Tetrahedron 66 (2010) 2037–2043

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Trifluoroacetyl as a protecting group for HYNIC: stability in the presence of electrophiles and application in the synthesis of ^{99m}Tc-radiolabelled peptides

M. Bashir-Uddin Surfraz [a], Robert King [b], Stephen J. Mather [b], Stefano C.G. Biagini [c,*], Philip J. Blower [d,*]

[a] Cancer Research Group, Department of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom
[b] Cancer Research UK, Department of Nuclear Medicine, Dominion House, St Bartholomew's Hospital, London
[c] Functional Materials Group, School of Physical Sciences, University of Kent, Canterbury, Kent CT2 7NH, United Kingdom
[d] Division of Imaging Sciences, King's College London, Guy's Hospital Campus, St Thomas' Street London, SE1 9RT, United Kingdom

ARTICLE INFO

Article history:
Received 23 September 2009
Received in revised form 1 December 2009
Accepted 11 January 2010
Available online 18 January 2010

Keywords:
^{99m}Tc
HYNIC
Peptide
Trifluoroacetyl
Protecting group

ABSTRACT

TrifluoroacetylHYNIC-peptides have recently been shown to label directly with ^{99m}Tc as efficiently as their non-trifluoroacetylated analogs. In this work, the trifluoroacetyl (Tfa) moiety has been evaluated as a protecting group for HYNIC against reaction with strong electrophiles. Fmoc-(trifluoroacetylHYNIC)-lysine, the chosen model starting material, was found to be resistant against acetaldehyde and benzylchloroformate challenges, at 1 mol equiv and a 1000 M excess, respectively. In contrast, the Fmoc-(HYNIC)-lysine derivative, with a free hydrazine group, was quantitatively converted to the corresponding hydrazone after a 1 h incubation with acetaldehyde. Fmoc-(trifluoroacetylHYNIC)-lysine was also found to be stable over a wide pH range (3.6–10) to the acetaldehyde challenge. High efficiency ^{99m}Tc-radiolabelling (99%) was achieved in the presence of acetaldehyde using Fmoc-(trifluoroacetylHYNIC)-lysine, as compared to a poor radiolabelling yield (34%) obtained with the non-trifluoroacetylated analog. These findings firmly establish the trifluoroacetyl group as a convenient and effective protecting group for HYNIC, and as a promising alternative to currently available labelling strategies.

1. Introduction

The 6-hydrazinonicotinyl group, known as HYNIC[1–12] is an important bifunctional complexing agent used in medical imaging. It has been used extensively to functionalise peptides through a coupling step with an active ester of a Boc-protected version of HYNIC.[9,13,14] This approach normally yields a mixture of peptide-HYNIC conjugates if there is more than one attachment point available on the peptide. Alternatively, using a technetium-binding protected amino acid such as Fmoc-(HYNIC-Boc)-lysine **1** (Fig. 1) allows incorporation of the ^{99m}Tc-binding HYNIC moiety into peptides during solid-phase peptide synthesis (SPPS) at a single specified location in the sequence, resulting in a homogeneous radiolabelled peptide.[15–18] A drawback of HYNIC is that the nucleophilic hydrazine group, if used without protection, also participates in undesired side-reactions. For example, oligomeric HYNIC species are formed when using active ester derivatives of HYNIC,[19] and problems can be caused by low molecular weight aldehyde and ketone contaminants present during the radiolabelling process. The presence of these contaminants is unavoidable in a commercial pharmaceutical manufacturing environment as they are extracted from various rubber and plastic materials and are also used in common disinfectants.[13,14] Although such contaminants are present at trace level, their concentrations can be significant taking into account the low concentration of HYNIC-peptide conjugates used during radiolabelling. The single location strategy depends critically on both the protection and deprotection of the HYNIC hydrazine functionality: Rajopadhye et al. have investigated the relative merits of the well-established protecting groups 9-Fluorenylmethoxycarbonyl (Fmoc); benzyloxycarbonyl (Cbz) and *tert*-butyloxycarbonyl (Boc).[9] The group concluded that the Boc moiety was the preferred protecting group on the basis of isolated final yields, and indeed the Boc functionality is still the most widely used group for HYNIC protection, although its removal remains problematic. Boc deprotection requires conditions (e.g., neat trifluoroacetic acid, or 5 M hydrochloric acid), which can be incongruous with sensitive peptides and proteins. One of the main side-products formed during the deprotection of the Boc group from a HYNIC compound using trifluoroacetic acid is the formation of a trifluoroacetylHYNIC derivative. Recently we have shown that trifluoroacetyl derivatives of HYNIC can be labelled directly and efficiently with technetium-99m without exposing the free hydrazine group.[19–21] In this report we wish to establish the value and limitations of the trifluoroacetyl

* Corresponding authors. Tel.: +44 1227 823521; fax: +44 1227 827558 (S.C.G.B); tel.: +44 207 188 9513; fax: +44 207 188 5442 (P.J.B); *e-mail addresses:* S.Biagini@kent.ac.uk (S.C.G. Biagini), Philip.Blower@kcl.ac.uk (P.J. Blower).

0040-4020/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2010.01.038

group as a protecting group for HYNIC against electrophilic attack. The Fmoc-(trifluoroacetylHYNIC)-lysine derivative **3** (Fig. 1) was chosen as a model compound[15–21] and we tested the resistance of the Tfa moiety against: (a) acetaldehyde and (b) carboxybenzylchloride. Acetaldehyde was chosen instead of formaldehyde, as it is less volatile and the corresponding hydrazone formed accounts for a larger increase in mass, easily followed-up by mass spectrometry. Carboxybenzylchloride was chosen as an example of a strong electrophile.

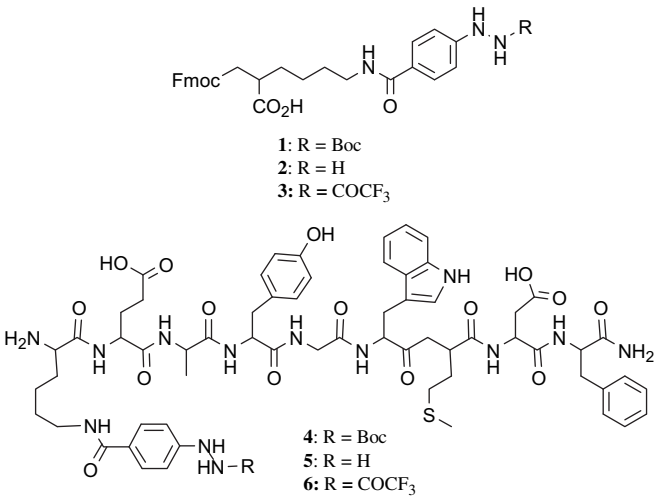


Figure 1. Structures of Fmoc-(HYNIC)-lysine compounds **1**–**3** and nanogastrin-HYNIC-peptides **4**–**6**.

2. Results

2.1. RP HPLC-MS analysis of ^{99m}Tc-tricine complexes formed with HYNIC derivatives

Radiolabelling experiments performed at 97 °C were scaled up (using ^{99}Tc instead of ^{99m}Tc) to meet the requirements for mass spectrometry detection. Table 1 illustrates the results obtained using three different methods with variable ionisation conditions and optimised for analysing technetium-peptide conjugates (Method A and B) and organic compounds (Method B and C). Both Fmoc-(HYNIC)-lysine **2** and Fmoc-(trifluoroacetylHYNIC)-lysine **3** form the same Tc–tricine complexes and gave identical UV profiles. The HPLC profiles for Fmoc-(HYNIC)-lysine compounds **2** and **3** showed two sets of two peaks. The first two peaks, eluting at 29.3 and 29.6 min, respectively, corresponded to a Tc-complex involving two tricines while the other two eluting at 30.4 and 30.7 min, respectively, accounted for 1 tricine–Tc complex.

2.2. Acetaldehyde challenges

In a time course experiment, an aqueous solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** was treated with 1 equiv of acetaldehyde at room temperature. This experiment was then repeated with 1000 equiv of acetaldehyde under similar conditions. Samples were taken at different time intervals and analysed by negative mode RP HPLC-MS (electrospray). As illustrated in Figure 2, Fmoc-(trifluoroacetylHYNIC)-lysine **3** was almost unreactive to an equimolar amount of acetaldehyde after a 2 h incubation, with 98% of **3** remaining. Reaction with a 1000-fold excess of acetaldehyde showed an increase in the yield of hydrazone **7** (13%) after 2 h, with 87% of unreacted starting material **3** left (Fig. 2, and ESI Table 1). In a parallel control reaction in which an aqueous solution of unprotected Fmoc-(HYNIC)-lysine **2** was treated with 1000 equiv of acetaldehyde, after 1 h, the reaction went to completion as confirmed by RP HPLC-MS with a single peak, eluting at 23.4 min, corresponding to hydrazone **7**.

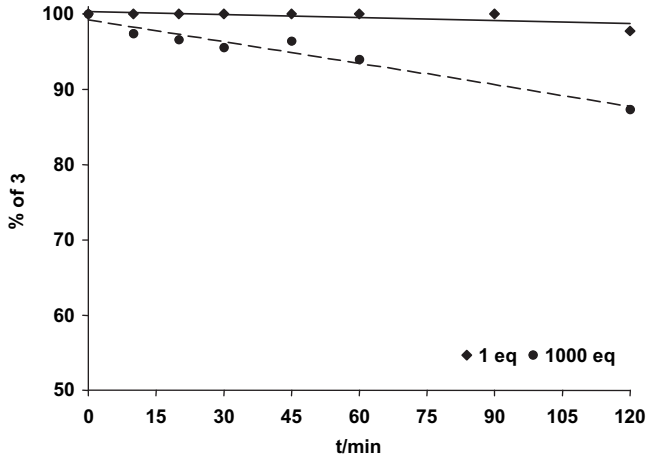


Figure 2. Time course study showing the percentage of Fmoc-(trifluoroacetylHYNIC)-lysine **3** remaining after incubation with 1 equiv and 1000 equiv of acetaldehyde, respectively.

2.3. Benzylchloroformate challenge

In a time course experiment, a solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** in DMSO was incubated with 1 equiv of benzylchloroformate at room temperature. This experiment was then repeated with 1000 equiv of benzylchloroformate using a mixture of 1,4-dioxane/water (1:1, v/v) as solvent.[22] Samples were taken at different time intervals and analysed by negative mode RP HPLC-MS. As illustrated in Figure 3, Fmoc-(trifluoroacetylHYNIC)-lysine **3** was not greatly affected by an equimolar amount of benzylchloroformate after a 2 h incubation, with approximately 95% remaining. The conversion over time was slow affording only 11% of the Cbz-derivative **8** after 24 h (Fig. 4, eluting at 27.0 min corresponding to *m*/*z* 636.0 for $[M-H]^-$). Reaction with a 1000-fold excess benzylchloroformate caused a significant decrease over time with only 46% of **3** remaining after 2 h.

Furthermore, two additional peaks were observed eluting at 32.3 min and 32.8 min, growing in intensity over time. They were attributed to Fmoc-lysine derivatives **9** and **10**, respectively (Fig. 4, corresponding to *m*/*z* 732.0 and 770.1, respectively for $[M-H]^-$).

Table 1
Mass spectrometry analysis of Tc-tricine complexes formed with HYNIC derivatives

HYNIC derivative	*m*/*z* (intensity)				
	Method A (ES-)	Method B (ES^+)		Method C (ES^+)	
	1 tricine Tc-complex	1 tricine Tc-complex	2 tricines Tc-complex	1 tricine Tc-complex	2 tricines Tc-complex
Fmoc-(HYNIC)-lysine **2**	—	776.0 (100%)	955.0 (60%)	776.0 (10%)	955.0 (100%)
Fmoc-(HYNIC-Tfa)-lysine **3**	—	776.0 (100%)	955.0 (60%)	776.0 (10%)	955.0 (100%)

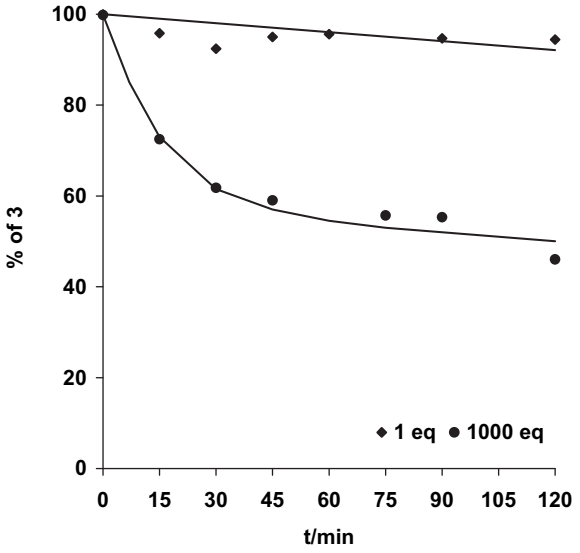


Figure 3. Time course study showing the percentage of Fmoc-(trifluoroacetylHYNIC)-lysine **3** remaining after incubation with 1 equiv and 1000 equiv of benzyl-chloroformate, respectively.

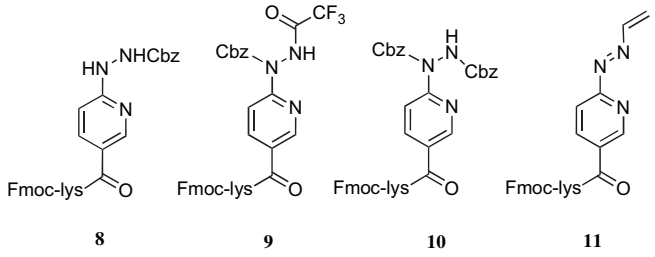


Figure 4. Structures of Fmoc-(HYNIC)-lysine derivatives **8**–**11**.

2.4. pH dependence of acetaldehyde reactivity

A solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** in citrate buffer (pH 3.6) was treated with 1 equiv of acetaldehyde at room temperature. Samples were taken at different time intervals and analysed by negative mode RP HPLC-MS (electrospray). The same experiment was then repeated at different pH using citrate buffer (pH 5.6), citrate/phosphate buffer (pH 7), phosphate buffer (pH 8) and ethanolamine buffer (pH 10). In all cases, at least 92% of starting material **3** remained after incubation with acetaldehyde for 2 h (Fig. 5). The results obtained at pH 3.6 and 5.6 were almost identical; for each pH, after 30 min, 1 h and 2 h, respectively there was 97%, 95.5% and 92% of starting material **3** left. After 2 h, the reaction yielded 7% of hydrazone **7** and 1% of Fmoc-(HYNIC)-lysine **2**, at pHs 3.6 and 5.6. At pHs of 7 and 8, after 2 h, the yields of hydrazone **7** were both around 1.5%. When the reaction was performed in water, a yield of 2.5% was observed for the hydrazone **7** after 2 h. Finally, at pH 10, yields of 1.8% and 1.9% of hydrazone **7** were observed after 1 h and 2 h incubations, respectively. At this pH, the reaction also yielded 3.3% of Fmoc-(HYNIC)-lysine **2** after 2 h.

2.5. Stability of Fmoc-Lys-HYNIC-Tfa in water or water/acetaldehyde at room temperature and at 90 °C

Fmoc-(trifluoroacetylHYNIC)-lysine **3** was chosen as a model compound to investigate the degree of stability and protection the trifluoroacetyl group can provide for HYNIC. Samples taken from four experiments were analysed and quantified by negative mode RP HPLC-MS (electrospray). A solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** in water was first stirred at room temperature for 1 h and the compound was found to be completely stable under such conditions. The outcome was identical after a longer incubation time of 24 h, with a single peak eluting at 26.3 min, accounting for 100% yield of **3** and corresponding to *m*/*z* 598.0 for $[M-H]^-$ (ESI, Fig. 3, Chart A). Analysis of a solution of **3** heated at 95 °C for 1 h revealed that 67% of Fmoc-(trifluoroacetylHYNIC)-lysine **3** remained, with the rest converted to Fmoc-(HYNIC)-lysine **2** as a second peak eluting at 22.7 min corresponding to *m*/*z* 502.0 for $[M-H]^-$ (ESI, Fig. 3, Chart B). This experiment was repeated once more and the resulting solution was allowed to cool down to room temperature before addition of 1 equiv of acetaldehyde. After incubation for 5 min, analysis of the resulting solution showed the presence of a third peak eluting at 23.4 min with *m*/*z* 528.0 for $[M-H]^-$ corresponding to a 13% yield of hydrazone **7** (Fig. 1). The two other peaks eluting at 22.7 min and 26.3 min were assigned to Fmoc-(HYNIC)-lysine **2** and Fmoc-(trifluoroacetylHYNIC)-lysine **3**, with the corresponding yields of 19% and 68%, respectively (ESI, Fig. 3, Chart C). Finally, when a solution of **3** was heated with 1 equiv of acetaldehyde at 95 °C for 1 h, only 40% of starting material remained (ESI, Fig. 3, Chart D), with formation of the hydrazone **7** (24%) and its corresponding by-product **11** (see Fig. 4, eluting at 23.8 min with to *m*/*z* 526.0 for $[M-H]^-$, 36% yield).

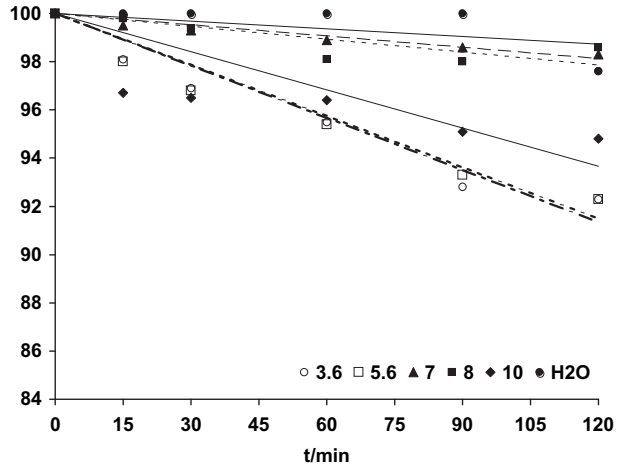


Figure 5. Time course study showing the percentage of Fmoc-(trifluoroacetylHYNIC)-lysine **3** remaining after incubation for 2 h with 1 equiv of acetaldehyde at different pHs.

2.6. ^{99m}Tc-radiolabelling of Fmoc-(HYNIC)-lysine compounds 2 and 3

Both Fmoc-(HYNIC)-lysine **2** and its trifluoroacetyl derivative **3** were radiolabelled with ^{99m}Tc after incubation with tricine as co-ligand at 95 °C for 30 min, using optimised radiolabelling conditions previously reported.[17–20] In parallel experiments, **2** and **3** were incubated separately in an equimolar aqueous solution of acetaldehyde for 24 h before being subjected to ^{99m}Tc-radiolabelling under similar conditions. Labelled Fmoc-(HYNIC)-lysine **2** and trifluoroacetyl derivative **3** gave virtually identical radiochemical yield/purity (99%) and radiochromatograms in the absence of acetaldehyde (Fig. 6a and c). Incubation of **2** with acetaldehyde (1 equiv, 24 h, RT) caused a significant drop in radiolabelling efficiency (only 34%), with additional radioactive peaks possibly accounted for by non-specific binding and/or multiple coordination of **2** to Tc (Fig. 6b). In contrast, the radiolabelling efficiency and radiochromatograms for Fmoc-(trifluoroacetylHYNIC)-lysine **3** with acetaldehyde (Fig. 6d) and without (Fig. 6c) are almost identical.

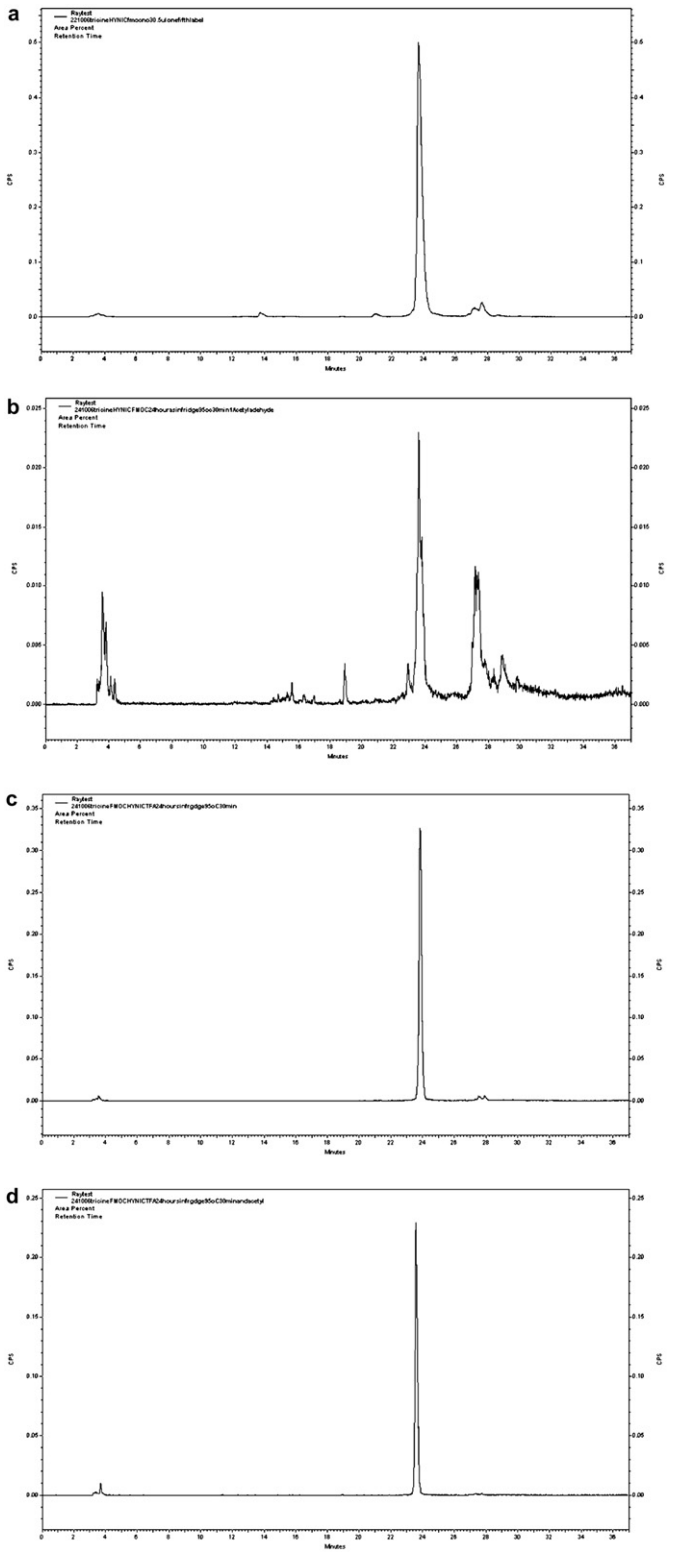
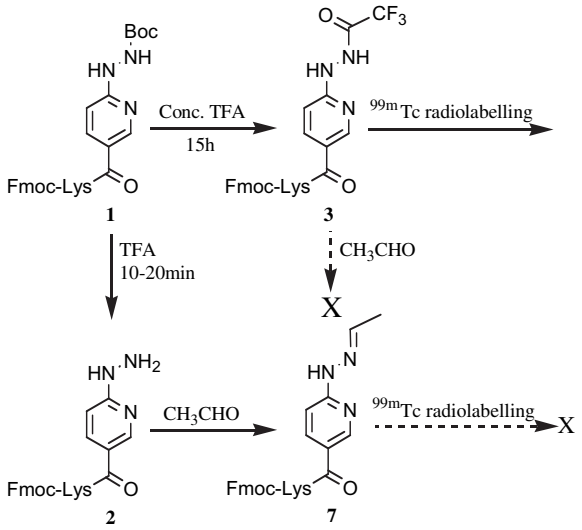


Figure 6. a) HPLC radiochromatogram for the ^{99m}Tc labelling of Fmoc-(HYNIC)-lysine **2**; (b) HPLC radiochromatogram for the ^{99m}Tc labelling of Fmoc-(HYNIC)-lysine **2** after a 24 h incubation with 1 equiv of acetaldehyde; (c) HPLC radiochromatogram for the ^{99m}Tc labelling of Fmoc-(trifluoroacetylHYNIC)-lysine **3**; (d) HPLC radiochromatogram for the ^{99m}Tc labelling of Fmoc-(trifluoroacetylHYNIC)-lysine **3** after a 24 h incubation with 1 equiv of acetaldehyde, all with tricine as co-ligand and analysed by HPLC Method 3.

3. Discussion

Transformation of the HYNIC-peptide into the corresponding hydrazone results in low radiolabelling efficiency, as shown by the observed reactivity of acetaldehyde with the 'free' HYNIC functionality, and by the effect on subsequent ^{99m}Tc labelling. When a solution containing a mixture of Fmoc-(HYNIC)-lysine **2** (43%) and Fmoc-(trifluoroacetylHYNIC)-lysine **3** (57%) was incubated with 1 equiv of acetaldehyde for 5 min, the formation of corresponding hydrazone **7** (17% overall yield) was observed, along with concomitant reduction of the pool of **2** available in solution. In contrast, no decrease in Fmoc-(trifluoroacetylHYNIC)-lysine **3** was observed. In a separate experiment, Fmoc-(HYNIC)-lysine **2** was fully converted to the corresponding hydrazone **7** when treated with 1000 equiv of acetaldehyde for 1 h (Scheme 1). Furthermore, ^{99m}Tc-radiolabelling of a solution of Fmoc-(HYNIC)-lysine **2** incubated with 1 equiv of acetaldehyde for 24 h afforded a poor radiolabelling yield of 34%.

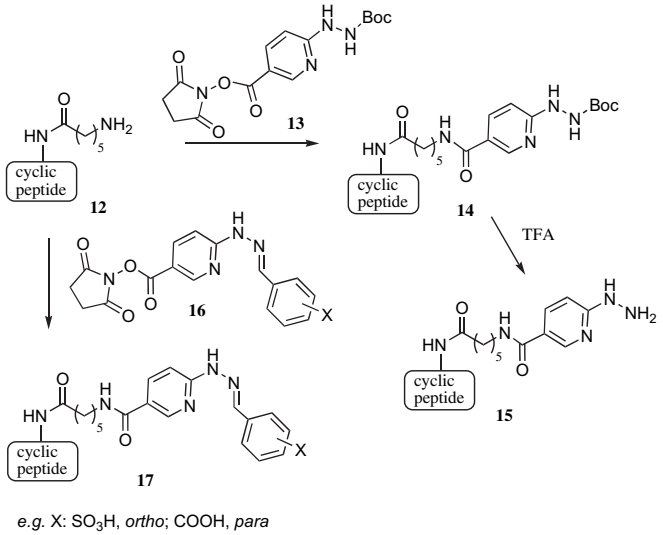


Scheme 1. Conversion of Fmoc-Lys-HYNIC-Boc **1** to the corresponding Tfa-HYNIC **3**[20] and hydrazone derivative **7**. Tfa-HYNIC **3** is resistant to electrophiles but will radiolabel with high efficiency.

To circumvent hydrazone by-product formation in radiolabelling kits, Harris et al. altered their initial post-labelling strategy, which entailed coupling the Boc-protected HYNIC-NHS ester **13** with their cyclic peptide **12** followed by Boc-removal with TFA of peptide **14** to afford the targeted HYNIC-peptide **15**.[9] Instead, they demonstrated a HYNIC protection post-labelling approach (Scheme 2) through the synthesis of a small library of aromatic hydrazone peptide conjugates **17** of the 6-hydrazinonicotinyl-modified cyclic peptide **12**.[13] This was achieved using pre-synthesised succinimidyl esters of HYNIC aromatic hydrazones **16** as reagents to yield stable hydrazone conjugates **17**, which do not react appreciably with formaldehyde under simulated manufacturing conditions, but were still reactive enough under acidic conditions to form ternary ligand complexes with ^{99m}Tc.

This reported HYNIC protection strategy circumvents hydrazone impurity formation during radiolabelling due to the presence of aldehyde and ketone contaminants[9] but has its limitations, since, it involves additional synthesis of the NHS-precursors **16**, used in slight excess to afford **17**. As reported by Edwards et al., the unreacted NHS ester **16** remaining has to be converted into the corresponding nicotinamide, a compound responsible for lowering the radiolabelling yield and purity of the desired ^{99m}Tc-radiolabelled peptide product.[13,14] In comparison, the synthesis of trifluoroacetylHYNIC compounds can be obtained in a quantitative

yield by prolonging the incubation with TFA during the removal of the Boc-protecting group (Scheme 1) and removal of peptides from resins during automated solid-phase peptide synthesis (SPPS).[20] Therefore the trifluoroacetyl protection approach for HYNIC is applicable to either strategy-site-specific labelling of peptides through SPPS, or post-labelling of small peptides and high molecular weight proteins.



Scheme 2. Hydrazone protection strategy for cyclic HYNIC-peptide **15** by Harris et al.[13]

According to Harris et al., the target HYNIC-peptide conjugate was prone to unwanted formation of the corresponding hydrazone particularly under basic conditions.[13] In contrast, almost 95% of Fmoc-(trifluoroacetylHYNIC)-lysine **3** remained after incubation with 1 equiv of acetaldehyde for 2 h at pH 10. Furthermore, the ^{99m}Tc-labelling strategy using hydrazones **17** is critically dependant on the hydrolysis of the latter, a step which is pH dependent and requires an acidic medium.[13,14] In practical terms however a pH$\leq$3.5 cannot be envisaged as tricine tends to protonate and is therefore unable to stabilise the ^{99m}Tc-tricine complex(es) formed. According to Edwards and colleagues, at pH$\geq$5.0, hydrazone **17** remains highly stable in solution and is not prone to ^{99m}Tc-HYNIC complex formation.[14] In contrast, our procedure to label the trifluoroacetylHYNIC compounds is performed at pH 6. We have also shown the stability of Fmoc-(trifluoroacetylHYNIC)-lysine **3** in different buffer solutions and in water (Fig. 5), over a wider range of pH conditions (3.6–10) with 92–99% of starting material left after a 2 h incubation in presence of acetaldehyde. This shows that the trifluoroacetylHYNIC functionality is ideal for ^{99m}Tc-radiolabelling at pH$\geq$3.5.

When Fmoc-(trifluoroacetylHYNIC)-lysine **3** was treated for 2 h with either 1 equiv or 1000 equiv of acetaldehyde (ESI, Table 1), the outcome was similar (98% and 87% of **3** remaining, respectively, Fig. 2). Fmoc-(HYNIC)-lysine **2** in comparison, was fully converted to the corresponding hydrazone **7** after only 1 h incubation with 1000 equiv of acetaldehyde. Labelling of Fmoc-(trifluoroacetylHYNIC)-lysine **3** with ^{99m}Tc using tricine as co-ligand, in the presence of acetaldehyde, was achieved (in 30 min at 95 °C) at pH 6 in high radiolabelling yield of 99% (Fig. 6). In comparison, Fmoc-(HYNIC)-lysine **2** afforded a radiolabelling yield of 34%. This illustrates that the trifluoroacetyl group can provide a high level of protection against aldehydes and yet remains sufficiently reactive to allow labelling to occur under mild conditions.

It was decided to further test the protective value of the Tfa moiety to a challenge by benzylchloroformate (Cbz-Cl) (Fig. 3), a standard reagent used for Cbz-protection of amines[22–24] and also used successfully on a HYNIC derivative.[9] Fmoc-(trifluoroacetylHYNIC)-lysine **3** was treated with 1 equiv of benzylchloroformate and after 2 h there remained 94% of **3**. In presence of 1000 equiv of benzylchloroformate, however only 46% of **3** remained (Fig. 3). We postulate that this is due to a partial hydrolysis of benzylchloroformate, which leads to the release of hydrogen chloride in the medium, which in turn leads to the acid-catalysed removal of the trifluoroacetyl group. Interestingly, mass spectrometry (Table 2) showed the presence of two new products, which were tentatively assigned by ES-MS as the Cbz-protected HYNIC derivatives **9** and **10** (Fig. 4, Table 2).

Table 2
RP HPLC-MS characterisation of Fmoc-(HYNIC)-lysine derivatives

Compound	RP HPLC method	Retention time(s)/min	MS (ES$^-$)		
			m/*z*	Intensity/%	Assignment
2	1	22.7	502.0	100	[M−H]$^-$
	2	29.7	1005.0	70	[2M−H]$^-$
			1507.9	5	[3M−H]$^-$
3	1	26.3	598.0	80	[M−H]$^-$
	2	34.0	1197.7	100	[2M−H]$^-$
			1795.7	50	[3M−H]$^-$
7	1	23.4	528.0	70	[M−H]$^-$
	2	30.7	1057.0	100	[2M−H]$^-$
			1585.7	50	[3M−H]$^-$
8	1	27.0	636.0	100	[M−H]$^-$
			1273.1	50	[2M−H]$^-$
			1910.0	10	[3M−H]$^-$
9	1	32.2	732.0	100	[M−H]$^-$
			1465.0	25	[2M−H]$^-$
10	1	32.5	770.1	100	[M−H]$^-$
			1541.1	20	[2M−H]$^-$
11	1	23.8	526.0	100	[M−H]$^-$
			1053.0	50	[2M−H]$^-$
			1579.9	20	[3M−H]$^-$

Finally, we observed that heating a solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** at 95 °C for 1 h partially (33%) removes the trifluoroacetyl group (ESI Fig. 3, Chart B). The key aspect of our methodology however, is that under standard radiolabelling conditions deprotection *only* occurs accompanying labelling, and pleasingly this was observed when a set of radiolabelling experiments performed at 20 °C resulted in Tc-radiolabelling (99% yield) (Fig. 6c and d). This outcome strengthens the notion that reduced Tc (+5 oxidation state) is first involved in the removal of the trifluoroacetyl protecting group, followed by HYNIC complexation. At this stage we suspect a Lewis acid-like catalysis mechanism for the removal of the trifluoroacetyl group from Fmoc-(trifluoroacetylHYNIC)-lysine **3** by Tc, we will investigate this further in due course.

4. Conclusion

We have shown by mass spectrometry that labelling of trifluoroacetylHYNIC compounds affords the same Tc-complexes as their HYNIC derivatives. Additionally we have demonstrated that the trifluoroacetyl-HYNIC functionality is resistant against acetaldehyde and benzylchloroformate challenges, and is stable over a wide range of pHs (3.6–10). High efficiency ^{99m}Tc-radiolabelling, using Fmoc-(trifluoroacetylHYNIC)-lysine, was also achieved in the presence of acetaldehyde. Added to the fact that trifluoroacetyl-HYNIC-peptides can now be synthesised in high yields, this is a step towards the efficient production of kit-based ^{99m}Tc labelled HYNIC-peptides.

5. Experimental procedures

Buffers were prepared as follows: *citrate buffer*, *pH* 3.6: to 37 ml of citric acid (0.1 mol dm^{-3}) was added 13 ml of sodium citrate

(0.1 mol dm^{-3}) followed by addition of 50 ml of water; *citrate buffer*, *pH* 5.6: to 13.7 ml of citric acid (0.1 mol dm^{-3}) was added 36.3 ml of sodium citrate (0.1 mol dm^{-3}) followed by addition of 50 ml of water; *citrate/phosphate buffer*, *pH* 7: to 6.5 ml of citric acid (0.1 mol dm^{-3}) was added 43.6 ml of disodium hydrogen phosphate (0.2 mol dm^{-3}) followed by addition of 50 ml of water; *phosphate buffer*, *pH* 8: sodium dihydrogen phosphate (0.2 g) was dissolved in 90 ml of water and titrated with 1 mol dm^{-3} HCl until pH 8, followed by addition of 10 ml of water; *ethanolamine buffer*, *pH* 10: to ethanolamine (61 μl) was added 90 ml of water and the resulting solution titrated with drops of 1 mol dm^{-3} sodium hydroxide until pH 8, followed by addition of 10 ml of water.

Solvents and chemicals used were of Analar quality and purchased from the following suppliers: acetaldehyde, benzylchloroformate and ethanolamine: Acros Organics, UK; citric acid, sodium citrate, disodium hydrogen phosphate, sodium dihydrogen phosphate and sodium hydroxide: Aldrich Chemicals, U.K.; trifluoroacetic acid and tricine: Sigma, U.K.; 1 M hydrochloric acid: BDH Chemicals Ltd, UK. $Na^{99m}TcO_4$ was eluted from a $^{99}Mo/^{99m}Tc$ generator (Mallinckrodt, Petten, the Netherlands) with 0.9% saline.

Electrospray ionisation mass spectra (ES-MS) were obtained with a Finnigan Mat LCQ ion trap mass spectrometer coupled to a Hewlett-Packard 1100 HPLC system for LC-MS. Time course of reactions was followed by RP HPLC-MS (ES^-) using either a Phenomenex Polymer PRP-1 column (150×2 mm, 5 μm), with HPLC method 1 or 2. HPLC method 1: mobile phase: linear gradient of increasing solvent B (ACN/water; 70/30% v/v) in 0.05% aqueous TFA: 0–5 min 5% solvent B, 5–35 min 5–90% solvent B, 35–40 min 90–100% solvent B, 40–45 min 100–5% solvent B; flow rate: 0.2 ml/min; detection: UV absorbance at 214 nm and 254 nm; HPLC method 2: mobile phase: linear gradient of increasing solvent B in 0.05% aqueous TFA: 0–10 min 5% solvent B, 5–40 min 10–90% solvent B, 40–45 min 90–100% solvent B, 40–45 min 100–5% solvent B; flow rate: 0.2 ml/min; detection: UV absorbance at 214 nm and 254 nm. RP LC-MS analysis of technetium complexes was performed using the following mass spectrometry methods: *Method A*: Peptide analytical mode, negative mode ionisation with tube lens offset (skimmer) and capillary voltage set at −50 V and −16 V, respectively; *Method B*: Peptide analytical mode, positive mode ionisation with tube lens offset (skimmer) and capillary voltage set at +30 V and +19 V, respectively; *Method C*: organic analytical profile mode, positive mode ionisation with tube lens offset (skimmer) and capillary voltage set at 0 V and +15 V, respectively; *Method D*: organic analytical profile mode, negative mode ionisation with tube lens offset (skimmer) and capillary voltage set at −30 V and −10 V, respectively.

5.1. Acetaldehyde challenges

To a solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** in DMSO (2.5 mg in 140 μl, 4.1 μmol) was added 350 μl of water followed by a 0.41 M aqueous solution of acetaldehyde (either: 1 equiv 4.1 μmol; or 1000 equiv 4.1 mmol). The resulting solution was left stirring at room temperature. A 20 μl aliquot of the reaction mixture was sampled at different time intervals and diluted with 480 μl of a mixture of DMSO/water (1:1; v/v), and 80 μl of the resulting solution was injected for analysis by RP LC-MS using Method 1 and Method D in tandem.

5.2. Benzylchloroformate challenges

To a solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** in DMSO (2.5 mg in 140 μl, 4.1 μmol) was added either: 1 equiv of benzylchloroformate [350 μl of water followed by a 0.41 M solution of benzylchloroformate in DMSO (10 μl, 4.1 μmol)]; or 1000 equiv [benzylchloroformate (596 μl, 4.1 mmol) in a mixture of 1,4-dioxane/water (800 μl, 1:1; v/v)]. The resulting solution was left stirring at room temperature. A 20 μl aliquot of the reaction mixture was sampled at different time intervals and diluted with 480 μl of a mixture of DMSO/water (1:1; v/v), and 80 μl of the resulting solution was injected for analysis by RP LC-MS using Method 1 and Method D in tandem.

5.3. Acetaldehyde challenge at different pHs

To 300 μl of the appropriate buffer was added 40 μl of DMSO followed by a 0.375 M solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** in DMSO (50 μl, 18.7 μmol). Finally was added a 1.87 M solution of acetaldehyde in buffer (10 μl, 18.7 μmol). The resulting solution was left stirring at room temperature. A 20 μl aliquot of the reaction mixture was sampled at different time intervals and diluted with 780 μl of a mixture of DMSO/water (1:1; v/v), and 100 μl of the resulting solution was injected for analysis by RP LC-MS using Method 2 and Method D in tandem.

5.4. Stability of Fmoc-Lys-HYNIC-Tfa in water or water/acetaldehyde at room temperature and at 90 °C

To a solution of Fmoc-(trifluoroacetylHYNIC)-lysine **3** in DMSO (2.5 mg in 140 μl, 4.1 μmol) was added 360 μl of water. The resulting solution was either: *A* left stirring at room temperature for 2 h; or *B* left stirring at 90 °C for 1 h; or *C* left stirring at 90 °C for 1 h, cooled to room temperature, then treated with a 0.41 M aqueous solution of acetaldehyde (10 μl, 4.1 μmol) for 1 min; or *D* treated with a 0.41 M aqueous solution of acetaldehyde (10 μl, 4.1 μmol), and the resulting solution left stirring at 90 °C for 1 h. A 20 μl aliquot of the reaction mixture was sampled and diluted with 480 μl of a mixture of DMSO/water (1:1; v/v), and 80 μl of the resulting solution was injected for analysis by RP LC-MS using Method 1 and Method D in tandem.

5.4.1. Radioanalytical methods. Radiolabelled HYNIC compounds were analysed by HPLC using a Beckman System Gold running 24 Karat proprietary software and a Beckman 168 UV detector in series with a GABI radioactivity monitor (Raytest), running HPLC Method 3: Phenomenex Jupiter C18 300Å column, 250×4.60 mm 5 μm, flow rate 1 ml/min, UV detection at 220–350 nm were employed with the following gradient: ACN in 0.1% aqueous TFA: 0–5 min 0% ACN, 5–25 min 0–60% ACN, 25–30 min 60% ACN, 30–35 min 60–100% ACN, 35–37 min 100–0% ACN. ITLC was performed on silica gel (ITLC-SG, Gelman Sciences, Ann Arbour, Mich.) with saline as eluent for detection of ^{99m}Tc-pertechnetate and ^{99m}Tc-labelled co-ligands, and 50% acetonitrile-water solution for determination of ^{99m}Tc colloid ('reduced hydrolysed technetium').

5.4.2. ^{99m}Tc-radiolabelling with tricine as co-ligand. In a screw top 2.5 ml polypropylene Corning vial, 3 μg of HYNIC compound in water was incubated with 0.5 ml of a solution of tris(hydroxymethyl)methylglycine (tricine; 100 mg/ml in water), 0.5 ml of $^{99m}TcO_4^-$ solution (>200 MBq), and 10 μl of stannous chloride dihydrate solution (3 mg/ml in ethanol) for 30 min at 95 °C or 15 min at 20 °C. To meet MS requirements, the labelling experiment was scaled up as follows: In a screw top 2.5 ml polypropylene corning vial, 14 μg of HYNIC compound in water was incubated with 100 μl of a solution of tricine (100 mg/350 μl in water), 50 μl $^{99}TcO_4^-$ solution (1×10^{-8} mol), 5 μl $^{99m}TcO_4^-$ solution (3 MBq), and 5 μl stannous chloride dihydrate solution (6 mg/ml in ethanol) for 30 min at 95 °C or 15 min at 20 °C.

5.4.3. Acetaldehyde challenge to ^{99m}Tc-radiolabelling with tricine as co-ligand. In a screw top 2.5 ml polypropylene corning vial, 3 μg of Fmoc-(HYNIC)-lysine **2** or Fmoc-(trifluoroacetylHYNIC)-lysine **3**

was incubated with 20 μl of an aqueous solution of acetaldehyde (0.5 μmol dm^{-3}) in 0.5 ml of a solution of tris(hydroxymethyl)methylglycine (tricine; 100 mg/ml in water) for 24 h at 20 °C. Then 0.5 ml of $^{99m}TcO_4^-$ solution (>200 MBq), and 10 μl of stannous chloride dihydrate solution (3 mg/ml in ethanol) were added and the resulting solution heated for 30 min at 95 °C.

Acknowledgements

We thank Kevin Howland for his assistance and advice in the LC-MS work, and the EPSRC and Cancer Research UK for funding.

References and notes

1. Abrams, M. J.; Juweid, M.; Tenkate, C. I.; Schwartz, D. A.; Hauser, M. M.; Gaul, F. E.; Fuccello, A. J.; Rubin, R. H.; Strauss, H. W.; Fischman, A. J. *J. Nucl. Med.* **1990**, *31*, 2022–2028.
2. Steffens, M. G.; Oosterwijk, E.; Kranenborg, M.; Manders, J. M. B.; Debruyne, F. M. J.; Corstens, F. H. M.; Boerman, O. C. *J. Nucl. Med.* **1999**, *40*, 829–836.
3. Verbeke, K.; Hjelstuen, O.; Debrock, E.; Cleynhens, B.; Deroo, M.; Verbruggen, A. *Nucl. Med. Commun.* **1995**, *16*, 942–957.
4. Ultee, M. E.; Bridger, G. J.; Abrams, M. J.; Longley, C. B.; Burton, C. A.; Larsen, S. K.; Henson, G. W.; Padmanabhan, S.; Gaul, F. E.; Schwartz, D. A. *J. Nucl. Med.* **1997**, *38*, 133–138.
5. Blankenberg, F. G.; Katsikis, P. D.; Tait, J. F.; Davis, R. E.; Naumovski, L.; Ohtsuki, K.; Kopiwoda, S.; Abrams, M. J.; Darkes, M.; Robbins, R. C.; Maecker, H. T.; Strauss, H. W. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6349–6354.
6. vanderLaken, C. J.; Boerman, O. C.; Oyen, W. J. G.; vandeVen, M. T. P.; Edwards, D. S.; Barret, J. A.; vanderMeer, J. W. M.; Corstens, F. H. M. *J. Nucl. Med.* **1997**, *38*, 1310–1315.
7. Ono, M.; Arano, Y.; Mukai, T.; Uehara, T.; Fujioka, Y.; Ogawa, K.; Namba, S.; Nakayama, M.; Saga, T.; Konishi, J.; Horiuchi, K.; Yokoyama, A.; Saji, H. *Nucl. Med. Biol.* **2001**, *28*, 155–164.
8. Eisenhut, M.; Mohammed, A.; Mier, W.; Schonsiegel, F.; Friebe, M.; Mahmood, A.; Jones, A. G.; Haberkorn, U. *J. Med. Chem.* **2002**, *45*, 5802–5805.
9. Rajopadhye, M.; Harris, T. D.; Yu, K.; Glowacka, D.; Damphousse, P. R.; Barrett, J. A.; Heminway, S. J.; Edwards, D. S.; Carroll, T. R. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 955–960.
10. Liu, S.; Edwards, D. S.; Looby, R. J.; Harris, A. R.; Poirier, M. J.; Barrett, J. A.; Heminway, S. J.; Carroll, T. R. *Bioconjug. Chem.* **1996**, *7*, 63–71.
11. Liu, S. *Adv. Drug Deliv. Rev.* **2008**, *60*, 1347–1370.
12. Bartholoma, M.; Valliant, J.; Maresca, K. P.; Babich, J.; Zubieta, J. *Chem. Commun.* **2009**, 493–512.
13. Harris, T. D.; Sworin, M.; Williams, N.; Rajopadhye, M.; Damphousse, P. R.; Glowacka, D.; Poirier, M. J.; Yu, K. *Bioconjug. Chem.* **1999**, *10*, 808–814.
14. Edwards, D. S.; Liu, S.; Harris, A. R.; Poirier, M. J.; Ewels, B. A. *Bioconjug. Chem.* **1999**, *10*, 803–807.
15. Greenland, W. E. P.; Howland, K.; Hardy, J.; Fogelman, I.; Blower, P. J. *J.Med. Chem.* **2003**, *46*, 1751–1757.
16. King, R. C.; Surfraz, M. B. U.; Biagini, S. C. G.; Blower, P. J.; Mather, S. J. *Dalton Trans.* **2007**, 4998–5007; Blower, P. J.; Surfraz, M.; King, R.; Biagini, S.; Mather, S. J. *20th Annual Congress of the European-Association-of-Nuclear-Medicine*; Springer: Copenhagen, DENMARK, 2007, pp S148–S148.
17. King, R.; Surfraz, M. B.-U.; Finucane, C.; Biagini, S. C. G.; Blower, P. J.; Mather, S. J. *J. Nucl. Med.* **2009**, *50*, 591–598; King, R.; Surfraz, M.B.; Biagini, S.C.G.; Blower, P. J.; Mather, S.J. in 20th Annual Congress of the European-Association-of-Nuclear-Medicine, Copenhagen, DENMARK, 2007, pp S180–S180.
18. Surfraz, M. B. U.; King, R.; Mather, S. J.; Biagini, S.; Blower, P. J. *J. Inorg. Biochem.* **2009**, 971–977; Surfraz, M.; King, R.; Mather, S.; Biagini, S.; Blower, P. *20th Annual Congress of the European-Association-of-Nuclear-Medicine*; Springer: Copenhagen, DENMARK, 2007, pp S148–S148.
19. Surfraz, M. B. U.; Biagini, S. C. G.; Blower, P. J. *Dalton Trans.* **2008**, 2920–2922.
20. Surfraz, M. B. U.; King, R.; Mather, S. J.; Biagini, S. C. G.; Blower, P. J. *J. Med. Chem.* **2007**, *50*, 1418–1422.
21. Surfraz, M.; King, R.; Mather, S.; Biagini, S.; Blower, P. *20th Annual Congress of the European-Association-of-Nuclear-Medicine*; Springer: Copenhagen, DENMARK, 2007, pp S179–S179; Surfraz, M. B. U.; King, R.; Mather, S. J.; Biagini, S. C. G.; Blower, P. J. In *Technetium, Rhenium and Other Metals in Chemistry and Nuclear Medicine*; Mazzi, U., Ed.; SGEditoriali: Padova, 2006; Vol. 7, pp 89–294.
22. Miller, D. J.; Surfraz, M. B. U.; Akhtar, M.; Gani, D.; Allemann, R. K. *Org. Biomol. Chem.* **2004**, *2*, 671–688.
23. Surfraz, M. B. U.; Akhtar, M.; Allemann, R. K. *Tetrahedron Lett.* **2004**, *45*, 1223–1226.
24. Schelhaas, M.; Waldmann, H. *Angew. Chem. Int. Ed.* **1996**, *35*, 2056–2083.