Action of Lombazole, an Inhibitor of Fungal Ergosterol Biosynthesis, on *Staphylococcus epidermidis*

DANIEL BARUG,* HELENA B. BASTIAANSE, JOHANNA M. VAN ROSSUM, AND ANTONIUS KERKENAAR

Institute of Applied Chemistry, The Netherlands Organization for Applied Scientific Research, 3700 AC Zeist, The Netherlands

Received 21 February 1986/Accepted 27 May 1986

Lombazole had no effect on respiration at any tested concentration and had little effect on the K⁺ permeability of *Staphylococcus epidermidis*. Of the major metabolic processes investigated in this bacterium, only de novo synthesis of the cell envelope was inhibited by lombazole well in advance of an effect on growth. The time course of inhibition indicated that lombazole exerted its primary effect via inhibition of lipid synthesis; other induced changes, such as reduced synthesis of lipoteichoic acid and cell wall components, were considered to be secondary effects. Although the precise site of action in *S. epidermidis* has to be established, the absence of alterations in lipid patterns after treatment with lombazole suggests the toxicant may affect an essential step in lipid biosynthesis. In *Candida albicans*, lombazole inhibited the sterol C-14 demethylation step in the ergosterol biosynthesis pathway.

The N-substituted imidazole derivative lombazole is an antimicrobial agent used in the treatment of acne (7). Like other imidazoles (5), the compound is active against fungi as well as gram-positive bacteria.

Members of the imidazole series, such as clotrimazole, miconazole, ketoconazole, and bifonazole, have been identified as inhibitors of ergosterol biosynthesis in fungi (6, 20). Since sterols are rare in most bacteria (23), it is doubtful that the antibacterial activity of imidazoles originates from interference with sterol biosynthesis. As yet, the basis for bacterial susceptibility has not been well established.

An ultrastructural study by Barug and de Groot (4) has shown that lombazole primarily disturbs the plasma membrane structure of *Staphylococcus epidermidis*. Biochemical information on the antibacterial mode of action of lombazole is confined to a preliminary report by Barug and Bastiaanse (3). Giving no experimental details, these authors suggested that the toxicant might interfere with lipid biosynthesis.

This paper examines the primary action of lombazole on *S. epidermidis*. The conclusions of the present study could probably be extended to encompass other imidazole derivatives, on the condition that lombazole, too, is an inhibitor of ergosterol biosynthesis in fungi. For this reason, the effect of this toxicant on sterol biosynthesis in *Candida albicans* was studied.

**MATERIALS AND METHODS**

**Microorganisms.** *C. albicans*, *S. epidermidis*, and the other bacterial isolates used have been previously described (1, 2).

**In vitro susceptibility tests.** MICs of lombazole were determined by the agar dilution test as previously described (2) with peptone glucose agar or Diagnostic Sensitivity Test (DST) agar (Oxoid Ltd., London, England). Final concentrations of lombazole (kindly supplied by Bayer AG, Wuppertal, Federal Republic of Germany) were 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 500 μg/ml.

The MIC of lombazole for anaerobically grown *S. epidermidis* was determined in the medium and under the conditions described by Jacobs and Coni (15).

**Growth studies.** Stationary-phase cultures of *S. epidermidis* grown at 37°C in synthetic amino acid (SAA) medium (14) were diluted into fresh, warm SAA medium to an optical density at 535 nm (OD₅₃₅) of 0.1 and grown with shaking to approximately the mid-exponential phase. The cell suspensions were then diluted into fresh, warm SAA medium to an OD₅₃₅ of 0.1 and grown for about 40 min until the OD₅₃₅ was 0.2. At this time, lombazole was added as a solution in acetone (final concentration of acetone, ≤0.05%). The same concentration of solvent was added to controls. Growth was monitored by measuring the OD₅₃₅.

*C. albicans* cells from cultures in the early stationary phase were diluted with fresh, warm Kimmig broth (2) to an OD₅₃₅ of 0.1. The cultures were grown for about 1 h, and lombazole was then added as described above.

**Measurement of K⁺ release.** Essentially the method of Fuller et al. (11) was followed to determine K⁺ release. Exponentially growing cells of *S. epidermidis* were harvested by centrifugation, washed twice with Tris hydrochloride buffer (0.05 M; pH 7.4), resuspended in the same buffer at a density of 5 × 10⁶ CFU/ml, and exposed to lombazole at 1, 5, 10, 50, and 100 μg/ml. After 1 h of incubation at 37°C, cells were removed by filtration on membrane filters (type SM, 0.2 μm; Sartorius, Göttingen, Federal Republic of Germany), and K⁺ in the filtrate (extracellular K⁺) was determined by flame emission spectroscopy at 766.5 nm (Instrumentation Laboratory Video 12 atomic absorption spectrophotometer). Maximal K⁺ release was measured after a cell suspension was heated in a boiling water bath for 30 min.

**Measurement of respiration.** The effect of lombazole on respiration was measured with a Warburg respirometer (type V 85; B. Braun Apparatebauer, Melsungen, Federal Republic of Germany). *S. epidermidis* cells from a culture in the early stationary phase were harvested by centrifugation, washed twice with potassium phosphate buffer (0.3 M; pH 7.0), and resuspended in the same buffer (OD₅₃₅, 1.8). Aliquots (2.0 ml) of the cell suspension were added to the main compartment of Warburg flasks. After equilibration, glucose and
TABLE 1. Antibacterial activity of lombazole

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mediuma</th>
<th>Incubation temp (°C)</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus epidermidis</td>
<td>a</td>
<td>37</td>
<td>5</td>
</tr>
<tr>
<td>&quot;Corynebacterium acnes&quot;</td>
<td>a</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>b</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>Mycobacterium phlei</td>
<td>b</td>
<td>30</td>
<td>0.2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>b</td>
<td>&gt;500</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>b</td>
<td>30</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>b</td>
<td>24</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

a Determined by the agar dilution test.

b a, DST agar (pH 7.4); b, peptone glucose agar (pH 6.9).

Lombazole (final concentrations, 1, 10, 30, and 100 µg/ml) were tipped in from the side arms, and oxygen uptake was measured during a 4-h period at 37°C.

Isotope experiments. [U-13C]glycerol (171 mCi/mmol), N-acetyl-D-[1-14C]glucosamine (57.9 mCi/mmol), [2-14C]thymidine (54 mCi/mmol), [2-14C]uridine (57 mCi/mmol), and [1-U-14C]phenylalanine (509 mCi/mmol) were all obtained from Amersham International, Amersham, England. Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer with either a toluene-based 2,5-diphenyloxazole (POPO) and 5,5’-diphenyl-2,2’-p-phenyleneoxid(oxazole) (POPOP) scintillator solution or Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.).

Measurement of macromolecular syntheses. Macromolecular syntheses in S. epidermidis were measured by the incorporation of isotopically labeled glycerol, N-acetylglucosamine, thymidine, uridine, and phenylalanine into material precipitated by ice-cold 5% (wt/vol) trichloroacetic acid (TCA) by the method of Greenway and Dyke (12). Cells were grown as described for growth studies, except that SAA medium was supplemented with unlabeled precursor (10 µg/ml). 14C-precursor (0.2 µCi/ml) was added approximately 40 min before the addition of solvent (control) or lombazole, and the incorporation of radioactivity was monitored until 90 to 120 min later.

Measurement of initial uptake of macromolecular precursors. Exponentially growing cells of S. epidermidis (OD553, 0.4) were added to flasks containing lombazole (final concentrations, 1 and 5 µg/ml) and the labeled macromolecular precursor to be tested. Initial uptake activity was measured as total cell-associated radiocounts after incubation for 2 to 4 min by the procedure of Chou and Pogell (8).

Glycerol incorporation into lipids and lipoteichoic acid. S. epidermidis cells were grown as described for growth studies and pulse-labeled with [14C]glycerol for 5 min at different times after the addition of solvent (control) or lombazole, followed by rapid cooling. Cells were harvested by filtration on membrane filters (type SM, 0.45 µm; Sartorius) after the pH of the growth medium was adjusted to 2.0 with concentrated HCl (32), washed with ice-cold medium and then with potassium phosphate buffer (0.05 M; pH 7.6), frozen, and freeze-dried.

Lipids were extracted from freeze-dried cells by the method of White and Fremaux (32), which involves acidification of the growth medium before harvest and heating bacteria suspended in isopropanol. Separation of the extracted lipids was conducted by thin-layer chromatography on precoated silica gel 60 F-254 plates (E. Merck AG, Darmstadt, Federal Republic of Germany) with chloroform-methanol-water (70:25:4, vol/vol/vol) and petroleum ether-diethyl ether-acetic acid (82:18:2, vol/vol/vol) for polar and neutral lipids, respectively. Radioactive spots on the chromatograms were detected with a radiochromatogram scanner (Berthold model LB 2723). The Rₘ of the different lipids were compared with those of standards separated with the same solvent systems and visualized with primuline (19).

For determination of lipoteichoic acid, freeze-dried cells were extracted by the procedure of Kessler and Shockman (18), which uses 45% aqueous phenol at 68°C with the addition of 0.01 M Mg²⁺ to the extraction mixture.

Sterol extraction and analysis. For sterol extraction, large-scale incubations of C. albicans were carried out in the absence and presence of lombazole at concentrations of 1 and 5 µg/ml. After treatment for 1 and 2 h, the whole contents (1.5 liters) of the conical flasks (5 liters) were removed for harvesting of the cells. The latter were disrupted with glass beads (0.45 to 0.55 mm in diameter) by the method of Hazen and Cutler (13) and saponified with 16% KOH in 80% methanol under nitrogen (17). After extraction with hexane, the free sterols were separated by gas-liquid chromatography and analyzed by mass spectrometry as previously described (17).

RESULTS

MICs. The action of lombazole on the growth of some gram-positive and gram-negative bacteria is shown in Table 1. In contrast to the latter, the former proved to be susceptible. The MIC for S. epidermidis grown anaerobically in medium with or without hemin was the same as that for aerobically grown cells.

Effect on growth of S. epidermidis. Figure 1 shows the effect of lombazole on the growth of S. epidermidis in shake cultures. At a final concentration of 5 µg/ml, the rate of growth was undisturbed for about 20 min; thereafter, growth became increasingly inhibited.

Effect on K⁺ release from S. epidermidis. Lombazole had very little effect on the K⁺ permeability of S. epidermidis. No extensive loss of cellular K⁺ occurred after treatment with the oxidant. At 1 and 5 µg/ml, lombazole was even without any effect on K⁺ leakage. At the highest concentra-

FIG. 1. Effect of lombazole on the growth of S. epidermidis in SAA medium (initial pH, 6.5) at 37°C. Growth was monitored by measuring the OD553. Solvent (control) or lombazole was added at the time indicated (arrow). Symbols: ○, control; □, lombazole at 1 µg/ml; △, lombazole at 5 µg/ml.
tion tested (100 μg/ml), the loss of cellular K⁺ in 1 h was about 20% of the maximal K⁺ release, compared with 10% in control cells.

**Effect on respiration in S. epidermidis.** Even at the highest concentration tested (100 μg/ml), lombazole did not inhibit the rate of glucose oxidation in S. epidermidis.

**Effect on macromolecular syntheses in S. epidermidis.** The effect of lombazole on the incorporation of the following radioactive precursors into macromolecules of S. epidermidis was studied: cell envelope synthesis, [¹⁴C]glycerol and N-acetyl[¹⁴C]glucosamine; protein synthesis, [¹⁴C]phenylalanine; RNA synthesis, [¹⁴C]uridine; and DNA synthesis, [¹⁴C]thymidine. Incorporation of these precursors was plotted against the increase in the OD₅₃₅ of the bacterial cultures to observe how the effects of lombazole differed from those on growth (12). Results from experiments on the incorporation of glycerol clearly demonstrated lower incorporation during lombazole treatment (Fig. 2A). This occurred within only 10 min of incubation in the presence of the toxicant, well in advance of any effect on growth. Inhibition of the incorporation of N-acetylglucosamine by lombazole would appear to be a later effect (Fig. 2B). Evidently, for similar increases in OD₅₃₅, the effect on the incorporation of glycerol was far more pronounced. Syntheses of protein, RNA, and DNA appeared only significantly affected by the same level of toxicant upon prolonged treatment. Results for DNA synthesis are shown in Fig. 2C.

Repeated experiments substantiated the aforementioned data on the effect of lombazole on macromolecular biosyntheses in S. epidermidis.

**Effect on initial uptake of cell envelope precursors in S. epidermidis.** Since the incorporation of exogenous glycerol and N-acetylglucosamine into the cell envelope of S. epidermidis involved transport into the cells, the effect of lombazole on the initial uptake of these precursors was studied. Initial uptake, defined as total cell-associated counts after cells are separated from the labeled precursors in the incubation medium (8), is generally used as a rough measurement of transport into bacteria.

The initial uptake of glycerol and N-acetylglucosamine in the presence of lombazole at 1 and 5 μg/ml appeared to be identical to that in the absence of the toxicant.

**Effect on lipid and lipoteichoic acid syntheses in S. epidermidis.** The effect of lombazole on biosyntheses of lipids and lipoteichoic acid was studied in S. epidermidis cells pulse-labeled with [¹⁴C]glycerol (Table 2). Of the radioactivity precipitated by TCA, 36 to 42% was located in the lipid fraction and 6 to 9% was located in lipoteichoic acid, which consists of a 1,3-phosphodiester-linked glycerophosphate polymer (26). The amount of lipoteichoic acid may be somewhat low, since the cells were extracted without disintegration (9). The remainder of the radioactive glycerol label may in part be accounted for by glycerol teichoic acid from the cell wall (26).

The incorporation of radioactivity into the total lipid fraction of S. epidermidis was inhibited up to 25% after only 10 min of incubation with lombazole at a concentration of 1

---

**FIG. 2.** Effect of lombazole on macromolecular syntheses in S. epidermidis, as measured by the incorporation of radioactive precursors into TCA-precipitated material. (A) Incorporation of [¹⁴C]glycerol; (B) incorporation of N-acetyl[¹⁴C]glucosamine; (C) incorporation of [¹⁴C]thymidine. Solvent (control) or lombazole (final concentration, 5 μg/ml) was added at the time indicated (arrow). Symbols: ○, control; △, lombazole at 5 μg/ml.
and 24-methylene-24,25-dihydrolanosterol (eburicol), respectively (25). The fragmentation pattern of the minor sterol with a \( t_R \) of 1.36 was consistent with that reported by Ragsdale (25) for 4α,14α-dimethylergosta-8,24(28)-dien-3β-ol (obtusifoliol).

The peak at \( t_R \) 1.29 was found to be composed of two sterols with molecular ion peaks at \( m/z \) 412 and 398. The fragmentation pattern of the first one conformed to that of 4α,14α-dimethyl-5α-cholesta-8,24-dien-3β-ol (4α,14α-dimethylzymosterol) (28). The second one had a mass spectrum identical to that of 14α-methyl-5α-cholesta-8,24-dien-3β-ol (14α-methylzymosterol) (24).

Although somewhat less pronounced, alterations in sterol composition were detected after only 1 h of incubation in the presence of lombazole at 1 and 5 \( \mu g/ml \).

### DISCUSSION

Under anaerobic conditions in the absence or presence of hemin, the MIC of lombazole for \( S. epidermidis \) appeared to be the same as that under aerobic conditions. Since anaerobically grown \( S. epidermidis \) cells require exogenous hemin to form cytochromes (15), these results indicate that the antibacterial activity of lombazole cannot be attributed

![Radioactivity scans](https://example.com/radioactivity_scans.png)

**FIG. 3.** Radioactivity scans of thin-layer chromatograms of lipids extracted from untreated and lombazole-treated \( S. epidermidis \) cells pulse-labeled with \([^{14}C]\)glycerol. The amounts of radioactivity applied to the plates were the same. (A) Control; (B) lombazole at 5 \( \mu g/ml \). Shaded spots correspond to lipids visualized by spraying the plate with primuline after scanning. (1) Phosphatidylglycerol; (2) neutral lipid fraction. F indicates the solvent front.
to an effect on respiratory activity. This was confirmed by
direct respiration measurements.

As strongly suggested by the K⁺ release experiments,
lombazole does not cause direct membrane damage in S.
epidermidis. Imidazole derivatives apparently differ in their
interaction with membranes. For example, in S. aureus,
ketoconazole has been reported to have little effect on the
membrane permeability of K⁺, whereas miconazole, at high
concentrations, does cause an extensive loss of cellular K⁺
(27).

In S. epidermidis, lombazole inhibited de novo cell enve-
lope synthesis, as measured by the incorporation of labeled
glycerol and N-acetylglucosamine. The toxicant exerted its
most pronounced effect on the incorporation of the former
precursor, with which lipids, lipoteichoic acid and, most
probably, cell wall glycerol teichoic acid were found to be
labeled. The time course of inhibition indicates that lipid
biosynthesis is the primary target of lombazole. It was
inhibited very rapidly after the addition of the toxicant to the
cultures and before other effects on bacterial growth and
metabolism were evident. Further analysis of the lipid pat-
terns revealed no effect of lombazole on the quantitative
distribution of glycerol label, suggesting that the toxicant
may block an essential step in lipid biosynthesis.

The greater part of the glycerol label appeared to be
associated with phosphatidylglycerol. This major phospha-
lipid in membranes of staphylococci (26) and of other grampositive bacteria has been shown to be the donor of
glycerophosphate units of the poly(glycerophosphate) moi-
ety of lipoteichoic acids (31). Thus, the primary effect of
lombazole adequately accounts for the reduced synthesis of
lipoteichoic acid in S. epidermidis after prolonged incubation
in the presence of the toxicant. Lipoteichoic acids are found
associated with the membrane in a wide variety of grampositive bacteria. Several important functions, including the
binding of divalent cations, the control of autolytic enzymes,
and the adhesion of bacteria to surfaces, have been ascribed
to these polymers (31).

FIG. 4. Effect of lombazole on the growth of C. albicans in
Kimmig broth (initial pH, 6.8) at 30°C. Growth was monitored by
measuring the OD₅₃₅. Solvent (control) or lombazole was added at
the time indicated (arrow). Symbols: O, control; □, lombazole at 1
µg/ml; ●, lombazole at 5 µg/ml.

Although to a lesser extent than with lipid biosynthesis,
lombazole has also been shown to inhibit the incorporation of
N-acetylglucosamine into, most probably, cell wall compo-
nents of S. epidermidis. Since wall polymers are all
synthesized from intracellular precursors by membrane-bound enzymes, alterations in cell wall synthesis are consid-
ered likely to be secondary effects resulting from the inhibit-
ion of lipid synthesis by the toxicant.

In C. albicans, lombazole inhibited ergosterol biosyn-
thesis. The accumulation of sterols which all retained the
14α-methyl group indicates the inhibition of sterol C-14
demethylation by lombazole in a manner similar to that
found for other imidazole derivatives (20).

In the route preferred by C. albicans from lanosterol to
ergosterol, methylation at C-24 occurs after nuclear
demethylations, with loss of the methyl group at C-14
preceding loss of the methyl groups at C-4. Saturation of the
24(28) double bond occurs immediately after Δ⁵→Δ⁷
isomerization, followed by introduction of the 5(6) and
22(23) double bonds (22). It has been suggested by Fryberg
et al. (10) that methylation at C-24 can precede nuclear demethylation and that saturation of the 24(28) double bond also can occur very early in the pathway. The presence of 24-methylenesterols in lombazole-treated C. albicans cells is in line with the former suggestion. The absence of 4α,14α-dimethyl- and 14α-methylsterols in lombazole-treated cells is in agreement with the effects of miconazole on the release of methyl groups at C-4 can occur before loss of the methyl group at C-14.

The antifungal effects of imidazole derivatives are well documented (20). On the other hand, investigations into the antibacterial mode of action of these compounds are scarce. Van den Bossche et al. (30), who studied the mode of action of miconazole in S. aureus, have suggested that the antibacterial activity may result from interference with the biosynthesis of polyisoprenoids. This was based on the fact that the inhibition of S. aureus by miconazole was associated with a reduced incorporation of radioactivity, derived from 14C-labeled mevalonate, into C-55 isoprenoid alcohol and into K vitamins. It should be stressed, however, that this effect might well be secondary, since it was noted after long incubation (24 h) with miconazole. Interestingly, 1 and 2 h of treatment with lombazole did not produce any adverse effects on the incorporation of labeled mevalonate into S. aureus by miconazole was associated with a change in lipid organization in the membranes, resulting in a change in lipid organization in the membranes, may be part of its antibacterial property. As evidence, they put forward the calculated conformation of miconazole in a dipalmitylophosphatidylcholine monolayer, which is supposed to modify the lipid layer organization. This is in agreement with the effects of miconazole on the release of glucose from unilamellar vesicles composed of egg phosphatidylcholine and on the differential scanning calorimetric spectra of multilamellar vesicles of dipalmitoylcholine. Although these arguments may appear sound, there is a notable inconsistency. In the genus Staphylococcus and in other genera of gram-positive bacteria (16), methyl iso- and anteiso-branched fatty acids are normally the predomi-
nant components of membrane lipids, instead of saturated and monounsaturated straight-chain fatty acids. Since the properties of phospholipids containing branched-chain fatty acids differ from those containing unbranched fatty acids (21), the significance of the evidence mentioned above, in view of the action of miconazole towards staphylococci, is difficult to assess.

ACKNOWLEDGMENTS
We thank G. Versluis for assistance in gas chromatography,

LITERATURE CITED