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Potential Radiosensitizing Agents. Dinitroimidazoles[1]

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New compounds of the nitroimidazole series have been synthesized as radiosensitizers which selectively sensitize hypoxic cells to the lethal effect of radiation. The reaction of 2,4(5)-dinitroimidazole (**2**) with chloroethanol or hydrochloric acid yielded 4(5)-nitro-5(4)-chloroimidazole (**3**), which upon reaction with ethylene oxide yielded the 4-nitro-5-chloroimidazole-1-ethanol (**6**). Reaction of **2** with ethylene oxide resulted in a mixture of two compounds, the 2,4-dinitroimidazole-1-ethanol (**4**) and 2,3-dihydro-5-nitroimidazo[2,1-*b*]oxazole (**5**). The structure of the new heterocyclic compound **5** was confirmed by ^{1}H NMR, mass spectrum, and X-ray crystallography. These agents were tested for their ability to sensitize hypoxic *Escherichia coli* cells to killing by ionizing radiation. Compound **4** was found to be the most active agent of this series of compounds.

In the radiotherapy of cancer, the relative resistance of hypoxic cells present in solid tumors is a serious limitation in attempts to increase the therapeutic ratio between tumor and normal tissue damage.[2] Ionizing radiation inactivates cells in solid tumors according to a multicomponent survival curve which is related to the variation in oxygen concentration within the tumor mass.[3] Employment of hyperbaric oxygen chambers, fast neutrons, or π^- meson beams are being considered as approaches to overcome this problem of hypoxic resistance in radiotherapy. However, another logical approach which has received more attention recently is that of chemical sensitizers designed to selectively sensitize hypoxic neoplastic cells to the lethal effect of radiation.[4] The rationale for the radiosensitizing drugs is that these agents are not rapidly metabolized and can diffuse from the end of the blood capillaries to the hypoxic cells in tumors. Although the ability of these compounds to sensitize hypoxic cells is directly related to the electron affinity of the molecule, the molecular aspects of the mechanism of action are at present vague. The search for the radiosensitizing compounds was greatly facilitated by the work of Adams and his associates[5-7] and of Chapman and his co-workers[8-12] who showed that a variety of electron-affinic compounds were capable of sensitizing hypoxic bacterial and mammalian cell populations to the lethal effect of radiation.

A series of nitrobenzene analogues have been tested against Chinese hamster cells for their ability to sensitize hypoxic cells to X irradiation.[13] Cellular radiosensitization was found to correlate with drug electron affinity, as measured by the Hammett σ constant.[13] Several nitrofurans were also shown to possess significant radiosensitization properties when tested in vitro.[9] However, both the nitrobenzenes and nitrofurans failed to achieve significant radiosensitization in vivo.[4] A series of nitropyrroles have also been reported recently;[14] the most effective derivative of this class was *N*-(hydroxyethyl)-2-cyano-5-nitropyrrole. These latter studies also indicated that *N*-(hydroxyethyl) substitution decreased toxicity relative to N-CH_3, N-C_2H_5, and N-C_3H_7 substitution. The role of the partition coefficient in radiosensitization of hypoxic cells has also been examined;[15] lipophilicity seemed to have an insignificant effect on in vitro radiosensitization but may possess a greater role in vivo.

Metronidazole (Flagyl), a 5-nitroimidazole derivative, has been reported to sensitize tumor hypoxic cells both in vitro and in vivo.[11,16] Asquith et al.[17] have reported a 2-nitroimidazole (azomycin) derivative, 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol (Ro-07-0582, misonidazole), to be a most effective radiosensitizing agent. Misonidazole has since been found to be an effective radiosensitizer of hypoxic cells in at least 16 different animal tumors[18] and is currently under clinical trials.[19] However, the high doses of misonidazole required for activity were found to be a limiting factor because of resulting neurotoxicity. Convulsions and peripheral neuropathy were encountered in a relatively large number of patients.[19] We, therefore, have undertaken a systematic approach to design and synthesize various analogues of nitroimidazoles in order to study the relationship between structure and biological activity that might lead to a more advantageous sensitizing agent. Initially, a series of nitroimidazoles were examined in *E. coli* cells and compared with misonidazole for toxic side effects on end points such as mutagenesis, cell killing, and inhibition of the synthesis of the inducible enzyme β-galactosidase.[20] These results indicated that 2,4(5)-dinitroimidazole (**2**) was the most promising agent for further study because it exhibited good radiosensitization coupled with low toxicity and mutagenicity.[20] Compound **2** was initially synthesized in an effort to increase the electron affinity of the 2-nitroimidazole nucleus by inserting an additional electron-

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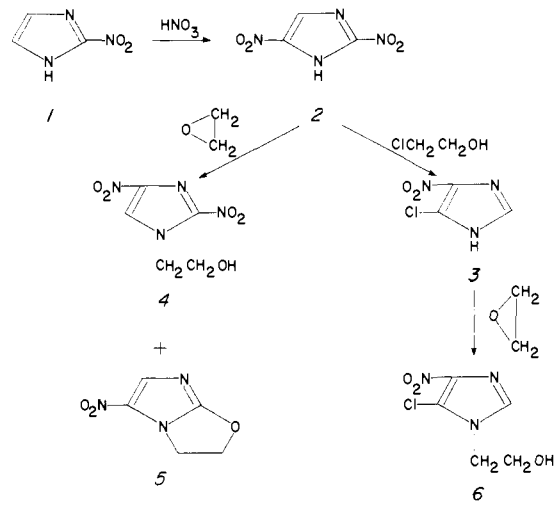
Scheme I



withdrawing group such as NO_2. The electron-affinity has been related to the ability of these compounds to sensitize hypoxic cells toward radiation.[21] Compound **2**, however, is a strongly acidic compound with a pK_a of 2.86 and would be expected to be highly ionized at physiological pH. In the present study, we have therefore extended the modifications of the nitroimidazole nucleus by alkylating the 1 position of **2** to change its strong acidic character. The resulting derivatives were tested for radiosensitization against *E. coli* cells as the in vitro test system.

Chemistry. The synthesis of 2-nitroimidazole (**1**) was accomplished by initially synthesizing 2-aminoimidazole sulfate according to the published procedure of Storey et al.,[22] which was then oxidized by a modification of the procedure of Beaman et al.[23] This modification involved the use of fluoboric acid instead of hydrochloric acid. The reaction under these conditions was complete within 2 h. This procedure also avoided the extensive extractions of large volumes of the reaction mixture. However, subsequently we were able to procure **1** directly from Aldrich Chemical Co., Milwaukee, Wis, which responded to our request of synthesizing **1**.

The electrophilic substitution of **1** by an additional NO_2 group was carried out in the presence of Ac_2O with fuming HNO_3 at 100 °C to yield 2,4(5)-dinitroimidazole[24] (**2**, Scheme I). In an effort to introduce an hydroxyethyl group in **2**, we initially attempted the reaction of chloroethanol with **2** under a variety of conditions by modifying the reaction temperatures and the duration of heating. However, the only product isolated from this reaction was 4(5)-nitro-5(4)-chloroimidazole (**3**), the structure of which was confirmed by proton NMR and mass spectrometry. Similarly, the sodium salt of **2** upon reaction with chloroethanol also yielded **3**. Compound **3** was also obtained upon heating **2** with hydrochloric acid. In another attempt to achieve hydroxyethylation, **2** was reacted with ethylene oxide. Unlike the requirement of a catalytic amount of base in reactions with ethylene oxide, **2** reacted with it directly. Paradoxically, the presence of base seemed to hinder this reaction. The major products isolated from this reaction were 2,4-dinitroimidazole-1-ethanol (**4**, 50%) and 2,3-dihydro-5-nitroimidazo[2,1-*b*]oxazole (**5**, 18%). A minor, yellow-colored polymeric product was also formed, which did not melt up to 350 °C and was insoluble in most organic solvents. No attempts were made to further characterize this polymeric material. The structure of **5** was initially assigned as the imidazo[2,1-*b*]oxazole nucleus based upon proton NMR and mass spectral data and was finally confirmed by X-ray crystallographic studies.

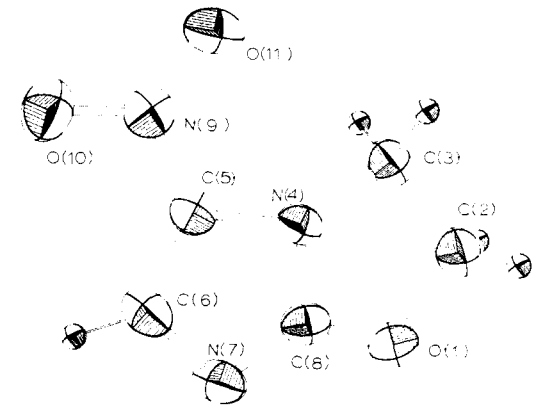


Figure 1. Stereodrawing of 2,3-dihydro-5-nitroimidazo[2,1-*b*]-oxazole.

Subsequently, the reaction of ethylene oxide with **2** has been found to be of a general type; a number of substituted oxiranes reacted with **2** to produce the corresponding isomeric imidazo[2,1-*b*]oxazoles and 1-(2-hydroxyalkyl)-2,4-dinitroimidazoles.[25]

The mixture consisting of **4** and **5** was separated by column chromatography utilizing silica gel. The imidazo[2,1-*b*]oxazole **5** was eluted with chloroform and was found to be unstable upon heating in various solvents. The analytically pure sample was obtained by crystallization from ethyl ether in a cold room. Compound **5** was found to decompose gradually to the yellow polymeric material if left in chloroform solution at room temperature overnight. Reaction of **3** with ethylene oxide resulted in the formation of its 1-(2-hydroxyethyl) analogue **6** in the presence of a catalytic amount of sodium hydroxide. This reaction did not occur in absence of the base.

X-ray Studies. The structure of **5** was solved by multiple tangent refinement using 200 E calculated from a Wilson plot. Isotropic refinement of the 11 nonhydrogen atoms led to $R = 0.096$. The hydrogen atoms were easily located in a difference Fourier map. Refinement was continued (nonhydrogen atoms, anisotropic temperature factors; hydrogen atoms, isotropic temperature factors) until the largest shift was less than 0.1 its estimated standard deviation. The final R value is 0.042 and the largest peak on a final difference map is 0.15 $e^-/Å^3$. A strong double bond exists between atoms C(8) and N(7) (1.312 ± 0.004 Å, Figure 1). The double bond between C(5) and C(6) (1.360 ± 0.003 Å) is delocalized into the C(5)–N(4) (1.383 ± 0.003 Å) and C(5)–N(9) (1.400 ± 0.003 Å) bonds. Since the hydrogen atoms were unambiguously located on a difference map and behaved nicely during isotropic refinement, there is little doubt about the assignment of their positions.

Biological Results. Compounds **2**, **4**, **5**, and **6** were evaluated for their ability to sensitize hypoxic *E. coli* cells. The biological results are shown in Figure 2. Misonidazole (□) was included as a reference in this test system for comparative study. Control curves for the oxic (●) and hypoxic (x) cell-surviving fraction after irradiation are also shown. Since misonidazole at a 1 mM concentration has been shown to increase the sensitivity of hypoxic *E. coli* cells significantly,[20] the newly synthesized compounds were also tested at this concentration. Under the conditions of irradiation employed in these experiments for radiosensitization, no cell killing was observed when cells were exposed to each of the agents alone at 1 mM concentrations. Also, no increased radiosensitivity was observed in oxic cells in the presence of these agents. Compound **4**, a 2,4-dinitroimidazole derivative (▲), was the most effective

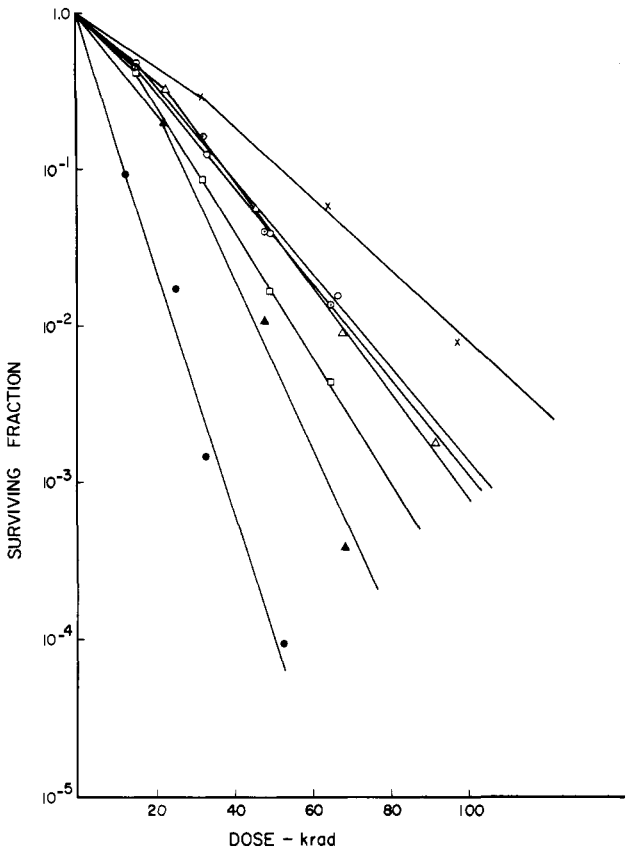


Figure 2. The effect of nitroimidazoles on *E. coli* cells: (●) oxic cells; (○) 1 mM 2,4(5)-dinitroimidazole; (△) 1 mM 2,3-dihydro-5-nitroimidazo[2,1-*b*]oxazole; (⊙) 4-nitro-5-chloroimidazole-1-ethanol; (□) 1 mM misonidazole; (▲) 1 mM 2,4-dinitroimidazole-1-ethanol; (x) hypoxic cells.

agent in increasing the sensitivity of hypoxic cells toward irradiation. It was found to be a more efficient radiosensitizer than misonidazole at equimolar concentration. Compound **2** (○), a strongly acidic compound with a pK_a of 2.86, was less effective as a radiosensitizer. It may be reasoned that **2** would be expected to be highly ionized at pH 7.4, whereas its 1-(2-hydroxyethyl) derivative **4** would not be. Compounds **5** (△) and **6** (⊙) were found to be less effective than **4** in sensitizing hypoxic *E. coli* cells toward irradiation, indicating that the presence of a 2-nitro group in this series of compounds increases the radiosensitizing ability of these agents. It has been shown that the relative effectiveness of the various compounds for hypoxic cell radiosensitization is a function of the electron affinities of the compounds.[26] The electron-spin resonance data on spin distribution in the nitroimidazole radicals indicate that the electron affinities of **2** and **4** are very similar whereas **5** is comparatively less electron affinic.[27] The electron affinity of **4**, as measured by one-electron reduction potential,[28] was found to be −229 mV and was the highest reported to date in this series of compounds. Thus, the high electron affinity of **4** also correlated with the potent radiosensitizing ability of this compound in this test system.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. The infrared spectra were obtained using a Beckman IR 10 spectrophotometer with KBr pellets of solids. The ^{1}H NMR spectra were determined with a Varian A-60 spectrophotometer using Me_4Si as an internal standard. Mass spectra (70 eV) were run on a Hitachi Perkin-Elmer RMU-6E spectrometer using direct-inlet injection. The elemental analyses were performed by Integral Microanalytical Laboratories, Raleigh, N.C. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

Radiosensitization Studies. The radiosensitization studies were carried out in a bacterial system using *E. coli* of strain AB1157. The bacteria were grown in standard bacteriological broth in an exponential growth at the beginning of the experiment. The bacteria were then collected by centrifugation and resuspended in a buffered balanced salt solution with or without the drug at a concentration of approximately 10^6 cells/mL; experimental details have been described earlier.[29] The drugs were dissolved in the buffer to provide solutions of 1 mM concentration. For aerated survival curves, the bacteria were irradiated in suspension in the buffer with air bubbled through the medium throughout the irradiation process. For hypoxic irradiation, the cells were bubbled with nitrogen for 15 min before irradiation and during the irradiation process. After irradiation, the cells were plated on agar plates and assayed for viability by their ability to form colonies.[29] Determination of the potency of the radiosensitizing agents was based upon the reduction of survival of the hypoxic bacterial cells in comparison to the oxic cells in the exponential region of this curve.

X-ray Crystallographic Studies. A single crystal of **5** was grown from a solution in diethyl ether at 4 °C. The crystal showed symmetry and systematic extinctions consistent with the space group $P2_1/c$ with a = 11.736(2), b = 5.320(1), c = 14.148(3) Å, and β = 135.66(1)°. Complete three-dimensional X-ray intensity data were collected on a Picker FACS-I diffractometer using Ni-filtered Cu Kα radiation to a 2θ limit of 125° using a 10:20 scan. The intensities of 983 reflections were corrected for a 7% linear rise in intensity during data collection and for Lorentz polarization and absorption. Of these, 989 had $|F| > 2\sigma(F)$ and were used in the solution and refinement of the structure.

2-Nitroimidazole (1). 2-Aminoimidazolium sulfate[23] (1.57 g, 5.94 mmol) was dissolved in 10 mL of H_2O and 7 mL of 50% fluoboric acid in a round-bottom flask. The solution was cooled to −20 °C in an ice–salt bath. A solution of sodium nitrite (4.1 g, 59.4 mmol) in 10 mL of H_2O was added dropwise to the cooled 2-aminoimidazolium sulfate solution. The mixture was stirred at −10 °C for 30 min and then added to a solution of $CuSO_4 \cdot 5H_2O$ (29.7 g, 119 mmol) in 200 mL of H_2O. An additional 4.1 g of sodium nitrite was added to this mixture and allowed to stir for 2 h at room temperature. The pH of the mixture was then adjusted to approximately 2.0 with dilute HNO_3. The mixture was extracted with EtOAc (200 mL) in a liquid–liquid extraction apparatus. The EtOAc layer was dried (Na_2SO_4) and the solvent removed in vacuo to leave a yellow-colored residue, which was collected and washed with 10 mL of Et_2O to yield 0.6 g (45%), mp 279–283 °C dec. Recrystallization from EtOH raised the melting point to 286–288 °C (lit.[23] mp 287–288 °C).

2,4(5)-Dinitroimidazole (2). A mixture of 3 mL of HNO_3 (90%) and 1 mL of Ac_2O was cooled to 0 °C in an ice–salt bath. Compound **1** (0.226 g, 2 mmol) was added in small portions to this mixture while stirring. The solution was heated for 2 h at 100 °C and then for 30 min at 115 °C. The solution was cooled, poured into 30 mL of ice–H_2O, and extracted with EtOAc (3 × 30 mL). The EtOAc layer was dried (Na_2SO_4), solvent was removed in vacuo, and the pale yellow colored residue was collected and washed with 10 mL of cold Et_2O to yield 0.2 g (63%): mp 278–280 °C dec, lit.[24] 266–268 °C; IR (KBr) 3100 (NH), 1490 and 1310 cm^{-1} (NO_2); ^{1}H NMR (Me_2SO-d_6) 8.62 (s, $C_{4(5)}$-H), 9.57 ppm (br s, NH); mass spectrum m/e 158 (M^+). Anal. ($C_3H_2N_4O_4$) C, H, N.

4(5)-Nitro-5(4)-chloroimidazole (3). A solution of **2** (0.38 g, 2.4 mmol) in 12 mL of chloroethanol was refluxed at 135 °C for 24 h. The excess of chloroethanol was then removed in vacuo and the residue was extracted with EtOAc. The solvent was dried ($MgSO_4$) and removed in vacuo to give **3**, which was recrytallized (EtOAc) to yield 0.226 g (64%) of white crystals: mp 222 °C, lit.[24] 214–216 °C; IR (KBr) 3013 (NH), 1490 and 1360 cm^{-1} (NO_2); ^{1}H NMR (Me_2SO-d_6) 8.3 (s, C_2-H), 7.55 ppm (br s, NH); mass spectrum m/e 147 (M^+). Anal. ($C_3H_2ClN_3O_2$) C, H, N.

Compound **3** was also conveniently synthesized by refluxing **2** (1.11 g, 7 mmol) in 20 mL of concentrated HCl for 4 h. Upon cooling, **3** crystallized out and then filtered to yield 0.655 g (64%).

Reaction of 2,4(5)-Dinitroimidazole with Ethylene Oxide. A solution of **2** (1.0 g, 6.3 mmol) in 50 mL of EtOH was heated at 70 °C and 7 mL (0.14 mol) of ethylene oxide was added to the solution. The reaction mixture was refluxed for 2 h, an additional 7 mL of ethylene oxide was added, and then the mixture was stirred overnight at room temperature. The solvent was removed in vacuo to leave a residual oil consisting of a mixture of **4** and **5**. A solution of the residual oil in 5 mL of EtOAc was applied on a silica gel (50 g) column (2.5 × 30 cm) and initially eluted with $CHCl_3$. The first six fractions of 50 mL each were collected, and the solvent was removed in vacuo at 20 °C to leave an oil which was crystallized from Et_2O in a cold room to yield 0.175 g (18%) of **5** in colorless crystals: mp 107–108 °C; IR (KBr) 1500 and 1330 cm^{-1} (NO_2); ^1H NMR ($CDCl_3$) 7.65 (s, C_6-H), 5.22 (t, CH_2O), 4.57 ppm (t, NCH_2); mass spectrum m/e 155 (M^+), 139 (M – O), 109 (M – NO_2). Anal. ($C_5H_5N_3O_3$) C, H, N.

Compound **4** was obtained from the silica gel column by changing the solvent to EtOAc. The EtOAc fractions (3 × 50 mL) were collected, and solvent was removed in vacuo to leave a residue, which was crystallized (EtOAc/$CHCl_3$) to yield 0.64 g (50%) of **4**: mp 102–103 °C; IR (KBr) 3340 (OH), 1530 and 1310 cm^{-1} (NO_2); ^1H NMR (CD_3OD) 8.44 (s, C_5-H), 4.71 (t, CH_2O), 3.96 ppm (t, NCH_2); mass spectrum m/e 156 (M – NO_2). Anal. ($C_5H_6N_4O_5$) C, H, N.

1-(2-Hydroxyethyl)-4-nitro-5-chloroimidazole (6). A solution of **3** (0.15 g, 1 mmol) in 25 mL of absolute EtOH was heated to 70 °C, and 30 mg of NaOH was added followed by 7 mL of ethylene oxide. The mixture was refluxed for 7 h and then allowed to stir at room temperature overnight. The solvent was removed in vacuo, and the residue was recrystallized (EtOAc) to yield 75 mg (39%) of **6**: mp 138–140 °C; IR (KBr) 3350 (OH), 1500 and 1330 cm^{-1} (NO_2); ^1H NMR (Me_2SO-d_6) 8.46 (s, C_2-H), 4.12 (t, CH_2O), 3.75 ppm (t, NCH_2). Anal. ($C_5H_6ClN_3O_3$) C, H, N.

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Synthesis of a Proposed Thymic Factor

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<Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn, a proposed serum thymic factor, has been synthesized. The protected precursor, <Glu-Ala-Lys(*i*-Noc)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn, was prepared by a combination of solid phase and solution methods. The benzyl blocking groups were removed by HF and the *i*-Noc blocking group was removed by catalytic hydrogenation.

Bach and associates[1,2] have proposed the amino acid sequence V for a serum thymic factor (STF) isolated in their laboratory. We report herein the synthesis of a compound having this peptide sequence. The nonapeptide V was prepared by a fragment-coupling procedure as indicated in Scheme I. The protected heptapeptide <Glu-Ala-Lys(*i*-Noc)-Ser(Bzl)-Gln-Gly-Gly-OMe[3] (Ia) was prepared using the solid-phase method of peptide

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