TcO(PnAO-1-(2-nitroimidazole)) [BMS-181321], a New Technetium-Containing Nitroimidazole Complex for Imaging Hypoxia: Synthesis, Characterization, and Xanthine Oxidase-Catalyzed Reduction

Karen E. Linder,* Yee-Wai Chan, John E. Cyr, Mary F. Malley, David P. Nowotnik, and Adrian D. Nunn

Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Bldg. E1.483, Lawrenceville, New Jersey 08543-4000

Received August 9, 1993•

A technetium(V)oxo nitroimidazole complex that shows promise for imaging regional hypoxia in vivo, [BMS-181321, TcO(PnAO-1-(2-nitroimidazole))] (1) was prepared from 3,3,9,9-tetramethyl-1-(2-nitro-1*H*-imidazol-1-yl)-4,8-diazaundecane-2,10-dione dioxime, a 2-nitroimidazole-containing derivative of propyleneamine oxime (PnAO). The ^{99}Tc complex [^{99}Tc]Oxo[[3,3,9,9-tetramethyl-1-(2-nitro-1*H*-imidazol-1-yl)-4,8-diazaundecane-2,10-dione dioximato]-(3-)-*N*,*N*′,*N*″,*N*‴]technetium (V) was synthesized both from pertechnetate and $[TcO(Eg)_2]^-$ (Eg = ethylene glycol). A new synthetic route to TcO(PnAO) (2) is also described. ^{99}TcO(PnAO-1-(2-nitroimidazole)) was characterized by ^{1}H NMR, IR, and UV/vis spectroscopy, HPLC, FAB mass spectrometry, and X-ray crystallography. Electrochemistry of 1 reveals that the nitro redox chemistry found in the ligand is maintained upon coordination to technetium but shifts to a slightly more positive potential. Using chiral HPLC (Chiracel OD), ^{99m}Tc (1) was resolved into its two enantiomers. However, the two isomers were found to racemize quickly ($t_{1/2}$ < 2 min) in the presence of water. Localization of 1 is believed to be mediated by enzymatically catalyzed reduction of the nitroimidazole group, so the in vitro reaction of ^{99}Tc(1) with the nitroreductase enzyme xanthine oxidase (XOD) was studied. XOD catalyzed the quantitative reduction of the nitroimidazole group on the molecule under anaerobic conditions in the presence of hypoxanthine. No reaction was noted using a non-nitro-containing complex (2). The rate of reduction of the Tc–nitroimidazole complex (1.5 ± 0.16 nmol/min per unit XOD) was faster than that observed previously for the nitroimidazole BATOs (BATO = boronic acid adduct of technetium dioxime) and was about two-thirds that of fluoromisonidazole, a compound that has proven useful for imaging hypoxia in humans when labeled with ^{18}F. These data suggest that BMS-181321 (1) has the potential to be recognized by nitroreductase enzymes in vivo, thus satisfying one of the criteria required for this potential hypoxia imaging agent.

Introduction

We are currently developing a ^{99m}technetium-labeled nitroimidazole complex [BMS-181321] that may allow delineation of hypoxic tissue in vivo.[1-5] Such an imaging agent could be clinically useful in (for example) the identification and management of tissue at risk in myocardial ischemia, in the development of interventional strategies for revival of jeopardised tissue in stroke, and in early assessment of tumors that are potentially resistant to radiotherapy and/or chemotherapy because of their hypoxic status.

There has been considerable interest in imaging hypoxia with radiolabeled derivatives of nitroimidazoles. Other groups have reported the binding of 14-C-,[6-9] 3-H-,[10-12] 82-Br-,[13,14] and 18-F-labeled[15-19] 2-nitroimidazole derivatives to hypoxic cells or tissues in cerebral ischemia,[11] myocardial infarction,[12,16,17] and in malignant tumors or tumor spheroids.[6-10,18,19] Several iodine-labeled[20-23] nitroimidazole derivatives have also been reported, and the uptake of ^{123}I-labeled iodoazomycin arabinoside in some human tumors has been demonstrated.[69] Fluoromisonidazole, labeled with 18-F,[12,15-19] has been used to image hypoxia in humans[19] in conjunction with positron emission tomography (PET). However, the high cost and limited availability of PET imaging equipment and radionuclides make it desirable to develop a technetium-99m (^{99m}Tc)-labeled hypoxia imaging agent which can be used with widely-available single photon emission computed tomography (SPECT) imaging equipment.

We have previously reported studies on Tc–nitroimidazoles from the BATO class of compounds[24] TcX(dioxime)$_3$BR (X = Cl, OH; R = a nitroimidazole derivative, BATO[25,26] = boronic acid adduct of technetium dioxime). However, we found that these nitro-BATO compounds were more difficult to reduce (both electrochemically and enzymatically) than was misonidazole,[6-11] a widely studied 2-nitroimidazole compound known to localize in hypoxic tissue. As reduction of the nitroimidazole moiety is believed to be required[27-29] for trapping in hypoxic tissue, this was considered a problem. On the basis of this experience, we began to screen other technetium cores and found that BMS-181321 [^{99m}TcO(PnAO-1-(2-nitroimidazole)), 1, Figure 1], a 2-nitroimidazole derivative of the well-known class of technetium(V)oxo propyleneamine oxime (PnAO) complexes[30-33] exhibited preferential localization in hypoxic tissue, in both whole animal[3,5] and in vitro models.[2,4] The synthesis and characterization of the long-lived ^{99}Tc analog of this complex is described here, as is some of the ^{99m}Tc chemistry. Separation of the ^{99m}Tc complex into its enantiomers is also reported.

This report also describes an in vitro enzyme assay performed to determine whether ^{99}TcO(PnAO-1-(2-nitroimidazole)) can be enzymatically reduced. Nitroimidazoles are known to form adducts with other molecules when treated with high doses of radiation or when reduced

• Abstract published in *Advance ACS Abstracts*, December 15, 1993.

0022-2623/94/1837-0009$04.50/0

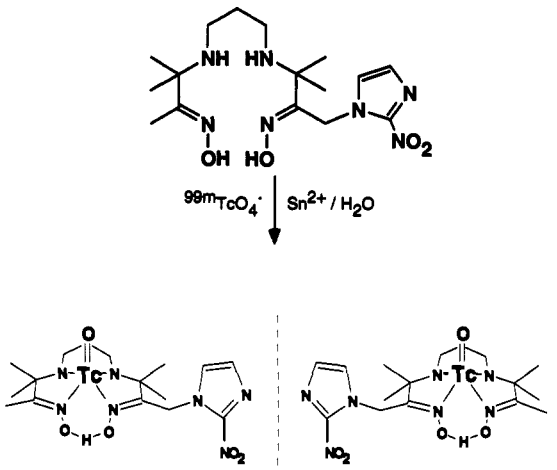

Figure 1. Structure of PnAO–1-(2-nitroimidazole) ligand and the two enantiomersof TcO(PnAO–1-(2-nitroimidazole)) (BMS-181321) (1).

either enzymatically or chemically in the absence of oxygen.[6,9,10,34,36–38] Enzymatically catalyzed in vivo reduction of the nitro group, followed by binding of the reactive reduced species to cellular components, has been proposed as a mechanism by which radiolabeled nitroimidazoles might be preferentially retained in hypoxic tissue. The assay described here is based on methods previously reported in the literature[39–44] and uses xanthine oxidase (XOD) as a model nitroreductase, hypoxanthine as the reducing substrate, and ^{99}TcO(PnAO–1-(2-nitroimidazole)) as the electron acceptor. In vivo, XOD catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid:

$$\text{xanthine} + O_2 + H_2O \rightarrow \text{urate} + H_2O_2$$

In the absence of oxygen, these reactions will not proceed, unless some other source of oxidant (such as a nitroimidazole) is added as an electron source. Others have reported that nitro compounds such as misonidazole,[41,43] fluoromisonidazole,[44] niridazole,[39] metronidazole,[40,42] benzonidazole, and the *N*-oxide SR-4233[45] are suitable substrates for XOD. The results in this report demonstrate that ^{99}TcO(PnAO–1-(2-nitroimidazole)) is also recognized by this enzyme. This is of note because technetium is not a naturally occurring element, and literature examples where technetium complexes are recognized as substrates for enzymes are rare. It has been proposed[70] that the ease of in vivo reduction determines the relative ability of technetium cations to localize in myocardial tissue, and some groups appear to be using redox potential as a guiding principle with some success (see for example refs 70–73). However, definitive demonstrations of enzymatically catalyzed metabolism are still relatively rare. Except for our previously reported studies on the XOD-catalyzed reduction of nitroimidazole BATOs,[24] documented examples of enzymatically catalyzed metabolism of technetium complexes, namely TcO(ECD),[47] an ester derivative of Tc(4-MeO-SAL$_3$TAME)$^+$,[48] and Tc(CNC(CH$_3$)$_2$(COOCH$_3$)$_6$)$^+$,[49] have all involved deesterification reactions in serum or tissue homogenate.

Results and Discussion

Synthesis and Characterization of ^{99}TcO(PnAO–1-(2-nitroimidazole)). The Tc complex ^{99}TcO(PnAO–1-(2-nitroimidazole)) (1) was prepared both by stannous tartrate reduction of TcO_4^- (a route that has been used to prepare other Tc PnAO-type complexes)[33,50] and by ligand exchange from TcO(ethylene glycol)$_2^-$. TcO(Eg)$_2^-$ was also used to prepare TcO(PnAO); this starting material[51] has not been used previously for the synthesis of PnAO compounds. Characterization of the ^{99}Tc nitro complex by elemental analysis, NMR, IR, mass spectrometry, and X-ray crystal structure analysis indicates strong similarity to the previously characterized ^{99}TcO(PnAO) (2). The Tc=O stretch at 918 cm^{-1} falls within the range observed for other Tc=O amine oxime compounds.[33,50] A strong protonated molecular ion is seen under FAB (+) conditions, as well as fragments corresponding to the loss of oxygen and NO_2. The NMR spectrum of ^{99}Tc-PnAO–1-(2-nitro) is similar to that of ^{99}TcO(PnAO)[33] but exhibits somewhat more complicated splitting due to the inequivalence introduced by the nitroimidazole moiety. The nitroimidazole ring protons appear as a pair of singlets at 7.1 and 7.5 ppm. The absence of splitting by adjacent nitroimidazole protons has been reported by others.[52] The methylene protons on the carbon that links the nitroimidazole moiety to the PnAO core, which appear as a singlet in the free ligand, become a pair of doublets centered at 5.7 ppm in the Tc complex. The two protons on the central carbon of the propylene bridge are clearly inequivalent; they appear as two multiplets at δ 1.75 and 2.4 (1H each). A similar result is seen for TcO(PnAO).

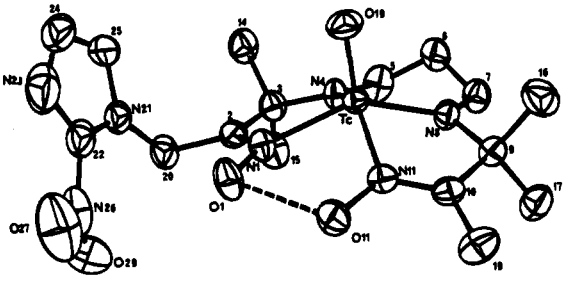

Figure 2. ORTEP[46] of (1), showing 50% probability ellipsoids.

Crystal Structure. The structure of 1 (Figure 2) is similar to that reported for a number of other PnAO derivatives.[32,33] Selected bond lengths and angles are tabulated in Table 1. The complex has square-pyramidal geometry about the five-coordinate Tc(V) center, with the oxygen occupying an apical position. The plane of the nitroimidazole group is approximately perpendicular to the plane through the four coordinated nitrogen atoms; the nitro group is *trans* with respect to the Tc-oxo core. The six-membered chelate ring has a flattened boat conformation in which the atoms Tc, O19, and C6 lie +0.36, 1.99, and +0.71 Å above the plane of its two C–N bonds. The two amines and one oxime are deprotonated, giving the complex an overall neutral charge. There is a strong *intra*molecular hydrogen bond bridge between the two oxime oxygens (O1···O11 2.440(4) Å). There is no evidence of interaction between the nitroimidazole side chain and the technetium core. As the nitro group must be accessible to the enzymes that are required for in vivo hypoxia trapping, this absence of interaction in the solid state is an important finding. We have found that in solution (vide infra), the nitro group of ^{99}Tc 1 can be reduced using the nitroreductase enzyme xanthine oxidase as a catalyst. This result suggests that the nitroimidazole side chain remains accessible to the enzyme in aqueous solution.

Electrochemistry. The electrochemistry observed for 1 and other[53–56] nitroimidazole complexes is strongly

Table 1. Bond Lengths and Bond Angles in TcO(PnAO-1-(2-nitroimidazole)) (1)[a]

Selected Bond Distances in Angstroms			
Tc-N1	2.085(3)	C6-C7	1.511(6)
Tc-N4	1.911(3)	C9-C16	1.534(5)
Tc-N8	1.916(3)	C9-C17	1.531(7)
Tc-N11	2.080(3)	C10-C18	1.483(6)
Tc-O19	1.678(3)	C20-N21	1.475(6)
N1-O1	1.357(5)	N21-C22	1.350(7)
N1-C2	1.279(4)	N21-C25	1.373(6)
N4-C3	1.482(4)	C22-N23	1.299(7)
N4-C5	1.471(6)	C22-N26	1.429(7)
N8-C7	1.463(6)	N23-C24	1.354(8)
N8-C9	1.479(5)	C24-C25	1.342(10)
N11-O11	1.375(5)	N26-O27	1.234(6)
C2-C3	1.497(7)	N26-O28	1.213(6)
Selected Bond Angles in Degrees			
N1-Tc-N4	77.4(1)	C5-C6-C7	113.3(4)
N1-Tc-N11	86.9(1)	N11-C10-C18	122.5(4)
N1-Tc-O19	107.1(1)	C2-C20-N21	111.8(3)
N4-Tc-N8	94.0(1)	C20-N21-C22	130.4(4)
N4-Tc-O19	110.2(1)	C20-N21-C25	125.2(5)
N8-Tc-N11	76.9(1)	C22-N21-C25	104.0(4)
N8-Tc-O19	108.8(1)	N21-C22-N23	114.3(5)
N11-Tc-O19	110.5(1)	N21-C22-N26	124.2(5)
O1-N1-C2	120.0(3)	N23-C22-N26	121.4(5)
C10-N11-O11	117.3(3)	C22-N23-C24	103.8(5)
N1-C2-C3	116.2(3)	N23-C24-C25	111.0(5)
N1-C2-C20	120.6(4)	N21-C25-C24	106.8(5)
N4-C3-C2	106.6(3)	C22-N26-O27	117.6(5)
N4-C3-C14	109.2(3)	C22-N26-O28	118.6(4)
C14-C3-C15	110.9(3)	O27-N26-O28	123.8(5)

[a] Numbers in parentheses are estimated standard deviations in the least-significant digits.

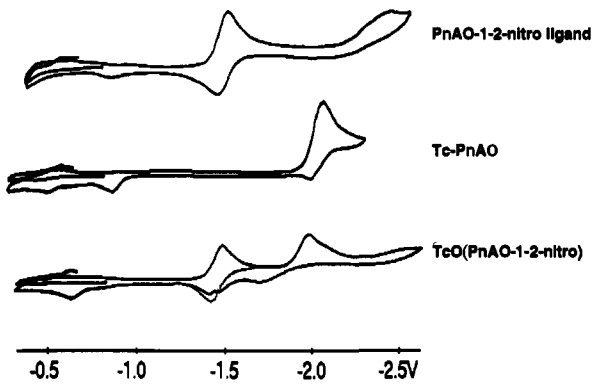

Figure 3. Electrochemistry of PnAO-1-(2-nitroimidazole) ligand, ^{99}TcO(PnAO) (2), and ^{99}TcO(PnAO-1-(2-nitroimidazole)) (1).

influenced by solvent. In aprotic solvents such as DMF, a quasireversible one-electron reduction occurs, forming a nitro radical anion.[53,54] However, in aqueous media, a single, irreversible multielectron reduction is seen.[55,56] This process is very sensitive to the nature of supporting electrolyte, pH, and concentration, making comparison of E_{pc} values between compounds very difficult. For this reason, we have chosen to focus primarily on nonaqueous electrochemistry. Cyclic voltammograms of ^{99}TcO(PnAO-1-(2-nitroimidazole)) in DMF (Figure 3) show a reversible reduction process at E_{pc} = -1.48 V. On the basis of electrochemical results with other nitroimidazole compounds, this process can be assigned to reduction of the nitro group. In addition, a totally irreversible reduction process at E_{pc} = -1.99 V can be assigned to reduction of the Tc-PnAO core, by comparison to the cyclic voltammetry results for TcO(PnAO). If this Tc-PnAO core reduction is traversed, the nitro reduction in TcO(PnAO-1-(2-nitroimidazole)) is made irreversible. Direct current polarography electron counting experiments comparing

Table 2. Resolution of the Enantiomers of ^{99m}TcO(PnAO-1-(2-nitroimidazole)) (1)[a]

% hexane	R_t (peak no. 1) (min)	R_t (peak no. 2) (min)	R_s
50	15.9	18.1	0.84
60	18.6	21.4	0.89
70	24.0	28.2	1.18

[a] Chiracel OD column (150 × 4.6 mm) eluted with hexane/isopropyl alcohol (IPA) at 0.5 mL/min.

nitro and core reduction limiting currents to that of the one-electron Ru(III)/Ru(II) reduction of Ru(acac)$_3$ indicate that 1.04 and 1.17 electrons are transferred for the nitro and core reductions, respectively.

The E_{pc} value for TcO(PnAO-1-(2-nitroimidazole)) (-1.48 V) is 0.01 V positive to that of misonidazole, a "gold standard" of 2-nitroimidazole chemistry. Under similar conditions, the uncomplexed PnAO-1-(2-nitroimidazole) ligand undergoes a one electron reduction at -1.52 V. This result demonstrates that complexation to the metal shifts the reduction potential of the pendant nitroimidazole slightly positive. As the Tc(V)oxo(3+) core is expected to be electron withdrawing, this shift is not unexpected.

^{99m}Tc Complex (1). The ^{99m}TcO(PnAO-1-(2-nitromidazole)) complex of 1 is readily prepared using methods useful for the preparation of ^{99m}TcO(PnAO).[30,31] At pH 8.2, $^{99m}TcO_4^-$ is easily reduced by stannous tartrate in the presence of ligand to give ^{99m}Tc 1 in greater than 90% radiochemical purity, as determined by HPLC. Alternatively, the complex can be prepared using stannous DTPA (Techneplex) as the reductant. Material prepared by either route coelutes with samples of analytically pure ^{99}Tc 1 on two different HPLC systems. Retention times for a $^{99/99m}$Tc mixture on Nucleosil C-8 (60/40 ACN/0.1 M NH_4OAc, pH 4.6, 1.0 mL/min) are identical at 3.60 min (void volume = 1.73 mL). On a 10-μm PRP-1 reversed-phase column (Hamilton) eluted with 65/35 ACN/0.1 N NH_4OAc at 2.0 mL/min, the two compounds coeluted at a retention time of 2.6 min (void volume = 2.06 mL).

Separation and Characterization of Enantiomers. While the ligand used to prepare 1 is achiral, it forms two Tc complexes which are an enantiomeric pair (Figure 1). It is well-known that stereochemistry is an important consideration in pharmaceuticals,[57] and there are already some examples of stereochemistry having an influence on biodistribution of Tc complexes.[58] Therefore, we needed to separate and evaluate the individual enantiomers to determine whether these enantiomers differed in their imaging capabilities. Racemates of 1 coelute in conventional HPLC, but chiral HPLC, which is well established for the resolution of enantiomers,[59] was useful for resolving the enantiomers of ^{99m}Tc 1 (Chiracel OD column, hexane/IPA eluent) (Table 2). At 50/50 hexane/IPA, HPLC fractions containing relatively pure peak no. 1 or no. 2 could be obtained. Reanalysis of these samples on the chiral column at several times after isolation revealed that the purifed compounds underwent racemization. No other degradation was noted. In Figure 4, the log of the enantiomeric excess (% major (isolated) peak-% of the other enantiomer) is plotted against time for two separate runs for each enantiomer. The straight line relationship indicates pseudo-first-order kinetics, with the $t_{1/2}$ of ~25 min. As expected for a racemization process, the rate of racemization is the same in both directions.

The stereochemical difference between the two enantiomers of 1 results from the relative position of the oxygen

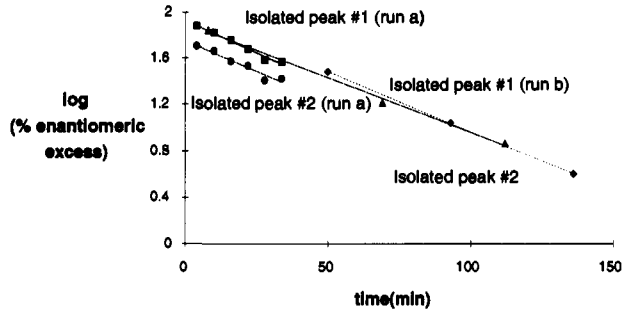

Figure 4. Racemization of the isolated enantiomers of ^{99m}TcO-(PnAO-1-(2-nitroimidazole)) as monitored by chiral HPLC (Chiracel OD column (50:50 hexane/IPA, 0.5 mL/min).

Table 3. Effect of DMF on Initial Rate of Xanthine Oxidase-Catalyzed Aerobic Oxidation of Xanthine

% DMF	initial rate (AU/min)	% of no DMF control
0	0.032 (±0.002) n = 3	100
5	0.025 (±0.002) n = 3	78
10	0.0186	58

atom in the Tc=O "core". If the ligand portions of the two enantiomers are rotated so that they overlap, this oxygen atom in the two molecules will be found either above or below the plane of the four ligating nitrogen atoms. Therefore, racemization occurs by a process in which the net result is oxo core inversion. The mechanism for this process has not been determined. However, we have noted previously that certain PnAO ligands can form two Tc complexes which interconvert, with oxo core inversion being one possible explanation for the observed results.[60] In that series, water played an important role in complex interconversion; therefore, we examined the effect of added water on the rate of racemization of TcO(PnAO-1-(2-nitroimidazole)). Water was added to isolated peak no. 1 fractions to give solutions which contained 14% water. The rate of racemization was markedly increased; it was complete 2 min after water addition. With such rapid racemization in the presence of water, we could not prepare samples of individual enantiomers in a form suitable for in vivo administration. This result suggests that the two enantiomers in racemic 1 should rapidly interconvert in vivo.

Enzyme Studies. Effect of DMF on Enzyme Activity. The ^{99}Tc complexes have very low solubility in water but are quite soluble in DMF. Before studies on the enzymatic reduction of Tc-PnAO complexes were carried out, the effect of aqueous DMF on xanthine oxidase was tested. Table 3 shows the effect of DMF on the rate of XOD-catalyzed oxidation of xanthine under aerobic conditions. Enzyme activity in this assay drops as the concentration of DMF is increased. At 5% DMF, activity was 78% of that observed in the absence of DMF. DMF also caused a decrease in activity when the XOD-catalyzed anaerobic reduction of misonidazole was studied. In enzyme solutions that were 5% in DMF, the rate of misonidazole disappearance was 60% of that observed in 0% DMF (Table 4). These results were deemed significant but tolerable, so all assays using the ^{99}Tc complexes 1 and 2 contained 5% DMF.

Reduction of ^{99}TcO(PnAO-1-(2-nitroimidazole)) (1). The ability of ^{99}TcO(PnAO-1-(2-nitroimidazole)) to serve as a substrate for XOD is demonstrated by Figure 5, which shows a typical UV/vis trace of the reaction of this PnAO-nitroimidazole complex with XOD and hypoxanthine under anaerobic conditions. During this reaction, hypoxanthine is first oxidized to xanthine and then to uric acid, as determined by HPLC (data not shown). At the same time, the absorbance peak at 326 nm (which is attributable to the nitroimidazole group) disappears. Figure 6 compares the rate of loss of the nitro absorbances of misonidazole and ^{99}Tc 1 under identical assay conditions. Data for metronidazole (a 5-nitroimidazole) in 0.2% DMF are also shown; this compound was found to be much less reactive in this assay. Half-life data for the complexes are given in Table 5. In control experiments performed in the absence of XOD or the absence of hypoxanthine, no reaction was seen over 16 h, except a 2-4% decrease in absorbance at all wavelengths. The non-nitro-containing complex TcO(PnAO) (2) was also tested, to study the reactivity of the PnAO core, which is common to both 1 and 2. No reaction was observed. These results suggest that it is the nitroimidazole group in TcO(PnAO-1-(2-nitroimidazole)) that is affected in the presence of XOD, and not the PnAO core.

Table 4. Effect of DMF on Rate of Xanthine Oxidase-Catalyzed Anaerobic Reduction of Misonidazole

% DMF	reaction half-life (min)	% of no DMF control
0	37 (±2) n = 4	100
0.2	44 (±4) n = 3	84
5.0	60 (±3) n = 3	62

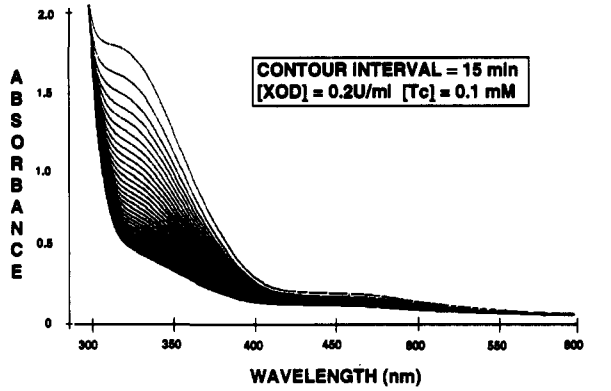

Figure 5. Loss of ^{99}TcO(PnAO-1-(2-nitroimidazole)) in the presence of xanthine oxidase and hypoxanthine in 5% DMF.

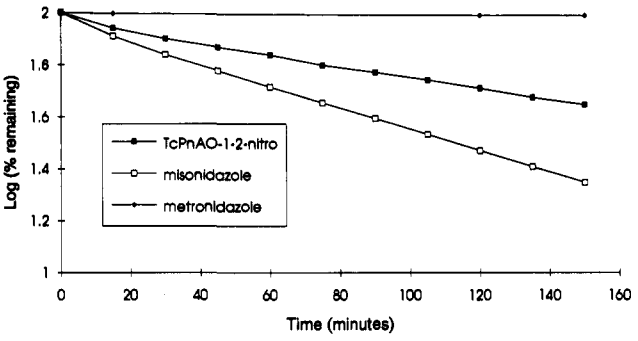

Figure 6. Comparison of rate of reduction of ^{99}TcO(PnAO-1-(2-nitroimidazole)), misonidazole and metronidazole (0.2% in DMF) under anaerobic conditions. (Initial concentrations = 0.2 units/mL in XOD, 100 μM in nitro compound, 4 mM in hypoxanthine, and 5% in DMF.

Nature of the Products. The nature of the products formed in the studies described above has not been established. However, it is presumed that enzymatically catalyzed *reduction* of the nitro group has occurred, for the following reasons:

Table 5. Half-Lives for Nitro Group Loss in Xanthine Oxidase Enzyme Assay

complex	initial [Tc] or [nitro compound] (mM)	[DMF] (%)	$T_{1/2}$ for nitro loss (min)
TcO(PnAO-1-(2-nitro)	0.1	5	120 (±13) (n = 3)
TcO(PnAO-1-(2-nitro) no enzyme control	0.1	5	no reaction seen (n = 2)[a]
TcO(PnAO-1-(2-nitro) no hypoxanthine control	0.1	5	no reaction seen (n = 2)
TcO(PnAO) non-nitro control	0.1	5	no reaction seen[a]
$TcOH(DMG)_3BBNO_2$	0.077	5	240 (n = 2)
$TcOH(DMG)_3B$-propene-NO_2 NO_2	0.077	5	185
misonidazole	0.1	5	60 (±3) (n = 3)
metronidazole	0.1	0.2	>20 h (n = 2)

[a] 2–4% drop in absorbance observed in all wavelengths, suggestive of minor precipitation.

(1) During the reaction, hypoxanthine is oxidized to xanthine and uric acid (as determined by HPLC analysis of products). This oxidation must be coupled to a reduction. Under anaerobic conditions, this reaction did not occur unless a nitro compound was added. If the non-nitro-containing TcO(PnAO) compound was substituted, neither the PnAO compound nor the hypoxanthine underwent any change. These results suggest that nitro group reduction is coupled to the oxidation of hypoxanthine.

(2) In the absence of enzyme, no reaction occurs, so the changes observed are not due to a nonenzymatic reaction between hypoxanthine and test compound.

(3) In the absence of hypoxanthine (reductant), no reaction is observed.

The nature of the reduced product(s) changes over time. At time points immediately following complete reduction of the nitro group of ^{99}TcO(PnAO-1-(2-nitroimidazole)), UV/vis absorbances due to the the PnAO core of the molecule are still at least partially retained, because the broad absorbances centered at 360 and 460 nm in TcO(PnAO) are still present in solutions of enzymatically reduced ^{99}TcO(PnAO-1-(2-nitroimidazole)). However, these solutions bleach over 24 h from orange to colorless, a phenomenon that is not noted in corresponding TcO(PnAO) reactions. The nature of the products formed has not been studied. This result stands in contrast to that obtained previously with another class of technetium nitroimidazoles, namely the Tc nitroimidazole–BATO complexes.[24] Even after complete disappearance of the nitroimidazole-based absorbance centered at 326 nm, all other absorbances attributable to the BATO core remained.

Correlation with Literature Results. The rates that we observed for the XOD-catalyzed reduction of misonidazole in phosphate buffer (initial rate of 6 nmol/min per unit of XOD) are comparable to initial rates reported by Josephy et al.[43] (6 nmol/min per unit), Clarke et al.[42] (8 ± 2 nmol/min per unit) and Prekeges (5.7 ± 0.7 nmol/min per unit),[44] despite the fact that we used hypoxanthine, rather than xanthine, as the electron donor in this system. Our metronidazole results are also similar to literature values; Clarke et al.[42] have reported very slow reduction of metronidazole in the presence of XOD; this is consistent with our observed half-life of ≫20 h. The rate observed for nitro group loss with TcO(PnAO-1-(2-nitroimidazole)) is about two-thirds that reported for fluoromisonidazole,[44] a compound that has proved useful for imaging hypoxia in humans when labeled with the positron emitting isotope ^{18}F.[19] The Tc complex is reduced at a rate that is 40% that of misonidazole in 5% DMF (Table 6). As addition of DMF lowers the activity of XOD, it is reasonable to presume that 1 would be reduced more rapidly in water. The rate of nitro group loss from 1 was significantly faster than that observed with the nitroimidazole-substituted BATO complexes $TcOH(DMG)_3BBNO_2$, and $TcOH(DMG)_3B$-propene-NO_2 (Table 5) previously studied in these laboratories.[24]

Table 6. Reaction Rates for Nitro Complexes

nitro substrate	reducing substrate	reaction rate (nmol/min per unit XOD)	N
TcO(PnAO-1-(2-nitro) in 5% DMF	hypoxanthine	1.5 ± 0.16[a]	3
misonidazole in 5% DMF	hypoxanthine	3.7 ± 0.2[a]	3
misonidazole in 0% DMF	hypoxanthine	6.0 ± 0.2[a]	4
misonidazole in 0% DMF	xanthine	6,[b] 5.7 ± 0.7[c]	2
fluoromisonidazole in 0% DMF	xanthine	2.4 ± 0.27[c]	15

[a] This work. [b] Reference 62. [c] Reference 44.

The enzyme assay described in this report is only a model for potential nitroreductase activity in vivo. The relevance of XOD-catalyzed reduction of 1 to the hypoxia localization that we have noted in animal studies is not clear. For example, despite the fact that XOD levels in rabbit heart are reported to be very low,[61] ^{99m}TcO(PnAO-1-(2-nitroimidazole)) has been shown to preferentially localize in hypoxic myocardial tissue in the rabbit,[3] suggesting that XOD is not specifically required for the localization of 1 in hypoxic tissue. It should also be noted that although the $t_{1/2}$ value observed in this assay appears to be relatively slow (~2 h under the conditions used), trapping of the complex in hypoxic tissue of ischemic brain[5] and heart[3] in vivo appears to be rapid. Several other enzymes besides XOD have been reported to use nitro compounds as electron acceptors; which enzyme system is responsible for the trapping of ^{99m}TcO(PnAO-1-(2-nitroimidazole)) in vivo is not clear at this point.

Conclusion: In summary, we have isolated and fully characterized the ^{99}Tc standard of a potential new hypoxia imaging agent, ^{99m}TcO(PnAO-1-(2-nitroimidazole)) [BMS-181321]. The complex is a neutral oxo complex of technetium(V) that is structurally similar to known examples of propyleneamine oxime technetium complexes, except for the presence of a nitroimidazole-containing side chain. The nitroimidazole moiety in the complex was found to maintain the electrochemistry required for biological activity, and studies revealed that coordination to the Tc=O core shifted the redox potential slightly positive to that of misonidazole. X-ray crystal structure

analysis revealed that the nitroimidazole side chain is oriented away from the complex core, an orientation which (if maintained in solution) should assure accessibility to nitroreductase enzymes. Under anaerobic conditions, xanthine oxidase was found to catalyze reduction of ^{99}TcO(PnAO–1-(2-nitroimidazole)) at a rate that is about two-thirds of that reported previously for fluoromisonidazole (in phosphate buffer). Under identical conditions, TcO(PnAO) was unaffected.

This work demonstrates that it is possible to design technetium complexes that are recognized as biological substrates for nitroreductases, despite the presence of a chelated metal that is not native to in vivo systems. This result is heartening, as in vivo metabolism of the nitroimidazole group of TcO(PnAO–1-(2-nitroimidazole)) must occur for the compound to show any utility for imaging of hypoxia. In addition, studies aimed at resolution of the two enantiomers of ^{99m}TcO(PnAO–1-(2-nitroimidazole)) have demonstrated that, although the two isomers can be resolved under nonaqueous conditions, rapid racemization occurs in the presence of water. This result precludes testing of the isolated enantiomers in vivo, as rapid racemization is expected.

Experimental Section

The PnAO–1-(2-nitroimidazole) ligand (BMS-181032, 4,8-diaza-3,3,9,9-tetramethyl-1-(2-nitro-1*H*-imidazol-1-yl)undecane-2–10-dione dioxime, Figure 1) was prepared in house. The PnAO ligand was synthesized by the method of Vassian et al.[63] Misonidazole was obtained as a gift from Prof. K. Krohn, University of Washington. Metronidazole was purchased from Aldrich. ^{99}Tc is a weak β-emitter (0.29 keV, half-life 2.12×10^5 y); all reactions with technetium were carried out in laboratories approved for the use of radioactivity. Ammonium pertechnetate (NH_4TcO_4) [Oak Ridge National Laboratories] was recrystallized from aqueous H_2O_2. $^{99m}TcO_4^-$ in saline was obtained from an NEN/DuPont technetium generator. [NBu_4]$TcOCl_4$[64] was recrystallized from CH_2Cl_2/hexane. The complex TcO(ethylene glycol)$_2^-$ ($TcO(Eg)_2^-$)[51] was generated "in situ" using the procedure of Brenner et al.[65] Ru(acac)$_3$ was purchased from Strem. Bu_4NBF_4 (Aldrich) was recrystallized twice from MeOH/H_2O or acetone/ether; Bu_4NPF_6 (Aldrich) was recrystallized from acetone/absolute ethanol. Both supporting electrolytes were dried thoroughly and stored under vacuum. All other chemicals and solvents were reagent grade and used as received.

Reversed-phase high-pressure liquid chromatography (HPLC) measurements were made on 15-cm Hamilton PRP-1 or Nucleosil C8 columns (10 μm) using a mobile phase of acetonitrile (ACN)/0.1 M NH_4OAc, pH 4.6, a flow rate of 1–2 mL/min, and either UV detection at 230 nm or radiation detection. Infrared spectra (KBr) were obtained on a Sirius 100 FT-IR. Proton NMR data (270 MHz) were obtained with a JEOL-GX-270 spectrometer. UV/visible spectra were recorded using a Hewlett-Packard diode array spectrophotometer (Model 8451A). Fast atom bombardment (FAB) mass spectra were obtained on a VG-ZAB-2F spectrometer from a nitrobenzyl alcohol matrix. Elemental analyses were performed in-house by the Bristol-Myers Squibb Microanalytical Department.

^{99}TcO(PnAO–1-(2-nitroimidazole)) (1) from TcO_4^-. This compound was prepared in a manner similar to that described[33,50] for the synthesis of several congeners of ^{99}TcO(PnAO). The ligand PnAO–1-(2-nitroimidazole)·2.5H_2O (86.4 mg, 0.225 mmol) was dissolved in 10 mL of saline and 10 drops of 3 M HCl, adjusted to pH 6.3 with NaOH and added to a stirred solution of NH_4TcO_4 (26.6 mg, 0.148 mmol) in saline (4 mL). The pH of the solution was adjusted to pH 8.5–9.0 with 0.1 M $NaHCO_3$ and 0.1 M KOH. Diethyl ether (60 mL) was added, and a suspension of stannous tartrate (83.6 mg, 0.313 mmol) in saline (5 mL) was added dropwise, with stirring. After 10 min, the organic phase was removed, and the aqueous layer was extracted into 2 × 50 mL of ether. The combined ether layers were dried over anhydrous Na_2SO_4, reduced to ~2 mL, and purified on a silica gel column eluted with diethyl ether. The eluant was reduced to ~1 mL by rotary evaporation and chilled to −18 °C. Medium orange crystals (25.8 mg, 35%) precipitated overnight. The resulting analytically pure product was filtered, washed with cold ether, and vacuum dried for four hours. Anal. Calcd C, H, N. FAB MS: m/z (assignment) 496 $(M + H)^+$, 494 $(M - H)^-$, 480 $(M + H - O)^+$, 449 $(M - NO_2)^+$. IR (KBr): (Tc=O) 918, (N=O) 1373, 1539 cm^{-1}. ^{1}H NMR (270 MHz, CD_2Cl_2): δ 1.39, 1.44, 1.46, 1.49 (s, 12H, $C(CH_3)_2$), 1.75 (m, 1H, CH_2CHCH_2), 2.4 (m, 1H, CH_2CHCH_2), 2.34 (s, 3H, $N{=}CCH_3$), 3.35 (m, 2H, NCH_2CH_2); 3.47 (m, 2H, NCH_2CH_2), 5.72, 5.65 (dd, J_{AB} = 14.3 H, 2H $N{=}CCH_2$); 7.09 (s, 1H, imidazole), 7.47 (s, 1H, imidazole).

^{99}TcO(PnAO–1-(2-nitroimidazole)) (1) from TcO(ethylene glycol)$_2^-$. Ethylene glycol (50 μL, 0.84 mmol) was added to a stirred solution of MeOH (1 mL) and TBA[$TcOCl_4$] (50.4 mg, 0.1 mmol). A methanolic solution of sodium acetate (0.75 M, 0.54 mL) was then added dropwise. The solution turned deep purple, indicating the in situ formation[65] of TBA[$TcO(Eg)_2$]. A solution of PnAO–1-(2-nitroimidazole)·2.5H_2O (42.2 mg, 0.1 mmol) in 10 mL of MeOH was then added. The reaction was stirred for 15 min, concentrated under a stream of nitrogen until yellow product began to precipitate, and then cooled at 4 °C for 1 h. Product (28.3 mg, 57%) was collected by filtration, washed with 0.5 mL of cold methanol, recrystallized from CH_2Cl_2/ether/hexane, and dried in vacuo overnight. The product thus isolated was 99% pure, as determined by HPLC. Crystals suitable for X-ray crystallographic analysis were prepared by slow growth from CH_2Cl_2/ether at room temperature in the dark.

^{99}TcO(PnAO) (2) from TcO(Eg)$_2^-$. To a stirred solution of [TBA]$TcOCl_4^-$ (59 mg, 0.118 mmol) were added 1 mL of MeOH, 150 μL of neat ethylene glycol, and 1.6 mL of 0.75 M sodium acetate solution in MeOH. Addition of PnAO·HCl (54 mg, 0.177 mmol) caused the resulting purple solution to turn deep orange. The reaction mixture was treated with 10 mL of CH_2Cl_2, stripped to an orange oil by rotary evaporation, and purified on a 1.5- × 10-cm flash silica gel column eluted with CH_2Cl_2. Hexanes (15 mL) were added to the first red fraction, and the solvent was removed by rotary evaporation until precipitation of product occurred. The resulting red crystals were washed well with hexanes and dried in vacuo to yield 32 mg of pure **2** (71% yield overall). The product thus isolated was identical to that prepared by the method of Jurisson et al.,[33] as determined by HPLC, TLC, and UV/vis. Anal. Calcd C, H, N. FAB MS m/z (assignment): 385 $(M + H)^+$, 383 $(M - H)^-$. IR (KBr): (Tc=O) 924 cm^{-1}. ^{1}H NMR (270 MHz, CD_2Cl_2): δ 1.39, 1.45 (s, 12H, $C(CH_3)_2$), 1.83 (m, 1H, CH_2CHCH_2), 2.45 (m, 1H, CH_2CHCH_2), 2.26 (s, 6H, $N{=}CCH_3$), 3.37 (t, 2H, NCH_2CH_2), 3.55 (m, 2H, NCH_2CH_2).

^{99m}TcO(PnAO–1-(2-nitroimidazole)) (1). The ^{99m}Tc complex was prepared by reconstituting a freeze-dried kit containing 2.0 mg of PnAO–1-(2-nitro) ligand at pH 8.2 with 2.0 mL of a normal saline/generator eluant mixture containing 30 mCi of $^{99m}TcO_4^-$. The reaction was initiated by adding 50 μL of saturated stannous tartrate solution in saline via Hamilton syringe. The reaction was complete after 10 min at room temperature. Alternatively, a 0.15-mL aliquot from a Squibb Techneplex (Tc-DTPA) kit that had been reconstituted with 4.0 mL of saline was used as the source of stannous ion. The radiochemical purity of the complex was >94%, as determined by HPLC. The material thus prepared coeluted from HPLC columns with ^{99}TcO(PnAO–1-(2-nitroimidazole)).

Electrochemistry. Cyclic voltammetry (CV) experiments were performed using a Princeton Applied Research (PAR) Model 174A polarographic analyzer at a Model 303 static mercury drop electrode; data were recorded on a RE0074 X–Y recorder. The reference electrode was $Ag/AgNO_3$ with an acetonitrile filling solution saturated with LiCl. The counter electrode was a platinum wire. All CV and DC polarography solutions were 0.1 M in tetrabutylammonium tetrafluoroborate (Bu_4NBF_4) or tetrabutylammonium hexafluorophosphate (Bu_4NPF_6) supporting electrolyte, 0.2–0.7 mM in sample, and deoxygenated by bubbling solvent-saturated nitrogen or argon through the solution for 15 min. Variations in the reference potential were corrected for by determining the CV of a Ru(acac)$_3$ standard on a daily basis. All measured potentials were corrected according to an absolute peak reduction potential for Ru(acac)$_3$ of −1.210 V vs $Ag/AgNO_3$ at Hg (−0.790 V vs SCE at Pt). DC polarography

experiments were conducted on the same instrumentation and electrodes as described for CV. Polarograms were measured at 1-, 2-, and 5-s drop times with scan rates of 10, 5, and 2 mV/s, respectively.

Crystal Structure Analysis. Crystals of racemic 1 were obtained from methylene chloride/diethyl ether. Unit cell parameters were obtained through a least-squares analysis of the experimental diffractometer settings of 25 high angle reflections using Mo Kα monochromatic radiation (λ = 0.71069 Å): a = 12.393(3), b = 11.770(3), c = 15.026(4) Å; β = 113.07(2) deg; V = 2016(2) Å^3. Space group $P2_1/c$ was assigned on the basis of systematic absences on Weissenberg films and confirmed by the full structure analysis. The crystal density, D_{obs} = 1.63 g-cm^{-3} was measured by flotation in carbon tetrachloride/bromoform mixtures (D_{calc} = 1.629 for Z=4, $TcC_{16}H_{26}N_7O_5$). A total of 2795 reflections were measured on an Enraf-Nonius CAD4 diffractometer at 23 °C with the θ-2θ variable scan technique and were corrected for Lorentz polarization factors and for absorption by the DIFABS[66] method. Background counts were collected at the extremes of the scan for half the time of the scan. Two standard reflections were monitored for decay; no decrease of intensity was observed during the course of the measurements. Calculations utilized the SDP program package with minor local modifications.[67] The structure was solved by direct methods and refined on the basis of 2299 "observed" reflections with $I \geq 3\sigma(I)$. Although all hydrogen positions were evident in difference maps, only the hydroxyl proton was introduced in its observed position. All other protons were introduced in idealized positions, and their scattering was taken into account in the terminal stages of refinement. Least-squares weights, $w = \sigma^{-2}(F_o)$ were calculated with the assumption that $\sigma^2 = \epsilon^2 + (\rho I)^2$ where ϵ is the statistical counting error and ρ = 0.04. The function minimized in the least squares refinements was $\Sigma w(|F_o| - |F_d|)^2$. R is defined as $\Sigma||F_o| - |F_d||/\Sigma|F_o|$ while $R_w = [\Sigma w(|F_o| - |F_c|)^2/\Sigma w|F_o|^2]^{1/2}$. Refined variables were the coordinates and anisotropic temperature factors of all non-hydrogen atoms. The refinements converged at R = 0.031, R_w = 0.039. The final difference map contained no significant features.

Chiral HPLC Separation of Enantiomers of 1. Enantiomers of ^{99m}Tc 1 were separated using a Chiracel OD (150 × 4.6 mm) column eluted with hexane/isopropyl alcohol (IPA) at 0.5 mL/min. Column performance was monitored using *trans*-stilbene oxide as a standard. Samples of ^{99m}Tc 1 were labeled as described above and purified on reversed-phase resin using the procedures described in ref 68. After purification, the radioactive concentration of ^{99m}Tc 1 in ethanol was typically 7–12 mCi/mL. For studies of the rate of racemization, 1 was eluted from the column with a solvent ratio of 50:50 hexane/IPA; the individual peaks were collected as 0.5-mL fractions. Aliquots (25 μL) of isolated peaks were reinjected onto the column at several times after isolation. The effect of water on enantiomer racemization was studied by adding 2 drops of water (~0.07 g) to an isolated peak in 50:50 hexane/IPA (0.5 mL), giving a solution with ~14% water. In separate studies, samples were analyzed 0.33, 0.66, 2, and 23 min after water addition.

Enzyme Solutions. Xanthine oxidase (XOD, xanthine: oxygen oxidoreductase, EC 1.1.3.22; isolated from cow milk) was obtained from Boeringer Mannheim as a suspension in ammonium sulfate solution (3.2 mol/L in $(NH_4)_2SO_4$, pH ca. 8, [EDTA] = 10 mmol/L). The solution had a specific activity of about 1 unit/mg of protein, and contained ~20 units/mL. Xanthine (20 mg/L) and hypoxanthine (0.01 M) solutions were prepared by stirring the purines (Sigma) in water or phosphate buffer at a near boil until dissolved. All pH 7.4 phosphate buffer (0.025 or 0.1 M) contained 20 mg/L of $Na_2EDTA{\cdot}2H_2O$. Solutions were freshly prepared for each assay. The specific activity (units/mL) of the stock xanthine oxidase suspension was assayed by measuring the rate of XOD-catalyzed formation of uric acid from xanthine, following the procedure of Bray.[35] On the basis of these assay results, 0.5 unit of enzyme was added per run. One unit of activity was defined as that required to catalyze the aerobic oxidation of 1 μmol/min of xanthine to urate at 25 °C. The molar absorbtivity of urate was taken as 1.22 × 10^4 cm^{-1}.

Effect of DMF on XOD-Catalyzed Oxidation of Xanthine. Xanthine oxidase suspension (10 μL) was diluted to 1 mL with 0.025 M phosphate/EDTA buffer (pH 7.4). To a quartz cuvette was added 1.0 mL of xanthine solution (20 mg/L of water) followed by 1.75 mL of phosphate buffer and 150 μL of DMF, to give a final DMF concentration of 5%. The reaction was initiated by adding 100 μL of diluted enzyme solution. Absorbance at 292 nm was monitored for 10 min, and data converted to [change in absorbance units/min]. Similar runs were performed with 0 and 10% DMF. Appropriate UV/vis blanks contained water in the place of xanthine solution.

Effect of 0, 0.2, and 5% DMF on XOD-Catalyzed Reduction of Misonidazole and Metronidazole. To a 3-cm^3 quartz cuvette was added 1 mL of hypoxanthine (0.01 M, 10 μm) in pH 7.4 phosphate/EDTA buffer (0.1 M) and misonidazole (0.25 μm) dissolved in either 5 or 125 μL of DMF or in PO_4^- buffer. The volume was brought to 2 mL with PO_4^- buffer, and the cuvette was sealed with a rubber septum and purged of oxygen by passage of a stream of ultra high purity argon through the solution. Meanwhile, to a 5-mL siliconized vial was added 1.05 mL of PO_4^- buffer followed by about 150 μL of XOD suspension. The vial was crimp sealed, and deoxygenated by passing a flow of argon over the surface of the enzyme solution for exactly 15 min. The final solution was either 0, 0.2, or 5% in DMF. The same procedure was used for metronidazole, but only solutions that were 0.2% DMF were tested. To initiate the reaction, 500 μL of the deoxygenated enzyme solution (0.5 units) was added to the degassed misonidazole solution via gas-tight noncoring syringe. The final assay solution (2.5 mL) was 0.2 units/mL in XOD, 100 μM in nitro compound and 4 mM in hypoxanthine. The cuvette was inverted to mix, and then monitored by UV/vis (vs phosphate buffer/DMF buffer blank) to follow the disappearance of the nitro group absorbance. Both the spectrum of the solution from 280 to 450 nm, and absorbance at 326 nm were recorded every 5 min. Data were automatically stored to disk for later retrieval and analysis. Absorbance at 326 nm was converted to concentration, and plots of log[% remaining] vs time were prepared using Excel software.

XOD-Catalyzed Reduction of ^{99}TcO(PnAO–1-(2-nitroimidazole)) (1) and ^{99}TcO(PnAO) (2). The Tc complex (0.2 μm) in DMF (125 μL) was added to a 3-cm^3 quartz cuvette containing 1.0 mL of 0.01 M hypoxanthine solution in 0.1 M phosphate/EDTA buffer (pH 7.4) and 0.875 mL of 0.1 M phosphate/EDTA buffer. The cuvette was sealed and deoxygenated as described above. XOD suspension was diluted and deoxygenated as described above. To initiate the reaction, 500 μL of this enzyme solution (0.5 unit) was added to the degassed nitro solution via a gas-tight noncoring syringe. The final assay solution (2.5 mL) was 0.2 units/mL in XOD, 100 μM in nitro compound, 4 mM in hypoxanthine, and 5% in DMF. The spectrum of the resulting solution was recorded from 280 to 600 nm every 15 min for 10 h. Data were stored to disk for later retrieval and analysis as above. Control reactions (in the absence of enzyme or in the absence of hypoxanthine) were also carried out, substituting phosphate buffer for reagents not added.

Acknowledgment. We gratefully acknowledge Natarajan Raju and Kondareddiar Ramalingam for synthesis of the PnAO–1-(2-nitroimidazole) and PnAO ligand, John Di Marco for assistance in the X-ray crystallography, and Al Bauer for writing the Basic program for the automatic data collection from the UV/vis spectrometer. We also thank Professor Ken Krohn (University of Washington) for a generous gift of misonidazole.

Supplementary Material Available: Atomic positional parameters, thermal parameters, bond distances and bond angles for ^{99}TcO(PnAO–1-(2-nitroimidazole)) (1) (5 pages). Ordering information is given on any current masthead page.

References

(1) Linder, K. E.; Cyr, J.; Chan, Y. W.; Raju, N.; Ramalingam, K.; Nowotnik, D. P.; Nunn, A. D. Chemistry of a Tc-PnAO-Nitroimidazole Complex that Localizes in Hypoxic Tissue. *J. Nucl. Med.* **1992**, *33*, 919 (abstr).

(2) (a) Rumsey, W. L.; Patel, B.; Kuczynski, B.; Narra, R. K.; Chan, Y. W.; Linder, K.E.; Cyr, J.; Raju, N.; Ramalingam, K.; Nunn, A.

D. Potential of Nitroimidazoles as Markers of Hypoxia in Heart. Proceedings of the 20th Conference of the International Society of Oxygen Transport to Tissue, August 23–27, 1992, Mainz, Germany. (b) Rumsey, W. L.; Cyr, J. E.; Raju, N.; and Narra, R. K. A Novel [99m]Technetium-Labeled Nitroheterocycle Capable of Identification of Hypoxia in Heart. *Biochem. Biophys. Res. Commun.* **1993**, *193*, 1239–1246.

(3) Di Rocco, R. J.; Bauer, A.; Kuczynski, B. L.; Pirro, J. P.; Linder, K. E.; Narra, R.K.; Nunn, A. D. Imaging Regional Hypoxia with a New Technetium-Labeled Imaging Agent In Rabbit Myocardium After Occlusion of the Left Anterior Descending Coronary Artery. *J. Nucl. Med.* **1992**, *33*, 865 (abstr).

(4) Rumsey, W. L.; Patel, B.; Linder, K. E. The Oxygen Dependence of Mitochondrial Oxidative Phosphorylation and Its Relation to the Retention of a Novel ^{99m}Technetium-Labelled Nitroheterocycle. Proceedings of the 20th Conference of the International Society of Oxygen Transport to Tissue, August 23–27, 1992, Mainz, Germany.

(5) Di Rocco, R. D.; Kuczynski, B. L.; Pirro, J. P.; Bauer, A.; Linder, K. E.; Ramalingam, K; Cyr, J.; Chan, Y. W.; Raju, N.; Narra, R. K.; Nowotnik, D. P.; Nunn, A. D. Imaging Ischemic Tissue at Risk of Infarction in Stroke. *J. Cerebral Blood Flow Metab.* **1993**, *13*, 755–762.

(6) Varghese, A. J.; Gulyas, S.; Mohindra, J. K. Hypoxia-dependent Reduction of 1-(2-nitro-1-imidazoyl)-3-methoxy-2-propanol by Chinese Hamster Ovary Cells and KHT Tumor Cells In-vitro and In-vivo. *Cancer Res.* **1976**, *36*, 3761–3765.

(7) Garrecht, B. M.; Chapman, J. D. The Labeling of EMT-6 Tumors in BALB/C Mice with [C-14]-Misonidazole. *Br. J. Radiol.* **1983**, *26*, 745–753.

(8) Horowitz, M.; Blasberg, R.; Molnar, P.; et al. Regional [^{14}C]-misonidazole Distribution in Experimental RT-9 Brain Tumors. *Cancer Res.* **1983**, *43*, 3800–3807.

(9) Koch, C. J.; Stobbe, C. C.; Baer, K. A. Metabolism Induced Binding of 14-C Misonidazole to Hypoxic Cells. *Int. J. Oncol. Biol. Phys.* **1984**, *10*, 1327–1331.

(10) Urtasun, R. C.; Chapman, J. D.; Raleigh, J. A.; Franko, A. J.; Koch, C. J. Binding of ^{3}H-misonidazole to Solid Human Tumors as a Measure of Tumor Hypoxia. *Int. J. Radiat. Oncol. Biol. Phys.* **1986**, *12*, 1263–1267.

(11) Hoffman, J. M.; Rasey, J. S.; Spence, A. M.; et al. Binding of the Hypoxic Tracer [H-3] Misonidazole in Cerebral Ischemia. *Stroke* **1987**, *18*, 168–176.

(12) Martin, G. V.; Caldwell, J. H.; Krohn, K. A.; et al. Enhanced Binding of the Hypoxic Cell Marker [3-H] Fluoromisonidazole in Ischemic Myocardium. *J. Nucl. Med.* **1989**, *30*, 194–201.

(13) Rasey, J. S.; Krohn, K. A.; Freauff, S. Bromomisonidazole: Synthesis and Characterization of a New Radiosensitizer. *Radiat. Res.* **1982**, *91*, 542–554.

(14) Jette, D. C.; Weibe, L. I.; Chapman, J. D. Synthesis and in-vivo Studies of the Radiosensitizer 4-[^{82}Br]bromomisonidazole. *Nucl. Med. Biol.* **1983**, *10*, 205–210.

(15) Jerabek, P. A.; Patrick, T. B.; Kilbourn, M. R.; Dishino, D. D.; Welch, M. J. Synthesis and Biodistribution of ^{18}F-labeled Fluoronitroimidazoles: Potential in-vivo Markers of Hypoxic Tissue. *Int. J. Appl. Radiat. Isotop.* **1986**, *37*, 599–605.

(16) Martin, G. V.; Rasey, J. S.; Caldwell, J. C.; et al. Fluoromisonidazole Uptake in Ischemic Myocardium. *J. Nucl. Med.* **1987**, *28*, 668 (abstr).

(17) Shelton, M. E.; Dence, C. S.; Hwang, D.-R.; Welch, M. J.; Bergmann, S. R. Myocardial Kinetics of Fluorine-18 Misonidazole: A Marker for Hypoxic Myocardium. *J. Nucl. Med.* **1989**, *30*, 351–358.

(18) Rasey, J. S.; Nelson, N. J.; Chin, L., Evans, M. L.; Grunmaum, Z. Characteristics of the Binding of Labeled Fluoromisonidazole in Cells in Vitro. *Radiat. Res.* **1990**, *122*, 301–308.

(19) Koh, W. J.; Rasey, M. L.; Evans, J. R.; Grierson, J. R.; Lewellen, T. K.; Graham, M. M.; Krohn, K. K.; Griffin, T. W. Imaging of Hypoxia in Human Tumors with [^{18}F]fluoromisonidazole. *Int. J. Radiat. Oncol. Biol. Phys.* **1992**, *22*, 199–212.

(20) Martin, G. V.; Biskupiak, J. H.; Caldwell, J. H.; Grierson, J. R.; Krohn, K. A. Iodovinylmisonidazole: A Metabolic Marker for Myocardial Ischemia. *J. Nucl. Med.* **1990**, *31*, 833.

(21) Jette, D. C.; Wiebe, L. I.; Flanagan, R. J.; et al. Iodoazomycin Riboside, a Hypoxic Cell Marker. Synthesis and in vitro Characterization. *Radiat. Res.* **1986**, *105*, 169–179.

(22) Biskupiak, J. E.; Grierson, J. R.; Rasey, J. S.; Martin, G. V.; Krohn, K. A. Synthesis of an (Iodovinyl)misonidazole Derivative for Hypoxia Imaging. *J. Med. Chem.* **1991**, *34*, 2165–2168.

(23) Mannan, R. H.; Somayaji, V. V.; Lee, J.; Mercer, J. R.; Chapman, J. D.; Wiebe, L.I. Radioiodinated 1-(5-Iodo-5-deoxy-β-D-arabinofuranosyl)-2-nitroimidazole (Iodoazomycin Arabinoside:IAZA): A Novel Marker of Tissue Hypoxia. *J. Nucl. Med.* **1991**, *32*, 1764–1770.

(24) Linder, K. E.; Chan, Y.-W.; Cyr, J. E.; Nowotnik, D. P.; Eckelman, W. C.; Nunn, A.D. The Synthesis, Characterization and In-vitro Evaluation of Nitroimidazole-BATO Complexes; New Technetium Compounds for Imaging Hypoxic Tissue. *Bioconjug. Chem.* **1993**, *4*, 326–333.

(25) Treher, E. N.; Francesconi, L. C.; Gougoutas, J. Z.; Malley, M. F.; Nunn, A. D. Monocapped Trisdioxime Complexes of Technetium(III): Synthesis and Structural Characterization of TcX(dioxime)$_3$B-R. *Inorg. Chem.* **1989**, *28*, 3411–3416.

(26) Linder, K. E.; Malley, M. F.; Gougoutas, J. Z.; Unger, S. E.; Nunn, A. D. Neutral, Seven-Coordinate Complexes of Technetium(III): Synthesis and Characterization. *Inorg. Chem.* **1990**, *29*, 2428–2434.

(27) Kedderis, G. L.; Miwa, G. T. The Metabolic Activation of Nitroheterocyclic Therapeutic Agents. *Drug Metab. Rev.* **1988**, *19*, 33–62.

(28) Chapman, J. D.; Baer, K.; Lee, J. Characteristics of the Metabolism-induced Binding of Misonidazole to Hypoxic Mammalian Cells. *Cancer Res.* **1983**, *43*, 1523–1528.

(29) Whitmore, G. F.; Varghese, A. J. The Biological Properties of Reduced Nitroheterocycles and Possible Underlying Biochemical Mechanisms. *Biochem. Pharmacol.* **1986**, *35*, 97–101.

(30) Troutner, D. E.; Volkert, W. A.; Hoffman, T. J.; Holmes, R. A. A Tetradentate Amine Oxime Complex of Tc-99m. *J. Nucl. Med.* **1983**, *24*, P10.

(31) Troutner, D. E.; Volkert, W. A.; Hoffman, T. J.; Holmes, R. A. A Neutral Lipophilic Complex of Tc-99m with a Multidentate Amine Oxime. *Int. J. Appl. Radiat. Isot.* **1984**, *35*, 467–470.

(32) Fair, C. K.; Troutner, D. E.; Schlemper, E. O.; Murmann, R. K.; Hoppe, M. L. Oxo[3,3′-(1,3-propanediyldiimino)bis(3-methyl-2-butanone oximato)(3-)-N,N′,N″,N‴]technetium(V), [TcO(C_{13}-$H_{25}N_4O_2$)]. *Acta Crystallogr.* **1984**, *C40*, 1544–1546.

(33) Jurisson, S.; Schlemper, E. O.; Troutner, D. E.; Canning, L. R.; Nowotnik, D. P.; Neirinckx, R. D. Synthesis, Characterization, and X-ray Structural Determinations of Technetium(V)-Oxo-Tetradentate Amine Oxime Complexes. *Inorg. Chem.* **1986**, *25*, 543–549.

(34) Chapman, J. D.; Franko, J. A.; Sharplin, J. A. A Marker for Hypoxic Cells in Tumors with Potential Clinical Applicability. *Br. J. Cancer* **1981**, *43*, 546–550.

(35) Bray, R. C. In *The Enzymes*, 3rd ed.; Boyer, . D., Ed.; Academic Press: New York, 1975; Vol. 12, part B, p 300.

(36) Varghese, A. J.; Whitmore, G. F. Binding of Nitroreduction Products of Misonidazole to Nucleic Acids and Protein. In *Radiosensitizers: Their Use Clin. Management of Cancer [Conf. Proc.]*; Brady, L. W., Ed.; Masson: New York, 1980; p 57.

(37) McClelland, R. A.; Fuller, J. R.; Seaman, N. E.; Rauth, A. M.; Battistella, R. 2-Hydroxylaminoimidazoles - Unstable Intermediates in the Reduction of 2-Nitroimidazoles. *Biochem. Pharmacol.* **1984**, *33*, 303–309.

(38) McClelland, R. A.; Panicucci, R.; Rauth, A. M. Products of the Reductions of 2-Nitroimidazoles. *J. Am. Chem. Soc.* **1987**, *109*, 4308–4313.

(39) Morita, M.; Feller, D. R.; Gillete, J. R. Reduction of Niridazole by Rat Liver Xanthine Oxidase. *Biochem. Pharmacol.* **1971**, *20*, 217–226.

(40) Chrystal, E. J. T.; Koch, R. L.; Goldman, P. Metabolites from the Reduction of Metronidazole by Xanthine Oxidase. *Mol. Pharmacol.* **1980**, *18*, 105–111.

(41) Clarke, E. D.; Wardman, P.; Goulding, K. H. Anaerobic Reduction of Nitroimidazoles by Reduced Flavin Mononucleotide and by Xanthine Oxidase. *Biochem. Pharmacol.* **1980**, *29*, 2684–2687.

(42) Clarke, E. D.; Goulding, K. H.; Wardman, P. Nitroimidazoles as Anaerobic Electron Acceptors for Xanthine Oxidase. *Biochem. Pharmacol.* **1982**, *31*, 3237–3242.

(43) Josephy, P. D.; Palcic, B.; Skarsgard, L. D. Reduction of Misonidazole and its Derivatives by Xanthine Oxidase. *Biochem. Pharmacol.* **1981**, *30*, 849–853.

(44) Prekeges, J. L.; Rasey, J. S.; Grunbaum, Z.; Krohn, K. Reduction of Fluoromisonidazole, A New Imaging Agent for Hypoxia. *Biochem. Pharmacol.* **1991**, *42*, 2387–2395.

(45) Walton, M. I.; Wolf, C. R.; Workman, P. Molecular Enzymology of the Reductive Bioactivation of Hypoxic Cell Cytotoxins. *Int. J. Radiation Oncol. Biol. Phys.* **1989**, *16*, 983–986.

(46) Johnson, C. K. ORTEP. Report ORNL-3794; Oak Ridge National Laboratory: Oak Ridge, TN, 1965.

(47) Edwards, D. S.; Cheesman, E. H.; Watson, M. W.; Maheu, L. J.; Nguyen, S. A.; Dimitre, L.; Nason, T.; Watson, A. D.; Walovitch, R. Synthesis and Characterization of Technetium and Rhenium Complexes of N,N′-1,2-ethylenediylbis-L-cysteine. Neurolite and its Metabolites. in *Technetium and Rhenium in Chemistry and Nuclear Medicine, Vol. 3*; Nicolini, M., Bandoli, G., Mazzi, U., Eds.; Raven Press: New York, 1990; pp 433–444.

(48) Pilcher, G. D.; Coveney, J. R.; Marmion, M. E.; Nosco, D. L.; Strubel, T. W. Biological Characteristics of Tc-99m Complexes with Ester Analogues of 1,1,1-tris(4-methoxysalicylaldimino-methyl)ethane (4-MeO-SAL$_3$TAME). *J. Nucl. Med.* **1989**, *30*, 937 (abstr).

(49) Kronauge, J. F.; Davison, A; Roseberry, A. M.; Costello, C. E.; Maleknia, S.; Jones, A. G. Synthesis and Identification of the Monocation Tc(CPI)$_6^+$ in Tc(CNC(CH$_3$)$_2$COOCH$_3$)$_6$Cl and Its Hydrolysis Products. *Inorg. Chem.* **1991**, *30*, 4265–4269.

(50) Jurisson, S.; Aston, K.; Fair, C. K.; Schlemper, E. O.; Sharp, P. R.; Troutner, D. E. Effect of Ring Size on Properties of Technetium Amine Oxime Complexes. X-ray Structures of TcO$_3$(Pent(AO)$_2$, Which Contains an Unusual Eight-Membered Chelate Ring, and of TcOEn(AO)$_2$. *Inorg. Chem.* **1987**, *26*, 3576–3582.

(51) DePamphilis, B. V.; Jones, A. G.; Davison, A. Ligand Exchange Reactivity Patterns of Oxotechnetium(V) Complexes. *Inorg. Chem.* **1983**, *22*, 2292–2297.

(52) Nagarajan, K.; Sudarsanam, V.; Parthasarathy, P. C.; Arya, V. P.; Shenoy, S. J. Nitroimidazoles: Part X - Spectral Studies on Isomeric 1-substituted 4- and 5- nitroimidazoles and Some 2-nitroimidazoles. *Indian J. Chem. Sect. B* **1992**, *21B*, 1006.
(53) Tocher, J. H.; Edwards, D. I. Electrochemical Characteristics of Nitro-Heterocyclic Compounds of Biological Interest. *Free Rad. Res. Commun.* **1988**, *4*, 269–276.
(54) Breccia, A.; Berrilli, G.; Roffia, S. Chemical Radiosensitizaton of Hypoxic Cells and Redox Potentials: Correlation of Voltammetric Results with Pulse Radiolysis Data of Nitro-Compounds and Radiosensitizers. *Int. J. Radiat. Biol.* **1979**, *36*, 85–89.
(55) O'Neill, P. One Electron Reduction Potentials of Nitroimidazoles: Correlation with Polarographic Half-Wave Potentials. *Anal. Proc. (London).* **1980**, *17*, 282–283.
(56) Greenstock, C. L.; Ruddock, G. W.; Neta, P. Pulse Radiolysis and ESR Studies of the Electron-Affinic Properties of Nitroheterocyclic Radiosensitizers. *Radiat. Res.* **1976**, *66*, 472.
(57) Maher T. J.; Johnson D. A. Review of Chirality and Its Importance in Pharmacology. *Drug. Develop. Res.* **1991**, *24*, 149.
(58) Nowotnik D. P.; Jurisson, S. Structure and Stereochemistry in Technetium Coordination Complexes. In *The Chemistry of Technetium in Medicine*; Steigman, J.; Eckelman, W.C., Eds.; National Academy Press: Washington, D.C., 1992; pp 111–130.
(59) Krstulovic, A. M. *Chiral separations by HPLC. Applications to pharmaceutical compounds*; Ellis Horwood Ltd.: Chichester, 1989.
(60) Cyr, J.; Linder, K. E.; Nanjappan, P.; Raju, N.; Ramalingam, K.; Nowotnik, D. P.; Nunn, A. D. Tc-PnAO Complexes that are Substituted at the Central Carbon Atom. Proceedings of 9th International Symposium on Radiopharmaceutical Chemistry, April 6–10, 1992, Paris. *J. Labeled Compd. and Radiopharm.* **1993**, *32*, 4–6.
(61) de Jong, J. W.; van der Meer, P.; Nieukoop, A. S.; Huizer, T.; Stroeve, R. J.; Bos, E. Xanthine Oxidoreductase Activity in Perfused Hearts of Various Species, Including Humans. *Circ. Res.* **1990**, *67*, 770–773.
(62) Rauth, A. M. Pharmacology and Toxicology of Sensitizers: Mechanism Studies. *Int. J. Radiat. Oncol. Biol.* **1984**, *10*, 1291–1300.
(63) Vassian, E. G.; Murmann, R. K. Aromatization of an Aliphatic Amine Oxime Ni(II) Complex by Molecular Oxygen. *Inorg. Chem.* **1967**, *6*, 2043.
(64) Cotton, F. A.; Davison, A.; Day, V. W.; Gage, L. D.; Trop, H. S. Preparation and Structural Characterization of Salts of Oxotetrachlorotechnetium(V). *Inorg. Chem.* **1979**, *18*, 3024–3029.
(65) Brenner, D.; Davison, A.; Lister-James, J.; Jones, A. G. The Synthesis and Characterization of a Series of Isomeric Oxotechnetium(V) Diamido Dithiolates. *Inorg. Chem.* **1984**, *23*, 3793–3797.
(66) Walker, N.; Stuart, D. *Acta Crystallogr., Sect. A* **1983**, *39*, 159.
(67) SDP. Structure Determination Package. ENRAF-NONIUS, Bohemia, NY 11716.
(68) Jurisson, S. S.; Hirth, W.; Linder, K. E.; Di Rocco, R. J.; Narra, R. K.; Nowotnik, D.P.; Nunn, A. D. Chloro→Hydroxy Substitution in Technetium BATO [TcCl(Dioxime)$_3$BR] Complexes. *Nucl. Med. Biol.* **1991**, *18*, 735–744.
(69) Parliament, M. B.; Chapman, J. D.; Urtasun, R. C.; McEwan, A. J.; Golberg, L.; Mercer, J. R.; Mannan, R. H.; Wiebe, L. I. Non-invasive Assessment of Human Tumour Hypoxia with ^{123}I-iodoazomycin Arabinoside: Preliminary Report of a Clinical Study. *Br. J. Cancer* **1992**, *65*, 90–95.
(70) Deutsch, E.; Ketring, A. R.; Libson, K.; Vanderheyden, J.-L.; Hirth, W. W. The Noah's Ark Experiment: Species Dependent Biodistributions of Cationic ^{99m}Tc Complexes. *Int. J. Nucl. Med. Biol.* **1989**, *16*, 191–232.
(71) Deutsch, E. A.; Vanderheyden, J.-L.; Gerundini, P.; Libson, K.; Hirth, W. W.; Colombo, F.; Savi, A.; Fazio, F. Development of Non-reducible Technetium-99m(III) Cations as Myocardial Perfusion Imaging Agents: Initial Experience in Humans. *J. Nucl. Med.* **1987**, *28*, 1870–1880.
(72) Dilworth, J. A.; Archer, C. M.; Latham, I. A.; Bishop, P. T.; Higley, B. The Synthesis of Technetium-nitride Cations as Potential Myocardial Agents. *J. Nucl. Med. Allied Sci.* **1989**, *33*, 281–282.
(73) Lahiri, A.; Higley, B.; Crawly, J. C. W.; Chiu, K. W.; Edwards, B.; Smith, T.; Griffiths, D. V.; Archer, C. M.; Latham, I. A.; Kelley, J. D. Novel Functionalized Diphosphine Complexes of Tc-99m for Myocardial Imaging in Man. *J. Nucl. Med.* **1989**, *30*, 818 (abstr).