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Bioorganic & Medicinal Chemistry 13 (2005) 4713–4720
Bioorganic & Medicinal Chemistry

Synthesis and evaluation of bifunctional chelating agents derived from bis(2-aminophenylthio)alkane for radioimaging with ^{99m}Tc

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Received 31 March 2005; revised 20 April 2005; accepted 28 April 2005
Available online 26 May 2005

Abstract—Novel bifunctional chelating agents bearing an aromatic rigid backbone have been synthesized and characterized on the basis of spectroscopic techniques. These macrocyclic multidentate chelating agents were conjugated with monoclonal antibody which forms stable complexes with ^{99m}Tc with high radiochemical purity.

1. Introduction

Bifunctional chelating agents (BFCs), the central molecules meant for attaching the radionuclide to biomolecules, have gained widespread interest because of their utility in radiodiagnostic and radiotherapeutic applications.[1–3] Bifunctional chelating agent is key to successful application of receptor–ligand (biomolecule) based targeted radiopharmaceuticals and there is continued interest in the design and development of new bifunctional chelating agents for effective coordination of radioactive metals.[4–9] Bifunctionalized EDTA was the first chelating agent evaluated for scintigraphy.[10] The metal complex with EDTA had very low stability under in vivo conditions. Alternatively, bifunctionalized macrocyclic chelating agents based on cyclam and cyclene framework have been successful for in vivo applications, as complexes of macrocyclic chelating agents are more stable than acyclic chelating agents.[11,12] As a general rule, complexes with preorganized structures have higher stability under physiological conditions. Accordingly, many modifications have been introduced in the structures of chelating moieties, such as introduction of bulky methyl and ethyl groups and synthesis of cyclohexane and cyclopentane ring based chelating agents.[13] Recently, many groups have started exploring the aromatic ring based chelating agents. In aromatic BFCs, besides the increased rigidity in framework, the introduction of a nitro group (required for functionalization by converting in amine group) is comparatively easier.[14]

Considering the above aspects, we herein report the synthesis and evaluation of macrocyclic bifunctional chelating agents based on an aromatic ring (**5a–c**) (N_2S_2 and N_2S_2O system) derived from bis(2-aminophenylthio)alkane. The initial complexation studies with these BFCs were performed with ^{99m}Tc radionuclide.

2. Results and discussion

Designing and synthesis of bifunctional chelating agents (BFCs) are crucial for efficient in vivo application of radiolabeled bioconjugates. BFCs, having rigid backbones as exemplified by the classical *trans*-cyclohexylethylenediaminetetraacetic acid (CDTA) ligand, provide enhanced kinetic inertness relative to flexible parent ligands.[15] Similarly, for a series of Y(III) complexes of eight coordinate DTPA type ligands which included cyclohexyl and benzyl moieties in the backbone, the observed acid catalyzed dissociation rate constant varied by up to 3 orders of magnitude.[16] In this study, macrocyclic BFCs with increased rigidity for complex stability besides requisite flexibility for complexation along with variation in ring size and donor atoms were studied for radiodiagnostic

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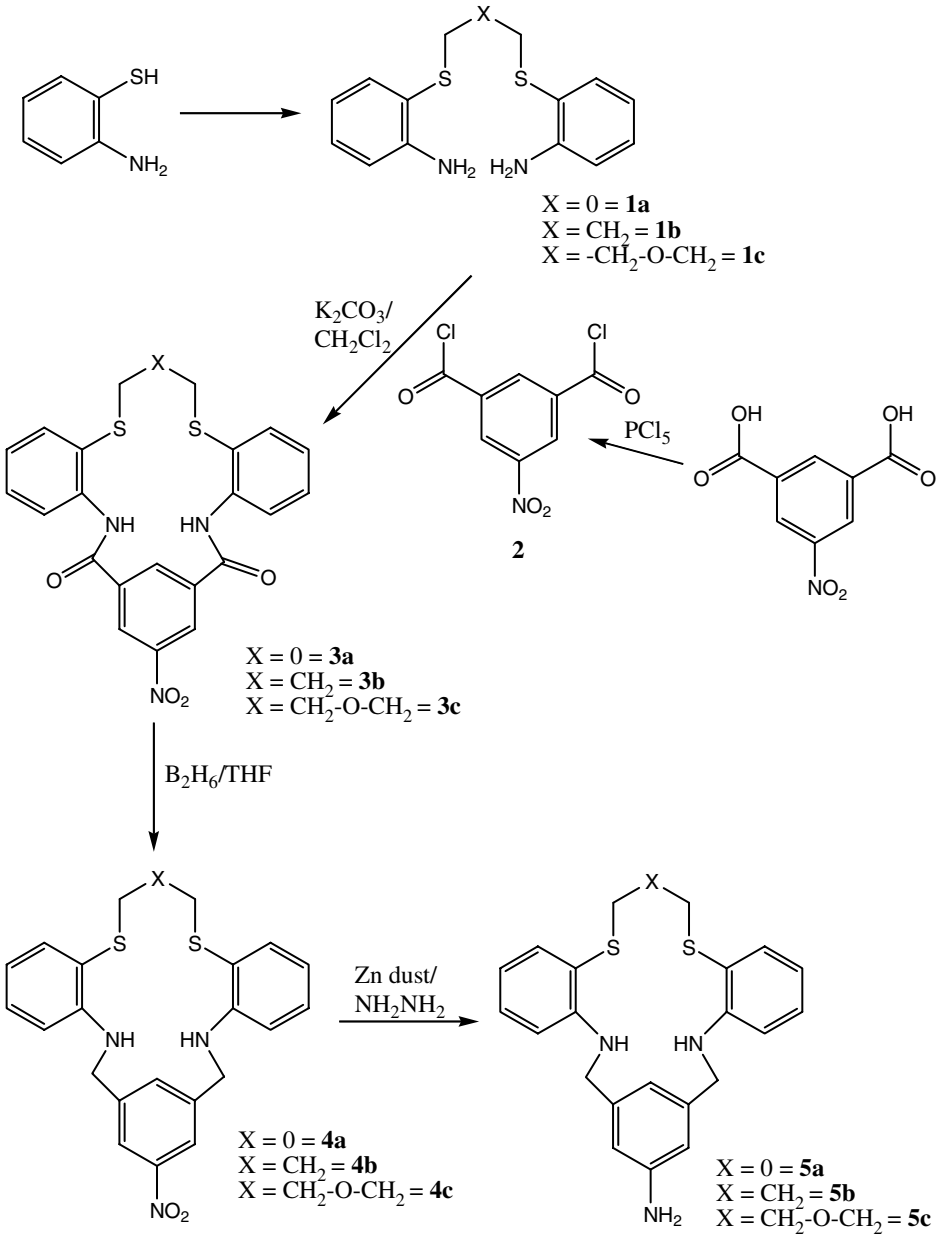
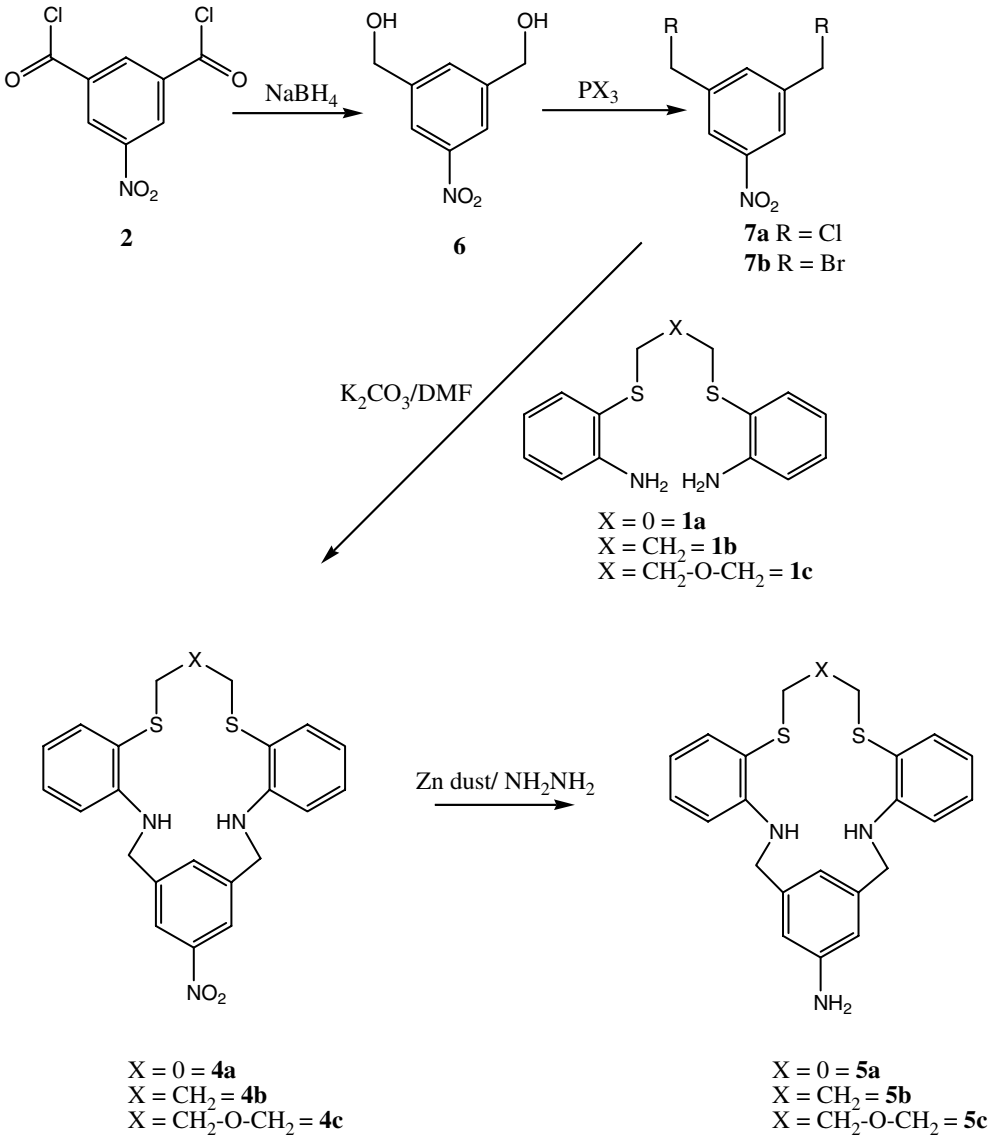
doi:10.1016/j.bmc.2005.04.073

and radiotherapeutic applications. Bifunctional chelating agents **5a**–**c** were synthesized by two different synthetic routes as depicted in Schemes 1 and 2.

The bis(2-aminophenylthio)alkanes were prepared from 2-aminothiophenol by the reaction of respective dibromoalkane and ditosylates. In one of the earlier attempts to cyclize the diamines (**1a**–**c**) with bromoacetyl bromide, 3-oxo-3,4-dihydro-2*H*-1,4-benzothiazine was obtained quantitatively.[17] Another approach to obtaining the macrocycles prepared using derivatives of 5-nitroisophthalic acid are shown in the schemes. As described in Scheme 1 the 5-nitroisophthalic acid was converted into diacidchloride (**2**) with PCl_5 and used for cyclization of **1a**–**c** to obtain **3a**–**c**. The slow addition of 5-nitroisophthaloyl dichloride (**2**) to a solution of diamine **1a** in dichloromethane containing a suspension of K_2CO_3 (anhydrous) and TBA·HSO_4 (tetrabutylammonium hydrogen sulfate as phase transfer catalyst) gave macrocycle **3a** (yield 42%) mp 245 °C. **3a**–**c** were selectively reduced by diborane to synthesize **4a**–**c** macrocycles. Reduction of the nitro group of **4a**–**c** with $Pd/C/H_2$ yielded **5a**–**c** in very low yield besides separation of product from reaction mixture being tedious. The Gowda and Gowda[18] method for reduction of nitro group was the only successful method by which the product was easily obtained in high yield. In Scheme 1, the cyclization was performed under high dilution conditions to increase the yield of macrocycle. The cyclization reaction was followed by selective reduction of the carbonyl and nitro groups present on the macrocyclic moiety and as both these reactions involved the low yielded macrocycles, this effectively lowered the final yield of macrocyclic BFCs. Alternatively another



Scheme 1.



Scheme 2.

synthetic route was followed in which the reduction of carbonyl group was done prior to cyclization reaction as depicted in Scheme 2. The 5-nitroisophthaloyl dichloride (**2**) was reduced to dialcohol by sodium borohydride, which was further converted into dihalide with phosphorus trihalides. The synthesis of dihalides from diol, the dibromo product **7b**, was obtained in very high yield compared to dichloro **7a**. The cyclization of **1b** with bis-3,5-bromomethylnitrobenzene in dry DMF at 120 °C in the presence of K_2CO_3 and $TBA{\cdot}HSO_4$ gives **4b** (62%).

All the chelating agents (**5a**–**c**) are stable at room temperature and do not require any specialized condition for long-term storage. The radiocomplexation studies of these BFCs (**5a**–**c**) with ^{99m}Tc were performed in a biphasic state. Complexation of chelating agent with ^{99m}Tc was done by reduction of pertechnetate with stannous chloride ($SnCl_2{\cdot}2H_2O$) in the presence of chelating agents. Radiocomplexation was completed in 40–50 min with a radiochemical purity of 92% (**5a**), 94% (**5b**), and 98% (**5c**) as monitored by ITLC-SG using different solvent conditions. The complexes were stable in dilute saline solution and in serum under physiological conditions as expected for backbone substituted rigid macrocyclic chelating agents. In serum, neither transchelation of radioactive metal ions nor metal ion transfer to serum proteins was observed. The final chelating agents (**5a**–**c**) are bifunctional bearing a free amine group that was used for conjugation with monoclonal antibodies by converting it to –NCS, which can be extended to couple with other biologically active peptides.

The synthesis of bifunctional chelating agent (**5a**–**c**) is shown in Schemes 1 and 2. Synthesis of bifunctional chelating agents was performed as described in Section 4.1. The –NCS derivatives were found to be stable when stored at −20 °C in 0.3 M HCl.

Conjugation of mAbs with chelates was performed in the ratio of 1:20. UV absorbance and the ^{57}Co binding assay indicated that 2.1 ± 0.1 chelate molecules were conjugated per antibody molecule.

The conjugated antibodies were labeled with a specific activity 20–30 mCi/mg of protein. Labeling efficiencies were measured by ascending paper chromatography on ITLC-SG strips. Results of radiolabeling of the immunoconjugates were found to be 98.5 ± 0.30%. ITLC-SG results in acetone showed that 1.5–2% or less free pertechnetate ran with the solvent front (R_f = 0.7–1.0). This indicated that pertechnetate was reduced almost entirely. Using 10% NH_4Ac and methanol 1:1 as a solvent for migration showed all the activity on the base of ITLC-SG strips indicating the radiolabeled immunoconjugate, not the other species.

Radiolabeled immunoconjugates were challenged with (25–100 mM) DTPA and cysteine to test the stability of the radiolabeled antibodies. Macrocyclic chelating agents—ior egf/r3—showed only 2–3% transcomplexation. Approximately more than 97% of the radioactivity remains associated with the antibody after 24 h challenge at 37 °C with DTPA. Immunoreactivity of immunoconjugate was determined on a phase grafted with antigens. The immunoreactivity of ^{125}I-labeled ior egf/r3 and CB-CEA-1 using the iodogen method[19] was examined as a reference.

Immunoreactivity was greater than 80% for ^{99m}Tc-labeled anti-EGFr conjugated with chelating agents. These values are equivalent to those of ^{125}I-labeled counterparts, indicating that biological activities were not compromised after modification with a bifunctional chelating agent.

3. Conclusions

In conclusion, we here describe the synthesis of new aromatic bifunctional chelating agents (**5a**–**c**) with different central cavity sizes and donor atoms. The complexes formed by these chelating agents are highly stable under physiological conditions. New chelating agents can be effectively used for targeted scintigraphy with ^{99m}Tc by conjugation with biomolecules because biological activities were not compromised after modification with bifunctional chelating agent.

4. Experimental

4.1. Materials and methods

All chemicals and reagents used in present study were of analytical grade. TLC was run on the silica gel coated aluminum sheets (silica gel 60 F_{254}, E. Merck, Germany) and visualized in UV light (254 nm). Melting point was determined with a Buchi B540 instrument. The corresponding 3,5-bis-bromoethylnitrobenzene was synthesized by following the reported procedure.[20] 2-Aminothiophenol was procured from Acros Organics (USA) and distilled prior to use. 5-Nitroisophthalic acid was synthesized by nitration of isophthalic acid essentially following the reported procedure.[21,22] IR spectra were recorded on the FT-IR Perkin Elmer Spectrum BX spectrophotometer. NMR spectral characterization was carried out on a Bruker 400 MHz NMR instrument operating near 400 (^{1}H) and 100 (^{13}C) MHz, and a Bruker 300 MHz NMR instrument operating near 300 MHz (^{1}H) and 75 MHz (^{13}C). The FAB-MS spectra were recorded at Central Drug Research Institute, India, on a JEOL SX 102/DA-6000 mass spectrometer using *m*-nitrobenzyl alcohol as matrix. EI-MS spectra were recorded on a JEOL SX102/DA (KV 10 mA) instrument. Elemental analysis was done on the Elementar Analysensysteme GmbH VarioEL system. Radiocomplexation and radiochemical purity were checked by instant strip chromatography (silica gel impregnated paper chromatography) with ITLC-SG (Gelman Sciences, Ann Arbor, MI, USA). The gamma scintillation counting was done on an ECA (Electronic Corporation of India Ltd) Gamma Ray Spectrometer K2700B.

4.2. Synthesis

4.2.1. 1,2-Bis(2-aminophenylthio)ethane (1a). The 1,2-dibromoethane (3.7 g, 20 mmol) was added dropwise to a refluxing solution of 2-aminothiophenol (5 g, 40 mmol) and sodium ethoxide (3.7 g, 50 mmol) in dry ethanol (20 mL). The refluxing reaction mixture was stirred for 6–7 h and it was monitored by TLC. On completion of reaction the solvent was removed in vacuo and the reaction mixture was cooled to 0 °C. Water (50 mL) was added and the reaction mixture was extracted with dichloromethane (20 mL × 4). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo to get a solid product, which on recrystallization from ethanol gave white colored solid (4.3 g, 78%), mp 72–73 °C. IR (KBr pellets, cm^{-1}) 3385, 3356, 3290, 3018, 2988, 2925, 1617, 1582, 1479, 1446, 749. ^{1}H NMR (60 MHz, $CDCl_3$) δ ppm: 6.70–7.50 (m, 8H, ArH), 4.30 (br s, 4H, 2× NH_2, exchanged with D_2O), 2.80 (s, 4H, 2× S–CH_2). Anal. Calcd for $C_{14}H_{16}N_2S_2$: C, 60.83; H, 5.83; N, 10.13; S, 23.20. Found: C, 60.63; H, 5.76; N, 10.60; S, 23.01. FAB-MS: Found: *m/z* 276 $[M]^{+\cdot}$. Calcd for $C_{14}H_{16}N_2S_2$: 276. EI-MS: 276, 138, 124 (base peak), 94.

4.2.2. 1,3-Bis(2-aminophenylthio)propane (1b). The 1,3-dibromopropane (4.02 g, 20 mmol) was added dropwise to a refluxing solution of 2-aminothiophenol (5 g, 40 mmol) and sodium methoxide (3.2 g, 50 mmol) in dry methanol (20 mL). The refluxing reaction mixture was stirred for 6–7 h and it was monitored by TLC. On completion of reaction the solvent was removed in vacuo and the reaction mixture was cooled to 0 °C. Water (50 mL) was added and the reaction mixture was extracted with dichloromethane (20 mL × 4). The organic layer was dried over Na_2SO_4 and evaporated to dryness in vacuo to get a crude product, which was further purified by column chromatography [column of SiO_2 (100 g); preadsorption of the residue at SiO_2 (ca. 8 g) with ethyl acetate; elution with petroleum ether/ethyl acetate 60:40 (v/v)] to obtain transparent oily liquid (4.5 g, 82%). IR (NaCl plates, cm^{-1}) 3434, 3355, 3018, 2988, 2925, 1606, 1478, 1448, 1301, 748. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.20 (dd, J_{ab} = 7.5 Hz, J_{ac} = 1.5 Hz, 2H, ArH), 7.00 (dt, J_{ab} = 7.6 Hz, J_{ac} = 1.4 Hz, 2H, ArH), 6.68 (dd, J_{ab} =8.0 Hz, J_{ac} = 1.2 Hz, 2H, ArH), 6.64 (dt, J_{ab} =

7.6 Hz, J_{ac} = 1.2 Hz, 2H, ArH), 4.10 (br s, 4H, 2× NH_2, exchanged with D_2O), 2.80 (t, J = 7.1 Hz, 4H, 2× S–CH_2–), 1.75 (quintet, J = 7.1 Hz, 2H, S–C–CH_2–). Anal. Calcd for $C_{15}H_{18}N_2S_2$: C, 62.03; H, 6.25; N, 9.64; S, 22.08. Found: C, 62.14; H, 6.26; N, 9.82; S, 21.88. FAB-MS: Found: m/z 290 $[M]^{+\cdot}$. Calcd for $C_{15}H_{18}N_2S_2$: 290. EI-MS: 290, 166, 138, 124 (base peak), 94.

4.2.3. 1,5-Bis(2-aminophenylthio)-3-oxapentane (1c). The diethyleneglycolditosylate (8.2 g, 20 mmol) was added dropwise to a refluxing solution of 2-aminothiophenol (5 g, 40 mmol) and sodium methoxide (3.2 g, 50 mmol) in dry methanol (20 mL). The refluxing reaction mixture was stirred for 6–7 h and it was monitored by TLC. On completion of reaction the solvent was removed in vacuo and the reaction mixture was cooled to 0 °C. Water (50 mL) was added and the reaction mixture was extracted with dichloromethane (20 mL × 4). The organic layer was dried over Na_2SO_4 and evaporated to dryness in vacuo to get a crude product, which was further purified by column chromatography [column of SiO_2 (100 g); pre-adsorption of the residue at SiO_2 (ca. 8 g) with ethyl acetate; elution with petroleum ether/ethyl acetate 60:40 (v/v)] to obtain viscous liquid (4.56 g, 72%). IR (KBr pellets, cm^{-1}) 3432, 3349, 3058, 2921, 2855, 1607, 1479, 1105, 748. ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 7.30 (d, J = 7.5 Hz, 2H, ArH), 7.10 (t, J = 7.5 Hz, 2H, ArH), 7.00 (d, J = 7.5 Hz, 2H, ArH), 6.60 (t, J = 7.8 Hz, 2H, ArH), 4.00 (br s, 4H, 2× NH_2, exchanges with D_2O), 3.30 (t, J = 7.1 Hz, 4H, 2× O–CH_2–), 2.73 (t, J = 7.0 Hz, 4H, 2× S–CH_2). ^{13}C NMR ($CDCl_3$) δ ppm: 32.15, 67.32, 112.71, 114.06, 114.97, 127.81, 133.97, 148.19. Anal. Calcd for $C_{16}H_{20}N_2OS_2$: C, 59.96; H, 6.29; N, 8.74; O, 4.99; S, 20.01. Found: C, 59.66; H, 6.08; N, 8.44; S, 20.00. EI-MS: Calcd for $C_{16}H_{20}N_2OS_2$: 320. Found: m/z 320, 319, 227, 195, 136 (base peak), 166, 94, 57.

4.2.4. 5-Nitroisophthaloyl dichloride (2). 5-Nitroisophthalic acid (5 g, 23.7 mmol) and phosphorus pentachloride (PCl_5) (10 g, 48 mmol) were mixed in a dry flask and heated to 120 °C. The molten solid reaction mixture was stirred at the same temperature for 2 h. $POCl_3$ formed was distilled off under vacuum and residual mixture on cooling gave colorless solid acid chloride product (Note: in some repeat reactions, acid chloride remained as a colorless syrupy liquid) (6.5 g, 92%)). IR (KBr pellets, cm^{-1}): 3088m, 1756vs, 1622m, 1535s, 1349s, 1145s, 1005s, 734m, 703s, 680s.

4.2.5. 3,5-Bis-hydroxymethylnitrobenzene (6). 5-Nitroisophthaloyl dichloride (2 g, 8.0 mmol) in dry diglyme (10 mL) was added dropwise with stirring (mechanical) to sodiumborohydride (0.92 g, 24 mmol) solution in diglyme (20 mL) at 0 °C under nitrogen atmosphere. After complete addition of acid chloride, the reaction mixture was stirred at ambient temperature for 7 h. The reaction mixture was cooled to 0 °C and treated with 1 N HCl (aq) to destroy excess $NaBH_4$. The resulting mixture was concentrated to remove diglyme, added distilled water (100 mL), and extracted with ethyl acetate (40 mL × 5). The organic phase was dried over Na_2SO_4 and concentrated to afford a thick liquid which was chromatographed over silica gel (EtOAc/Pet ether 40:60) to obtain a light yellowish solid product (1 g, 70%). mp 91–92 °C. IR (KBr pellets, cm^{-1}) 3290, 3207, 1533, 1342, 1035, 773, 746, 678. ^1H NMR (300 MHz, DMSO-d_6) δ: 8.0 (s, 2H), 7.6 (s, 1H), 5.5 (t, J = 5.5 Hz, 2H, exchanged with D_2O), 4.6 (d, J = 5.5 Hz, 4H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 148.7, 145, 131.3, 119.8, 62.7. Anal. Calcd for $C_8H_9NO_4$: C, 52.46; H, 4.95; N, 7.65; O, 34.94. Found: C, 52.28; H, 4.89; N, 7.71. EI-MS: Calcd for $C_8H_9NO_4$: 183. Found: m/z 183 $[M]^{+\cdot}$.

4.2.6. 3,5-Bis-chloromethylnitrobenzene (7a). 3,5-Bis-hydroxymethylnitrobenzene (1 g, 5.4 mmol) was dissolved in ether (30 mL) with stirring and cooled to 0 °C. PCl_3 (1 mL) dissolved in ether (20 mL) was added to the above reaction mixture and stirred at 30–35 °C for 36 h. The reaction mixture was poured onto crushed ice and extracted with ether. The organic layer was washed with Na_2CO_3 sol and water, and dried over Na_2SO_4. Whitish solid product is obtained on removal of solvent (200 mg, 20%). mp 67–68 °C. IR (KBr pellets, cm^{-1}): 2923m, 1529s, 1361s, 1213m, 777w, 746w, 686s. ^1H NMR (60 MHz, $CDCl_3$) δ: 8.2 (d, J = 3.6 Hz, 2H), 7.8 (s, 1H), 4.7 (s, 4H).

4.2.7. 3,5-Bis-bromomethylnitrobenzene (7b). 3,5-Bis-hydroxymethylnitrobenzene (1 g, 5.4 mmol) was dissolved in ether (30 mL) with stirring and cooled to 0 °C. PBr_3 (1 mL) dissolved in ether (20 mL) was added to the above reaction mixture and stirred at the same temperature for 6 h and then at ambient temperature for 12 h. The reaction mixture was poured onto crushed ice and extracted with ether (20 mL × 5). The organic layer was washed with Na_2CO_3 solution and water, and dried over Na_2SO_4. White solid product is obtained on removal of solvent (1.6 g, 97%). mp 102–103 °C (lit.[23] mp 103–104 °C). IR (KBr pellets, cm^{-1}): 3067vw, 3033vw, 2919w, 1530vs, 1361m, 1214,656m, 607m, ^1H NMR (300 MHz $CDCl_3$) δ: (d, J = 1.5 Hz, 2H), 7.75 (t, J = 1.5 Hz), 4.52 (S, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 148.6, 140.4, 135.2, 123.6, 30.6. Anal. Calcd for $C_8H_7Br_2NO_2$: C, 31.10; H, 2.28; N, 4.53. Found: C, 31.28, 2.44; N, 4.36. EI-MS: Calcd for $C_8H_7Br_2NO_2$: 308. Found: m/z 308 $[M]^{+\cdot}$.

4.2.8. General procedure for the synthesis of macrocycles 3a, 3b, and 3c. 1,3-Bis(2-aminophenylthio)propane (2.9 g, 10 mmol) in 150 mL dry dichloromethane and 5-nitroisophthaloyl dichloride (2.5 g, 10 mmol) in 150 mL dichloromethane were added simultaneously dropwise to a vigorously stirring reaction mixture of K_2CO_3 (2.8 g, 20 mmol) and TBA·HSO_4 (20 mg) in 200 mL dry dichloromethane at ambient temperature. After complete addition, the reaction mixture was further stirred for an additional 1 h. Solvent was removed under reduced pressure and the obtained solid product was washed with water and dried under vacuum, which resulted in a puff-colored solid product.

4.2.8.1. Macrocycle 3a. (1.9 g, 42%.) IR (KBr pellets, cm^{-1}) 3276m (N–H str), 1680s (C=O str), 1653s, 1579s, 1513s, 1436s, 1348s, 757m, 722w, 681w. ^1H NMR

(300 MHz, DMSO) δ ppm: 10.1 (s, 1H, ArH), 8.9 (d, $J = 1.24$ Hz, 2H, ArH), 8.6 (d, $J = 8.32$ Hz, 2H, ArH), 7.6 (dd, $J = 7.7$ Hz, $J = 1.48$, 2H, ArH), 7.4 (dt, $J = 8.04$, $J = 1.44$ Hz, 2H, ArH), 7.1 (dt, $J = 7.4$ Hz, $J = 1.44$ Hz, 2H, ArH), 2.8 (s, 4H, 2× S–CH_2–). Anal. Calcd for $C_{22}H_{17}N_3O_4S_2$: C, 58.52; H, 3.97; N, 9.31; O, 14.17; S, 14.20. Found: C, 58.31; H, 3.89; N, 9.45; S, 14.19. FAB-MS: Calcd for $C_{22}H_{17}N_3O_4S_2$: 451. Found: *m/z* 452 $[M+H]^+$.

4.2.8.2. Macrocycle 3b. (2.4 g, 52%.) IR (KBr pellets, cm^{-1}) 3311m, 1681s, 1580s, 1517s, 1434, 1347m, 756m, 733m. ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 9.7 (s, 1H, ArH), 9.1 (d, $J = 1.24$ Hz, 2H, ArH), 8.6 (d, $J = 8.32$ Hz, 2H, ArH), 7.6 (dd, $J = 7.7$ Hz, $J = 1.48$, 2H, ArH), 7.4 (dt, $J = 8.04$, $J = 1.44$ Hz, 2H, ArH), 7.1 (dt, $J = 7.4$ Hz, $J = 1.44$ Hz, 2H, ArH), 2.7 (t, $J = 7.9$ Hz, 4H, 2× S–CH_2–), 1.8 (quintet, $J =$ (merged), 2H, 2× S–C–CH_2–). Anal. Calcd for $C_{23}H_{19}N_3O_4S_2$: C, 59.34; H, 4.11; N, 9.03; O, 13.75; S, 13.78. Found: C, 59.17; H, 4.03; N, 9.23; S, 13.82. FAB-MS: Calcd for $C_{23}H_{19}N_3O_4S_2$: 465. Found: *m/z* 466 $[M+H]^+$.

4.2.8.3. Macrocycle 3c. (2.9 g, 59%.) IR (KBr pellet, cm^{-1}) 3430w, 3247w, 2922m, 2858m, 1646s, 1527vs, 1440, 1351m, 731m, 664m. ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 9.7 (s, 1H, ArH), 9.1 (d, $J = 1.24$ Hz, 2H, ArH), 8.6 (d, $J = 8.32$ Hz, 2H, ArH), 7.6 (dd, $J = 7.7$ Hz, $J = 1.48$, 2H, ArH), 7.4 (dt, $J = 8.04$, $J = 1.44$ Hz, 2H, ArH), 7.1 (dt, $J = 7.4$ Hz, $J = 1.44$ Hz, 2H, ArH), 3.6 (t, $J = 4.88$ Hz, 4H, 2× O–CH_2–), 3.0 (t, $J = 4.88$, 4H, 2× S–CH_2–). Anal. Calcd for $C_{24}H_{21}N_3O_5S_2$: C, 58.17; H, 4.27; N, 8.48; O, 16.14; S, 12.94. Found: C, 58.02; H, 4.20; N, 8.62; S, 12.98. FAB-MS: Calcd for $C_{24}H_{21}N_3O_5S_2$: 495. Found: *m/z* 496 $[M+H]^+$.

4.2.9. General procedure for the synthesis of macrocycles 4a, 4b, and 4c from 3a, 3b, and 3c. To a solution of **3a** (1.15 g, 2.5 mmol in 20 mL of dry THF) 88 mL of 1 M diborane in THF at 0 °C was added with syringe under argon atmosphere and the reaction mixture was stirred for 1 h. The temperature of the reaction mixture was raised to 60 °C and the solution allowed to reflux for 24 h. After cooling, 20 mL of water was carefully added into the solution to destroy the excess diborane. The solvent was evaporated under reduced pressure, the residue was dissolved in 8 mL of ethanol, and the resultant solution was saturated with HCl(g) and refluxed for 2 h. On cooling, the precipitated product was collected and washed with water and methanol (5 mL) to give compound **4a** (522 mg, 49%).

4.2.10. General procedure for the synthesis of macrocycles 4a, 4b, and 4c. 1,3-Bis(2-aminophenylthio)propane **1b** (2.9 g, 10 mmol) in 100 mL DMF and bis-3,5-bromomethylnitrobenzene **7b** (3.09 g, 10 mmol) in 100 mL DMF were added simultaneously dropwise from separate dropping funnels under nitrogen atmosphere at 120 °C with vigorous stirring (using mechanical stirrer) dry K_2CO_3 (2.8 g, 20 mmol) and TBA·HSO_4 (20 mg) in 100 mL DMF. The reaction mixture was stirred at the same temperature for 7 h and on completion of the reaction (TLC) the DMF was removed under vacuum, the solid left triturated with water thoroughly, and the water phase decanted away. Residual solid was again washed with methanol (20 mL) and dried under vacuum to afford a yellow solid product (**4b**).

4.2.10.1. Macrocycle 4a. (2.2 g, 52%.) mp 222–224 °C IR(KBr, cm^{-1}): 3384m (N–H str), 1586m, 1524s (NO_2 asym str), 1498s, 1350s (NO_2 sym str), 1311s, 743s. ^1H NMR (300 MHz, DMSO-d_6) δ ppm: 8.1 (s, 2H, ArH), 7.5 (s, 1H, ArH), 4.5 (d, $J = 6$ Hz, 4H, Ar–CH_2–N), 7.2 (d, $J = 6.9$ Hz, 2H, ArH), 6.9 (t, $J = 7.44$ Hz, 2H, ArH), 6.4 (t(merged), $J = 7.4$ Hz, 2H, ArH), 6.3 (d, $J = 8.1$ Hz, 2H, ArH), 2.8 (s, 4H, S–CH_2–). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 147.9, 142.7, 137.0, 130.3, 129.2, 120.3, 115.9, 114.8, 109.8, 44.1, 33.7. Anal. Calcd for $C_{22}H_{21}N_3O_2S_2$: C, 62.39; H, 5.00; N, 9.92; O, 7.55; S, 15.14. Found: C, 62.28; H, 4.98; N, 10.01; S, 15.26. EI-MS: Calcd for $C_{22}H_{21}N_3O_2S_2$: 423. Found: *m/z* 429 $[M+Li]^+$. FAB-MS: Calcd for $C_{22}H_{21}N_3O_2S_2$: 423. Found: *m/z* 423 $[M]^{+\cdot}$.

4.2.10.2. Macrocycle 4b. (2.8 g, 64%.) mp 206–207 °C IR(KBr, cm^{-1}): 3388m (N–H str), 1585s, 1520s (NO_2 asym str), 1499s, 1352s (NO_2 sym str), 1320s, 745s. ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 8.06 (s, 2H, ArH), 7.6 (s, 1H, ArH), 4.5 (d, $J = 5.5$ Hz, 4H, 2× Ar–CH_2–N), 5.6 (s (broad), 2H, NH), 7.3 (d, $J = 7.0$ Hz, 2H, ArH), 7.0 (t, $J = 7.3$ Hz, 2H, ArH), 6.5 (t, $J = 7.2$ Hz, 2H, ArH), 6.1 (d, $J = 7.9$ Hz, 2H, ArH), 2.7 (t, $J = 7.9$ Hz, 4H, 2× S–CH_2–), 1.7 (quintet (merged), 2H, 2× S–C–CH_2–). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 142.2, 135.6, 129.9, 128.6, 120.2, 117.5, 109.9, 46.3, 34.7, 30.4. Anal. Calcd for $C_{23}H_{23}N_3O_2S_2$: C, 63.13; H, 5.30; N, 9.60; O, 7.31; S, 14.66. Found: C, 62.98; H, 5.21; N, 9.89; S, 14.78. EI-MS: Calcd for $C_{23}H_{23}N_3O_2S_2$: 437. Found: *m/z* 443 $[M+Li]^+$. FAB-MS: Calcd for $C_{23}H_{23}N_3O_2S_2$: 437. Found: *m/z* 437 $[M]^{+\cdot}$.

4.2.10.3. Macrocycle 4c. (3.2 g, 68%.) mp 159.5–160 °C IR(KBr, cm^{-1}): 3365m (N–H str), 2921m, 2850w, 1587s, 1529s (NO_2 asym str), 1497s, 1447m, 1350s (NO_2 sym str), 1317s, 1259m, 1085s, 751s. ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 8.1 (s, 2H, ArH), 7.8 (s, 1H, ArH), 7.4 (d, $J = 7.35$ Hz, 2H, ArH), 7.1 (t, $J = 7.5$ Hz, 2H, ArH), 6.6 (t, $J = 7.5$ Hz, 2H, ArH), 6.6 (t, $J = 7.29$ Hz, 2H, ArH), 6.3 (d, $J = 8.0$ Hz, 2H, ArH), 5.6 (s (broad), 2H, NH), 4.4 (d, $J = 5.3$ Hz, 4H, 2× Ar–CH_2–N), 3.4 (t, $J = 6.9$ Hz, 4H, 2× O–CH_2–), 2.8 (t, $J = 6.9$, 4H, 2× S–CH_2–). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 149.2, 148.3, 142.5, 136.7, 130.4, 120.9, 117.8, 117.1, 110.5, 69.2, 47.9, 34.6. Anal. Calcd for $C_{24}H_{25}N_3O_3S_2$: C, 61.65; H, 5.39; N, 8.99; O, 10.26; S, 13.17. Found: C, 61.52; H, 5.28; N, 9.02; S, 13.89. EI-MS: Calcd for $C_{24}H_{25}N_3O_3S_2$: 467. Found: *m/z* 473 $[M+Li]^+$. FAB-MS: Calcd for $C_{24}H_{25}N_3O_3S_2$: 467. Found: *m/z* 467 $[M]^{+\cdot}$.

4.2.11. General procedure for the synthesis of macrocycles 5a, 5b, and 5c. Compound **4b** (437 mg, 1 mmol) was suspended in ethyl acetate/tetrahydrofuran 50:50 (25 mL) in a nitrogen flushed flask. Zinc dust (130 mg) and hydrazine hydrate 99% (5 mL) were added and the reaction mixture was stirred at room temperature under N_2

atmosphere for 8 h. On completion of reaction (TLC), the reaction mixture was decanted carefully, leaving behind zinc dust adhered to walls of the flask. Organic solvent was removed under reduced pressure and the reaction mixture kept still for 1 h. The solid precipitated was filtered through filter paper by decantation so that most of the solid remained in the flask. Solid so obtained was triturated in water, filtered, and dried under vacuum to obtain a pale whitish solid product.

4.2.11.1. Macrocycle 5b. (350 mg, 85%.) mp 159–160 °C. IR(KBr, cm^{-1}) 3373w, 3278m, 2846w, 1585s, 1500s, 1450m, 1319m, 1286m, 746s. ^1H NMR (300 MHz DMSO-d_6) δ ppm: 7.2 (d, $J = 7.4$ Hz, 2H, ArH), 6.9 (t, $J = 7.6$ Hz, 2H, ArH), 6.3 (d, $J = 8.1$ Hz, 2H, ArH), 6.4 (merged peaks, 3H, ArH), 5.8 (t, $J = 5.58$ Hz, 2H, ArH), 5.05 (s), 4.1 (d, $J = 5.59$ Hz, 4H, 2× N–CH_2–), 2.7 (t, $J = 7.7$ Hz, 4H, 2× S–CH_2–), 1.6 (quintet (merged), 2H, S–C–CH_2–). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 148.6, 148.5, 140.6, 135.0, 129.4, 115.7, 115.4, 111.0, 110.2, 109.8, 46.1, 33.3, 29.9. Anal. Calcd for $C_{23}H_{25}N_3S_2$: C, 67.77; H, 6.18; N, 10.31; S, 15.73. Found: C, 67.12; H, 6.08; N, 11.04; S, 15.76. FAB-MS: Calcd for $C_{23}H_{25}N_3S_2$: 407. Found: m/z 407 $[M]^{+\cdot}$.

4.2.11.2. Macrocycle 5a. mp 205–206 °C. IR (KBr, cm^{-1}) 3367w, 2923w, 1654w, 1586s, 1499s, 1450m, 1316m, 746m. ^1H NMR (300 MHz, DMSO-d_6) δ ppm: 7.3 (d, $J = 7.4$ Hz, 2H, ArH), 6.9 (t, $J = 7.6$ Hz, 2H, ArH), 6.2 (d, $J = 8.1$ Hz, 2H, ArH), 6.3 (merged peaks, 3H, ArH), 5.8 (t, $J = 5.58$ Hz, 2H, ArH), 5.05 (s, NH), 4.1 (d, $J = 5.59$ Hz, 4H, 2× N–CH_2–), 2.8 (s, $J = 7.7$ Hz, 4H, 2× S–CH_2–). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 148.6, 148.5, 140.6, 135.0, 129.4, 115.7, 115.4, 111.0, 110.2, 109.8, 46.1, 33.3. Anal. Calcd for $C_{22}H_{23}N_3S_2$: C, 67.14; H, 5.89; N, 10.68; S, 16.29. Found: C, 66.98; H, 5.68; N, 11.01; S, 16.31. FAB-MS: Calcd for $C_{22}H_{23}N_3S_2$: 393. Found: m/z 393 $[M]^{+\cdot}$.

4.2.11.3. Macrocycle 5c. IR (KBr, cm^{-1}) 3373w, 3278m, 2846w, 1585s, 1500s, 1450m, 1319m, 1286m, 746s. ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 7.4 (d, $J = 7.4$ Hz, 2H, ArH), 7.1 (t, $J = 7.6$ Hz, 2H, ArH), 6.2 (quintet (merged peaks), 6H, ArH), 6.8 (s, 1H, ArH), 5.4 (br s, 2H, exchanges with D_2O), 4.2 (s, 4H, 2× N–CH_2–), 3.4 (t, $J = 7.7$ Hz, 4H, 2× O–CH_2–), 2.8 (t, $J = 7.8$ Hz, 4H, 2×S–CH_2–). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 148.6, 148.5, 140.6, 135.0, 129.4, 115.7, 115.4, 111.0, 110.2, 109.8, 69.2, 47.9, 34.6. Anal. Calcd for $C_{24}H_{27}N_3OS_2$: C, 65.87; H, 6.22; N, 9.60; O, 3.66; S, 14.65. Found: C, 65.23; H, 6.03; N, 9.89; S, 14.89. FAB-MS: Calcd for $C_{24}H_{27}N_3OS_2$: 437. Found: m/z 437 $[M]^{+\cdot}$.

4.3. Radiocomplexation of BFCs (5a–c) with technetium

Macrocycle **5c** (100 μg) was dissolved in $CHCl_3$ (100 μL) in a shielded vial and stannous chloride (100 μg; 1 mg dissolved in N_2 purged 1 mL of 10% acetic acid) was added followed by addition of (<1 h) freshly eluted saline solution of sodium pertechnetate ($NaTcO_4$) (74 MBq, 100 μL). The pH of the reaction mixture was marked to 7 with 0.1 M $NaHCO_3$ solution and the solution vortexed to mix it properly. The vial was allowed to stand for 50 min at room temperature. The yield of complexation and radiochemical purity of the ^{99m}Tc–BFC complex were determined by ascending thin layer chromatography on ITLC-SG strips using 0.9% NaCl aqueous solution (saline) as developing solvent and simultaneously in pyridine/acetic acid/water (PAW) 3:5:1.5 and acetone. Each TLC was cut in 0.5 cm segments and counts of each segment were taken. By using this method the percentage of complex formed between ^{99m}Tc and BFC could be calculated.

4.4. In vitro serum stability assay

The fresh human serum was prepared by allowing blood collected from healthy volunteers to clot for 1 h at 37 °C in a humidified incubator maintained at 5% carbon dioxide, 95% air. Then the samples were centrifuged at 400g and the serum was filtered through 0.22 μm syringe filter into sterile plastic culture tubes. The above freshly prepared technetium radiocomplexes ^{99m}Tc–**5a**–**c** (10 MBq) were incubated in fresh human serum under physiological conditions, that is, at 37 °C at a concentration of 100 nmol/ml and then analyzed by ITLC-SG at different time intervals to check for any dissociation of complex. Percentage of free pertechnetate at a particular time point estimated using saline and pyridine/acetic acid/water (PAW) 3:5:1.5 as mobile phase represented percentage dissociation of the complex at that particular time point in serum.

4.5. Conjugation

The amino group of the bifunctional chelating agents (**5a**–**c**) was converted to isothiocyanato group by reacting with thiophosgene at pH 2. Compound (**5a**–**c**, 1.5 mmol) in 5 mL of 3 M HCl was added to 3 mL $CSCl_2$ (85% in CCl_4) at room temperature. The reaction was stirred vigorously for 4 h. The aqueous phase was washed with $CHCl_3$ (4 × 5 mL) under a fume hood to remove excess $CSCl_2$ and then purified by reverse phase HPLC. Conjugation of EGFr monoclonal antibody was performed by adding the 25 μL of 20 mM chelate solution to 300 μL of a solution containing 3 mg of antibody in 0.1 M sodium phosphate, pH 7. Saturated trisodium phosphate solution (40 μL) was added to make the pH 8.5. The reaction mixture was incubated at 37 °C for 60 min and then subjected to centrifuged column gel chromatography, which removed the unreacted chelate and changed the buffer to 0.1 M sodium acetate, pH 5.5. UV absorbance at 280 nm for the centrifuged column effluent was used to determine the antibody concentration, and ^{57}Co assay to obtain the bound chelate concentration. Briefly, 1 nmol of conjugated antibody, 2 nmol of ^{59}Co, and a tracer of ^{57}Co were mixed and incubated at pH 5.5 at room temperature for 30 min. An aliquot was applied to thin layer chromatography with ITLC-SG (Gelman Sciences, Ann Arbor, MI) with ammonium acetate/CH_3OH 1:1 (v/v) solution as eluent. Free cobalt migrates to $R_f = 1.0$ while labeled cobalt with immunoconjugate stays at $R_f = 0$. The ^{57}Co binding assay indicates that 2.1 ± 0.1 chelate molecules were conjugated per antibody molecule.

Acknowledgment

The senior research fellowship (SRF) provided by the Council of Scientific and Industrial Research (CSIR), New Delhi, to one of the authors (B.S.C.) is highly acknowledged.

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