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Effect of Different Nitroheterocyclic Compounds on Aerobic, Microaerophilic, and Anaerobic Bacteria

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The antibacterial activities of different nitroheterocyclic compounds were assessed by an agar dilution method against aerobic, microaerophilic, and anaerobic bacteria. Nitronaphthofurans inhibited the multiplication of aerobic bacteria at low concentrations (MIC for 50% of strains tested [MIC_{50}], 1 mg/liter). Under anaerobic growth conditions the MICs were found to be even lower. The rough, DNA repair-deficient mutants of *Salmonella typhimurium* were more susceptible, whereas nitroreductase-deficient strains were resistant. Microaerophilic campylobacter isolates could be divided into two groups, one of which was as susceptible as aerobic bacteria (MIC_{50}, 1 mg/liter) and the other of which was more highly susceptible (MIC_{50}, 0.015 mg/liter). All anaerobic bacteria tested were susceptible to nitronaphthofurans (MIC_{50}, 0.125 mg/liter). Nitrothiazole exerted antibacterial activities similar to those of the nitronaphthofurans. Metronidazole, a nitroimidazole derivative, and nitrofurans were definitely less active. Nitrobenzofurans showed relatively high MICs.

Nitroheterocyclic compounds are known for their diverse antimicrobial activities (11). A broad spectrum of pathogenic organisms, i.e., worms, protozoa, fungi, and bacteria, are susceptible to such agents. Nitrofurans (19) and nitroimidazoles (5, 6) are common therapeutic agents in human and veterinary medicine. Others, such as nitrothiazoles, are rarely employed (17), although they display marked antibacterial activities against aerobic, microaerophilic, and anaerobic bacteria (13, 15). Nitrofurans are poorly investigated, although a broad range of microorganisms such as bacteria, protozoa, as well as worms seem to be susceptible to them (2, 7, 8, 22, 24; M. Bonnin, Y. Michel-Briand, R. Royer, and J.-P. Buisson, Ann. Pharm. Fr., in press; R. Royer and J.-P. Buisson, Ann. Pharm. Fr., in press).

In this report the antibacterial activities of certain derivatives of nitroheterocyclic compounds against aerobic bacteria (*Escherichia coli* and *Salmonella typhimurium*), microaerophilic campylobacter isolates, and anaerobic bacteria (*Bacteroides* spp. and *Clostridium* spp.) were examined, and their activities were compared.

MATERIALS AND METHODS

Bacteria. Of 11 strains of anaerobic bacteria, 7 were donated by H. Werner, University of Tübingen, Tübingen, Federal Republic of Germany. They were stored in liquid nitrogen. Before use two subcultures were made on viande-levure agar. The other strains were fresh isolates and were characterized by cultural and biochemical methods. All these bacteria were subcultured in thioglycolate broth at 37°C for 48 h.

The reference strains *Campylobacter jejuni* NCTC 11168 and *Campylobacter coli* NCTC 11353, as well as 13 other campylobacter strains of both species, were obtained from V. Sticht-Groh, University of Würzburg, and characterized by the method of Skirrow and Benjamin (26). They were stored in liquid nitrogen and subcultured twice on FBP agar (brucella agar supplemented with $FeSO_4 \cdot H_2O$, sodium *meta*-bisulfite, and sodium pyruvate). Before testing they were grown in alkaline peptone water for 24 h under microaerophilic conditions (Campy Pak; BBL Microbiological Systems, Cockeysville, Md.).

Most of the aerobic bacteria were fresh isolates taken from a routine bacteriology laboratory. Additionally, some other strains were examined. *Escherichia coli* ATCC 25922 served as a control. The smooth strain LT2 and the semirough (SR) strain of *S. typhimurium* were a gift from G. Schmidt (Forschungs-Institut Borstel, Borstel, Federal Republic of Germany). By conjugation plasmid p124 coding for tetracycline resistance was transferred to strain LT2 by J. Hacker, University of Würzberg). From this strain a nitroreductase-deficient (NR) mutant was obtained by cultivating the bacteria in increasing doses of niridazole. This clone was also unable to reduce nitrofurazone (18). The deep rough and excision repair-deficient strain TA-1538 (*rfa uvrB*$^-$) of *Salmonella typhimurium* (1) and its ampicillin-resistant derivative TA-98 were obtained from B. N. Ames (University of California, Berkeley). The NR mutant TA-98 was sent by H. S. Rosenkranz (Case Western Reserve University, Cleveland, Ohio). All aerobic bacteria were subcultured in Mueller-Hinton broth for 24 h.

Substances. Synthesis and chemical characterization of nitronaphthofurans (R-6597, 2-nitro-naphtho 2, 1-*b* furan; R-7000, 7-methoxy-2-nitro-naphtho 2,1-*b* furan) and nitrobenzofurans (R-5144, 2-nitro-benzofuran; R 5255, 5-methoxy-2-nitrobenzofuran) have been reported previously (22–24). Nitrofurantoin and nitrofurazone were obtained from Röhm Pharma, Darmstadt, Federal Republic of Germany. The nitrothiazole derivatives were kindly supplied by O. Zak, CIBA-GEIGY AG, Basel, Switzerland. Metronidazole was a gift from Bayer AG, Leverkusen, Federal Republic of Germany (Fig. 1). Tetracycline and ampicillin were obtained from Serva Feinbiochemica, Heidelberg, Federal Republic of Germany.

The nitro-containing compounds that were insoluble in water were dissolved in *N*,*N*-dimethylformamide and then further diluted in water.

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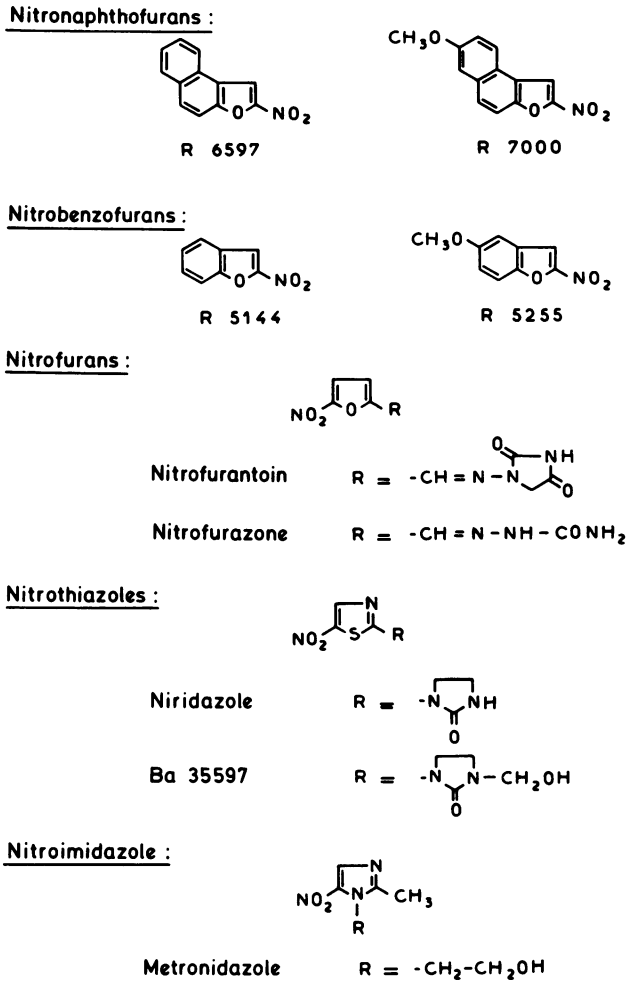

FIG. 1. Chemical structures of nitro-containing compounds.

Susceptibility testing. The MICs were determined by an agar dilution method, with Mueller-Hinton agar used as the nutrient for aerobic bacteria (15), Wilkins-Chalgren agar used for anaerobic bacteria (27) or Mueller-Hinton agar supplemented with 7% sheep blood used for campylobacter isolates (14). Agar plates containing drugs were used not later than 48 h after preparation.

Appropriate dilutions were prepared so that 10^4 to 10^5 viable cells of a bacterial strain were transferred with a replicator to an agar plate. The aerobic bacteria were incubated for 24 h under normal atmospheric conditions or in an anaerobic jar provided by a Gas Pak system (BBL). (*Pseudomonas aeruginosa* ATCC 27853 was cocultured as a control. This strain did not grow under anaerobic conditions.) The microaerophilic campylobacter isolates were incubated for 24 h in a microaerophilic milieu (Campy Pak; BBL), whereas the anaerobic bacteria were held for 48 h under anaerobic conditons, before the MICs were recorded.

RESULTS

Aerobic bacteria. Metronidazole was inactive against aerobic bacteria, even under anaerobic conditions. The nitrofurans were moderately active. Nitrofurazone showed MICs that were somewhat lower than those of nitrofurantoin. Anaerobic growth conditions did not markedly reduce

TABLE 1. Antibacterial activities against aerobic bacteria

Organism	MIC (mg/liter)[a]										
	Nitronaphthofurans		Nitrobenzofurans		Nitrofurans		Nitrothiazoles		Nitroimidazole (metronidazole)	Tetracycline	Ampicillin
	R-7000	R-6597	R-5255	R-5144	Nitrofurantoin	Nitrofurazone	Niridazole	Ba35597			
E. coli											
ATCC 25922	0.5 (0.06)	1.0	8.0	8.0	8.0 (8.0)	4.0	8.0 (2.0)	16.0	>128.0 (>128.0)	2.0	8.0
10432/84	>128.0 (0.5)	>128.0	>128.0	64.0	64.0 (16.0)	64.0	>128.0 (128.0)	>128.0	>128.0 (>128.0)	>128.0	128.0
S. typhimurium											
TA-1538	0.0037 (0.0037)	0.0037	0.25	2.0	1.0	1.0	0.25 (0.125)	0.06	128.0 (64.0)	2.0	0.5
TA-98	0.0037 (0.0037)	0.0037	0.5	2.0	1.0 (0.5)	2.0	0.25 (0.125)	0.125	>128.0 (>128.0)	2.0	>128.0
TA-98 NR	0.5	1.0	>128.0	>128.0	64.0		128.0	128.0	>128.0 (>128.0)	1.0	
LT2	0.5 (0.06)	1.0	8.0	8.0	16.0 (8.0)	8.0	4.0 (1.0)	8.0	>128.0 (>128.0)	2.0	2.0
LT2 (p124)	0.5 (0.125)	1.0	8.0	8.0	16.0 (8.0)	8.0	8.0 (2.0)	4.0	>128.0 (>128.0)	>128.0	2.0
LT2(p124) NR	>128.0 (1.0)	>128.0	8.0	16.0	64.0 (16.0)	64.0	>128.0 (>128.0)	128.0	>128.0 (>128.0)	>128.0	2.0
SR	0.5 (0.125)	1.0	8.0	8.0	16.0 (8.0)	8.0	8.0 (0.5)	8.0	>128.0 (>128.0)	2.0	2.0
1366/74	1.0 (0.125)	1.0	8.0	8.0	16.0 (4.0)	8.0	4.0 (1.0)	8.0	>128.0 (>128.0)	64.0	2.0
5962/84	2.0 (0.125)	1.0	8.0	8.0	16.0 (8.0)	8.0	2.0 (1.0)	8.0	>128.0 (>128.0)	16.0	128.0
5894/84	0.5 (0.125)	1.0	8.0	8.0	32.0 (8.0)	8.0	2.0 (1.0)	8.0	>128.0 (>128.0)	2.0	2.0
6719/84	1.0 (0.125)	1.0	8.0	8.0	16.0 (8.0)	8.0	2.0 (1.0)	2.0	>128.0 (>128.0)	2.0	2.0
6519/84	1.0 (0.125)	2.0	8.0	8.0	16.0 (8.0)	8.0	2.0 (1.0)	4.0	>128.0 (>128.0)	2.0	2.0
174/85	0.5 (0.06)	1.0	8.0	8.0	16.0 (8.0)	8.0	4.0 (0.5)	4.0	>128.0 (>128.0)	2.0	2.0
5568/84	>128.0	>128.0	>128.0	16.0	64.0 (8.0)	32.0	>128.0 (>128.0)	128.0	>128.0 (>128.0)	2.0	2.0
2400/80	>128.0 (1.0)	>128.0	>128.0	32.0	128.0 (16.0)	64.0	>128.0 (>128.0)	128.0	>128.0 (>128.0)	2.0	2.0

[a] Values in parentheses are MIC obtained by incubation in an anaerobic atmosphere.

TABLE 2. Antibacterial activities against *Campylobacter* spp.

Organism	MIC (mg/liter)							
	Nitronaphthofurans		Nitrobenzofurans		Nitrofurans		Nitrothiazole (niridazole)	Nitroimidazole (metronidazole)
	R-7000	R-6597	R-5255	R-5144	Nitrofurazone	Nitrofurantoin		
C. coli NCTC 11353	0.0075	0.0075	1.0	1.0	2	2	0.0075	2
C. jejuni 4629/79	0.015	0.015	1.0	1.0				
C. coli 5380/80	0.0075	0.0075	0.5	0.5				
C. coli 85/80	0.015	0.015	1.0	1.0				
C. coli 6405/80	0.015	0.015	2.0	0.5				
C. jejuni 3680/79	0.015	0.06	2.0	1.0				
C. jejuni 5276/79	0.015	0.0075	0.5	0.5	2	1		1
C. jejuni 5224/79	0.015	0.0075	0.5	0.5	2	1		1
C. jejuni NCTC 11168	0.5	0.5	8.0	2.0	2	2	1	128
C. jejuni 2296/79	1.0	1.0	2.0	1.0				
C. coli 06/80	1.0	0.5	1.0	1.0				
C. coli 3482/80	0.125	0.25	1.0	1.0				
C. coli 1000/80	1.0	1.0	1.0	2.0				
C. jejuni 3015/80	2.0	1.0	1.0	2.0				
C. jejuni 3389/80	2.0	1.0	2.0	2.0				

the MICs. The nitronaphthofuran derivatives were more active. Under anaerobic growth conditions the MICs were found to decrease even further. Nitrobenzofurans were comparatively less active. Here the MICs were of the same order as the nitrothiazole compounds.

The deep rough, DNA repair-deficient strains TA-1538 and TA-98 of *S. typhimurium* were much more susceptible to the nitroheterocyclic compounds than were the smooth isolates. However, the NR mutant TA-98 definitely lost its susceptibility to nitroheterocyclic substances. Similarly, the other NR strain, LT2(p124), was resistant. The original strain, however, was found to be susceptible. *S. typhimurium* 2400/80 and 5568/84, as well as *E. coli* 10432/84, were resistant to most nitro-containing agents and, therefore, possibly deficient in nitroreductase activity.

It was found that the pattern of resistance to nitroheterocyclic compounds did not correlate with that of tetracycline and ampicillin (Table 1).

Microaerophilic bacteria. The microaerophilic campylobacter isolates differed considerably in their susceptibilities to nitroheterocyclic compounds. Some strains, such as the reference strain *C. coli* NCTC 11353, were highly susceptible, whereas other strains were relatively resistant, for example, *C. jejuni* NCTC 11168. A division into a susceptible and a relatively resistant population among the strains tested could not be observed with nitrobenzofurans or nitrofurans. All strains showed similar high MICs (Table 2).

Anaerobic bacteria. The nitrobenzofurans were inactive, whereas the nitronaphthofurans, as well as the nitrothiazole derivatives, showed high activity, more so than metronidazole and the nitrofurans. Single strains were not markedly different in their susceptibilities to nitroheterocyclic compounds, whereas to tetracycline or ampicillin they exhibited varying degrees of susceptibility (Table 3).

DISCUSSION

It is known that all nitro-containing compounds are not similar with respect to their mutagenic activities (21). This is also true for their antibacterial activities.

As demonstrated in previously published reports (22;

TABLE 3. Antibacterial activities against anaerobic bacteria

Organism	MIC (mg/liter)										
	Nitronaphthofurans		Nitrobenzofurans		Nitrofurans		Nitrothiazoles		Niridazole (metronidazole)	Tetracycline	Ampicillin
	R-7000	R-6597	R-5255	R-5144	Nitrofurantoin	Nitrofurazone	Niridazole	Ba35597			
Bacteroides fragilis											
ATCC 25285	0.06	0.5	≧16.0	≧16.0	1.0	1.0	0.0075	0.015	0.25	0.06	8.0
3676	0.5	0.25	≧16.0		1.0	2.0	0.015	0.015	0.5	0.25	16.0
6869	0.25	0.25	≧16.0		2.0		0.015	0.015	0.5	8.0	16.0
94	0.125	0.25	≧16.0	≧16.0	2.0	2.0			0.25	32.0	128.0
Bacteroides thetaiotaomicron											
ATCC 10852	0.125	0.25	8.0	≧16.0	2.0	4.0	0.015	0.03	0.5	0.5	0.32
85	0.06	0.25	≧16.0	≧16.0	2.0				0.25		
Bacteroides vulgatus											
ATCC 8482	0.06	0.25	≧16.0	≧16.0	2.0	4.0	0.06	0.06	0.5	0.25	2.0
85	0.06	0.125	≧16.0		1.0				0.25		
Bacteroides variabilis 85	0.125	0.125	≧16.0		2.0				0.25		
Clostridium difficile 13720/71	0.125	0.25	≧16.0		4.0	4.0	0.03	0.03	0.5	1.0	1.0
Clostridium perfringens Es 17a/80	0.5	0.25	≧16.0	≧16.0	4.0	4.0	0.015	0.015	0.25	1.0	1.0

Royer and Buisson, in press), nitronaphthofurans display much better antimicrobial properties than do the nitrobenzofurans. This observation was confirmed in this study, because not only aerobic bacteria, such as *E. coli* and *S. typhimurium* (Table 1), but also microaerophilic (Table 2) and anaerobic (Table 3) bacteria were found to be more susceptible to 2-nitro-naphthofurans R-7000 and R-6597 than to 2-nitrobenzofurans R-5255 or R-5144, respectively (Fig. 1). Nitrofurans, such as nitrofurantoin and nitrofurazone (Fig. 1), are comparatively less active than the nitronaphthofurans (Tables 1 to 3). Nitrothiazoles (Fig. 1), which are known for their excellent antibacterial activity (13, 15), show MICs similar to those of the nitronaphthofurans. Metronidazole, a nitroimidazole derivative (Fig. 1), is only active against microaerophilic and anaerobic bacteria (Tables 2 and 3). Aerobic bacteria are totally resistant (Table 1), although it has been reported that facultatively anaerobic bacteria such as *E. coli* may respond to metronidazole, at least under strict anaerobic conditions (16). Other common antibiotics such as tetracycline and ampicillin that are not chemically related to the nitro-containing agents may have similar inhibitory effects on aerobic bacteria (Table 1) but show different effects on microaerophilic or anaerobic (Table 3) bacteria.

The in vitro activity of nitronaphthofurans, however, depends on certain conditions. In a broth dilution method MICs for aerobic bacteria were found to be lower (2) than those obtained by an agar dilution method (Table 1). Inoculum concentrations, as well as incubation conditions, were found to influence results. Especially in an anaerobic milieu, low MICs for facultative anaerobes were obtained (Table 1). Furthermore, the activity of niridazole, a nitrothiazole derivative (Fig. 1), was found to increase under anaerobic growth conditions (Table 1) (12), whereas other nitro-containing compounds did not show this dependency.

The lipopolysaccharide of the bacterial cell wall seems to be a barrier to the penetration of nitro-containing compounds, which not only affects the mutagen (1) but also its antibacterial activity. The deep rough strains TA-1538 and TA-98 of *S. typhimurium* are much more susceptible than the SR variant or the smooth (LT2) strains, respectively (Table 1). Another possible explanation for the pronounced susceptibility of strains TA-1538 and TA-98 could be their lack of a DNA repair system, so that even minor DNA damage may have bactericidal effects. Similar repair-deficient mutants of *E. coli* also have an increased susceptibility to metronidazole (28).

Another essential prerequisite for the expression of the antibacterial activity of nitro-containing compounds is the ability of the bacterial target cells to produce nitroreductases. The NR strain *S. typhimurium* TA-98, which is deficient in classical nitroreductase (21), is definitely less susceptible or even completely resistant to the different nitro-containing compounds, except for the nitrofurans (Table 1). Nitroreductase deficiency has also been shown to occur in strain LT2(p124), which is consequently resistant to nitro-containing compounds. A lack of the activating enzyme may also be responsible for the resistance of strains *E. coli* 10432/84 and *S. typhimurium* 2400/80 and 5568/84. It is postulated that the reduction of the nitro group of the antibacterial substance may lead to toxic intermediates which are able to damage the bacterial DNA (9, 20, 28). The resistance of certain strains of microaerophilic campylobacter isolates to these compounds (Table 2) may be due to a deficiency of nitroreductase. Different enzymatic systems exist in bacteria that are more or less susceptible to the different nitro-containing compounds. In aerobic bacteria, at least two nitroreductase systems, an oxygen-sensitive system and an oxygen-insensitive system, have been described (4). In anaerobic bacteria at least three distinct nitroreductases have been found (3). Anaerobic bacteria in particular seem to possess highly active enzymatic systems, for example, that of ferredoxin (20), which renders these bacteria (Table 3) much more susceptible to certain nitro-containing compounds than aerobic bacteria (Table 1). Since these enzymatic processes are essential for the energy metabolism of anaerobic bacteria, resistance to nitro-containing compounds may be extremely rare.

The nitrofurans, however, are peculiar among the nitro-containing compounds, because the MICs are uniform in the nitro-susceptible and nitro-resistant strains (Tables 1 and 2) (13) of aerobic (Table 2) and anaerobic (Table 3) bacteria. Whereas the nitronaphthofurans, nitrothiazoles, and nitroimidazoles are much more active against anaerobic bacteria (Table 3) than against aerobic bacteria (Table 2), nitrofurans and nitrobenzofurans do not exert a strong inhibitory effect on anaerobic bacteria. An inhibition of protein synthesis rather than a damage of the bacterial DNA seems to be the major mechanism of action of nitrofurans.

In conclusion, one can say that several different nitro-containing compounds display marked antibacterial properties. If one considers that this group of compounds inhibits not only bacteria but also other microorganisms, the nitro-containing chemicals possess a high antimicrobial potential which is hardly equalled by any other chemical group. In addition to the nitrofurans, nitrothiazoles, and nitroimidazoles (10), the nitronaphthofurans are of special interest, although their mutagenic and carcinogenic properties (25) will definitely limit their future clinical use.

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