The Synthesis and *in Vitro* Evaluation of a ^{99m}Technetium–Nitroimidazole Complex Based on a Bis(amine–phenol) Ligand: Comparison to BMS-181321

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We have developed a ^{99m}Technetium complex for imaging of hypoxic tissue (BMS-181321). Recently, another nitroimidazole derivative, based upon a bis(amine–phenol) ligand, was described in the patent literature. To compare this compound to BMS-181321, we have synthesized the ligand, prepared its ^{99m}Tc complex, and evaluated its performance in two *in vitro* assays of bioefficacy: membrane permeability and uptake in normoxic and anoxic cardiocytes. In attempting to reproduce the synthesis of the ligand described in the patent application, we found that one intermediate could not be made by the method described, and alternative routes were investigated. Complexation of the bis(amine–phenol) nitroimidazole with ^{99m}Tc gave an apparent single complex; this appeared as a broad peak on HPLC analysis. Purification by a solid-phase method gave a complex with 95% radiochemical purity. This complex was not permeable to cultured bovine brain endothelial cells nor did it show preferential uptake in anoxic myocytes.

Introduction

The development of imaging agents which aid in the identification of tissue at risk as a result of ischemia is an important clinical goal. Much of the recent research in this area has focused on the development of new radiopharmaceuticals which are derivatives of nitroimidazoles. Preliminary studies with ^{14}C-[1-4] and ^{3}H-labeled[5,6] nitroimidazoles demonstrated that these compounds display preferential binding to hypoxic cells. ^{82}Br,[7,8] ^{18}F,[9-12] and I-125/123-labeled[13-17] 2-nitroimidazole derivatives have been evaluated for the identification of hypoxic tissue, primarily in brain, heart, and tumors.

As the preferred radionuclide for use in nuclear medicine is ^{99m}Tc, the development of a technetium nitroimidazole derivative for the identification of hypoxic tissue would be a useful contribution to this field. Our initial studies involved the preparation and evaluation of nitroimidazole–BATO complexes [BATO = boronic acid adduct of technetium dioxime, TcX(dioxime)$_3$BR (X = Cl, OH). BR = a nitroimidazole boronic acid derivative[18]]. Results of *in vitro* studies with these BATOs were promising,[19] but they did not provide adequate discrimination between normoxic and hypoxic tissue *in vivo* (unpublished results). One factor which might contribute to the poor *in vivo* performance of the nitroimidazole BATOs is inadequate permeability to lipophilic membranes. As the reductive enzymes, which are thought to be responsible for the entrapment of nitroimidazoles in hypoxic cells[20] are located within cells, compounds must cross the plasma membrane to reach these enzymes. BATO complexes have displayed poor permeability to membranes in an *in vitro* model of the blood–brain barrier.[21]

As the ligand propyleneamine oxime (PnAO) forms a technetium complex which is able to cross cell membranes,[21] we prepared a nitroimidazole derivative of PnAO. The technetium complex of this compound, termed BMS-181321 (Figure 1), displays preferential localization in both *in vitro* and *in vivo* models of hypoxia.[22-24] During the course of our studies with this complex, we became aware of a patent application[25] which describes a technetium–nitroimidazole derivative based upon a bis(amine–phenol) tetradentate chelating moiety (Figure 2). We have prepared the bis(amine–phenol)–nitroimidazole (BAPN, Figure 2) and evaluated its potential for imaging hypoxic tissue. We found that we could not reproduce the synthesis as described in the patent application, and we now describe an alternate method for the preparation of this ligand. We evaluated the ^{99m}Tc complex of this ligand in our *in vitro* model for membrane permeability and by using uptake studies in isolated cardiac myocytes. The results are compared to those obtained with BMS-181321.

Results and Discussion

Synthesis of the Bis(amine–phenol)–Nitroimidazole (BAPN). Literature Procedure. The published procedure[25] for the synthesis of BAPN is shown in Scheme 1. We could not reproduce the reduction of the diamide to diamine (step iii). The lithium aluminum hydride reduction of 2-allylmalonamide using THF as a solvent afforded a complex mixture of products. The NMR spectrum of the crude reaction mixture indicated the presence of the diamide as the major component. Table 1 lists the alternate reaction conditions that we investigated to produce the diamine from the diamide. None were successful. We believe that the problem is related to the poor solubility of the starting material. Either unreacted starting material was recovered or, when more forcing conditions were used, degraded diamine. As will be described below, we were able,

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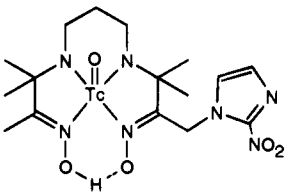


Figure 1. The structure of BMS-181321.

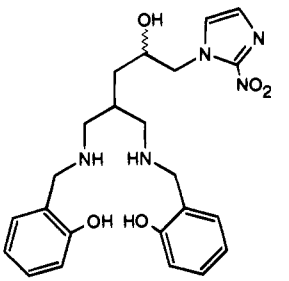


Figure 2. The structure of BAPN.

under considerably modified conditions, to isolate the diamine by reduction of the diamide. This involved *in situ* protection of the amine groups as soon as they were formed. This intermediate could (in principle) be deprotected, and the route described in the published procedure continued to completion. However, we opted to use an alternative route which we felt was superior to the literature method for the synthesis of BAPN from this N-protected intermediate.

A number of alternative routes for the synthesis of BAPN were explored. These involved the intermediate *o*-hydroxybenzylamine and are described in the next section.

Possible Routes of Synthesis of BAPN via *o*-Hydroxybenzylamine. The reaction pathways of the two routes discussed in this section are shown in Schemes 2 and 3. In both cases, the strategy involves the condensation of *o*-hydroxybenzylamine with a malonic ester derivative to give a diamide, which is reduced to the tetradentate diamine diphenol ligand. The plan for attachment of the nitroimidazole in these two schemes is the same as in the published procedure; alkylation of 2-nitroimidazole by an epoxide. The major difference between the two schemes involves the point in the scheme when the nitroimidazole is attached; in Scheme 2, the nitroimidazole is attached at an early stage in the synthesis, whereas in Scheme 3, the nitroimidazole is linked to the bis(amine–phenol) in the penultimate step (the final step being N/O deprotection). The route shown in Scheme 3 was taken to completion, although the BAPN obtained by this method was not fully purified and characterized.

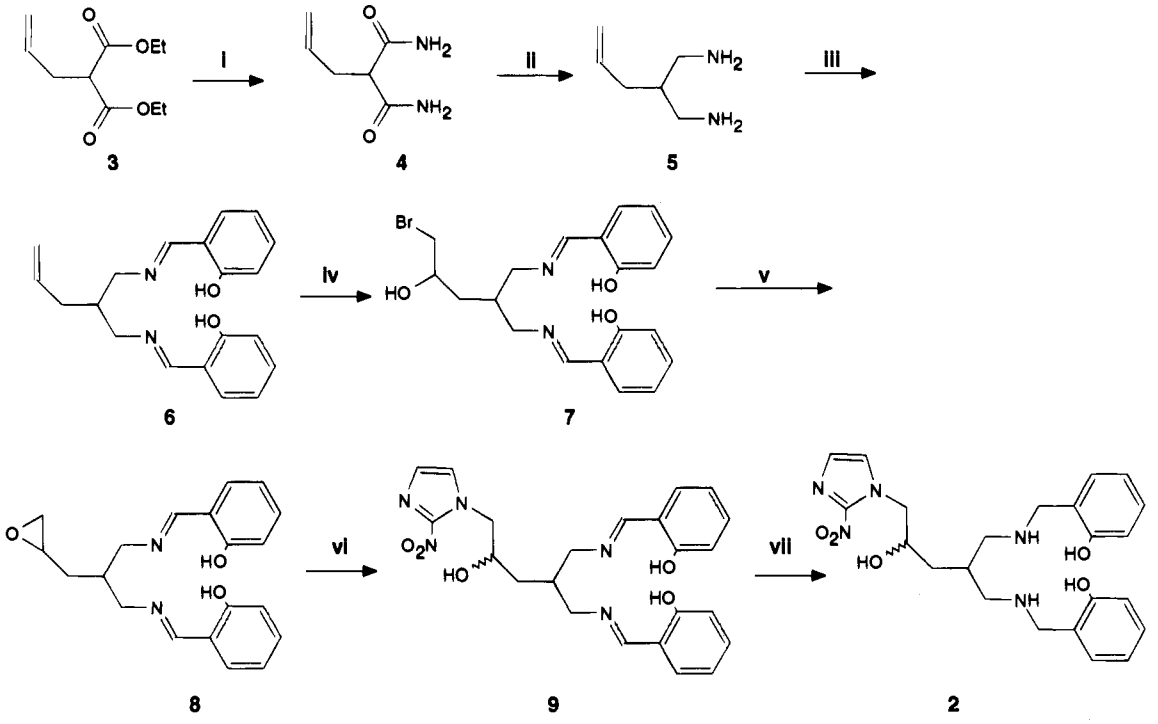
The route, part of which is shown in Scheme 2, represents an attempt to bypass the problematic synthesis of 2-allyl-1,3-diaminopropane, by preparing the diamine *after* attachment of the nitroimidazole group. The intention was to react the nitroimidazole-substituted diethyl malonate (**11**) with *o*-hydroxybenzylamine, which could then be reduced to give BAPN. At the outset, diethyl 2-allylmalonate was oxidized with *m*-chloroperoxybenzoic acid (mCPBA) to afford epoxide **10**, which was reacted with 2-nitroimidazole in DMF at 80 °C in the presence of Proton Sponge to give the diester **11** and the lactone **12**. These products were separated on a silica gel column in yields 25% and 50%, respectively. Diethyl 2-(3-(2-nitro-1*H*-imidazol-1-yl)-2-hydroxypropyl)malonate (**11**) on treatment with *o*-hydroxybenzylamine in refluxing methanol gave a complex mixture of products. As other synthetic strategies were proving to be more successful, the condensation reaction between **11** and *o*-hydroxybenzylamine was not investigated further.

In Scheme 3, *o*-hydroxybenzylamine (**14**) was prepared by the catalytic reduction (Pd–C, 10%) of commercially-available salicylaldoxime. Coupling the benzylamine with diethyl allylmalonate afforded the required diamide (**15**) in 50% yield. Reduction of the diamide with $LiAlH_4$ in THF provided the diamine (**16**) in 28% yield after column chromatography. Purification of the diamine proved to be difficult, so the impure diamine was converted to its tetra-*t*-Boc derivative. Repeated purification yielded the product which appeared as a single spot on TLC analysis. The tetra-*t*-Boc olefin (**16**) was converted to the epoxide (**17**) with mCPBA, and the epoxide (**17**) was purified using flash column chromatography. The epoxide was reacted with 2-nitroimidazole, and the *t*-Boc groups were removed with methanolic HCl. After neutralization, BAPN was isolated as a yellow solid in <10% yield following purification by flash silica gel column chromatography. The ^1H NMR spectrum and HPLC retention time of this compound were identical to BAPN prepared by the method described below.

The Synthesis of BAPN via the Protected Diamine. The route of synthesis which eventually provided us with fully characterized BAPN is shown in Scheme 4. The first part of the scheme is similar to the published method. An essential improvement was in the reduction of the allyl diamide to the allyldiamine. We were able to make this work successfully by *in situ* conversion of the diamine as its bis-*t*-Boc derivative, which inhibited degradation of the diamine and made it possible to isolate the diamine from other reaction components.

DME was found to be a good solvent for the reduction of the diamide (**4**) with $LiAlH_4$. The solubility of the diamide in DME at room temperature was poor, so a solution of the diamide was obtained at reflux temperature, and this was added to a slurry of lithium aluminum hydride. The amine from the reaction mixture was isolated as a bis-*t*-Boc derivative (**18**) using di-*tert*-butyl dicarbonate. *t*-Boc-protection of the amine was also useful in subsequent steps, which differ from the published method. Reaction of the bis-N-protected allyldiamine (**18**) with *m*-chloroperoxybenzoic acid gave the epoxide (**19**) in 92% yield, avoiding the need to make the bromohydrin intermediate (**7**) in the published procedure. Also, in the literature procedure, the nitroimidazole group was attached to the "propanediamine" fragment after the two phenol groups. In our method, the nitroimidazole group was attached to the "propanediamine" fragment prior to the attachment of two phenol groups. The choice of reaction conditions was crucial in this step. Epoxide (**19**) was first reacted with the sodium salt of 2-nitroimidazole in DMF. Workup afforded only a water-soluble product which was found (by HPLC analysis) to be unreacted 2-nitroimidazole. Attempted opening of the epoxide with 2-nitroimidazole using potassium carbonate in DMF also failed. However, nucleophilic ring opening of the

Scheme 1. Reaction Scheme Described in the Literature[25]



Reaction key:

	Reagents/conditions	Published yield
i	NH_3/catalytic amt. Na/MeOH/RT/1 week	94%
ii	$LiAlH_4$/anhydrous THF/RT and reflux.	65%
iii	Salicylaldehyde/RT/3 h.	83%
iv	NBS/THF/water - RT/2 days.	32%
v	K_2CO_3/MeOH/water - RT.	76%
vi	Nitroimidazole/DMSO/80°C.	28%
vii	$NaBH_4$/EtOH/0°C/2 h.	63%

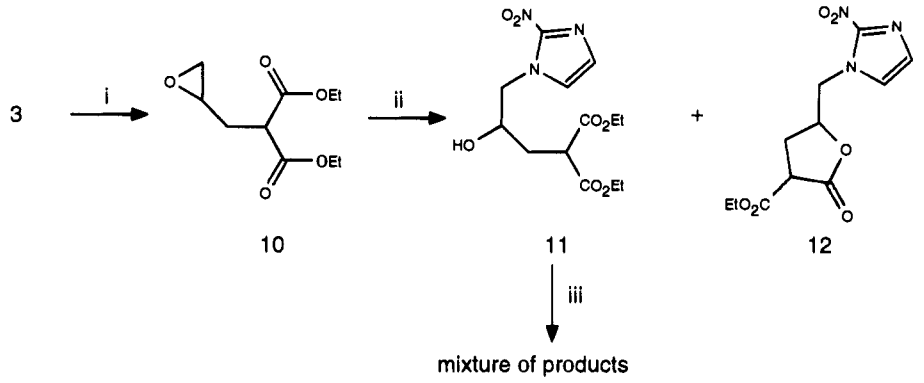
Table 1. Reaction Conditions Used in Attempts To Prepare 2-Allyl-1,3-propanediamine from 2-Allylmalonamide

diamide (mmol)	solvent (mL)	$LiAlH_4$ (mmol)	reaction temperature	reaction time (h)	observations
22	THF/100	40	reflux	8	complex products with diamide as the major component (diamide was recovered as a white solid)
22	THF/150	93	reflux	24	complex products with diamide as the major components (diamide was recovered as a white solid)
22	THF/50	50 mL, 1 M in THF	reflux	60	complex products with diamide as the major component (diamide was recovered as a white solid)
22	DME/100	40	reflux	8	crude NMR of the diamine, some diamide was recovered; no pure diamine was obtained
22	DME/100	40 (pellet)	reflux	60	crude NMR of the diamine, some diamide was recovered; no pure diamine was obtained
22	DME/50	100 mL, 0.5 M in DME	reflux	60	crude NMR of the diamine, some diamide was recovered; no pure diamine was obtained
22	diglyme/100	40	reflux	8	MS and crude NMR of the diamine, no diamide was recovered; vacuum distillation of the product failed to give pure diamine (probably due the instability of the amine)
22	diglyme/50	100 mL, 0.5 M in diglyme	reflux	24	crude product was use to react with salicylaldehyde and a complex mixture resulted; no pure imine was obtained

epoxide (**19**) with nitroimidazole was achieved, providing **20** in 35% yield, when the epoxide was reacted with 2-nitroimidazole in ethanol using catalytic amount of potassium carbonate. Removal of the *t*-Boc groups was followed by the condensation with salicylaldehyde. The resultant imine (**9**) was reduced with $NaBH_4$ in methanol to give BAPN (**2**).

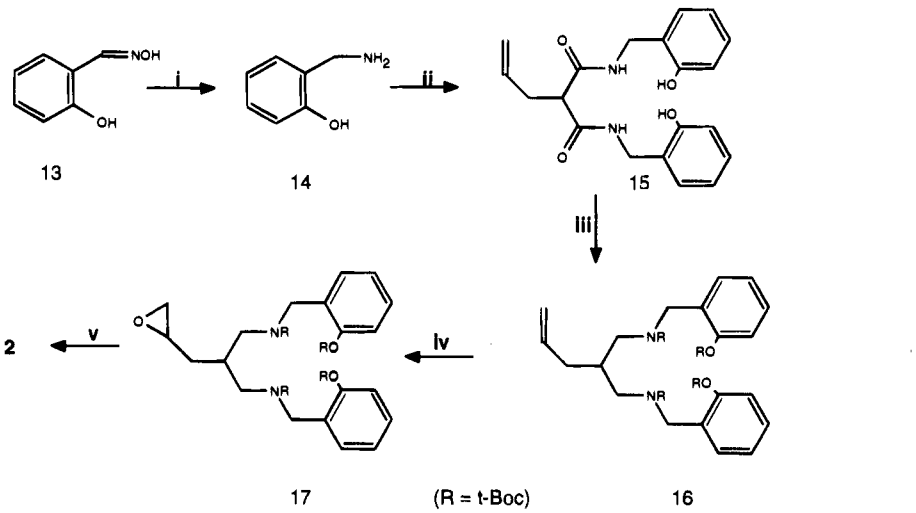
Synthesis and Analysis of the ^{99m}Tc Complex of BAPN. ^{99m}Tc complexes of the bis(amine–phenol) ligand systems are not novel. Pillai and co-workers

Scheme 2. Alternate Reaction Pathways: (a) Addition of the Nitroimidazole at an Early Stage[a]



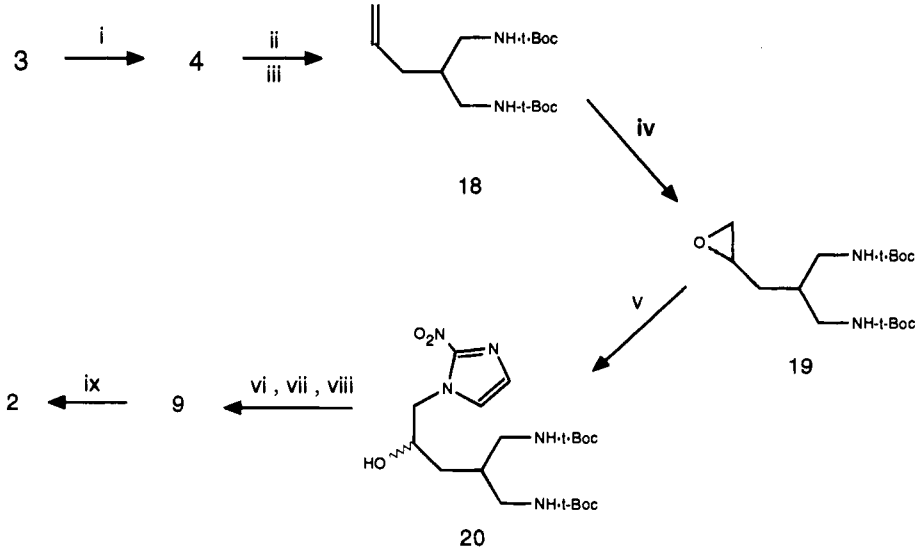
[a] (i) *m*-CPBA, CH_2Cl_2; (ii) 2-nitroimidazole, Proton Sponge, DMF; (iii) *o*-hydroxybenzylamine, CH_3OH.

Scheme 3. Alternate Reaction Pathways: (b) Addition of the Nitroimidazole at the Final Stage[a]



[a] (i) $LiAlH_4$,THF or Pd–C, H_2; (ii) diethyl allylmalonate, MeOH, reflux, 48 h; (iii) (a) $LiAlH_4$, THF, reflux, 24 h, (b), di-*tert*-butyl dicarbonate, Et_3N, THF, reflux; (iv) mCPBA, $NaHCO_3$, CH_2Cl_2, 6 h, room temperature; (v) (a) 2-nitroimidazole, $NaHCO_3$, DMF, 100 °C, 2 h, (b) methanolic HCl, NH_4OH.

Scheme 4. The Synthesis of Fully Characterized BAPN[a]



[a] (i) NH_3, CH_3OH; (ii) $LiAlH_4$, DME; (iii) (*t*-Boc)$_2$O, Na_2CO_3, dioxane–water; (iv) mCPBA, CH_2Cl_2; (v) 2-nitroimidazole, K_2CO_3, C_2H_5OH; (vi) CH_3OH, HCl; (vii) CH_3OH, NH_3; (viii) salicylaldehyde, C_2H_5OH; (ix) $NaBH_4$, CH_3OH.

have published details of the synthesis of several bis(amine–phenol) and bis(imine–phenol) ligands and their ^{99m}Tc complex.[26-30] The structures of some of the reported bis(amine–phenol) ligands are shown in Figure 3. The ligands with a propylene bridge between the amines have the same ligand structure as BAPN. All four ligands form ^{99m}Tc complexes with an initial high (>85%) radiochemical purity (RCP), when formed by standard stannous reduction (using stannous tartrate) of pertechnetate in the presence of 1.4×10^{-4} M solution

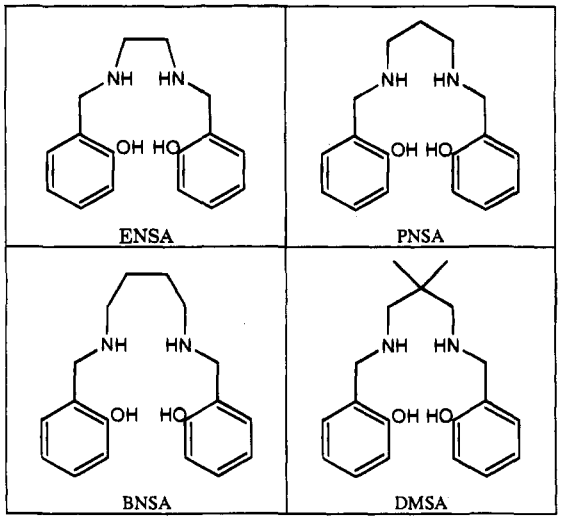


Figure 3. Structures of the bis(amine–phenol) ligands described by Corlija et al.[27]

Table 2. The Loss of Radioactivity from an Aqueous Solution of ^{99m}Tc BAPN over Time

reaction time (min)	total counts eluted from HPLC with 2 μL injection	% recovery (relative to $T = 0$)
0[a]	459403	100
3	331011	72
15	283062	62
81	164265	36

[a] Analyzed before Sn tartrate added.

of ligand at pH 9.0.[27] The complexes with a propylene bridge between the amines (PNSA and DMSA) are reasonably stable up to 2 h after reconstitution, whereas the complexes from ligands with ethylene and butylene bridges degrade quickly.[28] Reversed-phase HPLC traces of products of ^{99m}Tc complexation of PNSA indicate that several complexes may be formed; the proportion of each complex appears pH dependent, with one complex predominating at pH 10.5.[27] The structure of the ^{99}Tc complex of PNSA was reported as a O=TcOTc=O dimer.[31]

We prepared the ^{99m}Tc complex of the BAPN using conditions similar to those of Corlija et al.[27] (stannous reduction of pertechnetate under slightly alkaline conditions). The product eluted as a broad tailing band from a PRP-1 reversed-phase HPLC system (80/20 MeOH/0.1 M NH_4OAc pH 4.6, 2 mL/min, void volume = 1.06 min) with a retention time of 2.5 min. The complex was purified on PRP-1 resin and eluted with the free ligand in 95% EtOH. Ethanol fractions appeared to be stable. However, in aqueous solution, the complex gradually came out of solution, with radioactivity being deposited on the vial wall. The loss of radioactivity from solution was followed over time, as shown in Table 2.

The deposited radioactivity could be removed by an ethanol wash; HPLC analysis of this ethanol wash indicated that it was unchanged complex. These data indicate that the ^{99m}Tc complex of BAPN is lipophilic, with poor water solubility. Without further study, we do not know whether BAPN forms a single technetium complex or a mixture of complexes. Although the ^{99m}Tc complex of BAPN was observed as a single peak in our preliminary HPLC analysis (suggesting a single complex), the breadth of the peak may be the result of a mixture of complexes (possibly isomers) or chemial modification during elution from the column.

Table 3. Comparison of the Retention of BMS-181321 and ^{99m}Tc BAPN in Isolated Cardiac Myocytes under Anoxic and Normoxic Conditions

	retention (% of total) of ^{99m}Tc complexes in isolated cardiac myocytes ($n = 1$)		
	normoxia	anoxia	anoxic/oxic
^{99m}Tc BMS 181321	17.6	53.4	3.03
^{99m}Tc–BAPN	38.2	41.4	1.08

***In Vitro* Evaluation of Efficacy of the ^{99m}Tc Complex of BAPN.** While the exact mechanism by which nitroimidazoles are selectively trapped in hypoxic tissue is not known, one hypothesis is generally accepted.[32] This proposed mechanism involves an enzymatic one-electron reduction of the nitroimidazole to a radical anion in both normoxic and hypoxic cells. Within the normoxic cell, oxidation of this reduced compound (back to the nitroimidazole) should occur readily, whereas in the oxygen-deficient environment within hypoxic cells, re-oxidation is relatively slow. As a result, further reduction of the radical anion can take place in hypoxic cells, leading to the production of reactive species, which bind to cellular components. Although not well-documented in the literature, it was apparent to us that it is also necessary that the nitroimidazole can readily traverse the cell membrane so that it can participate in these processes within cells. We have previously described two *in vitro* assays of efficacy for putative hypoxia localization agents; these are an endothelial monolayer system for the assessment of membrane permeability[21] and an isolated cardiac myocyte system, which examines trapping of compounds under anoxic and normoxic conditions.[23] We have examined the ^{99m}Tc complex of the BAPN in both of these assays.

In the cultured bovine brain endothelial cell monolayer system,[21] the permeability index (P_i) of the ^{99m}Tc complex of BAPN was −0.25. The P_i for BMS-181321 is 63.2. The negative P_i value found for the ^{99m}Tc complex of BAPN indicates that it is much less permeable than the "nonpermeable" standard, sucrose. An appreciable amount of the ^{99m}Tc complex of BAPN was found to stick to the cell monolayers. These data indicate that the ^{99m}Tc complex of BAPN does not efficiently cross the intact cell membrane and therefore fails one of the criteria we believe to be essential for a successful hypoxia imaging agent.

The ^{99m}Tc complex of BAPN was compared to BMS-181321 in the assay based on isolated cardiac myocytes. The results, shown in Table 3, demonstrate that the ^{99m}Tc complex of BAPN was not selectively retained under anoxic conditions, whereas BMS-181321 had a 3:1 anoxic/normoxic ratio (results of BMS-181321 in this assay were reported previously[23]). These data provide further evidence that the ^{99m}Tc complex of BAPN is likely to be inferior to BMS-181321 as an imaging agent of hypoxic tissue.

Conclusions

We found that the published synthesis of BAPN is irreproducible. In the crucial step (reduction of a bis-amide to a bis-amine), the method given (and several variations on the conditions described) failed to yield the desired product. Instead, this reduction was carried out successfully by a modification which involved *in situ* protection of the product.

BAPN reacted with ^{99m}Tc to give an apparent single complex. This complex was evaluated in two *in vitro* assays which we have developed to assess the efficacy of putative hypoxic cell imaging agents. Unlike BMS-181321, the ^{99m}Tc complex of BAPN did not cross cultured endothelial monolayers, nor did it display selective retention in anoxic isolated cardiac myocytes, indicating that BMS-181321 has greater potential than does the ^{99m}Tc complex of BAPN as a radiopharmaceutical for imaging of hypoxic tissue.

Experimental Procedures

Materials and Reagents. All reagents were purchased from Aldrich, and all solvents were obtained from J. T. Baker. ^{99m}Pertechnetate was obtained from a NEN/Dupont ^{99}Mo/^{99m}Tc generator. Stannous tartrate was obtained from Sigma, while a Techneplex (Squibb Diagnostics) lyophilized kit was used as the source of stannous DTPA, for the preparation of BMS-181321. Water was purified using a Millipore MilliQ system. All HPLC solvents were filtered and degassed prior to use. Anocell aluminum oxide anapore inserts were purchased from Whatman, Inc.

HPLC analysis of the nonradioactive compounds reported herein was performed using a two-pump Rainin HPX solvent delivery system controlled by Gilson 712 software. The detector was a Gilson Dynamax UV-D set at 230 nm. The Microsorb C18 column (3 μm, 100 × 0.46 mm) was eluted with acetonitrile/water containing 0.1% trifluoroacetic acid. Each analysis consisted of a linear gradient of 0–50% acetonitrile in water for 50 min. Proton NMR data (270 MHz) were obtained with a JEOL-GX-270 spectrometer. Fast atom bombardment (FAB) mass spectra were obtained on a VG-ZAB-2F spectrometer from a glycerol/thioglycerol matrix. Positive-ion FAB high-resolution mass spectra were obtained on a JEOL-SX spectrometer from a glycerol/thioglycerol matrix. Elemental analyses were performed in-house by the Bristol-Myers Squibb Microanalytical Department.

Synthesis of BAPN Shown in Schemes 2 and 3. Synthesis of Diethyl 2-(2,3-epoxypropyl)malonate (10). *m*-Chloroperoxybenzoic acid (13.0 g, ca. 80%, 59.93 mmol) was added to a solution of diethyl 2-allylmalonate (10.0 g, 49.94 mmol) in dry CH_2Cl_2 (50 mL), and the resultant solution was stirred at room temperature for 15 h under a nitrogen atmosphere. A white precipitate was formed, and this was removed by filtration. The epoxide **10** was obtained as a colorless oil on evaporation of the filtrate on a rotary evaporator. The yield of the epoxide was near quantitative (10.2 g) and purity, as determined by HPLC, was 95%: ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 6H, CH_2CH_3), 2.02 and 2.25 (2m, 2H, $CHCH_2CH$), 2.51, 2.78, 3.03, and 3.52 (4m, 4H, C(=O)C*H* and epoxide ring *H*), and 4.21 (m, 4H, CH_2CH_3).

Synthesis of Diethyl 2-(3-(2-Nitro-1*H*-imidazol-1-yl)-2-hydroxypropyl)malonate (11). A solution of the epoxide **10** (10.0 g, 46.24 mmol) in dry DMF (30 mL) was treated with 2-nitroimidazole (7.85 g, 69.47 mmol) in the presence of Proton Sponge (15.0 g, 70.09 mmol). The reaction mixture was heated at 80 °C under a nitrogen atmosphere for 15 h. After cooling to room temperature, the reaction mixture was poured into water and extracted with ethyl acetate (3 × 50 mL). The combined organic layer was then washed with water (2 × 50 mL), dried, and evaporated under vacuum to provide a thick paste which was treated with silica gel (5 g) and CH_2Cl_2 (5 mL). After evaporation of the solvent, the silica gel impregnated with the compound was loaded onto a silica gel column and eluted with CH_2Cl_2:CH_3OH (98:2). The fractions with a compound showing R_f 0.58 [silica gel, CH_2Cl_2:CH_3OH (95:5)] were collected and evaporated to afford the lactone **12** in 50% yield (6.5 g) as a colorless thick oil: ^{1}H NMR ($CDCl_3$) δ 1.32 (m, 3H, CH_2CH_3), 2.18, 2.45, and 2.82 (3m, 2H, $CHCH_2CH$), 3.72 (2m, 1H, NCH_2CHO), 4.21 (m, 2H, CH_2CH_3), 4.59 [m, 1H, C(=O)C*H*], and 7.14 and 7.28 (2s, 2H, nitroimi.-*H*); MS *m/e* 284 $(M + H)^+$. The fractions with compound showing R_f 0.50 [silica gel, CH_2Cl_2:CH_3OH (95:5)] were collected and evaporated to afford **11** as a cream-colored solid in 25% (3.81 g) yield: mp 75–77 °C; ^{1}H NMR (DMSO-d_6) δ 1.22 (m, 6H, CH_2CH_3), 1.78 and 1.99 (2m, 2H, $CHCH_2CH$), 3.74 [m, 1H, C(=O)C*H*], 4.18 (m, 4H, CH_2CH_3), 4.48 (m, 1H, C*H*OH), 5.20 (m, 2H, NCH_2), and 7.05 and 7.49 (2s, 2H, imidazole*H*); MS *m/e* 330 $(M + H)^+$.

Synthesis of *o*-Hydroxybenzylamine (14). A solution of salicylaldoxime (**13**, 13.7 g, 0.1 mol) in ethanol (40 mL) was treated with concentrated HCl (10 N, 30 mL, 0.3 mol) followed by Pd/C (10%, 1.5 g). The mixture was hydrogenated under 55 psi of hydrogen in a Parr hydrogenator until the absorption of hydrogen ceased (about 2 h). The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure to a volume of 20 mL. This solution was washed with dichloromethane, and the aqueous solution was diluted with water (20 mL) and cooled in an ice bath. Concentrated ammonium hydroxide solution was slowly added until the pH was raised to about 9.0. The precipitate which formed was collected and washed with ice-cold water, dried, and recrystallized from isopropyl ether to yield colorless crystals: yield 10.5 g (85%); mp 128–129 °C (lit.[33] mp 129.0–129.5 °C); ^{1}H NMR (DMSO-d_6) δ 4.3 (s, 2H, CH_2N), 7.1 and 7.6 (m, 4H, ArH); HPLC t_R 12.91 min.

Synthesis of 2-Allyl-*N,N'*-bis(2-hydroxybenzyl)malondiamide (15). A solution of **14** (9.84 g, 80 mmol) and **3** (8.0 g, 40 mmol) in dry methanol (40 mL) was refluxed for 48 h. The cooled reaction mixture was concentrated to a paste, which was chromatographed on a flash silica gel column. Elution with 98:2 dichloromethane–methanol furnished the diamide as a colorless solid. The product was recrystallized from isopropyl ether/methanol: yield 7.76 g (50%); mp 155–156 °C; ^{1}H NMR (DMSO-d_6) δ 2.8 (m, 2H, $CH_2CH=CH_2$), 3.8 (m, 1H, O=CC*H*C=O), 4.59 (d, 4H, $ArCH_2N$), 5.4 (m, 2H, $CH=CH_2$), 6.1 (m, 1H, $CH=CH_2$), 7.0–7.6 (m, 8H, ArH), 8.9 (m, t, 2H, O=CN*H*CH_2), and 10.04, (s, 2H, ArO*H*); HPLC t_R 43.53 min.

Synthesis of 2-Allyl-*N,N'*-bis(2-hydroxybenzyl)-1,3-propanediamine (16). A solution of **15** (7.12 g, 20 mmol) in dry THF (100 mL) was added dropwise to a suspension of $LiAlH_4$ (3.8 g, 100 mmol) in dry THF (100 mL). Following addition, the reaction mixture was stirred under reflux for 24 h. The solution was cooled in an ice–salt bath and was treated carefully with a saturated solution of potassium sodium tartrate until the precipitate which formed had begun to settle. The THF layer was separated and filtered, and the residue was washed thoroughly with hot THF (5 × 100 mL). The combined organic layer was washed with saturated sodium chloride solution and then dried with anhydrous sodium sulfate. Evaporation of the solvent yielded a brown oil (5.8g). The oil (5.0 g) was dissolved in THF (50 mL) and was treated with di-*tert*-butyl dicarbonate (22.0 g, 100 mmol) followed by triethylamine (7.2 g, 100 mmol). The mixture was refluxed for 20 h and then concentrated to a paste. The brown paste was chromatographed on a flash silica gel column. Elution with 95:5 hexanes/ethyl acetate yielded a colorless gum which was homogeneous on TLC (9:1 hexane/EtOAc; R_f 0.72): yield 4.0 g (28%); MS $(M + H)^+$ 727 (tetra-*t*-Boc derivative). Deprotection by standard methods yielded the diamine: ^{1}H NMR (D_2O as HCl salt) δ 2.1 (m, 2H, $CH_2CH=CH_2$), 2.3 (m, 1H, NCH_2CHCH_2N), 3.0 (d, 4H, NCH_2), 4.2 (s, 4H, $ArCH_2$), 5.1 (m, 2H, $CH=CH_2$), 5.5 (m, 1H, $CH=CH_2$), 6.9 and 7.2 (m, 8H, ArH); ^{1}H NMR (free amine, $CDCl_3$) δ 1.8 (m, 1H, NCH_2CHCH_2N), 2.1 (m, 2H, $CH_2CH=CH_2$), 4.0 (s, 4H, $ArCH_2$), 5.1 (m, 2H, $CH=CH_2$), 5.7 (bs merging with a multiplet, 5H, N*H*, O*H* and $CH=CH_2$), and 6.8–7.2 (m, 8H, ArH); HPLC t_R 27.32 min.

Synthesis of 2-(2,3-Epoxypropyl)-*N,N'*-bis(2-hydroxybenzyl)-1,3-propanediamine (17). A solution of **16** (4.0 g, 5.5 mmol) in dichloromethane (20 mL) was treated with a solution of $NaHCO_3$ (1.7 g, 20 mmol) in water (10 mL). *m*-Chloroperoxybenzoic acid (1.9 g, 11 mmol) was added to this stirred mixture, and stirring was continued at room temperature until the starting material disappeared on TLC analysis (6 h). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (5 × 10 mL). The combined organic layer was washed with saturated sodium bicarbonate (2 × 20 mL) and then dried (sodium sulfate). Evaporation of solvent yielded an oil which was purified on a

flash silica gel column to yield an oily product which was homogeneous on TLC (90:10 hexane/EtOAc): yield 2.17g (54%); MS $(M + H)^+$ 743.

Synthesis of *N,N'*-Bis(2-hydroxybenzyl)-2-(2-hydroxy-3-(2-nitro-1*H*-imidazol-1-yl)propyl)-1,3-propanediamine (BAPN, 2). A solution of **17** (2.17 g, 2.9 mmol) in dry DMF (10 mL) was treated with solid sodium bicarbonate (0.84 g, 10 mmol) and 2-nitroimidazole (0.68 g, 6 mmol). The reaction mixture was heated with stirring under nitrogen for 2 h at 100 °C by which time all the epoxide starting material was consumed, as demonstrated by TLC. DMF was removed under reduced pressure, and the residue was poured into ice-cold water and extracted with dichloromethane (5 × 20 mL). The combined organic extracts was washed with water and dried (anhydrous sodium sulfate). Evaporation of the solvent yielded a brownish gum which was treated with saturated methanolic HCl (10 mL). The solution was concentrated and coevaporated with chloroform (5 × 10 mL). The residue was dissolved in water (10 mL) and then extracted with dichloromethane (5 × 20 mL). The combined organic extracts were dried and chromatographed on a flash silica gel column. Elution with 9:1 dichloromethane/methanol yielded the product as a yellow solid: yield 0.14 g (10%); mp >70 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.45 (m, 2H, CH_2CHOH), 2.0 (m, 1H, NCH_2CHCH_2N), 2.7 (m, 4H, NCH_2), 3.8 (s and m, 5H, ArCH_2 and CHOH), 4.1 (m, 1H, imid-CH_a), 4.4 (m, 1H, imid-CH_b), 5.0 (bs, 4H, OH and NH), and 6.8–7.2 (m, 10H, Ar-H and imid-H); ^{1}H NMR (DMSO-d_6) δ 1.4 (m, 2H), 2.0 (m, 1H), 2.7 (m, 4H), 3.9 (s merging with a m, 5H), 4.2 (m, 1H), 4.4 (m, 1H), 6.7 (m, 4H), 7.1 (m, 5H), and 7.7 (s, 1H); MS $(M + NH_4 - H_2O)^+$ 455; HPLC t_R 26.61 min.

Synthesis of BAPN Shown in Scheme 4. Synthesis of 2-Allylmalondiamide (4). Diethyl 2-allylmalonate (100 g, 650 mmol) was dissolved in methanol (500 mL), and the solution was cooled to 0 °C in an ice bath. Ammonia was bubbled into the ice-cold solution. The reaction mixture was sealed and stirred at room temperature for 20 h. Solvent and ammonia were removed by rotary evaporation, and the residue was washed with ether to give **4** as a white solid. This was used for the next stage without further purification: yield 76.5 g (92%); mp 168–169 °C (lit.[25] mp 168.5–169 °C); ^{1}H NMR (DMSO) δ 2.4 (m, 2H, $CH_2CH{=}CH_2$), 3.0 (t, 1H, COCHCO), 5.0 (m, 2H, $CH_2CH{=}CH_2$), 5.7 (m, 1H, $CH_2CH{=}CH_2$), 7.0 (bs, 2H, $CONH_2$), 7.3 (bs, 2H, $CONH_2$).

Synthesis of 2-Allyl-*N,N'*-bis(*tert*-butyloxycarbonyl)-1,3-propanediamine (18). A warm solution of **4** (14.2 g, 100 mmol) in dimethoxyethane (800 mL) was added over a period of 1 h to a slurry of $LiAlH_4$ (11.8 g, 300 mmol) in dimethoxyethane (300 mL). The reaction mixture was stirred at 50 °C for 48 h. Excess $LiAlH_4$ was destroyed by the addition of 10% NaOH and water, and the mixture was allowed to stir for 2 h. Di-*tert*-butyl dicarbonate (50.0 g, 230 mmol) was added, and the reaction mixture was stirred for 24 h. The reaction mixture was filtered, and the filtercake was washed with CH_2Cl_2 (250 mL). The filtrate and the washings were collected and evaporated on a rotary evaporator to afford a thick viscous oil, which was purified by column chromatography (silica gel, hexane:ethyl acetate, 9:1). Fractions containing the product were collected and evaporated to give a thick oil which solified on standing. Trituration with pentane gave **18** as a white solid, which was crystallized from hexane: yield 11.9 g (38%); mp 84–87.5 °C; MS $(M + H)^+$ 315; ^{1}H NMR ($CDCl_3$) δ 1.4 (s, 18H, *t*-Boc), 1.7 (m, 1H, CH), 2.85 and 3.22 (m, 4H, $CH(CH_2NH)_2$), 5.0 (m, 2H, $CH_2{=}C$), 5.75 (m, 1H, $CH{=}CH_2$). Anal. Calcd for $C_{16}H_{30}N_2O_2$: C, 61.12; H, 9.62; N; 8.91. Found: C, 60.95; H, 9.17; N, 8.90.

Synthesis of 2-(2,3-Epoxypropyl)-*N,N'*-bis(*tert*-butyloxycarbonyl)-1,3-propanediamine (19). *m*-Chloroperoxybenzoic acid (5.0 g, 22 mmol) was added in portions to a cooled (0 °C) solution of **18** (5.0 g, 16 mmol) in CH_2Cl_2 (30 mL), and the reaction mixture was stirred for 24 h. The precipitated *m*-chlorobenzoic acid was removed by filtration, and the filtrate was diluted with diethyl ether (200 mL). Excess *m*-chloroperoxybenzoic acid was decomposed by the addition of sodium sulfite solution (20%, 10 mL), and the organic layer was washed with a saturated solution of sodium bicarbonate and water and dried (Na_2SO_4). Evaporation of ether gave **19** as a viscous oil which was used in the next step without further purification: yield 5.2 g (98%); MS $(M + H)^+$ 330; ^{1}H NMR ($CDCl_3$) δ 1.5 (m, 3H, CH and CH_2CH), 2.45 and 2.80 (m, 2H, epoxide), 3.0–3.4 (m, 5H, 1H epoxide and $(CH_2NH$-*t*-Boc$)_2$), 5.0 and 5.5 (m, 2H, NH-*t*-Boc).

Synthesis of 2-(2-Hydroxy-3-(2-nitro-1*H*-imidazol-1-yl)propyl)-*N,N'*-bis(*tert*-butyloxycarbonyl)-1,3-propanediamine (20). **19** (1 g, 3 mmol) was added to a mixture of 2-nitroimidazole (500 mg, 4.5 mmol) and potassium carbonate (70 mg, 0.5 mmol) in ethanol (50 mL), and the mixture was refluxed under nitrogen for 12 h. The reaction mixture was cooled and filtered. The precipitate was washed with water, and the resultant off-white solid was air-dried. The crude product was chromatographed over silica gel (CH_2Cl_2:CH_3OH, 95:5). The UV–visible fractions were collected and evaporated to give **20** as a white solid: yield 0.42 g (28%); mp 185–186 °C; MS $(M + H)^+$ 443; ^{1}H NMR (DMSO) δ 1.2 (m, 2H, $CHCH_2$-CHOH), 1.75 (m, 1H, $CHCH_2CHOH$), 2.9 (m, 4H, $CH(CH_2$-$NHtBoc)_2$), 3.9 (m, 1H, CHOH), 4.1–4.4 (m, 2H, $CHOHCH_2N$), 5.0 (m, 1H, OH), 6.65 (m, 2H, NH), 7.1 and 7.52 (s, 2H, ArH).

Synthesis of 2-(2-Hydroxy-3-(2-nitro-1*H*-imidazol-1-yl)propyl)-*N,N'*-propylenebis(salicylidinimine) (9). To generate the free diamine, the bis-*t*-Boc derivative **20** (0.9 g, 2 mmol) was treated with methanolic HCl (5 mL), and the mixture was stirred for 30 min. Methanol was removed on a rotary evaporator, and the residue was neutralized with methanolic ammonia. The methanolic solution was concentrated on a rotary evaporator, and the residue was dried under vacuum to yield a light yellow solid. This solid was used in the next step without further purification: yield 0.38 g (80.0%). Salicylaldehyde (0.5 g, 4 mmol) was added to a solution of the diamine (0.38 g, 1.6 mmol) in absolute ethanol (5 mL), and the reaction mixture was maintained at 45 °C for 3 h. Ethanol was removed on a rotary evaporator, and the residue was purified by flash silica gel chromatography (hexane–ethyl acetate, 8:2). UV–visible fractions were collected, and the bisimine (**9**) thus obtained was used in the next step without further purification: yield 0.38 g (53%); MS $(M + H)^+$ 452; ^{1}H NMR ($CDCl_3$) δ 1.7 (m, 2H, $CHCH_2CHOH$), 2.5 (m, 1H, $CHCH_2CHOH$), 3.7 (m, 4H, $(CH(CH_2)_2N{=}CH$), 4.1 (m, 2H, $CHOHCH_2N$), 6.8–7.4 (m, 8H, ArH), 8.4 (s, 2H, N=CH).

Synthesis of *N,N'*-Bis(2-hydroxybenzyl)-2-(2-hydroxy-3-(2-nitro-1*H*-imidazol-1-yl)propyl)-1,3-propanediamine (BAPN, 2). The bisimine **9** (0.38 g, 0.8 mmol) was dissolved in methanol (10 mL), and the solution was cooled to 0 °C. Sodium borohydride (100 mg, 3 mmol) was added in portions to the stirred solution. The reaction mixture was stirred for 2 h, and then methanol was removed on a rotary evaporator. The residue was purified by column chromatography (silica gel, eluted with 200 mL of CH_2Cl_2/ethyl acetate, 9:1 and then 8:2). The compound was further purified by preparative HPLC using acetonitrile and water with 0.1% TFA: yield 0.2 g (87%); HRMS $(M + H)^+$ found 456.2238, calcd 456.4700.

TFA salt: ^{1}H NMR (D_2O) δ 1.7 (m, 2H, $CHCH_2CHOH$), 2.42 (m, 1H, $CHCH_2CHOH$), 3.1 (m, 4H, $CH(CH_2)_2NH$), 4.2–4.4 (m, 2H, $CHOHCH_2N$), 4.72 (s, $ArCH_2$), 6.8–7.4 (m, 10H, ArH and imidazoleH); ^{13}C NMR (D_2O) δ 31.26 ($CH(CH_2NH)_2$), 33.69 (CH_2CHOH), 47.67 and 47.78 (CH_2Ph), 48.35 and 48.72 ($CH(CH_2NH)_2$), 55.16 ($CHOHCH_2$), 67.58 (CHOH), 115.73, 116.93, 117.01, 120.78, 131.87, and 132.03 (ArC), 127.96 and 128.92 (imidazole C=C), 144.50 (imidazole NO_2C).

Free base: ^{1}H NMR ($CDCl_3$) δ 1.52 (m, 2H, $CHCH_2CHOH$), 2.65 (m, 1H, $CHCH_2CHOH$), 2.62 (m, 4H, $CH(CH_2)_2NH$), 3.9 (s overlapped with m, 5H, CH_2Ar and CHOH), 4.10 and 4.55 (m, 2H, $CHOHCH_2N$), 6.7–7.18 (m, 10H, ArH and imidazoleH).

Preparation of the ^{99m}Tc Complex of BAPN. BAPN (bis-CF_3COOH salt) (2 mg) was dissolved in saline (1 mL), and 0.1 M $NaHCO_3$ (0.5 mL) and ^{99}Mo/^{99m}Tc generator eluant (0.5 mL, 17–30 mCi) were added. The pH of the resultant solution was 9.0. A 50 μL aliquot of a saturated solution of stannous tartrate in saline was added. Two minutes after the addition of the stannous salt, the reduction of pertechnetate was essentially complete, as determined by HPLC analysis. The

product eluted as a broad tailing band from a PRP-1 reversed-phase column (80/20 MeOH/0.1 M NH_4OAc pH 4.6, 2 mL/min, void volume = 1.06 min) at a t_R of 2.5 min (BMS-181321 has an t_R of 7.5 min on same system). The complex was purified on PRP-1 resin[34] and eluted with the free ligand in 95% EtOH. A small aliquot of the ethanol fraction was blown to dryness with a nitrogen stream and redissolved in 100 μL of DMF, followed by 900 μL of normal saline. After dilution with normal saline, the radiochemical purity was approximately 90% (by HPLC analysis).

Preparation of the ^{99m}Tc BMS-181321. This complex was prepared by the method described previously.[22] In summary, eluate from a ^{99}Mo/^{99m}Tc generator is reduced at pH 8.2 with a suitable stannous salt (for example, tartrate or DTPA) in the presence of 2 mg of the ligand 4,8-diaza-1-(2-nitro-1*H*-imidazol-1-yl)undecane-2,10-dione dioxime.[35] The radiochemical purity (RCP), as determined by HPLC analysis,[22] was routinely >90%.

Permeability to Endothelial Monolayers. Details of this procedure are described elsewhere.[21] In summary, the measurement of bovine brain microvessel endothelial permeability *in vitro* was adapted from models by Audus and Borchardt[36,37] and Pardridge et al.[38] except that anocell inserts were used in place of transwells containing polycarbonate filters. A study of the permeability of a single test compound utilized 12 anocell inserts; four wells containing monolayers, 0.4 mL of media with 10% plasma-derived horse serum, 5 μCi of [^{3}H]water, 2 μCi of [^{14}C]sucrose, and 20 μCi of the ^{99m}Tc complex; four wells containing the same as above, but without the monolayers; and four wells containing the same but without monolayers or the 10% plasma-derived horse serum. The fraction of radioactivity transported from the donor to the acceptor well at each time point over the first 10 min of the study was calculated. This procedure[21] provides a permeability index (P_i) for test substrates, which ranks the permeability of a compound based on a scale in which water (a highly permeable compound) = 100 and sucrose (a nonpermeable compound) = 0.

Studies with Isolated Cardiac Myocyte. Calcium-tolerant ventricular myocytes were isolated from hearts of male Sprague–Dawley rats (200 g) according to the procedure of Wittenberg and Robinson[39] with modifications described previously.[40] Cells were used immediately following morphological analysis (using a hemocytometer) of viability and were maintained at 37 °C during the experiments. The number of quiescent, rod-shaped cells ranged from 70 to 90% within a total population of (5–9) × 10^6 cells.

The isolated myocytes were maintained in either a normoxic or anoxic state. Glucose oxidase (Glu. Ox.) plus catalase (5/5 mg) was added to argon-treated cells to provide anoxia. Cells were suspended [(6.5–7.5) × 10^4 cells/mL] in isolation media and aliquots were added to incubation vials maintained at 37 °C.

After incubation with a test compound for 1 h, triplicate aliquots of the myocytes were obtained and separated from the suspending media by passing the cells through 99% dibutyl phthalate by centrifugation at 12 000 rpm for 30 s. The three phases were separated and assayed for radioactivity. The results are expressed as a percentage of the radioactivity retained in the sampled aliquot.

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