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On the Mechanism of Action of the Myxobacterial Fungicide Ambruticin

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The myxobacterial fungicide, ambruticin, kills the yeast, *Hansenula anomala*, with high efficacy (MIC 0.05 μg/ml), but only when the cells are growing. The earliest effect, observed almost immediately after the addition of the antibiotic, is a transient but substantial increase of intracellular glycerol, followed by an accumulation of triacylglycerols and free fatty acids. At about the time when free fatty acids accumulate, the cells become leaky to low molecular weight compounds. We assume that this leakage kills the cells. The mechanism of action of ambruticin thus appears to be the same as that of the phenylpyrroles, *e.g.*, pyrrolnitrin, *viz.*, interference with osmoregulation.

Ambruticin (Fig. 1) is the first antibiotic from myxobacteria for which a chemical structure could be presented[1]. It came from in a strain of *Sorangium cellulosum*, and was isolated and characterized in the laboratories of Warner & Lambert around 1970. Later, ambruticin variants containing a free or methyl substituted amino group instead of an OH-group were discovered in our laboratories at the GBF[2]. They, too, were found in *S. cellulosum* strains, and are distinguished from the original ambruticin, which was then labeled ambruticin S (for slow, referring to its chromatographic behavior) as ambruticins VS (for very slow). Still later, we discovered the jerangolids, again in *S. cellulosum* strains. The jerangolids are shorter versions of ambruticin S[3].

The ambruticins were of interest because of their antifungal effects, which were quite impressive. The *in vitro* inhibitory spectrum and efficacy of ambruticins S and VS-3 were determined in earlier studies[1,4~6]. The two variants behave in many respects identically. They are active against various yeasts, zygomycetes, oomycetes, ascomycetes, and deuteromycetes including important pathogens for plants (*e.g.*, *Botrytis cinerea*), animals and humans. Of particular interest was the good activity against *Trichophyton* and *Microsporum* species, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatididis*, and *Aspergillus* species. On the other hand, *Sporothrix schenckii*, *Cryptococcus neoformans*, *Candida albicans*, and many other fungi turned out to be resistant, so that the effect of the ambruticins is clearly selective. Of the fungi with well established genetics, *Neurospora crassa* is sensitive, while *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are not. *In vitro*, typical MIC values for sensitive strains are between 0.01~0.5 μg/ml. The yeast, *Hansenula anomala*, responds very nicely and was used in this study as the experimental organism. The practical potential of ambruticin S was also assessed in animals. Mice infected with *C. immitis* at an LD_{70} could all be cured with a dose of 50 mg p.o. per kg administered every 8 hours for 2~3 weeks[7]. About 70% of the animals were then free of the pathogen. No macroscopic toxic effects were seen. Development of ambruticin S into a drug was eventually given up, because its activity spectrum, and in particular its low efficacy against *C. albicans*, was deemed too narrow. In the age of HIV infections, this decision may deserve reconsideration.

From the beginning the mechanism of action of the new compound was studied, but without conclusive results[6,8]. SIMPKIN proposed the hypothesis, that in *Candida parapsilosis* ambruticin reduces carbohydrate utilization leading to a decrease in energy production and, as a result, a

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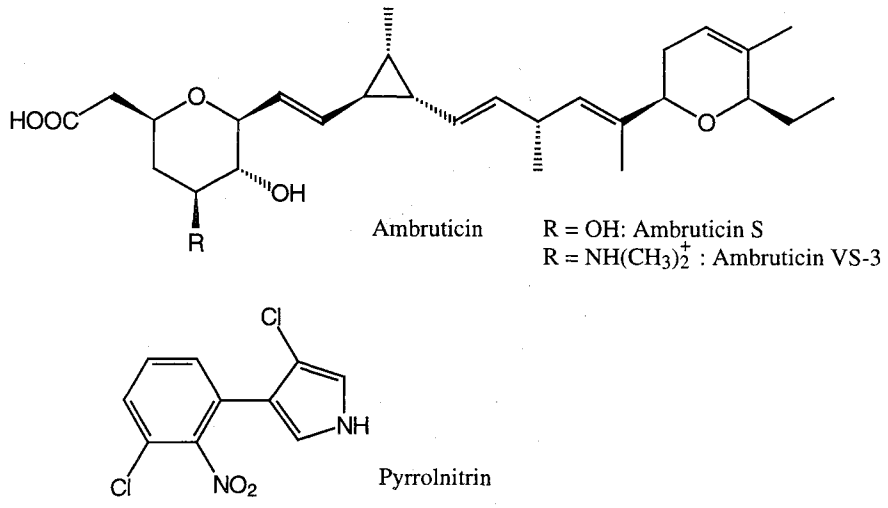
Fig. 1. Chemical structures of ambruticins S and VS-3 and of pyrrolnitrin.



break-down of energy-dependent transport, *e.g.*, of amino acids. When the effects of the jerangolids were investigated, it was discovered that those compounds lead to leakage of treated *H. anomala* cells, and that there was a complete cross-resistance to ambruticin, which was not astonishing, but also a considerable cross-resistance to pyrrolnitrin, a chemically totally different compound[3] (Fig. 1). Unfortunately this did not help much, because the mechanism of action of pyrrolnitrin was also not known at the time. However, recently colleagues at Novartis reported new results on the mechanism of phenylpyrrols[9,10]. Those compounds interfere apparently with the stress-response system and lead to an accumulation of glycerol in sensitive organisms, in this case *N. crassa*. As will be reported below, ambruticin has indeed the same or very similar effects as pyrrolnitrin in *H. anomala* and very likely attacks some enzyme in the complex stress-response network.

Materials and Methods

Experimental Organism and Culture Conditions

The yeast *Hansenula anomala* was grown in Malt extract medium (malt extract, 3%; peptone from casein, tryptically digested, 0.3%) at 30°C on a rotary shaker at 160 rpm. Experiments were usually done in 20~30 ml of Minimal medium (MM) in 100-ml-Erlenmeyer flasks at 30°C and 130 rpm. MM contained in 1 liter of deionized water: 2 g NH_4NO_3, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2 \cdot 6H_2O$, 0.1 g yeast extract (Difco), 1 ml trace element soln[11]; 10 g glucose·H_2O, dissolved in 30 ml deionized water and sterilized by filtration, was added to autoclaved basal medium. Cells grown overnight in Malt extract medium were harvested by centrifugation at 4,500 *g* for 2 minutes, washed twice with sterile deionized water, resuspended to a defined cell density, and transfered into MM. The cultures were preincubated for 1~2 hours before experiments were started. Casamino acid-peptone medium (CAP) consisted of 10 g casamino acids (Difco), 3 g Bacto peptone (Difco), 1 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2 \cdot 6H_2O$, 1 ml trace element soln, in 1 liter of deionized water. All media were sterilized by autoclaving. The pH was not adjusted and was around 5.0.

Inhibitors

Ambruticin VS-3[2] and pyrrolnitrin were dissolved in MeOH. The solns were stored at −20°C and diluted 1 : 10 with autoclaved Tris buffer (20 mM, pH 7.0) before being added to the cultures.

Determination of MIC under Various Conditions

The MIC of ambruticin VS-3 was determined in 96-well microtiter plates with 135 μl of MM per well and 10^6 cells/ml. To this 15 μl ambruticin VS-3 soln in concentrations between 0.005 and 10 μg/ml were added. After 24 hours at 30°C, the optical density (OD) was determined at 620 nm in a microtiter plate reader. To measure the effect of cell density on MIC, the inoculum

was varied between 10^2 and 10^7 cells/ml. The influence of growth phase on MIC was determined by adding ambruticin VS-3 at 0, 2, 4, and 6 hours after inoculation. The influence of pH was shown in MM buffered with 40 mM MES (pH 5.0 and 6.0) or HEPES (pH 7.0 and 8.0).

Determination of Survival and Regeneration of Cultures

Cultures in 20 ml MM with 10^6 cells/ml were kept on an ice bath or at 30°C. Ambruticin VS-3 was added to give different concentrations. After various times, samples were diluted with deionized water and plated on MM agar. Colonies were counted after 2 days at 30°C. In regeneration experiments, 200 μl of the adsorber resin XAD-16 (Rohm & Haas) was added to a 2 ml culture with 0.5 μg/ml ambruticin VS-3. After various times, aliquots were diluted and plated as above. Resistance of clones was tested on MM agar with 0.5 μg/ml ambruticin VS-3.

Determination of Cell Leakage

A 30 ml culture in MM with 2×10^7 cells/ml and 5 μCi [1-^{14}C]-isoaminobutyric acid (specific activity 11.7 mCi/mmol) was incubated for 36 hours at 30°C under shaking at 130 rpm. The cells were then centrifuged down, washed with 20 mM Tris buffer (pH 7.0), and resuspended in 5 ml Tris buffer. 25 ml MM or Tris buffer, respectively, were inoculated with 1 ml cell suspension and incubated for 2 hours, when ambruticin VS-3 was added at various concentrations. One MM culture was put on an ice bath. Release of radioactivity from the cells was shown by centrifuging at various times 500 μl samples at 15,800 g, transferring 250 μl aliquots of the supernatant to 3 ml scintillation liquid, and measuring radioactivity in a liquid scintillation counter.

Production of $^{14}CO_2$ from Labeled Glucose

To 20 ml cultures with 10^7 cells/ml in 50 ml screw cap flasks with a central vial containing a CO_2 absorbing liquid (2-methoxyethanol/ethanolamine 2 : 1, v/v), 3 μCi [U-^{14}C]-glucose (specific activity 293 mCi/mmol) was added. Ambruticin VS-3 was added 2 hours later to give concentrations of 0.05 and 1.0 μg/ml, respectively. Samples of 50 μl were taken from the CO_2-absorbing liquid at various times and added to 3 ml scintillation cocktail. Radioactivity was measured in a liquid scintillation counter.

Glucose Uptake

To a 100 ml culture in MM with only 4 g/liter glucose and 5×10^7 cells/ml, 5 μCi [U-^{14}C]-glucose (specific activity 12.1 mCi/mmol) was added. After 2 hours ambruticin VS-3 was added to give 0.1 μg/ml. Samples of 10 ml were taken 15 minutes before and 1, 15, 30, 60, 120, and 240 minutes after the addition of the antibiotic. The samples were centrifuged at 4,200 g, and 1 ml of the supernatant was frozen at −20°C. Finally, 250 μl samples of the thawed supernatant were used to determine radioactivity as above. An identical experiment was performed in glucose-free CAP medium.

Measurement of Metabolic Activity

Metabolically active cells reduced the dye, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (WST-1) (Boehringer Mannheim) with their dehydrogenases to give a red formazan (absorption maximum 420~480 nm). The wells of 96-well microtiter plates were filled with 180 μl MM containing 10^7 cells/ml. After 1 hour preincubation at 30°C, 20 μl WST-1, and 1 hour later 22 μl ambruticin VS-3 soln (to give 0.1 and 1.0 μg/ml, respectively) were added. Metabolic activity was determined at various times as the absorption difference at 450 nm between sample and control without ambruticin.

Determination of Macromolecular Syntheses

The general procedure was as follows. To 20 ml MM with 10^7 cells/ml, 3 μCi of the respective radioactive precursor, and 1 hour later, ambruticin VS-3 to give 0.01, 0.1 or 1.0 μg/ml, or pyrrolnitrin to 0.5 μg/ml, respectively, were added. At various times, 500 μl samples were transferred to 1 ml 10% (w/v) ice-cold TCA and frozen at −20°C overnight. The thawed samples were passed through glass fiber filters, which were then washed consecutively with 3 ml 10% TCA, 5 ml 0.01 M HCl, and 5 ml deionized water. The dried filters were placed in 3 ml liquid scintillation liquid, and radioactivity was measured in a liquid scintillation counter. For DNA and RNA determination, the cultures also contained 10 mmol unlabeled nucleotides (Ade, Thy, Gua, Cyt or Ade, Ura, Gua, Cyt, respectively). For DNA, the precursor was [8-^{14}C]-adenine (specific activity 54.7 mCi/mmol). The samples were transferred to 0.5 ml 2 M NaOH to hydrolyze RNA. On the following day, DNA was precipitated by adding 1 ml ice-cold 25% (v/v) perchloric acid and filtered as above. For RNA, the precursor was [2-^{14}C]-uracil (specific activity 60.0 mCi/mmol). In this case, only 2 μCi of the precursor was used. For protein, the precursor was [U-^{14}C]-leucine (specific activity 310.8 mCi/mmol). The culture contained 2 mg/ml unlabeled L-leucine. For cell wall glycoproteins, the precursor was [1-^{14}C]-mannose (specific activity 0.3 mCi/mmol). The culture contained 0.05 mg/ml unlabeled mannose and 0.01 mg/ml KH_2PO_4.

Cross-resistance

The MIC of various antifungal antibiotics was determined for *H. anomala* and a spontaneous resistance mutant R^{Amb} in 96-well microtiter plates. Each well contained 150 μl MM with 10^6 cells/ml. The highest concentration of the antibiotic to be tested was added to the first well, which was then diluted in steps of 1 to 2. After 24 hours of incubation at 30°C, the OD was measured at 620 nm.

Determination of Intracellular Glycerol

At various times, 10 ml samples were taken from a 200 ml culture with 5×10^7 cells/ml and 1 μg/ml ambruticin VS-3 and centrifuged at 4,200 *g*. The pellet was washed with deionized water, resuspended in 1 ml deionized water, boiled for 10 minutes, and frozen at −20°C. After thawing, 1 g glass beads (0.5 mm diameter) were added. The samples were vigorously shaken for 5 minutes in a dismembrator and centrifuged at 15,800 *g*. Glycerol was determined from the supernatant by HPLC (Shodex sugar-column with Ionpack KS-801, flow rate 0.75 ml/minute, solvent Milli-Q™ water at 80°C; according to VAN ECK *et al.*[12]). Detection was by refractometry at an Rt of 12.0~12.5 minutes. The glycerol peak could be augmented by external glycerol, and was positive in the glycerol-dehydrogenase (EC 1.1.1.6) test (Sigma). Further, radioactive glycerol was seen in the same peak from the glucose-uptake experiment: During the HPLC run, 188 μl samples were collected every 15 seconds, 100 μl aliquots were transferred to 2 ml scintillation liquid, and radioactivity was determined in a liquid scintillation counter.

Analysis of Cellular Lipids

To a 100 ml culture in MM with 2×10^7 cells/ml and 0.25 μg/ml sodium acetate, 5 μCi [2-^{14}C]-acetate (specific activity 55 mCi/mmol), and 2 hours later ambruticin VS-3 to give 0.1 μg/ml were added. At various times, the cells were harvested from 10 ml samples by centrifugation at 4,200 *g* and resuspended in 2.5 ml MeOH. To this, 2 ml $CHCl_3$ was added, and lipids were extracted under shaking at 150 rpm at room temperature. The organic phase was washed consecutively with 1 ml 1 M NaCl, 3 ml MeOH - 1 M NaCl (5 : 1), and 2.5 ml deionized water, then evaporated in a nitrogen stream. The residue was dissolved in 25 to 50 μl $CHCl_3$. Separation of lipids was done by TLC (HPTLC silica 60 F_{254} ready-made plates, Merck Darmstadt) with heptane - diethyl ether - AcOH (70 : 30 : 1) as the solvent[13]. The lipids were visualized under UV light (254 nm) after spraying with rhodamine B 0.01% (w/v) in EtOH - 1 M NaCl (1 : 1). The Rf of free fatty acids was 0.41~0.47, that of triacylglycerols 0.83. For further identification, the triacylglycerol spot was extracted with $CHCl_3$ and fatty acid methyl esters were produced by adding MeOH - HCl concentrated (10 : 1). Alternatively, the triacylglycerols were saponified with NaOH in ethanol to give the fatty acids and their ethyl esters. The free fatty acids were also esterified with MeOH - HCl concentrated (10 : 1). The lipid derivatives thus created were identified by TLC as above.

Results

Influence of Culture Parameters on MIC. Reversibility of Effect

The MIC of ambruticin VS-3 for *Hansenula anomala* in Minimal medium (MM) was found to be 0.05 μg/ml. The size of the inoculum did not influence the MIC between 10^2~10^6 cells/ml. At 10^7 cells/ml the MIC rose slightly to 0.075 μg/ml. The MIC of ambruticin VS-3 did not change between pH 5 and 8, which is contrary to what was found for ambruticin S whose MIC increased with a rising pH[6,8]. The difference may be due to the amphoteric nature of ambruticin VS-3. Nor was the MIC influenced by the growth phase at which the inhibitor was added (between 0 and 6 hours preincubation).

When liquid cultures exposed to 1 μg/ml ambruticin (20-fold MIC) were diluted and plated, the number of cfu decreased from 10^6 cells/ml by 3 logs within 2 hours (Fig. 2). The cfu decreased further during the following hours and fell below 10^2 cells/ml after 5 hours.

However, a decrease of cfu was seen only when metabolically active cells were treated. Cultures kept at 0°C or cells suspended in Tris buffer retained their ability to form colonies (Fig. 2). The same was observed with *Microsporum fulvum*[6]. *Candida parapsilosis*, on the other hand, was less susceptible when in exponential rather than in stationary phase[8].

The neutral adsorber resin, XAD-16, binds ambruticin efficiently and is used during fermentation to extract the product from the broth. When added (10%, v/v) to cultures of *H. anomala* containing 1 μg/ml ambruticin, it mitigates the decline of cfu. Still, even upon addition after only 15 and 30 minutes of exposure, the cfu count initially declined from 10^6 to 10^4 viable cells/ml. But later, after 1~2 days, the cfu count recovered to the level of the control without ambruticin (Fig. 3). Addition of XAD-16 later than 4 days of treatment resulted no longer in recovery. The control with ambruticin but without XAD-16 remained below 10 cfu/ml for 8 days. The cells in the recovering cultures were fully sensitive to ambruticin: 2×10^8 cells/ml yielded

Fig. 2. Effect of ambruticin VS-3 on survival of *H. anomala* in Minimal medium (MM) with 0.01 μg/ml (○), 0.1 μg/ml (△), 1.0 μg/ml (▽) at 30°C and 1.0 μg/ml at 0°C (◇).

Control without the antibiotic at 30°C (□).

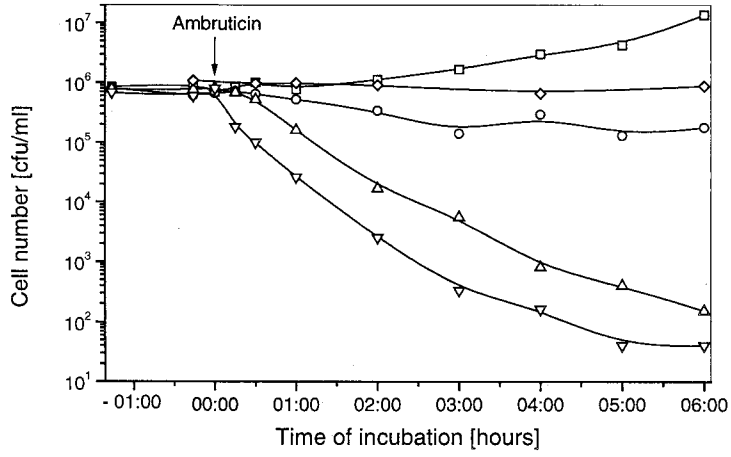
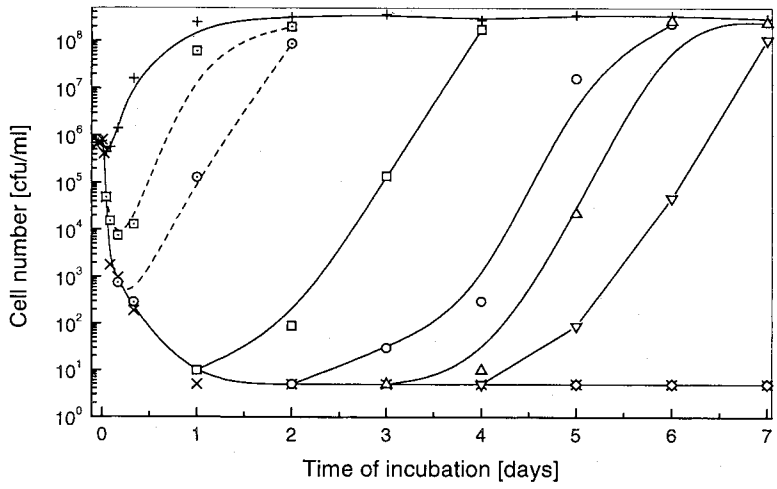


Fig. 3. Recovery of cultures of *H. anomala* in MM with 1.0 μg/ml ambruticin VS-3 upon the addition of the adsorber resin XAD-16 after 1 hour (⊡), 4 hours (⊙), 1 day (□), 2 days (○), 3 days (△), 4 days (▽), 5 days (◇).

Controls without ambruticin (+) and with ambruticin but without XAD-16 (×). Cfu/ml below detection limit (10 cfu/ml) is shown as 5 cfu/ml.



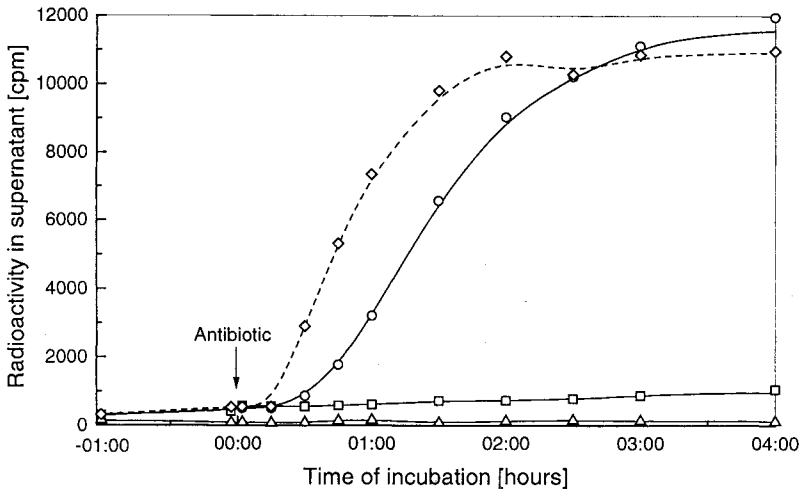
but $0\sim5\times10^2$ colonies on ambruticin agar. The recovering cultures grew probably from cells that were metabolically inactive during the period of ambruticin exposure.

Leakage of Ambruticin-treated Cells

In *M. fulvum*, treatment with ambruticin S did not lead to a leakage of UV-absorbing material over 120 minutes at 32°C[6]. *C. parapsilosis* did not release low molecular weight material during the first 30 minutes after addition of the antibiotic[8]. When we loaded *H. anomala* cells with the non-metabolizable amino acid ^{14}C-isoaminobutyrate, we realized that 0.1 μg/ml ambruticin resulted in a release of radioactivity into the medium beginning, however, only

Fig. 4. Release of ^{14}C-isoaminobutyrate from *H. anomala* after exposure to 0.1 μg/ml ambruticin VS-3 in MM (○), or 1.0 μg/ml in buffer (△), or 30.0 μg/ml ampothericin B in MM (◇).

Control in MM without ambruticin (□). All experiments were done at 30°C.



after 45 minutes (Fig. 4). Cells kept at 0°C (data not shown) or in Tris buffer at 30°C with 1 μg/ml ambruticin, and an ambruticin-resistant mutant at 10 μg/ml ambruticin (data not shown) did not set free radioactivity. The same was seen with 0.5 μg/ml pyrrolnitrin, only slightly delayed with respect to the ambruticin effect. Also in this case, cells in buffer did not release radioactivity (data not shown). In contrast to amphotericin B (30 μg/ml) which induced an almost immediate release of radioactivity, the effect of ambruticin on cell permeability thus appears not to be a primary effect.

Inhibition of Metabolic Activity and Macromolecular Syntheses

Glucose uptake at MIC was normal for 30~60 minutes when it gradually declined (data not shown). Production of CO_2 from glucose stopped 2 hours after the addition of 1 μg/ml ambruticin (20-fold MIC) (Fig. 5). Also, general metabolic activity remained normal for 60~90 minutes with 20-fold MIC. Incorporation of various radioactively labeled precursors into macromolecules, *viz.*, adenine into DNA (Fig. 5), uracil into RNA, leucine into proteins, mannose into cell wall polymers (data not shown) showed a uniform pattern: labeling of the polymers was impeded not before 30~60 minutes after the addition of 1.0 μg/ml of the antibiotic. Thus it may be concluded that ambruticin does not directly interfere with any of those syntheses nor with uptake of the precursors or energy metabolism.

Cross-resistance

Mutants resistant to ambruticin could easily be obtained (about 1 out of 10^8 cells). Those mutants showed cross resistance to jerangolid and pyrrolnitrin[3]. The latter antibiotic is produced by various *Pseudomonas* species[14,15], but also by certain strains of the myxobacteria *Myxococcus fulvus*[16] and *Sorangium cellulosum* (unpublished). Despite the difference in chemical structure (Fig. 1), both compounds act additively, so they probably attack the same target (Table 1). No further cross resistances could be found among 22 other antifungal compounds, like 5-fluorouracil, amphotericin B, nystatin, camptothecen, cycloheximide, miconazole, and soraphen.

Glycerol Accumulation. Changes in Lipid Content

Upon the addition of 0.1 μg/ml ambruticin to growing cultures of *H. anomala*, the intracellular glycerol content rose up to 10-fold within 30 minutes (Fig. 6). In contrast to the effects of ambruticin mentioned above, this was a fast reaction starting immediately upon the addition of the antibiotic. After reaching a maximum between 30 minutes and 1 hour, the glycerol content decreased, and, after 3 hours, became low again. When U-^{14}C-glucose was fed to the culture, the produced glycerol was radioactive, so the

Fig. 5. Effects of 1.0 μg/ml ambruticin VS-3 on metabolic activity of *H. anomala* in MM at 30°C.

Inhibition of DNA-synthesis (○). Release of $^{14}CO_2$ from labeled glucose (▽). Controls without the antibiotic are shown by the dotted lines (□/△).

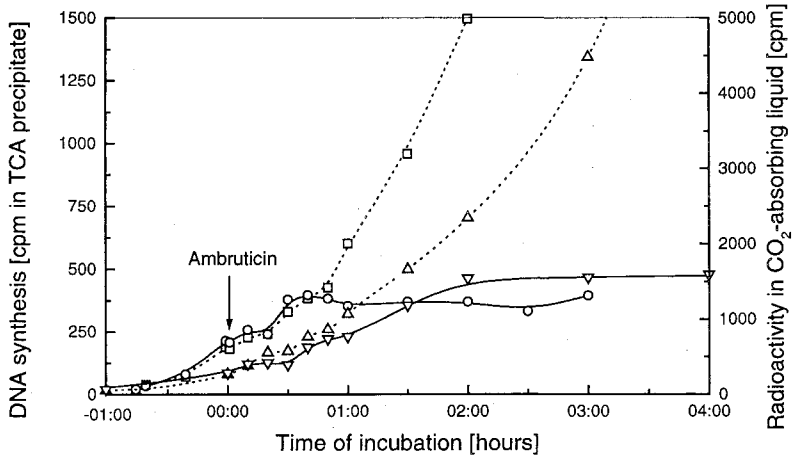


Table 1. Ambruticin VS-3 (Amb) and pyrrolnitrin (Pyr) act additively on *H. anomala*.

Amb [μM]	0	0,04	0,06	**0,10**	*0,20*	0,50
Pyr [μM] 0	0,589	0,607	0,605	0,388	*0,093*	0,046
0,04	0,530	0,573	0,584	0,396	0,105	0,059
0,06	0,570	0,596	0,606	0,272	0,067	0,046
0,10	0,535	0,576	0,534	0,229	0,064	0,049
0,12	0,619	0,627	0,619	0,152	0,064	0,053
0,20	0,560	0,512	0,352	**0,115**	0,054	0,054
0,40	*0,059*	0,065	0,062	0,059	0,069	0,054
0,50	0,100	0,090	0,065	0,058	0,045	0,054

Cell growth is shown as OD_{620} at different combinations of ambruticin and pyrrolnitrin. The MIC of ambruticin was 0.20 μM and that of pyrrolnitrin 0.40 μM, respectively (shown in italic letters), the MIC of the mixture of ambruticin and pyrrolnitrin was 1/2-fold MIC of each (shown in bold letters).

accumulated glycerol was clearly synthesized *de novo*. A similar increase of intracellular glycerol was seen, when 0.1 μg/ml pyrrolnitrin was added to the culture. In this case, however, there was no decline with time. No accumulation of glycerol was observed in the ambruticin-resistant mutant.

With a delay, there also was a change in the cellular lipids (Fig. 7). About 30 minutes upon the addition of ambruticin, the cells started to accumulate triacylglycerols, and at about 60 minutes free fatty acids. The concentration of the free fatty acids reached a maximum at about 3 hours. Similar changes in the lipid pattern were seen with 0.1 μg/ml pyrrolnitrin, although with somewhat lower peaks. The resistant mutant did not show a change in the lipid pattern with 1 μg/ml ambruticin (data not shown). It synthesized slightly higher amounts of triacylglycerols already normally. No changes were seen in sterol biosynthesis.

Discussion

After soraphen, ambruticin is the second fungicide from myxobacteria with a unique mechanism of action. While soraphen blocks specifically and efficiently fungal acetyl-CoA carboxylase[17], ambruticin appears to interfere with the osmoregulation system of susceptible fungi, as do the phenylpyrroles, *e.g.*, pyrrolnitrin, a compound that was discovered in pseudomonads, but is also occasionally found in myxobacteria. The following observations appear to support that assumption. There is a substantial cross-resistance between pyrrolnitrin and ambruticin[3], and both compounds act additively, which suggests a common target. The fastest biochemical response seen in *Hansenula anomala* is an intracellular increase of glycerol, which

Fig. 6. Intracellular accumulation of glycerol by *H. anomala* after the addition of 0.1 μg/ml ambruticin VS-3 (○) or 0.1 μg/ml pyrrolnitrin (□).

Control without antibiotic (□).

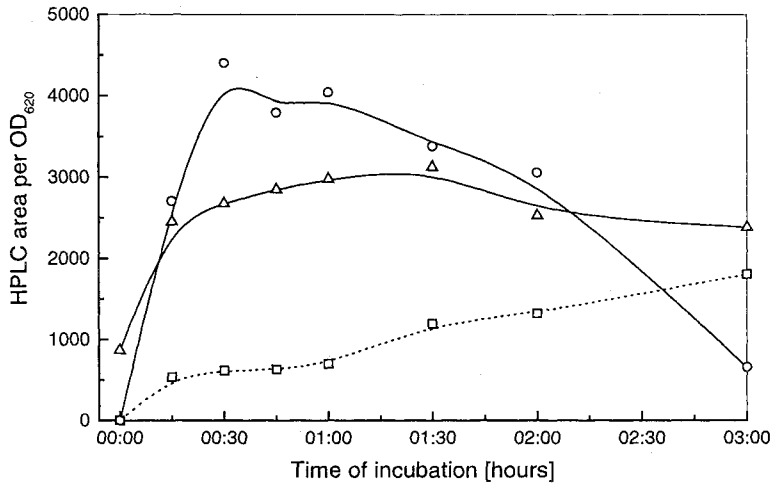
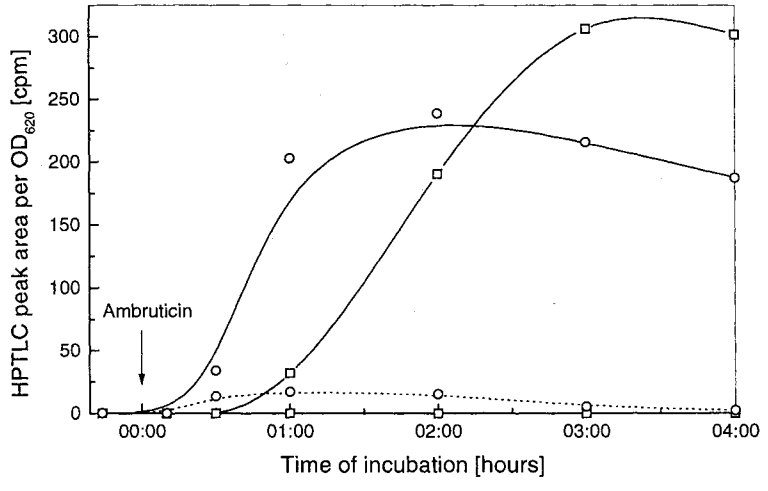


Fig. 7. Intracellular accumulation of triacylglycerols (○) and free fatty acids (□) by *H. anomala* after the addition of 0.1 μg/ml ambruticin VS-3.

Controls without the antibiotic are shown by the dotted lines.



began almost immediately upon the addition of the inhibitor. The glycerol arose by *de novo* synthesis from glucose. How this increased glycerol synthesis is achieved is presently not known. In *Neurospora crassa* apparently a protein kinase involved in stress response is modulated by phenylpyrroles[9,10]. With a time delay of about 30 minutes, in *H. anomala* synthesis of triacylglycerols was much stimulated, and after another 30 minutes free fatty acids appeared in the cells. Similar effects were seen when cultures were treated with pyrrolnitrin. Free fatty acids were virtually absent in untreated cells. Nor did an ambruticin resistant mutant show glycerol accumulation or free fatty acids in the presence of 10-fold higher ambruticin concentrations. By the time free fatty acids accumulated the

cells became leaky for low molecular weight substances, and this is probably what kills the cells. As all these reactions require metabolic activity, it is understandable that resting cells, either in growth medium at 0°C, or suspended in Tris buffer, were not killed by ambruticin. The same fact may explain, why after removal of ambruticin from the culture by the addition of the adsorber resin, XAD-16, the cultures recover up to the fourth day: there may always be a few cells in a culture (around or below 100 in a population of 10^6 cells/ml) that are metabolically inactive and therefore do not respond to ambruticin. The alternative explanations, *viz.*, that ambruticin is not taken up by resting cells or has first to be transformed into an active form by a biochemical reaction, can not strictly be ruled out but appear less likely in the light of the ambruticin effects listed above. A direct interference with the cell membrane, as in the case of the polyene antibiotics, can be ruled out, because the cells became leaky only after some time, and metabolically inactive cells were not sensitive at all. On the other hand, the early effects observed after ambruticin exposure of *Hansenula* are typical stress response reactions in fungi[18]. The inhibitor apparently leads to an overreaction with changes in the plasma membrane that lead to the death of the yeast.

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