Nitrosoimidazoles: Highly Bactericidal Analogues of 5-Nitroimidazole Drugs

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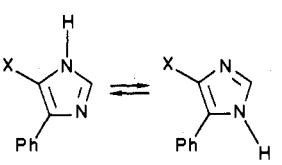
It is believed that metronidazole and related 5-nitroimidazoles are activated by reduction of the nitro group and that the active species has a nitrogen functionality of intermediate oxidation state. However, the preparation and isolation of the active forms of the 5-nitroimidazoles used therapeutically have proven elusive. To pursue this problem we have prepared both 1-methyl-4-phenyl-5-nitrosoimidazole (3) and 1-methyl-4-nitroso-5-phenylimidazole (5) from 4(5)-nitroso-5(4)-phenylimidazole (1). We have also prepared the homologous nitroimidazoles. *Escherichia coli* mutants with defects in DNA repair were found to be sensitive to both 1-methyl-4-phenyl-5-nitroimidazole (4) and metronidazole, but fairly resistant to 1-methyl-4-nitro-5-phenylimidazole (6), a finding in accord with the relative biological activity of 4- and 5-nitroimidazoles examined previously. In contrast, all three nitroso compounds are considerably more bactericidal than their analogous nitro compounds under both aerobic and anaerobic conditions, a finding that provides direct evidence that reduction of the nitro group is responsible for activation of the nitroimidazoles. Further evidence is also consistent with the possibility that the nitrosoimidazoles are themselves biologically active species derived from nitroimidazoles, although a more conservative interpretation is simply that they are more facilely converted to such active species.

Metronidazole and related 5-nitroimidazoles are valuable drugs for the treatment of several protozoal diseases as well as for treating infections due to anaerobic bacteria.[1] Several types of evidence strongly suggest that reduction of the nitro group is obligatory for the biological effects that are responsible for the therapeutic activity of these drugs.[2-5] Yet this hypothesis remains unproven because the postulated active functionality resulting from reduction of the nitro group has not been isolated. The amine derived from reduction of the metronidazole has been shown to form under conditions where the 5-nitroimidazoles are bactericidal, but this compound itself has no bacterial activity.[4] Attempts to synthesize such other forms of therapeutically active 5-nitroimidazoles as those with the hydroxyamino[5] and the nitroso functionalities have not been successful.

The failure to synthesize compounds of intermediate oxidation state has frustrated attempts to determine the mode of action of metronidazole and related drugs. However, a small number of phenyl-substituted nitrosoimidazoles are known, including 4(5)-nitroso-5(4)-phenylimidazole (1),[6] a nitrosoimidazole in which the carbon atom adjacent to that bearing the nitroso substituent is blocked by a phenyl group. This compound and its nitro homologue, 4(5)-nitro-5(4)-phenylimidazole (2),[7] offer an opportunity to compare the biological activity of a nitroimidazole with that of its corresponding nitrosoimidazole.

Because 1 and 2 lack a substituent at N_1, they each exist in two tautomeric forms:



1, X = NO_2
2, X = NO_2

Thus in the case of 2, one tautomer is formally a 5-nitroimidazole, the other a 4-nitroimidazole. The nitroimidazoles used therapeutically are 5-nitroimidazoles that have substituents at N_1 and C_2 but lack a substituent at C_4;[8] the 4-nitroimidazoles have no therapeutic value.[9,10] The distinction between the 4- and 5-nitroimidazoles is lost when 2 is used, and furthermore, the equilibrium between the tautomers of 2 in neutral aqueous solution probably differs from that of 1.[11-13] These considerations limit the value of using a comparison between 1 and 2 as a means of understanding the possible relationship between a therapeutic 5-nitroimidazole and its putative nitroso functionality.

In order to compare the properties of the 4- and 5-nitroimidazoles with analogous nitrosoimidazoles, we prepared both 1-methyl-4-phenyl-5-nitroimidazole (4)[7] and 1-methyl-4-nitro-5-phenylimidazole (6) from 2 and both 1-methyl-4-phenyl-5-nitrosoimidazole (3) and 1-methyl-4-nitroso-5-phenylimidazole (5) from 1 (see Table I). This paper describes the properties of these three pairs of homologous nitro and nitroso compounds and compares their bactericidal potency in experiments designed to gain insight into the mechanisms that are responsible for the bactericidal activity of the nitroimidazoles.

Chemistry

4(5)-Nitro-5(4)-phenylimidazole (2) has been prepared previously,[7] but the route of its synthesis was not fully described. When prepared by our method, 2 had properties (Table I) that are consistent with both the proposed structure and previous descriptions.[7] The assignment of the structures of 1-methyl-4-phenyl-5-nitroimidazole (4) and 1-methyl-4-nitro-5-phenylimidazole (6) is also in accord with previous experience. Thus, methylation of 2 favors formation of 4 under neutral conditions but 6 under basic conditions.[14-16] Additionally, the relative melting

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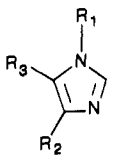
Table I. Structures of Isomeric Phenyl-Substituted Nitrosoimidazoles and Nitroimidazoles and a Comparison of Their Properties



compd	R_1	R_2	R_3	retention time,[a] min	absorbance max,[b] nm	mp, °C
1	H	C_6H_5	NO	21.5	360	195 dec
2	H	C_6H_5	NO_2	21.4	330	295–300
3	CH_3	C_6H_5	NO	26.9	364 312	128–130
4	CH_3	C_6H_5	NO_2	25.5	336 280	73–75
5	CH_3	NO	C_6H_5	20.5	342	185 dec
6	CH_3	NO_2	C_6H_5	22.5	308	178–180

[a] Retention time is for the reverse-phase liquid chromatographic system described in the Experimental Section. [b] In aqueous solutions.

Table II. Stability of the Nitrosoimidazoles in Various Solutions

solution	half-life,[a] min		
	1	3	5
0.1 M KPO_4 buffer, Ph 7.0	330 ± 25	540 ± 20	>600
BHI spent medium[b]	-	40 ± 3	160 ± 5
E. coli SR58 in BHI medium (10^8 cfu/mL)[c]	1–3	3–4	4–6
B. fragilis in BHIS medium (10^9 cfu/mL)[c]	<2	<2	<2

[a] Half-life was measured by the time required for a 50% decrease in UV absorbance at the absorbance maximum of each nitrosoimidazole. This decrease was found to be first order for each compound in the concentration range 0.05–1.0 mM in both phosphate buffer and BHI spent medium. The first-order half-lives were calculated from a linear regression of the data and expressed as the mean ± its standard deviation. In the presence of bacteria, compounds decomposed with an initial slow phase followed by a rapid phase. Accordingly, half-lives were estimated during the rapid phase from inspection of the data. [b] BHI medium from which bacteria were removed by centrifugation and filtration after the maximal logarithmic growth of *E. coli* had been supported. [c] Bacteria were allowed to grow and stability tests were carried out under anaerobic conditions.

points of 4 (73–75 °C, lit.[7] 74.5–75.5 °C) and 6 (178–180 °C) are consistent with similar isomeric pairs,[14] as is the shorter wavelength ultraviolet absorbance maximum of the 4-nitroimidazole 6 compared to that of the 5-nitroimidazole 4 (Table I).[16] Furthermore, 4 shows a hypsochromic shift (along with broadening) of the absorbance maximum at acid pH (10 nm in 0.1 N HCl/EtOH), as do other 5-nitroimidazoles, whereas 6 does not.[12,16]

4(5)-Nitroso-5(4)-phenylimidazole (1) is quite reproducibly prepared from 4(5)-phenylimidazole by using the method of Brothers and McClelland,[11,17] and methylation of 1 leads to the isomers 1-methyl-4-phenyl-5-nitrosoimidazole (3) and 1-methyl-4-nitroso-5-phenylimidazole (5). Once purified, all three nitrosoimidazoles appear to be stable in solid form and decompose comparatively slowly in aqueous solution at pH 7 (Table II), but fairly rapidly in acidic solutions, in the presence of sulfhydryl compounds, or in bacterial cultures (Table II). Like other *C*-nitrosoimidazole monomers,[11,13] 1, 3, and 5 are green in both solid form and aqueous solution and have weak $n \rightarrow \pi^*$ UV absorption bands at approximately 670 nm.

Structural assignment of the isomers 3 and 5 is difficult because few nitrosoimidazoles have been prepared and no isomeric pairs have been characterized. The NMR and mass spectra of each isomer are compatible with either structure. It seems reasonable to assume, however, that these monomeric nitrosoimidazoles would have properties comparable to those of the better-chracterized homologous nitroimidazoles because both pairs of compounds have an electron-withdrawing substituent on the heteroaromatic imidazole ring. Thus, treatment of 1 with diazomethane under neutral conditions yielded only one product with a UV absorption maximum above 300 nm, which was considered to be 3. On the other hand, methylation of 1 with methyl iodide under more basic conditions yielded an additional product, which was assigned the structure 5. In terms of these provisional assignments, the properties of 5 and 3 bore a relationship that was comparable to that of 6 and 4 (Table I).

This provisional assignment was confirmed by reducing the nitrosoimidazoles to aminoimidazoles and oxidizing them to nitroimidazoles. Thus, oxidation of 3 with either sodium hypochlorite[18,19] or ozone[19] resulted in the formation of 4 in yields of approximately 5% and 1%, respectively. Although the reaction with each oxidant was rapid and yielded many decomposition products, compound 6 was never detected among these products. Furthermore, when either 4 or 3 was reduced with hydrogen in the presence of a palladium catalyst,[4] each was converted almost quantitatively to the same product, which was assigned the structure 1-methyl-4-phenyl-5-aminoimidazole [MS, m/z 174 (MH^+); UV (aqueous) λ_{max} 280 nm; LC retention time 18.5 min]. Another distinct product formed almost quantitatively when 6 was reduced by the same method; it was assigned the structure 1-methyl-4-amino-5-phenylimidazole [MS, m/z 174; UV λ_{max} 280 nm; retention time 22.5 min].

Results and Discussion

Metronidazole and related nitroimidazole drugs are apparently activated as the result of nitro group reduction,[2,3] a reaction that has been proposed to proceed through a series of potentially active intermediates analogous to those that form in the reduction of such nitro aromatic compounds as nitrobenzene.[20] Although the intermediates shown in Scheme I are consistent with the available evidence, it is uncertain which, if any, has biological activity. Thus, although the nitroimidazole radical anion (II, Scheme I) appears from EPR studies[21,22] to form

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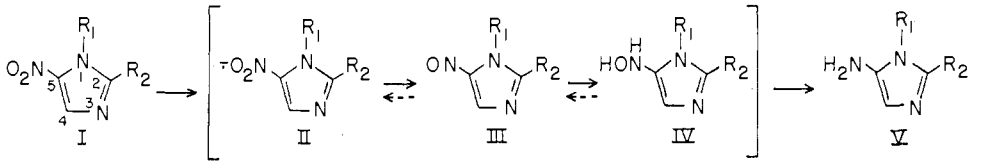
Scheme I[a]



[a] Reduction of therapeutic 5-nitroimidazoles I to the amine V proceeds potentially through the radical anion (II), the nitroso (III), and the hydroxyamino (IV) intermediates. At least one of these intermediates (in brackets) is proposed to be the biologically active form of I. Metronidazole is structure I with $R_1 = CH_2CH_2OH$ and $R_2 = CH_3$.

Table III. Minimal Inhibitory Concentration of Various Nitroimidazoles[a]

strain (phenotype)[b]	MIC, mM			
	2	4	6	metronidazole
B. fragilis	0.03	<0.002	0.5	0.006
C. perfringens	0.25	0.015	>0.5	0.013
E. coli AB1157 (Uvr^+Rec^+)	>0.5	>0.5	>0.5	>0.5
E. coli SR58 ($RecA^-UvrB^-$)	0.13	0.008	0.5	0.1
E. coli AB1885 ($UvrB^-Rec^+$)	>0.5	>0.5	>0.5	>0.5
E. coli EE128 (Uvr^+RecA^-)		0.03	>0.5	0.6
E. coli EE349 ($UvrB^-Rec^+$)		0.10	≥0.5	>0.6

[a] Determined under anaerobic conditions as described in the Experimental Section. [b] For further details, see Yeung et al.[23]

under certain conditions, it has not been possible to associate this labile intermediate with any of the biological activities of the nitroimidazoles (or nitrofurans). The 5-aminoimidazole (V), on the other hand, has been synthesized and found to form as the result of bacterial reduction of a nitroimidazole; yet it does not have the biological activities of the nitroimidazoles.[4] Other possible intermediates, e.g., the 5-nitroso (III) and the free 5-hydroxyamino (IV) functionality,[5] have never been investigated directly because they have never been prepared.

Minimal inhibitory concentrations (MIC) of the nitroimidazoles are compared under anaerobic conditions for several metronidazole-sensitive and -resistant strains in Table III. The 5-nitroimidazole **4** is found to be only up to approximately 20 times more potent than metronidazole. Furthermore, **4** is generally considerably more potent than the 4-nitroimidazole **6**, a relationship that is consistent with past experience.[9,10] As might be expected because it is a tautomer, **2** tends to have antibacterial potency between that of **6** and that of **4**. The MIC is not useful for testing the antibacterial potency of the nitrosoimidazoles, however, because such compounds decompose so rapidly under the test conditions (Table II) that their inhibitory effect on bacterial growth is lost long before the 1–2-day MIC test is completed.

The antibacterial activities of the various nitro- and nitrosoimidazoles were therefore monitored in terms of their effects on the viability of anaerobic stationary-phase cultures of *Escherichia coli* strain SR58, a strain whose defective DNA repair system makes it sensitive to metronidazole[23] (Figure 1). In this test system the nitroimidazoles cause a monotonic loss of bacterial viability that continues for several hours; 0.1 mM **4** is found to have approximately the same bactericidal activity as 0.6 mM metronidazole and greatly surpasses that of 1.0 mM **6** (a concentration of **6** that is close to its limit of solubility in this medium). In this test system **2** has a bactericidal activity between those of **4** and **6**.

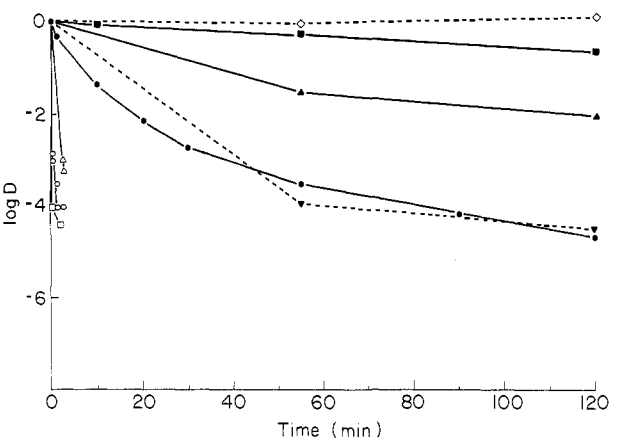


Figure 1. The effect of various concentrations of nitroimidazoles and nitrosoimidazoles on the viability of stationary-phase cultures (5×10^8 cfu/mL) of *E. coli* SR58. Bacteria were incubated anaerobically at 37 °C in BHI broth with the compounds indicated and assayed periodically for viability. The decrease in viability (*D*) is the ratio of the concentration of surviving bacteria at any time to the concentration of viable bacteria at the start of the experiment. Symbols: ◇, no addition; ▼, 0.6 mM metronidazole; △, 0.05 mM **1**; ▲, 0.5 mM **2**; ○, 0.002 mM **3**; ●, 0.1 mM **4**; □, 0.02 mM **5**; ■, 1.0 mM **6**.

Although the 5-nitroimidazoles used clinically are generally substituted only at the N-1 and C-2 positions,[8] the results in Figure 1 indicate that a substituent at C-4, as in **4**, does not necessarily interfere with bactericidal activity. Indeed the potencies of **2** and **4** are quite similar to that of metronidazole for a variety of bacterial strains (Table III). Thus **2** and **4**, like metronidazole, appear to serve as reservoirs from which the actual mediator of bactericidal activity is generated, presumably by the action of nitroreductase. It seems reasonable, therefore, that **4** is representative of such therapeutic nitroimidazoles as metronidazole and that the activation of **2** and **4** proceeds by a mechanism analogous to that by which metronidazole is activated.

The data in Figure 1 also illustrate that the potency of the nitrosoimidazoles is enhanced over that of their corresponding nitroimidazoles. Thus, for example, 2 μM **3** has essentially the same bactericidal effect within 2 min as 100 μM **4** has after 2 h, and the potency of **1** is similarly greater than that of **2**. The comparison of potency between **5** and **6** is particularly striking, as the latter compound is essentially devoid of bactericidal activity. In these experiments it was found that the nitrosoimidazoles caused no further change in bacterial viability after the first few minutes (presumably because they decompose so rapidly in bacterial cultures, Table II); hence, to simplify the presentation of data, bacterial viability after the first 5 min of exposure to the nitrosoimidazoles has been omitted from Figure 1.

The bactericidal activities of the nitroimidazoles and their corresponding nitrosoimidazoles were also compared

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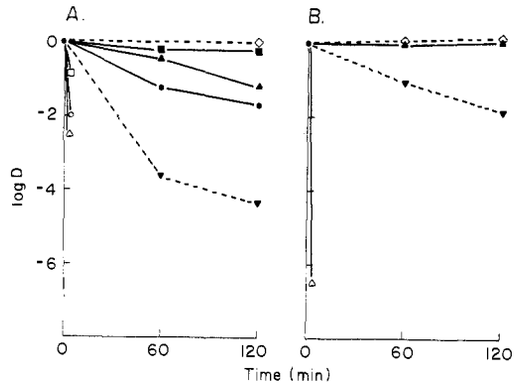

Figure 2. The effect of various nitroimidazoles and nitrosoimidazoles on the viability of stationary-phase cultures of *B. fragilis*. Bacteria were incubated at 37 °C in BHIS medium under anaerobic conditions in the presence of the compounds indicated and assayed at times indicated for bacterial viability. The decrease in viability (*D*) is defined as in the legend to Figure 1. A. Cultures of wild-type *B. fragilis* in stationary phase (5×10^9 cfu/mL) were treated as follows: ◇, no addition; ▼, 0.6 mM metronidazole; △, 1.0 mM **1**; ▲, 1.0 mM **2**; ○, 0.5 mM **3**; ●, 0.5 mM **4**; □, 0.5 mM **5**; ■, 5.0 mM **6**. B. Cultures of *B. fragilis* resistant to metronidazole in stationary phase (5×10^8 cfu/mL) were treated under similar conditions with some of the same compounds, and the data are portrayed with the same symbols as in panel A.

by using *Bacteroides fragilis*, an anaerobe known to be sensitive to metronidazole[24] (Figure 2A). The results with these bacteria confirm qualitatively the findings with *E. coli*. The less striking relative potency of the nitrosoimidazoles in the *B. fragilis* system can be attributed to the supplemented brain–heart infusion medium (BHIS), which contains cysteine and dithiothreitol, compounds known to react rapidly with the nitrosoimidazoles. (The nitroso compounds completely lost their bactericidal activity when incubated for 5 min in BHIS medium before the test bacteria were added.) Nevertheless, a metronidazole-sensitive strain of *Clostridium perfringens*,[25] although cultured in BHIS medium, was also found to be significantly more sensitive to each nitrosoimidazole than to its corresponding nitroimidazole.

The bactericidal activity of the nitrosoimidazoles seems to be restricted to those bacteria that are sensitive to metronidazole. Thus *E. coli* strain AB1157, the wild-type progenitor of the sensitive SR58 strain,[23] was resistant to the nitrosoimidazoles at concentrations up to 0.5 mM (not shown), in addition to metronidazole and **2** (Table III). One notable exception is a strain of *B. fragilis* with diminished nitroreductase activity.[24] This strain has diminished sensitivity to metronidazole[26] and to **2** (Figure 2B), but, like the metronidazole-sensitive strain of *B. fragilis*, is very sensitive to nitrosoimidazole **1**. Such a result would be expected, however, because resistance to metronidazole in this case is presumably the result of the loss of nitroreductase activity and hence due to the loss of the ability to generate the drug's active species. (Under such circumstances it might be expected that both strains of *B. fragilis* would be equally susceptible to **1**. It must be recognized, however, that the two strains of *B. fragilis* are independent isolates, which are not necessarily genetically related; hence the two strains cannot be assumed to be equally sensitive to the active form of the nitroimidazoles.)

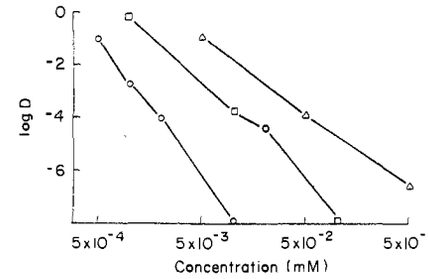

Figure 3. The decrease in viability of *E. coli* SR58 when incubated in stationary-phase culture with various concentrations of nitrosoimidazoles. Incubations were at 37 °C in BHI medium under anaerobic conditions with the compounds indicated. The decrease in viability (*D*) is expressed as described in the legend to Figure 1. The compounds tested were **1** (△), **3** (○), and **5** (□).

The bactericidal effects of various concentrations of **1**, **3**, and **5** on anaerobic cultures of *E. coli* SR58 in stationary phase are compared on the basis of a decrease in bacterial viability measured 15 min after the addition of the nitrosoimidazole (Figure 3), a time previously shown to be sufficient for the nitrosoimidazole to decompose completely. Under these conditions, the culture's viability decreases more than 10^6-fold after exposure of the culture to either 0.1 mM **5** or 0.01 mM **3**. At other concentrations as well, it appears that **3** is consistently approximately 10-fold more potent than **5**, a difference in potency that may be underestimated because **3** seems to decompose more rapidly than **5** under these conditions (Table II). Nevertheless, in spite of this consideration and the uncertainty in measuring the concentration of **5** (see Materials), it is clear that all three nitrosoimidazoles are quite potent against *E. coli* SR58 and that both **5** and **3** are more potent than **1**. Therefore the relative potency of the three nitrosoimidazoles is not comparable to that found for their homologous nitroimidazoles (Table III, Figure 1).

The presence of oxygen is known to interfere with the reductive activation of the nitroimidazoles,[2,3,21,22,27] whereas the effect of oxygen on the activity of the nitrosoimidazoles cannot be predicted. The effect of oxygen can be inferred, however, by comparing a compound's bactericidal activity under both aerobic and anaerobic conditions in tests with a facultative aerobe such as *E. coli*. In planning such tests it must be recognized that *E. coli* have the ability to scavenge dissolved oxygen and hence at high densities to make a liquid culture anaerobic even when it is exposed to air. Thus, in an initially well-aerated culture of *E. coli* SR58 in stationary phase (about 10^9 cfu/mL), the potency of the nitroimidazoles was found after a few minutes to be essentially indistinguishable from potency measured under conditions that were deliberately made anaerobic.

The bactericidal effect of the nitrosoimidazole **1** was therefore compared to that of the nitroimidazole **2** under experimental conditions designed to ensure that *E. coli* were well exposed to air, namely, by suspending them with the test compound in agar, which was then spread across the surface of petri plates in a thin (<0.4 mm) layer. This procedure ensured that anaerobic conditions did not develop during incipient colony formation and also allowed **1** to exert its bactericidal effect before it decomposed. Under these conditions (Figure 4), the bactericidal effect of **2**, which had been observed under anaerobic conditions

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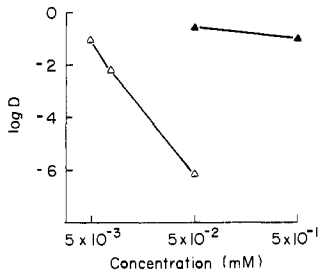

Figure 4. The effect on viability of treating *E. coli* SR58 with various concentrations of **1** and **2** under aerobic conditions. Bacterial cultures in agar ($10-10^9$ cfu/mL) were treated with **1** (△) or **2** (▲) at the concentrations indicated. After the plates had been incubated aerobically overnight at 37 °C, the colonies that developed were enumerated. Further details are found in the Experimental Section. The decrease in viability (D) is defined in the legend to Figure 1.

(Figure 1), was essentially eliminated, whereas the potency of **1** was somewhat greater than that found under anaerobic conditions (Figure 3). This greater measured potency of **1** under aerobic test conditions cannot be attributed to an alteration in the stability of **1**, because the compound decomposes in a bacterial culture under aerobic conditions with a half-life (1–2 min) that is indistinguishable from that observed under anaerobic conditions (1–3 min, Table II).

Although the 5-nitrosoimidazole **3** appears from these assays to be between 2 and 5 orders of magnitude more bactericidal than the active 5-nitroimidazole **4**, the limitations of these estimates must be recognized. First, the nitrosoimidazoles are degraded rapidly in the bacterial culture and so their potency tends to be underestimated. Second, the nitroimidazoles and the nitrosoimidazoles clearly have different requirements for activation; hence it is difficult, for theoretical reasons, to compare their potencies. In this connection the nitrosoimidazoles, possible active forms of the nitroimidazoles, may have a different effect when added to the bacterial medium than when they are generated intracellularly by bacterial nitroreductases. Nevertheless it can be concluded that the nitrosoimidazoles act more rapidly and are several orders of magnitude more potent than the comparable nitroimidazoles.

That **4** is found to be considerably more potent than **6** is also consistent with the properties of 4- and 5-nitroimidazoles studied previously. Thus others have postulated that the low potency of the 4-nitroimidazoles is associated with their more negative one-electron-reduction potential and hence their greater resistance to reductive activation.[28-30] The similarity in the bactericidal activities of the 4-nitrosoimidazole **5** and the 5-nitrosoimidazole **3** is therefore consistent with the view that these compounds are distal to the limiting step in nitro group activation.

Compounds **1**, **3**, and **5**, which are formally two-electron-reduction products of nitroimidazoles, may actually be the bactericidal forms of their respective homologous nitroimidazoles. Indeed these nitrosoimidazoles have the characteristics previously deduced[2,3] for the active form of metronidazole, namely, they are derived from a nitroimidazole by nitro group reduction, they decompose rapidly in the bacterial environment, and they are rapidly lethal for bacteria sensitive to 5-nitroimidazoles. Furthermore, the superior potency of a nitrosoimidazole compared to its corresponding nitroimidazole is compatible with the expected inefficiency of the reductive activation of the nitroimidazoles.[2-4,25] In addition, the rapid reaction of these nitroso compounds with sulfhydryl compounds may explain why sulfhydryl compounds, which react only slowly with metronidazole under biological conditions,[31,32] can nevertheless diminish the bactericidal effects of metronidazole.[32] Finally, the nitrosoimidazoles are extremely bactericidal under both aerobic and anaerobic conditions, suggesting that they require neither oxidation nor reduction to be activated.

Other evidence tends to exclude other proposed intermediates (Scheme I) as the active species resulting from reduction of the nitroimidazoles. Thus, the observation that **1** is at least as bactericidal under aerobic conditions as under anaerobic conditions makes the radical anion (II, Scheme I) an unlikely ultimate mediator of the bactericidal effect, because the radical anion should not form so readily under both reducing and oxidizing conditions. In addition, the radical anion reacts rapidly with oxygen to yield a nitroimidazole, in what has been described as a "futile cycle";[21,22] furthermore, the radical anion of other nitro heterocyclic antibiotics reacts neither with reduced glutathione nor with DNA.[33] There is little direct evidence regarding the possibility that the hydroxyamino functionality (IV, Scheme I) is the ultimate mediator of the biological activity of the nitroimidazoles. However, a 2-(hydroxyamino)imidazole (the four-electron-reduction product of a 2-nitroimidazole) has been synthesized and found to require the presence of oxygen in order to become biologically active,[34] suggesting that the 2-hydroxyamino functionality is more reduced than the biologically active form. All such evidence is indirect, however, and it must be recognized that our assay, based on bacterial viability, is not sufficiently sensitive to detect electron transport or other fast processes that might be necessary to convert a nitrosoimidazole to its active form.

In summary, our results are consistent with the possibility that the 5-nitrosoimidazole **3** is the active form of **4** as well as with the more general inference that the nitrosoimidazole is the active form of the 5-nitroimidazole drugs used therapeutically. It must be emphasized, however, that our findings do not show that the nitrosoimidazoles are the ultimate active form of the therapeutic nitroimidazoles. Indeed there is no indication that only one intermediate is responsible for all the various biological effects of these compounds. A conservative interpretation of the existing data is simply that these nitrosoimidazoles are more facilely converted to the biologically active form than are the corresponding nitroimidazoles, a further indication that the initial reduction of the nitroimidazoles is the limiting step in their activation.

Experimental Section

Synthetic Methodology. Ultraviolet (UV) and visible spectra were recorded by using a Hewlett-Packard (Palo Alto, CA) Model 8451A diode-array spectrophotometer. ^{1}H NMR spectra (80 MHz) were recorded on a Varian CFT20 Fourier-transform NMR

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spectrometer and mass spectra (MS) with a VG Analytical ZAB-SE mass spectrometer using ammonia chemical ionization. Melting points, uncorrected, were determined with an Arthur H. Thomas Co. (Philadelphia, PA) Model 40 micro hot stage apparatus.

High-pressure liquid chromatography (LC) using a C-8 reverse-phase analytical column (Supelco, Inc., Bellefonte, PA, Cat. No. 5-8297) was performed either on a Hewlett-Packard 1082B liquid chromatograph equipped with a 1040A diode-array UV spectrophotometric detector or on a Waters Associates (Milford, MA) high-pressure liquid chromatograph system with a UV absorbance detector (Model 440). Data were recorded and areas under the curve computed by a Hewlett-Packard 52398B LC terminal. Unless otherwise noted, compounds were eluted (1.5 mL/min) by means of a solvent program that began isocratically with 5% methanol in 0.1 M phosphate buffer at pH 7.4 for 3 min and then increased linearly to 50% methanol during 20 min. Preparative reverse-phase liquid chromatography, at a flow rate of 6.0 mL/min with the same solvent program, was carried out with a Supelco C-18 semipreparative reverse-phase column (Supelco Cat. No. 5-8368).

Materials. **Metronidazole** was a gift of G. D. Searle and Co. (San Juan, Puerto Rico).

4(5)-Nitro-5(4)-phenylimidazole (2) was synthesized from 4(5)-(*p*-aminophenyl)-5(4)-nitroimidazole (prepared by the method of Grant and Pyman[35]) by using the general procedure of Robison and Robison.[36] To a solution of 500 mL of concentrated sulfuric acid and 250 mL of water at 0 °C was added 19 g (0.27 mol) of sodium nitrite. The solution was cooled to −5 °C, after which 119 g (152 mL, 0.9 mol) of 50% hypophosphorous acid was added. Gas evolution and foaming of the solution was controlled by adding the reagents cautiously. The solution was then cooled and kept at −10 to −15 °C, while 10.2 g (0.05 mol) of 4(5)-(*p*-aminophenyl)-5(4)-nitroimidazole, which had been suspended (partially dissolved) in 500 mL of glacial acetic acid, was added. The solution was allowed to remain for 2 h at −10 °C and then at 5 °C for 48 h, during which time some gas evolved and the solution turned yellow. The solution was adjusted to pH 3 with 50% sodium hydroxide (added slowly at 0 °C), to yield a brown precipitate. More precipitate, including salts, formed while the solution remained at 5 °C. The solid was collected by filtration, washed 10 times with 500 mL of water, and dried for 24 h at <1 Torr, to give approximately 10 g of a brown product (mp 280–290 °C). A 500-mg portion of this crude product was sublimed (1 Torr and 200 °C) to yield 150 mg of light yellow microscopic needles (mp 295–300 °C, sublimation >180 °C), which indicated an overall yield of 30%: NMR (Me_2SO-d_6) δ 7.90 (s, 1 H, C_2H), 7.4–7.6 (m, 5 H, phenyl H), 3.3 (br s, >10 H, HOD + NH); MS, m/z 160 (MH^+ − NO, 100), 207 (MNH_4^+, 95), 190 (MH^+, 50); UV (EtOH) λ_{max} 228, 318 nm.

1-Methyl-4-phenyl-5-nitroimidazole (4) was prepared by treatment of 100 mg of 4(5)-nitro-5(4)-phenylimidazole (mp >290 °C) under neutral conditions as described by Walsh et al.[37] The crude product, a deep yellow oil, was added to a 12.5 × 2.5 cm silica gel column (BioSil A, 100–200 mesh, BioRad Laboratories, Richmond, CA) and eluted with 10 column volumes of ether, yielding two yellow bands. When the contents of the second band were collected and dried in air after removal of the ether, there remained 100 mg of pale-to-bright-yellow microscopic rectangular platelets, mp 65–70 °C, for a yield of 90%. Analysis of this material by LC indicated that it contained less than 0.1% of ultraviolet-absorbing compounds, including **2** and **6**. Recrystallization of this material from dimethoxyethane/water afforded thick yellow needles, mp 73–75 °C (lit.[7] mp 74.5–75.5 °C), with a recovery of slightly less than 50%: NMR ($CDCl_3$) δ 7.7–7.8 (m, 2 H, phenyl H), 7.4–7.5 (m, 3 H, phenyl H), 7.55 (s, 1 H, C_2H), 4.00 (s, 3 H, Me); MS, m/z 204 (MH^+, 100), 221 (MNH_4^+, 70), 174 (MH^+ − NO, 10), 159 (MH^+ − $NOCH_3$, 5); UV (EtOH) λ_{max} 228, 266, 328 nm.

1-Methyl-4-nitro-5-phenylimidazole (6) was prepared by treatment of 10 mg of 4(5)-nitro-5(4)-phenylimidazole (mp >290 °C) with methyl iodide under basic conditions as described by Walsh et al.[37] After recrystallization of crude product (a light-green solid) from ether, 5 mg (45%) of small colorless platelets, mp 178–180 °C (sublimation >150 °C), was obtained: NMR, mass spectroscopic, and LC analysis failed to detect any impurity, including **4**; NMR ($CDCl_3$) δ 7.45–7.55 (m, 6 H, phenyl H and C_2H), 3.53 (s, 3 H, Me); MS, m/z 221 (MNH_4^+, 100), 238 (MH-$[NH_3)_2^+$, 70), 204 (MH^+, 60), 174 (MH^+ − NO, 50), 159 (MH^+ − $NOCH_3$, 5); UV (EtOH) λ_{max} 238, 300 nm.

4(5)-Nitroso-5(4)-phenylimidazole (1) was prepared from 9.9 g of 4(5)-phenylimidazole as described by Brothers and McClelland,[11,17] except that the crude product was recrystallized twice from 15 mL of acetone, affording 7.5 g (62% yield) of small metallic green platelets, which discolored suddenly at 195 °C and further decomposed at 210 °C: NMR (Me_2SO-d_6) δ 8.12 (s, 1 H, C_2H), 8.4–8.5 (m, 2 H, phenyl H), 7.55–7.65 (m, 3 H, phenyl H), 3.3 (br s, >10 H, HOD, NH); MS, m/z 160 (MH^+ − N, 100), 191 (MNH_4^+, 70), 174 (MH^+, 60); UV (0.1 mM in 1% Me_2SO/H_2O) λ_{max} 240, 360 (ϵ 7600), 670 (20–50) nm.

1-Methyl-4-phenyl-5-nitrosoimidazole (3) and 1-methyl-4-nitroso-5-phenylimidazole (5) were prepared by methylation of 1 under slightly basic conditions.[14] A mixture of 10 mg (0.058 mmol) of 4(5)-nitroso-5(4)-phenylimidazole (dec >190 °C), 4.0 μL (9.2 mg, 0.066 mmol) of methyl iodide (99.5%, Aldrich Chemical Co.), and 5.2 mg of potassium carbonate was partially dissolved in 1 mL of acetone and then refluxed in a screw-capped vial for 30 min. The solution became green-brown after 2 min and red-brown after 0.5 h; when cooled and filtered it was found by LC to contain **3** and **5** (structural assignments are described under Chemistry) in the ratio of 5:1, as well as a small amount of **1**. The two products were separated by preparative reverse-phase LC (see Table I), and the combined yield of the isolated products was 40–50% (see below).

One fraction yielded 4 mg of **3** in the form of a bright green powder, mp 118–125 °C (sublimation >100 °C), that by LC contained <0.1% of either **1** or **5** and whose overall purity, as estimated by LC and NMR, was greater than 90%. Compound **3** was further purified by sublimation (1 Torr, 100 °C) to yield microscopic green needles: mp 128–130 °C (sublimation >100 °C); NMR ($CDCl_3$) δ 8.40–8.55 (m, 2 H, phenyl H), 7.55 (s, C_2H), 7.45–7.55 (m, 4 H, phenyl H, incl C_2H), 3.66 (s, 3 H, Me); MS, m/z 174 (MH^+ − N, 100), 172 (MH^+ − NH_2, 65), 188 (MH^+, 45), 205 (MNH_4^+, 15), 159 (MH^+ − NCH_3, 15); UV (0.1 mM in 1% Me_2SO/H_2O) λ_{max} 230, 312, 364 (ϵ 8600), 670 (60) nm.

Another fraction yielded approximately 1 mg of **5** as a light green amorphous powder, which decomposed at 180–185 °C. Because analysis by LC and NMR indicated that the purity of this material in different preparations varied between 50% and 80%, the molar extinction coefficient cited below can only be estimated. Nevertheless, LC analysis indicated that no preparation of **5** contained detectable amounts (<0.1%) of either **1** or **3**; that no other compound had UV absorption above 300 nm indicated that other nitrosoimidazoles were not among the contaminants. Furthermore, all batches of **5** had the same bactericidal potency when the concentration of **5** was determined from the UV absorption maximum at 342 nm: NMR ($CDCl_3$) δ 8.2–8.4 (m, 2 H, phenyl H), 7.5–7.8 (m, 4 H, phenyl H and C_2H), 3.81 (s, 3 H, Me); MS (impure sample), m/z 159 (MH^+ − NCH_3, 100), 205 (MNH_4^+, 90), 174 (MH^+ − N, 70), 188 (MH^+, 60); UV (~0.1 mM in 1% Me_2SO/H_2O) λ_{max} 228, 290 (sh), 342 (ϵ 8000 ± 2000), 670 (30–50) nm.

Bacterial Cultures. Test bacteria included two strains of *B. fragilis*, which differed in their sensitivity to metronidazole,[24,26] a strain of *C. perfringens*,[25] and strains of *E. coli* that included the wild type (AB1157) and several DNA repair mutants (SR58, AB1885, EE128, and EE349) with known genotypes.[23]

E. coli strains were cultivated anaerobically in brain–heart infusion (BHI) medium (BBL Microbiology Systems, Cockeysville, MD) or aerobically in trypticase soy medium (BBL Microbiology Systems). The anaerobes *B. fragilis* and *C. perfringens* were cultivated in supplemented brain–heart infusion medium (BHIS), a BHI medium autoclaved with 10 μg/mL hemin (Nutritional Biochemical Co.) and 0.5 μg/mL vitamin K_1 (Sigma Chemical Co.) to which was added a filtered (0.45-μm Millex) aqueous solution

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of L-cysteine and dithiothreitol, so that the final concentrations of these ingredients were 500 μg/mL and 300 μg/mL, respectively.

An anaerobic environment for bacterial incubations was provided in Gaspak jars containing palladium catalysts for scavenging oxygen (BBL Microbiology Systems), either by using Gaspak envelopes (BBL Microbiology Systems) or by sealing the Gaspak jars inside a glovebox made anaerobic with a gas mixture consisting of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Culture media used for propagation of bacteria under anaerobic conditions were stored in the anaerobic glovebox and after inoculation were flushed with an anaerobic gas mixture (85% argon, 10% hydrogen, 5% carbon dioxide) dispensed through a V.P.I. anaerobic culture system (Bellco Glass, Inc., Vineland, NJ).

Since test compounds were solubilized in dimethyl sulfoxide (Me_2SO) in order to achieve the desired concentrations in the culture media, control media always included appropriate concentrations of Me_2SO (either 1% or 5%). Such concentrations of Me_2SO were shown in other experiments to have no effect on bacterial growth or viability.

Antibacterial Studies. The minimal inhibitory concentration (MIC) of each nitroimidazole was determined by diluting the compound serially in 2-fold steps with BHIS medium containing 5% Me_2SO and dispensing aliquots (0.5 mL) of each dilution into the microwells of multiwell plates (Becton Dickinson and Co., Oxnard, CA). Each microwell was then inoculated with 50 μL of a bacterial suspension (10^7 cfu/mL) obtained from a bacterial culture grown to stationary phase. The plates were incubated anaerobically at 37 °C in Gaspak jars, and growth was determined after 17 h.

The effects of test compounds on the viability of bacteria were evaluated by using two methods. In one, from 1 to 5 mL of a bacterial culture, in stationary phase, was incubated with a test compound under anaerobic conditions in a butyl-rubber-stoppered culture tube maintained at 37 °C. Periodically, aliquots were withdrawn, diluted serially in 10-fold steps with 0.9% saline, and viable bacteria assayed by enumerating the number of colonies formed on solid media after incubation at 37 °C for 24–48 h under either anaerobic or aerobic conditions. To assay bacterial viability at time points less than 1 min, we terminated the bactericidal effect by diluting the reaction mixture with 100 volumes of 0.9% saline. That this dilution effectively terminated the bactericidal activity of the nitrosoimidazoles was shown when the same relative bacterial survival was observed in parallel experiments that employed a diluent containing cysteine and dithiothreitol at concentrations known to cause the rapid decomposition of nitrosoimidazoles[11,17] (Table II).

The second method of assaying bacterial survival was designed to ensure that the bacteria were exposed to the test compound under conditions that were as aerobic as possible. For this procedure, a culture of *E. coli* SR58 in stationary phase was diluted serially in 10-fold steps (between 10 and 10^9 cfu/mL). Aliquots (200 μL) of these culture dilutions were combined with 25-μL aliquots of test compound dissolved at various concentrations in Me_2SO and 2.5 mL of a molten agar solution (0.6% agar in 0.9% saline maintained at 45–50 °C). Such combinations of test bacteria and test compounds in agar solution were then poured over the surface of a petri plate (100 × 15 mm) containing trypticase soy agar, and the plates were incubated aerobically overnight at 37 °C. The concentrations of bacteria used with a given concentration of test compound were varied in making up such plates to ensure that at least one plate after incubation would contain a number of colonies (30–300) that was satisfactory for enumeration.

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