4. B. I. Sikic, M. N. Ehsan, W. G. Harker, N. F. Friend, B. W. Brown, R. A. Newman, M. P. Hacker and E. M. Acton, *Science* **228**, 1544 (1985).
5. K. Ishiguro, K. Takahashi, K. Yazawa, S. Sakiyama and T. Arai, *J. biol. Chem.* **256**, 2162 (1981).
6. J. W. Lown, A. V. Joshua and J. S. Lee, *Biochemistry* **21**, 419 (1982).
7. A. Begleiter and J. B. Johnston, *Biochem. biophys. Res. Commun.* **131**, 336 (1985).
8. J. Westendorf, G. Groth, G. Steinheider and H. Marquardt, *Cell Biol. Toxic.* **1**, 87 (1985).
9. K. Wassermann, L. A. Zwelling, T. D. Mullins, L. E. Silberman, B. S. Andersson, M. Bakic, E. M. Acton and R. A. Newman, *Cancer Res.* **46**, 4041 (1986).
10. E. M. Acton, G. L. Tong, T. H. Smith, D. L. Taylor, D. G. Streeter, J. H. Peters, G. R. Gordon, J. A. Filppi, R. L. Wolgemuth, F. C. Giuliani and S. Penco. *J. med. Chem.* **29**, 2120 (1986).
11. J. H. Peters, G. R. Gordon and E. M. Acton, *Proc. Am. Ass. Cancer Res.* **25**, 293 (1984).
12. J. H. Peters, G. R. Gordon, D. Kashiwase, J. W. Lown, S-F. Yen and J. A. Plambeck, *Biochem. Pharmac.* **35**, 1309 (1986).
13. T. Imanari, S. Tanabe and T. Toida, *Chem. pharm. Bull., Tokyo* **30**, 3802 (1982).
14. M. Hussain, J. E. Chaney, G. A. Digenis and W. J. Layton, *J. labelled Compounds Radiopharm.* **22**, 983 (1985).

Biochemical Pharmacology, Vol. 37, No. 2, pp. 361–363, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Regioselective formation of a misonidazole–glutathione conjugate as a function of pH during chemical reduction

(*Received* 4 *October* 1986; *accepted* 30 *June* 1987)

Misonidazole (1-2[hydroxy-3-methoxypropyl]-2-nitro-1*H*-imidazole; Fig. 1), also known as MISO, is an experimental drug under investigation as a radiosensitizing [1, 2] and chemosensitizing [3] agent. Our previous studies concerning the metabolism of MISO by hypoxic rat livers showed that a MISO–glutathione conjugate (MISO–GSH) is formed as a major metabolite, and that high doses of MISO result in depletion of hepatic glutathione (GSH) [4, 5]. Furthermore, it was noted that formation of the MISO–GSH appears to correlate with the metabolic reduction of MISO [5]. In addition to GSH reactions, it has been demonstrated that MISO undergoes reductive metabolism in hypoxic tissue to yield reactive species which extensively alkylate tissue protein and RNA [5, 6]. Covalent binding to biological nucleophiles by reduced nitroimidazoles [7] has led to an interest in nitroimidazole reduction chemistry, and particular attention has been directed toward the characterization of the reactive intermediate. Among the reports that have appeared in the literature concerning the chemistry of 2-nitroimidazoles under reductive conditions, McClelland and Panicucci [8] have postulated a plausible mechanistic explanation for the reductive activation. McClelland and his group have proposed a mechanism where the neutral hydroxylamine (the four-electron reduction product) of a nitroimidazole derivative undergoes an acid-catalyzed and a non-catalyzed loss of the hydroxyl group, resulting in a cation that is a resonance stabilized nitrenium ion. This mechanism complements and helps to explain our observations regarding the formation of MISO–GSH under various reaction conditions.

A system developed by Varghese [9] for the chemical synthesis of MISO–GSH led to the production of a mixture of conjugates with GSH being attached to the C-4 or C-5 positions of the imidazole ring (Fig. 1). In our investigations concerned with the chemical synthesis of MISO–GSH, it was found that the selectivity for the formation of the C-4 or C-5 conjugate isomers was influenced significantly by the pH of the reaction mixture. The work described herein characterizes the influence of the reaction medium on the regioselective binding of GSH to a reductively-generated, MISO-derived electrophile. Our observations of the pH-dependent, regioselective formation of the MISO–GSH adduct have provided an opportunity to probe the apparent similarity of the reductive activation of MISO with the mechanism proposed by McClelland and Panicucci [8]. In our study, the use of tritiated MISO facilitated MISO–GSH isolation and quantitation.

Methods

Proton nuclear magnetic resonance spectra were recorded in D_2O solution on an NMC-360-MHz spectrometer

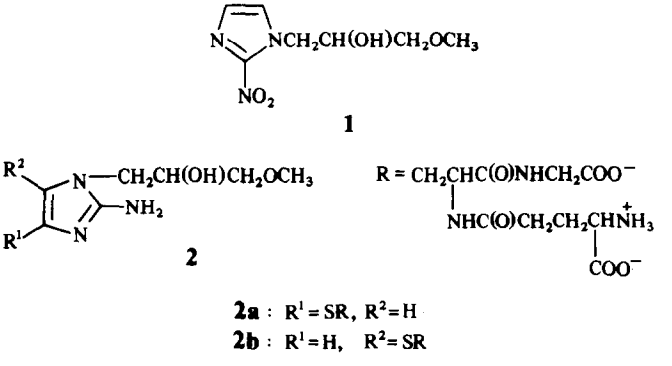

Fig. 1. Structures of MISO (1), MISO-anine (2), and glutathione.

(HF-NMR). Chemical shifts are reported in parts per million (ppm) assuming a chemical shift of 4.65 ppm for the HOD absorption. A Varian model 5020 HPLC fitted with a Vari-Chrome variable wavelength detector set at 254 nm was used for analytical and purification procedures. A 30 cm × 4 mm C-18 10 μm Varian MicropaK reverse phase column at a flow rate of 1 ml/min was used for analytical separations. A 25 cm × 7 mm C-18 10 μm Alltech reverse phase semipreparative column at a flow rate of 5 ml/min was used to isolate the MISO–GSH for NMR analysis. The columns were eluted with a 0.1 M formic acid buffer (pH 2.65) against a methanol gradient. The methanol gradient, which was started 5 min after initiation of the chromatographic run, increased linearly to 5% over 18 min, then to 25% over the next 25 min, and held constant at 25% for 5 min. Under analytical conditions, the products of interest had the following retention times: MISO-amine (1-[2-aminoimidazol-1-yl]-3-methoxy-2-propanol; Fig. 1), 17 min; MISO–GSH, 27 min; and MISO, 44 min.

The HPLC effluent was collected for radiometric analysis at 0.5-min intervals using an ISCO model 1850 fraction collector. Percent yields of MISO–GSH, which were based on the amount of MISO used as starting material, were determined radiometrically. Radiometric determinations were carried out in 2 ml of Scinti Verse I (Fisher Chemical Co.) using an LKB Wallace 1212 Mini Beta liquid scintillation counter. Counting efficiency was determined by external standardization and using appropriate quench curves. All reagents were used as obtained from commercial sources, and were reagent grade or better. Misonidazole was supplied by Dr. W. E. Scott from Hoffmann-La Roche, Inc. In this study, [2-^{3}H](2-hydroxy-3-methoxypropyl)-2-nitroimidazole ([2-^{3}H]MISO) was prepared as previously reported [10] and was diluted with unlabeled MISO to a specific activity of 0.75 mCi/mmol. Stability studies conducted on [2-^{3}H]MISO showed this compound to be isotopically stable under physiological conditions [10]. Additional [2-^{3}H]MISO stability determinations showed that tritium exchange from the molecule ranged from a minimum of 1.21 ± 0.23% (pH 2.75) to a maximum of 1.85 ± 0.25% (pH 10.85) under the reaction conditions used in this study.

For the MISO–GSH synthesis, GSH (1.86 mmol) was dissolved in sodium phosphate buffer (0.01 M, 15 ml) at a pH of 3.0, 4.5, 6.0, 7.0, 7.4, 8.0, 9.0, or 11.0. The solutions of MISO (0.74 mmol in 15 ml of water) and buffered GSH were flushed with nitrogen and drawn up into separate 20-ml syringes that had also been flushed with nitrogen. A suspension of 2.98 mmol of zinc dust in 0.25 ml of distilled water was placed in a three-neck, round-bottom flask flushed with nitrogen and fitted with a reflux condenser, a pH electrode, and a rubber septum. The MISO and GSH solutions were added via separate syringes through the rubber septum. The reaction was allowed to proceed under nitrogen at room temperature for 40 hr at a constant pH. The reaction mixture was drawn up through a Sherwood–Brunswick "MONOJECT 305" 5 μm stainless steel filter needle into a disposable Becton–Dickinson syringe. The filtrate was lyophilized, and the resultant solid was reconstituted to 4 ml in water. Purification of the MISO–GSH from the reconstituted solution was accomplished by semipreparative HPLC. Four consecutive 1-ml injections were required for each synthetic batch. The two MISO–GSH isomers, which eluted as a single peak, were collected and pooled, and the total yield was determined radiometrically. The proportion of each of the two positional isomers of the MISO–GSH was determined by NMR analysis. NMR spectra of the combined MISO–GSH isomers were obtained in 100% D_2O. The H_2O was removed by repeated lyophilization of the isomer mixture followed by reconstitution in 100% D_2O. The diagnostic signals in the MISO–GSH isomers were the singlet signals at $\delta = 6.99$ and $\delta = 6.83$ ppm corresponding to the protons at carbon-4 and carbon-5 respectively [9]. The percent ratio of isomers was determined from the H-NMR peak areas corresponding to the protons at positions C-4 and C-5 of the imidazole ring. The remaining peaks correlated well with the assignments for the MISO–GSH made by Varghese [9].

Results and discussion

While carrying out the chemical synthesis of the conjugates of interest, it was found that, by varying the pH of the reaction mixture, the overall yields as well as the ratio of the two resultant isomers could be changed significantly. Satisfactory (60–80%) yields of the MISO–GSH were obtained in the pH range from 6.0 to 8.0, and smaller amounts (<10%) of the MISO–GSH adducts were produced at pH 3.0 or pH 11.0. The MISO-amine, the six-electron reduction product of MISO, was also produced in the acidic reactions (63% at pH 4.0). Other unidentified products (possibly decomposition products [11] or products resulting from nucleophilic displacement of the nitro-substituent [12]) were observed in the reactions carried out at or above pH 7. It was revealed using HF-NMR that, at either high or low pH ranges (Fig. 2), there was a marked regioselectivity for the formation of a specific isomer. The degree of regiocontrol that the pH of the reaction medium exerts on the formation of the MISO–GSH is illustrated in Fig. 3 as the percent ratio of isomers versus the pH of the reaction medium.

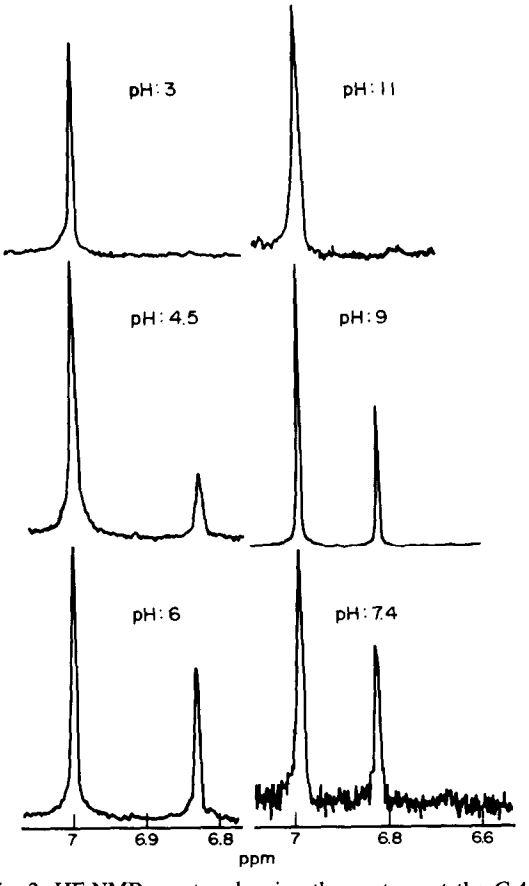

Fig. 2. HF-NMR spectra showing the protons at the C-4 and C-5 positions of the imidazole ring. These data illustrate the regioselectivity for the formation of the specific MISO–GSH isomers in the pH range from 3 to 11. The percent of the total MISO–GSH made up by each of the isomers was determined from the peak areas corresponding to the protons at positions C-4 and C-5 of the imidazole ring.

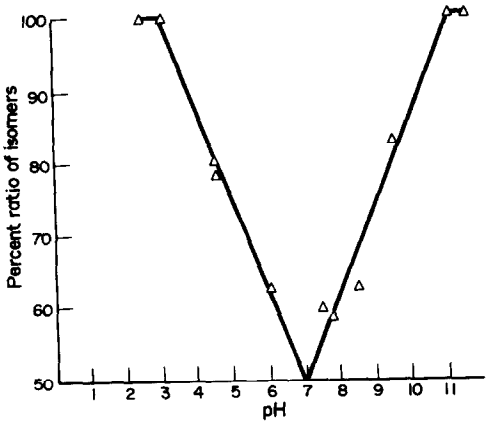

Fig. 3. Graphic representation of the effect of the reaction medium pH on the selective binding by GSH to the C-4 and C-5 positions of the imidazole ring.

These results can be explained in light of the observations of McClelland and Panicucci [8]. Via a comprehensive evaluation of the properties of the competing species (GSH, GS^-, HO^-, HOH), one can account for each of the products formed at the corresponding pH ranges. At low pH, for example, the nucleophilic attack on C-5 by GSH (as the less reactive sulfhydryl form) competes with the acid catalyzed reduction of MISO to the chemically stable MISO-amine, thus yielding a mixture of the MISO-amine and the single MISO–GSH isomer. As the pH is increased, water becomes a more competitive nucleophilic species. Nucleophilic attack of water on C-5 of the intermediate proposed by McClelland's and Panicucci [8] would form a 5-hydroxyimine which could then be attacked at C-4 by GS^-, resulting in the C-4 MISO–GSH adduct, after dehydration. McClelland has observed that, at high pH, the amount of decomposition of the reactive intermediate (probably by ring opening reactions) increases, which would account for our observed low yields of conjugates under these conditions. This rapid decomposition of the reactive intermediate at a high pH may be responsible for an inefficient attack of GS^- to the C-5 position. Thus, the activation pathway proposed by McClelland and coworkers provides a plausible explanation for our results.

Development of the chemical concepts responsible for the pH-dependent regioselectivity observed in our study is an important step in understanding the reductive chemistry and metabolic activation of 2-nitroimidazoles. An understanding of the mechanism(s) whereby 2-nitroimidazoles are reduced to therapeutic or toxic derivatives may provide a rational basis for new drug design. Since the C-5 position appears to be the more reactive site with GSH, for example, the use of a C-4 substituted MISO analog should still yield a highly reactive intermediate via a 4-electron reduction. This reactive species should retain the ability to deplete tissue GSH, which is thought to be related to the chemo- and radiosensitizing properties of the drug. It is also possible that the C-4 substituted 2-nitroimidazoles may lack the potential toxicity that would result from decomposition to 1,2-dicarbonyl compounds [13–15] such as glyoxal, which has been proposed to be a by-product of MISO reduction [16, 17].

A C-5 substituted MISO derivative should have biological properties that contrast with those of the C-4 substituted analog. Blocking the reactive C-5 position should produce an agent with little ability to deplete tissue GSH, but which should retain the ability to scavenge free electrons (such as those generated by ionizing radiation) to generate nitroimidazole radicals.

The next logical step in evaluating these predictions is the synthesis of C-4 and/or C-5 substituted MISO analogs. These syntheses are currently underway in our laboratory.

Acknowledgements—This study was supported in part by the University of New Mexico College of Pharmacy, the Department of Chemistry, NSF Grant CHE-8201374, and by NIH-DRR Minority Biomedical Research Support Grant 85-6000-642 awarded to the University of New Mexico School of Medicine, and by PHS Grant RO1 CA40284, awarded by the National Cancer Institute, DHHS.

* *College of Pharmacy* ENRIQUE CHACON*
‡ *Department of Chemistry, and* CARY J. MORROW‡
§ *Department of Radiology* ALBERTO A. LEON§
The University of New Mexico JERRY L. BORN*
Albuquerque, NM 87131, *U.S.A.* BRIAN R. SMITH*†

† Author to whom correspondence should be addressed.

REFERENCES

1. J. M. Brown, *Radiat. Res.* **64**, 633 (1975).
2. J. J. Clement, M. S. Gorman, I. Wodinsky, R. Catane and R. K. Johnson, *Cancer Res.* **40**, 4165 (1980).
3. D. G. Hirst and J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 639 (1982).
4. B. R. Smith and J. L. Born, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1365 (1984).
5. B. R. Smith, *Biochem. Pharmac.* **33**, 1379 (1984).
6. G. G. Miller, J. Ngan-Lee and J. D. Chapman, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 741 (1982).
7. A. M. Rauth, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1293 (1984).
8. R. A. McClelland and R. Panicucci, *J. Am. chem. Soc.* **107**, 1762 (1985).
9. A. J. Varghese, *Biochem. biophys. Res. Commun.* **112**, 1013 (1983).
10. J. L. Born and B. R. Smith, *J. labelled Compounds Radiopharm.* **3**, 429 (1983).
11. R. A. McClelland, J. R. Fuller, N. E. Seaman, A. M. Rauth and R. Batistella, *Biochem. Pharmac.* **33**, 303 (1984).
12. P. Goldman and J. D. Wuest, *J. Am. chem. Soc.* **103**, 6224 (1981).
13. R. Shapiro and J. Hachmann, *Biochemistry* **5**, 2799 (1966).
14. K. Takakashi, *J. Biochem., Tokyo* **81**, 395 (1977).
15. A. J. Varghese and G. F. Whitmore, *Cancer Res.* **43**, 78 (1983).
16. J. A. Raleigh and S. F. Liu, *Biochem. Pharmac.* **32**, 1444 (1983).
17. J. A. Raleigh and S. F. Liu, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1337 (1984).