Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 13 (2005) 4315–4322
Bioorganic & Medicinal Chemistry

An estradiol-conjugate for radiolabelling with ^{177}Lu: an attempt to prepare a radiotherapeutic agent

Sharmila Banerjee,[a,*] Tapas Das,[a] Sudipta Chakraborty,[a] Grace Samuel,[a] Aruna Korde,[a] Meera Venkatesh[a] and M. R. A. Pillai[b]

[a]*Radiopharmaceuticals Division, Bhabha Atomic Research Centre, Mumbai 400085, India*
[b]*Industrial Application and Chemistry Section, International Atomic Energy Agency, Wagramerstrasse, A-1400 Vienna, Austria*

Received 8 December 2004; revised 24 March 2005; accepted 5 April 2005
Available online 29 April 2005

Abstract—^{177}Lu is presently being considered as one of the most promising radionuclide for targeted therapy owing to its suitable decay characteristics. ^{177}Lu in high radionuclidic purity (99.99%) and moderate specific activity (100–110 TBq/g) was produced using enriched (60.6% ^{176}Lu) Lu_2O_3 target. The macrocycle 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) is known to form stable complexes with lanthanides. Herein, we describe a novel attempt to introduce ^{177}Lu in the estradiol moiety through a steroidal–BFCA (Bifunctional Chelating Agent) conjugate. The preparation of a steroid conjugate via coupling of 6α-amino-17β-estradiol with a C-functionalized DOTA derivative viz. *p*-NCS-benzyl-DOTA as a BFCA and thereafter the radiolabelling of the conjugate with ^{177}Lu is reported. Biological activity of the resultant estradiol–DOTA conjugate after radiolabelling was studied by carrying out preliminary in vitro cell uptake studies with MCF-7, human breast carcinoma cell line expressing estrogen receptors as well as binding studies with anti-estradiol antibodies.

1. Introduction

The use of radiolabelled derivatives of potent estrogens, possessing receptor affinity towards use as diagnostic agents, has been extensively and amply demonstrated.[1–3] In this direction, a plethora of studies have been reported, which describe the preparation of radiolabelled estradiol derivatives using ^{77}Br, ^{123}I and ^{125}I.[4–6] However, investigations directed towards development of steroidal derivatives suitable for use as radiotherapeutic agents for breast cancers have possibly received inadequate attention. This could be attributed to the significant chemical challenge posed while incorporating radioisotopes to steroidal substrates.[7–9] Additionally, greater than 30% of breast cancers are known to be estrogen receptor negative in which case the present modality has limited applicability. In connection with our present working areas of interest which includes development of radiotherapeutic agents using ^{177}Lu as the radioisotope, we have reported peptides, nitroimidazoles, etc. as carrier molecules for conjugation with suitable bifunctional chelating agents (BFCA) towards targeted delivery of radiation to specific diseased sites.[10,11] However, for development of potential radiotherapeutic agents for targeting specific tumours overexpressing steroidal receptors, steroidal substrates could be envisaged as vectors for covalent attachment with chelating moieties for use in the post-labelling method for complexation with ^{177}Lu. In this direction, the work reported herein exemplifies yet another strategy for introduction of the radioisotope in the steroidal moiety. The three challenging facets of the present work include derivatization of the steroidal substrate of choice for chemical coupling with a suitable BFCA, identification of the chelating moiety and optimization of the protocol for introducing ^{177}Lu in the steroid-BFCA conjugate.

The essential criterion guiding the choice of BFCA for sequestering the radioisotope and subsequent conjugation with biomolecules for use in radioimmunotherapy (RIT) is the in vivo stability of the biomolecule–BFCA conjugate.[12] On the other hand, radionuclides that provide ideal characteristics for utilization in RIT

Keywords: ^{177}Lu; *p*-NCS-benzyl-DOTA; BFCA; 17β-estradiol; Estradiol–BFCA conjugate.
* Corresponding author. Tel.: +91 22 2559 5371; fax: +91 22 2550 5345; e-mail: sharmila@apsara.barc.ernet.in

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.04.009

include β^--emitters such as ^{90}Y ($t_{1/2}$ = 2.7 d, E_{max} = 2.21 MeV), ^{153}Sm ($t_{1/2}$ = 1.9 d, E_{max} = 0.81 MeV), ^{188}Re ($t_{1/2}$ = 17 h, E_{max} = 2.12 MeV) and most recently ^{177}Lu ($t_{1/2}$ = 6.71 d, E_{max} = 0.497 MeV).[13] However, the use of high-energy β^--emitters are usually accompanied by undesirable side effects. These disadvantages have necessitated the development of low energy and hence better-tolerated β^--emitters such as ^{177}Lu. ^{177}Lu also possesses imageable low energy, low abundance γ-emissions (113 keV, 6.4% and 208 keV, 11%) which provide additional advantages towards its use as an ideal candidate for RIT.[14,15] ^{177}Lu is a potential radionuclide for targeted radiotherapy owing to its availability in relatively high specific activity and excellent radionuclidic purity from moderate flux reactors using commercially available enriched Lu (60.6% in ^{176}Lu) target. The long half-life of the radioisotope also provides logistic advantages. Further, it has been documented that polyazamacrocycles are ideal chelating species for complexation with lanthanides.[12] The advantages provided by macrocycles such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) in forming thermodynamically stable complexes with ^{177}Lu coupled with its high kinetic inertness which constitutes a desirable feature for in vivo usage provide impetus to the choice of suitable DOTA derivatives as a BFCA in the present study.[16]

In our earlier work[17] on 17β-estradiol (E2) immunoassay, we have used estradiol-6-carboxymethyl oxime (CMO)-bovine serum albumin (BSA) as the immunogen to raise antibodies and E2-3-CMO-histamine-^{125}I as the radiotracer. It was observed that the use of a similar bridge in both the tracer as well as in the immunogen greatly influenced the sensitivity of the binding assay. Antibodies raised using a common bridge recognize the chemical bridge in addition to the steroid molecule and therefore decreases the sensitivity of the assay. To circumvent this problem of poor sensitivity, there was a need to prepare a derivative of 17β-estradiol with a different bridge at the C_6 position wherein the radioisotope could be introduced. It has also been demonstrated that the immunoreactivity of the steroidal substrate is retained after functionalization in the C_6 position.[18] In this connection, the synthesis of 6α-amino derivative of 17β-estradiol has been achieved. With 17β-estradiol as the available precursor, derivatization of the C_6 position appeared to be more feasible as compared to the C_7 and C_{11} positions in an attempt to prepare a potential agent for radiotherapy. Besides being distant from the C_3 and C_{17} positions, the C_6 position in the estradiol molecule have the additional advantage of being the benzylic position with respect to the aromatic ring and therefore becomes a suitable site for modification and attachment of a BFCA. Functionalization at an alternate position viz. C_7 has also been documented to proceed via the C_6 keto derivative in a rather elaborate synthetic protocol.[19] In the present strategy, the conversion of 17β-estradiol to its 6-keto derivative has been achieved involving the benzylic oxidation of the diacetate. The attachment of a DOTA derivative as the BFCA in the strategy envisaged herein, requires the relatively less sterically hindered viz. the α disposition of a suitable functionality at the C_6 position.[19,20] It was therefore pertinent to investigate the feasibility of covalently coupling the 6α-amino-estradiol with a suitable DOTA derivative. Since, *para*-thiocyanato-benzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10- tetraacetic acid (*p*-NCS-benzyl-DOTA), the activated synthon from the precursor *para*-amino-benzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (*p*-NH_2-benzyl-DOTA) was readily available and the covalent coupling with the amino substituent has been observed to be feasible, it was logical to effect the coupling of the aforementioned steroidal amino derivative with that of *p*-NCS-benzyl-DOTA. Subsequent optimization of the parameters for radiolabelling of the conjugate with ^{177}Lu constituted the next challenge. The ^{177}Lu labelling of *p*-NCS-benzyl-DOTA has been initially standardized in our laboratory. Herein, we report the synthesis of an estradiol–DOTA conjugate, its labelling with ^{177}Lu and preliminary studies towards evaluation of its biological activity.

2. Results and discussion

2.1. Production of ^{177}Lu

^{177}Lu was produced with moderate specific activity (100–110 TBq/g, 2.7×10^3–3.0×10^3 Ci/g) and excellent radionuclidic purity (99.985%) by irradiating enriched (60.6% in ^{176}Lu) Lu_2O_3 target for 7 d at a thermal neutron flux of 3×10^{13} n/cm^2/s. The radionuclidic purity of the irradiated target after radiochemical processing was determined by recording the gamma ray spectra. The major gamma peaks observed were at 72, 113, 208, 250 and 321 keV, all of which correspond to the photopeaks of ^{177}Lu.[21] This was further confirmed from the decay of counts per second values at those peaks according to the half-life of ^{177}Lu.

It is worthwhile to note that there is a possibility of formation of ^{177m}Lu ($t_{1/2}$ = 160.5 d) on thermal neutron bombardment of Lu_2O_3 target.[22,23] However, gamma ray spectrum of the irradiated Lu target after chemical processing did not show any significant peak corresponding to the photopeaks of ^{177m}Lu (128, 153, 228, 378, 414, and 418 keV), which is attributable to its long half-life and comparatively low cross-section (σ = 7 barns).[21] When the gamma ray spectrum of the irradiated sample was recorded after complete decay of ^{177}Lu activity (55–65 d post-EOB), photopeaks attributable to trace level of ^{177m}Lu could be observed. The average level of radionuclidic impurity burden in ^{177}Lu due to ^{177m}Lu was found to be 5.5 kBq of ^{177m}Lu/37 MBq of ^{177}Lu (150 nCi/1 mCi) at EOB, which corresponds to 0.015% of the total activity produced.

2.2. Characterization of ^{177}Lu-*p*-NCS-benzyl-DOTA complex

The characterization as well as the estimation of the extent of complexation of ^{177}Lu-*p*-NCS-benzyl-DOTA complex were carried out by using paper chromatography technique. In paper chromatography using 10%

ammonium acetate/methanol (1:1, v/v) as the eluting solvent, it was observed that the complex moved towards the solvent front ($R_f = 0.7–0.8$) while the uncomplexed ^{177}Lu under identical conditions showed no movement from point of spotting ($R_f = 0$).

2.3. Optimization of complexation yield of ^{177}Lu-*p*-NCS-benzyl-DOTA complex

The reaction conditions for complexation of *p*-NCS-benzyl-DOTA with ^{177}Lu were optimized prior to the radiolabelling of the conjugate. Various parameters such as ligand concentration, pH of the reaction, incubation time and temperature were varied in order to maximize the complexation yield of ^{177}Lu-*p*-NCS-benzyl-DOTA. The ligand concentration was varied from 25 to 250 μg/mL and it was observed that while only ~27.0% complexation yield was achieved with 25 μg/mL ligand concentration, it was increased to 98.9% when 100 μg/mL ligand concentration was used. Further increase of ligand concentration did not show any appreciable increase in the extent of complexation and hence, 100 μg/mL ligand concentration, which was identical with 40 μg ligand in a reaction volume of 400 μL was taken as an optimum ligand concentration. The complexation was studied over a wide pH range of 2–10 in order to determine the optimum pH required for complexation. It was observed that maximum complexation was achieved at pH ~ 5 and hence, pH 5 was chosen as the optimum pH for complexation. The reaction mixture was incubated at room temperature for different time periods ranging from 10 min to 1 h and >98% complexation was achieved within 10 min. Therefore, 10 min reaction time was chosen as the optimum time for complexation. As very good complexation yield was achievable within 10 min at room temperature, the effect of higher temperature on the reaction kinetics was not studied.

2.4. Characterization of ^{177}Lu labelled *p*-NCS-benzyl-DOTA-estradiol conjugate

The characterization of ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex was carried out by a combination of paper chromatography and paper electrophoresis techniques. In paper chromatography using normal saline (0.9% w/v aqueous NaCl), both uncomplexed ^{177}Lu and ^{177}Lu-*p*-NCS-benzyl-DOTA complex remained at point of spotting ($R_f = 0–0.1$), while ^{177}Lu complex of the estradiol–BFCA conjugate exhibited a migration towards the solvent front ($R_f = 0.8–1.0$). Hence, from this chromatographic study, it was possible to distinguish and characterize the ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex from the other two possibilities namely, ^{177}Lu-*p*-NCS-benzyl-DOTA complex and uncomplexed ^{177}Lu.

On the other hand, in paper electrophoresis carried out using 0.025 M phosphate buffer of pH 7.5, at a potential gradient of ~10 V/cm for 75 min, the ^{177}Lu complex of the *p*-NCS-benzyl-DOTA-estradiol conjugate showed a migration of 3–4 cm towards anode. On the other hand, both uncomplexed ^{177}Lu and ^{177}Lu-*p*-NCS-benzyl-DOTA complex did not show any appreciable migration under identical conditions.

2.5. Optimization of complexation yield of ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex

Various reaction parameters were varied in order to determine the optimum conditions to obtain maximum complexation yield of ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex. Variation of the ligand concentration between 100 and 500 μg/mL showed that reasonably good complexation yield of 80–85% was achieved when 300 μg/mL of the conjugate was used. Further increase of ligand concentration did not have any appreciable effect on the extent of complexation and therefore, 300 μg/mL conjugate was used for all subsequent optimization studies. It is well reported that ^{177}Lu complexation with biomolecules are mostly carried out below pH 7[24,25] and as the present BFCA also showed maximum labelling yield at pH ~ 5, no pH variation for the labelling of conjugate was attempted and it was carried out by maintaining the pH of the reaction mixture ~5. The complexation studies carried out at room temperature showed that the reaction kinetics is too slow to achieve good complexation yield within a reasonable time and hence, the incubation at higher temperature was attempted. It was observed that a maximum of 80–85% complexation was achieved when the conjugate was incubated with ^{177}Lu at 37 °C for a period of 2 h.

2.6. Stability of ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex

The stability of the ^{177}Lu labelled *p*-NCS-benzyl-DOTA-estradiol conjugate was studied by using the similar quality control techniques employed for the determination of the complexation yield. It was observed that the complex exhibited good stability when stored at room temperature as it maintained its radiochemical purity to the extent of 77% for a period of 7 d after preparation.

2.7. Purification of ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex

Purification of the ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex was achieved by using Sep-pak® column as well as by HPLC technique.

2.7.1. Sep-pak® purification. In Sep-pak® column purification, it was observed that >95% of ^{177}Lu-*p*-NCS-benzyl-DOTA complex was eluted with 0.1 M ammonium acetate buffer, pH ~ 5.5. In the actual purification, ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex was retained in the column on elution with aqueous ammonium acetate buffer, while radiolabelled *p*-NCS-benzyl-DOTA was eluted out. The desired complex was subsequently eluted in radiochemically pure form from the column using ethanol. The presence of 15–20% of the loaded activity in the ammonium acetate buffer fraction and 80–85% of the activity in the ethanol fraction gave an indirect support in favour of the extent

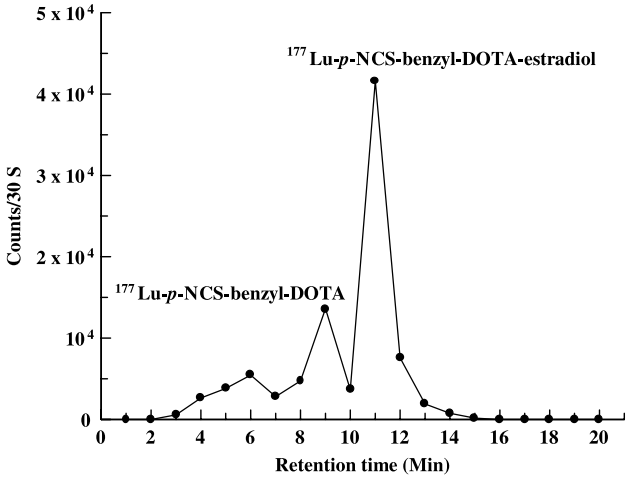

Figure 1. HPLC pattern of ^{177}Lu-*p*-NCS benzyl-DOTA-estradiol complex using water (A) and methanol (B) as mobile phase (0–4 min 90% A, 4–10 min 90% A to 10% A, 10–15 min 10% A to 90% A, 15–20 min 90% A).

of complexation determined by chromatographic methods.

2.7.2. HPLC purification. The HPLC pattern of ^{177}Lu labelled *p*-NCS-benzyl-DOTA-estradiol conjugate is shown in Figure 1. It is evident from the figure that while ^{177}Lu-*p*-NCS-benzyl-DOTA complex exhibit a retention time of 9 min, ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex eluted from the column at 11 min. Apart from purification of ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex, this method also helped in precise determination of the extent of complexation achieved.

2.8. In vitro binding studies

2.8.1. Antibody binding studies. The anti-estradiol antibody used for the binding studies was characterized in our laboratory and has shown 10% cross-reactivity with estrone and <1% cross-reactivity with all the other structurally related molecules. The crude reaction mixture showed a binding of 8–9% with a 1:5 diluted antibody. While on Sep-pak® purification, the extent of binding increased to 14%, a further improvement to a maximum of 38% was achieved on HPLC purification with 300 ng of the ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex (125 Bq/ng). This compares well with our earlier studies, wherein 60 pg of the radioiodinated estradiol (65 kBq/ng) exhibited a binding of 40% with 1:75,000 diluted antibody.[17] The binding observed indicates that there is no major change in the molecule with respect to antibody recognition after incorporation of the BFCA. The specific binding is further confirmed by the low nonspecific binding of <1% observed when ^{177}Lu-*p*-NCS-benzyl-DOTA complex was reacted with the antibody.

2.8.2. Cell binding studies. A binding of (13.2 ± 0.8)% ($n = 3$) was observed with 5×10^4 MCF-7 cells for 1 μg of the ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex. On Sep-pak® purification, the cell uptake improved to (17.1 ± 1.6)% ($n = 3$). No further increase in cell uptake was observed on HPLC purification. It was observed that the tracer uptake in the cells decreased to (8.3 ± 2.0)% ($n = 3$) on the addition of 100 μg of cold estradiol. Similar results were observed when these experiments were carried out in 24-well plates where cells were plated one day prior to the experiments. The decrease in cell uptake on the addition of estradiol indicated the specificity of the radiolabelled conjugate for the MCF-7 cell lines. Blank experiments have been carried out with ^{177}Lu-labelled BFCA under similar experimental conditions. No retention of the activity in the cell pellet was observed, ruling out the possibility of carrier-mediated uptake.

3. Conclusion

With a view to preparing a ^{177}Lu labelled estradiol derivative, 17β-estradiol has been modified and coupled with a suitable BFCA, *p*-NCS-benzyl-DOTA. To the best of our knowledge, this steroidal conjugate derivatized at C_6 is a novel agent of its kind. The radiolabelling of the estradiol–BFCA conjugate with ^{177}Lu has been standardized and the preliminary studies to explore the biological behaviour of the complex are carried out with respect to antibody binding and cell uptake studies. This conjugate could serve as an entry point to the preparation of a wide variety of therapeutic agents making use of radioactive lanthanides and pseudo-lanthanides viz. ^{153}Sm, ^{166}Ho, ^{90}Y possessing a wide spectrum of β^--energies as would be required for varied tumour types.

4. Experimental

4.1. Materials and methods

17β-Estradiol and sodium cyanoborohydride were purchased from Aldrich Chemical Company, USA. *p*-NCS-benzyl-DOTA, 4HCl was obtained from M/s Macrocyclics, USA. Isotopically enriched Lu_2O_3 (60.6% ^{176}Lu) was obtained as a part of a Co-ordinated Research Project of the International Atomic Energy Agency (IAEA), Vienna, Austria. Flexible and preparative silica gel plates IB-F were obtained from Bakerflex Chemical Company, Germany. Whatman 3 chromatography paper used for paper chromatography and paper electrophoresis was purchased from Whatman International Limited, UK. Sep-pak® (Vac C-18, 1 cc) column cartridges were obtained from Waters Corporation, USA.

Gamma ray spectra of ^{177}Lu were recorded using HPGe detector coupled to a 4K multichannel analyzer (MCA) system. A ^{152}Eu reference source, obtained from Amersham Inc., USA, was used for both energy and efficiency calibration of the detector. High performance liquid chromatography (HPLC) system used was obtained from JASCO, Japan and coupled with a radioactive detector and data acquisition software developed in-house. Fourier Transform Infra Red (FT-IR) spectra were recorded using JASCO FT/IR-420 spectrophoto-

meter. Proton NMR spectra were recorded on a 300 MHz Varian VXR 300S spectrophotometer.

MCF-7 cell line was procured from National Centre for Cell Sciences, Pune, India. Antibodies against estradiol derivative were raised in rabbits in-house in our laboratory.

4.2. Production of ^{177}Lu

For production of ^{177}Lu, isotopically enriched (60.6% in ^{176}Lu) Lu_2O_3 target was irradiated at Dhruva reactor at Bhabha Atomic Research Centre, Mumbai, India for 7 days at a thermal neutron flux of 3×10^{13} n/cm^2/s. The irradiated target was dissolved in 1 M HCl by gentle warming. The solution was evaporated to near-dryness and reconstituted in double distilled water. Assay of the radioactivity was carried out by measuring the ionization current of the solution in a pre-calibrated well-type ion-chamber (0.423 pA/mCi). Appropriately diluted sample solutions were counted for 1 h and the radionuclidic purity was estimated by recording gamma ray spectrum of the irradiated target using HPGe detector coupled with a 4K multichannel analyser (MCA) system.

4.3. Synthesis and characterization of 6α-amino-17β-estradiol and its coupling with *p*-NCS-benzyl-DOTA

The synthesis of 6α-amino-17β-estradiol (IV) was achieved by a four-step process using 17β-estradiol as the starting material and it was subsequently coupled with *p*-NCS-benzyl-DOTA to obtain the *p*-NCS-benzyl-DOTA-estradiol conjugate (V). The scheme for the synthesis of *p*-NCS-benzyl-DOTA-6α-amino-17β-estradiol conjugate (V) is shown in Figure 2. The characterization of all the intermediates (I–IV) as well as the final product (V) were achieved by spectroscopic techniques, such as FT-IR and ^{1}H NMR spectroscopy.

4.3.1. 17β-estradiol diacetate (I). 17β-Estradiol (7.5 g, 0.028 M) was dissolved in a mixture of 105 mL of pyridine and 27 mL (0.265 M) of acetic anhydride. The mixture was refluxed for 3 h, cooled to room temperature and poured slowly in ice cold water with vigorous stirring. The precipitate was filtered, washed with dilute HCl (pH 4) and dried. The crude product (9.56 g, 97.36%) thus obtained was purified by two successive recrystallizations from ethanol whereby 7.53 g (76%) of the purified product (I) was obtained.

Melting point: 126 °C. FT-IR (KBr, ν cm^{-1}): 1767 (C_{17}-$OCOCH_3$), 1729 (Ar-$OCOCH_3$). ^{1}H NMR ($CDCl_3$, δ ppm): 0.82 (3H s, C_{18}-H_3), 1.24–1.58 (10H m, C_7, C_{11}, C_{12}, C_{15}, C_{16}-H_2), 1.71–1.78 (1H m, C_{14}-H), 1.84–1.90 (2H m, C_8-H and C_9-H), 2.06 (3H s, C_{17}-$OCOCH_3$), 2.28 (3H s, Ar-$OCOCH_3$), 2.84–2.86 (2H t, C_6-H_AH_B, J = 4.2 Hz), 4.69 (1H q, C_{17}-H, J = 7.7, 9.0 Hz), 6.78–6.85 (2H m, Ar-H), 7.26–7.29 (1H m, Ar-H).

4.3.2. 6-Keto-17β-estradiol diacetate (II). 17β-Estradiol diacetate (I) (2.5 g, 0.007 M) was dissolved in 9 mL of glacial acetic acid and poured into a solution of chromium trioxide (2.1 g, 0.02 M) in 50 mL of 88% aqueous

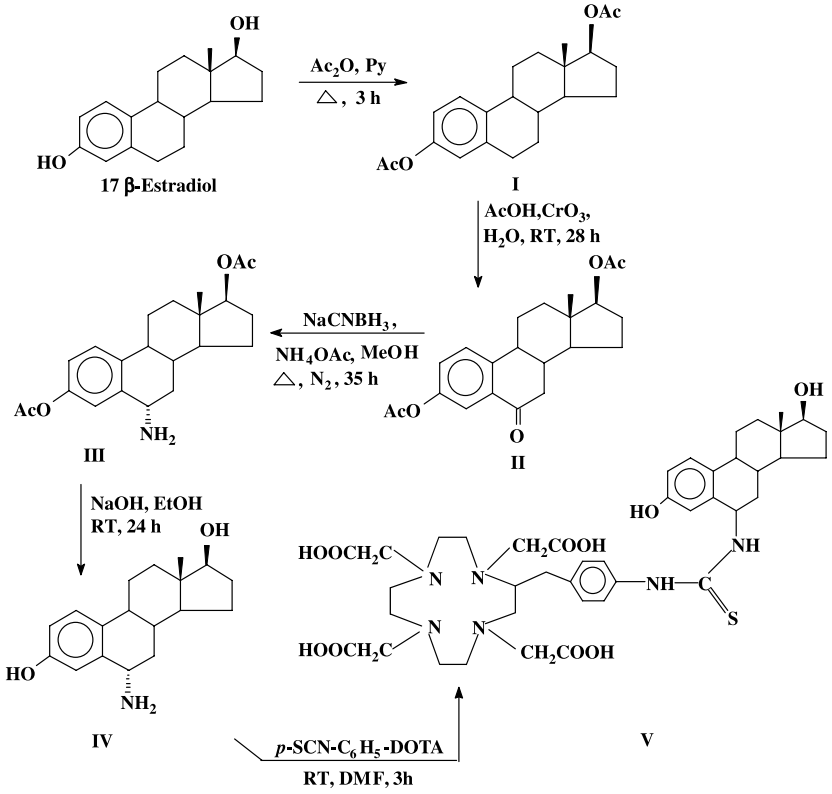

Figure 2. Synthesis of *p*-NCS-benzyl-DOTA-6α-amino-17β-estradiol conjugate.

acetic acid. The reaction mixture was stirred at room temperature for 28 h. Subsequently, it was diluted with 150 mL of distilled water and extracted with diethyl ether (6 × 75 mL). The organic layers were pooled together and thoroughly washed with saturated aqueous sodium bicarbonate solution until the organic layer appeared light pink in colour. The organic layer was further washed with 1 N aqueous sodium carbonate solution followed by distilled water and dried over anhydrous sodium sulfate. Evaporation under vacuum afforded 2.03 g (78.14%) of crude 6-keto-17β-estradiol diacetate. The crude product was purified by silica-gel column chromatography using ethyl acetate–petroleum ether (1:9, v/v) as eluting solvent to give a white crystalline solid (II).

Melting point: 173 °C. FT-IR (Nujol, ν cm^{-1}): 1769 (C_{17}-OCOCH$_3$), 1730 (Ar-OCOCH$_3$) 1677 (C_6-CO). ^{1}H NMR ($CDCl_3$, δ ppm): 0.83 (3H s, C_{18}-H_3), 1.20–1.92 (11H m, C_8, C_9, C_{14}-H and C_{11}, C_{12}, C_{15}, C_{16}-H_2), 2.07 (3H s, C_{17}-OCOCH_3), 2.31 (3H s, Ar-OCOCH_3), 2.53–2.55 (1H m, C_7-H_AH_B), 2.73–2.79 (1H dd, C_7-H_AH_B, J = 16.8, 3.5 Hz), 4.72 (1H q, C_{17}-H, J = 9.2, 7.8 Hz), 7.24–7.55 (3H m, Ar-H).

4.3.3. 6α-Amino-17β-estradiol diacetate (III). The conversion of 6-keto-17β-estradiol diacetate (II) to 6α-amino-17β-estradiol diacetate was achieved by reductive amination using sodium cyanoborohydride as the reducing agent.[18] The reaction was carried out by refluxing a mixture of 6-keto-17β-estradiol diacetate (0.75 g, 0.002 M) and ammonium acetate (2.7 g, 0.035 M) in 60 mL of dry methanol under nitrogen atmosphere. Sodium cyanoborohydride (0.66 g, 0.01 M) was added to the refluxing mixture in five equal portions at an interval of 30 min between two successive additions. The refluxing was continued for 35 h. The solvent was evaporated to obtain the crude product. The crude product was dissolved in 60 mL of 1 N HCl and extracted with ethyl acetate to remove the unreacted 6-keto-17β-estradiol diacetate. The aqueous layer was then neutralized with 1 N aqueous NaOH solution to yield the free amine from the hydrochloride salt and re-extracted with ethyl acetate. The organic layers were pooled together, dried over anhydrous sodium sulfate and concentrated under vacuum to obtain pure 6α-amino-17β-estradiol diacetate (0.5 g, 66.49%).

Melting point: 220 °C. FT-IR (Nujol, ν cm^{-1}): 1769 (C_{17}-OCOCH$_3$), 1730 (Ar-OCOCH$_3$), 2923, 2852, 1611, 1462, 1246 and 1021 (–NH$_2$). ^{1}H NMR (CD_3OD, δ ppm): 0.77 (3H s, C_{18}-H_3), 1.20–1.85 (11H m, C_8, C_9, C_{14}-H and C_{11}, C_{12}, C_{15}, C_{16}-H_2), 2.01 (3H s, C_{17}-OCOCH_3), 2.2–2.3 (2H m, C_7-H_AH_B), 2.50 (3H s, Ar-OCOCH_3), 3.64–3.7 (1H dd, C_6-H_β, J = 4.4, 10.7 Hz), 4.65–4.70 (1H br t, C_{17}-H), 6.69–6.85 (2H m, Ar-H), 7.17–7.21 (1H m, Ar-H).

4.3.4. 6α-Amino-17β-estradiol (IV). The deacetylation of 6α-amino-17β-estradiol diacetate (III) was effected by stirring a mixture of 6α-amino-17β-estradiol diacetate (0.5 g, 0.0013 M) dissolved in 20 mL ethanol and 4 mL of 40% aqueous NaOH for 24 h at room temperature. At the end of the reaction, the reaction mixture was acidified at 0 °C with 1 N HCl to pH 6. Concentration of the reaction mixture in vacuo afforded 6α-amino-17β-estradiol as a solid.

Melting point: 159–161 °C. FT-IR (KBr, ν cm^{-1}): 3394 (–OH), 2927, 2861, 1670, 1617, 1459, 1222 and 1021 (–NH$_2$). ^{1}H NMR (CD_3OD, δ ppm): 0.77 (3H s, C_{18}-H_3), 1.20–1.78 (11H m, C_8, C_9, C_{14}-H and C_{11}, C_{12}, C_{15}, C_{16}-H_2), 2.2–2.4 (2H m, C_7-H_AH_B), 3.63–3.71 (1H dd, C_6-H_β, J = 4.0, 8.1 Hz), 4.67–4.72 (1H m, C_{17}-H), 6.55–6.65 (1H m, Ar-H), 6.97–7.15 (1H m, Ar-H), 7.32–7.36 (1H m, Ar-H).

The stereoselective amination of the 6-keto derivative to the less sterically hindered 6α-amine via reductive amination was achieved using ammonium acetate and sodium borohydride in methanol. The high resolution ^{1}H NMR of the unhydrolyzed as well as the hydrolyzed 6-amino derivative shows that the H_β proton exhibits a one-proton dd with J values of the order of 4.4 Hz and 10.7 Hz suggestive of an axial equatorial and diaxial coupling respectively with the adjacent methylene protons at C_7. Owing to the puckering of the B-ring in presence of the aromatic A-ring, the H_β proton at C_6 acquires a pseudo-axial-orientation resulting in a slight deviation from true axial equatorial and diaxial coupling constants.

4.3.5. *p*-NCS-benzyl-DOTA-6α-amino-17β-estradiol conjugate (V). 6α-Amino-17β-estradiol (0.01 g, 0.035 mM) and *p*-NCS-benzyl-DOTA, 4HCl (24 mg, 0.035 mM) were dissolved in 5 mL of distilled *N*,*N*-dimethyl formamide and the mixture was stirred at room temperature for 3 h after adjusting the pH to 9 using 1 N NaOH solution. The pH was maintained at 9 throughout the reaction. The progress of the reaction was monitored by TLC in 30% ammonia in methanol. On completion of the reaction, the solvent was removed to yield the crude product. The purification of the steroidal–BFCA conjugate (V) was carried out by preparative TLC on silica gel plates using 10% ammonia in methanol. The compound exhibited a R_f value of 0.2–0.3 in this solvent system.

FT-IR (KBr, ν cm^{-1}): 3382 (–OH), 2932, 2867, 1670, 1619, 1463, 1233 and 1021 (–NH$_2$). ^{1}H NMR (CD_3OD, δ ppm): 0.90 (3H s, C_{18}-H_3), 1.25–1.59 (11H m, C_8, C_9, C_{14}-H and C_{11}, C_{12}, C_{15}, C_{16}-H_2), 2.0–2.05 (1H m, C_7-H_AH_B), 2.1–2.2 (1H m, C_7-H_AH_B), 3.31 (8H s, –CH_2COOH), 3.52–3.58 (15H m, DOTA-CH_2), 3.65–3.68, (1H m, C_6-H), 4.0 (2H br t, Ar-CH_2-DOTA), 7.1–7.37 (6H m, Ar-H), 7.75–7.77 (1H m, Ar-H).

The appearance of the ^{1}H NMR signals in the conjugate (V) corresponding to the *p*-NCS-benzyl-DOTA moiety is indicative of the desired derivatization.

4.4. Radiolabelling of *p*-NCS-benzyl-DOTA with ^{177}Lu

For ^{177}Lu labelling of *p*-NCS-benzyl-DOTA, a stock solution of the ligand was prepared in 0.1 M ammonium acetate buffer of pH ~ 5.5 with a concentration of

1 mg/mL. To 40 μL of this stock solution, 320 μL of the 0.1 M ammonium acetate buffer (pH ~ 5.5) and 40 μL of $^{177}LuCl_3$ (100–200 MBq) were added. The pH of the mixture was adjusted to ~5 using 1 M aqueous NaOH solution. Finally, the reaction mixture was incubated at room temperature for 10 min.

4.5. Radiolabelling of *p*-NCS-benzyl-DOTA-estradiol conjugate (V) with ^{177}Lu

Coupled product (V) (300 μg) was dissolved in 0.2 mL of DMF, followed by the addition of 0.2 mL of 0.1 M ammonium acetate buffer (pH ~ 5.5) and 40 μL $^{177}LuCl_3$ solution (100–200 MBq). The volume of the reaction mixture was made up to 1 mL using normal saline and its pH was adjusted to ~5. Finally, the reaction mixture was incubated at 37 °C for 2 h.

4.6. Quality control techniques

The characterization of the radiolabelled products was achieved and the radiolabelling yields were determined by paper chromatography and paper electrophoresis techniques.

4.6.1. Paper chromatography. The test solutions (5 μL) were spotted at 1.5 cm from one end of Whatman 3 mm chromatography paper strips (12 × 2 cm). The strips were developed in normal saline, dried, cut into 1 cm segments and activity was measured.

4.6.2. Paper electrophoresis. The complex solutions (5 μL) prepared were spotted on pre-equilibrated Whatman 3 mm (35 × 2 cm) chromatography paper at 15 cm from the cathode. Paper electrophoresis was carried out for 75 min under a potential gradient of ~10 V/cm using 0.025 M phosphate buffer, pH 7.5. The strips were dried, cut into 1 cm segments and activity was counted.

4.7. Purification of ^{177}Lu labelled *p*-NCS-benzyl-DOTA-estradiol conjugate

Purification of the ^{177}Lu labelled *p*-NCS-benzyl-DOTA-estradiol conjugate was achieved by using Sep-pak® column as well as by high performance liquid chromatography (HPLC) technique. HPLC studies also helped in the precise determination of the extent of complexation.

4.7.1. Sep-pak® column purification. The complex solution (20 μL) was loaded in a Sep-pak® column previously conditioned with 2 mL of 0.1 M ammonium acetate buffer of pH ~ 5.5. The column was initially eluted with 2 × 1 mL of 0.1 M ammonium acetate buffer and followed by 2 × 1 mL ethanol. The aqueous and the ethanolic fractions were measured for radioactivity.

4.7.2. HPLC purification. HPLC of the ^{177}Lu labelled conjugate was carried out by using a C-18 reverse phase HiQ-Sil (5 μM, 4 × 250 mm) column at a flow rate of 1 mL/min. Gradient elution technique with water (A) and methanol (B) as the mobile phase was used for the separation (0–4 min 90% A, 4–10 min 90% A to 10% A, 10–15 min 10% A to 90% A, 15–20 min 90% A).

4.8. In vitro binding studies

4.8.1. Antibody binding studies. ^{177}Lu-labelled *p*-NCS-benzyl-DOTA-estradiol conjugate was incubated with antibodies raised against estradiol-6-carboxymethyl-oxime-bovine serum albumin (BSA) conjugate. The antibody used for the present study was validated in a separate radioimmunoassay system for serum estradiol estimation with adequate controls as reported.[17] 0.1 mL of the diluted complex (~1 μg) was incubated with 1:5 diluted antiserum in 0.05 M phosphate buffer containing 0.1% BSA. 0.1 mL of 2% γ globulin was added in all the tubes to facilitate precipitation. The incubation was carried out for 2 h at room temperature, after which 1 mL of 22% polyethylene glycol in saline was added to precipitate the ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex bound to antibody. The precipitate was separated by centrifugation and the radioactivity in the precipitate was measured. Blank studies were simultaneously set up without the antiserum to rule out any nonspecific binding. To ascertain the immunoreactivity of the ^{177}Lu-*p*-NCS-benzyl-DOTA-estradiol complex, ^{177}Lu-*p*-NCS-benzyl-DOTA complex was also incubated with the antiserum under similar conditions. The binding of the crude reaction mixture as well as the purified fractions was studied.

4.8.2. Cell binding studies. MCF-7, human breast cancer cell line that is known to possess estrogen receptors,[26] was used for cell uptake studies of the radiolabelled conjugate. These cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum, 2% glutamine and antibiotics and maintained in humidified atmosphere of 5% CO_2 at 37 °C. Confluent monolayers were detached by trypsinization and cells were suspended in plain DMEM. 5×10^4 cells per tube were incubated with 50 μL (~1 nM) of the radiolabelled conjugate in binding buffer (DMEM with 0.2% bovine serum albumin) for 2 h at 37 °C. An identical concentration of the radiolabelled conjugate was also incubated in presence of 100 nM of cold estradiol. The total reaction volume was 0.3 mL. After the incubation, the cells were washed with plain DMEM and centrifuged. The supernatant was discarded and the washing was repeated to remove any loosely bound activity. Similar experiments were also carried out in 24-well plates, where cells were plated one day prior to the experiments in order that the MCF-7 cells are grown adherent instead of being in the cell suspension. The radioactivity associated with the cells was measured in a NaI(Tl) scintillation counter.

Acknowledgements

The authors acknowledge Dr. S. V. Thakare and Mr. K. C. Jagadeesan for their valuable help in carrying out irradiations. The assistance provided by International Atomic Energy Agency (IAEA), Vienna, in the supply of enriched ^{176}Lu target as a part of a Co-ordinated

Research Project is gratefully acknowledged. The authors express their sincere thanks towards the help rendered by Sophisticated Analytical Instruments Facility (SAIF), Indian Institute of Technology, Mumbai, India for the high resolution ^{1}H NMR studies and National Centre of Cell Sciences, Pune, India for the cell lines.

References and notes

1. Katzenellenbogen, J. A.; Heiman, D. F.; Carlson, K. E.; Lloyd, J. E. In *Receptor-Binding Radiotracer*; Eckelman, W. C., Ed.; CRC: Boca Raton, 1982, p 93.
2. Katzenellenbogen, J. A. In *Drugs and the Pharmaceutical Sciences, Radiopharmaceuticals, Chemistry and Pharmacology*; Nunn, A. D., Ed.; Marcel Dekker: New York, 1992, pp 297–364.
3. Hostetler, E. D.; Jonson, S. D.; Welch, M. J.; Katzenellenbogen, J. A. *J. Org. Chem.* **1999**, *64*, 178.
4. Katzenellenbogen, J. A.; Carlson, K. E.; Heiman, D. F.; Goswami, R. *J. Nucl. Med.* **1980**, *23*, 550.
5. McElvany, K. D.; Carlson, K. E.; Welch, M. J.; Senderoff, S. G.; Katzenellenbogen, J. A. The Las Alamos Medical Radioisotope Group. *J. Nucl. Med.* **1982**, *23*, 420.
6. Kabalka, G. W.; Shoup, T. M.; Daniel, G. B.; Goodman, M. M. *Nucl. Med. Biol.* **2000**, *27*, 279.
7. Hom, R. K.; Katzenellenbogen, J. A. *J. Org. Chem.* **1997**, *62*, 6290.
8. Hom, R. K.; Katzenellenbogen, J. A. *Nucl. Med. Biol.* **1997**, *24*, 485.
9. Skaddan, M. B.; Wuest, F. R.; Katzenellenbogen, J. A. *J. Org. Chem.* **1999**, *64*, 8108.
10. Banerjee, S.; Das, T.; Chakraborty, S.; Samuel, G.; Korde, A.; Srivastava, S.; Venkatesh, M.; Pillai, M. R. A. *Nucl. Med. Biol.* **2004**, *31*, 753.
11. Das, T.; Chakraborty, S.; Banerjee, S.; Mukherjee, A.; Samuel, G.; Sarma, H. D.; Nair, C. K. K.; Kagiya, V. T.; Venkatesh, M. *Bioorg. Med. Chem.* **2004**, *12*, 6077.
12. Liu, S.; Edwards, D. S. *Bioconjugate Chem.* **2000**, *12*, 7.
13. Volkert, W. A.; Hoffman, T. J. *Chem. Rev.* **1999**, *99*, 2269.
14. Chakraborty, S.; Das, T.; Unni, P. R.; Sarma, H. D.; Samuel, G.; Banerjee, S.; Venkatesh, M.; Ramamoorthy, N.; Pillai, M. R. A. *Nucl. Med. Commun.* **2002**, *23*, 67.
15. Pillai, M. R. A.; Chakraborty, S.; Das, T.; Venkatesh, M.; Ramamoorthy, N. *Appl. Radiat. Isot.* **2003**, *59*, 109.
16. Kodama, M.; Koike, T.; Mahatma, A. B.; Kimara, E. *Inorg. Chem.* **1991**, *31*, 1270.
17. Korde, A.; Venkatesh, M.; Balakrishnan, S. A.; Lal, R. *J. Radioanal. Nucl. Chem. Articles* **1996**, *206*, 219.
18. Tiefenauer, L. X.; Bodmer, D. M.; Frei, W.; Andres, R. Y. *J. Steroid Biochem.* **1988**, *32*, 251.
19. Mull, E. S.; Sattigeri, V. J.; Rodriguez, A. L.; Katzenellenbogen, J. A. *Bioorg. Med. Chem.* **2002**, *10*, 1381.
20. Hussey, S. L.; He, E.; Peterson, B. R. *Org. Lett.* **2002**, *4*, 415.
21. Firestone, R. In *Table of Radioisotopes*; Shirley, V. S., Ed.; John Wiley & Sons: New York, 1996, p 2112.
22. Knapp, F. F.; Ambrose, K. R.; Beets, A. L.; Luo, H.; McPherson, D. W.; Mirzadeh, S. Nuclear Medicine Program Progress Report for Quarter Ending, September 30, 1995; Oak-Ridge National Laboratory, 1995, pp 6–16.
23. Neves, M.; Kling, A.; Lambrecht, R. M. *Appl. Radiat. Isot.* **2002**, *57*, 657.
24. Milenic, D. E.; Garmestani, K.; Chappell, L. L.; Dadachova, E.; Yordanov, A.; Ma, D.; Schlom, J.; Brechbiel, M. W. *Nucl. Med. Biol.* **2002**, *29*, 431.
25. Stimmel, J. B.; Kull, F. C., Jr. *Nucl. Med. Biol.* **1998**, *25*, 117.
26. McManaway, M. E.; Jagoda, E. M.; Eckelman, W. C.; Larson, S. M.; Francis, B. E.; Gibson, R. E.; Reba, R. C.; Lippman, M. E. *Cancer Res.* **1986**, *46*, 2386.