The antibacterial activity of alamethicins and zervamicins

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There is considerable evidence (Shaw & Taylor 1986) that many genera of fungi produce peptides of molecular weight about 2000. In some cases the physiological function of these metabolites is known (Sakamoto et al. 1986) but such information is, in general, uncommon. Many of these compounds were discovered because they inhibited the growth of other organisms, but quantitative data on such antibiosis are lacking despite the definition of 'units' in some cases (Upjohn 1969).

Our interest in these compounds arose when it was discovered (Brewer et al. 1972) that they were frequently encountered among metabolic products of Trichoderma sp. isolated from soil of pastures that did not support good performance of grazing ruminants. It was also observed that these compounds affected the physiology of rumen bacteria, for example, their ability to produce volatile fatty acids. For these reasons studies were started on the production of experimental quantities of these interesting compounds, but it was soon discovered that the development of an antibacterial assay to follow their purification was not simple. A systematic investigation of the biological activity of these peptaibols was therefore initiated and the results of this work are reported. A preliminary account of these investigations has been published (Brewer et al. 1979).

Materials and Methods

ORGANISMS

Micrococcus luteus Schroeter (HLX 701, held at the Atlantic Research Laboratory) was maintained and cultivated as described by Brewer et al. (1970). The cellulolytic culture of rumen micro-organisms used in this work has been described (Brewer et al. 1982). It was maintained by weekly transfer on rumen diluent (Kurihara et al. 1968) using rumen fluid that had not been centrifuged, without added soluble sugars, but with a paper strip (Whatman No. 1, 0.7 cm wide) clamped in the bung and dipped 3 cm into the culture medium (Mann 1968). Inocula (0.1 ml) were taken from such 40 h cultures when the paper was just beginning to disintegrate.

Butyribrio fbrisolvens Bryant & Small (strain D1); Megasphaera elsdenii (Gutierrez et al.) Rogosa (B159); Selenomonas ruminantium
BIOASSAY OF PEPTAIBOLS USING *Mic. luteus*

Samples of the antibiotics (1–5 mg, accurately weighed) were dissolved in ethyl alcohol (95%, benzene-free, 0.2 ml) and the solution diluted to 1 ml with sterile sodium bicarbonate solution (1%, w/v). This solution (0.05 ml) was applied to filter paper discs of diameter 1.25 cm (Schleicher & Schuell, Inc., Keene, NH, USA). The discs containing the test solutions were placed on the surface of Nutrient agar (Difco) contained in Petri plates. The test solutions were then allowed to diffuse at 35°C for 24 h, when the plates were overlaid with a suspension (ca 10^5 cells/ml) of *Mic. luteus* in nutrient agar at 45°C (Brewer et al. 1982) such that the overlay covered the agar but did not submerge the paper discs. The cultures were incubated at 35°C for 24 h and the clear zones of inhibition measured (two diameters normal to each other). All test solutions were assayed in duplicate with positive and negative controls. Samples in solution (0.05 ml), e.g. fermentation broths, were applied to the discs directly.

**DETERMINATION OF ANTICELLULOYTIC ACTIVITY OF PEPTAIBOLS**

The antibiotic (1–5 mg) was dissolved in ethyl alcohol (0.2 ml) and the solution diluted to 2 ml with 1% (w/v) sodium bicarbonate solution. Air was displaced from the solution by blowing a stream of nitrogen (oxygen < 0.01%) through the liquid for 10 min. This solution was used to make a two-fold dilution series using degassed water (conductivity < 100) down to the 1:64 level. These solutions (0.5 ml) were passed through a 0.22 μm filter (Millipore) into the rumen diluent (9.5 ml). Paper strips were added to the tubes, carbon dioxide passed into the tubes for 10 min (to degas the paper) and the tubes inoculated with the rumen culture (0.1 ml). The cellulolytic effect was measured visually by the degree of integrity of the paper at 24 h intervals after inoculation. In control cultures the paper below the surface of the medium had completely disappeared 48 h after inoculation.

FERMENTATION EXPERIMENTS WITH AXENIC CULTURES OF RUMEN BACTERIA

The experimental methods used to test the effects of alamethicins on the four species of rumen bacteria were those described by Brewer et al. (1984). Volatile fatty acids and other soluble acid products of glucose fermentation were determined using the method of Salanitro & Muirhead (1975) on the supernatant fluids obtained after centrifugation of 7 d cultures at 17 300 g for 10 min.

**ANTIBIOTICS**

Alamethicins were produced by cultivation of *Trichoderma* spp. The fermentation conditions and isolation procedure have been reported (Brewer et al. 1987). Zervamicins Ia, Ila and IIb were generous gifts from Prof. K.L. Rinehart Jr.

**Results**

**BIOASSAY OF ALAMETHICINS USING *Mic. luteus***

Alamethicins inhibited the growth of 2.5 x 10^4 cells of *Mic. luteus* for 18 h at 25°C when they were added to the culture in the concentration range 10–20 μg/ml. Similar variability was observed in disc diffusion experiments. Thus, when the paper discs were impregnated with 450 μg of alamethicins the mean diameter of the zones of inhibition was 34 ± 7 mm (n = 16). It was found that this variability was due to the time the impregnated discs were in contact with the agar before inoculation, and to the temperature of the agar during diffusion. Experimental results of different diffusion times at different temperatures are summarized in Table 1. The values given are the gradients of linear least squares fits of the relationship between the diameters of the zones of inhibition and the time of diffusion before inoculation. The indices of fit (r²) of the fitted curves lie in the range 0.9–0.95. Better fits were obtained when the data were fitted to curves of the type y = ae^{bx}. Extrapolation of the latter curves revealed that a considerable time was required...
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Table 1. Gradients of linear least mean square fits of the relationship between diffusion time and the diameters of the zones of inhibition of Micrococcus luteus by alamethicins

<table>
<thead>
<tr>
<th>Concentration of alamethicin (μg/disc)</th>
<th>Diffusion temperature (°C)</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>23</td>
<td>$1.3 \times 10^{-4}$</td>
<td>$-4.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>97</td>
<td>0.14</td>
<td>0.24</td>
</tr>
<tr>
<td>450</td>
<td>0.25</td>
<td>0.34</td>
</tr>
</tbody>
</table>

for the systems to come to equilibrium, e.g. the calculated zone diameters for a solution of 97 μg of alamethicin in 0.05 ml in 50 h was 23 mm and in 100 h, 33 mm. Hence the results in Table 1 collected over 48 h are a reasonably reliable indication of the effect of temperature and time of diffusion before inoculation.

An accurate assay, therefore, requires precise specification of these parameters. Clearly the longer the diffusion time, the bigger the inhibition zone and the greater the accuracy; but this is offset by the need for a reasonably rapid analysis, the speed of which can be only marginally increased by raising the temperature (a factor of 2 per 30°C). We have chosen to allow our assay solutions to diffuse for 24 h at 35°C and have found that, for accurate results, this time should not vary by more than ±0.1 h. Under these conditions the index of fit to an equation of the type:

$$\text{zone diameter (in mm)} = 12.5 - 3.9 \times e^{0.139 \times \text{μg alamethicin on disc}}$$

was 0.994 ($n = 308$). This curve is shown in Fig. 1 from which it is clear that solutions of alamethicin in the range 0.1-0.5 mg/ml (i.e. 5-25 μg on the disc) are optimum from the point of view of accuracy of the assay.

INHIBITION OF CELLULOLYSIS BY ALAMETHICINS AND ZERVAMICINS

The values given in Table 2 for the inhibition of cellulolysis by alamethicins and zervamicins refer to concentrations that resulted in no observable digestion of the paper in 7 d. Control cultures grown under the same conditions without antibiotics had completely digested the paper in 48 h. All of the antibiotics had inhibitory effects at 25% of the concentrations given in Table 2. During the course of this work, samples of alamethicins containing different proportions of the component antibiotics (Brewer et al. 1979) have been examined, but the results were very similar to those given in Table 2. Unfortunately, only small quantities of zervamicins IIA and IIB, which differ only in the substitution of a 2-ethylalanine residue for a 2-methylalanine, were available, but the two-fold difference in activity seems to be reproducible.

INHIBITION OF GROWTH OF RUMEN BACTERIA BY ALAMETHICINS

The concentration ranges required to inhibit growth of $2.5 \times 10^7$ cells of the four species of rumen bacteria for 18 h are given in Table 2. At the concentrations given in the table, alamethicins were bacteriostatic with respect to M. elsdenii, S. dextrinosolvens and S. ruminantium in the sense that growth was observed in all of these cultures after incubation for 7 d. In the case of
Table 2. Minimum concentrations of peptaibols that inhibit growth or cellulolysis by rumen bacteria measured at 7 d

<table>
<thead>
<tr>
<th>Organism</th>
<th>Alamethicins (µg/ml)</th>
<th>Zervamicin-Ic (µg/ml)</th>
<th>Zervamicin-IIa (µg/ml)</th>
<th>Zervamicin-IIb (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenomonas ruminantium</td>
<td>&gt; 100</td>
<td>&lt; 200</td>
<td>ND</td>
<td>ND*</td>
</tr>
<tr>
<td>Megasphaera elsdenii</td>
<td>&gt; 60</td>
<td>&lt; 120</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Butyribrio fibrisolvens</td>
<td>&gt; 0.5</td>
<td>&lt; 2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Succinivibrio dextrinosolvens</td>
<td>&gt; 200</td>
<td>&lt; 400</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bacteroides succinogenes†</td>
<td>&gt; 50</td>
<td>&lt; 100</td>
<td>&gt; 40</td>
<td>&lt; 80</td>
</tr>
</tbody>
</table>

* Experiment not done.
† Principal species present.

B. fibrisolvens, a Gram-positive organism, higher concentrations of alamethicins than those given in Table 2 appeared to be bactericidal. Microscopic examination of the inhibited cells of all four species did not reveal abnormal morphology.

EFFECTS OF ALAMETHICINS ON GLUCOSE FERMENTATION BY RUMEN BACTERIA

The changes effected by alamethicins on the acidic fermentation products produced by the four species of rumen bacteria are given in Table 3. The concentrations of alamethicins given in Table 3 were the greatest at which the antibiotics were bacteriostatic, i.e. in all cases growth was observed after incubation for 7 d. The results show that there was a decline in the production of acetate by S. dextrinosolvens (the utilization/production of acetate by S. ruminantium was erratic in our hands) but by contrast the utilization of acetate by M. elsdenii fell from 15 µmol/ml to about 7 µmol/ml. This latter decline was attended by a decline in its production of butyrate, valerate and caproate. Under the fermentation conditions used to generate the data in Table 3, propionic acid is produced by S. ruminantium and B. fibrisolvens but in the presence of alamethicins the former utilized this acid and its production by the latter declined by about 60%.

Discussion

The results of the experiments to determine the rate of diffusion of alamethicins in agar gels can be used to calculate a diffusion coefficient. The relationship (Fig. 1) between the zone diameters and the weight of alamethicin applied to the discs shows that the minimum detectable amount in this system is about 4 µg per disc. The concentration gradient can therefore be found by removing the disc from which the alamethicin has partly diffused and reassaying it. Similarly, measurement of the zone diameters at different times permits calculation of the rate of diffusion of 4 µg of the antibiotics. Thus the

Table 3. Effect of alamethicin on major products of glucose fermentation by four non-cellulolytic rumen bacteria

<table>
<thead>
<tr>
<th>Product change (µmol/ml) of acidic products by</th>
<th>Alamethicin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Selenomonas ruminantium</td>
</tr>
<tr>
<td>Product</td>
<td>0</td>
</tr>
<tr>
<td>Formate</td>
<td>-1.3</td>
</tr>
<tr>
<td>Acetate</td>
<td>-3.0</td>
</tr>
<tr>
<td>Lactate</td>
<td>20.5</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.7</td>
</tr>
<tr>
<td>n-butyratel</td>
<td>-0.5</td>
</tr>
<tr>
<td>n-valerate</td>
<td>-0.8</td>
</tr>
<tr>
<td>n-caproate</td>
<td>2.3</td>
</tr>
</tbody>
</table>

A negative sign indicates a decrease in concentration with growth.
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well-known relationship

\[ ds = -D \cdot A \cdot dc/dx \cdot dt \]

(where \( ds/dt \) is the diffusion rate and \( dc/dx \) is the concentration gradient) can be used to calculate \( D \) in the case where \( A \) is a diffusion boundary of 1 cm². For the results obtained at 35°C the value of \( D \) is about 0.01/cm²/h and this implies from the Stokes/Einstein equation (Glasstone et al. 1941) that the size of the migrating particle is large (Stearn et al. 1940). It is known that in the crystal (Brewer et al. 1979; Fox & Richards 1982) alamethicin molecules are associated and it has been suggested that the mode of this association accounts for their remarkable effects on the conductivity of membranes (Hanke et al. 1983). Practically, an important consequence of this association is the requirement for precise control of diffusion time and temperature in any biological or quasi-biological system under investigation.

The value found for the limiting concentration (under defined conditions) of alamethicin (80 µg/ml) for growth inhibition of Mic. luteus was similar to the concentrations reported by other workers that initiated a 50% haemolysis of erythrocytes (Irmscher & Jung 1977) and for the release of membrane-bound enzymes, e.g. adenylate cyclase from cardiac sarcolemmal vesicles (Besch et al. 1977). It is at least two orders of magnitude greater than the concentration required to induce voltage-dependent conductivity in synthetic lipid bilayers (Hanke et al. 1983). The reasons for this difference are not clear but it has been assumed to be due to the greater complexity of, e.g. the bacterial cell envelope because of the greater sensitivity of Gram-positive bacteria (Argoudelis et al. 1974). Thus the sensitivity (Table 2) of the Gram-positive bacterium \( B. \ fibrisolvens \) is not only in accord with this assumption, but also offers an opportunity to investigate the physiological properties of the membrane of this functionally important component of the rumen flora.

Our earlier report (Brewer et al. 1979) of the inhibition by alamethicins of the production of volatile fatty acids by rumen bacteria has been disputed (Brückner & Graf 1983). It seems likely that these discrepant results are due to differences in both species and numbers of protozoa and bacteria used in the experiments reported, because it is known (Hungate 1966) that this population depends on the diet of the donor animal. The results in Table 3 demonstrate this point for there is a marked reduction in butyrate, valerate and caproate production by \( M. \ elsdenii \) but their formation by \( S. \ dextrinosolvens \) is enhanced in the presence of alamethicins.

References


FOX, R.O. & RICHARDS, F.M. 1982 A voltage-gated ion channel model inferred from the crystal structure of alamethacin at 1.5Å resolution. Nature 300, 325–330.


