Journal of Labelled Compounds and Radiopharmaceuticals
J. Labelled Cpd. Radiopharm. **43**, 403-412 (2000)

Lysine Conjugates for the Labelling of Peptides with Technetium-99m and Rhenium

Duncan H. Hunter* and Leonard G. Luyt
Department of Chemistry, The University of Western Ontario
London, Ontario, Canada N6A 5B7
Phone: 519-679-2111 ext.6347, Fax: 519-661-3022
E-mail: dhunter@julian.uwo.ca

SUMMARY

A novel method of preparing ^{99m}Tc labelled peptides, through the incorporation of a lysine analogue containing a metal chelation centre, is proposed. α-CBZ-Lysine has been conjugated to an N_2S_2 bifunctional chelator yielding **1**, which has been chelated with both rhenium and technetium-99m and can yield a single isomer product. A Fmoc protected lysine analogue **2**, has been prepared, and is ready for incorporation into a peptide by standard peptide synthetic methods, with the potential of the forming a single isomer ^{99m}Tc labelled peptide.

Key words: lysine, technetium-99m, rhenium, labelling, peptides

INTRODUCTION

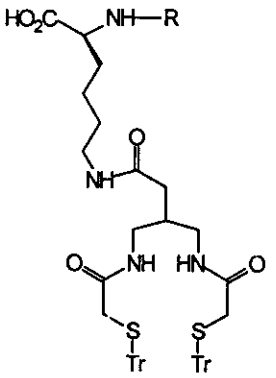
The radiolabelling of peptides with technetium-99m is a method to achieve target specific imaging agents. Three approaches are currently being investigated: direct-labelling, pre-formed chelation, and indirect-labelling. While each has advantages and disadvantages, the indirect-labelling approach, where a bifunctional chelator is attached to a peptide followed by ^{99m}Tc labelling, is considered to be the most practical method (*1, 2*).

While indirect-labelling has been successfully applied to the ^{99m}Tc-labelling of peptides, it still suffers from potential difficulties. If a peptide contains more than one nucleophilic site, attachment of a bifunctional chelator may occur at random or multiple locations on the peptide, resulting in a number of radiopharmaceutical

Received 5 May 1999
Revised 24 November 1999
Accepted 24 November 1999

complexes. An example of this occurred when a MAG3 bifunctional chelator was utilized in the ^{99m}Tc-labelling of four small peptides (*3*). Another difficulty is the potential for forming isomers after metal chelation, which can result in isolation and characterization difficulties.

In an alternative indirect-labelling approach, the peptide would be synthesized incorporating a lysine with a bifunctional chelator already attached. Upon chelation, the resulting ^{99m}Tc complex will exist with a single-radiolabel in a pre-defined location. Additionally, the chelation site will be situated away from the peptide backbone. The proposed amino acid derivatives **1** and **2** are lysine derivatives with an N_2S_2 core known to chelate technetium-99m and rhenium. The resultant stable anionic complexes are a mixture of anti/syn isomers, which epimerize to a single isomer (*4*). This article presents the preparation of **1** and **2**, and demonstrates the metal chelating ability of **1**.



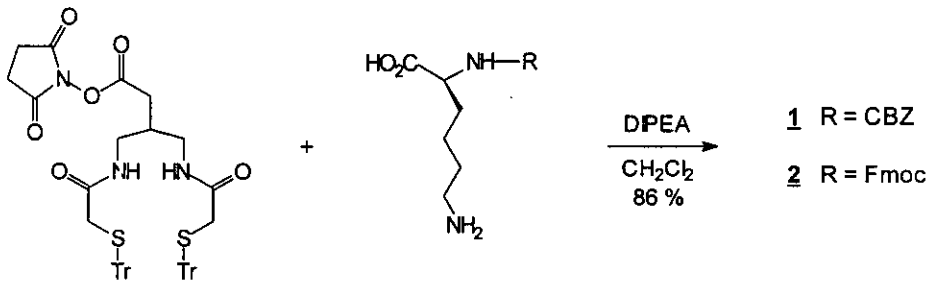
1 R = CBZ

2 R = Fmoc

RESULTS AND DISCUSSION

Lysine derivative **1**, is intended for application as a terminal amino acid in a peptide synthesis. The deprotection of the thiols will yield a N_2S_2 conjugate that is ready for rhenium or technetium chelation. As well, **1** itself, was used as a model for studying rhenium and technetium chelation.

Compound **1** was prepared by reaction of N-(α)-carbobenzoyloxy-lysine (R = CBZ) and the N-hydroxysuccinimidyl ester **3**, in dichloromethane with N,N-diisopropylethylamine present as a non-nucleophilic base (Scheme 1). The purified product **1** was characterized by ^{1}H and ^{13}C NMR spectroscopy, with assignments based on 2-dimensional spectra.

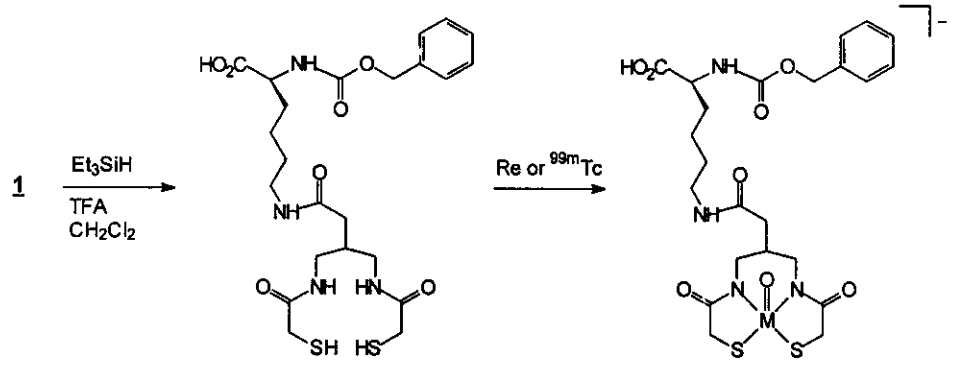


3

Scheme 1

As shown in Scheme 2, the trityl protecting groups on 1 were removed using trifluoroacetic acid with triethylsilane as the cation scavenger. After work-up and removal of the triphenylmethane via hexane trituration, 4 was recovered in 90% yield. Further purification was not carried out so as to avoid product oxidation. A ^{1}H NMR spectrum was acquired indicating the formation of the thiols by the presence of a SH triplet at 2.00 ppm with a coupling constant of 8.9 Hz.

Chelation of the lysine conjugate was first carried out with rhenium using *trans*-bis(ethylenediamine)dioxorhenium(V) chloride as the rhenium exchange ligand. The product was extracted and precipitated as the tetraphenylarsonium salt in 83% yield. HPLC analysis, shown in Figure 1a, was performed using an anion



4

5 M = Re

6 M = ^{99m}Tc

Scheme 2

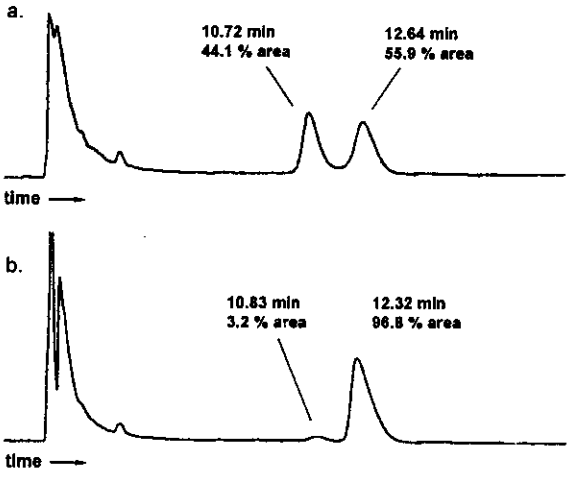
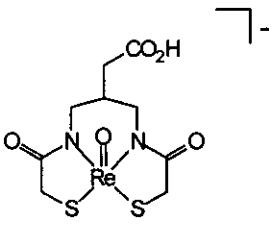


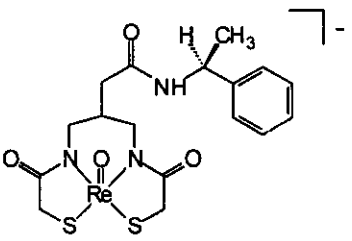
Figure 1. HPLC chromatograms (UV detector): a) ***syn*** and ***anti-5***; b) epimerization yielding primarily ***anti-5***.

exchange column and indicates the presence of two isomers in a 44:56 ratio, with retention times of 10.7 and 12.6 minutes respectively. The highly UV absorbing tetraphenylarsonium cation is eluted just after the solvent front.

Epimerization of this mixture of *anti* and *syn* isomers was carried out at room temperature using 0.5 M NaOH solution, with HPLC analysis (Figure 1b) of the product indicating that epimerization to the later eluting isomer has occurred. This product was characterized by ^{1}H and ^{13}C NMR spectroscopy. Selected ^{1}H NMR spectroscopy data is presented in Table 1 as a comparison to the chelation centre of two similar rhenium compounds 7 and 8 (*4*). The four protons next to the sulphur are all non-equivalent, due to the chiral centre of the lysine, the pro-chiral centre of the chelator, and the five-membered ring from the chelation. As a result, these protons are present as two distinct AB patterns. The four protons next to the chelator amides are also all non-equivalent. As a result they exist as two multiplets at 2.80 ppm and 3.86 ppm. As described in Table 1, the ^{1}H NMR spectrum for the chelation core is consistent with this thermodynamically preferred isomer being the *anti*-epimer. This is most obvious for the axial protons of the NCH_2 where the *anti*-isomers consistently have a chemical shift of between 2.6 and 2.9 ppm, while for the



7



8

Table 1. Comparison of ^{1}H NMR (300 MHz, δ ppm) spectroscopy for protons near the rhenium chelation site (*4*).

	5	7		8	
	anti	*anti*	*syn*	*anti*	*syn*
NCH_2 (axial)	2.80	2.63	3.31	2.87	3.42
NCH_2 (equatorial)	3.86	3.78	3.88	3.92	3.86
SCH_2	3.65 / 3.77 3.65 / 3.78	3.57 / 3.64	3.48 / 3.79	3.62 / 3.74 3.62 / 3.76	3.76 / 3.84 4.04 / 4.09

syn isomer the chemical shift is typically above 3.3 ppm. The fact that this epimer is the later eluting isomer on an anion-exchange column shows consistency with the chromatographic behaviour of the previously described ***anti-7*** (*4*).

Negative-ion FAB-MS of ***anti-5*** gave the parent ion peaks at *m/z* = 741 (100%) and 739 (69%), and a precise mass consistent with a formula of $C_{23}H_{30}N_4O_8S_2{}^{187}Re$. FTIR showed an absorption at 972 cm^{-1} indicating the presence of a rhenium-oxo bond.

Chelation of the CBZ-lysine conjugate 4 was also achieved using pertechnetate-99m, with stannous chloride as the reducing agent. When the chelation was performed in the presence of sodium acetate, a mixture of two isomers was formed. As shown in Figure 2a, the radiometric γ trace from HPLC analysis of this mixture indicates a 7:3 preference for the later eluting *anti* isomer. The radiochemical yields were consistently greater than 80% (uncorrected). Retention times of 11.9 and 13.6 minutes respectively, are later than the rhenium analogues, in part due to the radiometric detector being located in series after the UV detector.

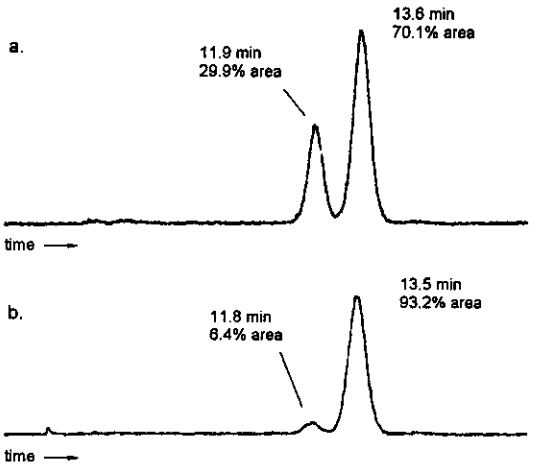


Figure 2. HPLC chromatograms (radiometric γ detector): a) ***syn*** and ***anti-6***; b) primarily ***anti-6***.

Epimerization of the mixture of [^{99m}Tc]-isomers (**6**) was possible by the addition of 1 M NaOH solution at room temperature. After 20 minutes, greater than 93% of the compound exists as the *anti* isomer (retention time of 13.5 minutes) as shown by the radiometric γ trace in Figure 2b. Chelation of the dithiol **4** with technetium-99m in the presence of 1 M NaOH gave very low yields, and a mixture of many products.

Scheme 1 illustrates the conjugation of the N_2S_2 chelator to N-α-Fmoc-lysine (R = Fmoc, 9-fluorenylmethoxycarbonyl). This was accomplished as for the preparation of **1**, yielding **2** in a 59% yield after purification by column chromatography. The product was characterized by ^{1}H and ^{13}C NMR spectroscopy, with assignments based on 2-dimensional spectra.

The Fmoc-lysine conjugate **2** is ready to be used in a peptide synthesis scheme, as the lysine carboxylic acid is free for amide coupling. Following standard Fmoc peptide synthetic procedures, the trityl protecting groups will be present until the desired peptide sequence has been completed, at which point the thiol groups may be deprotected using acid.

EXPERIMENTAL

3,3-Bis(triphenylmethylthioacetamidomethyl)propanoic acid N-hydroxysuccinimidyl ester (**3**) and *trans*-bis(ethylenediamine)dioxorhenium(V) chloride were prepared according to literature procedures (*4, 5*). NMR spectra were collected on a Varian Gemini-300 spectrometer, infrared spectra in the range 4000-600 cm^{-1} on a Bruker IFS 5500 FT-IR with a Spectra-Tech diffuse reflectance accessory (DRIFT) and negative ion fast atom bombardment mass spectrum (FABMS) on a Finnigan MAT 8200 spectrometer. Melting points were taken on a Fisher-Johns apparatus and are uncorrected. HPLC was performed using a Dionex DX 300 with a Dionex IonPac AS9-SC 4.0 x 250 mm anion exchange column, under an isocratic eluent at 1.0 mL/min (20% CH_3CN / 80% 0.1 M NaCl, 1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$ solution). The UV detector was set at 254 nm and the gamma counter window was at 140 ± 30 keV. $Na^{99m}TcO_4$ was obtained from a commercial $^{99}Mo/^{99m}Tc$ generator as supplied by DuPont.

N-(α)-Carbobenzyloxy-N-(β)-3,3-bis(triphenylmethylthio-acetamidomethyl)propanamide-L-lysine (1). A flask was charged with **3** (200.4 mg, 0.233 mmol), N-(α)-carbobenzoyloxy-lysine (65.4 mg, 0.233 mmol), 41 μL N,N-diisopropylethylamine (0.237 mmol), and 50 mL CH_2Cl_2. After the mixture was refluxed for twenty hours, and cooled to to RT, the reaction mixture was washed with 0.1 M HCl (x2), sat'd NaCl, and the organic layer was dried over $MgSO_4$. After filtering, solvent removal yielded 226 mg of crude material; recrystallized from benzene/hexanes to yield **1** (205 mg, 86% yield) as colourless fluffy crystals: mp 97° C (dec); ^1H NMR ($CDCl_3$) δ: 1.36 ppm (m, 2H, γ-lys C*H*$_2$), 1.47 (m, 2H, δ-lys C*H*$_2$), 1.64 to 1.84 (m, 5H, β-lys C*H*$_2$, C(O)-C*H*$_2$, C*H*), 2.53/2.97 (m x 2, 4H, NH-C*H*$_2$), 3.02 (s, 4H, S-C*H*$_2$ x 2), 3.16 (m, 2H, ϵ-lys C*H*$_2$), 4.24 (m, 1H, C*H*-CO_2H), 4.99 (s, 2H, Ph-C*H*$_2$), 5.71 (d, 1H, O-C(O)-N*H*), 6.91 (m, 2H, S-CH_2-C(O)-N*H* x2), 7.18 to 7.41 (ar, 35H), 7.57 (m, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 22.03 (γ-lys $\underline{C}H_2$), 28.58 (δ-lys $\underline{C}H_2$), 31.18 (β-lys $\underline{C}H_2$), 36.06 (S-$\underline{C}H_2$), 36.33 (C(O)-$\underline{C}H_2$-CH), 37.65 ($\underline{C}H$), 38.80 (ϵ-lys $\underline{C}H_2$), 39.82 (NH-$\underline{C}H_2$), 39.97 (NH-$\underline{C}H_2$), 53.81 ($\underline{C}H$-CO_2H), 66.70 (Ph-$\underline{C}H_2$), 67.65 ($\underline{C}Ph_3$), 126.94 (ar), 127.80 (ar), 128.00 (ar), 128.09 (ar), 128.42 (ar),

129.43 (ar), 136.26 (ar), 143.86 (ar), 156.15 (NH-C(O)-O), 170.21 (C=O), 172.35 (C=O), 174.21 (C=O).

N-(α)-Carbobenzyloxy-N-(β)-3,3-bis(thioacetamidomethylene) propanamide-L-lysine (4). To 1 (113 mg, 0.110 mmol) dissolved in 10 mL methylene chloride and cooled on an ice bath, trifluoroacetic acid (5 mL) and triethylsilane (36 μL, 0.225 mmol) were added and the reaction was stirred at 0° C for 30 minutes. After washing twice with water, and drying, the solvent was removed. The resulting mixture was repeatedly triturated with hexanes to remove the triphenylmethane, yielding 4 in 90% yield (53 mg): ^{1}H NMR ($CDCl_3$) δ: 1.52 to 1.80 ppm (m, 6H, γ-lys C*H*$_2$, δ-lys C*H*$_2$, β-lys C*H*$_2$), 2.00 (t, 2H, S*H*, J = 8.9 Hz), 2.10 (m, 3H, C(O)-C*H*$_2$, C*H*), 3.07 (m, 2H, one of NH-C*H*$_2$ x 2), 3.21 (d, 4H, C*H*$_2$-S x 2, J = 8.9 Hz), 3.29 (m, 4H, one of NH-C*H*$_2$ x2 plus ϵ-lys C*H*$_2$), 4.25 (m, 1H, C*H*-CO_2H), 5.07 (s, 2H, Ph-C*H*$_2$), 5.92 (d, 1H, O-C(O)-N*H*), 7.07 to 7.31 (ar, 5H), 7.85 (m, 2H, S-CH_2-C(O)-N*H* x2), 7.94 (m, 1H, N*H*), 9.95 (br, 1H, CO_2*H*).

Tetraphenylarsonium N-(α)-carbobenzyloxy-N-(β)-(3,3-Bis(thio-acetamidomethyl)propanamide oxorhenate (V))-L-lysine (5). A sample of 4 from the deprotection of 119 mg of 1 (0.116 mmol) was immediately dissolved in degassed methanol/water containing 0.5 M sodium acetate, and 48 mg *trans*-bis(ethylenediamine)dioxorhenium(V) chloride (0.118 mmol) was added. After refluxing for 18 hours, the initially clear yellow solution resulted in a clear orange solution. Methanol removal was followed by addition of tetraphenylarsonium hydrate (51 mg, 0.122 mmol) to yield an oil which was extracted from the aqueous solution with methylene chloride (x2). The combined organic layers were washed with 0.1 M HCl, sat'd NaCl solution, filtered through cotton wool, evaporated to dryness finally at 0.1 mm Hg (RT) to yield 108 mg (83%) of 5 as a pale red solid. ^{1}H NMR spectroscopy indicated the presence of two isomers; HPLC: 10.72 min. (44.1% area), 12.64 min. (55.9% area), Ph_4As^+ elutes just after the solvent front.

Epimerization to *anti*-N-(α)-Carbobenzyloxy-N-(β)-(3,3-Bis(thio-acetamidomethyl)propanamide oxorhenate(V))-lysine (*anti*-5). The mixture of isomers, 5 (104 mg, 0.092 mmol), was dissolved in 10 mL of methanol and 5 mL of

0.5 M NaOH was added, followed by stirring at RT for one hour. The solution was neutralized with 1 M HCl; the methanol was removed by a rotary evaporator resulting in precipitation of ***anti-5***, which was filtered and dried at 0.025 mm Hg (RT) yielding 84 mg of pale red crystals (81%): mp 128 - 130° C; ^{1}H NMR ($CDCl_3$) δ: 1.49 to 1.84 ppm (m, 6H, γ-lys C*H*$_2$, δ-lys C*H*$_2$, β-lys C*H*$_2$), 2.26 (m, 2H, C(O)-C*H*$_2$), 2.57 (m, 1H, C*H*), 2.80 (m, 2H, one of N-C*H*$_2$ x 2), 3.11 (m, 1H, ϵ-lys C*H*$_2$), 3.30 (m, 1H, ϵ-lys C*H*$_2$), 3.65/3.78 (AB, 2H, S-C*H*$_2$, J = 17.4 Hz), 3.65/3.77 (AB, 2H, S-C*H*$_2$, J = 17.4 Hz), 3.86 (br. d, 2H, one of N-C*H*$_2$ x 2), 4.39 (m, 1H, C*H*-CO_2H), 5.03 (s, 2H, Ph-C*H*$_2$), 5.73 (d, 1H, O-C(O)-N*H*), 6.03 (br. t, 1H, N*H*), 7.07 to 7.84 (ar, 25H); ^{13}C NMR ($CDCl_3$) δ: 22.59 (γ-lys C$\underline{H}_2$), 28.81 (δ-lys C$\underline{H}_2$), 32.91 (β-lys C$\underline{H}_2$), 38.96, 39.31, 39.51, 40.07, 40.87 (C$\underline{H}$), 54.25 (C$\underline{H}$-CO_2H), 54.50 (N-C$\underline{H}_2$), 54.82 (N-C$\underline{H}_2$), 66.62 (Ph-C$\underline{H}_2$), 120.36 (ar), 127.94 (ar), 128.43 (ar), 129.39 (ar), 131.31 (ar), 132.89 (ar), 134.91 (ar), 136.44 (ar), 155.91 (NH-C$\underline{(O)}$-O), 171.27 (C$\underline{O}_2H$), 173.82 (NH-C$\underline{(O)}$-CH_2), 196.40 (N-C$\underline{(O)}$), 196.74 (N-C$\underline{(O)}$); FT-IR (diffuse reflectance) 972 cm^{-1} (Re=O), 3300 (NH); HPLC: 10.83 min. (3.2% area), 12.32 min. (96.8% area), Ph_4As^+ elutes just after the solvent front; HRMS FAB (negative ion) *m/z*: calc'd for $C_{23}H_{30}N_4O_8S_2{}^{187}Re$ 741.1063, found 741.1077.

N-(α)-Carbobenzyloxy-N-(β)-(3,3-Bis(thio-acetamidomethyl)propanamide oxotechnetate-99m(V))-L-lysine (6). A 3 mL vial was charged with 259 μCi $Na^{99m}TcO_4$, 20 μL 1mM **4**, 20 μL 1 M sodium acetate, 0.04 mg $SnCl_2$ in 20 μL H_2O, and 20 μL 10 mM sodium gluconate. The vial was sealed and heated at 100° C for 30 minutes, then cooled for 10 minutes. A 92.7 μCi aliquot was injected onto an HPLC (radiometric γ detector) and indicated two products: 11.9 min. (29.9% area), 13.6 min. (70.1% area). The eluent from 11 to 14 minutes was collected and counted at 76.2 μCi giving a radiochemical yield of 82% (uncorrected).

The remaining reaction contents were subjected to 20 μL 1 M NaOH at RT for 20 minutes, followed by neutralization. The reaction mixture containing 100.5 μCi was injected onto an HPLC (radiometric γ detector): 1.7 min (0.5% area), 11.8 min. (6.4% area), 13.5 min. (93.2% area). The eluent from 11 to 14 minutes was collected and counted at 84.8 μCi giving a radiochemical yield of 84% (uncorrected).

N-(α)-(9-Fluorenylmethoxycarbonyl)-N-(β)-3,3-bis(triphenylmethylthio-acetamidomethyl)propanamide-L-lysine (2). The procedure was as per the preparation of **1**, using 144 mg of **3** (0.17 mmol), and 62 mg of N-(α)-(9-fluorenylmethoxycarbonyl)-L-lysine (0.17 mmol). After work-up, the crude product was purified by column chromatography (Silica 60, 200x20 mm), with the residual starting material being eluted with ethyl acetate and **2** being eluted with methanol. The solvent was evaporated and dried at 0.25 mm Hg (RT) yielding 110 mg of **2** as an off-white fluffy solid (59% yield): ^{1}H NMR ($CDCl_3$) δ: 1.40 ppm (m, 2H, γ-lys C*H*$_2$), 1.51 (m, 2H, δ-lys C*H*$_2$), 1.79 (m, 5H, β-lys C*H*$_2$, C(O)-C*H*$_2$, C*H*), 2.54/3.01 (m x 2, 4H, NH-C*H*$_2$ x 2), 3.01 (s, 4H, S-C*H*$_2$), 3.19 (m, 2H, ϵ-lys C*H*$_2$), 4.13 (t, 1H, O-CH$_2$-C*H*), 4.27 (m, 3H, C*H*-CO$_2$H, O-C*H*$_2$), 5.82 (d, 1H, O-C(O)-N*H*), 6.89 (m, 2H, S-CH$_2$-C(O)-N*H* x 2), 7.15 to 7.40 (ar, 36H), 7.55 (m, 1H, N*H*), 7.72 (ar, 2H); ^{13}C NMR ($CDCl_3$) δ: 22.05 (δ-lys <u>C</u>H$_2$), 28.64 (δ-lys <u>C</u>H$_2$), 31.28 (β-lys <u>C</u>H$_2$), 36.02 (S-<u>C</u>H$_2$), 36.45 (C(O)-<u>C</u>H$_2$), 37.67 (<u>C</u>H), 38.79 (ϵ-lys <u>C</u>H$_2$), 39.84 (NH-<u>C</u>H$_2$), 39.99 (NH-<u>C</u>H$_2$), 47.08 (O-CH$_2$-<u>C</u>H), 53.80 (<u>C</u>H-CO$_2$H), 66.82 (O-<u>C</u>H$_2$), 67.68 (<u>C</u>Ph$_3$), 119.89 (ar), 125.11 (ar), 126.93 (ar), 127.02 (ar), 127.64 (ar), 127.90 (ar), 128.08 (ar), 129.42 (ar), 141.18 (ar), 143.85, 156.12 (NH-<u>C</u>(O)-O), 170.20 (<u>C</u>=O), 172.22 (<u>C</u>=O), 174.26 (<u>C</u>=O).

ACKNOWLEDGMENTS

The authors wish to thank Doug Hairsine for running mass spectra, and NSERC (Canada) for financial support.

REFERENCES

1. Fischman, A. J., Babich, J. W., and Strauss, H. W. *J. Nucl. Med.*, **34**(12): 2253-2263 (1993).
2. Liu, S., Edwards, D. S., and Barrett, J. A. *Bioconjugate Chem.*, **8**: 621-636, (1997).
3. Hnatowich, D. J., Qu, T., Chang, F., Ley, A. C., Ladner, R. C., and Rusckowski, M. *J. Nucl. Med.*, **39**(1): 56-64 (1998).
4. Luyt, L. G., and Hunter, D. H. *Bioconjugate Chem.*, **10**(3): 470-479 (1999).
5. Johnson, N. P., Lock, C. J. L., Wolkinson, G. *J. Chem. Soc.*, 1054-66 (1964).

