Microbial Transformation of Methyl 5(6)-Butyl-2-Benzimidazolecarbamate

J. R. VALENTA, C. J. DiCUOLLO, L. R. FARE, J. A. MILLER and J. F. PAGANO
Research and Development Division, Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19101

Received for publication 22 August 1974

The anthelmintic agent methyl 5(6)-butyl-2-benzimidazolecarbamate (Parbendazole) was transformed to two of its animal metabolites, methyl 5(6)-(4-hydroxybutyl)-2-benzimidazolecarbamate and methyl 5(6)-(3-carboxypropyl)-2-benzimidazolecarbamate, by the filamentous fungus Cunninghamella bainieri ATCC 9244. The transformation pathway was shown to be through the 4-hydroxybutyl product to the 3-carboxypropyl product. The reaction favored accumulation of the latter product.

Methyl 5(6)-butyl-2-benzimidazolecarbamate (Parbendazole) is a potent broad-spectrum anthelmintic agent that was discovered in our laboratories in 1967 (1). The metabolism of this agent was studied in farm animals, and several urinary metabolites were observed. To expedite the synthesis of sufficient quantities of these metabolites for toxicity studies, their preparation by microbial transformation of the parent compound was investigated. The isolation of the animal metabolites (C. J. DiCuollo, J. A. Miller, and J. F. Pagano, Abstr. 155th Meet., A27 Sect. N, Amer. Chem. Soc. 1968) and confirmation of structure of the animal and microbial metabolites (3) have been described previously. The present paper describes the microbial transformation studies, the isolation of two of the metabolites, and a proposed microbial transformation pathway.

MATERIALS AND METHODS
Transformation studies. One hundred fungi, including both yeast and filamentous types, and bacteria were examined for their ability to convert methyl 5(6)-butyl-2-benzimidazolecarbamate to its animal metabolites. Transformation studies were carried out in a novel microfermentation apparatus of our own design (Fig. 1) (J. R. Valenta and J. F. Pagano, Bacteriol. Proc., A33, p. 5, 1969). Microbial species selected for use were cultured in 250-ml shake flasks, using 50 ml of medium per flask. Ten milliliters of the vegetative cultures was added to 35 ml of sterile liquid media in 50-ml serum bottles with rubber vacuum stoppers. Bacteria were grown in Trypticase soy broth (BBL), yeasts were grown in Mycophil broth (BBL), and filamentous fungi were grown in Sabouraud broth (BBL) or potato-dextrose (Difco). Fungal cultures were homogenized before use. The bottles of inocula were stored at 4 °C in styrofoam trays. The trays and bottles were numbered to correspond to the microfermentation apparatus units. Each microfermentation apparatus unit held 50-15-ml autoclavable poly-propylene bottles (Fig. 1), which served as microfermentation vessels. Five milliliters of the liquid media that had been used to prepare inocula was dispensed by Cornwall syringe into each beaker. A polyurethane foam pad inside the snap-on screened cover pressure sealed the beakers. A layer of cheese-cloth between the polyurethane foam and the beakers prevented adherence during autoclaving. A template with 3/8-inch (about 0.9 cm) diameter holes numbered to correspond to the beaker positions was placed on the unit. Disposable plastic syringes (2.5 ml; 1.5-inch (about 3.8 cm) needles, 20 gauge for bacteria and yeast and 18 or 13 gauge for homogenized filamentous fungi) were used to inject 0.5 ml of vegetative culture inocula through the pad into the beakers. Inoculated units were incubated on a triple tier gyrotry shaker (New Brunswick Scientific Co., model G-53) at 200 rpm, 26 °C, for 24 to 48 h. The substrate for these studies was [methyl-14C] 5(6)-butyl-2-benzimidazolecarbamate (see Fig. 3I), 17.62 μCi/mg. One milliliter of substrate solution (0.017 mg of 14C-labeled substrate per ml plus 0.483 mg of non-radioactive substrate per ml; total activity, 0.3 μCi/ml; total substrate, 0.5 mg/ml) was added to each 5 ml of vegetative culture in the microfermentation apparatus by syringe. Cultures were incubated for 4 days. Samples were examined by autoradiography after chromatography.

Cell suspension reactions. Duplicate sets of 50-ml vegetative shake flask cultures of microorganisms selected from the transformation studies were grown. The medium and growth conditions for each microorganism were the same as those utilized in the microfermentation apparatus. After incubation, the cells were harvested from each of the cultures in one set, washed, and then resuspended in 50 ml of sterile 1.5% glucose solution. The pH of each cell suspension was adjusted to that of its corresponding vegetative broth culture. Twenty-five milligrams of non-radioactive methyl 5(6)-butyl-2-benzimidazolecarbamate, 10 mg/ml in water as a fine suspension, was added to the
broth cultures and cell suspensions. Reaction mixtures were returned to the shaker and incubated for 3 days. They were clarified by centrifugation before chromatographic analysis. The transformation products observed were confirmed as being chromatographically the same as those observed in the 14C-labeled substrate study by mixed-spot analysis utilizing each of the detection methods described.

The effect of pH on the transformation of methyl 5(6)-butyl-2-benzimidazolocarbamate by one of these microorganisms, Cunninghamella bainieri ATCC 9244, was studied in 50-ml mycelial suspensions prepared from 4-day-old vegetative shake flask cultures. The substrate concentration and incubation conditions already described were used. The reactions were initially adjusted, at 1-unit increments, over the pH range 3.0 to 7.0. They were examined periodically during incubation, and the pH was maintained at the original value by adding acid or base as required.

Analysis. After incubation, the reaction mixtures were adjusted to pH 6.0 and extracted with an equal volume of chloroform. In some cases reaction mixtures were clarified before extraction. Extracts of 14C-labeled substrate reactions were allowed to air dry, whereas extracts of nonradioactive substrate reactions were evaporated to dryness in vacuo at 30 C. The residues were examined by chromatographic methods. Thin-layer chromatography was carried out on silica gel plates (Eastman Chromagram no. 6061), which were developed in chloroform-methanol-water (70:20:2, vol/vol). 14C-transformation products were detected as dark spots on developed X-ray film that had been exposed to chromatograms for 4.5 days. Nonradioactive drug and transformation products were detected as dark-blue spots on a pale blue-white background when plates were exposed to vapor from 5.25% sodium hypochlorite solution (Clorox) and then sprayed with 1% potassium iodide-2% soluble starch solution. Both detection systems were used when radioactive and nonradioactive products were subjected to mixed-spot analysis.

Transformation of methyl 5(6)-butyl-2-benzimidazolocarbamate. Cunninghamella bainieri ATCC 9244 was grown in submerged culture in a medium containing 2% dehydrated potato extract (Difco) and 2% glucose. The medium was adjusted to pH 6.5 before autoclaving. Nine liters of medium was inoculated with 1 liter of a homogenized vegetative shake flask culture of C. bainieri ATCC 9244. Incubation conditions in the fermenter (New Brunswick Scientific Co., model FS-314) were 200 rpm, 30 C, and aeration at the rate of 1 volume of air/volume of medium per min. After incubation for 48 h, the cells were harvested from the fermentation by continuous-flow centrifugation, washed with water to remove residual medium, and resuspended in 9.5 liters of 1.5% sterile glucose solution. The mycelial suspension was returned to the fermenter and adjusted to pH 6.0. Incubation conditions were the same as those used for growth. Five grams of methyl 5(6)-butyl-2-benzimidazolocarbamate in 0.5 liters of 1.5% glucose solution, as a fine suspension, was added just below the surface of the mycelial suspension. The mycelial suspension was agitated during substrate addition. The final concentration of substrate in the reaction mixture was 0.5 mg/ml. The pH of the reaction mixture was maintained at 5.5 to 6.0 by means of an automatic pH controller (model pH-161, New Brunswick Scientific Co.). Incubation was terminated when chromatographic analysis indicated an acceptable concentration of the desired transformation products. The reaction mixture was clarified by continuous-flow centrifugation, and the mycelia and insoluble products were washed with water. The water wash was added to the centrifugate solution, and the resultant solution was concentrated in vacuo at 40 C to 1.5 liters. The concentrate was adjusted to pH 9.0 with 1 N NaOH and exhaustively extracted with chloroform. It was then adjusted to pH 5.0 with 2 N HCl, and the extraction process was repeated. Each of the extraction lots was dried over anhydrous sodium sulfate and evaporated in vacuo at 30 C to a minimal volume. The transformation products crystallized slowly from the extract concentrates when cooled in the refrigerator. A small sample of a minor microbial transformation product was isolated from the pH 9.0 chloroform extract [Fig. 2(II) and 3(III)] by preparative thin-layer chromatography. The major microbial transformation product from the pH 5.0 chloroform extract [Fig. 2(III) and 3(III)] was recrystallized from hot 95% ethanol.
Transformation pathway. The pathway of transformation of methyl 5(6)-butyl-2-benzimidazolecarbamate by C. bainieri ATCC 9244 to the two observed products was studied. Each of the products was added both separately and combined to 25-ml mycelial suspensions prepared from 4-day-old vegetative shake flask cultures grown in potato extract-dextrose medium. The final concentration of each was 0.1 mg/ml in both cases. Incubation conditions and analyses of the reaction mixtures were carried out as previously described.

RESULTS AND DISCUSSION

Methyl 5(6)-butyl-2-benzimidazolecarbamate (Parbendazole) was transformed by a variety of the microorganisms examined, including bacteria, yeasts, and filamentous fungi. Although many chromatographically different transformation products were observed, only two of the animal urinary metabolites were produced. The filamentous fungus C. bainieri ATCC 9244, one of the several microbial species producing both animal metabolites, accumulated these products in reasonably good yields in the absence of other detectable transformation products (Fig. 2). C. bainieri ATCC 9244 was selected for further study. Transformation of methyl 5(6)-butyl-2-benzimidazolecarbamate by C. bainieri ATCC 9244 was optimal at pH 5.0 to 6.0. The ratio of the two products, however, was relatively constant over the entire pH range studied.

A scaled-up transformation of 5 g of methyl 5(6)-butyl-2-benzimidazolecarbamate by C. bainieri ATCC 9244 yielded 418 mg of crystalline material (pH 5.0 chloroform extracts), which was identified as methyl 5(6)-(3-carboxypropyl)-2-benzimidazolecarbamate [Fig. 3(III)]. The structure of this product was confirmed by synthesis (3). The pH 9.0 chloroform extract yielded 300 mg of material, which consisted of parent and two transformation products. Preparative thin-layer chromatography of this material yielded a small sample of the second transformation product. It was identified as methyl 5(6)-(4-hydroxybutyl)-2-benzimidazolecarbamate [Fig. 3(II)] by comparison with an authentic sample (3). No effort was made to quantitate or recover the remaining II or III from the pH 9.0 chloroform extract. The relative insolubility of methyl 5(6)-butyl-2-benzimidazolecarbamate in water at pH 5.0 to 6.0 suggested that only a part of that added was available for transformation in reaction mixtures. Detectable amounts of substrate were found in the solids recovered by centrifugation of the reaction mixtures. Thus it was not possible to determine precisely the reaction yields.

The earlier observation that ratios of the two products in reaction mixtures of C. bainieri ATCC 9244 remained relatively constant, even at suboptimal pH (below pH 5.0 and above pH 6.0), suggested that a sequential transformation pathway involving both products might be operating.

Consideration of known microbial transformations suggested a possible pathway. Methyl 5(6)-butyl-2-benzimidazolecarbamate (I) is converted by C. bainieri ATCC 9244 to methyl 5(6)-(4-hydroxybutyl)benzimidazolecarbamate (II), which is then further oxidized to methyl 5(6)-(3-carboxypropyl)-2-benzimidazolecarbamate...
Numerous examples of substitution of a hydroxyl group for a hydrogen on a terminal methyl group have been reported among steroid transformations. Similar examples are known for conversion of a terminal alcohol group to a carboxyl. For example, \textit{Methanobacterium omelianskii} oxidizes butanol to butyric acid in 97% yield (2). The proposed transformation pathway was consistent with subsequent experimental data. Methyl 5(6)-(4-hydroxybutyl)-2-benzimidazolecarbamate (II) was transformed to methyl 5(6)-(3-carboxypropyl)-2-benzimidazolecarbamate (III) by \textit{C. bainieri} ATCC 9244, whereas the latter was essentially unaffected. When a mixture of the two products was treated in the same way, the concentration of the 3-carboxypropyl product increased as the concentration of the 4-hydroxybutyl product diminished.

\textbf{ACKNOWLEDGMENTS}

We are grateful to our colleagues at Smith Kline & French Laboratories: D. Blackburn and his associates in the Radiosynthesis Laboratory for \([\text{methyl}^{13}C]5(6)-\text{butyl}-2\)-benzimidazolecarbamate; and G. Dunn and his associates in the Medicinal Chemistry Department for confirmation of metabolite structure by chemical synthesis.

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