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On the isolation and evaluation of a novel unsubstituted 5-nitroimidazole derivative as an agent to target tumor hypoxia

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ABSTRACT

The presence and extent of hypoxic regions in cancerous tissue bears a negative influence on the effectiveness of radiation therapy and chemotherapy of the cancer hence estimation of hypoxia is an important problem. Several ^{99m}Tc-labeled nitroimidazole-based non-invasive agents have been tried for this purpose but none had optimal characteristics and the search continues. Herein we report, for the first time to the best of our knowledge, the isolation, $^{99m}Tc(CO)_3$ labeling and evaluation of an unsubstituted 5-nitroimidazole derivative obtained as a side product during the synthesis of 4-nitroimidazole derivative. The $^{99m}Tc(CO)_3$-labeled complex of 5-nitroimidazole derivative could be prepared in excellent yield under mild conditions. Its evaluation in fibrosarcoma tumor bearing Swiss mice showed uptake and slow clearance of injected activity from tumor. The tumor-to-muscle ratio was found to be very high but tumor-to-blood ratio greater than 1 could not be obtained throughout the limited time point study. The study revealed that complex under investigation has features similar to other 2-nitroimidazole complexes so far as the retention of injected activity in tumor is concerned.

Tumour hypoxia plays a major role in reducing the efficacy of therapeutic modalities like chemotherapy and radiation therapy in combating cancer.[1,2] Therefore, both quantitative and qualitative estimation of hypoxia becomes an important parameter in planning the therapeutic strategy for a better clinical outcome. Nitroimidazole-based non-invasive imaging agents are for long being used for this purpose. The positron emission tomography (PET) tracer [^{18}F]FMISO was the first nitroimidazole and till date the gold-standard agent for imaging hypoxia.[3] Wiebe et al. investigated a series of ^{123}I-labeled 2-nitroimidazole derivatives for imaging with the more widely available SPECT technique, culminating with ^{123}I-iodoazomycin arabinoside (IAZA) currently undergoing clinical trials.[4] However, due to the short half life, high cost and limited availability of cyclotron produced isotopes such as ^{18}F and ^{123}I, the development of ^{99m}Tc-based agents for this purpose has gained importance. ^{99m}Tc with its optimal nuclear characteristics, easy availability and versatile chemistry is an ideal diagnostic radionuclide. While in ^{99m}Tc-BATO-nitroimidazole (BATO = Boronic acid adduct of technetium dioxime), one of the earlier studied agents, the nitroimidazole moiety was found to be enzymatically reduced in absence of oxygen, it was observed that the rate of reduction was much slower than with the corresponding unchelated boronic acid nitroimidazole molecule. The cause of this slower reduction rate was attributed to the steric hindrance offered by the bulky technetium complex to the nitroimidazole moiety.[5] This study revealed the necessity of a spacer arm between the nitroimidazole and metal centre. Several other ^{99m}Tc-labeled nitroimidazole-based agents were explored, which have thus far not achieved the optimal characteristics to target hypoxia, and hence the search continues.[6–15]

Most of the agents studied to target hypoxia are based on derivatives of 2-nitroimidazole (nitro group substituted at the second position in the imidazole ring). However, no reports are available till date on unsubstituted 5-nitroimidazole derivatives, a possible reason being the non-availability of 5-nitroimidazole. The inherent instability of the 5-nitroimidazole is due to the tautomeric equilibrium with 4-nitroimidazole wherein the latter is favored due to higher acidity of the –NH– proton of the 5-nitro isomer. Such a tautomerism in the case of metronidazole, a 5-nitroimidazole with a methyl substitution at C-2, is also possible albeit to a limited extent. The presence of the methyl substituent at C-2 possibly reduces the acidity of the –NH– protons induced by the nitro group at the C-5 position. Therefore metronidazole is readily available and studies using metronidazole have been reported.[12,15] A careful survey of single electron reduction potential

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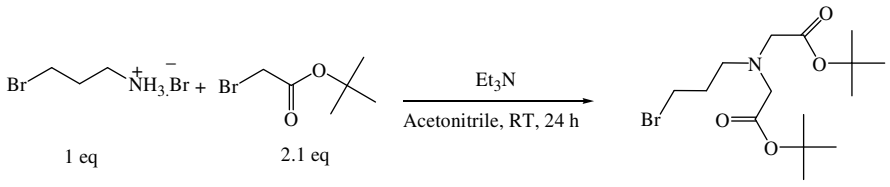
data reported by Wardman reveal that the substituent on the nitroimidazole ring is one factor which determines the value of the one electron reduction potentials of different nitroimidazoles.[16] An electron donating substituent like a methyl group on the nitroimidazole ring (as with metronidazole) was found to shift the reduction potential to more negative values compared to an analogous nitroimidazole with no substitution. In connection with our research efforts to prepare and study differently substituted nitroimdazoles for targeting tumor hypoxia, it was therefore felt pertinent to investigate the potential of unsubstituted 5-nitroimidazole derivative as a hypoxia marker. It is but logical to assume that reduction potential of unsubstituted 5-nitroimidazole will be less negative in comparison with its methyl substituted counterpart, ie 2-methyl-5-nitroimidazole. Hence, hypothetically, unsubstituted 5-nitroimidazole could be a better choice to target hypoxic cells than 2-methyl-5-nitroimidazole reported earlier. The present study could also reveal whether the effect of the substitution in the nitroimidazole ring could cause a significant change in the in vivo hypoxia targeting potential of nitroimidazole-based molecules. During the course of a study, which was primarily aimed at preparing a 4-nitroimidazole iminodiacetic acid, with a three-carbon spacer arm, we observed that a 5-nitroimidazole derivative was formed, in significant yield, during the course of the reaction and was successfully isolated. This derivative, which has not been earlier explored, was subsequently radiolabeled with the $[^{99m}Tc(CO)_3]^+$ core. Preliminary bioevaluation of this complex was carried out in tumor bearing animal model.

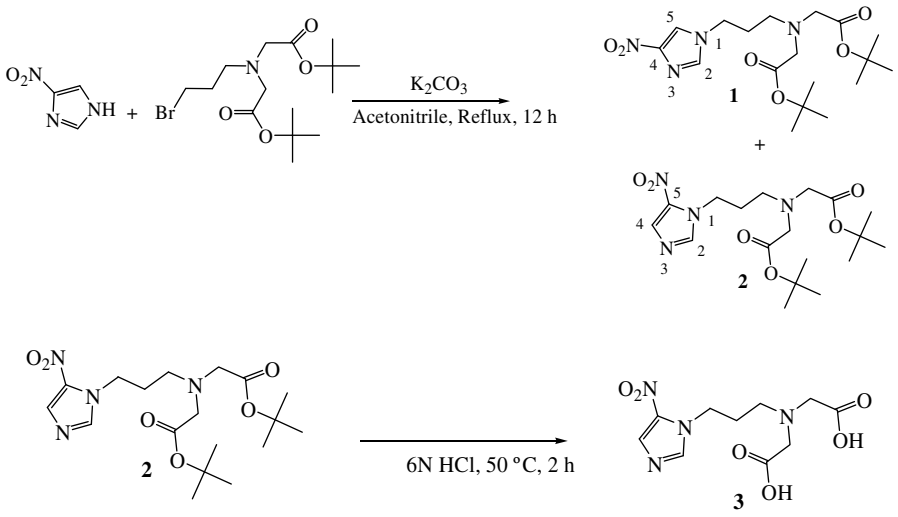
It has been documented that tautomeric inter-conversion of the 5-nitro and 4-nitro imidazoles takes place under either acidic or basic conditions. During the N-alkylations 4-nitroimidazole with alkyl halides, while acidic conditions favor the 5-nitro orientation, the 4-nitro orientation is favored under basic conditions.[17] However, reaction yields are generally poor under acidic conditions and they cannot be utilized when the reactants are acid sensitive. The 5-nitroimidazole derivative reported herein was obtained as a side product during the synthesis of 4-nitroimidazole iminodiacetic acid derivative under basic conditions. The synthesis was carried out in two steps. A bifunctional chelating agent (BFCA), *N*,*N*-bis[*tert*-butoxycarbonylmethyl]-3-bromopropyl amine was prepared in the first step, via reaction between 3-bromopropylamine hydrobromide and *tert*-butylbromoacetate in presence of triethyl amine as base (Scheme 1).[18] The BFCA was then coupled to the 4-nitroimidazole ring in the second step to obtain the *tert*-butylester derivative of 4-nitroimidazole (Scheme 2).[19] The progress of the reaction was followed by thin layer chromatography (TLC). Contrary to our expectation of a single product, TLC in diethyl ether showed two spots corresponding to two products. The two ester derivatives were separated by silica gel column chromatography, with ether where the minor product was obtained in 15% overall yield. ^{1}H NMR analysis (300 MHz Varian VXR 300S spectrophotometer, USA) revealed that one of the products was the expected 4-nitroimidazole ester derivative while the other was a 5-nitroimidazole ester.

The unambiguous structure assignments were made considering the chemical shift values of the nitroimidazole-*N*-(alkyl)–CH_2– proton as reported by Rao et al.[20] It is expected that this proton should be more downfield in 5-nitroimidazole derivative, due to the proximity of nitro group, than in the 4-nitroimidazole derivative. In the present case the ^{1}H NMR spectra of the two esters prepared, substantiates the findings of Rao et al., where this proton appears at 4.57 ppm and 4.28 ppm in the two NMR spectra. Based on this, while the compound which exhibited a 2 H-triplet at δ 4.57 ppm was confirmed to be the 5-nitroimidazole ester, the compound which showed a similar peak at δ 4.28 ppm was identified as the 4-nitroimidazole isomer. Additional evidence supporting the present structural assignment can be obtained from the chemical shift values of C4 and C5 protons. The C4 proton which is between the nitro group and a sp^2 nitrogen in 5-nitro isomer (7.99 ppm) is expected to be more downfield than the C5 proton (7.87 ppm) placed between the nitro group and a sp^3 nitrogen in the 4-nitro isomer. The target compound, iminodiacetic acid derivative of 5-nitroimidazole (**3**), was obtained by the hydrolysis of corresponding *tert*-butyl ester derivative with 6 N HCl as shown in Scheme 2. The ligand **3** was obtained in almost quantitative yield. All the compounds synthesized were characterized by ^{1}H NMR and IR (Jasco FT/IR-420, Japan).

The 5-nitroimidazole derivative was then labeled with ^{99m}Tc via the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core. The core was prepared following a reported procedure.[21] Under optimized conditions, the labeling was carried out by mixing 0.5 mL of phosphate buffer saline (pH 7.4) containing 0.5–1 mg of ligand [10^{-3} M] with 0.5 mL of freshly prepared $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core [3.7 MBq] in a 10 mL capacity vial. The mixture was then heated at 70 °C for 30 min, after which the vial was cooled in ice. Schibli et al. had earlier shown the tridentate coordination of iminodiacetic acid with $Re(CO)_3$ core, analogue of $^{99m}Tc(CO)_3$ core, resulting in a negatively charged complex.[22] Since the ligand presently being studied is also an iminodiacetic acid, it could be logical to assume that the resultant complex will be uninegative. The characterization of the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core and the complex was carried out by HPLC. HPLC analyses were carried out on a JASCO PU 2080 Plus dual pump HPLC system, Japan, with a JASCO 2075 Plus tunable absorption detector and a radiometric detector system, using a C18 reversed phase HiQ Sil (5 μm, 4 × 250 mm) column. The solvents used for HPLC were filtered through Millipore filter paper and contained 0.1% trifluoroacetic acid. The $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core appeared as a broad peak at 17.42 ± 0.2 min and that of the complex appeared as a sharp peak at 15.67 ± 0.1 min (Fig. 1a and b). Both the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core and the complex could be prepared in more than 95% yield, as determined by HPLC. The octanol–water partition coefficient ($Log P_{o/w}$) of the complex was determined, applying multiple back extraction method described by Troutner et al.,[23] and found to be 0.39. In vitro serum stability studies of the labeled complex were performed using a method adapted from a protocol reported earlier.[24] About 50 μL of the labeled compound was added to 0.5 mL of human serum and this mixture was incubated at 37 °C for 3 h. Aliquots were withdrawn at intervals of 1, 2 and 3 h, ethanol was added to precipitate the serum protein, centrifuged and the supernatant was analyzed by HPLC to assess stability of the complex. No significant re-oxidation of the complex to $^{99m}TcO_4^-$ was observed upon incubation in human serum at 37 °C.



Scheme 1.



Scheme 2.

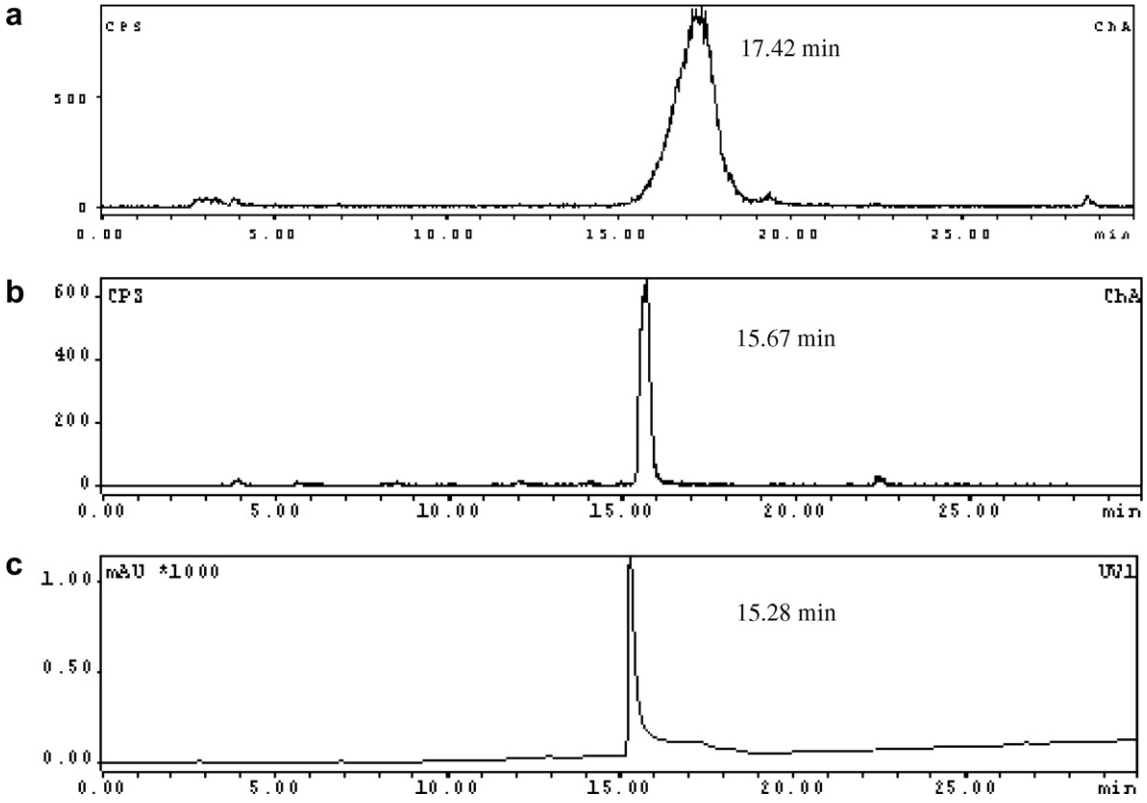


Figure 1. HPLC profile of (a) $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core (b) $^{99m}Tc(CO)_3$-5-nitroimidazole complex and (c) $^{185}Re(CO)_3$-5-nitroimidazole complex.

For the purpose of characterization by comparison, the corresponding rhenium analog, 5-nitroimidazole-$Re(CO)_3$, was prepared and analyzed by HPLC using the inactive isotope ^{185}Re. The rhenium analog was prepared following a reported procedure,[25] by reacting **3** with one equivalent of $Re(CO)_5Br$ in methanol in presence of triethyl amine as base. It could be observed that the HPLC profile of the rhenium analog (Fig. 1(c)) prepared in macroscopic level matched with that of 5-nitroimidazole-$^{99m}Tc(CO)_3$ prepared in the no-carrier-added level (Fig. 1(b)) suggesting that the complexes formed in both the cases are probably the same.

Swiss mice (~25 g body weight) were used to develop in vivo tumor models for the biodistribution studies. Solid tumors were propagated in the animals by subcutaneous administration of HSBM1C1 murine fibrosarcoma cell line (~10^6 cells per animal) in the dorsal region. The tumor size was allowed to reach approximately 10 mm diameter after which the animals were used for the experiment. All procedures performed herein were in strict accordance with the national laws pertaining to the conduct of animal experiments.

For the in vivo distribution studies, about 0.1 mL of the labeled product (~100 μCi) per animal was administered intravenously. The animals were sacrificed at various time points (30, 60 and 180 min p.i.) after which the relevant organs and tissues were excised for measurement of associated activity. Radioactivity mea-

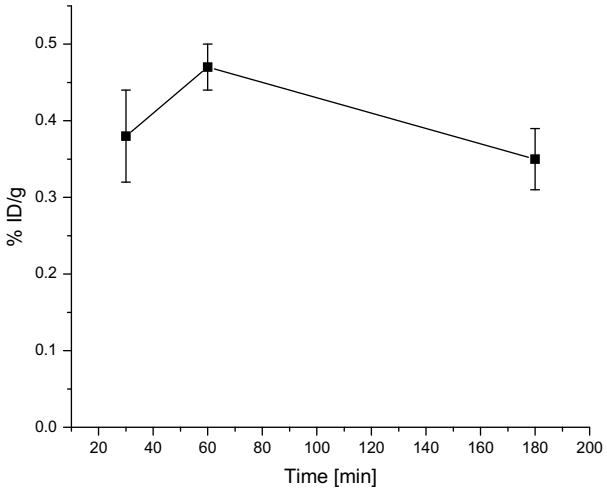


Figure 2. Uptake and retention of injected activity in tumor with time ($n = 3$).

surements were carried out in a flat-bed type NaI(Tl) scintillation counter with optimal energy window for ^{99m}Tc. The accumulated activity was expressed in terms of percentage of total injected dose associated with the specific organ/tissue per gram.

The biodistribution studies carried out for limited time (three time points, $n = 3$) showed uptake and slow clearance of activity from tumor (Fig. 2) with a maximum uptake of 0.47% ID/g observed at 60 min p.i. This retention characteristics of activity in tumor by the complex under investigation was found to be similar to those of BMS181321 and BRU59-21,[6,7] though absolute uptake in tumor cannot be directly compared as the tumor models were different. However, the slow clearance of activity may be considered an indication of efficient reduction of the 5-nitroimidazole complex within the tumor mass. Though clearance of activity from the blood pool was fast, the tumor-to-blood ratio above 1 could not be obtained in the studied time-frame (Fig. 3). A very high tumor-to-muscle ratio is observed throughout the period of study. The predominance of hepatobiliary clearance was evident from high activity in liver and low activity in kidney (Fig. 4). Liver activity was found to be high even at 3 h p.i. This could be explained considering the lipophilicity ($\mathrm{Log}P = 0.39$) of the complex. However, the wash-out from liver was fast, as could be observed from the increase in activity in intestine with time (Fig. 4). There was no significant accumulation of activity in other organs.

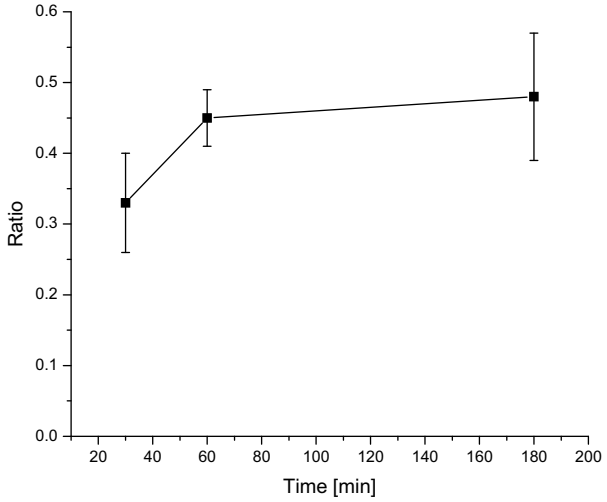


Figure 3. Variation of tumor-to-blood ratio with time. (The ratios are calculated using the %ID/g in respective organs/tissue).

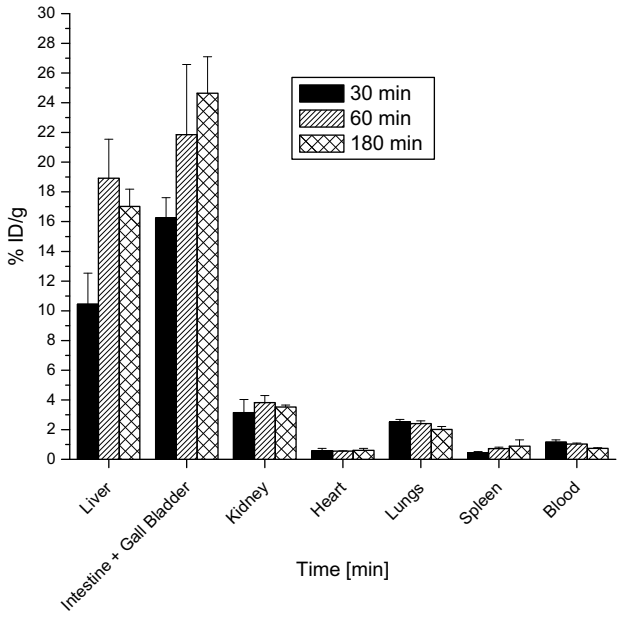


Figure 4. Clearance of injected activity from different organs/tissue with time ($n = 3$).

A comparative study of the biodistribution patterns of the ^{99m}Tc(CO)$_3$-labeled unsubstituted 5-nitroimidazole and ^{99m}Tc(CO)$_3$-labeled 2-methyl-5-nitroimidazole reported by our group earlier[12] revealed no significant difference in the uptake and retention of the two complexes in tumor (Fig. 5). Similarly, considering the clearance patterns of the two complexes, it can be observed that the former showed slower clearance of activity from the liver than the latter. This could be attributed to the higher lipophilicity of unsubstituted 5-nitroimidazole ($\mathrm{Log}P_{o/w} = 0.39$) compared to 2-methyl-5-nitroimidazole ($\mathrm{Log}P_{o/w} = -0.82$). The tumor-to-blood ratio and tumor-to-muscle ratio of the two complexes also showed similar trend.

To conclude, an unsubstituted 5-nitroimidazole derivative was synthesized and its potential to target hypoxia was investigated. The ligand was labeled with ^{99m}Tc via. $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core in excellent yield. The corresponding ^{185}Re analogue was also prepared and compared using HPLC techniques (both at the "no-carrier-ad-

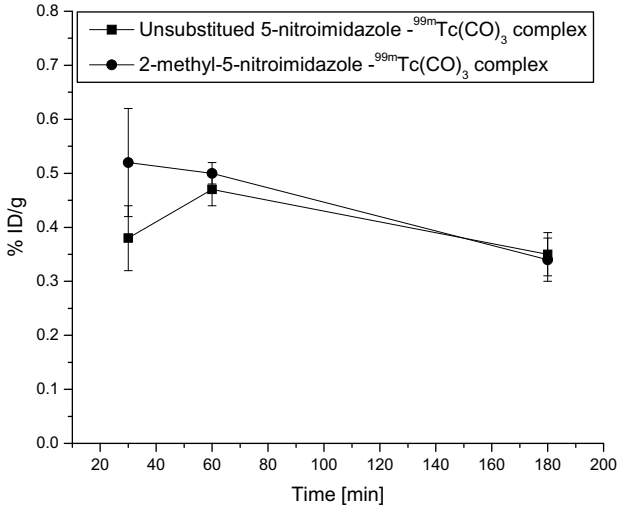


Figure 5. Comparison of uptake and retention of injected activity in tumor shown by unsubstituted 5-nitroimidazole (present study) and substituted 5-nitroimidazole.

ded" level and macroscopic level). Preliminary biodistribution studies in animal model bearing solid tumor showed that the $^{99m}Tc(CO)_3$-complex gets accumulated and is retained in the tumor mass, similar to some of the reported 2-nitroimidazole complexes. However, the present study which documents the isolation of an unsubstituted 5-nitroimidazole and its usage in designing a radiolabeled agent for targeting hypoxia, did not show any significant advantage of unsubstituted 5-nitroimidazole over that of substituted 5-nitroimidazole with respect to its biological behavior.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.069.

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18. To 3-bromopropylamine hydrobromide (0.5 g, 2.3 mmol) in 15 mL acetonitrile was added three equivalents of triethylamine. The reaction mixture was stirred at room temperature for 15 min and then 2.1 equivalents of *tert*-butylbromoacetate (0.94 g) was added. The reaction is then continued for 24 h at room temperature. The progress of the reaction was monitored by TLC. Upon completion of the reaction, the solvent was evaporated under vacuum, 15 mL water was added and then extracted with two 15 mL portions of chloroform. The chloroform layer was then concentrated and the target compound **1** separated by silica get column chromatography eluting the chloroform. Overall yield of **1** was found to be 82% (0.841 g). R_f (Ethyl acetate) = 0.83. ^{1}H NMR ($CDCl_3$, δ ppm) 1.46 (s, 18 H, –$N(CH_2CO_2CCH_3)_2$); 2.03 (q, 2 H, $BrCH_2CH_2CH_2N$-); 2.90 (t, 2H, $BrCH_2CH_2CH_2N$); 3.47 (s, 4H, -$N(CH_2CO_2CCH_3)_2$); 3.5 (t, 2H, $BrCH_2CH_2CH_2N$-). IR (neat, cm^{-1}) 2980 (m); 2939 (m); 1741 (vs); 1444 (m); 1371 (m); 1252 (s); 1190 (vs); 1144 (w). 1030 (s); 993 (w); 918 (w); 864 (w); 731 (w); 649 (w); 565 (w).
19. To 4-nitroimidazole (218 mg, 1.93 mmol, 1.2 eq) in 15 mL of acetonitrile, anhydrous K_2CO_3 (2 equiv) was added and the suspension stirred for 15 min. To this mixture *N,N*-bis[(*tert*-butoxycarbonyl)methyl]-3-bromopropylamine] (**1**) (0.595 g, 1.6 mmol) was added and the reaction mixture refluxed for 12 h. The progress of the reaction was monitored by TLC. After removing the solvent, the residue was dissolved in 20 mL water and extracted with 2 × 15 mL portions of chloroform. The combined chloroform layer was washed with brine and dried. The 4- and 5-nitroimidazole *tert*-butylester derivative formed are separated by silica gel chromatography, using diethyl ether as the eluant. The 5-nitroimidazole derivative (**2**) could be obtained in an overall yield of 15% (0.098 g). R_f (Diethyl ether) = 0.72; ^{1}H NMR ($CDCl_3$, δ ppm) 1.46 (s, 18H, –$N(CH_2CO_2CCH_3)_2$); 1.93 (q, 2H, nitroimidazole–$CH_2CH_2CH_2N$–); 2.71 (t, 2H, nitroimidazole–$CH_2CH_2CH_2N$–); 3.37 (s, 4H, –$N(CH_2CO_2CCH_3)_2$); 4.57 (t, 2H, nitroimidazole–$CH_2CH_2CH_2N$–); 7.82(d, 2H, nitroimidazole C2–H,); 8.0 (d, 2H, nitroimidazole C4-H). IR (Neat, cm^{-1}) 3130 (m); 2980 (m); 2931 (m); 1733 (vs); 1540 (s); 1497 (m); 1336 (m); 1222 (m); 1147 (vs); 1070 (m); 987 (m); 938 (w); 854 (m); 823 (m); 760 (m); 658 (m). The purified ester **2** was dissolved in 1 mL of methanol and to this solution 7 mL of 6 N HCl was added. The reaction was kept stirring at 50 °C for 2 h. The solvent was removed under vacuum to yield the hydrochloride salt of iminodiacetic acid 3 as a white powder. The yield was quantitative. ^{1}H NMR (D_2O, δ ppm) 2.27 (m, 2H, nitroimidazole–$CH_2CH_2CH_2N$–); 3.42 (m, 2H, nitroimidazole-$CH_2CH_2CH_2N$–); 4.04 (s, 4H, –$N(CH_2CO_2CCH_3)_2$); 4.52 (m, 2H, nitroimidazole–$CH_2CH_2CH_2N$–); 8.26 (d, 2H, nitroimidazole C2–H,); 8.46 (d, 2H, nitroimidazole C4–H). IR (Neat, cm^{-1}) 3130 (m); 2974 (m); 2847 (m); 1763 (vs); 1724 (s); 1559 (m); 1499 (m); 1392 (m); 1350 (m); 1230 (m); 1180 (m); 1140 (s); 1014 (w); 861 (m); 827 (m); 758 (w); 654 (m).
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