# **Bioreductive Markers for Hypoxic Cells: 2-Nitroimidazoles with Biotinylated 1-Substituents**

Richard J. Hodgkiss,<sup>\*,‡</sup> John Parrick,<sup>†</sup> Manuchehr Porssa,<sup>†</sup> and Michael R. L. Stratford<sup>‡</sup>

Gray Laboratory of the Cancer Research Campaign, P.O. Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, U.K., and Department of Chemistry, Brunel University, Uxbridge, Middlesex UB8 3PH, U.K.

Received September 6, 1994<sup>®</sup>

The interference by oxygen with the bioreductive metabolism and binding within cells of 2-nitroimidazoles has been used to identify hypoxic cells. Three novel compounds were synthesized with a 1-substituent containing a biotin moiety. Bound adducts of these compounds could be identified in hypoxic cells in vitro by the biotin binding proteins, avidin or streptavidin, labeled with fluorescein. The metabolism and discrimination of these compounds between welloxygenated and hypoxic cells was evaluated by flow cytometry. Ester or amide links between the 2-nitroimidazole and the biotin were degraded in the presence of mouse serum, but a compound with a C5 hydrocarbon link was stable, and this compound was suitable for evaluation in an *in vivo* tumor model.

It is thought that the presence of poorly oxygenated (and therefore radioresistant) cells in human tumours  $1^{-3}$ reduces the efficacy of radiotherapy and in some cases may contribute to failure to control the disease. A simple method of measuring tumor hypoxia could facilitate optimization of treatment schedules for individual patients, with the use of modalities designed to overcome or even take advantage of the presence of hypoxic cells in their tumors.

The interference by oxygen with the bioreductive metabolism of nitroaromatic compounds, such as nitroimidazoles, has been used to identify and label hypoxic cells. A number of isotopically-labeled 2-nitroimidazoles have been investigated as potential hypoxia markers<sup>4-8</sup> but require the whole-body administration of substantial amounts of radioactivity. Fluorinated 2-nitroimidazoles have also been proposed for use with nuclear magnetic resonance detection,<sup>9</sup> although the sensitivity of currently available equipment needs to be improved for routine clinical use to be practical. Recent work has focused on the use of 2-nitroimidazoles with immunogenic side chains,  $10^{-12}$  the bioreductivelybound adducts of which can be identified in tumors by immunohistochemical techniques.

The current series of 2-nitroimidazoles extends this principle by incorporating a biotin moiety into the 1-substituent. The two biotin binding proteins, avidin and streptavidin, have a very high affinity and specificity for biotin and are widely used to locate biotin-labeled molecules in biological systems. As with other bioreductive hypoxia markers, the selective binding of these probes in hypoxic cells was expected to be conferred by the oxygen-sensitive reductive metabolism of the 2-nitroimidazole.

# Chemistry

The ester 2 was prepared by heating biotin 1 with 2-(2-nitroimidazol-1-yl)ethanol<sup>13</sup> in the presence of N,N'carbonyldiimidazole. The biotin diamide 5 was synthesized by reaction between the acid  $4^{14}$  and amine  $3^{15}$ using N.N'-carbonyldiimidazole as coupling agent (Scheme 1).

In order to prepare the required biotin-imidazole 9, monoprotected N-tritylbiotinol  $6^{16}$  was reacted with tosyl chloride to give the tosylate 7, and this was used to alkylate the anion of 2-nitroimidazole, in the presence of a crown ether,  $^{17}$  to give **8** (Scheme 2). The trityl group was then removed from 8 by treatment with trifluoroacetic acid to give the required compound 9 in a good yield.

# **Biological Results and Discussion**

In Vitro Assays. Both 2 and 9 (0.1 mmol dm<sup>-3</sup>) became metabolically bound specifically to hypoxic mammalian cells in vitro, with low binding to welloxygenated cells. Bioreductively bound adducts of 2 and 9 could be readily detected in cells using an avidin-FITC conjugate (Figure 1), but only adducts of 9 could be detected with streptavidin-FITC (data not shown). Binding of 5 to cells was inefficient with little oxic: hypoxic differential (Figure 1). The amount of fluorescent staining observed with the avidin-FITC staining reagent was concentration dependent (Figure 2a), with a maximum hypoxic:oxic ratio of nearly 10 at a reagent dilution of 1:1000 (Figure 2b). This dilution was used throughout this study.

HPLC measurements showed 2 (180  $\mu$ mol dm<sup>-3</sup>) to be rapidly destroyed by mouse plasma in vitro with a half-life of ca. 0.5 min at 20 °C. The diamide 5 (50  $\mu$ mol dm<sup>-3</sup>) was also unstable in mouse plasma with a halflife in vitro of 2 h at 20 °C. Significant amounts of 9 could be detected in mouse plasma, with at least two metabolites, 2 h after administration of 0.45  $\mu$ mol g<sup>-1</sup> in vivo.

The high hypoxic:oxic differential binding of both 2 and **9** in vitro initially suggested that both compounds had potential as hypoxia markers, but 2 was unsuitable for use in vivo because of the susceptibility of the ester linkage to degradation by esterases in serum. Compound 9 was therefore identified as the best compound for further studies in vivo. In view of the structural similarities of these compounds, the poor performance of 5 was unexpected. The identical 2-nitroimidazole

<sup>\*</sup> To whom correspondence should be addressed. Telephone: 0923-828611. Fax: 0923-835210. <sup>†</sup> Brunel University. <sup>‡</sup> Mount Vernon Hospital.

<sup>\*</sup> Abstract published in Advance ACS Abstracts, October 15, 1994.





<sup>a</sup> Reagents: (i) N,N'-carbonyldiimidazole/DMF; (ii) 2-(2-nitroimidazol-1-yl)ethanol/DMF; (iii) H<sub>2</sub>NCH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>/DMF.





<sup>a</sup> Reagents: (i) tosyl chloride/pyridine; (ii) Na salt of 2-nitroimidazole/15-crown-5/CH<sub>3</sub>CN; (iii) TFA.



**Figure 1.** The time course of binding of compounds 2, 5, and 9 to  $(\bullet)$  hypoxic and  $(\bigcirc)$  well-oxygenated cells, detected with an avidin-FITC conjugate, normalized to the same photodetector gain.

moiety was expected to be metabolized in a similar manner in all three compounds. The detection system was apparently effective, as above-background levels of bound metabolites of 5 were detected in both hypoxic and oxic cells.

In Vivo Assays. Bound metabolites of 9 could be readily detected in cell suspensions made from tumors



**Figure 2.** (a) The dependence of the fluorescent staining on the dilution of the stock avidin-FITC reagent (2 mg mL<sup>-1</sup>) in the assay: (•) hypoxic cells or (O) oxic cells exposed to 9 (100  $\mu$ mol dm<sup>-3</sup>) for 4 h; ( $\Delta$ ) control cells not treated with 9. (b) Ratios of hypoxic:oxic fluorescence for different dilutions of the avidin-FITC reagent.

exposed to the drug *in vivo* (Figure 3). A region has been set on each panel in Figure 3 so that only 1% of the background staining, determined from the aneuploid cells from tumors not exposed to **9**, would be within this



**Figure 3.** Typical flow cytometric distributions of single cells from CaNT tumors treated *in vivo* with **9**, with animals breathing air or carbogen, and of cells from tumors not treated with **9**, following staining with avidin-FITC. Mean proportions and standard errors of an euploid cells from three tumours, with fluorescent staining in the region indicated were no-drug control (background)  $1.2 \pm 0.6\%$ ; air-breathing 16.5  $\pm 4.0\%$ ; carbogen-breathing  $3.4 \pm 1.4\%$ .

region. Tumors in mice breathing air to which **9** had been administered (0.45  $\mu$ mol g<sup>-1</sup> ip) contain many hypoxic aneuploid cells with above-background fluorescent staining. The amount of fluorescent staining obtained is reduced to near-background levels if the mice breathe carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>), demonstrating increased oxygen availability to the previously hypoxic cells in the tumor. Either avidin-FITC or streptavidin-FITC could be used in the detection system.

Some background binding of the detection reagents to the normal stromal cell complement of the tumor may reflect high levels of biotin adsorbed onto the surface or in the cytoplasm of these cells. This artefact can be eliminated by treating the tumor material with pepsin  $(0.2 \text{ mg mL}^{-1})$  in 2 M HCl to remove the plasma membrane and cytoplasm. The resulting cell nuclei have much lower background binding of the reagents and can be used successfully in the staining procedure (Figure 4). Similar regions have been set on each panel in Figure 4 to those in Figure 3, except that both the diploid and aneuploid cells are included. Abovebackground levels of staining were observed for tumors treated with **9** in mice breathing air, and the levels of staining of the tumor cells were considerably reduced if the mice breathed carbogen (Figure 4).

#### Conclusions

These data illustate both the potential of biotinylated probes for hypoxic cells and some important features of these molecules that contribute to their activity as hypoxia markers. Bound adducts of 2 could be identified in mammalian cells with avidin-FITC, but not with streptavidin-FITC in this detection system. Mouse



**Figure 4.** Typical flow cytometric distributions of cell nuclei from CaNT tumors treated *in vivo* with **9**, with animals breathing air or carbogen, and of cell nuclei from tumors not treated with **9**, following staining with avidin-FITC. Mean proportions and standard errors of all cell nuclei from three tumours, with fluorescent staining in the region indicated, were no-drug control (background)  $0.85 \pm 0.45\%$ ; air-breathing  $11.7 \pm 7.5\%$ ; carbogen-breathing  $0.69 \pm 0.17\%$ .

serum contains high levels of esterases, and it is therefore not surprising that 2 was rapidly degraded by mouse serum. The amide linkage on 5 was also susceptible to degradation, presumably by proteases in serum. However, the C5 linkage between the 2-nitroimidazole and biotin moieties of 9 appeared to be relatively stable and was long enough for the biotin to bind to either avidin or streptavidin.

In preliminary experiments, 9 has shown considerable potential as a hypoxia marker *in vivo*. The reduction in the amount of bound probe in tumor cells which was observed when the animals breathed carbogen instead of air demonstrates that binding of the probe *in vivo* can be modulated by oxygen and indicates the amount of hypoxia in the tumor. The effect of breathing carbogen on tumor hypoxia has also been demonstrated with our NITP probe for hypoxia<sup>11</sup> and by our measurements of the increased radiosensitivity of tumors in mice breathing carbogen compared with air.

### **Experimental Section**

**Preparative Chemistry.** Melting points were determined on an Electrothermal Digital apparatus and are uncorrected. IR spectra were obtained as KBr disks on a Perkin-Elmer 1420 spectrophotometer. The <sup>1</sup>H NMR spectra were recorded on a Varian CFT-20 spectrometer. Low-resolution electron-impact mass spectra were obtained at 70 eV using a modified MS-902 spectrometer. Elemental analyses were carried out by MEDAC Ltd., Brunel University. Silica gel 60 (May and Baker,  $40-60 \,\mu$ m) was used for column chromatography while for TLC, silica gel 60A (Whatman, 250  $\mu$ m thick layer) was employed. All solvents were distilled prior to use. The identity of known compounds was established by comparison of their spectroscopic properties with those in the literature.

D-(+)-Biotin 2-(2-Nitroimidazol-1-yl)ethyl Ester (2). N,N'-Carbonyldiimidazole (1.13 g, 7 mmol) in DMF (5 mL) was

added to a stirred solution of biotin 1 (1.4 g, 5.73 mmol) in DMF (20 mL). After 1 h of stirring, 2-(2-nitroimidazol-1-yl)ethanol<sup>13</sup> (1.10 g, 7 mmol) in DMF (12 mL) was added and the reaction mixture stirred for a further  $2^{1/2}$  h. The resultant suspension was heated to ca. 90 °C, the mixture was stirred for a further 2 h and then evaporated under reduced pressure, and the residue was chromatographed on silica gel with a mixture of ethyl acetate-methanol (9:1). The resultant solid was crystallized from a mixture of chloroform and petroleum ether to give 2 as an off-white powder (1.1 g, 50%): mp 104-106 °C; IR  $v_{\text{max}}$  3280, 1750, 1710, 1550, and 1370 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.42–1.70 (6H, m, side chain-H), 2.31 (2H, m, CH<sub>2</sub>CO<sub>2</sub>), 2.89 (3H, m, 4- and 6-H), 4.51 (6H, m, 3a-H, 6a-H, and im- $CH_2CH_2$ ), 6.29 (1H, s, exchanged with  $D_2O$ , NH), 6.33 (1H, s, exchanged with  $D_2O$ , NH), 7.09 (1H, d, J = 1 Hz, imidazole-H), and 7.14 (1H, d, J = 1 Hz, imidazole-H); MS m/z 383 (M<sup>+</sup>, 20), 240 (10), 226 (60), and 143 (100). Anal. (C<sub>15</sub>H<sub>21</sub>N<sub>5</sub>O<sub>5</sub>S) C, H, N.

 $N^{1}$ -D-(+)-Biotinyl-N^2-[(2-nitroimidazol-1-yl)acetyl]-1,2ethanediamine (5). To a solution of 2-(2-nitroimidazol-1-yl)acetic acid  $4^{14}$  (0.30 g, 1.75 mmol) in DMF (2 mL) was added  $N_{*}N'$ -carbonyldiimidazole (0.35 g, 2.15 mmol) in DMF (2 mL) and the mixture stirred for 1 h. Then a solution of biotinylethylenediamine  $3^{15}$  (0.45 g, 1.6 mmol) in DMF (6 mL) was added and the mixture stirred for  $3^{1/2}$  h. The reaction mixture was evaporated under reduced pressure and the residue crystallized from propan-2-ol as a light yellow powder (0.32 g, 46%): mp 125–128 °C; IR  $\nu_{max}$  3300, 3110, 1700, 1650, 1580, 1540, and 1370 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 1.45 (6H, m, side chain-H), 2.07 (2H, m, CH<sub>2</sub>CONH), 3.01 (3H, m, 4- and 6-H), 3.11 (4H, m, HNCH<sub>2</sub>CH<sub>2</sub>NH), 4.26 (2H, m, 3a- and 6a-H), 5.03  $(2H, s, im-CH_2)$  6.24 (1H, s, exchanged with D<sub>2</sub>O, NH), 6.27 (1H, s, exchanged with  $D_2O$ , NH), 7.14 (1H, d, J = 0.8 Hz, imidazole-H), 7.54 (1H, d, J = 0.8 Hz, imidazole-H), 7.57 (1H, br s, exchanged with D<sub>2</sub>O, CONH), and 7.72 (1H, br s, exchanged with  $D_2O$ , CONH); MS m/z 439 (M<sup>+</sup>, 10), 368 (4), 313 (10), 226 (60), and 149 (100). Anal.  $(C_{17}H_{25}N_7O_5S.H_2O)$ C. H. N.

1-(4,4'-Dimethoxytrityl)-D-(+)-biotinol Tosylate (7). Tosyl chloride (8.8 g, 45 mmol) was added to a cooled solution of  $6^{16}$  (13.3 g, 25 mmol) in pyridine (20 mL) and the mixture kept at 0 °C for 24 h. Ice water was then added to the reaction mixture, and the resultant gummy solid was dissolved in chloroform and washed with Na<sub>2</sub>CO<sub>3</sub> solution and water. The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated and the residue chromatographed on silica gel, using 1% methanol in chloroform to give 7 as yellow needles (13.1 g, 76%): mp 67-69 °C; IR v<sub>max</sub> 3400, 3200, 1690, 1575, 1350, 1250, and 1170 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.31–1.42 (8H, m, side chain-H), 2.31 (2H, m, 6-H), 2.42 (3H, s, TsCH<sub>3</sub>), 3.09 (1H, m, 4-H), 3.76 (6H, s,  $2 \times OCH_3$ ), 3.94 (2H, t, J = 6.1 Hz,  $CH_2OTs$ ), 4.26 (2H, m, 3a- and 6a-H), 5.35 (1H, s, exchanged with D<sub>2</sub>O, NH), 6.75  $(4H, d, J = 9.1 Hz, 2 \times 3$ - and 5-H Ar), 7.16 (4H, d, J = 9.1Hz,  $2 \times 2$ - and 6-H Ar), 7.23 (5H, s, Ph), 7.49 (2H, J = 8.4 Hz, 3- and 5-H of Ts), and 7.77 (2H, d, J = 8.4 Hz, 2- and 6-H of Ts); MS m/z 686 (M<sup>+</sup>, 12), 667 (5), 511 (16), and 484 (100). Anal.  $(C_{38}H_{42}N_2O_6S_2.1/_2H_2O) C, H, N.$ 

1-[2,2a,4,5,5a,6-Hexahydro-5-(4,4'-dimethoxytrityl)-3H-4-oxothieno[3,4-d]imidazol-2-yl]-5-(2-nitroimidazol-1-yl)pentane (8). A mixture of 7 (11.9 g, 17.3 mmol), 15-crown-5 (4.63 g, 21 mmol), and the dry sodium salt of 2-nitroimidazole (2.83 g, 21 mmol) in acetonitrile (300 mL) was heated under reflux for 18 h. The mixture was evaporated under reduced pressure and the residue chromatographed on silica gel with a mixture of chloroform-methanol (9:1) giving a solid which crystallized from CHCl<sub>3</sub>/petroleum ether as light yellow prisms of 8 (8.2 g, 75%): mp 110–112 °C; IR  $\nu_{max}$  3420, 3100, 1695, 1520, and 1360 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO- $d_6$ )  $\delta$  1.38 (6H, m, side chain-H), 1.82 (2H, quintet, J = 7.1 Hz,  $CH_2$ ), 2.31 (1H, dd, J= 12.2 Hz and J = 5.4 Hz, 6-H), 2.48 (1H, d, J = 12.2 Hz, 6-H), 3.01 (1H, m, 4-H), 3.79 (6H, s,  $2 \times OCH_3$ ), 4.31 (2H, m, 3a- and 6a-H), 4.38 (2H, t, J = 7.1 Hz,  $CH_2$ -im), 5.1 (1H, s, exchanged with  $D_2O$ , NH), 6.81 (4H, d, J = 9.2 Hz,  $2 \times 3$ - and 5-H Ar), 7.08 (1H, s, imidazole-H), 7.18 (4H, d, J = 9.2 Hz, 2  $\times$  2- and 6-H Ar), 7.26 (1H, s, imidazole-H), and 7.30 (5H, s,

Ph); MS m/z 627 (M<sup>+</sup>, 80), 520 (15), and 303 (100). Anal. (C<sub>34</sub>H<sub>37</sub>N<sub>5</sub>O<sub>5</sub>S.H<sub>2</sub>O) C, H, N.

1-[2.2a,4,5.5a,6-Hexahydro-3H-4-oxothieno[3,4-d]imidazol-2-yl]-5-(2-nitroimidazol-1-yl)pentane (9). Compound 8 (5 g, 8 mmol) was stirred with TFA (20 mL) for 10 min and then treated with cold water. The mixture was evaporated under reduced pressure and the residual solid crystallized from MeOH/ether to give 9 as colorless plates (2.2 g, 85%): mp 181–183 °C; IR  $\nu_{max}$  3280, 3110, 1705, 1520, and 1350 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO- $d_6$ )  $\delta$  1.35 (6H, m, side chain-H), 1.78 (2H, quintet, J = 7.3 Hz, CH<sub>2</sub>), 2.59 (1H, d, J = 12.2 Hz, 6-H), 2.83 (1H, dd, J = 12.2 Hz and J = 5.4 Hz, 6-H), 3.11  $(1H,\,m,\,4\text{-}H),\,4.16\,(1H,\,m,\,3a\text{-}H),\,4.29\,(1H,\,m,\,6a\text{-}H),\,4.38\,(2H,\,M)$ t, J = 7.3 Hz,  $CH_2$ -im), 6.24 (1H, s, exchanged with  $D_2O$ , NH), 6.30 (1H, s, exchanged with  $D_2O$ , NH), 7.16 (1H, d, J = 1 Hz, imidazole-H), and 7.65 (1H, d, J = 1 Hz, imidazole-H); MS m/z 270 (M<sup>+</sup>, 15), 196 (65), 152 (31), and 97 (100). Anal. (C<sub>13</sub>H<sub>19</sub>N<sub>5</sub>O<sub>5</sub>S) C, H, N.

**Biology.** Dimethyl sulfoxide (DMSO) and other reagents were BDH AnalaR grade. Special gases (air + 5% CO<sub>2</sub>, nitrogen + 5% CO<sub>2</sub>) were obtained from British Oxygen Co. V79 379A Chinese hamster cells were maintained as exponentially-growing suspension cultures in Eagle's Minimal Essential Medium (MEM) modified for suspension cultures +7.5% foetal calf serum. For use in tissue culture experiments, **9** was dissolved in DMSO at 10 mmol dm<sup>-3</sup>, and appropriate quantities of this stock solution were added to the growth medium so that the DMSO was 1% or less of the final volume. At this concentration, DMSO is not toxic to mammalian cells over the incubation times used in these experiments.

In Vitro Assays. Cells were treated with drugs as stirred suspension cultures at 37  $^{\circ}C$ ,<sup>18</sup> following equilibration with either air +5% CO<sub>2</sub> or nitrogen +5% CO<sub>2</sub> by passing the gas over the cultures for 1 h, in the absence of drug. After various periods of incubation with drugs, cell samples were removed, centrifuged, and washed with PBS to remove residual drug, resuspended in a small volume of PBS, and fixed in 70% ethanol.

In Vivo Assays. The moderately well differentiated mammary adenocarcinoma CaNT of spontaneous origin<sup>19,20</sup> was maintained by serial passage in CBA mice, and for this work was implanted as rear dorsal subcutaneous tumours in adult male mice. The CaNT tumor has a volume doubling time of 2.8 days. Tumors were used at a mean diameter of 10 mm calculated from three orthogonal measurements.

Drugs were administered to mice at a dose of  $0.45 \ \mu mol g^{-1}$ as intraperitoneal injections in peanut oil + 10% DMSO. This method of administering drugs has given satisfactory results with a similar 2-nitroimidazole (NITP) probe for hypoxia.<sup>11</sup>

Two hours after administration of drugs, the animals were sacrificed by cervical dislocation and tumors excised, minced, and digested with 1 mg mL<sup>-1</sup> collagenase and 0.5 mg mL<sup>-1</sup> DNAase for 30 min at 37 °C. Any remaining fragments of tumor were then broken up by pipetting and the cell suspension filtered through 35  $\mu$ m cloth to remove large cell clumps. The cell suspension was then centrifuged, resuspended in a small volume of PBS, and fixed in 70% ethanol.

Staining and Flow Cytometry. For flow cytometry, alcohol-fixed cells were resuspended in PBS and incubated for 1 h with fluorescein avidin–DCS (avidin–FITC) or fluorescein streptavidin–DCS (streptavidin–FITC) (Vector). Cells were stained using a range of avidin–FITC dilutions, and it was found that at a 1:1000 dilution of the stock reagent (2 mg mL<sup>-1</sup>) into PBS the largest hypoxic:oxic ratio was obtained. The staining reagent was removed by centrifugation, and the cells were suspended in PBS with 0.5 mg mL<sup>-1</sup> RNAase and 10  $\mu$ g mL<sup>-1</sup> propidium iodide for quantitative DNA staining.

**Acknowledgment.** This work was supported by the Cancer Research Campaign.

#### References

 Bush, R. S.; Jenkin, R. D. T.; Allt, W. E. C.; Beale, F. A.; Bean, H.; Dembo, A. J.; Pringle, J. F. Definitive Evidence for Hypoxic Cells Influencing Cure in Cancer Therapy. *Br. J. Cancer* 1978, 37, Suppl. III, 302-306.

- Churchill-Davidson, I.; Foster, C. A.; Wiernik, G.; Collins, C. D.; Piezez, N. C. D.; Skeggs, D. B. L.; Purser, P. R. The Place of Oxygen in Radiotherapy. Br. J. Radiol. 1966, 39, 321-331.
  Gray, L. H.; Conger, A. D.; Ebert, M.; Hornsey, S.; Scott, O. C.
- (3) Gray, L. H.; Conger, A. D.; Ebert, M.; Hornsey, S.; Scott, O. C. A. The Concentration of Oxygen Dissolved in Tissues at the Time of Irradiation as a Factor in Radiotherapy. *Br. J. Radiol.* 1953, 26, 638-648.
- Raleigh, J. A.; Franko, A. J.; Koch, C. J.; Born, J. L. Binding of Misonidazole to Hypoxic Cells in Monolayers and Spheroid Culture: Evidence that a Side-Chain Label is Bound as Efficiently as a Ring Label. Br. J. Cancer 1985, 51, 229-235.
  Chapman, J. D.; Franko, A. J.; Sharplin, J. A. A Marker for
- (5) Chapman, J. D.; Franko, A. J.; Sharplin, J. A. A Marker for Hypoxic Cells in Tumours with Potential Clinical Applicability. Br. J. Cancer 1982, 45, 694-699.
- (6) Franko, A. J.; Chapman, J. D. Binding of <sup>14</sup>C-Misonidazole to Hypoxic Cells in V79 Spheroids. Br. J. Cancer 1982, 45, 694-699.
- (7) Garrecht, B. M.; Chapman, J. D. The Labelling of EMT-6 Tumours in BALB/C Mice with <sup>14</sup>C misonidazole. Br. J. Radiol. 1993, 56, 745-753.
- (8) Rasey, J. S.; Krohn, K. A.; Grunbaum, Z.; Conroy, P. J.; Bauer, K.; Sutherland, R. M. Further Characterisation of 4-Bromomisonidazole as a Potential Detector of Hypoxic Cells. *Radiat. Res.* 1985, 102, 76-85.
- (9) Raleigh, J. A.; Franko, A. J.; Treiber, E. O.; Lunt, J. A.; Allen, P. S. Covalent Binding of a Fluorinated 2-Nitroimidazole to EMT-6 Tumours in BALB/C Mice. Detection by F-19 Nuclear Magnetic Resonance at 2.35 T. Int. J. Radiat. Oncol. Biol. Phys. 1986, 12, 1243-1245.
- (10) Raleigh, J. A.; Miller, G. G.; Franko, A. J.; Koch, C. J.; Fuciarelli, A. F.; Kelley, D. A. Fluorescence Immunohistochemical Detection of Hypoxic Cells in Spheroids and Tumours. Br. J. Cancer 1987, 56, 395-400.
- (11) Hodgkiss, R. J.; Jones, G.; Long, A.; Parrick, J.; Smith, K. A.; Stratford, M. R. L. Flow Cytometric Evaluation of Hypoxic Cells

- (12) Lord, E. M.; Harwell, L.; Koch, C. J. Detection of Hypoxic Cells by Monoclonal Antibody Recognizing 2-Nitroimidazole Adducts. *Cancer Res.* 1993, 53, 5721-5726.
- (13) Threadgill, M. D.; Webb, P. Selective reductions of 1-(carbonyl) substituted 2-nitroimidazoles with alkali metal borohydrides and with borane-tetrahydrofuran. Synth. Commun. 1990, 20, 2319-2325.
- (14) Webb, P.; Threadgill, M. D. Labelled compounds of interest as antitumour agents. Part iii. Synthesis of deuterium and tritium isotopomers of etanidazole (SR 2508). J. Labelled Compd. Radiopharm. 1990, 28, 265-271.
- (15) Garlick, R. K.; Giese, R. W. Avidine binding of radiolabelled biotin derivatives. J. Biol. Chem. 1988, 263, 210-215.
- (16) Alves, A. M.; Holland, D.; Edge, M. D. A chemical method of labelling oligodeoxyribonucleotides with biotin: A single step procedure using a solid methodology. *Tetrahedron Lett.* 1989, 30, 3089-3092.
- (17) Long, A.; Parrick, J.; Hodgkiss, R. J. An Efficient Procedure for the 1-Alkylation of 2-Nitroimidazoles and the Synthesis of a Probe for Hypoxia in Solid Tumours. Synthesis 1991, 709-716.
- (18) Begg, A. C.; Hodgkiss, R. J.; McNally, N. J.; Middleton, R. W.; Stratford, M. R. L.; Terry, N. H. A. Fluorescent Markers for Hypoxic Cells: a Comparison of Two Compounds on Three Cell Lines. *Br. J. Radiol.* **1985**, *58*, 645-654.
- (19) Hewitt, H. B.; Blake, E.; Porter, E. H. The Effect of Lethally Irradiated Cells on the Transplantability of Murine Tumours. Br. J. Cancer 1973, 28, 123-135.
- (20) Denekamp, J.; Harris, S. R. The Response of a Transplantable Tumour to Fractionated Irradiation 1. X-rays and the Hypoxic Cell Radiosensitizer Ro-07-0582. *Radiat. Res.* 1976, 66, 66– 75.