Chemical Reduction of the Radiosensitizer Misonidazole by Zinc or Glucose

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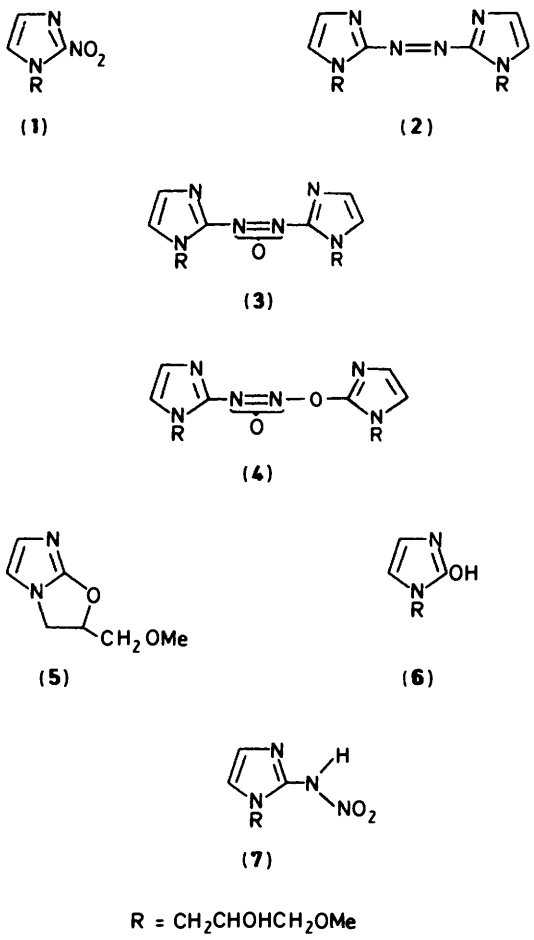
Misonidazole, 1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol, was reduced by zinc dust or glucose in almost neutral or alkaline solutions. T.l.c. of the reduction mixtures has revealed the presence of several products. Two of them have been identified as the azo and the azoxy derivatives of misonidazole. When the reduction was carried out in alkaline solution, another reaction, competitive with the reduction, was observed. This reaction, involving the loss of the nitro group, led to two products, the former deriving from a nucleophilic substitution by OH^- and the latter from an intramolecular displacement. This kind of denitrative process must be considered when the reduction of misonidazole is performed at basic pH. In such conditions two other reduction products have been identified and a possible reaction mechanism for their formation is suggested.

The nitroimidazole drug misonidazole (**1**) is an effective radiosensitizer of hypoxic mammalian cells both *in vitro* and *in vivo*.[1] In addition to its radiation-sensitizing effects on such cells, misonidazole exhibits a preferential cytotoxicity toward hypoxic cells in the absence of radiation. This supports the idea that hypoxic toxicity may result from the formation of active nitroreduction products which might be present free in the cells as well as being bound to cellular targets such as DNA, RNA, and proteins.

The chemical nature of the reduced metabolites of (**1**) as well as of nitroaromatic drugs in general, is still unclear. Evidence for the reduction of misonidazole to the amine derivative has been presented by Varghese[2] who detected such a metabolite in the urine of patients receiving the drug. Several attempts have been made to clarify the mechanism which is the basis of the biological effects of misonidazole under hypoxic conditions. To this end, and in particular to isolate reduction products with damaging biological activities, researchers have reduced misonidazole by chemical,[3,4] electrochemical,[5,6] enzymatic,[7] and radiolytic techniques.

Previous studies by Varghese *et al.*[8] have shown that reduction of (**1**) with zinc dust in aqueous solutions leads to products which are similar to those formed by cellular reduction. The exact conditions of the reduction are important to the products observed.[3,4]

We have reinvestigated these conditions, taking an interest also in any secondary products formed during the reduction. This paper describes a detailed study of the chemical products of misonidazole obtained both in aqueous ammonium chloride and in alkaline solutions, using zinc dust or glucose as reducing agents.



Results and Discussion

When misonidazole was reduced with zinc dust in aqueous ammonium chloride or in more alkaline conditions, it yielded azo (**2**) and azoxy (**3**) derivatives, respectively, as the main products. These compounds were confirmed by the data obtained from elemental analysis and u.v.-visible, n.m.r., and i.r. spectra. The u.v.-visible and n.m.r. spectra of both compounds were in excellent agreement with the literature.[3] The i.r. spectra are shown in Figure 1. Unlike Josephy *et al.*[3] we succeeded in obtaining mass spectra of (**2**) and (**3**) without performing acetylation. Using zinc dust with aqueous calcium chloride as a reducing agent, the above authors have isolated and characterized (**2**) and (**3**) with a final weight ratio of 1:3. Using the same conditions we were able to produce almost exclusively the azo derivative with complete conversion of misonidazole.

Thus, we carried out a study of reaction conditions in order to obtain appreciable yields of both reduction products. T.l.c., followed by densitometric quantitation, was used to monitor the progress of reduction in the different conditions used.

The most significant results obtained are reported in Figure 2. Figure 2(*a*) shows that in almost neutral solutions the production of the azo derivative is always higher than that of the azoxy. Moreover, the yield of azo increases with temperature; thus, the conditions of Figure 2(*a*) were chosen for its production. In order to increase the yield of (**3**), the reducing capability of the medium was decreased by using glucose in

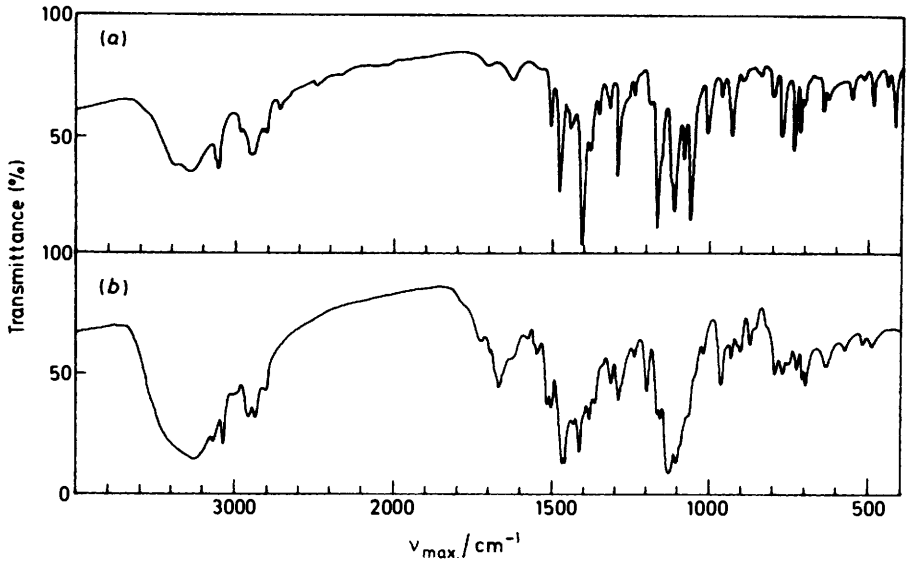


Figure 1. I.r. spectra of the derivatives (**2**) (*a*) and (**3**) (*b*) recorded in solid phase using the KBr pellet technique

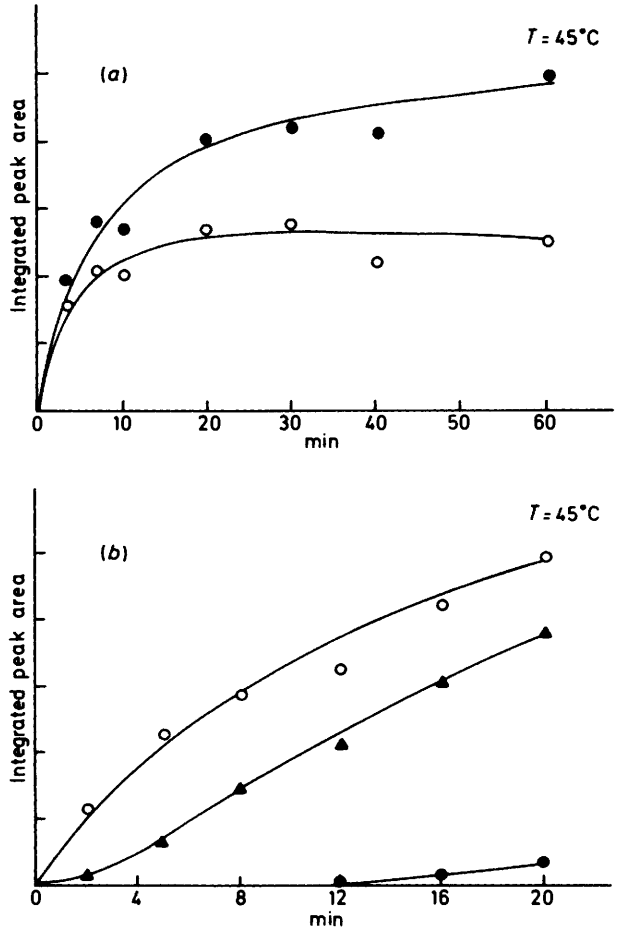


Figure 2. Time course for the production of (**2**) (●), (**3**) (○), and (**4**) (▲), in aqueous Zn–NH_4Cl (*a*) and in aqueous glucose–KOH 1M (*b*). The course of the reactions was monitored by reading the quenching of fluorescence at 254 nm on silica gel layers GF_{254} (Merck) after chromatographic separations, by a Camag t.l.c./h.p.t.l.c. densitometer. The vertical axis is arbitrarily scaled

basic solution to prevent further reduction of the azoxy to the azo derivative. As shown in Figure 2(*b*), under these conditions the yield of (**3**) was finally higher than that of (**2**). However, t.l.c. revealed the presence of other products, including fluorescent compounds. In particular, we noticed a u.v.-absorbing species, (**4**), arising from a reaction parallel to the one giving the azoxy compound. In order to isolate and characterize (**4**), we studied the best conditions for its preparation. It was observed that a decrease in the basicity of the medium leads almost solely to the formation of (**4**). For this reason, we chose glucose in 0.1M NaOH as the reducing agent for (**4**), and zinc dust in 2M KOH for (**3**). The reaction temperature of both preparations was kept at 50 °C or less, because at higher temperatures there are numerous products of side reactions, significantly decreasing the yield of (**4**) and (**3**).

Once the conditions to achieve (**4**) were established, it was prepared and purified. The compound, which is unstable at acidic pH, when analysed gave a highest *m/z* value of 199; this value could not be attributed to the molecular ion of (**4**), since the parent peak in the mass spectrum of misonidazole, from which (**4**) originates by reduction, is 201. The fragmentation pattern, shown in Figure 3(*a*), was very similar to that observed in the mass spectrum of misonidazole. In particular, the loss of fragments *m/z* 17 (OH), 31 (OMe), 45 (CH_2OMe), and 74 (OMe + CH_2CHO) from the ion at *m/z* 199, are responsible for the peaks at *m/z* 182, 168, 154, and 125, respectively. These mass spectral data provided confirmatory evidence that the peak at *m/z* 199 corresponded to an ion still containing the imidazole ring and the N-1 side-chain of (**1**) intact.

In order to obtain the molecular weight of (**4**) it was submitted to c.i. mass analysis, using methane as reactant gas; this gave a *m/z* value of 371 for the 'quasi molecular ion' (MH^+). This result suggested that (**4**) was a dimeric molecule formed by a condensation reaction taking place in basic solution. Having obtained the molecular formula of (**4**) and elucidated the main fragmentation pathways of the spectrum, the chemical structure (**4**) shown was proposed.

Since most of the reactions examined occurred in basic media we investigated the chemical stability of (**1**) in such conditions. In particular, we recorded the changes in the absorption spectra of solutions of misonidazole both in aqueous and alcoholic potassium hydroxide, thermostatted at different temperatures (37—60 °C). During the course of the reaction, the loss of the

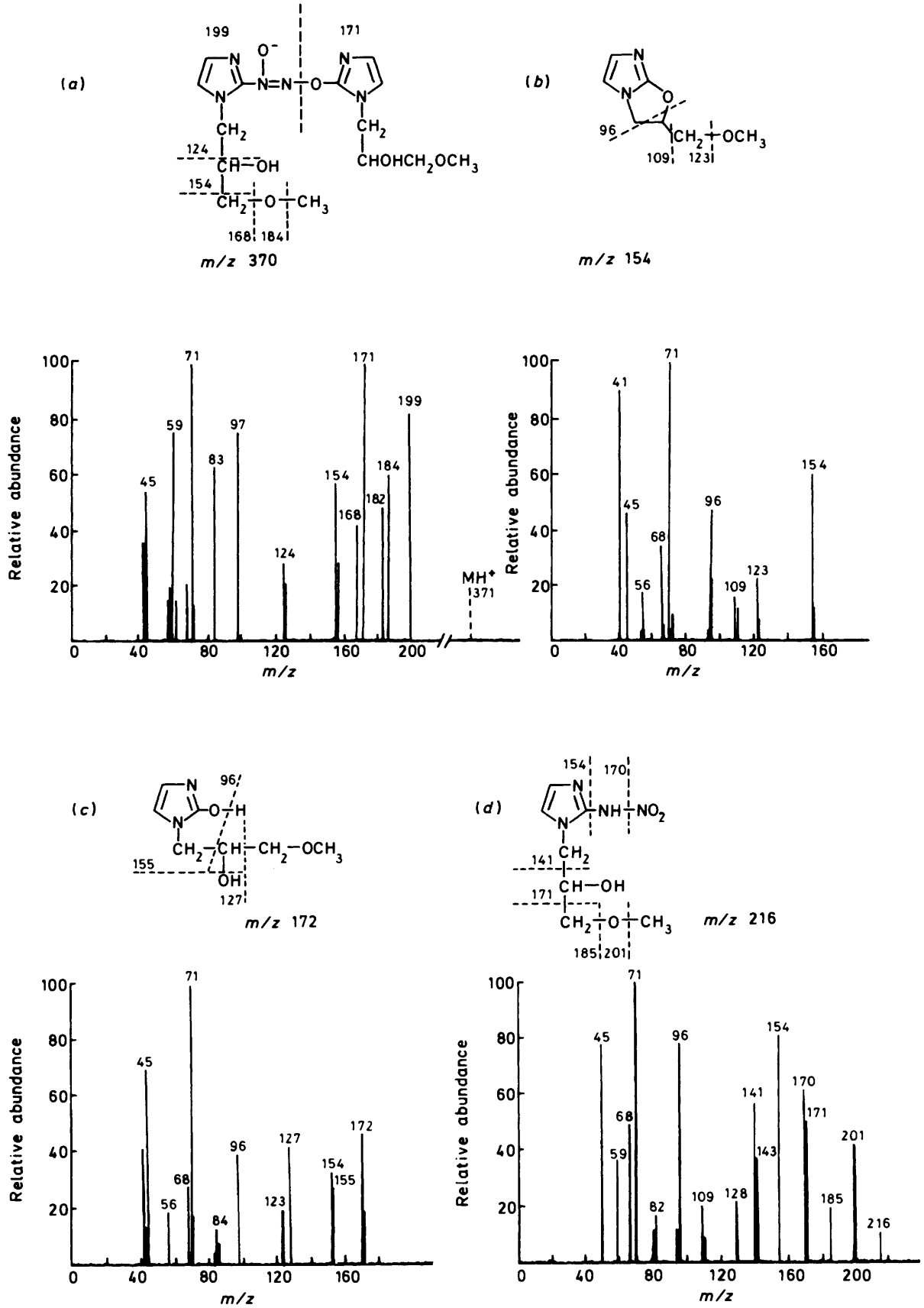
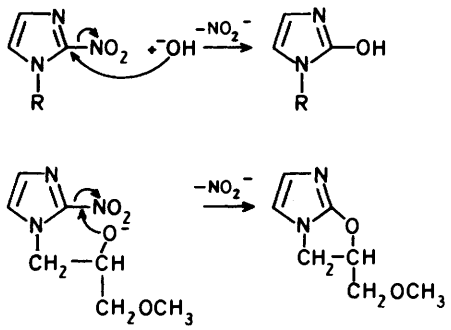
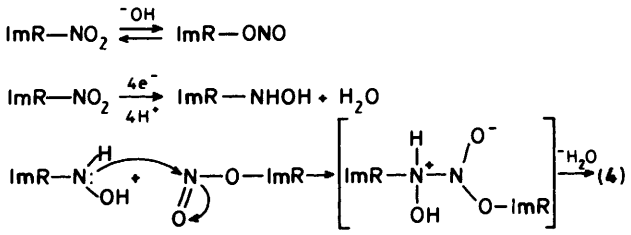


Figure 3. Mass spectra of (**4**), (**5**), (**6**), and (**7**) obtained by solid-probe injection and e.i. ionization (70 eV). Probe temperatures: 100 °C [(*a*) and (*b*)], 170 °C [(*c*) and (*d*)]. In Figure 3(*a*) the dashed line represents the 'quasi molecular ion' obtained by c.i. mass analysis



Scheme 1.



Scheme 2.

characteristic absorbance of (**1**) at 325 nm was accompanied by an increase in absorbance at 283 nm and by the production of nitrite ion. The mass spectral data provided evidence that in both cases two compounds were produced, the former with a molecular ion at *m/z* 154 and the latter, less volatile, with a molecular ion at *m/z* 172.

According to the observed fragmentation pathways the structures (**5**) and (**6**) respectively, were assigned to the two compounds.

Thus, it can be assumed that in basic media two competitive reactions may occur: one a nucleophilic substitution and the other an intramolecular displacement, according to Scheme 1.

Our results agree with those obtained for 2-nitroimidazoles for which an intramolecular displacement of the nitro group (presumably as nitrite ion) has already been reported.[9] In view of all the above results, we suggest the reaction shown in Scheme 2 for the formation of (**4**) from misonidazole, where Im denotes the imidazole ring and R the N-1 side-chain of (**1**).

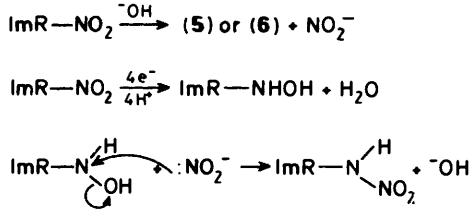
The reaction is initiated by a base-catalysed rearrangement of the nitro group to nitrite followed by a condensation of the latter with the hydroxylamine, deriving from the reduction of misonidazole.

During the preparation of both (**3**) and (**4**) we noticed the formation of a u.v.-absorbing secondary product with a molecular ion at *m/z* 216. On the basis of the fragmentation pattern shown in Figure 3(*d*), the molecular structure (**7**) was assigned. The mechanism shown in Scheme 3 was proposed for the production of (**7**).

The addition of sodium nitrite to the reduction mixture of misonidazole greatly increased the yield of (**7**), thus supporting the above reaction scheme.

Since under hypoxic conditions cellular metabolism of misonidazole leads primarily to products resulting from the reduction of the nitro group, knowledge of the exact chemical structure of such products is essential for the understanding of the biological activity of the drug.

The reduction chemistry of nitroimidazoles is not fully explained, although the results obtained support the idea that this reduction may be similar to that of nitroaromatic compounds and may proceed from the nitro compound,



Scheme 3.

through a nitroso derivative, to hydroxylamine and amine. In addition, second-order reactions of the intermediates may lead to azoxy, azo, and hydrazo derivatives.[9] Actually, some of the reduction intermediates (*e.g.* nitroso and hydroxylamine) are too unstable to be isolated. Furthermore, depending on the pH of the solution and on the temperature, these compounds may undergo rearrangements, decompositions, and inter-reactions leading to several final products.[10] Hence, it can be stated that reduction of nitroimidazoles involves a more complex system of reduction events in comparison to nitroaromatics.

In particular, this work has shown that in basic media condensations of hydroxylamine derivatives with products deriving either from the loss or the isomerization of the nitro group may occur, besides condensations leading to azo and azoxy derivatives. That kind of reaction [yielding (**4**) and (**7**)] might take place when aqueous solutions of (**1**) are γ-irradiated. In fact, radiolysis of such solutions leads to nitrite ions arising from ·OH attack on misonidazole,[11] in addition to the reduction products such as azo, azoxy, and hydroxylamine derivatives.[1]

Hence, reactions similar to those suggested in this paper might occur during γ-irradiation of misonidazole and our results could be useful to characterize further the nature of some radiolytic products not yet identified and the mechanism of their formation.

Experimental

Misonidazole was kindly supplied by Hoffman La Roche (Basel, Switzerland) and was purified by repeated crystallization from acetone, checking the purity by t.l.c.

Analytical reagent-grade products were used without further purification.

Compound (**2**) was prepared by adding misonidazole (0.5 g) and zinc dust (0.5 g) to a stirred aqueous solution of 0.1M NH_4Cl (50 ml) at 45 °C. The reaction was stopped when the mixture was yellow-orange by cooling to room temperature and filtering off the excess of zinc. The filtered solution was evaporated under reduced pressure and extracted with chloroform.

Preparative t.l.c. on Florisil (Merck, Darmstadt, GFR) layers (2 mm thick) activated at 105 °C for 60 min, using methanol–dichloromethane (1:10, v/v) as eluant, gave the pure azo derivative (65 mg, 13%) (Found: C, 49.9, H, 6.4, N, 24.6, Calc. for $C_{14}H_{22}N_6O_4$: C, 49.7; H, 6.6; N, 24.8%); *m/z* 338 (M^+, 5%), 293 ($M^+ - CH_2OMe$, 11), 279 ($M^+ - CH_2CH_2OMe$, 36), 265 ($M^+ - CHOHCH_2OMe$, 14), 154 (ImR^+, 6), 71 ($^+CH{=}CHCH_2OMe$, 77), and 57 ($EtCO^+$, 100). The spectrum was obtained using solid-probe injection and e.i. ionization (70 eV) at a probe temperature of 170 °C (a higher temperature could cause carbonization).

Compound (**3**) was prepared by adding misonidazole (0.5 g) and zinc dust (2.5 g) to a stirred aqueous solution of 2M KOH (25 ml) thermostatted at 50 °C. After 30 min the mixture was cooled, filtered to remove the excess of zinc, and neutralized with a solution of $HClO_4$ (4M). The filtered solution was evaporated

and extracted with chloroform–methanol (8:1). The azoxy derivative (45 mg, 9%) was purified [from (**2**), (**4**), and (**7**)] by preparative t.l.c. as described for (**2**) and recrystallized from water (Found: C, 48.1; H, 6.1; N, 23.6. Calc. for $C_{14}H_{22}N_6O_5$: C, 47.45; H, 6.26; N, 23.72%); M.s. (probe temperature 300 °C): *m/z* 354 (M^+, 3%), 279 (M^+ – $CHOHCH_2OMe$, 17), 265 (M^+ – $CH_2CHOHCH_2OMe$, 8), 154 (ImR^+, 5), 71 ($^+CH{=}CHCH_2OMe$, 49), and 57 ($EtCO^+$, 100).

Compound (**4**) was prepared by reduction of misonidazole with glucose. A stirred mixture of (**1**) (0.5 g), glucose (2.0 g), and 0.1M NaOH (40 ml) was kept at 45 °C for 15—20 min. After cooling, the mixture was freeze-dried and extracted with methanol–dichloromethane (2:1). Preparative chromatography using a column packed with silica gel (Kieselgel 60, 70—230 mesh, Merck) (eluting with acetone), and recrystallization from methanol gave pure compound (**4**).

Compounds (**5**) and (**6**) were synthesized by warming at 60 °C a solution of misonidazole (10^{-3}M) either in aqueous or alcoholic KOH (0.2M) until the disappearance of the characteristic absorption maximum of (**1**) at 325 nm. The freeze-dried residue was taken for mass spectrometric analysis.

Compound (**7**) was prepared as described earlier for (**3**).

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