Solid-Phase Synthesis of Peptide Radiopharmaceuticals Using Fmoc-*N*-ϵ-(Hynic-Boc)−Lysine, a Technetium-Binding Amino Acid: Application to Tc-99m-Labeled Salmon Calcitonin

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Labeling of proteins with metallic radionuclides for use in radiopharmaceuticals involves covalently attaching a bifunctional chelator. In principle, use of smaller peptides allows this chelator to be incorporated during solid-phase peptide synthesis (SPPS) with total site specificity. To realize the advantages of this approach, a lysine−hynic conjugate Fmoc-*N*-ϵ-(Hynic-Boc)−Lys was synthesized for incorporating the well-known technetium-99m-binding hydrazinonicotinamide ligand into peptides during SPPS. It was used to synthesize a technetium-99m-labeled salmon calcitonin with the hynic-linked amino acid in place of lysine-18. A trifluoroacetate group protected the hynic during alkaline oxidation to the cyclic disulfide and was readily removed by mild acid treatment. The peptide was efficiently labeled (91−98% radiochemical yield) with Tc-99m in the presence of tricine and $SnCl_2$ with high specific activity (>100 MBq/μg). The product showed good serum stability and specific affinity for human calcitonin receptors. Fmoc-*N*-ϵ-(Hynic-Boc)−Lys is a highly versatile technetium-binding amino acid for incorporation into peptides during SPPS. This allows total flexibility and control in the site of attachment and is suitable for a combinatorial approach to peptide radiopharmaceuticals.

Introduction

Imaging and therapy with radiolabeled peptides is now one of the fastest-growing aspects of nuclear medicine. Radiolabeled octreotide, a small peptide that binds to somatostatin receptors expressed in many cancers, is the leading example, but several others have followed, both for tumor imaging and noncancer applications.[1] Current methods for linking metallic radionuclides such as technetium-99m and indium-111 to peptides for these purposes derive from methods developed earlier for labeling monoclonal antibodies. These typically entail covalent attachment of a bifunctional chelator to a protein, usually at a lysine residue, followed by labeling with the radiometal. Currently popular and convenient prosthetic groups used in this way for Tc-99m and In-111 are the hydrazinonicotinamide (hynic) and diethylenetriamine pentaacetic acid (DTPA) groups, respectively.

The recent shift in emphasis toward smaller peptides as targeting agents brought the possibility to exploit solid-phase peptide synthesis (SPPS) rather than rely on proteins of biological origin. Radiolabeling methods that are adequate for antibodies (because it is statistically unlikely that modifying one or two of many lysine residues will interfere with the target-binding site) can be problematic for small peptides. If there is more than one possible conjugation site (as is the case with salmon calcitonin, vide infra), several labeled products may be formed with different biodistribution, some of which may have lost target affinity. Such a mixture is unacceptable for clinical use.

In principle, use of SPPS offers the opportunity to incorporate the metal-binding group as part of the amino acid sequence on the resin, allowing the chelator to be inserted specifically at a predetermined location in the sequence. For example, technetium-chelating amino acid sequences such as gly-gly-cys[2] or poly-his[3] are often incorporated during SPPS or recombinant protein production. These sequences are not ideal for the purpose of metal chelation, because they merely represent the best achievable for chelating the TcO^{3+} and $Tc(CO)_3^+$ cores, respectively, using only "standard" amino acids (i.e., those coded through tRNAs). Neither of these sequences guarantees both convenient kit-based synthesis and site-specific attachment of the radiolabel. A simple solution has been to incorporate a synthetic chelator or a radiolabeled chelate as the last step of SPPS. The ligands DOTA[4] and DTPA[5] were incorporated in this way for labeling with copper and indium isotopes, respectively, and a diaminodithiol chelator was incorporated in a similar manner for labeling with technetium-99m.[6] This approach has the limitation that the chelator has to be at one end of the peptide chain, which is frequently essential to the biological activity of the peptide.

A more versatile approach would be to synthesize a chelator incorporating a pendant-protected (e.g., Fmoc) amino acid functionality. This would offer several advantages over both conventional post-SPPS conjuga-

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tion and conventional chelating amino acid sequences. It would allow the flexibility to incorporate the chelator with total site specificity in place of any native amino acid or, in addition to native amino acids, not just terminally or at one or more lysines. Essential lysines could be left unmodified. It could be used for any metal since a specifically designed synthetic chelator can be used, rather than relying on coded amino acids, and it would be suitable for synthesis of combinatorial libraries of peptide radiopharmaceuticals from which optimal imaging agents for specific targets can be selected.

A few such reagents have been reported previously, but in no case has their successful use for synthesis of peptide radiopharmaceuticals been demonstrated. A diamidodithiol chelator[7] has been introduced into a peptide in this way, but radiolabeling of the conjugate was not reported. Iminodiacetic acid (IDA)[8] and EDTA[9−11] have similarly been introduced into peptides, but these chelators do not form stable complexes with clinically useful radionuclides. Here we report the synthesis of a derivative of L-lysine incorporating the well-known technetium-binding ligand hynic. We also report its use in the synthesis of a Tc-99m-labeled peptide, salmon calcitonin (sCT), for evaluation as an imaging agent for calcitonin receptor expression.

Salmon calcitonin is a 32-amino acid neuropeptide with a disulfide loop. It has higher affinity for human calcitonin receptors than human calcitonin[12] and is more stable in vivo. Calcitonin receptors are particularly abundant (ca. 10^6 per cell) in osteoclasts[12] and have recently been detected in various tumors and tumor-derived cells including human ovarian small cell carcinoma,[13] breast cancer,[14−16] prostate cancer,[17] and neuroblastoma,[18] as well as bone tumors (osteoclastoma)[19] and bone cancers and metastases with increased osteoclast activity.[20,21] A suitably labeled calcitonin analogue might therefore have applications in imaging bone diseases and cancer. Indeed, I-123-labeled salmon calcitonin was taken up at sites of active Paget's disease in humans, and a Tc-99m-labeled calcitonin analogue was taken up in MCF-7 breast tumor xenografts in nude mice.[22,23] Unfortunately, I-123-labeled sCT is too unstable in vivo for effective use as an imaging agent.[22] Conjugates incorporating Tc-99m-binding groups such as hynic were therefore considered more promising. However, sCT has two lysine residues and, thus, presents three sites (including the amino terminus) for conventional conjugation with *N*-hydroxysuccinimide (NHS)−hynic, giving rise to the possibility of mixtures of radiolabeled species. Solid-phase synthesis of an analogue of sCT incorporating hynic at a predefined specific location therefore presents an excellent example to validate the use of the hynic-linked amino acid for synthesis of a peptide radiopharmaceutical.

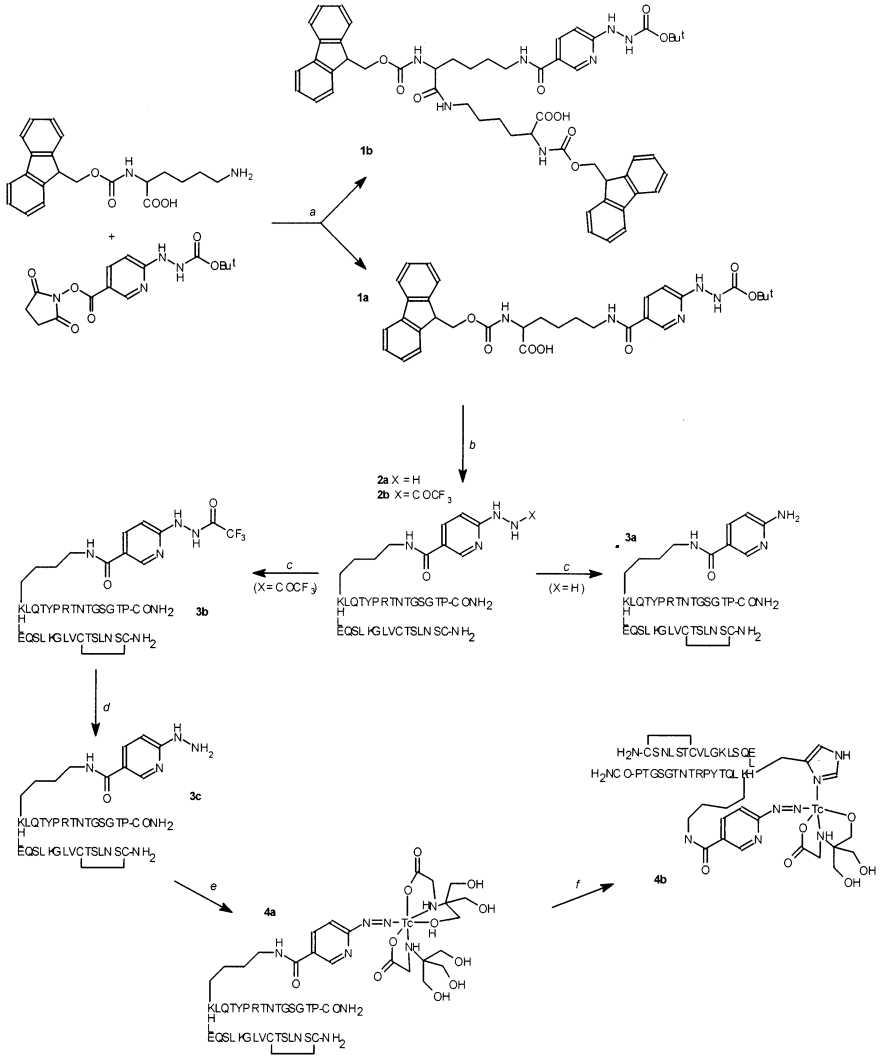
Results

α-Fmoc-protected lysine was treated with the NHS ester of Boc-protected hynic to give the α-Fmoc-protected amino acid **1a** (Scheme 1 and Figure 1), which was purified by normal-phase HPLC. Elemental analysis and IR, NMR, and mass spectra were in accord with the proposed structure (Figure 1). Elemental (C, H, N) analysis and ^{1}H NMR both indicated the presence of residual methanol in the vacuum-dried solid. On analytical HPLC, the product **1a** eluted as a narrow band at 26.9 min, which gave a positive mode electrospray mass spectrum (ES-MS) showing the expected ion at *m*/*z* 604 (100%) together with fragment ions corresponding to loss of the Boc group and loss of C_4H_8 from the Boc group (Table 1). A second minor peak eluted at 31.4 min with ES-MS (Table 1) consistent with the structure **1b**. This species was not present in sufficient quantity (<8% mole-for-mole) to be detected in the ^{1}H NMR, and no peptides incorporating it could be detected among products of SPPS.

The utility of **1a** in synthesizing peptides for radiolabeling with Tc-99m was demonstrated through synthesis of a Tc-99m-labeled sCT using standard SPPS methods. **1a** was included in the SPPS as the 18th amino acid, replacing lysine-18 present in sCT. After deprotection and cleavage of the 32-amino acid sequence from the resin by treatment with TFA for 3 h, two principal peptide fractions were purified by reversed-phase (RP) HPLC. These were identified by ES-MS (Table 1) as reduced sCT−hynic (**2a**, see Scheme 1) (*m*/*z* 1785.3, calcd 1785.6), in which the Boc group protecting the hynic had been removed along with all other protecting groups, and reduced sCT−hynic−TFA (**2b**), in which it had been replaced by a trifluoroacetyl group (*m*/*z* 1833.4, calcd 1833.6). These two peptides were oxidized with air in 0.1 M $NaHCO_3$, pH 8.2, under high dilution to form the respective disulfide-cyclized peptides **3a** and **3b**. HPLC−ES-MS showed that the peptide containing the deprotected hynic group (**2a**) did not give a product with the expected *m*/*z* (Table 1) but one 15 Da lighter. This was tentatively ascribed to loss of NH due to N−N bond cleavage in the hydrazide group under these alkaline conditions (*m*/*z* 1777.3, calcd 1777.0). However, the peptide-containing TFA-protected hynic (**2b**) quantitatively gave the required cyclic disulfide **3b** (*m*/*z* 1832.5, calcd 1832.6), which after HPLC purification was suitable for prolonged storage. It showed no significant deterioration after 42 days stored frozen at −20 °C in 10 mM sodium bicarbonate (pH 8.2).

Prior to radiolabeling, the TFA-protecting group was readily removed by treating the peptide solution with 2 volumes of 0.1% TFA (pH 2.5) for 3 h. HPLC−ES-MS showed that this deprotection reaction was at least 50% complete under these unoptimized conditions and gave no detectable decomposition products. The deprotected peptide could then be labeled in 1 h to high specific activity (e.g., 100 MBq/μg) with Tc-99m using pertechnetate eluate from a technetium generator in the presence of $SnCl_2$ and tricine to give the labeled peptide. This was shown by RP HPLC to be a single radioactive peptide species (Figure 2), which was assumed to have the structure **4a** shown in Scheme 1. The only radioactive contaminant detectable by HPLC was a variable amount of unreduced pertechnetate (2−9%). Instant thin layer chromatography (ITLC) confirmed the HPLC estimate of unreduced pertechnetate and showed that the amount of "reduced-hydrolyzed Tc-99m" or "Tc-99m colloid" was below 2%. There was no significant adsorption of radioactivity to the surface of the microcentrifuge tube in which the labeling was performed. A control in which native sCT (i.e., without hynic) was subjected to the same labeling conditions showed no HPLC peak corresponding to a labeled peptide.

Scheme 1[a]



[a] Synthesis of **1a** and its use in peptide synthesis and Tc-99m labeling. (a) DMSO, rt, overnight. (b) SPPS, standard Fmoc chemistry with **1a** in place of Fmoc-lysine-18; final cleavage from resin/deprotection with TFA, 3 h. (c) Aerial oxidation, 0.1 M sodium bicarbonate (pH 8.2). (d) 0.1% TFA (pH 2.5), rt, 3 h. (e) Tricine (pH 3.5), $SnCl_2$ and $Na^{99m}TcO_4$ generator eluate, rt, 1 h.

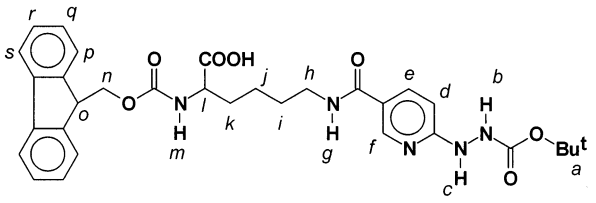


Figure 1. Structure and ^{1}H NMR assignments of *N*-α-Fmoc-*N*-ϵ-(Hynic-Boc)−Lys, **1a**. *a*: 1.41 9H s; *b*: 8.55 1H d (J < 2); *c*: 8.64 1H s; *d*: 6.50 1H d (J = 8); *e*: 7.96 1H d (J = 8); *f*: 8.92 1H s; *g*: 8.31 1H s; *h*: 3.20 2H m; *i*: 1.45 2H m; *j*: 1.28 2H m; *k*: 1.58, 1.72 2 × 1H m (prochiral); *l*: 3.73 1H m; *m*: 6.69 1H s; *n,o*: 4.21−4.31 m; *p*: 7.68 2H d (J = 6.9); *s*: 7.87 2H d (J = 7.4); *r*: 7.31 2H t (J = 7.4); *q*: 7.35 2H t (J = 7.0). Signals attributable to methyl and hydroxyl groups of methanol were also present at 3.34 and 3.73 ppm, respectively.

Chromatographic analysis after incubation of the labeled peptide **4a** in human serum confirmed that it is serum-stable for at least 30 min. RP HPLC analysis after incubation in serum showed a major peak at the same elution time (25.9 min) as that of the freshly labeled peptide. The peak was broadened, but this could be attributed to the effect of the high concentration of serum proteins on the elution or to substitution of tricine by other small metal-affine molecules, rather than dissociation of technetium from the peptide. No radioactivity coeluted with the serum proteins, which eluted later (30−50 min), and there was no increase in the fraction of residual pertechnetate (1.6 min). This stability was confirmed when the serum incubation was analyzed by size-exclusion chromatography. Labeled peptide eluted at 22.0, pertechnetate at 25.3, and UV-absorbing serum constituents from 10.5 to 30 min. No new radioactive peaks or changes in ratio of the peptide and pertechnetate peaks were observed after 30 min incubation in serum.

To check whether the labeled peptide retains affinity for human calcitonin receptors, a semiquantitative binding assay was performed with cultured human MCF7 breast cancer cells. This assay showed that receptor binding of approximately 1 nM of ^{99m}Tc-labeled peptide occurred and was effectively inhibited by 1 μM unlabeled sCT, suggesting strong and specific binding to hCtRs (Table 2). As part of the validation of the semiquantitative binding assay, a quantitative assay had previously established the receptor affinity K_D of commercial I-125-sCT as 43.5 pM (SEM 4.97) (consistent

Table 1. HPLC and ES-MS Data for Compounds Synthesized in This Work

	HPLC system	elution time/min	found *m/z*	calc. *m/z*[a]	ES-MS peak assignment
1a	B	26.9	604.1 (100)	604	[*N*-α-Fmoc-*N*-ϵ-(**Hynic-Boc**)−Lys + H]$^+$
			1207.1 (16)	1207	[(*N*-α-Fmoc-*N*-ϵ-(**Hynic-Boc**)−Lys)$_2$ + H]$^+$
			548.1 (53)	548	[*N*-α-Fmoc-*N*-ϵ-(**Hynic-Boc**)−Lys + H]$^+$ − C_4H_8
			504.3 (30)	504	[*N*-α-Fmoc-*N*-ϵ-(**Hynic-Boc**)−Lys + H]$^+$ − Boc
1b	B	31.4	954.3 (100)	954	[*N*-α-Fmoc-*N*-ϵ-(*N*-α-Fmoc-*N*-ϵ-(**Hynic-Boc**)−Lys)-Lys + H]$^+$
			898.3 (33)	898	[*N*-α-Fmoc-*N*-ϵ-(*N*-α-Fmoc-*N*-ϵ-(**Hynic-Boc**)−Lys)-Lys + H]$^+$ − C_4H_8
			854.4 (85)	854	[*N*-α-Fmoc-*N*-ϵ-(*N*-α-Fmoc-*N*-ϵ-(**Hynic-Boc**)−Lys)-Lys + H]$^+$ − Boc
2a	C	25.7	1785.3 (18)	1785.6	[reduced sCtLys^{18}−Hynic + 2H]$^{2+}$
			1190.6 (100)	1190.7	[reduced sCtLys^{18}−Hynic + 3H]$^{3+}$
2b	C	26.6	1833.4 (100)	1833.6	[reduced sCtLys^{18}−Hynic−TFA + 2H]$^{2+}$
			1222.7 (59)	1222.7	[reduced sCtLys^{18}−Hynic−TFA + 3H]$^{3+}$
3a	C	27.1	1777.3 (100)	1777.0	[sCtLys^{18}−Hynic−NH + 2H]$^{2+}$
			1185.6 (40)	1185.0	[sCtLys^{18}−Hynic−NH + 3H]$^{3+}$
3b	C	27.7	1832.5 (100)	1832.6	[sCtLys^{18}−Hynic−TFA + 2H]$^{2+}$
			1221.9 (34)	1222.0	[sCtLys^{18}−Hynic−TFA + 3H]$^{3+}$
3c	B	26.3	1784.6 (100)	1784.5	[sCtLys^{18}−Hynic + 2H]$^{2+}$
			1190.1 (82)	1190.0	[sCtLys^{18}−Hynic + 3H]$^{3+}$
4b	B	23.3	1920.8 (100)	1920.5	[sCtLys^{18}−Hynic−Tricine + Tc − H]$^{2+}$ (Tc^{III})
			1280.9 (43)	1280.7	[sCtLys^{18}−Hynic−Tc−Tricine + Tc]$^{3+}$ (Tc^{III})
4b	E	25.9			
4b	F	22.0			

[a] Principal isotope peak for m/z < 1000; average m/z for m/z > 1000.

Table 2. sCT Receptor Binding of Labeled Peptide **4a**, Expressed as Radioactivity Bound to Wells of Multi-Well Plates with and without Confluent MCF7 Cells, in the Presence and Absence of Cold Peptide[a]

labeled species	MCF7 cells present/absent	nominal cold sCT concn[b]	activity bound to well/cpm (SEM)	
			preparation 1	preparation 2
^{99m}Tc−**4a** (1 nM)	present	0	27525 (610)	23638 (490)
^{99m}Tc−**4a** (1 nM)	present	1 μM	586 (38)	726 (90)
^{99m}Tc−**4a** (1 nM)	absent	0	758 (45)	941 (145)
^{99m}Tc−**4a** (1 nM)	absent	1 μM	701 (29)	588 (83)
sCT (1 nM) + ^{99m}Tc/tricine[c]	present	0		4908 (1136)
sCT (1 nM) + ^{99m}Tc/tricine[c]	present	1 μM		3867 (858)
sCT (1 nM) + ^{99m}Tc/tricine[c]	absent	0		1565 (698)
sCT (1 nM) + ^{99m}Tc/tricine[c]	absent	1 μM		3690 (2046)

[a] Data for radiolabel not covalently attached to peptide are also given as a control [b] Native sCT added as inhibitor. [c] Control with native sCT subjected to the same labeling conditions as **4a**. The ^{99m}Tc is not bound to peptide under these conditions.

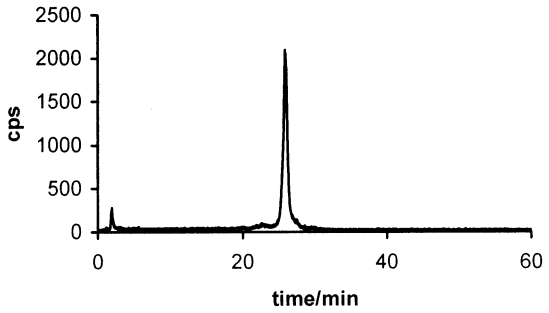


Figure 2. Radio-HPLC of unpurified Tc-99m-labeled peptide **4a**. A single radioactive peptide species is detected. The only detectable radiochemical impurity is unreacted $^{99m}TcO_4^-$ (<9%).

with literature values reported in the range 7−15000 pM[24]). In the semiquantitative assay used for the Tc-99m-labeled peptide, the specific-to-nonspecific binding ratio exhibited by I-125-sCT at comparable concentration was in the range 30−60, comparable to the range of 30−50 found for the Tc-99m-labeled peptide. Significant nonspecific binding to the plastic was excluded by inspection of the counts bound to the empty wells. Another control in which native sCT (i.e., without hynic) was subjected to the same radiolabeling conditions showed no specific binding of radioactivity to the cells (Table 2). Binding of radioactivity to cells was therefore not due to either nonspecific labeling of peptide or to non-peptide-bound technetium.

The structure of the radioactive peptide **4a** (Scheme 1), represented by the single major HPLC peak (Figure 2), was assumed on the basis of previous work[25−28] to correspond to the bis(tricine) complex **4a** shown in the scheme. However, when **3b** was labeled with Tc-99 (at stoichiometric rather than tracer levels) to enable ES-MS to be performed, a molecular dication with m/z 1920.8 (calcd 1920.5) and the corresponding trication with an additional proton were observed (Table 1). These observations are consistent with the mono(tricine) structure **4b**.

Discussion

The new lysine−hynic conjugate **1a** is a versatile reagent for incorporating the Tc-99m-binding hynic moiety into peptides during SPPS at any chosen position in the sequence. It is a prototype for a series of such chelators designed for binding different radionuclides. Such reagents lend themselves to the synthesis of a wide variety of peptide-based radiopharmaceuticals, and would be especially useful in the synthesis of combinatorial libraries of peptides from which optimized peptide radiopharmaceuticals can be developed.

The example of a salmon calcitonin analogue, used here to demonstrate its application, is a fairly demand-

ing one that is toward the higher end of the size range of peptides typically produced by SPPS and that has a disulfide loop. It shows that the method produces a peptide species with a radiolabel incorporated at a single specific location with retention of receptor binding affinity and with sufficient serum stability to warrant in vivo evaluation as an imaging agent. The location of the hynic-linked residue at amino acid 18 in the sequence was chosen because it is in the central domain of the peptide, whereas both terminal domains of the peptide are more highly conserved and are instrumental in receptor binding.[12,29] Analogues of calcitonin with modifications in this central domain (e.g., at lysine-18) have been shown to retain receptor affinity even when relatively large groups are attached,[30] and indeed **4a** retains strong and specific cell binding. Although in this case the hynic modification corresponded to lysine-18 of sCT, clearly the hynic could equally have been positioned at other locations had retention of both lysines been necessary.

The example given also shows that, under the mildly alkaline conditions necessary to form the disulfide bond, the unprotected hynic group is unstable. Mass spectrometry indicates that this instability is associated with the loss of 15 mass units (e.g., NH), suggesting cleavage of the N−N bond to give the corresponding amino−nicotinamide **3a**. Such a reaction, though not without precedent,[31,32] is not well-known and is associated with harsher conditions than those used here. Nevertheless we have not investigated it further. We have shown, however, that it can be easily prevented by protection with the trifluoroacetate group, which can be incorporated during the cleavage of the peptide from the SPPS resin. It is conveniently removed, once the oxidation step is complete, by incubation in mildly acidic conditions to afford the free hynic groups ready for Tc-99m radiolabeling. Although this protection strategy has not explicitly been reported previously, there has been a report of formation of a "TFA salt" when deprotecting a Hynic-Boc-labeled peptide with TFA, but no further details were given.[33,34] Although further optimization of both protection and deprotection is likely to improve the efficiency of each step, the present conditions are nevertheless adequate for the production of high specific-activity-labeled peptide.

ES-MS of the Tc-99-labeled peptide suggests that the technetium atom is incorporated into the peptide with concomitant displacement of five hydrogen atoms from the hynic−peptide−tricine. This is consistent with the structure **4b** shown in the scheme. In the structure shown, three of the hydrogens are lost from the hydrazide group and two from the tricine (although the location of all the hydrogen atoms and the coordination of uncharged ligands around the technetium cannot be determined from the ES-MS data). This is in agreement with previous studies of hynic−Tc-labeled peptides and with the known body of technetium-coordination chemistry.[27,28,35] Higher or lower oxidation states of technetium would have been expected to give molecular ions with *m*/*z* slightly higher or lower, respectively, although the precision of the measurements is only sufficient to give an indicative rather than definitive value for the technetium oxidation state. The observation of the molecular ion of **4b** rather than **4a** in the ES-MS might reflect the structure of the labeled species present in the radiopharmaceutical, or it may be the result of the loss of a tricine ligand in the mass spectrometer. The present results cannot distinguish these possibilities. Moreover, although the Tc-99 and Tc-99m labeling were performed under identical conditions except that the concentration of technetium was higher for Tc-99, these two preparations were not analyzed together on the same HPLC system, and hence, it is assumed, but not proven, that the two labeled species have the same structure. It has been shown that relatively harsh solution conditions (e.g., 100 °C for 10−30 min[25,26]) are required to induce replacement of one of the tricine ligands by other ligands such as phosphines. It seems likely, therefore, that the tricine ligand is displaced in the electrospray mass spectrometer, and that the radiopharmaceutical has the structure **4a**. Because there is a histidine residue adjacent in the sequence to the hynic-containing residue, we surmise that it is this histidine residue (his-17) that displaces a tricine ligand to give **4b**. This reaction would be analogous to that previously observed when bis(tricine)−Tc−hynic−peptide conjugates are treated with nitrogen heterocycles[26] or phosphine ligands[25] at high temperature. Further work is required to clarify this.

Conclusions

We have demonstrated using the example of Tc-99m-labeled sCT, which is a demanding one in that it is a relatively large peptide with a disulfide loop, that Fmoc-*N*-ϵ-(Hynic-Boc)−Lys **1a** is a highly versatile technetium-binding amino acid for incorporation into peptides during SPPS. Using the Fmoc-*N*-ϵ-(Hynic-Boc)−Lys **1a** allows total flexibility and control in the site of attachment and will be suitable for a combinatorial approach to the development of peptide radiopharmaceuticals.

Experimental Section

IR spectra were measured using a Thermo Nicolet Corporation Avatar 360 FT-IR spectrometer. Elemental analysis for C, H, and N was performed by the University of Kent analytical service. ^{1}H NMR spectra were obtained in DMSO-d_6 at 270 MHz using a JEOL GSX 270 FT spectrometer and at 600 MHz using a Varian Unity Inova 600 instrument, with tetramethylsilane as internal standard. SPPS was performed using a Shimadzu PSSM-8 peptide synthesizer. Electrospray ionization mass spectra (ES-MS) were obtained with a Finnegan Mat LCQ ion trap mass spectrometer coupled to a Hewlett-Packard 1100 HPLC system. ITLC was performed using 1 cm × 8 cm Gelman Sciences ITLC SG strips with a mobile phase of 1% NaCl in water (to separate pertechnetate from labeled peptide and reduced-hydrolyzed technetium) or 1% aqueous NaCl−acetone−acetonitrile (2:1:1) (to distinguish labeled peptide from reduced-hydrolyzed technetium). The strips were scanned with a Bioscan Mini-Scan at 0.25 mm s^{-1} and a Bioscan Flow-Count gamma detector (LabLogic, U.K.). Several systems were used for HPLC analysis. These are detailed in Table 3.

6-Chloronicotinic acid, hydrazine hydrate, triethylamine, di-*tert*-butyl dicarbonate, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, and $SnCl_2$ dihydrate were obtained from Aldrich, U.K. Fmoc-Lys-OH (*N*-α-Fmoc-Lys) was obtained from Novabiochem, U.K. TFA and tricine were obtained from Sigma, U.K. $Na^{99m}TcO_4$ was eluted from a $^{99}Mo/^{99m}Tc$ generator (Mallinckrodt, Petten, the Netherlands) with 0.9% saline. Solid $NH_4{}^{99}TcO_4$ was obtained from Amersham plc, dissolved in water, and filtered before use. 6-Boc-hydrazinopyridine-3-NHS (NHS−Hynic-Boc) was synthesized by the literature method[36]

Table 3. HPLC Conditions Used in This Work

system	instrumentation	column	flow ml/min	solvent A	solvent B	gradient	detection
A	LKB 2152 LC Controller, Kontron 422 pump, 1 mL sample loop, Applied Biosystems 759A UV Detector	Phenomenex Hypersil Silica (250 × 21.2 mm, 5 μm)	3.0	MeOH		isocratic	280 nm
B	Hewlett-Packard 1100 system, 200 μL sample loop	Hamilton PRP-1 C-18 (150 × 2.1 mm, 10 μm)	0.2	0.05% TFA/H_2O	0.0425% TFA/MeCN	0, 5; 5, 5; 30, 100; 35, 100; 40, 5.	214 nm
C	As for B	Vydac C18 (250 × 2.1 mm, 5μm)	0.2	0.05% TFA/H_2O	0.0425% TFA/MeCN	0, 5; 5, 5; 30, 100; 35, 100; 40, 5	214 nm
D	As for A	Vydac C18 (250 × 10 mm, 5μm)	4.0	0.05% TFA/H_2O	0.0425% TFA/30:70 H_2O:MeCN	0, 10; 10, 10; 15, 30; 45, 70; 55, 100; 60, 100	214 nm
E	LKB 2100 system, Bioscan Flow-Count γ detector, 100μL sample loop	Hamilton PRP-1 (150 × 2.1 mm, 10μm) and PRP-1 guard column (25 × 2.1 mm, 10 μm)	0.5	0.1% TFA/H_2O	0.085% TFA/30:70 H_2O:MeCN	0, 10; 5, 10; 10, 30; 60, 100	210 nm and γ
F	As for E	TosoHaas size exclusion TSKgel G3000SWXL (300 × 7.8 mm, 5μm)	0.5	PBS pH7.2		isocratic	210 nm and γ

using DMSO as the solvent, and the reaction mixture was used without purification.

Synthesis of Fmoc-*N*-ϵ-(Hynic-Boc)−Lysine, 1a. To the NHS−Hynic-Boc reaction mixture (1.79 mmol in 15 mL DMSO) was added *N*-α-Fmoc-Lys (220 mg, 0.597 mmol). The mixture was stirred overnight at room temperature and became clear yellow after 1 h, then cloudy after 3 h. The reaction mixture was added slowly to 60 mL of water to give a suspension (pH 4), which was stirred overnight at room temperature. The crude precipitated product was filtered off, washed with 50 mL of water, and dried under vacuum to give 460 mg of an off-white solid. This crude product was dissolved in methanol (60 mg/mL) and purified by normal-phase preparative HPLC (0.800 mL per run) using system A (Table 3). The broad second peak (42−53 min) was collected and evaporated to dryness under vacuum to give an off-white solid, 229 mg (64%). The product was analyzed by RP HPLC (system B, Table 3) and in-line ES-MS (positive mode, *m*/*z* range 150−2000). Elemental analysis calculated for $C_{32}H_{37}N_5O_7$·MeOH·$2H_2O$: C, 59.0; H, 6.7; N, 10.4. Found: C, 59.2; H 5.8; N, 10.5. The presence of residual MeOH was supported by NMR (see below). IR (Nujol, cm^{-1}): 3271 (OH), 1700 (C═O), 1643 (C═O), 1600 (C═O), 1274, 1244, 1157. ^{1}H 270 MHz NMR data and assignments, aided by 2D homonuclear ^{1}H COSY 600 MHz NMR, are shown in Figure 1.

SPPS of sCtLys^{18}-Hynic−TFA. The peptide was synthesized using standard Fmoc SPPS using 50 mg of NovaSyn TGR (Novabiochem) resin in DMF. Each Fmoc amino acid was added at a ×10 molar excess (except for *N*-α-Fmoc-*N*-ϵ-(Hynic-Boc)−Lys, **1a**, at position 18, which was used at a ×5 molar excess to save reagent) and activated using HBTU, HOBT, and DIEA in stoichiometric ratio (reagent−amino acid) of 1:1, 1:1, and 2:1, respectively. Fmoc groups were removed with 30% piperidine in DMF. Once all 32 amino acids had been added, the resin was washed with 5 × 1 mL of methanol and vacuum-dried. The side-chain protecting groups were removed, and the peptide was simultaneously cleaved from the resin by treatment with TFA−H_2O−EDT−TIS (940:25:25:10) for 3 h followed by elution with 2 × 0.5 mL TFA and precipitation with 3 × 15 mL ice-cold diethyl ether. The dried precipitate was reconstituted with 2.5 mL of H_2O. The product was analyzed by RP HPLC (system C, Table 3) and ES-MS (*m*/*z* range 500−4000). Prior to disulfide bond formation, the two major products peaks (**2a**, **2b**) were purified together by preparative RP HPLC (system D, Table 3). Fractions containing the two main peptide peaks (**2a**, **2b**) were combined (28.0 and 30.5 min) and freeze-dried.

Disulfide Bond Formation. The freeze-dried peptide was reconstituted in 15 mL of degassed 0.1 M sodium bicarbonate (pH 8.2) and oxidized by standing in air for 6 days at 4 °C. The solution was then analyzed by RP HPLC (system C, Table 3) and ES-MS, *m*/*z* range 150−4000. Preparative RP HPLC (system D) was used to isolate the second of the two peaks observed (corresponding to **3b**, sCtLys^{18}-Hynic−TFA), which was then freeze-dried. The peptide content was assayed by a modified Lowry method (Sigma Protein Assay Kit).

Deprotection and Radiolabeling. Freeze-dried **3b** sCtLys^{18}-Hynic−TFA was reconstituted with 10 mM sodium bicarbonate (pH 8.2) to a concentration of approximately 0.27 mg/mL. For radiolabeling, the hynic group was first deprotected by incubating 7 μL of peptide solution (containing approximately 2 μg of **3b**) with 14 μL of 0.1% aqueous TFA at room temperature for 3 h (pH 2.5) in a polypropylene microcentrifuge tube. The completion of the deprotection under these conditions was monitored by RP HPLC−ES-MS (system C, Table 3). Tricine (50 μL of 100 mg/mL aqueous solution, pH 3.5) was added, followed by $SnCl_2$ (5μL of 0.2 mg/mL aqueous solution). Then 100 μL of $Na^{99m}TcO_4$ (typically approximately 250 MBq) in saline was added, and the mixture was left at room temperature for 60 min (pH 3.5). The product was analyzed by ITLC and RP HPLC (system E, Table 3). Radioactivity adsorbed to the reaction tube was assayed after removal of the contents.

Receptor Binding. A cell binding assay, validated with commercial I-125-labeled sCT (Amersham plc, U.K.) using cultured MCF7 human breast cancer cells expressing calcitonin receptors measured at ca. 26 000 per cell (comparable with literature estimates[12] for this cell line), was used to assess receptor binding of the labeled peptide.[37] A 24-well plate was divided into four quadrates of six wells. The two upper quadrates contained confluent MCF7 cells, and the two lower quadrates contained no cells and were used as a control for nonspecific binding to the plastic. The plate was washed three times with PBS. Fetal calf serum (100 μL) was added to the two left quadrates, and foetal calf serum (100 μL) containing 2 μM of unmodified sCT was added to the two right quadrates. Foetal calf serum containing 2 nM of Tc-99m-labeled peptide (100 μL) was then added to all wells. The plate was sealed and incubated at room temperature with shaking for 30 min and then washed three times with phosphate-buffered saline. A 1% solution of SDS (500 μL) was added to each well, and after shaking for 15 min, the contents of the wells were transferred to counting tubes and counted for 1 min using a LKB Wallac 1282 Compugamma CS Universal Gamma Counter, with automatic dead time and decay correction.

Serum Stability. A 40 μL aliquot of the labeling reaction mixture was diluted in 160 μL of fresh human serum. A similar dilution was prepared with water in place of serum. The mixtures were left at room temperature for 30 min, and then each was analyzed by RP HPLC (system E). All eluted radioactivity was collected and assayed to ensure that no radioactivity remained on the column. The incubation was also analyzed by size-exclusion HPLC (system F).

Tc-99 Peptide Labeling. A reconstituted sample of **3b** was deprotected with dilute TFA as described for technetium-99m labeling above. To 100 μL of the resulting solution of sCtLys^{18}−

Hynic (9.17 μg, 2.57 nmol) was added 50 μL of aqueous tricine (100 mg/mL, pH 3.5), followed by 5 μL of aqueous $SnCl_2$ dihydrate (0.2 mg/mL). Then 4.53 μL of aqueous $NH_4{}^{99}TcO_4$ (0.1 mg/mL, 0.453 μg, 2.50 nmol) was added. The mixture was left at room temperature for 1 h (pH 3.5). The mixture was then analyzed by RP HPLC−ES-MS (HPLC system B, Table 3; *m*/*z* range 150−4000). A control without $NH_4{}^{99}TcO_4$ was prepared and analyzed similarly.

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