Convenient Route for Synthesis of Bifunctional Chelating Agent: 1-(*p*-Aminobenzyl)ethylenediaminetetramethylphosphonic acid–Folate Conjugate (Am-Bz-EDTMP–Folate)

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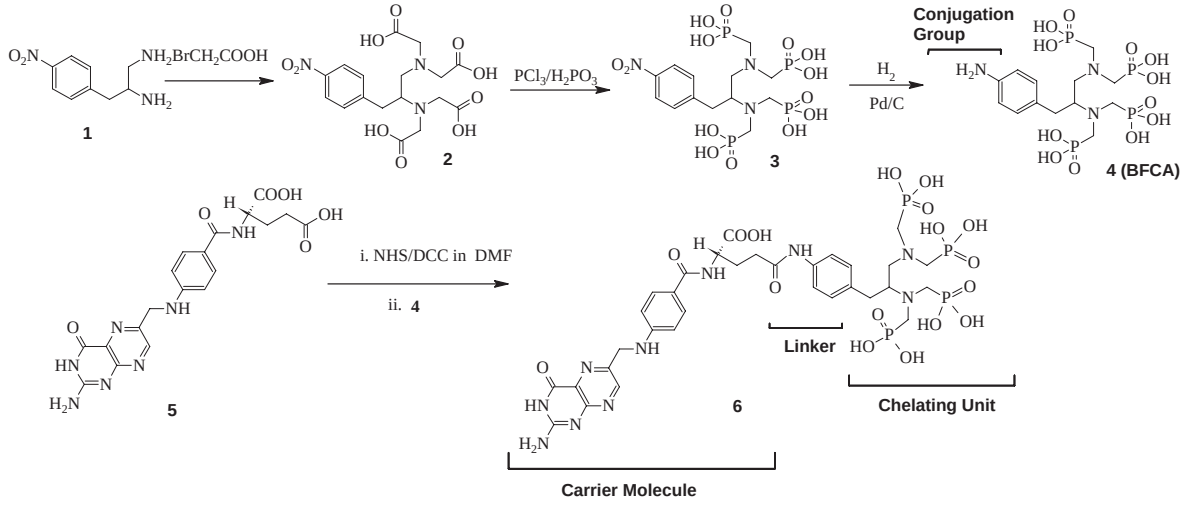
The present work describes a convenient and efficient approach for the synthesis of bifunctional chelating agent, 1-(*p*-aminobenzyl)ethylenediaminetetramethylphosphonic acid (Am-Bz-EDTMP). The key steps involved are functionalization of L-phenylalanine to give ethylenediaminetetraacetic acid derivative and its conversion to phosphonic acid followed by the reaction with NHS-folate to give 1-(*p*-aminobenzyl)-EDTMP–folate conjugate to be used for targeted radio imaging and therapy. Radiolabeling with ^{111}In and ^{153}Sm of Folate–Bz-EDTMP conjugate showed very high stability under physiological conditions.

The folate receptors are over expressed in a wide variety of human tumors. Conjugates of folate have been shown to be selectively taken-up by tumor cells via the folate receptor.[1] It has been shown that the natural receptor mediated endocytosis pathway for folic acid, can be exploited to selectively and non-destructively deliver folate-conjugated small molecules, macromolecules and drug carriers into cultured tumor cells.[2] The development of a target specific radiopharmaceutical often requires synthesis of bifunctional chelating agents. According to the bifunctional approach the radionuclide is conjugated to the carrier molecule by a bifunctional chelating agent (BFCA), which consists of three parts: a chelating unit for strong coordination to the metallic radionuclide, a conjugation group for covalent attachment to the carrier-molecule and a linker as pharmacokinetic modifier. The ideal BFCA must exhibit high labeling efficiencies to achieve good radiolabeling yields (>90%) and consequently high specific activity of the radio-conjugate. They should form stoichiometrically well-defined complexes that exhibit thermodynamic and kinetic stability with respect to dissociation and stabilization of the current oxidation state of the radionuclide.[3] In this context, our group is involved in synthesis of bifunctional chelating agents with prior knowledge of stability constant of classical chelating agent such as EDTMP, well known for its applications in nuclear medicine field.

Radiolabeling is mostly classified into three categories: direct labeling,[4] pre-labeling and post-labeling.[5] In the post-labeling approach, the BFCA is first attached to the carrier-molecule and then the radionuclide is coupled to the free chelating group of the BFCA. Most suitable for clinical application might be the post-labeling approach as it combines the ease and effectiveness of direct labeling with the well-defined chemistry of pre-labeling. The objective of this study is to prepare a synthetically useful chelate with higher coordinating ability and a reactive moiety for conjugation chemistry with folate starting from an optically active amino acid, L-phenylalanine, to form stable complexes with ^{111}In, ^{99m}Tc, and other metal ions of +3 or more oxidation number, with lesser structural forms and more stable under physiological conditions.

1-(*p*-Nitrobenzyl)ethylenediaminetetraacetic acid (*p*-NO_2-Bz-EDTA, **2**)[6] was synthesized from 1-(*p*-nitrobenzyl)ethylenediamine **1**[7] by standard amine alkylation technique employing excess bromoacetic acid/NaOH. Purification of **2** was accomplished by HPLC by using 0.05% TFA and MeOH on C_{18}-RP column. Finally, in order to generate *p*-NO_2-Bz-EDTMP, **3**[8] carboxylic functions were converted into phosphonic functions ac-



Scheme 1.

cording to the method of Krüger and Bauer,[9,10] by using H_3PO_3/PCl_3 in toluene. Reduction of NO_2 group with H_2/Pd–C in basic media unmasked the amino group of *p*-NH_2-Bz-EDTMP with a purity of above 97%. 1-(*p*-Aminobenzyl)ethylenediaminetetramethylenephosphonic acid (**4**, BFCA) thus obtained was reacted with NHS-ester of folic acid **5**[11] in anhydrous dimethylformamide to give *p*-NH_2-Bz-EDTMP–folate conjugate **6** (Scheme 1).[11] The compound was purified by HPLC and characterized by NMR and mass spectrometry. Complexation of folate–EDTMP **6** (aq solution, 0.01 mM, 20 μL) was carried out with $^{111}InCl_3$ (carrier free, 20 μL in sodium acetate buffer) and the pH was marked to **7** with sodium acetate buffer. Radiochemical purity was checked after 3 h on a TLC system (1:1 MeOH–10% NH_4OAc) and was above 97%. **6** was radiolabled with ^{153}Sm under the same conditions as ^{111}In except the incubation time samarium was 5 h at room temperature.

The rates of decomplexation of the metal chelate (complexes of ^{111}In and ^{153}Sm) were studied in serum under physiological conditions over a 7-day period. 5.7 μL of the ^{111}In-Folate–EDTMP complex solution was mixed with 2 mL of healthy human serum to study the transchelation of indium metal ion. The rate of transchelation was determined by polyacrylamide gel electrophoresis under physiological conditions.[12] Over 7-day period no measurable loss (less than 0.5% per day) of metal ion from the EDTMP–folate conjugate was observed.

In conclusion, we described a simple, efficient synthesis pathway to a new bifunctional chelating agent having tetraphosphonic ligands. The BFCA was successfully linked to folic acid and a 1:1 conjugation was achieved. The key steps in synthesis were the dramatizations of simple amino acid to synthesize first bifunctional EDTA followed by introduction of phosphonic functions and finally conjugation with NHS-ester of folic acid. Complexation studies concerning indium-111 and samarium-153 showed that the chelating agent thus developed has excellent stability in human serum and deserves further investigation as a potential therapeutic bone agent for targeted therapy.

References and Notes

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6 Preparation of compound **2** (*p*-NO_2-Bz-EDTA): compound **1** (1.0 g, 3.73 mmol) was dissolved in H_2O (5 mL) and allowed to stir at 70 °C. An aqueous solution of bromoacetic acid (2.10 g, 15.1 mmol) was added in equal portions over 3 h, while the reaction mixture was maintained at pH 10.2 using pH stat (10 M sodium hydroxide solution). The reaction mixture was further allowed to stir for 5 h at 60 °C thereafter it was neutralized by 3 M hydrochloric acid and solvent was removed under reduced pressure to dryness. Obtained yellowish product **2** was purified by preparative HPLC (C_{18}, 5 μ, 20 × 250 mm^2). The major peak at retention time 13.94 min. was collected using gradient solvent: NH_4OAc (pH 6.0, 0.1 M) solution and methanol as solvent. ^1H NMR (250 MHz, D_2O), δ 8.23 (d, 2H, $J = 8.0$ Hz), 7.64 (d, 2H, $J = 8.0$ Hz), 4.12 (s, 8H), 3.92 (d, 2H), 3.88 (m, 1H), 3.20 (t, 2H). FAB-MS: found: 196 $[M + H^+]$ (calcd for $C_9H_{13}N_3O_2$: *m/e* 195) Elemental Anal. Calcd for $C_9H_{13}N_3O_2$: C, 55.37; H, 6.71; N, 21.52; O, 16.39%. Found: C, 55.40; H, 6.73; N, 21.60; O, 16.50%.

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8 Preparation of compound **4** (*p*-NH_2-Bz-EDTMP): To a solution of *p*-NO_2-Bz-EDTA (**2**, 0.5 g; 1.17 mmol) dissolved in 10 mL of toluene phosphorous acid (0.42 g; 5.15 mmol) was added with stirring. The reaction mixture was refluxed while phosphorous trichloride (0.94 g; 6.14 mmol) was added drop wise to the refluxing mixture at 80 °C. After 3 h, toluene was removed following addition of deionised water. The filtrate was concentrated under vacuum. The concentrated product was precipitated by addition of methanol/ethanol to give *p*-NO_2-Bz-EDTMP, **3**. ^1H NMR (250 MHz, D_2O) δ 7.55 (d, 2H, ArH), 7.12 (d, 2H, ArH), 3.75 (d, 1H), 3.53 (dd, 1H, $J = 15.0$, 7.5 Hz), 3.46 (dd, 1H, $J = 15.0$, 7.5 Hz), 3.39 (dd, 1H, $J = 14.0$, 7.5 Hz), 3.26 (dd, 1H, $J = 14$, 7.5 Hz), 3.0842–3.0153 (m, 8H); FAB-MS: found: 572 $[M + H^+]$ (calcd *m/e* 571) Elemental Anal. Calcd for $C_{13}H_{25}N_3O_{14}P_4$: C, 27.33; H, 4.41; N, 7.36%. Found: C, 27.30; H, 4.44; N, 7.38%. *p*-NO_2-Bz-EDTMP was converted to *p*-NH_2-Bz-EDTMP, **4**, by reduction of NO_2 group with H_2 using Pd/C in aqueous sodium hydroxide (pH 11.0) to give *p*-NH_2-Bz-EDTMP with a purity of above 97%. The product was characterized by mass spectrometry. FAB-MS: found: 542 $[M + H^+]$ (calcd: *m/e* 541) Elemental Anal. Calcd for $C_{13}H_{27}N_3O_{12}P_4$: C, 28.85; H, 5.03; N, 7.76%. Found: C, 28.81; H, 5.06; N, 7.72%.

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11 Preparation of compound **6** (*p*-NH_2-Bz-EDTMP–Folate) **6**: 10 g (22.65 mmol) of folic acid **5**, were dissolved in 150 mL of dimethylformamide (DMF). A slight excess of NHS (2.67 g, 24.94 mmol) and dicyclohexylcarbodiimide (DCC, 4.67 g, 22.65 mmol) were then added. The reaction was allowed to stir at room temperature for 4 h. The dicyclohexylurea (DHU) was removed by filtration. The DMF solution of the NHS-folate was stored at −20 °C until use. Conjugation of folate with compound **4**: To a solution of folate-NHS ester (obtained above) in anhydrous DMF (78.74 mL containing 2 g NHS ester; 3.7 mmol) compound **4** (2 g, 3.69 mmol) was added slowly within 15 min and reaction was further stirred at room temperature for 2 h. The progress of the reaction was followed by analytical HPLC. After full consumption of NHS-folate (2 h) pH was set to 8.5 with 2 M Na_2CO_3 and the resultant solution was filtered to remove and the clear orange solution was concentrated under vacuum and purified by preparative HPLC on C-18 column (5 μ, 20 × 250 mm^2) with a gradient (eluent A, 0.1 M ammonium acetate buffer at pH 6.6, eluent B acetonitrile; gradient 0 min at 4% B, 10 min at 12% B and 15 min at 15% B at a flow rate of 10 mL/min; t_R 6.3 min) to remove bis-conjugated side product and to obtain EDTMP–folate conjugate in a yield of 49%. Analytical HPLC on C-18 reversed phase column (5 μ, 4.6 × 250 mm^2) revealed a single peak with a retention time of 18.5 min (eluent, 10 mM ammonium acetate buffer (pH 6.0) at 75% and acetonitrile 25%; flow rate of 0.7 mL/min) ^1H NMR (250 MHz, D_2O): δ 8.46 (s, 1H), 7.59 (d, 2H, ArH), 7.41 (d, $J = 8.3$ Hz, 2H, ArH), 7.10 (d, 2H, ArH), 6.34 (d, $J = 8.3$ Hz, 2H, ArH), 4.24 (dd, $J = 4.4$, 8.4 Hz, 1H), 4.15 (s, 2H), 3.75 (d, 1H), 3.53 (dd, 1H, $J = 15.0$, 7.5 Hz), 3.46 (dd, 1H, $J = 15.0$, 7.5 Hz), 3.39 (dd, 1H, $J = 14.0$, 7.5 Hz), 3.26 (dd, 1H, $J = 14$, 7.5 Hz), 3.0842–3.0153 (m, 8H), 2.28–1.84 (m, 4H); ^{31}P NMR: (D_2O): 17.0448, 15.7118; FAB-MS: found: 965 $[M + H^+]$ (calcd for $C_{32}H_{44}N_{10}O_{17}P_4$: *m/e* 964). Elemental Anal. Calcd for $C_{32}H_{44}N_{10}O_{17}P_4$: C, 39.84; H, 4.60; N, 14.52%. Found: C, 39.81; H, 4.63; N, 14.50%.

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