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Evaluation of new positively charged 11- and 12-carbon ^{99m}Tc-labeled fatty acid derivatives for myocardial imaging

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Radiolabeled fatty acids are used as tracers for myocardial metabolic imaging and currently ^{123}I-iodophenyl pentadecanoic acid (IPPA) or ^{123}I-β-methyl-iodophenyl pentadecanoic acid (BMIPP) are the agents of choice. However, ^{123}I being a cyclotron-produced isotope, ^{99m}Tc-labeled fatty acids are more desirable substitutes to ^{123}I-labeled fatty acids. Toward this, two fatty acids, having 11 and 12 carbon atoms respectively, modified with cysteine were synthesized in a four-step procedure. These ligands were then radiolabeled with ^{99m}Tc using the $[^{99m}TcN(PNP6)]^{2+}$ core. Formation of the complexes and determination of radiochemical yields were ascertained by HPLC technique. *In vivo* distribution of the complexes was carried out in Swiss mice and the results are compared with ^{125}I-IPPA. Both the complexes showed fast clearance from the myocardium till 10 min post injection (p.i.) followed by retention in the myocardium till 30 min p.i. However, compared with the result obtained with ^{125}I-IPPA, the amount of activity retained in the myocardium by the present complexes were low. Both the complexes showed rapid clearance from liver, lungs, and blood unlike the case with ^{125}I-IPPA.

Keywords: $[^{99m}TcN(PNP)]^{2+}$ core; myocardial imaging; fatty acids; IPPA; radiolabeling

Introduction

Fatty acids are the major source of energy for the normal myocardium. However, under ischemic conditions the myocardial cells switch to glucose metabolism for their energy needs. Fatty acids undergo prolonged metabolic stunning in patients with reversible ischemia, thereby helping in early diagnosis of coronary artery disease in high-risk patients.[1,2] The ^{123}I-labeled fatty acids such as ^{123}I-Iodophenylpentadecanoic acid (IPPA) and ^{123}I-Beta-methyl iodophenylpentadecanoic acid (BMIPP) are the agents used clinically for myocardial imaging.[3,4] However, high cost and limited availability of cyclotron-produced ^{123}I, makes ^{99m}Tc-labeled fatty acids more desirable for the purpose. A number of ^{99m}Tc-fatty acids have been designed and evaluated with different ^{99m}Tc cores; however, the desired *in vivo* biological characteristics could not be achieved in these complexes.[5–15]

Among the different cores of ^{99m}Tc, the $[^{99m}TcN(PNP)]^{2+}$ core has shown excellent *in vivo* characteristics suitable for myocardial imaging.[16,17] This is a pseudo octahedral complex, with two positions in a square basal plane being occupied by phosphorus atoms of the long chain PNP ligand (*cis*-arrangement) and the other two *cis*-positions are labile, which can be easily replaced by a suitable π-donor bidentate ligand having donor groups, such as SS/SO/SN. Thus, the amino acid cysteine is a useful bi-functional chelator, where a bio-molecule, like fatty acid, can be attached either at –COOH or at $-NH_2$ group, and the other two groups, SH and NH_2/COOH, can be used for labeling with $[^{99m}TcN(PNP)]^{2+}$ core.[18] The overall charge of the complex depends on the groups in cysteine that are coordinated to the $[^{99m}TcN(PNP)]^{2+}$ core, with the overall charge being positive, if –SH and $-NH_2$ group of cysteine are involved in coordination, or neutral, if –SH and –COOH groups are coordinated to the $[^{99m}TcN(PNP)]^{2+}$ core. The *in vivo* behavior of the complexes prepared using this metal fragment can be suitably modified by changing the lateral alkyl groups on the tertiary alkyl phosphine and nitrogen atom of the long chain PNP ligand.

The uptake and retention of the fatty acid complexes in myocardium is a complex function of the chain length of the fatty acid, which mainly decides the lipophilicity of the overall complex, the chelating molecule, which decides the overall charge of the complex on chelation with $[^{99m}TcN(PNP)]^{2+}$ core, and the nature of the PNP ligand are factors which governs the *in vivo* pharmacokinetics of the fatty acid complex.

The $[^{99m}TcN(PNP)]^{2+}$ core, with PNP3 and PNP5 as the bidentate phosphorus ligand, has been used earlier for the preparation of neutral and positively charged 11 and 12 carbon fatty acid complexes.[5] The charged complexes reported therein

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have shown considerable promise in terms of longer retention in the myocardium compared with the neutral counterparts. This study was aimed at studying the uptake and retention characteristics of two new positively charged [^{99m}TcN(PNP)]-fatty acid (11 and 12 carbon) complexes prepared from a new bidentate phosphine ligand (PNP6) and fatty acid modified with a cysteine residue. The fatty acid was modified with cysteine so that the complex will have an overall positive charge. The two complexes are characterized and evaluated in normal Swiss mice.

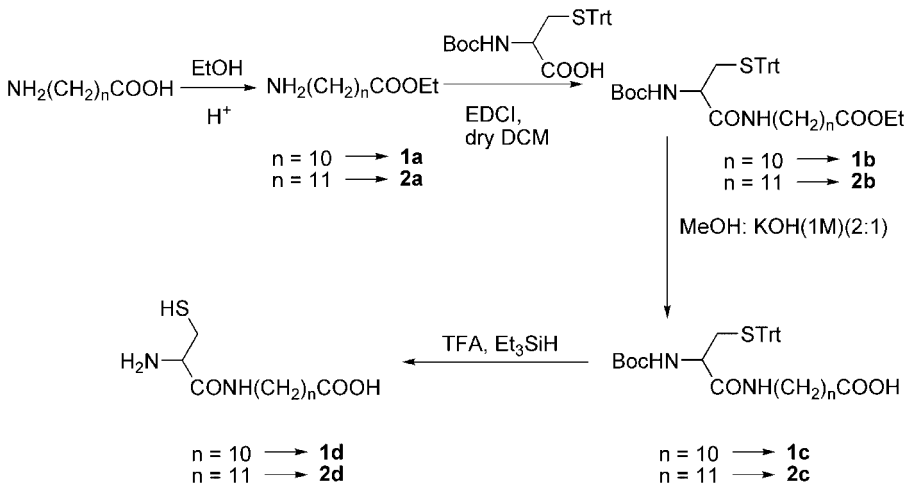
Results and discussion

Two fatty acids, having chain length of 11 and 12 carbon atoms respectively, were modified with cysteine at ω-position following a four-step synthetic procedure (Scheme 1). Initially, the acid group of the amino fatty acid was protected, by converting it to an ethyl ester, followed by coupling with *N*-Boc, *S*-Trt-cysteine using *N*-ethyl, *N*′-(3-dimethylamino) carbodiimide hydrochloride (EDCI) as a coupling agent. To obtain the target molecule, the ester group was removed by alkaline hydrolysis followed by simultaneous de-protection of the trityl and Boc groups using trifluoroacetic acid and triethyl silane.

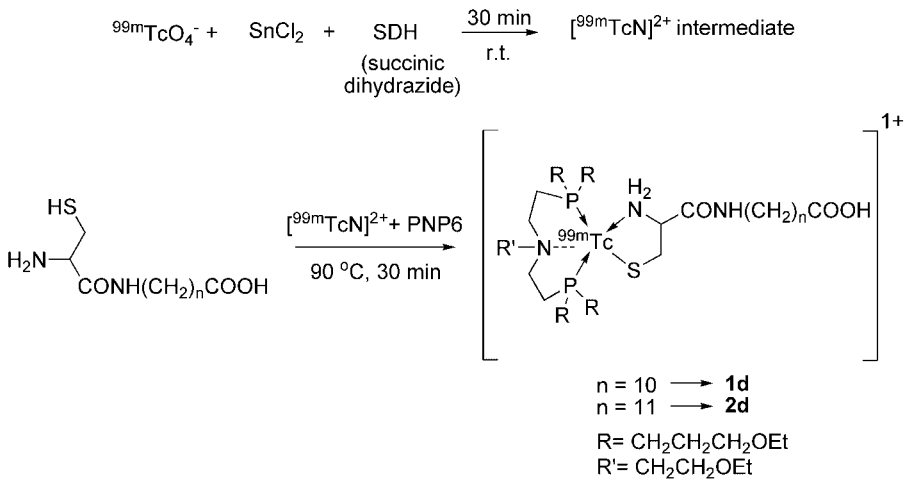
The two fatty acid derivatives thus prepared act as a bidentate donor ligand, with free thiol and amino groups in a suitable stereochemical orientation, suitable for coordination with ^{99m}Tc via the [^{99m}TcN(PNP)]$^{2+}$ core. The radiolabeling strategy involved prior preparation of [^{99m}TcN]$^{2+}$ intermediate following a reported protocol.[18] To the freshly prepared [^{99m}TcN]$^{2+}$ intermediate, both PNP6 ligand and respective fatty acid-cysteine derivative were added simultaneously to obtain the desired complex (Scheme 2).

The [^{99m}TcN(PNP)]$^{2+}$ core as well as the fatty acid complexes were characterized by HPLC using appropriate solvent system as mentioned in experimental section. The [^{99m}TcN(PNP)]$^{2+}$ core eluted at 16.1 min (Figure 1(a)), whereas the 11 and 12 carbon fatty acid complexes were eluted at 20.3 min and 19.1 min, respectively (Figure 1(b,c)). The peak area measurements indicated that both the complexes could be prepared in over 80% complexation yield.

Similar complexes reported earlier[18] were shown to possess pseudo octahedral structure. Hence, the complexes prepared in this study were also envisaged to exhibit similar geometry with an overall uni-positive charge. The syn- and anti-isomers of the fatty acid complexes are possible due to the presence of chiral carbon in cysteine residue,[18] however, the two isomers could be



Scheme 1. Synthesis of fatty acid–cysteine derivatives.



Scheme 2. Synthesis of [^{99m}TcN(PNP)]–fatty acid complexes.

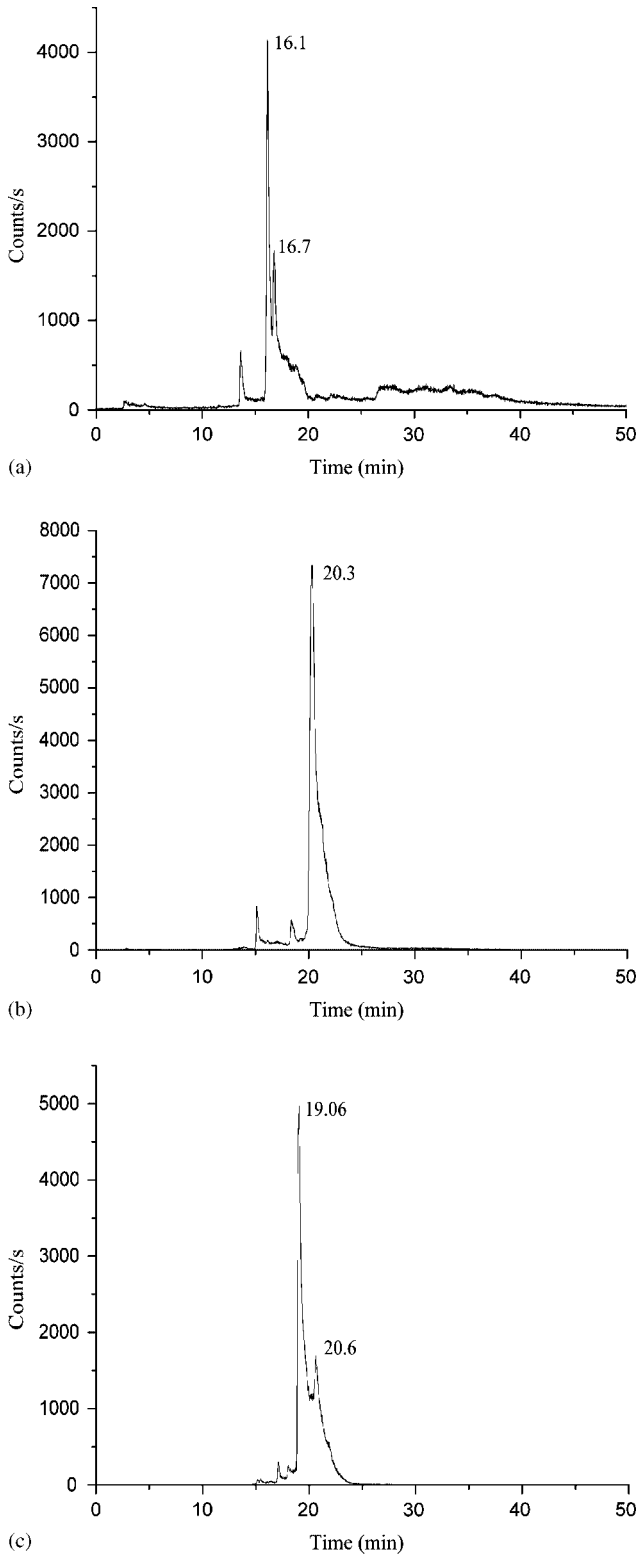


Figure 1. HPLC profile of (a) $[^{99m}TcN(PNP6)]^{2+}$ core, (b) 11-carbon fatty acid complex, and (c) 12-carbon fatty acid complex.

observed only if they are sufficiently resolved in the HPLC column. Thus, while two separate peaks (at 19.1 and 20.6 min) could be observed in the HPLC elution profile of 12 carbon fatty acid complex, such a pattern was not visible in the case of 11 carbon fatty acid complex. The stereochemistry of each isomer could be

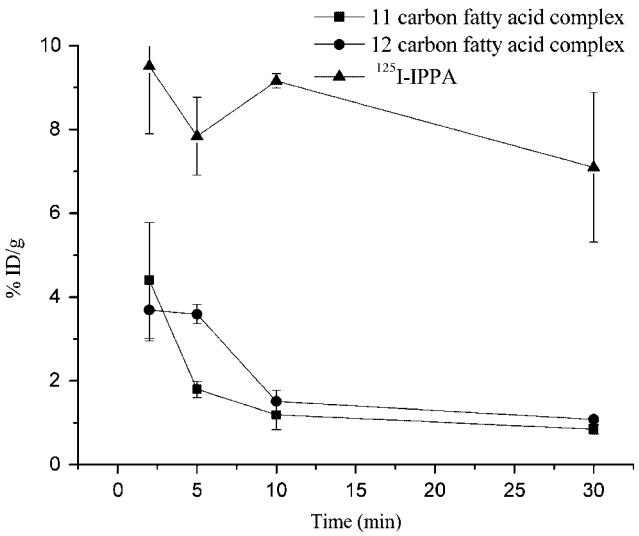


Figure 2. Uptake and retention characteristics of radiolabelled fatty acids in the myocardium of Swiss mice.

ascertained only after preparing the complexes at macroscopic level,[19] and such a study is beyond the scope of this work. The major isomer was isolated by HPLC and used for further studies.

The purified complexes on challenging with excess of cysteine, at 37°C, were found to be stable and showed no trans-chelation. Both the complexes were also found to be stable on incubation with human serum at 37°C for 30 min. The Log $P_{o/w}$ values of the two complexes were found to be 0.8 and 0.83, for 11 and 12 carbon fatty acid complexes, respectively.

The biodistribution studies of the complexes in normal Swiss mice showed uptake and rapid clearance from the myocardium up to 10 min and thereafter the rate of clearance was observed to drop off sharply (Figure 2). This was similar to the observation made by E. Cazzola *et al.*,[5] wherein the positively charged fatty acid complexes were reported to exhibit ~50 % retention of the initial activity in the myocardium even after 30 min post injection (p.i.). However, the uptake and retention values observed in the myocardium with the present complexes were significantly low in comparison with values obtained with ^{125}I-IPPA[6] in the same species (Figure 2).

For the 11 carbon fatty acid complex, the heart-to-blood ratio improved with time attaining a maximum of 3.44 ± 0.76 at 30 min p.i., which was observed to be better than the value obtained with ^{125}I-IPPA (Figure 3(a)) at same interval of time. However, for 12-carbon fatty acid complex, the ratio did not improve above 1 throughout the period of study. The heart-to-lung and heart-to-liver ratios also did not exceed 1 and similar trend was obtained with ^{125}I-IPPA (Figure 3(b,c)). The radioactivity profile of the two complexes in other organs is shown in Figure 4. Major clearance of both the complexes was through hepatobiliary route. Activities from other organs were also found to clear with time.

Experimental

The compounds 11-amino undecanoic acid, triethyl silane, succinic dihyhdrazide, and stannous chloride were obtained from Aldrich, USA. The compounds 12-amino dodecanoic acid, *N*-Boc *S*-trityl cysteine and *N*-ethyl, *N*′-(3-dimethylamino) carbodiimide hydrochloride (EDCI) were purchased from Fluka, Germany. All other reagents used were of analytical grade. Solvents such as

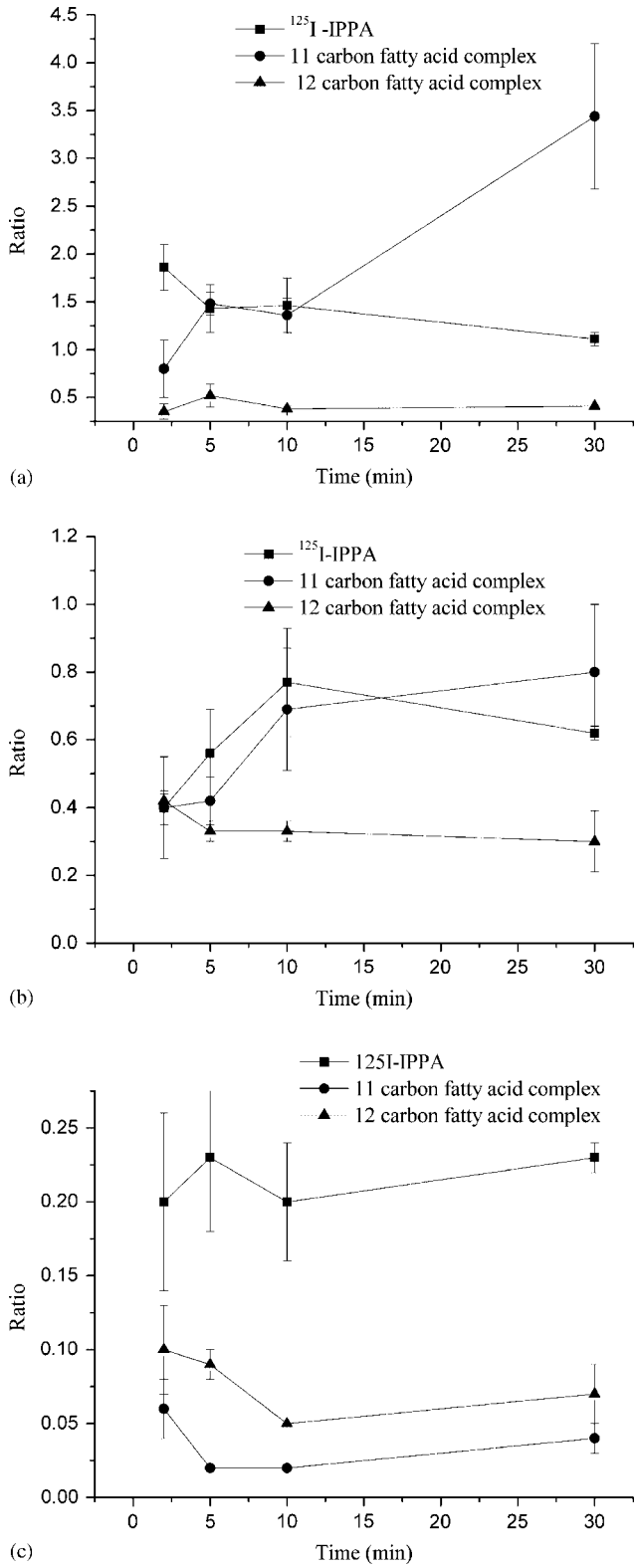


Figure 3. Variation in (a) heart/blood, (b) heart/lungs, and (c) heart/liver ratios of different radiolabelled fatty acids.

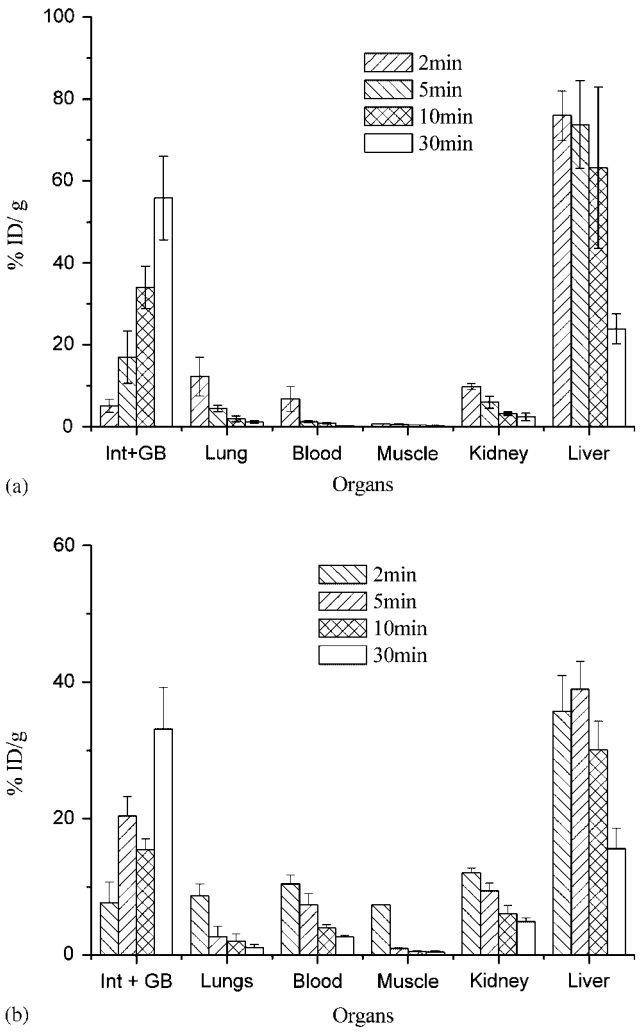


Figure 4. Activity distribution pattern of (a) 11-carbon fatty acid complex and (b) 12-carbon fatty acid complex in different organs in Swiss mice.

ethanol and dichloromethane were dried as per the standard procedure prior to use. Sodium pertechnetate ($Na^{99m}TcO_4$) was eluted with saline just before use from a ^{99}Mo-^{99m}Tc gel generator, supplied by Board of Radiation and Isotope Technology, India. Bis[(diethoxypropylphosphanyl)ethyl]ethoxyethylamine (PNP6) was obtained as a gift from Prof. Adriano Duatti, University of Ferrara, Italy. Silica gel plates (Silica Gel 60 F_{254}) were obtained from Merck, India. HPLC characterization of the prepared complexes were carried out on a JASCO PU 1580 HPLC system, with a JASCO 1575 tunable absorption detector and radiometric detector system, using a C18 reversed phase HiQ Sil (5 μm, 4 × 250 mm) column. The IR spectra were recorded on a JASCO FT-IT/420 spectrophotometer, Japan. ^{1}H-NMR spectra were recorded on a 200-MHz Bruker spectrophotometer. Mass spectra were recorded on Varian Prostar Mass Spectrometer, USA using electron spray ionization (ESI) in negative mode.

Synthesis

General procedure for the synthesis of ω-amino fatty acid ethyl ester

About 2.3 mmol of ω-amino fatty acid was added to ethanol (20 ml). To this, concentrated sulphuric acid (0.1 ml) was added and the reaction mixture refluxed overnight. On completion of the reaction, excess ethanol was removed under vacuum, water was added, and the pH of the solution was brought to 8 using 5% $NaHCO_3$ solution. The resultant solution was extracted with

chloroform (3 × 10 ml) and dried over anhydrous sodium sulphate. The filtrate on evaporation of the solvent gave the required ω-amino fatty acid ester.

*11-amino fatty acid ester (**1a**)*. Yield: Quantitative (525 mg). IR (neat, cm^{-1}): 3370(b); 2918 (s); 2850 (s); 1735 (s); 1599(w); 1466 (m); 1375 (m); 1180 (s); 1114 (m); 1034 (m); 917 (w); 859 (w); 726 (m).

*12-amino fatty acid ester (**2a**)*. Yield: 96% (540 mg). IR (neat, cm^{-1}): 3365 (b); 2922 (s); 2851 (s); 1737 (s); 1594 (m); 1466 (m); 1373 (m); 1181 (s); 1113 (m); 1034 (m); 917 (w); 860 (w); 722 (m).

General procedure for the preparation of ω-(N-Boc, S-trityl cysteinyl) fatty acid ester

About 0.5 mmol of amino fatty acid ester and *N*-Boc, *S*-trityl cysteine (233 mg, 0.5 mmol) in dry dichloromethane (15 ml) was stirred magnetically and cooled to 0°C in an ice bath. To the cooled reaction mixture, EDCI (106 mg, 0.55 mmol) was added and the stirring was continued for 1 h at 0°C. The reaction was then brought to room temperature and stirring continued overnight. The progress of the reaction was followed by TLC (5% ethyl acetate: 95% chloroform v/v, $R_f = 0.6$). On completion of the reaction, the reaction mixture was washed with water (3 × 10 ml) and dried over anhydrous sodium sulphate. The crude product obtained after removing dichloromethane was purified by silica gel column chromatography (5% ethyl acetate: 95% chloroform v/v) to yield the target compound.

*11-(N-Boc, S-trityl cysteinyl) undecanoic ester (**1b**)*. Yield: 88% (297 mg), $R_f = 0.6$. IR (neat, cm^{-1}): 3313 (b); 3057 (w); 2924 (s); 2852 (s); 1734 (s); 1713 (s); 1656 (s); 1530 (b); 1491 (m); 1444 (m); 1366 (m); 1248 (w); 1167 (s); 1033 (w); 742 (s); 699 (s). ^{1}H-NMR ($CDCl_3$, δ ppm) 7.14–7.41 [$(C_6\underline{H}_5)_3C$–, 15H, m]; 6.12 (–CHCONHCH$_2$–, 1H, s); 4.92–4.96 (–NHCHCH$_2$S–, 1H, m); 4.04-4.15 (–COOCH$_2$CH$_3$, 2H, q); 3.80–3.83 (–NHCHCH$_2$S–, 1H, m); 3.09–3.15 (–CH$_2$CH$_2$NHCO–, 2H, m); 2.62–2.68 (–CHCH$_A$CH$_B$S–, 1H, m); 2.44–2.54 (–CHCH$_A$CH$_B$S–, 1H, m); 2.22–2.29 (–CH$_2$CH$_2$COOEt, 2H, t); 1.49–1.55 (–CH$_2$CH$_2$COOEt and –CH$_2$CH$_2$NHCO–, 4H, m); 1.39 [$(C\underline{H}_3)_3C$–, 9H, s]; 1.19–1.26 [$(C\underline{H}_2)_6$ and COOCH$_2$CH$_3$, 15H, m].

*12-(N-Boc, S-trityl cysteinyl) dodecanoic ester (**2b**)*. Yield: 82% (282 mg), $R_f = 0.6$. IR (neat, cm^{-1}) 3309 (b); 3057 (w); 2925 (s); 2852 (s); 1736 (s); 1713 (s); 1656 (s); 1530 (b); 1494 (m); 1444 (m); 1366 (m); 1167 (s); 1033 (w); 742 (s); 699 (s). ^{1}H-NMR ($CDCl_3$, δ ppm) 7.14–7.41 [$(C_6\underline{H}_5)_3C$–, 15H, m]; 6.09 (–CHCONHCH$_2$–, 1H, s); 4.89–4.93 (–NHCHCH$_2$S–, 1H, m); 4.04–4.15 (–COOCH$_2$CH$_3$, 2H, q); 3.80–3.83 (–NHCHCH$_2$S– 1H, m); 3.09–3.18 (–CH$_2$CH$_2$NHCO–, 2H, m); 2.62–2.65 (–CHCH$_A$CH$_B$S–, 1H, m); 2.44–2.53 (–CHCH$_A$CH$_B$S–, 1H, m); 2.22–2.29 (–CH$_2$CH$_2$COOEt, 2H, t); 1.49–1.59 (–CH$_2$CH$_2$COOEt and –CH$_2$CH$_2$NHCO–, 4H, m); 1.39 [$(C\underline{H}_3)_3C$–, 9H, s]; 1.12–1.26 [$(C\underline{H}_2)_7$ and COOCH$_2$CH$_3$, 17H, m].

General procedure for the synthesis of ω-(N-Boc, S-trityl cysteinyl) fatty acid

The coupled fatty acid ester (0.15 mmol) was treated with 1 M KOH solution (300 µl, 0.3 mmol) and methanol (600 µl). The reaction mixture was stirred at room temperature for 2 days. The completion of the reaction was monitored by TLC (10% ethyl acetate:90% chloroform v/v). On completion of the reaction, methanol was removed under vacuum, water (5 ml) was added and the pH of the reaction mixture was adjusted to 3 using 2 N HCl. The white precipitate obtained was filtered and dried under vacuum.

*11-(N-Boc, S-trityl cysteinyl) undecanoic acid (**1c**)*. Yield: 80% (78 mg), $R_f = 0.2$. IR (neat, cm^{-1}) 3313 (b); 3057 (w); 2924 (s); 2852 (s); 1685 (s); 1656 (s); 1530 (b); 1491 (m); 1444 (m); 1366 (m); 1248 (w); 1167 (s); 1033 (w); 742 (s); 699 (s). ^{1}H-NMR ($CDCl_3$, δ ppm): 7.14–7.41 [$(C_6\underline{H}_5)_3C$–, 15H, m]; 6.32 (–CHCONHCH$_2$–, 1H, s); 5.22 (–NHCHCH$_2$S–, 1H, bs); 3.80–3.83 (–NHCHCH$_2$S–, 1H, m); 3.09–3.15 (–CH$_2$CH$_2$NHCO–, 2H, m); 2.62–2.68 (–CHCH$_A$CH$_B$S–, 1H, m); 2.44–2.54 (–CHCH$_A$CH$_B$S–, 1H, m); 2.22–2.29 (–CH$_2$CH$_2$COOH, 2H, m); 1.49–1.55(–CH$_2$CH$_2$COOH and –CH$_2$CH$_2$NHCO, 4H, m); 1.39(–C(CH$_3$)$_3$, 9H,s); 1.19–1.26[$(C\underline{H}_2)_6$, 12H, m]. MS (ESI): Mass (calculated) $C_{38}H_{50}N_2O_5S$ 646.8; *m/z* (observed) 645.5.

*12-(N-Boc, S-trityl cysteinyl) dodecanoic acid (**2c**)*. Yield: 75 % (74 mg), $R_f = 0.2$. IR (neat, cm^{-1}) 3309 (b); 3057 (w); 2925 (s); 2852 (s); 1685 (s); 1656 (s); 1530 (b); 1494 (m); 1444 (m); 1366 (m); 1167 (s); 1033 (w); 742 (s); 699 (s).

^{1}H-NMR ($CDCl_3$, δ ppm): 7.14–7.42 [$(C_6\underline{H}_5)_3C$–, 15H, m]; 6.22 (–CHCONHCH$_2$–, 1H, bs); 5.12 (–NHCHCH$_2$S–, 1H, bs); 3.80–3.83 (–NHCHCH$_2$S–, 1H, m); 3.09–3.18 (–CH$_2$CH$_2$NHCO–, 2H, m); 2.62–2.65 (–CHCH$_A$CH$_B$S–, 1H, m); 2.44–2.53 (–CHCH$_A$CH$_B$S–, 1H, m); 2.22–2.29 (–CH$_2$CH$_2$COOH, 2H, m); 1.49–1.59 (–CH$_2$CH$_2$COOH and –CH$_2$CH$_2$NHCO, 4H, m); 1.385 (–C(CH$_3$)$_3$, 9H,s); 1.12–1.26 [$(C\underline{H}_2)_7$, 14H, m]. MS (ESI): Mass (calculated) $C_{39}H_{52}N_2O_5S$ 660.9; *m/z* (observed) 659.5.

General procedure for the synthesis of ω-cysteinyl fatty acid

The ω-(*N*-Boc, *S*-trityl cysteinyl) fatty acid (0.08 mmol, **1c** or **2c**) was stirred with trifluoroacetic acid (2 ml) for 2 h at room temperature. To the yellow solution, triethyl silane was added drop-wise until it the solution turned colorless. Stirring was continued for another 15 min. A white precipitate separated out and the solvent was removed under vacuum to give the desired product (**1d** or **2d**), which was subsequently dried under vacuum and used as such for radiolabeling without further characterization.

Radiolabeling

In a typical labeling procedure, freshly eluted $Na^{99m}TcO_4$ (50 mCi, 750 µl) was added to a mixture of succinic dihydrazide (5 mg), stannous chloride (0.1 mg), and ethanol (250 µl) in a vial. On keeping the reaction mixture at room temperature for 20 min, $[^{99m}TcN]^{2+}$ intermediate was formed. To the same vial, PNP6 ligand (~2.5 mg) and respective fatty acid ligand (~5 mg, **1d** or **2d**), each dissolved in nitrogen-purged ethanol (250 µl), were added simultaneously and the reaction mixture heated at 90°C for 30 min. Thereafter, the reaction mixture was cooled over ice and characterized by HPLC.

Quality control

HPLC

The radiochemical purity of the $[^{99m}TcN(PNP)]^{+2}$ core as well as the complex was assessed by HPLC using a C18 reversed phase column. Water (A) and methanol (B) were used as the mobile phase and the following gradient elution technique was adopted for the separation (0 min 50% A, 15 min 0% A, 35 min 0% A). Flow rate was maintained at 1 ml/min. The test solution (25 µl) was injected into the column and the elution was monitored by observing the radioactivity profile.

Partition coefficient (LogP_o/w)

The HPLC-purified labeled compound (0.1 ml, 5 μCi) was mixed with water (0.9 ml) and of octanol (1 ml) on a vortex mixer for about 1 min and then centrifuged for 5 min to effect the separation of the two layers. Equal aliquots of the two layers were withdrawn and measured for the radioactivity. The readings thus obtained were used to calculate the Log $P_{o/w}$ value of the complex.

Stability studies

Cysteine challenge. In challenge studies, purified fatty acid complex (50 μl, 10 μCi), 10 mM cysteine solution (50 μl) and saline (400 μl) were mixed in a 5-ml vial and incubated at 37°C for 30 min. Thereafter, the sample was analyzed by TLC [EtOH: $CHCl_3$: benzene: 0.5 M ammonium acetate (1.5:2:1.5:0.5) v/v] for possible degradation of the original complex (11 carbon fatty acid complex: $R_f = 0–0.1$, 12 carbon fatty acid complex: $R_f = 0–0.1$).

Serum stability. To assess the stability of the fatty acid complex in human serum, purified fatty acid complex (50 μl, 10 μCi) was incubated with human serum (450 μl) at 37°C for 30 min. Thereafter, the serum proteins were precipitated by addition of ethanol (500 μl), the solution was centrifuged, and the supernatant was analyzed by TLC to determine the stability of the complex in serum.

In vivo *evaluation studies*

All procedures performed herein were in accordance with the national laws pertaining to the conduct of animal experiments. Normal Swiss mice (20–25 g body weight) were used for the *in vivo* distribution studies. All the mice involved in the study were kept under fasting condition for 6–7 h prior to the experiment, with water given *ad libitum*. The HPLC-purified radiolabeled preparation (100 μl, 20 μCi) was administered intravenously through tail vein of each animal. Individual sets of animals ($n = 3$) were utilized for studying the biodistribution at different time points (2, 5, 10, and 30 min). The animals were sacrificed immediately at the end of the respective time point and the relevant organs and tissue were excised for measurement of associated activity. The organs were weighed and the activity associated with each was measured in a flat-bed type NaI(Tl) counter with suitable energy window for ^{99m}Tc (140 keV ± 10%). For the sake of comparison, the activity retained in each organ/tissue was expressed as a per cent value of the injected dose per gram (%ID/g).

Conclusions

Two new positively charged fatty acid complexes with a $[^{99m}TcN(PNP6)]^{2+}$ core were prepared and evaluated in normal Swiss mice. Although the results obtained in this study were similar to previously reported positively charged fatty acid complexes labeled with $[^{99m}TcN(PNP3)]^{2+}$ or $[^{99m}TcN(PNP5)]^{2+}$ core, no significant improvement in the uptake and retention of the prepared complexes in the myocardium was obtained.

Acknowledgements

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