ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1999, p. 582–588 Vol. 43, No. 3
0066-4804/99/$04.00+0

Antimicrobial Activities of Synthetic Bismuth Compounds against *Clostridium difficile*

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Received 9 June 1998/Returned for modification 30 September 1998/Accepted 17 December 1998

Clostridium difficile **is a major nosocomial pathogen responsible for pseudomembranous colitis and many cases of antibiotic-associated diarrhea. Because of potential relapse of disease with current antimicrobial therapy protocols, there is a need for additional and/or alternative antimicrobial agents for the treatment of disease caused by *C. difficile*. We have synthesized a systematic series of 14 structurally simple bismuth compounds and assessed their biological activities against *C. difficile* and four other gastrointestinal species, including *Helicobacter pylori*. Here, we report on the activities of six compounds that exhibit antibacterial activities against *C. difficile*, and some of the compounds have MICs of less than 1 μg/ml. Also tested, for comparison, were the activities of bismuth subcitrate and ranitidine bismuth citrate obtained from commercial sources. *C. difficile* and *H. pylori* were more sensitive both to the synthetic bismuth compounds and to the commercial products than were *Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*, and the last three species were markedly resistant to the commercial bismuth salts. Testing with human foreskin fibroblast cells revealed that some of the synthetic compounds were more cytotoxic than others. Killing curves for *C. difficile* treated with the more active compounds revealed rapid death, and electron microscopy showed that the bismuth of these compounds was rapidly incorporated by *C. difficile*. Energy dispersive spectroscopy X-ray microanalysis of *C. difficile* cells containing electron-dense material confirmed the presence of internalized bismuth. Internalized bismuth was not observed in *C. difficile* treated with synthetic bismuth compounds that lacked antimicrobial activity, which suggests that the uptake of the metal is required for killing activity. The nature of the carrier would seem to determine whether bismuth is transported into susceptible bacteria like *C. difficile*.**

In the majority of patients, predisposition to infection with *Clostridium difficile* is induced by the disruption of the normal intestinal flora by antibiotic therapy (5). While vancomycin and metronidazole treatment of antibiotic-associated diarrhea and pseudomembranous colitis caused by this bacterium is effective, relapses do occur in up to 20% of patients, and these patients require further antimicrobial therapy (19). In view of such difficulties, other approaches to treatment and cure have been sought, such as biotherapy (18). There clearly is a need to develop new treatments for this potentially life-threatening disease.

Bismuth compounds have proven utility as fungicides and antitumor agents and have been used to treat a variety of medical disorders for more than 200 years (4, 13). Most obvious has been the widespread use of bismuth compounds, mainly colloidal bismuth subcitrate (CBS) and bismuth subsalicylate (BSS), in the treatment of duodenal ulcers (25, 26), gastritis, chronic diarrhea, traveler's diarrhea (11, 22), and acute diarrhea in children (20). A preliminary report (12) suggested that children with *C. difficile* colitis responded to treatment with BSS, and this concept was supported by an experimental model of *C. difficile* colitis in hamsters (6). However, the mechanism of therapeutic activity of bismuth compounds is not known, and their further development will require a fundamental understanding of the chemistry involved. The complex chemical nature of the common commercial bismuth compounds CBS and BSS has restricted the characterization and impeded the systematic development of new compounds. In addition to the many reports relating to BSS and CBS, some investigators have endeavored to synthesize new bismuth-containing compounds which might have heightened antibacterial activities (1, 2, 3, 8, 9, 10, 24). Asato et al. (3) produced three compounds with activity against *Helicobacter pylori*. The activities of those compounds were comparable to that of CBS. The compounds also possessed urease inhibitory activity. Domenico et al. (10) have explored the enhancement of antibacterial activities of bismuth-salts in the presence of lipophilic thiol chelators, mixtures which likely contain compounds earlier described by our laboratory (1, 2). In this context we initiated the present study to address structure-function relationships of bismuth-chelate complexes (thiabismuth heterocyles) against selected bacterial species as a way of elucidating the mechanism of action of bismuth against selected bacteria. To facilitate these studies we synthesized and tested a systematic series of structurally simple thiabismuth heterocycles (1, 2) to assess the potential structure-activity relationship of bismuth. The thermodynamically favored chelated environment and the prominent thiophilicity of bismuth make these compounds ideal initial candidates for such a study in that they are likely to retain their structures in solution for the duration required for biological testing. In this report we demonstrate the antimicrobial activities of these compounds against *C. difficile* and, in comparison, their activities against four species of gram-negative bacteria. Also compared are the activities of these compounds with those of three CBS compounds and ranitidine bismuth citrate.

(Part of this work was presented at the 64th Conjoint Meeting on Infectious Diseases of the Canadian Association for Clinical Microbiology and Infectious Diseases, Hamilton, On-

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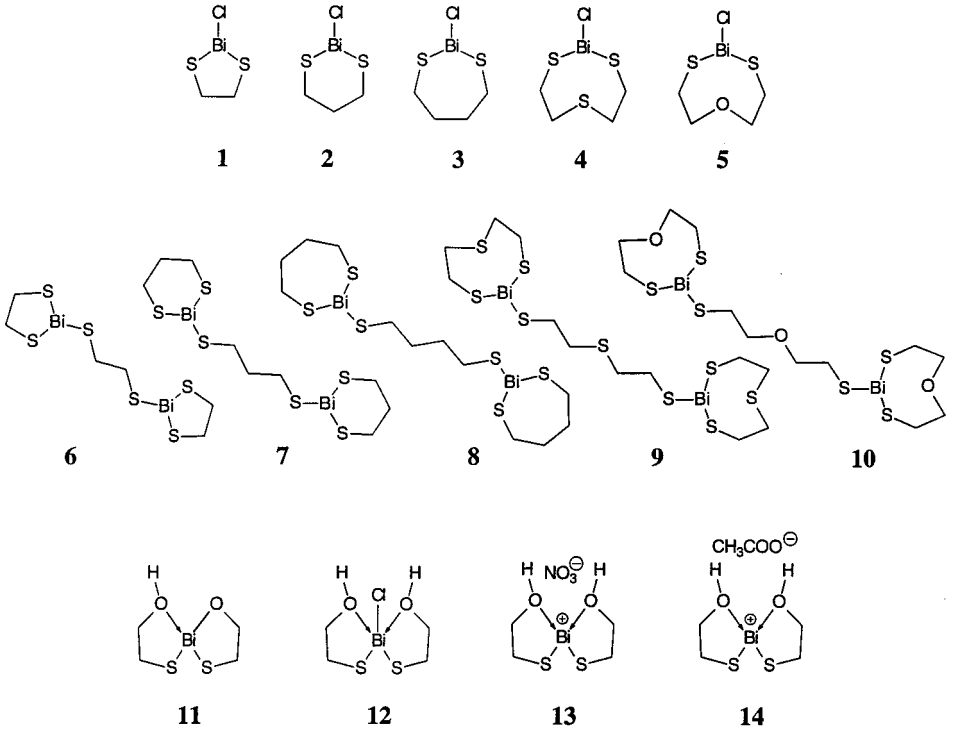

FIG. 1. Structural drawings of the synthetic thiobismuth compounds under investigation.

tario, Canada, 10 to 14 November 1996 [12a], and at the Second International Meeting on the Molecular Genetics and Pathogenesis of the Clostridia, Onzain, France, 22 to 25 June 1997 [12b].)

MATERIALS AND METHODS

Bismuth compounds. Fourteen synthetic compounds prepared in our laboratory (Fig. 1) were tested for their antibacterial activities. The identities and structures of the compounds were confirmed previously by comprehensive characterization, including characterization of their X-ray crystal structures and characterization by mass spectrometric analysis (atmospheric pressure chemical ionization) and nuclear magnetic resonance spectroscopy, which revealed maintenance of the heterocyclic structure in solution (1, 2). These bismuth compounds were relatively insoluble in water, organic solvents, acids, and bases; however, we prepared aqueous saturated solutions of each compound by stirring the compound in distilled water for 6 h, followed by filtration through sterile, 0.22-μm-pore-size filters. The elemental bismuth concentration in such aqueous filtrates was determined by inductively coupled plasma mass spectrometry (ICPMS) (Philip Analytical, Halifax, Nova Scotia, Canada) and was expressed as elemental bismuth in parts per million or micrograms per milliliter. For comparison, three water-soluble CBS samples, CBS 007, CBS 6002, and CBS 023, provided by Biopharm Laboratories, Inc., Laval, Quebec, Canada, and another water-soluble compound, ranitidine bismuth citrate (RBC), provided by Glaxo Research and Development Ltd., Hertfordshire, United Kingdom, were tested for their antimicrobial activities.

Antibacterial activity assay. Two main assay systems were used to determine the antibacterial activities of the synthetic compounds. The first method was the agar dilution method described by the National Committee for Clinical Laboratory Standards in its guidelines (16). Twofold dilutions of the aqueous compound preparations described above were made in 2 ml of sterile distilled water and were added to 18 ml of molten Wilkins-Chalgren agar (Difco Laboratories, Detroit, Mich.) at 50°C and then poured into petri plates. The bismuth concentrations in the plates usually ranged from 0.2 to 10 μg/ml, depending upon the magnitude of the compound's antibacterial activity. *C. difficile* VPI 10463 was grown anaerobically overnight in cooked meat medium (Difco) from which 1 ml was subcultured to 10 ml of boiled brain heart infusion (BHI) broth (Difco). This was incubated for 6 h at 37°C (late logarithmic phase of growth) and was then standardized to an optical density of 0.09 to 0.10 (equivalent to a 0.5 McFarland standard) at a transmission wavelength of 625 nm. Ten-microliter volumes of the standardized inoculum were delivered to the surface of the agar plates with an electronic pipet. The number of viable bacteria contained in such an inoculum was approximately 3.5×10^4 organisms. The plates were incubated for 18 h in an anaerobic glove box (Forma Scientific, Marrietta, Ohio) and were read for growth or no growth. Anaerobic control plates contained no bismuth compound, and aerobic control plates served to check for any aerobic contamination and inoculum opacity that might be misinterpreted as growth on the anaerobic plates. The second method for assessment of the antimicrobial activities of the bismuth compounds was the standard tube dilution assay, which was performed as follows. Aqueous filtrates of the compounds were serially diluted in 2.5-ml volumes of BHI broth to obtain known concentrations of elemental bismuth per milliliter. To each tube was added 2.5 ml of a 1/100 dilution of the standardized broth culture (0.5 McFarland standard). Control tubes contained broth with bacteria but no bismuth compound. These tubes were incubated anaerobically for 48 h. The MIC was the lowest concentration of elemental bismuth that inhibited growth. A loopful of fluid from those tubes showing no growth was subcultured onto blood agar plates for anaerobic incubation to test for viability, and the lowest concentration of bismuth that demonstrated no viability represented the minimum bactericidal concentration (MBC) of the compound.

The sensitivity testing methods used to determine the level of antimicrobial activity against gram-negative bacteria were as described above except for variations in the media and the sizes of the inocula. *H. pylori* 1134 was grown at 37°C in brucella broth (Difco) supplemented with 7% bovine serum under microaerophilic conditions (5% CO_2, 7% O_2) and was adjusted by dilution to obtain an optical density of 0.35 at a transmission wavelength of 625 nm. This inoculum density and dilutions of it (including the 0.5 McFarland standard) were used to test *H. pylori* on brucella agar plates. For the tube dilution tests, the tubes were equilibrated under microaerophilic conditions for 24 h at 37°C, capped, and incubated with shaking for a further 72 h. Four-hour BHI broth cultures of *Escherichia coli* C600, *Pseudomonas aeruginosa* p60-45, and *Proteus mirabilis* 2 were diluted to obtain an optical density at 625 nm of 0.1 (0.5 McFarland standard) for the inoculum used to test these species by the agar dilution method.

Bactericidal activity of compound 1. To determine the dynamics of bacterial killing by compound 1, two series of tubes containing 2.5-ml volumes of undiluted compound 1 (in water) or sterile distilled water (control), respectively, were placed in the anaerobic glove box at 37°C 6 h before the addition of equal volumes of a 6-h culture of *C. difficile* grown anaerobically in BHI broth. At 5, 10, 15, 20, 25, and 30 min of incubation, samples were removed from each tube and microdilutions were made in BHI broth for triplicate determination of viable counts on blood agar within the glove box. At the same time the sampled tubes were placed in an ice bath within the glove box. To determine whether the antimicrobial activity of compound 1 might be reversible by washing the exposed bacteria, the tubes were removed from the glove box and 1-ml samples from each tube were centrifuged in an Eppendorf centrifuge for 3 min. The supernatants were discarded, and the bacterial pellets were resuspended in 1 ml of BHI broth. These cells were then washed by three further cycles of centrifugation and

TABLE 1. MICs and minimum cytotoxic concentrations of six active bismuth compounds tested against *C. difficile* and FSK cells, respectively

Compound	MIC (μg/ml)[a]	MCC (μg/ml)[b]	MCC/MIC[c]
1	1	0.28	0.28
4	2.5	0.45	0.18
6	0.05	0.41	8.2
9	0.5	0.63	1.26
12	0.5	5.5	11
13	2.5	8.6	3.4

[a] The MIC was determined by the agar dilution method. The MIC is based on the concentration of elemental bismuth as determined by ICPMS.

[b] MCC, minimum cytotoxic concentration. The minimum cytotoxic concentration was determined by the FSK cell cytotoxicity assay and is based on the concentration of elemental bismuth as determined by ICPMS.

[c] Ratio of cytotoxicity to inhibitory activity.

resuspension in BHI broth followed by a final resuspension in 1 ml of broth. Serial microdilutions of the resuspended bacteria were made in broth, and viable counts were determined in triplicate on blood agar. The latter manipulations were done under aerobic conditions before the blood agar plates were returned to the anaerobic incubator.

Cytotoxicity assay. Compounds showing significant antibacterial activity were tested for cytotoxicity with human foreskin (FSK) cells. The aqueous filtrates of the compounds were serially diluted in minimal essential medium containing 1% calf serum. The culture medium was removed from a 96-well tissue culture plate containing FSK cells growing as a monolayer and was replaced with 100 μl of the diluted bismuth compounds. The FSK cells were incubated for 22 h at 37°C in an atmosphere of 5% CO_2. Cytotoxicity was recorded as rounding of the fibroblast cells, and the cytotoxic titer was the last dilution that resulted in a rounding of at least 50% of the cells.

TEM. One milliliter of an overnight cooked meat culture of *C. difficile* VPI 10463 was inoculated into 10 ml of prereduced BHI broth, and the culture was incubated at 37°C in the anaerobic glove box for 2 h, at which time 2 ml of compound 1 stock solution (72 μg of elemental bismuth per ml) was added (time zero). One-milliliter samples were removed from this culture at time zero and at 1, 2, 3, 4, and 18 h for electron microscopy. Control samples were taken from cultures of *C. difficile* in which water replaced the bismuth compound. The samples were centrifuged for 5 min in an Eppendorf centrifuge, and the supernatant was discarded. The sample pellets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h at room temperature, washed three times with buffer for 30 min, and postfixed with 1% osmium tetroxide. The samples were stained en bloc with 1% uranyl acetate, dehydrated with acetone, and embedded in Taab 812–Araldite 504. Thin sections, without further staining or poststained with uranyl acetate and lead citrate, were viewed with a Philips 300 transmission electron microscope operating at 60 kV. Whole-mount preparations of selected cultures of bacteria, treated or untreated with bismuth, were micropipetted into Formvar carbon-coated 300-mesh grids. Grids with adherent bacteria received no staining or were negatively stained with 1% aqueous uranyl acetate prior to observation by transmission electron microscopy (TEM).

Energy dispersive spectroscopy (EDS) X-ray microanalysis. A bismuth-treated bacterial culture was prepared as described above for TEM. Suspensions of *C. difficile* taken 4 h after the addition of bismuth were fixed, postfixed, dehydrated, and embedded as described above for TEM. Thick-thin sections (150 to 250 nm thick) were cut and placed on naked 300-mesh nickel grids before the grids were transferred to a low-background beryllium specimen holder. The sections were analyzed in a Philips EM400 transmission electron microscope that was operated at 100 kV and that was equipped with a LINK Pentafet energy detector and a Link eXL analyzer. The spectra were acquired for 100 s at 1,000 counts per second over an energy range of 0 to 20 keV from areas of the section most distant from the grid bar.

RESULTS

Inhibitory activities of bismuth compounds. Of the 14 compounds tested, 8 (compounds 1, 4, 6, 9, 11, 12, 13, and 14) showed significant antibacterial activity. Table 1 shows the amount of elemental bismuth in aqueous filtrates of the six most active compounds, the MIC of each compound for *C. difficile*, and the minimum cytotoxic concentration of bismuth tested with FSK cells. To determine whether any chemical constituent other than bismuth used in the synthesis of these compounds possessed antibacterial activity, key components of the synthesis reactions (e.g., 2-mercaptoethanol, 1,2-ethanedithiol, and 2-mercaptoethyl sulfide) were assayed for their antibacterial activities by spotting 10-μl volumes of serially diluted chemical onto a blood agar plate seeded with a 1:8 dilution of 6-h culture of *C. difficile*. Pure 2-mercaptoethanol demonstrated slight antibacterial activity (at a 1:4 dilution of the chemical), but similarly assayed 1,2-ethanedithiol and 2-mercaptoethyl sulfide had no inhibitory activity against *C. difficile* growth. By comparison, compound 1 had activity at a 1:1,024 dilution or greater when it was similarly assayed.

The stabilities of the most active compounds were tested by assaying the antibacterial activities of aqueous solutions held at 4°C over a period of 28 days. The results revealed considerable stability over the test period, and variations in activity represented a 1-dilution fluctuation, which is an inherent variable with doubling dilution assays. Stock solutions held at room temperature also demonstrated stability, and exposure of the compounds to 100°C for 10 min did not reduce their antibacterial activities. Because of its simple structure and high level of antimicrobial activity, compound 1 (2-chloro-1,3-dithia-2-bismolane) was chosen as a model compound in several of the experiments described below.

Comparison of MICs for *C. difficile* and other test organisms. Experiments comparing the sensitivities of *C. difficile*, *E. coli*, *H. pylori*, *P. aeruginosa*, and *P. mirabilis* to our most active synthetic bismuth compounds also included three lots of CBS compounds (CBS 6002, CBS 007, and CBS 023) and RBC (Table 2). Although a range of bacterial dilutions above and below the 0.5 McFarland standard inoculum density was tested by the agar dilution method, the results recorded here are based on the complete inhibition of growth observed when this standard inoculum was used. The synthetic bismuth compounds were more active against *C. difficile* and *H. pylori* than against the other species tested, and all of the synthetic bismuth compounds were more active than the CBS compounds and RBC against all the organisms tested. Tube dilution assays with aqueous bismuth solutions provided results approximating those obtained by the agar dilution method (data not shown). The MICs were also the MBCs for most compounds assayed, but occasional MBCs were twice the MICs, i.e., 1 doubling dilution difference. To determine whether the sensitivity of *C. difficile* VPI 10463 to these bismuth compounds was representative for the species, 32 additional strains from clinical sources were tested by the agar dilution method (Table 3). The results imply that the sensitivities of these strains were comparable to that of strain VPI 10463.

TABLE 2. Antimicrobial activities of 10 bismuth compounds against five species of bacteria assayed by the agar dilution method[a]

Compound	MIC (μg/ml)				
	C. difficile	*H. pylori*	*P. aeruginosa*	*P. mirabilis*	*E. coli*
1	1	1	2.5	2.5	>5
4	2.5	5	1	2.5	5
6	0.05	0.5	>0.5	>0.5	>0.5
9	0.5	5	2.5	5	10
12	0.5	2.5	2.5	10	10
13	2.5	1	1	5	5
CBS 007	6.0	6.0	>180	>180	>180
CBS 6002	8.5	8.5	>255	>255	>255
CBS 023	6.8	6.8	>203	>203	>203
RBC	9.0	9.0	>270	>270	>270

[a] Plates were prepared from aqueous bismuth solutions, and the MIC was expressed as micrograms of elemental bismuth per milliliter as determined by ICPMS.

TABLE 3. MICs of six synthetic bismuth compounds and one CBS compound tested against multiple strains of *C. difficile* and determined by the agar dilution method

Compound	MIC (μg/ml) for *C. difficile* VPI 10463	No. of additional strains tested	MIC (μg/ml)[a]		
			Range	50%	90%
1	2.5	32	2.5–5.0	2.5	5.0
4	5.0	32	5.0	5.0	5.0
6	0.25	24	0.25–0.50	0.25	0.50
9	2.5	24	1.0–2.5	2.5	2.5
12	1.0	32	0.5–2.5	1.0	1.0
13	1.0	32	1.0–5.0	2.5	2.5
CBS 023	6.8	24	3.4–13.5	6.8	13.5

[a] The MIC is based on the concentration of elemental bismuth as determined by ICPMS. 50% and 90%, MICs at which 50 and 90% of strains, respectively, are inhibited.

Bactericidal activity of compound 1. Failure to culture bacteria from most broth cultures showing no turbidity by the tube dilution method indicated that the synthetic compounds were bactericidal rather than bacteriostatic. Figure 2 shows that *C. difficile* is killed rapidly following exposure to compound 1. Washing of the cells did not alter the antimicrobial activity of compound 1 over this time period, suggesting that the compound was rapidly taken up by or bound to *C. difficile*. The control viable count remained constant over the time course of the experiment and differed little whether the counts were determined entirely in the glove box or on the bench. Similar killing curves were obtained when compounds 4, 6, 9, 12, and 13, the three CBS compounds, and RBC were tested under anaerobic conditions (data not shown); but the washing steps were not deemed necessary to determine the rate of killing. It was noted that *C. difficile* broth cultures became black in color within 5 to 10 min following the addition of compounds 1 and 12 and at somewhat later times for cultures treated with compounds 4, 6, and 9. The CBS- and RBC-treated broth cultures did not blacken, but after 18 h of incubation the settled cells were black. The blackening was restricted to the bacterial cells, since centrifugation of such cultures resulted in a colorless supernatant.

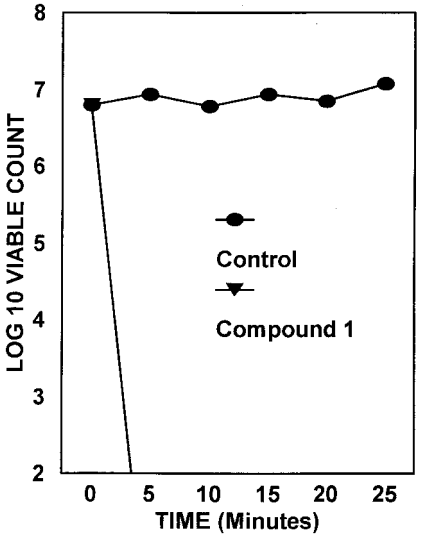

FIG. 2. Killing curve of synthetic bismuth compound 1 tested against *C. difficile* VPI 10463. Equal volumes of a 6-h *C. difficile* broth culture were added to aqueous compound 1 and sterile water (control) under anaerobic conditions at 37°C. At 5-min intervals the cultures were placed on ice and subsequently washed by three cycles of centrifugation and resuspension in broth. After the final wash, the bacteria were resuspended in broth and viable counts were determined in triplicate on blood agar plates.

Binding of compound to bacteria. If broth cultures of *C. difficile* were mixed with aqueous compound 1 or 4 and immediately centrifuged, there appeared a bright yellow bacterial pellet that was similar in color to the original powdered compound. This phenomenon was unrelated to the black color described above. The appearance of black cells required anaerobic incubation of the culture. This suggested that the compound either bound to the bacterial cell or precipitated out of solution. To be assured that the yellow pellet represented compound bound to the bacteria rather than an independent precipitate induced by some feature of the bacterial broth culture, 1 ml of a filtered supernatant of a centrifuged 6-h broth culture of *C. difficile* was added to 1 ml of compound 1. No significant yellow pellet was produced upon centrifugation. Subsequent electron microscopy of the pellets obtained immediately after the interaction of compound 1 and bacteria revealed electron-dense bismuth particles that adhered to the bacterial surface (see below). *E. coli*, *P. aeruginosa*, and *P. mirabilis* were similarly tested and were found to bind to compounds 1 and 4, as did a suspension of FSK cells.

TEM. Observation of whole-mount, air-dried bacteria from a 6-h BHI broth culture exposed to a lethal concentration of compound 1 showed particles believed to be the bismuth compound that adhered to the surfaces of the cells, whether or not the cells were treated with a negative stain. Thin-sectioned bacteria with and without section poststaining showed electron-dense particles that adhered to the surfaces of the bacteria and also inside the cells, often in association with the inner side of the cytoplasmic membrane (Fig. 3a). Such particles were not observed in untreated control preparations (Fig. 3b). To determine whether an increment in intracellular bismuth might be observed within a logarithmically growing culture, *C. difficile* was grown in BHI broth for 2 h, at which time a subinhibitory concentration of compound 1 was added. The samples were immediately removed from the treated culture at 0, 1, 2, 3, 4, and 18 h and were examined by electron microscopy (Fig. 3c). The observations indicate a rapid attachment and uptake of compound 1, even at time zero, and a marked increment in the amount of intracellular bismuth by 3 and 4 h. The 18-h sample showed a mixture of healthy looking bacterial cells with little electron-dense material and obviously lysed cells whose membranes still enclosed large quantities of electron-dense material (Fig. 3d). Bismuth compound 1 did not seem to penetrate the spores which were seen within some bismuth-impregnated bacteria (Fig. 3e). So far, preliminary electron microscopy observations (data not shown) of *C. difficile* treated with the synthetic bismuth compounds indicate that intracellular bismuth is observed only with those synthetic bismuth compounds that demonstrate antibacterial activity.

EDS X-ray microanalysis. The scanning of a thin section of *C. difficile* containing electron-dense material revealed peaks characteristic of bismuth (Fig. 4). Other elements represented in the analysis were nickel (from the grid), carbon, osmium from the fixative, and uranium from the uranyl acetate. These data further confirm that the intracellular electron-dense material is bismuth.

DISCUSSION

In the study described in this paper we have focused on six synthetic bismuth compounds, specifically, compounds 1, 4, 6, 9, 12, and 13, that have marked inhibitory activities against

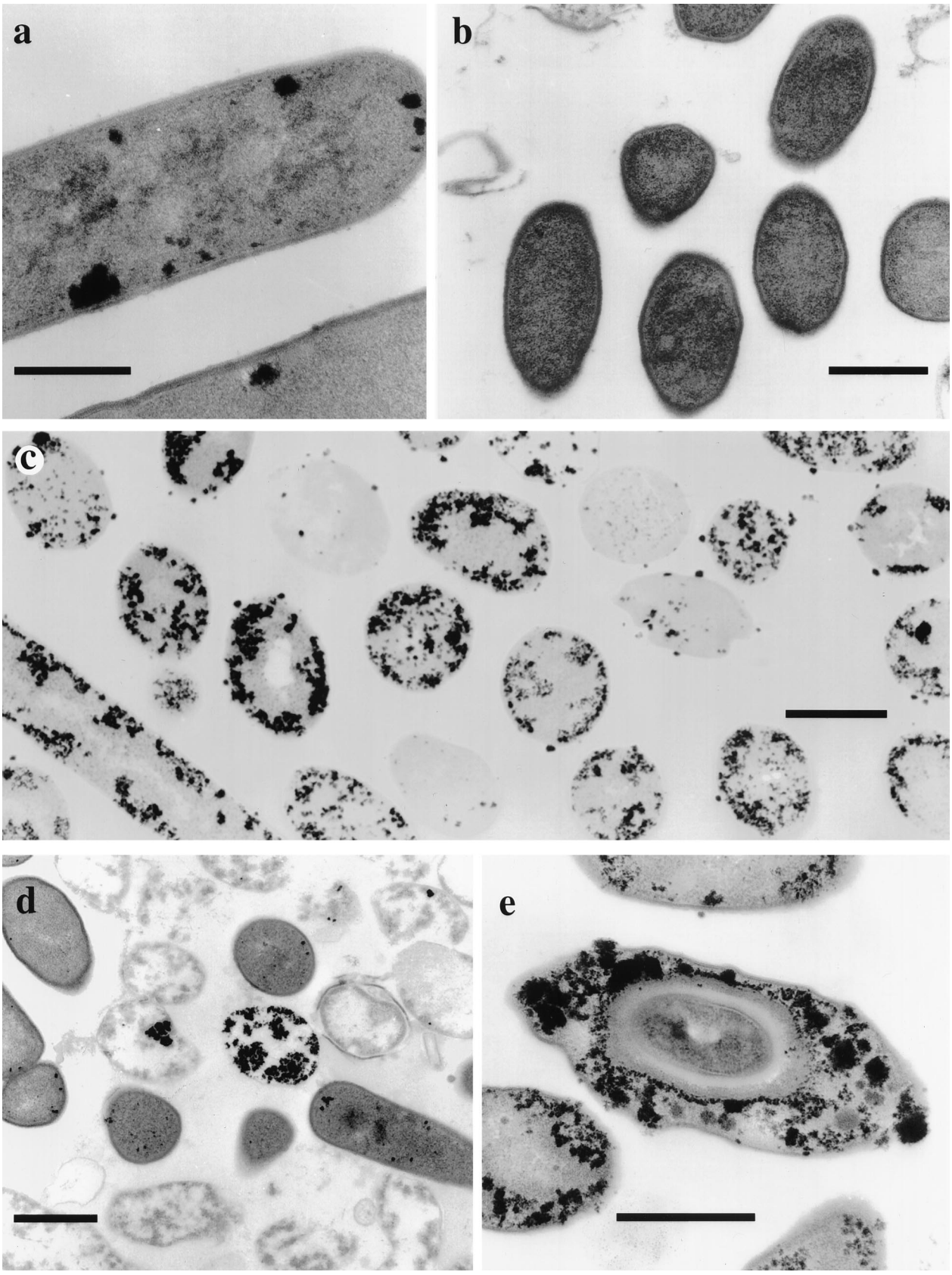

FIG. 3. (a) Thin section of *C. difficile VPI* 10463 after exposure of the bacterium to compound 1 for 10 min and viewed by TEM. Electron-dense bismuth is found inside the cell, often in association with the cytoplasmic membrane. Bar, 300 nm. (b) Thin section control; distilled water replaced compound 1. Bar, 600 nm. (c) Thin section of bacteria from a 2-h culture of *C. difficile* incubated anaerobically with compound 1 for a further 4 h. Note the large amount of intracellular bismuth. Bar, 600 nm. (d) Thin section of bacteria from the culture described in the legend for panel c but incubated for a total of 18 h. Note the healthy looking bacteria containing little or no bismuth as well as lysed cells containing large amounts of bismuth. Bar, 600 nm. (e) Thin section of a spore-containing cell of *C. difficile* from the culture described in the legend for panel c after 3 h of growth in the presence of compound 1. Bar, 600 nm.

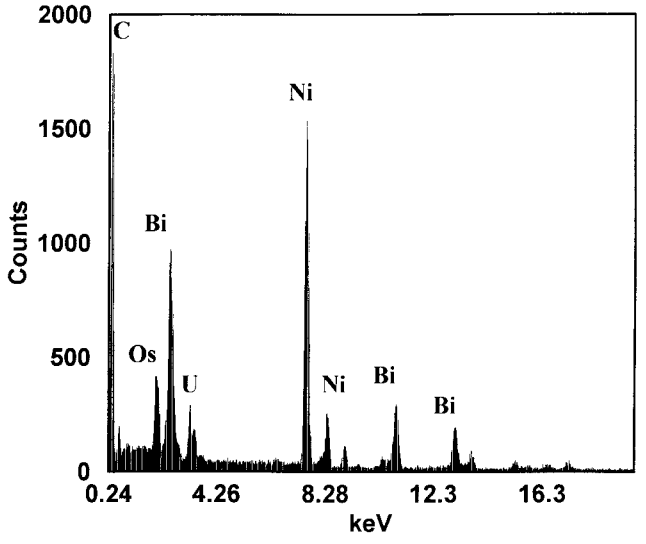

FIG. 4. EDS X-ray microanalysis performed with a thin section of *C. difficile*. Bacteria from a 2-h culture of *C. difficile* were incubated anaerobically with compound 1 for a further 4 h (see legend to Fig. 3c). The intracellular, electron-dense material reveals peaks characteristic of bismuth. The spectra were acquired for 100 s at 1,000 counts per second over an energy range of 0 to 20 keV from areas of the section most distant from the grid bar.

C. difficile. Among four comparator species of bacteria, we have shown that these compounds are also active against *H. pylori* but have lesser activities against *E. coli*, *P. aeruginosa*, and *P. mirabilis*. In general, most of these compounds have very low solubilities in water, but aqueous solutions with antimicrobial activity are achievable and filtered solutions sustained their antimicrobial activities for a 28-day test period. It should be noted, however, that there were occasional deviations in MICs between different stock solutions of compounds (especially compound 6, which has a very low saturated solution concentration) and that the values presented in Tables 1 and 2 are more relative than absolute. Such variation is seen when the MIC data in Tables 2 and 3 are compared. These experiments were performed with different lots of compound and were done several months apart. What is significant in Table 3 is the consistency of MICs of all compounds tested for 33 strains of *C. difficile*. This shows that strain VPI 10463 is not unique in its sensitivity to these compounds. The surprising precipitation of at least two of the compounds onto the surfaces of *C. difficile*, *E. coli*, *P. aeruginosa*, and *P. mirabilis* cells indicates a peculiar interaction of these bismuth compounds with their environment and, in particular, the bacterial surface. In this context, Sox and Olson (21) described the binding of *E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *H. pylori* to BSS. The binding resulted in variable degrees of bacterial killing. Killing curves for *C. difficile* established for six of the synthetic compounds, the three CBS compounds, and RBC indicated that killing was rapid and that the dynamics of killing were similar for all compounds. Results of the agar dilution method suggest that the CBS compounds and RBC have lower antimicrobial activities (MICs, 6 to 9 μg/ml) than the synthetic compounds (MICs, 0.05 to 2.5 μg/ml) against *C. difficile* and that the former compounds have no effect on the *P. aeruginosa*, *P. mirabilis*, and *E. coli* strains tested (MICs, > 180 μg/ml).

The astounding avidity of *C. difficile* for bismuth was further illustrated by electron microscopy, which showed that electron-dense bismuth was both bound to the outside of the bacterial cells and distributed throughout the cytoplasm. Large, localized bismuth deposits, confirmed by X-ray dispersion analysis, were sometimes seen embedded within the cell wall, and it is difficult to speculate how such large particles might gain entrance into the interior of the bacteria. Possibly, soluble bismuth enters through the cell wall and membrane to assemble into larger masses at select intracellular sites.

The mechanism by which bismuth actually kills *C. difficile* (or any bacterium) is not understood. Electron microscopy studies of *Yersinia enterocolitica* treated with BSS show bismuth deposits in the cell wall but not the cytoplasm (14), and work with *H. pylori* indicates distortion of the bacterial morphology with little or no intracellular accumulation of bismuth (14, 17). Preliminary electron microscopy studies in our laboratory (unpublished data) indicate that *H. pylori* does incorporate compound 1 but that the other gram-negative bacteria tested demonstrate little or no incorporation of this compound. We are not aware of electron microscopy studies that have described the interaction of bismuth compounds with gram-positive bacteria. Whether such incorporation is more prevalent in gram-positive bacteria, in particular, is under study. The presentation of intracellular electron-dense material in compound 1-treated *C. difficile* closely resembles that in uranium-treated *P. aeruginosa* (23); however, unlike uranium, compound 1 did not seem to be massively incorporated by our strain of *P. aeruginosa* (data not shown).

While the mechanism of action of bismuth may differ for the various bacterial species examined in this study, we did note several common features among the series of bismuth compounds that seemed to correlate with their biological activities. For example, compound 1 and the tethered version of compound 1 (compound 6) were particularly active. The addition of one or more carbon atoms to the ring of compound 1 (compounds 2 and 3 and tethered compounds 7 and 8) abolished the biological activity, whereas the addition of a sulfur atom (thioether) to the ring of compound 3 (compounds 4 and 9), but not the addition of an oxygen atom (compounds 5 and 10), restored the biological activity. These observations emphasize both the importance of sulfur in the chelation of bismuth and the role of the carrier itself in the delivery of bismuth to biological targets. This knowledge may aid in the design of carrier molecules for the delivery and bioactivity of future bismuth chelates.

There is little doubt that the antimicrobial activities of these compounds are attributed to their bismuth contents and not to the reagents used in their syntheses. Domenico et al. (10) reported that replacement of bismuth with arsenic and antimony in their compounds reduced the compounds' antibacterial activities, and we have also confirmed that the arsenic and antimony analogues of compound 1 have substantially lower levels of activity (unpublished data). Although our compounds demonstrated cytotoxicity, some were less cytotoxic than others. A comparison of the antibacterial activity versus the cytotoxicity showed that compounds 6, 12, and 13 produced the more desirable ratios; however, FSK cells may be more susceptible to these compounds than other tissue culture lines more representative of the colonic epithelium. FSK cells were used in these experiments because of their availability and extreme sensitivity to bacterial toxins. The development of more soluble and less toxic bismuth compounds with high levels of antimicrobial activity is under investigation in our laboratory, although increased water solubility may not necessarily accompany increased bioactivity. It may be that the lipophilicity of these compounds is a virtue that allows them to cross membranes. Certainly, the water-soluble CBS compounds have lower levels of activity compared with those of the synthetic compounds.

Direct comparisons of the MICs for the six bacterial species tested are somewhat difficult because of the considerably different microbial physiologies, but it is not unreasonable to conclude that our synthetic compounds demonstrate higher levels of antimicrobial activity than the CBS and RBC compounds tested. On the basis of the analysis of elemental bismuth for the latter compounds, they would seem to have lower levels of activity than the synthetic compounds against *C. difficile* and *H. pylori* and little or no activity against *E. coli*, *P. aeruginosa*, and *P. mirabilis*. Thus, the potential therapeutic significance of bismuth compounds may be their selectivity for *C. difficile* and *H. pylori*. Reports of other studies (7, 10, 14) in which the sensitivity of *C. difficile* to bismuth compounds has been tested indicate that *C. difficile* is among the most sensitive of many bacterial species tested.

In conclusion, we have shown that a systematic series of bismuth compounds have significantly varied antimicrobial activities against *C. difficile*, implicating a relationship between the structure of the bismuth compound and its activity. We have shown that bismuth compound 1 is readily, if not aggressively, incorporated into *C. difficile* and that these compounds, in general, have a selective activity against *C. difficile. H. pylori* is also sensitive to these compounds, but the other gram-negative bacteria tested were much less sensitive to the synthetic compounds, CBS, RBC.

ACKNOWLEDGMENTS

We acknowledge S. H. S. Lee, Department of Microbiology, Queen Elizabeth II Health Sciences Centre, Victoria General Site, Halifax, Nova Scotia, Canada, for supplying FSK cells and the constructive comments and criticism of S. J. O. Veldhuyzen van Zanten, Department of Gastroenterology, Queen Elizabeth II Health Sciences Centre, in the preparation of the manuscript. We are indebted to S. Belfry, Electron Microscopy Unit, University of New Brunswick, Fredericton, New Brunswick, Canada, for performing the X-ray microanalysis.

We thank the Natural Sciences and Engineering Research Council of Canada (Strategic Projects grant STP01 93114), Biopharm Laboratories, Inc., and other private industries for financial support.

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