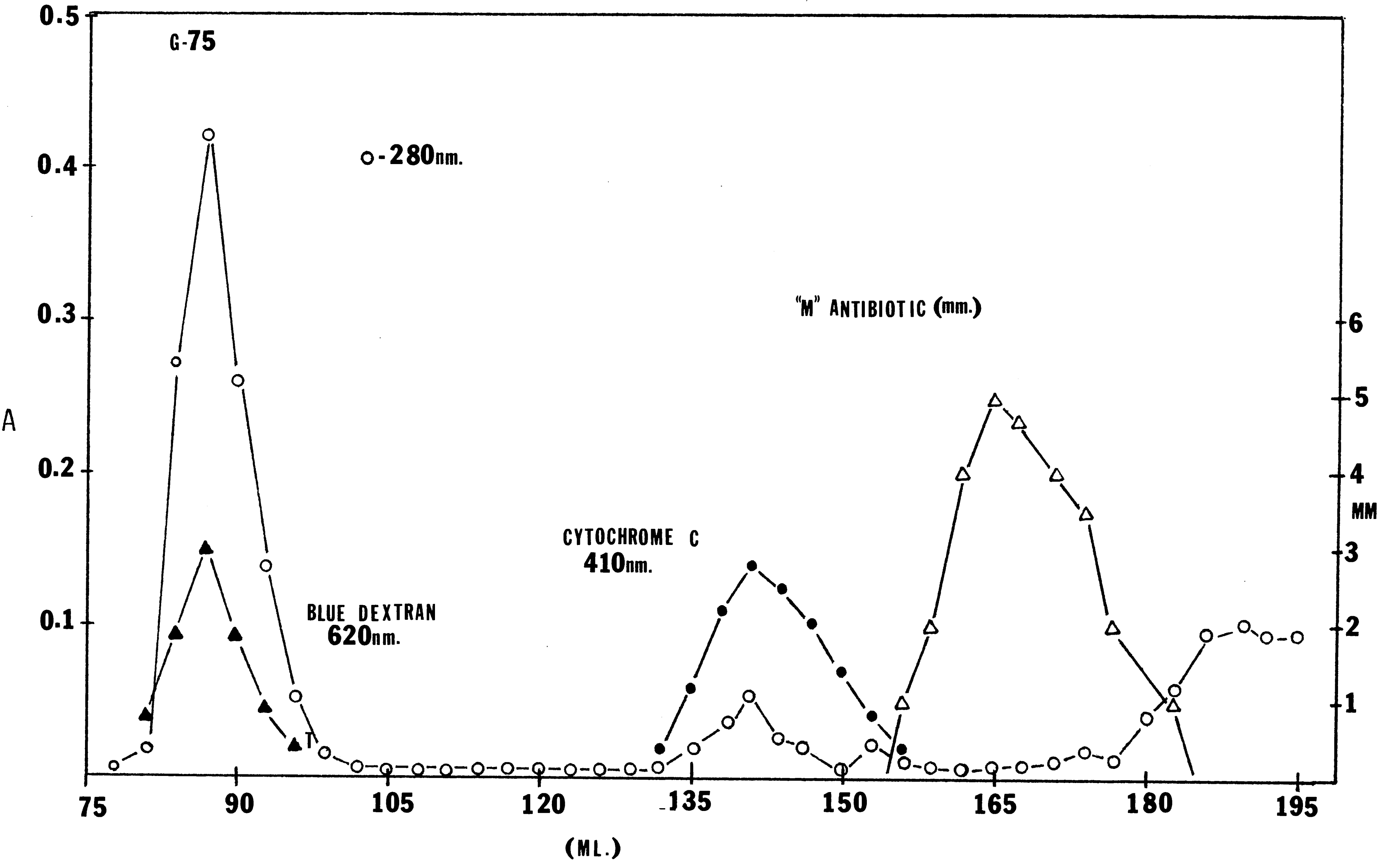
Results of the first fractionation using G-75 are given in Figure 6. The peak of antibiotic activity is seen to occur just beyond the cytochrome C marker, thus indicating a molecular weight for the lytic antibiotic of approximately 12,000 daltons. This figure is an approximation only; to get a more accurate determination one must use a gel with a fractionation range such that the molecule being studied would have a molecular weight close to the mid-range in addition to using several reference substances.

It was not intended to determine molecular weight with any degree of precision, but rather to establish a range wherein it would fall. In addition, it would provide further evidence of differentiation from the factor of Adefarati which had a molecular weight of approximately 198 daltons. Two filtration gels which would fit the specifications are Sephadex G-50 (fractionation range 1,500 to 30,000) and Bio-Rad P-10. Sephadex gels consist of cross-linked dextran chains, and Bio-Rad P gels consist of polyacrylamides. Bio-Rad P-10 was chosen for purification because it would have less interaction with polar carbohydrates than a dextran gel. Since the antibiotic appeared after the cytochrome C, cytochrome C was used as the void volume marker rather than blue dextran. The preparation chosen for filtration using Bio-Rad P-10 was the acetone precipitate of the spent culture medium filtrate.

The active fractions were collected immediately after the cytochrome C had eluted. Immediate testing of each fraction for activity was done using an

Figure 6. Profile of gel filtrated GAC acetone precipitate with Sephadex G-75. Two milliters of a 100 mg/ml sample was added to the top bed and eluted with degassed glass-distilled water. The column attained a 75 ml void volume at a rate of 1.0 ml/min. The markers, blue dextran and cytochrome C were measured spectrophotometrically at 610 nm and 410 nm, respectively. A 280 nm profile was done on all tubes and antibiotic activity was measured by antibiotic diffusion disc assay. All active fractions were bioautographed for migrating activity, collected, and lyophilized.

○, 280 nm; ▲, 620 nm blue dextran;
●, 410 nm cytochrome C; △, M antibiotic


G-75
o-280nm.
BLUE DEXTRAN
620nm.
CYTOCHROME C
410nm.
"M" ANTIBIOTIC (mm.)
A
0.5
0.4
0.3
0.2
0.1
75
90
105
120
135
150
165
180
195
(ML.)
MM.
6
5
4
3
2
1

agar diffusion disc assay. Fractions were pooled and lyophilized. Assay of the lyophilizate, and confirmation of its identity were done at a concentration of 8 mg/ml, using chromatography followed by bioautography. The active factors had an R_f of 0.81, and there was no indication of "origin" activity. This indicates that a certain degree of purity had already been achieved.

The lytic activity of this preparation was tested using a growing culture of Bacillus No. 4. At the same time its activity was compared with that of the acetone precipitate lyophilizate. Results are shown in Figure 7. It is clear that this preparation is quite active lytically, but is not highly more active than the crude acetone precipitate. Both resulted in an A_{540} of essentially zero in one hour (from $A_{540} = 0.2$) whereas the assay using one-half the concentration of P-10 filtrate (4.0 mg/ml) did not achieve complete lysis. The final pH of the medium with the 8 mg/ml P-10 filtrate added was 6.4, and that with the acetone precipitate added was 5.6--a significant difference and an additional indication of a significant degree of purification, since the acid factor of Adefarati is still present in the crude acetone precipitate.

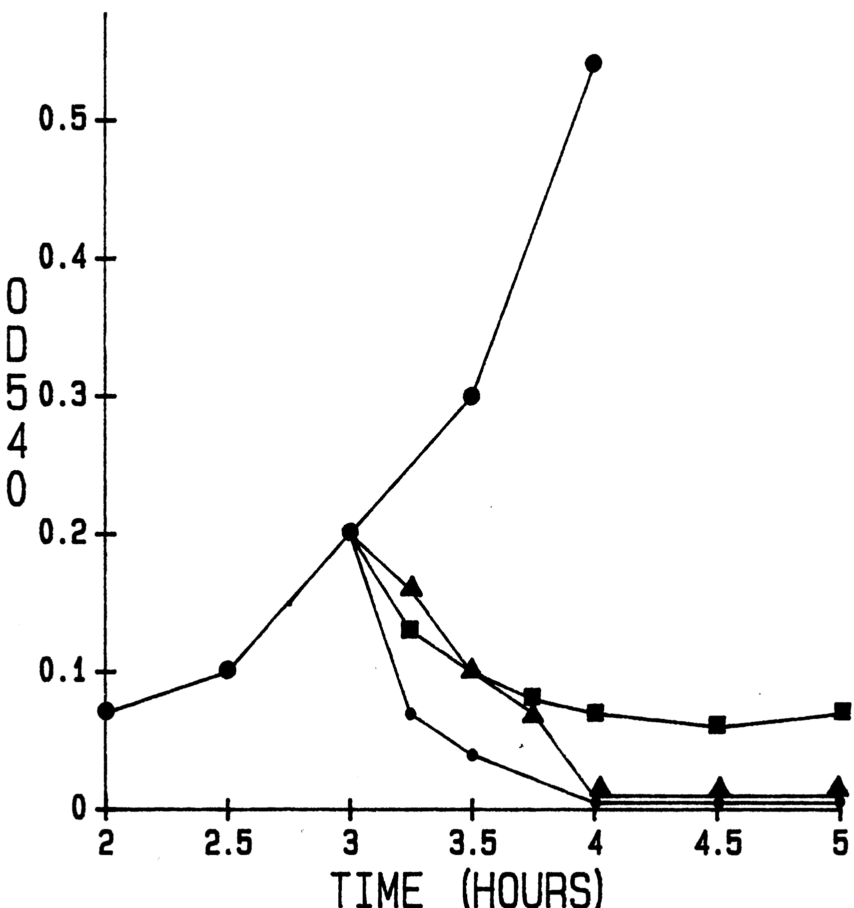
Characterization of Lytic Antibiotic Using Chromatography on Paper and Silica D Paper

Verification of purity and further characterization was done chromatographically using paper and Silica G paper. The relatively pure P-10 fraction was chromatographed as well as some of the earlier (and more crude) preparations, not only to demonstrate purity but also to learn something of the nature of the latter and whether more than one component might have contributed to the activity.

Four preparations, all of which were lytically active (at least to some extent) were tested. In detecting the location (migrating properties) of the

Figure 7. Treatment of growing Bacillus sp. with G-25 active samples gel filtered with Bio-Rad P-10 in TSB. Lyophilized antibiotic samples were extracted with glass-distilled water at a volume of 0.5 ml and added to 4.5 ml of log-phase cultures.

■, 4 mg/ml P-10; •, 8 mg/ml P-10;
▲, 8 mg/ml GAC acetone precipitate;
●, control



OD540
0.5
0.4
0.3
0.2
0.1
0
2
2.5
3
3.5
4
4.5
5
TIME (HOURS)

agents on the chromatogram, it was necessary to do bioautography. To gain information on the chemical nature of the active materials, seven detection reagents were employed, namely, anisidine, p-anisidine phthalic acid, anthrone, and periodate-anisidine for various types of carbohydrates; ninhydrin for compounds containing a free amino group (this could include compounds related to carbohydrates, such as hexoseamines); bromcresol green for organic acids; and vanillin for polyethers.

Only those spots which were shown by bioautography to correspond with the lytic antibiotic (separated at this point from the acidic, low molecular weight factor of Adefarati, which had nearly the same R_f value as the lytic factor) were used for characterization of the molecule. Absorbent paper rather than pure cellulose paper was used for chromatography because the spots with the former were more rounded and compact, probably because of fewer hydrophilic interactions, and consequently better resolution.

Four lytically active samples were tested with each reagent; they were in ascending order of purification: (1) GAC culture filtrate lyophilizate; (2) methanol extract of GAC culture filtrate (pH 2.8); (3) acetone precipitate of GAC culture filtrate; and (4) acetone precipitate of GAC culture filtrate, filtered through Sephadex G-25 followed by Bio-Rad P-10.

These preparations, according to the evidence I have at present, should contain the following: (1) all antimicrobial factors described so far by this laboratory, namely, "origin" and "migrating" antibiotics of Champlin (1981), the latter consisting of a lytic factor, and the strongly acidic compound of Adefarati, related to citric acid; (2) both migrating antibiotics; (3) both migrating antibiotics; and (4) the lytic factor only. The principal rationale for this was to contrast the purity of the P-10 fraction with the other preparations. As expected, the P-10, the acetone precipitate, and the crude culture filtrate each

had a lytically active factor, with an R_f of 0.80, whereas the methanol extract showed a weaker and smaller zone with an R_f of 0.90.* The higher R_f than previously observed may have resulted from a different pH, or the use of a different stationary phase in the chromatography. The P-10 fraction produced the largest and clearest zone of all, while in decreasing order of activity were the acetone precipitate, GAC crude filtrate, and the methanol extract.

The reagent p-anisidine phthalic acid was used to detect hexoses, pentoses, methyl pentoses, and uronic acids (see Figures 8 and 9). The methanol extract showed numerous yellow or green spots, and a small brown spot at the antibiotic zone R_f. This indicates that methanol extraction does not separate the antibiotic from many other similar compounds (which have no antibiotic or lytic activity in the culture filtrate). It would not be expected to; methanol extraction was used by Champlin to separate his "origin" antibiotic from the "migrating" antibiotic. The small size of the spot is indicative of a lower degree of purification.

The acetone precipitate also contained a number of compounds showing yellow or green spots with p-anisidine phthalic acid, but the spot with an R_f at the antibiotic zone (0.80) was a large brown compact spot outlined with a white periphery. The crude filtrate (least pure of the lot) showed a spot at the proper R_f, but with a very light whitish brown color; the lightness of the color was undoubtedly because of the slight amount of antibiotic in the crude preparation.

The P-10 fraction was found to be free of contaminating sugars except for some light brownish color at the solvent front. Only one large brown spot

---

*In Champlin's thesis (p. 80) he states that "The heavy white precipitate obtained in this manner (with acetone) can be washed with methanol ... evaporation of methanol leaves a yellow oily residue...." Adefarati (p. 28) states that "The isoamyl phase was air blown dry, redissolved in about 10 ml glass-distilled water, and lyophilized to dryness. This yielded a yellowish, oily substance which had been found earlier to have relatively high antibiotic activity."

Figure 8. Silica gel paper chromatography of extracted lytic "M" antibiotic and sprayed with p-anisidine-phthalic acid. All depositions were 20 μl of an 80 mg/ml concentration.

1--methanol extraction of GAC acetone precipitate (pH 2.8)
2--GAC acetone precipitate
3--GAC spent filtrate
4--P-10 gel filtrate

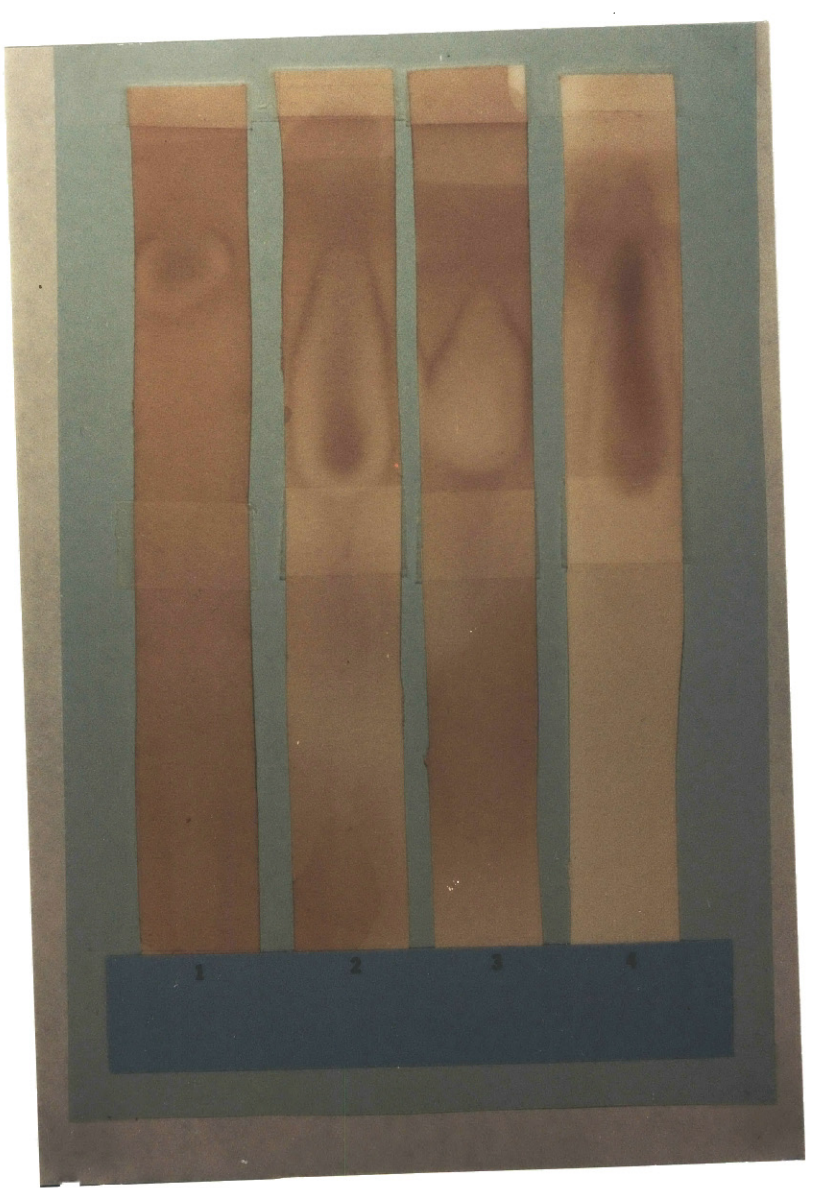

1
2
3
4

Figure 9. Chromatogram on Whatman paper #1 of uronic acid controls, glucuronic acid and glucuronic acid lactone, and P-10 gel filtrate sprayed with p-anisidine-phthalic acid.

1, 2--10 and 5 µl of glucuronic acid (10 mg/ml)
3, 4--10 and 5 µl of glucuronic acid lactone (10 mg/ml)
5, 6, 7--10, 5, and 1 µl of P-10 gel filtrate

1
2
3
4
5
6
7

was present with an R_f of 0.80, again with a narrow white outline at the periphery. Evidently, gel filtration of the acetone precipitate separates the antibiotic from all other sugars that may be in the crude GAC culture filtrate. The brown color of the spot with p-anisidine phthalic acid indicates that a uronic acid moiety, rather than a hexose or a pentose, is present in an unknown linkage on the molecule of the lytic factor.

Since there was some indication from previous experiments that free amino groups may be present, the four preparations were tested with 0.1% ninhydrin in acetone; this should reveal amino sugars, amino uronic acids, or amino acids--any molecule with a free amino group. Free amino sugars combined with ninhydrin result in a reddish-purple color, while amino uronic acids give a light brown color turning purple on standing (Champlin, 1981).

It was observed (see Figure 10) that all the preparations except the methanol extract showed a weak brown color, turning a light purple color at an R_f of 0.80. The P-10 filtered fraction and the acetone precipitate gave similar reactions, while the GAC (crude spent medium) was weakly positive. There was no indication of any other ninhydrin positive spots on the chromatograms.

Bromcresol green is an acid-base indicator which is used to locate organic acids on paper chromatograms. The indicator is yellow on the acid side and blue on the basic side, with a color change interval of 3.6 to 5.2.

At an R_f of 0.80 (see Figure 11), spots with a yellow color surrounded by blue were produced by all preparations except that the methanol extract spot was at an R_f of 0.90. The solvent front turned blue, while the rest of the chromatograms were a neutral green. The blue color at the solvent front appeared in a control chromatogram run in the same solvent system; therefore, there may be impurities in the solvents giving it a pH greater than 6.0. The blue color at the periphery of the yellow spots may be attributed to the same

Figure 10. Chromatogram of glucosamine control and P-10 gel filtered "M" antibiotic dipped in 0.1% ninhydrin.

1, 2--10 and 5 μl of glucosamine (10 mg/ml)
3--P-10 gil filtered "M" antibiotic (20 mg/ml)

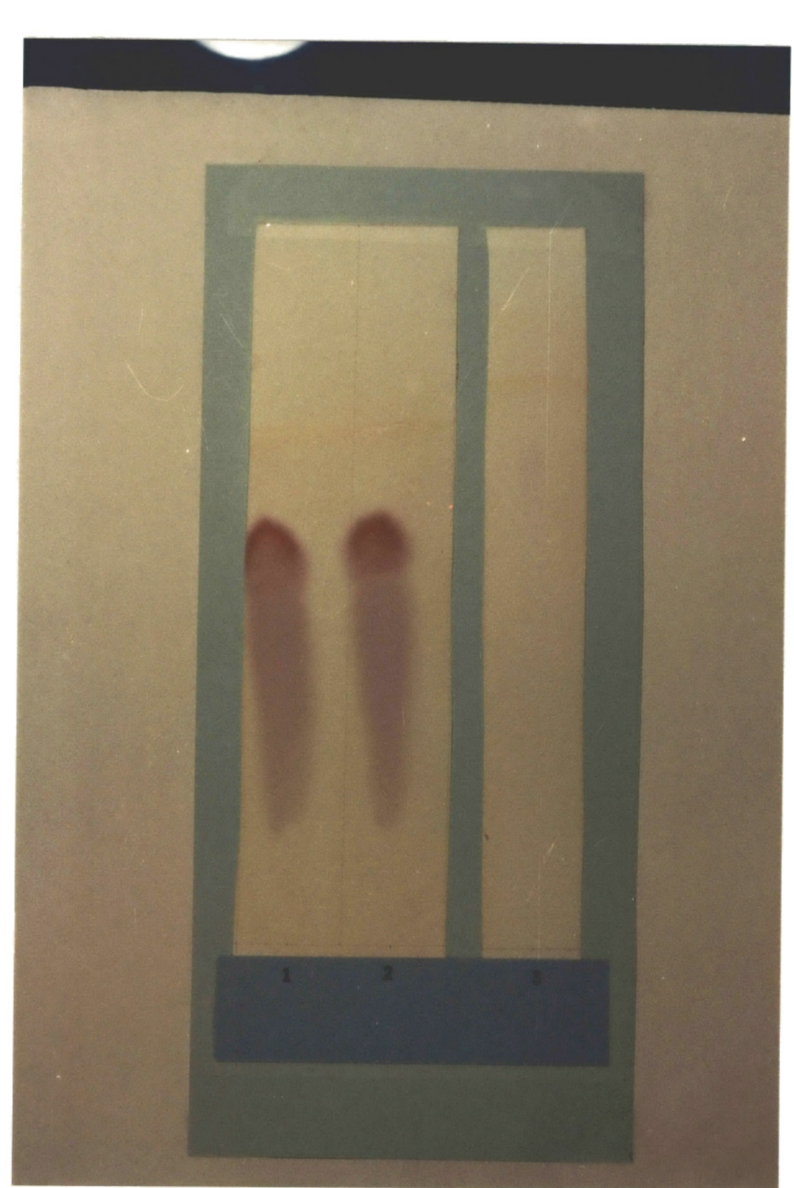

1
2

Figure 11. Silica gel paper chromatograms of GAC extracted "M" antibiotic sprayed with acid and base indicator bromcresol blue.

1--GAC acetone precipitate
2--GAC spent filtrate
3--GAC acetone precipitate extracted with methanol at pH 2.8
4--P-10 gel filtered "M" antibiotic

1
2
3
4

hypothetical substance(s). The reagent for polyethers (tested for because they have a lytic action against growing bacterial cells) proved to be negative with all four preparations.

Specific Tests With Bio-Rad P-10 Filtrate

Since ninhydrin and anisidine phthalic acid indicated that this preparation was relatively pure, additional reagents were used to specifically identify it further, if possible. Since the substance possibly is or may contain a uronic acid, periodate-anisidine was used, which is useful in distinguishing uronic acids from aldonic acids, and in detecting amino sugars. Uronic acids produce a red to brown color, and aldonic acids result in no color are white (distinguishable from background). In turn, amino sugars are brown to yellow. It was found that the spot with an R_f of 0.80, from the Bio-Rad P-10 filtrate turned a very light brown; this is indicative of the possibility of an amino sugar or a uronic acid.

The anthrone reagent spray, which detects ketoses, and oligosaccharides containing ketoses, proved to be negative. The other reagent spray used was anisidine in methanol and n-butanol, with the addition of 1% sodium dithionite. None of the reagents used could confirm the presence of a uronic acid, because of the light color of the spots and dark background produced upon heating (except for p-anisidine phthalic acid). Therefore, Whatman No. 1 paper was tried; it is said to be more resistant to browning than the silicated paper used previously. Additionally, it would be useful to include known compounds similar to suspected compounds in the extracts are seeking.

Three such standards were tested in our system: glucuronic acid (Sigma), glucuronic acid lactone (Sigma), and glucosamine. An amino uronic acid was not available. Depositions of the known standards were made at 1 and 5 μl; and P-10 gel filtrate of the lytic antibiotic depositions were made at 10, 5, and

1 µl. All samples were run in the same solvent system as before and were then sprayed with p-anisidine phthalic acid or ninhydrin. Unfortunately, negative controls were not run.

In contrast to the P-10 preparation on silicated paper chromatograms, the P-10 samples sprayed with p-anisidine phthalic acid on Whatman No. 1 paper were a greenish brown color rather than the dark brown spots seen on silicated paper. The uronic acids (glucuronic acid and glucuronic acid lactone) were a reddish brown. The greenish brown color is indicative of an aldohexose (glucose), a reducing sugar.

The ninhydrin appearance of glucosamine was a dark purplish reaction with a reddish purple tip (indicative of a primary amine, which glucosamine is). The P-10 fraction, however, gave a weak purple reaction which disappeared after about 30 sec. This reaction is typical of a secondary amine. The P-10 fraction was also negative (no reaction indicated) when sprayed with anisidine.

Mode of Action of Lytic Antibiotic

There are at least two classes of antibiotics (based on mode of action) which are capable of lysing (reducing the turbidity of a cell suspension because cells are broken up) growing bacterial cells: (1) those antibiotics which, by various mechanisms, inhibit the synthesis of peptidoglycan--this includes the beta-lactam antibiotics, vancomycin, and others; and (2) the membrane directed antibiotics, whose primary site of action is destruction of the cell membrane. Polymyxin B is one of the latter. Either way, the cell envelope is damaged, cell contents are released, and the cell is broken up and destroyed.

As a preliminary experiment to gain insight into the mode of action of the lytic antibiotic, 1 and 10 µg each of penicillin G, vancomycin, and polymyxin B were added to exponentially growing culture of Bacillus No. 4. Also, a 10-mg

preparation of acetone precipitate was added. Rates of decrease in absorbance were monitored in all. Results are given in Figure 12.

With both concentrations of penicillin and vancomycin, the Bacillus No. 4 cells continued growing for 15 min after addition of the antibiotics, after which the absorbance fell precipitously in both cultures with 10 μg, and in the culture with 1 μg penicillin added. The culture with 1 mg vancomycin added continued growing at the same rate for 15 more min after which its A_{540} fell, but not as rapidly as in the other cultures. A minimum OD of 0.02 to 0.03 was reached in the cultures with 10 μg penicillin or vancomycin. The culture with 1 μg vancomycin may have leveled off after reading an OD of about 0.35.

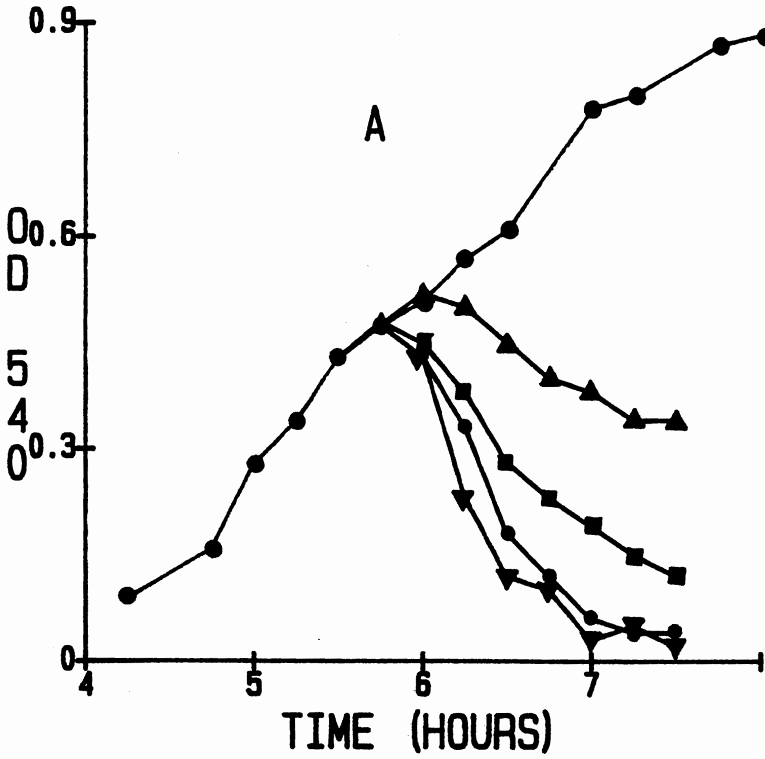
Ten micrograms of polymycin B lowered the OD by about 10% in 15 min, and to zero in about 1 hr. In contrast, 1 μg allowed growth to continue, at almost the same rate as the control, for 45 min before the OD fell to about 0.32. The disparity between the two concentrations of polymycin B seems to be considerably greater than that for penicillin, but of the same order of magnitude as the disparity with vancomycin. That in itself tells nothing about the mode of action, but it would have been interesting to compare the concentration of "acetone precipitate" used in this study with one tenfold higher.

One fact that might be worthy of note is that no impure preparation, obviously with a low concentration of the active factor (quite possibly lower than that of penicillin G, vancomycin, or polymyxcin B) immediately stopped growth, in contrast to the continued growth, at almost the control rate, in the cultures with 1 μg of one of the three known antibiotics.

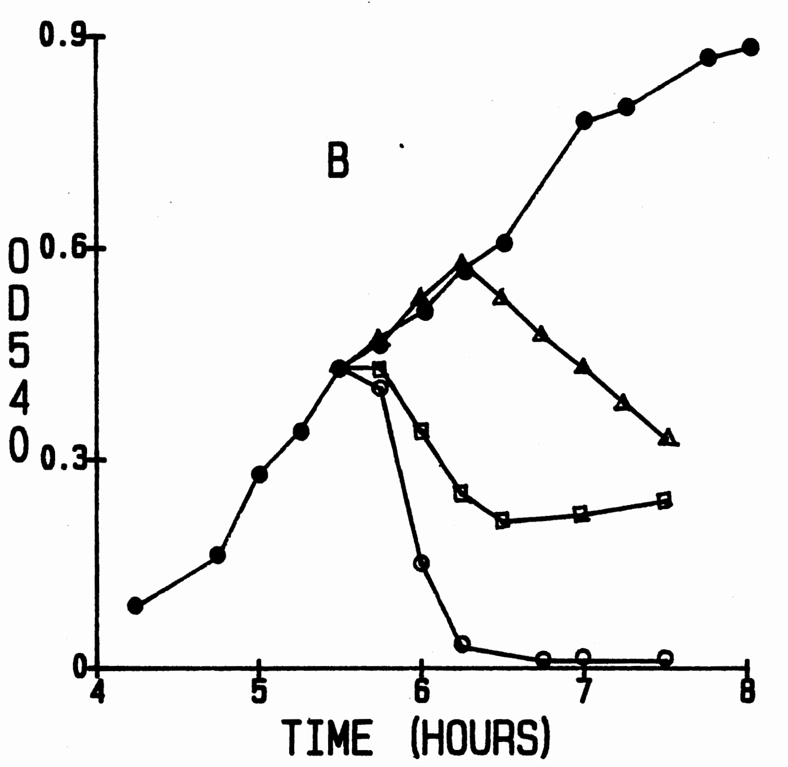
The lay time before lysis, in the cases of penicillin and vancomycin, may be attributed to the time required for these antibiotics to incorporate themselves into the metabolic reactions involved in peptidoglycan synthesis (Tomasz, 1979).

Figure 12. Comparison of the activity of the acetone precipitate to other cell wall and cell membrane antibiotics against growing _Bacillus sp._

- ● , 10.0 μg/ml vancomycin
- ▲ , 1.0 μg/ml vancomycin
- ▼ , 10.0 μg/ml penicillin G
- ■ , 1.0 μg/ml penicillin G
- ○ , 10.0 μg/ml polymyxin B
- △ , 1.0 μg/ml polymyxin B
- □ , 1.0 mg/ml GAC acetone precipitate
- ● , Untreated control



A
OD540
0.9
0.6
0.3
0
4
5
6
7
TIME (HOURS)



B
OD540
0.9
0.6
0.3
0
4
5
6
7
8
TIME (HOURS)

CHAPTER IV

DISCUSSION

It was initially believed that the acid-activated factor (maximum activity around pH 3.0) isolated and characterized by Adefarati, and the M antibiotic of Champlin (1981) were one and the same compound. Adefarati (1983) believed that the supernatant after acetone precipitation of the spent medium contained much more activity (based on disc assay and chromatography-bioautography) than the precipitate. In subsequent work, the precipitate was discarded, and a purification scheme was developed which led to the compound of relatively low molecular weight having acidic properties as revealed by mass spectrometry and and possessing a methyl group. It seemed reasonable that this acidic antimicrobial compound was an isomer of citric acid, since its molecular weight fell in that range. Mass spectrometry showed that it contained six atoms of carbon, eight atoms of hydrogen, and seven atoms of oxygen (Adefarati, 1983), giving it a molecular weight of 192. Before the dual nature of the M antibiotic was noticed, we felt it was important to demonstrate that the acid factor was not citric acid; that is why the experiment on relative toxication of various TCA cycle intermediates, using disc assays, was done. Our results showed conclusively that the factor was not citric acid.

In studies of the effects of this factor on growing and nongrowing cells of Bacillus No. 4, and of Micrococcus lysodeiktecus, Adefarati did not note any lytic activity against either of these organisms. In fact, more spontaneous lysis of

nongrowing cells (with no factor added) occurred than if the low molecular weight acid antibiotic was added. This lack of lytic ability was not given serious consideration, and was not thought to be an essential property of the "M" antibiotic, probably because, at least with Bacillus No. 4, the role of autolytic enzymes in bringing about cell lysis was believed to be critical. It is known that in penicillin-induced lysis, autolysins play a key role (Tomasz, 1979). This author states that: "... lysis of penicillin-treated bacteria seems to be an enzyme-catalyzed process rather than the direct product of mechanical-osmotic pressure on the weakened cell wall."

Tomasz further described some characteristics of beta-lactam antibiotics as a group. Different members of the group, particularly at their minimal inhibitory concentrations (MIC's) were selective, and differed in their physiological effects. Tomasz pointed out that the generally accepted model of the mode of action of penicillin (which usually involves lysis) is in the light of recently acquired experimental evidence, is untenable, and quite oversimplified. One of the reasons for this is that the classical model does not allow for any role of autolytic enzymes.

Therefore, it becomes important to determine if the component of the M antibiotic that has lytic activity is an actual chemical entity, and not just a particular set of conditions that allows the autolytic enzyme(s) of Bacillus No. 4 to be fully active.

It certainly seems that the rapid lysis induced in an exponentially growing culture by the acetone precipitate is the result of, or effect of, a specific chemical compound in the acetone precipitate. This discussion will attempt to consider carefully, within the framework of the exprimental results obtained, the evidence concerning the existence of an actual antibiotic.

Selectivity of morphological and physiological effects induced in E. coli by various beta-lactam antibiotics, particularly at the MIC, has been noted in several laboratories (Greenwood, D., and O'Grady, F., 1973; Greenwood, D., and O'Grady, F., 1973; and Spratt, B. G., 1975). This selectivity is based on the preferential binding of various beta-lactam to different penicillin-binding proteins (PBP's).

Champlin (1981), in his section on Mechanistic Studies, noted that the onset of cell lysis occurred very shortly after treatment of the cells--within 5 min or less. This was done with a unique preparation (a water extract of a lyophilized spent culture filtrate), and therefore contained all antimicrobial factors produced by B. bassiana. A similar rapid lysis was noticed in this project using the acetone precipitate from GAC spent culture filtrate; the acetene precipitation should have separated the lytic factor from the low MW acidic factor. Both of these active principles were found in Champlin's M antibiotic.

The rapid lysis is in marked contrast to the continued growth after addition of penicillin, or vancomycin, or polymyxcin B (two concentrations). In these cases, growth continued for 15 to 45 min at almost the control rate after addition of the antibiotic. This difference in timing of lytic activity may have important implication for the mode of action of the lytic factor.

The morphological changes undergone by the Bacillus sp. when treated with the spent SFB culture filtrate lyophilizate were described in some detail by Champlin (1981). Cells show a rapid loss in phase density, while intracellular granules (volutin) gradually become more distinct. Vacuolization and marked lateral swelling occur prior to cell lysis, at which time the granules are released.

Champlin also described the appearance in the electron microscope; these pictures provided evidence of cell wall degradation (Champlin, 1981). On the other hand, Champlin states that rapid clearing of liquid cultures and increased

cell permeability are consistent with the bacteriocidal activity which would be expected for a membrane-directed surfactant antibiotic. It is of interest that nongrowing Bacillus sp. cultures also showed lysis immediately (within 5 min or less) when treated with the crude preparation. This lack of a requirement for cell growth for activity of the lytic antibiotic is in contrast with the situation with, say, penicillin, another beta-lactam antibiotic. Champlin interprets it as support for the involvement of a membrane-directed antibiotic.

There is also some evidence that one or both of Champlin's antibiotics are acting (primary site) at the level of the cell wall. More likely is the possibility that the disorganized cell membrane (alternative primary site of attack) leads to the activation of one or more constitutive autolytic enzymes (Tomasz, 1979).

Champlin also showed that upon separation of his crude preparation into the M and O antibiotics, the M antibiotic produced rapid lysis of exponentially growing Bacillus cells, but the O antibiotic produced only a slight reaction. Ten µg/ml polymyxin B sent the OD of these cells from 0.25 to 0.05 in just over one hour. The O antibiotic produced slight lysis of nongrowing Bacillus cells, whereas 10 µg/ml polymyxin B resulted in slightly more lysis, and the M antibiotic, still more.

In no cases were intact cell wall "ghosts" observed. After membrane dissolution with the crude preparation, or with the M and O antibiotics alone, one conclusion of Champlin was that the cell wall was in some way being degraded, but that neither of the antibiotics acts directly on the cell wall. Dissolution of the cell wall is a result of the activity of the constitutive autolytic system via a secondary effect of membrane disorganization (the site of attack of the lytic antibiotic) was proposed as the mode of action by Champlin.

What made me suspect that the M antibiotic described by Champlin actually consisted of two distinct and separate factors was the fact that the purest

preparation achieved by Adefarati had no lytic activity. The question arises, then, regarding the confusion about the nature of the M antibiotic. In my opinion, one factor was that activity (as seen on a disc assay) and extractability by various solvents as a function of pH were not adequately characterized. Of course a major factor, and probably the main reason, was the close similarity of R_f values, in the solvent system used for chromatography, of the lytic factor and the acid factor.

We have demonstrated this similarity by chromatographing the lytic factor and the acid factor independently. Since the molecular weights and the chemical nature of the two molecules are quite different, the similarity in R_f values appears to be coincidental.

The molecular weight of the lytic factor has been shown, using two independent approaches (dialysis and gel filtration) to be in the neighborhood of 12,000. A molecular weight of this magnitude indicates the possibility of an oligomeric molecule--one which may contain as many as 100 amino acid residues, or hexose units (polysaccharide complexes with lipids, or with proteins, are a possibility). It cannot be a substance such as a simple sugar or uronic acid.

The Bio-Rad P-10 filtrate probably represents as high a degree of purity as has been achieved for the substance. The various spots seen on chromatograms with this preparation may represent monomers that make up a portion of the lytic antibiotic molecule.

Relative purity of the P-10 filtrate is indicated by the fact that its activity against log-phase cells of Bacillus No. 4 (measured spectrophotometrically) was greater than that of the acetone precipitate, or G-25 filtrate, at the same weight concentration. On the other hand, as indicated by zone size on the agar diffusion assay, the diameter was only slightly greater than in the less pure preparation. The edges showed undulation, and there was no secondary zone.

It may be that the lytic antibiotic does not diffuse well through agar (because of its molecular size), or that it forms strong bonds with paper, hindering its diffusion in the disc assay. Its molecular weight is in a range so that it could be a small protein. Ninhydrin reaction was weakly positive for an aminouronic acid. On cellulose paper, however, ninhydrin did not react with the antibiotic. The possibility that the compound is a cyclic peptide should have been considered. If the molecule is of this type, it could be revealed by hydrolysis followed by paper chromatography in the Heathcote-Jones system.

Acetone precipitation therefore is a good method of separating the two M antibiotics but does not purify the lytic compound. Thus, gel filtration provided a means of fractionating the acetone precipitate by molecular weight. Sephadex G-75 showed that antibiotic activity peaked approximately around a molecular weight of 12000 daltons as indicated by the cytochrome C marker. A 280 mm scan of the antibiotic peaked showed no protein peaks; however, this was reinforced by a Bio-Rad protein assay of active collected fractions. An anthrone test of the active peak which gives total hexose content showed a strong positive test. Therefore, assuming the antibiotic was a large carbohydrate-type antibiotic, Bio-Rad P-10 seemed to be useful since it has a good fractionative range between 5000 to 20000 daltons and resolution was increased by lengthening the column and decreasing the flow rate used by Champlin. The peaks were sharper and paper chromatography indicated no origin antibiotic was present. A 100 mg/ml sample of acetone precipitate yielded around 10 mg of antibiotic on P-10 gel filtration. The sample was hightly active against Bacillus No. 4 at 8 mg and 4 mg/ml but produced only a 4 to 5 mm zone of inhibition on disc assay which was highly undulated. Champlin produced a similar zone with 200 mg/ml of G-25 fractionated GAC.

For an indication of purity and general characterization of the lytic antibiotic, paper chromatography compounded with silica G was used because of less interaction with the more polar cellulose components of paper and the antibiotic. Some tailing of the zone still existed but not to the extent found on paper.

Through numerous reagent sprays, all indications characterize the compound as a uronic acid. Uronic acids are defined as carbohydrate derivatives possessing both aldehyde (or hemiacetals) and carboxyl groups (Theander, 1980). Some of the more common types are D-glucuronic acid, D-mannuronic acid, and D-galacturonic acid, and occur as important building units in many polysaccharides, particularly pectins and alginic acid (Theander, 1980). Some poisonous substances and metabolic products are eliminated in the urine as glucosid uronic acid. The reagents used for uronic acid were anisidine, p-anisidine-phthalic acid, and periodate-anisidine.

The anthrone test of the active peak showed no hexose impurities and the bromcresol blue test for acids and bases indicated the compound was possibly an acid. It is interesting to note that Champlin's ninhydric spray of his butanol extracted preparation was ninhydric negative which further indicates the butanol extraction did not isolate the lytic compound.

Another indication that separates uronic acids from other carbohydrates is that uronic acids, because they can exist in nonionized and ionized forms, will produce tailing or double spotting unless the solvent system is buffered. This may be why a long tailing effect existed with the GAC and acetone precipitate (Theander, 1980).

The ninhydrin reagent indicated that the antibiotic could be an amino uronic acid. Amino acids are not shown to be associated with fungi but uronic acids have been known to be present in cell wall polysaccharides in cell walls of

numerous fungi (Gancedo, 1966; Bartnicki-Garcia, 1968). For some phytopathogenic fungi studied, they have been found to synthesize uronic acids at a relatively high concentration toward the end of the growth phase and produce uronic acids in both the mycelium and culture liquid (Martinez, 1986). These studies were exclusive to those pectinolytic phytopathogenic fungi, which does not include Beauveria, but does show that some fungi are capable of uronic acid extracellular production. There has been no indication of uronic acid production by Beauveria in the literature.

The fact that polymyxin B (a surfactant-type antibiotic) and the lytic factor show similar morphological effects (lateral swelling and vacuolization, possibly due to membrane disorganization) points to possible similarity in mode of action. In both cases, membrane dissolution takes place. No intact cell wall "ghosts" can be seen with the lytic antibiotic, indicating that wall dissociation also takes place.

It is interesting to speculate on just how the misconceptions about the M antibiotic actually arose (they were bolstered by the fact that the R_f values were so similar). The pH of the spent medium (whether adjusted to a certain value or not) is a key factor in determining extractability by various solvents. Lowering the pH to 3.0 or decreasing it naturally to 2.5 (as it does in unbuffered SFB) facilitates extraction of the acid factor with organic solvents (n-butanol, isoamyl alcohol, methanol) since it suppresses ionization of the acid, resulting in a neutral molecule that is more soluble in relatively nonpolar solvents.

This same nonionized molecule is more toxic for susceptible bacteria at pH 3 than at pH 7, because in general only uncharged molecules traverse the cell membrane, and get inside the cell, where their toxicity takes effect. Hence Adefarati observed increasing zone sizes with a lowered pH, an effect he referred to as "acid activation."

Another fact about the lytic antibiotic is that it can be deactivated by a low pH (3.0) as seen in Figure 5. It was first thought that the antibiotics were more active at a lower pH, but that was only true with samples on disc diffusion assay which contained the "acid" factor. This was the reason SDA (pH 5.6) was used for disc diffusion assay rather than a medium or more neutral pH.

I have shown that the lytic factor and the acid-activated antibiotic, in the chromatographic systems used, have essentially the same R_f. The misunderstandings that stemmed from this coincidence provides a warning not to depend upon R_f values alone as evidence that two compounds are the same.

The chemical nature of the lytic factor remains something of a puzzle. The great difference in molecular weight between the lytic factor and the acid factor certainly facilitates their separation. Adefarati unknowingly accomplished this by treating the spent medium at a low pH (3.0). The supernatant retained most of the acid factor (uncharged small molecules escaped acetone precipitation, evidently), and consequently resulted in a large zone of inhibition in the disc assay. It was easy to follow Adefarati's reasoning and experimentation from there, which led to a high degree of purification and characterization of the acid factor.

Early in this work, while still believing that the acid factor was the M antibiotic, I demonstrated that the acid factor produced significantly larger zones of inhibition at a given pH than did citric acid. This has some significance because the question had been raised as to its possible identity with citric acid. We had hoped to test the possibility that its mode of action somehow involved competitive inhibition of an enzyme in the TCA cycle (possibly acotase). Based on mass spectroscopy results, it was concluded that the compound might be an isomer of acetric acid containing a methyl group (Adefarati, 1983).

The lytic factor remains another problem. Because of its molecular weight, it can be separated from the acid factor by dialysis or gel filtration. The purest preparation was after filtration through Bio-Rad P-10. Future workers will be able to produce this factor in this form, after which studies on the molecular structure, the antimicrobial spectrum, and the mode of action against susceptible organisms can be done.

CHAPTER V

SUMMARY

In summary, this investigation of antibacterial compounds produced by a mutant strain of Beauveria bassiana characterizes the purified form of a lytic antibiotic which migrates on paper chromatography within a solvent system of water:n-butanol:isopropanol (65:12.5:22.5).

The lytic compound was precipitated with five volumes of acetone from spent GAC filtrate which had been lowered to pH 3.0. This procedure separated the lytic antibiotic from the other migrating antibiotic which remained in the acetone supernatant. The acetone precipitate was then desalted and separated from larger proteins by passage through G-25 gel filtration and further purified by Bio-Rad P-10 gel filtration. The active fractions on P-10 eluted close to the cytochrome C marker. Therefore, the compound could be between 5000 to 10000 daltons.

On disc diffusion assay, using SDA (12% agar), the lytic compound showed little activity (4 to 5 mm) with characteristic undulated edges around the zone throughout the purification process. This indicated that the compound may have been too large to diffuse freely through the agar and/or the antibiotic was binding to the paper disc. Binding to paper may have also been seen in paper chromatography which revealed spots with long tailing.

Reagent sprays of P-10 samples indicate a fairly pure compound which either is or contains moieties of uronic acid or hexoses. There was a small

amount of free amino acid detected but no protein or polyether was indicated by any of the sprays.

Comparing the mode of action of the lytic compound and other cell wall and membrane-type antibiotic against growing Bacillus sp. found the compound to be similar in activity to polymyxin B. The difference between the two is that polymyxin B produced "ghosts" (release of cellular material from inside the cell) and the lytic antibiotic did not. We can only assume at this time that the lytic antibiotic is activating autolysins within the inner membrane.

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APPENDIX

LYTIC ANTIBIOTIC PURIFICATION

GAC SPENT FILTRATE

|

pH TO 3.0

|

ADD 5 VOLUMES ACETONE

|

ALLOW TO STIR GENTLY 24 HOURS
AT 0-5°C

|

LYOPHILIZE PRECIPITATE
DISCARD SUPERNATANT

|

GEL FILTRATION THROUGH G-25 100mg/ML. (H_2O)
OF THE ACETONE PRECIPITATE

|

SAVE AND POOL ACTIVE FRACTIONS (DISC
DIFFUSION ASSAY)

|

LYOPHILIZE ACTIVE FRACTIONS

|

P-10 GEL FILTRATION

|

POOL ACTIVE FRACTIONS AND LYOPHILIZE

n-BUTANOL EXTRACTION

50ml. SAMPLE OF LYOPHILIZED GAC SPENT FILTRATE

5.0 ml. OF ddH_2O

PLACE IN 50 ml. CONICAL CENTRIFUGE TUBE

ADJUST pH TO 2.5 WITH 3 N HCL

ADD 25.0 ml. OF n-BUTANOL

VORTEX 1 MINUTE

ALLOW PHASES TO SEPARATE

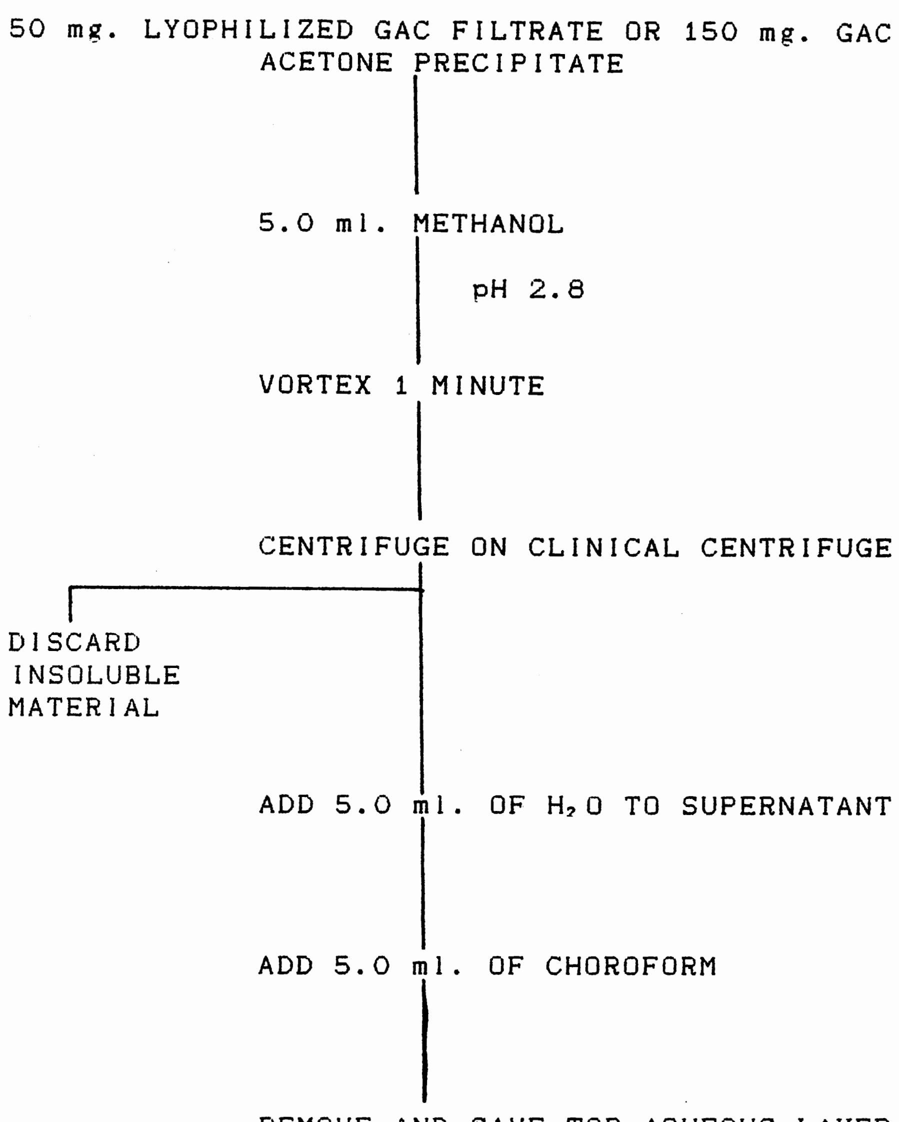
REMOVE n-BUTANOL PHASE

EVAPORATE WITH NEGATIVE PRESSURE TILL 5.0 ml. n-BUTANOL REMAIN

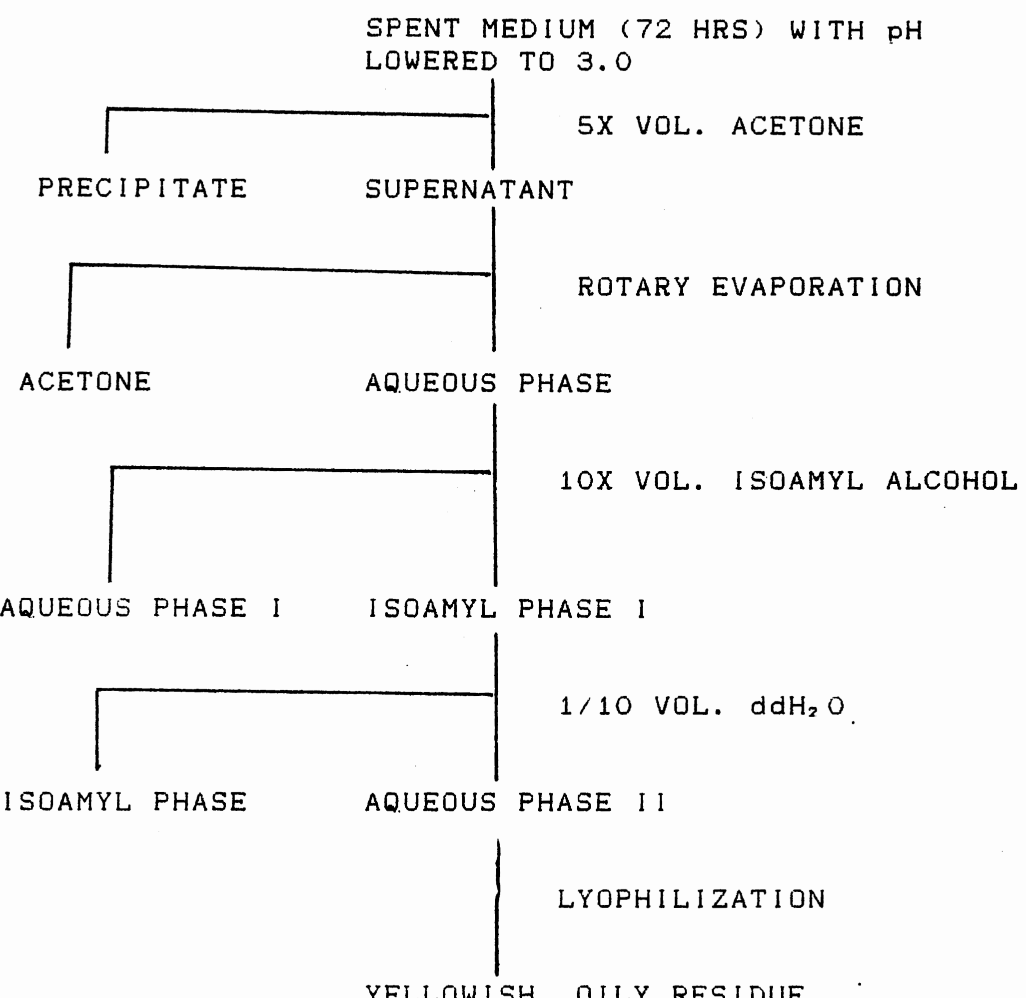
ADD 5.0 ml. ddH_2O

VORTEX 1 MINUTE

SAVE AQUEOUS PHASE


METHANOL FRACTIONATION OF LYTIC ANTIBIOTIC
50 mg. LYOPHILIZED GAC FILTRATE OR 150 mg. GAC ACETONE PRECIPITATE
5.0 ml. METHANOL
pH 2.8
VORTEX 1 MINUTE
CENTRIFUGE ON CLINICAL CENTRIFUGE
DISCARD INSOLUBLE MATERIAL
ADD 5.0 ml. OF H2O TO SUPERNATANT
ADD 5.0 ml. OF CHOROFORM
REMOVE AND SAVE TOP AQUEOUS LAYER

PURIFICATION OF ACID ANITIBIOTIC (ADEFARATI, 1984)



VITA 2

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